The Role of Glycosylphosphatidylinositol phospholipase D (GPI-PLD) in Type One Hypersensitivity

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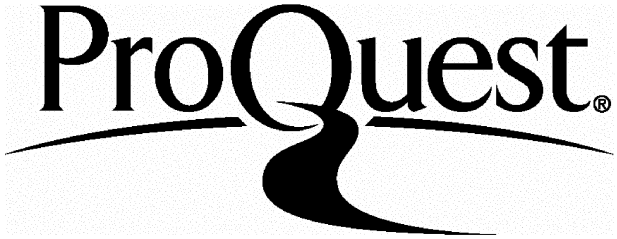

PATENT APPLICATION:

Materials and Methods relating to the treatment of conditions involving mast cells, basophils and eosinophils

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'The Scientific Mind does not so much provide
the right answers as to ask the right questions'

Claude Lévi-Strauss

DEDICATION:

A very special thanks goes to my parents. I thank you for passing on your genes, for teaching me to reach for the stars, and for being there when I fell. I love you and I thank you.

And to my fiancé, Paul. You have the patience of a saint and a heart of gold. I love you and I look forward to spending the rest of my life with you.

SUMMARY.

GPI-anchored proteins are a diverse group of molecules, which are ubiquitous in nature. The core structure of the GPI-anchor is highly conserved across a number of different species, a feature that implies a biological significance. Glycosylphosphatidylinositol-phospholipase D (GPI-PLD) is a 115 kDa glycoprotein which is found in high concentrations in mammalian serum. GPI-PLD hydrolyses GPI anchors, and it has been suggested that the enzyme is involved in the selective release of GPI-anchored proteins from the cell surface.

GPI-PLD and GPI-anchored proteins have been implicated in a number of pathological situations, including T cell activation, insulin-mediated signalling and parasite immune evasion

That GPI-PLD might play a role in the IgE-dependent activation of mast cells was suggested following experiments using a polyclonal antibody against bovine GPI-PLD. Inclusion of this antibody in experiments to activate mast cells lead to inhibition of the cell's activation. However, this research was never completed.

The research undertaken in this thesis has employed the Rat Basophilic Leukaemia cell line (RBL-2H3) as an analogue of Type One Hypersensitivity. IgE-dependent and independent stimulation of the cells was achieved, after which time the cells were employed in a variety of experiments to determine the role of GPI-PLD in their activation.

Techniques were used to determine whether the cells generated the enzyme, It was determined that the cells did not generate mRNA, but contained the protein. The source of the enzyme was the Foetal Bovine Serum in the culture medium, and techniques were employed to inactivate or remove the enzyme from the serum. The effect on the cells' behaviour was ascertained.

Conclusions drawn were that RBL-2H3 cells employ a serum component, with characteristics of a GPI-PLD enzyme, in IgE-mediated activation.

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LIST OF ABBREVIATIONS.

A260	Absorbance at 260 nm wavelength
Apo-Al	Apolipoprotein A1
cm	centimetre
°C	Degrees centrigrade
cDNA	Complementary deoxyribonucleic acid
Con A	Concavalin A
DAF	Decay Accelerating Factor
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNP	Dinitrophenyl
DNP-albumin	Dinitrophenyl-albumin
dNTPs	Deoxyribonucleoside triphosphate(s)
DTT	Dithiothreitol
EDTA	Ethylenediaminetetracetic acid
EGTA	Ethyleneglycol bis (β-amino ethyl ether) N,N,N',N', tetracetic acid
EMEM	Eagles Minimum Essential Medium
FBS	Foetal Bovine Serum
g	Gram
GPI	Glycosylphosphatidylinositol
GPI-PLC	Glycosylphosphatidylinositol-Phospholipase C
GPI-PLD	Glycosylphosphatidylinositol-Phospholipase D
kDa	Kilo Dalton
IgE	Immunglobulin E
IP3	Inositol 1,4,5 triphosphate
IPG	Inositol phosphoglycan
M	Molar
MAb	Monoclonal Antibody
MDCK	Madin-Darby canine kidney
mg	Milligram
min	Minute
ml	Millilitre
mm	Millimetre
mM	Millimolar
MW	Molecular weight
ng	Nanogram
Nm	Nanometre
OA	Ovalbumin
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Phosphatidyl Choline
PCA	Passive Cutaneous Anaphylaxis
PCR	Polymerase chain reaction
PE	Phosphatidyl Ethanolamine
PHA	Phytohaemaglutinin
PI-3-K	Phosphatidylinositol-3-kinase
PI-PLC	Phosphatidylinositol-Phospholipase C
PIP-2	Phosphatidylinositol 2-phosphate
PLD	Phospholipase D
pmol	Picomole
'	Prime
PS	Phosphatidyl Serine
RBL	Rat Basophilic Leukaemia
r-GPI-PLD	recombinant GPI-PLD

RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
SCF	Stem Cell Factor
SDS	Sodium dodecylsulphate
SM	Sphingomyelin
TCR	T Cell Receptor
TEMED	N,N,N',N;-tetramethylethylenediamine
Tm	Melting temperature
Tris	Tris (hydroxylaminomethane)
Tris-HCl	Tris Hydrochloric Acid
U	Units
µg	Microgram
µl	Microlitre
µM	Micomolar
UV	Ultra violet
v	Volume
w	Weight

Chapter 1

Introduction to Allergic Disease and Type One Hypersensitivity

1 General Introduction to Allergic Disease

1.1 Allergic Disease

1.1.1 Human Immune System

The human immune system has evolved into a highly elaborate system that acts, simply, to maintain and protect 'self' and to neutralise or destroy 'non-self'. Through the ability to differentiate between and self and foreign, the human immune system has proven itself to be remarkably efficient in minimising human disease.

1.1.2 Hypersensitivity

Hypersensitivity is an example of a disorder of the human immune system. The consequence of any shift in the delicate balance of the immune system is the development of immune responses that are either inadequate or detrimental to the host, ultimately leading to a pathological situation.

Hypersensitivity is defined as 'an adaptive immune response, which occurs in an exaggerated or inappropriate form, typically leading to inflammatory reactions and tissue damage'. Hypersensitivity typically involves an initial exposure to the 'offending' antigen followed by an exaggerated response on subsequent exposure (Roitt *et al.*, 1996).

In 1998, the Coombs and Gell classification scheme defined six types of hypersensitivity (www.hon.ch/Library/Theme/allergy.glossary/gcc.html):

I. Immediate hypersensitivity;
II. Cytotoxic antibody;
III. Immune complex;
IV. Delayed hypersensitivity;
V. Stimulating antibody;
VI. Antibody dependent cell mediated cytotoxicity (ADCC).

The research performed for this thesis has focused on the study of Type One, immediate hypersensitivity.

1.1.3 Type One Hypersensitivity

The term allergy, meaning 'changed reactivity of the host when meeting an agent on a second or subsequent reaction', was first used in the early 1900's by von Pirquet (von Pirquet, 1906). This original definition emanated from an observation that the injection of proteins and sera could result in a variety of harmful immune reactions.

In 1923, Coca and Cooke used the term atopy, which literally means strange disease, as a descriptive term that could be associated with allergic individuals. Atopy was originally used to differentiate between the familial syndrome of asthma and hay fever and the so-called mixed reactions (Coca & Cooke, 1923). Nowadays, the terms atopy and allergy are interchangeable and broadly encompass a number of complaints, including hay fever, rhinitis, atopic asthma and atopic dermatitis.

It has only been in recent years that Von Pirquet's definition of atopy has evolved to become synonymous with Type One Hypersensitivity. Further fine-tuning has resulted in the current definition as 'a helper T cell 2 (TH-2) driven immune response, directed against a common environmental protein'. The innocuous protein in question is termed an antigen, or allergen (Roitt *et al.*, 1996). Type One Hypersensitivity is a consequence of the beneficial defence mechanism employed in the attack against parasites, the mechanisms of which will be discussed later in Section 1.3.4.

1.1.4 Significance of Allergic Disease

The past century has seen a dramatic rise in the reported cases of allergic disease, including rhinitis, asthma, eczema and anaphylaxis, reaching epidemic proportions in many parts of the world. The most detailed information, regarding the numbers of individuals suffering with allergic diseases, is collected in the United States. In the USA alone, an estimated 20% of the population is affected by allergic disease, which equates to approximately fifty million individuals (www.aaaai.org/public/fastfacts/statistics.stm). An overview of the different atopic diseases, including the symptoms observed, is provided in Table 1.1.

1.2 Biochemistry of the Type One Hypersensitivity Reaction

1.2.1 Introduction

The complexity of multi-cellular organisms necessitates exquisite levels of co-ordination and control, even in pathological situations. An extracellular environment, including cells, plasma and signalling molecules, surrounds each individual cell, and each cell has the potential to recognise a large number of extracellularly-initiated signalling events. From the plethora of information it receives, the cell is required to precisely translate an extracellular signal into an amplified intracellular signal, ultimately leading to the appropriate immune response.

The process by which the Type One hypersensitivity response is initiated and perpetuated is no exception and the complexity of the cells and mediators involved is complex. The Type One hypersensitivity reaction is summarised in Figure 1.1.

TABLE 1.1

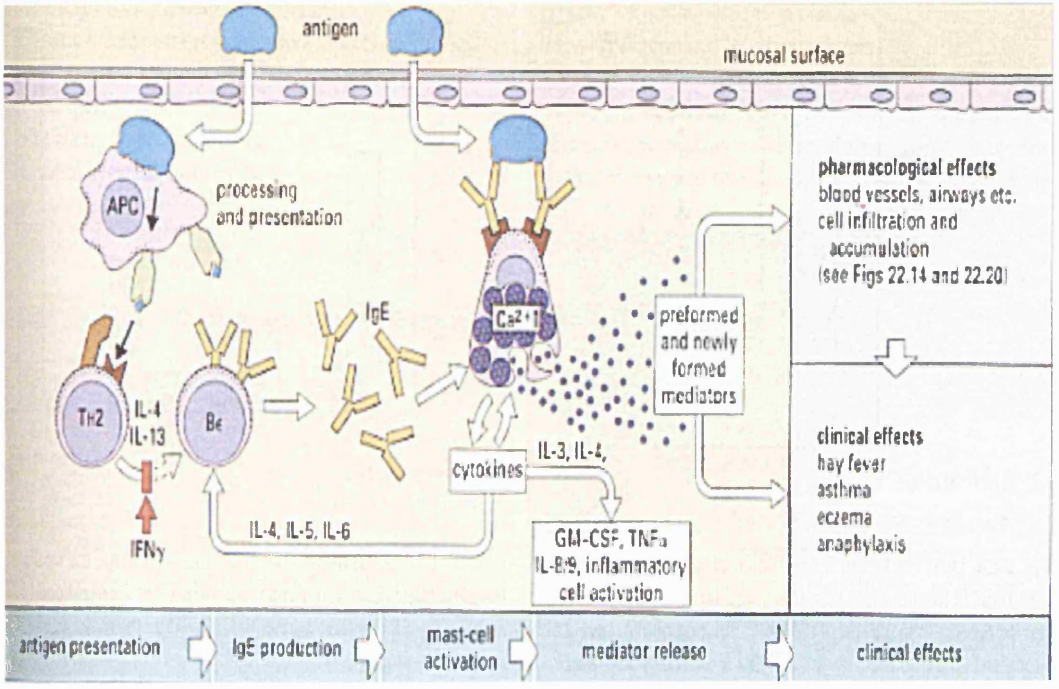
SUMMARY OF THE SYMPTOMS OF ALLERGIC DISEASES

Disease	Symptoms
Allergic Rhinitis	Sneezing, itching and watery eyes, runny nose and burning palate and throat, nasal congestion and sinusitis Seasonal allergies may also trigger asthma
Eczema or atopic dermatitis	Dermatitis is an itchy rash, often on hands, arms, legs and neck Eczema refers to a weeping dermatitis
Contact Dermatitis	Red, itchy and inflamed skin, as a result of direct contact with the allergen
Urticaria or Hives	Pale red and swollen areas of skin (wheals) Hives are usually itchy, but may also burn or sting.
Allergic asthma	Spasm (bronchospasm) of the muscles surrounding the walls of the lung airways (bronchi). Bronchospasm causes narrowing of the airways which leads to shortness of breath, wheezing, coughing, and congestion. Airways can also be narrowed, due to accumulated mucus and swelling that is associated with inflammation of the bronchi.
Anaphylaxis	Anaphylaxis is characterized as an acute reaction resulting from the release of mediators, causing profound end organ dysfunction (cardiac, respiratory, GI, dermatologic, neurologic). Symptoms include anxiety, itching of the skin, headache, nausea and vomiting, sneezing and coughing, abdominal cramps, hives and swelling of tissues such as lips and joints, diarrhea, shortness of breath and wheezing, low blood pressure, convulsions, and loss of consciousness.

(http://www.immune.com/allergy/allabc.html)

FIGURE 1.1

MAST CELL DEGRANULATION



(Taken from Roitt et al, 1996)

Figure Legend

Antigen enters the body, is processed by antigen-presenting cells (APCs), and expressed on the surface, in association with MHC molecules. APCs, in coalition with TH2 cells and B cells upregulate the production of antigen-specific IgE, which is bound to its high-affinity receptor (FcεRI) on the surface of mast cells, and other effector cells. Antigen-mediated cross-linking of this receptor, during subsequent encounter with antigen, promotes cross-linking of the IgE/ FcεRI complexes. Through a complex sequence of signalling events, the mast cell undergoes degranulation, with the release of numerous mediators and cytokines. These degranulation products initiate the clinical allergic response.

As has already been discussed, Type One hypersensitivity reactions represent the immune response to common proteins, known as allergens. Typical allergen sources include grass pollens, house dust mites, latex, moulds and animal sheddings. These allergens are omnipresent and typically cause no adverse effects in non-allergic individuals. What separates allergic from non-allergic individuals is the intensity of the immune reaction that is provoked by allergen exposure.

The cell of pivotal importance is the mast cell, through its primary role in the initiation and perpetuation of the allergic reaction. Briefly, immunoglobulin E (IgE) is bound to its high-affinity receptor (FcεRI) on the surface of mast cells and other effector cells. Exposure to allergen in a sensitised individual leads to a cross-linking of the IgE (bound to FcεRI), which promotes mast cell degranulation and the initiation of an inflammatory response.

1.2.2 Role of Type One Hypersensitivity in Asthma and Eczema

A paradox in the study of Type One hypersensitivity has been the relative importance of the Type One Hypersensitivity reaction in diseases such as asthma and eczema. Asthma and eczema are hypersensitivity diseases, but each can be sub-divided into either atopic (extrinsic) or non-atopic (intrinsic) in nature. In the case of intrinsic asthma or eczema, the patients display no elevated levels of serum IgE (Bochner *et al.*, 1994) and no skin reactivity to the common allergens. For many years it was believed that the lack of skin reactivity was simply because the 'correct' allergen had yet to be found in the non-atopic individuals.

The establishment of animal models, combined with a greater knowledge of allergic disease, however, has altered this theory. The current theory postulates that the asthmatic reaction may be more dependent on the IL-5-mediated recruitment and degranulation of eosinophils, than on the IgE-mediated degranulation of mast cells (Hogan *et al.*, 1997). Studies of experimental asthma, using genetically modified T or B cell-deficient mice, have indicated that the disease state was largely independent of antibody (Corry *et al.*, 1998). It was determined that B-cell deficient mice, when challenged with antigen, developed airway hyperreactivity, typical of an asthmatic airways response. Considering that B cells are largely responsible for the generation of antibodies, the conclusion was made that antibodies were not directly involved in this experimental asthma system.

These data contrast to previous data concerning IgE in experimental asthma (Oshiba *et al.*, 1996). A model was established in which mice were injected with ovalbumin-specific IgE (anti-OVA-IgE) or anti-OVA-IgG isotypes. After cutaneous challenge with ovalbumin, the mice developed an immediate hypersensitivity-type skin wheal, whereas airway challenge

promoted airway hyper-reactivity. Neither reaction was observed after injection with either IgG2 or IgG3 isotypes. Although this data does not directly demonstrate a cause-and-effect relationship between IgE and the asthmatic response, it challenges data that suggests that IgE is unimportant. Evidently, further analysis is required to determine whether IgE plays a pivotal role in asthmatic disease.

Other studies have questioned the relative importance of IL-4 and IL-13 in experimental asthma, despite the critical role of these cytokines in Type One hypersensitivity. A study using IL-4 deficient mice showed that, although eosinophil recruitment and airway hyperreactivity was attenuated by the absence of IL-4, the final airway pathology was not affected (Hogan *et al.*, 1997). By comparison, a different study, using anti-IL-4 antibodies to neutralise the effect of the cytokine, showed reduced airway hyperreactivity and eosinophil accumulation in response to antigen challenge (Corry et al., 1996). The importance of IL-13 was studied using a mouse model of asthma, specific anti-IL-13 antibodies, and antigen challenge. The results indicated that the blockade of IL-13 led to significant inhibition of the asthmatic response. It was further hypothesised that IL-13 was of greater importance than IL-4 in asthma, and that IL-13 alone can effectively stimulate airway hyperreactivity independent from antigen-specific IgE and eosinophils (Wills-Karp *et al.*, 1998)

Clearly, the significance of each of the different cell types, the antibodies and the cytokines in airway hyperreactivity has yet to be elucidated. Furthermore, the results appear to be dependent on the animal model under investigation.

None of the studies to date has conclusively proven that the pathology of diseases such as asthma is entirely independent from Type One Hypersenstivity. It is important, however, to note that the study of intrinsic diseases has been largely neglected in this Introduction, which has focussed on the more classic immediate allergic responses in preference to asthma and eczema.

1.2.3 Cells Involved in the Type One Hypersensitivity Reaction

It was originally believed that both mast cells and basophils had an equal prominence in the Type One hypersensitivity response. This theory, however, was refuted with the discovery of significant differences between the two cell types.

There are many structural and functional similarities between mast cells and basophils. Both are metachromatically staining cells, with surface IgE receptors and the capacity to release allergic mediators upon appropriate stimulation (Schwartz *et al.*, 1979). In addition, both mast

cells and basophils were demonstrated, *in vitro*, to be derived from a CD34+ progenitor cell (Kirshenbaum *et al.*, 1991). However, mast cells and basophils have conspicuous differences that separate them as distinct, and individual, cell types.

Mast cells originate in the bone marrow, circulate in the blood for a short time and terminally differentiate in peripheral tissues. They are widely distributed throughout mammalian tissues and are particularly concentrated in connective tissues, such as skin, where they are concentrated along small blood vessels (Benditt & Lagunoff, 1964). They are also located in the sub-epithelial connective tissue of the respiratory and digestive tracts (Gartner & Hiatt, 1997). Circulating mast cell precursors express the CD34 epitope and differentiate into metachromatic, tryptase-positive mast cells under the influence of stem cell factor (Castells, 1997).

Basophils, however, terminally differentiate in the bone marrow and have a smaller repertoire of mediators. Experimentation has suggested that the mechanism controlling the degranulation of mast cells is significantly different in basophils (MacGlashan *et al.*, 1983). Firstly, the concentration of anti-IgE required to promote degranulation is 30 fold higher for mast cells, compared with basophils, and this is unrelated to the quantity of surface receptors. Secondly, the role of cAMP in degranulation appears to be different in mast cells from basophils. It is currently believed that basophils have a greater prominence in the initiation and perpetuation of the Late Phase allergic reaction (Guo *et al.*, 1994).

A number of other different cell types play a role in the perpetuation of an allergic reaction, as illustrated in Figure 1.1, and these cells are largely dependent on the initial signal from mast cells.

Techniques have permitted the purification of rodent and human mast cells and the establishment of immortal cell lines and these will be discussed in greater detail in Chapter 2. The current ability to produce pure mast cells, or mast cell lines, has permitted investigation of the fine details of the intracellular biological mechanisms that control mediator release from mast cells free from the complications of contaminating cells.

1.2.4 Receptor Mediated Signalling Systems

As already outlined, the Type One hypersensitivity reaction follows a sequential pathway, from the surface cross-linking of the IgE/FcεRI complexes by antigen, through to ultimate cell degranulation. Broadly speaking, the reaction which occurs in mast cells can be summarised by the following, sequential steps.

1 Antigen induced receptor activation;

2 Signal transduction;

3 Signal translation and amplification;

4 Secretion of granule components.

It should be noted that, where possible, the discussions that follow are based on studies of rat cells. Although the mechanisms of activation of human and rat cells are broadly similar, there is some variation. However, as the cell system under investigation for this doctoral thesis utilised a rat cell line, it is more appropriate to discuss what is understood in the rat.

1.3 Antigen Induced Receptor Activation

1.3.1 Antigen Recognition

Antigen recognition is a central event in the initiation of the allergic response and is dependent on the existence of immunoglobulin E (IgE) on the mast cell surface. All immunoglobulin molecules are elegantly constructed in a manner that serves two primary functions. One region of the molecule contains an antigen-recognition site that confers the capacity to recognise and bind a wide variety of antigenic determinants. A second, separate region contains a receptor-recognition site, which confers the capacity to interact with the host cell and thereby mediate the biologic effects of the antigen recognition. Immunoglobulin E is no exception; the molecule contains both functional regions, permitting both antigen recognition and receptor recognition.

1.3.2 Antigens and Allergens

An antigen (antibody generator) is defined as any substance that elicits an immune reaction involving T lymphocytes or B-lymphocytes. Allergens are those antigens that specifically initiate the allergic cascade.

Biochemically, the characteristics of an antigen dictate the direction of the immune response that it elicits. The immune system has evolved to determine the characteristics of the antigen and to instigate the most appropriate response. CD4+ T cells can be broadly subdivided into two categories, termed type one helper (TH-1) and type two helper (TH-2) (Mosmann & Coffman, 1989). In general, these effector subtypes are mutually antagonistic, with inflammatory responses typically dominated by the effects of one cell type in preference to the other.

TH-l and TH-2 cells derive from a common precursor, variously called TH-P or TH-O, and are most commonly separated according to their cytokine production, as shown in Figure 1.2. TH-1 cells produce interleukin (IL) 2 and 3, interferon (IFN)-γ and tumour necrosis factor (TNF)-α, whereas TH-2 cells produce IL- 4, 5, 6, 9 and 13. Certain antigens promote initiation of the TH-1 mediated pathway, whereas others promote initiation of the TH-2 mediated pathway.

For example, bacterial antigens promote the TH-1 pathway, ultimately leading to macrophage activation and to the production of IgG2a and IgG3 that mediate antibody dependent cell cytotoxicity and complement activation. By comparison, antigens such as grass pollens or *Der p I*, from house dust mites, direct the human immune system to the TH-2 pathway. This results in the accumulation and activation of mast cells and eosinophils, stimulation of B cells and the production of IgE and IgG1 (Parronchi *et al.*, 1991). The significance of the TH-2 pathway in the initiation of mast cell activation will be discussed in more detail in Section 1.3.7.

1.3.3 Immunoglobulin E

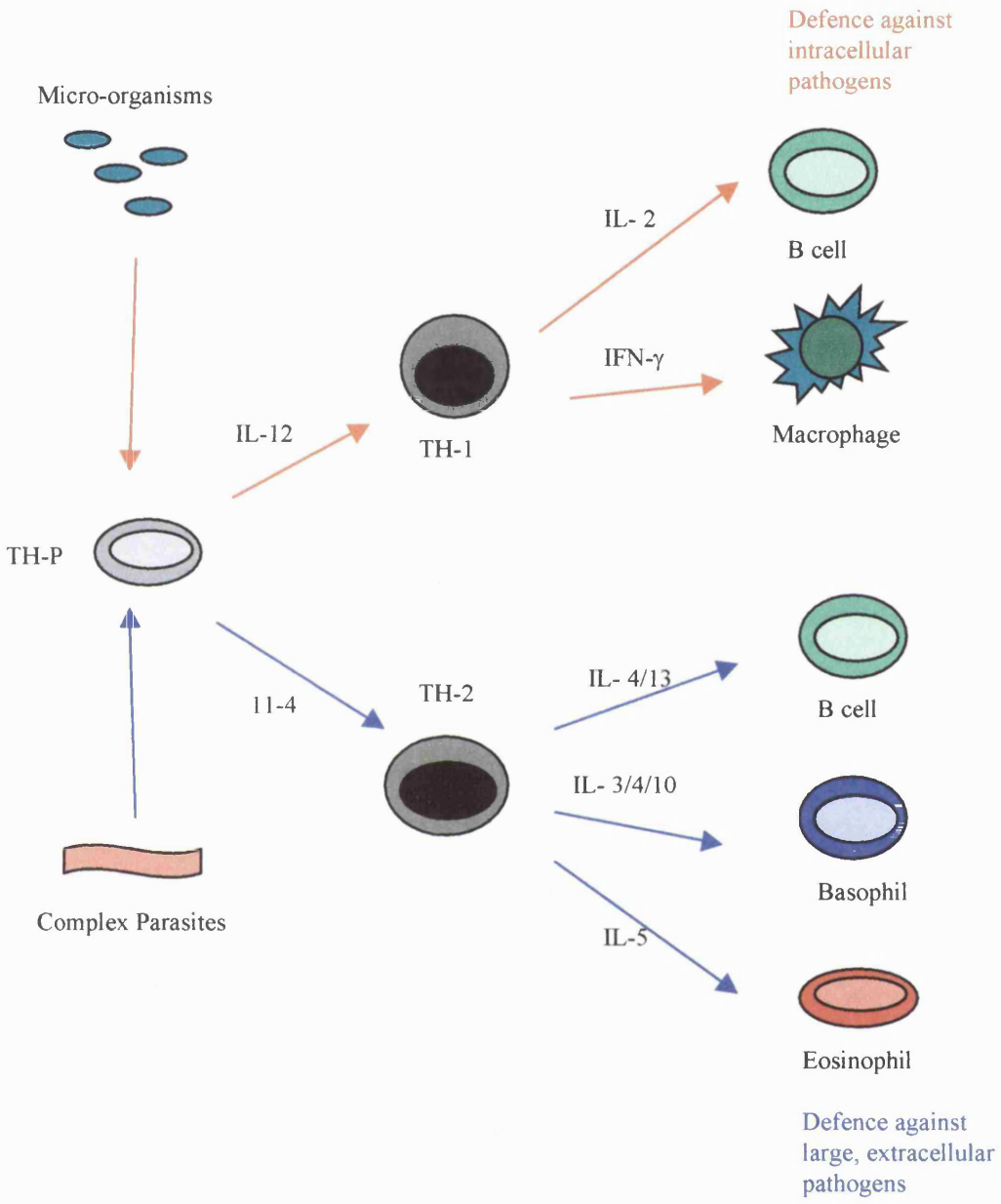
Prausnitz and Küstner presented the first evidence of an unusual serum component in 1921, with the proposal of the existence of an 'atopic reagin' in the serum of allergic individuals. This reagin, when injected into the skin of another individual, had the capacity to induce an allergic-type wheal and flare reaction in the recipient (Prausnitz & Küstner, 1921).

Investigation of this reagin indicated features that distinguished it from the existing immunoglobulin classes (A, D, G and M). Despite the identification of antigenic determinants in common with these immunoglobulins, this reagin also contained sequences that were novel. In addition, unlike the alternative immunoglobulins, the atopic reagin lacked the capacity to form a precipitate and to form complexes when combined with complement and specific antigens *in vitro*. With reference to the official classifications or the nomenclature of human immunoglobulins (Bennich *et al.*, 1964), this novel protein was classified as immunoglobulin E (IgE) (Bennich *et al.*, 1968). The structure of IgE is shown schematically in Figure 1.3.

Produced by B cells, IgE comprises the least abundant class of immunoglobulin, with levels as low as 50 - 300 ng/ml in serum (Sutton & Gould, 1993). In many individuals, severity of allergic disease correlates roughly with serum IgE levels, although even with levels of IgE reaching 100 times in excess of normal, the absolute quantities of IgE still remain relatively small (Corry & Kheramand, 1999). The suggestion was made that tight control over the quantities of circulating IgE is necessary, to minimise the potential damage that IgE-mediated inflammation can cause. Immunoglobulin E is of paramount importance in the allergic

FIGURE 1.2

SIMPLIFIED SCHEMATIC OF T-CELL DIFFERENTIATION



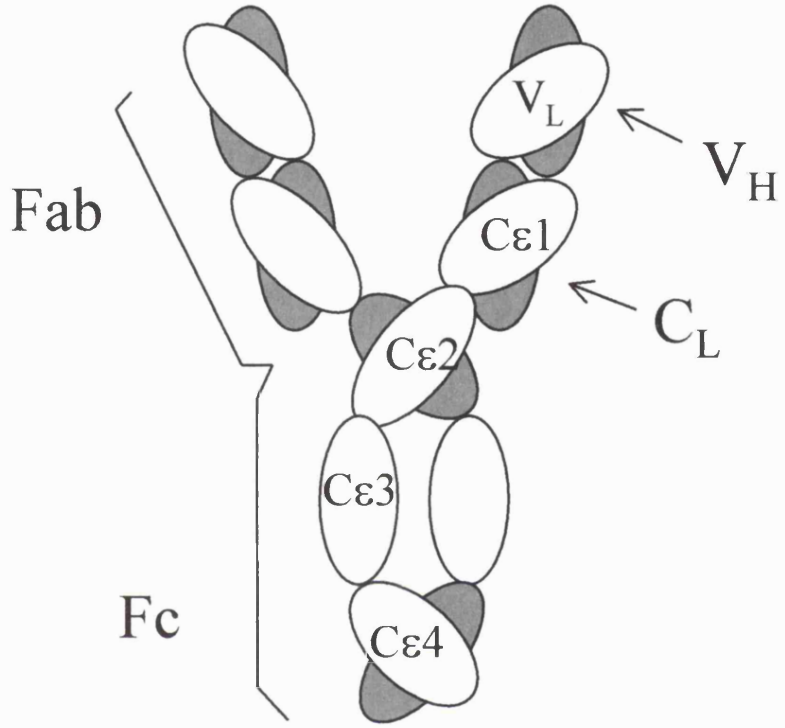
(Adapted from Romagnani, 1996)

Figure Legend

TH-l and TH-2 cells derive from a common precursor, the so-called THP cell. The differentiation of TH1 or TH2 cells is dependent on the signals received at the time of antigen recognition. TH1 cells are induced by IL-12, and secrete IL-2, IL-3, IFN-γ and TNF-α; ultimately eliciting antibodies (IgG2a and IgG3), complement activation and cytotoxic cellular responses. TH2 cells are induced by IL-4, secrete IL- 4, IL-5, IL-6, IL-9 and IL-13, which elicit antibody classes (IgE, IgG1) and cellular mechanisms that promote the destruction of larger organisms that cannot be taken intracellularly.

FIGURE 1.3

SCHEMATIC REPRESENTATION OF IMMUNOGLOBULIN E (IgE)



(Taken from from Sutton & Gould, 1993)

Figure Legend

The structure of human IgE. The molecules consists of two identical light (L) chains (κ and λ) and two identical ε heavy (H) chains. The schematic also indicates the receptor binding domain (Fc) and the antigen-binding domain (Fab), which are central to the function of IgE

reaction, and an understanding of the complex interaction between IgE and mast cells, and the role of IgE and the allergic cascade, is essential.

1.3.4 Beneficial Effects of IgE in Helminth Infections

The beneficial role of IgE was first demonstrated in the immunological defence against helminths (macroparasitic worms). Due to their large size, macroparasites cannot be phagocytosed, meaning that any immune response has to occur extracellularly. The immune response, simply, involves the recruitment of large numbers of mast cells and eosinophils to the location of the parasite. The B cells produce vast quantities of IgE, which serves to coat (opsonise) the parasite, followed by an antibody-dependent cell cytotoxicity (ADCC) response against the parasite by the eosinophils. The net result is the *in situ* release of cell-derived mediators and cytokines that damage the outer surface of the parasite and encourage its destruction (Jarrett & Miller, 1982).

1.3.5 Detrimental Effects of IgE in Type One Hypersensitivity

The first evidence of the detrimental effects of IgE in allergic disease was suggested as a result of Prausnitz and Küstner's proposed 'atopic reagin' (IgE) in the serum of allergic individuals. The concurrent ability to generate a wheal and flare reaction, the classic allergic indicator, upon injection of the atopic reagin into a naive recipient strengthened the case that IgE was involved in allergic disease (Prausnitz & Küstner, 1921).

Once IgE could be quantitatively determined from serum samples, its relationship with the atopic status of the donor could be determined. It was discovered that the atopic status of the individual did not necessarily reflect higher serum IgE levels, except in the case of atopic asthma and eczema. In these diseases, the serum IgE levels could be as high as 100 times the normal level (Roitt *et al.*, 1996). This further supported the importance of IgE in allergic disease.

The exact role of IgE in allergic disease, however, required further investigation and it was essential to directly determine the answers to three main questions. The first concerned upregulation of IgE upon initiation of the allergic reaction and the second concerned the nature of the interaction between IgE and those cells involved in the allergic reaction. The breakthrough to these two questions came with the discovery of IgE-specific receptors on the surface of mast cells. A third question remained to be answered however, concerning the cascade of reactions that occurred during the course of an allergic reaction. This was resolved by the demonstration that the high affinity receptor, FcεRI, was tightly integrated into the

intracellular signalling system of the mast cells and, upon activation, promoted degranulation of the cell.

1.3.6 The Role of T Cells in the Allergic Cascade

Traditionally, the immune response was sub-divided into two categories, humoral or cellular, describing the respective contributions of antibodies or cells. It has become increasingly hard to separate these two categories, as the prominent effects of interactions between antibodies and cells have become apparent.

The existence and regulation of IgE in allergic disease, for example, is highly regulated by the cellular immune system, with a particular prominence of CD4+ T cells. As mentioned previously (Section 1.3.2), CD4+ T cells can be broadly subdivided into two categories, termed type one helper (TH-1) and type two helper (TH-2) (Mosmann & Coffman, 1989). It is the TH-2 group that is closely associated with the production and up-regulation of IgE in response to the appropriate stimulation. TH-2 cells secrete IL-4, IL-5, IL-6, IL-9 and IL-13. These TH-2 type cytokines have been shown to co-ordinate the responses against large, extracellular pathogens, including parasites (Locksley, 1994). As previously discussed, the immune responses against parasitic infections reveal extensive similarities with the immune responses in allergic disease. That a predominantly TH-2 immune response would also be observed in allergic disease seemed an appropriate assumption.

TH-1 cells and cytokines, by comparison, exert an inhibitory effect over the allergic response. TH-1 cells secrete IL-2, IFN-γ, TNF-α, and other cytokines, which antagonise IgE responses. For example, IL-2 and IFNγ both suppress IL-4 induced IgE synthesis *in vitro*. (Nakanishi *et al.*, 1995; Pene *et al.*, 1988).

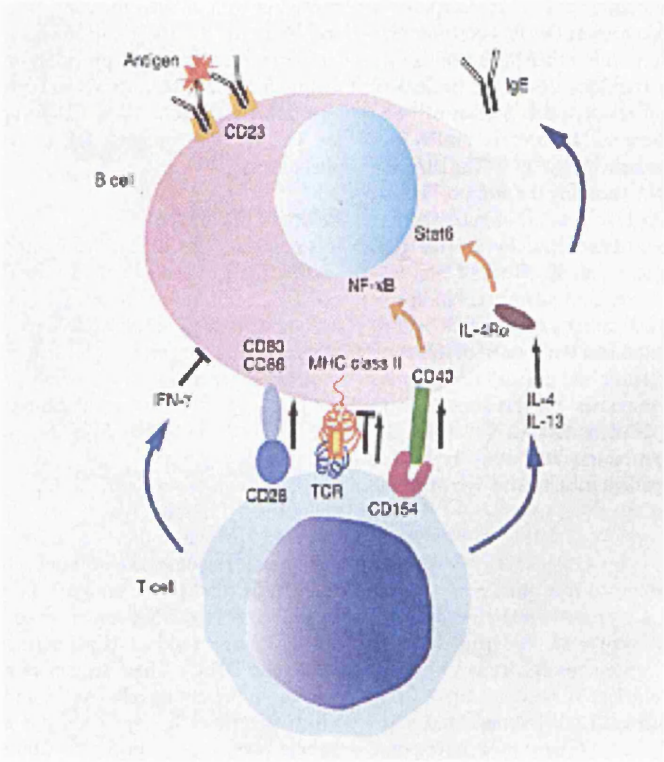
The principle role of the TH-2 cells in the initiation and perpetuation of the allergic cascade is in their control over the other cells types. One of the most important areas of control is in their capacity to induce up-regulation of IgE production by B cells. The following Section deals with this aspect of the immune system.

1.3.7 The Role of T and B cells in the Up-Regulation of IgE

B cells are responsible for the small quantities of IgE present in serum, although these are inadequate for the complete induction of the allergic cascade. Initiation of this cascade, necessitates the generation of larger quantities of IgE and this is largely controlled by interactions between B and T cells. A schematic, illustrating the T cell control over IgE up-regulation in B cells, is illustrated in Figure 1.4. This shows that three primary interactions

FIGURE 1.4

FEEDBACK CONTROL OF IgE SYNTHESIS AND UPREGULATION



(Taken from Corry & Kheradmand, 1999)

Figure Legend

Three primary interactions between T cells and B cells are involved in the process by which the synthesis of IgE is upregulated. These interactions are; the T cell receptor (TCR) with the MHC Class II on B cells, CD54 with CD40 on B cells, and CD28 with either CD 80 or CD86 (previously termed B7-1 and B7-2) on B cells. These interactions promote the upregulation of the synthesis of antigen-specific IgE by B cells, through the role of cytokines and transcriptional up-regulation.

between T cells and B cells are involved in the process by which the synthesis of IgE is upregulated. These primary interactions include:

1 the T cell receptor (TCR) with the Major Histocompatability Complex (MHC) class II on B cells;
2 CD154 with CD40 on B cells;
3 CD28 with either CD 80 or CD86 (previously termed B7-1 and B7-2) on B cells.

1.3.7.1 TCR and MHC Class II

Antigen recognition by T lymphocytes is central to the generation and regulation of an effective immune response. The TCR is a heterodimeric glycoprotein, formed of either α and β or γ and δ units, that enables T cells to recognise a diverse array of antigens. It is surface- expressed, and associated with a collection of polypeptides, called CD3 (Clevers *et al.*, 1988).

The MHC, encodes two sets of highly polymorphic cell surface molecules, termed MHC class I and II. The class II complexes are closely associated with TCR-mediated cell signalling in Type One Hypersensitivity. MHC class II complexes are glycoprotein heterodimers, consisting of heavy (α) chain and light (β) chains, a transmembrane sequence and a cytoplasmic sequence. Located within this complex is a peptide binding groove, in which processed antigen-derived peptides are presented to the TCR (Clevers et al., 1988). The interaction of the TCR with antigen-containing MHC class II serves to up-regulate the cellular response to the offending antigen.

The interaction between TCR on T cells and MHC Class II on B cells appears to involve exquisite levels of control. Experimentally, it was observed that, under otherwise neutral conditions, T cells develop according to the strength of the signals they receive from antigen presenting cells (APCs). Intermediate signals from APCs promote their differentiation into TH-1 cells, whereas high or low signals promote their differentiation into TH-2 cells (Constant *et al.*, 1995). *In vivo*, this can be related to the 'strength' of the affinity of the antigens and the MHC molecule. Allergenic peptides with low or high affinity for MHC molecules should confer relatively weak or strong signals through the same TCR, thereby promoting TH-2 cell differentiation.

Likewise, the method of presentation of the antigen is likely to influence the strength of the resultant T and B cell response. Antigens administered through the respiratory route were shown to be highly immunogenic, whereas antigens administered through alternative routes were less immunogenic and were more likely to induce a state of tolerance, whereby an

immune response is not initiated. It is likely that this state of tolerance is related to the method of presentation of the antigen to the B cell and, therefore, most likely involves APCs (McWilliam *et al.*, 1995). The exact reason for this relative immunogenicity of antigens is not entirely understood.

1.3.7.2 CD54 and CD40

The CD40 antigen is expressed on the B cell surface following antigen recognition. Interaction with its ligand, CD154 on T cells, promotes both proliferation B cells and IgE class switching. The importance of the CD40 antigen was demonstrated in a CD-40-deficient mouse model, in which no IgE-mediated responses were mounted following antigen challenge.

Further evidence was provided by the use of a T cell receptor deficient mouse model, in which antigen challenge failed to stimulate upregulation of IgE production. The addition of an anti-CD40 antibody, however, led to IgE production. It was suggested that the anti-CD40 antibody effectively cross-linked CD40 antigen on the B cell surface, effectively by-passing the need for the CD54 on the T cell (Ferlin *et al.*, 1996). Although the cross-linking of CD40 alone is sufficient to elicit production of polyclonal IgE (a non-specific response), when combined with signals from the B cell receptor the response becomes antigen specific (Kawabe *et al.*, 1994).

1.3.7.3 CD 28 and CD80/86

The CD28 receptor on T cells provides another level of control over IgE production. CD28 interacts with both CD80 (B7-1) and CD86 (B7-2) antigens on B cells. However, there appears to be a level of control at this stage, depending on whether CD28 binds either CD80 or CD86. The CD80 and CD86 antigens appear to direct the T cell immune response towards either TH-1 or TH-2, respectively (Kuchroo *et al.*, 1995).

Blocking antibody experiments were performed, using a murine model of allergic asthma, and an antibody against CD86. Administration of the antibody in antigen-stimulated mice significantly reduced both the symptoms of allergic asthma, serum IgE levels and IL-4 production. No concomitant effect was observed on IFN-γ levels, which would be indicative of an effect on the TH-1 pathway (Keane-Myers *et al.*, 1998). It was concluded that the activation of CD86 leads to activation of the TH-2 pathway of the immune system, in preference to the TH-1 pathway.

1.3.8 The Role of T Cells in Cytokine Production

Of all the known cytokines, the two of greatest importance in the regulation of IgE synthesis are IL-4 (Finkelman *et al.*, 1988) and IL-13 (Punnonen *et al.*, 1993). IL-4 and IL-13 are

produced by a limited number of cells, primarily T cells, and also by granulocytes (mast cells, basophils, and eosinophils). IL-13 is also produced by the natural killer (NK) cells, in response to stimulation by either IL-2 or phorbol myristate acetate, but only in the absence of IFN-γ (Hoshino *et al.*, 1999). The fact that these two cytokines are produced by mast cells and basophils suggests some form of autocrine loop, whereby the cells both produce, and respond to, the cytokines of interest.

Both IL-4 and IL-13 recognise the α chain of the IL-4 receptor. The amino acid sequences of these two cytokines show thirty percent homology. Analysis of the predicted secondary structure of the cytokines suggests that both would have a highly helical structure, which would almost certainly affect their interaction with the IL-4 receptor (Zurawski *et al.*, 1993). Interaction with the IL-4 receptor on the surface of B cells is known to initiate a cascade reaction, ultimately leading to the transcription of the mRNA for IgE and epsilon class switching. Epsilon class switching is a recombination event, in which DNA encoding the antigen-binding domain (Fab) is paired with a non antigen-binding (Fc) region. The resultant IgE is antigen specific.

1.3.9 Class Switching and IgE Upregulation

The result of the upregulation of IgE synthesis, and IgE class switching, is the production of large quantities of IgE with antigenic specificity. This antigen-specific IgE is adsorbed onto the surface of mast cells, from where it can respond to the appropriate antigen and initiate the allergic cascade.

1.3.10 Clonal Selection and Expansion

Individual lymphocytes are genetically programmed to recognise a particular antigen. As a result, lymphocytes recognising one antigen constitute a minute fraction of the lymphocyte population. Researchers had difficulty envisaging how such a small quantity of cells could generate an adequate immune response. The answer was revealed to be clonal selection. This involves the proliferation of the cells following their recognition of a specific antigen, thereby amplifying the response.

1.3.11 Interaction of IgE with Cells

The powerful action of IgE in Type One Hypersensitivity was found to be dependent on the high affinity interaction of IgE with host cells. The most important receptor in the Type One Hypersensitivity reaction was the high affinity receptor, FcεRI.

1.3.12 IgE-Specific Receptors - High and Low Affinity

The first evidence of the existence of an IgE-specific receptor came from a series of experiments with leukocyte preparations. The leukocytes were purified to partial purity and were bathed in a serum suspension containing so-called 'antigen-responsive agents' (reagins) (VanArsdel & Sells, 1963). It was determined that the leukocytes must have the capacity to 'extract' the reagins from the serum, although the mechanism by which this was achieved was not clear. Clearly, with the re-classification of 'reagins' as IgE molecules it was apparent that leukocytes had the capacity to remove IgE from serum suspensions.

As greater separation of the individual cell types from the partially pure leukocyte suspension was achieved, basophils were identified as the cell with the reagin-extracting capability (Ishizaka *et al.*, 1970). The similarities between basophils and mast cells led researchers to believe that mast cell also had the capacity to adsorb IgE onto their surface.

Two types of receptors have been identified: FcεRI (high affinity) and II (low affinity). Only the high affinity form has been demonstrated to play a direct role in the activation of mast cells and basophils.

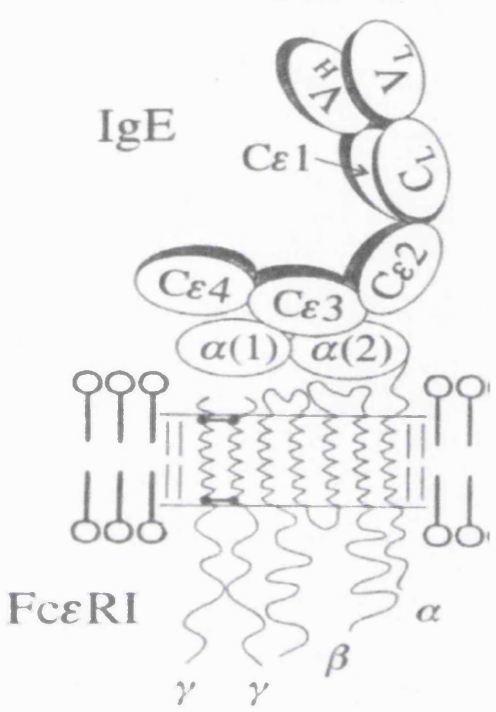
1.3.13 Structural Characteristics of High Affinity IgE Receptor - FcεRI

The FcεRI receptor is a member of the immunoglobulin superfamily, a group that includes the B and T cell receptors and several receptors with affinity to IgG (Kinet, 1989). These receptors have a number of characteristics in common. Structurally, they all contain immunoglobulin-type domains in the α chain and conserved immuno-receptor tyrosine-based activation motifs (ITAMs) in the cytoplasmic tails of their β and γ chains (Cambier, 1995(b)). The ITAM consists of six conserved amino acids; the first two are negatively charged aspartic acid or glutamic acid, the third and fifth amino acids are tyrosine, and the fourth and sixth are leucine or isoleucine (Reth, 1989). The significance of the ITAM in mast cell degranulation will be discussed in more detail in Section 1.3.20.

The FcεRI receptor is a complex structure, which is schematically illustrated in Figure 1.5. Analysis indicates that it is typically composed of four units, termed the αβγ2 tetramer. The alpha (α) chain consists of two extracellular domains, one intracellular domain and a short cytoplasmic tail (Ra *et al.*, 1989). It is surface-located, heavily glycosylated, and contains the site responsible for binding to the IgE. The beta (β) chain contains four transmembrane domains and is involved with amplification of the signal (Dombrowicz *et al.*, 1998). The two disulphide-linked gamma (γ) chains each contain a transmembrane domain and a cytoplasmic

FIGURE 1.5

SCHEMATIC REPRESENTATION OF FcεR1 AND ITS INTERACTION WITH IgE



(Taken from Sutton & Gould, 1993)

Figure Legend

The FcεR1 receptor is typically composed of 4 units, termed the αβγ2 tetramer. The alpha (α) chain consists of two extracellular antigen-binding domains, one intracellular domain and a short cytoplasmic tail. The beta (β) chain contains four transmembrane domains, and the two disulphide-linked gamma (γ) chains each contain a transmembrane domain and a cytoplasmic tail. The cytoplasmic γ chains are associated with the signalling machinery internal to the cell

tail. The γ chains are associated with the signalling machinery internal to the cell (Bach *et al.*, 1973). The association of the receptor chains with the intracellular signalling machinery is believed to occur through the ITAMs of the γ chain, which act as phospho-acceptors, presumably controlling intracellular phosphorylation.

In addition, it has recently been shown that FcεRI can also exist as an αγ3 trimer in humans (Garman *et al.*, 1998). As this trimer still possesses the α binding unit and γ signalling units, its biologic function should not be impaired

The importance of the FcεR receptor in the allergic cascade was first revealed from comparative studies of FcεRI-deficient mice strains with wild type strains. The genetically deficient mice were generated through gene targeting of either the α or the β chain. Whilst the wild-type mice displayed local or systemic anaphylactic responses from the injection of antigen-specific IgE followed by injection with the specific antigen, no such responses were observed in the mutant mice strains (Dombrowicz *et al.*, 1993).

1.3.14 Relationship between Serum IgE Levels and FcεRI Expression

Experimentally, it was shown that the surface expression of FcεRI on mast cells and basophils is dependent on the serum concentrations of IgE (Yamaguchi *et al.*, 1997). The connection was initially made when it was found that genetically IgE-deficient mice had dramatically lower levels, over five times lower, of the high affinity receptor. Subsequently, experiments were performed with mouse umbilical cord mononuclear cells, which had been stimulated to develop into mast cells. Prior to differentiation, the cells were incubated with variable levels of IgE and the receptor expression was determined, both by flow cytometry and western blot analysis. The results showed a direct relationship between the IgE concentration and the surface expression of the receptors. Similar results were subsequently generated with mature mast cells, indicating that this was not a phenomenon solely associated with an immature phenotype.

Examination of the regulation of FcεRI expression suggested that the surface expression of FcεRI expression is directly related to the interaction between IgE and FcεRI (MacGlashan *et al.*, 1999). A mutant IgE fragment was created (R334S), which had a significantly reduced affinity to FcεRI (Henry *et al.*, 1997). Upregulation of FcεRI expression required up to thirty times more of the mutant IgE, compared with the wild-type, to effect the same level of FcεRI expression on basophils. This relationship is likely to be dependent partially the on the increased stability of occupied receptors, compared with the un-occupied receptors, which effectively reduces their degradation (Turner & Kinet, 1999).

An additional finding from this study was that the increased levels of receptor expression, in response to increased serum IgE concentrations, were associated with an enhancement of IgE-mediated cell degranulation. The levels of antigen required for stimulation were reduced, accompanied by increased quantities of mediators secreted from the mast cells. The conclusion was drawn that any mechanism that results in the substantial elevation of serum IgE levels may also result in significantly elevated IgE-dependent effector cell function (Yamaguchi *et al.*, 1997).

1.3.15 Interaction of IgE with the FcεRI Receptor

The optimal conditions for the interaction of IgE with recipient cells were studied in detail, both through the use of mammalian tissue sections (Feigen *et al.*, 1962), and cell suspensions (Levy & Osler, 1966). It was determined that the interaction of IgE with the high affinity receptor was time, temperature and pH-dependent. This led to the conclusion that the process was not simply a physical adsorption of the antibody onto an inert cell surface.

More recently, the precise kinetic characteristics of the receptor have been determined. FcεRI has a high binding affinity for the IgE molecule (Ka = 10^9 to 10^{10} M in RBL cells), which appears to protect the IgE from destruction by serum proteases (Isersky *et al.*, 1979). Therefore, although the half-life of serum IgE is only a few days (Ogawa *et al.*, 1971), mast cells can remain sensitised by IgE for many weeks. The interaction of IgE with its receptor was shown to be both reversible (Ishizaka & Ishizaka, 1974), and highly specific, with the FcεRI receptors showing no capacity to interact with other subclasses of immunoglobulins (Kulczycki *et al.*, 1974(b)).

Advances in technology permitted detailed mapping of the interaction of IgE with the FcεRI receptor. Mutational analysis showed that the third Ig constant domain (Cε3) of IgE contains the contact point between antibody and ligand (Henry et al., 1997). A number of mutations were created in both the N-terminal 'linker' region and the Cε3 domains of IgE, and the binding of these mutants to FcεRI was studied. Through the use of these mutants, the arginine residue at position 334 was identified as a contact residue in the IgE structure. In addition, X-ray crystallography of the IgE molecule revealed that the postulated symmetry of the IgE molecule is perturbed by a bend in the linker region between the Cε2 and Cε3 domains. The unusual conformation of IgE dictates that FcεRI can only bind to one chain of the IgE structure (Keown *et al.*, 1998).

Crystallisation analysis of FcεRI (Garman *et al.*, 1998) revealed that, within the α unit, the two domains are positioned at an acute angle relative to one another. The resulting structure is a convex surface at the top of the molecule and a marked cleft enclosed by the two globular immunoglobulin domains, D1 and D2 (see Figure 1.6). It is almost certain that four tryptophan residues at the top surface of this structure form a hydrophobic patch, which is the putative contact point between FcεRI and the IgE molecule.

1.3.16 Low Affinity IgE Receptor; FcεRII

The low affinity form FcεRII has a significantly lower affinity for IgE than the high affinity receptor (Ka $=10^6$ to 10^7 M) (Metzger *et al.*, 1983). Its identity was confirmed to be identical to an antigen associated with B cell differentiation, called CD23. The primary role of CD23, which is located on the surface of B cells, is believed to be as a regulator in the upregulation of IgE production by B cells. The full extent of its physiological role remains to be elucidated (Capron *et al.*, 1986). Aside from their ability to bind IgE, the high and low affinity receptors share little in common. It is known that CD23 does not directly participate in mediating the degranulation of mast cells.

1.3.17 FcεRI Dependent Mast Cell Degranulation

1.3.17.1 Antigen recognition by IgE, and bridging of FcεRI

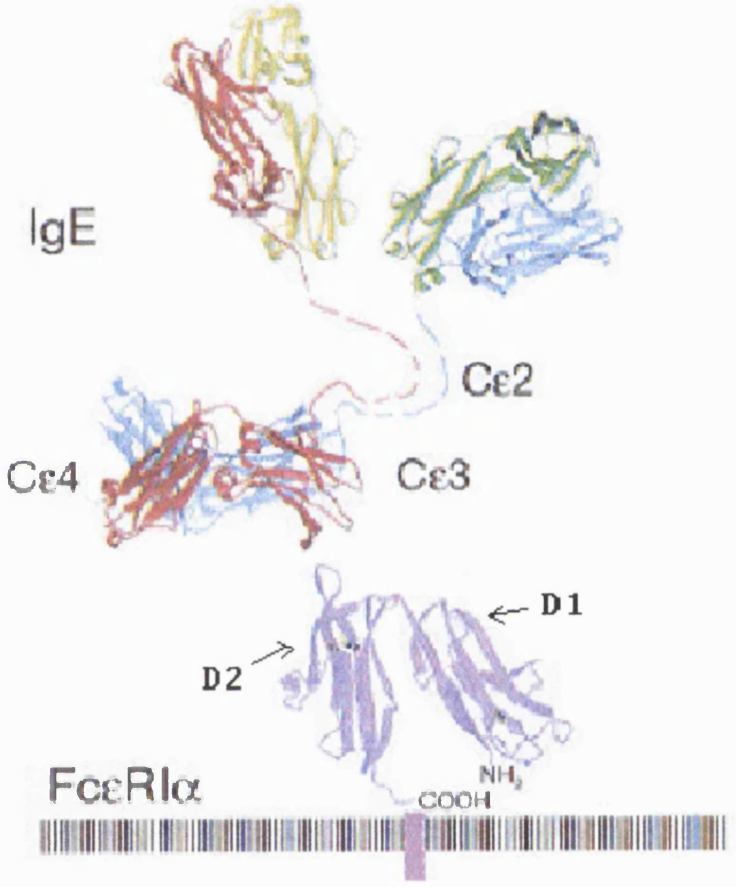
The recognition of antigen by IgE, or IgE with FcεRI, though necessary, is not sufficient to induce an allergic reaction (Feigen *et al.*, 1962). In order for the immune system to adequately attack the foreign object, it is essential that the binding event be translated into a signal that is interpreted by the host cell as an initiation to an immunological event.

However, the activation of mast cells and basophils by IgE/antigen complexes is considerably more complex than the binding of IgE to the cell surface, followed by IgE cross-linking. This results from the requirement of mast cells to remain quiescent, despite the binding of IgE to FcεRI, but to become activated when the IgE binds to an 'appropriate' antigen. It is the act of allergen recognition by IgE, rather than the binding of IgE to its receptor, that is physiologically relevant in the activation and degranulation of mast cells.

It was originally believed that cross-linking of the IgE molecules, with the resultant cross-linking of the FcεRI receptors, provided the initiating event for the degranulation of mast cells. Physiologically, cross-linking of the IgE molecules can be achieved by antigens and carbohydrate-binding agents such as lectins (Keller *et al.*, 1973).

FIGURE 1.6

SCHEMATIC OF IgE INTERACTION WITH FcεR1



(Taken from Turner & Kinet, 1999)

Figure Legend

The interaction between IgE and its receptor occurs through the Cε3 domain on IgE and the α chains of FcεR1. The two extracellular α domains form a convex surface at the top of the receptor, and a marked cleft enclosed by the two globular immunoglobulin domains, D1 and D2.

It has since been demonstrated that the direct bridging of FcεRI receptors is sufficient to stimulate degranulation. Initially, research was performed using antibodies directed against the mast cell membranes and, although the specific target of the antibodies was not clear, it was suggested that the bridging of a membrane component was sufficient. (Ishizaka & Ishizaka., 1977). The creation of more specific bridging-antibodies, directed against the FcεRI receptor, showed that it was possible to stimulate degranulation with unoccupied receptors (Ishizaka *et al.*, 1978; Isersky *et al.*, 1978). The main condition for degranulation was that the antibody caused the bridging of the receptors. Evidence exists that monovalent antigen, which can only bind to a single IgE/FcεRI unit, has the capacity to activate the cell. However, monovalent antigen rapidly dissociates from the IgE molecule, thereby terminating the activation of the cell. This supports the hypothesis that only through receptor clustering will the cell be completely activated (Metzger *et al.*, 1994).

Since bridging of cell-bound IgE molecules brings receptor molecules into close proximity, local changes in membrane structure are almost certainly responsible for the activation of the enzyme sequence that leads to histamine release (Ishizaka *et al.*, 1978).

The α unit of the FcεRI is highly glycosylated and this is believed to play a role in the interaction of the receptors following antigen cross-linking. *In vitro*, de-glycosylated FcεRα aggregates spontaneously, in the absence of antigen. It was concluded, therefore, that the glycosylation of potential α chain interaction surfaces prevents premature aggregation and permits interaction with the synthetic machinery. Binding of multivalent antigen, however, overcomes the intrinsic resistance of α chains to interaction (Letourneur *et al.*, 1995).

1.3.17.2 FcεRI-Independent Mast Cell Degranulation

The degranulation of mast cells can also be achieved by bypassing the FcεRI receptor. This can be accomplished, for example, by anaphylatoxins, C3a and C5a (Johnson *et al.*, 1975), basic substances, such as compound 48/80 (Enerback, 1966), ionophores (Pressman, 1976) or deuterium oxide (Gillespie *et al.*, 1972). These agents largely act independently from the IgE-mediated signalling systems and this type of degranulation will not be further discussed. There is one exception to this group of agents, which was further investigated for this thesis, the calcium ionophore, A23187 and its mode of action is discussed in Chapter 2.

1.3.18 Signal Transduction, Following Receptor Cross-linking

Signal transduction is concerned with the mechanism by which the surface events directly promote activation of the signalling machinery in the cell. Essentially, the process of signal

transduction, following cross-linking of the FcεRI receptors, is highly dependent on the activation of tyrosine kinases and a sequence of phosphorylation events. These events, in turn, are dependent on a panel of tyrosine kinase enzymes, including Lyn, Syk, Btk and Itk, which directly interact with the receptor subunits or which are independent. Activation of these tyrosine kinases most typically occurs through phosphorylation and their activation promotes the initiation of a number of signalling cascades, which will be discussed further in Section 1.3.19.

1.3.19 Role of Tyrosine Kinases in the Degranulation of Rat Mast Cells

The event following the antigenic cross-linking of the FcεRI receptor is the initiation of a chain of phosphate transfer events. These are schematically illustrated in Figures 1.7(a) and (b). The link of FcεRI with tyrosine kinases was demonstrated through experiments, which showed that the surface engagement of the FcεRI receptor promoted the phosphorylation of several distinct proteins (Benhamou *et al.*, 1990). The phosphorylation was rapid, correlated with the antigen dose and was detectable one minute after stimulation.

The identity of the tyrosine kinases responsible for the phosphorylation was demonstrated by study of two mast cell lines, RBL-2H3 from rat, and PT18 from mouse (Eiseman & Bolen, 1992). These cell lines were stimulated to degranulate by two methods. The first involved passive sensitisation of the cells with anti-DNP IgE and cross-linking of the IgE molecules with the DNP antigen. The second method involved direct cross-linking of receptor components, thereby removing the need for the IgE passive sensitisation. The tyrosine-mediated phosphorylation of proteins was determined by anti-phospho-tyrosine immunoblotting procedures.

Results from both cell lines showed that a tyrosine kinase was associated in a 'receptor complex', with the FcεRI receptor. The activity of this kinase was enhanced following stimulation of the mast cells by either method of activation. The kinetics of the activation were, however, slightly different between the different methods and between the different cell lines. Identification of the kinase was possible through co-precipitation of the receptor complex and peptide mapping analysis. In the RBL-2H3 and PT18 cell lines, the tyrosine kinase was identified as p56-Lyn and p62-cYes respectively.

1.3.20 Tyrosine Kinases and the FcεRI Receptor

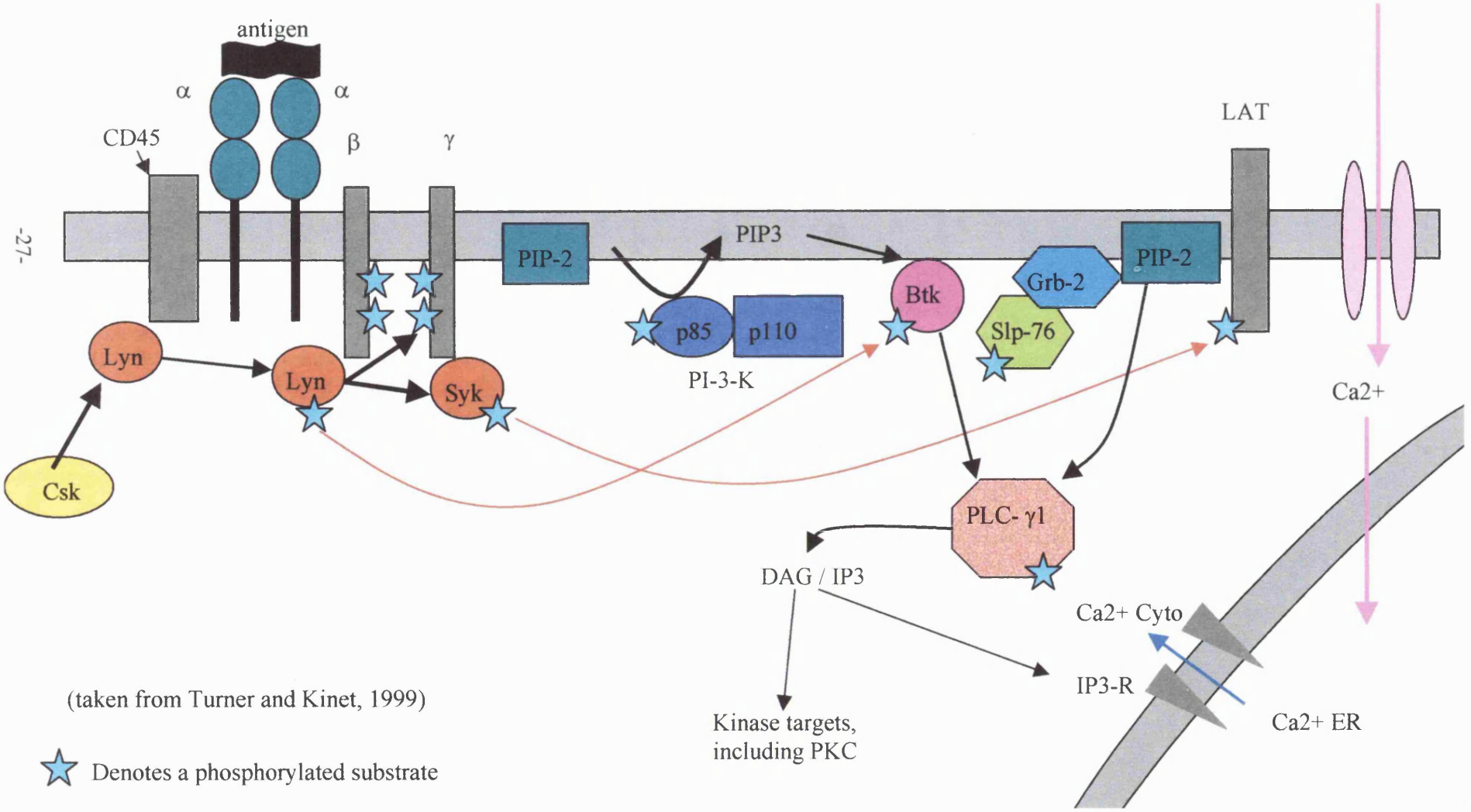
As discussed previously, the β- and γ-chains of the FcεRI receptor complex contain ITAMs. The ITAM sequence was known to be found primarily in those receptors involved in signal transduction. In addition, the cytoplasmic location of the motif suggested a role in

Figure Legend

Briefly, the cascade is initiated at the cell membrane, upon the antigenic cross-linking of the FcεR1 receptor. FcεR1 is associated with tyrosine kinases, including Lyn and Syk, which are activated by tyrosine phosphorylation, involving accessory proteins. Down-stream, the activation of Btk is accomplished through the binding of a PIP-3, which is derived from the PI-3-K-mediated phosphorylation of PIP-2. Btk, in turn activates phospholipase Cγ1 (PLCγ1), with the assistance of LAT, GRB-2 and Slp-76 accessory proteins. PLCγ1 catalyses the breakdown of membrane phospholipids to generate IP_3, which is responsible for calcium release from intracellular stores, and DAG, which promotes the activation of protein kinase C.

FIGURE 1.7A

IgE / FcεR1 MEDIATED MAST CELL SIGNALLING (EARLY AND MIDDLE EVENTS)



(taken from Turner and Kinet, 1999)

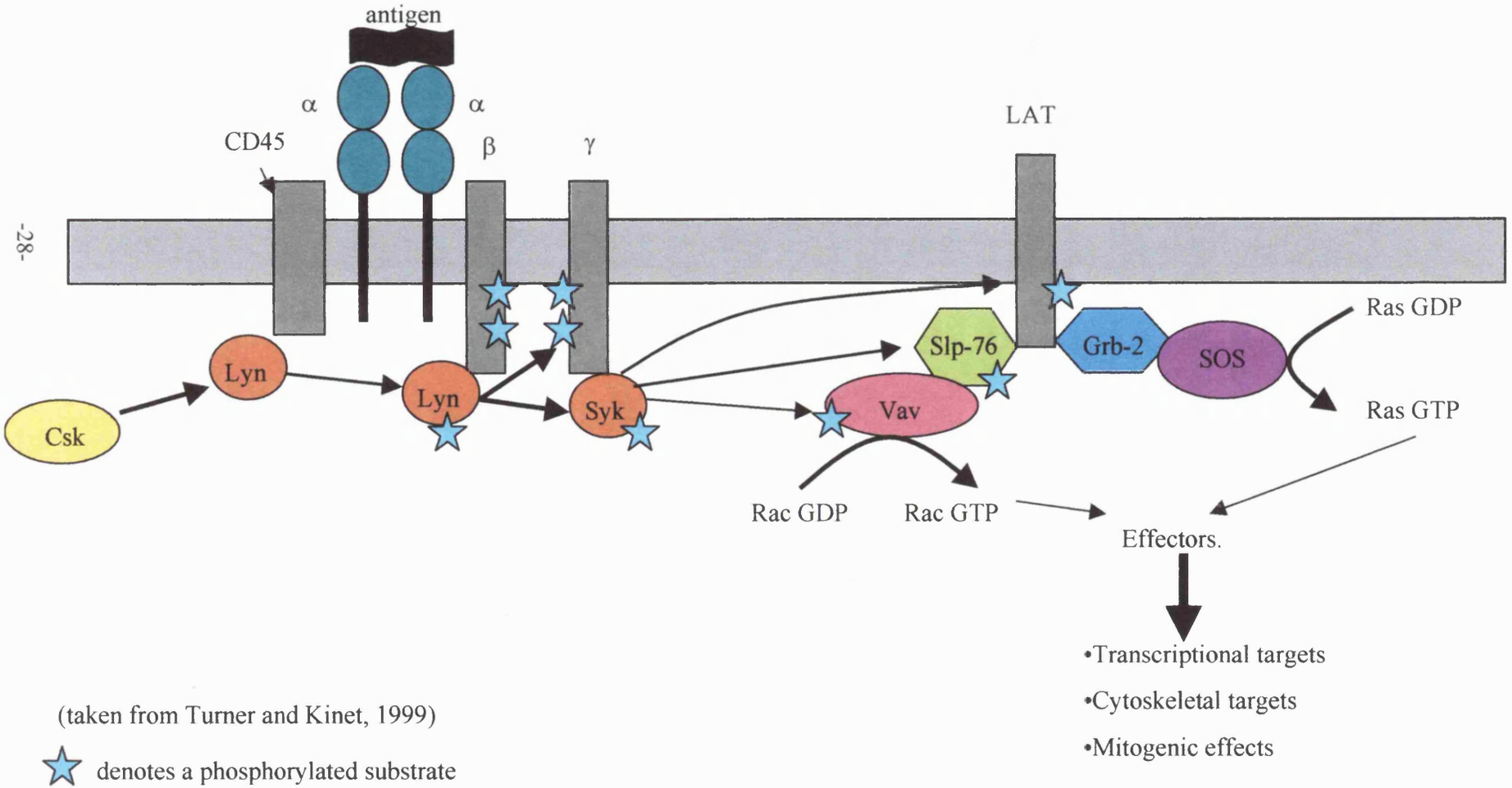
Denotes a phosphorylated substrate

Figure Legend

The late events in the signalling cascade are dependent on the receptor-mediated activation of Lyn and Syk. These phosphorylated enzymes promote the activation of two pathways, both dependent on LAT molecules. The first involves Grb-2 in combination with the SOS adaptor protein, which activates the Ras-GTP pathway. The second involves Slp- 76, which recruits and activates Vav, a guanine nucleotide exchange factor. Vav / SLP-76 is responsible for the activation of the Rac-GTP system. Both Rac and Ras-GTP are responsible for the activation of a number of downstream signalling cascades, including other GTPase pathways, PI-3-kinase, and the Raf/MEK/ERK cascade. These influence transcriptional targets, which induce the expression of various genes, and ultimately, the degranulation of the cell.

FIGURE 1.7B

IgE / FcεR1 MEDIATED MAST CELL SIGNALLING (LATE EVENTS)



(taken from Turner and Kinet, 1999)

denotes a phosphorylated substrate

intracytoplasmic signalling (Cambier, 1995). A number of studies were performed, including mutagenesis, to ascertain the role of the ITAM motif. It was demonstrated that its sequence contained all structural information necessary for the signal transduction leading to protein tyrosine phosphorylation, calcium mobilisation and cytolysis. Of critical importance in this function were the two tyrosine residues, although other residues contained within the sequence almost certainly contribute to the efficacy of the ITAM's function (Romeo *et al.* 1992).

The tyrosine residues are phospho-acceptor sites, responsible for the action of receptor-associated protein tyrosine kinases (PTKs) (Vonakis *et al.*, 1997). As such, the phospho-ITAM complexes provide a docking site for cytoplasmic proteins that contain the Src-homology-2 (SH2) domain, since the SH2 domain has high affinity for phosphorylated tyrosine residues. It is this association between the receptor units and the PTKs that provides the link between the receptor and subsequent signal transduction cascades.

1.3.21 Receptor Kinases; Lyn and Syk

The β and γ chain ITAM sequences have slight structural differences between them, which are reflected in the kinases for which they have the greatest affinity. There are two principal species of FcεRI-associated PTK, namely the *src*-type kinase p56-Lyn and the p72-Syk kinase (Jouvin *et al.*, 1994). Lyn is found associated with FcεRIβ and, although p72-Syk has the capacity to bind both β and γ, it has higher affinity for the γ unit (Turner and Kinet, 1999). In the context of intracellular signalling, it is clear that both β and γ are essential. It is important to note that Lyn is naturally bound to the receptor under resting conditions, almost certainly as a result of its myristoylated state that constitutively targets the enzyme to the membrane. This myristoylation almost certainly also controls the activity of the kinase, rendering it inactive until required.

1.3.22 Activation of Lyn Kinase

Following the bridging of the FcεRI receptor, Lyn is activated, although precisely how this is achieved isn't entirely clear. It is believed that Csk and CD45 regulate the activation process of src family kinases, at least in certain cell types. Lyn contains an autoregulatory c-terminal tyrosine that can be phosphorylated by Csk kinase. Following phosphorylation, Lyn binds to its own SH2 domain, thereby forming an internal loop that is keeping the kinase inactive. Activation is achieved through the de-phosphorylation of this regulatory tyrosine, by CD45 phosphatase (Trowbridge & Thomas, 1994).

This model of activation is supported in several studies. For example, in human basophils, antibodies against CD45 inhibited FcεRI mediated histamine release (Hook *et al.*, 1991).

These antibodies, called HB 9AB6 and HB 10AB2, were identified as being anti-CD45 in specificity through binding studies and specific immunoprecipitation. The use of the CD-45 deficient Jurkat cell line provided additional evidence, as cross-linking of FcεRI on these cells resulted in diminished phosphorylation of FcRβ and FcRγ. Both functions were restored after reconstitution with a chimeric molecule containing the phosphatase domain of CD45 (Adamczewski *et al.*, 1995). A further experiment, using NIH 3T3 cells (that lack expression of CD45) provided less convincing evidence. Clustering of FcεRI resulted in Lyn-dependent phosphorylation of FcRβ and FcRγ, suggesting that CD45 is not required for Lyn activation (Scharenberg *et al.*, 1995). It is possible that a different phosphatase plays the role of CD45 in NIH 3T3 cells.

It is understood that Lyn may undergo intermediary stages, both inactive and active, and that it must undergo auto-phosphorylation in order to achieve full activity. The catalytic activity of Lyn is directed towards the β- and γ-chain ITAMs and an important feature of the Lyn kinase is that it has the capacity to transphosphorylate ITAMs in other receptor complexes (Pribluda *et al.*, 1994). In this case, it can trans-phosphorylate Syk kinase, on tyrosine-518.

1.3.23 Amplification Role of Lyn Kinase

The Lyn kinase is also associated with the role of the FcεRIβ chain of the receptor, which was shown to be responsible for the amplification of the activation signal. Through quantitative determination of the activation of the Syk kinase, and calcium mobilisation, the relative contribution of the FcεRIβ chain was measured. It was determined that the β chain amplified the activity of the FcεRI receptor between five and seven fold and the amplification involved enhanced recruitment of the Lyn kinase and an increase in the phosphorylation of FcεRI-γ (Lin *et al.*, 1996).

1.3.24 Recruitment and Activation of Syk Kinase

Following activation of Lyn, Syk is recruited to the FcεRI receptor γ chain. The significant role of Syk kinase in mast cell degranulation was illustrated in a variant of the RBL-2H3 cell line, TB1A2, in which Syk was not detected, either by immunoblotting and *in vitro* kinase reactions. Cross-linking of the FcεRI receptor in these mutant cells failed to stimulate cell degranulation, and no intracellular phosphorylation was detected. Experiments were performed to establish the expression of Syk in these TB1A2 cells and it was shown that following transfection, the cells responded to FcεRI-cross-linking, undergoing both phosphorylation and degranulation (Zhang *et al.*, 1996).

Evidence exists which supports the theory that Lyn can undergo auto-phosphorylation (El-Hillal *et al.*, 1997). Experiments using 3T3E cells (NIH 3T3 cells transfected with the complete FcεRI receptor which essentially undergo the same early tyrosine-kinase activation events observed in antigen-stimulated RBL-2H3 cells) used anti-phosphotyrosine immunoblotting to reveal the pattern of phosphorylation of Syk after stimulation. It was revealed that the activation of Syk is accomplished through phosphorylation, both by Lyn and by other activated Syk units.

This pattern of auto-phosphorylation helps to explain the exponential degranulation response of the mast cell, following antigen-mediated receptor cross-linking. Essentially, once an activated Syk unit is introduced into a population of non-activated Syk units, phosphorylation events ensure that the inactive units are rapidly converted into active units. Hence, even a weakly active antigenic binding can stimulate a rapid and vigorous cellular degranulation response. Once activated, Syk is able to interact with its targets, one of which is phospholipase Cγ1.

1.3.25 Tec Family Tyrosine Kinases

Two further tyrosine kinases have been shown to be involved in the signal transduction cascades and these are members of the Tec family of non-receptor tyrosine kinases, namely Itk and Btk (Bruton's tyrosine kinase). Members of this family are distinguished by the presence of a large, unique, terminal amino acid sequence containing a pleckstrin homology (PH) domain and single SH2 and SH3 domains. In addition, an auto-regulatory C-terminal tyrosine is absent (Desiderio & Siliciano, 1994).

PH domains were identified by their similarities with pleckstrin, a major protein kinase C substrate found in platelets. They consist of approximately 120 amino acids, and are found in several biologically-important kinases. Analysis of the binding specificity of kinases to particular phospho-inositide proteins was performed and it was determined that Btk has a high affinity and specificity for PtdIns(3,4,5)P_3. This affinity is believed to be, at least partially, due to a cluster of basic residues contained within the β1-β2 region of the PH domain (Rameh *et al.*, 1997).

1.3.26 Btk Tyrosine Kinase

As discussed, the activation and membrane recruitment of Btk is accomplished through the binding of a phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3, or PIP-3) moiety, via its PH domain (Salim *et al.*, 1996). PtdIns(3,4,5)P_3 is normally absent from quiescent cells, and only appears in response to cell stimulation. In the case of Btk, the PtdIns(3,4,5)P_3 substrate is derived from pre-existing phosphatidylinositol-4,5-biphosphate (or PIP-2), which is

phosphorylated by the enzyme phosphatidylinositol-3-OH kinase (PI-3-K) (Carpenter & Cantley, 1996). The activation of PI-3-K, in turn, is believed to be reliant on Syk. PI-3-K is composed of a regulatory unit (P85) and a catalytic unit (P110). It is currently believed that Syk is responsible for the phosphorylation of the P85 unit which, in turn, recruits the P110 unit to the membrane, where it is active and in contact with its substrates. One of these products, as indicated above, is the PtdIns(3,4,5)P_3, which activates the Btk enzyme. Once activated, Btk becomes an accessible substrate for Lyn, which is responsible for the phosphorylation, on tyrosine 551, and the full activation of Btk (Scharenberg *et al.*, 1998).

1.3.27 Itk tyrosine kinase

A further Tec family kinase, Itk, is expressed in mast cells and is activated through FcεRI (Kawakami *et al.*, 1994). Experiments have shown that that there is functional redundancy between Btk and Itk (Hata *et al.*, 1998). Using cultured mast cells derived from Btk-deficient mice (btk-null), FcεRI-mediated cross-linking showed diminished, but not entirely eliminated, release of both histamine and cytokines, thus suggesting that an alternative activator may replace the Btk function. In addition, over-expression of Itk in Btk-deficient mast cells can partially restore the signalling defects.

1.3.28 Signal Translation and Amplification

Clearly, Btk and Itk are important in the activation and membrane recruitment of protein tyrosine kinases and in the subsequent intracellular signalling cascade.

Signal translation and amplification events are effected by the initial signals that enter the cell (signal transduction) and are translated into the up or down-regulation of signalling systems. The end result of up regulation of the mast cell is degranulation.

Essentially, following the activation of Lyn and Syk, a number of intracellular transduction cascades are initiated. The initiation of the cascade is reliant on the recruitment of signalling molecules to the activated receptor complex at the cell surface. The presence of enzymes, primarily the tyrosine kinases, promotes the post-translational phosphorylation/modification of the signalling molecules leading to their activation.

1.3.29 Role of Phospholipase Cγ1 in Signal Translation and Amplification

One of the primary players of FcεRI-mediated intracellular signalling is phospholipase Cγ1 (PLC-γ1). This enzyme catalyses the breakdown of membrane phospholipids to generate two second messengers, inositol-1,4,5-triphosphate (Ins(1,4,5)P_3) and diacylglycerol (DAG).

$Ins(1,4,5)P_3$ is responsible for calcium release from intracellular stores and DAG promotes the activation of various protein kinase C (PKC) isoforms (Turner & Kinet, 1999).

Following activation of the receptor, PLC-γl is recruited to the membrane and tyrosine-phosphorylated prior to activation. An analogous situation occurs in T cell signalling, which involves a protein called 'linker for activation of T cells' (LAT) adapter protein (Zhang *et al.*, 1998). In T cells, LAT is associated with ZAP-70, a kinase shown to be analogous to the Syk protein in mast cells. ZAP-70 is a highly tyrosine-phosphorylated protein and is found in association with both PLC-γ1 and the p85 unit of PI-3-K. In addition, phosphorylated LAT is responsible for the membrane recruitment of multiple signalling molecules.

The existence of a LAT protein in mast cells was recently confirmed by immuno-histochemistry (Fachetti *et al.*, 1999) and it was assumed that, as with T cells, the mast cell LAT was associated with Syk and was responsible for the membrane recruitment of PLC-γ1. Once within the membrane, PLC-γl undergoes phosphorylation, primarily by Syk, Itk or Btk. Once activated, the catalytic action of PLC-γ1 is directed towards membrane phospholipids generating the two second messengers, $Ins(1,4,5)P_3$ and DAG.

1.3.30 Elevation of Intracellular Free Calcium

The elevation of intracellular free calcium (Ca_i^{2+}) is an essential component of FcεRI-mediated mast cell degranulation and is directly related to the activation of phospholipase Cγ1 (PLC-γ1) and the generation of inositol-1,4,5-triphosphate ($Ins(1,4,5)P_3$).

$Ins(1,4,5)P_3$ binds to, and opens the Ins(1,4,5)P3 receptor calcium channels on the surface of intracellular calcium stores that are primarily located in the endoplasmic reticulum. The opening of these channels promotes the release of the stored calcium. This calcium, however is insufficient to sustain calcium signalling, and the cell is required to extract calcium from extracellular sources (Parekh *et al.*, 1997). This was, experimentally, proven by the addition of calcium chelators to cell preparations (which effectively remove calcium from the extracellular milieu) which inhibited mast cell degranulation.

It has been demonstrated that mast cells do not express voltage-operated or ligand-gated calcium channels. This would be the most typical entry point for calcium from the extracellular milieu. The influx of calcium from extracellular sources is believed to occur through specialised structures by a process called capacitative store entry (Putney & McKay, 1999). The calcium channels are defined by their opening in response to any store-depletion stimulus

and they operate by raising and sustaining calcium flow into the mast cell. The mechanism by which this influx is initiated remains to be elucidated.

1.3.31 The Role of Adaptor Proteins in Signal Translation and Amplification

In addition to the tyrosine kinases, an additional group of proteins, called adaptor proteins, play a significant role in the signalling cascade following FcεRI cross-linking. Adaptor proteins are composed of modular protein-protein interaction domains and lack intrinsic enzymatic activity. Inherently, their role in the FcεRI mediated signalling pathways is to facilitate the function of the key players and in this capacity they are essential. Of the adaptor proteins, Grb-2 has a major role in the activation of the GTPase pathways.

1.3.31.1 Grb-2 Adaptor

The Grb-2 adapter contains two SH3 (proline-directed) domains and one SH2 (phosphotyrosine-directed) domain. Grb-2 does not interact directly with the ITAMs within the FcεRI structure. Its role is to link their upstream phosphotyrosine unit with effector molecules, forming complexes within the SH3 domain. This association is mediated by either the Shc adapter or by the LAT phosphoprotein. In mast cells, several proteins can form complexes with the SH3 domain of GRB-2, although not all are involved in FcεRI-mediated signalling (Turner *et al.*, 1995). The two prominent Grb-2 effector molecules are Sos and Slp-76.

1.3.31.2 Sos and Slp-76

Sos is the mammalian homologue of the *Drosophila* "Son of Sevenless" protein (Turner *et al.*, 1995). It is a guanine nucleotide exchange factor that promotes GTP-loading, and hence activation of the GTPase Ras and its effector pathways. Ras, in turn, is involved with a number of downstream signalling cascades, including other GTPase pathways, PI-3-kinase, and the Raf/MEK/ERK cascade (Marshall, 1996).

Slp-76 is a critical effector in antigen-mediated mast cell signalling and undergoes rapid tyrosine phosphorylation upon FcεRI aggregation (Hendricks-Taylor *et al.*, 1997). In mast cells, one function of Slp-76 is the recruitment of Vav, a guanine nucleotide exchange factor. Upon cell stimulation, Vav is tyrosine-phosphorylated and associates with a number of signalling proteins, including Slp-76. Phosphorylated Vav catalyses the GDP/GTP exchange and subsequent activation of the GTPase, Rac-1 (Crespo *et al.*, 1997). Thus, the involvement of Slp-76 in mast cell signalling is largely through its activation of further GTP-regulated pathways through Vav (Teramoto *et al.*, 1997).

1.3.32 GTP-binding Regulatory Proteins in Signal Translation and Amplification

The GTP-binding regulatory protein that is involved in enzyme activation is called the stimulatory G protein (Gs). The function of a Gs depends critically on its subunit structure. The classical Gs is composed of three polypeptides; an α *chain* (Gsα), which binds and hydrolyses GTP, and a dimer, consisting of a β *chain* and a γ *chain* (Gβγ), which anchors the Gs to the cytoplasmic face of the plasma membrane.

In its inactive form, Gs exists as a trimer, with GDP bound to the α unit. When activated, the guanyl-nucleotide-binding site on Gsα is altered, allowing GTP to bind in place of GDP. The binding of GTP is thought to cause Gsα to dissociate from Gβγ, allowing Gsα to bind tightly to an adenylate cyclase molecule, which is thus activated to produce cyclic AMP.

Within less than a minute, the Gsα hydrolyses its bound GTP to GDP, causing Gsα to dissociate from the adenylate cyclase (which thereby becomes inactive) and re-associate with Gβγ, to reform an inactive Gs molecule. It is well established that GTPase has available a wide range of effector molecules, each of which in turn regulates complex networks of signalling pathways such as the mitogen-activated protein (MAP) kinase cascades.

1.3.33 GTP-binding Regulatory Proteins in FcεRI Mediated Signalling

The GTP-proteins with greatest significance in IgE / FcεRI-mediated signalling are Ras, Rac and Rho.

1.3.33.1 Ras

To date, the structure of Ras is not entirely understood. It is known, however, to consist of a single polypeptide chain which confirms that it does not represent classical G proteins. The activation of Ras-type GTPases is a feature of FcεRI-mediated activation of mast cells (Turner & Cantrell, 1997).

In addition to the release of inflammatory mediators from cytoplasmic granules, there is a significant nuclear component to the activation of mast cells following FcεRI-mediated cell activation. Expression of various genes is induced, leading to *de novo* synthesis of cytokines, such as IL-4, GM-CSF, and TNFα. Experimental data places the GTPases Ras and Rac-1 in a critical position in the regulation of the nuclear component of mast cell end function. Ras mediates its diverse effects via multiple effector pathways, described below:

1.3.33.2 Ras / Raf-1 / MEK / ERK Cascade

Raf-1 is recruited to the plasma membrane by active Ras-GTP. This recruitment results in the activation of Raf-1 and subsequent activation of the mitogen-activated protein (MAP) kinases, Erk1 and Erk2 by the Erk-activating kinases. Activation of the Ras/Raf-1/MEK/ ERK cascade is necessary and sufficient for FcεRI induction of the transcription factor, Elk-1. Elk-1 is an immediate early gene regulator, which is dependent on phosphorylation of its C terminus prior to activation (Whitmarsh *et al.*, 1995). In FcεRI-mediated signalling, this phosphorylation absolutely requires MEK, acting down-stream from Ras.

1.3.33.3 NFAT

Ras function is required, but not sufficient, for activation of 'nuclear factor of activated T cells' (NFAT), by a mechanism distinct from the Raf-1 / MEK pathway. Upon stimulation, cytoplasmic NFAT members translocate to the nuclei and form regulatory complexes with cytokine gene promoters including Fos and Jun (Rao, 1994). Although the mechanisms are not understood, the activation of NFAT implicates Ras in the transcriptional activation of the genes for IL-4, GM-CSF and TNF.

1.3.33.4 Rac

In addition to Ras and Raf, the Rac-1 GTPase is a critical component of the regulation of NFAT (Turner & Cantrell, 1997). Expression of active Rac-1 protein dramatically potentiates FcεRI induction of NFAT, whereas expression of an inhibitory Rac-1 mutant severely abrogates the NFAT response to FcεRI stimulation. The mechanism for this regulation is currently unknown (Turner *et al.*, 1998), although evidence exists that Rac-1 can activate certain MAP kinase family members. Whether these kinases are involved in NFAT activation in mast cells remains to be established.

1.3.33.5 Rho

There is a consensus that Ras responses are coupled to signalling networks mediated by Rho family GTPases, such as Rac-1, CDC42 and RhoA (Qiu *et al.*, 1995).

The role of these Rho-family GTPases in antigen receptor responses is not well characterised, but there is an increasing awareness, from biochemical and genetic analyses, that these molecules will have important functions in the immune system.

1.3.33.6 Protein Kinase C

Diacylglycerol (DAG), released by the breakdown of membrane phospholipids by phospholipase Cγ1, has two potential signaling roles. Firstly, it can be cleaved, producing

arachidonic acid, which can be used in the synthesis of prostaglandins and leukotrienes (as discussed in Section 1.4.2).

Secondly, DAG can activate protein kinase C (PKC). PKC is a calcium-dependent, serine-threonine specific, protein kinase. DAG molecules associate with membrane-phosphatidylserine molecules, and these complexes bind to PKC. This results in an increased affinity of the enzyme for calcium, inducing transient activation of the enzyme. The activated PKC enzyme promotes the phosphorylation of number of proteins within the target cell. Within seconds, the diacylglycerol is phosphorylated to form phosphatidate, or further cleaved to arachidonic acid.

Following activation, PKC transfers the terminal phosphate group from ATP to specific serine or threonine residues on target proteins, which vary depending on the cell. In the mast cell, PKC, in combination with calcium, induces degranulation in mast cells and it is known that mast cells contain the α, β, δ, ε and ζ isoforms of PKC (Kawakimi *et al.*, 2000). However, the precise targets for the calcium/PKC signals within the secretory machinery of mast cells are not known.

1.3.33.7 Phospholipase A2

MAP-kinase is responsible for the activation of cytoplasmic phospholipase A2 (PLA-2), through phosphorylation of a serine residue. Following activation, PLA-2 is translocated to the membrane. Both PKC and PLA-2 are involved in the release of arachidonic acid-derived mediators (Section 1.4.2) (Kawakimi *et al.*, 2000).

1.4 Secretion of Granule Components

The direct result of the sequential steps of mast cell activation is the release of mediators, from the cell into the extracellular milieu. Immuno-histochemistry and sub-cellular fraction techniques were employed to determine the mediators (Schwartz *et al.*, 1980). These were broadly grouped into three types, pre-formed, newly synthesised and cytokines. The diverse array and role(s) of these mediators in the allergic reaction is shown in Table 1.2.

1.4.1 Pre-formed Mediators

The secretory granule of the human mast cells contains a crystalline complex of pre-formed inflammatory mediators, ionically bound to a matrix of proteoglycan. The recognition that the mast cell secretory granule contains acid hydrolases, implicates this organelle as a modified primary lysosome. The existence of the modified lysosome suggests distinct intracellular and extracellular functions for the mast cell granule (Schwartz *et al.*, 1980). The principal

TABLE 1.2

MEDIATORS RELEASED FROM MAST CELLS UPON DEGRANULATION, AND THEIR FUNCTIONS

Substance	Type of Mediator	Function
Histamine	Preformed	Increases vascular permeability, vasodilation, smooth muscle contraction of bronchi, chemokinesis
N-acetyl-β-glucosaminidase	Preformed	Function unknown
Heparin	Preformed	Anti-coagulant and anti-complementary activity
Trypsin-chymotrypsin	Preformed	Protease, proinflammatory
Eosinophil chemotactic factor	Preformed	Eosinophil and Neutrophil chemotaxis and deactivation
Neutrophil chemotactic factor	Preformed	Neutrophil chemotaxis and deactivation
Leukotrienes C_4 and D_4	Newly synthesised	Broncho-constrictor, and promotes mucous secretion in airways
Prostaglandin D_2	Newly synthesised	Many functions
IL-4	Cytokine	Promotes IgE synthesis, eosinophil chemotaxis
IL-13	Cytokine	Similar effects as IL-4
IL-5	Cytokine	Eosinophil recruitment, particularly implicated in asthma
TNF-α	Cytokine	Putative role in amplifying allergic reaction, through the activation of transcription factors.
IL-3	Cytokine	Pro-inflammatory cytokines
IL-8	Cytokine	Involved in granulocyte chemotaxis and activation

(Schwartz et al, 1980)

component of the mast cell granule is heparin, which is a highly sulphated glycosaminoglycan, bearing a strong negative charge. In addition, the granule contains histamine, N-acetyl-β-D-glucosaminidase and peroxidase, as shown in Table 1.2.

When mast cell activation occurs, the granules swell and lose their crystalline nature. The mediator complex becomes solubilised and the individual mediators including histamine, protease and proteoglycans are expelled into the local extracellular environment by a process of compound exocytosis (Church *et al.*, 1997).

1.4.2 Newly Synthesised Mediators

The newly synthesised mediators are leukotrienes and prostaglandins, the most important of which are prostaglandin D2 (PGD-2) and leukotriene C4 (LCT-4). These mediators are generated by the activation of phospholipase A2 (PLA-2) an intracellular, calcium-dependent enzyme. The activation of PLA-2 induces the liberation of membrane-derived arachidonic, fatty acid. Arachidonic acid is oxidised, primarily by either cyclo-oxgenase (prostaglandin endo-peroxide synthase) to form PGD-2, or by 5-lipoxygenase to form LCT-4 (Church *et al.*, 1997). The production of both PGD-2 and LCT-4 involves the formation of intermediate molecules and the messengers are ultimately metabolised to inactive products.

1.4.3 Mast Cell Cytokines

Cytokines, generated by both resident and recruited cells, are responsible for the initiation and co-ordination of many local processes, including allergic inflammation and tissue remodelling. Although synthesis and secretion of all of these cytokines is upregulated after FcεRI-mediated cell activation, the presence of preformed cytokines suggests that they are available for rapid secretion upon cell stimulation. The most important cytokines that are generated by human mast cells are IL-3, IL-4, IL-5, IL-6, IL-8 and IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), TNF-α, and RANTES. The function of these cytokines is described in Table 1.2.

1.4.4 Ultrastructural Observations of Mast Cell Degranulation

Early in the investigation of degranulation of mast cells, it was determined that the process was not cytolytic in nature. That is, the process by which mast cells release their mediators into the extracellular milieu did not involve destruction of the cell membrane. The study of potassium efflux indicated that the degranulation process was an active secretory process that is not intrinsically injurious to the mast cell (Lichtenstein *et al.*, 1968).

The ultra-structural sequence of mast cell degranulation was studied in human lung mast cells, largely using transmission electron microscopy (TEM) techniques. In these studies, mast cells were stimulated to release by exposure to a number of different secretagogues and the process of granule mediator release was carefully monitored (Dvorak, 1998).

It was found that an orderly, and reproducible, sequence of ultra-structural changes occurred in the degranulation process. This process was termed piecemeal degranulation. The earliest event observed was a swelling of the granules, which resulted in a decrease in the density of granule matrix. This was followed by fusion of the swollen granules into chains, which then enlarged, becoming intra-cytoplasmic. The channels and, less commonly, individual swollen granules then fused with the plasma membrane. This permitted the release of the granule contents, including histamine, into the extra-cellular milieu. The openings at the cell surface became progressively wider, accompanied by increasingly prominent filaments in the intervening cytoplasm and by the convolution of the plasma membrane into folds and projections.

The study of the release of non-granule or newly formed mediators was less extensive and comparatively more difficult to follow (Dvorak *et al.*, 1984). Newly formed mediators are derived from arachidonic acid, the majority of which is localised in the lipid bodies of mast cells, as identified through tracing experiments using tritiated arachidonic acid and purified lung mast cells. Lipid bodies, as characterised by TEM, are morphologically roughly spherical, often located close to the nucleus and associated with cytoplasmic filaments. The lipid bodies were larger in diameter, and typically less numerous, than the cytoplasmic granules. It was observed that the lipid bodies did not degranulate in a similar sequence to the cytoplasmic granules. During the degranulation of mast cells, the number of lipid bodies did not appear to alter, no structures representing fused lipid bodies were observed and the morphology of the bodies was not seen to alter. In addition, only a very small percentage of the radiolabelled arachidonic acid was discharged to the extracellular environment. To date, the mechanism by which the lipid-derived mediators are transferred to the extracellular milieu remains to be elucidated (Dvorak, 1998).

1.4.5 The Role of the Cytoskeleton in Mast Cell Degranulation

Experimental observations support the view that a functional cytoskeleton is important for degranulation to occur. Agents that disaggregate microtubules, including colchinine and vinblastine, inhibited the release of histamine. By comparison, Deuterium oxide (D_20), which is known to stabilise microtubules, markedly enhanced the histamine release. It is believed that

microtubules are necessary for the actual secretion of histamine rather than for the initial antigen-induced activation of the process (Gillespie *et al.*, 1972).

Agents that disrupt microfilaments, such as cytochalasin B, do not promote degranulation on their own. They do, however, enhance FcεRI-mediated degranulation. Upon addition of latrunculin to FcεRI-stimulated mast cells, the inhibition of actin polymerization was associated with increased cell degranulation. Furthermore, latrunculin promoted an increase in the detectable phospholipase activity, increased tyrosine phosphorylation of Syk and increased tyrosine phosphorylation of the FcεRI receptor, by Lyn. These results indicated that the very earliest signaling events are enhanced, suggesting that actin microfilaments may interact, either directly or indirectly, with the receptor itself and that they may regulate the signaling process at the level of receptor phosphorylation (Frigeri & Apgar, 1999).

In addition, the role of intermediate filaments in mast cell degranulation was investigated. Permeabilised mast cells were stimulated with calcium, and intracellular effects were observed. Upon stimulation, protein kinase C was translocated from the cytosol to the membrane, promoting the phosphorylation of vimentin. Vimentin is a component of the intermediate filament system, and its phosphorylation induced the disruption of the intermediate filaments, resulting in the increased mobility of mast cell granules (Tasaka, 1994).

The precise role of the cytoskeleton in cell degranulation remains to be established. However, it was suggested that the cytoskeleton acts as a barrier between the plasma membrane and the granule membrane. Alterations in the distribution of the skeleton may facilitate the initiation of the fusion of the plasma membrane and granular membrane, ultimately leading to cell degranulation (Tasaka, 1994). In addition, the cytoskeleton probably provides a structural framework along which the enzyme-containing granules are moved to the cell membrane (Gillespie *et al.*, 1972).

1.4.6 Termination of the Allergic Signalling Cascade

Prevention of the allergic reaction from continuing indefinitely, by termination of the allergic cascade, is largely under the control of the FcεRI receptor. Activation of the FcεRI receptor persists only as long as the receptor is engaged and phosphorylated (Paolini *et al.*, 1991). Receptor-mediated signalling is terminated completely by receptor disengagement from multivalent antigen, indicating that rapid response mechanisms are in place to stop the induction of signalling pathways in the absence of continued stimulation.

However, although there is no clear evidence against the theory that dissociation of the antigen from the receptor is responsible for the deactivation, it is unlikely that this process is the primary controlling factor *in vivo*. This is because it is likely that the removal of antigen from its receptor is a slow process. A more plausible explanation is that the allergic cascade is controlled by the state of phosphorylation of the receptor, under the control of phosphatase enzymes. Specific phosphatase enzymes, likely to be involved, are the protein tyrosine phosphatases SHP1 and SHP2. Both these enzymes are associated with the FcεRI receptor, although SHP2 is only co-precipitated with the receptor upon activation. In addition, both phosphatases are phosphorylated upon receptor cross-linking (Kimura *et al.*, 1997). As with many of the processes of Type One Hypersensitivity, although the key players in the process of termination of the allergic cascade have been identified, the entire process is far from clear.

1.4.7 Conclusions

In conclusion, the activation of mast cells follows a complex sequence of events. Briefly, the cascade is initiated at the cell membrane, upon the cross-linking of the FcεRI receptor, which is intimately associated with tyrosine kinases. Some kinases, such as Lyn and Syk, are receptor associated, and interact with ITAM motifs within the receptor's protein sequence. Such kinases are activated by tyrosine phosphorylation. Others, such as Btk are non-receptor associated and are activated by interaction with phosphoinositides.

The activation of tyrosine kinases promotes the intracellular events, leading to amplification of the initial signal event. Key players in the intracellular signalling are phospholipase Cγ1, which is responsible for the production of the second messengers, Ins(1,4,5)P_3, which is responsible for increased intracellular calcium, and DAG, which activates PKC. In addition, adaptor protein complexes, such as Grb-2/Sos serve to up-regulate GTPase pathways and Raf/MEK/ERK cascade. The end result of this sequence of events is the full activation of the mast cells and their ultimate release of mediators into the external environment by the process known as degranulation.

1.5 Aims of the Research

The purpose of the research carried out for this doctoral thesis was to identify whether a novel pathway existed, involving the enzyme glycosylphosphatidylinositol-phospholipase D (GPI-PLD), or its products. The potential importance of GPI-PLD in the allergic cascade will be introduced in Chapter 3. If it can be proven that GPI-PLD occupies a central role in the allergic cascade, it may be possible to develop a specific therapeutic that could target either the enzyme, or its products, thereby preventing the downstream signalling events.

Chapter 2

The Rat Basophilic Leukaemia Cell Line (RBL-2H3) as an Experimental Mast Cell System

2.1 Summary

The aim of the research described in this Chapter was to characterise the RBL-2H3 mast cell line, which was used for the studies performed during this thesis. The RBL-2H3 cell line is considered to be analogous to human mast cells and was selected in preference to tissue mast cells. There are a number of complications with the use of tissue mast cells including difficulties with isolation, and heterogeneity in both their constitution and in their response to stimulation.

It was of importance to confirm that the RBL-2H3 cells used showed responses that were phenotypically similar to those previously confirmed in isolated RBL cell lines. In addition, the parameters of the IgE-mediated stimulation of the RBL-2H3 cell line required determination, specifically the optimal concentrations of the IgE and the cross-linking agent, the optimal time of incubation, and the quantification of the mediators released upon degranulation.

These parameters were studied and optimised. It was confirmed that the RBL-2H3 cell line employed in this thesis was phenotypically similar to the RBL-2H3 cell line previously characterised.

2.2 Introduction

2.2.1 Mast cell degranulation as an analogue of the Type One Hypersensitivity reaction

As outlined in the introductory Chapter, mast cells play a pivotal role in the initiation and perpetuation of the acute allergic response (Type One Hypersensitivity). Briefly, mast cell degranulation is dependent on the cross-linking of FcεR1-bound IgE molecules on the mast cell surface. To minimise indiscriminate degranulation of mast cells, it is essential that the binding of antigen by IgE / FcεR1 is recognised as a specific interaction, which is translated into an initiation signal. This signal feeds into a number of intracellular signalling systems and ultimately leads to the release of mast cell mediators into the extracellular milieu. Degranulation is a highly complex process, employing a number of enzymes and second messengers, including tyrosine kinases, protein kinase C, cellular oncogenes, phospholipid metabolites, calcium and cAMP.

2.2.2 *In vitro* study of Type One Hypersensitivity

The aim of the research performed for this thesis was to study the role of an enzyme, glycosylphosphatidylinositol-phospholipase D (GPI-PLD) in the process of mast cell degranulation. In order to perform these studies, it was necessary to establish a methodology for the degranulation of mast cells *in vitro*.

As indicated in the Introduction, which focussed mainly on the biochemistry of rat mast cells, the responses of mast cells are likely to be species specific. The research for this thesis aimed to discover the response of human mast cells and to elucidate its relevance to human allergic disease. Clearly, the *in vitro* experiments should optimally be performed using human mast cells.

For the production of mast cells for experimentation, a number of alternatives are currently available, including the extraction of tissue mast cells, culture of mast cells derived from bone marrow progenitor cells, or culture of a mast cell line. A rat mast cell line was selected in preference to human tissue mast cells, for reasons described below.

2.2.3 Purification of Mast cells from Tissues.

The selection of a rat mast cell line, RBL-2H3, was based largely on two observations that emanated from the study of human tissue mast cells. The first lies in the method of extraction of human mast cells from tissues and the second lies in the heterogeneity observed in the extracted mast cells.

The extraction of a specific cell type from a tissue mass presented extensive problems to researchers, since the conditions of extraction had to minimise cell damage and death. Extraction in an environment that closely resembles the tissue norm is essential, and extremes of pressure, pH and toxic chemicals must be avoided. In addition, the risk exists that the cells will undergo apoptosis in response to extraction procedures. All of these complicating factors needed to be addressed before a suitable method for cell extraction could be determined (Shortman *et al.*, 1972). An inherent problem in the study of tissue-derived mast cells resulted from their being present in low concentrations, typically 0.5 to 1% in most tissues. A refined methodology, for the extraction of human mast cells from tissues, developed and improved over time.

2.2.3.1 Mechanical Dispersion of Mast Cell-containing tissues

Initial extraction techniques employed mechanical dispersion of the target tissue, followed by separation of the resultant cell mass into a cell suspension, by cell sieving (Gould *et al.*, 1972). This resulted in complications in separating the mast cells from the other tissue cells and resulted in a mast cell suspension that was contaminated with a broad variety of cells. Any research data derived from the use of these cells had to consider the potential influences of the contaminating cell types on the process of degranulation.

2.2.3.2 Enzymatic Digestion and gradient-centrifugation

A better purification was developed through the use of enzymatic digestion, using combinations of pronase, chymopapain, collagenase or elastase. The digested tissue suspension was sieved through a metal grid to generate a more uniform cell suspension. The buffered cell suspension was then layered onto an isopycnic and isokinetic gradient, such as Ficoll-paque, and subjected to centrifugation. The cells that collected at the interface were enriched with mast cells, as determined by microscopy and histamine detection (Gould *et al.*, 1972).

Both techniques were initially employed for purification of lung tissue, and the mast cell-enriched preparations were contaminated with macrophages, lymphocytes, lung parenchymal cells and lung stromal cells (Lewis *et al.*, 1974). The behaviour of the extracted mast cells was studied, including their response to IgE-mediated stimulation and pharmacological agents, and the results were compared with those obtained from whole tissues. It was found that the extracted cells were functionally comparable with the whole tissue mast cells (Peters *et al.*, 1982). However, purity greater than 60% was difficult to achieve, and, with each successive step of the methodology, loss, alteration or mutation of the cells was observed.

2.2.3.3 Counter-current elutriation and affinity chromatography separation techniques

Mast cell purification was greatly improved by the addition of counter-current elutriation and affinity chromatography. Following enzymatic dispersion and gradient separation (Schulman *et al.*, 1982; MacGlashan *et al.*, 1982), the cell suspension is further purified by counter-current elutriation, a negative selection procedure that separates cells according to their size (Pretlow & Pretlow, 1975). This is followed by affinity chromatography, which takes advantage of unoccupied IgE receptors on the mast cell surface. These are primed with anti-penicillin IgE antibodies and the cells are then selectively adsorbed onto benzyl-penicilloyl (BPO)-Sepharose. Non-mast cells are removed by washing the column, and mast cells are selectively eluted from the column using an excess of N-ε- benzyl-penicilloyl-N-α-formyl-L-lysine. This methodology has permitted the purification of mast cell suspensions to almost 100% purity.

Sequential use of all these methods has resulted in mast cells of between 65 and 98% purity. The cells can be shown to be functionally intact by dye exclusion, survival in culture and response to pharmacological agonists. In addition, IgE-mediated simulation of the purified cells promoted the release of histamine and arachidonic acid metabolites, indicating that the cells are functionally responsive (MacGlashan *et al.*, 1982).

2.2.3.4 Drawbacks of the Mast Cell extraction procedure

The major drawback of method of mast cell purification is the volumes of lung tissue required for extraction. On average, a million mast cells are extracted per gram wet weight of tissue. The quantity of tissue that would be required for this thesis would be, therefore excessive and impractical. In addition, the batch to batch variation of each cell extraction may result in very small differences difficult to detect by conventional testing methodologies.

Furthermore, there was no data regarding the effect of storage on the extracted cells, and it was unclear how long the extracted cells would survive *in vitro*. As the tissue mast cells were not an adapted cell line, their ability to grow in long-term culture is likely to be limited. The protracted and laborious extraction technique would be necessary each time a cell experiment was performed, placing an enormous strain on time and resources. The use of a cell line would reduce this burden.

2.2.4 Heterogeneity of mast cells extracted from tissues

Of greater importance in the decision to select the RBL-2H3 mast cell line was the marked heterogeneity that was observed in mast cells extracted from humans, rodents and primates.

2.2.4.1 Heterogeneity of mast cells from different tissues

A comparison of the studies of these different tissues revealed marked morphological, histochemical and functional differences among mast cells, extracted from different tissues (Dvorak *et al.*, 1992). For example, variability was observed in the histamine content, size, mediator content (Enerback, 1966) and responsiveness of the mast cells to stimulation, in mast cells extracted from different locations (Patella *et al.*, 1995). The conclusion was drawn, that the local microenvironment plays an important role in regulating the characteristics of the resultant mast cells.

2.2.4.2 Heterogeneity of mast cells extracted from different locations within the same tissue

Heterogeneity is also evident within the mast cells extracted from individual organs. Within the gastrointestinal tract, for example, morphologically and functionally distinct populations of mast cells have been identified. Mast cells were extracted from the peritoneal cavity (PMC), the lamina propria (LPMC) and the mucosa (MMC) of the small intestine, and comparisons between the different mast cells were made. Whereas LPMC were identified as being morphologically and functionally identical to MMC, profound differences were observed between MMC and PMC, most typically in their response to stimulation. For example, MMC typically release significantly smaller quantities of mediators in response to both IgE-mediated, and ionophore-mediated stimulation, than LPMC and PMC (Pearce *et al.*, 1982). Whether the differences between PMC and MMC reflect differences in mast cell differentiation, local environment effects or distinct pathways of cell development remains to be determined.

2.2.4.3 Heterogeneity of mast cells extracted from the same anatomical location

2.2.4.3.1 Heterogeneity of Cell Size of Extracted Tissue Mast Cells

Mast cells were extracted from a single tissue, using the method described above (2.2.3.1 – 2.2.3.4), and a number of different characteristics were studied. Firstly, heterogeneity was observed in the sizes of the cells extracted. The majority of the mast cells had a diameter of 12 to 15 microns, although the sizes ranged from between 10 to 18 microns (Schulman *et al.*, 1983)). It was believed that, in the case of peritoneal mast cells, the size was dependent on the stage of maturation of the cell. Using a method called zonal sedimentation, separation of rat peritoneal mast cells into four sequential stages of mast cells maturation (I to IV) was achieved (Pretlow & Cassady, 1970). The smallest mast cells (stages I and II) are capable of cell division, whereas histamine was only detected in stage IV cells, which are also larger and more mature. It is clear, therefore, that the extraction procedure would produce a variety of mast cells at different stages of maturation and that this would have an effect on the degranulation responses and make comparison difficult.

2.2.4.3.2 Heterogeneity of Granule Content of Extracted Tissue Mast Cells

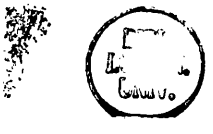
Secondly, heterogeneity was observed in the mediators detected in the extracted mast cells. Significantly, the histamine content varied between different mast cells, dependent on their diameter. The relationship between the cell size and the histamine content, however, is not proportional (Schulman *et al.*, 1983). The typical human mast cell contains approximately 3.6 picograms (pg) of histamine. In mast cells of less than 10 μm diameter, the average histamine content was 2.5 pg, compared with medium sized cells (14 μm) or large cells (greater than 18 μm), which contained 3.4 and 10.1 pg, respectively (Lichtenstein *et al.*, 1983).

For *in vitro* analysis, the larger (greater than 11 μm diameter) cells were preferentially purified. As discussed in Section 2.2.3.4, the weight of tissue that would be required for this research would be very large. If in addition, the extraction procedure selects larger cells, then the loss of the smaller cells would lead to a lower overall concentration of cells. This would lead, in turn, to a need for unacceptable, and difficult to obtain, quantities of tissue and was a primary factor in the decision to use a mast cell line.

A further example of the heterogeneity of human mast cells lies in the profile of granule proteins, the bulk of which are protease enzymes. Mast cells can be typically characterised according to the proteases they contain, and three classes of cells have been identified (Weidner & Austen, 1993):

1. those containing only tryptase (MCt);
2. those containing only chymase (MCc);
3. those containing both tryptase and chymase (MCtc).

Within these three sub-classes, additional heterogeneity exists, at the level of the cytokine expression. The distribution of cytokines IL-4, 5 and 6 has been studied, by immuno-histochemistry, in comparison with the tryptase and chymase content of the cells. The results indicated that, preferentially, IL-4 is restricted to the MCtc subset, while IL-5 and 6 are found almost exclusively in the MCt subset (Bradding *et al.*, 1995). The heterogeneity of human mast cells suggests specific biological functions, depending on the location of the mast cell, with the cytokines and proteases as an example of the different responses that could be mounted by differentially located cells. The potential exists that the variation in cytokine expression might affect the tissue mast cells' degranulation responses, which would alter the experimental results from one batch of cells to another.



2.2.4.3.3 Heterogeneity of Degranulation responses of Extracted Tissue Mast Cells

Mast cell degranulation is normally completed within 3 to 15 minutes, independent of cell size, although a marked difference was observed in the percentage of mediators released depending on the cell size. Typically, IgE-mediated degranulation of human mast cells showed a positive linear correlation between the quantity of mediators released and the cell size. The small mast cells released less than 10% of the total histamine, while large mast cells released over 50% (Schulman *et al.*, 1983). Similarly, the release of prostaglandin D2 was strongly dependent on the diameter of the mast cell, with larger cells releasing, on average, 10 times more PGD_2 than the small cells.

It was theorised that an increased surface density of IgE molecules on larger cells could be responsible for the correlation between the cell size and the quantity of mediators released. The higher density of IgE molecules would reflect a greater capacity for surface cross-linking, with a resultant amplified signal transduction, leading to increased release. To test this theory, a study was made of the number of IgE molecules on the surface of mast cells of different diameters (Schulman *et al.*, 1983). The results showed no difference between the number of acid-elutable IgE molecules on large and small mast cells. A comparison of the surface area of mast cells and the observed constancy of the number of surface IgE molecules per mast cell, revealed that the surface density of IgE on large mast cells is lower than that on small mast cells.

In addition, the number of IgE molecules was not adversely affected by the extraction procedure. Comparisons were made of the surface IgE molecules on mast cells extracted by mincing, mincing with enzymatic digestion, or with more stringent extraction procedures. It was determined, whatever the extraction procedure, that the smaller diameter mast cells routinely possessed between 1.5 to 3 times more surface IgE molecules per μm^2 surface area, than the large diameter cells.

It is possible, however, that the heterogeneity of the quantity of mediators released from different-sized mast cells is associated with the stage of maturation of the cell. As discussed earlier in Section 2.2.4.3.1, the mast cell size appears to be dependent on its stage of development with histamine only being detected in the larger and more mature stage IV cells. The correlation between cell size and degranulation responses is not unexpected. However, variation in degranulation responses would lead to difficulties in interpreting results from different experiments.

2.2.4.3.4 Heterogeneity of Passive Sensitisation of Extracted Tissue Mast Cells

Mast cells are receptive to passive sensitisation, which involves bathing the cells in a solution containing antigen-specific IgE. Addition of the specific-antigen to the sensitised mast cells promotes the specific degranulation of the cells. Marked heterogeneity in the susceptibility of the mast cells to passive sensitisation is observed.

Studies of human leukocyte suspensions, which were bathed in serum obtained from ragweed-sensitive allergic donors and then incubated with ragweed antigen, were performed. Results indicated that 50% of the donor leukocytes failed to show reactivity to the rag weed antigen, despite the other 50% of the donors showing appropriate degranulation responses (Levy & Osler, 1966). It is possible that the extraction procedure impairs the mast cells' ability to respond to passive sensitisation.

In order to maintain consistency of the experimentation for the experiments for this thesis, it was essential to use mast cells that showed uniform responses to IgE-mediated stimulation in *in vitro* experiments. It was decided that an extensive analysis of each batch of mast cells (from different donor tissues) would be necessary to determine the cell responses in comparison to previous batches.

2.2.5 Relation of heterogeneity of mast cells to the atopic status of the donor

The atopic status of the donor of the tissue mast cells was not available. Based on the premise that atopic individuals are hypersensitive to specific antigens, it is likely that the *in vitro* mast cell responses observed would differ from those in non-atopic individuals. This could introduce a further level of variability and further complications in interpreting experimental data.

2.2.6 Consequences of the heterogeneity of human tissue mast cells

The discovery of significant heterogeneity in extracted human tissue mast cells, suggests the potential for differential *in vivo* function, possibly dependent on the location of the cell and both the normal and the pathological response required. For example, mast cells derived from lung tissue have to be adapted to combat airborne allergen challenge, whereas gastrointestinal mast cells require adaptations to encounter both intestinal allergens, and parasitic infections. Mast cells are ubiquitous throughout connective tissues, along epithelial surfaces and in close proximity to blood vessels. The presence of a vast array of mediators together, combined with the marked heterogeneity of the cells, helps to explain the mast cell's potential to contribute to a plethora of biological events. The cells within the different tissues, therefore, have adapted to

provide the most effective defence. It is more difficult to explain, however, the variation observed in mast cells extracted from the same anatomical location (Bradding *et al.*, 1999).

2.3 Development of mast cells from their progenitors

The development of techniques to derive mature mast cells from mast cell progenitors presented an alternative to the tissue extraction of mast cells. The techniques were developed with murine mast cells, derived from mononuclear cells and extracted from bone marrow aspirates. The resultant mast cells were purified by density-gradient centrifugation over Percoll or Ficoll-paque and then were cultured with recombinant IL-3 (rIL-3). This technique produced a pure mast cell population (Saito *et al.*, 1987). Mast cells were identified through staining with toluidine blue and Wright-Giemsa, using electron microscopy, and were characterised according to their protease profile.

Recombinant (r)IL-3 derived mast cells were shown to be analogous to gastro-intestinal mast cells, as they preferentially contained chondroitin sulphate. The production of mature cells, analogous to heparin-containing connective tissue mast cells, was promoted by the addition of mouse 3T3 fibroblast cells in addition to rIL-3 (Dayton *et al.*, 1988; Levi-Shaffer *et al.*, 1986). It was concluded that the phenotype of the mature mast cells was greatly influenced by the microenvironment in which the precursor cells were placed.

These culture methods were, however, difficult to reproduce with human progenitor cells. After two weeks of culture with rIL-3, a suspension of human bone marrow cells and cord blood cells, produced cells with characteristics of basophils, eosinophils, neutrophils and macrophages. Within three weeks, however, the majority of non-adherent cells had become eosinophils (Saito *et al.*, 1988). Mast cells were absent in all cultures studied.

The differentiation of mast cells from their cord-blood progenitors was reliant on the presence of both T-cell conditioned medium and mouse 3T3 (Swiss/3T3) fibroblasts. Human fibroblasts alone, by comparison, promoted the differentiation of the precursors preferentially into basophils or eosinophils (Furitsu *et al.*, 1989). Using affinity chromatography techniques, it was confirmed that the factor of significance, promoting mast cell differentiation, in the 3T3 conditioned medium was the c-kit ligand, stem cell factor (SCF) (Mitsui *et al.*, 1993).

Extensive analysis, including electron microscopy, confirmed the 3T3 cultured cells as mast cells (Furitsu *et al.*, 1989). Although the cells were smaller than normal tissue mast cells, being between 7 and 12 μm diameter, phenotypically they were similar to normal mast cells. The cells typically contained 2 μg of histamine per million cells, and released histamine in response

to IgE-mediated stimulation. They were shown to contain both chymase and tryptase, which suggested that they were analogous to mast cells found in normal dermis and intestinal sub-mucosa (Mitsui *et al.*, 1993).

It was discovered that the progenitor cells in human bone marrow aspirates, responsible for the mast cells and basophils, were CD34+ cells. Removal of the CD34+ cells from the bone marrow aspirates ablated the production of both basophils and mast cells, whereas removal of T cell, B cell, macrophage or eosinophil precursor did not (Kirshenbaum *et al.*, 1991).

2.3.1 Complications with using artificially differentiated human mast cells

A complication of using artificially differentiated mast cells is that the cells have been created in the absence of interaction with their extracellular environment. A plethora of soluble and cellular stimulatory and inhibitory conditions are present in this environment, all of which may influence the development of the cell (Bressler *et al.*, 1997). It is, therefore, impossible to conclude that the cells' responses in an *in vitro* situation accurately represents the responses observed in extracted tissue mast cells.

2.4 Development of cell lines as analogues of tissue cells

The research performed for this thesis endeavoured to elucidate the existence and role of GPI-PLD in mast cell physiology. Clearly, the aforementioned heterogeneity of mast cells presents a challenge to the interpretation of the data. The possibility that an artificial cell line might provide less heterogeneity than the human tissue mast cells, was evaluated. A number of different mast cell lines exist, including human mast cell lines, mouse mastocytomas and rat basophil leukaemia cell lines. For example, the human mast cell line, HMC-1, was derived from a patient with mast cell leukemia (Butterfield *et al.*, 1990). Solid tumors of these cells were serially passed in mice using 5-azacytidine transformed cells.

This cell line was shown to display a phenotype similar to the MCt (Section 2.2.4.3.2), as judged by the expression of surface antigens, and the cytochemical analysis (Nilsson *et al.*, 1994). Both heparin and chondroitin sulfate were found to be present in approximately equal amounts. The HMC-1 cell line, however, lacked surface expression of the high-affinity IgE receptor. Analysis of the mRNA from these cells showed an absence of both alpha- and beta-chain mRNA, although the gamma-chain of the IgE-receptor complex was strongly detected. Despite the lack of high affinity receptor, HMC-1 has been successfully employed in a number of different experimental studies, including receptor-expression (Sillaber *et al.*, 1997) and cytokine metabolism studies (Macchia et al, 1995).

The choice of the RBL-2H3 cell line, in preference to a human mast cell line, was based on the pilot experiments that were performed in collaboration with Dr Alasdair Gilfillan, Hoffman-la-Roche. These experiments, which are discussed in greater detail in Chapter 3, involved the analysis of the role of GPI-PLD in mast cell function. The experiments were performed using RBL-2H3 cells, and demonstrated that GPI-PLD might be involved in the Type One hypersensitivity reaction (Lin *et al.*, 1995). It seemed of importance, therefore, to pursue the role of GPI-PLD in mast cell function using the RBL-2H3 cell line. The development and the characteristics of the RBL-2H3 cell line are discussed in greater detail in Section 2.11.

2.5 Experimental aims

The experimental aim of the research performed for this thesis was to determine the existence, and role of GPI-PLD in the function of mast cells. Most importantly, to determine whether GPI-PLD was a participant in the signalling machinery activated upon IgE-mediated stimulation of mast cells.

The reasons for the selection of the RBL-2H3 cell line have already been discussed. Prior to any research into GPI-PLD, however, it was essential to establish a consistent culture of the RBL-2H3 cells, and to verify the cells' characteristics, particularly in relation to their IgE mediated degranulation. This is a complex process, which involves the specific interaction of a number of components. It was essential to establish a consistent methodology for the IgE-dependent stimulation of the RBL-2H3 cells and to accurately measure the quantities of mediators released as a result of the cells' degranulation.

2.6 Culture of RBL-2H3 cells

The culture of RBL-2H3 cells was dependent on supplemented culture medium and growth in a humidified, carbon dioxide-enriched incubator. In addition, the RBL-2H3 cells required specific culture flasks and plates to permit attachment of the cells. Culture of the RBL-2H3 cells was significantly impeded if the cells were maintained as a suspension, rather than as a confluent monolayer.

2.7 Non-IgE-mediated Degranulation of RBL-2H3 cells

2.7.1 Lysis, using Triton X-100

The selection of Triton X-100 as a positive control was used as a measure of the complete mediator content of the RBL-2H3 cells. The mechanism by which Triton X-100 releases mediators from cells is discussed in greater detail in Section 2.11.4.1.

2.7.2 *In vitro* degranulation, using Calcium Ionophore A23187

The primary experimental aim of the research described in this Chapter was to establish a consistent method for the IgE-dependent degranulation of RBL-2H3 cells. It was also of importance, however, to establish an IgE-independent method for cell degranulation.

Experiments performed in later Chapters required the manipulation of the cells' culture environment, and determination of whether the IgE mediated degranulation of the cells was affected by this manipulation. The inclusion of a positive control in these experiments, in the form of an IgE-independent method of cell stimulation was also essential. If a reduction in the quantity of mediators released by IgE-dependent route was accompanied by a reduction in the IgE-independent route, it could be concluded that the manipulation of the cells' environment was non-specific. That is, the manipulation affected the complete functioning of the RBL-2H3 cells, rather than the IgE system specifically.

The calcium ionophore, A23187, was selected as an IgE independent positive control, to permit the selective release of mediators from the mast cells by an IgE-independent route. A23187 was isolated from *Streptomyces chartreusensis* and belongs to the family of monocarboxylic antibiotics (Chaney *et al.*, 1974). It was shown to release mediators from partially purified rat mast cells (Caswell *et al.*, 1972). The mechanism by which A23187 releases mediators from cells is given in greater detail in the Section 2.11.4.2.4.

2.8 IgE-mediated degranulation of RBL-2H3 cells, *in vitro*

2.8.1 Binding of IgE to the surface of the RBL-2H3 cells

It has been demonstrated that RBL-2H3 cells are responsive to IgE-mediated degranulation (Barsumian *et al.*, 1981). However, as discussed in Section 1.3.15, an initial and prerequisite event in IgE-mediated degranulation of mast cells is the binding of IgE to the surface of the cells (Kulczycki *et al.*, 1974). An important difference between the RBL cells and their human mast cell counterpart is that the FcεR1 receptors on the surface of the RBL cells are naturally unoccupied. This results from their lack of contact with serum IgE (Iserksy *et al.*, 1978).

It is estimated that, with a serum IgE concentration of 200 ng/ml, greater than 50% of the FcεR1 receptors on normal blood basophils would be occupied (Ishizaka *et al.*, 1973). Although the same data has not been calculated for mast cells, their contact with serum IgE would suggest an equivalent occupation of their IgE receptors. Although the IgE on the surface of human basophils and mast cells can be removed by incubation with lactic acid at pH 4.0 (Ishizaka & Ishizaka, 1974), reduction in cell viability was observed after lactic acid exposure. Incubation for as little as 10 minutes at pH 4.0, led to a reduction in the viability of the cells

from greater than 90% to between 40 and 60% (as determined by the erythrocin B exclusion test). By comparison, since the FcεR1 receptors on RBL-2H3 cells were unoccupied, this surface IgE removal was unnecessary. This reduced the amount of manipulation, and potential damage, of the RBL-2H3 cells.

The experiments performed for the study of the role of GPI-PLD in the IgE-mediated degranulation of RBL-2H3 cells, therefore, were dependent on an appropriate selection of an IgE isoform, whose cross-linking will induce the activation of RBL-2H3 cells and the release of the cell mediators.

2.8.2 Selection of IgE isoforms, for the IgE-mediated degranulation of RBL-2H3 cells

The artificial creation of IgE isoforms with specific characteristics improved the field of *in vitro* mast cell stimulation. These IgE isoforms are reviewed in greater detail in Section 2.11.5.2. Three possible IgE isoforms were considered. The first two were IgE isoforms with no particular antigen-specificity, which were cross-linked by anti-IgE antibodies. The generation of antibodies with no particular antigenic-specificity is discussed in Section 2.11.5.2.3. In this instance, the antigen specificity of the IgE was of reduced importance, since the antibody was designed to target a non-antigenic epitope on the IgE molecules. The first combination experiment was rat serum, containing IgE, in combination with a monoclonal mouse anti-rat IgE and the second was rat myeloma IgE with a monoclonal goat anti-Rat IgE.

Following experiments using IgE with no particular antigenic-specificity, an IgE with specificity against a particular antigen, dinitrophenyl-albumin, was selected.

In each case of IgE and cross-linker employed, the relative success of the IgE isoform, in promoting degranulation of the RBL-2H3 cell was determined by analysing the quantities of mediators released.

2.8.3 Determination of mediators released upon RBL-2H3 degranulation

The direct result of mast cell activation may be measured by the release of mediators into the extracellular milieu. To accurately determine the efficacy of the IgE passive sensitization on RBL-2H3 cells, it was important to select a mast cell mediator, from the many that are released, that could be quantitatively identified. The different mediators may be determined by immuno-histochemistry and sub-cellular fraction techniques and these may be broadly grouped into three types: pre-formed, newly synthesized and cytokines (Schwartz *et al.*, 1980).

The mediator selected was N-acetyl-β-glucosaminidase, alternatively termed β-hexosaminidase, which is an exo-glycosidase enzyme. This enzyme is responsible for the release of terminal β-N-Acetyl-D-glucosaminide and galactosaminide residues (Stoward & Pearse, 1991). Immuno-staining determined that a high percentage of N-acetyl-β-glucosaminidase is located in the secretory granules of human mast cells (Lagunoff *et al.*, 1970), and is released in parallel with histamine upon IgE-mediated mast cell stimulation (Schwartz *et al.*, 1979). The absence of any delay in time for the release of N-acetyl-β-glucosaminidase or histamine indicated that both mediators were pre-formed. It is important to note that the function of N-acetyl-β-glucosaminidase in the Type One Hypersensitivity reaction is currently not understood.

The N-acetyl-β-glucosaminidase mediator was selected for two main reasons. Firstly, because it is released rapidly upon degranulation and does not require additional synthesis pathways. Secondly, detection of N-acetyl-β-glucosaminidase is straightforward and rapid. The detection of N-acetyl-β-glucosaminidase relies on the cleavage of a synthetic chromogenic substrate, p-nitrophenyl-N-acetyl-D-glucosaminide, under acidic conditions (Lagunoff *et al.*, 1970). Catalysis of the terminal p-nitrophenyl in acid conditions liberates p-nitrophenol, which is detected in alkaline conditions, at 405 nm wavelength, by spectrophotometry (Walker *et al.*, 1961).

2.9 Experimental Methods

2.9.1 Routine Culture and Maintenance of the RBL-2H3 cell line

2.9.1.1 Origination of the RBL-2H3 cell line

The RBL-2H3 cell lines used in this study were kindly provided by both Dr. A. Gilfillan, Hoffman la-Roche, and by Niamh O'Luanaigh, University College London.

2.9.1.2 Long-term storage of RBL-2H3 cells in liquid nitrogen

A freezing solution was prepared, containing 10% (v:v) DMSO and 30% (v:v) FBS in normal supplemented EMEM (Eagles Minimum Essential Medium (EMEM) supplemented with 10% volume:volume (v:v) heat inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) (Sigma Chemicals Ltd). The freezing solution was maintained on ice until required.

Adherent RBL-2H3 cells were washed with sterile phosphate buffer saline (PBS) (0.01 M phosphate buffer, 0.0027 potassium chloride and 0.137 M sodium chloride, pH 7.4) (Sigma Chemicals), and detached from the culture flasks using a cell scraper (Life Technologies Ltd.). Cells were removed into a sterile universal (BDH Chemicals) and centrifuged at 1,000 G for

greater than 5 minutes. The DMSO-containing supplemented EMEM was added to the cell pellet and the cells were gently triturated by pipette, to ensure thorough resuspension. The cell density was determined, using a Neubauer haemocytometer (BDH Chemicals), and the cell concentration was adjusted to a final concentration of 1 million cells per ml. The suspension was aliquoted into 2 ml cryotubes (Life Technologies Ltd), and the cells were allowed to freeze slowly at -80°C for at least one day, prior to long-term storage in liquid nitrogen.

2.9.1.3 RBL-2H3 thawing and maintenance

The RBL-2H3 cells, stored in freezing media (Eagles Minimum Essential Medium (EMEM) supplemented with 10% volume:volume (v:v) heat inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, diluted 6:1:3 with DMSO(v:v) and FBS (v:v) (Life Technologies Ltd), were removed from liquid nitrogen and placed immediately at 37°C, to effect rapid thawing.

The cells were centrifuged at 1,000 G for greater than 5 minutes, and the freezing media was removed by pipette. The cell pellet was resuspended in fresh culture media (Eagles Minimum Essential Medium (EMEM) supplemented with 10% volume:volume (v:v) heat inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) (Life Technologies Ltd). Cells were incubated at 37°C in an humidified atmosphere of 95% air and 5% carbon dioxide.

2.9.1.4 Sub-culturing of the cell line

Non-adherent RBL-2H3 cells were removed from the culture flask by pipette and the confluent cell monolayer was washed with fresh supplemented EMEM. Adherent cells were detached from the culture flask using a cell scraper (Life Technologies Ltd.), prior to dilution of the suspension into fresh culture flasks (Life Technologies Ltd.). Cells were normally amplified at a split ratio of 1 in 10.

2.9.1.5 Plating out RBL-2H3 cells onto culture plates

RBL-2H3 cells were grown to confluence, after which time the adherent cells were removed from the culture flask using a cell scraper (Life Technologies Ltd.). The cell density was determined, using a Neubauer haemocytometer (BDH Chemicals), and was adjusted according to experimental requirements. Typically, for experiments using 24 well plates, the cell density was adjusted to between 1 and 2 x 10^5 cells per well in a 1 ml volume. The cells were cultured overnight at 37°C, in a humidified 5% CO_2 incubator.

2.9.2 Non-IgE mediated degranulation of RBL-2H3 cells

2.9.2.1 Using Triton X-100

The total mediator content of the cell was determined by lysis of the cells with a 5% Triton X-100 (t-octylphenoxyl polyethoxyethanol) (Sigma Chemicals Ltd) solution and detection of the β-hexosaminidase (as discussed in Section 2.9.3).

2.9.2.2 Stimulation of RBL-2H3 cells with Calcium ionophore A23187

Stimulation of cells was achieved using a 500 μM A23187 solution (Sigma Chemicals Ltd). The time of incubation varied between different experiments.

2.9.3 IgE-mediated degranulation of RBL-2H3 cells

The overnight culture media was removed by pipette and replaced with 300 μl supplemented medium (Eagles Minimum Essential Medium (EMEM) supplemented with 10% volume:volume (v:v) heat inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) (Life Technologies Ltd.). The RBL-2H3 cells were incubated at 37°C in a humidified 5% CO_2 incubator for 2 hours, after which time the medium was removed and the cells were washed twice with HEPES buffered saline (140 mM Sodium Chloride, 10 mM HEPES, pH 7.4) (Sigma Chemicals Ltd).

Degranulation was achieved by the addition of 200 μl of the appropriate cross-linker (dependent on the selected IgE, as discussed in the section below (Section 2.9.3.1) and incubation at 37°C in a humidified 5% CO_2 incubator for an appropriate amount of time.

2.9.3.1 Types of immunoglobulin E and cross-linkers employed

2.9.3.1.1 Rat serum and anti-rat IgE antibody

The first combination of IgE and cross linker involved a rat serum preparation, containing IgE, in combination with a monoclonal mouse anti-rat IgE cross-linker, MAS 314pf. The MAS 314pf anti-IgE antibody was an IgG1 isotype, with specificity for the ε heavy chain of rat IgE.

2.9.3.1.2 Rat Myeloma IgE and anti-rat IgE antibody

Secondly, a purified rat IgE myeloma protein was obtained, and the cross linker employed was a monoclonal goat anti-Rat IgE (AB 688).

2.9.3.1.3 Rat kappa IgE, with reactivity against dinitrophenyl-hapten, and DNP albumin

Thirdly, a rat IgE isoform, with reactivity against dinitrophenyl-hapten was obtained. This kappa IgE isoform was derived from a non-secreting LOU/C rat IR983F fusion line, clone LO-

DNP-30, with specificity against *Nippostrongylus brasiliensis*-DNP. The cross linker employed was dinitrophenol-albumin (DNP-albumin).

2.9.4 Colorimetric detection of β-hexosaminidase

The degranulation of RBL-2H3 cells was quantified by detection of a granule enzyme, β-hexosaminidase. Following degranulation or lysis of the RBL-2H3 cells, fifty microlitre aliquots of the cell supernatants were transferred to a 96 well plate (Life Technologies Ltd). 100 µl of PNP-substrate solution (10 mM p-nitrophenyl-N-acetyl-β-D-glucosamine, in a 0.1 M citrate buffer (pH 4.0)) (Sigma Chemicals Ltd.) was added to each sample and the plate was incubated for 30 minutes at 37°C. The reaction was stopped by addition of 100 µl of 0.4 M glycine, pH 10.6 (Sigma Chemicals Ltd) (Yasuda *et al.*, 1995). Hydrolysis of the substrate produced p-nitrophenolate, a coloured product, which was detected at 405 nanometres wavelength on an ELISA plate reader (Thermomax Microplate reader, Molecular Devices).

2.9.5 Inhibition of IgE-stimulated mediator release from RBL-2H3 cells using EGTA

The RBL-2H3 cells were prepared as for a standard IgE-mediated cell stimulation assay, as discussed in Section 2.9.1.5. The IgE-mediated stimulation was performed as described in Section 2.9.3, until the point at which the DNP-albumin solution is added to the cell monolayer.

EGTA and DNP-albumin (Sigma Chemicals Ltd) were diluted into HEPES AGM buffer (25 mM HEPES, 110 mM Sodium Chloride, 5 mM Potassium Chloride, 0.3 mg/ml Human Serum Albumin, 2 mM Calcium Chloride, 1 mM Magnesium Chloride, pH 7.4) (Sigma Chemicals Ltd). The EGTA/DNP solution was then substituted for the standard DNP-albumin solution and 200 µl were pipetted into each well. The plates were incubated for 2 hours, after which time 50 µl aliquots of solution were removed for analysis in the β-hexosaminidase detection assay (Section 2.9.3).

2.10 Results

2.10.1 Culture of the RBL-2H3 cells

Culture of the RBL-2H3 cells was successfully achieved. To confirm that the characteristics of the RBL-2H3 cell line were consistent, two different batches of RBL-2H3 cells were obtained. To ensure that the second batch of RBL-2H3 cells had a similar reactivity to IgE-mediated stimulation, experiments were performed to determine the concentrations of IgE and DNP-albumin, and the times of incubations required for cell degranulation. A comparison of the results from the two different batches of RBL-2H3 cells is shown in Table 2.1

TABLE 2.1

COMPARISON OF DEGRANULATION EXPERIMENTS WITH TWO DIFFERENT BATCHES OF RBL-2H3 CELLS

	UNITS	EXPERIMENTAL DETAILS	RBL-2H3 BATCH ONE (RBL-2H3 (1))	RBL-2H3 BATCH TWO (RBL-2H3 (2))
OPTIMAL IgE CONCENTRATION	(μg/ml)	DNP-albumin at 0.1 μg/ml (2 hours)	3	3 (see Figure 2.5a)
		DNP-albumin at 1 μg/ml (2 hours)	3	9 (see Figure 2.5b)
OPTIMAL DNP-ALBUMIN CONCENTRATION	(μg/ml)	IgE at 3 μg/ml (3 hours)	Approximately 0.1 (see Figure 2.6)	Approximately 0.1
OPTIMAL TIME OF DNP –ALBUMIN INCUBATION	(minutes)	IgE at 3 μg/ml (2 hours) DNP at 0.1 μg/ml	120	Greater than 90 minutes (see Figure 2.7a)
		IgE at 3 μg/ml (2 hours) DNP at 1 (μg/ml		Greater than 60 minutes (see Figure 2.7b)

These results confirmed that, despite there being differences between the two batches of cells, essentially both sets of RBL-2H3 cells are responsive to IgE mediated stimulation. In addition, both batches release maximal quantities of β-glucosaminidase with 3 micrograms per ml IgE and DNP at between 0.1 and 1 micrograms per ml. The time required for DNP-albumin incubation varies between 60 to 120 minutes for the two batches, and although the optimal time differs, both require greater than 60 minutes of incubation. Almost all of the experiments discussed in this section were performed with the second batch of RBL-2H3 cells. Where the first batch was used, this is noted.

2.10.2 IgE-independent release of mediators from RBL-2H3 cells

2.10.2.1 Release of mediators through Triton lysis of the RBL-2H3 cells

The results of the lysis of an RBL-2H3 (Batch 1) cell suspension with a 5% Triton X-100 solution is shown in Figure 2.1. The results are expressed as the optical density (OD) of the product, β- glucosaminide, detected at 405 nm wavelength. The β-glucosaminide product is released by the reaction catalysed by β-glucosaminidase, an enzyme released by the degranulation of RBL-2H3 cells. This plot shows the optical density of β- glucosaminide in comparison with RBL-2H3 cell number after 30 minutes of incubation in the detection assay.

These results illustrate that Triton X-100 is an effective agent for the complete lysis of the RBL-2H3 cells. The plot shows a linear relationship (R = 0.99) between β-glucosaminidase with increasing numbers of RBL-2H3 cells.

2.10.2.2 Release of mediators through Ionophore-mediated stimulation of the RBL-2H3 cells

The graph in Figure 2.2 shows the results from the stimulation of RBL-2H3 with calcium ionophore A23187, at concentrations from 5 to 500 μM. The cells were incubated for time periods of between 5 and 60 minutes. The results are expressed as an OD reading at 405 nm wavelength, as measured by the detection of β-glucosaminide, after a 30 minute detection period.

The plot shows that, following incubation with 500 μM ionophore A23187, the degranulation of the RBL-2H3 cells increased approximately linearly with time, with a peak OD reading of 2.8 after 60 minutes of incubation. Incubation with 50 μM A23187 produced a maximum OD reading of 0.2 or 0.4 respectively. When compared with the 0.2 OD reading from the negative control, HEPES AGM without A23187, these results indicated that 50 μM A23187 did not produce a significant degranulation response in RBL-2H3 cells. A

FIGURE 2.1

TRITON LYSIS OF RBL-2H3 CELLS

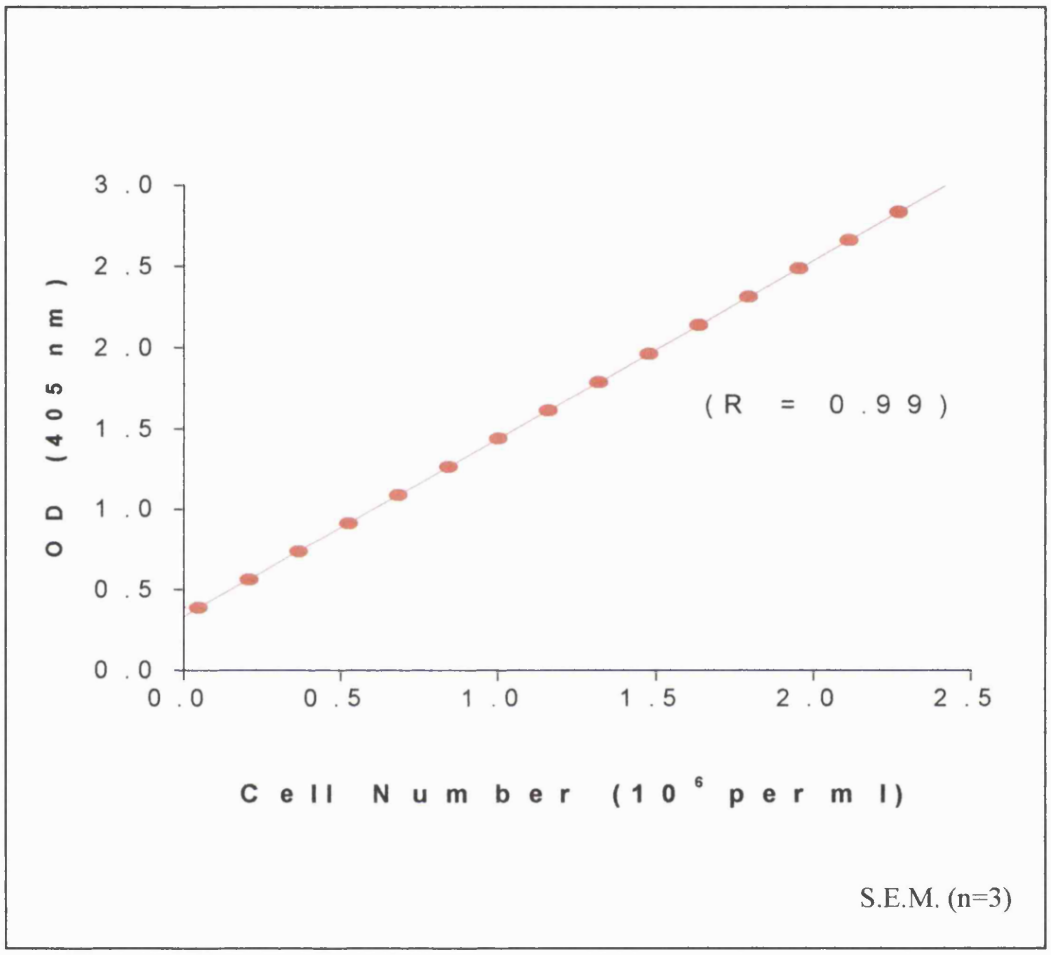


Figure Legend

The plot in Figure 2.1 shows the results from the lysis of a serial dilution of RBL-2H3 cells with a 5% Triton solution. The results are expressed as an optical density (OD) reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase, an enzyme released by the degranulation of RBL-2H3 cells. The β-glucosaminidase assay was incubated for 30 minutes.

The plot shows a linear relationship between the cell number versus the optical density reading from the β- glucosaminidase assay.

FIGURE 2.2

TIME COURSE OF INCUBATION OF RBL-2H3 CELLS WITH CALCIUM IONOPHORE A23187

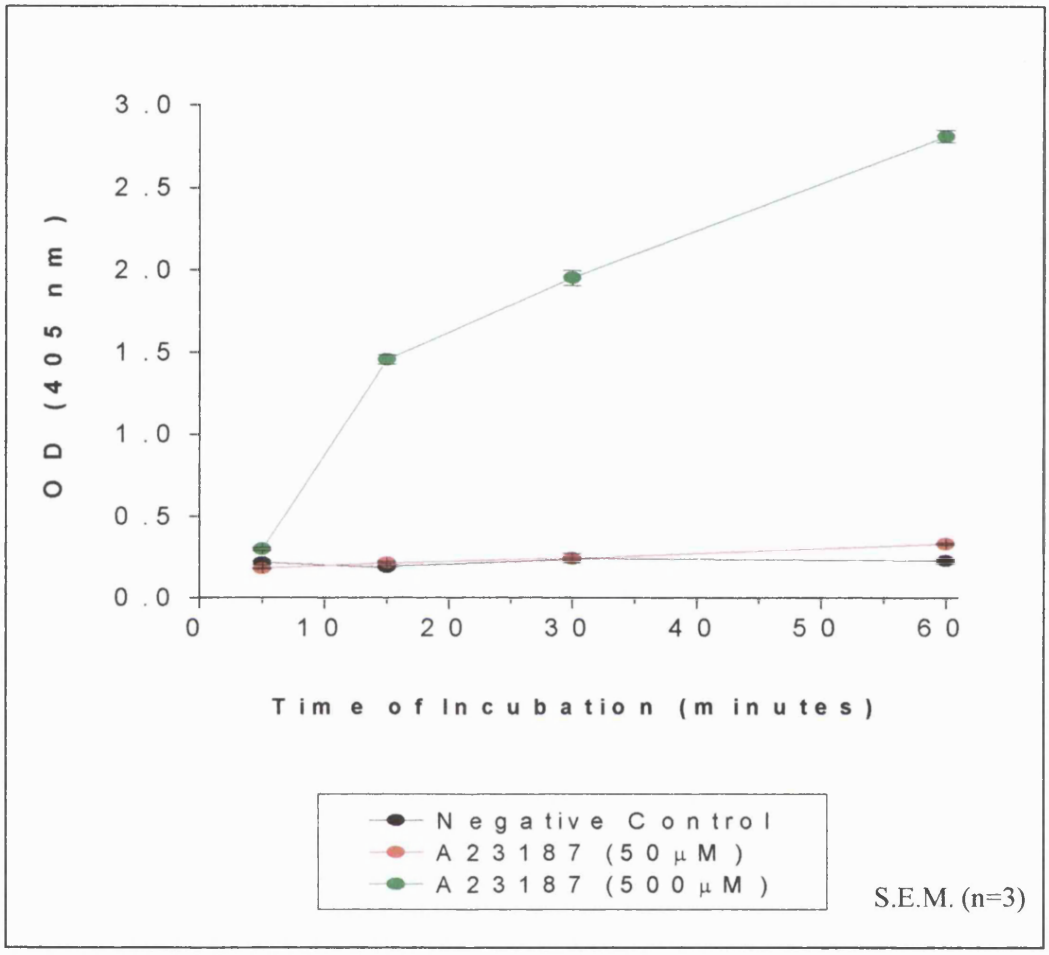


Figure Legend

The plot in Figure 2.2 shows the results from the stimulation of RBL-2H3 cells with 200 μl calcium ionophore A23187 (concentration range from 5 to 500 μM), and negative controls. The cells were incubated for time periods of between 5 and 60 minutes. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase after a 30 minute detection period.

The plot shows that after incubation with 500 μM ionophore A23187, the degranulation of the RBL-2H3 cells increased linearly, reaching a peak after 60 minutes of incubation.. 50 μM of A23187 did not effect significant degranulation responses in RBL-2H3 cells (data not shown).

concentration of 500 μM A23187 was used, therefore, in all subsequent stimulation experiments.

2.10.3 IgE-mediated stimulation of RBL-2H3 cells

2.10.3.1 Optimal number of cells per well, on a 24 well plate

The optimal number of cells required for a fully confluent monolayer in the well of a 24 well plate, were ascertained by microscopy. It was determined that 200,000 RBL-2H3 cells gained confluence in a 24 well plate after overnight incubation.

2.10.3.2 Stimulation of RBL-2H3 cells using rat IgE and monoclonal anti-IgE antibodies

Figures 2.3 and 2.4 show the data obtained from the passive sensitisation of RBL-2H3(1) cells with either purified rat IgE myeloma protein (2.3) or rat serum (2.4), followed by stimulation of the sensitised cells with monoclonal anti-rat IgE antibodies.

The plots in Figure 2.3 show the results from the incubation of RBL-2H3 cells with purified rat IgE myeloma protein, at a concentration of between 0.5 and 4 μg/ml (in EMEM). Following the incubation with IgE, the cells were incubated with monoclonal goat anti-rat IgE antibody, at a dilution of 1 in 250 (plot a) or 1 in 1000 (plot b) diluted in HEPES AGM. Negative controls were included, in which RBL-2H3 cells were incubated with EMEM containing no IgE, and cells were also lysed by the addition of a 5% Triton X-100 solution. The results are expressed as an OD reading at 405 nm wavelength, as measured by the detection of β-glucosaminide after 30 minutes of incubation in the detection assay.

The data indicated that incubation of RBL-2H3 cells with purified IgE myeloma protein generated OD readings which were equivalent to, or possibly lower than, readings obtained when the cells were incubated without IgE. When incubated with a 1 in 250 dilution of goat anti-rat IgE antibody, the OD readings ranged from 0.17 to 0.29, compared with 0.3 from those cells without IgE myeloma protein. When incubated with a 1 in 1000 dilution of goat anti-rat IgE antibody, the OD readings ranged from 0.2 to 0.33, compared with 0.26 from those cells without IgE myeloma protein. The data, therefore, showed that this combination of myeloma IgE and goat anti-rat IgE cross linker were not able to promote degranulation of RBL-2H3 cells. In addition, it is possible that the myeloma IgE and goat anti-rat IgE cross linker marginally inhibited the degranulation process.

The plots in Figure 2.4 show the results from the incubation of RBL-2H3 cells with rat serum (between 5 and 200 μl, diluted in 300 μl EMEM). Following the incubation with IgE, the cells

FIGURE 2.3 a

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING PURIFIED RAT IgE MYELOMA PROTEIN AND MONOCLONAL GOAT-ANTI-RAT IgE

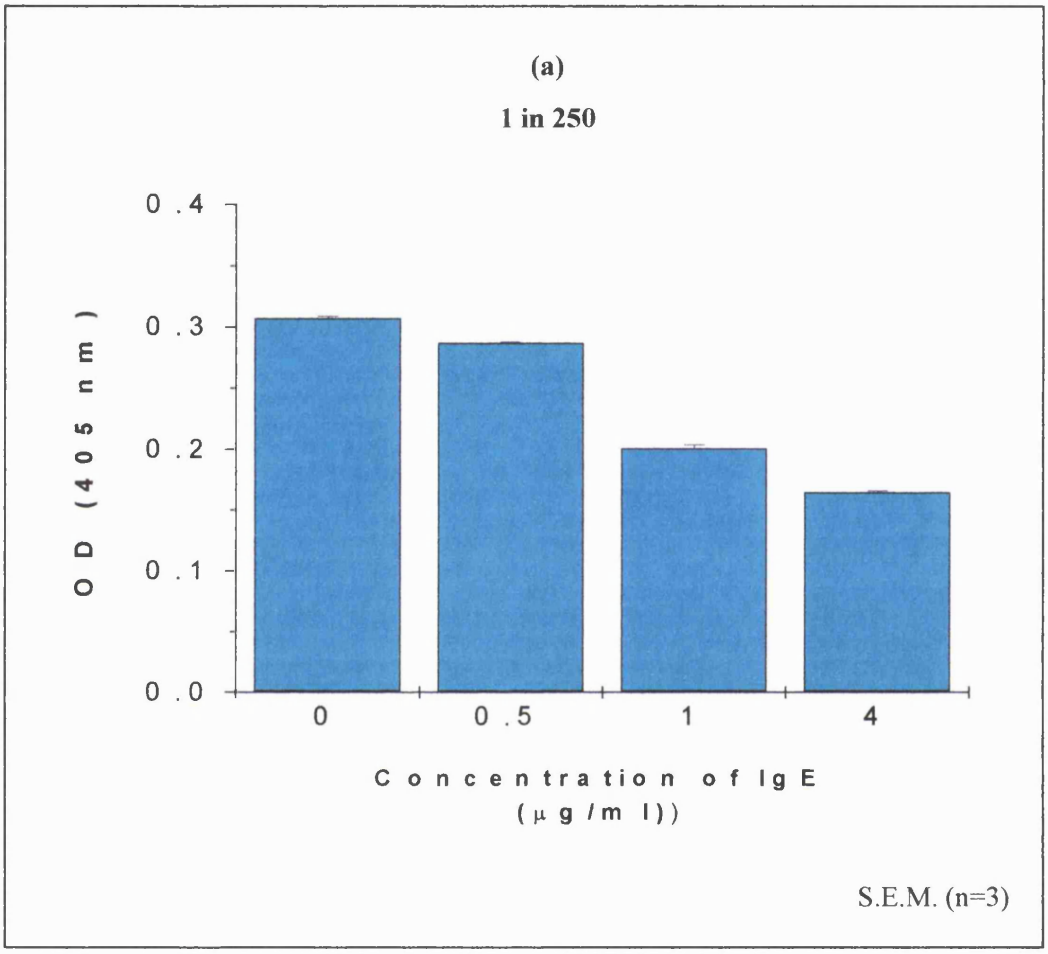


Figure Legend

The plot in Figures 2.3 a shows the results from the incubation of 200,000 RBL-2H3 cells with purified rat IgE myeloma protein (diluted in EMEM), at a concentration of between 0.5 and 4 μg/ml (in 300 μls), for 2 hours. Subsequently, the cells were incubated with monoclonal goat anti-rat IgE antibody diluted 1 in 250 in HEPES AGM for 60 minutes. Negative controls were included, in which 200,000 cells were incubated with EMEM containing no IgE, and cells were also lysed by the addition of a 5% Triton X-100 solution. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase.

Data indicated that incubation of RBL-2H3 cells with purified IgE myeloma protein produced OD readings which were equivalent to, or possibly lower than, the OD readings obtained when the cells were incubated without IgE. The conclusion was that this combination of myeloma IgE and goat anti-rat IgE cross linker did not promote degranulation of RBL-2H3 cells, and may have actually been marginally inhibitory.

FIGURE 2.3 b

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING PURIFIED RAT IgE MYELOMA PROTEIN AND MONOCLONAL GOAT-ANTI-RAT IgE

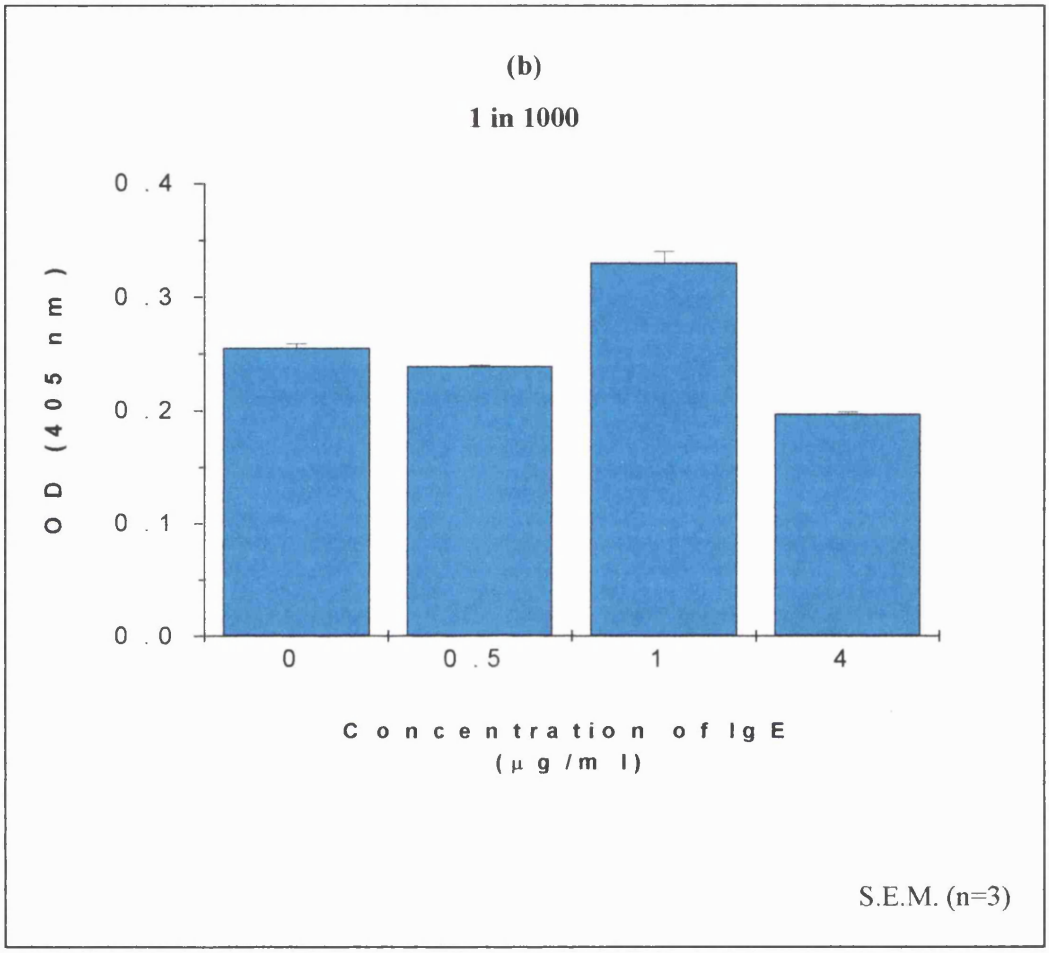


Figure Legend

The plot in Figures 2.3 b shows the results from the incubation of 200,000 RBL-2H3 cells with purified rat IgE myeloma protein (diluted in EMEM), at a concentration of between 0.5 and 4 μg/ml (in 300 μls), for 2 hours. Subsequently, the cells were incubated with monoclonal goat anti-rat IgE antibody diluted 1 in 1000 in HEPES AGM for 60 minutes. Negative controls were included, in which 200,000 cells were incubated with EMEM containing no IgE, and cells were also lysed by the addition of a 5% Triton X-100 solution. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase.

Data indicated that incubation of RBL-2H3 cells with purified IgE myeloma protein produced OD readings which were equivalent to the OD readings obtained when the cells were incubated without IgE. The conclusion was that this combination of myeloma IgE and goat anti-rat IgE cross linker did not promote degranulation of RBL-2H3 cells.

FIGURE 2.4 a

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING RAT SERUM AND MONOCLONAL MOUSE ANTI-RAT IgE

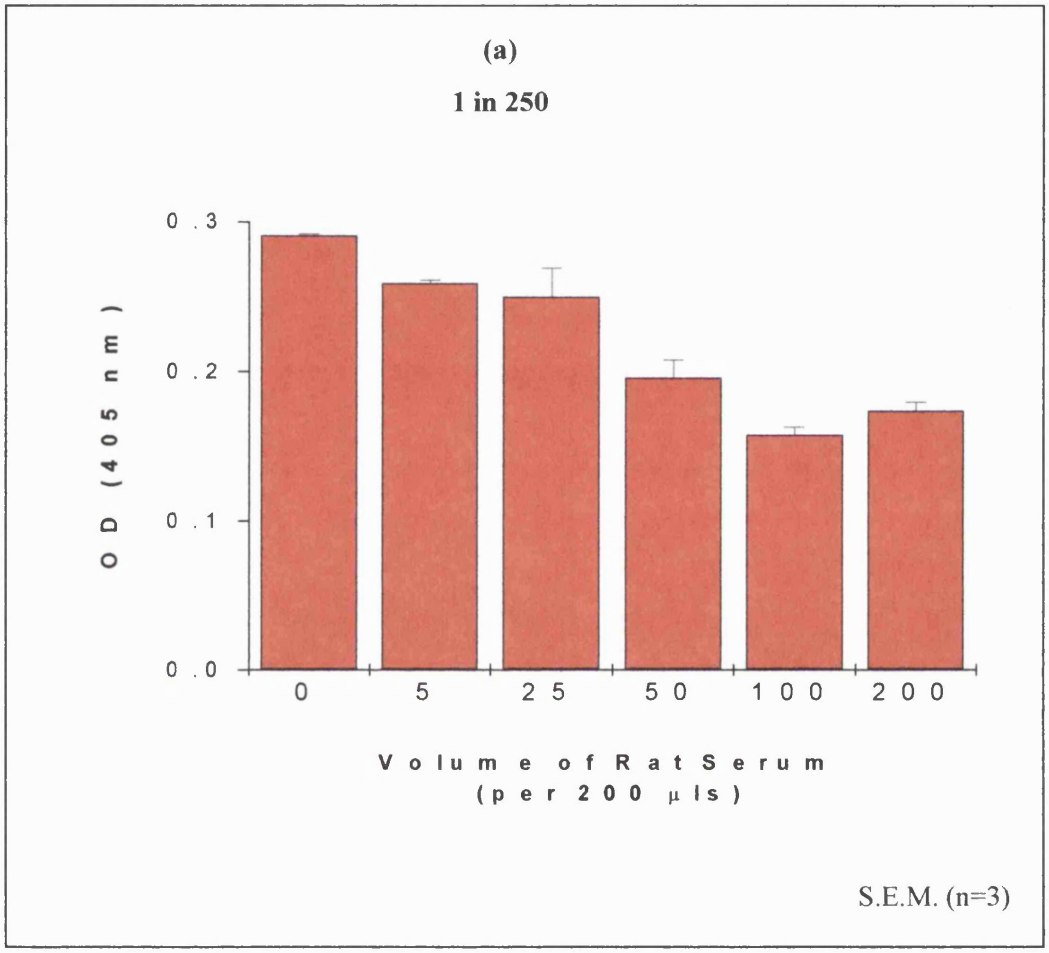


Figure Legend

The plot in Figure 2.4 a shows the results from the incubation of 200,000 RBL-2H3 cells with rat serum (between 5 and 200 µls, diluted in 300 µls EMEM), for 2 hours. Subsequently, the cells were incubated with with monoclonal mouse anti-rat IgE antibody, at a dilution of 1 in 250, diluted in HEPES AGM for 60 minutes. Negative controls were included, in which 200,000 cells were incubated with EMEM containing no IgE, and the cells were also lysed by the addition of a 5% Triton X-100 solution. The results are expressed as an optical density reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β- glucosaminidase, after a 30 minute incubation period.

Data indicated that incubation of RBL-2H3 cells with rat serum produced OD readings which were equivalent to the OD readings obtained when the cells were incubated without serum. The conclusion was that this combination of rat serum and mouse anti-rat IgE cross linker not was able to promote the degranulation of RBL-2H3 cells.

FIGURE 2.4 b

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING RAT SERUM AND MONOCLONAL MOUSE ANTI-RAT IgE

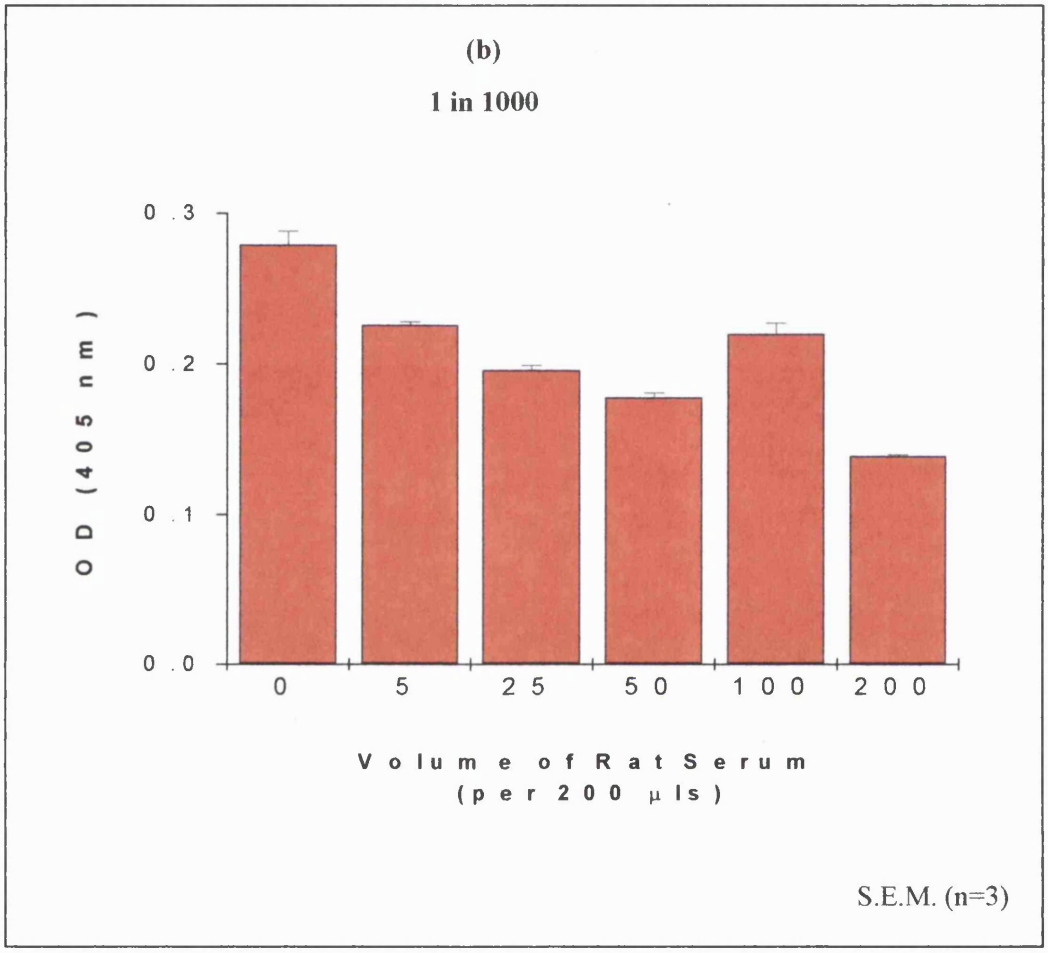


Figure Legend

The plot in Figure 2.4 b shows the results from the incubation of 200,000 RBL-2H3 cells with rat serum (between 5 and 200 µls, diluted in 300 µls EMEM), for 2 hours. Subsequently the cells were incubated with monoclonal mouse anti-rat IgE antibody, at a dilution of 1 in 1000, diluted in HEPES AGM for 60 minutes. Negative controls were included, in which 200,000 cells were incubated with EMEM containing no IgE, and the cells were also lysed by the addition of a 5% Triton X-100 solution. The results are expressed as an optical density reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β- glucosaminidase, after a 30 minute incubation period.

Data indicated that incubation of RBL-2H3 cells with rat serum produced OD readings which were equivalent to the OD readings obtained when the cells were incubated without serum. The conclusion was that this combination of rat serum and mouse anti-rat IgE cross linker not was able to promote the degranulation of RBL-2H3 cells.

were incubated with monoclonal mouse anti-rat IgE antibody, at a dilution of 1 in 250 (plot a) or 1 in 1000 (plot b), diluted in HEPES AGM. Negative controls were included, in which 200,000 cells were incubated with EMEM containing no IgE, and the cells were also lysed by the addition of a 5% Triton X-100 solution. The results are expressed as an OD reading at 405 nm wavelength, as measured by the detection of β-glucosaminide, terminated after 30 minutes of incubation.

Data indicated that incubation of RBL-2H3 cells with rat serum produced OD readings which were equivalent to those obtained when the cells were incubated without serum. When incubated with a 1 in 250 dilution of mouse anti-rat IgE antibody, all the OD readings fell approximately 0.2, compared with 0.29 from those cells without IgE myeloma protein. When incubated with a 1 in 1000 dilution of mouse anti-rat IgE antibody, the OD readings also fell approximately 0.2 compared with 0.28 from those cells without IgE myeloma protein. The data showed, therefore, that this combination of rat serum and mouse anti-rat IgE cross linker were not able to promote degranulation of RBL-2H3 cells.

It was concluded that neither combination of IgE with anti-IgE was an effective stimulator of degranulation of RBL-2H3 cells.

2.10.3.3 Cross-linking using anti-DNP-IgE and DNP-albumin

2.10.3.3.1 Optimal Concentration of anti-DNP-IgE

The plots in Figure 2.5 show the results from passive sensitisation of RBL-2H3 cells with Rat IgE with reactivity against dinitrophenyl-hapten (0.1 to 9 μg/ml), followed by stimulation with DNP-albumin at a concentration of either 0.1 μg/ml (plot a) or 1 μg/ml (plot b). The results are expressed as an OD reading at 405 nm wavelength which represents the OD of the product from the reaction catalysed by β-glucosaminidase after 30 minutes of incubation in the detection assay.

Data in plot 2.5 (a) shows that the degranulation response was approximately linear with 0.1 μg/ml DNP-albumin, with the OD values rising with increasing concentrations of IgE. At 0.1 μg/ml of IgE, no β-glucosaminidase release was detected when compared with those cells incubated without IgE. By 0.3 μg/ml of IgE, the degranulation response was small, with OD readings of approximately 0.2 in comparison with a reading of approximately 0.1 from incubation of RBL-2H3 cells without IgE. Incubation of cells with greater than 1 μg/ml of IgE, however, produced β-glucosaminidase levels significantly above the baseline, with a maximum OD reading of approximately 0.8 after incubation with 9 μg/ml IgE.

FIGURE 2.5 a

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING DNP-ALBUMIN (0.1 μg/ml) AND A VARIABLE CONCENTRATION OF ANTI-DNP IgE

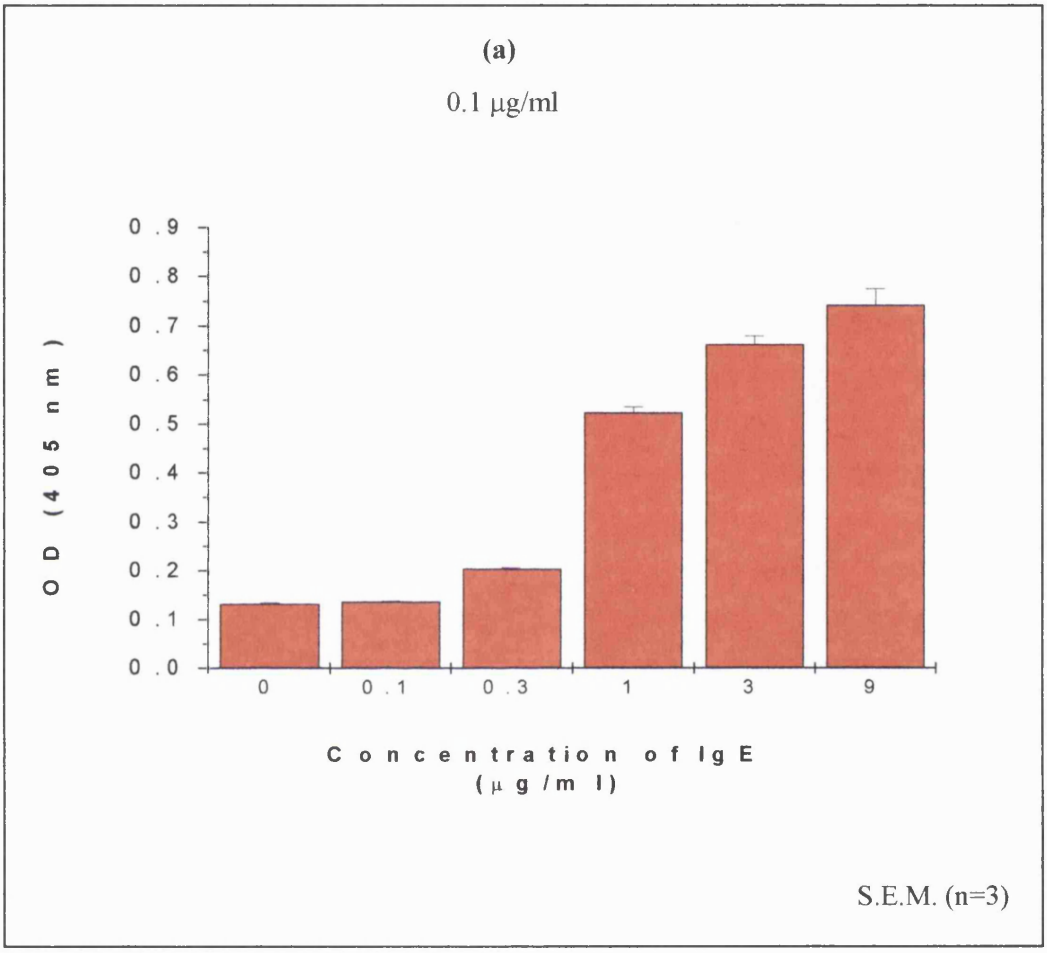


Figure Legend

The plot in Figure 2.5 a shows the results from the passive sensitisation of 200,000 RBL-2H3 cells with 300 μls Rat IgE, with reactivity against dinitrophenyl-hapten (concentration range from 0.1 to 9 μg/ml) diluted in EMEM, and incubation for 3 hours. Subsequently, the RBL-2H3 cells were stimulated with 200 μls DNP-albumin at a concentration of either 0.1 μg/ml and incubated for 2 hours. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase after a 30 minute incubation period.

Data shows that the quantities of β- glucosaminidase released upon degranulation rose with increasing quantities of IgE, when the cells were incubated with 0.1 μ g/ml of DNP-albumin.

FIGURE 2.5 b

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING DNP-ALBUMIN (1 μg/ml) AND A VARIABLE CONCENTRATION OF ANTI-DNP IgE

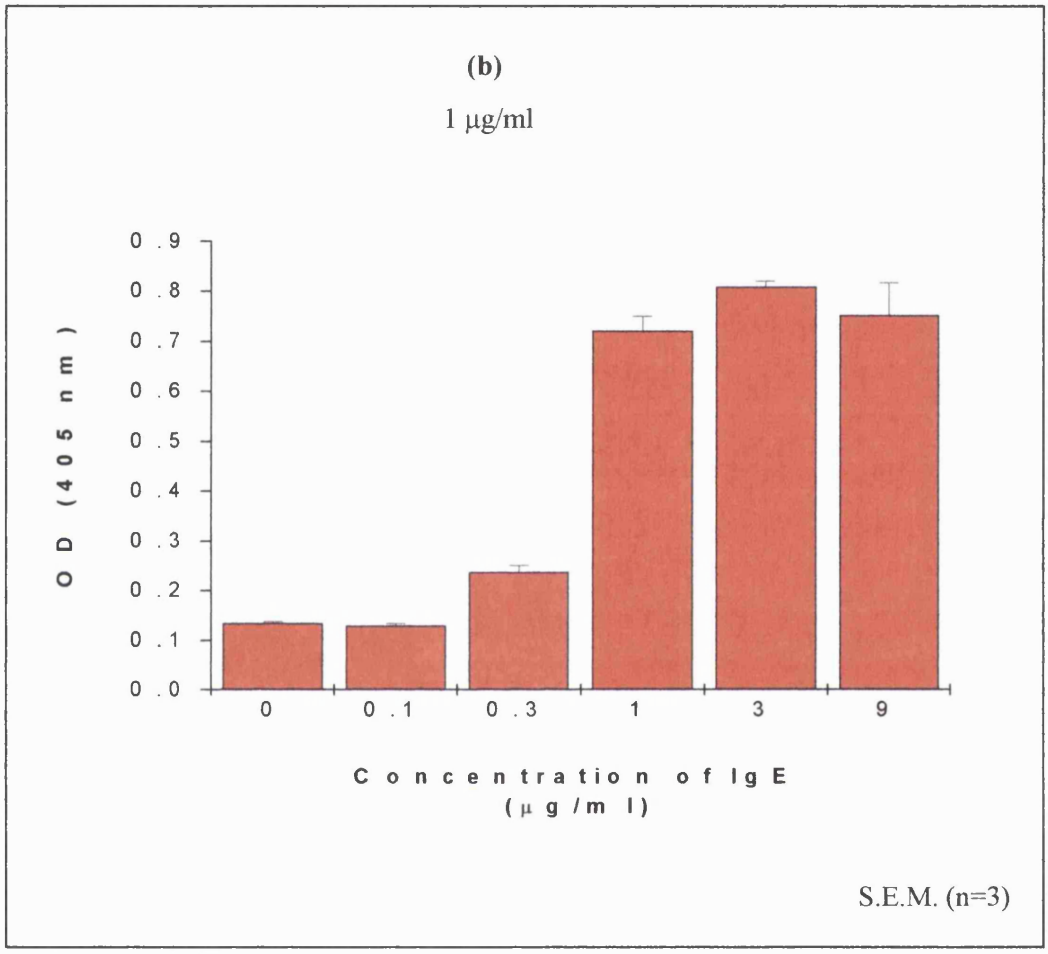


Figure Legend

The plot in Figure 2.5 b shows the results from the passive sensitisation of 200,000 RBL-2H3 cells with 300 μls Rat IgE, with reactivity against dinitrophenyl-hapten (concentration range from 0.1 to 9 μg/ml) diluted in EMEM, and incubation for 3 hours. Subsequently, the RBL-2H3 cells were stimulated with 200 μls DNP-albumin at a concentration of 1 μg/ml and incubated for 2 hours. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase after a 30 minute incubation period.

Data shows that the quantities of β- glucosaminidase released upon degranulation rose with increasing quantities of IgE, whether the cells were incubated with 1 μ g/ml of DNP-albumin.

Data in plot 2.5 (b) shows that the degranulation response was approximately a sigmoid response with 1 μg/ml DNP-albumin, with the OD values rising with increasing concentrations of IgE, reaching a plateau at greater than 3 μg/ml IgE. As with plot 2.5 (a), neither 0.1 nor 0.3 μg/ml of IgE produced levels of β-glucosaminidase significantly greater than cells incubated without IgE. Incubation of cells with greater than 1 μg/ml of IgE, however, produced β-glucosaminidase levels significantly above the baseline, with a maximum OD reading of approximately 0.8 after incubation with 3 μg/ml IgE.

2.10.3.3.2 Optimal concentration of DNP-albumin

The plot in Figure 2.6 shows the results from the stimulation of 200,000 RBL-2H3 (Batch 1) cells with 300 μl Rat IgE, with reactivity against dinitrophenyl-hapten, at a concentration of 3μg/ml, following a 3 hour incubation. Subsequently, the RBL-2H3 cells were stimulated with 200 μl dinitrophenyl–albumin (DNP-albumin) in a concentration range from 0.0001 to 100 μg/ml and incubated for 2 hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, with the reaction terminated after 30 minutes.

The plot shows a bell shaped curve, with the peak of degranulation achieved at a concentration of approximately 0.11 μg/ml DNP-albumin. Above and below this concentration of DNP-albumin, the degranulation response is reduced, and at concentrations between 0.001 μg/ml and 100 μg/ml the degranulation response is almost undetectable.

2.10.3.3.3 Time of Incubation with DNP-albumin

The plots in Figure 2.7 (a and b) shows the results from the passive sensitisation of 200,000 RBL-2H3 cells with 300 μl anti-DNP IgE (3μg/ml) diluted in EMEM, and incubated for 3 hours. Subsequently, the cells were stimulated by the addition of 200 μl DNP-albumin at a concentration of either 0.1 μg/ml (a) or 1 μg/ml (b) and incubated for 2 hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

The results show a sigmoid shaped curve, with increasing quantities of β-glucosaminidase released as the time of incubation with DNP-albumin was increased. Less than approximately 15 minutes of incubation, whether with 100 or 1 μg/ml DNP-albumin, the quantities of β-glucosaminidase released were small and not significantly above the levels released from those cells without the IgE incubation. After 30 minutes of incubation, however, the OD values had

FIGURE 2.6

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING ANTI-DNP IgE AND A VARIABLE CONCENTRATION OF DNP-ALBUMIN

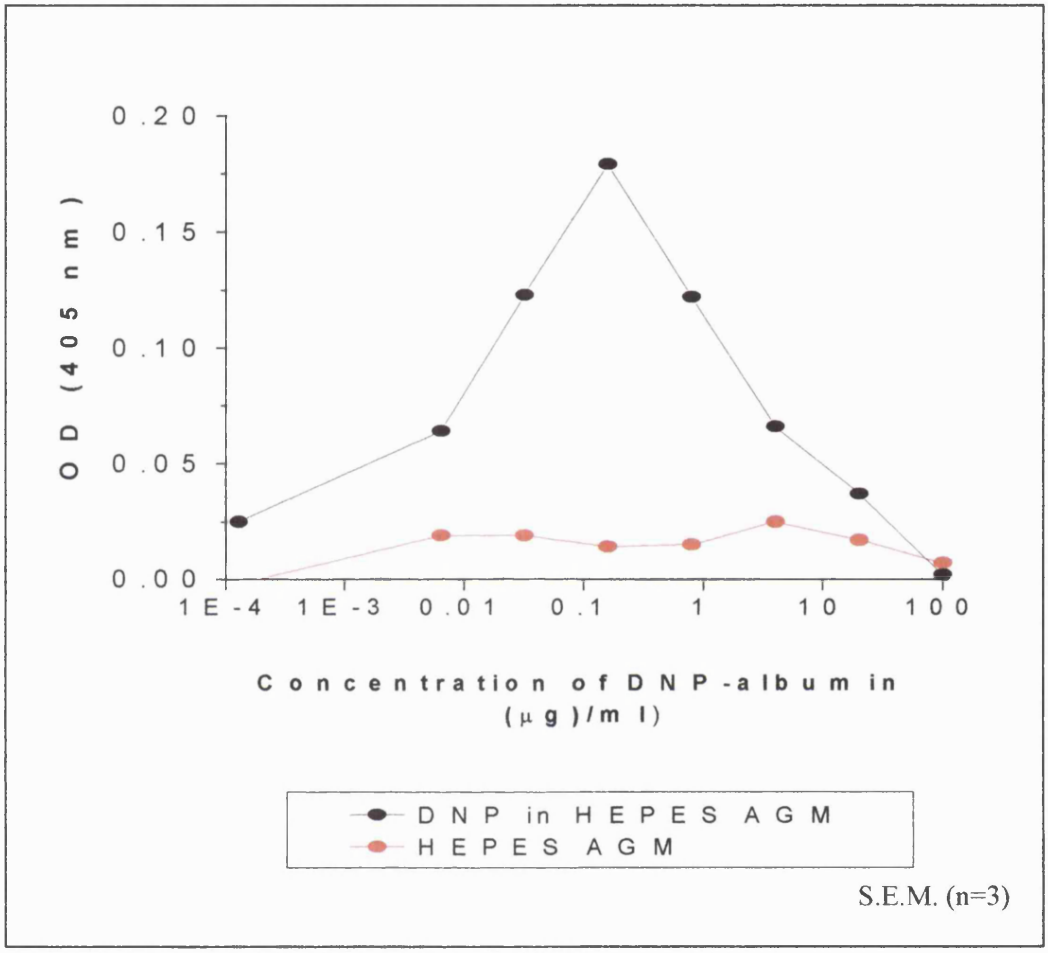


Figure Legend

The plot in Figure 2.6 shows the results from the stimulation of 200,000 RBL-2H3 cells with 300 µls Rat IgE, with reactivity against dinitrophenyl-hapten, diluted in EMEM to a concentration of 3 µg/ml, and incubated for 3 hours. Subsequently, the RBL-2H3 cells were stimulated with 200 µl dinitrophenyl–albumin (DNP-albumin) in a concentration range from 0.0001 to 10 µg/ml, and incubated for 2 hours. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase, terminated after 30 minutes.

The plot shows a bell shaped curve, with the peak of degranulation achieved at a concentration of approximately 0.11 µg/ml DNP-albumin. Above and below this concentration of DNP-albumin, the degranulation response is significantly reduced, and by 0.001 µg/ml and 10 µg/ml, the response is virtually zero.

FIGURE 2.7 a

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING ANTI-DNP IgE AND DNP-ALBUMIN, DNP-ALBUMIN INCUBATIONS FROM 5 TO 120 MINUTES

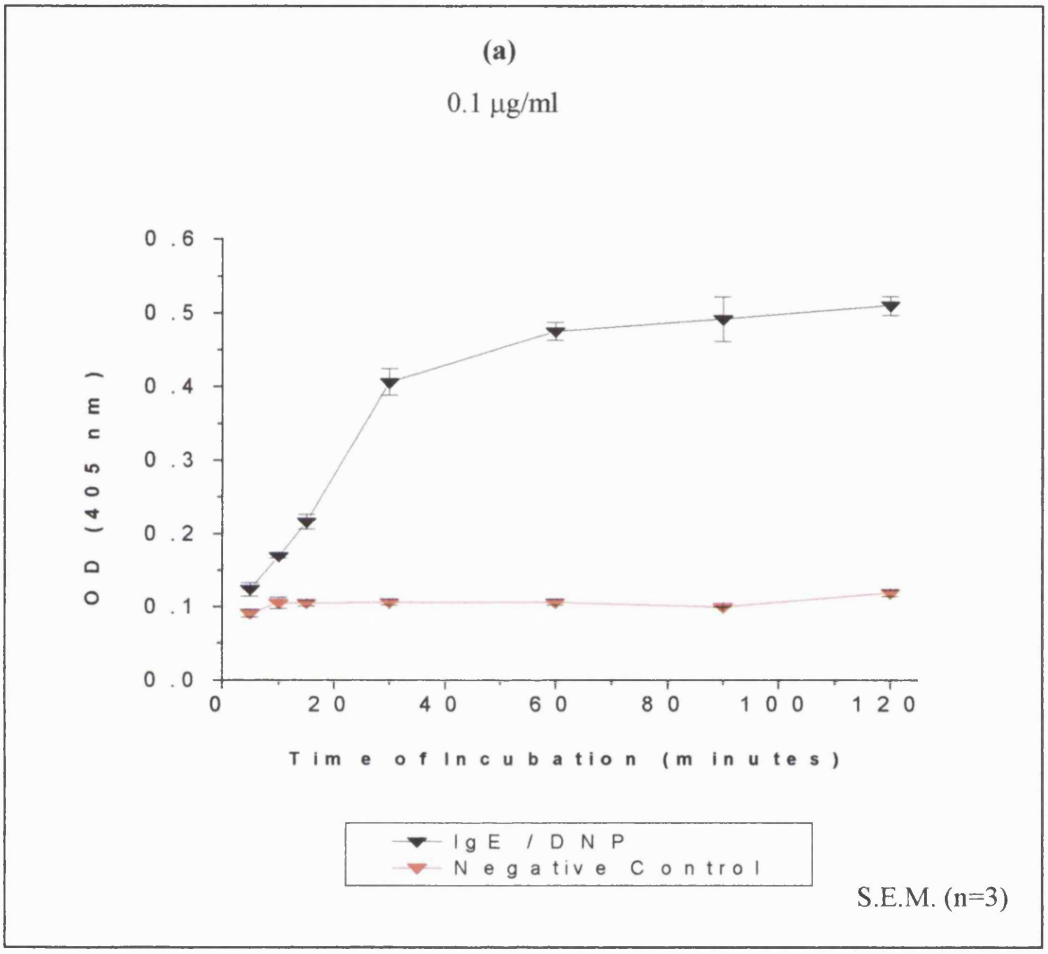


Figure Legend

The plot in Figure 2.7 a shows the results from the passive sensitisation of 200,000 RBL-2H3 cells with 300 µls Rat IgE, with reactivity against dinitrophenyl-hapten (3µg/ml) diluted in EMEM, and incubation for 3 hours. Subsequently, the cells were stimulated by the addition of 200 µls DNP-albumin at a concentration of 0.1 µg/ml, and incubation for 2 hours. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase, terminated after 30 minutes.

The results show a sigmoid shaped curve, with increasing quantities of β-glucosaminidase released as the time of incubation is extended.

FIGURE 2.7 b

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING ANTI-DNP IgE AND DNP-ALBUMIN, DNP-ALBUMIN INCUBATIONS FROM 5 TO 120 MINUTES

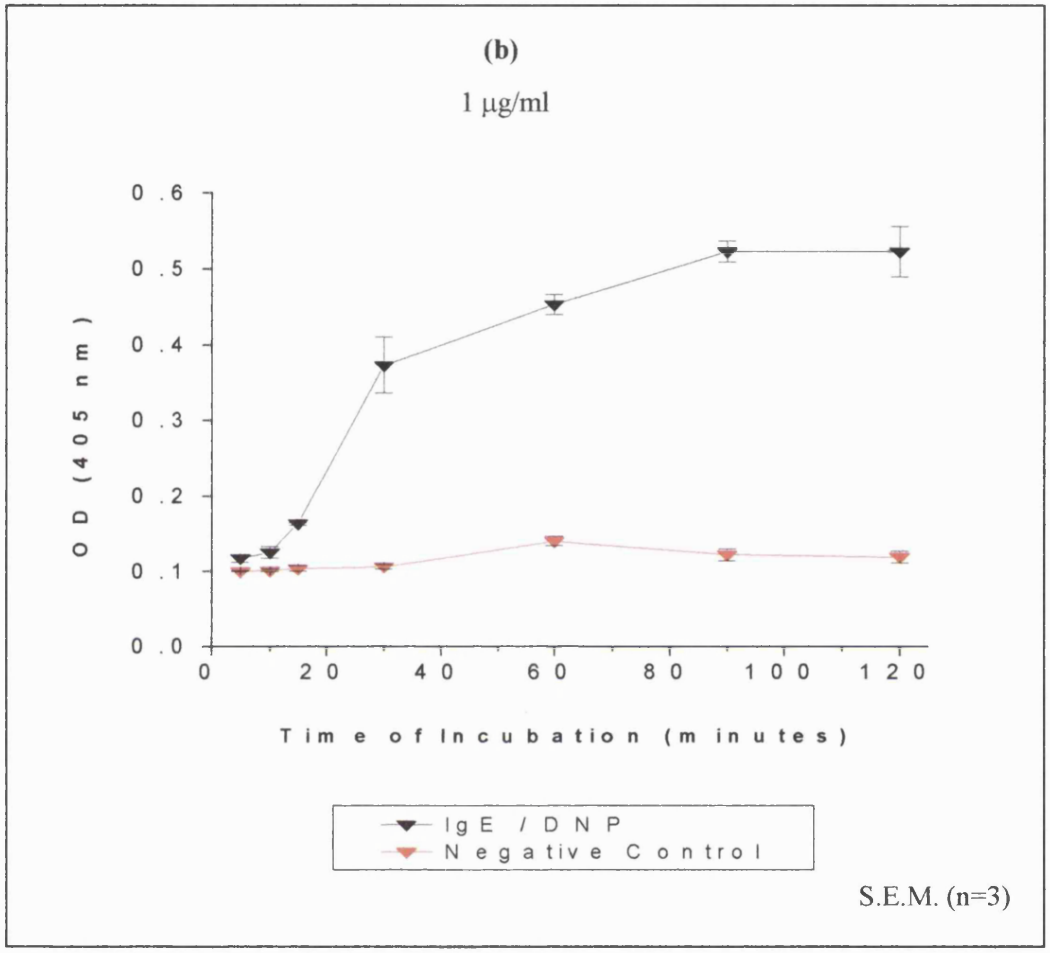


Figure Legend

The plot in Figure 2.7 b shows the results from the passive sensitisation of 200,000 RBL-2H3 cells with 300 µls Rat IgE, with reactivity against dinitrophenyl-hapten (3µg/ml) diluted in EMEM, and incubation for 3 hours. Subsequently, the cells were stimulated by the addition of 200 µls DNP-albumin at a concentration of 1 µg/ml, and incubation for 2 hours. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase, terminated after 30 minutes.

The results show a sigmoid shaped curve, with increasing quantities of β-glucosaminidase released as the time of incubation is extended.

risen significantly above baseline, reaching a maximum of approximately 0.5 in both instances after 90 minutes of incubation. In neither experiment did incubation of the RBL-2H3 cells without IgE promote degranulation after incubation with DNP-albumin.

2.10.3.3.4 Optimal time of IgE Incubation

The plot in Figure 2.8 shows the results from the incubation of 200,000 RBL-2H3 cells with anti-DNP IgE (3 μg/ml) diluted in EMEM, for time periods of between 5 and 180 minutes, followed by stimulation with DNP-albumin (1 μg/ml) and incubation for 2 hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after a 30 minute incubation in the detection assay.

Following 5 minutes of incubation with IgE, the OD reading had reached 1.5, compared with readings of approximately 0.2 with the negative controls. After 30 minutes of incubation with IgE, the assay has essentially reached saturation, with an OD reading of approximately 2. None of the RBL-2H3 cells incubated without either IgE or DNP-albumin released significant quantities of β-glucosaminidase, supporting the assumption that the stimulation of RBL-2H3 cells requires both IgE and the appropriate cross-linker.

2.10.3.3.5 Optimal time of β-glucosaminidase assay

The data in Figure 2.9 shows the results from the incubation of the supernatant of stimulated RBL-2H3 cells in the β-glucosaminidase assay, and incubation from between 0 and 120 minutes. The supernatant was derived from the passive sensitisation of 200,000 RBL-2H3 cells with 300 μl anti-DNP Rat IgE for 3 hours, followed by stimulation with 200 μl of DNP-albumin at 1 μg/ml for 3 hours. The supernatants were pooled and 50 μl aliquots were incubated in the β-glucosaminidase assay. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase.

The data shows that the OD readings obtained with RBL-2H3 supernatants increased linearly with increasing time of incubation in the β-glucosaminidase assay, to a maximum of 2.6 and 1.3 with neat and 10% supernatants respectively. With increasing time, the value of the negative control (HEPES AGM) rose linearly, although the maximum value gained was only 0.3. A comparison of the data in Figures 2.9 and 2.10 was performed. Figure 2.10 shows the results from the incubation of the supernatant from RBL-2H3 cells in the β-glucosaminidase assay, and incubation from between 0 and 120 minutes. In this experiment the supernatant was

FIGURE 2.8

IgE-MEDIATED CELL STIMULATION OF RBL-2H3 CELLS USING ANTI-DNP IgE AND DNP-ALBUMIN, IgE INCUBATIONS FROM 5 TO 180 MINUTES

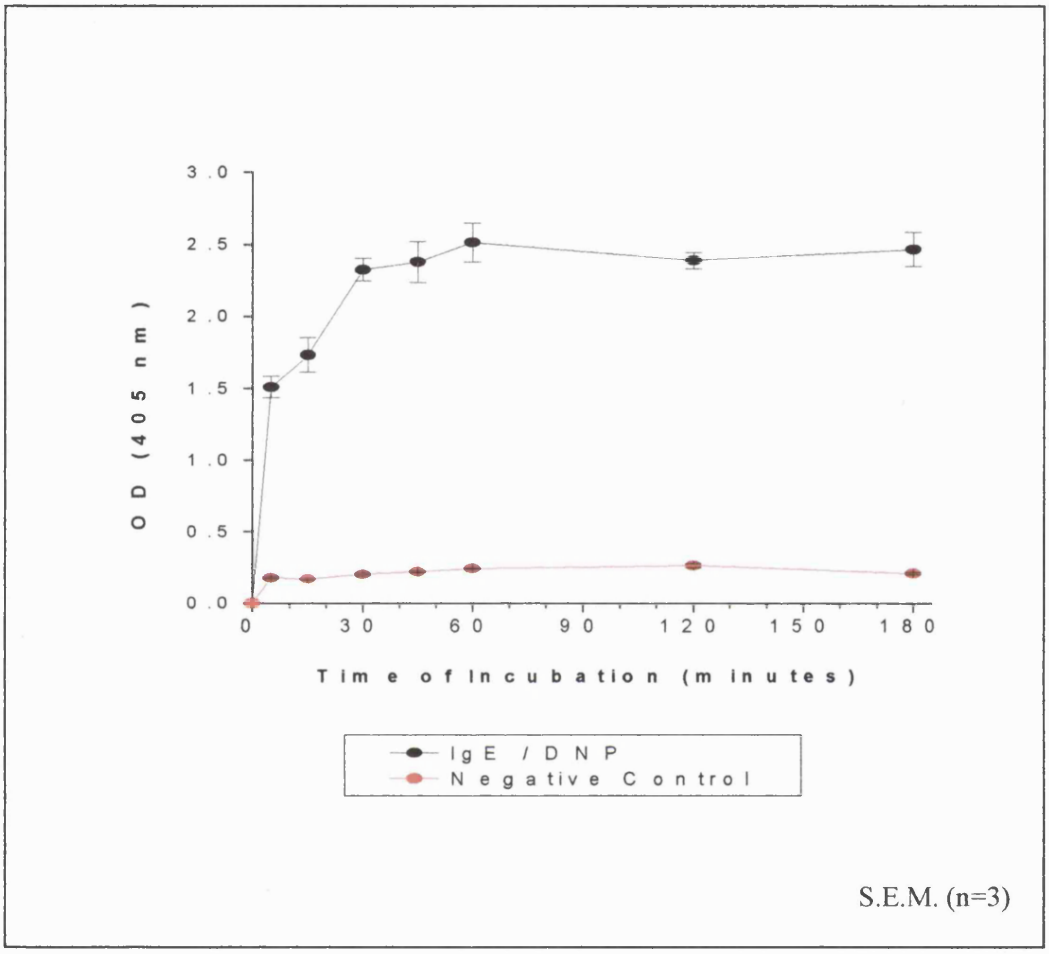


Figure Legend

The plot in Figure 2.8 shows the results from the stimulation of 200,000 RBL-2H3 cells with Rat IgE, with reactivity against dinitrophenyl-hapten (3 μg/ml) diluted in EMEM, incubated for time periods of between 5 and 180 minutes, followed by stimulation with DNP-albumin (0.1 μg/ml) and incubation for 2 hours. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β- glucosaminidase, with the β- glucosaminidase assay terminated after 30 minutes .

Data shows that, after 30 minutes of incubation with IgE, and stimulation with DNP-albumin for 3 hours, the RBL-2H3 cells release the maximal amount of β-glucosaminidase. From 30 to 180 minutes, the assay has essentially reached saturation.

FIGURE 2.9

DILUTION OF IgE-STIMULATED RBL-2H3 CELL SUPERNATANT, VERSUS TIME OF INCUBATION IN THE β- GLUCOSAMINIDASE ASSAY

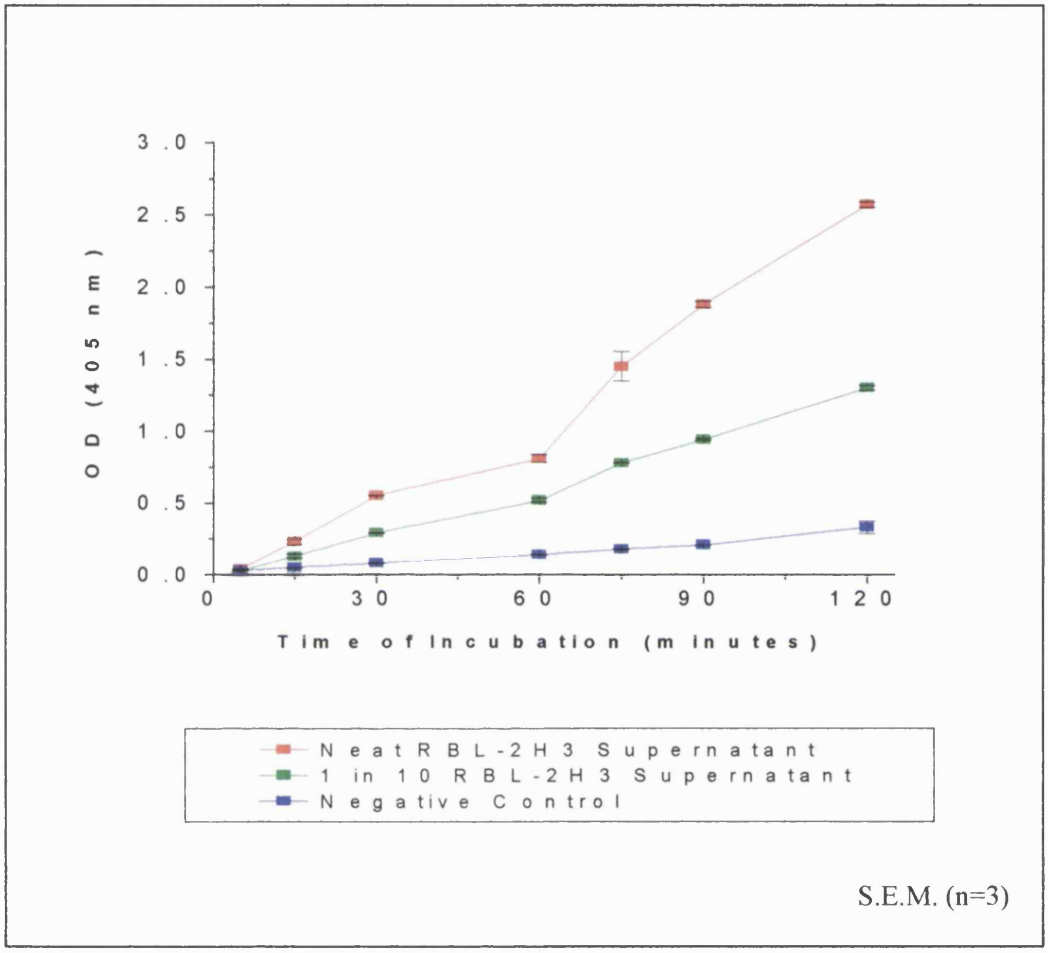


Figure Legend

The data in Figure 2.9 shows the results from the incubation of the supernatant of stimulated RBL-2H3 cells in the β- glucosaminidase assay, and incubation from between 0 and 120 minutes. The supernatant was derived from the passive sensitisation of 200,000 RBL-2H3 cells with 300 µls anti-DNP Rat IgE for 3 hours, followed by stimulation with 200 µls of DNP-albumin at 1 µ g/ml for 3 hours. The supernatants were pooled and 50 µl aliquots were incubated in the β- glucosaminidase assay. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β- glucosaminidase, after a 30 minute incubation period.

The data shows that the OD readings obtained with RBL-2H3 supernatants increased linearly with increasing time of incubation in the β- glucosaminidase assay. With increasing time also, the value of the negative control (HEPES AGM) also rose linearly, although the maximum values gained were relatively small.

FIGURE 2.10

VARIOUSLY STIMULATED RBL-2H3 CELL SUPERNATANTS, VERSUS TIME OF INCUBATION IN THE β-GLUCOSAMINIDASE ASSAY

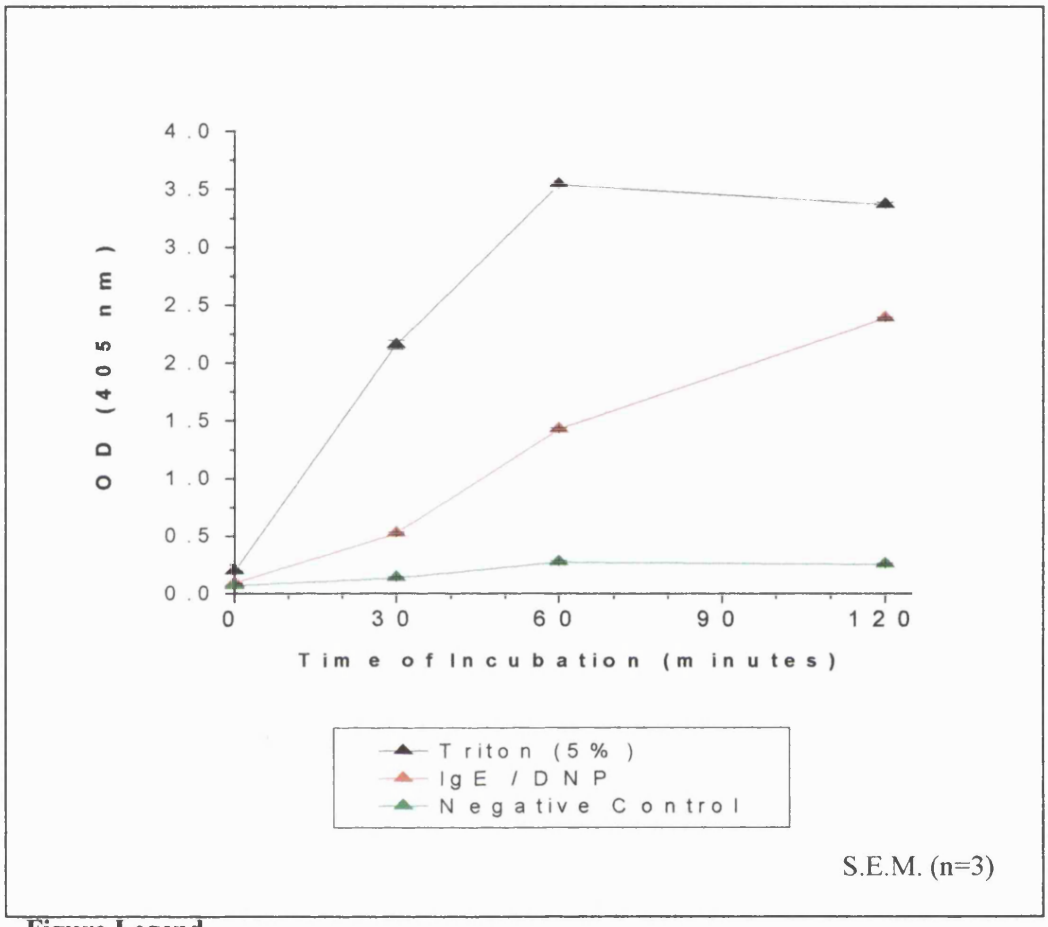


Figure Legend

The data in Figure 2.10 shows the results from the incubation of the supernatant of stimulated RBL-2H3 cells in the β- glucosaminidase assay, and incubation for 0 and 120 minutes. The supernatants were derived from the passive sensitisation of 200,000 RBL-2H3 cells with anti-DNP Rat IgE for 3 hours. Subsequently, the RBL-2H3 cells were incubated with (DNP-albumin at 1 μg/ml for 3 hours. Positive controls involved lysis with 5% Triton X-100, and the negative controls involved incubation without either IgE or DNP-albumin. The supernatants were pooled and 50 μl aliquots were incubated in the β- glucosaminidase assay. The results are expressed as an OD reading at 405 nm wavelength, and represent the OD of the product from the reaction catalysed by β-glucosaminidase.

The data shows that the OD readings obtained with IgE-stimulated RBL-2H3 supernatants increased linearly with increasing time of incubation in the β-glucosaminidase assay. Those cells incubated with IgE and DNP reached a maximum OD after incubation for 120 minutes, whereas the negative controls remained small during the entire time. The RBL-2H3 that had undergone triton lysis reached a maximum OD after 60 minutes, after which time the readings did not rise significantly.

either derived from IgE-mediated cell stimulation, as outlined in the details of Figure 2.9, or from Triton lysis of the RBL-2H3 cells, or from negative controls. The supernatants were pooled and 50 µl aliquots were incubated in the β-glucosaminidase assay. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase.

The data shows that the OD readings obtained with RBL-2H3 supernatants increased linearly with increasing time of incubation in the β-glucosaminidase assay. However, after 60 minutes of incubation in the β-glucosaminidase assay, the results from the Triton-lysed cell supernatants had reached a plateau. No further extension of incubation increased the OD reading. It was decided, therefore, that the β-glucosaminidase assay should be terminated after 60 minutes of incubation, to avoid saturating the assay, and make that the experimental results difficult to compare.

2.11 Discussion

2.11.1 Introduction to the Rat Basophil Leukaemia Cell Line

The Rat Basophil Leukaemia (RBL) cell line was selected for experimentation during the course of this thesis, based on evidence that it was analogous to 'normal' tissue mast cells. This cell line emerged from investigations using the potent leukaemia-promoting compound, β-chlorethylamine [2-(α-chlor-β-isopropylamine) ethylnapthalene hydrochloride] (β-chlor). A study of Wistar rats, fed a diet of β-chlor for the first three months of life, resulted in over 90% of the animals developing myeloid or lymphatic leukaemia (Leonard, 1968). A 15-month-old rat proved to be particularly interesting, as it developed a pure basophilic leukaemia, the first of its kind reported in a laboratory animal. The resultant RBL tumour was maintained by serial passage in neonatal Wistar rats (Eccleston *et al.*, 1973). The RBL tumour was dispersed to individual cells by mincing the tumour mass, followed by filtration through sterile glass wool, and then adapting the cells to suspension cell culture. After four months in culture, the basophil leukaemia cells displayed a stable phenotype, when compared with the original tumour cells (Kulczycki *et al.*, 1974).

RBL cells show many features that make them ideal candidates for *in vitro* research, including homogeneity, resistance to damage and capacity for long-term storage in liquid nitrogen. In addition, the cells grow attached to plastic surfaces and can be easily maintained in a culture incubator (Kulczycki *et al.*, 1974).

2.11.2 Development of sub-lines of the RBL cell line

Between 1973 and 1975, four further cell lines were derived, by the limiting dilution method, from the original RBL tumour, and were named RBL-I to RBL-IV consecutively (Barsumian *et al.*, 1981). RBL-I, II, III and IV were all shown to possess similar levels of IgE receptors and a similar capacity to bind IgE. However, when stimulated to release allergic mediators by an Immediate Hypersensitivity-type reaction, there was large variability in the quantity of mediators released. Early reports suggested that these RBL I to IV sub-lines were an inadequate substitute for 'normal' mast cells.

Further sub-culture of the IV cell line resulted in the RBL-IV HR+ line, developed in 1976, from which the RBL-2H3 cell line was derived (Barsumian *et al.*, 1981). Preliminary experiments showed that the RBL-2H3 line was consistent in its release of histamine (over 40% release) after stimulation with either IgE or ionophore. Further study was, however, necessary to determine whether this cell line was a suitable replacement for human tissue mast cells.

2.11.3 Experimental Responses of the RBL-2H3 cell line

As discussed in the Introduction, a primary characteristic of the mast cell is its responsiveness to IgE-mediated stimulation. It was vital to ascertain that the RBL-2H3 cell lines used for the duration of this thesis were similarly responsive. To this end, a number of experiments were performed to determine the reactivity of RBL-2H3 cells to IgE passive sensitisation, and to cross-linking with various agents.

The results indicated that the RBL-2H3 cells were stable over the course of the experimentation and routinely release measurable levels of mediators upon both IgE-dependent and IgE-independent stimulation. The experiments that were performed will be discussed in greater detail, with reference to the results gained during the course of this doctoral thesis.

2.11.4 Non-IgE-mediated release from RBL-2H3 cells

2.11.4.1 Triton-mediated release of mediators from RBL-2H3 cells

The experimental use of Triton X-100, indicated that the detergent was an effective reagent for the complete lysis of RBL-2H3 cells. Triton X-100 is a widely used non-ionic surfactant, which is employed in experimental situations for the recovery of membrane components. Simply, Triton X-100 breaks down the membrane structure of the RBL-2H3 cells, causing the contents of the cells to be released (Collins & Salton, 1979). The mediators detected represent the entire complement of cell contents.

2.11.4.2 Ionophore A23187-mediated release of mediators from RBL-2H3 cells

These studies demonstrated the non-toxic release of mediators from RBL-2H3 cells by the calcium ionophore, A23187. The results, illustrated in Figure 2.2, support these findings by showing that A23187 leads to the release of mediators from RBL-2H3 cells in a time and concentration dependent manner. In conclusion, for the research performed for this thesis, the A23187 ionophore was selected as an IgE-independent positive control for the release of mediators from RBL-2H3 cells.

These experimental results support previous studies in which RBL-2H3 cells were shown to be responsive to ionophore A23187-mediated stimulation. Experiments showed that the addition of A23187 to cultured RBL-2H3 cells resulted in calcium influx, phospholipase activation, and histamine release (Urata & Siraganian, 1985).

2.11.4.2.1 Introduction to Ionophores

Ionophores are organic compounds that facilitate the transport of ions from one aqueous medium to another, through a natural or artificial lipid membrane (Pressman *et al.*, 1976). These compounds were first identified in microorganisms, although several synthetic compounds have also been found to have ionophore-like properties, and have been employed in experimental studies.

Ions are charged particles that are naturally unable to cross hydrophobic lipid barriers. The primary function of an ionophore lies in their ability to form hydrophilic structures within the membrane structure, in which the ions can pass. Originally, it was believed that the ionophores formed static channels within membranes, as illustrated through experimentation with gramicidin, which formed ion-conducting channels across lipid bilayers and membranes (Kilbourn *et al.*, 1967). An alternative theory emerged, however, when experiments were performed with membranes that were physically too thick to accommodate channels (Pressman *et al.*, 1976). The ionophore-aided passage of ions through such thick membranes suggested that certain ionophores functioned as mobile ion carriers. The term 'ionophore' literally means ion bearer, to emphasize the dynamic aspect of the transport mechanism. The term quasi-ionophore is used to describe ionophores that form channels.

Mobility is reliant on the ionophores' ability to form lipid soluble complexes with polar cations, of which potassium, sodium, calcium and magnesium are the most biologically significant. Ionophores are diverse in structure, but typically contain strategically placed oxygen atoms, which form a central ring or cavity into which the cation is inserted (Pressman *et*

al., 1976). The exterior of the ionophore comprises various hydrocarbon groups, including ether, carboxyl and hydroxyl, thereby protecting the hydrophillic interior of the molecule.

2.11.4.2.2 Calcium ionophore A23187, a carboxylic ionophore

Carboxylic ionophores have a carboxyl group at the C-terminus of the molecule and one or two hydroxyl groups or an amino group, in the case of A23187, at the N–terminus. A23187 is a unique ionophore, since it predominantly selects divalent ions in preference to monovalent ions (Pfeiffer *et al.*, 1974). This is most probably due to the inclusion of two nitrogen atom ligands in the structure. A23187 is capable of stimulating a number of calcium-dependent biological reactions without disturbing pre-existing balances of sodium and potassium. In addition, it can transport magnesium, although this ion is rarely implicated in cellular control mechanisms.

2.11.4.2.3 Role of the cytoskeleton in the A23187-mediated degranulation of RBL-2H3 cells

Histamine release from RBL-2H3 cells was inhibited by microtubule-depolymerising agents and microtubule-stabilising agents, indicating that the exocytotic mechanism stimulated by A23187 was dependent on a functional microtubule cytoskeleton. By comparison, histamine release was enhanced by the addition of the microfilament-disrupting agents, cytochalasins A, B and D. Although cytochalasins are reported to inhibit glucose uptake by cells (Griffin *et al.*, 1982), their ability to enhance RBL-2H3 degranulation is believed to be related to increased calcium uptake as a result of altered cell membrane fluidity.

RBL-2H3 cells were stimulated with ionophore A23187 or anti-IgE, and the cell surface and cytoskeletal changes were examined by both fluorescence microscopy (FM) and scanning electron microscopy (SEM) (Sahara *et al.*, 1990). After exposure of the cell to either secretagogue, the cells underwent rearrangement of the cytoskeleton. The changes seen were more pronounced with anti-IgE than with the ionophore. Cytoskeletal changes were associated with histamine release, which was dependent, both on dose of the secretagogue and on the extracellular calcium concentration. It was demonstrated that the cytoskeletal component, actin, was most closely associated with the alteration upon stimulation.

2.11.4.2.4 Mechanism by which A23187 promotes the degranulation of RBL-2H3 cells

Histamine release, following the addition of A23187 to mast cells, was also inhibited by the addition of the metabolic inhibitor, antimycin A, which blocks oxidative phosphorylation. It is believed that the inhibitory activity of antimycin A is related to its ability to decrease cellular ATP levels (Fewtrell & Metzger, 1981). Histamine release was also inhibited by the addition of

protease inhibitors. The concentrations at which these inhibitors were effective were high and it is possible that they alter the membrane function, resulting in an inhibition of calcium influx into the cell.

A23187 appears to function by transporting calcium into the cell and thereby initiating a reaction, dependent on intact metabolic processes, which results in the secretion of histamine (Foreman *et al.*, 1973). It was suggested that magnesium is exchanged for calcium during this ionophore-mediated shift, in order to maintain the ionic balance, although this magnesium shift was not thought to be essential for cell degranulation.

The ionophore-mediated degranulation of rat peritoneal mast cells, after exposure to A23187, was also studied using both light and electron microscopy. The mast cell was an ideal candidate for study, as the large size of its secretory granules allowed light microscopic observation of granule extrusion in living cells. Under phase contrast light, isolated rat peritoneal mast cells were observed to extrude whole secretory granules when exposed to A23187. Secretion was abolished when the cells were treated with EDTA or were suspended in a calcium-free environment (Cochrane & Douglas, 1974). This type of secretion of whole granules is called exocytosis. Electron microscopy of peritoneal mast cells following exposure to A23187 confirmed the results obtained with light microscopy. In addition, electron microscopy further established that the type of exocytosis was sequential, or compound exocytosis (Kagayama *et al.*, 1974).

2.11.5 IgE-mediated stimulation of RBL-2H3 cells

2.11.5.1 Introduction

As discussed in the Introductory Chapter, a primary characteristic of the mast cell is its responsiveness to IgE-mediated stimulation and it was vital that the RBL-2H3 cell lines used for the duration of this thesis were similarly responsive. A number of experiments were performed to determine the reactivity of RBL-2H3 cells to IgE passive sensitisation, and the sensitivity of the mast cells to undergo degranulation after the addition of a specific cross-linking agent. Results from these experiments confirmed that mast cell degranulation is significantly more complex than the simple cross-linking of surface IgE molecules, and the complexity of the degranulation of RBL-2H3 cells is more fully described in the following sections.

2.11.5.2 Passive sensitisation of RBL-2H3 cells with IgE

It is well-documented (Section 1.3.12) that free IgE has the capacity to associate, at high affinity, with its receptors. To achieve this *in vitro*, however, requires incubating the cells in a

solution containing IgE, a process which is called 'passive sensitisation' (Van Arsdel & Sells, 1963). Passive sensitisation is defined as 'the acquisition of reactivity to a specific antigen following incubation of the cells with the serum of an allergic individual'. Clearly, the specific detection of a particular antibody on the recipient cell surface is not directly possible. The success of passive sensitisation is judged in terms of the amount of mediators released from the passively sensitised leukocytes after interaction with the antibody-specific antigen (Levy & Osler, 1966).

Studies involving passive sensitisation of human leukocytes with ragweed allergen successfully demonstrated that the leukocytes retained the full capacity for degranulation during, and subsequent to, *in vitro* passive sensitisation. That is, passive sensitisation with antigen-specific IgE enables the cell to degranulate in response to specific antigen, but does not adversely affect the cells' function.

Of paramount importance in the establishment of the RBL line as an analogue of Type One Hypersensitivity was the full investigation of IgE-mediated degranulation in RBL-2H3 cells. It was essential, firstly, to select an IgE that could be used to passively sensitise the RBL-2H3 cells.

2.11.5.2.1 Sources of IgE: Human IgE-secreting immunocytomas

The experimental study of IgE was limited by the inability to identify and purify immunoglobulin proteins in sufficient quantities. In 1967, a myeloma was identified in a human patient, which was shown to secrete immunoglobulin-type molecules. These immunoglobulin-secreting myelomas were termed immunocytomas. The immunoglobulin, however, lacked the antigenic determinants of the pre-existing A, D, G or M classes (Johansson & Bennich, 1967), and a variety of studies were performed to elucidate its characteristics.

The first indication of the nature of this novel myeloma protein was demonstrated through its ability to inhibit the Prausnitz and Köstner reaction in a human subject, a characteristic that had previously been demonstrated by the so-called 'atopic reagin', which was also under investigation at that time. The myeloma protein was injected intradermally either before, or together with, a sensitising reagin previously shown to induce the characteristic wheal and flare response. The inclusion of the myeloma protein with the sensitising agent completely inhibited the wheal and flare response (Stanworth *et al.*, 1967 (b)). The official classification of the atopic reagin as immunoglobulin E (IgE) (Bennich, 1968), enabled the characteristics of this myeloma protein to be re-evaluated and it was determined that the myeloma protein belonged to the 'E' class (Ogawa *et al.*, 1968).

The immunocytomas were capable of secreting huge quantities of IgE. In one human IgE myeloma, for example, serum IgE levels were raised to 30 mg/ml, compared with atopic serum IgE levels of 10 μg/ml (Ogawa *et al.*, 1971). Clearly, the IgE-secreting capacity of the tumour was immense, and provided researchers with an ideal resource for study of the IgE protein.

2.11.5.2.2 Sources of IgE: Rat IgE-secreting immunocytomas

In rats, immunocytomas were identified in an inbred albino LOU / Wsl strain, in which tumours spontaneously arose in the ileocoecal lymph node of the gastrointestine after approximately 12 months of life (Bazin *et al.*, 1972). In rare cases, the mass of proliferating cells could be identified as plasma cells although, in the majority of cases, the tumours were poorly differentiated.

Study was specifically directed towards two particular rat strains, LOU/M/Wsl and LOU/C/Wsl. To determine the specific type of immunoglobulin secreted by the rat tumours, serum derived from 250 ileocoecal immunocytomas was purified, most typically through ammonium sulphate precipitation, DEAE chromatography and gel filtration. The serum concentrations of IgE were variable, although levels as high as 50 to 60 mg/ml were found (Bazin *et al.*, 1973 (a)). Agarose electrophoresis, immuno-diffusion and immuno-electrophoretic techniques were used to determine the class of immunoglobulin secreted by these tumours. The studies revealed that 184 of the 250 tumours studied secreted monoclonal immunoglobulin antibodies, including IgG, IgM and Bence Jones proteins (immunoglobulin proteins without heavy chains). Sixty three of the tumours secreted IgE (Bazin *et al.*, 1973 (b)).

The high incidence of IgE secretion could be attributed to the origin of the tumours, in the ileocoecal region of the intestine. IgE-secreting plasma cells are localised in the gastro-intestinal and respiratory mucosa, and their regional lymph nodes in man (Ishizaka & Ishizaka, 1971). Although, in the rat, numbers of IgE-secreting plasma cells in the ileocoecal region are fewer, the numbers are dramatically raised with infection by *N. braziliensis*. It is possible, therefore, that a pre-existing raised IgE level encouraged the production of IgE-secreting cells in the gastrointestine. Malignant tumours originating in the gastrointestinal region may be derived, therefore, from IgE-secreting plasma cells.

2.11.5.2.3 Purification of IgE from IgE-secreting immunocytomas

The discovery of the IgE-secreting myelomas permitted both the isolation and characterisation of the IgE proteins and the production of anti-IgE antibodies. Anti-IgE antibodies were created by intramuscular injection of purified IgE, emulsified in complete Freunds adjuvant, into the

host animal. A second injection was administered a few days later, consisting of a higher dose of IgE in incomplete Freunds adjuvant. Purification methods were employed, to remove both non-anti-IgE immunoglobulin contaminants and antigenic contaminants (Isersky *et al.*, 1974).

The use of myeloma-derived IgE was limited in *in vivo* studies with mast cells. It was determined that none of the IgE myelomas produced IgE with the capacity to bind antigen (Stanworth, 1971). It appeared that the myeloma IgE represented a pathological counterpart to normal IgE, as was similarly observed with the over-production of the other immunoglobulin classes in plasmocytic and lymphocytic neoplastic disorders.

The lack of antigenic reactivity limited the use of the myeloma IgE in antigen-mediated cell stimulation experiments. However, the use of the myeloma IgE in the creation of anti-IgE antibodies facilitated the extraction of IgE from alternative sources.

2.11.5.2.4 Purification of IgE from alternative sources

The purification of IgE from many different sources was achieved through the use of immuno-absorbent columns. These columns were created by the adsorption of anti-IgE antibodies onto absorbent agents, such as Sepharose, which were then packed into columns (Iserksy *et al.*, 1974).

Animals infected with parasites provided some valuable information regarding the production of parasite-specific IgE. As discussed in Section 1.3.4, helminth infections provide a very effective stimulus for the production of high titres of IgE (Jarrett, 1974). The purified IgE from parasite infected rats was shown to have similar properties, including sensitivity to heat, and cross-reactivity with anti-IgE antibodies, to the myeloma-derived IgE (Kulczycki *et al.*, 1974). The IgE was also recognised by the IgE receptors on mast cell surfaces (Ogawa *et al.*, 1971).

The parasite-derived IgE, however, was of limited use in the IgE-mediated degranulation of mast cells. When the antigenic specificity of IgE derived from *Nippostrongylus brazilensis*-infected rats was determined, it was found that the IgE molecules possessed specificity against numerous antigenic determinants, including both helminth and non-helminth antigens (Ogilvie, 1967). Therefore, any mast cells bathed in this IgE would become coated with IgE with specificity against numerous antigens. As discussed, specific mast cell degranulation requires the cross-linking of IgE molecules with particular antigenic specificity. It is unlikely, therefore, that these mast cells would undergo antigen-specific degranulation. Furthermore, the production of an IgE with cross-reactivity against a specific antigen was of paramount importance.

2.11.5.3 Production of antigen-specific IgE by mouse IgE hybridomas

The generation of antibodies, with affinity to a particular antigen, was achieved by the fusion of normal, antibody-secreting plasma cells with murine plasma-cell tumours. This lead to the creation of stable hybrid cell lines, commonly called hybridomas, which express characteristics of both parental cell lines (Köhler & Milstein, 1975).

The first demonstration of an IgE-producing myeloma cell was reported in 1978. Using the technique described by Köhler and Milstein, spleen/mesenteric lymph node cells were sensitised by intraperitoneal injection of ovalbumin (OA) (Böttcher *et al.*, 1978). The OA sensitised cells were then fused with IgG1(k)-synthesising myeloma cells. The hybrid cells were positively selected, and existence of IgE anti-OA antibodies was determined. Of more than 2,000 hybrid cells tested, only one produced the IgE anti-OA antibodies (IgE-14-205).

The characteristics of the hybrid (IgE-14-205) anti-OA antibody were determined and it was confirmed that the antibody behaved like a typical IgE. Characteristics included loss of activity after heating at 56°C for 2 to 4 hours, and positive results in anti-rat IgE immuno-diffusion experiments. Specificity against ovalbumin was confirmed by cross-linking studies, in which passive cutaneous anaphylaxis was effected by the addition of ovalbumin but not other antigens.

Following the creation of IgE with anti-OA specificity, the production of IgE antibodies with specificity for alternative antigens was attempted. The production of IgE-secreting hybridomas with dinitrophenol (DNP) specificity was first documented in 1980 (Bottcher *et al.*, 1980). In this instance, murine spleen cells were primed with DNP-keyhole limpet haemocyanin (DNP-KLH) and fused with myeloma cells. Of the 1,000 clones tested, 80 clones with IgE-anti-DNP secreting capability were identified, a much larger percentage than the previous study with ovalbumin.

As before, the characteristics of the hybrid IgE anti-DNP antibody were determined, and it was confirmed that the antibody behaved like a typical IgE. Characteristics included positive results with immuno-diffusion experiments against anti-mouse IgE antiserum, and removal of PCA capability by pre-incubation of the anti-DNP antibody with anti-mouse IgE serum. Specificity against ovalbumin was confirmed by cross-linking studies, in which passive cutaneous anaphylaxis was only effected by the addition of ovalbumin but no other antigens.

Further anti-IgE antibodies were created using this method. IgE with anti-DNP specificity was specifically purified and concentrated from hybrid H1-DNP-ε-26.82. The purification method involved ammonium sulphate precipitation, affinity chromatography against DNP-BSA-Sepharose 4B, ion exchange chromatography and gel filtration. The IgE was then employed in *in vitro* studies (Liu *et al.*, 1980) and it was found that the IgE retained biological activity even after the extensive purification.

2.11.5.4 *In vitro* use of murine anti-DNP-IgE with RBL-2H3 cells

Previous studies had shown that the FcεR1 receptor had the capacity to bind IgE from either mouse or rat (Taurog *et al.*, 1977). This meant that either rat or mouse-derived IgE could be used for passive sensitisation of rat cells. It was important to ascertain whether this was also the case with the hybridoma-derived IgE. If there was no cross-reactivity between mouse and rat IgE, then the use of murine anti-DNP-IgE antibodies with the RBL-2H3 cell line would not be possible and it would be necessary to create anti-hapten IgE-producing hybridomas from rat myeloma cells.

The anti-DNP IgE derived from H1-DNP-ε-26.82 was used to passively sensitise RBL-2H3 cells, after which time, degranulation of the cells was promoted by the addition of either antigen (DNP-BSA) or anti IgE antibodies (NMS-adsorbed-RAME). The conclusion was drawn that the artificially-created mouse IgE antibodies were an effective substitute for normal serum-rat IgE in IgE mediated degranulation, in that they could effect degranulation of rat mast cells, following their cross-linking.

2.11.5.5 IgE and cross-linkers unable to promote measurable RBL-2H3 cell degranulation

Difficulties were encountered in the choice of the IgE, for the passive sensitisation of the mast cells, and in the choice of cross-linking agent. A number of IgE and cross-linker combinations proved incapable of promoting measurable degranulation of RBL-2H3 cells. Experimental results indicated that neither purified rat IgE myeloma protein with monoclonal goat anti-rat IgE antibody (described in Section 2.9.3.1), nor IgE-containing rat serum with monoclonal mouse anti-rat IgE antibody (described in Section 2.9.3.2) was an effective combination to promote measurable mediator release from RBL-2H3 cells.

There are a number of explanations for these results, including an insufficient concentration of IgE, insufficient time for passive sensitisation, or insufficient time for cross-linking. It is unlikely that the IgE concentration was too low or was unable to bind effectively to the receptor. In addition, the FcεR1 receptor has the capacity to bind IgE from either mouse or rat,

as well as hybridoma-derived IgE (Taurog *et al.*, 1977). The species from which the IgE was derived should, therefore, not affect the ability of the IgE molecules to bind to the RBL-2H3 FcεR1 receptor.

In addition, as indicated in the results from the successful experiments with anti-DNP IgE and DNP-albumin, the concentrations of IgE and the time of incubation was sufficient for passive sensitisation of the RBL-2H3 cells. It is, therefore, unlikely that the RBL-2H3 cells were insufficiently coated with IgE molecules.

2.11.5.5.1 Stimulation of RBL-2H3 cells by monomers, dimers, trimers and oligomers

The most likely explanation for the inefficacy of these IgE and anti-IgE combinations is the inability of the anti-IgE antibodies to cross-link a sufficient number of receptors. Each anti-IgE antibody is designed to cross link only two IgE molecules, regardless of the affinity of IgE with anti-IgE antibody. To determine the efficacy of artificial oligomers of IgE at stimulating the degranulation of normal tissue mast cells and RBL-2H3 cells, artificial dimers, trimers and oligomers were formed by treatment of IgE monomers with the cross-linking agent, dimethyl suberimidate.

The results obtained from the first studies indicated that the minimal cross-linking unit required for degranulation in either normal or tumour mast cells was the dimer. Monomers were incapable of stimulating degranulation, whereas dimers and oligomers showed comparable levels of mediator release (Segal *et al.*, 1977). More recently, results have confirmed that the dimer was the minimal unit required for normal mast cell degranulation, but questioned the results gained with RBL-2H3 cells. The data concluded that oligomers are routinely more effective at stimulating degranulation in the RBL-2H3 cell line, when compared with dimers or trimers of IgE (Fewtrell & Metzger, 1980). It would appear that the RBL-2H3 cells have a tightly regulated mechanism of discriminating between different polymeric bridging antibodies, which may explain the inability of the dimeric anti-IgE antibodies to stimulate degranulation from the RBL-2H3 cells. By comparison, DNP-albumin acts as an oligomeric antigen. Each unit of DNP-Albumin has the capacity to cross-link several IgE / FcεR1 complexes, thereby stimulating the degranulation of the RBL-2H3 cells.

2.11.5.5.2 Inability of the cross-linking antibodies to gain access to the binding site on the FcεR1 bound IgE molecule

An alternative theory suggests that the inability of these anti-IgE antibodies to promote measurable mediator release was a result of the inability of the cross-linking agent to gain

access to the specific binding site on the receptor-bound IgE molecule. As discussed previously, the IgE molecule is bound to the FcεR1 receptor through the Cε3 domain on the IgE molecule (Henry et al., 1997), and a schematic representation of the interaction between IgE and the FcεR1 receptor is shown in Figure 1.5.

A significant proportion of the surface IgE molecule is, therefore, occupied with its interaction with its receptor. If the target of the anti-IgE antibody is an epitope located in the region in which the antibody interacts with its receptor, the anti-IgE antibody will be unable to bind and will, therefore, be unable to promote degranulation of the target cell.

2.11.6 Anti-DNP IgE and DNP-albumin mediated degranulation of RBL-2H3 cells

2.11.6.1 Introduction

Degranulation of the RBL-2H3 cells was effected by passive sensitisation with rat IgE with reactivity against dinitrophenyl-hapten, followed by the addition of dinitrophenol-albumin (DNP-albumin).

Optimisation of the experiments to stimulate degranulation of RBL-2H3 cells, using this combination of IgE and cross-linking agent, was dependent on a number of factors. These included the viability of the cells, the capacity of the IgE to bind to the FcεR1 receptor and the capacity of the DNP-albumin to cross-link to IgE antibodies. Each of these variables was thoroughly investigated and an explanation of the results obtained is given below.

The data generated from experimentation with the RBL-2H3 cell line supported previous results that showed that mediator release was only achieved through multiple bridging of the receptor molecules. This multiple bridging of surface IgE molecules was effected using DNP-albumin as the cross-linking agent.

2.11.6.2 Negative Controls

The RBL-2H3 cells were unable to release mediators in the absence of either the IgE, or the DNP-albumin. Without IgE on the surface of the cells, the DNP-albumin had nothing to cross-link. Furthermore, without DNP-albumin, the HEPES-AGM contained no reagent with a capacity to cross-link the IgE molecules on the cell surface. The routinely low negative controls supported the assumption that the cells require both IgE and an IgE cross-linking agents to effect degranulation by an IgE-mediated route.

2.11.6.3 Optimal number of cells per well

The culture of the RBL-2H3 cells was highly dependent on the cell density. When the cell density was too low, the cultures often did not attain confluence. It was concluded that RBL-2H3 cells required a minimum number of adjacent cells surrounding them, probably to maintain adequate cellular contact and for the production of adequate concentrations of cytokine or growth factors. With too few cells, the remaining cells could not establish adequate contact, which resulted in inefficient proliferation. Furthermore, when the cell density was too high, after overnight incubation, the cells had often detached from the surface of the culture flask or plate leaving foci of clear flask which were visible under the microscope. Typically, contact between cells inhibits proliferation, but the results showed that above a certain RBL-2H3 cell density, detachment occurred rather than a simple reduction in cell proliferation.

2.11.6.4 Optimisation of RBL-2H3 degranulation with anti-DNP-IgE and DNP-albumin

The results showed that a concentration of IgE greater than 3 μg/ml was sufficient to passively sensitise RBL-2H3 cells and to induce the release of cell mediators upon contact with an adequate concentration of DNP-albumin. The results of stimulation using variable concentrations of DNP-albumin indicated that a peak of release was obtained with concentrations between than 0.1 and 1 μg/ml. Below 0.1 μg/ml, there was an insufficient number of units to cross link the receptors and thereby stimulate maximal release. Similarly, an excess of molecules also reduced the efficacy of release. At excessive concentrations the anti-DNP-IgE molecules would be in a 1:1 ratio with the antigen, and cross-linking could not occur, leading to a reduction in the levels of mediator released.

These results compare favourably with data presented in previous literature, including the studies performed by Metzger's group (Taurog *et al.*, 1977). These earlier experiments were performed with newly discovered murine mastocytoma cells lines, AB-CBF1 and MCT-1, although the MCT-1 line was subsequently re-classified as being of rat in origin and appears to be the RBL- cell line (Siraganian & Metzger, 1978). These cells were stimulated to undergo degranulation following exposure to various combinations of IgE and cross linking agents, including rat IgE with anti-IgE, and anti-ovalbumin IgE with ovalbumin. Briefly, their experimental technique involved passive sensitisation of 1 to 2 x 10^6 cells per ml for 1 hour in supplemented media containing 3 to 10 μg/ml of IgE. The cells were then washed with a buffered solution and incubated with cross-linking agents, prepared in buffer, for 30 to 60 minutes at 37°C, after which time mediator release was determined. Typically, the optimal concentration of ovalbumin required to cross-link the IgE molecules was between 1 and 10 μg/ml.

A comparison can also be made with the information provided from the pilot experiments, using the RBL-2H3 cells. Stimulation of RBL-2H3 cells was effected by incubation of the cells with 0.5 μg/ml of anti-trinitrophenol (TNP) IgE, followed by incubation with TNP-ovalbumin at a concentration of 10 ng/ml). Although these concentrations of IgE and ovalbumin are approximately 10-fold lower than the concentrations that were used for research performed for this thesis, the figures are comparable.

Experimental evidence supports the theory that higher valency antigens promote degranulation with greater potency than lower valency antigens (Collins *et al.*, 1996). RBL-2H3 cells were sensitised with one of three monoclonal IgE antibodies that bind to trinitrophenylated (TNP)-proteins with varying affinity. Degranulation was effected by exposure of the cells to the specific TNP-human serum albumin (HSA) and the relative success of the degranulation was determined. It was found that the higher the valency of the antigen, the more effective it was at stimulating degranulation. It is possible, therefore, that the TNP was of a higher valency than DNP, and could therefore effect degranulation of the RBL cells at lower concentrations.

Based on the data presented in this and other papers, it can be concluded that the results obtained using the RBL-2H3 cell line compare favourably with previous experimental results using the same cell line. It was responsive to passive sensitisation with anti-DNP IgE, and released measurable quantities of β-glucosaminidase upon contact with DNP-albumin.

211.6.5 Variation in RBL-2H3 releasability through time

During the course of experimentation, variation in the results was observed. It was essential to include adequate negative controls and to closely monitor whether the standard IgE-stimulation methodology required adjustment to achieve optimal release. Many factors undoubtedly contributed to the variability in the mediators detected upon the degranulation of the RBL-2H3 cells, including variation in the solutions prepared. For example, the dose response of the sensitised RBL-2H3 cells to DNP-albumin showed a narrow optimal range of between 0.1 and 1 μg/ml. Minor variation in this optimal concentration might dramatically affect the stimulation of the cells and, therefore, the quantity of mediators released. In addition, it was possible that different batches of fetal calf serum, or other components of the culture medium, may influence the release activity of the cells.

2.11.6.6 Similarities in the Degranulation of RBL-2H3 cells versus human tissue mast cells

The experiments that were performed confirmed that the RBL-2H3 cells were responsive to passive sensitisation with IgE, and could be stimulated to degranulate upon addition of antigen or anti-IgE antibody (Siraganian *et al.*, 1982). These results indicated that the RBL-2H3 cells contain a fully functional IgE-mediated degranulation response.

A further area that requires discussion is the extent to which the RBL-2H3 cell line represents an analogy to human mast cells, with particular reference to their respective degranulation responses.

The biochemical pathway of mast cell degranulation, as discussed in Chapter 1, is an extremely complex and specific pathway, involving a number of intracellular signalling pathways and second messengers. The complete degranulation pathway has yet to be elucidated, in either normal mast cells or tumour (RBL-2H3) cells. Therefore it is not possible, at this stage in time, to determine whether the IgE-mediated degranulation response of RBL-2H3 cells is biochemically comparable with human tissue mast cells. However, a number of studies have been performed, which suggest that sufficient similarities exist between rat and human mast cells. The following section (2.11.7) describes these studies.

2.11.7 Rat Mast cells versus human mast cells

A pivotal series of experiments were designed to ascertain whether the FcεR1-mediated cell signalling in human and mast cells was functionally comparable. The early experiments involved the transfection of human α units into rat cells with the aim of determining whether the human α subunit could effectively substitute for the rat α subunit. If this were possible, it would suggest that the human α unit was functionally identical to the rat α unit, and produce further evidence that might indicate that other parts of the FcεR1 mediated signal transduction were similar. Furthermore, it would support the argument that a study of a rat mast cell line would provide information that is comparable to the human mast cell.

2.11.7.1 Surface expression of human and rat FcεR1 sub-units

Transfection of any of the individual units of rat FcεR1 (α, β or γ) into COS cells was successfully achieved, as confirmed by Northern blotting. However, none was expressed on the cell surface when transfected individually. Surface expression was only observed after transfection of all four FcεR1 units, to create the αβγγ tetramer (Blank *et al.*, 1989). By comparison, after transfection of the human FcεR1 receptor units into rat mastocytoma cells, successful surface expression of the α unit was dependent only on the presence of the γ unit,

and the β unit was superfluous (Miller *et al.*, 1989). Transfection of the α unit together with the β unit did not produce surface expression of the α unit.

It would appear, therefore, that there is a fundamental difference in the requirements of surface expression of the human FcεR1 receptor, versus the rat FcεR1 receptor. Whether this was reflected in differences between the human and rat mast cell signalling remained to be determined.

2.11.7.2 Transfection of FcεR1 α units

Despite differences in the surface expression of the FcεR1 receptor, it was considered important to elucidate whether the human α unit could substitute for the rat α unit, and *visa versa*. As indicated by the previous experiments, surface expression of α alone was difficult to achieve. Efficient transfection of the α unit alone required the creation of a chimeric α unit. This was effected by substituting the transmembrane and cytoplasmic domains of the α subunit with those coding for the p55 IL-2 receptor, which had been shown previously to be expressed efficiently on cell surfaces. Transfection with the chimeric α receptor resulted in efficient cell surface expression of α on COS cells (Hakimi *et al.*, 1990).

These results suggested that unknown elements in the transmembrane or cytoplasmic sequences of the α subunit prevented surface expression, a prevention that could be ablated by the presence of the γ subunit. The α-transfected COS cells showed the capacity to bind both human and rat IgE, through the co-existence of both rat and human FcεR1 α units, although there was variation in the affinity of binding between different isotypes of IgE. It was concluded that the surface binding of IgE is entirely dependent on the existence of the α subunit of the high affinity receptor.

Experiments were devised, which involved the transfection of the P815 rat mastocytoma cell line with a plasmid containing the cDNA sequence of the human α unit of FcεR1, in combination with either, or both, of the sequences for the rat β and γ units. Cells transfected with either $\alpha_{(human)}\beta\gamma_{(rat)}$ or $\alpha_{(human)}\ \gamma_{(rat)}$ expressed as many as 1×10^5 to 1.5×10^6 receptors on their cell surface. These experiments confirmed that the human α unit could be effectively transfected into a rat mast cell line and were useful in understanding the specificity of binding. They did provide, however, no information as to the subsequent biochemical events. Neither the COS, nor the P815 cell line degranulates in response to FcεR1 cross-linking. Downstream events in the signalling from the human/rat receptors were, therefore, not studied (Miller *et al.*, 1989).

2.11.7.3 Signalling differences between human and rat mast cells

To fully elucidate the nature of the signalling events, it was necessary to study degranulation in a transfected mast cell line. Transfection experiments were performed to introduce the human α subunit of the FcεR1 receptor into RBL-2H3 cells. Following successful transfection, it was observed the α subunit formed complexes with the endogenous β and γ sub-units of the rat cells. Furthermore, the existence of the human α receptor unit provided the RBL cells with ability to respond to human stimuli.

IgE-mediated aggregation of human FceRIα subunits resulted in a series of biochemical events that were indistinguishable from the events mediated by the rat FcεRIα (Gilfillan *et al.*, 1992). These events included a rapid increase in the formation of inositol phosphates, an increase in intracellular calcium, selective phosphorylation of tyrosine residues, all of which ultimately contribute to the cells' degranulation.

The existence of cell lines, combining characteristics of both human and rat receptors, allowed experimentation without the laborious task of extracting and purifying human cells. For example, these cell lines have been used to determine the efficacy of binding of anti-human monoclonal antibodies to human FcεR1 α subunits, without the need for human cells (Lowe *et al.*, 1995).

2.12 Conclusions

The results described in Chapter 2 provide evidence that the RBL-2H3 cells employed in my research were stable over the course of the experimentation. The cells routinely released measurable levels of mediators upon passive sensitisation with anti-DNP IgE, and cross-linking with DNP-albumin. The standard experimental methodology for the IgE-mediated degranulation of RBL-2H3 cells involved overnight incubation of 200,000 RBL-2H3 cells in a 24 well plate, to allow the cells to adhere prior to experimentation. Subsequently, the cells were exposed to 3 μg/ml of IgE, prepared in supplemented EMEM media, and incubated for a minimum of 2 hours. The cells were washed thoroughly, exposed to DNP-albumin, at a concentration of 100 to 1000 ng/ml and then incubated for 2 to 3 hours. The release of mediators was quantitatively measured by the detection a mast cell mediator, β-glucosaminidase. The β-glucosaminidase assay was routinely terminated after a 30 minute incubation, after which time the OD of the β-glucosaminide product was measured at 405 nm wavelength.

The additional observation that rat receptor units could functionally substitute for human receptor units supports the theory that RBL-2H3 cells are sufficiently comparable with normal human mast cells. RBL-2H3 cells were chosen, therefore, as the ideal method of studying Type One Hypersensitivity reactions in an *in vitro* situation.

Chapter 3

Expression of GPI-PLD in RBL-2H3 Cells

3.1 Summary

The aim of the research performed in this chapter was to determine the expression of GPI-PLD in the cultured RBL-2H3 cells. Three techniques were selected for study: a radioactive GPI-PLD activity assay, Western Blotting and RT-PCR, to detect the enzyme's activity, protein and mRNA respectively.

No evidence of GPI-PLD mRNA was detected in the cultured RBL-2H3 cells, and thus it was concluded that these cells do not express the RNA for the GPI-PLD protein. However, GPI-PLD protein was detected by Western blotting and its activity was confirmed using the radioactive activity assay.

Analysis of the culture medium determined that the only source of GPI-PLD for the cells was the Foetal Bovine Serum that was used to supplement the medium. It was proposed that the cells take the GPI-PLD from the serum, in which it is present in high quantities, after which time the enzyme undergoes intracellular processing.

3.2 Introduction

3.2.1 Phospholipase enzymes

Glycosyl-phospholipase enzymes, of which GPI-PLD is an example, are part of a much larger group of enzymes, the phospholipases. Phospholipases are widely distributed in nature, and serve many biological functions. The main property that they have in common is their capacity to catalyze the cleavage of phospholipids, and they are classified on the basis of the type of bond they cleave, and the location of that bond (Low, 1990).

- A–type phospholipases hydrolyze the carboxylic ester linkages to produce fatty acids and lysophospholipids.
- C–type phospholipases hydrolyze the phosphodiester linkage to produce, for example, 1,2-diacylglygerol or alkylglycerol, and a polar head group
- D–type phospholipases hydrolyze the phosphodiester linkage to produce phosphatidic acid and the polar head group with an exposed hydroxyl group.

The majority of the phospholipase enzymes have the capacity to hydrolyse most phospholipids, and are often referred to as non-specific, or broad-specificity. Even though they have absolute specificity for the type of bond hydrolysed, non-specific phospholipases have little ability to discriminate between different types of phospholipids. Several phospholipases, however, have a requirement for a particular structural feature of the phospholipid. These phospholipases are called specific-phospholipases, of which glycosylphosphatidylinositol-phospholipases C and D (GPI-PLC and GPI-PLD) are examples. Prior to discussion of the enzymes, however, it is necessary to introduce the substrates against which they are catalytically active.

3.2.2 Substrates of phospholipase enzymes

3.2.2.1 The phospholipid bilayer

The phospholipid bilayer is a dynamic entity, composed of over 1000 distinct lipid species that are constantly being remodelled (Hjelmstad & Bell, 1991). The Fluid Mosaic model (Singer & Nicholson, 1972) proposed a construction whereby the phospholipid bilayer provides a structural matrix into which molecules are inserted. Two types of membrane proteins have been described, and their primary difference lies in their method of insertion into the bilayer. Intrinsic membrane proteins pass through the membrane, and are anchored by non-covalent hydrophobic interactions between the phospholipids and hydrophobic sequences within the protein. By comparison, extrinsic proteins are 'fixed' on one side of the bilayer, and use a hydrophobic terminal structure to interact with the phospholipids, allowing the hydrophilic portion to remain free from the lipids.

3.3 Phosphatidylinositol Phospholipase C (PI-PLC)

The existence of a novel extrinsic attachment was suggested through research into bacterial PI-PLC enzymes. PI-PLC enzymes were amongst the first specific phospholipases identified, through their involvement in the disease, anthrax. An unanticipated side-effect of anthrax was a significantly raised concentration of alkaline phosphatase (ALP) activity in the blood stream, a phenomenon called alkaline phosphatasemia (Smith, 1955).

Investigation of this phosphatasemia showed that it could be reproduced by injection of a soluble factor derived from *Bacillus anthracis* (Slein & Logan, 1960). A crude toxin preparation was injected intravenously into rabbits, and blood was drawn at intervals. The blood samples were analysed for a variety of serum components, including glucose, glycoproteins, cholesterol, alkaline phosphatase and serum enzymes. As little as 4 hours after injection of the toxin preparation, ALP levels had risen as much as 4 fold, compared with normal levels. The activity decreased slowly, but was still significantly higher than normal 71 hours after injection. Similar effects were observed after intravenous injection of the *B. anthracis* toxin into rats, mice and guinea pigs.

This toxic effect was also observed after intravenous injection of a culture filtrate derived from *Bacillus cereus*, inducing a marked increase in the blood ALP levels (Slein & Logan, 1962). Fractionation techniques, using N,N'-diethyl-aminoethyl cellulose, were designed to separate the phospholipase enzymes from the culture filtrate. The resultant fractionate contained two phospholipase enzymes, one of which displayed the capacity to induce alkaline phosphatasemia, which was termed phosphatasemia factor (PF), while the other enzyme inhibited the phosphatasemia. Intravenous injection of relatively large amounts of the purified PF resulted in the depletion of bone ALP (Slein & Logan. 1963).

Originally, this enzyme was termed lecthiniase, because of the ability of lecithin to inhibit its activity. The lecithinase was classified as a phospholipase, through its ability to cleave both phosphoryl choline, phosphoryl ethanolamine and phosphoryl inositol from the phosphatase group in a number of substrates. Upon closer examination, however, it was revealed that the PF enzyme specifically hydrolysed phosphatidyl inositol (PI), but had no activity against phosphatidyl Choline (PC), sphingomyelin (SM) or phosphatidyl ethanolamine (PE) (Slein & Logan, 1965). It was, therefore, suggested that ALP was attached to the membrane via a phosphatidylinositol (PI) structure (Izekawa, 1976), and that the target of the enzyme was this PI. The enzyme was, therefore, re-classified as phosphatidylinositol-phospholipase C, and represented a phospholipase with specificity for the PI structure. PI-PLC enzymes have been

identified in other bacterial species, including *Staphylococcus aureus* (Low & Finean, 1977 (b)) and *Bacillus thuringiensis* (Taguchi *et al.*, 1980)

The association between PI and ALP could either exist as a non-covalent binding, between a specific binding site on the enzyme molecule and the PI molecule, or as a covalent linkage. The suggestion of a non-covalent link was dismissed, largely due to two observations. Firstly, non-covalent interactions can be disrupted, by exposure to high ionic strength, divalent cations or extremes of pH. None of these disruptions promoted the release of significant amounts of ALP. In addition, non-covalent linkage is also inconsistent with the observation that the free-ALP, released after exposure to PI-PLC, could not re-associate with membranes (Low & Zilversmit, 1980). The evidence, therefore, argued against a non-covalent binding of the ALP to membrane-located PI. Evidence that the link was covalent, however, required analysis of the mechanism by which the phospholipase enzymes dissociated PI-linked substrates.

3.4 GPI anchored proteins, as substrates of PI-PLC

In vitro treatment of membranes with bacterial PI-PLC resulted in the release of various membrane proteins, including 5'-nucleotidase (Taguchi & Ikezawa, 1978) and acetylcholinesterase (AChE) (Low & Finean, 1977(b)). Analysis of these proteins, however, showed that they contained a structure that was different from the PI structure. The evidence to support the theory that an alternative structure existed was difficult to obtain, as experimental studies had specifically removed the protein from its anchoring structure. In order to determine the nature of the anchor, it was essential to identify a wide range of substrates in which this novel structure was thought to occur, and to purify these proteins without the removal of the anchor. This was achieved through the identification of the substrates, Thy-1 and mfVSG, whose characterisation will be discussed more fully in the subsequent Sections.

3.5 Structural analysis of glycoproteins

3.5.1 Thy-1 glycoproteins

Thy-1 is a cell surface glycoprotein, of relative molecular mass 18,000 daltons, one third of which represents carbohydrate (Morrison *et al.*, 1984). Thy-1 is a major constituent of thymocytes and neurons (Reif & Allen, 1966). The rat protein was selected for study because it is present in high quantities in cells, and because large quantities of rat thymus and brain could be obtained. The protein sequences of both rat and mouse Thy-1 have been analysed, and the mature protein consists of 111 to 112 amino acids, respectively (Williams & Gagnon, 1982). However, Thy-1 was susceptible to cleavage from the cell surface by *S. aureus* PI-PLC enzyme, which supported the idea that the protein contained a PLC-sensitive segment, in addition to the mature protein sequence (Boothroyd, 1985).

Initial analysis of the protein sequence indicated that Thy-1 did not contain a typical transmembrane sequence. However, examination of the cDNA of Thy-1 revealed a sequence of 31 codons that were not present in the mature protein sequence. These 31 amino acids contained an extremely hydrophobic stretch of 20 amino acids, including six leucine residues, although it was not believed that this stretch represented a transmembrane anchor (Seki *et al.*, 1985 (a)). The conclusion was drawn that Thy-1 is created as a molecule of 142 amino acids, 31 of which are removed, during the transformation into the mature product. However, pulse chase experiments, designed to study the transformation of the immature protein to the mature protein, found no evidence of post-translational processing. It was concluded that these 31 nucleotides provided a signal sequence, which directed the immature protein to the biosynthetic machinery responsible for attaching the non-protein tail, with the con-current loss of the 31 nucleotides (Seki *et al.*, 1985 (b)).

Extraction of the intact Thy-1 protein was possible as a consequence of the ability of Thy-1 to bind to non-ionic detergents. The C-terminal of Thy-1 protein was extracted using Brij 96 detergent, with purification through a Biogel column. However, the protein could not be analysed by standard gas chromatography analysis because of the presence of the detergent in the Thy-1 extract. This problem was bypassed by the development of a thin layer analysis method (Williams & Tse, 1985). Results from this technique indicated that, in addition to the known Thy-1 protein, mannose, myo-inositol, glucosamine, galactosamine, phosphate, glycerol and stearic acid were detected.

More detailed information concerning the anchor of Thy-1 was provided through the specific release of the protein from the fatty acid chains using *S. aureus*-derived PI-PLC (Homans *et al.*, 1988), after which the anchor was subjected to one and two dimensional NMR, gas chromatography mass spectroscopy and exoglycosidase digestion. The structural information that was obtained as a result of this analysis is discussed in Section 3.7.

3.5.2 Variable Surface Glycoprotein from *Trypanosoma Bruceii*

The study of the novel anchor was further advanced through research into the surface coating of *Trypanosoma bruceii,* the parasite responsible for the cattle disease, nagana. The related parasites, *T. rhodesiense* and *T. gambiense*, cause sleeping sickness in humans (Borst & Rudenko, 1994). Variable Surface Glycoprotein (VSG) proteins form a dense coating on the surface of each individual parasite. The ability of trypanosomes to periodically shed and replace this coating with a different sub-type of VSG, a process called antigenic variation, is

an important factor in the ability of trypanosomes to evade the host's immune system. The process by which antigenic variation was accomplished seemed a novel area of research.

VSG, derived from *T bruceii*, was chosen an ideal candidate for study. The parasite is relatively easy to cultivate in large quantities, and the VSG can be purified to homogeneity. Two forms of VSG, membrane-form (mf) and soluble (s), were specifically extracted from the surface of parasite (Cardoso de Almeida *et al.*, 1983), and analysis of the anchor bearing mfVSG was, therefore, possible.

3.6 Chemical analysis of the membrane attachment of VSG

Structural analysis of the membrane-form (mf)VSG indicated that it contained unusual chemical components, including a glycan, containing mannose, glucosamine and variable amounts of galactose (Holder & Cross, 1981) and an ethanolamine-amide, linked to the C-terminal amino acid of the target protein (Holder, 1983). The anchor was termed the Glycosyl-Phosphatidyl-Inositol (GPI) anchor, with the proteins being termed GPI-anchored proteins (Ferguson *et al.*, 1985).

Following the detailed analysis of Thy-1 and mfVSG, a number of other GPI-anchored proteins were identified. These included alkaline phosphatase (ALP) (Malik and Low, 1986), membrane-form bovine erythrocyte acetylcholinesterase (mfAChE) (Hoener *et al.*, 1990), decay accelerating factor (DAF) and basic fibroblast growth factor (bFGF) (Metz *et al.*, 1994). These substrates were extracted, purified and characterised.

3.7 Structure of the GPI-anchor

The complete structure of the GPI-anchor was first determined using the VSG substrate (Ferguson *et al.*, 1988) after which time the mammalian Thy-1 antigen was analysed (Homans *et al.*, 1988). A number of analytical techniques have been employed, including gas chromatography, mass spectroscopy, specific chemical cleavages and sequential digests with exoglycosidases, chromatography and NMR spectroscopy (Ferguson *et al.*, 1988). Essentially the same backbone structure was identified in both proteins. Subsequently, the composition and arrangement of the chemical constituents of the GPI-anchor has accumulated from many different structure studies. The GPI anchor contains a number of components, which are linked to one another in a precise sequential arrangement, and is schematically illustrated in Figure 3.1.

FIGURE 3.1

SCHEMATIC OF THE STRUCTURE OF THE GPI-ANCHOR

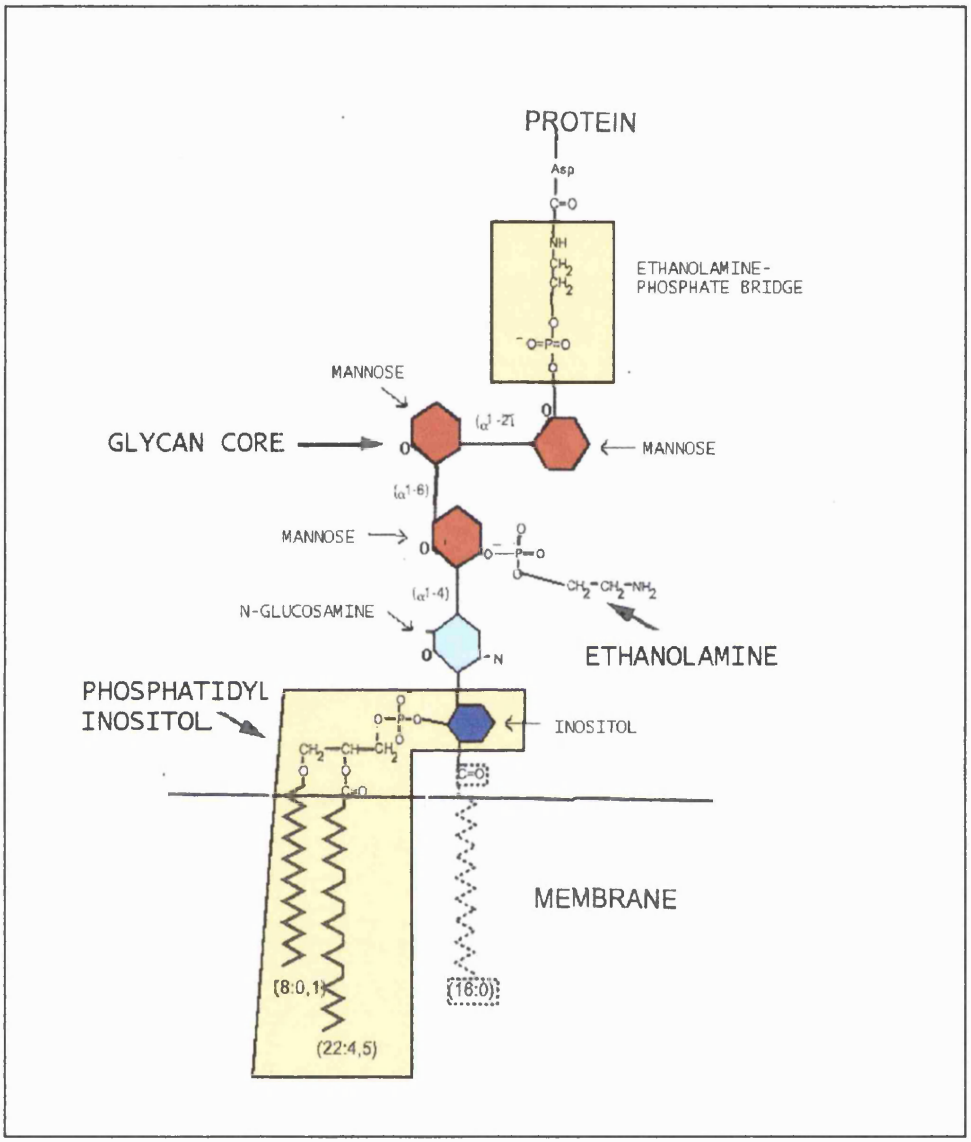


Figure Legend

GPI-anchored proteins are inserted into the membrane bilayer by fatty acid chains. The fatty acids chains are linked to an inositol phosphate residue, which is glycosidically linked to a deaminated N-acetyl glucosamine residue. The sequence typically follows with three residues of α-mannose, and ethanolamine phosphate is linked to the terminal mannose. The protein is linked to the GPI-anchor from the α-carboxyl group of the terminal amino acid to the amino group of the phosphoethanolamine. Variation in the core GPI structure includes the addition of ethanolamine phosphate residues or α-linked galactosyl side-chain residues, palmitoylation of the hydroxyl on the inositol ring and variation in the fatty acids.

3.8 Synthesis of the GPI-anchor

The process of synthesis of GPI-anchored proteins has been mapped in great detail. A great deal of information has been elucidated from the study of a disease of the blood system, paroxysmal nocturnal haemoglobinuria (PNH). Clinically, PNH is manifested by a number of complications with haematopoesis, including intravascular haemolysis and unusual venous thromboses. Biochemically, PNH is characterized by the existence of sub-populations of haemopoetic cells that exhibit deficiencies in GPI-anchored proteins. Approximately 15 proteins have been found to be lacking, or markedly deficient, on the abnormal blood cells (Rosse *et al.*, 1997). PNH has been shown to be due to defects in GPI anchor biosynthesis (Brown *et al.*, 1992). Present data strongly suggests that the abnormal PNH cells are unable to synthesize sufficient quantities of the GPI core (GlcNAc-PI), as a result of defects in the biosynthetic pathway (Mahoney *et al.*, 1992). In addition, a panel of T cell mutants that are unable to create GPI-anchored proteins was of great benefit in the elucidation of the synthesis of GPI-anchored proteins (Sugiyama *et al.*, 1991). The synthesis of the GPI-anchor core follows a stepwise addition, and is outlined in Figure 3.2.

Briefly, the process begins with the formation of a glycosidic linkage between N-acetyl-glucosamine and the inositol group of phosphatidylinositol. The inositol detected in the PI is typically D-myo-inositol (Ferguson *et al.*, 1985). The glucosamine then undergoes deamination, forming GlcN-PI (Doering *et al.*, 1989). Subsequently, three residues of α-mannose, derived from dolichol-phosphate-mannose (Menon *et al.*, 1990(a)), are added to the chain, forming Man(3)-GlcN-PI. Ethanolamine phosphate is then linked to a terminal mannose, forming EtN-P-Man(3)-GlcN-PI (Menon et al., 1990(b)). This structure, a GPI precursor, is called glycolipid A'.

Glycolipid A' then undergoes a complex series of fatty acid remodeling (Masterson *et al.*, 1990) resulting in the production of glycolipid A, which contains sn-1,2-dimyristoylglycerol. Glycolipid A may be modified, by palmitoylation of the inositol, generating glycolipid C. It has been conclusively demonstrated that both glycolipid A and C are competent for transfer to a VSG substrate *in vitro* (Mayor *et al.*, 1991). However, there is only evidence that glycolipid A can directly attach to proteins *in vivo*, despite substantial evidence that palmitoylated GPI-anchors exist.

Transcribed proteins appear to be rapidly attached to the GPI-anchor precursors by transamidation of the amino group of the phosphoethanolamine to the α carboxyl group of the terminal amino acid in the target protein (Bangs *et al.*, 1985). The protein is rapidly added *en bloc* to the GPI anchor, directly following its translation. The ethanolamine-amino acid

Figure 3.2 is a schematic representation of the step-wise synthesis of GPI-anchored proteins.

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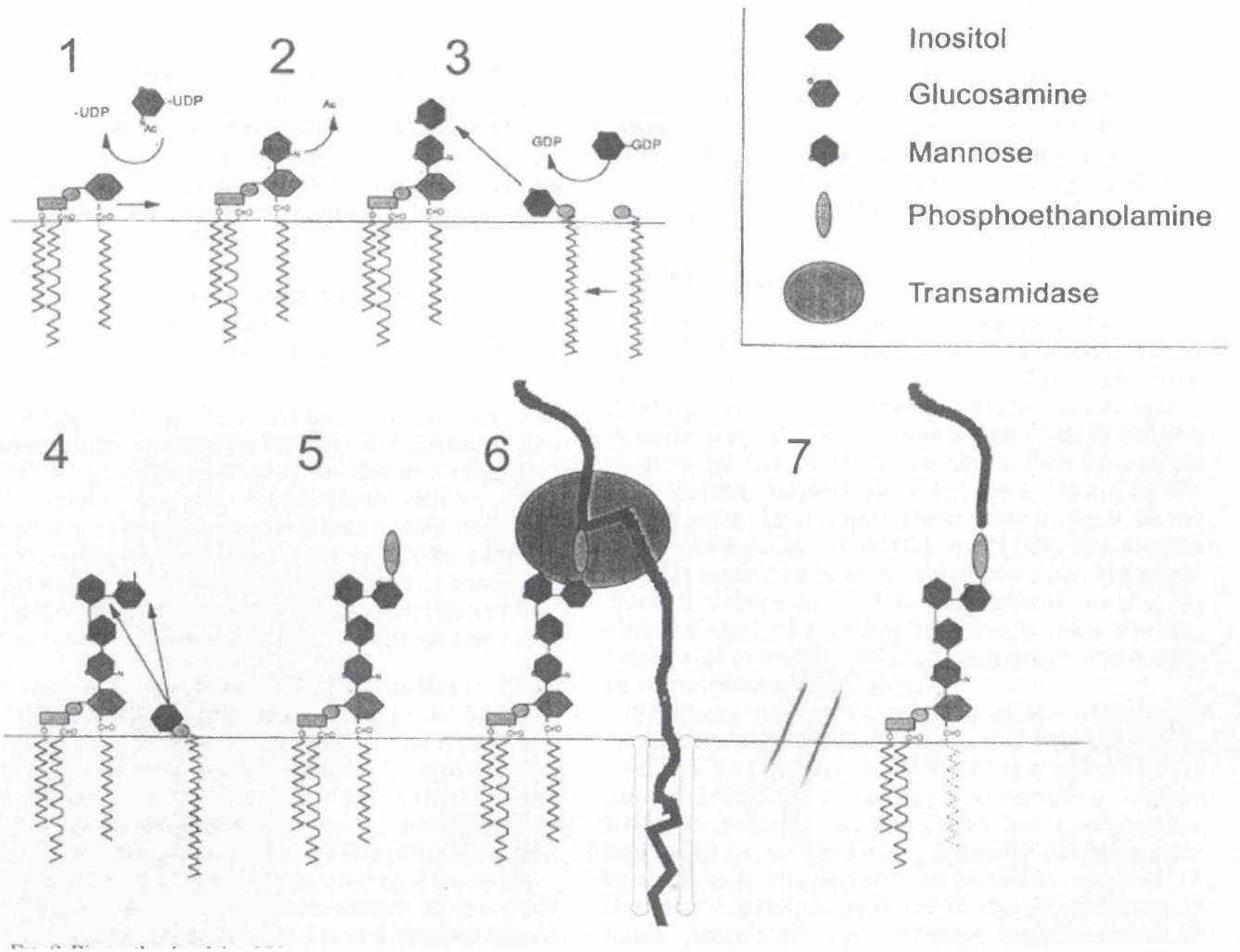
Glycolipid A' then undergoes a complex series of fatty acid remodeling resulting in the production of glycolipid A, which contains sn-1,2-dimyristoylglycerol.

Transcribed proteins appear to be rapidly attached to the GPI-anchor precursors by transamidation of the amino group of the phosphoethanolamine to the α carboxyl group of the terminal amino acid in the target protein. The protein is rapidly added *en bloc* to the GPI anchor, directly following its translation.

FIGURE 3.2

SEQUENTIAL CREATION OF THE GPI ANCHOR



attachment has been demonstrated in VSG (Ferguson *et al.*, 1985), Thy-1 (Williams & Tse, 1985) and human erythrocyte AChE (Haas *et al.*, 1986). Variation in the terminal amino acid has been demonstrated, and is dependent on the protein. For example, aspartic acid, serine or asparagine residues are found in different forms of the VSG protein (Boothroyd, 1985) whereas cysteine is the terminal amino acid in Thy-1 (Williams & Tse, 1985). Finally, the GPI-anchor may be α-galactosylated, during the passage of the protein through the endoplasmic reticulum and the Golgi apparatus (Mayor *et al.*, 1992).

3.8.1 Target of GPI-specific catalysis

The phosphatidylinositol unit is responsible for anchoring the protein to the membrane, and is the specific target for GPI-specific phospholipases. The majority of the GPI-anchored proteins studied to date are released from the membrane by treatment with bacterial PI-PLC and purified PI-PLC (Low & Zilversmit, 1980; Taguchi & Ikezawa, 1987. In addition, many of the proteins are susceptible to cleavage by GPI-PLC, but only following detergent treatment of the membranes in which the proteins are located.

3.8.2 Modifications to the core GPI anchor

Analysis of GPI-anchors from many different sources indicated that the core structure of the GPI-anchor is highly conserved across a number of different species (Davitz *et al.*, 1989), a feature which implies that this structure plays a significant biological role. Despite this highly-conserved nature of the anchor, however, cell-specific and / or species-specific heterogeneity has been found.

The existence of an additional ethanolamine phosphate, for example, appears to be a feature common to all non single-cell eukaryotic anchors. This residue has been detected in many other substrates, including bovine AChE (Haas *et al.*, 1986), human AChE (Roberts *et al.*, 1987) human DAF (Medof *et al.*, 1986) and hamster brain Scrapie prion protein (Stahl *et al.*, 1987).

Variation also exists in the fatty acid composition of GPI-anchors. For example, the majority of parasite VSG anchors contain myristic acid, whereas other protozoan GPI anchors appear to contain a mixture of myristic and palmitic acids (Ferguson & Cross, 1985). GPI-anchors from higher eukaryotes show a variety of saturated and unsaturated fatty acids, with a predominance of stearic acid. However, ethyl- and ester- linked alkyl chains have also been detected (Roberts *et al.*, 1987).

Additionally, the hydroxyl on the inositol ring may be substituted with an additional fatty acid, palmitic acid. This may be an important regulatory point in GPI anchor biosynthesis, as the existence of this palmytic acid imparts resistance to all PI-PLC enzymes that have been tested (Roberts *et al.*, 1988(a)). It would appear, however, that palmitoylated anchors are susceptible to hydrolysis by PLD enzymes (Brown *et al.*, 1992).

Furthermore, a number of α-linked galactosyl side-chain residues may be attached to the core region. The majority contain two or four galactosyl residues, while the remainder contain either none, one or greater than five residues (Ferguson *et al.*, 1988). In mammalian GPI-anchors, the two most extensively characterised, Thy-1 and AChE, both lack side chains composed of α-galactosyl residues. Instead, the rat brain Thy-1 contains additional mannose residues and an N-acetylgalactosamine residue (Homans *et al.*, 1988). Once again, it would appear that addition of carbohydrate side chains is a standard feature of the GPI-anchored proteins.

3.9 Variety of GPI-anchored proteins

GPI-anchored proteins comprise an extraordinarily diverse group of membrane molecules (Deeg *et al.*, 1995) which are ubiquitous in nature. The group includes complement regulatory factors, cell adhesion molecules, ecto-enzymes, differentiation antigens, tumour markers, prion proteins, and the majority of protozoan parasitic cell surface antigens (Metz *et al.*, 1991).

3.10 Characterisation of a novel GPI-specific phospholipase enzyme

3.10.1 Catalysis of the mfVSG anchor by PLC-type enzymes

Analysis of the VSG was facilitated by radiolabelling, typically by the substitution of a fatty acid with tritiated [^{3}H] myristic acid (Bangs *et al.*, 1985). The radiolabelled form was then subjected to cleavage by enzymes from various sources. It was demonstrated that the purified mfVSG was susceptible to cleavage (Bulow & Overath, 1986), and it was considered important to determine the agents responsible for this.

The conversion of mfVSG to sVSG involved cleavage of the phosphodiester bond. An enzyme of 37 - 39 kDa MW was identified which was capable of cleaving this bond. The enzyme was called VSG lipase or hydrolase (Hereld *et al.*, 1986), and was found to have high specificity for mfVSG (Bulow & Overath, 1986). This lipase was subsequently reclassified as a PLC-type enzyme.

3.10.2 GPI-PLD, a novel GPI-specific phospholipase enzyme

Data obtained from three groups almost simultaneously suggested that the GPI-anchor of mfVSG was susceptible to cleavage by an additional enzyme in addition to the PLC. The

experiments were all performed with an enzyme derived from human serum or plasma (Davitz *et al.,* 1987, Low and Prasad, 1988, Cardoso de Almeida *et al.*, 1988). The indication that this enzyme was different from PLC was based on three major lines of evidence.

The first line of evidence was dependent on the existence of a cryptic epitope, the 'cross-reacting' determinant (CRD) epitope, consisting of a carboxyl terminal myo-inositol plus phosphate, which is exposed as a result of PLC-mediated catalysis of GPI anchors. By comparison, hydrolysis with a phospholipase D (PLD) enzyme generates products with a myo-inositol minus phosphate (Shak *et al.*, 1988). Analysis of the cleavage products of VSG after treatment with the novel enzyme indicated that no phosphate was associated with the inositol group, which pointed to the catalysis being PLD-driven (Davitz *et al.*, 1987).

Secondly, analysis of the reaction products determined that the major product was primarily phosphatidic acid. As the product of PLC-mediated cleavage is dimyristyl-glycerol, and the product of PLD-mediated catalysis is phosphatidic acid, it was postulated that the enzyme activity in question was a PLD. Furthermore, the addition of phosphatase inhibitors in the reaction buffers precluded the existence of a PLC in combination with a phosphatase, which would remove the terminal phosphate from dimyristyl-glycerol, leaving phosphatidic acid (Low & Prasad, 1988)

Thirdly, inhibition of the enzyme also showed marked differences in comparison with the GPI-PLC. GPI-PLC is stimulated by dithiothreitol, and strongly inhibited by PCMPSA (p-hydroxymercuriphenylsulphonic acid), and has no indication of a need for metal ions to function adequately. By comparison, the postulated PLD enzyme is inhibited by dithiothreitol and insensitive to PCMPSA. It is also sensitive to chelating agents, such as EGTA and 1,10-phenanthroline, which suggests a role for metal ions in its function (Low & Prasad, 1988).

This evidence lent further weight to the argument of a novel enzyme, with characteristics of a phospholipase D. The association with the GPI-anchor lead to the designation of this enzyme as a GPI-PLD. The different characteristics of GPI-PLD, compared with PI-PLC and GPI-PLC are briefly summarised in table 3.1.

3.11 Characteristics of GPI-PLD

Serum samples isolated from all mammals studied to date, including human, rat, ox and rabbit, contain a glycosylphosphatidyl-specific phospholipase D type enzyme (GPI-PLD). GPI-PLD consists of a single polypeptide chain, of 110 kDa molecular weight and comprises approximately 0.02% of human serum proteins. GPI-PLD exhibits specificity for the GPI

TABLE 3.1

CHARACTERISTICS OF GPI-PLD IN COMPARISON WITH PI-PLC AND GPI-PLC

Characteristic	Phosphatidylinositol-specific phospholipase C (PI-PLC)	Glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC)	Glycosylphosphatidylinositol-specific phospholipase D (GPI-PLD)
Source for Experimentation	Bacteria	*Trypanosoma brucei,* *Trypanosoma. cruzi* Plants Existence in Mammals is not proven to date	Mammalian Plasma and Serum
Phospholipids Hydrolysed	Membrane bound Phosphatidylinositol (PI) Glycosylphosphatidylinositol (GPI)	Non-membrane bound Glycosylphosphatidylinositol	Non-membrane bound Glycosylphosphatidylinositol Acylated Glycosylphosphatidylinositol
Phospholipids Not hydrolysed	Phosphatidyl Choline (PC) Phosphatidyl Ethanolamine (PE) Phosphatidyl Serine (PS) Sphingomyelin (SM) Phosphatidylinositol phosphate (PIP) Phosphatidylinositol 2-phosphate (PIP2) Acylated Glycosylphosphatidylinositol	Phosphatidylinositol (PI) Phosphogylcan (PG) PC, PE, PIP, PIP2 Acylated glycosylphosphatidylinositol Membrane bound GPI	Phosphatidylinositol PC, PE Membrane bound GPI

(adapted from Hooper & Turner, 1992; Brodbeck & Bütikofer, 1994)

anchor, and the GPI-precursors, glycolipids A and C (Doering *et al.*, 1989), which contain the core of the GPI anchor, as outlined in Section 3.7. It was concluded, therefore, that the glucosamine-phosphatidylinositol, is the minimum unit required for substrate recognition by the enzyme.

3.12 Analysis of the *Gpld* gene

The most complete genetic information on the *Gpld* gene was derived from study of the mouse glucagonoma cell line, αTC6, from which a full length cDNA sequence was cloned and sequenced. The location of the *Gpld*1 gene was identified in the proximal region of chromosome 13, by linkage analysis of restriction fragment length variants (LeBoeuf *et al.*, 1998). The cDNA for the *Gpld*1 gene contains an open reading frame of 2511 base pairs, counting from the second methionine, based on the bovine protein sequence (Genbank accession no. M60894) (Scallon *et al.*, 1991). This open reading frame includes a 270 base pair 5' un-translated sequence and a 655 base pair 3' non-translated sequence, which includes the stop codon and a poly A tail. Translation results in a putative protein of 837 amino acids, including a 23 amino acid signal sequence. Upon removal of the signal sequence, the mature 814 amino acid protein has a predicted molecular weight of 90,776 daltons. The cDNA sequence was cloned into pCDM8 vector, and expressed in COS cells. The generation of a protein, which generated a phosphatidic acid product upon the hydrolysis of mfVSG, confirmed the identity of the gene sequence as that which generates the GPI-PLD.

A more complete discussion of the GPI-PLD enzyme is given in the discussion sections of this, and subsequent chapters. The characteristics of the enzyme are discussed with reference to the experimental results gained during the course of experimentation.

3.13 Existence of GPI-PLC in mammalian tissues

Throughout the course of the research performed for the duration of this thesis, the possibility existed that the enzyme responsible for the activity assay results was GPI-PLC, rather than GPI-PLD. Only two papers have reported the existence of a GPI-PLC enzyme in a rat liver fraction (Fox *et al.*, 1987; Stieger et al, 1991), through the demonstration of activity against mfVSG. The product of mfVSG hydrolysis was diacylglycerol, which would have been generated by PLC-mediated hydrolysis. The conclusion was made that rat liver membranes contained a GPI-PLC enzyme.

The experimental details from these studies can, however, be questioned. Firstly, it was demonstrated that the solutions employed in the experiments did not contain phosphatase inhibitors, such as sodium fluoride. The existence of a phosphatase enzyme would serve to

remove the phosphate from the phosphatidic acid, leaving the diacylglycerol product, which would be identified as the product of PLC-mediated hydrolysis. Experiments to confirm the results from these studies revealed that, in the absence of phosphatase inhibitor, the product of catalysis was primarily diacylglycerol, whereas the product of hydrolysis in the presence of sodium fluoride was phosphatidic acid. It was concluded that the enzyme fractions that were separated from rat and bovine liver membranes consisted of a GPI-PLD and a phosphatase enzyme (Heller *et al*, 1992).

Secondly, the reported PLC enzyme was highly sensitive to the calcium concentration, and was inhibited by both 1,10-phenanthroline and bicarbonate (Stieger *et al.*, 1991), characteristics typically observed with the PLD enzymes (Low & Prasad, 1988). Finally, the conclusions from the first series of experiments (Fox *et al.*, 1987), were made before the identification of the novel GPI-PLD enzyme (Davitz *et al.*, 1987, Low and Prasad, 1988, Cardoso de Almeida *et al.*, 1988). It is entirely possible, therefore, that the researchers did not consider that an alternative to the PLC enzyme might exist.

All of the information that has been collected, in reference to mammalian phospholipase enzymes, challenges the existence of GPI-PLC in mammals (Davitz *et al.*, 1987), and states that the only purified and well-characterized GPI-cleaving enzyme in mammals is GPI-PLD (Tujioka *et al.*, 1998). Experimentation during the course of this thesis has, therefore, made the assumption that a mammalian GPI-PLC does not exist, and that any evidence of activity against GPI-anchored substrates in mammalian mast cells is mediated by GPI-PLD.

3.14 Why was GPI-PLD implicated in Allergic Disease?

The possibility that GPI-PLD might play a role in IgE-dependent activation of a rat mast cell line was suggested following pilot studies using a polyclonal antibody against bovine GPI-PLD. RBL-2H3 cells were stimulated to degranulate by an IgE-mediated process. The inclusion of this antibody in such stimulation experiments led to the inhibition of the cells' degranulation. The data is illustrated in Figure 3.3. The graph in Figure 3.3 shows the percentage of histamine released from TNP-OVA stimulated mast cells, in cells that had been incubated with, and without, polyclonal anti-GPI-PLD antibody. It was concluded that the percentage of histamine released was reduced by the increasing concentrations of anti-GPI-PLD antibody. By 1000 μg/ml of antibody, almost no histamine was detectable.

The suggestion that GPI-PLD might be implicated in mast cell signalling was not unusual. GPI-anchored proteins have been implicated in a number of other cellular processes, including caveolar-mediated signalling, T cell activation, parasite immune evasion and in the disease,

FIGURE 3.3

INCLUSION OF POLYCLONAL ANTI-GPI-PLD ANTIBODY
IN THE IgE MEDIATED STIMULATION OF RBL-2H3 CELLS

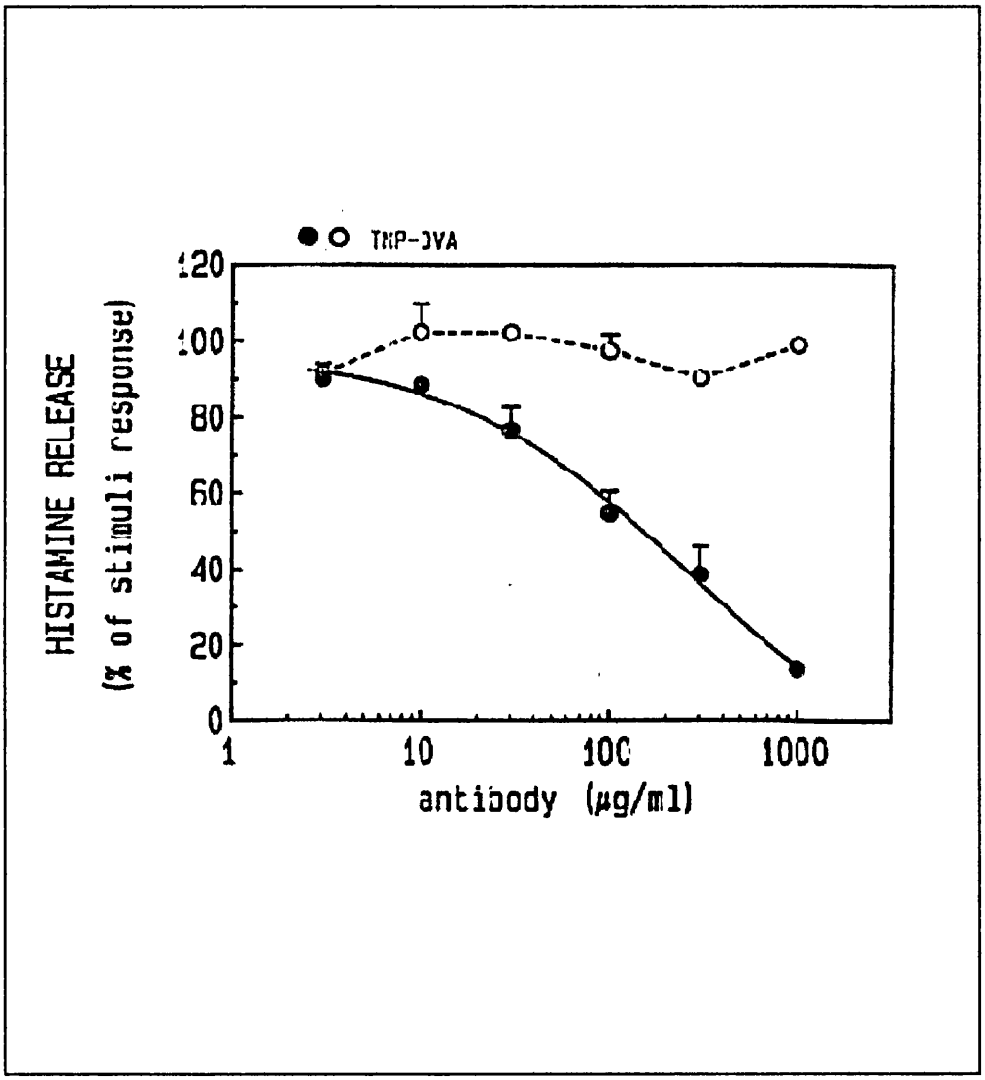


Figure Legend
The graph in figure 3.3 shows the percentage of histamine released from TNP-ova stimulated mast cells, in cells that had been incubated with and without the polyclonal anti-GPI-PLD antibody. Precise details of this experiment are unclear, as the research was never published in its entirely. However, it can be concluded that the percentage of histamine released was reduced by the increasing concentrations of anti-GPI-PLD antibody. By 1000 µg/ml of antibody, almost no histamine was detectable

(Provided by Dr A Gilfillan, Hoffman-la-Roche)

paroxysmal nocturnal haemoglobinuria. The significance of GPI-PLD, or GPI-derived second messengers will be reviewed in greater detail in the discussion chapter, with reference to the signalling processes observed in Type One Hypersensitivity.

3.15 Conclusions

In conclusion, the research discussed during the subsequent two chapters was directed at the study of Glycosylphosphatidylinotisol phospholipase D (GPI-PLD), and its putative role in Type One Hypersensitivity in the rat mast cell line, RBL-2H3. GPI-PLD is defined as a glycolipid-specific phospholipase enzyme, of 110 kDa molecular weight, which is found in mammalian serum and plasma. The target of this phospholipase is the GPI-anchor, a membrane attachment, comprised of phosphatidylinositol, glucosamine, three mannose residues and a terminal ethanolamine phosphate to which the protein is attached. GPI-anchored proteins comprise an extraordinarily diverse group of membrane molecules (Deeg *et al.*, 1995) which are ubiquitous in nature.

GPI-PLD has the ability to hydrolyse the phosphodiester linkage of the GPI-anchor, to produce phosphatidic acid and a polar head group. However, the enzyme is inactive against intact cells, and enzyme activity requires that the GPI-anchored proteins be dissociated from the membrane by detergent treatment. Other enzymes capable of hydrolysing this GPI-anchor are PI-PLC and GPI-PLC, although the product of the catalysis is dimyristyl-glycerol.

GPI-anchored proteins have been implicated in a number of cellular situations. It was, therefore, considered of interest to determine the role of GPI-anchored proteins in allergic disease. Prior to this, however, it was essential to determine the existence of the GPI-PLD enzyme, and its activity, in the RBL-2H3 cell line under investigation.

3.16 Experimental Aims

As discussed, the pilot studies on which this thesis was based described the inhibition of IgE-mediated cell stimulation of RBL-2H3 cells when an anti-GPI-PLD antibody was included (Section 3.14). It was, therefore, concluded that the GPI-PLD occupied a pivotal role in the IgE-mediated stimulation of RBL-2H3 cells. Prior to ascertaining the role of GPI-PLD in the function of RBL-2H3 cells, however, it was considered of importance to determine the expression of the enzyme in relation to RBL-2H3 cells. It was necessary to ascertain whether the cells indeed contained the enzyme, in a functional state, and whether they produced it *in situ.*

Three principle methods were employed for the detection and expression of GPI-PLD in RBL-

2H3 cells, and in the culture medium. Firstly, a GPI-PLD activity assay was selected, to ascertain whether the cells contained functional enzyme activity. Secondly, Western Blot analysis was employed to support the results of the activity assay, through the specific detection of the protein. Thirdly, an analysis of the cells' RNA, using RT-PCR techniques, was designed to ascertain whether the cells synthesised the enzyme. This introductory Section concerns the choice of experimental methods.

3.17 GPI-PLD activity assay

The GPI-PLD activity assay is based on the ability of GPI-PLD enzyme to hydrolyse the GPI-anchor of the membrane-form variant surface glycoprotein (mfVSG), which is labeled with tritiated myristic acid. The products from this reaction are radioactive dimyristyl phosphatidic acid and soluble VSG. As both of the products and the mfVSG differ in their solubility in organic solvent, they can be easily separated by simple solvent extraction. The intact mfVSG and soluble VSG are soluble in water, whilst the radioactive dimyristyl phosphatidic acid is soluble in organic solvent. Detection of the radioactive product enables researchers to ascertain the efficacy of the hydrolytic reaction, which is a direct reflection of the quantities of phospholipase enzyme (Hooper & Turner, 1992). In addition, it was previously demonstrated that the GPI-PLD enzyme was sensitive to chelating agents, including EGTA and 1,10-phenanthroline (Low & Prasad, 1988). The effect of these chelating agents on the activity of any enzymes detected in the FBS and RBL-2H3 cells was, therefore, studied.

3.18 Analysis of the Protein components in Foetal Bovine Serum and RBL-2H3 membrane samples

3.18.1 SDS Polyacrylamide gel electrophoresis

The separation of the proteins contained within the FBS samples, and the RBL-2H3 membrane extractions was effected by vertical electrophoresis on a polyacrylamide gel. Polyacrylamide gels are composed of chains of polymerized acrylamide, cross-linked by N,N'-methylenebisacrylamide. The range of proteins that may be effectively separated on a polyacrylamide gel is dependent on the concentration of both polyacrylamide and the cross-linking agent, with the most typical ratio being 1 polyacrylamide: 29 bisacrylamide. In these experiments a 5% acrylamide separating gel was selected, as it was shown to effectively separate proteins of molecular weights from 57 to 212 kDa. The 5% separating gel was overlain with a 4% stacking gel, whose role was to effect the concentration of the samples into a tight band, called a stack, prior to their separation (Maniatis *et al.*, 1982).

To ensure that the protein mixture was separated into its differently-sized components, with minimal aggregation, the samples were boiled with a strongly anionic detergent, SDS, in

combination with a reducing agent prior to being loaded onto the gel. Boiling of the proteins serves to break down any secondary protein structures, after which time the denatured proteins bind to SDS, and become negatively charged. The amount of SDS that binds to the protein is proportional to its molecular weight, independent of its sequence. The SDS-polypeptide complexes, therefore, migrate through the gel according to their molecular weight. A marker panel, consisting of proteins of known molecular weight is run alongside the unknown samples, against which any protein bands can be compared, to determine their approximate molecular weights. A GPI-PLD positive control was included in each experiment.

Electrophoresis was performed in a discontinuous buffer system, in which the buffer in the electrophoresis units was of a different pH and ionic strength from the buffer used in the preparation of the gel. The protein samples were moved through the gel by the current that is created when a current is passed across the electrodes. Firstly, the samples pass through the porous stacking gel, and are deposited at the interface with the separating gel. Upon increasing the current applied across the apparatus, the samples separate according to their charge (Maniatis *et al.*, 1982).

3.18.2 Detection of the protein concentration

In order that the results gained from the western blot procedure could be compared, it was necessary to standardize the quantities of protein that were loaded onto the SDS-PAGE gels. To this end, the Coomassie plus protein assay was selected as a suitable method for the detection of protein in biological samples. The Coomassie plus protein assay reagent is a pre-prepared aqueous solution containing Coomassie dye, methanol, phosphoric acid and solubilizing agents. The assay is simple, reliable, and the protein concentration can be quantified by the comparison with protein standards (Sedmak & Grossberg, 1977). Ideally, the protein standards would consist of a pure sample of the protein being assayed. However, in most cases this was not possible, and a BSA standard was regarded as a suitable alternative.

The Coomassie detection system relies on the following principle:

$$\text{Protein} + \text{Coomassie G-250} \Rightarrow \text{Protein-Dye complex}$$

$$\text{465 nm (green or red/brown)} \Rightarrow \text{595 nm (blue)}$$

3.18.3 Coomassie detection of proteins on an SDS-PAGE gel

The efficient separation of proteins after SDS-PAGE electrophoresis is essential if the individual protein bands are to be identified. To this end, it was important to select a staining agent that could effectively highlight the protein bands on an SDS-PAGE gel. As with the protein detection assay, a Coomassie-based stain was selected. PhastGel Coomassie tablets

contain coomassie, a triphenylmethane anionic dye, which has the capacity to form dye complexes with proteins in a gel matrix. The staining solution was prepared in a methanol and acetic acid solution. After an appropriate level of staining was achieved, a de-staining agent removed excess dye, leaving dark blue proteins bands against a clear gel background. The de-staining agent was a methanol: glacial acetic acid: water mixture. The sensitivity of the method is estimated to be approximately 20 to 30 ng of protein per protein band (Neuhoff *et al.*, 1985).

3.18.4 Western Blotting of SDS-PAGE gels

Western blotting involves the transfer of electrophoretically-separated proteins onto a solid support, followed by the detection of particular sequences of amino acids. In this case, the proteins that were in the SDS-PAGE gel were immobilized on a nitrocellulose membrane (Towbin *et al.*, 1979). A nitrocellulose membrane was selected since it required minimal handling, and binds proteins efficiently, irrespective of their size or charge (Burnette *et al.*, 1981). The membrane was precisely aligned with the SDS-PAGE gel, and sandwiched into a cassette arrangement, which was transferred to a vertical electroblotting unit, as discussed in section 3.23.3.9.

The target proteins were then detected using anti GPI-PLD antibodies. Two antibodies were employed; a mouse anti-human monoclonal or a rabbit anti-bovine polyclonal anti GPI-PLD antibody. HRP-conjugated goat anti-mouse immunoglobulins or HRP-conjugated swine anti-rabbit immunoglobulins, respectively, were used to detect these primary antibodies. The horse radish peroxidase attached to the secondary antibodies was detected using the ECL detection method (Section 3.18.5). Western blotting can detect as little as 1 to 5 nanograms of an average sized-protein (Maniatis *et al.*, 1982).

3.18.5 Electrochemiluminescence detection (ECL) of horseradish peroxidase-conjugated antibodies

The ECL Plus detection system was designed for the detection of immobilised proteins, bound by horseradish peroxidase (HRP)-conjugated antibodies. HRP and peroxidase combines with the ECL substrate, Lumigen PS-3 acidran, generating acridinium ester intermediates. Under slightly alkaline conditions, these intermediates react with peroxide, generating high intensity chemiluminescence, which can be detected by exposure of a photographic film. The ECL Plus system was selected, as it has been demonstrated to be both sensitive and reliable, when compared with alternative detection systems. In addition, the avoidance of radioactive substrates meant that the ECL Plus system was distinctly more user-friendly (Whitehead *et al.*, 1979)

3.19 Detection of mRNA in RBL-2H3 samples by RT-PCR

RT-PCR was selected as the most appropriate method for the detection of the expression of mRNA. Alternatives to RT-PCR include the RNAase protection assay, or Northern blotting. However, RT-PCR was the preferred choice, as this straightforward method detects RNA with high sensitivity.

3.19.1 Preparation of RNA from biological samples

RNAzol B is a pre-prepared solution, designed specifically for the preparation of DNA-free RNA from cell samples. The separation of RNA from other cell components is based on the principle that the RNA forms complexes with guanidinium thiocynanate and water, while abolishing the hydrophilic interactions between DNA and proteins. The RNAzol B promotes separation of the RNA into the aqueous phase, while DNA and proteins separate into the organic phase (Chomczynski *et al.*, 1987). To obtain good preparations of RNA, it was necessary to minimize the activity of RNAse enzymes. Glass and plastics were treated with DEPC, which is a strong, but not absolute, inhibitor of RNAses (Fedorcsak & Ehrenberg, 1966).

3.19.2 Reverse Transcription (RT)

The total RNA that was purified using RNAzol B provides a template for the creation of DNA, which can then be amplified by the polymerase chain reaction (PCR). The reaction is dependent on the reverse transcriptase enzyme, which produces a complementary DNA (cDNA) copy of each RNA strand in the RNA mixture. The reverse transcriptase is dependent on random hexamers, which align with the RNA at various points along the sequence, and provide a free 3' terminus that can be extended by the RT enzyme (Maniatis *et al.*, 1982).

3.19.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction is a method for the *in vitro* amplification of specific DNA fragments (Saiki *et al.*, 1985). PCR amplification is dependent on the nucleotide sequence on either side of the target sequence being known, from which specific oligonucleotide primers can be designed. The primers are complementary to the sequences on the opposite strand on either side of the target region. After heat denaturation of the DNA, the single nucleotide strands are annealed to the primer sequences, and the sequence is extended from the primers, by the Taq DNA polymerase enzyme. After repeated cycles of heat denaturation of template DNA, annealing of the oligonucleotide primers to their complementary sequences and extension of the annealed primers with DNA polymerase, the result is an exponential amplification of the target DNA.

3.19.4 Design of the *Gpld* specific primers

The sequence from the rat GPI-PLD gene has only recently been elucidated, and it was necessary to use human and murine *Gpld*-specific primers for the majority of the PCR experiments performed during this thesis. The sequences of the oligonucleotide primers used for the PCR amplification of the RNA preparations are given in Table 3.2. As indicated in Table 3.3, however, significant homology exists between the sequences of the human and murine *Gpld* genes. It was, therefore, considered likely that the rat sequence would be similarly homologous and that the human/mouse specific *Gpld* primers would detect rat GPI-PLD mRNA. The recent elucidation of the rat sequence, as shown in Table 3.3, has proven this theory correct, as the rat *Gpld* cDNA shows significant homologies when compared with the human and murine DNA.

Details of the standard cycling details for the GPI-PLD specific primers are outlined in Table 3.4, and the details of the annealing temperatures employed are shown in Table 3.5.

3.19.5 Positive controls employed in the RT-PCR reactions

The positive controls that were employed in the RT and PCR amplifications were a human GPI-PLD positive human cDNA and cDNA derived from rat liver RNA. The GPI-PLD-positive human cDNA was derived from a human superscript cDNA liver library. The liver cDNA library, derived from a nine year old caucasian, was directionally cloned into an eukaryotic expression vector, pCMV Sport. The GPI-PLD specific sequence was identified by screening the expression vector with a human GPI-PLD cDNA probe.

The second positive control was rat liver RNA, which was extracted using the RNAzol extraction methodology (Section 3.24.2). The liver tissue was homogenised prior to the addition of the RNAzol, after which the extraction was essentially identical to the extraction of RNA from the RBL-2H3 monolayer. The RNA was reverse transcribed into cDNA, as discussed in Section 3.24.2.5.

3.20 Experimental Methods

3.20.1 Detection of GPI-PLD activity using the radioactive mfVSG assay

3.20.1.1 Origin of the radiolabelled mfVSG

Radiolabelled (^{3}H) mfVSG was kindly prepared and provided by Dr Kia-Joo Puan, University College London.

TABLE 3.2

OLIGONUCLEOTIDE PRIMERS USED IN THE POLYMERASE CHAIN REACTION AMPLIFICATION OF GPI-PLD

Name	Target?	GC Content	Sequence	Orientation	Company
P6	Human GPI-PLD	48%	TTGTTGGAGGACTGGATGATATGGC	Sense (740 – 765)	Cruachem
P7	Human GPI-PLD	48%	AACCAAAGTAGACATACACGGCACC	Antisense (1447– 1471)	Cruachem
MP1	Mouse GPI-PLD	60%	GTGCTGGACTTCAACCAGGATGGGC	Sense (1639 – 1644)	Oswel DNA Service
MP2	Mouse GPI-PLD	60%	AGCAGAGCAGGCTGTGTCTTCTCGG	Antisense (2372 - 2397)	Oswel DNA Service
RATG2	Rat GPI-PLD	60%	GTTGCAGTCACTGGTCCCGTTCTCC	Sense (833 – 858)	Cruachem
RATG1	Rat GPI-PLD	60%	TGTCTGTAGGCAGGCTGTGGTCTGG	Antisense (30-55)	Cruachem

TABLE 3.3

ALIGNMENTS OF PRIMER AND HUMAN, MOUSE AND RAT GLPD GENE TEMPLATES

Primer	**Sequence**		**Mismatches**
Rat GPI-PLD Primer P6 Human GPI-PLD)	5′ -TCCTCGGAGGTCTAGATGACATGGC- 3′ 5′ -TTGTTGGAGGACTGGATGATATGGC- 3′	Sense	5/25
Rat GPI-PLD Primer P7 (Human GPI-PLD)	3′ -TTGGTATCATCTGTATGTGTCTCGG- 5′ 5′ -AACCAAAGTAGACATACACGGCACC- 3′	Antisense	1/25
Rat GPI-PLD Primer MP1 (Mouse GPI-PLD)	5′ -GTGCTGGACTTCAACAAGGATGGGC- 3′ 5′ -GTGCTGGACTTCAACCAGGATGGGC- 3′	Sense	1/25
Rat GPI-PLD Primer MP2 (Mouse GPI-PLD)	3′ -TCGTCTCGTCCGACACAGAAGAGTC- 5′ 5′ -AGCAGAGCAGGCTGTGTCTTCTCGG- 3′	Antisense	1/25
Rat GPI-PLD Primer RATG1 (Rat GPI-PLD)	3′ -CAACGTCAGTGACCAGGGCAAGAGG- 5′ 5′ -GTTGCAGTCACTGGTCCCGTTCTCC- 3′	Sense	0/25
Rat GPI-PLD Primer RATG2 (Rat GPI-PLD)	5′ -TGTCTGTAGGCAGGCTCTGGTCTGG- 3′ 5′ -TGTCTGTAGGCAGGCTGTGGTCTGG -3′	Antisense	0/25

TABLE 3.4

STANDARD CYCLING DETAILS FOR PRIMERS

	Temperature	Time	Number of Cycles
	40 °C	2 minutes	1
Denaturation	94 °C	4 minutes	
Annealing	* °C	30 seconds	
Extension	72 °C	40 seconds	29
Denaturation	94 °C	25 seconds	
Annealing	* °C	2 minutes	1
Extension	72 °C	5 minutes	

* denotes the annealing temperature, as outlined in Table 3.5

TABLE 3.5

DETAILS OF THE ANNEALING TEMPERATURES EMPLOYED IN THE POLYMERASE CHAIN REACTION

The Annealing Temperatures for the Primers were calculated as follows

$$Tm\ (°C) = 69.3 + 0.41 \times (\%\ GC) - (650 / \text{Primer length})$$

$$Ta = Tm - 5°C$$

Primer Pair	**Target**	**Annealing Temperature (°C)**
P6 / P7	Human GPI-PLD	58
RATG2 / RATG1	Rat GPI-PLD	62.9
MP1 / MP2	Mouse GPI-PLD	62.9
β-Actin	Rat β-actin	60

3.20.1.2 Preparation of the RBL-2H3 membrane fraction

3.20.1.2.1 Using cultured cells (derived from culture flask)

Prior to preparation of the membrane fraction, it was necessary to remove all culture media from the culture flask in order to prevent contamination of the RBL-2H3 cell membrane. The adherent cells were then washed thoroughly, twice, with sterile PBS (0.01 M phosphate buffer, with 0.0027 M KCl, 0.137 M NaCl, pH 7.4) (Sigma Chemicals Ltd). Fresh PBS was added, and adherent cells were detached from the flask using a cell scraper (Life Technologies Ltd). The total cell count was determined using a Neubauer haemocytometer (BDH Chemicals) and the cells were pelleted by centrifugation at 1,000 G for greater than 5 minutes. The cell pellet was then resuspended with between 100 and 500 µl of solubilising buffer (150 mM NaCl, 10 mM HEPES, 0.5% Nonidet P40 and 0.1% sodium azide, pH 7.0, and then vortexed thoroughly for greater than 3 minutes. The suspended cells were then incubated on ice for 30 minutes and re-centrifuged at 12,0000 g for 5 minutes to pellet the cell debris.

The supernatant was removed to a fresh eppendorf tube and stored at –20°C. Freeze-thawing cycles were avoided and samples were kept on ice to minimise damage by endogenous protease enzymes.

3.20.1.2.2 From cultured cells (derived from a 24 well plate)

When starting material was RBL-2H3 cells in a 24 well plate (Life Technologies Ltd.), it was more practical to extract the membrane fraction directly from the plate wells. The 24 well plate was placed, and maintained on ice, during the extraction procedure. Prior to preparation of the membrane fraction, the RBL-2H3 cells were washed twice with sterile PBS, to remove the cell culture medium. After washing, two alternative protocols were followed for the extraction of the cells from the monolayer:

Protocol 1:

Two hundred and fifty µl of PBS were pipetted onto the monolayer and the cells were gently scraped from the plastic surface using the tip of the pipette. The cell suspension was centrifuged at 1000 rpm for 5 minutes to pellet the cells. The supernatant was removed, and 250 µl of solubilising buffer was added to the cell pellet.

Protocol 2:

Following the PBS wash, as outlined above, 250 µl of solubilising buffer was pipetted directly onto the cell monolayer. The cells were incubated for 2 minutes, after which time the liquid was removed to an eppendorf tube.

Following the cell removal protocol, the microtitre plate was held up to the light to establish whether the cells had been removed from the plastic. The sample in the eppendorf tube was vortexed thoroughly for greater than 3 minutes, incubated on ice for 30 minutes and centrifuged at 12,0000 g for 5 minutes, to pellet cell debris. The supernatant was removed to a fresh eppendorf tube and the GPI-PLD activity in the samples was determined (Section 3.21).

3.21 Radioactive GPI-PLD activity assay

A master assay reaction mix of mfVSG for ten reactions was prepared, to minimise variability, which contained Tris-maleate buffer (200 mM, pH 7.0), nonidet P-40 (1% weight:volume (w:v)) and de-ionised water at a ratio of 2:1:7. 30 mM mfVSG was then added. Samples were diluted 1:1 (v:v) with the reaction mix in an eppendorf tube and were incubated at 37°C for 10 minutes. The reaction was terminated by the addition of 500 µl 1M NH_4OH-saturated-butanol (BDH Chemicals Ltd), followed by thorough vortexing. The samples were then centrifuged at 12,000 rpm for 5 minutes to effect separation of the phases. Three hundred µl of the butanol phase were removed into 2 ml of scintillation fluid (Packard Bioscience BV) and the quantity of [^{3}H]-dimyristyl phosphatidic acid produced was determined using a scintillation counter (Beckman LS5000CE).

0.2 Units/ml of GPI-PLD (Roche Diagnostics Ltd), diluted in solubilising buffer, were used as a positive control. In addition, an identical aliquot of radioactivity was counted in the scintillation counter, to determine the total quantity of radioactivity in each assay.
The GPI-PLD activity in this study was arbitrarily defined as 1 Unit (U) being equal to the hydrolysis of 1% substrate per minute.

3.22 Inhibition of GPI-PLD activity assay, using EGTA and 1,10-phenanthroline

Inhibition of the GPI-PLD enzyme was effected by the addition of either 1,10-phenanthroline or EGTA. A 100 mM stock solution of 1,10-phenanthroline was prepared in 95% ethanol, and adjusted to pH 7.4. A 500 mM stock solution of EGTA was prepared in water, and adjusted to pH 8.0. The 1,10-phenanthroline or EGTA was added to the samples, prior to addition of the VSG reaction mix. The activity assay was performed as described in Section 3.21.

3.23 Polyacrylamide gel electrophoresis and Western Blotting

3.23.1 Preparation of the membrane fraction from RBL-2H3 cells

To ensure that the RBL-2H3 membrane preparation was not contaminated, the culture media was removed and the adherent RBL-2H3 cells were washed thoroughly, twice, with sterile PBS. Fresh PBS (approximately 5 ml) was added and adherent cells were detached from the

flask using a cell scraper. The density of cells was determined using a Neubauer haemocytometer, and the cells were then pelleted by centrifugation at 1,000 G for greater than 5 minutes.

The cell pellet was resuspended in an appropriate volume of suspension buffer (100 mM sodium chloride, 10 mM Tris HCl (pH 7.6), 1 mM EDTA (pH 8.0), 1 μg/ml aprotinin and 100 μg/ml phenylmethylsulphonyl fluoride (PMSF) (Sigma Chemicals Ltd). PMSF is labile in aqueous solutions and was, therefore, made up in isopropanol (BDH Chemicals), stored at -20° and added to the suspension buffer immediately prior to experimentation.

3.23.2 Protein detection assay using the Coomassie Plus Detection system

Prior to use, the Coomassie Plus Reagent (Pierce & Warriner Ltd) was allowed to equilibrate at room temperature, and the solution was then gently mixed by inversion of the bottle. Samples or standards were diluted as appropriate and pipetted into individual wells on a 96 well microtitre plate. A minimum volume of 300 μl total volume per microtitre plate well was required to gain an accurate spectrophotometer reading and the experimental volumes were adjusted dependent on the concentration of protein:

- Between 25 to 2,000 μg/ml, the samples were diluted 30:1 (v:v) with Coomassie Plus reagent.
- Between 1 to 25 μg/ml, the samples were diluted 1:1 (v:v) with Coomassie Plus reagent.

Microtitre plates were transferred directly to the spectrophotometer plate reader (Thermomax microplate reader, Molecular Devices). Samples were shaken to effect thorough mixing and the absorbance at 595 nm was read. A freshly prepared BSA standard curve (Sigma Chemicals Ltd) was assayed each time and the protein concentration was determined by comparison against the curve.

3.23.3 Separation of proteins on an SDS PAGE gel

3.23.3.1 Assembly of the glass plates

Prior to use, the glass plates, spacers and comb (Flowgen) were cleaned thoroughly with mild detergent and water, rinsed with 70% ethanol (BDH Chemicals Ltd) and dried thoroughly. On a level bench surface, the two glass plates were placed back to back, with the spacers aligned with the edge of the glass. The plates were tightly fastened into a gel casting unit.

3.23.3.2 Preparation of the 4% stacking SDS-PAGE gel

A 5% acrylamide gel was mixed in a beaker using a magnetic stirrer to ensure complete mixing of the solutions. A 36 ml volume consisted of 6 ml Protogel (30% w:v acrylamide, 0.8% methylene-bisacrylamide w:v in solution), 10.5 ml Protogel buffer (375 mM Tris-HCL, 0.1% w:v SDS, pH 8.8) (National Diagnostics), 19 ml of water, 360 µl of 10% (w:v) ammonium persulphate (freshly made) and 30 µl of TEMED (Sigma Chemicals Ltd).

Following addition of the ammonium persulphate, polymerisation was initiated and the gel had to be poured quickly before it began to set. This was effected by drawing the acrylamide gel into a 50 ml syringe and injecting it into the gap between the glass plates. Sufficient space was left at the top for the stacking gel and comb. Using a pipette, the gel was over-lain with 2 ml butanol, both to ensure a flat surface, and to prevent oxygen from diffusing into the gel and preventing polymerisation. After polymerisation was complete (approximately 30 minutes), the butanol was removed and any remaining was drained from the gel using a paper towel.

A 4% stacking acrylamide gel was prepared in a 15 ml Falcon Tube (BDH Chemicals) by the addition of 1.4 ml Protogel, 2.5 ml Protogel stacking buffer, (125 mM Tris, 0.1% w:v SDS, pH 6.8) (National Diagnostics), 6.1 ml deionised water, 50 µl 10% (w:v) ammonium persulphate (freshly prepared) and 10 µl TEMED (Sigma Chemicals Ltd). The Falcon tube was inverted to ensure complete mixing of the solutions and the mixture was then drawn into a 30 ml syringe. The acrylamide solution was overlain onto the separating gel, a clean comb was inserted into the top, taking care to avoid trapping air bubbles. The gel was allowed to completely polymerise (30 minutes).

3.23.3.3 Setting up the electrophoresis apparatus

The glass plates and gel were removed from the gel casting apparatus and transferred to the Vertical Electrophoresis unit (Flowgen G3-0500), orientating the smaller glass plate innermost. The plates were fastened into the unit. The upper and lower chambers of the unit were filled with 1 x Tris-glycine-SDS buffer (250 mM Tris-base, 1.92 M glycine, 1% (w:v) SDS) (National Diagnostics), ensuring that there was no leakage from the upper into the lower chamber. The gel comb was removed, and the wells were thoroughly washed out, using the 1 x Tris-glycine-SDS buffer, to remove any extraneous SDS gel.

3.23.3.4 Selection of defined molecular weight markers

The samples were compared against Full Range Rainbow Recombinant protein molecular weight markers (Amersham). These markers consist of a mixture of individually coloured recombinant proteins (at 150 µg/ml) of 10,000 to 250,000 kDa molecular weight. Twenty µl of marker was sufficient for a 20 x 20 cm gel.

3.23.3.5 Quantities of protein required for adequate detection

The quantities of protein required for adequate detection on the SDS gel differed depending on the number of bands that required detection:

- When a single band of protein was anticipated, up to 10 μg of protein was sufficient;
- When multiple protein bands were anticipated, 100 μg of protein was sufficient.

Samples were diluted 1:1 (v:v) with 2 x protein loading buffer (0.5M Tris-Cl (pH 6.8), 4.4% SDS (w:v), 20% glycerol (w:v), 2% 2-mercaptoethanol (w:v), 0.01 mg/ml bromophenol blue). The samples were then boiled for 10 minutes to remove secondary structures and then centrifuged briefly to collect the fluid at the bottom of the eppendorf tube.

Using a fine tipped pipette tip, the samples were carefully loaded onto the gel, ensuring that the wells did not overflow. Unused wells were filled with an equivalent volume of sample buffer, to maintain a uniform electrical resistance across the gel. The lid was fitted onto the Vertical electrophoresis unit and the entire apparatus was connected to a power pack.

3.23.3.6 Separation of proteins by electrophoresis

Stacking of the proteins was effected at 90V for approximately 1 hour - until the samples had formed tight bands and were collected uniformly at the junction between the stacking and separating gels. Separation of the samples was effected at 150 to 200 V for approximately 3 to 4 hours (Consort E835 Microcapter electrophoresis power supply) until the dye front reached near to the bottom of the glass plates.

3.23.3.7 Disassembly of the glass plates

The glass plates were removed from the electrophoresis unit and the spacers were carefully removed from between the plates. Using a flat strong metal blade, the glass plates were prized apart, taking care to avoid tearing the gel or cracking the glass. The excess stacking gel was then removed using a scalpel. Subsequent treatment of the gel was dependent on whether it was to be directly stained with Coomassie stain (Sections 3.23.3.8.1 to 3.23.3.8.4), or whether Western blotting of the gel (Sections 3.23.3.9.1 to 3.23.3.10.8) was to be performed.

3.23.3.8 Direct Coomassie staining of the SDS-PAGE gel

3.23.3.8.1 Preparation of the SDS-PAGE gel

If the gel was to be stained, the entire glass plate plus adherent gel was transferred to a staining

tray and was submerged in 1 x Tris-glycine-SDS buffer (National Diagnostics). Taking care to avoid damage to the gel, it was carefully detached from the glass plate. The buffer was then poured away and replaced with the stain solution (described below).

3.23.3.8.2 Preparation of the Coomassie staining solution

Coomassie staining was performed using PhastGel Blue R tablets, which contain Coomassie R 350 dye in a readily soluble form. A 0.2% Coomassie staining solution was prepared by dissolving one PhastGel Blue R tablet in 80 ml of water, followed by stirring for 5-10 minutes, to ensure that the tablet had dissolved. The volume was then made up to a final volume of 200 ml by the addition of 120 mls of methanol. This solution was then passed through a 0.45 micron filter (Pall Corporation), to remove any undissolved Coomassie blue.

3.23.3.8.3 Method of Coomassie staining

A final 0.1% staining solution was created by mixing 1:1 (v:v) with freshly prepared 20% glacial acetic acid. Sufficient 0.1% Coomassie solution was poured into the staining tray to entirely cover the SDS-PAGE gel. Shaking was avoided, as it was found to promote tearing of the gel. The tray was covered with Saranwrap and kept at room temperature for at least 60 minutes to ensure adequate staining of the gel.

De-staining was performed, without shaking, using a solution of methanol:glacial acetic acid:water (3:1:6 v:v:v) (BDH Chemicals). The destain solution was replaced at regular intervals until the stained blue-coloured bands in the gel became clearly visible.

3.23.3.8.4 Drying the gel

The gel was removed from the staining solution and transferred onto two pieces of 3M Whatman paper, which were slightly larger than the gel. The gel was overlain with Saranwrap, and air bubbles were carefully removed. The gel was transferred to the gel drying apparatus (Biotech model 583 gel dryer). The dryer plastic overlay was placed on top of the gel and the vacuum (Biotech Hydrotech vacuum pump) was started. The gel was dried for approximately 3 hours at a temperature of 60°C.

3.23.3.9 Transfer of the proteins to a nitrocellulose membrane

3.23.3.9.1 Assembly of the transfer cassette

The Electro-blotting Unit (Flowgen Unit B4-0200) was filled with approximately 4.5 litres of Transfer buffer (39 mM glycine, 48 mM Tris-base, 0.037% (w:v) SDS (National Diagnostics) and 20% (v:v) methanol) (BDH Chemicals), and a magnetic stirrer was placed at the bottom of the unit. Six pieces of Whatman 3 MM paper and 1 piece of nitrocellulose membrane

(Amersham Life Science Ltd) were cut to the exact size of the SDS-PAGE gel (to ensure that any paper overhangs would not promote short-circuiting and reduce the transfer of proteins from the gel to the membrane).

The membrane and paper were completely immersed in de-ionised water and soaked for at least 5 minutes. The 3 M paper was soaked in a shallow tray, containing 1 x Transfer buffer. The black (anode) grid was lain on the bench surface and overlain with one piece of foam pad, soaked in 1 x transfer buffer, and three pieces of soaked 3 M paper. Any bubbles between the layers were removed by using a plastic 10 ml pipette as a roller. The nitrocellulose membrane was then aligned with the 3 MM Whatman paper. Air bubbles were removed, and one corner of the membrane was marked, and aligned, with a soft pencil.

After separation of the glass plates, the acrylamide gel remained attached to one side of the glass. With the gel still attached to the large glass plate, the plate was inverted and placed precisely onto the nitrocellulose membrane. The gel was carefully detached from the plate onto the membrane. The final three pieces of Whatman paper, and another soaked foam pad, were lain over the membrane, air bubbles were removed, and the white (cathode) grid was added. The cassette was held together with clamping clips, and was transferred to the Electro-blotting Unit, ensuring that the membrane faced the positive electrode. The lid was replaced on the unit, ensuring close connection between the electrical connectors and the unit was transferred to a magnetic stirrer in the 4°C cold room. Protein transfer was effected by either 55V for approximately 1 hour, or 30V overnight (Consort E835 Microcapter electrophoresis power supply).

After incubation, the cassette was removed from the Electro-blotting Unit and disassembled. The membrane was transferred to a tray containing 1 x Transfer buffer and the proteins were detected using antibodies as described below.

3.23.3.10 Protein detection using antibodies

3.23.3.10.1 Primary antibodies

The primary antibodies that were employed for the detection of the GPI-PLD protein were a monoclonal mouse anti-human GPI-PLD (IgG1 isotype) (Transduction Laboratories), and a polyclonal rabbit anti-bovine GPI-PLD (Hoffman la Roche).

3.23.3.10.2 Secondary antibodies

The primary antibodies that were employed for the detection of the GPI-PLD protein were an HRP-conjugated goat anti-mouse Igs (Harlan Seralab) and an HRP-conjugated swine anti-rabbit

Igs (Dako).

3.23.3.10.3 Protein detection on a nitrocellulose membrane

3.23.3.10.4 Blocking unoccupied sites on the membrane

The nitrocellulose membrane was immersed in a 5% (w:v) non-fat dried milk in 0.1% (v:v) (Marvel) PBS-Tween solution to effect blocking of the non-specific binding sites on the membrane. Incubation was performed either overnight at 4 °C, or for 1 hour at room temperature.

3.23.3.10.5 Primary antibody incubation and wash

Incubation with primary antibody, to minimise the quantities of antibody required, was performed using a plastic bag. A length of plastic bagging was cut, and opened along one long end. The membrane was removed from the blocking buffer and introduced flat into the bag. The edges of the bag were sealed, using a heat sealer (BDH Laboratories), leaving approximately one-quarter of the upper edge unsealed.

Ten ml of primary antibody solution was prepared, by dilution in a 0.1% (v:v) PBS-Tween solution (Sigma Chemicals). The antibody was carefully poured into the sealed bag and any air bubbles were carefully removed, prior to completing the sealing of the bag. The bag was then transferred to a rotary shaker (R100 Rotatest Shaker (Luckman)) and incubated at room temperature for one hour. At regular intervals, the bag was moved to ensure complete coverage of the membrane by the liquid.

After the primary antibody incubation (1 hour), the bag was cut open and the membrane was transferred to a plastic tray. The membrane was completely immersed in 0.1% (v:v) PBS-Tween solution and incubated at room temperature on a rotary shaker. The PBS-Tween buffer was replaced four times, with incubation times of approximately 15 minutes for the first and 5 minutes for the others.

3.23.3.10.6 Second antibody and wash

The membrane was sealed into a plastic bag, as discussed in Section 3.23.3.10.5, containing 10 ml of secondary antibody in a 0.1% (v:v) PBS-Tween solution. The membrane was incubated on a rotary shaker at room temperature for 1 hour.

The second wash step was identical to the first with the exception that each of the four incubation times was extended to approximately 20 minutes.

3.23.3.10.7 Electrochemiluminesence (ECL) detection of horse-radish peroxidase-conjugated antibodies

Prior to use, the ECL-Plus detection reagents (Amersham Life Sciences Ltd) were allowed to equilibrate to room temperature. An aliquot (final volume dependent on the dimensions of the membrane (0.1 ml per cm^2)) of the detection reagent was made up, composed of solutions A and B mixed in a 40:1 (v:v) ratio. Excess wash buffer was drained from the membrane, and it was placed onto a piece of Saranwrap, with the proteins uppermost. The detection reagent was pipetted onto the membrane, ensuring that it was entirely covered, and was incubated at room temperature for 5 minutes. Excess detection reagent was drained from the membrane by touching the edge of the membrane against 3 MM Whatman paper. The membrane was then wrapped in a fresh piece of Saranwrap, any air bubbles were smoothed away and it was then placed, proteins uppermost, into an x-ray film cassette. The Saranwrap edges were secured to the cassette using tape, to ensure that the membrane did not move during detection.

A sheet of autoradiography film (Fuji Photo Film Company Ltd) (with one corner cut off to enable orientation) was placed on top of the membrane and the cassette was tightly closed. Following a 1 minute exposure, the autoradiography film was removed and was replaced with a second piece of autoradiography film to permit a second exposure. The film was then developed as described below, the results were assessed and the length of exposure of the film was adjusted until the desired band intensity was achieved.

3.23.3.10.8 Development of the photographic film

After removal from the x-ray cassette, the auto-radiography film was quickly immersed into developing solution (Photosol) for at least a minute. The film was thoroughly rinsed with water, after which time it was immersed into fixing solution (Photosol) until the film cleared.

The film was rinsed with water, to remove excess fixative, and then with a weak detergent solution to prevent hard-water marks from clouding the detail. The film was then hung to air dry.

3.24 RNA extraction, treatment and amplification by RT-PCR

3.24.1 Diethyl pyrocarbonate (DEPC) treatment of glassware and solutions

Glass and plasticware were soaked overnight in a 0.1% (v:v) solution of DEPC in water. The solution was then discarded and the containers were rinsed with ethanol and autoclaved before use. In addition, all solutions were prepared using DEPC-treated water. The water was treated by the addition of DEPC, to a final concentration of 0.1%. The samples were incubated, either

for 1 hour at 37°C or overnight at room temperature. Following incubation, the water was autoclaved.

3.24.2 RNAzol B extraction of RNA

3.24.2.1 Preparation of RNA from RBL-2H3 cells

Prior to RNA extraction, the culture medium was removed and the RBL-2H3 cells were washed thoroughly with PBS. Adherent cells were detached from culture flask using a cell scraper and the cell density was determined using a Neubauer hamocytometer. The cells were centrifuged at 1,000 G for greater than 5 minutes, after which time the supernatant was removed. The cell pellet was resuspended with RNAzol B (1 ml RNAzol B per 5 x 10^6 cells) (Biogenesis Ltd).

The mixture was transferred to a 1.5 ml eppendorf tube and chloroform was added to a final concentration of 10%. The tube was shaken vigorously for 15 seconds and the samples were maintained on ice for 5 minutes. The samples were centrifuged at 12,000 G for 15 minutes at 4°C and the upper layer was carefully removed to a fresh tube. The RNA was precipitated by the addition of isopropanol (Sigma Chemicals Ltd), at a ratio of 1:1 (v:v), and storage at -20°C overnight.

After overnight incubation, the samples were centrifuged at 12,000 x g, at 4°C, for 15 minutes. The supernatant was removed and the RNA pellet was carefully resuspended in an adequate volume of water (normally between 20 and 40 µl).

3.24.2.2 Spectrophotometric determination of RNA concentration

The principle of this method (Maniatis *et al.*, 1982,) is based on the absorption of ultra violet light by the bases of nucleic acid. This method was routinely employed for nucleic acid samples greater than 500 ng. The principal disadvantage of the method is its failure to distinguish between degraded and undegraded nucleic acid.

The RNA suspension was diluted 400-fold in sterile water in a quartz cuvette. The absorbance readings at 260 nm were measured using (V-560, UV/VIS Spectrophotometer, Jasco). Purified RNA was quantitated by absorbance at 260 nm, assuming that one A260 nm unit corresponds to 40 µg/ml of RNA.

3.24.2.3 DNAse treatment of extracted RNA

For each DNAse treatment, a reaction mix was created, consisting of 10µl of 10 x buffer, 15 µl of DNAse (1 U per ml) (Promega UK Ltd) and 2.5 µl RNAsin per reaction (Life Technologies Ltd). 17.5 µl of the resultant reaction mix was aliquoted into individual eppendorf tubes. RNA

was added to a final concentration of 15 μg per reaction and the volume was made up to 100 μl with DEPC-treated water. The tubes were sealed with Nescofilm and were incubated at 37°C for 1 hour in a water bath. Removal of the DNAse from the sample was achieved by phenol extraction.

3.24.2.4 Phenol / chloroform extraction

The sample was diluted 1:1 (v:v) with a 10 mM Tris solution, pH 8.0. An equal volume of phenol (Sigma Chemicals Ltd) was then added and the eppendorf tube was vortexed thoroughly to mix the solutions. The tube was then centrifuged at 12,000 g for 5 minutes and the upper layer was collected into a clean tube. A further equal volume of chloroform (Sigma Chemicals Ltd) was added and the tube was vortexed thoroughly to mix the solutions. Again, the samples were centrifuged at 12,000 G for 2 minutes and the upper layer was collected into a clean tube. Precipitation of the RNA was effect by addition of 3M sodium acetate (Sigma Chemicals Ltd), to a final concentration of 300 mM, and 2.5 volumes of ethanol and storage at –20°C overnight.

Following overnight incubation, the RBL-2H3 samples were centrifuged at 12,000 g for 30 minutes at less than 4°C. Taking care to minimise disturbance to the RNA-containing pellet, the supernatant was removed and the sample was resuspended in an adequate volume (typically 15 to 20 μl) of DEPC treated water. The OD reading of the RNA preparation was ascertained at 260 nm wavelength. (Section 2.12.13).

3.24.2.5 Reverse transcription of RNA

A master reaction mix was made, containing 7 μl of 5 x Buffer, 3.5 μl of 0.1M dithiothreitol, 1 μl of RNAsin (Life Technologies Ltd), 2 μl of 5 mM dNTP and 1 μl of random hexamers (1 μg/ml) (Promega UK Ltd) per sample. 14.5 μl of the reaction mix were aliquoted into individual eppendorf tubes. RNA was added to a final concentration of 5 μg, and the final volume was made up to 33.5 μl with DEPC-treated water. The solutions were carefully vortexed to ensure adequate mixing and then subjected to a pulse of centrifugation to collect the suspension at the bottom of the tube.

The tubes were incubated at 65°C for 5 minutes, to denature the secondary structure in the RNA. After this incubation, 1.5 μl of reverse transcriptase (Life Technologies) was added. The tube tops were sealed with Nescofilm, transferred to a water bath incubated at 42°C for 1½ hours. The resultant complementary DNA (cDNA) samples were then stored at –20°C until required.

3.24.3 Polymerase Chain Reaction

A master reaction mix was made, containing 5 µl 10 x Buffer, 3 µl of 25 mM magnesium chloride, 0.5 µl of 10 mM dNTP mix (Promega UK Ltd), 0.5 µl of Primer 1 (50 pMol/µl), 0.5 µl of Primer 2 (50 pMol/µl) and 0.5 µl DEPC-treated water. 10 µl of reaction mix was aliquoted into individual eppendorf tubes. 5 µl of RBL-2H3-extracted cDNA, derived from the reverse transcription reaction (Section 2.12.6) was added to each PCR reaction.

With samples containing DNA, rather than cDNA, 1 µl of DNA was added to the reaction mix and an additional 0.5 µl of 10 mM dNTP was added (to compensate for the lack of dNTP otherwise derived from the RT incubation). The volume was made up to 49 µl with DEPC-treated water and 1 µl of Taq polymerase (Promega U.K. Ltd)_was added. The tubes were centrifuged briefly at less than 4,000 rpm to ensure the reagents are all mixed. The samples were then transferred to the PCR machine (Techne Genius) and the correct cycling programme was initiated.

3.24.4 Gel electrophoresis of the RT-PCR products

10 µl of gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF and 30% ethanol in water) (Sigma Chemicals Ltd) was added to the amplified product mix. Separation of the product was effected by electrophoresis at 100 to 150 volts for 30 minutes to 1 hour,.on a 1 to 1.5% agarose gel in 1 x TBE buffer (0.89 M Tris Borate, pH 8.3 plus 20 mM Na_2EDTA) in a horizontal gel electrophoresis unit (Biorad Sub-cell GT, plus Biorad power/pac 300). Visualisation of the DNA was effected by the inclusion of 1.5 µl ethidium bromide per 100 ml of agarose gel. The DNA was visualised and photographed on an UV trans-illuminator (UVP trans-illuminator TS-15E) and cDNA bands of correct size were located by comparison with molecular weight markers.

3.25 Results

3.25.1 GPI-PLD Activity Assay in RBL-2H3 preparations

The plot in Figure 3.4 shows the results from the GPI-PLD activity assay, with preparations extracted from RBL-2H3 cells. Extraction of the membrane from the RBL-2H3 cells was accomplished, after thorough washing of the cells, to remove contaminating serum GPI-PLD enzyme. The cells were incubated in solubilising buffer, containing Nonidet P-40 detergent, and the membrane was separated from the other cellular components by centrifugation. Following centrifugation, the supernatant was removed, and the GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Membrane samples extracted from approximately 3.1 million cells per aliquot, were diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 10 minutes at 37°C.

After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

The data in Figure 3.4 is expressed as counts per minute (cpm), and shows the results from the RBL-2H3 membrane preparations in comparison with the total number of radioactivity counts available in each reaction (1250 cpm), and the negative control (solubilising buffer). The radioactivity count from the RBL-2H3 preparation (75 cpm), was only marginally higher than the 50 cpm gained with the negative control. According to the calculation (Section 3.21), 0.34 units of GPI-PLD were detected in this sample of RBL-2H3 membrane. This result indicated that the proposed incubation time of 10 minutes was insufficient to adequately detect GPI-PLD activity in RBL-2H3 preparations, in comparison with the background readings. An activity assay time course, allowing for an increased time of incubation of the RBL-2H3 preparations with the mfVSG substrate was, therefore, performed.

The plot in Figure 3.5 shows the results from the GPI-PLD activity assay with GPI-PLD preparations from RBL-2H3 cells, following termination of the assay after time intervals from 1, 20, 40 and 60 minutes. The membrane was prepared by incubation with solubilising buffer, as described above, and the GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Each aliquot of membrane extracted from the RBL-2H3 cells contained approximately 3 million cells. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The plot shows the radioactivity count from the RBL-2H3 preparations in comparison with a solubilising buffer negative control. The results clearly show that the difference between RBL-2H3 and blank rises linearly with increasing time of incubation, to a maximum of 600 counts per minute, after 60 minutes. Once again, according to the calculation (Section 3.21) the units of GPI-PLD that were detected were 0.03, 1.74, 4.71 and 9.51 at 1, 20, 40 and 60 minutes, respectively. The conclusion was that GPI-PLD activity assay with the RBL-2H3 membrane preparation required an incubation of greater than 20 minutes, before the levels of hydrolysed products were sufficient for adequate detection.

3.25.2 Inhibition of Activity in RBL-2H3 membrane preparations by EGTA and 1,10-phenanthroline

The plots in Figure 3.6 show the results from the inclusion of EGTA (3.6 a) and 1,10-phenanthroline (3.6 b) in the GPI-PLD activity assay with solubilised RBL-2H3 preparations.

FIGURE 3.4

GPI-PLD ACTIVITY ASSAY WITH PREPARATIONS FROM RBL-2H3 CELLS

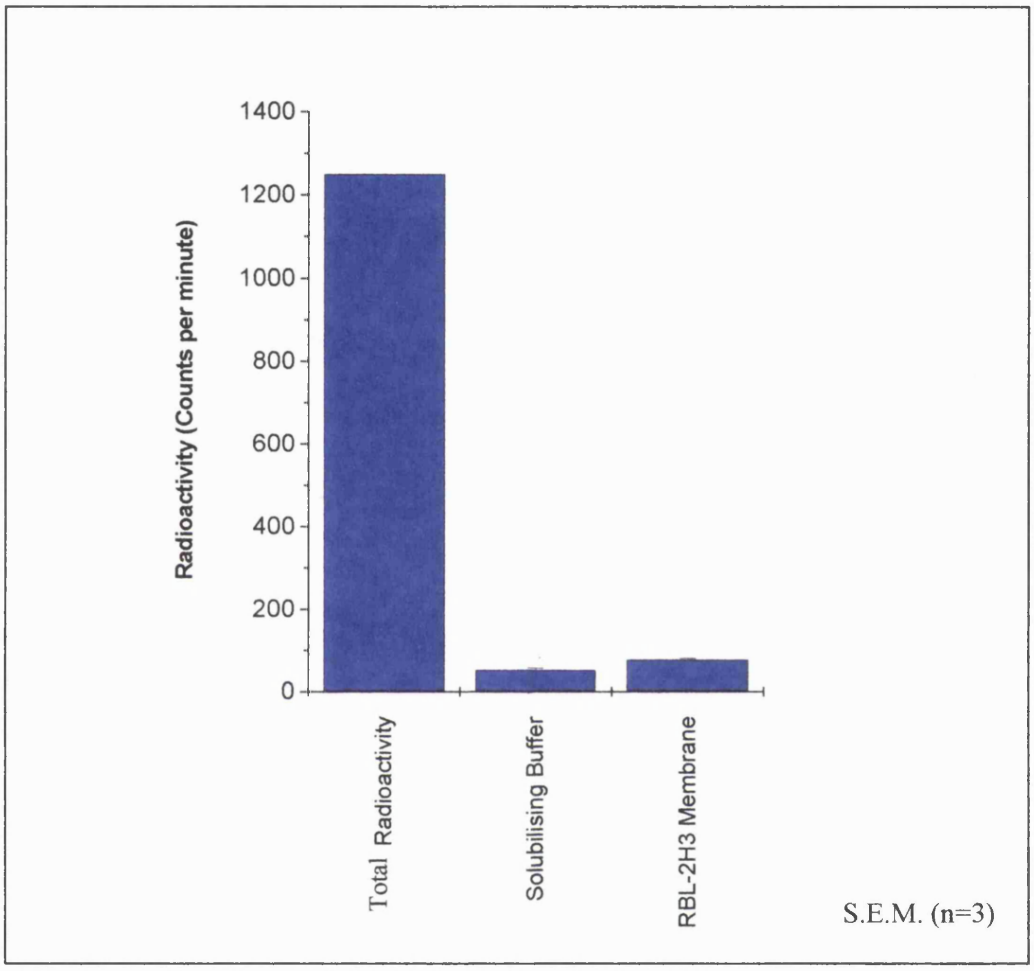


Figure Legend

The plot in figure 3.4 shows the results from the GPI-PLD activity assay using membrane prepared from RBL-2H3 cells (approximately 3 million cells per reaction). The membrane was extracted, using a detergent-containing buffer, from a thoroughly washed monolayer of RBL-2H3 cells. The GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Membrane samples were diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 10 minutes at 37°C. After termination of the activity assay, with butanol, the [^{3}H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter. The data is expressed as counts per minute (cpm),

The data shows the radioactivity count from the RBL-2H3 preparations in comparison with the total number of radioactivity counts available in each reaction, and the negative control (solubilising buffer). Note that the value gained from the RBL-2H3 preparations is only marginally higher than the result gained with the negative control.

FIGURE 3.5

VARIABLE TIME OF INCUBATION IN THE GPI-PLD ACTIVITY ASSAY WITH MEMBRANE PREPARATIONS FROM RBL-2H3 CELLS

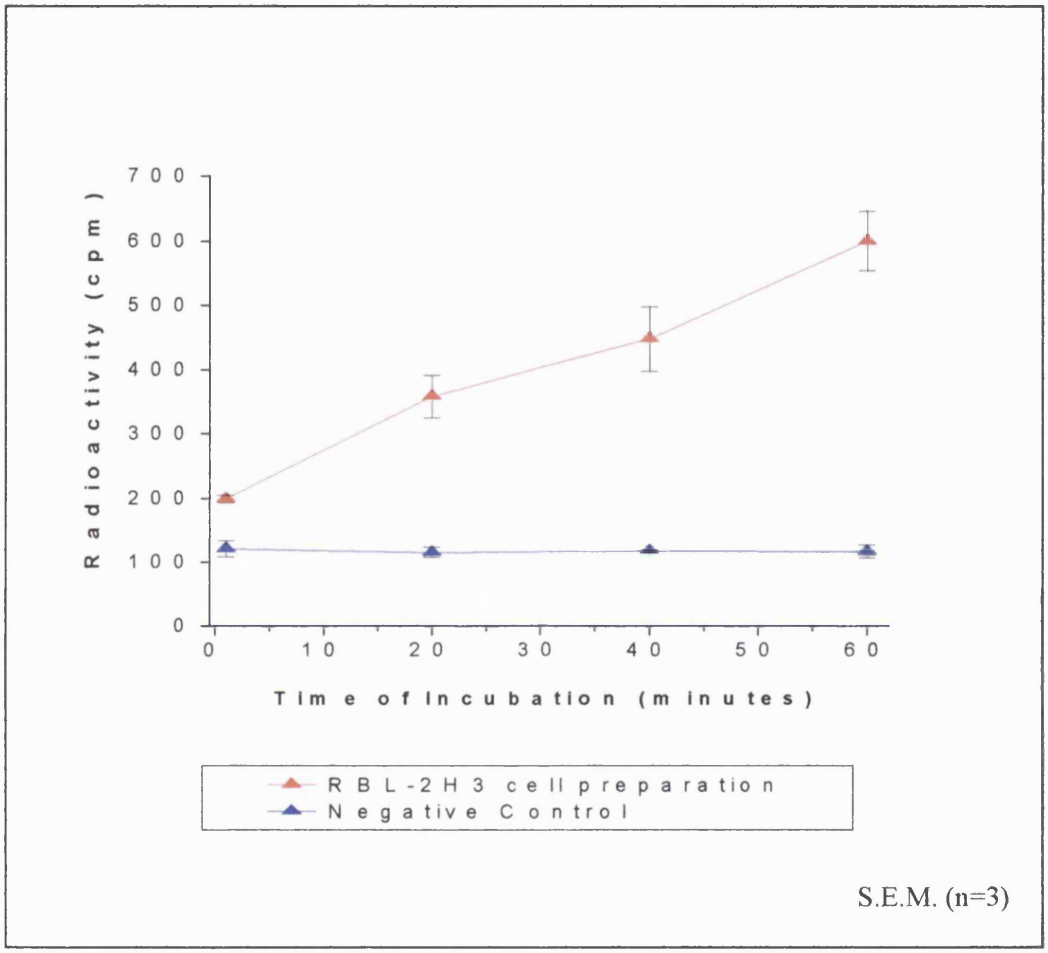


Figure Legend

The plot in figure 3.5 shows the results from the GPI-PLD activity assay with GPI-PLD using membrane, prepared from RBL-2H3 cells (approximately 3 million cells per reaction). The membrane was extracted, using a detergent-containing buffer, from a thoroughly washed monolayer of RBL-2H3 cells. The GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Membrane samples were diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 1, 20, 40 and 60 minutes at 37°C. After termination of the activity assay, with butanol, the [^{3}H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter. The data is expressed as counts per minute (cpm),

The plot shows the radioactivity count from the RBL-2H3 preparations in comparison with a solubilising buffer blank. The results clearly show that the difference between RBL-2H3 and blank rises with increasing time of incubation.

FIGURE 3.6 a

INHIBITION OF GPI-PLD ACTIVITY IN RBL-2H3 PRERARATIONS BY EGTA

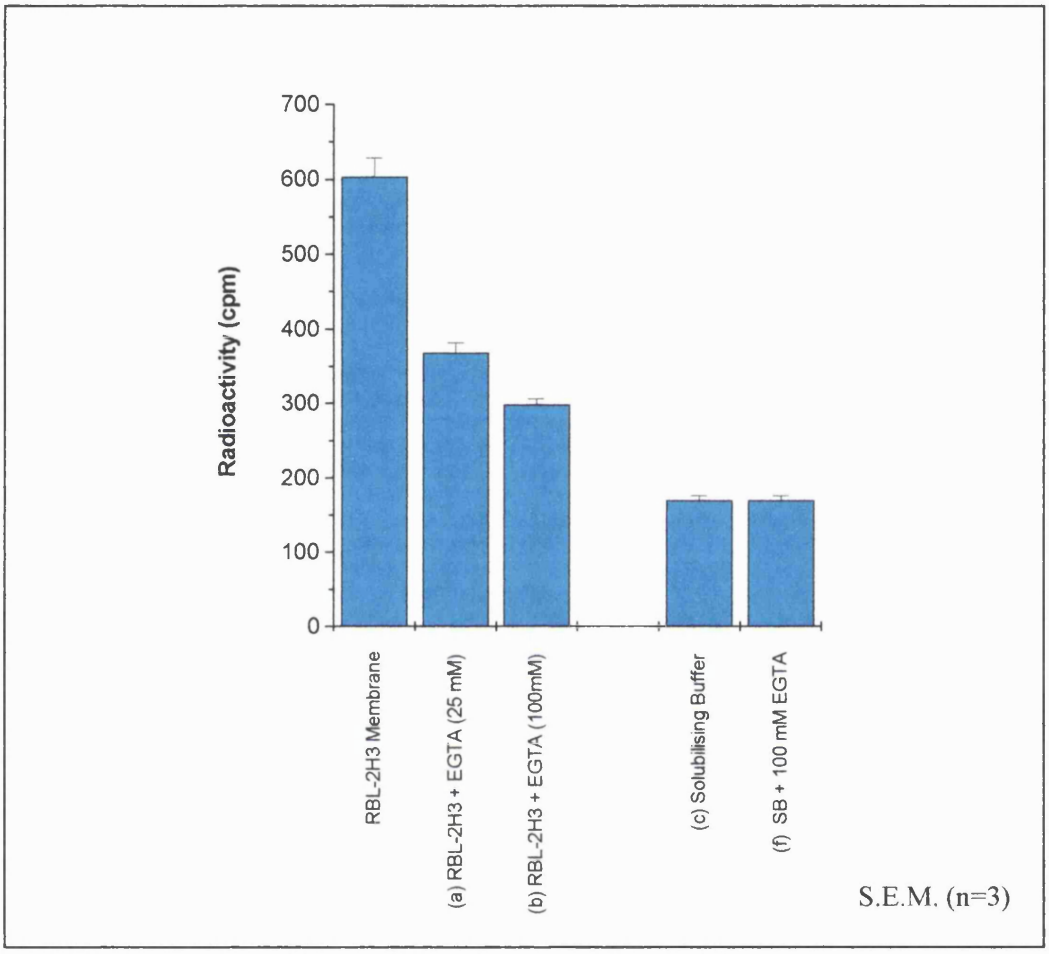


Figure Legend

The plot in figure 3.6a shows the results of the inclusion of EGTA in the GPI-PLD activity assay with RBL-2H3 preparations. The membrane fraction was extracted, using a detergent-containing buffer, from a thoroughly washed monolayer of RBL-2H3 cells. The GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Membrane samples, each containing the equivalent of approximately 2.8 million cells, were diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 30 minutes. After termination of the activity assay, with butanol, the [^{3}H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter. The data is expressed as counts per minute (cpm),

The experiment was performed using an aliquot of the same RBL-2H3 preparation, with the inclusion of (a) 25 mM and (b) 100 mM EGTA. In addition, the solubilising buffer (c) negative control is shown, with (d) 100 mM EGTA.

FIGURE 3.6 b

INHIBITION OF GPI-PLD ACTIVITY IN RBL-2H3 PRERARATIONS
BY 1,10-PHENANTHROLINE

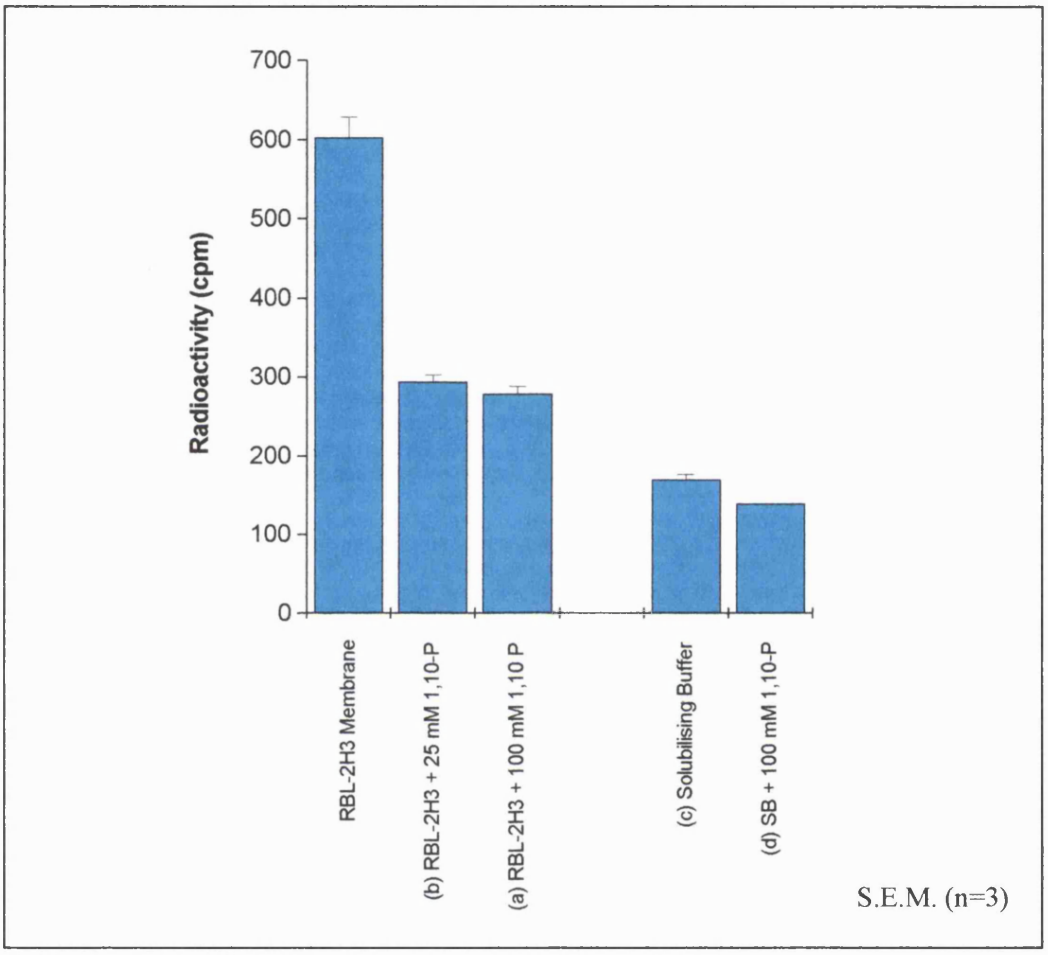


Figure Legend

The plot in figure 3.6b shows the results of the inclusion of 1,10-phenanthroline in the GPI-PLD activity assay with RBL-2H3 preparations. The membrane fraction was extracted, using a detergent-containing buffer, from a thoroughly washed monolayer of RBL-2H3 cells. The GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Membrane samples, each containing the equivalent of approximately 2.8 million cells, were diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 30 minutes. After termination of the activity assay, with butanol, the [^{3}H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter. The data is expressed as counts per minute (cpm),

The data was performed using an aliquot of the same RBL-2H3 preparation, with the inclusion of (a) 100 mM and (b) 25 mM 1,10-phenanthroline. In addition, the solubilising buffer (c) negative control is shown, with (d) 100 mM 1,10-phenanthroline.

The RBL-2H3 membrane was prepared as discussed previously (Section 3.20.1), and the GPI-PLD activity assay was performed with 30 mM mfVSG substrate. Each aliquot of RBL-2H3 cells contains membrane extracted from approximately 2.8 million cells. The reaction was terminated, using ammonium-saturated butanol, after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The result in Figure 3.6a was generated using the same RBL-2H3 preparation, with the inclusion of (a) 25 mM and (b) 100 mM EGTA, prior to the GPI-PLD activity assay. In addition, the solubilising buffer (c) negative control is shown, with (d) 100 mM EGTA. The results show that EGTA inhibited the GPI-PLD activity of RBL-2H3 preparations. Without inhibition, 12 units of GPI-PLD activity were detected in the RBL-2H3 membrane preparation. By comparison, the inclusion of either 25 or 100 mM EGTA reduced the GPI-PLD activity to 7.3 and 5.9 units, respectively. EGTA, at a concentration of 100 mM, did not affect the negative control.

The result in Figure 3.6b was generated using the same RBL-2H3 preparation, with the inclusion of (a) 100 mM and (b) 25 mM 1,10-phenanthroline prior to the GPI-PLD activity assay. The results show that 1,10-phenanthroline inhibited the GPI-PLD activity of RBL-2H3 preparations. Without inhibition, 12 units of GPI-PLD activity were detected in the RBL-2H3 membrane preparation. 1,10-phenanthroline reduced the GPI-PLD activity to 5.8 and 5.5 units at 25 mM and units at 100 mM concentrations, respectively. The solubilising buffer (c) negative control is shown, with (d) 100 mM 1,10-phenanthroline. 100 mM 1,10-phenanthroline did not affect the negative control.

Using this methodology for the detection of GPI-PLD activity, the conclusion was made that RBL-2H3 cells contain activity, and therefore, contain the GPI-PLD enzyme, and that it was susceptible to inhibition by EGTA and 1,10-phenanthroline. To confirm this observation, it was necessary to detect the GPI-PLD protein. The method by which this was accomplished was the Western Blot procedure.

3.25.3 GPI-PLD detection in RBL-2H3 Membrane preparations through Western Blot analysis

Figure 3.7 shows the results from Western Blot analysis of RBL-2H3 membrane preparations, with polyclonal anti bovine-GPI-PLD antibody, in comparison with molecular weight markers. The protein concentration in the RBL-2H3 samples was determined using the Coomassie plus protein detection assay, and 200 μg of protein was loaded into each well on the SDS-PAGE gel.

FIGURE 3.7

WESTERN BLOT ANALYSIS OF RBL-2H3 MEMBRANE PREPARATIONS USING POLYCLONAL BOVINE ANTI-GPI-PLD ANTIBODY

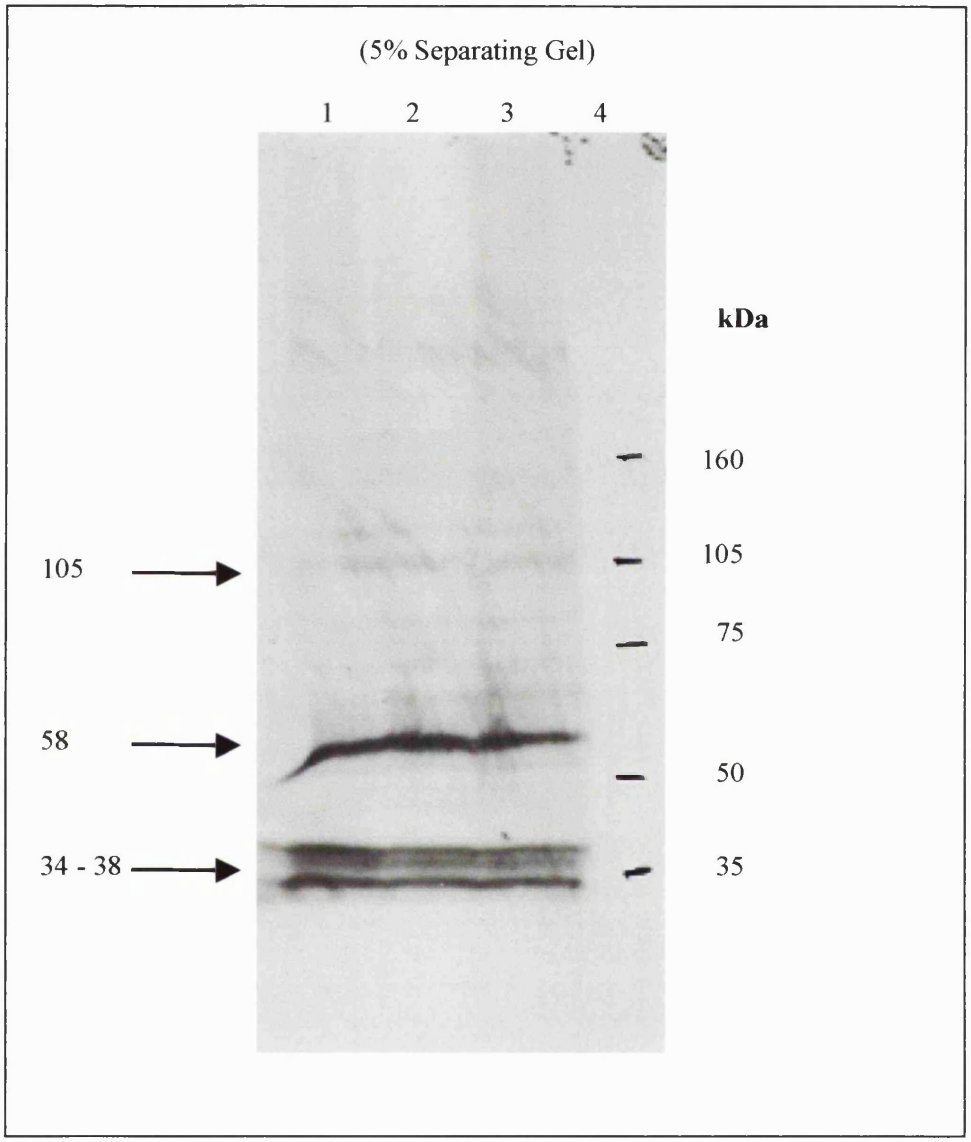


Figure Legend

Figure 3.7 shows the results from Western Blot analysis of RBL-2H3 membrane preparations, with polyclonal anti bovine-GPI-PLD antibody, in comparison with molecular weight markers (the markers are not visualised by the Western Blot procedure, and, therefore, after protein separation, the position of the markers was pencilled onto the nitrocellulose membrane). The photographic film was exposed to the ECL+-treated nitrocellulose membrane for 5 minutes.

Lanes 1, 2 and 3 show preparations from RBL-2H3 cells which were frozen at different times, but were defrosted and cultured simultaneously, prior to membrane preparation. Note the strong bands at approximately 58 kDa, and a cluster of bands at approximately 38, 36 and 34 kDa. Note also a more weakly-staining band at approximately 105 kDa molecular weight.

The three lanes show separation of membrane preparations from RBL-2H3 cells. The three different RBL-2H3 preparations represent cells that were frozen at different times, but were defrosted and cultured simultaneously, prior to membrane preparation. GPI-PLD positive-bands were detected approximately molecular weights of approximately 58 kDa, and a cluster of bands at approximately 38, 36 and 34 kDa. A more weakly-staining band was also detected at around 105 kDa.

By comparison, the results in Figure 3.8 show the Western Blot analysis of RBL-2H3 membrane preparations, with monoclonal anti human-GPI-PLD antibody, in comparison with molecular weight markers. The result shows that the monoclonal antibody has not detected GPI-PLD the RBL-2H3 membrane preparations.

In addition, Figure 3.8 shows the GPI-PLD positive control was detected as a ladder of different molecular weights. The suppliers' information regarding the GPI-PLD positive control states that, after Western blotting with the monoclonal antibody, the positive control should be detected as a single band, of 110 kDa molecular weight (www.translab.com). This ladder of differently sized proteins, therefore, suggests that the positive control has undergone degradation during the course of the experiment. The reason for this degradation was not determined.

3.25.4 Detection of GPI-PLD mRNA expression using the RT-PCR methodology

3.25.4.1 β-actin specific primers

The photograph in Figure 3.9 shows the amplified products from the RT-PCR reaction using β-actin specific primers. Total RNA was extracted from RBL-2H3 cells using RNAzol B, followed by treatment with DNAse. Following reverse transcription with random hexamers, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 amplification cycles, with an annealing temperature of 60°C).

The products were separated on a 1.5% agarose gel, alongside a 100 bp DNA ladder (Lanes 1 and 9). Lanes 2 to 5 contain the RT-PCR products from RBL-2H3 cells. Lanes 2 and 3 contain un-treated RNA with (2) and without (3) RT. Lanes 4 and 5 contain DNase treated RNA with (4) and without (5) RT. The positive control, rat liver RNA was included, with (6) and without (7) RT, and lane 8 contains an additional negative control, containing water instead of RNA.

Bands, of approximately 100 bp, were visualised in lanes 2, 4 and 6, representing the 102 bp product expected from this pair of primers. These samples were the RBL-2H3 and liver

FIGURE 3.8

WESTERN BLOT ANALYSIS OF RBL-2H3 MEMBRANE PREPARATIONS USING MONOCLONAL BOVINE ANTI-GPI-PLD ANTIBODY

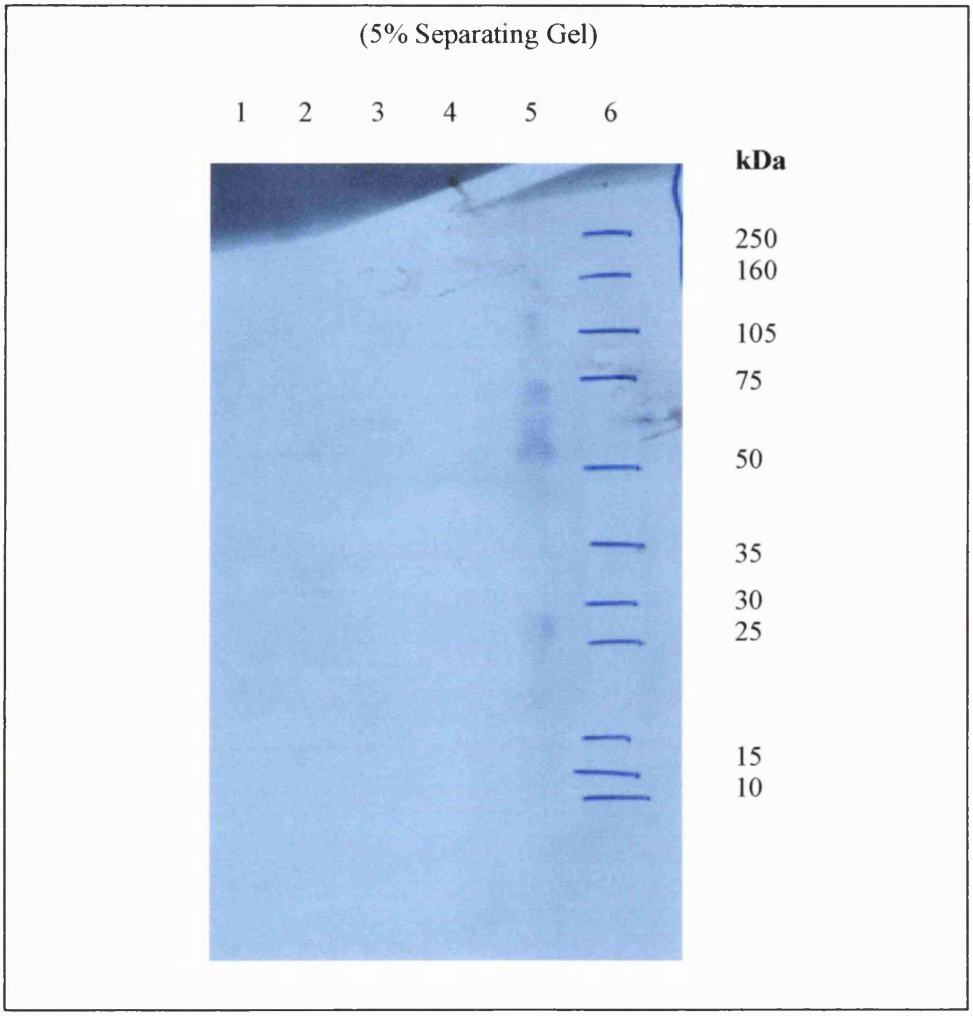


Figure Legend

Figure 3.8 shows the results from Western Blot analysis of RBL-2H3 membrane preparations, with monoclonal anti human-GPI-PLD antibody, in comparison with molecular weight markers (the markers are not visualised by the Western Blot procedure, and, therefore, after protein separation, the position of the markers was pencilled onto the nitrocellulose membrane). The photographic film was exposed to the ECL+-treated nitrocellulose membrane for 5 minutes.

Lanes 1 contains 10% FBS in EMEM and lane 2 contains EMEM without FBS. Note the faintly stained band at approximately 60 kDa. Lanes 3, 4 and 5 contain preparations from RBL-2H3 cells which were frozen at different times, but were defrosted and cultured simultaneously, prior to membrane preparation. Lane 6 contains the markers.

Note that the monoclonal antibody has not detected GPI-PLD in the RBL-2H3 membrane preparations. In addition, although the positive control is supposed to be a single band, at 110 kDa, the positive control in this case is a ladder of different molecular weights.

FIGURE 3.9

RT-PCR AMPLIFIED cDNA USING β-ACTIN SPECIFIC PRIMERS
(ACTIN F AND ACTIN R)

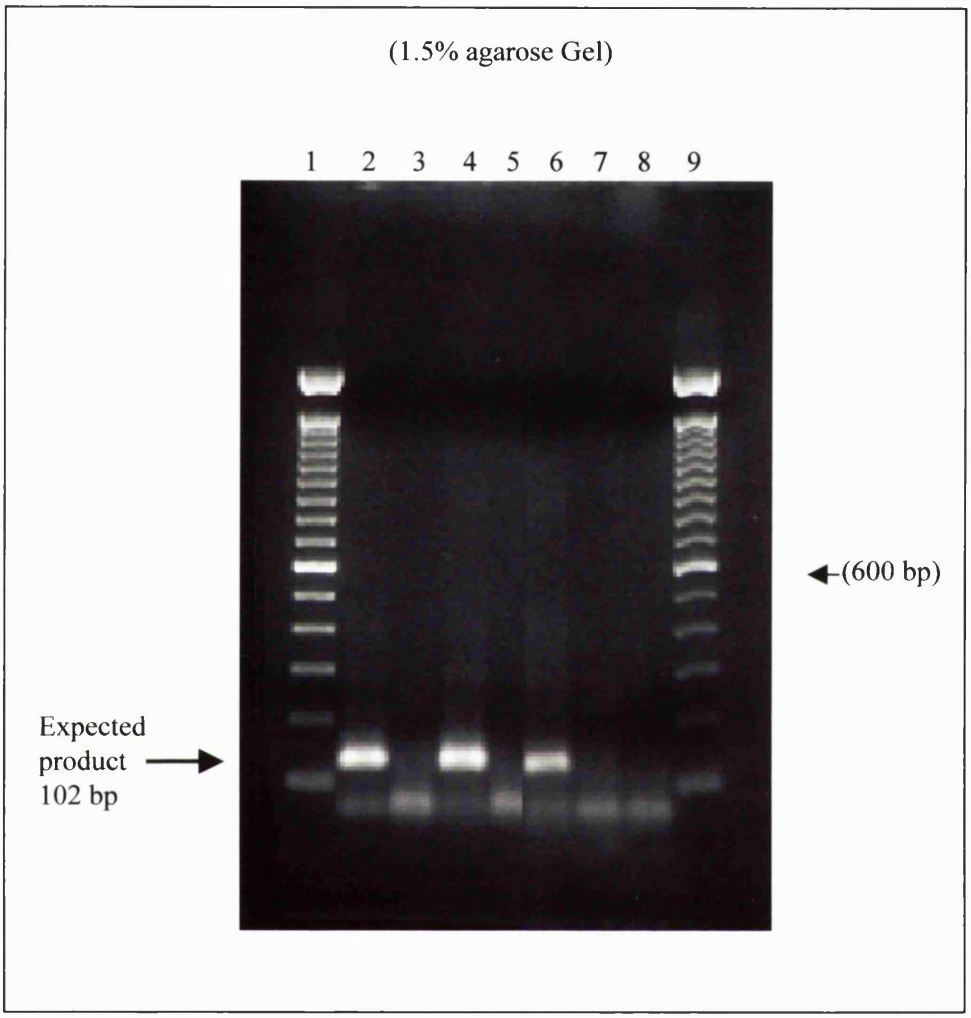


Figure Legend

The photograph in Figure 3.9 shows the amplified products from the RT-PCR reaction using β-actin specific primers. The mRNA was extracted from RBL-2H3 cells using RNAzol B, followed by treatment with DNAse. Following reverse transcription with random hexamers, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 amplification cycles, with an annealing temperature of 60°C).

The products were separated on a 1.5% agarose gel, alongside a 100 bp DNA ladder (lanes 1 and 9). Note that an RT-PCR product (of approximately 100 bp) was visualised in the lanes containing both DNAse treated and untreated samples with RT (lanes 2 and 4) and in the rat liver positive control with RT (lane 6). By comparison, no RT-PCR product was visualised in those lanes in which RT was not included (lanes 3, 5 and 7). The absence of RNA also precluded the existence of a RT-PCR product (lane 8)

This result confirms that the RNA from RBL-2H3 cells, and from liver RNA, was successfully extracted, and was amplified by the RT-PCR methodology

samples in which RT was included in the PCR reaction. This result confirms that RNA from RBL-2H3 cells was successfully extracted, and was amplified by the RT-PCR methodology.

3.25.4.2 Human GPI-PLD specific primers

The photograph in Figure 3.10 shows the amplified products from the RT-PCR reaction using human GPI-PLD specific primers. The RNA was extracted, DNAse treated and reverse transcribed, as discussed in Section 3.24.2.5. The RBL-2H3 samples were then amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 58°C).

The products were separated on a 1.5% agarose gel, alongside a 100 bp DNA ladder (Lanes 1 and 8). Lanes 2 to 5 contain the RT-PCR products from RBL-2H3 cells. Lanes 2 and 3 contain un-treated RNA with (2) and without (3) RT. Lanes 4 and 5 contain DNase treated RNA with (4) and without (5) RT. Lane 6 contains a full length human liver GPI-PLD cDNA, as a positive control and lane 7 contains the negative control, containing water instead of mRNA.

Note that an approximately 730 bp sized band was visualised only in the lane containing product from the A1 positive control. This represents the 730 bp product expected from this pair of primers. Once again, however, the absence of RT-PCR product in the samples extracted from RBL-2H3 cells confirmed that these cells do not contain the GPI-PLD mRNA.

3.25.4.3 Mouse GPI-PLD specific primers

The photograph in Figure 3.11 shows the amplified products from the RT-PCR reaction using mouse GPI-PLD specific primers. The RNA was extracted, and treated with DNAse, as discussed in Section 3.24.2.3. Following reverse transcription, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 62.9°C).

The products were separated on a 1.5% agarose gel, in comparison with a 100 bp DNA ladder (Lane 7). Lanes 1 to 4 contain the RT-PCR products from RBL-2H3 cells. Lanes 1 and 2 contain un-treated RNA with (1) and without (2) RT. Lanes 3 and 4 contain DNase treated RNA with (3) and without (4) RT. Lane 5 contains the rat liver positive control, and lane 6 contains the negative control.

Two bands were visualised in the lane containing rat liver, corresponding to approximately 730 and approximately 950 base pairs. Unfortunately, the resolution of the markers on this electrophoresis gel is not particularly clear, and it is difficult to determine the size of the RT-PCR product more accurately. However, the presence of an approximately 750 base pair-sized

FIGURE 3.10

RT-PCR AMPLIFIED cDNA USING HUMAN GPI-PLD SPECIFIC PRIMERS (P6 AND P7)

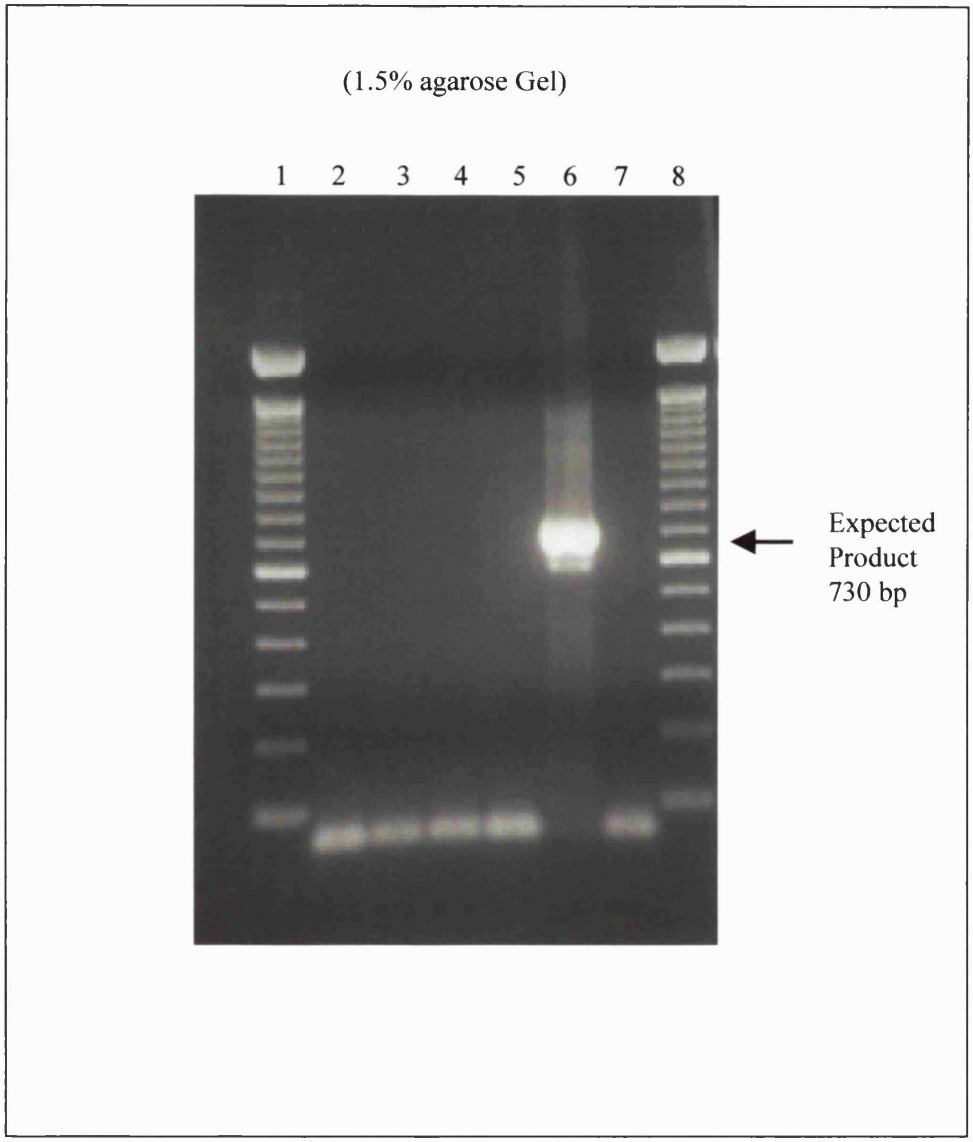


Figure Legend

The photograph in Figure 3.10 shows the amplified products from the RT-PCR reaction using human GPI-PLD specific primers. The RNA was extracted, DNAse treated and reverse transcribed, as discussed in Figure 3.9. The RBL-2H3 samples were then amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 58°C).

The products were separated on a 1.5% agarose gel, against a 100 bp DNA ladder. No RT-PCR product was detected in any of the RBL-2H3 mRNA samples, either with (lanes 2 and 4) or without RT (lanes 3 and 5), or in the negative control (lane 8). However an approximately 730 kB sized band was visualised in the lane containing product from the positive control.

FIGURE 3.11

RT-PCR AMPLIFIED cDNA USING MOUSE GPI-PLD SPECIFIC PRIMERS
(MP1 and MP2)

Figure Legend

The photograph in Figure 3.11 shows the amplified products from the RT-PCR reaction using mouse GPI-PLD specific primers. The RNA was extracted, and treated with DNAse, as discussed in Figure 4.9. Following reverse transcription, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 62.9°C).

The products were separated on a 1.5% agarose gel, against a 100 bp DNA ladder. No RT-PCR product was detected in any of the RBL-2H3 RNA samples, either with (lanes 1 and 3) or without RT (lane 2 and 4), or in the negative control (lane 6). However, two bands were visualised in the lane containing rat liver, corresponding to approximately 950 and approximately 750 base pairs.

band would represent the 758 product, as predicted by the position of the primers on the gene sequence. The existence of an additional product, at approximately is 950 bp could be explained by an additional site on the *Gpld* gene against which the primer has aligned, thereby producing a second PCR product.

The confirmation that a correctly sized RT-PCR product was detected with the liver positive control established that the RT-PCR was successfully performed. Once again, the lack of positive RT-PCR results with any of the RNA samples extracted from RBL-2H3 cells confirms that the mRNA detected by these primers is not contained within this cell line.

3.25.4.4 Rat GPI-PLD specific primers

The photograph in Figure 3.12 shows the amplified products from the RT-PCR reaction using rat GPI-PLD specific primers. The RNA was extracted from RBL-2H3 cells using RNAzol B, followed by treatment with DNAse. Following reverse transcription with random hexamers, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 58°C).

The products were separated on a 1.5% agarose gel, alongside a 100 bp DNA ladder (Lane 7). Lanes 3 to 6 contain the RT-PCR products from RBL-2H3 cells. Lane 1 contains the negative control, containing water instead of RNA, whereas Lane 2 contains the rat liver positive control. Lanes 3 and 4 contain un-treated RNA with (3) and without (4) RT. Lanes 5 and 6 contain DNase treated RNA with (5) and without (6) RT. Unfortunately, the clarity of the markers on this electrophoresis gel is not particularly defined, and it is difficult to determine the size of the RT-PCR product accurately. However, an approximately 800 bp sized band was visualised in the lane containing product from the rat liver positive control. This product represents the 828 bp sized product anticipated from this pair of primers. This result confirms that the RNA samples extracted from RBL-2H3 cells did not contain the sequence corresponding to these primers, but the result from the positive control confirmed that the RT-PCR was successful.

3.26 Conclusions

Overall, the results from the RT-PCR experiments show that both the RNA extraction technique, and the RT-PCR methodology, was successful. The positive controls were positive in each case, and the negative controls were negative. In no case was a GPI-PLD-specific sequence detected in the RNA extracted from RBL-2H3 cells.

After analysis of the RT-PCR results, it was concluded that RBL-2H3 cells did not produce the

FIGURE 3.12

RT-PCR AMPLIFIED cDNA USING RAT GPI-PLD SPECIFIC PRIMERS
(RATG1 and RATG2)

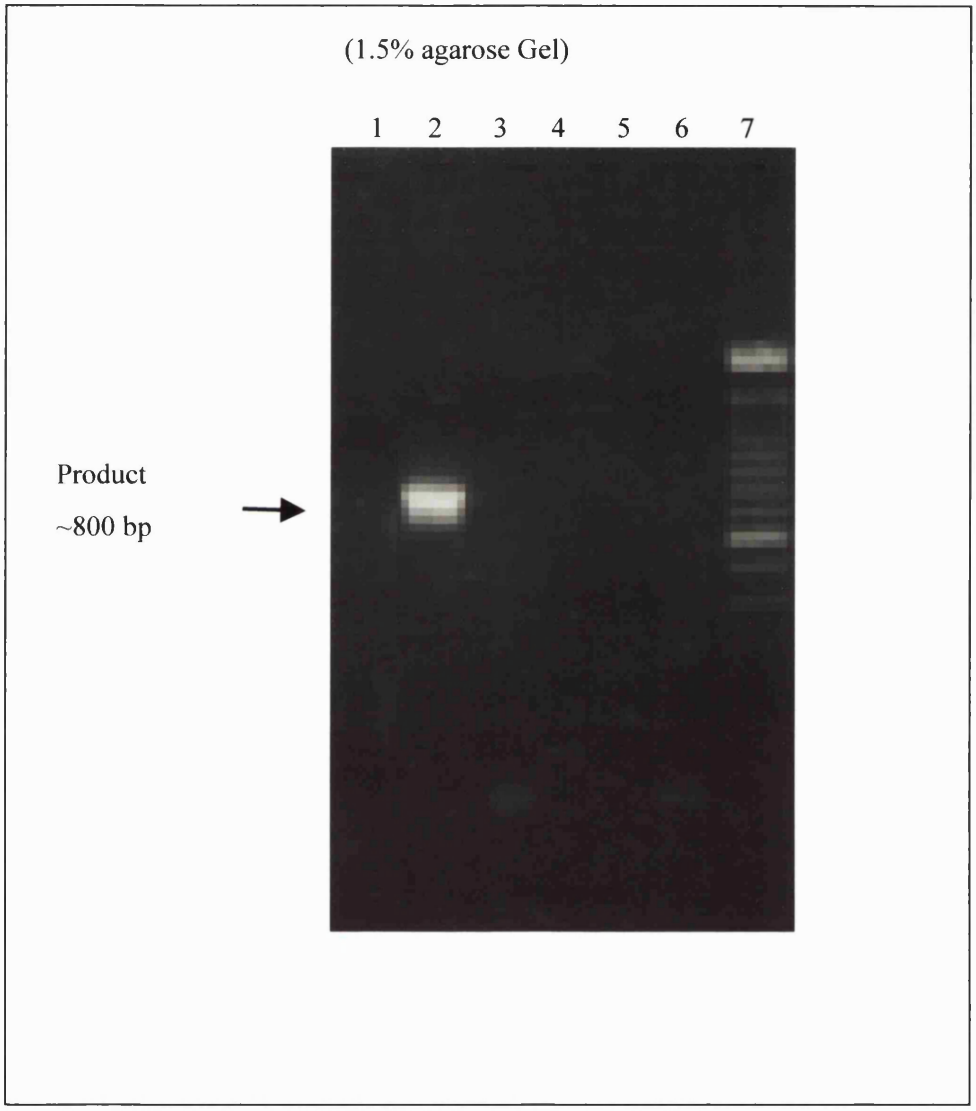


Figure Legend

The photograph in Figure 3.12 shows the amplified products from the RT-PCR reaction using rat GPI-PLD specific primers. The RNA was extracted from RBL-2H3 cells using RNAzol B, followed by treatment with DNAse. Following reverse transcription with random hexamers, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 58°C).

The products were separated on a 1.5% agarose gel, alongside a 100 bp DNA ladder. No RT-PCR product was detected in any of the RBL-2H3 RNA samples, either with (lanes 3 and 5) or without RT (lanes 4 and 6), or in the negative control (lane 1). However, an approximately 800 bp sized band was visualised in the lane containing product from the rat liver positive control (Lane 2)

mRNA for the GPI-PLD enzyme. Considering that the activity assay and the Western blot had showed the existence of the enzyme, it was important to determine the source of the protein in RBL-2H3 cells. The literature reports high levels of GPI-PLD in serum. Therefore, the source of GPI-PLD that was most strongly implicated was the serum in which the RBL-2H3 cells were cultured and it was important to determine the existence of GPI-PLD activity and protein in the FBS samples.

3.27 GPI-PLD activity in Foetal Bovine Serum

The plot in Figure 3.13 shows the results from the GPI-PLD activity assay with FBS (10% in EMEM), following termination of the assay after 15 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter. The data shows the radioactivity count from the FBS sample in comparison with the total number of radioactivity counts, and the negative control (0% FBS in EMEM). The data shows that the GPI-PLD activity in the 10% FBS in EMEM sample had a radioactivity reading of approximately 1000 cpm, in comparison with a 0% FBS in EMEM (blank) of 100 cpm. According to the calculation (Section 3.21) this is equal to 9.4 units of GPI-PLD in a 10% FBS sample.

By comparison, the plot in Figure 3.14 shows a time course of incubation of the 10% FBS in EMEM samples, with termination of the assay after time intervals from 1, 20, 40 and 60 minutes. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The plot shows the radioactivity count from the 10% FBS in EMEM in comparison with a 0% FBS in EMEM blank. The results clearly show that the difference between 10% FBS and 0% FBS blank rises to a plateau, at 20 minutes, after which time the assay is saturated. Once again, according to the calculation (Section 3.21) the units of GPI-PLD that were detected were 0.14, 10.2, 20.7, 31.0 at 1, 20, 40 and 60 minutes, respectively.

3.28 Inhibition of Activity in FBS membrane preparations by EGTA and 1,10-phenanthroline

The plot in Figure 3.15 shows the results of the inclusion of EGTA and 1,10-phenanthroline in the GPI-PLD activity assay with 10% FBS samples. The reaction was terminated after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The data was performed using an aliquot of the same FBS preparation in each assay, with the

FIGURE 3.13

GPI-PLD ACTIVITY ASSAY USING MIMUMUM ESSENTIAL MEDIUM SUPPLEMENTED WITH (10%) FOETAL BOVINE SERUM

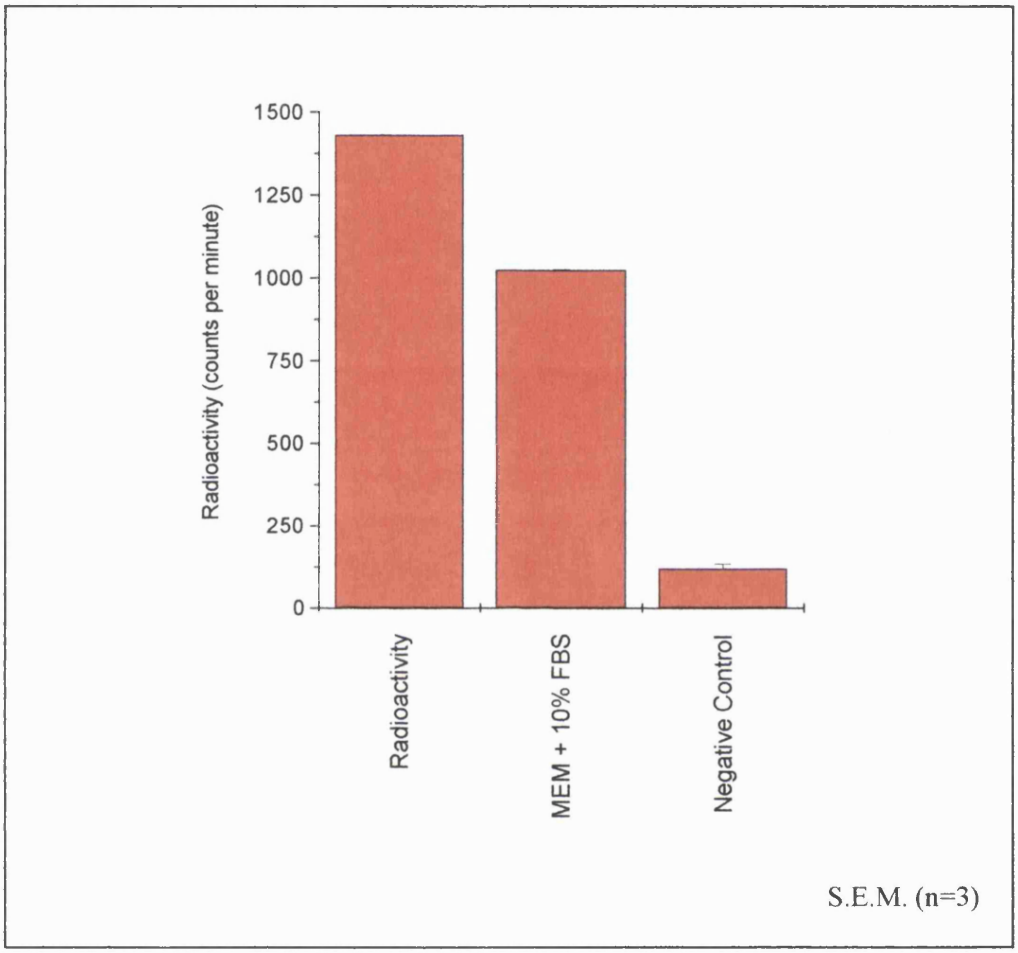


Figure Legend

The plot in figure 3.13 shows the results from the GPI-PLD activity assay with FBS (10% in EMEM) following termination of the assay after 15 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter. The data shows the radioactivity count from the FBS samples in comparison with the total number of radioactivity counts available in each reaction and the negative control (0% FBS in EMEM).

The data shows that the GPI-PLD activity in the 10% FBS in EMEM sample had a radioactivity reading of approximately 1000 cpm, in comparison with a 0% FBS in EMEM (blank) of 100 cpm.

FIGURE 3.14

VARIABLE TIME OF INCUBATION IN THE GPI-PLD ACTIVITY ASSAY WITH MIMUMUM ESSENTIAL MEDIUM SUPPLEMENTED WITH (10%) FOETAL BOVINE SERUM

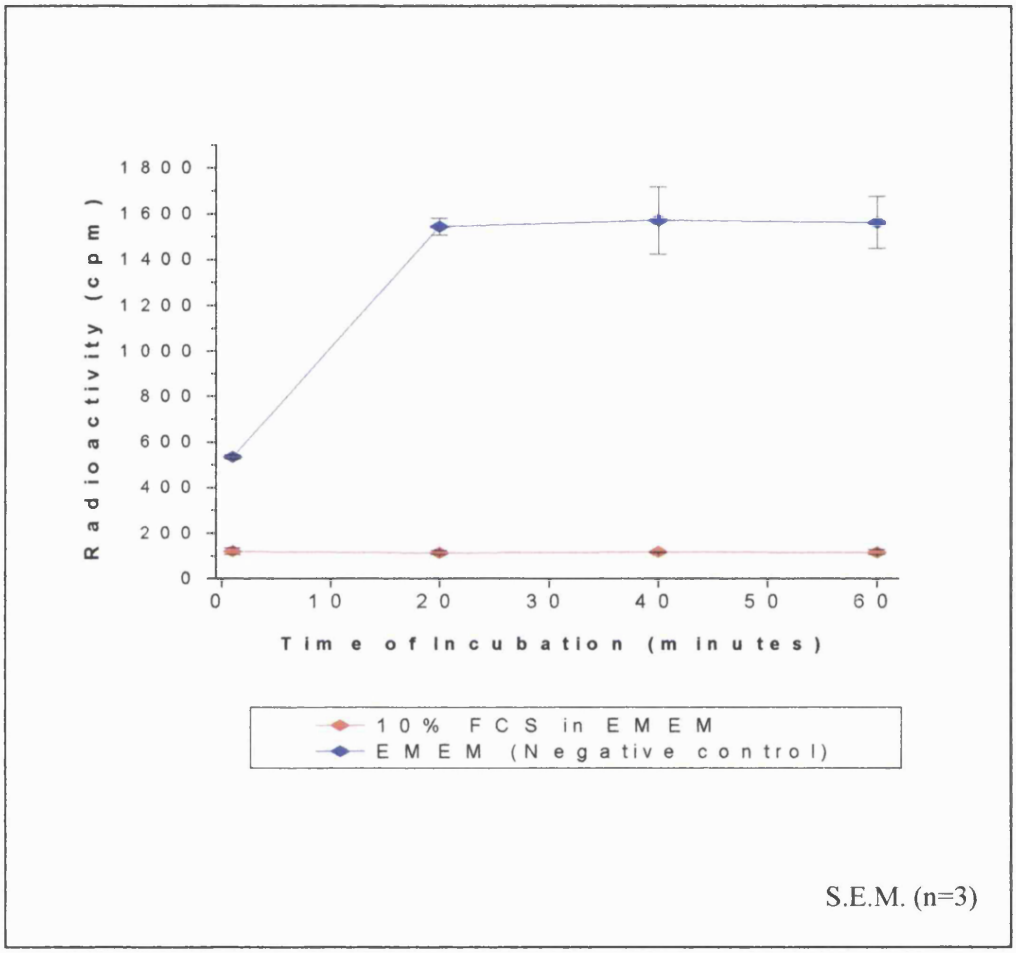


Figure Legend

The plot in figure 3.14 shows the results from the GPI-PLD activity assay with 10% FBS in EMEM, following termination of the assay after time intervals from 1, 20, 40 and 60 minutes. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The plot shows the radioactivity count from the 10% FBS in EMEM in comparison with a 0% FBS in EMEM blank. The results clearly show that the difference between 10% FBS and 0% FBS blank rises to a plateau, at 20 minutes, after which time the assay is saturated.

FIGURE 3.15

INHIBITION OF GPI-PLD ACTIVITY IN MEM WITH 10% FOETAL BOVINE SERUM USING EGTA AND 1,10-PHENANTHROLINE

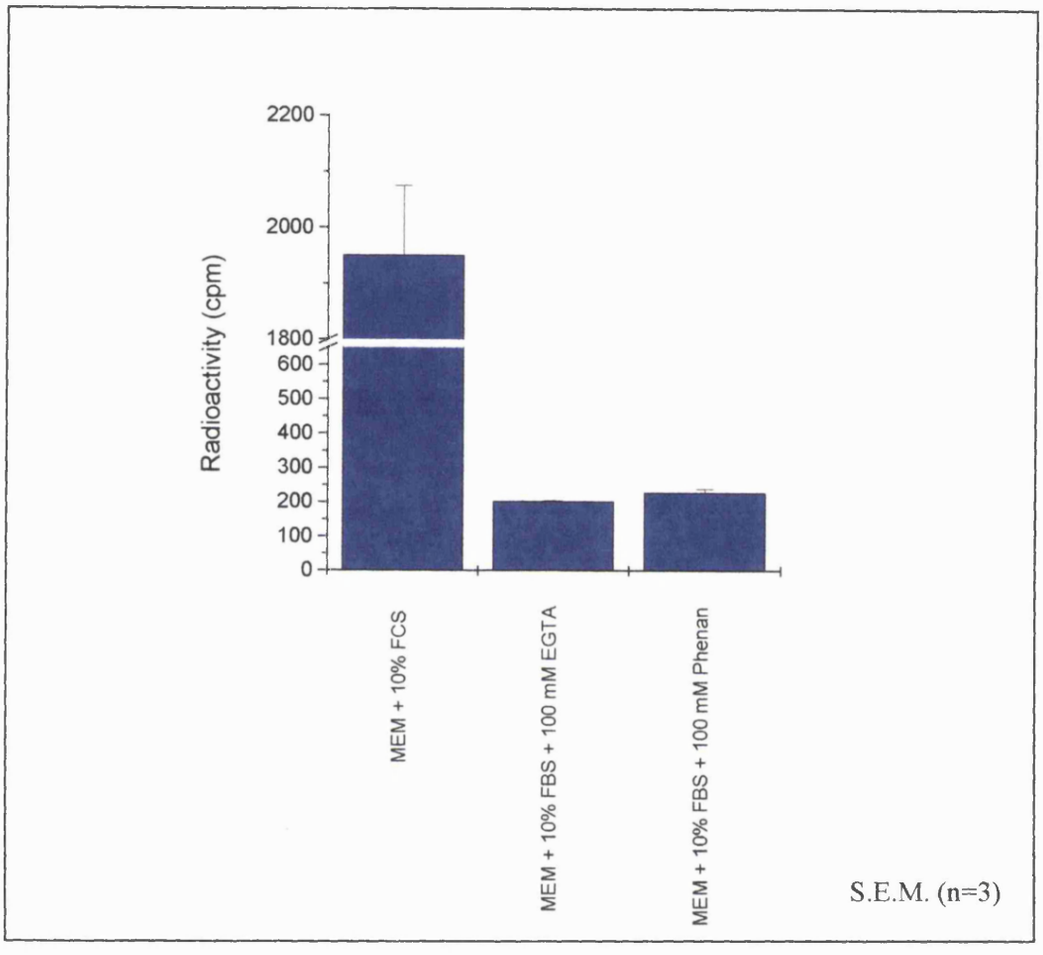


Figure Legend

The plot in figure 3.15 shows the results of the inclusion of EGTA and 1,10-phenanthroline in the GPI-PLD activity assay with 10% FBS samples. The reaction was terminated after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The data was performed using an aliquot of the same FBS preparation in each assay (1), with either 100 mM EGTA (2) or 100 mM 1,10-phenanthroline (3). Note the break in the y axis, to compensate for the large differences between the 10% FBS with and without inhibitors. Clearly, the inhibitors have effected a dramatic reduction in the activity of the GPI-PLD enzyme. Neither inhibitor affected the negative control.

inclusion of 100 mM EGTA or 100 mM 1,10-phenanthroline. Without inhibition, 38 units of GPI-PLD activity were detected in the 10% FBS preparation. By comparison, the inclusion of 100 mM EGTA reduced the GPI-PLD activity to 4 units, whereas 1,10-phenanthroline reduced the GPI-PLD activity to 4.5 units. Clearly this is a dramatic reduction in the activity of the GPI-PLD enzyme, and demonstrates that the FBS-derived GPI-PLD is highly susceptible to inhibition by EGTA or 1,10-phenathroline. Neither inhibitor affected the negative control.

3.29 Western Blot Analysis of Foetal Bovine Serum

Figure 3.16 shows the results from Western Blot analysis of Foetal Bovine Serum (10% in supplemented EMEM) with monoclonal anti human-GPI-PLD antibody, in comparison with High Molecular Weight markers. 300 μg of protein was loaded into each well on the SDS-PAGE gel, with the exception of the un-supplemented EMEM, in which the protein concentration was almost negligible.

Lane 1 shows un-supplemented EMEM, whereas lanes 2, 3 and 4 show three different samples of 10% FBS, prepared in EMEM. The Western Blot procedure has not detected any GPI-PLD protein in EMEM without FBS. A weakly staining band, of approximately 115 kDa, is visible in lanes 2, 3 and 4. In addition, strongly GPI-PLD positive bands were observed at the interface of the stacking and separation gels.

By comparison, Figure 3.17 shows the results from Western Blot analysis of Foetal Bovine Serum (10% in supplemented EMEM) with polyclonal anti human-GPI-PLD antibody, in comparison with molecular weight markers. In this instance, the polyclonal antibody has not detected any GPI-PLD protein in any of the samples.

3.30 Discussion

3.30.1 Introduction

The results show that the RBL-2H3 cells contain the GPI-PLD protein, and it's enzymatic activity, despite finding no evidence for the production of the enzymes' mRNA. That the RBL-2H3 cells do not produce mRNA for GPI-PLD, yet contain the protein, assumes that they derive the enzyme from an extracellular source. Analysis of the medium in which the cells were cultured, supported the existence of the GPI-PLD enzyme in the Foetal Bovine Serum (FBS), which was used to supplement the culture medium. The conclusion was made that the source of the protein in the RBL-2H3 cells was the FBS.

FIGURE 3.16

WESTERN BLOT ANALYSIS OF FOETAL BOVINE SERUM USING MONOCLONAL ANTI GPI-PLD ANTIBODY

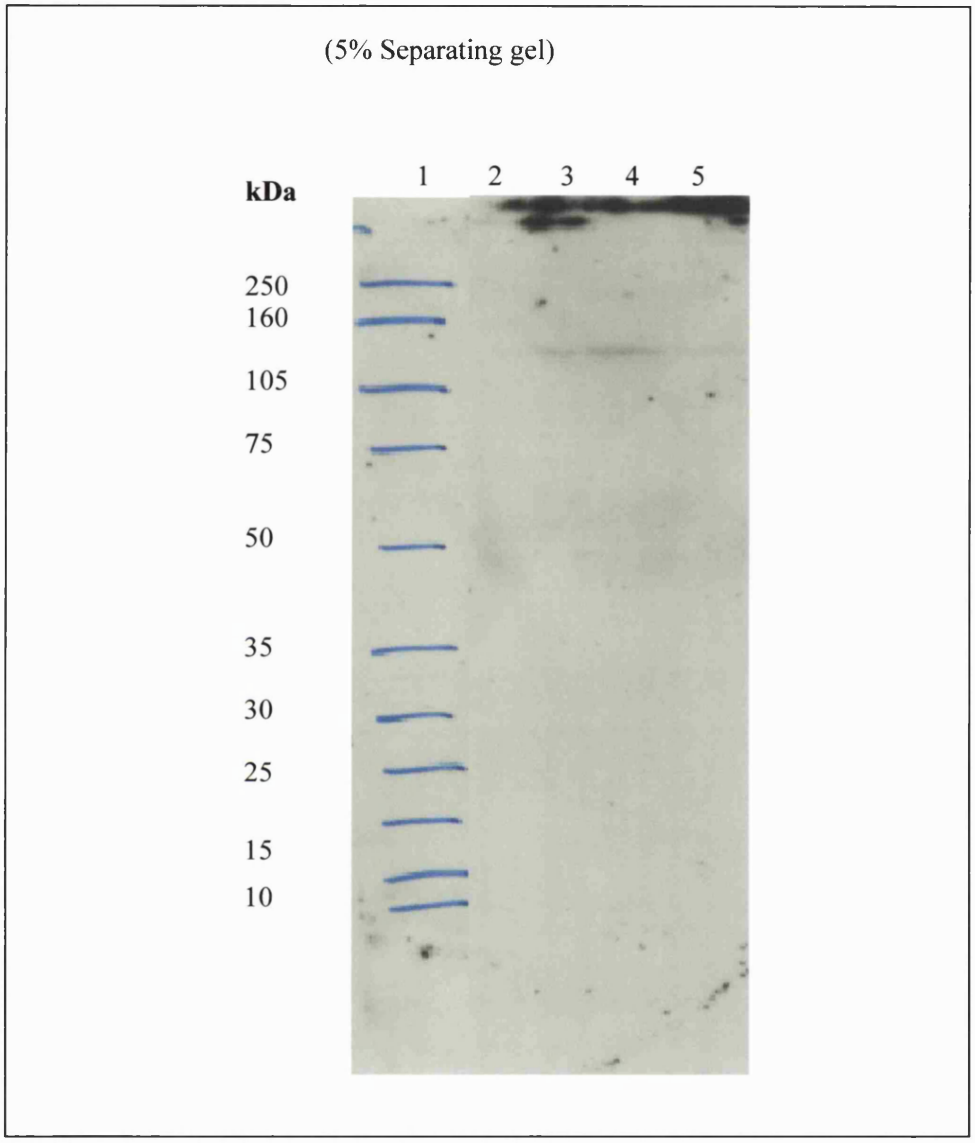


Figure Legend

Figure 3.16 shows the results from Western Blot analysis of Foetal Bovine Serum (10% in supplemented EMEM) with monoclonal anti human-GPI-PLD antibody, in comparison with molecular weight markers (the position of the markers was pencilled onto the nitrocellulose membrane). 300 μg of protein was loaded into each well

Lanes 1 shows un-supplemented EMEM, whereas lane 2, 3 and 4 show three different samples of 10% FBS, prepared in EMEM. The Western Blot procedure has not detected any GPI-PLD protein in EMEM without FBS. A weakly staining band (approximately 115 kDa) is visible in lanes 2, 3 and 4. In addition, strongly GPI-PLD positive bands were observed at the interface of the stacking and separation gels.

FIGURE 3.17

WESTERN BLOT ANALYSIS OF FOETAL BOVINE SERUM USING POLYCLONAL ANTI GPI-PLD ANTIBODY

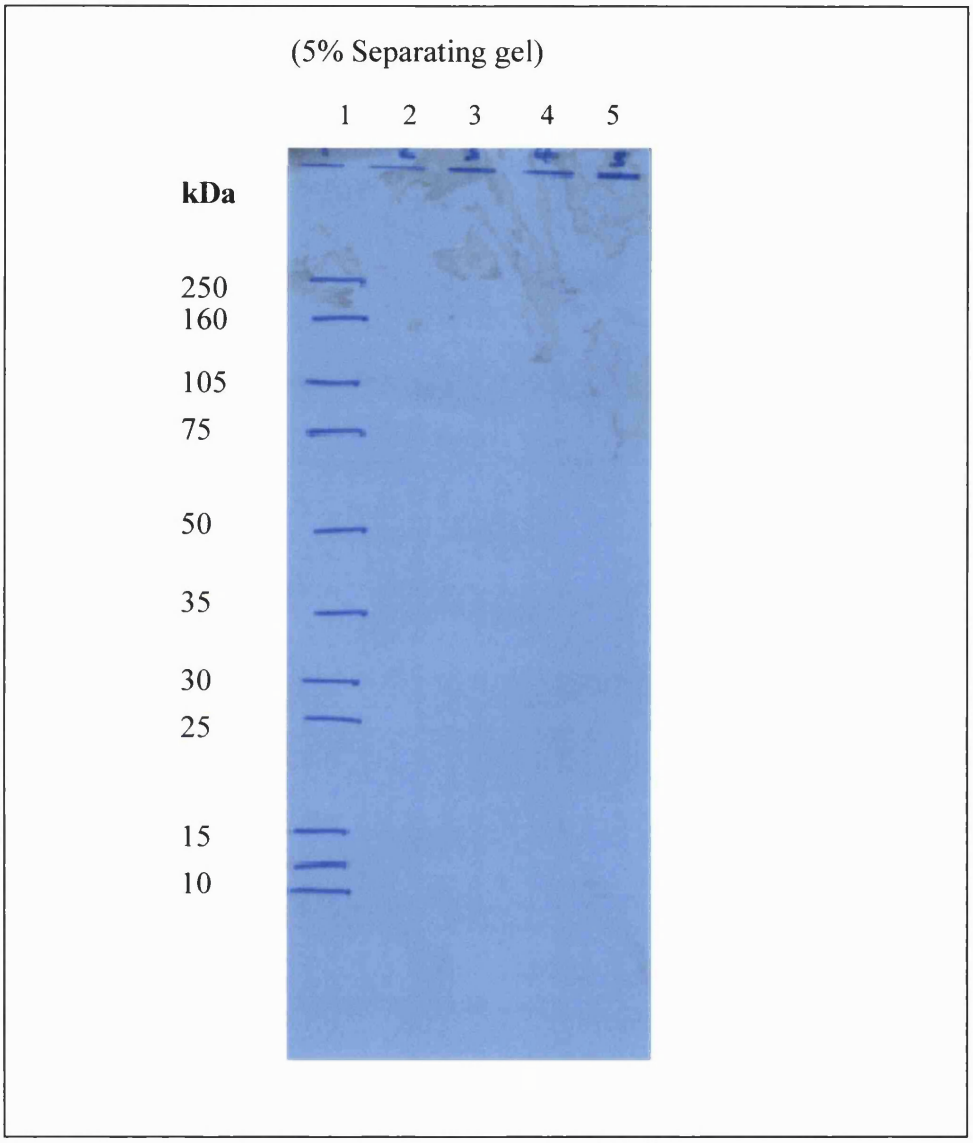


Figure Legend

Figure 3.17 shows the results from Western Blot analysis of Foetal Bovine Serum (10% in supplemented EMEM) with polyclonal anti human-GPI-PLD antibody, in comparison with molecular weight markers (the position of the markers was pencilled onto the nitrocellulose membrane).

Lanes 1 shows un-supplemented EMEM, whereas lane 2, 3 and 4 show three different samples of 10% FBS, prepared in EMEM. The Western Blot procedure has not detected any GPI-PLD protein in either the EMEM without FBS, or any of the 10% FBS supplemented EMEM samples.

3.30.2 Existence of GPI-PLD in serum

The first evidence of the existence of a novel GPI-PLD enzyme was suggested in 1987, from experiments using HeLa cells (Davitz *et al.*,1987). HeLa cells were chosen because they were shown to release large quantities of the GPI-anchored decay accelerating factor (DAF) into the culture medium, and it was considered of interest to determine whether a phospholipase enzyme was responsible for the release of DAF. HeLa cells were incubated with radiolabelled mfVSG, after which time hydrolysis of the GPI-anchor of mfVSG was effected. Chromatographic analysis of the products of hydrolysis indicated that the product was a phosphatidic acid, which confirmed that a phospholipase D was responsible. An un-anticipated result from this experiment was that the GPI-anchor hydrolysis occurred in the presence or absence of the HeLa cells, and analysis of the culture conditions revealed that the foetal bovine serum, included in the cell's culture medium, also contained a component with the capacity to degrade the mfVSG.

Further studies confirmed the existence of GPI-PLD in serum samples isolated from all mammals studied to date, including human, rat, ox and rabbit (Low & Prasad, 1988). In all instances, enzyme activity was demonstrated by reactivity against non-membrane associated GPI-anchored substrates, and confirmation that the product of hydrolysis was phosphatidic acid.

Purification of the enzyme was first accomplished by cellulose, sepharose and wheat-germ lectin chromatography (Cardoso de Almeida *et al.*, 1988). The observation that the enzyme bound to the wheat germ lectin-sepharose indicated that it was a glycoprotein. The separation method was extended, incorporating 'fast protein, peptide and polynucleotide liquid chromatography' (FPLC) and phenyl- and wheat-germ column chromatography procedures (Davitz *et al.*, 1989). With time, the procedure has become a complex methodology, and includes an initial precipitation with a polyethylene glycol solution, and filtration (Huang *et al.*, 1990). The enzyme that was detected after this extensive separation protocol had a molecular weight of between 100 and 110 kDa, and displayed enzyme activity against a radiolabelled mfVSG substrate.

Purification methods enabled the creation of a panel of GPI-PLD specific monoclonal antibodies, which were employed in immuno-affinity chromatography. From this, it was possible to quantify the concentration of GPI-PLD in normal serum. Current information suggests that there is some variation in the concentrations of enzyme from different serum samples. For example, estimates vary from 5 to 10 μgs/ml in mammalian serum (Davitz *et al.*,

1989), and between 7 to 40 μgs/ml in bovine serum (Huang *et al.*, 1990).

In vivo, GPI-PLD is an amphiphillic protein, which associates with hydrophobic serum components. Typically, GPI-PLD is found in close association with the high-density apolipoprotein A1, the significance of which will be discussed later in Chapter 4 (Hoener et al., 1993).

3.30.3 Production of GPI-PLD

Following the initial observation that a GPI-PLD existed in mammalian serum, it was of interest to determine where the enzyme was produced. The high levels of GPI-PLD in serum, however, had the potential of hampering studies through the contamination of tissues with serum (Low & Prasad, 1988).

3.30.3.1 Localisation of GPI-PLD mRNA in Liver

In order to define the specific tissues in which the GPI-PLD protein was produced, it was essential to identify the mRNA from which the enzyme was transcribed. The identification of the cDNA from which the GPI-PLD protein is derived was first accomplished using bovine DNA. This cDNA sequence was recently termed *Gpld*. Using the amino acid sequence from the purified bovine serum GPI-PLD, four degenerate oligonucleotide probes were made. Bovine DNA libraries were screened, using these oligonucleotide probes, and GPI-PLD positive DNA clones were identified (Scallon *et al*, 1991).

Using the cloned DNA sequence from bovine serum GPI-PLD (Scallon *et al.*, 1991), a number of human cDNA libraries were screened, and a GPI-PLD cDNA sequence was identified in human liver. Upon characterisation, the 2.9 kilobases of liver cDNA were shown to encode a protein of 817 amino acids, with 82% homology to the bovine sequence (Tsang *et al.*, 1992). A related sequence was also identified in a human pancreas cDNA library, the significance of which will be discussed in Section 3.30.3.2.

Support for the theory that liver tissues represent a source of the GPI-PLD mRNA came from studies of patients with cirrhosis (Raymond *et al.*, 1994). Primary biliary cirrhosis is a chronic liver disease, which involves the obstruction of the bile ducts inside the liver tissues, ultimately leading to the replacement of the ducts by connective tissue. (www.uperaje.com). Measurement of serum GPI-PLD levels in patients with cirrhosis indicated that levels of enzyme were significantly lower than in healthy individuals. In addition, serum GPI-PLD levels showed significant reductions following the removal of the liver (Maguire *et al.*, 1995). These results suggested that the reduced liver function that was associated with cirrhosis or liver

removal led to a reduced production of the GPI-PLD enzyme, and that other tissues did not have the capacity to compensate for this loss. That the enzyme activity was not totally ablated with liver removal, however, indicated that secondary sources might exist (Rhode *et al.*, 1999).

It is important to also mention that the reduced levels of circulating GPI-PLD in liver disease might be related to the serum levels of ApoAl, with which the GPI-PLD is associated. ApoA1 is a lipoprotein that is synthesised in the liver. Reduced production of ApoA1 might lead to reduced circulation of GPI-PLD, without necessarily any alteration in the levels of the enzyme itself. However, studies with mice suggested that this was not the case. In certain mouse strains, a diet rich in fat and cholesterol was shown to decrease the levels of circulating high density lipoproteins (HDLs), including apoAI and ApoAII (leBoeuf et al, 1998). Analysis of the GPI-PLD levels in these mice, after the altered diet, did not show a concomitant decrease in the levels of serum GPI-PLD. It is possible that the reduced serum ApoA1 levels in the mice were still sufficient to form complexes with the GPI-PLD proteins. Alternatively, upon reduction of the HDL levels, it is possible that GPI-PLD formed association with alternative lipids. The role of ApoA1 in the regulation of serum GPI-PLD levels remains to be established.

3.30.3.2 Localisation of GPI-PLD mRNA in Pancreas

Another research group identified the production of GPI-PLD in pancreatic islets (Metz *et al.*, 1991). Once again, the existence of the GPI-PLD enzyme activity was demonstrated by the hydrolysis of radiolabelled mfVSG, and identification of the products of catalysis by thin layer chromatography (TLC). GPI-PLD activity was found in both pancreatic micrososomes, and in two mouse pancreatic islet cell lines, αTC6 (glucagonoma) and (βTC3) insulinoma lines.

However, without the demonstration of the GPI-PLD mRNA in pancreas, these results were insufficient to claim that the protein was produced in this tissue. To confirm the existence of GPI-PLD mRNA in pancreatic cells, a normal human pancreatic islet cDNA library was examined, with reference to the cloned DNA sequence from bovine serum GPI-PLD (Scallon *et al.*, 1991). Using the bovine sequence, GPI-PLD-specific oligonucleotide primers were generated, and the existence of the mRNA in islet cDNA was confirmed by PCR (Metz *et al.*, 1991).

3.30.3.3 Do two different *Gpld* genes exist?

There is currently some speculation as to whether the pancreas and liver-associated forms of GPI-PLD represent two distinct sub-types of the GPI-PLD enzyme (Tsang *et al.*, 1992). Support for the existence of two isotypes came from the extraction of bovine, rat and human liver membrane GPI-PLD enzymes (Genbank accession numbers L11701 and L11702).

Chromatographic analysis of the enzyme fractions on Fractogel TSK-DEAE columns revealed two peaks of anchor-converting activity, termed P1 and P2, which eluted at 50 – 80 mM and 120 – 170 mM sodium chloride concentration, respectively (Heller *et al.*, 1992). It appears that P1 represents a membrane-bound enzyme fraction, whereas P2 was only loosely associated with the membrane. Comparison of the P1 and P2 forms against serum GPI-PLD indicated that the serum form eluted at 120 – 170 mM sodium chloride, suggesting that P2 was serum-derived.

Challenge to the theory that two forms of the enzyme exist comes from analysis of the genetic sequences of the two postulated GPI-PLD 'isotypes'. Analysis revealed only 42 single-base changes between the two forms. This could be explained by genetic polymorphisms in the *Gpld* gene, or by minor discrepancies in the sequencing technique (LeBoeuf *et al.*, 1988).

3.30.4 Secretion of the GPI-PLD protein

The study of GPI-PLD to date shows that, although the enzyme is found in high concentrations in serum, only a limited number of tissues are capable of producing the mRNA. It must be assumed, therefore, that the enzyme is secreted into the circulation, from where it would be available to all of the body's tissues.

Evidence that GPI-PLD might be secreted from cells was gained from *in vitro* experiments, using cell lines. For example, a plasmid containing the bovine GPI-PLD sequence (pBJ1682) was introduced into COS-1 cells. Expression of the transfected enzyme was confirmed by immunofluoresence of permeabilised cells, and by Western blot analysis, and enzyme activity was monitored by the degradation of radiolabelled mfVSG. Analysis of the cells lysates indicated that 48 hours after transfection, only a small fraction of the GPI-PLD was associated with the cells, and it was concluded that the majority of the enzymatic activity was secreted into the extracellular milieu (Scallon *et al.*, 1991).

Additional information, concerning the secretion of GPI-PLD, came from the study of the mouse pancreatic islet cell line, βTC3 (Deeg & Verchere, 1997), which were shown to be phenotypically similar to normal β cells (d'Ambra *et al.*, 1990). The use of a cell line permitted the study of specific pancreatic cells without the influence of contaminating cells. The cells were cultured in a GPI-PLD-free serum substitute, to remove contaminating serum-derived GPI-PLD.

GPI-PLD activity was demonstrated by the hydrolysis of a radiolabelled mfVSG substrate, and confirmation that the product of hydrolysis was phosphatidic acid. Secretion from βTC3 cells

was also shown to be stimulated by the addition of a number of insulin secretagogues, including glucose, phorbol myristic acid and isobutylmethylxanthine, and GPI-PLD release occurred in parallel with the release of insulin.

Two conclusions were made, regarding the release of GPI-PLD from the βTC3 cell line. Firstly, the overlap between the secretion of insulin and GPI-PLD suggested that there are common elements in the signal transduction pathways that mediate the release of the two proteins, although the nature of these pathways was not established. Secondly, the release of GPI-PLD is almost certainly dependent on both a constitutive and a regulated pathway. Evidence for a regulated pathway was derived from study of the cellular and extracellular GPI-PLD activity. Stimulation of βTC3 cells promoted the decrease of the cellular GPI-PLD activity, with a concomitant increase in the activity detected in the extracellular medium. The possibility that GPI-PLD exists in the insulin secretory granule was provided by immuno-histochemistry, which showed a punctate staining pattern for both insulin and GPI-PLD. Evidence for a constitutive pathway came from inhibition of GPI-PLD secretion by the addition of the protein synthesis inhibitor, cyclohexamide. This inhibition suggested that at least a certain proportion of GPI-PLD is synthesised *de novo*. Both the regulated and the constitutive theories, however, require further experimentation (Deeg & Verchere, 1997).

3.30.5 Production of GPI-PLD by mast cells

As discussed, a number of studies have been performed to determine the production of GPI-PLD by mammalian tissues. One such study was concerned with human tissues, in which the researchers concluded that mast cells had the capacity to produce the GPI-PLD enzyme (Metz *et al.*, 1992). The studies employed an anti-GPI-PLD monoclonal antibody, 612C, in immuno-staining procedures using human tissues. Results indicated that 612C stained sparsely distributed cells in the lung interstitium, gastric sub-mucosa and dermis. These cells corresponded to cells that also stained with toluidine blue, a dye routinely used in the identification of mast cells granules. These studies, however, were designed to detect the protein, rather than the existence of GPI-PLD-specific mRNA.

Other studies, using RNA probes generated against bovine GPI-PLD, and screening of bovine tissues have provided evidence of production of the enzyme in sub-populations of mast cells found in the adrenal gland, lung and liver (Stadelman *et al.*, 1993). Using an alcian blue 8GX, the GPI-PLD positive cells were identified as mast cells. To date, however, this is the only evidence that mast cells may produce the GPI-PLD enzyme, and these results are contradicted by the data collected during the course of this thesis.

The observation that normal tissue mast cells may produce the GPI-PLD protein *in situ* lead us to believe that the GPI-PLD RNA would, likewise, be detected in the RBL-2H3 cell line. This was not the case. The experimental results collected during this thesis revealed that the RBL-2H3 cell line does not produce the GPI-PLD RNA, although it does contain an enzymatically active GPI-PLD protein. It was, therefore, assumed that the protein was derived from the serum. This is an important observation. The aim of the research performed in this thesis was to determine the role of the GPI-PLD enzyme in the Type One Hypersensitivity cascade. It is, therefore, important to note that a potentially key enzyme in the allergic cascade is derived from the serum, rather than being created in an intracellular location.

3.30.6 Uptake of serum GPI-PLD by RBL-2H3 cells

The uptake of GPI-PLD from the extracellular culture medium into cells has been observed in one other cell line; the mouse neuroblastoma cell line, N2A, in which the uptake of bovine-serum GPI-PLD was studied. The results demonstrated that both intact GPI-PLD, and trypsin-treated fragments of the enzyme, were taken up in a concentration- and time-dependent way. Cell-associated GPI-PLD activity also increased in a linear fashion (Hari *et al.*, 1997).

In addition, experimental studies have been performed, to determine the production of GPI-PLD in the placenta (personal communication with Ms S Deborde, Department of Molecular Pathology, UCL). As was observed with the RBL-2H3 cell line, the RNA for the GPI-PLD was not detected in any of the placental components, using the RTR-PCR methodology. However, an active GPI-PLD protein was detected within the foetal syncitiotrophoblast, and it was assumed that the enzyme was taken up from the reserves of maternal serum GPI-PLD.

The observation that the RNA for GPI-PLD is detected in very few mammalian cell types, and that both RBL-2H3 cells and the placental tissues may derive their active GPI-PLD protein from the serum leaves a vital question unanswered. Why is the GPI-PLD protein not created intracellularly? Experimental evidence to answer this question was provided from studies in which the GPI-PLD protein was over-expressed in mammalian cells.

The GPI-PLD enzyme was over-expressed in macrophages, and the resultant effects on cellular signalling were determined. Results showed that the over-expression of GPI-PLD promoted the GPI-PLD-mediated cleavage of the GPI anchors in the early secretory pathway. The phosphatidic acid produced by GPI-PLD was rapidly converted into diacylglycerol (DAG), through the action of an intracellular phosphatase (Singer *et al.*, 1997). The raised DAG levels resulted in the translocation of intracellular PKCα to the surface membrane, ultimately promoting the phosphorylation, and activation, of PKCα (Tsujioka et al 1999). Furthermore,

PKCα was also translocated to the membrane of the endoplasmic reticulum, as determined by sucrose-density-gradient centrifugation and immunofluorescence microscopy.

Taken together, these results place GPI-PLD in the signalling cascade initiated through the generation of diacylglycerol. Although the DAG is a by-product of GPI-PLD mediated catalysis, being generated from the action of a phosphatase on phosphatidic acid, nevertheless DAG plays a pivotal role in the activation of PKCα. Through the activation of protein kinase C a number of intracellular signalling pathways are likely to be affected.

Firstly, the role of PKC in the IgE-mediated degranulation of mast cells was discussed in Section 1.3.33.6. The potential exists that the activation of mast cells might be as a result of the GPI-PLD-mediated generation of DAG, and the resultant activation of PKC. Through the activation of the PKC enzyme, mast cells could undergo degranulation, without the initial IgE-FcεR1 cross linking event. Clearly, the non-specific activation of mast cells, with the release of allergic mediators, would be an unwanted event.

Secondly, it was recently suggested that PKC regulates the translocation of newly synthesized proteins, including docking protein α, across the endoplasmic reticulum membrane (Gruss *et al.*, 1999). Indeed, the phosphorylation of microsomal proteins by PKC enzymes improved their translocation efficiency *in vitro*. Overall, it appears likely that the PKC enzymes are involved in the early stages of cellular secretion.

This evidence suggests that, if the GPI-PLD were present in an intracellular location in the mast cells, it may promote the non-specific activation of the cells. The evidence provided from these experimental studies, therefore, support the results generated in this chapter. That is, it is preferential for the mast cells to take up the GPI-PLD protein from the serum, rather than creating the protein intracellularly.

3.30.7 Intracellular Location of GPI-PLD

The intracellular location of GPI-PLD was studied through the use of labelled anti-GPI-PLD antibodies, which were employed in immuno-histochemical analysis of a variety of cell lines. A punctate staining pattern was observed in a variety of myeloid cell lines, including human myeloblasts and mouse macrophages (Xie & Low, 1994), and in a pancreatic βTC3 cell line (Deeg & Verchere., 1997). This punctate pattern was taken to mean that the enzyme is concentrated in limited intracellular locations, such as secretory vesicles (Xie & Low, 1994).

Secondly, centrifugation techniques were employed to separate washed rat liver tissue into different cellular fractions, according to the density of the fraction. Using this technique, a nuclear fraction, heavy mitochondrial fraction, light mitochondrial or lysosomal fraction, microsomal fraction and supernatant were purified. Analysis of fraction-specific marker enzymes determined the purity of the fraction, and the GPI-PLD activity in each fraction was then quantified.

The results indicated that the majority of enzyme activity was detected in the light mitochondrial or lysosomal fraction, suggesting a lysosomal or peroxisomal localisation for GPI-PLD. However, the subcellular distribution pattern of GPI-PLD showed more similarities with the lysosomal marker enzymes, including β-N-acetyl-D-glucosaminidase, rather than the peroxisomal marker enzyme, catalase (Hari *et al.*, 1996). The observation that β-N-acetyl-D-glucosaminidase may be co-localised with GPI-PLD, combined with the observation that β-N-acetyl-D-glucosaminidase is released concomitantly with histamine, as discussed in Section 2.8.3, raises the possibility GPI-PLD may be located in the same granules as histamine. The relevance of this observation in the study of mast cell degranulation certainly warrants further investigation.

The third line of evidence for the intracellular location of the GPI-PLD enzyme was provided from the mouse neuroblastoma cell line, N2A, in which the uptake of purified bovine-serum GPI-PLD was previously demonstrated (Section 3.30.6). Chloroquine is a weak base, which is rapidly taken up by cells. It accumulates primarily in the lysosomes (de Duve *et al.*, 1966) and serves to neutralise these compartments. As a consequence of the reduced acidity of lysosomes, the activity of the lysosomal proteolytic enzyme is inhibited (Wibo & Poole, 1974), resulting in reduced levels of protein degradation. Experimentally, the inclusion of chloroquine in the uptake of radiolabelled GPI-PLD into N2A cells resulted in a reduced degradation of the GPI-PLD, as detected by SDS-PAGE. The conclusion was made, therefore, that GPI-PLD was located in the lyosomal compartment of the cell, in which it would be in contact with chloroquine (Hari *et al.*, 1997).

3.30.8 Evidence of intracellular processing of GPI-PLD in RBL-2H3 cells

The results gained with the Western Blotting technique on RBL-2H3 membrane preparations provided evidence for the existence of the enzyme in these cells. However, in RBL-2H3 cells, the polyclonal anti-GPI-PLD antibody recognized 5 different bands; a weakly staining band at around 105 kDa, and four strongly staining bands at approximately 58, 38, 36 and 34 kDa. These bands may be compared with the native enzyme, which has a molecular weight of 110 kDa (Huang *et al.*, 1990).

It was assumed that either the protein was cleaved during the preparation of the RBL-2H3 membrane, or that it was intracellularly processed. The inclusion of protease inhibitors in the buffer in which the cell membrane was prepared minimized the chance that the reduced band sizes was as a result of proteolytic cleavage during the preparation of the membrane fraction.

In vitro, GPI-PLD can be cleaved with trypsin, typically generating three peptides of molecular weights of approximately 42, 38 and 33 kDa. Intermediate fragments, of 82 and 53 kDa have also been detected by SDS-PAGE and Western blotting (Heller *et al.*, 1994). The pattern of fragments is illustrated in Figure 3.18. Under non-denaturing conditions, the fragments remain tightly, but non-covalently, associated with each other.

The presence of proteolytically cleaved forms of GPI-PLD was also demonstrated in the N2A cell line, into which radiolabelled 110 kDa GPI-PLD protein was incorporated. After as little as one day of culture, almost no 110 kDa protein was detected by SDS-PAGE, but a number of smaller fragments, ranging from 39 to 101 kDa, were observed. Clearly, cleavage of the native GPI-PLD is not restricted to *in vitro* situations (Hari *et al.*, 1997), and there is no reason to believe that GPI-PLD would not undergo processing in the RBL-2H3 cell line.

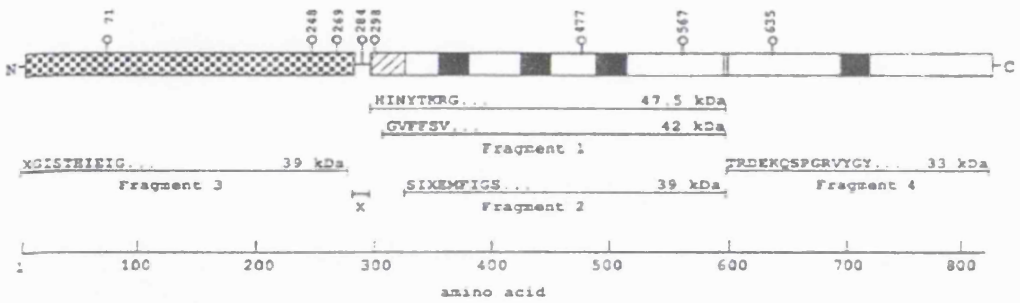
The molecular weights reported in these experimental studies compare well with the results gained by Western Blot analysis of RBL-2H3 membrane preparations. As stated, polyclonal anti-GPI-PLD antibody recognised 5 different bands; a weakly staining band at around 105 kDa, and four strongly staining bands at approximately 58, 38, 36 and 34 kDa from the RBL-2H3 membrane preparation. Trypsin cleavage was typically found to generate three peptides of molecular weights of approximately 42, 38 and 33 kDa, which compares roughly with the three smaller bands in RBL-2H3 membrane preparations (38, 36 and 34 kDa), and the approximately 58 kDa band could represent the intermediate 53 kDa band reported.

Analysis of the hydrolysing capacity of these different fragments revealed that the N-terminal fragment, with a molecular weight of 38.6 kDa, had the capacity to hydrolyse GPI-anchored substrates (Heller *et al.*, 1994). Therefore, the proteolytically cleaved GPI-PLD enzyme fragments in RBL-2H3 cells should still possess the ability to hydrolyse the mfVSG substrate.

Similar cleavage products were observed with the N2A cell line, in which a number of smaller fragments, ranging from 39 to 101 kDa, were GPI-PLD positive by Western blot analysis. It could also be argued that cell-specific variation in the processing of the 110 kDa

FIGURE 3.18

MODEL FOR THE TRYPTIC DIGESTION OF GPI-PLD



	Amino acids (from..to)	Calculated molecular mass (kDa) (amino acid composition)	Apparent molecular mass (kDa) (SDS/PAGE, before deglycosylation)	Apparent molecular mass (kDa) (SDS/PAGE, after deglycosylation)
Fragment 1	His296..Arg589	31 658	43 600	35 400
	or Gly303..Arg589	30 883		
Fragment 2	Ser326..Arg589	28 185	38 100	30 500
Fragment 3	Cys1..Lys275	31 360	38 600	29 600
Fragment 4	Thr590..Asp816	24 303	29 200	25 600
Fragment X	Val276..Lys295	2 169	antigenic site	

(Heller et al, 1994)

Figure legend

The bars in Figure 3.18 correspond to the four major fragments isolated after the trypsin treatment of GPI-PLD. The amino acids, and the potential N-glycosylation sites, are numbered according to the amino acid sequence deduced from the sequence of a bovine liver cDNA (Scallon et all, 1991). Below each fragment, the first 8 to 14 amino acids obtained by N-terminal amino acid sequence analysis are shown together with the apparent molecular mass of the fragment determined with SDS-PAGE (5 to 15 % polyacrylamide). Finally, the table lists the putative length of each fragment, and compares the molecular mass calculated from the amino acid sequence, with that derived from SDS-PAGE before and after deglycosylation.

▪ denotes the fragment catalytically activee after denaturation / renaturation, ° denotes a potential N-glycosylation site, ▨ variable tryptic cleavage site, ▪ calcium binding motif

GPI-PLD protein might affect the size of the products detected, although this was not determined with the RBL-2H3 cell line.

Sequence analysis of the GPI-PLD protein, as deduced from its cDNA (Scallon *et al.*, 1991) indicates that the protein contains a total of 67 potential trypsin-cleavage sites, comprising 33 arginine and 34 lysine residues. That only a small number of cleavage products are created by the incubation of GPI-PLD with trypsin *in vitro* suggests that the enzyme is relatively insensitive to cleavage by proteases (Heller *et al.*, 1994). It would appear that the enzyme is protected from proteolytic cleavage, possibly by glycosylation, and by the secondary and tertiary structures. The identity of the protease(s) responsible for the intracellular cleavage is under investigation (Li *et al.*, 1994).

3.30.9 Western Blotting of FBS samples

The results gained from the Western blot analysis of Foetal Bovine serum showed that a GPI-PLD positive band, of approximately 120 kDa molecular weight, was detected after the ECL methodology. These results supported the evidence reported in the literature, and the results from the GPI-PLD activity assay, that mammalian serum contains GPI-PLD. However, considering the high concentration of GPI-PLD in serum, up to 10 μg per ml, the fact that the GPI-PLD positive bands were only faintly positive was disappointing.

The strongly stained bands that were observed at the interface of the stacking and separation gels suggested that a significant proportion of the FBS protein had accumulated this interface. The reason for this accumulation was not determined, although it is possible that the quantity of protein that was loaded onto the gel (approximately three hundred micrograms of protein) may have been in excess of what could be effectively separated on the SDS-PAGE gel. This quantity was loaded to improve the chances of detecting the GPI-PLD protein, as no GPI-PLD protein was detected when a smaller quantity of protein was loaded onto the acrylamide gel. As a consequence of the accumulation of proteins at the interface, the actual volume of proteins that separated on the gel was less than the three hundred micrograms originally loaded onto the gel. The proportion of GPI-PLD within the separated FBS proteins would be fairly limited, which may explain the faint positive staining of the GPI-PLD protein.

Nevertheless, the existence of the faint GPI-PLD positive band was evidence that the FBS contained the GPI-PLD protein. Without the addition of FBS, the MEM culture media contained neither GPI-PLD Activity in the radioactivity assay, nor the protein in Western Blot analysis. This FBS was routinely used for the *in vitro* culture of RBL-2H3 cells, and it can, therefore, be assumed that the source of GPI-PLD for RBL-2H3 cells was the FBS.

3.30.10 Western Blotting of FBS samples versus RBL-2H3 membrane samples

The results in Figures 3.7, 3.8, 3.16 and 3.17 indicated that, despite the fact that the GPI-PLD in RBL-2H3 cells was derived from the FBS, the monoclonal and polyclonal antibodies employed in the Western blot procedure did not detect GPI-PLD in both RBL-2H3 and FBS samples. The polyclonal anti-GPI-PLD antibody only detected the GPI-PLD in the RBL-2H3 membrane preparation, whereas the monoclonal anti-GPI-PLD antibody only detected the FBS-derived GPI-PLD.

As discussed previously (Section 3.19.4), the mRNA sequence of the human and bovine sequences showed significant (82% homology), and it is, therefore, unlikely that an anti-human GPI-PLD antibody would not recognize a bovine GPI-PLD protein, and *vice versa.* However, in the case of Western blotting, the antibody probes are designed to hybridize with specific antigenic epitopes, displayed by the target protein. The hybridization of a specific antibody to a specific target sequence is dependent upon a number of different variables, including the conformation of the target protein. Consequently, it is not possible to predict the efficiency with which a given antiserum will detect different epitopes of an immobilized, denatured target protein. Monoclonal antibodies may be unsuitable for use as probes in western blots, where the target proteins are thoroughly denatured. Polyclonal antisera, on the other hand, are undefined mixtures of individual immunoglobulins, whose specificity, affinity, and concentration are often unknown (Maniatis *et al.*, 1982).

As discussed (Section 3.30.7), it appears that the GPI-PLD protein is processed in RBL-2H3 cells, so that the resulting proteins detected by Western blotting were approximately 105, 58, 38, 36 and 34 kDa, in comparison with the full length 110 kDa protein detected in FBS. It would appear that the processing of the GPI-PLD in RBL-2H3 cells has altered the ability of the monoclonal antibody to recognize the protein, while concomitantly enhancing the ability of the polyclonal antibody to detect the protein fragments.

3.30.11 Inhibition of GPI-PLD enzyme activity by EGTA and 1,10-phenanthroline

Experiments were performed to determine whether the activity was susceptible to inhibition. Previous studies have indicated that GPI-PLD activity could be distinguished from other divalent cation-dependent PI-PLC or GPI-PLC enzymes by the fact that the activity could be inhibited by incubation with 1,10-phenanthroline, a transition metal chelator (Low & Prasad, 1988). Non-chelating analogues of 1,10-phenanthroline, such as 1,7-phenanthroline, did not have the capacity to inhibit the action of GPI-PLD (Stieger *et al.*, 1991). Our results supported this data, with an inhibition of activity in both RBL-2H3 membrane preparations and FBS, by

EGTA and 1,10-phenanthroline.

Inhibition by EGTA and 1,10-phenanthroline is believed to occur through these agents' ability to chelate transition metals. Analysis of GPI-PLD characteristics suggested that the activity of GPI-PLD is dependent on divalent cations, including calcium and zinc. EGTA was demonstrated to have the capacity to inhibit GPI-PLD, an inhibition that was ablated by increasing the calcium concentration (Low *et al.*, 1988).

A computer search of the GPI-PLD sequence in the Genbank database revealed homology with integrins. GPI-PLD is the only protein so far identified which contains the sevenfold repeat structure, characteristically found in the N-terminal extracellular domain of integrin α subunits (Li & Low, 1999). This region contains a motif called the integrin E-F-Hand site (Szebenyi, 1991), which was found at 4 different locations in the GPI-PLD protein (residues 357 – 379 (I), 426 – 448 (II), 489 – 511 (III) and 694 – 716 (IV)). This sequence consists of Asp-X-(Asp or Asn)-X-Asp-Gly-X-X-Asp, and represents a divalent cation binding motif, detected in several calcium and magnesium binding proteins, including calmodulin, troponin C and parvalbumin. Although the integrin EH hand units differ from conventional EF-hand motifs, in that they are missing a co-ordinating residue at position 12, experimental and modelling studies indicate that the region containing the sevenfold repeat of the α subunits, does have the capacity to bind calcium ions (Tuckwell *et al.*, 1992). To date, no sequence has been identified by which zinc may interact with GPI-PLD.

GPI-PLD was shown to bind four or five calcium ions per mole, with high selectivity as determined by equilibrium dialysis (Li *et al.*, 1994). The experimental evidence that GPI-PLD does, indeed, bind calcium ions, combined with the evidence of EF hand motifs led to the conclusion that the EF hand are responsible for the observed calcium binding and have an important structural or functional role. Determinating the role of calcium in the function of the enzyme, however, was difficult. The removal of calcium ions, using chelating agents, would serve to remove other divalent cations, such as zinc, which are known to be essential for optimal catalytic activity. Recently, however, significant advances have been made, in determining the role of calcium in enzyme function. To date, it is believed that calcium serves to maintain the structural conformation of the protein. The significance of this, along with the experimental details from which this conclusion was drawn are discussed in Chapter 4 (Li *et al.*, 1999).

3.31 Conclusions

In conclusion, the results showed that the RBL-2H3 cells contain the GPI-PLD protein, and it's enzymatic activity, despite finding no evidence for the production of the enzymes' mRNA.

From these results, however, it is impossible to conclude that the RBL-2H3 cells are incapable of expressing the mRNA. Whether the GPI-PLD mRNA is expressed under different culture conditions, or under extreme stress, for example, was not determined.

GPI-PLD protein is known to be in high concentrations in mammalian serum, and enzyme activity was detected in the Foetal Bovine serum, in which the RBL-2H3 cells were cultured. It was assumed, therefore, that under the experimental conditions employed, the RBL-2H3 cells derive their GPI-PLD activity from their culture medium. The uptake of serum GPI-PLD into specific cells has previously been studied *in vitro*, and was shown to occur in a concentration- and time-dependent way. Upon uptake, the 110 kDa protein typically undergoes intracellular processing.

To date, confirmation of the production of GPI-PLD mRNA has been limited to liver and pancreatic tissues, although whether the two tissues contain different isotypes of the *Gpld* gene remains controversial. Evidence exists that normal tissue mast cells may naturally produce the GPI-PLD mRNA (Stadelman *et al.*, 1993).

As discussed previously, the purpose of the research performed for the duration of this thesis was to ascertain the role of GPI-PLD in the function of RBL-2H3 cells. The demonstration that RBL-2H3 cells derive their GPI-PLD from the serum reserves presented the opportunity to modify the serum GPI-PLD, thereby modifying the exposure of the cells. The following chapter describes the techniques employed to manipulate the profile of GPI-PLD in the FBS. Following manipulation of the FBS, it was important to determine whether the behavior of the cells was altered, particularly in relation to their responses to IgE-mediated stimulation.

Chapter 4

Role of GPI-PLD in IgE-mediated Degranulation of RBL-2H3 cells

4.1 Summary

Following the discovery that RBL-2H3 do not synthesise mRNA for GPI-PLD, but do contain the protein and the enzyme activity, and that GPI-PLD protein is abundant in Foetal Bovine Serum, it was proposed that RBL-2H3 cells take up the GPI-PLD from the extensive reserves in the FBS.

The following research was aimed at the analysis of the contribution of GPI-PLD to the RBL-2H3 cell degranulation responses. Of particular importance was the determination of the role of GPI-PLD in IgE-mediated degranulation.

After consideration of a number of alternatives to FBS, and methods to remove GPI-PLD from FBS, an alkaline inactivation method was selected, which lead to the almost complete inactivation of GPI-PLD activity in FBS. The addition of the alkaline inactivated FBS to the supplemented EMEM culture medium, in place of normal FBS, and culture of RBL-2H3 cells in this EMEM was followed by characterisation of any alterations in the phenotype of the RBL-2H3 cells.

It was discovered that RBL-2H3 cells cultured in GPI-PLD inactive FBS-containing media lost the ability to respond to surface IgE cross-linking. The degranulation of the RBL-2H3 cells, as measured by the quantity of β-glucosaminidase, was dramatically reduced, when compared with cells that were cultured in EMEM containing normal FBS.

Finally, those cells that had been cultured in alkaline inactivated FBS were re-suspended in either normal FBS, or a recombinant GPI-PLD protein. The addition of normal FBS re-established the ability of the cells to respond to IgE mediated stimulation. Results indicated that the recombinant protein was taken up by the RBL-2H3 cells. However, the recombinant enzyme appeared to be of a lower specific activity, compared with the native GPI-PLD enzyme, and the presence of the recombinant GPI-PLD appeared to reduce the ability of the RBL-2H3 cells to respond to IgE-mediated stimulation.

Further investigation is required before the association between the GPI-PLD enzyme and the IgE mediated degranulation of RBL-2H3 cells is conclusively demonstrated. However, the demonstration of the link between the GPI-PLD enzyme and this mast cell line has served to advance the scientific knowledge in this field.

4.2 Introduction

The following research was aimed at the analysis of the contribution of GPI-PLD to the RBL-2H3 cell degranulation responses. Of particular importance was the determination of the role of GPI-PLD in IgE-mediated degranulation.

As discussed in section 3.14, the pilot studies on which this thesis was based involved the degranulation of RBL-2H3 cells in response to cross-linking surface IgE receptors. Briefly, the addition of a polyclonal bovine anti-GPI-PLD antibody to the degranulation experiments led to an ablation of the degranulation responses, and it was concluded that the IgE-mediated response of RBL-2H3 cells involved the GPI-PLD enzyme.

As a consequence of the results gained in Chapter 3, the decision was made to focus on the Foetal Bovine Serum. As discussed, it was determined that the RBL-2H3 cells do not synthesise mRNA for GPI-PLD, but do contain the protein and the enzyme activity. The GPI-PLD protein and enzyme activity, however, was shown to be abundant in Foetal Bovine Serum. The conclusion from this research was that the RBL-2H3 cells are incapable of producing GPI-PLD protein *in situ* and, therefore, take up the enzyme from the extensive reserves in the FBS. Once this was determined, the potential existed for removing, or inactivating the serum GPI-PLD.

4.3 Introduction to the use of Serum Supplements in Culture Media

Prior to a discussion of the removal or inactivation of serum GPI-PLD, however, it is important to understand the importance of the FBS within the cell culture medium. Most culture media formulations contain a pH-balanced mixture of salts, amino acids, sugars, vitamins and other trace essential nutrients. Un-supplemented media, however, is incomplete, and would support only short-term survival of cultured cells. Long-term culture of cells can only be achieved through the addition of supplements to the media, in the form of serum, or other humoral fluids. Serum is a complex mixture, and the addition of serum introduces an ambiguous factor into cell culture. However, many commercially available sera are of a high uniform quality, thereby reducing this ambiguity (Shah, 1999).

FBS was selected in preference to other commercial serum sources, as it is the most widely used serum in animal cell culture, and has been shown to reliably support the growth of a variety of eukaryotic cells (Shah, 1999). It has been estimated that FBS contains over 1000 different components, including proteins, electrolytes, non-protein nitrogenous substances, lipids, carbohydrates, attachment factors, hormones, binding factors, enzymes, inhibitory substances and other miscellaneous components. In addition, FBS contains high concentrations

of growth stimulatory factors, and low concentrations of growth inhibitory factors, thereby promoting the growth of the cultured cells more efficiently than other available serum supplements.

4.4 Removal of GPI-PLD activity from the RBL-2H3 culture medium

Four methods were considered, as a means of limiting the exposure of the RBL-2H3 cells to the active GPI-PLD enzyme in the culture medium. These were;

- Use of serum-free substitutes
- Reduction of the serum concentration
- Filtration of the FBS to remove GPI-PLD
- Inactivation through incubation in alkaline conditions.

Each of these different approaches will be discussed in turn.

4.4.1 Replacement of FBS with commercially available Serum-free substitutes

4.4.1.1 Introduction to Serum-Free Supplements

In the 1950's it was demonstrated that, under certain circumstances, a synthetic medium could replace the serum that was employed in cell culture. Serum-free medium has been defined as 'a complete nutrient formulation capable of supporting cellular proliferation and/or biological production without serum'. Essentially, the components of a serum-free supplement are precisely defined according to the requirements of the cell being cultured. Variation is observed, for example, according to the normal proliferation of the cell, the conditions of incubation, and the preferred culture medium. All serum-free supplements must, however, contain fat and water-soluble hormones, growth and attachment factors, and transport proteins (www.bmb.psu.edu). Serum free formulations may also contain purified material, recombinant proteins, growth factors, and tissue or organ extracts (www.lifetech.com).

The advantage of a using serum-free supplement is primarily that the precise chemical composition of the culture medium can be adjusted, according to the experimental specifications. Study of a defined serum nutrient, for example, is possible through the addition of the pure nutrient to a serum-free supplement (www.lifetech.com). In this instance, therefore, RBL-2H3 cells could be cultured in a defined, GPI-PLD free culture medium, to determine the role of GPI-PLD in cell function.

4.4.1.2 Nutridoma Serum-free supplements

'Nutridoma' is a family of chemically defined supplements that can completely replace serum in cell culture media, and have been successfully employed in the culture of a variety of cell

lines, including lymphoblastoid, myeloma and hybridoma cell lines. To date four Nutridoma serum-free media have been developed (CS, SP, NS and HU), each with a defined composition, and developed for the culture of a specific cell line(s). For example, Nutridoma-SP was developed for the culture of murine Sp2/0 myeloma cell lines, whereas Nutridoma-NS was designed for use with NS-1 and P3X63-Ag8.653 myeloma cell lines (Roche Molecular Biochemicals Specifications sheet).

Examination of the characteristics of the Nutridoma serum-free supplements revealed that GPI-PLD activity was not detected (Deeg & Verchere, 1997). The potential existed, therefore, that FBS could be replaced with Nutridoma, to examine the behaviour of RBL-2H3 cells in a GPI-PLD-free environment. As the nature of cell culture in Nutridoma was studied in greater detail, however, a number of potential problems were identified.

4.4.1.3 Problems with the use of Nutridoma in cell culture

Examination of the technical information that was provided with the Nutridoma serum-free supplements revealed a number of issues that might be in conflict with the use of this supplement as an alternative to FBS. Firstly, cells grown in serum-free media often display altered morphology and behavior, including higher susceptibility to damage, and the cells may become more rounded and smooth. Of significance also, is the reduced attachment to culture flasks and plates. Indeed, cells grown in Nutridoma-supplemented medium are usually in suspension, although this is dependent on the type of plastic of the culture flask.

Although the manufacturers state that these changes do not reflect a change in the cells' viability, the reduced adherence and greater fragility would almost certainly affect the ability of the cells to undergo the IgE-mediated stimulation procedure, as discussed in Section 2.6. The IgE-mediated degranulation of RBL-2H3 cells is dependent on an adherent cell monolayer, and the methodology involves a number of washing procedures. The likelihood that the cells cultured in serum-free medium might not be able to withstand this procedure meant that the use of Nutridoma might not be suitable. In addition, the changes in the cell appearance could reflect an alteration in the phenotype of the cells, which may alter their ability to respond to IgE-mediated stimulation.

A second potential problem is a consequence of the reduced protein content of Nutridoma, which necessitates a reduction in the concentration of the other media supplements, including the antibiotics and L-glutamine, by as much as 50%. The purpose of the use of a serum-free supplement is to remove GPI-PLD activity from the RBL-2H3 culture media, but to retain consistency of the other parameters of the supplemented medium. The alteration in the

Nutridoma-supplemented media could have adverse affects on the cell culture, which may be entirely independent from the lack of GPI-PLD. It would, of course, be extremely difficult to determine whether changes in the phenotype of the RBL-2H3 cells were as a result of the lack of GPI-PLD in the serum, or as a result of the alteration of an alternative serum factor (Roche Molecular Biochemicals Specifications sheet).

Nutridoma had previously been used in studies performed by colleagues, and the results indicated that the cell behaviour was clearly altered by incubated in Nutridoma-supplemented medium, in comparison with normal FBS-supplemented medium. The studies were designed to determine the role of signalling in T cell lines. Those cells cultured in Nutridoma-supplemented medium responded to stimulation with exactly the opposite responses when compared with normal. For example, exposure of T cells to phorbol myristic acid typically upregulated the IL-4 production. After culture in Nutridoma, IL-4 production by T cells after exposure to PMA was down-regulated (Dr E Filley, Department of Bacteriology, UCL, unpublished observations).

Although this discussion of the potential problems of culture with Nutridoma serum-free supplements was not directly related to the RBL-2H3 cell line, it was considered inadvisable to perform experiments with RBL-2H3 cells in a serum-free supplement which appeared to induce unusual responses in cell lines. An alternative was, therefore, sought.

4.4.1.4 Aim-V serum-free supplements

Aim-V medium is another serum-free, lymphocyte medium that was considered for the FBS-free culture of RBL-2H3 cells. Aim-V was originally developed for use in adsorptive immunotherapy, a novel cancer treatment. However, Aim-V was found to successfully support the growth of many cell types, and has since been used in a variety of studies. These include lymphoid regulation and cytokine studies, cultivation of human macrophages (Helsinki *et al.*, 1988) cultivation of lymphokine-activated killer (LAK) cells (Lafrenier *et al.*, 1989), and lymphocyte receptor studies (Owen-Schaub *et al.*, 1989).

In the case of macrophage cultivation, for example, the Aim-V media was described as a mixture of HEPES-buffered Dulbecco's Modified Eagle Medium and Ham's Nutrient Mixture F12 that had been supplemented with purified human albumin, transferrin, insulin, and a mixture of purified factors. The medium did not contain additional mitogenic stimulants, growth factors or differentiation-inducing agents. The macrophages were cultivated in this media for greater than 2 weeks. The viability, and behaviour, of the cells was determined by trypan blue exclusion, phagocytotic capacity, light and scanning electron microscopy,

cytochemistry, and phenotypic analysis. The results showed that the macrophages were phenotypically normal after culture in this serum-free medium (Helinski *et al.*, 1988).

The technical information that was provided with the Aim-V supplement was less detailed than that provided with Nutridoma, and it was, therefore, unclear as to whether similar culture problems were observed with this supplement (www.lifetech.com). It seemed of interest to determine whether alterations in the behaviour of the RBL-2H3 cells would result from culture in a serum-free supplemented medium. Before this was attempted, however, it was of interest to determine whether this supplement contained any detectable GPI-PLD activity. To this end, the radioactive GPI-PLD activity assay was used to determine whether Aim-V had the capacity to hydrolyse the mfVSG substrate.

4.4.2 Reduction of serum concentration

Several studies into the role of GPI-PLD in other cell systems have employed serum starvation techniques. The serum was removed from the supplemented culture medium, and any change observed in the behaviour of the serum-starved cells was interpreted as being as a result of the lack of serum proteins.

With RBL-2H3 cells, it was firstly important to determine whether the cells would survive extended periods of time in a serum-free medium. This was simply achieved by removing the FBS from the culture medium, and evaluating the behaviour of the cells through time. Secondly, it was important to determine the GPI-PLD activity in a reduced concentration of FBS. Although it was not ideal to reduce the concentrations of serum below the suggested 10% concentration, it was of interest to determine the GPI-PLD activity detected with a significantly lower concentration of FBS.

4.4.3 Filtration of Foetal Bovine Serum

The use of immuno-absorbent columns, to remove GPI-PLD from solution, has been employed in previous studies (Xie *et al.*, 1994). These techniques were shown to remove up to 99% of the GPI-PLD enzyme.

Although the column might remove up to 99% of the GPI-PLD, the remaining 1% could be equivalent to 0.4 μg/ml (based on a serum concentration of 40 μg/ml in bovine serum (Huang *et al.*, 1990)). Although the concentration of GPI-PLD that is required by a typical cell has not been determined, it is possible that this 0.4 μg/ml is sufficient for complete cell function. In combination with the issues that arose over the GPI-PLD-free nature of the serum after passage through the immunoabsorbent columns, it was decided that this method would not be pursued.

An alternative to immuno-absorbent column was the Vivaspin concentrator column, which had been successfully employed by colleagues. Vivaspin concentrator columns are designed for the purification and concentration of small volumes in a swing-bucket or fixed-angle centrifuge. The columns consist of a plastic, disposable column, containing a longitudinal polysulphone membrane, which provides optimal conditions for the centrifugation of solutions. These columns are designed to selectively filter proteins of a defined molecular weight this weight being dependent on the pore size of the membrane (from 5,000 – 100,000 molecular weight cut-off). Furthermore, the membrane is inert, non-cytotoxic and will not denature biological solutions, such as FBS (VivaScience Ltd. technical information sheet). The choice of the membrane pore-size depends on the sample being concentrated.

The selection of a Vivaspin column with a membrane pore size of 100 kDa, for example, would permit the passage of proteins smaller than 100 kDa, whilst retaining proteins larger than 100 kDa. GPI-PLD protein is 110 kDa protein, and in principle it should be retained in the upper reservoir of the column, whilst permitting the passage of smaller serum proteins. The eluted solution should, however, contain the majority of the serum proteins, whilst being selectively depleted in the higher molecular weight compounds. It was, therefore, considered interesting to determine whether the GPI-PLD protein could be removed from FBS samples by passage through a Vivaspin concentrator column.

4.4.4 Alkaline inactivation of Foetal Bovine Serum

A method that was reported in the literature involved the incubation of Foetal Bovine Serum in alkaline conditions. This incubation ultimately led to a loss of the GPI-PLD activity, as measured by incubation of alkaline-treated serum samples with radiolabelled GPI-anchored substrates (Kung *et al.*, 1997). If it were possible to reproduce this alkaline-incubation method, the treated FBS could then be used to supplement the RBL-2H3 culture media. This method, therefore, provided the opportunity of altering the GPI-PLD activity in the RBL-2H3 culture medium, whilst removing the need to replace the FBS entirely.

In attempting this alkaline inactivation method, it is acknowledged that it is crude. The probability clearly exists that this technique will lead to the inactivation of serum components in addition to the GPI-PLD. Prior to commencing with this alkaline incubation method, the current literature was reviewed. To date, no documented evidence was found for the mechanism by which the GPI-PLD activity was lost following alkaline incubation. In addition, no attempts have been made to determine the effect on other serum components. These issues are discussed in greater detail in later Sections.

However, the precise aim of the research performed in this chapter was to determine the role of GPI-PLD in Type One Hypersensitivity, through manipulation of the Foetal Bovine Serum. The loss of enzyme activity, through incubation of FBS at an alkaline pH was, therefore, considered to be an appropriate area of research. Using the results gained from this type of crude experiment, it would be possible to design more precise experiments, from which the role of the enzyme may be more accurately demonstrated.

In addition to the incubation of FBS in alkaline conditions, it was considered important to determine whether incubation at an alternative pH would also inactivate GPI-PLD. To this end, samples of FBS were independently incubated at pH 3, 5, 7, 9, 11, and the effect on the activity of GPI-PLD was ascertained, using the radioactivity assay described previously (Section 3.17).

4.4.4.1 Culture of RBL-2H3 cells in pH-11 treated FBS-supplemented media

Once a consistent method for removing active GPI-PLD from the FBS had been found, in the form of inactivation by incubation at pH 11, it was important to determine the effect on the behaviour of the RBL-2H3 cells. To achieve this, the FBS in the culture medium was replaced with pH-11-treated FBS. In all other respects, the culture medium (supplemented EMEM) remained identical.

Following the culture of the RBL-2H3 cells in this altered medium, any alterations in the phenotype of the cells were determined. In each case, comparisons were made between RBL-2H3 cells cultured in normal FBS containing medium, and those cells cultured in the pH 11-treated FBS containing medium. These included:

- Determination of GPI-PLD activity in cell membrane preparations.
- Determination of reactivity to IgE-mediated stimulation.
- Determination of reactivity to Ionophore-mediated stimulation.

4.5 Addition of GPI-PLD activity to RBL-2H3 cells cultured with pH-11 treated FBS

Following the culture of RBL-2H3 cells in the pH-11 treated FBS, it was important to ascertain whether the cells had been irreparably damaged. To this end, it was important to investigate whether any behavioural changes in the RBL-2H3 cells were reversed by the addition of normal GPI-PLD. Two different approaches were made:

- replacing the pH-11 treated FBS with normal FBS (section 4.5.1)
- the addition of a recombinant GPI-PLD protein to the pH-11 treated FBS (Section 4.5.2.2)

Each of these alternatives, and the benefits and drawbacks of their use in an experimental study requires further discussion.

4.5.1 Addition of normal FBS to RBL-2H3 cells cultured with pH-11 treated FBS

Firstly, it was important to determine whether the mast cells were irreparably damaged by their incubation with pH 11-treated FBS. To this end, the RBL-2H3 cells were transferred back into a medium containing normal, untreated, FBS. The cells were allowed to grow in this normal medium for a determined period of time, after which their response to IgE-mediated stimulation was ascertained.

4.5.2 Addition of purified GPI-PLD to RBL-2H3 cells cultured with pH-11 treated FBS

The addition of fresh untreated FBS to the culture medium is an appropriate method to determine whether the cells were irreparably damaged by incubation with the alkaline-treated FBS. It was, however, important to determine whether any behavioural changes observed in the cultured RBL-2H3 cells were as a consequence of the loss of the GPI-PLD activity, rather than an alternation in an alternative, un-measured, serum component. A method by which this could be achieved involved the replacement of the GPI-PLD activity with a purified GPI-PLD protein. This would retain the alkaline-treated profile of the FBS, whilst effectively regaining the GPI-PLD activity.

4.5.2.1 Replacement of GPI-PLD activity in alkaline-treated FBS with purified GPI-PLD protein

GPI-PLD has been successfully purified from serum samples in a number of different studies (Huang *et al.*, 1990). The purified enzyme is between 100 and 110 kDa molecular weight. However, the method is highly complex, involving up to eleven sequential steps, as outlined below:

1. 9% polyethylene glycol precipitation.
2. Q-Sepharose anion-exchange column chromatography.
3. S-300 gel filtration.
4. Wheat germ lectin-Sepharose chromatography.
5. Hydroxylapatite Ultragel agarose chromatography.
6. Zinc-chelated Fractogel column chromatography.
7. Mono Q-high performance liquid chromatography (HPLC).
8. Superose 12 (gel filtration) HPLC.

9. Immunoaffinity chromatography, using the partially purified GPI-PLD protein as an antigen, against which monoclonal antibodies were created.
10. Wheat germ lectin-Sepharose.
11. Mono Q-fast protein liquid chromatography.

Unfortunately, this method requires experimental expertise not found in our laboratory, and it was considered inappropriate to attempt to learn the techniques within the time constraints of this research thesis.

It was, therefore, considered appropriate to use an alternative GPI-PLD, and the possibility existed that a recombinant protein may be an effective substitute for the native GPI-PLD protein.

4.5.2.2.1 Creation of a recombinant GPI-PLD protein

The generation of recombinant GPI-PLD proteins has been described in a number of alternative studies, and these recombinant proteins were shown to possess enzyme activity against GPI-anchored substrates (Tujioka *et al.*, 1998). It was, therefore, assumed that a recombinant protein could effectively substitute for a serum-isolated GPI-PLD protein.

The generation of a recombinant GPI-PLD protein was performed in collaboration with Dr Julian Schofield, University College London. A full length GPI-PLD cDNA was isolated from a human superscript cDNA liver library (Gibco BRL), which was produced using the superscript plasmid system for cDNA synthesis and plasmid cloning (D'Allessio *et al.*, 1990). Liver cDNA, derived from a nine year old Caucasian, was directionally cloned into an eukaryotic expression vector, pCMV Sport (Gibco brl). After transformation into Electromax DH12S cells, independent clones were amplified in semi-solid culture. This particular library was selected because the GPI-PLD was known to be expressed within the liver (Scallon *et al.*, 1991). The GPI-PLD sequence was detected by hybridisation against a ^{32}P-labelled PCR product.

The cDNA fragment containing the GPLD1 sequence was removed from the pCMV vector, and inserted into a mammalian pcDNA 3.1 expression vector, HisB (Invitrogen Corporation). These pcDNA 3.1 vectors are designed for high level constitutive expression in a variety of mammalian cell lines, under the control of the human immediate early cytomegalovirus (CMV) promoter. In addition, the expressed protein can be easily purified from mammalian cells, by the addition of a histidine tag. The pcDNA 3.1 / GPI-PLD plasmids were transfected into CHO cells, using the PerFect Transfection Kit (Invitrogen Corp.).

The presence of a neomycin-resistant marker, within the pcDNA 3.1 DNA plasmid, permitted the selection of GPI-PLD positive clones, by growth of the CHO cells in neomycin-containing medium. The positive clones were selected, and amplified in culture. The results showed that transfection was successful, with a number of positive clones identified.

The GPI-PLD protein was extracted from intact cells, using the X-press protein purification system (Invitrogen Corp.). This system is reliant on the existence of an N-terminal histidine tag, which is selectively adsorbed by the Invitrogen ProBond Resin. The Xpress recombinant protein was eluted from the ProBond resin by washing with either a pH 4.0 buffer, under denaturing conditions, or by an imidazole gradient in pH 6.0 buffer, under native conditions. The size of the protein generated by this method was determined by separation by both native and denaturing gel electrophoresis. In addition, the GPI-PLD activity in the separate fractions that were eluted from this purification method was determined using the GPI-PLD activity assay, with radiolabelled mfVSG as the substrate.

4.5.2.2.2 Addition of recombinant GPI-PLD protein to RBL-2H3 cell culture

It was essential to determine the effect of the addition of recombinant GPI-PLD protein to RBL-2H3 cells cultured with pH 11-treated FBS. However, the culture of RBL-2H3 cells with r-GPI-PLD was limited by two major factors. Firstly, the small quantities of recombinant protein limited the amounts that could be added to the cultured cells. Ideally, the recombinant protein would have been added to a standard culture flask of alkaline FBS-treated RBL-2H3 cells, to provide a large number of cells for experimentation. With the limited volumes of r-GPI-PLD, however, this was not possible, and it was necessary to perform the addition of the recombinant protein to cells cultured in a 24 well plate.

The second limitation, based on the use of a 24 well plate for culture of the cells, was the time of culture of the cells. As discussed in Chapter 2 (section 2.11.6.3) the growth of RBL-2H3 cells on plastic surfaces was limited by a minimal and maximal quantity of cells. The minimal quantity of cells, approximately 100,000 per well on a 24 well plate, represented the number at which the cells would adequately grow to confluence. Below this number, there were insufficient cells to maintain adequate quantities of growth factors and cytokines and, although the cells would adhere to the plastic surface, very little proliferation was observed. By comparison, the maximal quantity of cells, approximately 200,000 per well on a 24 well plate, represented the number at which the cells were fully confluent. Above this number, contact inhibition lead to detachment of the excess cells, which were washed away during experimentation. With excessive numbers of cells in a well, the volumes of toxic cell by-

products were raised, and the cells began to appear less healthy. The methodology for the experiment to add r-GPI-PLD to RBL-2H3 cells had to consider both of these limitations.

In addition, it was considered of importance to determine whether the RBL-2H3 cells had even taken up the recombinant protein from the culture medium. Without the demonstration that the r-GPI-PLD could be effectively absorbed by the culturing cells, it was not possible to attribute any observed alterations in the RBL-2H3 cell behaviour to the existence of the r-GPI-PLD in the cells.

The most effective method for establishing the uptake of the recombinant protein was a direct membrane extraction performed on the RBL-2H3 cells, immediately following the IgE-mediated stimulation experiments. The GPI-PLD activity in the extract was determined using radio-labelled mfVSG as a substrate.

4.6 Transfection of the GPI-PLD protein into RBL-2H3 cells

A number of studies have described the transfection of GPI-PLD constructs, both transient and stable, permitting researchers to closely examine the role of the enzyme in the cellular behaviour. It was, therefore, considered to be appropriate to attempt transfection of a recombinant GPI-PLD protein into RBL-2H3 cells, for two primary reasons.

Firstly, transfection of a recombinant GPI-PLD into RBL-2H3 cells cultured with pH-11 treated FBS may prove whether the recombinant protein can compensate for the lack of active GPI-PLD in the culture medium.

Secondly, previous studies have accomplished transfection of mutated forms of the GPI-PLD protein (Stadelman *et al.*, 1997). If the expression of a mutated form of GPI-PLD could be achieved in RBL-2H3 cells, it would have been interesting to determine whether the behaviour of the cells was altered. This may ultimately confirm whether or not GPI-PLD is directly involved with the Type One Hypersensitivity process in this mast cell line.

The transfection method that was selected was the PerFect Transfection Kit (Invitrogen), which employs a lipid-carrier mechanism, by which the expression vector is introduced into the cells. A control plasmid, pcDNA3.1/His/lacZ, is supplied with the kit along with a panel of eight lipids, consisting of either a single cationic lipid, a combination of two cationic lipids, or a combination of a cationic lipid and L-dioleoyl phosphatidylethanolamine (DOPE). The details of the different lipids are outlined in Table 4.1. The PerFect Transfection Kit has been tested in a variety of different cell lines, and it is clear that different lipids are optimal for different cell

TABLE 4.1

DETAILS OF THE LIPIDS USED IN THE TRANSFECTION EXPERIMENTS

Lipid	**Composition**	**Moles of Positive Charge per μg lipid mix**	**Average Molecular Weight**
Pfx-1	1:1 mix of two cationic lipids	2.20×10^{-9}	2,492
Pfx-2	Single cationic lipid	2.08×10^{-9}	964
Pfx-3	1:1 mix of cationic lipid and DOPE	1.52×10^{-9}	1025
Pfx-4	1:1 Mix of two cationic lipids	1.73×10^{-9}	847
Pfx-5	1:1 Mix of two cationic lipids	3.00×10^{-9}	1470
Pfx-6	1:1 Mix of cationic lipid and DOPE	1.07×10^{-9}	840
Pfx-7	1:1 Mix of cationic lipid and DOPE	1.56×10^{-9}	1011
Pfx-8	Single cationic lipid	3.05×10^{-9}	2617

lines. For example, in COS-7 cell (Monkey Fibroblasts), Pfx-6 effected the highest transfection efficiency, whereas Pfx-4 was more successful in CHO cells (Hamster Fibroblasts). Prior to any experiments involving the GPI-PLD protein it was, therefore, necessary to test each lipid, to determine which promotes the highest transfection efficiencies in the RBL-2H3 cell line. The presence of a functional expression vector is confirmed by β-galactosidase expression.

This method of transfection was selected because it had already been successfully used for the generation of the recombinant GPI-PLD which was used in previous experiments during this Chapter, as discussed in Section 4.5.2.3. Transfection can be a very laborious process, which can involve a number of modifications to the published methodology before the expression vector is successfully transferred into a specific cell line. It seemed sensible, therefore, to use a method that had already proved successful in our laboratory.

4.7 Experimental Methods

The methods employed for the duration of this chapter are outlined in Chapter 2 and 3. They include:

- Routine Culture and Maintenance of the RBL-2H3 cell line (Section 2.9.1)
- Non-IgE mediated degranulation of RBL-2H3 cells (Section 2.9.2)
- IgE-mediated degranulation of RBL-2H3 cells (Section 2.9.3)
- Colorimetric detection of *β*-hexosaminidase (Section 2.9.4)
- Detection of GPI-PLD activity using the radioactive mfVSG assay (Section 3.20.1)
- RNA extraction, treatment and amplification by RT-PCR (Section 3.24)

In addition, the following methods were employed, specific to the research performed in this chapter.

4.7.1 Concentration of protein samples using Vivaspin concentrator columns

Up to 4 ml of the sample to be concentrated was pipetted into the upper reservoir of the Vivaspin concentrator column and the lid sealed tightly. The column was then transferred to a 15 ml capacity Falcon tube (17 mm diameter). Ensuring adequate counter balance, the tubes were placed into a swing-bucket centrifuge (Heraeus Labofuge AE), maintained at 4°C, and spun at up to 12,000 x g, with the speed dependent on the nature of the sample.

Centrifugation was continued until the desired final volume was achieved. The concentrated sample (retentate) was recovered from above the membrane, using a fine-tipped pipette tip, taking care to ensure that contact with the membrane was avoided. The filtered sample (eluate) was recovered from the Falcon tube. Samples were stored at -20°C until required.

To determine any alteration of the FBS following this filtration technique, both the retentate and the eluate samples were separated by SDS-PAGE electrophoresis, in comparison with untreated FBS and molecular weight markers. A total of 200 μg of protein was loaded onto on a 5% polyacrylamide gel, and the protein separation was determined by staining the SDS-PAGE gel with Coomassie Blue.

4.7.2 Incubation of Foetal Bovine Serum under acid or alkaline conditions

The pH of the Foetal Bovine Serum was adjusted to pH 11 using 1M sodium hydroxide, and incubated for 1 hour at 37°C in a shaking water bath. After this time, the pH was adjusted back to 7.4, using 1M hydrochloric acid. Determination of the activity of the GPI-PLD was performed using the radioactive GPI-PLD activity assay, as described in Section 3.20.1. Prior to inclusion in the culture medium, the pH-11-treated FBS was filtered through a 0.2 μ filter, to ensure its sterility.

In addition to the incubation at pH 11, samples of FBS were also incubated at pH 3, 5, 7 and 9. The altered pH was achieved by the addition of either 1M sodium hydroxide or 1M hydrochloric acid, as applicable, and incubation for 1 hour at 37°C in a shaking water bath. After this time, the pH was adjusted back to 7.4, using either 1M sodium hydroxide or 1M hydrochloric acid. Again, the GPI-PLD activity in the samples was determined using the radioactive GPI-PLD activity assay, as described in Section 3.20.1, and the samples were filtered through a 0.2 μ filter before use in cell culture.

4.7.3 Culture of RBL-2H3 cells in pH treated-FBS, and analysis of alterations in cell phenotype

To determine the effect of culture of RBL-2H3 cells in pH-11-treated FBS, the supplemented EMEM was replaced with EMEM in which the FBS had been inactivated. Prior to the addition of the FBS to the medium, however, the serum was filtrated through a 0.2 μ filter, to remove any contamination that may have been introduced during the alkaline treatment. With the exception of the pH-11 treated FBS, the EMEM was prepared as in previous experiments (Eagles Minimum Essential Medium supplemented with 10% volume:volume (v:v) heat-inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin).

The cells were split to produce a density of 2 million cells per 20 ml fresh EMEM. The EMEM was supplemented with either normal FBS, or alkaline-treated FBS, at a concentration of 10%

(v:v), in addition to 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

When cell confluency was achieved, non-adherent cells were removed by washing the monolayer twice with fresh supplemented EMEM (containing untreated or alkaline-treated FBS, as applicable). The adherent cells were detached from the plastic surface, using a cell scraper. The cell density was determined using a Neubauer haemocytometer and the cells were split at the same density as was originally determined, in 20 ml fresh supplemented EMEM.

As appropriate, aliquots of cell suspension were withdrawn for analysis. Three analyses were routinely performed:

- IgE-mediated stimulation (Section 2.9.3)
- radioactive GPI-PLD activity assay (Sections 3.20 to 3.21)
- RNA extraction, for the RT-PCR protocol (Section 3.24).

The splitting, sub culture, and removal of cells for analysis, was continued for as long as was deemed appropriate.

4.7.4 Adding back normal FBS to RBL-2H3 cells cultured in pH-treated FBS

Adherent 'inactive' cells were washed with sterile PBS and detached from the culture flasks using a cell scraper. The cell density was determined, using a Neubauer haemocytometer. The cells were then split at a density of 1 million cells per 20 ml of fresh culture medium, containing normal FBS-supplemented EMEM. The 'active' and 'inactive' cells were cultured in an identical fashion, with 'active' and 'inactive' FBS respectively. Once confluence was achieved, the cells were split, at a density of 1 million cells per 20 ml fresh EMEM. After the cells had been split twice, a standard IgE cross-linking assay (Section 2.9.3) was performed using active, inactive and reconstituted cells.

4.7.5 Incubation of RBL-2H3 cells with recombinant GPI-PLD protein

As only small quantities of recombinant GPI-PLD (r-GPI-PLD) were available, the methodology for the addition of r-GPI-PLD to RBL-2H3 cells required small volumes of cells.

RBL-2H3 cells were grown to confluence, after which time the adherent cells were removed from the culture flask using a cell scraper. The cell density was determined, using a Neubauer haemocytometer. RBL-2H3 cells were plated onto a 24 well plate at a density of 100,000 cells per well in a 500 µl volume. Both RBL-2H3 cells that had been cultured in untreated FBS, and cells that had been cultured in alkaline-treated FBS, were plated out.

r-GPI-PLD was added to selected 'inactive' wells, at a concentration of either 100 or 200 μg per well. The cells were cultured overnight at 37°C in a humidified 5% CO_2 incubator, taking care to ensure that contamination of the plates was minimised. The cells were monitored and when confluence had been achieved, an IgE-mediated cross-linking experiment was performed, as described in Section 2.9.3.

4.7.6 Transfection of the Expression Vector into RBL-2H3 cells

RBL-2H3 cells were grown to confluence, after which time the adherent cells were removed from the culture flask using a cell scraper. The cell density was determined, using a Neubauer haemocytometer. RBL-2H3 cells were plated onto two 12 well plates at a density of 100,000 cells per well in a 1 ml volume. The cells were maintained in normal, supplemented EMEM (Eagles Minimum Essential Medium (EMEM) supplemented with 10% volume:volume (v:v) heat inactivated Foetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin) (Sigma Chemicals Ltd). The cells were cultured at 37°C in a humidified 5% CO_2 incubator, taking care to ensure that contamination of the plates was minimised, and were maintained until approximately 50 to 60% confluence was achieved.

The lyophilised expression vector, pcDNA3.1/His/lacZ, was reconstituted to a final concentration of 1 mg/ml in sterile water, and stored at – 20 °C until needed. Prior to use, the vector is further diluted, to a final concentration of 4 μg/ml, in serum-free buffer. Each lipid was diluted to a final concentration of 24 μg/ml in serum-free culture medium (EMEM (Eagles Minimum Essential Medium (EMEM) supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin). The DNA and lipid were combined to produce a final ratio of 1:6 (w/w) plasmid to lipid, serum-free medium, and a final volume of 2 mls.

Following incubation, the culture medium was removed and the adherent RBL-2H3 cells were washed with sterile phosphate buffer saline (PBS) (0.01 M phosphate buffer, 0.0027 potassium chloride and 0.137 M sodium chloride, pH 7.4) (Sigma Chemicals), to remove any contaminating serum. 1 ml of plasmid : lipid mixture was added to two culture wells, until a duplicate set of each lipids was plated out. The cells were then returned to the incubator and were incubated at 37 °C for 4 hours. Following this incubation, the transfection solution was removed, and replaced with 1 ml of complete medium per well. The cells were returned to the incubator and left for 24 hours, after which time the transfection efficiency was determined.

The transfection efficiency was determined by assaying for the expression of the β-galactosidase gene, which is located on the pcDNA3.1/His/lacZ plasmid. The culture medium

was removed from the cells, and the adherent RBL-2H3 cells were washed with sterile phosphate buffer saline (PBS) (0.01 M phosphate buffer, 0.0027 potassium chloride and 0.137 M sodium chloride, pH 7.4) (Sigma Chemicals).

Following washing, 0.5 ml of a fixing solution (2% formaldehyde and 0.2% gluteraldehyde in PBS without cations, pH 7.4), was added to each culture well. This fixing solution was created for each experiment, and discarded immediately after use. The cells were fixed in this solution for 1 minute at room temperature, after which time they were washed with 0.5 ml of sterile phosphate buffer saline.

Stock solutions of the X-gal staining reagents were created as follows:

- 20 mg/ml X-Gal in dimethylformamide
- 0.4 M potassium ferricyanide ($K_3Fe(CN)_6$) in water
- 0.4 M potassium ferrocyanide ($K_4Fe(CN)_6$-$3H_20$) in water
- 2 mM magnesium chloride hexahydrate in water

Each of these stock solutions was stable, when stored at – 20°C.

A working X-gal solution was created, containing 1 mg/ml X-Gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide and 2 mM magnesium chloride hexahydrate in a PBS solution, pH 7.4. Once again, this working solution was created fresh for each experiment, and was discarded immediately after use. 0.5 ml of this staining solution was added to each well, and the cells were incubated at room temperature overnight. Following incubation, the cells were examined under the microscope, and those cells that have been successfully transfected with pcDNA/His/lacZ were identified as being blue. The percentage of blue cells was determined, using a Neubauer haemocytometer.

4.8 Results

4.8.1 Removal of GPI-PLD activity from the RBL-2H3 culture medium

4.8.1.1 Aim-V serum-free supplements

The plot in Figure 4.1 shows the results from the GPI-PLD activity assay with the Aim-V serum supplement, in comparison with Foetal Bovine Serum samples. Both FBS and Aim-V samples were diluted to a final concentration of 10%, in EMEM. The data shows the radioactivity count from the FBS sample in comparison with the Aim-V sample, and a negative control (0% FBS in EMEM). The samples were incubated, in a 1:1 ratio with the activity assay reaction mix, containing 30 mM mfVSG in total, and the reaction was terminated after 10 minutes incubation at 37 °C. The data is expressed as counts per minute (cpm), after a one-minute detection

FIGURE 4.1

CONCENTRATION OF GPI-PLD IN AIM-V SERUM SUBSTITUTE

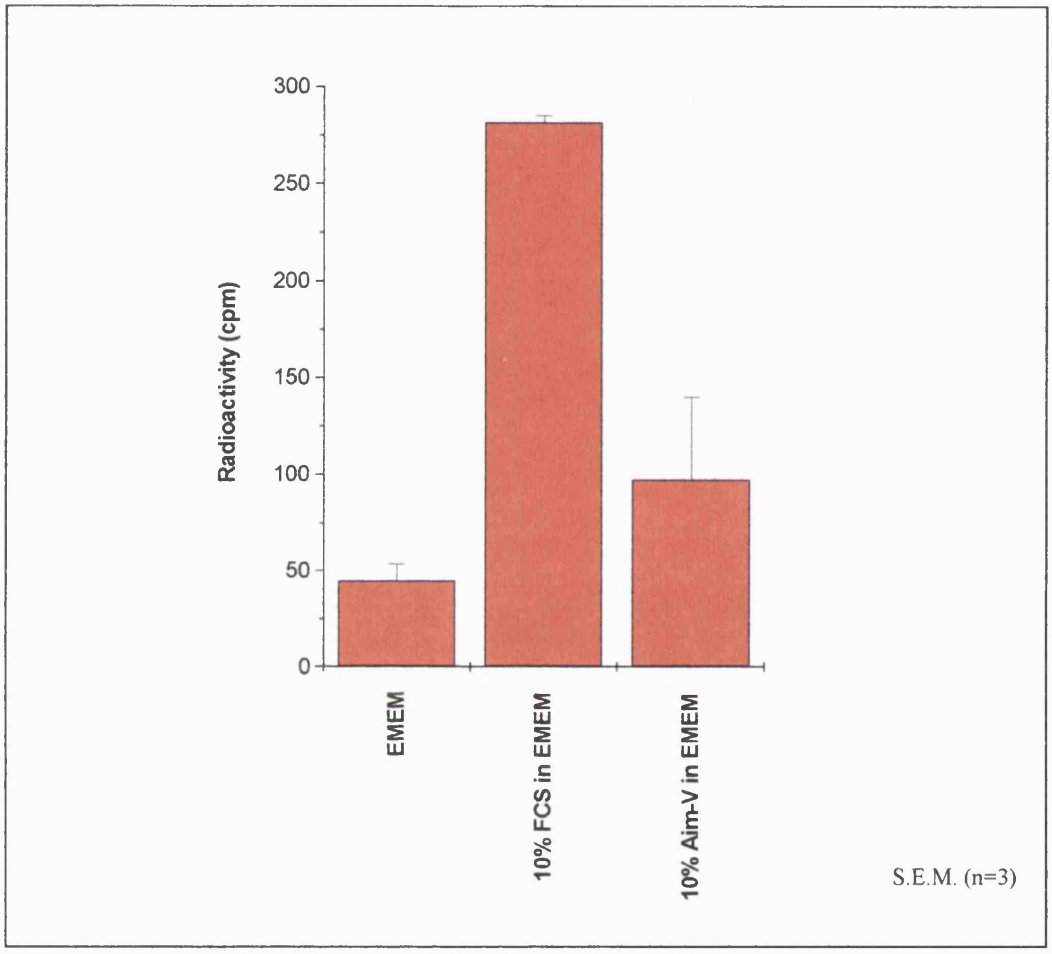


Figure Legend

The plot in Figure 4.1 shows the results from the GPI-PLD activity assay with the Aim-V serum supplement, in comparison with Foetal Bovine Serum samples (10% in EMEM), and EMEM as the negative control.

The samples were incubated, in a 1:1 ratio with the activity assay reaction mix, containing 30 mM mfVSG in total, and the reaction was terminated after 10 minutes incubation at 37 °C. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

Data showed that, in comparison with the negative control, GPI-PLD activity was detected in the Aim-V serum-free samples.

programme in the scintillation counter, and represents the radioactivity detected from the [3H]-dimyristyl phosphatidic acid product.

Overall, the values are quite low, as a result of the short incubation time. As discussed in Chapter 3, later experiments with the GPI-PLD activity typically involved an incubation of greater than 30 minutes, in order to adequately determine the GPI-PLD activity in the samples. However, the point of importance from this data is that GPI-PLD activity was detected in the serum-free Aim-V sample. According to the calculation (Section 3.21), 0.81 units of activity were detected in the 10% Aim-V sample, in comparison with 3.64 units in the 10% FCS sample. Although the error bar showed quite a wide variation between the different samples detected, this result was evidence that this serum-free supplement contained detectable GPI-PLD activity.

4.8.1.2 Reduction of serum concentration

Initially, experiments were performed to determine whether the RBL-2H3 cells were viable in a serum-free culture medium. After as little as overnight culture, a significant percentage of cells had died, as determined by microscopy (data not shown). Clearly, a culture medium with a complete absence of serum components was not sufficient for the culture of RBL-2H3 cells. The alternative approach, therefore, was to determine whether, at significantly reduced serum concentrations, the levels of GPI-PLD would fall below what would be considered suitable for the function of the RBL-2H3 cells. Analysis of the GPI-PLD activity in a serial dilution of FBS samples is shown in Figure 4.2.

FBS samples were diluted in EMEM, and mixed, in a 1:1 ratio with the reaction mix, containing 30 mM mfVSG in total. The samples were incubated at 37 °C, and the reaction was terminated after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter, and represents the radioactivity detected from the [3H]-dimyristyl phosphatidic acid product.

The plot shows that the GPI-PLD activity detected rose with the increasing concentration of FBS, and was starting to reach a plateau by approximately 10% FBS. According to the calculation (Section 3.21) the units of GPI-PLD activity that was detected in the samples was 0.12 at 0% FBS, 4.60 (1%), 11.83 (4%), 14.97 (7%) and 16.84 (10%).

With reference to the results in Figure 4.2, the decision was made that culture of RBL-2H3 cells in the FBS-reduced medium would not be an effective method for reducing the GPI-PLD in the culture medium. Although the quantities of GPI-PLD required for a cell to maintain

FIGURE 4.2

GPI-PLD ACTIVITY IN A REDUCED CONCENTRATION OF FBS

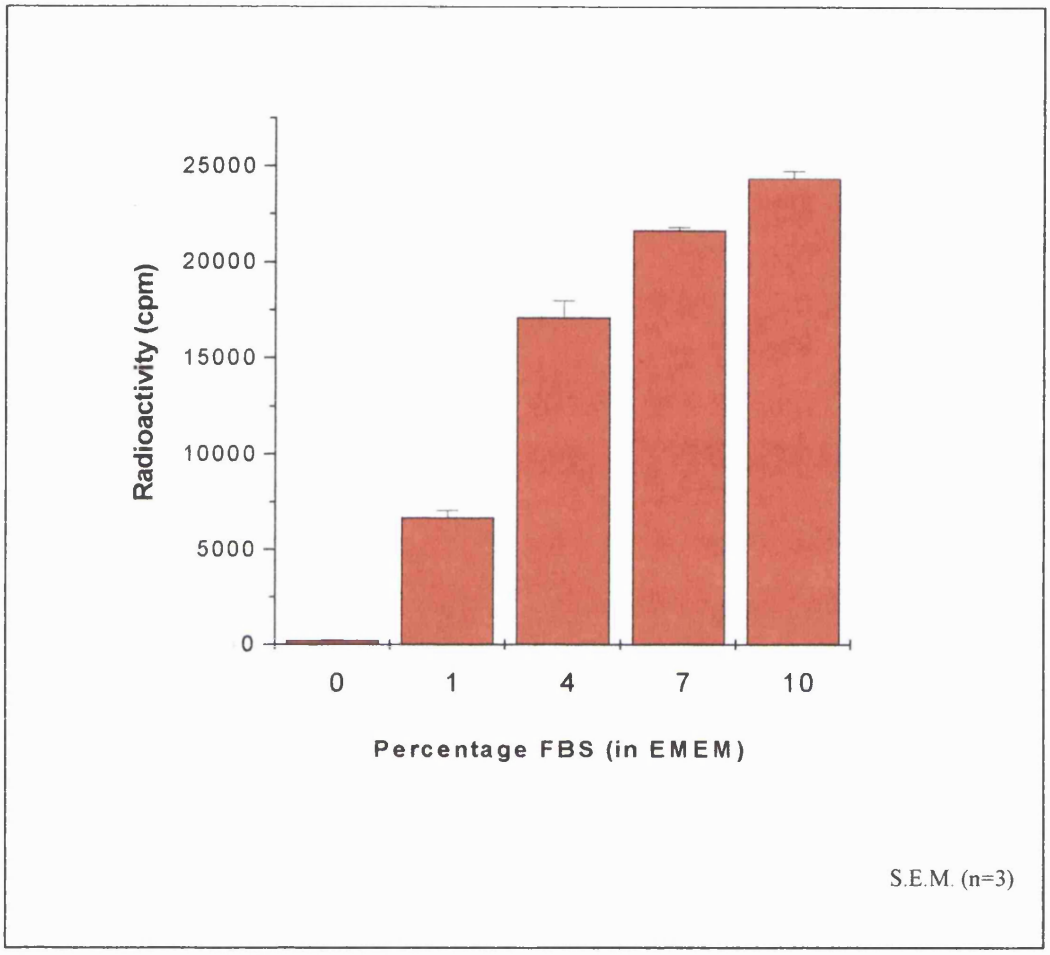


Figure Legend

The plot in Figure 4.2 shows the results from the GPI-PLD activity assay with 0 to 10% FBS (in EMEM), following termination of the assay after 30 minutes. The FBS samples were diluted in EMEM, and mixed, in a 1:1 ratio with the reaction mix, containing 30 mM mfVSG in total. The samples were incubated at 37 °C. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The plot shows that the GPI-PLD activity detected rose with the increasing concentration of FBS, and was starting to reach a plateau by approximately 10% FBS.

adequate function has never been determined, it can be assumed that even the 4.60 units of GPI-PLD per millilitre of 1% FBS would be sufficient. In addition, at a FBS concentration of 1%, it is likely that the loss of other proteins within the FBS would be sufficient to affect the growth and/or behaviour of the cells. An alternative to a reduction in the concentration of FBS was, therefore, sought.

4.8.1.3 Filtration of FBS through VivaSpin Concentrator columns

The plots in Figures 4.3 and 4.4 show the analysis of FBS samples following their passage through a 100 kDa cut-off Vivaspin concentrator column. Briefly, neat FBS samples were diluted 1 in 10 with un-supplemented EMEM. 4 ml of sample was then pipetted into the upper reservoir of the Vivaspin column, the lid sealed tightly, and the column was transferred to a 15 ml capacity Falcon. The samples were the centrifuged at 1,000 rpm, at 4°C. The samples were centrifuged until the volume retained in the upper reservoir was 400 µl, which represented a 10 fold concentration of the sample.

As a consequence of the dilution of the FBS samples, prior to their filtration, it was not possible to compare the GPI-PLD activity detected in the filtered samples with normal un-treated FBS. Any difference in the detected enzyme activity could be ascribed to the different preparation of the samples. To determine the result of the VivaSpin filtration procedure, therefore, the proteins detected in the sample were separated by SDS-PAGE gel electrophoresis, and visualisation of the proteins by Commassie Staining. Firstly, the protein concentration in the different FBS samples was analysed using the Coomassie plus protein detection assay. The results are shown in Figure 4.3.

Samples were diluted 30:1 (v:v) with Coomassie Plus reagent, mixed thoroughly, and the colour of the resultant solution was determined by spectrophotometry, at 595 nm wavelength. A freshly prepared BSA standard curve was assayed each time and the protein concentration was determined by comparison against the curve.

The results in Figure 4.3 essentially show that the filtration of the FBS sample appears to have concentrated a majority of the proteins in the upper reservoir, rather than effecting their passage through into the lower reservoir. The protein detected in an un-concentrated FBS sample was calculated as approximately 40 µg of BSA equivalent. The protein detected in the upper reservoir, after filtration, was 70 µg BSA equivalent, in comparison to approximately 13 µg, which was detected in the lower reservoir. It appeared, therefore, that the GPI-PLD in the FBS sample (and other proteins greater than 100 kDa) had not been selectively concentrated by the filtration procedure.

FIGURE 4.3

PROTEIN DETECTION IN VIVASPIN CONCENTRATED FOETAL BOVINE SERUM SAMPLES

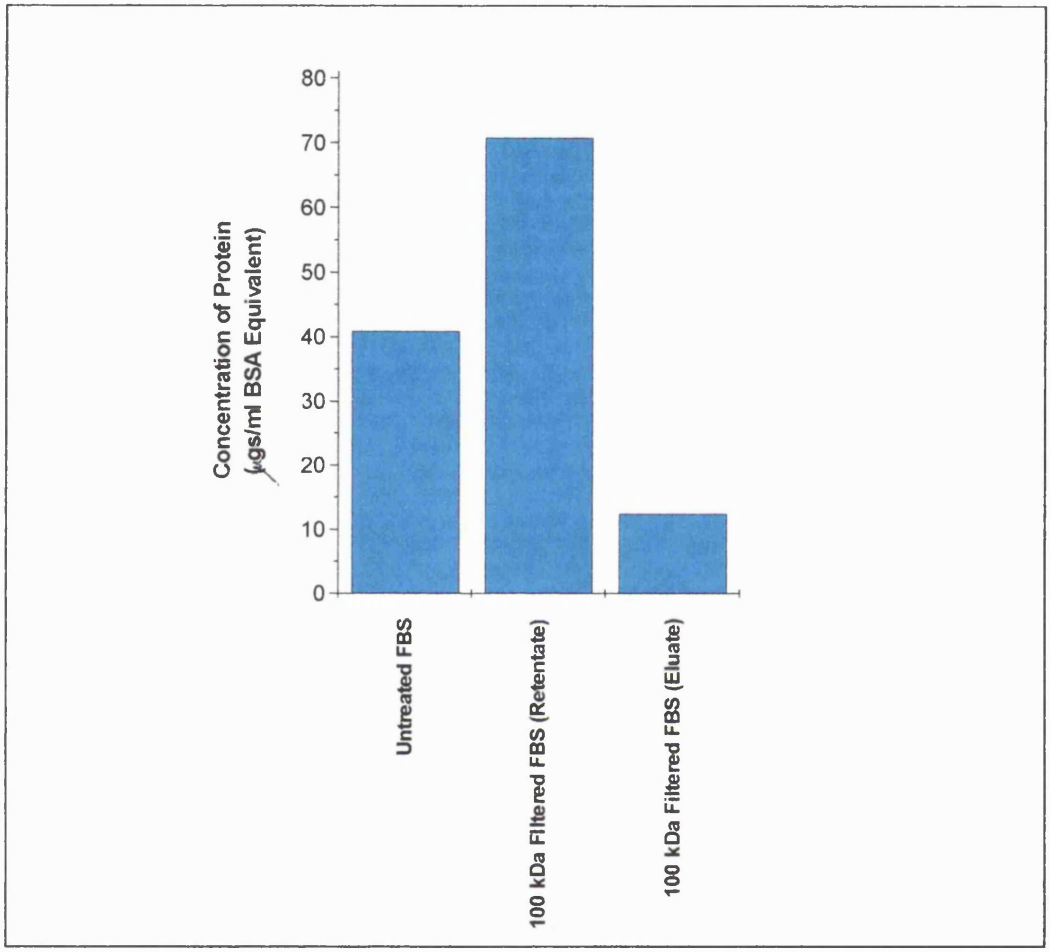


Figure Legend

The plot in Figure 4.3 shows the results from the Coomassie plus protein detection assay, using the FBS samples that were filtered through the Vivaspin concentrator columns. Filtered FBS samples (from both the upper and lower reservoirs) were diluted 30:1 (v:v) with Coomassie Plus reagent, mixed thoroughly. The colour of the resultant solution was determined by spectrophotometry at 595 nm wavelength. A freshly prepared BSA standard curve was assayed each time and the protein concentration was determined by comparison against the curve.

The results in Figure 4.3 shows that the filtration of the FBS sample has retained a majority of the proteins in the upper reservoir, rather than effecting their passage through into the lower reservoir. It appeared, therefore, that the GPI-PLD in the FBS sample (and proteins greater than 100 kDa) had not been selectively concentrated by the filtration procedure.

FIGURE 4.4

COOMASSINE STAIN OF VIVASPIN CONCENTRATED FOETAL BOVINE SERUM SAMPLES

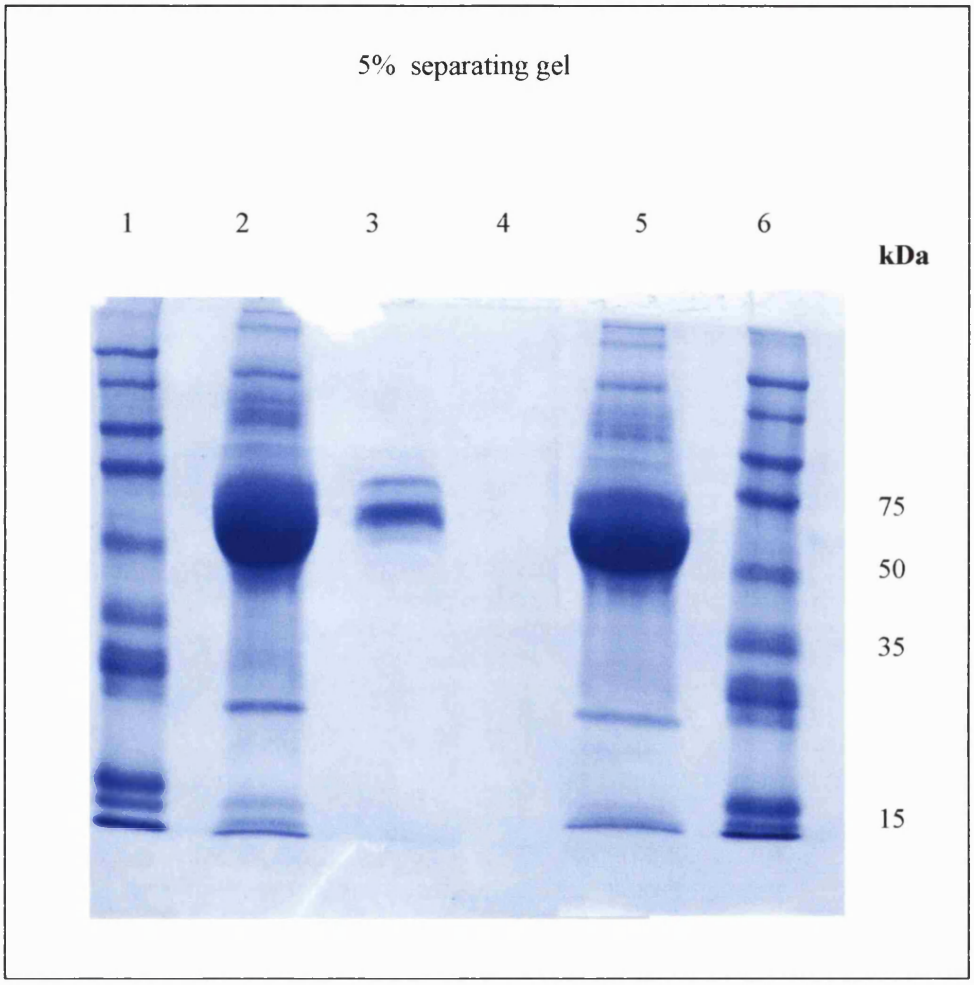


Figure Legend

Figure 4.4 show the separation of the filtered FBS samples by SDS-PAGE. 100 μg of protein was loaded into each well, with the exception of the the eluate from the Vivaspin filtration of 10% FBS in EMEM (lane 4) and the unsupplemented EMEM (lane 5), in which the protein concentration was insufficient for the loading of 100 μg of protein. Separation of the proteins was effected on a 5% acrylamide gel, and separation at 200 V for three hours. The gel was stained with Coomassie stain for 1 hours, and the proteins were visualised by the destain solution.

The proteins were separated in comparison with a molecular weight marker (Lanes 1 and 6). Lanes 2 and 3 contain the FBS samples from the upper (2) and lower (3) reservoirs of the filtration column. Lane 4 contains unsupplemented EMEM, and Lane 5 contains normal untreated FBS. In comparison with the untreated FBS, the quantity of protein that has passed through the Vivaspin membrane, as detected in the lower reservoir, was minimal. A majority of the proteins have been retained in the upper reservoir, retained by the membrane.

The separation of the FBS samples by filtration was revealed by SDS-PAGE analysis of the filtered FBS samples. These results are shown in Figure 4.4. Using the information derived from the Coomassie plus protein detection assay, as discussed above, 100 μg of protein was loaded into each well, with the exception of the unsupplemented EMEM and the eluate from the Vivaspin filtration of 10% FBS in EMEM (lower reservoir). In these cases, the protein concentration that was detected in the samples was insufficient for the loading of 100 μg of protein. Separation of the proteins was effected on a 5% acrylamide gel, and electrophoresis at 200 V for three hours. The gel was stained with Coomassie stain for 1 hours, and the proteins were visualised after incubation in the destain solution.

The proteins were separated in comparison with a molecular weight marker (Lanes 1 and 6). Lanes 2 and 3 contain the FBS samples from the upper (2) and lower (3) reservoirs of the filtration column. Lane 4 contains unsupplemented EMEM, and Lane 5 contains normal untreated FBS. In comparison with the untreated FBS, the quantity of protein that has passed through the Vivaspin membrane, as detected in the lower reservoir, was minimal. Only two bands were visualised by Coomassie protein staining, of approximately 60 and 70 kDa. A majority of the proteins have been retained in the upper reservoir, above the membrane.

4.8.1.4 pH 11-treatment of Foetal Bovine Serum

The plot in Figure 4.5 shows the GPI-PLD activity detected in FBS samples, following their incubation at pH 11 for one hour. Briefly, the pH of the FBS samples was adjusted using a 1M Sodium Hydroxide solution. The samples were incubated at 37 °C on a rotary shaker, for one hour, prior to the addition of 1M Hydrochloric acid, to return the samples to pH 7.4. Prior to inclusion in the GPI-PLD activity assay, the FBS samples were diluted 1 in 10 with EMEM. Samples were mixed, in a 1:1 ratio, with the reaction mix, containing 30 mM mfVSG in total. The samples were incubated at 37 °C, and the reaction was terminated after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The plot in Figure 4.5 shows the results from the GPI-PLD activity assay with two different samples of pH 11-treated FBS, prepared in two different experiments. The samples are shown in comparison with a normal, untreated FBS sample (10% in EMEM) and an EMEM negative control. The units of activity that were detected in the samples (using the calculation discussed in Section 3.21) was 14.2 units for the untreated sample, compared with 0.03 for one of the alkaline treated sample. The radioactivity detected in the other alkaline-treated sample was below the radioactivity detected in the negative control, and it was, therefore, not possible to

FIGURE 4.5

INACTIVATION OF GPI-PLD AFTER INCUBATION AT pH 11

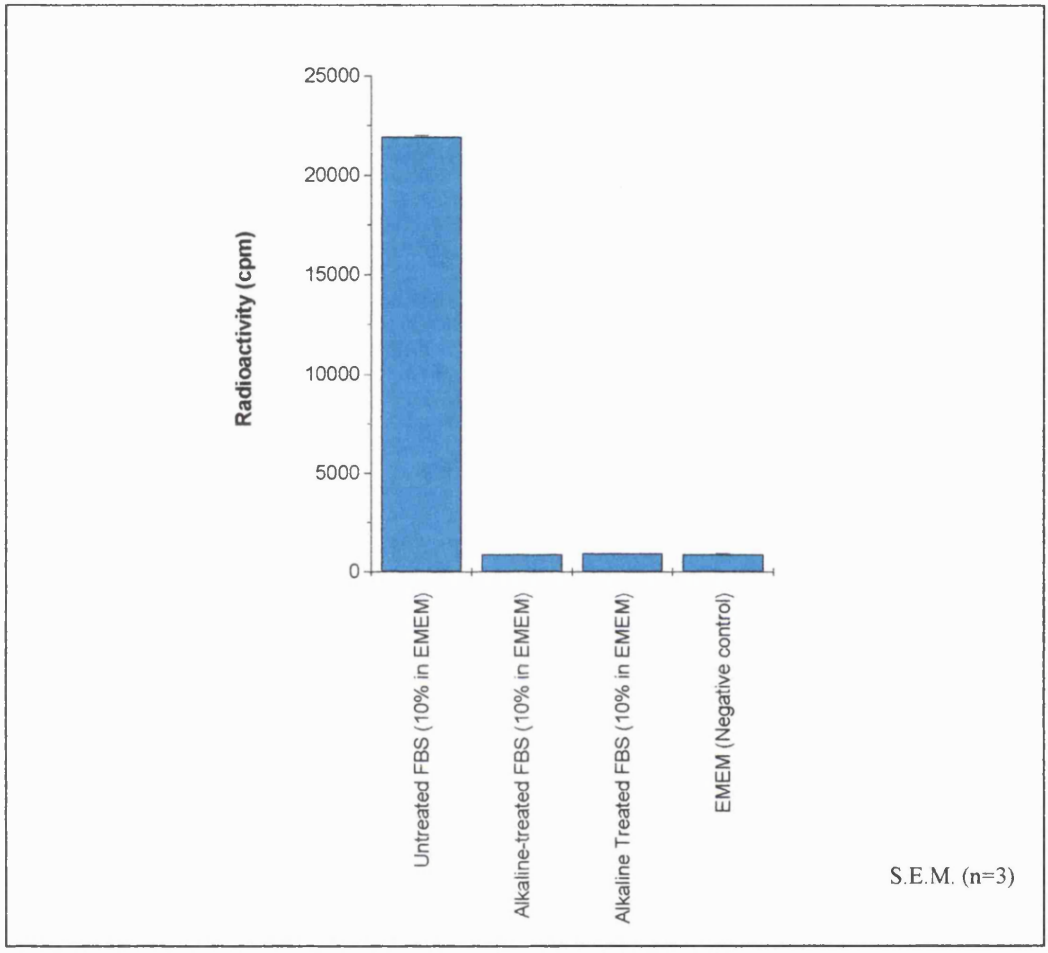


Figure Legend

The plot in Figure 4.5 shows the results from the GPI-PLD activity assay, using both normal FBS and pH 11-FBS (diluted 1 in 10 with EMEM), in comparison with an EMEM negative control. Samples were mixed, in a 1:1 ratio, with the reaction mix, containing 30 mM mfVSG in total. The samples were incubated at 37 °C, and the reaction was terminated after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

Data shows the results from the GPI-PLD activity assay with two different samples of pH 11-FBS, prepared in two different experiments. This result showed that, on two different occasions, the FBS that was incubated at pH 11 for one hour had lost the ability to hydrolyse the mfVSG substrate. As a result, the radioactivity detected from the [3H]-dimyristyl phosphatidic acid product was reduced, almost to zero, in these samples, compared with the untreated FBS samples.

determine the units of activity. This result confirmed that, on two different occasions, the FBS that was incubated at pH 11 for one hour had lost the ability to hydrolyse the mfVSG substrate.

4.8.1.5 Treatment of FBS at alternative pH

In addition to the alkaline inactivation method, samples of FBS were also incubated at pH 3, 5, 7 and 9. Although the original reference material had determined that GPI-PLD activity was lost after incubation at pH 11 for one hour (Kung *et al.*, 1997), no attempt was made to determine whether the protein was susceptible to incubation at other pH's. This experiment was designed to indicate the optimal pH range for GPI-PLD activity.

Analysis of the GPI-PLD activity in the treated FBS samples in shown in Figure 4.6. Briefly, the FBS samples were incubated at different pHs for 1 hour, before being returned to pH 7.4. Prior to inclusion in the GPI-PLD activity assay, the FBS samples were diluted 1 in 10 with EMEM. Samples were mixed, in a 1:1 ratio, with the reaction mix, containing 30 mM mfVSG in total. The samples were incubated at 37 °C, and the reaction was terminated after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The plot in Figure 4.6 shows a bell-shaped curve of GPI-PLD activity detected in different FBS samples. Essentially, those samples incubated at either pH 5 or 9 showed similar GPI-PLD activity to those samples maintained at pH 7. By comparison, when the FBS samples were incubated at pH 3 or 11, the GPI-PLD activity detected in these samples was significantly reduced, and was almost equal to the results gained with the negative controls (EMEM buffer, without FBS).

The units of GPI-PLD activity that were detected in each sample, after a 30 minute incubation were calculated using the equation discussed in Section 3.21. Accordingly, 15.17 (pH 5), 19.93 (pH 7) and 18.31 (pH 9). 0.19 units of activity were detected in the sample incubated at pH 3, while the sample incubated at pH 11 generated 0.46 units.

4.9 Culture of RBL-2H3 cells with pH 11-treated FBS-supplemented medium

The FBS in the culture medium was replaced with pH 11-treated FBS, after filtration through a 0.2 μ filter, and the EMEM culture medium was prepared as normal. The RBL-2H3 cells were split at a density of 1 million cells per flask, in 20 mls of fresh culture medium, and were split every two days. The cells were cultured for a total of 14 days, prior to the establishment of a variety of experiments. In order to determine the effect of the pH 11-treated FBS on the behaviour of the RBL-2H3 cells, a series of experiments were performed.

FIGURE 4.6

INACTIVATION OF GPI-PLD AFTER INCUBATION AT DIFFERENT pH

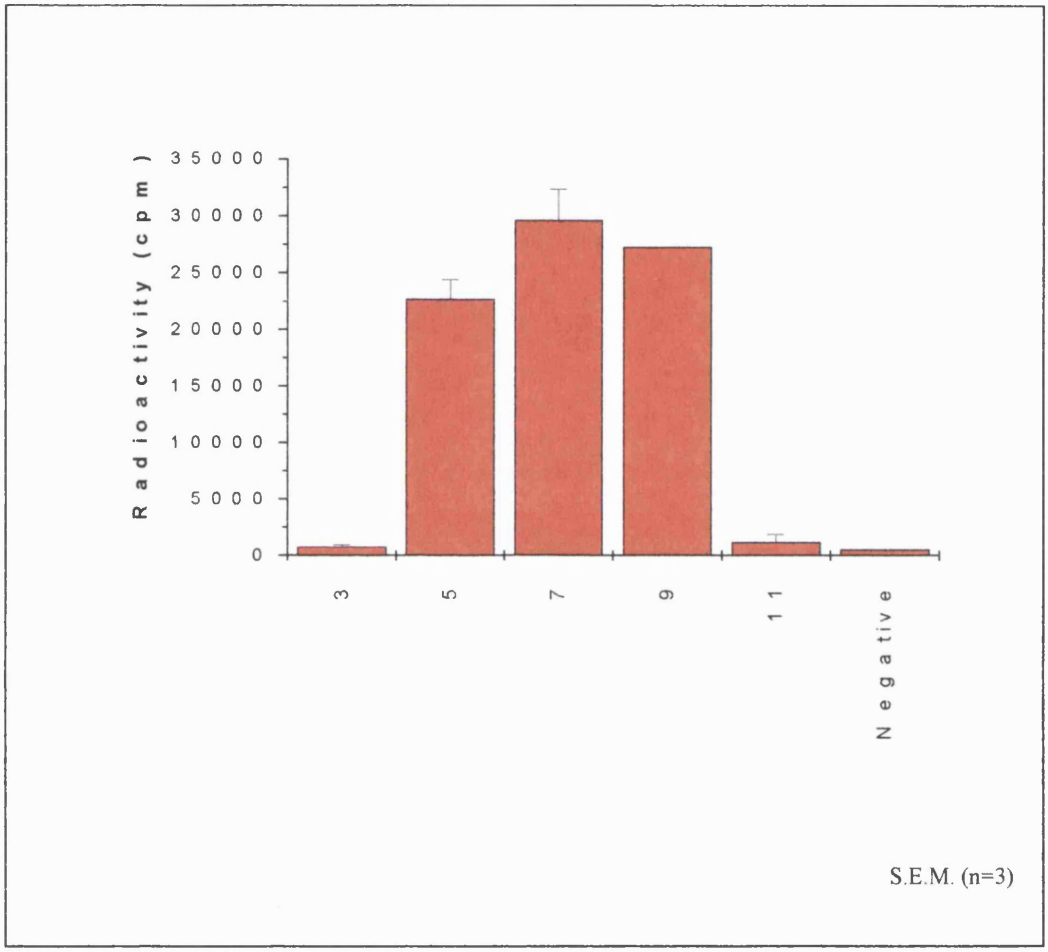


Figure Legend

The plot in Figure 4.6 shows the results from the GPI-PLD activity assay, using both Briefly, the FBS samples were incubated at different pHs for 1 hour, before being returned to pH 7.4. Prior to inclusion in the GPI-PLD activity assay, the FBS samples were diluted 1 in 10 with EMEM. Samples were mixed, in a 1:1 ratio, with the reaction mix, containing 30 mM mfVSG in total. The samples were incubated at 37 °C, and the reaction was terminated after 30 minutes incubation. The data is expressed as counts per minute (cpm), after a one-minute detection programme in the scintillation counter.

The plot in Figure 4.6 shows a bell-shaped curve of GPI-PLD activity detected in different FBS samples. Essentially, those samples incubated at either pH 5 or 9 showed similar GPI-PLD activity to those samples maintained at pH 7. By comparison, when the FBS samples were incubated at pH 3 or 11, the GPI-PLD activity detected in these samples was significantly reduced, and was almost equal to the results gained with the negative controls

4.9.1 GPI-PLD activity of RBL-2H3 cells cultured with pH 11-treated FBS

Figure 4.7 shows the results from the GPI-PLD activity assay from RBL-2H3 cells, after culture in pH 11 treated FBS-supplemented EMEM. Prior to extraction of the membrane fraction, the RBL-2H3 cell monolayer was washed thoroughly, with 1 x PBS. The cells were incubated with solubilising buffer, containing Nonidet P-40 detergent, and the membrane was separated from the other cellular components by centrifugation. Following centrifugation, the supernatant was removed, and the GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Membrane samples extracted from approximately 2.8 million cells per aliquot, were diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 30 minutes at 37°C. After termination of the activity assay, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

The plot in Figure 4.7 shows that, after culture with pH 11-treated FBS medium, the response in the GPI-PLD activity was significantly reduced. The radioactivity that was detected from the [3H]-dimyristyl phosphatidic acid, the product of the PLD-mediated hydrolysis of mfVSG, was approximately 1700 cpm in those cells cultured in normal FBS medium. By comparison, the activity detected in cells cultured with pH 11-treated FBS medium was 300 cpm, whereas the EMEM negative control was 200 cpm. The units of GPI-PLD activity, calculated according to the equation in Section 3.21, equated to 1.03 units for the 'active' cells, and 0.09 units for the 'inactive' cells. However, it is important to note that the 'inactive' cells appeared to contain a residual GPI-PLD activity, as the units of GPI-PLD activity were not zero.

4.9.2 IgE-mediated degranulation of RBL-2H3 cells, cultured with pH 11-treated FBS

Figure 4.8 shows the results from the IgE-mediated stimulation of 200,000 RBL-2H3 cells, maintained in pH-11 treated FBS-supplemented EMEM. The RBL-2H3 cells, maintained in both normal and treated-FBS containing medium, were plated onto a 24 well plate at a density of 200,000 cells per well, in a 1 ml volume. After overnight incubation, the cells were washed thoroughly with 1 x PBS, and incubated with 300 µl anti-DNP IgE (3µg/ml) diluted in EMEM, and incubated for 2 hours. Subsequently, the cells were incubated with 200 µl of DNP-albumin (1 µg/ml), in HEPES buffered saline, and incubated for two hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

As shown in Figure 4.8, after two weeks of culture with pH 11-treated FBS, the RBL-2H3 cells had lost the capacity to respond to IgE-mediated stimulation. In normal culture medium, the

FIGURE 4.7

LOSS OF GPI-PLD ACTIVITY FROM RBL-2H3 CELLS CULTURED IN pH 11-TREATED FBS

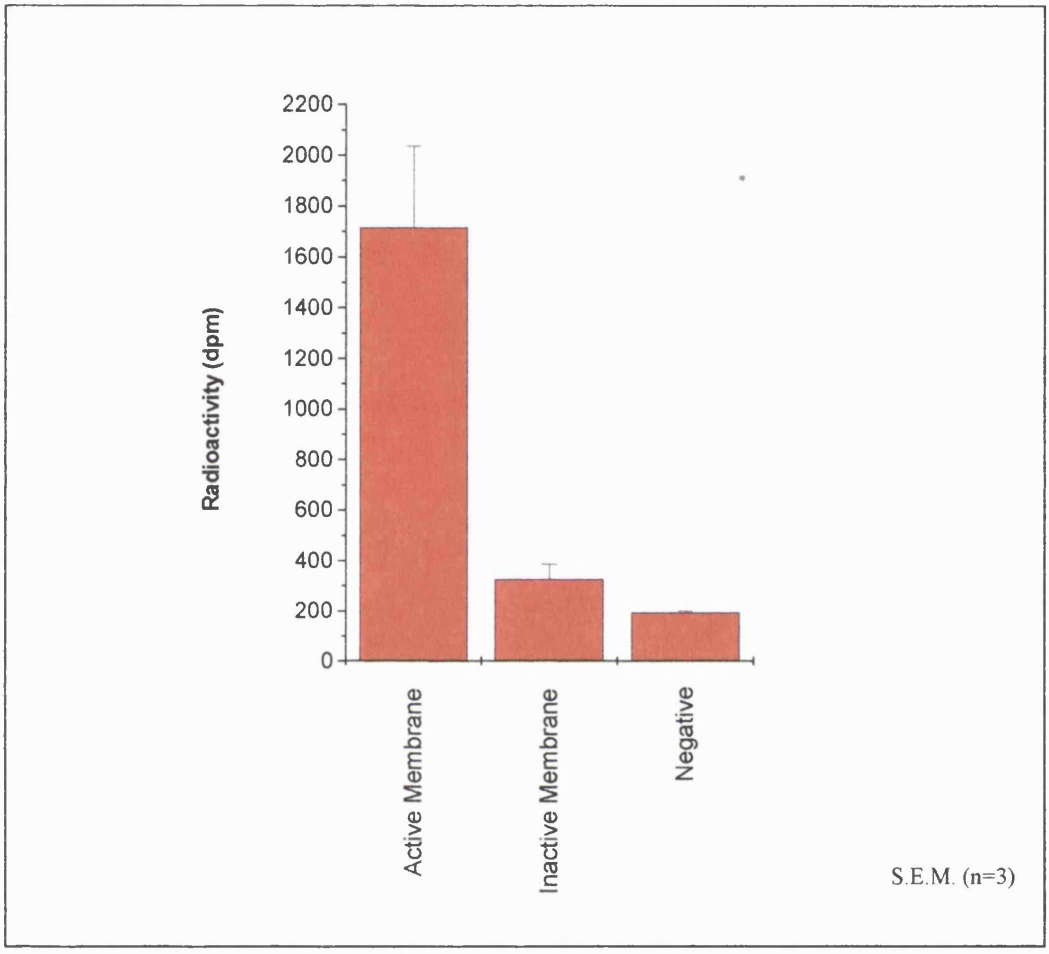


Figure Legend

Figure 4.7 shows the results from the GPI-PLD activity assay from RBL-2H3 cells, maintained in pH 11-FBS containing culture medium, in comparison to those cells cultured in normal media. The membrane fraction was prepared from the washed RBL-2H3 cells using with solubilising buffer, and by centrifugation. The GPI-PLD-containing supernatant was removed, and was employed in the GPI-PLD activity assay, with 30 mM mfVSG substrate. Membrane samples containing the membrane derived from approximately 2.8 million cells per aliquot, was diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 30 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

Data shows that, after culture in pH 11-FBS medium, the response in the GPI-PLD activity was significantly reduced. However, a minimal amount of GPI-PLD activity was detected in these cells, in comparison with the negative control.

FIGURE 4.8

IgE MEDIATED CROSS-LINKING OF RBL-2H3 CELLS AFTER INCUBATION WITH AND WITHOUT pH 11 INACTIVATED FOETAL BOVINE SERUM

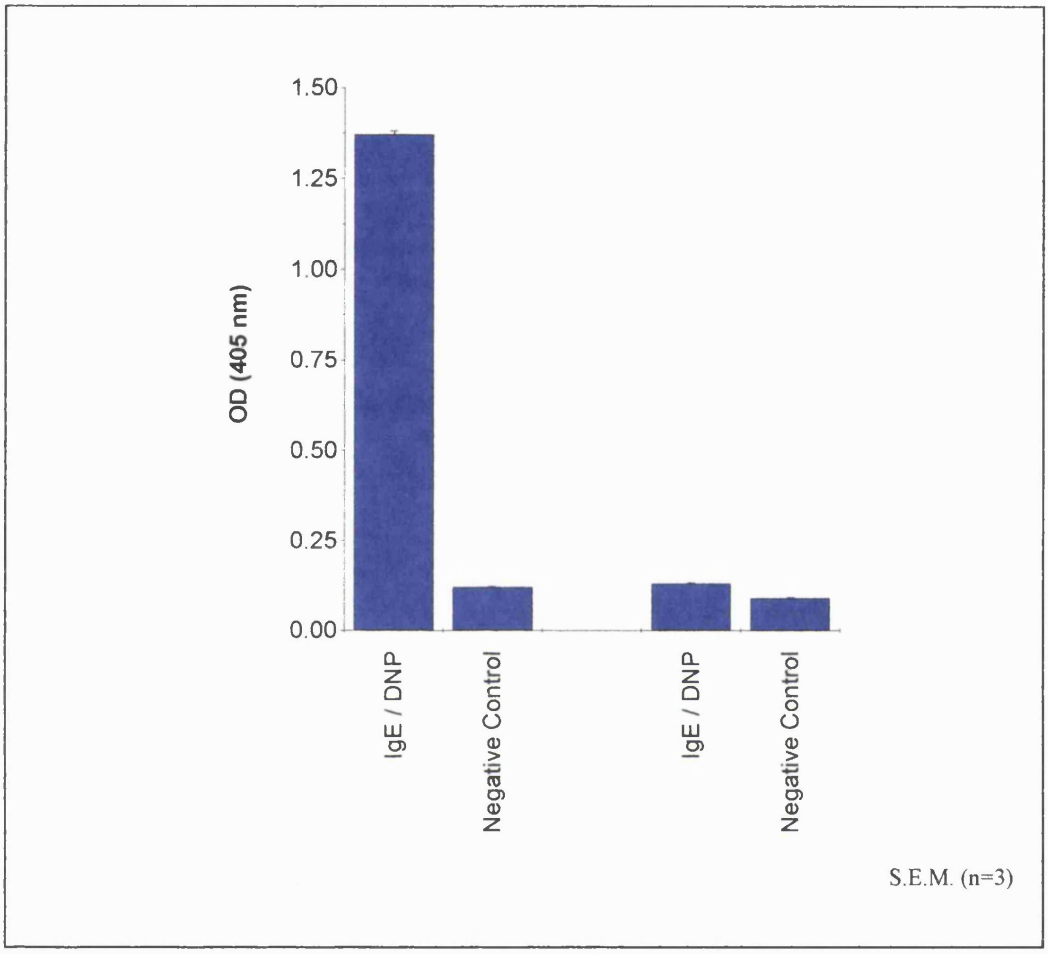


Figure Legend

Figure 4.8 shows the results from the IgE-mediated stimulation of 200,000 RBL-2H3 cells, maintained in either normal, or pH 11-treated FBS-containing medium. The pH 11-treated FBS was employed in the GPI-PLD activity assay, the results of which are discussed in Figure 4.5. This result confirmed that the pH 11-treated FBS had lost the ability to hydrolyse the mfVSG substrate. The culture medium was prepared normal, with FBS replaced by pH 11-treated FBS. RBL-2H3 cells were split at a density of 1 million cells per flask, in 20 mls of fresh culture medium, and were split every two days.

After 14 days of culture, the cells were subjected to IgE-mediated stimulation, using 200,000 cells on a 24 well plate. The cells were stimulated with anti-DNP IgE (3μg/ml), and incubated for 2 hours, followed by DNP-albumin (1 μg/ml), and incubation for two hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay. The results show that, after two weeks of culture in pH 11-treated FBS, in which minimal GPI-PLD activity was detected, RBL-2H3 cells had lost the capacity to respond to IgE-mediated stimulation.

optical density reading was 1.35. By comparison, after culture with pH 11-treated FBS, the OD reading was approximately 0.125. The negative control has been included on the graph, to illustrate that the IgE-mediated degranulation result from those cells culture with pH 11-treated FBS was roughly equal to the result from un-stimulated cells.

4.9.2.1 Increased liability of mediators release from RBL-2H3 cells without stimulation

With increasing time of incubation in the pH 11-treated FBS containing medium, however, it became apparent that the RBL-2H3 cells were releasing mediators without stimulation. An example of this is shown in Figure 4.9. This Figure shows the results from the IgE-mediated stimulation of 200,000 RBL-2H3 cells, maintained in alkaline FBS containing culture medium for 3 weeks.

The RBL-2H3 cells, maintained in both normal and treated-FBS containing medium, were plated onto a 24 well plate, at a density of 200,000 cells per well, in a 1 ml volume. After overnight incubation, the cells were washed thoroughly with 1 x PBS, and incubated with 300 µl anti-DNP IgE (3µg/ml) diluted in EMEM, and incubated for 2 hours. Subsequently, the cells were incubated with 200 µl of DNP-albumin (1 µg/ml), in HEPES buffered saline, and incubated for two hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

The OD reading obtained with the normal RBL-2H3 cells is smaller than anticipated. Compared with Figure 4.8, for example, the values are typically greater than 1. However, Figure 4.9 illustrates that, after three weeks of culture with pH 11-treated FBS, the OD values gained with the negative controls (unstimulated cells) were higher than anticipated. In comparison with the negative controls, degranulation of the RBL-2H3 cells in response to IgE-mediated stimulation was almost negligible after culture with pH 11-treated FBS. However, it appeared that the cells were becoming 'leaky' and releasing mediators without stimulation.

4.9.2.2 Time course of alterations in RBL-2H3 behaviour after culture in pH-11 treated-FBS

Following the observation that, after culture with pH 11-treated FBS the RBL-2H3 cells lost both the capacity to respond in the GPI-PLD activity assay, and the ability to degranulate in response to IgE-mediated stimulation, it seemed important to study the loss of these characteristics through time.

FIGURE 4.9

IgE MEDIATED CROSS-LINKING OF RBL-2H3 CELLS AFTER INCUBATION WITH AND WITHOUT pH 11 INACTIVATED FOETAL BOVINE SERUM

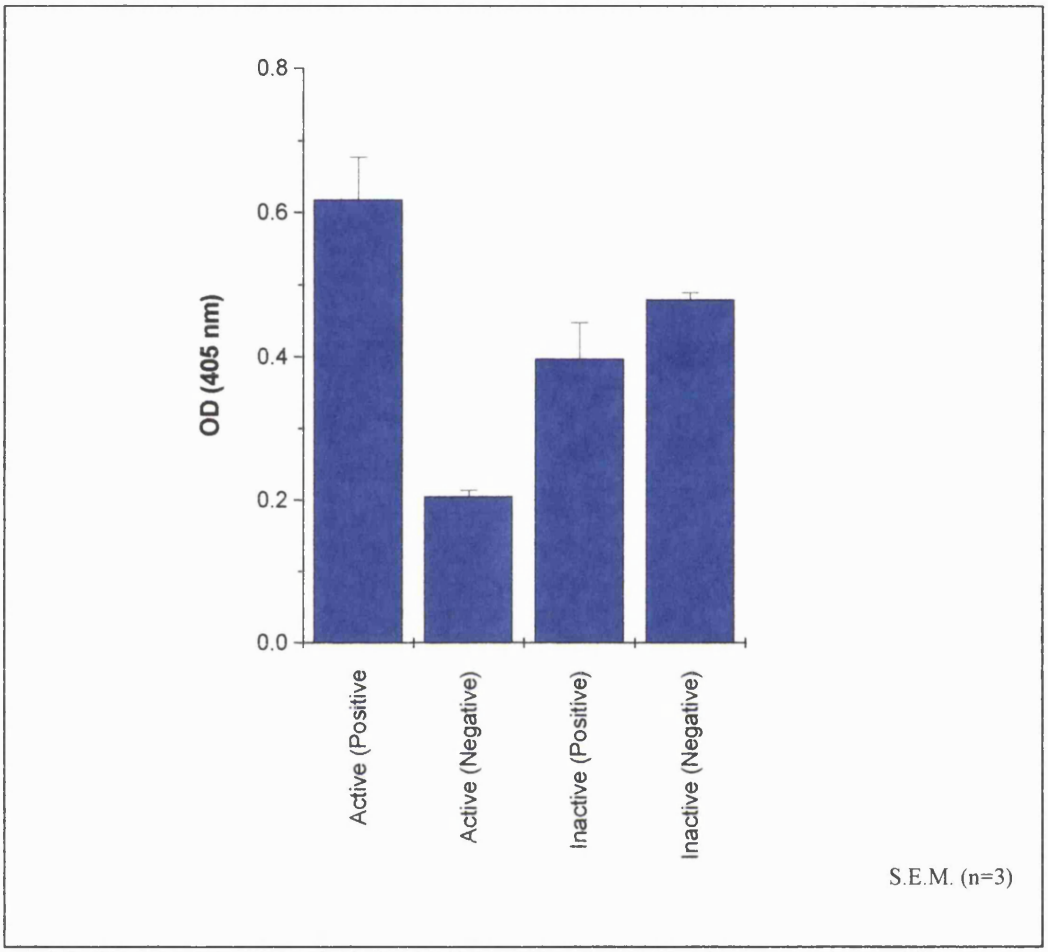


Figure Legend
Figure 4.9 shows the results from the IgE-mediated stimulation of 200,000 RBL-2H3 cells, maintained in pH 11 FBS containing culture medium for 3 weeks.

The cells were stimulated by the addition of anti-DNP IgE (3μg/ml), and incubation for 2 hours, followed by addition of DNP-albumin (1 μg/ml) and incubation for two hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

Data shows that, after three weeks of culture with pH 11-FBS, the OD values gained with the negative controls (unstimulated cells) were higher than anticipated. It appeared that the cells were becoming 'leaky' and releasing mediators without stimulation. However, the specific response of the RBL-2H3 cells to IgE-mediated stimulation, after subtraction of the negative controls was almost negligible after culture with pH 11-FBS.

Figures 4.10 and 4.11 show the changes in the behaviour of the RBL-2H3 cells, after culture in the medium containing the pH-11 treated FBS. Prior to cell splitting the adherent monolayer was washed thoroughly with sterile 1 x PBS, and the adherent cells were removed from the culture flask using a cell scraper. The density of cells was determined, using a Neubauer hamocytometer. The cells were split every day, at a density of 2 million cells per ml in fresh cultured medium. Aliquots of cells, equivalent to 1 million cells each, were removed daily. Using these cells the activity of the GPI-PLD in the sample was determined using the radioactive GPI-PLD activity assay. In addition, the cells were stimulated to undergo IgE-mediated degranulation, and any change in responsiveness was noted.

4.9.2.2.1 Time course of the loss of GPI-PLD activity in RBL-2H3 cells

The plot in figure 4.10 shows the change of GPI-PLD in extracted RBL-2H3 membranes, after culture with the altered FBS. Prior to extraction of the membrane fraction, the RBL-2H3 cell monolayer was washed thoroughly, with 1 x PBS. The GPI-PLD containing membrane fraction was extracted, by incubation with a detergent-containing solubilising buffer, and the membrane was separated from the other cellular components by centrifugation. The GPI-PLD activity contained within the supernatant was ascertained in the GPI-PLD activity assay, with 30 mM mfVSG substrate.

As shown in figure 4.10, the loss of GPI-PLD activity occurred rapidly, following the transferral of the RBL-2H3 cells into the alkaline-treated FBS-containing medium. The radioactivity that was detected in the samples of normal RBL-2H3 cells, with the equivalent of 1 million cells per aliquot, was approximately 4500 decays per minute. According to the calculation (Section 3.21), this equated to 3.1 units of GPI-PLD activity. By comparison, after as little as 2 days of cultured with the alkaline-treated FBS, the radioactivity count had fallen to 1500 decays per minute, which was equivalent to 1.1 units of activity. With continuing culture, the GPI-PLD activity remained at approximately 1000 dpm and, after 8 days of culture, 0.71 units of GPI-PLD were detected. The fact that the units of activity did not fall to zero in this time, however, suggested that the cells retained a fraction of GPI-PLD activity, which was not affected by the altered culture conditions.

4.9.2.2.2 Time course of the loss of IgE-degranulation in RBL-2H3 cells

The plot in figure 4.11 shows the change in responsiveness of the RBL-2H3 cells to IgE-mediated stimulation, following their transferral into the alkaline-treated FBS-containing medium. Briefly, aliquots of RBL-2H3 cells were plated onto a 24 well plate, at a density of 200,000 cells per well. After overnight incubation, the cells were washed thoroughly with 1 x PBS, and incubated with 300 µl anti-DNP IgE (3µg/ml) diluted in EMEM, and incubated for 2

FIGURE 4.10

LOSS OF GPI-PLD ACTIVITY IN RBL-2H3 MEMBRANE PREPARATIONS, THROUGH TIME, AFTER INCUBATION WITH AND WITHOUT pH 11 TREATED FOETAL BOVINE SERUM

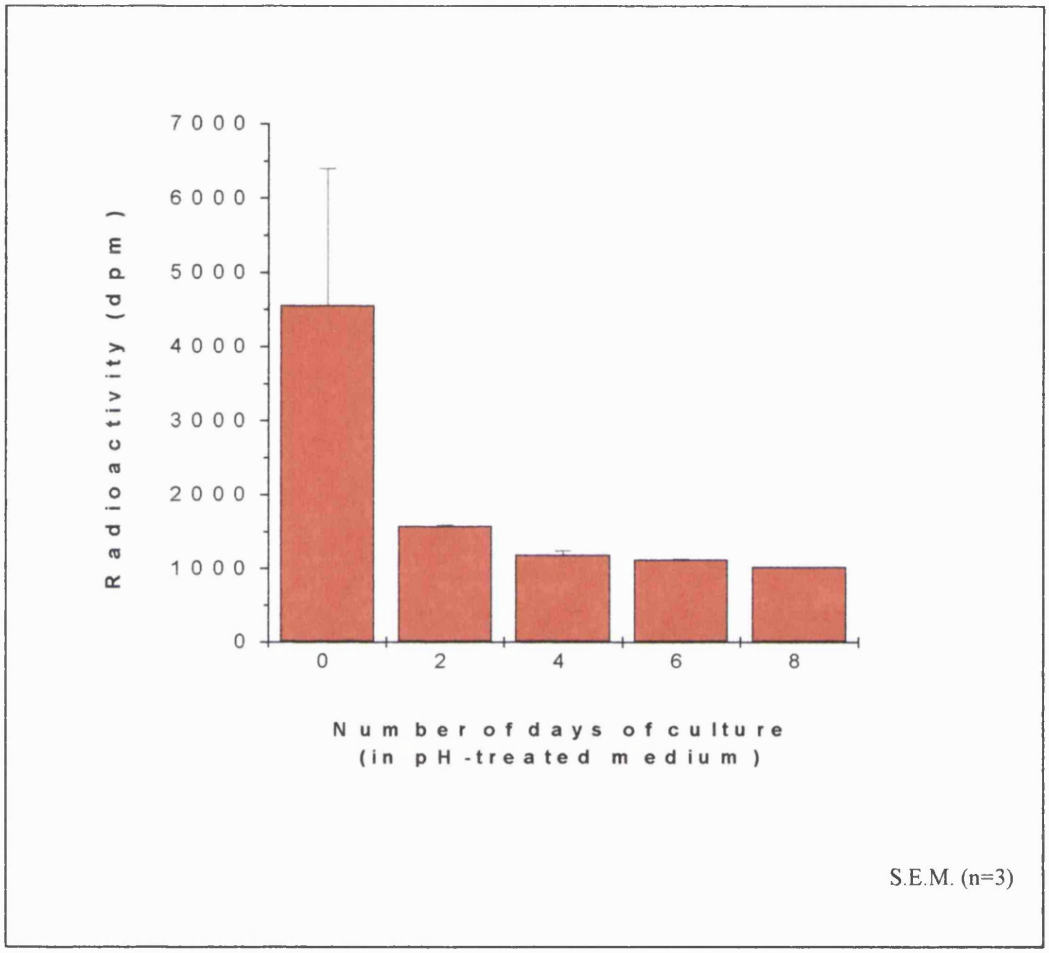


Figure Legend

The plot in Figure 4.10 shows the loss of GPI-PLD activity from RBL-2H3 cells, through time, after transfer of the cells into pH 11-treated FBS containing EMEM. The membrane fraction was extracted from the cells, by incubation with solubilising buffer. The GPI-PLD activity in the sample was ascertained, using 30 mM of mfVSG substrate. Each aliquot of RBL-2H3 cells contained approximately 2.8 million cells per aliquot. Each aliquot was diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 30 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

The loss of GPI-PLD activity occurred rapidly after the RBL-2H3 cells were transferred into the pH 11-treated FBS-containing medium. The radioactivity that was detected in the membrane samples of normal RBL-2H3 cells was equal to 4500 cpm to approximately 1000 cpm after as little as two days culture. The fact that the units of activity did not fall to zero in this time, however, suggested that the cells retained a fraction of GPI-PLD activity, which was not affected by the altered culture conditions.

FIGURE 4.11

LOSS OF RBL-2H3 REACTIVITY TO IgE-MEDIATED STIMULATION, THROUGH TIME, AFTER INCUBATION WITH AND WITHOUT pH 11 TREATED FOETAL BOVINE SERUM

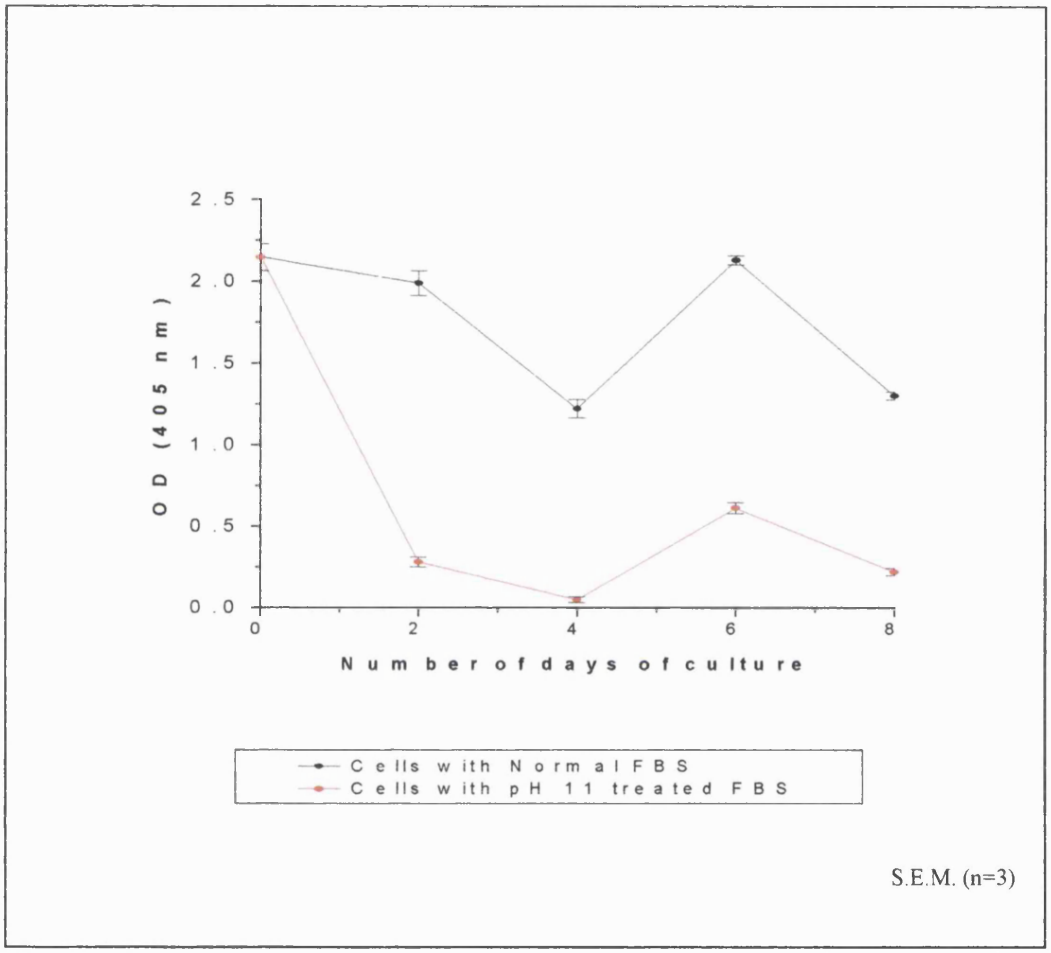


Figure Legend

The plot in figure 4.11 shows the change in responsiveness of the RBL-2H3 cells to IgE-mediated stimulation, following their transferral into the pH 11-treated FBS-containing medium. Briefly, aliquots of RBL-2H3 cells were plated onto a 24 well plate, at a density of 200,000 cells per well. After overnight incubation, the cells were washed thoroughly with 1 x PBS, and incubated with 300 µl anti-DNP IgE (3µg/ml) diluted in EMEM, and incubated for 2 hours. Subsequently, the cells were incubated with 200 µl of DNP-albumin (1 µg/ml), in HEPES buffered saline, and incubated for two hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

Although a wide variation was observed between the OD readings gained from different IgE-mediated stimulation experiments, the overall pattern of results was the cells maintained in pH 11 treated FBS rapidly lost the ability to respond to IgE mediated stimulation, falling from OD 2.2 to OD 0.2 in two days. Those cells maintained in normal culture medium retained the ability to undergo IgE-mediated degranulation.

hours. Subsequently, the cells were incubated with 200 µl of DNP-albumin (1 µg/ml), in HEPES buffered saline, and incubated for two hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

Although a wide variation was observed between the OD readings gained from different IgE-mediated stimulation experiments, the overall pattern of results was the OD reading, representing the detection of the β-glucosaminidase mediator, fell in the cells that were cultured in medium in which the GPI-PLD activity detected was significantly reduced. After two days of culture in the altered medium, the OD reading fell to between 0.2 and 0.6. By comparison, the OD readings observed from the cells cultured with normal FBS varied between approximately 1.2 and 2.2.

4.9.2.3 RT-PCR analysis of the cells cultured in pH-11 treated FBS

The results in Figure 4.12 show the amplified products from the RT-PCR reaction with mouse GPI-PLD specific primers, and RNA extracted from RBL-2H3 cells cultured with pH 11-treated FBS. The RNA was extracted from a RBL-2H3 cell monolayer, and treated with DNAse, as discussed in Section 2.12.4. Following reverse transcription, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 62.9 °C).

The products were separated on a 1.5% agarose gel, in comparison with a 100 bp DNA ladder (Lane 1). Lanes 2 to 5 contain the cDNA made from the RBL-2H3 cells. Lanes 2 and 3 contain RNA, without DNAse treatment, with (2) and without (3) RT. Lanes 4 and 5 contain DNase treated mRNA with (4) and without (5) RT. Lane 6 contains the rat liver positive control, and lane 7 contains the negative control.

Two bands were visualised in the lane containing rat liver, corresponding to approximately 740 and approximately 900 base pairs. The resolution of the markers on this electrophoresis gel was not particularly clear, and it was difficult to determine the size of the RT-PCR product more accurately.

The confirmation of a correctly sized RT-PCR product from the liver positive control established that the RT-PCR was successfully performed. Once again, the lack of positive RT-PCR results with any of the mRNA samples extracted from RBL-2H3 cells confirms that the mRNA detected by these primers was not contained in the RBL-2H3 cell line. The results from this experiment, therefore, found no evidence for the production of GPI-PLD specific mRNA,

FIGURE 4.12

RT-PCR AMPLIFIED cDNA USING MOUSE GPI-PLD SPECIFIC PRIMERS
WITH 'INACTIVE' RBL-2H3 CELLS
(MP1 and MP2)

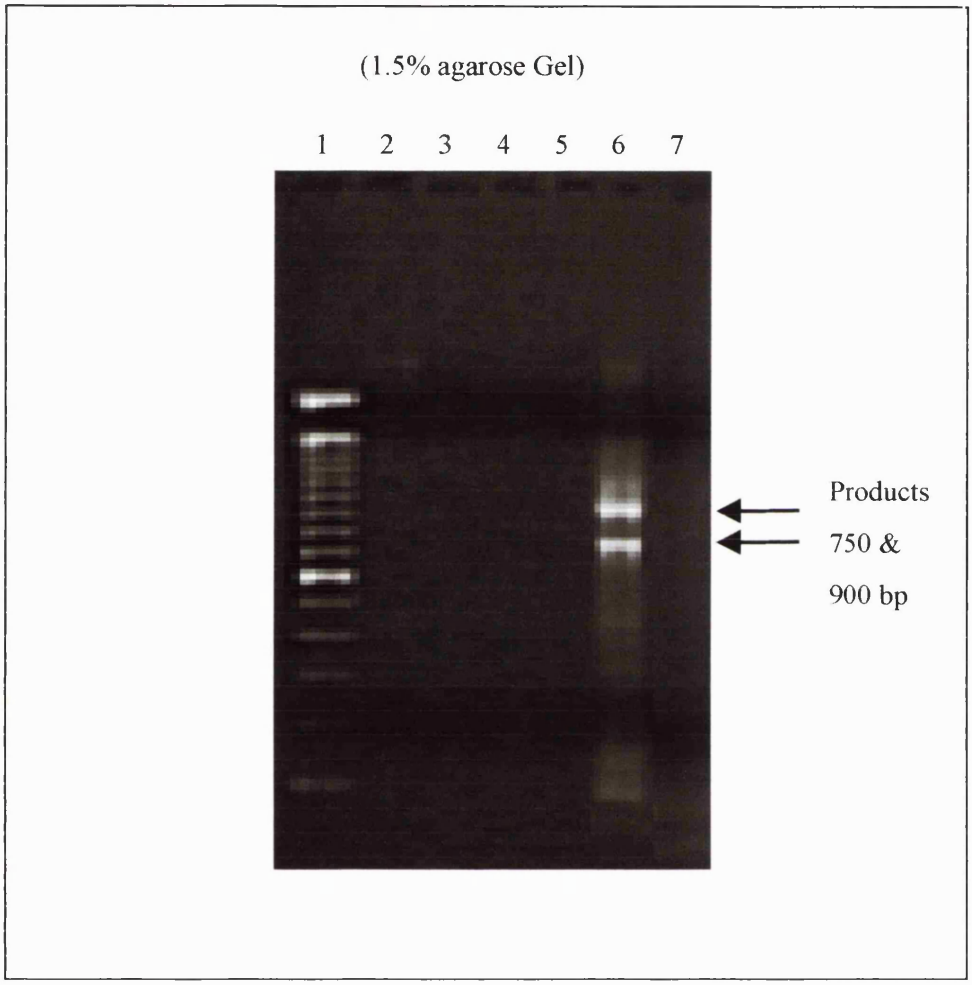


Figure Legend
The results in Figure 4.12 show the amplified products from the RT-PCR reaction using mouse GPI-PLD specific primers, and RNA extracted from RBL-2H3 cells cultured in pH 11-treated FBS. The mRNA was extracted from a RBL-2H3 cell monolayer, and treated with DNAse. Following reverse transcription, the RBL-2H3 samples were amplified by the polymerase chain reaction (30 cycles, with an annealing temperature of 62.9°C). The products were separated on a 1.5% agarose gel, in comparison with a 100 bp DNA ladder (Lane 1). Lanes 2 to 5 contain the RT-PCR products from RBL-2H3 cells. Lanes 2 and 3 contain mRNA, without DNAse treatment, with (2) and without (3) RT. Lanes 4 and 5 contain DNAse treated mRNA with (4) and without (5) RT. Lane 6 contains the rat liver positive control, and lane 7 contains the negative control.

The only PCR products that were visualised were in the lane containing the rat liver positive control. These products were approximately 620 and 700 bp in size. No evidence of PCR product was found in any of the sample derived from RBL-2H3 cells

which established that the cells did not up-regulate the production of GPI-PLD mRNA in the altered culture conditions.

4.10 Ionophore-mediated stimulation of RBL-2H3 cells cultured in pH-11 treated FBS

In addition to the experiments to determine the changes in the responsiveness of the RBL-2H3 cell to IgE-mediated stimulation, it was also considered important to determine the effect on the ionophore-mediated stimulation of the RBL-2H3 cells. Once again, after transfer into pH 11-treated FBS media, the cells were split every two days, at a density of 2 million cells per flask, in 20 mls of fresh medium. The cells were cultured for a total of seven days. Following this culture, both normal and pH 11-treated FBS cells were plated out onto a 24 well plate, at a density of 200,000 cells per well, in a 1 ml volume, and cultured overnight.

The cells were stimulated to degranulate in response to both IgE and ionophore mediated stimulation. In addition, lysis of the cells was effected by the addition of a 5% Triton X-100 solution. The results from this experiment are shown in Figure 4.13. The β-glucosaminidase assay was incubated for 30 minutes, and the results are expressed as an optical density reading, at 405 nm wavelength.

The results in Figure 4.13 are expressed as a percentage of the total cell mediator content, from the Triton result. The negative control has been subtracted from the data, for ease of comparison. Data shows that, after 7 days of culture in the pH 11-treated FBS culture medium, the RBL-2H3 cells had lost the ability to respond to ionophore-mediated stimulation, in addition to the loss of IgE-reactivity. The cells cultured in normal EMEM media released approximately 43% and 58% of the total β-glucosaminidase content in response to ionophore- and IgE mediated stimulation, respectively. By comparison, the cells cultured with pH 11-treated FBS medium released approximately 16% and 13% of the total β-glucosaminidase content in response to ionophore- and IgE-mediated stimulation, respectively. This expected result appears to suggest that, although the IgE-reactivity of the RBL-2H3 cells is ablated by incubation with the pH-11 treated FBS, the reactivity to ionophore-mediated stimulation is also affected.

The plot in figure 4.14 show the results from the ionophore mediated stimulation of RBL-2H3 cells, through time, following their transferral into pH 11 treated FBS containing culture medium. These results show a striking similarity to the loss of IgE responsiveness of the RBL-2H3 cell in the altered culture medium.

FIGURE 4.13

INHIBITION OF STIMULATION BY IONOPHORE IN RBL-2H3 CELLS CULTURED IN GPI-PLD INACTIVE MEDIUM

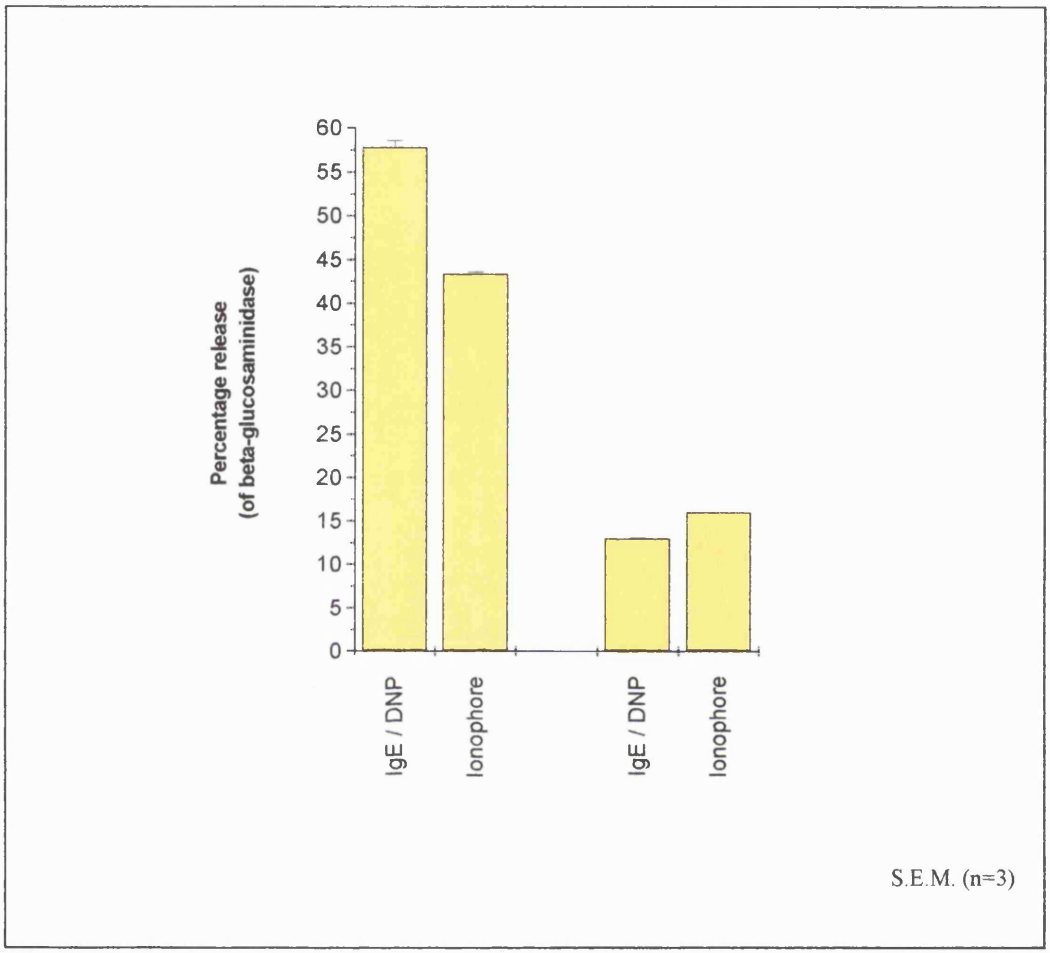


Figure Legend

The graph in Figure 4.13 shows the response of RBL-2H3 cells, cultured in both normal and pH 11-FBS medium, to IgE and ionophore-mediated stimulation. In addition, lysis of the cells was effected by the addition of a 5% Triton X-100 solution. As previously, after transfer into pH 11-FBS media, the cells were split every two days, at a density of 2 million cells per flask, in 20 mls of fresh medium. The cells were cultured for a total of seven days. Following this culture, both normal and pH 11-FBS cells were plated out onto a 24 well plate, at a density of 200,000 cells per well, in a 1 ml volume, and cultured overnight. The β-glucosaminidase assay was incubated for 30 minutes, and the results are expressed as an optical density reading, at 405 nm wavelength.

The results in Figure 4.13 are expressed as a percentage of the total cell mediator content. The negative control has been subtracted from the data, for ease of comparison. Data shows that, after 7 days of culture in the normal culture medium, the RBL-2H3 cells had lost the ability to respond to ionophore-mediated stimulation, in addition to the loss of IgE-reactivity.

FIGURE 4.14

LOSS OF RBL-2H3 REACTIVITY TO IONOPHORE STIMULATION, THROUGH TIME, AFTER INCUBATION WITH AND WITHOUT pH 11 TREATED FOETAL BOVINE SERUM

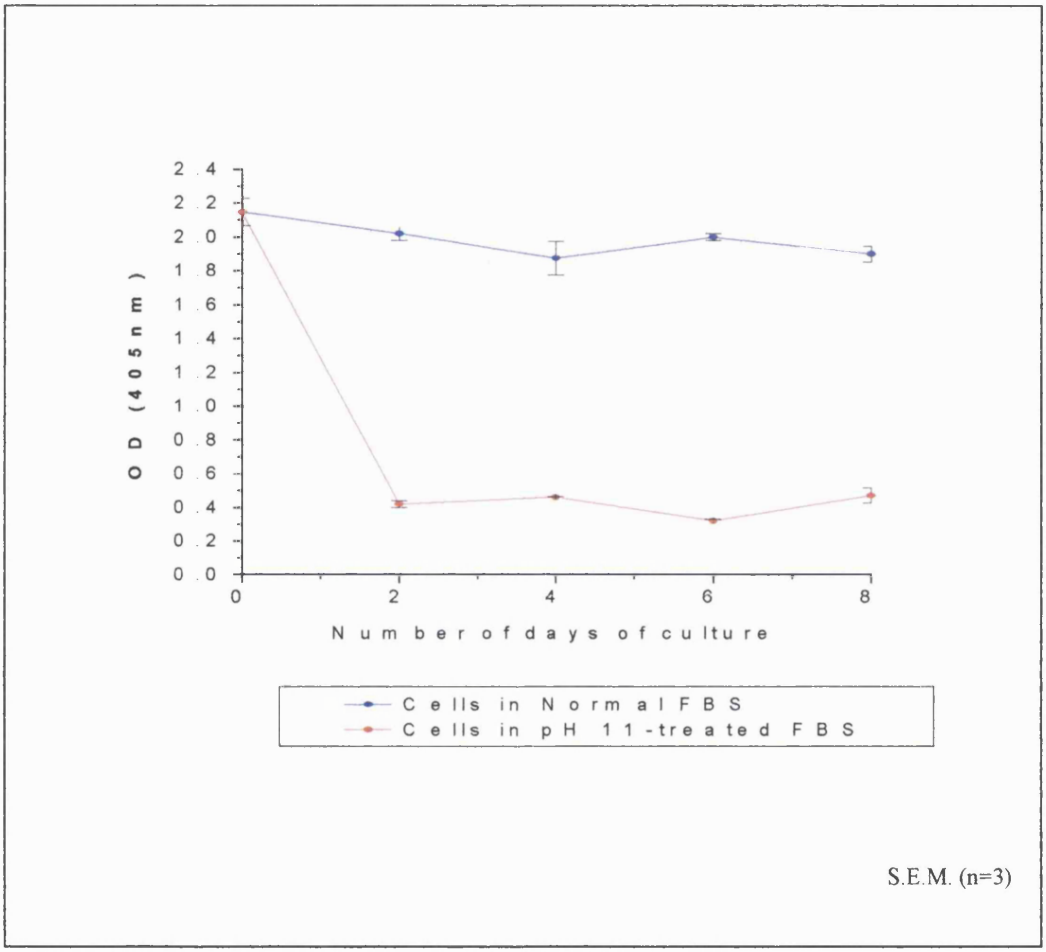


Figure Legend

The plot in figure 4.14 shows the change in responsiveness of the RBL-2H3 cells to Ionophore mediated stimulation, following their transferral into the pH 11-treated FBS-containing medium. Briefly, aliquots of RBL-2H3 cells were plated onto a 24 well plate, at a density of 200,000 cells per well. After overnight incubation, the cells were washed thoroughly with 1 x PBS, and incubated with 500 μM ionophore A23187, diluted in HEPES AGM. The cells were incubated for 2 hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

As with the IgE mediated stimulation the overall pattern of results was the cells maintained in pH 11 treated FBS rapidly lost the ability to respond to ionophore mediated stimulation, falling from OD 2.2 to OD 0.4 in two days. Those cells maintained in normal culture medium retained the ability to undergo ionophore-mediated degranulation.

Briefly, aliquots of RBL-2H3 cells were plated onto a 24 well plate, at a density of 200,000 cells per well. After overnight incubation, the cells were washed thoroughly with 1 x PBS, and incubated with 500 μM ionophore A23187, diluted in HEPES AGM. The cells were incubated for 2 hours. The results are expressed as an OD reading at 405 nm wavelength and represent the OD of the product from the reaction catalysed by β-glucosaminidase, after 30 minutes of incubation in the detection assay.

In comparison with the IgE mediated stimulation results, less variation was observed between the OD readings gained from different ionophore stimulation experiments. As with the IgE mediated stimulation, however, the overall pattern of results was the cells maintained in pH 11 treated FBS rapidly lost the ability to respond to ionophore mediated stimulation, falling from OD 2.2 to OD 0.4 in two days. Those cells maintained in normal culture medium retained the ability to undergo ionophore-mediated degranulation.

4.11 Addition of GPI-PLD activity to RBL-2H3 cells cultured with pH-11 treated FBS

4.11.1 Addition of normal FBS to RBL-2H3 cells cultured with pH-11 treated FBS

To determine whether the RBL-2H3 cells were irreversibly altered by their culture with pH 11 treated FBS, an aliquot of these RBL-2H3 cells were transferred into culture medium containing normal untreated FBS. The cells cultured in normal and pH-11 treated FBS were split every two days, at a density of 2 million cells per flask, in 20 mls of fresh medium. The cells were cultured for a total of seven days, at 37°C in an humidified atmosphere of 95% air and 5% carbon dioxide. Following this culture, both normal, pH 11-treated FBS and reconstituted RBL-2H3 cells were plated out onto a 24 well plate, at a density of 200,000 cells per well, in a 1 ml volume, and cultured overnight.

The cells were stimulated to degranulate in response to three stimuli. Firstly, degranulation was effected by IgE-mediated stimulation. Briefly, following the overnight incubation of the 200,000 cells, the cell monolayer was washed thoroughly with 1 x PBS, and incubated with 300 μl anti-DNP IgE (3μg/ml) diluted in EMEM, and incubated for 2 hours. Subsequently, the cells were incubated with 200 μl of DNP-albumin (1 μg/ml), in HEPES buffered saline, and incubated for two hours. Triton lysis of the cells was effected by the addition of a 5% Triton X-100 solution. Ionophore mediated stimulation was effected by the addition of 500 μM of Ionophore A23187 in HEPES buffered saline. All stimuli were incubated with the cells 2 hours, alongside the IgE-mediated stimulation. The results from this experiment are shown in

Figure 4.15. The β-glucosaminidase assay was incubated for 30 minutes, and the results are expressed as an optical density reading, at 405 nm wavelength.

As a result of the variation in the results obtained after Triton X-100 lysis of the different cell monolayers, it was considered most appropriate to express the results as a percentage of the total cell mediator content. That is, the OD derived from the IgE-mediated stimulation was divided by the OD derived from the Triton lysis of the cells. The negative control has been subtracted from the data, for ease of comparison.

The data in Figure 4.15 shows that, after 7 days of culture in the normal culture medium, the RBL-2H3 cells showed the ability to respond to both IgE- and ionophore-mediated stimulation. Data from the IgE-mediated degranulation of both normal cells and reconstituted cells showed that the percentage of mediators released was approximately 20%, in comparison with 10% release from inactive cells. The ionophore mediated stimulation also appears to have been partially reconstituted by the transfer of the 'inactive' cells into normal medium, with the percentage value rising from 3% to 7.5% for the 'inactive' and reconstituted cells respectively.

4.11.2 Addition of purified GPI-PLD to RBL-2H3 cells cultured with pH-11 treated FBS

4.11.2.1 Creation of recombinant GPI-PLD protein

The results from the analysis of the recombinant GPI-PLD are shown in Figures 4.16, 4.17 and 4.18. Typically, all detectable GPI-PLD activity was eluted in the first fraction, at a concentration of 50 mM of imidazole buffer (50 mM imidazole in HEPES buffered saline, pH 6.0). The plot in Figure 4.15 shows the results from the GPI-PLD activity assay with recombinant GPI-PLD. R-GPI-PLD samples were diluted in imidazole buffer, in a 100 µl volume. In addition, 1,10-phenanthroline was included in the serial dilution of the putative r-GPI-PLD, to a final concentration of 500 µM, in order to determine whether this inhibitor would affect the activity of the enzyme. The 1,10-phenanthroline was incubated with the r-GPI-PLD for 60 minutes, prior to the activity assay. The samples were diluted 1:1 with the mfVSG-containing reaction mix, and incubated for 40 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected.

Figure 4.16 shows that, as the quantity of r-GPI-PLD increased, the GPI-PLD activity detected increased in a linear fashion. After 40 minutes incubation, the activity of GPI-PLD that was detected, after a one-minute counting programme in the scintillation counter 1.59 with 100 µg of recombinant protein.

FIGURE 4.15

ADD BACK OF NORMAL FOETAL BOVINE SERUM TO INACTIVE RBL-2H3 CELLS

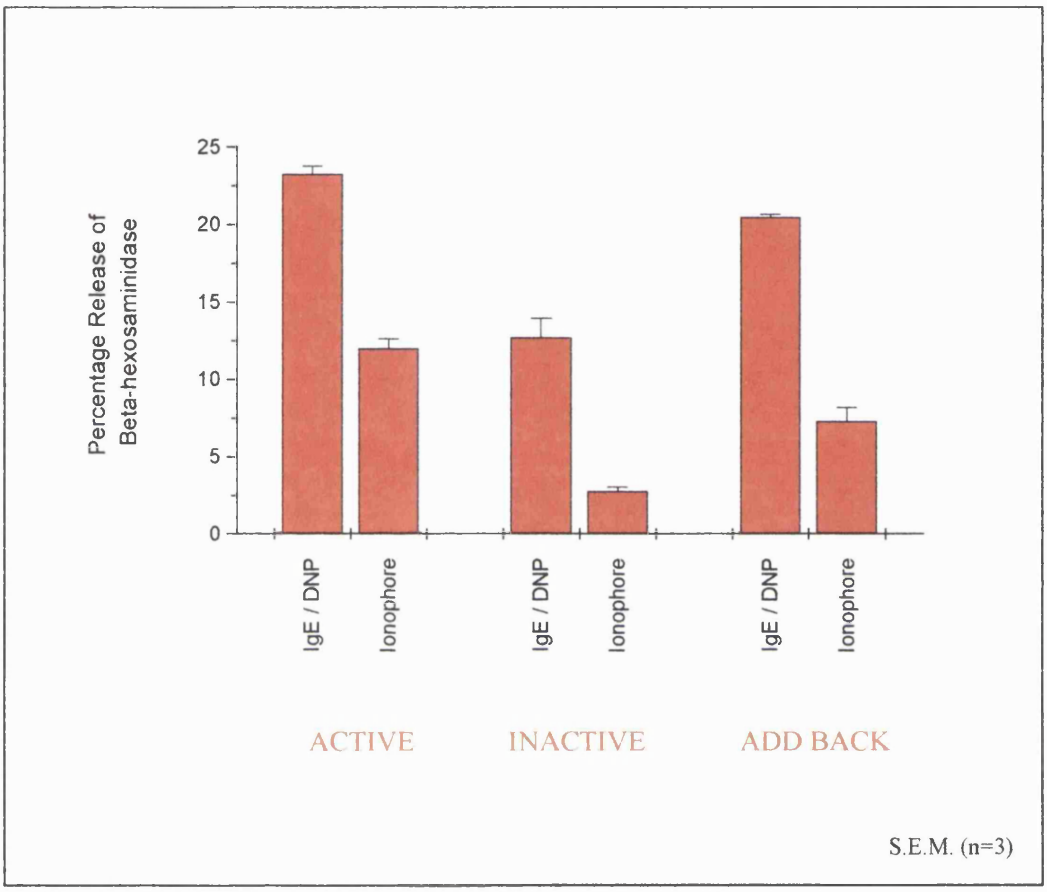


Figure Legend

The plot in figure 4.15 shows the results from the incubation of RBL-2H3 cells, cultured with pH-11 treated medium in normal, untreated culture medium. The cells were split, every two days, at a density of 2 million cells per flask, in 20 mls of fresh medium. The cells were cultured for a total of seven days. Following this culture, both normal, pH 11-FBS and reconstituted RBL-2H3 cells were plated out onto a 24 well plate, at a density of 200,000 cells per well, in a 1 ml volume, and cultured overnight. The cells were then stimulated to undergo both IgE-mediated and ionophore mediated degranulation. Cell lysis was promoted by the addition of a 5% Triton X-100 solution. All stimuli were incubated with the cells for 2 hours. The β-glucosaminidase assay was incubated for 30 minutes, and the results are expressed as an optical density reading, at 405 nm wavelength.

The release results are expressed as a percentage of the total cell content of β-glucosaminidase. The data from the negative control has been subtracted from the data, for ease of comparison. The data shows that, after 7 days of culture in the normal culture medium, the RBL-2H3 cells had partially regained the ability to respond to both IgE- and ionophore mediated stimulation.

FIGURE 4.16

GPI-PLD ACTIVITY ASSAY WITH RECOMBINANT GPI-PLD

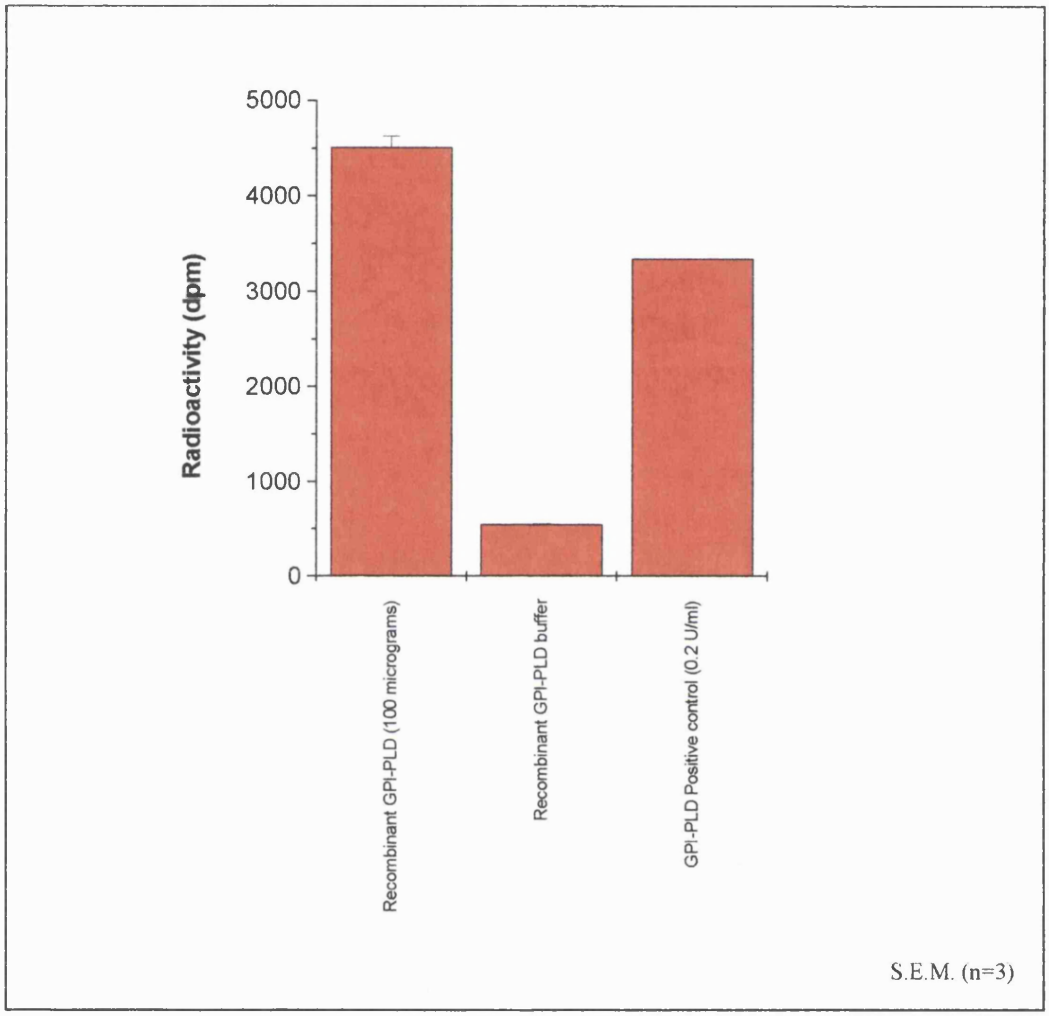


Figure Legend

The plot in Figure 4.16 shows the results of the GPI-PLD activity assay the recombinant GPI-PLD protein, following termination of the assay after 30 minutes incubation. This is in comparison with a negative control (Buffer 6), and a GPI-PLD positive control (0.2 U/ml)

The data is expressed as decays per minute (dpm), after a one-minute detection programme in the scintillation counter.

The inclusion of 500 μM 1,10-phenanthroline in the r-GPI-PLD activity assay effectively inhibited the activity of the enzyme. The activity detected with 100μg of recombinant protein, for example, was reduced from 4.11 Units without inhibition to 0.05 units with inhibition. The activity that was detected after incubation with 1,10-phenanthroline was, on average, less than 0.05 units, regardless of the concentration of the protein (data not shown)

The size of the recombinant GPI-PLD was determined by separation by denaturing SDS-PAGE, using a 5% acrylamide gel, after staining with Coomassie Blue. The results are shown in Figure 4.17. Lanes 3 and 4 contain the GPI-PLD purified from 2 different batches of CHO cells, in comparison with molecular weight markers (Lane 2), and the diluent buffer (Lane 1). Three protein bands were visualised in both lanes 3 and 4, of approximately 50, 70 and 75 kDa molecular weight. Not protein of 110 kDa was detected.

In addition, the samples were run out on a native SDS-PAGE, using a 5% acrylamide gel, and stained with Coomassie Blue. The results are shown in Figure 4.18. Once again, lanes 3 and 4 contain the GPI-PLD purified from 2 different batches of CHO cells, in comparison with molecular weight markers (Lane 2), and the diluent buffer (Lane 1). In this instance, two protein bands were detected in both lanes 3 and 4, of approximately 80 and 120 kDa molecular weight. This result is in comparison with the GPI-PLD protein synthesised after transfection into CHO cells, which was typically 115 kDa, whereas H5 cells produced a 98 kDa protein (Tujioka *et al.*, 1998). The significance of these different sized proteins will be discussed in greater detail in a Section 5.1.4.4.3.

4.11.2.2 Incubation of RBL-2H3 cells with recombinant GPI-PLD protein

4.11.2.2.1 GPI-PLD extraction from RBL-2H3 monolayers

The methodology for the extraction of GPI-PLD from RBL-2H3 cell pellets was discussed in Section 3.20.1. An example of the GPI-PLD activity detected in the membrane extracted from RBL-2H3 cell pellets is shown in Figure 3.4. The method, which involved centrifugation of a cell suspension, was shown to successfully extract GPI-PLD from RBL-2H3 cells. When starting material was RBL-2H3 cells in a 24 well plate, however, it was more practical to extract the membrane fraction directly from the plate wells.

Briefly, following the overnight culture of 200,000 cells per well, in a 24 well plate, the cell monolayer was washed thoroughly with 1 x PBS. The cells were detached from the plastic by either:

FIGURE 4.17

DENATURING SDS-PAGE OF RECOMBINANT GPI-PLD

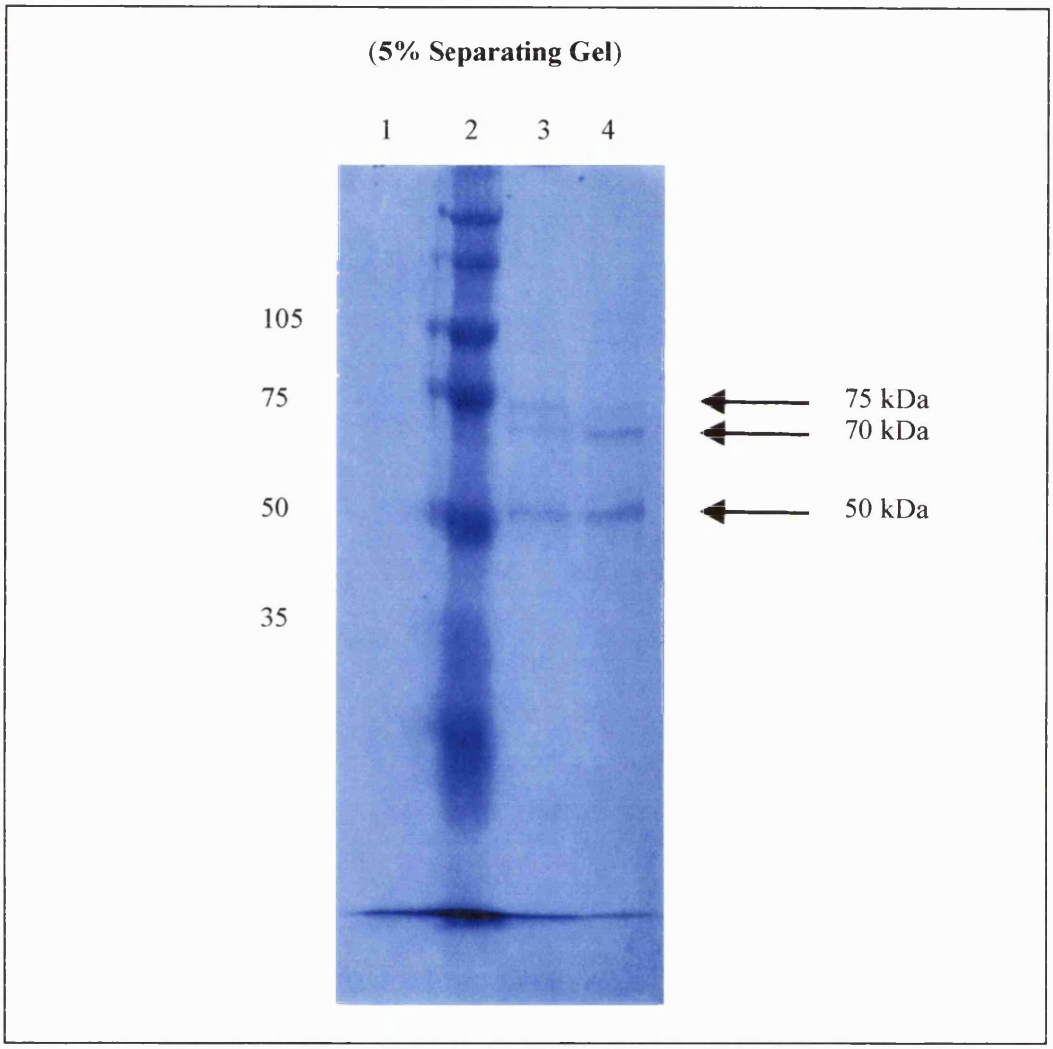


Figure Legend

Figure 4.17 shows the recombinant GPI-PLD protein after separation by a denaturing SDS-PAGE, on a 5% acrylamide gel. The proteins were visualised by Coomassie Blue stain. The protein was separated in comparison with molecular weight markers (Lane 2). Lane 1 contains the negative control, and lanes 3 and 4 contain the GPI-PLD purified from 2 different batches of CHO cells.

Three protein bands were visualised in lanes 3 and 4 by the Coomassie stain, of approximately 50, 55 and 75 kDa molecular weight.

FIGURE 4.18

NATIVE SDS-PAGE OF RECOMBINANT GPI-PLD

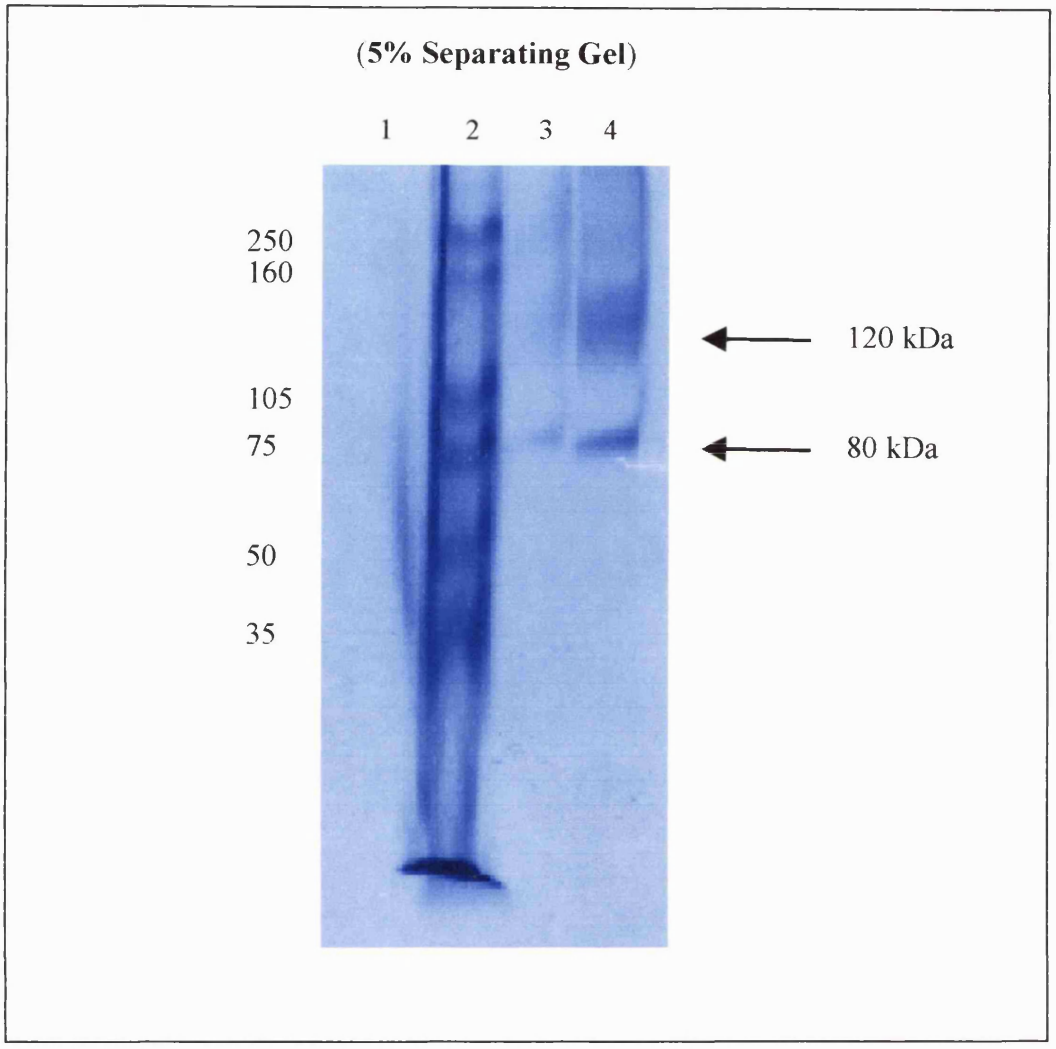


Figure Legend

Figure 4.18 shows the recombinant GPI-PLD protein after separation by a native SDS-PAGE, on a 5% acrylamide gel. The proteins were visualised by Coomassie Blue stain. The protein was separated in comparison with molecular weight markers (Lane 2). Lane 1 contains the negative control, and lanes 3 and 4 contain the GPI-PLD purified from 2 different batches of CHO cells.

Two protein bands were visualised in lanes 3 and 4 by the Coomassie stain, of approximately 80 and 120 kDa molecular weight..

- the addition of 250 µl of PBS onto the monolayer and the cells were gently scraped from the plastic surface using the tip of the pipette. The cell suspension was centrifuged at 1000 rpm for 5 minutes to pellet the cells. The supernatant was removed, and 250 µl of solubilising buffer was added to the cell pellet.
- 250 µl of solubilising buffer was pipetted directly onto the cell monolayer. The cells were incubated for 2 minutes, after which time the liquid was removed to an eppendorf tube.

Following the cell removal protocol, the microtitre plate was held up to the light to establish whether the cells had been removed from the plastic. The sample in the eppendorf tube was vortexed thoroughly for greater than 3 minutes, incubated on ice for 30 minutes and centrifuged at 12,0000 g for 5 minutes, to pellet cell debris. The GPI-PLD-containing supernatant was removed to a fresh eppendorf tube and the GPI-PLD activity in the samples was determined. This methodology was performed using RBL-2H3 cells cultured in either normal supplemented EMEM, or pH-11 treated supplemented EMEM.

Figure 4.19 shows the results from the GPI-PLD activity assay from RBL-2H3 cells removed from a 24 well culture plate. Samples were diluted 1:1 with a reaction mix containing 30 mM mfVSG, and incubated for 30 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

The results showed that the method in which the solubilising buffer was pipetted directly onto the cell monolayer was most successful. Using the calculation described in Section 3.21, the units of activity that were detected in these preparations were 2.14 units from the cells maintained in normal culture medium, and 0.29 units for those cells maintained with pH 11-treated FBS medium. By comparison, only 0.36 units was detected in the normal cells, after the cells were scraped from the plastic surface. The data, therefore, shows both the relative success of the extraction procedure, and also confirms the difference in the GPI-PLD in 'active' and 'inactive' RBL-2H3 cells. Note that 'active' denotes those cells that were responsive to IgE-mediated stimulation, after incubation in EMEM containing normal FBS. 'Inactive' denotes those cells that were unresponsive to IgE-mediated stimulation, after incubation in EMEM containing alkaline inactivated FBS.

4.11.2.2.2 Analysis of RBL-2H3 cells after incubation with recombinant GPI-PLD

The recombinant GPI-PLD was added to cells that had been cultured with pH 11-treated FBS for one week. As shown in figure 4.11, it had previously been determined that one week was

FIGURE 4.19

GPI-PLD ACTIVITY ASSAY WITH MEMBRANE PREPARATIONS EXTRACTED FROM CELL MONOLAYERS IN A 24 WELL PLATE

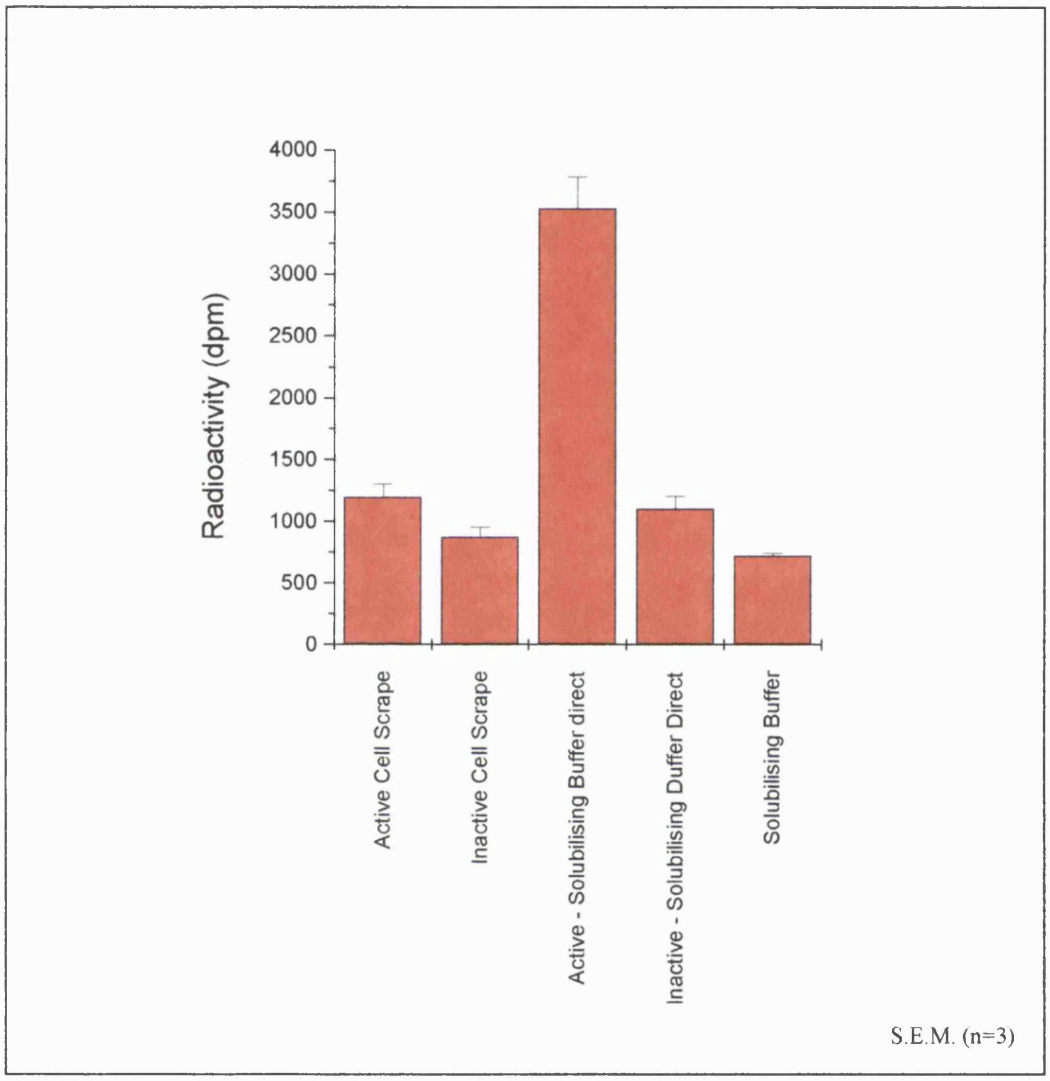


Figure Legend

The plot in Figure 4.19 shows the results from the GPI-PLD activity assay from RBL-2H3 cells removed from a 24 well culture plate. Samples were diluted 1:1 with a reaction mix containing 30 mM mfVSG, and incubated for 30 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

The results showed that the method in which the solubilising buffer was pipetted directly onto the cell monolayer was most successful, with 3500 cpm being detected in these samples. The data also confirms the difference in the GPI-PLD in 'active' and 'inactive' RBL-2H3 cells. Note that 'active' denotes those cells that were responsive to IgE-mediated stimulation, after incubation in EMEM containing normal FBS. 'Inactive' denotes those cells that were unresponsive to IgE-mediated stimulation, after incubation in EMEM containing pH 11 treated FBS.

sufficient for the RBL-2H3 cells to lose the ability to respond to IgE-mediated stimulation. Note that in all subsequent discussion, those cells that showed reactivity to IgE mediated stimulation (cultured with normal EMEM) are referred to as 'active' cells, and those cells that showed reduced reactivity to IgE-mediated stimulation (cultured with pH 11-treated FBS) are referred to as 'inactive'.

Both active and inactive RBL-2H3 cells were grown to confluence, after which time the adherent cells were removed from the culture flask using a cell scraper. The cell density was determined, using a Neubauer haemocytometer. RBL-2H3 cells were plated onto a 24 well plate at a density of 100,000 cells per well in a 500 µl volume. Both RBL-2H3 cells that had been cultured in untreated FBS, and cells that had been cultured in alkaline-treated FBS, were plated out.

r-GPI-PLD was added to selected 'inactive' wells, at a concentration of either 100 or 200 µg per well. The cells were cultured overnight at 37°C in a humidified 5% CO_2 incubator, taking care to ensure that contamination of the plates was minimised. The cells were monitored. When confluence had been achieved, the cells were used in an IgE-mediated cross-linking experiment. This involved incubation of the RBL-2H3 cells with anti-DNP IgE (3µg/ml), and incubation for 2 hours. Subsequently, the cells were incubated with 200 µl of DNP-albumin (1 µg/ml), and incubated for two hours. The β-glucosaminidase assay was incubated for 30 minutes, and the results are expressed as an optical density reading, at 405 nm wavelength.

4.11.2.2.2.1 IgE mediated stimulation of RBL-2H3 cells cultured with recombinant GPI-PLD

As shown in Figure 4.20 and 4.22, the addition of recombinant GPI-PLD protein into cells cultured with pH 11-treated FBS medium showed that the cells did not regain the capacity to respond to IgE mediated stimulation. In Figure 4.20, the OD gained from the degranulation of normal cells was approximately 2.65, in comparison with an OD reading of 0.9 gained with the cells cultured with pH 11-treated FBS. Although the value gained with the pH 11-treated FBS cells was higher than anticipated, comparison with those cells incubated with recombinant GPI-PLD revealed that neither 100 nor 200 µg of protein had raised the OD reading of the degranulated β-hexosaminidase above 1.5.

The results shown in 4.22 show a second experiment in which recombinant GPI-PLD was added to RBL-2H3 cells. In this case, the OD gained from the degranulation of normal cells was approximately 2.75, in comparison with an OD reading of 1.5 gained with the cells cultured with pH-11 treated FBS. Once again, the value gained with the pH 11-treated FBS

FIGURE 4.20

RESULTS FROM CROSS LINKING 'INACTIVE' RBL-2H3 CELLS AFTER INCUBATION WITH RECOMBINANT GPI-PLD
(1)

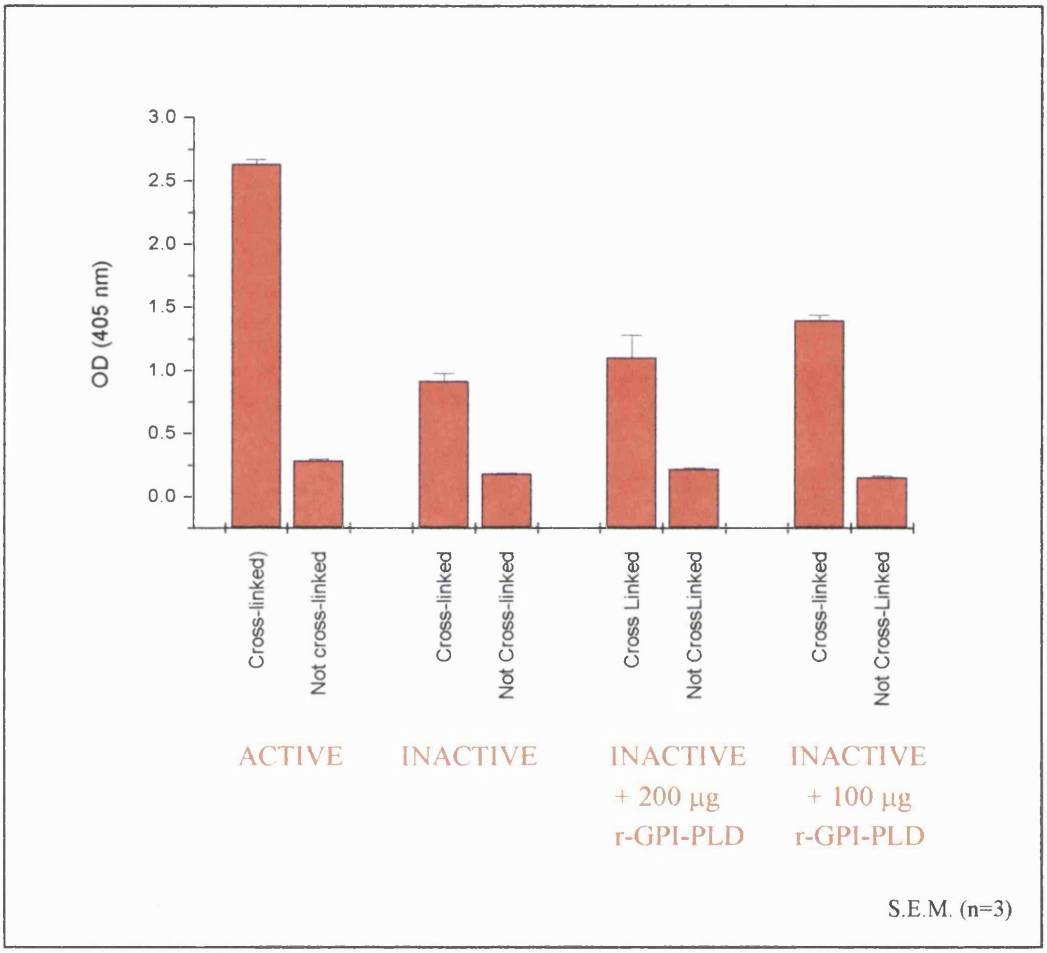


Figure Legend

The plot in Figure 4.20 shows the results from the incubation of RBL-2H3 cells, cultured in pH-11 treated medium, with the recombinant GPI-PLD protein. RBL-2H3 cells were plated onto a 24 well plate at a density of 100,000 cells per well in a 500 µl volume. Both RBL-2H3 cells that had been cultured in untreated FBS, and cells that had been cultured in pH 11-treated FBS, were plated out.

r-GPI-PLD was added to selected 'inactive' wells, at a concentration of 200 µg per well. The cells were cultured overnight at 37°C in a humidified 5% CO_2 incubator, taking care to ensure that contamination of the plates was minimised. The cells were monitored and when confluence had been achieved (after 36 hours), an IgE-mediated cross-linking experiment was performed. The β-glucosaminidase assay was incubated for 30 minutes, and the results are expressed as an optical density reading, at 405 nm wavelength.

As shown in Figure 4.20 the addition of recombinant GPI-PLD protein into cells cultured with pH 11-FBS medium showed that the cells did not regain the capacity to respond to IgE mediated stimulation.

FIGURE 4.21

RESULTS FROM GPI-PLD ACTIVITY ASSAY WITH RBL-2H3 CELLS AFTER ADDITION OF RECOMBINANT GPI-PLD
(2)

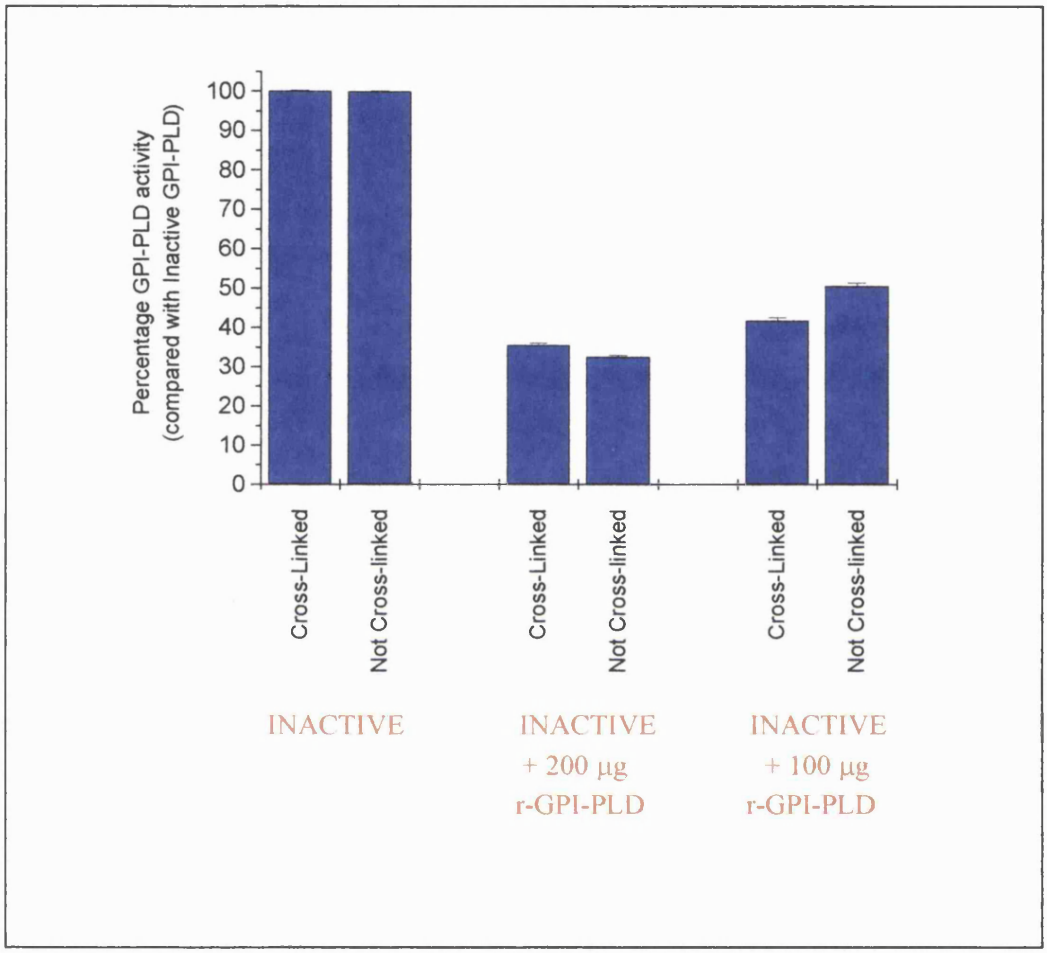


Figure Legend

The plot in Figure 4.21 show the result from the GPI-PLD activity assay on the RBL-2H3 cells cultured with either 100 or 200µg of recombinant GPI-PLD protein. Following the IgE mediated degranulation of the 200,000 RBL-2H3 cells, the cells were thoroughly washed, and GPI-PLD was extracted from the cell membrane. Solubilising buffer was pipetted directly onto the cell monolayer, and the membrane fraction was separated from other cell debris by centrifugation. RBL-2H3 membrane samples were diluted 1:1 with a reaction mix containing 30 mM mfVSG, and incubated for 30 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

The plot in both Figure 4.21 shows that the GPI-PLD activity from the cells incubated with the recombinant protein was reduced to between 30 to 55% of the activity detected in those cells not incubated with the r-GPI-PLD. That is, there appears to be less GPI-PLD activity in these cells, despite the addition of the catalytically active recombinant protein.

cells was higher than anticipated. However, the addition of recombinant GPI-PLD appeared to reduce the degranulation of the RBL-2H3 cells, and the maximum OD reading gained from cells after IgE-mediated stimulation was approximately 0.75.

4.11.2.2.2.2 GPI-PLD Activity of RBL-2H3 cells after incubation with recombinant GPI-PLD

Following the IgE mediated degranulation of the 200,000 RBL-2H3 cells, the cells were thoroughly washed, and GPI-PLD was extracted from the cell membrane, by the method discussed in section. Solubilising buffer was pipetted directly onto the cell monolayer, as described in section 4.11.2.2.1, and the membrane fraction was separated from other cell debris by centrifugation.

The extracted membrane samples were diluted 1:1 with a reaction mix containing 30 mM mfVSG, and incubated for 30 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter. The results are shown in Figure 4.21 and 4.23.

For ease of discussion, the results are shown as percentages, representing the quantity of GPI-PLD detected in the RBL-2H3 membrane samples in comparison with the GPI-PLD activity detected in the cross-linked 'inactive' cells. The GPI-PLD activity in the cross-linked 'inactive' cells is defined as 100%.

The plot in both Figure 4.21 and Figure 4.23 shows that the GPI-PLD activity detected in those inactive cells incubated with recombinant GPI-PLD was significantly reduced, when compared with the normal 'inactive' cells. In Figure 4.21, the GPI-PLD activity from the cells incubated with the recombinant protein was between 30 to 55% of the activity detected in the inactive cell membrane extraction. By comparison, the data in Figure 4.23 shows that the GPI-PLD activity from the cells incubated with r-GPI-PLD was between 40 and 65%. These results demonstrate that the cells incubated with the recombinant GPI-PLD protein had 'lost' GPI-PLD activity, compared with the 'inactive' cells.

4.12 Transfection of the GPI-PLD protein into RBL-2H3 cells

The results from the transfection experiment are shown in Table 4.2. Essentially, in each of the preliminary experiments that were performed with the RBL-2H3 cells, the cells did not survive the methodology. Following the incubation with the DNA : lipid mixture, a majority of the cells had detached from the plastic well, and each well contained a large quantity of cell debris. The subsequent staining with the X-gal reagent, and examination under the microscope,

FIGURE 4.22
RESULTS FROM CROSS LINKING 'INACTIVE' RBL-2H3 CELLS AFTER INCUBCATION WITH RECOMBINANT GPI-PLD
(2)

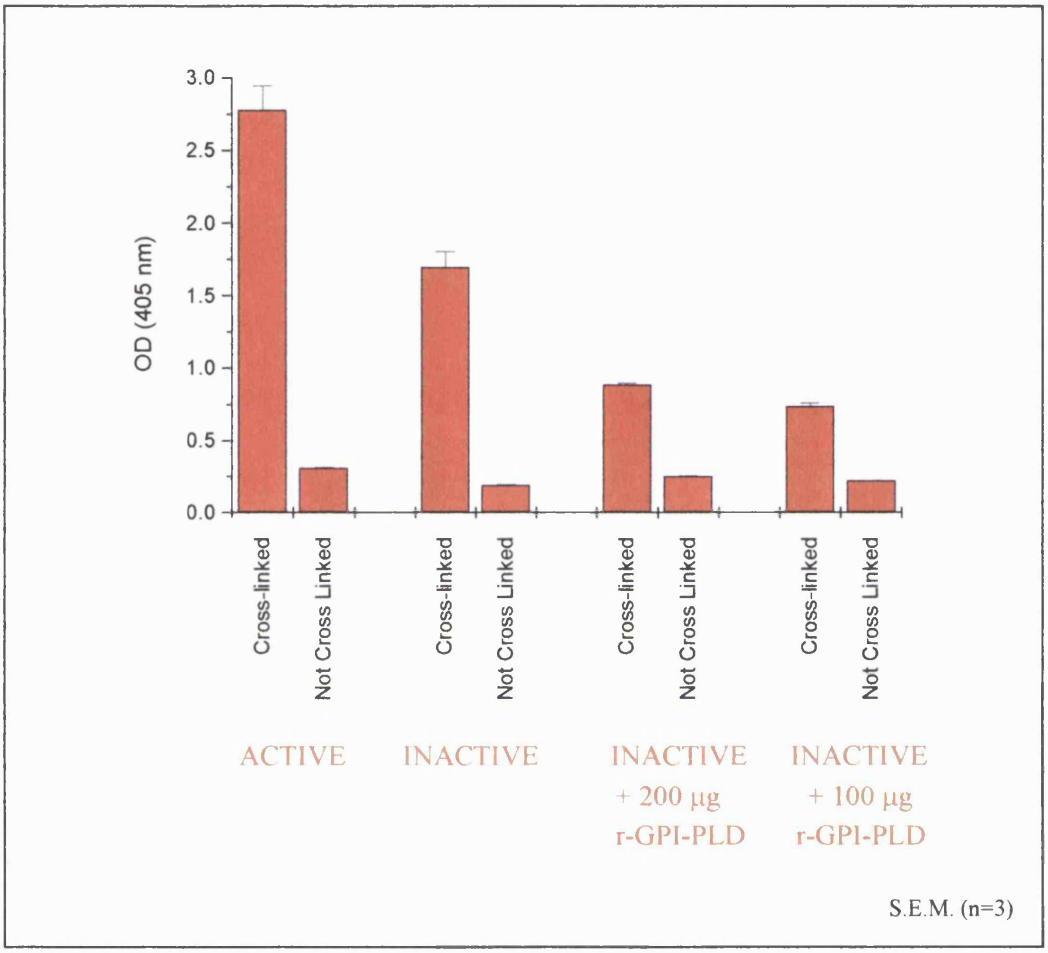


Figure Legend
The plot in Figure 4.22 shows the results from the incubation of RBL-2H3 cells, cultured in pH-11 treated medium, with the recombinant GPI-PLD protein. RBL-2H3 cells were plated onto a 24 well plate at a density of 100,000 cells per well in a 500 µl volume. Both RBL-2H3 cells that had been cultured in untreated FBS, and cells that had been cultured in pH 11-treated FBS, were plated out.

rGPI-PLD was added to selected 'inactive' wells, at a concentration of 200 µg per well. The cells were cultured overnight at 37°C in a humidified 5% CO_2 incubator, taking care to ensure that contamination of the plates was minimised. The cells were monitored and when confluence had been achieved (after 36 hours), an IgE-mediated cross-linking experiment was performed. The β-glucosaminidase assay was incubated for 30 minutes, and the results are expressed as an optical density reading, at 405 nm wavelength.

The results shown in 4.22 show that the addition of recombinant GPI-PLD appeared to reduce the degranulation of the RBL-2H3 cells.

FIGURE 4.23

RESULTS FROM GPI-PLD ACTIVITY ASSAY WITH RBL-2H3 CELLS AFTER ADDITION OF RECOMBINANT GPI-PLD
(2)

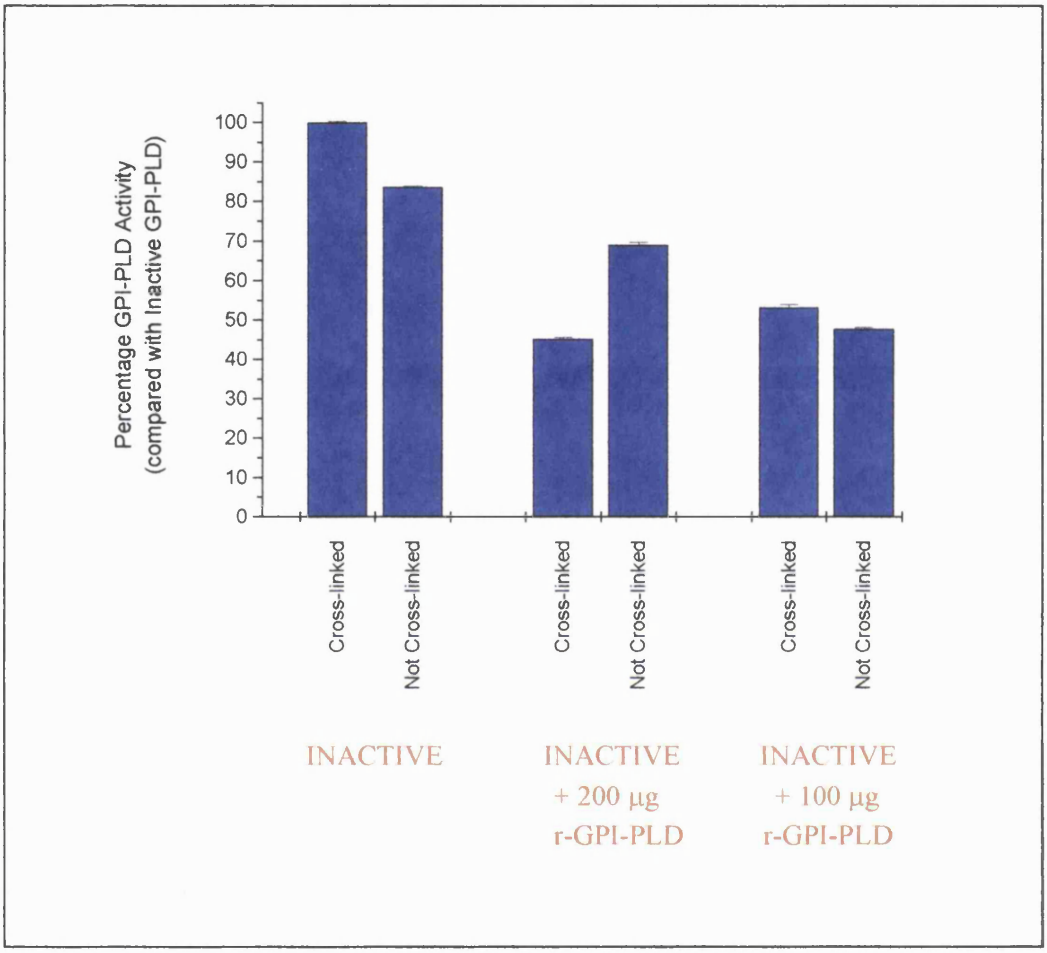


Figure Legend

The plot in Figure 4.23 show the result from the GPI-PLD activity assay on the RBL-2H3 cells cultured wit 200 µg recombinant GPI-PLD protein. Following the IgE mediated degranulation of the 200,000 RBL-2H3 cells, the cells were thoroughly washed, and GPI-PLD was extracted from the cell membrane. Solubilising buffer was pipetted directly onto the cell monolayer, and the membrane fraction was separated from other cell debris by centrifugation. RBL-2H3 membrane samples were diluted 1:1 with a reaction mix containing 30 mM mfVSG, and incubated for 30 minutes at 37°C. After termination of the activity assay, with butanol, the [3H]-dimyristyl phosphatidic acid product was detected, using a one-minute counting programme in the scintillation counter.

Similar to the results in Figure 4.21, the plot in Figure 4.23 shows that the quantity of GPI-PLD activity detected in those inactive cells incubated with recombinant GPI-PLD was reduced to between 40 and 65%. Once again, this reveals that the cells have lost GPI-PLD activity, despite being incubated with the active r-GPI-PLD protein.

TABLE 4.2

RESULTS FROM THE TRANSFECTION OF RBL-2H3 CELLS WITH PCDNA3.1/HIS/LACZ AND DIFFERENT LIPIDS

Lipid	**Nature of the Lipids**	**Approximate Percentage of intact RBL-2H3 cells**	**Blue Cells?**
Pfx-1	1:1 mix of two cationic lipids	10 %	0
Pfx-2	Single cationic lipid	15 %	0
Pfx-3	1:1 mix of cationic lipid and DOPE	5 %	0
Pfx-4	1:1 Mix of two cationic lipids	10 %	0
Pfx-5	1:1 Mix of two cationic lipids	10 %	0
Pfx-6	1:1 Mix of cationic lipid and DOPE	20 %	0
Pfx-7	1:1 Mix of cationic lipid and DOPE	5 %	0
Pfx-8	Single cationic lipid	5 %	0

revealed very few intact cells. In addition, not one blue cell was detected, which suggested that those cells that had survived the transfection had not successfully taken up the expression vector.

4.13 Discussion

4.13.1 Removal of GPI-PLD activity from Foetal Bovine Serum

4.13.2 Replacement of serum with commercially available serum substitutes

The results from the GPI-PLD activity using Aim-V suggested that this serum-free supplement might contain activity against the mfVSG substrate. For example, in one experiment alone, 0.81 units of activity was detected, in comparison with 3.64 units in the 10% FCS sample. Wide variation in the experimental results obtained with the detection of GPI-PLD activity in Aim-V suggested that the quantity of GPI-PLD activity might fluctuate between different aliquots of the serum substitute. Nevertheless, this result precludes the use of Aim-V as a GPI-PLD-free serum supplement.

According to the information supplied by Life technology, Aim-V contains L-glutamine streptomycin sulphate (50 μg/ml) gentamycin sulphate (10 mg/ml) and Human Serum Albumin (0.25%). It is unclear as to why this supplement should contain GPI-PLD activity, unless some of the enzyme has co-purified along with the Human Serum Albumin. By comparison, the Nutridoma supplements contain a greater array of components, including albumin, insulin, transferrin, highly purified serum albumin and other defined organic and inorganic compounds. Nutridoma-NS contains, in addition, a cholesterol source. If the Aim-V serum supplement also contains cholesterol, it is possible that other serum lipids might have contaminated the supplement. As will be discussed in a later section (Section 4.13.3.2.3,) GPI-PLD is closely associated with serum lipoproteins, and the possibility exists, therefore, that some of this enzyme has contaminated the supplement.

As a consequence of the detection of GPI-PLD activity in the Aim-V serum supplement, it was decided not to use of these supplements as a replacement for FBS in culture medium. The potential problems with the use of Nutridoma were outlined in Section 4.4.1.3, and include altered cell morphology and behaviour, and a higher susceptibility to damage. The likelihood that the cells cultured in serum-free medium might not be able to withstand the IgE-mediated degranulation procedure meant that the use of Nutridoma might not be suitable. In addition, the changes in the cell appearance could reflect an alteration in the phenotype of the cells, which may alter their ability to respond to IgE-mediated stimulation. In addition, the establishment of the culture of RBL-2H3 cells has been finely adjusted, for optimal cell growth and behavior. In addition, it would be extremely difficult to determine whether changes in the phenotype of the

RBL-2H3 cells were as a result of the lack of GPI-PLD in the serum, or as a result of the alteration of an alternative serum factor (Roche Molecular Biochemicals Specifications sheet).

4.13.3 Reduction of serum concentration

As discussed in Section 4.8.1.2, according to the current literature, certain cell lines are able to survive short-term culture in medium without serum supplementation. Long-term growth, however, is not possible without the addition of either FBS, or a serum-free supplement. The results from the growth of the RBL-2H3 cells indicated that this cell line does not survive well in the absence of serum, and the majority of cells died after as little as overnight culture in supplemented EMEM without FBS.

To determine whether as little as 1% serum would be sufficient to permit the growth of the cells, whilst limiting the quantity of GPI-PLD protein in the culture medium, a GPI-PLD activity assay was performed on a serial dilution of FBS samples. The results gained from the GPI-PLD activity assay with showed that with as little as 1% FBS, 4.6 units of GPI-PLD activity were detected, after a 30 minute incubation period.

This relatively high level of GPI-PLD activity in a small volume of FBS was not surprising. As stated in Section 4.4.3, GPI-PLD can be at levels as high as 40 μg/ml in bovine serum (Huang *et al.*, 1990). After one hundred-fold dilution of the neat FBS, up to 0.4 μg/ml of GPI-PLD might remain. Although the quantity of GPI-PLD that is required for complete cell function is unclear at present, it may be assumed that 0.4 μg /ml of GPI-PLD would be sufficient.

The aim of this research was the growth of RBL-2H3 cell in the absence of GPI-PLD activity. Based these results, the removal of GPI-PLD would only be achieved by the complete elimination of FBS from the culture medium, and this was not suitable for cell survival. It was necessary, therefore, to choose an alternative method, whereby the quantity of FBS that was included in the culture medium remained the same, whilst removing the GPI-PLD enzyme activity. To achieve this, two methods were attempted. The first involved the removal of the GPI-PLD protein by passage through a VivaSpin concentrator column. The second method involved the removal of the GPI-PLD enzyme activity, using an alkaline inactivation method.

4.13.3.1 Filtration of the serum

The choice of a Vivaspin concentrator column, for the selective removal of GPI-PLD from the Foetal Bovine Serum was made. VivaSpin columns are designed to concentrate proteins of higher molecular weight than the membrane of choice. That is, the selection of a column with a membrane pore size of 100 kDa, would be designed to concentrate proteins of a molecular

weight greater than 100 kDa. Thus, the 110 kDa GPI-PLD protein would be retained in the upper reservoir, whilst the majority of the FBS components would pass through into the lower reservoir. The resultant 'eluate' would be essentially similar to the original FBS, with the exception of a few high molecular weight components.

The initial results that were obtained with the Vivaspin concentrator column were promising, with a significant reduction in the GPI-PLD activity detected. On examination of the 'eluate' in comparison with the original FBS, however, it was revealed that the majority of the proteins had been retained in the upper reservoir of the column. Effectively, the Vivaspin column was removing almost the entire protein content of the FBS, and allowing only a small quantity of FBS components to pass through the membrane. The solution that was collected in the lower reservoir, after concentration, essentially contained two bands, of approximately 60 and 70 kDa molecular weight. The identity of these two protein bands was not determined.

The conclusion that was drawn was that the high protein concentration of the FBS had served to clog the membrane in the Vivaspin column, effectively preventing the passage of the proteins, irrespective of their molecular weight. This method was, therefore, not considered to be appropriate for the selective removal of the GPI-PLD from the FBS samples.

4.13.3.2 Removal of GPI-PLD activity by incubation at pH 3 and pH 11

As discussed, the aim of the research performed in this chapter was to determine the role of GPI-PLD in Type One Hypersensitivity. Thus far, all methods to remove the GPI-PLD protein, or its activity from the RBL-2H3 cell culture have proved ineffective. However, a successful method was found in the literature, which involved the incubation of the Foetal Bovine Serum at pH 11 for one hour. Following this incubation, the activity of the enzyme was dramatically reduced (Kung et al., 1997).

The experiments performed in this chapter have confirmed this finding. To extend the knowledge of this experiment, the FBS was exposed to pH 3, 5, 7, 9 and 11 for one hour, and the effect on the detectable GPI-PLD activity was determined, using the radioactive activity assay. The results confirmed that the FBS that was incubated at either pH 3 or 11 for one hour had lost the ability to hydrolyse the mfVSG substrate. By comparison, incubation at pH 5 or 9 had slightly reduced the detectable GPI-PLD activity, although not significantly, whereas the activity was unaffected by incubation at pH 7.

These results confirm the optimal pH range for the GPI-PLD enzyme, which was previously stated as being between pH 4 and 8.5 (Davitz et al., 1989). That GPI-PLD activity in FBS was slightly reduced at pH 5 and 9, and significantly reduced after treatment at pH 3 or 11 was,

therefore, not unexpected. However, no attempt was made to discuss the potential reasons for the susceptibility of the GPI-PLD protein to extremes of pH. The aim of the following sections, therefore, is to discuss what is currently understood regarding the conformation of the enzyme, with particular attention paid to how the pH changes might affect the ability of the enzyme to perform the hydrolysis of the radio-labelled GPI-anchored substrate.

4.13.3.2.1 Relationship between GPI-PLD protein conformation and enzyme activity

The conformation of the enzyme appears to be of paramount importance in the activity of GPI-PLD (Heller *et al.*, 1994). As discussed previously (Section 3.30.7), GPI-PLD is susceptible to cleavage by trypsin, generating distinct, and reproducible, fragments. Furthermore, the trypsin-cleaved fragments remain associated with one another. Separation and analysis of trypsin-cleaved GPI-PLD fragments led to the conclusion that the active portion of the enzyme is associated with N-terminal fragment containing approximately 275 amino acid residues (Heller *et al.*, 1994).

To confirm that the N terminus is associated with enzyme activity, COS cells were transfected with an N-terminal 1.06 kb fragment of GPI-PLD cDNA. Examination of the activity of this N terminal fragment, however, revealed no activity against GPI-anchored substrates. It was, therefore, suggested that the C-terminus of the protein has a role in maintaining the structural integrity of the enzyme, thereby permitting the catalytic activity of the N-terminus. To investigate this possibility, a number of mutated GPI-PLD sequences were transfected into COS cells, and the effect on the enzyme activity was ascertained (Stadelman *et al.*, 1997).

Using the mfVSG substrate to determine the GPI-PLD activity generated from the mutated proteins, it was revealed that the deletion mutations and substitutions that were directed against portions of the C terminal end of the protein dramatically reduced the enzyme's activity. Deletions of as little as six amino acids resulted in a total loss of enzyme activity. Equally, the addition of an extra 10 amino acids, to a deletion mutant in which eight amino acids had been removed, did not restore enzymatic activity. This result indicated that a specific amino acid sequence was more important than simply the length of the C-terminus. A particular significance was ascribed to the tyrosine residue at position 811, whose removal led to a drastically reduced enzymatic activity (Stadelman *et al.*, 1997).

Further evidence that the conformation of the enzyme is paramount to it's activity was the demonstration that cleavage of the native GPI-PLD protein apparently results in a significant increase in the total enzyme activity, when assayed at detergent levels above the critical

micellar concentrations. The raised activity of this fraction was thought to be due to a decreased sensitivity to detergent, rather than a specific activation of the enzyme itself (Heller *et al.*, 1994). It has been suggested that, *in vivo*, GPI-PLD undergoes a cleavage, possibly resulting in a fragment with the ability to act on membrane-bound substrates (Brodbeck *et al.*, 1994), although this remains to be proven.

That the entire protein is responsible for maintaining the catalytic activity of a specific terminus is not unusual. It is known that the tertiary and quaternary structures of proteins are of vital importance in their complete function. A possible explanation for the loss of activity in the GPI-PLD mutants may include mis-folding of the protein. Therefore, the loss of activity that resulted from the exposure to highly acid or alkaline conditions can be explained by the perturbation of the tertiary structure of the protein, although the precise way in which the GPI-PLD protein was affected by the extreme pH was not determined. It was also not determined whether the protein returns to its native configuration once it has been removed from the extreme pH was not determined. However, it might be assumed that the more extreme the incubation conditions, the more irreparable the damage to the protein, which would prevent re-folding.

4.13.3.2.2 Role of lipids and apo-lipoproteins on GPI-PLD activity

The role of plasma constituents on the activity of GPI-PLD was analysed, through the inclusion of plasma (venous or capillary) in the GPI-PLD activity assay. It was determined that the GPI-PLD activity decreased with increasing plasma content. Preliminary findings ruled out proteins and complex plasma constituents, and the inhibitor was shown to possess hydrophobic tendencies (Low & Huang, 1993). The suggestion was made, therefore, that the plasma constituent that was responsible for the inhibition of GPI-PLD was a lipid.

In vitro, GPI-PLD is not active against membrane-attached GPI-anchored proteins. Enzyme activity requires that the integrity of the lipid bilayer be disturbed by detergents. However, it was noted that when the detergent levels rose above a certain concentration, typically greater than 0.016%, the activity of GPI-PLD was inhibited. This concentration was called the critical micellar concentration (CMC) (Hoener & Brodbeck, 1992).

To determine the effect of detergent on GPI-PLD activity, the molecular weights of GPI-PLD were analysed by gel filtration. In the presence of detergent, bovine and human serum GPI-PLD eluted with apparent molecular masses of 300 and 350 kDa. By comparison, in the absence of detergent, the main GPI-PLD activity peaks eluted with an apparent molecular mass of 550 kDa. By SDS-PAGE analysis, the molecular weight of the individual enzyme subunits

was confirmed as being approximately 100 kDa. It was concluded that GPI-PLD naturally exists as high molecular weight aggregates, and that detergent caused the dissociation of these aggregates.

The hydrophobic nature of the GPI-PLD-containing aggregates lead to the suggestion that GPI-PLD was an amphiphillic protein, which associates with hydrophobic serum components, such as lipoproteins. Structural study of the GPI-PLD sequence indicated that the C-terminal domain confirmed that the protein contains large stretches of hydrophobic amino acids (Hoener & Brodbeck, 1992). Analysis of the secondary structure predicts that the enzyme contains four amphipathic helixes (Chou et al., 1978), which have also been identified in a number of proteins that are known to interact with lipids and lipoproteins (Deeg, 1994).

Isolation of different lipoprotein fractions from serum was accomplished by ultra-centrifugation, and detection of GPI-PLD in the different fractions was performed by Western blot analysis. The results from the Western blot clearly showed that the bulk of enzyme was contained in the high-density lipoprotein fraction. When the lipoproteins were isolated from plasma using a dextran sulphate and immunoaffinity chromatography procedure, the majority of the GPI-PLD was associated with the apolipoprotein-A1 fraction (Deeg, 1994).

In the absence of Apo-A1, GPI-PLD forms high molecular weight aggregates, which are virtually inactive against substrates. By comparison, enzyme / Apo A1 complex efficiently hydrolyses solubilised GPI-anchored substrates, which suggests a significant role of apo-A1 as a co-factor in the activity of the enzyme. (Hoener et al., 1993). It would appear that the association of the enzyme with the serum lipoproteins serves to regulate the activity of the enzyme.

It would be interesting, therefore, to determine whether the interaction between GPI-PLD and apo-A1 is disturbed by the incubation of the FBS at extreme pH. Conformational changes of apo A-1 have been studied by differential scanning calorimetry and ultraviolet difference spectroscopy, as a function of temperature and pH. Preliminary analysis suggested that native apo A-1 has a loosely folded tertiary structure, which is in contrast with the complex folding of the GPI-PLD protein. In addition, the results showed that the lipoprotein underwent reversible denaturation between pH 6.5 to 11.8, which suggests that incubation of the apo-A1 at pH 11 would not dramatically alter its conformation. However, the results also revealed a high degree of exposure of hydrophobic areas in the native protein molecule. Whether these hydrophobic areas are associated with the hydrophobic GPI-PLD protein, and are perturbed by the presence of excess hydroxyl ions at pH 11 remains to be established (Tall *et al.*, 1976).

4.13.3.2.3 Role of calcium on the enzyme activity

It is believed that the function of calcium is to maintain the structural conformation of GPI-PLD. GPI-PLD was shown to bind four or five calcium ions per mole of protein (Li et al., 1994). As a consequence of the contact of GPI-PLD with serum calcium ions, it was assumed that enzyme would be fully saturated under physiological conditions. Hence, it was assumed that these ions must play an important role in the behaviour of the enzyme, and it was considered of importance to determine that role. Furthermore, the possibility exists that any alteration in the calcium concentration, by incubation at pH 3 or 11, might affect the activity of the enzyme.

The literature described a complex methodology, which involved replacement of the divalent cations within the GPI-PLD protein with alternative cations (Li & Low, 1999). The GPI-PLD protein was incubated in a buffered solution, containing either EDTA, EGTA, or 1,10-phenanthroline, after which time the chelators were removed by centrifugation through Biogel filtration columns. The resultant GPI-PLD was then incubated with manganese, cobalt or zinc cations in an EGTA-containing buffer, and the enzyme activity was determined. Results from this procedure showed that the enzymatic activity of purified calcium-free GPI-PLD was completely restored by the addition of zinc ions, and partially restored with cobalt. The addition of manganese resulted in a substantial reduction in the enzyme activity. The restoration of function with these alternative ions confirmed that calcium could be removed from the protein without affecting its enzymatic activity. This result suggested that calcium bound to GPI-PLD has a structural or regulatory role, but is not directly required for GPI hydrolysis (Li & Low, 1999).

To more fully evaluate role of the calcium binding sites, mutant GPI-PLD sequences were constructed, in which the predicted calcium binding sites were either deleted, or altered by single substitutions. These experiments were based on the theory that if any of the bound calcium ions have a fundamental structural role in the newly synthesized GPI-PLD, then mutation would result in a non-functional protein. Conversely, if the calcium binding sites were playing an accessory role in the mature protein, then any mutation would probably not affect the enzymatic activity, nor the amount of GPI-PLD secreted into the medium.

The mutated sequences were successfully expressed in COS-1 cells. It was found that point mutations in the third and fourth calcium-binding motifs, effected a substantial reduction in the amount of GPI-PLD that was detected in the extracellular medium. However, the relative activity of the secreted GPI-PLD was similar to the wild-type form of the enzyme. The

conclusion that was drawn was that calcium ions were important in the secretion of the mature GPI-PLD, although they were not directly related to the activity of the enzyme.

Thus, it may be concluded that although the possibility exists that pH 3 or 11 may alter the interaction of calcium ions with the GPI-PLD protein, it is unclear as to whether this would dramatically affect the activity of the enzyme. It is possible that the removal of the calcium might affect the tertiary structure of the protein, or may affect protein secretion, and thereby alter the detectable enzyme activity. This, however, was not determined.

The possibility that an alternative ion plays an essential role in the activity of GPI-PLD remains to be determined. Although, zinc has been shown to be essential for optimal catalytic activity (Li et al., 1999), no sequence has been identified by which zinc may interact with GPI-PLD, and the mechanism by which zinc may regulate the enzyme activity remains to be determined. This remains an interesting area of research.

4.14 Altered behaviour of the RBL-2H3 cells following culture with pH-11 treated FBS

Following the culture of the RBL-2H3 cells with pH-11 treated FBS, a number of experiments were performed, to determine whether the behaviour of the cells was altered. These experiments included:

- Detection of GPI-PLD activity in the cells
- Determination of responsiveness to IgE-mediated stimulation
- Determination of responsiveness to Ionophore-mediated stimulation
- RT-PCR, to detect GPI-PLD RNA.

A number of conclusions can be drawn from these experiments, all of which suggest that the behaviour of the RBL-2H3 cells was dramatically altered through incubation in the pH-11 treated culture medium.

4.14.1 Loss of detectable GPI-PLD activity in the RBL-2H3 membrane fraction

The results from the analysis of the RBL-2H3 cells indicated that the GPI-PLD activity detected in the membrane fraction was reduced, through time. Essentially, the results showed that the GPI-PLD activity detected in the RBL-2H3 cells was lost rapidly, and that after as little as two days of culture, the activity was significantly reduced.

There are various explanations for the loss of GPI-PLD activity from the RBL-2H3 cells upon extended periods in the alkaline treated FBS-containing culture medium. The first proposal is

that the cells partition the GPI-PLD between their progeny cells, during the mitosis that naturally occurs during cell culture. Alternatively, the possibility exists that there is a constant efflux between the cells and the environment, and that as the 'active' GPI-PLD is lost to the medium, the serum GPI-PLD reserves are 'inactive', thus denying the cells the uptake of functional enzyme.

Experimental evidence would support the first proposal. As discussed previously (section 3.30.6), the uptake of GPI-PLD into a cultured neuroblastoma cell line, N2A, has been studied. In this cell line, it was confirmed that the enzyme was taken up in a concentration- and time-dependent way, and that there was minimal efflux of the enzyme to the cell culture supernatant (Hari et al., 1997). On this basis, we may assume that the RBL-2H3 cells behave in a similar way, and that the GPI-PLD that is absorbed by the cells is not in constant efflux with the extracellular GPI-PLD. On this premise, therefore, we must assume that the RBL-2H3 cell partition the active GPI-PLD protein between their progeny cells. Upon transfer into pH 11 treated FBS, the cells' supply of active GPI-PLD is removed, thereby reducing the quantities of active GPI-PLD that are detected in the progeny cells.

4.14.2 Loss of IgE-responsiveness of the RBL-2H3 cells

The results in figure 4.8, showed that after two weeks of culture with pH 11-treated FBS, the RBL-2H3 cells had lost the capacity to respond to IgE-mediated stimulation. The loss of activity through time was studied by monitoring the IgE-reactivity every two days. The results from this time course are shown in Figure 4.11. Using this technique, it was determined that the loss of reactivity was lost rapidly, with a significant reduction in the quantity of β-glucosaminidase detected after as little as two days. This appears to parallel the loss of GPI-PLD activity, which was also rapidly lost from the RBL-2H3 cells upon their transferral into culture medium containing pH 11–treated FBS.

4.14.3 Loss of Ionophore-responsiveness of the RBL-2H3 cells

As discussed in section, the ionophore A23187 was used as a positive control in all cell stimulation experiments. It was important, therefore, to determine whether the cells cultured in pH-11 treated FBS had lost the capacity to respond to ionophore-mediated stimulation. The results shown in Figure 4.13 indicated that this was the case.

The plot in figure 4.14 show the results from the ionophore mediated stimulation of RBL-2H3 cells, through time, following their transferral into pH 11 treated FBS containing culture medium. Once again, these results show a striking similarity to the loss of IgE responsiveness of the RBL-2H3 cell in the altered culture medium. This result appears to suggest that,

although the IgE-reactivity of the RBL-2H3 cells is ablated by incubation with the pH-11 treated FBS, the reactivity to ionophore-mediated stimulation is likewise affected.

We propose that the pathway of IgE-mediated degranulation converges, at some point, with the pathway of ionophore mediated degranulation. That the pathways of these stimuli converge, therefore, suggested that certain components, such as GPI-PLD, might be found in both pathways.

A number of lines of evidence support the proposal that the IgE and the ionophore mediated pathway might converge. For example, it was found that stimulation of RBL-2H3 cells, through either the IgE or ionophore-mediated pathways induced similar morphological changes. Prior to stimulation, the cells were elongated and spindle shaped, with their surfaces covered with small short microvilli, as studied by both fluorescence and scanning electron microscopy. After IgE-mediated stimulation, the cells flattened and spread over the surface of the culture dish, the plasma membrane became pliated and deep folds and ruffles were observed. These changes were dependent on calcium. The quantities of histamine released, and the changes in cell shape and actin were directly dependent on the concentration of antigen or ionophore used. Upon stimulation with A23187, similar changes were observed, although the surface changes were less dramatic. It was suggested that the morphological changes observed with A23187 were less pronounced as a consequence of the reduced involvement of the surface membrane by the ionophore. By comparison, the surface morphology is of great importance in IgE-mediated stimulation (Section 1.3.17.1) (Sahara *et al.*, 1990).

The distribution of the cytoskeleton, upon stimulation of the RBL-2H3 cells, was also studied. It was found that the distribution of polymerised actin paralleled the morphological changes observed in the RBL-2H3 cells, upon stimulation with either IgE or ionophore. For example, polymerised actin was detected in the surface folds, as little as 5 minutes after stimulation. Once again, the changes induced by IgE mediated stimulation were more rapid that those seen with ionophore (Sahara *et al.*, 1990).

The second line of evidence that phospholipases might play a role in ionophore-mediated stimulation comes from studies which demonstrated that both IgE-mediated and ionophore-mediated stimulation are dependent on inositol phospholipids. The research was performed with the RBL-2H3 cell line and is, therefore, directly relevant to the research performed during this thesis. The putative role of inositol phospholipids in IgE-mediated signalling is discussed in greater detail in Section 5.1.2.2.3. Briefly, receptor-mediated stimulation of RBL-2H3 cells promotes the hydrolysis of membrane inositol phospholipids, and appears to be a direct

consequence of aggregation of IgE receptors. As a consequence of the hydrolysis of as much as 50% of the cellular inositol phospholipids, histamine secretion was effected (Beaven *et al*, 1984).

Similarly, the hydrolysis of inositol phospholipid is effected by ionophores A23187 and ionomycin. The response was dependent on both the concentration of ionophore and the presence of external calcium ions, and correlated with the magnitude of the secretory response. Although this hydrolysis was less extensive than that induced by aggregation of IgE receptors, it certainly appears to play a role in the ionophore-mediated degranulation of RBL-2H3 cells (Lo *et al*. 1987). The downstream effects of these inositol phospholipids was not determined, but it is believed that they may be involved with the PKC mediated signalling cascade.

Thus, inositol phospholipids have been implicated in both IgE-dependent and ionophore-dependent degranulation of RBL-2H3 cells.

4.14.5 RT-PCR analysis of the cells cultured in pH-11 treated FBS

RT-PCR analysis was performed on the RBL-2H3 cells, to determine whether the cells had begun to synthesise GPI-PLD specific RNA. No evidence was found of a GPI-PLD specific RNA, and it was concluded that the cells did not up-regulate the production of GPI-PLD mRNA in the altered culture conditions.

4.15 Addition of GPI-PLD activity to RBL-2H3 cells cultured with pH-11 treated FBS

4.15.1 Addition of normal FBS to RBL-2H3 cells cultured with pH-11 treated FBS

The addition of normal FBS cells to those cells cultured in altered EMEM had the capacity to promote the degranulation of cells that were previously unresponsive to IgE mediated stimulation. It could be concluded that the cells were not irreversibly damaged by their culture with altered serum.

4.15.2 Addition of recombinant GPI-PLD protein to RBL-2H3 cells cultured with pH-11

4.15.2.1 Creation of the recombinant GPI-PLD

The creation of the recombinant GPI-PLD was successful. The resultant protein was active against the mfVSG substrate, and was inhibited by 1,10-phenanthroline. The size of the protein was determined by separation of the recombinant product(s) by native SDS-PAGE gel electrophoresis. Using this separation technique, two protein bands were detected, one of

approximately 80 and a smear at around 120 kDa molecular weight. It is likely that the larger smear represent the mature 110 kDa GPI-PLD protein (Huang *et al.*, 1990), with variation in glycosylation patterns producing the smear of differently sized products. The 80 kDa band may represent an incompletely processed immature form of the recombinant protein.

By comparison, when the protein(s) were separated by reducing SDS-PAGE gel electrophoresis, three protein bands were visualised, of approximately 50, 70 and 75 kDa molecular weight. As discussed in Section 3.30.7, it is known that the GPI-PLD protein contains a number of trypsin-sensitive sites (Scallon *et al.*, 1991). GPI-PLD is susceptible to processing *in vitro* and *in vivo*, a process which typically generates a number of intermediate-sized fragments, ranging from 39 to 101 kDa (Hari *et al.*, 1997). Under non-denaturing conditions, the processed fragments remain tightly, but non-covalently, associated with each other (Heller *et al.*, 1994), as confirmed by the native gel of the recombinant GPI-PLD protein created for this research. We may assume, therefore, that the r-GPI-PLD, of approximately 120 kDa, and with activity against GPI-anchored substrates, represents a functional GPI-PLD protein.

4.15.2.2 Incubation of RBL-2H3 cells with recombinant GPI-PLD protein

4.15.2.2.1 GPI-PLD extraction from RBL-2H3 monolayers

The results from the extraction of RBL-2H3 membrane from the cultured cell monolayers confirmed that the membrane was most effectively extracted when the solubilising buffer was pipetted directly onto the adherent monolayer.

This was not surprising, considering the small diameter of the culture wells. It was impossible to effectively scrape the cells from the plastic surface and, therefore, a significant proportion of the cells remained in the culture plate. By comparison, pipetted the solubilising buffer directly onto the cell monolayer effected the direct lysis of the cells, and the lysed preparation could be removed for further experimentation.

4.15.2.2.2 Analysis of RBL-2H3 cells after incubation with recombinant GPI-PLD

4.15.2.2.2.1 IgE mediated stimulation of RBL-2H3 cells cultured with recombinant GPI-PLD

Following a one-week culture of the normal RBL-2H3 cells with pH-11 treated FBS-supplemented EMEM, it was confirmed that the cells showed reduced responses to IgE-mediated stimulation. The quantity of β-glucosaminidase that was detected following cell degranulation was significantly reduced, when compared with the normal RBL-2H3 cells. The

recombinant GPI-PLD protein was added to 'inactive' cells, and the cells were cultured overnight, and any changes in the responsiveness of the cells to IgE-mediated stimulation was determined.

Unfortunately, it was found that the 'inactive' RBL-2H3 cells did not regain the ability to respond to IgE-mediated stimulation, following culture with r-GPI-PLD. The β-glucosaminidase that was detected from the degranulated cells was approximately equivalent to the quantity released from the 'inactive' cells. An explanation for this was only given when the GPI-PLD activity of the different cells was analysed.

4.15.2.2.2.2 GPI-PLD Activity of RBL-2H3 cells after incubation with recombinant GPI-PLD

The results from the experiments to determine the GPI-PLD activity in those cells incubated with r-GPI-PLD revealed that the cells lost detectable GPI-PLD activity, when compared with those cells incubated without r-GPI-PLD.

It was suggested that the loss of GPI-PLD activity was related to the IgE-mediated stimulation of the cells. The possibility existed that the presence of the recombinant protein during the IgE-mediated stimulation effected the release of the active GPI-PLD protein, thereby reducing the detectable GPI-PLD activity. However, a comparison of the cross-linked and not cross-linked data suggests that this was not the case. The detected GPI-PLD activity was similar, whether the cells had undergone cross-linking or not. It was, therefore, concluded that the cross-linking process was not responsible for the reduced GPI-PLD activity detected in the 'inactive' RBL-2H3 cells incubated with recombinant GPI-PLD protein.

As a consequence of the reduced GPI-PLD activity in all of the RBL-2H3 cells incubated with recombinant GPI-PLD protein, it was concluded that the cells did take up the r-GPI-PLD from the culture medium. However, it was also concluded that the r-GPI-PLD has a lower specific activity, when compared with the native GPI-PLD protein. During the culture period, the less active r-GPI-PLD must, somehow, effect the displacement of the more active native GPI-PLD protein, and thereby reduces the GPI-PLD activity detected in these cells.

In this instance, therefore, it is unsurprising that the cells cultured with r-GPI-PLD protein did not regain the capacity to respond to IgE-mediated stimulation. Indeed, the results in Figure 4.22 suggest that those cells incubated with r-GPI-PLD had a reduced capacity to respond to IgE mediated stimulation. The proposal that GPI-PLD may be involved in the IgE-mediated stimulation cascade is, therefore, supported by this data. That is, the introduction of a GPI-PLD

with a lower specific activity than the native GPI-PLD protein appears to affect the ability of the cells to respond to IgE mediated stimulation.

4.15.2.2.2.3 Lower specific activity of the recombinant GPI-PLD protein

The data in Figure 4.16 confirmed that the r-GPI-PLD was catalytically active against the mfVSG substrate. However, a number of minor differences exist between the native and the recombinant GPI-PLD protein, which may effect the reduced specific activity of the recombinant protein.

As stated in Section 4.5.2.2.1, the recombinant protein was created using a CHO cell line. Although the protein displays characteristics of the normal serum GPI-PLD protein, including activity against a radiolabelled GPI-anchored substrate and sensitivity to 1,10-phnenanthroline, the possibility exists that the CHO cells have produced an altered protein. For example, the patterns of glycosylation may be different from the serum protein, which may affect its activity against the GPI-anchored substrates.

In addition, the role of both Apo-A1 and calcium in the activity of the GPI-PLD protein were discussed in Sections 4.13.3.2.2 and 4.13.3.2.3, respectively. It is possible that the recombinant protein does not associate with either Apo-A1 or calcium in the correct conformation, and that this effects a change in the activity of the enzyme against GPI-anchored substrates.

However, to date, the precise explanation for the reduced activity of the r-GPI-PLD protein, in comparison with the native GPI-PLD protein is unclear.

4.16 Transfection of the GPI-PLD protein into RBL-2H3 cells

A number of explanations may be given for the death of the RBL-2H3 cells during the transfection method. The most likely explanation was the lipid concentration was too high. To permit the passage of the expression plasmid, a minimum quantity of lipid is essential, as it is complexed with the DNA, resulting in an overall net positive charge. It is thought that the positive charge of the lipid:DNA complex facilitates interaction with the negatively charged cell surface. Excess lipid, however, is toxic to the cells, resulting in changes in cell morphology, slow growth rates and eventual cell death. The literature, therefore, suggests that certain cell lines might benefit a change in the ratio of lipid to plasmid.

An alternative reason for the cell death might have been the incubation period in which the cells were maintained in serum-free culture medium. The problems associated with the serum-free culture of RBL-2H3 cells have been discussed previously (Section 4.8.1.2), and it is clear

that the absence of serum might have weakened the cells, leading to their death in later stages of the protocol. Increasing the cell density for transfection might have alleviated the problems associated with excess lipid quantities, or serum-free incubation. Unfortunately, time constraints meant that this was not attempted.

However, it was clear the initial experiments that the RBL-2H3 cells responded extremely badly to this methodology, and that it was unlikely that the optimisation suggestions would be sufficient to prevent cell death. An alternative method of transfection would probably be more suitable.

Unfortunately, time constraints restricted the opportunity to explore the transfection of the RBL-2H3 cell line. The time and expertise required to establish, maintain and characterise an RBL-2H3 cell line expressing a transfected GPI-PLD protein is an extensive procedure. To further express altered versions of the GPI-PLD proteins is an additional complication. This was, therefore, considered a suitable place to terminate the experimentation performed during this thesis.

4.17 Conclusions

The aim of these experiments was to study the role of GPI-PLD in mast cell function, with particular significance placed upon the IgE-mediated degranulation of these cells. The results that were gained were consistent with a role for GPI-PLD in IgE-dependent degranulation, but did not prove its role conclusively.

The removal of GPI-PLD activity from the FBS, which was proven as the source of GPI-PLD for the cultured RBL-2H3 cells, resulted in the loss of reactivity to both IgE- and Ionophore-mediated stimulation. Experiments in which the GPI-PLD was replaced with a recombinant GPI-PLD protein, however, failed to re-establish reactivity to IgE-mediated stimulation. It was revealed, however, that the recombinant GPI-PLD had a lower specific activity than the native GPI-PLD protein.

The physiological relevance of the GPI-PLD enzyme in mast cells is, therefore, incompletely understood. However, a number of experiments have been suggested during the course of this chapter from which more complete picture of the role of GPI-PLD in this RBL-2H3 cell line may be derived

Chapter 5

Discussion

5.1 Introduction to the role of GPI-PLD in cellular systems

The aim of this thesis was to determine the involvement of the GPI-PLD enzyme in Type One Hypersensitivity. As discussed in Chapter 3, GPI-anchored proteins have been implicated in a wide variety of different cellular systems. A large body of research in other cellular systems has led to the theory that GPI-PLD is a key enzyme in a GPI / IPG signalling systems, located within caveolae. This chapter aims to review these experimental studies, to discuss what is currently understood regarding the role of GPI-PLD in other intracellular signalling pathways, and to relate this information to the postulated role of GPI-PLD in IgE-mediated mast cell signalling.

5.1.1 The GPI-signalling system and caveolae

5.1.1.1 Introduction to caveolae and GPI-anchored proteins

The term 'caveolae intracellularis' was first coined in 1955, with the description of small vesicles on the surface of epithelial cells, indenting the membrane and cytoplasm, and in contact with the extracellular milieu (Yamada, 1955). The later identification of caveolar structures on the surface of cells including smooth muscle cells (Forbes *et al.*, 1979) and fibroblasts (Bretscher & Whytock, 1977) led to the suggestion that most cell types possess caveolae, or caveolae-like structures.

Upon examination of the caveolar structure of endothelial cells, by high resolution scanning (Peters *et al.*, 1985) and freeze-etch techniques (Izumi *et al.*, 1989), it was noted that the caveolar membrane surface was coated with striated filaments. Separation of these striated filaments from the membrane was effected by detergent extraction, and the filament was identified as a 22 kDa protein. Upon comparison with known protein sequences, this 22 kDa protein was identified as a substrate for v-src tyrosine kinase in RSV virus transformed chick embryo fibroblasts (Glenney & Zokas, 1989). Independently this protein had also been identified as a major component of the vesicles that originated from the trans-Golgi network (TGN) of epithelial cells, and it was termed vesicular integral membrane protein of 21 kDa, or VIP-21 (Kurzchalia *et al.*, 1992). Through its association with caveolae, the protein was renamed caveolin (Rothberg *et al.*, 1992).

A number of techniques were developed for the purification of caveolae, most commonly involving solubilisation with Triton X-100 at 4°C, followed by either a density-gradient separation or by specific adsorption techniques (Hooper, 1998). This method permitted the discovery that caveolin co-precipitated with a lipid-rich mixture, containing glycosphingolipids (GSLs), sphingomyelin and cholesterol. Further analysis determined that caveolae are enriched with lipid-modified proteins, receptors and structural components. Significantly, a number of

the proteins that co-purified with caveolin were shown to contain a GPI-anchor, which led to the investigation of the association between the GPI-anchor and caveolar function.

The first evidence that GPI-anchored proteins might have an unusual association with lipid membranes came from the study of the Thy-1 antigen. Analysis of the surface-mobility of Thy-1 indicated that a substantial proportion of the protein units were immobile (Ishihara *et al.*, 1987). The further observation that, routinely, between 10 and 20% of the Thy-1 in T-lymphoma cells could not be solubilised by detergent was taken to suggest that this fraction was a constituent of the detergent-resistant membrane fraction. Using immunogold-labelling techniques, the surface location of different GPI-anchored proteins was assessed, including the folate receptor (5-methyltetrahydrofolate), Thy-1 and prion proteins. In all cases, the proteins were found to be highly clustered, and a substantial fraction of the clusters were located near, or within, caveolae (Ying *et al.*, 1992). The association of GPI-anchored proteins with caveolae was found to be independent of the cell type and the maturity of the cell. It was, therefore, concluded that the formation of clusters was an intrinsic property of the GPI-anchor, rather than the cell.

Opponents of this theory believe that the evidence gained is an experimental artefact, and that GPI-anchored proteins do not preferentially locate in caveolae under physiological conditions. To prove this, a variety of GPI-anchored proteins were studied using electron microscopy and immunohistochemistry. The results showed that the GPI-anchored proteins were diffusely distributed over the cell surface, both within and out of caveolae. The addition of clustering agents, however, dramatically altered the location of the GPI-anchored proteins, with a higher percentage of GPI-anchored proteins being detected in, or near to, caveolae (Mayor *et al.*, 1994). The conclusion was, therefore, that the GPI-anchored proteins have a diffuse surface distribution, which may be altered by the addition of clustering agents.

Clearly, there is conflict between the data. To date, the majority of the experimental data supports the conclusion that certain GPI-anchored proteins are associated with caveolae. The extent to which this represents a physiological situation, however, remains to be established.

5.1.1.2 Putative association of caveolar components with intracellular signalling cascades

Further studies to determine the nature of the caveolin protein indicated that it was susceptible to phosphorylation. This was demonstrated through the use of anti-phosphotyrosine monoclonal antibodies, which were designed to detect phosphorylated antigens in Rous sarcoma virus-transformed chick embryo fibroblasts. When employed in affinity chromatography, these

antibodies precipitated a number of antigens, including the caveolin protein. The existence of phosphorylated tyrosine residues on caveolin indicated that in transformed cells, the protein was susceptible to PTK activity (Glenney & Zokas, 1989).

In transformed cells, the α caveolin isoform was phosphorylated on tyrosine 14 by the viral oncogene, v-Src tyrosine kinase. V-Src is derived from the viral transformation of the cellular c-src. The demonstration that c-src co-precipitates with caveolin in normal cells led to suggestion that caveolin may be phosphorylated by c-src in a normal healthy cell (Li *et al.*, 1996). The potential exists that the phosphorylation of caveolin represents a link between the extracellular environment and intracellular tyrosine kinase signalling pathways, although further proof is required.

The potential that caveolin forms a link between the extracellular milieu and the intracellular signalling pathways led researchers to determine the relationship between caveolae and other intracellular components. In epithelial cells, for example, analysis of the purified caveolin-associated constituents of epithelial cells revealed that detergent-extracted caveolar complexes contain many proteins associated with intracellular signalling systems. These include GTP-binding proteins, an apical calcium-regulated phospholipid binding protein called annex II, which has a demonstrated role in exocytic fusion events, and the tyrosine kinases, c-Yes, which was identified as a member of the Src family of non-receptor type protein-tyrosine kinase (Sargiacomo et al, 1993).

More extensive characterisation of epithelial cells was performed, using micro-sequencing and immuno-blotting techniques. These techniques revealed a number of cytoplasmic signalling molecules associated with caveolin, including Src-like kinases, the alpha and beta subunits of heterotrimeric GTP-binding protein, and members of the Ras-related GTP-binding protein family (Lisanti et al, 1994; Chang et al, 1994).

The transmembrane nature of caveolin, coupled with its capacity to undergo phosphorylation, led to the proposal that caveolin might function as a transmembrane adaptor molecule, coupling caveolae-associated GPI-anchored proteins with cytoplasmically-oriented signalling molecules (Sargiacomo *et al.*, 1993). This remains to be established.

Using these purification and analytical techniques, a number of GPI-anchored proteins were found to be associated with caveolae. Furthermore, several of these GPI-anchored proteins are associated with intracellular tyrosine kinases. DAF, for example, was shown to be associated with both p56-lck and p59-fyn in DAF transfected murine thymocytes (Shenoy-Scaria *et al.*,

1992). It is believed that the interaction of GPI-anchored proteins with intracellular tyrosine kinases compensates for the lack of transmembrane signalling domains in the GPI-anchor (Stefanova *et al.*, 1991).

A detergent-free extraction method was developed, which permitted identification of caveolae components without contamination of other membrane components. This extraction protocol incorporated the adsorption of polycationic colloidal silica particles onto the membrane surface, which were subsequently extracted to high purity by sedimentation (Schnitzer *et al.*, 1995).

Determination of the components of the silica-separated caveolar membranes from epithelial cells revealed a number of molecules that are known to have significance in intracellular signalling cascades, including phosphoinositides and sphingomyelin (Liu *et al.*, 1997). Sphingomyelin is a minor lipid component, which is involved in the regulation of cell growth and proliferation. The hydrolysis of sphingomyelin is initiated by cytokines, including TNF-α and IL-1β, resulting in the generation of ceramide, which activates a ceramide-activated protein kinase (Berridge, 1993). The demonstration that caveolae are enriched in sphingomyelin, therefore, demonstrates that caveolae are linked with the ceramide signalling cascade, although any further details are unclear. Likewise, phosphoinositides mediate several receptor transduction pathways, including G-protein coupled receptor and tyrosine kinase receptor pathways (Liu *et al.*, 1994). In addition, the hydrolysis of phosphatidylinositol 4,5-bisphosphate, by phospholipase C, generates diacylglycerol, and inositol-4,5-trisphosphate, whose significance in the Type One hypersensitivity reaction have already been discussed (Section 1.3.29 and 1.3.30).

In addition to these lipid-based signalling molecules, caveolae were found to be enriched in a number of other molecules. These included non-receptor tyrosine kinases, such as lyn, src and fyn, as confirmed using tyrosine kinase assays and confocal immunofluorescence microscopy. The PDGF β-receptor, protein kinase C, PI-3-kinase, and phospholipase Cγ were also found to be concentrated in caveolae, as quantified using enzyme linked immunoabsorbant assays on the purified caveolar fractions (Liu *et al.*, 1997).

Although a number of signalling molecules were detected in the purified caveolar fractions, the evidence for actual signal transduction in caveolae was lacking. Without evidence of the activation of signalling cascades through these molecules, it was possible that they were functionally inactive. In order to investigate whether signal transduction could occur specifically in caveolae, a panel of ligands was tested, including endothelin-1, platelet derived growth factor (PDGF), insulin and epithelial growth factor (EGF). All of these ligands are

known to activate various signalling pathways in endothelial cells. In addition, the ligands are known to bind to molecules that are known to reside in caveolae. The pattern of tyrosine phosphorylation of proteins in response to these ligands was determined by immunofluorescence microscopy.

Upon stimulation of endothelial cells with these ligands, the strongest signal was generated through PDGF. Following PDGF stimulation, extensive tyrosine phosphorylation was visualized on the surface of the cell, and the pattern of phosphorylation appeared to be located in discrete surface domains. A number of differently sized proteins appeared to be phosphorylated, with molecular weights ranging from 40 to 200 kDa, and the phosphorylation was inhibited by pre-treatment of the cells with a specific tyrosine kinase inhibitor, genistein. Using confocal immunofluorescence microscopy, tyrosine phosphorylated proteins were detected in cell surface structures that also labeled with caveolin, which confirmed that the location of the PDGF derived phosphorylation was in the caveolae.

Finally, the use of *in vitro* kinase assays permitted the identification of the intrinsic tyrosine kinase activity in caveolae. A number of proteins were phosphorylated by these intrinsic proteins, ranging from 18 to 116 in molecular weight. More importantly, the use of tyrosine kinase assays on endothelial cell caveolae preparations confirmed that one of the major proteins being phosphorylated was caveolin (Liu *et al.*, 1997). This result supported the data derived from transformed cells, in which caveolin was phosphorylated by viral-src.

A fundamental question in signal transduction is how substrates are rapidly and specifically phosphorylated by specific protein kinases. Signalling molecules have the capacity to activate a diverse group of effectors in different pathways, and it has become of importance to determine how the activation of intracellular pathways may be controlled. The restricted compartmentalization of certain kinases and their substrates into specialized microdomains within the membrane bilayer would provide at least some specificity. As such, the compartmentalization of membrane elements into caveolae would establish a level of organization over the initiation of signalling (Liu *et al.*, 1997).

5.1.1.3 Putative role of caveolae and caveolar-associated GPI-anchored proteins in mast cell degranulation

Within the study of intracellular signalling, a considerable amount of research has been devoted to understanding how calcium functions as a second messenger, and the significance of calcium in mast cell signalling was discussed in Section 1.3.30. Of particular significance in IgE-

mediated mast cell degranulation is inositol-1,4,5-triphosphate Ins(1,4,5)P_3, under the control of phospholipase Cγ1.

Two major proteins involved in the movement of calcium are the 1,4,5 triphosphate (IP3) sensitive calcium channel, and the ATP dependent calcium pump (Berridge, 1993). The cellular location of both proteins was studied by immuno-cytochemistry, fluorescence microscopy and electron microscopy, in a number of cell types, including endothelial cells, cardiac muscle cells and epidermal keratinocytes. Both the IP3 calcium channel (Fujimoto *et al.*, 1992) and the calcium pump (Fujimoto, 1993) were found to be associated with caveolae. As mentioned in Section 1.3.30, it is known that Ins(1,4,5)P_3 binds to, and opens, the Ins(1,4,5)P3 receptor calcium channels on the surface of the endoplasmic reticulum, thereby promoting the release of stored calcium. That this receptor may also be located in caveolae reveals a potential association between caveolae and mast cell degranulation, which certainly warrants greater attention. Precisely how caveolae function in calcium metabolism is an exciting new topic for investigation.

A second possible role for caveolae in mast cell degranulation involves the prominent role of the cell membrane and the cytoskeleton in the early stages of IgE-mediated mast cell activation, as discussed in the Section 1.4.5. Through the association of caveolae with both intracellular signalling cascades, and the cytoskeleton, it was suggested that caveolae might provide a link between the extracellular and intracellular environments in mast cells.

Finally, the role of the src family of tyrosine kinases in the IgE mediated degranulation of mast cells is well documented, and was discussed in Section 1.3.22. Lyn, for example, is activated through the bridging of the FcεR1 receptor, and serves to recruit another tyrosine kinase, Syk to the signalling cascade. The demonstration that both GPI-anchored proteins, and the FcεR1 mediated signalling cascades are associated with tyrosine kinases suggests that overlap might occur between the two pathways. However, tyrosine kinases have been implicated in a number of other cellular signalling cascades, and a great deal more information is required before the association between GPI-anchored protein-mediated signalling and mast cell signalling is proven.

5.1.2 GPI-derived second messengers

The hydrolysis of GPI anchored proteins by phospholipase enzymes results in the production of a lipid portion, namely phosphatidic acid and a water-soluble protein to which inositol is attached. Substantial evidence exists for a role of phosphatidic acid in intracellular signalling cascades, as discussed in the following section (Section 5.1.2.1). The role of GPI-derived

inositol compounds in signalling cascades is, however, incompletely understood, and is discussed in section 5.1.2.2.

5.1.2.1 Phosphatidic Acid in Intracellular signalling pathways

Phosphatidic Acid is rapidly produced during the receptor-stimulated breakdown of phosphoinositide molecules, with consequent formation of second messengers such as inositol trisphosphate. Exogenously-applied phosphatidic acid elicits a transient rise in intracellular calcium in a variety of cultured cells, through the release of calcium from intracellular stores (Moolenaar et al., 1986). In contrast, other phospholipids, such as phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositol, failed to cause a raise in intracellular calcium levels (Kawase & Suzuki, 1988).

Phosphatidic acid also induces a rapid and dose-dependent increase in production of inositol phosphates in cultured human keratinocytes. The production of inositol phosphates could be inhibited by pre-treatment of the cells with either pertussis toxin or 12-O-tetradecanoylphorbol 13-acetate, which suggested that a GTP-binding protein and a protein kinase C might be involved in PA mediated cellular responses (Ryder *et al.*, 1993).

The effects of phosphatidic acid in keratinocytes could be reproduced by the addition of exogenous phospholipase D, stimulating the production of inositol phosphates (Ryder *et al.* 1993). Although this second messenger may be formed by phospholipase D-mediated catalysis, or by the phosphorylation of diacylglycerol, this result suggested that the PLD has a significant role in the production of phosphatidic acid in human keratinocytes.

5.1.2.2 Inositol phosphoglycans in Intracellular Signalling cascades

The generation of molecules containing both protein and inositol, from the phospholipase-mediated catalysis of GPI-anchored proteins, is widely acknowledged. In the discussion of the role of inositol phosphoglycans (IPG) in intracellular signalling, however, it is not believed that inositol-containing molecules are derived from GPI-anchored proteins. Chemical analysis to determine the components in the IPG mediators showed that these molecules contain components not found in the classical GPI-protein anchor. The components included galactosamine residues and chiro-inositol (Mato *et al.*, 1987(b)). The suggestion was, therefore, made, that an alternative 'free' GPI anchor existed, which was not covalently linked to proteins. The precise chemical details of these free GPI, or the IPG molecules remains, however, unresolved.

An intracellular pool of free-GPI molecules has been detected in several higher eukaryotic cells, including T lymphocytes. This pool contained a number of chemically-different GPI-like structures, variously including a phosphatidylinositol linked to between 3 and 10 carbohydrate residues, including glucosamine, mannose, galactose and ethanolamine. These structures also contained chiro-inositol (Gaulton & Pratt, 1994). Whether this intracellular pool represents the biosynthetic precursors to IPG molecules remains to be established (Menon *et al*, 1990 (b)).

Nevertheless, the action of a phospholipase enzyme on such free GPIs would result in the production of 'protein-free' inositol molecules (Jones *et al.*, 1997). In all subsequent discussions of IPG molecules, it must be assumed that these compounds are derived from free GPI-anchors.

5.1.2.2.1 Inositol phosphoglycans in Insulin-mediated cell signalling

The most conclusive evidence for a role for inositol phosphoglycans (IPGs) in intracellular signalling cascades, has come from the study of insulin-mediated cell signalling. Firstly, the addition of insulin to either myocyte or hepatic cells, resulted in the generation of a number of molecules. The re-addition of these molecules to un-stimulated cells resulted in cellular responses that closely resembled the responses observed upon insulin-mediated stimulation (Cheng & Larner, 1985). These included the inhibition of lipolysis and glucose production, and the inhibition of several enzymes, including glucose-6-phosphate, adenylate cyclase (Saltiel & Sorbara-Cazann, 1987) and cAMP dependent protein kinase (Villalba *et al.*, 1988).

Two of these insulin-mimicking substances were specifically purified from hepatic plasma membranes, and analysis of these substances suggested characteristics with similarity to GPI-anchors. Firstly, production of these two mediators by insulin could be reproduced by the addition of *S. aureus*-derived PI-PLC to hepatocytes. As it is known that PI-PLC cleaves membrane-bound PI and GPI structures, it was concluded that the precursors of the insulin mimics were both membrane-located, and contained a PI or GPI anchorage (Saltiel & Cuatrecasas, 1986). Radiolabelling of the PI-PLC sensitive precursors was achieved, typically through the addition of tritiated glucosamine, and these radiolabelled glycolipids were incorporated into cultured myocytes. Insulin treatment of these myocytes stimulated the rapid hydrolysis of the radiolabelled glycolipid, resulting in the generation of two soluble complex carbohydrates, and diacylglycerol (Saltiel *et al.*, 1986).

Simple chemical analysis of the complex carbohydrates indicated that they contained glucosamine and inositol. More complete analysis of these mediators was performed using a variety of techniques, including radiolabelling techniques, gas and paper chromatography, and

mass spectroscopy. It was determined that the glycolipid was sensitive to nitrous acid deamination, which indicated that the molecule contained an amino group attached to a glucosamine. The glycolipid incorporated radiolabelled palmytic acid, in preference to myristic acid, oleic acid and arachidonic acid (Mato *et al.*, 1987(a)).

Despite having chemical similarities with GPI-anchors, however, significant differences were found between the chemical composition of GPI-anchors and the IPGs. For example, an IPG was found to contain up to four galactosamine residues and a phosphatidyl-chiro-inositol glycosididally linked to a non-N-acetylated glucosamine, none of which was detected in GPI-anchors (Mato *et al.*, 1987 (b)).

A second line of evidence for the role of IPG second messengers in insulin signalling came from the use of IPGs that were created artificially. These molecules were generated by the treatment of *T. brucei* VSG with bacterial PI-PLC enzymes (Misek & Saltiel, 1992). These IPGs specifically, and in a dose-dependent manner, mimicked the action of insulin in insulin-sensitive cells. For example, the artificial IPGs inhibited lipolysis, glucose-6-phosphate activity and fructose-1,6-biphosphate activity in rat adipocytes. In addition, the IPG promoted the dephosphorylation of a 70 kDa phosphoprotein, an event that was also observed after insulin stimulation (Misek & Saltiel, 1994).

Thirdly, antibodies have been created with specificity against IPG molecules. These antibodies were employed in *in vivo* and *in vitro* assays, and were shown to inhibit several of the insulin-induced effects. For example, using intact BC3H1 myocytes, anti-IPG antibodies blocked the stimulation of pyruvate dehydrogenase. The uptake of glucose, the generation of diacylglycerol and the generation of insulin mediators was, however, unaffected. The conclusion was drawn that the antibodies could inhibit certain aspects of insulin signalling, but not the entire process (Romero *et al.*, 1989). The fact that the antibodies were only active outside the cell almost certainly limited their ability to inhibit intracellular processes.

5.1.2.2.2 Inositol phosphoglycans and other cellular systems

Since the discovery of the role of inositol phosphogylcans in insulin signalling, a diverse group of hormones have been shown to stimulate the production of IPG mediators from cells. For example, IPGs have been implicated in interleukin 2-dependent signalling. In both T cell lines (CTLL-2) (Merida *et al.*, 1990) and B cell lymphoma lines (Eardley *et al.*, 1991), the addition of IL-2 induced the rapid hydrolysis of an inositol-containing glycolipid, yielding two second messengers; an inositol phosphate-glycan and either a myristylated diacylglycerol or myristylated phosphatidic acid.

The TGF-β signalling system also appears to generate IPG second messengers. Rabbit articular chondrocytes were stimulated with TGF-β1, after which time it was demonstrated that GPI-anchored proteins had undergone hydrolysis, and IPG mediators were detected (Vivien *et al.*, 1993).

Data such as this strongly implicates IPGs as messenger molecules in a number of cellular systems. However, to date the precise structural details of the IPG molecules remains to be established. It is, therefore, impossible to determine the biological activities of specific IPG molecules, and significant advances are required before the numerous putative roles of IPG molecules are determined.

5.1.2.2.3 Inositol phospholipids and IgE-mediated signalling

Receptor-mediated stimulation of RBL-2H3 cells promotes the hydrolysis of membrane inositol phospholipids, which appears to be a direct consequence of aggregation of IgE receptors. The reaction is dependent on the number of receptors aggregated and the size of the receptor clusters. Under optimal stimulatory conditions, as much as 50% of the membrane inositol phospholipids may be hydrolysed during the course of stimulation (Beaven et al, 1986). The precise function of these inositol phospholipids in the pathway of IgE-mediated stimulation, however, remains to be determined (Lo *et al.*, 1987), and this data forms only a beginning of the elucidation of this pathway.

5.1.2.2.4 Inositol phosphoglycans and Caveolae

The theory that certain GPI-anchored proteins are located in caveolae (Section 6.1.1.1), led to the suggestion that IPG-mediated signalling might be dependent on caveolae or potocytosis. However, since all GPI-anchored proteins appear to be located on the external surface of plasma membranes, any IPG molecules generated would rapidly diffuse away from the cell surface. The concentration of the IPG molecules within caveolae, which may be closed to the external environment, could solve this problem. From this concentrated pocket, the mediators could be specifically transferred into the cell, from where they could interact with intracellular signalling pathways (Anderson *et al.*, 1993). Whether this occurs in a physiological situation remains to be established.

5.1.2.2.5 Situation or species specific IPG molecules

The proposition that IPG molecules might function as second messengers in biological signalling cascades posed a problem. Without specificity in the IPG molecules, it would be almost impossible to regulate their activity, and to direct the initiation of a specific pathway. It

has, therefore, been suggested that IPG molecules may be species or situation-specific, although the nature of this specificity is unknown, to date.

Although a core glycan structure has been observed in GPI-anchors, the variety that is also seen in the different GPI-anchored structures led to the suggestion that the IPG molecules that are derived from these anchors may also show variety. For example, the GPI-anchors may contain additional ethanolamine phosphate or galactosyl residues (Roberts *et al.*, 1987), substitution with palmytic acid (Roberts *et al.*, 1988(a)), and variation in the fatty acid chains. The potential for the formation of species or situation-specific IPG molecules, therefore, exists.

The possibility that IPG molecules might be created for the specific initiation of the Type One Hypersensitivity cascade is an attractive proposal, as the capacity for developing specific anti-IPG antibodies would be realistic. To this end, the laboratory has been involved in research, to determine the nature of the IPGs derived from different disease situations. As mentioned previously, however, until the precise characteristics of the inositol phosphoglycan molecules have been elucidated, it is impossible to define specific differences between different molecules.

5.1.3 GPI-anchored proteins in T cell activation

Further evidence for a role of GPI-PLD in mammalian cell signalling cascades came from the study of T cells. It was observed that a number of proteins of importance in T lymphocyte signalling are anchored by GPI-anchors, including Thy-1 (Low & Kincade., 1985) and T-cell activating protein (TAP) (Reiser *et al.*, 1986). Several lines of evidence have suggested that the GPI anchor plays an important role in the T-Cell-Receptor (TCR)-independent activation of T lymphocytes.

One line of evidence was derived from studies in which T cells were treated with the PI-PLC enzyme, purified from *Staphylococcus aureus* culture supernatants, which is known to remove GPI-anchored proteins from the cell surface (Low & Kincade, 1985). Following PI-PLC treatment, an examination was made of the cells' viability and responsiveness. Firstly, it was confirmed that treatment with PI-PLC did not alter the viability or proliferation of the cells, as confirmed by trypan blue exclusion. Secondly, it was demonstrated that PI-PLC treated T cells were functionally impaired (Stiernberg *et al.*, 1987). When stimulated with the mitogens, phytohaemagglutinin (PHA) and concavalin A (Con A), the cells had a diminished ability to respond. However, neither the specific GPI-anchored proteins involved, nor the mechanism for the reduced responses was established (Presky *et al.*, 1990).

A second line of evidence involved the experimental cross-linking of surface GPI-anchored proteins. A rat monoclonal antibody, G7, was created, which was shown to recognise a cell surface molecule of 28 – 32 kDa that appeared to be identical to the Thy-1 antigen (Gunter *et al.*, 1984). G7 was shown to induce both IL-2 and IL-2 receptor expression in normal murine T cells and T cell hybridomas. Furthermore, it was demonstrated that the cross-linking of Thy-1 triggered a rapid rise in the cytoplasmic calcium concentration in normal murine T cells (Kroczek *et al.*, 1986), independently from the T cell receptor.

Further evidence for a role of the GPI-anchor in T cell activation was provided from experiments performed on a number of T cell clones in which the biosynthesis of selected GPI-anchored proteins was altered. Hybridoma cells were created, for example, which were deficient in the Ly-6A.2 antigen. These hybridoma cells were unable to respond to specific antigen stimulation, or stimulation by anti-TCR antibodies (Yeh *et al.*, 1988). It was concluded that the GPI-anchored Ly-6A.2 plays a critical role as a signal-transducing molecule. It was difficult to conclude that the GPI-anchored proteins were entirely responsible for the altered cell phenotype, however, since the mutagenesis might have altered other critical cell functions.

Collectively, these experiments illustrate that GPI-anchored proteins might be significant in activating T cells, although any specific mechanism of action remains unclear. Additionally, none of these experiments determined whether the signal was directly generated from the GPI-anchored proteins, or whether the proteins were acting in co-ordination with additional intracellular signalling units. Unlike transmembrane proteins, the interaction between GPI-anchored proteins and intracellular signalling cascades is unclear.

5.1.4 Regulation of GPI-PLD activity

Through the dissemination of the GPI-PLD protein in the circulating serum, the enzyme gains access to GPI-anchored proteins all over the body. It was immediately unclear, therefore, why all GPI-anchored proteins were not immediately hydrolysed by the circulating GPI-PLD.

The apparent resistance of the GPI-anchors to GPI-PLD hydrolysis is controlled by three main factors; regulation of the enzyme, regulation of the substrate and regulation of the products of the hydrolysis. There is a great deal of uncertainty regarding the mechanism of regulation of the GPI-PLD enzyme in vivo, and the following Sections aim to discuss what is currently understood.

5.1.4.1 Role of the Secondary Protein Structure in GPI-PLD activity

As discussed in Chapter 4, the activity of the GPI-PLD enzyme many be related to the conformational structure of the protein. It is believed that the loss of enzyme activity, following the incubation of the protein at alkaline pH, may be related to the nature of the protein folding *in vivo*. A number of other factors, however, contribute to the regulation of the activity of the GPI-PLD protein, including phosphorylation, glycosylation and substrate availability.

5.1.4.2 Regulation of GPI-PLD activity by phosphorylation

A role for GPI-PLD in intracellular signalling cascades requires that the protein sequence has the capacity to interact with intracellular elements. To this end, the protein sequence of GPI-PLD has been extensively studied, and comparisons have been made with protein motifs of known function (Ogiwara *et al.*, 1996). Within the amino acid sequence of GPI-PLD, several potential sites of phosphorylation, or protein modification, were identified, which may be of significance in the interaction of the GPI-PLD enzyme with intracellular signalling cascades. These sites include a tyrosine kinase phosphorylation site, a cAMP dependent protein site and multiple sites for both protein kinase C (PKC) and casein II kinase phosphorylation.

In vitro phosphorylation of GPI-PLD was studied through the use of two different kinases; the commercially available catalytic subunit of protein kinase A (PKA), which is a cAMP-dependent protein kinase (Civenni *et al.*, 1999), and a partially purified tyrosine kinase from T lymphocytes (Arni et al., 1996). The phosphorylation of GPI-PLD was demonstrated through the incorporation of a radiolabelled phosphate. The radiolabelled products were identified by phase separation in Triton X-100, followed by sequential chromatography steps, and comparison with defined molecular weight markers (Civenni et al., 1999).

Using these techniques, the GPI-PLD, partially purified from both human and bovine serum, was efficiently phosphorylated by the PKA catalytic subunit. Radiolabelled phosphate was incorporated into the 110 kDa intact form of GPI-PLD as well as into the proteolytically processed forms of 93, 63 and 56 kDa.

Furthermore, the phosphorylation was associated with a 40% decrease in enzyme activity, as measured by the hydolysis of GPI-anchored bovine erythrocyte acetylcholinesterase. De-phosphorylation of the phosphorylated GPI-PLD, using calf intestinal alkaline phosphatase, restored the enzyme activity to 100%.

Several theories have been suggested, to explain the decrease in enzyme activity. For example, it was suggested that the decrease in activity was due to a change in the enzyme conformation,

caused by the introduction of an additional negative charge. Alternatively, the newly introduced phosphate group may be localized close to the active site and interfere with substrate binding.

Treatment of the purified GPI-PLD with trypsin, which resulted in the generation of 42 and 32 kDa fragments (Heller et al., 1994), resulted in the complete removal of radiolabelled phosphate. This suggested that the phosphorylation occurred on a site that was affected by trypsin. This phosphorylation result was, therefore, compared with what was currently understood regarding the fragments generated by trypsin cleavage (Heller et al., 1994; Li et al., 1994), and it revealed that a small peptide, between amino acid residues 276 and 295 is released by trypsin treatment. This peptide also contains a putative PKA-dependent phosphorylation site at Thr-286. It was reasonable to conclude, therefore, that the phosphorylation of GPI-PLD was targeted at Thr-286.

However these researchers made a fundamental mistake in determining the potential phosphorylation sites of cAMP-dependent protein kinase A, since they used the original K/R - -S/T motif to identify potential phosphorylation site. It is now known that the PKA-specific motif is RR-S/T. The GPI-PLD protein sequence contains only one such motif, which is conserved across the 4 species for which the cDNA sequence has been described. The conserved motif is RRFS, and the phoshorylation would occur on the serine residue (position 716 – Human; 713-mouse; 719-rat; 715- ox). This residue occurs within the C-terminal peptide fragment of 33kDa. Close examination of the result from the orignal research reveals a very faint radolabelled band of 33 kDa, which was missed.

GPI-PLD also contains a single potential tyrosine phosphorylation site, at Tyr-189. Using partially purified tyrosine kinase from T lymphocytes (Arni et al., 1996), the phosphorylation of GPI-PLD was studied, using anti-phosphotyrosine antibodies and Western blotting. The results showed that the 110 kDa GPI-PLD protein was not phosphorylated by this tyrosine kinase. By comparison, the 93 kDa fragment was susceptible to phosphorylation. This suggested that the tyrosine phosphorylation site was not accessible in native GPI-PLD but became exposed after proteolytic processing. In contrast to PKA dependent phosphorylation, no significant change in GPIPLD activity was measured after tyrosine phosphorylation (Civenni *et al.*, 1999).

Whether the phosphorylation of GPI-PLD plays a significant role in the regualtion of the enzyme's activity *in vivo*, remains to be established. However, the phosphorylation of GPI-PLD represents an intriguing possibility for controlling the biological behaviour of the enzyme. The cellular location of GPI-PLD is currently unclear, although arguments have been made that

it may be located in an intracellular compartment (Section 5.1.4.3). If is it to be believed that GPI-PLD is associated with the surface, however, it is possible that the enzyme is concentrated in caveolae. In this instance, the observation that both the GPI-PLD protein, and a variety of caveolar components, are sensitive to phosphorylation (as discussed in Section 5.1.1.2) suggests a means by which these membrane microdomains might regulate interaction with intracellular signalling cascades. This represents a significant area of research.

5.1.4.3 Regulation of GPI-PLD activity by glycosylation

As mentioned earlier (Section), the GPI-PLD protein that is generated from the *GPLD1* gene consists of 814 amino acids, and has a molecular weight of approximately 90 kDa. However, extensive purification of the serum GPI-PLD enzyme, revealed that the serum enzyme was approximately 110 kDa molecular weight (Huang *et al.*, 1990). A number of lines of evidence have suggested that the enzyme undergoes glycosylation *in vivo*.

Firstly, treatment of the enzyme with N-glycosidase F resulted in the generation of a 91 kDa product, with decreased mobility on SDS-PAGE gels. This suggested that the protein was glycosylated, although the precise details were unclear (Hoener *et al.*, 1992).

Secondly, sequence analysis predicts that there were 8 potential sites for N-glycosylation. The significance of these glycosylation sites in the GPI-PLD sequence was revealed by a transfection study, in which human GPI-PLD was both expressed as a stable form in mammalian CHO cells, and as a transient form in H5 insect cells. The biosynthesis and secretion of GPI-PLD was analyzed by pulse-chase experiments.

In the CHO cells, the GPI-PLD was initially synthesized as a 105-kDa form and secreted as a 115-kDa form. In the H5 cells, by comparison, the protein was secreted as an immature 98-kDa form. The difference of the two forms was related to the nature of the post-transcriptional processing in the two cell lines. Firstly, when the differently-sized GPI-PLD proteins were treated with the Endo H enzyme (endo-β-N-acetylhexosaminidase H), which removes terminal mannose residues, the 105 kDa form was converted to a 92 kDa form, whereas the 115 kDa protein was unaffected. The conclusion was that the 105-kDa form contains oligosaccharides that are sensitive to Endo H. By comparison, the oligosaccharides on the mature 115-kDa form appear to have undergone additional processing, producing complex-type sugar chains that are resistant to Endo H.

Secondly, a panel of anti-GPI-PLD antibodies was created, with specificity against the C terminal amino acid sequence. In immunoblot experiments, these antibodies had the capacity

to recognize 105-kDa and 98-kDa forms of GPI-PLD, whereas the 115-kDa form was immuno-reactive only after denaturation.

These immunoblot experiments, in combination with the results from the Endo H treatment, led to the conclusion that the 98-kDa form was an incompletely processed molecule that had some additional oligosaccharide chains. A similar pattern of modification was observed for other glycoproteins synthesized in insect cells (Jarvis & Finn, 1995). It was possible that these terminal oligosaccharides were masking the C-terminal site that was recognized by the antibody panel. On examining the sequence of the antibodies, however, it was demonstrated that they did not incorporate any potential glycosylation sites, and therefore should be unaffected by the presence of these additional oligosaccharides. The alternative conclusion was that the terminal glycosylation was inducing a conformational change of the GPI-PLD protein, with the result that the C-terminal epitopes could not be recognized by the antibodies. Once again, a conformational change as a result of glycosylation has been observed in other glycoproteins (Cyster *et al.*, 1991).

In an *in vitro* assay, the 98-kDa form, but not the 115-kDa form, was able to release a significant amount of GPI-anchored ALP from intact membranes. The two forms had the same GPI-anchor cleavage activity in the presence of detergents. Overall, the conclusion that was drawn from this series of experiments was that GPI-PLD undergoes a conformational change after post-translational modification, which affects its immunoreactive properties. Since the immature enzyme is capable of cleaving substrate without the need for detergents, it may be assumed that the glycosylation, and subsequent conformational change alters the activity of the enzyme (Tujioka *et al.*, 1998).

It must be mentioned that, although these experiments conclusively prove that the immature mammalian GPI-PLD protein undergoes glycosylation, the glycosylation patterns of the GPI-PLD protein in an insect cell would not represent what occurs in a mammalian cell. Insect expression systems are invaluable, experimentally, as they permit the generation of large quantities of protein. However, to determine the precise details of glycosylation of the mammalian GPI-PLD protein, it would be necessary to repeat the experiments discussed above using a mammalian expression system.

5.1.4.4 Regulation of GPI-PLD activity by substrate availability

As previously mentioned (Section 5.1.4.4), the existence of high quantities of the GPI-PLD protein in the circulating serum means that if the GPI substrates were susceptible to the actions of the circulating enzyme, they would all be cleaved within seconds. As this does not happen,

it must be assumed, therefore, that the GPI substrates are protected from catalysis. A number of factors affect the presentation of these substrates, including protection of the site of catalysis by the insertion of the anchor into the membrane, and inhibition of catalysis by certain components of the membrane.

In vitro, GPI-PLD is only catalytically active against detergent-solubilised GPI-anchors. The potential effect of detergent on the activity of the GPI-PLD, was discussed in section (Section 4.13.3.22). However, the presence of detergent also has a significant effect on the presentation of the GPI-anchored protein. When inserted into a membrane bilayer, whether natural or artificial, the GPI-PLD sensitive site on the GPI-anchor is not accessible to the enzyme (Figure 4.2). The effect of the detergent is simply to release the GPI structures from the membrane, permitting access of the GPI-PLD to the specific site of action on the anchor, between the inositol and the phosphatidic acid (Davitz et al, 1989; Low & Huang, 1991).

The importance of the membrane structure on the accessibility of GPI-anchors to GPI-PLD was also demonstrated through the use of cholesterol-binding drugs. The association of certain GPI-anchored proteins with caveolae has already been reviewed (Section 5.1.1.1). A distinctive characteristic of caveolae is that they are enriched with lipids, including cholesterol, and cholesterol-binding agents were shown to disrupt the caveolar structure. The addition of such cholesterol-binding agents, such as such as streptolysin-O (Xie & Low, 1995) or saponins (Bergman & Carlsson, 1994), were found to promote the action of GPI-PLD. This supported the theory that the GPI-anchor in its natural state was resistant to the action of the GPI-PLD enzyme, but that disruption of the caveolae-structure could remove this resistance.

5.1.4.5 Regulation of GPI-PLD activity by extrinsic factors

As discussed previously (section 4.13.3.22), serum GPI-PLD is closely associated with Apolipoprotein A1. It was considered important, however, to determine the role of other lipids on the activity of the enzyme.

In vitro, the inclusion of dimyristoyl phosphatidic acid, lysophosphatidic acid and Lipid A into a preparation containing mfVSG prevented the action of GPI-PLD on the GPI-anchored substrate, even in the presence of Nonidet P-40 detergent. Inhibition was also effected by synthetic analogues of lipid A and phosphatidic acid (Low & Stutz, 1999). The effect of these lipids was observed at concentrations as low as 1 μM. The minimal structure required for effective inhibition was determined to consist of a phosphomonoester residue, linked to a 2 or 3 carbon backbone that was O or N acylated on the α or β carbon, with a fatty acid containing at least 10 carbon atoms.

It was not possible to demonstrate that these lipids directly interact with the GPI-anchor. It was suggested, therefore, that the inhibition of GPI-PLD activity was due to a direct interaction with the enzyme. This, however, remains to be proven (Low & Huang, 1993). Significantly, phosphatidic acid, lysoPA and Lipid A are naturally occurring constituents of plasma membranes, and their effect on the action of GPI-PLD is, therefore, likely to occur *in vivo*. In addition, phosphatidic acid is one of the products from the hydrolysis of the GPI-anchor. The inhibition of GPI-PLD by this lipid, therefore, suggests a mechanism by which the hydrolysis is inhibited by increasing concentrations of its product.

5.1.3 Putative role of free GPI molecules and caveolae in IgE-mediated signalling cascades.

Figure 5.1 shows a putative role for caveolae and molecules containing GPI-anchors in the IgE mediated mast cell degranulation cascade. Essentially, all of the components that have been discussed in this chapter, with reference to the role of GPI-PLD in IgE mediated stimulation of mast cells, have been drawn together in a figure. GPI-anchored proteins and free GPI molecules are shown located in the caveolae, in close proximity to a membrane-bound GPI-PLD enzyme.

Briefly, the figure shows the IgE molecule attached to the FcϵR1 receptor. Cross linking of two or more receptor-associated IgE molecules would promote initiation of the downstream Tyrosine kinases pathways. Upon activation of the cell by receptor cross-linking, it is postulated that the GPI-PLD promotes the cleavage of the GPI-anchor, thereby releasing free proteins and phosphatidic acid. By comparison, inositol phosphoglycan molecules are generated from the GPI-PLD mediated hydrolysis of the free GPI molecules. These IPG molecules may be released into the extracellular environment, or may be translocated into the intracellular space. The precise function of the IPG molecule in IgE mediated degranulation is yet to be determined.

Figure Legend
Figure 5.1 shows a putative role for caveolae and molecules containing GPI-anchors in the IgE mediated mast cell degranulation cascade.

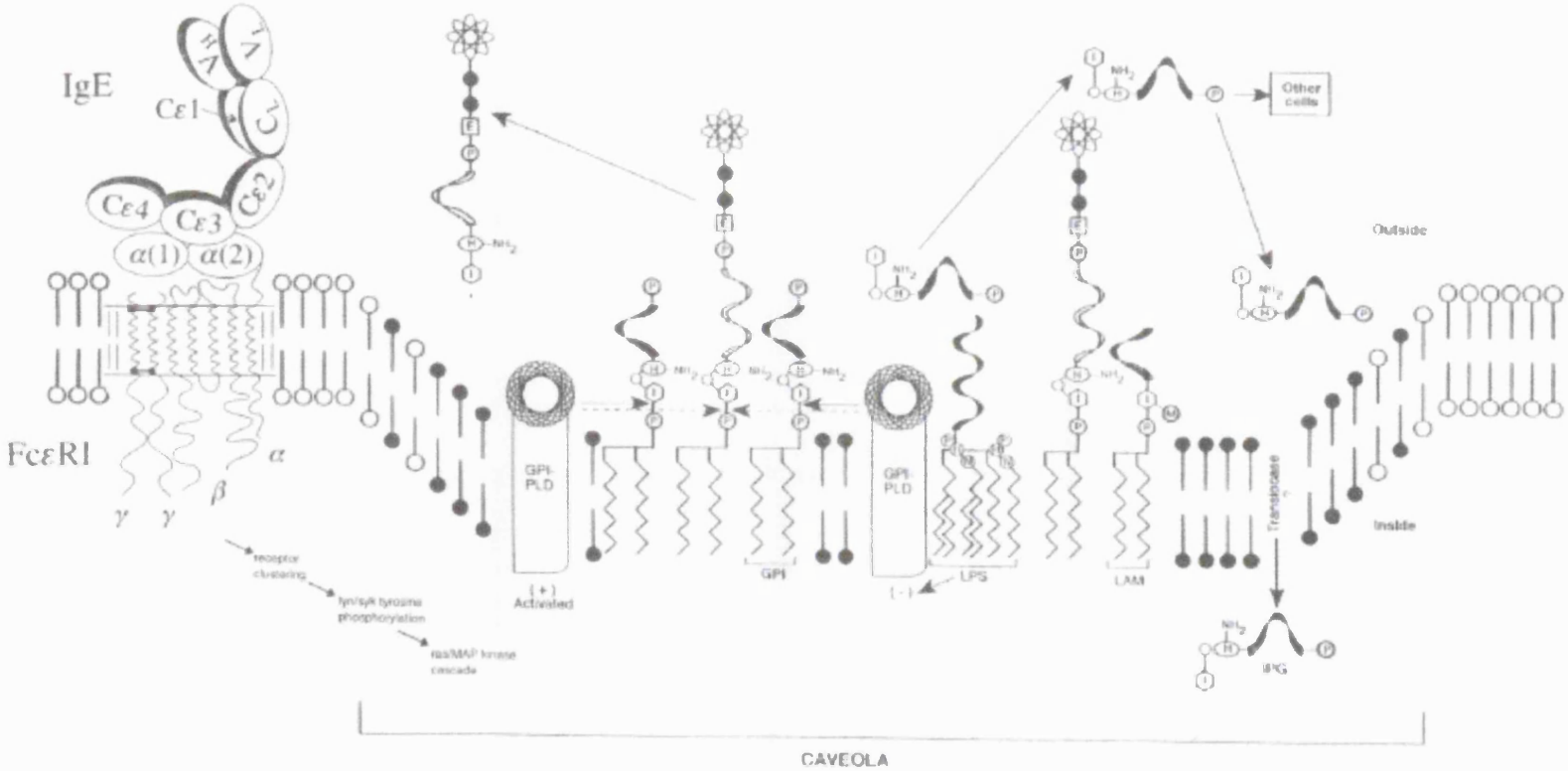
Briefly, the figure shows the IgE molecule associated with the FcεR1 receptor. Cross linking of at least 2 IgE molecules could promote the initiation of the downstream Tyrosine kinases pathways.

In addition, GPI-anchored proteins and free GPI molecules are shown, located in the caveolae, in close proximity to a membrane-bound GPI-PLD enzyme.

Upon activation of the cell by receptor cross-linking, it is postulated that the GPI-PLD promotes the cleavage of the GPI-anchor, thereby releasing free proteins, phosphatidic and from the GPI-anchored proteins, and Inositol Phosphoglycan molecules from the free GPI molecules. These IPG molecules may be released into the extracellular environment, or may be translocated into the intracellular space. The precise function of the IPG molecule in IgE mediated degranulation is yet to be determined.

FIGURE 3.18

PROPOSED ROLE FOR CAVEOLAE AND GPI-PLD IN THE IgE MEDIATED MAST CELL DEGRANULATION CASCADE



Chapter 6

Conclusions

6.1 Summary of the Experimental Results

In summary, the aim of the research performed in this thesis was to study the role of the enzyme, glycosylphosphatidylinositol-phospholipase D (GPI-PLD) in the process of mast cell degranulation. Before this was possible it was necessary to establish a methodology for the degranulation of mast cells in a laboratory environment. A mast cell line, RBL-2H3, was selected in preference to the extraction of human tissue mast cells, and the methodologies that were employed for the maintenance and stimulation of the RBL-2H3 cell line were described in Chapter Two.

Of paramount importance in the establishment of the RBL-2H3 line was to confirm both that the cells were responsive to IgE-mediated stimulation, and that the characteristics of the cell line were phenotypically similar to those previously confirmed in other RBL cell lines. IgE-mediated degranulation of the RBL-2H3 cells was effected by passively sensitising the cells in a solution containing IgE with specificity against dinitrophenol-albumin, followed by cross-linking the surface IgE with DNP-albumin. The quantities of mediators that were released upon degranulation were determined. Chapter Two described the parameters of the IgE-mediated stimulation experiments, including the optimal concentrations of the IgE and the DNP-albumin, the optimal time of incubation, and quantification of the mediators released upon degranulation.

The results indicated that the RBL-2H3 cells were stable over the course of the experimentation, and routinely released measurable levels of mediators upon passive sensitisation with anti-DNP IgE, and cross-linking with DNP-albumin. It was also confirmed that the RBL-2H3 cell line employed in this thesis was phenotypically similar to the RBL-2H3 cell line previously characterised. Subsequent research, employing the RBL-2H3 cell line, was considered sufficiently analogous to the situation observed during the *in vivo* degranulation of human tissue mast cells.

Chapter Three described the research that was performed to determine the expression of GPI-PLD in RBL-2H3 cells. Three techniques were selected for study of GPI-PLD; detection of the expressed mRNA by RT-PCR, detection of the enzyme activity using a radioactive GPI-PLD activity assay, and detection of the protein by Western Blotting. The results found no evidence of GPI-PLD mRNA in the RBL-2H3 cells, and thus it was concluded that these cells do not express the GPI-PLD gene. However, GPI-PLD protein was detected by Western blotting and its activity was confirmed using the radioactive activity assay. Analysis of the culture medium determined that the only source of GPI-PLD for the cells was the Foetal Bovine Serum that was used to supplement the medium. It was assumed that the cells take the GPI-PLD from the

serum, in which it is present in high quantities, after which time the enzyme undergoes intracellular processing.

With reference to the work performed in Chapter 3 it would be of interest to determine the nature of the uptake of GPI-PLD into the RBL-2H3 cells. A number of methods have been described in other cell types, including the tracking of radiolabelled GPI-PLD protein through pulse chase experiments. In addition, a variety of histochemical techniques have been described, which can variously locate the enzyme on the cell surface, or in an intracellular compartment.

Chapter Four described the research performed to ascertain the role of GPI-PLD in the degranulation of RBL-2H3 cells, with particular emphasis placed on the role of the enzyme in the IgE-mediated degranulation of the cells. Following the observation that the RBL-2H3 cells take up GPI-PLD from the FBS, the possibility existed that the serum GPI-PLD might be altered, to permit the study of the cells in a GPI-PLD-free environment.

After consideration of a number of alternatives to FBS, and methods to remove GPI-PLD from FBS, an alkaline inactivation method was selected. This method led to the almost complete inactivation of GPI-PLD activity in FBS, as determined by the radioactive GPI-PLD activity assay. The addition of the alkaline inactivated FBS to the EMEM culture medium, in place of normal FBS, and culture of RBL-2H3 cells in this medium, was followed by characterisation of any alterations in the phenotype of the RBL-2H3 cells. Significantly, it was discovered that RBL-2H3 cells cultured in GPI-PLD inactive FBS-containing media lost the ability to respond to surface IgE cross-linking. The degranulation of the RBL-2H3 cells, as measured by the quantity of β-glucosaminidase, was dramatically reduced, when compared with cells that had been cultured with normal FBS. Degranulation was re-established upon transferring the RBL-2H3 cells back into normal culture medium.

An future line of research involves research into the nature of the FBS. Given the complex nature of the FBS, and the time constraints of this thesis, it was impossible to determine whether other components, of significance for the culture of RBL-2H3 cells, where also altered by this alkaline incubation. It would also be of great interest to determine the nature of the inactivation of the GPI-PLD protein.

Finally, those cells that had been cultured in alkaline inactivated FBS were transferred into medium containing recombinant GPI-PLD protein. However, the addition of the recombinant GPI-PLD failed to re-establish IgE mediated degranulation responses, although it also appeared

that the cells had, indeed, taken up the recombinant protein. It was not possible to conclude, therefore, that the recombinant GPI-PLD protein could re-establish the IgE-mediated degranulation of RBL-2H3 cells.

In conclusion, the results were consistent with a role for GPI-PLD in IgE-dependent degranulation, but did not prove its role conclusively. The research performed, however, suggest a number of lines of research that can be pursued.

Conclusions

Although a number of issues need to be resolved with the RBL-2H3 cell line, the ultimate goal of this research would be to determine the expression and role of GPI-PLD in human tissue mast cells. As discussed, the mechanisms and manifestations of Type One Hypersensitivity reactions differ widely between the various animal species and humans. The purification of RBL-2H3 cells was discussed in Chapter 2, and the methodology is well established. It would be interesting to repeat the majority of experiments performed during this thesis with human mast cells.

Although the advent of animal models of allergic disease have provided a wealth of information on the induction and regulation of Type One Hypersensitivity, we are far from having a complete understanding of this important topic. Throughout the course of this thesis, the gaps in existing knowledge have been evident, and the challenge to future researchers is to reveal the nature of this complex signalling cascade.

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