IDENTIFICATION OF PHOSPHATIDYLINOSITOL TRANSFER PROTEIN AS AN ESSENTIAL REQUIREMENT FOR INOSITOL LIPID SIGNALLING IN HL60 CELLS

Phosphatidylinositol transfer protein is a requirement for G-protein-mediated inositol lipid signalling in HL60 cells.

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A thesis submitted for the degree of
Doctor of philosophy
in the
University of London

Department of Physiology
University College London
September 1994
Abstract

Ligand binding to G-protein-linked receptors triggers the activation of the phosphoinositide specific phospholipase C (PLC). Activated PLC catalyses the hydrolysis of phosphatidylinositol(4,5)bisphosphate to generate two intracellular second messengers, inositol 1,4,5-trisphosphate and diacylglycerol. Purification and molecular cloning have revealed that PLCs are made up of at least three families β, γ and δ. Members of the PLC β family are regulated by heterotrimeric G-proteins, either α-subunits of the Gq family or by βγ subunits.

It has previously been established that when HL60 cells are permeabilized with the cytolytic toxin, streptolysin O, cytosolic proteins leak out of the cells and this is associated with a loss of GTPγS and fMetLeuPhe-mediated activation of PLC. In this thesis cytosol-depleted cells were used as a reconstitution system to identify the cytosolic component(s) necessary for inositol lipid signalling. Two reconstituting factors were isolated from rat brain cytosol. The major reconstituting factor was identified as a 35 KDa protein phosphatidylinositol-transfer protein (PI-TP). The minor reconstituting factor proved to be PLC β1.

PI-TP is a ubiquitous protein found in all mammalian tissues examined to date. It was originally identified by its ability to bind and transfer PI and PC from one membrane compartment to another in vitro. Here it is shown that PI-TP can restore responsiveness to GTPγS and fMetLeuPhe to activate inositol lipid signalling and this reconstitution is Ca2+ and Mg-ATP dependent.

PI-TP and PLC β1 synergise to restore GTPγS-stimulated inositol phosphate production. Examination of the kinetics of reconstitution with PI-TP shows that PI-TP increases the rate of inositol phosphate production by GTPγS or fMetLeuPhe-regulated PLC β. The data suggest that the function of PI-TP in inositol lipid signalling cannot be entirely explained by its established biochemical in vitro transfer activity but plays a more direct but as yet undetermined role. Substrate presentation to the inositol lipid kinases) as a mechanism to allow PI-TP to enhance G-protein-regulated PLC β activity is discussed.
Abbreviations and symbols

approx.         approximately
ATP             Adenosine 5'-triphosphate
βME             beta mercaptoethanol
BSA             Bovine serum albumen
°C              degrees centigrade
cAMP            Adenosine 3',5'-cyclic-monophosphate
DAG             1,2 Diacylglycerol
DFP             Di-isopropylfluorophosphate
cDNA            complementary Deoxyribonucleic acid
DTT             Dithiothreitol
DMSO            Dimethyl sulphoxide
EDTA            Ethylenediamine tetra-acetic acid
                (disodium salt)
EGTA            Ethyleneglycol-bis-(b-aminoethylether)
                N,N,N',N' tetra-acetic acid
FMetLeuPhe      N-formyl-methionyl-leucyl-phenylalanine
FPLC            Fast protein liquid chromatography
GDP             Guanosine 5'-diphosphate
GTP             Guanosine 5'-triphosphate
GTPγS           Guanosine 5'-O-(3-thiophosphate)
Gpp[NH]p         Guanosine 5' -[βγ-imido]triphosphate
Gpp[CH₂]p        Guanosine5' -[βγ-methylene]triphosphate
HEPES           N-[2-Hydroxyethyl]piperazine-N'
                [2-ethanesulphonic acid]
PBS             Phosphate buffered saline
pCa             -Log₁₀ Ca²⁺ [M]
PA              Phosphatidic acid
PI              Phosphatidylinositol
PIP             Phosphatidylinositol-4-phosphate
PIP₂            Phosphatidylinositol-4,5-bisphosphate
PIP₃            Phosphatidylinositol-3,4,5-trisphosphate
PIPES           Piperazine-N,N' -bis[2-ethylsulphonic acid]
PKA             Cyclic AMP-dependent protein kinase
PKC             Ca²⁺ -associated, phospholipid dependent protein kinase (protein kinase C)
PMSF            Phenyl-methyl-sulphonyl-fluoride
<table>
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<tr>
<td>PVDF</td>
<td>Polyvinyl difluoride</td>
</tr>
<tr>
<td>S.A.</td>
<td>Specific activity</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
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<tr>
<td>Temed</td>
<td>N,N,N',N'-Tetra methyl ethylene diamine</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TLCK</td>
<td>N α-p-Tosyl-L-Lysine Chloromethyl Ketone</td>
</tr>
<tr>
<td>Tris-HCl</td>
<td>Tris(hydroxymethyl)aminomethane-hydrochloride</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Polyoxyethylene sorbitan monolaurate</td>
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Chapter 1

Introduction

Hormones, growth factors, neurotransmitters and other agonists can bind to specific receptors on the external surface of the cell membrane to cause a physiological response. These extracellular signals are transmitted across the cell membrane by receptors, leading to the generation of a variety of second messengers such as diacylglycerol (DAG) and inositol phosphates derived from inositol phospholipids. Second messengers can initiate subsequent biochemical reactions such as the phosphorylation and dephosphorylation of proteins eventually leading to the modulation of many cellular processes, for example proliferation, phagocytosis or secretion.

The work described in this thesis was carried out in a human promyelocytic cell line HL60 cell which can be differentiated towards a neutrophil-like cell. This cell line is a useful model system for studying many of the lipid signalling pathways since unlike neutrophils they can be prelabelled to equilibrium with phospholipid precursors. These cells can be induced to undergo differentiation with a variety of stimuli. Differentiation with DMSO or dibutyryl cAMP leads to the expression of membrane receptors for the chemotactic peptide fMetLeuPhe (1) and the acquisition of most known functions of the neutrophil such as chemotaxis, lysosomal enzyme secretion, phagocytosis, respiratory burst activity and bacterial killing. The neutrophil has provided many new insights in the past which have relevance to other cell types. For instance, receptor-mediated PLD activation (2) and G-protein regulation of inositide-specific phospholipase C (PLC) (3) were first shown in neutrophils. For this reason, conclusions drawn from studies in HL60 cells can provide a prototype for cell activation by agonists that utilise these phospholipases in other cell-types.
Receptors present on neutrophils include GTP-binding (G)-protein-linked receptors and receptors for the Fc domain of IgG (Fc\(\gamma\)R). Fc\(\gamma\)Rs activate their downstream effectors via tyrosine phosphorylation whereas G-protein-linked receptors such as fMetLeuPhe can stimulate PI-3-kinase, phosphatidylinositol specific PLC, phospholipase D (PLD) and phospholipase A\(_2\) (PLA\(_2\)) to generate multiple second messengers (Fig. 1.1). The function of individual second messengers has been evaluated by controlling their endogenous production or by externally adding them to permeabilized cells. The second messengers may converge or diverge to finely tune specific responses in the neutrophil, for example an increase in Ca\(^{2+}\) stimulated by PLC activation, may not evoke a response itself, but facilitate the activation of another signalling pathway such as PLA\(_2\) (4).

1.0.1 G-proteins

1.0.1.1 Heterotrimeric G-proteins

The superfamily of GTP-binding proteins that have intrinsic GTPase activity can be subdivided into heterotrimeric and monomeric G-proteins. Heterotrimeric G-proteins (G-proteins) have a role in transducing receptor-generated signals across the plasma membrane to the cytosol. G-proteins are composed of three distinct subunits \(\alpha\), \(\beta\), and \(\gamma\). The \(\beta\)- and \(\gamma\)-subunits exist as a tightly associated complex that functions as a unit and associates with many \(\alpha\)-subunits. In the resting state the \(\alpha\)-subunit binds GDP and is strongly associated to \(\beta\gamma\) complex. Receptor occupation stimulates the exchange of GDP for GTP whereupon the \(\alpha\)-subunit dissociates from \(\beta\gamma\). Subsequently both can serve as regulators of effector proteins. The \(\alpha\)-subunits possess intrinsic GTPase activity which catalyses the hydrolysis of GTP to form GDP leading to the reassociation of \(\alpha\) and \(\beta\gamma\) (5).

G-proteins are categorised on the basis of their \(\alpha\)-subunit. To date, cDNA that encodes 21 distinct G-protein \(\alpha\)-subunits have been cloned and they have been divided into four separate groups \(G_i\), \(G_q\), \(G_{12}\) and \(G_s\). In addition, at least four distinct \(\beta\)- and six \(\gamma\)-subunits have been described (6).
Fig. 1.1 Multiple second messengers derived from signalling pathways triggered by the G-protein-coupled fMetLeuPhe receptor and Fcγ receptor.

Rapid activation of three phospholipases and the activation of tyrosine phosphorylation in addition to the activation of phosphatidylinositol-3-kinase are known to be regulated by occupied receptors. The products of the individual reactions which function as second messengers are indicated.
The G-protein families can be further classified as pertussis toxin-sensitive or pertussis toxin-insensitive depending on whether the $G_\alpha$ subunit possesses a cysteine residue four amino acids from the carboxy-terminal. This residue constitutes the site of pertussis toxin-mediated ADP-ribosylation which leads to an uncoupling of the G-protein from its receptor (6).

All four heterotrimeric G-protein families are represented in the HL60 cell but only members of the $G_i$ and $G_q$ families have been shown to regulate PLC. The $G_i$ family consists of $G_{i1-3}$, $G_{iA-B}$, $G_{i1-2}$, $G_q$ and $G_\zeta$. It is represented in the HL60 cell by $G_{i2}$ and $G_{i3}$ which transduce fMetLeuPhe receptor signalling (7). All of this family of G-proteins are substrates for pertussis toxin except $G_\zeta$. The $G_q$ family has at least five members $G_{11}$, $G_{14}$, $G_{15}$, $G_{16}$ and $G_q$ (6) and of these $G_{11}$, $G_q$, $G_{15}$ and $G_{16}$ are present in HL60 cells. The $\alpha$-subunits of members of the $G_q$ family lack the the site for pertussis toxin modification (8) making them pertussis toxin insensitive. $G_q$ and $G_{11}$ are 88% homologous whereas $G_{15\alpha}$ and $G_{16\alpha}$ are more distantly related having 57% and 58% amino acid identity respectively. Most G-proteins present in HL60 cells are also found in a wide variety of cell and tissue types (8), except $G_{16}$ and $G_{15}$ which are exclusively expressed in haematopoietic cells (9, 10). Furthermore, $G_{16}$ expression is down regulated when HL60 cells are terminally differentiated toward neutrophil-like cells with DMSO (9). This may indicate that $G_{16}$ regulates a cell-specific signalling pathway which is not present in neutrophils.

1.0.1.2 Monomeric G-proteins

The eukaryotic monomeric GTP-binding proteins are collectively referred to as the Ras superfamily which can be subdivided into the Ras, Rho, ARF and Rab families. Ras proteins are essential components of receptor-mediated signal transduction pathways stimulating proliferation and differentiation (11). Rho regulates the assembly of focal adhesions and actin stress fibres in response to growth factors (12). ARF was originally identified as a cofactor for the efficient ADP-ribosylation of $G_8$ by cholera toxin (13). But, it is now clear that ARF proteins along with Rab proteins have a part to play in endocytic and exocytic pathways of vesicular traffic (14, 15).
Members of the Ras and Rho families have been identified in neutrophils. Rap1A, a Ras protein, is a substrate for protein kinase A (PKA) and therefore may play a role in mediating the inhibitory effects of cAMP-elevating agents upon receptor-mediated cell activation (16). Rac 1 and Rac 2, members of the Rho family, are a requirement for NADPH oxidase activation, mediator of the respiratory burst (17). Rac 1 and Rac 2 expression increases when HL60 cells are differentiated towards neutrophils (18).

1.1 Phospholipase C

PLC catalyses the hydrolysis of phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-bisphosphate (PIP$_2$) in vitro but the main substrates for PLC in vivo are the PIP and PIP$_2$ which are concentrated at the plasma membrane (19, 20). These polyphosphoinositides are derived from PI by the sequential action of PI-4-kinase and PIP-5-kinase (20) (Fig. 1.2). These enzymes and their opposing phosphatases have been isolated and localised to the plasma membrane and cytosol (21). PIP and PIP$_2$ are made up of a saturated stearoyl residue in the $sn$-1 position and a polyunsaturated arachidonyl residue in the $sn$-2 position (22). PLC cleaves polyphosphoinositides at an ester link to generate two second messengers diacylglycerol (DAG) and inositol trisphosphate (IP$_3$) (Fig. 1.3). Phosphatidylcholine-specific PLC has been described which cleaves PC to generate DAG and phosphocholine but it will not be discussed here.

Both DAG and the inositol moiety of IP$_3$ can be recycled to replenish PIP$_2$ at the plasma membrane during continued stimulation. DAG is rapidly phosphorylated to PA by DAG kinase which can be subsequently converted to CMP-PA via CTP-phosphatidate cytidylyltransferase. IP$_3$ can be phosphorylated to $I(1,3,4,5)P_4$ which is not a ligand for the IP$_3$ receptor (23). Ultimately, both inositol phosphates are dephosphorylated to myo-inositol by phosphatases. PI is synthesised from myo-inositol and CMP-phosphatidate by CMP-phosphatidate:inositol phosphatidyltransferase (PI synthase) (Fig. 1.3). Recycling of the inositol moiety of IP$_3$ is blocked by Li$^+$ ions at the level of inositol polyphosphate 1-phosphomonoesterase and inositol monophosphate phosphomonoesterase (24).
PLC can cleave PIP$_2$ to generate cyclic counterparts to the inositol phosphates mentioned above i.e. clns(1:2)P, clns(1:2,4)P$_2$ and clns(1:2,4,5)P$_3$. There is no evidence for enzymatic interconversion of the cyclic and noncyclic inositol phosphates in cell extracts. The proportion of these cyclic products generated and their physiological relevance is unknown (24).

It was first established in 1985 that PLC could be regulated by a G-protein which was assigned the generic title G$_p$ (3). Recent studies have led to a more complete picture of G-protein regulation of PLC, which is outlined below. In neutrophils G-proteins couple a number of receptors to PLC such as fMetLeuPhe (25), C5a (26), IL-8(27) and an ATP purinergic receptor (28-31). These belong to a superfamily of G-protein-coupled receptors. These G-protein-linked receptors typically have seven membrane spanning domains which form three extracellular and three intracellular loops and possess a cytosolic carboxy-terminal tail (32). A recent study of the fMetLeuPhe receptor has found that the second intracellular loop and the carboxy terminal tail are essential for its coupling to G-proteins (33).

1.1.1 Location of PLC

Because it was recognised that membrane-associated PLC could be regulated by G-proteins (3) PLC was thought to be tightly associated with the membrane. But, most PLC enzymes have been purified from cytosolic fractions (34, 35). Of the three members of the PLC family purified i.e. PLC$\beta$, PLC$\gamma$, and PLC$\delta$ (see section 1.1.2), PLC$\beta$ is the only membrane-associated PLC although it has also been found in the cytosol. PLC$\beta$ was purified from washed particulate fractions of bovine brain and was found to be absolutely identical to that purified from the cytosol on the basis of molecular weight, monoclonal antibody reactivity, tryptic peptide mapping (36) and by nucleotide sequence (37).

Apart from membrane-associated PLC there have been PLCs isolated from cytoskeleton and nucleus. Vaziri and Downes (38) identified a G-protein regulated PLC (85 KDa) that was specifically associated with a component of detergent-insoluble cytoskeleton in turkey erythrocytes. In addition, PLC$\beta$1 and other components of the inositol lipid signalling pathway have been localised to the cytoskeleton-like matrix of the nuclei of swiss 3T3 cells (39, 40). This nuclear inositol signalling pathway can
Fig. 1.2 Structure of the phosphoinositides. Reproduced from Meldrum et al (60).
Fig. 1.3 Cycle of reactions that interconnect the products of receptor-stimulated inositol lipid metabolism.

PIP₂ is initially hydrolysed to generate diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP₃⁺) by phospholipase C. The top half of the cycle (inositol lipid cycle) illustrates how the DAG is removed. The DAG is phosphorylated to phosphatidate (PA) by DAG kinase and this is recycled back to phosphatidylinositol (PI) via CDP-diacylglycerol (CDP-DAG). The bottom half of the cycle (inositol phosphate cycle) illustrates the metabolism of I(1,4,5)P₃ (IP₃⁺). (IP₃⁺) can be metabolized by two separate routes: dephosphorylation to I(1,4)P₂ or phosphorylation to I(1,3,4,5)P₄. I(1,3,4,5)P₄ is then dephosphorylated to I(1,3,4)P₃, an inactive isomer of I(1,4,5)P₃. Sequential dephosphorylation of the inositol phosphates leads to the release of free inositol, and thus combines with CDP-diacylglycerol to regenerate PI. Key to symbols: [a] phospholipase C; [b] DAG kinase; [c] CTP phosphatidate cytidyl transferase; [d] PI synthase; [e] PI-4-kinase; [f] PI-4-P phosphatase [g] PI-4-P 5-kinase; [h] PI(4,5)P₂ 5-mono-esterase; [i] I(1,4,5)P₃ kinase; [j] phosphatase specific for removing the 5'-phosphate from either I(1,4,5)P₃ or I(1,3,4,5)P₄; [k-m] phosphatases with differing specificity for the different inositol phosphates. The site of lithium ions inhibition is shown.
be activated by insulin-like growth factor 1 without any effect on plasma membrane polyphosphoinositides.

1.1.2 Isoforms of PLC

A number of distinct PLC enzymes have been purified from a variety of mammalian tissues (41). Comparison of nucleotide sequences has indicated that mammalian PLCs can be divided into three types PLC β (150-154 KDa), PLC γ (145-148 KDa), and PLC δ (85-88 KDa) (34, 35, 42) and each type has more than one subtype (43, Table 1.1). The mammalian PLC β family has four members, PLC β1-4 (44-47) and in addition there are two PLC β-like enzymes from Drosophila, Dros Norp A and Dros p21 (48, 49). PLC β4, the most recently purified PLC β from bovine retina is most likely a mammalian homologue of Dros NorpA since PLC β4 had the greatest sequence identity to this PLC (47). Recently, a novel PLC β has been purified from Xenopus Oocytes that possesses 60 % identity to mammalian PLC β3 (50). There are two members of the mammalian PLC γ family identified to date PLCγ1 and PLCγ2 (51, 52). Recently a PLC γ has been identified in Drosophila designated PLC γd (53). There are three members in the PLC δ family PLC δ(1-3) (54-56).
<table>
<thead>
<tr>
<th>Name</th>
<th>Size (kDa)</th>
<th>Source (Ref)</th>
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<tr>
<td>PLC α Family</td>
<td></td>
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</tr>
<tr>
<td>δ1</td>
<td>85</td>
<td>Rat and Bovine Brain (37, 59)</td>
</tr>
<tr>
<td>δ2</td>
<td>85</td>
<td>Bovine Brain (54, 60)</td>
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<tr>
<td>δ3</td>
<td>84</td>
<td>Human Fibroblast cDNA (56)</td>
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Table 1.1 Classification of PLC isozymes from different tissues
PLC β, PLC γ and PLC δ are discrete gene products. There is very little homology between each of them except at two regions, one of 170 amino acids and the other of 260 amino acids. These regions have been assigned X and Y and are approx. 60% and 40% identical respectively, between the three PLCs (Fig. 1.4). The X and Y regions are considered to constitute, separately or jointly, the catalytic domain of the enzyme (37, 61). All three PLCs contain an amino-terminal approx. 300 amino acids long that precedes the X region. PLC β is unique in possessing a 450 amino acid carboxy-terminal. PLC γ has a long sequence of approx. 400 amino acids separating the X and Y regions which contains src homology domains SH2 and SH3 not present in either PLC β or PLC δ (51). SH2 and SH3 domains were initially identified as noncatalytic regions common to a variety of src family tyrosine kinases but subsequently have been found in a large number of unrelated proteins such as GAP, PI-3-kinase and tyrosine phosphatases. These domains govern protein-protein interactions i.e. SH2 has the ability to target a molecule to specific tyrosine phosphorylated sequences present in other proteins such as receptor tyrosine kinases and the SH3 domains target molecules to cytoskeletal elements (62).

PLCs with molecular weights of 62-68 KDa, originally referred to as PLC αs, have been purified. The cDNA corresponding to one of these smaller enzymes has been cloned and sequenced but the deduced amino acid sequence revealed that there were no X and Y regions (41). Recent studies have suggested that the cDNA actually encodes a thiol:protein disulphide oxidoreductase that carries no PLC activity (63) and some of the 62-68 KDa enzymes may be proteolytic fragments derived from PLC β, PLC γ and PLC δ (41).

### 1.1.3 Regulation of PLC β

Research over the last several years has revealed the existence of distinct mechanisms of activation of two families of PLC. The consensus now is that PLC β isozymes are activated by G-proteins while PLC γ isozymes are activated by the phosphorylation of specific tyrosine residues. The mechanism of activation of PLC δ has not yet been elucidated.

The detection of the G-proteins involved in the activation PLC β isoforms (originally referred to as Gp) was the result of the convergence of the research of several laboratories. In 1990 Taylor et al (64) tested the ability of cholate extracts of liver plasma membrane treated with GTP
Fig. 1.4 Linear representation of three types of mammalian PLC isoforms.

Open boxes denote the regions of approx. 170 and approx. 260 amino acids, respectively, of similar sequences found in all eukaryotic PLCs. The numbers above each box refer to the first and last amino acids. The degree of sequence identity with the corresponding region of PLC β1, PLC γ1 or PLC δ1 is indicated by percentage. Reproduced from Sue Ghoo Rhee (61).
analogues to activate partially purified PLC from the same membranes and found the extracts effective. Assaying for PLC-activating-activity through a number of chromatography steps, they purified a 42 KDa G-protein α-subunit that was not a substrate for pertussis toxin and was different from any G-protein α-subunit known at that time (65). The α-subunit was later resolved into two proteins of 42 KDa and 43 KDa (66).

Independently, Pang and Sternweis were purifying a 42 KDa G-protein α-subunit from rat brain using an affinity matrix of immobilised G-protein βγ-subunits (67). This α-subunit was not recognised by antisera for any of the known α-subunits, nor was it a pertussis toxin substrate. Subsequently, it was found to share sequence homology with two proteins identified by Strathmann and Simon designated Gaq and Gap11, members of a novel G-protein family Gq (8). Very soon Sternweis and his colleagues were able to demonstrate fluoride activation of partially purified PLC with their Gaq proteins (68). The mixture of Gaq and Gap11 specifically activated PLCβ1 but not PLCγ1 or PLCδ1 when reconstituted in vitro in the presence of GTPγS (66).

Further investigation of the specificity of the interaction between different G-protein α-subunits and PLC was assessed by introducing cDNAs corresponding to various α-subunits into Cos-7 cells and measuring inositol phosphates formed after stimulation with ALF₄⁻ (69). Transfection with Gaq or Gap11 cDNA resulted in a marked increase of inositol phosphate production, but co-transfection of either α-subunit cDNA with PLCβ1 cDNA resulted in even higher levels. PLCβ1 was seen to be specifically activated by Gaq and Gap11 while other Ga cDNAs such as Ga12, Gapβ, Gapα and transducin were inactive.

The relative abilities of members of the Gq family to activate PLCβ isoforms was determined by the same method. PLCβ1, while activated by all Gq family α-subunits was preferentially activated by Gaq and Gap11. Similarly, PLCβ2 was capable of being activated by all Gq α-subunits although the response was attenuated. PLCβ2 differed from PLCβ1 in having a preference for Gap16 (70). A comprehensive study by Hepler et al (71) using recombinant proteins revealed an order of potency of PLC activation by Gaq and Gap11, PLCβ1 = PLCβ3 >> PLCβ2. This was confirmed by Jhon et al (72) and extended to include Gap16. Since Gap16 and PLCβ2 are specifically expressed in HL60 cells, it follows that the pertussis toxin-insensitive response observed in neutrophils and HL60
cells may be a result of $G_{16}$ regulating PLC $\beta_2$. The receptors driving this have not been described.

While there are pertussis toxin-insensitive routes of neutrophil activation, fMetLeuPhe receptor-mediated activation of PLC in HL60 cells is pertussis toxin-sensitive (73). The fMetLeuPhe receptor of HL60 cells interacts with $G_{i2}$ and $G_{i3}$ (74) yet recombinant $G_{i2}$ and $G_{i3}$ failed to activate any PLC $\beta$ isoforms in an in vitro reconstitution assay (71). The explanation for this apparent anomaly comes from the breakthrough of Camps et al (75). They demonstrated that PLC from partially purified HL60 cytosol could be activated with $\beta\gamma$-subunits purified from bovine brain retinal transducin ($\beta\gamma_i$). Subsequently, co-transfection assays revealed $\beta\gamma$-subunit activation was isozyme-specific i.e. PLC $\beta_2$ is more sensitive to $\beta\gamma$ activation than PLC $\beta_1$ (76). In addition, Katz et al (77) illustrated that receptor-mediated release of $\beta\gamma$ from $G_i$ can activate PLC $\beta_2$ in a model system, and this activation could be inhibited by pertussis toxin treatment. In addition $G_{i2}$ was inhibitory, presumably by scavenging the free $\beta\gamma$ complex.

By reconstituting purified protein components with lipid vesicles containing $[^3H]P_2$, Park et al (78) have shown the order of potency of $\beta\gamma$ subunit activation of PLC $\beta$ isoforms to be PLC $\beta_3 > PLC \beta_2 > PLC \beta_1$. In the light of this information it is likely that PLC $\beta_2$ and PLC $\beta_3$ present in HL60 cells, can be regulated by the $\beta\gamma$-subunits of $G_{i2}$ and $G_{i3}$. Indeed, there is evidence that $G_{i6}$ and free $\beta\gamma$-subunits may cooperatively regulate PLC $\beta_2$. The presence of $G_{i6}$ and $\beta\gamma$-subunits in cos-7 cells led to a marked synergistic activation of PLC $\beta_2$ (79). Thus, PLC $\beta$ isozymes can be regulated by pertussis toxin sensitive and insensitive mechanisms.

Stimulation of G-protein effectors with the $\beta\gamma$ complex was not without precedence. $\beta\gamma$ subunits were first recognised as effector activators of atrial muscarinic K$^+$ channels (80). Since then, many more diverse effectors have been described such as adenylate cyclase, PLC, PLA$_2$, tyrosine kinase (81) and PI-3-kinase (82). Most recently, studies have been carried out to examine how important the make-up of the $\beta\gamma$ complex is in affecting their ability to activate PLC $\beta$. Since there are at least four $\beta$ subunits and six $\gamma$ subunits, there are many permutations theoretically possible. Although many of these combinations appear to be allowed there is some selectivity in the interactions of $\beta$ and $\gamma$ and functional differences have been described between different complexes.
(83). Co-transfection assays using various combinations of $\beta\gamma$-subunits have suggested that the nature of the $\beta$ subunit may play a role in determining the preference of the $\beta\gamma$ complex for effectors while the nature of the $\gamma$ subunits may be important for determining the relative affinity of the $\beta\gamma$ complexes for a specific $\alpha$-subunit.

1.1.4 Regions of PLC $\beta$ involved in G-protein interaction

Smrcka and Sternweis (84) demonstrated that a $Ga_q/\alpha_1$ mixture gave an additive response with $\beta\gamma$ subunits during activation of PLC $\beta$3. PLC $\beta$3 was activated by this $\alpha$-subunit mixture even at saturating concentrations of $\beta\gamma$ subunits, indicating that there are separate sites on PLC $\beta$ for the interaction of G-protein $\alpha$- and $\beta\gamma$-subunits. Moreover, they were able to show that the interaction differs depending on the PLC $\beta$ isoform. The region of the PLC $\beta$ required for $Ga_q$ activation was localised to the carboxy-terminal. It corresponds to residues 903-1142 which can be subdivided into two sequences, one extending from residues Thr-903-Gln-1030 that are required for both particulate fraction association as well as for activation by $\alpha$-subunits and the other extending from residues Gln-1030 to Leu 1142 that was only required for interaction with $\alpha$-subunits (85, 86).

On the other hand, the section of PLC $\beta$2 required for activation by the $\beta\gamma$ complex has been isolated to the N-terminal region of PLC $\beta$2 with amino acid sequences extending to the end of the Y region (79). A more well-defined site of interaction has recently been proposed; a novel domain of approx. 100 residues has been described that is considered to be a $\beta\gamma$-complex-binding site. It is called the pleckstrin homology (PH) domain since it was originally identified in the protein pleckstrin (87). Within the PH-domain, the $\beta\gamma$-complex-binding site has been localised to the C-terminal portion and sequences immediately distal to it (88). PH domains have been found in a whole host of proteins, for example Ras-GAP, Sos, $\beta$ARK and isoforms of PLC. PLC $\beta$(1-4) have one PH domain at the amino terminus which would explain its activation by $\beta\gamma$. But, what cannot be so easily explained is that PLC $\gamma$ and PLC $\delta$ also possess PH-domains but are not known to be activated by G-proteins (89). There is one exception, activation of PLC $\gamma$ by EGF is pertussis toxin-sensitive in hepatocytes (90).
1.1.5 GAP activity of PLC β

Deactivation of monomeric, GTP-binding proteins such as Ras and Rho is promoted by GTPase activating proteins (GAPs). The observation that the rate of GTP hydrolysis of many G-protein α-subunits is slower \textit{in vitro} than \textit{in vivo} led Bernstein et al (91) to investigate whether $G_{a_q}$ requires a GAP for its efficient deactivation. Purified M1 cholinergic receptor and a mixture of $G_q$ and $G_{11}$ were reconstituted in lipid vesicles. Addition of purified PLC β1 caused a rapid burst of hydrolysis of $G_{q/G_{11}}$-bound GTP, 50-fold higher than in its absence. Furthermore, PLC β1 stimulated the receptor-mediated steady state GTPase activity of $G_{q/11}$ up to 20-fold. The study concluded that PLC β1 acts as a GAP for $G_{q/11}$ its physiological regulator.

1.1.6 Regulation of PLC γ

The first indication of the mode of activation of PLC γ came from its amino acid sequence (92). The sequence showed that PLC γ possessed domains homologous to the products of various tyrosine kinase-related oncogenes. Subsequently PLC γ was revealed as being a substrate for receptor tyrosine kinases such as the platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors (93, 94). It is now established that, growth factors such as EGF and PDGF activate PLC γ and other proteins by their receptors dimerising upon ligand occupation enabling the cytoplasmic kinase domains to phosphorylate each other on specific tyrosine residues. Autophosphorylation acts as a molecular switch to create docking sites for the SH2 domains of PLC γ1 thereby promoting the recruitment of the enzyme from the cytosol to the plasma membrane where it subsequently becomes a target for activation (95). High affinity binding of the SH2 domain requires that the phosphotyrosine be embedded within a specific amino acid sequence of the receptor (96). Once bound, PLCγ1 is phosphorylated by the active receptor on specific serine and tyrosine residues at its carboxy-terminal region. An increase in PLC γ1 phosphorylation has been correlated to an increase in PI turnover in 3T3 cells (97). There are four sites of tyrosine phosphorylation on PLCγ i.e. Tyr-771, Tyr-783, Tyr-1254 and to a lesser extent Tyr-472 (98, 99). Site directed mutagenesis has allowed the essential residues to be determined. Tyr-783 and to a lesser extent Tyr-1254 are essential for PLCγ activation in intact cells. However, mutation at residues Tyr-771, Tyr-783 and Tyr-1254 does not change the catalytic activity of PLCγ1.
measured *in vitro* (93). The series of events subsequent to PLCγ1 phosphorylation which lead to full activation are not fully understood but a model has been proposed by S.G.Rhee whereby PLCγ1, after binding to a tyrosine kinase receptor and sustaining tyrosine phosphorylation, undergoes a conformational change that allows the intrinsic SH3 domain to bind to the membrane cytoskeleton, simultaneously positioning the proposed catalytic X and Y regions at the cytoplasmic face of the cell membrane. (19).

The other member of the PLCγ family PLCγ2, also contains SH2 and SH3 domains. Moreover, the equivalents of Tyr-771 and Tyr-783 of PLCγ1 are conserved, but an equivalent of Tyr-1254 is not present. It has been speculated that the mechanism of activation of PLCγ2 also involves tyrosine phosphorylation. PDGF can stimulate the phosphorylation of PLCγ2 when overexpressed in rat-2 cells or in NIH 3T3 fibroblasts (100, 101) but is unlikely to be the natural activator since PDGF receptors are not normally found in haematopoietic cells where PLCγ2 is specifically expressed (52).

Cross-linking of the Fc receptors for IgG (FcγR) on U937 cells, mediated by immune complexes lead to a rapid tyrosine phosphorylation of PLCγ1 (102). These receptors are not intrinsic tyrosine kinases but recruit cytosolic tyrosine kinases such as *fyn* and *lck* to activate PLCγ1 by a mechanism similar to growth factor receptors. Human neutrophils do not contain PLCγ1 in substantial amounts and therefore it is expected that the relevant isoform that is regulated by the FcγRs in neutrophils would be PLCγ2.

**1.1.7 Inhibition of PLCγ1 and PLCβ1**

There is evidence that PLCγ1 activity in intact cells may be tightly regulated by a negative modulator that binds it or its substrate PIP2 (103). Candidates for the former include several proteins that co-immunoprecipitate with PLCγ1 antibodies. A candidate for the latter is profilin, a cytosolic actin-binding protein which binds four to five molecules of PIP2 with high affinity and inhibits hydrolysis of this lipid by unphosphorylated PLCγ1 *in vitro* but not by PLCγ1 phosphorylated by purified EGF receptor kinase (104).
Studies in Jurkat cells (a human leukaemic T cell line) have shown that agents that lead to an increase in the concentrations of cAMP such as forskolin or activators of protein kinase C such as PMA inhibit PLCγ1 activity (but not PLC β1 or PLC δ1). Both lead to a reduction in the extent of tyrosine phosphorylation of PLC γ1. Incubation with either agent results in the phosphorylation of a serine residue of PLC γ1 at the 1248 position. It has been surmised that PKC, activated by an increase in the concentration of DAG after PIP2 hydrolysis, phosphorylates PLCγ1 and thereby provides a negative feedback signal to limit the magnitude and duration of receptor signalling. The fact that the same serine residue is phosphorylated by PKC and PKA (105) might imply that it is a common mechanism of PLC γ1 inhibition. Phosphorylation at this position could make PLCγ1 an unfavourable substrate for tyrosine kinase or a more readily accessible target for tyrosine phosphatase.

PLC β1 can be phosphorylated by PKC but not PKA at Ser-887 both in vitro and in vivo. The catalytic activity of PLC β1 is unaffected by phosphorylation when assayed in vitro. However, it has not been determined whether PKC-mediated PLC β1 phosphorylation has any affect on its interaction with Gαq or Gβγ (106).

### 1.1.8 Consequences of PLC activation

PLC catalysed hydrolysis of PIP2 generates two second messengers DAG and IP3. IP3 induces a biphasic rise in intracellular calcium, an initial transient phase followed with a more sustained elevation of the intracellular Ca2+ concentration (107). The earlier rise in Ca2+ is a direct result of IP3 binding to a specific IP3 receptor. This receptor is a ligand-activated calcium channel located at intracellular Ca2+-storing organelles. Subsequent to IP3 binding, the channel opens to release Ca2+ into the cytosol. There is a distinct all or nothing response due to the fact that Ca2+ exerts a positive feedback effect on the IP3 receptors (107). IP3 alone or in conjunction with inositol (1,3,4,5) tetrakisphosphate (IP4) a phosphorylated derivative of IP3 (catalysed by I(1,4,5)P3-kinase) (23) initiates the second phase. This later phase is caused by sustained Ca2+ entry across the plasma membrane. It appears that Ca2+ entry across the plasma membrane is somehow coupled to the depletion of intracellular Ca2+ stores, a process designated as "capacitative calcium entry" by Putney (108). Three fundamental mechanisms for the signal for capacitative calcium entry have been investigated A) A diffusible messenger is
released when the intracellular Ca\(^{2+}\) pool is depleted and this diffuses to the plasma membrane to activate Ca\(^{2+}\) entry. There is a candidate termed the calcium influx factor (CIF) described by Randriamampita and Tsien (109). B) The IP\(_3\) receptor interprets the amount of Ca\(^{2+}\) leaving intracellular stores and in some way transduces the signal to IP\(_4\) receptors, predominantly located at the plasma membrane which allow Ca\(^{2+}\) influx (23). C) Emptying of the intracellular Ca\(^{2+}\) pool causes a conformational change in the organelle and this change is transmitted to the plasma membrane calcium channels either by direct coupling or via cytoskeleton (110).

The IP\(_3\)-Ca\(^{2+}\)-signalling pathway has been implicated in a variety of functions in a multitude of cell types for example fertilisation and early development in *Xenopus Oocytes*, smooth muscle contraction stimulus-response coupling in many secretory cells such as HL60 cells and is predicted to have a fundamental role in modulating neural activity and the neural plasticity responsible for memory (107).

The immediate downstream effector of DAG, the other second messenger associated with PIP\(_2\) hydrolysis is the serine/threonine specific PKC. PKC is a key enzyme in signal transduction evidenced by the fact PKC activators such as phorbol esters, interfere dramatically with proliferative and differentiation events in the cell by promoting oncogenic transformation. PKC represents at least nine isoforms (PKC \(\alpha-\Theta\)), their cDNAs having been cloned from different species or cell lines (111). They have identical catalytic domains and vary in their regulatory domains. PKC isoforms can be divided into two groups, the Ca\(^{2+}\)-dependent PKCs (\(\alpha, \beta\), \(\beta\).2 and \(\gamma\)) and the novel PKCs which are Ca\(^{2+}\)-independent (\(\delta, \epsilon, \zeta, \eta, \Theta\)).

**1.2 PI-3-Kinase**

FMetLeuPhe can stimulate the accumulation of phosphatidylinositol-(3,4,5)trisphosphate (PI(3,4,5)P\(_3\)) and phosphatidylinositol-(3,4)bisphosphate (PI(3,4)P\(_2\)) in neutrophils (112). Similarly, growth factors such as EGF (113) and activated oncogenes have this ability in a variety of cells types (114). PI(3,4,5)P\(_3\) is generated by the phosphorylation of PI(4,5)P\(_2\) at the 3-position of the inositol ring catalysed by phosphoinositide (PI)-3-kinase and rapidly dephosphorylated to PI(3,4)P\(_2\) (112). Mammalian PI-3-kinase is a heterodimer composed of an 85 KDa regulatory subunit (p85) and a catalytic subunit of 110 KDa.
p85 possesses two src homology SH2 domains and one SH3 domain and has no PI-3-kinase activity whereas p110 shares homology throughout its C-terminal half to the S. Cerevisiae protein VPS34p (115). VPS34p is a PI-3-kinase activity in yeast required for vacuolar protein sorting (116). Two forms of p85 (p85α and p85β) have been characterised (117) although so far only the p85α is found to be associated with p110 in PI-3-kinase from bovine brain (115).

1.2.1 Regulation of PI-3-kinase

PI-3-kinase was originally characterised as an enzyme that associated with tyrosine kinases including activated growth factor receptors e.g. PDGF receptor to mediate mitosis (118). The current model of PI-3-kinase activation by receptor tyrosine kinases is that when the receptor is activated by ligand-binding it autophosphorylates, facilitated by constitutive tyrosine kinase activity. Subsequently, the phosphorylated receptor recruits PI-3-kinase via the SH2 domains of p85. As a consequence, PI-3-kinase appears to become tyrosine phosphorylated and activated, but the order in which this happens remains unclear. Several transforming non-receptor tyrosine kinases with SH2 domains also have the ability to activate PI-3-kinase by forming complexes through phosphotyrosyl containing peptide sequences (96).

Tyrosine kinase-sensitive PI-3-kinase may be regulated by small GTP-binding proteins. For instance, PI-3-kinase can be activated by Rho in GTPγS-stimulated platelet lysates (119). In this case it has not been established if there is a direct interaction or if there is a intermediary protein involved. It is feasible that there is a direct interaction since p85 possesses a rho/GAP-like domain that has been shown to be necessary for its GTPase-activating functions (117). However, there is evidence of PI-3-kinase being a direct target of Ras (120). Ras in the active, GTP-bound conformation has been shown to bind p110 through the Ras effector site. Furthermore, transfection of Ras into COS cells results in a large elevation of 3′ phosphorylated phosphoinositides.

Pertussis toxin inhibition of the accumulation of PI(3,4,5)P3 induced by fMetLeuPhe in neutrophils indicated the involvement of the heterotrimeric G-protein Gi (121). Since tyrosine phosphorylation seemed fundamental to PI-3-kinase activation, G-protein-mediated activation of PI-3-kinase was originally considered to be via G-protein-dependent regulation of protein
tyrosine kinase activity, but this has proved not to be the case. Firstly, Stephens et al (122) demonstrated that the majority (greater than 90 %) of fMetLeuPhe-stimulated PI-3-kinase activity in neutrophils was directly regulated by G-proteins. This G-protein-mediated response was completely resistant to a panel of tyrosine kinase inhibitors confirming that PI-3-kinase activation was not a downstream effect of a protein tyrosine kinase. Subsequently, Stephens et al (82) have partially purified a distinct isoform of PI-3 kinase from neutrophils and U937 cells that is regulated by G-protein βγ-subunits. Preliminary studies show that βγ-sensitive PI-3-kinase a) is immunologically distinct from tyrosine kinase-sensitive PI-3-kinase b) cannot be activated by Gaα, Gaβ2, Gaγ1, or Gaq c) has a molecular mass of 210-220 KDa and d) can be inhibited by wortmannin, a specific inhibitor of tyrosine kinase-sensitive PI-3 kinase at higher IC50 than tyrosine kinase specific PI-3-kinase. Their data suggest that regulation of PI-3-kinases is directly analogous to the pattern of PLC regulation i.e. different receptor transduction systems specifically regulate dedicated isoforms of effector protein.

1.2.2 Function of PI-3-kinase

The physiological role of PI(3,4,5)P3 in cells is still the subject of much research, but the rate of accumulation of PI(3,4,5)P3 has been associated with superoxide production and actin polymerisation in neutrophils (121). Recently wortmannin was demonstrated to be a specific inhibitor of PI-3-kinase by binding irreversibly to the 110 KDa catalytic subunit of the enzyme (123). A recent study with wortmannin has confirmed that PI-3-kinase mediates the respiratory burst in neutrophils and has a modulatory but not primary role in cytoskeletal rearrangements (124). PI-3-kinase also mediates histamine secretion in rat basophilic leukaemia 2H3 cells (123). Finally, an increase of PI(3,4,5)P3 in PC12 pheochromocytoma cells in response to growth factors correlated with an increase in the activation of MAP kinase (mitogen activating protein kinase) implying that PI(3,4,5)P3 may activate MAP kinase kinase (118).

1.2.3 PI-directed PI-3kinase

PI-3-kinase can catalyse the phosphorylation of PI, PI-4-P and PI(4,5)P2 in vitro but it preferentially phosphorylates PI(4,5)P2 in vivo (112). The resultant PI(3,4,5)P3 is metabolised by a predominantly membrane-associated 5-phosphatase that can be distinguished from PI(4,5)P2-5-
phosphatase (112). PI(3,4)P₂ can be dephosphorylated to generate PI-3-P but this pathway is only secondary to PI(3,4,5)P₃ production. Here lay an anomaly since PI-3-P preferentially accumulates in unstimulated intact neutrophils and there is a high basal rate of synthesis of PI-3-P that does not change in response to agonists that activate PI-3-kinase (122). The explanation came with the discovery of a novel mammalian PI-3-kinase that was specific for PI and distinct from the more established PI(4,5)P₂-directed enzyme. The PI-directed PI-3-kinase was purified from bovine adreno-reticulosa cell lysates. It was a) not recognised by antibodies against PI(4,5)P₂-directed PI-3-kinase b) not activated by tyrosine phosphorylated receptor peptide and c) not inhibited by wortmannin. It is thought that this enzyme may have a closer relationship to VPS34p than PI(4,5)P₂-directed PI-3 kinase (125).

1.3 Phospholipase A₂

1.3.1 Identification of arachidonic acid as a second messenger

When neutrophils or HL60 cells are stimulated with fMetLeuPhe, arachidonic acid (AA) is released from phospholipids catalysed by cytosolic phospholipase A₂ (29, 126, 127). The role of AA had, up until recently, been considered to be solely a precursor of the eicosanoids. Now, AA is considered a novel intracellular second messenger.

Very broadly, the PLA₂ enzymes can be divided into two groups, the "secreted" and the "cytosolic" forms. They differ in their location, molecular weight and substrate preference. Neutrophils secrete these PLA₂s during degranulation in response to chemotactic and phagocytic stimuli. The secreted PLA₂s have a molecular weight of 13-18 KDa (128). The secretory PLA₂s do not demonstrate any selectivity among the fatty acids in the sn-2 position of phospholipids.

In contrast, cytosolic PLA₂ specifically cleaves AA from the sn-2 position of a glycerophospholipid releasing the corresponding lyso-phospholipid (129). Phosphatidylinositol (29), phosphatidylcholine, phosphatidylethanolamine and triglycerides are all potential substrates (130). The molecular weight of PLA₂ is in the range of 85-110 KDa and has been identified in many cell types e.g. U937 (131), RAW 264.7 cell (132), rat brain (133) and platelets (134). Cytosolic PLA₂ is also present
in HL60 cells (135). In quiescent cells PLA_2 is present in the cytosol but in response to elevated Ca^{2+} (> 300 nM) translocates to membranes by virtue of a 45 amino acid Ca^{2+}-dependent phospholipid binding motif. PLA_2 shares this property with PKC, GTPase activating protein and PLC_γ1 (131).

1.3.2 Regulation of PLA_2

FMetLeuPhe-stimulated AA release is G-protein-dependent. The evidence for this is that a) GTP_γS and NaF are potent activators of AA release in permeabilized neutrophils (136), HL60 cells (135) and platelets (137) b) GTP_γS and fMetLeuPhe are synergistic in activating PLA_2 in acutely permeabilized neutrophils (138) and c) pertussis toxin treatment results in the inhibition of fmetLeuPhe-mediated release of AA in guinea pig human neutrophils (29, 127), rod outer segments (139) and HL60 cells (29).

Since PI-PLC is also activated via a G-protein thus elevating Ca^{2+} and activating PKC, the question arises whether PLA_2 activation is a consequence of the products of PLC activation or if it is activated by a distinct G-protein. There is substantial evidence for the latter. Under conditions were the Ca^{2+} concentration is controlled with EGTA buffers and PLC activity is inhibited by the removal of MgATP from the assay, PLA_2 activation is still possible with fMetLeuPhe and GTP_γS in human neutrophils (138) and differentiated HL60 cells (135). In addition, treating cells with neomycin prevented inositol phosphate but not AA production in both thyroid cells and human platelet membranes (137, 140). Furthermore, AA release but not PLC activity was inhibited by pertussis toxin in FRTL5 thyroid cells (140).

Assuming PLA_2 is directly activated by a G-protein it remains to be established whether it is the α-subunit or the βγ-complex that is the transducer of PLA_2 activation in neutrophils. In rod outer segments the activation of PLA_2 is mediated by βγ-subunits of transducin. The α-subunit of transducin slightly stimulated PLA_2 activity alone and inhibited the βγ complex-induced increase in AA presumably by reassociating with it (139). The fact that PLA_2 activation is inhibited by pertussis toxin in many cell types (4) could be interpreted that Gi/Go is the G-protein linking the receptor to PLA_2.
Over the last few years the role of PLA$_2$ phosphorylation has come under scrutiny. Most recently, Lin et al (141) have demonstrated that PLA$_2$ is a substrate for MAP kinase and MAP kinase-mediated phosphorylation is essential for PLA$_2$ activity. First, they showed that purified PLA$_2$ can be stoichiometrically phosphorylated by MAP kinase and this enhanced the activity of the enzyme. Second, PLA$_2$ containing a mutation at the consensus site for MAP kinase (ser-505) failed to be phosphorylated. Third, phosphorylation of PLA$_2$ isolated from phorbol-treated cells by MAP kinase was dramatically reduced as compared with that from untreated cells. With this information they have proposed a model whereby a ligand binds to its cognate receptor and activates PLC causing an increase in the intracellular concentration of Ca$^{2+}$. Increased Ca$^{2+}$ causes PLA$_2$ to translocate from the cytosol to the membrane. At the same time DAG activates PKC, giving rise to the activation of MAP kinase. Activated MAP kinase can then phosphorylate PLA$_2$ at serine-505, causing an increase in PLC$_{A2}$ activity. Both the Ca$^{2+}$-dependent membrane association of PLA$_2$ and phosphorylation of PLA$_2$ are required for the full activation of PLA$_2$ (141).

1.3.3 Function of arachidonic acid

Studies with intact and permeabilized neutrophils and HL60 cells show a strong correlation between the ability of agonists to induce AA release and exocytosis (29, 138). Secretion is independent of cyclooxygenase and lipooxygenase pathways indicating that AA is acting as an intracellular second messenger.

In addition, there is evidence that AA has a modulatory role in in the activation and maintenance of O$_2^-$ generation by NADPH oxidase (142). The precise site of action of of AA mediating the respiratory burst within neutrophils remains to be positively identified but activation of the PKC isoform PKC$_{\gamma}$ has been suggested (143). On the other hand, Dana et al (144) have demonstrated that while PKC and PLA$_2$ are both necessary for NADPH oxidase activation in human neutrophils, PLA$_2$ operates downstream from PKC.

Cleavage of phosphatidic acid by PLA$_2$ will generate both AA and lyso-phosphatidic acid (LPA). LPA is emerging as an important intercellular messenger. The best documented example of LPA production and release comes from studies of platelet activation. LPA accumulates rapidly in
thrombin-stimulated platelets, predominantly through the PLA$_2$ attack of newly formed PA in the inner leaflet of the plasma membrane. Since LPA is quite water soluble most of it is released extracellularly via secretion or diffusion from leaky platelets. LPA has specific G-protein coupled receptors on a variety of cell types but not HL60 cells. Activation of LPA receptors can evoke a wide range of biological effects including Ca$^{2+}$ release from intracellular stores, stimulation of fibroblast proliferation, platelet aggregation, cellular motility and tumour cell invasiveness (145).

1.4 Phospholipase D

1.4.1 Location of PLD

In 1984 it was established that fMetLeuPhe stimulation of intact human neutrophils gave rise to an elevation of phosphatidic acid (PA) that was not derived by phosphorylation of DAG (a product of PLC activity) but directly from a phospholipid (2). Phospholipase D (PLD) catalyses the hydrolysis of phosphatidylcholine (PC) at the terminal phosphate diester bond to generate PA plus choline (146). This fMetLeuPhe-induced rise in PA is rapid and transient only lasting 20 sec, after which the levels decline slowly as PA is converted to DAG via phosphatidate phosphohydrolase (147). PLD was originally discovered in plants (148) but later it was identified in mammalian tissues (149) where it was localised to the microsomal fraction of rat brain and lung. Since then, PLD has been detected in many cell types including human neutrophils (2, 150). It seems most likely that there are distinct isoforms of PLD which may be specific to particular tissues or alternatively one cell could contain multiple forms of PLD in a similar fashion to the phospholipase C isoforms (41). Partially purified PLD from disparate sources demonstrate different properties. For example, there is a rat brain PLD that is membrane bound, cation-independent, activated by oleic acid and other surfactants and hydrolyses PC and PE in vitro (151). Another form of PLD is present in plasma which is soluble, sensitive to EGTA and hydrolyses PI (152). Furthermore, neutrophils express two forms of PLD which hydrolyse PI, one was soluble, Ca$^{2+}$-dependent with a neutral pH optimum, the other was found in azurophilic granules, Ca$^{2+}$-independent and had an acidic pH optimum (153, 154).
Since PLD has been associated with both the particulate and cytosolic fractions of various cell types, attempts have been made to purify it from both sources (155, 156). Most recently, Brown et al (157) have partially purified a PLD activity as a peripheral protein from a 0.4 M NaCl extract of HL60 cells. In contrast to other workers (156) they have found no cytosolic PLD activity.

1.4.2 Regulation of PLD

PLD can be activated by G-proteins via receptors such as fMetLeuPhe in intact neutrophils (2) and GTPγS in permeabilized HL60 cells (158). GTPγS-induced activation can occur at very low concentrations of Ca²⁺ but it is enhanced by micromolar Ca²⁺ concentrations. Ligand occupied tyrosine kinase receptors also have the ability to activate PLD (159). The observation that receptor-induced activation of PLD in intact neutrophils can be inhibited with tyrosine kinase inhibitors indicated that these disparate receptors share tyrosine phosphorylation as a common pathway to activate PLD (160). In addition, pervanadate induced an increase in tyrosine phosphorylation that was associated with a concomitant increase in PLD activity (160). Together with receptor-mediated activation of PLD it is possible for calcium alone in micromolar concentrations or PMA in the presence of Mg.ATP to activate PLD (2, 161-163). In MDCK cells PMA and nucleotide receptors have been shown to utilise the PKC isoform PKCa to regulate PLD activity (164).

GTPγS, while stimulating PLD activity in HL60 cells will also activate PLC (165). It was of interest to establish whether PLD was activated as a consequence of PLC-catalysed PIP₂ hydrolysis or directly by a discrete pathway. Geny et al (158) dissociated PLC activity from PLD activity by eliminating the PLC activity through metabolic inhibition in HL60 cell and GTPγS-induced activation of PLD remained although the magnitude of the response was reduced. This was confirmed in MDCK cells were elimination of PLC activity with neomycin had little or no effect on PLD activity (164). Furthermore, PMA could not substitute for GTPγS in the activation of PLD in permeabilized cells. In fact, GTPγS and PMA activate PLD synergistically (158).

G-protein linked receptors, PKC and Ca²⁺ can each independently activate PLD but there is also co-operative interaction between their individual pathways (158, 166).
1.4.3 PLD is activated by a small GTP-binding protein ARF

Activation of PLD in HL60 cells by GTPγS and fMetLeuPhe requires the presence of both the cytosolic and membrane components (167, 168). HL60 cells depleted of their cytosol by prolonged incubation with streptolysin O lost the ability to activate PLD with GTPγS or fMetLeuPhe. Cytosol prepared from rat and bovine brain or HL60 cells restored GTPγS-dependent PLD activity when added back to the permeabilized cells. This demonstrated that cytosolic proteins played an essential role in the activation of PLD. Cells, depleted of cytosol were used as a assay system to find the reconstituting factor. Bovine brain cytosol was sequentially chromatographed on a series of columns and a 16 KDa cytosolic component that had reconstituting ability was isolated. The amino acid sequence of the reconstituting factor was determined and was identified as the small GTP-binding protein, ADP-ribosylating factor ARF-1 (169). This protein is one of a highly conserved family of GTPγS-binding proteins with a similar degree of relatedness to both the RAS-superfamily and the heterotrimeric G-protein α-subunits (170).

1.4.4 Function of PLD activation

PA production is considered to be the primary role of PLD activation, whereby PA acts as a second messenger mediating cellular responses particularly secretion. Intact human neutrophils stimulated with fMetLeuPhe demonstrate a time course for PA formation that coincides with that of secretion (171). In addition, inhibiting PA production with ethanol (which diverts PA to inactive phosphatidylethanol) suppresses secretion in many secretory cells including mast cells (172), neutrophils (173) and HL60 cells (171). Since PLD is activated by ARF which is associated with vesicular trafficking, it is possible that PLD activity may be intimately linked to membrane fusion events. PA has also been implicated in the activation of PI(4)P-kinase (174) and stimulation of DNA synthesis leading to cell proliferation (175).
1.5 Reconstitution of the inositol lipid signalling pathway in HL60 membranes and permeabilized cells

G-protein-regulated inositol lipid signalling in HL60 cells is the subject of this thesis. The components of the pathway under investigation consist of the fMetLeuPhe receptor, G-protein and the PLCs. It would seem as if these three proteins constitute the whole pathway since activation of PLC has been successfully demonstrated \textit{in vitro} with purified M1 receptor, G-protein subunits and PLC \( \beta 1 \) (176). But, one must be careful when extrapolating the activity of enzymes in the intact cell from \textit{in vitro} studies.

The lipid composition of the plasma membrane has an influence on the ability of individual phospholipases and PI-3-kinase to hydrolyse specific substrates. When phospholipases are assayed \textit{in vitro} the substrates are presented in synthetic vesicles often in the presence of surfactants. For this reason studies of phospholipase activities \textit{in vitro} may have limited relevance to their activity \textit{in vivo}. For example, PLC has the ability to hydrolyse PI, PIP and PIP\(_2\) \textit{in vitro} but \textit{in vivo} PLC only recognises PIP\(_2\) and PIP, PI being in some way obscured (177). In addition, the surface pressure of membranes can influence PLC substrate specificity. In a recent study using a controlled monolayer system, PLC activity towards both PIP and PIP\(_2\)-containing monolayers was shown to be surface pressure-dependent and at pressures approaching those of biological membrane structures, PIP\(_2\) hydrolysis is favoured over PIP hydrolysis (178). For these reasons endogenous membrane preparations are more relevant for the examination of PLC activity.

Cockcroft and Stutchfield (73) demonstrated Ca\(^{2+}\)-dependent G-protein and fMetLeuPhe receptor-mediated activation of PLC in HL60 cell membranes. They repeated these studies in "acutely permeabilized" HL60 cells, where the activator and permeabilizing agent (streptolysin O) are added to the cell at the same time. Streptolysin O makes holes in the plasma membrane large enough for cytosolic proteins such as lactate dehydrogenase to leak from the cells and allows the rapid introduction of GTP\(\gamma\)S into the interior of the cell (179). PLC activation in this permeabilized HL60 cell system showed the same Ca\(^{2+}\)-dependence as HL60 membranes and the same rank order of potency for guanine nucleotide analogues. In addition the receptor-mediated activation of PLC
could be demonstrated in "acutely-permeabilized" differentiated HL60 cells (73).

PLC activity can be assayed by monitoring the release of [\(^3\)H]-IP\(_3\) and [\(^3\)H]-IP\(_2\) from the membranes of [\(^3\)H]-inositol labelled HL60 cells. The amount of product released can be expressed as a function of the total inositol lipids. Using this approach it was noted that the extent of stimulated polyphosphoinositide hydrolysis in membranes was different to that in "acutely-permeabilized" HL60 cells (73). In fact, there was a 10-fold disparity between the two preparations, the permeabilized cells demonstrating the greater response. Clearly there was a loss of an essential component(s) during the preparation of HL60 membranes that was present in "acutely-permeabilized" cells.

The first approach to determine what the essential cytosolic component(s) might be, was to set up an assay for potential candidates. To this end, HL60 cells were permeabilized for 40 min to allow all of the cytosol to leak from the cells and the cells washed to remove leaked cytosol. This generated "ghost" cells that had a markedly reduced endogenous response to GTP\(_{\gamma}\)S. The "ghost" cells retain G-proteins and the substrate for PLC. The pool of PIP\(_2\) is normally replenished from the much larger pool of PI by PI-kinase and PI(4)P-5-kinase which are also membrane-associated. Cytosol-depleted cells were used as the assay system to ascertain what cytosolic component(s) could restore responsiveness to GTP\(_{\gamma}\)S in activating PLC.

Because it was assumed that all of the components required for efficient inositol lipid signalling were known, the initial hypothesis was that the cytosolic component was an isoform of PLC. This theory was compounded when the time course of the release of PLC activity into the extracellular medium upon cell permeabilization apparently had some correlation with the progressive loss of response to GTP\(_{\gamma}\)S-mediated activation of PLC after permeabilization (180).

PLCs from rat brain were best characterised at that time, therefore rat brain cytosol was selected as a source of PLCs. Thomas et al (180), demonstrated that crude rat brain cytosol could restore GTP\(_{\gamma}\)S-mediated activation of PLC in cytosol-depleted HL60 cells in a time- and concentration-dependent manner. To identify which of the isoforms of PLC present in brain cytosol is activated by the G-protein, rat brain cytosol was fractionated on a heparin affinity column and all of the
fractions were assayed for in vitro PLC activity. Three major peaks of activity were identified. The fractions constituting each peak were pooled, concentrated and tested for their ability to restore GTPγS-stimulated PLC activation in cytosol-depleted cells. Two peaks were effective at reconstitution, one eliciting a much stronger response than the other. The following chapters describe the purification of these two reconstituting factors from rat and bovine brain. The characteristics of the major reconstituting factor are described and its role in inositol lipid signalling discussed.
Chapter 2

Materials and methods

2.1 Materials

Amersham
Myo-[2-\textsuperscript{3}H]inositol, specific activity 16.6 \( \mu \text{Ci/mmol} \)
Phosphatidyl[2-\textsuperscript{3}H]inositol 4,5-bisphosphate
Scintillation fluid (PCS)
ECL western blotting detection kit

BioRad
Electrophoresis apparatus-mini protean II dual slab cell
Transfer apparatus-mini TRANS-BLOT-electrophoretic transfer cell
SDS-PAGE reagents

Gifts
Anti-PLC \( \beta \)1 monoclonal antibody \quad \text{S.G. Rhee}
Phosphatidylinositol II \quad \text{K. Wirtz}
Phosphatidylcholine transfer protein \quad \text{K. Wirtz}
Nonspecific lipid transfer protein \quad \text{K. Wirtz}
Pre-nonspecific lipid transfer protein \quad \text{K. Wirtz}

Murex diagnostics
Streptolysin O

Pharmacia
Heparin Sepharose column (100ml-HiTrap)
Heparin Sepharose CL-6B (for rat cytosol)
Phenyl Superose columns (HR5/5 and HR 10/10 )
Superdex 75 (HR 16/60), gel filtration column
Superose 12 (HR 10/30), gel filtration column
NAP 10, Sephadex G-25 columns

Sigma
Ammonium formate
Bovine serum albumin
Dowex 1 X 8 resin, 100-200 dry mesh, 8% cross-linked
HEPES
Phosphatidylinositol-4,5-bisphosphate
PIPS
Routine laboratory reagents

Whatman
DE52, weak anion exchange column

Miscellaneous
Conductivity Meter
War ing blender
Filtration apparatus
Immobilon PVDF
Pertussis toxin
Rotatest shaker, model 100
Scintillation fluid
(Ultimagold)

Radiometer, Copenhagen
Phillips
Amicon and Centricon
Millipore
Porton Products, Porton Down,
Luckam limited
Sailsbury, Wiltshire, England
Canberra Packard

2.2 Purification of factor X from rat brain

2.2.1 Preparation of rat brain cytosol

7 g of rat brain were homogenised in 10-15 ml 20 mM PIPES, 3 mM KCl pH 6.8 supplemented with 5 mM EGTA, 5 mM EDTA, 10 mM benzamidine, 1 mM DTT, 2 μM pepstatin A, 5 μg/ml aprotinin, 100 μM TLCK, 0.1 mM leupeptin and 1.0 mM PMSF in a glass homogeniser. The homogenate was centrifuged for 60 min at 100,000 X g. The supernatant was filtered through a 45 μm filter.
2.2.2 Fractionation of rat brain cytosol on a heparin affinity column

A 40 ml bed volume heparin sepharose column was washed extensively in 20 mM PIPES, 3 mM KCl pH 6.8 prior to use. Approx. 20 ml of filtered rat brain cytosol was loaded onto the column, and eluted with a 300ml 0-500 mM NaCl gradient. The column was run at 0.25 ml/min and 50 X 6 ml fractions were collected. The fractions were assayed for PLC activity (see 2.7.2). Three major peaks emerged, peak I, II and III (Fig.3.3).

2.3 Purification of PLC B1 from bovine brain membrane

2.3.1 Preparation of bovine brain membrane

One frozen bovine brain was thawed and homogenised in a Waring blender in ice cold 20 mM Tris-HCl, 3mM KCl pH 7.6 (Tris buffer) supplemented with 5 mM EDTA, 5 mM EGTA, 10 mM benzamidine, 1 mM dithiothreitol, 1 µg/ml soyabean trypsin inhibitor, 5 µg/ml aprotinin, 2 µM pepstatin A, 100 µM TLCK, 0.1 mM Leupeptin and 1 mM PMSF at 2 ml/g tissue. The homogenate was centrifuged overnight at 10,000 X g at 4°C to pellet the membranes. Membrane pellets could be stored at -70°C.

Five such bovine brain pellets were resuspended in 1:1 v/v 20 mM Tris-HCl, 3 mM KCl, 5mM EDTA, 5mM EGTA pH 7.6 and washed by centrifugation overnight at 10,000 X g at 4°C.

2.3.2 KCl extraction and ammonium sulphate precipitation

The washed pellet (approx. 1L) was resuspended using a blender in 1:1 v/v 20 mM Tris-HCl, 4 M KCl, pH 7.6 giving a final concentration of 2 M KCl and centrifuged at 10,000 X g overnight at 4°C. To concentrate the proteins the supernatant was decanted and subjected to 0-60% ammonium sulphate precipitation i.e. 361g/L ammonium sulphate was added to the supernatant, and stirred at 4°C for 1 hour and then centrifuged at 10,000 X g for two hours. The resultant salt pellet was resuspended in 20 mM Tris buffer and dialysed until the conductivity was below the equivalent of 25 mM NaCl.
Fig. 2.1 Columns and salt gradients used in the purification of PLC β1.

A] DE52 column, proteins were eluted with a linear NaCl gradient
B] heparin sepharose column, proteins were eluted with a partially linear ascending NaCl gradient
C] phenyl superose column, a two step descending KCl gradient eluted the proteins
D] Mono Q, a two step ascending NaCl gradient eluted proteins.
2.3.3 DE52 weak anion exchange column

Buffer A: 20 mM Tris-HCl, 3 mM KCl, pH 7.6, 0.02 % (w/v) sodium azide
Buffer B: 20 mM Tris-HCl, 3 mM KCl, 1 M NaCl, pH 7.6, 0.02 % (w/v) sodium azide

A 400 ml bed volume DE52 column was equilibrated in buffer A. The column was loaded at 2 ml/min and the proteins are eluted with a linear 0-1 M NaCl gradient with a volume of 1.4L (Fig. 2.1A). The column was run at 2 ml/min, collecting 140 x 10ml fractions. 3 μl of very 3rd fraction was assayed for 5 min at 37°C for in vitro PLC activity. Those fractions with maximal PLC activity, were pooled and concentrated to 50 ml by ultrafiltration. The conductivity of the sample was equivalent to approximately 100 mM NaCl.

Column Conditions

Flow: 2 ml/min
Fraction size: 10 ml
Fraction collector: 5 min
Chart Recorder: 0.1 cm/min

Gradient

0-50 min 0 % Buffer B
50-1400 min 0 %-100 % Buffer B

2.3.4 Heparin affinity column

Buffer A: 20 mM Tris-HCl, 3 mM KCl pH 7.6, 0.02 % (w/v) sodium azide
Buffer B: 20 mM Tris-HCl, 3 mM KCl, 1 M NaCl, pH 7.6, 0.02 % (w/v) sodium azide

The concentrated sample from DE52 was passed through a 45 μm disposable filter and chromatographed using FPLC on heparin sepharose (CL-613, 100 ml Hitrap, Pharmacia custom column). The column was equilibrated with 10 % Buffer B (100 mM NaCl), the sample was loaded and the gradient run as described below and in Fig. 2.1B. Fractions were assayed for PLC activity and three peaks emerged. Those fractions with PLC B1 activity were identified by western blotting with an anti-PLC B1 antibody. Those fractions containing PLC B1 (between 400 mM and 650 mM NaCl) were pooled and concentrated to 50 ml.
2.3.5 *Phenyl superose hydrophobic interaction column*

Buffer A: 20 mM Tris-HCl, 3 mM KCl pH 7.6, 0.02 % (w/v) sodium azide  
Buffer B: 20 mM Tris-HCl, 3 M KCl pH 7.6, 0.02 % (w/v) sodium azide

The phenyl superose (HR 10/10) FPLC column was equilibrated with 100 % Buffer B. In addition, the KCl concentration of the sample was brought to 3 M KCl with solid salt and filtered through 45 µm disposable filters before loading. Chromatography proceeded with the gradient described below and Fig. 2.1C. When the fractions were assayed for *in vitro* PLC activity there was one peak. The fractions were pooled and dialysed to remove the high salt concentration before being chromatographed on the final column.

**Column Conditions**

<table>
<thead>
<tr>
<th>Flow:</th>
<th>1.5 ml/min</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6 ml</td>
</tr>
<tr>
<td>Fraction collector:</td>
<td>4 min</td>
</tr>
<tr>
<td>Chart Recorder:</td>
<td>0.1 cm/min</td>
</tr>
</tbody>
</table>

**Gradient**

<table>
<thead>
<tr>
<th>0-25 min</th>
<th>100 % Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-90 min</td>
<td>100-40 % Buffer B</td>
</tr>
<tr>
<td>90-175 min</td>
<td>40-0 % Buffer B</td>
</tr>
<tr>
<td>175-200 min</td>
<td>0 % Buffer B</td>
</tr>
</tbody>
</table>
2.3.6 Mono Q

Buffer A: 20 mM Tris, 3 mM KCl, pH 7.6, 0.02 % (w/v) sodium azide
Buffer B: 20 mM Tris, 3 mM KCl, 1 M NaCl, pH 7.6, 0.02 % (w/v) sodium azide

The dialysed sample from phenyl superose was filtered, loaded onto the FPLC Mono Q (HR 5/5) column and the gradient was run as described below and Fig. 2.1D. Fractions with in vitro PLC activity were pooled and concentrated. PLC β1 was stored frozen at -20°C. The protein was approx. 70 % pure by analysis by SDS-PAGE.

Column Conditions

<table>
<thead>
<tr>
<th>Flow:</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Fraction size:</td>
<td>1.0 ml/min</td>
</tr>
<tr>
<td>Fraction Collector:</td>
<td>1 min</td>
</tr>
<tr>
<td>Chart Recorder:</td>
<td>0.2 cm/min</td>
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</tbody>
</table>

Gradient

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Buffer B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5</td>
<td>0 %</td>
</tr>
<tr>
<td>5-20</td>
<td>40 %</td>
</tr>
<tr>
<td>20-25</td>
<td>100 %</td>
</tr>
<tr>
<td>25-30</td>
<td>100 %</td>
</tr>
</tbody>
</table>

2.4 Purification of PI-TP

2.4.1 Preparation of bovine brain cytosol and ammonium sulphate precipitate

One frozen bovine brain was thawed and homogenised in a Waring blender in ice cold Tris buffer supplemented with 5 mM EDTA, 5 mM EGTA, 10 mM benzamidine, 1 mM DTT, 1 μg/ml soyabean trypsin inhibitor, 5 μg/ml aprotinin, 2 μM pepstatin A, 100 μM TLCK, 0.1 mM Leupeptin and 1 mM PMSF (2 ml/g tissue). The homogenate was centrifuged overnight at 10,000 X g at 4°C. A 40-60 % ammonium sulphate precipitate was prepared from the supernatant and the precipitate was resuspended and dialysed overnight in Tris buffer at 4°C.
2.4.2 DE52 weak anion exchange column

Buffer A: 20 mM Tris, 3 mM KCl pH 7.6, 0.02 % (w/v) sodium azide  
Buffer B: 20 mM Tris, 3 mM KCl, 2 M NaCl pH 7.6, 0.02 % (w/v) sodium azide

The dialysed solution prepared from the ammonium sulphate pellet was chromatographed on a 200 ml DE52 weak anion exchange resin. Proteins were eluted with a linear NaCl gradient (0-700 mM) at 4°C. The active fractions were pooled and concentrated by ultrafiltration to 25 ml.

Column Conditions

Flow Rate: 1 ml/min  
Fraction Size: 7.5 ml  
Fraction collector: 7.5 min  
Chart Recorder: 0.1 cm/min

Gradient

1-50 min 0 % Buffer B  
50-600 min 0-40 % Buffer B

2.4.3 Heparin affinity column

Buffer A: 20 mM Tris, 3 mM KCl pH 7.6, 0.02 % (w/v) sodium azide

Ensuring that the conductivity of the sample was equivalent to 90 mM NaCl or below, it was filtered and chromatographed isocratically with Buffer A on heparin sepharose (using FPLC, 100 ml HiTrap, Pharmacia).

Column Conditions

Flow Rate: 10 ml/min  
Fraction Size: 10 ml  
Fraction collector: 1 min  
Chart Recorder: 0.1 cm/min

Gradient

0-30 min 0 % Buffer B
2.4.4 Gel filtration

Buffer A: 20 mM PIPES, 3 mM KCl, 137 mM NaCl, pH 6.8, 0.02 % (w/v) sodium azide

Fractions from the heparin sepearose column were pooled and concentrated by ultracentrifugation to 0.2 ml and passed through a FPLC Superose 12 (HR 10/30) gel filtration column at 4°C. Fifty fractions were collected after a void volume of 7 ml. In subsequent purifications of PI-TP a Superdex 75 (HR 16/60) gel filtration column was used which has a higher capacity and 2 ml samples could be loaded and sixty fractions were collected after a void volume of 40 ml.

Column conditions for Superose 12

Flow Rate: 0.5 ml/min
Fraction Size: 0.2 ml
Fraction collector: 0.4 min
Chart Recorder: 0.1 cm/min

Column conditions for Superdex 75

Flow Rate: 0.12 ml/min
Fraction Size: 0.8 ml
Fraction collector: 6.66 min
Chart Recorder: 0.05 cm/min

2.4.5 Phenyl superose hydrophobic interaction column

Buffer A: 20 mM Tris, 3 mM KCl, pH 7.6, 0.02 % (w/v) sodium azide
Buffer B: 20 mM Tris, 3 mM KCl, 1 M (NH₄)₂SO₄, pH 7.6, 0.02 % (w/v) sodium azide

Active fractions from gel filtration were pooled and brought to 340 mM (NH₄)₂SO₄ with 3.4 M (NH₄)₂SO₄ stock solution prior to being loaded onto the FPLC phenyl superose (HR 5/5) hydrophobic interaction column. The column was initially washed with 34 % Buffer B to remove unretained material. The PI-TP is eluted by stepping the salt concentration down to 0% Buffer B.

Column Conditions

Flow Rate: 0.5 ml/min
Fraction Size: 2.5 ml
Fraction collector: 5 min
Chart Recorder: 0.1 ml/min

Gradient

0-25 min 34 % Buffer B
25-60 min 0 % Buffer B

All fractions were desalted on Nap-10, sephadex G-25 columns prior to being assayed for PI-TP activity because high salt would interfere with the reconstitution assay (Fig. 4.10). Each 2.5 ml fraction was loaded onto a NAP-10 column which had been prewashed with 25 ml 20 mM PIPES, 137 mM NaCl, 3 mM KCl pH 6.8 with 0.02 % sodium azide. Proteins were eluted in 3.5 ml of wash buffer. Once the fractions with the PI-TP were identified by the in vitro assay for PI-transfer (section 2.8.3) or by SDS-PAGE (section 2.10) they were pooled and concentrated to 200-600 μg/ml by placing the sample in centricons and centrifuging at 9,000 x g for 1-2 hours. At this stage PI-TP was apparently pure by SDS-PAGE.

2.5 Cells

2.5.1 Culturing, labelling and differentiation of HL60 Cells

HL60 cells were grown in RPMI 1640 medium supplemented with 10 % foetal calf serum, glutamine (2 mM) and antibiotics (penicillin at 50 i.u./ml and streptomycin at 50 μg/ml). Cells were diluted every 2-3 days to a density of 0.3 X 10⁶ and grown at 37°C in humidified 95 % air and 5 % CO₂.

To label inositol lipids, cells were harvested by centrifugation and resuspended in 0.8 X 10⁶ cells/ml in Medium 199 supplemented with L-glutamine, antibiotics and [³H]-inositol (1 μCi/ml). Insulin (5 μg/ml) and transferrin (5 μg/ml) were added as growth factors [181]. Cells were grown for 48 hrs to reach equilibrium. To differentiate to neutrophil-like cells that express fMetLeuPhe receptors, cells were grown in the presence of 300 μM dibutyryl cyclic AMP for 48 hr.
2.5.2 Preparation of HL60 cell membranes and cytosol

All procedures were carried out at 4°C. The cells (2 \( \times \) 10^8) were washed three times in 20 mM Hepes, 137 mM NaCl, 3 mM KCl, 0.1 mM EGTA, pH 7.2, and resuspended in 10 ml of the same buffer. The cells were incubated with DFP, 2 mM final concentration, on ice for 5 min and centrifuged at 450 \( \times \) g to remove the cells from the DFP (the supernatant was decanted into 1 M NaOH). The cells were resuspended in 20 mM Hepes, 250 mM sucrose and 100 \( \mu \)M EGTA, 100 \( \mu \)M EDTA at pH 7.2 and sonicated for 2 \( \times \) 20 sec with an MSE probe set at medium. The sonicated cells were centrifuged at 2000 \( \times \) g at 4°C for 5 min to pellet unbroken cells. The supernatant was decanted and centrifuged at 100,000 \( \times \) g for 60 min. This supernatant is the HL60 cytosol and the pellet the membrane fraction which can be resuspended PIPES buffer (section 2.6.1) and used immediately or stored frozen at -70°C.

2.5.3 Measurement of fMetLeuPhe-stimulated hydrolysis of inositol lipids in intact HL60 cells

\(^{[3]}\)H-inositol-labelled HL60 cells were washed three times by centrifugation at 450 \( \times \) g for 5 min in 20 mM Hepes, 137 mM NaCl, 3 mM KCl, 1 mM MgCl\(_2\), 1 mM CaCl\(_2\), 1 mg/ml BSA, 1 mg/ml glucose pH 7.2 (Hepes buffer) at room temperature and resuspended in Hepes buffer supplemented with 10 mM LiCl. Typically the assay was carried out in a volume of 400 \( \mu \)l. 200 \( \mu \)l of cells were transferred to tubes with 200 \( \mu \)l fMetLeuPhe at required concentrations and incubated at 37°C for 10 min. The reaction was quenched with 1.5 ml of chloroform:methanol (1:2) followed by 0.5 ml chloroform and 0.5 ml H\(_2\)O, vortexed and centrifuged at 2000 \( \times \) g for 5 min and the aqueous phase analysed for released inositol phosphates (see section 2.6.7).

2.6 Reconstitution assays

2.6.1 Permeabilization of HL60 cells

50 ml (50 \( \times \) 10^6) of \(^{[3]}\)H-Inositol-labelled HL60 cells were centrifuged at 450 \( \times \) g for 5 min and the medium decanted. The cells were resuspended in 40 ml of 20 mM PIPES, 3 mM KCl, 137 mM NaCl pH 6.8 with 1 mg/ml of BSA and 1 mg/ml glucose (PIPES buffer). Then they were washed twice, resuspended in 4.5 ml and incubated for 20 min at 37°C to
equilibrate. To permeabilize the cells, 0.5 ml of 10 times concentrated permeabilizing cocktail (0.6 i.u./ml streptolysin O, 1 mM MgATP, pCa7, final concentrations) was added to the cells. These cells were incubated for either 10, 40 or 60 min at 37°C as indicated in the figure legends.

Note: For a period of about 9 months during this investigation the cells were DFP-treated (2 mM DFP for 5 min on ice) prior to permeabilization. There did not seem to be any advantage (or disadvantage) to doing this so the practice was suspended. In another deviation from the quoted protocol, protease inhibitors (10 mM benzamidine, 1 mM dithiothreitol, 1 μg/ml soyabean trypsin inhibitor, 5 μg/ml aprotinin, 2 μM pepstatin A, 100 μM TLCK, 0.1 mM Leupeptin) were included in the permeabilizing cocktail over a period of six months, but again there did not seem to be any significant beneficial effect and it was therefore discontinued.

2.6.2 Reconstitution of cytosol-depleted cells

The permeabilized cells were washed in 50 ml ice cold PIPES buffer to remove cytosol and were now referred to as cytosol-depleted cells. These cells were resuspended in the required volume of PIPES buffer supplemented with 4 mM MgATP, 20 mM LiCl, 4 mM MgCl₂, pCa 6. Free calcium concentration was maintained at 1 μM (unless indicated otherwise) with calcium-EGTA buffers with 3 mM EGTA.

When fluoride was used as an activator, calcium-EGTA buffers were excluded, because activation by fluoride is dependent on the presence of Al³⁺ (30 μM) which would be chelated by EGTA [182, 183]. In this case the concentration of free calcium was approx. 1-5 nM [184]

20 μl of cytosol-depleted cells suspended in assay buffer were added to reaction tubes on ice containing 20 μl of fractions or other samples to be tested and 5 μl of activator as indicated. The tubes were transferred from 4°C to 37°C to initiate the reaction and were incubated for 20 min. At the end of the incubation period the tubes were transferred back to ice and the reaction was quenched with 1 ml ice cold saline (0.9 % NaCl) and centrifuged at 2000 X g (4°C) to pellet the cells. 0.9 ml of the supernatant was analysed for inositol phosphates.
2.6.3 Measurement of progressive loss of responsiveness of PLC upon permeabilization

[3H]-inositol-labelled HL60 cells were permeabilized with streptolysin O (0.6 i.u./ml) in the presence of 1 mM MgATP at pCa 7 in a single tube. At the times indicated in the figures, 50 µl aliquots were removed and transferred to tubes with two times concentrated assay buffer (20 mM PIPES, 2 mM MgATP, 2 mM MgCl₂, 10 mM LiCl, final concentrations at pCa 6) supplemented with 1.2 i.u./ml streptolysin O plus GTPγS (10 µM final concentration) or fMetLeuPhe (1 µM final concentration) at 37°C. (If fMetLeuPhe-mediated activation of PLC activity was being assayed, the cells were washed in glucose free buffer and metabolically inhibited with 5 µM antimycin-A and 6 mM deoxyglucose for 5 min prior to permeabilization). After 20 min incubation at 37°C, samples were quenched with 500 µl chloroform:methanol (1:1, v/v) which gives a single phase. A two phase system was generated by the addition of 250 µl of H₂O. Each sample was vortexed rigorously and centrifuged at 2000 X g. The inositol phosphates in the aqueous phase were analysed as described in section 2.6.9.

2.6.4 Time courses

Equal volumes of cytosol-depleted cells (which had been permeabilized for either 10 or 40 min as indicated) and sample (e.g. PI-TP or PLC) were added to reaction tubes at 4°C. The tubes were transferred to 37°C and the reaction initiated by addition of 3 µl of fMetLeuPhe (1 µM final concentration) or GTPγS (10 µM final concentration). 20 µl aliquots were removed at different time points and quenched with 500 µl chloroform:methanol (1:1) followed by 250 µl of H₂O. Each tube was vortexed, centrifuged and 400 µl of the aqueous phase analysed for inositol phosphates as described (see 2.6.9).

2.6.5 Acutely permeabilized cells stimulated by fMetLeuPhe

[3H]-inositol-labelled differentiated HL60 cells were centrifuged at 450 X g for 5 min and washed twice in PIPES buffer without glucose, resuspended in 5 ml and allowed to equilibrate at 37°C for 30 min. The cells were metabolically inhibited with 5 µM antimycin-A and 6 mM deoxyglucose for 5 min, washed once in PIPES buffer and resuspended in the required volume. 20 µl aliquots of cells were added to reaction tubes with 20 µl of
two times concentrated assay buffer, supplemented with 1.2 i.u./ml streptolysin O and 5 μl of fMetLeuPhe (10μM), incubated at 37°C for 10 min and quenched with ice cold saline.

2.6.6 PMA treatment of intact HL60 cells

Prior to permeabilization, HL60 cells were pretreated with 10 nM PMA for 30 min at 37°C in a volume of 4 ml. The cells were then washed in PIPES buffer, after which, permeabilization and reconstitution continued as described above (see sections 2.6.1 and 2.6.2)

2.6.7 Preparation of calcium buffers

In order to control Ca²⁺ at required concentrations, it is necessary to make Ca²⁺ buffers. EGTA, an anionic chelator with a preference for Ca²⁺ over Mg²⁺ was used. A series of buffers were prepared that had a fixed concentration of EGTA and different concentrations of Ca²⁺ from the method of Tatham and Gomperts (185). Two solutions A) 100 mM EGTA, 20 mM PIPES pH 6.8 and B) 100 mM Ca²⁺-EGTA, 20 mM PIPES pH 6.8 are added together at different ratios to achieve the required stock calcium concentration. Calcium buffers are stored at 100 mM EGTA at -20°C until required. The buffers are then used at pH 6.8 at a final concentration of 3 mM EGTA and a free Mg²⁺ concentration of 2 mM.

2.6.8 Preparation of MgATP

A stock solution of 100 mM MgATP was prepared, aliquoted and stored at -20°C until required. To make up 10 mls of stock, 0.605 g Na₂ATP was dissolved in 7 ml distilled water, 1 ml MgCl₂ (1 M) and 1 ml Tris-HCl (1 M). While monitoring the pH, another ml of Tris-HCl was titrated into the MgATP solution to bring the pH to pH 7. The final stock is 100 mM MgATP, 20 mM Tris-HCl pH 7. Cells were routinely permeabilized in the presence of 1 mM MgATP and the reconstituting factors were assayed at 2 mM MgATP.
2.6.9 Analysis of inositol phosphates

Inositol phosphates were separated from free inositol and glycerophosphoinositol by passage through Dowex 1 X 8 resin. 0.9 ml from saline quenched or 0.4 ml from chloroform:methanol quenched assays were loaded onto 0.5 ml columns. Free inositol was washed off with 3 X 2 ml wash of H$_2$O and subsequently glycerophosphoinositol was washed off with 3 X 2 ml of 60 mM sodium tetraborate, 5 mM sodium formate. IP$_1$, IP$_2$ and IP$_3$ were eluted together with 1 M ammonium formate, 0.1 M formic acid [186]. If IP$_1$ was to be separated from the IP$_2$ and IP$_3$ it was eluted from the columns with 0.2 M ammonium formate before IP$_2$ and IP$_3$ were eluted together with 1 M ammonium formate.

2.6.10 Conversion of Dowex resin to the formate form and their regeneration

Before use, the Dowex resin was washed with 1 M NaOH for 60 min followed by 60 min exposure to 1 M formic acid and finally sequential washes with distilled water.

After use, Dowex columns could be regenerated with 3 X 2 ml washes of 2 M ammonium formate, 0.1 M formic acid followed by 5 X 2 ml washes of H$_2$O.

2.6.11 Expression of data

The results illustrated are representative of at least three experiments. All determinations except the time-course experiments were carried out in duplicate.

The increase in total inositol phosphates (IP$_1$, IP$_2$ and IP$_3$) is expressed as a function of the total radioactivity incorporated in the inositol lipids. The lipids were extracted from the cells. First, a single-phase mixture was made with chloroform, methanol and H$_2$O with the final proportions 0.5:1.0:0.4 ml. A two-phase system was produced by addition of 0.5 ml of H$_2$O and 0.5 ml of chloroform and centrifuged at 2000 X g. The organic phase was removed and evaporated before the addition of scintillation fluid and counted on a liquid scintillation counter.
2.7 In vitro PLC assay

2.7.1 Preparation of [³H]-PIP₂ vesicles

In a modification of a published procedure [44], the *in vitro* assay was carried out in a total volume of 50 µl containing 20 mM PIPES, 100 mM NaCl, 0.6 % sodium cholate, 2 mM CaCl₂, 5 mM ß-mercaptoethanol at pH 6.8 (reaction buffer).

Stock of [³H]-PIP₂ substrate was prepared in reaction buffer as follows: 5 mg of PIP₂ was dissolved in 1 ml of chloroform:methanol 1:1 and dispensed into a 50 ml glass tube followed by 12 µCi [³H]-PIP₂ in chloroform:methanol:H₂O (20:10:1) and dried down under nitrogen. The lipids were resuspended in 28 ml 2.5 times concentrated reaction buffer. The [³H]-PIP₂ vesicles were made by sonicating with an MSE probe for 5 X 5 sec bursts. The vesicles were aliquoted and stored at -20°C until required.

2.7.2 In vitro assay of PLC activity

20 µl of [³H]-PIP₂ vesicles in reaction mix (154 µM PIP₂, 0.035 µCi/mmol [³H]-PIP₂ final concentration) were added to 30 µl of sample to be tested and incubated at 37°C for 10 min or less depending on the predicted activity of the sample. The reaction was quenched with 250 µl of chloroform:methanol:conc. HCl (50:50:1), vortexed and 75 µl of 0.1 M HCl added to separate the phases. Samples were centrifuged for 5 min at 2000 X g. The nonhydrolysed PIP₂ remain in the organic phase and the product of the reaction, inositol phosphates are localised in the aqueous phase. 100µl of the aqueous top phase was removed and the radioactivity counted by liquid scintillation counting. The PLC preparation was diluted such that less than 25% of the substrate was hydrolysed. Under these conditions the assay is linear [44]. Total PIP₂ was calculated by simply counting the radioactivity in 20 µl of PIP₂ vesicles.

2.8 PI-transfer assay

The PI-transfer activity of PI-TP was monitored by measuring the transfer of radiolabelled PI from microsomes to liposomes (187).
2.8.1 Preparation of [\textsuperscript{3}H]-inositol-labelled microsomes

Three fresh rat livers were chopped into small pieces and homogenised with Potter-Elvehjem dounce homogeniser in 0.25 M sucrose, 1 mM EGTA, 10 mM Tris-HCl at pH 7.4 (SET buffer) to give a final volume of 200 ml. The homogenate was centrifuged at 2000 X g at 4°C for 5 min to sediment unbroken cells. To pellet the mitochondria the supernatant was centrifuged at 10,000 X g for 15 min and the supernatant decanted. To pellet the microsomes, this post mitochondrial supernatant was centrifuged at 100,000 X g for 60 min. The microsomal pellet was resuspended in 50 mM Tris-HCl, 2 mM MnCl\textsubscript{2}, pH 7.4 to give a total volume of 40 ml and homogenised with a Potter-Elvehjem dounce homogeniser. 300 \mu Ci [\textsuperscript{3}H]-inositol were added to the homogenate and incubated at 37°C for 90 min and centrifuged at 100,000 X g at 4°C for 90 min. This pellet was resuspended to a volume of 100 ml in 10 mM Tris-HCl, 2 mM inositol, pH 8.6 and centrifuged at 100,000 X g for 60 min. The pellet was washed once more in 1 mM Tris-HCl, 2 mM inositol pH 8.6 and centrifuged at 100,000 X g at 4°C for 60 min. Finally, the pellet was resuspended in 20 ml SET buffer and the protein concentration adjusted to 10-20 mg/ml, aliquotted and stored at -70°C until required.

2.8.2 Preparation of liposomes

To prepare PC:Pl (98:2) liposomes, 235\mu l of PC (100mg/ml in chloroform) and 100\mu l of Pl (10mg/ml in chloroform) were dried down under nitrogen in a glass tube. The lipids were sonicated extensively in 30 ml of SET buffer giving a total lipid concentration of 1 mM. The resultant liposomes could be stored at 4°C for a week with no deterioration.

2.8.3 In vitro assay of Pl-TP activity

To assay transfer activity, the microsomes were diluted by 10-20 fold to bring the protein concentration to 1.25 mg/ml. The ratio of liposomes to microsomal protein in the assay is 1 \mu mol to 1.25 mg.

Typically 5 \mu l of sample was analysed, diluted to 50 \mu l in SET buffer and incubated with 100 \mu l [\textsuperscript{3}H]-inositol-labelled microsomes (0.125 mg) and 100 \mu l liposomes (0.1 \mu mol) for 30 min at 25°C. The microsomes were precipitated by the addition of 50 \mu l ice-cold 0.2 M sodium acetate, pH 5 in 0.25 M sucrose and vortexed. The aggregated microsomes were
pellet by centrifugation at 11,500 X g for 15 min at 4°C. 100 μl of the liposome-rich supernatant was transferred to scintillation vials, 3 ml of scintillation fluid added and measured for radioactivity by liquid scintillation counting. The percentage transfer was calculated from the total counts that transferred from the microsomes to the liposomes.

2.8.4 Monitoring the release of PI-TP from HL60 cells

50 ml of unlabelled HL60 cells (5 X 10^7) (labelled cells can also be used) were permeabilized with 0.6 i.u./ml streptolysin O in a volume of 5 ml. 200 μl samples were removed at different time intervals after permeabilization, quenched with 200 μl of ice cold 0.9 % saline and centrifuged at 2000 X g for 5 min at 4°C. 25 μl of the supernatant was assayed for PI-TP activity as described above (see 2.8.3).

2.9 Protein quantitation

2.9.1 Pierce Kit

Protein concentration was calculated from a BSA standard curve using a colour absorbance kit (BCA kit, Pierce) that consists of two reagents A and B. 10 μl of samples were incubated with 200 μl of reagent mix (reagent A:reagent B, 50:1 ) at room temperature for 30 min, and absorbance read at 540 nm. Those samples with little or no protein remained a light green whereas those with high protein concentration became purple.

2.10 Gels

2.10.1 SDS-PAGE gel

30 ml of running gel was mixed to the desired percentage of acrylamide from 30 %/0.8 % acrylamide/bisacrylamide solutionin 563 mM Tris pH 8.8. 150 μl of 20 % SDS, 100 μl of freshly prepared 10 % ammonium persulphate, and 20 μl of Temed were mixed with the running gel. The gel was cast on a Biorad casting stand and overlaid with water-saturated isobutanol and allowed to set for > 30 min. The isobutanol was washed off the gel and overlaid with stacking gel (1.67 ml 30 %/0.8 % acrylamide/bis acrylamide, 1.25 ml Tris-HCl pH 6.8, 7.03 ml H2O, 50 μl
of 20 % SDS, 50 μl of 10 % ammonium persulphate and 10 μl Temed). A 10- or 16-well comb was introduced to the stacking gel immediately and the gel was allowed to set for > 15 min. Once set, the gel was connected to the inner cooling core and placed in the buffer chamber, filled with running buffer (25 mM Tris base, 192 mM glycine, 0.1 % SDS, pH 8.3).

Samples and molecular weight markers were boiled with sample buffer (Note:- 8 ml of 2 times concentrated sample buffer: 0.8 ml of 20 % SDS, 0.8 ml glycerol, 0.8 ml 0.5 M Tris-HCl pH 6.8, 4 ml H2O, 0.05 % bromophenol blue and 0.8 ml of 1 M DTT or β-mercaptoethanol to reduce all disulphide bonds) for 3-5 min prior to loading. The combs were removed from the stacking gel and the samples were loaded into the wells. The gels were run at 150 V until the dye-front reached the bottom of the gel. The gels were stained by either coomassie or silver staining.

2.10.2 Coomassie Staining

The gel was submerged in coomassie stain (1.2 mg Coomassie R250, 250 ml acetic acid, 500 ml methanol, 500 ml H2O) usually overnight and then de-stained for 3-4 hours, to reveal stained protein bands, in methanol:acetic acid:H2O (30:10:60 by volume).

2.10.3 Silver Staining

When the gel had run it was immediately immersed in fixing solution (ethanol:acetic acid: H2O, 40:10:50) and left on a tilting plate at room temperature for 30 min to precipitate proteins and allow SDS to diffuse from the gel. The gel was incubated in 30 % ethanol, 0.5 M sodium acetate, .0013 % gluteraldehyde and 8 mM sodium thiosulphate for a further 30 min. Following this, the gel was washed three times in deionised water and placed in silver solution (12 mM silver nitrate, 0.02 % (v/v) formaldehyde) for 40 min. To visualise the proteins, the gel was incubated in developing solution (236 mM sodium carbonate, 0.01 % (v/v) formaldehyde) for anything from 1 to 15 min until the proteins can be seen clearly. To stop developing, the bathing solution was changed to 39 mM EDTA for 10 min. The developed gels could be preserved in 10 % glycerol. A photograph of the gel is taken at this stage.
2.11 Western blotting

2.11.1 Transfer of proteins from SDS-Gel to PVDF

The unstained gel was equilibrated in transfer buffer (25 mM Tris base, 192 mM glycine, pH 8.3) for 30 min. A "blotting sandwich" was assembled. Filter paper, presoaked in transfer buffer, was placed on a wet fibre pad and on top of that, the gel. The PVDF "Immobilon" membrane was immersed in methanol (to ensure the wetting of a hydrophobic surface) and then transfer buffer prior to placing it on the gel. The sandwich was completed with another layer of presoaked filter paper and fibre pad. Once assembled, the sandwich was submerged in an electrophoretic transfer cell (mini Trans-Blot, Biorad) filled with transfer buffer, making sure the orientation was correct i.e. the proteins travelled from the negative to the positive electrode. Transfer was carried out overnight at 30 V. To confirm the transfer of proteins from the gel to the PVDF membrane, the membrane was incubated with Ponceau-S a nonpermanent red protein dye which was subsequently washed off with tap water and then PBS (1.5 g di-sodium hydrogen orthophosphate anhydrous, 96 g sodium dihydrogen orthophosphate, 84 g NaCl in 1 L, pH 7.5). The efficiency of the transfer was monitored by coomassie staining the gel (2.10.2).

2.11.2 Detection of the antigen

Non-specific protein binding was blocked with 5 % non-fat milk protein in PBS. The blocked blot was washed in PBS supplemented with 0.05 % (v/v) Tween 20 (PBS-Tween 20), 3 X 15 min and probed with the appropriate primary antibody (anti-plcB1 at 1/1000 S.G.Rhee monoclonal, anti-PI-TP at 1/2500 polyclonal from our rabbits) in a volume of 10 ml for 60 min. The excess primary antibody was washed off with PBS-Tween 20 three times for 15 min. The blot was then incubated with a secondary horse radish peroxidase (HRPO)-labelled anti-mouse or anti-rabbit antibody (against monoclonal or polyclonal primary antibodies respectively) for 60 min. Finally the blot was washed 3 X 15 min in PBS-Tween 20.
2.11.2.1 ECL Detection

ECL-western blotting is a light emitting non-radioactive method for detection of immobilised specific antigens, conjugated directly or indirectly with HRPO-labelled antibodies. Luminol, a constituent of the ECL detection solution, is oxidised by HRPO and hydrogen peroxide in alkaline conditions, generating an excited state. Light is emitted as luminol decays to its ground state. Enhancers are also present in the detection solution which increase the light output (approx. 1000 fold) and extend the time of light emission. The maximum light emission is at a wavelength of 428 nm which can be detected by short exposure to blue-light sensitive autoradiography film (Hyperfilm ECL).

The blot was drained and put in a container protein-side up, incubated with 10 ml of detection solution (5 ml of detection-solution-1 mixed with 5 ml of detection-solution-2 immediately before use), for 1 min at room temperature. The blot was drained, wrapped in "Clingfilm" and exposed to Hyperfilm ECL for 15 sec-1 min depending on the strength of the signal and minimising the signal to noise ratio.

2.11.2.2 3,3'-Diaminobenzidine Detection

3,3'-diaminobenzidine is a sensitive substrate for immunocoupled HRPO. It is converted *in situ* to a brown precipitate. 6 mg of diaminobenzidine tetrahydrochloride was dissolved in 9 ml of 10 mM Tris-HCl pH 7.6 and to this added 1 ml of 3 % CoCl₂. The solution was filtered through a 0.45 μm filter to remove any precipitate. Immediately before exposing the blot, 10 μl of H₂O₂ was added to the 3,3'-diaminobenzidine solution. The blot was exposed to the solution (100 μl/cm²) at room temperature with gentle agitation. It was necessary to closely monitor the reaction because the antigen was detected within 1-3 min and the developing needed to be stopped by washing in PBS, otherwise the background would overshadow the bands. It was necessary to photograph the blot immediately because the stained bands fade on exposure to light.
Chapter 3

Characterisation of a partially purified cytosolic factor that restores G-protein-stimulated phospholipase C activation in cytosol-depleted cells

3.1 Introduction

Incubation of HL60 cells with streptolysin O, a bacterial derived cytolytic toxin, produces lesions in the plasma membrane large enough for cytosolic proteins to leak out. Lactate dehydrogenase, considered a true cytosolic protein, is released into the external medium within 10 min (165). In contrast, PLC activity (monitored as *in vitro* activity) is lost at a slower rate maximal after 20 min (180).

GTP\(_{7}S\), added in the presence of streptolysin O, can gain entry to the cell interior within 10 sec (179) and activate PLC catalysed hydrolysis of PIP\(_2\) (165). When GTP\(_{7}S\) or fMetLeuPhe are added to the cells at the same time as the permeabilizing agent the cells are described as "acutely-permeabilized" cells. Fig. 3.1 illustrates that as HL60 cells are permeabilized with streptolysin O for increasing lengths of time, prior to incubation with GTP\(_{7}S\), there is a diminution in GTP\(_{7}S\) activation of PLC activity. After 40 min permeabilization, the ability of GTP\(_{7}S\) to stimulate PLC activity is considerably reduced. There is always a residual response after extended permeabilization. Cells were routinely permeabilized for 40-60 min, centrifuged to remove the leaked cytosol and used in a reconstitution assay to identify the cytosolic factor(s) required for the restoration of GTP\(_{7}S\)-stimulated PLC activity and are referred to as cytosol-depleted cells (Fig. 3.2). These cytosol-depleted cells retain the enzymes required for the synthesis of PIP\(_2\) from PI.
Fig 3.1 Loss of GTPγS-stimulated PLC activity after addition of streptolysin O.

[3H]-inositol labelled HL60 cells were washed in PIPES buffer and permeabilized with 0.6 i.u./ml streptolysin O in the presence of 1 mM MgATP at pCa 7 in a volume of 4 ml. At the indicated time intervals 20 μl aliquots were removed and assayed for GTPγS-stimulated PLC activity. Samples were incubated for 20 min at 37°C with assay buffer (PIPES buffer supplemented with 2 mM MgATP, 10 mM LiCl, 5 mM MgCl₂ at pCa 6, final concentrations) in the absence (open circles) or presence (closed circles) of 10 μM GTPγS. The assay was quenched with 1 ml of 0.9 % ice cold saline and the cells sedimented by centrifugation. The supernatant was analysed for inositol phosphates.
Step 1 HL60 cells are incubated with [³H]-inositol for 48 hrs to label the inositol lipids.

Step 2 Cells are permeabilized with streptolysin O and washed to remove the leaked cytosol.

Step 3 Cytosol-depleted cells are incubated with reconstituting factors (column fractions, PI-TP, PLC) in the presence of a G-protein- or receptor-activator.

Fig. 3.2 Protocol used for reconstitution of G-protein-stimulated PLC activity.
The exit of PLCs from the permeabilized cells was thought to account for the loss of the cells' responsiveness to GTP\(_7\)S. Thomas et al (180) partially purified, what they believed to be, a G-protein-regulated isoform of PLC from rat brain cytosol that restored responsiveness to GTP\(_7\)S in cytosol-depleted cells and designated it PLC\(\epsilon\). It is this entity which is characterized in this chapter and is referred to as factor X.

3.2 Fractionation of Rat Brain cytosol

To separate factor X from other PLC isoforms, rat brain cytosol was fractionated on a heparin sepharose column. PLC activity in the resultant column fractions was assayed. Out of a total of 16 rat brain cytosol fractionations approx. sixty percent of the chromatograms had three major peaks of PLC activity, peak I peak II and peak III, peak I typically having a shoulder to the right of it (Fig. 3.3). In the other forty percent, peak II and peak III overlapped to some extent. It was previously established that Peak II contained PLC\(_1\) and Peak III contained PLC\(_\beta1\) + PLC\(_\delta1\) as determined by immunoblotting (180). The PLC activity in peak I was not identified by antibodies against PLC isoforms \(\beta1\), \(\gamma1\) or \(\delta1\). The fractions making up this peak are described as containing factor X (PLC\(\epsilon\)).

In some experiments factor X partially purified from bovine brain cytosol was used and are indicated in the figure legends. This purification procedure involved two additional steps to factor X isolation from rat brain cytosol. A 40-60% ammonium precipitation of the bovine brain cytosol was prepared, dialysed and chromatographed on DE52 prior to fractionation on heparin sepharose (chromatography carried out by Dr. G. M. H. Thomas).

The fractions constituting each peak from heparin sepharose were pooled and concentrated to approx. 2-5% of their original volume. Table. 3.1 compares the extent of GTP\(_7\)S-stimulated reconstitution given by the each of the peaks. Peak I and Peak III were capable of restoring responsiveness to GTP\(_7\)S in cytosol-depleted cells, peak I (factor X) being most efficient. PLC\(_\beta1\) was responsible for Peak III-induced reconstitution (180). Peak II did not reconstitute, this is not surprising since PLC\(_\gamma1\) has not been known to be regulated by G-protein-linked receptors. In experiment 1 (Table 3.1) rat brain cytosol was capable of substantial hydrolysis of inositol lipids in the absence of GTP\(_7\)S. This has been
observed on a number of occasions and is most likely due to the presence of the 100 KDa fragment of PLC β1 (chapter 6, fig. 6.2A). However GTPγS enhances inositol lipid hydrolysis to over 20%. In experiment 1 and experiment 2 the extent of the response to factor X approached that given by whole rat brain cytosol.

3.3 Guanine nucleotide stimulation

Factor X reconstitution is dependent on the concentration of GTPγS (Fig. 3.4A). In the absence of factor X, GTPγS stimulated endogenous PLC activity to a small extent, in addition, factor X had some activity independent of GTPγS. The response to GTPγS plateaued at 10 μM and for this reason 10 μM GTPγS was used routinely for subsequent experiments. Reconstitution was also dependent on the concentration of factor X (Fig. 3.4B). The response to GTPγS in permeabilized cells increased with the amount of factor X added and did not saturated with the maximum protein available. In addition to GTPγS, other guanine nucleotide analogues supported factor X reconstitution. Fig. 3.4C demonstrates the rank order of potency for the guanine nucleotides as: GTPγS > > Gpp(NH)p > Gpp(CH2)p > GTP.

3.4 FMetLeuPhe-mediated inositol lipid signalling

Differentiated HL60 cells express fMetLeuPhe receptors (188). Just as the loss of cytosol from undifferentiated HL60 cells led to a decline in the cells response to GTPγS, so too cytosol-depletion led to a decline in the fMetLeuPhe response in differentiated HL60 cells (Chapter 6, Fig. 6.6).

To investigate whether factor X could restore responsiveness to fMetLeuPhe as it did to GTPγS, it was introduced to cytosol-depleted differentiated cells (Fig. 3.5A). FMetLeuPhe alone elicited a very small response that was Ca2+ dependent and addition of factor X enhanced the fMetLeuPhe response at all Ca2+ concentrations (pCa8-5). The percentage inositol lipids hydrolysed in cytosol-depleted cells approached that seen for "acutely-permeabilized" cells (see inset to Fig. 3.5A).

While fMetLeuPhe does stimulate PIP2 hydrolysis in the presence of factor X, GTPγS is capable of stimulating a far greater response (Fig. 3.5B). GTPγS is capable of stimulating all available G-proteins in the permeabilized cells indiscriminately, and since it is poorly hydrolysable, G-protein activation of PLC cannot be terminated. On the other hand, fMetLeuPhe is a receptor-directed agonist which transduces its signal via
Fig. 3.3 Fractionation of rat brain cytosol on heparin sepharose

Rat brain cytosol was loaded onto a heparin sepharose column and the proteins eluted with a linear NaCl gradient. Fractions were assayed for in vitro PLC activity. Three peaks of activity were identified, labelled peak I, peak II and peak III.
% hydrolysis of inositol lipids

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<tr>
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<th>-GTPγP</th>
<th>+GTPγS</th>
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<tr>
<td>No additions</td>
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</tr>
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<tr>
<td>PeakI (factor X)</td>
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<tr>
<td>PeakII (PLC γ1)</td>
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<tr>
<td>PeakIII (PLC β1 plus PLC δ1)</td>
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<td>PeakI (factor X)</td>
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<tr>
<td>PeakII (PLC γ1)</td>
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</tr>
<tr>
<td>PeakIII (PLC β1 plus PLC δ1)</td>
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<td>3.7</td>
</tr>
</tbody>
</table>

Table 3.1 A comparison of the extent of GTPγS-stimulated reconstitution of cytosol-depleted cells by different isoforms of PLC isolated from rat cytosol.

Fractions making up the peaks of PLC activity (peaks I, II and III) were pooled, concentrated and assayed for GTPγS-mediated PLC reconstitution. The specific activity of factor X was 366 nmoles and 456 nmoles PIP2 hydrolysed/min/ml for experiment 1 and experiment 2 respectively. The specific activity of PLC β1 was 500 nmoles and 560 nmoles PIP2 hydrolysed/min/ml for experiment 1 and experiment 2 respectively.
Fig. 3.4 Restoration of PLC responsiveness to guanine nucleotides in cytosol-depleted HL60 cells with factor X. [A] GTPγS concentration dependence [B] factor X concentration dependence [C] G-protein stimulation with GTP, Gpp(NH)p and Gpp(CH2)p

[A] 20 μl of cytosol-depleted cells were assayed in the absence (open circles) or presence (closed circles) of 20 μl of bovine brain factor X (S.A. 1.35 μmol PIP2 hydrolysed/min/ml) and 5 μl of GTPγS to give the final concentrations indicated and [B] 20μl of cytosol-depleted cells were assayed in the absence (open circles) and presence (closed circles) of GTPγS (10 μM) and 20 μl factor X at different dilutions. [C] 20 μl of cytosol-depleted cells were incubated with GTPγS (10 μM), Gpp(NH)p (1 mM), Gpp(CH2)p (1 mM) or GTP (1 mM) in the absence or presence of 20μl of bovine brain factor X (S.A. 1.35 μmol PIP2 hydrolysed/min/ml), in a total assay volume of 45 μl.

Samples were incubated for 20 min at 37°C, then the assay was quenched with 0.9 % saline, centrifuged and the supernatant analysed for inositol phosphates.
Fig. 3.5 [A] FMetLeuPhe-stimulated factor X reconstitution in the presence of different concentrations of calcium [B] Comparison of GTPγS-stimulated and FMetLeuPhe-stimulated factor X reconstitution.

Differentiated HL60 cells were permeabilized for 60 min. 20 μl of the cytosol-depleted cells were incubated with 1 μM FMetLeuPhe in the absence (open circles) or presence (closed circles) of rat brain factor X (S.A. 420 nmol PIP₂ hydrolysed/min/ml) at the indicated Ca²⁺ concentrations with a total assay volume of 45 μl. Stimulation of acutely permeabilized HL60 cells with FMetLeuPhe at pCa6 is illustrated in the inset; control (open bar), FMetLeuPhe (solid bar). [B] Cytosol-depleted differentiated HL60 cells were incubated with either GTPγS (10 μM) or FMetLeuPhe (1 μM) in the absence (open bars) or presence (solid bars) of rat brain factor X (S.A. 80 nmol PIP₂ hydrolysed/min/ml).
specific G-proteins (G_{i2} and G_{i3}) (7). Therefore a smaller population of G-proteins are activated and GTP will be hydrolysed rapidly due to the endogenous GTPase activity in the \( \alpha \)-subunit (6).

3.5 Fluoride-mediated stimulation of PLC activity

Fluoride, in the presence of micromolar concentrations of aluminium, is an activator of heterotrimeric G-proteins (189). It is believed that fluoride in a complex with aluminium (\([\text{AlF}_4^-] \) binds in the nucleotide site of the G-protein next to the \( \beta \)-phosphate of GDP, acting as an analogue of the \( \gamma \) phosphate of GTP (183). Fig. 3.6A illustrates that the intact cell can be activated by fluoride. The response increased with increasing concentrations of fluoride up to 10 mM, but at concentrations above 10 mM it was inhibitory. Cytosol-depleted cells, reconstituted with factor X were also stimulated by fluoride (Fig. 3.6B). In the absence of factor X while fluoride had very little activity, factor X alone elicited some response. The profile of fluoride-mediated reconstitution was very similar to the intact cell response in that concentrations of fluoride below 10 mM caused stimulation of PLC activity whereas above 10 mM caused inhibition of PLC activity.

3.6 ATP dependence

\( \text{PIP}_2 \), is the major substrate for PLC in vivo (19, 177). In contrast to the adenylate cyclase signalling system where the catalytic unit of adenylate cyclase has a high concentration of substrate (ATP) at all times (21), \( \text{PIP}_2 \) is in very low concentration (1/100 that of ATP). During stimulation the cells must control the rate of synthesis to match increased demand for \( \text{PIP}_2 \) resulting from PLC hydrolysing activity. \( \text{PIP}_2 \) is synthesised from PI by the sequential action of isoforms of PI-4-kinase and PI-4-P-5-kinase in the presence of MgATP, at the plasma membrane (20). There is also evidence of \( \text{PIP}_2 \) synthesis at the nucleus (40). Fig. 3.7 illustrates the MgATP requirement for factor X-induced reconstitution. It was only in the presence of MgATP plus GTP\( \gamma \)S that factor X could fully reconstitute cytosol-depleted cells.
Fig. 3.6 Concentration dependence of fluoride-stimulation of [A] intact cell [B] factor X-reconstituted cytosol-depleted cells.

[A] [³H]-inositol labelled HL60 cells were washed in Hepes buffer and incubated with different concentrations of fluoride plus Al³⁺ (10 μM) at 37°C for 10 min. The assay was quenched with chloroform:methanol. [B] Cytosol-depleted cells were washed and incubated with factor X from bovine brain (S.A. 1.35μmol PIP₂ hydrolysed/min/ml) with (closed circles) or without (open circles) indicated concentrations of fluoride plus Al³⁺ (10 μM) for 20 min with assay buffer in the absence of EGTA buffers. The assay was quenched with ice cold saline, centrifuged and the supernatant analysed for inositol phosphate.
Fig. 3.7 PLC reconstitution is dependent on the presence of MgATP.

Cytosol-depleted HL60 cells were reconstituted with bovine brain factor X (1.35 μmol PIP2 hydrolysed/min/ml) in the presence or absence of MgATP (2 mM) and/or GTPγS (10 μM). The assay was quenched with ice cold saline, centrifuged and the supernatant analysed for inositol phosphates.
3.7 Influence of PMA treatment on reconstitution with factor X

Phorbol 12-myristate 13-acetate (PMA) an activator of the majority of the PKC isoforms, has been shown to attenuate the ability of GTP\(\gamma\)S and agonists to stimulate hydrolysis of polyphosphoinositides in a number of cells types including HL60 cells (190, 191). In Fig. 3.8 HL60 cells were pre-treated with 10 nM PMA for 30 min at 37°C prior to permeabilization. PMA caused inhibition of factor X reconstituting ability across the GTP\(\gamma\)S concentration range by approx. 60 %. PMA also slightly inhibited the endogenous GTP\(\gamma\)S-induced response in the absence of factor X. This reconstitution system shows the same sensitivity to PMA pre-treatment as HL60 cell membranes and "acutely-permeabilized" HL60 cells (191).

3.8 Discussion

The observation that the percentage of PIP\(_2\) hydrolysed in the presence of GTP\(\gamma\)S in "acutely-permeabilized" HL60 cells was approx. 10-fold greater than that in membrane preparations, indicated that there was an essential component for inositol lipid signalling in the cytosol that was absent in membrane preparations (73). When HL60 cells are permeabilized with streptolysin O there is a loss of GTP\(\gamma\)S responsiveness over time. Thomas et al (180) observed that rat brain cytosol restored responsiveness to GTP\(\gamma\)S in cytosol-depleted cells. To find out exactly what the essential component(s) of the rat brain cytosol was, it was fractionated on heparin sepharose and all the fractions were assayed for in vitro PLC activity. One peak of PLC activity (peak I) was particularly effective at GTP\(\gamma\)S-mediated reconstitution. It was assumed that it was the PLC activity in this peak that was restoring responsiveness to GTP\(\gamma\)S and in the absence of positive identification by immunoblotting with the then available antibodies it was referred to as factor X.

Introducing factor X to cytosol-depleted HL60 cells restored properties to the cells that mimicked intact and "acutely permeabilized" cells. To start with, factor X-reconstituted cells could be stimulated by GTP\(\gamma\)S in a concentration dependent manner just as in "acutely permeabilized cells". Moreover, other guanine nucleotides could stimulate PLC activity and do so with the same rank order of potency as in "acutely permeabilized" cells (165). In addition, GTP\(\gamma\)S-mediated reconstitution exhibited the same Ca\(^{2+}\)- and ATP-dependence as "acutely permeabilized cells" (165). G-protein activation of factor X dependent reconstitution could also be mediated by fluoride. In both intact and cytosol-depleted cells,
Fig. 3.8 Effect of PMA pretreatment of HL60 cells on GTPγS-stimulated factor X reconstitution.

HL60 cells were pretreated with 100 nM PMA for 30 min at 37°C in a volume of 4ml. The cells were then washed in PIPES buffer to remove the PMA, permeabilized for 40 min and stimulated with different concentrations of GTPγS in the absence and presence of bovine brain factor X (1.35 μmol PIP₂ hydrolysed/min/ml). Control for untreated cells, open triangles; Control for PMA-treated cells, closed circles; factor X response in untreated cells, open circles; factor X response in PMA-treated cells, closed circles.
concentrations of fluoride above 10 mM proved inhibitory. This may be due to a fluoride-induced inhibition of PI-kinases at the plasma membrane causing a diminution of substrate supply to PLC substrate.

The receptor-directed agonist fMetLeuPhe also stimulated factor X reconstitution in differentiated HL60 cells. FMetLeuPhe-induced reconstitution of cytosol-depleted cells was Ca^{2+}-dependent similar to fMetLeuPhe-stimulated intact and "acutely permeabilized" cells (192, 193).

FMetLeuPhe-stimulated PIP_{2} hydrolysis in intact HL60 cells (194) and GTP_{γS}-stimulated PIP_{2} hydrolysis in "acutely permeabilized" cells and HL60 membranes were inhibited by pretreating the cells with the phorbol ester, PMA (191). PMA is an activator of protein kinase C (111). There is some controversy as to which protein is the target of PKC to cause inhibition of inositol lipid signalling. It has been demonstrated that in human platelet and astrocytoma cells the G-protein G_{i} (190, 195) is phosphorylated by PKC, uncoupling it from PLC. But there is also evidence that PKC can phosphorylate a receptor i.e. β-adrenoreceptor, resulting in the uncoupling of the receptor from the G-protein linked to adenylate cyclase in turkey erythrocytes (196). Others have implicated the PLC itself as the target for PMA-induced phosphorylation (191). In order to find the target for PMA in HL60 cells, the cells were pretreated with PMA prior to permeabilization. If PMA phosphorylated the cytosolic PLC, factor X should overcome that inhibition. In this reconstituted system PMA inhibited GTP_{γS}-stimulated hydrolysis. Since factor X did not overcome the inhibition it must be concluded that the target of protein kinase C must be situated at the membrane. This would be in agreement with the fact that protein kinase C translocates to the plasma membrane on activation (197) and that PMA pretreatment can inhibit inositol lipid signalling in HL60 cell membranes. The possible candidates for phosphorylation are A) a G-protein or B) a PLC at the plasma membrane leading to uncoupling of the inositol signalling pathway.

In summary, factor X restored responsiveness to PLC activating agents in cytosol-depleted HL60 cells with characteristics that were similar to previously described G-protein regulation of PLC in "acutely-permeabilized" cells and HL60 membranes. The data indicated that factor X was the essential component of cell cytosol for inositol lipid signalling that was lost in HL60 membrane preparations. At this stage the evidence
suggested that factor X was an unidentified G-protein-regulated isoform of PLC, but it needed to be purified.
Chapter 4

Identification of the PLC reconstitution factor as phosphatidylinositol transfer protein

4.1 Introduction

Factor X from bovine and rat brain cytosol possessed an unidentified PLC activity that was assumed to be responsible for its ability to reconstitute cytosol-depleted HL60 cells. For this reason column fractions were only assayed for in vitro PLC activity throughout its subsequent purification. Bovine brain cytosol was fractionated on DE52 and heparin sepharose, the unidentified PLC activity was chromatographed sequentially on cibacron-blue affinity, alkyl superose hydrophobic interaction and gel filtration columns, chromatofocused on Mono-P and finally purified to homogeneity by chromatography on Mono Q (carried out by Dr G.M.H. Thomas). PLC activity recovered from any column was unable to reconstitute GTPγS-mediated activation of cytosol-depleted HL60 cells. The amino acid sequence of the protein was determined and it was identified as the 100 KDa fragment of the PLC isoform PLC β1 (150 KDa). PLC β1 has a calpain-sensitive site which when cleaved generates a 100 kDa fragment and a 45 kDa fragment. The 100 KDa fragment retains the ability to catalyse the hydrolysis of PIP₂ in vitro but does not possess the motif for interaction with the α-subunit of G₉ and therefore could not reconstitute GTPγS-activated cytosol-depleted HL60 cells (see chapter 6).

Because of the inability of the PLC to reconstitute, it was decided to return to the original source of factor X i.e. rat brain cytosol, to purify the true reconstituting activity. This time, column fractions were assayed for both in vitro PLC activity and GTPγS-stimulated PLC reconstitution.
4.2 The reconstituting factor is not an isoform of PLC

Rat brain cytosol was fractionated on heparin sepharose as described in section 2.2 and the column fractions constituting factor X were concentrated to 0.2 ml and gel filtered on a superose-12 column. Every fraction was assayed for GTP\(_\gamma\)S-stimulated reconstitution as well as \textit{in vitro} PLC activity (Fig. 4.1). The PLC activity separated completely from GTP\(_\gamma\)S-stimulated reconstituting activity. When the column was calibrated with several proteins of known molecular weight, the elution behaviour of the reconstituting factor indicated that it had an apparent molecular weight of approx. 17 KDa.

4.3 Purification and identification of the reconstituting factor from bovine brain

Once it was established that the reconstituting activity was not a PLC, bovine brain cytosol was used for purification since it was available in greater quantity.

\textit{4.3.1 Purification}

A purification scheme was developed (Fig. 4.2) where bovine brain was homogenised with a cocktail of protease inhibitors and centrifuged to remove the particulate fraction. The reconstituting activity was precipitated from the cytosol by ammonium sulphate (40%-60% saturation), dialysed and loaded onto a DE52 weak anion exchange column and eluted with a linear salt gradient. Fractions were assayed for restoration of GTP\(_\gamma\)S activity in cytosol-depleted cells and \textit{in vitro} PLC activity. The peak of reconstituting activity was narrower than the peak for \textit{in vitro} PLC activity (Fig. 4.3). The active fractions were pooled and dialysed, concentrated to 25 ml and loaded onto an FPLC HiTrap heparin sepharose column. The proteins were eluted isocratically at zero salt. This differs from the linear gradient used in the original fractionation of PLCs from rat brain cytosol (Fig. 3.3) because it was recognised that the active peak eluted without salt. PLC reconstitution and a portion of the PLC \textit{in vitro} activity co-eluted (Fig. 4.4). The active fractions making up the peak were pooled and concentrated, 0.2 ml aliquots were loaded onto a Superose-12 gel filtration column. The region of reconstitution did not coincide with the peak of \textit{in vitro} PLC activity as expected. The chromatogram (Fig. 4.5A) was very similar to that for the equivalent...
Fig. 4.1 Dissociation of PLC activity and PLC reconstitution after gel permeation chromatography on Superose 12

Following fractionation of rat brain cytosol on heparin sepharose, peak 1 containing reconstituting activity (see Fig.3.3) was concentrated to 200 µl and loaded onto a Superose 12 column and eluted with 20 mM PIPES, 137 mM NaCl, 3 mM KCl pH 6.8. Fractions were assayed for in vitro PIP$_2$-hydrolysing activity (open circles) as well as GTP$_{y}$S-stimulated reconstitution in cytosol-depleted cells (closed circles). Molecular size was estimated by reference to a calibration curve constructed using proteins of known molecular size. The estimated size of the reconstituting factor was 17 KDa.
Brain Cytosol

\[\downarrow\]

Ammonium sulphate fractionation (40–60%)

\[\downarrow\]

DE52 weak anion exchange

\[\downarrow\]

Heparin sepharose

\[\downarrow\]

Superdex 75

\[\downarrow\]

Phenyl superose

---

*Fig. 4.2 Purification scheme for the reconstituting factor from bovine brain cytosol.*
sample of rat brain cytosol filtered on the same column (compare Fig. 4.1 with Fig. 4.5A). Aliquots of the fractions with reconstituting ability were analysed on a 12.5% SDS polyacrylamide gel (fig.4.5B). The reconstituting activity was then purified to apparent homogeneity on phenyl superose hydrophobic interaction column (fig. 4.6A). Aliquots of the fractions were run on a 12.5% SDS-polyacrylamide electrophoresis gel (SDS-PAGE). From Fig. 4.6B it can be seen that reconstituting activity resides in a single protein with an apparent molecular mass of 35 KDa.

4.3.2 Identification of reconstituting activity.

The 35 KDa protein was excised from the gel, digested and the amino acid sequence determined by Justin Hsuan and Nicholas Totty from the Ludwig Institute. The sequence was found to match closely that of rat phosphatidylinositol-transfer protein (PI-TP), a polypeptide of 271 amino acids with a mass of 31,911 daltons (Fig. 4.7). Comparison of several peptide sequences with the Swissprot and NBRF databases revealed near identity to rat brain PI-TP. The protein sequences obtained, are shown underlined in the rat protein, and differences are shown in bold type. The unidentified residue is aligned with position 138 of the rat sequence, but no significant phenylthiohydantoin amino acid could be detected at this cycle.

The molecular mass assessment of 31,911 daltons is not in agreement with the predicted molecular weight of 17 KDa determined by gel filtration. This anomaly in apparent molecular weight is a characteristic of PI-TP that has been previously observed (187, 198).

4.3.3 Confirmation that PI-TP is the reconstituting factor

In subsequent purifications of PI-TP from bovine brain, GTPγS-mediated reconstitution and in vitro PI-transfer activity were monitored (Fig.4.8). The PI-TP assay monitors the transfer of radiolabelled PI from donor membranes (microsomes) to acceptor membranes (liposomes). PI-transfer and PLC reconstitution were closely associated through out the purification.

But, examination of the protein separation on DE52 (see Fig 4.8A) revealed that reconstitution does not completely correspond to the in vitro PI-TP activity. The peak for PI-TP activity is much broader than the reconstitution peak. Although in most purification runs only the
Fig. 4.3 Chromatography of bovine brain cytosol on DE52 weak anion exchange column

Cytosol from one bovine brain was precipitated with 40-60% ammonium sulphate, dialysed and loaded onto a 400 ml DE52 weak anion exchange column. The column was eluted with a linear NaCl (0-700 mM) gradient. Fractions were assayed for in vitro PLC activity (open circles) and PLC reconstitution (closed circles).
Fig. 4.4 Heparin sepharose chromatography

Reconstituting fractions from DE52 were pooled, concentrated to 25 ml and loaded onto a 100 ml HiTrap heparin sepharose column. Proteins were eluted isocratically with zero salt. All fractions were assayed for both in vitro PIP$_2$ hydrolysing activity (open circles) and PLC reconstitution (closed circles).
Fig. 4.5 Gel filtration on Superose 12

[A] The fractions active in reconstituting cytosol-depleted cells from heparin sepharose were pooled and concentrated to 0.2 ml and filtered through a Superose 12 gel permeation column. Fractions were assayed for *in vitro* PLC activity (open circles) and PLC reconstitution in cytosol-depleted cells (closed circles).

[B] Fractions 37-43 were electrophoresed on a 12.5 % SDS-polyacrylamide gel and the gel stained with coomassie blue.
Fig. 4.6 Phenyl superose hydrophobic interaction

[A] Active fractions from superose 12 gel filtration column were chromatographed on phenyl superose hydrophobic interaction column. Fractions were assayed for PLC reconstitution (closed circles). [B] Fractions 2-9 from phenyl superose were analysed on a 12.5% SDS polyacrylamide gel. The gel was stained with coomassie blue. The molecular weight markers are 66, 45, 36, 29, 24, 20 and 14.2 KDa.
Fig. 4.7 Identification of the reconstituting factor as PI-TP by sequence analysis

The sequence of rat PI-TP is outlined above. The underlined sequences are those bovine brain peptide sequences determined by Justin Hsuan and Nick Totty (Ludwig Institute). The amino acids of the rat PI-TP that differ from the bovine brain PI-TP are in bold type and above is the bovine amino acid. There is an unidentified residue in the sequence of bovine brain that is aligned with position 138 of the rat sequence.
Fig. 4.8 Purification of PLC reconstituting activity coincides with in vitro PI-TP activity.

Bovine brain cytosol was chromatographed on [A] DE52 anion exchange resin, [B] Hi-Trap heparin sepharose, [C] Superose 12, a gel-permeation column and [D] phenyl superose hydrophobic interaction column. Fractions were assayed for PLC reconstitution (closed circles) and in vitro PI-TP activity (open triangles).
reconstituting peak was used for the next chromatography step i.e. heparin sepharose, the remainder of the PI-TP peak was retained and purified to homogeneity using the same sequential chromatography on A) heparin sepharose, B) gel filtration and C) phenyl superose (Fig.4.9). The phenyl superose fractions were examined by SDS-PAGE to confirm that it ran as a 35 KDa protein (Fig. 4.9C). This purified PI-TP was equally active in the reconstitution assay (Fig.4.9D).

Therefore, it seemed as if there was an inhibitory factor at the earlier stages of purification that was responsible for concealing the reconstituting activity of PI-TP. But, it is also possible that PI-TP inhibition may have been simply due to salt concentration since fractions from DE52 were not dialysed before being assayed. To test whether increased NaCl concentration was inhibitory in the reconstitution system rat brain cytosol was assayed for GTPγS-mediated stimulation of PLC activity in the presence of different salt concentrations (Fig.4.10). As the salt concentration increased there was a decline in response to rat brain cytosol. Close examination of Fig.4.8A reveals that inhibition begins at about half way along the NaCl gradient (350 mM NaCl). This translates to 230 mM NaCl in the reconstitution assay. This concentration of salt would be adequate to cause inhibition. It is possible that high NaCl concentrations facilitate the dissociation of PLC β isoforms from HL60 cell plasma membranes. Once separated from the membrane PLC β would no longer be capable of interaction with the G-proteins and therefore be refractory to GTPγS stimulation.

4.4 Characterisation of reconstitution of cytosol-depleted HL60 cells with PI-TP

4.4.1 PI-TP concentration-dependence

Fig. 4.11A demonstrates the concentration dependence of PI-TP for both the in vitro transfer activity and GTPγS-stimulated reconstitution. In the absence of PI-TP, GTPγS stimulated some degree of activation of membrane associated PLC. PI-TP alone had little effect, reconstitution was dependent on the presence of GTPγS plus PI-TP and increased with increasing concentrations of PI-TP. When the maximal amount of available protein was added, saturation of the response was not observed.
Fig. 4.9 Partially purified PI-TP activity from DE52 that does not reconstitute PLC activity will do so when purified to apparent homogeneity

Fractions from DE52 Fig.4.8A that had in vitro PI-TP activity but were not active for reconstitution were pooled and concentrated to 25 ml. Following in vitro PI-TP activity (closed circles) the sample was sequentially chromatographed on [A] heparin sepharose and [B] gel filtration and finally [C] phenyl superose. The bottom panel shows the corresponding fractions analysed for protein content by SDS-PAGE (stained with coomassie blue), demonstrating the correlation between in vitro PI-TP activity and the apparently pure 35 KDa PI-TP in fractions 7 and 8. [D] 20 µl samples of fraction 8 from phenyl superose (50 µg/ml final concentration) were incubated with 20 µl cytosol-depleted cells for 20 min at 37°C in the absence (open bar) or presence (solid bar) of GTPγS (10 µM) in a total assay volume of 45 µl. Final concentration of PI-TP was 22 µg/ml.
Fig. 4.10 High concentrations of NaCl can inhibit PLC reconstitution in cytosol-depleted cells

HL60 cells were permeabilized for 40 min and reconstituted with rat brain cytosol in the absence (open circles) or presence of 10 μM GTPγS (closed circles) with increasing concentrations of NaCl. The concentrations quoted are final concentrations in the assay taking into consideration that PIPES buffer has a NaCl concentration of 137 mM.
The reconstituting activity of the purified protein declined if stored at 4°C, therefore freshly prepared PI-TP was routinely used.

4.4.2 Ca^{2+} dependence of PLC reconstitution with PI-TP

Activation of "acutely-permeabilized" cells by GTPγS shows a Ca^{2+} concentration dependence (165). Examination of Ca^{2+}-dependence of PI-TP reconstitution of cytosol-depleted cells revealed an identical pattern. PI-TP reconstitution of PLC activity can occur at pCa 7, the resting concentration found inside living cells, but increasing the Ca^{2+} concentration to 1 μM greatly enhanced the response (Fig.4.11B).

4.4.3 ATP dependence

PIP₂ is the predominant substrate for PLC in vivo, and since PI-TP is known to transfer only PI (199, 200), it was expected that PLC reconstitution would be totally dependent on the presence of MgATP as a source of phosphate groups for the conversion of PI to PIP and PIP₂. Fig.4.11C illustrates the MgATP requirement for reconstitution. It is clear that only in the presence of MgATP and GTPγS did PI-TP produce maximal activation of PLC. When HL60 cells are reconstituted with PI-TP in the presence of GTPγS the principal inositol phosphates formed are IP₂ and IP₃ (Fig.4.12) confirming that when PLC is presented with PI, PIP and PIP₂ in the plasma membrane it only hydrolyses the polyphosphoinositides. So PI has to be converted to its phosphorylated forms prior to hydrolysis.

4.4.4 FMetLeuPhe-mediated inositol lipid signalling

When differentiated HL60 cells are permeabilized they lose their responsiveness to GTPγS and in addition to fMetLeuPhe (Fig. 6.6). When PI-TP was introduced to cytosol-depleted cells it restored activity to fMetLeuPhe (Fig.4.13) demonstrating that PI-TP can reconstitute the receptor-stimulated PLC.
Fig. 4.11 [A] Concentration dependence of PI-TP for PLC reconstitution and PI-TP activity [B] Ca\(^{2+}\) dependence of PI-TP-mediated reconstitution with GTP\(\gamma\)S [C] MgATP-dependence of PI-TP-mediated reconstitution

[A] Purified PI-TP was assayed for PLC reconstitution in cytosol-depleted HL60 cells in the presence (closed circles) or absence (open circles) of GTP\(\gamma\)S (10 \(\mu\)M) at pCa 6. PI-TP was also assayed for in vitro PI transfer (open squares).

[B] Cytosol-depleted HL60 cells were incubated with Ca\(^{2+}\) buffered in the indicated range in the presence of GTP\(\gamma\)S (10 \(\mu\)M) and PI-TP (200 \(\mu\)g/ml final concentration). Control, open circles; GTP\(\gamma\)S alone, closed circles; PI-TP alone, open triangles; PI-TP plus GTP\(\gamma\)S, closed triangles.

[C] HL60 cells were permeabilized for 40 min, washed in PIPES buffer and reconstituted with PI-TP (200 \(\mu\)g/ml final concentration) in the absence or presence of MgATP (2 mM) and GTP\(\gamma\)S (10 \(\mu\)M) for 20 min at 37\(^\circ\)C.

The assays were quenched with ice cold saline, centrifuged and the supernatant analysed for inositol phosphates.
Fig. 4.12 The products of inositol lipid hydrolysis are IP$_2$ and IP$_3$ when HL60 cells are reconstituted with PI-TP

$[^{3}H]$-inositol labelled HL60 cells were permeabilized for 40 min and reconstituted with purified PI-TP (30 µg/ml final concentration) in the absence (open bar) and presence of GTPγS (10 μM) (solid bar). The assay was incubated for 20 min at 37°C and quenched 1 ml with ice cold saline. Each sample was centrifuged and 0.9 ml of the supernatant loaded onto dowex columns. The columns were washed with deionised water and tetraborate A) IP$_1$ was eluted with 0.2 M ammonium formate and B) IP$_2$ + IP$_3$ were eluted with 1 M ammonium formate.
4.4.5 Influence of PMA treatment of HL60 cells on PI-TP-mediated reconstitution

PMA has been shown to inhibit GTPγS-stimulated PLC activation in "acutely-permeabilized" HL60 cells and HL60 membranes (191). In Fig.4.14, HL60 cells were pre-treated with 100 nM PMA for 30 min at 37°C prior to permeabilization. PMA pretreatment caused inhibition of PI-TP reconstitution at the highest concentrations of PI-TP but there was not a complete inhibition. This would imply that there is a PMA-sensitive and PMA-insensitive element to PI-TP reconstituting activity.

HL60 cells contain two isoforms of PLC β, PLC β2 and PLC β3, which leak from permeabilized cells leaving a residual amount in the membrane (201). Both have been shown to be regulated by the α-subunit of G₁₆ a pertussis toxin-insensitive G-protein that is specifically expressed in human haematopoietic cells (9). In addition, receptor-mediated release of βγ-subunits from a pertussis toxin-sensitive G-protein can activate PLC β2 and PLC β3 (77). PMA activates PKC which in turn can phosphorylate the pertussis toxin-sensitive G-protein Gᵢ in several cells types (190, 195). Members of the Gᵢ family (G₁₂ and G₁₃) present in HL60 cells could be the target for PMA-induced phosphorylation leading to their uncoupling from the PLC. This would leave only that fraction of the response mediated by G₁₆, which has not been shown to be a target of PKC. This might explain the limited inhibition of PI-TP reconstitution by PMA pretreatment.

4.4.6 PI-TP is present in HL60 cells

Reconstitution is heterologous in so far as cytosol-depleted HL60 cells are reconstituted with a protein purified from bovine brain. It was necessary to establish that HL60 cells contained PI-TP and that it did leak from cells upon permeabilization with streptolysin O. Fig. 4.15A. demonstrates that permeabilization leads to a time-dependent release of PI-TP into the medium. The release was maximal after 10 min. The question then was, could PI-TP be recovered from HL60 cytosol and be re-introduced to the permeabilized cells to restore GTPγS-stimulated PIP₂ hydrolysis. HL60 cytosol from 500 ml of cells was passed through a Superdex 75 gel filtration column to separate PI-TP from PLC activity (Fig.4.15B). Fractions were assayed for PI transfer and the active fractions were pooled and concentrated. The concentrate was assayed for the restoration of
Fig. 4.13 Agonist-stimulated PI-TP reconstitution.

[³H]-inositol labelled, differentiated HL60 cells were permeabilized for 60 min, resuspended in assay buffer and incubated in the absence (open bars) or presence (closed bars) of PI-TP (175 μg/ml final concentration). The cells were stimulated with fMetLeuPhe at pCa 6 in a total assay volume of 45 μl.
Fig. 4.14 Effect of PMA pretreatment on PI-TP reconstitution of cytosol-depleted cells

HL60 cells were pretreated with 10 nM PMA for 30 min at 37°C in a volume of 4 ml. The cells were washed in PIPES buffer to remove the PMA, permeabilized for 40 min and incubated with increasing concentrations of PI-TP, the highest concentration being 175 μg/ml in the absence and presence of GTPγS (10 μM). Control for untreated cells, open triangles; Control for PMA treated cells, closed triangles; GTPγS response in untreated cells, open circles; GTPγS response in PMA treated cells, closed circles.
Fig. 4.15 [A] Time course of release of PI-TP from HL60 cells on permeabilization with streptolysin O [B] Fractionation of HL60 cell cytosol on Superdex 75 gel filtration column [C] Reconstitution of cytosol-depleted cells with PI-TP from HL60 cytosol

[A] HL60 cells were permeabilized with 0.6 i.u./ml streptolysin O in a volume of 5 ml. Samples (200 µl) were removed at indicated times after permeabilization, quenched with 200 µl of ice cold saline and centrifuged. 25 µl of the supernatant was analysed for the presence of in vitro PI-TP activity (closed circles).

[B] HL60 cytosol from 500 ml of cells was gel filtered on a Superdex 75 column. Fractions were assayed for PI transfer (closed circles) and the active fractions (44-46) were pooled and concentrated.

[C] The concentrated PI-TP from HL60 cells (30 µg/ml final assay concentration) was assayed for GTPγS activated PLC activity in cytosol-depleted HL60 cells, control (open bars), HL60 PI-TP (closed bars).
GTPγS-mediated PLC activation in cytosol-depleted cells (Fig. 4.15C). PI-TP from HL60 cell cytosol was capable of homologous reconstitution with GTPγS.

4.5 Conclusion

HL60 cells permeabilized with streptolysin O lose their ability to be stimulated with GTPγS. Two reconstituting activities were identified in rat and bovine brain cytosol that could restore responsiveness to GTPγS. They were PLCβ1 and an unidentified activity (factor X). The unknown protein was purified on the basis of its ability to reconstitute inositol lipid signalling. A protein coinciding with reconstituting activity was identified on a gel, excised, digested and the polypeptides sequenced. The amino acid sequence corresponded to mammalian PI-TP. PI-TP is a ubiquitous, cytosolic protein that can bind and transfer PI and to a lesser extent PC from one membrane compartment to another in vitro (202).

GTPγS-stimulation of PIP2 hydrolysis and PI-transfer was linear with increasing concentrations of PI-TP. The characteristics of PI-TP reconstitution were defined with regard to Ca2+ dependence and MgATP requirement and were found to mimic closely the responses seen in studies with "acutely-permeabilized" cells (165). This implies that even though PLC leaks from the cells on exposure to streptolysin O the fraction of PLCβ2 and PLCβ3 that remains in the cell after 40 min of permeabilization is sufficient to restore responsiveness to the cells. Since the system is heterologous it was necessary to show that PI-TP is lost from HL60 cells on permeabilization and having demonstrated that to show that the endogenous HL60 PI-TP was capable of reconstitution (Fig. 4.15).

GTPγS responses in "acutely-permeabilized" cells were inhibited when the cells were pretreated with PMA prior to permeabilization, in a concentration and time-dependent manner (191). PMA inhibited the maximal stimulation achieved with GTPγS i.e. it had an effect on \( V_{\text{max}} \) rather than the half maximal concentration required for activation. This was consistent with the notion that a subset of G-proteins are phosphorylated via PMA. PI-TP reconstitution in cytosol-depleted cells similarly is inhibited to a certain extent by PMA, leaving a residual response that is refractory to PMA pretreatment, perhaps the \( \text{G}_{16} \alpha \)-subunit-mediated response.
What is PI-TP doing in this reconstitution system?

In the plasma membrane only about 1% of lipids are inositol phospholipids and these for the most part are phosphorylated (20, 203). This small pool of PIP$_2$ is rapidly degraded by PLC when the cell is activated via a receptor or GTP analogue. PIP$_2$ must be replenished by the phosphorylation of PI. The main path of PI production is via the PI synthase enzyme which is found predominantly in the endoplasmic reticulum (ER) (204, 205). PI must in some way be transferred from the ER to the plasma membrane.

The *in vitro* transfer properties of PI-TP make it an ideal candidate for maintaining the PI levels of intracellular membranes. It has been shown to transfer PI from regions of high concentration to regions of low concentration (206). If PI-transfer is the function of PI-TP *in vivo*, it could be envisaged that if substrate supply is limited in the immediate proximity of the activated PLC, PI-TP would transfer PI down its concentration gradient from the ER to the plasma membrane and thereby be critical in maintaining a steady supply of substrate for the enzyme.
Chapter 5

The major reconstituting factor in rat brain cytosol is also phosphatidylinositol-transfer protein

5.1 Introduction

The major reconstituting activity in bovine brain cytosol was positively identified by amino acid sequencing as PI-TP a 35 KDa protein present in high concentration in mammalian cells (chapter 4). It was expected that the reconstituting activity present in rat brain cytosol would also be PI-TP. Purification of the major reconstituting activity from rat brain cytosol is described here and is identified as PI-TP.

5.2 Purification and identification of the major reconstituting activity from rat brain

Rat brain cytosol was fractionated on heparin sepharose with a linear NaCl gradient (0-500 mM) (as described in section 2.2.2). Fractions were assayed for in vitro PLC activity and in vitro Pi-transfer activity. The PI-TP activity and PLC activity co-eluted (Fig. 5.1). Fractions from peak I were chromatographed on a Superdex 75 gel filtration column and fractions were assayed for PLC reconstitution as well as in vitro PLC and PI-TP activity. Fig.5.2A illustrates how Pl-transfer activity aligns with PLC reconstitution in fractions 40-50. In vitro PLC activity eluted much earlier at fractions 8-16. Examination of fractions 40 to 49 on a 12 % SDS polyacrylamide gel (Fig.5.2B) confirmed that a 35 KDa protein coincides with PLC reconstitution. The reconstituting factor was positively identified as PI-TP by its immunoreactivity with an affinity purified polyclonal antibody against recombinant rat PI-TP (Fig.5.2C)
Fig. 5.1 Fractionation of rat brain cytosol on heparin sepharose.

Rat brain cytosol was loaded onto a 40 ml heparin sepharose column and proteins were eluted with a linear NaCl (0-500 mM) gradient. PLC activity (open triangles) and PI-transfer (open circles) were monitored. The dotted line represents optical density at 280 nm.
Fig. 5.2 [A] Dissociation of in vitro PLC activity and PLC reconstitution after gel permeation chromatography on Superdex 75 [B] SDS-PAGE of the fractions containing reconstituting activity [C] confirmation of the identity of the reconstituting activity by western blotting.

[A] Peak I from rat brain cytosol fractionated on heparin sepharose was concentrated to 2 ml and loaded onto a Superdex 75 column and eluted with PIPES buffer (see section 2.4.4). Fractions were assayed for in vitro PIP$_2$-hydrolysing activity (open circles), GTP$\gamma$S-stimulated reconstitution in cytosol-depleted cells (closed circles) and in vitro PI-transfer activity (open triangles).

[B] 30 $\mu$l samples of fractions 40-49 were electrophoresed on a 12.5% SDS-polyacrylamide gel. The gel was stained with coomassie blue. The molecular weight markers are 66, 45, 36, 29,24, 20 and 14.2 KDa and the apparent molecular mass of the protein corresponding to reconstituting activity was 35 kDa.

[C] 30 $\mu$l samples of fractions 40-49 were electrophoresed on a 12.5% SDS-polyacrylamide gel and the proteins transferred to PVDF membrane. The membrane was probed for PI-TP with a polyclonal antibody raised against recombinant PI-TP. The antigen was detected using 3,3'-diaminobenzidine.
[A] PLC in vitro
- PLC Reconstitution
- PI Transfer

[DPM (X10^-3)]

Fraction No.

[B]

Fraction No.

[C]

Fraction No.
The PLC activity that co-purified with PI-TP from rat brain cytosol after fractionation on heparin sepharose was not identified by monoclonal antibodies against PLC β1, PLC γ1 or PLC δ1 (207). Fig.5.3 illustrates that the analogous PLC activity in bovine brain cytosol is the 100 KDa fragment of PLC β1. The bovine brain PLC activity was separated from PI-TP by gel filtration on Superose 12 (see section 2.4.4). Proteins in fractions with in vitro PLC activity were resolved on an 8% SDS polyacrylamide gel and the proteins transferred to PVDF membrane and subsequently probed with a monoclonal antibody against PLC β1 (a gift from S.G. Rhee). A 100 KDa protein was clearly detected, confirming that the PLC activity is the 100 KDa fragment of PLCβ1.

Knowing this, I wished to establish whether the PLC activity in rat brain was also PLCβ1100. Fractions containing in vitro PLC activity (8-16) from gel filtration (Fig 5.2) were run on an 8% SDS gel (Fig. 5.4A). An almost identical gel was run that included a sample of PLC β1 purified from bovine brain membrane (as described in section 2.3). The proteins were transferred to PVDF membrane and probed with a monoclonal antibody against PLC β1 (a gift from S.G. Rhee). While recognising the PLC β1 control sample, the antibody did not recognise the PLC in the gel filtered fractions (Fig. 5.4B). The identity of this PLC remains to be elucidated although it quite likely to be a breakdown product of PLC β3 which is present in rat brain (72). In addition, fragments of PLC β3 have previously been recovered from rat brain cytosol (J. Exton personal communication).

5.3 Conclusion

Just as the major reconstituting factor in bovine brain proved not to be an isoform of PLC but PI-TP the rat brain reconstituting factor was also identified as PI-TP. It was purified on the basis of its ability to reconstitute cytosol-depleted cells and this was synonymous with its ability to transfer PI in vitro.

The contaminating PLC that co-purified with PI-TP for the initial stages of PI-TP purification from bovine brain cytosol was identified as a 100 KDa fragment of PLC β1 (150 KDa). It is likely that the PLC in rat brain cytosol that was originally proposed as the reconstituting activity (66) is also a breakdown product of an isoform of PLC β. While it was not recognised by a monoclonal antibody directed towards a region within the N-terminal of PLC β1 there is the possibility that the PLC may be a fragment of
Fig. 5.3 Confirmation that PLC \( \beta_{1100} \) is the PLC activity that co-purified with PI-TP from bovine brain cytosol.

Fractions containing PLC activity from Superose 12 gel filtration chromatography were analysed on an 8 % SDS polyacrylamide gel. The proteins on the gel were transferred to PVDF and probed with a monoclonal antibody against PLC \( \beta_1 \) (a gift from S.G.Rhee). The antigen was detected with an ECL kit.
Fig. 5.4 PLC $\beta_{1100}$ is not the PLC activity that co-purified with PI-TP from rat brain cytosol.

[A] Fractions containing PLC activity from rat brain cytosol gel filtered on Superdex 75 (Fig. 5.2), were run on an 8 % SDS-polyacrylamide gel. The gel was stained with coomassie blue.

[B] An identical gel was run and an additional sample of PLC $\beta_1$ included as a positive control. The proteins were transferred to PVDF membrane and probed with a monoclonal antibody raised against PLC $\beta_1$ (a gift from S.G. Rhee). The antigen was detected with 3,3'-diaminobenzidine.
PLC β3 which also resides in the rat brain. Antibodies capable of recognising fragments of PLC β1 or PLC β3 were not available to confirm this.
Chapter 6

Examination of the influence of PI-TP on the rate of inositol phosphate production by PLC β isozymes

6.1 Introduction

Rat brain cytosol can restore responsiveness to GTPγS in cytosol-depleted HL60 cells. Fractionation of rat brain cytosol on heparin sepharose generated two reconstituting peaks of activity, one more active than the other (180). The most active reconstituting factor was identified as PI-TP and the less active as PLC β1. It was of interest to investigate how the two reconstituting activities influence GTPγS-stimulated reconstitution when they are introduced to the cytosol-depleted cells simultaneously.

PI-TP has the ability to transfer PI or PC from one membrane compartment to another in vitro depending on the ratio of PI:PC in the two compartments (202). The ability of PI-TP to restore G-protein-mediated inositol lipid signalling in cytosol-depleted cells suggested that if substrate was limited at the plasma membrane in activated cells the role of PI-TP would be to maintain PI concentrations. For this reason it was not expected that PI-TP would have any effect on the initial rate of PIP₂ hydrolysis. But when the influence of PI-TP on the rate of inositol phosphate production was examined the data suggested that the function of PI-TP in inositol lipid signalling might be more complex than predicted by its in vitro function.

6.2 Purification of PLC β1 from bovine brain membranes.

In order to have sufficient material to examine PLC β1 reconstitution in detail, PLC β1 was purified from bovine brain membranes by a modification of a published procedure (208). Bovine brains were homogenised in Tris buffer supplemented with EDTA, EGTA and protease
inhibitors. The homogenate was centrifuged overnight at 4°C to separate the cytosol from the particulate fraction. The pellet was washed, resuspended in 2 M KCl and centrifuged again to extract that PLC associated with the membrane. The PLCs were ammonium sulphate precipitated (60 % saturation) and dialysed. The dialysate was loaded onto a DE52 weak anion exchange column and the proteins eluted with a linear NaCl gradient (Fig. 6.1A). Fractions that eluted with the gradient and had in vitro PLC activity were pooled and chromatographed on an FPLC heparin sepharose column with a NaCl gradient (Fig. 6.1B). Active fractions were pooled and brought to 3 M KCl with solid salt and loaded onto a phenyl superose, hydrophobic interaction column. Proteins were eluted with a descending KCl gradient (Fig. 6.1C). Finally, PLC β1 was purified on a Mono Q (strong anion exchange) column (Fig. 6.1D). The protein was approx. 70 % pure by SDS-PAGE.

6.3 Characterisation of PLC β1-mediated reconstitution.

Fig. 6.2A illustrates the concentration dependence of PLC β1 for GTPγS-mediated reconstitution. In the absence of GTPγS, PLC β1 hydrolysed a small proportion of inositol lipids but this hydrolysis was markedly enhanced with the addition of GTPγS (10 μM). The response saturated with 25 μg/ml PLC β1. It was consistently observed that PLC β1 was weaker than PI-TP in reconstitution. PLC β1 is a 150 KDa protein which is susceptible to calpain (a Ca^{2+}-dependent protease) giving rise to 100 kDa and 45 KDa fragments (86). The 100 KDa fragment (PLC β1_{100}) retains catalytic activity but is not capable of regulation by σ-subunits of Gq (86). As mentioned in the previous chapter (chapter 5) PLC β1_{100} proved to be the contaminating PLC during the purification of PI-TP from bovine brain cytosol. Fig. 6.2B shows that PLC β1_{100} is capable of hydrolysing some inositol lipids but this activity was not GTPγS-regulated.

It was reported in section 3.1, that permeabilization of HL60 cells led to an attenuation in their response to GTPγS. Fig. 6.3 shows that the decline of responsiveness to GTPγS is accompanied by a concomitant release of PI-TP into supernatant. The presence of PI-TP was detected by monitoring the transfer of labelled PI from microsomes to liposomes.
Fig. 6.1 Purification of PLC R1 from bovine brain membranes.

2 M KCl extract from the particulate fraction of 5 bovine brains was precipitated with 60% ammonium sulphate, dialysed and chromatographed on [A] DE52 weak anion exchange resin, [B] Hi Trap heparin sepharose by FPLC, [C] phenyl superose HR 10/10 by FPLC and [D] Mono Q by FPLC. Fractions from each chromatography column were assayed for *in vitro* PLC activity (closed circles). The dotted line represents optical density at 280 nm.
Fig. 6.2 [A] Concentration dependence of PLC β1 in reconstituting GTPγS-activated cytosol-depleted HL60 cells. [B] Effect of increasing concentrations of PLC β1100 on GTPγS activation of PLC in cytosol-depleted cells.

[A] 20 µl of PLC β1 (to give the indicated final concentrations) were incubated with 20 µl of cytosol-depleted cells suspended in assay buffer in the absence (open circles) or presence (closed circles) of 5 µl GTPγS for 20 min at 37°C. [B] Different concentrations of PLCβ1100 were incubated with cytosol-depleted HL60 cells in the absence (open circles) or presence (closed circles) of GTPγS (10 µM) for 20 min at 37°C. Samples were quenched with 1 ml ice-cold saline, centrifuged and the supernatant analysed for PLC activity.
Fig. 6.3 Loss of GTPγS-stimulated PLC activity after addition of streptolysin O and release of PI-TP into the medium.

[3H]-inositol labelled HL60 cells were washed in PIPES buffer and permeabilized with 0.6 i.u./ml streptolysin O in the presence of 1 mM MgATP at pCa 7 in a volume of 4 ml. At the indicated time intervals 50 µl aliquots were removed and assayed for PLC activity in the absence (open circles) or presence (closed circles) of 10 µM GTPγS. Samples were also assayed for PI transfer activity (open squares).
6.4 Interplay between PI-TP and PLCβ1

Since PI-TP and PLC β1 are both capable of restoring GTPγS-stimulated PLC activity it was of interest to examine their effect on reconstitution when present together. Fig. 6.4 illustrates that when PI-TP and PLC β1 are added together, the response is not additive but synergistic giving nearly 20% hydrolysis of inositol lipids. This synergy was only observed if the concentration of PI-TP was kept low.

The time course of inositol lipid hydrolysis in the presence of PI-TP and PLC β1 alone and in combination was examined. Cytosol-depleted HL60 cells could be activated with GTPγS to stimulate a linear increase in inositol lipid hydrolysis, in the absence of PI-TP or PLC β1 (Fig. 6.5). The effect of PI-TP or PLC β1 individually was to enhance the rate of hydrolysis stimulated by GTPγS. Furthermore, when PI-TP and PLC β1 were added simultaneously, the initial rate of hydrolysis was greater than that observed with either protein alone and the absolute extent of hydrolysis was also increased. The synergy between PI-TP and PLC β1 was observed at all time points.

6.5 Kinetics of PI-TP enhanced inositol lipid signalling

When GTPγS is introduced into cytosol-depleted cells it will indiscriminately stimulate all available G-proteins. Because GTPγS is poorly hydrolysed, activation cannot be terminated and the resulting hydrolysis of inositol lipids is supramaximal. The plasma membrane represents 20-30% of total cell membrane (203). With this in mind, it would not be surprising that PI-TP would be required to sustain inositol lipid hydrolysis since PI is concentrated at the endoplasmic reticulum and golgi rather than at the plasma membrane. By using a powerful stimulus such as GTPγS, it was a possibility that the reconstitution system was artificially made PI-TP-dependent and the requirement for PI-TP was not essential when a receptor-directed ligand was used as the stimulus. Fig. 6.6 shows that depletion of cytosol from cells by permeabilization leads to a decline in the responsiveness to fMetLeuPhe, a receptor-directed agonist. This indicated that cytosolic component(s) are also essential for signalling through the fMetLeuPhe receptor.
HL60 cells were washed in PIPES buffer and permeabilized in the presence of 1 mM Mg ATP at pCa 7 for 40 min. 20μl of cytosol-depleted cells were added to tubes containing 15μl PI-TP (20 μg/ml) and/or 15 μl PLC β1 (34.5 μg/ml, specific activity of PLC β1, 2.2 μmol PIP₂ hydrolysed /min/mg) in the absence (open bar) or presence (closed bar) of GTPγS (10 μM) in a final assay volume of 55 μl. After incubation at 37°C for 20 min, the reaction was terminated with 1 ml of ice cold 0.9 % saline and analysed for released inositol phosphates.

Fig. 6.4 Reconstitution of inositol lipid hydrolysis with PI-TP and PLC-β1 in cytosol-depleted HL60 cells is synergistic.
Fig. 6.5 Time-Course of reconstitution with PI-TP and PLC-β1 alone and in combination.

HL60 cells were permeabilized for 40 min at pCa 7, washed and subsequently incubated at 37°C in the presence of GTPγS (10 μM); PI-TP (800 ng per assay); PLC β1 (280 ng per assay). 20 μl aliquots were removed at different time points, quenched with chloroform:methanol and the aqueous phase analysed for inositol phosphates. Control, open circles; GTPγS, closed circles; PLCβ1 and GTPγS, open triangles; PI-TP and GTPγS, closed triangles; PI-TP, PLCβ1 and GTPγS, open squares.
PI-TP and PLC β1 were tested for their ability to reconstitute the fMetLeuPhe response in cytosol-depleted cells. In initial experiments, the cells were permeabilized for 60 min and PI-TP was not always able to reconstitute fMetLeuPhe-mediated activation of PLC despite the fact that GTPγS always worked. PLC β1 was unable to reconstitute fMetLeuPhe-mediated PLC activation. FMetLeuPhe receptors interact with Gi2 and Gi3 (7) leading to the dissociation of the G-protein βγ subunits from the α-subunits allowing the activation of the endogenous PLCs (PLC β2/β3) (78). PLC β2 and PLC β3 enzymes are membrane-associated and only begin to elute from the cells after 10 min of permeabilization and by 45 min the majority have left the cells (201). This being the case, there was very little PLC β2/β3 present in 60 min permeabilized cells that was available for activation by the fMetLeuPhe receptors. Permeabilization time was reduced to 10 min allowing a larger fraction of the PLCs to remain in the cell while the majority of PI-TP would have leaked out.

When HL60 cells were permeabilized for 10 min, responsiveness to fMetLeuPhe could be reliably reconstituted with PI-TP. Following the time course of fMetLeuPhe activation (Fig. 6.7), the response was linear for 20 min, after which a plateau was observed.

The kinetics of the endogenous PLC (PLC β2/β3) activation with GTPγS in the absence and presence of PI-TP was also examined. Addition of GTPγS alone stimulated inositol lipid hydrolysis at a linear rate over a 60 min period. When PI-TP was added with GTPγS, there was a lag period of no longer than 2 min that is probably due to the entry of PI-TP into the permeabilized cell. The rate of hydrolysis was linear for 20 min, after which it began to slow down.

6.6 Discussion

Of the three PLC families (β, γ and δ) only the PLC β family has been demonstrated to be regulated by heterotrimeric G-proteins. They can be regulated by either the α-subunit of the Gq family or the βγ-subunits of the Gi family (76, 77). HL60 cells express two isoforms of the PLC β family, PLC β2 and PLC β3 (201). They express G-proteins Gi2 and Gi3 as well as members of the Gq family i.e. Gq, G11 and G16. G16 is of particular interest because it is highly expressed in undifferentiated HL60 cells with the level declining by 90% upon differentiation. In contrast, the levels of Gi2 and Gi3 increase upon differentiation (9, 74, 7).
Fig. 6.6 Loss of fMetLeuPhe-stimulated PLC activity after addition of streptolysin O

Differentiated, [³H] inositol-labelled HL60 cells were washed in PIPES buffer and permeabilized with 0.6 i.u./ml streptolysin O in the presence of 2 mM MgATP at pCa 7 in a volume of 4 ml. At the indicated time intervals 50 µl aliquots were removed and assayed for fMetLeuPhe-stimulated PLC activity. Samples were incubated for 20 min at 37°C with assay buffer in the absence (open circles) or presence (closed circles) of 1 µM FMLP. The assay was quenched with chloroform:methanol (1:1) and the aqueous phase analysed for PLC activity.
Fig. 6.7 Time-Course of PLC activation by fMetLeuPhe in 10 min permeabilized cells.

HL60 cells were permeabilized for 10 min at pCa 7, washed and subsequently incubated in assay buffer at 37°C in the absence or presence of fMetLeuPhe (1 µM) and/or 5 µg PI-TP (250 µg/ml) as indicated. Aliquots were removed at different time points and quenched with chloroform methanol. Control, open triangles; PI-TP, closed triangles; fMetLeuPhe, open circles; fMetLeuPhe and PI-TP, closed circles.
Fig. 6.8 Time-Course of PLC activation by GTPγS in 10 min-permeabilized cells.

HL60 cells were permeabilized for 10 min at pCa 7, washed and subsequently incubated at 37°C in the presence of GTPγS (10 μM) and/or PI-TP (4.4 μg per assay, 220 μg/ml) in a final volume of 500 μl. 20 μl samples were quenched with chloroform:methanol at the indicated time intervals. Control, open triangles; PI-TP, closed triangles; GTPγS, open circles; GTPγS and PI-TP, closed circles.
When "acutely-permeabilized" HL60 cells are stimulated with GTP\(_\gamma\)S the hydrolysis of PIP\(_2\) is catalysed by endogenous PLC \(\beta2\) and PLC \(\beta3\). This response diminished with increasing time of exposure to streptolysin O. PLC \(\beta1\), which is predominantly regulated by \(\alpha\)-subunits of the \(G_q\) family was able to restore some of the GTP\(_\gamma\)S responsiveness. Since GTP\(_\gamma\)S can indiscriminately activate all available G-proteins in this system, activated \(G_{0q}\) and \(G_{o11}\) could stimulate the exogenously added PLC \(\beta1\). The observation that the truncated form of PLC-\(\beta1\) (PLC \(\beta1_{100}\)) was unable to reconstitute, supports the concept that it is the G-protein \(\alpha\)-subunits that are active in regulating the exogenous PLC-\(\beta1\).

Differentiated HL60 cells express G-protein-coupled fMetLeuPhe receptors and signalling through the fMetLeuPhe receptor is pertussis toxin-sensitive (73). This implies that the \(G_i\) family are responsible for activation of the PLC \(\beta2/\beta3\) when the receptor is occupied by fMetLeuPhe and therefore the dominant mechanism of activation is via \(\beta\gamma\) subunits. PLC \(\beta1\) was unable to restore responsiveness to fMetLeuPhe which was not unexpected since PLC \(\beta1\) is poorly activated by \(\beta\gamma\)-subunits (78).

The observation that the rate of lipid hydrolysis stimulated by GTP\(_\gamma\)S was linear with time for at least an hour was not anticipated. It had been expected that in the absence of PI-TP the rate of inositol lipid hydrolysis stimulated by GTP\(_\gamma\)S would fall as the amount of PLC substrate was depleted to the point that it is no longer saturating. The function of PI-TP would then have been to replenish PI from intracellular membranes. In fact PI-TP increases the rate of activation of PLC in cytosol-depleted cells 8-fold, from 5\% per hour in its absence to 40 \% per hour in its presence. The notion that PI-TP is functioning in some manner other than PI transfer is strongly supported by the fact that the rate of fMetLeuPhe-mediated inositol lipid hydrolysis is also increased with PI-TP. It is unlikely that substrate would have been limiting in the latter case because typically there is only 3-7\% of inositol lipid hydrolysed.

PI-TP not only increased the apparent activity of endogenous PLC \(\beta2\) and PLC \(\beta3\) but was also capable of enhancing the apparent activity of exogenously added PLC \(\beta1\). The implications of these results are considered in the final discussion.
Chapter 7

Influence of a variety of phospholipid transfer
proteins on G-protein-mediated inositol lipid
signalling in cytosol-depleted HL60 cells

7.1 Introduction

PI-TP is one of three categories of phospholipid transfer protein (PL-TP) so far identified. The PL-TPs are soluble proteins that share the ability to transfer phospholipids between one membrane compartment and another in vitro. They differ in their distribution, substrate specificity, isoelectric point, and molecular weight (Table 7.1). PI-TP has been found in every mammalian tissue investigated and can bind and transfer PI and PC (187). PC-TP has been found predominantly in liver and exclusively transfers PC from one membrane to another (209). Non-specific lipid transfer-protein (nsL-TP) was originally isolated from rat liver and transfers phospholipids in the rank order PI > PE = PC > SM and has also been shown to transfer glycolipids and cholesterol in vitro (210, 211). NsL-TP is derived from a precursor protein, pre-nsL-TP which possesses a 20 amino acid extension at its N-terminal which is susceptible to proteolysis generating mature nsL-TP. Recombinant pre-nsL-TP has been demonstrated to have in vitro lipid transfer activity identical to mature nsL-TP (212). This chapter examines whether any of these PL-TPs could substitute for PI-TP in reconstituting GTPγS-stimulated PLC activity in cytosol-depleted HL60 cells.
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<th>Substrate specificity</th>
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<td><em>Drosophila</em></td>
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<td></td>
<td>(221)</td>
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<tr>
<td>PG-TP</td>
<td></td>
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<td>(202)</td>
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<tr>
<td>Lung</td>
<td></td>
<td>PG</td>
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<tr>
<td>nsL-TP</td>
<td></td>
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<tr>
<td>Rat liver</td>
<td>8.7</td>
<td>PC,PE,PI,PS</td>
<td>cholesterol</td>
<td>(222)</td>
</tr>
<tr>
<td>Bovine liver</td>
<td>9.55,9.75</td>
<td></td>
<td>cholesterol</td>
<td>(211)</td>
</tr>
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</table>
PI-TP preferentially transfers PI but is capable of transferring PC from donor to acceptor vesicles (223). Because there is one negative charge difference between the two lipids, Pl-occupied PI-TP can be distinguished from PC-occupied PI-TP as each has a different isoelectric point, 5.3 and 5.6 respectively (224). The Pl- and PC-forms of PI-TP were assayed for their ability to restore GTPγS-mediated inositol lipid signalling in cytosol-depleted cells. In addition, recombinant mouse PI-TP and bovine brain PI-TP both prepared in the K.W. Wirtz laboratory (Utrecht) were assayed for PLC reconstitution.

7.2 Results

It has been shown in chapter 4 that PI-TP purified from bovine brain cytosol, restored responsiveness to GTPγS in cytosol-depleted HL60 cells. PI-TP purified independently from bovine brain cytosol by another procedure (sequential chromatography on DE52, Sephadex G-75, hydroxyapatite and Mono Q) referred to here as PI-TP-II (a gift from K.W. Wirtz) was tested alongside PI-TP purified as described in chapter 3 (Pl-TP-I). Both proteins restored GTPγS-stimulated PLC activity (Fig.7.1).

Alternative phospholipid transfer proteins were examined for their ability to substitute for PI-TP in the reconstituting assay. PC-TP was not capable of substituting for PI-TP. NsL-TP and its precursor pre-nsL-TP can transfer PI in vitro albeit at a slower rate than PI-TP (Klaas Jan de Vries (Utrecht) unpublished observations). Because these two proteins transfer PI at a slow rate the assay was incubated for 60 min rather than the usual 20 min. However, they could not reconstitute GTPγS-stimulated PLC activity (Fig.7.1).

To confirm that PI-TP is responsible for GTPγS-mediated reconstitution and not a trace contaminant, recombinant PI-TP (rPI-TP) expressed in E.Coli was examined in the permeabilized cell system. A comparison of the concentration dependence of native PI-TP (nPI-TP) and rPI-TP is shown in Fig.7.2. The extent of the response to the two proteins is very similar. The response for rPI-TP plateaus at about 500 µg/ml final concentration in the assay.

Pl-occupied and PC-occupied PI-TP (a gift from K.W. Wirtz) were assayed for reconstitution of GTPγS-stimulated PLC activity in cytosol-depleted cells. These samples of PI-TP had been stored in 50 % glycerol at -20°C for a year prior to their use in this experiment. Freshly prepared native
Fig. 7.1 Assay of PI-TP, PC-TP, nsL-TP and pre-nsL-TP for their ability to reconstitute PLC activity in 10 min-permeabilized HL60 cells.

HL60 cells were washed in PIPES buffer and permeabilized with 0.6 i.u./ml streptolysin O in the presence of 1 mM MgATP at pCa 7 for 10 min. 20 µl of cytosol-depleted cells resuspended in assay buffer were added to tubes containing 20 µl of transfer protein as indicated. Final concentrations of proteins in the assay: PI-TP-I, 200 µg/ml; PI-TP-II, 65 µg/ml; PC-TP, 175 µg/ml; nsL-TP, 75 µg/ml; pre-nsL-TP, 110 µg/ml. Following incubation at 37°C for 60 min, the reaction was terminated with chloroform:methanol and analysed for released inositol phosphates.
Fig. 7.2 Recombinant PI-TP concentration dependence in the reconstitution of GTPγS-stimulated PLC activity.

Recombinant PI-TP (rPI-TP) was assayed for PLC reconstitution of 10 min-permeabilized HL60 cells. For comparison, the concentration dependence of native bovine brain cytosol PI-TP (nPI-TP) was also included. Data are derived from a separate experiment. rPI-TP in the absence of GTPγS, open circles; rPI-TP in the presence of GTPγS, closed circles; nPI-TP in the absence of GTPγS, open triangles; nPI-TP in the presence of GTPγS, closed triangles.
Fig. 7.3 A comparison of reconstituting activity of freshly purified PI-TP with stored forms PI- and PC-occupied PI-TP.

Freshly prepared PI-TP (65% PI-occupied, 35% PC-occupied), PI- and PC-occupied PI-TP were introduced to 40 min permeabilized cells in the absence or presence of GTPγS (10 μM) and incubated for 20 min at 37°C. Freshly purified PI-TP 35 μg/ml; PC form of PI-TP 245 μg/ml; PI-form of PI-TP 538 μg/ml final concentrations in the assay.
PI-TP which is approx. 65% PI occupied (201, 223) was included as a positive control. PI- and PC-occupied PI-TP reconstitute GTPγS-stimulated PLC activity but were less active than freshly prepared native PI-TP (Fig. 7.3).

7.3 Discussion

PI-TP can restore responsiveness to GTPγS-mediated inositol lipid signalling in cytosol-depleted HL60 cells. Other phospholipid transfer proteins were introduced into cytosol-depleted HL60 cells to establish if they had any reconstituting ability. PC-TP, a protein known to exclusively transfer PC, was inactive in reconstituting cytosol-depleted cells indicating that PC-TP and PI-TP are not interchangeable in this system and the PI-binding and possibly PI-transfer ability are a fundamental requirement for PI-TP reconstituting activity. NsL-TP and pre-nsL-TP have low affinity binding sites and can slowly transfer PI as well other phospholipids. Nonetheless, neither nsL-TP or pre-nsL-TP showed any activity in the reconstitution system.

rPI-TP purified from transformed E.Coli cell lysate was as good as the native form of the protein in restoring responsiveness to GTPγS in cytosol-depleted cells. The nucleotide sequence of the cDNA encoding the mouse PI-TP has a high similarity (98%) with that of rat PI-TP. Furthermore the predicted amino acid sequence is 99.6% identical to rat PI-TP and has a molecular weight of 35 KDa and an isoelectric point of 5.4 whether occupied by PI or PC (225). The protein concentrations of rPI-TP required for reconstitution are very similar to those concentrations of native PI-TP that achieve reconstitution. The availability of milligram quantities of rPI-TP allowed saturation of the response not achieved by native protein which has only been available in microgram amounts.

PI-TP can be occupied with either PI (PI-form) or PC (PC-form), and these two can be differentiated by their isoelectric points. They were indistinguishable in their ability to restore GTPγS-mediated PIP2 hydrolysis in cytosol-depleted HL60 cells. The PI and PC-forms of PI-TP were not as efficient at reconstitution as the freshly prepared PI-TP but they had been stored for over a year and probably a proportion of the PI-TP had become inactive. PI-TP has consistently been seen to lose activity with time and for this reason it was freshly prepared for all of the experiments described in previous chapters.
Chapter 8

Discussion

Early studies had established cytosol was necessary to obtain a robust activation of PLC activity by fMetLeuPhe receptors or GTP\(\gamma\)S in HL60 cells. The identity of the cytosolic factor(s) was the subject of this thesis. The data presented here demonstrate that PI-TP is essential for efficient PLC activation.

Cytosol-depleted HL60 cells were used as an assay system to determine exactly which cytosolic protein(s) have the ability to reconstitute inositol lipid signalling. This assay system retains PI-kinases, phospholipids, and some phospholipases. It has an advantage over synthetic \textit{in vitro} systems in that both enzymes and substrates are in their native environment. While the synthetic system is extremely useful when known components are being characterised it does not lend itself to the unveiling of unknown elements. The permeabilized cell system facilitated the detection of PI-TP as an important reconstituting factor. Since rat brain cytosol could reconstitute cytosol-depleted HL60 cells it was used as the initial source for the purification of the reconstituting factor.

Rat brain cytosol was fractionated on heparin sepharose generating three peaks of PLC activity. Western blotting identified the PLCs in the second and third peaks but the PLC in the first peak did not react with any of the PLC antibodies available (PLC\(\beta\)1, PLC\(\gamma\)1, PLC\(\delta\)1), and was designated factor X. When factor X was added to cytosol-depleted HL60 cells GTP\(\gamma\)S- and fluoride-mediated PLC activation were restored. Guanine nucleotide analogue activation mimicked "acutely-permeabilized" cells by demonstrating the same rank order of potency. Finally, fMetLeuPhe receptor-mediated activation of PLC was restored to differentiated HL60 cells depleted of cytosol and this response was enhanced with micromolar concentrations of Ca\(^{2+}\). It was not unreasonable to assume that the reconstituting factor was a PLC in the light of the fact that a) reconstitution coincided with PLC activity when rat brain cytosol was
fractionated on heparin sepharose and b) another phospholipase C, partially purified PLC β1 was capable of a degree of reconstitution (180).

Up until this time, any attempt to purify the PLC resulted in a loss of reconstituting activity. However, fractions from each chromatography column had only been assayed for in vitro PLC activity. At this point the approach was modified and column fractions were directly examined for reconstituting activity. Factor X from rat brain cytosol was chromatographed on a gel filtration column and the fractions were assayed for both reconstituting activity as well as PLC activity. Unexpectedly, the PLC activity separated completely from the reconstituting activity. Bovine brain cytosol was subsequently used as a source for the full purification of the reconstituting factor because it was available in larger amounts. The reconstituting factor was purified to apparent homogeneity by sequential chromatography and identified on a gel. Justin Hsuan and Nicholas Totty from the Ludwig institute determined the amino acid sequence of the protein and found that it corresponded to mammalian PI-TP.

The unidentified PLC activity from bovine brain was purified to homogeneity and identified as a 100 KDa fragment of PLC β1 (Dr. G.M.H. Thomas unpublished observations). It had in vitro catalytic activity but did not have the ability to be activated by the G-protein presumably because it does not possess the motif to interact with Gq. The unidentified PLC in rat brain did not react with PLC β1 antibodies, it is possible that that this PLC may be a fragment of PLC β3 which has been recently identified in rat brain cytosol (J. Exton personal communication).

8.1 Introduction to PI-transfer protein-Historical

The notion of a transfer protein for phospholipids was mooted as far back as 1968 when Wirtz and Zilversmit (226) observed an exchange of PC and to a lesser extent PE between rat or bovine liver mitochondria and liver microsomes. Later (227), they demonstrated that phospholipid exchange was dependent on a protein present in a pH 5.1 extract of liver cytosol.

In the 1970s there was a huge increase in information regarding phospholipid exchange proteins. It emerged there are three main categories of transfer protein which differ in their distribution, substrate specificity, isoelectric point, and molecular weight (Table 7.1). Firstly
there is PC-TP (209), a 24-28 KDa protein that exclusively transfers PC from one membrane to another (most likely the transfer activity first seen by Wirtz and colleagues). Secondly, nsL-TP, a 14 KDa protein originally described by Bloj and Zilversmit (210) which is predominantly found in liver (211). Substrate specificity of nsL-TP extends to phospholipids (PI, PC, PE, PS), glycolipids and cholesterol (210, 211). Finally, there is PI-TP the protein of interest in this discussion which predominantly binds and transfers PI. PI-TP is also able to bind and transfer PC and has a very low affinity towards phosphatidyglycerol (PG) and sphingomyelin (223). This protein does not transfer phosphatidylethanolamine, phosphatidylserine or cardiolipin. Transfer of PA by PI-TP has been reported (206) but this has not been independently confirmed. Recently a second isoform of PI-TP has been cloned from a rat brain cDNA expression library. The deduced amino acid sequence showed 77 % identity to that of the rat PI-TP and has been designated PI-TP β (228).

PI-TP appears to be universally distributed throughout eukaryotic organisms (202). It has been identified in cytosol fractions of mammalian, avian, reptile, amphibian and insect tissues and also in the fungus Neurospora crassa and all of them are cross reactive with antibodies raised against bovine brain PI-TP (198). Cross reactivity of PI-TP from disparate sources with bovine antibodies indicates its conserved nature. PI-TP from Saccharomyces cerevisiae yeast (Sec14p) has proved an exception and does not share any sequence homology with mammalian PI-TP (229, 230). In addition there has been an unusual PI-TP identified in the subrhabdomeric cisternae of the photoreceptor cell of Drosophila i.e. Drosophila retinal degeneration B protein (rdgB). It differs from the PI-TPs so far mentioned in that the PI-TP activity constitutes the 281 amino-terminal residues of an integral membrane protein, yet it is > 40 % identical to rat brain PI-TP. Truncated rdgB protein, which constitutes the amino terminal possesses in vitro PI-TP activity (221).

The primary sequence of rat PI-TP predicted from rat cDNA clones yields a molecular weight of 31,911 (229). This is considerably less that that calculated from the migration of the denatured rat protein and other mammalian PI-TPs on polyacrylamide gel i.e. 35 KDa. On the other hand, it is greater than that calculated from molecular sieve chromatography (approx. 17 KDa) some have explained the non-ideal chromatographic behaviour to an unusual association of PI-TP with dextran beads (187, 201, 231).
Two forms of PI-TP have been identified which have distinct isoelectric points but are identical in every other way i.e. molecular weight, amino acid composition, immunological reactivity, sensitivity to heat denaturation, activity and phospholipid specificity (187, 216). The different isoelectric points reflect whether the protein is occupied by PI or PC (224). PI-occupied PI-TP is more acidic than PC-occupied PI-TP having isoelectric points of 5.3 and 5.6 respectively in rat brain (201). Both forms are readily interchangeable when incubated with phospholipid vesicles made-up of either PI or PC (224). PI an PC are accommodated on the same hydrophobic binding site on PI-TP (232). It follows that the binding sites for PI and PC are mutually exclusive i.e. PI and PC cannot be bound to the protein simultaneously (200).

### 8.2 Phospholipid Specificity of PI-TP

The distinguishing feature of PI-TP is its dual substrate specificity. Van Paridon et al (223) estimated that the affinity of bovine brain PI-TP is 16-fold higher for PI than for PC. This difference in affinity is caused by the interaction of the protein with the polar headgroups.

From kinetic analysis it was estimated that PI-TP exchanges its bound PI 20 times faster for a PI than PC, and its bound PC 5 times faster for PI than for PC (223). It would seem unlikely that PI-TP would bind PC at all since it so strongly prefers to bind PI, but the PI to PC ratio in cell membranes is low e.g 0.11 for bovine brain homogenate and in bovine brain approx. 65 % of PI-TP was found to contain an endogenous PI molecule (201, 223).

PI absolutely requires an intact inositol ring for it to be preferentially transferred by PI-TP. Any modification of the inositol moiety of PI leads to an inhibition of its transfer. PI derivatives where the inositol ring underwent partial periodate oxidation and subsequent borohydride reduction were transferred at an order of magnitude less than PI (200).

PI-TP-mediated transfer of phosphorylated derivatives of PI have been examined in small unilamellar vesicle assay systems. Bovine brain PI-TP exhibited no transfer activity toward PIP (199). Similarly human platelet PI-TP did not transfer PIP or PIP₂ (198).
8.3 Influence of donor and acceptor membrane composition, pH, charge and divalent cations on in vitro PI-TP activity

PI and PC transfer by PI-TP is sensitive to the polar headgroups of donor and acceptor membrane phospholipids. The observation that increasing the concentration of PI in acceptor vesicles led to an inhibition of the rate of PI transfer by PI-TP was initially considered to be due to a specific interaction of PI-TP with PI. But, incorporation of high concentrations of the negatively charged non-transferable PA into acceptor vesicles also caused an inhibition in the rate of phospholipid transfer by PI-TP. Even though the overall rate of transfer of phospholipids was inhibited, PI-TP substrate specificity remained unchanged indicating that membrane interaction and phospholipid transfer are two separate activities (200, 233).

Van Paridon et al (233) demonstrated that increased acidic phospholipids (PIP and PIP₂) in the acceptor membrane causes an increase in association of PI-TP with this membrane due to a decrease in the dissociation constant. The presence of Mg²⁺ or neomycin, a positively charged polyamine antibiotic, which binds acidic phospholipids reduced the affinity of PI-TP for vesicles containing PIP₂ presumably by shielding the negative surface charge of PIP₂ and thereby eliminated transfer inhibition (233).

PI-TP activity is pH-dependent. If the pH of the environment is greater than the isoelectric point of PI-TP (approx. 5.5) the protein is negatively charged causing it to be repelled from the acceptor vesicles. Conversely, if the pH is below the isoelectric point of PI-TP it will become positively charged and strongly associate with the acceptor vesicle obliterating all transfer. It has been noted that at pH 7.4 the association constant increases for vesicles containing increasing amounts of acidic lipid, since at this point PI-TP would be negatively charged the increased affinity points to a membrane interaction site on PI-TP which is positively charged i.e under this condition the attraction between the interaction site and the vesicles prevails over the repulsive forces (233).

Helmkamp (234) investigated the importance of the fatty acid composition of the constituent phospholipids of the acceptor vesicles in influencing PI-TP activity. Vesicles consisting of unsaturated and saturated PC were examined for their ability to be transferred by PI-TP. PI-TP could interact with both cis- and trans-monosaturated phospholipid
bilayers, however a saturated dimyristoyl phospholipid bilayer inhibited PI-TP activity.

PI-TP activity is also sensitive to the fluidity of the membranes with which it interacts. Helmkamp (234) found that donor to acceptor transfer of PI varied inversely with the viscosity of the acceptor vesicle.

Finally, PI-TP preferentially transfers phospholipids from highly curved vesicles. Van der waals interactions between phospholipid molecules are less efficient in highly curved single bilayer vesicles than in large multilamellar liposomes. The effect of curvature was reflected in the dissociation rate constant of the PC-TP-interface complex which was 100 times lower for multilamellar liposomes than for vesicles (235).

8.4 Phospholipid exchange versus net transfer

Since the discovery of PI-TP it has been of interest whether the protein simply exchanges one phospholipid for another or whether it is capable of net transfer of phospholipids. Net transport of phospholipids would imply that PI-TP could leave an acceptor membrane unoccupied having deposited PI or PC.

The overriding evidence suggests that PI-TP mediates a true molecular exchange reaction in the sense that the flow of PI and PC from donor to acceptor membranes equals that in the opposite direction Fig. 8.1(236). However, PI-TP can mediate the transfer of PI from rat liver microsomes or pure PI monolayers to vesicles consisting of only PC (206). Similarly, in a vesicle-vesicle assay PI-TP will mediate the transfer of PC to vesicles that contain PI but lack PC (236). But, the flux of PI or PC is always counterbalanced by the transfer of an equal amount of PC or PI in the opposite direction. Therefore, it is not a true net movement of phospholipid equivalent to that mediated by PC-TP. PC-TP can deposit PC at a membrane compartment consisting of non-transferable phospholipids (236, 237).

8.5 Tissue distribution and intracellular localisation of PI-TP

PI-TP has been found in all tissues investigated. In one study thirteen rat tissues (238) were examined; the brain exhibited the highest specific transfer activity and adipose tissue and skeletal muscle exhibited the lowest. Immunoblots of cytosolic fractions with an antibody against
Fig. 8.1 In vitro activity of PI-TP

PI-TP transfers PI or PC from donor to acceptor membranes. Exchange requires equivalent bidirectional fluxes i.e. as many donor phospholipid molecules are transferred to the acceptor membrane as acceptor phospholipids to the donor membranes. PI-TP is always occupied.
bovine PI-TP showed that an immunoreactive PI-TP band (Mol.wt. 35-36 KDa) was present in each rat tissue. An additional band (Mol.wt. 41 KDa) was detected in rat testis that was not appreciably different from the smaller molecular weight protein. Within the brain, the greatest concentration of PI-TP was in the pons-medulla and lowest in the pituitary gland (238). Subfractionation of total rat brain showed PI-TP was highly enriched in synaptosomes and myelin fractions (219).

Using immunofluorescence techniques Snoek et al (218, 239) have studied the intracellular localisation of PI-TP in Swiss mouse 3T3 fibroblast cells. In quiescent cells PI-TP is broadly distributed throughout the cell and structural labelling is barely visible. The labelling pattern changes on stimulation with PMA (PKC activator) or bombesin (activates PLC via a receptor) as PI-TP becomes associated with the golgi. In semi-quiescent (serum-starved) cells, PI-TP is homogeneously distributed in the cytosol but is also associated with the perinuclear golgi system in a closely packed stack pattern. Stimulation with PMA or bombesin induces PI-TP redistribution. In exponentially growing cells the total amount of PI-TP labelling is increased in both the cytosol and in the golgi system. Golgi localisation was confirmed by treating the cells with brefeldin A. Brefeldin A caused a redistribution of PI-TP labelling throughout the cell as a result of the dissociation of the golgi. PI-TP was also detected in the nucleus of exponentially growing cells although this could not be confirmed in isolated nuclei (218).

PI-TP has five PKC substrate consensus sequence sites in its primary structure i.e. potential sites for PKC-mediated phosphorylation. PI-TP phosphorylation accompanied PMA stimulation in Swiss 3T3 cells but it does not seem to be a prerequisite for redistribution because bombesin induces redistribution of PI-TP without detectable phosphorylation of the protein. The in vitro PI-transfer activity of PI-TP is unaffected by phosphorylation. However, the role of phosphorylation in the localisation of PI-TP cannot be dismissed since PI-TP is phosphorylated to a certain extent in the absence of any activating agents in semi-quiescent cells (239).
8.6 PI-TP-mediated reconstitution of cytosol-depleted HL60 cells

Despite the wealth of knowledge regarding PI-TP, its physiological role in the intact cell has remained elusive. There has been a lot of speculation regarding its involvement in the inositol lipid signalling pathway but up until now there has been no strong evidence to confirm this. The results presented here demonstrate that PI-TP is essential for efficient PLC β-mediated signalling in HL60 cells. PI-TP purified from bovine and rat brain was shown to reconstitute fMetLeuPhe receptor- and GTPγS-mediated activation of PLC in cytosol-depleted cells. In addition, PI-TP-mediated reconstitution exhibited Ca^{2+} and ATP dependence that closely mimicked the responses seen in "acutely-permeabilized" cell studies (165).

Although PI-TP was purified to apparent homogeneity there was always the chance that reconstitution was due to a contaminant rather than PI-TP itself. This possibility was discarded when recombinant mouse PI-TP (rPI-TP) (a gift from Prof. Karel Wirtz) was shown to reconstitute GTPγS-mediated PLC activity. The concentration of rPI-TP necessary to generate a response was not appreciably different from concentrations of native protein used routinely.

Reconstitution of permeabilized HL60 cells is a property unique to PI-TP amongst the transfer proteins. PC-TP, nsL-TP and pre-nsL-TP were not able to restore GTPγS-mediated activation of PLC activity. This would indicate that the PI/PC dual specificity of PI-TP is relevant to its physiological role.

Since reconstitution is heterologous i.e. PI-TP from bovine brain was reconstituting HL60 cells it was of interest to show that endogenous PI-TP leaked from HL60 cells upon permeabilization with streptolysin O. Moreover, when partially purified PI-TP from HL60 cell cytosol was introduced to cytosol-depleted cells homologous GTPγS-stimulated reconstitution was induced.

In addition to PI-TP, PLC β1 purified from a 2 M KCl extract of bovine brain membrane was able to restore GTPγS-stimulated inositol lipid signalling but not that stimulated by fMetLeuPhe. HL60 cells do not contain PLC β1, therefore PLC β1-mediated reconstitution most likely reflects substitution for the endogenous PLC β isoforms i.e. PLC β2 and PLC β3. GTPγS will activate members of the G_q and G_i families. The α-subunits of the G_q would be able to activate exogenously added PLC β1.
On the other hand fMetLeuPhe can only activate the $G_i$ family and the released $\beta\gamma$-subunits are poor activators of PLC $\beta1$ (78). It is therefore not surprising that PLC $\beta1$ can be regulated by GTP$\gamma$S but not by the receptor. Reconstituting ability does not extend to the 100 KDa proteolytic fragment of PLC $\beta1$. This fragment retains PIP$_2$ hydrolysing activity in vitro but lacks the site for interaction with the $\alpha$-subunits of $G_q$, therefore it cannot be activated by GTP$\gamma$S. The site for interaction with $\beta\gamma$-subunits should be present but since PLC $\beta1$ is a poor substrate for the $\beta\gamma$-complex it was unlikely to reconstitute (78).

The fact that PLC $\beta1$ reconstitutes cytosol-depleted cells might have inferred that it was PLC that was limiting. But, PI-TP alone can restore GTP$\gamma$S-activated inositol lipid signalling. This indicates that the residual PLC in the cytosol-depleted cell, a fraction of that in an intact cell, is sufficient to produce a robust response. This is a similar situation to that in avian erythrocytes where studies have shown that a very small population of PLCs associated with the cytoskeleton (2 %) are sufficient to couple to all the available purinergic receptor-regulated G-proteins (38).

Since both PI-TP and PLC $\beta1$ had the ability to reconstitute cytosol-depleted cells it was of interest to examine how these reconstituting factors interacted. When they were introduced to the cells together the response was synergistic. This co-operativity indicates that both PLC and PI-TP are required for efficient inositol lipid signalling.

**8.6.1 What is PI-TP doing in the cell?**

Over the last 20 years many investigators have proposed a role for PI-TP in inositol lipid signalling (198, 202, 223, 240). The in vitro properties of PI-TP make it ideally suited to maintaining the steady state concentration of PI in cell membranes i.e. PI-TP exhibits dual specificity toward PI and PC with a preferred affinity for PI and tends to transport PI from where it is in high concentration to were it is in low concentration (206). The linearity of reconstitution with respect to PI-TP concentration would indicate that PI-TP has an important role to play in inositol lipid signalling. PI-TP concentration may dictate directly the concentration of PI available for polyphosphorylation at the plasma membrane and therefore the activity of activated PLC. In such a model it is envisaged that fMetLeuPhe- or GTP$\gamma$S-stimulated PIP$_2$ hydrolysis ultimately causes a depletion of PI at the plasma membrane decreasing the PI:PC ratio. PIP$_2$
hydrolysis also leads to an increase in intracellular Ca$^{2+}$ and a raised intracellular pH (107, 241). Both of these phenomenon will result in an increased dissociation rate of PI-TP, contributing to enhanced transfer activity. PI-TP transfers PI from intracellular membranes to plasma membrane to replenish PI levels. When the cell is persistently stimulated, PI-TP would continually supply PLC with substrate.

This model can be supported by the observation that while PI is uniformly distributed in the intracellular membranes of cells its phosphorylated derivatives are predominantly at the plasma membrane (20). Polyphosphoinositides make up only 5% of total PI yet the stimulated cell can hydrolyse a percentage of PIP$_2$ in excess of this. Maximum occupation of muscarinic receptors of rat parotid cells leads to the complete turnover of the cellular pools of PIP and PIP$_2$ approx. 1-2 times per min for at least 30 min (242). Similarly in some of the experiments described in this thesis GTP$\gamma$S was capable of stimulating the hydrolysis of over 20% of the inositol lipids in the presence of PI-TP. This indicates that there is net synthesis of PIP and PIP$_2$ from their precursor PI. PI synthesis requires PI synthase and CTP-phosphatidate cytidylyltransferase, enzymes that are predominantly present in the endoplasmic reticulum remote from the plasma membrane (204). PI must therefore be recruited from its site of synthesis to the plasma membrane as a substrate for the lipid kinases. The only drawback to this model is that the PI precursor PA needs to be cycled from the plasma membrane to the endoplasmic reticulum. To date, no specific PA transport protein has been described.

### 8.6.2 Cellular lipid pools

There has been an alternative model of phosphoinositol lipid recycling that has gained credence, whereby a pool of PI is specifically dedicated to the supply of substrate to the plasma membrane. This concept was first put forward by Hokin and Hokin in 1964 (243) in a study of $[^{32}P]$-labelled avian salt glands. They found that cell stimulation seemed to instigate the turnover of an inositol lipid pool that was only a minor fraction of the total quantity of PI present.
Later in 1979, Fain and Berridge (244) demonstrated preferential breakdown of recently labelled lipid in the salivary gland of blowflies when stimulated with an agonist. A double labelling technique was used where PI was labelled to equilibrium with $^{32}$P-orthophosphate and briefly with $^3$H-inositol. Further evidence came from Monaco (245), where WRK1 mammary tumour cells were labelled with $^{32}$P orthophosphate during vasopressin stimulation or in quiescent cells. Subsequently, the cells were stimulated with vasopressin which led to a much more rapid degradation of the PI synthesised during the initial stimulation than of the PI labelled in unstimulated cells.

However Michell et al (246) attempted to repeat the work of Monaco in WRK1 rat mammary tumour cells using a different double-labelling technique. The cells were labelled to equilibrium with $^{14}$C-inositol and subsequently the cells were incubated with $^3$H-inositol for a short time to label selectively any inositol lipid pools that have a particularly rapid turnover. When the cells were stimulated with vasopressin they found no indication of selective degradation of the $^3$H-labelled lipids.

This model of inositol lipid pools necessitates that PI resynthesis occurs at or near the plasma membrane. This would imply that all the enzymes necessary for the production of PI be present at or near the plasma membrane. PI synthase activity has been detected at the plasma membrane of GH$_3$ rat pituitary tumour cells (247), rabbit proximal tubule cells (248), 1321 N1 astrocytoma cells (249) and in turkey erythrocyte membranes which are virtually devoid of endoplasmic reticulum (250). But this is still a contentious issue since others cannot confirm the presence of PI synthase at the plasma membrane (204, 251). However, if it were true that PI synthase is present in the plasma membrane the need for a transfer protein is obviated.

It must be acknowledged that PI synthesis also requires CTP phosphatidate cytidylyltransferase and there is only one report of minimal plasma membrane activity of this enzyme in rat liver (252). However, there are several studies that have failed to find any such activity (204).
8.6.3 PI-TP increases the rate of IP₃ production by PLC β isozymes

It would be anticipated that in the cytosol-depleted cell (PI-TP-depleted), the rate of GTPγS-mediated PIP₂ hydrolysis would fall as the amount of substrate is run down, to the point that it is no longer saturating. Significantly, this was not the case, GTPγS activation of PLC in the absence of PI-TP is linear with time for at least 60 min. This result strongly argues that the PLC is not limited by the supply of the substrate. It was calculated that in the absence of PI-TP, PLC hydrolysed 5% of the total cellular inositol lipid per hour. But when PI-TP was present, the initial rate of hydrolysis is greatly enhanced to 40% lipid hydrolysed per hour, an 8-fold increase in rate. This would indicate that the function of PI-TP is not merely to replenish the plasma membrane with PLC substrate but to play a more direct role in inositol lipid signalling. The fact that the rate of fMetLeuPhe-induced hydrolysis of PIP₂ is increased in the presence of PI-TP would further support the idea that PI-TP has some property additional to PI transfer. It is very unlikely that the substrate would have been limiting in the latter case because typically there is only 3-7% of inositol lipid hydrolysed.

PLC β1 has been shown to reconstitute in cytosol-depleted cells. If it is assumed that the substrate is not limiting then PLC β1 must reconstitute because PLC enzyme is limiting. PI-TP in the presence of GTPγS can increase the activity of the endogenous PLC β2/β3 and when PI-TP is added in conjunction with PLC β1, has the ability to enhance the activity of this exogenous enzyme. This would imply that the rate of IP₃ production in cells will be dictated by the concentration of the PI-TP.

What could PI-TP be doing to increase the rate of inositol phosphate production. Other transfer proteins have been shown to enhance lipid metabolism for example, nsL-TP activates microsomal enzymes involved in the conversion of sterol intermediates to cholesterol and steroid hormones presumably by making the substrates available to the enzymes. In addition, PC-TP from bovine and rat liver can stimulate the microsomal conversion of DAG to PC up to 10-fold and may be doing this by directly interacting with cholinephosphotransferase or specifically removing the newly synthesised PC molecule from the enzyme (253). PI-TP does not have the ability to enhance de novo synthesis of PI (238).
8.6.4 *A possible role for PI-TP in the regulation of lipid kinases*

The explanation for the increase in the rate of IP$_3$ induced by PI-TP may lie in the regulation of the PI-kinase pathway. It has been shown that PI-TP reconstitution is MgATP-dependent. PI is initially converted to PIP by PI-4-kinase followed by its conversion into PIP$_2$ by PI(4)P-5-kinase. The permeabilized cell system described here is dependent on all of these enzyme activities and thus can be regarded as a single entity. The rate limiting step could be anyone of those steps occurring between phosphorylation of PI and the dissociation of IP$_3$ and DAG from PLC. If PI-TP accelerated any of these, the effects observed could be explained.

A rapid decrease in PIP and PIP$_2$ levels immediately subsequent to agonist stimulation has been observed in several cell types (21). In vasopressin-stimulated rat hepatocytes polyphosphoinositides levels reach a minimum of about 50-70 % of control levels after 30 sec, after which, they return towards and sometimes surpass control levels (254). In rat pituitary cells under continual agonist stimulation, the increase in net synthesis of polyphosphoinositides can continue for at least 30 min (255).

Imai and Gershengorn (255) determined that rapid breakdown of PIP$_2$ can be separated from PI metabolism in thrombin-stimulated rat pituitary cells. PIP$_2$ hydrolysis is a rapid, transient event whereas PI turnover is more persistent. The increase in PIP$_2$ synthesis has been shown to be a G-protein regulated event in NRK fibroblasts and human neutrophils (256, 257). In general, there seems to be a lag period between receptor occupation and an acceleration in the production of IP$_3$ in the region of 10 sec-3 min (21, 255-257).

Increasing PIP$_2$ synthesis must result from a rise in either one or both of the lipid kinases and could occur by either positive intervention of a cofactor or a change in substrate presentation to the lipid kinases. It is possible that PI-TP acts as a cofactor to PI-4-kinase (Fig.8.2). A soluble activator of PI-4-kinase is not without precedence. PIK-A49, a 49 KDa activator of PI-4-kinase has been isolated from carrot cells (258). PI-TP may activate PI-kinase in mammalian cells, in an analogous fashion to this protein. Alternatively, PI-TP may enhance PI-4-kinase by presenting PI to the enzyme in a conformation that is preferred to that of PI already present in the plasma membrane and thereby dictate the overall activity of the inositol lipid signalling pathway.
Fig 8.2 A model of the mechanism of action of PI-TP: PI-TP presents PI to PI-4-kinase

In this model PI is transferred from the endoplasmic reticulum and presented to PI-4-kinase in a conformation that is preferred over PI already in the plasma membrane. PI-TP can thereby increase PI-4-kinase activity and consequently dictate the rate of inositol phosphate production. R, receptor; G, G-protein; PIP<sub>2</sub>, phosphatidylinositol 4,5 bisphosphate; PIP5K, phosphatidylinositol(4)phosphate-5-kinase; PIP, phosphatidylinositol(4)phosphate; PI4K, phosphatidylinositol-4-kinase; PI-TP, phosphatidylinositol-transfer protein; PI, phosphatidylinositol; DAG, diacylglycerol; IP<sub>3</sub>, inositol 1,4,5 trisphosphate.
While PI-4-kinase is an attractive target for PI-TP-induced activation, Stephens et al (257) have proposed that it is PIP-5-kinase that is under agonist control. They carried out a study in "acutely-permeabilized" human neutrophils monitoring the accumulation of PIP$_2$ labelled with [$\gamma^{32}$P] ATP. It was demonstrated that while PIP is under substantial substrate cycling there are very low levels of substrate cycling of PIP$_2$. It was clear that agonists can stimulate a PI(4)P-5-kinase activity via a pertussis toxin sensitive G-protein and this activation is independent of PKC or Ca$^{2+}$.

8.6.5 Yeast PI-TP and vesicular trafficking

PI-TP was isolated from *Saccharomyces cerevisiae* yeast strain by Daum and Paltauf (220). It was found to be identical to the *SEC14* gene product (sec14p) which is essential for the export of secretory proteins from trans golgi membranes (259) and cell growth (230). Sec14p closely resembles PI-TP from mammalian tissues with respect to size, isoelectric point, substrate specificity and inhibition by negatively charged phospholipids (260). But surprisingly, the deduced amino acid sequence of sec14p displays no homology with mammalian PI-TP (230). All the same, yeast has been considered a useful biological model to study the role of PI-TP *in vivo* by a combination of biochemical and molecular genetic techniques.

Recently, detailed information has emerged concerning the mode of action of sec14p (261). Sec14p maintains the critical PC content of yeast golgi membranes not by removing or delivering PI, but by suppressing the CDP-choline pathway of PC synthesis specifically at the golgi. Sec14p is envisaged as acting as a golgi phospholipid sensor. Overexpression of the sec14p lowers PC concentration in the golgi and conversely underexpression results in an increase of PC which seems to be detrimental to golgi secretion. It has not been determined why the CDP-choline pathway is regulated by sec14p and the PE methylation pathway is not. If mammalian PI-TP is functioning in a manner similar to yeast PI-TP its role may not be to present PLC substrate to the plasma membrane but to control the phospholipid composition of the vesicles destined for the plasma membrane (Fig 8.3).

What relationship does sec14p have to mammalian PI-TP? In a study addressing this question rat PI-TP was expressed in yeast cells to see if it could substitute for sec14p. Rat PI-TP rescued sects, a temperature sensitive mutant, at the nonpermissive temperature of 37°C but not the
Fig. 8.3 A model for the mechanism of action of PI-TP: PI-TP interacts with the golgi

In this model PI-TP acts in a similar manner to sec14p. PI-TP regulates the phospholipid composition of vesicles formed in the golgi that are destined for the plasma membrane. PM; plasma membrane; ER, endoplasmic reticulum; PI-TP, phosphatidylinositol transfer protein.
lethal SEC14 null mutation (262). This infers that PI transfer activity is relevant but not sufficient for the essential function of sec14p. PI-TP may also possess some property additional to PI transfer. This notion can be supported by the observation that nsL-TP while possessing the ability to transfer PI in vitro, is inactive in the reconstituting system.

It has recently become apparent that mammalian PI-TP has a role in regulated exocytosis in PC12 cells. Regulated secretion consists of sequential priming and triggering steps which depend on ATP and Ca^{2+} respectively, and require distinct cytosolic proteins. Hay and Martin (231) have found that PEP3, one of three priming proteins identified, is in fact PI-TP. Sec14p can substitute for PI-TP but is not quite as efficient. This may further advance the hypothesis that both act through a common mechanism that is essential in both regulated and constitutive secretion.

The priming step in exocytosis is ATP dependent and in vitro PI transfer is not, which may indicate that PI is phosphorylated at the golgi and PI-4-kinase activity has been detected there (263). It also has been shown that there is a correlation between cellular polyphosphoinositide levels and vesicle competence for calcium regulated exocytosis that is not associated with signalling (264). PIP may target the vesicle to the plasma membrane or the cytoskeleton. Profilin and gelsolin both actin binding proteins effectively bind to polyphosphoinositides (265, 266).

Further support for PI-TP having a role in the formation of transport vesicles comes from the rdgB protein from Drosophila. It has been implicated in having a direct role in membrane movement from the subrhabdometric cisternae to the rhabdomere and in so doing facilitating phospholipid transport. RdgB mutants express light-enhanced retinal degeneration which might be due to a lethal lack of phospholipid at the rhabdomere. It is interesting that a double rdgB and norpA mutant will not undergo retinal degeneration (221).

8.6.6 Is PI-TP a multifunctional protein?

If PI-TP is fulfilling only one single role, PI-TP regulation of vesicular flow would be inexorably linked to its ability to reconstitute GTP\gamma S-mediated activation of PLC in cytosol-depleted HL60 cells. In this model it can be envisaged that PI-TP regulates golgi function allowing competent vesicles
to emerge from the golgi that would be in a conformation accessible to lipid kinases at plasma membrane.

A recent paper has been published that puts in some doubt the relevance of the role of sec14p to mammalian PI-TP and indicates that PI-TP may be a multifunctional protein. Lopez et al. (267) have reported the characterisation of a sec14p homolog from the dimorphic yeast strain *Yarrowia lipolytica* (sec14p^YL). This protein shares PI/PC-transfer ability with sec14p and is similarly localised to golgi-like cytoplasmic structures. Furthermore, sec14p^YL expression in *Saccharomyces cerevisiae* can compliment the lethality of *SEC14* mutants indicating that it fulfils all the essential sec14p functions in a heterologous system. The surprising distinction between sec14p and sec14p^YL is their *in vivo* function. Whereas sec14p is essential for cell viability in *Saccharomyces cerevisiae*, sec14p^YL was neither required for cell viability nor secretory function in *Yarrowia lipolytica*. The role of sec14p^YL in *Yarrowia lipolytica* is in mediating differentiation from the yeast to the mycelial form. It would seem that PI-TP function is utilised in diverse ways by different organisms and if so PI-TP may also have diverse functions in the same cell.

In a multifunctional model PI-TP could influence a number of processes in a manner analogous to calmodulin. Calmodulin is a ubiquitous calcium-binding protein that is known to be involved in a diverse range of mechanisms. It is activator of a whole host of enzymes such as adenylate cyclase, phosphodiesterase, myosin light chain kinase to name but a few (268) and is essential for secretion in mast cells and neutrophils (184). Similarly PI-TP by influencing the rate of production of PIP$_2$ could support inositol lipid signalling through PLC$\beta$ isoforms, PLC$\gamma$ isoforms, exocytosis and would be predicted to be a requirement for the PI-3-kinase signalling pathway (Fig. 8.4).

In conclusion, PI-TP has been identified as an essential requirement for inositol lipid signalling. It has the ability to restore responsiveness to fMetLeuPhe and GTP$_\gamma$S to activate PLC in cytosol-depleted HL60 cells. Moreover, it has been shown to increase the rate of receptor or G-protein-mediated inositol phosphate production by the endogenous isoforms of PLC$\beta$. The mechanism of action remains ambiguous but a couple possibilities have been considered. PI-TP may be acting as a cofactor within the inositol lipid signalling pathway presenting PI to PI-4-kinase in a conformation that is preferred by the enzyme. On the other hand, PI-TP could regulate the supply of PI to the plasma membrane by
PI-TP may act as a multifunctional protein supporting a number of cellular processes through its influence on the production of PIP_2. Any system that utilises PIP_2 would have a requirement for PI-TP e.g. the PI-3-kinase signalling pathway, exocytosis and inositol lipid signalling through PLC β and PLC γ isoforms.
interacting with the golgi. This might explain its role in the priming step of exocytosis and inositol lipid signalling. But since it has been shown that PI-TP can carry out different functions in different organisms these two mechanism may not be mutually exclusive. By increasing the supply of PIP$_2$, PI-TP can be predicted to be required in supporting signalling pathways such as PLC $\gamma$ or PI-3-kinase alongside exocytosis.
Acknowledgements

First and foremost I would like to thank Shamshad Cockcroft, my supervisor, for her unerring enthusiasm and encouragement and making my time in her laboratory so enjoyable. Thanks too, to Geraint Thomas for a fruitful collaboration and his helpful advice throughout. A big thank you to Mandy Fensome, Andy Ball, Blandine Geny & Jackie Whatmore, my colleagues in the lab who all contributed to the realisation of this thesis. I am grateful to the BBSRC for providing funding. Finally, thank you to Paul Tyndall for his contribution to the graphics in this thesis and more importantly for his constant moral support for the last three years.
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