THE ROLE OF K⁺ CHANNELS IN THE SIGNAL TRANSDUCTION MECHANISMS OF ANTIGEN-INDUCED DEGRANULATION IN THE RAT BASOPHILIC LEUKAEMIA (RBL-2H3) CELL LINE

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A thesis submitted for the degree of Doctor of Philosophy

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In the name of Allah the most compassionate and the most merciful

To:

My dear wife and daughter, Leila and Maryam,
My dear parents,
The martyrs of my country during the revolution and war,
and my sponsor.
Abstract

To investigate whether potassium channels are involved in the signal transduction mechanisms of antigen-stimulated mediator release in the rat basophilic leukaemia cell line (RBL-2H3), the effect of some potassium channel modulators on antigen-stimulated β-hexosaminidase secretion, membrane potential changes, $^{86}$Rb$^+\,$-efflux and [Ca$^{2+}$]$_i$ have been evaluated. Quinidine, a nonspecific K$^+$ channel blocker, dose-dependently inhibited antigen induced $^{86}$Rb$^+$-efflux, membrane repolarisation and secretion. Ba$^{2+}$ inhibited antigen-induced secretion, but it had no effect on resting and antigen-stimulated $^{86}$Rb$^+$-efflux, and it caused a massive depolarisation by itself. These data indicate that the inhibitory effect of quinidine may be due to the inhibition of K$^+$ channels, whereas the effect of Ba$^{2+}$ might be either direct, by interfering with Ca$^{2+}$ influx, or indirect, by causing depolarisation. Cetiedil and charybdotoxin inhibited β-hexosaminidase release with an IC$_{50}$ of 84μM and 130nM respectively. Some recently synthesised cetiedil-related compounds (“UCL compounds”) have also been tested. All the UCL compounds inhibited the antigen-stimulated β-hexosaminidase release and $^{86}$Rb$^+$-efflux and their potency was as follows: UCL1608 > 1710 > 1617 > 1348 > 1349 > 1495. Charybdotoxin inhibited repolarisation and $^{86}$Rb$^+$-efflux with a similar IC$_{50}$ (~90nM) to that for inhibition of secretion. Margatoxin 10nM (K$_{v1.3}$ blocker) and tetrodylammonium 5mM (BKca blocker) did not affect antigen-induced secretion significantly. Since charybdotoxin is capable of inhibiting BKca, IKca and K$_{v1.3}$, these results suggest that the outward rectifier K$^+$ channel in RBL cells may have some similarities to IKca but not to BKca or K$_{v1.3}$. This is consistent with the known inhibitory effect of cetiedil and the UCL compounds on the IKca channels in erythrocytes. Based on my $^{86}$Rb$^+$-efflux studies, it can be proposed that the K$^+$-efflux pathway cannot be entirely voltage-dependent and also that it may comprise two components, one Ca$^{2+}$-dependent and the other Ca$^{2+}$-independent. It can be concluded that at least two kinds of K$^+$ channels are activated by antigen; Ca$^{2+}$-independent (possibly G-protein-linked) channels whose activation is not necessary for degranulation and Ca$^{2+}$-dependent channels which help to maintain the electrochemical driving force for Ca$^{2+}$ influx.
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2. Discrimination of the inhibitory effects of Ba\(^{2+}\) and quinidine on rat basophilic leukaemia cell line activation(1). (Abstract)
   In the 26th annual meeting of the European Histamine Research Society (Seville, Spain, May 14-17, 1997)

3. Discrimination of the inhibitory effects of Ba\(^{2+}\) and quinidine on rat basophilic leukaemia cell line activation(2). (Abstract)
   In the 26th annual meeting of the European Histamine Research Society (Seville, Spain, May 14-17, 1997)

4. Characteristics of antigen-induced \(^{86}\)Rb\(^+\) -efflux from sensitised rat basophilic leukaemia (RBL-2H3) cells: Evidence for potassium channels involvement. (Abstract)
   In Young Physiologist Meeting, University of Cambridge, June 17-18, 1997

5. Some characteristics of the antigen-induced Ca\(^{2+}\) oscillations in individual RBL-2H3 cells. (Abstract)
   In the 27th annual meeting of the European Histamine Research Society (Lodz, Poland, May 20-23, 1998)

6. Quantification of the antigen-induced Ca\(^{2+}\) oscillations in individual RBL-2H3 cells. (Abstract)
   In the 27th annual meeting of the European Histamine Research Society (Lodz, Poland, May 20-23, 1998)
## Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ATP]_i</td>
<td>Intracellular concentration of ATP</td>
</tr>
<tr>
<td>[ion]_i</td>
<td>Intracellular concentration of an ion</td>
</tr>
<tr>
<td>[ion]_o</td>
<td>Extracellular concentration of an ion</td>
</tr>
<tr>
<td>[pH]_i</td>
<td>Intracellular pH</td>
</tr>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>Anti-IgE</td>
<td>Antibody raised against immunoglobulin E</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BAPTA-AM</td>
<td>Acetoxy methyl ester form of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>BDA</td>
<td>bis(2-hydroxyethyl)dimethylammonium</td>
</tr>
<tr>
<td>BHQ</td>
<td>2',5'-di(tert-butyl)-1,4-benzohydroquinone</td>
</tr>
<tr>
<td>Bis-oxonol</td>
<td>bis-(1,3-diethylthiobarbiturate)trimethoxonol</td>
</tr>
<tr>
<td>BK_c^2</td>
<td>High conductance Ca^{2+}-activated K^+ channel</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine 3',5' monophosphate</td>
</tr>
<tr>
<td>CBP</td>
<td>Calcium binding protein</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide 3-chlorophenylhydrazone</td>
</tr>
<tr>
<td>ChTX</td>
<td>Charybdotoxin</td>
</tr>
<tr>
<td>Con-A</td>
<td>Concanavalin A</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium release activated calcium channels</td>
</tr>
<tr>
<td>CTMC</td>
<td>Connective tissue mast cells</td>
</tr>
<tr>
<td>CTX</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>D_2O</td>
<td>Heavy water (deuterium oxide)</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNP-BSA</td>
<td>Dinitrophenyl-bovine serum albumin</td>
</tr>
<tr>
<td>DNP-HSA</td>
<td>Dinitrophenyl-human serum albumin</td>
</tr>
<tr>
<td>DNP-Lys</td>
<td>Dinitrophenyl-Lysine</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetra-acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol-bis(β-amino-ethyl ether)N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>F(ab)_2</td>
<td>Bivalent antigen binding fragment of antibody</td>
</tr>
<tr>
<td>Fab</td>
<td>Antigen binding fragment of antibody</td>
</tr>
<tr>
<td>Fc</td>
<td>Constant structure fragment of antibody</td>
</tr>
<tr>
<td>FceRI</td>
<td>High affinity receptor for IgE</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
</tr>
<tr>
<td>GABA</td>
<td>Gama amino butyric acid</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GDPβS</td>
<td>Guanosine-5'-O-(2-thiodiphosphate)</td>
</tr>
<tr>
<td>GppNHP</td>
<td>5'-guanylylimidodiphosphate</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanosine triphosphate (GTP) binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>GTPγS</td>
<td>Guanosine-5'-O-(3-thiotriphosphate)</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid</td>
</tr>
<tr>
<td>IbTX</td>
<td>Iberiotoxin</td>
</tr>
<tr>
<td>i_CRIC</td>
<td>Ca^{2+} release activated Ca^{2+} conductance</td>
</tr>
</tbody>
</table>
IgE Immunoglobulin E
IgG Immunoglobulin G
IKCa Intermediate conductance Ca^{2+}-activated K^+ channel
IP Inositol Phosphate
IP_3 Inositol 1,4,5 triphosphate
IP_4 Inositol 1,3,4,5-tetrakis-phosphate
IR Inward rectifier
K_a A current
K_{ATP} ATP-sensitive K^+ channel
K_{Ca} Ca^{2+}-activated K^+ channel
K_M M current
K_V Voltage-activated K^+ channel
M.W. Molecular weight
MC^T Mast cells containing tryptase
MC^TC Mast cells containing both tryptase and chymase
MgTX Margatoxin
MMC Mucosal mast cell
NECA 5’-(N-ethylcarboxamido) adenosine
NMG N-methyl-D-glucamin
OR Outward rectifier
PA Phosphatidic acid
PBS Phosphate buffered saline
PC Phosphatidyl choline
PE Phosphatidyl ethanolamine
PIP_2 Phosphatidylinositol 4,5-bisphosphate
PKC Protein kinase C
PLA_2 Phospholipase A_2
PLC Phospholipase C
PLD Phospholipase D
PMA Phorbl 12-myristate 13-acetate (=TPA)
PTX Pertussis toxin
RBL Rat basophilic leukaemia cell line
RBL-2H3 Rat basophilic leukaemia cell line (2H3 subtype)
SEM Standard error of mean
SKCa Small conductance Ca^{2+}-activated K^+ channel
S-MEM Minimum essential medium
TEA Tetraethylammonium
TPA 12-O-tetradecanoylphorbol ester (=PMA)
TPP^+ Tetraphenylphosphonium ion
UCL 1348 (+)-2-cyclohexyl-2-(3-thienyl) ethanoic acid 2-(hexahydro-1H-azepin-1-yl) ethyl ester monohydrochloride
UCL 1349 (-)-2-cyclohexyl-2-(3-thienyl) ethanoic acid 2-(hexahydro-1H-azepin-1-yl) ethyl ester monohydrochloride
UCL 1495 Triphenyl acetic acid-2-N[5-ethyl-2-methylpiperidinoethyl] ester monooxalate
UCL 1608 1-[(9-benzyl)fluoren-9-yl]-4-(N-homopiperidino)-but-2-ynyl oxalate
UCL 1617 N-[2-(N'-triphenylethanamido) ethyl]-5-ethyl-2-methylpiperidine
UCL 1710 1-[(9-benzyl)fluoren-9-yl]-4-(N-5-ethyl-2-methylpiperidino)-but-2-ynyl oxalate
UK 66914 (N-[4-[1-hydroxy-2-[4-(4-pyridyl)-1-piper=azinyl]ethyl]phenyl]methanesulphon-amide
CHAPTER 1 INTRODUCTION

1.1 Mast cells and basophils

1.1.1 Definition

Mast cells and basophils are immune effector cells that contribute to the specific immune response against helminth parasites and certain bacterial infections, the initiation of anaphylactic reactions to allergens, inflammation, asthma and tumour surveillance. The name of mast cell (Mastzellen = well-fed cells) was given to the cell by Ehrlich, who for the first time discovered that blue aniline dye is taken up by the granules of these cells, and he believed that the granules contained phagocytosed material. However it has been well established that granules are full of histamine and other stored materials for release. Mast cells are resident cells in all connective tissue, but basophils, which were also described by Ehrlich, are circulating cells. Basophils, like other granulocytes, differentiate and mature in bone marrow, circulate in the blood, and are not normally found in connective tissue [1, 2].

The plasma membrane of mast cells and basophils contains a receptor glycoprotein, which binds the immunoglobulin E (IgE) monovalently via its Fc region with high specificity and affinity [3, 4, 5, 6]. The receptor glycoprotein forms the physiologically critical molecular link between an exogenous stimulus (antigen-IgE complex) and the signal transduction mechanism for exocytosis, and it is called FcεRI.

Basophils, like mast cells, are granulated and the granules contain histamine and other inflammatory mediators. A common precursor of both cell types (stem cell)
Chapter 1  Introduction

originates in the bone marrow. However, during development they diverge from each other at an early stage. Based on morphological features and cell surface differentiation markers, basophils resemble more closely other blood granulocytes, rather than mast cells. It has been established that tissue mast cells do not originate from circulating basophils.

1.1.2 The role of mast cell in immunity and inflammation

The physiological function of mast cells remains largely unknown, but theories include rejection of parasites, regulation of wound healing, regulation of microvasculature and angiogenesis, and limitation of delayed hypersensitivity reactions [7, 8]. There is no human condition associated with a complete lack of mast cells. The important pathophysiological process involving mast cells and basophils is the immediate hypersensitivity reactions. Likewise, a role for mast cells has been shown in several inflammatory conditions including asthma, atopic dermatitis, psoriasis, contact hypersensitivity reactions and fibrosis reactions [9, 10].

1.1.3 Heterogeneity

Although different mast cells share some common characteristics, it is known that they do not represent a homogenous population. The differences observed are reflected in secretory function, staining properties, location, granular contents, morphology, life span, development and response to stimuli and drugs. There are two distinct subpopulations of rat mast cells: serosal, also known as the connective tissue mast cells, and mucosal mast cells. However, the classification of human mast cells is more complicated.

1.1.3.1 Heterogeneity of mast cells in the rat

Despite a common precursor, the two subpopulations are distinct and it is unlikely that one is derived from the other [reviewed in 11, 12, 13, 14, 15]. However transdifferentiation has been reported. Thus, upon interactions with other cells including fibroblasts, nerves, epithelial and endothelial cells and others, pluripotential mast cell precursors may undergo final differentiation into a tissue-specific phenotype. The characteristics of the two different subpopulations of mast cells in the rat are summarized in table 1.1.
### Table 1.1: Characteristics of rat mast cells subtypes:

#### 1.1.3.2 Human mast cells heterogeneity:

It may not be possible to classify human mast cells by one or two phenotypic characteristics [16]. However, the most frequently used criterion is the difference in neutral protease content. Some human mast cells contain measurable levels of both tryptase and chymase (MC\textsuperscript{Tc}), whereas others contain tryptase but no detectable chymase (MC\textsuperscript{T}). In some respects, but not all, human MC\textsuperscript{T} seem similar to rat MMC, and human MC\textsuperscript{Tc} resemble murine CTMC. Table 1.2 shows the features of two subtypes of human mast cells.
Table 1.2: Characteristics of human mast cells subtypes.

1.2 Rat basophilic leukaemia cell line

1.2.1 Historical background

Development of a basophilic leukaemia in the rat following treatment with β-chlorethylamine was first reported by Eccleston et al. in 1973 [17]. These rat basophilic leukaemia (RBL) cells were cultured *in vitro* by two different groups in the same year [6, 18]. RBL cells are mononuclear cells (10-12μm) containing predominantly chondroitin di-B proteoglycan and low amounts of histamine (0.1-1.0 pg/cell) as well as RMCP-II protease in their granules. Compared with serosal mast cells, the granules in RBL cells are smaller and less electron-dense. Furthermore, these cells stain with alcian blue but not safranin [19] and they are not activated by compound 48/80 [20, 21, 22]. On the basis of these criteria RBL cells seem very closely related to mucosal mast cells [19, 23]. The paucity of granules and the small quantity of histamine in RBL cells might reflect the incomplete differentiation of rapidly growing cells in culture media. Although the original cell lines had IgE receptors and contained histamine [6, 24, 25, 26], because of defects at different parts of the pathways related to histamine release, they were quite variable in histamine release [27]. In 1981 Barsumian et al. cloned some variants of RBL cells and they found out that the RBL-2H3 variant is a good releaser line without any deficiency.
The RBL-2H3 subline typically expresses $2-3 \times 10^5$ FceRI per cell on the cell surface [27]. IgE-mediated activation is inhibited during mitosis, but not A23187-induced activation, indicating an impaired transmembrane signaling during mitosis [28, 29].

### 1.2.2 Similarity and advantages

RBL-2H3 cells have been widely used as an *in vitro* model, to investigate the mechanisms of immediate hypersensitivity reactions. Because of the availability of large numbers, high purity, and the capability to be grown in cell culture, this cell line provides a unique opportunity for exploring the molecular details of IgE-mediated cell activation to an extent that would be difficult with normal cells. Although there are only limited comparative data, FceRI structure and IgE-mediated secretion in RBL-2H3 cells, human basophils and rodent mast cells share several important characteristics [30 and references therein].

Unlike normal mast cells and basophils from actively sensitised animals, whose FceRI are variably occupied with different IgEs, the RBL-2H3 cells can be passively sensitised with a specific exogenous monoclonal IgE. The most important differences between RBL-2H3 and normal mast cells include responsiveness to crosslinked IgE oligomers, desensitisation, potentiating effect of phosphatidylserine and effects of metabolic inhibitors [30]. RBL-2H3 cells do not show desensitisation when calcium is added back as long as 90 min after stimulation in its absence [30]. In contrast to rat peritoneal mast cells, RBL-2H3 cells respond poorly to dimeric IgE. Phosphatidylserine potentiates stimulated secretion from normal mast cells whereas it has no effect on stimulated secretion from RBL-2H3 cells. In normal mast cells, antimycin has no effect on spontaneous release and inhibits antigen-stimulated secretion completely at a concentration of 1μM. However, in RBL-2H3 cells, antimycin 10nM increases the spontaneous release, while it completely inhibits the antigen-induced secretion (100 more potent than with normal mast cells). In contrast to normal mast cells, RBL-2H3 cells treated with optimal concentrations of antigen secrete slowly over 15-30 minutes [31, 32]. The slow response in RBL-2H3 cells enables considerably better time-resolution of cellular events.
1.2.3 Mechanisms of Activation

1.2.3.1 Sensitisation and Activation

Activation by IgE/antigen interaction is very complex. The simple binding of IgE to FcεRI, so-called sensitisation, does not activate mast cells and causes no known perturbation. However, the cells become activated when the IgE bound to receptors is crosslinked by multivalent antigen (indirectly aggregating the IgE receptors) or when the IgE receptors are aggregated directly by other procedures. This process in turn induces exocytosis. Thus, the addition of polymerised IgE molecules [33, 34], anti-IgE antibodies [35] and concanavalin A [36] can aggregate IgE bound to receptors and cause secretion. Exocytosis can also be induced by calcium ionophores independently of IgE receptor crosslinking [37, 38, 39].

1.2.3.2 IgE molecule

The molecular structure of IgE (MW = 190 kDa) is shown diagramatically in Figure 1.1. The IgE molecule consists of two identical light (L) chains (κ or λ) and two identical ε heavy (H) chains [reviewed in 40]. Although it shares the general structure of other classes of antibodies, it is distinguished from them by the sequence of its (ε) heavy-chain constant region. Each heavy chain of IgE is composed of 5 domains, four constant domains Ce1 - Ce4 and one variable domain VεH. The light chain includes Cl and VL domains. The Ce2 domain replaces the hinge region of the other antibody classes. The constant region fragment (Fc) contains Ce2 - Ce4 in which the receptor binding sites are located. The two antibody-binding fragments (Fab) recognise and bind to the specific antigen. They are composed of Ce1 - VεH and CeL - VL, which are linked by a disulphide bridge between CeL to Ce1. Based on resonance energy transfer fluorophore measurements, it became apparent that IgE is bent. The IgE does not undergo any change in its molecular shape upon binding to FcεRI. Although IgE is rich in carbohydrate, the carbohydrate components are not required for its binding to the receptor.

The receptor binding site is located in the region from glutamine-301 in the carboxy-terminal portion of the Ce2 domain to arginine-376 in the amino-terminal portion of the Ce3 domain (a 76 amino acid residue segment in the interface between Ce2 and Ce3 domains) so that the recombinant peptide containing this segment can sensitize mast
cells to degranulation by anti-IgE [41]. It has been suggested that only one of the two ε chains (Ce3) interacts with the receptor directly [40, 41].

IgE is a minor component of serum immunoglobulins (0.004%) with a normal concentration of 50-180 ng/ml. It has the shortest half life, highest fractional catabolic rate and lowest synthetic rate of the Ig classes. The high affinity binding permits a dramatic amplification of IgE-antigen activation.

Based on fluorescence resonance energy transfer measurements, the IgE structure in solution as well as the receptor-bound IgE are in a bent conformation [42].

Figure 1.1: The IgE structure.
1.2.3.3 FceRI receptor

Immunoglobulin (Ig) molecules can bind to Fc receptors which are a type of immunoreceptor that respond to foreign antigen via a bound Ig molecule. A specific Ig class can bind to a particular Fc receptor through its constant Fc segment, and the variable domains in the two Ig Fab segments provide specific antigen recognition [43].

FceRI is the high affinity receptor for IgE which consists of four transmembrane polypeptides with the composition αβγ2 (Fig. 2). Although IgE binds to the α-chain, the β- and γ-chains are required for insertion of the α chain into the membrane and for signal transduction [44]. Since the fully assembled complex is required for the cell response, omitting just one of the three subunits is sufficient to prevent FceRI expression and function [44]. Like other Fc receptors, an “immune receptor tyrosine kinase activation motif” (ITAM) found within the cytoplasmic domain of the γ subunit is necessary for signal transduction to occur from FceRI receptors [45]. β chain has also been shown to act as an amplifier and enhance signalling through the γ chain [46]. In spite of heavy N-glycosylation of the receptor, the carbohydrate component is not required for IgE binding [40].

The α subunit is composed of two extracellular Ig-like domains (α1-α2 domains), a single transmembrane sequence, and a cytoplasmic domain containing 20 to 31 amino acids. Spectroscopic analysis suggests that the α domains lie parallel to the cell membrane. Although both IgE and the receptor have two binding sites in their structure, because of the configuration of α domains (parallel to cell membrane) and the conformation of IgE (bent), IgE can bind to the receptor monovalently only on the convex face. After IgE binding, the Fab arms of the IgE point outwards from the cell surface. The β subunit consists of four membrane spanning domains, with the amino and carboxy termini exposed to the cytoplasmic side of the membrane. The γ subunits include two identical disulfide-linked chains. Although the β subunit lacks any detectable homology with any other known sequences, the γ subunit has two regions which exhibit homology with the ζ subunit of the T cell receptor. The IgE binding site of the receptor is situated in the α2 domain near the interface with α1 domain. The regions in α1 are required for high affinity binding [47].
1.2.3.4 Binding specificity and affinity

IgE binds monomerically [48] and tightly to FceRI ($K_a \sim 10^{10} \text{ M}^{-1}$) and dissociates very slowly. Thus the formation of an IgE-FceRI complex can be considered irreversible [18, 25, 49]. Figure 1.2 shows the interaction between IgE and FceRI.

The binding specificity of the receptor for the ε isotype is virtually perfect, so that any other isotype cannot compete effectively against the binding of IgE. However, the species specificity is imperfect. For instance, mouse IgE can bind to rat mast cells and basophils, and rodent IgE can bind to human receptors. In contrast, RBL-2H3 cells fail to bind human IgE.

![Figure 1.2: IgE molecule bound to the FceRI receptor](image-url)
1.2.3.5 Antigen stimulation (IgE-crosslinking)

Crosslinking of FcεRI with antigen or any aggregator (see below) initiates a biochemical cascade leading to cell degranulation and mediator secretion, which is similar to other immunoreceptor activation processes such as those involved in B and T lymphocytes stimulation. Early studies suggested that symmetric, bivalent aggregation of IgE-bound receptors is sufficient to trigger cell activation [50]. However, Fewtrell and Metzger showed that highly purified covalent IgE dimers stimulate RBL-2H3 cells poorly while covalent trimers were more effective, and longer oligomers even more effective still. In contrast, rat peritoneal mast cells can be effectively stimulated by covalent dimers [51].

In RBL-2H3 cells, a relatively small number (fewer than $10^3$ per cell) of trimers or oligomers were sufficient to trigger significant degranulation: this figure corresponds to a small fraction of the $2-3 \times 10^5$ FcεRI receptors per RBL-2H3 cell. This means that the threshold for RBL-2H3 cell activation requires only 1% of the FcεRI receptors to be crosslinked. For maximum secretion to occur, only 10% of the FcεRI receptors need to be crosslinked [49, 52]. Moreover, it has been shown that crosslinking becomes maximal only at a very high concentration of antigen (>1μg/ml), whereas the degranulation response reaches its maximum at 7ng/ml [53].

Although it is well established that IgE crosslinking does not produce any substantial changes in the size or stoichiometry of the subunits of the receptor, small alterations in the state of phosphorylation have been described [54, 55]. However, it is not clear whether this phosphorylation plays an important role in the signal transduction mechanisms of secretion.

It has been reported that the aggregated FcεRI receptors are only transiently active and the addition of monovalent haptens (DNP-Lysine) inhibits antigen-induced RBL-2H3 cell degranulation [56, 57]. The effect of such monovalent hapten might be mainly due to prevention of formation of new aggregates rather than dissociation of the preexisting ones. However, Kent et al [58] have shown that brief exposure of the RBL-2H3 cells to covalently cross-linked IgE oligomers and then addition of excess monomeric IgE to prevent further aggregation can cause stable FcεRI aggregation resulting in sustained signaling. They concluded that aggregated receptors can remain active for a long time. Moreover, Seagrave et al. [59] have reported that within the first 1-2min following FcεRI
crosslinking, addition of DNP-Lys effectively deaggregates FceRI receptors as assayed by the release of multivalent antigen from the cell surface. However, at later time points, addition of hapten cannot deaggregate receptors or cause the release of multivalent antigen from cell surface [59].

1.2.3.6 Other crosslinking agents

A variety of other cross-linking ligands are also capable of eliciting secretion. These include chemically polymerised IgE, IgG antibodies against IgE, antireceptor antibodies, antibody against hapten-IgE complex and the plant lectin concanavalin A which aggregates IgE via sugar residues in the Fc region.

1.2.3.6.1 Polymerised IgE

Segal et al. showed that chemically cross-linked dimers and higher oligomers of IgE are able to elicit mast cell degranulation [50]. In their study, rat IgE was reacted at alkaline pH with the homobifunctional chemical cross-linking reagent dimethyl suberimidate, which reacts specifically with amino groups. They fractionated the resulting mixture by gel filtration to obtain purified dimers, trimers and oligomers of cross-linked IgE. As has been mentioned above, Fewtrell and Metzger [51] used the same highly purified cross-linked IgE on RBL-2H3 cells. IgE dimers are an ineffective stimulus, but IgE trimers and oligomers induce considerably more release than dimers.

1.2.3.6.2 Anti-IgE

Apart from antigen, specific anti-Fc antibodies (anti-IgE) can crosslink IgE-bound to its receptors. Anti-IgE-induced activation requires the presence of IgE on the receptor and it does not have any effect on non-sensitised RBL-2H3 cells. Using a monoclonal anti-IgE antibody A2, Conrad et al. have shown that this antibody is a poor activator of secretion [60]. This is in agreement with a study using chemically crosslinked IgE dimers which do not lead to secretion. Anti-IgE stimulation cannot be halted by monovalent conjugated hapten DNP-Lys [57].
1.2.3.6.3 Antibody against FcεRI receptor

Anti-FcεRI antibody is also able to stimulate RBL-2H3 cells. Since it can crosslink the receptor itself, it does not need the receptor-bound IgE. Ishizaka & Ishizaka [35] obtained anti-FcεRI by sensitization of a rabbit with immune precipitates composed of rat IgE receptor. Anti-FcεRI and its F(ab)2 can induce mast cell degranulation. However, Fab fragments could bind to the receptor and inhibit passive sensitisation of mast cells with IgE antibodies, but failed to induce histamine release. The skin reaction induced by anti-FcεRI can be overcome if mast cells have been saturated with IgE. It has been demonstrated that antibodies against extracellular α domains can induce RBL-2H3 cells to degranulate [47]. However, antibodies against the β subunit of the receptor fail either to prevent IgE-binding or induce cell activation [61].

1.2.3.6.4 Concanavalin A

Concanavalin A (Con-A), a lectin isolated from the jack bean, can cause receptor crosslinking via sugar residues in the Fc region. The characteristics of cell activation by Con-A are very similar to antigen-induced activation and it seems that both stimuli activate mast cells through the same signal transduction pathways, although their binding sites are different. The effect of Con-A can be inhibited by α-methyl-D-mannose indicating that the sugar binding site of Con-A is responsible for its effect [32, 36]. Like antigen, Con-A cannot induce histamine release from non-sensitised RBL-2H3 cells [20].

1.3 Signal transduction pathways of exocytosis in RBL-2H3 cells

1.3.1 Changes in mobility and distribution of aggregated receptors

According to fluorescent labeling and photobleaching techniques FcεRI receptors are independently mobile and the translational diffusion coefficient is \(2 \times 10^{-10} \text{ cm}^2 \text{s}^{-1}\) which is similar to other membrane proteins [62]. IgE binding to the receptor does not affect the receptor mobility. However, cross-linking of the receptor causes a substantial decrease in mobility. The antigen-induced immobilization of IgE receptor complexes is rapid (\(t_{1/2} = 1.0 \text{min}\)) and does not exhibit a significant temperature dependence [63].
Despite the close correlation between the degree of receptor immobilization and secretion, it is unlikely that immobilization is secondary to the other biochemical perturbations, since this immobilization can occur in the absence of extracellular calcium or when cellular ATP is depleted. Immobilization does not appear to be necessary for the other changes to take place.

### 1.3.2 Changes in cellular cytoskeleton

Crosslinking of IgE receptors causes an association of IgE-receptor complexes with the cellular cytoskeleton in RBL-2H3 [64]. The extent of the cytoskeletal association follows the extent of bridging. Transformation of the cellular surface from a microvillous to a lamellar topography has been shown when RBL-2H3 cells are activated by antigen. These changes appear to be mediated by activation of PKC.

### 1.3.3 Biochemical events

Aggregation of IgE-bound receptors leads to many biochemical changes which are similar to those observed in many other secretory cell systems [56, 65]. These changes include tyrosine phosphorylation [66, 67], cholera toxin-sensitive G-protein \((G_\alpha)\) activation [68, 69] and stimulation of phospholipase C (PLC) [21], phospholipase D (PLD) [70] and phospholipase \(A_2\) (PLA\(_2\)) [71].

#### 1.3.3.1 Tyrosine phosphorylation

Upon FceRI crosslinking, tyrosine kinase activity is induced within 5-15s resulting in tyrosine phosphorylation of the receptor subunits and other intracellular substrates like PLC\(\gamma_1\) and PLC\(\gamma_2\) as well as the syk tyrosine kinase itself [72, 73]. Since FceRI has no sequence motifs indicative of intrinsic phosphotyrosine kinase (PTK) activity, the IgE-crosslinking may activate nonreceptor kinases [44]. Several tyrosine kinase inhibitors such as genistein and herbimycin A inhibit antigen-induced cellular responses in permeabilised cells [72, 74]. In contrast, \(Na_3VO_4\), a tyrosine phosphatase inhibitor, is capable of stimulating secretion and membrane responses in permeabilised RBL-2H3 cells [74, 75]. Eiseman and Bolen [76] have reported that FceRI is preassociated with src-like kinase- p53/56\(^{lyn}\) in RBL-2H3 cells. More recently Yamashita et al. [77] have shown that p53/56\(^{lyn}\) kinase is activated by receptor aggregation. It has been shown that
piceatannol, a specific syk-protein tyrosine kinase inhibitor, can inhibit antigen-induced RBL-2H3 cell activation, but it has no effect on receptor subunit phosphorylation. Due to this observation, Oliver et al. suggested that receptor-mediated lyn activation leads to receptor subunit phosphorylation, but syk causes the FceRI-mediated cellular response [78]. By analogy with T cells, it is likely that lyn activation and resulting receptor phosphorylation may normally facilitate the association of syk with the receptor and its activation through an autophosphorylation mechanism.

PLC activation by 5’-(N-ethylcarboxamido)adenosine (NECA), an adenosine analogue, is not mediated through tyrosine kinase activation, because NECA fails to induce any tyrosine phosphorylation activity and also herbimycin A, a tyrosine kinase inhibitor, has no inhibitory effect on the NECA-stimulated PIP$_2$ hydrolysis [72]. It has been reported that NECA activates a PLC isozyme other than PLC$\gamma_1$ through a PTX-sensitive G-protein [79, 80].

1.3.3.2 Receptor phosphorylation

FceRI aggregation induces immediate phosphorylation of $\beta$ (tyrosine and serine) and $\gamma$ (tyrosine and threonine) subunits by at least two different non-receptor kinases. Phosphorylation of tyrosines on the $\beta$ and $\gamma$ subunits of the FceRI by lyn tyrosine kinase is among the earliest biochemical events that follow receptor aggregation [76, 81, 82]. Tyrosine phosphorylation occurs within “immune receptor tyrosine kinase activation motifs” (ITAM) which are present in the carboxy-terminal cytoplasmic tail of the $\beta$ chain and in the cytoplasmic tail of the $\gamma$ chain. Recently it has been demonstrated that the $\gamma$ subunit is phosphorylated at threonine by protein kinase C-$\delta$ activation [83].

1.3.3.3 G-protein activation

Using GTP and its nonhydrolysable analogues such as GTP$\gamma$S in permeabilised RBL-2H3 cells, it has been shown that the FceRI mediated signal transduction pathway in RBL-2H3 cells is controlled by GTP-binding protein activation [84, 85]. Likewise, cholera toxin treatment potentiates antigen-stimulated Ca$^{2+}$-influx and secretion in RBL-2H3 cells. These effects were unrelated to the stimulation of adenylate cyclase [69, 86].

McCloskey [69] reported that cholera toxin also induces inositolphosphate (IP) production. However, Narasimhan et al. [71, 86] found that this effect was most likely
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secondary to the increased Ca\(^{2+}\) influx in cells treated with antigen and toxin. Moreover, antigen-stimulated IP hydrolysis in the intact or permeabilised cells is resistant to pertussis or cholera toxin [79, 86]. In contrast, IP production by NECA can be inhibited by both pertussis or cholera toxin, indicating that NECA activates PLC through a special kind of G-protein (G\(_i\)) [79].

Depletion of guanine nucleotides with mycophenolic acid incubation leads to over 60% reduction of GTP levels and nearly 50% inhibition of antigen-stimulated mediator release as well as a 50% decrease in Ca\(^{2+}\)-influx rate, whereas antigen-induced IP production is only slightly reduced [87]. These observations indicate a role for G proteins in RBL-2H3 activation by antigen via the Ca\(^{2+}\) influx pathway.

GDP\(_\beta\)S, a GDP analogue which stabilises GTP-binding proteins in their inactive form, can inhibit GTP\(_\gamma\)S-induced secretory and membrane (transformation of cellular surface) responses in RBL-2H3 cells, whereas it has little effect on antigen-induced responses. On the other hand, tyrosine kinase inhibitors such as genistein block the secretory and membrane responses to antigen, without altering the GTP\(_\gamma\)S-induced responses [74]. Overall it can be expected that GTP\(_\gamma\)S and antigen activate separate pathways leading to functional responses in permeabilised RBL-2H3 cells. The involvement of G-proteins in the activation of PLC is based on indirect evidence. It includes stimulation of IP hydrolysis in intact cells by NaF (or the combination of NaF and AlCl\(_3\)) or in permeabilised cells by GTP\(_\gamma\)S [22].

The receptor-mediated activation of cellular PLA\(_2\) is controlled by a G-protein and it can be enhanced by GTP\(_\gamma\)S. However, pretreatment of cells with cholera toxin or pertussis toxin does not affect PLA\(_2\) activation by antigen (or GTP\(_\gamma\)S) [71].

1.3.3.4 Activation of phospholipases

The coupling events between FceRI-crosslinking and PLC activation and other biochemical and functional responses have not been clearly defined. However, it is likely that protein tyrosine kinase activation, through FceRI crosslinking, has a crucial role in the sequence of events leading to the calcium pulse, possibly through tyrosine phosphorylation and activation of PLC\(_\gamma\) isozymes.

Phospholipase C activation hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) and yields two intracellular second messengers: 1,4,5 inositol trisphosphate (IP\(_3\)) which is released into the cytosol, and 1,2 diacylglycerol (DAG) which remains in the cell
membrane. IP$_3$ by binding to its high affinity receptor (K$_D$=8nM) on the endoplasmic reticulum, releases calcium from intracellular stores [65]. IP$_3$ is also phosphorylated to yield inositol 1,3,4,5 tetrakisphosphate (IP$_4$) by a cytosol 3'-kinase.

The credentials of IP$_3$ as a second messenger in RBL-2H3 cells include its rapid action (release of Ca$^{2+}$ in permeabilised cells is complete within 6s) and its ability to induce physiological responses upon injection into single cells [88, 89, 90]. IP$_3$ and IP$_4$ production precedes the onset of the increase in [Ca$^{2+}$], and the generation of these molecules is sustained long enough to support the presistent calcium signal in RBL-2H3 cells.

The second messenger DAG, activates protein kinase C (PKC), which phosphorylates substrate proteins. DAG and phorbol esters (artificial PKC stimulators) reduce the requirement for Ca$^{2+}$ and its threshold concentration to support the enzyme activity. DAG causes the translocation and attachment of PKC to plasma membrane and induces phosphorylation of cellular proteins to produce physiological responses.

Protein kinase C contains two subunits, the regulatory subunit which binds with a high affinity to phorbol esters and phosphatidylserine and the catalytic subunit which has a full serine/threonine kinase activity. However, PKC activators, such as PMA have no effect on [Ca$^{2+}$], and do not cause any histamine release [91]. Moreover preincubation of RBL-2H3 cells with these reagents abolishes the IP hydrolysis and the [Ca$^{2+}$], response induced by antigen. In contrast, PMA has no significant effect on maximal antigen-stimulated histamine release [91]. The lack of effect of PMA-incubation on the A23187-induced Ca$^{2+}$ signal indicates that Ca$^{2+}$ influx is not significantly regulated by activated PKC [91].

In addition to the PLC activation, the PLD pathway has an important role in DAG generation in RBL-2H3 cells. PLD catalyses phosphatidylcholine (PC) hydrolysis to produce phosphatic acid (PA), which is converted to DAG by PA phosphohydrolase [92]. Free arachidonic acid can be released from arachidonic acid-containing DAG by the action of DAG lipase. C$_2$-ceramide, which blocks Ca$^{2+}$ influx and translocation of Ca$^{2+}$-dependent PKC isozymes, is capable of inhibiting antigen-induced PLD activation and secretion [93]. These data indicate that the PLD activation depends on Ca$^{2+}$ influx.

Phospholipase A$_2$ (PLA$_2$) stimulation is also an early response to receptor aggregation in RBL-2H3 cells. The time-course of PLA$_2$ activation precedes histamine release. PLA$_2$ utilizes IP, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) to produce arachidonic acid and lysophospholipids [94]. The latter products may cause
changes in membrane structure facilitating granular fusion. The PLA$_2$ activation requires continued receptor aggregation but not Ca$^{2+}$ influx [95]. PLA$_2$ inhibitors block arachidonic acid and histamine release in RBL-2H3 cells [31].

1.3.3.5 Polyphosphoinositide breakdown

Using IgE polymers of different sizes, it has been shown that the rate of inositol phospholipid (IP) hydrolysis and the peak level of [Ca$^{2+}$]$_i$ is correlated to the number and size of aggregated receptors. The maximum secretory response needs 15-20% of FceRI receptors to be occupied. However, the hydrolysis of inositol phospholipid is increased proportionally to the number of receptors occupied until all receptors are saturated [49]. The observations indicate that RBL-2H3 cells have an excess number of receptors (more than 80%) and excess capacity for IP hydrolysis over the requirement for maximal release.

Upon optimal stimulation by antigen as much as 50% of membrane IP is consumed, whereas A23187 (200-1000nM) causes less than 5% IP hydrolysis [96]. Subsequent addition of antigen after A23187 provokes substantial IP hydrolysis, with no further increase in [Ca$^{2+}$]$_i$ or secretion [21]. Interestingly, low concentrations of A23187 (<200nM) which cause an increase in the [Ca$^{2+}$]$_i$, up to1000nM cannot induce a secretory response. Above this concentration of A23187, which can result in 1-5% IP breakdown, histamine release is induced. Based on these data, Lo et al. proposed that the Ca$^{2+}$ signal alone is not a sufficient stimulus for secretion and at least 1-2% IP hydrolysis is necessary to initiate secretion [96].

After antigen stimulation, blockade of Ca$^{2+}$ influx and of the rise in [Ca$^{2+}$]$_i$ by La$^{3+}$ cannot impair the IP breakdown [91]. However, preincubation with La$^{3+}$ inhibits IP hydrolysis dose-dependently (Table 1.3) [21]. In the absence of Ca$^{2+}$, DNP-BSA induces a rapid but transient production of IP [49]. Increased [Ca$^{2+}$], by influx can enhance phospholipid hydrolysis and amplify antigen-induced secretion. Moreover, Zn$^{2+}$ and Co$^{2+}$ can inhibit histamine release and Ca$^{2+}$ signal without affecting IP hydrolysis indicating that IP breakdown is dependent on receptor activation and not the Ca$^{2+}$ signal [20, 21].

However, the kinetics of the IP hydrolysis and the dependency on external Ca$^{2+}$ differ with the various crosslinking agents [89]. It is clear that IP breakdown can be evoked independently of Ca$^{2+}$ by some stimuli in RBL-2H3, but the stimulatory response needs to be amplified in the presence of external Ca$^{2+}$, which in turn, results in secretion
In parallel, the IP response, calcium signal and secretion are very sensitive to changes in temperature [97].

<table>
<thead>
<tr>
<th>(La^{3+}) concentration</th>
<th>% Inhibition of IP hydrolysis</th>
<th>% Inhibition of histamine release</th>
</tr>
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<tbody>
<tr>
<td>10(\mu M)</td>
<td>20%</td>
<td>50-90%</td>
</tr>
<tr>
<td>100(\mu M)</td>
<td>57%</td>
<td>97%</td>
</tr>
<tr>
<td>500(\mu M)</td>
<td>&gt;97%</td>
<td>&gt;97%</td>
</tr>
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Table 1.3 Effect of preincubation of RBL-2H3 cells with \(La^{3+}\) on the IP hydrolysis and histamine release. Cells were incubated with different concentration of \(La^{3+}\) 10min before antigen stimulation. (Data are obtained from ref. 21)

IP hydrolysis parallels the rise in \([Ca^{2+}]_i\), and secretion due to stimulation with different concentrations of antigen [21]. Moreover, manipulations that increase (e.g. presence of heavy water [49]) or decrease (e.g. raising the temperature to 40\(^\circ\)C [97], depletion of cellular ATP [96] or the addition of hapten [88]) the IP hydrolysis rate lead to analogous changes in the intensity of the \(Ca^{2+}\) response and secretion (i.e. increase in IP hydrolysis results in increase in the intensity of \(Ca^{2+}\) response and secretion).

1.3.4 Calcium movement

1.3.4.1 Calcium requirement

Early observations indicated that the presence of external calcium is essential for secretion [98, 99, reviewed in 100]. Moreover, elevation of the intracellular calcium concentration \([Ca^{2+}]_i\), can induce the secretory response [37]. The IgE-mediated release is also accompanied by an influx of calcium into RBL-2H3 cells [101] and an increase in \([Ca^{2+}]_i\); [20]. Furthermore, RBL-2H3 cell degranulation can be induced by \(Ca^{2+}\) ionophores [37, 38, 102]. Above all, \(La^{3+}\) and addition of excess EGTA can inhibit \(Ca^{2+}\) influx and secretion induced by antigen [20, 91]. Finally, if \(Ca^{2+}\) is removed from the extracellular buffer while the stimulated cells are degranulating, release is immediately halted [56, 103].
Likewise, phosphatidyl inositol breakdown is dependent on the presence of external calcium, but not on the rise of \([\text{Ca}^{2+}]\), in the range of 100nM to 1\(\mu\)M and it is not inhibited by La\(^{3+}\) at concentrations which block calcium influx.

However, some stimuli such as GTP\(_{\gamma}\)S (in permeabilised cells) [104, 105] and nerve growth factor [106] do not require a rise in \([\text{Ca}^{2+}]\). Moreover, antigen can stimulate significant histamine release in PMA-pretreated RBL-2H3 cells, a condition in which there is no detectable increase in \([\text{Ca}^{2+}]\). This may be due to an unknown signal induced by antigen that can synergise with PMA-activated PKC to cause exocytosis [91]. Furthermore, it has been shown that the RBL-1B3 Tg\(^{-}\) cell line can respond to antigen with a modest rate of IP hydrolysis but no detectable Ca\(^{2+}\) signal. Notably, the response of this cell line is dependent on extracellular Ca\(^{2+}\) [22]. Finally, using chlortetracycline as an indicator of the Ca\(^{2+}\) release from stores, Marecotte et al. have shown that antigen activation results in a decrease in chlortetracycline fluorescence even in the absence of Ca\(^{2+}\) influx (in high K\(^{+}\) buffer or in the absence of extracellular Ca\(^{2+}\)), indicating antigen-induced Ca\(^{2+}\) release from stores [107].

1.3.4.2 Increase in intracellular calcium concentration

Using Quin-2 loaded cells, it has been reported that in unstimulated RBL-2H3 cells the intracellular calcium concentration \([\text{Ca}^{2+}]\) is about 105nM and is not affected by changes in the concentration of extracellular calcium ((\([\text{Ca}^{2+}]\))\(_{o}\)) from 0.1 to 2mM. Incubating the cells in Ca\(^{2+}\)-free medium, however, causes \([\text{Ca}^{2+}]\) to fall to 57nM over 1 hour [20]. The addition of antigen to IgE-primed cells results in an initial sharp rise in \([\text{Ca}^{2+}]\), that reaches a maximum of 5-10 fold above resting levels by 1 min (the calcium spike). After approximately 5 min, the calcium level declines to a point that is significantly higher than resting level. The increase in calcium concentration persists over at least 20 min (the calcium plateau) [108]. It is well established that the calcium spike, which does not require external Ca\(^{2+}\), is related to calcium release from internal stores and that the calcium plateau is sustained by the influx of calcium from the medium [65].

Mucosal mast cells and RBL-2H3 secrete much more slowly than serosal mast cells, taking 15-30 min to reach maximum secretion, and the prolonged response depends on calcium influx and a rise in \([\text{Ca}^{2+}]\), [31]. In contrast, rat peritoneal mast cells achieve maximum response within 1-2 min [32]. In the absence of extracellular Ca\(^{2+}\), antigen
causes a very limited or no increase in [Ca^{2+}]_i in RBL-2H3 cells, but the full response can be restored by reintroduction of Ca^{2+} to the external medium [20].

The rise in [Ca^{2+}]_i induced by antigen and Ca^{2+}-ionophores can be inhibited equally by depletion of intracellular ATP with the metabolic inhibitor antimycin A, in the absence of glucose [96, 109]. In the case of Ca^{2+}-ionophores, which permit the redistribution of Ca^{2+} ions down the concentration gradients of free Ca^{2+}, the ATP dependency is hard to explain. Lo et al. have suggested the possibility that an ATP-dependent site for Ca^{2+} entry may be involved in Ca^{2+} influx-induced by both antigen and ionophore [96]. Another possibility, as introduced by Putney, is that the replenishment of depleted stores is carried out by an ATP-dependent uptake of Ca^{2+} from extracellular and intracellular spaces [110].

A high extracellular K^+ concentration is capable of inhibiting Ca^{2+} influx and secretion induced by antigen and Ca^{2+}-ionophores without altering the release of Ca^{2+} from intracellular stores by antigen. This inhibition is possibly due to the neutralization of the negative membrane potential which provides part of the necessary electrochemical gradient for Ca^{2+} influx [111, 112]. However, this inhibitory effect can be reversed by increasing external Ca^{2+} concentration [112]. In spite of inhibiting Ca^{2+} influx, K^+-depolarisation does not affect the A23187- and antigen-induced Ca^{2+} efflux. The inhibitory effects of ATP-depletion and K^+-depolarisation on Ca^{2+}-ionophore-induced RBL-2H3 cell activation are indistinguishable. Overall, intracellular ATP and the maintenance of a sufficient electrochemical gradient are necessary for Ca^{2+} uptake induced by both stimuli. It should be noted that phospholipid breakdown is not affected in a depolarising medium [89]. Notably, in high K^+-depolarised RBL-2H3 cells, a significant (50%) decrease in total cell calcium has been reported [112].

Not only [Ca^{2+}]_i (from 100nM to 0.3-1μM) but also total cell Ca^{2+} (from 0.5mM to 2.5mM) concentration is increased by antigen stimulation in RBL-2H3 cells, possibly because of uptake from the cytosol into IP_3-insensitive stores i.e. mitochondria [20, 112].

Low concentrations of A23187 (<200nM) or PMA (10-100nM) each of which alone cannot elicit secretion from RBL-2H3 cells, can stimulate cell degranulation when combined [91]. This observation led to the proposal of a dual signal hypothesis in RBL-2H3 cells, when PKC activation and an increase in [Ca^{2+}]_i act in synergy to provide a sufficient stimulus for RBL-2H3 cell activation. Similar results have been observed with unresponsive RBL-2H3 cell clones which secrete in response to A23187 and PMA [22]. However, the combination of ionophores and PKC activators do not precisely mimic the
actions of antigen. For instance, for an increase in $[\text{Ca}^{2+}]_i$ to less than 1000nM, IgE oligomers are more potent secretagogues than the combination of ionophore and PMA [91]. Thus there is an indication that some additional path(s) of signal transduction (i.e. via G-proteins) is/are involved in the amplifying the effect of antigen and other crosslinking agents. Similarly, antigen-induced histamine release can occur in RBL-2H3 cells pretreated with PMA, where IP hydrolysis and the rise in $[\text{Ca}^{2+}]_i$ are strongly inhibited. This means that antigen can stimulate an effector which can synergise with PKC activation and result in secretion, even without IP hydrolysis and Ca$^{2+}$ influx.

The IP hydrolysis, rise in $[\text{Ca}^{2+}]_i$ and secretion are markedly enhanced in the presence of 30% D$_2$O [49]. By contrast D$_2$O has no significant effect on the A23187-induced response. An effect of D$_2$O on binding of IgE oligomers to FceRI receptors has been ruled out as a possible explanation for its enhancing effect on the antigen-induced response [49]. IP hydrolysis and secretion are significantly inhibited by organic solvents including ethanol, 1-propanol and dimethyl sulphoxide [0.1-0.5%v/v] [49]. These effects have been allowed for in this study.

1.3.4.3 Calcium influx

Apart from the release of calcium from intracellular stores by IP$_3$, Ca$^{2+}$ uptake from the cell exterior via the activation of a calcium permeable pathway or channel contributes to the substantial increase in total cell calcium that occurs with cell stimulation. There is a very close correlation between secretion, Ca$^{2+}$ uptake and a sustained increase in $[\text{Ca}^{2+}]_i$. Using Quin-2 as a calcium buffer, Fewtrell and Sherman have measured the unidirectional Ca$^{2+}$ influx and calculated a Km of about 0.7mM and a Vmax of 0.9 nmol of Ca$^{2+}$/ 10$^6$ cells / min [52]. This data is almost identical with the value of 1 nmol of Ca$^{2+}$/ 10$^6$ cells / min (equivalent to 0.91mM/cell/min) obtained by Beaven et al by measuring $[\text{Ca}^{2+}]_i$ in Quin-2 loaded cells [20]. The Ca$^{2+}$ influx rate in unstimulated cells equals 0.06nmol/ 10$^6$ cells / min which is 15-fold slower than the stimulated Ca$^{2+}$ influx rate. Thus, the Ca$^{2+}$ influx increases 15-fold after stimulation which accounts for the 12-fold increase in $[\text{Ca}^{2+}]_i$ (from 100nM to 1.2μM). With a relatively low antigen concentration, Ca$^{2+}$ influx reaches a plateau after a few minutes and for the maximum response as few as 10% of FceRI receptors need to be aggregated [52]. The response is maintained for at least an hour. The antigen-stimulated Ca$^{2+}$ uptake (i.e. by a polyvalent hapten conjugate such as DNP-BSA) can be halted immediately after the
addition of a monovalent hapten conjugate (i.e. DNP-Lys) [52]. Thus, IP hydrolysis and the peak of \([Ca^{2+}]_i\) (due to the Ca\(^{2+}\) release from stores by IP\(_3\)) but not Ca\(^{2+}\) influx and secretion are coupled closely with the magnitude of aggregated receptors indicating that excess crosslinking leads to desensitisation of the early events.

### 1.3.4.4 Calcium efflux

A rapid increase in \(^{45}Ca^{2+}\) efflux was observed after Fc\(_e\)RI receptor aggregation (antigen and other crosslinking agents) in RBL-2H3 cells which had been previously equilibrated with \(^{45}Ca^{2+}\). At first, it was thought that this was the consequence of secretion or displacement of \(^{45}Ca^{2+}\) by external Ca\(^{2+}\) [30]. This change is inhibited by metabolic inhibitors but not by omitting extracellular Ca\(^{2+}\). Moreover, non-secreting RBL-2H3 cell lines show the antigen-stimulated Ca\(^{2+}\) efflux [30] and the stimulated Ca\(^{2+}\) efflux still occurred in high K\(^+\)-depolarised RBL-2H3 cells, a situation in which Ca\(^{2+}\) uptake and secretion are greatly inhibited [112]. The data suggest that the stimulated Ca\(^{2+}\) efflux is not a result of secretion. The stimulated Ca\(^{2+}\) efflux and secretion are closely correlated [30]. The rate of Ca\(^{2+}\) efflux from resting RBL-2H3 cells has been calculated to be \(>60\times10^5\) atoms /cell/min, equivalent to a clearance rate of 0.05mM /cell/ min [20]. The Ca\(^{2+}\) efflux rate after addition of La\(^{3+}\) or EGTA has been measured as approximately 1.2 nmol Ca\(^{2+}\)/10\(^6\) cells/min. These data, overall, indicate that the Ca\(^{2+}\) efflux rate is increased to balance the stimulated influx. It has been suggested that the calcium signal is a dynamic balance between ATP-dependent increases in influx and efflux across the plasma membrane [20].

### 1.3.4.5 Calcium oscillations

Ca\(^{2+}\) oscillation, a cyclical fluctuation in \([Ca^{2+}]_i\), has been reported in a variety of cell types including non-excitable cells [review in 113]. RBL-2H3 cell activation by antigen but not by calcium ionophores leads to an asynchronous \([Ca^{2+}]_i\) fluctuation rather than a regular sinusoidal pattern. Likewise, irregular \([Ca^{2+}]_i\) spiking can also be observed. These oscillations are not constant in frequency, amplitude, shape and lag time [109, 114]. This heterogeneous response in individual cells, the so-called Ca\(^{2+}\) fingerprint, is not likely to be due to cell cycle differences or to the presence of a variety of cell clones in a population [109, 115]. Transient oscillations (3 or 4 spikes only) can be observed when Ca\(^{2+}\) influx is inhibited (e.g. in the absence of extracellular Ca\(^{2+}\), or in the presence of
La\(^{3+}\), or on depolarising the cells in high K\(^+\) buffer), but prolonged oscillations (>30min) are resumed as soon as Ca\(^{2+}\) influx is restored [109, 116]. However, increasing the time of preincubation of the cells with EGTA leads to the depletion of Ca\(^{2+}\) stores and abolition of Ca\(^{2+}\) oscillations. Based on these results, it seems that the oscillations occur as the result of modulation of [Ca\(^{2+}\)], by exchange with intracellular stores [109], but Ca\(^{2+}\) influx is required to sustain Ca\(^{2+}\) oscillations. In addition, since changes in membrane potential cannot abolish Ca\(^{2+}\) oscillation when RBL-2H3 cells are depolarised in high K\(^+\) solution, it is not likely that variations in plasma membrane potential cause the Ca\(^{2+}\) oscillations [109, 113]. However, it cannot be ruled out that fluctuation in membrane potential might follow or even potentiate the oscillations. Ca\(^{2+}\) oscillations can occur in the absence of glucose and in the presence of metabolic inhibitors: the condition in which mitochondrial Ca\(^{2+}\) uptake is prevented, indicating that mitochondrial Ca\(^{2+}\) uptake is not required for the oscillations [109]. It has been proposed that RBL-2H3 cells might have several efficient Ca\(^{2+}\) buffering systems and the calcium extrusion via the plasma membrane may also contribute to Ca\(^{2+}\) oscillation. The latter is in agreement with antigen-induced \(^{45}\)Ca\(^{2+}\) efflux observation [109].

1.3.4.6 Proposed theories about calcium oscillation

To explain the mechanism of Ca\(^{2+}\) oscillations, several different models have been proposed. Since Ca\(^{2+}\) oscillations occur as a result of the release of Ca\(^{2+}\) from intracellular stores, most of models concentrate on the internal stores [113]. However, Ca\(^{2+}\) influx is essential for sustained Ca\(^{2+}\) oscillations. Based on \(IP_3\) levels the suggested models can be divided into three groups:

a) The \(IP_3\) levels fluctuate.

1. Receptor-controlled oscillator model (Cobbold theory): Activation of PKC by DAG and the rise in [Ca\(^{2+}\)], due to calcium release from internal stores, exerts negative feedback on the early stages [117]. The inhibitory effect of PKC on PLC decreases the \(IP_3\) levels and this, in turn, causes the falling phase of the Ca\(^{2+}\) spike. By lowering PKC activity, PLC produces more \(IP_3\) and causes the rising phase of the Ca\(^{2+}\) spike.

2. \(IP_3\)-Ca\(^{2+}\) crosslinking model (Meyer & Stryer theory): Ca\(^{2+}\) release from \(IP_3\)-sensitive stores by \(IP_3\) results in a positive feedback by Ca\(^{2+}\) on PLC. This
causes a surge of IP$_3$ production and Ca$^{2+}$ release. Depletion of IP$_3$-sensitive stores and sequestration of Ca$^{2+}$ into the IP$_3$-insensitive stores terminate the Ca$^{2+}$ spike. Further Ca$^{2+}$ spikes can be restored when IP$_3$-sensitive stores are replenished by Ca$^{2+}$ influx [118].

3. Reuptake of Ca$^{2+}$ into IP$_3$-sensitive stores by IP$_4$: IP$_3$ is metabolized to IP$_4$ which promotes Ca$^{2+}$ reuptake into IP$_3$-sensitive stores. Fluctuation of IP$_3$ levels and Ca$^{2+}$ sequestration by IP$_4$ result in Ca$^{2+}$ oscillations [119, 120].

b) IP$_3$ is increased but does not oscillate.

1. Negative feedback by Ca$^{2+}$ on IP$_3$-induced Ca$^{2+}$ release (Payne theory): Release of Ca$^{2+}$ from internal stores by IP$_3$ and a rise in [Ca$^{2+}$]$_i$ inhibit IP$_3$ production. By sequestration of Ca$^{2+}$, the inhibition is removed. Then IP$_3$ can initiate another Ca$^{2+}$ spike [121].

2. Ca$^{2+}$ induced Ca$^{2+}$ release (Berridge theory): IP$_3$ releases Ca$^{2+}$ from IP$_3$-sensitive stores which are continuously replenished by Ca$^{2+}$ influx. Ca$^{2+}$ is also sequestrated into IP$_3$-insensitive stores. Once IP$_3$-insensitive stores are full, [Ca$^{2+}$]$_i$ finally begins to increase and the stores release their contents by Ca$^{2+}$-induced Ca$^{2+}$ release. IP$_3$-insensitive stores become depleted, and the initiation of the next Ca$^{2+}$ spike needs these stores to be filled [122].

c) Ca$^{2+}$ oscillations do not require any increase in IP$_3$ levels.

1. Thapsigargin-induced Ca$^{2+}$ oscillations: Thapsigargin, an inhibitor of Ca$^{2+}$-ATPase, is capable of inducing Ca$^{2+}$ oscillations in parotid acinar cells. It was suggested that Ca$^{2+}$ oscillations in these cells, which are dependent on extracellular Ca$^{2+}$, can occur while IP$_3$-sensitive stores are completely depleted of Ca$^{2+}$. Since Ca$^{2+}$ oscillations are sensitive to caffeine and ryanodine, the IP$_3$-sensitive stores may not be involved in the generation of Ca$^{2+}$ oscillations [123].

2. Ca$^{2+}$ influx and membrane potential fluctuations: Since depolarisation inhibits agonist-stimulated Ca$^{2+}$ influx in non-excitable cells, the membrane potential fluctuations might account for Ca$^{2+}$ oscillations [124].
1.3.5 Plasma membrane potential change

1.3.5.1 Resting membrane potential

Although RBL-2H3 is a non-excitable cell, its resting membrane potential is close to the equilibrium potential of $K^+$, and the values obtained by several groups are: -94 mV [57], -90 mV [125], -70 mV [126]. The resting potential is proportional to $[K^+]_o$, and for a 10 fold change in $[K^+]_o$, the resting potential is changed by 54 ± 2 mV [125]. In contrast, the rat peritoneal mast cell resting membrane potential has been reported to fluctuate around 0 mV [125, 127]. The resting membrane potential in RBL-2H3 cells is maintained by $K^+$ permeability and it is not immediately affected by ouabain, a $Na^+/K^+$ ATPase pump inhibitor. In contrast, in rat peritoneal mast cells, the resting membrane potential is predominantly controlled by the $Na^+/K^+$ ATPase pump. It should be mentioned here that another indication of the heterogeneity of mast cells is the difference between mucosal and serosal mast cells in relation to the resting membrane potential, ionic conductance and changes in membrane potential after stimulation as well as other characteristics (this will be also discussed in section 1.5.4).

1.3.5.2 Measurement of changes in membrane potential in RBL-2H3-2H3

Using the tetraphenylphosphonium ion (TPP+) equilibration technique, Kanner and Metzger demonstrated that crosslinking of IgE receptors on RBL-2H3 cells depolarises the plasma membrane [57]. However, it was later suggested that the overall depolarisation is a reflection of the mitochondrial membrane depolarisation, which is possibly caused by mitochondrial calcium uptake following cell activation and an increase in the intracellular concentration of calcium [128]. The TPP+ method is limited by slow equilibration of the probe with the cells and by its responsiveness to the mitochondrial membrane potential in addition to that of the cell membrane. To overcome these problems, Mohr and Fewtrell [111] used the negatively charged potential sensitive fluorescent dye bis-(1,3-diethylthiobarbiturate)trimethineoxonol (bis-oxonol). In contrast to TPP+, bis-oxonol rapidly equilibrates with the cells and because of its negative charge, it does not accumulate in the mitochondria, and so it is not affected by changes in the mitochondrial membrane potential. Using the conventional intracellular microelectrode technique, antigen-induced depolarisation has been confirmed [129].
1.3.5.3 Membrane potential changes by antigen stimulation

Using the bis-oxonol technique, the membrane depolarisation elicited by an optimum dose of antigen, was found to have a half time of 1.4 min and was followed by partial repolarisation. A new steady state membrane potential was usually reached by 7 to 9 min after stimulation [53].

On the basis of indirect evidence, it is generally believed that the membrane depolarisation induced by antigen is related to calcium influx. This is supported by the evidence that prevention of calcium influx by La³⁺, addition of DNP-Lys, prior depolarisation in high extracellular K⁺ concentration or ATP depletion, all block antigen-induced depolarisation [128, 130]. The time course of the antigen-stimulated rise in cytoplasmic free ionised calcium is similar but not identical (somewhat faster) to the depolarisation response measured by bis-oxonol. Moreover, the antigen-induced depolarisation is abolished after the pretreatment of RBL-2H3 cells with PMA (50nM) [53]. In this condition, IP breakdown and Ca²⁺ influx are also abolished [91].

Absence of calcium in the external medium abolishes calcium influx and secretion, although in this case there is still an antigen-induced increase in bis-oxonol fluorescence, indicating that depolarisation still occurs in the absence of extracellular calcium [111, 131]. This is thought to be due to Na⁺ influx, because of the observation of antigen-induced Na⁺ influx in the absence but not in the presence of extracellular Ca²⁺ [57, 111]. When both Ca²⁺ and Na⁺ are omitted, the depolarisation is almost completely abolished [57, 111]. These observations indicate that the calcium permeability pathway does not have an absolute specificity for calcium. However, in the presence of millimolar extracellular calcium levels, this pathway tends to be more specific for calcium, because no Na⁺ influx is detected under these conditions.

A possible mechanism for repolarisation could be an inwardly rectifying chloride current [135] or an outward rectifier K⁺ current [161]. Since the repolarisation in parallel to secretion can be inhibited by quinidine, a K⁺ channel blocker, it has been suggested that K⁺ channels are involved and that the repolarisation is necessary for the secretory response [53].
Chapter 1  Introduction

1.3.6 Calcium permeability pathway

1.3.6.1 Voltage-gated calcium channel is not involved

The mechanism of the influx of extracellular calcium into the cell remains unclear. However, voltage-activated calcium channels are not involved, since the calcium influx and sustained increase in $[Ca^{2+}]_i$ are neither activated by depolarisation [108, 112, 130] nor blocked by a variety of calcium channel blockers, with the exception of nitrendipine (at micromolar concentrations) [132].

Using whole-cell recording, Lindau and Fernandez failed to detect IgE-operated ion channels in either rat peritoneal mast cells [133] or RBL-2H3 cells [125], although their RBL-2H3 experiments were performed at room temperature, where both degranulation and membrane depolarisation are small. More recently, it has been shown that the detection of any current induced by antigen in RBL-2H3 cells needs the preservation of cytoplasmic integrity and recording at 37°C [134].

1.3.6.2 CBP theory

It was proposed by Mazurek et al. that a putative channel is responsible for calcium influx, which is identical to a cromolyn-binding protein 60 kDa (CBP) [135] and that direct physical interaction between the aggregated IgE receptor and CBP suffices to open a calcium channel, and no second messenger or coupling proteins are required. However, these results have not been reproduced [103, 136]. Moreover, cromolyn fails to inhibit histamine release from intact RBL-2H3 cells [108], although it is capable of inhibiting secretion from permeabilised RBL-2H3 cells.

1.3.6.3 Involvement of second messengers

On the basis of electrophysiological and pharmacological evidence, it is suggested that in mast cell activation, calcium influx is mediated by second messenger-activated rather than classic voltage-activated calcium channels [137]. Among second messengers, $[Ca^{2+}]_i$ has a potential role in mediating calcium influx. Also IP$_3$, IP$_4$ and cAMP may be involved in calcium influx [137].
1.3.6.4 Potential-sensitive calcium permeability

Penner et al. [137] proposed that the amount of calcium influx is potential-dependent, because they observed that membrane hyperpolarisation of rat peritoneal mast cells promotes calcium influx, whereas upon depolarisation, less calcium influx occurs [137, 138]. This is in agreement with the inhibition of calcium influx and secretion by depolarisation of RBL-2H3 cells in high extracellular K⁺ concentration. Therefore, the driving force for calcium influx is highly dependent on the membrane potential as well as the concentration gradient.

1.3.6.5 Effect of extracellular sodium on calcium influx

Above all, the calcium influx requires extracellular sodium, in view of the following observations: 1) Secretion is inhibited by 30% (antigen-induced) or up to 50% (ionomycin-induced) in sodium-free buffer in which sodium is replaced by choline or bis(2-hydroxyethyl)dimethylammonium⁺ (BDA⁺) [108, 139, 140]. 2) Under these conditions, antigen or calcium ionophore induces a calcium spike around 70 to 80% of normal, but there is no calcium plateau. 3) Amiloride, a sodium transport inhibitor, inhibits antigen- and ionomycin-stimulated secretion by up to 35% at a concentration of 100μM. 4) A sodium ionophore, monensin, at a concentration of 50nM can produce a calcium plateau, and this effect is greatly attenuated in nominally calcium free medium [108]. 5) Monensin causes 40% serotonin release at 1 μM in Ca²⁺ free buffer. The induced release is not cytotoxic and is increased by raising of the pHᵢ and suppressed by lowering the [Na⁺]ₒ [139, 140]. However, monensin cannot induce β-hexosaminidase release, suggesting that monensin-induced serotonin release occurs by a mechanism different from exocytosis [139].

1.3.6.6 Na⁺/Ca²⁺ exchanger theory

Based on the above observations, Stump et al. [108] suggested that antigen binding activates a PKC-regulated sodium transport system such as a reverse Na⁺/Ca²⁺ exchanger, that causes an exchange of intracellular sodium for extracellular calcium. However, this suggestion has not been widely accepted. If a Na⁺/Ca²⁺ exchanger is a mechanism for calcium influx into RBL-2H3 cells, this would hyperpolarise instead of depolarise the plasma membrane, because this pump exchanges 3 internal sodium ions for
external calcium ion and is not electrically neutral. Likewise, the antigen-stimulated depolarisation is not affected in the absence of \([Na^+]_o\) [53]. An alternative theory might be that sodium influx first depolarises the membrane and then calcium enters via a \(Na^+/Ca^{2+}\) exchanger, but this is apparently not the case, because RBL-2H3 cells still depolarise in response to antigen in a sodium-free solution.

### 1.3.6.7 Role of \(Na^+/H^+\) exchanger

Since an increase in intracellular pH (pHi) and in \([Ca^{2+}]_i\) by monensin and inhibition of antigen-induced secretion by amiloride were reported (see above), there might be a role for \(Na^+/H^+\) exchanger in the signal transduction mechanism of secretion [139, 141]. In contrast, antigen and calcium ionophore operate in a different way to monensin, since they both cause cytosolic acidification and this might be due to activation of \(HCO_3^-\)-dependent systems rather than an \(Na^+/H^+\) exchanger [139, 142, 143]. Moreover, PMA, a protein kinase C activator, has no effect on pH in the concentration of 5-80nM [139].

### 1.3.6.8 Possible existence of a non-specific cation channel

It has been suggested that the increase in calcium permeability might occur through a \(Ca^{2+}\)-activated, non-specific cation channel which is activated by a rise in \([Ca^{2+}]_i\), as in neutrophils [144]. The driving force for calcium influx through these channels may be influenced by the membrane potential. This is in agreement with Penner et al.’s finding that the calcium influx is potential-dependent [137].

### 1.3.6.9 Store-operated calcium channel

The capacitative \(Ca^{2+}\) entry\(^1\) model was first proposed by Putney in 1986 [110]. According to this model, \(Ca^{2+}\) entry is regulated by the filling state of \(Ca^{2+}\) stores. In extreme terms, an empty store is open to the outside whereas a loaded store is not.

Hoth and Penner recently described the presence of a calcium current in mast cells which is activated by depletion of internal calcium stores [138, 145]. Since the calcium conductance is activated by three independent mechanisms (namely intracellular perfusion

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\(^1\) The term capacitative was used because the regulation of a capacitor’s conductivity by its state of charge is considered analogous to the way in which capacitative \(Ca^{2+}\) entry is thought to be determined by the filling state of \(Ca^{2+}\) stores.
of IP₃ [146], extracellular application of ionomycin and intracellular perfusion of calcium chelators) which release calcium from intracellular stores, they have termed this current Iₐ[RAC] (for calcium release-activated calcium channel) [147, 148, 149]. The current is a highly specific pathway for calcium entry into mast cells, but in the absence of divalent cations the pathway is able to pass monovalent cations [148, 150]. The Iₐ[RAC] is identified on the basis of its strong inward rectification, voltage-independent gating, positive reversal potential (>+30mV) and dependency on extracellular Ca²⁺ [147, 148]. This model would predict that application of calcium pump inhibitors, which inhibit the reuptake of leaked-out calcium into internal stores, cause the depletion of Ca²⁺ stores and result in a sustained increase in [Ca²⁺]ᵢ by activating Iₐ[RAC]. In fact this is the case, because cyclopiasonic acid and thapsigargin by inhibiting Ca²⁺-ATPase in the stores, deplete the stores and increase the [Ca²⁺]ᵢ , which, in turn, opens the calcium pathway and induces secretion [151].

Thimerosal, a sulfhydryl agent, which increases the sensitivity of IP₃ receptors to IP₃, has been shown to deplete the internal Ca²⁺ stores in the presence of basal concentration of IP₃ and to induce Ca²⁺ influx because of Iₐ[RAC] activation [152].

Using nystatin-perforated patch recording at 37°C, more recently Zhang and McCloskey have reported an inwardly rectifying Ca²⁺ channel (I₀) activated by antigen [134]. The stimulated current which has a time-dependent and voltage-independent opening, can be blocked by addition of hapten in large molar excess over antigen, indicating that the activation of I₀ needs a specific and reversible interaction of IgE with antigen. Moreover, this current is not blocked by the L-type Ca²⁺ channel blocker, nitrendipine (5µM), nor by the anti-asthmatic drug cromolyn sodium (0.5mM). However, La³⁺ and Zn²⁺ are capable of reversibly inhibiting I₀ with an IC₅₀ of 1.4µM and 42µM, respectively. This channel is permeable to Na⁺ in the absence of Ca²⁺ (nominal) and this is consistent with the result of a Na⁺ uptake study [131]. Since I₀ is not affected by a change in [Na⁺]ᵢ from 0 to 88mM, it is suggested that I₀ is not due to reversed Na⁺/Ca²⁺ exchange² [134]. Interestingly, Zhang and McCloskey reported Ca²⁺ current oscillation in eight of ninety-one cells. Although the I₀ occurs more slowly than the first Ca²⁺ oscillation, it is unlikely that Ca²⁺ current can be directly activated by Ca²⁺, because preincubation of cells with BAPTA-AM³ reduces or abrogates the Ca²⁺ signal without

² According to reverse Na⁺/Ca²⁺ exchanger theory, upon activation 3 internal Na⁺ ions exchange with 1 external Ca²⁺. If removal of [Na⁺] does not inhibit Ca²⁺ current, it can rule out the involvement of Na⁺/Ca²⁺ exchanger.

³ BAPTA-AM is the acetoxymethyl ester form of 1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid.
affecting the I_{Ca}. The current can also be carried by Ba^{2+} and Sr^{2+} (in the nominal absence of Ca^{2+}) and the I_{Ba} and I_{Sr} increase more sharply upon membrane hyperpolarisation and both are greater than the I_{Ca}. This is likely to be due to the inhibitory effect of Ba^{2+} and Sr^{2+} on the inward rectifier (IR) K^{+} channel which reduces the opposing effect of the IR K^{+} channel against hyperpolarisation. However, since preincubation of cells with BAPTA-AM reverses the order of conductance such that I_{Ca} > I_{Ba}, I_{Sr}, it has been suggested that [Ca^{2+}]_i inactivates the conductance more readily than does Ba^{2+}.

By directly measuring cytosolic Ca^{2+}, Parekh et al. [153] have shown that low concentrations (nanomolar) of IP3 induced substantial Ca^{2+} release without any activation of I_{CRAC}. However, micromolar concentrations of IP3 are required to activate CRAC channels. To explain these data, they suggested that there are at least two types of functional IP3 stores: one involved in Ca^{2+} release and another is responsible for I_{CRAC} activation.

**Characteristics of CRAC channels:**

1. Activation by depletion of internal Ca^{2+} stores
2. Its induction by IP3 is sensitive to heparin
3. Inward rectification with reversal potential >+30mV
4. Activation is dependent on [Ca^{2+}]_o
5. Very selective for Ca^{2+}
6. Na^{+}-permeant in the absence of divalent cations
7. Inactivation by increased [Ca^{2+}]_i or PKC activation
8. Ba^{2+}, Mn^{2+} and Sr^{2+} are less permeable than Ca^{2+}
9. Blocking efficacy of cations is La^{3+} > Zn^{2+} > Cd^{2+} > Be^{2+}, Co^{2+}, Mn^{2+} > Ni^{2+} > Sr^{2+} , Ba^{2+}
10. Ba^{2+}, Sr^{2+} and Mn^{2+} cause a permeation block
11. Single channel conductance has been estimated to be <1pS

**1.3.6.10 non-specific cation channel (50pS channel)**

Fasolato et al. [138, 154] described two main calcium influx pathways in rat peritoneal mast cells. First, I_{CRAC} or capacitative calcium entry, a heparin-sensitive current which is linked to the filling state of calcium stores and depletion of the pools. The second one is the non-specific cation channel, referred to as a 50pS channel, which may be activated by agonist stimulation of mast cells in parallel with I_{CRAC}. The activation of 50pS
channels even at low $[\text{Ca}^{2+}]_i$ and the inability of IP$_3$ to activate them [137, 149] distinguishes these channels from non-specific cation channels in neutrophils [144]. In whole-cell patch clamp experiments on rat peritoneal mast cells, the 50pS channel was activated by compound 48/80 and substance P, when the pipette solution contained 500µg/ml heparin to prevent CRAC channel opening [137, 149]. These compounds fail to activate RBL-2H3 cells. The role of 50pS channels has not yet been reported in RBL-2H3 cells.

Although the total current of CRAC channels is small (about 1-2pA), this pathway is the major determinant of changes in $[\text{Ca}^{2+}]_i$ following receptor-mediated release of $\text{Ca}^{2+}$ from internal stores, accounting for at least 70% of the plateau of $\text{Ca}^{2+}$ signal [137, 155]. The characteristics of 50pS channels in rat peritoneal mast cells have been shown as follows:

**Characteristics of non-specific cation channel (50pS channel):**

1. Activation by agonist like substance P or compound 48/80 but not by $[\text{Ca}^{2+}]_i$ or IP$_3$
2. Voltage-independent gating
3. Increasing $[\text{Ca}^{2+}]_i$ cause inhibition of the current
4. The amount of current is increased by enhancing $[\text{Ca}^{2+}]_o$
5. Under physiological conditions 3.6% of the current is carried by $\text{Ca}^{2+}$
6. Permeability ratio $P_{\text{Ca}^{2+}}/P_{\text{M}^+}$ is 0.7 at 2mM $[\text{Ca}^{2+}]_o$
7. Activation by GTP$\gamma$S
8. Inhibition of agonist activation by GDP$\beta$S or phorbol esters
9. Permeable to Mn$^{2+}$ and Ba$^{2+}$ (permeation block)

### 1.3.6.11 Mechanism of activation of store-depleted calcium channel

How store depletion results in the opening of the CRAC channel is not clear [148, 155]. Since this current can be induced by diverse measures that deplete internal $\text{Ca}^{2+}$ stores, even in the absence of any rise in $[\text{Ca}^{2+}]_i$, it can be concluded that the current is not induced by a rise in $[\text{Ca}^{2+}]_i$. It was suggested that IP$_4$ activates CRAC channels, but IP$_4$ is not capable of either $I_{\text{CRAC}}$ activation or enhancing of IP$_3$-induced $\text{Ca}^{2+}$ entry [138].
addition, $I_{\text{CRAC}}$ can be activated under conditions in which neither IP$_3$ nor IP$_4$ are expected to be generated [138, 147]. It has also been proposed that $I_{\text{CRAC}}$ is regulated by the filling state of the stores [148]. Bode and Netter have proposed a model to explain the store-dependent Ca$^{2+}$ entry [156]. Based on this model, the lumen of Ca$^{2+}$ stores could be connected to the extracellular space by a tubule-like structure. Upon store depletion by IP$_3$, the external Ca$^{2+}$ could enter the store lumen via such a continuity with the extracellular space. Thus, extracellular Ca$^{2+}$ would enter the cytosol by passing through the stores first [156]. It has also been suggested that a small G-protein induces the activation of the capacitative Ca$^{2+}$ entry following depletion of intracellular Ca$^{2+}$ stores. This G-protein can be inhibited by GTP$\gamma$S [157]. Moreover, recently a low-molecular weight Ca$^{2+}$ influx factor has been described which is produced upon store-depletion [158]. However, its role in the CRAC channel activation by FcεRI crosslinking remains to be established.

More recently, Parekh and Penner [159] have reported that a protein kinase, which is comparable to PKC, inactivates $I_{\text{CRAC}}$. $I_{\text{CRAC}}$ inactivation is increased by PMA (100nM) and is inhibited by staurosporin (2μM) and bisindolylmaleimide (500nM). This is consistent with the inhibition of the antigen-induced Ca$^{2+}$ response by PMA, a PKC activator [91]. Moreover, these authors have observed that the PKC inhibitors also potentiate the effect of 5'-N-ethylcarboxamido)adenosine (NECA)$^4$ on $I_{\text{CRAC}}$ activation and on Ca$^{2+}$ influx [159]. NECA-induced current is identical to the current activated by ionomycin or IP$_3$ in terms of inward rectification, voltage-independent gating and positive reversal potential (≥30mV) [159]. Regarding the activation of PLD and PKC by NECA in RBL-2H3 cells [160] and inactivation of $I_{\text{CRAC}}$ by PKC (Ca$^{2+}$-dependent inactivation of $I_{\text{CRAC}}$), it can be suggested that PKC activation is likely to be involved in Ca$^{2+}$ oscillation in these cells.

1.3.6.12 The importance of the electrochemical driving force for Ca$^{2+}$ permeability

In rat peritoneal mast cells, IP$_3$-induced Ca$^{2+}$ current and influx occurs only when the membrane potential is sufficiently negative [138, 149]. In these cells, a cAMP- and Ca$^{2+}$-activated Cl$^-$ channel can clamp the membrane potential to negative values, thus providing driving force for Ca$^{2+}$ influx through $I_{\text{CRAC}}$ and 50pS channels [137, 149].

$^4$ NECA can transiently activate PLC through A$_3$ adenosine receptors and G-protein activation. It cannot induce a sustained response in RBL-2H3 cells, possibly due to its failure for prolonged IP production. For more details, the reader is referred to Chapter 7.
As mentioned earlier, depolarisation causes inhibition of Ca$^{2+}$ influx and secretion induced by antigen. It has also been shown that hyperpolarisation in mast cells increases the IP$_3$-induced Ca$^{2+}$ current [137, 138]. Thus, as Penner et al. [137] proposed, Ca$^{2+}$ current through CRAC channels in mast cells is dependent on the electrochemical driving force for Ca$^{2+}$ influx and these cells need a negative membrane potential for maximum CRAC channel activation. It is important to know how RBL-2H3 cells maintain a sufficient electrochemical driving force for calcium influx. It might be explained by the existence of an outward K$^+$-conductance through K$^+$ channels in RBL-2H3 cells which cause repolarisation and provide an efficient driving force for calcium influx [53]. The demonstration of changes in $^{86}$Rb$^+$-efflux induced by antigen and calcium ionophore and its inhibition by quinidine supports this idea [161]. Moreover, the dose-response curves for the inhibition of secretion, repolarisation and the increase in $^{86}$Rb$^+$-efflux by quinidine are quite similar. This means that the repolarisation and K$^+$ efflux processes may be important in stimulating secretion.

1.3.7 Myosin phosphorylation

The antigen-induced secretion from RBL-2H3 cells is accompanied by a marked movement of granules to the cell membrane and cell shape change. Since these alterations appear to be caused by actin-myosin interaction, and as the increase in myosin phosphorylation and histamine release in activated RBL-2H3 cells are correlated in time, it has been suggested that myosin-phosphorylation and actin-myosin interaction might have a role in the antigen-triggered signal transduction mechanism of secretion. The myosin light chain is phosphorylated by a Ca$^{2+}$-calmodulin dependent protein kinase. This, in turn, results in an increase in the actin-activated MgATPase activity of myosin and possibly contributes to cell shape change and granule movement [162].

1.3.8 Cellular response

The sequence of events between the increase in intracellular calcium and the fusion of granules with the plasma membrane, resulting in the release of mediators, is poorly understood. The mechanism of granule mobility, granule/granule interaction or granule/plasma membrane association remains to be clarified. A hypothetical sequence of events during exocytosis of mast cell secretory granules has been proposed [163]. According to this theory, the granule first binds to the cell membrane at particular sites.
Then the fusion pore, an aqueous channel, forms connecting the granular interior to the extracellular media. Finally the fusion pore is replaced by a wider opening, and the granule which is no longer entirely surrounded by the membrane, can swell without stretching the granule membrane.

1.4 Classification and function of potassium channels

Potassium channels are membrane-spanning proteins which form pores between intracellular and extracellular media. They are highly selective for $K^+$. Potassium channels seem to be present in almost every eukaryotic cell. Compared with $Ca^{2+}$ channels, $K^+$ channels exist in many varieties in keeping with the diversity of function and electrical activity of different cells and organs. $K^+$ channels are diverse in their conductance and in their sensitivity to cellular factors which gate the flow of current through the channel [164]. Their function in excitable cells includes maintaining the normal cell resting potential and controlling the duration of the action potential. More generally, potassium channels contribute to the control of net potassium flux, cell volume, release of hormones and neurotransmitters, resting potential and excitability. The classification of $K^+$ channels and their nomenclature is based on the properties of the channels. The main classes include voltage-dependent, inward rectifier (anomalous rectifier), $Ca^{2+}$-activated, ATP-sensitive and neurotransmitter- and second messenger-regulated $K^+$ channels. Moreover, subtypes of these families are also described. Although many distinct $K^+$ channels are known, it is still not always possible to classify every potassium conductance.

1.4.1 Voltage-dependent potassium channels

These channels, which are gated by the membrane potential, give rise to a delayed rectifier ($K_V$) and a transient outward current: the A current, ($K_A$). The voltage sensors detect changes in membrane potential and trigger conformational changes of the channel protein, thereby generating gating currents and causing the channel to be activated for ion permeation [165]. The main differences between the A current and the delayed rectifier are in their inactivation rates and the threshold for activation. The delayed rectifier channels inactivate much more slowly and the threshold for activation is more positive than is characteristic for the A current.

The delayed rectifier is often responsible for the repolarisation of the action potential in excitable cells. The current is activated with a delay (1-100ms) at potentials
positive to about -20mV. These channels show little or no inactivation at depolarised potentials. \(K_v\) channels are blocked by both internal and external TEA in the mM-range and by 4-aminopyridine (4-AP) but not by \(Ca^{2+}\) and \(Na^+\) channel blockers [166].

The A current was first described in neurons [167] and was later shown to be present in the heart and portal vein. Although this current may not contribute to the resting input conductance, it has a major role in fast, repetitively-spiking cells. The A current which is a relatively fast, transient \(K^+\) current, is activated by depolarising steps more positive than -50mV. This current rapidly activates and also quickly inactivates. Repolarisation and regulation of action potential discharge frequency are two main functions of this current. It is blocked by dendrotoxin (DTX) and 4-AP (much less potent) [166]. Its blockade by 4-AP results in uncontrolled repetitive discharges. Thus, this channel can retard or even prevent the initiation of action potential discharge. \(K_A\) channels can be modulated by activation of certain neurotransmitter receptors (e.g. effect of noradrenaline on \(\alpha_1\) receptor) [168].

1.4.2 Calcium-activated potassium channels

This kind of channel increases the \(K^+\) permeability of a cell following an increase in \([Ca^{2+}]_i\). However, in some subtypes there may also be voltage-dependent gating i.e. the maxi-\(K\) \(Ca^{2+}\)-activated \(K^+\) channel (or \(BK_{Ca}\) channel). \(Ca^{2+}\)-activated \(K^+\) channels sometimes contribute to the repolarisation of the action potential and to a long-lasting after-hyperpolarisation which follows the spike.

Three different subtypes of \(Ca^{2+}\)-activated \(K^+\) channels have been identified: the large conductance channel (Maxi-\(K\) or \(BK_{Ca}\)), the small conductance channels (\(SK_{Ca}\)) and the intermediate conductance channels (\(IK_{Ca}\)). These channels differ in their single channel conductance, \(Ca^{2+}\) sensitivity, voltage-dependency, pharmacological properties and kinetic properties such as open and closed probabilities and their rate constants [review in 169].

1.4.2.1 \(BK_{Ca}\) channels

\(BK_{Ca}\) channels have been reported in skeletal muscles, neurons, pancreatic \(\beta\)-cells, smooth muscle and blood cells such as platelets and B-lymphocytes. They can be modulated by both \([Ca^{2+}]_i\) and the membrane potential and are very sensitive to
charybdotoxin and TEA. The $\text{BK}_{\text{Ca}}$ channel contributes to the repolarisation phase of
spikes and the regulation of $\text{Ca}^{2+}$ entry.

$\text{BK}_{\text{Ca}}$ has a very large conductance (150-300pS) in symmetrical 140mM KCl
solution (140mM KCl in both the external and pipette solution). In spite of such a large
unitary conductance, this class of $\text{Ca}^{2+}$-activated $\text{K}^+$ channels has an extremely high $\text{K}^+$
selectivity. The current is maximally activated by both voltage and micromolar levels of
$[\text{Ca}^{2+}]_\text{i}$. Voltage sensitivity is regulated by the transmembrane domain, which shares sequence
similarity with voltage-gated $\text{K}^+$ channels, whereas $\text{Ca}^{2+}$ sensitivity is associated with a cytoplasmic carboxyl
terminal domain [170]. The open probability of the channel increases on increasing the
voltage in the positive direction. $\text{Ca}^{2+}$ binding to the inner surface of the channel causes a
shift in the voltage dependency of gating so that the channel can open at more negative
membrane potentials. Thus, BK channel opening is regulated by a combination of both
$[\text{Ca}^{2+}]_\text{i}$ and voltage [171]. In addition to $[\text{Ca}^{2+}]_\text{i}$ and membrane potential, some $\text{BK}_{\text{Ca}}$
channels can be activated by G-proteins [172].

Since $\text{BK}_{\text{Ca}}$ channels contribute to repolarisation after membrane depolarisation
and elevation of extracellular $\text{Ca}^{2+}$, they are involved in regulation of neurotransmitter
secretion, in the control of muscle contractility, in the movement of ions across epithelial
tissues and in a variety of diverse cellular responses.

The distinguishing pharmacology of the $\text{BK}_{\text{Ca}}$ channel is the block by external
charybdotoxin [173, 174] or 0.1-1mM external TEA and no effect of 4-AP, apamin, or
glibenclamide. Charybdotoxin is a 4.3 kDa, highly basic peptide containing 37 amino
acids [175, 176]. It has no effect on $\text{Ca}^{2+}$ or $\text{Na}^+$ channels, but does inhibit $\text{BK}_{\text{Ca}}$, within
the nM-range, in a variety of cell types. Charybdotoxin also inhibits the $\text{IK}_{\text{Ca}}$ channels
[176] e.g. in erythrocytes [174, 177]. Charybdotoxin at 5nM blocks voltage-gated $\text{K}^+$
channel ($K_{\text{V}_{1.3}}$) in mouse thymocytes [178]. $K_{\text{V}_{1.3}}$ channels in human T lymphocytes can
also be inhibited by noxiustoxin (NtxTX) and margatoxin (MgTX) [179, 180].

$\text{MgTX}$, derived from the venom of the scorpion, *Centruroides margaritatus*, is a
peptide containing 39 amino acids with six cysteine residues. It has high homology with
NtxTX (79%). Since $\text{MgTX}$ is 20 fold more potent than $\text{ChTX}$ ($IC_{50}$=50pM) in blocking
$K_{\text{V}_{1.3}}$, has a slower dissociation rate and does not affect the $\text{Ca}^{2+}$-activated $\text{K}^+$ channel, it
represents an especially useful tool to identify and study the physiological role of $K_{\text{V}_{1.3}}$
channels [181].

$\text{NtxTX}$ can block the $\text{BK}_{\text{Ca}}$ channels, but it is 100 times less potent than
charybdotoxin [176, 182]. Iberiotoxin (IbTX), derived from the scorpion *Buthus tamulus*,
is a 4.3kDa peptide containing 37 amino acids. It has 68% homology with ChTX. IbTX and limbatustoxin (LbTX) can block BK_{Ca} without any effect on Kv and SK_{Ca} channels [176]. IbTX represents a selective and high affinity probe with which to explore the physiological role of BK_{Ca} channels in different tissues [175]. Limbatustoxin, purified from the new world scorpion, Centruroides limbatus, has been shown to have 57% and 70% homology with ChTX and IbTX, respectively [175].

1.4.2.2 IK_{Ca} channels

The conductance of IK_{Ca} channels ranges between 25 to 135pS. The Ca^{2+} sensitivity of IK_{Ca} channels is slightly more than that of BK_{Ca} channels, but not as great as for SK_{Ca} channels. There may be subgroups of IK_{Ca} channels because some of them are sensitive to TEA, while others are not. Charybdotoxin blocks IK_{Ca} channels with tenfold less potency (IC_{50} =30-100nM) than the block of BK_{Ca} (IC_{50} =3nM) [182]. There is no reported inhibitory effect of IbTX on IK_{Ca} channels [183]. Since they are different in voltage sensitivities, from no voltage dependency in mouse olfactory neurons to a definite voltage dependence in paramecium, they are divided into two subtypes.

a) Voltage-insensitive IK_{Ca} channels. The typical example of this subgroup is the K^{+} channel responsible for the Gardos effect in red blood cells. Moreover, the K^{+} channels which have a role in the regulatory volume decrease (RVD) in lymphocytes, Ehrlich cells and intestinal epithelial cells are similar to those in red blood cells. These channels are activated by a rise in [Ca^{2+}], but not by changes in voltage [184]. Voltage-insensitive IK_{Ca} channels do not show inactivation. It should be noted that the channel opening is dependent on [K^{+}]_{o}. In high external K^{+} and K^{+}-free conditions, channel opening is decreased and 1mM [K^{+}]_{o} is the optimum concentration for channel activation. This kind of IK_{Ca} channel can be inhibited by quinine, quinidine, cetiedil (μM-range) as well as by ChTX (nM-range).

b) Voltage-dependent IK_{Ca} channels. The activation of these channels by increased [Ca^{2+}], is potentiated with depolarisation. In neurons, the channel activation results in reducing excitability and tends to decrease the frequency of action potentials towards the end of the burst. The voltage-sensitive IK_{Ca} has a similar
characteristics to \( \text{BK}_{\text{Ca}} \) with the exception of lower conductance. They can also be inhibited by quinidine, TEA and ChTX but not by apamin.

### 1.4.2.3 \( \text{SK}_{\text{Ca}} \) channels

\( \text{SK}_{\text{Ca}} \) channels have been shown in hepatocytes, neurons, smooth and skeletal muscle. \( \text{SK}_{\text{Ca}} \) channels present in rat skeletal muscle exhibit a conductance of 12-20pS and account for the after hyperpolarisation in myotubes and sympathetic ganglion cells [185, 186]. The \( \text{SK}_{\text{Ca}} \) channel is very sensitive to apamin, a peptidyl toxin isolated from bee venom [182, 187]. The inhibitory effect of apamin is exclusive to \( \text{SK}_{\text{Ca}} \) channels, as it has no effect on \( \text{BK}_{\text{Ca}} \) and \( \text{IK}_{\text{Ca}} \) channels [188]. Blockade of \( \text{SK}_{\text{Ca}} \) channels reduces inhibition intrinsic to the cell leading to repetitive action potentials following excitatory stimuli. Leiurotoxin I, although having little homology with apamin, can also block \( \text{SK}_{\text{Ca}} \) channels by binding to the same binding site as apamin [189]. TEA (< 10mM) has no effect on \( \text{SK}_{\text{Ca}} \) channels. Compared to \( \text{BK}_{\text{Ca}} \) channels, \( \text{SK}_{\text{Ca}} \) channels are less voltage-sensitive but far more Ca\(^{2+}\)-sensitive (nearly ten times more). In contrast to \( \text{BK}_{\text{Ca}} \), \( \text{SK}_{\text{Ca}} \) channel open probability is not affected by membrane potential.

### 1.4.3 Inward rectifier potassium channels

In many cell types, this kind of channel is activated after a hyperpolarisation to a potential negative to \( E_{\text{K}} \) (about -85mV). Because of this characteristic, this current cannot be the only component which underlies the input conductance in the membrane potential range between -90 and -30mV. A high \( K^+ \) conductance, at negative voltages, allows cells to maintain a stable resting potential, but the greatly reduced conductance at positive potentials avoids short-circuiting the action potential. This conductance is highly selective for \( K^+ \) and is characterized by a lack of inactivation [190, 191]. Inward rectifier \( K^+ \) channel gating depends on the external \( K^+ \) concentration \([K^+]_o\) but not the intracellular \( K^+ \) concentration \([K^+]_i\), possibly because the cytoplasmic blocking cation can be repelled by potassium binding to the external \( K^+ \) binding site in the long pore [190]. The potassium current through these channels can be blocked by cytoplasmic cations such as magnesium and polyamines. These channels can be blocked by extracellular \( \text{Ba}^{2+} \) and \( \text{Cs}^+ \) ions [190]. The voltage- and time-dependent block of inward rectifier \( K^+ \) channels by extracellular \( \text{Na}^+ \), \( \text{Cs}^+ \) and \( \text{Ba}^{2+} \) suggests that these ions act as open channel pore blockers [190]. The physiological role of this channel is in the maintenance of cellular resting potential and in
the contribution of outward current to the final phase of action potential repolarisation (Table 1-4). Blockade of inward rectifier K⁺ channels leads to a change in resting potential and prolongation of the action potential due to these dual roles of the channel [192].

<table>
<thead>
<tr>
<th>Cell situation</th>
<th>Current</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly depolarised</td>
<td>No Current</td>
<td>Prolonged depolarisation</td>
</tr>
<tr>
<td>Potential more negative than (E_k)</td>
<td>Inward</td>
<td>Prevent hyperpolarisation</td>
</tr>
<tr>
<td>Potential just above (E_k)</td>
<td>Slightly outward</td>
<td>Keep the resting membrane potential close to (E_k)</td>
</tr>
</tbody>
</table>

Table 1.4 Inward rectifier K⁺ channel functions in different conditions.

1.4.4 Receptor- or second messenger- coupled potassium channels

Some K⁺ channels can be opened or closed by the action of neurotransmitters or second messengers. An example of these channels is a current which is inhibited by acetylcholine acting through muscarinic receptors. This current has been called the M current due to its inhibition by muscarinic agonists. Under non-stimulated conditions, this current is carried by \(K_M\) channels which shut off after muscarinic stimulation [193]. \(K_M\) channels are characterized by slow activation and deactivation kinetics, and they do not exhibit steady-state inactivation. At potentials positive to about -60mV, \(K_M\) channels open and remain open as long as the membrane potential does not hyperpolarise. The lack of inactivation of the \(K_M\) channel helps it contribute to the resting membrane potential. The M-current is slowly activated when the neuron is depolarised toward the threshold for action potential firing, tending to hyperpolarise the membrane back toward its resting value and reducing membrane excitability. Its suppression by muscarinic receptor activation leads to membrane depolarisation and increases the input resistance, which makes the cell more likely to fire action potentials. The M-current is coupled to a large number of neurotransmitter receptors including those for acetylcholine (muscarinic), luteinizing hormone-releasing hormone, angiotensin, 5-hydroxytryptamine (5-HT) and
somatostatin. Activation of all of the coupled receptors except those for somatostatin leads to $K_M$ channel closure.

Activation of 5-HT$_{1A}$, γ-aminobutyric acid (GABA)$_B$ or adenosine $A_1$ receptors leads to $K^+$ channel opening. In contrast, activation of adrenergic $\beta_1$, histamine $H_2$, corticotrophin-releasing factor and muscarinic receptors results in the closure of $K_{Ca}$ channels and thus a reduction in action potential after-hyperpolarisation [194]. Another example is the $S$ channel which is inhibited by an increase in cAMP through activation of the serotonin receptor [195, 196].

1.4.5 ATP-sensitive potassium channels ($K_{ATP}$)

This potassium channel is dependent on the physiological concentration of intracellular ATP, [ATP], providing a link between metabolism and electrical activity. It is opened when [ATP] is decreased and closed by high [ATP], [197, 198]. This channel is blocked by the intracellular perfusion of ATP or extracellular application of glibenclamide. The effect of ATP does not appear to reflect a phosphorylation [198] and the channel does not have a pronounced dependence on membrane potential or [Ca$^{2+}$]$_i$. These channels are found in the heart, insulin-secreting cells, skeletal muscle and central nervous system. The sensitivity to glibenclamide varies between different tissues.

In intact, resting, insulin-secreting cells, $K_{ATP}$ is the only operational channel. The closure of this channel by high extracellular glucose concentration initiates membrane depolarisation, which in turn, opens voltage-gated Ca$^{2+}$ channels and produces the upstroke of the spike potential and a sharp rise in [Ca$^{2+}$]$_i$ [199]. Then voltage- and Ca$^{2+}$-activated $K^+$ channels ($K_V$ and $BK_{Ca}$) open to repolarise the membrane, thereby closing Ca$^{2+}$ channels and producing the downstroke of the spike potential [200]. A resulting fall in [Ca$^{2+}$]$_i$ combined with repolarisation towards the high resting negative membrane potential causes both $BK_{Ca}$ and $K_V$ to close. As $K_{ATP}$ channels remain closed, subsequent depolarisation causes a new cycle of channel opening and closure. Since Ca$^{2+}$ channels, because of their voltage-dependency, are open in the depolarised state and closed at negative potentials, the repetitive changes in potential result in controlled waves of Ca$^{2+}$ influx.

$K_{ATP}$ activity is also regulated by G-protein(s). Application of GTP, GDP and GTPyS increase the channel opening reversibly and dose-dependently [200]. $K_{ATP}$ channel activity is also greatly reduced by quinine (100μM), aminoacridine (100μM), and 4-AP
TEA at <2mM has little effect on the channel and its IC$_{50}$ has been estimated to be 22mM [173]. Glybenclamide and tolbutamide have been shown to block the channel and decrease the K$^+$ permeability of the β cell membrane [201]. Moreover K$_{ATP}$ is the site of action of K$^+$ channel openers such as cromakalim, diazoxide, nicorandil and pinacidil [202].

1.4.6 Na$^+$-activated potassium channels

This kind of K$^+$ channel is activated by a rise in [Na$^+$]$_i$ and its activation is correlated with the size of inward sodium current. It has been reported in myocytes and neurons. Under physiological potassium concentrations the conductance of the K$^+$ channel is near 50pS. The reversal potential is at $E_K$ and depends on K$^+$ concentration and is not affected by different [Na$^+$]$_o$. These data suggest that the major ion carried by the sodium-activated K$^+$ channels is the potassium ion, and justifies the name K$_{Na}$ [203, 204].

1.4.7 Volume-activated potassium channels

Volume- or stretch-activated K$^+$ channels have been observed in several cell types such as neurons, skeletal muscles, myocytes, hepatocytes and endothelial cells. It has been proposed that membrane deformation modulates this kind of K$^+$ channel. The role of the cytoskeleton and signaling cascades in the activation of volume-activated K$^+$ channels remains to be understood [205].

1.5 Evidence for existence of K channels in RBL-2H3 cells

1.5.1 Inward rectifier potassium channel

The only significant K$^+$ channel in RBL-2H3 cells, first reported by Lindau and Fernandez, is a K$^+$-selective inward rectifier (IR) [125]. This conductance, which probably determines the resting membrane potential, is enhanced by increasing external potassium concentration and is blocked by sodium and barium in a voltage-dependent manner [125, 126, 206, 207]. The IR K$^+$ current is roughly proportional to the square root of the external K$^+$ concentration [125, 126, 206, 208]. The conductance increases with
hyperpolarisation until inactivation occurs [209]. This inactivation is due to blockade by Na$^+$ ions, since inactivation is nearly abolished in the absence of Na$^+$ [125]. With depolarisation of the cells between -80 and about -30mV, an outward current passes through IR K$^+$ channels [125, 208]. The IR K$^+$ current therefore provides a mechanism that would prevent excessive hyperpolarisation and resist small depolarisations of the cell.

The IR K$^+$ channels in RBL-2H3 cells are stabilized by cytoplasmic ATP or GTP, and are inhibited by activation of a pertussis-insensitive G-protein [208]. The average conductance of the IR K$^+$ channel is 2-3pS at 2.8mM [K$^+$]$_o$ and 26pS in symmetrical 160mM [K$^+$]$_o$ [125, 208]. Intracellular dialysis of G-protein activators, GTPγS, GppNHP, AlF$_4^-$, completely inhibits the inward rectifier conductance [208, 210].

Expression of the inward rectifier K$^+$ channel mRNA (from RBL) in Xenopus oocytes allowed Lewis et al. [211] to emphasize four characteristics of this channel: activation at potentials negative to E$_K$; decreased slope conductance near E$_K$; the dependency on [K$^+$]$_o$; and blockade by external Ba$^{2+}$ and Cs$^+$ [126, 209, 211]. Likewise, it has been shown that rubidium decreases the IR K$^+$ current, although this ion seems to be permeant [209]. Strontium induced an additional inward current between -20mV to -110mV, but this effect could be due to permeation of strontium through a non-specific cation channel.

Extracellular application of Ba$^{2+}$, Cs$^+$ and cell perfusion with K$^+$-free solution blocked the inward rectifier current in antigen-stimulated RBL-2H3 cells. It has been reported that tetraethylammonium (TEA) can inhibit the inward current by 50% at a concentration of 10-15mM [207, 208, 209]. However, this current is not affected by phorbol esters and internal magnesium [207, 209]. A rise in [Ca$^{2+}$]$_i$ results in closure of the inwardly rectifying K$^+$ channel [212, 213]. Modulation of the channel activity by [Ca$^{2+}$]$_i$ might be due to an alteration of the channel kinetics or the number of channels capable of being activated, via a direct inhibitory action or actions of membrane-bound enzymes. Mukai et al. suggested that the antigen-induced depolarisation in RBL-2H3 cells might be caused by closure of the inwardly rectifying K$^+$ channel due to elevation of [Ca$^{2+}$]$_i$ [212]. Interestingly, this is consistent with the rate of rise in [Ca$^{2+}$]$_i$, which is somewhat faster than the depolarisation in RBL-2H3 cells.

2',5'-di(tert-butyl)-1,4-benzohydroquinone (BHQ), a Ca$^{2+}$-ATPase inhibitor, like cyclopiazonic acid and thapsigargin, should deplete internal calcium stores and induce calcium influx in RBL-2H3 cells. However, in spite of depleting calcium stores, BHQ cannot increase calcium permeability [214]. It has been shown that BHQ inhibits the
inward rectifier potassium current and may cause depolarisation and inhibition of calcium influx. However, thapsigargin does not inhibit this current [206].

Regarding the physiological and molecular characteristics, Wischmeyer et al. [207] concluded that the inward rectifier potassium channel in RBL-2H3 cells belongs to a classical voltage-dependent IRK-type K⁺ inward rectifier which is negatively controlled by G proteins.

### Characteristics of IR K⁺ channels in RBL-2H3 cells:

1. Activation at potential negative to $E_K$.
2. Outward current at positive potentials just near to $E_K$.
3. Enhancement of conductance by increase in $[K^+]_o$.
4. Inhibition by an increase in $[Ca^{2+}]$.
5. Blockade by $Ba^{2+}$ [100μM] and $Cs^+$ [1mM].
6. Lack of inhibition by quinidine [100μM].

Lindau and Fernandez [125, 133] did not observe any significant antigen-stimulated change in the ionic conductance. The possible explanations for this finding are that:

- They performed the experiments at room temperature. It is well known that antigen activation is temperature-dependent, and at room temperature both degranulation and membrane depolarisation are greatly inhibited.
- The patch-clamp conditions used by these authors may also have led to the loss of a necessary soluble component from the cell cytoplasm due to diffusion into the patch pipette. Using conventional whole-cell recording, antigen stimulation failed to cause any ionic conductance change [134]. Despite preservation of cytoplasmic integrity, even with the perforated-patch recording, the addition of antigen at room temperature still failed to induce any conductance change [134].
- A holding potential of +5mV was used, in which RBL-2H3 cells are depolarised and antigen-induced Ca²⁺ influx is almost completely inhibited.
- Moreover, they emphasized that after stimulation the cell dialyzed with the patch pipette did not degranulate [133]. It has also been reported that intracellular dialysis of
GTPγS [100μM] induces little or no change in cell capacitance suggesting that GTPγS cannot result in exocytosis in this condition [208].

Inhibition of S-adenosylmethionine decarboxylase reduces spermidine and spermine levels as well as inward rectification by IR K⁺ channels in RBL-2H3 cells, indicating participation of intracellular polyamines in the regulation of IR K⁺ channels [215].

### 1.5.2 Outward rectifier potassium channel

Using the whole-cell patch-clamp technique, Jones et al. in 1987 [216] reported that antigen stimulates a low-amplitude inward current in parallel with a voltage-dependent outward current. Since both of these currents could be activated by IP₃ (10μM), they concluded that store depletion may activate the currents [89, 216].

In addition to IR K⁺ channel inhibition, GTPγS, GppNHP and AlF₄⁻ induce the appearance of a novel time-independent outwardly rectifying (OR) K⁺ conductance, which is distinct from the inward rectifier conductance [208]. The induced current is not activated by second messengers such as IP₃, IP₄, cAMP, [Ca²⁺], and it is permeable to both K⁺ and Rb⁺, with a permeability ratio, P_{Rb}/P_{K} of 0.67 [208]. Pretreatment with PTX inhibits the G-protein-induced current, but it has no effect on the inward rectifier channel, indicating that two different G-proteins mediate these two currents. CTX does not have any significant effect on the decay of IR K⁺ channels or the induction of OR K⁺ channels [208, 210].

The induced outward rectifier K⁺ current is blocked 50% by 50μM quinidine, whereas the IR conductance is not affected even by 100μM quinidine [208]. Likewise it has been shown that quinidine blocks antigen-stimulated ⁸⁶Rb⁺ efflux [161]. The IR K⁺ channel was completely inhibited by 100μM Ba²⁺, whereas 20mM Ba²⁺ did not completely inhibit induced OR K⁺ conductance [208]. Both IR and OR K⁺ channels can be inhibited by Cs⁺ but display different voltage dependencies [208]. The average conductance of OR K⁺ channels in symmetrical 160mM K⁺ was 8pS [208]. The induced OR K⁺ channel can be blocked by Ba²⁺ and Sr²⁺, but not by La³⁺, Cd²⁺ and Zn²⁺. TEA can inhibit both channels with an IC₅₀ of 10-15mM [208].

Application of NECA, ADP and ATP causes a rapid activation of an outwardly rectifying K⁺ conductance in RBL-2H3 cells [210]. This conductance is prevented by
guanosine 5'-[\beta\text{-thio}]diphosphate (GDP\beta S) or pretreatment with pertussis toxin indicating activation by G protein.

The conductance of NECA-induced OR K\textsuperscript{+} current is about half as large as that induced by ADP or ATP. Moreover, dexamethasone pretreatment can double the maximal conductance induced by NECA but not by ADP or ATP. Dexamethasone can also potentiate the effect of NECA-induced serotonin release and the rise in [Ca\textsuperscript{2+}]\textsubscript{i}, but it downregulates IgE-mediated signals\textsuperscript{5} [217]. These data suggest that NECA stimulates a separate receptor from that which is activated by ADP or ATP. However, signal transduction pathways for NECA and ADP/ATP receptors converge in activation of the same K\textsuperscript{+} channel [210].

It has been reported that antigen stimulation of RBL-2H3 cells caused an increase in inward current by 23±11% and the outward current by 45±6% [218].

More recently, Hoth reported that dialysis of RBL-2H3 cells with IP\textsubscript{3} (10\mu M) or application of ionomycin, which led to Ca\textsuperscript{2+} store depletion, activated an inward CRAC channel and an outward K\textsuperscript{+} current [219]. The outward current is mainly carried by K\textsuperscript{+} and is strongly dependent on [Ca\textsuperscript{2+}]\textsubscript{i}. The amplitude of the outward current was increased if external Ca\textsuperscript{2+} was reduced or replaced by Ba\textsuperscript{2+}. Since the outward current was activated by store-depletion and its activation was correlated with the activation of a CRAC channel, Hoth called it a store-depleted K\textsuperscript{+} conductance [219].

Similarly, it has also been shown that the activation of 5-HT receptors expressed in *Xenopus* oocytes, or direct IP\textsubscript{3} injection, stimulates Ca\textsuperscript{2+} entry as well as an outward rectifier K\textsuperscript{+} channel activated by depletion of Ca\textsuperscript{2+} stores [220].

---

**Characteristics of OR K\textsuperscript{+} channels in RBL-2H3 cells:**

2. Lack of activation by IP\textsubscript{3}, IP\textsubscript{4}, cAMP or increased [Ca\textsuperscript{2+}]\textsubscript{i}.
3. Blockade by quinidine (IC\textsubscript{50}=50\mu M).
4. Activation by adenosine analogues such as NECA, ADP and ATP.
5. Lack of complete inhibition by Ba\textsuperscript{2+} [even at 20mM].

\textsuperscript{5} Treatment with dexamethasone increases the expression of adenosine A\textsubscript{1} receptors in RBL-2H3 cells [79].

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1.5.3 $^{86}$Rb$^+$-efflux study

Labrecque et al in 1991 [161] reported that antigen can cause an increased $^{86}$Rb$^+$-efflux in RBL-2H3 cells. They proposed that the stimulated K$^+$ permeability contains two components:

1. PTX sensitive component: PTX can inhibit the antigen-stimulated $^{86}$Rb$^+$-efflux by 60%, but it has no effect on stimulated $^{86}$Rb$^+$-efflux by A23187. PTX can also inhibit GTP$\gamma$S and ADP-induced OR K$^+$ conductance in RBL-2H3 cells [210, 208]. Since PTX has no effect on antigen-stimulated Ca$^{2+}$ influx and degranulation [80], the PTX-sensitive component might not play an essential role in these responses.

2. Ca$^{2+}$-activated component: CTX potentiates antigen-induced $^{86}$Rb$^+$-efflux in parallel with enhancing Ca$^{2+}$ influx and secretion. La$^{3+}$, quinidine and the absence of [Ca$^{2+}$]o inhibit the stimulated efflux. The Ca$^{2+}$ ionophore A23187 can also induce an increase in $^{86}$Rb$^+$ efflux in RBL-2H3 cells.

Increased $^{86}$Rb$^+$-efflux is not stimulated by depolarisation of the cells with high K$^+$ solution or application of carbonyl cyanide 3-chlorophenylhydrazone CCCP (a proton ionophore). If the stimulated $^{86}$Rb$^+$-efflux is through a voltage-sensitive K$^+$ channel, so cell depolarisation should activate the channel and $^{86}$Rb$^+$-efflux. Therefore, these data suggest that the K$^+$ channels involved may not be voltage-sensitive. However, it has been reported that depolarisation inhibits antigen-stimulated $^{86}$Rb$^+$-efflux in RBL-2H3 cells [161].

1.5.4 K$^+$ channels in other histaminocytes

More recently McCloskey and Qian [222] have reported that bone marrow-derived mast cells (BMMC) [221], have two K$^+$ channels. These are an inward rectifier, constitutively active [222, 223], and a latent outward rectifier K$^+$ conductance, which is stimulated by activation of P$_2$ purinergic receptors linked to G proteins of the Gi family. However, rat peritoneal mast cells lack these conductances [222, 224]. Intracellular application of GTP$\gamma$S causes the disappearance of the IR K$^+$ and the induction of the latent OR K$^+$ channels in BMMC. However, NECA and ADP can only induce the OR K$^+$ channels, without affecting currents through IR K$^+$ channels [222].
BMMC and RBL-2H3 cells belong to the "mucosal" subtype of mast cells and both possess IR and OR K⁺ conductances as well as the same two receptor G-protein signal transduction pathways leading to opening of OR K⁺ channels.

In spite of numerous reports indicating the presence of K⁺ channels in mucosal mast cells, no potassium channel has been detected in rat peritoneal mast cells [125, 137, 222, 223]. The resting potential in rat peritoneal mast cells fluctuates between 0 and -30 mV and these cells possess a very small whole cell conductance of about 10-30 pS. Since this small conductance is mainly due to the seal resistance, it has been suggested that no ion channels of significant conductance are open in the cell membrane. Perhaps the resting membrane potential, the zero-current potential, is determined by other electrogenic ion transport systems, such as the Na⁺/Ca²⁺ exchanger or the Na⁺/K⁺ ATPase pump [125, 127]. Moreover, it has been shown that the plasma membrane potential of the rat peritoneal mast cells is set predominantly by the Na⁺/K⁺ ATPase pump [225], while in RBL-2H3 cells the plasma membrane potential is set by the Na⁺ pump and K⁺ conductances. These data suggest a possible difference in K⁺ channel expression between serosal and mucosal mast cells.

1.6 Aims of this study

Since the activation of CRAC channels and Ca²⁺ influx is inhibited when RBL-2H3 cells are depolarised, it seems that the maintenance of a sufficient negative membrane potential is necessary for antigen-induced Ca²⁺ influx. It has also been proposed that one function of the OR K⁺ channels is to provide a switching mechanism to control antigen-stimulated Ca²⁺ entry via changes in the membrane potential. Thus, in RBL-2H3 cells, functional K⁺ channels might serve as regulators of membrane potential and as a positive feedback mechanism for Ca²⁺ entry.

However, it is not likely that the IR K⁺ channels are involved in RBL-2H3 cell activation for the following reasons:

• IR K⁺ channels are closed following modest depolarisation.
• Elevation of [Ca²⁺], can inhibit IR K⁺ channels.
• Ba²⁺ completely blocks the IR K⁺ channel at a concentration of 50 μM. However, it has no significant effect on antigen-induced mediator release from RBL-2H3 cells at the same concentration.
In the light of the previous investigations, it was considered interesting to clarify whether potassium channels in RBL-2H3 cells are involved in the signal transduction pathways of exocytosis. To investigate this, β-hexosaminidase release measurement was used as a marker of the degranulation and the effect of 27 potassium channel modulators on the antigen-stimulated β-hexosaminidase release was determined.

Also the effect of K⁺ channel blockers was determined on antigen-induced depolarisation and repolarisation, as measured using bis-oxonol. In addition, the characteristics of antigen- and A23187-stimulated ⁸⁶Rb⁺ efflux and the effect of K⁺ channel blockers were investigated. Finally some features of antigen-stimulated Ca²⁺ oscillations and the inhibitory effect of some K⁺ channel blockers thereon were studied.
CHAPTER 2 MATERIALS AND METHODS

2.1 Materials and sources

<table>
<thead>
<tr>
<th>Materials</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td>4-aminopyridine (4-AP)</td>
<td>Sigma, Poole</td>
</tr>
<tr>
<td>4-methylumbelliferyl N-acetyl-β-D-glucosaminide</td>
<td>Sigma, Poole</td>
</tr>
<tr>
<td>5-(N-ethylcarboxyamido)adenosine (NECA)</td>
<td>Sigma, Poole</td>
</tr>
<tr>
<td>$^{86}\text{Rubidium (as RbCl)}$</td>
<td>New England Nuclear (NEN),</td>
</tr>
<tr>
<td></td>
<td>Hounslow</td>
</tr>
<tr>
<td>9-Aminoacridine (9-AA)</td>
<td>Sigma, Poole</td>
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<td>Amiodarone</td>
<td>Sigma, Poole</td>
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<tr>
<td>Apamin</td>
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<td>Barium chloride</td>
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<td>Bis-oxonol</td>
<td>Molecular probes, Cambridge</td>
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<td>Bovine serum albumin (BSA)</td>
<td>Sigma, Poole</td>
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<tr>
<td>n-Butanol</td>
<td>BDH Chemicals, Lutterworth</td>
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<td>Calcium chloride</td>
<td>BDH Chemicals, Lutterworth</td>
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<tr>
<td>Calcium ionophore A23187</td>
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<tr>
<td>Cromakalim</td>
<td>Sigma, Poole</td>
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<td>Diazoxide</td>
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<tr>
<td>Dibutyl phthalate</td>
<td>Sigma, Poole</td>
</tr>
<tr>
<td>Dimethylsulphoxide (DMSO)</td>
<td>Sigma, Poole</td>
</tr>
<tr>
<td>Dinitrophenyl human albumin (DNP-HSA)</td>
<td>Sigma, Poole</td>
</tr>
<tr>
<td>DNP-specific monoclonal mouse IgE</td>
<td>Sigma, Poole</td>
</tr>
<tr>
<td>EDTA (Ethylene diaminetetraacetic acid) 0.02% solution</td>
<td>Sigma, Poole</td>
</tr>
</tbody>
</table>
EGTA (Ethylene glycol-bis (ß-amoanethethyl ether) N,N,N',N'-tetra acetic acid)
Ethanol, absolute
Foetal calf serum (FCS)
Gelatin
Glibenclamide
Glucose
Gramicidin
HEPES, Free Acid (N-2-hydroxyethyl piperazine-N'-2-ethane sulphonylic acid)
n-Heptane
Histamine dihydrochloride salt
Hydrochloride acid
Iberiotoxin (IbTX)
Indo 1-AM
Lanthanum chloride
L-Glutamine 200mM solution
Lithium chloride
Magnesium chloride
Margatoxin (MTX)
Methanol
Minimum essential media medium (S-MEM)
Nicardipine
Nitrendipine
N-methyl D-glucogamine chloride (NMG-Cl)
o-Phthaldialdehyde (OPT)
Penicillin-Streptomycin solution
Perchloric acid
Phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}
Phthalic acid bis(2-ethylhexyl ester)
Potassium chloride
Propafenone
Quinidine hydrochloride monohydrate
Quinine sulphate

Sigma, Poole
BDH Chemicals, Lutterworth
Gibco, Paisley
Sigma, Poole
Sigma, Poole
BDH Chemicals, Lutterworth
Sigma, Poole
BDH Chemicals, Lutterworth
BDH Chemicals, Lutterworth
Sigma, Poole
BDH Chemicals, Lutterworth
Sigma, Poole
BDH Chemicals, Lutterworth
Calbiochem, Nottingham
BDH Chemicals, Lutterworth
Sigma, Poole
BDH Chemicals, Lutterworth
Molecular probes, Cambridge
BDH Chemicals, Lutterworth
Sigma, Poole
BDH Chemicals, Lutterworth
Sigma, Poole
Sigma, Poole
BDH Chemicals, Lutterworth
BDH Chemicals, Lutterworth
Gibco, Paisley
Sigma, Poole
Bayer, Leyverkensen
Sigma, Poole
Sigma, Poole
Sigma, Poole
BDH Chemicals, Lutterworth
Sigma, Poole
Sigma, Poole
Sigma, Poole
Rubidium chloride | Sigma, Poole
Sodium chloride | BDH Chemicals, Lutterworth
Sodium dihydrogen phosphate | Fisons, Loughborough
Sodium hydroxide solution | BDH Chemicals, Lutterworth
Tetraethylammonium (TEA) | Sigma, Poole
Thapsigargin | Sigma, Poole
Triton X-100 | BDH Chemicals, Lutterworth
Triton X-405 | BDH Chemicals, Lutterworth
Trizma® base (Tris[hydroxymethyl]aminomethane) | Sigma, Poole
Trypan blue 0.4% solution | Sigma, Poole
Trypsin-EDTA solution | Sigma, Poole

Additionally, cetiedil, charybdotoxin (ChTX), dequalinium, clotrimazole, UK 66914 were generously donated by Professor D.H. Jenkinson. UCL compounds were synthesised by Dr. C. Roxburgh, Dr. S. Athmani, Dr. W. Quaglia and Ms. Z. Miscony under the supervision of Professor C.R. Ganellin in the Department of Chemistry, University College London. The purity of the UCL compounds, assessed by HPLC, was >98% except UCL1495 whose purity was 78%.

<table>
<thead>
<tr>
<th>Name of compound</th>
<th>Chemical name</th>
</tr>
</thead>
<tbody>
<tr>
<td>UK 66914</td>
<td>(N-[4-[1-hydroxy-2-[4-(4-pyridyl)-1-piperazinyl]ethyl]phenyl]methanesulphonamide</td>
</tr>
<tr>
<td>UCL 1348</td>
<td>(+)-2-cyclohexyl-2-(3-thienyl) ethanoic acid 2-(hexahydro-1H-azepin-1-yl) ethyl ester monohydrochloride</td>
</tr>
<tr>
<td>UCL 1349</td>
<td>(-)-2-cyclohexyl-2-(3-thienyl) ethanoic acid 2-(hexahydro-1H-azepin-1-yl) ethyl ester monohydrochloride</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>Triphenyl acetic acid-2-N[5-ethyl-2-methylpiperidinoethyl] ester monooxalate</td>
</tr>
<tr>
<td>UCL 1617</td>
<td>N-[2-(N'-triphenylethanamido) ethyl]-5-ethyl-2-methylpiperidine</td>
</tr>
<tr>
<td>UCL 1710</td>
<td>1-[(9-benzyl)fluoren-9-yl]-4-(N-5-ethyl-2-methylpiperidino)-but-2-yne oxalate</td>
</tr>
<tr>
<td>UCL 1608</td>
<td>1-[(9-benzyl)fluoren-9-yl]-4-(N-homopiperidino)-but-2-yne oxalate</td>
</tr>
</tbody>
</table>
2.2 Buffer solutions

2.2.1 Tyrode’s buffers

For β-hexosaminidase release experiments, Tyrode’s solution was used. It contained 137mM NaCl, 2.7mM KCl, 1mM CaCl₂, 1mM MgCl₂, 5.6mM glucose, 10mM HEPES, 0.4mM NaH₂PO₄ and 0.05% BSA, adjusted to pH 7.4 with NaOH (1M). BSA (0.05% or 0.5mg/ml) was added to prevent adsorptive loss of the antigen DNP-HSA, except for membrane potential change measurement experiments in which 0.5mg/ml (0.05%) gelatin instead was added. Modified Tyrode’s solution is the same as Tyrode’s solution except that it contained 1.8mM CaCl₂ and 5mM KCl.

2.2.2 Ca²⁺ free Tyrode’s buffers

Two Ca²⁺ free buffers were used. These buffers contained 0mM CaCl₂ (for nominal Ca²⁺ free) or 0mM CaCl₂ + 100μM EGTA (for Ca²⁺ free). The other components were as in modified Tyrode’s solution.

2.2.3 Substituted buffers

LiCl, NMG-Cl (N-methyl-D-glucamine chloride), RbCl, CsCl, NaCl(K⁺ free), KCl(high K⁺) buffers contained 140mM of corresponding ions plus CaCl₂ 1.8mM, MgCl₂ 1mM, glucose 5.6mM, HEPES 10mM, BSA or gelatin 0.05%. Finally, pH was adjusted to 7.4 with 1M NaOH.

2.2.4 10mM CaCl₂ modified Tyrode’s buffer

To assess the effect of extracellular calcium concentration on the inhibitory effect of some drugs, the concentration of calcium in the buffer was increased to 10mM. The other components in the buffer are the same as modified Tyrode’s buffer.

2.2.5 β-hexosaminidase assay solution

For the β-hexosaminidase release assay, the substrate solution was 1mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide dissolved in 0.2M citrate buffer
containing 0.01% Triton X-100 adjusted to pH 4.5 with concentrated HCl (10.14M). The substrate was dissolved in dry DMSO and then transferred into the citrate buffer.

2.2.6 Tris Buffer

Tris buffer (0.2M) was made by dissolving 24.2 gram of (Tris[hidroxymethyl]aminomethane) in 1 liter of distilled water. This buffer was used to stop the enzyme reaction in the β-hexosaminidase release measurement experiments.

2.2.7 Preparation of stock solutions

Stock solutions of cetiedil, ChTX, IbTX, MTX, apamin, BaCl₂, 4-AP, TEA, CsCl, 9-aminoacridine and dequalinium were prepared in isotonic saline. Glibenclamide, clotrimazole, quinidine, calcium ionophore A23187, diazoxide, UK 66914, amiodarone, nicardipine, nitrendipine, propafenone, cromakalim, indo-1 AM and UCL compounds were dissolved in dry DMSO. Gramicidin and bis-oxonol were dissolved in ethanol. Cells were exposed to not more than 0.1% DMSO or ethanol.

2.3 Cell culture

2.3.1 Cell culture

RBL-2H3 cells were maintained in monolayer culture in minimal essential medium (S-MEM) supplemented by 10%FCS, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin at 37°C in a humidified atmosphere with 95% air-5% CO₂. To harvest adherent cells, the culture medium was discarded and the monolayer culture was washed with phosphate buffer saline (PBS) without Ca²⁺ and Mg²⁺ (commercially available from Gibco, Paisley). Then cells were harvested by trypsinisation: 5ml trypsin-EDTA solution (containing porcine trypsin 0.05% and EDTA 0.02% in a calcium and magnesium free buffer solution) was added into a 250ml tissue culture flask, and left for 3-5 min at 37°C. After this time, the flask was tapped to release the cells from the flask base. This cell suspension was centrifuged at 225g for 3 min. The cell pellet was resuspended in fresh supplemented S-MEM medium. Then cells were subcultured into a tissue culture flask allowing for a doubling time of 24 hours to produce confluent culture.
2.3.2 Cryo-preservation of RBL-2H3 cells

Harvested RBL-2H3 cells were suspended in a volume of supplemented S-MEM medium (as above) to give a cell density of $4 \times 10^6$ cells/ml and 0.9 ml aliquots of this cell suspension were placed into cryotubes. Then 0.1ml sterile DMSO was added into each cryotube and the cells were quickly frozen at -20°C. After 2 hours the frozen cells were moved into a -80°C freezer and the next morning they were inserted into liquid N$_2$ for storage.

Cells were defrosted by rapidly thawing out the cryotube in a water bath at 37°C. Then the defrosted cell suspension was transferred into a 10ml centrifuge tube and 9ml of medium was added on top of the cell suspension, very slowly, with continuous hand shaking. The cell suspension was centrifuged at 225g for 3min, and the cell pellet was resuspended in fresh supplemented S-MEM medium and cultured at $10^6$ cell/ml in the normal way.

2.3.3 Measurement of cell density and viability

The viability of cells was determined by a dye exclusion method. 150μl each of Trypan Blue solution (0.25% w/v in PBS) and cell suspension were mixed. A small volume of mixture was added to a Neubauer haemocytometer. The numbers of unstained (living) and stained (dead) cells in the central chamber were counted and the cell viability (% living) determined. The cell density (cell/ml) of the cell suspension was calculated from the number of cells in the central chamber and the chamber volume of $1 \times 10^{-4}$ ml.

2.4 Histamine release measurement

2.4.1 Cell preparation

The cells were passively sensitised overnight to prepare them for antigen challenge and measurement of histamine release [226]. Cells ($3 \times 10^5$ cells in 0.5ml S-MEM/well)
seeded in a 24-well tissue culture plate were incubated with 0.5\(\mu\)g/ml DNP-specific monoclonal mouse IgE antibody.

### 2.4.2 Histamine release experiment

Histamine release was measured by published methods [226, 227, 228] which are described below. Triplicate samples were used for each treatment in all experiments. After passive sensitisation, each well was washed twice with 0.05% BSA-Tyrode’s buffer, then incubated with the same buffer (450\(\mu\)l in each well) for 5 minutes at 37\(^\circ\)C. Then cells were challenged with 50\(\mu\)l stimuli (DNP-HSA or A23187 calcium ionophore) added at 10-fold the required final concentration for 15 minutes. At the end of experiment, 1.3ml cold buffer was added to each well to stop the cell response. Then the plate was centrifuged at 225g for 3 min.

To measure \(\beta\)-hexosaminidase release in parallel with histamine, 50\(\mu\)l of the supernatant of each well was taken and placed into a 96-well black plate\(^1\) (see section 2.5.3). The remaining supernatant from each well was transferred into plastic tubes taking care not to disturb the cell pellet at the bottom of the well. 200\(\mu\)l perchloric acid (4M) was added into each supernatant tube and 2ml perchloric acid (0.4M) was added into each well to lyse the cell pellet and release the residual histamine.

### 2.4.3 Histamine assay

The perchloric acid-treated samples were first centrifuged (at 225g for 3min) to remove protein (antigen and BSA) precipitated by the perchloric acid. The supernatants were then assayed for histamine by the automated doubled-extraction procedure using a Technicon Fluorescent Autoanalyser II.

This procedure involves the extraction of histamine into alkalised, salt-saturated butanol, after alkalisation of the sample, followed by the separation of the butanol-organic phase and the aqueous phase (wash). The organic phase was made less polar by mixing with n-heptane and the histamine was re-extracted into a stream containing diluted HCL. Finally the amine was condensed with o-phthaldialdehyde (OPT) under alkaline conditions to yield a highly intense fluorophore which was stabilized by acidification. The intensity of the fluorescence, as a measure of the histamine concentration in the sample,

\(^1\) This is a 96-well plate that composed of black material in order to prevent extraneous light interfering with plate reading.
was read by a fluoronephelometer and recorded on a pen recorder. The sensitivity of this procedure is 0.5ng/ml of histamine, and a minimal volume of 0.8ml is required. Test samples were passed through the system preceded by known standards and with marker histamine standards at intervals of 9-12 samples.

2.4.4 Data expression

Histamine release was expressed as a percentage of total cellular content of the amine.

\[
\% \text{ Net histamine release} = \frac{\text{HS}}{(\text{HS} + \text{HCP})} \times 100 \times \% \text{ Spontaneous release}
\]

Where, HS is the histamine content in the supernatant,

HCP is the corresponding histamine content in cell pellet,

\% Spontaneous release is the percentage of histamine release in the absence of any stimulant.

2.5 \(\beta\)-hexosaminidase release measurement

2.5.1 Cell preparation

Cells were passively sensitised overnight with 0.5μg/ml DNP-specific monoclonal mouse IgE antibody and were seeded in 96-well tissue culture plates (3×10^4 cells in 100μl S-MEM/well) in preparation for antigen challenge and measurement of \(\beta\)-hexosaminidase release as previously described [229].

2.5.2 \(\beta\)-hexosaminidase release experiment

Measurement of \(\beta\)-hexosaminidase release was carried out according to published literature [229, 230]. In detail, on the day of the experiment each well was washed twice with 0.05% BSA-Tyrode’s buffer. Then cells were preincubated with the same buffer for 5 minutes at 37° C. The test drugs were added for 15 minutes and then cells were
challenged with antigen (DNP-HSA) for another 15 minutes. Tyrode’s buffer containing 1% triton X-100 was also added into six wells (in a 96-well plate) to lyse cells to measure the total β-hexosaminidase content in each well.

2.5.3 β-hexosaminidase assay

At the end of the experiment, using a multichannel pipette, 50µl of the supernatant containing the secreted β-hexosaminidase from each well was transferred into the corresponding well of a 96-well black plate. 50µl of 1mM of substrate solution (4-methylumbelliferyl N-acetyl-β-D-glucosaminide) was also added to each well. The black plate was incubated at 37°C for 90 to 120 minutes. To terminate the enzyme reaction, 300µl of 0.2M Tris buffer (pH=11) was added to each well. Then fluorescence was measured by an automatic microtiter plate reader at the excitation wavelength of 365 and emission wavelength of 450nm. The instrument was equipped with facilities for direct transfer of data for processing on a computer spreadsheet.

2.5.4 Data expression

Release was expressed as a percentage of the total cell content of β-hexosaminidase. Spontaneous release was subtracted from the stimulated release by antigen or A23187, and final results were expressed as the net percentage release (see equation below). In all experiments, spontaneous release was not more than 8% of total β-hexosaminidase content in the cells. All treatments in any one experiment were done in triplicate. Experiments were also done in triplicate or more (shown in each figure) and the data was presented as mean ± SEM.

\[
\frac{\text{Supernatant reading}}{\text{Total reading}} \times 100 - \% \text{ Spontaneous release}
\]

The possible interference of all the compounds studied with the fluorescence reading at the selected wavelengths was measured. The test was done by determining the fluorescence of the tested compounds and antigen dissolved in Tyrode’s buffer solution at
the appropriate concentration. None of these compounds had a significant effect on that reading.

2.6 Membrane potential change measurement

2.6.1 Cell preparation

RBL-2H3 cells were sensitised overnight with 0.5μg/ml monoclonal DNP-specific monoclonal mouse IgE in a 250ml flask containing at least 15×10^6 cells. On the day of experiment, the sensitised cells were washed with PBS and were then dislodged by treatment with EDTA 0.02% solution for 15min at 37°C. To avoid proteolysis of IgE bound to their receptors we did not use trypsin/EDTA solution for harvesting. The cell suspension in the EDTA solution was then centrifuged at 225g for 3 min and the cell pellet was then resuspended in fresh supplemented S-MEM medium to a count of 10^6 cell/ml. The cells were then kept in medium at room temperature.

2.6.2 Membrane potential measurement by bis-oxonol

Changes in the membrane potential were monitored by using a potential-sensitive fluorescent dye, bis-oxonol. Recordings were performed on a LS-5 Perkin-Elmer spectrofluorometer operating in ratio mode (the excitation wavelength was 540nm and the emission wavelength was 580nm). 2ml of cell suspension (see the previous section) were washed twice with 0.05% gelatin modified Tyrode’s buffer. After the second wash, the cell pellet was resuspended in the same buffer and transferred into a quartz cuvette, and maintained at 37°C with constant stirring. After a 5min preincubation, bis-oxonol was added to the cells to give a final concentration of 50nM and this was allowed to equilibrate until the fluorescence reached a constant value (2-3min). The fluorescence increased 5-6 times when the dye was added to the cell suspension. The cells were then treated with drugs and/or stimulant. As shown in Fig.2.1, at the end of each experiment (at time 9min), gramicidin, at a final concentration of 1μg/ml, was added to the cell suspension to cause complete cell depolarisation. Bis-oxonol had no effect on the spontaneous and antigen-induced α-hexosaminidase release [53, 111].

2 See section 4.1 for the mechanism of action of bis-oxonol.
2.6.3 Quantification of the data

The cell depolarisation response is represented as fractional depolarisation \( (F_D) \). \( F_D \) stands for the ratio of the extent of stimulant-induced depolarisation to the total depolarisation by 1\( \mu \text{g/ml} \) gramicidin. Typical data and the measured quantities are shown in Fig. 2-1. Data are reported as fractional depolarisation \( (F_D) \) that equals the ratio \( D/G \) and as fractional repolarisation \( (F_R) \) which is given by the ratio \( R/D \).

\[
\begin{align*}
F_D &= \frac{D}{G} \\
F_R &= \frac{R}{D}
\end{align*}
\]

\( D = \) Antigen-induced depolarisation, \\
\( G = \) Gramicidin-induced depolarisation, \\
\( R = \) The repolarisation phase,

\[\text{Figure 2.1: Membrane potential change measured by bis-oxonol. Cells (10}^6 \text{cell/ml) were stimulated with DNP-HSA (1ng/ml) at time zero. } D = \text{the extent of antigen-induced depolarisation; } R = \text{the extent of repolarisation; } G = \text{the extent of gramicidin-induced depolarisation at the end of each experiment.}\]
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2.7 $^{86}$Rb$^+$ efflux measurement

$^{86}$Rb$^+$ was used, as it poses as a good substitute for K$^+$ in its passage through K$^+$ channels and has the advantage over radioactive K$^+$ (e.g. $^{42}$K$^+$) of having a much longer half-life (18 days). This enables a sufficient length of study before the β-emission decays beyond the countable range (normally 3-4 half-lives).

2.7.1 Cell preparation

Passively sensitised RBL-2H3 cells were prepared as described in section 2.6.1, but at the end, the cell pellet was resuspended in fresh medium with a count of 4x10$^6$ cell/ml.

2.7.2 $^{86}$Rb$^+$ uptake measurement experiment

To evaluate the t½ and to study the time course of $^{86}$Rb$^+$ loading, 7ml of cell suspension containing 4x10$^6$ cell/ml were loaded with $^{86}$Rb$^+$ (3μCi/ml)$^3$ at 37°C in a shaking incubator for 3 hours. During the incubation time, 0.5ml samples were taken at 15 min periods (as $^{86}$Rb$^+$ loading is a relatively slow process) from $^{86}$Rb$^+$ addition. The samples were centrifuged at 225g for 3min and the pellets were resuspended in 5ml perchloric acid (0.4M) and transferred into scintillation vials. The $^{86}$Rb$^+$ loaded into cells was counted in a liquid scintillation counter. Figure 2.2 shows the time course of $^{86}$Rb$^+$ loading in RBL cells. 50% total uptake occurred at 30 min and reaches a plateau at approximately 2 hours.

2.7.3 $^{86}$Rb$^+$ loading

Cells in suspension (4x10$^6$ cell/ml) were loaded with $^{86}$Rb$^+$ (3μCi/ml) at 37°C in a shaking incubator for 2 hours. As already noted, uptake has a doubling time of 30min and reaches a plateau at approximately 2 hours. Increases in loaded $^{86}$Rb$^+$ after 120min are not statistically significant (P<0.1).

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$^3$ Calculating on a daily base.
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Figure 2.2: Time course study of $^{86}$Rb$^+$ loading into RBL-2H3 cells. Cells ($4 \times 10^6$ cell/ml) were incubated with 3μCi/ml $^{86}$Rb$^+$ at 37°C. Samples were taken at 15 min intervals and spun down. The pellet was resuspended in perchloric acid (0.4 M) to lyse the cells. The "loaded $^{86}$Rb$^+$" was counted on a liquid scintillation counter. CPM changes after 120 min are not statistically significant (P<0.1).

2.7.4 $^{86}$Rb$^+$ efflux measurement experiment

For a typical experiment, 0.5 ml of cell suspension ($4 \times 10^6$ cell/ml) loaded with $^{86}$Rb$^+$ (see section 2.7.3) was centrifuged (at 225g for 3 min), resuspended in 2 ml modified Tyrode’s (0.05% BSA) and preincubated ($1 \times 10^6$ cell/ml) for 1 min at 37°C in a shaking water bath prior to the start of each experiment. Prior to initiation of the experiment, test drugs were pipetted into the test tubes at 100 times the test concentration and then the cell suspension was added into the test tube to dilute the test drug to the desired concentration. Cells were then incubated with drugs for 9 min before antigen stimulation. Each experiment was carried out for a total of 24 minutes, stimulant (antigen or A23187) being added after 8 minutes in order that a basal rate of $^{86}$Rb$^+$-efflux be established. Samples were taken every 2 min from 8 min before (to establish the spontaneous rate of $^{86}$Rb$^+$-efflux), to 16 min after, antigen stimulation$^4$ (to calculate the stimulated rate $^{86}$Rb$^+$-efflux). 100μl aliquots of the cell suspension were taken at each time point and sedimented (at 16000g for 10 sec) through 200μl phthalate oil (60% dibutyl phthalate and

---

$^4$ Secretion was measured for 15 min and 16 min for this experiment gives a desired comparison. 2 min time intervals have been chosen for logistical reasons.
40% phthalic acid bis(2-ethylhexyl ester) using an Eppendorf microfuge. 50μl of the supernatant on top of the oil was transferred into scintillation vials containing 5ml distilled water. In order to lyse the cells and obtain a standard for total $^{86}$Rb$^+$ uptake for each experiment, 5 ml perchloric acid 0.4M was added to a tube containing the same cell number after centrifuging and discarding the media. The $^{86}$Rb$^+$ released by cells was counted on a liquid scintillation counter.

2.7.5 Quantification of the data

Since a plot against time of the total amount of $^{86}$Rb$^+$ lost produces a curve, it was preferred to use a linearising equation which would allow an estimate of the rate constant to be obtained. A logarithmic function was used:

$$k = \frac{\ln (Q_- \Delta Q/Q_0)}{t}$$

k= rate of $^{86}$Rb$^+$-efflux  

t= time,  

$Q_0$= total $^{86}$Rb$^+$ uptake by the same number of cells during the loading time, which is the cpm recorded from the perchloric acid-treated cells.  

$\Delta Q$= the amount of $^{86}$Rb$^+$ released from cells into the supernatant which equals the cpm recorded from each sample at any time point.

Now, if $-\ln (Q_- \Delta Q/Q_0)$ is plotted against time, a line can be obtained whose slope is k (Fig.2.3). The other advantage of this equation is to normalise results from different experiments, irrespective of variation in the number of cells or $^{86}$Rb$^+$ loaded into the cells.

For each experiment the data from T=-8 min (8 min before addition of stimulant) to T=0 (just before stimulant addition) were plotted and the slope of the regression line for this period gave an estimate of the rate constant for spontaneous $^{86}$Rb$^+$ efflux. When test drug has been used, the calculated rate from T=-8 to T=0 was compared with control, to show the effect of drugs on the spontaneous $^{86}$Rb$^+$ efflux. The best software found to analyse these data was “Microcal Origin 4”.

To express the effect of test drugs on the stimulated $^{86}$Rb$^+$-efflux, two different methods have been used.
1. To express as a value, the initial rate of stimulated $^{86}$Rb$^+$-efflux which was obtained from the difference between the rate of $^{86}$Rb$^+$-efflux after cell stimulation and the rate of spontaneous $^{86}$Rb$^+$-efflux. The calculated initial rate constant of stimulated $^{86}$Rb$^+$-efflux for 10ng/ml DNP-HSA was 0.015298 ± 0.001291 min$^{-1}$. Therefore, the inhibitory effect of tested drugs were calculated by comparison of the stimulated rate of $^{86}$Rb$^+$-efflux with and without drugs.

2. To present the data graphically, data points from a linear regression fit were subtracted from the corresponding values for the efflux after stimulation (from $t=0$ to $t=16$) (Fig. 2.4). The advantage of this curve, in addition to showing the effect of drugs, is that it also gives an indication of the time course of stimulated $^{86}$Rb$^+$ efflux from the ‘control’ cells.

Figure 2.3: Antigen caused increase in $^{86}$Rb$^+$-efflux in sensitised RBL-2H3 cells. Cells were preincubated in modified Tyrode’s buffer 9 min before stimulation at 37°C. Samples were taken after one min from 8min before to 16 min after antigen addition. Antigen [DNP-HSA 10ng/ml] was added at 0 min. The red line represent the spontaneous $^{86}$Rb$^+$-efflux data points.
Figure 2.4: ^86Rb\(^\text{-}\)efflux stimulation by antigen [DNP-HSA 10ng/ml] in sensitised RBL cells. Stimulated ^86Rb\(^\text{-}\)efflux at each point is defined as the difference between the value for the efflux after triggering (from \(t=0\) to \(t=16\)) and the values calculated from a linear regression fit of basal efflux (the first five timepoints from \(t=-8\) to \(t=0\) on the red line in Fig.2.3).

2.8 Measurement of intracellular free calcium concentration \([\text{Ca}^{2+}]_i\)

2.8.1 The preparation of RBL-2H3 cells for the measurement of \([\text{Ca}^{2+}]_i\)

Free cells were prepared from a RBL-2H3 monolayer cell culture. They were firstly washed with PBS and then harvested by trypsinisation (See section 2.3.1). The cell suspension in trypsin-EDTA solution was centrifuged at 225g for 3min and then resuspended in fresh media. Cell counting indicated a harvested cell density of \(~5\times10^4\) cell/ml. 0.5ml of this cell suspension was then placed within the ‘calcium recording chamber’ which consisted of a glass ring (diameter 16mm; height 3mm) attached to a microscope slide coverslip using a silicon rubber elastomer ‘Sylgard’. During the culturing procedure the recording chamber was kept in a standard 35mm culture dish within a humidified incubator (5% CO\(_2\) and 95% air) at 37\(^\circ\)C. Cells were normally used within 48 hours after culture. The cells were sensitised to antigenic stimuli by incubating overnight at 37\(^\circ\)C with the DNP specific monoclonal mouse IgE antibody (0.5\(\mu\)g/ml).
2.8.2 Indo-1 loading

Cells were loaded with the Ca indicator dye, indo-1, by using the acetoxymethyl ester form; indo-1 AM [231]. Cells were incubated with 1μM indo1-AM for 30-45 min in the culture medium at 37°C. To assure adequate dispersion of both indo-1 and its DMSO solvent, 100μl of culture media was removed from the recording chamber and transferred into a 0.5ml Eppendorf tube where 0.5μl of indo-1 AM (1mM in DMSO) was added. This was then mixed using a vortex and returned into the recording chamber. The uncharged lipophytic indo-1 AM readily passes into the cell cytoplasm where endogenous esterases induce ester hydrolysis generating indo-1 free acid, which being charged is trapped within the cell. Indo-1 had no effect on the spontaneous and antigen-stimulated β-hexosaminidase release from RBL-2H3 cells (data shown in section 6.3.1).

2.8.3 The recording of [Ca^{2+}] from RBL-2H3 cells

The method used in this study was as previously described by Marsh et al. (232, 233, 234, 235). The recording chamber was placed onto the microscope stage of a Nikon inverted microscope (Diaphot) fitted with UV epifluorescence illumination. The recording chamber was continuously perfused with the modified Tyrode’s buffer (8-12 ml/min at 37°C). To record [Ca^{2+}], following indo-1 loading (see above), individual cells were excited with UV light (360nm) and emitted light at both 408 and 480 nm was recorded simultaneously using a series of dichroic mirrors and photomultiplier tubes (Thorn EMI QL30) (see Fig. 2.5) A home-built amplifier converted the anodal photomultiplier current to voltage and continuously output the ratio of the emissions at 408 and 480nm (408/480 ratio). The 408/480 ratio output was computer digitised at 2.5 Hz using a Digidata 1200 and ‘pClamp 6.0’ software (Axon Instruments) and the estimation of [Ca^{2+}], undertaken on-line after subjecting the data to a simplified algorithm (see below).

2.8.4 Calculation of [Ca^{2+}], and calibration of the system

[Ca^{2+}] was estimated from the acquired ratio data using the equation first described by Gryniewicz at al. in 1985 [231]:
\[ [\text{Ca}^{2+}]_i = \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \times K_d \left( \frac{F_v}{F_s} \right) \]

Where, Kd is the dissociation constant for \( \text{Ca}^{2+} \) and indo-1,

\( F_v/F_s \) is the maximum excursion at 450nm,

R is the recorded 408/480 emission ratio,

\( R_{\text{min}} \) is the minimal value of R at zero \([\text{Ca}^{2+}]_i\),

and \( R_{\text{max}} \) is the value of R at saturating \([\text{Ca}^{2+}]_i\).

\( R_{\text{min}} = 0.38, R_{\text{max}} = 4, K_d \left( \frac{F_v}{F_s} \right) = 1400\text{nM} \)

The measured constants used in the above equation were obtained in a separate set of experiments carried out by Dr. Steve Marsh (Pharmacology U.C.L) by applying a series of known intracellular calcium concentrations in the presence of indo-1 to the inside of sympathetic neurons using a whole cell patch pipette and then measuring the 408/480 ratio. This allowed construction of a calibration curve and estimation of the above constants.

Figure 2.5: Schematic diagram of \([\text{Ca}^{2+}]_i\) measuring equipment.
2.8.5 Quantification of the data

In this study antigen-induced changes in [Ca\(^{2+}\)] are oscillatory, asynchronous and irregular in individual RBL-2H3 cells. Figure 2.6 shows Ca\(^{2+}\) oscillations induced by 1ng/ml DNP-HSA in 18 different cells. The so-called Ca\(^{2+}\)-fingerprint is obvious and the antigen-induced changed in [Ca\(^{2+}\)] in individual cells is different in frequency, amplitude and shape (Fig. 2.6). Millard et al. have used a graph obtained from averaging the response of more than ten cells to quantify their data [114]. Their method is not suitable for this study as it does not allow the evaluation of the effect of drugs on the antigen-stimulated changes in [Ca\(^{2+}\)]. However in this study, to address this problem, the area under the curves (AUC) have been calculated, using the ‘Origin 4’ software program. To enable a comparison between the control and the effect of drugs and the estimation of the antigen-induced changes in [Ca\(^{2+}\)], I utilised an integration procedure on the [Ca\(^{2+}\)] response using the ‘Origin 4’ software program to generate the area under the curve (AUC). Before calculation of AUC, the resting [Ca\(^{2+}\)] on the curves was adjusted to zero, and data was presented solely for the increased [Ca\(^{2+}\)]. The equation used for calculating AUC was:

\[
\sum_{i=0}^{i=1535} = \frac{2(Y_i + Y_{i+1})}{\Delta X}
\]

In detail, an oscillation curve was divided into 1536 sections, the area for each section calculated as a trapezoid whose heights are Y\(_i\) and Y\(_{i+1}\) and its base equal to the interval time between each two samples (\(\Delta x=0.4\)second). Finally the calculated area for all sections were summed (see Fig. 2.7). The above equation is a general formula for this calculation method.

Data obtained from the ‘Origin 4’ software program or calculated from the above equation, using ‘Microsoft Excel’, were exactly identical.
2.9 Statistical analysis

All values are presented throughout this study as mean ± standard error of the mean (SEM) for the number of experiments carried out and cited under each graph or table. The points on the graphs or figures in the tables are the means of n experiments noted and the vertical bars show the SEM. Statistical comparison of results was carried out by using Student's paired t-test or Mann-Whitney U-test (where appropriate). Values of p > 0.05 are considered significant. Denotation by asterisks *, **, *** represent significance of p > 0.05, p > 0.01 and p > 0.001, respectively.

2.10 Data analysis

Concentration-response curves were fitted by the Hill equation using the method of least squares using the “Origin v.5” program.

\[
y = \frac{y_{\text{max}} [A]^{n_H}}{K^{n_H} + [A]^{n_H}}
\]

where:

- \( y \) = response as percentage of maximum
- \( y_{\text{max}} \) = maximum response
- \([A]\) = concentration of a stimulating agent (e.g. antigen) or of a blocker
- \( K \) = EC\(_{50}\) or IC\(_{50}\)
- \( n_H \) = Hill coefficient

The program provided estimates of \( y_{\text{max}} \), \( K \) and \( n_H \) with approximate standard deviations for each. The data were weighted using 1/var calculated from the standard error for each data point. When inhibition curves for K\(^+\) channel blockers were constructed, \( y \) was usually constrained to 100%.
Figure 2.6: Antigen induced \( \text{Ca}^{2+} \) oscillations in 18 different individual RBL-2H3 cells. Cells were loaded with indo-1 AM (1\( \mu \)M for 30 min). Cells were then challenged with DNP-HSA 1 ng/ml at time 0 second and the changes in \([\text{Ca}^{2+}]_i\) were monitored (see section 2.8.3 for details).
Figure 2.7: Area under the curve calculation for antigen induced Ca^{2+} oscillations in an individual RBL-2H3 cells. Cell was loaded with indo-1 AM (1μM for 30min). The cell was then challenged with DNP-HSA 1ng/ml at time 0 second and the changes in [Ca^{2+}], were monitored. The lower graph shows the data from a whole experiment, while the upper graph shows a part of the lower graph divided in respect of the interval time between each sample. Y and ΔX values are shown in the inset of the upper graph.
CHAPTER 3 Effect of K⁺ channel modulators on β-hexosaminidase release from RBL-2H3 cells

3.1 Introduction

Antigen- or A23187- induced secretion from RBL-2H3 cells can be evaluated by measuring the release of histamine, incorporated tritiated 5-HT ([^3]H 5-HT) or granular enzymes. Moreover, the fusion of secretory vesicles with the cell membrane increases the cell surface area, and this effect can be assayed by measuring the cell membrane capacitance [105, 163]. β-hexosaminidase is one of the granule enzymes which is released during secretion. To measure the enzyme content in the supernatant (released either from stimulated cells or from cells lysed with Triton X-100), a synthetic substrate is added to the supernatant. By the action of β-hexosaminidase released from cells, the substrate gives rise to products which are fluorescent. The amount of fluorescence is proportional to the concentration of enzyme and also the extent of secretion.

It has been well established that histamine and other inflammatory mediators are released together with β-hexosaminidase following antigen-induced degranulation [236, 237]. However, a few differences have been reported between β-hexosaminidase and 5-HT release. These include: 1) monensin can stimulate 40% 5-HT release but it has no effect on β-hexosaminidase release [139], and 2) high concentrations of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (20μM), a proton ionophore, induce a noncytotoxic 5-HT release in RBL-2H3 cells, while β-hexosaminidase release is unaffected [147]. Monovalent cation ionophore-induced 5-HT release does not require extracellular Ca²⁺ [147]. These results indicate that changes in the proton electrochemical gradients and alkalinisation of the granules by these ionophores cause secretory granule disturbances and 5-HT to diffuse out, but not granule exocytosis and β-hexosaminidase release.
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Perhaps these compounds open a fusion pore but do not promote real secretion. Possibly due to molecular size, 5-HT, but not β-hexosaminidase, can leak through the fusion pore.

Compared with the other methods, β-hexosaminidase release measurement is the most convenient.

3.2 Methods and materials

All materials and experimental procedures are outlined in the appropriate sections in chapter two. None of the compounds used in this study was fluorescent within the wavelengths under study and also none of them caused any significant change in the spontaneous release of β-hexosaminidase except DMSO (see section 3.3.2). Each experiment was carried out with triplicate samples for each treatment. DMSO was always used at less than 0.1% v/v to avoid inhibition of antigen-induced β-hexosaminidase release.

3.3 Results

3.3.1 Antigen-stimulated degranulation dose-response curves

In passively sensitised RBL-2H3 cells, crosslinking of FcεRI-bound IgE by antigen (DNP-HSA) induced a substantial increase in β-hexosaminidase release; up to 67% above spontaneous release (3-8%). As expected, DNP-HSA did not cause non-sensitised RBL-2H3 cells to release significant amounts of β-hexosaminidase. Figure 3.1A depicts the DNP-HSA-induced degranulation dose-response curve. Antigen-stimulated histamine release was also measured and the release achieved was closely correlated with the β-hexosaminidase data.

As shown in Fig. 3.1, the optimum concentration of DNP-HSA was 10ng/ml and the EC₅₀ values were 0.3±0.03 and 0.2±0.07ng/ml for histamine and β-hexosaminidase release, respectively. The results are in agreement with other reports [112, 218]. In later experiments, optimal and suboptimal concentrations of DNP-HSA (10, 1, 0.1, 0.01ng/ml) were used. Figure 3.1B also shows the effect of BSA (0.05%) in the buffer. BSA binds to the plasticware and saturates the non-specific binding sites, so that it can prevent the binding of DNP-HSA to plastic and increase the availability of antigen to the receptor-bound IgE. The presence of 0.05% BSA in the buffer solution shifts the dose-response curve to the left (Fig. 3.1B).
Figure 3.1: (A) Dose-response curves of antigen-stimulated β-hexosaminidase and histamine release, and (B) effect of the presence of BSA in the buffer on the antigen-induced release. After 5 min preincubation at 37°C, cells were stimulated DNP-HSA (for 15 min). Histamine and β-hexosaminidase were measured in the supernatant from the same experiments. Each point on the above curves represents the mean ± SEM of %net release in four experiments. Data have been fitted to the Hill equation with Bmax constrained to the maximum net release for each curve. Hill coefficient (nH) was 0.5 ± 0.03. Spontaneous release was 4.5 ± 0.5%. Student’s paired t-test, * p<0.05, ** p<0.01 and *** p<0.001. □ % net histamine release; ● % net β-hexosaminidase release; ▼ BSA 0.05% and ▲ without BSA.
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The difference was significant (Student’s paired t-test, p<0.05), and to induce the same amount of β-hexosaminidase release, a ten times higher concentration of antigen was needed in the absence of BSA.

3.3.2 Effect of the solvent/vehicle (dimethyl sulphoxide) on β-hexosaminidase release

In order to investigate the effect of dimethyl sulphoxide (DMSO), which had been used as a solvent for lipid-soluble compounds, cells were incubated with DMSO and then measured the percentage of spontaneous and antigen-induced β-hexosaminidase release (Fig.3.2). DMSO up to 0.1% v/v slightly (but not significantly, Student’s paired t-test, p<0.1) inhibited the antigen-induced release, but higher concentrations significantly inhibited the release, so that at the concentration of 5% v/v, DMSO completely inhibited antigen-stimulated release. DMSO at concentrations between 0.01-0.1%v/v significantly increased spontaneous release, and with higher concentrations, spontaneous release was inhibited almost completely. To dissolve some test drugs in DMSO, concentrations of more than 0.1% v/v were avoided.

3.3.3 Calcium ionophore A23187-induced secretion dose-response curves

A23187 is an ionophore that is predominantly selective for divalent over monovalent ions. Thus, it is capable of stimulating various Ca^{2+}-dependent biological reactions without disturbing the pre-existing balance of Na^{+} and K^{+}.

The dose-response curve for A23187-induced β-hexosaminidase and histamine release is shown in figure 3.3. The EC_{50} values of A23187 for stimulation of β-hexosaminidase and histamine release were 81.5±6.3 and 77.3±5.5nM, respectively. Maximum release induced by A23187 was 66% at the concentration of 1μM. Fewtrell et al. [38] reported that RBL-2H3 granule exocytosis by A23187 at concentrations lower than 0.5μM is a Ca^{2+}-dependent, non-cytotoxic effect. A23187-stimulated β-hexosaminidase release was almost identical to the histamine release.

3.3.4 Inhibition of the antigen-stimulated β-hexosaminidase release by quinidine

Quinidine, a non-specific K^{+} channel blocker, is capable of blocking the different K^{+} channels: BK_{Ca}, IK_{Ca} and SK_{Ca}. It also has equally potent activity in blocking other K^{+} channels such as K_{A}, K_{V}, K_{ATP}, as well as the cardiac Na^{+} channel and Ca^{2+} channel.
Chapter 3 Antigen-induced β-hexosaminidase release

0.01% 0.05% 0.10% 1% 5% 10%

Figure 3.2: Effect of dimethylsulphoxide (DMSO) on the spontaneous and antigen-induced β-hexosaminidase release. After 5min preincubation at 37°C, DMSO was added at different concentrations (%v/v). 15min later cells were challenged with antigen (DNP-HSA 10ng/ml) for a further 15 min. Each point represents the mean of β-hexosaminidase release in four experiments. Spontaneous release was 5.2 ± 0.5%. ■ Spontaneous, ■ antigen-induced induced β-hexosaminidase release with 10ng/ml DNP-HSA.
Figure 3.3: Dose-response curves of A23187-stimulated β-hexosaminidase and histamine release. After 5 min preincubation at 37°C, cells were stimulated with A23187 (for 15 min). Histamine and β-hexosaminidase were measured in the supernatant from the same experiments. Each point on the above curves represents the mean ± SEM of four experiments. Data have been fitted to the Hill equation with Bmax constrained to the maximum net release for each curve. Hill coefficient $n_H$ was $2.3 ± 0.1$. Spontaneous release was $5.6 ± 0.8\%$. ● % net histamine release; ■ % net β-hexosaminidase release.
Quinidine also inhibited β-hexosaminidase release. Figure 3.4 shows the inhibitory effect of quinidine on the antigen-induced β-hexosaminidase release in three different buffer solutions: A) ordinary Tyrode's buffer (2.7mM KCl, 1mM CaCl$_2$), B) modified Tyrode's buffer (5mM KCl, 1.8mM CaCl$_2$), C) 10mM CaCl$_2$ modified Tyrode's buffer (see sections 2.2.1 and 2.2.4). The inhibitory effect of quinidine on β-hexosaminidase release induced by a suboptimal concentration of antigen (0.01ng/ml DNP-HSA) was 50% at 45μM and about 70% at 80μM. However, degranulation mediated by higher concentrations of DNP-HSA was less susceptible to inhibition by quinidine. In the latter case, the highest concentration of quinidine (80μM) that was used, inhibited the release (mediated by 1ng/ml DNP-HSA) only by less than 40% (Fig.3.4).

Table 3.1 shows the IC$_{50}$ of quinidine for the inhibition of β-hexosaminidase release induced by different concentration of antigen. As shown in this table, the inhibitory effect of quinidine was very low when the highest concentration of antigen (100ng/ml) was used, so that quinidine is more potent against release induced by the low concentrations of antigen (0.001-0.1ng/ml). Comparing Fig. 3.4C and 3.4B suggests that increasing [Ca$^{2+}$]$_o$ reduces the effect of quinidine.

### 3.3.5 Inhibition of the antigen-induced β-hexosaminidase release by BaCl$_2$

Ba$^{2+}$ (0.5-5mM) is relatively non-specific in blocking K$^+$ channels. In addition to blocking both the inward rectifier and delayed rectifier, it can also inhibit the M current, BK$_{Ca}$, IK$_{Ca}$, K$_{ATP}$, SK$_{Ca}$ and A current. Because of a close similarity between the atomic radii of the two cations (Ba$^{2+}$ and K$^+$), Ba$^{2+}$ can enter the K$^+$ channel and bind to a blocking site within the pore. Barium (Ba$^{2+}$) inhibited antigen-triggered degranulation dose-dependently. In the present experiment on the inhibition of the enzyme release by Ba$^{2+}$, the IC$_{50}$ was about 2mM for all antigen concentrations (table 3.1). The effect of Ba$^{2+}$ on the antigen-induced β-hexosaminidase release in three different buffer solutions A) ordinary Tyrode's buffer (2.7mM KCl, 1mM CaCl$_2$), B) modified Tyrode's buffer (5mM KCl, 1.8mM CaCl$_2$) and C) 10mM CaCl$_2$ modified Tyrode's buffer (see sections 2.2.1 and 2.2.4) is shown in Fig. 3.5. Ba$^{2+}$ at concentrations up to 1 mM did not have any significant effect, whereas 10mM Ba$^{2+}$ completely inhibited the release induced by antigen. Increasing [Ca$^{2+}$]$_o$ enhanced the inhibitory effect of barium at 5mM (Fig. 3.5C).
Figure 3.4: Inhibitory effect of quinidine on antigen-stimulated β-hexosaminidase release. (A) ordinary Tyrode's buffer (2.7mM KCl, 1mM CaCl\(_2\)); (B) modified Tyrode's buffer (5mM KCl, 1.8mM CaCl\(_2\)); (C) 10mM CaCl\(_2\) modified Tyrode's buffer. After 5min preincubation at 37°C, quinidine was added at different concentrations and after 15 min cells were challenged with antigen (for 15min). Each point on the curves represents the mean ± SEM of %net β-hexosaminidase release in four experiments. Spontaneous release was 4.5 ± 0.5% and was not significantly altered by the highest concentration of quinidine. ■ without, ○ 20μM, ▲ 40μM and ▼ 80μM quinidine.
Figure 3.5: Inhibitory effect of BaCl₂ on antigen-stimulated β-hexosaminidase release. (A) ordinary Tyrode's buffer (2.7mM KCl, 1mM CaCl₂); (B) modified Tyrode's buffer (5mM KCl, 1.8mM CaCl₂); (C) 10mM CaCl₂ modified Tyrode's buffer. After 5min preincubation at 37°C, BaCl₂ was added at different concentrations and after 15 min cells were then challenged with antigen (for 15min). Each point on curves represents the mean ± SEM of %net β-hexosaminidase release in four experiments. Spontaneous release was 6.2 ± 0.4% and was not significantly affected by the highest concentration of Ba²⁺. ■ without, ○ 1mM, ▲ 5mM, ▼ 10mM BaCl₂.
3.3.6 Comparison of the inhibitory effect of quinidine and Ba\(^{2+}\) on the antigen-induced \(\beta\)-hexosaminidase release; effect of [Ca\(^{2+}\)]\(_o\)

An increase in [Ca\(^{2+}\)]\(_o\) from 1.8 to 10mM does not change the effect of Ba\(^{2+}\) [10mM] on antigen-stimulated mediator release. However, raising [Ca\(^{2+}\)]\(_o\) significantly (Student's paired t-test, \(p<0.01\)) reduced the inhibitory effect of quinidine, as shown in Fig. 3.6. Moreover, the effect of Ba\(^{2+}\) 5mM was enhanced in the presence of 10mM Ca\(^{2+}\). Quinidine was more potent in Tyrode's buffer containing 2.7mM KCl as compared with 5mM KCl. Thus the potency of quinidine may be increased by lowering [K\(^+\)]\(_o\).

3.3.7 A23187-induced release was inhibited by Ba\(^{2+}\) but not by quinidine

Ba\(^{2+}\) inhibited the \(\beta\)-hexosaminidase release induced by A23187. Its IC\(_{50}\) values were 0.156±0.005 and 1.20±0.03 and 2.74±0.07mM when RBL-2H3 cells were stimulated by 0.15, 0.5, 1\(\mu\)M A23187, respectively (Fig.3.7A). As seen, Ba\(^{2+}\) has a higher inhibitory effect on low doses of A23187, while the inhibitory effect of Ba\(^{2+}\) for all doses of antigen is nearly the same (IC\(_{50}\) = 2mM). By contrast, quinidine did not have any significant effect on A23187-induced \(\beta\)-hexosaminidase release (Fig.3.7 B) but it had an inhibitory effect on antigen-induced \(\beta\)-hexosaminidase release which was more pronounced at low concentrations of antigen. The difference between the effect of Ba\(^{2+}\) and quinidine on A23187- and antigen-induced secretion (in different [Ca\(^{2+}\)]\(_o\)) strongly suggests that these two agents have different modes of action. If it is accepted that inhibition by quinidine of antigen-stimulated \(\beta\)-hexosaminidase release is related to blocking of K\(^+\) channels and inhibition of the repolarisation phase, it can be proposed that maintaining the membrane potential near E\(_K\) helps to maintain the electrochemical driving force for Ca\(^{2+}\) influx. The lack of inhibition of A23187-induced degranulation by quinidine suggests that the Ca\(^{2+}\) influx induced by A23187 does not depend on this electrochemical driving force.
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Table 3.1: Comparison of IC_{50} values of quinidine and Ba^{2+} for the inhibition of antigen-induced β-hexosaminidase release from RBL-2H3 cells. After 5 min preincubation at 37°C, BaCl_{2} or quinidine was added at different concentrations and after 15 min cells were then challenged with antigen (for 15 min). Spontaneous release was 6.2 ± 0.4% and was not significantly affected by the highest concentration of Ba^{2+} or quinidine. The IC_{50} values were estimated by fitting the Hill equation to the data.

<table>
<thead>
<tr>
<th>Antigen [ng/ml]</th>
<th>α_{0} Net β-hexosaminidase release</th>
<th>IC_{50} for Quinidine [μM]</th>
<th>IC_{50} for Ba^{2+} [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.001</td>
<td>13.3 ± 3.6</td>
<td>37 ± 2</td>
<td>1.3 ± 0.07</td>
</tr>
<tr>
<td>0.01</td>
<td>28.4 ± 4.3</td>
<td>45 ± 4</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>0.1</td>
<td>37.9 ± 4.9</td>
<td>83 ± 10</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>1</td>
<td>61.7 ± 4.4</td>
<td>110 ± 16</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>10</td>
<td>58.5 ± 4.1</td>
<td>292 ± 78</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>100</td>
<td>57.3 ± 1.3</td>
<td>112 ± 16</td>
<td>2.3 ± 0.3</td>
</tr>
</tbody>
</table>

Table 82
Figure 3.6: Effect of $[Ca^{2+}]_0$ on the inhibitory action of $Ba^{2+}$ and quinidine on antigen-induced $beta$-hexosaminidase release from RBL-2H3 cells. After 5min preincubation at 37°C, BaCl$_2$ or quinidine was added at different concentrations and after 15 min cells were then challenged with antigen (DNP-HSA 10ng/ml for 15min). Each point on the above bars represents the mean ± SEM of %net $beta$-hexosaminidase release in 4-6 experiments. Spontaneous and antigen-induced releases were 5.7 ± 0.6% and 52 ± 3%, respectively. Spontaneous release was not affected by 80μM quinidine or 10mM $Ba^{2+}$. □ 1mM, ■ 1.8mM, □ 10mM CaCl$_2$. Student’s paired t-test, ** p< 0.01 and *** p<0.001.
Figure 3.7: Effect of Ba^{2+} (A) and quinidine (B) on the calcium ionophore A23187-stimulated β-hexosaminidase release. After 5 min preincubation at 37°C, BaCl\textsubscript{2} or quinidine was added at different concentrations and after 15 min cells were then challenged with A23187 (for 15 min). Each point on the above curves represents the mean ± SEM of %net β-hexosaminidase release of four experiments. Spontaneous release was 6.3 ± 0.5% and was not significantly influenced by 80 μM quinidine or 10 mM Ba^{2+}.
3.3.8 Effect of some K⁺ channel peptide toxins on antigen stimulation of β-hexosaminidase release

Since quinidine and Ba²⁺ are non-specific K⁺ channel blockers, it was necessary to study the effect of some more selective K⁺ channel blockers on antigen-triggered β-hexosaminidase release. Charybdotoxin is a blocker of BKCa, IKCa and KV1.3 channels. It inhibited antigen-stimulated β-hexosaminidase release in a dose-dependent manner with a predicted IC₅₀ of 133±52nM when RBL-2H3 cells were activated by DNP-HSA 10ng/ml (Fig.3.8, Table 3.2). The reported IC₅₀ for blockade of BKCa, IKCa and KV1.3 channels is 5-100nM in T lymphocytes, erythrocytes and skeletal muscles. The inhibitory effect of charybdotoxin, like quinidine, is more pronounced against the lower concentrations of antigen, so that its IC₅₀ was 61±4nM for 1ng/ml DNP-HSA.

However, apamin, a selective SKCa channel blocker; iberiotoxin, a selective blocker of BKCa channel and margatoxin, a selective blocker of KV1.3, did not affect β-hexosaminidase secretion even at 100nM (Table 3.2). The lack of inhibition of secretion by these toxins suggests that SKCa, BKCa and KV1.3 are not involved in RBL-2H3 cell activation.

3.3.9 Inhibition of the antigen-induced β-hexosaminidase release by cetiedil

Since antigen-induced β-hexosaminidase release was inhibited by quinidine and charybdotoxin but not by iberiotoxin, it seems possible that the K⁺ channel involved might be similar to the IKCa channel in red blood cells. For this reason, the effect of cetiedil on antigen-stimulated β-hexosaminidase release was evaluated. Cetiedil (Fig. 3.10) is the 2-(N-hexahydroazepinyl)ethyl ester of α-cyclohexyl-α-(3-thienyl) acetic acid.

It is a blocker of IKCa, volume-sensitive and G-protein-regulated K⁺ channels [239, 240, 241, 242]. Cetiedil inhibited dose-dependently β-hexosaminidase release by antigen. The IC₅₀, as shown in figure 3.9, was approximately 84±10μM when the cells were stimulated by 10ng/ml DNP-HSA. It is possible that a K⁺ channel similar to IKCa in erythrocytes may be involved in the antigen activation of RBL-2H3 cells, since the antigen-induced β-hexosaminidase release was inhibited by quinidine, charybdotoxin and cetiedil.
### Table 3.2: Effect of K⁺ channels toxins on the antigen-induced β-hexosaminidase release.

<table>
<thead>
<tr>
<th>K⁺ channel toxins</th>
<th>% Net β-hexosaminidase release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Charybdotoxin</td>
<td>53.4 ± 3.97</td>
</tr>
<tr>
<td>Iberiotoxin</td>
<td>64.0 ± 0.5</td>
</tr>
<tr>
<td>Apamin</td>
<td>60.8 ± 1.9</td>
</tr>
<tr>
<td>Margatoxin</td>
<td>63 ± 0.6</td>
</tr>
</tbody>
</table>

Passively sensitised RBL-2H3 cells were preincubated for 5min at 37°C and toxins at the concentration of 100nM were added for 15min. Thereafter, cells were challenged with DNP-HSA [10ng/ml] for another 15min. Spontaneous release was 7.1 ± 0.6% and was not significantly influenced by any of the toxins at 100nM. Student’s paired t-test, ** p<0.01.
Figure 3.8: Inhibitory effect of charybdotoxin on antigen-stimulated β-hexosaminidase release. After 5min preincubation at 37°C, charybdotoxin was added at different concentrations and after 15 min cells were then challenged with antigen (for 15min). Each point on the curves represents the mean ± SEM of %net β-hexosaminidase release in 4-6 experiments. Data have been fitted to the Hill equation and with Bmax constrained to the maximum net release for each curve. Hill coefficient (nH) was 0.84±0.05. Spontaneous release was 5.2±0.4% and was not significantly altered by 100nM charybdotoxin. ■ 1, ○ 10ng/ml DNP-HSA. Student’s paired t-test * p<0.05 and ** p<0.01.
Figure 3.9: Inhibitory effect of cetiedil on the antigen-stimulated β-hexosaminidase release. After 5 min preincubation at 37°C, cetiedil was added at different concentrations and after 15 min cells were challenged with DNP-HSA 10ng/ml (for 15 min). Each point on the curves represents the mean ± SEM of %net β-hexosaminidase release in 6 experiments. Data have been fitted to the Hill equation and with Bmax constrained to the maximum net release for each curve. Hill coefficient (n_H) was 0.54±0.04. Spontaneous release was about 5.2±0.4% and was not markedly affected by 100μM cetiedil. Student’s paired t-test ** p<0.01 and *** p<0.001.
3.3.10 Inhibitory effects of cetiedil-related compounds (UCL compounds) on antigen-stimulated release

The agents hereafter referred to as ‘UCL compounds’ were synthesised by the Department of Chemistry at University College London. The molecular structures of cetiedil and the UCL compounds are shown in figures 3.10 and 3.11.

3.3.10.1 Cetiedil enantiomers

Since cetiedil has an asymmetrical carbon, its enantiomers can be used to investigate the importance of the stereochemistry of the molecule in relation to the inhibitory effect on antigen-induced β-hexosaminidase release from RBL-2H3 cells. The optical enantiomers of cetiedil, (+) UCL 1348 and (-) UCL 1349 were tested. Although they did not show any significant effect up to 10μM, they inhibited antigen-induced β-hexosaminidase release at 100μM. The calculated IC\(_{50}\) was 29±1μM and 77±11μM for UCL 1348 and UCL 1349, respectively (Fig. 3.12). The (+) enantiomer was 2 times more potent than the (-) enantiomer against antigen-triggered secretion in RBL-2H3 cells.

Cetiedil and the UCL compounds are more potent in blocking erythrocyte K\(^+\) loss at low extracellular K\(^+\) concentration. They have lower IC\(_{50}\) at 0.1mM [K\(^+\)]\(_o\) than at 5.4mM [K\(^+\)]\(_o\) (See table 3.3) [243, 244].

Figure 3.13 shows the effect of different [K\(^+\)]\(_o\) on the dose-response curve of antigen-induced β-hexosaminidase release. As shown, in K\(^+\) free solution, the stimulated secretion was markedly (Student’s paired t-test, p<0.001) inhibited and with a rise of [K\(^+\)]\(_o\) to 0.2mM, the curve was significantly (Student’s paired t-test, p<0.01) shifted to the right. Since at low [K\(^+\)]\(_o\), the antigen-induced secretion was already markedly inhibited, it was not considered feasible to elucidate the effect of cetiedil in solutions containing different concentration of K\(^+\).

3.3.10.2 Triphenyl acetic acid ester derivatives (UCL 1495 and UCL 1617)

Increasing lipophilicity by non-polar substitutions in any part of the cetiedil molecule causes an increase in activity for the block of the IK\(_{Ca}\) channels in red blood cells [244].
Figure 3.10: Molecular structures of cetiedil and the related UCL compounds. The chiral carbon has been shown by *, this carbon (α carbon) of acetic acid is also substituted with triphenyl to increase the lipophilicity of the molecule. UCL 1495 and 1617 are α-triphenylacetic acid esters, but they are different in esteric and amide bonds.
Figure 3.11: Molecular structures of UCL 1710 and 1608 compounds. The α carbon is substituted with 9-phenyl 9-fluorenyl to increase the lipophilicity of the molecule. UCL 1710 and 1608 are different in N-ring which is homopiperidine for UCL 1608 and piperidine for UCL 1710.
Figure 3.12: Inhibitory effect of UCL 1348 and 1349 on antigen-stimulated β-hexosaminidase. (A) UCL 1349, (B) UCL 1348. After 5 min preincubation at 37°C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (for 15 min). Each point on curves represents the mean ± SEM of % net β-hexosaminidase release in four experiments. Spontaneous release was 6.5 ± 0.5% and was not altered by 100 μM of each compound. □ 0.01, ◦ 0.1, ▲ 1, ▼ 10 ng/ml DNP-HSA.
Figure 3.13: Dose-response curves of antigen-stimulated β-hexosaminidase at different [K⁺]₀. After 5 min preincubation at 37°C, cells were stimulated with DNP-HSA (for 15 min). Each point on the above curves represents the mean ± SEM of %net release in four experiments. Data have been fitted to the Hill equation and with Bmax constrained to the maximum net release for each curves. Hill coefficient (nᵢ) was 0.39±0.05. Spontaneous release was 6.5 ± 0.3% and was not affected at different [K⁺]₀. ■ 5 mM, ▼ 0.2 mM, ○ 0 mM [K⁺]₀. Student’s paired t-test * p<0.05, ** p<0.01 and *** p<0.001.
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UCL 1495 is the N-ethyl-(2-methyl-5-ethyl homopiperidine ester of triphenylacetic acid with a higher lipophilicity than cetiedil itself. It was reported to be 20 times more potent than cetiedil in blocking erythrocyte IK$_{\text{Ca}}$ channels [244].

UCL 1617 is an amide derivative of UCL 1495. UCL 1617 has a lower lipophilicity than UCL 1495 and it is 6 times less potent on IK$_{\text{Ca}}$ channel blocking in red blood cells [244].

As shown in figure 3.14 and Table 3.3, both UCL 1495 and 1617 inhibited antigen-stimulated β-hexosaminidase release from RBL-2H3 cells. The values of IC$_{50}$ for these compounds were 104±15µM and 23±1µM for UCL 1495 and 1617 respectively. Since increasing lipophilicity caused a decrease instead of an increase in the inhibitory effect of UCL 1495, these data are not consistent with those obtained from erythrocyte K$^+$ loss experiments. However, it should be noted that UCL 1617 had higher potency, indicating that the ester linkage might not be important on the secretion from RBL-2H3 cells.

3.3.10.3 9-benzyl 9-fluorenyl derivatives (UCL 1608 and UCL 1710)

The next compound tested was UCL 1608, in which the ester function is replaced by ethyne, and the substitution on α carbon is 9-benzyl 9-fluorenyl. Compared with UCL 1617, this compound has a higher lipophilicity and a lower IC$_{50}$ [1.5µM] for causing block of IK$_{\text{Ca}}$ channels in red blood cells. In view of these results, it was suggested that the ester linkage is not necessary for channel blocking activity [244].

UCL 1710, which combines features of UCL 1495 and UCL 1608, has a higher lipophilicity and a lower IC$_{50}$ (0.31µM) than UCL 1608 for inhibition of the erythrocyte K$^+$ loss induced by Ca$^{2+}$ ionophore (Table 3.3) [244].

Figure 3.15 shows the inhibitory effect of UCL 1608 and UCL 1710 on antigen-induced β-hexosaminidase release. UCL 1608 was the most potent of the tested UCL compounds with an IC$_{50}$ of 9.6±0.6µM. UCL 1710 had an IC$_{50}$ of 14±2µM which is similar to that for UCL 1617 (Table 3.3).

Figure 3.16 shows the dose-response curve for inhibition of antigen-induced β-hexosaminidase release by the UCL compounds and table 3.3 shows their characteristics and IC$_{50}$ values.
Figure 3.14: Inhibitory effect of UCL 1617 and 1495 on antigen-stimulated β-hexosaminidase release. (A) UCL 1617, (B) UCL 1495. After 5min preincubation at 37°C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (for 15min). Each point on the above curves represents the mean ± SEM of %net β-hexosaminidase release in four experiments. Spontaneous release was 7.2 ± 0.5% and was not affected by 100μM of each compound. ■ 0.01, ○ 0.1, ▲ 1, ▼ 10 ng/ml DNP-HSA.
Figure 3.15: Inhibitory effect of UCL 1710 and 1608 on the antigen-stimulated β-hexosaminidase release. (A) UCL 1710, (B) UCL 1608. After 5min preincubation at 37°C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (for 15min). Each point on the above curves represents the mean ± SEM of %net β-hexosaminidase release in four experiments. Spontaneous release was 5.2 ± 0.3% and was not affected by 100μM of each compound. ■ 0.01, ○ 0.1, △ 1, ▼ 10 ng/ml DNP-HSA.
Figure 3.16: Concentration-response curves of the UCL compounds for the inhibition of antigen-stimulated β-hexosaminidase release. After 5min preincubation at 37°C, UCL compounds were added at different concentrations and after 15 min cells were then challenged with antigen (10ng/ml DNP-HSA for 15min). Each point on the curves represents the mean ± SEM of %net β-hexosaminidase release in four experiments. Data have been fitted to the Hill equation and with B_max constrained to 100%. Hill coefficient (n_H) was 0.91±0.01. Spontaneous and antigen-induced β-hexosaminidase release were 6.6 ± 0.3% and 49.7 ± 1.2%, respectively. ■ UCL 1348, ● UCL 1349, ▲ UCL1495, ▼ UCL 1608, ◆ UCL 1710, ■ UCL 1617.
Table 3.3: Characteristics of cetiedil and UCL compounds and their blocking effects on IK_{ca} channels in erythrocytes [244] and on antigen-induced β-hexosaminidase release from RBL-2H3 cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>ΔlogP′</th>
<th>α-substitution</th>
<th>N-ring</th>
<th>IC_{50} 0.1mM [K]^+</th>
<th>IC_{50} 5.4mM [K]^+</th>
<th>IC_{50} secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cetiedil</td>
<td>4.89</td>
<td>Original</td>
<td>HP</td>
<td>26μM</td>
<td>100μM</td>
<td>84.3 ± 10.5μM</td>
</tr>
<tr>
<td>UCL 1348</td>
<td>4.89</td>
<td>Original</td>
<td>HP</td>
<td>25μM</td>
<td>59μM</td>
<td>29.8 ± 1.1μM</td>
</tr>
<tr>
<td>UCL 1349</td>
<td>4.89</td>
<td>Original</td>
<td>HP</td>
<td>24μM</td>
<td>54μM</td>
<td>77.5 ± 11.8μM</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>7.53</td>
<td>Triphenyl</td>
<td>P</td>
<td>1.2μM</td>
<td>30μM</td>
<td>104.6 ± 14.7μM</td>
</tr>
<tr>
<td>UCL 1617</td>
<td>6.34</td>
<td>Triphenyl</td>
<td>P</td>
<td>6.6μM</td>
<td>n.d.</td>
<td>23.4 ± 1.4μM</td>
</tr>
<tr>
<td>UCL 1710</td>
<td>9.5</td>
<td>9-benzyl, 9-fluorenyl</td>
<td>P</td>
<td>0.3μM</td>
<td>3μM</td>
<td>14.4 ± 2.2μM</td>
</tr>
<tr>
<td>UCL 1608</td>
<td>8.5</td>
<td>9-benzyl, 9-fluorenyl</td>
<td>HP</td>
<td>1.5μM</td>
<td>n.d.</td>
<td>9.6 ± 0.6μM</td>
</tr>
</tbody>
</table>

a) ΔlogP = predicted log octanol/water partition coefficient.
b) Substitution at α carbon of acetic acid ester
c) HP homopiperidine and P piperidine.
3.3.11 Effects of nitrendipine and nicardipine

The effect of some other potassium channel blockers such as nitrendipine and nicardipine were also studied. Nitrendipine is a blocker of the L-type voltage-gated calcium channel, of the outwardly rectifying potassium channel and of the Ca\(^{2+}\)-activated K\(^{+}\) channels in red blood cells. The experiments were done in a nearly dark room to avoid the effect of light on the highly photosensitive compound. Nitrendipine (5\(\mu\)M) had no effect on the antigen-induced secretion from RBL-2H3 cells. Nicardipine, an inhibitor of outward rectifier K\(^{+}\) channels \[245\] as well as L-type calcium channels, at the quoted IC\(_{50}\) values, had no significant effect on \(\beta\)-hexosaminidase release. Table 3.4 shows the effect of nitrendipine and nicardipine (10\(\mu\)M) on antigen-induced \(\beta\)-hexosaminidase release.

<table>
<thead>
<tr>
<th>Blocker</th>
<th>(K^{+}) channel blocker</th>
<th>(\beta)-hexosaminidase release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrendipine 10(\mu)M</td>
<td>Transient outward rectifier</td>
<td>0.7(\mu)M Cardiac myocytes</td>
</tr>
<tr>
<td>Nicardipine 10(\mu)M</td>
<td>Transient outward rectifier</td>
<td>1.5(\mu)M Cardiac myocytes</td>
</tr>
</tbody>
</table>

Table 3.4: Effect of nitrendipine and nicardipine on antigen-induced \(\beta\)-hexosaminidase release from passively sensitised RBL-2H3 cells. After 5min preincubation at 37\(^\circ\)C, cells were treated with either nitrendipine or nicardipine. DNP-HSA [10ng/ml] was then added for a further 15min. Data are represented as mean ± SEM in four experiments. Student's paired t-test, * p<0.05.
3.3.12 Effect of CsCl on antigen-induced secretion

Cesium is a non-specific K\(^+\) channel blocker at concentrations of 5-20mM. As shown in figure 3.17, CsCl, at concentrations lower than 10mM had little or no effect on the antigen-stimulated secretion in RBL-2H3 cells. However, at a concentration of 20mM, CsCl markedly inhibited the cell response (IC\(_{50}\) =20mM against DNP-HSA 1ng/ml).

3.3.13 Inhibitory effects of tetraethylammonium (TEA)

Tetra-\(n\)-ethylammonium has been recognized as a tool for separating K\(^+\) currents from those generated by other ions. TEA blocks most classes of K\(^+\) channels e.g. SK\(_{Ca}\), BK\(_{Ca}\) and some IK\(_{Ca}\) channels as well as KV and K\(_{ATP}\) with an IC\(_{50}\) of 0.3-10mM [246, 247, 248]. As shown in figure 3.18, TEA had no significant effect on antigen-triggered \(\beta\)-hexosaminidase release at concentrations of up to 20mM.

In addition to the small effect of Ba\(^{2+}\) (<1mM), the lack of effect of TEA at 20mM on antigen-stimulated secretion, provides further evidence that IR K\(^+\) channels are not involved in the signal transduction mechanisms of RBL-2H3 cell stimulation.

3.3.14 Inhibitory effects of 4-aminopyridine

4-aminopyridine (4-AP) is more selective and usually more potent than TEA in its K\(^+\) channel blocking activity. 4-AP can block voltage-dependent K\(^+\) channels such as the delayed rectifier and A current [249, 250]. However, with very few exceptions, 4-AP has been shown to be without effect on Ca\(^{2+}\)-activated K\(^+\) channels. Interestingly, 4-AP inhibited antigen-triggered \(\beta\)-hexosaminidase release with an IC\(_{50}\) of 3.6±0.06mM (Fig. 3.19). To rule out the possibilities that the inhibitory effect was due to a pH change in the external buffer, and since secretion is pH-dependent, the experiment was repeated by dissolving 4-AP in the buffer and adjusting pH to 7.4. The same result was obtained.
Figure 3.17: Inhibitory effect of CsCl on antigen-stimulated β-hexosaminidase release. After 5 min preincubation at 37°C, CsCl was added at different concentrations and after 15 min cells were then challenged with antigen (for 15 min). Each point on the above curves represents the mean ± SEM of %net β-hexosaminidase release in 4-6 experiments. Spontaneous release was 6.4±0.5% and was not altered by 20mM CsCl. ■ without, ○ 2mM, △ 5mM, ▽ 10mM, ♦ 20mM CsCl. Student’s paired t-test * p<0.05.
Figure 3.18: Effect of TEA on antigen-stimulated β-hexosaminidase release. After 5 min preincubation at 37°C, TEA was added at different concentrations and 15 min later, cells were challenged with antigen (1 ng/ml DNP-HSA) for another 15 min. Each point on curves represents the mean ± SEM of %net β-hexosaminidase release in 4-6 experiments. Spontaneous release was 7.7 ± 0.6 and was not affected by 20 mM TEA. ■ 1, ○ 10 ng/ml DNP-HSA.
Figure 3.19: Inhibitory effect of 4-AP on antigen-stimulated β-hexosaminidase release. After 5 min preincubation at 37°C, 4-AP was added at different concentrations and after 15 min cells were then challenged with antigen 10 ng/ml DNP-HSA for another 15 min. Each point on the curves represents the mean ± SEM of %net β-hexosaminidase release in more than four experiments. Data have been fitted to the Hill equation and with B_{max} constrained to 100%. Hill coefficient (n_H) was 3.8 ± 0.3. Spontaneous release was 5.2 ± 0.4% and was not affected by 5 mM 4-AP.
3.3.15 Effects of other potassium channel blockers on RBL-2H3 secretion

Glibenclamide, an ATP-sensitive $K^+$ channel blocker, at concentrations that block this channel ($<1\mu M$) did not affect $\beta$-hexosaminidase release. However, at much higher concentrations, it inhibited the release dose-dependently (Table 3.5).

Clotrimazole is known to be an $IK_{Ca}$ channel blocker in red blood cells with an $IC_{50}$ of 0.1$\mu M$ [251]. Clotrimazole inhibited secretion from RBL-2H3 cells stimulated by antigen but only at a much higher concentration (with an $IC_{50}$ of about 70$\mu M$) than that required for blocking the $IK_{Ca}$ channel. This result is not consistent with the results from the erythrocyte $IK_{Ca}$ channels, suggesting that a different channel subtype is involved.

Likewise, UK 69614 (a selective blocker of the delayed rectifier potassium channel in ventricular muscles and the Purkinje fiber), amiodarone (a potent inhibitor of inwardly rectifying $K^+$ channel in the heart and also possibly of outward rectifier $K^+$ channels as well as $Na^+$ channels in the heart) and propafenone (an inhibitor of different $K^+$ channels as well as voltage-gated $Na^+$ channels) did not have significant effects on the secretion from RBL-2H3 cells mediated by antigen (Table 3.5). Dequalinium, a $SK_{Ca}$ channel blocker [252], also had no effect on antigen-induced secretion, and this is consistent with the lack of inhibitory effect of apamin. 9-aminoacridine, a blocker of $K_{ATP}$ in pancreatic $\beta$-cells and $SK_{Ca}$ in guinea pig hepatocytes with an $IC_{50}$ of 70$\mu M$, had no effect on antigen-induced $\beta$-hexosaminidase release [198, 246].

3.3.16 Effect of $K_{ATP}$ channel openers on RBL-2H3 degranulation

Two potassium channel openers, cromakalim and diazoxide, selective ATP-sensitive $K^+$ channel openers, were also tested on RBL-2H3 cells to evaluate their effects on antigen-stimulated secretion. Neither of them affected the release. Table 3.6 shows that these agents, at concentrations much higher than their $IC_{50}$ on pancreatic $\beta$-cells and smooth muscles function, did not have any significant effect on antigen-induced $\beta$-hexosaminidase release.
### Table 3.5: Effect of some potassium channel blockers on antigen-induced β-hexosaminidase release from passively sensitised RBL-2H3 cells.

After 5min preincubation at 37°C, cells were treated with the potassium channel blocker for 15min. DNP-HSA [10ng/ml] was then added for a further 15min. Data are presented as mean ± SEM of %net β-hexosaminidase release in four experiments. Spontaneous release was 7.2 ± 0.4% and was not affected by tested compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K^+$ channel</th>
<th>Tissue</th>
<th>$IC_{50}$ for $K^+$ channel blocking</th>
<th>Control</th>
<th>With Blocker</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glibenclamide [20μM]</td>
<td>$K_{ATP}$</td>
<td>β-cells</td>
<td>4-22nM</td>
<td>57.0 ± 1.8</td>
<td>53.7 ± 2.4</td>
</tr>
<tr>
<td>UK 69614 [10μM]</td>
<td>$K_V$</td>
<td>ventricular muscles &amp; Purkinje fiber</td>
<td>0.3-1μM</td>
<td>60.8 ± 2.8</td>
<td>57.5 ± 4.7</td>
</tr>
<tr>
<td>Clotrimazole [10μM]</td>
<td>$I_{KCa}$</td>
<td>red blood cells</td>
<td>1-24μM</td>
<td>61.3 ± 4.1</td>
<td>55.8 ± 3.8</td>
</tr>
<tr>
<td>Amisodarone [50μM]</td>
<td>$K_R$</td>
<td>ventricular cells</td>
<td>20μM</td>
<td>64.2 ± 2.1</td>
<td>60.5 ± 2.5</td>
</tr>
<tr>
<td>Dequalinium [50μM]</td>
<td>$SK_{Ca}$</td>
<td>skeletal muscles sympathetic neuron</td>
<td>1-1.5μM</td>
<td>55.5 ± 2.3</td>
<td>52.9 ± 3.6</td>
</tr>
<tr>
<td>9-Aminoacridine [60μM]</td>
<td>$SK_{Ca}$</td>
<td>hepatocytes</td>
<td>70μM</td>
<td>54.3 ± 4.9</td>
<td>55.0 ± 5.2</td>
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<td></td>
<td>$K_{ATP}$</td>
<td>B cells</td>
<td>100μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Propafenone [10μM]</td>
<td>$K_C$, $K_{IR}$</td>
<td>Atrial myocytes</td>
<td>5-63μM</td>
<td>51.4 ± 1.6</td>
<td>50.6 ± 5.5</td>
</tr>
</tbody>
</table>
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Antigen-induced β-hexosaminidase release

<table>
<thead>
<tr>
<th>Compounds</th>
<th>K⁺ channel</th>
<th>Tissue</th>
<th>IC₅₀ for K⁺ channel blocking</th>
<th>Control</th>
<th>With Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazoxide [100µM]</td>
<td>K₁ATP</td>
<td>β-cells</td>
<td>20µM</td>
<td>64.3 ± 2.6</td>
<td>61.3 ± 2.0</td>
</tr>
<tr>
<td>Cromakalim [50µM]</td>
<td>Different K⁺ channel</td>
<td>smooth muscles</td>
<td>0.1-1µM</td>
<td>64.3 ± 2.6</td>
<td>64.7 ± 3.2</td>
</tr>
</tbody>
</table>

Table 3.6: Effect of two potassium channel openers on the antigen-induced B-hexosaminidase release from passively sensitised RBL-2H3 cells. After 5min preincubation at 37°C, cells were treated with the potassium channel opener for 15min. DNP-HSA [10ng/ml] was then added for a further 15min. Data are presented as mean ± SEM % net β-hexosaminidase release in four experiments.

3.4 Discussion

3.4.1 Secretion

In this study, β-hexosaminidase release has been used as a marker of stimulated exocytosis or secretion. β-hexosaminidase release was found to be well-correlated with histamine release in RBL-2H3 cells stimulated either by antigen or by the Ca²⁺ ionophore A23187 (Fig. 3.1 and 3.3). Figure 3.1B also shows that the presence of 0.05% BSA in the modified Tyrode’s buffer improves the antigen response, possibly by decreasing the adsorptive loss of antigen (DNP-HSA) due to non-specific binding. DNP-HSA did not stimulate non-sensitised RBL-2H3 cells to degranulate and it had no significant effect on the spontaneous β-hexosaminidase release from passively sensitised RBL-2H3 cells in the absence of extracellular Ca²⁺.

Mohr and Fewtrell have shown that the complete removal of [K⁺]ₒ inhibited both secretion and Ca²⁺ influx in RBL-2H3 cells [112]. The cells are slightly depolarised in K⁺ free solution and the antigen-induced secretion cannot be restored by increasing [Ca²⁺]ₒ. However, in the cells depolarised in high K⁺ solution, the inhibition of secretion and Ca²⁺ influx can be abolished by increasing [Ca²⁺]ₒ [112]. In the present work, antigen-stimulated β-hexosaminidase release was greatly inhibited in K⁺-free buffer indicating that the response is dependent on the [K⁺]ₒ. However, the mechanism involved has not been explored.
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The Ca\(^{2+}\) ionophore, A23187, also induced β-hexosaminidase release from RBL-2H3 cells by bypassing the first stages of FcεRI crosslinking-induced activation. A23187 is an electrically neutral exchange diffusion carrier, which increases [Ca\(^{2+}\)]\(_i\) [253]. However, the mode of action of Ca\(^{2+}\) ionophores may be more complicated, because the inhibition of A23187-induced secretion by ATP-depletion and depolarisation is hard to explain in such terms. Alternatively, it can be proposed that A23187 releases Ca\(^{2+}\) from the internal stores and the store depletion results in the opening of CRAC channels for the sustained influx of Ca\(^{2+}\) [147]. This hypothesis is consistent with the inhibition of A23187-induced secretion by ATP-depletion and depolarisation, because in both conditions, though the initial rise in [Ca\(^{2+}\)]\(_i\) can be observed, the sustained Ca\(^{2+}\) influx is abolished [96, 109, 112].

3.4.2 Effect of K\(^+\) channel modulators on antigen-induced secretion

In order to investigate the role of K\(^+\) channels in RBL-2H3 cell activation, cells were incubated with different concentrations of K\(^+\) channel modulators and then challenged with antigen. Table 3.7 shows the compounds tested and their IC\(_{50}\) for K\(^+\) channel blockade in other tissues together with the IC\(_{20}\) and IC\(_{50}\) measured for the effect on antigen-stimulated β-hexosaminidase release.

3.4.2.1 Effect of Ba\(^{2+}\) on the stimulated β-hexosaminidase release from RBL-2H3 cells

Barium (2mM) reduces antigen-induced 5HT-release from RBL-2H3 cells by 50% [53]. It has been proposed that Ba\(^{2+}\) blocks the outward rectifier potassium channels and this, in turn, inhibits the maintenance of a sufficient electrochemical driving force for Ca\(^{2+}\) influx [53]. In order to investigate further this hypothesis, the effect of Ba\(^{2+}\) on antigen- and A23187-induced β-hexosaminidase release was studied. Ba\(^{2+}\) inhibited the antigen-induced β-hexosaminidase release dose-dependently with an IC\(_{50}\) of about 2mM (Table 3.1 and Fig. 3.5). Raising the extracellular calcium concentration (from 1.8mM to 10mM) increased the inhibitory effect of Ba\(^{2+}\) slightly (Fig. 3.6).

Interestingly, Ba\(^{2+}\) more potently inhibited cell activation by A23187 than by antigen. The measured values of IC\(_{50}\) for Ba\(^{2+}\) in A23187- and antigen-induced secretion are 1.2mM and 2.5mM respectively. Moreover, Ba\(^{2+}\) has a higher inhibitory effect on the...
response to low doses of A23187 (IC$_{50}$ = 0.156±0.005mM for 150nM A23187), while the inhibitory effect of Ba$^{2+}$ for all doses of antigen was nearly the same (IC$_{50}$ = 2mM).

The higher potency of Ba$^{2+}$ against A23187-stimulated secretion is likely to be due to inhibition of $I_{CRAC}$ by Ba$^{2+}$ [147]. A23187 can cross the cell membrane and deplete the Ca$^{2+}$ stores and thereby activate the CRAC channels. It has been shown that Ba$^{2+}$ inhibits CRAC channels in rat peritoneal mast cells (a permeant blocker) [147]. However, antigen stimulates other parts of the signal transduction mechanism and this may explain the difference in sensitivity of the two types of release to inhibition by Ba$^{2+}$.

Moreover, Ba$^{2+}$ at a concentration of 100µM can completely inhibit inward rectifier K$^+$ channels in RBL-2H3 cells [125, 208, 209, 211]. However, Ba$^{2+}$ at concentrations lower than 1mM had no effect on antigen-induced β-hexosaminidase release. These results indicate that the inhibitory effect of Ba$^{2+}$ is not likely to be the result of IR K$^+$ channel blocking. Ba$^{2+}$ is also much less effective in blocking the OR K$^+$ current induced by GTPyS, so that even at 20mM, it did not completely inhibit the OR K$^+$ current [208].

In summary, it seems that the inhibitory effect of Ba$^{2+}$ may be due to a direct effect on the CRAC channel or on Ca$^{2+}$ influx, rather a consequence of blocking of IR or OR K$^+$ channels in RBL-2H3 cells.

3.4.2.2 Inhibitory effect of quinidine on RBL-2H3 cell activation

Quinidine, a non-selective K$^+$ channel blocker, has been shown to inhibit 5-HT release, the repolarisation phase and $^{86}$Rb$^+$-efflux in antigen-stimulated RBL-2H3 cells [53, 161]. The inhibitory effect of quinidine on 5HT release was 50% at 30µM and about 70% at 70µM. The outward rectifier (OR) K$^+$ current induced by GTPyS is also blocked by more than 50% by 50µM quinidine [208, 210]. However, it does not have any effect on the inward rectifier (IR) K$^+$ channel even at a concentration of 100µM [208].

In the present study, antigen-induced β-hexosaminidase release was inhibited by quinidine dose-dependently, with an IC$_{50}$ of 45µM and 83µM for stimulation by 0.01ng/ml and 0.1ng/ml DNP-HSA, respectively. Quinidine has a higher inhibitory effect against lower antigen concentrations. As already suggested, Ca$^{2+}$ influx induced by higher concentrations of antigen may be too high to be inhibited by K$^+$ channel blockade or inhibition of the repolarisation phase. However, antigen, at lower concentrations, does not cause maximum Ca$^{2+}$ influx, possibly allowing a great action of quinidine.
Quinidine did not have any significant effect on calcium ionophore-activated secretion. Raising the extracellular calcium concentration (from 1.8mM to 10mM) decreased the inhibitory effect of quinidine. These results are compatible with a potassium channel blocking effect of quinidine on the antigen response, since as the extracellular calcium concentration is increased, the necessity of maintaining an electrochemical driving force is diminished. This is consistent with the fact that the inhibitory effect of depolarising agents (e.g. high K⁺ solution) on RBL-2H3 activation and secretion can be overcome by increasing the extracellular calcium concentration.

Quinidine was more potent in Tyrode's buffer containing 2.7mM KCl as compared with 5mM KCl. Thus, the potency of quinidine may be increased by lowering [K⁺]o. This is consistent with the effect of quinidine on IKCa in human red blood cells [283].

3.4.2.3 Effect of charybdotoxin

Since quinidine is known to be a Ca²⁺-activated K⁺ channel blocker, the effect of charybdotoxin, a blocker of BKCa channels, on antigen-stimulated β-hexosaminidase release was evaluated. Charybdotoxin inhibited the stimulated secretion dose-dependently with an IC₅₀ of 61±4nM (stimulation by 1ng/ml DNP-HSA) (Fig. 3.8 and Table 3.2). Since ChTX also blocks IKCa and KV1.3, selective blockers of these channels were used to elucidate the effect of ChTX. Iberiotoxin, a selective inhibitor of BKCa, had no significant effect on stimulated degranulation at 100nM (Table 3.2). These results are also consistent with the lack of inhibitory effect of TEA, because TEA can also block BKCa channels in the low mM concentration range. Margatoxin, a specific blocker of voltage-activated KV1.3 channels, at a concentration of 100nM also did not affect the antigen-induced response in RBL-2H3 cells (Table 3.2). These results indicate that BKCa and KV1.3 are not likely to be involved in the antigen-induced RBL-2H3 cell activation.

Since charybdotoxin, but not TEA, inhibited the antigen-stimulated secretion in RBL-2H3 cells, it does not seem that the OR K⁺ channels are similar to BKCa channels. However, the data presented so far suggest that the OR K⁺ channels resemble IKCa channels.

3.4.2.4 Effect of cetiedil

Cetiedil has been reported to inhibit the IKCa channels in erythrocytes [254]. Moreover, it is capable of blocking the following channels: the volume-sensitive K⁺
channels in lymphocytes and hepatocytes [241, 255], G-protein linked K⁺ channels in atrial myocytes and submucosal plexius neurons [242] and the K⁺ channels activated by levromakalim in smooth and skeletal muscles [256].

Cetiedil inhibited antigen-induced β-hexosaminidase release from RBL-2H3 cells with an IC₅₀ of 84±10μM (Fig. 3.9). It has been reported that quinidine and cetiedil are more potent against IKᵥCa channel in low [K⁺]₀. The IC₅₀ of quinidine for blocking the erythrocyte IKᵥCa channels in K⁺ free and 5mM [K⁺]₀ are 5μM and 98μM respectively. However, since antigen-induced β-hexosaminidase release is greatly diminished in the absence of [K⁺]₀ and at low [K⁺]₀ (Fig.3.12), the effects of cetiedil and UCL compounds have not been evaluated at different [K⁺]₀.

3.4.2.5 Effect of cetiedil-related compounds

The effects of some cetiedil-related compounds, synthesised in the Dept. of Chemistry at the University College London (UCL compounds), on secretion from RBL-2H3 cells were also investigated. Like ChTX and cetiedil itself, cetiedil-related compounds inhibit IKᵥCa channels in red blood cells. Since ChTX had an inhibitory effect on the stimulated secretion from RBL-2H3 cells, it was useful to assess the effect of cetiedil and the UCL compounds on antigen-induced β-hexosaminidase release. Cetiedil and the UCL compounds were able to inhibit antigen-induced activation of RBL-2H3 cells (see Table 3.3). The criterion of selection of these UCL compounds was their ability to block the IKᵥCa channel in red blood cells. Therefore, those most potent in this respect were chosen because the inhibition of antigen-induced β-hexosaminidase release by ChTX and cetiedil (but not by TEA, IbTX and MgTX) suggested the possible involvement of channels might resemble the IKᵥCa channels in red blood cells.

Interestingly, the UCL compounds had an inhibitory effect, some of them with higher potency than cetiedil. It has been shown that the cetiedil enantiomers are equipotent in blocking IKᵥCa in rabbit red blood cells. However, (+) cetiedil seems to be more potent (by 2.6 times) than (-) cetiedil in inhibiting the volume-sensitive K⁺ channel in liver cells [243]. Similarly, the (+) enantiomer was 2 times more potent than the (-) enantiomer against antigen-triggered secretion in RBL-2H3 cells. However, these results are not consistent with the equi-activity of the cetiedil enantiomers in blocking the erythrocyte IKᵥCa.
All the UCL compounds inhibited the antigen-stimulated β-hexosaminidase release and their potency was as follows: UCL1608 > 1710 > 1617 > 1348 > 1349 > 1495 (Table 3.3).

3.4.2.6 Effect of nitrendipine and nicardipine

It has been reported that nitrendipine inhibits antigen-induced 5-HT release from RBL-2H3 cells with an IC<sub>50</sub> of 1.3μM [210]. However, in the present experiments this result could not be reproduced. Nitrendipine and nicardipine had very little effect at 10μM, whereas the reported IC<sub>50</sub> for their block of OR K<sup>+</sup> channels is about 0.7 and 1.5μM, respectively. It seems that the possible K<sup>+</sup> channel involved, under the present experimental conditions, may not be sensitive to nitrendipine or nicardipine.

3.4.2.7 Voltage-dependent K<sup>+</sup> channel blockers

UK 66914, a specific blocker of delayed outward rectifier and inward rectifier K<sup>+</sup> channels in isolated canine ventricular muscle and Purkinje fibres [257], did not affect spontaneous or antigen-stimulated β-hexosaminidase release (Table 3.5). TEA, a non-specific K<sup>+</sup> channel blocker, which mainly inhibits voltage-sensitive K<sup>+</sup> channels also had no effect on stimulated secretion (Fig. 3.18). TEA has been reported to inhibit the IR K<sup>+</sup> channels in RBL-2H3 cells with an IC<sub>50</sub> of 15mM [208, 209]. Again, the lack of inhibitory effect on the stimulated secretion by TEA counts against any role for IR K<sup>+</sup> channels in RBL-2H3 cell activation.

4-aminopyridine, another blocker of voltage-sensitive K<sup>+</sup> channels, caused an inhibition of antigen-induced degranulation by about 85% at a concentration of 5mM (Fig. 3.19). This inhibitory effect needs to be examined further by measuring, for example, the effect of 4-AP on the antigen-induced membrane potential changes and the Rb<sup>+</sup>-efflux in RBL-2H3 cells.

Taking the evidence as a whole, the possible K<sup>+</sup> channel involved in RBL-2H3 cell activation does not appear to be voltage-sensitive.

3.4.2.8 Other Ca<sup>2+</sup>-activated K<sup>+</sup> channel blockers

It has been shown that in RBL-2H3 cells quinidine inhibits antigen-induced 5-HT release and the repolarisation phase, as well as <sup>86</sup>Rb<sup>+</sup>-efflux [53, 161]. Since quinidine
mainly blocks Ca\(^{2+}\)-activated K\(^+\) channels, it was proposed that the involved K\(^+\) channels in these RBL-2H3 cell responses might be Ca\(^{2+}\)-activated. In the present study, other specific Ca\(^{2+}\)-activated K\(^+\) channel blockers were also examined.

Apamin, a blocker of SK\(_{Ca}\) channels, had no effect on antigen-induced \(\beta\)-hexosaminidase release, even at a concentration of 1\(\mu\)M (Table 3.2). Dequalinium, a SK\(_{Ca}\) channel blocker, was also ineffective even at a very high concentration, 50\(\mu\)M (Table 3.5).

Clotrimazole, which has an IK\(_{Ca}\) channel blocking activity, had no significant effect on stimulated \(\beta\)-hexosaminidase release at a concentration that completely blocks the related K\(^+\) channels in red blood cells. The lack of inhibition of antigen-induced secretion by clotrimazole is not consistent with the effects of ChTX and cetiedil, suggesting a difference in the pharmacological properties of the relevant K\(^+\) channels in RBL-2H3 and red blood cells.

3.4.2.9 Inward rectifier K\(^+\) channel blockers

Inward rectifier K\(^+\) channels have a major role in maintaining the resting membrane potential of RBL-2H3 cells. Ba\(^{2+}\) is a known inhibitor of the IR K\(^+\) channel in RBL-2H3 cells. Nevertheless, though it can completely inhibit IR K\(^+\) channels at 100\(\mu\)M, it had little effect on stimulated secretion in RBL-2H3 cells, at that concentration and even up to 1mM. However, Ba\(^{2+}\) inhibited antigen-induced secretion with an IC\(_{50}\) of about 2mM. The effects of Ba\(^{2+}\) on the RBL-2H3 cell responses will be discussed later in section 7.4.

CsCl also blocks the IR K\(^+\) channel in RBL-2H3 cells. It inhibits this current completely at 1mM, but more than 10mM was needed to have a significant effect on antigen-stimulated \(\beta\)-hexosaminidase release. Cs\(^+\) blocks IR K\(^+\) channels (at 2mM) [209, 211] and OR K\(^+\) channels (at 20mM) in RBL-2H3 cells [218]. In view of the inhibition of IR K\(^+\) current by 2mM Cs\(^+\) and the lack of effect of Cs\(^+\) on antigen-induced secretion these results provided further evidence that an IR K\(^+\) current is not involved in RBL-2H3 cell activation.

TEA has been reported to inhibit IR K\(^+\) channels in RBL-2H3 cells at a concentration between 10-15mM [208, 209]. However, as already mentioned, it did not inhibit antigen-induced degranulation. Since charybdotoxin, but not TEA, inhibited antigen-stimulated secretion in RBL-2H3 cells, it is unlikely that the possible K\(^+\) channels
are similar to BK$_{Ca}$ channels. However, the data presented so far suggest that the possible K$^+$ channels resemble IK$_{Ca}$ channels.

Amiodarone, an inhibitor of the IR K$^+$ channel in the heart [258], was tested and it did not have any inhibitory effect on antigen-mediated activation of RBL-2H3 cells.

Taken together, these results indicate that IR K$^+$ channels do not play an important role in the antigen-induced signal transduction mechanisms of RBL-2H3 cells.

3.4.2.10 ATP-sensitive K$^+$ channel blocker

Glibenclamide, a specific blocker of ATP-sensitive K$^+$ channels in β-cells of the pancreas and skeletal muscle, even at concentration of 10μM, had no significant effect on antigen-induced β-hexosaminidase release. Its reported IC$_{50}$ for ATP-sensitive K$^+$ channel blocking is 4-27nM. Therefore, K$_{ATP}$ channels do not seem to be involved in antigen-activated secretion in RBL-2H3 cells.

3.4.2.11 K$_{ATP}$ channel openers

Cromakalim, a non-specific K$^+$ channel opener, and diazoxide, a selective ATP-sensitive K$^+$ channel opener, did not induce any significant inhibitory effect on antigen-induced β-hexosaminidase release. Thus, it seems that the possible K$^+$ channel involved in antigen-stimulated RBL-2H3 cells is not sensitive to cromakalim or diazoxide.

To summarise, the inhibitory effect of quinidine, charybdotoxin, cetiedil and the UCL compounds may suggest that the potassium channels possibly involved in antigen-induced activation of RBL-2H3 cells may resemble the IK$_{Ca}$ channels in red blood cells.
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Table 3.7: The K channel modulators tested, their IC₅₀ on K⁺ channels and the measured IC₂₀ & IC₅₀ on antigen-stimulated β-hexosaminidase release in RBL-2H3.

<table>
<thead>
<tr>
<th>No</th>
<th>Name</th>
<th>Type of channel</th>
<th>IC₅₀</th>
<th>Inhibitory effect on RBL secretion</th>
<th>IC₂₀</th>
<th>IC₅₀</th>
<th>References*</th>
</tr>
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<tr>
<td>1</td>
<td>Quinidine</td>
<td>different K⁺ channels</td>
<td>15-400µM</td>
<td>SR=10µM</td>
<td>ER=15µM</td>
<td>ER=45µM</td>
<td>246, 259, 260</td>
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<td></td>
<td></td>
<td>Na⁺ channels</td>
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</tr>
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<td></td>
<td>Voltage-dependent Ca²⁺ channel</td>
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<td>2</td>
<td>BaCl₂</td>
<td>IR K⁺ channels</td>
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<td>SR=1mM</td>
<td>ER=1.5mM</td>
<td>ER=6mM</td>
<td>208</td>
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<td>5nM</td>
<td>&gt;10nM</td>
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<td>209, 211, 218</td>
</tr>
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<td></td>
<td>OR K⁺ channels</td>
<td>20nM</td>
<td>&gt;20nM</td>
<td></td>
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</table>

*-*Serotonin release  †-β-hexosaminidase (enzyme) release  ‖-Not tested  NS- No Significant effect  ND- not determined

*These references are related to the effect of K⁺ channel modulators and their IC₅₀ in other tissues.
Chapter 4 The antigen-induced membrane potential changes in RBL-2H3 cells

4.1 Introduction

The high electrical resistance of the cell membrane and the asymmetrical distribution of ions (notably Na\(^+\) and K\(^+\)) between the cell interior and its environment, result in an electrical potential difference across the cell membrane called the resting membrane potential. This is an essential part of the property of excitability in nerve and muscle cells and plays a key role in many other cell and tissue functions (e.g. absorption and secretion). Ions can cross the cell membrane either by simple diffusion transport through aqueous channels or via carrier-mediated processes. Under physiological circumstances, the membrane potential is an electrical force that may either assist or impede a given ion’s passage across the membrane, depending on the direction of movement and the sign of its charge. In other words, in calculating the transmembrane driving force for any ion, the membrane potential must be taken into account.

In excitable cells, an action potential is created by an appropriate stimulus via the opening of voltage-gated Na\(^+\) channels. In some non-excitable cells, some non-electrical stimuli cause membrane potential changes. For instance, β-cells are depolarised in response to a high concentration of extracellular glucose and this, in turn, opens voltage-activated Ca\(^{2+}\) channels.

The resting membrane potential of RBL-2H3 cells has been reported to be between -60 to -90mV [57, 125, 218, 271]. Antigen-stimulated changes in plasma membrane potential in these cells were first reported by Kanner and Metzger who used
the tetraphenylphosphonium ion (TPP⁺) equilibration method to measure membrane potential [57]. Since the distribution of TPP⁺, a lipophilic cation, between the cells and the medium is related to the membrane potential, the electrical potential can be determined by monitoring the concentration of [³H]TPP⁺ taken up. This method has two disadvantages, (1) the slow equilibration rate of TPP⁺ ion and (2) its responsiveness to mitochondrial membrane potential.

To overcome the disadvantages of the TPP⁺ method, bis-oxonol, a potential-sensitive fluorescent dye, has been used [53, 111]. This negatively charged probe does not have the problems associated with TPP⁺ because: firstly, its negative charge prevents significant accumulation in mitochondria which also causes lack of its responsiveness to mitochondrial depolarisation; and secondly, the dye rapidly equilibrates within the cells. Bis-oxonol fluorescence does not change as a result of mitochondrial depolarisation induced by rotenone (0.1 μM), a mitochondrial electron transport chain inhibitor, or in the absence of glucose by oligomycin (1.2 μM), an inhibitor of oxidative phosphorylation [111]. Thus, bis-oxonol can directly monitor membrane potential changes.

Changes in transmembrane electrical potential cause alterations in the intensity of fluorescence of bis-oxonol. Bis-oxonol follows an on/off mechanism, so that a rapid (milliseconds) response to quick potential changes occurs, as the probe molecules move between the hydrocarbon environment of the membrane and a more polar region that may be in the water near the membrane or just on the surface of the membrane [272].

In contrast to rat peritoneal mast cells, the resting membrane potential in RBL-2H3 cells is maintained by K⁺ permeability but not by Na⁺/K⁺ ATPase, as it is not affected by ouabain, a Na⁺/K⁺ ATPase pump inhibitor [111, 225]. Moreover, antigen stimulation of RBL-2H3 cells causes a rapid depolarisation of the cells and this depolarisation has the following features:

1. Its dose-response curve is parallel to that for secretion [53].
2. Its temperature dependency is similar to that reported for secretion and for a rise in [Ca²⁺] [53].
3. It can be prevented by La³⁺, or by addition of monovalent conjugated hapten (DNP-Lys)¹, or by prior depolarisation in high K⁺ or [ATP] depletion [111].
4. The replacement of external Na⁺ with choline does not affect the antigen-induced depolarisation.

¹ The antigen-stimulated Ca²⁺ uptake and degranulation (i.e. by a polyvalent hapten conjugate such as DNP-BSA) can be halted immediately after the addition of a monovalent hapten conjugate (i.e. DNP-Lys) [52, 56, 57].
5. Both the depolarisation and a rise in \([\text{Ca}^{2+}]\), induced by antigen are not affected by PTX pretreatment.

6. The antigen-induced membrane potential changes, IP hydrolysis and the rise in \([\text{Ca}^{2+}]\), are also inhibited by pretreatment with 50nM PMA [22, 53].

On the basis of this evidence, the depolarisation seems to be the result of antigen-induced \(\text{Ca}^{2+}\) entry into the cells. Preincubation of RBL-2H3 cells with \(\text{La}^{3+}\), addition of DNP-Lys, ATP-depletion or prior depolarisation in high K\(^+\) saline inhibit \(\text{Ca}^{2+}\) influx and secretion induced by antigen [128, 130]. In the absence of \(\text{Ca}^{2+}\) some increases in bis-oxonol fluorescence can still be observed, but it appears to be due to \(\text{Na}^+\) influx through \(I_{\text{CRAC}}\) in the absence of \([\text{Ca}^{2+}]_o\).

### 4.2 Methods and materials

All materials and experimental procedures are outlined in the appropriate sections in chapter two. Gelatin was used instead of BSA, to prevent adsorptive loss of the dilute antigen (DNP-HSA), since BSA interferes with bis-oxonol fluorescence, possibly due to bis-oxonol binding to BSA. DNP-HSA, gramicidin and the potassium channel blockers tested, except cetiedil (see section 4.3.11), had no effect on bis-oxonol fluorescence in a cell-free buffer solution.

### 4.3 Results

#### 4.3.1 KCl and RbCl depolarised RBL-2H3 cells

It has already been shown that a high K\(^+\) buffer solution depolarises RBL-2H3 cells either by using the TPP\(^+\) measurement method [131] or by using bis-oxonol [111]. Moreover, elevated concentrations (>5mM) of KCl and RbCl caused RBL-2H3 cell depolarisation. 140mM of each of these compounds caused complete cell depolarisation, and subsequent addition of gramicidin 1\(\mu\)g/ml caused no further changes in the cell membrane potential, thus showing that the cells were completely depolarised. Figure 4.1 shows the depolarisation induced by a high concentration of KCl or RbCl (75mM final concentration) in RBL-2H3 cells. This depolarisation is equivalent to that induced by gramicidin 1\(\mu\)g/ml (see below). As shown in Fig.4.1, the depolarisation induced by KCl or RbCl consists of two phases; one fast and the other slow. The fast phase lasted for the first minute and the slow phase started thereafter and continued for 12min.
Figure 4.1: Depolarisation of RBL-2H3 cells by high KCl and RbCl (>75mM). Cells (10^6 cell/ml) were incubated with 50nM bis-oxonol at 37°C with constant stirring. At time zero KCl or RbCl was added to the cells to give a final concentration of 75mM. Data are shown as mean ± SEM of % response which is the percentage of the maximum response at the final steady state. (n=6)
4.3.2 Gramicidin-induced depolarisation of RBL-2H3

Gramicidin, an antibiotic, has been found to induce cationic permeability by inserting itself in the membrane as a stationary ion-conducting channel [273]. Gramicidin has already been shown to be capable of completely depolarising RBL-2H3 cells [111, 271].

As shown in Fig. 4.2, gramicidin depolarised RBL-2H3 cells incubated in modified Tyrode’s buffer. The depolarisation-induced by 1μg/ml gramicidin was very fast with a half-time of 1.2min and the maximum depolarisation was reached in 8min. Further addition of gramicidin had no subsequent effect, indicating that gramicidin 1μg/ml induced the maximum depolarisation and a higher concentration of gramicidin could not cause more depolarisation.

4.3.3 Effect of gramicidin in different buffer solutions

Gramicidin had no effect on the membrane potential of a cell suspension in 140mM KCl or RbCl, in keeping with the fact that the cells were already fully depolarised. However, it was capable of depolarising the cells in CsCl [140mM], LiCl [140mM] and NaCl [140mM] buffers, whereas there was no sign of depolarisation by gramicidin in glucose [270mM], choline chloride [140mM] and NMG-Cl buffer solution. These results indicate that gramicidin increases the cell membrane permeability to monovalent ions and causes depolarisation thereby.

4.3.4 Antigen (DNP-HSA) activation depolarised passively sensitised RBL-2H3 cells

Using TPP^ and bis-oxonol, it has been reported that FceRI crosslinking by antigen stimulation can depolarise RBL-2H3 cells [53, 57, 111]. Labrecque et al. reported that a relatively rapid depolarisation with a t_d of 1.4 min, is elicited by the optimal concentration of antigen [53]. The antigen-induced depolarisation is followed by a partial repolarisation and a new steady state membrane potential is usually reached by 7-9min. In this study, depolarisation induced by 1 ng/ml DNP-HSA, which follows a delay of about 15 second, was very fast with a half-time of 40 second, reaching a maximum in 1min. Thus, my results are slightly different from those reported by Labrecque et al.[53]; the kinetics of the antigen-induced depolarisation were somewhat faster in this study (t_d of 40 second).
Figure 4.2: Depolarisation of RBL-2H3 cells by gramicidin. RBL-2H3 cells in modified Tyrode's buffer and 140mM CsCl, 140mM LiCl or 140mM NaCl buffers ■, but not in 140mM choline chloride, 140mM NMG chloride or 270mM glucose buffers ▲. At time zero, gramicidin was added to the cells to give a final concentration of 1μg/ml. Data are shown as mean ± SEM % response which is the percentage of the maximum response at the final steady state. (n=6)
Figure 4.3 shows the antigen-stimulated depolarisation and repolarisation as well as the depolarisation induced by gramicidin. As seen in Fig 4.3 the depolarisation fraction ($F_D$) which equals to $D/G$ is 0.4 for DNP-HSA 1 ng/ml. $F_D$ reduces with decreasing antigen concentration, so that it was nearly 0.2 for 0.1 ng/ml (Fig. 4.4). Moreover, stimulation of RBL-2H3 cells by 0.01 ng/ml DNP-HSA not only was unable to induce depolarisation, but it also caused a decline in bis-oxonol fluorescence which might be due to a hyperpolarisation. The repolarisation fraction ($F_R$) which is equivalent to $R/D$ is nearly 0.70 for 1 ng/ml DNP-HSA. It has been reported that the extent of repolarisation is fairly constant with different antigen concentrations [53]. However, in this study, the magnitude of repolarisation was inversely proportional to the concentration of antigen, and it was minimal with antigen excess (Fig. 4.5).

Perhaps, then, the factors causing depolarisation and repolarisation balance when RBL-2H3 cells are stimulated with 0.1 ng/ml DNP-HSA. Also, it appears that antigen at lower concentrations (e.g. 0.01 ng/ml) may hyperpolarise rather than depolarise RBL-2H3 cells. It may be suggested that at this concentration, a small rise in $[Ca^{2+}]_i$ may not be able to induce depolarisation, whereas $K^+$ channel opening may cause a small increase in the negativity of the membrane potential. This is consistent with the inhibitory effect of quinidine on the antigen-stimulated $\beta$-hexosaminidase release, since quinidine is more potent against low concentrations of antigen (1 and 0.1 ng/ml DNP-HSA) and its activity greatly decreases as the optimal antigen concentration is approached (10 ng/ml DNP-HSA).

To assess the effect of potassium channel blockers on the antigen-induced membrane potential changes, cells were stimulated with 1 ng/ml DNP-HSA, because at this concentration the maximum depolarisation can be observed.

$F_D = \frac{D}{G}$ is the ratio of the extent of the antigen-induced depolarisation to the total depolarisation by gramicidin in the same experiment.

$F_R = \frac{R}{D}$ is the ratio of the extent of the repolarisation phase to the extent of the depolarisation by antigen in the same experiment.
Figure 4.3: Membrane potential changes measured by bis-oxonol. Cells (10^6 cell/ml) were stimulated with DNP-HSA 1ng/ml at time zero. D = the extent of antigen-induced depolarisation; R = the extent of repolarisation; G = the extent of gramicidin-induced depolarisation at the end of each experiment. $F_D = \frac{D}{G} = 0.4$ and $F_R = \frac{R}{D} = 0.82$. Data are shown as mean ± SEM % response which is the percentage of the maximum depolarisation by gramicidin. (n=12)
Figure 4.4: The antigen-induced membrane potential changes in passively sensitised RBL-2H3 cells. Cells (10^6 cell/ml) were incubated with 50nM bis-oxonol at 37°C with constant stirring. Cells were then stimulated with different concentrations of DNP-HSA at time zero. Gramicidin was added 9 minutes after antigen stimulation. Data are shown as the mean of % response which is the percentage of the maximum depolarisation by gramicidin. (n=12) ■ 0.1, □ 1 and ▣ 10ng/ml DNP-HSA.
Figure 4.5: Dose-response curve for the antigen-induced depolarisation and repolarisation phase. Cells were incubated with 50nM bis-oxonol at 37°C with constant stirring. Cells were then challenged with different concentrations of antigen (DNP-HSA). $F_D$ and $F_R$ were calculated and plotted against antigen concentration. $F_R = R/D$ = the extent of the repolarisation phase / the extent of the antigen-induced depolarisation, $F_D = D/G$ = the extent of the antigen-induced depolarisation / the extent of the depolarisation induced by gramicidin. Data presented are mean ± SEM of calculated $F_D$ and $F_R$ from 4-6 experiments.
4.3.5 Antigen-induced depolarisation in Ca\(^{2+}\)-free buffer

Even in the absence of external Ca\(^{2+}\) an antigen-induced increase in bis-oxonol fluorescence was measured. This indicates that in Ca\(^{2+}\)-free buffer Na\(^{+}\) can depolarise RBL-2H3 cells. It has been proposed that CRAC channels are permeable to Na\(^{+}\) in the absence of external Ca\(^{2+}\) [160] and that Na\(^{+}\) uptake induced by antigen can be seen only in the absence of external Ca\(^{2+}\) [57, 111]. Importantly, the repolarisation phase after depolarisation induced in Ca\(^{2+}\)-free buffer is markedly inhibited (Fig. 4.6), which might suggest that the repolarisation phase is dependent on external Ca\(^{2+}\). It is more likely that the current responsible for the repolarisation can be regulated either by the external Ca\(^{2+}\) or by a rise in [Ca\(^{2+}\)]\(_i\) via Ca\(^{2+}\) influx. These results are compatible with the possible involvement of a Ca\(^{2+}\)-activated K\(^{+}\) channel in RBL-2H3 cells following antigen activation.

4.3.6 DNP-HSA had no effect on the membrane potential of RBL-2H3 cells in Na\(^{+}\)-Ca\(^{2+}\) free buffer

In the absence of Ca\(^{2+}\) and Na\(^{+}\) (NMG-Cl buffer or choline buffer) antigen had no effect on the membrane potential (Fig. 4.6). This result suggests the antigen-stimulated depolarisation in Ca\(^{2+}\)-free solution (containing Na\(^{+}\)) is more likely due to Na\(^{+}\) influx through CRAC channels. However, in NMG buffer containing 1.8mM Ca\(^{2+}\), the antigen-induced membrane potential changes was the same as in the normal condition, indicating that the external Na\(^{+}\) is not of critical importance for the stimulation of RBL-2H3 cells by antigen.

4.3.7 Effect of La\(^{3+}\) on the antigen-induced membrane potential changes

It has already been shown that La\(^{3+}\) can inhibit Ca\(^{2+}\) influx, depolarisation and secretion induced by antigen [111]. Incubation of RBL-2H3 cells with La\(^{3+}\) decreased the antigen-induced depolarisation and this effect was dependent on the incubation time, so that after 6min incubation, depolarisation was completely abolished. As seen in Fig. 4.7, when La\(^{3+}\) was added 2min before antigen stimulation, the depolarisation was markedly inhibited but there was no effect on the repolarisation phase. La\(^{3+}\), at a concentration which can completely abrogate the antigen-stimulated secretion (100μM), had no effect on the repolarisation phase when it was added at the peak of the antigen-induced depolarisation, as indicated by the reduction in bis-oxonol fluorescence (Fig. 4.7).
Figure 4.6: Role of extracellular Ca\(^{2+}\) and Na\(^{+}\) on the antigen-induced membrane potential changes in passively sensitised RBL-2H3 cells. Cells (10\(^6\) cell/ml) were stimulated with 1ng/ml DNP-HSA at time zero. Gramicidin was added 9 minutes after antigen stimulation. Data are shown as the mean of % response which is the percentage of the maximum depolarisation by gramicidin. (n=3-6) ■ normal Tyrode’s buffer, ■ Ca\(^{2+}\) free buffer and ■ choline or NMG buffer without Ca\(^{2+}\) and Na\(^{+}\).
Figure 4.7: Effect of La$^{3+}$ on the antigen-induced membrane potential changes in passively sensitised RBL-2H3 cells. Cells (10^6 cell/ml) were stimulated with 1 ng/ml DNP-HSA at time zero. ■ La$^{3+}$ (100μM) was added at the peak of depolarisation or at time 1 min. ■ Cells were incubated with La$^{3+}$ (100μM) for 2 min before antigen stimulation. Gramicidin was added 9 minutes after antigen stimulation. Data are shown as the mean of % response which is the percentage of the maximum depolarisation by gramicidin (n=3).
In view of these results, it can be inferred that the repolarisation is not a result of secretion and the loss of dye from the cell membrane. In other words, the antigen-induced depolarisation precedes secretion.

4.3.8 Con A-induced depolarisation of RBL-2H3 cells

Concanavalin A (40μg/ml) depolarised RBL-2H3 cells. A delay of about 30sec was observed before the onset of the response. The depolarisation reached a peak 2min after stimulant addition. Cells then repolarised by nearly 80-90% (Fig. 4.8). This indicates that other crosslinking agents can also depolarise RBL-2H3 cells and that the cells subsequently repolarise. The kinetics of the Con A-induced depolarisation were a little different from that induced by antigen so that Con A acts more slowly than antigen.

4.3.9 Ca^{2+} ionophore A23187-induced depolarisation of RBL-2H3 cells

Ca^{2+} ionophore A23187 (1μM) depolarised RBL-2H3 cells but the depolarisation was slower compared to antigen, reaching a peak 2.5min after stimulant addition. Thereafter cells repolarised by up to 36% to the resting membrane potential. Compared with the effect of DNP-HSA 1ng/ml, it is obvious that the repolarisation phase for A23187 was smaller, and this is possibly due to a huge Ca^{2+} influx induced by A23187 (Fig. 4.8).

4.3.10 Effect of quinidine on antigen-stimulated membrane potential changes

As already shown, quinidine dose-dependently inhibited the antigen-induced β-hexosaminidase release (Table 3.1 and Fig.3.4). Quinidine has been shown to inhibit the repolarisation phase dose-dependently [53]. It also increases antigen-induced depolarisation. These results have been confirmed here, as shown in Fig. 4.9 The IC_{50} of quinidine was 19±1μM for the inhibition of repolarisation. In the absence of antigen, quinidine induced a small increase in the resting membrane potential.
Figure 4.8: RBL-2H3 cell membrane potential changes induced by different stimuli. Cells (10⁶ cell/ml) were stimulated with 1ng/ml DNP-HSA ■, Con A ■ or Calcium ionophore A23187 ■ at time zero. Gramicidin was added 9 minutes after antigen stimulation. Data are shown as means of % response which is the percentage of the maximum depolarisation by gramicidin. (n=3-4)
Figure 4.9: Inhibition by quinidine of the repolarisation phase after the antigen-induced depolarisation. Cells were incubated in modified Tyrode’s buffer containing 0.05% gelatin at 37°C with continuous stirring for 5 min. Bis-oxonol was then added to give a final concentration of 50 nM. After 3-4 min, quinidine was added at different concentrations. 15 min later, cells were challenged with 1 ng/ml DNP-HSA. The repolarisation phase was measured by $F_R = R/D = \text{the extent of the repolarisation divided by the extent of the antigen-induced depolarisation.}$ (section 2.6.3). Data are presented as mean ± SEM of % inhibition, calculated from 4 experiments. Data have been fitted to the Hill equation with $B_{\text{max}}$ constrained to 100%. Hill coefficient ($n_H$) was 1.36±0.08.
4.3.11 Effect of BaCl\(_2\) on antigen-stimulated membrane potential changes

Labrecque et al. have reported that Ba\(^{2+}\) inhibits repolarisation and secretion in a similar dose-dependent fashion [53]. However, they added antigen and Ba\(^{2+}\) at the same time. In this case, the depolarisation induced by Ba\(^{2+}\) was superimposed on the antigen-induced depolarisation, and the repolarisation was also dampened.

To overcome these problems, in the present study the cells were incubated with Ba\(^{2+}\) for 15 min (exactly the same time as for the release experiments) and they were then challenged with antigen (DNP-HSA 1 ng/ml). Figure 4.10 shows the effect of Ba\(^{2+}\) on the resting membrane potential and on the antigen-induced membrane potential changes. As seen, Ba\(^{2+}\) even at a concentration of 0.1 mM induced a marked depolarisation and this effect was dose-dependent. A possible explanation for depolarisation induced by Ba\(^{2+}\) is the blockade of the IR K\(^+\) channels by Ba\(^{2+}\) (see chapter 7). Although the antigen-induced depolarisation was diminished by increasing Ba\(^{2+}\) concentration, it could still be seen even in the presence of 10 mM Ba\(^{2+}\). The repolarisation phase was also obvious with Ba\(^{2+}\) up to 1 mM, but with the higher concentrations of Ba\(^{2+}\) depolarisation was so marked that it became difficult to assess whether the repolarisation phase was inhibited by Ba\(^{2+}\).

4.3.12 Attempts to study the effect of cetiedil on antigen-stimulated membrane potential changes

Cetiedil inhibited the antigen-stimulated secretion with an IC\(_{50}\) of 84±10 μM (section 3.3.9). This made it of interest to evaluate the effects of cetiedil on the resting membrane potential and its changes induced by antigen (Fig.4.11). Cetiedil by itself at concentrations higher than 1 μM increased the bis-oxonol fluorescence, suggesting that like Ba\(^{2+}\), it may depolarise RBL-2H3 cells. Moreover, cetiedil had a pronounced effect on the repolarisation phase even at 1 and 5 μM. Surprisingly at concentrations of 10 and 50 μM, cetiedil abolished the antigen-induced depolarisation. However, when the effect of cetiedil was tested in the absence of cells (just buffer and bis-oxonol) or in the absence of dye (just the cell suspension) the changes induced by cetiedil were still seen. Therefore, the apparent effects of cetiedil on antigen-induced membrane potential changes are not interpretable because of the interference by cetiedil with the fluorescence of bis-oxonol.
Figure 4.10: Effect of Ba\(^{2+}\) on the resting membrane potential and antigen-induced membrane potential changes. Cells were incubated in modified Tyrode’s buffer containing 0.05% gelatin at 37°C with continuous stirring for 5min. Bis-oxonol was then added at the final concentration of 50nM. After 3-4 min, Ba\(^{2+}\) was added at different concentrations. 15min later, cells were challenged with 1ng/ml DNP-HSA. Data are shown as mean from 4-5 experiments.
Figure 4.11: Apparent effect of cetiedil (see technical problems in section 4.3.12) on the resting membrane potential and antigen-induced membrane potential changes. Cells were incubated in modified Tyrode's buffer containing 0.05% gelatin at 37°C with continuous stirring for 5 min. Bisoxonol was then added to give a final concentration of 50 nM. After 3-4 min, cetiedil was added at different concentrations. 15 min later, cells were challenged with 1 ng/ml DNP-HSA. Data are shown as mean from 4-5 experiments.
4.3.13 Effect of charybdotoxin on the antigen-stimulated membrane potential changes

Charybdotoxin, a blocker of IKCa, BKCa and KV1.3 channels, inhibited the antigen-induced β-hexosaminidase release with an IC50 of 61±4nM and 133±52nM for the stimulation by 1ng/ml and 10ng/ml DNP-HSA, respectively (see section 3.3.7). In order to assess the effect of charybdotoxin on the antigen-induced membrane potential changes, cells were incubated with charybdotoxin for 15min and cells were then stimulated with 1ng/ml DNP-HSA. Charybdotoxin had no effect on the resting membrane potential of RBL-2H3 cells even at 100nM. However, it was capable inhibiting the repolarisation phase with an IC50 of 84±7nM (Fig. 4.12). This is very close to the IC50 (61±4nM) for the inhibition of the antigen-stimulated secretion.

4.3.14 Effect of 4-aminopyridine on the antigen-stimulated membrane potential changes

4-aminopyridine (4-AP) inhibited the antigen-stimulated β-hexosaminidase release dose-dependently (see section 3.3.14). It was thought interesting to assess the effect of 4-AP on the antigen-induced membrane potential changes. Figure 4.13 shows that 4-AP inhibits the depolarisation-induced by antigen, suggesting that 4-AP may inhibit Ca2+ entry. The repolarisation phase was inhibited only partially. These data suggest that the effect of 4-AP may not be totally due to prevention of the repolarisation phase.
Figure 4.12: Inhibition by charybdotoxin of the repolarisation phase after the antigen-induced depolarisation. Cells were incubated in modified Tyrode's buffer containing 0.05% gelatin at 37°C with continuous stirring for 5min. Bis-oxonol was then added at the final concentration of 50nM. After 3-4 min, charybdotoxin was added at different concentrations. 15min later, cells were challenged with 1ng/ml DNP-HSA. The repolarisation phase was measured by $F_R = \frac{R}{D} = \text{the extent of the repolarisation phase divided by the extent of the antigen-induced depolarisation}$. (section 2.6.3). Data presented are mean ± SEM of % inhibition from 4 experiment. Data have been fitted to the Hill equation with Bmax constrained to 100%. Hill coefficient ($n_H$) was 0.36±0.01.
Figure 4.13: Effect of 4-AP on the antigen-induced membrane potential changes in passively sensitised RBL-2H3 cells. Cells (10⁶ cell/ml) were stimulated with 1ng/ml DNP-HSA at time zero. Gramicidin was added 9 minutes after antigen stimulation. Data presented are mean ± SEM of % response which is the percentage of the maximum depolarisation by gramicidin (n=4-8). ■ without, ■ incubated with 4-AP 5mM for 5min.
4.4 Discussion

4.4.1 Membrane potential changes induced by antigen

The resting membrane potential in RBL-2H3 cells has been reported to be between -60 to -90 mV by several groups [57, 125, 126]. Membrane potential is affected by changes in $[K^+]_o$ but not by ouabain, a Na$^+$/K$^+$ ATPase inhibitor, indicating that, in contrast to rat peritoneal mast cells, the resting membrane potential in RBL-2H3 cells is not determined by the Na$^+$/K$^+$ ATPase pump [125]. In addition, no potassium channel has been detected in rat peritoneal mast cells [137, 222, 223].

In this study, RBL-2H3 cells were depolarised by KCl, RbCl and gramicidin (Fig. 4.1 and 4.2) and the depolarisation by 1 μg/ml gramicidin was used as a standard to quantify the observed data.

The antigen-induced depolarisation has been studied in RBL-2H3 cells by various groups, using different methods [53, 57, 111, 129]. Following antigen stimulation, these cells were depolarised and this depolarisation is followed by a partial repolarisation which is inhibited by quinidine, a K$^+$ channel blocker [53]. The induced depolarisation is likely to be due to Ca$^{2+}$ influx, since it is inhibited by any procedure that inhibits Ca$^{2+}$ influx including; application of extracellular La$^{3+}$, prior depolarisation by high K$^+$ buffer, addition of excess DNP-Lys (hapten) and ATP-depletion [128, 130]. The observation that A23187 induced depolarisation in RBL-2H3 cells (Fig. 4.8) also suggests a relationship between Ca$^{2+}$ influx and depolarisation. However, in the absence of $[Ca^{2+}]_o$, antigen still depolarises RBL-2H3 cells, indicating that Na$^+$ influx through CRAC channels may be responsible for the depolarisation in the absence of $[Ca^{2+}]_o$ [57, 111, 148, 150]. This result is consistent with the lack of depolarisation in the Ca$^{2+}$/Na$^+$-free buffer (Fig. 4.6). The repolarisation phase is also greatly inhibited in Ca$^{2+}$-free buffer, suggesting an important role of the increase in $[Ca^{2+}]_i$ in producing the repolarisation phase.

In this study, membrane potential changes were monitored, using bis-oxonol, a fluorescent dye. The antigen-triggered depolarisation is dose-dependent and starts after a delay of 15 seconds and reaches a peak at 1 minute (Fig. 4.3). This response shows different kinetics to those reported by Labrecque et al. [53]. The depolarisation is followed by a partial repolarisation which is also antigen dose-dependent, though in the reverse direction; i.e. the repolarisation phase decreased with increasing concentration of antigen. The repolarisation phase of the antigen-induced membrane potential changes has
been suggested to be the result of the opening of $K^+$ channels, since it can be inhibited by Ba$^{2+}$ and quinidine [53].

However, it could be argued that the repolarisation phase might not be a genuine phenomenon but rather the result of exocytosis and loss of the dye bound to the cell membrane. It is very difficult to establish that this is not the case, because in any condition in which the stimulated secretion is inhibited, depolarisation is also inhibited. In the absence of depolarisation, the repolarisation phase cannot be observed.

The repolarisation phase has been reported with antigen stimulation of RBL-2H3 cells in $K^+$ free solution, and after the pretreatment of the cells with 5mM 4-AP [53]; these are conditions in which secretion induced by antigen is greatly inhibited, indicating that the repolarisation phase is not a consequence to secretion.

La$^{3+}$ (100μM) can immediately abrogate antigen-stimulated secretion, when it is added to the cell suspension. If La$^{3+}$ is added 5min before cell stimulation, the antigen-induced depolarisation is inhibited. However, when La$^{3+}$ is added at the peak of antigen-induced depolarisation, which abrogates secretion induced by antigen, the repolarisation phase still can be observed. This also suggests that the antigen-stimulated depolarisation precedes secretion.

Moreover, the increase in antigen concentration causes a progressive reduction in repolarisation (which reaches a maximum decrease with antigen excess), while the rate of secretion increases with higher concentrations of antigen. If secretion and the observed reduction in bis-oxonol fluorescence follow a cause and effect relationship, they should have the same dose-response curve. However, the curves are in opposite directions. This further suggests that the repolarisation phase (reduction in fluorescence of bis-oxonol) is not the result of antigen-induced secretion. One possible explanation as to why antigen excess reduces repolarisation is that Ca$^{2+}$ influx continues to rise during this phase so that depolarisation dominates and becomes too large to be offset by opening of $K^+$ channels.

It seems that the repolarisation has some relationship with the decline in [Ca$^{2+}$], following the initial rise, although the time course for [Ca$^{2+}$] decline in a cell suspension (20-30min) is much longer than that for the repolarisation phase (8-9min) [111]. Thus, the time course of the change in [Ca$^{2+}$] response is more prolonged than that of the membrane potential changes initiated by antigen.

It appears that the depolarisation and repolarisation are in a balanced condition at an antigen concentration of 0.1ng/ml DNP-HSA. In high concentrations of antigen, depolarisation is dominant and cells do not fully repolarise to the resting membrane
potential. In low concentrations of antigen, repolarisation is more dominant and antigen at very low concentrations may cause a hyperpolarisation. These results are compatible with the effect of quinidine on the antigen-induced β-hexosaminidase release: quinidine is more potent against lower concentrations of antigen than the higher concentrations of antigen.

4.4.2 Membrane potential changes induced by other stimuli

Addition of Con A to passively sensitised RBL-2H3 cells increased the fluorescence of bis-oxonol dye indicating that Con A may depolarise these cells, although with different kinetics compared to antigen (Fig. 4.8). A partial repolarisation was observed after Con A-induced depolarisation. It can be inferred that other crosslinking agents may induce membrane potential changes similar to those induced by antigen.

The A23187-stimulated depolarisation is not consistent with the supposition that it simply increases [Ca\(^{2+}\)]\(\text{[753]}\) and stimulates RBL-2H3 cells to degranulate. According to this hypothesis, the resting membrane potential should not be changed following A23187 stimulation. However, the observation that A23187 causes depolarisation provides further evidence for the release of Ca\(^{2+}\) from internal stores and activation of CRAC channels by A23187 [147]. The inhibition of A23187-induced secretion by depolarisation of cells in high K\(^+\) solution or ATP-depletion, are also consistent with the latter hypothesis [96, 109, 112].

4.4.3 Effect of K\(^+\) channel blockers on the antigen-induced membrane potential changes

4.4.3.1 Effect of quinidine

Quinidine dose-dependently inhibited repolarisation after antigen-induced depolarisation with an IC\(_{50}\) of 19±1μM, when RBL-2H3 cells were stimulated by DNP-HSA 1ng/ml. In chapter 3, it has been shown that quinidine was be able to inhibit antigen-stimulated β-hexosaminidase release. Quinidine has also been reported to inhibit 5-HT release with an IC\(_{50}\) of 30μM [53]. This is in agreement with the inhibitory effect of quinidine on antigen-induced ⁸⁶Rb\(^+\)-efflux [161] and on GTPγS -induced OR K\(^+\) current in RBL-2H3 cells [208]. This result supports the involvement of a type of OR K\(^+\) channel in the activation of RBL-2H3 cells by antigen. It seems that the opening of OR K\(^+\)
channels contributes to the maintenance of the electrochemical driving force for Ca$^{2+}$ influx.

### 4.4.3.2 Effect of Ba$^{2+}$

It has been reported that Ba$^{2+}$ inhibits antigen-induced serotonin release and repolarisation in RBL-2H3 cells [53]. Ba$^{2+}$ also depolarises these cells making it difficult to study the effect of Ba$^{2+}$ on the membrane potential changes induced by antigen. In this study, RBL-2H3 cells were incubated with Ba$^{2+}$ for 15min and were then challenged with antigen (DNP-HSA 1ng/ml). This was done to avoid superimposition of the antigen-induced depolarisation by the Ba$^{2+}$-induced depolarisation. As shown in Fig. 4.10, Ba$^{2+}$ (up to 1mM) had no effect on the repolarisation phase, and higher concentrations induced a very marked depolarisation which dampened the repolarisation phase. It seems that the inhibitory effect of Ba$^{2+}$ may be directly due to interference with Ca$^{2+}$ influx rather than direct K$^+$ channel blocking.

### 4.4.3.3 Effect of charybdotoxin

As shown in chapter 3, charybdotoxin dose-dependently inhibited antigen-induced β-hexosaminidase release. Its effects on the membrane potential changes have also been examined. Charybdotoxin inhibited the repolarisation phase with an IC$_{50}$ of 84±7nM when RBL-2H3 cells were stimulated by 1ng/ml DNP-HSA. These results are in keeping with the idea that the activation of K$^+$ channels is responsible for repolarisation and so may contribute to the overall response. The pharmacological properties of these channels resemble those of the IK$_{Ca}$ channels in red blood cells. However, more electrophysiological evidence is needed to prove the existence of IK$_{Ca}$ channels in RBL-2H3 cells.

These results are also consistent with the lack of effect of TEA and IbTX on antigen-induced β-hexosaminidase release, suggesting that the BK$_{Ca}$ channels are not involved in the RBL-2H3 cell activation. Moreover, the involvement of voltage-activated K$_{V1,3}$ channels can be discounted due to the lack of effectiveness of MgTX (section 3.3.8).
Chapter 5 Antigen-stimulated $^{86}$Rb$^+$-efflux from the RBL-2H3 cell line

5.1 Introduction

$^{86}$Rb$^+$ can serve as a useful indicator of K$^+$ fluxes because of its ability to pass through K$^+$ channels and pumps, and its advantage over $^{42}$K$^+$ of having a much longer half life (18.7 days) [274]. Moreover, exocytosis inhibited in the absence of extracellular K$^+$ can be restored by addition of $^{86}$Rb$^+$, indicating the chemical similarity of Rb$^+$ to K$^+$ [275]. As stated in chapter 2, loading of $^{86}$Rb$^+$ into RBL-2H3 cells by incubation of $4 \times 10^6$ cell/ml with $3 \mu$Ci/ml $^{86}$Rb$^+$ reached an equilibrium after approximately 2 hours. Labrecque et al. have reported that the rate of $^{86}$Rb$^+$ uptake is not affected by antigen stimulation [161]. It has been shown that $^{86}$Rb$^+$ uptake is inhibited by ouabain, a Na$^+$/K$^+$ ATPase inhibitor, while the addition of monensin, a Na$^+$ ionophore, increases $^{86}$Rb$^+$ uptake [275]. In contrast to rat peritoneal mast cells, $^{86}$Rb$^+$ uptake in RBL-2H3 cells is not inhibited by extracellular Ca$^{2+}$ [275]. Partial depolarisation has no effect on $^{86}$Rb$^+$ uptake [275].

$^{86}$Rb$^+$ efflux measurement can therefore provide evidence on the possible involvement of K$^+$ channels in RBL-2H3 cell stimulation, following FceRI crosslinking. In this chapter, the characteristics of antigen-induced $^{86}$Rb$^+$-efflux and the effect of some K$^+$ channel blockers will be discussed.

The antigen-induced $^{86}$Rb$^+$-efflux from RBL-2H3 cells was reported by Labrecque et al. in 1991 [161] who concluded that stimulated $^{86}$Rb$^+$-efflux consists of two components: a major component which is Ca$^{2+}$-dependent and is possibly due to the opening of Ca$^{2+}$-activated K$^+$ channels, and a minor component which is G-protein linked. The evidence for the existence of Ca$^{2+}$-activated K$^+$ channel is:

1) Antigen-stimulated $^{86}$Rb$^+$-efflux is inhibited by quinidine, a K$^+$ channel blocker, which also inhibits antigen-induced secretion and membrane potential changes, as discussed in chapters 3 and 4. Although, quinidine is a non-specific
K\(^+\) channel blocker, it has been well known to inhibit Ca\(^{2+}\)-activated K\(^+\) channels.

2) In the absence of extracellular Ca\(^{2+}\) or in the presence of La\(^{3+}\) 0.1mM, the stimulated efflux is greatly inhibited.

3) In addition to inhibition of Ca\(^{2+}\)-influx, depolarisation of RBL-2H3 cells by 1\(\mu\)M carbonyl cyanide 3-chlorophenylhydrazone (CCCP) also inhibits the antigen-induced \(^{86}\)Rb\(^+\)-efflux.

4) Cholera toxin (CTX) pretreatment can potentiate both the antigen-stimulated Ca\(^{2+}\)-influx and the stimulated \(^{86}\)Rb\(^+\)-efflux. Thus, it can be inferred that the stimulated Ca\(^{2+}\)-influx is closely related to the stimulated \(^{86}\)Rb\(^+\)-efflux.

5) Ionomycin can induce a rise in [Ca\(^{2+}\)]\(_i\) and stimulate an increase in \(^{86}\)Rb\(^+\)-efflux which can be inhibited by quinidine.

In all above conditions, it can be seen that Ca\(^{2+}\)-influx and \(^{86}\)Rb\(^+\)-efflux are closely correlated. However, it is difficult to be sure about the sequential order of occurrence of each event.

The evidence for the minor component of \(^{86}\)Rb\(^+\)-efflux which seems to be mediated by a G-protein is:

1) Pertussis toxin (PTX) pretreatment decreases the antigen-stimulated \(^{86}\)Rb\(^+\)-efflux to 40% of the control, although it has no effect on the ionomycin-induced \(^{86}\)Rb\(^+\)-efflux.

2) PTX pretreatment does not affect the increase in the stimulated \(^{86}\)Rb\(^+\) efflux caused by CTX, indicating that the two pathways are distinct from each other. These data indicate that the PTX-sensitive \(^{86}\)Rb\(^+\)-efflux pathway is not activated by ionomycin or by potentiation of Ca\(^{2+}\)-influx by CTX pretreatment.

3) Since PTX pretreatment has no effect on the ionomycin-induced \(^{86}\)Rb\(^+\) efflux, the G-protein pathway may be directly coupled to the IgE receptor.

Bearing in mind that PTX had no effect on the antigen-stimulated secretion and membrane potential changes, it has been suggested that the Ca\(^{2+}\)-activated K\(^+\) efflux pathway but not the PTX-sensitive pathway is closely related to antigen-stimulated secretion [161]. However, the PTX-sensitive component is about 60% of the total efflux.
The stimulated efflux is not due to a Na\(^+\)-dependent pathway, since it is not inhibited in a buffer in which Na\(^+\) was replaced by choline.

It has been shown [161] that the stimulated \(^{86}\text{Rb}^+\) efflux is not a consequence of secretion and loss of \(^{86}\text{Rb}^+\) from cytosol (also see below) or a decline in \(^{86}\text{Rb}^+\) reuptake.

5.2 Methods and materials

All materials and experimental procedures are outlined in the corresponding sections in Chapter 2.

5.3 Results

5.3.1 Antigen-stimulated \(^{86}\text{Rb}^+\)-efflux in RBL-2H3 cells

\(^{86}\text{Rb}^+\) loaded into RBL-2H3 cells was released spontaneously with a rate constant of 0.0150 ± 0.001min\(^{-1}\) (n=23). Two typical experiments are presented in Fig. 5.1, showing the spontaneous and antigen-stimulated \(^{86}\text{Rb}^+\)-efflux. Fig. 5.1A shows the spontaneous \(^{86}\text{Rb}^+\)-efflux in an experiment in which \(^{86}\text{Rb}^+\) loaded cells were suspended in modified Tyrode’s buffer and cells were not stimulated until the end of experiment. However, in Fig. 5.1B, under the same conditions, another batch of cells were challenged with DNP-HSA (10ng/ml) at time 0min. Although the addition of antigen had no effect on the spontaneous or basal \(^{86}\text{Rb}^+\)-efflux from non-sensitised cells, a very significant increase in \(^{86}\text{Rb}^+\)-efflux can be observed on antigen stimulation of sensitised RBL-2H3 cells (Fig. 5.1B). The rate constant for the net stimulated efflux was calculated by subtracting the rate constant of spontaneous efflux from the rate constant of the efflux after stimulation, and it came to 0.01239 ± 0.00208min\(^{-1}\) (n=23).

Labrecque et al. have reported that the increase in rate of \(^{86}\text{Rb}^+\)-efflux started after 1min lag time following antigen stimulation and continued for 9-10min, followed by a return to the basal (unstimulated) rate of \(^{86}\text{Rb}^+\)-efflux [161]. In this study, the stimulated \(^{86}\text{Rb}^+\)-efflux had biphasic kinetics in most of the experiments. The first phase starts from antigen addition until 10min, when the next phase begins and a further increase in the efflux can be seen (Fig. 5.2). Since in this study, no return to the basal rate has been observed, the data is not consistent with that reported by Labrecque et al [161].
Chapter 5

Antigen-induced $^{86}\text{Rb}^-$-efflux

Figure 5.1: Antigen stimulated increase in $^{86}\text{Rb}^-$-efflux from sensitised RBL-2H3 cells. (A) Spontaneous $^{86}\text{Rb}^-$ efflux and (B) the antigen-stimulated $^{86}\text{Rb}^-$ efflux. Cells (4x10$^6$ cells/ml) were incubated with 3μCi/ml at 37°C for 2 hours in a shaking incubator. Then cells were washed and incubated in modified Tyrode’s buffer. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) was added and sampling was continued for another 16 min.
5.3.2 Dose-response curve for the antigen-induced $^{86}$Rb$^+$ efflux

As shown in figure 5.3, the antigen-induced $^{86}$Rb$^+$ efflux is dose-dependent and the dose-response curve for the antigen-stimulated $^{86}$Rb$^+$-efflux resembles that for the stimulated secretion. The optimal concentration for maximal responses in both is 10ng/ml DNP-HSA. The rates of stimulated $^{86}$Rb$^+$-efflux induced by 1 and 100 ng/ml DNP-HSA are nearly similar. These data indicate that antigen-induced Ca$^{2+}$ uptake, secretion and depolarisation are closely related to the antigen-stimulated $^{86}$Rb$^+$-efflux.

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Figure 5.2: Antigen-induced stimulation of $^{86}$Rb$^+$-efflux from passively sensitised RBL-2H3 cells. Cells (4x10⁶ cells/ml) were incubated with 3µCi/ml at 37°C for 2 hours in a shaking incubator. Then cells were washed and incubated in modified Tyrode’s buffer at 37°C. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) was added and sampling was continued for another 16min. Data points from a linear regression fit (basal $^{86}$Rb$^+$ efflux) were subtracted from the corresponding values for the efflux after stimulation (in Fig. 5.1B). Each point was presented as mean ± SEM from eighteen experiments.
Figure 5.3: Comparison of dose-response curves for the antigen-induced $^{86}$Rb$^+$-efflux (A) and secretion (B) from passively sensitised RBL-2H3 cells. Cells were sensitised overnight with 0.5 µg/ml DNP-specific monoclonal mouse IgE. A) Cells loaded with $^{86}$Rb$^+$ were suspended in modified Tyrode's buffer. Samples were taken at 2min intervals. After 8min, antigen (DNP-HSA) was added and sampling was continued for another 8min. B) After 5min preincubation, cells were challenged with different concentrations of antigen (DNP-HSA) for 15 min. β-hexosaminidase release was measured in the supernatant and each point on the curve is mean ± SEM of % net β-hexosaminidase release in five experiments. Spontaneous release was 5.2 ± 0.4%.
5.3.3 Depolarisation had no effect on spontaneous $^{86}\text{Rb}^+$-efflux, but inhibited the antigen-stimulated $^{86}\text{Rb}^+$-efflux

It might be suggested that the stimulated $^{86}\text{Rb}^+$-efflux is due to the depolarisation induced by antigen, possibly accompanied by the opening of a voltage-dependent $K^+$ channel. The effect of depolarisation on both spontaneous and stimulated $^{86}\text{Rb}^+$-efflux was, therefore, evaluated. Since $K^+$ is the major permeant ion in most cell types, the resting membrane potential is generally close to the equilibrium potential for potassium ($E_K$). Thus, an increase in $[K^+]_o$ will result in depolarisation. RBL-2H3 cells were depolarised by resuspension in a modified Tyrode’s buffer in which $Na^+$ was replaced with $K^+$ (140 mM KCl). In table 5.1 the rates of spontaneous and stimulated $^{86}\text{Rb}^+$ efflux in two conditions (normal buffer and high $K^+$) were compared. As seen in table 5.1, the rate of spontaneous efflux in the two conditions is not significantly different, suggesting that depolarisation by high $K^+$ did not increase the basal efflux. It has been reported that CCCP 1 μM, which depolarised RBL-2H3 cells, failed to stimulate $^{86}\text{Rb}^+$ efflux [161]. It may be suggested that (1) the depolarisation induced by antigen or any depolarising agents is not sufficient to induce an increased $^{86}\text{Rb}^+$ efflux and that (2) the channel responsible for the stimulated $^{86}\text{Rb}^+$ efflux may be a voltage-independent $K^+$ channel.

<table>
<thead>
<tr>
<th>Buffer solution</th>
<th>Spontaneous $^{86}\text{Rb}^+$-efflux</th>
<th>Stimulated $^{86}\text{Rb}^+$-efflux</th>
<th>Net Stimulated $^{86}\text{Rb}^+$-efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode’s</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.0124 ± 0.002</td>
</tr>
<tr>
<td>High $K^+$</td>
<td>0.0152 ± 0.002</td>
<td>0.0232 ± 0.001</td>
<td>0.0079 ± 0.0008</td>
</tr>
</tbody>
</table>

Table 5.1: Effect of depolarisation induced by high $[K^+]_o$ on the spontaneous and antigen-stimulated $^{86}\text{Rb}^+$-efflux. $^{86}\text{Rb}^+$ loaded cells were incubated in either modified Tyrode’s or high $K^+$ buffer solutions. Data are presented as the rate constant (min$^{-1}$) from 5 experiments. The stimulated $^{86}\text{Rb}^+$-efflux rate constants were calculated from 0 to 16 min.
In high K⁺ solution, the antigen-triggered ⁸⁶Rb⁺ efflux is considerably inhibited (by 35%). However, in the same conditions, a greater degree of inhibition of antigen-induced ⁸⁶Rb⁺-efflux has been reported by Labrecque et al [161], reaching up to 100%. Since Ca²⁺ influx and secretion in high K⁺ solution is greatly inhibited [112], it may be suggested that the stimulated ⁸⁶Rb⁺-efflux is not the result of secretion and that it is also partly dependent on Ca²⁺ influx. It may also be suggested that depolarisation can only inhibit the PTX-insensitive Ca²⁺-activated component of ⁸⁶Rb⁺ efflux (40% of the total stimulated efflux). This is compatible with the previously cited evidence for the lack of an important role of PTX-sensitive ⁸⁶Rb⁺-efflux pathway in the antigen stimulation of RBL-2H3 cells.

5.3.4 A23187 stimulated ⁸⁶Rb⁺-efflux in RBL-2H3 cells

Ca²⁺ ionophore A23187 also induced an increase in basal ⁸⁶Rb⁺-efflux. Compared with the antigenic response, A23187-induced ⁸⁶Rb⁺-efflux is lower in magnitude and is monophasic, starting from A23187 addition and reaching a maximum 10min later. Unlike antigen response, a steady state is reached after 10min (Fig. 5.5). It can be concluded that A23187 can stimulate ⁸⁶Rb⁺ efflux by a rise in [Ca²⁺]ᵢ and this response is Ca²⁺-dependent. Although 1µM A23187 and DNP-HSA 10ng/ml induce the same amount of histamine or β-hexosaminidase release, they differ in their effects on stimulated ⁸⁶Rb⁺-efflux, possibly due to the existence of the two different K⁺ efflux pathways (see section 5.1). DNP-HSA stimulates both ⁸⁶Rb⁺-efflux pathways, but A23187 activates only the Ca²⁺-dependent ⁸⁶Rb⁺-efflux pathway.

5.3.5 Stimulation of ⁸⁶Rb⁺-efflux by NECA

5'-(N-ethylcarboxamido)adenosine (NECA), an adenosine analogue, transiently activates PLC, which leads to IP₃ production and a rise in [Ca²⁺]ᵢ [160]. It has been reported that although NECA does not induce significant secretion, it potentiates the antigen-induced rise in [Ca²⁺]ᵢ and mediator release [80, 79]. Qian and McCloskey reported that NECA induces an OR K⁺ channel activation in RBL-2H3 cells [210], so that NECA would be expected to increase the ⁸⁶Rb⁺-efflux. Indeed, this is the case. NECA [10µM] stimulated a rise in ⁸⁶Rb⁺-efflux that is slower in rate and magnitude than the antigen-induced one (Fig. 5.6).
Figure 5.4: Inhibition of the antigen stimulated $^{86}$Rb$^+$-efflux by depolarisation of RBL-2H3 cells in high $[K^+]_e$. $^{86}$Rb$^+$-loaded cells were washed and incubated in modified Tyrode’s buffer and 140mM K$^+$ buffer. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) was added and sampling was continued for another 16 min ($n=5$).

Figure 5.5: A23187- and antigen-stimulated $^{86}$Rb$^+$-efflux from RBL-2H3 cells. Cells were incubated in modified Tyrode’s buffer. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) or A23187 1μM was added and sampling was continued for another 16 min ($n=6$).
5.3.6 Inhibition of antigen-stimulated $^{86}\text{Rb}^+$-efflux in Ca$^{2+}$-free buffer

In order to see whether the antigen-induced $^{86}\text{Rb}^+$-efflux is dependent on [Ca$^{2+}$]$_o$, cells were stimulated in the absence of extracellular Ca$^{2+}$ (in both nominally Ca$^{2+}$-free and Ca$^{2+}$-free + 100μM EGTA). Table 5.2 and Figure 5.7 show that the response significantly depends on [Ca$^{2+}$]$_o$, but was not completely abolished in the absence of extracellular Ca$^{2+}$. Comparing figures 5.4 and 5.7, it can be seen that they are similar. Since in both conditions Ca$^{2+}$-influx is abolished, it can be proposed that antigen-stimulated $^{86}\text{Rb}^+$-efflux, is at least in part dependent on Ca$^{2+}$-influx.

<table>
<thead>
<tr>
<th>Buffer solution</th>
<th>$^{86}\text{Rb}^+$-efflux Rate (min$^{-1}$)</th>
<th>$^{86}\text{Rb}^+$-efflux Rate (min$^{-1}$)</th>
<th>$^{86}\text{Rb}^+$-efflux Rate (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode's</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.0124 ± 0.002</td>
</tr>
<tr>
<td>Ca$^{2+}$-free</td>
<td>0.0151 ± 0.001</td>
<td>0.0231 ± 0.001</td>
<td>0.00805 ± 0.0008*</td>
</tr>
</tbody>
</table>

Table 5.2: Effect of extracellular Ca$^{2+}$ on the spontaneous and antigen-stimulated $^{86}\text{Rb}^+$-efflux. $^{86}\text{Rb}^+$ loaded cells were incubated in either modified Tyrode's or Ca$^{2+}$-free buffer solutions. Data are represented as the mean ± SEM of rate constant (min$^{-1}$) from 6 experiments. (Student’s paired t-test, *p<0.05). The stimulated $^{86}\text{Rb}^+$-efflux rate constants were calculated from 0 to 16min.
Figure 5.6: NECA- and antigen-stimulated $^{86}$Rb$^+$-efflux from RBL-2H3 cells. Cells were incubated in modified Tyrode's buffer. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) or NECA 10μM was added and sampling was continued for another 16 min (n=3).

Figure 5.7: Effect of extracellular Ca$^{2+}$ on the spontaneous and antigen-stimulated $^{86}$Rb$^+$-efflux. $^{86}$Rb$^+$ loaded cells were incubated in either modified Tyrode's or Ca$^{2+}$-free buffer solutions. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) was added and sampling was continued for another 16 min (n=5).
5.3.7 La\(^{3+}\) inhibited the antigen stimulated \(^{86}\text{Rb}^+\) efflux

Since La\(^{3+}\), a blocker of CRAC channels, can inhibit Ca\(^{2+}\) influx and secretion in RBL-2H3 cells [91, 20], it was interesting to examine if La\(^{3+}\) affects the antigen-stimulated \(^{86}\text{Rb}^+\) efflux. Table 5.3 shows that La\(^{3+}\) 1μM had no effect on the basal \(^{86}\text{Rb}^+\) efflux, but it markedly blocked the antigen-stimulated \(^{86}\text{Rb}^+\) efflux. The question arose as to why the inhibitory effect of La\(^{3+}\) is much higher than the effect of the absence of [Ca\(^{2+}\)]. A possible answer to this is that La\(^{3+}\) can affect or block the two \(^{86}\text{Rb}^+\) efflux pathways (see section 5.1). The Ca\(^{2+}\)-dependent (PTX-insensitive) pathway is blocked by inhibition of Ca\(^{2+}\) influx in conditions such as the addition of La\(^{3+}\), and the use of Ca\(^{2+}\)-free or high K\(^+\) buffer solution. It also seems that La\(^{3+}\) is able to block the PTX-sensitive (Ca\(^{2+}\)-independent) \(^{86}\text{Rb}^+\) efflux pathway.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spontaneous (^{86}\text{Rb}^+)-efflux</th>
<th>Stimulated (^{86}\text{Rb}^+)-efflux</th>
<th>Net Stimulated (^{86}\text{Rb}^+)-efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode’s</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.0124 ± 0.002</td>
</tr>
<tr>
<td>La(^{3+}) 1μM</td>
<td>0.0153 ± 0.002</td>
<td>0.01845 ± 0.002</td>
<td>0.0032 ± 0.001*</td>
</tr>
</tbody>
</table>

Table 5.3: Effect of La\(^{3+}\) 1μM on the spontaneous and antigen-stimulated \(^{86}\text{Rb}^+\)-efflux. \(^{86}\text{Rb}^+\) loaded cells were incubated in modified Tyrode’s with La\(^{3+}\) 1μM at time -8min. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) was added and sampling was continued for another 16min. Data are presented as the mean ± SEM rate constant (min\(^{-1}\)) from 4 experiments. (Student’s paired t-test, * p<0.05). The stimulated \(^{86}\text{Rb}^+\)-efflux rate constants were calculated from 0 to 16min.
5.3.8 Ba\(^{2+}\) did not affect the antigen-stimulated \(^{86}\text{Rb}\) efflux significantly

Although Ba\(^{2+}\) inhibited antigen-induced secretion from RBL-2H3 cells, it had no significant effect on stimulated \(^{86}\text{Rb}\)\(^+\) efflux by antigen. Since Ba\(^{2+}\) induces cell depolarisation, it was thought it might induce a rise in \(^{86}\text{Rb}\)\(^+\) efflux. However, this was not the case, because Ba\(^{2+}\) (10mM) as well as high K\(^+\) buffer had no effect on spontaneous \(^{86}\text{Rb}\)\(^+\) efflux. These findings are compatible with a role for a voltage-independent K\(^+\) channel in the response of RBL-2H3 cells to antigen stimulation. As shown in figure 5.9, Ba\(^{2+}\) 10mM had no significant effect on antigen-stimulated \(^{86}\text{Rb}\)\(^+\) efflux. Since in the presence of Ba\(^{2+}\) 10mM antigen-stimulated secretion is greatly inhibited (Fig. 3.5), this result adds to the evidence that stimulated \(^{86}\text{Rb}\)\(^+\)-efflux is not a consequence of secretion.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spontaneous (^{86}\text{Rb})(^+)-efflux</th>
<th>Stimulated (^{86}\text{Rb})(^+)-efflux</th>
<th>Net Stimulated (^{86}\text{Rb})(^+)-efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode’s</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.012 ± 0.002</td>
</tr>
<tr>
<td>Ba(^{2+}) 10mM</td>
<td>0.0147 ± 0.001</td>
<td>0.02564 ± 0.002</td>
<td>0.010 ± 0.002*</td>
</tr>
</tbody>
</table>

Table 5.4: Effect of Ba\(^{2+}\) 10mM on the spontaneous and antigen-stimulated \(^{86}\text{Rb}\)\(^+\)-efflux. \(^{86}\text{Rb}\) loaded cells were incubated in modified Tyrode’s with Ba\(^{2+}\) 10mM at time -8min. Samples were taken every 2 min up to 8 min, then antigen (DNP-HSA 10ng/ml) was added and sampling was continued for another 16min. Data are represented as the rate constant (min\(^{-1}\)) from 6 experiments. (Student’s paired t-test, * not significant, p<0.1). The stimulated \(^{86}\text{Rb}\)-efflux rate constants were calculated from 0 to 16min.
Chapter 5

Antigen-induced $^{86}$Rb$^+$-efflux

Figure 5.8: Effect of La$^{3+}$ 1μM on the antigen-stimulated $^{86}$Rb$^+$-efflux. $^{86}$Rb$^+$ loaded cells were incubated in modified Tyrode's buffer without ■ or with La$^{3+}$ 1μM ○ at time -8min. Samples were taken every 2 min, antigen (DNP-HSA 10ng/ml) was added at time 0min and sampling was continued for another 16min (n=5).

Figure 5.9: Effect of Ba$^{2+}$ 10mM on the antigen-stimulated $^{86}$Rb$^+$-efflux. $^{86}$Rb$^+$ loaded cells were incubated in modified Tyrode's buffer without ■ or with Ba$^{2+}$ 10mM ○ at time -8min. Samples were taken every 2 min, antigen (DNP-HSA 10ng/ml) was added at time 0min and sampling was continued for another 16min (n=5).
5.3.9 Quinidine inhibited antigen- and A23187-stimulated $^{86}{\text{Rb}}^+$-efflux

Antigen-induced secretion, as well as repolarisation, is inhibited by quinidine, a known $K^+$ channel blocker [53]. It has already been shown [53] that quinidine is dose-dependently capable of inhibiting antigen- and A23187-induced $^{86}{\text{Rb}}^+$-efflux. These results were reproducible in our laboratory. As seen in figures 5.10 and 5.11, quinidine at a concentration of 80μM inhibited both A23187- and antigen-triggered $^{86}{\text{Rb}}^+$-efflux. However, quinidine had no effect on the basal $^{86}{\text{Rb}}^+$-efflux. As described in chapter 3, quinidine did not significantly affect A23187-induced β-hexosaminidase release. This raises the question of why the inhibition by quinidine of the A23187-induced increase in $K^+$ permeability (as gauged by the effect on $^{86}{\text{Rb}}^+$-efflux) did not influence the A23187-induced secretion. One possible answer is that the secretory response to antigen requires the maintenance of a sufficient electrochemical driving force for $Ca^{2+}$ influx by $K^+$ channel opening, but $Ca^{2+}$ ionophores do not require this for secretion.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spontaneous $^{86}{\text{Rb}}^+$-efflux</th>
<th>Stimulated $^{86}{\text{Rb}}^+$-efflux</th>
<th>Net Stimulated $^{86}{\text{Rb}}^+$-efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode’s</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.0124 ± 0.002</td>
</tr>
<tr>
<td>Quinidine 80μM</td>
<td>0.0149 ± 0.002</td>
<td>0.0162 ± 0.002</td>
<td>0.0013 ± 0.001**</td>
</tr>
</tbody>
</table>

Table 5.5: Effect of quinidine 80μM on the spontaneous and antigen-stimulated $^{86}{\text{Rb}}^+$-efflux. $^{86}{\text{Rb}}^+$ loaded cells were incubated in modified Tyrode’s buffer with quinidine 80μM at time -8min. Samples were taken every 2 min, antigen (DNP-HSA 10ng/ml) was added 8min later and sampling was continued for another 16min. Data are presented as the mean ± SEM of rate constant (min⁻¹) from 5 experiments. (Student’s paired t-test, ** p<0.01). The stimulated $^{86}{\text{Rb}}^+$-efflux rate constants were calculated from 0 to 16min.

5.3.10 Charybdotoxin was also able to inhibit the stimulated $^{86}{\text{Rb}}^+$-efflux

Charybdotoxin (ChTX) inhibited antigen-induced β-hexosaminidase release and repolarisation with an $IC_{50}$ of 84±7nM (see previous chapters). Since MgTX, IbTX and apamin had no effect on the secretion stimulated by antigen, it appears that the channel
involved in the signal transduction mechanism resembles the IKr channels in red blood cells. Figure 5.12 shows that 100nM ChTX completely inhibited the early phase of \(^{86}\text{Rb}^+\) efflux induced by antigen. The later phase was reduced by 50%. This is consistent with the effects of ChTX on secretion and repolarisation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spontaneous (^{86}\text{Rb}^+)-efflux</th>
<th>Stimulated (^{86}\text{Rb}^+)-efflux</th>
<th>Net Stimulated (^{86}\text{Rb}^+)-efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode's</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.01239 ± 0.002</td>
</tr>
<tr>
<td>Charybdotoxin 100nM</td>
<td>0.0159 ± 0.001</td>
<td>0.0222 ± 0.002</td>
<td>0.0063 ± 0.002**</td>
</tr>
</tbody>
</table>

Table 5.6: Effect of charybdotoxin 100nM on the spontaneous and antigen-stimulated \(^{86}\text{Rb}^+\)-efflux. \(^{86}\text{Rb}^+\) loaded cells were incubated in modified Tyrode's buffer with charybdotoxin 100nM at time -8min. Samples were taken every 2 min. Antigen (DNP-HSA 10ng/ml) was added 8min later and sampling was continued for another 16min. Data are presented as the mean ± SEM rate constant (min\(^{-1}\)) from 4 experiments. (Student’s paired t-test, ** p<0.01). The stimulated \(^{86}\text{Rb}^+\)-efflux rate constants were calculated from 0 to 16min.

5.3.11 Inhibitory effect of the cetiedil-related compounds (UCL compounds) on antigen-induced \(^{86}\text{Rb}^+\)-efflux

Since cetiedil and some related compounds ('UCL compounds') inhibit antigen-induced β-hexosaminidase release (see chapter 3), it was interesting to assess their effects on the stimulated \(^{86}\text{Rb}^+\)-efflux. Unfortunately, cetiedil and the UCL compounds showed fluorescence interference with bis-oxonol, so their effects on the antigen-stimulated membrane potential changes are not conclusive. As shown in table 5.7, the UCL compounds had no effect on basal \(^{86}\text{Rb}^+\)-efflux. However, all compounds at a concentration near to their IC\(_{50}\) (on antigen-mediated secretion) inhibited antigen-induced \(^{86}\text{Rb}^+\)-efflux. Like ChTX, some of the UCL compounds have more inhibitory effects on the early phase of antigen-stimulated \(^{86}\text{Rb}^+\)-efflux (Table 5.7). Unfortunately, because of insufficient supply of the UCL compounds, it was not feasible to carry out enough experiments to establish dose-response curves.
Figure 5.10: Effect of quinidine on the antigen-stimulated $^{86}$Rb$^+$ efflux. $^{86}$Rb$^+$ loaded cells were incubated in modified Tyrode's buffer without ■ or with quinidine $80\mu$M ■ at time -8min. Samples were taken every 2 min and antigen (DNP-HSA 10ng/ml) was added at time 0min and sampling was continued for another 16min (n=5).

Figure 5.11: Effect of quinidine on the A23187-stimulated $^{86}$Rb$^+$ efflux. $^{86}$Rb$^+$ loaded cells were incubated in modified Tyrode's buffer without ■ or with quinidine $80\mu$M ■ at time -8min. Samples were taken every 2 min. A23187 (1µM) was added at time 0min and sampling was continued for another 16min (n=3).
Figure 5.12: Effect of charybdotoxin on the antigen-stimulated $^{86}$Rb$^+$-efflux. $^{86}$Rb$^+$ loaded cells were incubated in modified Tyrode's buffer without □ or with charybdotoxin 100nM at time -8min. Samples were taken every 2min. Antigen (DNP-HSA 10ng/ml) was added at time 0min and sampling was continued for another 16min (n=5)
<table>
<thead>
<tr>
<th>Condition</th>
<th>Spontaneous $^{86}$Rb$^+$-efflux</th>
<th>Stimulated $^{86}$Rb$^+$-efflux</th>
<th>Net Stimulated $^{86}$Rb$^+$-efflux</th>
<th>% Inhibition at min 4</th>
<th>% Inhibition at min 16</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode's</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.01239 ± 0.002</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>UCL 1348 (100µM)</td>
<td>0.0141 ± 0.002</td>
<td>0.0218 ± 0.002</td>
<td>0.0065 ± 0.001*</td>
<td>72.4%</td>
<td>73.2%</td>
</tr>
<tr>
<td>UCL 1349 (100µM)</td>
<td>0.0129 ± 0.002</td>
<td>0.0204 ± 0.002</td>
<td>0.0075 ± 0.002**</td>
<td>48.5%</td>
<td>78.2%</td>
</tr>
<tr>
<td>UCL 1495 (100µM)</td>
<td>0.0146 ± 0.0009</td>
<td>0.0226 ± 0.001</td>
<td>0.008 ± 0.0004*</td>
<td>100%</td>
<td>33.8%</td>
</tr>
<tr>
<td>UCL 1617 (50µM)</td>
<td>0.0131 ± 0.001</td>
<td>0.0177 ± 0.001</td>
<td>0.0046 ± 0.001**</td>
<td>100%</td>
<td>73.6%</td>
</tr>
<tr>
<td>UCL 1710 (50µM)</td>
<td>0.0134 ± 0.0005</td>
<td>0.0135 ± 0.0007</td>
<td>0.0001 ± 0.0003***</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>UCL 1608 (10µM)</td>
<td>0.0137 ± 0.0002</td>
<td>0.0141 ± 0.0005</td>
<td>0.004 ± 0.0007**</td>
<td>64.6%</td>
<td>72%</td>
</tr>
</tbody>
</table>

Table 5.7: Effect of UCL compounds on the spontaneous and antigen-stimulated $^{86}$Rb$^+$-efflux. $^{86}$Rb$^+$-loaded cells were incubated in modified Tyrode’s buffer with or without the UCL compounds at time -8min. Samples were taken every 2min. Antigen (DNP-HSA 10ng/ml) was added 8min later and sampling was continued for another 16min. Data are presented as the mean ± SEM of rate constant (min$^{-1}$) from 3-5 experiments. (Student’s paired t-test, * p<0.05, ** p<0.01, *** p<0.001)
Figure 5.13: Effect of UCL 1348 (A) and UCL 1349 (B) on the antigen-stimulated $^{86}$Rb⁺-efflux. $^{86}$Rb⁺-loaded cells were incubated in modified Tyrode's without ■ or with the UCL compound at 100µM ○ at time -8min. Samples were taken every 2 min and antigen (DNP-HSA 10ng/ml) was added at time 0min and sampling was continued for another 16min(n=4)
Figure 5.14: Effect of UCL 1495 [100μM] (A) and UCL 1710 [50μM] (B) on the antigen-stimulated \(^{86}\)Rb\(^{-}\)-efflux. \(^{86}\)Rb\(^{-}\) loaded cells were incubated in modified Tyrode's buffer without ■ or with the UCL compound • at time -8min. Samples were taken every 2min. Antigen (DNP-HSA 10ng/ml) was added at time 0min and sampling was continued for another 16min (n=3)
Figure 5.15: Effect of UCL 1617 [50μM] (A) and UCL 1608 [10μM] (B) on the antigen-stimulated $^{86}$Rb$^+$-efflux. $^{86}$Rb$^+$ loaded cells were incubated in modified Tyrode's buffer without ■ or with the UCL compound ○ at time -8min. Samples were taken every 2min. Antigen (DNP-HSA 10ng/ml) was added at time 0min and sampling was continued for another 16min (n=4)
5.3.12 Effect of 4-aminopyridine on the antigen-induced $^{86}$Rb$^+$-efflux in RBL-2H3 cells

4-aminopyridine, at a concentration of 5mM, inhibited antigen-induced β-hexosaminidase release by 85%. It also inhibited antigen-stimulated depolarisation and the repolarisation phase (section 4.3.13). To study its effect on stimulated $^{86}$Rb$^+$-efflux, $^{86}$Rb$^+$-loaded RBL-2H3 cells were incubated with 5mM 4-AP for 8 min, and then were challenged with antigen (DNP-HSA 10ng/ml). Table 5.8 shows the effect of 4-AP on spontaneous and antigen-stimulated $^{86}$Rb$^+$-efflux. As shown, although 4-AP had no effect on the basal $^{86}$Rb$^+$-efflux, it inhibited antigen-induced $^{86}$Rb$^+$-efflux by nearly 50%. However, it cannot be concluded that 4-AP is directly affecting the OR K$^+$ channel, since it has some inhibitory effect on antigen-stimulated depolarisation. Thus its effect may be, at least in part, due to inhibition of Ca$^{2+}$ influx and depolarisation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Spontaneous $^{86}$Rb$^+$-efflux</th>
<th>Stimulated $^{86}$Rb$^+$-efflux</th>
<th>Net Stimulated $^{86}$Rb$^+$-efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified Tyrode's</td>
<td>0.0149 ± 0.001</td>
<td>0.0273 ± 0.002</td>
<td>0.01239 ± 0.002</td>
</tr>
<tr>
<td>4-AP [5mM]</td>
<td>0.0150 ± 0.001</td>
<td>0.0200 ± 0.0003</td>
<td>0.005 ± 0.0007***</td>
</tr>
</tbody>
</table>

Table 5.8: Effect of 4-AP 5mM on the spontaneous and antigen-stimulated $^{86}$Rb$^+$-efflux. $^{86}$Rb$^+$ loaded cells were incubated in modified Tyrode’s buffer with or without 4-AP 5mM added at time -8min. Samples were taken every 2 min, antigen (DNP-HSA 10ng/ml) was added 8min later and sampling was continued for another 16min. Data are presented as the mean ± SEM of rate constant (min$^{-1}$) from 3 experiments. (Student’s paired t-test *** p<0.001). The stimulated $^{86}$Rb$^+$-efflux rate constants were calculated from 0 to 16min.
5.4 Discussion

5.4.1 Antigen-induced $^{86}$Rb$^+$-efflux

Labrecque et al., in 1991 showed that antigen induced an increase in $^{86}$Rb$^+$-efflux in RBL-2H3 cells [161]. They have also shown that the stimulated $^{86}$Rb$^+$-efflux is inhibited in the absence of extracellular Ca$^{2+}$, by prior depolarisation in high K$^+$ buffer solution, or by application of La$^{3+}$. Since in all these conditions Ca$^{2+}$ influx is greatly inhibited, these authors suggested that antigen-stimulated $^{86}$Rb$^+$-efflux is dependent on Ca$^{2+}$ influx. As the stimulated $^{86}$Rb$^+$-efflux is not affected by the absence of Na$^+$, it is not likely to be due to a Na$^+$-dependent pathway.

The effects of pertussis toxin (PTX) and cholera toxin (CTX) on antigen-induced $^{86}$Rb$^+$-efflux have been reviewed in section 5.1, where it has been noted that this response consists of two components [161]:

1) A PTX-sensitive component: this component forms 60% of the whole antigen-induced response.
2) A PTX-insensitive (Ca$^{2+}$-sensitive) component: this component forms 40% of the whole antigen-induced response. Since PTX had no effect on the A23187-induced $^{86}$Rb$^+$-efflux, A23187 may activate just the PTX-insensitive component. CTX potentiates the antigen-stimulated $^{86}$Rb$^+$-efflux by about 40%. This is likely to be due to the increase in Ca$^{2+}$ influx produced by CTX.

In this study, some features of stimulated $^{86}$Rb$^+$-efflux in RBL-2H3 cells were investigated. Although antigen had no effect on spontaneous $^{86}$Rb$^+$-efflux from nonsensitised RBL-2H3 cells, a significant increase in $^{86}$Rb$^+$-efflux was observed after antigen stimulation of passively sensitised cells (Fig. 5.1). This effect of antigen is dose-dependent and the dose-response curve has the same shape as that for antigen-stimulated secretion (Fig.5.3).

To assess the role of external Ca$^{2+}$, antigen-stimulated $^{86}$Rb$^+$-efflux was studied under conditions in which Ca$^{2+}$ influx was greatly reduced. The effect of antigen was tested in a high K$^+$ depolarising solution, in the absence of external Ca$^{2+}$ or in the presence of La$^{3+}$. Suspending RBL-2H3 cells in high K$^+$ buffer solution causes a complete depolarisation which, in turn, reduces Ca$^{2+}$ influx. This depolarisation did not cause any increase in the rate of spontaneous $^{86}$Rb$^+$-efflux. While this might suggest that the K$^+$
channels involved may not be voltage-sensitive, it has to be remembered that the reduction in the negativity of the membrane potential would tend to increase $^{86}\text{Rb}^+$ efflux. The fact that there was little change in the spontaneous efflux may then mean that this increase was offset by a reduction in $K^+$ permeability. On the other hand, antigen-induced $^{86}\text{Rb}^+$-efflux was inhibited in the depolarised cells by 35% (Fig. 5.4 and Table 5.1). However, it has been reported that in this depolarised condition stimulated $^{86}\text{Rb}^+$-efflux is completely abolished [161]. It would seem from the present results that if the inhibition of $Ca^{2+}$ influx caused by depolarisation [112] inhibits just the PTX-insensitive ($Ca^{2+}$-sensitive) component, this would explain why 60% of the response can still be seen. This is also consistent with the results obtained in the absence of extracellular $Ca^{2+}$ when stimulated $^{86}\text{Rb}^+$-efflux was about 60% of the control value (Fig. 5.7, Table 5.2). When RBL-2H3 cells were incubated with $La^{3+}$ (1µM), a greater inhibition of antigen-induced $^{86}\text{Rb}^+$-efflux was observed (75-80%) (Fig. 5.8, Table 5.3). This greater inhibition with $La^{3+}$ is different from the results obtained in the absence of extracellular $Ca^{2+}$ and in high $K^+$ solution (35-40% inhibition), suggesting that $La^{3+}$ might have inhibitory effect on both components of stimulated $^{86}\text{Rb}^+$-efflux.

5.4.2 A23187-stimulated $^{86}\text{Rb}^+$-efflux

RBL-2H3 cell stimulation with A23187 [1µM] also induced an increase in $^{86}\text{Rb}^+$-efflux (Fig. 5.5), but the amplitude and rate of stimulated efflux were less than those observed with antigen at the optimal dose (DNP-HSA 10ng/ml). A23187 activates the CRAC channel by $Ca^{2+}$ store depletion and this, in turn, increases the [$Ca^{2+}$]. Therefore, as mentioned by Labrecque et al., the stimulation of $^{86}\text{Rb}^+$-efflux by A23187 is likely to be due to the PTX-insensitive ($Ca^{2+}$-sensitive) component as A23187-induced $^{86}\text{Rb}^+$-efflux is not affected by PTX [161].

5.4.3 NECA-stimulated $^{86}\text{Rb}^+$-efflux

Electrophysiological studies report that ADP, ATP and NECA can open an outward rectifier $K^+$ channel in RBL-2H3 cells [210]. NECA, via the $A_3$ receptor, activates a PLC isozyme (other than PLCγ1) via a G-protein, but not tyrosine phosphorylation [72]. A transient production of inositol phosphates has also been reported [79]. However, it is clear that the inability of NECA to induce a sustained IP production is the main reason for the lack of a secretory response to NECA on its own. Nevertheless,
the question of why NECA cannot induce a prolonged PLC activation still remains to be answered. NECA has also been shown to activate ICRA in the presence of PKC inhibitors[153]. Although NECA fails to induce mediator release from RBL-2H3 cells, it significantly potentiates antigen- and A23187-induced histamine release but not arachidonic acid release [80, 79].

More recently, Ali et al. have reported that NECA causes a dose-dependent sustained activation of PLD and PKC [160]. The reason for the different time-courses of reactions mediated by PLC (transient) and PLD (sustained) is the negative feedback regulation of PLC by PKC. Consistent with this, it has been shown that the synergetic interaction of adenosine is abrogated by down-regulation of PKC with phorbol ester [276].

The results of this study are compatible with the above findings. NECA [10μM] induced an increase in $^{86}$Rb$^+$-efflux that was lower in magnitude compared with that induced by antigen. Pretreatment of the cells with PTX inhibits the potentiation effect of NECA on antigen-induced mediator release but not the effect of antigen on its own [79, 80]. It has also been shown that NECA fails to induce a sustained Ca$^{2+}$ influx (the Ca$^{2+}$ spike returns to the basal level in 100sec) [ref. 160 and my results in section 6.3.12]. Since NECA cannot induce a sustained increase in [Ca$^{2+}$], the NECA-stimulated $^{86}$Rb$^+$-efflux may not be entirely through a Ca$^{2+}$-activated K$^+$ channel. Regarding these results, it can be inferred that NECA might activate just the PTX-sensitive component of $^{86}$Rb$^+$-efflux which is not sufficient for a sustained Ca$^{2+}$ influx and secretion.

In general, all my results are compatible with the hypothesis that antigen-stimulated $^{86}$Rb$^+$-efflux is a two-component response, only one of which (Ca$^{2+}$-sensitive) plays an important role in RBL-2H3 cell activation leading to secretion. However, quinidin is able to inhibit both components.

### 5.4.4 Effect of K$^+$ channel blockers on the antigen-stimulated $^{86}$Rb$^+$-efflux

In order further to investigate the role of K$^+$ channels in RBL-2H3 cell activation, the effect of quinidine, Ba$^{2+}$, charybdotoxin and cetiedil on antigen-induced $^{86}$Rb$^+$-efflux was examined.

---

1 PKC inhibitors are required to prevent inactivation of CRAC channels by PKC.
5.4.4.1 Effect of quinidine

Quinidine has been shown to inhibit 5-HT release, the repolarisation phase and $^{86}\text{Rb}^+$-efflux in antigen-stimulated RBL-2H3. It also inhibits the opening of the OR $K^+$ channel induced by G-protein activators in these cells [53, 161, 208, 210]. It is not a specific $K^+$ channel blocker, as it also inhibits $Na^+$ and $Ca^{2+}$ channels. Antigen-induced $\beta$-hexosaminidase release was inhibited by quinidine dose-dependently. Quinidine has a higher inhibitory effect against lower antigen concentrations (chapter 3). As already suggested, $Ca^{2+}$ influx induced by higher concentrations of antigen may be too great to be inhibited by $K^+$ channel blocking or by inhibition of the repolarisation phase. However, antigen at lower concentrations causes a sub-maximal $Ca^{2+}$ influx which can be inhibited more readily by quinidine. Quinidine 80$\mu$M almost completely inhibited $^{86}\text{Rb}^+$-efflux stimulated by antigen. Both antigen- and A23187-induced $^{86}\text{Rb}^+$-effluxes were inhibited by 80$\mu$M quinidine, which is consistent with the involvement of $K^+$ channels in these stimulated effluxes. The inhibition of antigen-induced $^{86}\text{Rb}^+$-efflux by quinidine, $La^{3+}$ and the absence of extracellular $Ca^{2+}$ suggests the efflux might be partly through $Ca^{2+}$-activated $K^+$ channels. Similarly, secretion by antigen is inhibited by quinidine, $La^{3+}$ or the absence of extracellular $Ca^{2+}$.

Thus, quinidine can block antigen-activated potassium channels which, in turn, prevents repolarisation, and this inhibits $K^+$-efflux as well as secretion. These data suggest that antigen-activation of RBL-2H3 cells needs the opening of potassium channels (possibly $Ca^{2+}$-activated) to maintain a sufficient electrochemical driving force for calcium influx.

Although quinidine inhibited A23187-induced $^{86}\text{Rb}^+$-efflux, it had no significant effect on $\beta$-hexosaminidase release stimulated by A23187. A possible explanation is that the provision of an electrochemical driving force for $Ca^{2+}$ influx (by $K^+$ channel activation) is not essential for A23187 stimulation.

5.4.4.2 Effect of $Ba^{2+}$

It has been proposed that $Ba^{2+}$ blocks the outward rectifier potassium channels and this, in turn, inhibits the provision of a sufficient electrochemical driving force for $Ca^{2+}$ influx [53]. In order to investigate further this hypothesis, the effect of $Ba^{2+}$ on the antigen and A23187-induced $\beta$-hexosaminidase release was studied. $Ba^{2+}$ inhibited antigen-induced $\beta$-hexosaminidase release dose-dependently with an IC$_{50}$ of about 2mM.
Similarly, it has been reported that $\text{Ba}^{2+}$ inhibits 5-HT release with an IC$_{50}$ of 2mM [161]. $\text{Ba}^{2+}$ completely blocks the IR K$^+$ channel in RBL-2H3 cells at a concentration of 100µM. However, $\text{Ba}^{2+}$ at concentrations lower than 1mM had no effect on antigen-induced β-hexosaminidase release. These results indicate that the inhibitory effect of $\text{Ba}^{2+}$ is not likely to be the result of IR K$^+$ channel blocking. Barium more potently inhibited cell activation by A23187 than by antigen. Also, $\text{Ba}^{2+}$ has a higher inhibitory effect on the response to low doses of A23187 (IC$_{50}$ =0.156±0.005mM for 150nM A23187), while the inhibitory effect of $\text{Ba}^{2+}$ for all doses of antigen is the same (IC$_{50}$ = 2mM). Raising the calcium concentration in Tyrode buffer (from 1.8mM to 10mM) slightly increased the inhibitory effect of $\text{Ba}^{2+}$ on antigen-induced response.

The effect of $\text{Ba}^{2+}$ on antigen-induced membrane potential changes and $^{86}\text{Rb}^+$-efflux from passively sensitised RBL-2H3 cells has also been investigated. $\text{Ba}^{2+}$ itself depolarised the cells and antigen-induced depolarisation was affected by this change. The repolarisation was not blocked by $\text{Ba}^{2+}$ up to 1mM. With $\text{Ba}^{2+}$ 5mM and 10mM, cell depolarisation is marked and no repolarisation could be seen. $\text{Ba}^{2+}$ did not have any significant effect on the antigen-induced $^{86}\text{Rb}^+$-efflux. It can be suggested that the lack of effect by $\text{Ba}^{2+}$ is possibly because the expected reduction in induced potassium permeability was masked by an increase due to depolarisation. However, neither suspending the cells in high K$^+$ solution nor $\text{Ba}^{2+}$ itself affected basal $^{86}\text{Rb}^+$-efflux. Since depolarisation by $\text{Ba}^{2+}$ would be expected to increase $^{86}\text{Rb}^+$-efflux, it is possible that there was a concomitant reduction in K$^+$ permeability (perhaps as a consequence of a fall in Ca$^{2+}$ entry). Further work would be needed to clarify this.

5.4.4.3 Effect of charybdotoxin

Since charybdotoxin inhibits the antigen-stimulated β-hexosaminidase release, its effects on the membrane potential changes and $^{86}\text{Rb}^+$-efflux, as well as increase in [Ca$^{2+}$], have also been examined. Interestingly, ChTX inhibited the repolarisation phase and $^{86}\text{Rb}^+$-efflux with an IC$_{50}$ of 84±7nM. At a high concentration, ChTX can inhibit various RBL-2H3 cell responses, indicating that the activation of the channel, responsible for repolarisation and $^{86}\text{Rb}^+$-efflux may resemble the IK$_{\text{Ca}}$ channels in red blood cells. However, direct electrophysiological evidence is needed to prove the existence of IK$_{\text{Ca}}$ channels in RBL-2H3 cells.
These results are consistent with the lack of effect of TEA and IbTX on antigen-induced β-hexosaminidase release, suggesting that the BK\(_{Ca}\) channels are not involved in RBL-2H3 cell activation. Moreover, the involvement of voltage-activated \(K_{V1,3}\) channels can be discounted due to the lack of effectiveness of MgTX.

### 5.4.4.4 Effect of cetiedil

Cetiedil, a blocker of several \(K^+\) channels (including \(IK_{Ca}\)), inhibited antigen-induced β-hexosaminidase release from RBL-2H3 cells. It has been reported that quinidine [283] and cetiedil [244] are more potent against the \(IK_{Ca}\) channel when tested in low \([K^+]_o\). However, since antigen-induced β-hexosaminidase release is greatly diminished in the absence of \([K^+]_o\) and at low \([K^+]_o\) (Fig.3.12), the effects of cetiedil and the UCL compounds have not been evaluated in different \([K^+]_o\). Also, due to fluorescence interference, the effect of cetiedil on the membrane potential changes could not be determined.

Cetiedil also inhibited antigen-induced \(Ca^{2+}\) oscillations in RBL-2H3 cells (chapter 6) as well as \(^{86}Rb^+\) efflux suggesting that inhibition of the stimulated \(^{86}Rb^+\)-efflux by cetiedil affects \(Ca^{2+}\) regulation. These results indicate that \(^{86}Rb^+\)-efflux and \(Ca^{2+}\) influx are related; each can affect the other.

### 5.4.4.5 Effect of cetiedil-related compounds

The effects of some cetiedil-related compounds, synthesised in the Dept. of Chemistry at University College London (UCL compounds), on RBL-2H3 cell activation were also investigated. The criterion of selection of these UCL compounds was their effectiveness as \(IK_{Ca}\) channel blockers in red blood cells, since the inhibition of antigen-induced β-hexosaminidase release by ChTX and cetiedil (but not by TEA, IbTX and MgTX) suggested that the channels involved may resemble the \(IK_{Ca}\) channel in red blood cells.

The UCL compounds inhibited antigen-induced \(^{86}Rb^+\)-efflux in the concentration range which inhibited the stimulated secretion. Table 7.2 shows the effect of the UCL compounds on antigen-induced β-hexosaminidase release and \(^{86}Rb^+\)-efflux in passively sensitised RBL-2H3 cells. Since creating a dose-response curve for each UCL compound was not feasible, the results obtained cannot be used to determine structure-response
relationships. However, it is clear that the inhibitory effects of these compounds on secretion and $^8$Rb$^+$-efflux are closely related. In contrast to what is seen in red blood cells [256], increasing lipophilicity does not seem to determine potency. Thus, UCL 1617 is a more potent inhibitor of secretion and $^8$Rb$^+$-efflux than is the more lipophilic UCL 1495.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$\Delta\log P$</th>
<th>N-ring</th>
<th>$\alpha$-substituted</th>
<th>$IC_{50}$</th>
<th>Inhibition of $^8$Rb$^+$-efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCL 1348</td>
<td>4.89</td>
<td>original</td>
<td>HP</td>
<td>30µM</td>
<td>73.2% at 100µM</td>
</tr>
<tr>
<td>UCL 1349</td>
<td>4.89</td>
<td>original</td>
<td>HP</td>
<td>78µM</td>
<td>78.2% at 100µM</td>
</tr>
<tr>
<td>UCL 1495</td>
<td>7.53</td>
<td>Triphenyl</td>
<td>P</td>
<td>105µM</td>
<td>33.8% at 100µM</td>
</tr>
<tr>
<td>UCL 1617</td>
<td>6.34</td>
<td>Triphenyl</td>
<td>P</td>
<td>23µM</td>
<td>73.6% at 50µM</td>
</tr>
<tr>
<td>UCL 1710</td>
<td>9.5</td>
<td>9-benzy, 9-flourenyl</td>
<td>P</td>
<td>14µM</td>
<td>100% at 50µM</td>
</tr>
<tr>
<td>UCL 1608</td>
<td>8.5</td>
<td>9-benzy, 9-flourenyl</td>
<td>HP</td>
<td>10µM</td>
<td>72% at 10µM</td>
</tr>
</tbody>
</table>

Table 7.2: Effect of UCL compounds on the antigen-stimulated β-hexosaminidase release and $^8$Rb$^+$-efflux from RBL-2H3 cells. For details see tables 3.3 and 5.7.

a) $\Delta\log P$ = predicted log octanol/water partition coefficient.
b) Substitution at $\alpha$ carbon of acetic acid ester
c) HP homopiperidine and P piperidine.

In addition to the lack of an inhibitory effect of clotrimazole (chapter 3), the different potencies of the cetiedil enantiomers and of UCL 1495 in inhibiting antigen-induced secretion suggest some differences between the IK$_{Ca}$ channels in red blood cells and the K$^+$ channels in RBL-2H3 cells. However, the higher potency of UCL 1617, 1710 and 1608 in inhibiting secretion in RBL-2H3 cells is similar to their potencies in IK$_{Ca}$ blocking action in red blood cells.
Chapter 6 The antigen-induced rise in \([Ca^{2+}]_i\) and \(Ca^{2+}\) oscillations in RBL-2H3 cells

6.1 Introduction

Critical evaluation of the role of calcium as a second messenger requires quantitative measurement of \([Ca^{2+}]_i\). The most common method for measuring \([Ca^{2+}]_i\) is to monitor the fluorescence of an intracellular \(Ca^{2+}\) indicator. \(Ca^{2+}\) indicators are loaded into intact cells either by incubating them with a membrane-permeant ester derivative or by intracellular application of the acid form of dyes in solution through a micropipette. In the former case, cytosolic esterases split off the ester groups and leave the membrane impermeant (acid) form trapped in the cytosol.

6.1.1 Fluorescent \(Ca^{2+}\) indicators

Fluorescent \(Ca^{2+}\) indicators are divided into two operational groups:

1 Single wavelength intensity-modulating dyes. In this class, changes in \([Ca^{2+}]_i\) cause changes in the intensity of their fluorescence excitation and emission without any alteration in the wavelength of spectral maxima. Quin-2, fluo-3 and rhod-2 are classified in this group [277]. Single wavelength monitoring allows a video camera attachment.

2 Dual wavelengths ratiometric dyes. In this group, changes in \([Ca^{2+}]_i\) also bring about changes in their fluorescence intensity, but the \(Ca^{2+}\)-free and \(Ca^{2+}\)-bound forms of the dye have distinct spectra. Fura-2 and indo-1 are the two most commonly employed ratiometric
Antigen-stimulated rise in $[\text{Ca}^{2+}]$ indicators. The use of ratiometric dyes minimises the effect of many artifacts that are unrelated to changes in $[\text{Ca}^{2+}]$, such as changes in cell thickness and loss of indicator from a cell by leakage. Fura-2 is excited alternately at two different wavelengths, and the emission is measured at a single fixed wavelength. In contrast, indo-1 is excited at a fixed wavelength and the emission is monitored simultaneously at two different wavelengths. Thus, indo-1, potentially, can give better temporal resolution. Dual-wavelength monitoring allows imaging and therefore spatial resolution [277]. The properties of this group offer some advantages over single-wavelength dyes as seen below:

**Advantages of ratiometric $\text{Ca}^{2+}$ indicator**

- a) Their higher fluorescence can be used to decrease intracellular dye concentration which can buffer $[\text{Ca}^{2+}]$, transient.
- b) Because of a slightly lower affinity for $\text{Ca}^{2+}$, these dyes do not approach saturation and loss of resolution at an elevated level of $[\text{Ca}^{2+}]_i$ (>1 μM).
- c) Ratiometric measurements give estimation of $[\text{Ca}^{2+}]_i$ independent of dye concentration.
- d) Higher selectivity for $\text{Ca}^{2+}$ over other divalent cations avoids falsely low readings of $[\text{Ca}^{2+}]_i$.

### 6.1.2 Antigen-induced $\text{Ca}^{2+}$ signal in RBL-2H3 cells

Measurements of $^{45}\text{Ca}^{2+}$ uptake have shown a large increase in $\text{Ca}^{2+}$ influx upon mast cell antigen stimulation [101, 278, 279]. The antigen-induced increase in $[\text{Ca}^{2+}]_i$ in RBL-2H3 cells was monitored for the first time by Beaven *et al.* in 1984 [20, 21]. These authors used quin-2, a single-wavelength intensity-modulating $\text{Ca}^{2+}$ indicator, and they measured $[\text{Ca}^{2+}]_i$ in a population of cells loaded with the dye. It was reported that the $[\text{Ca}^{2+}]_i$ increased from 104 ± 5 nM to a maximum 1181 ± 290 nM within 2 to 3 min after antigen stimulation, and it then decreased slowly over 30 min [20]. Antigen stimulation in RBL-2H3 cells results in a biphasic increase in $[\text{Ca}^{2+}]_i$. The early, transient phase predominantly reflects $\text{Ca}^{2+}$ release from internal stores, whereas the secondary, plateau phase reflects $\text{Ca}^{2+}$ influx into the cells.

The requirement of the antigen-induced $\text{Ca}^{2+}$ signal and secretion for the continuous presence of $[\text{Ca}^{2+}]_o$ suggests that these responses are mainly dependent on $\text{Ca}^{2+}$ influx.
6.1.3 Antigen-induced Ca\textsuperscript{2+} oscillations in RBL-2H3 cells

In 1988, Miller et al. [114] reported the first investigation of antigen-stimulated [Ca\textsuperscript{2+}]\textsubscript{i} changes in individual RBL-2H3 cells. The Ca\textsuperscript{2+} response reported in single RBL-2H3 cells is quite different from the Ca\textsuperscript{2+} response measured in a cell population. It has been shown that lag times from antigen addition to the first Ca\textsuperscript{2+} spike vary widely among individual cells and that the first Ca\textsuperscript{2+} spike in a single cell can occur much more quickly than that reported for a cell suspension [114]. Later in 1989 [109], when the ratio images from the antigen-stimulated RBL-2H3 cells could be acquired at 1 Hz, compared with 0.1 Hz in the previous work, these authors demonstrated that antigen-induced Ca\textsuperscript{2+} changes were in fact oscillatory.

Although antigen-induced Ca\textsuperscript{2+} oscillations in single cells differ in frequency, amplitude and shape, the response is clearly dose-dependent and increases with increasing antigen concentration. Moreover, the lag time to onset of the response decreases with increasing antigen concentration or in the absence of [Ca\textsuperscript{2+}]\textsubscript{o} [280]. Ca\textsuperscript{2+} oscillation can also be observed in depolarised cells and in the absence of [Ca\textsuperscript{2+}]\textsubscript{o}, suggesting that Ca\textsuperscript{2+} oscillation might be due to periodic Ca\textsuperscript{2+} release from internal Ca\textsuperscript{2+} stores. It has also been reported that the heterogeneity of antigen-induced Ca\textsuperscript{2+} oscillations in RBL-2H3 cells is not due to cell cycle difference or to the presence of different cell clones in a population [109, 115].

6.2 Methods and materials

All materials and experimental procedures are outlined in the appropriate sections in the second chapter. DNP-HSA, A23187, Con-A and the tested compounds, except quinidine, did not interfere with the recording of the indo-1 ratio. The fluorescence interference caused by quinidine prevented the study of the antigen-induced rise in [Ca\textsuperscript{2+}]\textsubscript{i}. Anion exchanger inhibitors such as probenecid and sulfinpyrazone have been reported to decrease dye loss from cells during experiments [109, 277]. However, anion exchanger inhibitors were not used, in order to prevent any effects on the signal transduction mechanism of antigen-induced RBL-2H3 activation, in the experiments reporting in this chapter. Ba\textsuperscript{2+}, charybdotoxin and cetiedil had no effect on the resting [Ca\textsuperscript{2+}]\textsubscript{i}. 
6.3 Results

6.3.1 Indo-1 had no effect on antigen-induced β-hexosaminidase release

Incubation of RBL-2H3 cells with 1μM Indo-1 for 30min (under exactly the same conditions as that for the procedure of measuring \([Ca^{2+}]_i\)), had no significant effect on spontaneous and antigen-induced β-hexosaminidase release from RBL-2H3 cells (Table 6.1). Moreover, 2μM Indo-1 after 1 hour preincubation with RBL-2H3 cells also had no significant effect on spontaneous and antigen-stimulated β-hexosaminidase release.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>%Spontaneous release</th>
<th>%Net Antigen-stimulated release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.6 ± 0.6</td>
<td>37.0 ± 1.4</td>
</tr>
<tr>
<td>Indo-1 [1μM]</td>
<td>4.1 ± 1</td>
<td>37.7 ± 3.0</td>
</tr>
</tbody>
</table>

Table 6.1: Effect of Indo-1 on the spontaneous and antigen-induced β-hexosaminidase release. Adherent cells in the culture media were incubated at 37°C with Indo-1 (1μM) for 30min and then challenged with antigen (DNP-HSA 1ng/ml) for 15min.

6.3.2 Heterogeneity in response to antigen in different individual cells

A marked heterogeneity in the antigen-induced Ca^{2+} oscillations in different single cells was seen, as illustrated in Fig. 6.1. As shown, antigen stimulation (DNP-HSA 1ng/ml) of RBL-2H3 cells in 18 individual cells caused a sharp increase in \([Ca^{2+}]_i\), and Ca^{2+} oscillations with diverse patterns unique to any individual cell (Ca^{2+}-fingerprint). The results shown in Fig.6.1 are divided into three groups (columns). Column 1 demonstrates a group of cells which show a sustained decline in \([Ca^{2+}]_i\), after the sharp increase, with little or no Ca^{2+} oscillations. Irregular, repetitive spiking activity is clearly
obvious in column 2. In this group, Ca\(^{2+}\) spikes are different in frequency, amplitude and shape among individual cells. Column 3 shows a group of cells which falls between the other two. Here the cells show Ca\(^{2+}\) oscillations which are superimposed on an elevated [Ca\(^{2+}\)]. However, both the frequency and amplitude of early Ca\(^{2+}\) spikes are often greater than the subsequent spikes and decrease or even disappear (cells O and C).

### 6.3.3 Antigen dose-dependently induced an increase in [Ca\(^{2+}\)] and Ca\(^{2+}\) oscillations

Although antigen-induced Ca\(^{2+}\) oscillations are diverse in frequency, amplitude and shape, the Ca\(^{2+}\) response is clearly dependent on the antigen concentration (Fig. 6.2). The magnitude of the Ca\(^{2+}\) responses generally increased whereas the average lag time decreased with increasing antigen concentrations. Stimulation of RBL-2H3 cells with sub-maximal concentrations of antigen (0.001, 0.01, 0.1 ng/ml DNP-HSA) caused Ca\(^{2+}\) spikes whose amplitudes were increased by the higher dose of antigen, and the frequency of the spikes also increased. The peak change in Ca\(^{2+}\) response can be observed with the addition of 1ng/ml DNP-HSA. However, as mentioned in the previous section, the Ca\(^{2+}\) response among individual cells ranges from irregular repetitive spiking to steady decay of first Ca\(^{2+}\) spike without any oscillation. Moreover, Ca\(^{2+}\) spikes stimulated by 1ng/ml DNP-HSA are quite high in amplitude and low in frequency, compared with lower antigen concentrations. Stimulation with 10ng/ml DNP-HSA shows a smaller Ca\(^{2+}\) response compared to 1ng/ml DNP-HSA. To enable a comparison between the effect of different concentrations of antigen on changes in [Ca\(^{2+}\)], the area under the curves (AUC) have been calculated. The dose-response curve of the calculated AUC is consistent with the bell-shaped dose-response curve for the antigen-stimulated mediator release. Fig. 6.3 shows a typical Ca\(^{2+}\) response to 1ng/ml DNP-HSA which lasted over 30min. Although the height of spikes has decreased with time, it is not clear to what extent this decrease is related to dye quenching and how much corresponds to an actual decline in the Ca\(^{2+}\) response.

Cells that had not been maximally stimulated showed rapid additional Ca\(^{2+}\) oscillations on addition of a further concentration of antigen (Fig. 6.4). These results are in agreement with those reported in RBL-2H3 cell populations [20].
Figure 6.1: Antigen induced Ca\(^{2+}\) oscillations in 18 different individual RBL-2H3 cells. Cells were loaded with Indo-1 AM (1µM for 30min). Cells were then challenged with DNP-HSA 1ng/ml at 37°C and the changes in [Ca\(^{2+}\)] were monitored (see section 2.8.3 for details).
Figure 6.2: Ca^{2+} oscillations induced by different concentrations of antigen in 15 typical individual RBL-2H3 cells. In each experiment, the Indo-1 loaded cell (passively sensitised) was challenged with different concentration of antigen as shown on each row (3 cells in each row were stimulated by DNP-HSA at a concentration which is stated at the right top of the row).
Figure 6.3: Antigen-stimulated Ca^{2+} oscillation recorded for 30min in a single cell. As shown, the Ca^{2+} oscillations induced by antigen last for more than 30min. Indo-1-loaded cells were challenged at 37°C with antigen (DNP-HSA 1ng/ml) 2min before recording. The 3 rows are consecutive recordings of Ca^{2+} oscillations from an individual cell (each 600sec).
Figure 6.4: Enhancement of the Ca\(^{2+}\) oscillations induced by low concentrations of antigen, following the addition of higher doses of antigen. Addition of higher concentrations of antigen to cells that were already stimulated by lower concentration of antigen cause a further change in the shape of Ca\(^{2+}\) oscillation and increase in \([\text{Ca}^{2+}]_i\). As shown, rapid additional Ca\(^{2+}\) oscillations were induced by a higher concentration of antigen. Indo-1-loaded cells were challenged with submaximal concentrations of antigen (DNP-HSA 0.01 or 0.1ng/ml). Each tracing in this figure shows the response of an individual RBL-2H3 cell.
6.3.4 Dose-response relationship of the antigen-stimulated rise of \([\text{Ca}^{2+}]_{\text{i}}\) in RBL-2H3 cells

As described in chapter 2, the data, obtained from the antigen-induced \(\text{Ca}^{2+}\) oscillations, were quantified by measuring the area under the curve (AUC) of the \(\text{Ca}^{2+}\) response for each individual cell. This allowed comparison among the heterogeneous \(\text{Ca}^{2+}\) responses in single cells. Fig. 6.5 shows that the dose-response curve for the calculated AUC of antigen-induced \(\text{Ca}^{2+}\) oscillations is closely related to that for the depolarisation. This is compatible with the hypothesis that antigen-stimulated depolarisation is caused by an increase in \([\text{Ca}^{2+}]_{\text{i}}\), through \(\text{Ca}^{2+}\) influx. Although the curves shown in figure 6.5 are similar in shape, the maximum concentration is different for these responses, 10ng/ml for the \(^8\text{Rb}^+\)-efflux and 1ng/ml for the \(\text{Ca}^{2+}\) response and depolarisation. Likewise, the EC\(_{50}\) values of antigen (DNP-HSA) were 0.2, 0.19, 0.2 and 1.9ng/ml for \(\beta\)-hexosaminidase release, depolarisation, \(\text{Ca}^{2+}\) response and \(^8\text{Rb}^+\)-efflux, respectively.

6.3.5 Lag time of antigen-induced \(\text{Ca}^{2+}\) oscillations

As shown in figure 6.6A, the lag-time decreases with increasing antigen concentration and shows greater variability at the lower concentration of DNP-HSA so that the error bar of the 0.001ng/ml point is quite large. It has been reported that the lag time is related to the degree of IgE-receptor crosslinking, receptor density, stage of cell growth, speed of second messenger production (e.g. IP\(_3\)) and the extent of \(\text{Ca}^{2+}\) buffering by the cell [114]. More recently, it has been shown that the delay in I\(_{\text{CRAC}}\) activation is inversely dependent on IP\(_3\) concentration in the patch pipette solution. At low concentrations of intracellular IP\(_3\) (3\(\mu\)M), CRAC channels are activated with longer latency and a wide variability in individual cells [153]. Thus, in the case of antigen, the lag time for \(\text{Ca}^{2+}\) oscillations might be dependent on the time required for tyrosine kinase and PLC activation as well as for the production and action of enough IP\(_3\) to release \(\text{Ca}^{2+}\) from internal stores and activate I\(_{\text{CRAC}}\).

6.3.6 The resting \([\text{Ca}^{2+}]_{\text{i}}\) and the peak changes in \([\text{Ca}^{2+}]_{\text{i}}\) induced by antigen

The mean resting \([\text{Ca}^{2+}]_{\text{i}}\) in RBL-2H3 cells, measured in 90 different cells, was 133 ± 24nM. In this study, the maximum increase in \([\text{Ca}^{2+}]_{\text{i}}\) induced by different concentrations of antigen was also measured. As shown in figure 6.6B, the dose-response...
curve of the antigen-induced peak changes in $[\text{Ca}^{2+}]_i$ is bell-shaped. The shape of this curve is similar to that for AUC, but is less steep.

6.3.7 **A23187 induced a rise in $[\text{Ca}^{2+}]_i$ without $\text{Ca}^{2+}$ oscillation**

Figure 6.7 shows a typical experiment in which the $[\text{Ca}^{2+}]_i$ change induced by the calcium ionophore A23187 ($1 \mu\text{M}$) was monitored. In contrast to antigen, A23187 did not induce $\text{Ca}^{2+}$ oscillations. A23187 induced an increase in $[\text{Ca}^{2+}]_i$ that had a delay of about 20-30 seconds, suggesting that this $\text{Ca}^{2+}$ ionophore increased $[\text{Ca}^{2+}]_i$ primarily by depleting internal $\text{Ca}^{2+}$ stores which, in turn, may activate CRAC channels [147]. Thus, it is unlikely that A23187 simply increases $[\text{Ca}^{2+}]_i$ by increasing $\text{Ca}^{2+}$ transport across the plasma membrane. This conclusion is corroborated by the inhibition of A23187-induced $\text{Ca}^{2+}$ influx and secretion in ATP-depleted and high $K^+$-depolarised cells [96, 111, 112].

6.3.8 **Thapsigargin also increased the intracellular $\text{Ca}^{2+}$ concentration without oscillations**

The effect of thapsigargin, a $\text{Ca}^{2+}$-ATPase pump inhibitor, was also investigated. Figure 6.8 shows that thapsigargin ($1 \mu\text{M}$) induced a $\text{Ca}^{2+}$ response without any oscillation. Thapsigargin inhibits the $\text{Ca}^{2+}$-ATPase pump, and due to the leakage of $\text{Ca}^{2+}$ from stores and inhibition of reuptake, causes store depletion. This can also lead to the activation of CRAC channels. There is a delay before the rise of $[\text{Ca}^{2+}]_i$ and this is likely to be due to the time taken for store depletion by thapsigargin.

6.3.9 **Concanavalin A-induced an increase in $[\text{Ca}^{2+}]_i$**

Concanavalin A (Con A) aggregates IgE receptors and induces mediator release from RBL-2H3 cells. As shown in Fig. 6.9, Con A ($20 \mu\text{g/ml}$) induced $\text{Ca}^{2+}$ oscillations in passively sensitised RBL-2H3 cells. These data indicate that $\text{Ca}^{2+}$ oscillations can also be induced by other IgE crosslinking agents.
Figure 6.5: Dose-response curves for four antigen-induced cell responses in passively sensitised RBL-2H3 cells. A) The calculated area under the curve (AUC) for the stimulated Ca\(^{2+}\) oscillations by antigen. B) The antigen-induced depolarisation. C) The antigen-stimulated mediator release by antigen (■ histamine release and ● β-hexosaminidase release). D) antigen-stimulated \(^{86}\)Rb\(^{-}\)-efflux. (for details of methods see chapter 2)
Figure 6.6: Dose-response curve for the lag time of the Ca\(^{2+}\) response (A) and the peak changes in [Ca\(^{2+}\)]\(_i\) induced by antigen (B). Indo-1 loaded RBL-2H3 cells were challenged with various concentrations of antigen (DNP-HSA). Lag time was determined by measuring the time between antigen addition and the initiation of the first Ca\(^{2+}\) spike. Maximum [Ca\(^{2+}\)]\(_i\) was determined by measuring the highest [Ca\(^{2+}\)]\(_i\) induced by antigen. Student's paired t-test, * p<0.05 and *** p<0.001.
Figure 6.7: A23187-induced rise in [Ca^{2+}]_{i} in RBL-2H3 cells: After 10 min preincubation at 37°C, an Indo-1 loaded RBL-2H3 cells were stimulated with calcium ionophore A23187 [1μM] at time 0 sec. This is a typical response of a RBL-2H3 cell from the results of 5 different cells on different days. The calculated AUC values of A23187-induced (1μM) Ca^{2+} increase was about 47,866.9 ± 13,154.01 nM.sec (mean ± SEM).
Figure 6.8: Thapsigargin-induced rise in $[\text{Ca}^{2+}]_i$ in RBL-2H3 cells. After 10min preincubation at 37°C, an Indo-1 loaded RBL-2H3 cell was stimulated with thapsigargin at time 0sec. This is a typical response of a RBL-2H3 cell from the results of 3 different cells on different days. The calculated AUC values of thapsigargin-induced (1μM) Ca$^{2+}$ increase was about 304581.5 ± 31763.8 nM.sec (mean ± SEM).

Figure 6.9: Con A-induced rise in $[\text{Ca}^{2+}]_i$ and Ca$^{2+}$ oscillations in RBL-2H3 cells. After 10min preincubation at 37°C, an indo-1 loaded RBL-2H3 cell was stimulated with Con A at time 0sec. This is a typical response of a RBL-2H3 cell from the results of 3 different cells on different days. The calculated AUC values of Con A-induced (1μM) Ca$^{2+}$ oscillation was about 223084.6 ± 20347.08 nM.sec (mean ± SEM).
6.3.10 Ca\textsuperscript{2+} oscillations induced by antigen in Ca\textsuperscript{2+}-free buffer

It has been shown that Ca\textsuperscript{2+} influx is necessary to sustain but not to initiate Ca\textsuperscript{2+} oscillations [109, 280]. Absence of extracellular Ca\textsuperscript{2+}, incubation of the cells with La\textsuperscript{3+} and depolarisation with high K\textsuperscript{+} solution, inhibit Ca\textsuperscript{2+} influx induced by antigen in RBL-2H3 cells. Ca\textsuperscript{2+} oscillations (1-4 spikes) can be seen in the absence of external Ca\textsuperscript{2+} [109]. As shown in figure 6.10, removal of extracellular Ca\textsuperscript{2+} abolished Ca\textsuperscript{2+} oscillations and the subsequent reintroduction of Ca\textsuperscript{2+} caused their resumption. Figure 6.11 shows three typical experiments in which cells were challenged with antigen (DNP-HSA 1ng/ml) in Ca\textsuperscript{2+}-free buffer solution containing 100\textmu M EGTA. Subsequent addition of extracellular Ca\textsuperscript{2+} led to an immediate increase in [Ca\textsuperscript{2+}], and the appearance of Ca\textsuperscript{2+} oscillations. As seen in figure 6.11, the lag time for the antigen-induced rise in [Ca\textsuperscript{2+}], in Ca\textsuperscript{2+}-free buffer was 55 ± 4 seconds, which is significantly longer than that in the normal buffer (38 ± 2 seconds, Student paired t-test, p < 0.01).

6.3.11 NECA induces a transient increase in [Ca\textsuperscript{2+}]

Ali et al. [79, 160] have shown that NECA induces a transient IP production and a transient rise in [Ca\textsuperscript{2+}]. Although NECA fails to induce mediator release, it potentiates antigen-stimulated secretion [79]. As shown in Fig. 6.12, NECA can induce a transient rise in [Ca\textsuperscript{2+}], which returned to its basal level within 100sec. This is consistent with the results obtained from a cell population of RBL-2H3 cells [79]. Again, this is indicative of the importance of the sustained rise of Ca\textsuperscript{2+} influx and oscillations for antigen-induced secretion in RBL-2H3 cells.

6.3.12 Effect of BaCl\textsubscript{2} on the antigen-induced Ca\textsuperscript{2+} oscillations

Ba\textsuperscript{2+}, a non-specific K\textsuperscript{+} channel blocker, inhibits antigen-induced 6-hexosaminidase release. It has been proposed that this inhibition of secretion is due to blockade of the outward rectifier K\textsuperscript{+} channels [53, 161] and this blockade in turn, reduces the electrochemical driving force for Ca\textsuperscript{2+} influx. Ba\textsuperscript{2+} blocks G-protein-linked IR K\textsuperscript{+} channels (0.1mM), although it cannot completely block the OR K\textsuperscript{+} channel even at a concentration of 20mM [209, 210]. As shown in Fig. 6.13, Ba\textsuperscript{2+} dose-dependently decreased the AUC of the Ca\textsuperscript{2+} response stimulated by antigen. It is likely that the effect
of Ba\(^{2+}\) on antigen-induced Ca\(^{2+}\) oscillations in RBL-2H3 cells may be due to a direct action on CRAC channels (see also chapters 3 and 5).

Figure 6.10: Continuous requirement of extracellular Ca\(^{2+}\) for the antigen-stimulated Ca\(^{2+}\) oscillation. After 10 min preincubation at 37°C, an Indo-1 loaded RBL-2H3 cell was stimulated with 1 ng/ml DNP-HSA. As seen extracellular Ca\(^{2+}\) was removed and reintroduced three times. This is a typical response of a RBL-2H3 cell from the results of 6 different cells on different days.
Figure 6.11: Antigen-induced transient $[Ca^{2+}]_i$ oscillations in the absence of extracellular Ca$^{2+}$. Indo-1 loaded RBL-2H3 cells were challenged with antigen (DNP-HSA 1ng/ml) in the absence of external Ca$^{2+}$. A few Ca$^{2+}$ spikes (1-4 spikes) were observed with a delay after antigen stimulation, but Ca$^{2+}$ spikes were dampened very fast. An increase in $[Ca^{2+}]_i$ was immediately observed after reintroduction of external Ca$^{2+}$. 
Figure 6.12: NECA stimulated a transient increase in [Ca$^{2+}$]. Indo-1-loaded cells were activated by NECA 10μM and changes in [Ca$^{2+}$] were measured by recording the ratio of emitted wavelengths of Indo-1. Three typical experiments are presented.


6.3.13 Effect of charybdotoxin on the antigen-induced \( \text{Ca}^{2+} \) response

Charybdotoxin (ChTX) blocks \( \text{IK}_{\text{Ca}} \), \( \text{BK}_{\text{Ca}} \) and \( \text{K}_{\nu 1.3} \) channels in different tissues [176, 281]. In this study, it has been shown that ChTX inhibited antigen-induced secretion, \(^{86}\text{Rb}^+\)-efflux and the repolarisation phase in RBL-2H3 cells. The effect of ChTX on the antigen-stimulated rise in \( [\text{Ca}^{2+}]_i \) was also investigated. As shown in Fig. 6.13, ChTX significantly (Mann-whitney test, \( p<0.01 \)) inhibited the antigen-induced \( \text{Ca}^{2+} \) response (measured by the AUC of \( \text{Ca}^{2+} \)/time curve). The inhibitory effect of charybdotoxin on \( \text{Ca}^{2+} \) oscillations is consistent with the inhibitory effect of ChTX on antigen-induced \( \beta \)-hexosaminidase release and on the repolarisation phase of antigen-induced depolarisation (with a similar IC\text{50} \approx 85\text{nM}). It is necessary to mention that both MgTX, a \( \text{K}_{\nu 1.3} \) channel blocker, and IbTX, a \( \text{BK}_{\text{Ca}} \) channel blocker, did not affect antigen-induced \( \beta \)-hexosaminidase release, suggesting that the channel involved resembles the \( \text{IK}_{\text{Ca}} \) channel in red blood cells.

6.3.14 Effect of cetiedil on the antigen-induced \( \text{Ca}^{2+} \) response

Cetiedil, a non-specific \( \text{K}^+ \) channel blocker, and some of its analogues (UCL compounds) inhibited antigen-induced \( \beta \)-hexosaminidase release and \(^{86}\text{Rb}^+\)-efflux from passively sensitised RBL-2H3 cells (see chapters 3 and 5). Unfortunately, assessing the effect of cetiedil on antigen-induced membrane potential changes was not feasible due to fluorescence interference. Cetiedil enantiomers and UCL compounds inhibited antigen-stimulated \(^{86}\text{Rb}^+\)-efflux. As shown in Fig. 6.14, although cetiedil 50\( \mu \)M had no significant effect on the calculated AUC of antigen-induced \( \text{Ca}^{2+} \) oscillations, it markedly (Mann-whitney test, \( p<0.05 \)) inhibited the antigen-induced rise in \( [\text{Ca}^{2+}]_i \) and \( \text{Ca}^{2+} \) response at a concentration of 100\( \mu \)M.

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Figure 6.12: The effect of $\text{Ba}^{2+}$ on the antigen-induced $[\text{Ca}^{2+}]_i$ oscillations. Indo-1 loaded RBL-2H3 cells were incubated with or without $\text{Ba}^{2+}$ (5 or 10mM) for 15min and they were then challenged with antigen (DNP-HSA 1ng/ml). The top diagram shows the effect of $\text{Ba}^{2+}$ on the average of the AUC of antigen induced $[\text{Ca}^{2+}]_i$/time curves. The lower panel shows the comparison of typical experiments with antigen on its own (A) and antigen in the presence of $\text{Ba}^{2+}$ 10mM (B). Mann-whitney test, * $p<0.05$ and *** $p<0.001$. n=34 for control and n=10 for each concentration of $\text{Ba}^{2+}$.
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Antigen-stimulated rise in \( [\text{Ca}^{2+}] \).

Figure 6.13: Effect of charybdotoxin on the antigen-induced \( \text{Ca}^{2+} \) response. Indo-1 loaded RBL-2H3 cells were incubated with 100nM charybdotoxin and they were then challenged with antigen (DNP-HSA 1ng/ml). \( n=34 \) for control and \( n=12 \) for charybdotoxin. (Mann-whitney test, \( ** \ p < 0.01 \)).

Figure 6.14: Effect of cetiedil on the antigen-induced \( \text{Ca}^{2+} \) response. Indo-1 loaded RBL-2H3 cells were incubated with cetiedil (50 or 100\( \mu \)M) and they were then challenged with antigen (DNP-HSA 1ng/ml). \( n=34 \) for control and \( n=8-9 \) for each concentration of cetiedil. (Mann-whitney test, \( * \ p < 0.05 \)).
6.4 Discussion

6.4.1 The increase in \([Ca^{2+}]_i\) and Ca\(^{2+}\) oscillations

In RBL-2H3 cells, antigen stimulation causes an increase in \([Ca^{2+}]_i\), which consists of two phases: an initial rise related to \(Ca^{2+}\) release from internal stores and a sustained rise or plateau reflecting \(Ca^{2+}\) influx into the cells. There is a concentration gradient for \(Ca^{2+}\) across the plasma membrane, with the extracellular \(Ca^{2+}\) concentration being approximately four orders of magnitude greater than the intracellular \(Ca^{2+}\) concentration in resting cells: 100nM \([Ca^{2+}]_o\), compared with 1mM \([Ca^{2+}]_o\). Once RBL-2H3 cells are stimulated, \([Ca^{2+}]_i\) is increased from about 100nM to more than 1200nM, which is still less than the \([Ca^{2+}]_o\) by three orders of magnitude. In this study, the resting \([Ca^{2+}]_i\) was found to be 133 ± 24nM. This is close to the 104 ± 5nM reported from experiments with cell suspensions [20, 79]. In rat peritoneal mast cells, the resting \([Ca^{2+}]_i\) has been shown to be 113 ± 24nM [154]. Using digital fluorescence ratio imaging of fura-2, the resting \([Ca^{2+}]_i\) in RBL-2H3 cells has been reported to be 80nM [114].

Using ratiometric \(Ca^{2+}\) indicators, the antigen-induced increase in \([Ca^{2+}]_i\) was measured in individual RBL-2H3 cells, and this showed that the \(Ca^{2+}\) response in a single cell is quite different from the response seen in a cell suspension. Asynchronous \(Ca^{2+}\) oscillations, or irregular spikes, were observed with different frequencies, magnitudes and shapes. Extracellular \(Ca^{2+}\) is not necessary for the initiation of these \(Ca^{2+}\) oscillations but it is essential for sustained \(Ca^{2+}\) oscillations. Omitting extracellular \(Ca^{2+}\) after cell stimulation causes an immediate abrogation of \(Ca^{2+}\) oscillations (Fig. 6.10). Although \(Ca^{2+}\) oscillations are not completely inhibited in depolarised RBL-2H3 cells [109, 113], the possible influence of membrane potential changes in \(Ca^{2+}\) oscillations cannot be ruled out.

The antigen-induced \(Ca^{2+}\) oscillations show heterogeneity, which is not likely to be due to cell cycle or clone differences [109, 115]. Indo-1, a ratiometric \(Ca^{2+}\) fluorescent dye, was used to measure the changes in \([Ca^{2+}]_i\). Indo-1 (up to 2μM) had no significant effect on spontaneous and antigen-induced secretion from RBL-2H3 cells (Table. 6.1). In this study, irregular, repetitive spiking as well as an initial rise followed by a sustained decline in \([Ca^{2+}]_i\), was seen. In spite of the heterogeneity in amplitude, frequency and shape, \(Ca^{2+}\) oscillations are related to the degree of antigen stimulation (Fig. 6.2).

In this study, irregular, repetitive \(Ca^{2+}\) spikes were observed with submaximal concentrations of antigen (DNP-HSA <1ng/ml). The magnitude and frequency of these
Antigen-stimulated rise in $[Ca^{2+}]_i$.

Oscillations was enhanced with increasing antigen concentration. The maximal concentration of antigen (DNP-HSA 1ng/ml) induced $Ca^{2+}$ spikes with a high magnitude and frequency. In some cells, an initial rise in $[Ca^{2+}]_i$ was followed by a sustained decline, and $Ca^{2+}$ spikes were superimposed on an elevated $[Ca^{2+}]_i$.

The lag time (the time between antigen addition and the onset of $Ca^{2+}$ oscillations) and peak changes in $[Ca^{2+}]_i$ are also dependent on antigen concentration (Fig. 6.6A). Lag time was variable at very low concentrations of antigen and significantly decreased with increasing antigen concentration (Student’s paired t-test, $p<0.05$). This is probably related to the amount of IP$_3$ production, since the lag time of I$_{CRAC}$ activation by intracellularly applied IP$_3$ is also inversely dependent on the IP$_3$ concentration in the patch pipette solution [153]. Millard et al. [114] have also found that the lag time between antigen addition and the initiation of the $[Ca^{2+}]_i$ increase differs from cell to cell. These authors reported that increasing antigen concentration caused a decrease in the variability and length of the lag time [114]. A similar observation was also reported by Lee and Oliver [280], who in addition, found that the lag time was increased in the absence of $[Ca^{2+}]_o$.

The inverse relationship between the lag time and antigen concentration observed in the present study is in agreement with the previously published data, but the antigen concentration required for minimum lag time is lower (10ng/ml) than that reported previously [114, 280].

A23187 induced an initial rise of $[Ca^{2+}]_i$ followed by a sustained decline. No $Ca^{2+}$ oscillations were seen (Fig. 6.7). The same was true for thapsigargin (Fig. 6.8), a $Ca^{2+}$-ATPase inhibitor, which depletes the internal $Ca^{2+}$ stores and activates CRAC channels. This result suggests that fluctuations in the filling state of the internal $Ca^{2+}$ stores may be involved in $Ca^{2+}$ oscillations.

Like antigen, Con A was found to be capable of producing $Ca^{2+}$ oscillations in passively sensitised RBL-2H3 cells (Fig. 6.9). Since other activators of the CRAC channel, like $Ca^{2+}$ ionophores or $Ca^{2+}$ ATPase inhibitors, fail to produce $Ca^{2+}$ oscillations, a possible important role for fluctuations in the level of PIP$_2$ metabolites in $Ca^{2+}$ oscillations can be suggested.

NECA and other adenosine analogues, by themselves, fail to induce secretion from RBL-2H3 cells [79]. However, they potentiate the release of histamine but not arachidonic acid [79, 80]. It has been proposed that NECA activates PLC through PTX- and CTX-sensitive G-proteins (designated G$_i$). I$_{CRAC}$ activation by NECA has also been observed in the presence of PKC inhibitors. The present results (Fig. 6.12) have shown
the NECA-induced rise in $[Ca^{2+}]_i$ in RBL-2H3 cells to be transient, possibly because the stimulation of PLC and IP$_3$ production is short-lived. The negative feedback resulting from the sustained activation of PLD and then PKC stimulation through the adenosine receptors may play an important role in halting the signal transduction pathways of secretion and $Ca^{2+}$ influx [160]. Likewise, the synergistic effect of adenosine is abrogated by down-regulation of PKC with phorbol ester pretreatment [276].

### 6.4.2 Effect of $Ba^{2+}$, charybdotoxin and cetiedil on the antigen-stimulated $Ca^{2+}$ oscillations

It has been shown that CRAC channel activation and a sustained increase in $[Ca^{2+}]_i$ needs a sufficient electrochemical driving force for $Ca^{2+}$ influx. Regarding the above results, it can be suggested that inhibition of outwardly rectifying K$^+$ channels causes RBL-2H3 cells to stay in a depolarised state which, in turn, inhibits antigen-induced $Ca^{2+}$ influx and secretion. The effect of $Ba^{2+}$, cetiedil and charybdotoxin on antigen-induced increases in $Ca^{2+}$ were measured (see next sections).

$Ba^{2+}$ (100μM) completely inhibits the IR K$^+$ current [125, 208, 209, 211], but cannot totally block the G-protein-linked OR K$^+$ channels even at a concentration of 20mM [208]. In the previous chapters, $Ba^{2+}$ has been shown to depolarise RBL-2H3 cells, but to have no significant effect on antigen-induced $^{86}Rb^+$-efflux. Thus, it can be deduced that the inhibitory effect of $Ba^{2+}$ on antigen-induced secretion cannot be due solely to blockade of the OR K$^+$ channels. It was interesting to evaluate the effect of $Ba^{2+}$ on the antigen-induced increase in $[Ca^{2+}]_i$. $Ba^{2+}$ dose-dependently inhibited antigen-induced $Ca^{2+}$ oscillations in RBL-2H3 cells. As already mentioned, $Ba^{2+}$ is a permeant blocker of CRAC channels [147]; it has the same permeability as $Ca^{2+}$ through CRAC channels at the resting membrane potential [150]. Since $Ba^{2+}$ had no effect on antigen-induced $^{86}Rb^+$-efflux and its inhibitory effect on antigen-stimulated mediator release was not affected by increasing extracellular $Ca^{2+}$ concentration, it can be concluded that the effect of $Ba^{2+}$ might be either direct, by interfering with $Ca^{2+}$ influx, or indirect, by causing depolarisation.

Since charybdotoxin inhibits antigen-stimulated β-hexosaminidase release, its effects on membrane potential changes and $^{86}Rb^+$-efflux, as well as on the increase in $[Ca^{2+}]_i$ have also been examined. Interestingly, ChTX inhibited the repolarisation phase and $^{86}Rb^+$-efflux with the same IC$_{50}$ (90nM). It was also able to inhibit $Ca^{2+}$ oscillations
(35% inhibition at 100nM). At a high concentration, ChTX can inhibit various RBL-2H3 cell responses, and its inhibitory effects might be due to the blockade of the K\(^+\) channels present in these cells. One of these channels, responsible for repolarisation and increased \(^{86}\text{Rb}^+-\text{efflux}\), has some similarities to the IK\(_{\text{Ca}}\) channel in red blood cells. However, electrophysiological evidence is needed to substantiate the existence of IK\(_{\text{Ca}}\)-like channels in RBL-2H3 cells.

A high concentration of cetiedil also inhibited antigen-induced Ca\(^{2+}\) oscillation in RBL-2H3 cells suggesting that inhibition of the stimulated K\(^+\) permeability by cetiedil affects the Ca\(^{2+}\) fluxes. These results indicate that increased K\(^+\) permeability and Ca\(^{2+}\) influx are related; each can be affected by the other. However, electrophysiological experiments on the effect of cetiedil and the UCL compounds on antigen-induced Ca\(^{2+}\) and K\(^+\) currents would be needed to confirm this hypothesis.
Chapter 7 Conclusion

7.1 Conclusion

The purpose of this study was to investigate whether $K^+$ channels in RBL-2H3 cells are involved in the antigen activation mechanism. In order to achieve this aim, appropriate experimental methods were applied to assess the effect of $K^+$ channel modulators on antigen-induced $\beta$-hexosaminidase release, membrane potential changes, $^{86}\text{Rb}^+$-efflux and increase in $[\text{Ca}^{2+}]_i$.

7.1.1 Effect of $K$ channel modulators on RBL-2H3 cell activation

The inhibitory effect of quinidine and $\text{Ba}^{2+}$ on antigen-induced secretion from RBL-2H3 cells has been suggested to be due to the $K^+$ channel blocking effect of these compounds. This is in keeping with the fact that antigen-induced depolarisation is followed by repolarisation, and antigen also induces an increase in $^{86}\text{Rb}^+$-efflux. Quinidine inhibited the repolarisation phase and antigen-induced $^{86}\text{Rb}^+$-efflux from these cells. However, $\text{Ba}^{2+}$ had no effect on resting and antigen-stimulated $^{86}\text{Rb}^+$-efflux and it caused a massive depolarisation by itself.

Although quinidine had no effect on A23187-stimulated secretion, $\text{Ba}^{2+}$ more potently inhibited cell activation by A23187 than by antigen. Moreover, an increase in extracellular Ca$^{2+}$ concentration reduced the inhibitory effect of quinidine on antigen-induced secretion. These results indicate that the effect of $\text{Ba}^{2+}$ may be due to interference with Ca$^{2+}$ influx rather than by direct $K^+$ channel blocking. Using a calcium ionophore or raising the extracellular calcium concentration decreased the inhibitory effect of quinidine
on antigen-induced secretion. When the extracellular calcium concentration is increased, the necessity of providing an electrochemical driving force is diminished, so that blockade of potassium channels has a smaller influence on Ca\(^{2+}\) entry. Thus, these results are consistent with a potassium channel blocking effect of quinidine. This is also consistent with the fact that the depolarisation induced by high K\(^{+}\) solution or CCCP inhibits RBL activation and secretion. This inhibition can be overcome by increasing the calcium concentration in Tyrode buffer (from 1.8mM to 10mM).

Since quinidine and Ba\(^{2+}\) are non-specific K\(^{+}\) channel blockers, it was appropriate to study the effect of some more selective K\(^{+}\) channel inhibitors on antigen-triggered \(\beta\)-hexosaminidase release. In this study, most of the specific K\(^{+}\) channel blockers tested, including blockers of voltage-sensitive, ATP-sensitive and inward rectifier K\(^{+}\) channels, had no effect on antigen-induced \(\beta\)-hexosaminidase release. This strongly suggests that the SK\(_{Ca}\) and BK\(_{Ca}\) channels, ATP-sensitive K\(^{+}\) channels, voltage-sensitive K\(^{+}\) channels, inward rectifier K\(^{+}\) channels and nitrendipine-sensitive outward rectifier K\(^{+}\) channels are not involved in the signal transduction mechanisms of RBL-2H3 cell activation.

However, cetiedil and charybdotoxin inhibited antigen-induced \(\beta\)-hexosaminidase release with an IC\(_{50}\) of 84\(\mu\)M and 130nM respectively. Some recently synthesised cetiedil-related compounds ("UCL compounds") have also been tested. All the UCL compounds inhibited antigen-stimulated \(\beta\)-hexosaminidase release and \(^{86}\)Rb\(^{+}\)-efflux and their potency was as follows: UCL1608 > 1710 > 1617 > 1348 > 1349 > 1495. Because these compounds interfere with the fluorescence signal, the effect of cetiedil and related UCL compounds on antigen-induced membrane potential changes could not be determined. Charybdotoxin inhibited repolarisation and \(^{86}\)Rb\(^{+}\)-efflux with a similar IC\(_{50}\) (~100nM) to inhibition of secretion. These results together with the observed lack of inhibitory effect of margatoxin (K\(_{v1.3}\) blocker), iberiotoxin (BK\(_{Ca}\) blocker) and TEA (BK\(_{Ca}\) and voltage-activated K\(^{+}\) channel blocker) on antigen-induced secretion, suggest that the possible K\(^{+}\) channel in RBL-2H3 cells may have some similarities to IK\(_{Ca}\) but not to BK\(_{Ca}\) or K\(_{v1.3}\) . This is consistent with the known inhibitory effect of cetiedil and the UCL compounds on IK\(_{Ca}\) channels in erythrocytes. However, since cetiedil and the UCL compounds are not selective inhibitors of IK\(_{Ca}\) channels, and the IC\(_{50}\) of charybdotoxin is also high, it should be kept in mind that the inhibitory effect of these compounds may be on K\(^{+}\) channels other than the IK\(_{Ca}\) subtype. It is also conceivable that a non-specific effect of these compounds on some other part of the signal transduction mechanism pathways of RBL-2H3 cell activation could be involved.
Chapter 7 Conclusion

Inhibition of CRAC channels is a particularly interesting possibility, since this study cannot rule out any direct effect of charybdotoxin, cetiedil and the UCL compounds on CRAC channel activation and on Ca^{2+} influx induced by antigen. Since both antigen-induced responses, \(^{86}\text{Rb}^+\)-efflux and the rise in \([\text{Ca}^{2+}]_i\), are dependent on each other, it is difficult to conclude from the present results whether the tested drugs have, at least in part, a direct effect on the CRAC channel. To study this, the effects of these compounds on the Ca^{2+} current through the CRAC channels and on the K^+ current postulated by Hoth [219] will have to be measured directly.

7.1.2 Some characteristics of increased \(^{86}\text{Rb}^+\)-efflux in stimulated RBL-2H3 cells

Antigen-stimulated \(^{86}\text{Rb}^+\)-efflux was dose-dependent and was correlated with the dose-response curve of \(\alpha\)-hexosaminidase release. In the absence of extracellular Ca^{2+}, the efflux was markedly (>35%) inhibited, whereas secretion was almost completely (95%) abolished. This suggests that there are two components to \(^{86}\text{Rb}^+\)-efflux, only one of which is dependent on extracellular calcium. Antigen-activated \(^{86}\text{Rb}^+\)-efflux, was nearly abolished by the addition of 1μM La^{3+}. Bathing cells in 140mM KCl did not change basal \(^{86}\text{Rb}^+\)-efflux but antigen-stimulated \(^{86}\text{Rb}^+\)-efflux was inhibited by ~30%; perhaps because of a reduction in Ca^{2+} influx. The present results suggest that at least two kinds of K^+ channels are activated by antigen; Ca^{2+}-independent (possibly G-protein-linked) channels whose activation is not necessary for secretion and Ca^{2+}-dependent channels which help to maintain an electrochemical driving force for Ca^{2+} influx.

A23187 stimulated \(^{86}\text{Rb}^+\)-efflux from the cells, but the amplitude of this stimulation was less than observed with antigen at the optimal dose. As already mentioned, A23187 depletes internal Ca^{2+} stores and activates CRAC channels. The activation of CRAC channels contributes to an increase in \([\text{Ca}^{2+}]_i\). Thus it seems that A23187 induces just the Ca^{2+}-sensitive (PTX-insensitive) component of \(^{86}\text{Rb}^+\)-efflux, as the A23187-induced \(^{86}\text{Rb}^+\)-efflux in RBL-2H3 cells is not inhibited by pertussis toxin [161].

Based on electrophysiological studies, it has been reported that ADP, ATP and NECA can open an outward rectifier K^+ channel in RBL cells [210]. It has been shown that NECA stimulates PLC (transient) and PLD (sustained) [160]. Perhaps, transient PLC activation is partly due to PKC activation (feedback inhibition of PLC) via sustained PLD stimulation (the metabolism of PA to DAG). 5’-(N-ethylcarboxyamido)adenosine
(NECA) also stimulated $^{86}$Rb$^+$-efflux. Since NECA induces a transient rise in $[Ca^{2+}]_i$ in RBL-2H3 cells (chapter 6), it seems that NECA stimulates just the PTX-sensitive component of $^{86}$Rb$^+$-efflux in these cells.

The present findings of inhibition of antigen-induced $^{86}$Rb$^+$-efflux by quinidine, La$^{3+}$ and the absence of extracellular Ca$^{2+}$ indicate that the efflux might partly be due to the opening of Ca$^{2+}$-activated K$^+$ channels. Similarly, secretion by antigen is inhibited by quinidine, La$^{3+}$ and the absence of extracellular Ca$^{2+}$. This again suggests that K$^+$ channel opening (possibly Ca$^{2+}$-activated) is involved in maintaining the electrochemical driving force for Ca$^{2+}$ influx during RBL-2H3 cell activation.

7.1.3 Antigen-stimulated Ca$^{2+}$ oscillations

Using ratiometric Ca$^{2+}$ indicators, the antigen-induced increase in $[Ca^{2+}]_i$ was measured in individual RBL-2H3 cells. Asynchronous Ca$^{2+}$ oscillations, or irregular spikes, with different frequencies, magnitudes and shapes were seen. Extracellular Ca$^{2+}$ is not necessary for the initiation of these Ca$^{2+}$ oscillations, whereas it is essential for them to be sustained. Omitting extracellular Ca$^{2+}$ after cell stimulation causes an immediate abrogation of Ca$^{2+}$ oscillations (Fig. 6.10). Although Ca$^{2+}$ oscillations are not completely inhibited in depolarised RBL-2H3 cells, a possible role for membrane potential changes in the Ca$^{2+}$ oscillations cannot be ruled out. Antigen can induce Ca$^{2+}$ oscillations with heterogeneity which is not likely to be due to cell cycle or clone differences [109, 115]. The antigen-induced rise in the $[Ca^{2+}]_i$ dose-response curve is bell-shaped and has a similar shape to that for the antigen-induced depolarisation. This is consistent with the idea that depolarisation is a result of Ca$^{2+}$ influx.

A23187 and thapsigargin induced an initial rise of $[Ca^{2+}]_i$ followed by a slow decline. No Ca$^{2+}$ oscillation was seen with a range of concentrations of these stimuli. Thus, since A23187 and thapsigargin cause a maintained store-depletion, and CRAC channel activation, these results suggest that fluctuations in the filling state of the internal Ca$^{2+}$ stores may be involved in the Ca$^{2+}$ oscillations.

Like antigen, Con A is capable of producing Ca$^{2+}$ oscillations in passively sensitised RBL-2H3 cells. It can be inferred that crosslinking agents can also induce Ca$^{2+}$ oscillations in these cells. NECA induced a transient rise in $[Ca^{2+}]_i$ without any oscillation. It has been proposed that NECA activates PLC through PTX- and CTX-sensitive G-proteins (designated $G_\alpha$). $I_{CRAC}$ activation by NECA has also been observed in
the presence of PKC inhibitors. The possible explanation of the transient rise in $[Ca^{2+}]_i$ is a short-lived stimulation of PLC and IP$_3$ production. Whether the negative feedback resulting from PKC activation plays an important role to stop the signal transduction, through adenosine receptors, remains to be determined.

$Ba^{2+}$, cetiedil and charybdotoxin inhibited antigen-induced Ca$^{2+}$ oscillations in RBL-2H3 cells at a concentration near to their IC$_{50}$ for the inhibition of secretion. As mentioned before, $Ba^{2+}$ might directly affect antigen-induced Ca$^{2+}$ influx. However, the effect of cetiedil and charybdotoxin may be due to K$^+$ channel blockade and a subsequent reduction in the electrochemical driving force for Ca$^{2+}$ influx.

Taken together, these data suggest that antigen-activation in RBL-2H3 cells needs the opening of at least one and more probably, two types of potassium channel to maintain a sufficient electrochemical driving force for calcium influx.

7.1.4 Can the OR K$^+$ channel in RBL-2H3 cells be a novel sub-type?

Relating the results of this study to the electrophysiological studies of others [219], it can be inferred that the K$^+$ channel involved in RBL-2H3 cell activation may be a new class of K$^+$ channel. This channel shows some similar pharmacological properties to the IK$_{Ca}$ channels. However, the lack of effect of clotrimazole and nitrendipine, and the different potencies of cetiedil enantiomers on antigen-induced β-hexosaminidase release, are not in keeping with the effect of these compounds on the IK$_{Ca}$ in red cells, suggesting that the channels are not identical. The recent study by Hoth [219] shows that an outward K$^+$ current is activated by IP$_3$-dependent store-depletion. Activation of this channel is simultaneous with I$_{CRAC}$ and the author called it the “store-depleted K$^+$ current”. However, the pharmacological properties and other characteristics of this channel remain to be studied. Hoth has also suggested that K$^+$ may pass through the CRAC channels [219].

7.1.5 Possibility of the existence of more than one OR K$^+$ channel in RBL-2H3 cells

The possible existence and involvement of more than one kind of OR K$^+$ channel in RBL-2H3 cell activation cannot be ignored. Since antigen-induced $^{86}$Rb$^+$-efflux consists of two (Ca$^{2+}$-sensitive and PTX-sensitive) components, this is indicative of the involvement of at least two kinds of K$^+$ channel. However, the available evidence (see
Chapters 5 and 6) suggests that only the Ca\(^{2+}\)-sensitive component of the K\(^+\) permeability increase is important in the signal transduction mechanism pathways of antigen activation of RBL-2H3 cells.

### 7.1.6 Is the OR K\(^+\) current carried through IR K\(^+\) channels?

Another possibility worth considering is that the IR K\(^+\) channel, due to its dual action, is an outward rectifier at a potential just positive to $E_K$. Thus this channel might open after antigen-induced depolarisation, which would shift the membrane potential into the range of conductance of the ‘IR’ K\(^+\) channels.

However, as mentioned in Chapter 1, the studies by McCloskey’s group [210] suggest that the OR and IR K\(^+\) channels in RBL-2H3 cells are distinct, having different G-protein linkage and pharmacological properties, so that this explanation seems unlikely.

### 7.2 Further studies

In order to further study the role of K\(^+\) channels in RBL-2H3 cell activation, electrophysiological studies are needed. As already mentioned, the best way to study antigen activation of RBL-2H3 cells is to use the perforated patch-clamp technique in cells maintained at 37\(^\circ\)C. The internal solution should contain K\(^+\) but not Cs\(^+\). Using this approach, it should be possible to study the effect of K\(^+\) channel modulators directly on the antigen-induced outward rectifier K\(^+\) channel. Ideally, [Ca\(^{2+}\)]\(_i\) should be measured at the same time, to study the possible relationship between activation of K\(^+\) current and its effect on Ca\(^{2+}\) oscillations. Using current clamped cells, the potential changes induced by antigen, A23187 and adenosine analogues could be compared in order to determine the exact amount of voltage change produced by these stimuli.

It would also be interesting to study the effect of A23187 and NECA on \(^{86}\text{Rb}\(^+\)-efflux from RBL-2H3 cells in Ca\(^{2+}\)-free buffer. This experiment would show if A23187 and NECA induce just one component of \(^{86}\text{Rb}\(^+\)-efflux (Ca\(^{2+}\)-sensitive component by the former and PTX-sensitive component by the latter). It is also worth considering measuring the effect of quinidine on NECA-induced \(^{86}\text{Rb}\(^+\)-efflux; the result of this experiment could clarify whether quinidine can inhibit both components of antigen-induced \(^{86}\text{Rb}\(^+\)-efflux. It would be important in future \(^{86}\text{Rb}\(^+\)-efflux experiments to further characterise the early and late phases of the antigen-induced \(^{86}\text{Rb}\(^+\)-efflux as described in Chapter 5.
Finally, since NECA causes PLD activation and PKC stimulation, it could be useful to study the effect of PKC inhibitors on the NECA-induced increase in \([\text{Ca}^{2+}]\). If PKC inhibition potentiates the \([\text{Ca}^{2+}]\) response induced by NECA, it could be inferred that PKC activation is the main reason for the short-lived RBL-2H3 cell activation induced by NECA. Also, a potentiated and prolonged NECA-induced response should be more amenable to study by the methods described in this thesis.
References


References


53. LABRECOQUE, G.F., HOLOWKA, D., & BAIRD, B. (1989). Antigen-triggered membrane potential changes in IgE-sensitized rat basophilic leukemia cells: evidence for a repolarizing response that is important in the stimulation of cellular
References


66. QUARTO, R. & METZGER, H. (1986). The receptor for immunoglobulin E:


78. OLIVER, J.M., BURG, D.L., WILSON, B.S., MCLAUGHLIN, J.L., & GEAHLEN,
References


References


References


References

and Ca^{2+}-activated K^+ channels in mouse pancreatic β-cells by external


