

Investigation of the Mechanism of Exocytosis from Permeabilised Guinea Pig Eosinophils

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ABSTRACT

The regulatory pathways controlling exocytosis vary among cell types. The work described here concerns the regulation of exocytosis in eosinophils. In order to investigate the late steps regulating exocytosis, and to exclude complications arising from events mediated by cell surface receptors, it is helpful to work with permeabilised cells. The bacterial cytolysin, streptolysin-O generates large lesions in the plasma membranes of cells and so provides a means to access the interior. The cytosol can then be manipulated with precision allowing regulation of such diverse entities as divalent cations, nucleotides and even proteins which leak out, or which can be applied from the exterior, and their effect on exocytosis measured. When permeabilised with streptolysin-O, secretion of hexosaminidase from guinea pig eosinophils can be induced by Ca^{2+} and $\text{GTP}\gamma\text{S}$ both of which have been shown to be necessary. Extending this I have measured secretion of aryl sulphatase and peroxidase and in accompanying sub-cellular fractionations, I found that only the peroxidase is an exclusive component of the dense secretory (crystalloid) granules.

If the application of the stimulus is delayed after permeabilisation, soluble proteins leak out and the secretory response runs down (over 30-50min). The rate at which the response runs down depends on the composition of the permeabilisation buffer. The inclusion of Ca^{2+} (10^{-5}M), or soluble proteins derived from brain homogenates slows the rate of the rundown, and when both are applied together the run down is almost abolished. Using this technique as a basis of bio-assay, I have achieved a partial purification of two active factors from porcine brain homogenates. In addition, I have applied recombinant cytosolic proteins, in particular RhoGDI, to test their roles in the regulation of exocytosis.

ABBREVIATIONS USED IN THESIS

| | |
|----------------------|--|
| ATP | Adenosine triphosphate |
| BSA | Bovine serum albumin |
| b-g | β -glucuronidase |
| BAL | Bronchial alveolar lavage |
| C5a, C3a | Complement factor 5a, 3a |
| DFP | Diisopropyl fluorophosphate |
| ECP | Eosinophil cationic protein |
| EDN (EPX) | Eosinophil derived neurotoxin (Eosinophil protein X) |
| EGTA | Ethylene glycol-O,O'-bis (2-aminoethyl)-N', N,N',N'-tetraacetic acid |
| EPO | Eosinophil peroxidase |
| ETYA | Eicosa-5,8,11,14, tetraynoic acid |
| FMLP | Formylmethionyl-leucyl-phenylalanine |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| GTP γ S | Guanosine 5'-O-3- (thiotriphosphate) |
| HBSS | Hanks balanced salt solution |
| HEX (Hexosaminidase) | N-acetyl- β -D-glucosaminidase |
| Ig | Immunoglobulins |
| IP ₃ | Inositol 1,4,5 triphosphate |
| LTB ₄ | Leukotriene b ₄ |
| mRNA | messenger ribonucleic acid |
| PAF | Platelet activating factor |
| pCa | $-\text{Log}_{10} [\text{Ca}^{2+}]$ |
| PIPES | Piperazine-N, N'-bis (2-ethane-sulfonic acid) |
| PKC | Protein kinase C |
| PMA | Phorbol 12-myristate 13-acetate |
| SL-O | Streptolysin-O |

THE DISCOVERY OF THE EOSINOPHIL

1.0 HISTORY

Over a century ago (1846), Wharton-Jones an anatomist at Charing Cross Hospital was able to distinguish granulated cells from red blood cells in the blood of several animal species including frogs. Using a simple light microscope, he observed the nuclei of these cells after the addition of either water or acids, ^{which caused cell swelling} ~~allowing the cells to swell up~~. It is unlikely that he was able to distinguish the eosinophil from other granulated cells because there were no stains [145].

With the help of staining techniques, Paul Ehrlich a young physician in Germany identified the eosinophil some 33 years after the work of Wharton-Jones. He found that these cells could be stained with a number of negatively charged chemicals, especially eosin, hence the name. Eosin, a brominated fluorescein derivative, stains eosinophils because they contain granules consisting of basic cationic proteins.

He also discovered that the site of origin of this cell is the bone marrow and even suggested that this cell type may exert its function on tissues. His knowledge and understanding of this cell is reflected in the papers he published in German and in a later ~~monograph~~ monograph in English [145]

1.1 Eosinophil lineage

Eosinophils like other myeloid cells are derived from haemopoietic stem cells. Certain features have led to the belief that these cells have a common link with basophils (they are derived from the same progenitor cells). Both of these cells contain so called Charcot-Leyden-Crystals, which are a major protein component of human eosinophils (but apparently absent in guinea pig eosinophils-[164]) and also contain major basic protein (MBP) although basophils contain a smaller percentage of this protein compared to

eosinophils [164]. Elevated counts of basophils and eosinophils have also been found in patients with allergic rhinitis [126] and in asthmatics [49]. It has been suggested however, that although these cells have their origins in the haemopoietic stem cell, there must be specific growth factors which divert them into two distinct pathways during differentiation. This is because when grown on agar, a single progenitor cell was able to develop into two distinct colonies of eosinophils and basophils [145]. Hence eosinophils are not basophils and basophils are not eosinophils.

1.2 Structure

Eosinophils are granule containing cells of about 8µm in diameter derived from the bone-marrow. Their nuclei can be bilobed or may even have three or more lobes. Guinea pig eosinophils can contain up to about 190 membrane-bound granules [102] which possess a crystalline core consisting mainly of major basic protein and a granular matrix containing three other basic cationic proteins amongst other protein markers. These cells also contain a second type of granule which lacks the crystalline core and can vary in size. This second granule makes up 5% of the eosinophil granule population (at least in human eosinophils) and is the location of the Charcot-Leydon crystal protein [43]. Electronmicrographs of human blood and tissue eosinophils revealed the presence of further small round or elongated granules of between 0.1 and 0.5 µm in diameter which stain very strongly for aryl sulphatase and phosphatase activities [127]. As well as these three types of granules, eosinophils contain lipid bodies which store arachidonic acid and enzymes such as cyclooxygenase [49] and 5-lipoxygenase [167]. Consequently, these contain the substrate and the enzymes for the production of prostaglandins and leukotrienes, the latter being present in bronchial alveolar lavage fluid (BAL fluid) of asthmatics [163]. Finally, eosinophils are known to have vesicotubular structures which contain cytochrome b₅₅₈ [50], a component of NADPH- oxidase which is involved in the production of reactive oxygen species.

1.3 Constituents of eosinophil granules

THE CRYSTALLOID GRANULE PROTEINS.

Eosinophil granules contain four basic cationic proteins, namely, major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil cationic protein (ECP) and eosinophil derived neurotoxin (EDN) also called eosinophil protein X (EPX)).

MBP

MBP which constitutes 50% of the granule protein, is found in the crystalline core of the dense secretory granule [97].

It consists of a single polypeptide chain of 117 amino acid residues whose molecular weight is 13.8kD and isoelectric point 10.9. Its richness in arginine residues (17 of them) accounts for its basic properties and ^{it} also contains 9 cysteine residues which provides the propensity for it to form disulphide bonds [164]. The reduced form of this protein has been found to be just as toxic to parasites as its native form [123]. Analysis of cDNA coding human MBP mRNA from an HL-60 cell line, indicated that MBP is translated as a preproprotein with an acidic pro-portion containing a 15-amino acid ^(aa) signal peptide that is followed by a pro-sequence of 90 aa dominated by glutamine and aspartate residues [165]. The calculated pI value of the pro-sequence is 6.2 suggesting that the pro-MBP is translated as a ^{non-basic and therefore possibly} nontoxic precursor which could prevent it from causing damage, while it is being processed in the endoplasmic reticulum. Since the pro-protein is apparently absent in mature granules, it is possible that the pro-protein is processed into the toxic form of MBP and crystallizes as the granule takes on its mature form [11]. MBP is not confined to eosinophils alone; small quantities of this protein have also been found in the granules of basophils [1]. It has been found to have elevated concentrations in the sera of pregnant women but its function in pregnancy is still unclear [105]. It is toxic to a number of helminthic parasites

such as schistosomulae of *Schistosoma mansoni* [25] and can also kill certain strains of *S. Aureus* and *Escherichia coli* [95]. MBP at low concentrations, causes damage to the airway epithelia of both guinea pigs and humans and this is considered to be an important component in the pathogenicity of asthma [68].

ECP and EDN

The similarity between ECP and EDN (EPX) and the evidence that genes encoding for both these proteins are found on the same chromosome (14) has given rise to the idea that they may have arisen from a gene duplication [59]. Both of these proteins consist of single polypeptide chains with isoelectric points ranging between 8.9 and 10.8. Their molecular weights seem to vary: the molecular weight of ECP being between 16 and 21.4kD and EDN between 15.5 and 18.6 kD. They both possess ribonuclease activity but EDN is about 50-100 times more active than ECP. ECP is more toxic to helminths than EDN [59].

EPO

Eosinophil peroxidase (EPO) is an abundant haem-containing protein in the granule matrix. It comprises a light chain of about 14kD and a heavy chain of about 58kD, both of which are derived and cleaved from the same mRNA strand. It shares about 68% amino acid identity with human neutrophil myeloperoxidase (MPO) and homologies with other peroxidase proteins. The human gene for EPO has been located on chromosome 17 [138]. In the presence of hydrogen peroxide, both EPO and MPO are able to oxidise halides to form halogens (X_2) and reactive acids such as hypohalous acid (HOX) and /or hypohalite (OX^-). Although the concentration of chloride (100mM) is a 1000 times that of bromide (20-100 μ M) in physiological conditions, bromide is the preferred halide for both enzymes. However, this preference is stronger for EPO than for MPO [144,155]. Electron spin resonance (ESR), indicates that the microenvironment of the haem groups

of the two enzymes are different [73]. When peroxidase inhibitors (such as azide or cyanide) are tested against these two enzymes, they are found to be more effective against EPO than against myeloperoxidase [21]. It has been shown [144] that thiocyanate is able to compete for the halide binding site and so it inhibits the effects of bromide and other halides even when they are in excess. This suggests that thiocyanate could actually be the preferred substrate for EPO. This has given rise to confusion, since EPO was initially described as the azide and cyanide insensitive peroxidase [7]. In early work however, the concentration dependence of inhibition by these compounds was apparently not tested.

The activity of this protein is toxic to micro-organisms, helminthic parasites and it causes damage to respiratory epithelium and pneumocytes [166]. It is interesting to find that a patient who lacks EPO shows no adverse clinical consequences [135].

OTHER GRANULE PROTEINS

The use of techniques such as in situ hybridization have shown that RNA coding for cytokines such as Granulocyte macrophage colony stimulating factor (GM-CSF) is produced after the cells have been stimulated with the Ca^{2+} ionophore A23187 [113]. Others have shown [69] that on being stimulated with this ionophore, IL-3 is produced in conjunction with GM-CSF. Subcellular fractionation studies show that GM-CSF is present in the crystalloid (dense) core granules [96]. Besides GM-CSF, eosinophils also express the mRNA for IL-5 in BAL cells obtained from asthmatic subjects [23]. Together these three cytokines are capable of prolonging the survival of eosinophils and are also present at various sites of inflammation [84]

Other cytokines found in these cells include IL-2, IL-4, IL-6, IL-8, Tumour necrosis factor $-\alpha$ (TNF- α) and Macrophage Inflammatory Protein -1α (MIP- α) [164].

1.4 The physiology of the eosinophil

The role of eosinophils!

In the 1960's James Hirsh and G.T Archer used cine films and photomicrographs to study the behaviour of eosinophils. They discovered that these cells were capable of undergoing degranulation and phagocytoses. They also noted that on degranulating, the cells released lysosomal enzymes as well as a peroxidase. With the use of high resolution electron micrographs they discovered crystalloid structures inside human and rat eosinophils [5,6]. The initial discovery of the potential for eosinophils to phagocytose led to the proposal that the main role of this cell is to ingest and dispose of foreign matter [136]. Another role they were thought to perform was the inactivation of histamine [4] and other mast cell derived mediators [54].

Eosinophils- Worm killers?

The role of eosinophils in helminthic infections is unclear and still a cause of controversy. However, the proliferation of both tissue and peripheral eosinophil numbers during infections by parasitic worms formed the basis for the theory of their role as worm-killers. Basten and Beeson [15] showed that the production of eosinophils in rats injected intravenously with *Trichinella spiralis* larvae was controlled by T-lymphocytes. They concluded that eosinophilia was a part of the immune response and that this is T-cell dependent [145]. The identification of the cytokines IL-3, IL-5 and Granulocyte macrophage colony stimulating factor (GM-CSF) has given an explanation for the control of eosinophilia by T-lymphocytes [119]. In spite of this, there is still the question of why the eosinophils increase in number during parasitic infections. To elucidate this function, both *in vivo* and *in vitro* studies have been performed.

In vitro studies

With the use of phase contrast and electron microscopic techniques Butterworth et al [26,27] demonstrated that the damage to Schistosomulae by human eosinophils is dependent upon the presence of antibodies from sera collected from patients with active *S. mansoni* infection. He and others [108,109,134] have shown that both rat and human eosinophils in the presence of antibodies attach to intact Schistosomulum as well as other parasites (*Trichinella spiralis* and *Nippostrongylus brasiliensis*) and destroy them. The eosinophils are seen first to adhere, then flatten and spread over the surface of the parasite after which the dense granule contents appear in the extracellular space between the parasite and the eosinophil, possibly by granule fusion with the plasma membrane. All the cells in contact with the parasite degranulate leaving their contents on the surface of the nematode which then undergoes a number of structural changes. The basal area of the tegument vacuolates and is then removed and subsequently disintegrates, the fragments being phagocytosed by unattached eosinophils [51]. The structural changes are believed to be mediated by the cationic proteins released by the eosinophils namely EPO, MBP, ECP and EDN which are certainly capable of causing damage when isolated and incubated with schistosomulae in the absence of eosinophils. For instance, MBP and ECP both cause a ballooning of the tegument which has a similar pattern to that induced by eosinophils [28,29]. The damage caused by these cationic proteins was thought to be due to their basic charge and further evidence for this idea was obtained when synthetic polycations generated similar patterns of damage on parasites [28].

In vivo studies

Eosinophils have been found to be in contact with the surface tegument of schistosomula of *S. haematobium* in the cutaneous tissue of immune monkeys. These areas are rich in eosinophils and had a large number of dead larvae as well. Secondly, the levels of ECP in the blood are elevated in

patients with filariasis which suggests that eosinophils would have been activated to degranulate releasing this protein [164]. The argument for eosinophil mediated responses is reasonable since rats undergoing rapid expulsion of *T. spiralis* release LTC₄ (a constituent of eosinophils), a process related to immediate-type hypersensitivity and associated with increased numbers of eosinophils in the gut mucosa [114]. By comparison with laboratory animals little is known about the sequence of events that control eosinophil mediated responses in man.

Although there is a lot of evidence (mainly *in vitro* studies) to support this role for eosinophils, recent work now brings this into question. Primary and secondary infections in mice with a reduced number of eosinophils (by pretreatment with anti-IL-5 antibody) and in mice with normal levels of eosinophils are similar. Thus the presence or effective absence of eosinophils did not seem to affect the parasitic infections. It must be noted however that this study has been limited to mice and other animal species would have to be tested. [66,85,142]

Eosinophils and wound healing

The presence and possible involvement of cells such as mast cells, neutrophils, macrophages, lymphocytes and eosinophils have been noted at wound sites but little is known about the involvement of eosinophils in the process of wound healing [125]. Eosinophils express TGF- α and β both of which are understood to be involved in the process of wound healing along with EGF [57,138,158,170,171].

1.5 The pathology of eosinophils.

Eosinophilia

A normal eosinophil count is generally considered to be less than 0.4×10^9 /litre of blood. However a study of over 700 medical student donors

in America gave counts of between 0.015 and 0.65×10^9 /litre indicating a wide variability of eosinophil cell number. Eosinophils are a minor component of the white cell population, generally $< 0.5\%$, though in some pathological conditions the proportion can rise to 50% or more. Some individuals have high eosinophil counts yet apparently no deleterious symptoms. Asthma is associated with eosinophilia of the blood and the lung tissue. In addition to tissue damage, major basic protein release from eosinophils has been found to reduce ciliary beat frequency in epithelial cells and this is thought to be a contributory factor on the pathogenicity of asthma. A more direct method by which the role of eosinophils have been studied in asthma, is by analyses of products from bronchial secretions. Transmission electron photomicrographs of bronchoalveolar fluid (BAL fluid) cells have revealed degranulation of mast cells and a loss of the granule core materials from eosinophils. BAL fluid from patients with asthma also has elevated amounts of ECP derived from eosinophils [52].

In 1951 Churg J and Strauss L [37] performed post mortem examinations on a number of asthmatic patients who had developed a disease with granulomas containing necrotizing vasculitic lesions and discovered that they had a large number of eosinophils which had invaded a number of tissues. This disease, Churg-Strauss syndrome is characterised by a history, sometimes familial of asthma. Some patients who died of the disease had tissue deposits of at least two eosinophilic cationic proteins (ECP and EDN) and in one patient eosinophil deposits were found in the heart [149].

Another disease which is associated with eosinophilia is Crohns disease (inflammatory bowel disease) which has clinical features including persistent abdominal pain and diarrhoea. In Crohns disease, eosinophils infiltrate the lamina propria and submucosa of the gut and they have been seen to have lost their crystalline cores suggesting degranulation [42]. The contents of the non crystalloid granule matrix are also detectable in the lamina propria of gut specimens of Crohn's patients but not in healthy subjects [58,150]

Hypereosinophilic syndrome (HES)

Patients presenting with hypereosinophilia of unknown cause or origin were described in 1968 [65]. In some of these patients there seems to be a predisposition to cardiac and neurological injury. In yet others, there are no evident disease symptoms. It is a rare condition which is 90% more prevalent in males than in females but has a worldwide occurrence. [128].

1.6 RECEPTORS

Eosinophils have receptors for several classes of ligands including immunoglobulins, peptides (cytokines), lipids (PAF, eicosanoids), complement and others. The binding of these ligands causes the eosinophil to respond in several ways including chemotaxis, respiratory burst, adhesion and degranulation. In this section I merely refer to responsiveness as a general term, but more detail concerning individual responses is recorded in table 1.

Receptors for immunoglobulins

Eosinophils express receptors for Immunoglobulins (A, D, E and G) [164].

FC γ R

Studies with monoclonal antibodies reveal three types of receptors for IgG (FC γ R) in human leukocytes [160], two of which are of low affinity and one high affinity receptor. The high affinity receptor FcRI (cd64 is 72kd) is expressed in monocytes only [62]. Of the two low affinity receptors, FcRII and FcRIII (cd16 and cd32), FcRIII (cd32, 40kd) is found in various cell types including eosinophils and monocytes, neutrophils, platelets and B cells [62]. The Fc γ R is thought to mediate a number of important cellular

functions such as degranulation, respiratory burst, phagocytosis and clearance of immune complexes [160].

FcεR

Most of the studies performed so far have indicated that the Ig-E receptor expressed on eosinophils is of low affinity (FcεRII -K_a 10⁻⁷ M) unlike that on mast cells or basophils (K_a 10⁻⁹ M) [30,32,71,78]. However more recently, immune labelling studies have shown that some patients with the hypereosinophilic syndrome (see above) express the high affinity receptor (the α subunit of FcεRI). Analysis with PCR also revealed bands for the β and γ chains for this receptor [56]. It is therefore possible that these cells possess both types of receptors though no affinity measurements for the binding of IgE of this receptor have been reported.

Fcα

Kita et al as well as others [81,82] have demonstrated that eosinophils have surface receptors for IgA. Stimulation of human eosinophils with immobilized secretory IgA, triggers the release of eosinophil derived neurotoxin (EDN). When these cells are pre-treated with pertussis toxin, EDN release induced by IgA is abolished.

Receptors for cytokines

Eosinophils can respond to cytokines such as GM-CSF, IL-3 and IL-5 in the picomolar range and the high affinity receptors for these have been characterized [41,103]. Since eosinophils contain [96] and can secrete these cytokines, this form of stimulation is likely to comprise a form of autocrine positive feedback effect, amplifying the cellular responses. Binding studies have revealed that there is a common component between the three receptors for these cytokines [164]. They are all heterodimers and

share a common β but have distinct α -chains [154] and this may explain the cross inhibition observed between them [103].

GM-CSF, IL-5, IL-3 have a number of functions in eosinophils including prolongation of the survival and viability of these cells in culture [84] as well as being involved in mediator release and in cell mobility [104].

Receptors for complement factors

CR3 which binds to complement factor C3bi is strongly expressed in these cells [164]. These receptors are involved in functions such as adhesion to endothelial cells [161,162] and killing of schistosomulae [31] both important functions of eosinophils. The expression of CR3 by eosinophils in culture can be enhanced by the cytokines IL-3, IL-5 and GM-CSF. IL-3 and GM-CSF are more effective than IL-5.

Other receptors

Receptors for the following have also been found on eosinophils; leukotriene b_4 (LTB₄), platelet activating factor (PAF), complement 5a (C5a) and complement 3a (C3a). On human eosinophils, both high and low affinity receptors are expressed for PAF [92]. The surfaces of peritoneal eosinophils and the alveolar cells of guinea pigs indicate that two distinct low affinity receptors exist for LTB₄, although this has not been confirmed for human eosinophils [164]. Pharmacological studies on the receptors for these ligands have indicated that they probably bind to a family of GTP binding proteins. They may be also be members of the rhodopsin superfamily of seven ^{trans} membrane ~~proteins~~ receptors since the inferred amino acid sequence of at least the C5a receptor indicates the presence of seven hydrophobic (putative membrane spanning) domains and the presence of an N-linked glycosylation site in the first extracellular 30aa [48].

| | |
|---------------------|--------------------------------------|
| h. blood | human blood |
| rat. perit | rat peritoneal eosinophils |
| horse blood | horse blood eosinophils |
| guinea pig perit. | guinea pig peritoneal eosinophils |
| gp eos | guinea pig eosinophils |
| inh | inhibition |
| IP metab | IP metabolism (inositol phosphate) |
| b-g | β -glucuronidase |
| membrane Ag's | membrane Antigens |
| o'nite | overnight |
| tox | toxin |
| Fmlp | formylmethionyl-leucyl-phenylalanine |
| PAF | platelet activating factor |
| tyr phosphorylation | tyrosine phosphorylation |
| PT | pertussis toxin |
| PLA ₂ | phospholipase A ₂ |
| cyto-B | cytochalasin B |
| ar-sulph | aryl sulphatase |
| alk. Pase | alkaline phosphatase |
| St2 | serum treated zymosan |
| compd 48/80 | Compound 48/80 |

TABLE 1

| Stimulus | Measured | max secretion | other measurements | observations | special conditions | cell source | ref. |
|---|---------------|---|---------------------------------|---|-------------------------------------|--------------------------------------|-------|
| h.anti IgE h.anti IgG antigen | EPO " " | 1.0% 1.2% 0.8% | | complete @ 60min | | h. (filariasis infected) blood | [78] |
| IgA-beads IgG-beads | EDN " | 10.6% 5.3% | tyr phosphorylation IP metab | genistein and herbimycin inh | | h. blood | [74] |
| IgE-beads IgG-beads IgA-beads | EPO " " | not recorded " " " " | | low ECP release low EPO, Hi ECP EPO ~ ECP | | h. blood | [159] |
| IgA-beads IgG-beads | EDN | 18% | | max at 4hr | IL3, IL5 & GM-CSF enhance | h. blood | [47] |
| anti- IgG-1, 2(a,b,c) anti-IgE anti-IgM | EPO " " | only by anti- IgG-2a & IgE: 26% others negligible | | | preinc with specific Ig isotypes | rat perit | [79] |
| IgA-beads | EDN " | 3% - no IL5 4.1% + IL5 | ultrastructure | 3 day preinc with IL: prolongs viability degranulate more readily | | h. blood | [84] |
| IgE-beads | b-g | 35% | | 1hr incubation | IL-1b enhances | h. blood | [13] |

| | | | | | | | | |
|--|---------------------------|---------------------------------|--|------------------|-------------|--|--------------------------------|-------|
| IgG-beads | a-sulph b-g a-sulph | 12% 55% 21% | " " " | " " " | " " " | IL-1b inhibits | | |
| IgG-beads | a-sulph | not calibrated | | | | IL-4(16h preincubation) inhibits | h.blood | [14] |
| IgE-beads | b-g | " " | | | | IL-4 no effect | | |
| IgG-beads | EPO | 9% | | | | chymostatin inhibits | h.blood | [107] |
| mABs vs eosinophil plasma membrane Ags | ECP | not calibrated | | | | | h.blood | [148] |
| A23187 LTB-4 C5a paf | EPO & EDN | not recorded | | | | ? recovery of EPO activity | h. blood | [10] |
| IL5 C5a paf PMA slgA | EDN " " " " | 18% 19% 34% 31% 18% | adhesion IP metab F-actin staining | max at 4hr | | ext matrix proteins prolong survival and inhibit secretion | h. blood | [83] |
| C3b | ECP EDN | 12% 18% | | 20min incubation | | IL5 enhances C3b (GM-CSF, IL-3, little effect) | h. blood (also neutrophils) | [33] |

| | | | | | | | |
|----------------------------------|-----|-----------------------|--|---------------------------------|---|------------------|-------------|
| IgA & IgG fmIp (10^{-5} M) | EDN | 21% | cAMP | 1 hour incubations | Pertussis tox inhibits | h. blood | [82] |
| A23187 | " | 14% | ADP-ribosylation | requires cytochalasin B | " " " | | |
| PMA (100nM) | " | 15% | IP metab | enhanced by cyto | not inhibited by PT | | |
| | | 5% | | " | " " " | | |
| fmIp 10-6M | EPO | 40% | O ²⁻ generation fmIp binding | time not stated | requires o'nite culture with mononuclear cells | guinea pig perit | [9] |
| A23187 | ECP | | | | inh by pemirolast | h. blood | [75] |
| A23187 & PLA ₂ | EPO | 10% | ultrastructure | | | horse blood | [64,6 5] |
| | " | 13% | | | denat. PLA ₂ also active! | | |
| PMA | EDN | 10.0% | tyr phosphorylation IP metab | | | h. blood | [74] |
| C5a | EPO | (uncalibrated) | H ₂ O ₂ prodn. | 30min | requires cyto-B | h. blood | [173] |
| paf | ECP | 600ng/10 ⁶ | ultrastructure | C5a, 10 ⁻⁷ M for max | " " | | |
| | EPO | (uncalibrated) | | 30min | " " | | |
| | ECP | 150ng/10 ⁶ | | paf, 10 ⁻⁵ for max | " " | | |
| C5a paf fmIp | EPO | not recorded | intracellular Ca ²⁺ shape change | C5a>fmIp>paf>IL-8 | | h. blood | [77] |

| | | | | | | | |
|-----------------------------------|-------------|--|---|--|--|----------|-------|
| IL-8 | | | | | | | |
| C5a C3a fm1p paf IL-8 | ECP | 750ng/10 ⁶ 750ng/10 ⁶ 500ng/10 ⁶ 300ng/10 ⁶ negligible | intracellular Ca ²⁺ | EC ₅₀ , 3.10 ⁻⁹ M EC ₅₀ , 3.10 ⁻⁸ M | cytochalasin-B enhances " " | h. blood | [151] |
| paf | EPO b-g | 43.0% 38.0% | | EC ₅₀ , (paf) 0.9nM | | h. blood | [91] |
| paf | EPO | ? | O ²⁻ generation, cytosol Ca ²⁺ | | PMA inhibits (IC ₅₀ , 2-10nM) | gp eos | [90] |
| paf | EPO aryl | 19.0% 23.0% | cytosol Ca ²⁺ IP3 production | EC ₅₀ , 3nM | 10min inc | gp eos | [88] |
| C5a fm1p LTB4 paf | b-g | ? | chemotaxis | | | h.blood | [116] |
| C5a fm1p paf | ECP EDN | not calibrated | O ²⁻ | | IBMX & theophylline inhibit | h. blood | [63] |

| | | | | | | | | |
|------------------------------------|--|----------------|---|-------------------------------|--|--|-----------------------|------------|
| fmlp with cytoB | EPO | not calibrated | | | | inhib by albuterol, no effect of salmeterol but blocks effect of albuterol | h.blood | [117, 118] |
| fmlp subst P | EPO | 7.7% 12.0% | O ²⁻ cytosol Ca ²⁺ | no effect on Ca ²⁺ | | 100μM 100μM, not SP-receptor mediated 100μM: lysis! | | [89] |
| mellitin | | 100.0% | aa metabolites | " | | | | |
| paf | EPO | ? | O ²⁻ cytosol Ca ²⁺ | | | PMA inhibits | | [90] |
| A23187 | b-g ar-sulph | 24.0% 29.0% | O ²⁻ | | | PMA enhances O2 metab PMA inhibits secretion | ? | [130] |
| PMA Ca ²⁺ ionophores | ECP | ? | | | | cyto-B enhances BAPTA inhibits PMA | h.blood (adherent) | [45] |
| paf A23187 | EPO | ? | | | | inhib by chymostatin chymostatin, no effect | h.blood | [107] |
| paf A23187 PMA StZ | EPO ECP ar-sulph b-g alk. Pase | | O ²⁻ | 10min | | max at 100nM EC ₅₀ , 1.5nM | | [92] |

| | | | | | | | | | | |
|--|------------|----------|-------|-----|-------|--|--|------------------------------------|---------|------|
| | histamine | EPO | aryl | EPO | | | | gp eos | [132] | |
| | A23187 | ar-sulph | 15% | 23% | | | | | | |
| | cmpd 48/80 | | 60% | 55% | | | | | | |
| | LTB4 | | 10% | 32% | | | | | | |
| | heparin | | 70% | 60% | | | | | | |
| | | | 30% | 90% | | | | | | |
| | | | | | | | | | | |
| | A23187 | b-g | aryl; | b-g | 30min | | | enhanced by active oxygen products | h.blood | [12] |
| | IgG-beads | ar-sulph | 50% | 40% | | | | | | |
| | | | 40% | 40% | 60min | | | | | |

1.7 Activation and Degranulation of eosinophils

Activation and degranulation in intact cells

Several different ligands have been observed to activate eosinophils. In some cases and under certain conditions these induce the cells to secrete, releasing the granule contents to the extracellular medium. In table 1, the data available to date on secretion from eosinophils induced by different stimuli is summarized.

Activation by immunoglobulins and cytokines

Sepharose beads coated with IgA, IgG or IgE, and their respective anti-immunoglobulins have been found to stimulate the release of EPO, EDN, Aryl sulphatase and β -glucuronidase mainly in experiments on human blood eosinophils. IgM however, is unable to stimulate these cells to secrete. Surprisingly, the amounts of secretion measured as a percentage of the total, though calibrated only on occasion in this work, seems to vary from one study to the next. This may be due to the varying conditions under which these studies were performed although this cannot be the only reason. In some studies the amounts of enzyme released is not expressed at all. What is clear, is that most investigations have been devoted to human blood cells; eosinophils from other species have received little attention.

Interestingly, GTP binding proteins sensitive to pertussis toxin have also been implicated in human eosinophil degranulation. Human eosinophils pretreated with pertussis toxin fail to respond to stimulation by immobilised IgA and give only a partial response to immobilised IgG measured as increase in PLC activity and secretion of EDN [82].

If the cells are pre-treated with pertussis toxin for a minimum of 2 hours, then enzyme release induced by IgA is abolished though there is only a partial loss in the release induced by IgG. It was also noted that the increase

in phospholipase C activity was abolished. Incubation of cell lysates with activated pertussis toxin (the catalytic subunit of this toxin exhibits ADP-ribosyltransferase activity toward the α -subunits of Gi-like proteins) in the presence of [32 P] NAD caused transfer of ADP-ribose and revealed the substrates in eosinophils as proteins of 41 and 44kD, the former having the same MW as the α subunit of the G_{i2} heterotrimer.

Irrespective of the stimulus for secretion, cytokines such as IL-5, IL-3 and GM-CSF enhance the extent of secretion induced by other ligands. In one study, guinea pig eosinophils were activated by recombinant human IL-5 causing the cells to migrate [38], illustrating that this cytokine may be required for recruitment of these cells probably to sites of inflammation. Further work revealed that IL-5 also prolongs the viability of the cells in culture. Electron micrographs of these cells in culture have shown that with time the cells lose material, either from the granule matrix or the crystalline core and that the cells become less dense (so called hypodense). One of the granule enzymes, EDN was measured in the supernatant of the cells. The ability of IL-5 to activate eosinophils and to cause their release of granule material emphasizes the diversity in the functions of this cytokine. The studies on the effects of other cytokines on this cell type are not so clear cut. For instance, IL-1b enhances secretion induced by IgE coated beads but inhibits secretion induced by IgG. IL-4 also inhibits secretion induced by IgG but has no effect on that due to IgE.

Activation by other factors

Besides immunoglobulins and cytokines the release of eosinophil enzyme markers can be stimulated by wide range of factors such as complement, Platelet activating factor, FMLP, Ca^{2+} ionophore and PMA. In general, a number of activated functions were measured in addition to secretion. These include release of O_2^- , IP metabolism, changes in shape, ultrastructure and changes in concentration of intracellular Ca^{2+} .

Ultrastructural studies have indicated that the addition of Ca^{2+} ionophore A23187 and of highly purified porcine pancreatic phospholipase A_2 to horse eosinophils can initiate morphological degranulation ^{and} alongside measurable release of EPO. Degranulation by both A23187 and phospholipase A_2 (which is a Ca^{2+} dependent enzyme) requires the presence of Ca^{2+} in the medium and this provoked the suggestion that the Ca^{2+} ionophore A23187 may cause degranulation by Ca^{2+} -dependent activation of the endogenous phospholipase A_2 . EPO release induced by A23187 is inhibited by eicosa-5,8,11,14,tetraynoic acid (ETYA) an inhibitor of both the lipooxygenase and cyclooxygenase pathways while indomethacin, (which blocks the cyclooxygenase pathway) is without effect. Thus the lipooxygenase pathway may be important for degranulation of these cells [65].

Unlike other activators, formylMethionyl-Leucyl-Phenylalanine (FMLP) had not been seen to be effective in inducing degranulation of eosinophils unless cytochalasin B ^{was} present. It was however realised [9] that when guinea pig eosinophils were cultured overnight in the presence of mononuclear cells then FMLP became a strong activator, also causing degranulation and release of EPO. Whether the secretion is induced by factors released by the mononuclear cells or directly by FMLP itself is far from clear.

Complement factors such as C3a, C5a and platelet-activating factor (PAF) are also potent activators of eosinophil secretion. Complement 5a and 3a appear to be the most effective stimuli for these cells however other studies have shown that both PMA and PAF are more effective. In the presence of these complement factors, there are characteristic changes in cell morphology, such as the flattening and the generation of pseudopodia-like projections. They can also cause intracellular Ca^{2+} transients and activate the release of reactive oxygen radical species [46,173].

Two extracellular matrix proteins fibronectin and laminin also been found to prolong the survival of these cells in culture however they are observed to inhibit secretion.

In general one must conclude that there is little general agreement or understanding of the basic processes regulating secretion in these cells. The loss or partial loss of secretion induced by IgA or IgG when these cells are pretreated with pertussis toxin, has implicated GTP binding proteins of the trimeric order in eosinophil secretion. Ca^{2+} may also have a role to play here, since Ca^{2+} ionophore has been observed to induce the release of EPO.

Activation and degranulation: in permeabilised and patched cells

Cell permeabilisation which provides a method of gaining access into cells and manipulating the composition of the cytosol is an important experimental tool in the investigation of secretion. When eosinophils are permeabilised with streptolysin-O, a bacterial cytolysin, secretion (measured as a release of hexosaminidase) and degranulation can be induced by provision of Ca^{2+} and a guanine nucleotide, both of which are necessary [39,120,121]. ATP is not required although in its presence the ^{efficacy} operational affinity of the two effectors is enhanced [39].

Tannic acid, a plant polymer has been used to arrest exocytosis from neuroendocrine cells [24]. The exact mechanism by which tannic acid works is unclear but it is thought to act at the surface of the cell, arresting exocytosis after granule fusion but at the same time allowing earlier secretory events to continue [120]. It has recently been shown that exocytosis from permeabilised eosinophils can also be arrested by this compound. In this study, purified cells were first permeabilised with SLO and secretion induced with $\text{GTP}\gamma\text{S}$ and Ca^{2+} after which tannic acid was added. Electron microscopic examination of ultrathin sections and also of freeze

fracture replicas revealed a large number of arrested fusion sites which accumulate with time.

An alternative technique, which allows access to the interior of single cells, is the application of the patch pipette in the whole cell mode. This technique provides access to the cytosol of the cell and has demonstrated along with permeabilisation that secretion from eosinophils of at least three animal species namely guinea pig [121], horse [141] and human [2] occurs by an exocytotic mechanism. This technique allows an individual cell to be attached to a glass patch pipette in a strictly non-leaky whole cell configuration permitting the direct manipulation of the cytosolic environment. The measured electrical capacitance is directly proportional to the membrane area and changes in membrane electrical capacitance can be used to measure the incremental changes in membrane area due to the fusion of secretory granules. Degranulation of guinea pig eosinophils occurs in two phases. The first phase in which individual stepwise increments cannot be resolved involves small granules and is partially dependent on Ca^{2+} . The second phase, in which individual fusions become apparent has an absolute requirement for $\text{GTP}\gamma\text{S}$ suggesting the involvement of a GTP binding protein [102]. Human eosinophils can degranulate in the presence of either Ca^{2+} or $\text{GTP}\gamma\text{S}$ but in the presence of both effectors, the increase in capacitance is much greater than the change induced by either effector alone, suggesting that together they may act synergistically [2].

Furthermore, it has been observed by this method that granule fusion events take place in at least two ways. When ^ahigh concentrations of $\text{GTP}\gamma\text{S}$ (80-160 μM) ^{is} ~~was~~ applied through the patch pipette into horse eosinophils, granule-granule fusion events ^{a re} ~~were~~ induced. Degranulation then takes place but only in a few large steps. On the otherhand when lower concentrations of $\text{GTP}\gamma\text{S}$ (5-20 μM) are applied then cumulative fusion occurs. Thus once the first fusion event between the granule and the plasma membrane has taken place, it induces other granules to fuse with this granule leading to the formation of a degranulation sac and a focal release of all the granule

material. Random fusion, ^{as} which occurs from mast cells has not been seen to take place from these cells [140].

The knowledge that exocytosis from guinea pig eosinophils can be initiated by GTPS in the effective absence of Ca^{2+} , prompted a survey of heterotrimeric and monomeric GTP-binding proteins in these cells. To test their expression and possible localisation on eosinophil granule membranes, whole cells were subfractionated in order to separate intracellular compartments. The expression of GTP-binding proteins was tested and the α subunits of the subtypes G_i , G_o , G_q and G_s were found to be expressed on the light membrane fractions (corresponding to both the Golgi and plasma membrane fractions). Although a number of rab proteins were also found, surprisingly, rab3 was absent. With the exception of syntaxin-3, none of the other components of the synaptic vesicle fusion complex were detected [93].

1.8 GUANINE NUCLEOTIDE BINDING PROTEINS

In common with other Ig- and related receptors (e.g. the T-cell receptor [72], activation of eosinophils by aggregated immunoglobulins can be prevented by treating the cells with herbimycin or genistein, inhibitors of tyrosine kinases [74]. However, secretion of EDN and the early stages of activation (phospholipase-C activation, Ca^{2+} mobilisation) are also prevented in cells which have been pretreated with pertussis toxin which transfers ADP-ribose (from NAD^+) to ^{the} α -subunits of G_i and G_o , and prevents their communication with receptors). This suggests an unusual, but perhaps not unique role for a heterotrimeric GTP-binding protein. Of course, FMLP induced secretion of EDN from human eosinophils can also be inhibited by this toxin [82] but this should cause no surprise since activation by FMLP is mediated by a seven-membrane pass receptor [156,172]. (Refer to table 1).

These observations are clearly at variance with equivalent results obtained from experiments on the activation of the high affinity receptor for IgE on

mast cells and basophils which is unaffected by pretreatment with pertussis toxin [137]. In these cells, transmission of the signals is clearly conveyed by protein tyrosine phosphorylation of such substrates as lyn, syk and pp60^{src}. which rapidly become phosphorylated following receptor crosslinking [16,17]. Neutrophil responses due to crosslinking of the type-II receptor (FcγRII) are unaffected in cells treated with pertussis toxin but are blocked by inhibitors of protein tyrosine kinases such as genistein [98]. Also, the coupling of Fc receptors leading to initiation of antibody-dependent cytotoxicity in NK cells is coupled to phospholipase-C by a mechanism involving protein tyrosine phosphorylation (probably coupled to PLC-γ) [157]. Although unclear, the apparent involvement of both G-protein and tyrosine kinase mediated events in eosinophils is not unique. In B-cells, PLC activation due to Ig-receptor aggregation is inhibited by both protein tyrosine kinase inhibitors and by pertussis toxin [60]. In permeabilised B-cells, Ig-receptor coupling to PLC requires the provision of a guanine nucleotide such as GTPγS [61].

It remains unclear whether activation of the early stages of the pathway for secretion from eosinophils requires the involvement of two parallel pathways involving protein tyrosine kinases or heterotrimeric GTP binding proteins or whether they act in series. One possible explanation is that in a prolonged process such as secretion due to activation of Ig receptors on eosinophils (which continues for more than one hour) the two different pathways of PLC activation are invoked at different times. One might expect abrupt events to be mediated through GTP-binding proteins, while slower events would be mediated by covalent modification (tyrosine phosphorylation) of signalling proteins. Certainly, tyrosine phosphorylation persists for many hours (up to 16) in eosinophils following exposure to aggregated IgA and IgG [74,82].

1.9 The Snaps and Snares

Clostridial neurotoxins, botulinum and tetanus inhibit neurotransmitter release strongly. It is now known that this inhibition is due to proteolysis of

key proteins in synaptic vesicles and in presynaptic membranes, namely syntaxin, SNAP-25 (synapse-associated protein of 25kD) and synaptobrevin (VAMP). Interestingly, in *in vitro* experiments, these three proteins form a complex with N-ethylmaleimide sensitive factor (NSF) and with three other NSF attachment proteins (α , β , γ SNAPS) [112]. This led to the 'SNARE hypothesis' the 'V' Snare, VAMP, present on the synaptic vesicle associates specifically with the 'T' Snares, syntaxin and SNAP-25 on the presynaptic membranes. Subsequently, the association of NSF and its attachment proteins leads to vesicle docking and the hydrolysis of ATP leads to vesicle fusion and release of contents. Both constitutive and regulated exocytosis are thought to occur in this fashion. In this hypothesis, Ca^{2+} -dependent neurotransmitter release (a form of regulated exocytosis) is thought to involve another protein synaptotagmin (a Ca^{2+} binding protein) which accounts for Ca^{2+} dependent exocytosis from neuronal cells. Thus far, it is not known precisely at which step ATP interacts with the release process. This along with other inconsistencies have led to suggestions for alternatives to the 'SNARE Hypothesis'. In an alternative model, Ca^{2+} and not ATP is the final stimulus for the fusion of granules [115,122]. There have also been suggestions that NSF may function as a chaperonin and not a fusogen [115].

Chapter 2

MATERIALS and METHODS

2.0 MATERIALS

SUBSTRATES (SIGMA)

1. O-phenylenediamine (OPD)
2. 4-methylumbelliferyl N-acetyl- β -D-glucosaminide
3. 4-methylumbelliferyl sulphate
4. Sodium thymidine 5'-monophosphate-*p*-nitrophenyl ester

PROTEASE INHIBITORS

5. Aprotinin (Boehringer Mannheim)
6. O-N- α -*p* tosyl-L-arginine methyl ester (TAME) (Sigma)
7. Leupeptin (Sigma)
8. diisopropylfluorophosphate (DFP) (Sigma)
9. phenylmethylsulfonyl fluoride (PMSF) (Sigma)
10. Pepstatin (Sigma)

OTHER MATERIALS

11. Hanks balanced salt solution (supplied by Sigma at 10x's stock concentration)
12. Heparin (from Sigma)
13. bovine serum albumin (fraction V, from Boehringer Mannheim)
14. DNAase (from Sigma)
15. HEPES (from Boehringer Mannheim)
16. horse serum (GIBCO-BRL)
17. 96-Well plates (Labsystems)
18. Percoll (Pharmacia)
19. Light Green (Sigma)
20. filter, 0.45 μ (Sigma)
21. TRIS.Cl (tris-hydroxymethylaminoethane-HCL) (Sigma)
22. hydrogen peroxide (Sigma, provided as a 30% solution)
23. Triton-X100 (BDH)
24. sulphuric acid (BDH)
25. Medium 199 (GIBCO-BRL)
26. sodium citrate (Fisons- AR grade)
27. lead acetate (BDH- Analar grade)
28. Brilliant Blue (Sigma)
29. ethanol (BDH)
30. phosphoric acid (BDH)
31. sucrose (BDH-Analar grade)
32. MgCl₂ (BDH)

33. adenosine triphosphate (ATP) (Boehringer Mannheim)
34. Nycodenz (Nycomed)
35. KCL (BDH-Analar grade)
36. NaCl (BDH-Analar grade)
37. dithiothreitol (DTT) (Alexis)
38. ethylene glycol-O,O'-bis (2- aminoethyl) -N',N',N'',N'''-tetraacetic acid (EGTA)
(Fluka)
39. creatine phosphokinase (Boehringer Mannheim)
40. creatine phosphate (Boehringer Mannheim)
41. CaCl₂ (BDH)
42. 2-deoxyglucose (Sigma)
43. antimycin-A (Sigma)
44. streptolysin-O (SL-O) (Murex diagnostics)
45. guanosine 5'- O - 3 thiotriphosphate (GTP_γS) (100mM Stock from Boehringer
Mannheim)
46. glycine (Sigma)
47. sodium dodecylsulphate (SDS) (Sigma)
48. piperazine- N, N'- bis (2-ethane-sulphonic acid) (Sigma)
49. acetic acid (BDH)
50. sodium acetate (BDH-Analar grade)
51. sodiumthiosulphate (BDH-Analar grade)
52. glutaraldehyde (Sigma)
53. formaldehyde (Sigma)
54. silver nitrate (BDH)
55. sodium carbonate (BDH-Analar grade)
56. ethylenediaminetetra-acetic acid disodium (EDTA) (Sigma)
57. triethanolamine (Boehringer Mannheim)
58. diethanolamine (Sigma)
59. ethanolamine (Sigma)
60. diethylaminoethyl (DEAE) Sepharose (Sigma)
61. carboxymethyl (CM-sephadex) (Sigma)

2.1 COLLECTION OF EOSINOPHILS (by peritoneal lavage)

In order to elicit peritoneal eosinophils, guinea pigs are injected with 1ml of horse serum intraperitoneally three times a week for at least 4 weeks before the cells are collected. [53].

The guinea pig is asphxiated with carbon dioxide, the belly of the animal is shaven and a small incision made in the abdomen. 100mls of Hanks balanced salt solution (HBSS) containing Heparin sodium salt at 10U/ml and 1mg/ml of Bovine serum albumin (BSA- fraction V) are introduced and the abdomen is gently massaged while the muscle is clamped. The abdomen is then cut further and a gloved finger used to stir the liquid gently around the gut. The lavage solution is removed and the cells are pelleted by centrifugation at 240g for 5mins at 20°. The pellet is resuspended in HBSS containing 2.5mg/ml BSA and 1-1.5mg of DNAase using a plastic pipette and the cells are then pelleted again by centrifugation as before. After resuspending, the cell suspension is filtered through a nylon mesh and then layered onto each of two discontinuous Percoll gradients (see below for details). These are centrifuged at 1500g for 20mins. The top 2 layers as well as about 2/3 of the third layer are then removed by aspiration and discarded. The interphase and the lower layers are carefully collected to <0.5mls and then diluted with the HBSS containing 10mM HEPES and 2.5mg/ml BSA. The cells are pelleted by centrifugation at 240g for 5mins at room temperature and then resuspended in the solution containing HEPES (10mM) and centrifuged as before. The supernatant is again discarded and the pellet resuspended in Medium 199 (containing 25mM HEPES with Earle's Salts) and 100units of antibiotic solution containing Streptomycin and Penicillin. The cells can be maintained in this solution at room temperature and used as required for up to a three days.

2.2 Preparing the Percoll gradient (for eosinophils)

Percoll is adjusted to 90% by the addition of HBSS. The pH of this solution is then adjusted to between 7.3 and 7.4 with two drops of HCl (1M).

Percoll gradient

Two tubes containing 3 discontinuous layers of Percoll at three densities (1.1, 1.09, 1.08 g/ml) are prepared at a volume of 8mls (per tube).

| DENSITY(g/ml) | PERCOLL(90%) | Solution with DNAase |
|---------------|--------------|----------------------|
| 1.1 | 4mls | 1.03mls |
| 1.09 | 4.8mls | 2.19mls |
| 1.08 | 2.84mls | 2.16mls |

2.3 Identifying and counting eosinophils

10 μ l of cell suspension is mixed with 90 μ l of Kimura stain after which a small sample of suspension is taken and counted using a Haemocytometer.

2.4 Kimura's Stain [80]

This stain is prepared by dissolving 0.05% Toluidine Blue, 1.8% NaCl and 98% ethyl alcohol in 100mls of water. 1.1mls of this solution is then mixed with 80 μ l of 0.03% Light Green (from Sigma), 0.5mls of 50% saturated saponin (saponin in ethanol) and 0.5 mls of 0.067M Phosphate buffer. This is then filtered through a 0.45micron filter (Sigma) and can be stored for a maximum of one week.

2.5 Removal of dead cells

Over the duration that the cells are kept, some of them die. To remove these, the cells are centrifuged at a slow speed (500rpm), a procedure which prevents the dead cells from forming a pellet with the rest. They can then be removed by aspirating off the solution and leaving the pellet behind. Fresh ^MMedium 199 (10mls) containing 100units of antibiotics is then used to resuspend the cells which can now be left at room temperature.

2.6 Trypan blue exclusion

0.4% trypan blue in 0.9% NaCl is diluted to a final concentration of 0.1% in isotonic phosphate buffered saline (PBS). 40 μ l of this is then added to 10 μ l of cell suspension. The dead cells take up the dye quickly and have a blue colouration. The number of living cells are counted using a haemocytometer and the dead cells can be removed by centrifugation before the cells are used for experiments.

2.7 MAST CELL Preparation

Rat peritoneal mast cells were prepared by standard procedures as described [55,153]

2.8 ENZYME ASSAYS

Eosinophil peroxidase (EPO) assay

The assay is based on the catalysis of oxidation of o-phenylenediamine (OPD) by hydrogen peroxide. The substrate solution consists of TRIS (400mM), hydrogen peroxide (0.00375%), Triton-X100 (1%) and OPD (5mM), pH8. 50 μ l of the substrate is mixed with 50 μ l of the enzyme. Colour develops within minutes at room temperature, the reaction is quenched by the addition of 100 μ l 4M H₂SO₄ and absorbance measurements made with a plate reader at 492nm [168]. The calibration samples are prepared from reagent blanks (zero) and cells, lysed with 0.2% Triton-X100 (100%). These calibration standards are performed in the same fashion for all enzyme assays. The data obtained are transferred to a simple spread sheet for analysis.

Hexosaminidase assay

The substrate for this assay is 4-methylumbelliferyl N-acetyl- β -D-glucosaminide (1mM) dissolved in 0.2M citrate at pH 4.5. 50 μ l are incubated with 50 μ l of enzyme for a minimum of one hour at 37°. The reaction is quenched with 150 μ l of 0.2M TRIS. Fluorescence emission measurement is performed on a Fluoroskan plate reader using filters 356>465 [39].

Aryl sulphatase B assay

The substrate is 10mM 4-methylumbelliferyl sulphate made up in 0.2% Triton-X100 in 0.2M acetate buffer at pH 5.6. Lead acetate (60mM) is added to suppress the activity of contaminating aryl sulphatase A [36]. 50 μ l of this substrate is incubated with 50 μ l enzyme^{at} 37° for a minimum of 1 hour after which the reaction is terminated by addition of 150 μ l of 0.2M TRIS. Fluorescence measurement is performed in the same fashion as in the Hexosaminidase assay.

Alkaline phosphodiesterase assay

Sodium thymidine 5'-monophosphate-p-nitrophenyl ester (10mM) is made up in 0.1M TRIS-HCL at pH 9 in the presence of 0.1% Triton [147]. 120 μ ls of this substrate are added to 30 μ ls of enzyme and then incubated for 2 hours at 37°. The reaction is quenched by the addition of 150 μ l of 0.2M TRIS-HCL. The absorbance reading of the product nitrophenolate is determined using a plate reader at 410nm.

2.9 Density measurements of Nycodenz fractions

The refractive index, η , of each sample obtained after subfractionation is measured by using a refractometer. The following equation is then used to derive the density (taking account of the diluent, sucrose, 0.25M) density, $\rho=3.410\eta-3.555$).

2.10 Bradford assay (for protein concentration measurement)

This reagent is prepared by dissolving 0.04M of Brilliant Blue in 95% ethanol. Once dissolved, deionized water and 85% Phosphoric acid are added and the solution filtered. 100 μ l of this reagent are added to 10 μ l of the sample and the absorbance measured with a plate reader at 620nm. Standards of BSA (between 0 - 150 μ g/ml) are also measured along with the samples. The data are then transferred into a program in which λ linear regression is performed on the standards and estimates made of protein concentration in the samples [22].

2.11 Subfractionation of eosinophils

The cells are pretreated with 2mM Diisopropylfluorophosphate (DFP, a serine protease inhibitor) for 5mins. They are then pelleted by centrifugation at 240g, and the supernatant discarded. To remove any excess DFP remaining, the cells are resuspended in 10mls of an ice cold buffer containing 0.25M sucrose, 10mM HEPES, 1mM EGTA at pH7.4 (Buffer A) which has been prefiltered and the cells are again pelleted and the supernatant discarded as before. Beyond this step, the cells are kept on ice. They are resuspended in cold buffer A containing 2mM MgCl₂ (prevents the granules from clumping together), 1mM ATP and a cocktail of protease inhibitors (Aprotonin, O-N- α -p tosyl-L-arginine methyl ester (TAME) and leupeptin dissolved in neat DMSO, 5 μ g/ml each, final concentration). The cell concentration is adjusted to between 10-15 x 10⁶ cells/ml for homogenization, using a ball bearing shearing device "cell cracker". Before homogenising, the cells are filtered through a nylon mesh. The suspension is then placed in one of two 2ml syringes which are then secured on to the cell cracker and then pumped back and forth 10-15 times. The disruption of the cells is checked by viewing a drop of the cells under a fluorescence microscope in the presence of a drop of 1mM ethidium bromide. Any residual intact cells and nuclei are pelleted by centrifugation for 10mins at 400g, 4°. The post-nuclear supernatant is then layered slowly onto a linear gradient of Nycodenz (0-50%) in sucrose (0.25M), buffered with HEPES (10mM) at pH7.4. The gradient with the sample is then centrifuged at

100,000g for 2 hours at 4°C in an SW41 (swing-out) rotor. This allows the separation by density of the different cellular components. After this, cell fractions are collected using a fraction collector equipped with a drop counter, typically 15 drops per tube (0.4-0.5mls). For the purpose of assay, a small portion of each sample is diluted 10 times and the enzyme assays described above are applied. The measurement of refractive indices and hence density is performed on the undiluted fractions. The rest of the samples are then frozen until required.

2.12 Preparation of the linear gradient for subfractionation (0-50%

Nycodenz)

The linear gradient of 0-50% Nycodenz is prepared by slowly mixing a light solution (consisting of 0.25M sucrose, 10mM HEPES and 1mM EGTA) with a dense solution (50% Nycodenz made up in 0.25M sucrose, 10mM HEPES and 1mM EGTA) (both solutions supplemented with the cocktail of proteinase inhibitors (as above)) using a three channel system operated by a peristaltic pump.

2.13 Homogenization buffer

This buffer is used for preparing brain homogenates. It consists of NaCl (140mM), PIPES (40mM), KCl (2.7mM), $MgCl_2$ (1mM), ATP (1mM) Dithiothreitol (DTT, 1mM), EGTA (0.3mM) and a cocktail of protease inhibitors (0.1mM PMSF, 1ug/ml Pepstatin and 1ug/ml Leupeptin in Ethanol). The buffer is adjusted to pH6.8.

2.14 Regeneration buffer

This buffer is used for measurement of factors active in regulating secretion during purification from brain cytosol. The level of ATP in the assay buffer is maintained by an ATP regeneration system which relies on the conversion of ADP to ATP catalysed by creatine kinase. It consists of the standard pH6.8 salts buffer (NaCl 140mM, KCl 2.7mM, PIPES 20mM, MgCl₂ 1mM) supplemented with 4mM creatine phosphate, creatine kinase (6mg/ml), ATP 1mM and CaEGTA (0.1mM final concentration) to buffer cytosolic Ca²⁺ to pCa8.

2.15 Preparation of brain cytosol (rat or guinea pig)

One rat brain is homogenized in 5mls of ice-cold homogenization buffer (about 5ml per rat, approx 2gm tissue per 5ml) using a Dounce homogenizer. The homogenate is then centrifuged in a mini-ultracentrifuge (Beckman Optima TL) at 45,000rpm (100,000g) for 1 hour using a TLA 45 rotor. The supernatants are pooled and dialysed (cut-off 12kD) overnight against 1 litre of homogenization buffer.

2.16 Ca²⁺ BUFFERS

Ca²⁺ buffers are prepared by addition of equimolar solutions of EGTA and precisely end-point titrated Ca.EGTA according to a computer programme [55,153]. The Ca²⁺ buffers are then diluted using the standard pH6.8 salts buffer (see above) such that the final concentration of the chelator is 3mM. The Ca²⁺ buffer system regulates Ca²⁺ in the range pCa8 -pCa5 (0.01-1μM).

2.17 SECRETION EXPERIMENT CONDITIONS

All secretion experiments are performed in 96-well microtitre plates. The cells and all reagents are made up in PIPES 20mM, NaCl 137mM and KCl 2.7mM containing 1mg/ml of BSA at pH 6.8. Unless otherwise stated the temperature for these measurements was at 37°C.

2.18 Metabolic inhibition

In all experiments, the cells were pretreated with metabolic inhibitors in order to deplete the cytosol concentration of ATP prior to permeabilisation as previously described [70]. This procedure allows a decision to be made, whether to make measurements in the absence of ATP, or whether to supply ATP and if so, to set the concentration with precision. Prior to permeabilisation, the cells were incubated at 37°C for 5mins in the presence of 2-deoxyglucose at 3mM and antimycin-A at 5µM.

2.19 The SL-O prebind protocol

Ice cold PIPES buffered saline containing 1.6iu/ml of Streptolysin-O (SL-O) incubated with the cells at ice temperature for 5mins to allow binding. After this, excess unbound SL-O and impurities are removed by centrifugation at 240g for 5mins at 4°C. Whilst the cells remain in cold conditions (eg. on ice), no permeabilisation takes place.

2.20 Secretion experiment

30µls of cells (with prebound SL-O at ice temperature) are added to an equal volume of the stimulus (Ca. EGTA buffers, to regulate pCa, and GTP-γ-S at concentrations indicated, with or without Mg.ATP (1mM)) in a 96-“V”-well microtitre plate maintained at 37°C. The cells are allowed to secrete for a period of 10mins (unless otherwise stated) after which the reaction is stopped by the addition of ice cold PIPES buffered saline pH6.8 (when mast cells are used) or the addition of ice cold HEPES buffered saline at pH7.0 (when eosinophils have been used). The cells are sedimented by centrifugation (using a microtitre plate adaptor) at 240g, 4°C for 5mins and samples of supernatant taken for enzyme assays. In all of these experiments, the samples were tested at least in duplicate (on occasion also in quadruplicate).

Note, in some of the early experimental work, the SL-O was added directly to the cell suspension together with the stimulus for secretion with the result that excess material and impurities were not removed.

2.21 Run down experiments (time course)

The prebind protocol was applied in all these experiments unless otherwise stated. 20 μ l of stimulus is pipetted into all positions of a 96-"V"-well microtitre plate which is then placed in the waterbath at 37 $^{\circ}$ C. Cells, treated as described above with SL-O, are suspended in 4mls of ice cold buffered saline and placed in a trough (on ice) containing 2mls of ATP regeneration buffer or buffered saline. In some instances, when exogenous protein is being tested, 2mls of buffered saline (for resuspending the cells) is replaced with 2mls of protein solution. Using an 8 channel pipette, sets of 60 μ ls of the cold cell suspension from the trough is pipetted into column 1 of the plate (at time = 0) of the microtitre plate which is maintained at 37 $^{\circ}$ C (sometimes 30 $^{\circ}$ C). At this time, the trough containing the cells with bound SL-O is also brought to 37 $^{\circ}$ C so that the cells become permeabilised and cytosolic proteins begin to leak out. After set time intervals, samples of 60 μ l are transferred into the subsequent columns of the microtitre plate to allow stimulation for secretion. All the cells are allowed to secrete for 10mins after transfer to the stimulus and the reactions are then stopped with ice cold 20mM HEPES buffered saline. In most of these experiments the samples were tested in quadruplicate.

2.22 SDS-PAG ELECTROPHORESIS

The polyacrylamide gels were run according to the method of Laemmli [94]. 4% stacking gel and 10% or 12% running gels were used. The running buffer was Tris, (25mM), SDS (0.1%) and Glycine (192mM), pH8. The gels were run at 60Volts through the stacking gel and 150Volts through the running gel.

2.23 Preparation of red blood cell liposomes

About 10mls of blood was washed twice by centrifugation with 30-40ml of isotonic saline solution. The washed cells are then taken up in 10mM phosphate buffered saline. After centrifugation the ghost samples obtained are mixed with three times the volume of Chloroform/Methanol (in a 2:1 ratio) and shaken. Equal volumes of water and chloroform are added and

the solution centrifuged. Two layers of material is obtained, an aqueous and an organic layer. Pure red blood liposomes are extracted from the organic layer and dried under a stream of nitrogen. It is made up in isotonic buffered saline when required. A maximum of 0.8 μ mol of phospholipid is obtained for every 10ml of blood used. The exact amount of cholesterol obtained is not known but thought to be less than the amount of phospholipid obtained [20].

2.24 Silver staining (non-diamine staining method)

a) Principle:

This kind of stain depends on the reaction of silver nitrate with protein sites in acidic conditions. The silver ion is then reduced to metallic silver by formaldehyde in the presence of sodium carbonate which maintains alkaline conditions during the development.

b) Method: [67].

The gel is fixed in a solution of 40% Ethanol and 10% acetic acid for a minimum of 30mins. After this, the gel is incubated an incubation solution consisting of 30% Ethanol, 0.5M Sodium acetate, 0.008M Sodium thiosulphate and 1.3mls of Glutaraldehyde (25% w/v) for at least 30mins (if desired, can be kept overnight). Following this, the gel is washed three times with distilled water for 5mins. The fixed and washed gel is then treated with solution of 0.006M Silver nitrate and 50 μ l of 30% formaldehyde in 250mls for 40mins. The stain is then developed in a solution consisting of 0.235M Sodium carbonate and 50 μ l of 30% formaldehyde in 250mls, until the protein bands are dark enough to be seen. The reaction is stopped with a solution containing 0.04M EDTA and washed in water. If the gel is to be kept for a long period of time, then 0.02% azide is added to the solution in which it is kept.

Chapter 3

INTERLUDE:

CHARACTERISATION OF STREPTOLYSIN-O IN PERMEABILISED RAT MAST CELLS

As has been remarked by Christian de Duve, prokaryotic [40] cells do not ingest foodstuffs. They are consigned to live inside their food supply and so they have to secrete enzymes and other proteins and these digest the foodstuffs extracellularly. Among proteins secreted from Gram positive bacteria such as Streptococci, Bacilli, Clostridia and Listeria are a number of cytolytic toxins and these include Streptolysin-O, Pneumolysin, Alveolysin and about 12 other toxins.

These toxins are all oxygen labile or thiol-activated and bind to cholesterol present in plasma membranes of animal cells and cause lysis. Lysis can be prevented if the toxin is incubated with cholesterol or some other sterol [3].

Streptolysin-O, a product of group A Streptococci is thought to bind to cholesterol containing membranes after which it then associates with itself to form arcs and rod shaped oligomers which penetrate the apolar domains of the membranes. Preformed toxin oligomers can bind tightly to phosphatidylcholine in the absence of cholesterol but the binding of the toxin monomers on target cell surfaces is thought to be initiated by cholesterol. The ends of two individual arcs when joined together form a ring and create a lesion of up to 65nm in diameter [18]. The ring shaped oligomers once embedded in plasma membranes form transmembrane channels of between 25 and 30nm in diameter. These membrane lesions are thought to be lined with protein and are probably formed when the Streptolysin-O polymer, which has both hydrophobic and hydrophilic regions repels lipids within the membrane [18].

The enormous lesions produced by SL-O have made it a useful tool for permeabilisation of animal cells [101] amongst them mast cells [70,94] and

eosinophils [39]. It allows the cytosol of the cell to be manipulated with some precision.

Molecules such as nucleotides, divalent cations and even proteins can be introduced into cells after permeabilisation with SL-O and their effects on cellular activities studied.

The loss of activity of SL-O in the presence of atmospheric oxygen was formally thought to be due to the formation of intramolecular disulphide bonds. However, cloning has revealed that SL-O contains a single cysteine residue as do three other toxins of this group namely pneumolysin, listerolysin and alveolysin which share between 42 and 65% primary sequence homology. It follows that S-S bridging due to oxidation cannot be the cause of this loss of activity. In spite of this, addition of thiol blocking agents completely inhibits their cytolytic activity, which suggests a functional role for the single cysteine residue which is located within a conserved 12aa peptide in the C-terminal region of all these toxins.

Although the substitution of a serine for the cysteine reduces the haemolytic activity, substitution by an alanine residue is without effect [131]. This highlights the possibility that although the cysteine residue is conserved in all four toxins, it may not be essential for activity. The current belief is that in an oxidising environment, the cysteine residue forms disulphide bonds with low molecular weight impurities and this could lead to reduced binding to cholesterol and hence the loss of pore-forming activity [19].

These toxins are all water soluble and consist of single polypeptide chains ranging from 50 to 80kD. The native toxins all require the presence of a reducing agent such dithiothreitol (DTT) or cysteine for maximum activity.

Streptolysin O, a 69kD protein is available commercially as a partially purified culture filtrate of a strain of *Streptococcus* from Wellcome Diagnostics (later Murex Biotech). It is supplied as a freeze dried powder

and maintains its lytic activity over long periods of time (many years). At the time I commenced this work, this product went out of production and was not available for a year or so. This meant that an alternative source was required and this provoked the need for other formulations of SL-O to be characterised.

In this chapter I have compared the effect of the commercial SL-O (from Murex Biotech) with two other reagents (a purified protein and a recombinant protein from E.coli) on exocytosis from mast cells. I selected to carry out this study on mast cells (rather than eosinophils) because this cell type has been particularly well characterised and documented [70,94].

When permeabilised in buffered saline solutions, exocytosis from mast cells measured as secretion of histamine or hexosaminidase has been found to require both a guanine nucleotide and Ca^{2+} [70]. ATP is not needed, although in its presence, the affinity for the two effectors mentioned is enhanced. If the simple NaCl based solution is substituted by isotonic buffered glutamate, then a Ca^{2+} -independent component of secretion can be induced and the cells can be stimulated by $\text{GTP}\gamma\text{S}$ alone (a non hydrolysable analogue of GTP). [99,100].

In my investigation of exocytosis from SL-O permeabilised mast cells, I observed that the purified and recombinant materials differed from the commercial (Murex) product and this difference suggested that the commercial product may contain additives which mask a Ca^{2+} -independent component of secretion.

RESULTS

3.1 Dependence of exocytosis on both Ca^{2+} and $\text{GTP}\gamma\text{S}$ using two SL-O formulations for cell permeabilisation.

In this experiment, I stimulated mast cells permeabilised with a range of Ca^{2+} (pCa8-pCa5) concentrations and a range of concentrations of $\text{GTP}\gamma\text{S}$ (0-100 μM). This style of experiment is called a $\text{Ca}^{2+}/\text{GTP}\gamma\text{S}$ matrix. I also tested the effect of providing ATP. It is important to note that for the experiments described in this chapter, the cells were stimulated in the presence of excess SL-O.

In figure 3.1, the use of purified SL-O (A) and the Murex-SL-O product (B) is compared. At concentrations of $\text{Ca}^{2+} > \text{pCa6}$, maximum secretion was expressed at 1 μM $\text{GTP}\gamma\text{S}$ irrespective of which SL-O preparation was used. If ATP (1mM) is provided, a substantial amount of Ca^{2+} -independent secretion occurs at Ca^{2+} concentrations $< \text{pCa6}$ only when the purified reagent is used to permeabilise the cells. Even in the absence of Ca^{2+} (3mM EGTA) this component still remains. Ca^{2+} independent secretion does not occur when the Murex SL-O is used, an observation which is in agreement with previous results [70].

To investigate if the Ca^{2+} independent component was being suppressed by the presence of impurities, the Murex SL-O was subjected to dialysis (12kD cut off) after which a small Ca^{2+} independent component could now be induced (fig 3.1c). The Ca-independent component of secretion from cells permeabilised with the dialysed Murex preparation is less than that from cells permeabilised by purified preparation, but it is present none-the-less. The low cut off of the dialysis membrane implies that the impurities suppressing the Ca^{2+} independent component are unlikely to be contaminating proteins although this cannot be ruled out.

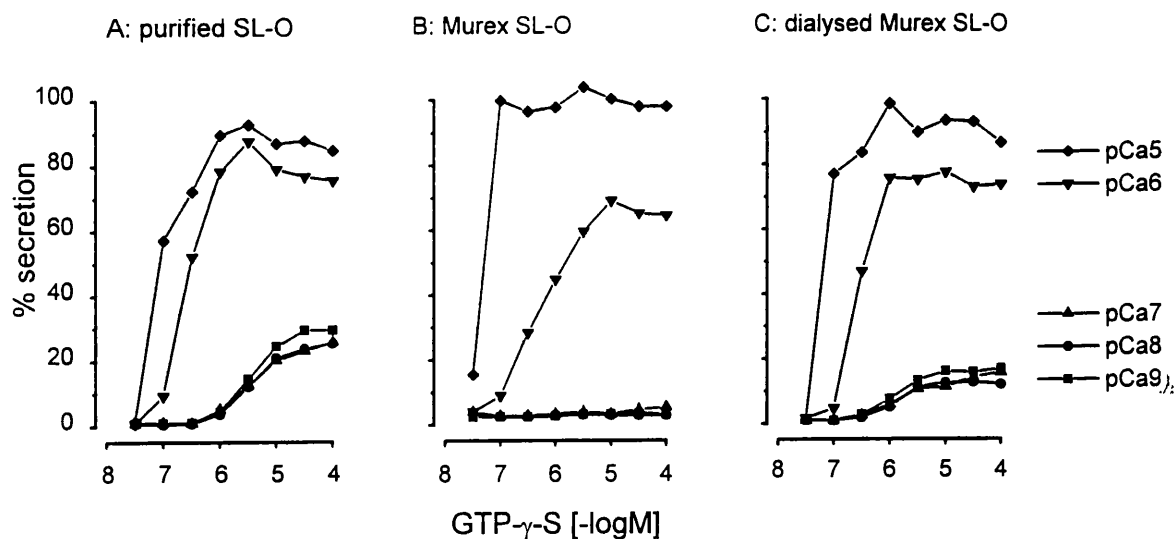


Fig 3.1

Dependence on GTP γ S and Ca²⁺ for exocytosis from mast cells permeabilised by various formulations of streptolysin-O.

Mast cells suspended in buffered salt solutions, were permeabilised with Streptolysin-O and stimulated to secrete by incubation at 37° for 10mins in the presence of GTP- γ -S and Ca²⁺ (buffered with EGTA) at the concentrations indicated. The cells were sedimented and the supernants assayed for secreted hexosaminidase. ATP was also supplied at 1mM.

3.2 When the prebind protocol is applied, Ca-independent secretion can be induced.

SL-O binds to cholesterol containing membranes at 0°C without making permeability lesions [111]. Mast cells were allowed to bind to Murex SL-O (undialysed preparation) or recombinant material for 5 minutes after which the unbound material and any additives and non adherent impurities were removed by centrifugation. The cells were then permeabilised by raising the temperature to 37°C in the presence of the stimulus.

Following this procedure, it can be seen from fig 3.2 that a Ca^{2+} independent component of secretion was expressed with both preparations though the Ca^{2+} independent component of secretion was less for cells permeabilised by the Murex product. In the absence of Mg.ATP this component is then completely lost. The non-induced release (EGTA, zero $\text{GTP}\gamma\text{S}$) due to the recombinant SL-O was always higher (by about 3-4%) than when Murex product was applied. I have no logical explanation for this.

3.3 A Ca-Independent Component Of Secretion Is still Induced When the Ultrafiltrate Of Murex SL-O Is Added To Purified SL-O.

From these experiments, it appears that the Ca^{2+} independent component of secretion could be masked by the presence of dialysable impurities present in the Murex SL-O. I therefore tested whether the ultrafiltrate from Murex SL-O could suppress the Ca^{2+} -independent secretion from cells permeabilised with the purified protein.

The ultrafiltrate was obtained by centrifugation of the Murex SL-O in an amicon (10kD cut off filter) and then added to the purified preparation at its working concentration. The prebind protocol was not applied in this particular experiment in order to ensure that the ultrafiltrate was present at the time of stimulation for secretion. The mast cells were permeabilised with either purified SL-O alone or purified SL-O reconstituted with ultrafiltrate

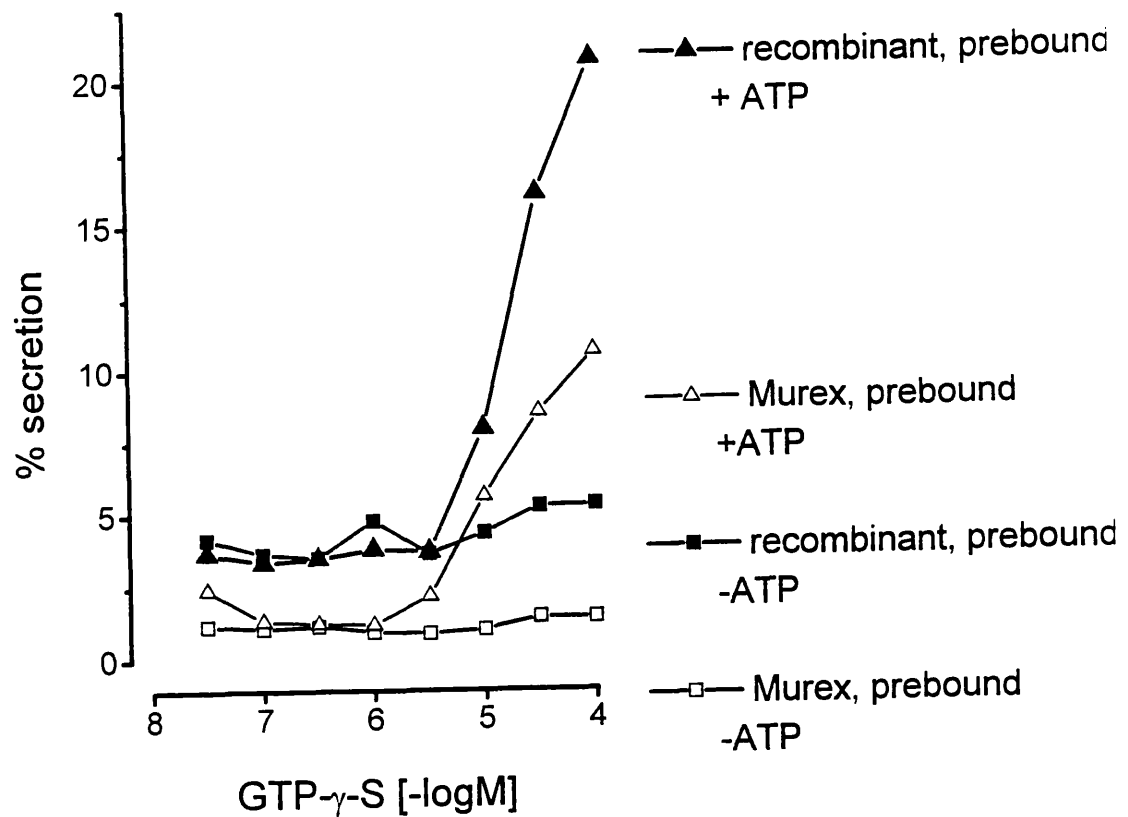


Fig 3.2

Ca²⁺ independent secretion from mast cells permeabilised by two formulations of SL-O using the prebind protocol.

Secretion was induced by GTP- γ -S at the concentrations indicated. The concentration of Ca²⁺ was suppressed by EGTA (3mM). ATP was required for Ca²⁺-independent secretion to occur. (note: 3mM EGTA corresponds to pCa9).

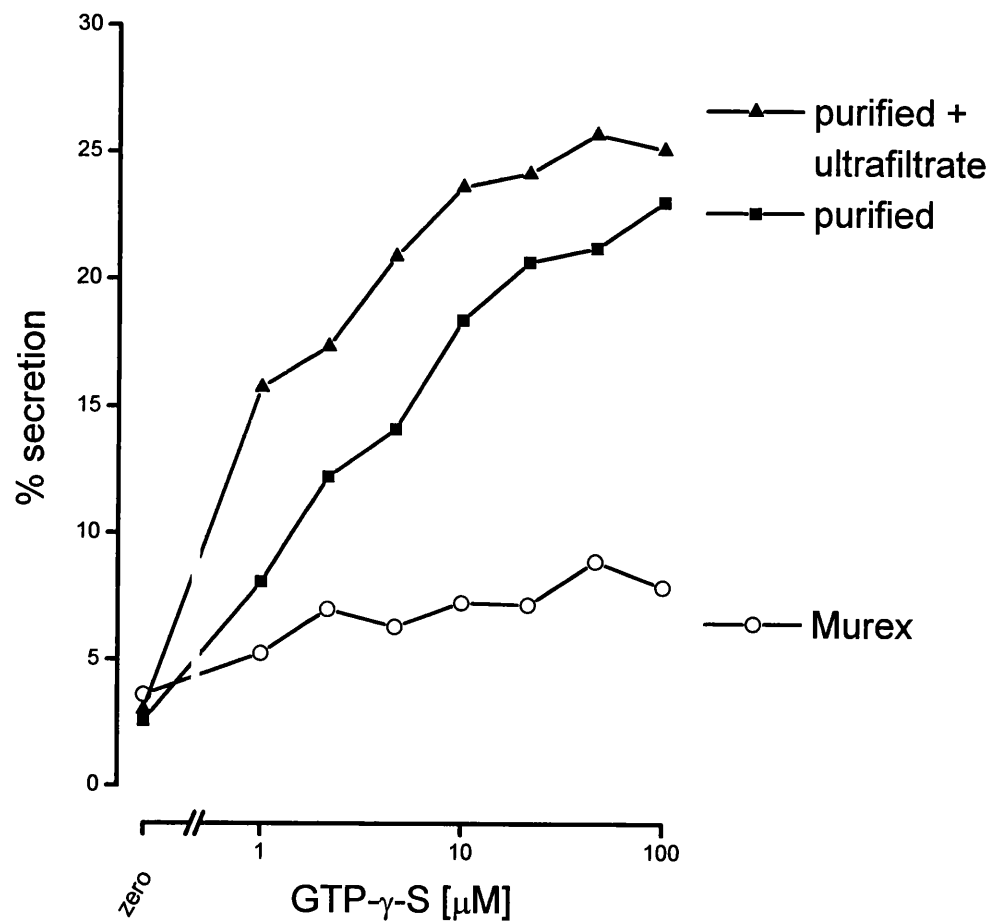


Fig3.3

Effect of SL-O ultrafiltrate on Ca^{2+} -independent secretion.

GTP- γ -S induced (Ca^{2+} -independent) secretion does not occur for cells permeabilised by Murex SL-O. However, the ultrafiltrate of Murex SL-O has little or no effect on secretion from cells permeabilised with purified SL-O. This experiment was carried out in the presence of EGTA (3mM) with no added Ca^{2+} .

from Murex SL-O. The cells were stimulated with a range of Ca^{2+} (pCa7-pCa5) and a range of concentrations of $\text{GTP}\gamma\text{S}$ (0-100 μM).

Fig3.3 shows that the addition of the ultrafiltrate to the purified preparation had no effect on the Ca-independent component of secretion. This result suggested that the 'masking agent' could be a protein (though unlikely) which had a molecular weight <12kD or an additive of formulation retained with the Murex product.

3.4 Does SL-O Damage intracellular Organelle Membranes?

The pores made by SL-O are enormous (30nm or more). Thus, SL-O itself (MW 69kD) should be able to enter the cytoplasm through its own pores. In principle, intracellular membranes which lack cholesterol should not serve as targets for the toxin. However to ensure that the Ca^{2+} independent component was not due to SL-O entering the cytoplasm through its own lesions and then releasing hexosaminidase by damaging the granule membranes, the effect of varying the concentration of recombinant SL-O under different conditions was tested (see fig 3.4). High concentrations of SL-O would be expected to cause more non-specific release.

In this experiment the prebind protocol was once again not applied so ensuring that excess SL-O was present at the time the cells were being stimulated.

In the absence of stimulus (see fig 3.4) only a minimal level of release occurred, rising to 4% at the highest concentration of SL-O (1 $\mu\text{g/ml}$). If the granule membranes had been damaged and the release of hexosaminidase occurred by leakage, then this would have been observed under these conditions.

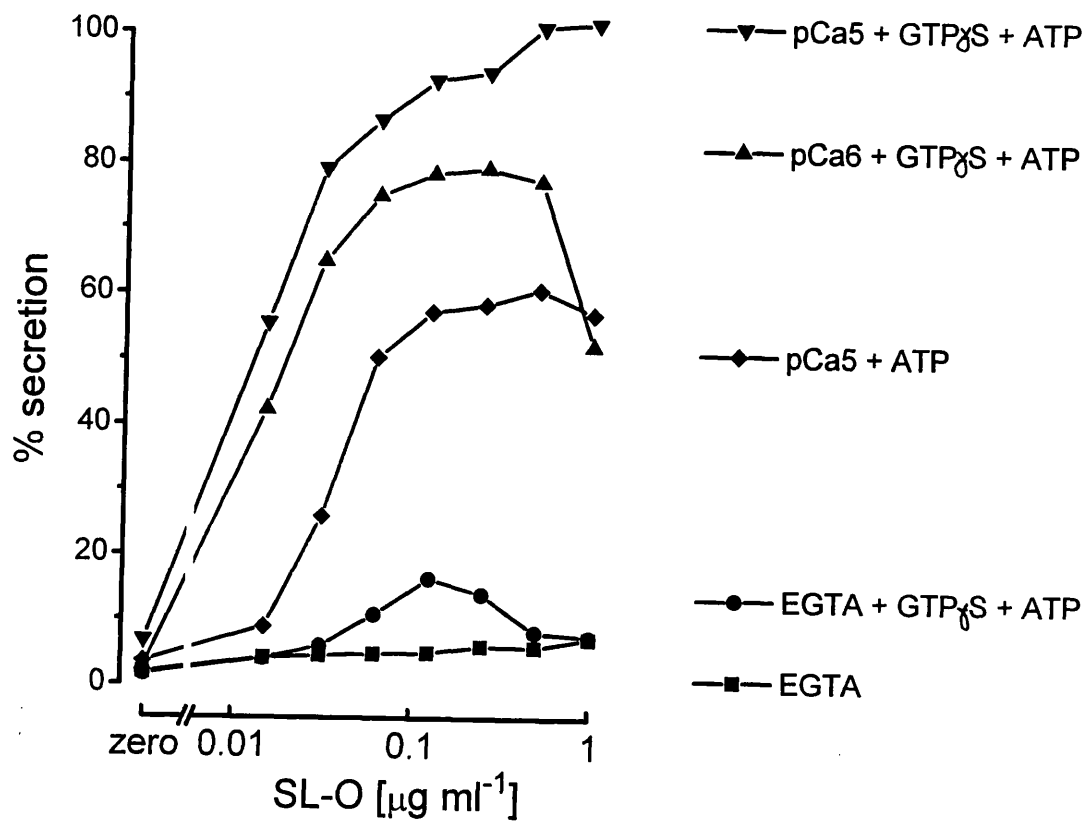


Fig 3.4

Secretion of hexosaminidase is dependent on the relevant effectors at all concentrations of recombinant SL-O.

The mast cells were permeabilised using serial dilutions of recombinant SL-O and stimulated with GTP γ S (10 μM), ATP (1mM) and Ca²⁺ at the concentrations indicated.

Once again, this provides further evidence that the granule membranes are not damaged by SL-O and that release of hexosaminidase is not due to leakage.

3.5 The Presence Of Cysteine And Phosphates In Murex SL-O Does Not Account For ^{the}Ca-Independent Component.

The contaminants that mask ^{the}Ca-independent component remain still unknown. It has been established that they are dialysable and are lost by centrifugation when using the prebind protocol to permeabilise cells (see fig.3.2). As supplied by Murex Biotech, SL-O contains cysteine and phosphate salts. Their effects were tested on exocytosis from permeabilised mast cells in particular, to find out whether they suppress the Ca-independent component of secretion (see fig.3.2). At the standard concentration of Murex SL-O (0.4iu/ml), one can estimate (from the specifications provided that the phosphate concentration is 16mM. The concentration of cysteine was calculated as 0.77mM. These two compounds were therefore tested both separately and together on exocytosis induced by Ca^{2+} and $\text{GTP}\gamma\text{S}$, using the purified SL-O as the permeabilising agent.

Cysteine at concentrations in the range 0 to 100mM had no effect on the Ca-independent component of secretion. The Ca-dependent component of secretion was very slightly inhibited at concentrations above 20mM (data not shown). When phosphate was applied alone, it was without effect when tested at concentrations up to 20mM. At very high concentrations, (100mM) phosphate inhibited both the Ca-independent component and the Ca-dependent component of exocytosis (data not shown). Phosphates are known to complex with Ca^{2+} and so there was a possibility that the inhibition could be due to complexation. However, with a log K_{app} of -2.38 at pH 6.8 [143], the concentration of free Ca^{2+} , regulated by EGTA is not substantially affected. Thus the inhibition by phosphate at high concentration is unlikely to be due to complexation with Ca^{2+} . I then tested the effect on secretion of applying phosphate (20mM) together with a range of cysteine

concentrations. The extent of secretion was inhibited slightly over the whole range of Ca^{2+} applied. However to cause even this small inhibition, it was necessary to use concentrations of cysteine and phosphate much greater than could be provided by SL-O. These experiments indicated that neither phosphate nor cysteine could be responsible for masking the Ca-independent component of secretion.

There must be other additives in the formulation not known to us. Besides the mention of cysteine, phosphate salts and bovine serum albumin (BSA), the manufacturers of Murex SL-O do not state the presence of other contaminants. They do state however that the preparation is only partially purified.

3.6 SDS-PAGE Electrophoresis Of SL-O And BSA With Or Without Red Blood Cell Liposomes.

To check if the impurities present in the Murex SL-O formulation are due to additives in BSA (fraction V), a 10% SDS Polyacrylamide gel electrophoresis was performed of Murex SL-O and BSA. The recombinant protein was also run.

In order to calibrate the system, different amounts of BSA protein were run on the gel. At least 20 peptides are visible (see lane 4). The Murex SL-O run in lane 6 presents an almost identical pattern which suggests that most of the contamination in the Murex formulation is from the BSA (fraction V).

The strong signal of BSA at about 70.6kD made it impossible to detect the signal for SL-O which has MW of about 69kD (though it is reported on running on SDS anywhere within the values 60 and 80 kD [3]).

SL-O binds to cholesterol containing membranes and I decided to exploit this characteristic by trying to extract pure SL-O from the Murex formulated material. Liposomes extracted from red blood cells were incubated together

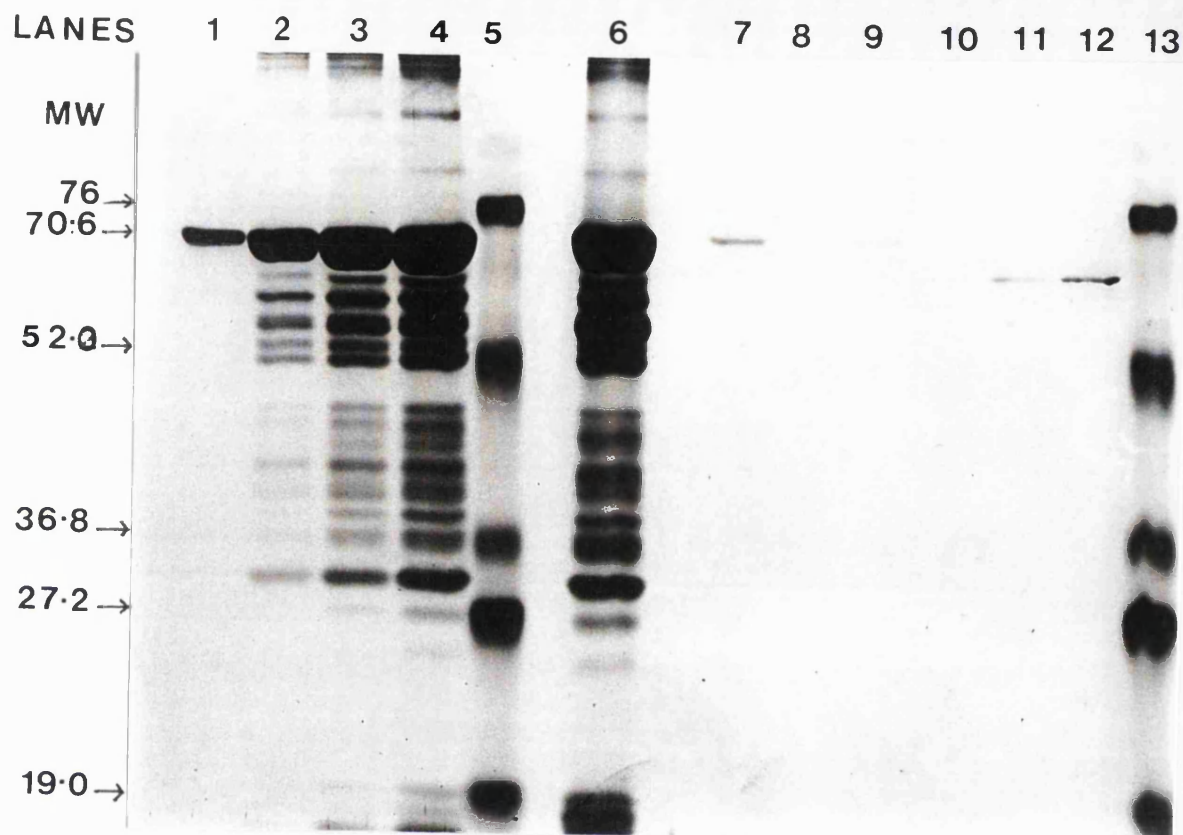


Fig 3.6

SDS polyacrylamide gel electrophoresis of bovine serum albumin (fraction V), recombinant and Murex SL-O.

Albumin (lanes 1-4), purified SL-O (lanes 11 and 12) and Murex SL-O (lane 6) were extracted directly into Laemmli sample buffer and applied to a 10% gel. Albumin (lanes 8-10) and Murex SL-O (lane 7) were extracted onto liposomes prepared from red blood cell lipids as described in chapter 2. Separated proteins were visualised using a silver stain and photographed. Lanes: 1 BSA 1.67µg; 2, BSA 8.33µg; 3, BSA 16.7µg; 4, BSA 33µg; 5, MW markers; 6, Murex SL-O 1.2iu; 7, Murex SL-O extracted with red cell lipids; 8, BSA 1.67µg extracted red cell lipids; 9, BSA 16.7µg extracted with red cell lipids; 10, BSA 167µg extracted with red cell lipids; 11, Purified SL-O 3µg; 12, Purified SL-O 6µg; 13, MW markers. The MW markers indicate (from top to bottom kDa) 76, 52, 36.8, 27.2, 19. The MW of bovine serum albumin estimated at 70.6 was also shown.

with SL-O for 10 minutes. This mixture was centrifuged and the pellet obtained washed four times to remove all the unbound material. When run on an SDS-PAGE gel under denaturing conditions, a single band was observed (lane 7). In order to ensure that BSA (other contaminating proteins) do not bind to the cholesterol-containing membranes, liposomes were treated with increasing concentrations of BSA (fraction V), then washed by centrifugation, extracted and run on the gel (lanes 8-10) with no detectable signal. Single bands were detected for the recombinant SL-O on SDS-PAGE (see lanes 11-12) (no added liposomes), with a MW of 67.6 kD. Although no detectable signal was present for BSA in the presence of liposomes it was impossible to assign the single signal in lane 7 definitely to Murex SL-O. This is because the molecular weights of SL-O and BSA are so similar (see lanes 4 and 6). Also, the recombinant material (lane 12) ran at a slightly lower molecular weight than that of the Murex product (lane 6). Having said that it is possible that the signal in lane 7 is SL-O. This is because of the large range of molecular weights at which SL-O has been observed to run on SDS gels [3].

3.7 Inhibition Of Secretion By Red Blood Liposomes In The Presence Of SL-O

Since SL-O can bind to cholesterol containing membranes, it is possible that cholesterol containing liposomes could compete with the cells for the binding of SL-O. In this case the permeabilising agent would be unable to bind to the cells and no lesions ^{would be} generated. The divalent cation Ca^{2+} and the guanine nucleotide analogue $\text{GTP}\gamma\text{S}$ would be unable to enter the cells and thus secretion induced by ~~this~~ two effectors will be inhibited. This idea was tested by incubating mast cells with increasing concentrations of Murex SL-O. Liposomes were sonicated to increase their surface area for binding. Increasing concentrations of liposomes were added to the cells ~~and~~ in the presence of Murex SL-O.

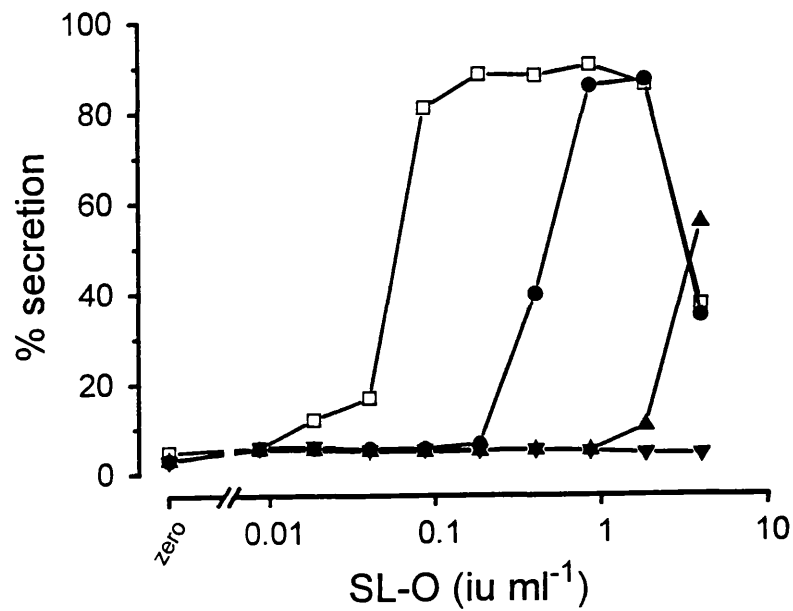


Fig 3.7

Inhibition of exocytosis from SL-O-permeabilised mast cells by red cell derived liposomes.

Mast cells were treated with Murex SL-O at the concentrations indicated in the presence of sonicated liposomes (as stated on page 41). Secretion was induced with Ca^{2+} (pCa5), $\text{GTP}\gamma\text{S}$ ($10\mu\text{M}$) and ATP (1mM). The black symbols indicate increasing concentration of lipids.

As shown in fig 3.7, addition of the liposomes had no effect on the maximum extent of secretion, but required progressively higher concentrations of the permeabilising agent. As the lipids were increased 10fold, an equivalent increment of SL-O was required to induce secretion. This emphasized the fact that although the Murex product is impure (as on the gel, lane6), it is the SL-O component of this formulation which permeabilises the cells.

Discussion

Streptolysin-O has become an important reagent in the investigation of secretion. It allows the precise regulation of the concentrations of low molecular weight solutes. We can also test the effect of allowing endogenous proteins to leak from the cells and the effect of applying exogenous proteins to the leaky cells. This is one of the main aims of my work.

The sudden and unexpected withdrawal of Murex SL-O from the Murex company threatened all hope of progress in this field. I therefore searched for alternative sources of this material and obtained supplies of purified protein (from Streptococcal culture filtrates) and a recombinant protein from Dr S Bhakdi in the University of Mainz.

I have noted that Ca-independent secretion (requiring Mg.ATP) can be induced by GTP γ S from mast cells permeabilised in NaCl based buffers if either the purified or recombinant SL-O are used. This was surprising as this result had previously been obtained only in Na-glutamate buffers. This difference could be due to impurities in the formulation.

Murex SL-O contains phosphate salts, cysteine, bovine serum albumin and proteinases [129]. At the working concentration of 0.4iu/ml the concentration of cysteine is 0.77mM and phosphate is present at 16mM. The amount of BSA present has not been disclosed.

Following dialysis and ultrafiltration, I found that cells permeabilised with Murex SL-O could undergo secretion in the absence of Ca²⁺. By applying the prebind protocol Ca²⁺ independent secretion was induced. The situation must be more complex than this, since the ultrafiltrate from the Murex preparation when added to the purified protein did not force secretion to occur in a Ca-dependent manner.

In conclusion the Murex product when dialysed or applied by the prebind protocol can be made to behave like the pure or recombinant products. However, when the reverse was done (i.e purified protein +ultrafiltrate from Murex SL-O) the pure proteins do not behave like the Murex product.

Eventually, after about a year, the Murex product (now from Murex Biotech) came back on stream. In view of the experience gained in comparing the various preparations, all subsequent work described in this thesis was carried out using the prebind method described in this chapter.

Chapter 4

THE STUDY OF THE REQUIREMENTS FOR EXOCYTOSIS FROM GUINEA PIG EOSINOPHILS.

Eosinophil granules contain a number of basic cationic proteins such as major basic protein (MBP), eosinophil peroxidase (EPO), eosinophil derived neurotoxin (EDN) and eosinophil cationic protein (ECP) [145]. The mechanism by which these are released is important because the release of the granule components is central to the toxic activities of these cells. Degranulation is thought to take four routes, necrosis, apoptosis, piece-meal degranulation (PMD) and secretion [164]. Eosinophils at sites of inflammation have centralised granules that have been disrupted, the plasma membranes and nuclei of these cells tend to be lysed. This is a state of cell death termed necrosis [44]. In culture, eosinophils can undergo programmed cell death but unlike necrosis, the integrity of the cell is kept intact [169]. This process of cell death can be decelerated in the presence of cytokines such IL-5, IL3 and granulocyte macrophage colony stimulating factor (GM-CSF) [146]. PMD is a process by which the contents of the granule are emptied either partially or completely into another vesicle. This vesicle then fuses with the plasma membrane to release their contents. The constituents of the granule core (MBP) or part of the granule matrix (for example EPO) or all of the granule contents may also be released.

Intact eosinophils cultured in the presence of IL-5 release some of their granule contents such as MBP and EDN over time and these cells also remain viable for longer periods [84].

Secretion from eosinophils has been studied by the use of other techniques such as patch-clamp and by permeabilisation. Both techniques have demonstrated that secretion from eosinophils of at least three animal species, namely guinea pig [39], horse [139] and human [2] occurs by an exocytotic mechanism.

Here I have studied the requirements for secretion from SL-O permeabilised eosinophils by the application of Ca^{2+} and a non-hydrolysable analogue of GTP ($\text{GTP}\gamma\text{S}$). As mentioned earlier, exocytosis requires the presence of both Ca^{2+} and $\text{GTP}\gamma\text{S}$ [39]. If NaCl based buffers are substituted by isotonic buffered ^{Na.}glutamate, then ^{+ke} Ca^{2+} independent component of secretion occurs in response to $\text{GTP}\gamma\text{S}$ alone [121]. In my study of exocytosis from permeabilised eosinophils, I discovered that in simple NaCl based buffers, exocytosis can be induced by $\text{GTP}\gamma\text{S}$ alone but this requires the presence of ATP. I have also studied the effect of permeabilising the cells under different conditions (e.g. in the presence of Ca^{2+} or ATP or both) and leaving the cells for a period of time before the application of the stimulus. This process allows the leakage of cytosolic factors from the permeabilised cells. Following a long time delay after permeabilisation, the cells are unable to respond to stimuli. The lack of responsiveness to stimulation after leakage has occurred is referred to as "Run Down". Understanding the requirements for this process is important. This is because one of the aims of my work has been the application of exogenous proteins to permeabilised eosinophils to test for their possible roles in the regulation of exocytosis.

I have successfully measured the release of eosinophil peroxidase (EPO) from permeabilised eosinophils for the first time (as far as I know). The release of other granule markers such as hexosaminidase and aryl sulphatase were also measured. I have also observed that when the cells are 'run-down' in the presence of Ca^{2+} and ATP they can maintain responsiveness to stimuli for a longer period of time.

RESULTS

4.1 Dependence of exocytosis from permeabilised eosinophils on both Ca^{2+} and $\text{GTP}\gamma\text{S}$.

This experiment was carried out at an early stage in the work, before I had realised that impurities present in commercial preparations of SL-O could affect the activation characteristics of secretion (see Chapter 3). It was therefore performed in the presence of excess SL-O and its purpose was to confirm that secretion from eosinophils, like mast cells requires the two effectors, Ca^{2+} and $\text{GTP}\gamma\text{S}$. The effect of adding ATP was also tested. The observation that Ca^{2+} and $\text{GTP}\gamma\text{S}$ are required for eosinophil secretion had been made previously but only with respect to the release of hexosaminidase [39].

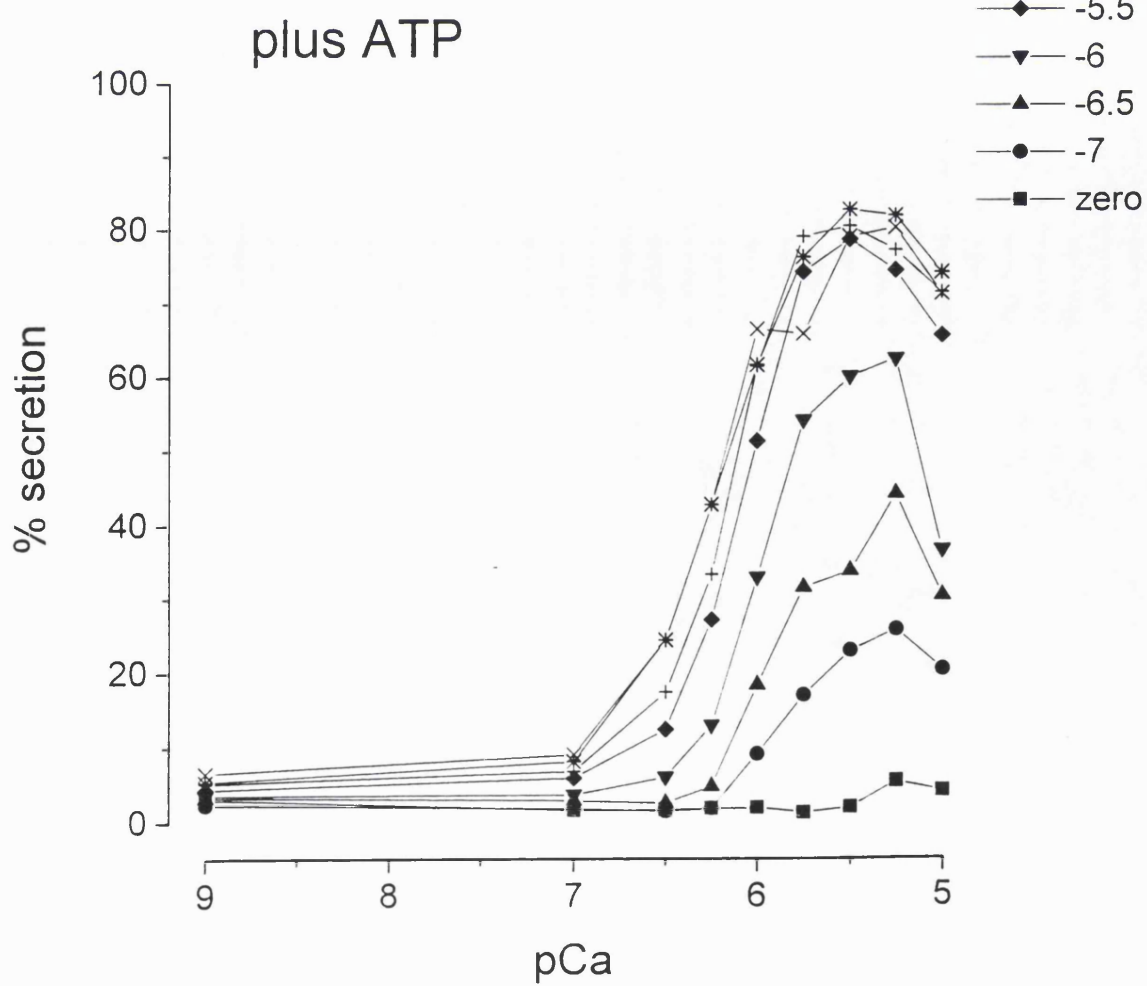
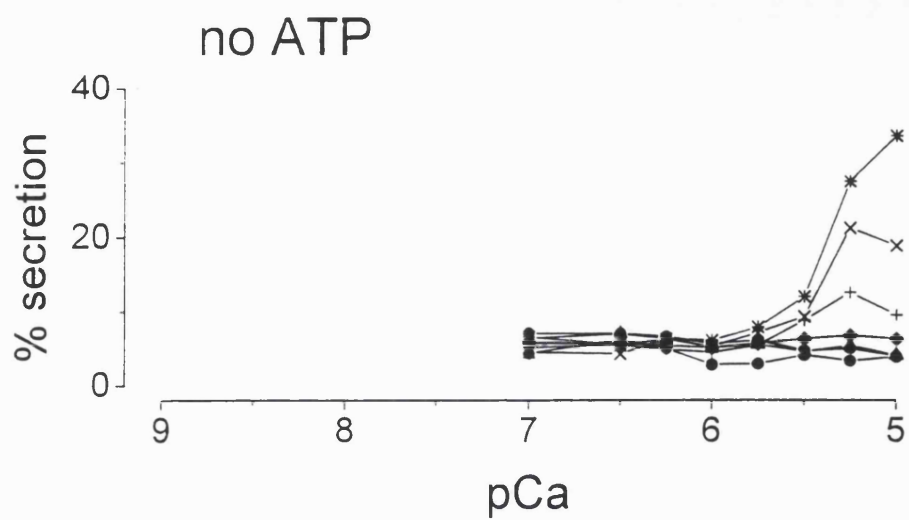
In this experiment (fig4.1), I stimulated the permeabilised eosinophils with a range of Ca^{2+} (pCa9-pCa5) concentrations and a range of concentrations of $\text{GTP}\gamma\text{S}$ (0-100 μM). This style of experiment is called a Ca^{2+} / $\text{GTP}\gamma\text{S}$ matrix. Secretion of eosinophil peroxidase (EPO), hexosaminidase, aryl sulphatase were measured from the supernatants after the cells had been allowed to secrete for 10minutes.

As shown in fig 4.1, when the cells are stimulated by 100 μM $\text{GTP}\gamma\text{S}$ and in the effective absence of Ca^{2+} (<pCa8), no secretion is achieved (<10%). Also, when the cells are stimulated by Ca^{2+} (pCa5) in the absence of $\text{GTP}\gamma\text{S}$, no secretion is achieved. In other words secretion requires both Ca^{2+} and $\text{GTP}\gamma\text{S}$. In the absence of ATP, secretion commences at 10 μM $\text{GTP}\gamma\text{S}$ and at concentrations of Ca^{2+} > pCa5.5. Similarly, very little Ca independent secretion occurs if ATP is provided, but in its presence, the EC_{50} for both Ca^{2+} and $\text{GTP}\gamma\text{S}$ are enhanced and the maximum release of EPO increases from about 35% (no ATP) to over 80% (plus ATP). When ATP is present the EC_{50} for both Ca^{2+} and $\text{GTP}\gamma\text{S}$ are reduced and

Fig 4.1

The requirement for both Ca^{2+} and $\text{GTP}\gamma\text{S}$ for exocytosis from permeabilised eosinophils.

The cells were permeabilised with SL-O in excess (not prebound) and stimulated for 10mins with a range of Ca^{2+} (pCa9-pCa5) and $\text{GTP}\gamma\text{S}$ (0-100 μM) concentrations and in the presence of ATP (zero or 1mM). Secretion was quenched with ice-cold HEPES buffered saline (pH 7).



maximum secretion is achieved at 3.16 μ M GTP γ S and Ca²⁺ at concentrations >pCa7. Irrespective of whether ATP is present or not, the highest concentration of Ca²⁺, pCa5, is seen to be slightly inhibitory to secretion but the reason for this is still unclear. Although not measured in this particular experiment, high concentrations of Ca²⁺, pCa5, also suppressed secretion of aryl sulphatase and hexosaminidase. It is worth remarking that in platelets, high concentrations of Ca²⁺ (>10 μ M) at first appeared to be inhibitory to lysosomal enzyme secretion. This effect can be annulled when protease inhibitors such as leupeptin and antipain are applied [8] suggesting that the loss of secreted enzyme activity is an artefact due to the action of calcium dependent proteases. The slight inhibition of secretion occurring at pCa5 in eosinophils could also be due to the action of proteases secreted from these cells.

The results obtained from this experiment are very similar to those reported previously which however concerned only the secretion of hexosaminidase [39]. These results imply that in NaCl based buffers at least, both Ca²⁺ and GTP γ S are required for exocytosis from permeabilised guinea pig eosinophils. ATP has the effect of enhancing the affinity for both effectors as well as enhancing the maximum extent of secretion. Since the prebind protocol was not applied in this experiment, there was excess SL-O present, together with the impurities and additives of formulation. This means that if there is a Ca²⁺ independent component of secretion, it may have been masked by additives in the Murex SL-O preparation (see chapter3).

4.2 Secretion From Eosinophils is maximum by six minutes after stimulation at 37°.

At the time I commenced my investigation, very little work had been performed on SL-O permeabilised eosinophils [39,121]. Previous experiments suggested that secretion of hexosaminidase from eosinophils reaches a maximum in about seven minutes and that secretion is faster at higher concentrations of GTP γ S (in the range 10^{-9} - 10^{-4} M) [39]. An experiment to measure the time course of secretion was therefore performed to verify and extend previously existing data.

The cells were stimulated at the time of permeabilisation (which once again was not applied by the prebind method) with Ca²⁺ buffered at pCa5 and a range of GTP γ S concentrations in the presence of 1mM Mg.ATP. The cells were allowed to secrete for different lengths of time after which the reaction was quenched with ice cold Hepes buffered saline.

At low concentrations of GTP γ S a delay (2mins at 10^{-7} M) is apparent before secretion commences (see fig 4.2). This delay declines as the concentration of the stimulus is increased so that at concentration of 10^{-5} M and above, secretion commences within 1minute. At nearly all the concentrations of GTP γ S, maximum secretion was observed by six minutes after stimulation. The time lag preceding the onset of secretion suggests that there may be an event that needs to occur before secretion commences.

The secretion of the three enzyme markers gave very similar results but only the data for EPO are shown.

4.3 Secretion From Permeabilised Eosinophils Takes At Least Eight Minutes To Begin At Room Temperature.

One possible way of obtaining better resolution in the time course experiments would be to slow down the process and a good way of doing

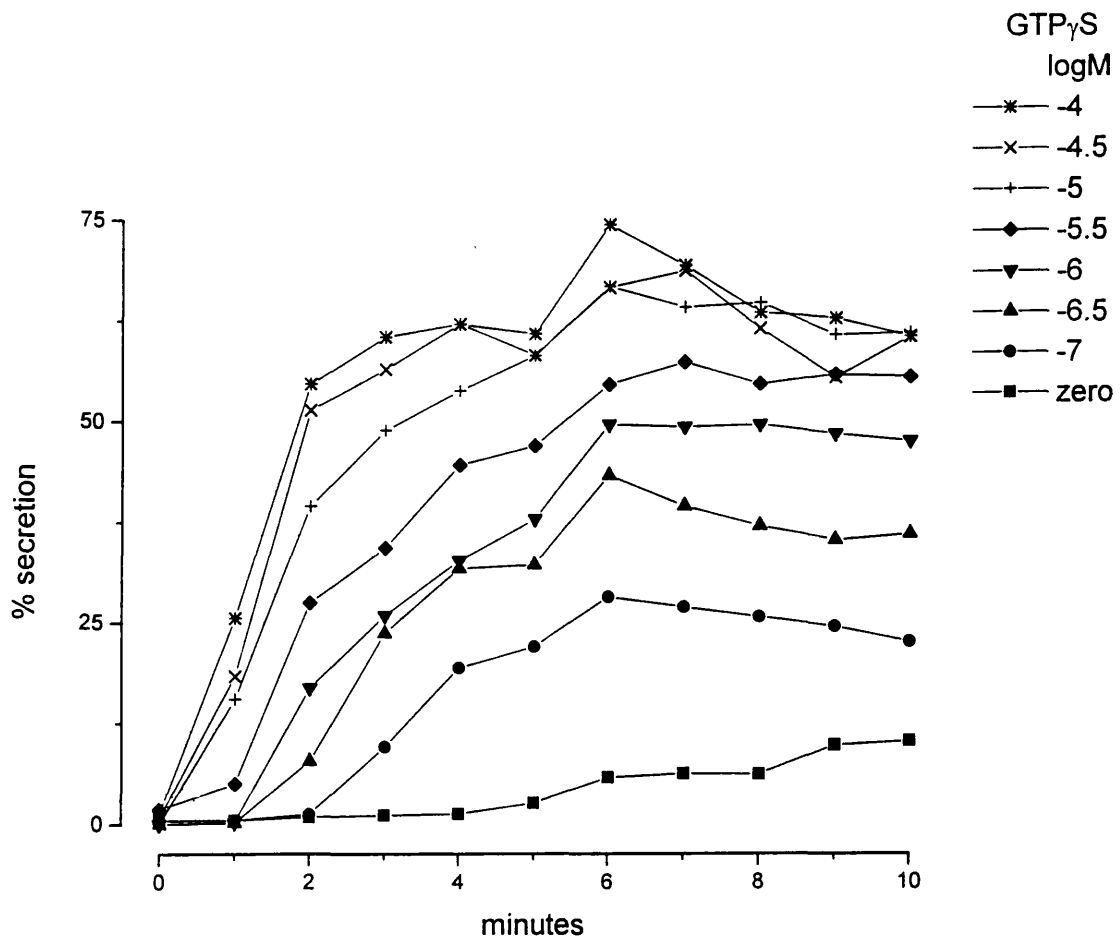


Fig 4.2

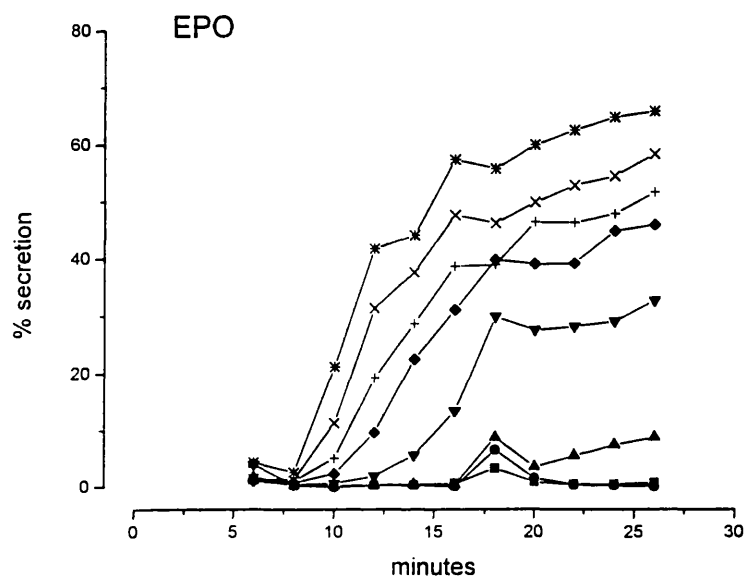
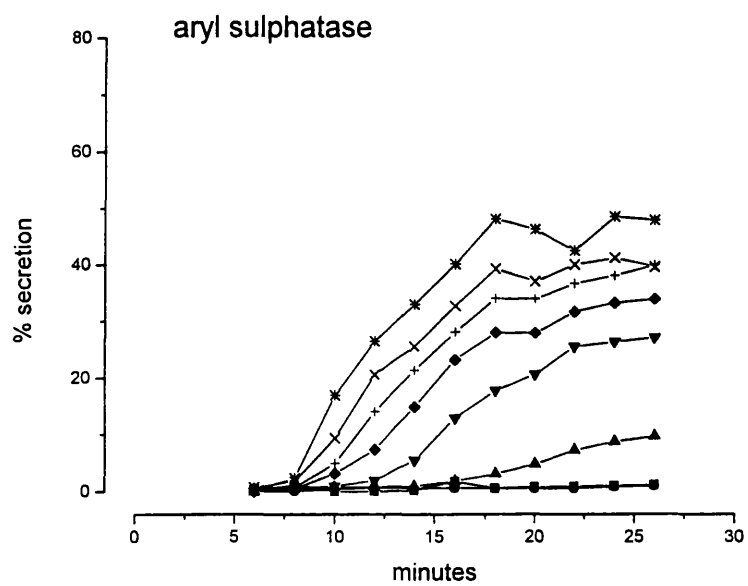
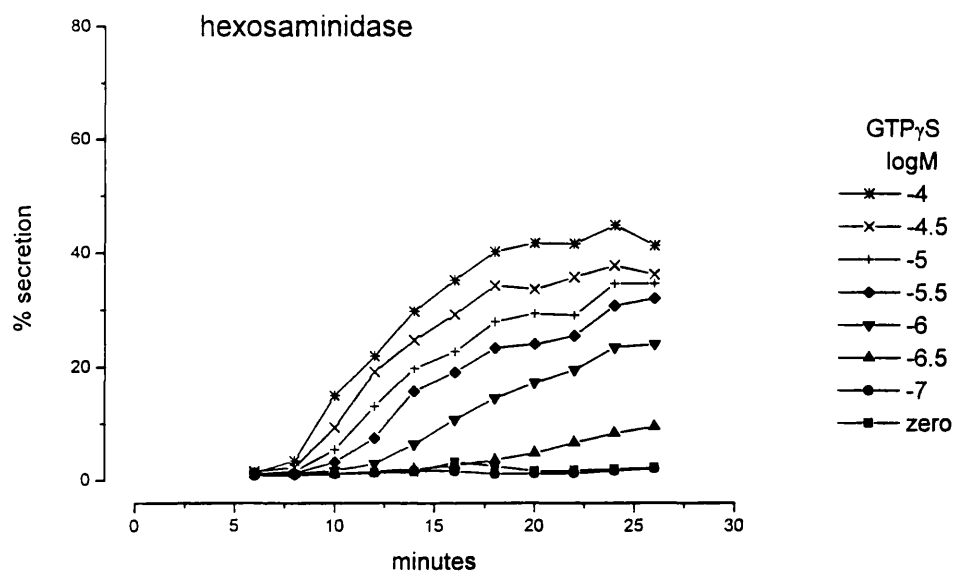
Secretion is complete within 6mins at 37°.

Cells were permeabilised with Murex SL-O in excess and stimulated with Ca^{2+} (pCa5) and a range of GTP γ S concentrations and ATP (1mM). The cells were sampled at the times indicated and processed for measurement of released EPO.

Fig 4.3

Secretion from eosinophils is delayed for up to 8mins when incubated at room temperature.

Again, the cells were permeabilised in the presence of excess SL-O and secretion was stimulated with Ca^{2+} (pCa5), ATP (1mM) and GTP γ S at concentrations between 0-100 μ M. After 10 mins incubation at room temperature the cells were sampled for measurement of secreted EPO, hexosaminidase and aryl sulphatase.



this should be by reducing the temperature as shown in fig 4.3. The first sample was taken six minutes after stimulation at which time no secretion had occurred at any concentration of GTP γ S applied.

As in the previous experiment (see fig 4.2) the time lag is dependent on the concentration of GTP γ S applied and at low concentrations of GTP γ S ($10^{-6.5}$ M, 0.316 μ M) the time lag was extended to 16mins. Similar results were obtained for all three enzyme markers (fig 4.3a,b,c) and the similarity between them suggests that they may reside in the same granule.

These results indicate that at room temperature, secretion is slow. For time course experiments, this is an advantage since it allows one to resolve the time lags which occur at the higher concentrations of GTP γ S ($\geq 10^{-5}$). The reasons underlying these time lags are unclear.

4.4 What is the effect of PKC on exocytosis from guinea pig eosinophils?

This experiment addresses the possibility that the enhancement in the EC₅₀'s for Ca²⁺ and GTP γ S, by ATP is mediated by PKC.

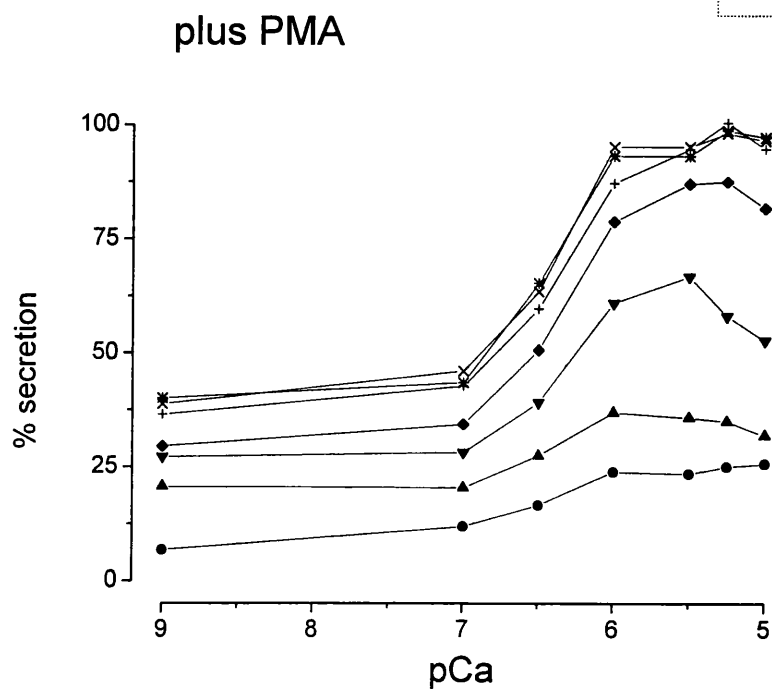
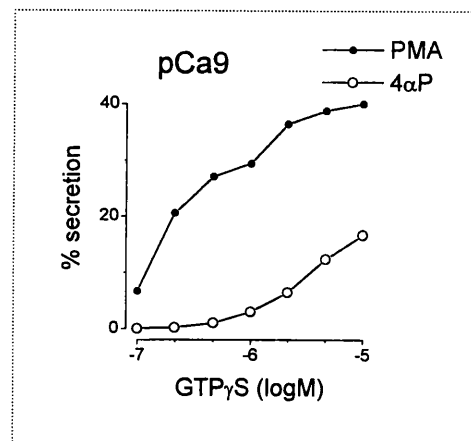
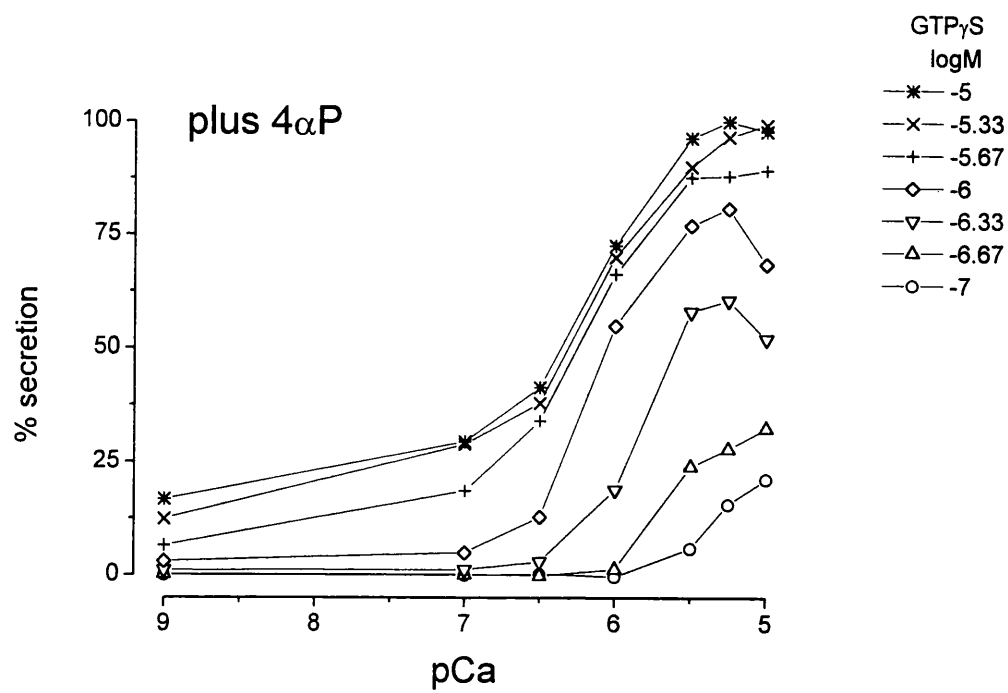
This experiment was performed in the presence of Mg.ATP and in the presence or absence of PMA (in the absence of PMA, a structurally inactive analogue of PMA (4 α P) was provided). The prebind protocol was applied in this experiment and in all other experiments performed after this unless otherwise stated.

In contrast with the initial experiments (fig 4.1), these results indicate (see fig4.4a,b) that at pCa9 (EGTA 3mM), a substantial component of secretion occurs even in the absence of PMA (~15%). In its presence the extent of this component of secretion is greatly enhanced. The presence of PMA also has the effect of enhancing the extent of secretion at all concentrations of

Fig 4.4

Ca²⁺ independent secretion can be induced in the absence of PMA using the prebind protocol.

Secretion was induced with GTP γ S (10 μ M), PMA (100nM: control = 4 α -phorbol), ATP (1mM) and a range of concentrations of Ca²⁺ (pCa9-pCa5). (Note: the prebind protocol was applied in this and all subsequent experiments, and incubations were allowed to proceed for 10 mins unless otherwise stated).



Ca^{2+} below pCa5.5, and then, as before, the highest concentration of Ca^{2+} causes slight inhibition of secretion at nearly all concentrations of $\text{GTP}\gamma\text{S}$.

These results indicate that a Ca^{2+} -independent component of secretion does occur in these cells but it requires the prebind protocol to be applied and thus implies that this component may have been masked by the excess SL-O material present in earlier experiments. Secretion can be induced with $\text{GTP}\gamma\text{S}$ in the presence of ATP (no Ca^{2+}) and this is not dependent on the action of PMA. In the presence of PMA, secretion is enhanced at all concentrations of Ca^{2+} including pCa9.

4.5 Cells Are Unable To Respond To Stimulation when A Long Delay Follows Permeabilisation; A Process Called “Run Down”.

One of the aims of my work is the application of exogenous proteins to permeabilised eosinophils to test their possible roles in the regulation of exocytosis. It is possible that it would take time for exogenous proteins to penetrate through SL-O induced lesions, find and adhere to their natural binding sites in the cells. For this reason, it was necessary, to investigate how the cells respond when the stimulus is applied late, after allowing time for the entry of exogenous proteins.

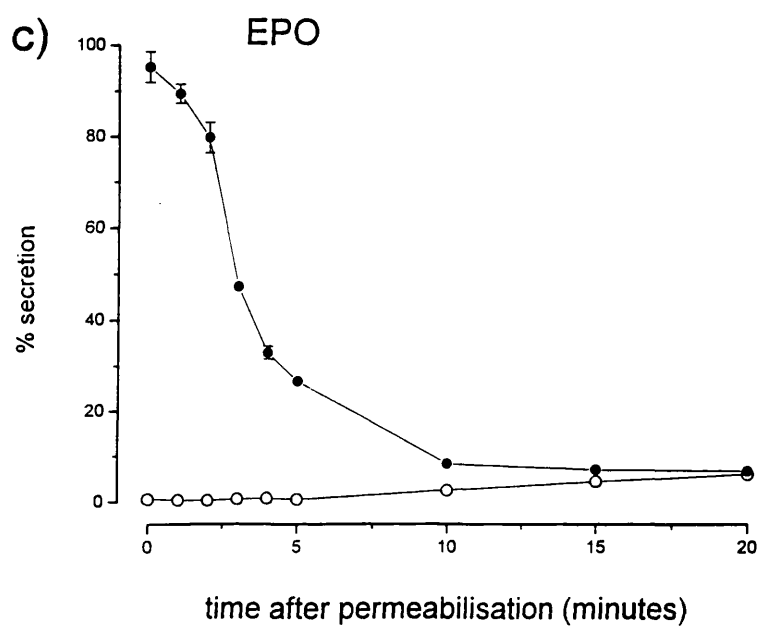
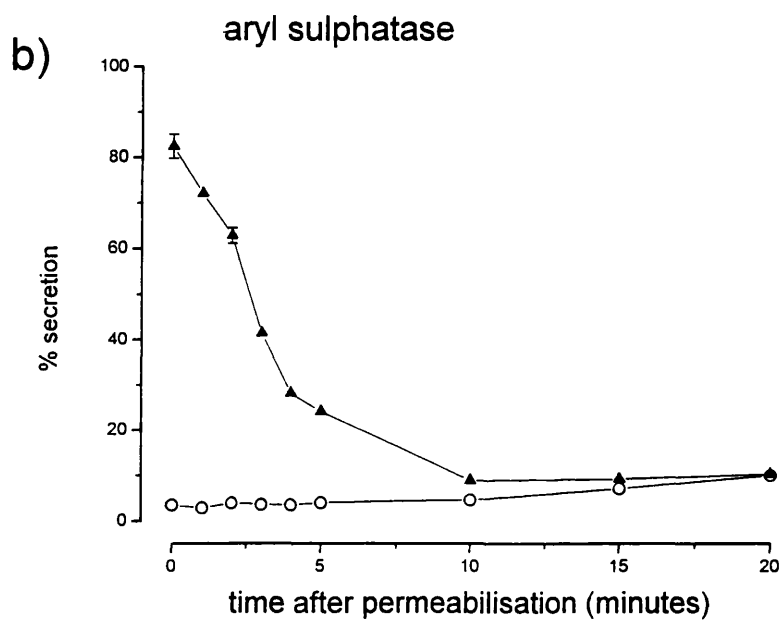
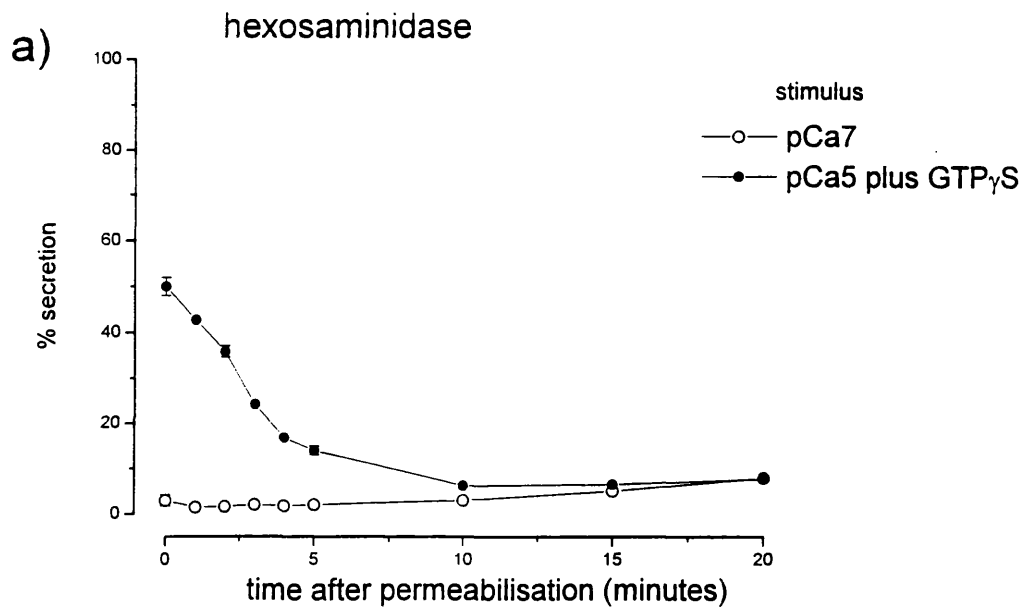
The experiment was performed in the presence of an ATP regeneration system. While it is not crucial to this experiment (this will be made evident in experiments presented later), for later work in which crude cytosolic extracts of brain homogenates are applied, this becomes important.

The experiment (see fig 4.5a,b,c) was performed by stimulation (with pCa5 and $100\mu\text{M}$ $\text{GTP}\gamma\text{S}$) of the cells at the time of permeabilisation (time-0) and then also after various time delays for up to 20mins. The cells were then allowed to secrete for 10mins. Thus cells stimulated 20mins after permeabilisation were allowed to secrete until time (t)=30mins.

Fig 4.5

Time course of run down of permeabilised eosinophils in the presence of an ATP regeneration system.

Cells were permeabilised in a buffer containing enough EGTA (0.1mM) to suppress Ca^{2+} to pCa7. At the times indicated, samples of cells were removed and transferred into solutions containing high concentrations of Ca^{2+} (pCa 5) and GTP γ S (100 μ M). After a further 10mins, the reaction was quenched and the supernatants processed for measurement of A) hexosaminidase, B) aryl sulphatase and C) EPO (Note: error bars have been removed where they are smaller than the points). *Each sample was performed in quadruplicate.*



Maximum secretion was obtained when the stimulus and the permeabilising agent were applied together (nearly 100% EPO release). The ability of the cells to respond to a late stimulus decreases fairly rapidly and 20mins following permeabilisation they were unable to respond at all. A similar result was obtained for all three enzymes measured. It is important to note that the time it takes for the cells to become refractory to stimulation is variable. On some occasions the cells became refractory to stimulation within 10mins and on others they remained viable for as long as 50mins.

The similarity in the rate of run down of the three enzymes is further evidence that their secretory components must reside in the same granule. It is important to note that although almost 100% of EPO was released, considerably less of hexosaminidase and aryl sulphatase was released.

4.6 There are variations to the time course of 'run down' in eosinophils.

The previous experiment indicates that eosinophils once permeabilised can remain responsive to stimulation for at least 20mins and sometimes even longer. However, the time course of run down is variable. The procedure is important for later work (to be described) and so the basic experiment was repeated many times. On a number of occasions, run down did not commence for about 5mins and on others the response even appeared to run up during the first few minutes following permeabilisation (see fig 4.6). This difference may be due to the leakage of a cytosolic factor or factors that is/are inhibitory to exocytosis. It must be stressed that 'run up' is not typical. The basic run down experiment must have been applied on at least 100 occasions and the run-up phenomenon was observed on not more than 10 occasions. An indication of the variability of the run down is illustrated in fig 4.6b. In a set of 12 randomly selected experiments the mean time taken for the response to decline to 50% was 8.71 minutes, standard error 1.2 minutes).

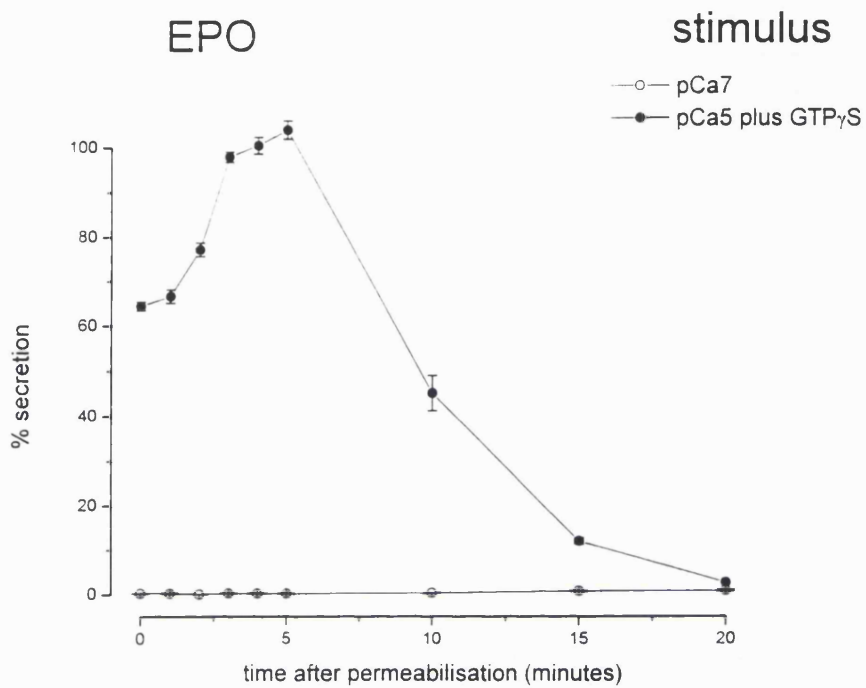
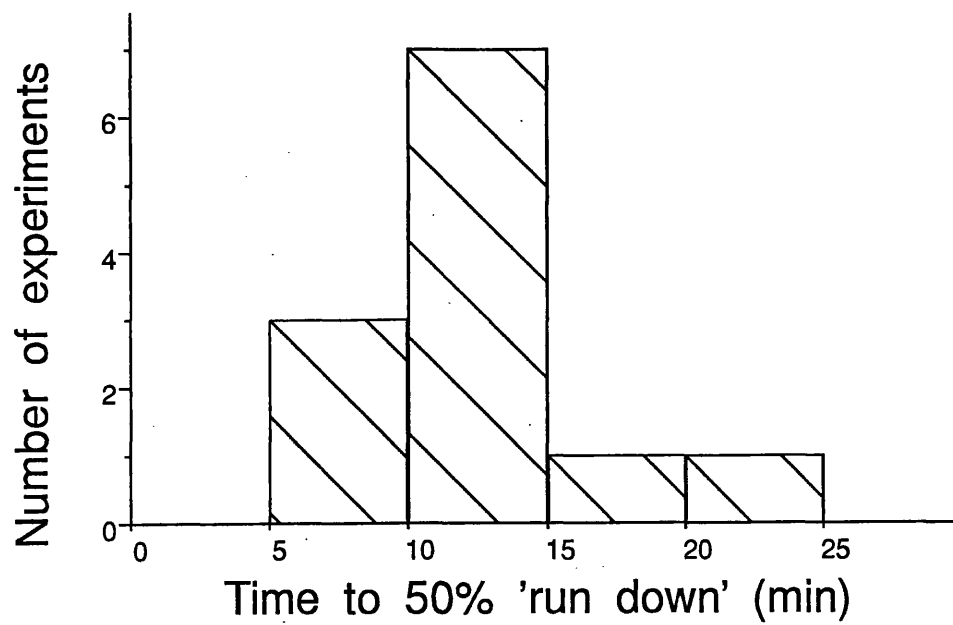


Fig 4.6

An example of the variability in the time course of run down.

The cells were permeabilised and stimulated as in fig 4.5. In this experiment, the extent of secretion increased when the stimulus was applied for up to 5mins, and declined thereafter.

Histogram of times taken for the response to pCa5/GTP- γ -S to fall to 50% of that at $t = 0$



4.7 Effect of ATP on the duration of responsiveness to stimulation.

Having studied the time course of run down, I decided to investigate the role of ATP in this process. I performed an experiment in which I tested the effect of removing and then adding back ATP during the 'run down process'.

The cells were allowed to run down in either low Ca^{2+} (pCa7) or high Ca^{2+} concentrations (pCa5) and then stimulated with $\text{GTP}\gamma\text{S}$ and Ca^{2+} as appropriate. When ATP was omitted in the run down process it was applied at the time of stimulation.

Fig 4.7 shows that when the cells are allowed to run down in the presence of Ca^{2+} alone (no ATP), they were unable to maintain their responsiveness for longer than 45mins. However, in the presence of low concentrations of Ca^{2+} (pCa7) and ATP (1mM), run down was retarded such that after 20mins, the cells could still secrete ~40% of their total content (more than in the absence of ATP). In the presence of both ATP and Ca^{2+} buffered at pCa5, run down was retarded for even longer. After 30mins, the cells were still viable and secreted ~30% of their content of EPO.

The data obtained from this experiment suggests that Ca^{2+} on its own cannot fully support a slow rate of run down. To achieve this, it is necessary to supply ATP.

4.8 Does the concentration of ATP applied during the 'run down' affect the process?

Although ATP is not essential for secretion from eosinophils, its possible effect in regulating the rate of run down had not been investigated. To this end, an experiment was performed in which the cells were stimulated at the time of permeabilisation and then after various time intervals in the presence of different concentrations of Mg.ATP.

Fig 4.8 illustrates that, maximum secretion can be induced with 1mM ATP. With time, these cells began to lose their responsiveness to stimulation such that 30mins following permeabilisation, they were fully refractory. Increasing the concentration above 1mM ATP had no effect on secretion at the time of permeabilisation, but run down was slower so that 20% secretion could be induced after 40mins in the presence of 10mM ATP. The amount of EPO released at the time of permeabilisation and at various time intervals was dependent on the concentration of ATP.

Hence, the length of time it takes for the cells to become refractory to stimulation is dependent on the concentration of ATP supplied.

4.9 What happens to the run down process when ATP is depleted suddenly?

The run down experiments performed thus far have indicated that in the presence of high concentrations of ATP, the cells lose their responsiveness to stimulation at a much slower rate. I therefore studied the run down process under conditions where ATP was suddenly withdrawn, just before applying the stimulus. The aim was to answer the question of whether ATP is a prerequisite for the run down.

Hexokinase was provided to the cells at the time of permeabilisation. It would equilibrate with the cells so that following addition of excess glucose,

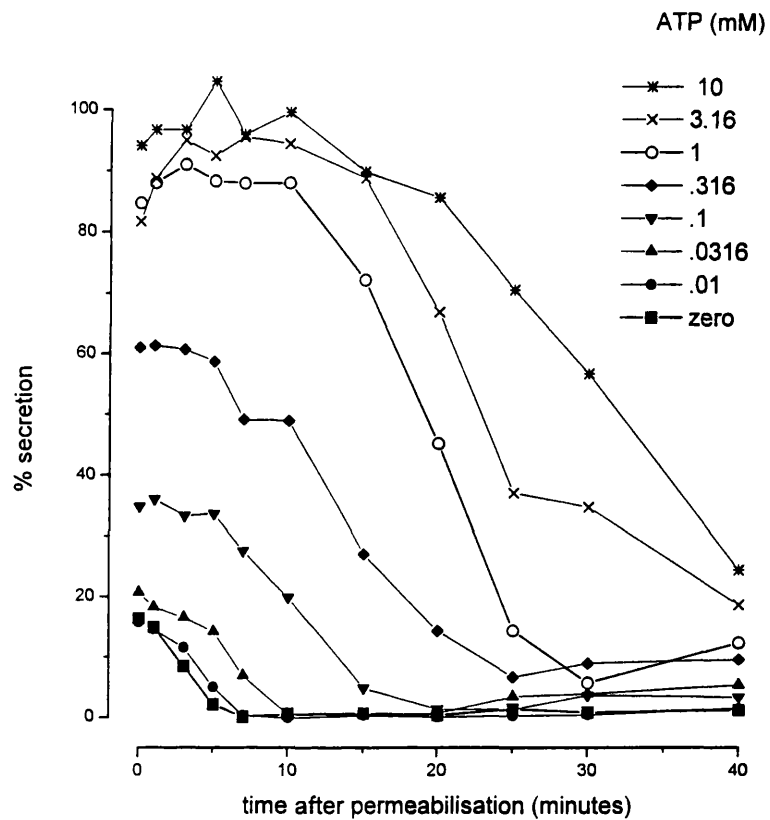


Fig 4.8

The rate of run down is dependent on the amount of ATP provided.

The cells were allowed to run down in the presence of a range of ATP concentrations and stimulated at the different time intervals indicated with $\text{GTP}\gamma\text{S}$ ($100\mu\text{M}$) and Ca^{2+} at $\text{pCa}5$.

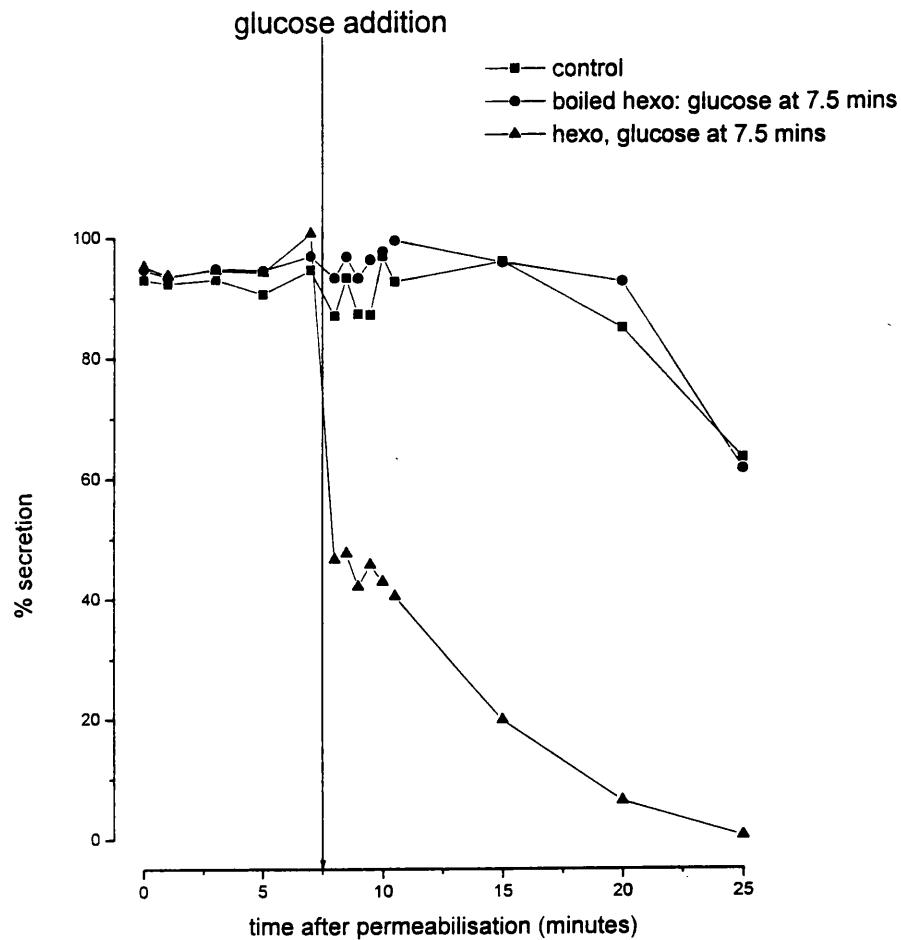


Fig 4.9

Sudden depletion of ATP accelerates rundown, but the cells still remain responsive for up to another 15mins.

The cells were permeabilised with SL-O in a buffer containing Mg.ATP (1mM), Ca^{2+} at low concentrations (pCa 7) and allowed to equilibrate with hexokinase (750units/ml). The cells were stimulated after time intervals indicated with Ca^{2+} (pCa5) and GTP γ S (100 μ M). 7.5mins following permeabilisation, glucose (10mM= 10 μ Moles/ml) was added in order to deplete the ATP (one unit of hexokinase will phosphorylate glucose at 1 μ Mole in 1min at 25° and pH 7.6). The cells were stimulated at the times indicated and sampled as described in the legend to fig 4.7.

the ATP would decline to zero in a few seconds. The cells were allowed to run down for 7.5mins in the presence of ATP (1mM), low Ca^{2+} (pCa7) and hexokinase after which excess amounts of glucose was added (10mM). Samples were withdrawn at 30secs intervals for the next 2.5mins and then at five minutes intervals subsequently until 25mins following permeabilisation. I also ran two necessary controls; 1) no added hexokinase and 2) boiled hexokinase.

When the cells were stimulated at the time of permeabilisation (time=0), almost 100% secretion occurred and this was maintained for 7.5mins. Glucose was added at this point and the run down was accelerated such that after 25mins the cells had become fully refractory (see fig 4.9). Although the loss of responsiveness to stimulation was accelerated when glucose was added, the cells were still able to respond to stimulation in the effective absence of ATP for another 17.5 mins. The rate of run down was slower when hexokinase was absent or when applied after boiling and the cells could respond to stimulation 25mins after permeabilisation. Run down was only slightly affected by the presence of glucose.

4.10 Cells allowed to 'run down' in the presence of Ca^{2+} and ATP maintain their responsiveness to stimulation for a much longer period.

Having studied the process of run down in the presence of ATP alone, I tested the effects of Ca^{2+} and ATP together. The cells were therefore allowed to run down in the presence of pCa8 (low Ca^{2+} concentrations) or pCa5 (high Ca^{2+} concentrations) but with ATP present under both conditions. They were then stimulated with $\text{GTP}\gamma\text{S}$ and samples taken for the measurement of the release of EPO.

The experiment illustrated in fig 4.10 shows that when the cells are permeabilised in the presence of Ca^{2+} and ATP and allowed to leak proteins before the application of stimulus, they maintain their responsiveness to stimulus for much longer. In the absence of Ca^{2+} , the initial level of secretion

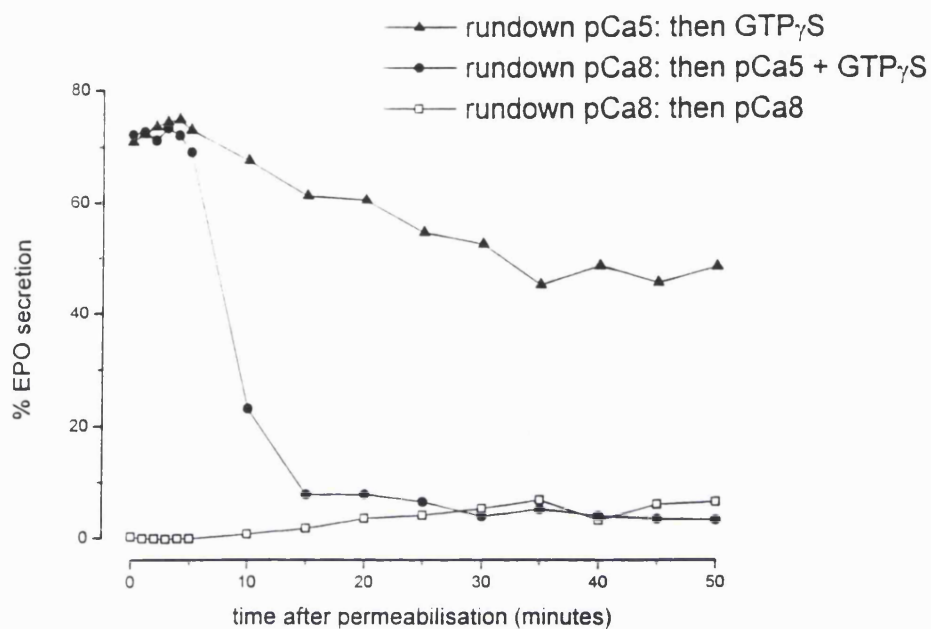


Fig 4.10

Run down is retarded by Ca²⁺.

The cells were permeabilised with SL-O in buffers containing Mg.ATP and in either low (pCa8) or high (pCa5) concentrations of Ca²⁺. After delays (indicated), the cells were stimulated by transfer to Ca²⁺ (pCa5) or GTP γ S (100 μ M) or both as appropriate. The cells were allowed to secrete for a further 10mins.

of ~70% was observed. Responsiveness then declined fairly rapidly and the cells were fully refractory to stimulation after 30mins. When run down in the presence of Ca^{2+} (pCa 5) however, the cells continued to respond to stimulation for up to 50mins after permeabilisation.

This result implies there may be a certain cytosolic factor or factors that are sensitive to Ca^{2+} . These may be retained only in its presence. It is this which may allow the cells to retain their responsiveness to stimulation for such a long time following permeabilisation. This factor has not been identified and its precise workings are not known. However, the phenomenon of Ca^{2+} regulated run down could be adapted as an assay procedure for Ca^{2+} sensitive factors for protein purification. It must be noted that the retarding run down requires the presence of both Ca^{2+} and ATP.

4.11 Subfractionation of guinea pig eosinophils

Previous experiments have indicated that although 100% EPO can regularly be released, the release of the other two enzyme markers (hexosaminidase and aryl sulphatase) are at a lower level. One possible explanation would be that these two enzymes are not exclusively confined to the crystalloid secretory granule. The subcellular distribution of these enzyme markers were studied by fractionating the cells using the technique described in the chapter 2. Enzyme assays were then performed.

Subfractionation experiments (see fig 4.11) indicated that EPO was confined to the granules of highest density (1.22g/ml). The other two enzyme markers, hex and aryl sulphatase were also found in these high density fractions. However, a substantial amount of ^(hexosaminidase)hex and a small but variable amount of aryl sulphatase are present in another set of fractions of lower density (between 1.12 and 1.15 g/ml).

The presence of some hexosaminidase and aryl sulphatase in a set of fractions of lower density than that of dense crystalloid granules may explain

Fig 4.11

Enzyme marker assays from subcellular fractionation of eosinophils.

Eosinophil post-nuclear supernatant was layered onto a linear gradient of Nycodenz (0-50% in 0.25M sucrose, buffered with HEPES) and centrifuged at 100,000g for 2 hours. Enzyme assays were performed on each fraction and their activities expressed as a percentage of the total.

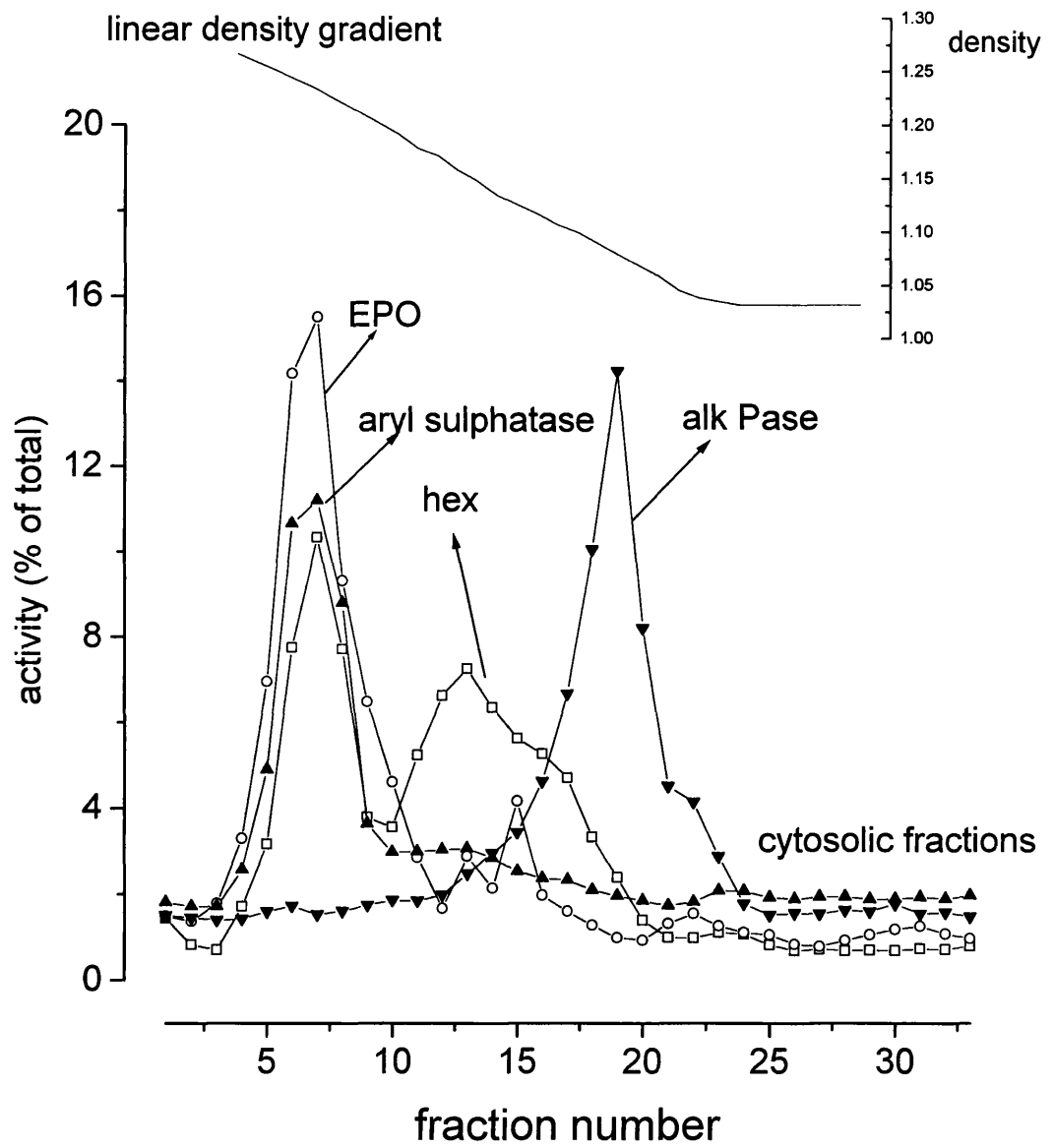
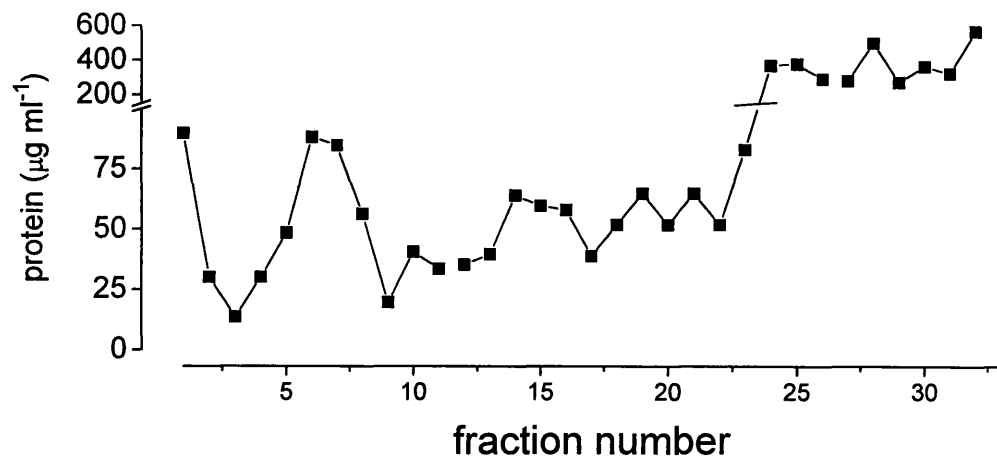
hex - hexosaminidase

perox - peroxidase

alk Pase - alkaline phosphodiesterase

cytosol - fractions containing
cytosolic proteins.

The upper part of this figure refers to the protein ($\mu\text{g ml}^{-1}$) concentration of each fraction collected.



the partial release of hexosaminidase and aryl sulphatase. Since eosinophils contain three types of granules, these fractions of lower density could be a second class of granules. Secondly, aryl sulphatase has been found in small granules in these cells [164]. It is equally possible that this lighter fraction is of lysosomal origin since these enzyme markers are also found in this organelle.

DISCUSSION

If the results of time course studies, Ca^{2+} /GTP γ S concentration response matrices and run down experiments are compared, it appears that the three enzyme markers (EPO, Hexosaminidase, Aryl sulphatase) reside in the same crystalloid granules and this is supported by data from cell subfractionation experiments (refer to fig 4.11) [93]. It was also noted that although 100% release of EPO was measured on many occasions considerably less of hexosaminidase and aryl sulphatase was released and this suggests that a small proportion of these two enzymes may be contained in a second organelle. This is interesting in itself because this second organelle (maybe another class of granule) could not be stimulated in the same way as the crystalloid granule and if such alternative granules can undergo exocytosis, then this process might be under different control. It must be noted that if aryl sulphatase is found in an organelle besides the crystalloid granule, then the proportion of enzyme present in the former would be small ($\leq 20\%$) because unlike hexosaminidase, up to 80% secretion can be induced on some occasions (see fig 4.5).

Exocytosis from eosinophils studied by applying the whole cell patch clamp technique indicated that this process is dependent on the presence of GTP γ S [121] and this is supported by data presented in this chapter. Although secretion can be induced in the absence of Ca^{2+} (with GTP γ S and ATP), it becomes important when the cells are permeabilised and allowed to leak cytosolic factors for a while before the application of stimulus.

One can conclude that exocytosis from eosinophils is certainly complex and the data presented here infers that there may be the involvement of a GTP binding protein and also a Ca^{2+} sensitive factor or factors in this process.

Chapter 5

Run down in the presence of exogenous factors

Calmodulin

Calmodulin is a Ca^{2+} binding protein with 148 amino acids present in all eukaryotic cells and involved in the activation of more than 20 enzymes which mediate a wide variety of physiological processes. These enzyme targets include myosin light chain kinase (MLCK), other protein kinases, phosphatases and nitric oxide synthases. This protein is characterised by an important motif, the EF hand which consists of a helix, a calcium-binding loop and a second helix. Calmodulin has 4 calcium binding domains and 8 helices. [87,110].

At the beginning of the 1980's studies performed with trifluoperazine (a drug that inhibits Calmodulin) in rat pancreatic islets suggested that this Ca^{2+} binding protein could be involved in the glucose induced secretion of insulin [86]. In contrast, the release of insulin stimulated by the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) in the presence of glucose was not affected [34]

In mast cells, trifluoperazine was found to inhibit the release of histamine induced by three secretagogues; antigen, compound 48/80 and Ca^{2+} ionophore A23187. The degree of inhibition was maximal when secretion was induced by antigen (60% inhibition) and least when induced by the ionophore (20% inhibition). Unfortunately, trifluoroperazine a member of the group of phenothiazine drugs (a group of tranquillizers and antihistamines) is known to affect the stability of membranes of cells independently of its effect on calmodulin and so the reason for its effect on secretion is not certain. In general, the role of calmodulin in exocytosis remains controversial, but there is strong evidence for its involvement in exocytosis from *Paramecium* [76]. More recently, purified calmodulin has been found to support the release of catecholamines from adrenal chromaffin cells. With the aid of stage specific assays which are diagnostic of an ATP-dependent

priming step and a Ca^{2+} dependent triggering step, the action of calmodulin was found to be confined to the triggering step [35]. It seems therefore to be important in exocytosis from at least two cell types.

α -SNAP

α -SNAP is a soluble protein to which NSF_A ^(N-methylmaleimide soluble factor) attaches when the former is bound to distinct sites in the plasma membrane. In permeabilised adrenal chromaffin cells, secretion of catecholamines can be supported by purified α -SNAP protein. Again a stage specific assay was used and it was shown that α -SNAP is required for both the ATP dependent priming stage and the Ca^{2+} dependent triggering step for the release of catecholamines [35].

RhoGDI

GTP binding proteins can be converted from an inactive GDP bound form to an active GTP bound form and this exchange is controlled by GDP/GTP exchange proteins (GEPs). These proteins can be divided into two classes which are inhibitory (GDP dissociation inhibitors (GDIs)) or stimulatory (GDP dissociation stimulators (GDSs)). Three of these GEPs have been isolated, cloned and their primary sequences determined. They are smgGDS, rhoGDI and rabGDI each of which associate with specific classes of the small monomeric GTPases. This association only occurs with the post-translationally modified forms of the monomeric proteins and the modifications include carboxy methylation and either a farnesyl (in Ras proteins) or a geranyl-geranyl moiety (Ras related proteins eg. rho, rac). Not only are these GEPs involved in the regulation of the exchange reaction between GTP and GDP but they are also involved in the translocation of small GTPases from the cytosol to their target sites in the plasma membrane [152].

A factor isolated from bovine brain cytosol consisting of a heterodimeric complex of Rac/rhoGDI has been demonstrated to support exocytosis from

permeabilised rat mast cells. This complex is able to retard the rate of run down. RhoGDI accelerates run down. One possible explanation for this could be that the RhoGDI detaches monomeric GTPases of the Rho family from their binding sites on the plasma membrane [124]. When purified rhoGDI (from bovine brain cytosol) was applied to rat peritoneal mast cells through a patch pipette [106], it had the effect of retarding the rate of secretion, increasing the time intervals between individual capacitance steps and decreasing the overall extent of secretion. The purified protein was found to be much more effective than the recombinant protein. Other groups have also implicated GTPases of the Rho family in mast cell exocytosis [133].

In this chapter, I have used the run down of secretion as a basis of assay to test separately the ability of these three proteins, calmodulin, α -SNAP, RhoGDI to support or suppress secretion from eosinophils. I discovered that while calmodulin and α -SNAP have no effect on secretion under these conditions, RhoGDI is an inhibitor as it accelerates the run down.

RESULTS

5.1 Does exogenous calmodulin accelerate or delay run down?

In this experiment, I applied calmodulin to permeabilised guinea pig eosinophils, using the standard run down procedure. Exogenous protein (50µg/ml) was presented to the cells at the time of permeabilisation. They were then stimulated at this time and at various times for up to 40mins. A parallel series of tests, in which the protein was absent, was run as a control. Mg.ATP was also supplied at a concentration of 1mM.

Fig 5.1a shows that from time 0 up to seven minutes following permeabilisation, the cells were able to secrete about 100% of EPO without any loss of responsiveness to stimulation. After this, they began to lose their response to stimulation fairly rapidly and after 30mins they were completely refractory. The presence or absence of calmodulin was without effect. A generally similar conclusion could be drawn when hexosaminidase was measured as the marker for secretion (see fig5.1b).

This experiment indicates that exogenous calmodulin does not alter the run down in eosinophils.

5.2 Does purified α -SNAP stimulate secretion from eosinophils?

Using the run down technique as a basis of assay as before, purified α -SNAP was tested in permeabilised eosinophils. Instead of testing the effect of the protein at different times during the run down, the cells were stimulated only at one time (10min), but on this occasion, a range of Ca^{2+} and $\text{GTP}\gamma\text{S}$ (a matrix) was applied.

Fig 5.2 a,b show that following run down (10mins), the cells were unable to respond to 1µM Ca^{2+} (pCa 6) even when $\text{GTP}\gamma\text{S}$ was added at maximum concentration (100µM). As the concentration of Ca^{2+} was increased above

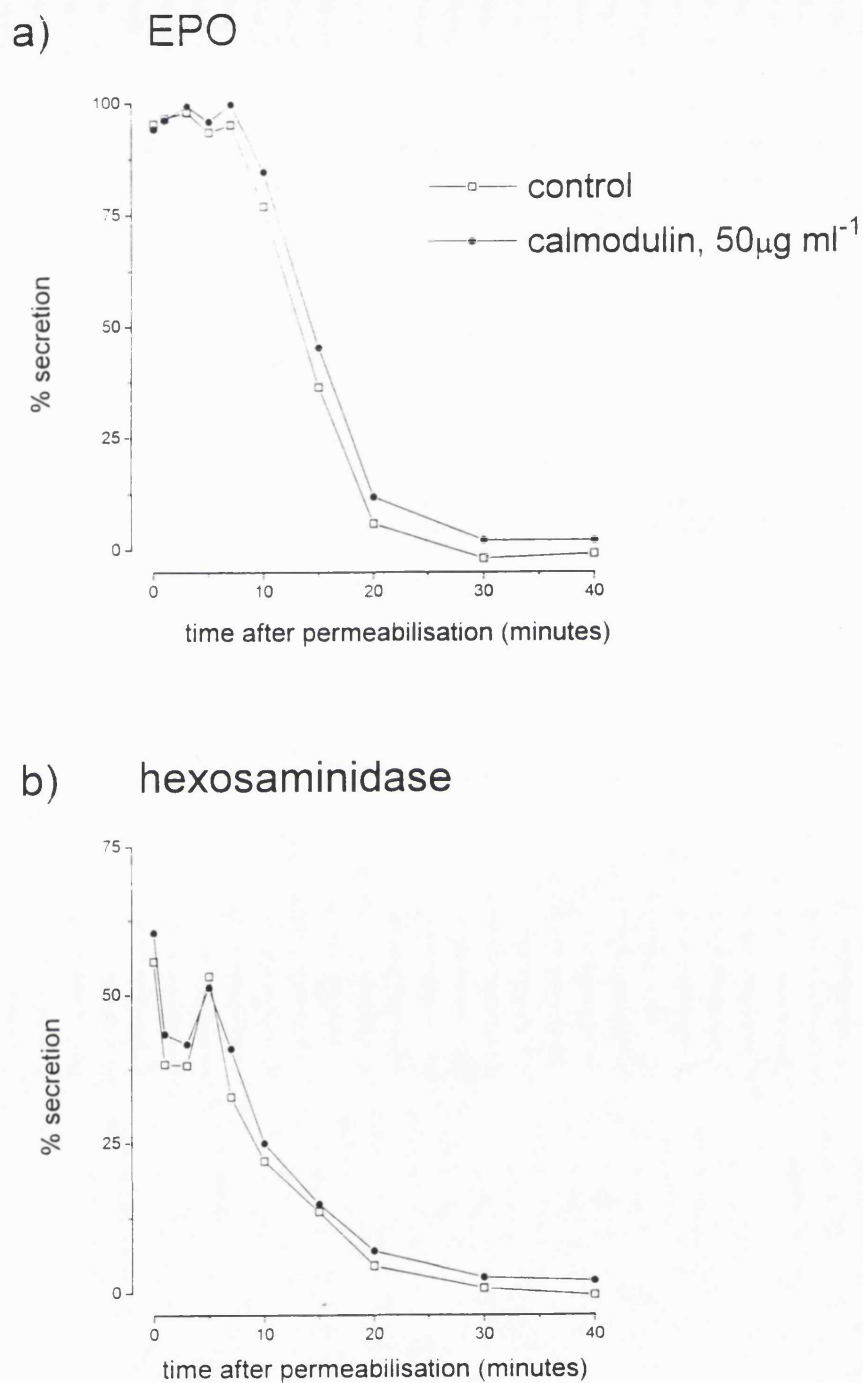


Fig 5.1

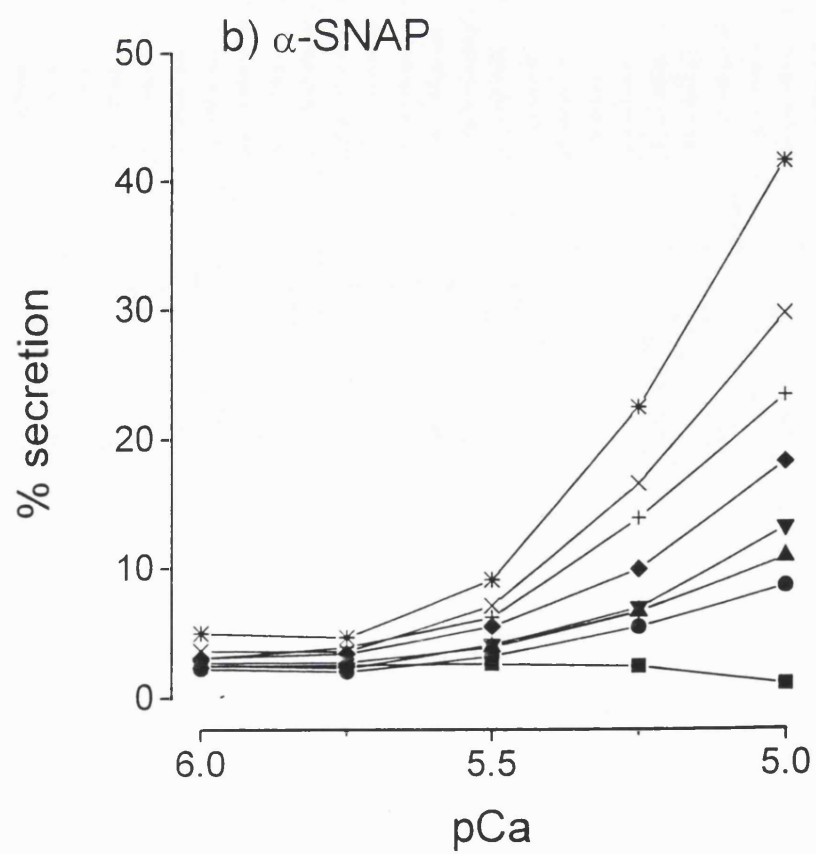
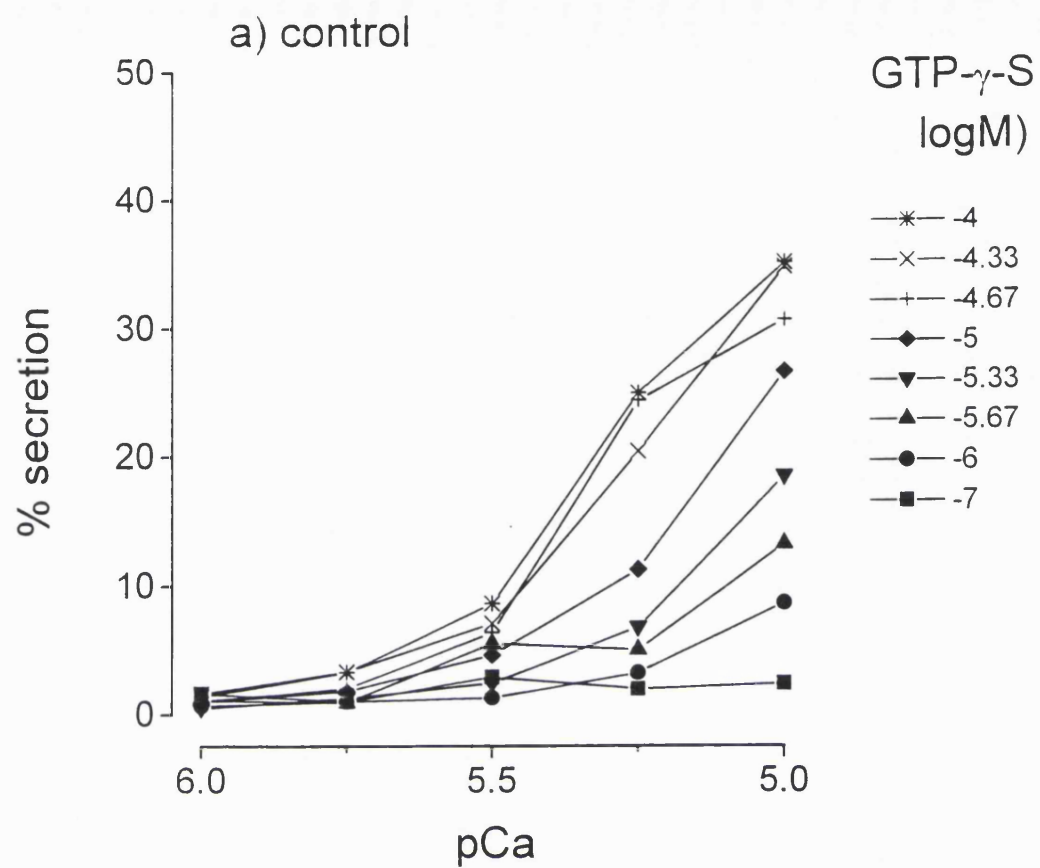
Time course of run down in the presence or absence of Calmodulin.

Cells were allowed to run down under standard conditions (ATP (1mM), low Ca^{2+} (pCa7)) and in the presence or absence of calmodulin (50µg/ml). At the time intervals indicated samples were taken and stimulated with Ca^{2+} at pCa5 and $\text{GTP}\gamma\text{S}$ (100µM). The cells were allowed to secrete for a further 10mins.

Fig 5.2

α -SNAP does not appear to affect the run down.

Cells were run down under standard condition in the presence or absence of α -SNAP (12 μ g/ml) for 10mins. Samples were taken and stimulated by a range of both Ca^{2+} and GTP γ S concentrations. Cells were allowed to secrete for a further 10mins.



pCa5.75, a low level response could be elicited. The addition of α -SNAP_A ^(12 μ g/ml) to permeabilised eosinophils had little effect on exocytosis. If anything, it seemed to be slightly inhibitory to secretion at certain concentrations of Ca^{2+} and $\text{GTP}\gamma\text{S}$.

Although in this experiment α -SNAP had little or no effect, it remains possible that the concentration of protein was too low. It has been shown [35] that purified α -SNAP applied to permeabilised adrenal chromaffin cells at 25 μ g/ml, enhances the extent of catecholamine release.

5.3 Run down in the presence of different concentrations of α -SNAP

In this experiment, the cells were allowed to run down in the presence of a range of concentrations of α -SNAP and then triggered to secrete with a maximal stimulus (pCa5 and GTPS at 100 μ M).

Fig 5.3 shows that when stimulated after 7mins, the control cells could secrete only 10% of EPO in response to maximum stimulus. As the concentration of α -SNAP was increased, the cells became slightly more responsive to stimulation, reaching a plateau between 1.5 - 6.25 μ g/ml. As the concentration was increased further from 6.25 to 150 μ g/ml, the response began to climb again. The optimum concentration was not reached in this experiment. From this, it appears that α -SNAP may be capable of contributing to the stimulation of exocytosis.

5.4 Does the response of these cells to α -SNAP improve when cells are allowed to secrete for longer periods?

This experiment is based on the observation that in chromaffin cells, the stimulatory effect of α -SNAP is more apparent when secretion (following application of the stimulus) is allowed to proceed for a longer period of time. To investigate whether this is also the case in eosinophils, the cells were

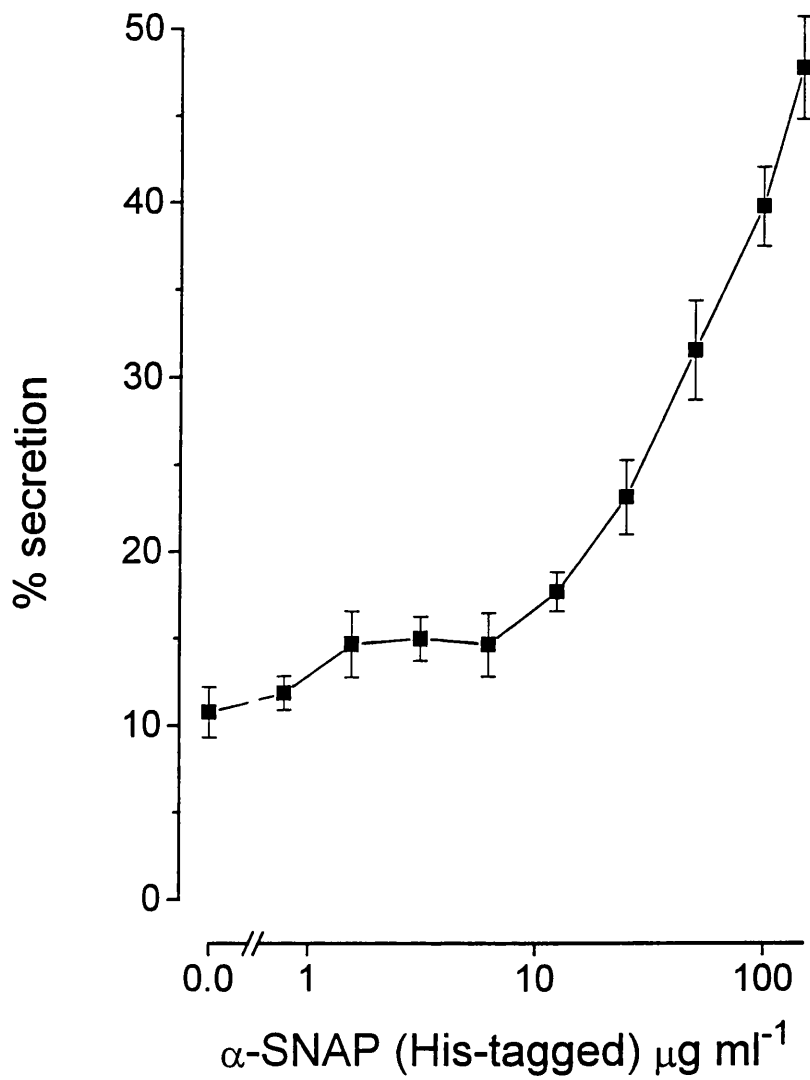


Fig 5.3

Secretion is enhanced by high concentrations of α -SNAP

The cells were allowed to run down in the presence of different concentrations of α -SNAP for 7mins. Samples were taken and stimulated with Ca^{2+} (pCa5) and $\text{GTP}\gamma\text{S}$ (100 μM) for 10mins.

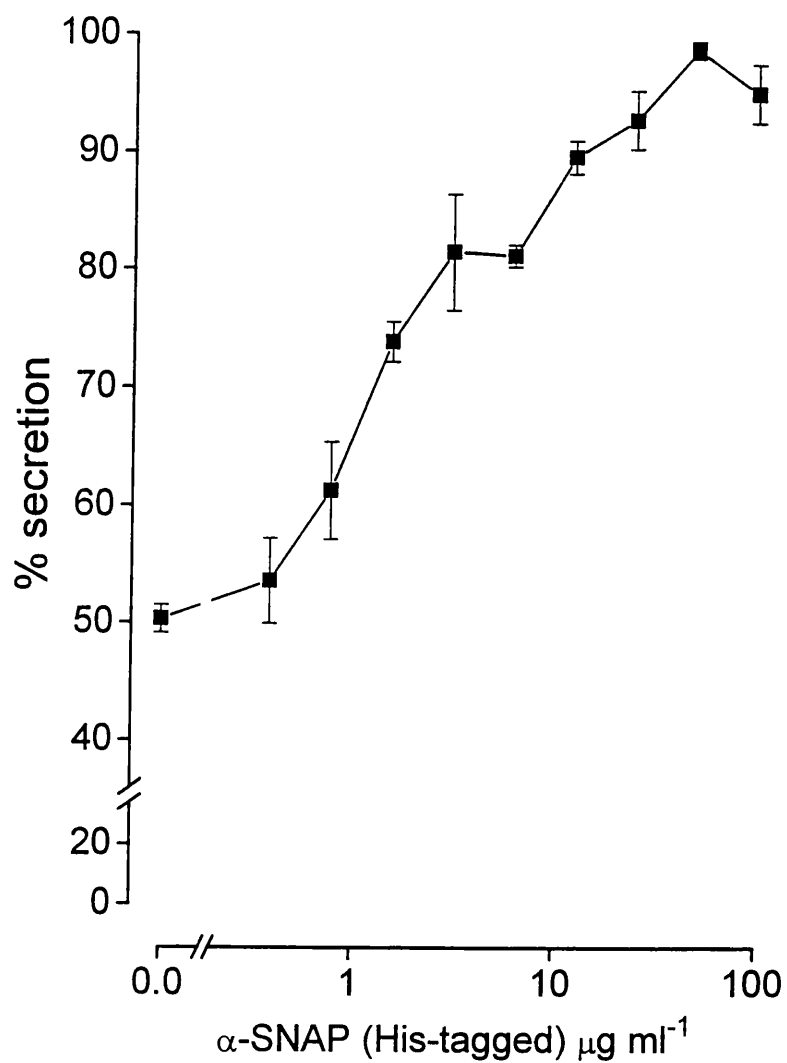


Fig 5.4

α -SNAP alone, fully restores responsiveness of the cells to stimulus.

Cells were allowed to run down in the presence of different concentrations of α -SNAP for 10mins. On this occasion the cells were stimulated (with pCa5 and GTP γ S (100 μ M)) for 20mins.

permeabilised in the presence of different concentrations of the protein and allowed to run down for 10mins before applying stimulus. After this time, stimulus was applied and the cells allowed to secrete for a period of 20mins instead of 10mins.

Fig 5.4 illustrates that in the absence of protein, 50% secretion was induced. As the concentration of α -SNAP was increased, the extent of secretion also increased. This appeared to occur in two phases. The first reached a maximum when about 3 μ g/ml of protein was applied and is maintained even when twice this amount of protein was applied. As the concentration was elevated above 6 μ g/ml, the extent of secretion again increased progressively so that at a concentration of 100 μ g/ml, 100% of EPO was released. Thus, it would appear that under these conditions, the loss of responsiveness due to protein leakage, may have been reversed. But is this real?

5.5 Is the effect of α -SNAP real?

The result from the experiment just described indicates that α -SNAP is able by itself fully to restore the responsiveness of the cells to stimulation. It seems unlikely that a single protein could have such a profound effect. To test its authenticity, a standard run down experiment was performed in which cells were permeabilised in the presence or absence of 3 μ g/ml of protein (i.e the plateau concentration) and also in the presence of a deletion mutant inactive form of α -SNAP (also at 3 μ g/ml).

In this experiment, (refer to fig 5.5) the cells were able to secrete close to 100% EPO for first 12mins following permeabilisation, after which they began to lose their responsiveness. Once again, α -SNAP caused some retardation of the run down so that 15mins following permeabilisation, it allowed 20% more secretion than the control. However, the mutant also enhanced secretion at this time point, although to a lesser degree. The cells

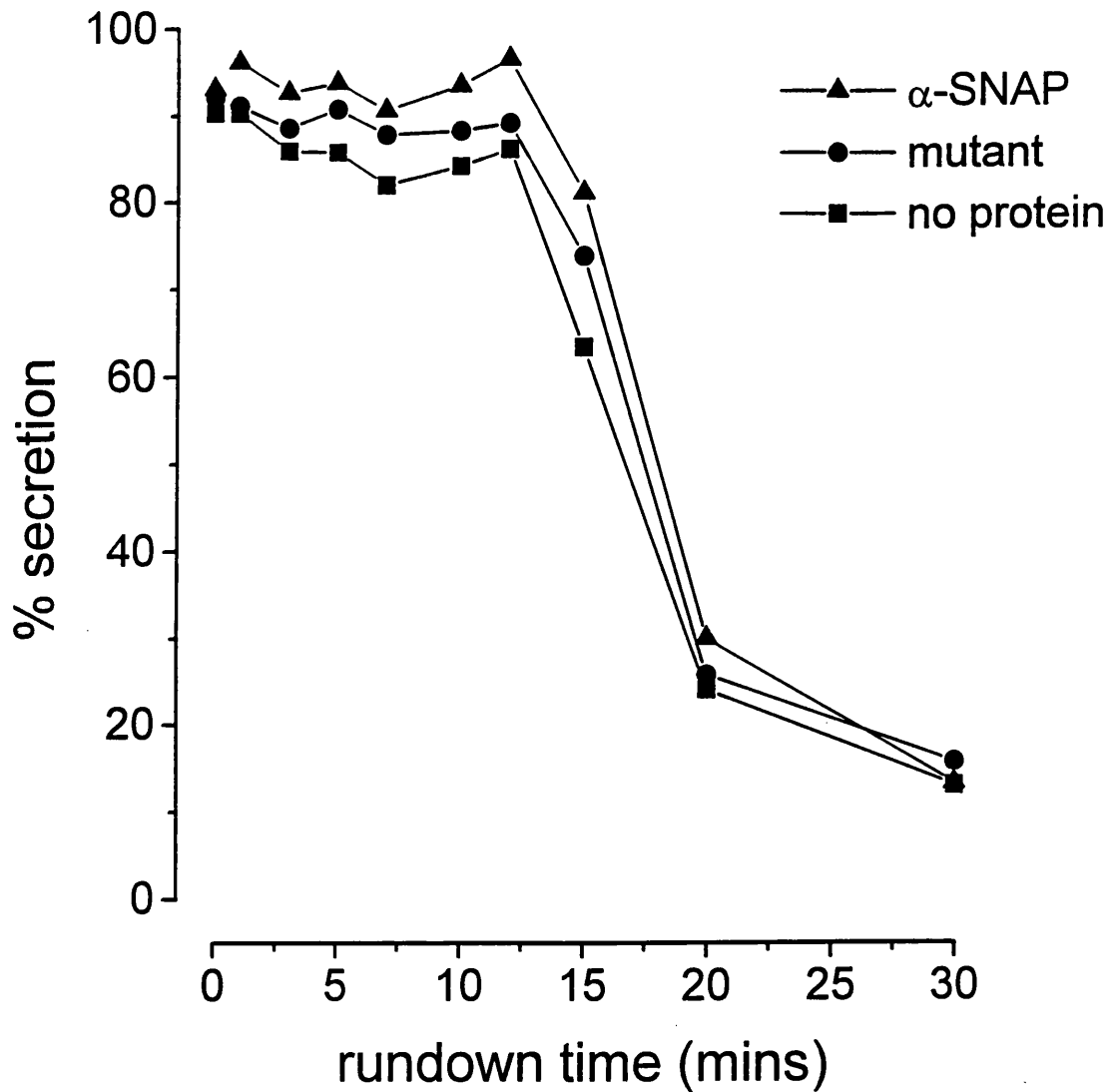


Fig 5.5

Run down is retarded in the presence of active and mutant α-SNAP (3μg/ml).

Cells were allowed to run down in the presence and absence of protein. At various time intervals, samples were taken and stimulated with pCa5 and GTPγS (100μM) for a further 10mins. (mutant - a deletion mutant Ala¹²¹-Arg²⁹⁵).

became refractory to stimulation by 30mins and this was unaffected by the presence or absence of protein.

5.6 Dose response of α -SNAP (mutant versus wild type)!

There remained some doubt as to whether α -SNAP was stimulatory to secretion. To test whether this is a real effect or due to a property of the protein (the presence of a Histidine₆ tag sequence in the tail of the protein), an experiment was performed in which different concentrations of both the mutant and the wild type proteins were tested.

After 17mins the cells were almost completely refractory to stimulation in the absence of protein (fig 5.6) and then as the concentration of the proteins were increased, the cells were stimulated to secrete once more. Interestingly, both the deletion mutant protein which is inactive in chromaffin cells and the active wild type protein were able to enhance secretion. At concentrations of protein greater than 1 μ g/ml the response of the cells to the presence of wild type protein was identical to that of the mutant protein.

This suggested that the stimulatory response of α -SNAP in eosinophils might be due to a property of the protein that is common to both the wild type and the mutant proteins. A likely possibility is the presence of the Histidine₆ tag sequence on both of these proteins.

5.7 SDS-PAG Electrophoresis of α -SNAP (wild type)

To check if the unpredictable behaviour of α -SNAP was due to contaminants in the purified protein, a 12% polyacrylamide gel electrophoresis was performed. Increasing concentrations of protein was then applied.

A strong signal of α -SNAP was observed in lane 2 at an estimated ^{molecular mass} ~~39kD~~ of 39kD (fig 5.7). As the concentration of α -SNAP was decreased, the intensity

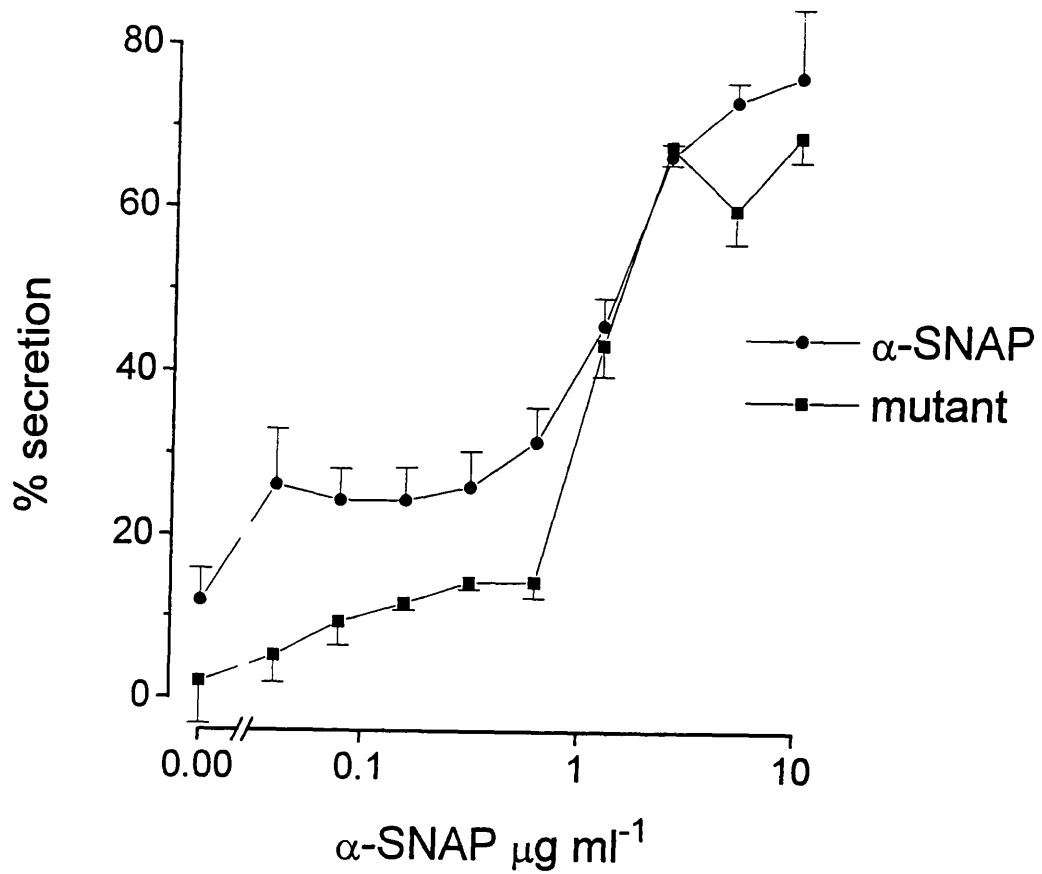


Fig 5.6

Mutant α-SNAP is almost as effective in enhancing secretion as wild type protein

Cells were allowed to run down in different concentrations of protein for 17mins. Samples were then withdrawn and stimulated with both Ca^{2+} (pCa5) and $\text{GTP}\gamma\text{S}$ (100 μM) for a further 10mins.

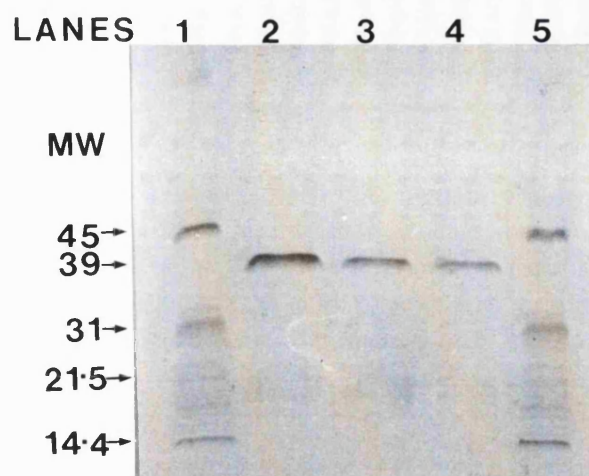


Fig 5.7

α -SNAP (wild type) preparation is homogeneous by SDS-PAGE electrophoresis.

α -SNAP (lanes 2- 4) was first extracted into Laemmli sample buffer and applied to a 12% gel. Lanes: 1, MW markers; 2, α -SNAP 20 μ g; 3, α -SNAP 10 μ g ; 4, α -SNAP 5 μ g ; 5, MW markers. MW markers indicate (from top to bottom kDa) 66, 45, 31, 21.5, 14.4. The ^{molecular mass} of α -SNAP estimated at 39kDa was also shown.

of this signal also decreased (see lanes 3 and 4). No other signals were observed in any of the lanes in which this protein was run. This suggested that the behaviour of the protein was not due to other contaminants or breakdown products. The molecular weight markers were run in lanes 1 and 5.

5.8 Does rhoGDI affect run down in eosinophils?

Thus far, neither of the proteins tested in eosinophils had been found to be effective in either stimulating or inhibiting exocytosis from these cells in a convincing manner. As stated in the introduction to the chapter, rhoGDI purified from bovine brain homogenates has been found to accelerate run down in rat peritoneal mast cells [124]. Also, when either the native or recombinant (purified from *E.coli*) forms of the protein were introduced into mast cells via a patch pipette, they retarded the onset and suppressed the extent of exocytosis, measured as change of membrane capacitance. However, the native protein was found to be much more effective than the recombinant material [106]. As the native protein was not available for my work, I tested the effect of different concentrations of recombinant rhoGDI on the process of run down in eosinophils. This was a cleaved GST fusion protein which was purified further by gel filtration. All the experiments with this protein were performed at 30°.

As shown in the experiment illustrated in fig 5.8, 10mins after permeabilisation and in the absence of protein, the cells were still able to secrete ~32% of the EPO content. As protein was added and increased above about 0.5µg/ml, the cells became progressively less responsive to the stimulus. In this experiment, a concentration of about 2.0µg/ml caused 50% inhibition, though it should be noted that the IC₅₀ value varied between batches of protein and ranged between 2 and 28µg/ml (n=4 experiments).

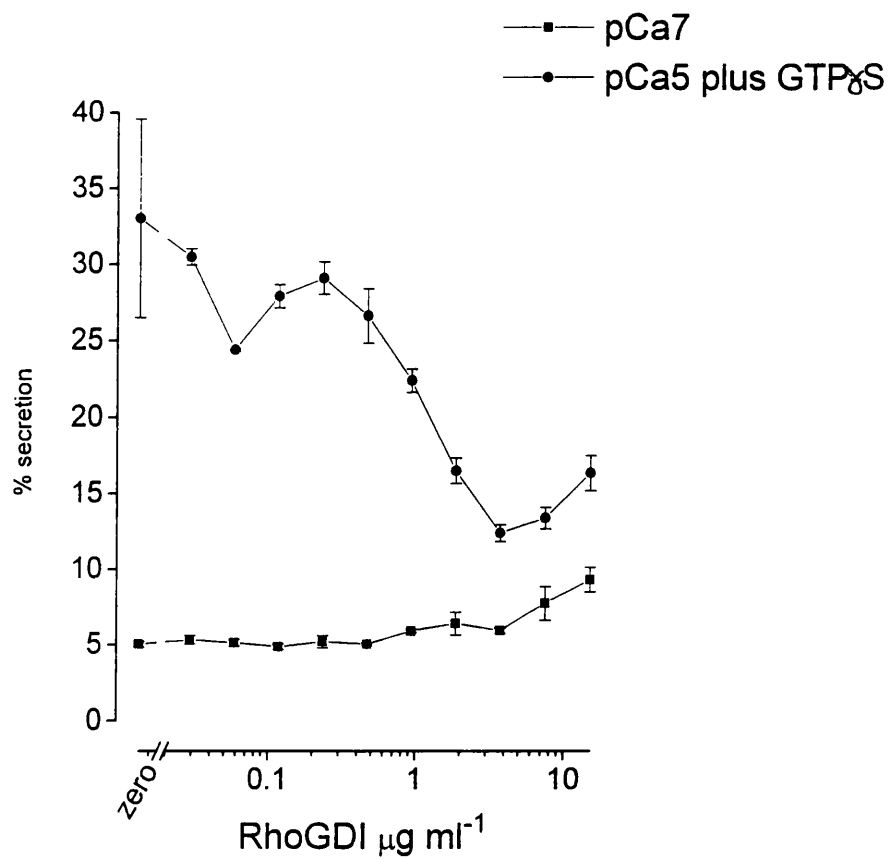


Fig 5.8

Recombinant RhoGDI can accelerate run down in eosinophils

The cells were allowed to run down in the presence of different concentrations of RhoGDI for 10mins at 30°C. Samples were then taken and stimulated with Ca^{2+} (pCa5) and $\text{GTP}\gamma\text{S}$ (100 μM) for 10mins. (Note: all experiments with RhoGDI were performed at 30°C).

5.9 Run down time course in the presence of boiled/ unboiled rhoGDI

To ensure that the inhibition was not due to some irrelevant quality of the recombinant protein, it would have been satisfying to have used an appropriate inactive mutant. Unfortunately, none were available to me. Instead, in a standard run down experiment I tested the effect of inactivating the wild type (recombinant) protein by boiling (at a concentration of 30µg/ml)

In this experiment (see fig 5.9), 60% EPO was released in the presence of boiled protein and after 3mins the cells began to run down. 10mins after permeabilisation they could only secrete 30%. In the presence of RhoGDI, about 50% secretion was achieved at time=0. Again, run down began after 3mins and the cells were able secrete 15% EPO 7mins later (time=10mins). When active rhoGDI was present run down was more rapid. Irrespective of the conditions, after 40mins, all the cells were completely refractory.

RhoGDI is known to bind to all the members of the Rho family which includes Rho, Rac and Cdc42 and probably competes for the binding sites of these proteins in the cells. Once bound to these proteins, it may then dislodge them from their binding sites, rendering them soluble and allowing them to leak out of the permeabilised cell.

5.10 RhoGDI tested in the Ca^{2+} /GTP γ S matrix assay

I also tested the effect on secretion of the RhoGDI in the Ca^{2+} / GTP γ S matrix assay.

After allowing the cells to run down for 20mins some secretion could be elicited at low concentrations of Ca^{2+} (pCa7) and this increased to 60% when maximum stimulation was applied (fig 5.10). In the presence of rhoGDI, this maximum level was reduced to 10%. The inhibition by rhoGDI strongly suggests a role or roles for small GTPases of the Rho family to which rhoGDI binds, in the regulation of secretion.

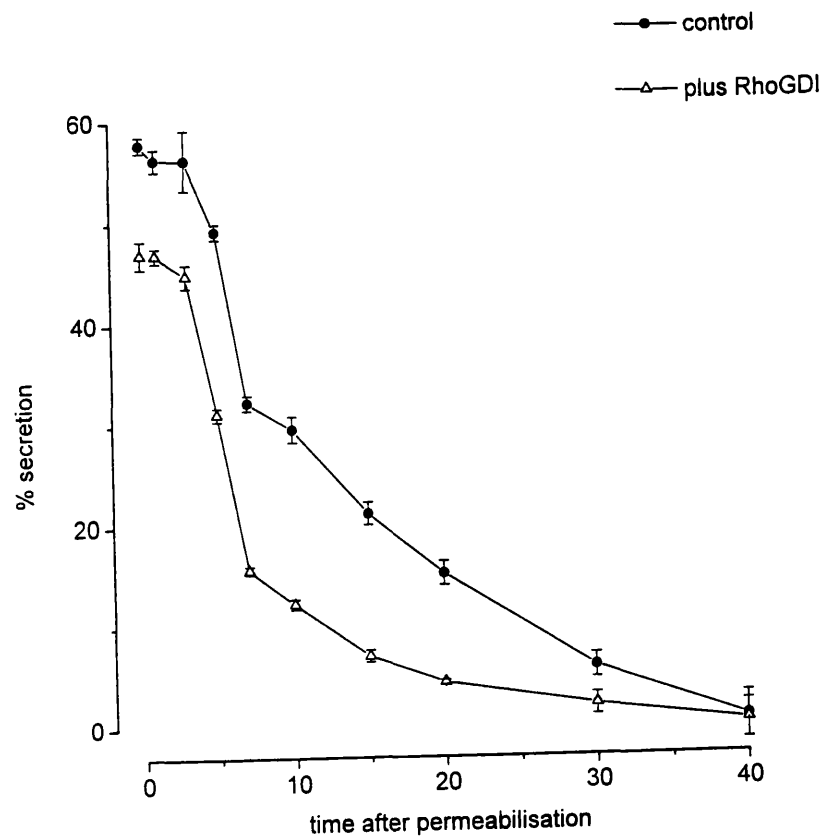


Fig 5.9

RhoGDI inactivated by boiling, does not retard run down

Cells were allowed to run down in the presence of active and inactive protein (30 $\mu\text{g/ml}$). Samples were withdrawn and stimulated with Ca^{2+} (pCa5) and $\text{GTP}\gamma\text{S}$ at time intervals indicated.

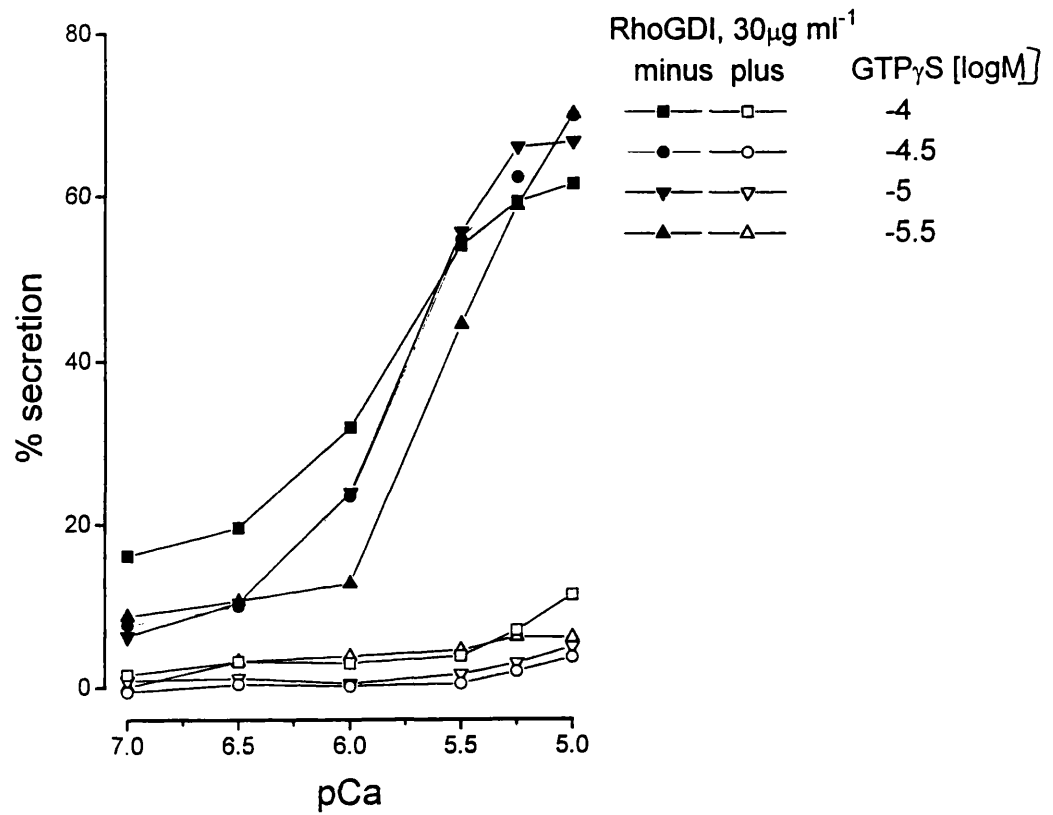


Fig 5.10

RhoGDI inhibits secretion induced by Ca^{2+} and $\text{GTP}\gamma\text{S}$.

The cells were allowed to run down in the presence or absence of protein for 20mins. Samples were then taken and stimulated with a range of Ca^{2+} and $\text{GTP}\gamma\text{S}$ concentrations for a further 10mins.

5.11 SDS-PAG Electrophoresis of recombinant rhoGDI

Recombinant rhoGDI prepared from E.coli, was purified further by gel filtration after which a 12% SDS Polyacrylamide gel electrophoresis was then performed of the fractions collected from this column. 4µg of crude recombinant protein following thrombin cleavage was run in lane A. RhoGDI (MW 28kDa) is present and there are two evident impurities which may be breakdown products or contaminants. Lanes 1 through 14 represent column fractions (G75 Superose 12) and a homogeneous signal due to RhoGDI is present in fraction 7 through 11. These fractions were pooled and used in the experiments described above.

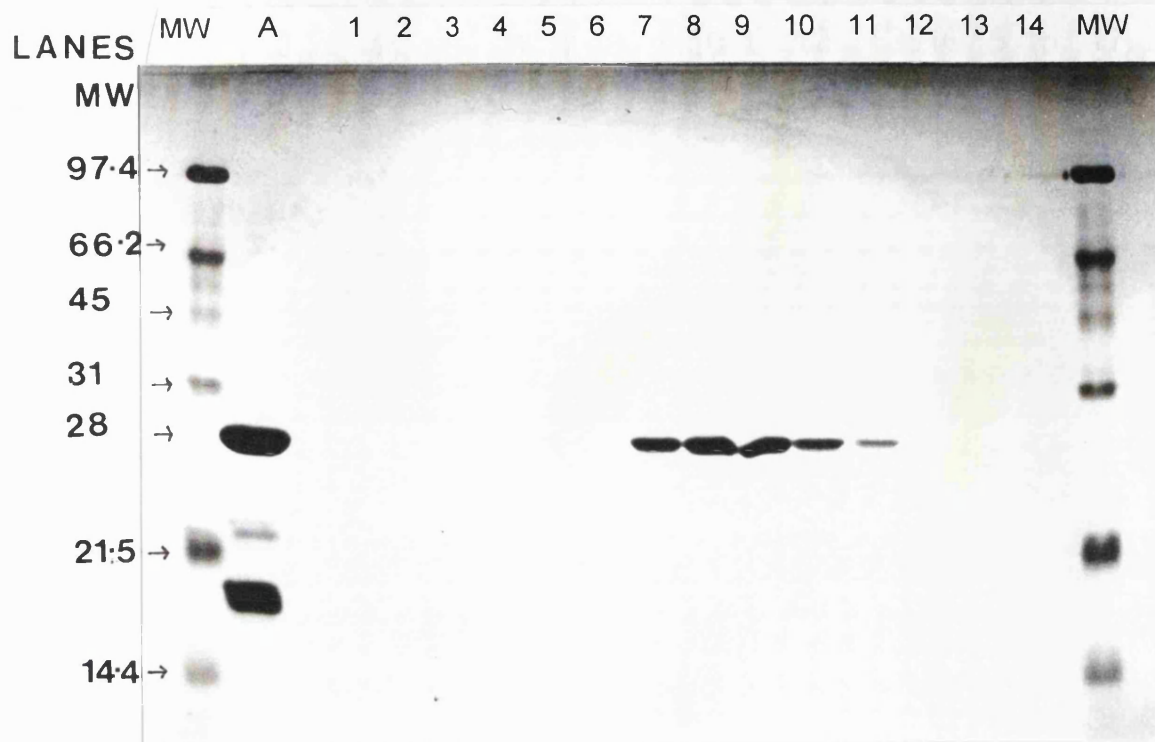


Fig 5.11

SDS-polyacrylamide gel electrophoresis of recombinant RhoGDI

RhoGDI was purified by gel filtration chromatography (on a G75 Superose 12 column) and samples of columns fractions 1 through 14 were extracted into Laemmli sample buffer under denaturing conditions and separated on 12% SDS polyacrylamide electrophoresis. Lane A represents the load, which had been eluted from glutathione-beads following cleavage of the RhoGDI/GST fusion product with thrombin. Separated proteins were visualised by silver staining and photographed. MW markers indicated (from top to bottom, kDa) 97.4, 66.2, 45, 31, 21.5, 14.4. The MW of RhoGDI is estimated at 28kDa as shown

DISCUSSION

The initial studies on exocytosis from eosinophils were concentrated on the requirements for low MW components and so little had been done on the involvement of specific proteins. To shed more light on this, three categories of proteins were introduced into permeabilisation eosinophils.

Calmodulin (a Ca^{2+} binding protein) is known to be involved in the process of exocytosis in both *Paramecia* [76] and in adrenal chromaffin cells [35].

When calmodulin was tested in guinea pig eosinophils, it had no observable effects. While it is possible that it may not be involved, there is also the possibility the role, if any, of Calmodulin is at a stage in the pathway upstream of where the Ca^{2+} and $\text{GTP}\gamma\text{S}$ stimuli operates.

Following this, I tested a protein known to be involved in the release of neurotransmitters^(α -SNAP). The effects were rather variable. Initial experiments showed that at concentrations below $10\mu\text{g/ml}$ of protein, it has little or no effect. If the time allowed for secretion was extended to 20mins, then α -SNAP appeared to enhance secretion. Surprisingly, when a mutant inactive form of this protein was tested alongside the native protein in these cells, their effects were more or less the same, suggesting that the effects of the native protein may have been artefactual. Both proteins have a His_6 sequence attached and it is therefore possible that the enhanced secretion is due to this.

Small GTP binding proteins of the Rho family have been implicated in the process of exocytosis in mast cells [106,124,133]. When RhoGDI was tested in the run down assay, it accelerated the run down. It is unlikely that the effect was due to the presence of other contaminating proteins.

The effect of RhoGDI on run down indicates that Rho-related proteins may be required for exocytosis from these cells. The inhibition may be due to

extraction of Rho related proteins from their binding sites in the plasma membrane. Since the Rho family consists of rhoA,B,C, rac1,2,cdc42,rhoG and TC10, the exact protein or proteins involved in exocytosis is not clear.

Chapter 6

PURIFICATION OF PROTEINS FROM BRAIN HOMOGENATES

The introduction of recombinant RhoGDI into permeabilised eosinophils and its acceleration of the run down, suggests a role for Rho related proteins in eosinophil pathway leading to exocytosis. Without knowing the precise identity of these proteins, understanding their place in exocytosis is difficult. In order to extend this study further, I decided to purify proteins by applying a number of protein purification techniques, using animal brain material as the source and by using the run down assay as a means of monitoring activities.

In this chapter, I describe the partial purification of an active component from rat brain cytosol which supports secretion from guinea pig eosinophils. This component is probably not the same as the Ca^{2+} sensitive factor described earlier which also retards the rate of run down in these cells.

When the cells are allowed to run down in the presence of either high Ca^{2+} conditions (pCa5) or with total cytosol, their responsiveness to stimulation is diminished by at least 50% 20mins following permeabilisation. If however the cells are allowed to run down in total rat brain cytosol proteins and high Ca^{2+} (pCa5), they can still secrete nearly all of ^{their} EPO content. In other words, there is little or no loss of responsiveness to stimulation even after 20mins suggesting an additive effect.

To start with, rat brain homogenates were used as a source of protein, however the material obtained was sufficient for a only single purification step and so for the purpose of scaling up it was replaced first with guinea pig brain and later on with porcine brain.

I found that rat brain cytosol was more active than porcine brain cytosol and that fresh brain material revealed three peaks of activity, one of which was absent after freezing.

Although neither of activity was taken to homogeneity, initial purification steps were established for a later study.

RESULTS

6.1 Does the addition of total rat brain cytosol affect run down?

I decided to test the effect of adding rat brain cytosol to the permeabilised cells in the run down assay, in the hope that proteins might be identified that are important for secretion. To this end, I tested the effect of allowing the cells to run down for up to 40mins in the presence or absence of dialysed rat brain cytosol. In the same run down assay, I also tested the effect of varying the concentration of Ca^{2+} (pCa7-pCa5). The cells were stimulated at the time of permeabilisation and then after various intervals for up to 40mins.

In these experiments, the presence of cytosol ATPases is likely to cause the depletion of ATP, so an ATP regeneration system was applied in order to maintain its concentration.

In the experiment illustrated in fig 6.1 maximum secretion of EPO could be induced for up to 5mins following permeabilisation, regardless of the condition in which the cells were allowed to run down. Thereafter, cells permeabilised under conditions of low Ca^{2+} (pCa8) and no added protein began to lose responsiveness. This decline was fairly rapid and by 20mins the cells became completely refractory to stimulation. Addition of dialysed cytosol proteins caused the run down to proceed at a slower rate so that 40mins following permeabilisation, the cells were still not completely refractory to stimulation and could still secrete up to 15% EPO. When the cells were run down in high concentrations of Ca^{2+} (pCa5) (no added proteins) they also maintained full responsiveness for the first 10mins and thereafter, run down was slow. Twenty minutes after permeabilisation, the cells could still secrete more than 50% of their EPO content and similar to the effect of added cytosol, 40mins after permeabilisation, the cells could still secrete about 10% EPO. The effect of adding Ca^{2+} (pCa5) and cytosol together was almost additive. Under these conditions full responsiveness to

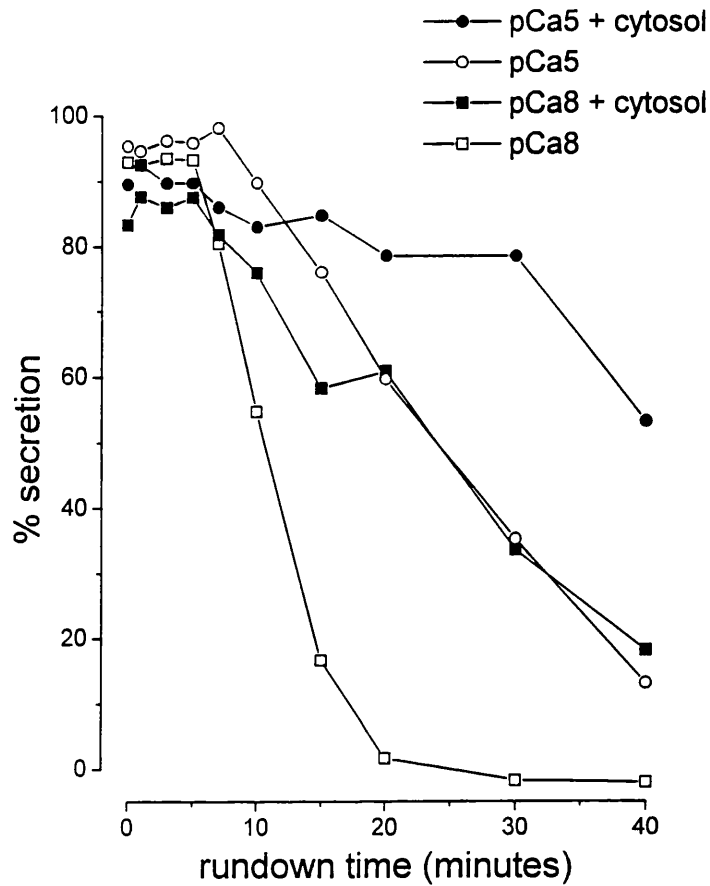


Fig 6.1

Ca²⁺ and total rat brain cytosol together, retard run down for longer.

Cells were allowed to run down in the presence of ATP with an ATP regeneration system, and in the presence or absence of dialysed brain cytosol with Ca²⁺ buffered at pCa8 and pCa5. Samples were taken at the times indicated and stimulated with Ca²⁺ (pCa5) plus GTP γ S (100 μ M).

stimulation was maintained for about 30mins and at 40mins, they were still able to secrete more than 50% of EPO.

These results indicate that there may be at least 2 factors in rat brain that can impede run down in eosinophils and that one of these factors is sensitive to the presence of Ca^{2+} .

6.2 How much protein is required for maximum stimulation of the cells?

The concentration dependency for total rat brain was determined by permeabilising the cells and leaving them in this state for 10mins in the presence of different concentrations of cytosol and in low levels of Ca^{2+} (pCa7).

Fig 6.2 shows that, run down was fairly rapid, thus 10mins following permeabilisation, the cells they were only able to secrete 10% EPO in the absence of added cytosol. The effect of adding total cytosol proteins was not evident until the concentration exceeded $75\mu\text{g/ml}$. A concentration of about $100\mu\text{g/ml}$ of total cytosol was sufficient to stimulate the cells to secrete up to 50% EPO. Above this, the cells became more responsive to stimulation such that at $300\mu\text{g/ml}$ of protein, maximum secretion of 90% EPO was observed. Increasing the concentration above $300\mu\text{g/ml}$ did not affect maximum secretion of EPO.

6.3 Can this factor or factors be purified?

To identify the most appropriate purification steps, a number of different purification procedures were examined, using rat brain cytosol as a small scale source.

Ion exchange Chromatography: I tested the use of both anion and cation exchanger under various conditions of pH. In the experiment illustrated in fig

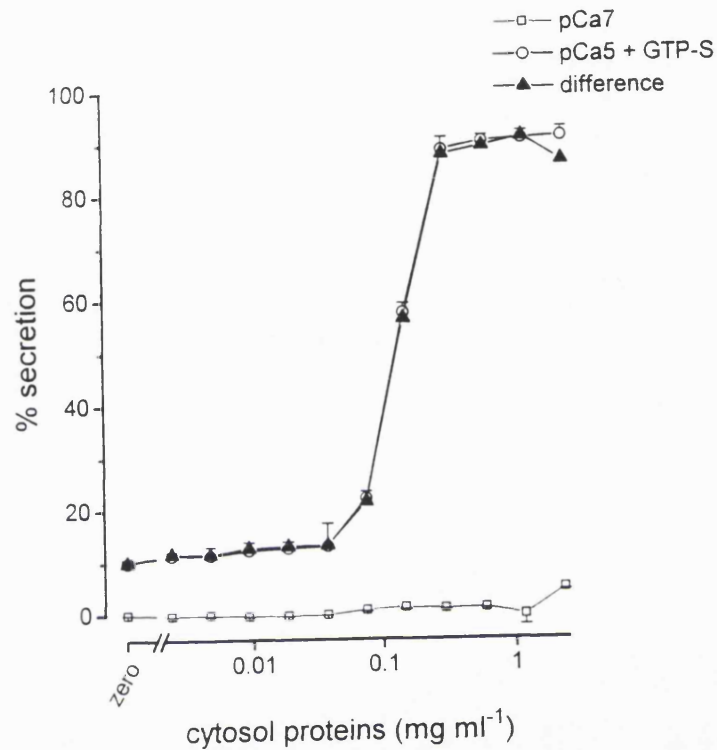


Fig 6.2

Total brain cytosol proteins (300μg/ml) maintain responsiveness almost completely over 10 minutes

Cells were run down for 10mins in the presence of an ATP regeneration system, Ca²⁺ at pCa7 and different concentrations of rat brain cytosol. Samples were withdrawn and stimulated with pCa5 and GTPγS for 10mins. Difference (▲) refers to subtracting secretion at pCa7 from secretion due to a maximal stimulus (pCa5 plus GTPγS).

6.3, 1ml of cold dialysed rat brain cytosol was loaded unto four 1ml DEAE sepharose columns equilibrated at pH 7.5, 8, 8.5 and 9 (using 20mM solutions of Triethanolamine, TRIS, Diethanolamine and Ethanolamine respectively). The columns were washed twice with 1ml of the appropriate solution and unbound protein was collected. The process was then repeated using the appropriate pH buffers made up to 1M NaCl to elute the bound protein.

For the purpose of assay, all the fractions collected from each column were transferred into Pipes buffered saline (homogenization buffer) at pH6.8 using small buffer exchange columns (nap 5 columns). The ATP regeneration sysytem was applied.

Fig 6.3a shows the effects of these crude fractions on cells run down in low Ca^{2+} (pCa8) conditions. Most of the active material must bind to the column since very little activity appears in the flow through (no salt wash) regardless of the pH. When eluted with 1M NaCl at pH 7.5 the active component is almost completely recovered. There is partial recovery at pH 8 but nothing at higher pH.

Fig 6.3b shows that for cells permeabilised under conditions of high Ca^{2+} (pCa5), some of the non-adherent proteins (flow through) are now capable of retarding run down. When the columns were washed with high salt buffers, active material was recovered at all the pH's tested with the best recovery obtained at pH 8. The recovery of active material from the DEAE column indicated that this might be a suitable procedure for further purification.

When a similar experiment was carried out using the weak cation exchanger CM-sepharose, no activities were recovered under any conditions.

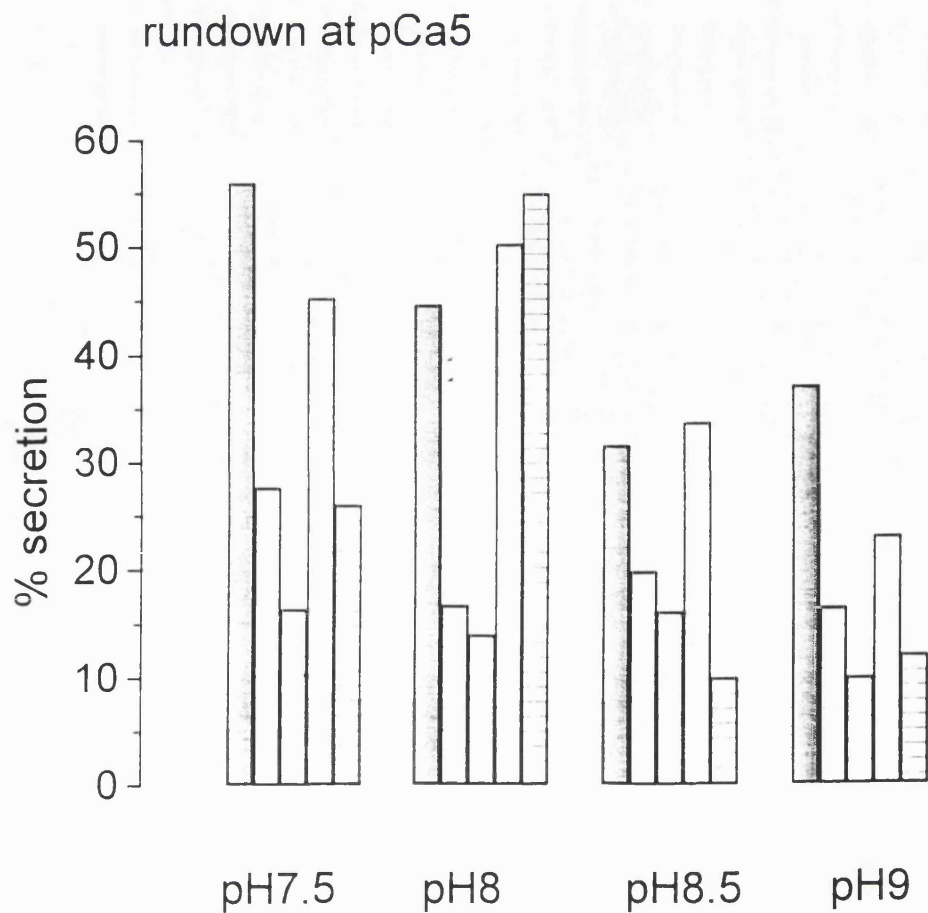
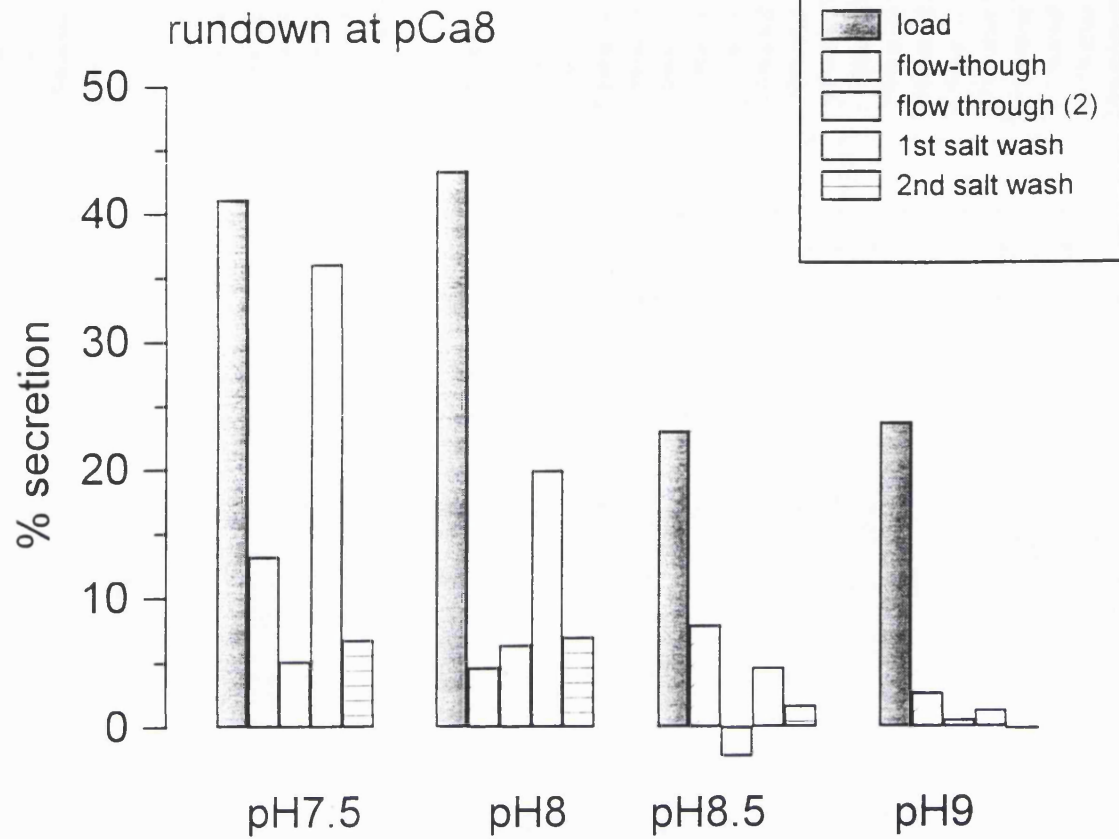
Although run down under conditions of both high and low Ca^{2+} had provided encouraging results, it was really too complicated to pursue the purification

Fig 6.3

Activity can be recovered from a weak anion exchange column (DEAE) trial size column

The cells were allowed to run down in the presence of fractions collected from trial-sized DEAE columns at various pH, and in either high (pCa5) or low (pCa8) concentrations of Ca^{2+} for 17mins. As before the ATP regeneration system was applied. Samples were then stimulated as appropriate for 10mins. All the samples collected from each column were transferred into PIPES-buffered saline at pH6.8.

DEAE trials



under both these conditions at the same time. Therefore I decided to pursue the purification of the activities supporting secretion from cells allowed to run down in low Ca^{2+} conditions. One of the reasons for this is, under high Ca^{2+} conditions, both the non-adherent proteins (flow through) and the adherent proteins can retard the run down so that finding the right conditions for the purification of both sets of active components might prove to be very complicated.

6.4 Ammonium sulphate precipitation

Ammonium sulphate was applied at increasing concentrations to precipitate out proteins which were then taken up, dialysed and tested in the run down assay. While proteins precipitated by ammonium sulphate at concentrations between 0-30% were found to have no activity in the run down assay, active materials were precipitated at salt concentrations of between 30 and 80% ammonium sulphate. Proteins which were precipitated by the addition of 30% ammonium sulphate were removed by centrifugation. The data from this series of tests are not shown.

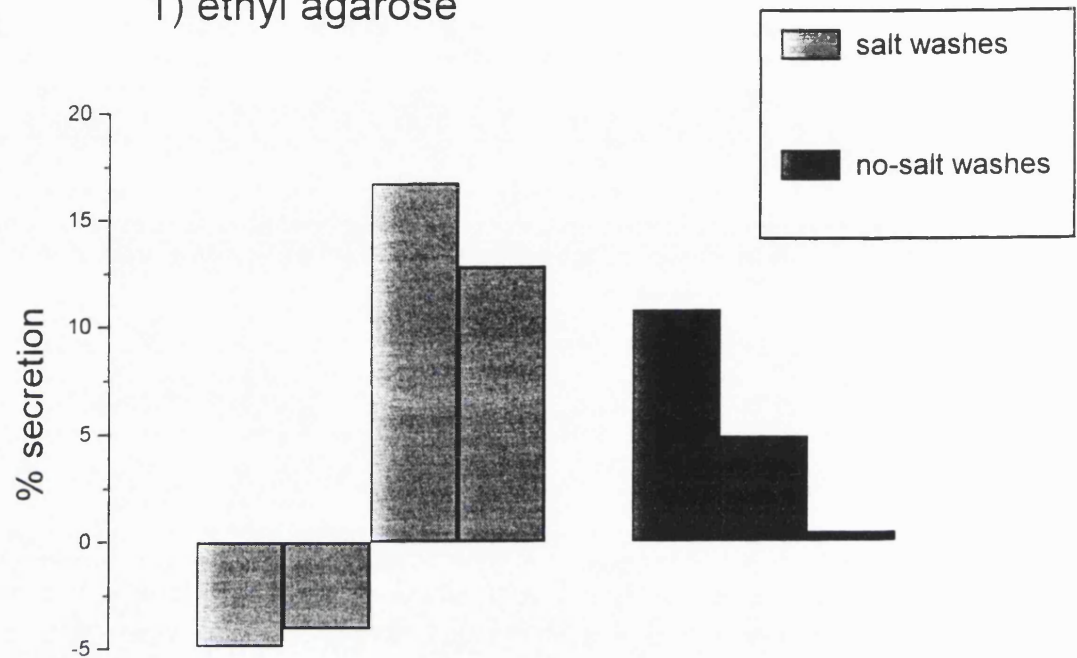
6.5 Can Hydrophobic interaction chromatography be used as a purification step?

Hydrophobic interaction chromatography (HIC) : In this experiment, 1ml samples of total rat brain cytosol were loaded onto 8 different hydrophobic columns (ethyl, propyl, butyl, pentyl, hexyl, octyl, decyl and dodecyl in agarose) and the bound proteins eluted with 20mM Pipes pH6.8. The unbound material (flow through) and the bound material (eluted samples) from each HIC column were then buffer exchanged as before and then tested in the run down assay.

Of the eight different hydrophobic affinity media tested, only the two extremes, ethyl (hydrophilic) and dodecyl (hydrophobic) bound released

HIC columns

1) ethyl agarose



2) dodecyl agarose

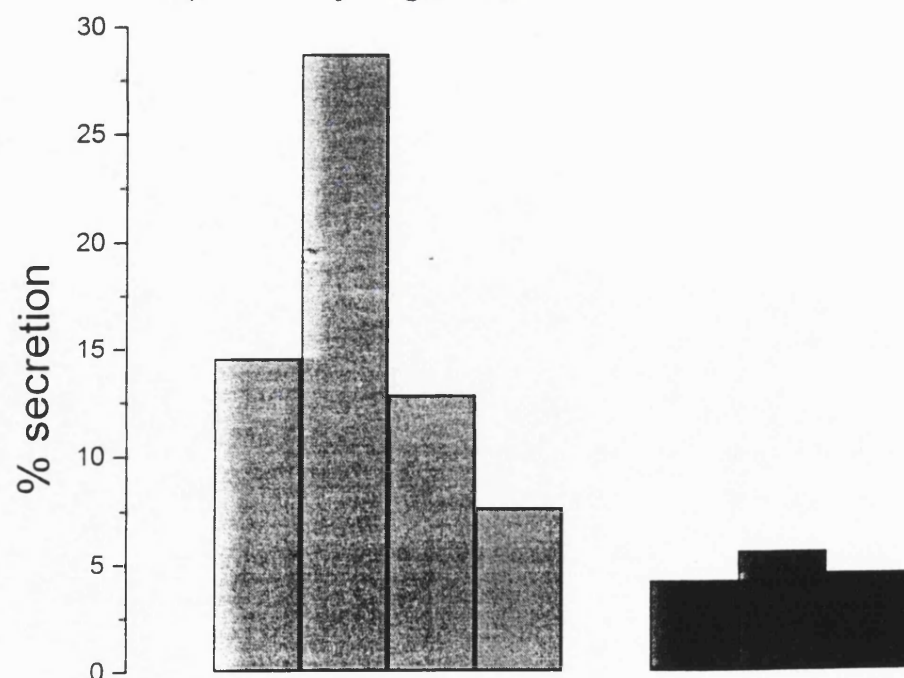


Fig 6.5

Hydrophobic interaction chromatography may be a useful for purification

The samples were buffer-exchanged into a solution of $\text{NH}_4)_2\text{SO}_4$ (1.2M) and PIPES (20mM, pH6.8) and loaded onto 8 hydrophobic interaction columns. The bound material was eluted with 20mM PIPES (low salt). Each sample was then buffer-exchanged into PIPES buffered saline (pH6.8) and applied to eosinophils in the standard run-down experiment (with the ATP regeneration system) for 12mins. Samples were stimulated with Ca^{2+} (pCa5) and $\text{GTP}\gamma\text{S}$ for a further 10mins.

active materials. This result suggests that the HIC columns tested might be useful in the purification process.

6.6 A proper run on a DEAE column

12mls of dialysed rat brain cytosol were loaded onto DEAE sepharose column in 20mM Triethanolamine (TEA) at pH 7.8 (no added NaCl) which was then eluted in the presence of an increasing salt gradient (0 to 1M NaCl, pH 7.8 buffered with 20mM TEA). 2.5ml size fractions were collected and each fraction exchanged into Pipes buffered saline pH 6.8 as before.

Two peaks of activity were obtained (see fig 6.6). Proteins in the larger peak eluted between fractions 20 and 30 (0.3-0.4M NaCl) enhanced secretion by more than 40%. The result confirmed that anion exchange chromatography will be a useful method for the purification of this activity. Unfortunately the activity is labile and has little effect when tested on the run down after 18 hours. The speed of the operation is critical.

6.7 Scaling up!

Rat or guinea pig brain material was insufficient for more than a single purification step, so I decided to use either fresh or frozen (and thawed) brains from piglets which were occasionally available.

Although the DEAE trial runs (using 1ml columns) with rat brain cytosol had indicated that the activity survives better at pH 8 (or lower) (refer to fig 6.3), a small amount of active unbound material had been detected at pH 7.8 when a proper run was performed on this column (fig 6.6). The binding capacity of the weak anion exchange column (DEAE-sepharose) can be increased by increasing the pH. Thus 20mM Diethanolamine was used at pH 8.5 and the experiment performed exactly as before (see fig 6.6).

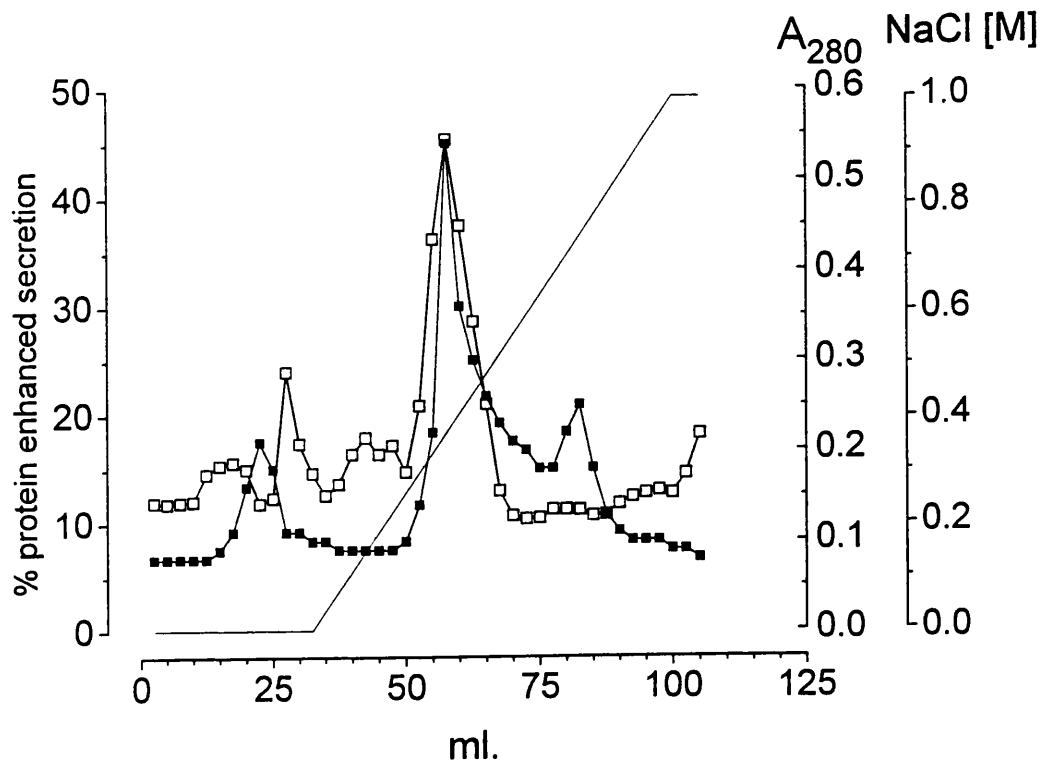


Fig 6.6

Chromatography of activities on DEAE column (rat brain proteins

The cytosol obtained from homogenized rat brain (in PIPES buffered saline (pH6.8)) was buffer-exchanged using PD10 columns into 20mM Triethanolamine (TEA pH7.8, low salt) and 16mls of this was loaded onto a DEAE column. Bound material was eluted with 20mM TEA and an increasing gradient of NaCl (0-1M) at 2.5ml/min. Eluted samples were passed through Nap5 columns for exchange into PIPES buffered saline (pH 6.8). Samples were then applied to permeabilised eosinophils in the standard run-down experiment (using a ATP regeneration system) for 10mins. Cells were induced to secrete with Ca^{2+} at pCa5 and GTP γ S for a further 10mins. The sloping line indicates the salt concentration between zero and 1M. □--□ indicates % protein enhanced secretion, ■--■ indicates protein concentration (mg/ml).

fresh pig brain cytosol

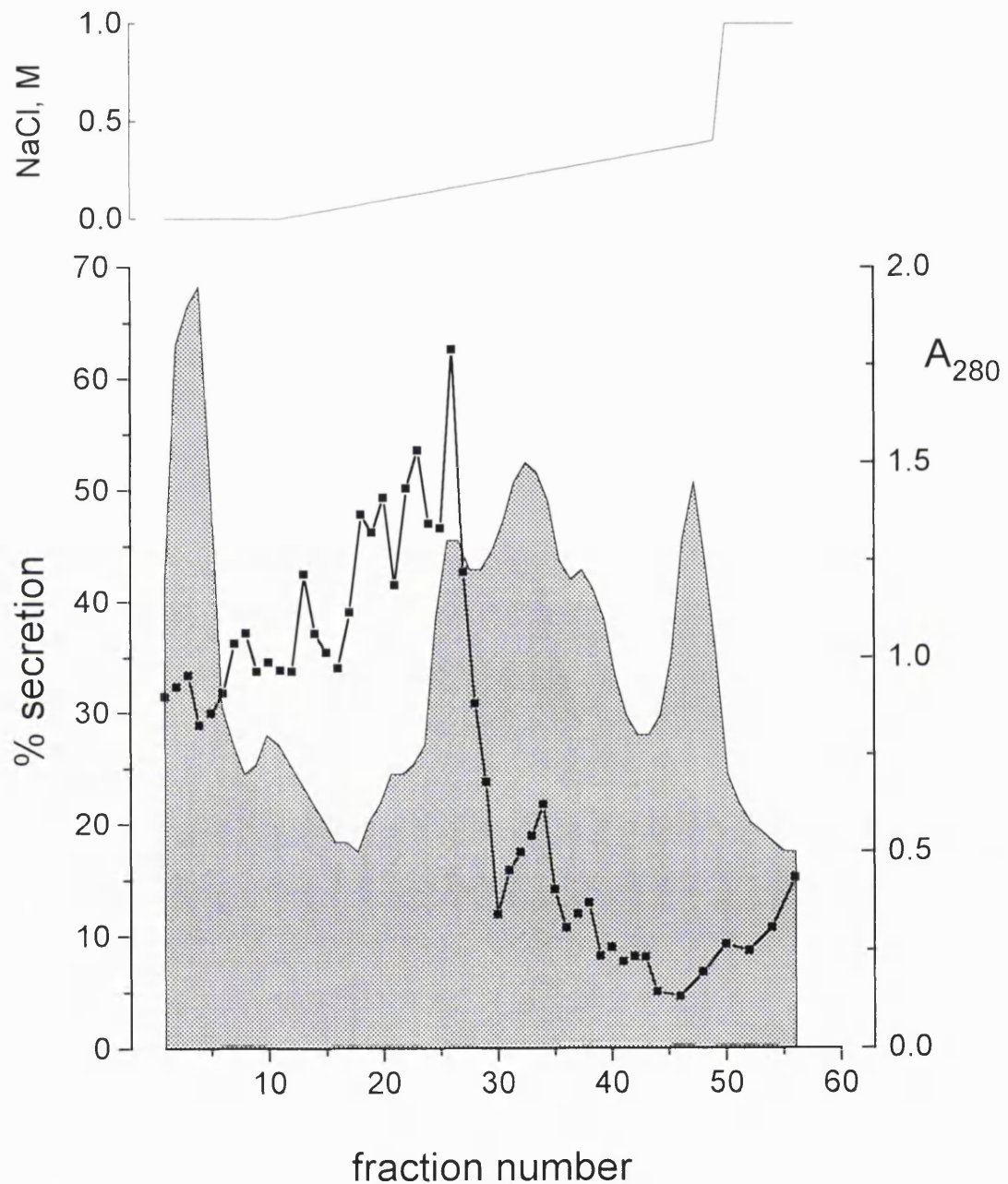


Fig 6.7

Chromatography of activity on DEAE column (using pig brain proteins)

Piglet brain cytosol was dialysed against 5 Litres of 20mM Diethanolamine (DEAE, pH 8.5, low salt) overnight. This was then loaded onto a DEAE column at 2mls/min. Bound material was eluted with 20mM DEAE pH8.5 and an increasing NaCl gradient (0-1M) at a flow rate of 5ml/min. 10mls fractions were collected and these were buffer-exchanged as before. Samples were applied to permeabilised eosinophils in the standard run-down experiment as described in fig 6.6. ■--■ indicates % secretion. The line enclosing the shaded area represents protein concentration.

Unlike the previous result, single sharp peaks of activity were not obtained. Instead, the active components were found to be spread out over at least 20 fractions (see fig 6.7). An activity inducing a maximum of 60% enhanced secretion was eluted at 0.2M NaCl. The lack of similarity between this and the previous data and might be due to the species difference but is more likely due to the difference in pH.

Although this result was not so well defined, it indicates further, that these activities can survive following elution from the DEAE column.

6.8 What next?

The trial run on the 1ml HIC columns had indicated that the activities could be eluted from the both the ethyl and the dodecyl columns. However when active fractions pooled from the anion exchange column (DEAE) ~~were~~ loaded onto the Ethyl column, no activity was released. I decided to fractionate the proteins using gel filtration.

In other experiments on the anion exchange column, two distinct peaks of activity had been observed when pig brain was fractionated (data not shown). Thus in this experiment, all the activity obtained from the DEAE column (see fig 6.7) was pooled into 2 samples (fractions 18-20 and 22-27) and loaded onto separate gel filtration columns (G75) which were then eluted in Pipes buffered saline (pH 6.8).

Fig 6.8a illustrates that after gel filtration (of fractions 18-20), two clear peaks of activity were eluted. These have MW's of 30kD and 12.9kD respectively. The first enhanced secretion by about 10% and the second by 5%.

A peak of activity with a calculated MW of 14.6kD (fig 6.8b) was eluted from the other gel filtration column (loaded with fractions 22-27). This enhanced

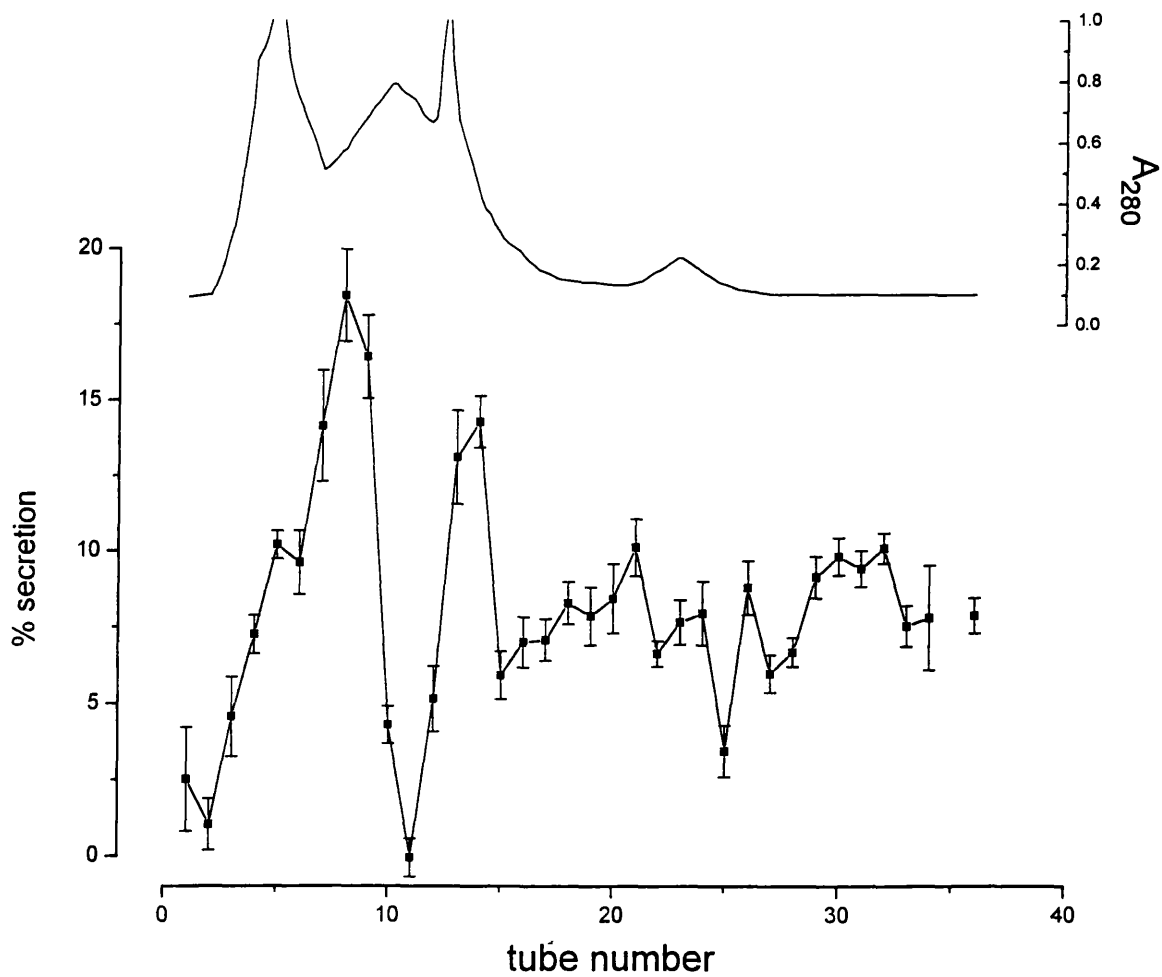


Fig 6.8a

Chromatography of activity on gel filtration G75

The active material eluted from the DEAE column was divided into two parts. The first consisting of *fractions 18-20* was buffer-exchanged into PIPES saline (pH 6.8) and concentrated to 2mls using a YM10 membrane in an Amicon pressure cell. This was loaded onto a G75 sephadex (gel filtration) column and eluted with PIPES buffered saline (pH 6.8) at a flow rate of 0.5ml/min. Fractions (1.6ml) were collected and applied to permeabilised eosinophils in the standard run-down experiment as described in fig 6.6.

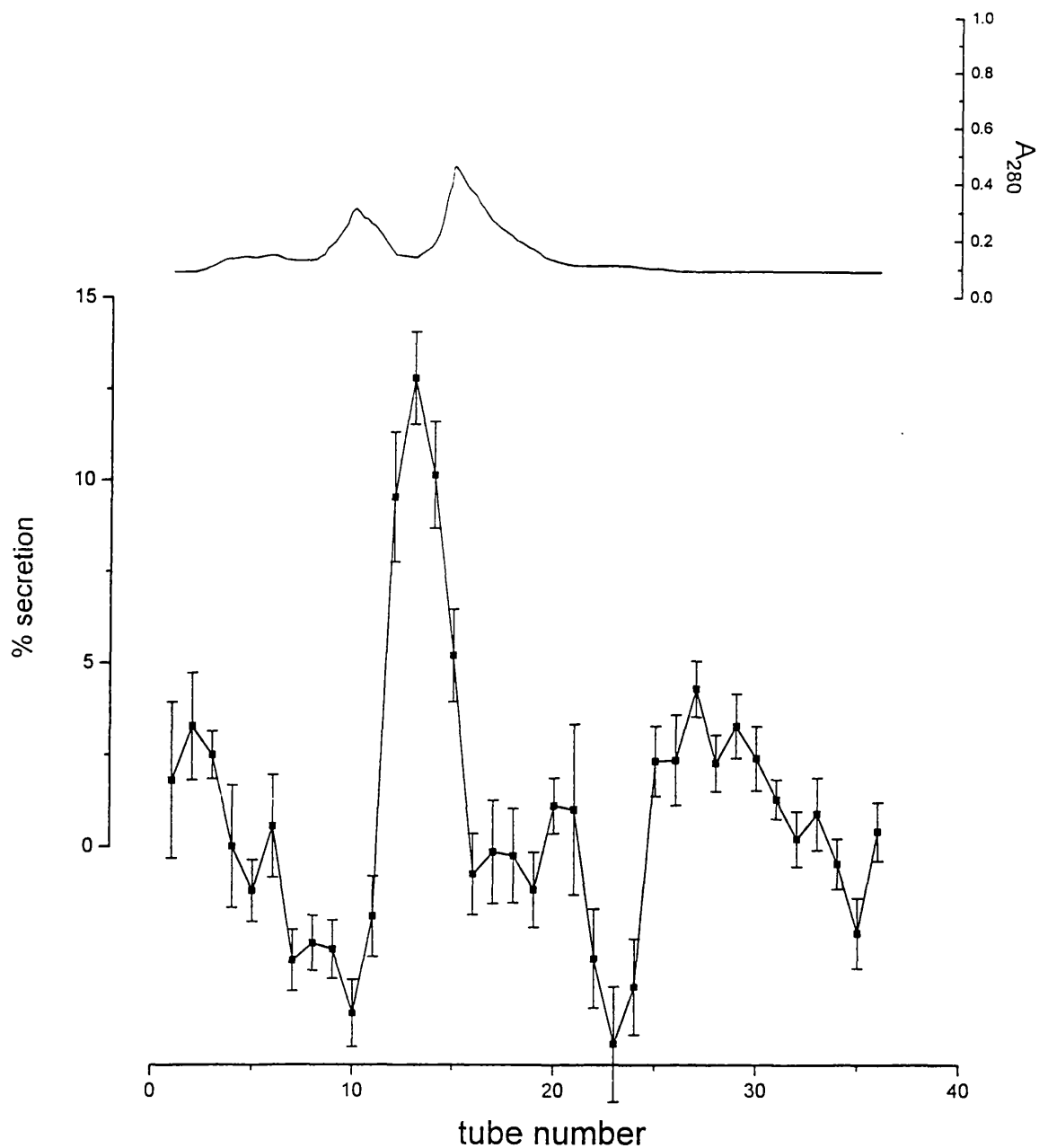


Fig 6.8b

Chromatography of activity on gel filtration G75

The second the active component from the DEAE column (*fractions 22-27*) was buffer-exchanged and concentrated as in **Fig 6.8a**. This was then loaded onto a G75 sephadex column and eluted as before. 1.6 ml size fractions were collected and applied to permeabilised eosinophils in the standard run-down experiment as described in fig 6.6.

secretion by about 10%. Thus three active components with varying molecular weights were obtained.

Chapter 7

CONCLUSION

The prevalence of eosinophils at various sites of inflammation and the release of their basic cationic proteins at these sites, has led to the investigation of their role in disease. Although an integral part of eosinophil behaviour, secretion itself, has received little attention. Having said that, the investigation of the binding of a number of ligands such as immunoglobulins and cytokines to their receptors on intact cells, which can lead to secretion, (refer to table 1 in chapter1) has added to our current understanding, although this is limited. This has given the impetus for work carried out in this thesis.

Our interest in the final steps leading to exocytosis, has governed the establishment of a permeabilised cell preparation which bypasses the many complications of receptor mediated processes. This method, although initially hampered by the Streptolysin-O crisis has led to the characterisation of these in a new light. It was discovered that the mechanism of secretion in eosinophils shares a number of similarities as well as differences with mast cells.

Secretion from both cell types can be induced by GTP γ S in the presence of Mg.ATP (no added Ca²⁺) in simple buffered salt solutions. Also, the loss of responsiveness which follows permeabilisation is accelerated by the presence of RhoGDI, a member of the Rho family of GTP binding proteins.

This process of run down is retarded in high concentrations of Ca²⁺ (pCa5, 1mM ATP) in eosinophils and this suggests that Ca²⁺ sensitive factor (possibly a Ca²⁺-sensor?) may leak from the cells when they are permeabilised in the absence of Ca²⁺. In mast cells however, run down is accelerated in these conditions. Thus although both cell types are from the

haemopoietic lineage, and their mechanisms of secretion are similar, there are clear differences of detail.

Using porcine brain cytosol as a source of soluble proteins, three components capable of enhancing secretion from eosinophils have been partially purified.

I hope that my work described in this thesis has shed some light on the mechanism of secretion from eosinophils.

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Bibliography

1. Ackerman, S. J., G. M. Kephart, T. M. Habermann, P. R. Greipp and G. J. Gleich. 1983. Localization of eosinophil granule major basic protein in human basophils. *J. Exp. Med.* 158: 946-961.
2. Aizawa, T., Y. Kakuta, K. Yamauchi, Y. Ohkawara, N. Maruyama, Y. Nitta, G. Tamura, H. Sasaki and T. Takishima. 1992. Induction of granule release by intracellular application of calcium and guanosine-5'-O-(3-thiotriphosphate) in human eosinophils. *J. Allergy Clin. Immunol.* 90: 789-795.
3. Alouf, J. E. 1980. Streptococcal toxins (streptolysin-O, streptolysin-S, erythrogenic toxin). *Pharmacol. Therap.* 11: 661-717.
4. Archer, G. T. 1968. The function of the eosinophil. *Bibl. Haematol.* 29: 71-85.
5. Archer, G. T. and J. G. Hirsch. 1963. Motion picture studies on degranulation of horse eosinophils during phagocytosis. *J. Exp. Med.* 118: 287-293.
6. Archer, G. T. and J. G. Hirsch. 1963. Isolation of granules from eosinophil leukocytes and the study of their enzyme content. *J. Exp. Med.* 118: 277-284.
7. Archer, R. K. and J. Broome. 1963. Studies on the peroxidase reaction of living eosinophils and other leucocytes. *Acta Haematol.* 29: 147-156.
8. Athayde, C. M. and M. C. Scrutton. 1990. Guanine nucleotides and Ca^{2+} -dependent lysosomal secretion in electroporabilised human platelets. *Eur. J. Biochem.* 189: 647-655.
9. Bach, M. K. and J. R. Brashler. 1992. FMLP is a potent activator of guinea-pig eosinophils but its activity is dependent on the prior overnight in vitro culture of the cells (facilitation). *Immunology* 75: 680-687.
10. Bach, M. K., J. R. Brashler, M. E. Saunders and M. J. Bienkowski. 1991. Pitfalls in the quantitative estimation of the secretion of granule proteins by eosinophils. *J. Immunol. Methods* 142: 243-250.
11. Barker, R. L., G. J. Gleich and L. R. Pease. 1988. Acidic precursor revealed in human eosinophil granule major basic protein cDNA [published erratum appears in J Exp Med 1989 Sep 1;170(3):1057]. *J. Exp. Med.* 168: 1493-1498.
12. Baskar, P. and S. H. Pincus. 1988. Oxidative requirement for degranulation of human peripheral blood eosinophils. *Infect. Immun.* 56: 1907-1911.
13. Baskar, P. and S. H. Pincus. 1992. Selective regulation of eosinophil degranulation by interleukin 1 beta. *Proc. Soc. Exp. Biol. Med.* 199: 249-254.
14. Baskar, P., D. S. Silberstein and S. H. Pincus. 1990. Inhibition of IgG-triggered human eosinophil function by IL-4. *J. Immunol.* 144: 2321-2326.

15. Basten, A. and P. B. Beeson. 1970. Mechanism of eosinophilia. II. Role of the lymphocyte. *J. Exp. Med.* 131: 1288-1305.
16. Benhamou, M., J. S. Gutkind, K. C. Robbins and R. P. Siraganian. 1990. Tyrosine phosphorylation coupled to IgE receptor-mediated signal transduction and histamine release. *Proc. Nat. Acad. Sci. (USA)*. 87: 5327-5330.
17. Benhamou, M., N. J. Ryba, H. Kihara, H. Nishikata and R. P. Siraganian. 1995. Protein-tyrosine kinase p72syk in high affinity IgE receptor signaling. Identification as a component of pp72 and association with the receptor gamma chain after receptor aggregation. *J. Biol. Chem.* 268: 23318-23324.
18. Bhakdi, S., J. Trantum-Jensen and A. Sziegoleit. 1985. Mechanism of Membrane Damage by Streptolysin-O. *Infect. Immun.* 47: 52-60.
19. Bhakdi, S., U. Weller, I. Walev, E. Martin, D. Jonas and M. Palmer. 1993. A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes. *Med. Microbiol. Immunol.* 182: 167-175.
20. Bligh, E. G. and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Canad. J. Biochem. Physiol.* 37: 911-917.
21. Bozeman, P. M., D. B. Learn and E. L. Thomas. 1990. Assay of the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase. *J. Immunol. Methods* 126: 125-133.
22. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
23. Broide, D. H., M. M. Paine and G. S. Firestein. 1992. Eosinophils express interleukin 5 and granulocyte macrophage-colony-stimulating factor mRNA at sites of allergic inflammation in asthmatics. *J. Clin. Invest.* 90: 1414-1424.
24. Buma, P., E. W. Roubos and R. M. Buijs. 1984. Ultrastructural demonstration of exocytosis of neural, neuroendocrine and endocrine secretions with an *in vitro* tannic acid (TARI) method. *Histochemistry* 74: 247-256.
25. Butterworth, A. E. 1984. Cell mediated damage to helminths. *Adv. Parasitol.* 23: 143-235.
26. Butterworth, A. E., R. F. Sturrock, V. Houba, A. A. Mahmoud, A. Sher and P. H. Rees. 1975. Eosinophils as mediators of antibody-dependent damage to schistosomula. *Nature* 256: 727-729.
27. Butterworth, A. E., R. F. Sturrock, V. Houba and P. H. Rees. 1974. Antibody-dependent cell-mediated damage to schistosomula in vitro. *Nature* 252: 503-505.
28. Butterworth, A. E., M. A. Vadas, D. L. Wassom, A. Dessein, M. Hogan, B. Sherry, G. J. Gleich and J. R. David. 1979. Interactions between human eosinophils and schistosomula of *Schistosoma mansoni*. II. The mechanism of irreversible eosinophil adherence. *J. Exp. Med.* 150: 1456-1471.

29. Butterworth, A. E., D. L. Wasson, G. J. Gleich, D. A. Loegering and J. R. David. 1979. Damage to schistosomula of *Schistosoma mansoni* induced directly by eosinophil major basic protein. *J. Immunol.* 122: 221-229.
30. Capron, M., T. Jouault, L. Prin, M. Joseph, J. C. Ameisen, A. E. Butterworth, J. P. Papin, J. P. Kusnierz and A. Capron. 1986. Functional study of a monoclonal antibody to IgE Fc receptor (Fc epsilon R2) of eosinophils, platelets, and macrophages. *J. Exp. Med.* 164: 72-89.
31. Capron, M., M. D. Kazatchkine, E. Fischer, M. Joseph, A. E. Butterworth, J. P. Kusnierz, L. Prin, J. P. Papin and A. Capron. 1987. Functional role of the alpha-chain of complement receptor type 3 in human eosinophil-dependent antibody-mediated cytotoxicity against schistosomes. *J. Immunol.* 139: 2059-2065.
32. Capron, M., M. J. Truong, D. Aldebert, V. Gruart, M. Suemura, G. Delespesse, B. Tourvieille and A. Capron. 1991. Heterogeneous expression of CD23 epitopes by eosinophils from patients. Relationships with IgE-mediated functions. *Eur. J. Immunol.* 21: 2423-2429.
33. Carlson, M., C. Peterson and P. Venge. 1993. The influence of IL-3, IL-5, and GM-CSF on normal human eosinophil and neutrophil C3b-induced degranulation. *Allergy* 48: 437-442.
34. Chakravarty, N. and E. H. Nielsen. 1985. Calmodulin in mast cells and its role in histamine secretion. *Agents Actions* 16: 122-125.
35. Chamberlain, L. H., D. Roth, A. Morgan and R. D. Burgoyne. 1995. Distinct effects of alpha-SNAP, 14-3-3 proteins, and calmodulin on priming and triggering of regulated exocytosis. *J. Cell Biol.* 130: 1063-1070.
36. Chang, P. L., N. E. Rosa and R. G. Davidson. 1981. Differential assay of arylsulfatase A and B activities: a sensitive method for cultured human cells. *Anal. Biochem.* 117: 382-389.
37. Churg, J. and L. Strauss. 1951. Allergic granulomatosis, allergic angiitis and periarteritis nodosa. *Am. J. Pathol.* 27: 277-301.
38. Coeffier, E., D. Joseph and B. B. Vargaftig. 1991. Activation of guinea pig eosinophils by human recombinant IL-5. Selective priming to platelet-activating factor-acether and interference of its antagonists. *J. Immunol.* 147: 2595-2602.
39. Cromwell, O., J. P. Bennett, A. B. Kay, I. Hide and B. D. Gomperts. 1991. Mechanisms of granule enzyme secretion from permeabilised guinea pig eosinophils: Dependence on Ca²⁺ and guanine nucleotides. *J. Immunol.* 147: 1905-1911.
40. De Duve, C. 1991. *Blueprint for a Cell: The nature and origin of life.* Portland Press,
41. DiPersio, J., P. Billing, S. Kaufman, P. Eghtesady, R. E. Williams and J. C. Gasson. 1988. Characterization of the human granulocyte-macrophage colony-stimulating factor receptor. *J. Biol. Chem.* 263: 1834-1841.

42. Dvorak, A. M. 1980. Ultrastructural evidence for release of major basic protein-containing crystalline cores of eosinophil granules in vivo: cytotoxic potential in Crohn's disease. *J. Immunol.* 125: 460-462.
43. Dvorak, A. M., L. Letourneau, G. R. Login, P. F. Weller and S. J. Ackerman. 1988. Ultrastructural localization of the Charcot-Leyden crystal protein (lysophospholipase) to a distinct crystalloid-free granule population in mature human eosinophils. *Blood* 72: 150-158.
44. Dvorak, A. M., M. C. Mihm, Jr., J. E. Osage, T. H. Kwan, K. F. Austen and B. U. Wintroub. 1982. Bullous pemphigoid, an ultrastructural study of the inflammatory response: eosinophil, basophil and mast cell granule changes in multiple biopsies from one patient. *J. Invest. Dermatol.* 78: 91-101.
45. Egesten, A., U. Gullberg, I. Olsson and J. Richter. 1993. Phorbol ester-induced degranulation in adherent human eosinophil granulocytes is dependent on CD11/CD18 leukocyte integrins. *J. Leukoc. Biol.* 53: 287-293.
46. Elsner, J., M. Oppermann, W. Czech, G. Dobos, E. Schopf, J. Norgauer and A. Kapp. 1994. C3a activates reactive oxygen radical species production and intracellular calcium transients in human eosinophils. *Eur. J. Immunol.* 24: 518-522.
47. Fujisawa, T., R. Abu Ghazaleh, H. Kita, C. J. Sanderson and G. J. Gleich. 1990. Regulatory effect of cytokines on eosinophil degranulation. *J. Immunol.* 144: 642-646.
48. Gerard, N. P. and C. Gerard. 1991. The chemotactic receptor for human C5a anaphylatoxin. *Nature* 349: 614-617.
49. Gibson, P. G., J. Dolovich, A. Girgis Gabardo, M. M. Morris, M. Anderson, F. E. Hargreave and J. A. Denburg. 1990. The inflammatory response in asthma exacerbation: changes in circulating eosinophils, basophils and their progenitors. *Clin. Exp. Allergy* 20: 661-668.
50. Ginsel, L. A., J. J. Onderwater, J. A. Fransen, A. J. Verhoeven and D. Roos. 1990. Localization of the low-Mr subunit of cytochrome b558 in human blood phagocytes by immunoelectron microscopy. *Blood* 76: 2105-2116.
51. Glauert, A. M., A. E. Butterworth, R. F. Sturrock and V. Houba. 1978. The mechanism of antibody-dependent, eosinophil-mediated damage to schistosomula of *Schistosoma mansoni* in vitro: a study by phase-contrast and electron microscopy. *J. Cell Sci.* 34: 173-192.
52. Gleich, G. J. 1990. The eosinophil and bronchial asthma: current understanding. *J. Allergy Clin. Immunol.* 85: 422-436.
53. Gleich, G. J. and D. Loegering. 1973. Selective stimulation and purification of eosinophils and neutrophils from guinea pig peritoneal fluids. *J. Lab. Clin. Med.* 82: 522-528.
54. Goetzl, E. J., S. I. Wasserman and F. Austen. 1975. Eosinophil polymorphonuclear leukocyte function in immediate hypersensitivity. *Arch. Pathol.* 99: 1-4.

55. Gomperts, B. D. and P. E. R. Tatham. 1992. Regulated exocytotic secretion from permeabilized cells. *Meth. Enzymol.* 219: 178-189.
56. Gounni, A. S., B. Lamkhieued, K. Ochiai, Y. Tanaka, E. Delaporte, A. Capron, J. P. Kinet and M. Capron. 1994. High-affinity IgE receptor on eosinophils is involved in defence against parasites. *Nature* 367: 183-186.
57. Grotendorst, G. R., Y. Soma, K. Takehara and M. Charette. 1989. EGF and TGF- α are potent chemoattractants for endothelial cells and EGF-like peptides are present at sites of tissue regeneration. *J. Cell Physiol.* 139: 617-623.
58. Hallgren, R., J. F. Colombel, R. Dahl, K. Fredens, A. Kruse, N. O. Jacobsen, P. Venge and J. C. Rambaud. 1989. Neutrophil and eosinophil involvement of the small bowel in patients with celiac disease and Crohn's disease: studies on the secretion rate and immunohistochemical localization of granulocyte granule constituents. *Am. J. Med.* 86: 56-64.
59. Hamann, K. J., R. L. Barker, R. M. Ten and G. J. Gleich. 1991. The molecular biology of eosinophil granule proteins. *Int. Arch. Allergy Appl. Immunol.* 94: 202-209.
60. Harnett, M. and K. Rigley. 1992. The role of G-proteins versus protein tyrosin kinases in the regulation of lymphocyte activation. *Immunology Today* 13: 482-486.
61. Harnett, M. M. and G. G. B. Klaus. 1988. G protein coupling of antigen receptor-stimulated polyphosphoinositide hydrolysis in B-cells. *J. Immunol.* 140: 3135-3139.
62. Hartnell, A., R. Moqbel, G. M. Walsh, B. Bradley and A. B. Kay. 1990. Fc gamma and CD11/CD18 receptor expression on normal density and low density human eosinophils. *Immunology* 69: 264-270.
63. Hatzelmann, A., H. Tenor and C. Schudt. 1995. Differential effects of non-selective and selective phosphodiesterase inhibitors on human eosinophil functions. *Br. J. Pharmacol.* 114: 821-831.
64. Henderson, W. R. and E. Y. Chi. 1985. Ultrastructural characterization and morphometric analysis of human eosinophil degranulation. *J. Cell Sci.* 73: 33-48.
65. Henderson, W. R., E. Y. Chi, A. Jorg and S. J. Klebanoff. 1983. Horse eosinophil degranulation induced by the ionophore A23187. Ultrastructure and role of phospholipase A2. *Am. J. Pathol.* 111: 341-349.
66. Herndon, F. J. and S. G. Kayes. 1992. Depletion of eosinophils by anti-IL-5 monoclonal antibody treatment of mice infected with *Trichinella spiralis* does not alter parasite burden or immunologic resistance to reinfection. *J. Immunol.* 149: 3642-3647.
67. Heukeshoven, J. and R. Dernick. 1988. Improved silver staining procedure for fast staining in PhastSystem Development Unit. I. Staining of sodium dodecyl sulfate gels. *Electrophoresis* 9: 28-32.
68. Hisamatsu, K., T. Ganbo, T. Nakazawa, Y. Murakami, G. J. Gleich, K. Makiyama and H. Koyama. 1990. Cytotoxicity of human eosinophil granule major basic protein to human nasal sinus mucosa in vitro. *J. Allergy Clin. Immunol.* 86: 52-63.

69. Hom, J. T. and T. Estridge. 1993. FK506 and rapamycin modulate the functional activities of human peripheral blood eosinophils. *Clin. Immunol. Immunopathol.* 68: 293-300.
70. Howell, T. W., S. Cockcroft and B. D. Gomperts. 1987. Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilised mast cells. *J. Cell Biol.* 105: 191-197.
71. Jouault, T., M. Capron, J. M. Balloul, J. C. Ameisen and A. Capron. 1988. Quantitative and qualitative analysis of the Fc receptor for IgE (Fc epsilon RII) on human eosinophils. *Eur. J. Immunol.* 18: 237-241.
72. June, C. H., M. C. Fletcher, J. A. Ledbetter, G. L. Schieven, J. N. Siegel, A. F. Phillips and L. E. Samelson. 1990. Inhibition of tyrosine phosphorylation prevents T-cell receptor-mediated signal transduction. *Proc. Natl. Acad. Sci. U. S. A.* 87: 7722-7726.
73. Kariya, K., E. Lee, M. Hirouchi, M. Hosokawa and H. Sayo. 1987. Purification and some properties of peroxidases of rat bone marrow. *Biochim. Biophys. Acta* 911: 95-101.
74. Kato, M., R. T. Abraham and H. Kita. 1995. Tyrosine phosphorylation is required for eosinophil degranulation induced by immobilized immunoglobulins. *J. Immunol.* 155: 357-366.
75. Kawashima, T., I. Iwamoto, N. Nakagawa, H. Tomioka and S. Yoshida. 1994. Inhibitory effect of pemirolast, a novel antiallergic drug, on leukotriene C4 and granule protein release from human eosinophils. *Int. Arch. Allergy Immunol.* 103: 405-409.
76. Kerboeuf, D., A. Le Berre, J. C. Dedieu and J. Cohen. 1993. Calmodulin is essential for assembling links necessary for exocytotic membrane fusion in *Paramecium*. *EMBO J.* 12: 3385-3390.
77. Kernen, P., M. P. Wymann, V. von Tscharner, D. A. Deranleau, P. Tai, C. J. Spry, C. A. Dahinden and M. Baggiolini. 1991. Shape changes, exocytosis, and cytosolic free calcium changes in stimulated human eosinophils. *J. Clin. Invest.* 87: 2012-2027.
78. Khalife, J., M. Capron, J. Y. Cesbron, P. C. Tai, H. Taelman, L. Prin and A. Capron. 1986. Role of specific IgE antibodies in peroxidase (EPO) release from human eosinophils. *J. Immunol.* 137: 1659-1664.
79. Khalife, J., M. Capron, J. Grzych, H. Bazin and A. Capron. 1985. Extracellular release of rat eosinophil peroxidase (EPO): Role of anaphylactic immunoglobulins. *J. Immunol.* 134: 1968-1973.
80. Kimura, I., Y. Moritani and Y. Tanizaki. 1975. Basophils in bronchial asthma with reference to reagin-type allergy. *Clin. Allergy* 3: 195-202.
81. Kita, H., R. I. Abu Ghazaleh, G. J. Gleich and R. T. Abraham. 1991. Regulation of Ig-induced eosinophil degranulation by adenosine 3',5'-cyclic monophosphate. *J. Immunol.* 146: 2712-2718.

82. Kita, H., R. I. Abu-Ghazaleh, G. J. Gleich and R. T. Abraham. 1991. Role of pertussis toxin-sensitive G proteins in stimulus- dependent human eosinophil degranulation. *J. Immunol.* 147: 3466-3473.
83. Kita, H., S. Horie and G. J. Gleich. 1996. Extracellular matrix proteins attenuate activation and degranulation of stimulated eosinophils. *J. Immunol.* 156: 1174-1181.
84. Kita, H., D. A. Weiler, R. Abu Ghazaleh, C. J. Sanderson and G. J. Gleich. 1992. Release of granule proteins from eosinophils cultured with IL-5. *J. Immunol.* 149: 629-635.
85. Korenaga, M., Y. Hitoshi, N. Yamaguchi, Y. Sato, K. Takatsu and I. Tada. 1991. The role of interleukin-5 in protective immunity to *Strongyloides venezuelensis* infection in mice. *Immunology* 72: 502-507.
86. Krausz, Y., C. B. Wollheim, E. Siegel and G. W. Sharp. 1980. Possible role for calmodulin in insulin release. Studies with trifluoperazine in rat pancreatic islets. *J. Clin. Invest.* 66: 603-607.
87. Kretsinger, R. H. 1976. Calcium binding-proteins. *Annu. Rev. Biochem.* 45: 239-266.
88. Kroegel, C., E. R. Chilvers, M. A. Giembycz, R. A. Challiss and P. J. Barnes. 1991. Platelet-activating factor stimulates a rapid accumulation of inositol (1,4,5)trisphosphate in guinea pig eosinophils: relationship to calcium mobilization and degranulation. *J. Allergy Clin. Immunol.* 88: 114-124.
89. Kroegel, C., M. A. Giembycz and P. J. Barnes. 1990. Characterization of eosinophil cell activation by peptides. Differential effects of substance P, melittin, and FMET-Leu-Phe. *J. Immunol.* 145: 2581-2587.
90. Kroegel, C., M. A. Giembycz, H. Matthys, J. Westwick and P. J. Barnes. 1994. Modulatory role of protein kinase C on the signal transduction pathway utilized by platelet-activating factor in eosinophil activation. *Am. J. Respir. Cell Mol. Biol.* 11: 593-599.
91. Kroegel, C., T. Yukawa, G. Dent, P. Chanez, K. F. Chung and P. J. Barnes. 1988. Platelet-activating factor induces eosinophil peroxidase release from purified human eosinophils. *Immunology* 64: 559-561.
92. Kroegel, C., T. Yukawa, G. Dent, P. Venge, K. F. Chung and P. J. Barnes. 1989. Stimulation of degranulation from human eosinophils by platelet-activating factor. *J. Immunol.* 142: 3518-3526.
93. Lacy, P., M. Tian, N. Thompson, R. Solari, I. Hide, T. M. Newman and B. D. Gomperts. 1995. A survey of GTP-binding proteins and other potential key regulators of exocytotic secretion in eosinophils: Apparent absence of rab3 and vesicle fusion protein homologues. *J. Cell Sci.* 108: 3547-3556.
94. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.

95. Lehrer, R. I., D. Szklarek, A. Barton, T. Ganz, K. J. Hamann and G. J. Gleich. 1989. Antibacterial properties of eosinophil major basic protein and eosinophil cationic protein. *J. Immunol.* 142: 4428-4434.
96. Levi-Schaffer, F., P. Lacy, N. J. Severs, T. M. Newman, J. North, B. D. Gomperts, A. B. Kay and R. Moqbel. 1995. Association of granulocyte-macrophage colony-stimulating factor with the crystalloid granules of human eosinophils. *Blood* 85: 2579-2586.
97. Lewis, D. M., J. C. Lewis, D. A. Loegering and G. J. Gleich. 1978. Localization of the guinea pig eosinophil major basic protein to the core of the granule. *J. Cell Biol.* 77: 702-713.
98. Liang, L. and C. K. Huang. 1995. Tyrosine phosphorylation induced by cross-linking of Fc gamma-receptor type II in human neutrophils. *Biochem. J.* 306: 489-495.
99. Lillie, T. H. W. and B. D. Gomperts. 1992. Nucleotides and divalent cations as effectors and modulators of exocytosis in permeabilised rat mast cells. *Phil. Trans. Roy. Soc. Lond. B.* 336: 25-34.
100. Lillie, T. H. W. and B. D. Gomperts. 1992. Guanine nucleotide is essential, Ca^{2+} is a modulator, in the exocytotic reaction of permeabilised rat mast cells. *Biochem. J.* 288: 181-187.
101. Lindau, M. and B. D. Gomperts. 1991. Techniques and concepts in exocytosis: Focus on mast cells. *Biochim. Biophys. Acta* 1071: 429-471.
102. Lindau, M., O. Nüsse, J. P. Bennett and O. Cromwell. 1993. The membrane fusion events in degranulating guinea pig eosinophils. *J. Cell Sci.* 104: 203-209.
103. Lopez, A. F., J. M. Eglinton, D. Gillis, L. S. Park, S. Clark and M. A. Vadas. 1989. Reciprocal inhibition of binding between interleukin 3 and granulocyte-macrophage colony-stimulating factor to human eosinophils. *Proc. Natl. Acad. Sci. U. S. A.* 86: 7022-7026.
104. Lopez, A. F., C. J. Sanderson, J. R. Gamble, H. D. Campbell, I. G. Young and M. A. Vadas. 1988. Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J. Exp. Med.* 167: 219-224.
105. Maddox, D. E., G. M. Kephart, C. B. Coulam, J. H. Butterfield, K. Benirschke and G. J. Gleich. 1984. Localization of a molecule immunochemically similar to eosinophil major basic protein in human placenta. *J. Exp. Med.* 160: 29-41.
106. Mariot, P., A. J. O'Sullivan, A. M. Brown and P. E. R. Tatham. 1996. Rho-guanine nucleotide dissociation inhibitor protein (Rho-GDI) inhibits exocytosis in mast cells. *EMBO J.* (in press):
107. Matsunaga, Y., H. Kido, K. Kawaji, K. Kamoshita, N. Katunuma and T. Ogura. 1994. Inhibitors of chymotrypsin-like proteases inhibit eosinophil peroxidase release from activated human eosinophils. *Arch. Biochem. Biophys.* 312: 67-74.
108. McLaren, D. J., C. D. Mackenzie and F. J. Ramalho Pinto. 1977. Ultrastructural observations on the in vitro interaction between rat eosinophils and some parasitic

helminths (*Schistosoma mansoni*, *Trichinella spiralis* and *Nippostrongylus brasiliensis*). *Clin. exp. Immunol.* 30: 105-118.

109. McLaren, D. J., F. J. Ramalho Pinto and S. R. Smithers. 1978. Ultrastructural evidence for complement and antibody-dependent damage to schistosomula of *Schistosoma mansoni* by rat eosinophils in vitro. *Parasitology* 77: 313-324.

110. Means, A. R., M. F. VanBerkum, I. Bagchi, K. P. Lu and C. D. Rasmussen. 1991. Regulatory functions of calmodulin. *Pharmacol. Ther.* 50: 255-270.

111. Miller, S.G. and H.H. Moore. 1992. Movement from trans-Golgi network to cell surface in semiintact cells. In *Reconstitution of Intracellular transport*. J.E. Rothman, editor. Academic Press, San Diego. 234-248.

112. Monck, J. R. and J. M. Fernandez. 1994. The exocytosis fusion pore and neurotransmitter release. *Neuron* 12: 707-716.

113. Moqbel, R., Q. Hamid, S. Ying, J. Barkans, A. Hartnell, A. Tsicopoulos, A. J. Wardlaw and A. B. Kay. 1991. Expression of mRNA and immunoreactivity for the granulocyte/macrophage colony-stimulating factor in activated human eosinophils. *J. Exp. Med.* 174: 749-752.

114. Moqbel, R., D. Wakelin, A. J. MacDonald, S. J. King, R. K. Grencis and A. B. Kay. 1987. Release of leukotrienes during rapid expulsion of *Trichinella spiralis* from immune rats. *Immunology* 60: 425-430.

115. Morgan, A. and R. D. Burgoyne. 1995. Is NSF a fusion protein? *Trends in Cell Biol.* 5: 335-339.

116. Morita, E., J. M. Schroder and E. Christophers. 1989. Differential sensitivities of purified human eosinophils and neutrophils to defined chemotaxins. *Scand. J. Immunol.* 29: 709-716.

117. Munoz, N. M., K. F. Rabe, A. J. Vita, K. McAllister, D. Mayer, M. Weiss and A. R. Leff. 1995. Paradoxical blockade of beta adrenergically mediated inhibition of stimulated eosinophil secretion by salmeterol. *J. Pharmacol. Exp. Ther.* 273: 850-854.

118. Munoz, N. M., A. J. Vita, S. P. Neeley, K. McAllister, S. M. Spaethe, S. R. White and A. R. Leff. 1994. Beta adrenergic modulation of formyl-methionine-leucine-phenylalanine-stimulated secretion of eosinophil peroxidase and leukotriene C4. *J. Pharmacol. Exp. Ther.* 268: 139-143.

119. Nathan, C. and M. Sporn. 1991. Cytokines in context. *J. Cell Biol.* 113: 981-986.

120. Newman, T. M., M. Tian and B. D. Gomperts. 1996. Ultrastructural characterisation of tannic acid arrested degranulation of GTP-g-S stimulated guinea pig eosinophils. *Eur. J. Cell Biol.* 70: 209-220.

121. Nusse, O., M. Lindau, O. Cromwell, A. B. Kay and B. D. Gomperts. 1990. Intracellular application of GTP-5'-O-(3-thiotriphosphate) induces exocytotic granule fusion in guinea pig eosinophils. *J. Exp. Med.* 171: 775-786.

122. O'Connor, V., G. J. Augustine and H. Betz. 1994. Synaptic vesicle exocytosis: molecules and models. *Cell* 76: 785-787.
123. O'Donnell, M. C., S. J. Ackerman, G. J. Gleich and L. L. Thomas. 1983. Activation of basophil and mast cell histamine release by eosinophil granule major basic protein. *J. Exp. Med.* 157: 1981-1991.
124. O'Sullivan, A. J., A. M. Brown, H. N. M. Freeman and B. D. Gomperts. 1996. Purification and identification of FOAD-II, a cytosolic protein that regulates secretion in streptolysin-O permeabilised mast cells, as a rac/rhoGDI complex. *Mol. Biol. Cell* 7: 397-408.
125. Ordman, L. J. and T. Gillman. 1966. Studies in the healing of cutaneous wounds. I. The healing of incisions through the skin of pigs. *Arch. Surg.* 93: 857-882.
126. Otsuka, H., J. Dolovich, D. Befus, J. Bienenstock and J. Denburg. 1986. Peripheral blood basophils, basophil progenitors, and nasal metachromatic cells in allergic rhinitis. *Am. Rev. Respir. Dis.* 133: 757-762.
127. Parmley, R. T. and S. S. Spicer. 1974. Cytochemical and ultrastructural identification of a small type granule in human late eosinophils. *Lab. Invest.* 30: 557-567.
128. Parrillo, J. E., J. S. Borer, W. L. Henry, S. M. Wolff and A. S. Fauci. 1979. The cardiovascular manifestations of the hypereosinophilic syndrome. Prospective study of 26 patients, with review of the literature. *Am. J. Med.* 67: 572-582.
129. Pimplikar, S. W., E. Ikonen and K. Simons. 1994. Basolateral protein transport in streptolysin O-permeabilized MDCK cells. *J. Cell Biol.* 125: 1025-1035.
130. Pincus, S. H., E. A. Whitcomb and C. A. Dinarello. 1986. Interaction of IL I and TPA in modulation of eosinophil function. *J. Immunol.* 137: 3509-3514.
131. Pinkney, M., E. Beachey and M. Kehoe. 1989. The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infect. Immun.* 57: 2553-2558.
132. Popper, H., G. Knipping, B. M. Czarnetzki, R. Steiner, G. Helleis and H. Auer. 1989. Activation and release of enzymes and major basic protein from guinea pig eosinophil granulocytes induced by different inflammatory stimuli and other substances. A histochemical, biochemical, and electron microscopic study. *Inflammation* 13: 147-162.
133. Price, L. S., J. C. Norman, A. J. Ridley and A. Koffer. 1995. Small GTPases, rac and rho, as regulators of secretion in mast cells. *Current Biology.* 5: 68-73.
134. Ramalho Pinto, F. J., D. J. McLaren and S. R. Smithers. 1978. Complement-mediated killing of schistosomes of *Schistosoma mansoni* by rat eosinophils in vitro. *J. Exp. Med.* 147: 147-156.
135. Romano, M., P. Patriarca, C. Melo, F. E. Baralle and P. Dri. 1994. Hereditary eosinophil peroxidase deficiency: immunochemical and spectroscopic studies and evidence for a compound heterozygosity of the defect. *Proc. Natl. Acad. Sci. U. S. A.* 91: 12496-12500.

136. Sabesin, S. M. 1963. A function of the eosinophil: Phagocytosis of antigen antibody complexes. *Proc. Soc. Exp. Biol. Med.* 112: 667-671.
137. Saito, H., F. Okajima, T. F. P. Molski, R. I. Sha'afi, M. Ui and T. Ishizaka. 1987. Effects of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and -independent histamine release from mast cells and basophils. *J. Immunol.* 138: 3927-3934.
138. Sakamaki, K., M. Tomonaga, K. Tsukui and S. Nagata. 1989. Molecular cloning and characterization of a chromosomal gene for human eosinophil peroxidase. *J. Biol. Chem.* 264: 16828-16836.
139. Sceppek, S. and M. Lindau. 1993. Focal exocytosis by eosinophils: Compound exocytosis and cumulative fusion. *EMBO J.* 12: 1811-1817.
140. Sceppek, S., R. Moqbel and M. Lindau. 1994. Compound exocytosis and cumulative degranulation by eosinophils and their role in parasite killing. *Parasitology Today* 10: 276-278.
141. Sceppek, S., O. Nüsse, G. Sachsse and M. Lindau. 1991. Horse eosinophils: A new model system for biophysical studies of exocytosis. *Eur. J. Cell Biol.* 54: 23
142. Sher, A., R. L. Coffman, S. Hieny and A. W. Cheever. 1990. Ablation of eosinophil and IgE responses with anti-IL-5 or anti-IL-4 antibodies fails to affect immunity against *Schistosoma mansoni* in the mouse. *J. Immunol.* 145: 3911-3916.
143. Sillen, L. G. and A. E. Martell. 1964. Stability constants of metal-ion complexes. *The Chemical Society. London.* pp 754:
144. Slungaard, A. and J. R. Mahoney. 1991. Thiocyanate is the major substrate for eosinophil peroxidase in physiologic fluids. Implications for cytotoxicity. *J. Biol. Chem.* 266: 4903-4910.
145. Spry, C. J. F. 1988. Eosinophils: A comprehensive review and guide to the scientific and medical literature. Oxford University Press, Oxford.
146. Stern, M., L. Meagher, J. Savill and C. Haslett. 1992. Apoptosis in human eosinophils. Programmed cell death in the eosinophil leads to phagocytosis by macrophages and is modulated by IL-5. *J. Immunol.* 148: 3543-3549.
147. Storrie, B. and E. A. Madden. 1990. Isolation of subcellular organelles. *Methods in Enzymol.* 182: 203-225.
148. Tai, P. C., M. Capron, D. M. Bakes, J. Barkans and C. J. Spry. 1986. Monoclonal antibodies to human eosinophil plasma membrane antigens enhance the secretion of eosinophil cationic protein. *Clin. exp. Immunol.* 63: 728-737.
149. Tai, P. C., M. E. Holt, P. Denny, A. R. Gibbs, B. D. Williams and C. J. Spry. 1984. Deposition of eosinophil cationic protein in granulomas in allergic granulomatosis and vasculitis: the Churg-Strauss syndrome. *Br. Med. J. Clin. Res. Ed.* 289: 400-402.

150. Tai, P. C., C. J. Spry, C. Peterson, P. Venge and I. Olsson. 1984. Monoclonal antibodies distinguish between storage and secreted forms of eosinophil cationic protein. *Nature* 309: 182-184.
151. Takafuji, S., K. Tadokoro, K. Ito and C. A. Dahinden. 1994. Degranulation from human eosinophils stimulated with C3a and C5a. *Int. Arch. Allergy Immunol.* 104 Suppl 1: 27-29.
152. Takai, Y., K. Kaibuchi, A. Kikuchi, T. Sasaki and H. Shirataki. 1993. Regulators of small GTPases. *Ciba. Found. Symp.* 176: 128-138.
153. Tatham, P.E.R. and B.D. Gomperts. 1990. Cell permeabilisation. In *Peptide Hormones - A Practical Approach*. K. Siddle and J.C. Hutton, editors. IRL Press, Oxford. 257-269.
154. Tavernier, J., R. Devos, S. Cornelis, T. Tuypens, J. Van der Heyden, W. Fiers and G. Plaetinck. 1991. A human high affinity interleukin-5 receptor (IL5R) is composed of an IL5-specific alpha chain and a beta chain shared with the receptor for GM-CSF. *Cell* 66: 1175-1184.
155. Thomas, E. L., P. M. Bozeman, M. M. Jefferson and C. C. King. 1995. Oxidation of bromide by the human leukocyte enzymes myeloperoxidase and eosinophil peroxidase. Formation of bromamines. *J. Biol. Chem.* 270: 2906-2913.
156. Thomas, K. M., H. Y. Pyun and J. Navarro. 1990. Molecular cloning of the fMet-Leu-Phe receptor from neutrophils [published erratum appears in *J Biol Chem* 1992 Jul 5;267(19): 13780]. *J. Biol. Chem.* 265: 20061-20064.
157. Ting, A. T., K. J. Einspahr, R. T. Abraham and P. J. Leibson. 1991. Fc gamma receptor signal transduction in natural killer cells. Coupling to phospholipase C via a G protein-independent, but tyrosine kinase-dependent pathway. *J. Immunol.* 147: 3122-3127.
158. Todd, R., B. R. Donoff, T. Chiang, M. Y. Chou, A. Elovic, G. T. Gallagher and D. T. Wong. 1991. The eosinophil as a cellular source of transforming growth factor alpha in healing cutaneous wounds. *Am. J. Pathol.* 138: 1307-1313.
159. Tomassini, M., A. Tsicopoulos, P. C. Tai, V. Gruart, A. B. Tonnel, L. Prin, A. Capron and M. Capron. 1991. Release of granule proteins by eosinophils from allergic and nonallergic patients with eosinophilia on immunoglobulin-dependent activation. *J. Allergy Clin. Immunol.* 88: 365-375.
160. Unkeless, J. C. 1989. Function and heterogeneity of human Fc receptors for immunoglobulin G. *J. Clin. Invest.* 83: 355-361.
161. Walsh, G. M., A. Hartnell, R. Moqbel, O. Cromwell, L. Nagy, B. Bradley, T. Furitsu, T. Ishizaka and A. B. Kay. 1990. Receptor expression and functional status of cultured human eosinophils derived from umbilical cord blood mononuclear cells. *Blood* 76: 105-111.

162. Walsh, G. M., A. Hartnell, A. J. Wardlaw, K. Kurihara, C. J. Sanderson and A. B. Kay. 1990. IL-5 enhances the in vitro adhesion of human eosinophils, but not neutrophils, in a leucocyte integrin (CD11/18)-dependent manner. *Immunology* 71: 258-265.
163. Wardlaw, A. J., H. Hay, O. Cromwell, J. V. Collins and A. B. Kay. 1989. Leukotrienes, LTC₄ and LTB₄, in bronchoalveolar lavage in bronchial asthma and other respiratory diseases. *J. Allergy Clin. Immunol.* 84: 19-26.
164. Wardlaw, A. J., R. Moqbel and A. B. Kay. 1995. Eosinophils: biology and role in disease. *Adv. Immunol.* 60: 151-266.
165. Wasmoen, T. L., M. P. Bell, D. A. Loegering, G. J. Gleich, F. G. Prendergast and D. J. McKean. 1988. Biochemical and amino acid sequence analysis of human eosinophil granule major basic protein. *J. Biol. Chem.* 263: 12559-12563.
166. Weiss, S. J., S. T. Test, C. M. Eckmann, D. Roos and S. Regiani. 1986. Brominating oxidants generated by human eosinophils. *Science* 234: 200-203.
167. Weller, P. F. and A. M. Dvorak. 1994. Lipid bodies: intracellular sites for eicosanoid formation. *J. Allergy Clin. Immunol.* 94: 1151-1156.
168. White, S. R., G. V. P. Kulp, S. M. Spaethe, E. van Alstyne and A. R. Leff. 1991. A kinetic assay for eosinophil peroxidase in eosinophils and eosinophil conditioned media. *J. Immunol. Meth.* 44: 257-263.
169. Williams, G., C. A. Smith, N. J. McCarthy and E. A. Grimes. 1992. Apoptosis: Final control point in cell biology. *trends Cell Biol.* 2: 263-267.
170. Wong, D. T., R. B. Donoff, J. Yang, B. Z. Song, K. Matossian, N. Nagura, A. Elovic, J. McBride, G. Gallagher, R. Todd and et al. 1993. Sequential expression of transforming growth factors alpha and beta 1 by eosinophils during cutaneous wound healing in the hamster. *Am. J. Pathol.* 143: 130-142.
171. Wright, N. A., C. Pike and G. Elia. 1990. Induction of a novel epidermal growth factor-secreting cell lineage by mucosal ulceration in human gastrointestinal stem cells. *Nature* 343: 82-85.
172. Ye, R. D., O. Quehenberger, K. M. Thomas, J. Navarro, S. L. Cavanagh, E. R. Prossnitz and C. G. Cochrane. 1993. The rabbit neutrophil N-formyl peptide receptor. cDNA cloning, expression, and structure/function implications. *J. Immunol.* 150: 1383-1394.
173. Zeck Kapp, G., C. Kroegel, U. N. Riede and A. Kapp. 1995. Mechanisms of human eosinophil activation by complement protein C5a and platelet-activating factor: similar functional responses are accompanied by different morphologic alterations. *Allergy* 50: 34-47.