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Pharmacological characterisation of the role of Ca2+ in human eosinophil activation

Grix, Suzanna Peta

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PHARMACOLOGICAL CHARACTERISATION OF THE ROLE OF Ca^{2+} IN HUMAN EOSINOPHIL ACTIVATION.

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ABSTRACT

This study has pharmacologically characterised the role of intracellular Ca^{2+} release and Ca^{2+} influx, as well as the signal transduction pathways involved in C5a- and fMLP-induced activation of human eosinophils.

C5a and fMLP caused concentration-dependent increases in $[Ca^{2+}]_i$, leukotriene C_4 (LTC_4), superoxide (O_2^-) and ECP release. C5a caused the release of Ca^{2+} from intracellular stores, via inositol 1,4,5-trisphosphate formation, as well as Ca^{2+} influx. EGTA markedly inhibited both C5a- and fMLP-induced O_2^- and LTC_4 generation, suggesting a requirement for extracellular Ca^{2+} for these responses.

Ni^{2+} and SK&F 96365, which block receptor-mediated Ca^{2+} entry (RMCE), but not the voltage-operated Ca^{2+} channel (VOC) blocker, nifedipine, caused marked inhibition of the C5a-induced Mn^{2+} influx response. Furthermore, SK&F 96365 and Ni^{2+}, but not nifedipine, inhibited both C5a- and fMLP-induced O_2^- and LTC_4 production. In contrast, ECP release in response to fMLP did not appear to require Ca^{2+} influx and C5a was only partially dependent upon RMCE. However, all of the responses appeared to be dependent upon tyrosine kinase activation, as they were inhibited by the erbstatin analogue.

Finally, RMCE is probably regulated by depletion of intracellular Ca^{2+} stores, as C5a-induced release of intracellular Ca^{2+} correlated closely with Ca^{2+} (Mn^{2+}) influx. Furthermore, eosinophils possess such a mechanism for regulating Ca^{2+} influx, as the endomembrane Ca^{2+}/Mg^{2+}-ATPase inhibitor, thapsigargin stimulated both Ca^{2+} and Mn^{2+} influx responses, as well as LTC_4 release.

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DEDICATION

I dedicate this thesis to my husband, Jonathan and my mother, Carol.

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AA	arachidonic acid
AC	adenylyl cyclase
AUC	area under the curve
BAL	Bronchoalveolar lavage
C5a	Complement fragment 5a
$[Ca^{2+}]_i$	cytosolic free Ca^{2+} concentration
cAMP	cyclic 3',5'-adenosine monophosphate
CICR	calcium-induced Ca^{2+} release
CIF	calcium influx factor
COX	cyclo-oxygenase
CR3	complement receptor 3 (Mac-1; CD11b/CD18)
Cs	cyclosporin
Cyt C	cytochrome C
DAG	diacylglycerol
ECP	eosinophil cationic protein
EDN	eosinophil-derived neurotoxin
EPO	eosinophil peroxidase
5-LOX	5-Lipoxygenase
FLAP	5-lipoxygenase activating protein
fMLP	N-formyl-methionyl-leucyl-phenyalanine
fura-2/AM	fura-2 acetoxymethyl-ester
G proteins	Guanine nucleotide-binding proteins
GM-CSF	Granulocyte/macrophage colony-stimulating factor

H_2O_2	hydrogen peroxide
HUVEC	human umbilical vein endothelial cells
ICAM-1	intercellular adhesion molecule-1
I_{CRAC}	Ca^{2+} release-activated Ca^{2+} current
Ig	Immunoglobulin
IL	interleukin
IP_3	inositol 1,4,5-trisphosphate
LFA-1;	leukocyte function antigen (CD11a/CD18)
LMWG	low molecular weight GTPase
LSI	leukotriene synthesis inhibitor
LT	leukotriene
MBP	major basic protein
MCP	monocyte chemotactic peptide
MIP	macrophage inflammatory protein
MTP	microtitre plate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NaF	sodium fluoride
O_2^-	superoxide
p150.95	CD11c/CD18
PA	phophatidic acid
PAF	platelet-activating factor
PG	prostaglandin
PI 3-kinase	phosphatidylinositol 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate

PKA	protein kinase A
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phosphoinositide-specific phospholipase C
PLD	phospholipase D
PMA	phorbol myristate acetate
PP	protein phosphatase
PTX	pertussis toxin
RBL-2H3	rat basophilic leukaemic cells
RMCE	receptor-mediated Ca^{2+} entry
ROC	receptor-operated Ca^{2+} channel
SMOC	second messenger operated Ca^{2+} channel
SOC	store-operated Ca^{2+} channel
SPA	Scintillation Proximity Assay
TK	tyrosine kinase
trp	transient receptor potential
VCAM-1	vascular cell adhesion molecule-1
VLA	very late antigen
VOC	voltage operated Ca^{2+} channels

1.1 INTRODUCTION

Eosinophils are polymorphonuclear leukocytes, which are normally present in relatively low numbers (<6 x 10^8/L) in peripheral blood (Spry, 1988). They were first identified by Paul Ehrlich in 1879, when he discovered that eosinophils could be stained using the negatively charged dye, eosin (Ehrlich, 1879a). It is now known that this is due to eosin binding to the highly cationic proteins present in eosinophil granules. He also proposed that eosinophils were derived from the bone marrow (Ehrlich, 1879b) and that they exerted their actions in tissues (Ehrlich and Lazarus, 1900).

1.1 Eosinophil morphology

Eosinophils are distinguished by a bilobed nucleus and by their characteristic granules (figure 1). They are usually oval or spherical, with a diameter of approximately 8μm and a volume of 275fl (Sokol *et al.,* 1987). However, they have been seen to develop pseudopods in human blood, sputum, bone marrow and nasal smears and are termed 'medusa cells' when this happens (Hanker *et al.,* 1981). The nucleus occupies 20% of the cell volume and the specific granules occupy 20% of the cytoplasm (Sokol *et al.,* 1987).

Primary, secondary and small granules are present in mature eosinophils. Primary granules are formed during the promyelocyte stage of eosinophil development. They are round, uniformly electron dense and contain lysophosphatase, which crystallises to form Charcot-Leyden crystals (Dvorak *et al.,* 1988; Ackerman *et al.,* 1993).

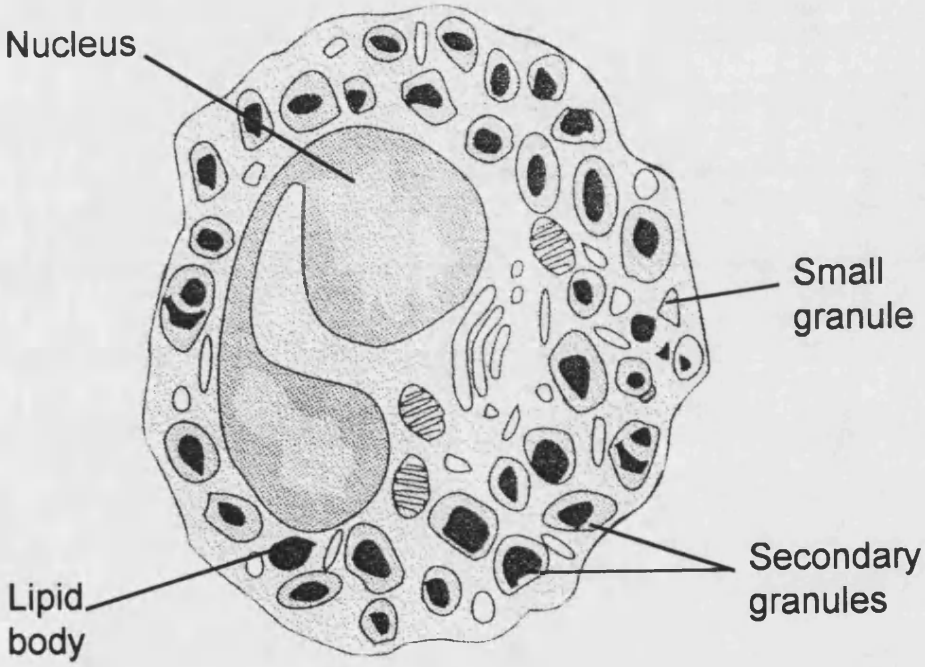


Figure 1

Diagram showing the morphology of the human eosinophil.

Eosinophil specific or secondary granules have an electron-dense crystalloid core within a less electron-dense matrix. They are present in mature eosinophils and may develop from large primary lysosomal granules, which are formed in the promyelocyte stage of development (Bainton and Farquhar, 1970). Secondary granules contain basic proteins (major basic protein (MBP), eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN)), hydrolases (collagenase and β-glucuronidase) and eosinophil peroxidase (EPO). MBP is stored in the crystalloid core of the granule, with the other basic proteins and hydrolytic enzymes stored in the surrounding matrix (Kroegel *et al.,* 1994).

Currently little is known about small granules, which are present in mature eosinophils, other than the fact that they contain arylsulphatase B and acid phosphatase (Gleich *et al.,* 1993).

Eosinophils also contain lipid bodies, which are non-membrane-bound lipid-rich inclusions in the cytoplasm. Their numbers increase in activated eosinophils and they have been shown to incorporate ^{3}H-arachidonate and appear to function as intracellular sites of arachidonic acid storage and metabolism (Weller and Dvorak, 1985).

1.2 Eosinophil surface markers and receptors

Eosinophils have many surface markers, including binding sites for immunoglobulins, lipid mediators, complement proteins, cytokines and adhesion molecules (Kroegel *et al.,* 1992). In common with other circulating white blood cells, eosinophils express surface class I human leukocyte antigen (HLA) and

the common leukocyte antigen (CD45) (Hartnell *et al.,* 1990). They also express Immunoglobulin (Ig) E receptors, similar but not identical to the $Fc_{\varepsilon}RII$ (CD23) receptors on lymphocytes and monocytes (Grangette *et al.,* 1989), as well as IgA receptors (Capron *et al.,* 1989). Binding of IgE leads to release of EPO and generation of platelet-activating factor (PAF), but does not cause ECP release (Capron *et al.,* 1989). In contrast, IgA challenge causes significant degranulation of eosinophils, including release of EPO and ECP (Capron *et al.,* 1989; Abu-Ghazaleh *et al.,* 1989). IgG receptor expression is limited on normal human eosinophils and IgM receptors are absent. The only IgG receptor that eosinophils express is $Fc_{\gamma}RII$, whereas neutrophils express $Fc_{\gamma}RII$ and $Fc_{\gamma}RIII$ (CD16) and monocytes express $Fc_{\gamma}RI$ and $Fc_{\gamma}RII$ (Kroegel *et al.,* 1994). This important difference in IgG receptor expression between neutrophils and eosinophils has been used to great advantage in the separation of these two cell types, using an immunomagnetic technique (Hansel *et al.,* 1991).

Eosinophils also express a number of adhesion molecules including selectins, β_1-integrins (very late antigen (VLA)) and β_2-integrins (the α-chains CD11a, CD11b and CD11c with their common β-chain CD18) (Hansel and Walker, 1992). Cytokine Binding sites for cytokines, including interleukin- (IL-) 3, IL-5 and granulocyte/macrophage colony-stimulating factor (GM-CSF), are also present on human eosinophils (Lopez *et al.,* 1991).

Eosinophils also possess surface receptors for complement factors (including C3a and C5a), chemotactic peptides (fMLP), prostaglandins, leukotrienes (LTB_4) histamine and PAF (Goers *et al.,* 1984; Gerard *et al.,* 1989; Raible *et al.,* 1992;

Fukuda *et al.,* 1992). The effects of these agents are dramatically enhanced if the eosinophils have undergone prior exposure to PAF (Zoratti *et al.,* 1992) or the cytokines IL-3, IL-5 or GM-CSF (Bourgoin *et al.,* 1991; Takafuji *et al.,* 1991). This process of sensitisation to a subsequent activating stimulus is known as 'priming' (Bruijnzeel *et al.,* 1992).

1.3 Role of the eosinophil in health and disease

Eosinophils were originally believed to be involved in removal and detoxification of the products from tissue reactions in allergic and granulomatous diseases (Spry, 1988). By the 1960s this view had adapted to incorporate the notion that eosinophils may inhibit the functions of mast cells by degrading histamine, as well as having a role in antigen presentation for antibody synthesis (Archer, 1968; Speirs, 1958). In the 1970s, it became clear that eosinophils also had a major role in the defence against parasitic infection. Butterworth *et al.* (1975) showed eosinophil-dependent killing of the schistosomula of *Schistosoma Mansonii* and later showed that this was mediated via the release of major basic protein (MBP) from eosinophil granules (Butterworth *et al.,* 1979). Furthermore, in experiments using polyclonal anti-serum raised against eosinophils, immunity to helminths was found to be abolished (Mahmoud *et al.,* 1975; Gleich *et al.,* 1993). Eosinophils have been linked with several diseases, particularly inflammatory skin diseases associated with oedema and certain endomyocardial diseases (Gleich *et al.,* 1993). However, the commonest causes of raised blood eosinophil counts in Western countries are asthma and hayfever (Spry, 1988).

1.4 The role of the eosinophil in asthma

Asthma is a condition often associated with atopy, particularly in children. Atopy occurs as a result of inappropriate synthesis of IgE, specific for particular external antigens (e.g. grass pollens). Atopic asthmatics who experience acute attacks upon exposure to the allergen to which they are sensitised, are termed 'extrinsic' asthmatics. 'Intrinsic' asthma refers to patients who are not atopic and where IgE-dependent mechanisms do not appear to operate. A third clinical category known as 'occupational' asthma refers to patients who become sensitised after exposure to particular proteins, or small molecular weight compounds (Corrigan and Kay, 1992).

A role for the eosinophil in bronchial asthma was originally observed in 1889, when peripheral blood eosinophilia was found to be associated with asthma, whilst airway eosinophilia linked with asthma was first described in 1922 (Gleich *et al.,* 1993). However, the emphasis for the role of eosinophils in asthma has changed over the last 20 years. This change of emphasis coincided with the understanding that asthma does not simply reflect acute airway obstruction, due to mast cell-mediated constriction of the airway smooth muscle. It is now understood that asthma is a chronic inflammatory disease with intermittent acute attacks. These acute bronchospastic episodes are thus superimposed on chronically inflamed airways tissues (Ackerman, 1989).

By the late 1970s, it was becoming clear that far from having a protective role in asthma, eosinophils were likely to have a pro-inflammatory effect in this disease. There was evidence for the presence of histaminase, an enzyme capable of

degrading mast cell-derived histamine, in eosinophils. However, greater amounts of this enzyme were to be found in neutrophils than in eosinophils. In addition, it was found that eosinophils produce large amounts of the inflammatory mediators PAF and leukotriene C_4 (LTC_4) (Spry, 1988). Clinically, increased numbers of eosinophils have been found in the peripheral blood, sputum and bronchial tissue of asthmatic patients (Horn and Karnovsky, 1986; Baigelman *et al.,* 1983; de Monchy *et al.,* 1985). Furthermore, a correlation between eosinophil counts and clinical severity of asthma has been widely documented (Taylor and Luksza, 1987; Bousquet *et al.* 1990; Janson and Herala, 1992).

Marked changes occur in the physiology of the airways of chronic asthmatic patients. The airways become hyperresponsive and this correlates with the degree of inflammation and eosinophilia present. The inflammation causes hyperresponsiveness as a result of both an inflamed thickened airway wall, which encroaches on the lumen, as well as release of bronchoconstrictor inflammatory mediators (Reed, 1988). The bronchoconstrictor effect of these inflammatory mediators is enhanced by the loss of the epithelial layer. The epithelium forms a protective barrier in the airways and its loss exposes sensory neurones, which release neurotransmitters. Many of the inflammatory mediators amplify the bronchoconstrictor effects of neurotransmitters on the airway smooth muscle. Additionally, neurotransmitters (particularly neuropeptides) stimulate mast cell mediator release and dilate blood vessels (Reed, 1988).

Eosinophils are capable of producing many of the pathophysiological changes

seen in asthma. Activation of eosinophils leads to the release of the potent cytotoxic pre-formed granule proteins and newly formed lipid mediators. These can cause submucosal oedema, enhancement of mucus secretion and non-specific airway hyperreactivity (Gleich *et al.,* 1993). Detailed studies of the effect of MBP on the respiratory epithelium of guinea-pigs showed that it caused both detachment of epithelial cells, as well as impairment of ciliary beating (Frigas *et al.,* 1980). This leads to reduced clearance of mucus from the airways. Furthermore, high concentrations of MBP are present in the sputum of many asthmatics and elevated sputum MBP levels are used as a marker for bronchial asthma (Frigas *et al.,* 1981). Similarly, ECP is also cytotoxic to the respiratory epithelium and sputum ECP levels are elevated in asthmatics (Virchow *et al.,* 1993).

EPO is also released from activated eosinophils and functions as a cationic toxin in the absence of hydrogen peroxide (H_2O_2) and as a peroxidase in the presence of H_2O_2 (Gleich *et al.,* 1993). EPO promotes the formation of hypohalous acid in the presence of H_2O_2 and halide ions (preferentially bromide) and may have an effect on acute changes in microvascular permeability (Yoshikawa *et al.,* 1993).

Eosinophil activation also leads to stimulation of a respiratory burst response, generating toxic oxygen radicals such as superoxide (O_2^-) and H_2O_2 (Dechatelat *et al.,* 1977). Reduced nicotinamide adenine dinucleotide (NADPH) oxidase, similar to that found in neutrophils, is also present in eosinophils (Bolscher *et al.,* 1990). NADPH oxidase is an electron transport chain found in lymphocytes and

in the wall of the endocytic vacuole of phagocytic cells. NADPH acts as an electron donor to reduce oxygen to O_2^- and H_2O_2 (Segal and Abo, 1993). In unstimulated cells, the enzyme consists of at least 3 cytosolic components (Rac, $p47^{phox}$ and $p67^{phox}$) and 3 plasma membrane subunits (cytochrome b_{558} (comprising $gp91^{phox}$ and $p22^{phox}$ subunits) and $p45^{phox}$). Upon cell stimulation the cytosolic components are rapidly transferred to the plasma membrane, so that the enzyme is assembled and activated (figure 2) (Thelen *et al.*, 1993).

Bronchoalveolar lavage (BAL) eosinophils were found to have a greater superoxide response than peripheral blood eosinophils (Sedgwick *et al.*, 1992), suggesting that release of toxic oxygen radicals may also be involved in the asthmatic inflammatory response.

Eosinophils also produce many of the inflammatory mediators present in the airways of asthmatics. These include cyclo-oxygenase- and 5-lipoxygenase-derived arachidonic acid metabolites, namely prostaglandins (PGs) (PGE_2, PGD_2, $PGF_{2\alpha}$ and thromboxane B_2 (TXB_2)) and leukotrienes (LTC_4), respectively (figure 3) (Kroegel and Matthys, 1993; Bruynzeel *et al.*, 1985). Leukotrienes are synthesised after liberation of arachidonic acid from arachidonyl-phospholipids within cell membranes or intracellular lipid bodies (Weller and Dvorak, 1985), probably via activation of a Ca^{2+}-dependent, arachidonate-selective phospholipase A_2 (Nigam *et al.*, 1995). Free arachidonic acid is converted to LTA_4 by 5-lipoxygenase, in association with a membrane-bound 5-lipoxygenase activating protein (FLAP) (Hatzelmann *et al.*, 1994). LTA_4 is then conjugated

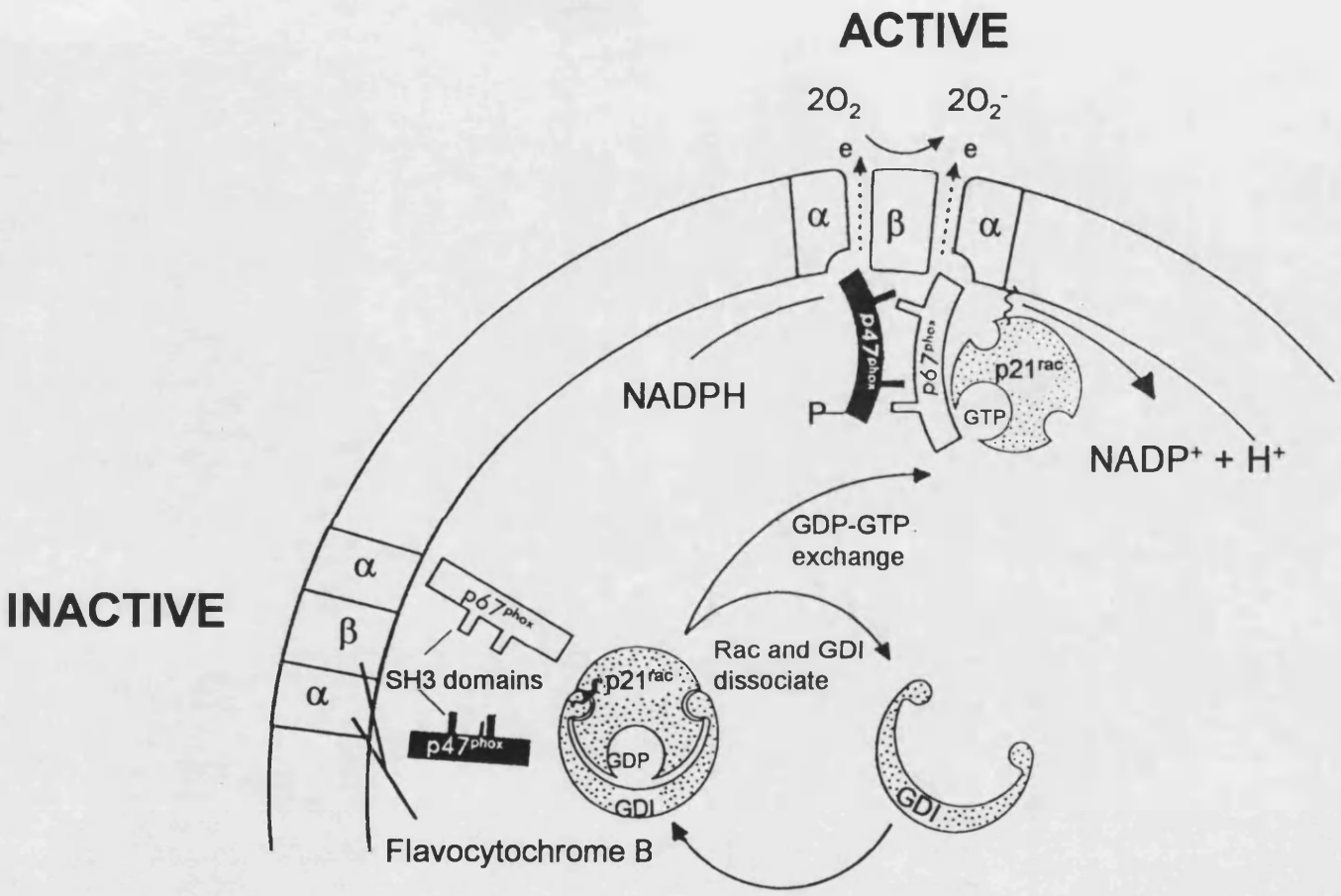


Figure 2

Activation of NADPH oxidase involves the formation of a complex of the cytosolic components of the enzyme (p47phox, p67phox and p21rac), which then associates with flavocytochrome B in the membrane (Adapted from Segal and Abo,1993).

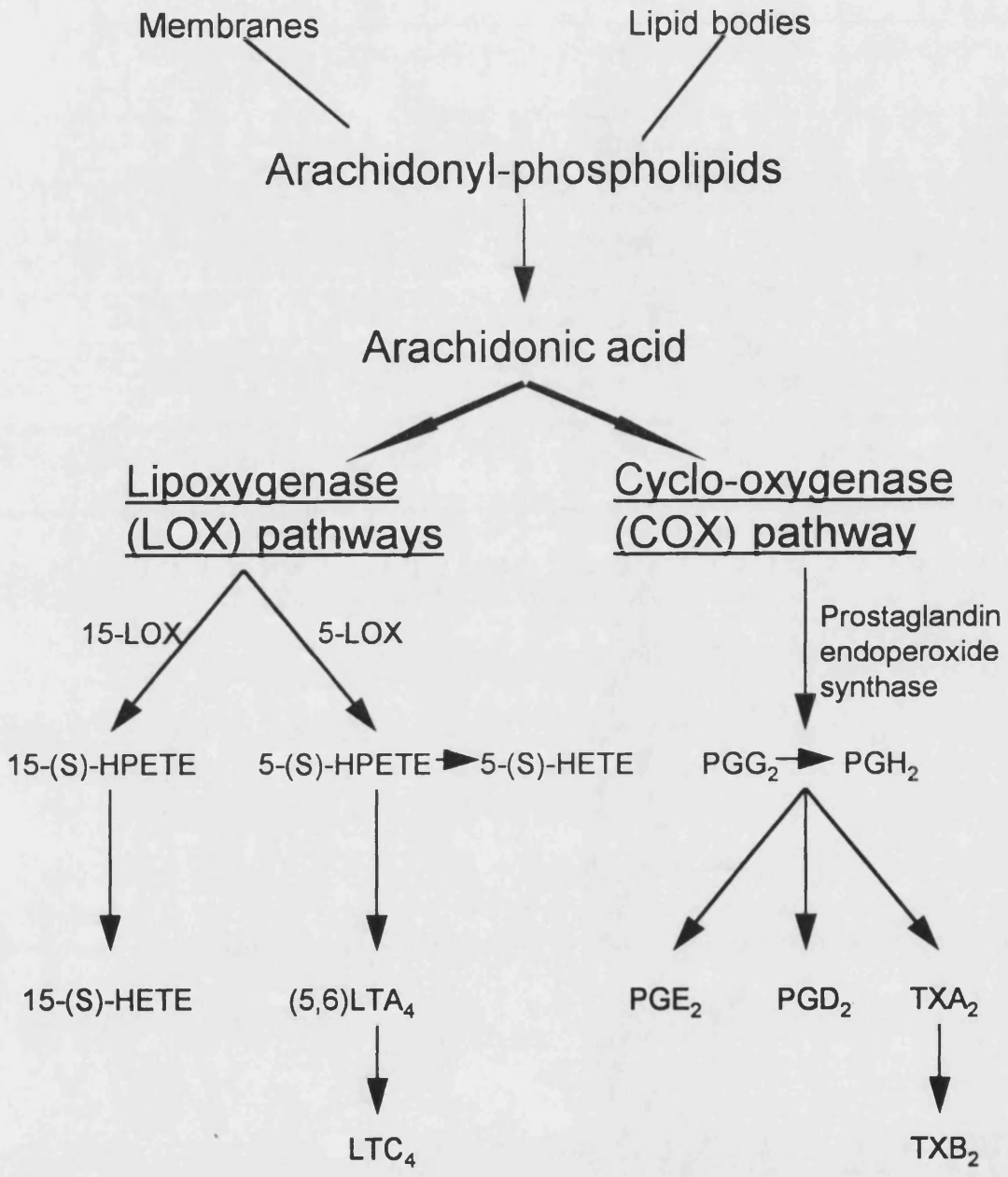


Figure 3

Human eosinophils metabolise arachidonic acid via the 5-lipoxgenase pathway to yield LTC_4, whilst metabolism via the cyclo-oxygenase pathway generates prostaglandins.

with glutathione, by LTC_4 synthase to form the sulphidopeptide LTC_4. LTC_4 may be converted to LTD_4 and LTE_4 by the removal of L-glutamate and glycine residues. Collectively, leukotrienes C_4, D_4 and E_4 constitute the agent formerly known as slow-reacting substance of anaphylaxis (SRS-A) (Samuelsson, 1983). In eosinophils, LTC_4 is released from the cell via a specific, saturable export mechanism (Lam *et al.*, 1989).

Human eosinophils have been shown to release LTC_4 *in vitro*, in response to a range of stimuli, including fMLP, opsonised zymosan, PAF and the Ca^{2+} ionophore, A23187 (Takafuji *et al.*, 1991). LTC_4 may contribute to the inflammatory process in the lung by causing bronchoconstriction, changes in vascular permeability and tone, as well as increased mucus secretion (Spry, 1988). In addition, eosinophils have been shown to produce large amounts of PAF, which is a potent bronchoconstrictor agent as well as being an extremely effective chemotactic stimulus for eosinophils (Kay, 1991). It is clear that eosinophils play an important role in the pathogenesis of the airway inflammation associated with asthma. In order to exert their inflammatory effects, eosinophils are first selectively recruited to the site of inflammation.

1.5. Recruitment of eosinophils to an inflammatory site

Eosinophils develop from bone marrow precursor cells which divide and differentiate, acquiring their characteristic granules. This process is under the control of a number of growth factors or colony stimulating factors derived from T-lymphocytes and mesenchymal cells, particularly GM-CSF, IL-3 and IL-5. Following the process of differentiation and maturation, which takes

approximately 5 days, the eosinophil leaves the bone marrow and enters the peripheral blood circulation ($t_{½}$~13-18 hours) before migrating into the tissue (Kroegel *et al.,* 1994).

The process of eosinophil transmigration from the blood to the tissues at the site of an inflammatory reaction consists of several sequential steps. Normally there will be random contact of the eosinophils with the endothelium as they circulate in the peripheral blood, particularly in the smaller blood vessels (i.e. capillaries). However, the endothelial cells near an inflammatory site become activated by inflammatory mediators and rapidly express surface selectins. Selectins are cell adhesion proteins that bind to carbohydrate/sugar residues on their corresponding adhesion molecule ligands. Eosinophils express two saccharide adhesion molecules, namely CD15 and CDw65, which bind to the endothelial selectins CD62 and ELAM-1, respectively (Kroegel *et al.,* 1994). The result of this initial reversible adhesion combined with the shear forces due to blood flow, leads to the leukocyte 'rolling' along the endothelial surface. The process of 'rolling' is sufficient to slow the eosinophil's progress, allowing time for eosinophil activation by locally-released inflammatory mediators (Pilewski and Albelda, 1993).

Eosinophils become activated as a result of the actions of locally released cytokines and inflammatory mediators, causing an increase in the number and affinity of eosinophil integrin adhesion molecules. The eosinophil β_1-integrin, VLA-4, binds to endothelial vascular cell adhesion molecule-1 (VCAM-1) receptors. Whereas the β_2-integrin receptors leukocyte function associated

antigen (LFA-1; CD11a/CD18), complement receptor 3 (CR3 or Mac-1; CD11b/CD18) and p150.95 (CD11c/CD18), bind to endothelial cell intercellular adhesion molecule-1 (ICAM-1) receptors. Integrin-mediated adhesion leads to a firmer adhesion or 'sticking' of eosinophils to the endothelium (Hansel and Walker, 1992)

Following firm adhesion to endothelial cells, eosinophils are stimulated by chemoattractants (e.g. IL-2, IL-5, RANTES and PAF) from the inflammatory area and migrate between the endothelial cells into the extracellular space ('diapedesis'). In order to allow transmigration, the eosinophils shed surface L-selectin receptors thus weakening the adhesion of the eosinophils to the endothelium (Walker *et al.,* 1993). The transmigrated cell moves along a chemotactic gradient, via increasing concentrations of chemotactic factors, as well as via the interaction of eosinophil integrins with matrix proteins, towards the inflammatory focus (Resnick and Weller, 1993). When the cell nears the inflammatory target, it is exposed to increasing concentrations of activating mediators and locally released cytokines, which activate the eosinophil causing it to release its cytotoxic granules and generate reactive oxygen species and inflammatory mediators (Kroegel *et al.,* 1994). The process of selective recruitment and subsequent survival and activation of eosinophils in the tissues appears to be predominantly controlled by cytokines.

1.6 Role of cytokines on eosinophil recruitment, survival and activation.

The understanding of the processes which control the selective recruitment of eosinophils to an inflammatory site has increased dramatically during the past

10 years. Identification of the adhesion molecule VLA-4, which is present on eosinophils, but not neutrophils, gave one possible mechanism for selective recruitment of eosinophils (Weller *et al.,* 1991). It is now recognised that cytokine-mediated regulation of adhesion molecule expression also plays an important role in this process. IL-5, PAF and fMLP have been shown to cause upregulation of MAC-1 and down-regulation of L selectin on eosinophils. PAF and FMLP also had a similar effect on neutrophils, whereas the effect of IL-5 was selective for eosinophils (Neeley *et al.,* 1993). The predominant source of IL-5 in asthma is the T helper (Th) lymphocytes ($CD4^+$). Recently, it has also been shown that eosinophils are themselves capable of synthesising IL-5 which may have autocrine and paracrine effects (Tanaka *et al.,* 1994; Robinson *et al.,* 1993).

In addition to IL-5, IL-3 and GM-CSF also appear to play a major role in modulating the activity and survival of eosinophils in the tissues. The cell surface receptors for IL-3, IL-5 and GM-CSF have specific α chains, which bind to their corresponding ligand with low affinity. High affinity receptors are formed by the association of the α chain with a common β chain. This can lead to cross-inhibition between the cytokines when a limited number of β chains are present (Nicola and Metcalf, 1991). These cytokines have been shown to stimulate chemotaxis (Quan *et al.,* 1993; Yamaguchi *et al.,* 1988), as well as enhance eosinophil survival (Yamaguchi *et al.,* 1991; Ohnishi *et al.,* 1993; Rothenberg *et al.,* 1988). Furthermore, it has been shown *in vitro* that blood eosinophils, from patients with allergic asthma, adhered to and transmigrated across a confluent layer of human umbilical vein endothelial cells, whereas eosinophils from normal

donors did not transmigrate. Prior incubation of normal eosinophils with IL-3, IL-5 and GM-CSF caused the cells to adhere and transmigrate. Thus, it appears that blood eosinophils from allergic asthmatics have undergone *in vivo* priming and this can be mimicked *in vitro* by incubating the cells with certain cytokines (Moser *et al.,* 1992). *In vitro*, bronchial epithelial cells (Soloperto *et al.,* 1991) and human lung fibroblasts (Vancheri *et al.,* 1989) have been shown to produce GM-CSF and these cell types may be a source of GM-CSF in asthmatic airways.

IL-3, IL-5 and GM-CSF prime eosinophils to subsequent activating stimuli. The respiratory burst response is enhanced and this process appears to involve tyrosine kinase activity and is a Ca^{2+}-independent process (van der Bruggen *et al.,* 1993). Similarly, IL-3 and IL-5 have been shown to enhance release of LTC_4 from eosinophils stimulated with soluble stimuli, such as C5a, FMLP and PAF (Takafuji *et al.,* 1991).

IL-5 has been detected in the serum and BAL fluid of asthmatics. In addition, increased numbers of IL-5 mRNA positive cells are present in the BAL and bronchial mucosa of asthmatic airways (Robinson *et al.,* 1993). This provides further support for cytokines, particularly IL-5, playing a major role in asthma.

Recently, there has been interest in a novel class of small cytokines (chemokines) and their potential role in allergic inflammation. Chemokines comprise 2 subfamilies, which are classified by the first 2 cysteine groups in their primary structure. The cysteine groups are either separated by 1 amino acid (CXC, e.g. IL-8) or are adjacent (CC, e.g. RANTES, monocyte chemotactic peptide (MCP) and macrophage inflammatory protein (MIP)) (Baggiolini and

Dahinden, 1994). Activation of eosinophils by RANTES and MIP-1α stimulates chemotactic responses (Rot *et al.,* 1992; Kameyoshi *et al.,* 1994), as well as generation of reactive oxygen species (Kapp *et al.,* 1994). In contrast, RANTES and MIP-1α have no effect on neutrophils. RANTES also upregulates expression of MAC-1 (CD11b/CD18) (Alam *et al.,* 1993), whilst the chemotactic responses are primed by IL-5 (Schweizer *et al.,* 1994). Similarly, MCP-3 was recently found to be a potent chemotactic stimulus for human eosinophils (Noso *et al.,* 1994; Dahinden *et al.,* 1994), whereas MCP-1 has no effect. Most recently, interest has focused on the identification of a new chemokine eotaxin, which was initially extracted from the BAL of actively sensitised guinea-pigs after allergen challenge (Griffiths-Johnson *et al.,* 1993). Both guinea-pig and human eotaxin have now been cloned (Jose *et al.,* 1994a; Ponath *et al.,* 1995). Eotaxin acts as a potent and specific chemoattractant in both guinea-pig and human eosinophils and appears to share a binding site with RANTES on guinea-pig eosinophils (Jose *et al.,* 1994b).

1.7 Signal transduction pathways involved in eosinophil activation

Relatively few studies have investigated the signal transduction mechanisms in human eosinophils, following agonist-receptor interactions. This has mainly been due to technical difficulties in obtaining sufficient cells of high purity, from normal healthy donors. Until recently, density gradient techniques using either metrizamide or Percoll were used for the preparation of eosinophils. However, the overlap of the density of eosinophils and neutrophils meant that in order to obtain high purity of eosinophils, only low yields were achieved (Hansel *et al.,*

1990). The advent of immunomagnetic cell separation has effectively overcome this problem. This technique does not rely on the density of the cells for separation, but utilises the fact that neutrophils, but not eosinophils, express the surface marker CD16. In a mixed granulocyte preparation, anti-CD16 micromagnetic beads bind to neutrophils. When the cells are added to a magnetised column, neutrophils are retained whereas the eosinophils are eluted (Hansel *et al.*, 1991). This technique permits both high yield and purity of eosinophils to be obtained, so that there are sufficient eosinophils from normal healthy donors to be able to investigate the biochemistry of these cells.

Agonist-induced stimulation of cells leads to activation of a complex cascade of intracellular messengers, which transduce the signal into a functional response. Classical signal transduction pathways, via heterotrimeric guanine nucleotide-binding proteins (G proteins), involve activation of adenylyl cyclase and phosphoinositide-specific phospholipase C (PLC) (Gilman, 1987; Berridge and Irvine, 1989). Activation of adenylyl cyclase stimulates the production of cyclic 3',5'-adenosine monophosphate (cAMP). The elevated levels of cAMP stimulate protein kinase A (PKA), which influences the activity of the cell by phosphorylating target proteins (Taylor, 1990).

The other major classical signal transduction pathway involves activation of PLC, which catalyses hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to form inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). DAG stimulates the activation of protein kinase C (PKC), whilst IP_3 binds to specific receptors present on the membrane of intracellular Ca^{2+} stores, stimulating the

rapid release of Ca^{2+} from this site (Berridge and Irvine, 1989; Ferris *et al.*, 1989).

Phospholipases A_2 and D are also involved in the signal transduction processes in many cells. Phospholipase A_2 is present in cell membranes and lysosomes and acts as a lipolytic enzyme to hydrolyse glycerol phosphatide, yielding lysophosphatide and arachidonic acid (White *et al.*, 1993). Phospholipase D (PLD) catalyses the hydrolysis of choline-containing phosphogycerides to form phosphatidic acid (PA) and choline. Conversion of PA by PA phosphohydrolase yields 1,2-diradyl-sn-glycerol (DG), which stimulates PKC (Mullmann *et al.*, 1990a). Interest in the role of PLD in activation of phagocytic cells has focused on the neutrophil. Cytokine priming in neutrophils activates tyrosine kinase, which leads to enhanced coupling between membrane receptors and PLD (Bourgoin *et al.*, 1992). Furthermore, PA appears to directly activate the neutrophil NADPH oxidase enzyme (Rossi *et al.*, 1990).

Agonist-induced activation of the respiratory burst response in eosinophils, mediated via activation of NADPH oxidase, is inhibited by elevation of cAMP (Dent *et al.*, 1994a; Barnette *et al.*, 1995). Eosinophils appear to have a substantial capacity to generate O_2^-, as stimulation of eosinophils with either the PKC activator, phorbol myristate acetate (PMA) or the calcium ionophore A23187, generates up to 3 times more O_2^-, than the equivalent number of neutrophils (Petreccia *et al.*, 1987; Cromwell *et al.*, 1985). Bach et al (1992) found that the signal transduction processes controlling respiratory burst responses appear to be dependent upon which agonist is used. PMA-induced

O_2^- release is blocked by several PKC inhibitors, whereas the response to IgG (coupled to Sepharose beads) was not inhibited. Conversely, wortmannin is extremely potent at inhibiting IgG- and PAF-mediated O_2^- release (IC_{50}<0.7nM), but is much less active against PMA-induced effects (IC_{50}=5μM). This suggested that at least 2 separate signal transduction pathways exist for O_2^- generation.

Recently, Wymann et al (1995) reported that while C5a-induced Ca^{2+} mobilisation and O_2^- release were inhibited by Ca^{2+} depletion, PMA-induced respiratory burst was not. In addition, the PMA response was more sensitive to inhibition by staurosporine, than C5a-mediated O_2^- generation.

In contrast to O_2^- generation, C5a- and PAF-induced EPO release are enhanced by staurosporine. PAF and C5a mediate their effects via G_i-like G proteins, as pertussis toxin pretreatment abolishes the respiratory burst response, as well as exocytosis of EPO (Kernen *et al.,* 1991).

PLD is also activated in human eosinophils by receptor (C5a) and non-receptor (PMA and A23187) agonists; suggesting that both PKC-dependent and -independent pathways for PLD activation are present in eosinophils (Minnicozzi *et al.,* 1990).

Phospholipase A_2, which catalyses the production of arachidonic acid, may also play a role in regulating eosinophil activation. Inhibition of PLA_2 by mepacrine was found to inhibit fMLP-induced O_2^-, LTC_4 and EPO release, whilst addition of exogenous arachidonic acid reversed this effect (White *et al.,* 1993).

Recently, there has been some interest in the role of phosphatidylinositol 3-

kinase (PI 3-kinase) during activation of the neutrophil respiratory burst response. Activation of PI 3-kinase, via tyrosine kinase-linked growth factor receptors, for mitogenic signals has been well documented (Cantley *et al.*, 1991). A recent report by Vlahos *et al.* (1995) found that inhibition of PI 3-kinase blocked fMLP, but not PMA-induced NADPH oxidase activity in human neutrophils.

There is also evidence that Ca^{2+} ions play an important role in regulating cell activity in human eosinophils. A variety of stimuli have been shown to elevate cytosolic Ca^{2+} levels in human eosinophils (Raible *et al.*, 1992). PAF and fMLP stimulate both release of Ca^{2+} from intracellular stores, as well as Ca^{2+} influx (Sedgwick *et al.*, 1992; Zoratti *et al.*, 1991). Kernen *et al.* (1991) demonstrated that the chemotactic peptide C5a is a potent Ca^{2+} mobilising stimulus and that the release of eosinophil peroxidase is a Ca^{2+}-dependent process. C5a- and PAF-induced $[Ca^{2+}]_i$ changes are inhibited by activation of PKC (using PMA) and by pertussis toxin (PTX) pretreatment (Kernen *et al.*, 1991).

It is clear that different responses involve different signal transduction pathways and that more than one pathway may be used for a particular response in human eosinophils. Moreover, different agonists appear to be able to mediate the same response via different second messenger systems (e.g. PMA- and IgG-induced respiratory burst).

1.8 Role of Ca^{2+} in cellular activation

An important determinant of cellular activation is the concentration of cytosolic

free Ca^{2+} ($[Ca^{2+}]_i$). Ca^{2+} plays a pivotal role in the excitation-contraction coupling of smooth muscle cells (Bolton, 1979; Rodger, 1987), as well as in stimulus-response coupling in inflammatory cells (Lew, 1989). However, in eosinophils very few studies have investigated the role of Ca^{2+}, and the relative contribution of release of Ca^{2+} from intracellular stores and Ca^{2+} influx.

The level of $[Ca^{2+}]_i$ in most unstimulated cells is approximately 100nM; this is some 10,000 fold lower than the extracellular Ca^{2+} concentration (Lew, 1989). This difference in Ca^{2+} concentration is maintained by a combination of homeostatic mechanisms, involving Na^+/Ca^{2+} exchange (Blaustein and Nelson, 1982), Ca^{2+} efflux via an ATP-dependent Ca^{2+} pump in the plasma membrane (Rickard and Sheterline, 1985) and sequestration of Ca^{2+} into intracellular stores via Ca^{2+}/Mg^{2+}-ATPase on endoplasmic membranes (Thastrup, 1990).

The source of elevation of $[Ca^{2+}]_i$ during cell activation may come from intracellular Ca^{2+} stores and/or influx of extracellular Ca^{2+} across the plasma membrane. Non-excitable cells, such as neutrophils, respond to agonist stimulation with a biphasic elevation in $[Ca^{2+}]_i$ comprising release of Ca^{2+} from intracellular Ca^{2+} stores, followed by the influx of extracellular Ca^{2+} into the cytosol (Montero *et al.,* 1991).

1.9 Types of intracellular Ca^{2+} store

Receptor stimulation leads to PLC-mediated generation of IP_3, which in turn activates Ca^{2+} release channels located within the membrane of intracellular Ca^{2+} stores (Krause *et al.,* 1989). The intracellular storage of Ca^{2+} in human

neutrophils is believed to be in specialised organelles, termed calciosomes, which contain the Ca^{2+}-binding protein calreticulin (Krause *et al.*, 1990). Virtually all non-muscle cells appear to possess intracellular stores with an IP_3-gated Ca^{2+} release channel (Berridge and Irvine, 1989; Burgess *et al.*, 1984; Satoh *et al.*, 1990), with at least 4 different IP_3 receptors sub-types so far identified (Berridge, 1993).

In addition to, or instead of IP_3 receptor-mediated intracellular Ca^{2+} release, many cell types possess ryanodine receptors. These receptors are able to bind the plant alkaloid ryanodine and were first described in the sarcoplasmic reticulum of skeletal muscle (RYR1) and cardiac muscle (RYR2) (Sorrentino and Volpe, 1993). Recently, a third ryanodine receptor has aroused a certain amount of interest, as it appears to be widely expressed and may be involved in calcium-induced Ca^{2+} release (CICR). CICR is thought to provide the positive feedback mechanism required to allow Ca^{2+} waves to propagate within the cell without fading. This process has been shown to occur in cardiac myocytes, mouse oocytes and pancreatic acinar cells (Taylor, 1994). A metabolite of NAD, cADP ribose has recently been found to act on certain types of ryanodine receptors to cause mobilisation of intracellular Ca^{2+} stores and may represent an endogenous regulator of these channels. cADP ribose has been found in several cell types, including sea urchin eggs (where its formation is stimulated by cGMP-dependent protein kinase), pancreatic acinar and β cells, as well as dorsal root ganglion cells (Taylor, 1994).

1.10 Ca^{2+} influx pathways

Ca^{2+} influx pathways may be divided into 2 major groups, according to their mechanism of activation (figure 4): voltage operated Ca^{2+} channels (VOCs) and receptor-mediated Ca^{2+} entry (RMCE).

Excitable cells (e.g. nerves and smooth muscle cells) mediate Ca^{2+} influx across the plasma membrane, via VOCs, as a result of membrane depolarisation. VOCs can be divided into 4 major subtypes, based on their electrophysiological properties, namely L-, T-, N- and P-type Ca^{2+} channels (Meldolesi and Pozzan, 1987). Clinically, VOC-blockers are useful therapeutic agents. Potent dihydropyridine inhibitors of L-type Ca^{2+} channels (e.g. nifedipine) have proved to be effective in treating cardiovascular disorders, such as hypertension. In addition to their activation by plasma membrane depolarisation, VOCs may be regulated by G proteins, protein kinases and potentially by other second messengers (Dolphin, 1990; Naccache *et al.,* 1990).

In contrast to excitable cells, non-excitable cells appear to use RMCE pathways as an important and abundant mechanism for Ca^{2+} entry (Meldolesi *et al.,* 1991). Guinea-pig eosinophils are reported to activate RMCE pathway(s) when stimulated by LTB_4 or PAF, as the Ca^{2+} responses are inhibited by EGTA and Ni^{2+}, but not by classical dihydropyridine VOC-blockers (Subramanian, 1992; Kroegel *et al.,* 1989a).

Benham *et al.* (1989) suggested an operational definition of RMCE as "any influx of Ca^{2+}, consequent on receptor occupation and not dependent on

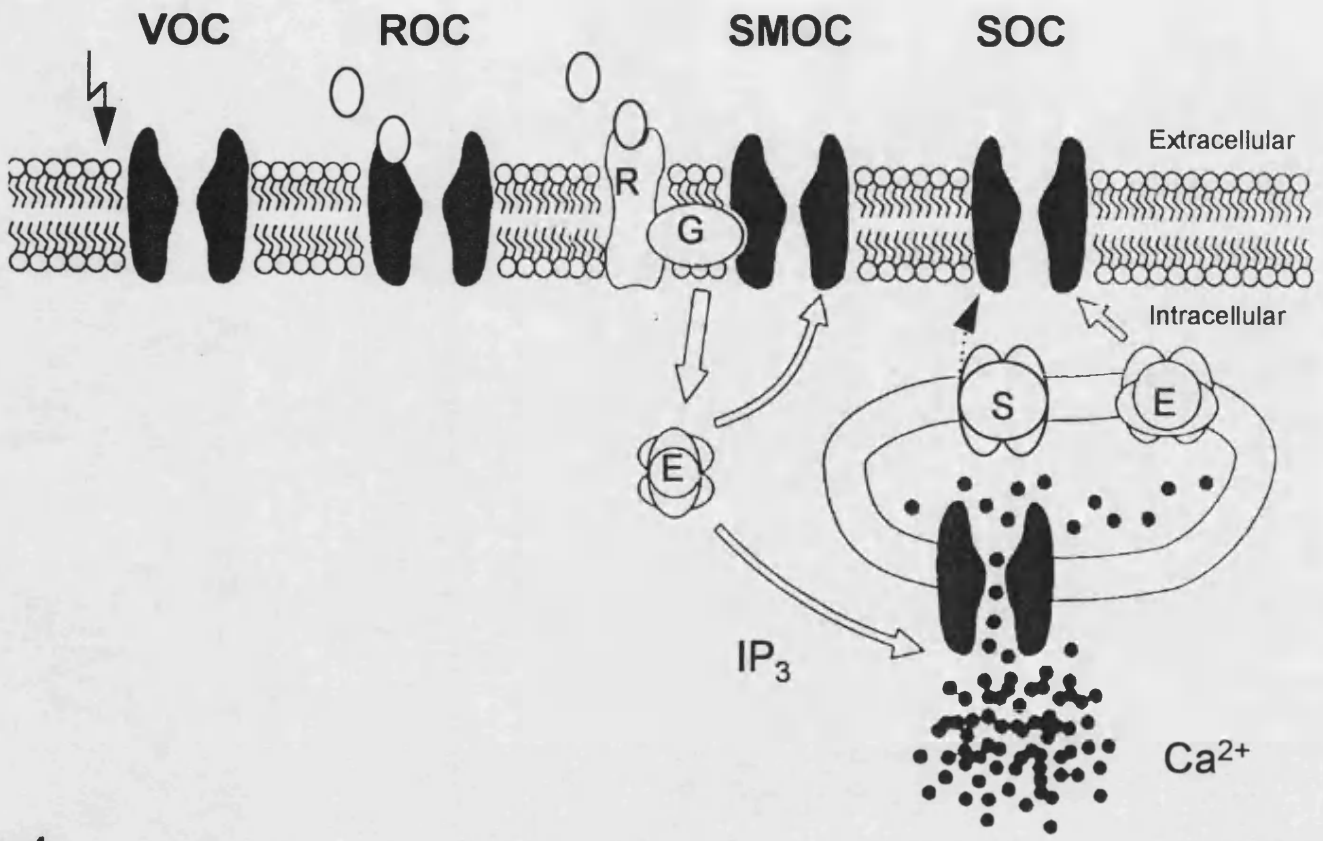
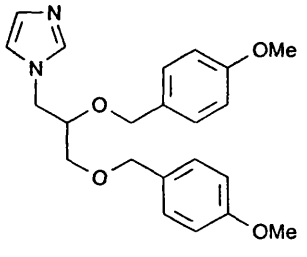


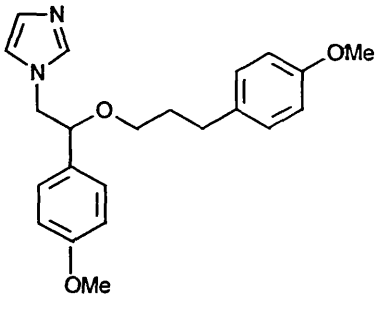
Figure 4.

Schematic representation of different types of Ca^{2+} channel, classified by their different routes of activation : VOCS (membrane depolarisation), ROCs (direct ligand interaction), SMOCs (generation of second messengers by effector enzymes (E)) and SOCs (depletion of intracellular Ca^{2+} stores, detected by a putative Ca^{2+} sensor (S)). Adapted from Penner et al., 1993.

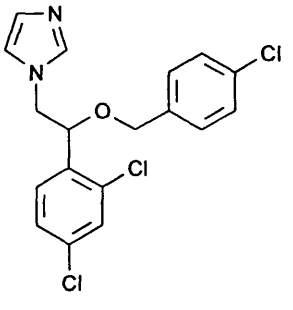
depolarisation, that generates a biologically significant, localised or general increase in $[Ca^{2+}]_i$". Currently, there are no potent and selective inhibitors of receptor-mediated Ca^{2+} influx available. The best available tools for the study of this influx pathway are inorganic cations (e.g. Ni^{2+}, La^{3+} and Cd^{2+}) and several imidazole compounds, although all of these agents are also reported to inhibit VOC-mediated Ca^{2+} influx. In this study, Ni^{2+} and the imidazoles, SK&F 96365 and econazole were used to investigate RMCE in human eosinophils. The structures of the imidazole RMCE inhibitors SC38249, SK&F 96365 and econazole are shown below:



SC38249



SK&F 96365



Econazole

Ni^{2+} has been shown to inhibit RMCE pathways, when used at relatively high (mM) concentrations, in HL-60 cells (Demaurex et al., 1992). The first imidazole reported to inhibit RMCE in platelets, smooth muscle cells and PC12 cells was SC 38249, but it was also found to inhibit Ca^{2+} efflux (Ciardo and Meldolesi, 1990). Subsequently, SK&F 96365 (1-β-[3-(*p*-methoxyphenyl)-propyloxy]-*p*-methoxyphenethyl-1H-imidazole hydrochloride), which blocked L-type VOCs in smooth muscle cells, as well as RMCE in a range of non-excitable cells was

identified (Merritt *et al.,* 1990). Both SK&F 96365 and an imidazole analogue, econazole, have been reported to inhibit RMCE in non-excitable cells, such as platelets, neutrophils, and HL-60 cells (Merritt *et al.*, 1990; Montero *et al.*, 1991; Alonso-Torre *et al.*, 1993).

Activation of RMCE has been proposed to occur via several different mechanisms (Meldolesi and Pozzan, 1987; Meldolesi *et al.,* 1991). Receptors may be directly coupled to the channel or linked via a G protein. This type of pathway is often termed receptor-operated Ca^{2+} (ROC) influx and requires receptor occupancy for activation (Fasolato *et al.,* 1994).

Alternatively influx may occur as a consequence of receptor occupation leading to the generation of a second messenger (second messenger operated Ca^{2+} (SMOC) influx) (Meldolesi and Pozzan, 1987). The final type of RMCE pathway is activated as a result of emptying of intracellular Ca^{2+} stores and is termed 'capacitative' Ca^{2+} influx (Putney, 1986; Putney, 1990) or store-operated Ca^{2+} (SOC) influx. The original model of capacitative Ca^{2+} entry proposed that refilling of intracellular Ca^{2+} stores during cell activation occured via a direct influx of extracellular Ca^{2+} into intracellular Ca^{2+} stores (Putney, 1986). Subsequent adaptation of this model suggested that extracellular Ca^{2+} enters into the cytosol of the cell, rather than directly into the Ca^{2+} storage organelles (Putney, 1990; Takemura *et al.,* 1989). The discovery of highly selective inhibitors of the endoplasmic reticulum Ca^{2+}-ATPase has contributed greatly to our understanding of the regulation of capacitative Ca^{2+} entry (Thastrup, 1990; Thastrup *et al.,* 1989). Inhibition of the endoplasmic reticular Ca^{2+} pump, by

agents such as thapsigargin and cyclopiazonic acid, causes emptying of intracellular stores without the generation of any known second messengers. The depletion of the intracellular Ca^{2+} stores stimulates a persistent elevation of $[Ca^{2+}]_i$ which is dependent upon the presence of extracellular Ca^{2+} (Thastrup *et al.*, 1990).

The first demonstration of a highly Ca^{2+}-selective current which was activated by store depletion was found in mast cells, by Hoth and Penner (1992) and was termed I_{CRAC} (Ca^{2+} release-activated Ca^{2+} current). The principle characteristics of this current are that it is activated by any mechanism that depletes intracellular Ca^{2+} stores, it has high selectivity for Ca^{2+} over K^+ or Na^+ (>1000 fold), a very low unitary conductance (<100 femtosiemens) and is voltage independent. This store depletion-activated Ca^{2+} influx pathway is present in many different cell types, including neutrophils (Montero *et al.*, 1991; Demaurex *et al.*, 1994), mast cells (Hoth and Penner, 1992), T cells (McDonald *et al.*, 1993; Premack *et al.*, 1994), endothelial cells (Hallam *et al.*, 1989) and oocytes (Snyder *et al.*, 1988) and appears to be an important and abundant mechanism for the regulation of $[Ca^{2+}]_i$ during cell activation.

1.11 Techniques for measuring $[Ca^{2+}]_i$

Detection of changes in $[Ca^{2+}]_i$ in cell populations has generally been made using either Ca^{2+}-sensitive fluorescent dyes or measurement of radiotracer Ca^{2+} fluxes.

The radioisotope $^{45}Ca^{2+}$ has been widely used to study the transport and

distribution of Ca^{2+} in both stimulated and unstimulated cells. This technique has been used successfully to detect receptor-mediated Ca^{2+} influx, yielding comparable results to those using fluorescent dyes. However, the usefulness of this technique is limited by the high rate of $^{45}Ca^{2+}$ uptake seen in unstimulated cells (Schilling *et al.,* 1989).

The advent of Ca^{2+}-sensitive fluorescent dyes, such as quin-2 and fura-2, has allowed direct real-time measurement of changes in $[Ca^{2+}]_i$. The esterified form (acetoxymethyl ester) of the indicator, which is uncharged and hydrophobic, is able to readily cross cell membranes. Once inside the cell cytosol, endogenous esterases cleave the acetoxymethyl ester from the dye, releasing the free acid form. The free acid is unable to cross cell membranes and is therefore trapped inside the cell. Once extracellular dye has been removed, fluorescence signals from the cytosolic dye can be used to measure $[Ca^{2+}]_i$.

The original fluorescent Ca^{2+}-indicator quin-2 was developed by Tsien (1980). It binds to Ca^{2+} (Kd=115nM) and undergoes a rapid 5-6 fold increase in fluorescent intensity. The subsequent improved dye, fura-2, also binds Ca^{2+} (Kd=224nM), but is 30 times more fluorescent than quin-2. Additionally, fura-2 is less sensitive to Mg^{2+} and less susceptible to photobleaching than quin-2. Furthermore, fura-2 exhibits a shift in excitation maximum, to a lower wavelength on Ca^{2+} binding, with little shift in the emission maximum. This has the advantage of allowing fura-2 to be used as a dual excitation indicator, with Ca^{2+}-free fura-2 measured at 380nm and Ca^{2+}-bound fura-2 measured at 340nm. The high level of quin-2 loading required in order to obtain a measurable

fluorescence signal causes problems with buffering of $[Ca^{2+}]_i$. The improvement in the fluorescent properties of fura-2 has the advantage that lower levels of fura-2 are loaded into the cell and effectively overcomes this problem (Grynkiewicz *et al.,* 1985). The only potential disadvantages associated with fura-2 relate to incomplete hydrolysis of the acetoxymethyl ester and its tendency to become compartmentalised in subcellular organelles (Thomas and Delaville, 1991).

AIMS AND OBJECTIVES

The aim of this study was to investigate the role of Ca^{2+} during agonist-induced activation of human eosinophils. A pharmacological approach was used to characterise the type of Ca^{2+} influx and signal transduction pathways used during C5a-, fMLP-, thapsigargin- and LTD_4-induced eosinophil activation. Measurement of activation was made by assaying for O_2^-, LTC_4 and ECP release, which represent respiratory burst, 5-LOX-mediated metabolism of arachidonic acid and degranulation responses, respectively. A comparison was made of the effect of agents which modulate $[Ca^{2+}]_i$, with the effects observed on the different functional responses.

2. MATERIALS AND METHODS

2.1 Purification of human eosinophils.

Human eosinophils were prepared essentially as previously described by Hansel *et al.* (1991). Human venous blood (50-100ml), from normal healthy donors, was taken into anti-coagulant (3.2% w/v trisodium citrate), diluted with an equal volume of Hepes-buffered, Hanks' balanced salts solution (HBSS), without Ca^{2+} or Mg^{2+} and overlaid on an isotonic Percoll solution (1.082 g/ml). After centrifugation (1000g, 20 min, 20°C), the supernatant and mononuclear cells at the Percoll interface were removed and discarded. The remaining granulocytes and erythrocytes were transferred to fresh tubes, to avoid contamination with mononuclear cells adhered to the sides of the tubes. Erythrocyte removal was carried out by either ammonium chloride or hypotonic lysis methods. The first method involved mixing the cells with ice-cold ammonium chloride lysis buffer (Hartnell *et al.,* 1990) and placing them on ice for 15 minutes. Hypotonic lysis required the cells to be mixed with 20ml of ice-cold double-deionised tissue culture water for 30 seconds, followed by addition of 20ml of 2x concentration HBSS; this process was repeated 3-4 times until all the red blood cells had been removed. After erythrocyte lysis, granulocytes were washed in RPMI 1640 medium, containing 5mM EDTA and 2% foetal calf serum (RPMI-FCS). The supernatant was removed and the cell pellet was resuspended with anti-CD16-conjugated micromagnetic beads (Miltenyi Biotec GmbH; 10μl beads per 2.5 x 10^7 granulocytes) diluted 1:4 in RPMI-FCS. The cells were incubated for 40 minutes at 4°C, with occasional gentle mixing and then loaded onto a magnetic

column (Becton-Dickinson) containing RPMI-FCS. Immunomagnetically labelled neutrophils were retained in the column, whereas the eosinophils were eluted at a flow rate of 1-1.5 ml per minute. The purified eosinophils were washed and resuspended in HBSS without Ca^{2+} or Mg^{2+}. Differential cell counts were performed, using Kimura stain (Kimura *et al.,* 1973), on whole blood, the granulocyte mixture after erythrocyte lysis and the purified eosinophil fraction. High yield (>95%) and purity (95% eosinophils) were obtained using this technique.

2.2 IP_3 assay.

Human eosinophils used for measuring IP_3 generation were prepared either from human venous blood from normal healthy donors, or from buffy coat preparations supplied by the Blood Transfusion Service. It was necessary to use the buffy coat preparations because of the very high number of cells required for this assay. However, comparable results were obtained using cells from either of these sources.

Purified human eosinophils (2-3 x 10^6 cells in a total reaction volume of 100μl) were resuspended in HBSS with Ca^{2+} and Mg^{2+} and pre-warmed at 37°C for 5 minutes Antagonist/vehicle was added 5 minutes prior to the addition of agonist/vehicle and the reaction was stopped by the addition of 20μl of ice-cold 20% perchloric acid. The cells were kept on ice for 20 minutes, then centrifuged (2000g, 15 minutes, 4°C) and the supernatants transferred to fresh tubes. It was necessary to use acidic conditions when extracting IP_3 from cells, to stop the association of IP_3 with proteins. The supernatants were neutralised by adding

1.5M KOH containing 60mM HEPES buffer and universal indicator, essentially as described by Palmer *et al.* (1986).

Measurement of IP_3 levels was made using the Amesham D-*myo*-Inositol 1,4,5-trisphosphate [^{3}H] assay system (TRK 1000). This assay involves competition between unlabelled IP_3 and a fixed amount of ^{3}H-IP_3 for binding sites on a bovine adrenal binding protein preparation. The tubes contained 100μl of each of the following : assay buffer (0.1M tris buffer, pH 9.0, with 4mM EDTA and 4mg/ml BSA), sample or IP_3 standard (0.19-25 pmole per tube), ^{3}H-IP_3 and binding protein. The tubes were vortex mixed, incubated for 15 minutes on ice, then centrifuged (2000g, 15 minutes, 4 °C). The supernatants were removed and discarded and the tubes carefully wiped with cotton buds to remove any adhering droplets of liquid. The pellets were resuspended in 1ml of 0.15M NaOH, incubated at room temperature for 10 minutes and 10ml of Optiphase scintillant added to each sample in scintillation vials. Counts were measured using an LKB β-scintillation counter and IP_3 levels were calculated from standard curve readings using GraphPad software.

2.3 Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$).

Human eosinophils (2 x 10^6 cells/ml) were loaded with the fluorescent Ca^{2+}-indicating dye, fura-2 acetoxymethyl ester (fura-2/AM) (0.5μM, 30 minutes, 20°C). Fura-2/AM is a cell permeant dye which becomes trapped inside cells due to esterase cleavage of the acetoxymethyl ester groups, resulting in the generation of membrane impermeant fura-2 acid. The cells were washed twice and resuspended (10^6 cells/ml) in Hepes-buffered Tyrodes (HBT) solution,

containing 0.1% BSA. Fluorescence measurements were made using a spectrofluorimeter (Biomedical Instruments Group, Univ. of PA or LS50, Perkin Elmer fluorimeter) using excitation wavelengths of 340nm and 380nm, an emission wavelength of 510nm and a slit width of 10nm. $[Ca^{2+}]_i$ was calculated from the ratio of fluorescence, using the equation described by Grynkiewicz *et al.* (1985):

$$[Ca^{2+}] = Kd \times \frac{(R - R_{min})}{(R_{max} - R)} \times \frac{S_{f2}}{S_{b2}}$$

The Kd for fura-2 is 224nM, R_{max} and R_{min} represent the fluorescence ratio values at saturating and Ca^{2+}-free conditions, respectively and S_{f2}/S_{b2} represents to fluorescence ratio of Ca^{2+}-free/ Ca^{2+}-bound fura-2 at 380nm. R_{max} was obtained by lysing the cells, using digitonin in the presence of 1mM $CaCl_2$ and R_{min} was acheived by subsequent addition of 10mM EGTA solution containing 0.1mM KOH. KOH was present in the EGTA solution because elevation of the final pH>8 increases the affinity of EGTA for Ca^{2+} (Thomas and Delaville, 1991).

2.4 Mn^{2+} influx assay.

Mn^{2+} influx studies were carried out using the isosbestic (Ca^{2+} insensitive) excitation wavelength of 360 nm, an emission wavelength of 510nm and a slit width of 10nm. Mn^{2+} has a 50 fold higher affinity for fura-2 than Ca^{2+} (Grynkiewicz *et al.,* 1985) and once bound quenches its fluorescence. The rate of Mn^{2+} influx was calculated as follows :

$$\Delta Mn^{2+} = F_0 - F_{40} + F_e$$

F_0 = fluorescent intensity 0 seconds after Mn^{2+} addition.

F_{40} = fluorescent intensity 40 seconds after Mn^{2+} addition.

F_e = Mn^{2+} quench of extracellular fura-2.

This represents the degree of quench of fura-2 fluorescence that occurred over a period of 40 seconds (essentially as previously described by Demaurex *et al.* (1994)), as the rate of quench is relatively linear during this time. Corrections for Mn^{2+} quench of leaked fura-2, were made by adding the membrane impermeant heavy metal chelator DTPA (2mM) at the end of each run. Any increase in fluorescence represented 'unquenching' of extracellular dye and this was subtracted from the agonist-induced quench effects (figure 5).

2.5 Measurement of superoxide production.

Superoxide (O_2^-) production was assayed by measuring the superoxide dismutase-inhibitable reduction of ferricytochrome C, via absorbance readings made at 550nm (Pick and Mizel, 1981), using a THERMOmax microtitre plate (MTP) reader (Molecular Devices, Palo Alto, CA). Each well contained eosinophils (2.5 x 10^5 cells/ml), cytochrome C (110µM), HBSS with Ca^{2+} (1.3mM) and cytochalasin B (5.5µM), in a total reaction volume of 250µl. Kinetic readings of O_2^- production were measured at 37°C, over a 30 minute period at 9 second intervals, with plate agitation between readings. The first 10 minutes of the reaction were used to calculate O_2^- generation as the rate of release was relatively linear during this time. O_2^- release was expressed as nmol of cytochrome C reduced/2.5 x 10^5 cells/10 minutes and was converted

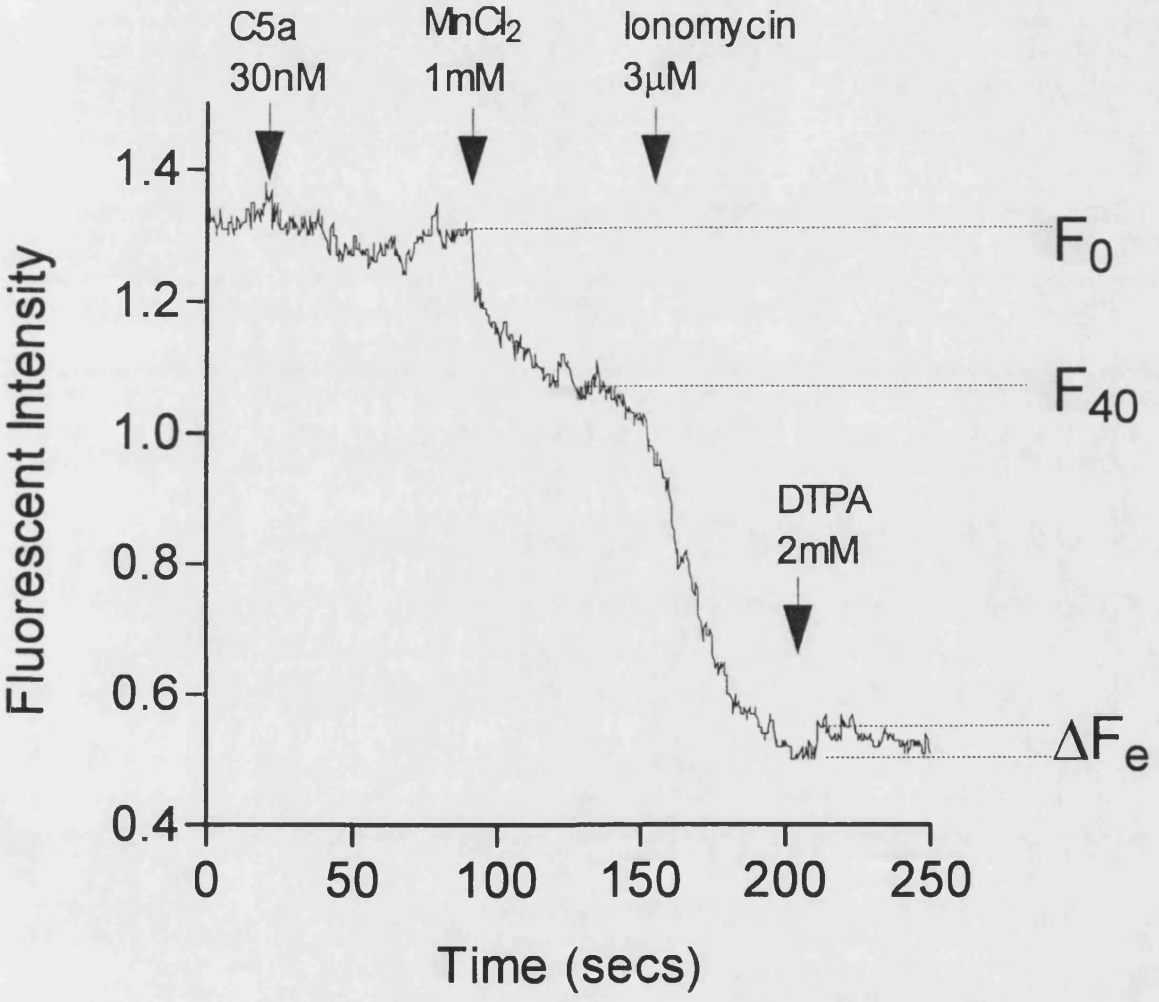


Figure 5

Example trace of agonist-induced Mn^{2+} influx. Agonist (e.g. C5a) was added 60 seconds prior to the addition of $MnCl_2$ (1mM). Ionomycin (3μM) was added 60 seconds after $MnCl_2$, to give maximal quench of fura-2 fluorescence. Subsequent addition of DTPA (2mM) caused a small increase in fura-2 fluorescence due to 'unquenching' of extracellular fura-2. Calculation of the rate of Mn^{2+} influx was calculated by measuring the extent of Mn^{2+} quench of fura-2 in 40 seconds (F_0 - F_{40}), with quench due to extracellular dye subtracted (ΔF_e).

from absorbance values using the following equation :

$$\Delta A = ECL$$

ΔA = change in absorbance (OD).

E= extinction coefficient for reduced cytochrome C at 550nm (21×10^3 cm^{-1} (Massey, 1959)).

C = concentration of cytochrome C (cyt C) reduced (moles/litre).

L=light path (0.78cm)

$$\Delta A = (21x10^3) \times mol/litre \times 0.78$$

$$\Delta A = (21x10^3) \times nmoles/nlitres \times 0.78$$

$$nmoles = (\Delta A \times nlitres)/(21x10^3 \times 0.78)$$

$$Volume = 250\mu l \ (= 2.5x10^5 \ nl)$$

$$nmoles = (\Delta A \times 2.5x10^5)/(21x10^3 \times 0.78)$$

$$nmoles \ cyt \ C \ reduced = \Delta A \times 15.26$$

ΔA measured as mOD per minute

$$nmoles \ cyt \ C \ reduced \ per \ minute = \Delta A \times 15.26 \times 10^{-3}$$

Values were adjusted to represent 1ml reaction volume (=$2.5x10^5$ cells) and 10 minute duration :

$$nmoles \ cyt \ C \ reduced/10 \ mins/2.5x10^5 \ cells = \Delta A \times 15.26 \times 10^{-3} \times 10 \times 4$$

$nmoles \ cyt \ C \ reduced/10 \ minutes/2.5x10^5 \ cells = 0.61 \times mOD/min$

Immediately after the assay the plates were centrifuged, the supernatants removed and transferred to fresh plates, covered with cling film and frozen

(-20°C) until it was convenient for an LTC_4 assay to be performed on the cell supernatants.

2.6 Measurement of LTC_4 release.

Previously frozen MTPs were removed from the freezer and allowed to thaw at room temperature. The cell supernatants were then assayed for the presence of LTC_4 by radioimmunoassay, essentially as described in the Amersham kit (TRK 910). The assay involves competition between 'cold' LTC_4 and a fixed amount of ^{3}H-LTC_4 for a limited amount of rabbit anti-human LTC_4 antibody. The antibody used also cross-reacts with LTD_4 (100%) and LTE_4 (30%). The cell supernatants or LTC_4 standards (12.5 - 800pg LTC_4 per tube) were mixed with equal amounts of ^{3}H-LTC_4 and a rabbit anti-human LTC_4 antibody and incubated for 1 hour, at 37°C in a shaking water bath. In early experiments the bound and unbound ^{3}H-LTC_4 were separated by addition of an activated-charcoal/dextran mixture and kept on ice for 10 minutes, followed by centrifugation (2000g, 15 minutes, 4 °C). The supernatants were decanted into scintillation vials, 10 ml optiphase scintillant added and counts were performed on an LKB β scintillation counter. In later experiments the assay was simplified by the availability of Scintillation Proximity Assay (SPA) reagents which removed the necessity to separate bound and unbound ^{3}H-LTC_4, as well as the need to add scintillant, this enabled the assay to be performed in 96 well plates. SPA reagent contains fluomicrospheres bound to a second antibody. Any radiolabelled-ligand bound to primary antibody will bind to the secondary antibody on the fluomicrospheres and will cause emission of light. Generic anti-rabbit IgG SPA beads were added

to the supernatant/^{3}H-LTC_4/anti-human LTC_4 antibody mixture, the plates were then shaken overnight on an orbital shaker and counts were performed using a Packard topcounter. LTC_4 levels were calculated from standard curve readings using GraphPad or Prism software. The $\%B/B_0$ standard curves obtained using either RIA or SPA techniques were the same (figure 6A).

2.7 Measurement of Eosinophil Cationic Protein (ECP) release.

Eosinophils were incubated in a plate shaker for 5 minutes at 37°C in a 96 well MTP, prior to the addition of antagonist/vehicle. Each well contained eosinophils (1.2×10^5 cells/ml), HBSS with Ca^{2+} (1.3mM) and cytochalasin B (5.5µM), in a total reaction volume of 250µl. The plate was incubated for a further 5 minutes prior to the addition of agonist/vehicle and the cells returned to the incubated plate shaker for 30 minutes. The MTP was centrifuged (350g, 5 mins, 4°C) and the supernatants transferred to a fresh MTP and frozen at -20°C, until it was convenient to perform the ECP radioimmunoassay. Frozen supernatants were thawed at room temperature and ECP levels were measured using a Pharmacia kit. Cell supernatants were mixed with equal amounts of ^{125}I-ECP and rabbit anti-human ECP antibody and shaken for 3 hours at room temperature. At this stage the method was altered from that described in the kit. Anti-rabbit SPA beads were added instead of the supplied decanting suspension of sepharose anti-rabbit IgG, which requires separation of bound and unbound ^{125}I-ECP and addition of scintillant. The plate was shaken overnight on an orbital shaker, then counted on a Packard Topcounter. ECP values were calculated from a standard curve using Prism software. The $\%B/B_0$ standard curves obtained using either

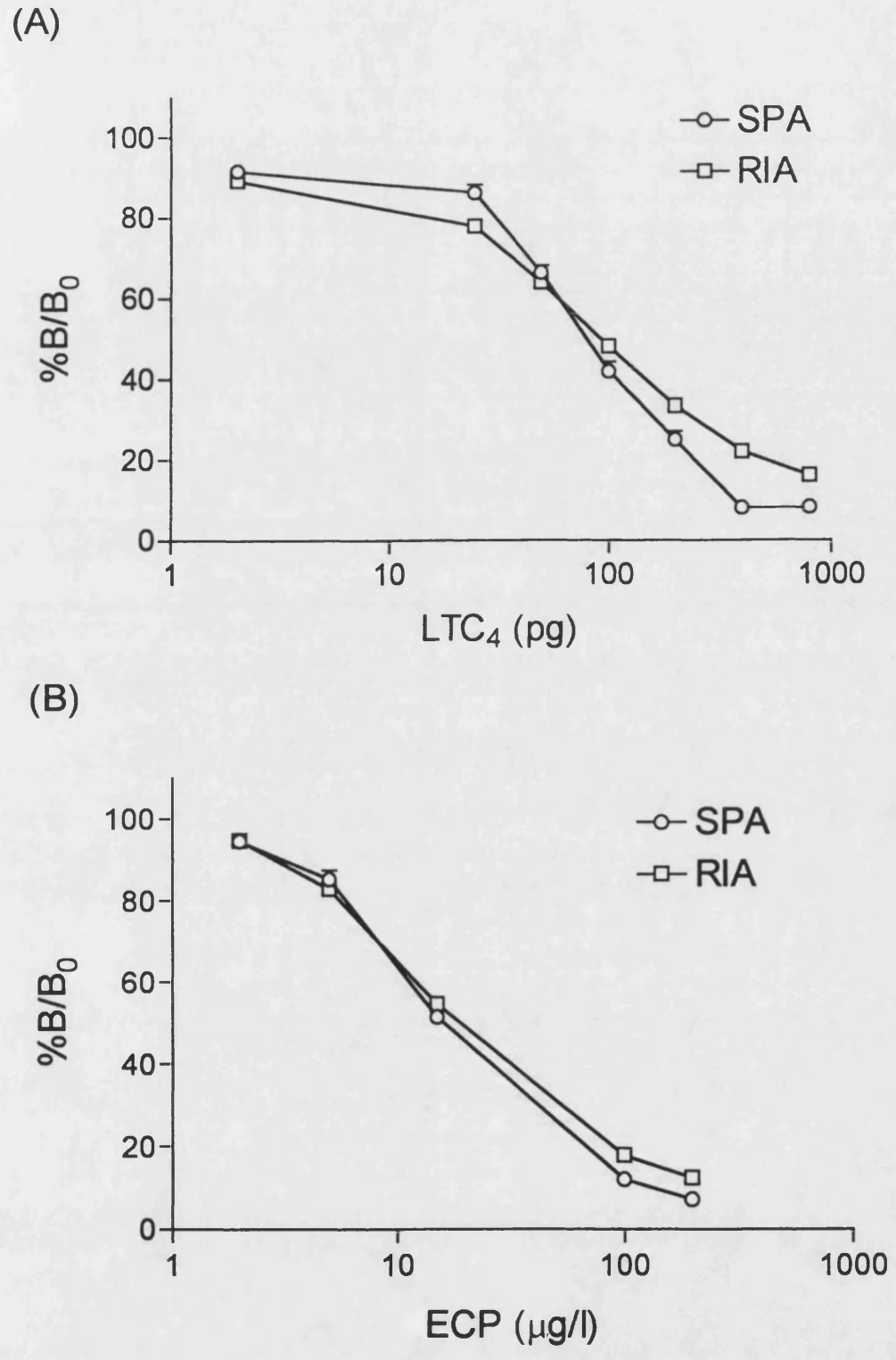


Figure 6

(A) Comparison of standard curves to LTC_4 and (B) ECP, when measured either by RIA or SPA techniques. Data represent mean ± standard deviation of duplicate standard solutions.

RIA (using the example values given in the kit instructions) or SPA techniques were the same (figure 6B).

2.8 Analysis of Data.

Results are expressed as either mean ± standard error of the mean (s.e.m) or mean ± 95% confidence intervals. Mean EC_{50} or IC_{50} values were calculated by linear regression analysis. Statistical significance was determined using Students' paired t-test on raw data, with $p<0.05$ taken as significant. Throughout this thesis * denotes $p<0.05$ and ** denotes $p<0.005$. P values reported are for comparisons of individual groups with control group. Since multiple comparisons were made with a single control group, type I error rates are likely to exceed those reported.

2.9 Materials.

Materials were obtained from the following sources:

Affiniti (formerly Biomol; Exeter, UK) : R59022, rolipram, U73122 and U73343.

Bayer : SK&F 96365 was synthesised 'in house' by Dr S Tudhope (Bayer PLC, Stoke Court, UK). BAY x1005 and WEB 2086 were supplied by the Chemistry department, (Bayer AG, Wuppertal, Germany) and cyclosporin A was supplied by Bayer Inc. (Westhaven, USA).

BDH Lab Supplies (Poole, UK) : $CaCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, KCl, NaCl and Na_2HPO_4.

Calbiochem (Nottingham,UK) : bisindolymaleimide (GF109203X; 3-[1-(3-dimethylaminopropyl)-indol-3-yl]-3-(indol-3-yl)-maleimide), calyculin A, erbstatin analogue (methyl 2,5 dihydroxcinnamate), fura-2/AM, manoalide, SK&F 525A and wortmannin.

Eurogenetics (Middlesex, UK) : CD16 microbeads.

GIBCO (Life Technologies; Paisley, Scotland, UK) : RPMI 1640 medium (L-glutamine free).

ICN Flow (Thame, UK) : foetal calf serum (FCS).

Molecular Probes (Oregon, USA) : benzamil

Porton Products (Maidenhead, UK) : pertussis toxin

R & D Systems (Abingdon, UK) : anti-CD11b and anti CD18

Sera-Lab (Crawley Down, UK) : HBSS (without phenol red) and HBSS (without phenol red, Ca^{2+} or Mg^{2+}).

Sigma (Poole, UK) : A23187, low endotoxin (<0.1ng/mg) bovine serum albumin (BSA), human recombinant complement fragment 5a (C5a), cromakalim, cytochalasin B, diltiazem, diethylenetriaminepentaacetic acid (DTPA), econazole, ethylenediaminetetaacetic acid (EDTA), ethylene glycol-bis(β-aminoether) N,N,N,N'-tetraacetic acid (EGTA), ferricytochrome C, fMLP, forskolin, HBSS (10x) with phenol red, HBSS (10x) without phenol red, Hepes, indomethacin, isoprenaline, leukotriene D_4 (LTD_4), nifedipine, okadaic acid,

Percoll, phorbol myristate acetate (PMA), salbutamol, sodium cromoglycate, sodium fluoride (NaF), sodium nitroprusside, staurosporine, thapsigargin and verapamil.

UCB (Belgium) : Cetirizine.

2.10 Solutions.

Unless otherwise stated, drug solutions were dissolved in either double deionised tissue culture water or dimethyl sulphoxide (DMSO) and were diluted as appropriate. The final concentration of DMSO present in the cell suspensions was $\leq$ 0.3%.

C5a was dissolved in phosphate buffered saline (PBS) containing 0.1% BSA and was immediately aliquoted and frozen (-20°C). Aliquots were removed from the freezer just prior to an experiment and kept on ice.

Fura-2/AM (10^{-3}M) was dissolved in DMSO and immediately aliquoted and frozen (-20°C). Solutions were stored in the dark and tubes were covered with aluminium foil when cells were loaded with fura-2/AM in order to prevent photobleaching.

The composition of Hepes-buffered tyrodes (HBT) solution was as follows : NaCl (11.5mM), KCl (5mM), $MgCl_2$ (1mM), Na_2HPO_4 (0.5mM) and Hepes (10mM), glucose (5.5mM) and 0.1% BSA.

3. RESULTS

3. ACTIVATION OF HUMAN EOSINOPHILS

3.1 Spontaneous eosinophil activation

Initial attempts to measure agonist-induced O_2^- release were hampered by the ability of eosinophils, in the absence of exogenous agonist, to produce substantial quantities of O_2^- equivalent to 5.0 ± 0.4 nmoles cytochrome C reduced/ 10 mins/ $2.5x10^5$ eosinophils (n=20). Under these conditions, no further O_2^- release was detected upon addition of C5a or FMLP (data not shown). Therefore it was necessary to determine the source of this spontaneous activation and how it could be removed, in order to allow measurement of agonist-induced eosinophil O_2^- release. Since it had previously been shown by Dri *et al.* (1991) that O_2^- release could be stimulated by eosinophil contact with ELISA plate plastic, the possibility that the spontanous activity seen in this study was due to contact of eosinophils with the MTP plastic was investigated. Pre-treating the MTP with the matrix protein laminin (3µg/ml, 2 hours, 37°C) markedly inhibited (69.7 ± 6.3 %) O_2^- release.

It was also clear from the work of Sedgwick *et al.* (1993) that it was possible to measure O_2^- release using MTPs without any spontaneous activity. The major difference between their assay conditions and those being used in this study being the presence of gelatin (0.1%) in their buffer. Addition of gelatin (0.05%) to the HBSS did indeed inhibit the spontanous activity (96.1 ± 1.3 %).

Finally, addition of 5.5µM cytochalasin B, which blocks polymerisation of

monomeric actin and thus blocks adhesion of the cells to the plastic, abolished spontaneous O_2^- release (figure 7A). Cell morphology studies confirmed that cytochalasin B did inhibit the adherence of eosinophils to the surface of the MTP plastic (figure 8). However, this adhesion process did not appear to involve MAC-1 or any of the β2 integrins, as anti CD18 and anti CD11b antibodies had no effect on the spontaneous O_2^- response (figure 7B).

There did appear to be some very variable spontaneous LTC_4 release (240.8 ± 128.4 pg LTC_4/2.5x10^5 cells/30 mins, n=5) and addition of cytochalasin B did appear to reduce this (31.2 ± 28.0 pg LTC_4 / 2.5x10^5 cells / 30 mins, n=5), although it did not acheive statistical significance. Similarly, a basal level of spontaneous ECP release (27.1 ± 6.2 ng ECP / 1.2x10^4 cells / 30 mins, n=4) was detected, however this was not inhibited (7.5 ± 21.3 %) by cytochalasin B (5.5μM). Subsequent studies investigating agonist-induced eosinophil activation were carried out in the presence of cytochalasin B (5.5μM).

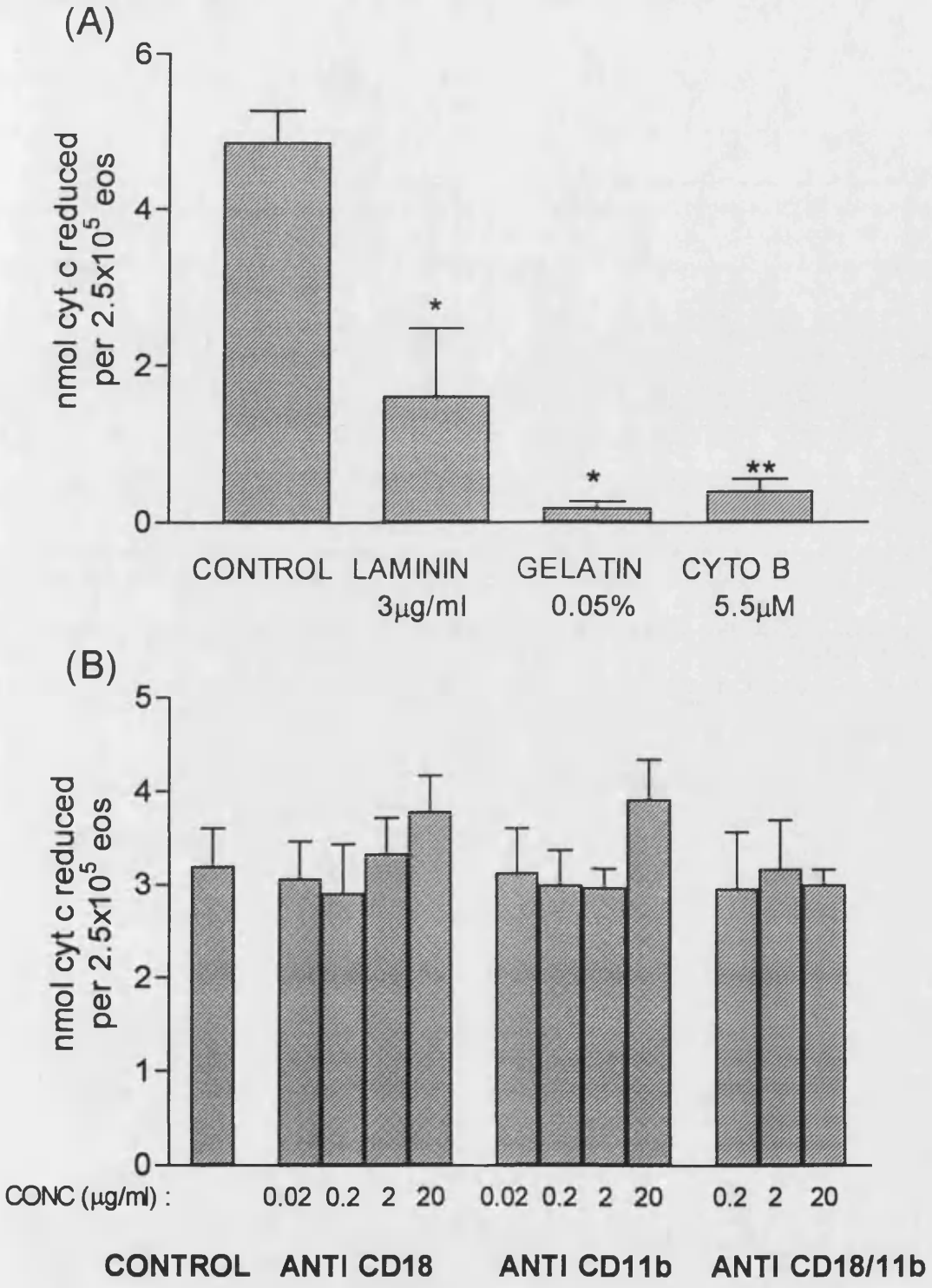
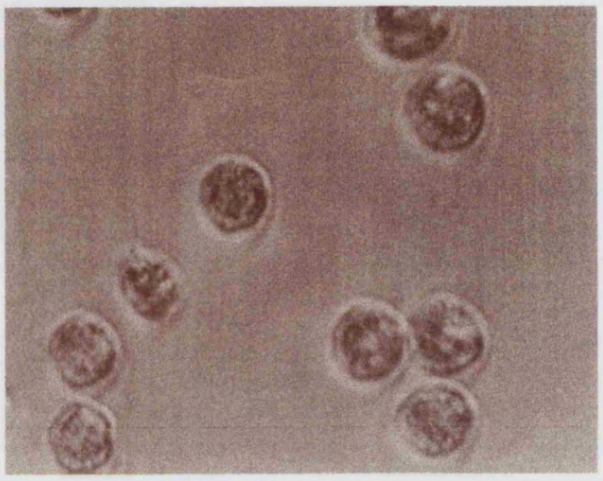
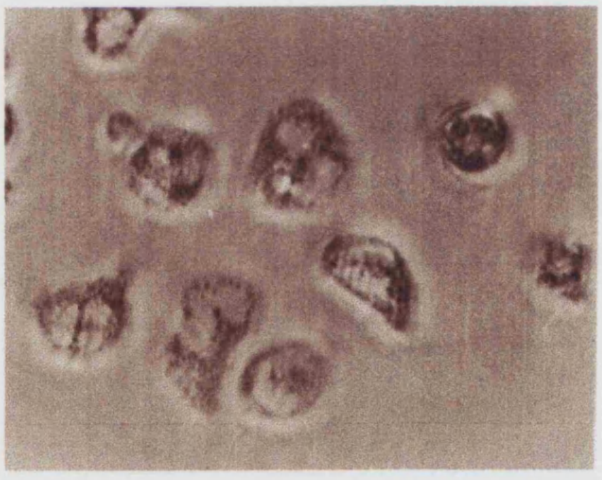


Figure 7

(A) Effect of pre-treatment of the microtitre plate (MTP) with laminin (3µg/ml, 2 hours, 37°C), or addition of gelatin (0.05%) or cytochalasin B (5.5µM) to the assay buffer (n=4-5 separate experiments) and (B) effect of anti CD18, anti CD11b (n=5) or a combination of anti CD18/CD11b (n=2) antibodies on spontaneous O_2^- release. * denotes $p<0.05$, ** denotes $p<0.005$.



+ cytochalasin B (5.5μM)



No cytochalasin B

Figure 8

Appearance of human eosinophils on the surface of a microtitre plate in the presence and absence of cytochalasin B (Original Magnification x900).

3.2 C5a-induced human eosinophil activation : effects on IP_3 generation, $[Ca^{2+}]_i$, Mn^{2+} influx, O_2^-, LTC_4 and ECP release.

C5a (0.03-30 nM) caused concentration-dependent increases in $[Ca^{2+}]_i$, (figure 9A) and rate of Mn^{2+} influx. $[Ca^{2+}]_i$, was elevated from a basal level of 74.9 ± 14.7 nM to a maximum of 538.5 ± 49.2 nM (n=4) by C5a (30nM). O_2^-, LTC_4 and ECP were released in response to C5a, being maximally stimulated at a concentration of 30 nM (figure 9B). The levels of O_2^-, LTC_4 and ECP released by C5a (30nM) were 11.6 ± 0.7 nmoles cytochrome C reduced/$2.5x10^5$ cells/10 mins, 985.5 ± 146.5 pg LTC_4/$2.5x10^5$ cells/30 mins and 121.9 ± 10.5 ng ECP/$1.2x10^4$ cells/30 mins, respectively. These levels of O_2^-, LTC_4 and ECP release are within the same range as the values obtained for the control groups reported in section 4. Mean EC_{50} values for C5a-induced eosinophil activation are shown below (table 1).

Table 1. Mean EC_{50} values for C5a-induced eosinophil activation

C5a-induced eosinophil activation	Mean EC_{50} (nM)	95% confidence limits
IP_3 release	16.2	3.9 - 66.6
$\Delta[Ca^{2+}]_i$	0.4	0.02 - 6.2
Mn^{2+} influx	0.1	0.02 - 0.5
O_2^- release	0.9	0.7 - 1.2
LTC_4 release	1.5	0.8 - 2.8
ECP release	2.6	1.3 - 5.1

Data represent mean of 4-7 separate experiments

C5a-induced increases in $[Ca^{2+}]_i$ and Mn^{2+} influx occur at lower concentrations

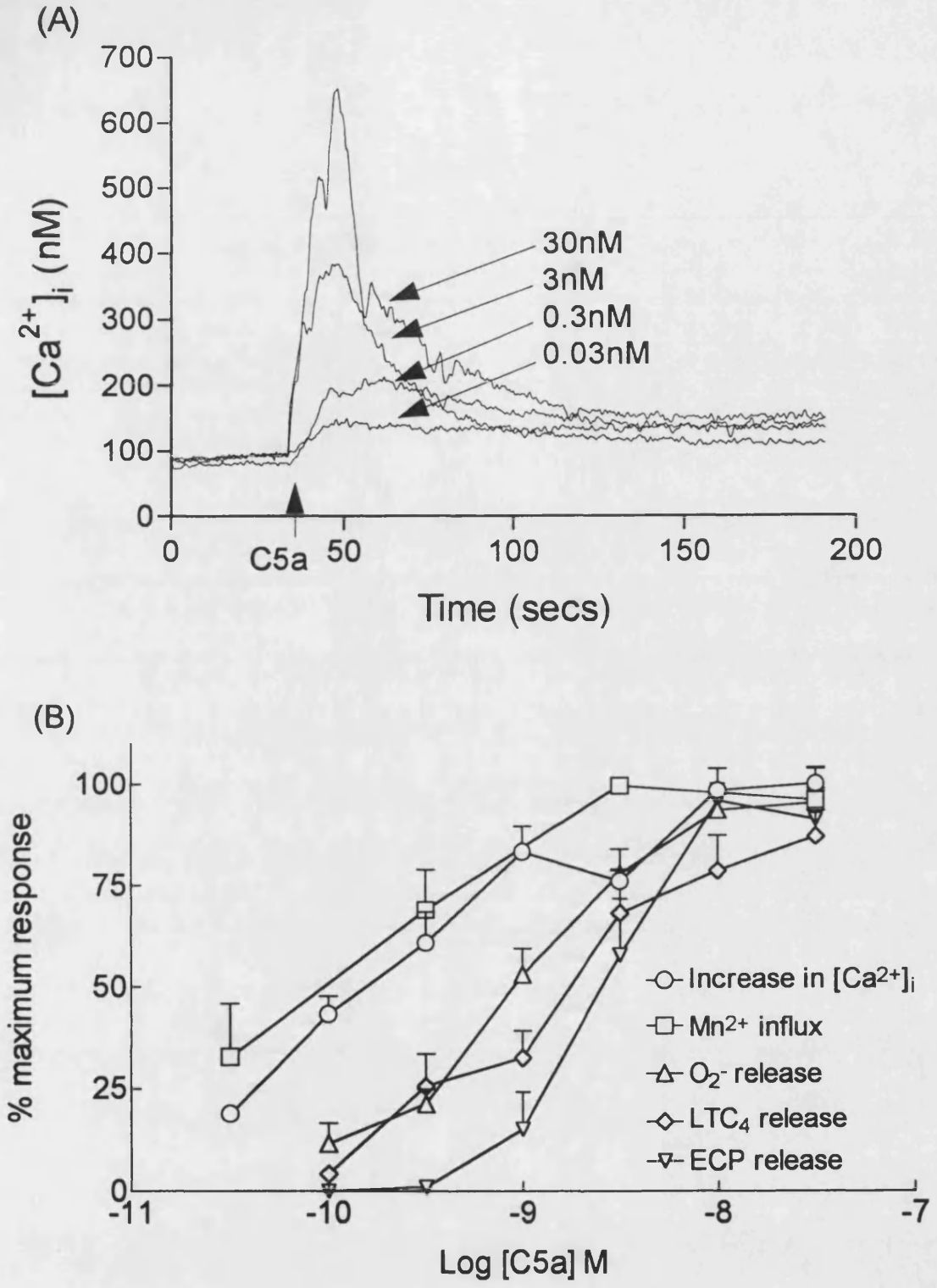


Figure 9

(A) Effect of increasing concentrations of C5a on Ca^{2+} mobilisation in fura-2-loaded human eosinophils and (B) comparison of concentration-effect curves for peak increase in $[Ca^{2+}]_i$, Mn^{2+} influx, O_2^-, LTC_4 and ECP release. Data represent mean ± s.e.m. of at least 4 separate experiments.

than O_2^-, LTC_4 and ECP release, suggesting that other signal transduction pathways are activated in addition to Ca^{2+} mobilisation.

IP_3 generation was also detected upon addition of C5a (30nM), with the peak increase detected when the reaction was stopped 5 seconds after agonist addition (figure 10A). All subsequent IP_3 measurements were made using this 5 second protocol. Comparison of the concentration-effect curves to C5a-induced IP_3 generation and increases in $[Ca^{2+}]_i$ (figure 10B) showed a large discrepancy in potency, with IP_3 release not detected until a concentration of 30nM C5a was added. However, this may purely be due to the fact that the 5 second time point was selected from data obtained using 30nM C5a and lower concentrations of C5a may require a different period of time to acheive their maximal IP_3 generation.

3.3 Thapsigargin-induced human eosinophil activation : effects on $[Ca^{2+}]_i$, O_2^-, LTC_4 and ECP release.

Thapsigargin (1nM-1μM), a selective endomembrane Ca^{2+}/Mg^{2+}-ATPase inhibitor, stimulated a concentration dependent Ca^{2+} influx response in human eosinophils (figure 11). This demonstrates the existence of a store-regulated Ca^{2+} entry pathway in human eosinophils. Interestingly, there was a substantial difference in the level of elevation of $[Ca^{2+}]_i$ depending on when Ca^{2+} was added to the cells. Addition of Ca^{2+} after thapsigargin stimulated a much larger increase in $[Ca^{2+}]_i$ than when thapsigargin was added to cells which were already in the presence of Ca^{2+} (figure 12A). When the concentration-effect curves were expressed as % maximum response, it was clear that the potency of

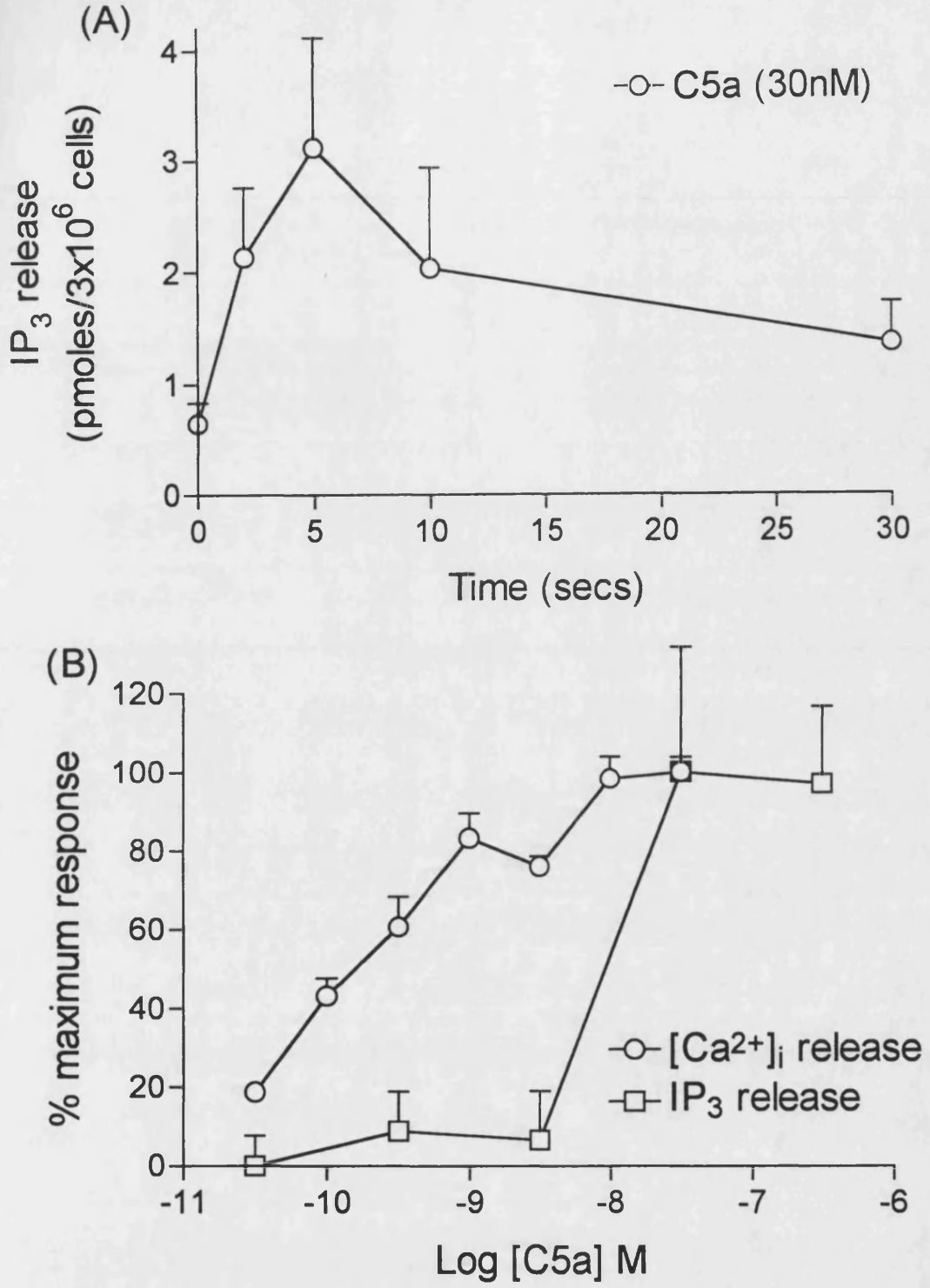


Figure 10

(A) Time-course of C5a (30nM)-induced IP_3 generation and (B) comparison of concentration-effect curves for C5a-mediated elevation of $[Ca^{2+}]_i$ and IP_3 production. Data represent mean ± s.e.m. of 4 separate experiments.

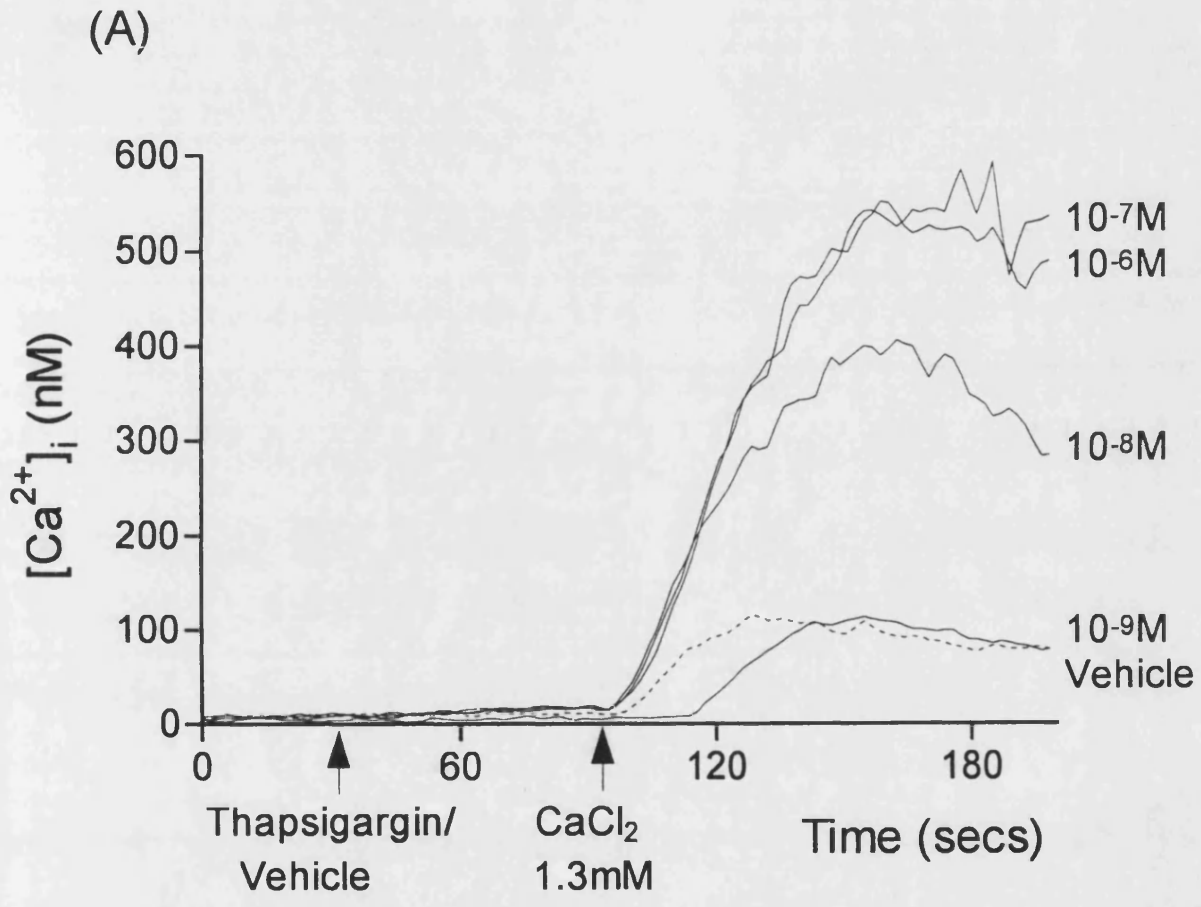


Figure 11

Effect of increasing concentrations of thapsigargin on $[Ca^{2+}]_i$ in fura-2 loaded human eosinophils (dashed line = vehicle effect).

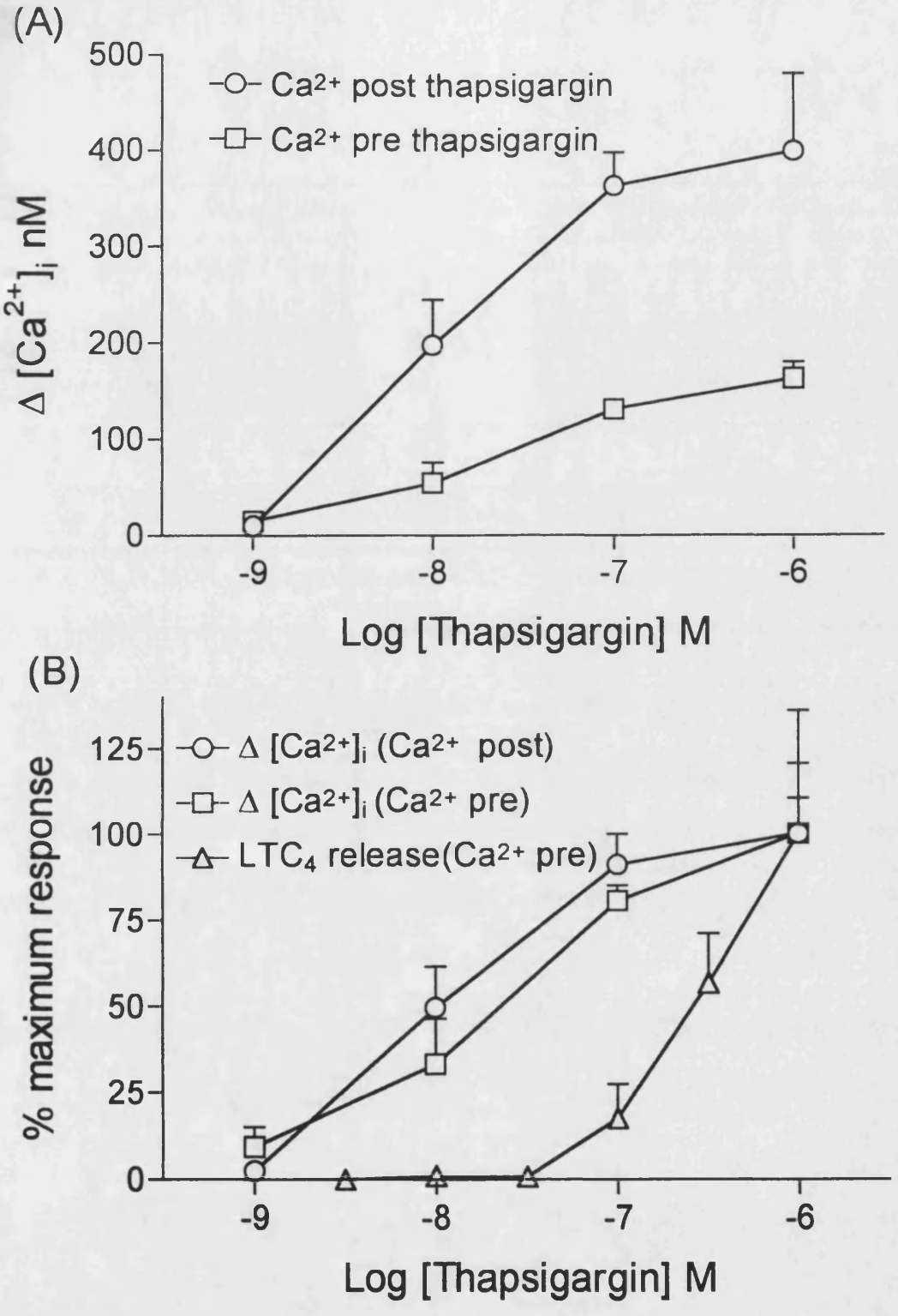


Figure 12

(A) Comparison of the extent of elevation of $[Ca^{2+}]_i$ stimulated by increasing concentrations of thapsigargin added either before or after the addition of 1.3mM $CaCl_2$. Any $[Ca^{2+}]_i$ rise seen with vehicle alone was subtracted from the thapsigargin-induced effects. (B) Comparison of concentration-effect curves to thapsigargin for elevation of $[Ca^{2+}]_i$ with LTC_4 release. Data represent mean ± s.e.m. of 3 separate experiments.

thapsigargin was unaffected by pre- or post-addition of Ca^{2+} (figure 12B). Thapsigargin did not stimulate any O_2^- or ECP release either in the presence or absence of cytochalasin B, but did cause substantial LTC_4 release both in the presence (457.3 ± 121.0pg $LTC_4/2.5x10^5$ cells/30 mins) and absence (488.12 ± 175.2 pg $LTC_4/2.5x10^5$ cells/30 mins) of cytochalasin B. However, the potency of thapsigargin for stimulation of LTC_4 release was lower than that for stimulating $[Ca^{2+}]_i$ rises. The amount of LTC_4 release is within the same range as the values obtained for the control groups reported in section 4. Mean EC_{50} values are shown in table 2.

Table 2. Mean EC_{50} values for thapsigargin-induced eosinophil activation

Thapsigargin-induced eosinophil activation	Mean EC_{50} (nM)	95% confidence limits
$\Delta[Ca^{2+}]_i$ (with Ca^{2+})	12.1	4.9 - 30.1
$\Delta[Ca^{2+}]_i$ (Ca^{2+} readdition)	9.7	7.6 - 12.3
LTC_4 release (with Ca^{2+})	228.9	168.4 - 310.9

Data represent mean of 3-4 separate experiments

3.4 FMLP-induced human eosinophil activation : effects on $[Ca^{2+}]_i$, O_2^-, LTC_4 and ECP release.

FMLP (1nM-1μM) caused concentration-dependent increases in $[Ca^{2+}]_i$, (figure 13A), from a basal level of 113.6 ± 11.8 nM to a maximum of 280.4 ± 28.0 nM (n=8). O_2^-, LTC_4 and ECP were released in response to FMLP, being maximally stimulated at a concentration of 1μM (figure 13B). The levels of O_2^-, LTC_4 and ECP released by FMLP (1μM) were 7.5 ± 0.8 nmoles cytochrome C

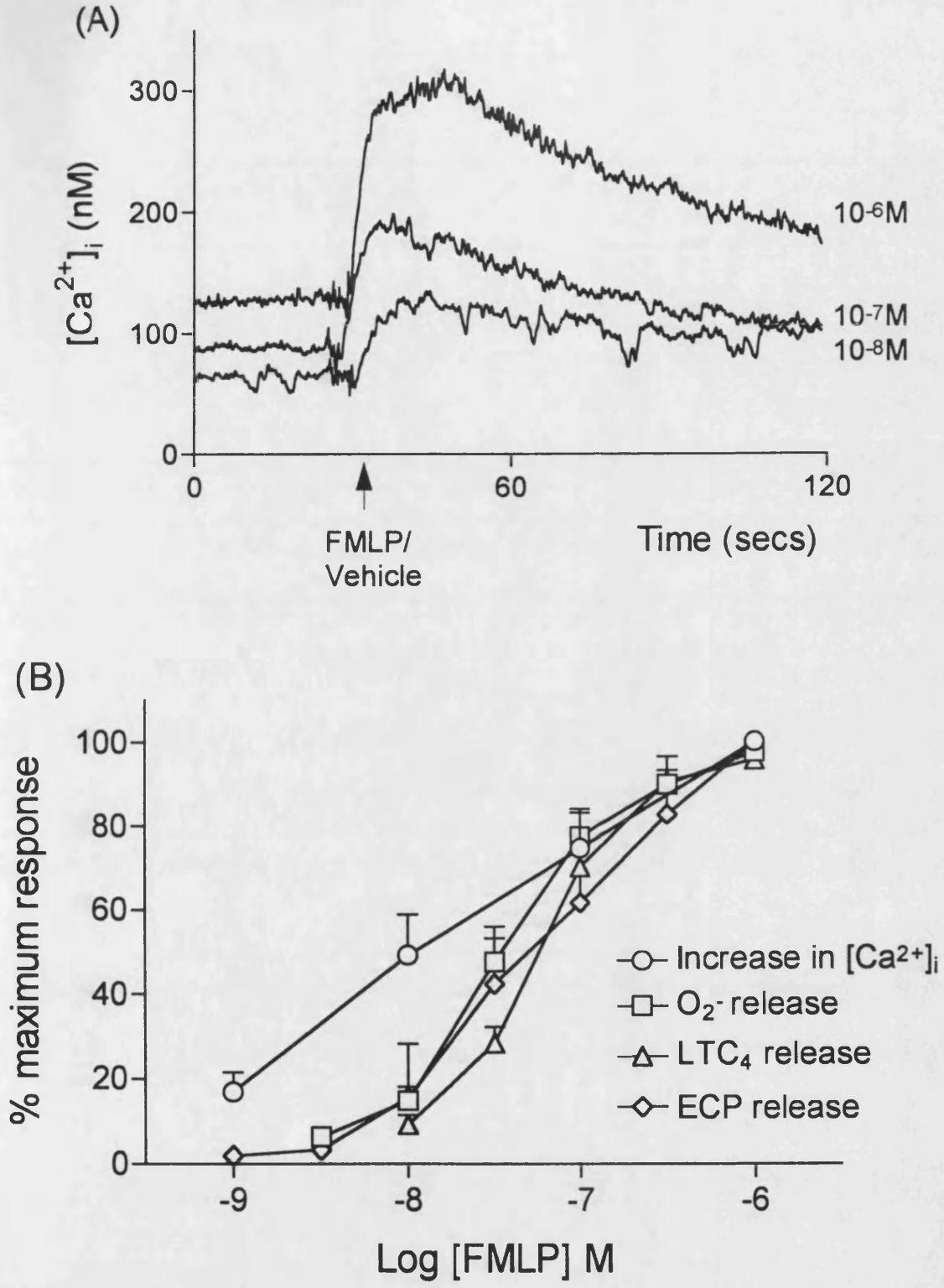


Figure 13

(A) Effect of increasing concentrations of FMLP on Ca^{2+} mobilisation in fura-2 loaded human eosinophils and (B) comparison of concentration-effect curves for peak increase in $[Ca^{2+}]_i$, O_2^-, LTC_4 and ECP release. Data represent mean ± s.e.m. of at least 4 separate experiments.

reduced/2.5x10^5 cells/10 mins, 1.08 ± 0.25 ng LTC_4/2.5x10^5 cells/30 mins and 112.4 ± 12.5 ng ECP/1.2x10^4 cells/30 mins, respectively. These levels of O_2^-, LTC_4 and ECP release are within the same range as the values obtained for the control groups reported in section 4. Mean EC_{50} values for FMLP-induced eosinophil activation are shown in table 3. A similarity between FMLP-induced eosinophil activation and C5a-mediated responses was that increases in $[Ca^{2+}]_i$ occured at lower concentrations than O_2^-, LTC_4 and ECP release and may suggest that other signal transduction pathways require activation in addition to Ca^{2+} mobilisation.

Table 3. Mean EC_{50} values for fMLP-induced eosinophil activation

FMLP-induced eosinophil activation	**Mean EC_{50} (nM)**	**95% confidence limits**
$\Delta[Ca^{2+}]_i$	11.1	4.1 - 30.0
O_2^- release	35.2	27.1 - 45.8
LTC_4 release	56.0	46.4 - 67.5
ECP release	35.6	5.9 - 216.9

Data represent mean of 3-10 separate experiments

3.5 LTD_4-induced human eosinophil activation : effects on $[Ca^{2+}]_i$, Mn^{2+} influx, O_2^- and ECP release.

LTD_4 (100nM) stimulated a rapid and transient peak elevation of $[Ca^{2+}]_i$ (figure 14A) from a basal level of 110.0 ± 6.5 nM to a peak value of 293.4 ± 20.2 (n=8), which desensitised to a repeated addition of LTD_4, but did not cross-desensitise with subsequent LTB_4 (100nM) addition (data not shown). In addition LTD_4

(100nM) stimulated Mn^{2+} influx in human eosinophils (figure 14B). However, despite these Ca^{2+} responses, LTD_4 (3nM-1μM) did not cause any release of O_2^- or ECP release.

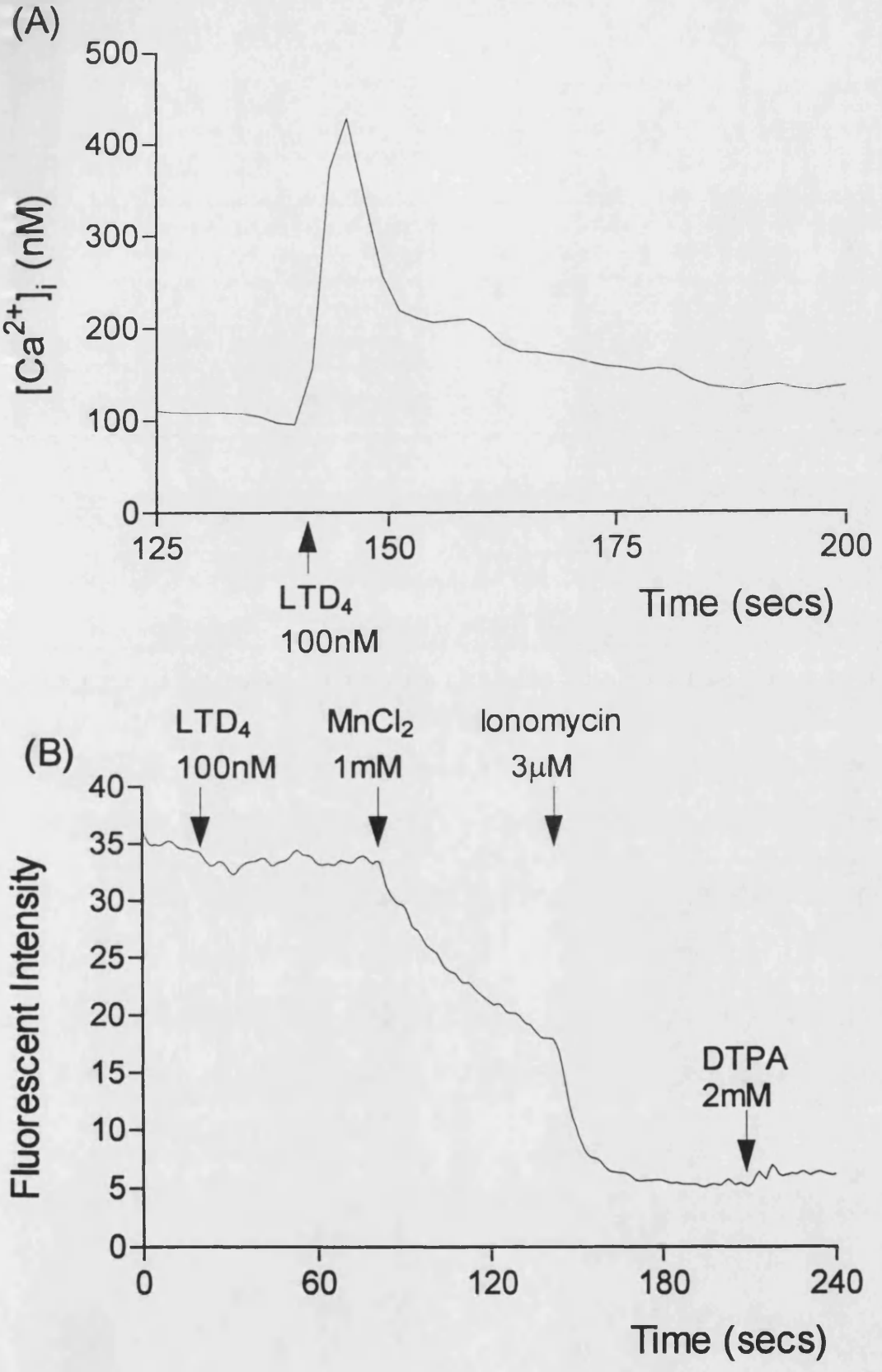


Figure 14

(A) Effect of LTD_4 (100nM) on $[Ca^{2+}]_i$ and (B) stimulation of Mn^{2+} influx by LTD_4 in fura-2 loaded human eosinophils. Traces are representative of 4 separate experiments.

4. RESULTS: MODULATION OF HUMAN EOSINOPHIL ACTIVATION

4.1 Spontaneous eosinophil activation

4.1.1 Investigation of the role of Ca^{2+} in spontaneous O_2^- release

A range of agents which modulate Ca^{2+}-dependent effects were tested against spontaneous O_2^- release. Chelation of extracellular Ca^{2+} by EGTA caused a concentration-dependent inhibition of the response, with a mean IC_{50} value of 1.1 (0.6-1.9) mM. This suggested that the spontaneous O_2^- release was dependent on the presence of extracellular Ca^{2+}. Investigation of the type of Ca^{2+} influx pathway that might be involved in the responses was carried out using agents which inhibit VOCs and RMCE; mean data are shown in figure 15A and below in table 4.

Table 4. Effect of agents that modulate Ca^{2+} influx, on spontaneous O_2^- release.

Inhibitor	Concentration (M)	% Inhibition of spontaneous O_2^- release Mean ± SEM
EGTA	$3x10^{-3}$	**92.4 ± 4.0****
$NiCl_2$	$3x10^{-3}$	**85.5 ± 4.5****
SK&F 96365	$1x10^{-5}$	**80.2 ± 16.7***
Nifedipine	$1x10^{-6}$	7.3 ± 27.4
Diltiazem	$1x10^{-5}$	16.9 ± 4.3
KCl	$5x10^{-2}$	12.5 ± 13
Benzamil	$3x10^{-5}$	**98.2 ± 1.8***

Data represent mean ± s.e.m. of 3-8 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

Both $NiCl_2$ and SK&F 96365 which block both RMCE and VOCs caused a

concentration-dependent inhibition of spontaneous O_2^- release, with IC_{50} values of 0.8 (0.2-3.9) mM and 3.5 (1.6-7.5) μM, respectively. In contrast, the potent and selective L-type VOC-blockers nifedipine and diltiazem had no effect against this response. This suggests that the O_2^- release involved Ca^{2+} influx via a RMCE, rather than a VOC-mediated pathway. However, depolarisation of the cell membrane by addition of KCl had no effect on the response. Finally, the Na^+/Ca^{2+}-exchange inhibitor benzamil caused marked inhibition of spontaneous O_2^- release at a concentration of 30μM.

4.1.2 Investigation of the second messengers involved in spontaneous O_2^- release.

Investigation of the second messengers that might be involved in the responses was carried out using a range of agents which modulate second messenger activation; mean data are shown in table 5 and are represented graphically in figure 15B.

Sodium fluoride (NaF, 30mM), which causes general activation of heterotrimeric G proteins, caused marked inhibition of spontaneous O_2^- release. This suggests that inhibitory G protein(s) may be activated by NaF and may stimulate generation of inhibitory second messengers.

Activation of PLC appeared to be involved, as U73122 (1μM), an inhibitor of PLC-dependent processes (Smith *et al.*, 1990), abolished the response. Whereas, U73343, a close structural but inactive analogue of U73122 was without effect, indicating some selectivity of action. In contrast, the

phospholipase A_2 (PLA_2) inhibitor manoalide had no effect.

Table 5. Effect of agents that modulate second messengers, on spontaneous O_2^- release.

Inhibitor	Concentration (M)	% Inhibition of spontaneous O_2^- release Mean ± SEM
NaF	$3x10^{-2}$	**84.7 ± 1.4****
U73122	$1x10^{-6}$	**96.7 ± 1.7****
U73343	$1x10^{-6}$	6.6 ± 10.6
Manoalide	$1x10^{-6}$	3.6 ± 4.2
Wortmannin	$1x10^{-8}$	**83.7 ± 13.1***
Forskolin	$3x10^{-5}$	35.2 ± 21.0
Nitroprusside	$1x10^{-6}$	9.9 ± 4.7
Staurosporine	$3x10^{-8}$	44.2 ± 29.4
Erbstatin analogue	$3x10^{-6}$	**88.6 ± 6.8***
Calyculin A	$1x10^{-7}$	-28.9 ± 22.9
Okadaic acid	$1x10^{-6}$	-2.3 ± 13.3
Cyclosporin A	$1x10^{-6}$	**75.4 ± 15.2***

Data represent mean ± s.e.m. of 3-4 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

Wortmannin (10nM) caused marked inhibition of the spontaneous O_2^- release. At this concentration wortmannin acts as a relatively selective PI-3 kinase inhibitor (Arcaro and Wymann, 1993), whereas inhibitory effects seen at higher concentrations (1μM) are generally due other effects, such as inhibition of tyrosine kinase and/or PLD (Naccache *et al.*, 1993).

Elevation of cGMP by sodium nitroprusside had no effect on the response, whilst elevation of cAMP by forskolin or inhibition of PKC by staurosporine caused modest inhibition, but were not statistically significant.

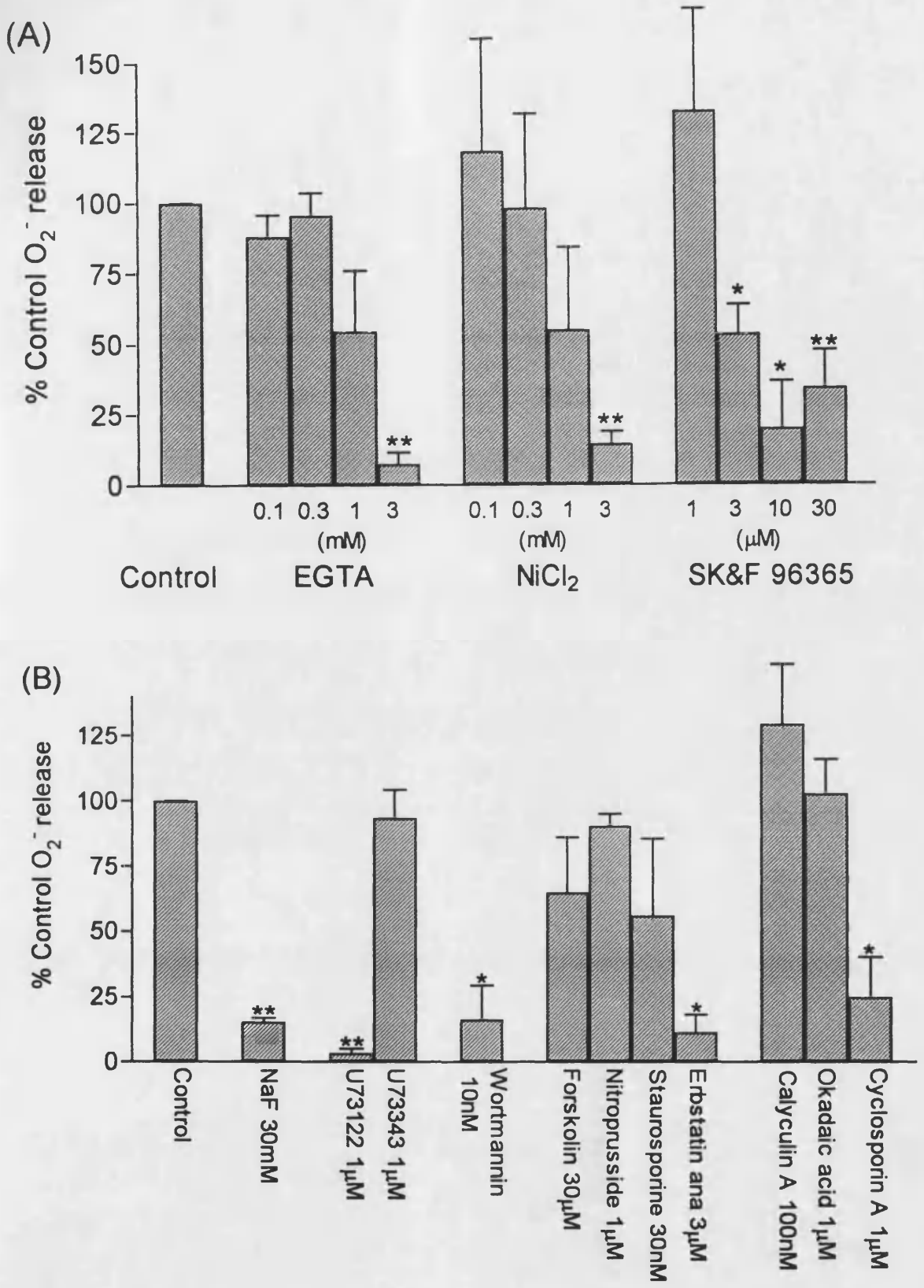


Figure 15

(A) Effect of agents which inhibit Ca^{2+}-dependent effects and (B) modulate second messengers, on spontaneous O_2^- release. Data represent mean ± sem of 3-4 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

There appears to be a role for tyrosine kinase in this response, as the erbstatin analogue caused concentration-dependent inhibition, with an IC_{50} value of 1.2 (0.6- 2.5) μM.

Investigation of the effect of phosphatase inhibitors found that the protein phosphatase 2B (PP2B) inhibitor cyclosporin A, caused marked inhibition of the spontaneous O_2^- response, whereas the PP1 and PP2A inhibitors okadaic acid and calyculin A were without effect.

4.1.3 Investigation of the effect of selected pharmacological reference agents on spontaneous O_2^- release

A range of pharmacological reference agents which possess anti-inflammatory activity or are used in asthma therapy, were tested for their effects against the spontaneous generation of O_2^- in human eosinophils; mean data are shown in table 6.

Table 6. Effect of anti-asthma/anti-inflammatory agents on spontaneous O_2^- release.

Inhibitor	Concentration (M)	% Inhibition of spontaneous O_2^- release Mean ± SEM
Cetirizine	$1x10^{-5}$	6.8 ± 6.9
Cromakalim	$1x10^{-5}$	20.1 ± 29.2
Sodium cromoglycate	$1x10^{-6}$	15.5 ± 17.3
Salbutamol	$1x10^{-6}$	12.2 ± 8.2
Indomethacin	$3x10^{-6}$	25.4 ± 27.0
BAY x1005 (n=2)	$1x10^{-5}$	5.1 ± 16.8

Data represent mean ± s.e.m. of 3-4 separate experiments, unless otherwise stated.

The β_2-agonist salbutamol, the potassium (K_{ATP}) channel opener cromakalim, the histamine antagonist cetirizine, as well as sodium cromoglycate had no significant effect on the spontaneous release of O_2^-. Also, release of prostaglandins or leukotrienes did not appear to contribute to the response as the cyclo-oxygenase (COX) inhibitor, indomethacin and the leukotriene synthesis inhibitor (LSI), Bay x1005 were without effect.

In summary, spontaneous O_2^- release appears to be dependent upon Ca^{2+} influx via a receptor-mediated entry pathway. In addition, the response is mediated via PLC activation, as well as PI-3 kinase, tyrosine kinase and PP2B activation.

4.2 C5a-induced human eosinophil activation

4.2.1 Investigation of the role of Ca^{2+} in C5a-induced eosinophil activation

A range of agents which modulate Ca^{2+}-dependent effects were tested against C5a-induced eosinophil activation; mean data are shown below in table 7 and are represented graphically in subsequent figures.

Table 7. Effect of agents that modulate Ca^{2+} influx, on C5a-induced increases in $[Ca^{2+}]_i$, Mn^{2+} influx, O_2^-, LTC_4 and ECP release.

Inhibitor	Conc (M)	% Inhibition of C5a-induced responses (Mean ± s.e.m.)				
		$\Delta[Ca^{2+}]_i$	Mn^{2+} influx	O_2^- release	LTC_4 release	ECP release
EGTA	$3x10^{-3}$	+34.7 ± 3.9**	nt	95.6 ± 2.8**	95.1 ± 3.6**	5.2 ± 5.5
$NiCl_2$	$3x10^{-3}$	58.3 ± 6.5**	73.6 ± 2.2**	97.9 ± 0.9**	79.5 ± 6.2**	77.1 ± 2.3**
SK&F 96365	$3x10^{-5}$	-1.9 ± 13.5	69.1 ± 6.7**	90.5 ± 5.5**	89.7 ± 5.3**	39.4 ± 2.3**
Nifedipine	$3x10^{-6}$	#-1.8 ± 14.5	#8.5 ± 12.9	24.4 ± 9.3*	28.8 ± 16.3	5.8 ± 5.5
Diltiazem	$1x10^{-5}$	nt	nt	1.7 ± 11.6	30.3 ± 15.2	5.5 ± 6.1
Verapamil	$3x10^{-6}$	nt	nt	5.2 ± 7.8	37.7 ± 12.1*	#-4.7 ± 7.2
Econazole	$3x10^{-5}$	nt	nt	91.6 ±17.9**	76.7 ±18.1**	nt
KCl	$5x10^{-2}$	nt	nt	82.1 ± 6.0**	82.2 ± 7.0**	55.3 ± 5.7**
Benzamil	$3x10^{-5}$	nt	nt	nt	63.5 ± 18.9*	11.5 ± 3.4

N.B. + denotes EGTA concentration of $4x10^{-3}$M used, # denotes inhibitor concentration of $1x10^{-5}$M used, nt = not tested. Data represent mean ± s.e.m. of 3-4 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

Chelation of extracellular Ca^{2+} using EGTA (4mM), prior to the addition of C5a caused 34.7 ± 3.9 % inhibition ($p<0.05$) of the initial peak increase in $[Ca^{2+}]_i$ (figure 16A). More detailed analysis of the data, comparing area under the curve (AUC) at different time points (figure 16B), revealed that during the first 30 seconds after stimulation (AUC 0-30 seconds) the response was inhibited

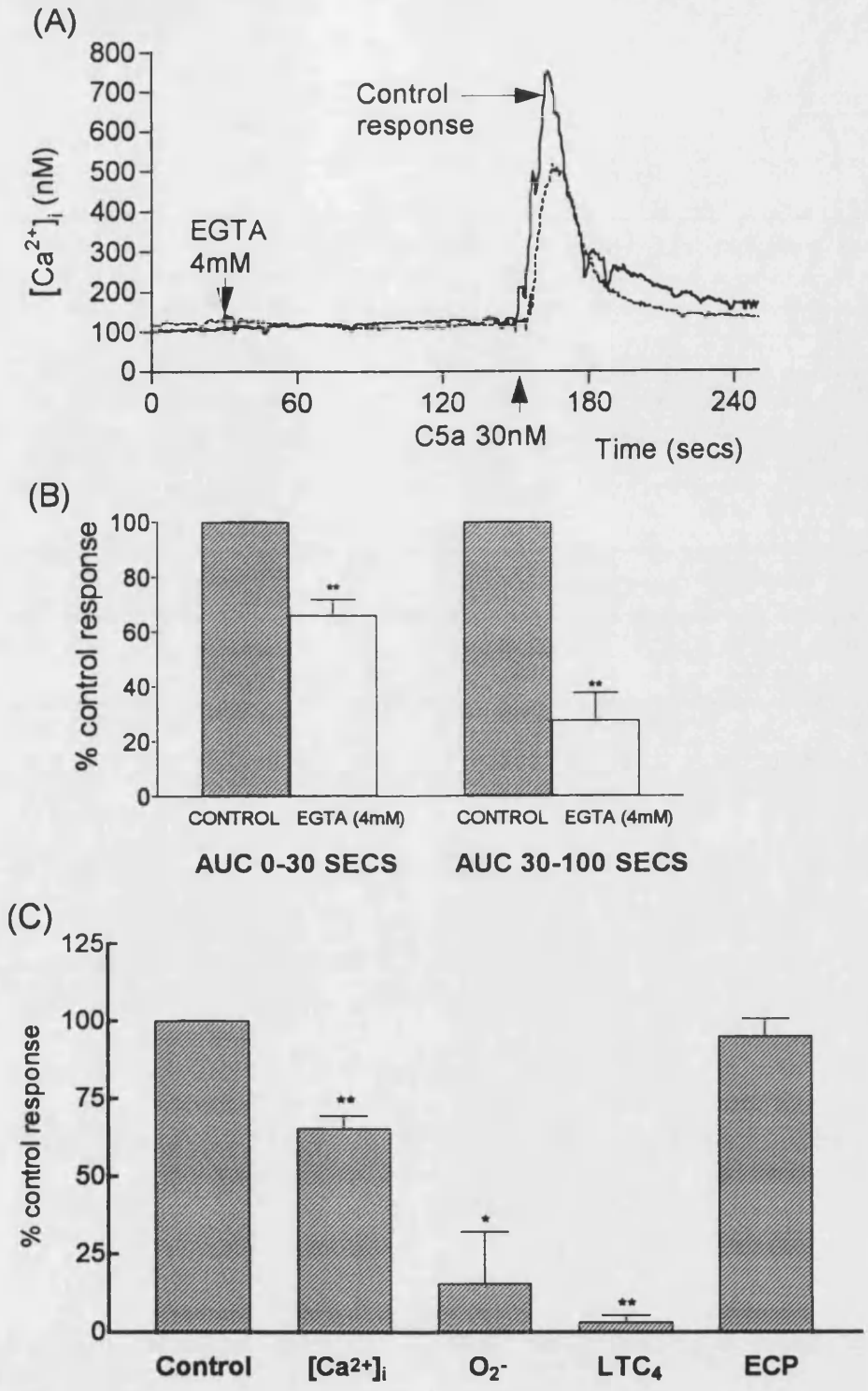


Figure 16

(A) Effect of EGTA on C5a-induced Ca^{2+} mobilisation compared with a vehicle-treated control response. (B) Analysis of the effect of EGTA (4mM) was made by measuring "area under the curve" (AUC) at early time points (0 to 30 seconds) and at later time points (30 to 100 seconds), after the addition of C5a (30nM). (C) Graphical representation of the effect of EGTA (4mM) against the peak increase in $[Ca^{2+}]_i$, O_2^-, LTC_4 and ECP release. Data are mean ± s.e.m. of 4-6 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

by 33.9 ± 5.7% ($p<0.005$), which was almost identical to the inhibition of the peak increase in Ca^{2+}. In contrast, the AUC between 30 seconds and 100 seconds (AUC 30-100 seconds) was markedly inhibited (72.2 ± 8.9 %, $p<0.005$). These data suggest that the C5a-induced transient peak $[Ca^{2+}]_i$ elevation response and AUC 0-30 seconds was predominantly due to release of Ca^{2+} from intracellular stores, whereas the sustained increase in $[Ca^{2+}]_i$ (AUC 30-100 seconds) was mainly due to Ca^{2+} influx across the plasma membrane. EGTA also caused marked inhibition of O_2^- and LTC_4 release, indicating a requirement for extracellular Ca^{2+} for these responses (figure 16C). In contrast, C5a-induced ECP release was not inhibited by 3mM EGTA.

Having established a requirement for extracellular Ca^{2+} for O_2^- and LTC_4 release, the pathway for Ca^{2+} influx was investigated. The mechanism of activation of Ca^{2+} influx in neutrophils is known to be via depletion of intracellular stores (Montero *et al.,* 1991; Demaurex *et al.,* 1994). It was therefore useful to compare the concentration-effect curves to C5a and evaluate whether release of intracellular stores correlated with Ca^{2+} influx. Measurement of C5a-induced peak increase in $[Ca^{2+}]_i$ and AUC 0-30 seconds appears to predominantly represent release of Ca^{2+} from intracellular stores. The concentration-effect curves for these responses were almost identical, with EC_{50} values of 0.36 (0.02-6.2) nM and 0.22 (0.06-0.76)nM, respectively (Figure 17A). Similarly, the concentration-effect curves for Mn^{2+} influx and AUC 30-100 seconds were comparable, with EC_{50} values of 0.09 (0.02-0.46) and 0.13 (0.07-0.25) nM, respectively (Figure 17B). Therefore, it appears that C5a-induced intracellular

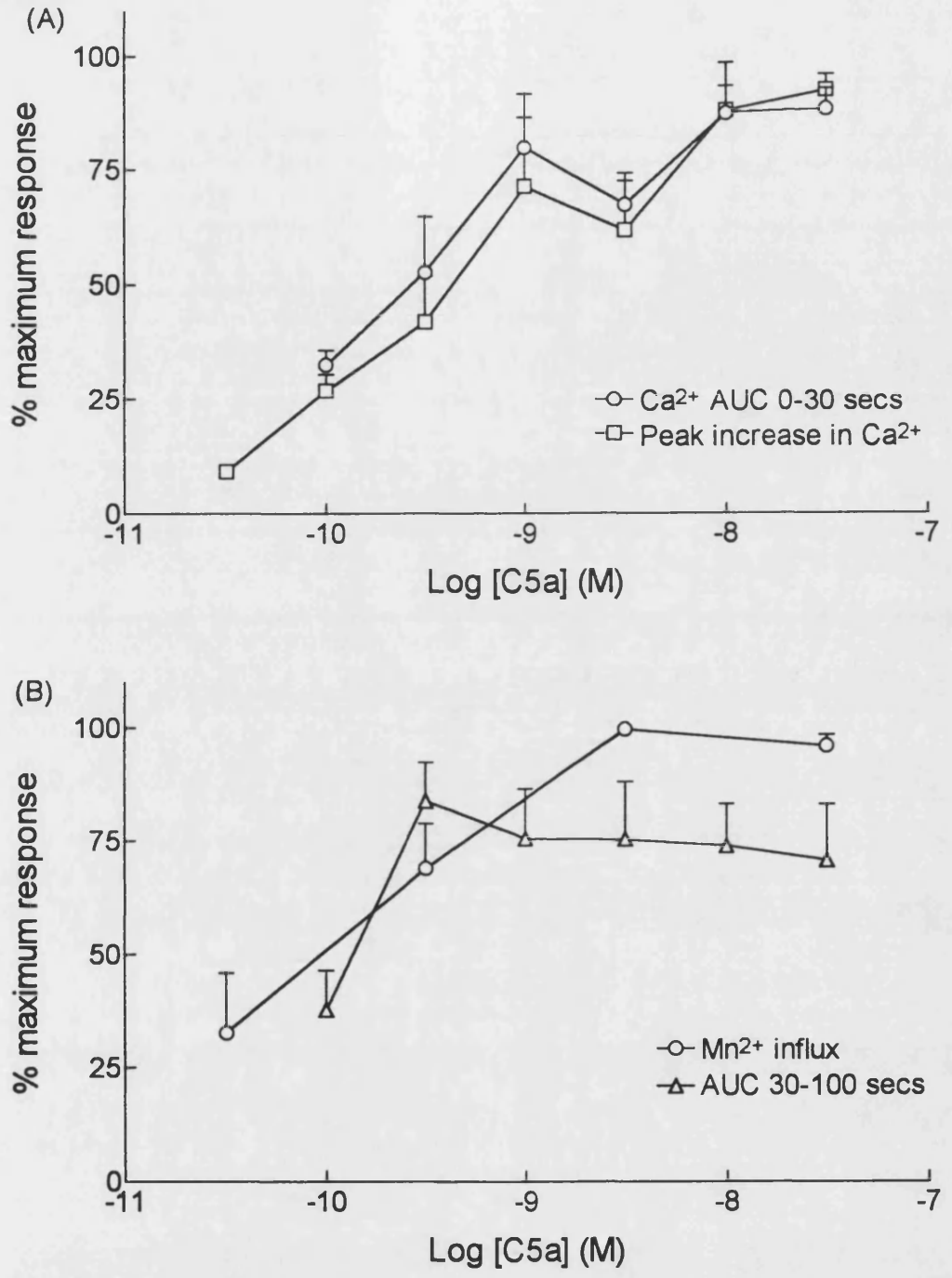


Figure 17

(A) Comparison of C5a-induced Ca^{2+} responses measured as either area under the curve (AUC) between 0 and 30 seconds after agonist addition or peak increase in $[Ca^{2+}]_i$ and (B) shows a comparison of C5a-induced Ca^{2+} responses measured as area under the curve (AUC) between 30 and 100 seconds after agonist addition and rate of Mn^{2+} influx. Data are represented as mean ± s.e.m. of 4 separate experiments.

Ca^{2+} release and Ca^{2+} influx occured at similar concentrations and that Mn^{2+} influx correlated closely with Ca^{2+} influx, when measured as AUC 30-100 seconds.

The inorganic cation Ni^{2+} (3mM), which blocks both RMCE and VOCs, caused marked inhibition of the rise in $[Ca^{2+}]_i$, Mn^{2+} influx, O_2^-, LTC_4 and ECP responses produced by C5a (30nM) (figure 18). However, some inhibition of the Ca^{2+} mobilisation response may be partly due to a small quench of fura-2 fluorescence, which was observed upon addition of $NiCl_2$ to the cells. The potent and selective VOC blocker, nifedipine (10μM), was inactive against either IP_3 release (18.1 ± 20.8 % inhibition, $p>0.05$, n=3), Ca^{2+} elevation or Mn^{2+} influx responses to C5a (figure 19) and at a concentration of 3μM had little or no effect against O_2^-, LTC_4 or ECP release. Similarly, diltiazem and verapamil, which represent different structural classes of VOC blocker also had little effect against O_2^-, LTC_4 and ECP release. The putative RMCE blocker, SK&F 96365 (30μM) had no significant effect on IP_3 generation (35.7 ± 31.1 % enhancement, $p>0.05$, n=4) or the Ca^{2+} mobilisation response (figure 20A), but caused marked inhibition of Mn^{2+} influx (IC_{50} = 19.0 (13.7 - 26.4) μM, figure 20B). Furthermore, SK&F 96365 (30μM) abolished the release of O_2^- and LTC_4 (figure 20C), as did its analogue, econazole, whereas ECP was only moderately reduced by SK&F 96365. Similarly, depolarisation of the cell membrane using KCl caused marked inhibition of O_2^- and LTC_4 release, but only caused just over 50% inhibition of ECP release. KCl also caused 39.4 ± 7.9 % inhibition (n=4, $p<0.05$) of IP_3 generation.

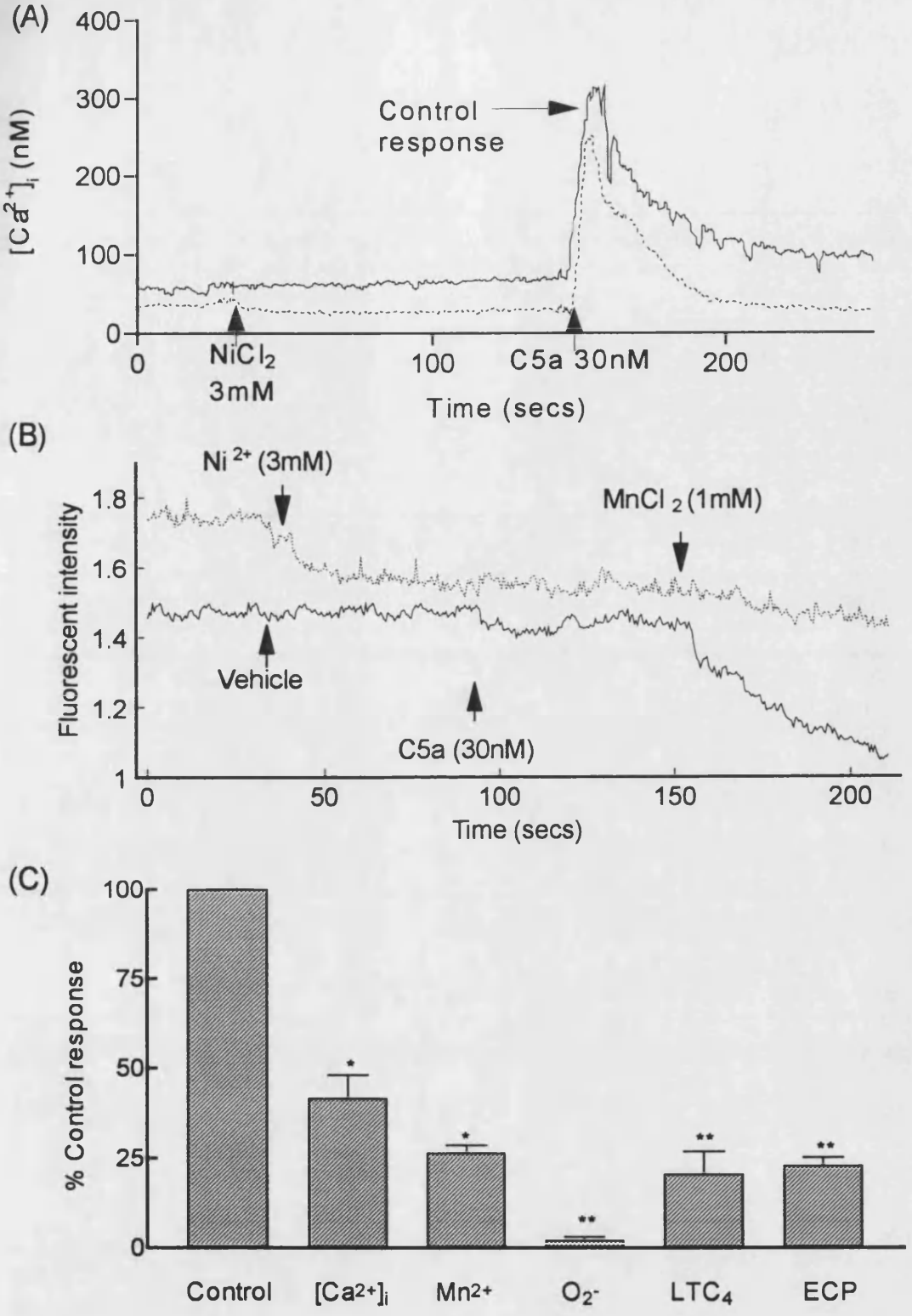


Figure 18

(A) Effect of the inorganic cation Ni^{2+} (3mM) on C5a-induced Ca^{2+} mobilisation and (B) on Mn^{2+} influx. Cells were pre-treated for 1 minute with either vehicle or $NiCl_2$ before addition of C5a (30nM) and $MnCl_2$ (1mM) was added 1 minute after the addition of C5a. (C) Graphical representation of these responses, as well as O_2^-, LTC_4 and ECP release. Data are mean ± s.e.m. of 4-6 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

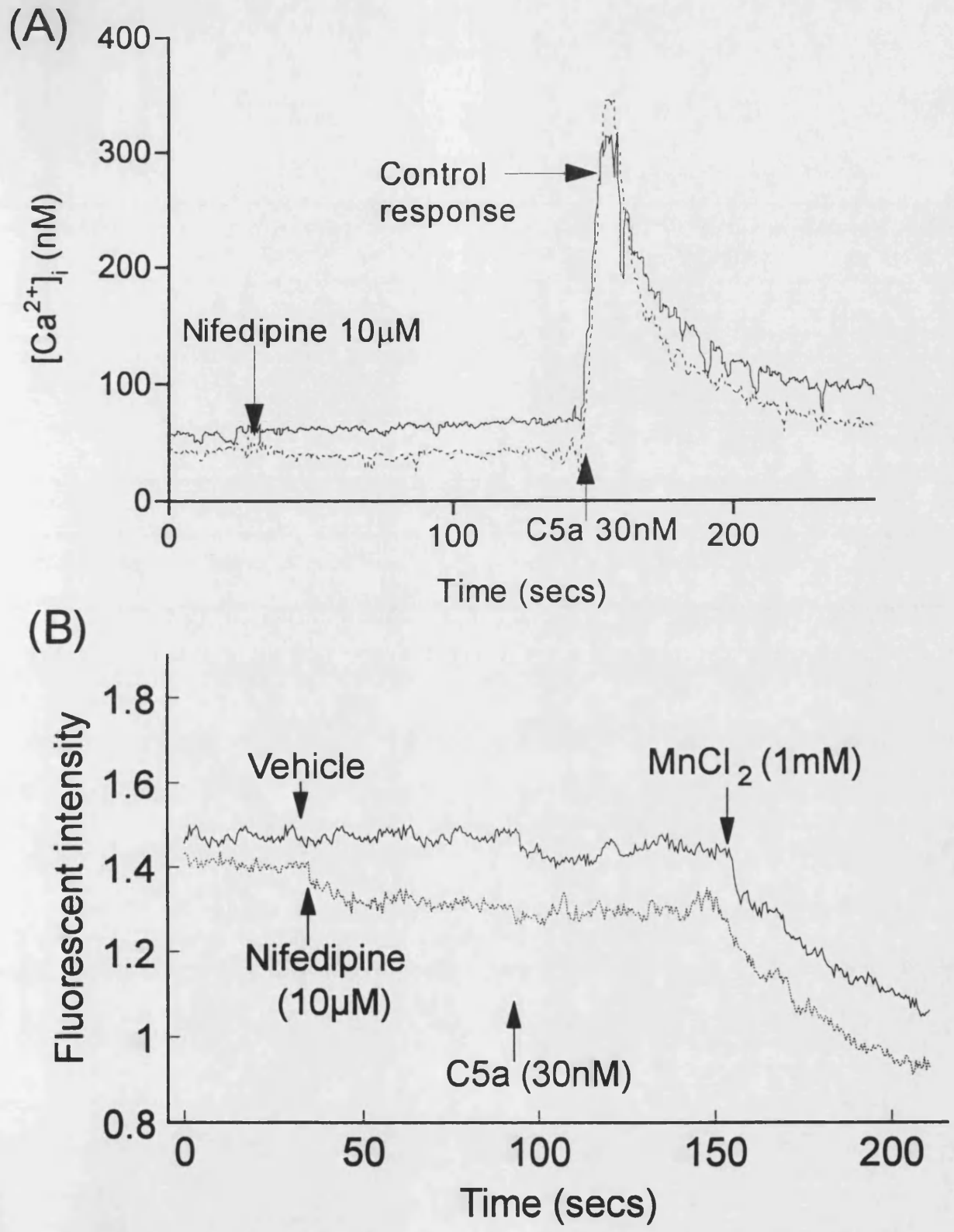


Figure 19

(A) Effect of the selective VOC blocker nifedipine (10μM) on C5a-induced Ca^{2+} mobilisation and (B) on Mn^{2+} influx. Cells were pre-treated for 1 minute with either vehicle or nifedipine, before addition of C5a (30nM) and $MnCl_2$ (1mM) was added 1 minute after the addition of C5a. Traces are representative of 3-4 separate experiments.

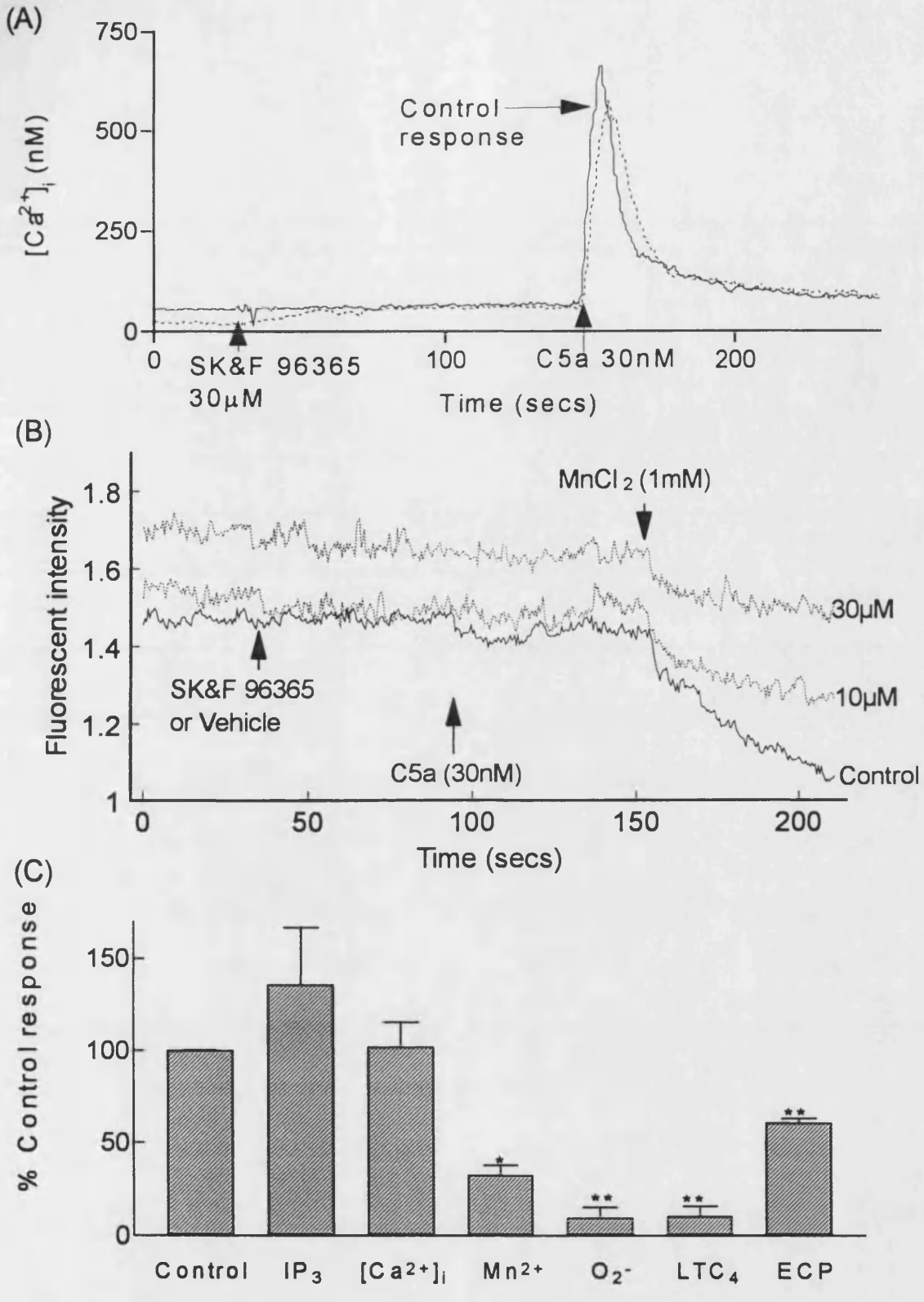


Figure 20

(A) Effect of the RMCE and VOC blocker, SK&F 96365 on C5a-induced Ca^{2+} mobilisation and (B) on Mn^{2+} influx. Cells were pre-treated for 1 minute with either vehicle or SK&F 96365 before addition of C5a (30nM) and $MnCl_2$ (1mM) was added 1 minute after the addition of C5a. (C) Graphical representation of these responses, as well as IP_3, O_2^-, LTC_4 and ECP release. Data are mean ± s.e.m. of 4-6 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

It is clear from these results that C5a stimulates release of intracellular Ca^{2+} and Ca^{2+} influx. The influx response appears to be mediated via RMCE, rather than VOCs and is required for the generation of O_2^- and LTC_4. However, conflicting results seem to occur with ECP release. Chelation of extracellular Ca^{2+} by EGTA did not inhibit the response, whereas the Ca^{2+} influx blocker $NiCl_2$ caused marked inhibition and SK&F 96365 moderate inhibition. The possibility that EGTA may affect the antibody binding in the ECP assay was tested, by carrying out the ECP standard curve in the presence and absence of EGTA, but was found to have no effect on the curves (data not shown). Another possibility was that the concentration of EGTA used was insufficient to chelate all of the extracellular Ca^{2+}. C5a-induced ECP release was therefore measured in cells resuspended in Ca^{2+}-free HBSS, which had different concentrations of $CaCl_2$ added to it. Using this protocol ECP release was reduced by 49.0 ± 7.2% ($p<0.05$, $n=4$) in the presence of 0.3mM $CaCl_2$ compared with the response obtained in 1.3mM $CaCl_2$. Based upon this result and the data obtained with the Ca^{2+} influx blockers there is evidence that the C5a-induced ECP response does have some extracellular Ca^{2+} dependence, however it is clearly less dependent on extracellular Ca^{2+} than either O_2^- or LTC_4 release.

The presence of cytochalasin B in the O_2^-, LTC_4 and ECP assays may have had an effect on their Ca^{2+} dependence and was therefore investigated. Cytochalasin B (5.5μM) had little or no effect (18.1 ± 9.3% enhancement, $p>0.05$, $n=4$) on the C5a-induced peak increase in $[Ca^{2+}]_i$ (Figure 21A). Furthermore, cytochalasin B had no significant effect on IP_3 generation (30.3 ±

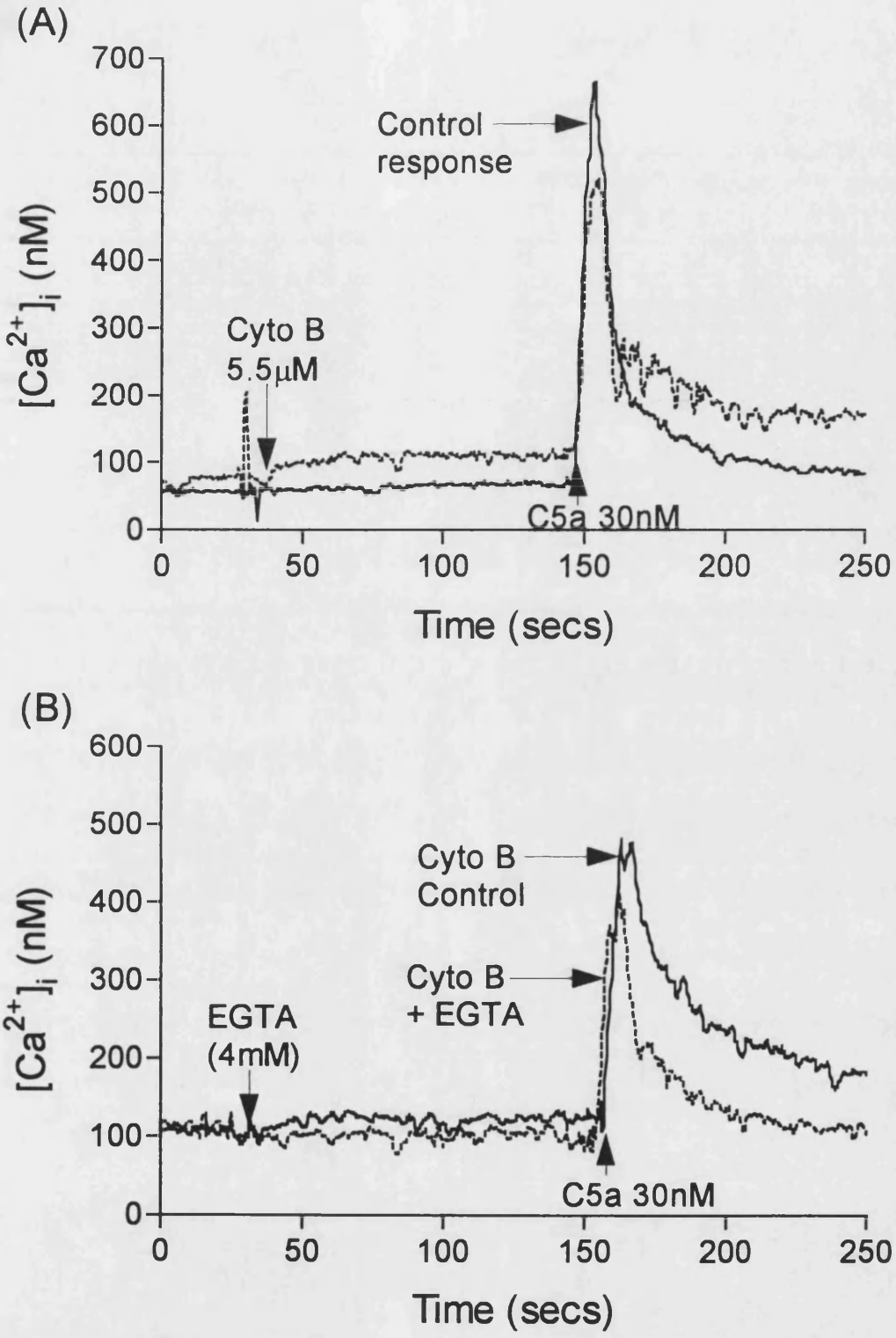


Figure 21

(A) Effect of cytochalasin B on C5a-induced Ca^{2+} mobilisation compared with a vehicle-treated control response and (B) a comparison of the C5a-induced Ca^{2+} mobilisation response in the presence and absence of EGTA, in cytochalasin B-treated cells.

30.0 % inhibition, p>0.05, n=3), suggesting that cytochalasin B had no effect on intracellular Ca^{2+} release. Measurement of AUC revealed that cytochalasin B caused enhancements of 21.8 ± 14.1% (AUC 0-30 seconds, p>0.05, n=4) and 90.7 ± 42.1 % (AUC 30-100 seconds, p>0.05, n=4). Thus, there appears to be a trend towards enhancement of Ca^{2+} influx in the presence of cytochalasin B, but this was variable and did not achieve statistical significance. Addition of EGTA (4mM) to cytochalasin B-treated cells caused a similar inhibition of the Ca^{2+} mobilisation response as was seen in the absence of cytochalasin B (Figure 21B).

4.2.2 Investigation of the second messengers involved in C5a-induced eosinophil activation

Investigation of the second messengers that might be involved in C5a-induced eosinophil activation was carried out using a range of agents which modulate second messengers; mean data are shown in table 8 and are represented graphically in figures 22 and 23.

Activation of heterotrimeric G proteins by NaF had no significant effect on C5a-induced LTC_4 release, but did cause partial inhibition of ECP release. Suggesting that there may be differences in the regulatory processes for these two responses.

The putative inhibitor of PLC-dependent processes, U73122 (1μM), inhibited C5a-induced IP_3 generation (66.0 ± 16.3 %, p<0.05, n=3), Ca^{2+} mobilisation (89.3 ± 0.2 %, p<0.005, n=3, figure 23A) and Mn^{2+} influx (73.1 ± 11.8 %, p<0.05,

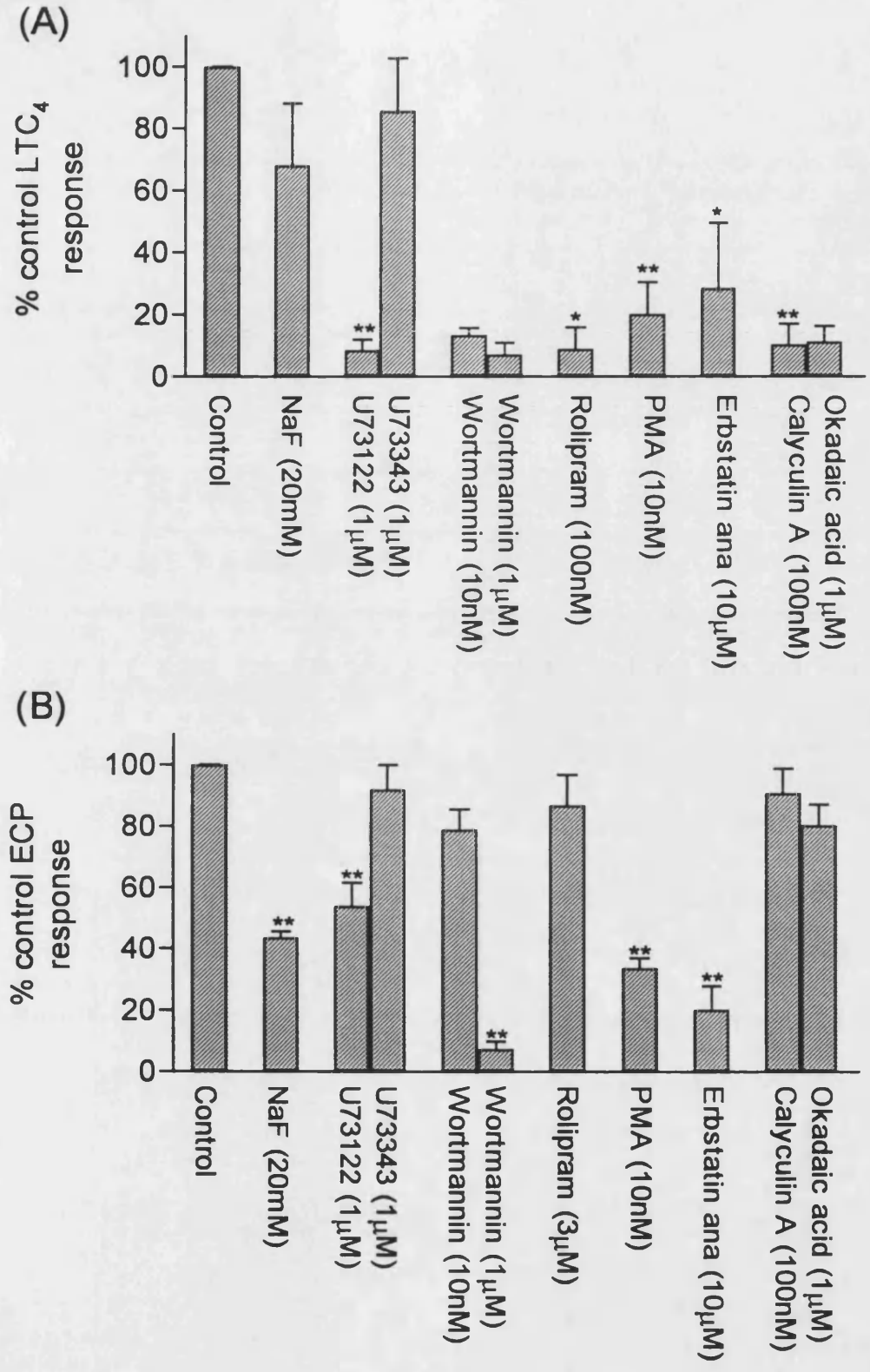


Figure 22

(A) Effect of agents which modulate second messengers, on C5a-induced LTC_4 release and (B) on ECP release. Data represent mean ± s.e.m of 2-6 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

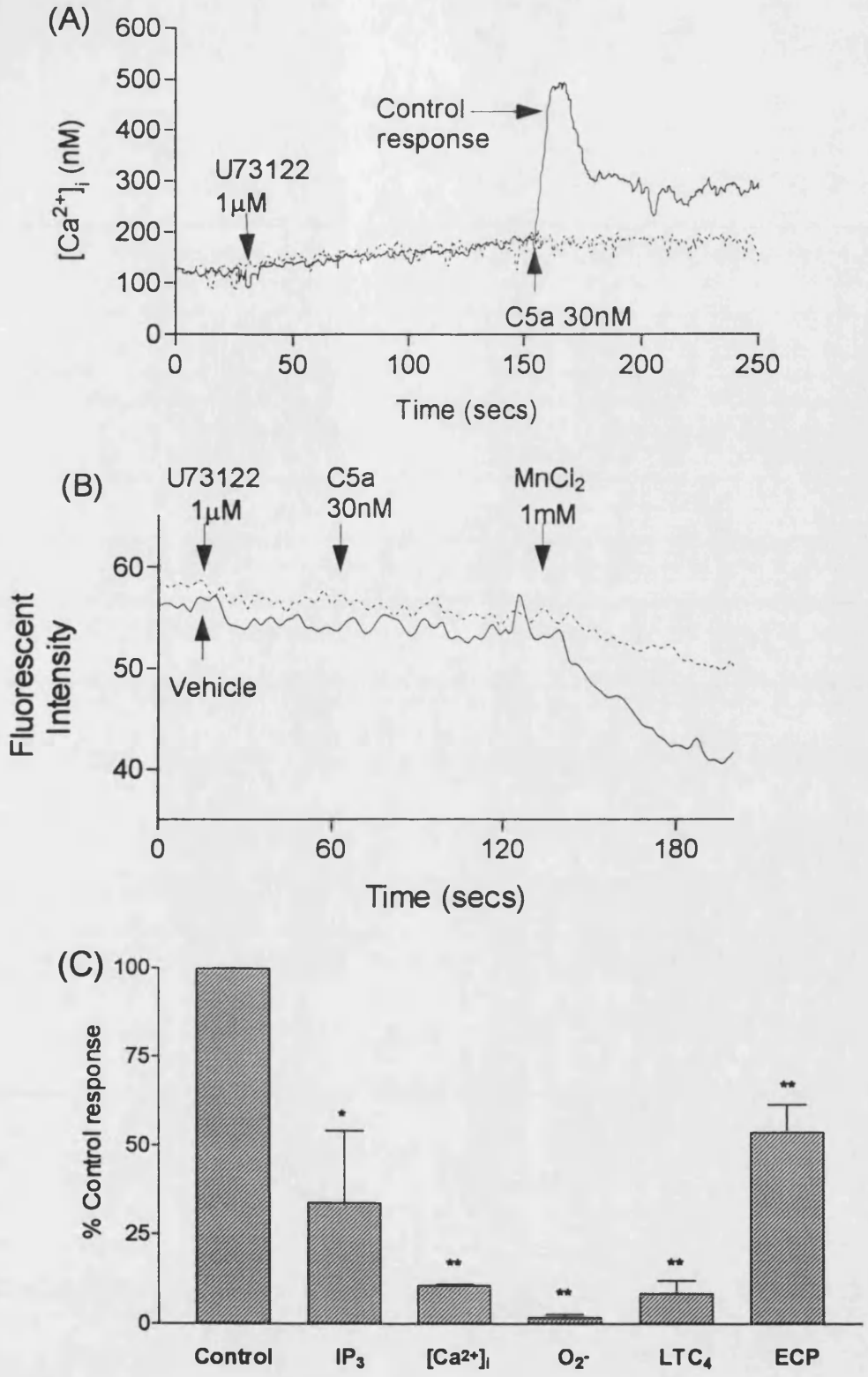


Figure 23

(A) Effect of the inhibitor of PLC-dependent process, U73122 on C5a-induced Ca^{2+} mobilisation and (B) Mn^{2+} influx, compared with a control response. Cells were pre-treated for 1 minute with either vehicle or U73122 before addition of C5a (30nM) and $MnCl_2$ (1mM) was added 1 minute after the addition of C5a and (C) graphical representation of these responses, as well as IP_3, O_2^-, LTC_4 and ECP release. Data are mean ± s.e.m. of 3-5 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

n=3, figure 23B). Similarly, the release of O_2^- and LTC_4 was markedly inhibited by U73122 (figure 23C). C5a-induced ECP release was significantly, but only partially inhibited by U73122. In contrast, U73343 (1μM), was without effect, suggesting some specificity of action. Thus, the O_2^- and LTC_4 responses appear to mediated predominantly via PLC-dependent processes, whereas ECP release requires activation of additional pathway(s).

Table 8. Effect of agents that modulate second messengers, on C5a-induced O_2^-, LTC_4 and ECP release.

Inhibitor	Conc (M)	% Inhibition of C5a-induced responses (Mean ± s.e.m.)		
		O_2^- release	LTC_4 release	ECP release
NaF	$2x10^{-2}$	nt	31.8 ± 19.9	**56.5 ± 2.1****
U73122	$1x10^{-6}$	**98.1 ± 0.9****	**91.4 ± 3.4****	**46.2 ± 7.6****
U73343	$1x10^{-6}$	9.6 ± 7.6	14.4 ± 17.3	8.1 ± 8.0
Wortmannin	$1x10^{-8}$	nt	$^{\$}$86.6 ± 2.2	21.2 ± 6.5
Wortmannin	$1x10^{-6}$	nt	$^{\$}$92.8 ± 3.7	**92.7 ± 2.4****
Forskolin	$3x10^{-5}$	29.5 ± 15.9	15.1 ± 30.1	-8.5 ± 5.6
Rolipram	$1x10^{-7}$	**92.4 ± 4.0****	**91.0 ± 7.0***	$^{\#}$13.3 ± 10.0
Nitroprusside	$1x10^{-6}$	-8.6 ± 6.9	**-28.5 ± 38.5***	-11.3 ± 7.1
PMA	$1x10^{-8}$	nt	**79.8 ± 10.3****	**66.4 ± 3.2****
Bisindolymaleimide	$1x10^{-7}$	-4.7 ± 3.6	-26.4 ± 37.5	**42.8 ± 4.8****
Erbstatin analogue	$1x10^{-5}$	nt	**71.6 ± 21.0***	**79.9 ± 7.6****
Calyculin A	$1x10^{-7}$	nt	**89.6 ± 6.5****	9.4 ± 8.0
Okadaic acid	$1x10^{-6}$	nt	$^{\$}$88.7 ± 5.0	19.9 ± 6.7
Cyclosporin A	$1x10^{-6}$	nt	$^{\$}$-72.7 ± 51.7	11.3 ± 8.2

$^{\#}$ denotes rolipram concentration of $3x10^{-6}$M used, nt = not tested. $^{\$}$ denotes mean ± s.d. of n=2 separate experiments, otherwise data represent mean ± s.e.m of 3-6 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

Further differences between C5a-induced LTC_4 and ECP release were observed with wortmannin. At a concentration of 10nM, inhibition of LTC_4, but not ECP

release was observed. However, at a concentration of 1μM, wortmannin inhibited both LTC_4 and ECP release. This suggests that the LTC_4 response involves activation of PI-3 kinase, whereas ECP does not involve PI-3 kinase, but may involve activation of tyrosine kinase and/or PLD.

Elevation of cAMP by forskolin had no significant effect on O_2^-, LTC_4 or ECP release. In contrast, inhibition of PDE IV activity by rolipram, abolished release of O_2^- and LTC_4, but had no effect on ECP. This once again suggests that there is a difference in the regulatory processes which control O_2^- and LTC_4 compared with ECP release. The difference in effectiveness of forskolin and rolipram, may suggest that the phosphodiesterase enzymes present in eosinophils are very active and rapidly breakdown cAMP, therefore inhibition of breakdown of cAMP would be a more effective means of elevating the level of cAMP in the cell.

Elevation of cGMP by sodium nitroprusside had no significant effect on O_2^- or ECP release, but did appear to cause a modest enhancement of LTC_4 release.

Activation of PKC by PMA (10nM) had no significant effect (29.7 ± 21.5 % inhibition, $p>0.05$, $n=6$) on the peak increase of $[Ca^{2+}]_i$ (figure 24A), but caused marked inhibition of both LTC_4 and ECP release (figure 24B). PMA (10nM) alone caused substantial O_2^- release (16.4 ± 1.1 nmoles cytochrome C/$2.5x10^5$ eosinophls/10 minutes, $n=4$). Inhibition of PKC, by bisindolymaleimide had no effect against LTC_4 release, but did significantly inhibit ECP release.

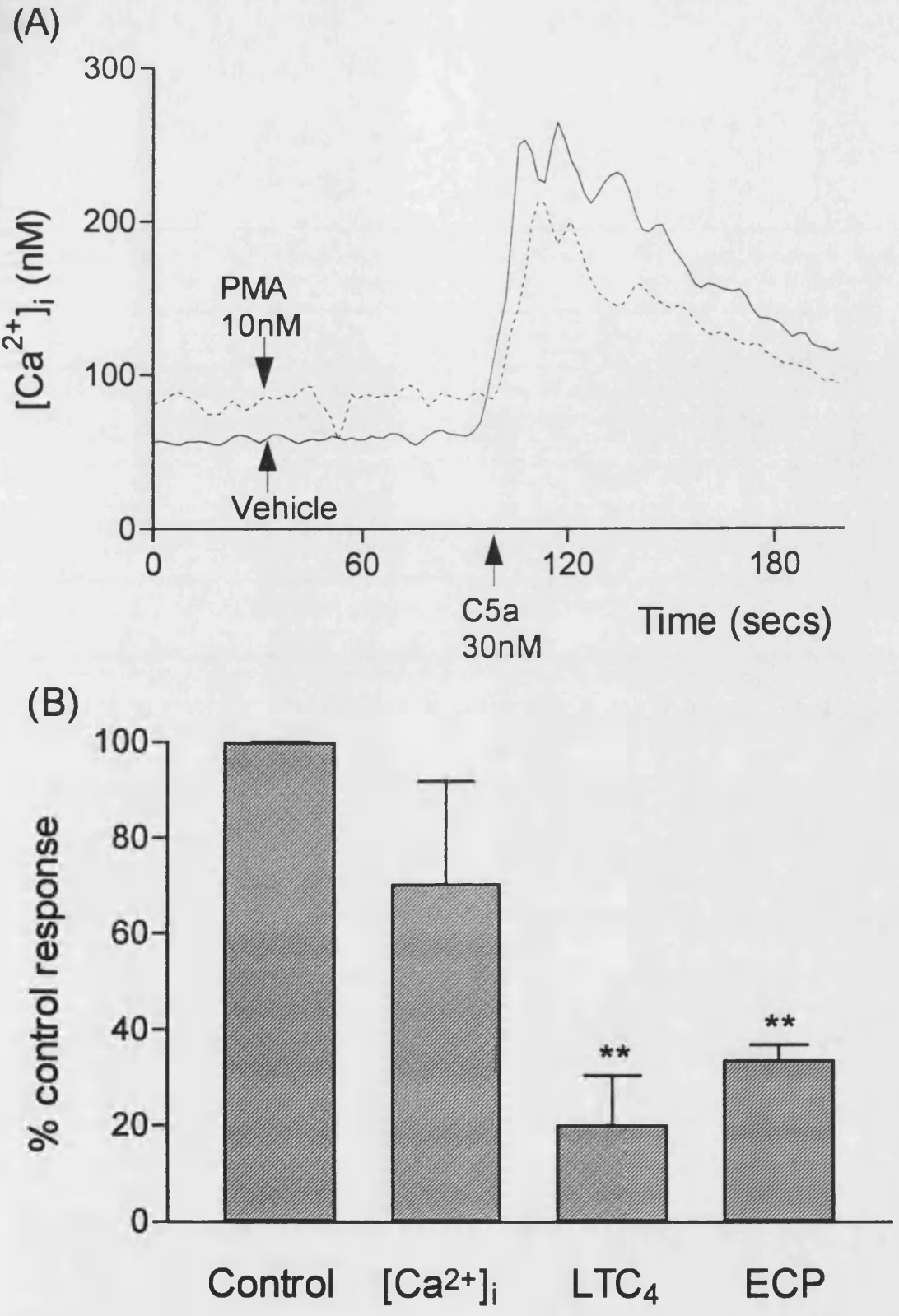


Figure 24

(A) Effect of the PKC activator, PMA on C5a-induced Ca^{2+} mobilisation and (B) graphical representation of this response, as well as LTC_4 and ECP release. Data are mean ± s.e.m. of 4-6 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

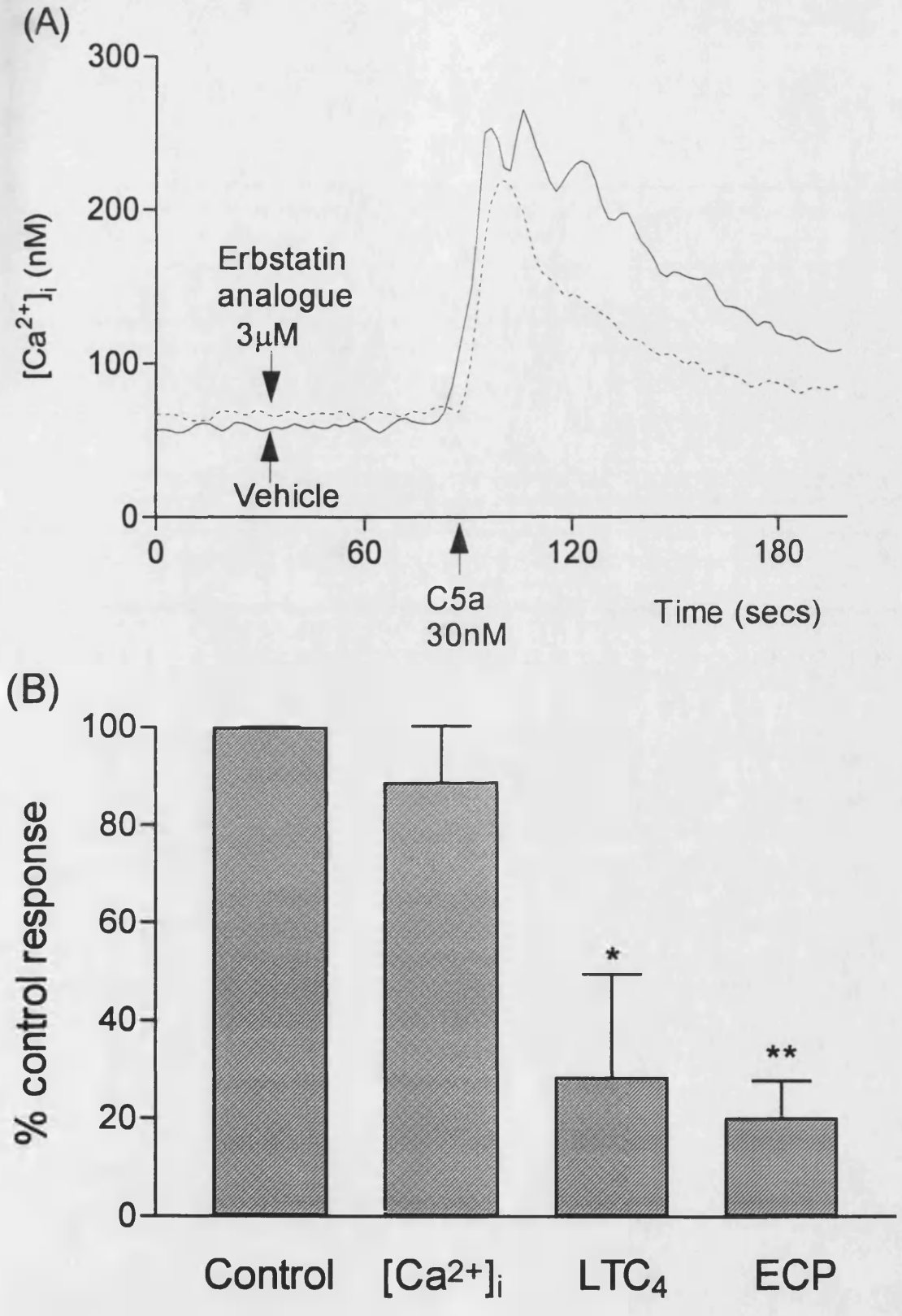


Figure 25

(A) Effect of the tyrosine kinase inhibitor, erbstatin analogue on C5a-induced Ca^{2+} mobilisation and (B) graphical representation of this response, as well as LTC_4 and ECP release. Data are mean ± s.e.m. of 3-4 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

No significant inhibition of the peak increase of $[Ca^{2+}]_i$ (figure 25A) was observed with the erbstatin analogue (3μM). Activation of tyrosine kinase did appear to be a common requirement for both LTC_4 and ECP release, as the erbstatin analogue inhibited both these responses (figure 25B), with IC_{50} values of <1μM (93.0 ± 6.4 % inhibition at 1μM, $p<0.005$, n=4) and 1.71(0.8 - 3.5) μM, respectively .

Investigation of the effect of phosphatase inhibitors found that calyculin A and okadaic acid inhibited LTC_4 release, with IC_{50} values of 17.8 (1.8 - 176) nM and 0.29 (0.11 - 0.76) μM, respectively. The low potency of okadaic acid compared with calyculin suggested that PP1 rather than PP2A was involved in the responses. In contrast, these agents had no effect against ECP release. The PP2B inhibitor, cyclosporin A had no effect against either LTC_4 or ECP release. In summary, C5a-induced O_2^- and LTC_4 release appear to be predominantly mediated via a PLC-dependent pathway. LTC_4 release involves activation of PI-3 kinase and tyrosine kinase, as well as PP1. In addition, activation of PKC and elevation of cAMP inhibited LTC_4 release. ECP release appears to be partially dependent upon PLC activation, but may also require tyrosine kinase and/or PLD activation and like LTC_4 release, involves stimulation of tyrosine kinase. Finally, ECP release was inhibited both by inhibition and activation of PKC.

4.2.3 Investigation of the effect of pertussis toxin (PTX) pre-treatment on C5a-induced eosinophil activation

Pretreatment of human eosinophils with PTX (0.2, 1 and 2 μg/ml: 90 minutes, 37°C, 10^7 cells/ml) caused a concentration-dependent inhibition of C5a-induced

increase in $[Ca^{2+}]_i$ (figure26A). PTX pre-treatment (1µg/ml) reduced the increase in $[Ca^{2+}]_i$ by 78.0 ± 13.1 % ($p<0.005$, n=4) and caused 43.9 ± 7.7 % ($p<0.005$, n=4) inhibition of C5a-induced Mn^{2+} influx (figure 26B). Similarly, concentration-effect curves to C5a, for ECP release were also inhibited by PTX pretreatment (figure 27A). PTX pretreatment (1µg/ml) caused 81.1 ± 9.9 % inhibition ($p<0.005$, n=4) of C5a (30nM)-induced ECP release. Comparison of the level of Ca^{2+} mobilisation and ECP release, with increasing concentrations of PTX revealed a close correlation between the degree of inhibition seen against these responses (figure 27B). This suggests that C5a mediates its effects via a PTX-sensitive G_i-like G protein and that this G protein is common for both Ca^{2+} and ECP responses.

4.2.4 Investigation of the effect of selected pharmacological reference agents on C5a-induced eosinophil activation

A range of pharmacological reference agents which possess anti-inflammatory activity or are used in asthma therapy, were tested for their effects against C5a-induced O_2^-, LTC_4 and ECP release in human eosinophils; mean data are shown in table 9.

The β_2-agonist salbutamol, the potassium channel opener cromakalim, the histamine antagonist cetirizine, as well as sodium cromoglycate had no significant effect on the C5a-induced release of O_2^-, LTC_4 or ECP release. The PAF receptor antagonist, WEB 2086 caused some inhibition of LTC_4 release,

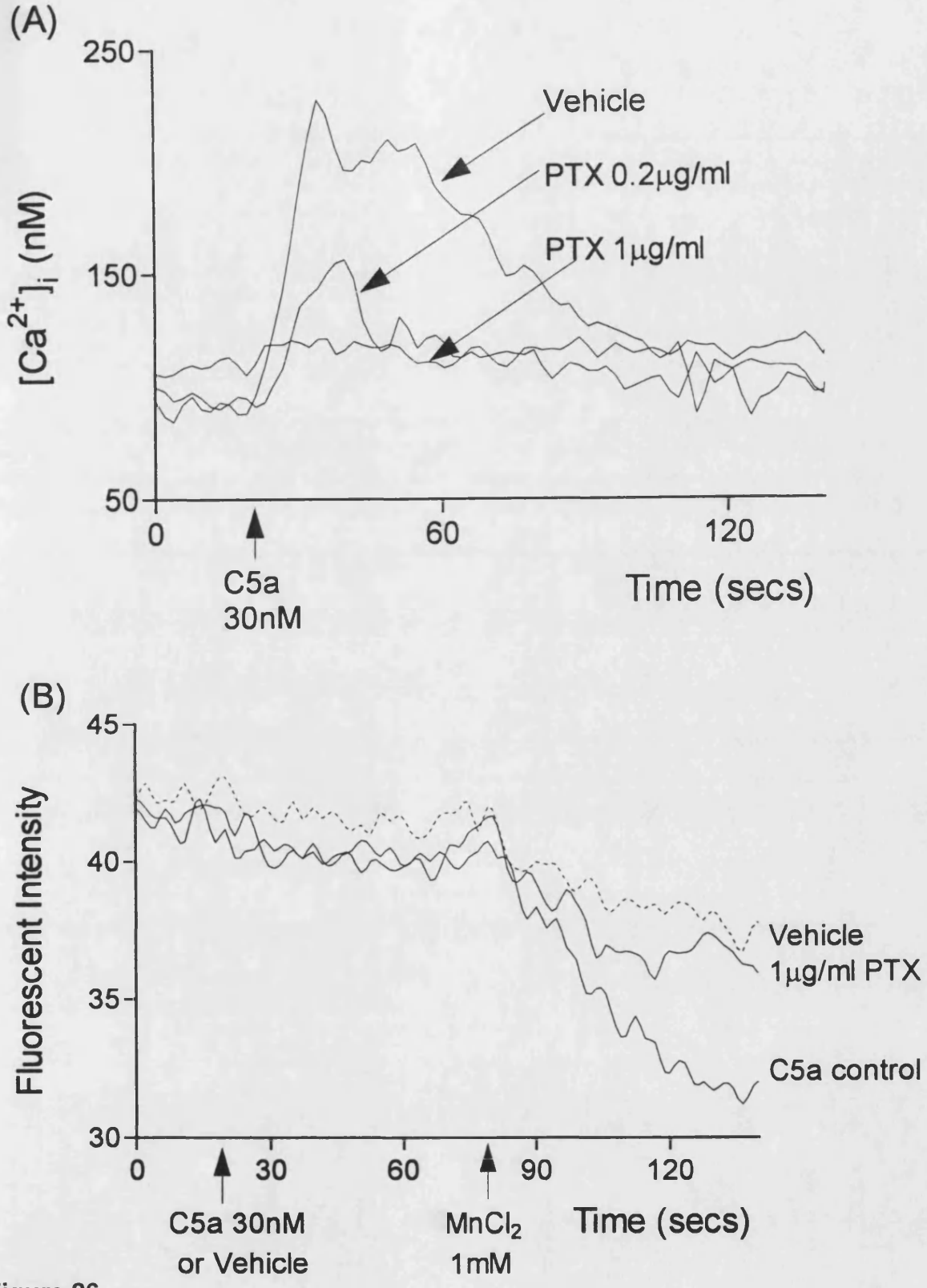


Figure 26

(A) Effect of the PTX pre-treatment on C5a-induced Ca^{2+} mobilisation and (B) Mn^{2+} influx, compared with a control response. Cells were pre-treated for 90 minutes at 37°C, with either vehicle or PTX. Traces are representative of 4 separate experiments.

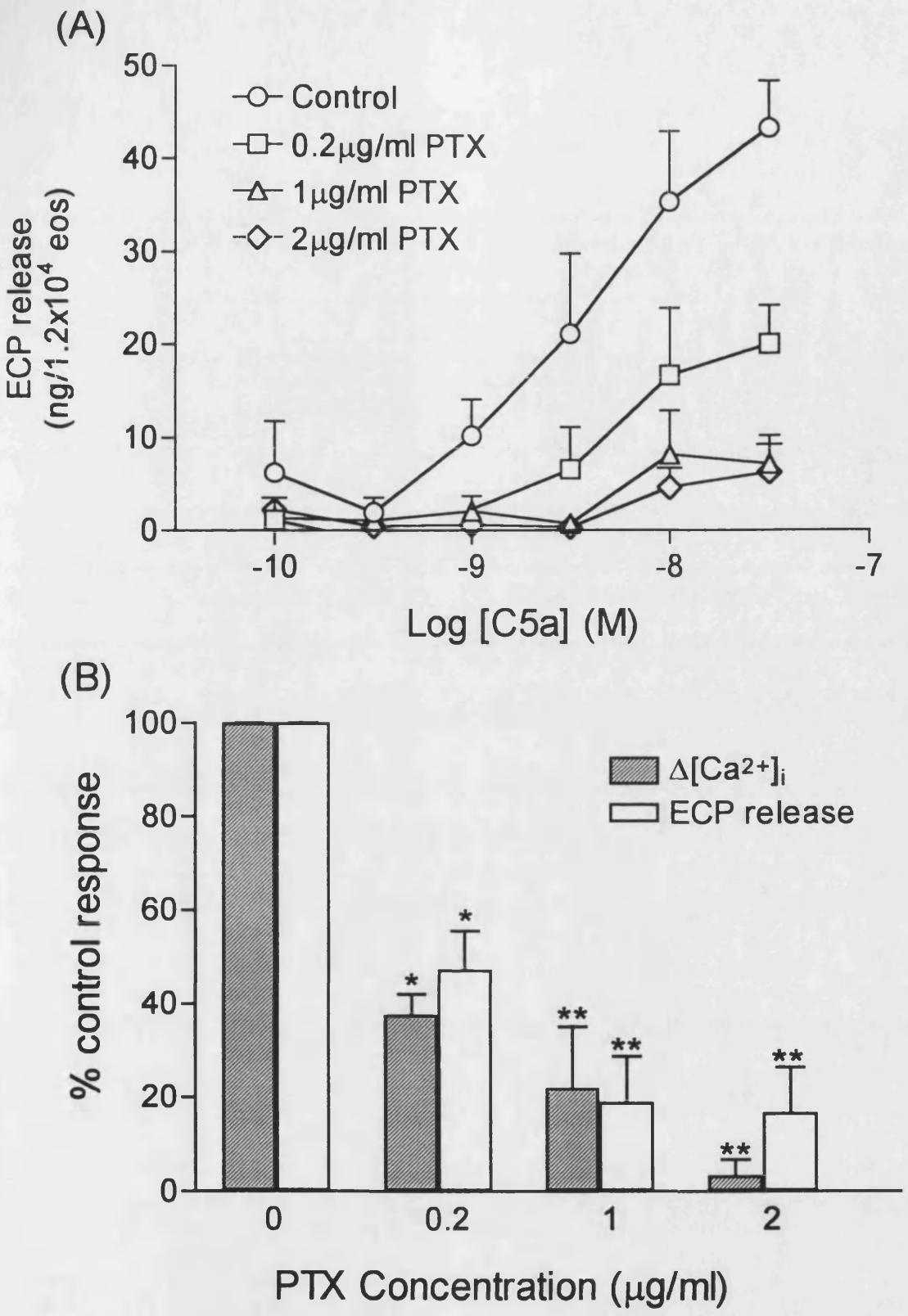


Figure 27

(A) Effect of the PTX pre-treatment on C5a concentration-effect curves for ECP release and (B) comparison of inhibition of C5a (30nM)-induced Ca^{2+} mobilisation and ECP release. Cells were pre-treated for 90 minutes at 37°C, with either vehicle or PTX. Data are mean ± s.e.m. of 4 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

although this was not statistically significant, as well as a very small inhibition of ECP release.

Table 9. Effect of anti-asthma/anti-inflammatory agents, on C5a-induced O_2^-, LTC_4 and ECP release.

Inhibitor	Conc (M)	% Inhibition of C5a-induced responses (Mean ± s.e.m.)		
		O_2^- release	LTC_4 release	ECP release
Cetirizine	$1x10^{-5}$	7.1 ± 9.0	0.5 ± 24.4	6.5 ± 4.0
Cromakalim	$1x10^{-5}$	-4.5 ± 5.1	18.6 ± 14.2	8.7 ± 4.3
Sodium cromoglycate	$1x10^{-6}$	-3.4 ± 5.8	-7.3 ± 16.3	nt
Salbutamol	$1x10^{-6}$	7.0 ± 5.2	6.4 ± 30.6	2.9 ± 4.5
WEB 2086	$1x10^{-5}$	nt	56.5 ± 18.9	**15.1± 2.7****
Indomethacin	$3x10^{-6}$	18.9 ± 10.5	**68.1 ± 10.5****	3.8 ± 9.2
BAY x1005	$1x10^{-5}$	nt	**88.2 ± 7.4****	7.8 ± 10.8

Data represent mean ± s.e.m of 3-6 separate experiments, nt = not tested.
* denotes $p<0.05$, ** denotes $p<0.005$.

The COX inhibitor, indomethacin was without effect against either O_2^- or ECP release, but was effective at inhibiting LTC_4 release. This may suggest that C5a stimulated synthesis of prostanoids which were stimulatory for leukotriene generation. The LSI, Bay x1005 selectively abolished the release of LTC_4, whilst having no effect on ECP release.

4.3 Thapsigargin-induced human eosinophil activation

4.3.1 Investigation of the role of Ca^{2+} in thapsigargin-induced eosinophil activation.

A range of agents which modulate Ca^{2+}-dependent effects were tested against thapsigargin-induced LTC_4 release (in the absence of cytochalasin B); mean data are shown below in table 10.

Table 10. Effect of agents that modulate Ca^{2+} influx, on thapsigargin-induced LTC_4 release.

Inhibitor	**Concentration (M)**	% Inhibition of thapsigargin-induced LTC_4 release Mean ± SEM
EGTA	$3x10^{-3}$	**97.9 ± 0.8****
$NiCl_2$	$3x10^{-3}$	**88.3 ± 3.6****
SK&F 96365	$1x10^{-5}$	**92.5 ± 2.8****
Nifedipine	$3x10^{-6}$	22.9 ± 15.3
Diltiazem	$1x10^{-5}$	-17.8 ± 10.4
Verapamil	$1x10^{-5}$	-5.3 ± 3.3
KCL	$5x10^{-2}$	**67.3 ± 10.3***
Benzamil	$3x10^{-5}$	**-124.9 ± 40.2***

Data represent mean ± s.e.m. of 4-8 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

Addition of the Ca^{2+} chelating agent, EGTA caused concentration-dependent inhibition of thapsigargin-induced LTC_4 release, with a mean IC_{50} value of 0.28 (0.20 - 0.39) mM. This indicated that these responses required extracellular Ca^{2+}. Investigation of the type of Ca^{2+} influx pathway involved in thapsigargin - induced LTC_4 production, revealed an identical profile to that seen with C5a-induced LTC_4 release. The RMCE blockers $NiCl_2$ (3mM) and SK&F 96365

(30μM) inhibited the thapsigargin-induced Ca^{2+} influx response, in fura-2-loaded cells, by 98.9 ± 0.8 % ($p<0.005$, figure 28A) and 69.4 ± 11.0 % ($p<0.05$, figure 29A). Furthermore, both $NiCl_2$ and SK&F 93365 also caused concentration-dependent inhibition of thapsigargin-stimulated LTC_4 release, with mean IC_{50} values of 0.09 (0.04 - 0.18) mM (figure 28B) and 3.0 (2.5 - 3.5) μM (figure 29B), respectively. In contrast, the VOC blockers nifedipine, diltiazem and verapamil were without effect against thapsigargin-mediated LTC_4 release. Additionally, membrane depolarisation by KCl also caused marked inhibition of leukotriene generation. Thus, RMCE rather than VOC-mediated Ca^{2+} entry is necessary for thapsigargin-induced LTC_4 release.

The Na^+/Ca^{2+} exchange inhibitor, benzamil (30μM) differed in its inhibitory profile against C5a and thapsigargin, as it inhibited C5a-induced LTC_4 release, but enhanced the response to thapsigargin.

4.3.2 Investigation of the second messengers involved in thapsigargin-induced eosinophil activation.

Investigation of the second messengers that might be involved in thapsigargin-induced eosinophil activation was carried out using a range of agents which modulate second messengers; mean data are shown in table 11.

Addition of U73122 (1μM), prior to the addition of thapsigargin caused marked inhibition (80.6 ± 6.8 %, n=2) of the Ca^{2+} influx response (figure 30A). In contrast, addition of U73122 after the addition of thapsigargin (figure 30B) only

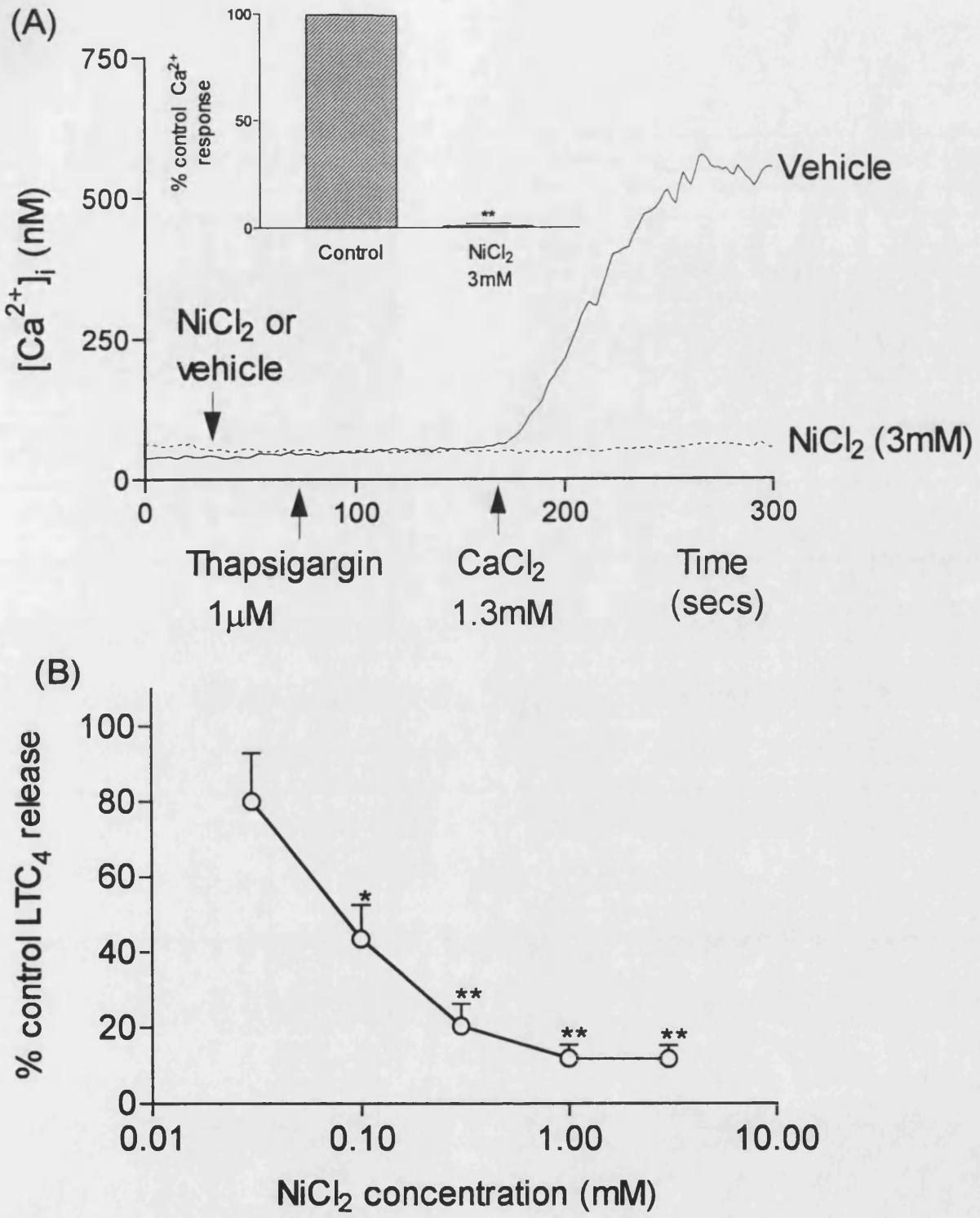


Figure 28

(A) Effect of $NiCl_2$ on thapsigargin-induced Ca^{2+} influx and (inset) graphical representation of this response (n=2). (B) Concentration-dependent inhibition of thapsigargin-induced LTC_4 release by $NiCl_2$. Data are mean ± s.e.m. of 8 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

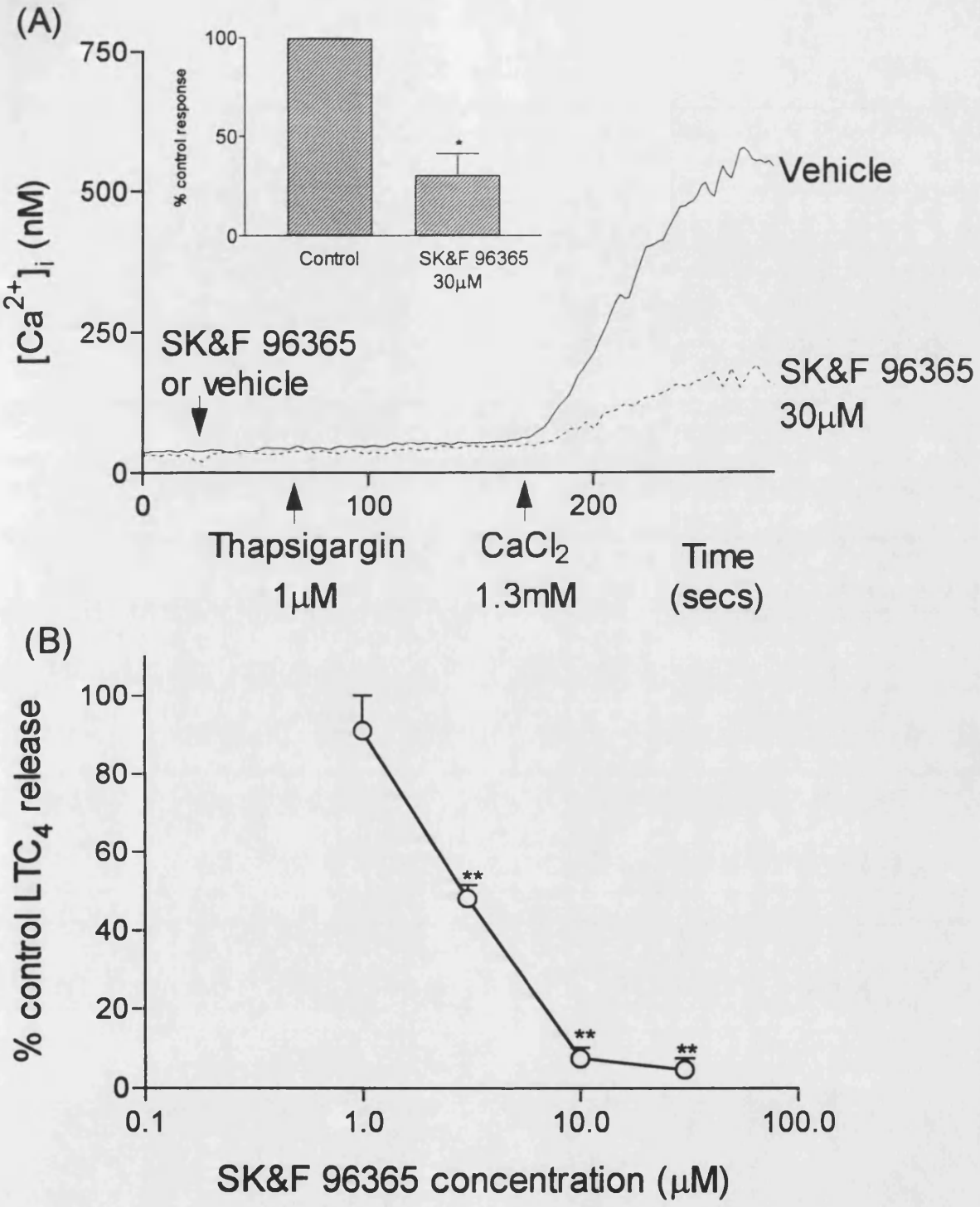


Figure 29

(A) Effect of SK&F 96365 (30μM) on thapsigargin-induced Ca^{2+} influx and (inset) graphical representation of this response (n=3). (B) Concentration-dependent inhibition of thapsigargin-induced LTC_4 release by $NiCl_2$ (n=8). Data are mean ± s.e.m. of 3-8 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

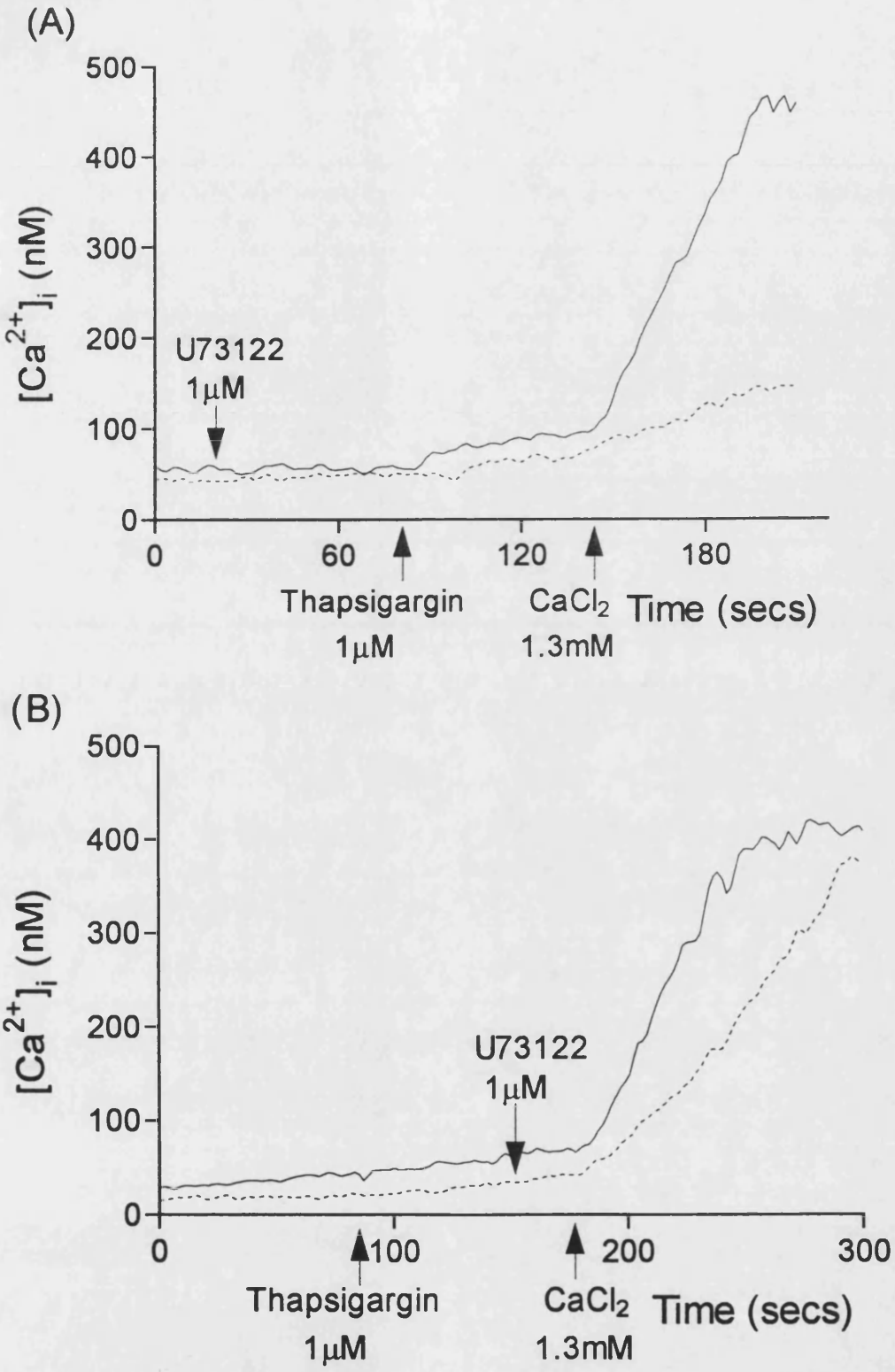


Figure 30

(A) Effect of U73122 (1μM) (dashed line) on thapsigargin-induced Ca^{2+} influx added 1 minute before thapsigargin or (B) added 1 minute after thapsigargin addition. Traces are representative of 2 separate experiments.

caused 31.3 ± 27.1 % (n=2) inhibition. This suggests that U73122 has an effect on the depletion process, rather than an effect on Ca^{2+} influx. U73122 (1μM) caused 99.5 ± 0.5 % inhibition (n=2) of LTC_4 release (in the presence of cytochalasin B), whereas U73343 (1μM) caused only 10.6 ± 36.2 % inhibition, suggesting some specificity of action.

Table 11. Effect of agents that modulate second messengers, on thapsigargin-induced LTC_4 release.

Inhibitor	**Concentration (M)**	**% Inhibition of thapsigargin-induced LTC_4 release Mean ± s.e.m.**
NaF	$2x10^{-2}$	**77.4 ± 6.1****
Forskolin	$3x10^{-5}$	-66.0 ± 26.9
Rolipram	$3x10^{-6}$	**58.0 ± 8.8****
Nitroprusside	$1x10^{-6}$	9.9 ± 4.7
PMA	$1x10^{-8}$	-16.6 ± 23.5
R59022	$1x10^{-5}$	**76.0 ± 2.7****
Bisindolymaleimide	$1x10^{-6}$	-256.8 ± 136.8
Erbstatin analogue	$3x10^{-6}$	**99.0 ± 0.6****
Calyculin A	$1x10^{-7}$	**68.9 ± 14.3***

Data represent mean ± s.e.m. of 4-5 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

Activation of heterotrimeric G proteins by NaF caused a significant reduction of thapsigargin-induced LTC_4 release, suggesting that it may be possible to activate inhibitory G proteins and so inhibit the effects of thapsigargin.

Elevation of cAMP by forskolin or cGMP by sodium nitroprusside had no significant effect on LTC_4 generation. In contrast, inhibition of PDE IV activity by rolipram, caused marked inhibition of release of LTC_4.

Activation of PKC, by PMA caused marked inhibition of the Ca^{2+} influx response, whether it was added before (61.2 ± 20.6 %) or after (73.0 ± 17.7 %) the addition of thapsigargin. This suggests that PMA was exerting its effects distal to the depletion of intracellular Ca^{2+} stores. However, somewhat surprisingly PMA had no effect against LTC_4 release; particularly since the diacylglycerol (DAG) kinase inhibitor R59022, which would also activate PKC, did cause substantial inhibition of thapsigargin-induced LTC_4 release. Furthermore, inhibition of PKC by bisindolymaleimide caused a trend towards enhancement of leukotriene generation.

In common with C5a-induced LTC_4 release, the thapsigargin-mediated responses were concentration-dependently inhibited by the tyrosine kinase inhibitor, erbstatin analogue and the phosphatase inhibitor, calyculin with IC_{50} values of 0.39 (0.19 - 0.82) μM and 65.5 (39.3 - 109) nM, respectively.

In summary, thapsigargin appears to cause depletion of intracellular Ca^{2+} stores via a PLC-dependent pathway and stimulated a Ca^{2+} influx response which is inhibited by activation of PKC. Leukotriene generation may be inhibited by activation of an inhibitory G protein, as well as by elevation of cAMP via inhibition of PDE IV. Finally, thapsigargin-induced LTC_4 release involves activation of tyrosine kinase as well as PP1 (and/or PP2A).

4.3.3 Investigation of the effect of pertussis toxin (PTX) pretreatment on thapsigargin-induced eosinophil activation.

PTX pretreatment of human eosinophils had only a small inhibitory effect

(29.8 ± 3.3 %, $p<0.005$, n=4) on the thapsigargin-induced increase in $[Ca^{2+}]_i$ and had no effect (-22.1± 24.2 % inhibition, $p>0.05$, n=3) on the Mn^{2+} influx response (figure 31). This suggests that thapsigargin-induced Ca^{2+} influx is not predominantly mediated via a PTX-sensitive G protein, nor is there activation of a tonic inhibitory PTX-sensitive G protein, as there was no enhancement of thapsigargin-induced Ca^{2+} influx.

4.3.4 Investigation of the effect of selected pharmacological reference agents on thapsigargin-induced eosinophil activation

A range of pharmacological reference agents which possess anti-inflammatory activity or are used in asthma therapy, were tested for their effects against thapsigargin-induced LTC_4 generation in human eosinophils; mean data are shown below in table 12.

Table 12. Effect of anti-asthma/anti-inflammatory agents, on thapsigargin-induced LTC_4 release.

Inhibitor	Concentration (M)	% Inhibition of thapsigargin-induced LTC_4 release Mean ± s.e.m.
Cetirizine	$1x10^{-5}$	**-44.2 ± 7.7***
Cromakalim	$1x10^{-5}$	**-65.0 ± 7.2****
Salbutamol	$1x10^{-6}$	-53.2 ± 17.9
Indomethacin	$3x10^{-6}$	9.6 ± 9.3
BAY x1005	$1x10^{-5}$	**96.3 ± 1.3****
WEB 2086	$1x10^{-5}$	**-50.9 ± 9.6***

Data represent mean ± s.e.m. of 4 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

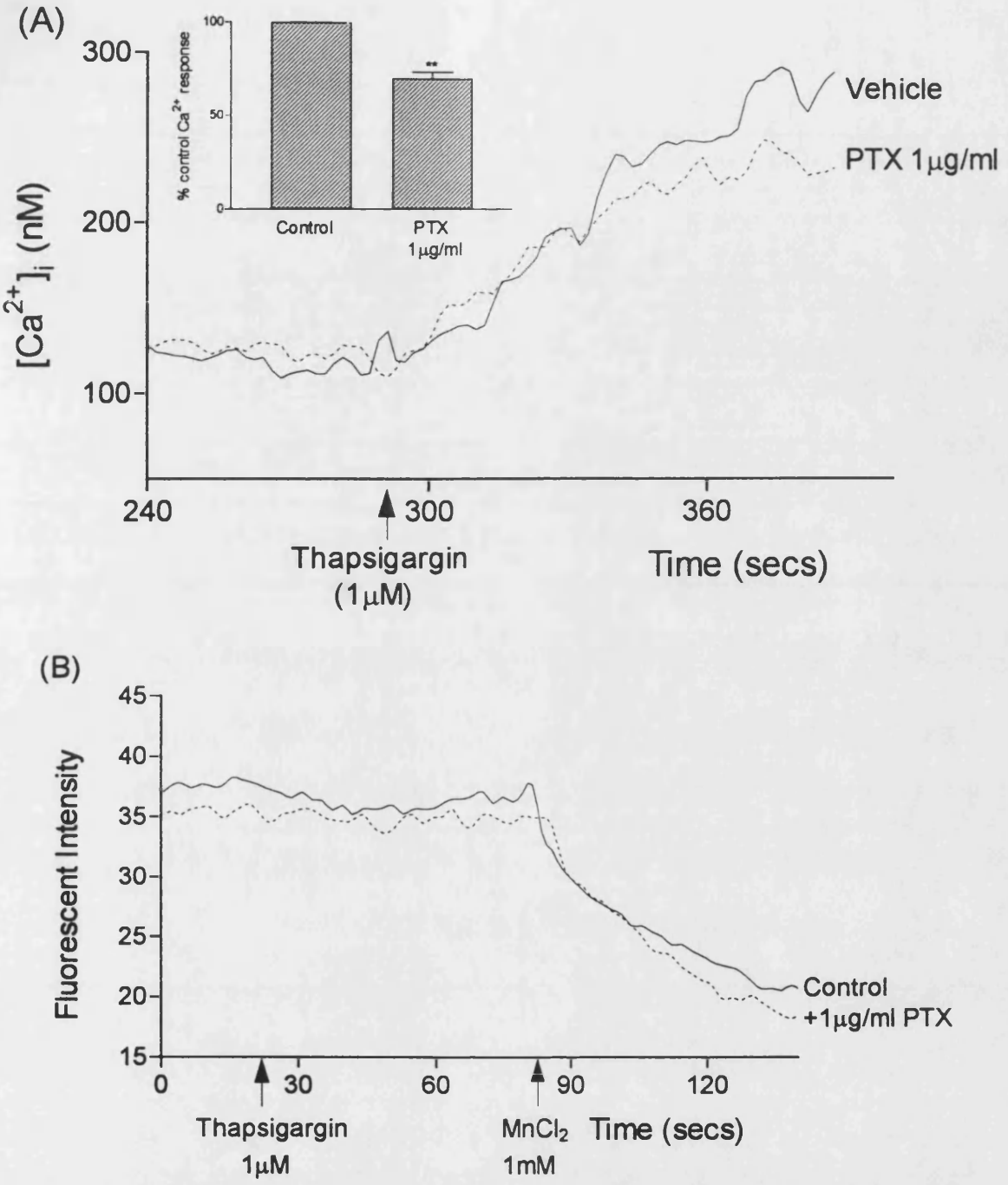


Figure 31

(A) Effect of the PTX pre-treatment on thapsigargin-induced Ca^{2+} influx, with graphical representation of this response inset (n=4) and (B) effect of PTX pre-treatment on Mn^{2+} influx, compared with a control response. Cells were pre-treated for 90 minutes at 37°C, with either vehicle or PTX. Traces are representative of 3-4 separate experiments are mean ± s.e.m. of 4 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

The β_2-agonist salbutamol, the potassium channel opener cromakalim, the histamine antagonist cetirizine, as well as the PAF antagonist WEB 2086 caused enhancement of thapsigargin-induced LTC_4 release. The COX inhibitor, indomethacin was without effect, whilst the LSI, Bay x1005 abolished the release of LTC_4.

4.4 FMLP-induced human eosinophil activation

4.4.1 Investigation of the role of Ca^{2+} in FMLP-induced eosinophil activation.

A range of agents which modulate Ca^{2+}-dependent effects were tested against FMLP-induced eosinophil activation; mean data are shown below in table 13.

Table 13. Effect of agents that modulate Ca^{2+} influx, on fMLP-induced O_2^-, LTC_4 and ECP release.

Inhibitor	Conc (M)	% Inhibition of FMLP-induced responses (Mean ± s.e.m.)		
		O_2^- release	LTC_4 release	ECP release
EGTA	$3x10^{-3}$	**74.6 ± 10.1***	**94.5 ± 2.4****	7.9 ± 15.9
$NiCl_2$	$3x10^{-3}$	**41.9 ± 10.8****	**83.1 ± 3.8****	21.7 ± 15.0
SK&F 96365	$3x10^{-5}$	**63.8 ± 6.0****	**90.3 ± 3.2****	3.4 ± 10.6
Nifedipine	$3x10^{-6}$	-0.5 ± 6.6	8.0 ± 16.3	0.6 ± 15.5
Diltiazem	$3x10^{-6}$	18.6 ± 7.7	**38.8 ± 12.5***	#5.5 ± 15.8
Verapamil	$3x10^{-6}$	**17.1 ± 4.9***	**36.9 ± 14.2***	#-8.2 ± 17.4
KCl	$5x10^{-2}$	**69.5 ± 9.7****	**64.1 ± 17.6***	32.2 ± 21.3
Benzamil	$3x10^{-5}$	nt	-64.8 ± 25.2	-21.1 ± 17.7

# denotes inhibitor concentration of $1x10^{-5}$M used. Data represent mean ± sem of 4-10 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

EGTA (3mM) caused concentration-dependent inhibition of O_2^- and LTC_4 release, with mean IC_{50} values of 0.75 (0.48 - 1.2) mM and 0.58 (0.47 - 0.73) mM, respectively, indicating a requirement for extracellular Ca^{2+} for these responses (figure 32A). In contrast, FMLP-induced ECP release was not inhibited by EGTA, up to a concentration of 3mM.

Having established a requirement for extracellular Ca^{2+} for O_2^- and LTC_4 release, the pathway for Ca^{2+} influx was investigated. $NiCl_2$ caused concentration-dependent inhibition of O_2^- release, with an IC_{50} value of 3.6 (1.3 - 9.7) mM and caused marked inhibition of LTC_4 release (figure 32B) with an IC_{50} < 0.1mM (71.5 ± 10.8 % inhibition at 0.1mM, $p<0.005$). Similarly, SK&F 96365 caused inhibition of O_2^- and LTC_4 release in a concentration-dependent manner, with mean IC_{50} values of 16.0 (12.1 - 21.1) μM and 11.0 (7.8 - 15.7) μM, respectively (figure 32C). In contrast, FMLP-induced ECP release was not inhibited by either $NiCl_2$ or SK&F 96365, providing further evidence that this response was not dependent upon Ca^{2+} influx. Furthermore, addition of KCl markedly inhibited O_2^- and LTC_4 release, but had no significant effect on ECP release. The VOC-blocking agents nifedipine, diltiazem and verapamil had little or no effect on O_2^-, LTC_4 or ECP release. Although there was some inhibition of LTC_4 release by diltiazem and verapamil at 3μM, no further inhibition was seen with a higher concentration. Finally, benzamil had no significant effect on FMLP-mediated LTC_4 and ECP release, although there was a trend towards enhancement of leukotriene release, which was similar to its effects on thapsigargin.

4.4.2 Investigation of the second messengers involved in FMLP-induced eosinophil activation.

Investigation of the second messengers that might be involved in FMLP-induced eosinophil activation was carried out using a range of agents which modulate second messengers; mean data are shown in table 14 and are represented graphically in figure 33.

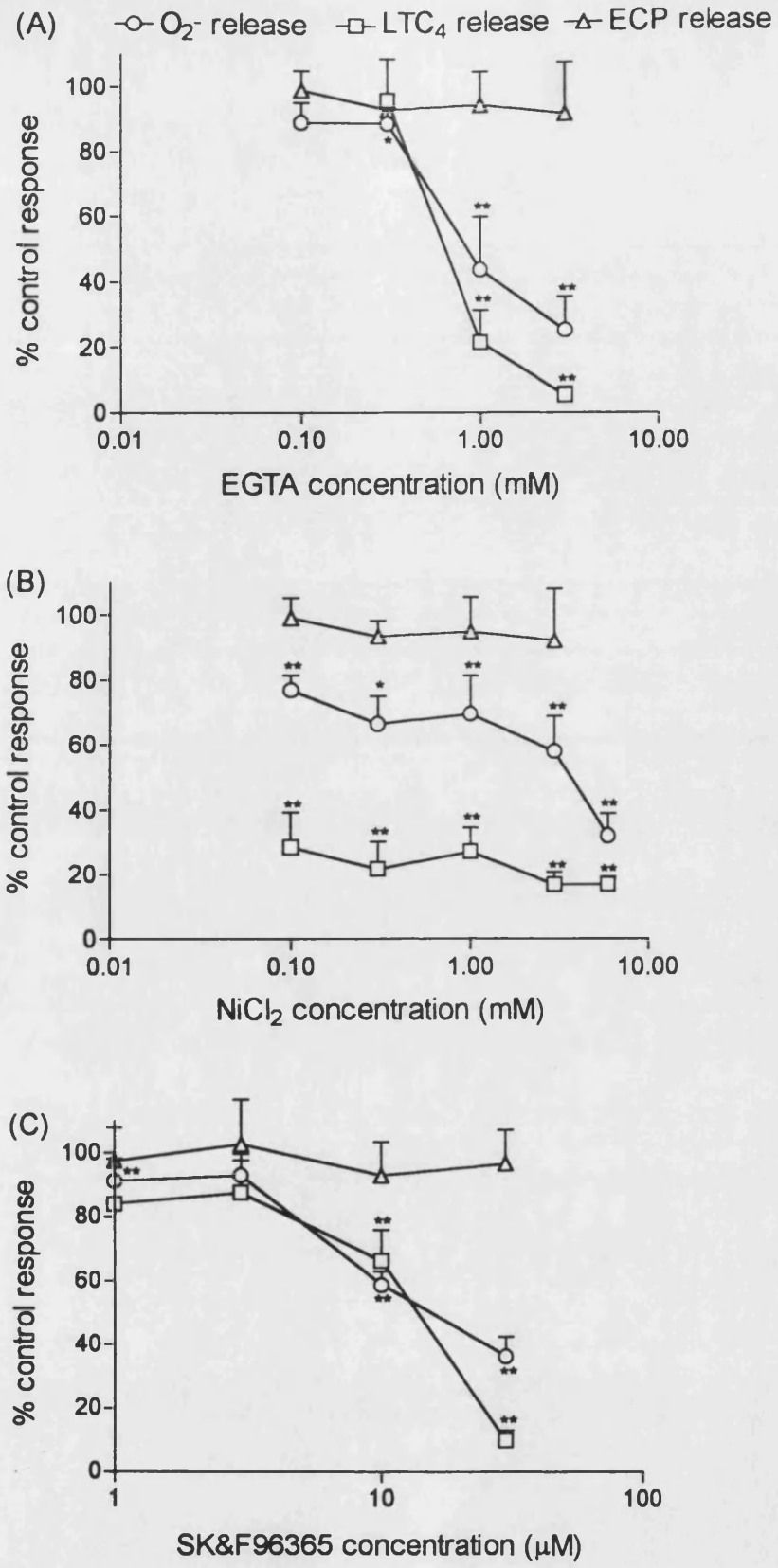


Figure 32

(A) The effect of increasing concentrations of EGTA, (B) $NiCl_2$ and (C) SK&F 96365 on FMLP-induced O_2^-, LTC_4 and ECP release in human eosinophils. Data represent mean ± s.e.m of 4-10 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

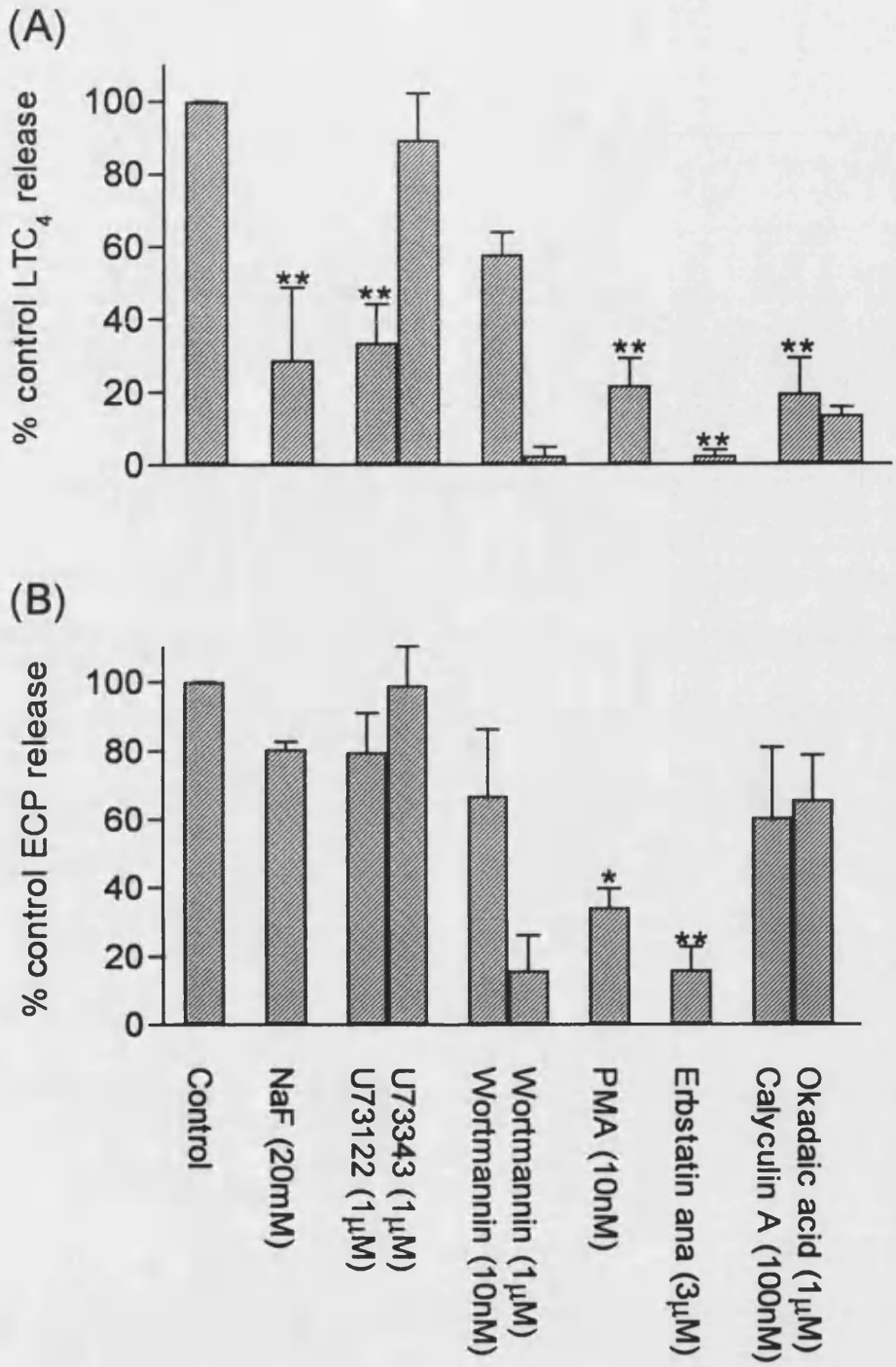


Figure 33

(A) Effect of agents which modulate second messengers, on FMLP-induced LTC_4 release and (B) ECP release. Wortmannin and okadaic acid data are mean ± s.d. of 2 separate experiments, otherwise data represent mean ± s.e.m of 4-10 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

Activation of heterotrimeric G proteins by NaF had a marked inhibitory effect on FMLP-induced LTC_4 release, but did not cause any inhibition of ECP release.

Table 14. Effect of agents that modulate second messengers, on fMLP-induced O_2^-, LTC_4 and ECP release.

Inhibitor	Conc (M)	% Inhibition of FMLP-induced responses (Mean ± s.e.m.)		
		O_2^- release	LTC_4 release	ECP release
NaF	$2x10^{-2}$	nt	**71.1 ± 5.8****	19.5 ± 17.2
U73122	$1x10^{-6}$	**92.1 ± 2.9****	**66.4 ± 10.5****	20.5 ± 11.5
U73343	$1x10^{-6}$	7.8 ± 6.9	10.5 ± 12.9	1.0 ± 11.2
Wortmannin	$1x10^{-8}$	nt	$42.2 ± 6.2	33.3 ± 19.5
Wortmannin	$1x10^{-6}$	nt	$97.5 ± 2.5	**84.1 ± 10.3***
Forskolin	$3x10^{-5}$	41.7 ± 12.4	-12.2 ± 15.0	-9.0 ± 12.9
Nitroprusside	$1x10^{-6}$	13.2 ± 6.5	-71.0 ± 34.9	-23.7 ±11.8
PMA	$1x10^{-8}$	nt	**78.3 ± 7.5****	**65.8 ± 5.6***
Bisindoly maleimide	$1x10^{-6}$	nt	-49.4 ± 29.3	47.5 ± 26.2
Erbstatin analogue	$1x10^{-5}$	nt	**96.4 ± 0.8****	**94.7 ± 1.2****
Calyculin A	$1x10^{-7}$	nt	**80.5 ± 9.9****	39.6 ± 20.4
Okadaic acid	$1x10^{-6}$	nt	$86.3 ± 2.2	34.5 ± 13.0
Cyclosporin A	$1x10^{-6}$	nt	$13.9 ± 12.1	-13.0 ± 11.9

N.B. $ denotes mean ± s.d. of n=2 separate experiments, otherwise data represent mean ± s.e.m of 4-10 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

FMLP-induced ECP release was not inhibited by U73122 (1μM), whereas O_2^- and LTC_4 release were substantially inhibited. U73343 (1μM) was without effect against any of the responses, suggesting some specificity of action. Thus the O_2^- and LTC_4 responses appear to be mediated predominantly via PLC-dependent processes, whereas ECP release requires activation of a different pathway(s).

Wortmannin caused marked inhibition of both LTC_4 and ECP release at a concentration of 1μM, but not at 10nM. This contrasts with the more potent effects of wortmannin against C5a-induced leukotriene generation, and suggests that FMLP-mediated effects may have a greater-dependence on tyrosine kinase and/or PLD activation, rather than PI-3 kinase.

The cAMP and cGMP elevators, forskolin and sodium nitroprusside had no significant effect on any of the responses, which was consistent with the data obtained for C5a-induced responses.

Activation of PKC by PMA markedly inhibited LTC_4 release and inhibition of PKC by bisindolymaleimide caused some enhancement of leukoriene generation, although this was not statistically significant. FMLP-induced ECP release was reduced by both activation and inhibition of PKC. This paradoxical effect was also seen with C5a-induced ECP release.

Activation of tyrosine kinase did appear to be a common requirement for both LTC_4 and ECP release, as the erbstatin analogue inhibited both these responses, with IC_{50} values of 1.1 (0.5 - 2.7) μM and 1.4 (0.9 - 2.2) μM, respectively .

Investigation of the effect of phosphatase inhibitors found that calyculin A and okadaic acid inhibited LTC_4 release, with IC_{50} values of 63.4 (44.7 - 89.9) nM and 0.21 (0.17 - 0.26) μM, respectively, but had no effect against ECP release. The low potency of okadaic acid compared with calyculin suggested that PP1 rather than PP2A was involved in leukotriene generation. The PP2B inhibitor,

cyclosporin A was without effect against either LTC_4 or ECP release.

In summary, FMLP-induced O_2^- and LTC_4 release appear to be predominantly mediated via a PLC-dependent pathway. LTC_4 release also involves activation of tyrosine kinase, PP1 and perhaps PLD, whilst activation of PKC inhibited the response. ECP release appears to be independent of PLC activation, but may be mediated via tyrosine kinase and/or PLD activation. Paradoxically, ECP release was inhibited both by inhibition and activation of PKC.

4.4.3 Investigation of the effect of pertussis toxin (PTX) pretreatment on FMLP-induced eosinophil activation.

Pretreatment of human eosinophils with PTX (0.2, 1 and 2 µg/ml: 90 minutes, 37°C, 10^7 cells/ml) caused concentration-dependent inhibition of fMLP-induced increase in $[Ca^{2+}]_i$ (figure 34). PTX pretreatment (1µg/ml) reduced the FMLP-mediated increase in $[Ca^{2+}]_i$ by 91.2 ± 4.5 % ($p<0.005$, n=4). Similarly, concentration-effect curves to FMLP, for ECP release were also inhibited by PTX pretreatment (figure 35A). PTX pretreatment (1µg/ml) caused 94.2 ± 3.5 % inhibition ($p<0.005$, n=4) of FMLP (1µM)-induced ECP release. Comparison of the level of Ca^{2+} mobilisation with LTC_4 and ECP release, using increasing concentrations of PTX, revealed a close correlation between degree of inhibition seen for these responses (figure 35B). This suggests that FMLP mediated its effects via a PTX-sensitive G_i-like G protein and that this G protein is common for Ca^{2+}, LTC_4 and ECP responses.

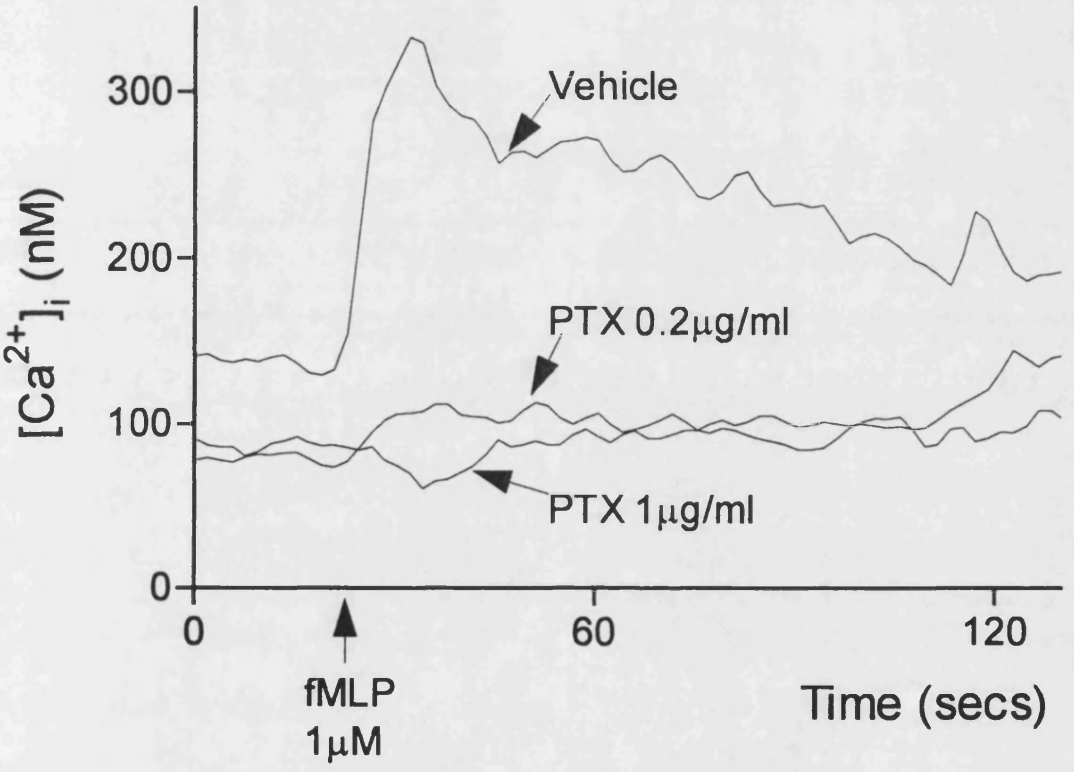


Figure 34

(A) Effect of PTX pretreatment on FMLP-induced Ca^{2+} mobilisation compared with a control response. Cells were pretreated for 90 minutes at 37°C, with either vehicle or PTX. Traces are representative of 4 separate experiments.

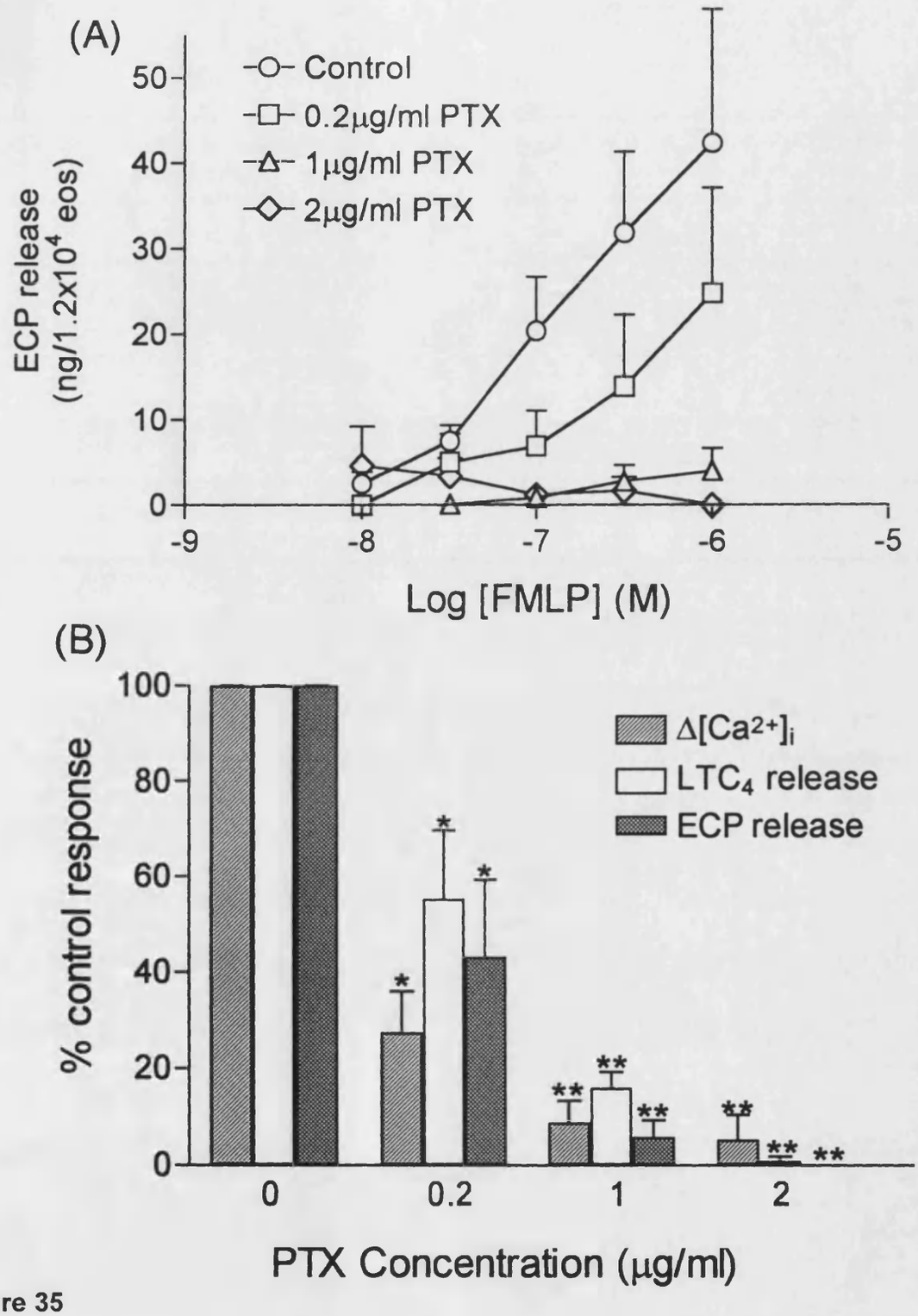


Figure 35

(A) Effect of PTX pretreatment on FMLP concentration-effect curves for ECP release and (B) comparison of inhibition FMLP (1μM)-induced Ca^{2+} mobilisation, LTC_4 and ECP release. Cells were pretreated for 90 minutes at 37°C, with either vehicle or PTX. Data are mean ± s.e.m. of 4 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

4.4.4 Investigation of the effect of selected pharmacological reference agents on FMLP-induced eosinophil activation.

A range of pharmacological reference agents which possess anti-inflammatory activity or are used in asthma therapy, were tested for their effects against FMLP-induced O_2^-, LTC_4 and ECP release in human eosinophils; mean data are shown in table 15.

Table 15. Effect of anti-asthma/anti-inflammatory agents, on fMLP-induced O_2^-, LTC_4 and ECP release.

Inhibitor	Conc (M)	% Inhibition of FMLP-induced responses (Mean ± s.e.m.)		
		O_2^- release	LTC_4 release	ECP release
Cetirizine	$1x10^{-5}$	**20.3 ± 4.9***	-17.9 ± 6.9	11.4 ± 6.9
Cromakalim	$1x10^{-5}$	35.4 ± 16.1	-5.9 ± 15.4	11.9 ± 3.7
Sodium cromoglycate	$1x10^{-6}$	32.2 ± 20.0	-16.4 ± 18.6	nt
Salbutamol	$1x10^{-6}$	29.0 ± 9.7	-15.3 ± 9.4	1.9 ± 13.5
WEB 2086	$1x10^{-5}$	nt	18.6 ± 11.3	8.7± 1.5
Indomethacin	$3x10^{-6}$	**27.3 ± 6.6***	-0.2 ± 15.1	9.2 ± 9.1
BAY x1005	$1x10^{-5}$	nt	**99.6 ± 0.4****	34.6 ± 18.8

Data represent mean ± s.e.m of 4 separate experiments, * denotes $p<0.05$, ** denotes $p<0.005$.

The β_2-agonist salbutamol, the potassium channel opener cromakalim, the histamine antagonist cetirizine, as well as sodium cromoglycate had no significant effect on FMLP-induced release of O_2^-, LTC_4 or ECP release. The PAF receptor antagonist, WEB 2086 and the COX inhibitor, indomethacin had little or no effect against LTC_4 or ECP release. Finally, the LSI Bay x1005 selectively abolished the release of LTC_4, whilst having no effect on ECP.

4.5 LTD_4-induced human eosinophil activation

4.5.1 Regulation of LTD_4-induced increases in $[Ca^{2+}]_i$ in human eosinophils.

Chelation of extracellular Ca^{2+} by EGTA (4mM) inhibited the LTD_4-induced rise in $[Ca^{2+}]_i$ by 57.8 ± 21.1 % ($p>0.05$, n=3; figure 36B), although this did not achieve statistical significance. This suggests that a large component of the peak increase in cytosolic free Ca^{2+} was due to Ca^{2+} influx. Preliminary data from a single experiment supported this view, as SK&F 96365 (30μM) and $NiCl_2$ (3mM) caused 65% and 95% inhibition, respectively of the Ca^{2+} mobilisation response.

Activation of PKC by PMA, caused 87.7 ± 11.1 % inhibition ($p<0.005$, n=5; figure 36A and B). This was much greater than the effect of PMA against C5a-induced elevation of $[Ca^{2+}]_i$, but was similar to the inhibition of the thapsigargin-induced response.

The potent PP1 inhibitor, calyculin (100nM) reduced the LTD_4-mediated Ca^{2+} mobilisation response by 41.6 ± 31.5 % ($p>0.05$, n=3), but this effect was not statistically significant. The tyrosine kinase inhibitor, erbstatin analogue (3μM) was without effect (3.4 ± 11.1 % inhibition, n=2; figure 36B).

4.5.2 Investigation of the effect of pertussis toxin (PTX) pretreatment on LTD_4-induced increases in $[Ca^{2+}]_i$ in human eosinophils.

Pretreatment of human eosinophils with PTX (0.2, 1 and 2 μg/ml: 90 minutes,

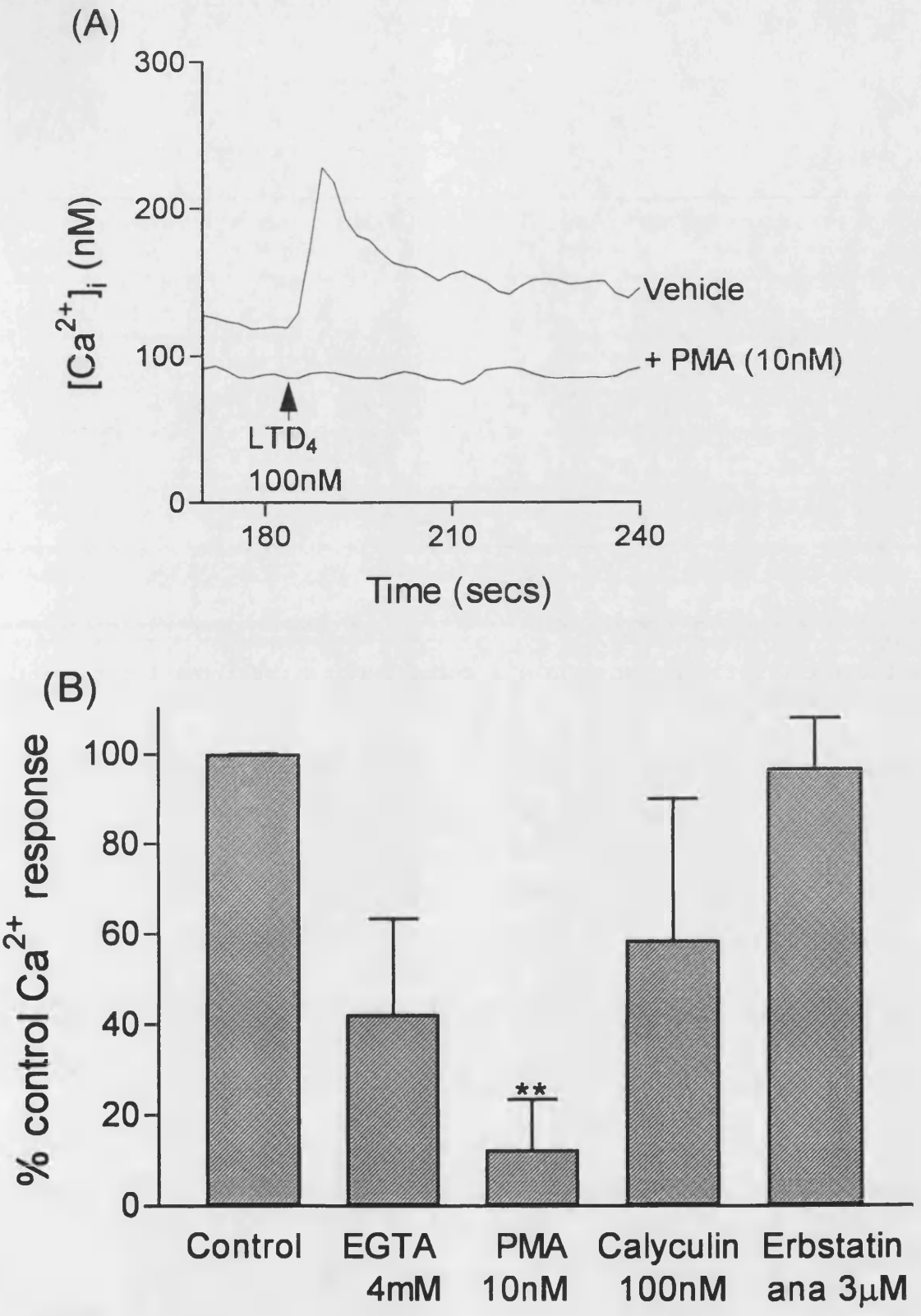


Figure 36

(A) Effect of the PKC activator, PMA on LTD_4-induced Ca^{2+} mobilisation and (B) graphical representation of this response, as well as the effect of EGTA, calyculin and erbstatin analogue on the Ca^{2+} mobilisation response. Data are mean ± s.e.m. of 2-5 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

37°C, 10^7 cells/ml) caused partial inhibition of LTD_4-induced increase in $[Ca^{2+}]_i$ (figure 37A). PTX pretreatment (1µg/ml) reduced the LTD_4-mediated increase in $[Ca^{2+}]_i$ by 46.1 ± 8.7 % ($p<0.005$, n=4; figure 37A inset). However, PTX pretreatment (1µg/ml) did not appear to have any effect on LTD_4-induced Mn^{2+} influx (12.5 ± 24.7 % inhibition, $p>0.05$, n=4; figure 37B). This contrasts with the marked inhibition of C5a- and FMLP-mediated effects and suggests that LTD_4 stimulates a different, PTX-insensitive G protein

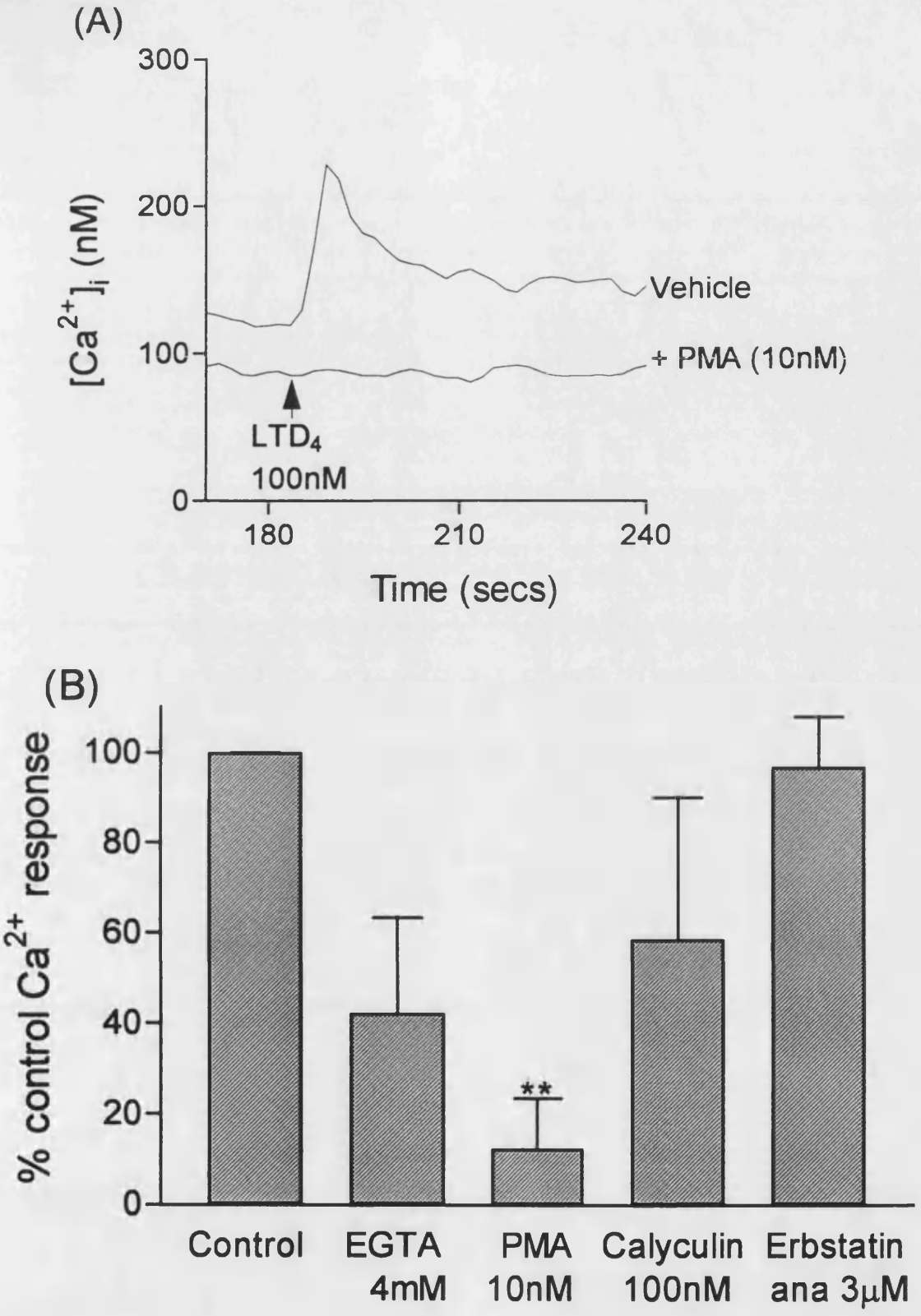


Figure 37

(A) Effect of PTX pretreatment on LTD_4-induced Ca^{2+} mobilisation, with graphical representation of this data (inset) and (B) Mn^{2+} influx, compared with a control response. Cells were pretreated for 90 minutes at 37°C, with either vehicle or PTX. Traces are representative of 4 separate experiments. Graphical data are mean ± s.e.m. of 4 separate experiments; * denotes $p < 0.05$; ** denotes $p < 0.005$.

5. DISCUSSION AND CONCLUSIONS

5.1 Spontaneous O_2^- release

The finding that eosinophils spontaneously released O_2^- was initially rather perplexing, as it delayed progress in measuring agonist-induced eosinophil activation. However, in the process of eliminating this spontaneous activity, it became clear that this response had an interesting pharmacological profile.

Dri *et al.* (1991) reported that eosinophils in contact with ELISA plastic exhibited a respiratory burst response. Furthermore, they found that different biological surfaces had a profound effect on the ability of eosinophils to release O_2^- in response to a range of physiological stimuli.

Examination of the morphology of the cells in this study, showed that the eosinophils were adhering to the plastic surface of microtitre plates and that spreading had taken place. In fact, their appearance was remarkably similar to that observed by Laudanna *et al.* (1993a), when they examined eosinophils adhered via cross-linking of VLA-4. They also reported similar effects with eosinophils adhered via β_2 integrins, but not when adhered to anti-class 1 MHC antigen-coated plates. Furthermore, cross-linking of VLA-4, LFA-1, CR3 or the common β_2 chain by immobilised monoclonal antibody also caused activation of the eosinophil respiratory burst response.

Thus, it is tempting to speculate that adhesion of the eosinophils to plastic may cause cross-linking or a sufficient conformational change in the surface integrins of the eosinophil to cause activation. However, the lack of effect of the anti-

CD11b and CD18 antibodies suggests that CD11b and CD18 integrins were not involved in the spontaneous respiratory burst response.

Interestingly, pretreatment of the MTPs with laminin abolished the spontaneous superoxide response. Functional laminin (VLA-6) receptors are known to be expressed on human eosinophils and are believed to be involved in the adhesive interaction of eosinophils with extracellular matrix proteins. These matrix proteins are present on sub-endothelial basement membranes and on interstitial connective tissue (Georas *et al.,* 1993).

Similarly, it seems likely that eosinophils adhered to gelatin, when it was included in the buffer. Neutrophils are known to adhere to gelatin (denatured collagen), which represents another component of the extracellular matrix (Perskin and Cronstein, 1992). During the process of transendothelial cell migration to the site of inflammation, eosinophils travel through extracellular matrix tissues, en route to the inflammatory site. Therefore, physiologically, it would appear to make good sense that in the absence of an inflammatory stimulus eosinophils do not exhibit a respiratory burst response when they are adhered to the extracellular matrix proteins.

Further circumstantial evidence that the spontaneous respiratory burst response was via integrin-mediated activation, was obtained by studying the signal transduction processes associated with this activity. Comparison of eosinophil O_2^- release, with integrin-mediated neutrophil activation of the respiratory burst response, demonstrated several similarities.

Firstly, stimulation of neutrophils with anti-CD18, anti-LFA-1 or gp150/95 mAbs stimulated a respiratory burst response, which was abolished by the microfilament disrupting agent, cytochalasin B (Berton *et al.,* 1992).

Secondly, activation of respiratory burst in human eosinophils, in response to antibodies to CD18 and LFA-1, was inhibited by wortmannin and tyrosine kinase inhibitors, but not by staurosporine (Laudanna *et al.,* 1993a). This profile of inhibition is identical to that observed with the spontaneous activity in human eosinophils, in the current study. In another study by Laudanna *et al.* (1993b), wortmannin and tyrosine kinase inhibitors prevented neutrophil spreading. They suggested that the process of spreading may be required for integrin-mediated generation of O_2^- and that wortmannin may act via inhibition of myosin light chain kinase (MLCK). However, it has subsequently been demonstrated that wortmannin is an extremely potent inhibitor of phosphatidyinositol (PI) 3-kinase. PI 3-kinase stimulates the production of phosphatidylinositol 3,4,5-trisphosphate ($PI(3,4,5)P_3$), which is known to modulate cytoskeletal rearrangement and it may be via this mechanism that wortmannin exerts its inhibitory effects (Arcaro and Wymann, 1993).

Thirdly, in neutrophils Jaconi *et al.* (1991) showed that integrin-mediated adherence and spreading stimulated the generation of $[Ca^{2+}]_i$ transients, which were abolished in the presence of EGTA. Furthermore, cytochalasin B inhibited both adhesion and the generation of these $[Ca^{2+}]_i$ transients. The requirement for extracellular Ca^{2+} for the spontaneous in human eosinophils, was demonstrated by the inhibitory effect of the Ca^{2+} chelating agent, EGTA.

Moreover, human eosinophils loaded with the Ca^{2+}-indicating fluorescent dye indo-1, adhered and spread onto glass coverslips and spontaneous oscillations of $[Ca^{2+}]_i$ were also observed (J. Schrenzel, personal communication).

The putative inhibitor of PLC-dependent processes, U73122, abolished the O_2^- response, suggesting that release of intracellular Ca^{2+}, via IP_3 generation was involved. One possibility for the activation of PLC may be via tyrosine kinase activity, as it has previously been shown in Chinese Hamster Ovary cells transfected with m5 muscarinic receptors, that Ca^{2+}-dependent tyrosine phosphorylation of PLCγ is capaple of stimulating IP_3 generation (Gusovsky *et al.,* 1993). Furthermore, eosinophils are believed to possess PLCγ, as it has recently be suggested that stimulation of degranulation responses in human eosinophils, by sIgA-coated beads is mediated via tyrosine kinase and PLCγ activation (Kato *et al.*, 1995).

Investigation of the type of Ca^{2+} influx pathway(s) that may be present in human eosinophils was carried out using a range of agents with different pharmacological activities. The profile expected for a RMCE pathway is for Ni^{2+} and SK&F 96365 to be inhibitory, whereas VOC-blockers (e.g. nifedipine, diltiazem and verapamil) would have no effect (Benham *et al.,* 1989).

Therefore, the spontaneous O_2^- response in eosinophils appeared to be dependent upon Ca^{2+} influx, via a receptor-mediated pathway, as SK&F 96365 and the inorganic cation, Ni^{2+} blocked the response, whereas the VOC blockers nifedipine and diltiazem were without effect.

SK&F 96365 is a useful tool for investigating Ca^{2+} influx pathways and has been used for distinguishing between different types of receptor-mediated Ca^{2+} influx channels, particularly in platelets and PC12 cells (Sargeant *et al.*, 1992; Fasolato *et al.*, 1990). SK&F 96365 is also known to have several non-specific effects, including blockade of potassium channels (Krautwurst *et al.*, 1993; Schwarz *et al.*, 1994) and cytochrome P-450 enzymes (Rodrigues *et al.*, 1987). The lack of potent and selective inhibitors of RMCE necessitate the use of agents such as Ni^{2+} and SK&F 96365 when this influx pathway is investigated. However, careful interpretation of the data obtained with these inhibitors is required and it is necessary to compare the effects of several agents that modulate Ca^{+} influx, rather than considering each agent in isolation.

Simchowitz and Cragoe (1990) have previously suggested that chemotactic peptide-induced $[Ca^{2+}]_i$ elevation and superoxide production in neutrophils, involves activation of Na^{+}/Ca^{2+} exchange and that this is blocked by benzamil. Thus, the inhibitory effect observed with benzamil might suggest a role for Na^{+}/Ca^{2+} exchange in this respiratory burst response. However, the overwhelming body of evidence from more recent studies suggests that fMLP stimulates receptor-mediated Ca^{2+} influx, via a store depletion-dependent mechanism and that this is the main route of Ca^{2+} entry in human neutrophils (Montero *et al.*, 1991; Demaurex *et al.*, 1994; Alvarez *et al.*, 1994). These data therefore cast doubt upon the use of benzamil as a specific inhibitor of Na^{+}/Ca^{2+} exchange.

NaF, which forms a complex with aluminium (AlF^{4-}) to stimulate heterotrimeric G

proteins (Watson and Arkinsall, 1994), caused marked inhibition of the spontaneous release of O_2^-. This suggests that an inhibitory G protein was activated, possibly Gs. Activation of Gs stimulates adenylyl cyclase activity, which leads to production of cAMP. Activation of PKA by cAMP has been shown to have an inhibitory effect on NADPH oxidase in human alveolar macrophages and thus inhibits O_2^- release (Dent *et al.,* 1994b). This hypothesis is valid, even though forskolin and salbutamol have no effect. It has previously been reported that although forskolin stimulates large increases in cAMP in most cell types, it is ineffective in guinea-pig eosinophils (Giembycz and Barnes, 1993). Moreover, it was suggested that this might possibly be due to a forskolin-insensitive form of adenylyl cyclase being present in these cells, or alternatively forskolin may be unable to cross the membrane of eosinophils.

The lack of effect observed with salbutamol is consistent with other groups who have investigated the effects of β-adrenoceptor agonists on the eosinophil respiratory burst response. Work carried out by Yukawa *et al.* (1990) demonstrated that β-adrenoceptors on eosinophils are functionally coupled to adenylyl cyclase. However, they also found that stimulation of β_2-adrenoceptors by salbutamol did not inhibit the respiratory burst response in either guinea-pig or human eosinophils. Rabe *et al.* (1993) found that salbutamol caused some inhibition of H_2O_2 production in guinea-pig eosinophils, but only at relatively high concentrations ($\geq 10^{-5}$M). The lack of effect of the β-adrenoceptor agonists has been suggested to result from rapid uncoupling of the β-adrenoceptors from adenylyl cyclase (Giembycz and Barnes, 1993).

Another possible mechanism of action for the inhibitory effects observed with NaF may be via inhibition of tyrosine kinase. In macrophages, NaF has been suggested to exert a direct effect on tyrosine kinase and this activity is unrelated to the formation of a complex with aluminium and an action on G proteins (Goldman and Zor, 1995). However, this seems unlikely, as subsequent experiments on the effect of NaF against fMLP-induced ECP release showed that the tyrosine kinase inhibitor, erbstatin analogue, but not NaF inhibited the response.

Finally, cyclosporin A (CsA) caused marked inhibition of the spontaneous O_2^- generation in human eosinophils. CsA has also been reported to have a similar effect in human neutrophils, although the mechanism of action for this effect is not clear. CsA is known to interact with cyclophilin to form an active complex that inhibits calcineurin (PP2B), which is believed to be the mechanism of action for its immunosuppressive effects in T cells (Morley, 1992). However Cyclosporin H, which does not inhibit calcineurin, does inhibit the release of O_2^- in human neutrophils. This suggests that CsA inhibits O_2^- generation via a mechanism distinct from inhibition of calcineurin. It does not appear that CsA exerts its effects directly on the NADPH oxidase enzyme, as it was without effect in a cell-free oxidase system, which suggests that it may regulate the signal transduction process in some way (Wenzel-Seifert *et al.,* 1991). However, the inhibitory effects observed in the neutrophil are not via inhibition of Ca^{2+} mobilisation, or via an effect on PKC (Wenzel-Seifert *et al.,* 1991; Wenzel-Seifert *et al.,* 1994).

5.2 Effect of cytochalasin B on human eosinophils

The inhibition of adherence of eosinophils to microtitre plates by cytochalasin B was consistent with its known inhibitory effects on the assembly of actin (Bengtsson *et al.*, 1991).

In this study, agonist-induced O_2^-, LTC_4 and ECP release was measured in the presence of cytochalasin B, which appears to act as a priming agent. In the absence of cytochalasin B eosinophils produced little or no O_2^-, LTC_4 or ECP, in response to agonist (data not shown). This is consistent with work carried out by Takafuji *et al.* (1991) where they found that eosinophils required a priming agent before C5a-induced LTC_4 release could be detected. Additionally, in agreement with the observations in this study, it has also been reported that a marked increase in ECP release is observed in the presence of cytochalasin B in response to either C5a (Takafuji *et al.*, 1994) or fMLP (White *et al.*, 1993).

Cytochalasin B alone had little effect on the basal level of $[Ca^{2+}]_i$ in human eosinophils. It also had no effect on C5a-induced IP_3 generation or intracellular Ca^{2+} release. In contrast, it did cause a trend towards enhancement of the C5a-induced Ca^{2+} influx response. These observations show some similarity with data obtained by Treves *et al.* (1987), who investigated the effect of cytochalasins on $[Ca^{2+}]_i$ in leukocytes. They found that cytochalasins alone caused some increases in $[Ca^{2+}]_i$ and the extent of this effect varied depending on the cell type studied. Neutrophils responded with a marked transient

mobilisation of intracellular Ca^{2+}, whereas lymphocytes exhibited a much smaller transient elevation of $[Ca^{2+}]_i$. Furthermore, cytochalasin B enhanced the duration of the fMLP-induced Ca^{2+} mobilisation response in human neutrophils, via an effect on Ca^{2+} influx rather than on intracellular Ca^{2+} release.

Interestingly, Sedgwick *et al.* (1992) observed that fMLP-stimulated BAL eosinophils (i.e. primed cells) have a much greater Ca^{2+} influx and respiratory burst response than those taken from peripheral blood. This is particularly interesting as there is also evidence that eosinophils from atopic individuals have an enhanced LTC_4 (Aizawa *et al.*, 1990) and respiratory burst (Koenderman and Bruijnzeel, 1989) response, compared with those from normal individuals.

Cytochalasin B may act by increasing the percentage of cells in the population that respond to agonist (Daniels *et al.*, 1994). This may be via increased surface expression of receptors, as a result of decreased internalisation of receptors in the presence of cytochalasin B (Bengtsson *et al.*, 1991). In neutrophils it has been suggested that the prolonged $[Ca^{2+}]_i$ transients reflect an increased fusion of specific granules with the plasma membrane. This causes new fMLP receptors, which are present on specific granule membranes, to be exposed on the outer surface of the cell (Treves *et al.*, 1987).

5.3 Effect of Ca^{2+} modulation on human eosinophil activation

Using the Ca^{2+}-chelating agent EGTA, it has been demonstrated in this study that C5a stimulates Ca^{2+} mobilisation responses which involve both release of Ca^{2+} from internal stores, via IP_3 generation, as well as Ca^{2+} influx across the

plasma membrane. Characterisation of the influx response using a range of Ca^{2+} channel blocking agents, suggests that Ca^{2+} influx occurs via a receptor-mediated entry pathway, as a result of emptying of intracellular Ca^{2+} stores. Furthermore, Ca^{2+} influx via a receptor-mediated pathway is required for C5a- and fMLP-induced O_2^- and LTC_4 release.

Previously, Kernen *et al.* (1991) have reported that C5a stimulates a rise in $[Ca^{2+}]_i$. In the current study, C5a (30nM) caused the generation of IP_3, which was maximal 5 seconds after agonist addition. U73122, an inhibitor of PLC-dependent processes, partially inhibited C5a-induced IP_3 generation but abolished both the Ca^{2+} mobilisation and Mn^{2+} influx responses. In addition, both C5a- and fMLP-induced O_2^- production and LTC_4 release were also blocked by U73122, but not by its inactive, analogue, U73343.

Smith *et al.* (1990) reported similar effects in human neutrophils against chemotactic peptide-mediated Ca^{2+} mobilisation and O_2^- release. Furthermore, fMLP-induced increases in $[Ca^{2+}]_i$ were reported to be more susceptible to inhibition by U73122 than IP_3 production. This discrepancy in potency was suggested to be due to non-cooperative binding of IP_3 to its intracellular receptor. This binding results in highly co-operative opening of endomembrane Ca^{2+} channels. Thus a small decrease in IP_3 concentration may be sufficient to completely inhibit IP_3-mediated release of intracellular Ca^{2+}.

An alternative explanation for the decreased potency of U73122 against C5a-induced IP_3 generation, compared with its effects against Ca^{2+} mobilisation, may result from differences in the cell concentrations for these assays. Measurement

of $[Ca^{2+}]_i$ was made using a concentration of $1x10^6$ cells/ml, whereas IP_3 generation was assayed using $2\text{-}3x10^7$ cells/ml. Bleasdale *et al.* (1990) reported a decrease in the potency of U73122 when the cell concentration was increased by 10 fold. This was suggested to occur as a result partitioning of U73122 into cell membranes, which would cause a reduction of the amount available for inhibition of PLC-mediated processes.

A report by Vickers (1993) suggested that U73122 may also have effects on phosphoinositide metabolism and so limit the usefulness of U73122 as a tool for evaluating PLC-mediated responses. However, the concentrations used in the latter study were much higher (15μM) than were used in either this study or that by Smith *et al.* (1990). It should also be stressed that in the current study, U73122 was used at a concentration that should not inhibit PLD-mediated effects (Naccache *et al.,* 1993). In addition, the combination of measurement of IP_3 generation, Ca^{2+} mobilisation and Mn^{2+} influx data, as well as the functional responses are consistent with a role for PLC in C5a-induced eosinophil activation, so it seems reasonable to interpret the effects of U73122 as acting against PLC-dependent processes in these experiments.

A rather surprising effect of U73122 was the observation that it inhibited thapsigargin-induced Ca^{2+} influx and LTC_4 production. Rather conflicting results concerning the effect of U73122 appear in the literature. In rabbit pancreatic acinar cells U73122 was reported to have no effect on thapsigargin-induced Ca^{2+} influx (Willems *et al.,* 1994), whereas in platelets it caused partial inhibition (Okamoto *et al.,* 1995). The inhibitory effect observed in platelets was not via

direct inhibition of Ca^{2+} influx, as the effect of an extract obtained from thrombin-stimulated platelets, which stimulates Ca^{2+} influx, was not inhibited by U73122 (Okamoto *et al.,* 1995). Similarly, in neutrophils stimulated with a cellular extract containing a factor which stimulates Ca^{2+} influx, U73122 inhibited the release of Ca^{2+} from intracellular stores, but not the influx response (Davies and Hallett, 1995). Contrary to the effects reported in platelets and neutrophils, a study by Berven and Barritt (1995) in hepatocytes, found that U73122 and its 'inactive' analogue U73343 caused inhibition of Ca^{2+} influx directly.

The data obtained in this study would tend to suggest that U73122 exerted its inhibitory effects on thapsigargin-induced Ca^{2+} influx via an action on the release of intracellular Ca^{2+} from the stores. This was demonstrated by the observation that U73122 only inhibited thapsigargin-induced Ca^{2+} influx when added before thapsigargin but not afterwards. Thus, U73122 was unable to inhibit Ca^{2+} influx once store depletion had already taken place. In unstimulated cells it is possible that a basal level of PLC activation and IP_3 generation is present. The action of IP_3 on the receptors present on the endomembrane of intracellular Ca^{2+} stores, may cause the leak of Ca^{2+} into the cytoplasm (Favre *et al.*, 1994). Normally, the endomembrane Ca^{2+}/Mg^{2+}-ATPase would replenish the Ca^{2+} stores, but in the presence of thapsigargin this would not occur. Thus the action of U73122 may be to prevent this 'leak pathway' and so prevent store depletion and activation of Ca^{2+} influx.

The peak increase in $[Ca^{2+}]_i$ in response to C5a appeared to predominantly represent release of Ca^{2+} from intracellular stores. This was demonstrated by

the removal of extracellular Ca^{2+}, using the Ca^{2+} chelator EGTA, which had only a small inhibitory effect on the response. When the Ca^{2+} mobilisation response was analysed as an area under the curve, it was clear that EGTA had a similar effect during the first 30 seconds (AUC 0-30 seconds) after agonist addition as against the peak elevation in $[Ca^{2+}]_i$. In contrast, marked inhibition of the sustained response (AUC 30-100 seconds) was observed, suggesting that this predominantly represented the Ca^{2+} influx response. The inhibitory effects of EGTA against C5a and fMLP-induced O_2^- and LTC_4 release appear to suggest that Ca^{2+} influx is required and that release of Ca^{2+} from intracellular stores alone is not sufficient to stimulate these functional responses.

Having established a role for extracellular Ca^{2+} for C5a-induced O_2^- and LTC_4 release in human eosinophils, the type of influx pathway that might be involved was investigated. The Ca^{2+} surrogate, Mn^{2+}, was used to study the influx response. Measurement of Mn^{2+} influx has proved to be a useful technique when studying influx pathways (Merritt *et al.,* 1989) as Mn^{2+} passes through Ca^{2+} channels and binds with high affinity to fura-2 (approximately 50 fold higher affinity than Ca^{2+}; Grynkiewicz *et al.* (1985)) to quench its fluorescence. Furthermore, as Mn^{2+} would not normally be present in the cytosol, any quench in cytosolic fura-2 fluorescence can be directly attributed to the passage of Mn^{2+} ions across the plasma membrane. In addition, the C5a-induced concentration-effect curve when measured as AUC 30-100 seconds (which predominantly represented Ca^{2+} influx) correlated closely with the Mn^{2+} influx concentration-effect curve.

Ca^{2+} influx can be stimulated by two main mechanisms, either by depolarisation of the plasma membrane, via voltage operated Ca^{2+} channels (VOCs), or via receptor-mediated Ca^{2+} entry (RMCE) pathways. VOCs are generally found in excitable tissue, whereas RMCE pathways are predominantly found in non-excitable cells (Meldolesi *et al.,* 1991). The data obtained in this study are consistent with the presence of RMCE rather than VOC-mediated Ca^{2+} influx for several reasons. Firstly, the potent VOC-blocker nifedipine was without effect against C5a-induced Mn^{2+} influx at concentrations 300-1000 times higher than are normally required to block VOCs. Moreover, different chemical classes of VOC-blocker, namely the dihydropyridine nifedipine, the benzothiazepine diltiazem and the phenylalkylamine verapamil had little effect against C5a- and fMLP-induced O_2^- and LTC_4 release. In contrast, depolarisation of the plasma membrane by KCl caused marked inhibition of these responses. This effect was presumably via inhibition of PLC activation and IP_3 generation, which has previously been shown to be inhibited by plasma membrane depolarisation in HL-60 cells (Pittet *et al.*, 1990).

Secondly, Ni^{2+} and SK&F 96365, which inhibit both RMCE and VOCs, block the C5a-induced Mn^{2+} influx response. Furthermore, SK&F 96365 and a structurally related RMCE inhibitor, econazole, caused concentration-dependent inhibition of both C5a- and fMLP-mediated O_2^- and LTC_4 generation. It was necessary to measure Mn^{2+} influx rather than AUC 30-100 seconds when assessing the effects of these agents against influx, as both SK&F 96365 and Ni^{2+} had effects on the basal $[Ca^{2+}]_i$ measurements. Ni^{2+} caused a small quench of fura-2 fluorescence upon addition to the cells. The kinetics of this effect would appear

to suggest that this was due to entry of Ni^{2+} into the cells rather than quench of extracellular dye, particularly as this was not reversed upon addition of DTPA. In contrast, SK&F 96365 appeared to cause some elevation of $[Ca^{2+}]_i$ when added to the cells. This is very similar to data obtained in human neutrophils, where SK&F 96365 caused some release of Ca^{2+} from intracellular stores as well as inhibition of Ca^{2+} influx (Merritt *et al.,* 1990). An alternative explanation for the rise in $[Ca^{2+}]_i$ caused by SK&F 96365 could be due to inhibition of Ca^{2+} extrusion from the cells, as it is known that a close analogue, SC 38249, has this activity (Ciardo and Meldolesi, 1990).

Thirdly, it has been reported that human neutrophils do not possess VOCs, but are believed to possess a single receptor-mediated Ca^{2+} entry pathway which is activated by depletion of intracellular Ca^{2+} stores (Demaurex *et al.,* 1994). The data obtained in this study, using thapsigargin, demonstrate for the first time that human eosinophils possess a 'store-regulated' mechanism for controlling Ca^{2+} influx. In human eosinophils C5a caused PLC activation, leading to IP_3 generation and release and emptying of Ca^{2+} from intracellular stores. The concentration-effect curves for peak elevation of cytosolic Ca^{2+}, AUC 0-30 seconds, AUC 30-100 seconds and rate of Mn^{2+} influx were almost identical, which suggests that the degree of emptying of the Ca^{2+} stores may affect Ca^{2+} (Mn^{2+}) influx (i.e. capacitative Ca^{2+} entry). Furthermore, the Ca^{2+} modulating agents show the same profile of effects against both C5a- and thapsigargin-induced increases in $[Ca^{2+}]_i$. In addition, the same inhibitors regulate the release of O_2^- and LTC_4 in response to C5a and fMLP in an identical fashion to the inhibition of thapsigargin-induced LTC_4 generation.

The requirement for extracellular Ca^{2+} for LTC_4 production, in human eosinophils, is consistent with studies carried out by Wong *et al.* (1991) using rat basophilic leukaemic cells (RBL-2H3). Their studies showed that addition of EGTA to the extracellular medium inhibited the translocation of 5-lipoxygenase to the endomembrane and leukotriene synthesis. They also found that a sustained Ca^{2+} influx response was required for LTC_4 generation.

Evidence for a fundamental requirement for a sustained Ca^{2+} influx response for LTC_4 generation was the observation that thapsigargin stimulated LTC_4 generation, but did not cause O_2^- or ECP release. Comparison of the concentration-effect curves for increases in $[Ca^{2+}]_i$ and LTC_4 generation, showed a marked difference in potency for the generation of these responses. The threshold concentration for LTC_4 release was virtually maximally effective for $[Ca^{2+}]_i$ elevation. This may suggest that a threshold level of $[Ca^{2+}]_i$ elevation was required or alternatively, that an additional signal needs to be generated, before the formation of leukotrienes occurs.

In RBL-2H3 cells Wong *et al.* (1992) found that a threshold level of Ca^{2+} was required for leukotriene generation in response to thapsigargin or the Ca^{2+} ionophore, ionomycin. Interestingly, antigen-induced leukotriene release was also Ca^{2+}-dependent, but required smaller increases in $[Ca^{2+}]_i$ for an equivalent response to that obtained with ionomycin, suggesting that an additional pathway(s) was involved in the antigen-mediated response.

The extent of the thapsigargin-induced rise in $[Ca^{2+}]_i$ was dependent upon whether store-depletion took place in the presence or absence of extracellular

Ca^{2+}, but the potency remained the same. One possible explanation for this could be that a negative feedback process is activated when Ca^{2+} enters the cytosol, as has been found in adrenal chromaffin cells (Fonteriz *et al.*, 1992). This could either be via a direct effect on the plasma membrane Ca^{2+} channels or possibly by inhibiting the generation of second messengers that are responsible for stimulating Ca^{2+} influx. Alternatively, in the presence of low $[Ca^{2+}]_i$ levels, there may be decreased degradation of messenger(s) which stimulate Ca^{2+} influx (Randriamampita and Tsien, 1995). An interesting experiment, would be to investigate whether the concentration-effect curve for LTC_4 generation is shifted to the left if a Ca^{2+} re-addition protocol is used, as well as comparing whether or not the threshold for generation of LTC_4 correlates with the rise in $[Ca^{2+}]_i$.

Perhaps more surprising was the requirement for extracellular Ca^{2+} for O_2^- release, in response to C5a and fMLP. PAF-induced O_2^- release in human eosinophils has been shown to be markedly inhibited, but not abolished, by EGTA (Zoratti *et al.*, 1991). In contrast, generation of O_2^- in guinea-pig eosinophils in response to LTB_4 (Subramanian, 1992) or PAF (Kroegel *et al.*, 1989b) has been reported to be independent of extracellular Ca^{2+}. Despite differences in the Ca^{2+} requirements for O_2^- generation, an interesting similarity between the studies carried out using guinea-pig eosinophils and our study using human eosinophils is the separation between the concentration-effect curves for the Ca^{2+} responses and functional responses.

Many studies have been carried out on human neutrophils and the signal

transduction pathways involved in the respiratory burst response. Generation of O_2^- in the neutrophil can be mediated by at least two signal transduction sequences, involving both Ca^{2+}-dependent and Ca^{2+}-independent pathways which act in concert (Thelen *et al.,* 1993; Wymann *et al.,* 1995). It is possible that different agonists may vary in the degree to which they stimulate these pathways. In this study, it is clear that in human eosinophils extracellular Ca^{2+} is required for C5a- and fMLP-induced O_2^- and LTC_4 release. However, the Ca^{2+} responses have a lower threshold and achieve a maximum effect at lower concentrations of C5a than are required for the functional responses. This suggests that although Ca^{2+} is required for these responses, other messenger(s) are also involved.

When measuring ECP release, it is clear that other signal transduction pathways must be activated by C5a and fMLP. C5a-induced ECP release appeared to be only partially dependent upon RMCE. Addition of U73122, KCl, SK&F 96365 or decreasing the extracellular [Ca^{2+}] to 0.3mM caused approximately 50% reduction of the degranulation response. This suggests that although activation of PLC and Ca^{2+} influx were required for part of this response, another pathway that was independent of PLC activation and Ca^{2+} influx was also involved. This was even more evident when eosinophils were stimulated with fMLP. FMLP-induced ECP release was not inhibited by any of the Ca^{2+} influx blockers or by U73122.

5.4 Signal transduction in human eosinophils

Activation of human eosinophils by C5a and fMLP, was found to be mediated via

a PTX-sensitive G protein(s). This is in agreement with the previous observation by Kernen *et al.* (1991) that C5a-induced EPO release and rises in $[Ca^{2+}]_i$ were abolished by PTX pretreatment, although interestingly the shape change response was only partially inhibited. Additionally both C5a and fMLP have recently been reported to induce release of IL-8 and GM-CSF from cytochalasin B-treated eosinophils via a PTX-sensitive route (Miyamasu *et al.,* 1995). Similarly, chemoattractant-mediated activation of neutrophils has also been shown to be via activation of PTX-sensitive G proteins (Becker *et al.*, 1985; Becker *et al.,* 1986; McDonald *et al.*, 1991).

In this study, a close correlation was observed between PTX-mediated inhibition of the C5a- and fMLP-induced Ca^{2+} mobilisation response and the functional responses (LTC_4 and/or ECP release). PTX acts by uncoupling G_i-like heterotrimeric G proteins (mainly $G\alpha_{i1}$, $G\alpha_{i2}$ and $G\alpha_{i3}$) from their receptor, by ADP ribosylating the α subunit (Watson and Arkinsall, 1994). Receptor-mediated activation of heterotrimeric G_i proteins causes them to separate into their α and $\beta\gamma$ components. Whilst the α subunit has an inhibitory effect upon adenylyl cyclase activation, it has been shown in neutrophils that the $\beta\gamma$ subunit stimulates activation of PLC_β (Watson and Arkinsall, 1994; Blank *et al.,* 1992). Antibodies to the different sub-types of $G_{i\alpha}$ are commercially available and could be used to identify which sub-type(s) of $G_{i\alpha}$ may be present in human eosinophils and are activated by C5a and fMLP.

Despite the fact that both C5a and fMLP mediate their effects through a PTX-sensitive G protein, it was clear that there are major differences in the signal

transduction pathways that regulate their functional responses. Schematic representations of the different signal transduction processes that appear to regulate C5a-induced LTC_4 and ECP generation, as well as fMLP-induced LTC_4 and ECP release, are shown in figures 38, 39, 40 and 41, respectively. It should be emphasised that these representations are both speculative and necessarily much simplified. They are mainly based upon the data obtained in this study and as such, only a limited range of agents have been tested. Clearly, it is necessary that other inhibitors, particularly of tyrosine kinase, PI 3-kinase and PLD are tested to confirm the data obtained in this study. Furthermore, biochemical measurement of the activity of these enzymes in eosinophils must also be made. However, it should also be stressed that these data are consistent with what is known about chemotactic peptide-induced activation in other cell types, particularly in the neutrophil.

The data obtained with the erbstatin analogue suggest that tyrosine kinase activity is extremely important for the generation of functional responses in human eosinophils, but does not appear to be involved in the Ca^{2+} mobilisation response. Similarly, it has previously been reported that stimulation of human eosinophils using Sepharose beads coated with secretory IgA or IgG, caused a degranulation response that was dependent upon activation of tyrosine kinase (Kato *et al.,* 1995). In common with these findings, there is a mounting body of evidence for a major role of tyrosine kinase in chemoattractant signalling in neutrophils. Naccache *et al.* (1990) reported that whilst erbstatin did not inhibit

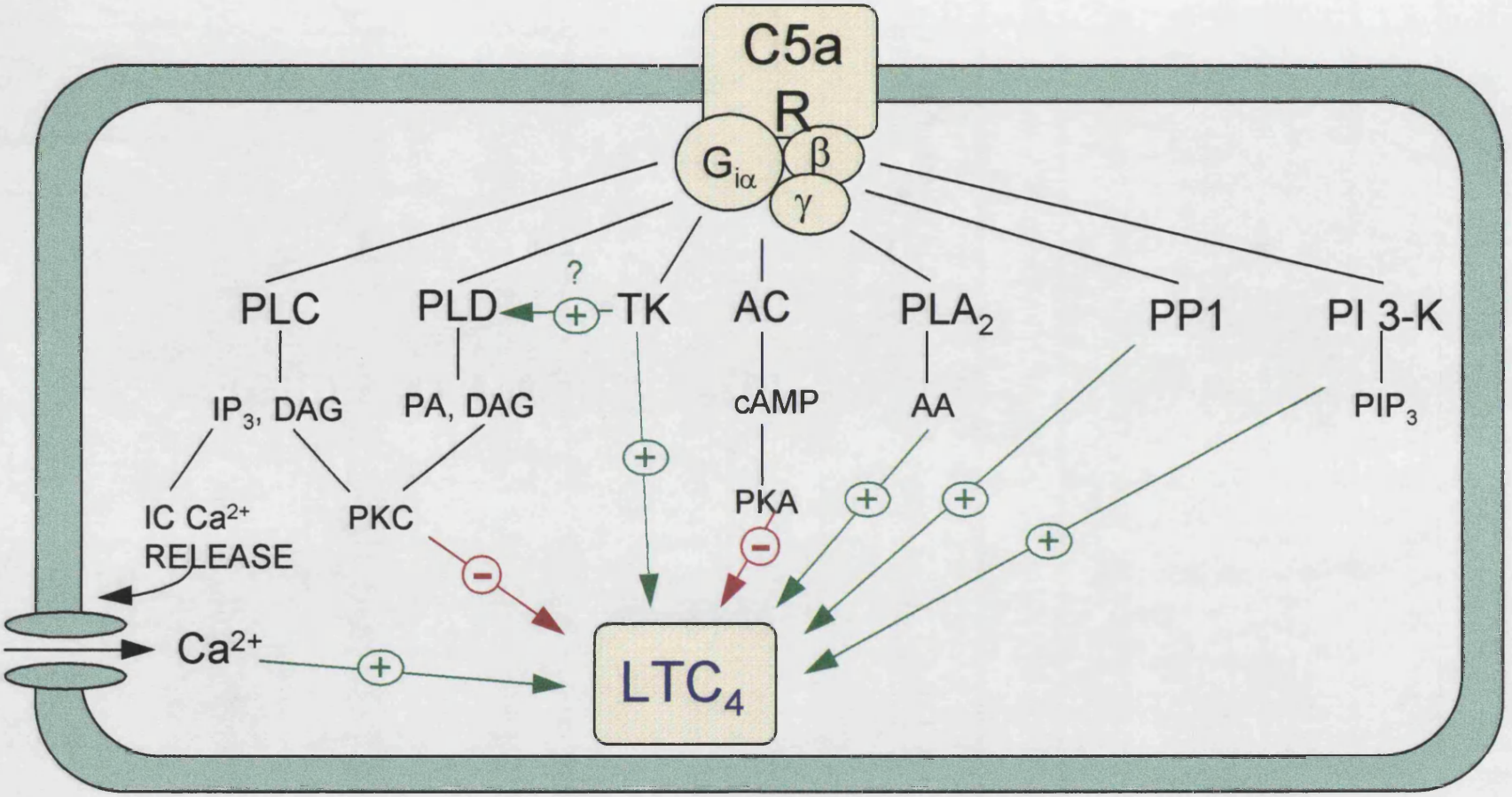


Figure 38

Schematic diagram of proposed signal transduction pathways that regulate C5a-induced LTC_4 production, in human eosinophils.

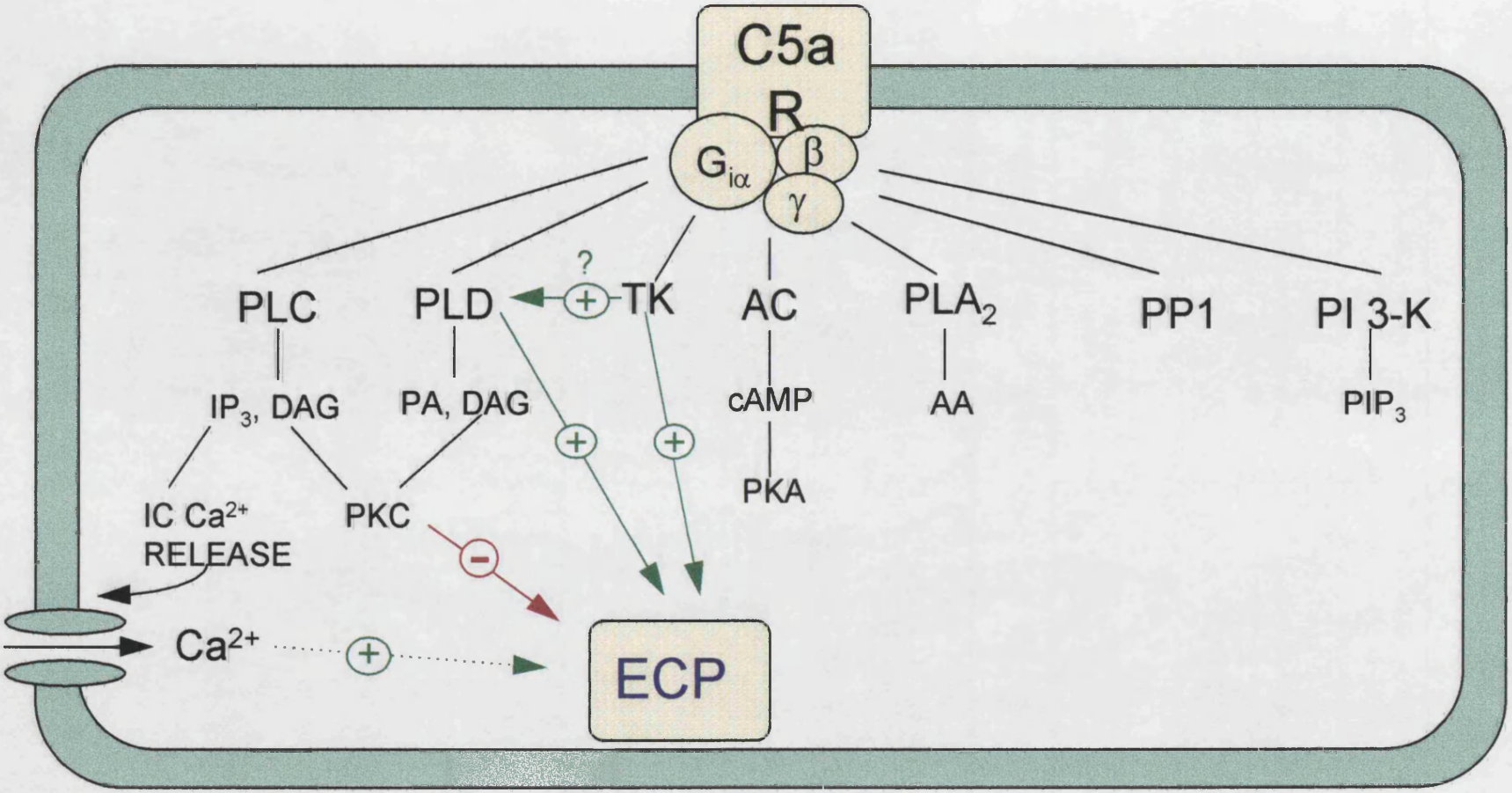


Figure 39

Schematic diagram of proposed signal transduction pathways that regulate C5a-induced ECP release, in human eosinophils.

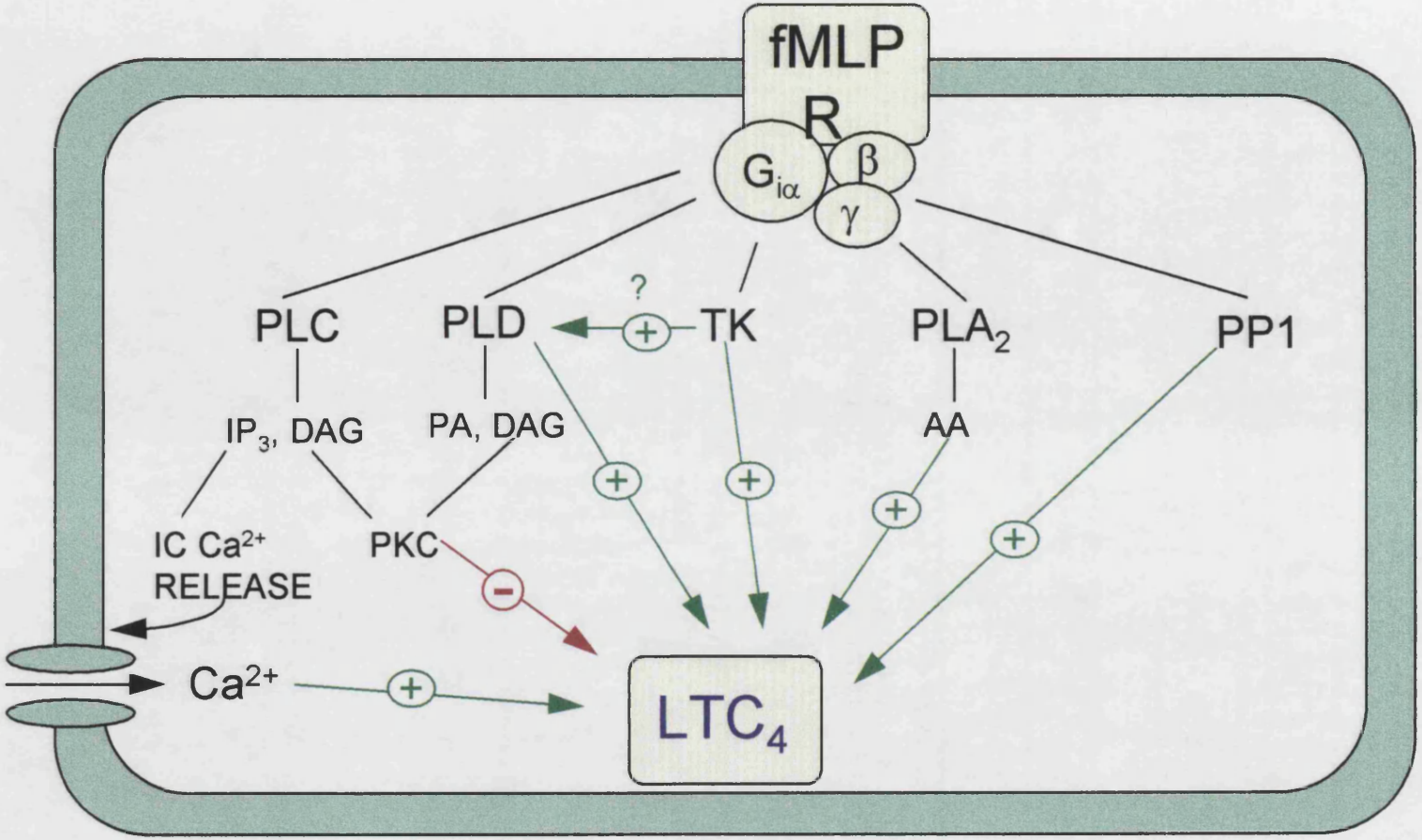


Figure 40

Schematic diagram of proposed signal transduction pathways that regulate fMLP-induced LTC_4 production, in human eosinophils.

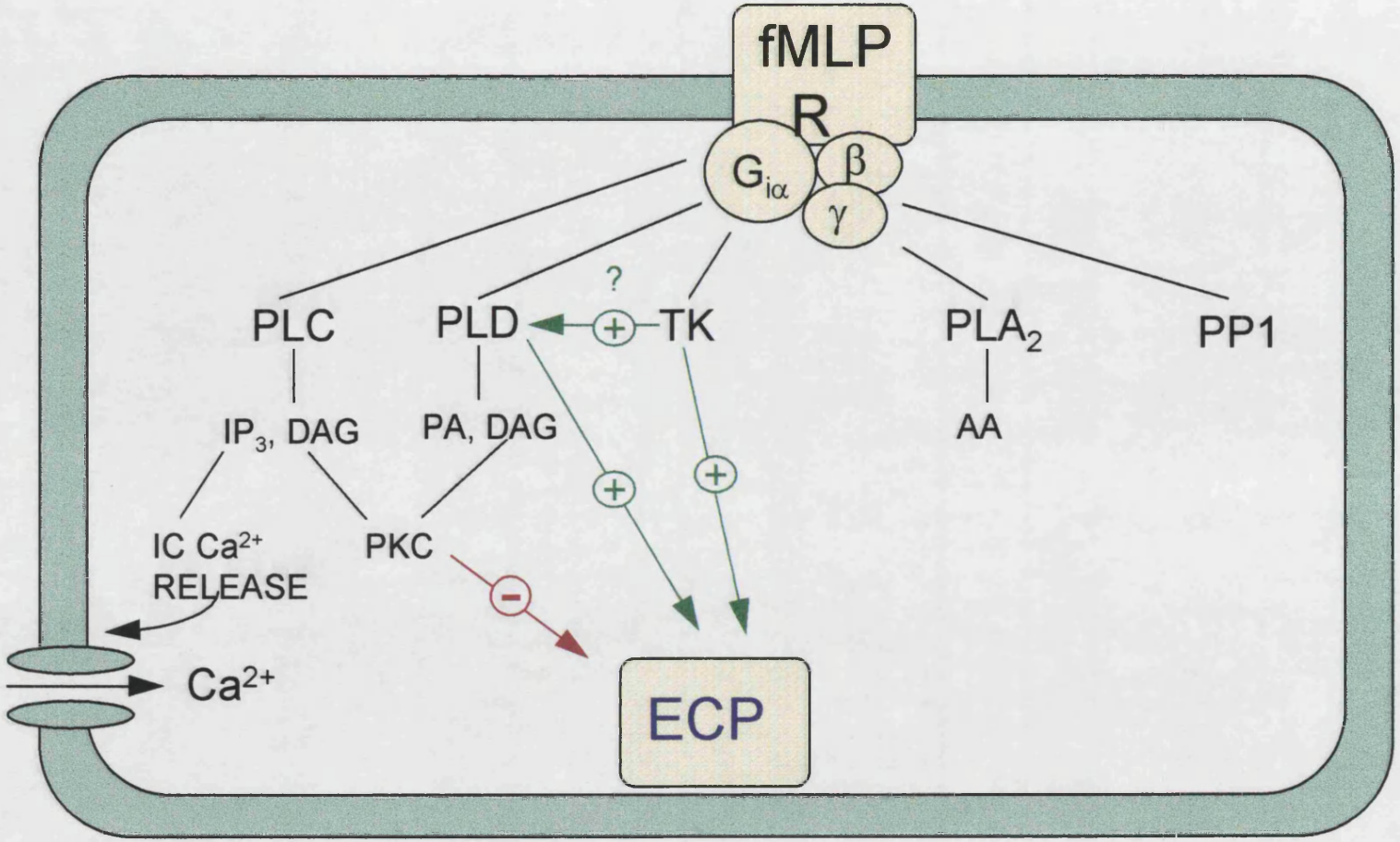


Figure 41

Schematic diagram of proposed signal transduction pathways that regulate fMLP-induced ECP release, in human eosinophils.

the increase in $[Ca^{2+}]_i$ elicited by C5a or fMLP in human neutrophils, it decreased the level of tyrosine phosphorylation stimulated by fMLP and abolished the respiratory burst response. More recently, it has been suggested that G proteins play a major role in the coupling of chemoattractant receptors with tyrosine kinases and that this coupling occurs in parallel to PLC activation in neutrophils (Rollet *et al.* 1994).

C5a and fMLP have been shown to activate both PLC and PLD in cytochalasin B-treated neutrophils (Kessels *et al.,* 1991; Kanaho *et al.,* 1991; Mullmann *et al.,* 1990b). It is now believed that receptor-mediated activation of PLD can occur as a consequence of tyrosine kinase stimulation (Yasui *et al.,* 1994; Uings *et al.,* 1992) and that protein phosphorylation by the kinase, increases the efficiency of coupling between the chemotactic peptide receptor and PLD (Garland, 1992). Naccache *et al.* (1993) found that wortmannin (≥50nM) caused inhibition of tyrosine phosphorylation in neutrophils and that this was dissociated from its effects against PLD. They further suggest that the inhibition of PLD observed with wortmannin was as a direct consequence of inhibition of tyrosine kinase.

Stimulation of human eosinophils by C5a has been shown to cause activation of PLD (Minnicozzi *et al.,* 1990). Thus, in human eosinophils, it remains to be seen whether the inhibition of the fMLP and C5a-induced ECP release, by wortmannin (1μM) was due to inhibition of tyrosine kinase or whether inhibition of subsequent PLD activation was the major pathway involved.

At low concentrations (≤10nM) wortmannin acts as a potent and specific inhibitor of PI 3-kinase (Arcaro and Wymann, 1993; Okada *et al.,* 1994). In RANTES-

stimulated T lymphocytes, chemotaxis and polarisation responses were found to involve activation of PI 3-kinase (Turner *et al.,* 1995). Similarly, activation of PI 3-kinase in neutrophils has been shown to be mediated via G protein-coupled receptors and is involved in acute activation of functional responses, particularly the respiratory burst response (Thelen *et al.,* 1994; Ding *et al.,* 1995; Vlahos *et al.,* 1995). In contrast, a recent study by Perkins *et al.* (1995) found that PI 3-kinase was not involved in the LTB_4-mediated respiratory burst response in guinea-pig eosinophils. The data obtained in this study suggest for the first time, in human eosinophils, that PI 3-kinase may be activated by C5a. Furthermore, this appears to be the first evidence that PI 3-kinase may be involved in the regulation of leukotriene generation.

Activation of PKC caused marked inhibition of both LTC_4 release and ECP generation in human eosinophils. This effect was probably as a result of its known inhibitory activity against the LTC_4 synthase enzyme (Ali *et al.,* 1994; Kargman *et al.,* 1994). In addition, activation of PKC has been shown to both enhance efflux of Ca^{2+} across the plasma membrane and inhibit Ca^{2+} influx in human neutrophils (McCarthy *et al.*, 1989), which would also inhibit LTC_4 generation.

The paradoxical effect that both activation of PKC, by PMA and inhibition of PKC, by bisindolymaleimide caused a reduction in both C5a- and fMLP-induced ECP release, is difficult to explain. One possibility may be that different isoforms of PKC are affected by these agents and that these isoforms phosphorylate different target proteins in the signal transduction pathway. It is possible that

PMA might stimulate PKC isoenzymes that are inhibitory for ECP release, whereas bisindolymaleimide could inhibit different isoenzymes of PKC that are stimulatory for ECP responses. Clearly, further investigation is required to identify which isoenzymes of PKC might be involved in the regulation of eosinophil activation.

Attempts to elevate cAMP levels using forskolin and the β_2-adrenoceptor agonist salbutamol, had no significant effect on the C5a- and fMLP-induced functional responses. This was presumably for the same reasons that have previously been discussed for their lack of effect against spontaneous O_2^- release. In contrast, inhibition of the breakdown of cAMP, by the type IV cAMP PDE inhibitor rolipram, abolished both O_2^- and LTC_4 generation in response to C5a. However, rolipram did not cause any inhibition of ECP release.

The inhibitory effect observed with the PDE IV inhibitor against O_2^- production is consistent with other reports on both guinea-pig and human eosinophils (Dent *et al.,* 1994a; Barnette *et al.,* 1995). However, the lack of effect of rolipram in this study against C5a-induced ECP release contrasts with its relatively potent effects against LTB_4-induced ECP release in guinea-pig esoinophils (Souness *et al.,* 1995). This may either reflect a species difference or may simply reflect differences between C5a- and LTB_4-mediated signal transduction pathways. The latter seems likely as C5a, but not LTB_4, has been shown to stimulate PLD and PI 3-kinase activation in eosinophils (Minnicozzi *et al.,* 1990; Perkins *et al.,* 1995).

The type IV PDE isoenzyme is the predominant form of cyclic nucleotide activity

in inflammatory cells, particularly in eosinophils (Torphy *et al.*, 1994). Therefore, a great deal of interest is currently focused on PDE IV inhibitors and it is thought that these agents may have a useful therapeutic potential in the treatment of asthma (Giembycz and Dent, 1992).

Inhibition of serine/threonine phosphatases markedly suppressed both C5a- and fMLP-induced O_2^- and LTC_4 generation, but not ECP release. Calyculin, which inhibits PP1 and PP2A, was more potent at inhibiting the functional responses than the more selective PP2A inhibitor, okadaic acid. Thus, it would appear that inhibition of PP1 may lead to prolonged phosphorylation of certain target proteins that would have an inhibitory effect on O_2^- and LTC_4 generation.

The effects of these inhibitors may be explained by causing a prolonged phosphorylation response as a result of C5a- or fMLP-induced PKC activation. This effect has been shown in neutrophils, resulting in enhanced fMLP-induced O_2^- release (Djerdjouri *et al.*, 1995; Lu *et al.*, 1992). Thus, enhancing the effect of PKC activation would cause inhibition of LTC_4 generation.

The lack of effect of okadaic acid and calyculin against ECP release could also be consistent with an effect on prolonging PKC-mediated phosphorylation events. The previous observation that both activation and inhibition of PKC inhibits ECP release, may suggest that some isoforms of PKC may have an inhibitory effect on ECP release, whereas others may have a stimulatory effect. The effect of calyculin or okadaic acid would be to prolong all phosphorylations stimulated by PKC (both stimulatory and inhibitory), resulting in a net lack of effect against ECP release. Alternatively, inhibition of PP1 may exert some

additional effect, which results in enhancement of agonist-induced ECP release.

This study has identified some of the signal transduction pathways that are involved in human eosinophil activation, with the main emphasis on classical signal transduction pathways, that are activated via heterotrimeric G protein-coupled receptors. However, recent studies in phagocytic cells have identified a major role for low molecular weight GTPases (LMWG) in the regulation of functional responses (Bokoch, 1995). LMWGs function as molecular switches that are regulated by their association with guanine nucleotides, such that the GTP-bound form is active and GDP-bound is inactive. LMWGs belong to the Ras superfamily, with 5 main sub-families comprising Ras, Rho, Rab, Arf and Ran (Bokoch, 1995).

It is now well established that Rac2 is required for the activation of NADPH oxidase (Knaus *et al.,* 1991). In addition, Arf (ADP-ribosylation factor) has been found to regulate PLD activity in human neutrophils via a mechanism that is dependent on the generation of polyphosphoinositides (Whatmore *et al.,* 1994). In human eosinophils, Rab1 was suggested to be involved in the process of degranulation (Aizawa *et al.,* 1992). Thus, it seems likely that LMWGs are involved in regulating signal transduction processes and it remains to be seen what role they may have in the regulation of eosinophil activation.

In summary, C5a and fMLP stimulate activation of human eosinophils via PTX-sensitive G protein-coupled receptors. Both agonists stimulate both PLC- and PLD-dependent pathways and require activation of tyrosine kinase for the generation of O_2^-, LTC_4 and release of ECP. However, C5a and fMLP appear to

have differences in the signal transduction pathways used to generate a particular response (e.g. LTC_4 release in response to C5a, but not fMLP, involves activation of PI 3-kinase). Moreover, stimulation of different responses by a particular agonist, involves different signal transduction pathways (e.g. C5a-induced LTC_4 release, but not ECP release, requires activation of PI 3-kinase).

5.5 Characterisation of LTD_4-induced increases in $[Ca^{2+}]_i$ in human eosinophils.

Stimulation of human eosinophils with LTD_4, caused increases in $[Ca^{2+}]_i$ which involved both release of Ca^{2+} from intracellular stores and Ca^{2+} influx, since EGTA caused partial inhibition of the response. However, LTD_4 did not cause any stimulation of the respiratory burst response or ECP release. This provided further evidence that, in addition to elevation of $[Ca^{2+}]_i$, other signal transduction pathways are also required for these responses.

Pretreatment of human eosinophils with PTX caused some inhibition of the LTD_4-induced increase in $[Ca^{2+}]_i$, but did not have any effect on the Mn^{2+} influx response. Increasing the concentration of PTX did not lead to complete inhibition of the response, suggesting that a PTX-insensitive component was also present. The finding that LTD_4 mediated its effects via both PTX-sensitive and insensitive pathways was consistent with the data obtained in other cell types including differentiated HL-60 cells (Baud *et al.,* 1987), U937 cells (Saussy *et al.,* 1989) and epithelial cells (Sjolander *et al.,* 1990).

In common with the findings in the current study, it has also been shown that PKC activation markedly attenuates the $[Ca^{2+}]_i$ response in U-937 cells (Pollock and Creba, 1990). The inhibitory effect of PMA may have been via heterologous desensitisation of LTD_4 receptors, as has previously been reported (Crooke, 1990). It is possible that calyculin, which caused rather variable inhibition of the LTD_4-induced Ca^{2+} mobilisation response, may have exerted its effects by prolonging the PKC-mediated phosphorylation events thereby causing some desensitisation of the response.

The observation that LTD_4 and LTB_4 did not cross-desensitise suggests that they act at different receptors, whilst LTC_4 had very little effect (data not shown). The presence of LTD_4 (now termed $CysLT_1$; Watson and Girdlestone, 1995) receptors on human eosinophils may have implications for the therapeutic effects of both LTD_4 receptor antagonists as well as LSIs. Until recently, it was believed that their major effect would be via inhibition of bronchoconstriction (Wensing *et al.,* 1994; Dahlen *et al.,* 1993). However, it has now been shown *in vitro,* that LTD_4 acts as a potent chemotactic agent on human eosinophils and that this effect is blocked by the selective $CysLT_1$ receptor antagonist, SK&F 104353 (Spada *et al.,* 1994). In addition, inhalation of LTE_4 stimulated the recruitment of granulocytes (predominantly eosinophils) into the airways mucosa of asthmatic patients (Laitinen *et al.,* 1993). This suggests that sulphidopeptide leukotrienes may be involved in the inflammatory response associated with asthma.

5.6 Current/potential anti-asthma agents

With the current emphasis on the role of eosinophils and inflammation in asthma, it is pertinent to investigate whether any of the currently used anti-asthma and anti-inflammatory agents are capable of modulating the activation of human eosinophils.

Cetirizine has previously been reported to to have anti-allergic properties in addition to its H_1 antihistamine effects (Naclerio, 1991). In a study by Okada *et al.* (1994), cetirizine was reported to partially inhibit the respiratory burst response in eosinophils from allergic patients but not from normal donors, as well as inhibiting chemotaxis. However, *in vivo* cetirizine had no effect on either the early or late asthmatic response after allergen challenge (de Bruin-Weller *et al.,* 1994). In this study, there was no evidence for an anti-inflammatory effect of cetirizine against either C5a- or fMLP-induced eosinophil activation.

The K_{ATP} channel opener, cromakalim, acts as a bronchodilator agent, although its therapeutic usefulness is limited by its cardiovascular side-effects (Nagai *et al.,* 1991; McPherson, 1993). In this study, cromakalim was not found to have any effect on the generation of O_2^-, LTC_4 or release of ECP in human eosinophils.

Sodium cromoglycate is believed to exert anti-inflammatory effects in addition to its mast cell 'stabilising' properties. It has been reported to inhibit inflammatory responses which are stimulated via IgE stimulation of $Fc_\varepsilon RII$, in a range of inflammatory cells (Tsicopoulos *et al.,* 1988). It was also reported to inhibit ECP release from eosinophils stimulated with anti-IgG antibodies

(Beauvais *et al.,* 1989). In contrast, PAF- and LTB_4-induced chemotaxis in human eosinophils was not inhibited by sodium cromoglycate (Bruijnzeel *et al.,* 1990). In this study, the release of O_2^-, LTC_4 and ECP in response to the chemotactic peptides C5a and fMLP were not inhibited by sodium cromoglycate.

Neither the PAF antagonist, WEB 2086, or the cyclo-oxygenase inhibitor, indomethacin, caused any inhibition of fMLP-induced LTC_4 or ECP release. However, both agents caused inhibition of C5a-induced LTC_4 release but had no effect on ECP release. The effect of indomethacin is unlikely to be due to non-specific inhibition of PLA_2 as it would also have inhibited the fMLP-induced response. A speculative explanation for the effects of these agents against C5a-induced stimulation of eosinophils could be that C5a-stimulated the generation of PAF and prostanoids, which contributed to the effect of C5a on eosinophils via a paracrine- or autocrine-mediated mechanism. Therefore, it might be interesting to assay for the generation of PAF and prostaglandins, in these cells and also to test a selective thromboxane synthase inhibitor, to determine whether release of thromboxane was involved in this response.

BAY x1005 acts as a potent and selective inhibitor of leukotriene synthesis via inhibition of 5-lipoxygenase-activating protein (FLAP) (Hatzelmann *et al.,* 1994). FLAP acts as a 'docking' protein in the endomembrane and 5-LOX translocation to this site is essential for its leukotriene synthesis activity (Ford-Hutchinson, 1991). The data obtained in this study confirmed that BAY x1005 selectively inhibits LTC_4 release from eosinophils and has no effect on ECP release.

Furthermore, the presence of $CysLT_1$ receptors on human eosinophils, which are stimulated by LTD_4 (a metabolite of LTC_4) to produce chemotactic responses, may enhance the potential anti-inflammatory profile of leukotriene synthesis inhibitors.

5.7 Summarising Discussion

In conclusion, C5a stimulated increases in $[Ca^{2+}]_i$, comprising release of Ca^{2+} from intracellular stores, via PLC-mediated IP_3 generation and Ca^{2+} influx across the plasma membrane, via a receptor-mediated Ca^{2+} entry pathway. Furthermore, receptor-mediated Ca^{2+} entry is required for production of O_2^- and LTC_4 in response to both fMLP and C5a. In contrast, C5a-induced ECP release appears to only be partially dependent upon RMCE, whereas the fMLP-induced response did not appear to require extracellular Ca^{2+}.

The RMCE pathway in human eosinophils is probably regulated by depletion of intracellular Ca^{2+} stores as intracellular Ca^{2+} release correlates closely with Ca^{2+} (Mn^{2+}) influx. Furthermore, it is clear that human eosinophils possess such a mechanism for regulating Ca^{2+} influx, as the Ca^{2+}/Mg^{2+} ATPase inhibitor, thapsigargin, stimulated a prolonged Ca^{2+} influx response.

Thus, Ca^{2+} influx appears to play an important role in regulating the activation of human eosinophils. Inhibition of Ca^{2+} influx would appear to be a potential strategy for the design of a therapeutic agent in asthma. In the light of the recent report that C5a and fMLP stimulate the release of IL-8 and GM-CSF from human eosinophils (Miyamasu *et al.*, 1995), it would be extremely relevant to investigate

whether this process is dependent upon RMCE. Inhibition of cytokine generation from human eosinophils may help reduce the further recruitment of cells to the site of inflammation and this would certainly enhance the therapeutic potential of a RMCE inhibitor in asthma.

Currently, there are no potent and selective agents that are able to inhibit this influx pathway. An increased understanding of the regulatory mechanisms that control receptor-mediated Ca^{2+} influx in non-excitable cells, as well as determination of the molecular identity of this channel, is still required.

Randriamampita and Tsien (1993) were the first to partially characterise a novel soluble mediator, which was generated in response to depletion of intracellular Ca^{2+} stores and could be extracted from Jurkat cells. This messenger caused a sustained Ca^{2+} influx response when applied extracellularly to macrophages, fibroblasts and astrocytoma cells and was termed calcium influx factor (CIF).

However, since this initial report, several groups have attempted to purify this extract and determine the chemical identity of CIF, so far without success. Jurkat cell extract contains at least 2 factors that regulate Ca^{2+} influx activity. One of these factors is a polar molecule, which is only active intracellularly, whilst the other is non-polar and active when applied extracellularly (Thomas and Hanley, 1995). Attempts to activate I_{CRAC} with Jurkat cell extracts have been reported to be unsuccessful, whilst activation of an unrelated non-selective cation channel has been reported (Clapham, 1995). Thus, it remains to be seen whether a pure CIF, which signals depletion of Ca^{2+} stores, will eventually be identified.

More recent hopes for identifying a Ca^{2+}-store-operated channel have focused on the *Drosophila* transient receptor potential (*trp*) Ca^{2+} channel. The *Drosophila trp* gene codes for a photoreceptor Ca^{2+} channel with properties analagous to those of I_{CRAC} (Vaca *et al.,* 1994).

Expression of *trp* protein in bacullovirus Sf9 insect cells demonstrated that this protein forms a channel that is highly Ca^{2+}-permeable and is activated by thapsigargin (Vaca *et al.,* 1994). Cloning of *trp* has recently lead to the identification of homologous proteins in *Xenopus* oocytes and mouse brain, which is the first evidence of a mammalian homologue of *trp*. Furthermore expression of *trp* in Xenopus oocytes lead to a significant increase in capacitative Ca^{2+} entry (Petersen *et al.,* 1995). It is hoped that the identification of mammalian homologues of *trp* will potentially lead to the cloning of store-operated Ca^{2+} channels.

Thus many advances in the understanding of the regulation and function of RMCE have recently been made. It remains to be seen whether different sub-types of channel will identified, analagous to the situation with VOCs. The identification of potent and specific inhibitors of RMCE pathway(s) will also be of great value in characterising these channels and identifying whether there are different sub-types of channel.

The ubiquitous presence of these Ca^{2+} channels on inflammatory cells makes them an attractive target for modulation of inflammatory responses. Future potent RMCE inhibitors may represent a novel mechanism for a new class of anti-inflammatory agent. Furthermore, the regulatory effect of RMCE inhibitors

on human eosinophil activation suggests that this class of agent may represent an exciting new therapy for the treatment of asthma.

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Appendix 1 : Work published during the course of this thesis.

Grix, S. P., Gardiner, P.J., Westwick, J. and Poll, C.T. (1994) Regulatory role for Ca^{2+} for superoxide and leukotriene C_4 production in human eosinophils. *British Journal of Pharmacology.* **112**: 85P

Grix, S. P., Gardiner, P.J., Westwick, J. and Poll, C.T. (1994) Receptor-mediated Ca^{2+} entry regulates C5a-induced activation of human eosinophils. *British Journal of Pharmacology.* **114**: 53P

Grix, S. P., Gardiner, P.J., Westwick, J. and Poll, C.T. (1995). Characterisation of thapsigargin-induced human eosinophil activation (proceedings from the 9th international conference on second messengers and phosphoproteins, in *Second messengers and phosphoproteins*, Raven press).

Grix, S.P., Gardiner, P.J., Westwick, J. and Poll, C.T. Receptor-mediated Ca^{2+} entry regulates superoxide (O_2^-) and leukotriene C_4 production in human eosinophils (full paper submitted to the British Journal of Pharmacology, September 1995).

Grix, S. P., Gardiner, P.J., Westwick, J. and Poll, C.T. Investigation of signal transduction processes involved in agonist-induced leukotriene C_4 generation in human eosinophils (Presented at the British Pharmacological Society meeting in December 1995).