MOLECULAR MECHANISMS OF STIMULUS-RESPONSE COUPLING IN HUMAN AND GUINEA PIG EOSINOPHILS. TRANSDUCTION PATHWAYS UTILIZED BY PLATELET ACTIVATING FACTOR AND THEIR RELATIONSHIP TO CELLULAR EFFECTOR FUNCTIONS

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To my wife

Nasim

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ABSTRACT

The molecular mechanisms of signal transduction in eosinophils by platelet activating factor (PAF) and the relationship to the cellular responses were investigated. PAF initiated eosinophil chemotaxis, granular enzyme secretion (eosinophil peroxidase, arylsulfatase B, β- glucuronidase), generation and release of prostanoids (the thromboxane TXB_2, and the prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$), and the production of oxygen radicals (measured as superoxide anion release, $\cdot O_2^-$) through the specific binding to two distinct classes of sites: a high affinity receptor (RI) with a dissociation constant K_D of approximately 0.3 nM (using [^{3}H]PAF as a radioligand) and 16 nM (using the PAF antagonist [^{3}H]WEB 2086) and a second receptor (RII) with an approximately 50- to 500- fold lower affinity. Occupation of the high affinity PAF- RI receptor related to degranulation and prostanoid release whereas occupancy of the PAF- RII receptor was associated with the generation of $\cdot O_2^-$ radicals. The receptor subtypes are differentially coupled to intracellular second messengers and enzymes. A pertussis toxin- sensitive guanine nucleotide binding protein of 41 kDa couples the PAF- RI receptor to phospholipase C (PLC) which, in turn, hydrolyses polyphosphoinositides to give inositol trisphosphate ($InsP_3$) and diacylglycerol (DAG). Both events are followed by an increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and activation of the protein kinase C (PKC) the latter being translocated from the cytosol to the eosinophil membrane. In contrast, stimulus activation coupling through the low affinity receptor was pertussis toxin-insensitive and appeared to occur without activation of the PLC, an elevation of $[Ca^{2+}]_i$, intracellular $Ins(1,4,5)P_3$, and DAG, indicating a transduction pathway different from the PAF- RI receptor. However, evidence for the involvement of the PKC was found. It is proposed that PAF utilizes two distinct transduction pathways in eosinophils operative in different functional

responses. These findings may have pathogenetic and therapeutic implications in inflammatory disease.

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ABBREVIATIONS

Ag,	antigen
ADP,	adenosine diphosphate
ATP,	adenosine trisphosphate
BAL,	bronchoalveolar lavage
BALF,	bronchoalveolar lavage fluid
BSA,	bovine serum albumin
C,	complement
°C,	degree Celsius
$[Ca^{2+}]_i$	intracellular calcium concentration
cAMP,	cyclic adenosine monophosphate
Ci,	curie
cpm,	counts per minute
d,	day
DAG,	1,2-diacylglycerol
dpm,	disintegrations per minute
DMSO,	dimethylsulfoxide
DNase,	deoxyribonuclease
DNP,	dinitrophenyl
DTT,	dithiothreitol
EC_{50}	50% effective concentration
ECF,	eosinophil chemotactic factor
ECP,	eosinophil cationic protein
EDN,	eosinophil-derived neurotoxin
EDTA,	ethylenediaminetetra-acetic acid
EGTA,	ethyleneglycol-bis(beta-aminoethylether)-tetra-acetic acid
EM,	electron microscopy
EPO,	eosinophil peroxidase
EPX,	eosinophil protein X (identical to EDN)
fMLP,	formyl-methionyl-leucyl-phenylalanine
g,	gram
GC/MS,	gas chromatography/mass spectrometry
GDP	guanosine diphosphate
GPC,	glycerophosphocholine
GPE,	glycerophosphoethanolamine
GTP,	guanosine triphosphate
h,	hour
HBSS,	Hanks' balanced salt solution
Hepes,	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HES,	hypereosinophilic syndrome
IC_{50},	50% ineffective concentration or 50% inhibiting concentration
ICS,	intracellular calcium stores
Ig,	immunoglobulin
InsP,	phosphatidyl inositol
$Ins(4,5)P_2$,	phosphatidylinositol-4,5-bisphosphate
Ins(1,4,5)P3,	phosphatidylinositol-1,4,5-trisphosphate
K_d,	dissociation constant
kDa,	kilodalton
LT,	leukotriene
m	meter
M,	molar
mAb,	monoclonal antibody

MBP,	major basic protein
min,	minute
ml,	milliliter
mo,	month
mol,	mole
MPO,	myeloperoxidase
n,	number in study or group
N,	normal (concentration of ionizable groups)
ND,	not determined
$\cdot O_2^-$	superoxide anion
OD,	optical density
OPD,	o-phenylenediamine
osM,	osmolar
osmol,	osmole
p,	probability
PAF,	platelet activating factor
PAF-RI,	high affinity PAF receptor
PAF-RII,	low affinity PAF receptor
PAGE,	polyacrylamide gel electrophoresis
PBS,	phosphate-buffered saline
PG,	prostaglandin
PGI_2,	prostacyclin
Pipes,	piperazine-N',N'-bus(2-ethane sulfonic acid)
PKC,	protein kinase C
PLA_2,	phospholipase A_2
PLC,	phospholipase C
PMA,	phorbol-12-myristate-13-acetate
PMN,	polymorphonuclear leukocytes
PTX,	pertussis toxin
R,	receptor
RIA,	radioimmunoassay
sec,	second
SD,	standard deviation
SDS,	sodium dodecyl sulfate
SE,	standard error
SEM,	standard error of mean
$t_{1/2}$,	half-time
TCA,	trichloroacetic acid
TLC,	thin layer chromatography
Tris,	tris(hydroxymethyl)aminomethane
TX,	thromboxane
U,	unit
wk,	week
ZAP,	zymosan-activated plasma

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CHAPTER ONE

1 INTRODUCTION

1.1 BACKGROUND

Inflammation may be defined as a non-specific protective reaction of tissues to injury, aimed at the maintenance of health and integrity of the individual and leading to the return to normal structure and function. However, excessive or dysregulated inflammation can destroy tissue, perpetuating many disease processes and leading to chronic inflammation and fibrosis. Typical examples include allergic inflammation, i.e asthma, or hypersensitivity pneumonitis, interstitial lung diseases and acute lung injury leading to adult respiratory distress syndrome. Successful therapeutic intervention in such diseases requires a complete understanding of the mechanisms involved. Thus, studies of fundamental mechanisms of inflammation are important endeavours and need to encompass the initiating process, the factors responsible for the attraction and accumulation of inflammatory cells, and the molecular events resulting within these cells. This thesis will focus on the eosinophil leukocyte which is now thought to represent a major effector cell not only in host defense against parasites but also in the pathogenesis of hypersensitivity reactions.

1.2 THE EOSINOPHIL LEUKOCYTE

The eosinophil granulocyte, although probably first observed by Wharton Jones in 1846 in unstained preparations of peripheral blood, was so named by Paul Ehrlich in 1879 because of the intense staining of its granules with the acidic dye eosin. Since then, the eosinophil has been the subject of intense investigation. Although its role in the host defence against parasitic infections has long been recognized, its significance in hypersensitivity disease is only now beginning to unfold.

1.2.1 Structure and morphology

The most distinguishing morphological features of the eosinophil are a bilobed nucleus and large eosinophilic granules which stain yellow-pink with eosin and other acidic aniline dyes (Miller *et al.*, 1966). These large elliptical secondary or specific granules possess an electron-dense crystalloid core that is embedded in a less electron-dense matrix (Fig. 1.1). They appear after the myelocyte stage and may develop from large spherical primary lysosomal granules (Bainton & Farquhar, 1970). In addition, some of these primary granules are thought to develop into smaller homogeneously dense cytoplasmic bodies containing lysophosphatase (Dvorak *et al.*, 1988). A third type of granule, the so called small granules, are less conspicuous. They form during the metamyelocyte stage, increase progressively in number with cellular maturation to become small and less electron-dense homogeneous cytoplasmic structures (Parmley & Spicer, 1974). All three granules are present in the mature eosinophil (Dvorak *et al.*, 1988) and contain different constituents (Table 1.1). Like other leukocytes, eosinophils contain several other organelles such as the endoplasmic reticulum, ribosomes, Golgi-apparatus and mitochondria of which the latter three seem to become more prominent in activated cells (Zucker-Franklin, 1974).

1.2.2 Granular proteins

The eosinophil stores several preformed proteins in the cytoplasmic granules which can be categorised on the basis of their function into enzymes and non-enzymatic basic proteins (Table 1.1). The arginine-rich basic proteins include the major basic protein (MBP), eosinophil cationic protein (ECP) the eosinophil-derived neurotoxin (EDN, also termed eosinophil protein X or EPX) and are the major constituents of the secondary granule. MBP comprises over 95 percent of the core whereas ECP and EDN are confined solely to the matrix of secondary granules.

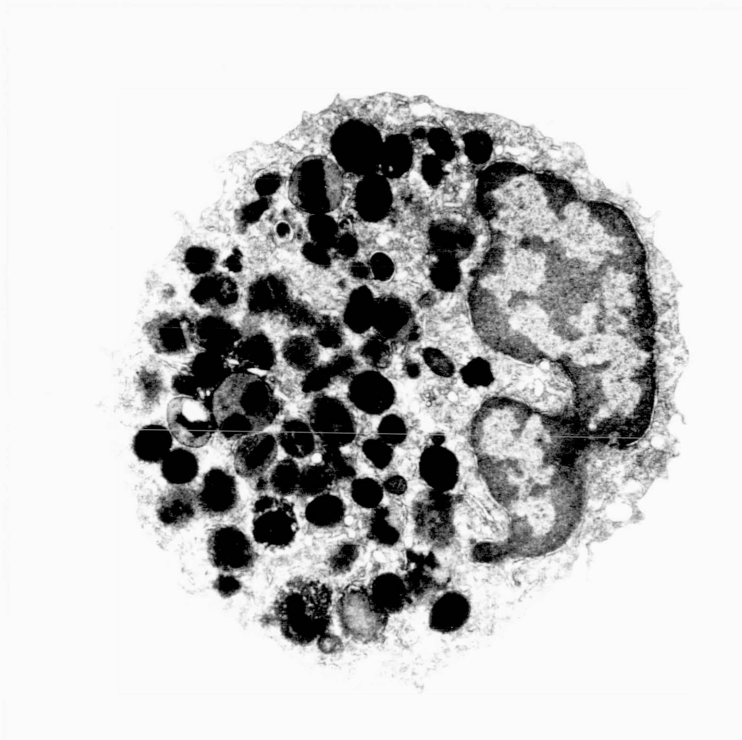


Figure 1.1: Electron micrograph of an eosinophil leukocyte. An eosinophil leukocyte obtained from the peritoneum of a guinea pig (original magnification x 5,000)

Primary Granules (Promyelocyte)
- Precursor proteins

Secondary Granules
- β-glucuronidase
- Collagenase
- Acid glycerophosphatase
- Alkaline phosphatase
- Histaminase
- Eosinophil peroxidase
- Major basic protein
- Eosinophil cationic protein
- Eosinophil Protein X
- Eosinophil-derived neurotoxin

Small Granules
- Arylsulfatase B
- Acid Phosphatase

Table 1.1: Constituents of the primary, secondary (specific), and small granules found in eosinophil leukocytes.

Functionally, the proteins are characterized as cytotoxic to an array of different targets (Gleich & Adolphson, 1986).

The secondary granules, which also contain a number of enzymes in their matrix resemble the neutrophil lysosomes in their composition. The principal differences between the two being that the eosinophil lacks lysozyme, and the type of peroxidase (EPO versus MPO), β-glucuronidase,and acid phosphatase also exceeds that of neutrophils two- to three-fold (Migler & DeChatelet, 1978; Weller & Goetzl, 1980; Gleich & Loegering, 1984). The eosinophil peroxidase(EPO) is a single chain protein of 75 kDa and exhibits microbiocidal and cytotoxic activities both in the presence or absence of a halide and H_2O_2(Jong & Klebanoff, 1980; Jong *et al.*, 1981; Weiss *et al.*, 1986)

1.2.3 Membrane proteins

Eosinophil leukocytes share many antigens found on other circulating white blood cells, such as the class I HLA antigens and the common leukocyte antigen CD45 (Kroegel *et al.*, 1991c; Hartnell *et al.*, 1990) although a specific epitope has not yet been identified. However, eosinophils can be differentiated from other leukocytes because they lack certain antigenic determinants. It is of particular significance that eosinophils do not express the IgG receptor FcγIII (CD16), an observation which has been used to distinguish between eosinophil and neutrophil populations. In addition, unlike monocytes which express FcγRI and FcγRII and neutrophils which express FcγRII and FcγRIII, eosinophils bear solely the FcγRII receptor (CDw32) on their membrane surface (Tosi & Berger, 1988; Kroegel *et al.*, 1991c). Interestingly, eosinophils challenged with anti-IgG secreted ECP but failed to release significant amounts of EPO (Capron *et al.*, 1989) or PAF (Capron *et al.*, 1988a)

IgE receptors on human eosinophils have been demonstrated by the rosette technique (using IgE myeloma protein bound to red cells) as well as by radioligand studies using ^{125}I-labelled IgE (Capron *et al.*, 1981; Capron *et al.*, 1985;

Walsh *et al.*, 1989). This receptor appears to be similar to the IgE receptors on T and B lymphocytes, platelets and macrophages. The IgE receptor on eosinophils ($Fc_{\varepsilon}RII$) is distinguished from the IgE receptors on mast cells and basophils($Fc_{\varepsilon}RI$) by its approximately 1000-fold lower binding affinity. In contrast, polymeric IgE contained in immune complexes is bound to eosinophils with a similar affinity as monomeric IgE to the mast cell and basophil $Fc_{\varepsilon}RI$ receptors. The number of $Fc_{\varepsilon}RII$ receptors appear to be increased on hypodense and normodense (see Chapter 1.2.6) eosinophils from patients with eosinophilia when compared to eosinophils from normal subjects. Functionally, binding of IgE leads to the release of EPO (Capron *et al.*, 1989) and generation of PAF (Capron *et al.*, 1988a) but fails to secrete ECP.

The same group (Capron *et al.*, 1988b) reported that human and animal eosinophils bind monomeric or secretory IgA, although with a relatively low affinity. However, challenge of eosinophils with IgA and particularly the IgA-dimer causes degranulation with the release of EPO (Capron *et al.*, 1988b) and ECP (Abu-Ghazaleh *et al.*, 1989).

Normal blood eosinophils do not express IgM receptors (Ottesen *et al.*, 1977; DeSimone *et al.*, 1982b; Walsh & Kay, 1986) although IgM binding to eosinophils can be induced by culturing them in the presence of cytokines (DeSimone *et al.*, 1982b). The functional effects of IgM binding to eosinophils are as yet not known.

In addition to immunoglobulin receptors, eosinophils express the leukocyte adhesion glycoproteins LFA-1 (CD11a), CR3 (CD11b),and the common β-chain (CD18) (Hartnell *et al.*, 1990; Kroegel *et al.*, 1991c). These surface proteins belong to a family of three receptors also termed integrins (Bernhard & Self, 1986). They are heterodimers composed of two non-covalently linked polypeptide chains: a distinct α-chain and a common β-chain (Springer & Anderson, 1986). The function of the α-chain is to confer receptor specificity whereas the β-chain directs the receptor protein into the proper configuration at the cell membrane. These leukocyte integrins facilitate the intercellular adhesion of eosinophils to microvascular endothelium (Dustin

et al., 1986; Lamas *et al.*, 1988; Wegner *et al.*, 1990) which is essential for the recruitment and migration of the cell into inflamed tissue. They may also be involved in binding of other ligands such as opsonized targets (Ross *et al.*, 1985; Fisher *et al.*, 1986; Myones *et al.*, 1988; Huizing *et al.*, 1989; Schleiffenbaum *et al.*, 1989).

Receptors for the complement factors C1, C3a, C3b, C3d and C4b have also been identified on human eosinophils (Anwar & Kay, 1977; Tai & Spry, 1980). However, eosinophils have far fewer of these receptors than neutrophils. The proportion of complement bearing eosinophils ranges from 30% in normal individuals to 40% in patients with parasitic infestations (Ottesen *et al.*, 1977). The chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) enhanced the number of C3b receptors (Kay *et al.*, 1979). In addition, ECF-A, histamine and its major catabolite, imidazole acetic acid, have been reported to increase the expression of C3b and C4 receptors on human eosinophils whereas C3d and IgG (Fcγ) receptors remained unaffected (Anwar & Kay, 1978).

As mentioned above, eosinophils express receptors for iC3b (CR3), a degradation product of C3b. Functionally CR3 appears to be similar to the receptor for C3b, causing binding of particles coated with iC3b to a phagocytic cell surface but failing to trigger ingestion in the absence of a second signal (Wright & Silverstein, 1982).

1.2.4 Eosinopoiesis

Recent evidence suggests that eosinopoiesis is closely related to the development of basophils (Ackerman *et al.*, 1982; Saito *et al.*, 1982; Ackerman *et al.*, 1983; Denburg *et al.*, 1985). The site of eosinophil production and development in mammals is the bone marrow (Hudson, 1968; Dresch *et al.*, 1977, Zucker-Franklin, 1985). There is evidence that the eosinophil descends from a totipotent hematopoietic stem cell (Abramson *et al.*, 1977; Fleishman *et al.*,1982) that gives rise to pluripotent stem cells (Ogawa *et al.*,1983; Leary *et al.*, 1984). Further differentiation results in a

progenitor cell committed to a single hematopoietic lineage (Dao *et al.*, 1977; Weller & Goetzl, 1980; Metcalf *et al.*, 1983). The progenitor cells have great proliferation potential and are sensitive to regulatory systems, leukopoietins, or colony-stimulating factors, which trigger differentiation to effector cells with various functions (Basten & Beeson, 1970; Metcalf *et al.*, 1986). Four different protein factors have been identified which promote and regulate the proliferation and differentiation of the eosinophil lineage: Interleukin (IL)-1, IL-3 (multi-colony stimulating factor, multi-CSF), IL-5 and the granulocyte-macrophage-colony stimulating factor (GM-CSF) (Silberstein *et al.*, 1989; Cosman, 1988; Clark & Kamen, 1987). IL-3 and GM-CSF support the growth of cells from relatively early pluripotent multilineage progenitors to mature cells whilst IL-5 supports the growth and proliferation of relatively late progenitors already committed to the eosinophil lineage.

1.2.5 Life cycle and kinetics

The mean eosinophil emergence time (time span between the end of the last premitotic S-phase in the proliferating pool and the egression of mature cells from the marrow) is between 3 to 5 days in humans (Parwesch *et al.*, 1976; Steinbach *et al.*, 1979). The eosinophil enters the circulation as a fully mature cell undergoing only minor biochemical changes. Eosinophils are thought to persist in the circulation with a mean half-life of between 6 and 18 hours (mean life between 12 and 26 hours) before entering the marginated pool (blood vessel wall) or infiltrating tissues. (Spry, 1971; Parwesch *et al.*, 1976; Steinbach *et al.*, 1979).

Only about 1% of the total eosinophils circulate in the blood. The vast majority of the cells accumulate in the parenchyma of certain organs and tissues preferentially below epithelial surfaces. Thus, outside of the bone marrow, most eosinophils are found in the skin, lungs, gastrointestinal tract, lower urinary tract and the uterus (Rytomaa, 1960). In disease, however, the number of eosinophils in both the peripheral blood and tissue can be dramatically increased. This may be due partly to

a shortening of the eosinophil generation time (Spry, 1971) and partly to the return of tissue eosinophils to the circulation (Dale, 1976).

1.2.6 Heterogeneity

Eosinophils comprise a heterogeneous population of cells when evaluated for their density. Eosinophils sedimenting at densities > 1.082 g/ml are designated normodense and eosinophils with a density < 1.082 g/ml are referred to as hypodense. Normodense eosinophils represent approximately 90% of the cells found in normal subjects (Fukuda *et al.*, 1985; Shult *et al.*, 1988; Prin *et al.*, 1984). In contrast, a significant proportion of peripheral blood eosinophils from patients with certain eosinophil-associated diseases such as allergic rhinitis (Frick *et al.*, 1988), asthma (Fukuda *et al.*, 1985; Shult *et al.*, 1988; Kloprogge *et al.*, 1989), allergic bronchopulmonary aspergillosis and helminthic parasite infestation (Prin *et al.*, 1984) are found to be less dense (Weller, 1984; Fukuda *et al.*, 1985; Kauffman *et al.*, 1987; Kloprogge *et al.*,1989). The mean percentage of hypodense eosinophils for patients with asthma in three studies were 35% (Fukuda *et al.*,1985), 41% (Shult *et al.*, 1988) and 65% (Kloprogge *et al.*, 1989), respectively, whereas for patients with allergic rhinitis a figure of 30% hypodense eosinophils has been reported (Frick *et al.*, 1988). In addition, the proportion of hypodense cells in bronchoalveolar lavage fluid (BALF) from asthmatics is consistently higher than that of blood eosinophils obtained simultaneously (Kroegel *et al.*, 1991c). In chronic eosinophilic pneumonia too the number of hypodense cells is higher in BALF than in blood (Kroegel & Costabel, 1991) and correlates with disease activity (Prin *et al*, 1986). Finally, the proportion of hypodense eosinophils in pleural fluids from patients with various lung diseases is dramatically increased (Winqvist *et al.*, 1982; Prin *et al.*,1983). The density of eosinophils appears therefore to depend on (1) the total number of eosinophils, (2) disease and disease activity, and (3) location of the cells.

The significance of hypodense eosinophils in certain diseases is not yet understood and is still a matter of speculation. Functionally, a number of differences between normodense and hypodense eosinophils have been demonstrated, which include differences in membrane receptor expression, cytotoxicity to immunoglobulin-coated targets, metabolic rate, and responsiveness upon stimulation with cell activators. For instance, the number of IgE receptors on low density eosinophils from patients suffering from hypereosinophilic syndrome (HES) is markedly increased (Parillo & Fauci, 1978; Capron *et al.*, 1984) whereas IgG receptor expression undergoes only a modest increase (Winqvist *et al.*, 1982). Low density eosinophils from HES patients are cytotoxic to IgE-coated schistosomula while normodense eosinophils are cytotoxic to IgG-coated schistosomula (Parillo & Fauci, 1978; Khalife *et al.*, 1986). Secretion of eosinophil peroxidase by low density eosinophils from HES patients is caused by stimulation with antigen or anti-IgE while normodense eosinophils from HES patients respond to anti-IgG (Khalife *et al.*, 1986). In addition, eosinophils from patients with parasitic diseases show a slightly reduced density and an increased cytotoxicity towards IgG-coated chicken erythrocytes (Bass *et al.*, 1980; DeSimone *et al.*, 1982a). Low density eosinophils obtained *in vitro* by incubation with GM-CSF are cytotoxic to IgG-coated schistosomula (Owen *et al.*, 1987). Furthermore, light density eosinophils from patients with HES generate and release greater amounts of leukotriene C_4 in response to IgG- or complement-coated targets and the monocyte-derived eosinophil activating factor (EAF) than do normal density eosinophils (Kauffman et *al.*, 1987; Fitzharris *et al.*, 1986; Kajita *et al.*, 1985). The amount of PAF released by light density blood eosinophils from HES patients upon stimulation with calcium ionophore A23187 (Jouvin-Marche *et al.*, 1984) is approximately 20-fold higher when compared to the amount produced by normodense eosinophils (Lee *et al.*, 1984). Low density eosinophils from patients with HES show an increased deoxyglucose uptake and oxygen consumption than normodense cells (Winqvist *et al.*, 1982; Prin *et al.*, 1983) indicating a higher metabolic activity in light density eosinophils. In contrast, these

cells produced a lower amount of reactive oxygen metabolites in response to phorbol myristate acetate (PMA), as assessed by NTB reduction and chemiluminescence (Prin *et al.*, 1984). Thus, in general, hypodense eosinophils appear to represent an activated eosinophil subtype.

1.2.7 Effector mechanisms

The eosinophil mediates its effector functions through different cellular mechanisms namely nonoxidative, oxidative, and humoral mechanisms.

Non-oxidative mechanisms. As mentioned previously, the three basic proteins as well as EPO have toxic properties independent of molecular oxygen species (Table 1.1). For instance, MBP (Butterworth *et al.*, 1979), ECP (McLaren *et al.*, 1981) and EPO in the presence of hydrogen superoxide and a halide (Jong *et al*,1981) can all kill helminthic parasites *in vitro.* In addition, these proteins are toxic for tumour cells and/or mammalian cells (Gleich *et al.*, 1979), including human pulmonary parenchyma and interstitial matrix (Gonzales *et al.*, 1987a; Davis *et al.*, 1984), cultured human lung epithelial cells (Ayars *et al.*; 1985), and guinea pig respiratory epithelium (Filley *et al.*, 1982; Frigas *et al.*, 1986). Furthermore, it has been demonstrated that tissue damage produced by MBP in human bronchial epithelium consisting of desquamation and destruction of ciliated cells (Frigas *et al.*, 1981; Hastie *et al.*, 1987), mimics the pathological findings in asthma (see below). Finally, ECP and MBP stimulate histamine release from mast cells and basophils (O'Donnell *et al.*, 1983; Zheutlin *et al.*, 1984).

The mode of action of these basic proteins is not yet known. Structurally, they contain a cluster of basic amino acids, such as lysine and alanine on one terminal as well as a long aliphatic amino acid chain on the other molecule terminus. Their physicochemical properties resemble hymenoptera venom toxins such as melittin or mastoparan (Kroegel *et al.*, 1987; Kroegel *et al.*, 1981; Perianin & Snyderman, 1989), the ninth component of complement (Podack and Tschopp, 1982),

perforin 1 from natural killer and cytotoxic T lymphocytes (Podack *et al.*, 1985), bacterial streptolysin-O and staphylococcal α-toxin (Bhakdi & Tranum-Jensen, 1985). These proteins share an affinity for plasma membranes to which they bind in a dual fashion: First, the proteins approach the cell through the formation of electrostatic bonds between their positively charged residues and anionic (acidic) groups on the external membrane surface. This interaction may then be followed by insertion of the lipophilic molecule fragment into the membrane lipid bilayer. The driving force for the hydrophobic interaction would be the entropic advantage gained from dissolution of both the hydrophobic core of the phospholipid bilayer and the apolar surface of the amphiphilic protein (Kroegel, *et al* 1981; Kroegel, 1984; McDowell *et al.*, 1985; Kroegel *et al.*, 1987; Abu-Ghazaleh *et al.*, 1991). This protein-membrane interaction leads to a number of consequences for the target cell including activation of phospholipase A_2 (Argiolas & Pisano, 1983; Kroegel *et al*, 1990a), formation of voltage-insensitive transmembrane ion channels (Podack *et al.*, 1985; Young *et al.*, 1986) followed by an enhanced membrane permeability to calcium (Pernian and Snyderman, 1989; Kroegel *et al.*, 1990a), and, at high concentrations, total membrane disruption (Kroegel *et al.*, 1981; Kroegel, 1984; Kroegel *et al.*, 1987). A recent study has, indeed, demonstrated that the eosinophil basic proteins, like melittin, form non-selective ion pores in liposome membranes (Young *et al.*, 1986).

Oxidative mechanisms. Eosinophils may also exert their effector function via two oxygen-dependent mechanisms (Weiss *et al.*, 1986; Petreccia *et al.*, 1987; Kanofsky *et al.*, 1988). One involves the generation of activated oxygen and toxic oxygen radicals (O_2 and $\cdot O_2^-$), and the other the production of hypobromous acid (HOBr), hydrobromic acid ($HBrO_3$) or hydriodic acid (HIO_3). These two events are linked, as newly formed superoxide spontaneously dismutates to H_2O_2. Both $\cdot O_2^-$ and H_2O_2 alone may have toxic effects (Weiss *et al.*, 1986). In addition, H_2O_2 serves as a cofactor in the EPO-mediated oxidation of both iodide (I^-) and bromide (Br^-) to their corresponding

hypohalous acids which, in turn, are able to halidate or oxidize a wide range of target molecules in cells and microorganisms.

Lipid mediator-mediated mechanisms. Eosinophils contribute to inflammation through the *de novo* synthesis and release of lipid mediators such as platelet activating factor (PAF) (Lee *et al.*,1984; Burke *et al.*, 1990), leukotriene B_4 (LTB_4) and C_4 (LTC_4) (Ziltner *et al.*, 1983; Henderson *et al.*, 1984; Shaw *et al.*, 1984; Bruynzeel *et al.*, 1987b; Kauffman *et al.*, 1987; Owen *et al.*, 1988; Tamura *et al.*, 1988; Burke *et al.*, 1990), prostaglandin E_2 (PGE_2) (Hubscher, 1975; Giembycz *et al.*,1990), PGF_1 and thromboxane A_2 (TXA_2) (Owen *et al.*, 1987, Morley *et al.*, 1979; Foegh *et al.*, 1986; Giembycz *et al.*, 1990a). Using combined capillary gas chromatography/mass spectrometry two more prostanoids, prostaglandin D_2 (PGD_2) and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) have been detected (Kroegel *et al.*, 1990b). Eosinophil-derived PAF, LTC_4 or TXA_2 may contribute to tissue inflammation and bronchoconstriction in late-phase asthmatic reactions where eosinophils are characteristically found (see below).

1.2.8 Priming

The presence of lymphocytes in asthmatic airways is well documented (Laitenen, 1985; Gonzales *et al.*, 1987b; Frew & Kay, 1988). T lymphocytes of the $CD4^+$, T_h/ inducer phenotype as well as macrophages and vascular endothelial cells secrete a variety of growth and differentiation factors called lymphokines or cytokines which include interleukin-1 (IL-1), IL-3, IL-5 and granulocyte/macrophage-colony-stimulating factor (GM-CSF). Human peripheral-blood-derived normodense eosinophils cultured for 7 days with IL-3 (Owen *et al.*, 1987) or GM-CSF (Silberstein *et al.*, 1986; Rothenberg *et al.*, 1988; Rothenberg *et al.*, 1989; Howell *et al.*, 1999) generate increased amounts of LTC_4 when challenged with calcimycin (the calcium ionophore A23187) and enhanced cytotoxicity toward schistosomula. Evaluation of the cell density revealed that exposure of eosinophils to the cytokines converted them to a hypodense phenotype. A similar effect on eosinophil releasability was observed with

IL-5 (Lopez *et al.*, 1988; Yamaguchi *et al.*,1988). In addition, Fujisawa *et al.* (1990) demonstrated that preincubation of normal eosinophils with IL-3, IL-5 or GM-CSF also enhanced the sIgA- and IgG-induced release of EDN. IL-5 was the most potent enhancer of immunoglobulin-mediated degranulation and its effect appeared within 15 minutes. Furthermore, GM-CSF and IL-5 by themselves induced a small but significant release of EDN. In contrast, other cytokines, such as IL-1, IL-2, IL-4, IL-6, IFN-γ, and TNF, had no effect on eosinophil degranulation. Finally, there is evidence that lipid mediators, at subthreshold concentrations may also have a priming activity (Nagy *et al.*, 1982; see also Chapter 10.8, page 215). Taken together, these findings suggest that eosinophil effector functions may be modulated by cytokines as well as other inflammatory mediators. Since alveolar macrophages obtained from asthmatic subjects spontaneously produce GM-CSF (Howell et al., 1989; Howell & Kay, 1990) and it's level is increased in BALF after antigen challenge (paper in preparation), cytokines may play an important role in the amplification of the eosinophilic inflammation in asthmatic airways.

1.3 EOSINOPHILS IN DISEASE

Eosinophils have been implicated in a wide spectrum of human disease ranging from parasitic infestations, and allergic reactions to vasculitic and granulomatous diseases (Schatz *et al.*, 1981; Hunninghake *et al.*, 1981; Spry *et al.*, 1983a; Spry *et al.*, 1983b; Davis *et al.*, 1984; Spry 1989). The potential role of the eosinophil in helminthic infections, interstitial lung disorders, and bronchial asthma including both their beneficial or protective and detrimental role will be outlined below.

1.3.1 Parasitic infestations

The association between helminth infestation and peripheral blood eosinophilia dates back to the beginning of this century. Four main findings have emerged over the past two decades. First, anti-eosinophil serum reduces both the

number of peripheral blood eosinophils and increases susceptibility to parasites (Gleich *et al.*, 1979). Secondly, eosinophils are directly involved in the killing of helminths (Butterworth *et al.*, 1977; Butterworth *et al.*,1979). Third, eosinophil granule proteins and oxygen metabolites are cytotoxic for parasites (Gleich *et al.*, 1974; Grove *et al.*, 1977; Butterworth, 1984).. Finally, eosinophils accumulate and degranulate around parasites *in vivo* (Hsu *et al.*, 1980; Kephart *et al.*, 1984). These data strongly support a role for the eosinophil in host defense against parasites.

The mechanism whereby eosinophils destroy parasites can be divided into recognition, attachment and killing phases. Recognition is facilitated by chemotactic factors produced by other participating inflammatory cells and by parasite-derived factors. In addition, IgG antibodies (Gryzch *et al.*, 1987) and complement proteins (Anwar *et al.*, 1979) facilitate the attachment of the eosinophil to the parasite. Adhering eosinophils flatten and release granule proteins (Glaudert *et al.*, 1978) and oxygen radicals (Kazura *et al.*, 1981; Jong *et al.*, 1981) onto the surface of the parasite eventually leading to its destruction.

1.3.2 Interstitial lung disorders

Although eosinophils are rarely found in the normal human lower respiratory tract, accumulation of eosinophils in the parenchyma is found in the course of various inflammatory diseases (Reynolds *et al.*,1977; Weinberger *et al.*, 1978). Besides the well known eosinophil-associated histiocytosis-X (Bassat *et al.*, 1978), and chronic eosinophilic pneumonia (Carrington *et al.*, 1969) other diseases such as idiopathic pulmonary fibrosis, sarcoidosis, hypersensitivity pneumonitis, and chronic interstitial disease associated with the collagen-vascular disorders can, on occasion, also be characterized by an accumulation of significant numbers of eosinophils in alveolar structures (Hunninghake *et al.*, 1981; Schatz *et al.*, 1981).

Although the cause of eosinophil accumulation in some of these disorders is unknown, increasing evidence suggests that the eosinophil can function as

an effector cell capable of mediating direct interstitial tissue destruction. In general, two different mechanisms may be involved. Firstly, the eosinophil collagenase has been shown to specifically cleave human lung collagens type I and III (Davis *et al.*, 1984, Hibbs *et al.*, 1982). Secondly, eosinophils damage parenchyma cells through their granular basic proteins and the generation of reactive oxygen radicals. For instance, eosinophils are cytotoxic for lung fibroblasts, mesothelial cells, and epithelial cells (Gleich *et al.*, 1979; Davies *et al.*, 1981; Davies *et al.*, 1984; Ayars *et al.*, 1985; Agosti *et al.*, 1987; Yukawa *et al.*, 1990b). Eosinophil mediated cytotoxicity could be partially inhibited by anti-oxidants (Motojima *et al.*, 1989; Yukawa *et al.*, 1990b) suggesting a role for both oxidative and non-oxidative effector mechanisms.

1.3.3 Bronchial asthma

Asthma is increasingly being recognized as a chronic inflammatory condition characterized by peripheral blood, bronchial tissue and sputum eosinophilia (Lowell, 1967, Franklin, 1974; Horn *et al.*, 1975; Ellis, 1908; Huber and Koessler, 1922; Houston, 1953; Guputa *et al.*, 1976; Dunhill, 1960; Naylor, 1962). The prominence of the eosinophil in asthmatic airways has even led to the suggestion that asthma would be better classified as 'chronic desquamating eosinophil bronchitis' (Reed, 1986).

1.3.3.1 Histopathology

The most striking histopathological feature of asthmatic airways is disseminated inflammatory cell infiltrates. These are predominantly eosinophils which penetrate the entire thickness of the bronchial wall, the nearby parenchyma and overflow into the bronchial lumen (Ellis, 1908; Huber & Koessler; 1922, Houston, 1953; Dunhill, 1960; Naylor, 1962; Horn *et al.*, 1975; Guputa *et al.*, 1976; Aita *et al.*, 1983). Although eosinophilic mucosal infiltrations are a characteristic finding in status asthmaticus (Cardell, 1956; Houston *et al.*, 1953; Earle, 1953) eosinophils are also

seen in patients with chronic bronchitis and bronchial inflammation (Glynn & Michaels, 1960; Salvato, 1968) indicating that bronchial eosinophilia is quantitative rather a qualitative abnormality. Another striking histological feature in asthma is the damaged and denuded epithelial cells (Laitinen *et al.*, 1985) usually found in conjunction with eosinophil products (Filley *et al.*, 1982). Desquamation of the epithelium can be extensive (Cardell, 1956; Houston *et al.*, 1953) with the basal layer becoming hyperplastic as it regenerates. The basement membrane is thickened (Huber & Koessler, 1922; Callerame *et al.*, 1971; Sobonya, 1984) with increased production of collagen and deposition of IgG, IgE, IgA, albumin and fibrinogen (Gerber *et al.*, 1971; McCarter & Vazquez, 1966; Callerame *et al.*, 1971). Eosinophils are associated with the areas of desquamation, bronchial plugging, and basement thickening in the late stage of asthma (Frigas and Gleich, 1986). The airway smooth muscle layer is usually increased by two- to three fold (Dunhill *et al.*, 1969; Huber & Koessler, 1922; Heard & Hossain, 1973) which results from hyperplasia of muscle cells rather than hypertrophy (Heard & Hossain, 1973; Takizawa & Thurlbeck, 1971). Furthermore, the tracheobronchial mucous glands are enlarged together with an increase in the number of goblet cells (Dunhill *et al.*, 1969; Glynn & Michaels, 1960; Huber & Koessler, 1922; Takizawa & Thurlbeck, 1971). Finally, tenacious, viscid, mucous plugs are present in asthmatic airways, extending from the trachea to the respiratory bronchioles (Dunhill, 1960).

1.3.3.2 Eosinophilia in asthma

In asthma the increase in circulating blood eosinophils is variable and their elevation is less pronounced when compared with other eosinophil-associated diseases. In fact, in some cases, eosinophils are not detected at all (Dahl & Venge, 1982; Bruynzeel *et al.*, 1987a). This may stem partly from the fact that asthmatics are usually under treatment with corticosteroids, a drug which effectively decreases the number of circulating eosinophils (Baigelman *et al.*, 1983; Anderson, 1969; Sabag *et*

al., 1978). In addition, the appearance of eosinophils in peripheral blood may be temporal occurring only at the time of the late-asthmatic response (Kay *et al.*, 1988; Durham *et al.*, 1988; Durham et al., 1985; Booij-Noord *et al.*, 1972) due to their relatively short blood half-life of between 13 (Parwaresch *et al.*, 1976) and 18 hours (Steinbach *et al.*, 1979). On the other hand, a recent study has shown that while the total number of eosinophils in patients with asthma remained constant the percentage of hypodense cells increased significantly (Frick *et al.*, 1989a; Frick *et al.*, 1989b). In addition, an inverse correlation between blood eosinophil count and the degree of bronchial hyperresponsiveness (Durham *et al.*, 1985; Taylor and Luksa, 1987) and airway resistance (Horn *et al.*, 1975) has been reported. Measurement of eosinophil-derived protein levels in serum as an indicator of eosinophil activation, however, did not prove useful. Most of the studies have failed to find a significant or consistent elevation of these proteins, a fact which may be due to their rapid degradation by serum proteases (Venge *et al.*, 1977; Dahl *et al.*, 1978).

Large numbers of eosinophils are found in sputum of asthmatics, particularly in aspirin-induced asthma (Vierira & Prolla, 1979; Dor *et al.*, 1984; Frigas *et al.*, 1981; Motojima *et al.*, 1987). Compact clusters of columnar cells containing numerous eosinophils, eosinophil granules as well as Creola bodies may also be seen (Naylor, 1962). In addition, it has been demonstrated that the concentration of MBP in sputum is consistently raised and the detection of this protein is specific for asthma (Frigas *et al.*,1981; Dor *et al.*, 1984). The amount of Creola bodies and EPO in sputa were also elevated in asthma although these proteins are not specific for this disease (Motojima *et al.*, 1987).

As stated above, eosinophils are seen in and around the wall of bronchi in patients with fatal asthma (Callerame *et al.*, 1971; Gough, 1961; Motojima *et al.*, 1987; Godard *et al.*, 1982). Moreover, marked bronchial eosinophilia is the most characteristic histopathological feature in status asthmaticus and is also noted soon after an asthmatic attack (Cardell, 1956; Houston *et al.*, 1953; Earle, 1960). In marked

contrast, eosinophils are not found in biopsy specimens from asthmatic subjects in remission (Kirby *et al.*, 1987). Thus, bronchial eosinophilia may only represent a transient feature appearing to occur in association with the active ongoing pathogenetic processes in asthma.

Increased numbers of eosinophils are also found in bronchoalveolar fluid (BALF) from asthmatics (Diaz *et al.*, 1984; Metzger *et al.*, 1985; Diaz *et al.*, 1986; Godard *et al.*, 1987; Kirby *et al.*, 1987; Wardlaw *et al.*, 1988; Bousquet *et al.*, 1990). The proportion of eosinophils, however, is not excessively increased. Furthermore, in asymptomatic patients and in patients treated with steroids the number of eosinophils is often normal (Godard *et al.*, 1987). Comparison of different asthmatic types have demonstrated that the eosinophil count is higher in atopic than in non-atopic asthmatics, and even greater in patients with aspirin-sensitive asthma (Dahl *et al.*, 1978).

Eosinophils have been indirectly implicated in asthma by demonstrating that eosinophil-derived products are found in bronchial tissue and in the BALF (Filley *et al.*, 1982; Frigas & Gleich, 1986; DeMonchy, 1985; Bousquet *et al.*, 1990). Histological sections of asthmatic lungs showed deposits of MBP along the lining of bronchioles close to the areas of damaged epithelium and mucus plugs (Filley *et al.*, 1982; Bousquet *et al.*, 1990).

1.3.3.3 Bronchial damage and eosinophils

Several observations suggest that the eosinophil is the central effector cell mediating epithelium damage in asthma (Frigas & Gleich, 1986). As already mentioned, eosinophil-derived products are found in the blood, sputum, BALF and bronchial wall of asthmatics indicating eosinophil secretion in this disease. These products show toxic properties towards a range of mammalian cells. For instance, MBP alone causes damage to both guinea pig and human respiratory epithelium which mimics the pathology of asthma (Gleich, 1986). At low doses, MBP causes exfoliation of epithelial cells and impairment of ciliary beating. At higher concentrations, MBP

detaches ciliated and brush cells and destroys individual cells exposing the basal cell layer. At an ultrastructural level, MPB disrupts the plasma membrane liberating the cellular contents (Gleich, 1986; Frigas *et al.*, 1980). Examination of asthmatic bronchial tissue sections by immunofluorescence technology shows deposition of MBP at the sites of epithelium damage (Gleich, 1986). ECP, another basic protein, also caused a dose-related damage to guinea pig tracheal epithelium as assessed by inverted microscopy. In addition, EPO at low concentrations either singly or in the presence of hydrogen peroxide and a halide caused ciliostasis, bleb formation, and exfoliation of epithelial cells (Motojima *et al.*, 1989). Finally, PAF-activated eosinophils also led to ciliostasis and disruption of respiratory epithelium *in vitro* (Read *et al.*, 1989; Yukawa *et al.*, 1990b). Taken together, these observations strongly suggest that the eosinophil represents an effector cell in asthma capable of damaging respiratory epithelium.

1.3.3.4 Eosinophils and bronchial hyperreactivity

A distinctive feature of asthma is the development of non-selective airway hyperresponsiveness to a wide variety of stimuli, such as histamine, cholinergic agonists, leukotrienes and physical stimuli. Although the underlying pathomechanism is not yet understood, there is evidence that bronchial hyperresponsiveness is associated with the development of the late response (Atkins *et al.*, 1978; Cartier *et al.*, 1982; Mapp *et al.*, 1985). The hypothesis that inflammation might be responsible for inducing bronchial hyperresponsiveness is further supported by the observation that stimuli which cause hyperresponsiveness also cause airway inflammation (Godard *et al.*, 1987; Chung, 1986; Schleuter, 1974). The involvement of inflammatory cells can also be deduced from the fact that corticosteroids which have potent anti-eosinophil effects can prevent the increase in bronchial hyperactivity during seasonal allergen exposure. Moreover, a correlation was found between the number of blood eosinophils and the FEV_1 in subjects with intrinsic asthma (Horn *et al.*, 1975), the late asthmatic response (Cookson *et al.*, 1989 and bronchial hyperresponsiveness

expressed in methacholine PC_{20} (Durham & Kay, 1985). A recent report on the effect of immunotherapy on bronchial responsiveness in pollen-allergic patients with a history of rhinoconjunctivitis and wheezing, suggests there is a correlation between serum ECP concentration and bronchial hyperresponsiveness (Rak *et al.*, 1988). In untreated patients the level of ECP increased significantly during the pollen season but this did not occur in patients receiving immunotherapy. These studies indicate that an association between eosinophils and bronchial hyperreactivity might exist.

The mechanism by which eosinophils induce bronchial hyper-responsiveness is uncertain and still a matter for speculation. Eosinophil-derived products may cause epithelial cell damage which is a characteristic histopathological feature of asthma (Dunhill, 1960; Guputa, 1976). Loss of respiratory epithelium leads to an increase in mucosal permeability (Hulbert *et al.*, 1981) and alters the osmolarity of the mucosal surface (Hogg & Eggleston, 1984). This may expose afferent sensory nerve endings and promote the release of neuropeptides, such as substance P (Barnes, 1987). Desquamation and dysfunction of bronchial epithelial cells may also result in the loss of epithelium-derived relaxing factor(s) (Flavahan *et al.*, 1988) causing hyper-responsiveness of airway smooth muscle (Gleich *et al.*, 1988).

1.3.3.5 Platelet activating factor and asthma.

PAF represents a heterogenous family of structurally related ether-linked phospholipids that are formed as a result of the concerted action of phospholipase A_2 and acetyltransferase on membrane alkylacyl phospholipids (Patterson *et al.*,1984; Vargaftig & Braquet, 1987; Barnes *et al.*, 1988a). It is generated and released by several inflammatory cells which have been implicated in asthma, including macrophages (Barnes & Chung, 1987), neutrophils (Chilton *et al.*, 1982; Ingraham *et al.*, 1982), lymphocytes (Rola-Preszynski *et al.*, 1987) and eosinophils (Lee *et al.*, 1982; Lee *et al*, 1984). In fact, eosinophils are the richest cellular source of PAF and in particular hypodense eosinophils of asthmatic patients release large amounts of this

important autocoid (Jouvin-Marche *et al.*, 1984; Lee *et al.*, 1984; Snyder *et al.*, 1985; Ojima-Uchimaya *et al.*, 1988; Cromwell *et al.*, 1990).

Direct measurement of PAF in biological fluids such as plasma or BALF has proved difficult since PAF is rapidly hydrolyzed to lyso-PAF by a plasma acetylhydrolase (Blank *et al.*, 1983) as well as taken up and metabolized by inflammatory cells (Malone *et al.*,1985; Chilton *et al.*, 1983) and bronchiolar and alveolar epithelial cells (Haroldsen *et al.*, 1987). However, PAF possesses many biological properties which are relevant to asthma. It induces bronchoconstriction (indirectly, since it has no direct effect on airway smooth muscle) (Schellenberg *et al.*, 1983; Mazzoni *et al.*, 1985), leads to airway microvascular leakage (O'Donnell *et al.*, 1987; Evans *et al.*, 1987) and it causes sustained increase in bronchial reactivity in several animal species (guinea pig, rabbit, dog, cat, sheep, primates) (Christman *et al.*, 1987; Chung *et al.*, 1987; Arnoux *et al.*, 1985; Barnes & Chung, 1987), including humans (Cuss *et al.*, 1986; Rubin *et al.*,1987). It is this latter property which distinguishes PAF from other putative mediators of asthma (Barnes *et al.*, 1988b) and this may relate to the interaction between PAF and eosinophils (see Discussion). In addition, systemically administered PAF-antagonists have been shown to block the asthmatic late response in passively sensitized sheep following antigen challenge (Stevenson *et al.*, 1987) and rabbits (Coyle *et al.*, 1987; Coyle *et al.*, 1988). Furthermore, two PAF antagonists, BN 52021 and WEB 2086 suppressed the bronchial eosinophil infiltration, the appearance of eosinophils in the bronchoalveolar fluid, the development of epithelial lesions, and the development of bronchial hyper-responsiveness in guinea pigs after antigen given by injection or inhalation (Braquet *et al.*, 1985; Lellouch-Tubiana *et al.*, 1987; Lellouch-Tubiana *et al.*, 1988; Coyle *et al.*, 1988). Finally, PAF has been shown to induce hyperplasia of vascular smooth muscle, presumably as a consequence of release of platelet-derived growth factor (Handley *et al.*, 1983). Although the possible significance of PAF in asthma is still an open issue, these experimental observations suggest that PAF may play a pathogenetic

role in the obstructive lung disease. In particular, its capacity to cause a sustained bronchial hyperresponsiveness, to cause hyperplasia of vascular smooth muscle as well as its potent eosinophil chemotactic activity elevates PAF above other autocoids as a mediator of asthma.

1.4 AIMS

Section 1.3 provides insight into the occurrence and the pathogenetic significance of eosinophils in human disease and particularly asthma. However, the factors and mechanisms involved in eosinophil accumulation are not yet understood. Furthermore, although signal transduction has been extensively studied in polymorphonuclear leukocytes, little information is available on the mechanisms of stimulus-response coupling in eosinophils underlying chemotactic migration, release of granular proteins or the *de novo* synthesis of lipid mediators. This is due, in part, to the difficulty in obtaining sufficiently large numbers of highly purified eosinophils necessary for these studies. One possible way of overcoming the problem is to use animal eosinophils which can be obtained in sufficiently high numbers and high purity. Therefore, this work focuses on the guinea pig peritoneal eosinophil as a model cell for the human eosinophil.

The general aims of this work are: (1) to establish a physiologic stimulus which activates eosinophils and could be used for further studies, and (2) to define the biochemical transmembrane signalling pathways operating in eosinophils. More specific aims are (3) to characterize possible binding sites for this ligand, (4) to examine the signalling mechanisms used by the stimulus, (5) and to relate these events to functional responses of the eosinophil.

Understanding the mechanisms of signal transduction may help to define the cell biology of the eosinophil. In addition, this information may yield further insight into how the eosinophil exerts its cytotoxic effector functions in human disease. More importantly, the knowledge of signal transduction mechanisms in eosinophils

may provide valuable clues for new therapeutic approaches in the management of allergic and other hypereosinophilic diseases.

CHAPTER TWO

2. MATERIALS AND METHODS

2.1. REAGENTS

Polyvinyl-pyrrolidene-coated silica gel (Percoll) was purchased from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Hanks balanced salt solution (HBSS) was bought from Flow Laboratories Ltd., Woodcock Hill, Rickmansworth, Herts, U.K. WEB 2086 and [^{3}H]WEB 2086 (specific activity: 15 Ci/mmol) were kindly donated by Boehringer Ingelheim (W. Germany). [14,15-^{3}H]Leukotriene C_4 (specific activity: 35 - 40 Ci/mmol) and [1,2-^{3}H]Hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (specific activity: 50 - 70 Ci/mmol) was purchased from NEN DuPont, Wilmington, USA. [^{3}H]Ins(1,4,5)P_3 (specific activity: 17.1 Ci/mmol) was obtained from New England Nuclear. [^{32}P]-γ-adenosine trisphosphate (specific activity: 3.9 - 2.5 Ci/mmol) was obtained from Amersham, U.K. Triton X-100 was purchased from BDH Ltd.(Poole, Dorset, U.K.). Fura-2 acetoxymethyl ester was obtained from Molecular Probes, (Eugene,OR). Platelet activating factor (C_{16}-PAF), leukotriene B_4, calcimycin (calcium ionophore, A23187), N-formyl-methionyl-leucyl-phenylalanine (FMLP), o-phenylene diamine dihydrochloride (OPD), phorbol myristate acetate (PMA), dimethyl sulfoxide (DMSO), piperazine-N,N'-bis-(2-ethanesulfonic acid), fetal calf serum (FCS), pertussis toxin, aprotinin, dipotassium 2-hydroxy-5 nitrophenol sulphate, sodium acetate, acetic acid, polymyxin B sulphate, DNAase, and Tris-HCL were from Sigma Chemicals Ltd.(Poole, Dorset, U.K.). All other reagents were of the highest commercial grade available. Recombinant C5a was generously provided by Dr. J. Westwick (Hunterian Institute, London,U.K.). Unlabelled 15(S), 9α,11β-trihydroxyprosta-5z,13E-dien-1-oic acid (9α,11β-PGF_2) standard was purchased from Biomol Res.Lab.(Philadelphia, USA). The other unlabelled

prostaglandin and prostaglandin metabolites standards were obtained from Sigma Chemical Co. (St.Louis, USA). The 3,3,4,4-tetradeuterated analogs of PGE_2, PGF_2 and 6-keto-$PGF_{1\alpha}$ employed as internal standards were purchased from Merck Isotopes (St.Louis, USA)

2.2 BUFFERS AND SOLUTIONS

Hepes buffer containing 10 mM Hepes, 145 mM NaCl, 5 mM KCl, 0.5 mM Na_2HPO_4 1 mM $MgCl_2$, 6 mM glucose, and 0.1% BSA was prepared weekly and stored at 4°C. Tyrode's buffer consisted of 140 mM NaCl, 1 mM MgCl2, 5 mM KCl, 0.5 mM Na_2HPO_4, 5.5 mM glucose, and 0.25% BSA. PIPES medium contained 25 mM piperazine-N,N'-bis-(2-ethane sulfonic acid), 110 mM NaCl, 5 mM KCl, 40 mM NaOH, and 5.4 mM glucose. PAG contained 25 mM PIPES, 110 mM NaCl, 5 mM KCL, 0.003% human serum albumin 30 and 0.1% glucose. PAGCM consisted of PAG with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Buffers were adjusted to pH 7.4 with 1N HCl or 1N NaOH as appropriate. Sodium acetate-acetic acid (0.5 M) was prepared by mixing 0.5 M sodium acetate and 0.5 M acetic acid until a pH of 5.7 was reached. Hypotonic lysis buffer contained 20 mM Tris-HCl, 1 mM EGTA, and 2000 U/ml aprotinin and was adjusted to pH 7.5 with 1N HCL. The assay buffer for [^{3}H]PAF binding experiments contained 140 KCL, 10 mM Tris, and 0.25% (w/v) BSA adjusted to pH 7.4 with HCl. The assay buffer for the phosphorylation of phosphatic acid consisted of 100 mM imidazole HCl, 100 mM NaCl, 25 mM $MgCl_2$, and 2mM EGTA, pH 6.6.

Pertussis toxin (Sigma, St.Louis, USA) was reconstituted with buffer at a concentration of 100 µg/ml or 500 µg/ml and used for treatment of intact eosinophils. For *in vitro* use with cell membranes pertussis toxin was preactivated in the presence of 20mM dithiothreitol and 2 mM ATP at 37°C for 60 min. The detergent solution for solubilization of lipids contained 7.5% (w/v) n-octyl-beta-glucopyranoside, 5 mM

cardiolipin dissolved in 1 mM diethylenetriamine-penta-acetic acid (DETAPAC), pH 6.6.

The OPD substrate solution was made up as a 10 mM stock solution in water, stored at -80°C and diluted immediately before use. Diacylglycerol kinase prepared from E.coli was dissolved in 10 mM potassium phosphate buffer pH 7.0 supplemented with 20% (v/v) glycerol and 2 mM mercaptoethanol. Dried 1,2-diacyl glycerol standards were reconstituted with 500 µl $CHCl_3$ to give a solution at 10 nmol per 100 µl. PAF (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) was dissolved in 0.9% NaCl containing 2 mg/ml bovine serum albumin at a final concentration of 1 mM. Aliquots(100 µl) were stored at -80°C and thawed for each experiment. Different concentrations of PAF were made up in HBSS immediately before use. Leukotriene B_4 was dissolved in 0.9% NaCl containing 0.2% BSA and 0.01 M EGTA. LTC_4 standard was dissolved in methanol (50 µg/ml) and stored at -70°C. Aliquots were stored at -80°C. Calcimycin was dissolved and stored at 10 mM in DMSO, and was diluted to the required concentrations in prewarmed HBSS immediately before being added to an equal volume of cells. PMA was prepared as a stock solution of 10 mM in DMSO and stored at -20°C. Required dilutions were made daily in cold deionized water. fMLP was stored as a stock solution of 10 mM and stored at -20°C. Dimethyl sulfoxide concentrations used did not affect either cell viability nor the enzyme assays.

2.3 PREPARATION OF EOSINOPHILS

2.3.1 Human eosinophils

Blood was obtained with informed consent from healthy donors and patients with hypereosinophilic syndrome. None of the donors was currently undergoing corticosteroid therapy. Buffy coat cells were obtained by sedimenting five volumes of blood with 1 volume of 6% dextran 110 (Fisons plc, Loughborough).in normal saline for 1 hour at 37°C. Cells were washed twice and resuspended in modified

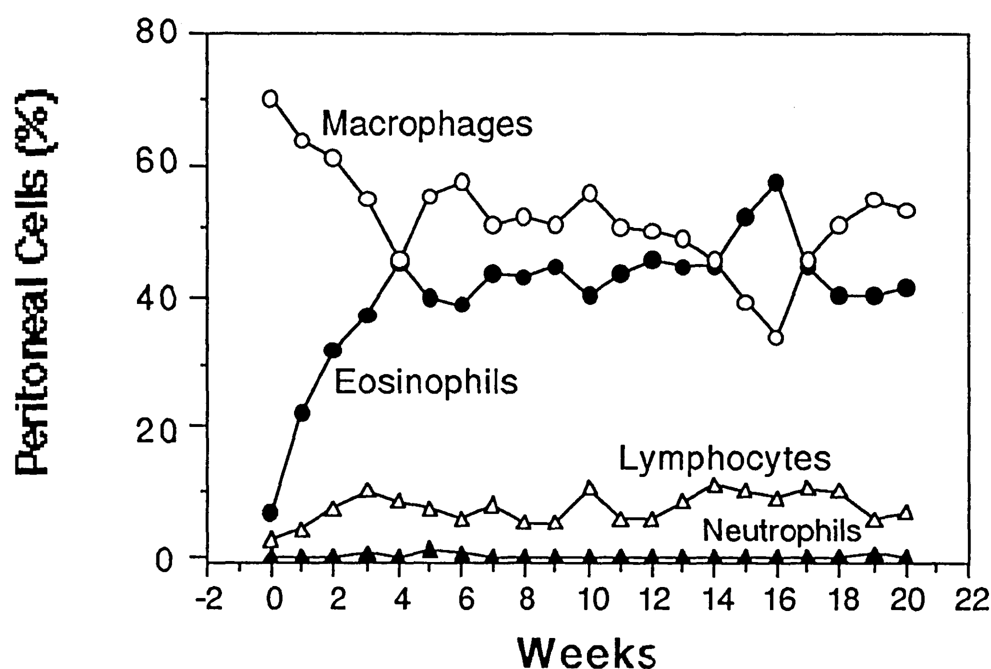


Figure 2.1: **Effect of normal human serum on the cell populations in guinea pig peritoneum.** Animals were treated with 1 ml of normal human serum per week and cells harvested by peritoneal lavage with 40-50 mls of 5% glucose. Total white cell and differential counts were performed using Kimura stain and a Neubauer counting chamber.

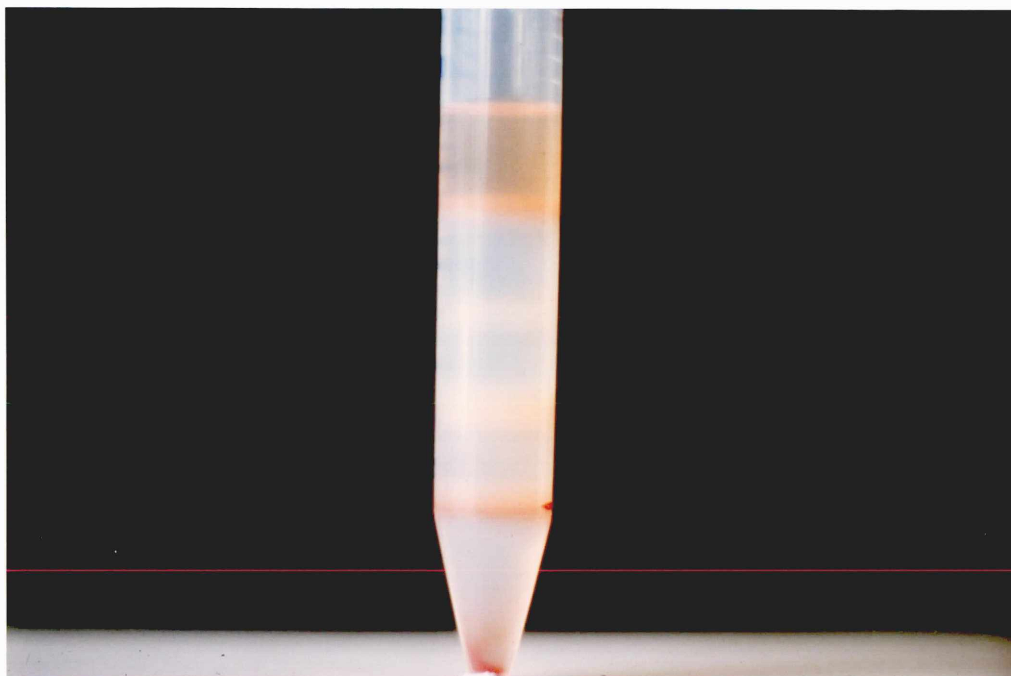


Figure 2.2: Purification of guinea pig eosinophils. Five step Percoll density gradient of guinea pig eosinophils with the top representing lower density cells (≥ 1.060g/ml) and the bottom representing higher densities (≤1.105 g/ml). Eosinophils with a purity > 90% were usually found at the 1.085 -1.100 g/ml density interfaces.

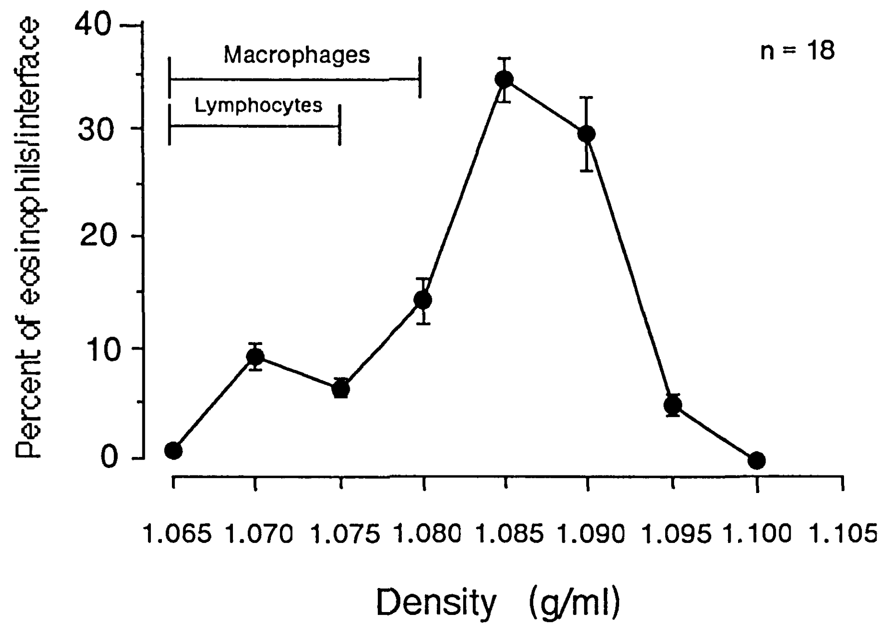


__Figure 2.3:__ **Density distribution profile of guinea pig eosinophils.** The density distribution of eosinophils obtained from human serum (1 ml.week^{-1}) treated guinea pigs by peritoneal lavage. Data shown represent the mean and SEM of 18 independent determinations.

HBSS buffer supplemented with 10% FCS. Eosinophils were purified by centrifugation over either metrizamide or Percoll density gradients. Percoll gradients were as described for the guinea pig eosinophil (see below, Chapter 2.3.2). Metrizamide gradients were prepared by the method of Vadas *et al.* (1979). Briefly, 2 ml of decreasing percentages of metrizamide (25,24,23,22 and 18% were layered into 16 ml polycarbonate centrifuge tubes and 5-10 x 10^7 buffy coat cells in 2 ml of modified HBSS were overlayered. The tubes were centrifuged for 1,200 x g for 45 minutes at 20°C, after which the cells were collected from each interface, washed twice in modified HBSS, stained with Kimura stain and counted in a modified Neubauer hemacytometer. Eosinophils of greater than 80% purity and 95% viability which were collected from the interface between the 22 and 23% layers were used in subsequent experiments. There was no difference between eosinophils purified by metrizamide and Percoll density gradients.

2.3.2 Guinea pig eosinophils

Male outbred Hartley guinea pigs (750 -1000 g) were treated with weekly intraperitoneal injections of 1 ml human serum (Litt, 1960; Pincus 1978). An eosinophil-rich exudate (35-65% eosinophils) was obtained by weekly peritoneal lavage with 50 ml of 5% glucose (Figure 2.1). The exudate from 8 -10 animals was cooled to 4°C, pooled, washed three times in HBSS and the cell number counted. Eosinophils were then purified using a discontinuous gradient (1.100, 1.090, 1.085, 1.080 and 1.070g/ml) of iso-osmolar Percoll as previously described (Gartner, 1980; Fukuda *et al.*, 1985). In some experiments, additional densities (1.075 g/ml and 1.095 g/ml) were used. Density was determined by refractometry and readings extrapolated from a standard curve. Gradients were formed using a peristaltic pump (LKB 2120 Varioperpex II pump, LKB-Beckman Instruments, Bromma, Sweden) and consisted of 1.5 ml 1.100, 2 ml 1.090, 2 ml 1.085, 2 ml 1.080 and 2 ml 1.070 g/ml in 15 x 100 mm polypropylene tubes (Falcon Plastics, Los Angeles, CA). The cells, suspended in

2 ml Percoll 1.070 g/ml supplemented with 5% FCS and 1.84 ng DNAse, were layered on top of the gradients and centrifuged at 1600 x g for 20 min at 15 °C. The resulting 6 fractions (Figure 2.2) were collected and washed twice in HBSS before they were counted in a hemacytometer. 80.1 ± 2.9% (n=57) of the total eosinophil number were usually found infractions 1.081-1.085 (92.9 ± 1.3%, mean purity ± SEM), 1.086-1.090 (99.3 ± 0.4%), and 1.091-1.100 (99.4 ± 0.3%). The fractions were pooled to give a final mean purity of 97.8 ± 0.5% and a mean viability of 99.2 ± 0.2% (n=37) as assessed by trypan blue exclusion. Cells were then washed in HBSS, and resuspended either in ice-cold HBSS or in HEPES buffer as indicated.

The majority of 84.4 ± 8.6% eosinophils were normodense (n=18) with a density > 1.081 g/ml (Figure 2.3) although the mean density of the cells shifted slightly as a function of repeated injection of the same animals with human serum. However, only normodense eosinophils could be used for the experiments since lower density interfaces were contaminated by peritoneal macrophages and lymphocytes.

2.4 PREPARATION OF GUINEA PIG NEUTROPHILS AND MONONUCLEAR CELLS

Peritoneal guinea pig macrophages (purity up to 90%) and lymphocytes (purity up to 80%) were obtained from Percoll gradients and usually found in the 1.065 g/ml, 1.070 g/ml and 1.075 g/ml density interfaces, respectively. A neutrophilia in guinea pig was induced by intraperitoneal injection of 0.12% glycogen in 0.9% saline 14 to 16 hours before lavage (Yamashita *et al.*, 1985). Neutrophil-rich peritoneal cell suspensions (70 - 80%) were subjected to Ficoll Paque separation and neutrophils with a purity > 98% and an eosinophil count of < 3% were used in the experiments.

2.5 QUANTITATION OF CELLS

Total white cell and differential counts were quantitated with Kimura stain (Kimura *et al.*, 1973) in a Neubauer counting chamber. The viability of

eosinophils isolated by metrizamide gradients was evaluated by trypan blue dye exclusion.

2.6 PREPARATION OF MEMBRANES

Purified eosinophils (2-3 x 10^8) were incubated in hypotonic lysis buffer (20 mM Tris-HCL, 1 mM EGTA, 2000 U/ml aprotinin, pH 7.5) for 30 min at 4°C and disrupted gently by sonication (Microson™ Ultrasonic Cell Disrupter, Farmingdale, N.Y., USA) with 2 x three 20-sec bursts in an ice / water bath. The disrupted cells were freed from remaining intact cells and nuclei by centrifugation at 16,000x g for 30 sec at 4°C (Matsumoto *et al.*, 1988). The supernatant was then centrifuged at 16.000 x g for 90 min at 4°C. The pellet was rinsed with incubation buffer, resuspended in the same buffer at a cell equivalent of approximately 1 x 10^7/100 µl and was resonicated with three 20-sec bursts as described above. These fractions were used for the [^{3}H]PAF binding assay and protein measurements.

2.7 PROTEIN ASSAYS

Protein concentrations were determined by the method of Bradford (1976) using a Bio-Rad Protein Assay Kit with human serum albumin as the standard. The protein content of the membrane and cytosolic fractions was 1-2 and 3-4 mg/ml, respectively.

2.8 TREATMENT WITH PERTUSSIS TOXIN

Purified eosinophils were resuspended in PAGCM and incubated at 37°C for 1 hour with either vehicle or 0.6 µg/ml to 5 µg/ml of pertussis toxin. The cells were washed three times, resuspended in PAGCM, and either stimulated with PAF, or processed for ADP-ribosylation (see 2.20, page 79).

2.9 ENZYME ASSAYS

2.9.1. Incubation procedure

Eosinophils (5 x 10^6) were suspended in 500 µl HBSS and incubated in triplicate with stimuli at 37°C for various periods in a shaking waterbath. The reaction was stopped by adding 500 µl ice cold HBSS followed immediately by centrifugation at 400 x g_{max} for 2 min. The supernatant containing released eosinophil enzymes was removed. To assess the total amount of cellular enzymes, the control cell pellet was washed in 2 ml HBSS, resuspended in 1 ml HBSS, and then sonicated for 1 min at 4°C (Ultrasonicator, Jencons Scientific Ltd., Leighton Buzzard, Bedfordshire, U. K.). Samples were frozen at -20°C and stored for up to seven days without loss of enzyme activity.

2.9.2 Eosinophil Peroxidase

Eosinophil peroxidase (EPO) was measured colorimetrically using 0.1 mM o-phenylenediamine (OPD) in a 0.05 M Tris-buffer pH 7.0 containing 0.1% Triton X-100 and 1 mM hydrogen peroxide as a substrate (Strath *et al.*, 1985). One hundred and fifty microliters of the eosinophil supernatant was added to 300 µl OPD substrate solution, incubated for 30 min at 37°C, and the reaction stopped by the addition of 200µl 4 M sulfuric acid. Absorbance was then determined at 492 nm using a thermostatically controlled spectrophotometer (PU 8620, Pye Unicam, Cambridge, U.K.) set at 37°C. The resulting brown color was stable for at least 150 min.

In some experiments enzyme activity was confirmed by a modified luminol-enhanced chemiluminescence method (Carlson *et al.*, 1985) using luminol (10mg/liter), hydrogen peroxide (17.6 mM) and Tris-buffer (100 mM, pH 8.0). The chemiluminescence produced by EPO catalyzed reaction between hydrogen peroxide and luminol was determined in a luminescence photometer (LKB-Wallace 1251 Luminometer, LKB Instruments Ltd. South Croydon, U.K.) at room temperature. The results of both assays were expressed as Units/mg referring to a standard curve using

horseradish peroxidase, Type I (hydrogen-peroxide oxireductase, EC 1.11.1.7). One unit of this enzyme will form 1.0 mg of purpuogallin from pyrogallol in 20 seconds at pH 6.0 at 20°C. To establish that the released peroxidase was truly of eosinophil origin, we tested total peroxidase activity from different cell populations according to the respective proportion of contaminating eosinophils. Guinea pig eosinophils (10^6) contained an EPO activity of 563 μU. In contrast, enzyme activity measured in the same number of macrophages, lymphocytes, and PMN was negligible. The results show that the assay using OPD as a substrate is specific for the eosinophil peroxidase, confirming previous reports (Strath *et al.*, 1985; Carlson *et al.*, 1985). The negative results observed with peroxidases of other cells can be explained by the different pH optimum and the low activity of myeloperoxidase on the hydrogen donor used.

In addition, we investigated the effect of the specific EPO inhibitor 3-amino-1, 2, 4-triazole (AMT) in our test system (Kroegel *et al.*, 1989a). The enzyme activity of an EPO containing supernatant obtained by sonication of 2 x 10^7 human eosinophils (18.9 mU) were assayed with different concentrations of AMT. AMT inhibits EPO activity in a dose-dependent manner. The IC_{50} of the AMT effect was 6.3 ± 0.9 mM. These results are in good agreement with the data previously reported (Strath *et al.*, 1985).

2.9.3 β-glucuronidase

β-glucuronidase was measured using phenolphthalein glucuronic acid as substrate 100 μl of the supernatant were added to 900 μl of a substrate solution consisting of 1 mM phenolphthalein glucuronic acid, 1% Triton X-100 in 50 mM acetate buffer, pH 4.5. After 12 hours incubation at 37°C in an incubator the reaction was stopped by adding 500 μl of a 0.75 M Na_2CO_3 solution. The resulting lilac color was stable for at least 60 min. Absorbance versus water as reference was then measured at 540 nm. Values were expressed as units referring to a standard as units/ml/minute, where 1 unit equals an absorbance decrease of 0.001 at 25°C.

2.9.4 Arylsulfatase B

Arylsulfatase activity was assessed spectrophotometrically by the formation of 4-nitrocatechol from dipotassium 2-hydroxy-5nitrophenyl sulphate (Weller & Austen, 1983). 100 µl of the enzyme containing supernatant was incubated at 37°C with 300 µl of 25 mM dipotassium 2-hydroxy-5 nitrophenyl sulfate in 0.5 sodium acetate-acetic acid, pH 5.7. The reaction was terminated after 60 min by the addition of 200 µl of 2N NaOH. The quantity of 4-nitrocatechol generated was determined by measuring the absorbance at 515 nm. The absorbance was plotted against a standard curve of a commercially available arylsulfate sulphohydrolase (E.C.3.1.6.1.) and the enzyme activity expressed as units. One unit hydrolyses 1.0 µM of nitrophenyl sulfate per hour at 37°C.

2.9.5 Alkaline Phosphatase

The activity of alkaline phosphatase was determined using p-nitrophenyl phosphate as substrate (Sommer, 1954): 350 µl alkaline buffered substrate solution containing 0.5 M glycine, 50 mM $MgCl_2$, 0.5 M NaOH and 1% p-nitrophenyl phosphate were incubated with 150µl supernatant for exactly 30 min at 37°C. The reaction was stopped by the addition of 100 µl of 2 N NaOH and absorbance was then determined spectrophotometrically at 410 nm. The results were expressed as a percentage of total enzyme release.

2.9.6 Acid Phosphatase

The concentration of released acid phosphatase in the supernatant was determined by the amount of 4-nitrophenol per unit time liberated during enzymatic hydrolysis of 4-nitrophenyl-phosphatein acid solution (Sommer, 1954). 300 µl of acid buffer/substrate solution containing 50 mM citrate buffer and 5.5 mM 4-nitrophenylphosphate, pH 4.8, was incubated with 150 µl supernatant at 25°C for 30 min.

After 30 min, 400 µl NaOH (100 mM) was added to stop the reaction and the increase in absorbance at 405 nm over the buffer control sample was measured. The yellow-green color obtained was stable for at least 4 hours. Enzyme activity per min was calculated using the equation:

$$\text{Acid phosphatase [U/min]} = \frac{\text{Absorbance x V}}{\text{T x } E_c \text{ x } V_s}$$

$$= \text{Absorbance x } 0.0084$$

where E_c the extinction coefficient of 4-nitrophenol (18.5 $cm^2/\mu M$ in alkaline solution at 405 nm),

T the incubation time in minutes,

V the incubation volume (ml) and

V_s represents the amount of supernatant in ml.

2.9.7 Lactate dehydrogenase

Lactate dehydrogenase (LDH) activity was determined by measuring the reduction of pyruvate to lactate at 25°C as described elsewhere (Wroblewski & LaDue, 1955). Briefly, 0.1 ml of each supernatant was added to 2.85 ml phosphate buffer (pH 7.5) containing 200 µg of reduced nicotinamide adenine dinucleotide (NADH). The resultant solution was then thoroughly mixed, incubated at 25°C for 20 min and then dispensed into disposable cuvettes (1 cm light pathlength). The reaction was initiated by adding 100 µl sodium pyruvate (22.7 mM) and the drop in absorbance at 340 nm monitored spectrophotometrically over 25 min at 25°C using distilled/de-ionized water as reference. LDH activity per minute was calculated from the linear part of the absorbance drop using the following equation:-

$$\text{LDH activity (U/ml)} = \frac{\text{Absorbance x } C_t}{\text{T x } E_a \text{ x V}}$$

where C_t is the temperature correction factor (1.0 at 25°C),

E_a is the absorbance equivalent to 1 U of LDH activity in a 3 ml volume with 1 cm lightpath at 25°C,

V represents the incubation volume(ml) in cuvette, and

T is the incubation time in minutes.

Values obtained were expressed as units/ml/minute, where 1 unit equals an absorbance decrease of 0.001 at 25°C.

2.10 SUPEROXIDE ANION PRODUCTION

Superoxide anion ($\cdot O_2^-$) production and release by resting and stimulated eosinophils was determined as the superoxide dismutase (SOD; 30 µg/ml) inhibitable reduction of ferricytochrome C (Johnston *et al.*, 1975). Each measurement was carried out in paired samples. Eosinophils (2 x 10^6) were allowed to equilibrate at 37°C for 3 min in pre-warmed HBSS containing 80 µM cytochrome C. SOD was added in the reference cuvette prior to the addition of the stimulant to both cuvettes (1 cm lightpath) and quickly stirred. Then the cells were left unstirred at 37°C and the reduction of ferricytochrome C was determined by continuously monitoring the absorbance at a wavelength of 550 nm. The activity of $\cdot O_2^-$ generation was expressed either as total reduction after 10 min or the rate per minute of ferricytochrome C reduction obtained from the linear portion of the curve in a photospectrometer (PU 8620, Pye Unicam, Cambridge, U.K.). The amount of superoxide anion production was calculated using a molar absorption coefficient of 17.7 x 10^3 and expressed as nmol ferricytochrome C reduction/3x10^6 eosinophils.

2.11 RADIOIMMUNOASSAY FOR EOSINOPHIL CATIONIC PROTEIN

The release of eosinophil cationic protein into the supernatant was detected by radioimmunoassay, described in detail elsewhere (Venge *et al.*, 1977). Briefly, 100 µl of the supernatant was incubated for two hours with 0.5 ml Sephadex-

bound anti-eosinophil cationic protein (50 mg/ml) before addition of 100 μl ^{125}I-eosinophil cationic protein (40,000 cpm). Incubation was continued for 20 hours at 22°C during rotation end over end. Finally, the samples were washed four times with 0.9% saline containing 0.2% CTAB (cetylmetylammoniumbromide) and the radioactivity of the pellet measured in a scintillation counter. The assays were performed by Dr.Per Venge (Uppsala, Sweden).

2.12 RADIOIMMUNOASSAYS FOR PROSTANOIDS

2.12.1 Thromboxane B_2

Eosinophils were resuspended in ice-cold HEPES-buffered PSS (10 mM K^+-HEPES, 124 mM NaCl, 4 mM KCl, 640 μM NaH_2PO_4, 660 μM K_2HPO_4, 5.2 mM $NaHCO_3$, 1.6 mM $CaCl_2$, 1 mM $MgCl_2$, and 5.6 mM glucose, adjusted to pH 7.4 at room temperature with 2 N HCl) at a concentration of 2 x 10^7 cells ml^{-1}. Assays were performed at 37°C in a shaking water bath (120 strokes min^{-1}) in a total volume of 125 μl and were initiated by addition of 25 μl (5 x 10^5 cells) of the eosinophil suspension to 87.5 μl pre-warmed HEPES buffer containing either vehicle, flurbiprofen or WEB 2086 at concentrations indicated in the text. PAF or calcimycin (12.5 μl) was added 5 min after the introduction of the eosinophils. Reactions were terminated by addition of 700 μl ice-cold HEPES-buffer supplemented with 8 uM flurbiprofen, an irreversible inhibitor of the cyclo-oxygenase enzyme complex (Rome & Lands, 1975). After thorough mixing the eosinophil suspension was rapidly pelleted by centrifugation at 12000 x g for 30 s in a Gilson microfuge and the resultant supernatant used as the source of elaborated prostanoids. The concentration of TXA_2 measured as its stable, non-enzymatic hydrolytic degradation product, TXB_2, was quantified by radioimmunoassay using a modified version of that described previously (Giembycz & Rodger, 1987). The conversion of TXA_2 to TXB_2 was assumed to follow 1:1 stoichiometry. Cross reactivity of all other prostanoids with the TXB_2

antiserum was equal to, or less than, 0.1% at B/B_0 = 0.5. The detection limit and sensitivity (IC_{50}) of the TXB_2 RIA were 5 fmol and 100 fmol, respectively (n=15).

2.12.2 Prostaglandin E

Determination of PGE followed in principle the procedure described in 2.12.1. The PGE_2 antiserum used in the experiments (Sigma) did not readily distinguish between PGE_1 and PGE_2. Data are, therefore, expressed as PGE-like immunoreactivity. Cross-reactivity of other prostanoids with the PGE_2 antiserum at B/B_0 = 0.5 was PGE_1 (41%), PGA_1 (4.1%), PGA_2 (3.3%), $PGF_{1\alpha}$ (1.3%), and $PGF_{2\alpha}$ (1.8%). The detection limit and sensitivity of the PGE RIA, using PGE_2 to generate the standard curve, were 50 fmol and 2000 fmol, respectively (n=9).

2.13 RADIOIMMUNOASSAY FOR LEUKOTRIENE C_4

Eosinophils (10^6) cells were stimulated at 37°C for 15 min in PAGCM (incubation volume 100 µl) and reaction was stopped by adding 250 µl ice-cold buffer followed immediate centrifugation (16,000 x gmax; 30sec). Supernatant was stored up to 1 week at -80°C prior to being assayed for LTC_4. The radioimmunoassay for LTC_4 was conducted as described above (2.12.1) using a rabbit anti-LTC_4 antiserum (Hayes *et al.*, 1983). Cross reactivity with LTD_4 and LTE_4 were 55% and 12%, respectively. The assay was performed in PBS buffer containing 0.1% (w/v)gelatin, pH 7.2. After addition of the antibody, samples and standards (7.8 pg/0.1 ml - 100 ng/0.1 ml) were incubated for 3 hours at 4°C. Separation of bound from free ligand was accomplished using dextran-coated charcoal. The detection limit of LTC_4 was 16 pg/0.1 ml.

2.14 GAS CHROMATOGRAPHY/MASS SPECTROMETRY

Gas chromatography/mass spectrometry (GC/MS) was used to determine the spectrum of prostanoids generated by eosinophils and offered the advantage of measuring several prostanoids simultaneously (Hubbard *et al.*, 1987).

2.14.1 Sample preparation

After incubation of the eosinophils for 5 min at 37°C, 10 volumes of ice-cold high-grade acetone were added to the samples followed by the addition of deuterated prostanoids (see below) to provide internal standards for identification of the lipids. Samples were then dried under a stream of nitrogen (approximately 20 min), and the residue was treated with 2% methoxamine-HCL dissolved in pyridine (acetylation grade) to prevent oxidation. Oximated samples that could not be analyzed within 48 hours were stored for periods up to 2 weeks at -20°C.

2.14.2 Derivatization

Samples were further processed by evaporation of excess pyridine under nitrogen, and the residue was subjected to sequential procedures for the synthesis of pentafluorobenzyl ester and trimethylsilyl ether derivatives (Hubbard *et al.*, 1986). Briefly, the oximated residue was treated with 25 µl each of 20% (v/v) solutions of diisopropylethylamine and pentafluorobenzyl bromide (silylation grade, Pierce Chemical Co., Rockford, IL) at 40°C for 20 min prior to reaction of the dried residue with 25 µl acetonitrile and 50 µl o-bis(trimethylsilyl)-trifluoroacetamide (Pierce, Rochford, IL) at 40°C for 15 min. The derivatized samples were then dried, extracted twice with 0.5 ml of hexane, and transferred to a clean silanized glass vial. After evaporation of the extraction solvent under nitrogen, the extract was dissolved in 30 µl of dodecane for injection into the capillary gas chromatography.

2.14.3 Sample extraction

Samples containing deuterated standards were mixed with 10 ml cold methanol in silanized 50 ml glass tubes and kept on ice for 60 min to facilitate precipitation of protein. After separation by centrifugation for 10 min at 1200 x g_{max}, supernatants were transferred to 50 ml silanized glass tubes and diluted with water to a

20% methanol solution. Samples were then loaded onto Sep-Pak C18 cartridges (Waters Associates, Milford, MA) previously conditioned with 10 ml methanol (JT Baker Chemical Co., Phillipsburg, NJ, HPLC grade) and 10 ml water (Baker, HPLC grade). Cartridges were washed with 10 ml of 20% methanol in water, eluted with 4 ml of 80% methanol in water into silanized glass vials and dried.

2.14.4 Standard curves

Standard curves of unlabelled prostaglandins were prepared by comparing the peaks to the deuterated analog employed as the internal standard. $PGF_{2\alpha}$ and $9\alpha,11\beta PGF_2$ were quantified on the basis of $^2H_4$-$PGF_{2\alpha}$ as the internal standard.

2.14.5 Analysis

Sample analysis was performed with a gas chromatograph (Finnigan Model 9611) interfaced with a Finnigan MAT 4610B EI/CI mass spectrometer supplied with a computerized data system (SUPERINCOS, San Jose, USA) programmed for simultaneous acquisition of ions at six different masses.

2.15 RADIOLIGAND BINDING ASSAYS

2.15.1 [^{3}H]WEB 2086 binding to intact eosinophils.

For the binding experiments 5×10^6 to 1.5×10^7 cells were resuspended in a final volume of 1 ml HEPES buffer containing [^{3}H]WEB 2086 at the indicated concentrations. Cells were incubated in a continuous shaking waterbath for 120 min. In competition experiments the final concentration of [^{3}H]WEB 2086 was 20 nM. Other substances were added as indicated. Nonspecific binding was determined in the presence of unlabelled WEB 2086 (10μM) or PAF (1 μM) and amounted to 20 - 30% of total binding. Triplicate incubations were carried out at 25°C for 90 minutes.

Bound and free radioligand were separated by rapid filtration through Whatman GF/C fibre filters presoaked in 1% BSA for 1 hour. Filters were then washed twice with 4 ml ice cold binding assay buffer and radioactivity was determined in a scintillation counter.

2.15.2 [^{3}H]PAF$_{16}$ binding to eosinophil membranes

Incubations were performed for 90 min in duplicate at 22°C in 1.8 ml Eppendorf tubes containing 40 µl of eosinophil membrane in incubation buffer (1-2 mg/ml; equivalent to 3 to 5 x 10^6 eosinophils). For saturation experiments increasing concentrations of [^{3}H]PAF (NEN, Du Pont, Wilmington, DE, USA; specific activity: 40.0 - 60.0 Ci/mmol) ranging from 0.1 nM to 10 µM were added together with 10 µM cold PAF to determine nonspecific binding. At the end of the incubation time, the mixture was rapidly filtered through a premoistened GF/C filter (Whatman Ltd., Maidstone,England) to separate bound from free ligand. The filters were then washed twice with 5 ml of cold assay buffer, air dried and placed into plastic vials containing 7 ml of scintillation fluid (Ready-Solv MP, Beckman Instr., Fullerton, CA, USA) for radioactivity counting. As previously reported by Marquis *et al.* (1988) some of the radioligand is retained by the GF/C filters. Therefore, matched paired samples without membranes were run as blanks. Thus, the net total non-specific binding to the membranes were given by the differences between the radioactivity retained by the filters in the presence and the absence of membranes in the incubation buffer. Between 5 and 10% of the radioactivity was retained by the filters and this amount was not altered by the presence of membranes or an excess of cold PAF in the filtrate.

2.16 INOSITOL 1,4,5-TRISPHOSPHATE MASS ASSAY

Eosinophils (7x10^6/assay) were incubated at 37°C in a shaking waterbath (120 strokes/min) for 5 min in 270 µl HEPES-buffer in the presence or absence of 300 nM WEB 2086. Reactions were started by the addition of PAF, LTB_4,

C5a, or fMLP in a volume of 30 μl. Incubations were terminated after appropriate intervals with 300μl ice-cold 1 M trichloroacetic acid (TCA) and placed on ice for 20 min followed by centrifugation at 10 000 x g_{max} for 5 min. One hundred twenty five μl of 10 mM EGTA and 500 μl of freon:tri-n-octylamine (1:1, v/v) were added to 500 μl of the supernatant from the TCA tissue extracts. The samples then were thoroughly mixed by repeated vortex-mixing and further centrifuged (10 000 x g_{max} for 1 min). Aliquots of the upper phase were removed, neutralised with 60 mM $NaHCO_3$ (100 μl) and taken for analysis. Inositol 1,4,5-trisphosphate concentration was measured using a recently described modified radioreceptor assay (Challiss *et al.*, 1988; Challiss *et al.*, 1990). In brief, 300 μl sample or 300 μl TCA-extracted HEPES buffer containing a known amount of D-Ins(1,4,5)P_3 (0.036 pmol) or DL-Ins(1,4,5)P_3 (0.3 nmol, to define non-specific binding) was added to 300 μl 100 mM Tris-HCl, 4 mM EDTA, pH 8.0, and 300 μl water containing [^{3}H]Ins(1,4,5)P_3 (approximately 7,000 dpm/assay). Three hundred microliter (0.2 - 0.4 mg protein) of an adrenal cortex binding protein preparation (Challiss *et al.*, 1988) was added and samples were incubated on ice for 30 min, prior to separation of bound from free radioligand by rapid filtration through Whatman GF/B filters. Radioactivity was determined by liquid scintillation spectrometry at a counting efficiency of approximately 62%. Since the concentration of Ins(1,4,5)P_3 was found to be very low in eosinophils (see Results) data are expressed as pmol/assay tube (i.e. Ins(1,4,5)P_3/7x10^6 eosinophils) which provides an accurate index of the actual Ins(1,4,5)P_3 concentration measured.

2.17 DETERMINATION OF $[Ca^{2+}]_i$

2.17.1 Mean $[Ca^{2+}]_i$ change.

Purified guinea pig eosinophils were incubated for 30 min at 37°C (10^7 cells/ml) in HBSS buffer containing 2.5 μM of fura-2-AM. The cells were then washed three times in 10ml calcium-free, HEPES buffered Tyrode's solution and resuspended to a final concentration of 10^6 cells/ml. Aliquots (2 ml) of the cell

suspension were dispensed into disposable cuvettes and the external calcium concentration ([Ca^{2+}]) adjusted to 1 mM with 1 M $CaCl_2$ Cuvettes were transferred to a thermostatically controlled Bowman spectrofluorimeter fitted with a stirring attachment for fluorescence reading at 37°C.

The intracellular calcium ion concentration ([Ca^{2+}]$_i$) was determined by the chelation method and formula described by Pollock *et al.* (1986). Maximal loading (F_{max}) was determined by lysing cells with 40 μM digitonin or 100 μM palmitoyl carnitine in the presence of 1 mM Ca^{2+}. Background fluorescence (F_{min}) was determined by adjusting the pH of the lysed cells to 8.5 with 20 mM Tris base followed by the addition of 10 mM EGTA. Leakage of fura-2 from eosinophils was shown to be small as determined by the quench in fluorescence signal produced by the addition of 1 mM Ni^{2+}. Cells were allowed to equilibrate at 37C for 2 min before agonists or vehicle were added. Where appropriate antagonists were added 1 min prior to the addition of the agonist. Fura-2 fluorescence was monitored continuously using monochromator settings of 339 nm(excitation) and 500 nm (emission).

The intracellular calcium concentration was calculated using the formula:

$$[Ca^{2+}]_i = K_d \frac{(F - F_{min})}{(F_{max} - F)}$$

where K_d is the dissociation constant for Ca^{2+} binding to the indicator and F the fluoresence in arbitrary units.

For fura-2 the K_d is 224 nM (Grynkiewicz *et al.*, 1985).

2.17.2 [Ca^{2+}]$_i$ changes in single cells

Purified eosinophils (1.5-2.5 x 10^6 per ml) were labelled with 1 μM fura-2AM (Calbiochem) for 30 min at 37°C in the absence of Ca^{2+} and Mg^{2+}. After repeated washing with HBSS buffer, the cells were resuspended in the same volume of PAGCM and loaded on to the microscope observation chamber. For each kinetic experiment, 15 μl of suspended eosinophils (approximately 25,000 cells) were placed

in the center of a siliconized (SigmaCote) cover slip which made up the base of the chamber. After a settling period of 10 minutes, the cells were overlaid with 1 ml of 37°C PAGCM buffer and the chamber was placed on the microscope scanning stage. The temperature in the observation chamber was measured by a probe placed next to the settled cells and brought to a steady 37°C.

$[Ca^{2+}]_i$ measurement were monitored under a phase contrast Zeiss Universal microscope (Thornwood, NY, USA) equipped with epifluorescence and a 200 Hz scanning stage as previously described in detail (MacGlashan, 1989). Fura-2 excitation was made with a Zeiss 400 nm dichromic mirror and 410 nm longpass filter in the epifluorescence assembly. The two excitation filters of 352 nm and 380 nm 1"bandpass (10 nm) interference, respectively, were controlled by a Macintosh computer equipped with appropriate software and hardware (MacGlashan, 1989). $[Ca^{2+}]_i$ was determined from the ratio of the fluorescent emission intensities. Calibration of the fura-2 signal was accomplished by examining the ratios before and after the cells were challenged with ionomycin. Four baseline images were spaced 30 sec apart prior to the edition of a stimulus, followed by 10 frames at 5 sec apart, 15 frames 10 sec apart, and 5 frames 30 sec apart.

Fura-2AM calibration was performed using fura-2-AM labelled cells in the presence of 2.5 µg/ml ionomycin under Ca^{2+}-free and Ca^{2+}-containing conditions. The fluorescence intensity at 352 nm and 380 nm of excitation subtracted from the background intensity, were used to determine R_{max}, R_{min} and β, which could be used in the equation relating ratio parameters to free Ca^{2+} concentration as described by Grynkiewicz (1985).

2.18 QUANTITATION OF 1,2-DIACYLGLYCEROL

The measurement of 1,2-diacyl glycerol (1,2-DAG) is based on a radioenzymatic assay employing diacylglycerol kinase which quantitatively converts

DAG to phosphatidic acid using [^{32}P]-γ-ATP as phosphate donor (Kennnerly *et al.*, 1979; Preiss *et al.*, 1986).

2.18.1 Sample preparation

Treated cell samples were extracted by a modification of the method of Bligh and Dyer (1959). First, sufficient 1.0 M NaCl was added to the samples to bring the aqueous volume to 0.8 ml. The monophase was mixed and 1.0 ml of $CHCl_3$ as well as 1.0 ml of 1 M NaCl was added to break the phases. The chloroform-phase was separated by brief (2 min) centrifugation at 5.000 x g_{max}. Exposure to mild acids was avoided to minimize migration of acyl groups.

2.18.2 Phosphorylation of phosphatidic acid

A sample (100 µl) from the chloroform phase was dried overnight using a vacuum dryer. The dried lipids then were solubilized in 20 µl of a 7.5% octyl-beta-D-glucoside/5 mM cardiolipin solution (in 10 mM imidazole/HCl, 1 mM diethylenetriamine-pentaacetic acid, DETAPAC, pH 6.6). Samples were sonicated in a bath sonicator (50/60Hz) for 2 min, thoroughly vortexed and left for 15 min at room temperature. Then 50 µl of reaction buffer (100 mM imidazole HCl, 100 mM NaCl, 25 mM $MgCl_2$ 2 mM EGTA), 10 µl/sample of diacylglycerol kinase (prepared from E.coli, in 5 mM potassium phosphate buffer pH 7.0 containing 10% (v/v) glycerol and 1 mM mercaptoethanol), 2 µl freshly prepared 100 mM dithiothreitol, and water to a total volume of 90 µl was added. The reaction was started by the addition of 10 µl of 10 mM [γ-^{32}P]ATP (specific activity 4.9 - 2.5 Ci/mmol), mixed with 5 mM ATP to give 1 µCi per aliquot and prepared in 100 mM imidazole, 1 mM DETAPAC, pH 6.6. Samples were mixed and the reaction allowed to proceed for 30 min at 25°C. The reaction was stopped by the addition of 20 µl 1% (v/v) perchloric acid and 450 µl $CHCl_3/CH_3OH$ (1:2, v/v) and extracted as described above (2.18.1). Samples were

washed twice with 1 ml perchloric acid (1%, v/v) and 150 μl of the $CHCl_3$ phase was removed and dried under N_2.

2.18.3 Thin-layer chromatography

The lipids were redissolved in 20 μl $CHCl_3/CH_3OH$ (95:5, v/v) and streaked onto 20x20 cm silica gel thin layer plates preactivated in acetone and air dried immediately before use. Plates were developed with $CHCl_3/CH_3OH/CH_3COOH$ (65:15:5, v/v/v), air dried and subjected to autoradiography overnight. The radioactive spot corresponding to phosphatidic acid (R_f: 0.35) was scraped into a scintillation vial to which 10 ml of Aquasol-2 scintillation fluid was added and radioactivity counted in a scintillation counter. The amount of 1,2-DAG present in the samples was calculated from the amount of [^{32}P]-phosphatidic acid produced and corrected for the volume of the aliquots and the specific activity of ATP employed.

2.19 PROTEIN KINASE C ASSAY

The cell pellet of PMA-stimulated eosinophils was resuspended in 200 μl of 50 μM digitonin in Tris-HCL (20 mM) buffer containing 2 mM EDTA, 0.5 mM EGTA, 2 mM phenyl-methylsulfonyl fluoride and 0.5mg/ml leupeptin (pH 7.5) and allowed to permeabilize for 10 min at 4°C as described (Pelech *et al.*, 1986). The tubes were then centrifuged (1 min, 12,000 x g) to separate the cytoplasm from the membrane fraction. The presence of EGTA and EDTA ensured that only the chelator stable form of the membrane-associated protein kinase C was determined. The membrane fraction was treated with 200 μl Tris-HCl buffer to solubilize membrane proteins. Protein kinase C activity was measured in triplicate. The reaction mixture contained 20 mM Tris-HCl, 0.75 mM $CaCl_2$, 10 mM $MgCl_2$, 0.2 mg/ml histone, 0.5 mg/ml leupeptin and 0.1 mM ATP (5 x10^6 cpm of [γ-^{32}P]ATP). Phosphatidylserine (24 μg/ml) and diolien (1.6 μg/ml) were added to some tubes to demonstrate phospholipid-dependent protein kinase activity. The reaction was halted by addition of

1 ml of trichloroacetic acid (25% v/v, 4°C). The precipitate was collected by filtration through a membrane filter (Whatman HA, 0.45 μm), the filters were washed five times (2 ml/wash) with trichloroacetic acid (5% v/v, 4°C) and radioactivity retained by the filter was determined. Protein kinase C activity was calculated from the difference between the ^{32}P incorporated into histone in the presence and the absence of added phospholipids and was expressed as picomoles of ^{32}P incorporated/min/10^6 eosinophils.

2.20 ADP-RIBOSYLATION

2.20.1 Ribosylation procedure

To determine whether the GTP-binding proteins were ADP-ribosylated by pertussis toxin (PTX)-treatment, eosinophils (approximately 10^8 cells/ml) incubated with or without PTX (1 μg/ml) were resuspended in hypotonic medium containing 20 mM Tris HCL, 1 mM EGTA, and 200U/ml aprotinin and were left for 30 min at 4°C. Cells were sonicated with 2 x three bursts for 20 sec each (Microson™ Ultrasonic Cell Disrupter, Farmingdale, NY, USA), homogenates were centrifuged at 16,000 x g for 60 min, and membrane containing pellets were resuspended in the same buffer. The GTP-binding protein (G_i) in the membrane fraction was then ADP-ribosylated by PTX (1 μg/ml) which had been preactivated with 20 mM dithiothreitol (DTT) and 2 mM ATP-Mg^{2+} at 37°C for 60 min. Thereafter, 20 μl of the membrane fraction (approximately 120 μg protein) was mixed with 5 μl [$\alpha^{32}P$]NAD (10 μCi) and 30 μl ADP-ribosylation mixture (15 mM MgCl2, 6 mM ATP, 6 mM EDTA, 60 mM thymidine, 6 mM DTT, 18 mM potassium phosphoenol pyruvate, 60 μg/ml pyruvate kinase, and 60 μM NAD in potassium phosphate buffer, pH 7.6). After incubation for 60 min at 30°C, reaction was stopped by adding 20 μl of Laemmli stopping buffer (Laemmli, 1970) and samples were boiled for 5 min.

2.20.2 SDS gel electrophoresis

Samples (25 µl) and standards were assayed in duplicate on a 10% sodium dodecyl sulfate-polyacrylamide gel overlaid with a 3% stacking gel. Protein separation was carried out overnight at room temperature with 5 mA of constant current followed by 1 hr at 20mA. Gels were stained with 0.025% (w/v) Coomassie blue R-250 (in 40% methanol and 7% acidic acid), dried and autoradiographed for 6 h to 21 days depending on the radioactivity. Radioactive spots corresponding to a 41 kDa protein were transferred into scintillation vials, 8 ml scintillation fluid added and the radioactivity counted in a scintillation counter for 4 min.

2.21 LIPID EXTRACTION AND SEPARATION

Intact eosinophils ($5x10^6$) or purified eosinophil membranes ($5x10^6$ cell equivalents) were incubated at 37°C for various periods of time in the presence of labelled 1-0-alkyl-2-acetyl-GPC µM/tube). Reactions were terminated by the addition of methanol/chloroform (2:1, v/v) and the lipids extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). Solvents were removed using a stream of nitrogen and the lipids resuspended in chloroform/methanol (1:1, v/v). The phosphoglyceride classes were separated on layers of Silica Gel G developed in chloroform/methanol/glacial acetic acid/water (50:25:8:4, v/v). The standard(1-0-alkyl-2-acetyl-GPC, 1-0-alkyl-2-acyl-GPC, 1-alkyl-2-hydroxy-GPC, phosphatidylinositol and phosphatidylethanolamine dissolved in chloroform/methanol (1:1, v/v) were visualized with I_2 vapor and the distribution of label in different products was determined by scanning the plate for radioactivity using a Bioscan System 200 Imaging Scanner (Bioscan, Washington, USA). The various products were then isolated and the radioactivity in the respective areas was determined by liquid scintillation spectroscopy.

2.22 DETERMINATION OF EC_{50} VALUES, K_i VALUES, DISSOCIATION CONSTANTS AND SCHILD ANALYSIS

To evaluate the effect of WEB 2086 on PAF-induced eosinophil degranulation, cells were incubated with the antagonist for 2 min before the start of the concentration-response curve. Negative log EC_{50} values from each concentration - response curve were derived by linear regression analysis of enzyme activity versus log concentration at concentrations immediately above and below the 50% response level. Differences in EC_{50} values in the presence and absence of WEB 2086 from paired experiments were analyzed by Student's paired t test with $P < 0.05$ regarded as significant. The apparent dissociation constant (K_B) of the receptor antagonist complex was calculated by the method of Furchott (1972) using the equation:-

K_B = [antagonist]/dose-ratio - 1.

Dose ratio was calculated to the following equation:

DR= antilog [(-log molar EC_{50} for PAF in the absence of antagonist) -(-log molar EC_{50} for PAF in the presence of antagonist)].

Schild analysis was obtained according to Arunlakshana and Schild (1959). pA_2 values were obtained with the aid of a computer and graphics plotter. The inhibition constant (K_i) for the competition experiments was derived from the equation of Cheng & Prussof (1973):

$$K_i = IC_{50}/(1 + L/K_D)$$

were the IC_{50} is the concentration of the antagonist which produces a 50% inhibition of the labeled ligand specific binding and L is the concentration of labelled ligand.

The rate of specific binding was calculated according to:

$$\%\ \text{specific binding} = 100 \times \frac{\text{specific binding in the presence of inhibitor}}{\text{specific binding in the absence of inhibitor}}$$

2.23 STATISTICAL ANALYSIS

For each stimulus, experiments were performed at least three times in duplicate or triplicate using cell preparations from different donors on different days. Results were expressed as mean ± SEM and were analyzed using Student's t-test for paired or unpaired variates, or Wilcoxon matched pairs sign-ranks test or Mann-Whitney U-test for paired and unpaired variates, respectively. Differences between more than two groups were assessed using Kruskal-Wallace ANOVA. Unless otherwise stated, significance was accepted when $P < 0.05$. All calculations were performed with the aid of a computer program (graph pad, USA, or NCSS, USA).

RESULTS

CHAPTER THREE

3 SPECTRUM OF PAF-INDUCED EOSINOPHIL RESPONSES

The first step towards understanding stimulus-response coupling is the characterization of the principal cell functions exhibited by stimulated eosinophils. Therefore, this chapter focuses on the effects of PAF and other stimuli on eosinophil functions, including degranulation, lipid mediator release and oxygen metabolism.

3.1 GRANULAR ENZYME RELEASE

3.1.1 Eosinophil Peroxidase

Purified eosinophils (4 x 10^6 cells/sample) obtained from the blood of patients suffering from HES were incubated with various concentrations of PAF at 37°C. After 10 min of incubation, the cells were centrifuged and the supernatant was assayed for enzyme activity of eosinophil peroxidase (EPO) and lactate dehydrogenase (LDH). As shown in Figure 3.1A, PAF induced EPO secretion in a dose-dependent manner with an EC_{50} of 1.26 nM. The enzyme could be detected at concentrations of 1 nM PAF and reached a peak at 100 nM PAF. The maximum release of EPO by PAF (10^{-7} M) was 22.6 ± 3.5%. However, with concentrations of PAF > 100 nM less enzyme release was observed. Lyso-PAF, the inactive precursor and metabolite of PAF, was ineffective at concentrations up to 10 μM (Figure 3.1A).

In order to evaluate the relative potency of PAF on human eosinophils we then compared its degranulating effect with that of calcium ionophore A23187 (calcimycin), opsonized zymosan, and PMA. Calcimycin caused a dose-dependent

cytolytic degranulation (Figure 3.1B) as indicated by the release of cytoplasmic LDH. In contrast, PAF at concentrations from 0.1 nM to 100 nM was more potent and did not cause any LDH release. The EC_{50} for calcimycin was 13.1 nM. PMA, a tumor-promoting agent which activates protein kinase C, also leads to EPO release at concentrations ranging from 10 pM - 100 nM (EC_{50} of 0.39 nM) without LDH secretion into the supernatant (Figure 3.1C). Furthermore, incubation of human eosinophils with varying particle concentrations of opsonized zymosan for 30 min releases EPO in a dose-dependent manner with a maximal effect at a concentration of 6 to 20 particles per cell (Figure 3.1D). Here again, no significant release of LDH was observed. The maximal percentage of total EPO release induced by opsonized zymosan (14.1%), ionophore (14.7%), and PMA (12.9%) was less than that seen with 100 nM PAF.

In order to evaluate the response of guinea pig eosinophils, the capacity of several potential stimuli to release EPO in these cells was examined. First, purified peritoneal eosinophils ($5x10^6$) were incubated for 15 min at 37°C with previously established optimal concentration of fMLP (10 μM), C5a (10 nM), substance P (10μM), melittin (100 nM), PAF (100 nM), lyso-PAF (100 nM), leukotriene B_4 (LTB_4 1 μM); PMA (100 nM), calcimycin (ionophore A23187; 1 μM), and opsonized zymosan (2×10^7 particles) and the supernatant was assayed for EPO activity. As demonstrated in Figure 3.2, eosinophils respond when challenged with several physiological and non-physiological stimuli, including peptides, lipids and particulate stimuli. Among the agonists studied, LTB_4 and PAF appeared to be the most effective. Interestingly, human eosinophils purified from the blood of asthmatics and patients with hypereosinophilia responded maximally to PAF but exhibited only a weak response to LTB_4 (see below).

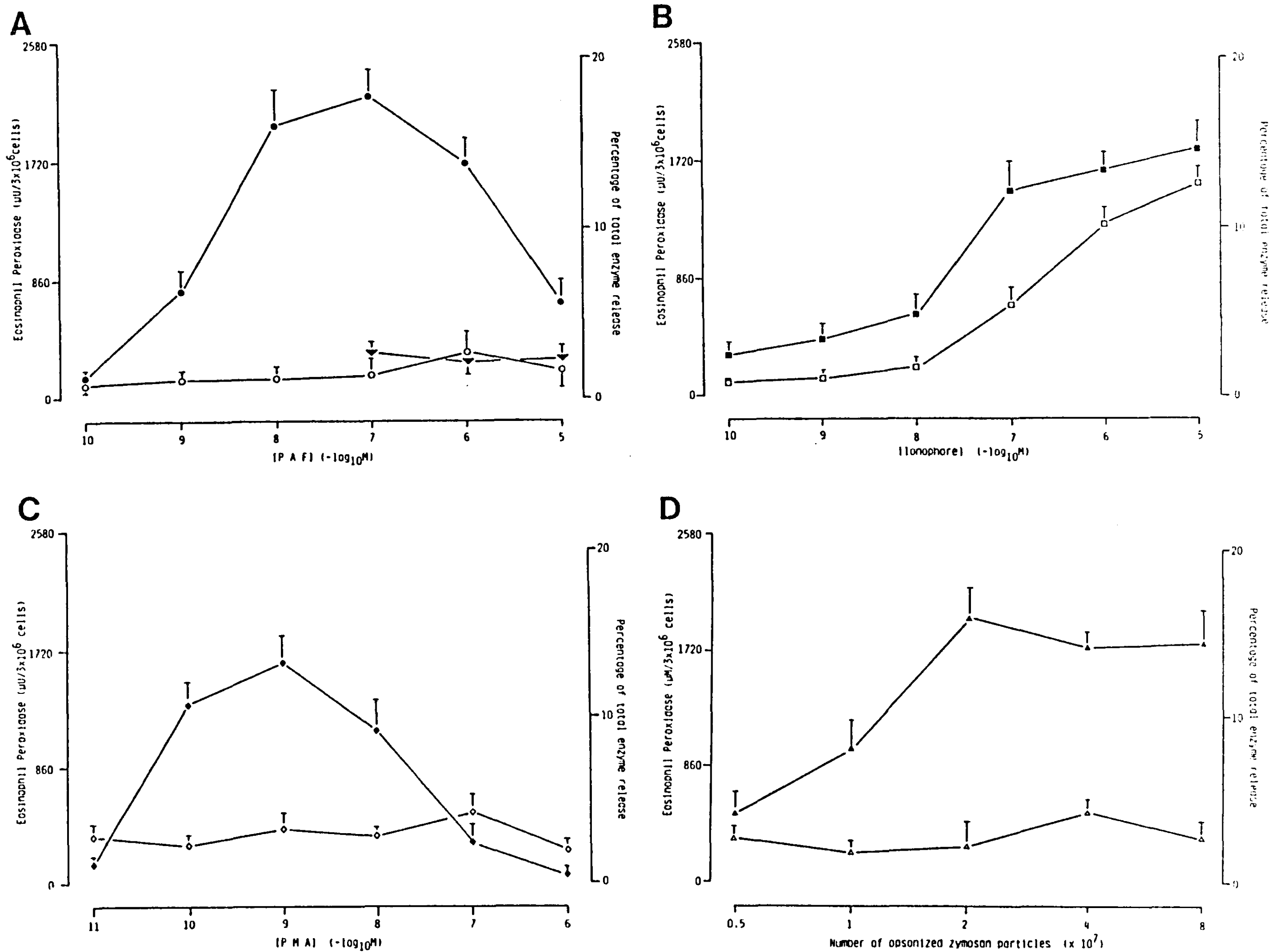


Figure 3.1: Release of eosinophil peroxidase (EPO) from purified human eosinophils. 3 x 10^6 purified human eosinophils were incubated with PAF (●), lyso PAF (▼), calcimycin, (■) phorbol myristate acetate, PMA (◆) and opsonized zymosan (▲) for 10 minutes. The supernatant was assayed for EPO (closed symbols) and LDH (open symbols). The data presented are the means ± SEM of triplicate assays from a single donor and similar results were obtained from 4 other donors. The total cellular EPO content was 4260 ± 534 mU/10^6 eosinophils (n=7).

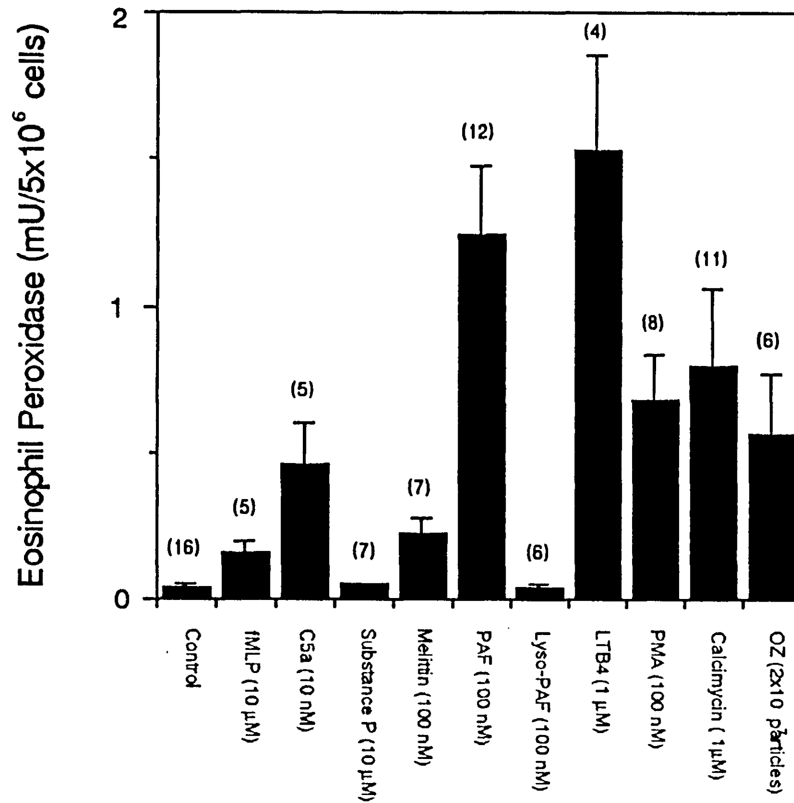


Figure 3.2: Agonist-induced EPO release from purified guinea pig eosinophils. Purified eosinophils were incubated with fMLP (10 µM), C5a (10 nM), substance P (10 µM), melittin (100 nM), PAF (100 nM), lyso-PAF (100nM), leukotriene B_4 (1 µM), PMA (100 nM), calcimycin (calcium ionophore, A23187, 1 µM) and opsonized zymosan ($2x10^7$ particles) and the supernatant assayed for EPO as described in the section 2.9.2 of the Methods. Each histogram represents the mean ± SEM of (n) observations indicated in the figure.

3.1.2 Eosinophil peroxidase and other granular enzymes

On the basis of these observations and because of its potential significance in asthma (Barnes *et al.* 1988a; Barnes *et al.* 1988b), PAF was chosen for further detailed studies. Eosinophils were stimulated with increasing concentrations of PAF under the conditions described above. Reaction was stopped by rapid centrifugation (12,000 x g_{max}, 30 sec) and the resulting supernatant was analyzed for enzyme activity of EPO, alkaline phosphatase, β-glucuronidase, arylsulfatase, acid phosphatase and lactate dehydrogenase. As shown in Figure 3.3, PAF induces eosinophil degranulation in a concentration-dependent fashion with a threshold stimulus concentration of 100 pM. The ability of PAF to degranulate eosinophils was maximal at 100 nM (n=9) and included both the enzymes stored in the specific granules (Fig. 3.3A) and the small granules (Fig. 3.3B). The corresponding EC_{50} values for the enzyme secretion varied between 2.3 to 5.6 nM (n = 6). The percentage of enzyme released after 10 minutes incubation with respect to total enzyme protein in the control cell pellet varied with each experiment between 9 and 25% (mean 15.7%, n = 13) for constituents stored in the specific granules and between 12 to 32% (mean 17.2%, n = 9) for enzymes released from the small granules. Concentrations of PAF higher than 100 nM were significantly less effective ($p < 0.05$; n=9). No release of cytoplasmic lactate dehydrogenase (LDH) was detected in the supernatant at concentrations up to 10^{-5} M PAF, suggesting a non-cytolytic release mechanism. Lyso-PAF did not promote enzyme exocytosis at any of the concentrations (10 pM - 10 μM) examined.

To determine the time course of enzyme secretion from human eosinophils induced by PAF, cells were incubated with 100 nM PAF for 2, 5, 15, 30, 60, 120, and 180 min. Reference to Figure 3.4 illustrates that enzyme was being released into the bathing buffer in the absence of a stimulus. After the addition of PAF and other stimuli the amount of secreted EPO increased rapidly in a time-dependent manner with a $t_{1/2}$ between 4 and 8 min. The release process reached a plateau 30 to 45 min post-challenge.

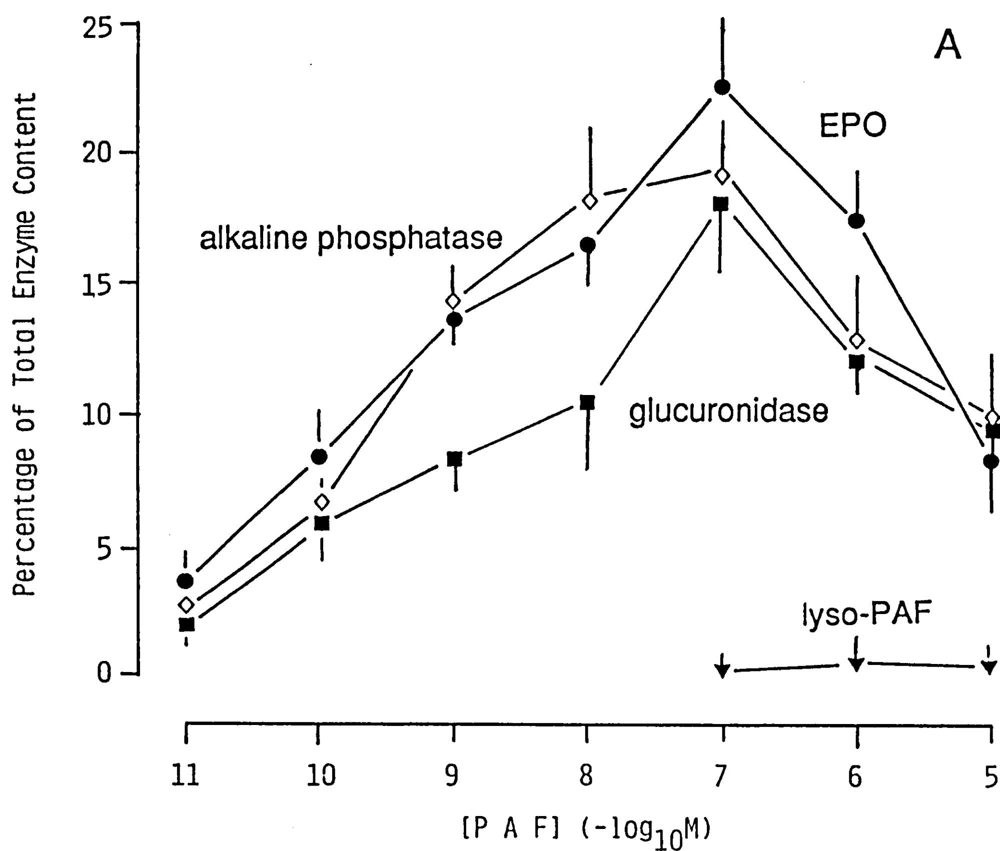


Figure 3.3A: PAF-induced enzyme secretion from guinea pig eosinophils. Eosinophils ($5x10^6$) were incubated with PAF (10 pM - 10 μM) and the reaction stopped by the addition of ice-cold buffer followed by rapid centrifugation. The enzymatic activities of EPO, β-glucuronidase, alkaline phosphatase are given in Panel A. The enzymes were assayed spectrophotometrically as described in section 2.9 of the Methods. The data are expressed as a percentage of total cellular enzyme content in the buffer control pellet and represent the mean ± SEM of six separate experiments.

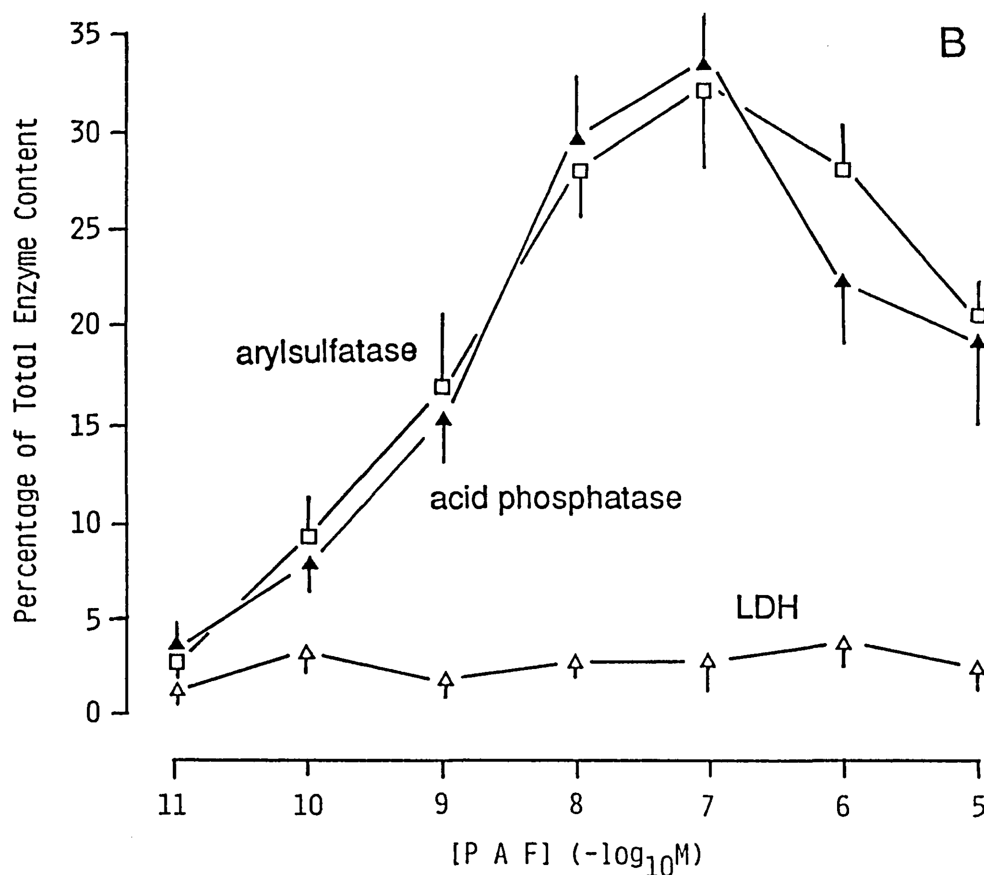


Figure 3.3B: PAF-induced enzyme secretion from guinea pig eosinophils. Eosinophils (5 x 10^6) were incubated with PAF (10pM - 10μM) and the reaction stopped by the addition of ice-cold buffer followed by rapid centrifugation. The enzymatic activities of EPO, β-glucuronidase, alkaline phosphatase are given in Panel A (see page 88) while acid phosphatase, arylsulfatase B and lactate dehydrogenase are shown in Panel B. The enzymes were assayed spectrophotometrically as described in section 2.9 of the Methods. The data are expressed as a percentage of total cellular enzyme content in the buffer control pellet and represent the mean ± SEM of six separate experiments.

3.2 GRANULAR PROTEIN RELEASE

Concurrent with the enzyme release we measured the extrusion of the non-enzymatic matrix protein, Eosinophil Cationic Protein or ECP. Purified human eosinophils (4 x 10^6) were stimulated for 2, 5, 10, 30, and 90 min with 100 nM PAF, the most effective concentration for PAF-induced eosinophil peroxidase (EPO) secretion (see above). As demonstrated in Fig. 3.5, human eosinophils from asthmatic subjects spontaneously released ECP into the bathing buffer. However, stimulation of cells with PAF (100 nM) caused an instantaneous increase in the release of ECP in a time-dependent fashion with a $t_{1/2}$ = 332 sec, reaching a plateau after approximately 30 min. Again the effect of PAF was inhibited in the presence of 1 μM WEB 2086 whereas lyso-PAF was ineffective. These results show that PAF causes the release of both granular enzymes and granular basic proteins from eosinophils.

3.3 PROSTANOID GENERATION

3.3.1 Spectrum of prostanoids generated by eosinophils

Combined capillary gas chromatography/mass spectrometry was used to examine the spectrum of prostanoids generated by resting and stimulated human blood eosinophils. A typical mass chromatogram obtained from eosinophils stimulated with PAF (1 μM) is depicted in Fig. 3.6. The figure represents unfiltered data with no foreground or background subtraction. Of the six prostanoids analyzed only PGE_2, PGD_2, $PGF_{2\alpha}$ and TXB_2 could be detected. The stable metabolite of prostacyclin, 6-keto-$PGF_{1\alpha}$, as well was the 11-keto reductase product of PGD_2, $9\alpha,11\beta$-PGF_2, were not observed. External prostanoid standards (6-keto-$PGF_{1\alpha}$ and $9\alpha,11\beta$-PGF_2 added to the cells before incubation could be fully recovered, excluding an inherent methodological problem and suggesting that eosinophils do not metabolize either of these prostanoids during the 5 min incubation.

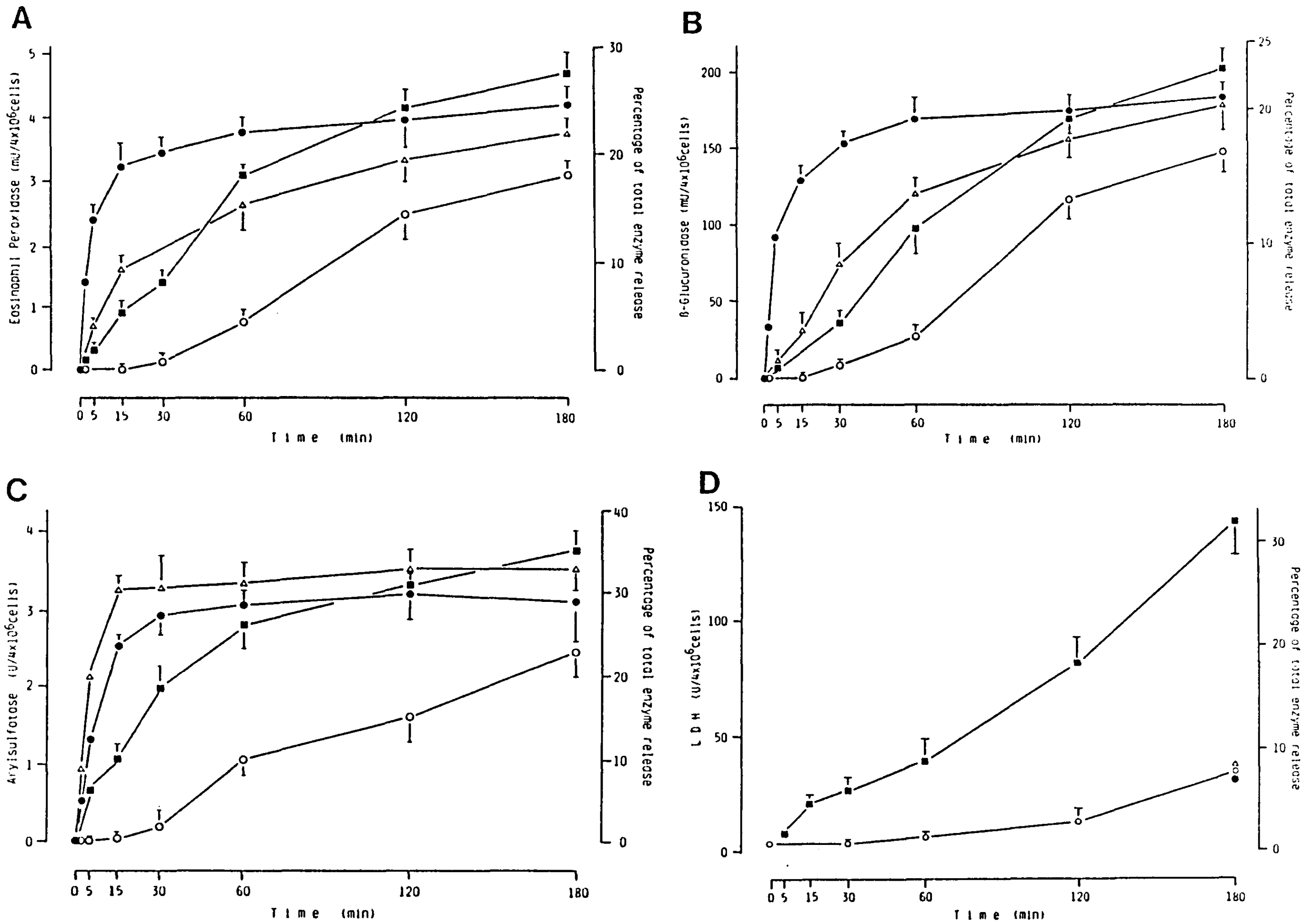


Figure 3.4: **Kinetics of enzyme release from human eosinophils.** Purified human eosinophils (4 x 10^6) were challenged with 100 nM PAF (●), 1 μM calcimycin (■), 2×10^7 particles of opsonized zymosan (Δ) or vehicle (O) and the reaction allowed to proceed for the times indicated. EPO (Panel A), β-glucuronidase (Panel B), arylsulfatase B (Panel C), and LDH (Panel D) were determined in the supernatant as described earlier (Section 2.9 of the Methods). Data are represented as Units/4 x 10^6 (left ordinate) and as percentage of total cellular enzyme content in the buffer control pellet (right ordinate). Each point represents the mean ± SEM of four separate preparations.

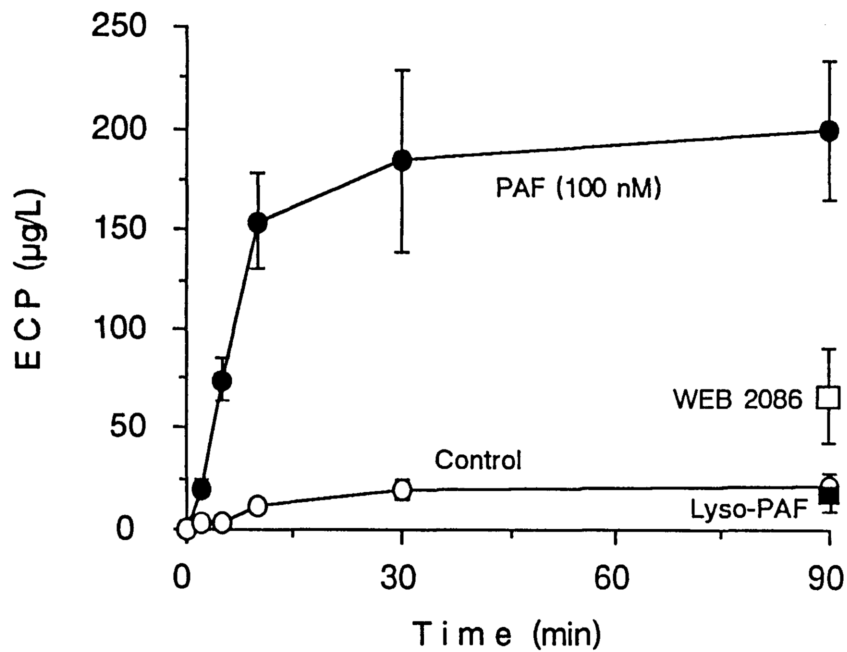


Figure 3.5: PAF-induced ECP release from human eosinophils. Purified human eosinophils (4 x 10^6) were incubated in HBSS as described in the Methods. They were challenged with buffer (control), lyso-PAF (100 nM) or PAF (100 nM) in the presence or absence of 1 µM WEB 2086 and the reaction allowed to proceed for the times indicated. The concentration of ECP was then determined by RIA. Data shown represent triplicate determinations of a representative experiment.

The quantitative profiles of the four prostanoids generated by resting human and guinea pig eosinophils are compared in Table 3.1A and 3.1B. TXB_2, the stable non enzymatic degradation product of TXA_2 was the principle prostanoid with an average of 8.4 ± 2.2 pg (n=6) and 57.6 ± 17 fg (n=9) being spontaneously generated by 10^6 human and guinea pig eosinophils, respectively (n=11). Minor amounts of $PGF_{2\alpha}$, PGE_2, and PGD_2 were also spontaneously synthesized. Stimulation of both cell types with calcimycin induced a 10- to 25-fold increase in prostanoid synthesis within 5 min (TXB_2: 753 ± 62 pg; $PGF_{2\alpha}$: 46.1 ± 12 pg; PGD_2: 20.4 ± 3.3 pmol; PGE_2: 23.0 ± 4.5 pmol). PAF also stimulated the biosynthesis of the four prostanoids with an increase between 2 and 11-fold over basal generation while fMLP and PMA were less effective and SP had no effect (data not shown). The magnitude of stimulated mediator generation in both human and guinea pig eosinophils differed for each of the prostanoids studied. The largest increase was observed with PGD_2 (7- to 25-fold) and the lowest with TXB_2 (2- to 12-fold). Quantitatively, human blood eosinophils synthesised approximately 50 to 100 x fold more prostanoids than guinea pig cells.

3.3.2 Dose-response curve of PAF-induced TXB_2 and PGE_2 release.

In order to further characterize the PAF induced effects on prostanoid release by eosinophils, both TXB_2 and PGE_1/E_2 were measured using a radioimmunoassay. Figure 3.7 shows the non-cumulative effect of PAF on the release of both prostanoids after incubation for 5 min at 37°C. PAF (1 pM to 1 µM) stimulated the release of TXB_2 and PGE-like activity in a concentration dependent manner with EC_{50} values (TXB_2: 35.7±10.9 nM, n=9; PGE_1/E_2 19.3 ± 9.3 nM, n=9) that did not differ significantly ($p>0.05$; Mann-Whitney U-test) from one another. The maximal effective concentration of PAF was 1 µM where the concentration of both prostanoids was increased approximately 4.5-fold over spontaneous release.

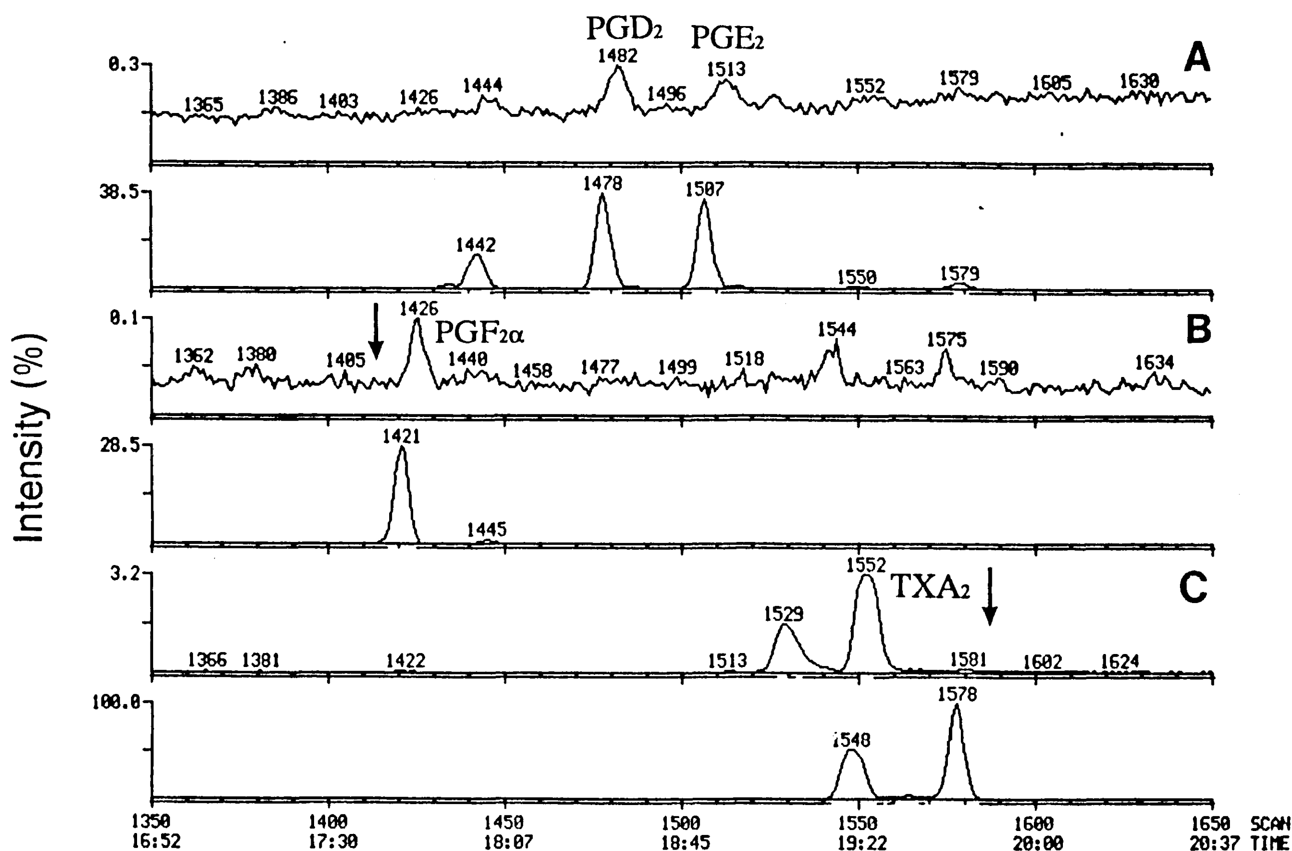


Figure 3.6: **Capillary gas chromatography-negative ion mass spectrometry of prostanoids generated by human eosinophils.** Fragment ions were monitored simultaneously at six different masses: m/z 524 (characteristic of PGD_2 and PGE_2); m/z 528 (derived from $^2H_4PGE_2$; panel A), m/z 569 (common ion generated from 9α,11β-PGF_2 and $PGF_{2\alpha}$; panel B), m/z 573 (fragment of $^2H_4PGF_{2\alpha}$; panel B), m/z 614 (fragment ion common to TXB_2 and 6-keto-$PGF_{1\alpha}$; panel C), and m/z 618 (fragment ion from $^2H_4$-6-keto-$PGF_{1\alpha}$; panel C) were monitored simultaneously. Signal intensity (ordinate) was normalized in each panel to that derived from the deuterated analogs employed as internal standards. Retention time in minutes and seconds are indicated along the abscissa. Arrows indicate the position at which 9α,11β-PGF_2(1411) and 6-keto-$PGF_{1\alpha}$(1586) would elute if they were present. The mass chromatograph is representative of the profiles obtained in 9 experiments. The sensitivity of the assay was 100 fg.

A. Human Blood Eosinophils

Prostanoids	TXB_2	PGE_2	PGD_2	$PGF_{2\alpha}$
Control	8.4±2.2§	0.2±0.1	0.5±0.2	0.3±2.3
Calcimycin (1 μM)	50.7±9.3	1.7±0.7	1.7±0.7	0.7±0.3
PAF (1 μM)	27.9±2.6	1.4±0.6	1.0±0.5	0.6±0.2
PMA (100 nM)	13.4±3.7	1.1±0.7	1.3±0.8	0.8±0.4
LTB4 (1 μM)	10.1±3.4	0.9±0.6	0.7±0.5	0.6±0.2

§ data in pg/10^6 eosinophils

B. Guinea Pig Eosinophils

Prostanoids	TXB_2	PGE_2	PGD_2	$PGF_{2\alpha}$
Control	57.6±17*	2.9±0.9	1.2±0.5	3.1±1
Calcimycin (1 μM)	753.5±97	23.0±4.5	20.4±3.3	46.1±12
PAF (1 μM)	120.7±40	8.1±3.4	9.6±4.0	4.8±1.9
fMLP (1 μM)	73.9±18	3.0±0.6	3.4±0.7	3.3±1.2
PMA (100 nM)	110.3±49	6.4±3.0	1.9±0.8	4.0±1.7
LTB4 (1 μM)	177.0±53	31.4±7.3	11.7±3.9	9.3±3.2

* data in fg/10^6 eosinophils

Table 3.1: Prostanoid biosynthesis by human and guinea pig eosinophils. Prostanoid biosynthesis from human and guinea pig eosinophils are shown in panels A and B, respectively. The quantity in each sample is expressed in pg/10^6 human eosinophils and fg/10^6 guinea pig eosinophils. Data are expressed as mean ± SEM of 6 (A) and 9 (B) independent experiments, respectively.

On a molar basis the assayable concentration of TXB_2 in the bathing buffer was approximately 30-fold higher than PGE_1/E_2 after stimulation with 1 μM PAF. In absolute units this corresponds to 10.4 ± 2.3 pmol.10^6cells^{-1}.825 μl^{-1} (or 12.6 μM) of TXB_2 compared to 334.2 ± 47.6 fmol.10^6.825 μl^{-1} (or 405 pM) for PGE_1/E_2. Lyso-PAF did not promote prostanoid release from eosinophils at any concentration (100 nM to 10 μM) examined (data not shown).

Pretreatment of the eosinophils with flurbiprofen (8 μM for 5 min), an irreversible inhibitor of the cyclo-oxygenase enzyme complex *in vitro* (Rome & Lands, 1975), abolished both the basal and PAF-elicited prostanoid release (see Figure 3.10). This observation is, thus, consistent with the hypothesis that both TXB_2 and PGE-like immunoreactivity were derived from the *de novo* cyclo-oxygenation of arachidonic acid in eosinophils.

Similarly, eosinophils pretreated with the selective thromboxane synthetase inhibitor, dazmagrel (Parry *et al.*, 1982), at concentrations of 0.5 to 500 μM for 5 min inhibited PAF-stimulated TXB_2 release in a concentration-dependent manner with an IC_{50} of 2.2 ± 0.1 μM (n=6). At 50 μM dazmagrel, a dose which inhibited the amount of PAF-induced TXB_2 release below the limit of detection of the RIA, PGE-like immunoreactivity was augmented approximately five-fold from 607 ± 43.3 fmol. 10^6 cells^{-1} to 2.81 pmol.10^6 cells^{-1} (n=6). As demonstrated in Figure 3.8, this increase in PGE-like activity induced by dazmagrel was concentration-dependent with an EC_{50} of 1.53 ± 0.13 μM (n=6) and was identical to the IC_{50} obtained for this drug for inhibiting TXB_2 release. Figure 3.8 indeed shows a clear inverse relationship between PAF (1 μM)-stimulated increase in PGE-like immunoreactivity on the one hand, and the disappearance of TXB_2 on the other. This relationship was not, however, stoichiometric because only 20% of the TXB_2 release was accounted for by the increase in PGE_1/E_2 seen in dazmagrel (50 μM)-pretreated cells.

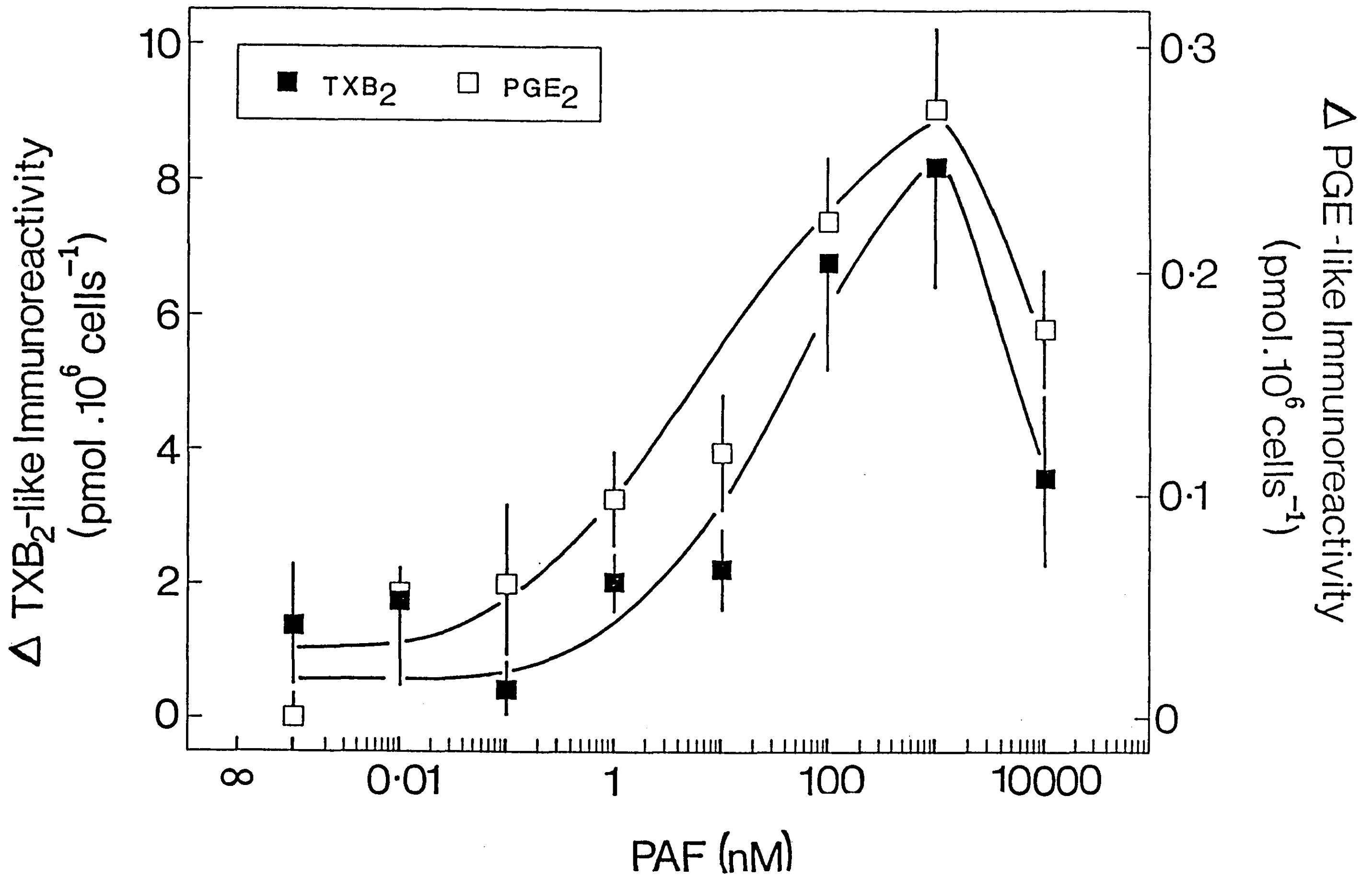


Figure 3.7: Effect of PAF on the generation of TXB_2- and PGE-like immunoreactivity. Guinea pig eosinophils were equilibrated for 5 min in prewarmed, HEPES-buffered PBS followed by the addition of PAF (1pM to 10 μM). Each incubation was allowed to proceed for an additional 10 min after which the reaction was quenched. Immunoreactive TXB_2 and PGE_1/E_2 were then determined by RIA (see 2.12). Each data point represents the mean ± SEM of nine observations obtained from three separate eosinophil preparations.

3.3.3 Time course

Since TXB_2 was the major cyclo-oxygenase synthesized by eosinophils this prostanoid was chosen as a marker to characterize the kinetics of eosinophil activation by PAF. As with enzyme secretion, significant amounts of TXB_2 were released in the absence of a stimulus (Fig. 3.9), indicating a basal rate of arachidonic acid utilization and metabolism. Addition of 1 μM PAF (Figure 3.9A) caused a rapid increase in the amount of TXB_2 released in a time-dependent fashion with a $t_{1/2} = 9.2 \pm 0.8$ sec (n=12). At 5 sec after the addition of PAF, the concentration of TXB_2 had increased to approximately 250% above that of the time-matched control samples. The release reaction reached a plateau 30 to 60 sec post-challenge at a time when the concentration of TXB_2 was 450% above unchallenged levels. Analysis of the kinetics (velocity of prostanoid release at time t sec post-PAF challenge) of PAF-promoted TXB_2 release from eosinophils showed that the release of TXB_2 occurred without a detectable lag period and was very rapid with a maximum rate of synthesis (19.4 ± 3.2 $pmol.min^{-1}10^6$ cells; n=12) being achieved 5 sec after challenge with PAF. Thereafter, the velocity of TXB_2 biosynthesis and secretion decreased reaching basal rate within 40 sec (Figure 3.8B).

3.3.4 Comparison of the effect of PAF with other stimuli

The histograms depicted in Figure 3.10 compare the ability of PAF to promote TXB_2 from eosinophils with a range of other stimulants. On an equimolar basis (100 nM), PAF was approximately twice as potent at generating TXB_2 as the complement fragment, C5a, and three-times more potent than the tripeptide chemoattractant, FMLP. Increasing the concentration of FMLP 1000-fold to 100 μM did result in an additional small increase in TXB_2-like immunoreactivity, amounting, however, to only of 42% of that generated by 100 nM PAF (data not shown).

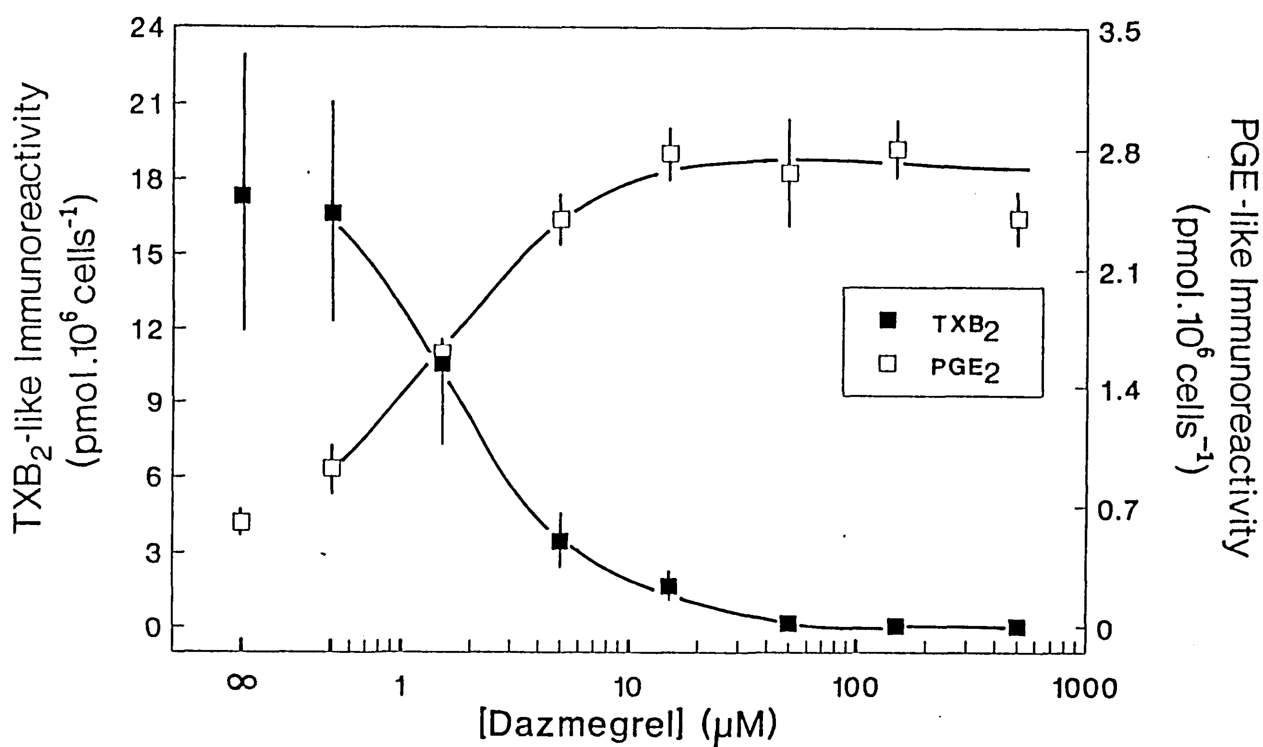


Figure 3.8: Effect of dazmegrel on PAF-induced TXB_2 and PGE_1/E_2. Eosinophils were incubated for 5 min in HEPES-buffered PBS containing either vehicle or dazmagrel (0.5, 1.5, 5, 15, 50, 100, and 500 μM, respectively). Cells were then challenged with PAF (1 μM) and the reaction allowed to proceed for an additional 10 min. The concentration of elaborated TXB_2 and PGE_1/E_2 was subsequently determined by RIA. Each data point represents the mean ± SEM of six observations obtained from three preparations.

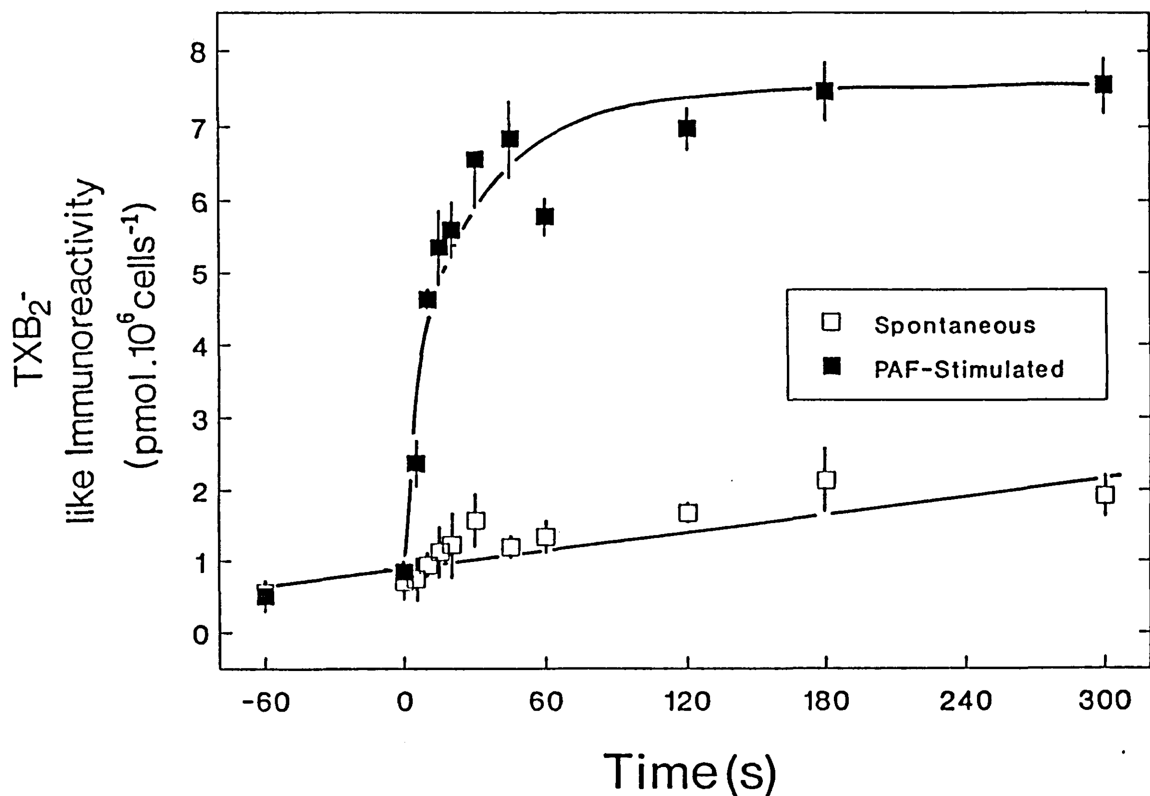


Figure 3.9: Time course and kinetics of PAF-stimulated TXB_2 release. Eosinophils were incubated as described in the legend to Figure 3.5. PAF or vehicle was added at 0 sec and the reaction allowed to proceed for the times indicated. The concentration of TXB_2 released was estimated by RIA. Graph (A) represents the profile of TXB_2 release with respect to time-matched, vehicle-treated eosinophils. In (B) the change in TXB_2 release (PAF-promoted vs spontaneous) is expressed as the rate during the first 60 sec of PAF-challenge. Data shown represent the mean ± SEM of 12 observations obtained from four separate eosinophil preparations.

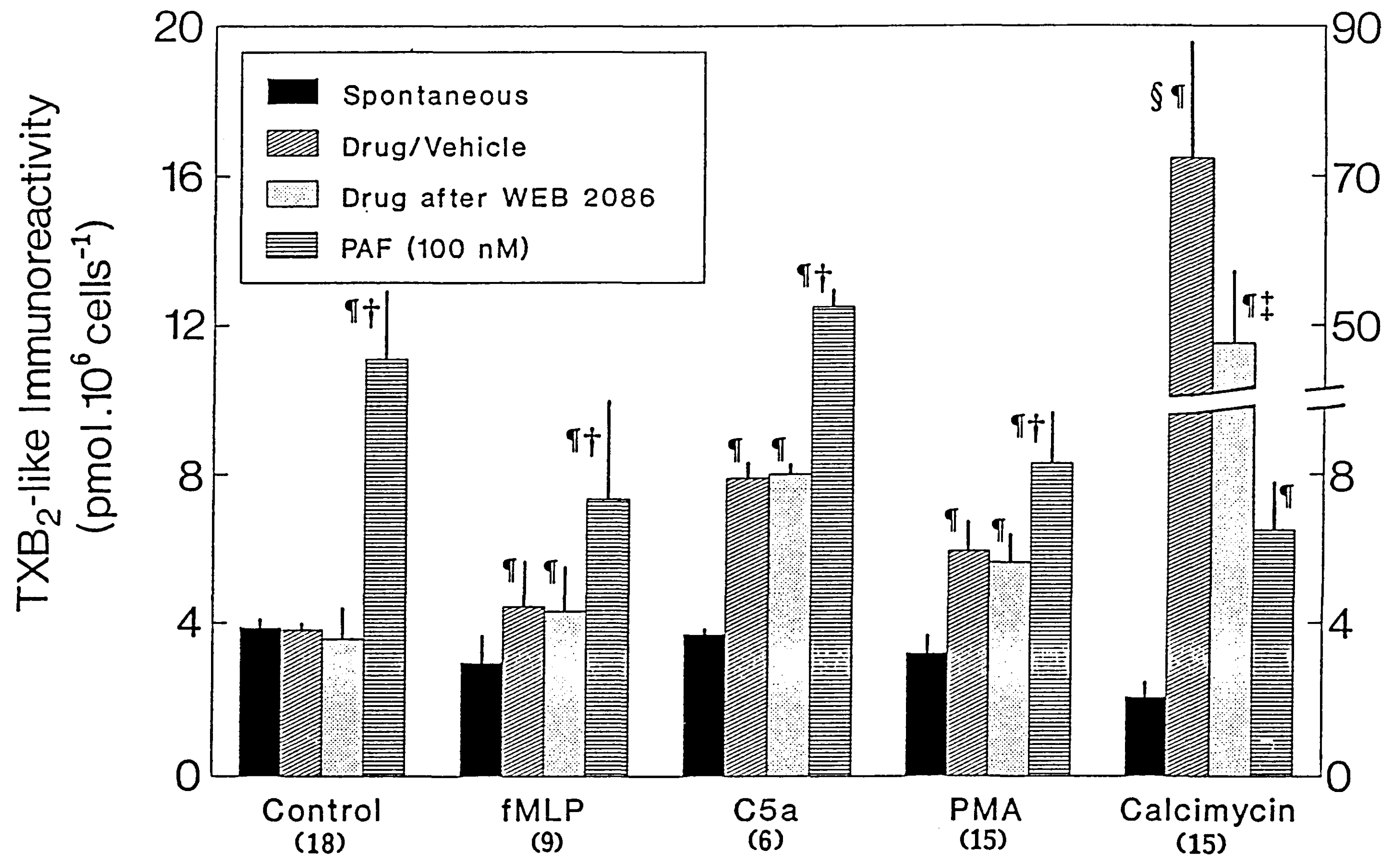


Figure 3.10: Comparison of PAF with FMLP, C5a, PMA and calcimycin on TXB2 release by eosinophils. Cells were incubated for 5 min in HEPES-buffered PSS (37°C) in the absence or presence of WEB 2086 (300 nM) and then challenged with PAF (100 nM), fMLP (100 nM), calcimycin (1 μM) or vehicle. Each incubation was allowed to proceed for 10 min, quenched with ice-cold buffer, and immunoreactive TXB2 measured by RIA as described in Methods. Each histogram represents the mean ± SEM of n observations given in parentheses. P, $p < 0.05$, TXB2 release was significantly greater than spontaneous; $p < 0.05$, PAF-stimulated TXB2 release was significantly greater than that induced by vehicle; $, $p < 0.001$, calcimycin-induced TXB2 secretion significantly greater than that induced by PAF; ++, $p < 0.01$ significant inhibition of calcimycin-induced TXB2 release by WEB 2086. Differences between data in each group were assessed by Kruskal-Wallace ANOVA.

Similarly, PMA induced TXB_2 release totalled 52% of that released by 100 nM PAF whereas the non-tumor-promoting phorbol, 4-α-phorbol, was inactive at 100 nM, 1 μM, and 10 μM (data not shown). Calcimycin, which was chosen as a reference agonist, was considerably more active than all stimuli examined, being approximately 10-fold more active than PAF (100 nM). In additional experiments, preincubation of the cells with WEB 2086 (300 nM for 5 min) abolished PAF-stimulated TXB_2 release and attenuated the release of TXB_2 induced by calcimycin (1 μM) by 32%. WEB 2086 did not antagonize the stimulatory effect of fMLP (100 nM), C5a (100 nM) and PMA (100 nM) confirming the selectivity of WEB 2086 for the PAF receptor (Figure 3.10).

3.4 RELEASE OF LEUKOTRIENE C_4

Guinea pig eosinophils (up to 5 x 106 cells.sample^{-1}) stimulated both with PAF (100 nM and 1 μM) and calcimycin (1 μM) and incubated for 15 min at 37°C failed to release any detectable amount of LTC_4 as assessed by RIA. This observation contrasts with the ability of human cells to generate this leukotriene and confirms previously published observations (Sun *et al.*, 1989a; Sun *et al.*, 1989b). In order to evaluate the effect of PAF and calcimycin on human eosinophils, we incubated blood cells obtained from asthmatics with vehicle, calcimycin (1 μM), LTB_4 (1 μM), lyso-PAF (1 μM), and PAF (1 μM) in the absence and presence of 1 μM WEB 2086 for 15 min at 37°C. The results are depicted in Figure 3.11. In contrast to guinea pig eosinophils, human eosinophils release LTC_4 when stimulated with PAF or calcimycin. Calcimycin was approximately 20-fold more effective in stimulating LTC_4 release (135 ± 37 pg versus 2.73 ± 0.5 ng/10^6 eosinophils; n=3). LTB_4 and lyso-PAF did not cause any significant leukotriene synthesis. In the presence of WEB 2086 (1 μM) only the PAF-induced LTC_4 synthesis was significantly inhibited whereas the other stimuli remained unaffected (Figure 3.11).

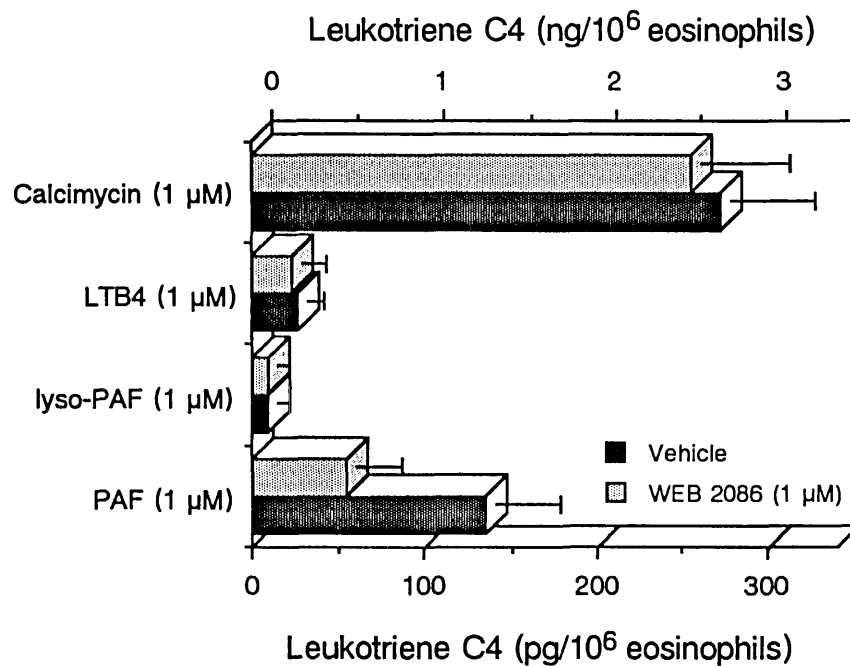


Figure 3.11: Effect of PAF, lyso-PAF, LTB_4 and calcimycin on leukotriene C_4 release by human eosinophils. Eosinophils were preincubated with vehicle or WEB 2086 (1 µM) for 5 min at 37°C. The reaction was started by the addition of the stimuli and terminated by placing the tubes in an ice-cold water bath and subsequent centrifugation at 2500 x g for 2 min. The resultant supernatant was stored at -20° C for mediator concentration and measured by RIA. Each data point represents mean ± SEM of six independent experiments.

3.5 GENERATION OF SUPEROXIDE ANIONS

Human blood eosinophils ($3x10^6$/ml) purified from patients with HES were incubated at 37°C in a disposable cuvette and superoxide anion ($\cdot O_2^-$) generation was monitored continuously at 550 nm as a measure of ferricytochrome C reduction. As demonstrated in Figure 3.12, PAF induces a time- and concentration dependent generation of $\cdot O_2^-$ by human eosinophils. Superoxide production was rapid ($t_{1/2}$ = 109 sec) with a maximum generation demonstrated at 3 x 10^{-5} M PAF and an EC_{50} of 8.4 ± 0.9 µM. The phorbol ester PMA, however, proved to be more potent with an EC_{50} of 147.4 ± 33.7 pM. While, WEB 2086 had no influence on the PMA-elicited $\cdot O_2^-$-production, the PAF-stimulated response was inhibited in the presence of WEB 2086 in a dose-dependent fashion (Figure 3.13), indicating a specific receptor-mediated process. The corresponding IC_{50} was calculated as 51.1 µM. Furthermore, lyso-PAF at concentrations up to 3 x 10^{-5} M did not induce $\cdot O_2^-$ generation by eosinophils. Finally the stereoisomer of PAF, enantio-PAF (3-0-hexadecyl-2-acetyl-sn-glycero-1-phosphocholine, C_{16}), was approximately 50% less effective than natural PAF. Ferricytochrome C reduction was 90 to 98% attenuated by 30 µg/ml superoxide dismutase (SOD) at each concentration of the stimuli.

The response of guinea pig eosinophils to PAF and PMA was comparable to that of human cells (Fig. 3.14). Other stimuli such as fMLP were less effective with respect to both maximal production of $\cdot O_2^-$ and the EC_{50} (≈23nM). Substance P or melittin did not cause any production of $\cdot O_2^-$ from guinea pig eosinophils.

3.6 EFFECTS OF DIVALENT CATIONS

Divalent cations such as Ca^{2+} and Mg^{2+} are thought to play an essential role as second messengers and cofactors for enzymatic reactions in many cells. Therefore the effect of these two cations on both prostanoid generation, EPO release, and $\cdot O_2^-$ production was examined. Purified eosinophils were washed three times in

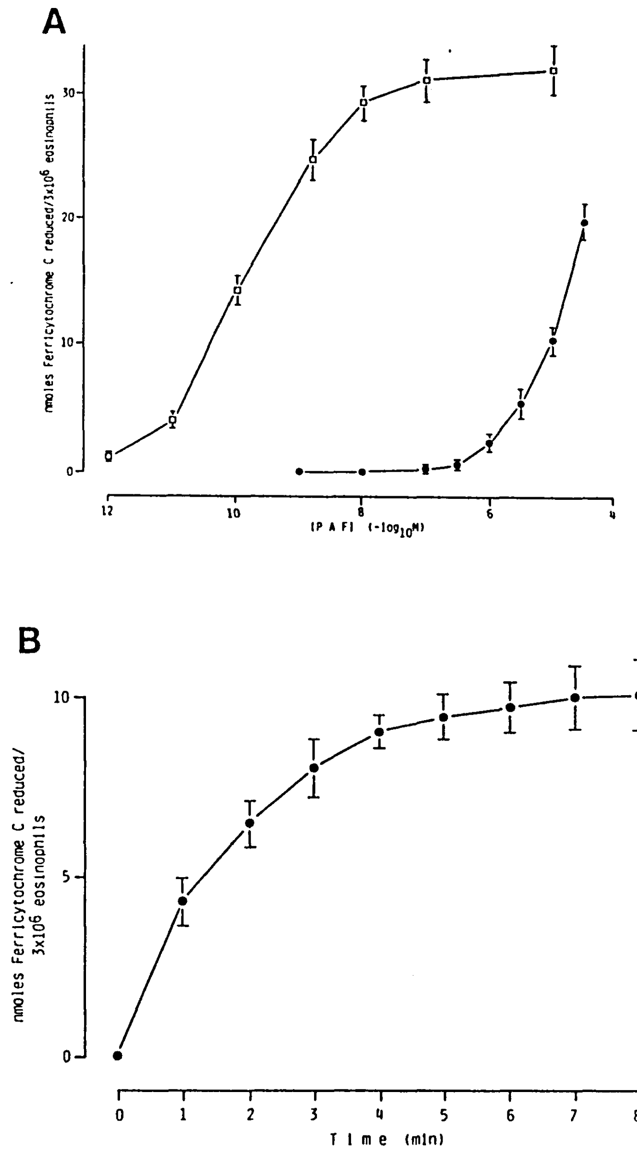


Figure 3.12: Superoxide production from human eosinophils by PAF and PMA. Panel A: Eosinophils (3 x 10^6/ml) were incubated in the presence of different concentrations of PAF (●) and PMA (□) for 10 min at 37°C. Ferricytochrome C reduction was 80 to 98% attenuated at each PAF concentration by 30 µg/ml superoxide dismutase. Results represent mean ± SEM for triplicate determinations for 1 preparation of eosinophils and are representative of results from 3 independent experiments. **Panel B:** Time course of ferricytochrome C reduction measured continuously at 550 nm in a suspension of 3x 10^6 cells/ml when eosinophils stimulated by 10^{-6} M PAF at 37°C in a 10 mm cuvette. Results are mean ± SEM for triplicate determinations.

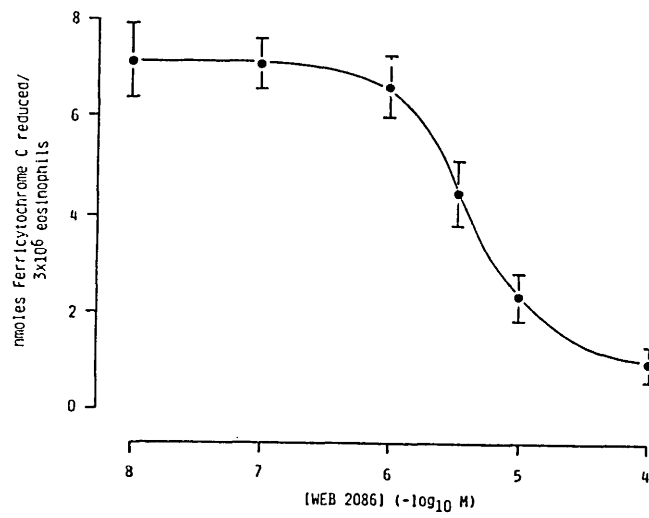


Figure 3.13: Inhibitory effect of WEB 2086 on PAF-induced superoxide generation by human eosinophils. 3 x 10^6 purified eosinophils were preincubated for 1 min with various concentrations of WEB 2086 before 30 μM PAF was added. Details as described in the legend of Fig. 3.12.

Ca^{2+}/Mg^{2+}-free HBSS. Twenty min prior to the addition of PAF or vehicle either $CaCl_2$ or $MgCl_2$ was added to give a final external ion concentration of 1 mM. As shown in Table 3.2, the absence of Mg^{2+} only slightly reduced both the prostanoid generation and EPO release stimulated by 1 µM PAF. In contrast, the absence of Ca^{2+} significantly diminished both eosinophil responses. Treatment of eosinophils with PAF (3 µM) in the absence of both divalent cations, however, almost completely inhibited the $\cdot O_2^-$ generation by the cells. Addition of 1 mM Ca^{2+} failed to increase $\cdot O_2^-$ production induced by PAF whereas 1 mM Mg^{2+} fully restored the cellular response. Interestingly, the stimulatory effects of PMA (100 nM) on all three cell functions were independent of concentration and composition of the extracellular divalent cation concentration (data not shown).

In order to evaluate the effect of calcium ions on PAF-induced enzyme secretion, purified human eosinophils were washed three times in Ca^{2+}-free HBSS buffer before stimulating the cells with PAF (10^{-7} M) for 2, 5, 15, 30, and 60 min. Under these conditions EPO release was reduced more than 50% at all time points (Figure 3.15). Furthermore, addition of EGTA (5 mM) almost completely inhibited PAF-induced degranulation of the cells. In contrast, PMA-induced degranulation was not inhibited either in the absence of Ca^{2+} or in the presence of EGTA (Figure 3.15). These results indicate, that enzyme release from human eosinophils induced by PAF is dependent on extracellular calcium whereas that induced by the protein kinase C activator PMA is not.

3.7 EFFECT OF FLURBIPROFEN

In order to examine potential positive or negative feedback mechanisms in eosinophils, the effect of the cyclo-oxygenase inhibitor, flurbiprofen, on eosinophil effector functions was investigated (see also Chapter 7.4). As shown above, stimulation of eosinophils with PAF in the presence of flurbiprofen (8 µM) abolished both basal and stimulated TXB_2 formation (see Chapter 3.3). In contrast, flurbiprofen

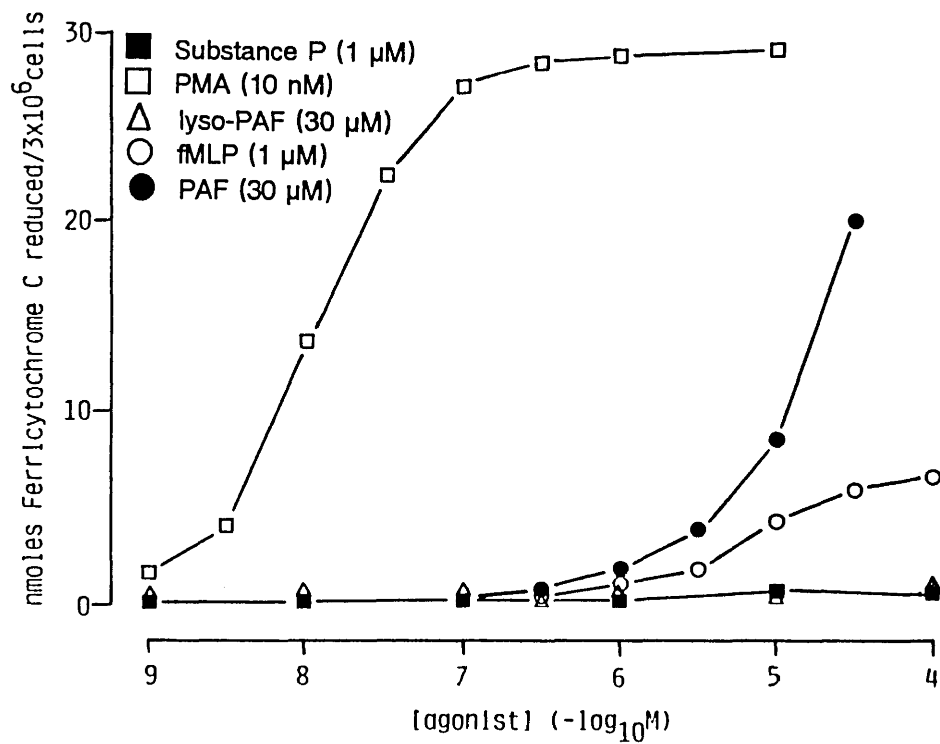


Figure 3.14: Superoxide anion generation from guinea pig eosinophils. Eosinophils (3×10^6 cells.ml^{-1}.sample^{-1}) were incubated for 10 min at 37°C in the presence of increasing concentrations of PAF (●), lyso-PAF (Δ), PMA (□), SP and melittin (■), fMLP (O). Kinetics of PAF-induced $\cdot O_2^-$ production induced by increasing concentrations of PAF were measured continuously at a wavelength of 550 nm. Ferricytochrome C reduction was 80 to 100% attenuated at each concentration by 30 μg/ml superoxide dismutase. Results represent mean ± SEM of triplicate determinations for one preparation of eosinophils and are representative of results from three independent experiments.

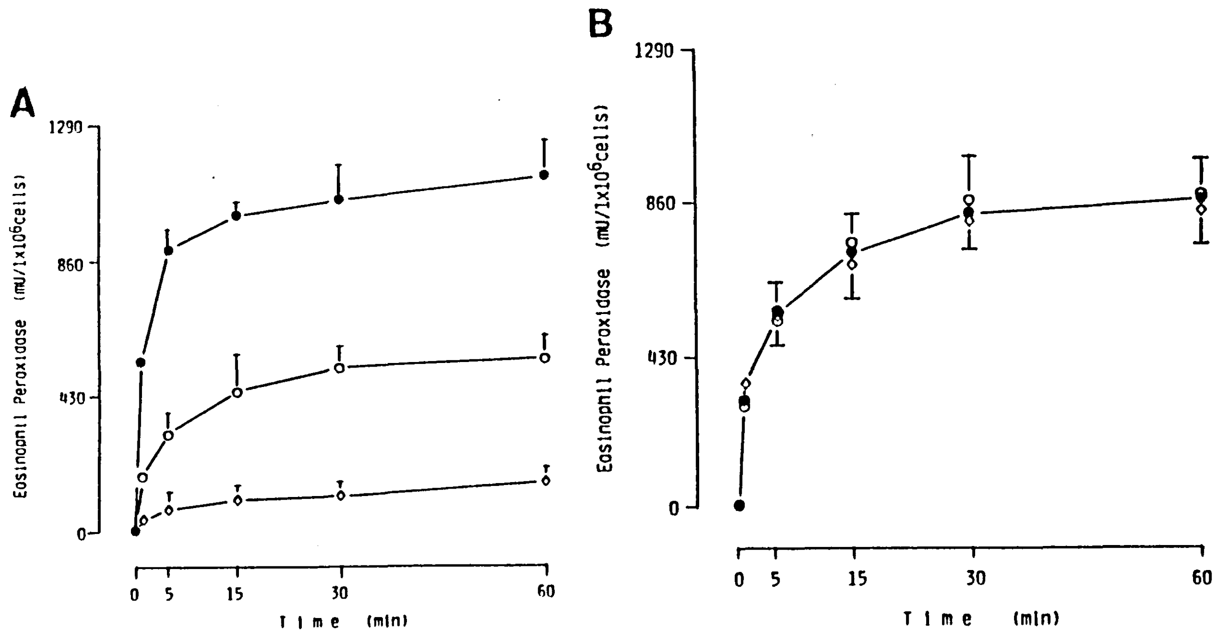


Figure 3.15: Effect of extracellular calcium on PAF- (Panel A) and PMA-induced (Panel B) EPO release by human eosinophils. Purified eosinophils (10^6) in calcium (1 mM) containing buffer (●), as well as calcium-free buffer in the absence (○), and in the presence of 5 mM EGTA (◇) were incubated with 100 nM PAF at 37°C for the intervals indicated in the figure. The reaction was stopped as described in the legend to Figure 3.3B (page 89). Data are expressed as mean ± SEM of triplicate determinations from a representative experiment.

Buffer	Δ $[Ca^{2+}]_i$ (nM)	EPO (mM/4x10^6 cells)	$\cdot O_2^-$ (nmol cytochrome reduced/10^6cells)	Δ TXB_2 (pg/10^6 cells)
Ca^{2+}/Mg^{2+} -free	73 ± 12	1.2±0.4	0.05±0.01	3.7±1
1 mM Ca^{2+}/Mg^{2+}	342 ± 22	7.2±0.9	4.9±0.4	65.0±23
1 mM Ca^{2+}	305 ± 29	5.2±0.8	0.07±0.04	59.6±19
1 mM Mg^{2+}	102 ± 18	2.8±0.5	4.3±0.5	8.3±2

Table 3.2: Effect of divalent cations on PAF and PMA-induced EPO release, superoxide anion generation, and prostanoid generation. Cells were stimulated in the presence and absence of 1 mM Ca^{2+} and/or Mg^{2+}, and supernatant was assayed as described in Methods. Data represent mean ± SEM of 4 independent experiments performed at least in duplicate.

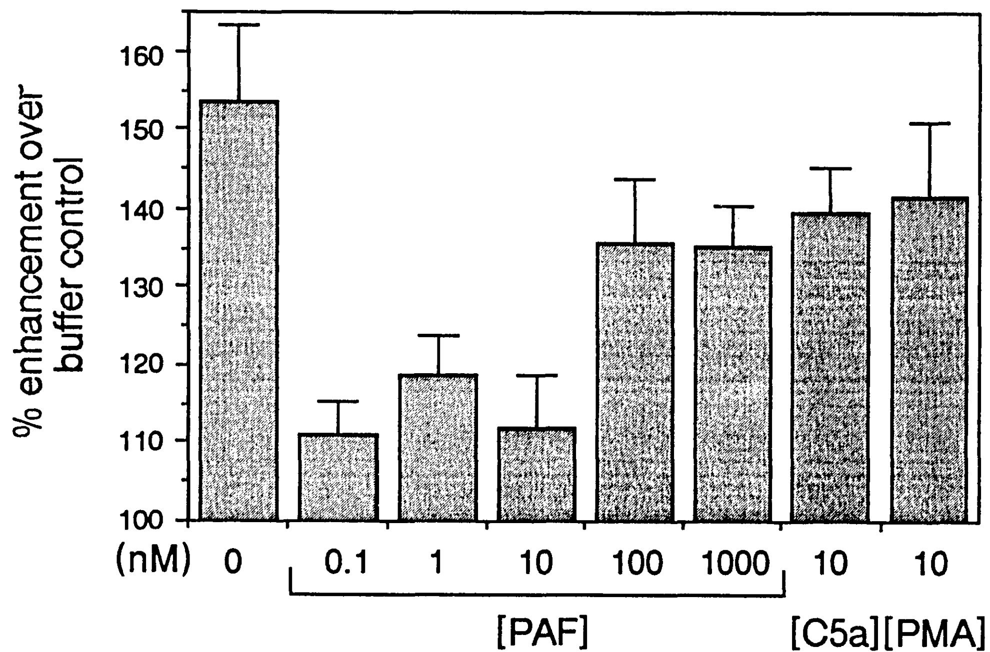


Figure 3.16A: Effect of PAF, C5a, and PMA on the enhancement of EPO release by fluribiprofen from guinea pig eosinophils. Cells ($10^7 ml^{-1}$) were preincubated with either 8 µM flurbiprofen or buffer 5 min before PAF (0.1 nM to 1 µM), C5a (10 nM) or PMA (10 nM) was added and reaction was allowed to proceed for 10 min at 37°C. The supernatant was assayed for EPO activity as described in the Methods. Each histogram shows the magnitude of enhancement induced by flurbiprofen in paired experiments where EPO release by cells without fluribprofen is set as 100%. Data are expressed as mean ± SEM of four observations obtained from three separate experiments.

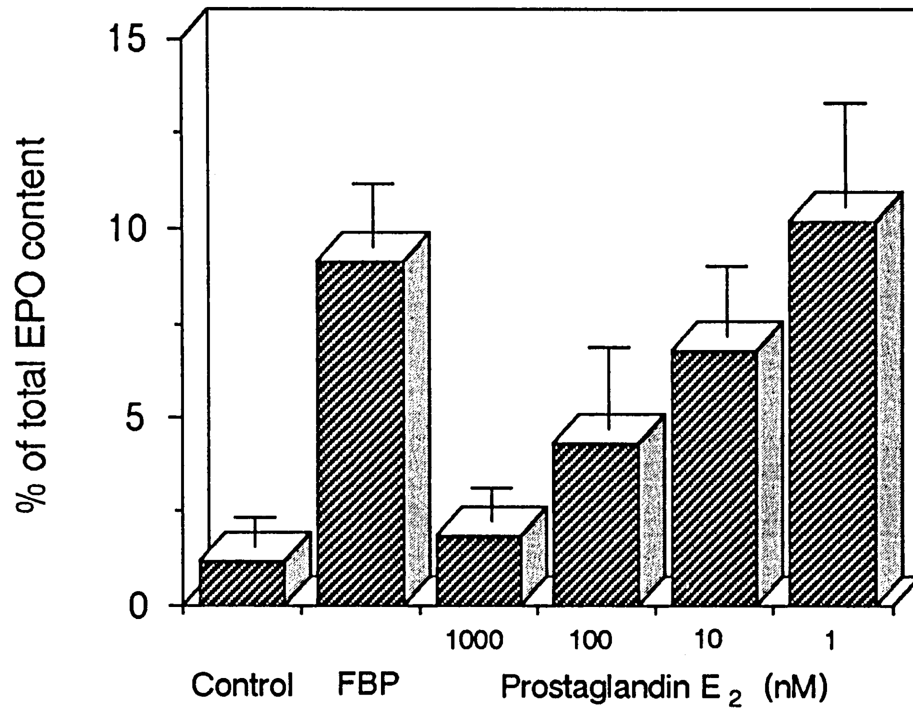


Figure 3.16B: Reversal of flurbiprofen (FBP)-induced EPO release by PGE_2
Eosinophils were preincubated with vehicle and PGE_2 (1 to 1000 nM) for five min prior to the addition of flurbiprofen (8 μM). EPO activity in the supernatant was measured as described in Methods. Data are expressed as percentage of total cellular EPO content. Each histogram represents 6 measurements obtained from 4 independent experiments.

Functional Response	[PAF]	IC_{50}	Maximal Inhibition	'n'
TXB_2 Release	100 nM	23 nM	61%	6
EPO Release	100 nM	19 nM	46%	5
$\cdot O_2^-$ Generation	3 uM	194 nM	63%	6
$[Ca^{2+}]_i$ Increase	100 nM	324 nM	>83%	9

Table 3.3: **Effect of PGE_2 on PAF-induced TXB_2 release, EPO secretion, $\cdot O_2^-$ generation, and increase in intracellular Ca^{2+}-concentration.**

caused an enhancement of both the spontaneous and PAF-stimulated EPO release (Fig. 3.16A). However, the magnitude of the flurbiprofen-promoted enhancement was reduced when cells were exposed to PAF (n=3). Consistent with these data, stimulation with other agonists such as C5a (10 nM) also showed no synergy between flurbiprofen and the stimulant. Furthermore, stimulation of eosinophils with PMA (10 nM) also reduced the flurbiprofen-induced enhancement of EPO secretion (n=3; Figure 3.16A, right). In contrast, treatment of eosinophils with flurbiprofen did not modify the $\cdot O_2^-$ generation induced by 1 μM PAF (7.0 ± 0.3 versus 7.4 ± 0.4 nmol cytochrome C reduced.min^{-1}.10^{6} cells^{-1}, n=4) and 1 μM PMA (25.5 ± 1.4 versus 25.7 ± 0.6 nmol cytochrome C reduced.min^{-1}.10^{6} cells^{-1}, n=4), respectively. However, the cell number in the incubation volume used for the measurement of $\cdot O_2^-$ was approximately 3 times less than that used for the EPO assay.

3.8 EFFECT OF PROSTAGLANDIN E_2

In a second set of experiments, the effects of PGE_2 on eosinophil functions was tested. Incubation of eosinophils with PGE_2 (1 nM to 1 μM) reversed the flurbiprofen-induced enhancement of spontaneous EPO release in a concentration-dependent manner with an IC_{50} of 51 nM (n=6) and an almost complete inhibition at 1 μM (Figure 3.16B). In addition, PGE_2 alone (1 nM to 10 μM) produced a concentration-dependent inhibition of EPO, TXB_2 and $\cdot O_2^-$ release promoted by PAF (Table 9.3). PGE_2 was approximately 3 and 8 times, respectively more potent at antagonising the production of TXB_2 from eosinophils than at inhibiting either EPO release or $\cdot O_2^-$ production. Taken together these results suggest that PAF-induced eosinophil activation is sensitive to inhibition by PGE_2. Given that inhibitors of Type IV cyclic nucleotide-dependent phosphodiesterase effectively attenuate eosinophil activation (Dent *et al.*, 1990) the action of PGE_2 may be related to its ability to stimulate adenylyl cyclase.

In contrast to PGE_2, TXA_2 had no direct or indirect effect on either spontaneous or stimulated eosinophil function. Cells challenged with the TXA_2 mimetic, U-46619, did not release superoxide anions at any concentration examined (1nM - 10 μM). However, U-46619 did potently contract guinea pig isolated trachea confirming that the drug was, indeed, active on a preparation known to express thromboxane receptors (Jones *et al.*, 1982). Experiments designed to determine if elaborated TXA_2 could augment its own release, were unsuccessful due to significant cross-reactivity of U-46619 (>70% at 10 μM) with the TXB_2 antiserum.

3.9 DISCUSSION

Increasing evidence suggests that the eosinophil leukocyte represents an effector cell capable of destroying not only parasites (Butterworth, 1977) but also contributing to the respiratory epithelium damage found in chronic asthma (Frigas & Gleich, 1986). Its cytotoxic effects have been demonstrated by numerous *in vitro* studies (for review see Spry, 1988). This eosinophil cytotoxicity might on the one hand have provided a phylogenetic advantage through its contribution to host defense mechanism against parasites. On the other hand, it seems that this advantage can, however, under certain circumstances turn into a disadvantage and provide the basis for a chronic asthmatic disorder.

As outlined in the Introduction, the eosinophil mediates this function through three different mechanisms which include the release of preformed toxic granular proteins as well as *de novo* generated reactive oxygen intermediates and bioactive lipid mediators. A number of different stimuli have been shown to induce at least one of these eosinophil functions, including soluble stimuli such as PMA, calcimycin, complement activation products or immune complexes, phagocytic stimuli such as zymosan-C3b, or large, non-phagocytosable stimuli such as sepharose-C3b or immune complex-coated agar layers (Ichikawa *et al.*, 1974; Ogawa *et al.*, 1981; Petreccia *et al.*, 1987; Owen *et al.*, 1988; Kroegel *et al.*, 1989a; Popper *et al.*, 1989).

The data presented in this chapter indicate that PAF stimulates the degranulation, superoxide anion release as well as the generation of prostanoids by both human and guinea pig eosinophils in a calcium-dependent and non-cytotoxic manner. Effective concentrations ranged from 100 pM to 10 μM PAF with a peak response seen at 100 nm and 1 μM PAF. In contrast, lyso-PAF, its precursor and metabolite, was inactive. Finally, the action of PAF on eosinophils is rapid, with a $t_{1/2}$ up to approximately 5 min for enzyme secretion and even 60 sec for prostanoid release.

The PAF-induced eosinophil effector functions were inhibited by WEB 2086, which had no effect on enzyme release induced by calcimycin, opsonized zymosan or PMA. These results indicate that WEB 2086 is a potent and specific antagonist for the PAF-induced degranulation of human eosinophils. We then went on to characterize the nature of this antagonism, shown in the next Chapter.

The stimulus specific nature of PAF-elicited degranulation of human eosinophils is further illustrated by the observation that pretreatment with PAF rendered the eosinophils unresponsive to subsequent exposure to this stimulus, though the cells remained fully responsive to opsonized zymosan or calcimycin. The attenuated capacity of eosinophils to release enzymes following a second addition of stimulus is probably due to desensitization which known to occur in neutrophils challenged with PAF and other stimuli (Smith *et al.*, 1983, Dent *et al.*, 1989a; Dent *et al.*, 1989b). In the neutrophils it has been reported that contact between cells and the chemotactic oligopeptide fMLP resulted in a reduced number of receptors for this ligand on the cell surface (Henson *et al.*, 1978). Consistent with this observation one might suggest that, following an initial exposure of eosinophils to PAF, the number of available membrane receptors is diminished, attenuating a subsequent response to the same stimulus. The decrease in receptor number may entail internalization and subsequent degradation of receptor-ligand complexes (see Chapter 4, page 119).

A characteristic feature observed for most of the responses of eosinophils to PAF, was a decrease in enzyme release above concentrations of 1 μM

PAF. This was seen for the release of granule enzymes as well as for prostanoid generation and release. The lack of LDH release by PAF indicates, but does not conclusively prove, that the decreased cell response was not due to a cytotoxic effect of high agonist concentrations. One potential explanation for this effect of PAF at higher concentrations is that simultaneous desensitization is occurring at the same rate as secretion and that this serves to limit granule extrusion. Henson has suggested that this mechanism may be responsible for the plateau in the secretory response in PAF-stimulated rabbit platelets (Henson *et al.*, 1978).

An interesting exception was the PAF-induced $\cdot O_2^-$ production in both human and guinea pig eosinophils. Although the effect was just as rapid as the other PAF-elicited eosinophil effector functions ($t_{1/2}$=109 sec), $\cdot O_2^-$ production failed to show a peak or plateau in response to PAF. In addition, $\cdot O_2^-$ occurred at considerably higher concentrations of PAF (>10 μM) with a maximal effective concentration of $3x10^{-5}$ M and an EC_{50} of 8.4 μM. These differences were specific for PAF, since other stimuli failed to show two different concentration-response relationships. Furthermore, in contrast to other eosinophil functions, PAF-induced $\cdot O_2^-$ was not dependent on the presence of extracellular Ca^{2+} ions. WEB 2086 however, inhibited $\cdot O_2^-$ production by PAF, but not that induced by PMA, in a dose-dependent manner (IC_{50} of 51.1 μM) again suggesting a receptor-mediated activation mechanism for the oxygen intermediate production.

The observation that two distinct forms of cellular response are elicited at different concentration ranges by the same stimulus strongly indicates two separate control mechanisms for eosinophil activation in response to PAF. One possible explanation is that two independent PAF-receptor subtypes exist on eosinophils. A second possibility may be that only one receptor type occurs on the cell surface but in two different affinity states. The existence of multiple PAF binding sites has also previously been put forward by others (Hwang & Lam, 1986; Hwang, 1988; Stewart

& Dustin, 1988; Murphy *et al.*, 1990). Additional evidence for this dual activation mechanisms by PAF in eosinophils will be presented in the following chapters.

PAF appears to represent a major natural stimulus for eosinophils. In recent years, for instance, it has been shown that PAF potently elicits directional locomotion of human eosinophils (Wardlaw *et al.*, 1986), induces leukotriene C_4 synthesis (Bruynzeel *et al.*, 1987b), enhances the cytotoxicity of normal human blood eosinophils for C3b- and IgG-coated schistosomula (MacDonald *et al.*, 1986), eosinophil adhesion to serum-coated plastic and that human eosinophils adhere to cultured human umbilical endothelial cells (Kimani *et al.*, 1988). The results presented in this Chapter extend the spectrum of biological activities of PAF on eosinophils to include eosinophil degranulation, the biosynthesis of lipid mediators and the generation of superoxide anions. Moreover, PAF appears to be the most potent of the physiological stimulus for human eosinophils whereas LTB_4 is considerably less active. It should here be emphasized here, however, that the latter lipid is, interestingly, more effective than PAF at stimulating peritoneal guinea pig eosinophils.

3.10 SUMMARY

The results presented in this chapter display the wide range of cellular responses exhibited by eosinophils challenged with PAF and other stimulants. As evident from the data shown above, PAF is a principal stimulus for human cells, whereas LTB_4 is a major physiologic stimuli for guinea pig eosinophils. Both cell types respond with granular enzyme release, biosynthesis of various prostanoids and production of $\cdot O_2^-$. Guinea pig eosinophils generate little or no LTC_4 while human eosinophils release substantial amounts. Furthermore, $9\alpha,11\beta$-PGF_2, a stable metabolite of PGD_2, was not detected in either human and guinea pig eosinophils indicating that eosinophils were not metabolizing PGD_2. Eosinophil effector functions appear to be under control of a negative feedback mechanism which involves PGE_2, possibly through a cAMP-dependent mechanism.

CHAPTER FOUR

4 CHARACTERIZATION OF PAF RECEPTORS

4.1 PAF RECEPTOR BINDING

In order to demonstrate whether PAF elicits its functional responses via a membrane receptor, binding studies were carried out using both intact cells and eosinophil membrane preparations.

4.1.1 Intact Eosinophils

4.1.1.1 Binding of [^{3}H]PAF

Preliminary experiments with [^{3}H]PAF as a radioligand on purified eosinophils showed inconsistent binding as well as a large total binding capacity of eosinophils, and a high level of non-specific labelling. These findings were consistent with observations on neutrophils and platelets (Valone *et al.*, 1982; Valone & Goetzl, 1983; O'Flaherty *et al.*, 1986), possibly reflecting the lipophilic nature of the ligand, which readily becomes metabolized and/or incorporated into the cell membrane (O'Flaherty *et al.*, 1986; O'Flaherty *et al.*, 1989) (see below).

4.1.1.2 Binding of [^{3}H]WEB 2086

In order to overcome these difficulties a tritium-labelled (2-position of the chlorophenyl ring) PAF antagonist, [^{3}H]WEB 2086, which is less lipophilic than PAF was employed (Casals-Stenzel *et al.*, 1987). Eosinophils (5 x 10^6) in Hepes buffer were incubated with 10 nM of the radioligand (specific activity of 15 Ci/mmol) were incubated at 25°C for 2, 10, 20, 30, 60, and 90 min. The percentage binding of [^{3}H]WEB 2086 was 7.9%, 16.3%, 25.5%, 30.2%, 32.5%, and 33.1%, respectively. The temperature-dependence of WEB 2086 binding to guinea pig eosinophils showed a

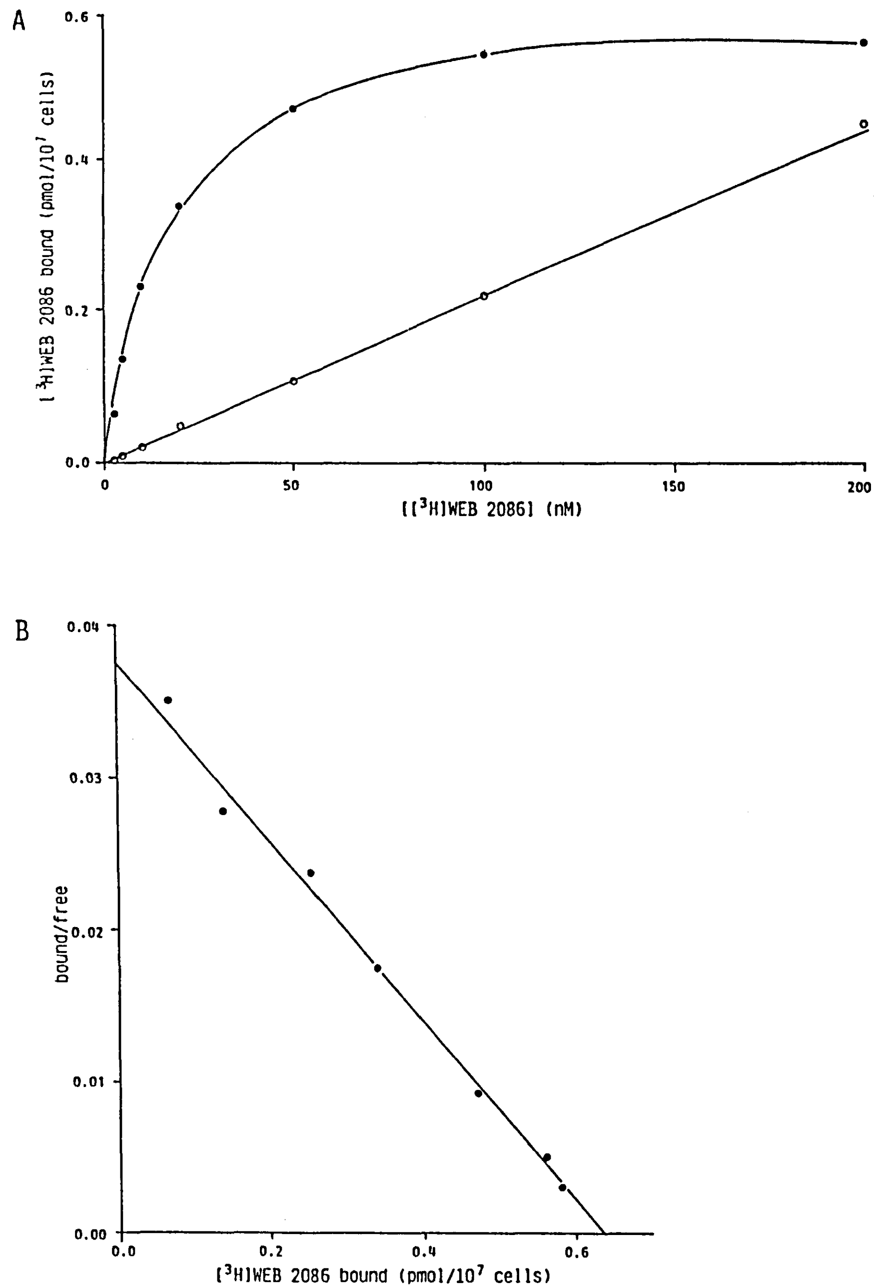


Figure 4.1: Binding of [^{3}H]WEB 2086 to guinea pig eosinophils. Specific binding (●) is obtained from the difference between total and non-specific binding (○) determined in the presence of 1 μM PAF and measured at 25°C for 90 min (**Panel A**). Inset (**Panel B**): Scatchard analysis of specific [^{3}H]WEB 2086 binding calculated from the same saturation curve. Each point represents the mean of triplicate determinations obtained from a single representative experiment.

maximum at 25°C. Thus, subsequent studies of the binding of [^{3}H]WEB 2086 were carried out for 90 min at 25°C.

4.1.1.3 Equilibrium analysis of [^{3}H]WEB 2086 binding.

In order to determine the affinity of WEB 2086 and the binding capacity (B_{max}) of eosinophils, cells were added to tubes containing increasing concentrations of [^{3}H]WEB 2086 (1 - 200 nM) in the presence and absence of a saturating concentration of unlabelled PAF (1 μM). A representative saturation isotherm is depicted in Fig. 4.1. Nonspecific binding increased linearly as a function of the radioligand concentration. In the absence of unlabelled PAF the isotherm conformed to a simple hyperbola. Scatchard analysis of the binding data revealed a linear function, indicating a homogeneous population of non-interacting binding sites (Fig. 4.1b, inset). The mean dissociation constant (K_D) of three independent eosinophil preparations was 16.1 ± 0.6 nM. The receptor density calculated from the maximal binding capacity, B_{max} (578 ± 38 fmol/10^7 eosinophils, n=3), and assuming a 1:1 stoichiometry was 34800 recognition sites per eosinophil. It should be noted that it was not possible to characterize the binding at higher concentrations of [^{3}H]WEB 2086 as the amount of labelled antagonist available was limited. To assess the specificity of binding of [^{3}H]WEB 2086 by eosinophils, the capacity of different non-labelled substances to displace the labelled antagonist was examined. Increasing quantities of C_{16}-PAF, WEB 2086 and a ginkgolide with PAF receptor antagonist activity, BN 52021, reduced the binding in a concentration-dependent manner with an inhibition constants of 13.4 nM (8.4-21.5), 0.23 nM (0.12-0.45), and 420 nM (283-630), respectively. In contrast, lyso-PAF up to a concentration of 10 μM did not displace the binding of [^{3}H]WEB 2086 (≈ 9%). Thus, of all the compounds studied, WEB 2086 appear to be the most potent antagonist. The binding parameters of [^{3}H]WEB 2086 for guinea pig eosinophils and human eosinophils are summarized in Table 4.1 (page 125).

4.1.2 Eosinophil Membranes.

In order to further characterize the proposed PAF binding sites, a radioligand binding assay was performed using [^{3}H]PAF. Since intact eosinophils (see Chapter 4.3, page 124) and other cells rapidly metabolize and incorporate PAF (Valone *et al.*, 1982; Valone & Goetzl, 1983; O'Flaherty *et al.*, 1986), membrane preparations from purified eosinophils were used. Binding was assayed at 22°C over a concentration range of 1 nM to 20 μM. Specific binding was > 78% of total binding at any concentration of [^{3}H]PAF tested and was a saturable process. As is clearly evident from Fig. 4.2, the Scatchard plot obtained with [^{3}H]PAF was curvilinear. LIGAND analysis of the binding data shown in the figure indicates the presence of two binding sites: a higher affinity site with an apparent K_d value of 0.33 ± 0.1 nM (n=3), and a lower affinity site with a K_d value of 11.5 nM ± 4.1 nM (n=3). Approximately 6% of the total binding sites detected (0.013 fmol/ng protein), were of the higher affinity (B_{max} 736 fmol/mg protein), and 94% were of the lower affinity (B_{max} 12.2 pmol/mg protein).

4.2 PHARMACOLOGIC CHARACTERIZATION

In order to further characterize the PAF receptor a number of pharmacological studies using WEB 2086 were carried out. Eosinophils (5 x 10^6) preincubated with vehicle or WEB 2086 at 30 nM, 100 nM, and 300 nM and challenged with increasing concentrations of PAF (10 fM - 10 μM) for 15 min and the secretion of EPO into the supernatant was measured. As shown in Figure 4.3, WEB 2086 gave rightward shifts in the PAF concentration-response curve. Schild regression of the data according to the equation 5 (for details see Chapter 2.22, page 80) produced a slope different from unity (Figure.4.3, inset). As shown in Figure 4.3, panel B, WEB 2086 at the same concentration also produced a shift to the right in the PAF-induced increase in intracellular free Ca^{2+} concentration (see also Chapter VII, page 149). Schild analysis of these data (panel B, inset) yielded a slope less than unity.

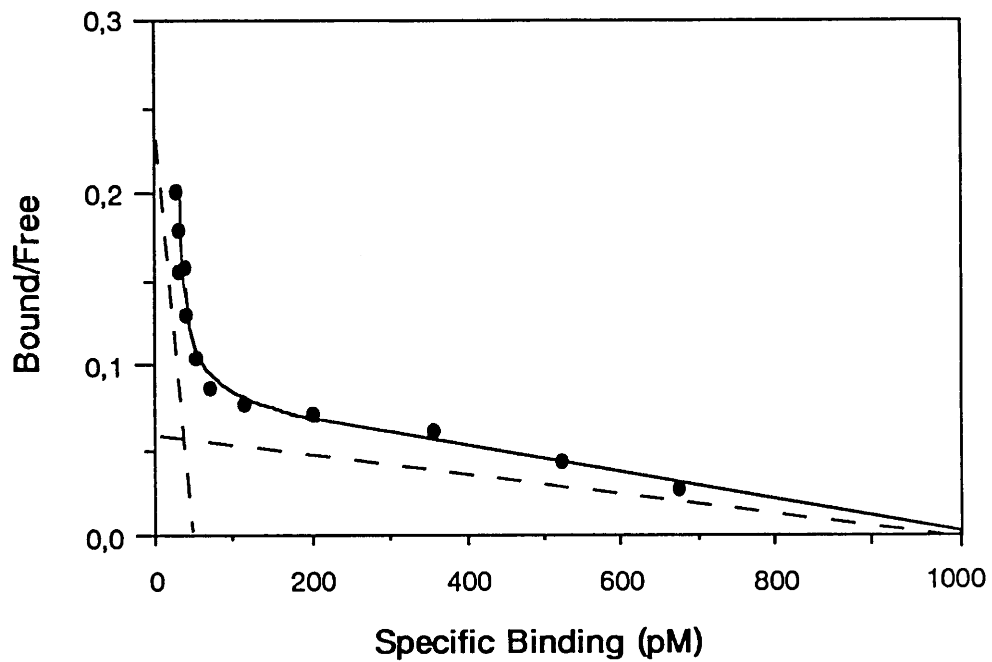


Figure 4.2: Scatchard plot for specific binding of [^{3}H]PAF to purified eosinophil membranes. Binding was determined at PAF concentrations between 1 nM and 20 μM. Data were plotted as described by Scatchard (1949) and analyzed with a modified LIGAND computer program. The two components of the curvilinear plots were reconstructed with data from LIGAND analysis and are shown as dotted lines under the binding plots.

Analysis of the PAF-induced (100 nM - 100 μM) O_2^- production by eosinophils (3 x 10^6) preincubated with WEB (1 μM, 3 μM, and 10 μM) also produced rightward shifts in the concentration-response curves (Figure 4.4). Again, the slope of the regression line was different from unity. Generally, the data suggest an inequilibrium between PAF, WEB 2086, and the putative receptor on eosinophils and allow only a rough estimate of the pA_2 values (Figures 3.4 and 4.4). The reason for the slope not being equal to unity may either indicate that (1) the antagonism is not competitive, (2) a drug-deposition mechanism or other nonequilibrium steady state obscures the competitive nature of the antagonism, (3) a heterogeneous receptor population subserving the same response, or (4) more than one drug property is expressed in the concentration used. Given a competitive antagonism, the slopes less than unity observed at lower agonist and antagonist concentrations may indicate that PAF is removed upon binding to eosinophils(see below), whereas at higher agonist concentrations the Schild-regression slope greater than unity may suggest a saturable removal mechanism.

4.3 PAF CATABOLISM

In order to establish the fate of the PAF used to challenge the guinea pig eosinophils, the kinetics of PAF catabolism in eosinophils were examined. Intact eosinophils (5 x 10^6; 96% pure) were incubated at 37°C in the presence of [^{3}H]PAF (specific activity: 60 mCi; final concentration 1 μM) for 15, 30, 60 and 120 min. The reaction was terminated by the addition of methanol/chloroform (2:1, v/v), lipids were extracted by the method of Bligh and Dyer (1959) and separated by TLC. As shown in Fig. 4.5, exogenous PAF (peak 2) is rapidly metabolized by intact eosinophils in a time-dependent manner. The first catabolite to be detected, 15 min post-challenge, was lyso-PAF (peak 1) comprising 17.8% of the total amount of exogenous PAF. At this time only 5.2% had been converted into 1-alkyl-2-acetyl-GPC. The amount of 1-alkyl-2-acetyl-GPC increases at a constant rate to 12.0%, 21.7%, and 39.2% after 30 min,

Binding Parameter	Guinea pig eosinophils	Human eosinophils
Dissociation constant (K_D)	16.1 nM	18.5 nM
Receptor number per cell	35,000	64,000
Inhibition constant (K_I)		
----PAF	0.23 nM	0.5 nM
----Lyso-PAF	>10,000 nM	>10,000 nM
----WEB 2086	13.4 nM	17.6 nM
-----BN 52021	420 nM	320 nM

Table 4.1: **Dissociation constants (K_D) and equilibrium constants (K_i) of [^{3}H]WEB 2086 binding to intact guinea pig and human eosinophils.** Data shown represent the mean of three independent experiments.

Receptor type	K_d	B_{max}	n
PAF-RI	0.33±0.1nM	736 fmol/mg	3
PAF-RII	11.5±4.1nM	12.2 pmol/mg	3

Table 4.2: **Binding parameters of the high-(RI)- and the low-(RII)-affinity PAF receptor on cell membranes obtained from purified guinea pig peritoneal eosinophils.** Data shown represent mean of three independent experiments.

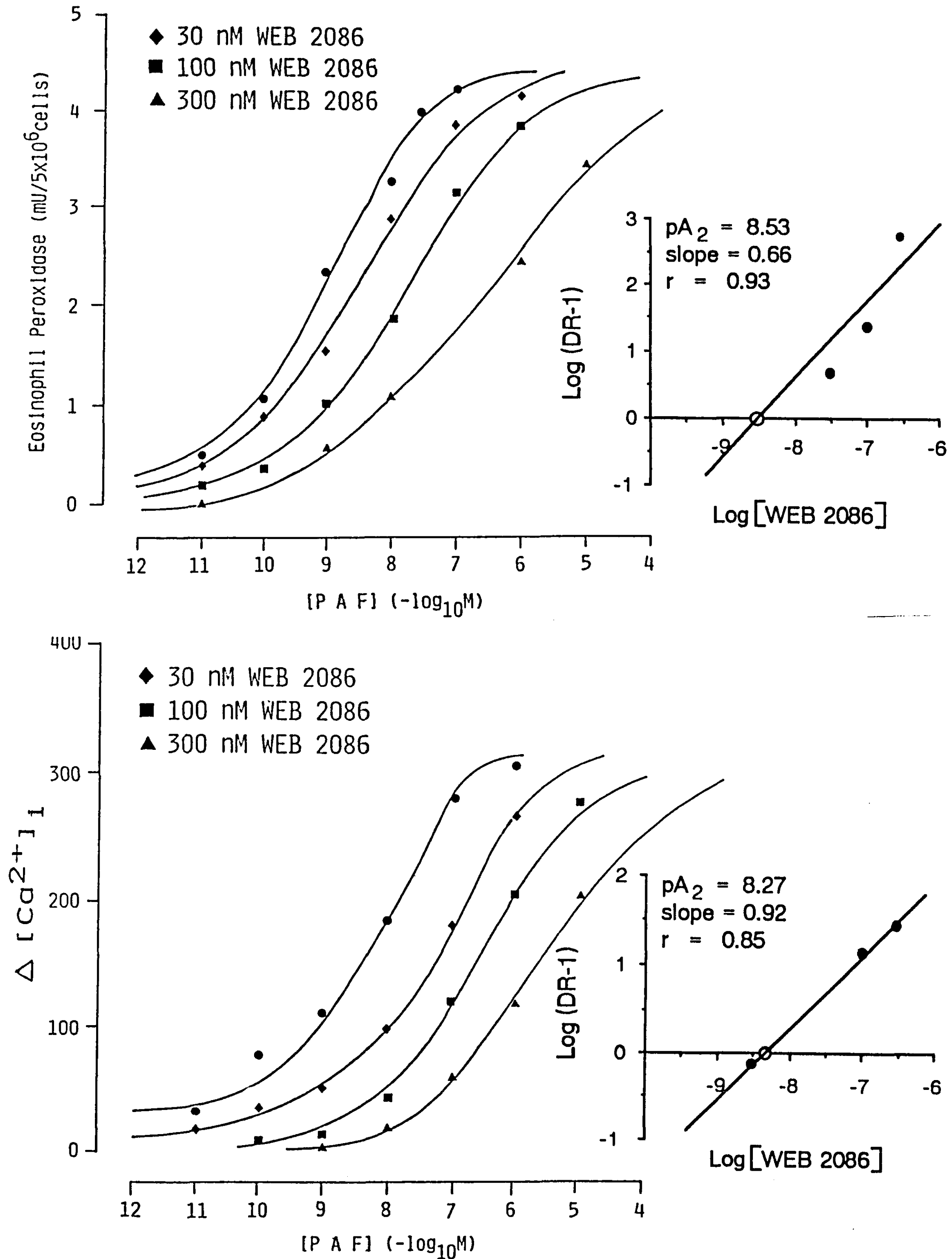


Figure 4.3: Effect of PAF antagonist WEB 2086 on PAF-induced responses. EPO secretion (Panel A) and rise in $[Ca^{2+}]_i$ (Panel B). Cells were preincubated with vehicle (●), 30 nM WEB 2086 (◆), 100 nM WEB 2086 (■), and 300 nM WEB 2086 (▲) prior to the addition of increasing concentrations of PAF. Mean values of three experiments are shown. Inset: Schild regression of the antagonism of WEB 2086 on eosinophils. Data are the mean of three determinations.

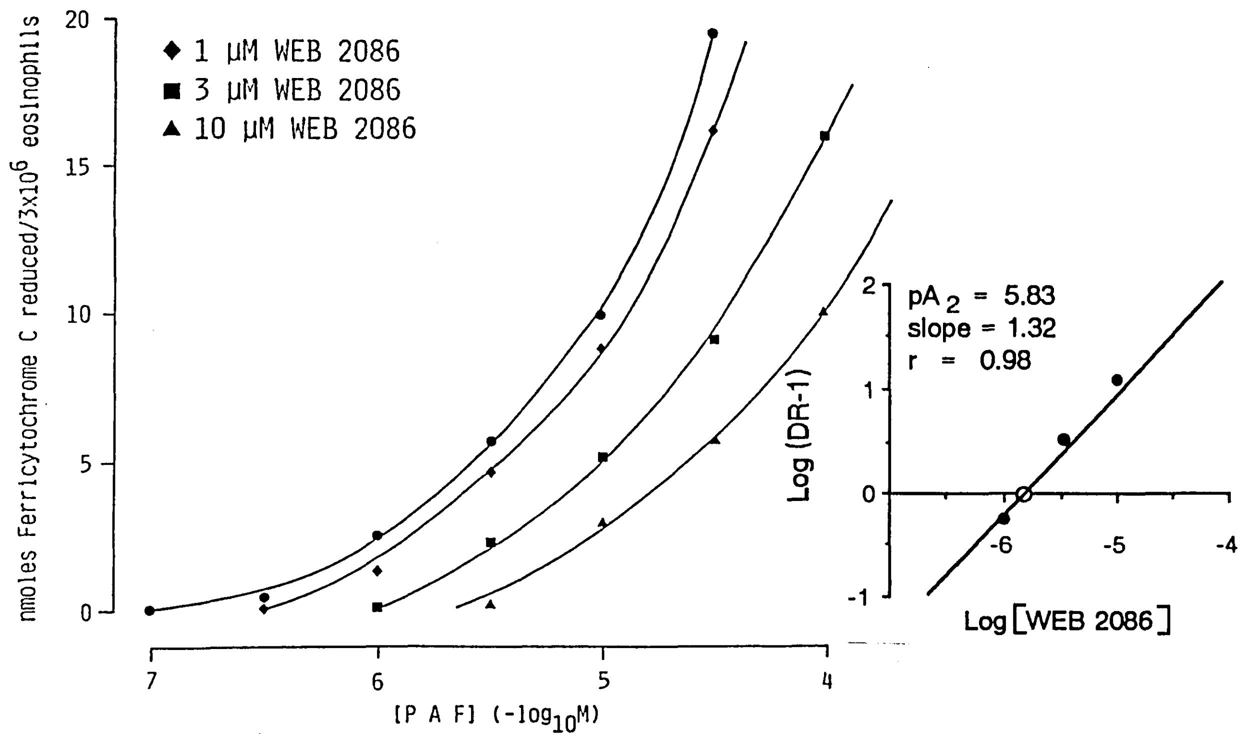


Figure 4.4: Effect of PAF antagonist WEB 2086 on PAF-induced superoxide anion production. Cells were preincubated with vehicle (●), 1 μM WEB 2086 (◆), 3 μM WEB 2086 (■), and 10 μM WEB 2086 (▲) before adding increasing concentrations of PAF. Data represent mean values of three experiments. Inset: Schild analysis calculated from the same data. Each data point represents the mean of three measurements.

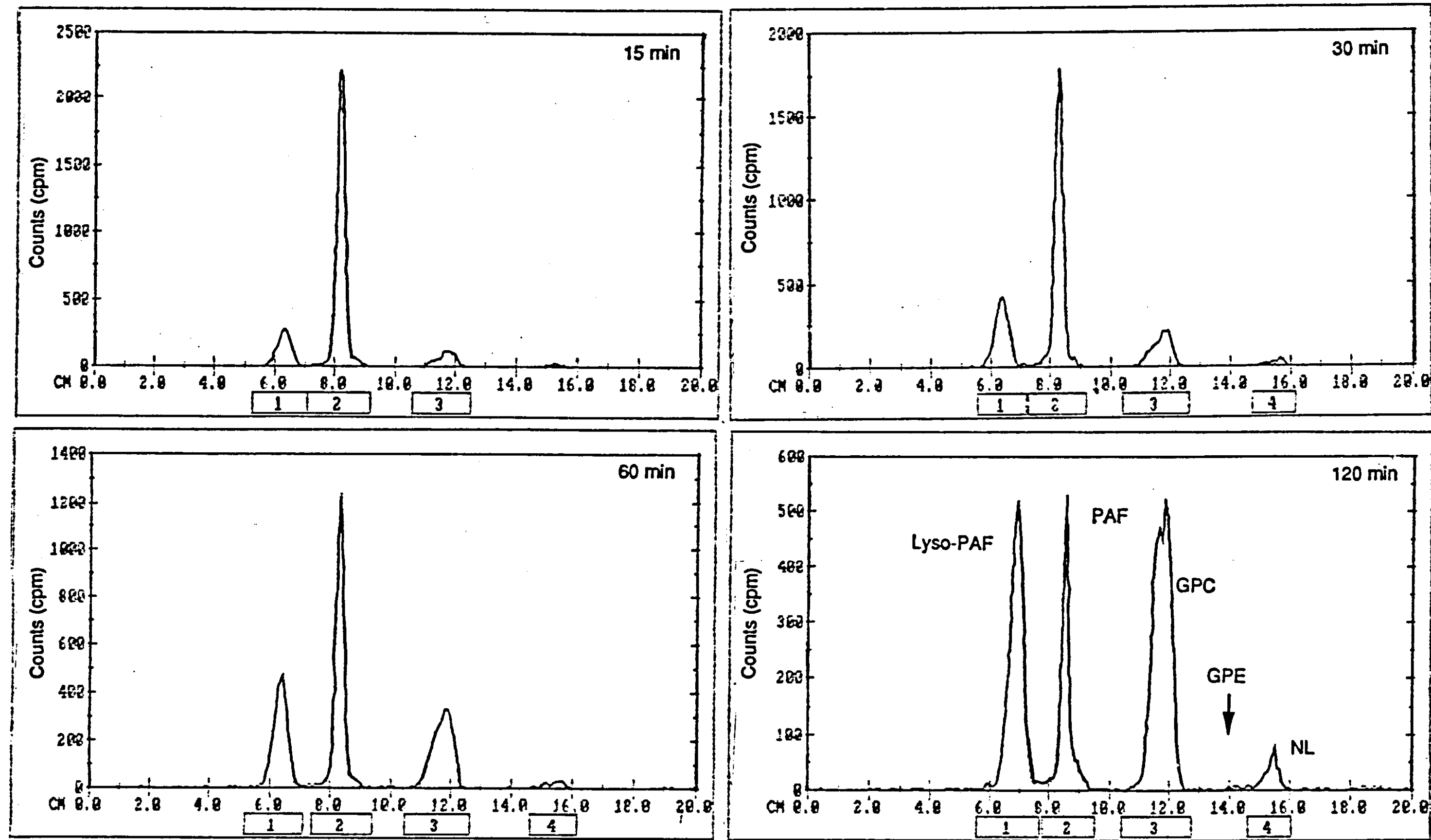


Figure 4.5: Kinetics of the catabolism of 1-[3H]-alkyl-2-acetyl-GPC (PAF) by intact eosinophils. Purified cells (5x106) were incubated at 37°C for 15, 30, 60 and 120 min in the presence of 1 μM [3H]PAF (60 mCi). Lipids were extracted from the whole sample (cell + supernatant) and separated by TLC as described in the Methods section (2.21, page 80). Standards (lyso-PAF, PAF, GPC, and GPE) were identified by I2 vapors. Traces shown were obtained by scanning the radioactivity on the TLC plates (20 min) using a Bioscan system. The results are representative of three separate experiments.

60 min and 120 min, respectively, while during the same period the percentage of lyso-PAF remained constant at approximately 30%. After 30 minutes post-PAF-challenge a small proportion (3.9%) of neutral lipids could be detected which increased at a constant rate to 5.6% at 120 min. Over a period of 120 min the concentration of exogenous PAF decreased steadily from 100% to 20.8%. The concentration of lyso-PAF rose during the first 30 minutes and then remained almost constant. It is interesting to note that the total radioactivity recovered decreased with time indicating a further catabolism of the molecule into both lipophilic and water-soluble units.

In a separate experiment, PAF catabolism in purified eosinophil membranes was investigated. Incubation with [^{3}H]PAF (1 μM) resulted in a small but consistent conversion of PAF into lyso-PAF and 1-alkyl-2-acetyl-GPC. However, the portion of metabolized PAF was less than in whole cells and did not exceed 20% of the added amount of exogenous PAF.

4.4 DISCUSSION

Receptors for PAF have been found in smooth muscle containing tissues, platelets, mononuclear cells and neutrophils from various species (Table 4.3). As has been demonstrated in the last Chapter, PAF can stimulate eosinophils at subnanomolar concentrations through a stereospecific and readily desensitizable mechanism. These findings suggest that PAF binds to specific receptors on eosinophils initiating a range of cellular effector functions. Confirmation of the presence of PAF receptors on both human and guinea pig eosinophils comes from direct studies with radiolabelled PAF or the PAF receptor antagonist WEB 2086 described in this Chapter. The receptors appear to be membrane associated and the receptor binding was rapid, reversible and saturable. Scatchard analysis of membrane-binding data is consistent with a two-receptor model. An estimated 94% of the receptors were of low ($K_d \approx 11.5$ nM) and 6% of high- ($K_d \approx 0.3$ nM) affinity.

Cell type	K_d (nM)	B_{max}	References
Intact cells			
Platelets			
Human	37	1400[a]	Hwang et al., 1983
	0.53	242	Kloprogge & Ackerman, 1984
	0.15	438	Chesney et al., 1984
	1.58	1,983	Inarrea et al., 1984
	0.26	245	Tuffin et al., 1985b
	9.2	1600	Tuffin et al., 1985b
	0.02	240	Ukena et al., 1988
Rabbit			
	0.9	19,386	Chesney et al., 1984
	0.5	400	Homma et al., 1987
Neutrophils			
Human			
	0.11	520,000	Valone & Goetzl, 1983
	0.2	1,100	O'Flaherty et al., 1989
	200	200,000	O'Flaherty et al., 1989
Mononuclear cells			
Human			
	5.7	11,100	Ng & Wong, 1988
Cell Membranes			
Platelets			
Human			
	0.25	300[b]	Hwang et al., 1983
Dog			
	0.23	911	Tarahoui et al., 1988
Human Lung			
	0.49	140	Hwang et al., 1985

a, binding sites per cell
b, binding sites per fmol/mg

Table 4.3: Characteristics of PAF binding sites on different cells and tissues detected by $[^3H]$PAF.

There is now an increasing body of evidence to support the concept of distinct PAF receptor subtypes. In 1986, two independent studies proposed the existence of two distinct PAF receptor subtypes in platelets and leukocytes (Lambrecht & Parnham, 1986; Hwang & Lam, 1986). Later, Hwang (1988) found that the relative potencies of PAF agonists and PAF antagonists on human platelets differ from those on neutrophils, and that the cellular responses to PAF in these cells could be differentiated by pertussis toxin, cholera toxin and the presence of monovalent cations Na^+ and Li^+. The expression of multiple receptor subtypes in the neutrophil has been further postulated based on the presence of pertussis toxin-sensitive and pertussis toxin-insensitive PAF-dependent activity in this cell type (Naccache *et al.*, 1985). A recent binding study identified a high ($K_d \approx 0.2$ nM) and a low ($K_d \approx 200$ nM) affinity receptor on neutrophils (O'Flaherty *et al.*, 1989). Even more recently, microinjection of size-fractionated mRNA from promyelocytic leukemia cell line HL60 into the Xenopus oocyte showed that PAF receptor activity was broadly distributed in several mRNA fractions ranging from 3.5 to 6 kb (Murphy *et al.*, 1990). This finding suggests that leukocytes may encode for distinct multiple PAF receptor subtypes.

The fate of PAF bound to eosinophils has not so far not been studied. In other cells, several studies have reported that exogenous PAF is rapidly metabolized by the cell (O'Flaherty *et al.*, 1986; O'Flaherty *et al.*, 1989). According to the experimental evidence presented in this Chapter, the same also appears to be true for eosinophils. Maximal PAF metabolism appeared to take place mainly within intact eosinophils since the rate of PAF catabolism in purified membrane subfractions was only half of that observed with the whole cell. This indicates that eosinophils metabolize a significant amount of PAF in the cytosol. As in neutrophils (O'Flaherty *et al.*, 1986), PAF may be sequentially attacked by cytosolic and membrane-bound enzymes as it sits on the inner leaf of the plasma membrane. In any case, the rapid catabolism of this highly hydrophobic ether lipid emphasizes that binding studies using PAF as a ligand have to be conducted and interpreted with great care. In addition, the

nature of the molecule may also explain the discrepancies in binding characteristics described in the literature (see Table 4.3).

4.5 SUMMARY

The conclusions drawn from the results presented in this chapter are five-fold. First, using both PAF and the PAF receptor antagonist WEB 2086, the data demonstrates for the first time, that eosinophils express PAF receptors on their cell surface. Of these ligands [^{3}H]WEB 2086 has an approximately 10-fold lower affinity for the receptor than [^{3}H]PAF. Secondly, the results confirm the initial proposal (see also Chapter III) that eosinophils may possess two distinct PAF binding sites. This indicates the presence of either a high and low affinity status of the same receptor or the expression of two distinct receptors, one with high affinity and the other with low affinity binding characteristics. During further discussions, the first receptor will be referred to as PAF-RI and the latter as PAF-RII. Thirdly, the data suggest that the site of WEB 2086 binding is, indeed, at the PAF receptor. Fourth, affinity of WEB 2086 for functional PAF receptors and actual [^{3}H]WEB 2086 binding is similar. Finally, PAF is rapidly catabolised by intact eosinophils and to a lesser extent by membranes into lyso-PAF and 1-alkyl-2-acetyl-GPC.

CHAPTER FIVE

5. IDENTIFICATION AND SIGNIFICANCE OF A GTP-BINDING PROTEIN

In a number of cells agonist-activated receptors transduce their signal through a guanine nucleotide (GTP)-binding protein or G-protein (Nakamura & Ui, 1985; Smith et al., 1986; Gilman, 1987). In order to assess the possible role of G-proteins in PAF-induced receptor activation the effects of pertussis toxin (PTX) on several eosinophil cell functions were examined. PTX is known to ADP-ribosylate the α subunit of the inhibitory G-protein (G_i) and other related G-proteins, inhibiting their receptor-mediated activation (Katada & Ui, 1982; Bokoch *et al.*, 1983).

5.1 EFFECTS OF PERTUSSIS TOXIN

Eosinophils from both guinea pig and human were pretreated with vehicle or PTX (0.5 µg/ml) for 60 min at 37°C in a shaking waterbath and challenged with PAF (1 µM) as described earlier (see Chapter 3.1, page 83). Due to the restricted numbers of human cells available, it was not possible to repeat all of these experiments in human eosinophils. However, PTX inhibited the release of LTC_4 and TXB_2 in a comparable fashion. This suggests that similar G-protein dependent mechanisms may be operating in the human cells.

5.1.1 EPO and TXB_2 release.

Supernatants from PAF and PMA-stimulated eosinophils (3 - 5 x 10^6 cells. sample^{-1}) were analysed for EPO and TXB_2 activity as previously described. As shown in Fig. 5.1, PTX pretreatment inhibited both net EPO release (69.1 ± 5.6% inhibition, n=3) and TXB_2 synthesis (55.2 ± 7.6% inhibition, n=8). In contrast, PTX-

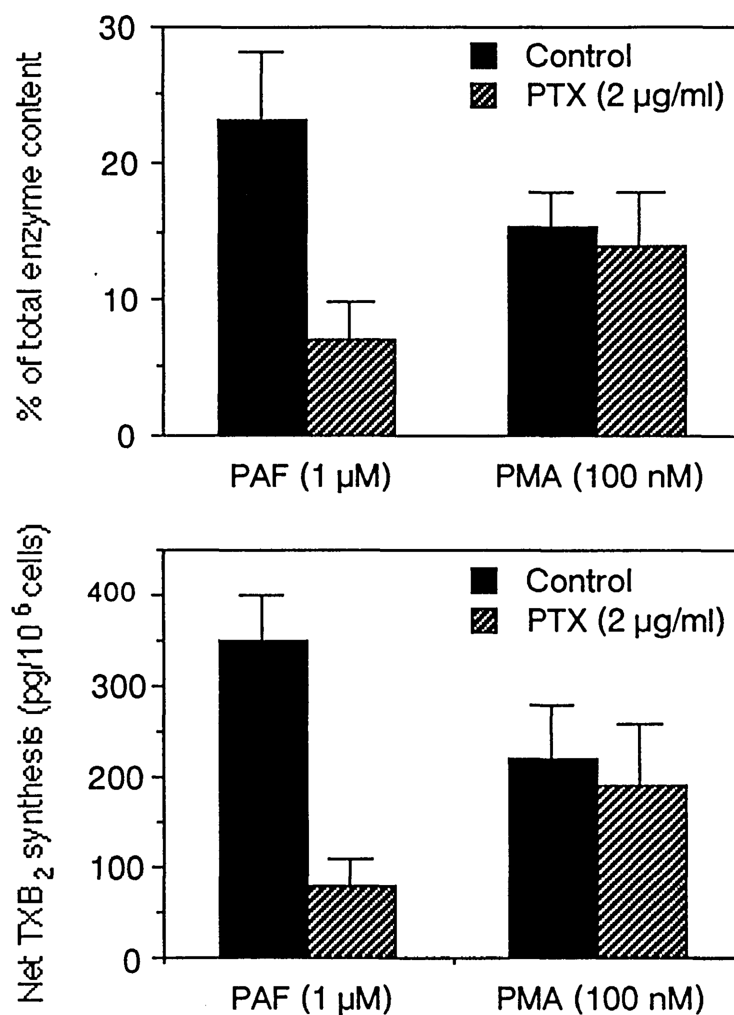


Figure 5.1: **Effect of pertussis toxin on PAF and PMA-induced eosinophil peroxidase (panel A) and thromboxane B_2 release (panel B).** Eosinophils were suspended in PAGCM buffer, incubated with vehicle or 0.5 μg/ml pertussis toxin for 60 min at 37°C and stimulated with 1 μM PAF and 100 nM PMA in a total volume of 500 and 100 μl, respectively. The resultant supernatant was assayed for EPO and TXB_2 activity as described in Chapter 2. Data points shown represent mean ± SEM of 3 (EPO) or 8 (TXB2) experiments.

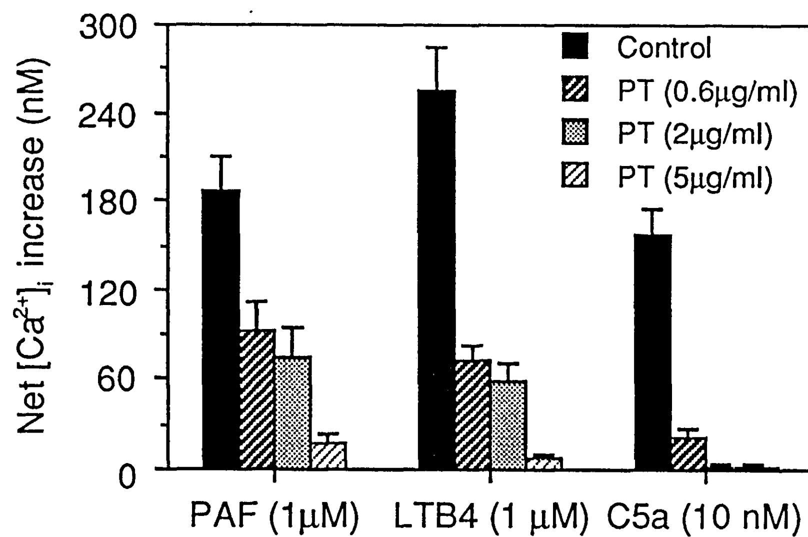
pretreatment had no significant effect on eosinophils stimulated with PMA. Finally, as demonstrated above, PAF at 30 μM did not induce TXB_2 generation. The viability of the cells was not affected by PTX-treatment (97.3 vs 95.6%, n=7) as assessed by trypan blue exclusion.

5.1.2 Increase in $[Ca^{2+}]_i$

As an additional parameter, the effect of PTX on the rise in $[Ca^{2+}]_i$ was examined. Figure 5.2 shows the results obtained from eosinophils preincubated with different concentrations of PTX (0.6 μg/ml, 2 μg/ml, and 5 μg/ml) and stimulated with increasing concentrations of PAF (100 pM - 1 μM). As clearly evident, PTX treatment inhibited the rise in $[Ca^{2+}]_i$ in a dose-dependent fashion at all of the concentrations studied. At 1 pM PAF, only 0.6 μg/ml PTX was required to abolish the cellular response. In additional studies, the effect of PTX on LTB_4 and C5a-induced eosinophil activation was studied. PTX at concentrations between 0.6 μg/ml and 5 μg/ml also inhibited the rise in $[Ca^{2+}]_i$ in eosinophils challenged with 1 μM LTB_4 and 10 nM C5a. At the concentrations examined, the inhibitory effect of PTX on LTB_4 (1 μM) and C5a (10 nM) promoted increase in $[Ca^{2+}]_i$ appeared to be slightly more pronounced than that by PAF (1 μM). These data suggest that receptor activation of eosinophils by PAF may be linked to a PTX-sensitive G-protein.

5.1.3 Superoxide anion generation

Generation and release of $\cdot O_2^-$ was studied as described under Chapter 3.5 using eosinophils pretreated with vehicle or PTX at 0.5 μg/ml (Figure 5.3) or 2 μg/ml. In contrast to the results obtained in 5.1.2, PTX did not not significantly affect PAF-induced production of $\cdot O_2^-$ by eosinophils ($p > 0.05$; Mann-Whitney U-test). These results suggest that, unlike PAF-induced EPO release and TXB_2 release $\cdot O_2^-$ generation is independent of a PTX-sensitive G-protein. In addition, the effect of PMA



<u>Figure 5.2:</u> **Effect of pertussis toxin on PAF-, LTB_4- and C5a-induced rise in $[Ca^{2+}]_i$ in eosinophils.** Eosinophils were pretreated with 0.6 μg/ml, 2 μg/ml and 5 μg/ml PT for 60 min as described in the legend to Figure 5.1. During the last 30 min of incubation fura-2AM was added to give a final concentration of 2.5 μM. $[Ca^{2+}]i$ was measured spectrofluorometrically under constant mixing at 37°C using monochromatic excitation wave length and emission detector set to 339 nm and 500 nm, respectively (see 2.17.1, page 74). Values given are mean ± SEM for n = 3.

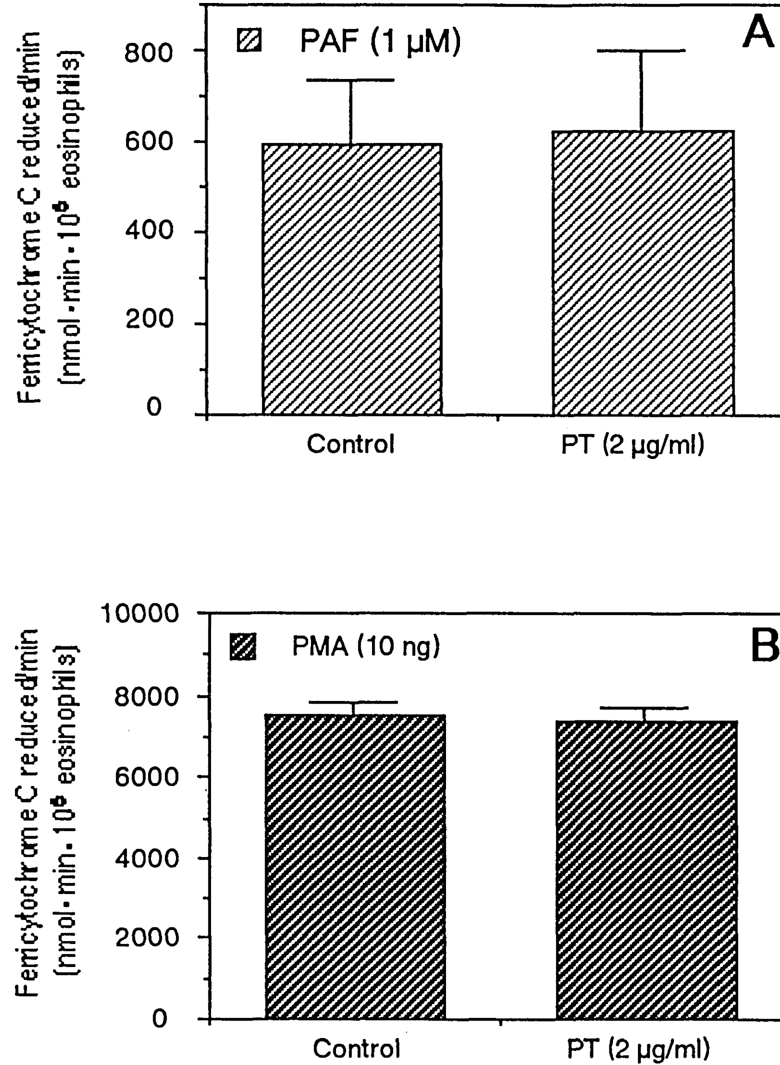


Figure 5.3: Effect of pertussis toxin on superoxide anion generation (nmol/min/10^6 cells) induced by PAF (Panel A) and PMA (Panel B). Eosinophils treated with vehicle or 0.5 µg/ml PTX were stimulated with PAF (30 µM) or PMA (100 nM) and $\cdot O_2^-$ generation was measured as the superoxide dismutase (30 µg/ml) inhibitable reduction of ferricytochrome C monitored spectrophotometrically at a wavelength of 550 nm. Values shown represent mean ± SEM from nine observations obtained from four separate experiments.

was not significantly altered by the PTX treatment suggesting that cell viability or other aspects of cell activation were not being affected by PTX.

5.2 CHARACTERIZATION OF A 41 KDa MEMBRANE PROTEIN

In order to verify that preincubation of eosinophils with PTX induced ADP-ribosylation of the α-subunit of a GTP binding regulatory protein in the cell membrane, preparations of membranes from untreated and PTX (2 μg/ml)-treated eosinophils were subjected to a second ADP-ribosylation in the presence of PTX (1 mg/ml) and [^{32}P]NAD for 60 min at 30°C. Membrane proteins were resolved by SDS-PAGE (3% stacking gel; 10% running gel) and subjected to autoradiography for 6h to 12 d at -80°C. The autoradiographs (Figure 5.4) indicate that both whole cell extracts of the human cells (lanes 1 and 2) and membrane preparations of guinea pig eosinophils (lanes 3 and 4) contained a substantial amount of a 41 kDa protein which was ADP-ribosylated in the presence of activated PTX (lanes 1 and 3). In contrast, pretreatment of the same cells or membranes with 2 μg/ml PTX for 60 min at 37°C, prevented the labelling of the 41 kDa protein with ^{32}P (lanes 2 and 4). These results further support the hypothesis that a GTP-binding protein may be involved in PAF-induced signal transduction in eosinophils.

5.3 DISCUSSION

Receptor proteins in the plasma membrane span the phospholipid bilayer and have outwardly facing domains responsible for agonist binding and inwardly facing domains that interact with other proteins or receptor subunits on the inner leaflet of the membrane bilayer. Conformational changes within the receptor protein initiated by binding of specific agonists are thought to be responsible for information transfer from the outer to the inner domain. In a number of cell systems a group of structurally and functionally related GTP-binding proteins are thought to be involved in receptor coupling (Smith *et al.*, 1986). Receptor-mediated effects on adenylate cyclase activity,

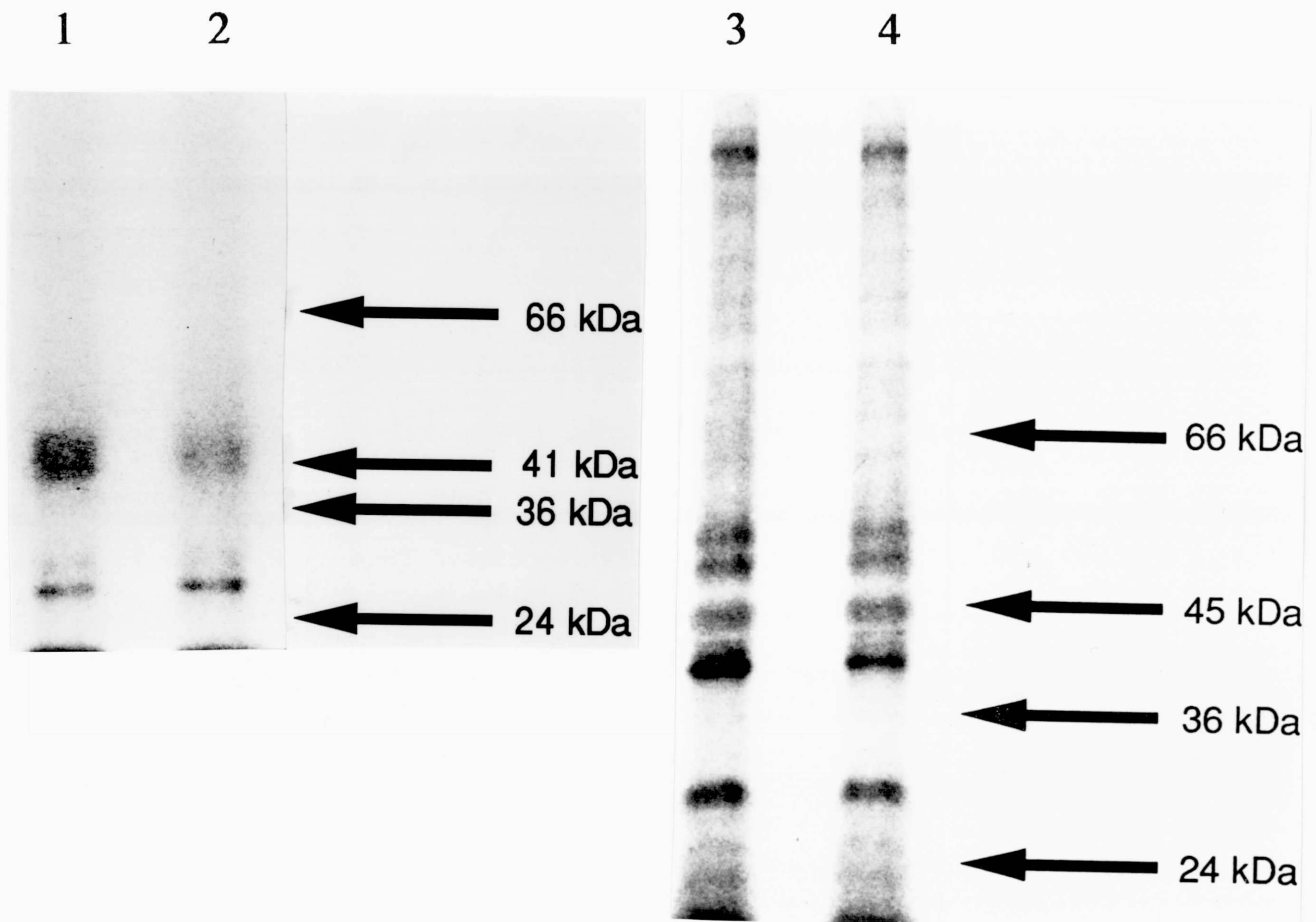


Figure 5.4: **Autoradiograph of PTX-mediated ADP-ribosylation of a 41 kDa protein in whole cell extracts and membrane preparations from human and guinea pig eosinophils.** Intact eosinophils (lanes 1 and 2) were incubated with 2 µg/ml PTX for 60 min at 37°C and either solubilized or the membranes isolated as described in the METHODS (see 2.6). Broken cells and membranes (approximately 120 ng protein) were incubated with 1 mg/ml preactivated PTX (20 mM dithiothreitol, 2 mM ATP; 60 min at 37°C) and ADP ribosylation was allowed to proceed for 60 min at 30°C in the presence of 10 µCi [^{32}P]NAD and an ADP-ribosylation mixture containing 15 mM $MgCl_2$, 6 mM ATP, 6 mM EDTA, 60 mM thymidine, 6 mM DTT, 18 mM potassium phosphoenol pyruvate, 60 µg/ml pyruvate kinase, and 60 µM NAD in potassium phosphate buffer, pH 7.6. The reaction was stopped by adding 20 µl of Laemmli stopping buffer, samples were boiled for 5 min and proteins resolved by SDS-PAGE (3% stacking gel; 10% running gel) followed by autoradiography. The arrow indicates the 41 kDa membrane protein. Molecular weight standards (x 10^3) are shown on the left.

for instance, are mediated by two GTP-binding proteins: G_s-protein for stimulatory signals (β-adrenergic agonists) and G_i-protein for inhibitory signals (α_2-adrenergic agonists).

GTP-binding proteins are heterotrimers consisting of α, β and γ chains. Most of the functional heterogeneity seems to reside in the α chains and at least twelve different α chains have been identified. In G_i the α subunit has a molecular weight of 41KDa while the α subunit of G_s has a molecular weight of either 45 or 52KDa. In resting cells, GTP-binding proteins are thought to be present in the membrane as inactive oligomeric complexes with GDP bound to the α subunit. Receptor activation causes replacement of the bound GDP by GTP followed by dissociation of the α-subunit from the $\beta\gamma$ complex. The α subunit activates the intracellular effector enzyme. The activated α-GTP complex possesses inate GTPase activity, hydrolyzes the α-bound GTP to GDP allowing the $\alpha\beta\gamma$ complex to reassociate. The α subunit is susceptible to irreversible ADP-ribosylation by either cholera toxin (G_s) or pertussis toxin (G_i) which prevents the modified α subunit from reassociating with the $\beta\gamma$ complex.

The data presented in this Chapter provide evidence for the first time that eosinophil possess a 41 kDa GTP-binding protein in their membranes. In the presence of PTX, this protein was specifically ADP-ribosylated, indicating that it represents either an inhibitory GTP-binding protein (G_i) or a closely related GTP-binding protein. Functionally, PTX inhibited EPO secretion and TXB_2 release in response to both PAF and LTB_4. In contrast, the toxin failed to modulate PMA - associated responses as well as the PAF-induced $\cdot O_2^-$ production. This evidence suggests a role for a G_i-like protein in the transduction of signals triggered by PAF-RI but not in PAF-RII-mediated signalling. The result lends further support to the hypothesis of two different signal transduction mechanisms utilized by high and low affinity receptors for PAF in eosinophils.

5.4 SUMMARY

The results presented in this chapter show for the first time that eosinophils possess a pertussis toxin-sensitive 41 kDa GTP-binding protein in their membranes which most likely represents a G_i-like protein. Since pertussis toxin selectively inhibits cellular responses associated with the high-affinity receptor proximal to the $[Ca^{2+}]_i$ changes, the G-protein appears to transduce PAF-RI signals into the cell. In contrast, the PAF-RII-associated functions appear to be transduced by a GTP-binding protein-independent mechanism. These findings provide further evidence for the dual nature of PAF receptors proposed on eosinophils. Similar results have also been obtained in human eosinophils (Kroegel *et al.*, 1990c).

CHAPTER SIX

6. INOSITOL 1,4,5- TRISPHOSPHATE PRODUCTION

6.1 INCORPORATION OF [2- ^{3}H]MYO-INOSITOL

In preliminary experiments designed to examine receptor- mediated phosphoinositide metabolism, guinea pig eosinophils were labelled under various conditions with [2-^{3}H]*myo*-inositol prior to stimulation with PAF (Fig. 6.1). Preliminary experiments demonstrated that PAF specifically stimulated the incorporation of [^{3}H]inositol into the chloroform: methanol- extracted phosphoinositide pool (i.e. largely Ptd Ins) in a concentration- , time- dependent, and WEB 2086- sensitive manner, neither [^{3}H]InsPs or added [^{3}H][^{14}C]InsP- standards could be recovered from the chloroform/methanol or neutralized TCA extracts using Dowex AG I- X8 formate columns. In view of this, an alternative method to determine receptor- mediated PtdIns(4,5)P_2 hydrolysis was adopted which allowed the determination of Ins(1,4,5)P_3 mass using a recently described radioreceptor assay (Challis *et al.*, 1988; Challis *et al.*, 1990).

6.2 ACCUMULATION OF Ins(1,4,5)P_3 MASS IN EOSINOPHILS

In order to evaluate whether eosinophil signal transduction pathways involve an accumulation of Ins(1,4,5)P_3 mass, purified eosinophils (7 x 10^6) were incubated with different stimuli at their, previously determined, maximally effective concentrations and Ins (1,4,5) P_3 mass accumulation measured. As shown in Figure 6.2, fMLP(100 μM), C5a (100 nM), leukotriene B_4 (1 μM), and PAF (1 μM) each promoted a significant accumulation of Ins(1,4,5)P_3 at 5 sec (0.40 ± 0.01; 0.56 ± 0.05; 1.23±0.06 pmol Ins(1,4,5)P_3/ 7x10^6 eosinophils, respectively) over basal values of 0.28 ± 0.02 (n=6). In contrast, the phorbol diester, PMA (1 μM) failed to increase the intracellular

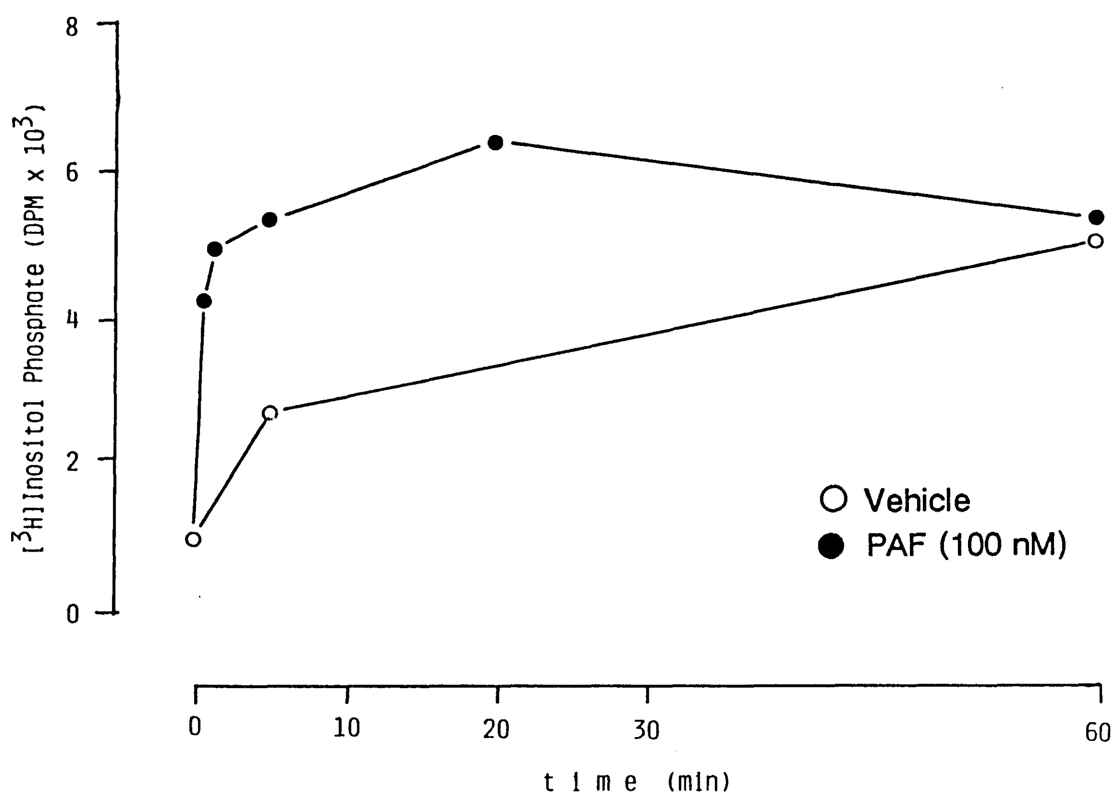


Figure 6.1: PAF- stimulated incorporation of [^{3}H]inositol into eosinophil membranes. Purified eosinophils (10^7) were incubated with 5 µCi [^{3}H]*myo*- inositol (NEN) in 500 µl HBSS buffer at 37°C in a shaking waterbath for the time periods indicated. The cells were then stimulated with PAF (100 nM) or buffer, reactions terminated by the addition of 1.6 ml chloroform/methanol (1:2, v/v), and membrane lipids extracted after the addition of chloroform and water (510 µl each). After vortex mixing and centrifugation (1000xg for 20 min) 200 µl of the lower chloroform phase was sampled, dried and counted for radioactivity. Data points given were obtained from one out of three experiment with similar results.

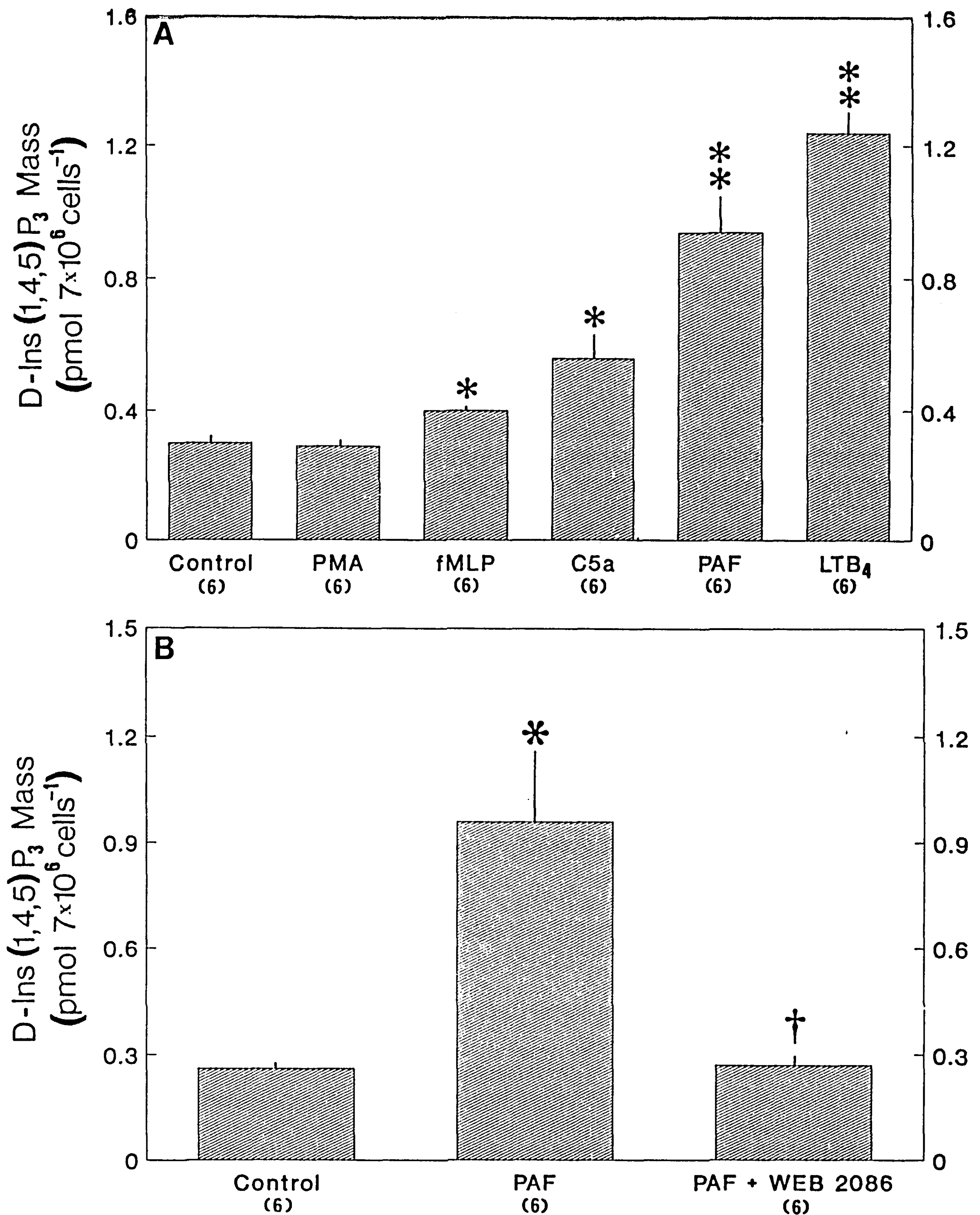


Figure 6.2: Comparison of different stimuli on Ins(1,4,5)P_3 mass accumulation (Panel A) and the effect of WEB 2086 on PAF-stimulated Ins(1,4,5)P_3 mass (Panel B). Eosinophils were incubated for 5 min in HEPES-buffered PBS (37°C) and challenged with phorbol myristate acetate (PMA, 1 μM), formyl- methionyl- leucyl- phenylalanine (fMLP, 100 μM), C5a (100 nM), platelet activating factor (PAF, 1 μM), and leukotriene B_4 (LTB_4, 1 μM) for 5 sec. Ins(1,4,5)P_3 mass was determined using a radioreceptor assay (see Methods). Each histogram represents the mean ± SEM of six observations obtained from three independent experiments. *, $P < 0.05$; ** $P < 0.01$, significantly greater than control; Kruskal- Wallace ANOVA. +, $P < 0.01$, significantly different from the effect of 1 μM PAF alone.

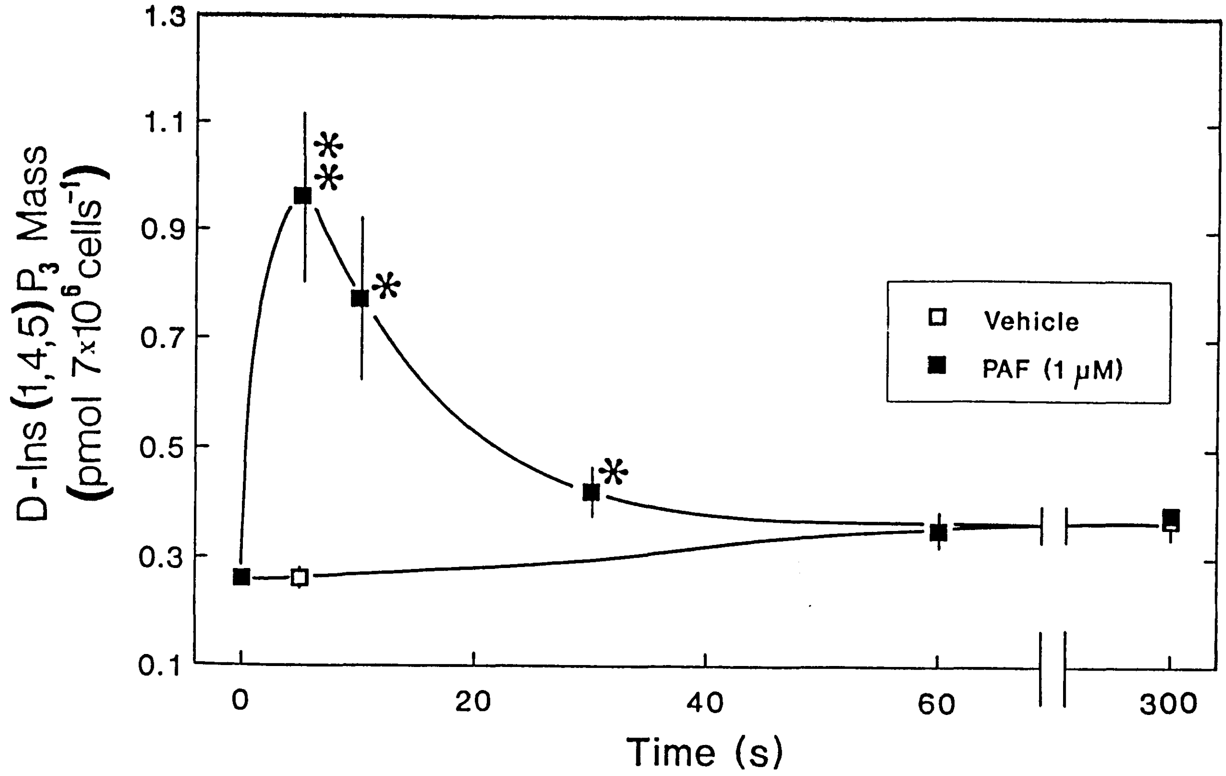


Figure 6.3: Time course of PAF-stimulated accumulation of Ins(1,4,5)P_3 mass.

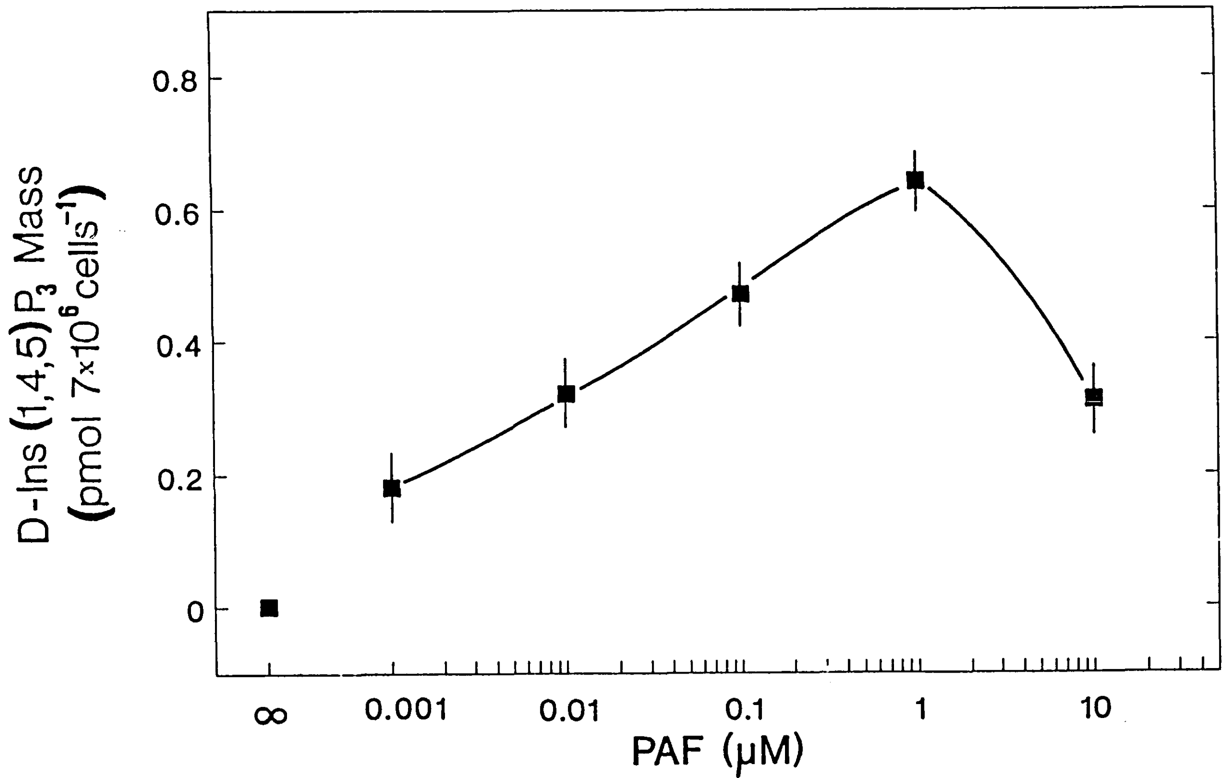


Figure 6.4: Concentration-response curve of PAF-stimulated accumulation of Ins(1,4,5)P_3 mass. Each data point represents mean ± SEM of eight observations obtained from four separate eosinophil preparations. *, $P < 0.05$; ** $P < 0.01$, significantly greater than control; Kruskal- Wallace ANOVA.

level of Ins(1,4,5)P3. Preincubation of eosinophils (5 min) with the PAF receptor antagonist WEB 2086 at 10 μM abolished the accumulation of Ins(1,4,5)3 induced by PAF (Figure 6.2b, right column). WEB 2086, itself, had no effect on the basal concentration of Ins(1,4,5)P3 at 5 min incubation (data not shown).

6.3 PAF- INDUCED Ins(1,4,5)P_3 ACCUMULATION

The effects of PAF on Ins(1,4,5)P_3 accumulation were consequently studied in more detail. Figure 6.3 shows the time- course of Ins(1,4,5)P_3 accumulation in eosinophils challenged with PAF. PAF (1 μM) induced a rapid accumulation of Ins(1,4,5)P_3 mass, with a 3.8- fold increase (0.96 ± 0.16 pmol/7x10^6 cells; n=6) over basal values (0.26 ± 0.01 pmol/7x10^6 cells; n=6) after 5 sec. The increase in Ins(1,4,5)P_3 concentration was transient returning to basal values by 60 sec. The Ins(1,4,5)P_3 concentration in control samples increased only slightly over 300 sec.

The formation of Ins(1,4,5)P_3 induced by PAF after 5 sec was concentration-dependent with a maximal effect seen with 1 μM PAF and a mean EC_{50} value of 9.6 μM ± 1.2 (n=6; Figure 6.4). Higher concentrations (10 μM) of PAF were significantly less effective at stimulating Ins(1,4,5)P_3 accumulation (Figure 6.4). Lyso- PAF, the inactive precursor and metabolite of PAF, had no stimulatory effect on intracellular levels of Ins(1,4,5)P_3.

6.4 DISCUSSION

There has been much recent interest in the receptor- activated breakdown of *myo*-inositol-containing phospholipids during cell activation and on the possible role of some of the products generated as intracellular messengers (Berridge & Irvine, 1984; Majerus *et al.*, 1988; Hansen *et al.*, 1986; Irvine *et al.*, 1986). Phosphatidylinositol which can be found on the inner leaflet of the plasmalemma, can be successfully

phosphorylated to yield Ins(4)P and Ins(4,5)P_2. It is proposed that occupation of a receptor activates a phospholipase C (PLC) that cleaves Ins(4,5)P_2 to yield diacylglycerol (see Chapter 8, page 167) and Ins(1,4,5)P_3 (Berridge, 1984; Nishizuka, 1984b). Ins(1,4,5)P_3 has been implicated as a second messenger in mobilizing intracellular Ca^{2+} (Berridge & Irvine, 1984) while diacylglycerol activates PKC (Nishizuka, 1984a). It is these two signals, $[Ca^{2+}]_i$ and PKC, which bring about the range of responses collectively known as cell activation.

In neutrophils, several studies have shown that agonists such as fMLP or LTB_4 trigger a PLC (Cockcroft *et al.*, 1984) that catalyses the hydrolysis of Ins(4,5)P_2 to give both diacylglycerol and Ins(1,4,5)P_3 (Cockcroft *et al.*, 1984; Cockcroft *et al.*, 1985; Smith *et al.*, 1985; Smith *et al.*, 1986). Indeed, it has even been possible to demonstrate that Ins(1,4,5)P_3 triggers calcium mobilization in permeabilized neutrophils (Prentki *et al.*, 1984). Furthermore the phosphorylation product of Ins(1,4,5)P_3, Ins(1,3,4,5)P_4 has been associated with the activation of receptor-operated calcium channels and an influx of extracellular Ca^{2+} (see Chapter 7, page 149). PTX was shown to inhibit this phosphoinositide remodelling, implying that a G_i-like protein is essential for the transduction process between receptor occupation and activation of PLC (Smith *et al.*, 1985).

In the present study, Ins(1,4,5)P_3 was used as a marker for the involvement of InsPs in eosinophil activation. The results showed, that agonists such as PAF together with LTB_4, fMLP and C5a induced a rapid and transient increase of this second messenger. In contrast, non-receptor mediated stimuli such as PMA did not raise the Ins(1,4,5)P_3 concentration above control levels. The dose-response curve of PAF elicited Ins(1,4,5)P_3 paralleled those of the PAF-induced degranulation and lipid mediator release. In contrast, Ins(1,4,5)P_3 synthesis revealed no correlation with the production of oxygen radicals again suggesting multiple independent activation mechanisms in eosinophils.

6.5 SUMMARY

The data presented in this chapter demonstrated for the first time that eosinophils challenged with PAF, or other receptor mediated stimuli responded with an accumulation of intracellular Ins(1,4,5)P_3. In contrast, the non-physiologic stimulus, PMA had no effect on the accumulation of intracellular Ins(1,4,5)P_3 mass. The amounts measured were comparable to the concentration of Ins(1,4,5)P_3 observed in other cells (Bradford & Rubin, 1986; Tarver *et al.*, 1987; Horstmann *et al.*, 1988; Shears, 1988). Given that Ins(1,4,5)P_3 is produced as an intermediate product of the InsPs cycle (Berridge & Irvine, 1984; Nishizuka, 1984) these data suggest that other metabolites of the inositol cycle may be also generated. As in other cell types, the major biological function of Ins(1,4,5)P_3 in eosinophils may be the liberation of other signalling messengers such as Ca^{2+} from the intracellular stores.

CHAPTER SEVEN

7. CHANGES IN INTRACELLULAR FREE CALCIUM ION CONCENTRATION

Changes in the intracellular free Ca^{2+} ion concentration ($[Ca^{2+}]_i$) have long been considered to play an important role in stimulus response coupling in various cells. In eosinophils, however, changes in $[Ca^{2+}]_i$ and their role in transmembrane signalling have never been investigated.

7.1 MEAN $[Ca^{2+}]_i$ CHANGE

In order to relate the receptor-mediated accumulation of Ins(1,4,5)P_3 to changes in $[Ca^{2+}]_i$, guinea pig eosinophils were loaded with fura- 2 (2.5 μM) and challenged with PAF (1 nM - 10 μM). Figure 7.1 illustrates the rise in $[Ca^{2+}]_i$ shown as traces of fluorescence output from a representative experiment. The effect of PAF on $[Ca^{2+}]_i$ was rapid and concentration- dependent. Peak $[Ca^{2+}]_i$ in response to 1 μM PAF was approximately 600 nM above basal levels of 108 ± 9 nM (n=36). A maximal increase in $[Ca^{2+}]_i$ stimulated by PAF (1 μM) was reached 15 to 20 sec (n=9) post-challenge followed by a gradual decline to basal values within 2 min. Lower concentrations of PAF increased the lag period before onset of the response as well as the time taken to reach peak $[Ca^{2+}]$i levels. The corresponding EC_{50} was calculated to be 13.1 ± 1.9 nM (n=8). Higher concentrations than 1 μM PAF led to a reduced peak calcium response in eosinophils. Lyso- PAF at concentrations ranging from 1 nM to 10 μM did not affect $[Ca^{2+}]_i$. As demonstrated in Figure 7.2, LTB_4, C5a and fMLP also induced an elevation of $[Ca^{2+}]_i$ in guinea pig eosinophils. On a molar basis, PAF, LTB_4 and C5a were equipotent while for fMLP 100- fold higher concentrations were needed to elicit a response.

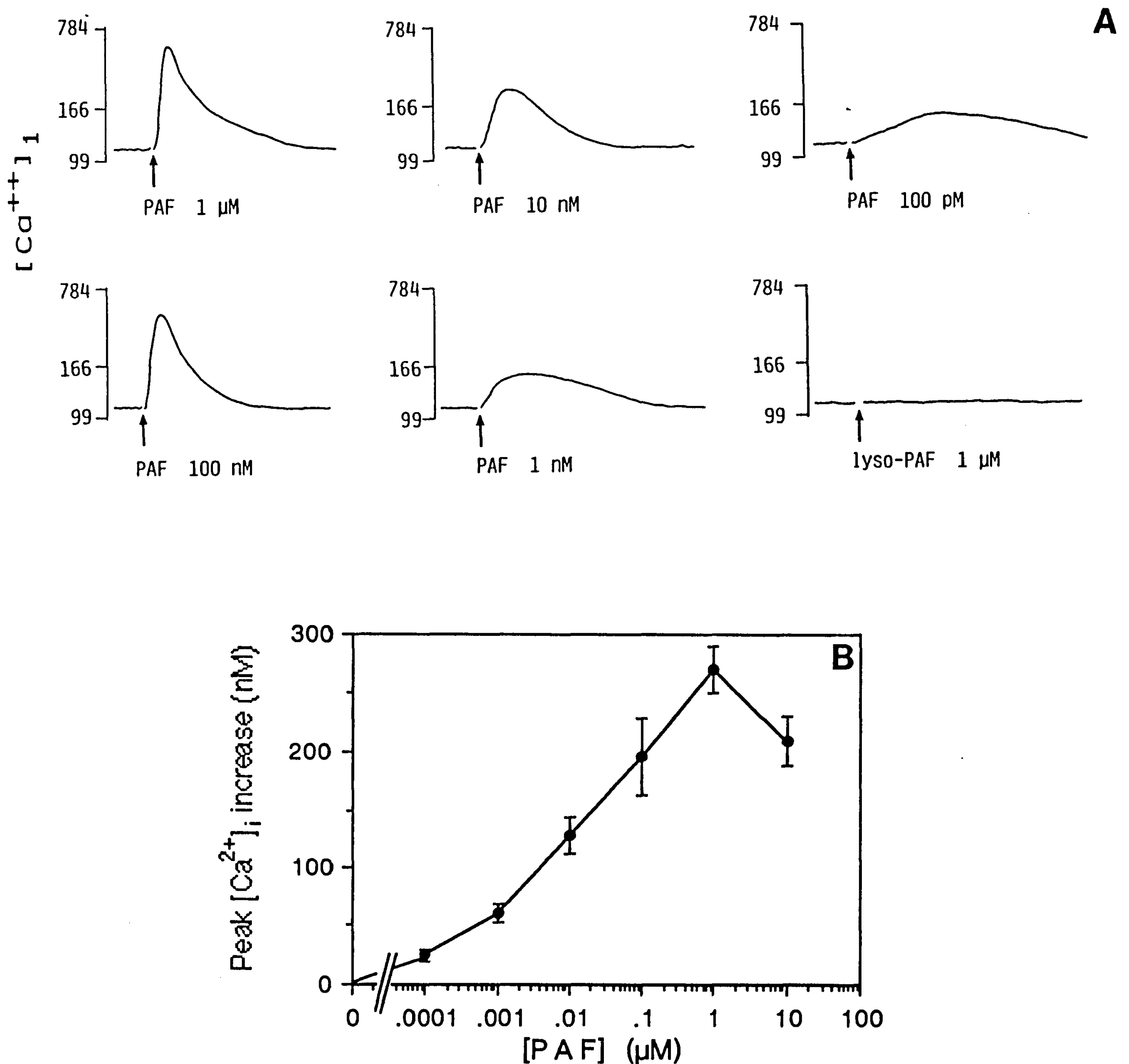


Figure 7.1: Concentration-response curve of PAF-induced elevation of $[Ca^{2+}]_i$. Purified eosinophils were labelled with fura-2 (2.5 µM; 30 min) in Ca^{2+} free, HEPES-buffered Tyrode's solution and adjusted to a final concentration of 1.5 x 10^6 cells/ml. Cells were challenged with PAF (1 nM - 1 µM) or lyso-PAF (1 µM) and changes in $[Ca^{2+}]_i$ were estimated fluorimetrically. In **panel A** the trace shown is a recording from a representative experiment using guinea pig eosinophils. The non-linear vertical scale is the result of transforming fluorescent output to eosinophil $[Ca^{2+}]_i$. **Panel B** depicts the concentration- dependency of the net peak increase in $[Ca^{2+}]_i$ induced by PAF (100 pM - 10 µM) in human blood eosinophils. Data were derived from nine independent eosinophil preparations and represent mean ± SEM.

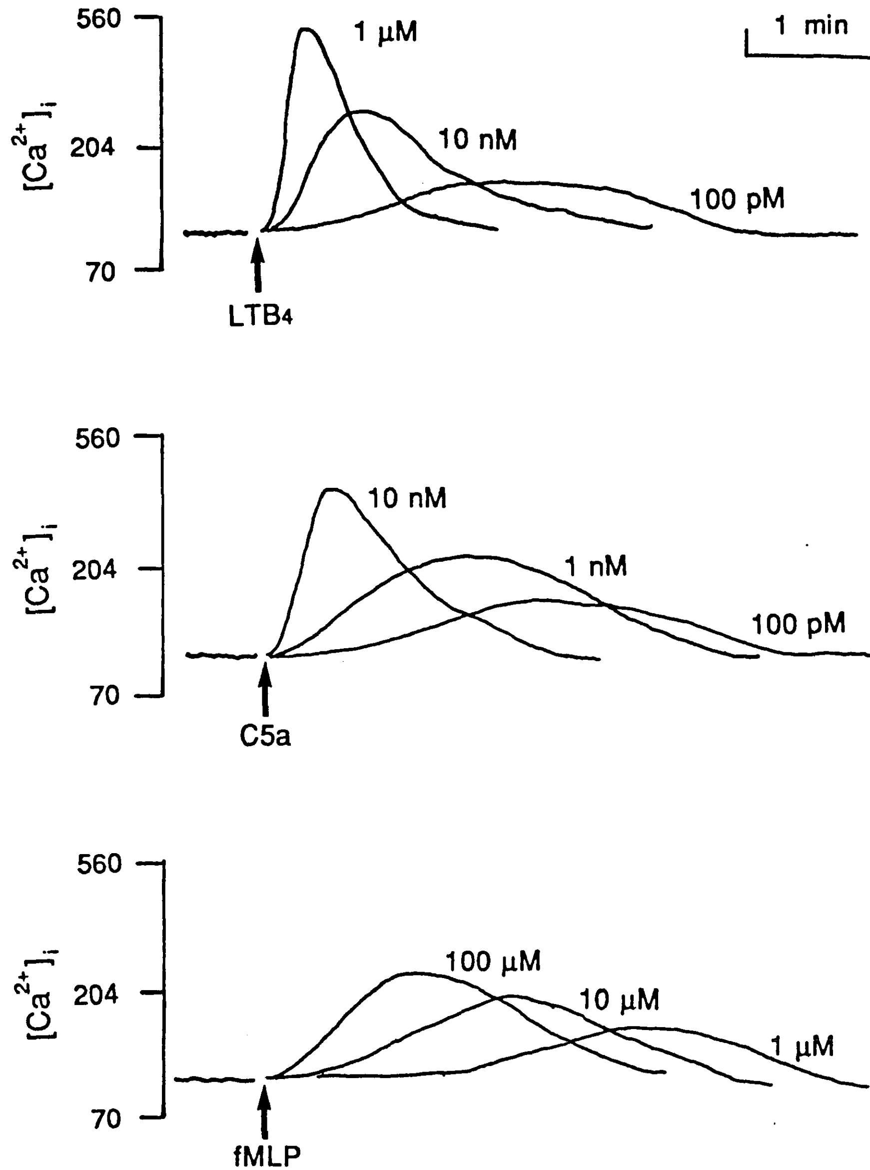


Figure 7.2: Kinetics of $[Ca^{2+}]_i$ induced by LTB_4, C5a, and fMLP. $[Ca^{2+}]_i$ was determined in stimulated fura-2-labelled guinea pig eosinophils as described in the legend to Figure 7.1 and Methods. The fluorimeter traces shown above were obtained from a representative experiment.

In addition, PAF, LTB_4 and C5a showed a similar time-course, whereas the response to fMLP characteristically showed a protracted onset and was longer lasting. PMA did not cause any detectable change in $[Ca^{2+}]_i$ at any concentration (1 nM - 10 μM) examined.

Preincubation of the eosinophils with WEB 2086 (300 nM) inhibited the PAF- induced rise in $[Ca^{2+}]_i$ but did not affect responses induced by LTB_4, C5a or fMLP (Fig.7.3) indicating that the $[Ca^{2+}]_i$ changes induced by PAF were selective and receptor mediated. Increasing concentrations of WEB 2086 (10 nM to 1 μM) attenuated the PAF (100 nM) -promoted rise in $[Ca^{2+}]_i$ in a concentration- dependent manner with a corresponding IC_{50} of 59.5 nM (data not shown).

Initial exposure to PAF (100 nM) rendered the cells unresponsive to a second application of PAF (100 nM) indicating homologous desensitization (Fig. 7.4). In contrast, initial exposure to PAF did not affect the responses produced by subsequent application of LTB_4 or fMLP. However, when eosinophils were first exposed to fMLP (100 μM) the response to both PAF and LTB_4 was inhibited, suggesting that fMLP causes a heterologous desensitization.

7.2 ROLE OF EXTRACELLULAR CALCIUM

In order to define the contribution of the extracellular Ca^{2+} ($[Ca^{2+}]_e$) to the increase in $[Ca^{2+}]_i$, eosinophils were stimulated with PAF in Ca^{2+}- free buffer in the presence of 5 mM EGTA. As shown in Figure 7.5, PAF-stimulated eosinophils still displayed a rise in $[Ca^{2+}]_i$ although the magnitude of the response was less than that seen at 1 mM Ca^{2+}. The mean inhibition for 100 pM - 1 μM PAF was 85.6 ± 5.4% ($p < 0.05$). Replacement of 1 mM $[Ca^{2+}]_e$ with 1 mM Ni^{2+} produced an instantaneous slight quench of the signal indicating a small leakage of intracellular fura-2. The initial quench was followed by a marked prolongation of the lag time (time between addition of stimulus and the onset of a measurable increase in $[Ca^{2+}]_i$ and a significant decrease in the peak elevation of $[Ca^{2+}]_i$ upon stimulation with PAF.

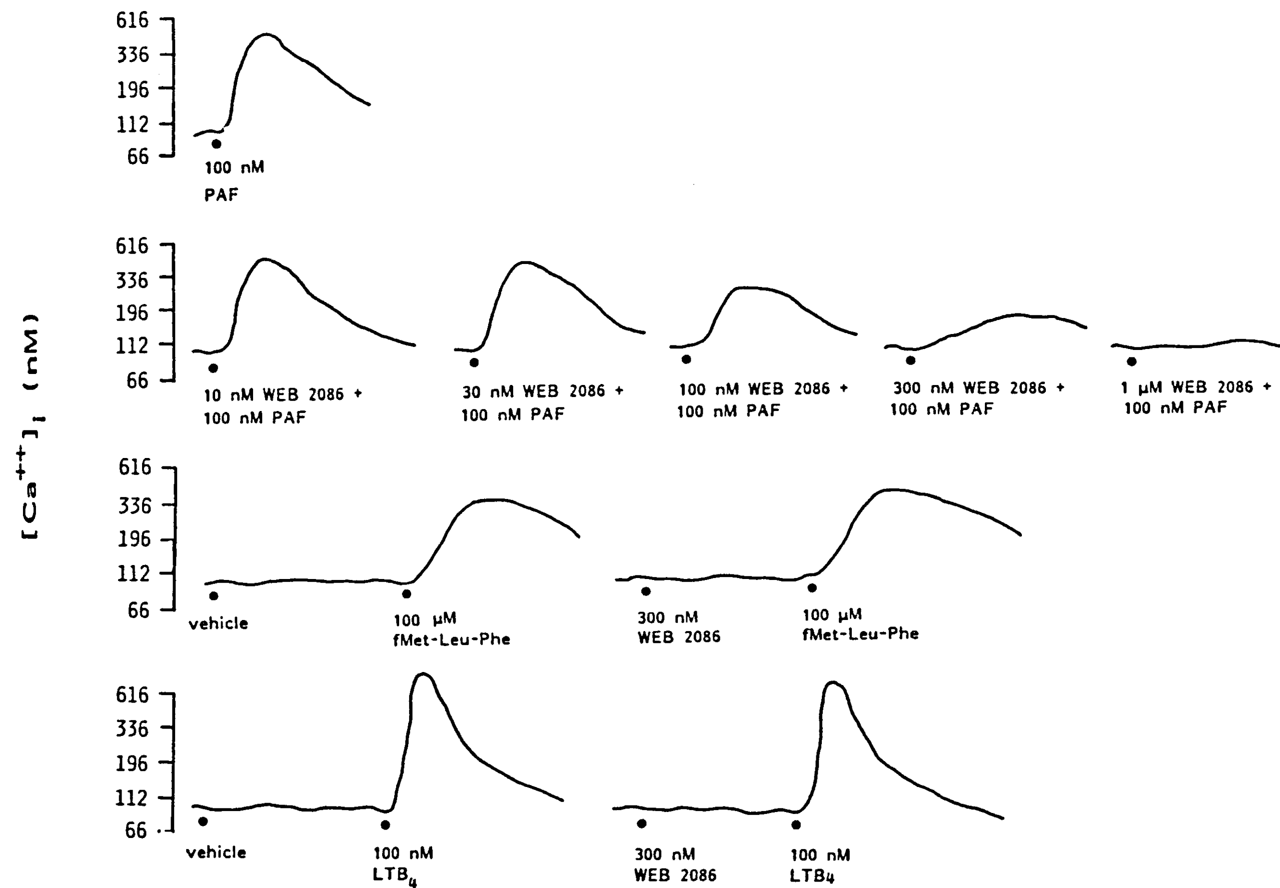


Figure 7.3: Effect of WEB 2086 on PAF-, fMLP, and LTB4 -induced increase in $[Ca^{2+}]_i$. Cells were treated as described in the legend to Figure 7.1. The fluorimeter traces shown above were obtained from a representative experiment.

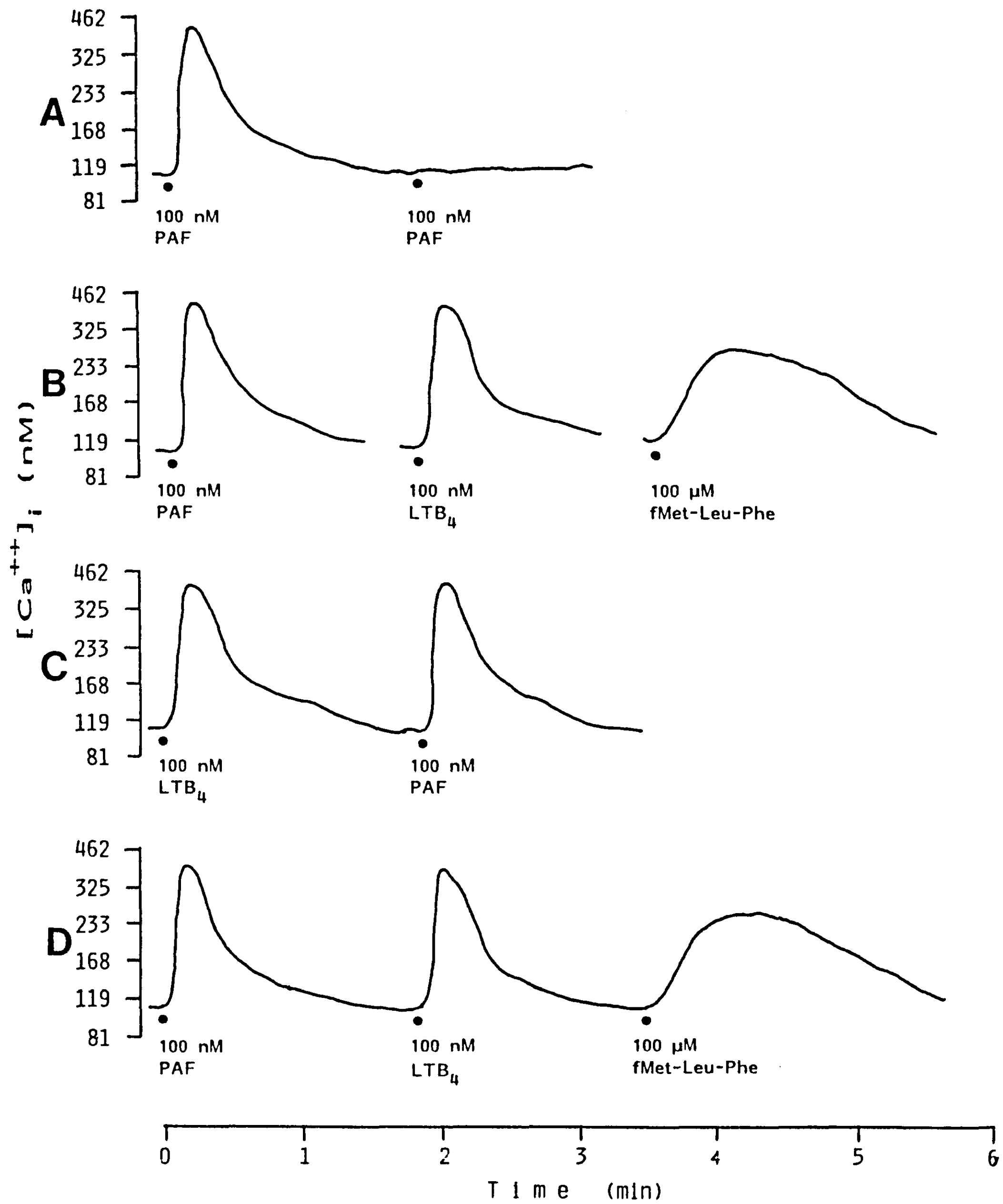


Figure 7.4: Comparison of repeated stimulation of the same eosinophil sample by PAF, LTB_4, or fMLP (A, C, and D) with their effect on independent cell samples (B). Cells were treated as described in the legend to Figure 7.1. The fluorimeter traces shown above were obtained from a representative experiment.

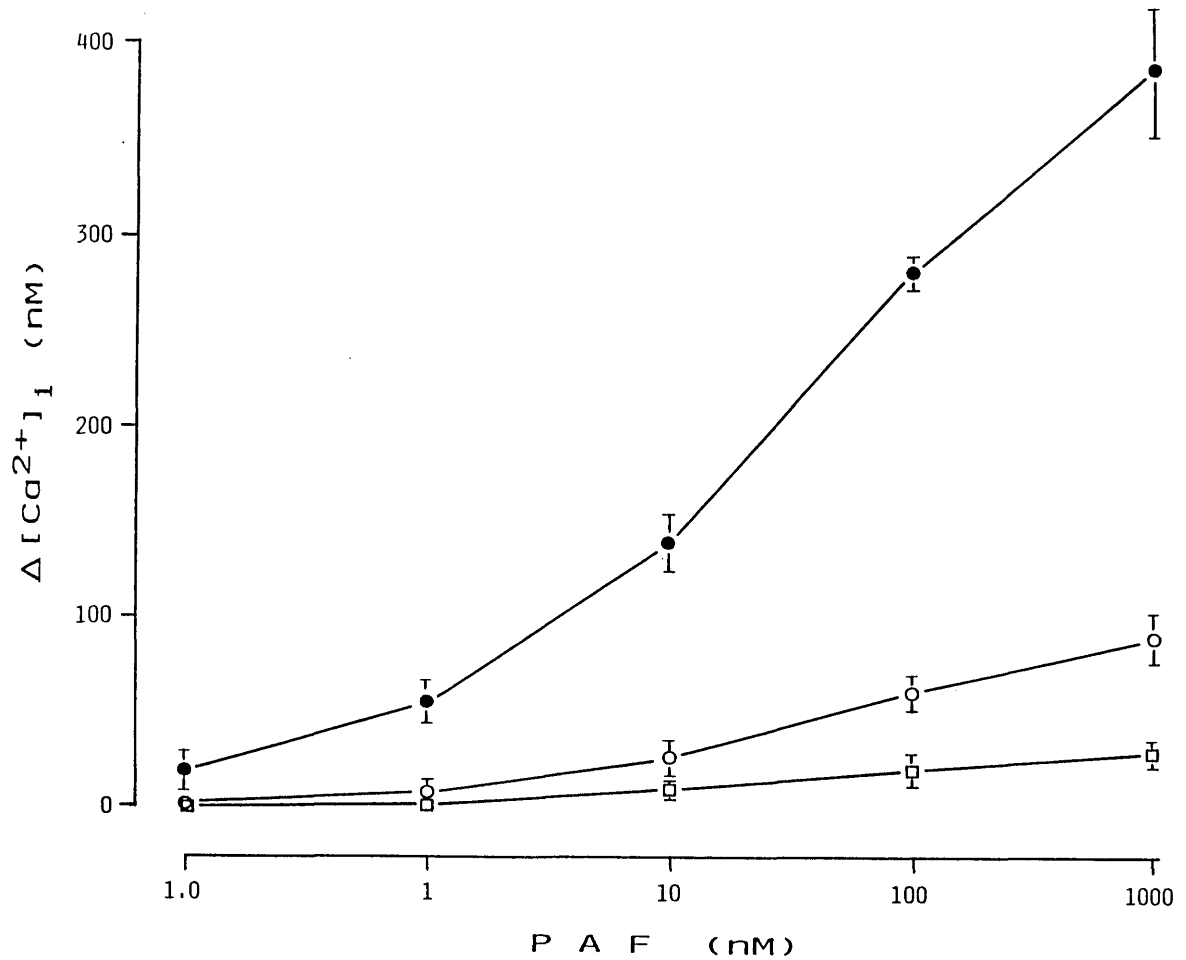


Figure 7.5: Dose- response curve of PAF-induced $[Ca^{2+}]_i$ changes in the presence of EGTA and Ni^{2+}. Dose- response curve of PAF - induced $[Ca^{2+}]_i$ in the presence of 1 mM Ca^{2+} (●), 5 mM EGTA (O), and 1 mM Ni^{2+} (□). $[Ca^{2+}]_i$ is the value obtained by subtracting basal pre-agonist value from the peak post-agonist value. Data are expressed as mean ± SEM of 9 determinations from three independent experiments.

Ni^{2+} ions, which are known to block calcium translocating pores (Hille, 1984), produced an almost complete inhibition (95.8 ± 2.1%; $p > 0.05$) of the PAF (100 pM-1 μM)-induced elevation in $[Ca^{2+}]_i$. These data suggest that the increase in $[Ca^{2+}]_i$ induced by PAF arose both from the release of Ca^{2+} from intracellular stores and an influx of exogenous Ca^{2+}. Finally, preincubation of eosinophils for 10 min with the dihydropyridine antagonist nimodipine up to concentrations of 10 μM did not affect the elevation of $[Ca^{2+}]_i$ by PAF, indicating that voltage- dependent Ca^{2+} channels are not involved. Exposure of eosinophils to 1 mM Mn^{2+} lead to a small, instantaneous sharp drop followed by a decline in fluorescence. As previously described for Ni^{2+}, the slight drop in fluorescence upon addition of Mn^{2+} reflects the immediate quenching of leaked extracellular fura- 2 while the subsequent decline indicates a moderate influx of Mn^{2+} into the cells displacing Ca^{2+} from the intracellular fura- 2 and quenching the fluorescence signal. In contrast to observations in platelets (Hallam & Rink, 1985; Poll & Westwick, 1986), PAF did not stimulate Mn^{2+} entry in eosinophils (data not shown).

7.3 CORRELATION BETWEEN $[Ca^{2+}]_i$ CHANGES AND $Ins(1,4,5)P_3$ ACCUMULATION.

Over a PAF concentration range of 1-1000 nM there was a significant correlation ($r=0.99$, $p < 0.01$) between the average net peak increase in $[Ca^{2+}]_i$ and the peak accumulation of $Ins(1,4,5)P_3$ in eosinophils. A close correlation was also found between these values for fMLP (100 μM), C5a (100 nM) and LTB_4 (1 μM) ($r = 0.89$, $p < 0.05$) indicating that $Ins(1,4,5)P_3$ may, indeed, be involved in the release of Ca^{2+} from intracellular stores.

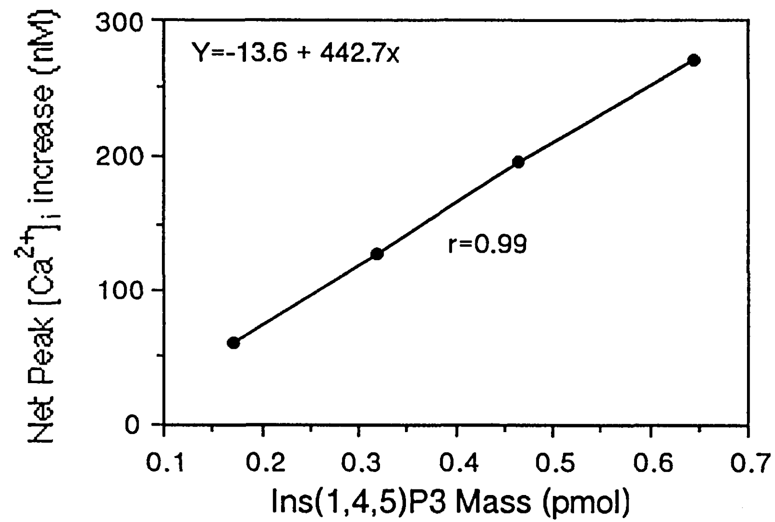


Figure 7.6: **Correlation between Ins(1,4,5)P_3 mass and net peak $[Ca^{2+}]_i$ change after challenge of eosinophils with PAF.** Eosinophils were challenged with PAF (1 nM - 1 μM) and the changes in Ins(1,4,5) P_3 and $[Ca^{2+}]_i$ measured. Each dot represents the mean of six experiments obtained by re-plotting the first four data points given in Figure 6.4 against the PAF-induced net peak $[Ca^{2+}]_i$ increase in guinea pig eosinophils.

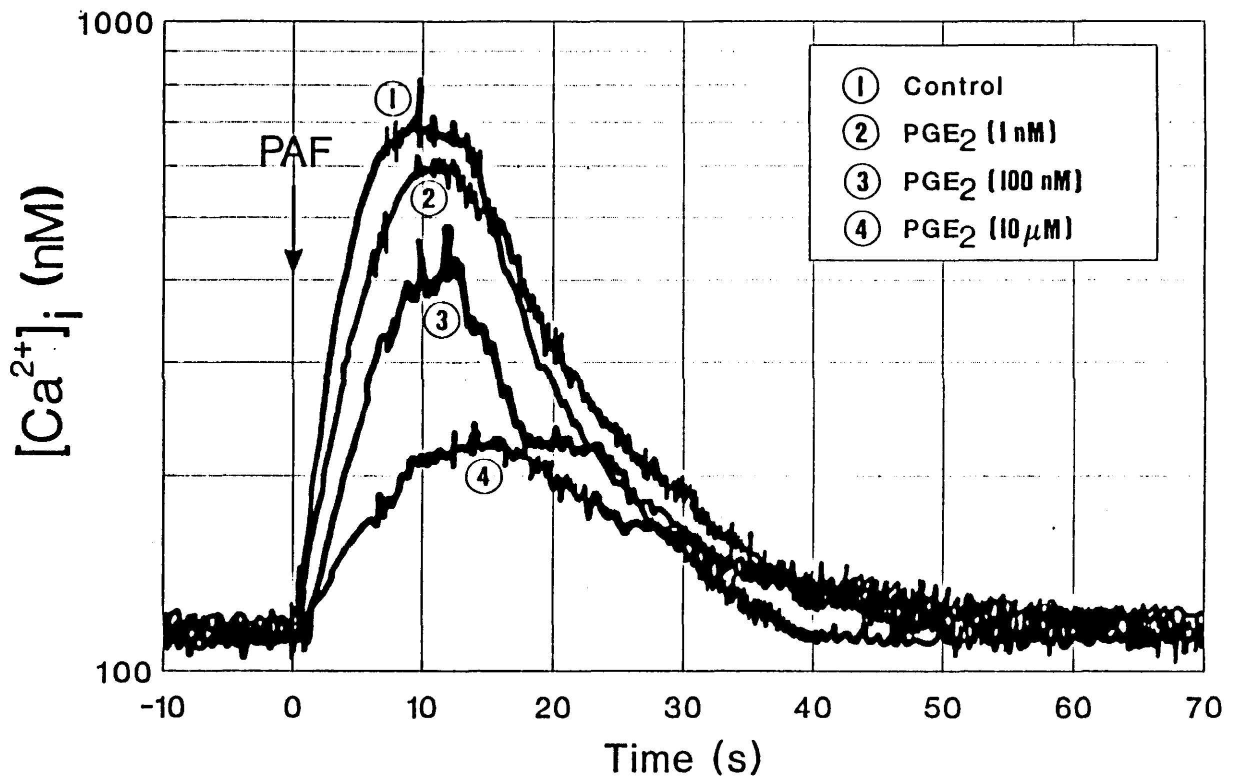


Figure 7.7: Effect of PGE_2 on PAF-induced rise in $[Ca^{2+}]_i$. Eosinophils were preincubated with PGE_2 (1 nM to 10 μM) for 5 min at room temperature prior to the addition of PAF (100 nM). The fluorimeter trace shown was obtained from a representative experiment.

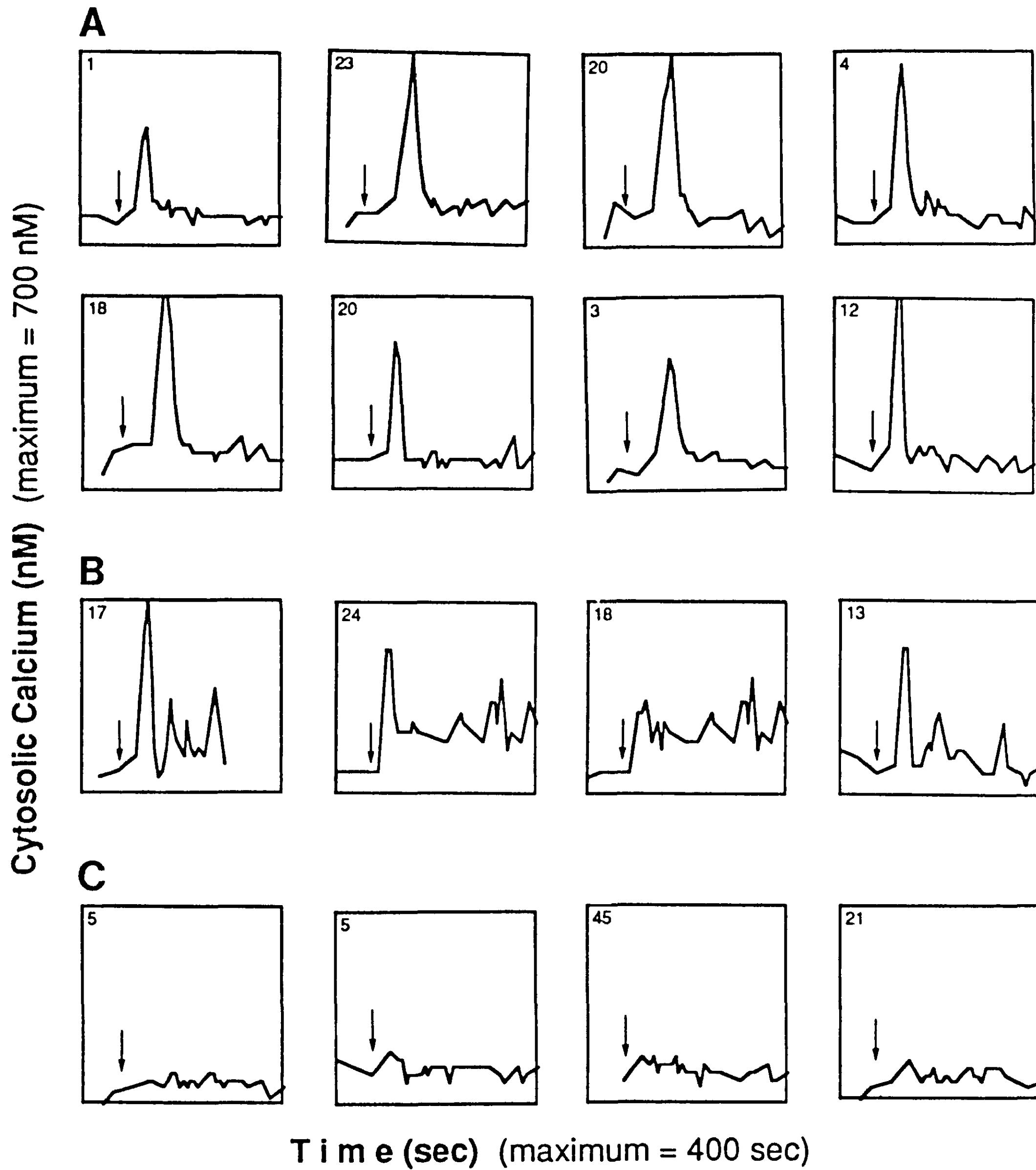


Figure 7.8: $[Ca^{2+}]_i$ changes in individual human blood eosinophils. The changes in $[Ca^{2+}]_i$ before and after the addition of 1 μM PAF (arrows). The traces shown represent characteristic examples taken from 133 individual guinea pig eosinophils obtained from three separate cell preparations. **Panel A:** Non-oscillating eosinophils. **Panel B:** Oscillating eosinophils. **Panel C:** Non-responding eosinophils.

7.4 EFFECT OF PROSTAGLANDIN E_2

Pretreatment of eosinophils with flurbiprofen affected neither the resting $[Ca^{2+}]_i$ (107.8 ± 8.7 nM Ca^{2+}, n=31), the magnitude or the time course of PAF-induced Ca^{2+}-mobilization at any concentration examined, nor the ability of PAF to induce this response (EC_{50}: 15.7 ± 3.5 nM, n=3). However, preincubation of the eosinophils with increasing concentrations of PGE_2 (1 nM to 10 µM) caused concentration-dependent inhibition of the PAF-induced rise in $[Ca^{2+}]_i$ with an IC_{50} = 324 nM (n=9) and a maximal inhibition (≈ 83%) being observed at 10 µM PGE_2 (Figure 7.7). The drug had no effect on the basal level of $[Ca^{2+}]_i$. TXB_2 failed to effect either basal or PAF-stimulated $[Ca^{2+}]_i$ levels. Together with the data described in Chapter 3.8 (see page 114), these results indicate that PGE_2 may regulate stimulus-response coupling in eosinophils.

7.5 $[Ca^{2+}]_i$ CHANGES IN SINGLE CELLS

In order to analyse the change in $[Ca^{2+}]_i$ induced by PAF (1 µM) in human eosinophils, 133 individual eosinophils were examined using digital video microscopy (MacGlashan, 1989). Representative $[Ca^{2+}]_i$ kinetics of 16 PAF-stimulated eosinophils are depicted in Figure 7.8. The average peak $[Ca^{2+}]_i$ of all individual cells examined obtained from three separate experiments was 291 ± 28 nM. The peak response to 1 µM PAF observed in individual cells was unimodal and normally distributed, indicating the presence of one population of eosinophils. In contrast, however, analysis of the decay rate of single cells revealed that approximately 15% of the cells showed more than one $[Ca^{2+}]_i$ peak. In addition, approximately 8% of the cells showed no distinct rise in $[Ca^{2+}]_i$ after stimulation. Although the majority of eosinophils showed similar kinetics, some cells responded differently with repeated $[Ca^{2+}]_i$ spikes while others did not respond at all. The significance of these findings for cell activation in eosinophils is as yet not known and is currently under

investigation. A possible explanation could be that eosinophils are composed of more than one population such as normodense and hypodense eosinophils, respectively.

7.6 DISCUSSION

In common with many other cell systems Ins(1,4,5)P_3 and Ca^{2+} appear to play a role as a second messenger in the activation sequence of the neutrophil (Westwick & Poll, 1988; Prentke *et al.*, 1984b). Changes in the level of $[Ca^{2+}]_i$ have been proposed to be key regulators of cell activation (O'Flaherty *et al.*, 1987; Goldman & Goetzl, 1984). A rapid increase in cytosolic calcium measured as changes in quin-2 or fura-2 fluorescence in response to various stimuli (Tsien *et al.*, 1982; Grynkiewicz *et al.*, 1985) has been demonstrated in neutrophils (DiVirgilio *et al.*, 1984; Pozzan *et al.*, 1983; Korchak *et al.*, 1984).. This Ca^{2+} originates primarily from cell- associated stores, since the response is only minimally affected by the removal of extracellular Ca^{2+}. Moreover, stimulus activation of neutrophils causes an immediate increase in $[Ca^{2+}]_i$ (lag < 1 sec) indicating that the rise in $[Ca^{2+}]_i$ is one of the earliest observable events in the activation sequence (Korchak *et al.*, 1984; Pozzan *et al.*, 1983).

The results in this Chapter demonstrate, that both human and guinea pig eosinophils, like neutrophils, underwent a rapid and transient increase in $[Ca^{2+}]_i$ in response to PAF and other stimuli such as LTB_4, fMLP or C5a. The magnitude and duration of the response was dependent on the agonist concentration used as well as on the presence of extracellular Ca^{2+}. PAF induced an increase at concentrations between 100 pM and 10 μM and the concentration-response curve correlated both with eosinophil cell functions and the rise in Ins(1,4,5)P_3 levels. These findings indicate that the rise in cytosolic free Ca^{2+} may represent an important intracellular signal to activate subsequent physiological cell responses.

In contrast, higher concentrations of PAF initiated the release of $\cdot O_2^-$ (see page 104) and yet failed to raise $[Ca^{2+}]_i$. This observation lends further support to the hypothesis that the two putative PAF receptors utilise distinct signal transduction

pathways. Interestingly a similar dissociation of $[Ca^{2+}]_i$ and $\cdot O_2^-$ has been observed in neutrophils challenged with the monoclonal antibody PMN7C3 (Apfeldorf *et al.*, 1985). This observation provides further evidence that an increase in $[Ca^{2+}]_i$ is not, in itself, a sufficient signal for superoxide anion production and is in agreement with analogous findings by others (Lew *et al.*, 1986). Evidently, some other, as yet unknown, factor(s) is (are) possibly required for a full cellular response (Korchak *et al.*, 1984).

When eosinophils were activated with the tumour promoter, phorbol myristate acetate, no increase in cytosolic Ca^{2+} was observed (see Chapter 7.1, page 149). Though, as demonstrated in Chapter 3, degranulation, lipid mediator release as well as superoxide anion generation were activated by PMA (see Figures 3.1, 3.10, and 3.12). These observations are comparable to studies examining the effect of PMA on neutrophils (Korchak *et al.*, 1984; Sha'afi *et al.*, 1983). It has been suggested that PMA bypasses the Ca^{2+} dependent segment of the activation cascade by directly activating protein kinase C (see Chapter 9, page 169).

7.7 SUMMARY

The data demonstrate that receptor-activated eosinophils responded with a stimulus- dependent rapid and transient increase in $[Ca^{2+}]_i$. The response to PAF was specifically inhibited by WEB 2086 and exposure of the cells to PAF induced a homologous desensitization. The rise in $[Ca^{2+}]_i$ constitutes both an influx of extracellular Ca^{2+} via receptor-operated channels and a release of Ca^{2+} from intracellular stores. Single cell analysis of human eosinophils revealed that the response to PAF was heterogeneous, ranging from non-responding eosinophils to cells with a $[Ca^{2+}]_i$ increase more than twice the mean. However, the significance of this finding is not clear. Although, in guinea pig eosinophils, LTB_4 may be slightly more effective than PAF in secreting granular enzymes, prostanoids as well as producing a

stronger $Ins(1,4,5)P_3$ signal, the time course of the elevation in $[Ca^{2+}]_i$ induced by either of the stimuli did not differ significantly.

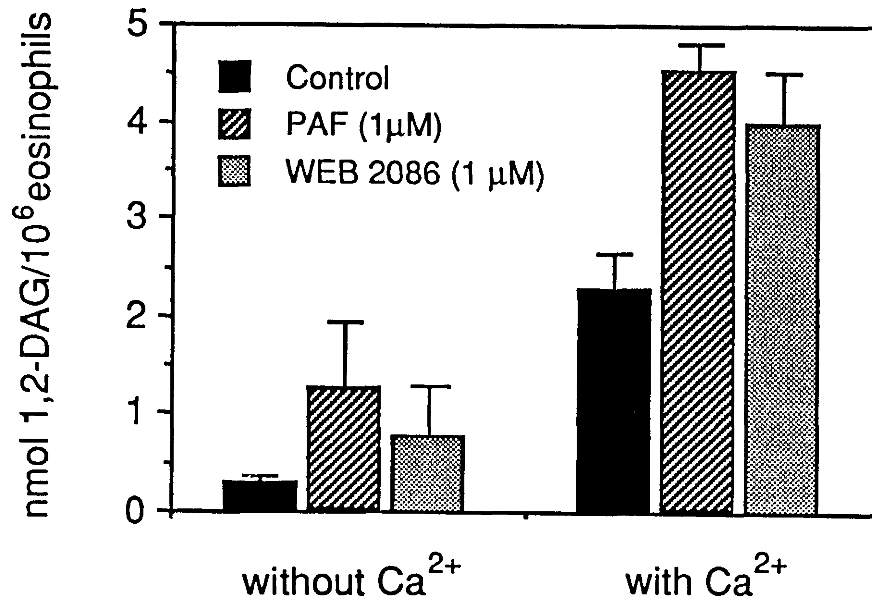
CHAPTER EIGHT

8 CHANGES IN INTRACELLULAR 1,2-DIACYL GLYCEROL CONCENTRATIONS

8.1 sn-1,2-DIACYLGLYCEROL LEVELS IN EOSINOPHILS

Pre-warmed (37°C; 5 min) purified eosinophils ($7x10^6$ cells per sample) were incubated for 10 sec with vehicle or PAF (1 μM) in a final volume of 500 μl. The reaction was stopped by the addition of 1 ml chloroform:methanol (1:2, v/v) and the lipids extracted and separated. The 1,2-DAG concentration was assayed as described in the Methods (Chapter 2.18, page 76). Resting eosinophils resuspended in Ca^{2+}-containing (1mM) HEPES- buffer were found to have 2.3 ± 0.3 pmol of 1,2-DAG per 10^6 cells (n=4). Stimulation with PAF lead to a 2-fold increase in the 1,2-DAG level (4.5 ± 0.3 pmol/10^6 cells) (Figure 8.1). In Ca^{2+}-depleted eosinophils incubated in the absence of extracellular Ca^{2+}, basal levels of 1,2-DAG were considerably lower (0.3± 0.1 pmol/10^6 cells). However, exposure of the cells to PAF (1 μM) caused an almost 4- fold rise in 1,2-DAG concentration to 1.2 ± 0.7 pmol/10^6 eosinophils, indicating that 1,2-DAG is generated by a Ca^{2+}- independent mechanism.

In order to examine the specificity of the PAF- induced increase in 1,2-DAG, eosinophils were preincubated with WEB 2086 (1 μM) for 5 min prior to the addition of PAF (1 μM). As shown in Fiure 8.1, WEB 2086 inhibited the PAF-induced 1,2-DAG synthesis by 20% and 33% respectively, indicating a specific PAF receptor mediated effect. WEB 2086 alone did not cause any detectable change of resting 1,2-DAG levels.



<u>Figure 8.1:</u> **Resting and stimulated 1,2-diacylglycerol levels in eosinophils in the presence and absence of Ca^{2+}.** Eosinophils (10^6 prewarmed at 37°C in the absence or presence of WEB 2086 were incubated for 10 sec with vehicle or PAF (1 µM). The amount of 1,2-DAG was measured by the quantitative conversion of 1,2-DAG to phosphatidic acid in the presence of diacyl glycerol kinase (see Methods, Section 2.18, page 76). Data represent mean ± SEM of duplicate values of two experiments.

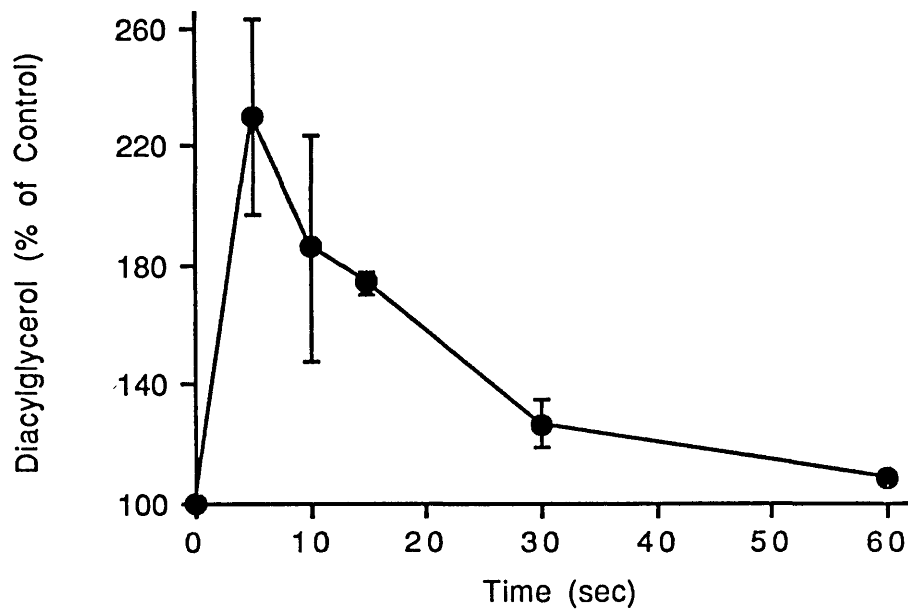


Figure 8.2: Kinetics of PAF- stimulated accumulation of 1,2-diacyl glycerol in eosinophils. Cells ($7x10^6$) were stimulated with 1 μM PAF for the time indicated before extraction, separation and quantification of lipids as described in Methods. Data shown are normalized to the resting 1,2-DAG values.

8.2 TIME COURSE OF 1,2-DAG-SYNTHESIS

Eosinophils were incubated with PAF (1 μM) as described above (8.1) and reaction stopped after 5, 10, 15, 30, and 60 sec. Addition of PAF induced a rapid and transient increase of intracellular 1,2-DAG concentration (Fig. 8.2). The amount of 1,2-DAG was already elevated 230 ± 33% over basal values at 5 sec, the earliest time point studied. By 60 sec the stimulated 1,2-DAG level reached a plateau while resting levels increased linearly as a function of time (data not shown). No stimulated 1,2-DAG increase was found after 60 sec.

8.3 DISCUSSION

The potential role of inositol phosphates in eosinophil activation has been discussed in Chapter 6. The results presented in this Chapter point out that 1,2-DAG may also be generated during PAF activation of eosinophils providing yet another mechanism by which lipid remodelling could play an essential role in signal transduction of the cell. 1,2-DAG is generated by the cleavage of phosphatidylcholine (Irving & Exton, 1987), InsP, and Ins(3,4)P_2 by PLC (Berridge & Irvine, 1984). The finding that 1,2-DAG can be further phosphorylated to generate phosphatidic acid explains why stimulation of the eosinophils led to an increase in phosphatidic acid. Stimulation of neutrophils with fMLP or concanavalin A triggers a marked rapid increase in the levels of phosphatidic acid (Serhan *et al.*, 1983; Korchak *et al.*, 1988) similar to that observed in eosinophils. This rapid elevation of phosphatidic acid preceded the relatively slow elevation of $[Ca^{2+}]_i$ noted in concanavalin A activated neutrophils. Furthermore, $\cdot O_2^-$ generation did not commence until both 1,2-DAG and $[Ca^{2+}]_i$ were of optimal levels suggesting a synergism between Ca^{2+} and PKC activation (Rider & Niedel, 1987).

1,2-DAG levels are also sensitive to Ca^{2+}, since depletion of calcium, for instance with EGTA, was shown to decrease the fMLP elicited increase in 1,2-DAG. However, as shown in Figure 8.1, the opposite appears to be true in guinea pig

eosinophils: The absence of Ca^{2+} reduced the concentration of both basal and stimulated 1,2-DAG. In any case, the results obtained in neutrophils and eosinophils suggest that the interrelation between Ca^{2+} and 1,2-DAG may provide a sensitive intracellular control mechanism for the regulation of the subsequent activation sequence.

8.4 SUMMARY

Eosinophils responded to stimulation with PAF not only with an accumulation of Ins(1,4,5)P_3 but also with an increase in the concentration of 1,2-DAG. Induction of both signalling systems are known to be initiated by the action of a membrane- associated phospholipase C, providing indirect evidence for a role of this enzyme in eosinophils. Both 1,2-DAG and Ins(1,4,5)P_3 may be directly and indirectly involved in the activation of the cellular protein kinase C.

CHAPTER NINE

9 THE ROLE OF PROTEIN KINASE C

Since its description in 1977 (Inoue *et al.*, 1977), it has become apparent that protein kinase C (PKC) plays an integral role in the activation processes of various different cell types (Nishizuka, 1986). However, to date no information has been available about the existence or possible role of PKC in eosinophils. In addition to measuring the cellular PKC activity directly, several substances have become available during recent years which allow a pharmacological evaluation of the functional significance of this enzyme in signal transduction.

9.1 EFFECTS OF PROTEIN KINASE C ACTIVATORS

It has been shown that the structural analogue of 1,2-diacylglycerol, phorbol myristate acetate (PMA), directly activates PKC by binding to the enzyme, thereby bypassing the proximal activation cascade (Nishizuka, 1984a; Nishizuka, 1986; Tauber, 1987). As shown in Chapter 3, incubation of eosinophils with PMA across a range of concentrations (0.1 pM to 1 µM) leads to secretion of EPO (Figure 3.1), TXB_2 release (Figure 3.7) and $\cdot O_2^-$ (Figure 3.8). Given its mode of action on cells, these data indicate that (1) eosinophils, truly, possess protein kinase C activity, and (2) that selective activation of this enzyme is itself a sufficient trigger to induce functional responses in eosinophils. PMA, however, affected neither the $[Ca^{2+}]_i$ nor the intracellular $Ins(1,4,5)P_3$ level (see Chapters 6.2 and 7.1).

For further characterization of the effect of PKC on eosinophil functions, eosinophils were preincubated with PMA at increasing concentrations (1 nM to 10 µM), washed three times, and stimulated with PAF. As shown in Fig. 9.1A, pretreatment of eosinophils with PMA attenuated the secretion of EPO in a concentration-dependent fashion with an IC_{50} value of 1.8 nM.

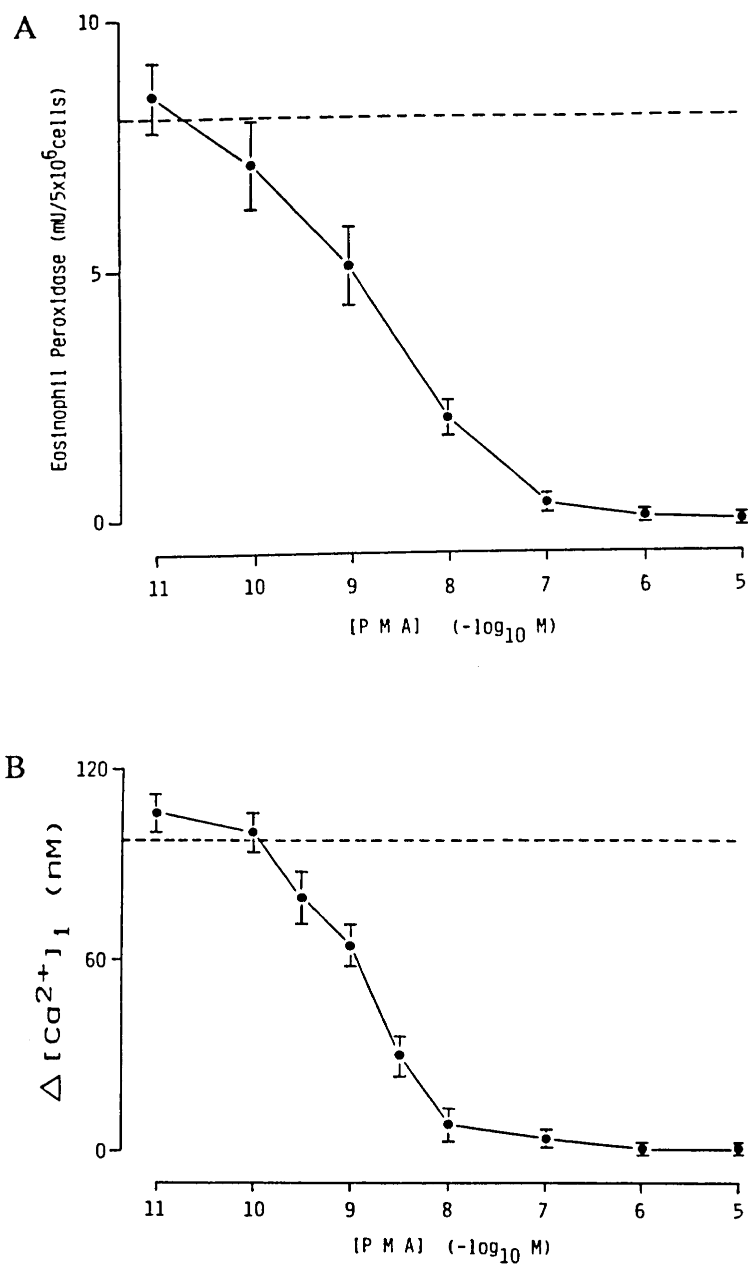


Figure 9.1: Effect of PMA preincubation on PAF-induced EPO release (A) and $[Ca^{2+}]_i$ (B). Eosinophils were incubated with PMA (10 pM to 10 μM) for 10 min (37°C), washed three times, resuspended, and the response to 100 nM PAF measured. The broken line indicates the value for the PAF-stimulated control preincubated with buffer instead of PMA. Data points shown represent the mean ± SEM of six observations obtained from three eosinophil preparations.

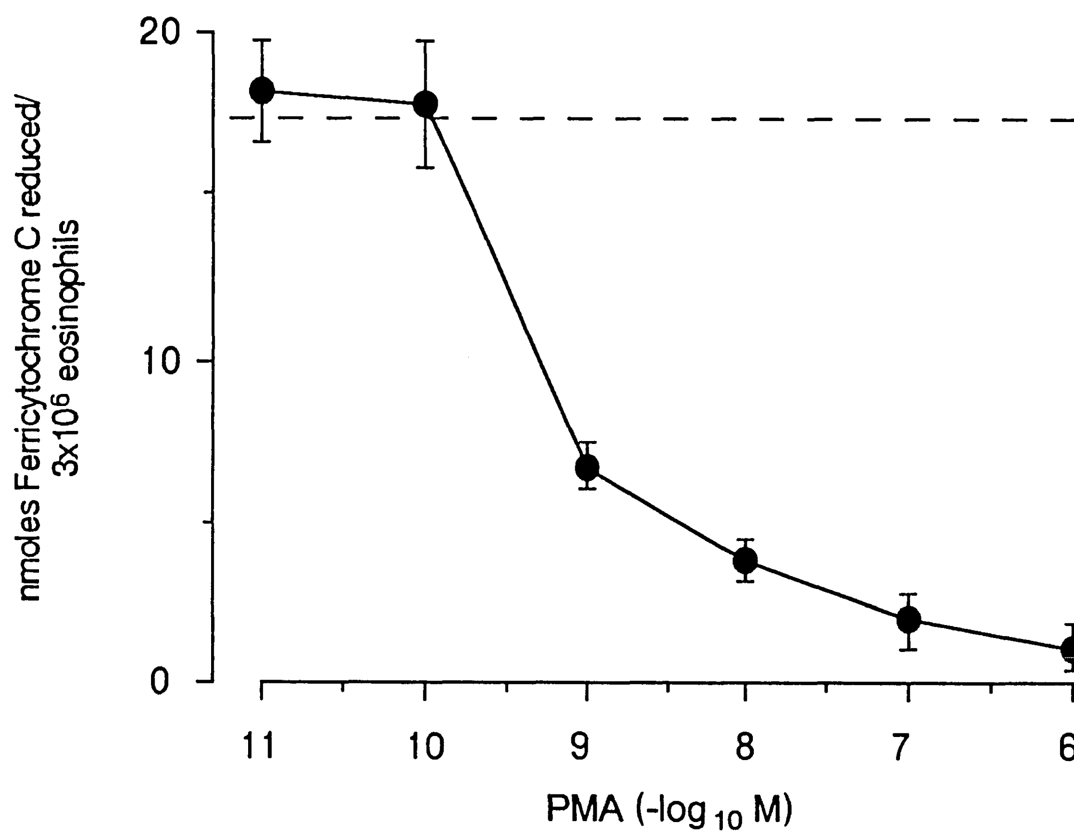


Figure 9.2: **Effect of PMA on PAF-induced superoxide anion production.** Eosinophils preincubated with PMA (1 pM - 1 µM) were stimulated with 30 µM PAF and the generation of $\cdot O_2^-$ was monitored continuously as described in Methods (Chapter 2.10).

Similar results were obtained when the effect of PMA on the PAF-promoted increase in $[Ca^{2+}]_i$ was studied. As demonstrated in Chapter 7 (see page 149), challenge of eosinophils with PAF resulted in a concentration-dependent mobilization of $[Ca^{2+}]_i$. When these cells were pretreated with PMA at concentrations ranging from 10 pM to 5 µM, the PAF-induced effect was inhibited in a concentration-dependent manner (Figure 9.1B). The IC_{50} value was calculated as 2.1 nM. Both eosinophil responses were abolished at concentrations of PMA > 1 µM. This inhibition occurred rapidly and was completed within 1 min, the first time point measured (data not shown). In addition, the inhibition was heterologous, in that PMA also attenuated activation of eosinophils by LTB_4 and C5a. In contrast to the effects of PMA, 4-α-phorbol 12,13-didecanoate, which does not activate protein kinase C, failed to inhibit PAF induced EPO secretion or $[Ca^{2+}]_i$ increase promoted by PAF.

Using a similar experimental design the effect of PMA on PAF-promoted $\cdot O_2^-$ was investigated (Figure 9.2). Cells ($3x10^6$) were exposed to PMA (1 pM to 1 µM) for 10 min prior to the addition of PAF (30 µM). As for $[Ca^{2+}]_i$ and EPO release induced by PAF, $\cdot O_2^-$ production elicited by PAF was also inhibited by prior treatment with PMA. This effect was concentration-dependent with a corresponding IC_{50} value of 10.3 nM (n=5).

9.2 EFFECTS OF PKC INHIBITORS

Although several drugs with a potential PKC inhibitory action have been described in recent years, the specificity of most is questionable. For the following studies, staurosporine, a microbial alkaloid (Tamaoki *et al.*, 1986), was chosen because it abolished the PKC-catalyzed phosphorylation of a human platelet derived 47 kDa substrate protein, whereas the other agents were either inactive or gave only partial inhibition (Watson *et al.*, 1988). Staurosporine (SPN) by itself at concentrations ranging from 30 nM to 300 nM had no effect on basal or PAF-induced increase in $[Ca^{2+}]_i$.

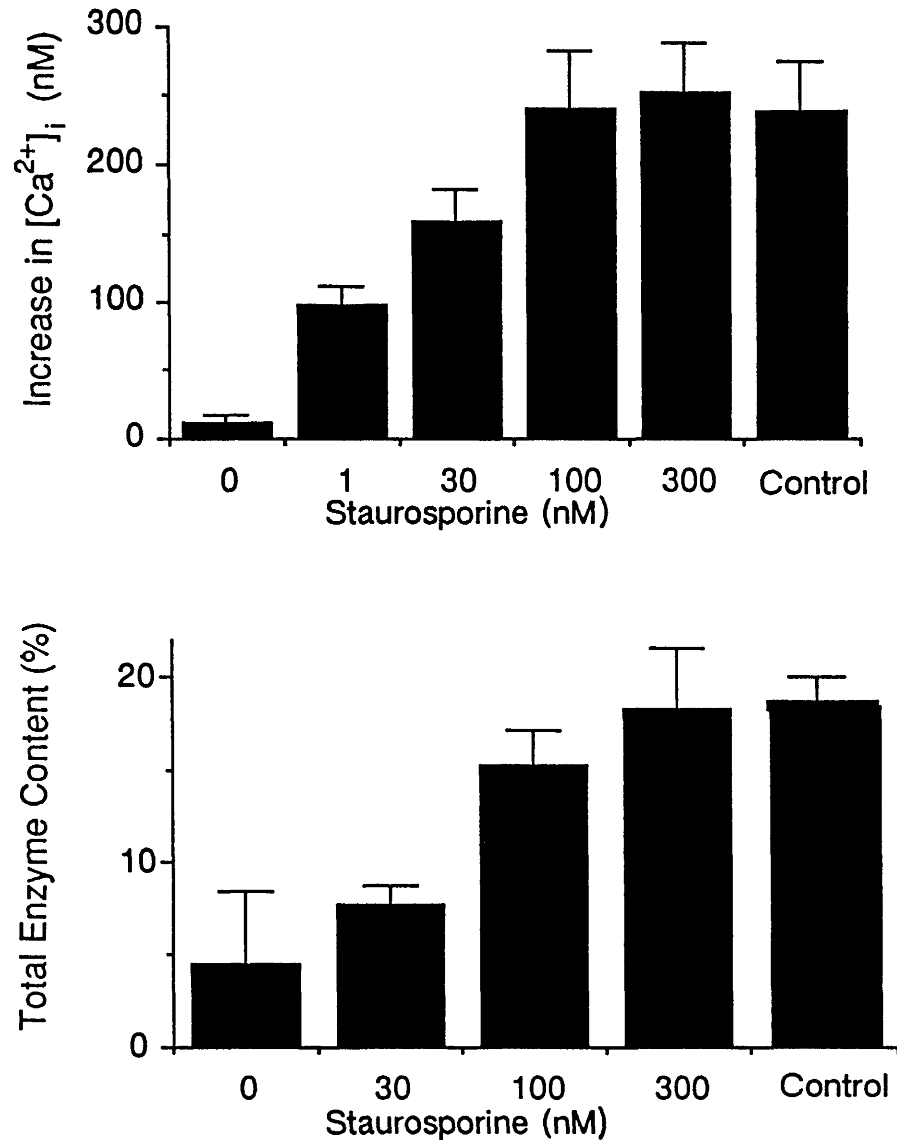


Figure 9.3: Effect of staurosporine on the PMA-induced inhibition of PAF-promoted rise in $[Ca^{2+}]_i$ (A) and EPO secretion (B). Purified eosinophils were preincubated twice (with buffer or staurosporine at 1, 30, 100, and 300 nM for 20 min followed by 10 nM PMA for 10 min), washed and stimulated with PAF (100 nM). Enzyme secretion is given as percentage of total cellular enzyme content. Histograms represent mean ± SEM from seven observations obtained from three independent experiments.

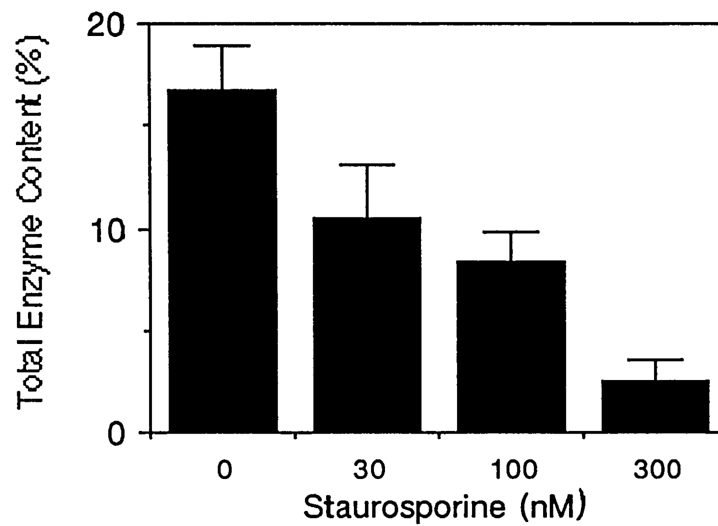


Figure 9.4: Effect of staurosporine on PAF-promoted EPO release. Purified eosinophils were preincubated with staurosporine at 30, 100, and 300 nM for 20 min, washed and stimulated with PAF (100 nM). Enzyme secretion is given as percentage of total cellular enzyme content. Data points shown represent six observations obtained from two independent experiments.

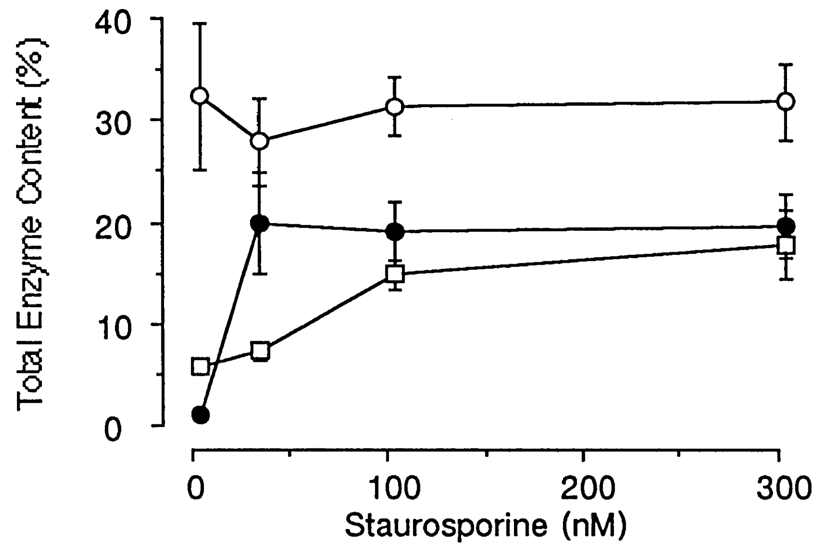


Figure 9.5: Effect of staurosporine treatment on PAF, calcimycin, and PMA pretreated eosinophils. As described in the legend to Figure 9.3, eosinophils were incubated with buffer and staurosporine (30 to 300 nM) for 20 min. Cells were then washed three times, and exposed to either PAF (100 nM; ●), PMA (10 nM; □), or calcimycin (1 µM; ○), washed again, and re-challenged with PAF (100 nM). Data were obtained in triplicate from two experiments.

However, as shown in Figure 9.3, prior treatment of the cells with staurosporine resulted in a reversal of PMA-induced inhibition of $[Ca^{2+}]_i$ increase (Figure 9.3A) and EPO release (Figure 9.3B), suggesting that inhibition of the PAF responses is mediated via PKC activation. This reversal of the inhibitory effects of PMA by staurosporine was concentration-dependent, with EC_{50} value of 9.7 nM and 53 nM, respectively. When eosinophils were treated with staurosporine at concentrations greater than 2.5 μM, the rise in $[Ca^{2+}]_i$ following the addition of PAF was reduced (data not shown). This may be due to the cytotoxic effect of this drug, or in addition to the inhibition of PKC there may be other mechanisms operating in addition to the inhibition of PKC at higher concentrations of staurosporine (Tamaoki *et al.*, 1986).

In contrast to its effect on basal $[Ca^{2+}]_i$ levels, the EPO release induced by PAF was inhibited by staurosporine alone (10-300 nM) in a concentration-dependent manner (Figure 9.4) and with an IC_{50} of 66 nM (n=6). Furthermore, staurosporine was able to reverse the PMA-induced inhibition of EPO secretion promoted by PAF. In addition, PAF causes homologous desensitization, i.e. preincubation with PAF (100 nM) abolished the response to a subsequent challenge with PAF. However, as demonstrated in Figure 9.5, incubation with staurosporine prior to the first exposure to PAF reversed this PAF-induced homologous desensitization (see also Chapter 7.1, page 152). In contrast, when the eosinophils were preincubated with staurosporine, treated with calcimycin (1 μM), followed by stimulation with PAF, staurosporine had no effect on the responses (Figure 9.5).

The presence of staurosporine (100 nM) did not alter the PMA-induced $\cdot O_2^-$ production (Table 9.1). Though staurosporine (100 nM) slightly enhanced the effect of PAF (30 μM), this enhancement was not statistically significant ($p > 0.05$, n=7).

Treatment	PMA (10 nM)	PAF (30 μM)
Control	1.21 ± 0.01*	0.55 ± 0.07
Staurosporine (100 nM)	1.32 ± 0.06	0.67 ± 0.07

* nmoles Ferricytochrome C reduced $\cdot 3 \times 10^6$ eosinophils$^{-1} \cdot$min^{-1}

Table 9.1: Effect of staurosporine on PAF and PMA-induced superoxide anion production in eosinophils. Data shown represent mean ± SEM of 6 determinations.

9.3 EFFECT OF PMA ON [^{3}H]WEB 2086 BINDING

Protein kinase C has been shown to exert negative feedback on signal transduction systems (Berridge, 1986) and several different mechanisms have been proposed including a decrease in the number of receptors expressed at the cell membrane. In order to see if PMA affected PAF receptor expression eosinophils were pre-incubated with PMA (100 nM to 1 nM) for 5 min at 37°C and washed three times in HBSS and the specific binding of [^{3}H]WEB 2086 determined. The results summarized in Figure 9.6 clearly show that PMA significantly reduced specific binding of [^{3}H]WEB 2086 to eosinophils. These data suggest, that PMA interferes with PAF-response-coupling on the receptor level by reducing receptor expression in eosinophils.

9.4 MEASUREMENT OF EOSINOPHIL PROTEIN KINASE C

In order to understand more about the molecular mechanism of PMA-induced PKC activation in eosinophils we studied the distribution of PKC in eosinophils. In unstimulated cells the majority of PKC activity was found in the cytosol (Table 9.2). In contrast, after stimulation with PMA (1 μM) for 10 min, most of the PKC activity was found in the particulate (membrane) fraction. However, no change in PKC activity could be observed when PAF was used as a stimulant.

9.5 DISCUSSION

Protein kinase C (PKC) is widely distributed in various cell types (Kuo *et al.*, 1980) and has been purified from brain and other tissues (Kikkawa *et al.*, 1982). The enzyme consists of a single polypeptide chain of molecular weight 77-80 kDa and comprises of two functional domains. Cleavage of the molecule by a thiol protease yields a hydrophobic domain that interacts with Ca^{2+} and phospholipids, and is responsible for the binding of the enzyme to membranes. The other more hydrophilic

	Histone H1 kinase activity (pmol/min/10^6 cells)	
	particulate fraction	soluble fraction
Untreated	not detected	4.65 ± 0.71
PMA-treated	0.69 ± 0.21	3.29 ± 0.09

Table 9.2: Effect of PMA on the distribution of PKC activity in eosinophils. The results shown represent 6 determination obtained from three independent experiments.

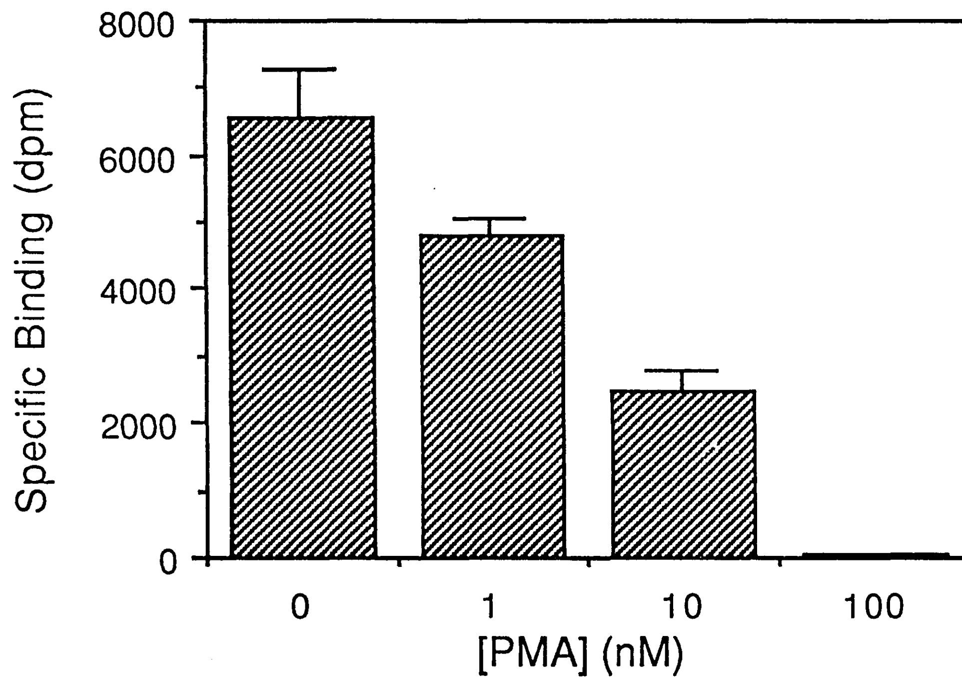


Figure 9.6: Effect of PMA on specific binding of [^{3}H]WEB 2086 to eosinophils. Cells were incubated with PMA (1 nM to 100 nM) for 5 minutes ate 37°C. Specific binding was calculated from the difference in cell-associated radioactivity between cells incubated in the presence of 1 µM unlabelled PAF and cells incubated in its absence. Cells were incubated with 100 nM [^{3}H] WEB 2086 at 37°C for 60 minutes. Data represent mean ± SEM obtained from three independent experiments.

fragment carries the catalytic site and is active in the absence of Ca^{2+}, phosphatidylserine and 1,2-DAG. In resting neutrophils, PKC activity to histones is mainly confined in the cytosol whereas stimulation with PMA enhances particulate associated PKC activity (Christiansen *et al.*, 1988). The results shown in this Chapter extend this observation to include a similar translocation of the PKC activity from the cytosol to membrane in eosinophils.

A number of phorbol ester-dependent isomers of the PKC, termed PKC- α to PKC- ε (Kikkawa *et al.*, 1987, Nishizuka, 1988) have been characterized and a differential increase in all enzyme levels is associated with cell differentiation (Makowske *et al.*, 1988) and stimulation (Sekiguchi *et al.*, 1987). In addition to these PKC isotypes, other cellular kinase activities have been described (Ryves *et al.*, 1989). The existence of multiple forms of the enzyme suggests that PKC may possess different substrate specificities (Sekuguchi *et al.*, 1987; Nishizuka, 1988) and that this may contribute to a more complex diversity in cellular signalling pathways.

Protein kinase C phosphorylates proteins on serine residues and classically requires Ca^{2+} and an acidic phospholipid such as phosphatidyl serine for this activity. In the presence of 1,2-DAG, the requirement for Ca^{2+} is reduced and the absolute level of kinase activity is enhanced above the activity observed in the presence of Ca^{2+} alone (Nishizuka, 1984a). 1,2-DAG is normally absent from membranes but agonist-elicited activation of PLC can yield elevated levels of 1,2-DAG. Translocation of PKC from cytosol to membranes is thought to be crucial for activation of the enzyme presumably through a direct association of 1,2-DAG and PKC. Moreover, Ca^{2+} controls PKC binding to membranes, and in the presence of 1,2-DAG controls both activity and binding (Bell *et al.*, 1986). These potential interactions between Ca^{2+}, 1,2-DAG and PKC may provide a network of positive and negative feedback regulatory mechanisms which may control both the magnitude and duration of the cellular response to activation by exogenous stimuli.

Tumour promoting phorbol esters insert into the cell membrane, substituting for 1,2-DAG and triggering translocation and activation of PKC. As demonstrated above, this mechanism has been proved to represent a sufficient trigger for activation of eosinophils. The presence of PMA activated the generation of $\cdot O_2^-$, release of granule contents as well as lipid mediator synthesis. Although a synergism between Ca^{2+} and PKC has been postulated, the response of eosinophils to phorbol esters were not affected by the absence of extracellular Ca^{2+}.

In addition to playing a role in the activation of cellular responses, PKC can also act as a negative modulator to responses elicited by Ca^{2+}-mobilizing receptors. Concordant with this role in cell signalling, experiments demonstrated in Chapter 7 have shown that PMA inhibit several PAF-induced eosinophil functions. Furthermore, the PKC inhibitor staurosporine, prevents this inhibitory effect of PMA. These results are in agreement with findings in neutrophils (Naccache *et al.*, 1985) and other cell systems (Watson *et al.*, 1985; Vegesna *et al.*, 1988; Winkler *et al.*, 1988) and underscore the central role of these kinases in the activation sequence.

However, interpreting the results from those experiments requires caution. The reason(s) being that phorbol esters are just as potent as non-physiological PKC activators and studies with cell-permeant DAGs, such as dioctanylglycerol, suggest that actions of 1,2-DAG and phorbol esters are not equivalent. For example DAG is rapidly broken down in the cell whereas phorbol esters are not (Nishizuka, 1986). Similarly, though a wide range of 'PKC inhibitors' have been described in recent years, most of them show only poor selectivity for the enzyme. Even staurosporine competes with ATP for the catalytic site of the enzyme suggesting an effect on cyclic-nucleotide-dependent kinases (Tamaoki *et al.*, 1986; Dawson *et al.*, 1987). Thus, in order to further define the regulatory function of PKC in cell activation, competitive agonists will have to be developed. Since staurosporine has such a high affinity for the catalytic sites of protein kinases (K_i = 0.3 nM), this molecule may be a suitable starting point for developing a specific inhibitor.

9.6 SUMMARY

The results presented in this chapter allow for the following conclusions. First, eosinophils possess PKC activity which is found mainly in the cytosol. Stimulation of eosinophils with PMA led to a redistribution and translocation of the PKC activity to the membrane. Second, although no direct change in enzyme activity was detected following PAF-challenge, the involvement of PKC in PAF-induced signalling is clearly evident from experiments using the PKC activator PMA and the PKC inhibitor staurosporine. These experiments indicate that PKC is involved not only in the PAF-promoted EPO release and change of $[Ca^{2+}]_i$ levels but also in the PAF-RII mediated generation of $\cdot O_2^-$. However, staurosporine inhibits the PAF-RI-mediated release of EPO but has no effect on the low affinity receptor induced $\cdot O_2^-$ production, further confirming the diversity of PAF transduction pathways in eosinophils. Finally, activation of PKC may be a crucial element in signal transduction, providing a negative feedback signal which serves to terminate cell activation. One other possible contributory mechanism may be that PKC reduces receptor expression on eosinophils.

CHAPTER TEN

10. DISCUSSION

The eosinophil leukocyte represents an important effector cell in the defense against infections by parasites, through the release of various inflammatory mediators, toxic proteins and oxygen radical scavengers. In order to effectively accomplish these functions the eosinophil is integrated into the complex immunological network, that consists of regulatory cells and humoral messengers. Like other leukocytes, the eosinophil possesses a highly regulated and differential sensory transduction system that selectively detects specific humoral signals which, in turn, activate a variety of cellular effector responses including chemotaxis, secretion, adherence, generation of lipid mediators and production of superoxide anion, enabling the eosinophil to destroy parasites. However, when antigen recognition and regulation of these functions is faulty the result can be disastrous for the host, propagating chronic inflammation with recurrent tissue damage followed under certain circumstances by fibrosis. For instance, it has been suggested that a biochemical lesion may differentiate leukocytes from asthmatic subjects with those from normals and that this difference may underlie the development of asthma (Garland, 1989). This suggests that an understanding of the molecular mechanisms regulating cellular effector functions may contribute a clearer definition of the role of the eosinophil in disease and could be valuable in designing new strategies for the treatment of eosinophil-associated disorders.

10.1 EOSINOPHIL ACTIVATING FACTORS

The number of substances with potential eosinophil activating properties is still comparatively small. Among them, calcimycin, or the Ca^{2+}-ionophore A23187,

a polyether antibiotic produced by *Streptomyces chartreusensis*, and the tumor promoting agent, PMA, represent valuable tools for evaluating the potential role of either Ca^{2+} or PKC and their interaction in eosinophil activation (see below). However, since these agents are non-physiologic and cytotoxic at certain concentrations (Henderson *et al.*, 1983, Fukuda *et al.*, 1985; Baskar & Pinkus, 1988) they have no pathophysiological significance *in vivo.* Other factors such as the mast cell-derived acidic tetrapeptides, Val-Gly-Ser-Glu and Ala-Gly-Ser-Glu (eosinophil chemotactic factor of anaphylaxis or ECF-A), which have occupied the work of several laboratories over many years and which were considered to be selective eosinophil chemoattractants (Goetzl & Austen, 1977), are now not only known to be ineffective *in vivo* (Bryant & Kay, 1977) but show negligible activity *in vitro* when compared to other stimuli (Wardlaw *et al.*, 1986; Kroegel *et al.*, 1981). In contrast, complement proteins such as C3a and C5a (assessed using opsonized parasites, zymosan or sepharose) have been implicated as eosinophil activators that facilitate chemotaxis (i.e. C5a) and binding of eosinophils to large surfaces thereby inducing secretion of granular contents (Ichikawa, *et al.*, 1974; Ogawa *et al.*, 1981; Yazdanbakhsh *et al.*, 1985; Henderson *et al.*, 1988; Spry, 1988).

Although the potential role of immunoglobulins in eosinophil activation is not yet fully established some immunoglobulin subtypes appear to have eosinophil activating properties. Using rosetting techniques, IgG receptors on eosinophils had been demonstrated as early as 1969 (Henson, 1969). More recent work has confirmed that mature eosinophils bear the $Fc_{\gamma}RII$ receptor (Fleit *et al.*, 1984; Hartnell *et al.*, 1990) which is known to bind to multivalent ligands (Anderson & Looney, 1986). Thus, the eosinophil tends to bind to large surfaces coated with antibody, such as parasites. IgG receptor activation causes the generation of LTC_4, respiratory burst and secretion of granular contents (Thorne *et al.*, 1982; Baskar & Pincus, 1988). In addition to $Fc_{\gamma}RII$ sites, $Fc_{\varepsilon}RII$ receptors have been found on eosinophils (Capron *et al.*, 1984; Capron *et al.*, 1985) and activation of this receptor appears to promote the

release of EPO, MBP and ECP (Capron *et al.*, 1989; Capron *et al.*, 1988a). However, using the monoclonal anti-Fc_{ε}RII antibody CDw32, several groups failed to confirm these data. Finally, although a receptor for it has not yet been identified, monomeric IgA and in particular secretory IgA have recently been shown to be potent eosinophil degranulating agents (Abu-Ghazaleh *et al.*, 1989).

Formyl-methionine-containing peptides such as fMLP are chemotactic for a number of cell types are similar to chemotactic factors produced by bacteria. Eosinophils appear to express specific fMLP receptors and respond to the tripeptide with chemotaxis, superoxide anion generation, and prostanoid production (Yazdanbakhsh *et al.*, 1987; Kroegel *et al.*, 1990a) although its effect appears to be small compared to other stimuli (see Chapters 3, 6, and 8).

In addition to the potential eosinophil activating factors mentioned above, there is increasing evidence that three mononuclear cell-derived cytokines, GM-CSF, IL-3, and IL-5, may play an important role in upregulating eosinophil effector functions induced by other stimuli (Silberstein *et al.*, 1986; Owen *et al.*, 1987; Silberstein *et al.*, 1989). However, cytokines themselves either fail to stimulate eosinophil activation or have only a minimal effect (Fujisawa *et al.*, 1990).

The short review of known eosinophil activating factors given above illustrates that many of the potential stimuli have either no direct stimulatory effect (cytokines), are non-physiologic agents (calcimycin, PMA) or are likely to be implicated in the host defense functions of eosinophils against parasites and bacteria (complement proteins, IgG, IgE, fMLP). Among them only IgE and to a lesser extent the complement factors may have some bearing on eosinophil activation in allergic inflammation.

During the past decade, however, increasing evidence that the catabolism of membrane phospholipids yields several bioactive products that participate in inflammation, has accumulated. Of these factors, PAF represents one of the most potent bioactive mediators known, exhibiting a variety of different effects in the

inflammatory tissue reaction underlying asthma (Barnes *et al.*, 1988a, Barnes *et al.*, 1988b). Indeed, several studies have demonstrated that PAF mimics many of the pathophysiological changes which occur during the asthmatic late response including bronchoconstriction (Denjean *et al.*, 1983, Vargaftig *et al.*, 1980; Vargaftig *et al.*, 1981), microvascular leakage (O'Donnell & Barrett, 1987; Evans *et al.*, 1987) and airway hyper-responsiveness in animals (Patterson *et al.*, 1984) and humans (Cuss *et al.*, 1986). In addition, PAF has been shown to be the most potent chemotactic stimulus for eosinophils (Wardlaw *et al.*, 1986; Sigal *et al.*, 1987), inducing eosinophil adherence (Lamas *et al.*, 1988; Kimani *et al.*, 1988) as well as eosinophil cytotoxicity against parasites (MacDonald *et al.*, 1986). The results presented here confirm the potent stimulatory action of PAF on eosinophils and extend its effects to include eosinophil degranulation, superoxide anion release, generation and release of TXA_2, PGE_2, PGD_2 and $PGF_{2\alpha}$. Thus, PAF, although not selective for eosinophils, appears to be a physiologic stimulus capable of promoting not only eosinophil diapedesis and migration but also various eosinophil effector functions implicated in allergic inflammation and tissue damage. The interaction of PAF and eosinophils in asthma may, therefore, have important implications for the pathophysiology of allergic disorders.

10.2 PAF-MEDIATED EOSINOPHIL EFFECTOR FUNCTIONS

As outlined above, eosinophils challenged with PAF respond with a wide range of different responses. PAF at a concentration ranging from 1 pM to 10 μM stimulates the release of TXB_2 and other prostanoids, granular enzymes, LTC_4 synthesis, adherence and chemotaxis (Table 10.1). In addition, all of these functional responses of eosinophils, with the exception of $\cdot O_2^-$ generation, are maximal at PAF concentrations ranging between 100 nM and 1 μM (Table 10.1), indicating a common activation mechanism for these effector functions.

	Eosinophil			
	Human		Guinea Pig	
	Concentration		Concentration	
Cellular Responses	Range	Maximal	Range	Maximal
Adherence	100 pM -10 μM	1 μM	n.d.	
Chemotaxis	1 nM - 10 μM	1 μM	10 nM - 1 μM	1 μM
Cytotoxicity	1 nM - 10 μM	1 μM*	10 nM - 100 nM	100 nM**
Degranulation#	100 pM - 10 μM	100 nM	10 nM - 10 μM	100 nM
Leuktriene C_4	100 nM - 10 μM	1 μM	n.g.	
Thromboxane A_2	1 nM - 10 uM	1 μM	1 nM - 10 μM	1 μM
Prostaglandin E_2	10 nM - 10 μM	1 μM	10 nM - 10 μM	10 μM
Superoxide anion	1 μM - 30 μM	30 μM	1 μM - 30 μM	30 μM

n.d. not done
n.g. not generated
* Complement-coated parasites
** Guinea pig bronchial epithelium
# eosinophil peroxidase

Table 10.1: PAF-induced responses by human and guinea pig eosinophils.

In contrast, however, production of $\cdot O_2^-$ occurred at concentrations (1 μM to 100 μM) of PAF that did not release appreciable amounts of TXB_2 or EPO. In addition, the maximal effective concentration of PAF (30 μM) was 30 to 100-fold higher than the respective concentrations for TXB_2 and EPO with an EC_{50} of 8.4 μM. Like the other responses of the eosinophils the generation of $\cdot O_2^-$ was rapid with a $t_{1/2}$ = 110 sec, reaching completion in less than 7 min. In spite of the obvious differences, both TXB_2 and EPO release as well as the production of $\cdot O_2^-$ were selectively antagonized by WEB 2086 (IC_{50} = 47.3 μM).

10.3 MOLECULAR MECHANISMS OF PAF-INDUCED EOSINOPHIL ACTIVATION

10.3.1 PAF-receptor interactions

The first event in the cell activation cascade is the interaction of PAF with its specific receptors on the cell surface. As estimated from the near instantaneous accumulation of 1,2-DAG, Ins(1,4,5)P_3 and mediator release, the process appears to be very rapid, commencing within the first second post PAF challenge.

PAF binds to two classes of sites on eosinophils: a high affinity receptor with a average dissociation constant K_d of 0.3 nM and a low affinity receptor with an average, K_d of 11.5 nM. Of the total population of receptors detected on the eosinophil, approximately 6% per cell were of high affinity and an estimated 94% per cell were of a the low affinity subset. When [^{3}H]WEB 2086 was used as a radioligand, an average K_d of 16.1 ± 0.6 nM for the high affinity receptor was determined, indicating a 50-fold lower affinity for the receptor antagonist. Binding of [^{3}H]PAF and [^{3}H]WEB 2086 to the receptor was found to be selective, saturable and reversible by addition of excess unlabelled agonist or WEB 2086 confirming that the site occupied by WEB 2086 is indeed the receptor for PAF. The K_d found for PAF binding to eosinophils is comparable to the respective binding constants in human

eosinophils, neutrophils and platelets (Dent *et al.*, 1988; Dent *et al.*, 1989a; O'Flaherty *et al.*, 1986; Tuffin *et al.*, 1985). A good correlation was found between the affinity of the binding sites derived from Scatchard analysis of equilibrium binding, and the K_i values, derived from the concentration of unlabelled antagonist required to displace 50% of [^{3}H]WEB 2086. In addition, the binding parameters correlated with both K_B and EC_{50} values obtained from several PAF-induced functional responses. Thus, binding of PAF to the putative receptors occurs within the same concentration range that induces eosinophil effector functions.

The fate of the PAF-receptor-complex on eosinophils is not known. However, as observed in other cells (Homma *et al.*, 1987; Valone *et al.*, 1982; O'Flaherty *et al.*, 1986), the PAF-receptor-complex on eosinophils is probably incorporated into the cells. PAF may then dissociate from the receptor molecule and undergo further metabolism. PAF is first catabolized by an acetyl hydrolase into alkyl lyso-PAF, which appears to represent a transient intermediate (Fig. 4.5, peak 1). Alkyl lyso-PAF is further degraded into alkylacyl-glycerophosphocholine (GPC) and neutral fatty acids, with exogenous PAF being converted at a steady rate of 0.3 to 0.5% per minute at 37°C. It is only after 120 min that small amounts of alkyl acyl-glycero phosphoethanolamine appear, suggesting a resynthesis of other phospholipid classes using moieties of the metabolized PAF molecule. This rapid uptake and metabolism of PAF may account for the high non-specific binding observed when intact eosinophils were used. The finding that the metabolism of the mediator in the membranes totalled approximately half of that observed in whole cells, suggests that PAF catabolism takes place in both the membrane and the cytosol.

Endocytosis of receptor-ligand complexes is a common phenomenon, which has been extensively studied in the case of the fMLP receptor on neutrophils (Sullivan & Zigmond, 1980; Niedel *et al.*, 1979) and the IgE receptor on rat basophilic leukemia cells (Furuichi *et al.*, 1986; Rivera *et al.*, 1986). The biological significance

of PAF-receptor internalization and degradation will be discussed further in Chapter 10.5.3 (page 207).

10.3.2 The role of GTP-binding proteins

Activation of a guanine nucleotide binding (G) protein is considered an essential step in coupling a large class of receptors to intracellular effector enzymes or ion channels. The G proteins that have been characterized to date comprise a family of heterotrimers containing a structurally distinct α-subunit and very similar or identical β- and γ-subunits. They serve as regulatory elements interposed between receptors and intracellular effectors (see Chapter 5.3). The three membrane-bound components are inactive when associated with a GDP molecule. A conformational change in the cell-surface receptor triggers the exchange of GTP for the bound GDP on the α subunit and results in dissociation of the α subunit from the β and γ subunits leading, in turn, to activation of an intracellular effector enzyme such as adenylate cyclase or PLC (see below).

Evidence for the existence of a G-protein in eosinophils was first derived by the successful ADP-ribosylation of a 41 kDa eosinophil membrane protein by PTX (Fig.5.4). The major substrate for this PTX catalyzed ADP-ribosylation is G_i, a guanine nucleotide regulatory protein involved in receptor mediated inhibition of adenylyl cyclase (Smith *et al.*, 1986). In addition, preincubation of eosinophils with PTX suppresses the rise in $[Ca^{2+}]_i$ induced by PAF, LTB_4 and C5a (Fig. 5.2, page 136). Furthermore, PTX also inhibits the PAF promoted release of EPO and TXB_2 while the activation of eosinophils by PMA (Fig. 5.1, page 134) or melittin (Kroegel *et al.*, 1990a) was not affected. Taken together, these observations indicate the existence of a G_i-like GTP-binding protein that is coupled to the PAF-RI receptor and suggest that in eosinophils this protein plays a crucial role in PAF signalling. In addition to PAF, the data show that the proposed LTB_4- and C5a-receptors on eosinophils are probably also linked to a similar GTP-binding protein.

In contrast to the PAF-RI mediated cellular responses, PTX had no effect on the PAF-induced generation of $\cdot O_2^-$ (Fig. 5.3, page 137). This finding is intriguing since in the first instance it supports the hypothesis that two selective PAF receptors are found on eosinophils and, secondly, it indicates that the two receptors may be differentially coupled. This raises the possibility that the PAF-signal may utilize at least two distinct transduction pathways within the same cell. It should be noted here, that neutrophils also appear to express two distinct PAF receptor subsets which differ in their sensitivity towards PTX (Naccache *et al.*, 1985; Naccache *et al.*, 1986; O'Flaherty *et al.*, 1986). The possible implications and mechanisms of these findings are discussed further in section 10.6 (page 209).

10.3.3 Putative role of phospholipase C

It is generally accepted that the GTP-binding proteins can directly activate membrane bound phospholipase C (Cockcroft & Gomperts, 1985; Dechmyn *et al.*, 1986). This ubiquitous enzyme hydrolyses mono-and polyphosphoinositides (i.e. Ins(4,5)P_2) yielding both 1,2-DAG and Ins(1,4,5)P_3 thereby linking the receptor-G-protein complex to intracellular second messenger systems. Although the activation of PLC in eosinophils was not measured directly, the PAF-promoted accumulation of 1,2-DAG and Ins(1,4,5)P_3(see 10.3.5 and 10.3.6, pages 165 and 166) provides indirect evidence that this enzyme is involved during PAF-response coupling in eosinophils.

10.3.4 Production of 1,2-Diacylglycerol

As demonstrated here for the first time, eosinophils stimulated with PAF respond with a rapid and transient, two- to four-fold increase in the concentration of 1,2-DAG over basal levels (Figures 8.1 and 8.2). Recently, it has been reported that activation of the CR3 receptor on human eosinophils also leads to an increase in 1,2-DAG (Koenderman *et al.*, 1990). However, in other inflammatory cells such as neutrophils, a rise in 1,2-DAG concentration has proved difficult to demonstrate.

There are several alternate explanations for these difficulties including the fact that the agonist-induced generation of 1,2-DAG may occur only in a small compartment of the cell, i.e. in the plasma membrane, with little effect on total cellular 1,2-DAG. The subcellular localization of newly formed 1,2-DAG is, however, important, since it allows protein kinase C to be selectively translocated onto, or near to, the plasma membrane. 1,2-DAG together with Ca^{2+} serves as a cofactor in the activation of PKC (Nishizuka, 1984a; Nishizuka, 1986; Rider & Niedel, 1987). In addition, further catabolism of 1,2-DAG either by diglyceride lipase or DAG kinase produces arachidonic acid and phosphatidic acid, respectively, which may also contribute to the Ca^{2+} mobilization and lipid mediator formation as well as activation of PKC (Sekiguchi *et al.*, 1987).

10.3.5 Phosphotidylinositol Metabolism

The data presented in chapter 6 show that eosinophils stimulated with PAF or other stimuli respond with the formation of one of these inositol phosphate metabolites, namely Ins(1,4,5)P_3. Ins(1,4,5)P_3 is capable of releasing Ca^{2+} from vesicular intracellular stores (Streb *et al.*, 1983; Prentki *et al.*, 1984). Accumulation of Ins(1,4,5)P_3 was rapid and transient, displaying a peak response within 5 sec and returning to basal values by 60 sec. In addition, the effect of PAF was concentration-dependent, non-cytotoxic and inhibited by WEB 2086, indicating that Ins(1,4,5)P_3 production by PAF was specific and receptor-mediated. Moreover, accumulation of Ins(1,4,5)P_3 was also observed with LTB_4, C5a, and fMLP whereas the protein kinase C (PKC) activator PMA, had no effect on Ins(1,4,5)P_3 accumulation or $[Ca^{2+}]_i$ at any concentration examined. The mass of Ins(1,4,5)P_3 measured in eosinophils, allowed the calculation of the absolute concentration of Ins(1,4,5)P_3 per 10^6 cells within the cytosolic compartment under resting and stimulated conditions. Assuming an average eosinophil radius of 13 μm and that approximately 50% of the eosinophil volume is cytosol (Beeson & Bass, 1977) our data indicate that PAF increases Ins(1,4,5)P_3 from

approximately 0.16 μM at resting conditions to 0.6 μM 5 seconds after the addition of PAF (1 μM). These values are comparable to the cellular Ins(1,4,5)P_3 concentrations in platelets (basal 0.2 μM, 1 μM upon maximal stimulation with thrombin after 10 sec) (Tarver *et al.*, 1987), in HL60 cells (0.4 μM basal to 1.6 μM within 35 sec of fMLP stimulation) (Shears., 1989), and neutrophils (basal 0.1 μM to 1 μM upon maximal stimulation) (Bradford & Rubin, 1986) but lower than values reported for AR42J pancreatoma cells where basal and substance P-stimulated Ins(1,4,5)P_3 concentrations were 2 μM and 35 μM, respectively (Horstmann *et al.*, 1988). The authors of this latter work, however, recognized that the putative Ins(1,4,5)P_3 may have been compartmentalized, or contaminated with an unknown inositol phosphate. The transient nature of Ins(1,4,5)P_3 accumulation in guinea pig eosinophils following PAF stimulation suggests that this inositol phosphate is rapidly metabolized to either Ins(1,4)P_2 or Ins(1,3,4,5)P_4 (Majerus *et al.*, 1988). The mechanism regulating Ins(1,4,5)P_3 metabolism in eosinophils, however, is not yet understood. Given the kinetics of the increase in both Ins(1,4,5)P_3 and $[Ca^{2+}]_i$ as well as the effect of Ins(1,4,5)P_3 on the release of Ca^{2+} from intracellular stores, the increase in $[Ca^{2+}]_i$ may itself provide a negative feedback signal to limit the accumulation of Ins(1,4,5)P_3. For instance in both R1N m5F cells (Wollheim & Biden, 1986) and human leukaemic HL-60 cells (Lew *et al.*, 1986b) it has been demonstrated that Ca^{2+} activates the Ins(1,4,5)P_3 3-kinase responsible for the conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 (Majerus *et al.*, 1988; Hansen *et al.*, 1986; Irvine *et al.*, 1986).

10.3.6 Calcium and PAF signalling in eosinophils

Calcium serves as a ubiquitous cofactor for various intracellular processes that are involved in sustaining cellular homeostasis as well as in secretory events following cell activation. The average basal $[Ca^{2+}]_i$ level in guinea pig eosinophils is 109 nM (n=33) and 96 nM in human blood eosinophils (n=3). However, PAF stimulation leads to transient 2-to 10-fold increase in $[Ca^{2+}]_i$ in

eosinophils. The response is heterogenous, with some individual cells failing to respond, yet other eosinophils displayed a $[Ca^{2+}]_i$ increase of more than twice the mean. Furthermore, some eosinophils appear to respond with repeated $[Ca^{2+}]_i$ oscillations of smaller magnitude after the initial peak.

On average the rise in $[Ca^{2+}]_i$ occurred after a lag time of approximately 3 to 5 seconds, reaching a peak after 15 to 20 seconds before returning to baseline levels in the following two minutes. In contrast, the time course of the PAF-elicited granular enzyme release showed a rapid onset within the first 5 min before reaching a plateau after 15 to 30 min. These data suggest that the rise in $[Ca^{2+}]_i$ represents an early event in eosinophil cell activation and precedes the eosinophil enzyme secretion. Thus our findings indicate that in eosinophils calcium may also play a role as a second messenger linking PAF receptor activation to the subsequent cellular response.

A $[Ca^{2+}]_i$ increase was also observed when eosinophils were challenged with LTB_4, fMLP, and C5a but not with PMA or substance P. The same cells were re-challenged with PAF, LTB_4 and fMLP two minutes later which resulted in a full response to each stimulus. This suggests that either each stimulus only used a minor part of the total $[Ca^{2+}]_i$ or that intracellular Ca^{2+} pools were rapidly restored. Alternatively, the repeated rise of $[Ca^{2+}]_i$ could be facilitated through a receptor-operated Ca^{2+} influx from the extracellular medium (see below).

It has long been appreciated that optimal degranulation of neutrophils or other cells is dependent upon the presence of extracellular calcium ions (Douglas, 1974; Williams, 1980). In addition, extracellular calcium as a source for the rise of the intracellular calcium level has been described in mouse macrophages (Hallam *et al.*, 1984), platelets (Conrad & Rink, 1986), and U937 cells (Ward & Westwick, 1988). The present results indicate that the same is true for PAF-stimulated eosinophils. Although calcium ions appear to be released from internal stores, a considerable amount of the Ca^{2+} necessary for the PAF-elicited increase in $[Ca^{2+}]_i$ in eosinophils came from an influx of exogenous Ca^{2+}. Electrically excitable cells such as muscle cells which

possess voltage-sensitive calcium-channels are inhibited by calcium antagonists including verapamil, diltiazem and the dihydropyridines. However, such channels appear not to be not present in eosinophils since PAF-induced elevation of $[Ca^{2+}]_i$ was insensitive by the dihydropyridine calcium entry blocker, nimodepine. Similar results have been obtained in neutrophils using nifedepin (Korchak, 1984). Thus, in non-excitable cells such as eosinophils and neutrophils, it appears likely that Ca^{2+} entry in PAF-stimulated eosinophils involves receptor-operated channels.

10.3.7 The role of protein kinase C.

Protein kinase C (PKC) comprises a number of isoenzymes (Nishizuka, 1988) and other cytosolic kinase subtypes (Ryves *et al.*, 1989) and plays a crucial role in cell surface signal transduction for a variety of biologically active substances (Nishizuka, 1984a; Nishizuka 1984b). It is activated directly either by diacyl-glycerol, a deacylated phosphatidylcholine, or by the tumour promoting agents such as PMA (or 12-0-tetradecanoyl phorbol-13 acetate) (Niedel *et al.*, 1983). Both agents intercalate into cellular membranes, bind with cytosolic PKC, causing this Ca^{2+}- and phospholipid-dependent enzyme to adhere to the membrane and leading to phosphorylation of related effector proteins (Nishizuka, 1986; Nishihira *et al.*, 1985, Wolfson *et al.*, 1985). In contrast, other more physiological stimuli such as PAF do not directly interact with PKC but rather bind with their respective cell surface receptors inducing the production of diacylglycerol, elevation of free cytosolic Ca^{2+}, and the synthesis of other second messengers that modulate kinase activity (Nishizuka, 1986; O'Flaherty, 1987). In human neutrophils, for instance, leukotriene C_4 and PAF have been shown to mobilize PKC at concentrations that correlate closely with their respective affinities for cellular LTB_4 or PAF receptors (O'Flaherty, 1987).

The first indication of possible PKC involvement in eosinophil activation came from the observation that PMA alone induced the release of granular enzymes (Figures 3.1 and 3.2), generation of TXB_2 (Figure 3.10) and $\cdot 0_2^-$ production

(Figure 3.14). The data shown in Chapter 9 extend these observations and demonstrate that PMA also inhibits PAF-induced eosinophil degranulation as well as Ca^{2+} mobilization (Figures 9.1 and 9.2, pages 170 to 171). Comparable results were obtained when eosinophils were stimulated with leukotriene B_4 or the complement protein C5a. This indicates that PKC represents an integral component of the activation cascade, utilized by PAF and other receptor agonists. In contrast, the calcimycin induced enzyme release was not effected by PMA pretreatment. In addition, preincubation with staurosporine, a relatively potent though not specific PKC inhibitor, reversed the PMA-induced inhibition in a concentration-dependent fashion with an EC_{50} of 9.7 nM. All together, these results provide indirect evidence that PAF-response coupling in eosinophils involves the activation of a PKC.

To further understand the mechanism of PKC activation we studied the effects of PMA on the intracellular distribution of the enzyme in eosinophils. In unstimulated eosinophils all the PKC activity appeared to be cytosolic. PMA at concentrations which induced granule protein secretion and respiratory burst, caused a translocation of PKC from the cytosol to the particulate fraction in eosinophils with approximately 15% of total enzyme activity becoming membrane-associated (Table 9.1, page 177). Translocation of PKC upon stimulation with phorbol esters has also been reported in other cell systems (Nishizuka, 1984a; Nishizuka 1984b; Nishizuka; 1986; Winkler 1988), indicating that translocation of the activated cytosolic enzyme to the particulate fraction may be a general biochemical mechanism of action for phorbol ester activation of PKC. These results suggest that the effect of PMA on PAF-mediated eosinophil responsiveness may be due to its ability to irreversibly activate PKC, leaving the enzyme unresponsive to a second stimulation. Unlike diacylglycerols, which undergo further metabolism in the cell, phorbol esters produce a sustained activation of PKC. Although, very prolonged treatment of cells with phorbol esters can result in loss of PKC activity (Huang *et al.*, 1989) it is evident that an acute, maximal stimulation of PKC by phorbol esters does not necessarily mimic the effects induced by

PAF-activation of the enzyme through 1,2-DAG formation. Agonists that induce a weak activation of PLC, and therefore, cause only a small increase of the diacylglycerol are also likely to cause only a minimal activation of PKC with a relative transient increased phosphorylation of a few target proteins. Alternatively, agonist-induced cell stimulation may cause activation of a receptor-specific kinase, as observed for the insulin receptor (Czech *et al.*, 1988). This might explain (1) why no significant activation of PKC was detected when eosinophils were treated with PAF, and (2) that eosinophils challenged consecutively with different stimulants showed a full responsiveness to each agonist. In contrast, maximal activation of the PKC by phorbol esters may result in prolonged phosphorylation of a large number of target proteins, such as receptor proteins or the PLC, with subsequent distortion of various biological responses.

Overall, these results demonstrate that in eosinophils PKC plays an integral role in signal transduction pathways utilized by PAF. Functionally the enzyme appears to be involved in both positive and negative feedback mechanisms. The finding that staurosporine reverses the PMA-induced inhibition of PAF-elicited EPO release and increase, as well as enhances the PAF induced EPO secretion, suggests that staurosporine may, in fact, inhibit the negative feedback functions of PKC rather than its permissive properties.

Recent work has demonstrated the existence of at least seven distinct mammalian protein kinase C enzymes, suggesting that PKC may comprise a functional diversity in cellular signalling pathways. Different isotypes may possess different substrate specificities and play discrete roles in the activation sequence. Studies in brain support this hypothesis where differential responses to activating agents have been shown to occur (Sekiguchi *et al.*, 1987). It may also be that the activation of PAF-RI or PAF-RII receptors also specifically activates certain isoenzymes resulting in distinct patterns of cellular responses (see Chapter 9.5, page 181).

10.4 RELATIONSHIP BETWEEN THE FORMATION OF SECOND MESSENGERS AND CELL RESPONSES

Within seconds of exposure to PAF, eosinophils begin to respond in a variety of ways. First the intracellular levels of Ins(1,4,5)P_3 and 1,2-DAG are elevated, both following a remarkably similar time course (Figures 6.3 and 8.2). The generation of these two intracellular signals is followed by a rise in $[Ca^{2+}]_i$, which reaches a peak after approximately 15 sec (1 μM PAF). As demonstrated in Chapter 7, the PAF-stimulated increase in $[Ca^{2+}]_i$ in guinea pig eosinophils results from both the release of intracellular Ca^{2+} stores and an influx of extracellular Ca^{2+}. Furthermore, this transient elevation in $[Ca^{2+}]_i$ appears to represent an integral step in signal-response coupling in these cells. The mechanisms responsible for the PAF-induced Ca^{2+} release from intracellular pools, however, remain speculative. Peak accumulation of Ins(1,4,5)P_3 stimulated by 1 μM PAF preceded the peak increase in $[Ca^{2+}]_i$ by approximately 10 to 15 sec confirming that the increase in $[Ca^{2+}]_i$ might be secondary to the formation of Ins(1,4,5)P_3. In addition, there was a significant correlation ($r=0.98$; $p < 0.01$) between the mean peak Ins(1,4,5)P_3 concentration and the average net peak $[Ca^{2+}]_i$ change in eosinophils stimulated with different concentrations of PAF. A significant correlation was also found when these parameters were plotted for other agonists ($r=0.87$; $p < 0.05$). These results are consistent with findings reported in neutrophils (Krause *et al.*, 1985; Spat *et al.*, 1986) and other biological systems (Berridge & Irvine, 1984) and support the hypothesis that Ins(1,4,5)P_3 regulates PAF-induced $[Ca^{2+}]_i$ increase in eosinophils.

The release of prostanoids such as TXB_2 generated by eosinophils is remarkably rapid with a $t_{1/2}$ = 9.2 sec. While maximal levels of TXB_2 are attained between 30 and 60 sec, its rate of production is maximal within the first 5 sec. In other words, maximal TXB_2 synthesis precedes the peak $[Ca^{2+}]_i$ by 10 seconds. This suggests that liberation and activation of arachidonic metabolism through PLA_2 and cyclo-oxygenase occurs at submaximal Ca^{2+} concentrations. Alternatively, direct

activation of PLC by the receptor/G-protein complex may cause a near-instantaneous liberation of arachidonic acid. The inositol lipids contain a high proportion of arachidonic acid in the 2-position of the glycerol moiety, and free arachidonic acid can be produced by further metabolism of 1,2-DAG through sequential degradation of diglyceride and monoglyceride lipases, providing an additional early source for the synthesis of TXB_2. In addition to being metabolized into prostanoids and leukotrienes, this early increase in free arachidonic acid may itself cause a direct release of Ca^{2+} from intracellular stores, independent of the production of Ins(1,4,5)P_3 and further may directly activate PKC (Nishizuka 1988).

While it is possible to hypothesize a cascade of distinct biochemical steps in eosinophils that lead to an increase in $[Ca^{2+}]_i$ and PKC activation, subsequent events of the cell response are less well defined. They appear to include the polymerization of actin filaments, solubilization of core material, active transport of this material from the cytoplasm to the membrane, and other so far unknown processes, eventually leading to the secretion of granular constituents. Enzyme release from guinea pig eosinophils challenged with PAF occurred within 2 min post-challenge and secretion was essentially complete by 15 min post-challenge. Analysis of the kinetic data yielded an average half-maximal release at 134 sec after stimulation. The apparent slower release process for granular proteins as opposed to the rapid release of lipid mediators may be attributable to the more complex cellular degranulation apparatus. Given the rapid generation of second messenger signals described above, these results point to the fact that both Ins(1,4,5)P_3 formation and increase in $[Ca^{2+}]_i$ occur prior to enzyme release, suggesting a role for both intracellular signals in mediating eosinophil enzyme secretion.

As discussed in more detail in Chapter 10.7, guinea pig eosinophils were more responsive to LTB_4 than to PAF. This difference was also observed for the formation of Ins(1,4,5)P3. Interestingly, however, the time course of the elevation in $[Ca^{2+}]_i$ induced by LTB_4 did not differ significantly from that induced by PAF. In

addition, the kinetics and magnitude of the rise in $[Ca^{2+}]_i$ elicited by C5a, the eosinophil chemotactic complement component, were found to be similar to those induced by PAF and LTB_4, although C5a was less effective in promoting enzyme release and Ins(1,4,5)P_3 accumulation. These results indicate that the magnitude of PtdIns(4,5)P_2 hydrolysis as judged by Ins(1,4,5)P_3 accumulation does not necessarily determine the magnitude of subsequent events in the transduction process. One possible explanation may be that LTB_4 generates an overabundance of Ins(1,4,5)P_3 which rapidly saturates Ins(1,4,5)P_3 binding sites in the eosinophil microsomes that are responsible for Ca^{2+} release while PAF and C5a do not. It has been reported, that the peak increase in $[Ca^{2+}]_i$ elevation depends on the rate of Ins(1,4,5)P_3 production in the first few seconds, rather than the eventual magnitude of its accumulation (Thomas *et al.*, 1984). Similar conclusions have been drawn from experiments using pancreatic acinar cells (Merrit *et al.*, 1986), which responded with a much greater accumulation of Ins(1,4,5)P_3 to caerulin than to carbachol, even though these two agents were equipotent in increasing cytosolic free Ca^{2+}.

Once the concentration of Ins(1,4,5)P_3 and the $[Ca^{2+}]_i$ have peaked, within 30 seconds post-challenge, the levels steadily return to basal values. Around this time, the rate of prostanoid release also declines. In addition, approximately 2 min post-challenge the generation and release of $\cdot O_2^-$ has attained its maximal level and subsequently declines. Degranulation is nearly complete 15 minutes post-challenge with only minor amounts of granular constituents being secreted at later time points. These data support the observation that stimulation of eosinophils by PAF is a self-limited process which produces both excitatory and inhibitory signals (see below). While the kinetics of most of these responses are concentration-dependent, with lower concentrations usually causing a slower eosinophil response, the order of subsequent cellular events remain unchanged.

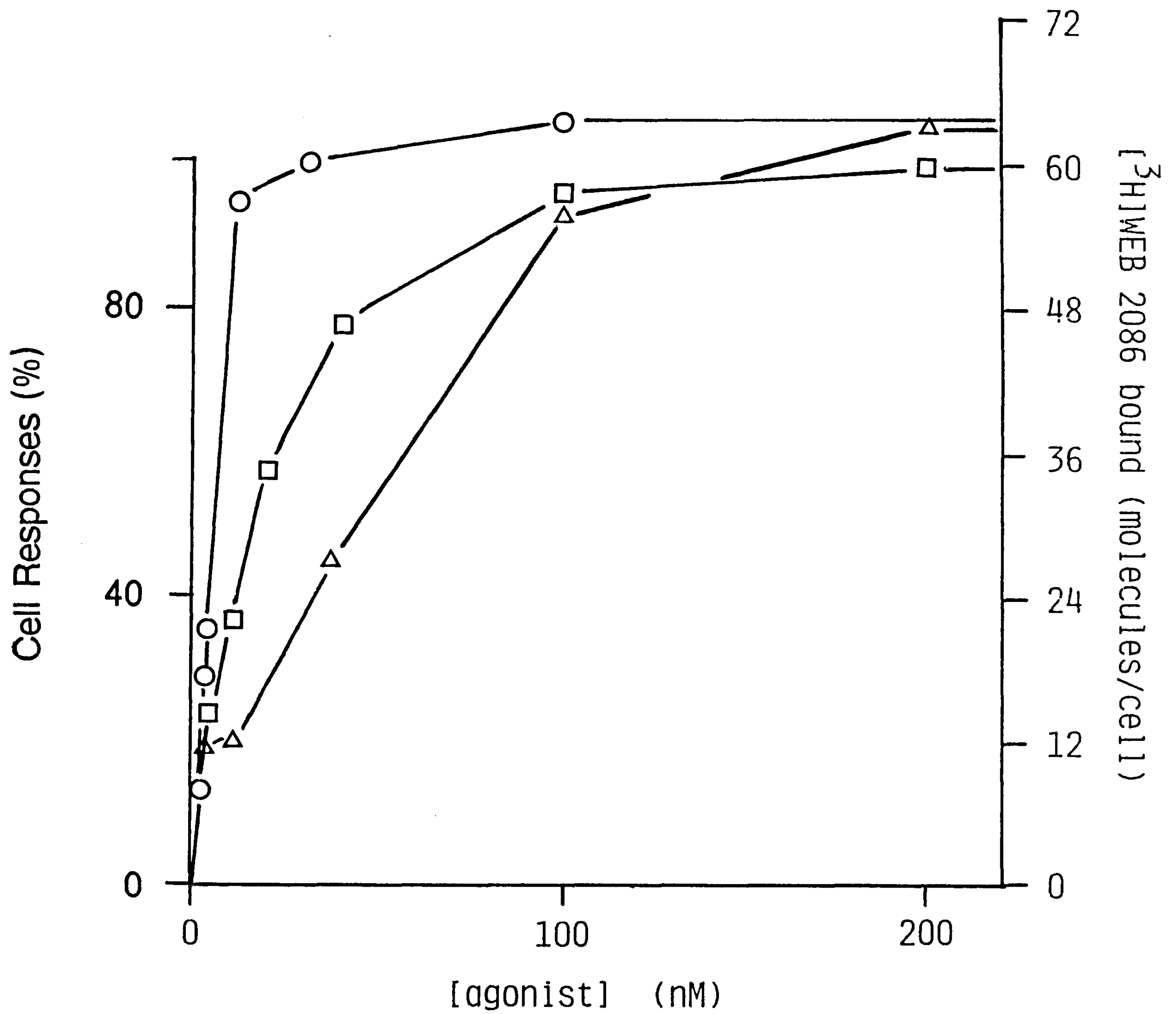


Figure 10.1: Relationship between [^{3}H]WEB 2086 binding to purified guinea pig eosinophils and cell responses. Correlation of [^{3}H]WEB 2086 binding to purified guinea pig eosinophils (○) with PAF-induced EPO secretion (□) and TXB_2 release (△). Data points shown in the Figure were adapted from results presented in Chapters 3 and 4.

The kinetic data summarized above suggest that binding of PAF causes a near instantaneous cellular response and that this response clearly occurs before a PAF-binding equilibrium is established. The number of receptors necessary to induce eosinophil activation, however, is not known. This direct link between occupation of PAF receptors and cellular response becomes evident when PAF binding is correlated to the release of EPO and TXB_2 (Figure 10.1). Within the limits of precision of the spectrophotometric radio-immunological measurements, it is obvious that the dose-response and PAF binding curves are superimposable suggesting that the occupation of PAF receptors is closely linked to degranulation of eosinophils. Interestingly, this correlation can be extended to other functional responses such as prostanoid release.

10.5 TERMINATION OF EOSINOPHIL ACTIVATION

In many biological systems, the initiation of cellular activation cascades by a specific ligand also activates inhibitory feedback pathways. Data presented here strongly indicate, that PAF serves not only as a potent eosinophil stimulant but also provides a signal for the reduction in cellular responsiveness. Accordingly, at least three different inhibitory signals in eosinophils are set in action by PAF:

(1) receptor desensitization,

(2) increase in cyclic adenosine monophosphate (cAMP), and

(3) internalization of PAF-receptor complexes.

10.5.1 Receptor Desensitization

10.5.1.1 Homologous desensitization

The stimulus specific nature of PAF-induced eosinophil activation is illustrated by the observation that pretreatment with PAF renders the eosinophils unresponsive to subsequent exposure to this stimulus, although the cells remained fully responsive to other stimuli. For instance, after initial exposure to PAF, eosinophils were unable to respond with a further elevation of the cytoplasmic Ca^{2+} level whereas

leukotriene B_4, C5a or fMLP produced a full response. The attenuated capacity of eosinophils to release enzymes or increase $[Ca^{2+}]_i$ following a second application of the same stimulus reflects homologous desensitization. Stimulus desensitization has previously been demonstrated in neutrophils with PAF and other stimuli (Henson *et al.*, 1978; Smith *et al.*, 1983; Chesney *et al.*, 1984) but had not previously been reported in eosinophils.

The inhibition of PAF-induced responses such as degranulation (Figure 3.2), prostanoid generation (Figure 3.9), accumulation of $Ins(1,4,5)P_3$ (Figure 6.3) and the increase in $[Ca^{2+}]_i$ (Figure 7.5) at agonist concentrations > 1 μM may also be attributable to cell desensitization. The lack of LDH release by PAF even at concentrations between 1 μM and 30 μM indicates that the decreased cell response was not due to a cytotoxic effect of high agonist concentrations. One possible explanation for this effect of PAF at higher concentrations is that simultaneous desensitization is occurring at the same rate as $Ins(1,4,5)P_3$ accumulation, $[Ca^{2+}]_i$ mobilization or secretion and that the former serves to limit the latter. This mechanism has been postulated by Henson (1976) to be responsible for the plateau in the secretory response in PAF-stimulated rabbit platelets. Simultaneous desensitization for other agents occurring at high stimulus concentrations has also been suggested for platelets (Hallam *et al.*, 1984), neutrophils (Niedel *et al.*, 1979; Sullivan & Zigmond, 1980) and smooth muscle cells (Findlay *et al.*, 1981). Interestingly, for $\cdot O_2^-$ release no simultaneous desensitization was observed and the precipitous fall in the ability of eosinophils to produce $\cdot O_2^-$ at concentrations of PAF above 30 μM was clearly associated with a cytotoxic effect of the stimulus.

Desensitization or supersensitivity are well known pharmacological phenomena. The biochemical basis of this desensitization process, however, is not yet understood. High agonist concentrations or prolonged exposure to the physiological agonists concentrations may result in a rapid loss of surface receptors due to internalization or endocytosis of stimulus-receptor complexes from the cell surface (Su

et al., 1980; Chuang & Costa, 1979; MacGlashan *et al.*, 1983; Sullivan & Zigmond, 1980; see below). Alternatively, there could be either a rapid structural modification of the receptor itself or a modulation of the activity of other target enzymes and regulatory proteins, i.e. through a phosphorylation catalyzed by a specific protein kinase C (Cohen, 1985). Indeed, homologous desensitization of β-adrenergic receptors and rhodopsin are due to a kinase that is specific for the agonist occupied receptor (Benovic *et al.*, 1986; Shichi & Somers, 1978). Furthermore, the observation that by 5 sec, 10 μM PAF caused a substantially lower accumulation of $Ins(1,4,5)P_3$ than 1 μM suggests that in eosinophils desensitization is due to alterations in some very early receptor-coupling event. This is in line with previous findings in the β-adrenoceptor-adenylyl cyclase system which shows that receptor specific desensitization is caused by an rapid reversible uncoupling of the receptor from the cyclase within the first few seconds (Su *et al.*, 1980).

10.5.1.2 Heterologous Desensitization

The data presented here also show that both PAF-RI and PAF-RII receptors are subject to heterologous desensitization. Short-term treatment of the cells with active phorbol esters resulted in a concentration-dependent attenuation of Ca^{2+}-mobilization, EPO release and $\cdot O_2^-$ production, promoted not only by PAF (Figures 9.1 and 9.2) but also by other stimuli such as LTB_4, C5a, and fMLP. These results suggest a possible role for PKC in the desensitization phase of receptor-mediated eosinophil activation. This conclusion is further supported by the finding that staurosporine reverses the inhibitory effect of PMA and enhances PAF-induced functional responses such as EPO release.

Although the exact mechanisms responsible for general desensitization through PKC-mediated signalling have so far not been elucidated, there are three possible explanations that should be discussed. The first mechanism involves the phosphorylation of the receptor itself with subsequent inhibition of agonist binding and

possibly uncoupling of the receptor from its G-protein. This process occurs for the β adrenergic receptors (Nishizuka, 1986). Secondly, PKC may phosphorylate the α-subunit of the G-protein, thus rendering it inactive, as has been demonstrated in platelet membranes (Katada *et al.*, 1985). The third conceivable mechanism involving desensitization by PKC may occur later in the signal transduction cascade at a site distal to receptor-coupled stimulation of PLC, namely at $Ins(1,4,5)P_3$ phosphatase. In platelets and brain tissue it has been demonstrated that PKC activation causes an increase in 5-phosphomonoesterase activity (Connolly *et al.*, 1985; Molina y Vedia & Lapetina, 1986). Consequently, phosphorylation of 5-phosphomonoesterase by PKC may have the effect of selectively diminishing the increase in $[Ca^{2+}]_i$, by accelerating the removal of $Ins(1,4,5)P_3$.

Some of the above mechanisms may also be effective in homologous receptor desensitization. Unlike diacylglycerols which undergo further metabolism in the cell, phorbol esters such as PMA produce a sustained activation of PKC. In addition, receptor activation may involve only a small portion of the total cellular PKC while phorbol esters may non-selectively activate all the available enzyme, hence rendering various, if not all, signal input mechanisms unresponsive to stimulation. Finally, PMA leads to down-regulation of PKC making it susceptible to proteolysis while receptor-mediated PKC activation is essentially reversible (Nishizuka, 1986; Nishizuka, 1988).

10.5.2 Increase in cyclic AMP

In the neutrophil fMLP elicits a rapid elevation of cyclic AMP (cAMP), peaking at 15 seconds after addition of the ligand (Korchak *et al.*, 1984). The β-adrenergic agonist isoproterenol, and prostaglandin E have also been shown to increase cAMP levels in eosinophils in response via their stimulatory action on adenylyl cyclase (Dent *et al.*, 1990; Yukawa *et al.*, 1990a). Though these agonists elevate cAMP in the eosinophil they do not lead to degranulation or generation of $\cdot O_2^-$. However,

preincubation of the cells with PGE_2, inhibited PAF-induced degranulation, elevation of $[Ca^{2+}]_i$, and $\cdot O_2^-$ in a concentration-dependent fashion. This suggests that instead of serving as a triggering mechanism, an elevation in cAMP levels apparently serves as an inhibitory feedback mechanism. One possible mechanism, which has been demonstrated in neutrophils, is the inhibition of the activation pathway at the level of phospholipase C, possibly via activation of the cyclic AMP-dependent protein kinase. Agents that increase cAMP were shown to inhibit remodelling of InsPs, arachidonate release and the subsequent calcium mobilization (Kato *et al.*, 1986; Della Bianca *et al.*, 1986; Takenaka & Ishitoya, 1985).

Further evidence to support this hypothesis comes from the experiments with the cyclo-oxygenase inhibitor flurbiprofen, which markedly enhanced the PAF-elicited responses. Thus, PAF-induced release of PGE_2 may act as an autocoid, binding to PGE_2 receptors on the eosinophil itself and raising cAMP levels secondary to PAF receptor activation. The mode of action of cAMP in eosinophils is not known. Nevertheless, in other cell systems, the rise in cAMP inhibits arachidonic acid release and remodelling of InsPs (Takenaka & Ishitoya, 1985; Della Bianca *et al.*, 1986), thereby reducing the amount of substrate available for $Ins(1,4,5)P_3$ and 1,2-DAG formation. In neutrophils, cyclic AMP apparently also inhibits cellular PKC activity (Di Virgilio *et al.*, 1987).

10.5.3 Internalization of PAF-receptor complexes

In many receptor systems (King & Cuatrecasas, 1981; Sibley *et al.*, 1987; Donabedian & Gallin, 1981) homologous desensitization is a consequence of decreased receptor expression. In neutrophils it has been shown that contact between cells and the chemotactic oligopeptide fMLP resulted in a reduced number of receptors for this ligand on the cell surface (Sullivan & Zigmond, 1980). Moreover, in rat basophilic leukemia cells, internalization of the IgE receptor occurred rapidly with a $t_{1/2}$ of 3 to 5 min, was Ca^{2+}-independent, and both receptor and ligand were degraded.

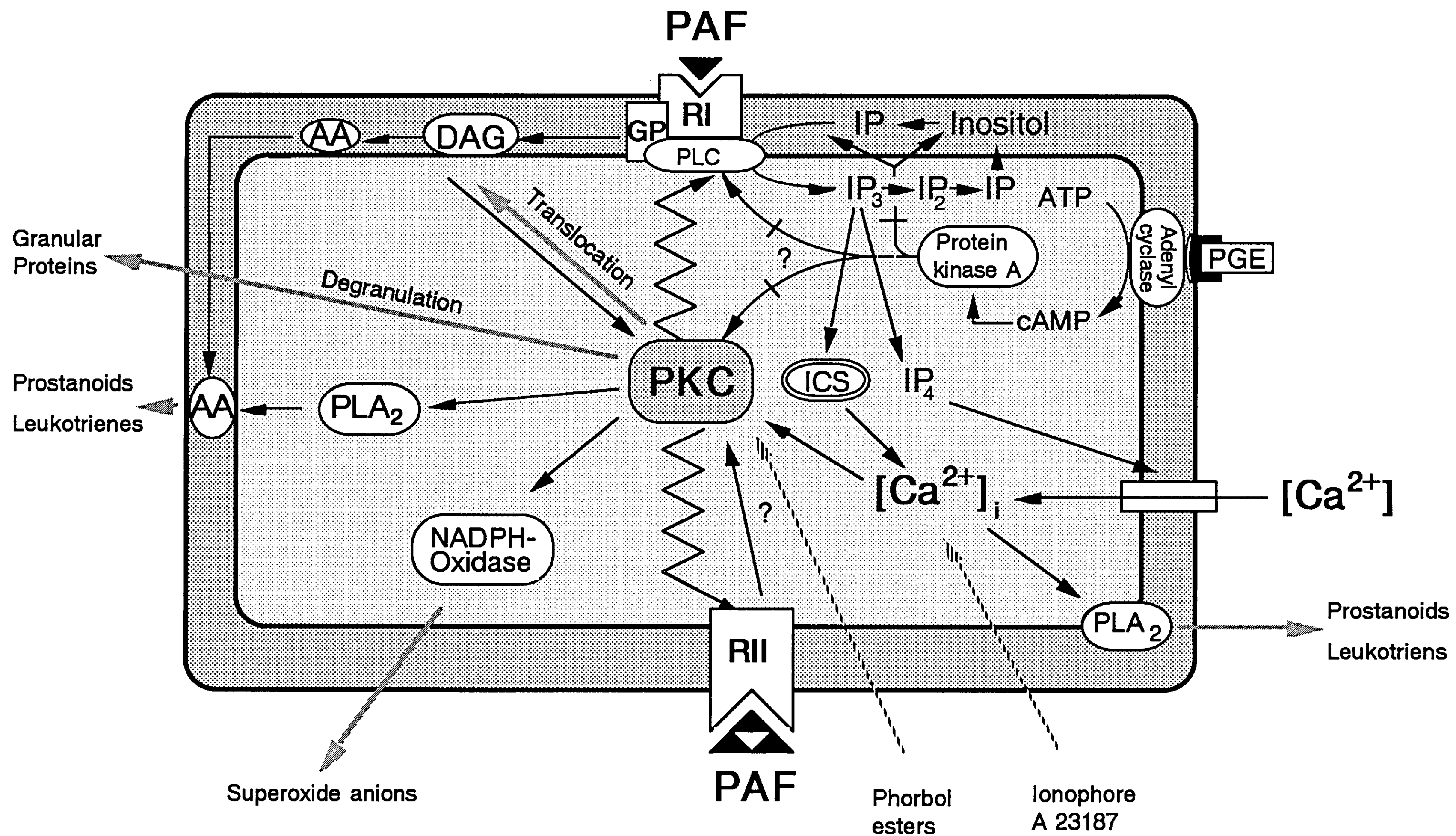


Figure 10.2: Schematic representation of the possible activation pathways in eosinophils stimulated with PAF. Key: AA, arachidonic acid; DAG, diacylglycerol; GP, G-protein; ICS, intracellular Ca2+ stores; IPx, inositol phosphates; PKC, protein kinase C; PLA2 phospholipase A2; PLC, phospholipase C; RI, high-affinity receptor; RII, low-affinity receptor.

Since both PAF and PMA, but not LTB_4, reduce the specific binding of $[^3H]$WEB 2086 to eosinophils (Figure 9.6), it is likely that receptor internalization and degradation can also be found in eosinophils. On the other hand, the observation that a reversible equilibrium of PAF binding occurs on eosinophils, denotes that not all receptors are internalized. Similar conclusions have also been drawn from IgE-binding experiments using rat basophilic leukemia cells (Isersky *et al.*, 1983; Furuichi *et al.*, 1986; Rivera *et al.*, 1986) and human basophils (Warner & MacGlashan, 1989). The observation that eosinophil responsiveness returns between one and two hours post-challenge, suggests that internalized receptors may be re-utilized.

10.6 THE DUAL NATURE OF THE PAF RECEPTOR

The possible activation mechanisms for eosinophils known to date are summarized in Figure 10.2. As is evident from the data presented throughout the dissertation, the eosinophil appears to express two receptor classes for PAF which differ in a number of aspects. Occupancy of the high-affinity receptor at 1 nM to 1 µM PAF evokes chemotaxis (Wardlaw *et al.*,1986), degranulation, and lipid mediator release whereas occupancy of the low-affinity PAF-RII receptor at 1 µM to 30 µM PAF elicits the release of superoxide anions. In addition, occupancy of PAF-RI leads to an increase in $[Ca^{2+}]_i$ and Ins(1,4,5)P_3, while no correlation could be found between the second messengers and $\cdot O_2^-$. Furthermore, both $\cdot O_2^-$ changes and the accumulation of Ins(1,4,5)P_3 mass decreased progressively at PAF concentrations greater than 1 µM and altogether ceased at concentrations greater than 10 µM PAF. In contrast, however, the most effective concentration for the PAF-induced $\cdot O_2^-$ was 30 µM, 30 to 300-fold greater than the PAF-RI-associated effector functions.

PAF-RI and PAF-RII also differed in their sensitivity towards PTX. While PAF-RI-coupled eosinophil functions were inhibited by PTX, PAF-RII-mediated $\cdot O_2^-$ release was not. This finding suggests that the two PAF receptors are coupled to distinct transduction mechanisms. While PAF-RI appears to utilize a G_i-like

protein-coupled PLC, leading to production of intracellular second messengers, the PAF-RII receptor appears to bypass most of these intracellular events. This is confirmed by the finding that production of $\cdot O_2^-$ occurs in the absence of extracellular Ca^{2+} (although not in Ca^{2+}-depleted cells) while PAF-RI-transduced functions essentially required Ca^{2+} (Figure 7.5). More than that, $\cdot O_2^-$ production was strongly dependent on the presence of extracellular Mg^{2+}, a fact which may, however, simply reflect its role as a cofactor for the NADPH oxidase (Heyneman & Vercauteren, 1984).

The fact that the phorbol esters are potent $\cdot O_2^-$ producers and that pretreatment of eosinophils with PMA inhibits the PAF-induced $\cdot O_2^-$ production implicates PKC in the signal transduction pathway utilized by PAF-RII. The molecular mechanisms coupling the receptor to PKC, however, are not known. It is possible to hypothesize that the receptor binds directly to a membrane bound PKC which then phosphorylates and activates the NADPH oxidase. Alternatively, other, so far unknown, signalling steps may link the PAF-RII receptor to the PKC. Another explanation could be that the PAF-RII is coupled to a different subspecies of PKC. This hypothesis is supported by the observation that PAF-RI receptor mediated EPO release is inhibited by staurosporine (Figure 9.4), whereas the PAF-RII transduced generation of $\cdot O_2^-$ is slightly enhanced (Table 9.1). It is intriguing that rabbit and human neutrophils also appear to express two subsets of PAF receptors that are distinguishable by their sensitivity to PTX (Naccache *et al.*,1985; Naccache *et al.*, 1986; O'Flaherty *et al.*, 1986; Lad *et al.*, 1987). The G_i-coupled receptor induces InsPs turnover, release of Ca^{2+} from intracellular stores, causes transient cell depolarization, an increase in cytoskeletal actin, secretion of granular enzymes, neutrophil aggregation and chemotaxis (Naccache *et al.*, 1985; Naccache *et al.*, 1986; Lad *et al.*, 1985; Lad *et al.*, 1987). However, in contrast to the PAF-RII site in eosinophils, the PTX-insensitive receptor appears to be associated with an apparent preexisting Ca^{2+} channel.

It is not clear whether the two PAF receptors identified on eosinophils represent distinct binding sites or whether they reflect two different affinity states of the same receptor. Recent experiments using immunoblotting on purified eosinophil membranes have failed to distinguish between the two binding sites (paper in preparation). However, β-adrenergic receptors are also capable of existing in two discrete states having either a high or low affinity for agonists (Kent *et al.*, 1980). Subsequent biochemical studies have shown that these two forms of the receptor corresponded to discrete molecular entities (Limbird *et al.*, 1980). Agonist binding forms a high affinity intermediate complex, consisting of receptor and a GTP binding protein. Binding of GTP then dissociates the complex to the low affinity form of the receptor (De Lean *et al.*, 1980; Stadel *et al.*, 1980). In contrast, however, to the two proposed PAF receptors on eosinophils, in this example only the high-affinity intermediate-complex is coupled to functional events.

A similar model has been proposed for fMLP peptide receptors. Binding of these peptides to human neutrophil membrane preparations best fits two classes of binding sites (Makin *et al.*, 1982; Koo *et al.*, 1982; Snyderman *et al.*, 1984). The K_d values for the sites are approximately 0.5 and 24 nM, respectively, with the high affinity state representing approximately 25% to 50% of the total number of receptors (Koo *et al.*, 1983). The high- and low-affinity binding sites were detectable in preparations of neutrophil membranes but only a single low-affinity site was found on intact neutrophils, suggesting that in whole cells, the sites might be rapidly interconverted, allowing detection of only a single class with the use of steady-state binding techniques (Koo *et al.*, 1983). Based on this observation, it was suggested that the fMLP receptors might be coupled to a GTP-binding protein and that binding of GTP causes a reversible conversion of high-affinity to low-affinity binding sites (Snyderman *et al.*, 1984; Koo *et al.*, 1983). The absence of detectable high-affinity receptors on intact cells using equilibrium binding is presumably due to the high levels of intracellular GTP or GDP (Snyderman *et al.*, 1984). This proposal was confirmed

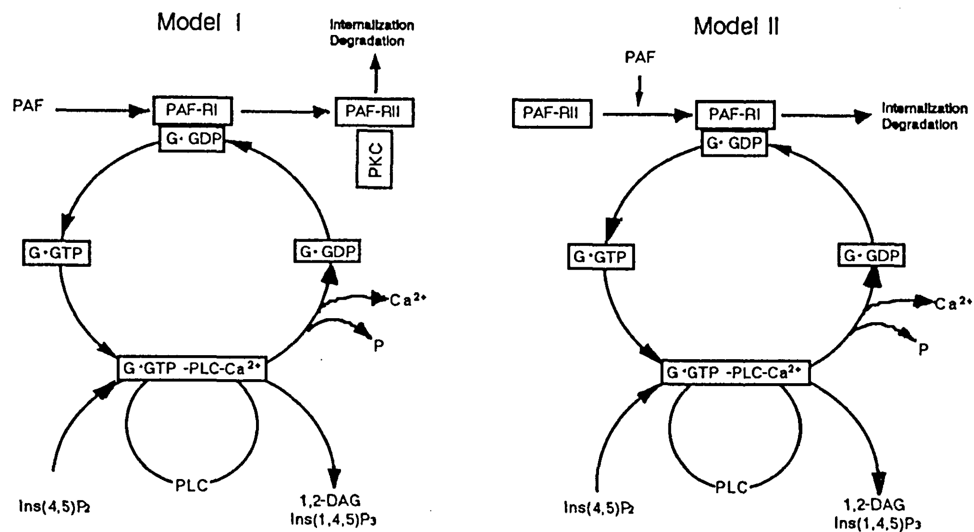


Figure 10.3: Hypothetical models of interconversion of the high-affinity (PAF-RI) and low-affinity receptor (PAF-RII). Key: G, G-protein, GDP, guanosine diphosphate; GTP, guanosine triphosphate; PLC, phospholipase C; P, inorganic phosphate.

by the discovery of two different GTP-dependent dissociation rates in whole neutrophils (Sklar *et al.*, 1987) and the modification of fMLP binding by guanine nucleotides (Preiss *et al.*, 1986). In other receptor systems, multiple binding sites have been shown to be due to aggregation, phosphorylation, or association with a GTP-binding protein.

It may, therefore, be possible that PAF binding to the high-affinity PAF-RI site in eosinophils results initially in a PAF-RI-G·GTP-PLC-Ca^{2+}-complex inducing the hydrolysis of Ins(4,5)P_2 to Ins(1,4,5)P_3 and 1,2-DAG. By analogy to other G-proteins, binding of GTP to the G-protein may uncouple the receptor, leaving behind a low-affinity site which could, if PAF concentrations reach sufficiently high levels, then activate a different pool of PKC. The low-affinity PTX-insensitive form of the receptor could also represent an intermediate form that is associated with rapid receptor internalization, since an increasing rate of [^{3}H]PAF incorporation was observed at high (> 10 μM) PAF concentrations (Fig. 10.3, model I). It can be hypothesized that when formation of this receptor state exceeds the internalization rates, this form of the receptor transmits signals for $\cdot O_2^-$ production. Furthermore, after the possible PAF-RI-induced translocation of PKC in the vicinity of the membrane the enzyme may be readily available for a direct interaction with the PAF-RII receptor. This hypothesis is supported by the finding that a precipitous decline of PAF-RI-related eosinophil functions correlates to an increase in PAF-RII transduced $\cdot O_2^-$ production.

Alternatively, PAF binding sites may occur originally as G_i protein-independent, low-affinity receptors. Exposure of eosinophils to PAF at low concentrations could then cause conformational changes leading to association with an adjacent G-protein, converting the receptor to the high affinity site and inducing the cellular functions. Once superoptimal PAF concentrations are reached the receptor G-protein coupling is inhibited and low affinity binding prevails which, when receptor

occupancy exceeds a critical threshold, transduces the signal for $\cdot O_2^-$ generation (Fig.10.3, model II).

The biological significance for the occurrence of two PAF binding sites is not clear. As suggested above, one of the two forms may simply reflect intermediate states in the receptor life cycle. This, for instance, has been proposed for the IL-2 receptor where only the high-affinity sites undergo receptor-mediated endocytosis (Weissman *et al.*, 1986). Moreover, the growth-promoting effects of IL-2 appear to be solely mediated by the interaction of ligand with high affinity IL-2 receptors whereas, to date, no biological function can be attributed to the low-affinity sites. However, in other receptor systems such as the LTB_4 or fMLP receptors on neutrophils, activation of the different receptor subtypes result in distinct functional responses (Goldman *et al.*, 1984). The division of eosinophil PAF receptors into two functionally distinct sites may, in fact, reflect an ingenious teleogical control mechanism for eosinophil effector functions. Hence, it can be hypothesized that the high-affinity receptor accounts for the chemotactic attraction, degranulation and mediator release taking place over longer distances, i.e. during migration of the cells into inflamed tissue. In contrast, the short-lived oxygen metabolites which can impose their toxic effects only locally require higher PAF concentrations for the production. Such concentrations of PAF could only be achieved at the site of inflammation, in close proximity to the antigen and surrounding antigen-processing mononuclear cells.

10.7 EOSINOPHIL ACTIVATION THROUGH PAF AS AN AUTOCOID

Not only is PAF a potent eosinophil activating factor, but eosinophils are also the major source of this mediator (Lee *et al.*, 1984). As with other cells, this observation suggests that cells capable of producing a certain lipid mediator also respond to it (see below). A major role of the lipids, therefore, may be to attract more effector cells to an inflammatory site through an amplification loop. In addition, PAF

may function as an autocoid, modulating the eosinophil response induced by other agonists such as C5a or fMLP. Such a function has been attributed to the mode of action of interleukin 2 in T helper cells. Evidence to support this hypothesis in eosinophils can be drawn from results presented in Chapter 3 where WEB 2086 partially inhibited TXB_2 release from eosinophils stimulated with calcimycin. Hence, the release of PAF by eosinophils upon stimulation may result in an autocrine response amplification loop. In addition, the fMLP-induced heterologous desensitization of the PAF-mediated increase in $[Ca^{2+}]_i$ (Fig. 7.4) may be caused, in part, by the release of PAF by fMLP thereby reducing the cellular response to a subsequent exogenous challenge with PAF. However, PAF synthesized in eosinophils tends to remain cell-associated (Lee *et al.*, 1984) an the possible role of PAF as an autocoid in eosinophil activation awaits further investigation.

10.8 PAF AS A PRIMING AGENT IN EOSINOPHIL ACTIVATION

An important and biologically relevant synergism exists between PAF and other inflammatory autocoids, including certain lipoxygenase and cyclo-oxygenase products of arachidonate metabolism. By design and implication in most *in vitro* experiments, a single mediator concept of inflammatory cell activation has emerged. However, during inflammatory tissue responses *in vivo*, several classes of inflammatory mediators are simultaneously produced and may collectively initiate either additive or synergistic effects. Indeed, for both platelets (Ostermann *et al.*, 1983) and neutrophils (Vercellotti *et al.*, 1988) it has been shown that subthreshold concentrations of PAF augment the cellular responses to suboptimal concentrations of other agonists. A couple of studies have now demonstrated a similar priming effect of PAF in eosinophils (Tamura *et al.*, 1987; Tamura *et al.*, 1989; Koenderman *et al.*, 1990). For instance, preincubation of human blood eosinophils with PAF for 30 min significantly enhanced the calcimycin-induced LTC_4 production (Tamura *et al.*, 1987). Interestingly, LTB_4 failed to enhance the eosinophil mediator production (Tamura *et*

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(Tamura *et al.*, 1989). These results indicate that PAF, in addition to its direct stimulatory actions, may also regulate inflammatory cells by its priming capacity.

Although the mechanism by which PAF is able to prime eosinophils remains to be established, one or more of the biochemical pathways outlined earlier in the Chapter may be involved. In a recent study examining the effects of PAF on serum-treated zymosan-induced respiratory burst, Koenderman and coworkers (1990) demonstrated that the priming effect of PAF is accompanied by 1,2-DAG accumulation and the activation of PKC.

In conclusion, the data indicate that PAF may not only serve as a direct activator of a secretory response but also be effective as a priming agent in eosinophils, an observation which has direct relevance to the treatment of pulmonary inflammation in asthma.

10.9 SPECIES DIFFERENCES IN EOSINOPHIL ACTIVATION

In the present study, the guinea pig eosinophil was chosen as a model cell to study eosinophil signal transduction. The main reason for this was its accessibility and the ease with which it can be isolated at the high purity necessary for biochemical investigations. However, there is evidence that guinea pig eosinophils differ from human eosinophils in certain aspects of their biological behaviour. For this reason the experiments were repeated with human eosinophils where feasible. The only major difference was the differential responsiveness of both eosinophil types to PAF and LTB_4. This extends previous studies showing that in human eosinophils PAF is 10- to 20-fold more potent at inducing chemotaxis than is LTB_4 *in vitro* (Wardlaw *et al.*, 1986). However, in guinea pig eosinophils, PAF is only one third as effective as LTB_4 (Aoki *et al.*, 1987). Furthermore, intradermal injection of PAF into healthy and atopic volunteers induces a cellular infiltrate consisting predominantly of eosinophils (Archer *et al.*, 1985; Henocq *et al.*, 1986; Henocq *et al.*, 1988) whereas leukotriene B_4 failed to induce a significant eosinophil accumulation as assessed by an

al., 1989). Similarly, PAF enhanced the respiratory burst of blood eosinophils induced by serum-treated zymosan (Koenderman *et al.*, 1990).

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improved skin window chamber technique (Bisgaard *et al.*, 1986). In the guinea pig, however, inhalation of either PAF or LTB_4 induced a marked increase in eosinophils in the lung (Aoki *et al.*, 1987; Silbaugh *et al.*, 1987; Coyle *et al.*, 1988). Interestingly, in spite of these differences in responsiveness, comparison of the $[Ca^{2+}]_i$ rise induced by PAF and LTB_4 in both guinea pig and human eosinophils, failed to show any significant differences (see also section 10.3.5).

PAF is a potent stimulus for prostanoid release in human eosinophils whereas LTB_4 is much less potent. In contrast, guinea pig eosinophils are 2-to 3-fold more sensitive to LTB_4 than PAF. Another point to note is that guinea pig and human eosinophils also differ in their ability to synthesise lipid mediators from arachidonic acid. While human eosinophils are known to synthesize LTC_4 (Aizawa *et al.*, 1987; Bruynzeel *et al.*, 1987b; Mahauthaman *et al.*, 1988), guinea pig eosinophils do not produce sulfidopeptide leukotrienes due to the absence of a specific LTA_4 glutathione S-transferase (Sun *et al.*, 1989b; Hirata *et al.*, 1990).

On the other hand, eosinophils from human and guinea pig sources share a variety of characteristics. Both cells express C3 receptors on their membrane surface (Henson, 1969; Tai & Spry, 1976). Likewise, both express PAF-receptors on their surface membrane, to which WEB 2086 binds with equal affinity (16.3 versus 18.2 nM). Finally, accumulation of eosinophils in both humans and guinea pig tissue follows a similar pattern. As demonstrated in human skin, PAF, antigen and anti-IgE challenge all produced a marked increase in numbers of eosinophils (Eidinger *et al.*, 1962; Henocq & Vargaftig, 1986; Henocq & Vargaftig, 1988; Henocq & Rihoux, 1990). Similarly, eosinophils are the predominant cell type in airways of guinea pigs challenged with PAF or antigen (Lelloch-Tubiana *et al.*, 1985; Coyle *et al.*, 1987; Lelloch-Tubina, *et al.*, 1988; Coyle *et al.*, 1988; Fugner, 1989).

10.10 IMPLICATIONS FOR THE PATHOGENESIS OF EOSINOPHILIC DISEASE

10.10.1 Eosinophil effector functions

Increasing evidence suggests that the eosinophil may play an important role as an inflammatory effector cell in the pathogenesis of asthma and other eosinophil-associated diseases (Gleich, 1986; Frigas & Gleich, 1986). Eosinophil effector functions are mainly mediated through the generation of oxygen radicals by the NADPH oxidase system (Weiss *et al.*, 1986; Kanofsky *et al.*, 1988) and through the release of granule contents, particularly the basic proteins which include eosinophil peroxidase, major basic protein and eosinophil cationic protein (Gleich & Adolfson, 1986). These proteins differ from other constituents by their strong positive charge and their high affinity to cellular membranes (Samoszuk *et al.*, 1988; Kroegel *et al.*, 1987). Membrane binding of these proteins results in both cell malfunction and cell disruption. The major basic protein or MBP, for instance, damages various mammalian cells, exfoliates bronchial epithelium cells and impairs the epithelial ciliary beating (Frigas *et al.*, 1980; Gleich, 1986). Recently, Flavahan and coworkers (1988) were able to show that MBP also induces bronchial muscle hyperactivity possibly by inhibiting the release of epithelium-derived relaxing factor (EpDRF).

Furthermore, eosinophil peroxidase, in the presence of hydrogen peroxide and a halide besides having a toxic effect on mast cells, pneumocytes and endothelial cells also stimulates both mast cells and basophils to release histamine (Gleich & Adolphson, 1986; Henderson et al., 1980; Samosuk et al., 1988). Aside from the the granular proteins, eosinophils also produce large amounts of leukotriene C_4, TXA_2 and PAF all of which may further contribute to the inflammatory tissue reaction as well as to bronchoconstriction (Aizawa *et al.*, 1987; Lee *et al.*, 1984; Kauffman *et al.*, 1987). Taken together, these observations provide evidence that eosinophils, indeed, possess the toxic potential of a potent effector cell.

I. Allergy
- Allergic rhinitis
- Asthma
- Atopic dermatitis
- Acute urticaria
- Food allergy
- Drug reactions

II. Infectious disease
1. Tissue-invasive helminths
   - Filariasis
   - Schistosomiasis
   - Strongyloides
   - Trichinosis
   - Toxocariasis
   - Ascariasis
   - Echinococcosis/Cysticercosis
2. Other Infections
   - Acute coccidioidomycosis
   - Afebrile tuberculosis
   - Cat scratch disease
   - Chlamydial pneumonia of infancy

III. Other cutaneous disease
- Bullous pemphigoid
- Herpes gestagionis
- Recurrent granulomatous dermatitis
- Scabies

IV. Other pulmonary diseases
- Transient pulmonary eosinophilic infiltrates (Löffler)
- Chronic eosinophilic pneumonia
- Hypersensitivity pneumonitis
- Allergic bronchopulm
- pulmonary aspergillosis
- Tropical eosinophilia
- Idiopathic pulmonary fibrosis

V. Connective tissue diseases
- Churg-Strauss syndrome
- Rheumatoid arthritis
- Eosinophilic fasciitis
- Sjögren`s syndrome

VI. Neoplastic and myeloproliferative diseases
- Solid tumours (mucin secreting, epithelial cell origin, serosa or bone metastases)
- Lymphomas, especially T cell type and Hodgkin`s disease
- T cell and acute lymphoblastic leukemias
- Occasional with myeloma (heavy chain disease)
- Hypereosinophilic syndrome

VII. Immunodeficiency diseases
- Selective IgA deficiency
- Combined immunodeficiency
- Nezelof syndrome
- Wiskott-Aldrich syndrome
- Hyper IgE syndrome
- Graft versus host reactions

Table 10.2: List of eosinophil-associated diseases (after Spry, 1988).

The results presented in this dissertation suggest that PAF may represent one of the most important natural stimuli for eosinophils. PAF has a variety of different effects on eosinophils which may help to explain some of the pathologic findings in asthmatic lungs and possibly in other eosinophil-associated diseases (Table 10.2). For example, PAF at physiologic concentrations elicits directional locomotion of human eosinophils *in vitro* in a time- and dose-dependent manner (Wardlaw *et al.*, 1986) and it induces chemotactic locomotion of eosinophils in animals (Lelloch-Tubiana *et al.*, 1985; Aoki *et al*, 1987) and humans *in vivo* (Henocq & Vargaftig, 1986). In addition, PAF stimulates eosinophil adhesion to human endothelial cells (Lamas *et al.*, 1988; Kimani *et al.*, 1988). Moreover, PAF-stimulated eosinophils have been shown to damage and exfoliate bronchial epithelium in guinea pig (Yukawa *et al.*, 1990b). PAF has further been shown to enhance the ability of normal human blood eosinophils to kill C3b- and IgG-coated schistosomula (MacDonald *et al.*, 1986). PAF also stimulates leukotriene C_4 synthesis (Bruynzeel *et al.*, 1986; Bruynzeel *et al.*, 1987b), superoxide anion generation as well as granular protein secretion. Finally, PAF leads to the generation and release of a variety of prostanoids from eosinophils.

10.10.2 Eosinophils, PAF and asthma

If the interaction between PAF and eosinophils does indeed play a role in the pathogenesis of asthma, the question that then arises is whether PAF is produced during asthma. PAF constitutes a heterogeneous family of structurally related ether-linked phospholipids that are formed as a result of the concerted action of phospholipase A_2 and acetyltransferase on membrane alkylacyl phospholipids (Barnes *et al.*, 1988a, Lee & Snyder, 1985; Patterson *et al.*, 1984). It is generated and released by several inflammatory cells which have been implicated in asthma, including macrophages (Barnes *et al.*, 1988b), neutrophils (Chilton *et al.*, 1982; Ingraham *et al.*, 1982; Shaw *et al.*, 1984) lymphocytes (Rola-Pleszynski *et al.*, 1987) and eosinophils (Lee *et al.*, 1982; Lee *et al.*, 1984). Direct measurement of PAF in biological fluids

such as plasma or BALF has proved difficult since PAF is rapidly hydrolysed to lyso-PAF by plasma acetylhydrolase as well as degraded and metabolized by inflammatory cells (Chilton *et al.*, 1983) and bronchiolar and alveolar epithelial cells (Haroldsen *et al.*, 1987). However, PAF possesses many biological properties which are relevant to asthma. For example, it causes bronchoconstriction (indirectly, since it has no direct effect on airway smooth muscle) (Mazzoni *et al.*, 1985), is the most potent cause of airway microvascular leakage to date (O'Donnell *et al.*, 1987; Evans *et al.*, 1987) and it causes a sustained increase in bronchial reactivity in several animal species (guinea pig, rabbit, dog, cat, sheep, primates) (Christman *et al.*, 1987; Chung *et al.*, 1987; Arnoux *et al.*, 1985; Barnes & Chung, 1987), including humans (Cuss *et al.*, 1986; Rubin *et al.*, 1987; Kaye & Smith, 1990). It is this latter property which distinguishes PAF from other putative mediators of asthma (Barnes *et al.*, 1988b) and this may probably relate to the interaction between PAF and eosinophils. Systemically administered PAF-antagonists have been demonstrated to block the asthmatic late response after antigen challenge of passively sensitized sheep (Stevenson *et al.*, 1987) and rabbits (Coyle *et al.*, 1987; Coyle *et al.*, 1988). Furthermore, BN 52021 as well as WEB 2086 suppressed the bronchial eosinophil infiltration, the appearance of eosinophils in the BALF, the development of epithelial lesions, and the development of bronchial hyper-responsiveness in guinea pigs after antigen administration by injection or inhalation (Braquet *et al.*, 1985; Lellouch-Tubiana *et al.*, 1988; Coyle *et al.*, 1988).

Taken together these observations suggest an interaction between PAF and eosinophils in the asthmatic airways may indeed represent a genuine pathogenetic principle in asthma: Under appropriate conditions (e.g. antigen stimulation), PAF might be generated by neutrophils, alveolar macrophages, mast cells, endothelial cells, or platelets (Braquet *et al.*, 1987) leading to adherence to vascular endothelium, diapedesis and eosinophil chemotaxis towards the bronchial lumen. An increasing PAF gradient within the tissue may trigger the eosinophils to degranulate, synthesize arachidonic metabolites or produce oxygen radicals. The basic proteins, in turn, either impair

epithelial cell function or lead to damage and loss of the epithelial cell layer. Impairment of epithelial cell function may be accompanied by loss of epithelial-derived relaxing factor which is believed to be involved in the development of bronchial hyperresponsiveness (Flavahan *et al.*, 1988). Damaged and denuded epithelium may expose unmyelinated C-fibers of afferent nerves, which are then susceptible to inflammatory mediators such as bradykinin. This leads to the release of sensory neuropeptides from collaterals, thus contributing to microvascular leakage, mucus secretion and bronchoconstriction. Bronchial oedema, mucous plugging and bronchoconstriction will eventually cause the symptoms of an acute asthma attack .

Although the biological function of eosinophils have been most intensively investigated in asthma, a number of other pulmonary diseases are also associated with eosinophil tissue infiltration. Davis and coworkers (1984) have demonstrated that eosinophils are cytotoxic *in vitro* for lung fibroblasts, mesothelial, and epithelial cells. Furthermore, stimulated eosinophils caused detachment and lysis of rat type II pneumocytes and cultured A549 cells, a human cell line with morphologic and synthetic characteristics of type II pneumocytes (Ayars *et al.*, 1985; Agosti *et al.*, 1987). Once again, the basic granular proteins appear to be involved in mediating these effects. For instance, it has been demonstrated that MBP detaches and lyses A549 cells (Ayars *et al.*, 1985). Furthermore, eosinophil granules contain a collagenase capable of degrading the type I and type III collagen components of the extracellular matrix, common in human lung parenchyma. These data indicate that eosinophils, in addition to their effects on bronchial epithelium, are able to damage interstitial tissue and thus, playing a pathogenetic role in eosinophil pneumonias. However, whether or not PAF is actually involved in eosinophil activation in these hypereosinophilic diseases remains to be elucidated.

10.11 IMPLICATIONS FOR FUTURE THERAPY OF EOSINOPHIL-ASSOCIATED DISEASE

The potential role of PAF in the activation of eosinophils in asthma offers new possibilities for clinical management of the disease. The results presented herein suggest that a promising strategy would be to interrupt the PAF-elicited cell activation of eosinophils. In principal, this could be achieved via two independent pharmacological approaches:

(1) Preventing PAF from binding to its respective receptors, or

(2) Inhibition of PAF-activated intracellular biochemical pathways.

(1). The first option, the use of specific PAF receptor antagonists such as WEB 2086 may be a valuable new pharmacological tool for preventing eosinophil activation *in vitro.* Studies in passively and actively sensitized animals have already demonstrated that PAF-antagonists can reduce both eosinophil infiltration and inflammation induced by specific antigen (Lelloch-Tubina, *et al.*, 1988; Coyle *et al.*, 1987). PAF-antagonists have also been shown to inhibit IgE-dependent bronchoconstriction in selected animal species (Touvay *et al.*, 1985). Thus, PAF antagonists too may prove beneficial for the therapeutic management of eosinophil-associated hypersensitivity diseases in human such as bronchial asthma.

In man, however, there is relatively little information on the effect of PAF antagonists available to date. A recent study demonstrated that the PAF ginkolide BN 52063 significantly inhibited antigen-induced late phase cutaneous response in atopic subjects without altering the early reaction (Roberts et al., 1989) suggesting that the drug may inhibit cellular infiltration into the skin . A preliminary report (Muino, 1990), relating to the follow-up of 17 selected asthmatics treated with a standardized mixure of PAF receptor antagonists BN 52020 to BN52024 (Braquet et al., 1985) at a dose of 2.5mg/kg

twice a day for more than a year, indicates that treatment of asthmatics with PAF antagonists may, actually, have beneficial effects. The study demonstrated by a clinical score that PAF antagonists of the ginkgolide-type inhibited bronchoconstriction, decreased hospitalization, reduced the use of corticosteroids and improved FEV_1 values. In addition, other parameters such as sputum and peripheral blood eosinophilia decreased dramatically to 9% and 42%, respectively, of the values before treatment. Serum IgE levels also decreased after approximately nine month of therapy. These changes are comparable to the effects of prednisone. Clinical trials currently under way to investigate the effects of PAF antagonist WEB 2086 in humans will provide further evidence of the effectiveness of such a therapeutic approach.

(2) Modulation of key biochemical events in eosinophil cell activation, is another proposal under consideration. Glucocorticoids, the most effective drugs for asthma available to date, show a profound inhibitory effect on eosinophil activation. Steroids reduce the number of peripheral blood eosinophils (Baigelman *et al.*, 1983, Anderson, 1969; Sabag *et al.*, 1979) presumably by decreasing the number of cells released from the bone marrow (Slavick *et al.*, 1985) and preventing eosinophil tissue infiltration most probably by affecting eosinophil chemotaxis (Hudson, 1968) and adherence to endothelium (Altman *et al.*, 1981). Steroids also inhibit the generation of superoxide anions by eosinophils when administered to normal subjects (Nelson, 1978). Moreover, steroids have been shown to inhibit eosinophil activation i*n vitro.* Studies have demonstrated that corticosteroids prevented the expression of Fc receptors (Oliver, 1982), the formation of lyso-PAF, leukotrienes, 15-HETE (Parente & Flower, 1985), and the release of EPO and superoxide anions (Yukawa *et al.*, 1989). However, corticosteroids induce a number of severe side effects which limit the use of this drug. Therefore,

future improvements in asthma therapy could be achieved with drugs capable of selectively "switching off" the cell activation of eosinophils, for instance by restricting the PAF-stimulated Ca^{2+}-influx and interrupting the intracellular Ca^{2+}-signal.

Although two calcium channel blocking agents, diltiazem and allopamil, have been shown to selectively inhibit platelet aggregation (Tuffin & Wade, 1985) their effect on eosinophils remains to be elucidated. In addition, a novel class of compounds with both calcium channel-blocking properties and PAF-antagonistic activity have recently been described (Hwang *et al.*, 1987). L-652-469 is the most potent of this class of dual antagonists but its effect on eosinophils is not yet known.

Another therapeutic approach could be to use compounds that selectively inhibit the cytosolic PKC or the InsPs cycle. However, the major practical problem with these compounds will most likely be their lack of specificity for eosinophils. The natural occurrence of two separate PAF receptors which are, moreover, differentially coupled to the cellular signal transduction apparatus may provide an advantage for the development of selective anti-eosinophil compounds.

10.12 FUTURE DIRECTIONS

The work presented in this thesis delineates cellular mechanism underlying transmembrane signalling in eosinophils. The data demonstrated that eosinophils can be activated through a G-protein- and Ca^{2+}-dependent signalling pathway. In addition, the data indicate that a G-protein-independend pathway may also operate suggesting that eosinophil activation can occur via multiple mechanisms. Indeed, recent findings have demonstrated that stimulation of neutrophils by PAF and other stimuli results in a activation of a phospholipase D (PLD) (Kanaho *et al.*, 1991).

Thus, direct production of 1,2-DAG, via the actions of PLC, may be supplemented by PLD-dependent conversion of phosphatidylcholine to phosphatidic acid and its subsequent hydrolysis to 1,2-DAG. The production of 1,2-DAG via this indirect pathway typically occurs over long periods (Kennerly, 1990) and it has been suggested that the 1,2-DAG derived from this alternative pathway may have a distinct role in regulating cell responsiveness.

Furthermore, studies carried out in the neutrophil, have indicated that PAF leads to the activation of tyrosine kinase and the increased phosphorylation of tyrosine residues (Gomez-Cambronero *et al.*, 1991). Again kinetics of this response are quite distinct from the rapid responses of the PLC pathway and it is not clear which aspect of cellular response this pathway may regulate. However, it is now clear that the leukocyte common antigen, CD45, represents a protein tyrosine phosphatase (PTPase) (Koretzky *et al.*, 1991; Tonks *et al.*, 1988) making CD45 a prototype for membrane receptor-linked PTPases that may participate directly in yet another novel signal transduction mechanism involving the dephosphorylation of target proteins. Thus, one of the major tasks of the next few years will be the integration of these diverse signal transduction pathways and an understanding of their respective roles in eosinophil activation.

Investigations into signal transduction have traditionally focused on the role of a single second messenger or pathway, resulting from the actions of a single agonist and limits of such an oversimplified view are rapidly becoming apparent. The diversity of cellular signal transduction mechanisms is further illustrated by the description and characterisation of various subtypes of G-proteins (Neer & Clapham, 1988; Bourne *et al.*, 1991; Kaziro *et al.*, 1991; Strathmann & Simon, 1991), PLC (Rhee et al., 1989; Rhee et al., 1991) and PKC (Nishizuka, 1986) in mammalian cells. These key proteins may all be differentially activated depending on the type of agonist or the number of stimuli acting simultaneously on the cell.

In considering the situation *in vivo*, it should be emphasized that a cell is likely to be exposed to several stimuli concomitantly or sequentially with possible reciprocal amplification or inhibition loops regulating its activation state and determining its functional responses. As biochemical, immunological and molecular techniques progress, these and similar considerations will increasingly dominated signal transduction research and will lead to the emergence of a more complete and integrated picture of cellular activation mechanisms.

The diversity of cell activation mechanisms both with respect to the diversity of potential agonists and the intracellular mechanisms must be considered when trying to relate *in vitro* observations to the complexity of *in vivo* phenomena. In this context, understanding of the effects and mode of actions of cytokines on eosinophils is still in its early infancy. The difficulties in estimating the pathophysiological role of cytokines is mainly due to the fact that:

a) novel cytokines are constantly being discovered,

b) a single cytokine has multiple effects on a range of various cell types, and

c) there is redundancy as many cytokines have identical effects.

Since cytokines not only modulate the function of mature eosinophils in inflammatory responses but are also involved in directing the development and differentiation of the cells, future research in this field may help to identify the factor(s) responsible for hypereosinophilia and eosinophil accumulation in tissue.

Understanding of the cell biology of the eosinophil will also have to include further characterization of surface antigens including adhesion molecules and other functionally important membrane antigens. The past year has seen the detection of an increasing number of eosinophil membrane epitopes and these findings have already begun to shift our understanding of eosinophil function in immunological responses. It appears that adhesion molecules such as LFA-1 (CD11a/CD18), CR3 (CD11b/CD18) (Hartnell *et al.,* 1990; Kroegel *et al.,* 1991c; see also page 36), and the very late activation antigen-4a (VLA-4α) (Bochner *et al.*, 1991; Walsh *et al.*, 1991) may play a

crucial role in eosinophil-endothelial cell interaction, margination and migration into tissues. Further studies in this field will not only help to unveil the principles of motility in general but also lead to a better understanding of intracellular mechanisms underlying surface receptor expression. In addition to the expression of adhesion molecules, eosinophils from patients with HES show CD4 antigens (Lucey *et al.*, 1989a) and class II proteins of the major histocompatibilty complex on their membrane (Lucey *et al.*, 1989b), suggesting a potential role as antigen-presenting cells, possibly pertinent to antigens that appear at mucosal surfaces. With the use of CD4 as a receptor, eosinophils may also respond to lymphocyte-derived lymphocyte chemotactic factor (Rand *et al.*, 1991).

While the work on this thesis was completed, Honda and coworkers (1991) reported the cloning and expression of a complementary DNA for a PAF receptor from guinea pig lung. Examination of the amino acid sequence suggests that the PAF receptor will be coupled to a G-protein which ma then link the receptor to a PLC. This exciting new work opens up new avenues of research. Factors which regulate gene expression of the receptor in eosinophils and other cells may now be investigated, and the distribution of the receptor studied by *in situ* hybridization. The availability of the cloned receptor cDNA may also lead to a better understanding of the coupling of PAF receptors and may help characterize the proposed receptor subtypes.

CHAPTER ELEVEN

11. SUMMARY

Eosinophils may play a crucial role in the pathogenesis of asthma through the release of their toxic granular proteins and bioactive lipid mediators. However, little is known about the signal transduction mechanisms underlying eosinophil activation. The aim of this work was to study the intracellular molecular events following receptor occupation and their relationship to the cellular responses of the eosinophil. In the initial studies platelet activating factor (PAF) was found to be a potent stimulus for eosinophil leukocytes inducing chemotaxis, granular enzyme secretion (eosinophil peroxidase, arylsulfatase B, β-glucuronidase), generation and release of prostanoids (thromboxane TXB_2, and prostaglandins PGE_2, PGD_2, $PGF_{2\alpha}$), and the production of oxygen radicals (measured as superoxide anion release $\cdot O_2^-$). PAF binds to two distinct classes of sites: a high affinity receptor (PAF-RI) with a dissociation constant K_D of approximately 0.3 nM (using [^{3}H]PAF as a radioligand) and 16 nM (using the PAF antagonist [^{3}H]WEB 2086) and a second receptor (PAF-RII) with an approximately 50- to 500-fold lower affinity. Occupation of the high affinity PAF-RI receptor correlated with several functional responses including degranulation and prostanoid release. In contrast, occupancy of the PAF-RII receptor solely correlated with the generation of $\cdot O_2^-$ radicals.

The PAF receptors utilized different signal transduction pathways with distinct intracellular second messengers and enzymes. The RI receptor was found to interact with a pertussis toxin-sensitive guanine nucleotide binding protein. Treatment of eosinophils with pertussis toxin catalyzed the ADP-ribosylation of a 41 kDa membrane protein indicating the existence of a G_i-like protein in eosinophils. This protein couples the receptor to a phosphatidylinositol phosphodiesterase, or

phospholipase C, which in turn, hydrolyses polyphosphoinositides to give inositol trisphosphate (Ins(1,4,5)P_3) and 1,2-diacylglycerol (1,2-DAG). The rapid accumulation of Ins(1,4,5)P_3 peaked only 5 seconds after PAF binding and preceded the maximal increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by approximately 15 sec. Although Ins(1,4,5)P_3 may mediate the release of Ca^{2+} into the cytoplasm the increase in $[Ca^{2+}]_i$ also constituted an influx of Ca^{2+} from the extracellular medium. In parallel with the accumulation of Ins(1,4,5)P_3, a rise in intracellular 1,2-DAG could be measured. Both Ca^{2+} and 1,2-DAG may be involved in the activation of the protein kinase C (PKC). Activation of PKC by phorbol myristate acetate (PMA) caused translocation of the cytosolic enzyme to the eosinophil membrane and resulted in the inhibition of the rise of $[Ca^{2+}]_i$, prostanoid generation as well as degranulation, demonstrating the significance of PKC in eosinophil activation.

In contrast to the intracellular events induced by the PAF-RI receptor, transmembrane signaling through the low affinity receptor was pertussis toxin-insensitive. In addition, production of $\cdot O_2^-$ occurred without an elevation of $[Ca^{2+}]_i$, intracellular Ins(1,4,5)P_3 and 1,2-DAG, indicating a transduction pathway different from the PAF-RI receptor. However, as with PAF-RI, transmembrane signaling by the PAF-RII receptor was also inhibited after PKC preactivation, suggesting a role for this enzyme in the signal transduction pathways of both PAF receptor types.

In conclusion, the data presented demonstrate that PAF may be an important stimulus for the recruitment and activation of eosinophils. It is proposed that PAF activates eosinophils through both a high and a low affinity receptor which utilize at least two distinct signal transduction pathways operative in eosinophils and which lead to different functional responses. These findings may have pathogenetic and therapeutic implications in inflammatory disease.

CHAPTER TWELVE

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