THE MECHANISM OF ACTION AND INTERACTION OF LEUKOTRIENE B_4 AND PLATELET-ACTIVATING FACTOR AS MEDIATORS OF NEUTROPHIL ACTIVATION.

A Thesis presented for the Degree of Doctor of Philosophy to the Faculty of Science in the University of Glasgow

by

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Much of the information presented in this thesis has been reported in the following publications.

MacIntyre D.E. & Rossi A.G. (1985)
Inhibition of agonist-induced Ca^{2+} flux in human neutrophils by tumour promoting phorbol esters.
Br. J. Pharmacol., 85: 377P (abstract)

MacIntyre D.E. & Rossi A.G. (1985)
Phosphoinositide metabolism and cytosolic free Ca^{2+} in leukotriene B_4 (LTB_4)-induced activation of human neutrophils.
Br. J. Pharmacol., 86: 654P (abstract).

Rossi A.G. & MacIntyre D.E. (1986)
The effect of pertussis toxin on agonist-induced phosphoinositide hydrolysis and changes in cytosolic free Ca^{2+} in human neutrophils.
Br. J. Pharmacol., 88: 253P (abstract).

Rossi A.G. & MacIntyre D.E. (1986)
Pertussis toxin inhibits the transduction processes activated by LTB_4 and PAF in human neutrophils.
Proc. 6th. International Conference on Prostaglandins and related compounds. p287 (abstract).

Rossi A.G., MacIntyre D.E. & McMillan R.M. (1987)
Cytosolic free calcium thresholds for human neutrophil activation.
Cell Calcium Metabolism - 87: 214P (abstract).

Rossi A.G., MacIntyre D.E. & McMillan R.M. (1987)
Human neutrophil activation: cytosolic free calcium, ($[Ca^{2+}]_i$) thresholds for aggregation, degranulation, leukotriene B_4 synthesis and phosphatidate formation.
Biochem. Soc. Trans., (in press).

Rossi A.G., McMillan R.M. & MacIntyre D.E. (1987)
Agonist-induced calcium flux, phosphoinositide metabolism, aggregation and enzyme secretion in human neutrophils.
Agents & Actions., (in press).

Rossi A.G., McMillan R.M. & MacIntyre D.E. (1987)
Are PAF and LTB_4 mediators of neutrophil activation?
Br. J. Pharmacol., (in press) (abstract).

Rossi A.G., MacIntyre D.E., Jones C.J.P. & McMillan (1987)
Do leukotriene B_4 and platelet-activating factor mediate activation of human polymorphonuclear leucocytes?
J. Cell Sci., (Submitted).

In addition, the following publications were completed before or during my postgraduate research work. The work was performed using human platelets and many of the experimental techniques are similar to those used when examining human neutrophils.

Drummond A.H., MacIntyre D.E., McNicol A. & Rossi A. (1985)
12-O-tetradecanoyl-phorbol-13-acetate (TPA) inhibits agonist-induced phosphoinositide metabolism and Ca^{2+} flux in human platelets.
Br. J. Pharmacol., 84: 27P (abstract).

McNicol A., Bushfield M., MacMillan L.J., Rossi A. & MacIntyre D.E. (1985)
Protein kinase C inhibits agonist-induced cAMP formation, phosphoinositide metabolism and Ca^{2+} flux in human platelets.
Thrombosis. Haemostas., 54: 038 (abstract).

MacIntyre D.E., Bushfield M., MacMillan L.J., Moffat K.J., Murdoch F.A., Thomson A., Rossi A.G. & McNicol A.(1986)
Receptors and receptor mechanisms of endogenous platelet stimulatory and inhibitory mediators.
Agents and Actions., 20 (Suppl) : 45-62

MacIntyre D.E., Bushfield M., Gibson I., MacMillan L., McNicol A. & Rossi A.G. (1986)
Human platelet receptors and receptor mechanisms for stimulatory and inhibitory lipid mediators.
Proc. NATO INSERM Adv. Res. Workshop Biol. Eicosanoids p34 (abstract).

MacIntyre D.E., Bushfield M., MacMillan L., Moffat K., Murdoch F., Thompson A., Rossi A.G. & McNicol A. (1986)
Platelet receptors for endogenous stimulatory and inhibitory mediators.
Proc. Cologne Artherosclerosis Conference 3., p 3 (abstract).

MacIntyre D.E., Bushfield M., Gibson I., MacMillan L., McNicol A. & Rossi A.G. (1987)
Human platelet receptors and receptor mechanisms for stimulatory and inhibitory mediators.
INSERM Proc. (Submitted).

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ABBREVIATIONS.

The more commonly-used abbreviations used throughout this thesis are listed below.

LTB_4	leukotrieneB4
PAF	platelet-activating factor
FMLP	formylmethionylleucylphenylalanine
PMA	phorbol-12-myristate-13-acetate
PtdA	phosphatidic acid
PtdIns	phosphatidylinositol
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
Ins $(1,4,5)P_3$	inositol 1,4,5 triphosphate
DAG	1,2-diacylglycerol
OAG	1-oleoyl-2-acetyl-glycero
PG	prostaglandin
$[Ca^{2+}]i$	cytosolic free calcium concentration
Quin2	2-methyl-6-methoxy-8-nitroquinoline
Quin2AM	quin2-acetoxymethylester
Kd	dissociation constant
EC_{50}	agonist concentration that elicits 50% of the maximum response produced by that agonist
IC_{50}	antagonist (inhibitor) concentration that inhibits the response to an agonist by 50%
G protein	guanine nucleotide-binding regulatory protein
AMP	adenosine 5' monophosphate
ADP	adenosine 5' diphosphate

ATP	adenosine 5′ triphosphate
cAMP	adenosine 3′5′cyclic phosphate
GTP	guanosine 5′ triphosphate
cGMP	guanosine 3′5′cyclic phosphate
EGTA	ethylene glycol tetra-acetic acid
EDTA	ethylene diamine tetra-acetic acid
DMSO	dimethylsulphoxide
RIA	radioimmunoassay
HPLC	high performance liquid chromatography
M.wt.	molecular weight

SUMMARY.

Human neutrophils can be stimulated by a plethora of soluble and particulate stimuli, the majority of which interact with specific recognition sites (receptors) located on the plasma membrane. These agonists evoke a series of cellular responses including chemotaxis, aggregation, degranulation, superoxide generation and the formation of numerous lipid products, for example LTB_4 and PAF, which are derived from membrane phospholipids. LTB_4 and PAF interact with specific receptors on, and are themselves potent activators of, human neutrophils. Therefore, these lipids have the potential to act as endogenous mediators or amplifiers of neutrophil activation. The mechanisms by which agonist receptor occupancy lead to such cellular activation remain to be fully established. It remains possible that in neutrophils, as in some other cells, reactivity may be regulated by the production of at least two second messenger molecules, 1,2-diacylglycerol (DAG) and $\left[Ca^{2+}\right]i$ that are produced as a consequence of phospholipase C catalysed phosphoinositide hydrolysis. The precise nature and role of this receptor mediated sequence of events in the human neutrophil, particularly concerning LTB_4 and PAF, still remains to be fully elucidated and is frequently controversial.

In this study I attempted to investigate the mechanism of action and interaction of the arachidonic acid metabolite, LTB_4, and the ether lipid, PAF, as mediators of neutrophil activation by comparing the responses elicited by these lipids to those evoked by other neutrophil stimuli, namely the chemotactic tripeptide, FMLP, the

calcium ionophore, ionomycin and the phorbol ester, PMA. I also examined the possible involvement of G proteins and the role of protein kinase C activation as stimulatory and regulatory mechanisms in the neutrophil. In addition, I explored the role of PAF and LTB_4 as endogenous mediators or amplifiers of neutrophil activation induced by other agonists.

Neutrophil reactivity was assessed by:

a. Examining ultrastructural features using electron microscopy.

b. Monitoring aggregation photometrically using a standard platelet aggregometer.

c. Determining NAG and lysozyme release using a fluorimetric and a spectrophotometric technique respectively.

d. Measuring LTB_4 generation by specific radioimmunoassay and the authenticity confirmed using reverse phase HPLC.

e. Observing changes in $[Ca^{2+}]i$ using the fluorescent calcium indicator dye, quin2.

f. Following inositol phospholipid metabolism in cells prelabelled with $[^{32}P]$-orthophosphate and monitoring changes in the levels of $[^{32}P]$-PtdA, $[^{32}P]$-PtdIns, $[^{32}P]$-PIP and $[^{32}P]$-PIP_2.

Using these in vitro techniques, the major observations and conclusions are listed below:

1. The ultrastructural features associated with neutrophils activated by FMLP, PAF and LTB_4 were similar. Control, unstimulated cells were generally rounded with smooth contours and the occasional extension of fine projections. Exposure of cells to agonists caused a marked plasma membrane ruffling followed by cell polarization and the formation of large lamellipodia. Pretreatment of neutrophils with the fungal metabolite, cytochalasin B, caused a marked vacuolation

and subsequent additions of agonists resulted in the formation of blebs giving the cells a bizarre appearance. Caution ought to be a priority when using or interpreting data generated by cytochalasin B.

2. LTB_4, PAF and FMLP all caused a reversible, concentration-dependent neutrophil aggregation. The aggregatory response produced by ionomycin and PMA was also concentration-dependent but was slower in onset and irreversible.

3. The receptor directed agonists caused a cytochalasin B dependent release of NAG (an azurophil granule marker) and lysozyme (an azurophil and specific granule marker). The calcium ionophore induced the release of both markers independent of pretreatment with cytochalasin B. PMA could release lysozyme in the absence of the fungal metabolite but the phorbol ester only induced a limited cytochalasin B dependent release of NAG.

4. Ionomycin elicited a concentration-dependent generation and release of LTB_4. FMLP and PAF only released barely detectable levels of the arachidonic acid metabolite.

5. All agonists except PMA caused a rapid concentration-dependent elevation of neutrophil $\left[Ca^{2+}\right]i$. Ionomycin induced a greater maximal increase than the receptor directed agonists.

6. FMLP, PAF and ionomycin caused a concentration-dependent formation of $\left[^{32}P\right]$-PtdA. The responses induced by FMLP and PAF were not calcium dependent whereas $\left[^{32}P\right]$-PtdA formation produced by the calcium ionophore required the presence of external Ca^{2+}. LTB_4 failed to elicit the formation of $\left[^{32}P\right]$-PtdA or the degradation of $\left[^{32}P\right]$-PtdIns, $\left[^{32}P\right]$-PIP or $\left[^{32}P\right]$-PIP_2. The results presented in this study suggest that LTB_4 may elicit cellular activation apparently independent of

phosphoinositide hydrolysis.

7. The concentration-response curves for FMLP, PAF and LTB_4-induced elevation of $[Ca^{2+}]i$ lie to the left of those for aggregation and degranulation. FMLP and PAF also caused an elevation of $[Ca^{2+}]i$ at concentrations lower than those required to elicit $[^{32}P]$-PtdA formation. These data indicate that $[Ca^{2+}]i$ elevation <u>per se</u> cannot mediate human neutrophil responses to these agonists. Consequently, there may exist other mediator(s) that act in concert with $[Ca^{2+}]i$ elevation to promote neutrophil activation.
8. From the cumulated ionomycin concentration-response curves the $[Ca^{2+}]i$ thresholds for the various indices of neutrophil activation were estimated to be approximately 600nM for aggregation, 600nM for NAG release, 500nM for lysozyme release, 800nM for $[^{32}P]$-PtdA formation and 200nM for LTB_4 biosynthesis.
9. Preincubation of cells with the phorbol ester, PMA, inhibited FMLP-, PAF- and LTB_4-induced elevation of $[Ca^{2+}]i$ and FMLP- and PAF-induced $[^{32}P]$-PtdA formation. These results indicate that protein kinase C activation may subserve a bidirectional role in regulating cellular responsiveness.
10. Pertussis toxin-attenuated FMLP-, PAF- and LTB_4-induced aggregation, enzyme release, changes in $[Ca^{2+}]i$ and also inhibited FMLP- and PAF-induced $[^{32}P]$-PtdA formation. The toxin, however, did not inhibit neutrophil activation elicited by the calcium ionophore or by the phorbol ester. These observations suggest that a pertussis sensitive process, presumably a guanine nucleotide regulatory binding protein (G protein) is involved in receptor mediated activation of human neutrophils.

11. The selective 5-lipoxygenase inhibitor, Revlon 5901, which attenuates LTB_4 biosynthesis did not significantly inhibit agonist induced aggregation, NAG release and lysozyme release. The PAF receptor antagonist, kadsurenone, inhibited aggregation and degranulation, induced by the ether lipid but failed to inhibit the same neutrophil responses induced by other stimuli. Therefore, the release of LTB_4 and PAF is unlikely to contribute to the activation of isolated neutrophils induced by other agonists.

This investigation has addressed a number of aspects and questions in relation to the control of neutrophil activation induced by a number of stimulatory agonists, especially with the arachidonic acid metabolite, LTB_4 and the ether lipid, PAF. A detailed understanding of the mechanisms of action and interaction of such lipid mediators of neutrophil responsiveness may facilitate the development of therapeutic agents to specifically regulate or modulate neutrophil reactivity, thought to be important in various inflammatory conditions.

INTRODUCTION

CHAPTER 1: THE NEUTROPHIL AND ITS ROLE IN INFLAMMATORY PROCESSES.

1. GENERAL INTRODUCTION.

The neutrophilic polymorphonuclear leucocyte (neutrophil) is the predominant leucocyte in human blood (approximately two-thirds of the total white cell population) and the first cell to appear at the site of acute inflammation. The main function of the neutrophil in the body is cellular defence against invading bacteria which it engulfs, digests and usually completely destroys. However, it is also suspected to have a function in general tissue repair and maintenance (Wilkinson & Lackie. 1979). Although the neutrophil has a unique structure and an impressive armamentarium of responses for bacteriostatic and bacteriocidal purposes, other leucocytes may have a similar or related array of responses. With this proviso and the fact that the main subject of this thesis is about human neutrophils, it is necessary to distinguish it from other leucocytes which will be referred to, for comparative purposes, on occasion. The term granulocytes is used collectively for the leucocytes of the myeloid series such as polymorphonuclear leucocytes comprising three cell types with differing staining properties with Romanowsky stains; the neutrophils have cytoplasmic granules that have a low affinity for both acidic and basic dyes, those of the eosinophil stain red or orange and the basophilic granules stain blue-black with Wright's stain. The mononuclear phagocyte, also of the myeloid series, is the term used to describe the monocyte which circulates in the blood differentiating into the macrophage once it enters the tissues. The lymphocyte, a white blood cell of the lymphoid series and most common mononuclear cell, is involved in immunoglobulin generation (B-cells) or cell mediated immunity (T-cells).

2. NEUTROPHIL MORPHOLOGY.

Mature human neutrophils are easily identified from other circulating cells using Wright stained blood smears visualised under a light microscope. They are normally spherical and range in size from 12-15μm in diameter. The neutrophil has a characteristic multi-lobed (usually between 2-5 lobes) deep purple-staining nucleus. The lobes are rich in heterochromatin and are surrounded by a relatively wide perinuclear cisterna, however, the functional significance of this nuclear segmentation is unclear. The pinkish cytoplasm is filled with small violet granules and with relatively few other cytoplasmic organelles.

Using electron microscopy and by cytochemical staining for myeloperoxidase (Graham & Karnovsky, 1966) two types of granules can be distinguished in human neutrophils. The azurophil (or primary) granules are larger (0.5μm), spherical or oval shaped and appear dark peroxidase positive whereas the specific (or secondary) granules, although more numerous, are smaller (0.2μm), round or dumb-bell shaped and stain peroxidase negative.

The subcellular structures, with the notable exception of the granules, are fairly scarce within the neutrophil. The few (20-30 per cell) mitochondria are tubular shaped, usually over 1μm long, between 100-150nm in diameter and are seen as round or oval cross-sections. The endoplasmic reticulum, both rough and smooth, is sparse and the Golgi-apparatus is small and presumably quiescent. Actin filaments constitute the major structural elements in the cell and comprise 10% of the total neutrophil protein. Immunological techniques have shown the presence of myosin distributed throughout the cytoplasm. (See figure 1).

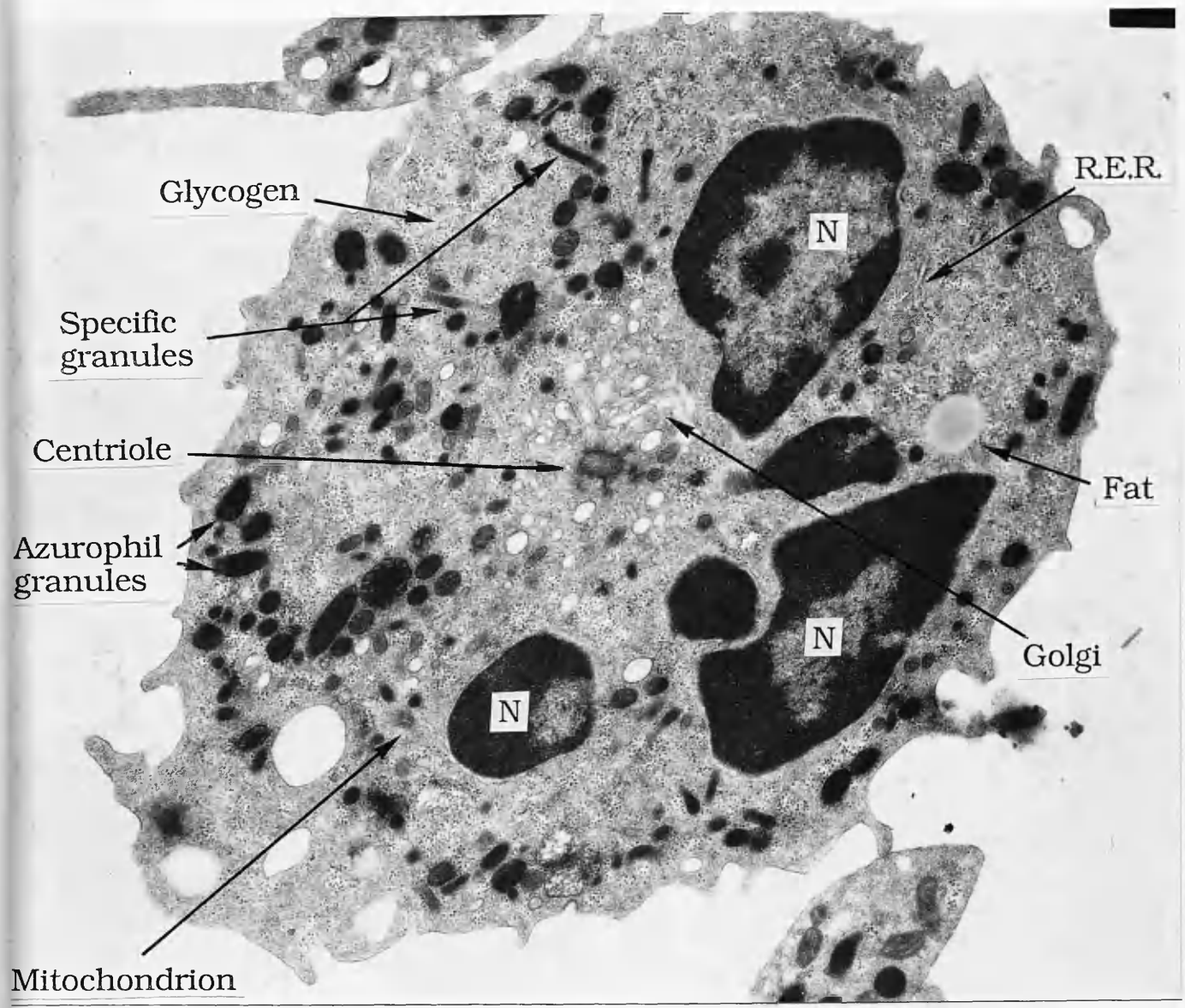

Figure 1.

The mature human polymorphonuclear neutrophil.

Several lobes of the nucleus (N) and numerous azurophil and specific granules, as well as glycogen granules, are scattered throughout the cytoplasm. A small golgi complex, a centriole, a fat globule, some rough endoplasmic reticulum (REM) and a few mitochondria are also visible. (EM x 27,500)

3. FORMATION AND LIFE HISTORY OF THE NEUTROPHIL.

The neutrophil spends its life in three distinct phases; the bone marrow, the blood and within the tissues. Collectively, the bone marrow is the largest organ in the body comprising about 4.5% of total body weight where approximately 75% of all the nucleated cells are committed to the production of leucocytes. The marrow phase commences with the proliferation of committed stem cells, comprising of about 5 different mitotic stages and lasting up to 10 days with mature cells being released 1-2 days later (Cronkite & Vincent, 1969; Athens, 1970). The bone marrow being a massive store of mature neutrophils (Dancey et al., 1976) not only maintains the physiological concentrations but can release elevated levels of neutrophils (neutrophilia) whenever the need arises, e.g. during inflammation or bacterial invasion.

The earliest stage of differentiation begins with pluripotential stem cells producing the first stage of the maturing neutrophil; the myeloblast. This cell is not capable of self-renewal and contains a large nucleus with dispersed chromatin exhibiting two to five nucleoli. The comparatively thin rim of cytoplasm contains the Golgi-apparatus, the endoplasmic reticulum, free ribosomes and a high number of mitochondria. The next cell in the maturation process is the promyelocyte being the largest of the series and responsible for the production of the azurophil granules. This cell contains a large nucleus, abundant rough endoplasmic reticulum, a strongly developed Golgi-apparatus and numerous mitochondria. The azurophil granules are formed and packaged in a similar manner to the formation of storage granules in other secretory cells (Meldolesi et al., 1978).

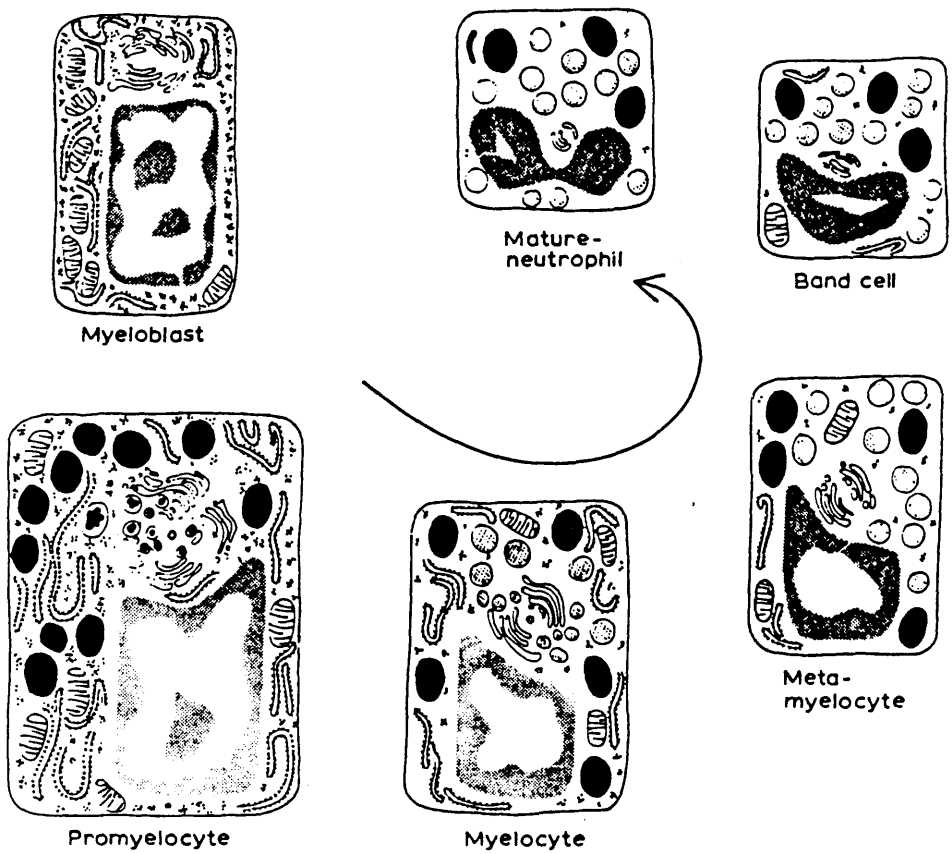

Figure 2.

Diagrammatic representation of the stages of maturation of the human neutrophil.

The myelocyte, which is formed directly from the promyelocyte, is much smaller and responsible for the formation of the specific granules; no azurophil granules are formed in this cell. The next stage results in the development of the metamyelocyte and band forms which are smaller, nonproliferating and nonsecretory cells, which ultimately develop into the mature neutrophil. (Bainton , 1980 ; Baggiolini , 1980) (figure 2).

Neutrophils have a short sojourn in blood, hence the number of cells in circulating peripheral blood is relatively low. There are approximately 10^9 neutrophils/l circulating in the blood of the normal adult. Neutrophils circulate, on average, for only 10 hours, whereas platelets circulate for 10 days and the erythrocytes for 120 days. The neutrophils then leave the blood vessels and enter the tissues where the cell's duration in this environment is uncertain but may be up to two days.

4. NEUTROPHILS IN VIVO.

Pathophysiological Role.

The main physiological function of the highly specialised neutrophil is to defend the body from invading organisms and other noxious agents. The body's natural reaction to an external destructive influence, e.g. trauma due to a cut, burn or bacterial infection, is to undergo an immediate response known as inflammation. The cardinal signs of the inflammatory response, i.e. heat, redness, swelling, pain and loss of function; and the relationship between leucocytes and inflammation have been known for decades. However, leucocytes have also been implicated in the more prolonged chronic inflammatory response (Metchnikoff, 1887; Weissmann, 1982). The morphological changes associated with these inflammatory symptoms are local vasodilation, an increased permeability to body fluid containing a high protein content, the accumulation of neutrophils by

chemotaxis and diapedesis and damage or necrosis of tissue. An inflammatory response can be acute as in septic arthritis or gout, sub-acute or chronic as in different forms of rheumatic disease such as the progressive systemic sclerosis or rheumatoid arthritis. It is still unclear precisely what and how many factors affect, mediate or modulate both acute and chronic inflammatory reactions, but it is certain that neutrophils play a central and pivotal role in the physiological and pathological manifestions of inflammation. The neutrophil, once recruited to the site of injury, undergoes various responses to remove the offending material by phagocytosis, degranulation and superoxide formation (discussed in the following sections). Over-production of lytic enzymes and reactive oxygen radicals can not only affect foreign matter but can destroy host tissue.

There have been numerous in vivo models used to show the involvement of polymorphonuclear leucocytes in various inflammatory conditions and to demonstrate their important role in the mediation of immunologically induced tissue injury. The first lesion shown to be dependent on neutrophils was the Arthus reaction (Humphrey, 1955; Williams et al., 1986). The specific depletion of neutrophils by either heterologous antineutrophil antisera or nitrogen mustard could attenuate or abort the Arthus reaction in a number of species. Although there was the presence of antigen, antibody and complement components in the vessels of antiserum-treated animals, no microscopic evidence of vascular injury could be detected (Parish, 1969; Cochrane & Janoff, 1974; Crawford et al., 1985; Fletcher et al., 1986).

Other experimental models showing that immunological injury and tissue damage are dependent on neutrophils include the proteinuria

associated with acute nephrotoxic vasculitis in rats and rabbits (Cochrane et al., 1965), necrotizing arteritis of serum sickness in rabbits (Kniker & Cochrane, 1965), the inflammation occurring during reverse passive Arthus (DeShazo et al., 1972; Williams et al., 1986) and pulmonary inflammation in monkeys (Revak et al., 1985) to name only a few. A great deal of evidence, derived from in vitro experiments examining the release of degradative substances, have implied that neutrophil-mediated tissue damage during inflammatory reactions involves numerous substances including lysosomal enzymes and toxic oxygen metabolites (Johnson & Ward, 1981; Till et al., 1982; Klebenoff and Clark, 1978).

5. NEUTROPHIL REACTIVITY.

Neutrophils when exposed to a plethora of particulate and soluble stimuli, both *in vivo* or *in vitro*, will become activated and will evoke a series of cellular reactions. They undergo chemokinesis and chemotaxis, become adhesive and phagocytose appropriate material, release many destructive enzymes and produce reactive oxygen species. Also formed are numerous lipid products including platelet-activating factor (PAF) and leukotriene B_4 (LTB_4) which are derived from membrane phospholipids possibly via activation of phospholipase A_2. These newly-formed lipids have the potential to act as potent independent activators of neutrophils or as endogenous amplifiers of neutrophil reactivity.

5.1 Chemokinesis and Chemotaxis.

Polymorphonuclear leucocytes have to actively leave the microcirculation and enter the tissues in order to perform their main physiological function of destroying invading bacteria. In the presence of an appropriate stimulus, neutrophils flowing in the

bloodstream will clump together, stick to the endothelial wall, crawl between the endothelial cells, progress through the interstitial matrix and eventually reach the site of injury. Neutrophils are among the most motile mammalian cells and are capable of migrating in the direction of a chemical gradient. This process is known as chemotaxis. This phenomenon must be distinguished from chemokinesis in which cells move with more kinetic energy but in a random manner (Zigmond, 1978). Many factors, which vary in size and chemical composition, have been shown to be chemotactic for neutrophils. These include a fragment of the fifth component of complement (C5a) and C5a-des-Arg (Shin et al., 1968), the bacterial derived tripeptide, FMLP (Schiffmann et al., 1975), the arachidonate metabolite, LTB_4, (Palmer et al., 1980) and numerous denatured proteins: all probably acting via membrane receptors.

Neutrophil locomotion can be investigated either by measuring changes in the distribution of a population of cells or by microscopically observing the motion of individual cells either directly or by use of time-lapse cinematography. Measuring the migration of a cell population can be achieved by use of an agarose gel matrix to allow the neutrophils to move between an underlying petri-dish and the gel; the movements are then analysed visually (Nelson et al., 1978). However, the most commonly used technique for measuring population movements is the Boyden chamber method. (Boyden, 1962). In this system neutrophils are added to an upper compartment and a potential chemoattractant is added to the lower section separated by a Millipore filter with 0.65 to 5μm pores (too small to allow passive diffusion of cells). Chemotaxis can be quantified microscopically by (a) measuring the distance that the cells have moved or (b) counting the number of

cells that have migrated through the filter. Microscopic cinematography has allowed the detailed analysis of neutrophils in locomotion not only to observe morphological changes but also to quantify parameters such as the rate of locomotion, the orientation of movement, the frequency and magnitude of turns and to assess variations among cells within a population or in a particular cell at different times (Zigmond , 1974). In vivo models of chemotaxis have also been reported: for example by making a skin window (a small superficial skin lesion) and removing and counting the migrated cells. This technique does not distinguish between chemotaxis and chemokinesis, hence the information received is difficult to interpret.

Unstimulated neutrophils are usually rounded with none or very few extended pseudopodia or lamellipodia (a thin extrusion of granule free cytoplasm) (Zigmond & Hirsch, 1973). A neutrophil when set in motion by a given stimulus has a polarized morphology with a characteristic extended pseudopodium at the front; a midsection containing the nucleus and most of the cytoplasmic granules and mitochondria; and a knoblike tail (see results section for micrographs). These techniques have been exploited to show that leucocyte motility is influenced by the pH (Bryant et al., 1966) and temperature (Nahas et al., 1971). Chemotaxis requires glycolytic energy (Carruthers, 1967) but is relatively insensitive to variations in the ionic composition of the external milieu; i.e. when cells are suspended in medium in the absence of potassium or sodium, although there is a notable decrease in locomotion in the absence of calcium (Gallin & Rosenthal, 1974; Showell & Becker, 1976; Wilkinson, 1975). Locomotion is inhibited by greater than 10% increases in the osmotic strength of the medium whereas a reduction of about 50% of the normal osmotic levels does not significantly attenuate chemotaxis (Lotz & Harris , 1956).

The precise molecular mechanism of chemotaxis is less well understood but it has been suggested that microtubules, actin and myosin are probably involved in the overall locomotory process. (For reviews see Zigmond, 1978; Wilkinson, 1982; Lackie, 1986).

5.2 Adhesion and Aggregation.

In order to function efficiently, neutrophils have a remarkable capacity to be selectively adhesive within their life cycle. It is presumed that the neutrophil is relatively adherent during its rather long developmental phase in the bone marrow. Once the cell has entered the blood it forms transient loose adhesions to the capillary walls, however, when activated by an appropriate stimulus, the cells will stop flowing, accumulate and stick to the endothelial wall, eventually reaching the site of insult within the tissues. The mechanisms that control and produce this calcium and magnesium dependent (Allison & Lancaster, 1964) adhesiveness are poorly understood.

Adhesion can be examined by a variety of techniques including microscopic observation of the adhered neutrophil either by use of the scanning electron microscope or by cross-sectional analysis. By far the most widely used techniques for quantifying neutrophil adhesion is by particle-counting, using Coulter counters, or by exploiting an in vitro assay similar to the one used for measurement of platelet aggregation. Suspensions of neutrophils, in an appropriate medium, and upon exposure to an agonist, become sticky and form cell-cell aggregates, which can be monitored using a standard platelet aggregometer. This technique relies on the fact that the extent of light transmission through a suspension of neutrophils will be less than through several large clumps of neutrophil aggregates.

Consequently, aggregation can be monitored as increments in light transmission. Although increased neutrophil-neutrophil aggregation may be a rather artificial *in vitro* index of neutrophil reactivity, it may have a parallel in augmented neutrophil-endothelium adhesiveness *in vivo* and may provide useful information concerning the importance of different agonists in neutrophil responsiveness.

5.3 Phagocytosis.

Phagocytosis is the process whereby microorganisms, immune complexes and other small particulate matter are ingested by the neutrophil. Once the neutrophil has attached itself to the organism, pseudopodia are extended and wrapped around each side of the bacterium so that it is interiorized into a pouch (or phagosome) of cell membrane. As viewed by transmission electron microscopy the cytoplasmic granules seem to fuse with the phagosome and releases its repertoire of lytic enzymes which usually destroy the invading bacterium. Phagocytosis is an energy requiring process, which is mostly derived from glycolysis and, as shown by immuno-cytochemical methods, the cytoplasmic contents of the protrusions that wrap around the particle are rich in actin and actin-binding protein (Stendahl et al., 1980). Nonspecific factors, such as pH, ionic strength, calcium concentration, osmotic pressure and temperature have profound effects on neutrophil phagocytosis (Murphy, 1976). The ability and rate at which neutrophils phagocytose a particle partly depends upon the surface characteristics of the particle. Although the optimal surface characteristics have not been fully investigated, surface charge and hydrophobicity profoundly influence digestion. Facilitation of phagocytosis is achieved by coating the particles with humoral factors known as

opsonins which effectively render the particle 'primed' for ingestion. There are two classes of opsonins, the heat-labile and the heat-stable factors. The C3b fragment of serum complement is characteristically an example of a heat-labile opsonin. Immunoglobulins particularly IgG comprise the heat-stable opsonins and must be intact to promote phagocytosis. The IgG antibody is composed of an Fab region which binds to specific antigenic sites on the particle and the Fc portion binds to specific receptor sites on the neutrophil membrane.

5.4 Degranulation.

Once the neutrophil has engulfed the invading organism the storage granules fuse with the membrane of the phagosome and release their contents. The microbicidal factors and lytic enzymes within the phagolysosome (as it is now called) can destroy and digest the organism. Although granule contents are usually secreted into the phagocytic vacuole (covert secretion) there are certain conditions where the enzymes are secreted into the external milieu (overt secretion); this process has been linked with various chronic inflammatory conditions e.g. rheumatoid arthritis.

When cells are in suspension and activated by an agonist it is virtually impossible to detect any enzyme release, however overt secretion is promoted by the pharmacological tool, cytochalasin B. This compound has profound and intriguing biological effects and is isolated from fungal moulds such as Helmintosporium dematodium. At low concentrations it inhibits hexose and nucleoside transport whereas at higher concentrations (1-10μM) its main effect is on the motor apparatus of the cell, inhibiting phagocytosis and chemotaxis and enhancing overt secretion. It is widely believed that

cytochalasin B exerts its effects by disruption of microfilaments resulting in disorganised contraction instead of controlled local effects. Since effects of cytochalasin B are vast and numerous and the precise mechanism of action is unknown, caution must be taken when using this compound (Tanenbaum, 1978).

The contents of the storage organelles have been extensively examined by differential centrifugation and subcellular fractionation followed by biochemical analyses. The azurophil granules contain a remarkable armamentarium of lytic enzymes and other constituents with antibacterial properties characteristic of lysozomes. They contain a variety of acid hydrolases (e.g. B N-acetylglucoasaminidase (NAG) and B glucuronidase), neutral proteinases (elastase, cathepsin G and proteinase 3) and two microbicidal enzymes (myeloperoxidase & lysozyme). The function of the specific granules is more difficult to pin-point. However, their contents have been investigated and shown to contain lysozyme, collagenase, lactoferrin and various vitamin B_{12}-binding proteins. Zonal differential sedimentation experiments performed with human neutrophils have shown the existence of a third population of smaller subcellular storage particles (C particles and secretory vesicles) (Baggiolini et al., 1978). The composition of these small organelles are possibly heterogeneous containing some acid hydrolases and neutral proteinases which also are found in the azurophil granules. Gelatinase, a metallo-proteinase, which was discovered in neutrophils by Sopata & Dancewicz (1974), has been localized exclusively in these small structures (Murphy et al., 1980; Dewald et al., 1982). Therefore, gelatinase has proven to be a useful specific marker for these tiny "granules". Table 1 shows an up-to-date resumé of the contents of the three storage organelles which presumably is not exhaustive (Baggiolini & Dewald, 1985).

Table I. Subcellular localization of enzymes and other constituents which are stored in human neutrophils[1]

Class of constituents	Azurophil granules	Specific granules	Smaller storage organelles[1]
Microbicidal enzymes	myeloperoxidase lysozyme	lysozyme	
Neutral proteinases	elastase cathepsin G proteinase 3	collagenase	gelatinase plasminogen activator (?)
Acid hydrolases	N-acetyl-β-glucosaminidase (NAG) cathepsin B cathepsin D β-glucuronidase β-glycerophosphatase a-mannosidase		N-acetyl-β-glucosaminidase (NAG) cathepsin B cathepsin D β-glucuronidase β-glycerophosphatase a-mannosidase
Other		lactoferrin vitamin B_{12}-binding proteins	

1 Attributions which are still uncertain are followed by (?)

2 Heterogeneous population of organelles including the C particles and secretory vesicles which are postulated as the carrier of gelatinase.

Lysozyme (muramidase).

Since the initial observation of the bacteriolytic activity of lysozyme by Fleming in 1922, much progress has been made in understanding this powerful lytic enzyme. Lysozyme cleaves the β-1-4 bond between N-acetylglucosamine and N-acetylmuramic acid residues of murein in bacterial cell walls. Lysozyme is capable of destroying some gram-positive organisms that do not have a peptidoglycan covering. One such organism is the bacterium Micrococcus lysodeikticus, the rapid lysis of which is used as a sensitive assay for lysozyme.

Lactoferrin and Vitamin B_{12}-binding proteins.

The iron-binding protein, lactoferrin, found in neutrophilic specific granules (Masson et al., 1969; Baggiolini et al., 1970) may constitute 10% of the total cell protein but there is no evidence to suggest that lactoferrin has any bacteriocidal properties. Due to its iron-binding ability, it may retard or block bacterial growth and may chelate iron liberated from haemoglobin degradation at inflammatory sites. The role of the vitamin B_{12}-binding proteins, also present in the specific granules, is unknown. However, they may have a bacteriostatic action by preventing folic acid production; which requires vitamin B_{12} and is necessary for bacterial growth.

Acid Hydrolases.

Comprises the major class of granule enzymes (see table 1) which are optimally active in acid conditions (usually below pH 5) and are most likely to be secreted into the phagocytic vacuole hence involved in intracellular degradation. The two most widely used azurophil granule markers are β-N-acetyl-glucosaminidase and β-glucuronidase which are detected by simple fluorimetric techniques.

Neutral Proteinases.

There are five main neutral proteinases found in human neutrophils; elastase, collagenase, gelatinase, cathepsin G and proteinase 3 which are maximally active at physiological pH and probably play a major role in extracellular matrix digestion. Interestingly, cathepsin G has been identified as the active ingredient of 'neutrophilin', the cationic protein released by neutrophils that activates human platelets (Selak et al., 1986).

Myeloperoxidase and superoxide generation.

Although phagocytosis is unaffected by anaerobiosis, bacteriocidal activity requires oxygen to function optimally and neutrophil activation is associated with a striking burst of oxidative metabolism. The mechanisms involved are not fully understood but it is thought that the generation of the extremely reactive and unstable intermediates, superoxide and hydrogen peroxide, are involved and that the azurophil enzyme, myeloperoxidase, plays an important role.

Hydrogen peroxide is produced from a two step synthesis. The superoxide free radical is formed from molecular oxygen and NADPH catalysed by the membrane bound enzyme NADPH oxidase (Baboir et al., 1973).

$$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + 2\cdot O^-_2 + H^+$$

Two molecules of the superoxide anion in the presence of protons combine either spontaneously or catalysed by the enzyme, superoxide dismutase, to form hydrogen peroxide. (McCord & Fridovich, 1969).

$$2\cdot O^-_2 + 2H^+ \xrightarrow{\text{superoxide dismutase}} O_2 + H_2O_2$$

The hydrogen peroxide serves as substrate, in the presence of the

chloride ion, for myeloperoxidase.

$$H_2O_2 + Cl^- \xrightarrow{\text{myeloperoxidase}} H_2O + OCl^-$$

The resultant hypochlorous acid is not only harmful to bacteria but may also produce OH radicals, halogen atoms and singlet oxygen.

These reactive species are extremely important in the destruction of invading organisms and are indispensable constituents of the host defence system. The above scheme for generating free radicals represents only part of the body's capacity for producing these toxic molecules; indeed, they are produced by various enzymatic lipid peroxidation pathways (e.g. during the formation of prostaglandins and leukotrienes). Although the body has a variety of efficient measures for removing these reactive species (e.g. superoxide dismutase and peroxide-metabolising enzymes; catalase and glutathione peroxidase) they are thought to contribute towards the pathogenesis of certain chronic inflammatory conditions since they not only kill bacteria but can also damage cells belonging to the host.

CHAPTER 2: NEUTROPHIL AGONISTS AND THEIR RECEPTORS.

Neutrophils can be modulated by a variety of different compounds which act to promote (stimulatory agents) or retard (inhibitory agents) cellular reactivity. However, this section is mainly concerned with the role of stimulatory agonists. The majority of these agonists exert their effects by interacting with specific receptor molecules located on the cell surface membrane. Although a number of receptors have been identified and characterised, the signal transduction mechanisms that link receptor occupancy to neutrophil activation remain largely unknown. This report will concentrate primarily on the best characterised agonists and receptors, with particular emphasis to PAF and LTB_4, rather than presenting a comprehensive list of all neutrophil stimuli.

1. C5a and C5a-des-arg.

The first chemotactic agent to be identified and characterised was the cleavage fragment of the complement component of C5 called C5a (Shin et al., 1968; Snyderman et al., 1969; Ward & Newman, 1969). This peptide, as well as being a potent activator of human neutrophils, also exhibits anaphalatoxin activity, contracts smooth muscle and causes mast cells to degranulate (Jensen et al., 1969; Snyderman & Pike, 1984). C5a has been located in the synovial fluid of patients suffering from conditions such as inflammatory arthritis (Ward & Zvaifler, 1971) and in the exudates of experimentally-induced inflammation (Snyderman et al., 1971). Human C5a is a glycopolypeptide that contains a complex carbohydrate moiety and a polypeptide region of 74 amino acid residues (Fernandez & Hugli, 1976). However,

once formed in human serum, C5a is rapidly metabolised to a C5a-des-arg by an endogenous carboypeptidase B enzyme. Using the Boyden chamber technique, Fernandez et al., (1978) showed that pure C5a was a potent neutrophil chemotactic agent over the concentration range of 0.04 to 1.7×10^{-8}M. In contrast C5a-des-arg, when applied alone, was devoid of chemotactic activity. However, C5a-des-arg, if combined with non-activated normal human serum, the chemotactic activity was restored although it was still 10 fold less active than C5a. This serum requirement was dependent on the technique employed for chemotactic measurement because Chenoweth et al., (1979), using the agarose system, showed that the EC_{50} for C5a in human neutrophils was between $1\text{-}3 \times 10^{-9}$M and the EC_{50} for C5a-des-arg, in the absence of serum, was 6×10^{-8}M. Using $[^{125}I]$-labelled C5a, Chenoweth & Hugli, (1978) first demonstrated the presence of specific receptors for this ligand on human neutrophils. The dissociation constant (Kd) for C5a binding occurred at 3-7nM at 0^{o}C and the number of receptors per cell was estimated to be between 100,000-300,000. Subsequently, it has been reported by Huey & Hugli, (1985), using human neutrophils, that the C5a receptor complex is actively internalised. With the development of better photoaffinity, cross-linking and electrophoretic techniques, it is believed that the C5a receptor molecule is an oligoprotein with an M.wt. between 40-48K (Huey & Hugli, 1985; Johnson & Chenoweth, 1985; Rollins & Springer, 1985).

2. FORMYL-METHIONYLLEUCYLPHENYLALANINE (FMLP)

Many small synthetic peptides such as N-formylmethionyl-leucylphenylalanine (FMLP) are highly potent activators not only of neutrophils but also of monocytes, macrophages, basophils and eosinophils. There is considerable evidence to show that these peptides exert their biological effects by acting on specific

receptor sites. Many microorganisms, for example; E. Coli, initiate protein synthesis with N-formylmethionine residues which eventually may be cleaved from the protein chain (Marasco et al., 1984). Since eukaryotic cells do not use N-formyl peptides in protein synthesis, except in the mitochondria, these peptides could represent a distinct prokaryotic metabolite that would allow these leucocytes to differentiate between foreign microorganisms and the host's own cells.

The interaction of N-formylmethionylpeptides with their corresponding receptor is probably the best studied agonist-receptor interaction in leucocytes. The biochemical nature of the receptor was initially determined by covalently linking a radiolabelled hexapeptide to the receptor of neutrophil plasma membranes (Niedel et al., 1980). The receptor was identified as a polypeptide that migrated as a wide band on SDS-polyacrylamide gel electrophoresis with an apparent M.wt. between 55 and 70K. It was then shown by Schmitt et al., (1983) that the broad band was comprised of two distinct entities with a M.wt. of 50K and 60K. The receptor is thought to exist as a glycoprotein complex with two N-linked oligosaccharide chains bound to a 33 -35K fragment that retains the N-formyl peptide binding site and consists of two distinct proteins (Dolmatch & Niedel, 1983; Painter et al., 1982; Malech et al., 1985). The protein fragment of the receptor may span the membrane phospholipid bilayer and the oligosaccharide chains are probably located on the external surface of the plasma membrane. It is estimated that there are between 50,000-80,000 receptor sites on the human neutrophil surface (Niedel et al., 1979; Sklar et al., 1984; Zigmond et al., 1982) and approximately an equal number expressed intracellularly (Fletcher & Gallin 1983; Jesaitis et al., 1984). It is believed that the number of surface receptors for

FMLP may increase during neutrophil activation and that this raised number of receptors may arise from mobilisation of the intracellular pool of receptors, synthesis of new receptors or by recycling of agonist-mediated internalised receptors (Gallin & Seligmann, 1984; Omann et al., 1987a). The N-formyl peptide receptor in human neutrophils was shown by Lane & Snyderman, (1984) to exist in two affinity states. They measured a Kd of 0.53 ± 0.01nM for the membrane-bound receptor. An average of 28% of the total receptor number was of the high affinity state. It has been shown that guanine nucleotides could modulate the conversion of high affinity receptors to the low affinity state without affecting the total receptor numbers or the overall receptor affinities (Koo et al., 1983). These observations offer some indirect evidence to implicate the involvement of guanine nucleotide binding regulatory proteins (G proteins) in stimulus-response coupling mechanisms in the neutrophil (see Chapter 3).

3. LEUKOTRIENE B_4 (LTB_4)

Leukotriene B_4 (5-D-(S),12-D-(R)-dihydroxy-6,8,10,14-eicosatetraenoic acid; 5,12-diHETE: LTB_4; (figure 3) is the major product of arachidonic acid metabolism in human neutrophils and is one of the most potent chemoattractant agents yet discovered. Borgeat & Samuelsson, (1979a; b; c) showed that isolated rabbit and human neutrophils when stimulated with the calcium ionophore, A23187, in the presence of arachidonic acid, were capable of generating several dihydroxy fatty acids. By using reverse-phase HPLC and structural analysis techniques, they revealed that the predominant product formed was the 5,12-diHETE with 4 double bonds at carbons 6,8,10 and 14. This compound was termed LTB_4 (Samuelsson et al.,

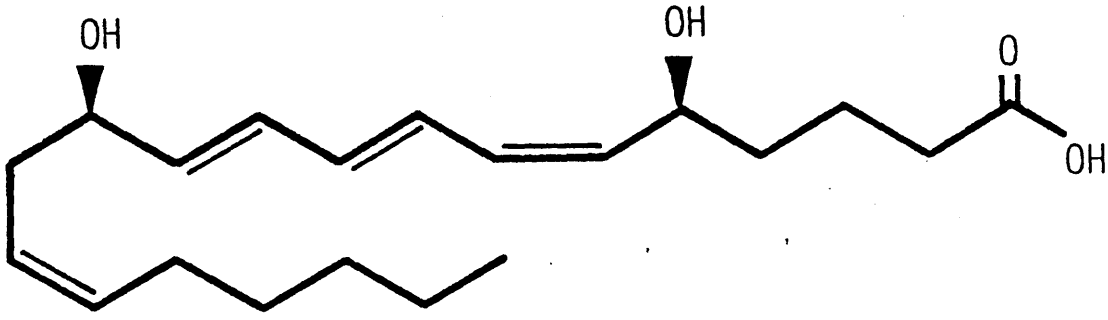

Figure 3.
Chemical structure of leukotriene B_4 (LTB_4).

1979; Samuelsson & Hammarstrom, 1980).

It was subsequently shown that LTB_4 is an extremely potent neutrophil agonist eliciting a whole series of neutrophil responses including chemokinesis, chemotaxis, aggregation and degranulation (Ford-Hutchinson et al., 1980; Bray, 1983). Although LTB_4 is probably the major arachidonic acid metabolite produced in the human neutrophil, numerous other products can be derived from arachidonic acid. The biosynthetic pathway for the formation and metabolism of LTB_4 is shown in figure 4.

Arachidonic acid is liberated from membrane phospholipids principally via activation of the calcium-dependent phospholipase A_2 (PLA_2) (O'Flaherty., 1987). Prevention of arachidonic acid release or inhibition of arachidonic acid metabolism has considerable therapeutic potential since its many biologically active products exhibit a plethora of physiological responses which have pathological consequences. Although no direct inhibitors of PLA_2 have yet reached clinical evaluation, some putative inhibitors such as p-Bromophenacyl bromide (BPB) and mepacrine have proved useful as research tools in various <u>in vitro</u> investigations. It is well established that steroids such as hydrocortisone and dexamethasone inhibit arachidonic acid release. The mechanism of action of these steriodal anti-inflammatory agents is believed to be the result of the synthesis of lipocortin, a peptide that inhibits PLA_2 activity (Flower & Blackwell, 1979). Since neutrophils and platelets only have a limited capacity to synthesise proteins, it is hardly surprising that these steroids have only a minor effect on these cells. Whether all the effects of the steroidal anti-inflammatory agents can be attributed to lipocortin inhibition of PLA_2 still remains to be established.

Arachidonic acid can be converted via cycloxygenase and other

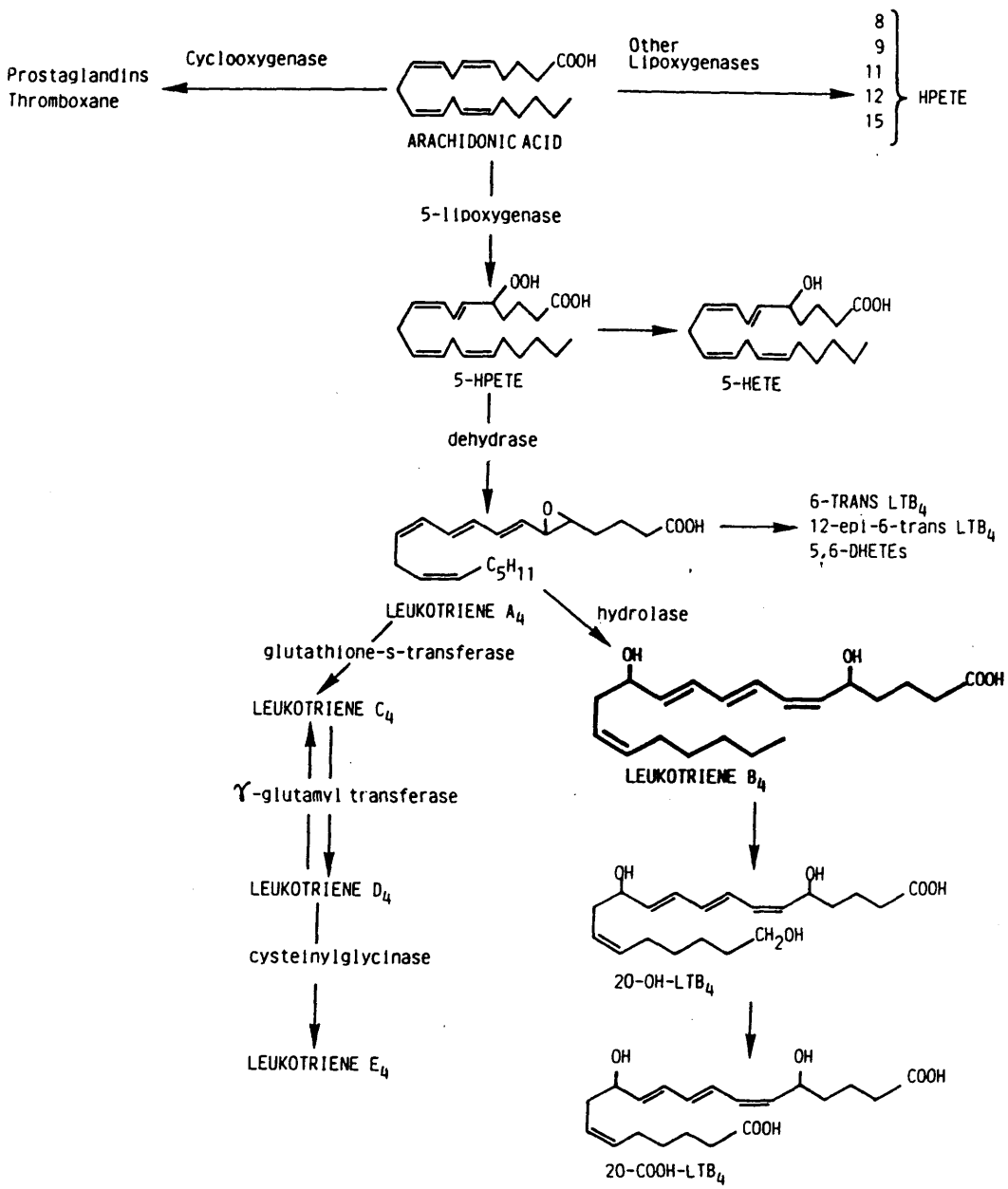

Figure 4.

Arachidonic acid metabolism; demonstrating the biosynthesis and deactivation of LTB_4.

enzymatic pathways to yield a number of prostaglandins and thromboxanes which have extremely powerful and varied biological effects (for a review see Johnson et al., 1983). In neutrophils, however, arachidonic acid is predominantly acted upon by the calcium-dependent and cytosolic enzyme 5-lipoxygenase to yield a 5-D-(S)-hydroperoxy-6,8,11,14 eicosatetraenoic (5-HPETE) intermediate. 5-HPETE can be further metabolised to the corresponding 5-hydroxy acid (5-HETE), which has only modest chemotactic activity in comparison to LTB_4. Alternatively, 5-HPETE can be converted by a dehydrase enzyme to the highly unstable allylic 5,6-epoxide derivative known as leukotriene A_4 (LTA_4). Although there exists a number of different types of lipoxygenases in a wide variety of cell systems, including human neutrophils, the precise function of these enzymes and their corresponding products are largely unknown. For example, the predominant lipoxygenase in the platelet is the 12-lipoxygenase (Turner et al., 1975; Nugteren, 1975) which catalyses the formation of 12-HPETE/12-HETE (Hamberg & Samuelsson, 1974; Johnson et al., 1983).

LTA_4 can be non-enzymatically transformed to 6-trans-LTB_4, 12-epi-6-trans-LTB_4 and 5,6-DiHETEs or can be enzymatically hydrolysed to the biologically active LTB_4. In human neutrophils LTB_4 is metabolised by w-oxidation to 20-OH-LTB_4 followed by a further transformation to the decarboxylic acid 20-COOH-LTB_4 (Hansson et al., 1981).

Other products of LTA_4 metabolism are the peptidyl-leukotrienes, often referred to as the slow-reacting substances of anaphylaxis (SRS-A). Peptidyl-leukotrienes (leukotriene C_4 (LTC_4), leukotriene D_4 (LTD_4), leukotriene E_4 (LTE_4) and leukotriene F_4 (LTF_4)) exert a number of biological effects such as bronchoconstriction and secretion of pulmonary mucus, suggesting a possible role for these

arachidonic acid metabolites in asthma and other inflammatory conditions (Piper, 1983: 1984).

The majority of investigations concerning LTB_4 generation have been performed using the calcium ionophore, A23187, a potent but non-physiological stimulus and such LTB_4 biosynthesis has been shown to exist in most leucocytic cells. Other more physiological agonists such as C5a, FMLP and PAF produce only a fraction of the amount generated by the calcium ionophore (Claesson et al., 1981; Salmon et al., 1982). LTB_4, as well as being a potent activator of neutrophils *in vitro*, also exerts a large number of effects *in vivo*. Smith et al., (1980) demonstrated that administration of LTB_4 into the peritoneal cavity of guinea pigs stimulates an accumulation of neutrophils and to a lesser extent eosinophils and macrophages, which lasts up to five hours after the initial injection. LTB_4 also causes a profound transient neutropenia and accumulation of neutrophils into the dermis and skin chambers of a variety of species (Camp et al., 1983; Lewis & Austen, 1984; Higgs et al., 1981; Bray, 1983). Simultaneously, LTB_4 also causes a neutrophil-dependent increase in peripheral vascular permeability especially in the presence of a vasodilator such as PGE_2 (Wedmore & Williams, 1981). Since LTB_4 is generated by neutrophils and is a potent stimulatory agonist, this lipid may function to amplify neutrophil responses elicited by other stimuli both *in vivo* and *in vitro*.

Prevention of LTB_4 biosynthesis may provide a useful way of determining the precise role of LTB_4 as an endogenous mediator of neutrophil responsiveness. The major problems with studies using putative 5-lipoxygenase inhibitors are toxicity and lack of specificity. Some investigations using isolated human neutrophils and various inhibitors have often produced conflicting and contrasting results

concerning whether products of arachidonic acid metabolism play an essential role in cellular activation (Smolen & Weissmann, 1980; Marone et al., 1983; Smith et al., 1986) or whether agonist-induced neutrophil responsiveness is independent of LTB_4 synthesis (Palmer & Salmon, 1985; Ozaki et al., 1986). With the development of more specific 5-lipoxygenase inhibitors, the precise role of LTB_4 in neutrophil activation may be evaluated.

There is also evidence that products of arachidonic acid may act as second messengers in cellular systems (Bevan & Wood, 1987). It has recently been shown that an eicosanoid, probably 12-HPETE, may act as a second messenger at a synapse in the marine mollusc, Aplysia californica. 12-HPETE is thought to be responsible for the inhibitory effects of FMRFamide (a neuroactive peptide) on 5HT-induced depolarization of Aplysia sensory neurones. (Piomelli et al., 1987). Whether products of arachidonic acid metabolism in other cell types including neutrophils act as second messengers like cyclic nucleotides, calcium, inositol phosphates and 1,2-diacylglycerol (Chapter 3) remains to be established.

LTB_4 is thought to exert its effects by interacting with specific LTB_4 receptors. O'Flaherty et al., (1981b) provided indirect evidence, suggesting that LTB_4 is acting via receptors when they demonstrated that neutrophil degranulation elicited by LTB_4 was subject to homologous desensitisation. Subsequently, specific human neutrophil LTB_4 receptors were shown independently by Goldman & Goetzl, (1982) and by Kreisle & Parker, (1983) by quantifying the binding of $[^3H]$-LTB_4. Goldman & Goetzl, (1984) showed that human neutrophils possess a high affinity receptor with a Kd of 0.39nM and 4400 sites per cell and a low affinity binding site with a Kd of 60nM and 270000 sites per cell. It has been suggested that the high and

low affinity may be associated with chemotactic and secretagogue activity respectively, since higher concentrations are usually required for lysosomal enzyme release than for cell locomotion. Caution must be taken when interpreting LTB_4 binding studies because LTB_4 is rapidly metabolised and other products of arachidonic acid metabolism can interact with the same receptor. For example 5-HETE, 6-trans-LTB_4 and 12-epi-6-trans-LTB_4 can competitively inhibit the binding of LTB_4. Other compounds at concentrations which produce a chemotactic response such as C5a and FMLP did not interfere with the binding of LTB_4. There are also marked differences in the radioligand binding data between human and rat neutrophils. Rat neutrophils exhibited only high affinity binding of LTB_4 with a Kd of 4.5nM and 6400 sites per cell. It was also demonstrated in the same investigation that rat neutrophils did not respond to LTB_4 by chemotaxis but at high concentration the neutrophils did aggregate (Kreisle et al., 1985). These observations indicate that the various effects of LTB_4 in leucocytes may result from interaction with distinct classes of receptors.

4. PLATELET-ACTIVATING FACTOR (PAF)

Platelet-activating factor (PAF) also known as PAF-acether or 1-O-alkyl-2-acetyl-sn-glycero-phosphocholine is a collection of ether phospholipids derived from membrane phospholipids. The ether lipid molecule possesses a long chain O-alkyl ether residue on the sn-1 position which may vary in length from $(CH_2)_{15-17}$ and an ester linked acetic acid moiety at the sn-2 position. At the sn-3 position, the polar head group in all naturally occurring ether lipids is that of an O-phosphocholine group (Demopoulos et al., 1979; Benveniste et al., 1979) (figure 5). PAF is synthesised by numerous cell types from many different species and exerts a wide spectrum of biological responses

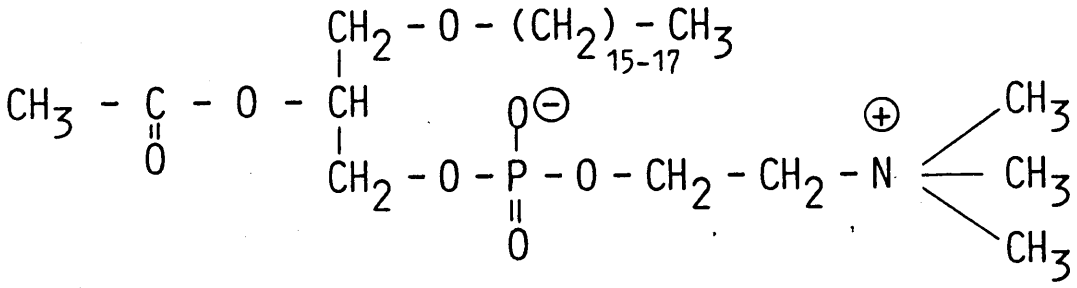

Figure 5.

Chemical structure of platelet-activating factor (PAF).

both *in vitro* and *in vivo* (Benveniste & Arnoux, 1983).

Barbaro and Zvaifler, (1966) first reported a leucocyte-dependent release of histamine from rabbit platelets but it was not until 1971 that Siragarian and Osler demonstrated that a soluble intermediate was responsible from this reaction. From its biological activity this compound was called PAF and shown to be released from rabbit basophil sensitised with IgE antibody (Benveniste et al., 1972). The production of PAF has been shown to exist in many other blood-borne cells such as neutrophils, peritoneal macrophages, alveolar macrophages, monocytes and platelets. Large amounts of PAF were recovered from calcium ionophore stimulated perfused isolated rat kidneys in the absence of blood cells (Pirotzky et al., 1980). Human endothelial cells are probably another source of PAF. A recent and interesting observation is that there is a natural occurrence of PAF in the protozoan *Tetrahymena pyriformis*. However, the significance of its presence in this microorganism is unknown (Lekka et al., 1986).

PAF is not stored within the cell but synthesised by the cell upon activation. Stimulation of neutrophils by agonists such as calcium ionophore, FMLP, C5a, zymosan and LTB_4 have been shown to generate this ether lipid (Camussi et al., 1981; Betz & Henson, 1980; Mueller et al., 1983). The predominant biosynthetic pathway for the production of PAF is thought to be via the combined efforts of two enzyme systems; phospholipase A_2 and acetyltransferase. It is generally agreed that the major precursor of PAF is a resident membrane phospholipid called 1-O-alkyl-2-acyl-glycero-phosphocholine containing a long chain residue in the sn-2 position (often arachidonic acid) (Swendsen et al., 1983). This precursor molecule is thought to be produced by *de novo* synthesis according to the sequence of events outlined in figure 6. Upon stimulation of the

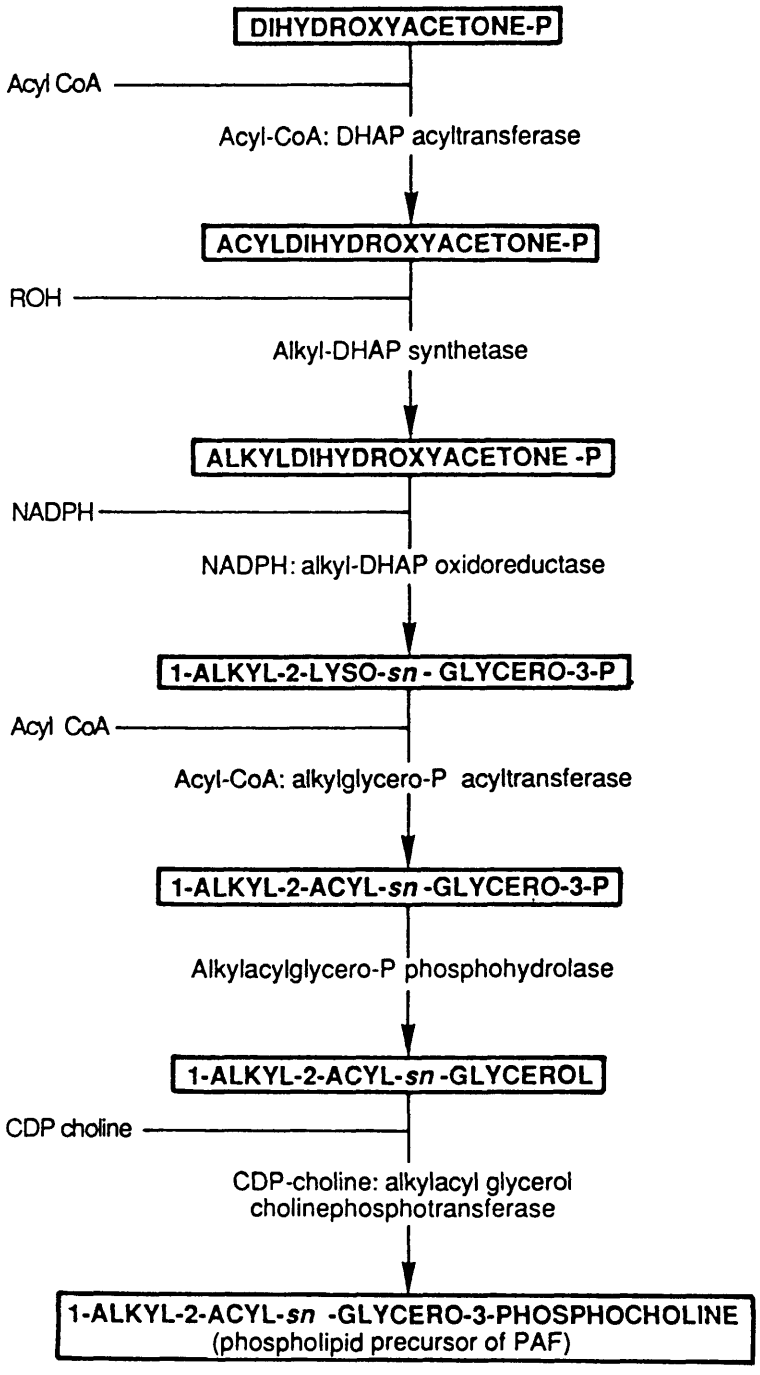

Figure 6.

The de novo biosynthetic pathway for the formation of 1-alkyl-2-acyl-sn-glycero-3-phosphocholine, the inactive phospholipid precursor of platelet-activating factor (PAF).

cell, PLA_2 cleaves the pre-existing precursor at the sn-2 position to yield lyso-PAF and a free fatty acid. If the free fatty acid is arachidonic acid, it can be utilised for eicosanoid (e.g. LTB_4) production. The lyso-PAF is then acetylated by 1-O-alkyl-2-lyso-glycero-phosphocholine acetyltransferase from acetyl-CoA to form PAF. This sequence of events is often referred to as the deacylation-reacylation pathway. PAF is then rapidly inactivated, independently from cell stimulation, by the reverse pathway catalysed by acetyl hydrolase and acetyl transferase (figure 7).

Although I have outlined the major biosynthetic pathway for PAF production, one must consider other possible pathways. An alternative pathway is by the transfer of a phosphocholine group into the 1-O-alkyl-2-acetyl-glycerol catalysed by a specific CDP-cholinephospho-transferase. This scheme has been characterised in human neutrophils (Alonso et al., 1982), rat platelets (Blank et al.,1984) and in a rat spleen preparation (Renooij & Snyder, 1981). The formation and availability of the substrate alkylacetylglycerol is not established but may be derived from de novo synthesis using dihydroxyacetone and fatty alcohol as the starting material. Mueller et al., (1983) using rabbit neutrophils showed that this pathway is probably only a minor source of PAF and that most is derived via the deacylation-reacylation pathway. It is also possible that PAF formation could be initiated by activation of phospholipase C which could attack the parent phosphoglycerides to yield a 1-O-alkyl-2-acyl glycerol; the long chain acyl residue from this molecule could then be replaced by an acetyl residue and the choline moiety could be added by the CDP-cholinephosphotransferase system.

PAF is one of the most potent activators of platelets and neutrophils so far described. PAF-induced neutrophil activation was

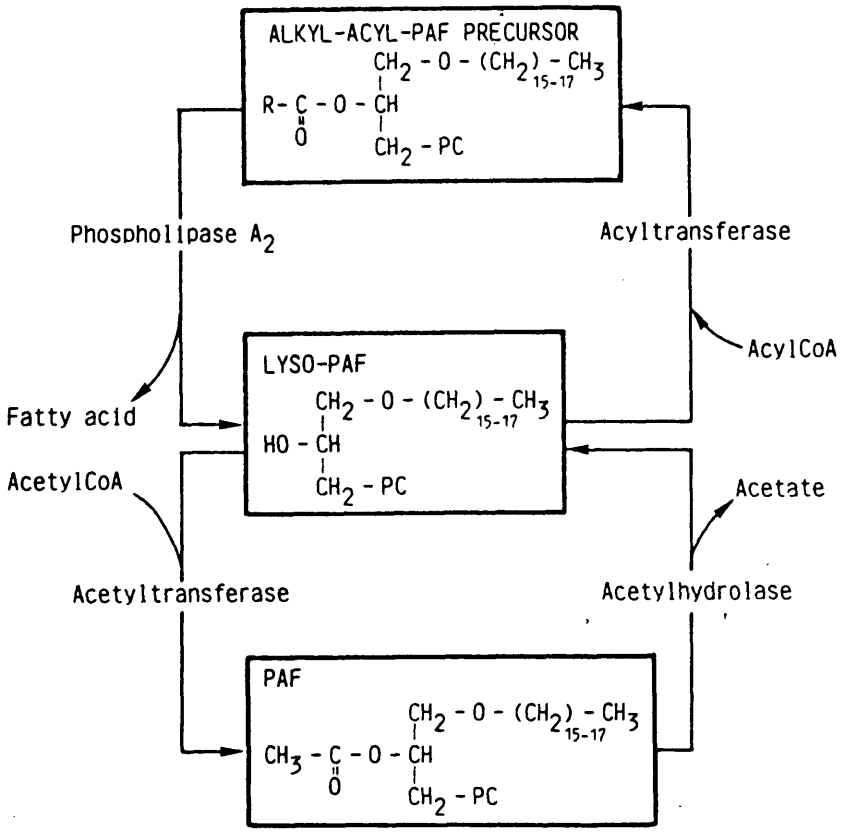

Figure 7.

The deacylation-reacylation pathway for platelet-activating factor (PAF).

PC - phosphorylcholine and R - a long chain hydrocarbon.

first reported by Henson, (1981) who demonstrated that intravenous injection of PAF causes a neutropenia with the formation of neutrophil aggregates. It has subsequently been shown that this ether lipid, at concentrations varying between 0.1-1μM, induces neutrophil aggregation, degranulation, chemotaxis and the release of superoxide anions (O'Flaherty et al., 1981a; Czarnetzi & Benveniste, 1981; Pirotzky et al., 1985). Intravenous injection of PAF leads to a number of systematic effects, which are sometimes species-dependent, including vasoconstriction, a particularly powerful bronchoconstriction, hypotension, pulmonary hypertension, increased vascular permeability, thrombocytopenia, neutropenia and death. Intradermal administration of PAF causes oedema and hyperalgesia with accumulation of both neutrophils and mononuclear cells (Benveniste & Vargaftig, 1983). With its wide spectrum of biological effects it is hardly surprising that this lipid has been implicated in a variety of pathological conditions. There is abundant literature indicating that PAF may be causally involved in thrombosis, acute inflammation, asthma and systemic anaphylaxis, cardiac anaphylaxis, endotoxic shock and in gastrointestinal ulcerations. The vast majority of these conditions are thought to be mediated partly by platelets or leucocytes but the precise role and participation of these cells still remains to be elucidated. PAF, like LTB_4, has the potential to act as an endogenous mediator or amplifier of neutrophil activation elicited by other agonists. Due to the lack of specific PAF biosynthesis inhibitors, the exact role of PAF awaits clarification. However, with the development of specific PAF antagonists such as kadsurenone and BN 52021, some light may be shone on this matter.

It is widely believed that PAF exerts its repertoire of biological effects by interacting with specific PAF receptors located on the plasma membrane. The involvement of specific PAF receptors was first demonstrated independently by Wykle et al., (1981) and by Heymans et al.,

(1981). They showed that the naturally occurring stereoisomer (R-PAF), but not S-PAF, was effective in stimulating the various responses associated with platelets and neutrophils both in vivo and in vitro. Radioligand binding analyses have revealed high affinity receptors in human neutrophils (Valone & Goetzl, 1983; O'Flaherty et al., 1986), human platelets (Valone et al., 1982), rabbit platelets (Hwang et al., 1983), guinea pig smooth muscle (Hwang et al., 1983) and human lung membranes (Hwang et al., 1986a).

Valone & Goetzl, (1983) showed that the binding of PAF in human neutrophils was maximal within 20-30 mins at 37°C, exhibiting a high affinity site (Kd of 0.11nM) with approximately 5.2×10^6 PAF binding sites per cell. A lower affinity binding site was also observed which the authors attributed to a non-specific uptake into cellular compartments. O'Flaherty et al., (1986b)demonstrated using human neutrophils that PAF binding fits a two receptor model; with Kd values for high and low affinity sites of 0.2nM and 500nM respectively. Lambrecht & Parnham, (1986) using the specific PAF receptor antagonist, kadsurenone, have shown that this agent can discriminate between PAF receptors in leucocytes and macrophages. These PAF receptors have provisionally been designated PAF_1 and PAF_2 receptors respectively.

5. OTHERS.

There are numerous other agents that cause the activation of human neutrophils that probably produce their effects by interacting with specific receptors. For example, crystal-induced chemotactic factor, various immune complexes, C3b opsonised and Ig coated particles, interleukin 1 and concanavalin A.

Non-physiological activators of human neutrophils such as phorbol

esters (e.g. PMA) and calcium ionophores (e.g. A23187 and ionomycin) have been used as pharmacological tools to probe the mechanisms underlying neutrophil reactivity. These compounds are thought to bypass ligand-receptor interactions; the phorbol esters directly stimulate protein kinase C and the ionophores cause an increase in cytosolic-free calcium ions, $\left[Ca^{2+}\right]i$ (Chapter 3).

CHAPTER 3. STIMULUS-RESPONSE COUPLING.

1. General Introduction.

Neutrophils, in common with other cells, are able to respond to changes in their environment; a process which is usually achieved by external chemical stimuli acting upon membrane associated specific recognition sites known as "receptors". These external stimuli (or agonists) are diverse in nature and may be soluble or particulate. Consequently, the receptor types are also numerous and equally diverse in their recognition capacity. Agonist-receptor combination can stimulate, inhibit or modulate cellular activity but the mechanisms linking the receptors to the cellular response, a phenomenon often referred to as stimulus-response coupling, are less well understood. The generalised concept of stimulus-response coupling is depicted schematically in Figure 8.

Receptors have been the focus of intense investigation within the last few decades and, as a result, some receptors have been identified, partially or completely purified, sequenced, cloned and classified. Receptors are usually proteins consisting of monomeric or oligomeric subunits located on the external surface of the plasma membrane or integrated into the phospholipid bilayer. The agonists combine with specific ligand binding sites in the receptor and information is transmitted through the plasma membrane via a particular transduction mechanism. These transducers are thought to be a family of heterotrimer proteins known as guanine nucleotide binding regulatory proteins or "G

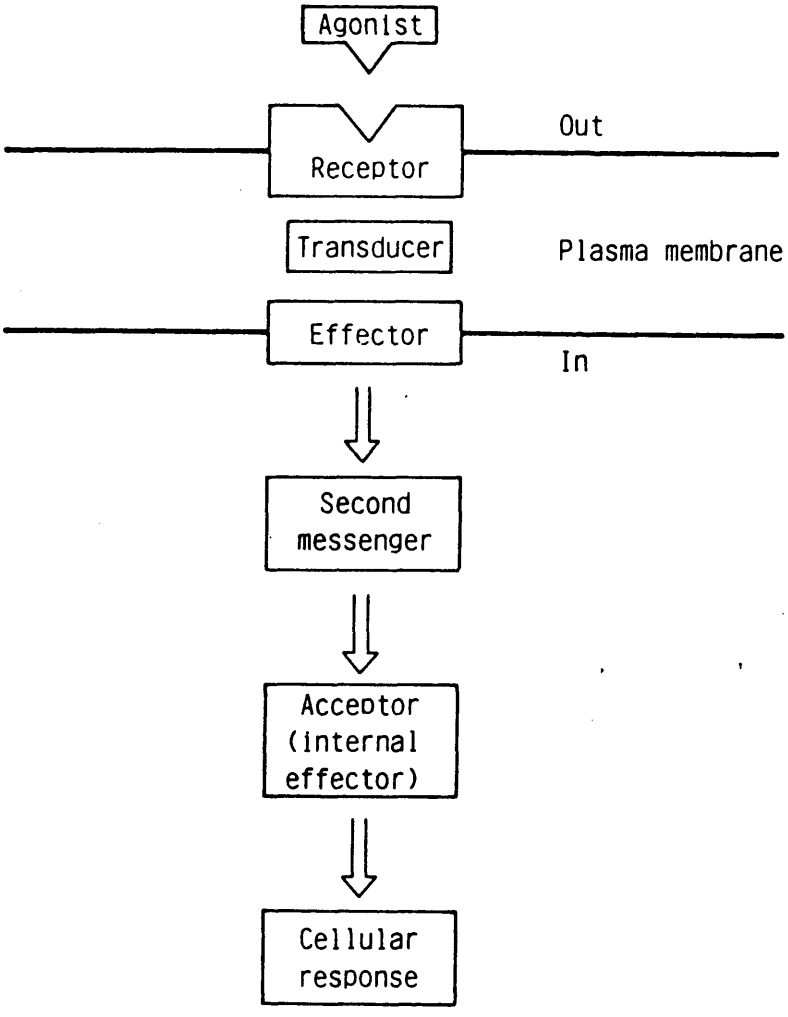

Figure 8.

Schematic representation of the general mechanism of stimulus-response coupling.

See text for explanation.

proteins" because they require guanosine triphosphate (GTP) to function. The G protein-GTP complex stimulates an effector, usually an enzyme, which amplifies the signal by converting precursor molecules into intracellular second messengers. Second messenger molecules convey information by combining with specific intracellular target entities or acceptors. These target sites are often proteins which undergo a conformational change or kinases which catalyse the phosphorylation of other target proteins. Collectively these events result in alteration of the rates of key biochemical reactions that govern the cellular responses. Although numerous agents have been proposed to be second messengers, remarkably few agents satisfy the criteria necessary to be formally categorised as such. These criteria are:

a) The putative second messenger and the enzymes responsible for its formation should be present within the cell.

b) The time-course for the formation of the second messenger should be consistent with agonist effect.

c) Addition of the putative second messenger or structural analogues should mimic the effect of the agonist response.

d) A mechanism for the inactivation of the second messenger should be present.

e) An intracellular target entity for the second messenger should be identified.

2. Cyclic AMP

Since the discovery of cyclic AMP by Sutherland and Rall

(1960), and the proposal that this nucleotide may be the intracellular mediator of the effects of glucagon and adrenaline in the liver, cyclic AMP has been the subject of much research. The formation of intracellular cyclic AMP through stimulation of the enzyme, adenylate cyclase, is the second messenger pathway currently best understood and thought to play an important role in many biological systems ranging from prokaryotes to complex eukaryotes. The pathway is schematically represented in Figure 9. Agonists combine with the specific stimulatory (R_S) or inhibitory (R_I) receptors to either cause an increase or decrease in cyclic AMP via stimulation or inhibition of adenylate cyclase which enzymatically converts ATP to cyclic AMP in the presence of Mg^{2+} ions.

Extensive research on the transduction process mediating adenylate cyclase activity has shown that two G proteins (N_S and N_I) are intimately involved (Ross & Gilman, 1980; Jakobs et al., 1984). N_S is associated with agonist-induced stimulation of adenylate cyclase and N_I mediates agonist-induced inhibition of the enzyme. N_S and N_I are oligomeric proteins comprising of three non-identical subunits termed as alpha (41-45K M.wt.), beta (35K M.wt.) and gamma (10K M.wt.). The beta and gamma subunits of these regulatory proteins appear to be similar, whereas the alpha subunits, which provide the GTP binding site, vary in molecular size (Codina et al., 1984; Hildebrandt et al., 1985; Housley, 1984).

The existence of the stimulatory G protein, N_S, was first shown in avian erythrocyte membranes using GTP photoaffinity analogues and shown to be a heat stable protein of 42K M.wt. (Pfeuffer, 1977; 1979). Stimulation of N_S facilitates binding

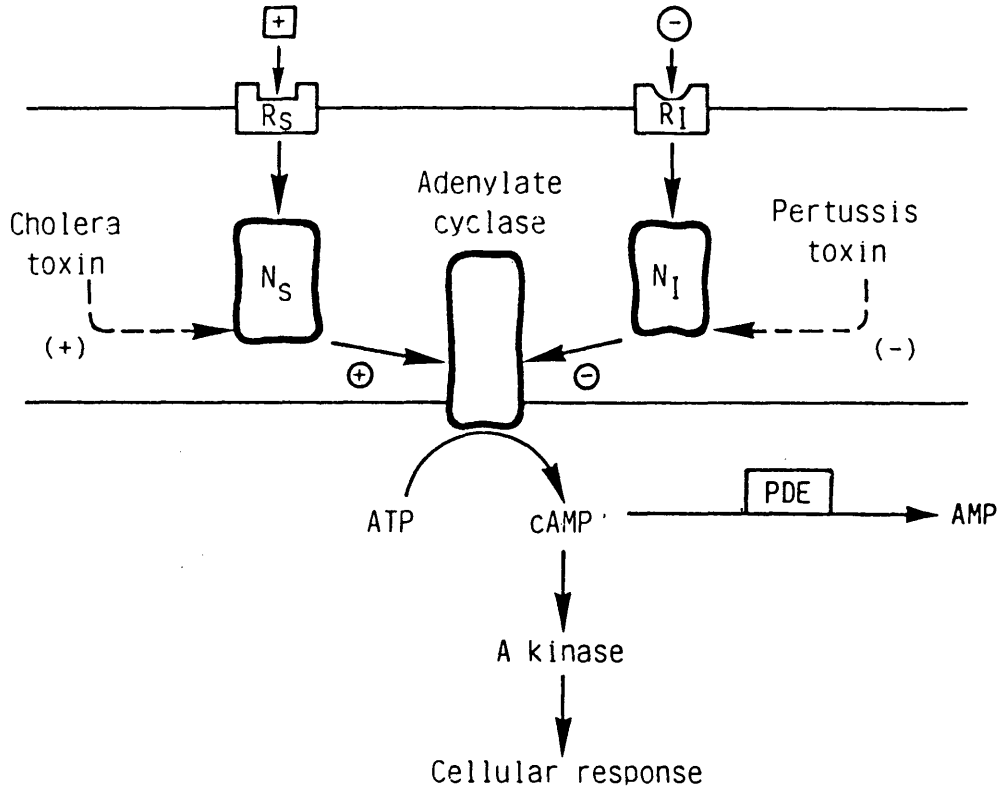

Figure 9.

Schematic representation of receptor-mediated control of cyclic AMP metabolism and proposed site of action of cholera toxin and pertussis toxin.

See text for explanation.

of GTP to the alpha subunit, which then dissociates from its beta and gamma subunits, thereby activating the catalytic unit (Gilman, 1984; Schramm & Selinger, 1984; Taylor & Merrit, 1986). The subunits remain dissociated for as long as GTP is bound to the alpha subunit; it is the $alpha_S$ that activates adenylate cyclase activity and thereby elevating intracellular levels of cyclic AMP. The activity of the N_S complex is terminated by the hydrolysis of the GTP to GDP by a GTPase inherent in the alpha subunit (Cassel & Selinger, 1976; Jakobs et al., 1984). GTPase activity has been shown to be present in the human neutrophil (Hyslop et al., 1984) as well as many other systems. This GTPase activity is a target for the toxin produced by the Cholera bacillus. Cholera toxin causes the NAD-dependent ADP ribosylation of the alpha subunit of the N_S which subsequently inhibits the inherent GTPase activity. This, in turn, prevents the hydrolysis of GTP, hence the N_S complex is permanently activated so that the cell continually produces cyclic AMP.

The presence of an inhibitory G protein, N_I, was first proposed in the late 60's from the ability of GTP to inhibit adenylate cyclase in rat adipocytes membranes (Creyer et al., 1969). The interaction of agonists with distinct receptors that exhibit a reduction of cyclic AMP accumulation stimulate N_I in a similar GTP-dependent manner as N_S. When GTP binds to the alpha subunit of N_I, the alpha subunit dissociates; however, the mechanisms by which adenylate cyclase activity is inhibited are not understood. It has been suggested that the beta-gamma subunits of N_I may mediate the inhibition of adenylate cyclase indirectly by favouring the reassociation of the N_S complex (Gilman,

1984; Smigel et al., 1984). This G protein too can be influenced by a naturally occurring toxin isolated from the bacterium Bordetella pertussis (Sekura et al., 1985). Pertussis toxin or Islet-activating protein ADP-ribosylates, and hence inhibits, the alpha subunit of N_I. This inhibition of the inhibitory G protein will thus cause a net increase in cyclic AMP (Gilman, 1984; Ui, 1984). Stable (non-hydrolysable) analogues of GTP (GppNHp and GTPγS) can also modulate G protein function causing persistent activation whereas analogues of GDP (GDPβS) result in inhibition (Jakobs & Aktories, 1983).

Adenylate cyclase has recently been purified from rabbit myocardium and shown to be a glycoprotein (150K M.wt.) located in the plasma membrane (Pfeuffer et al., 1985). The catalytic subunit of adenylate cyclase can be activated directly, hence by-passing the receptors and the G proteins, by using the diterpene forskolin (isolated from the roots of Coleus forskolii). Forskolin has proven to be an extremely useful compound in examining the adenylate cyclase system in numerous cell types.

Other important enzymes controlling the levels of cyclic AMP metabolism is cyclic AMP phosphodiesterases which hydrolyses cyclic AMP to the inactive nucleotide 5'-AMP. Cyclic nucleotide phosphodiesterases have been shown to exist in multiple forms in a variety of mammalian cells which are structurally distinct and have different substrate affinity and specificity (Wells & Hardman, 1977; Appleman et al., 1982). Most cyclic AMP phospho-diesterase isoenzymes so far described have been categorised according to their substrate specificity: cyclic nucleotide phosphodiesterase with low affinity for both cyclic AMP and cyclic GMP, a high affinity cyclic AMP phosphodiesterase and a high affinity

cyclic GMP phosphodiesterase. The former two enzymes have been shown to exist in human neutrophils (Smith & Peters, 1980; 1981) whilst the latter enzyme could not be detected (Grady and Thomas, 1986).

Many cellular responses consequent upon receptor stimulation by hormones or agonists in numerous biological systems are regulated or modulated by changes in intracellular cyclic AMP concentrations. However, the precise role of cyclic AMP in neutrophils is somewhat controversial and confusing. Agents that cause a sustained rise in the levels of cyclic AMP in human neutrophils cause an inhibition of cellular activation. The E-type prostaglandin receptor or the β-adrenergic receptor when stimulated by PGE1 or isoprenaline respectively,which activate adenylate cyclase and increase cyclic AMP content, attenuate agonist-induced chemotaxis, superoxide production and lysosomal enzyme secretion (Stephens & Snyderman, 1982; Bergman et al., 1978; Rivkin et al., 1975; Simchowitz et al., 1980). Paradoxically, however, agents that cause neutrophil activation also cause a small transient rise in cyclic AMP. Such agents include latex particles, FMLP, C5a, immune complexes, zymosan-treated serum, opsonized zymosan, calcium ionophores such as A23187, PAF and LTB_4 (Herlin et al., 1978; Simchowitz et al., 1980; Smolen & Weissmann, 1981; Pryzwansky et al., 1981; Claesson, 1982; Hopkins et al., 1983). Indeed, this transient rise in cyclic AMP led several workers to postulate that cyclic AMP elevation constituted a fundamental requirement for neutrophil activation (Simchowitz et al., 1980). On the other hand, Smolen et al., (1980) observed that sub-threshold concentrations of FMLP incapable of inducing cellular activation could still elicit normal rise in cyclic AMP. They also reported that

superoxide generation and lysosomal enzyme release were markedly inhibited in the presence of low extracellular Na^+ or Ca^{2+} or in high K^+ concentrations whereas cyclic AMP elevation was unaffected. From these observations they concluded that rises in cyclic AMP were not responsible for neutrophil responsiveness. Much evidence has emerged, using specific phosphodiesterase inhibitors (e.g. 1-isobutyl 3-methyl xanthine) or agents that act directly on the adenylate cyclase complex, indicating the latter conclusion to be correct, suggesting that cyclic AMP has an inhibitory effect or only a modulatory role in leucocytes (Simchowitz et al., 1983; Marone et al., 1980; Lad et al., 1985c). The rise in cyclic AMP associated with neutrophil agonists may not be directly coupled to adenylate cyclase. Indeed, Verghese et al., (1985) have demonstrated that chemoattractants raise intracellular cyclic AMP in human polymorphonuclear cells by a unique Ca^{2+}-dependent mechanism which may involve transient inhibition of phosphodiesterase activity.

Many actions of cyclic AMP are thought to be mediated through activation of a cyclic AMP-dependent protein kinases, which subsequently cause phosphorylation of a number of different proteins. (Flockhart & Corbin, 1982; Krebs, 1984; Berridge, 1985). The precise site of cyclic AMP-dependent reduction or modulation of neutrophil activation remains largely unknown. One possible mechanism, that has been suggested, for cyclic AMP-induced inhibitory effects is by interfering with the production, metabolism and effect of other second messenger molecules (Berridge, 1985). Strong evidence has been presented, using platelets, suggesting that cyclic AMP may interfere with phospholipase C activity (Lapetina et al., 1977) or with the consequent

superoxide generation and lysosomal enzyme release were markedly inhibited in the presence of low extracellular Na^+ or Ca^{2+} or in high K^+ concentrations whereas cyclic AMP elevation was unaffected. From these observations they concluded that rises in cyclic AMP were not responsible for neutrophil responsiveness. Much evidence has emerged, using specific phosphodiesterase inhibitors (e.g. 1-isobutyl 3-methyl xanthine) or agents that act directly on the adenylate cyclase complex, indicating the latter conclusion to be correct, suggesting that cyclic AMP has an inhibitory effect or only a modulatory role in leucocytes (Simchowitz et al., 1983; Marone et al., 1980; Lad et al., 1985c). The rise in cyclic AMP associated with neutrophil agonists may not be directly coupled to adenylate cyclase. Indeed, Verghese et al., (1985) have demonstrated that chemoattractants raise intracellular cyclic AMP in human polymorphonuclear cells by a unique Ca^{2+}-dependent mechanism which may involve transient inhibition of phosphodiesterase activity.

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production of inositol phosphates (Watson et al., 1984) and elevation of $[Ca^{2+}]i$ (Feinstein et al., 1983; Zavoico & Feinstein, 1984; MacIntyre et al., 1985b; Bushfield et al., 1985). There is some evidence in human neutrophils showing a relationship between cyclic AMP and $[Ca^{2+}]i$ elevation (DeTogni et al., 1984; Kato et al., 1986). These authors showed that, although cyclic AMP did affect agonist-induced $[Ca^{2+}]i$ transients, this could not fully explain cyclic AMP inhibition. Increased levels of cyclic AMP produced by PGE1 or by the cyclic AMP stable analogue; dibutyryl cyclic AMP and the phosphodiesterase inhibitor; theophylline, caused an inhibition of FMLP-induced phosphatidyl-inositide metabolism in human neutrophils (DellaBianca et al., 1986; Kato et al., 1986). Similar observations were made using guinea pig neutrophils where elevated levels of cyclic AMP induced by PGE2, dibutyryl cyclic AMP and forskolin inhibited arachidonic acid release and inositol phospholipid hydrolysis elicited by the chemotactic tripeptide (Takenawa et al., 1986).

Although there are potentially many other mechanisms whereby cyclic AMP could exert its inhibitory effect in human neutrophils, the precise biochemical consequence of elevated cytoplasmic cyclic AMP remains to be elucidated.

3. Calcium.

It was over 100 years ago that Sydney Ringer first systematically investigated the effects of removal of particular ions from the bathing medium surrounding various tissues. His experiments demonstrated the requirement for extracellular Ca^{2+} for the normal continued beating of an isolated frog heart (Ringer, 1883).

Many elegant studies followed showing that removal of external calcium affected various physiological processes which led to proposal that Ca^{2+} ions play a critical role in excitation-contraction coupling of muscle tissue (Heilbrunn, 1940; Sandow, 1952; Katz, 1969). It was subsequently shown that this ubiquitous ion was necessary for acetylcholine-induced secretion of catecholamines in the perfused adrenal medulla of the cat (Douglas & Rubin, 1961). From their studies they proposed that Ca^{2+} might act as a general effector of agonist stimulated secretion and thus coined the term stimulus secretion-coupling. With the knowledge that calcium plays a crucial role in contractile tissue and secretory cells, it soon became apparent that this divalent cation was an important coupling agent in neutrophil movement and secretion. Calcium is thought to act either by direct combination with a target enzyme, e.g. phospholipase A_2 (PLA_2), or by interaction with a Ca^{2+} acceptor protein, calmodulin, which can modulate enzyme activity (Fecheimer & Zigmond, 1983) and modulate cellular activation.

The proposal that intracellular Ca^{2+} is involved in neutrophil activation was originally made from circumstantial or indirect evidence by analogy with other cell types. It was shown that removal of external Ca^{2+} by suspending neutrophils in calcium-free buffer and/or in the presence of the calcium chelator EGTA reduced, but did not abolish, agonist-induced neutrophil activation. Numerous reports showed that the presence of external calcium is required for maximal agonist-induced adhesion and aggregation (O'Flaherty et al., 1978), chemokinesis and chemotaxis (Becker & Showell, 1972; Boucek & Snyderman, 1976; Estensen et al., 1976), lysosomal enzyme release (Goldstein et al.,

1975; Showell et al., 1977) and superoxide production (Hallett et al., 1981). Further indirect evidence implicating calcium as an important second messenger came from the observations that calcium ionophores, A23187 and ionomycin, could stimulate (Goldstein et al., 1974; Koza et al., 1975; Cockcroft et al., 1980a) and that the calcium antagonist TMB-8 or the inhibitor of calmodulin, trifluoperizine, could inhibit (Smolen et al., 1981; Naccache et al., 1980) neutrophil responsiveness.

The use of radiolabel $^{45}Ca^{2+}$ has proved useful in demonstrating Ca^{2+} fluxes in a variety of cells including neutrophils. Activation of neutrophils with chemotactic agents resulted in large uptake of $^{45}Ca^{2+}$ and in cells previously loaded with this radiolabel a net efflux of $^{45}Ca^{2+}$ was observed (Boucek & Snyderman, 1976; Naccache et al., 1977; Bennett et al., 1980). Caution must be taken when interpreting these results because neutrophils, when exposed to such stimuli, will undergo conformational and morphological changes such as cell movement, extrusion of pseudopods and degranulation. Such changes alter the cell surface area and increase calcium binding sites. Consequently, this prevents simple conclusions to be drawn concerning calcium fluxes across cell membranes (Sha'afi & Naccache, 1983; Foreman et al., 1977).

The fluorescent probe chlortetracycline (CTC) has been employed to investigate the possible involvement of calcium in a variety of cells including neutrophils. This hydrophobic molecule partitions into cell membranes, interacts with divalent cations and the resultant complex fluoresces. Several workers have shown that chemotactic agents (e.g. FMLP, C5a) cause a decrease in CTC fluorescence which has been attributed, perhaps

optimistically, to release of membrane associated calcium (Naccache et al., 1979a, 1979b; Takeshinge et al., 1980; Smolen & Weissmann, 1982). Current evidence, however, suggests that this is a misleading concept and that CTC does not only measure plasma membrane bound Ca^{2+} (Millman et al., 1980) but also measures Ca^{2+} located in other membrane bound organelles. Although CTC can be readily incorporated in neutrophils, for various reasons including lack of specificity (CTC also binds Mg^{2+} ions), lack of sensitivity (Caswell, 1979), the autofluorescence of neutrophils (Cross et al., 1982; Gabig, 1983), interference of oxygen metabolites with the dye (Whitin et al., 1981), pH dependence and difficulty in quantifying the fluorescence (Gains, 1980), it is inadequate as an indicator of $[Ca^{2+}]i$.

The direct measurement of cytosolic-free calcium concentrations, $[Ca^{2+}]i$, would be a better way of demonstrating the involvement of Ca^{2+} in neutrophil activation. This has been made possible only within the last few years by the introduction of various chemical $[Ca^{2+}]i$ indicators which include photoproteins (e.g. aequorin and obelin), organic dyes (e.g. arsenazo III) and fluorescent indicators (e.g. Quin2, Fura-2, Indo-1).

The photoproteins, especially aequorin, have a number of characteristics that make them good indicators of $[Ca^{2+}]i$ (Hallett & Campbell, 1982a; 1983; Cobbold et al., 1983). They do not cause any calcium buffering, they are sensitive enough to resolve small agonist-induced changes in $[Ca^{2+}]i$ and not toxic to the cell. However, one particular problem is that the proteins are consumed with repeated $[Ca^{2+}]i$ measurements because Ca^{2+} binding is not reversible. The main disadvantage of this technique

appears to be the technical difficulties of introducing the protein into the cytosol. A number of procedures have been developed in order to overcome this access problem including microinjection of the indicator directly to the cytosol (Morgan & Morgan, 1982) or by cell permeabilisation (Sutherland et al., 1980; Johnson et al., 1985). A particular technique adopted for measuring $[Ca^{2+}]i$ in neutrophils is to conceal the photoprotein in human erythrocytes 'ghosts' and induce them to fuse with the cells using Sendai virus (Hallett & Campbell, 1982b). These hybrids have shown rises in $[Ca^{2+}]i$ induced by FMLP and concanavalin A but no elevation could be detected using unopsonised particles (Campbell & Hallett, 1983).

By far the most widely used indicator of neutrophil $[Ca^{2+}]i$ is the fluorescent probe quin2 (Tsien, 1980; 1981; Tsien et al., 1982a;b). This calcium-sensitive dye is introduced into the cells in the form of the non-polar derivative, quin2-acetoxymethyl ester (quin2-AM). This ester can readily permeate the plasma membrane and, once located in the cytoplasm, it is hydrolysed into quin2 by cytoplasmic esterases. Quin2 is water-soluble, cannot pass the membrane and so remains within the cell. It will avidly bind intracellular calcium ions with a 1:1 stochiometry and undergoes fluorescence changes in response to calcium binding. This tetra-carboxylic acid is relatively indifferent to Mg^{2+} ions and changes in pH within the physiological range; and is thought to be distributed evenly throughout the cytosol.

White and colleagues first reported changes in $[Ca^{2+}]i$ using quin2 loaded rabbit neutrophils. They showed that resting $[Ca^{2+}]i$ varied between 100-200nM in different experiments and that FMLP, C5a and LTB_4 all caused a rapid increase in $[Ca^{2+}]i$ reaching maximal values by 15 seconds (White et al., 1983b). Chemotactic tripeptide

-induced changes in $[Ca^{2+}]i$ were subsequently shown in human neutrophils (Pozzan et al., 1983). The resting $[Ca^{2+}]i$ in unstimulated human neutrophils reported by various workers is maintained between 100-140nM depending on the experimental conditions used. Rapid concentration-dependent increases in $[Ca^{2+}]i$ in human neutrophils can be induced by many agonists including FMLP, C5a, LTB_4, PAF and ionomycin (for a review, see Westwick & Poll, 1986). In contrast, the phorbol ester, PMA, was found to cause neutrophil activation without elevating $[Ca^{2+}]i$ (Sha'afi et al., 1983).

Although quin2 has been extensively used in a wide variety of cell systems and a wealth of useful information can be credited to this fluorescent indicator, it does possess certain weaknesses and pitfalls. Quin2 has a poor quantum yield. Consequently, the dye has a poor fluorescent intensity resulting in only a two fold increase in fluorescence. The dye is excited by ultraviolet light irradiation which also generates considerable autofluorescence of the cells. This background autofluorescence has to be overcome by high concentrations of quin2 (0.1-mM) which causes significant increases in calcium buffering and blunting of agonist-induced elevation of $[Ca^{2+}]i$ (Tsien et al., 1982a). Indeed, under certain loading conditions and with a high concentration of cytosolic quin2, the indicator has been shown to inhibit neutrophil chemotaxis (Elferink & Deierkauf, 1985), exocytosis and superoxide production (Lew et al., 1984a). With large increases in $[Ca^{2+}]i$ ($>1\mu M$) quantification of accurate $[Ca^{2+}]i$ levels becomes difficult because quin2 becomes almost saturated. The fluorescence intensity is also dependent on a number of variables including illumination intensity, emission collection efficiency and tissue

thickness. In order to obtain accurate $[Ca^{2+}]i$ measurements, all these factors have to be taken into account and minimised or standardised for each particular cell system (Tsien et al., 1985; Rink & Pozzan, 1985).

With the development of newer fluorescent indicators such as fura-2 or indo-1, some of the problems associated with quin2 can be largely overcome (Grynkiewicz et al., 1985). Fura-2 displays a 30 fold greater fluorescence intensity in comparison with quin2; therefore it can be used at lower cytosolic loading concentrations. These lower concentrations (10-100μM) offer less calcium buffering enabling more accurate measurement of $[Ca^{2+}]i$ transients. Ca^{2+}-dye interaction not only increases the fluorescence intensity but also shifts the excitation and emission wavelengths. Thus, by measuring the ratio of free dye to Ca^{2+}-bound dye, a more precise measure of $[Ca^{2+}]i$ can be attained that is independent of dye concentration, illumination intensity and the amount of tissue in the light path. Fura-2 also has a larger dissociation constant (Kd) for Ca^{2+} than its predecessor, quin2, thus allowing measurements of larger rises in $[Ca^{2+}]i$. A better selectivity for Ca^{2+} than for Mg^{2+} and other heavy metals is also associated with these new indicators (Grynkiewicz et al., 1985). The observations made in human neutrophils using quin2 still await investigation and confirmation with fura-2.

The neutrophil is surrounded by an external calcium concentration approximately 10000-fold greater than its resting $[Ca^{2+}]i$. Consequently, the cell has adopted various homeostatic mechanisms in order to maintain this low $[Ca^{2+}]i$. This is achieved by a number of calcium extrusion and sequestration mechanisms. Most cells possess two main mechanisms, a Ca^{2+}, Mg^{2+}-ATPase pump and an

Na^+/Ca^{2+} exchange system.

Ca^{2+}, Mg^{2+}-ATPase pumps have been reported to be present in the plasma membrane (Ochs & Reed, 1983; Volpi et al., 1983; Lagast et al., 1984) and in lysosomal membranes (Klempner, 1985) of neutrophils. This pump has been shown to be activated by calmodulin-dependent kinase and cyclic AMP-dependent kinase. Recently, Lagast et al., (1984) and Rickard & Sheterline, (1985) have shown that PMA-stimulated guinea pig neutrophils activate this Ca^{2+} transporter, suggesting that protein kinase C may also regulate $\left[Ca^{2+}\right]i$ levels. Since the pump can be found on intracellular membranes such as lysosomal, mitochondrial and sarcoplasmic reticulum membranes, this mechanism may also be involved in Ca^{2+} sequestration.

Another mechanism by which cells have the potential to maintain low $\left[Ca^{2+}\right]i$ is by the extrusion of Ca^{2+} from the cell via an Na^+/Ca^{2+} electrogenic exchange system (Cauvin et al., 1983). The presence of this antiporter in human neutrophils has not been reported yet. However, Sha'afi & Naccache, (1983) failed to demonstrate the presence of an Na^+/Ca^{2+} exchanger in rabbit neutrophils. The development of amiloride analogues with increased specificity towards this antiporter (Kaczorowski et al., 1985) may help determine the precise function of this electrogenic exchange, if indeed present, in human neutrophils.

The mechanism and nature underlying agonist-induced elevation of $\left[Ca^{2+}\right]i$ has been the subject of intense research not only in the neutrophil but also in numerous other cell systems. It is thought that the increase in neutrophil $\left[Ca^{2+}\right]i$ is derived partly from the external milieu and partly from mobilisation of internal

stores. Quantification of the amount resulting from both sources has been attempted using quin2 loaded neutrophils suspended in the presence and absence of external Ca^{2+}. For example, White et al., (1983b) showed that in rabbit neutrophils FMLP-induced elevation of $[Ca^{2+}]i$ occurred independently of external Ca^{2+}. However, in human neutrophils, it is thought that the majority (> 75%) of the agonist-induced rise in $[Ca^{2+}]i$ derives mainly from influx of extracellular Ca^{2+} (Pozzan et al., 1983).

The translocation of extracellular Ca^{2+} into the cytosol is thought to occur through the opening of specific calcium channels located in the plasma membrane. There exist voltage-operated channels (VOCs) found in electrically excitable cells (Bolton, 1979). In tissues such as cardiac and smooth muscle cells, membrane depolarization results in the opening of VOCs which results in a net influx of external Ca^{2+} leading to activation, i.e. contraction. Although neutrophils can undergo membrane depolarization (Korchak & Weissmann, 1980) it is insufficient to cause activation and elevation of $[Ca^{2+}]i$. Membrane depolarization caused by elevation of the extracellular K^+ concentration or hyperpolarization produced by K^+ ionophores (e.g. valinomycin and nigericin) fails to cause chemotaxis, degranulation or an increase in $[Ca^{2+}]i$ (Showell et al., 1977; Romeo et al., 1975). In addition, classical calcium antagonists (e.g. diltiazem and nifedipine) which inhibit agonist-induced responses in depolarization activated tissue by allegedly blocking VOCs (Triggle, 1982) fail to attenuate agonist-induced activation of neutrophils. Therefore, it is highly unlikely that increases in $[Ca^{2+}]i$ occur via VOC in human neutrophils.

The other main type of calcium channel, which opens as a direct consequence of receptor occupation and are consequently termed receptor

operated calcium channels (ROCs), are thought to be involved in human neutrophil activation. The precise way that these ROCs open remains largely unknown. However, three reports have recently been published which might account for receptor operated Ca^{2+} entry. Kuno & Gardner, (1987) working with T-lymphocytes have shown that $Ins(1,4,5)P_3$, a product of phosphoinositide metabolism and mainly thought to be involved with the mobilisation of intracellular Ca^{2+} stores, produces influx of external Ca^{2+} through a specific channel in the plasma membrane. Irvine & Moor, (1986) have demonstrated that $Ins(1,3,4,5)P_4$ derived from $Ins(1,4,5)P_3$ phosphorylation may be involved in the translocation of external Ca^{2+} to the cytosol in sea urchin eggs. Another mechanism has been proposed by V-Tscharner et al., (1986) using quin2 and fura-2 loaded human neutrophils, suggesting ROCs may be opened by calcium itself. They have reported that an initial FMLP or PAF-induced rise in $\left[Ca^{2+}\right]i$ induces the opening of other calcium, or more accurately divalent cations, channels on the neutrophil membrane. These channels are not opened by $Ins(1,4,5)P_3$ or by the agonist directly but only by the elevation of $\left[Ca^{2+}\right]i$. It also remains possible that rises of $\left[Ca^{2+}\right]i$ may result from other yet unidentified mechanisms. The elevation of $\left[Ca^{2+}\right]i$ due to mobilisation of Ca^{2+} from internal stores has been mainly attributed to a product of inositol phospholipid metabolism, $Ins(1,4,5)P_3$.

4. Inositol Phospholipid Metabolism.

4.1. General

Since the original observations made by Hokin & Hokin in 1953 that neurotransmitters and hormones cause a turnover of membrane phosphoinositide lipids, our knowledge has vastly expanded within the last few years, suggesting that the 'phosphoinositide cycle' is much more complex than was previously believed (for reviews see Berridge, 1985; Hokin, 1985; Abdel-Latif, 1986). Figure 10 shows the pathways and proposed pathways involved in the phosphoinositide cycle thought to occur in a wide variety of cell systems including human neutrophils.

Inositol-containing lipids are relatively minor phospholipid constituents of mammalian cells, comprising less than 10% of the total phospholipid content (Marcus et al., 1969). 'Phosphoinositides' is the collective term used for three lipids; phosphatidylinositol (PtdIns), the major inositol phospholipid comprising about 90% of total, and the phosphorylated derivatives phosphatidylinositol 4 phosphate (PIP) and phosphatidylinositol 4,5 bisphosphate (PIP_2) (Figure 11).

The interconversion of the phosphoinositides is achieved by the presence of specific kinases and phosphatases. PtdIns kinase phosphorylates PtdIns at position 4 on the sugar inositol ring to yield PIP which is further phosphorylated at position 5 via PIP kinase to produce PIP_2. The reverse reaction can also occur enzymatically via PIP and PIP_2 phosphatases.

These inositol phospholipids, especially PIP_2, have a high

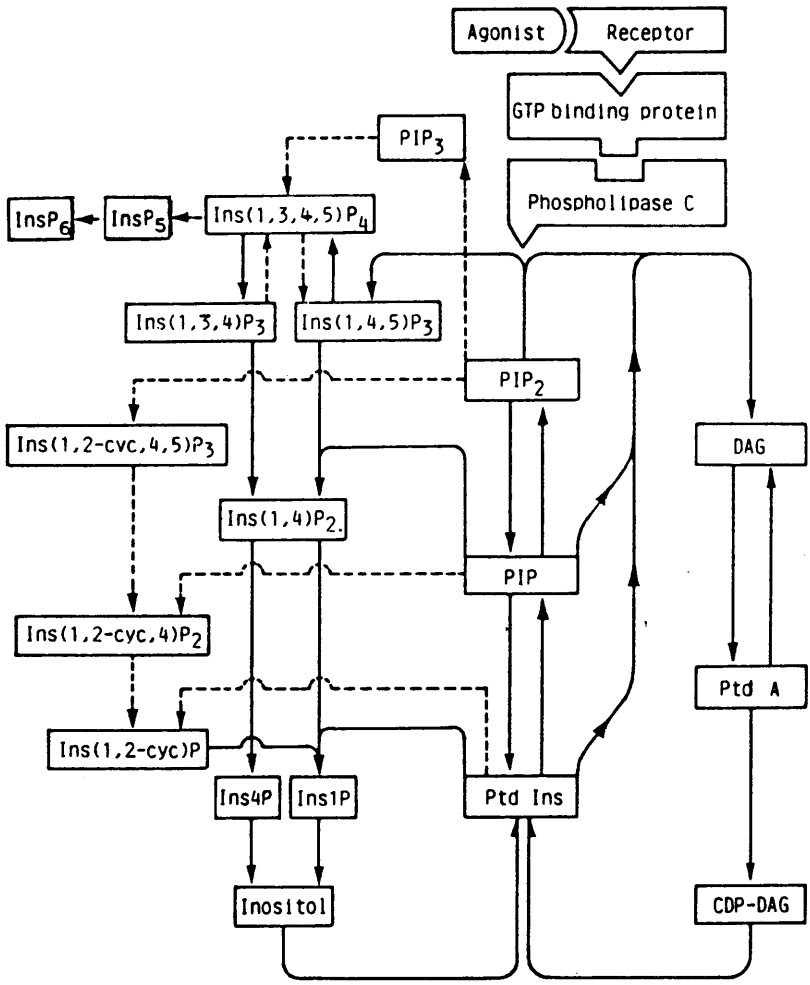


Figure 10.

The Complex Nature of the Phosphoinositide Cycle.

Agonistic-receptor combination results in the activation of phospholipase C presumably via a transducer (GTP binding protein) mechanism to yield a number of inositol phosphates and 1,2-diacylglycerol (DAG). These products may then be incorporated into cycles leading to the resynthesis of the phosphoinositides.

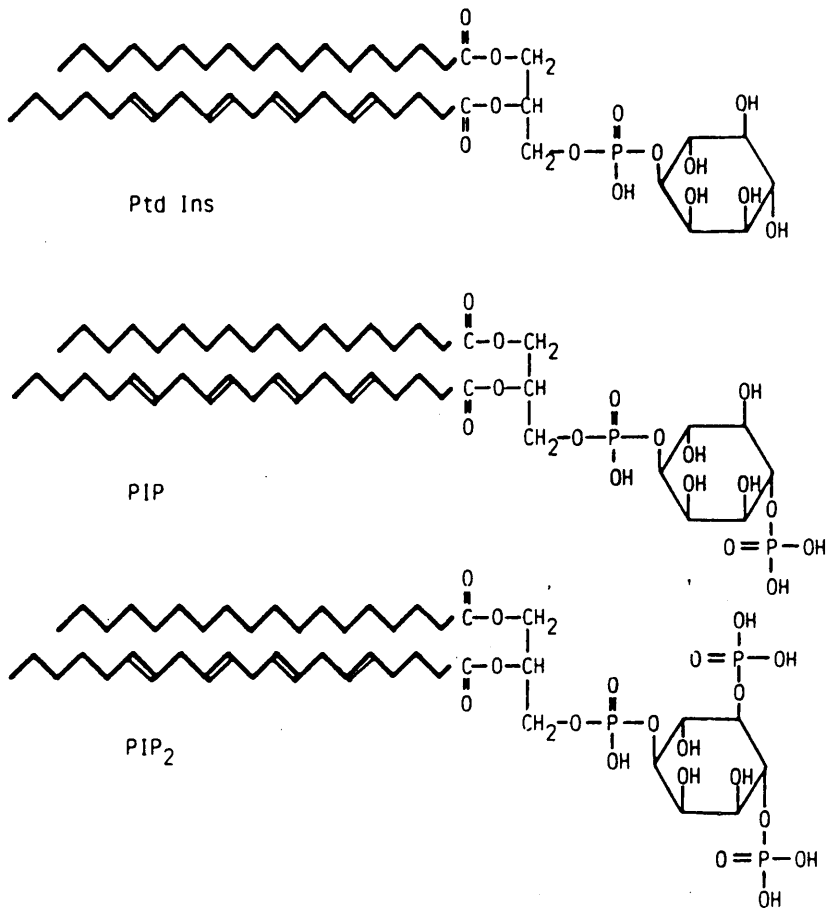


Figure 11.

Structures of the Phosphoinositides.

Phosphatidyl inositol (Ptd Ins), phosphatidyl inositol 4-phosphate (PIP) and phosphatidyl inositol 4,5-bisphosphate (PIP_2).

affinity for Ca^{2+} (Hirasawa & Nishizuka, 1985) and much of the membrane-bound Ca^{2+} is probably associated with these lipids (Buckley & Hawthorn, 1972). Indeed, it has been suggested that hydrolysis of PIP_2 could represent a receptor-linked mechanism whereby membrane-bound calcium might be released into the cytosol (Broekman, 1984). However, PIP_2 also exhibits a slightly lower affinity for Mg^{2+} where the cellular Mg^{2+}: Ca^{2+} ratio is 1000 : 1, suggesting that a substantial amount of PIP_2 is likely to bind Mg^{2+} (Fain, 1982). Bearing this in mind, the precise contribution Ca^{2+} released during the hydrolysis of PIP_2 has in agonist-induced elevation of $\left[Ca^{2+}\right]i$ still remains to be established.

There is considerable evidence suggesting that the principle phosphoinositide which is acted upon by the phosphodiesterase, phospholipase C, is PIP_2 (Berridge, 1983; Martin, 1983; Drummond et al., 1984). Consequently, the initial reaction resulting from agonist receptor combination appears to be the hydrolysis of PIP_2 via phospholipase C to form inositol 1,4,5 triphosphate ($Ins(1,4,5)P_3$) and 1,2-diacylglycerol (DAG) (Berridge, 1985). It has also been shown that minor pathways exist for the conversion of PtdIns and PIP to inositol 1 phosphate (Ins 1P) and inositol 1,4 bisphosphate ($Ins(1,4)P_2$) respectively, although these inositol phosphates are thought to be less important than the formation of $Ins(1,4,5)P_3$ (Figure 12). These inositol phosphates can be sequentially dephosphorylated to form inositol; the enzymes responsible are believed to be inhibited by lithium and such blockade has been useful in monitoring phosphoinositide turnover in a number of cell systems (Hallicher & Sherman, 1980; Huang & Detwiter, 1986; Drummond, 1987). The other product of PIP_2 hydrolysis, DAG, can be enzymatically converted by DAG kinase to yield phosphatidic acid

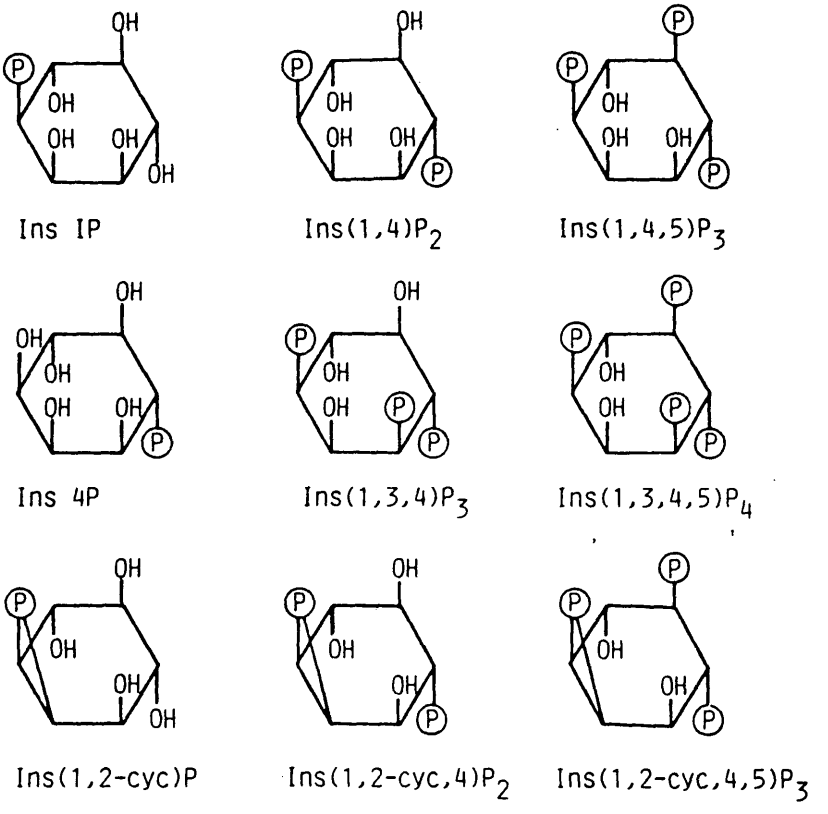


Figure 12.

Structures of the Inositol Phosphates.

Inositol 1 phosphate (Ins 1P),
Inositol 1,4 bisphosphate (Ins (1,4) P_2),
Inositol 1,4,5 trisphosphate (Ins (1,4,5) P_3),
Inositol 4 phosphate (Ins 4P),
Inositol 1,3,4 trisphosphate (Ins (1,3, 4P)$_3$),
Inositol 1,3,4,5 tetrakisphosphate (Ins (1,3,4,5)P_4),
Inositol 1,2-(cyclic) phosphate (Ins (1,2-cyc)P),
Inositol 1,2-(cyclic) 4 bisphosphate (Ins (1,2-cyc,4)P_2) and
Inositol 1,2-(cyclic) 4,5 trisphosphate (Ins (1,2-cyc,4,5)P_3).

(PtdA) which is then acted upon by CTP-PtdA-cytidyl transferase to form CDP-DAG (Figure 13). This latter product can combine with free inositol to resynthesise the phosphoinositides, thereby completing the cycle.

Recent reports have further complicated this cycle with the discovery of an isomer of Ins(1,4,5)P_3 known as inositol (1,3,4) trisphosphate (Ins(1,3,4)P_3) (Irvine et al., 1985; Burgess et al., 1985). Ins(1,4,5)P_3 is thought to be rapidly phosphorylated by the activity of an ATP-dependent kinase (Ins(1,4,5)P_3-3-kinase) to inositol 1,3,4,5 tetrakisphosphate (Ins(1,3,4,5)P_4) which is then converted to Ins(1,3,4)P_3 (Batty et al., 1985; Irvine et al., 1986). It has been suggested that Ins(1,3,4,5)P_4 may be formed by receptor-linked PLC catalysed hydrolysis of an appropriate phospholipid, phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Batty et al., 1985). It has also been shown that under special experimental procedures Ins(1,3,4,5)P_4 can be further phosphorylated to $InsP_5$ and $InsP_6$ (Heslop et al., 1985). There is also evidence for the existence of cyclic inositol phosphates produced from the inositol phospholipids (Wilson et al., 1985a,b.). It is conceivable that inositol 1,2-(cyclic) phosphate (Ins(1,2-cyc)P), inositol 1,2-(cyclic) 4-bisphosphate, (Ins(1,2-cyc4)P_2) and inositol 1,2-(cyclic) 4,5-trisphosphate, (Ins(1,2-cyc,4,5)P_3) could be produced from PtdIns, PIP and PIP_2 respectively.

Many of the above pathways have been studied using a variety of techniques including measurement of the mass, or, more recently, analysis by HPLC, of the intermediates, but by far the most widely used technique is by prelabelling the cells with radioactive species. For example $[^{32}P]$-orthophosphate prelabelling, which is incorporated into the cellular ATP pool and labels the phosphoinositides and PtdA. Other prelabelling procedures include $[^{3}H]$- or $[^{14}C]$-

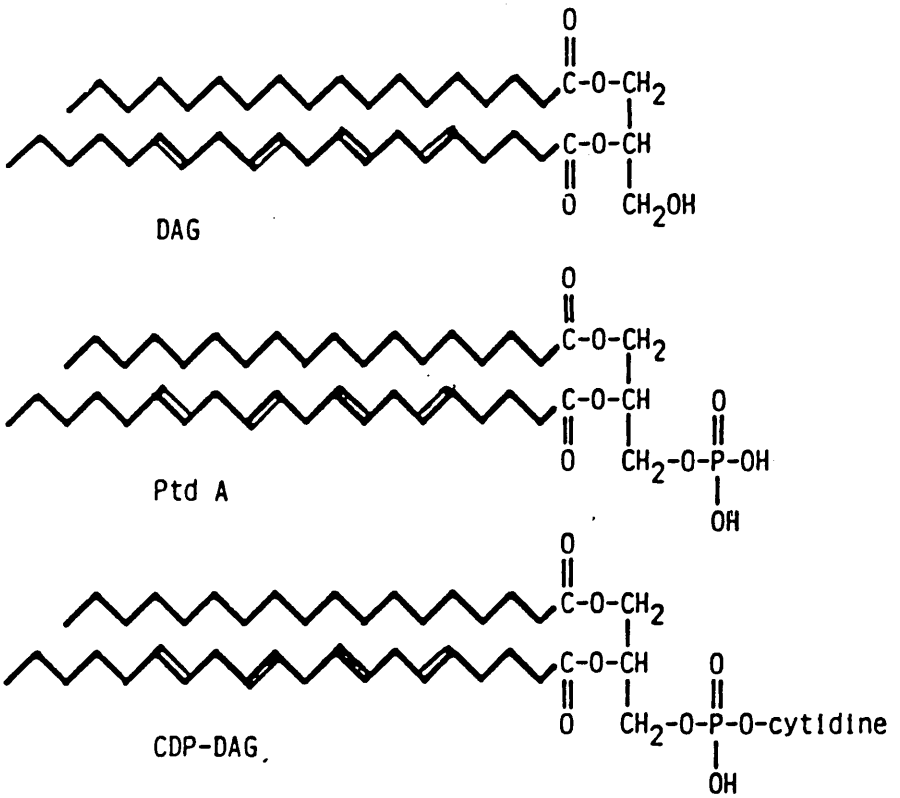


Figure 13.

Structures of the Diglycerides.

1,2-diacylglycerol (DAG), phosphatidic acid (Ptd A) and cytidine diphosphate diacylglycerol (CDP-DAG).

arachidonic acid or glycerol or $[^3H]$-inositol. However, depending on the cell system under investigation, there are different problems associated with each of these prelabelling procedures. Ever since Karnovsky and Wallach in 1961 first demonstrated phosphoinositide metabolism in stimulated neutrophils undergoing phagocytosis, there have been a number of investigations showing receptor-activated phosphoinositide metabolism in human neutrophils, the majority of which have been centred on FMLP as agonist. Whether phosphoinositide turnover in neutrophils is dependent on the presence of external calcium has been a matter of controversy. Early studies in Cockcroft's laboratory suggested that FMLP-induced breakdown of PtdIns and the formation of PtdA were dependent on the presence of calcium in the medium (Cockcroft et al., 1980b; 1981; Cockcroft, 1984). However, Dougherty et al., (1984) showed that, although PtdIns metabolism and PtdA formation are indeed Ca^{2+}-dependent, agonist-induced PIP and PIP_2 metabolism was independent of the presence of external Ca^{2+}. Rossi et al., (1985) have shown that FMLP causes rises in $[Ca^{2+}]i$ in quin2-loaded cells at concentrations significantly lower than FMLP-induced PtdA formation and superoxide production suggesting that phosphoinositide hydrolysis is not involved in the rise of $[Ca^{2+}]i$. On the other hand, DiVirgilio et al., (1985) have shown that FMLP stimulates $Ins(1,4,5)_3$ production in human neutrophils under conditions where calcium levels, as monitored by quin2, do not rise.

Examination of phosphoinositide hydrolysis induced by other ligands in neutrophils has proven to be equally as difficult and confusing. LTB_4 can cause an elevation of $[Ca^{2+}]_i$ in neutrophils to much the same extent as FMLP. However, Volpi et al., (1984) could not detect significant stimulation of phosphoinositide hydrolysis

in rabbit neutrophils by this arachidonic acid metabolite even although the chemotactic tripeptide could elicit phosphoinositide metabolism. The ether lipid, PAF, has been shown to cause a phosphoinositide turnover in rabbit neutrophils. However, Naccache et al., (1986) have also shown that higher concentrations of PAF are required to cause phosphoinositide turnover than are required to cause an elevation of $\left[Ca^{2+}\right]i$. These observations along with others (discussed later) have led to the hypothesis that there may exist at least two distinct receptors for PAF. Clearly then, phosphoinositide hydrolysis plays a role in neutrophil activation but for each particular agonist the precise functional significance still awaits elucidation.

4.2. Inositol Phosphates.

The gap between inositol lipid metabolism and mobilisation of intracellular stores of calcium is thought to be bridged by the formation of the second messenger molecule, $Ins(1,4,5)P_3$, the water-soluble product of PIP_2 hydrolysis. The first indirect evidence was put forward by Berridge, (1983) who demonstrated the early (within 5 seconds) liberation of $Ins(1,4,5)P_3$ in insect salivary glands. More direct evidence came from Streb et al., (1983) who demonstrated that the addition of exogenous $Ins(1,4,5)P_3$ to permeabilised pancreatic acinar cells could release Ca^{2+} from an intracellular non-mitochondrial store. Prentki et al., (1984) showed that the addition of $Ins(1,4,5)P_3$ to digitonin permeabilised human neutrophils also caused the release of Ca^{2+} from non-mitochondrial stores. This has subsequently been shown in a variety of other cell types including hepatocytes (Burgess et al.,

1984a), peritoneal macrophages (Hirata et al., 1984), Swiss 3T3 cells (Irvine et al., 1984), vascular smooth muscle (Somlyo et al., 1985), skeletal muscle (Vergara et al., 1985) and platelets (O'Rourke et al., 1985). It is thought that Ins(1,4,5)P_3 releases intracellular calcium from the smooth endoplasmic reticulum by acting upon specific acceptor sites. Indeed, Spat et al., (1986) have demonstrated, in permeabilised guinea pig neutrophils and hepatocytes, that Ins(1,4,5)P_3 binds to specific saturable sites. In addition, Wilson et al., (1985b) have reported that addition of the cyclic phosphate Ins(1,2-cyc,4,5)P_3 causes release of calcium from intracellular stores in permeabilised platelets and Limulus photoreceptor cells.

The role of the many other inositol phosphates (Figure 12) in agonist-induced cellular activation remains unknown. However, as already mentioned, Ins(1,3,4,5)P_4 may be involved in the translocation of external Ca^{2+} across the plasma membrane (Irvine & Moor, 1986).

4.3. 1,2-Diacylglycerol (DAG)

The production of another primary product of inositol phospholipid hydrolysis is DAG. This neutral lipid has been proposed to act as a second messenger molecule by stimulating a Ca^{2+}-dependent and phospholipid (particularly phosphatidylserine)-dependent protein kinase C (Nishizuka, 1984; 1986).

DAG is formed at the plasma membrane only transiently and disappears within a few seconds due to its phosphorylation to PtdA.

Indeed, this derivative of DAG was once believed to be a prime candidate for causing the elevation of $[Ca^{2+}]i$. It was reported that PtdA could act like a calcium ionophore, thereby elevating $[Ca^{2+}]i$ (Streb et al., 1983). However, others have subsequently shown convincing evidence disputing these observations (Holmes & Yoss, 1983; Putney, 1986) hence it is unlikely that PtdA is a second messenger molecule, at least in terms of Ca^{2+} signalling.

Protein kinase C affinity for Ca^{2+} is increased by kinetically small quantities of DAG, hence this kinase can be fully activated at resting $[Ca^{2+}]i$ without a net increase in $[Ca^{2+}]i$ (Kishimoto et al., 1980). Protein kinase C activation can be mimicked by either synthetic DAG (e.g. 1 oleoyl 2 acetyl glycerol, OAG) or by the tumour promoters known as phorbol esters (e.g. phorbol 12 myristate 13 acetate, PMA) (Castagna et al., 1982). It is thought that protein kinase C activation plays an important role in neutrophil responsiveness. PMA can stimulate a variety of neutrophil responses including superoxide production and degranulation (McPhail & Snyderman, 1983). Addition of subthreshold concentrations of PMA or OAG with subthreshold calcium ionophore can cause a marked activation of human neutrophils (Robinson et al., 1984, Penfield & Dale, 1984). These observations suggest that stimulation of protein kinase C can activate human neutrophils either independently or synergistically with an elevation of $[Ca^{2+}]i$ (Michell, 1983).

More recently there is evidence to suggest that protein kinase activation may have a regulatory effect on agonist-induced activation in a number of cell systems (MacIntyre et al., 1985a; Vicentini et al., 1985). Preincubation of neutrophils with increasing concentration of PMA prior to the addition of an agonist,

e.g. FMLP, PAF and LTB_4, can inhibit neutrophil activation produced by these agonists (Naccache et al., 1985a;b). The precise mechanism and role of this negative feedback system is not fully understood although there is some evidence to suggest that protein kinase C is inhibiting the possible transduction processes involved in neutrophil activation.

Stimulation of protein kinase C is believed to cause the phosphorylation of a number of proteins usually with an M.wt. greater than 40K. However, the precise number of proteins or function of this phosphorylation remains to be determined. Ohtsuka et al., (1986) showed, using intact guinea pig neutrophils, the involvement of protein kinase C in the phosphorylation of a 46K protein which parallels the activation of NADPH oxidase. This observation may explain why PMA seems to be a good stimulator of superoxide production in neutrophils. Another interesting observation made by Touqui et al., (1986) is that protein kinase C may be phosphorylating a 40K anti-phospholipase A2 protein indistinguishable from lipocortin (DiRosa et al., 1984). Other proteins believed to be phosphorylated by protein kinase C include $Ins(1,4,5)P_3$ phosphatase (Connolly & Majerus, 1986), glucose transporter (Witters et al., 1985) and tyrosine hydroxylase (Vulliet et al., 1985) to name only a few.

4.4. Involvement of G Proteins in Phosphoinositide Metabolism.

There is evidence to suggest that G proteins play a role either directly or indirectly in receptor coupled phosphoinositide metabolism by regulating phospholipase C activity (for a review,

see Litosch & Fain, 1986).

The first clues to the involvement of G proteins in this case came from observations that guanine nucleotides modulate the binding of agonists linked to stimulation of phosphoinositide turnover. This phenomenon was shown with muscarinic cholinergic agonists (Florio & Sternweiss, 1985), vasopressin (Cantau et al., 1980) and a1-adrenergic amines (Goodhardt et al., 1982). This was also shown with FMLP activated neutrophil plasma membranes where a high affinity form of the receptor can be converted to a low affinity form by guanine nucleotides (Koo et al., 1983; Snyderman et al., 1984). More convincing evidence came from studies by Gomperts, (1983) where non-hydrolyseable analogues of GTP caused the Ca^{2+}-dependent release of histamine from permeabilised mast cells. Further work by Cockcroft & Gomperts, (1985) and others (Smith et al., 1985) have shown that GTP analogues could stimulate phosphoinositide hydrolysis in neutrophil plasma membranes. Similar observations have been made in numerous other cell systems including permeabilised platelets (Haslam & Davidson, 1984), hepatocyte membranes (Wallace & Fain, 1985), blowfly salivary gland membranes (Litosch et al., 1985), smooth muscle membranes (Sasaguri et al., 1985) and permeabilised GH3 cells (Martin et al., 1986). These results suggest that GTP binding protein participates in the coupling of receptor activation to phospholipase C activity.

There probably exist numerous G proteins involved in signal transduction mechanisms, the best characterised being N_S and N_I which function in the adenylate cyclase system and transducin which transduces the effect of light activation on rhodopsin

stimulation of cyclic GMP phosphodiesterase activity on rod outer segments. The identity of the G protein (often referred to as Np) coupled to PLC has not yet been identified but may resemble these other better studied G proteins.

Pertussis toxin which causes the NAD-dependent ADP ribosylation of the 41K M.wt. alpha-subunit of N_I and inhibits its action in the adenylate cyclase system can also be utilised to probe G protein involvement in PLC activation. Pretreatment of neutrophils with pertussis toxin inhibited FMLP-induced phosphoinositide turnover, $\left[Ca^{2+} \right]i$ elevation and cellular activation, including arachidonic acid release, degranulation and superoxide generation in neutrophils isolated from a variety of species (for review, see Omann et al., 1987a). These reports suggest that the G protein involved in neutrophil activation may be N_I or a related protein. Indeed, Bokoch & Gilman, (1984) have shown that the effect of this bacterial toxin on neutrophils could be correlated with ADP ribosylation of a 41K membrane protein that comigrated on SDS poly-acrylamide gels with purified rat liver $N_I$alpha subunit. These workers also showed that sodium fluoride, a stimulator of G proteins that causes the dissociation of the alpha from the beta/gamma subunits, was able to cause a pertussis toxin sensitive release of arachidonic acid from neutrophils. This evidence strongly suggests that G proteins are involved in FMLP-induced neutrophil activation; whether they are involved in neutrophil responsiveness induced by other receptor agonist still remains to be fully established.

5. Changes in Membrane Potential.

It is worth mentioning that human neutrophils, in common with

other cells, possess a resting membrane potential which changes with the addition of certain agonists. The transmembrane potential can be measured in small cells such as neutrophils by preloading with the fluorescent dye 3-3'-dipentyloxacarbocyanine (di-O-C_5) or with the lipophilic dye triphenylmethylphosphonium ion ($TPMP^+$). The resting potential of human neutrophils measured by such dyes ranges from between -25mV to -75mV. This variation in the resting potential may depend on the age of the neutrophil (Seligmann et al., 1981a) or on the particular dye used. Korchak & Weissmann, (1978) have shown that the resting membrane potential is dependent on the K^+ concentration across the membrane since elevating the external concentration of this cation causes a marked depolarization. These workers also showed that stimulation of neutrophils with immune complexes or concanavilin A causes a rapid hyperpolarization which is not dependent on the presence of external Ca^{2+} but requires the presence of extracellular Na^+ for an optimal membrane potential response. It has also been shown by Korchak and Weissmann, (1978) that FMLP causes a triphasic response consisting of a transient increase in cell associated $TPMP^+$ followed by a decrease and finally a prolonged increase interpreted as a hyperpolarization, depolarization and a hyperpolarization respectively. Seligmann et al., (1980) using di-O-C_5 reported that FMLP stimulated a biphasic response; an apparent depolarization followed by a hyperpolarization. These discrepancies between the two groups may have resulted from differences in dye incubation periods (Seligmann et al., 1981a). Using di-O-C_5 Seligmann & Gallin, (1980) observed that low concentrations of PMA and A23187 cause a transient hyperpolarization. However, they also demonstrated that intermediate concentrations of these agonists produced a

similar hyperpolarization followed by a depolarization and that high concentrations of either secretagogue caused a large depolarization. An interesting observation made by Seligmann et al., (1981b) using di-O-C_5 is that neutrophils respond heterogeneously to FMLP. They showed that the majority (65%) of cells respond to FMLP by depolarization and the remaining cells either do not respond at all or cause a hyperpolarization of the membrane potential. Although these changes in membrane potential occur rapidly and precede superoxide generation and degranulation, (Korchak & Weissmann, 1980; Cohen & Chovaniec, 1978a;b) their significance in stimulus-response coupling is unknown. It is unlikely that alterations in the membrane potential play a major role in neutrophil activation but movement of ions across the plasma membrane may have a modulatory role in neutrophil activation by altering, for example, intracellular pH (for review, see Simchowitz & Roos, 1985).

DAG has been shown to induce an Na^+/H^+ exchange via activation of protein kinase C and the resultant increase in intracellular pH appears to be intimately linked with stimulus-response coupling. Brass & Joseph, (1985) have shown that increasing the extracellular pH lowered the apparent Km of $Ins(1,4,5)P_3$ towards the Ca^{2+} stores by around 40% in permeabilised platelets. More recently, Siffert & Akkerman, (1987) demonstrated that suppression of Na^+/H^+ counter transport by ethylisopropyl-amilioride or by lowering extracellular Na^+ reduces or even completely abolishes thrombin-induced elevation of $[Ca^{2+}]i$ in human platelets. They concluded that an increase in intracellular pH evoked by activation of Na^+/H^+ exchange is an important signal in stimulus-response coupling and forms an essential step in the cascade of events

required to increase $[Ca^{2+}]i$ in platelets. They have also reported that thrombin-induced cleavage of PIP_2 was unaffected by treatments that blocked Na^+/H^+ exchange for increased intracellular pH and concluded that, although Na^+/H^+ exchange is a prerequisite for $[Ca^{2+}]i$ mobilisation, it is not necessary for the stimulus-induced hydrolysis of PIP_2 (Siffert et al., 1987). Whether pH changes are modulating agonist-induced changes in $[Ca^{2+}]i$ and phosphoinositide hydrolysis in the human neutrophil is currently the focus of much attention.

OBJECTIVES.

The major objectives of this study were to investigate the mechanisms of action and interaction of the arachidonic acid metabolite, LTB_4, and the ether lipid, PAF, as mediators of human neutrophil activation.

The parameters examined were:

(a) Ultrastructural changes.

(b) Aggregation.

(c) Degranulation by measuring (i) NAG release

(ii) Lysozyme release.

(d) LTB_4 generation.

(e) Changes in $[Ca^{2+}]i$.

(f) Phosphoinositide hydrolysis.

This was attempted by:

(1) Comparing the various neutrophil responses induced by LTB_4 and PAF with those elicited by the chemotactic tripeptide, FMLP, the phorbol ester, PMA and the calcium ionophore, ionomycin.

(2) Examining the possible involvement of "G proteins" in neutrophil activation by utilising pertussis toxin.

(3) Investigating the role of protein kinase C activation in agonist-induced elevation of $[Ca^{2+}]i$ and phosphatidic acid production (an indirect index of phosphoinositide hydrolysis).

(4) Exploring the role of LTB_4 and PAF as endogenous mediators or amplifiers of neutrophil responsiveness by employing a selective 5-lipoxygenase inhibitor and a specific PAF receptor antagonist.

METHODS AND MATERIALS

CHAPTER 4: METHODS AND MATERIALS.

1. PREPARATION OF HUMAN NEUTROPHILS.

Blood was obtained by ante-cubital venepuncture from healthy adult volunteers who denied taking any drugs known to affect neutrophil function. The blood samples were immediately mixed with 3.8% trisodium citrate (9 parts blood to 1 part anticoagulant) and then centrifuged (850g; 5 min; room temperature) in a bench minor centrifuge. The platelet rich plasma was discarded and the erythrocyte / leucocyte ("buffy coat") layer was mixed with 1-2 volumes gelatin solution (2.5% gelatin in 154mM saline) and incubated at 37°C for 30-45 min. The supernatant was removed and centrifuged (1,000g; 5 min; room temperature) and the resultant cell pellet was resuspended in lysis buffer (C) to remove contaminating erythrocytes (Henson, 1971). This cell suspension was then centrifuged (850g; 3 min; room temperature) and the resultant cell pellet resuspended in Hanks Balanced Salt Solution. This procedure was repeated and the cells were finally resuspended in appropriate buffer at the desired neutrophil concentration. The cells were stored on ice until use.

2. COUNTING OF NEUTROPHILS.

The cells were counted and their viability assessed by:

(a) Trypan blue exclusion, using a standard haemocytometer under a Leitz labovert light microscope.

(b) Coulter counter; where 20µl aliquots of cell suspensions were added to 20ml of Isoton and counted at predetermined settings (amplitude 8 ; aperture current 0.354) for human neutrophils.

The cells were approximately 95-98% viable, 90-98% pure and were free from any neutrophil aggregates.

3. BUFFERS.

BUFFER A

NaCl	124mM
KCl	4mM
NaH_4PO_2	0.64mM
K_2HPO_4	0.66mM
$NaHCO_3$	5.2mM
Hepes	10mM
Glucose	5.6mM
$CaCl_2$	1.6mM

The above were dissolved in glass-distilled water and the pH was adjusted to 7.4 using NaOH (1N).

BUFFER B (phosphate free buffer)

NaCl	140mM
KCl	5mM
$NaHCO_3$	2.8mM
$MgCl_2$	1mM
$MgSO_4$	0.06mM
Hepes	15mM
Glucose	5.6mM
$CaCl_2$	1.6mM

The above were dissolved in glass-distilled water and the pH was adjusted to 7.4 using NaOH (1N).

BUFFER C (lysis buffer)

NH_4Cl	150mM
$KHCO_3$	10mM
EDTA	1mM

The above were dissolved in glass-distilled water.

BUFFER D (LTB_4 radioimmunoassay dilution buffer)

NaH_2PO_4	40mM
NaCl	0.9% (w/v)
Bovine gamma globulin	0.1% (w/v)

The above were dissolved in glass-distilled water and the pH was adjusted to 7.4 using NaOH (1N).

BUFFER E (LTB_4 stopping "cocktail")

BW755C	1mM
Indomethacin	1mM
EGTA	100mM
Sodium azide	1% (w/v)

The above were dissolved in glass-distilled water and the pH was adjusted to 7.4 using NaOH (1N).

4. ELECTRON MICROSCOPY STUDIES.

Neutrophils were dispersed into aliquots of 0.5ml (containing

10^7cells) and challenged with an agonist. At a desired time the reaction was stopped and fixed by addition of 0.5ml aliquots of prewarmed (37°C) 2.5% glutaraldehyde (0.25ml) and 0.1M sodium cacodylate buffer (0.25ml), pH 7.4, which were mixed together immediately before use to give a final glutaraldehyde concentration of 1.25%. The cells were allowed to fix in suspension for 1 hour and then centrifuged gently (300g; 2 min; room temperature). The resultant supernatant was decanted and 0.5ml of fresh human serum was added to the cells, which were resuspended and then centrifuged (850g; 3 min; room temperature). The serum was removed and carefully replaced by 0.5ml fresh fixative (2.5% glutaraldehyde) and left for 2 hours (Payne & Satterfield, 1980). The samples were washed, at room temperature, in 0.1M sodium cacodylate containing 3mM $CaCl_2$ three times over a period of 24 hours.

Much of the following procedure was performed with the assistance of Dr. Caroline Jones from the Pathology Department of the University of Manchester. The samples were diced into 1mm cubes then post-fixed in 1% osmium tetroxide in 0.1M sodium cacodylate at 4°C for 1 hour, washed in buffer, and dehydrated in an ascending series of alcohols. Two changes of propylene oxide were then given and samples transferred to equal parts of propylene oxide and Taab epoxy resin for 1 hour at room temperature. They were then left in 1 part propylene oxide to 3 parts resin overnight, given three changes of fresh resin at 48°C and polymerised in gelatin capsules at 60°C for 72 hours. Ultrathin sections were cut on a Reichert OMuIII ultramicrotone, picked up on copper grids (300 mesh), and double stained with uranyl acetate and lead citrate prior to examination in a Philips 301 electron microscope at an accelerating voltage of 60 KV.

5. AGGREGATION.

Neutrophil aggregation was measured photometrically in a standard Malin Clinical Aggregometer. To a cuvette containing a teflon-coated stirring bar revolving at 600 r.p.m., was added 0.6ml of neutrophil suspension (2.5×10^7cells/ml) in buffer A. After a 2 min delay, to allow warming of cells to 37°C, the neutrophils were challenged with an agonist and the resultant changes in light transmission recorded on a Linseis recorder.

6. DEGRANULATION.

(a) Sample Generation.

Neutrophil suspensions (0.6ml of 10^7cells/ml) in buffer A, in the presence or absence of cytochalasin B (5µg/ml; 10 min), were challenged with an agonist for 10 min at 37°C. The reactions were terminated by centrifugation (9,000g; 20 sec) in a micro-centrifuge. The supernatant was removed and stored at -20°C.

(b) NAG Measurement.

NAG activity was measured fluorimetrically as the formation of 4-methylumbelliferone by a modification of the technique of Gordon, (1975) for measuring platelet lysosomal enzymes. The reaction mixture contained 100µl of cell supernatant or cell pellet, 100µl of citrate buffer (0.3M; pH 4.8) and 100ul of 4-methylumbelliferyl-N-acetyl B-D-glucosaminide (3mM). The reaction mixture was incubated for 60 min at 37°C and then stopped by boiling for 2-3 min. The mixture was then diluted with 0.7ml glass-distilled water and centrifuged (9,000g; 20 sec.) to remove any remaining cell fragments. A sample (100µl) was added to 1.9ml glycine-NaOH

buffer (50mM glycine in 39mM NaOH; pH 10.4). Fluorescence was monitored in a Perkin-Elmer LS3 Fluorescence Spectrometer using excitation and emission wavelengths of 370 and 450nm respectively.

(c) Lysozyme Measurement.

Lysozyme was determined as the rate of lysis of the bacterium Micrococcus lysodeikticus. The rate at which the bacterial suspension (substrate) clears is thus a measure of the lysozyme content within a sample. An aliquot (20µl) of sample was added to 1ml of substrate and the decrease in absorbance at 550nm was determined in a Shimadzu recording spectrophotometer. The change in absorbance was determined between 1-3 min of the reaction at a constant temperature of 25°C.

The cell pellets, in both enzyme assays, were digested with 0.2% Triton X-100 and the results were expressed as a percentage of the total enzyme activity.

7. LTB_4 MEASUREMENT.

(a) Sample generation.

Neutrophil suspensions(0.6ml of 1 x 10^7cells/ml) in buffer A, in the presence or absence of cytochalasin B (5µg/ml; 10 min) were challenged with agonist for 10 min. The reactions were terminated by addition of 10% stopping "cocktail" (buffer E) and immediately centrifuged (9,000g; 2 min) to remove any cell fragments. The samples were stored at -20°C prior to assay for LTB_4 by specific radioimmunoassay (RIA) or for authentication by reverse phase HPLC.

(b) Radioimmunoassay.

The LTB_4 concentration was estimated by RIA essentially as described by Carey & Forder, (1986). Authentic LTB_4 standard

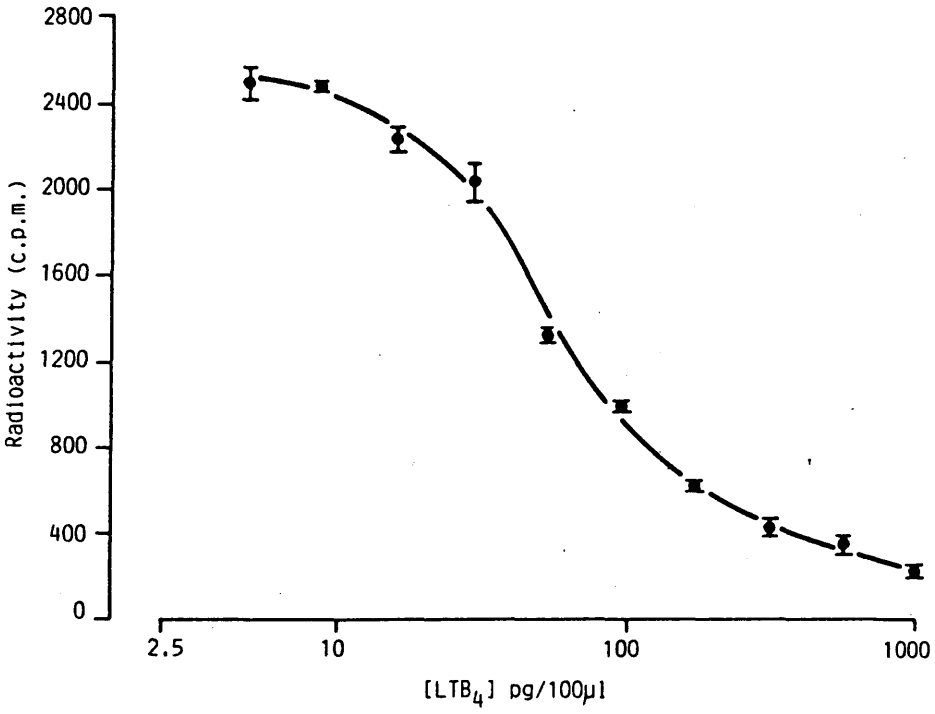


Figure 14.

Typical LTB_4 radioimmunoassay standard curve.

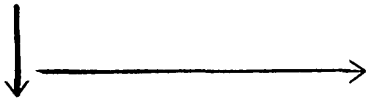
concentrations (0.1-10ng/ml) were prepared in buffer D. To each assay tube was added 200µl of 1/1000 dilution (in 0.1% bovine gamma globulin, D) of anti-LTB_4 antiserum (raised in New Zealand white rabbits) and 100µl of 1/666 dilution of $[^3H]$-LTB_4 (approximately 2000cpm/sample). Samples (100µl) or standard (100µl) were mixed and incubated for 18-24 hours at 4^oC. After the incubation period, 200µl of dextran-coated charcoal (0.5% w/v dextran T70 and 1% w/v charcoal in buffer D) was added. The samples were mixed and the charcoal sedimented by centrifugation (2,000g; 15 min; 4^oC). Aliquots (450µl) of the supernatant were taken to assess antibody bound $[^3H]$-LTB_4 which was counted (1 min) in an LKB Wallac 1218 Rackbeta scintillation counter. The results were processed and the standard calibration curves in each experiment were plotted as radioactivity bound (cpm) against LTB_4 (pg/100µl)(Figure 14) using an IBM personal computer.

(c) Reverse phase HPLC

Extraction Procedure.

In order to remove contaminants from the samples (1ml of 10^7cells/ml) they were purified by extraction through Bond Elut 1ml C_{18} mini-extraction columns. The solvent/sample addition was gently forced through the columns by vacuum suction. The extraction procedure is summarised as follows:

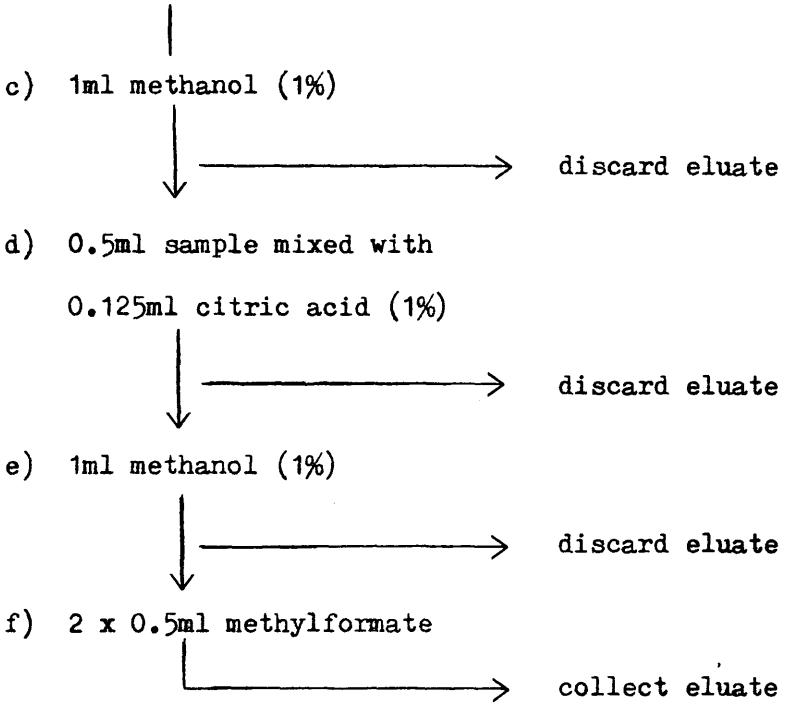
a) 1ml ethylacetate (100%)



discard eluate

b) 1ml methanol (100%)

discard eluate



The collected extract was dried under N_2 and reconstituted in methanol (60%) and stored overnight in a glass vial at $-20^{\circ}C$. The vial was placed in a WISP prior to HPLC separation.

HPLC Separation.

An internal standard containing a mixture of cold LTB_4, $[^3H]$-LTB_4, $[^3H]$-5HETE, $[^{14}C]$-arachidonic acid and $[^3H]$-LTB_4 metabolites or test sample were eluted using a gradient from 60% to 95% methanol at a flow rate of 1.5ml/min on a Spherisorb 25cm x 4.9cm column.

The internal standard (80µl) was applied to the column and a total of 60 fractions, one every 0.5 min, were collected for liquid scintillation counting. This procedure was repeated for 200µl of test sample and the resultant separation identified by Ultraviolet detection and by comigration with the authentic standards. The collected fractions were evaporated to dryness using a Savant Aquavac

and redissolved in 0.5ml buffer D and volumes removed for quantitative analysis by RIA.

8. PHOSPHOLIPID STUDIES.

(a) Measurement of $[^{32}P]$-PtdA and $[^{32}P]$-PtdIns

Neutrophils (5 x 10^7cells/ml) suspended in a phosphate free buffer B were incubated (37°C, 90 min) with carrier-free $[^{32}P]$-orthophosphate (30μCi/ml) and 0.025% bovine serum albumin. Neutrophils were then pelleted and resuspended (2.5 x 10^7cells/ml) in fresh albumin free buffer and 0.4ml aliquots were dispensed into plastic tubes at 37°C. The cells were allowed to equilibrate for 10 min before being challenged with an agonist. At the appropriate time, reactions were terminated and lipids extracted essentially by method B of Lloyd et al., (1972).

Briefly, the neutrophil sample was transferred into a glass test-tube containing 2ml of chloroform:methanol:10N HCl (25:50:4, v/v/v) at room temperature. Neutrophil lipids were extracted by partitioning of the aqueous and organic phases following the addition of 625μl chloroform and 625μl water, vortexing and then centrifuging at 1,000g for 3-5 min. The lower organic phase was removed into a glass vial, dried at 40°C under N_2 using a Techne Dri-Block, and stored at -20°C until use.

The lipids were redissolved in 100μl of chloroform/methanol 9:1 v/v) and spotted on silica-gel t.l.c. plates (10cm x 10cm) for two dimensional separation of phospholipids (Yavin & Zutra, 1977). This t.l.c. system employs three basic solvents; in the first dimension- chloroform:methanol:40% aqueous methylamine (13:6:1.5,

v/v/v) and in the second dimension- diethylether:glacial acetic acid (19:1, v/v) and chloroform:acetone:methanol:glacial acetic acid:water (10:4:2:3:1, v/v/v/v/v). Phospholipid samples were applied to the lower left hand corner of the t.l.c. plates, using a 10µl disposable micropipette. They were then placed in pre-equilibrated chromatographic chambers containing Whatman I filter paper at both ends. The solvent front was allowed to run to within 1cm from the edge of the plate; they were then removed and dried in warm air. Each plate was then exposed to concentrated HCl fumes followed by sequential drying in warm and cool air. The plates were then placed in the second solvent running in the second dimension (origin at the lower right corner). Following this, the plates were removed, dried in cool air and placed in the third solvent in the same dimension as the second solvent. After the solvent front had run to 1cm from the edge of the plates, they were dried thoroughly in a stream of cool air. The major phospholipids separated by this method are shown in figure 15. Individual spots were detected visually by exposure to I_2 vapour for 5 min and/or by autoradiography on X-Omat S X-ray film (Kodak) for 16-24 hours. Spots corresponding to PtdIns and PtdA were identified by comparison with reference standards, scraped into vials and counted for radioactivity in a liquid-scintillation spectrometer.

(b) Measurement of $[^{32}P]$-PIP and $[^{32}P]$-PIP_2

Neutrophils (5×10^7cells/ml) suspended in phosphate-free buffer B with 0.025% bovine serum albumin were incubated (37°C; 45 min) with carrier-free $[^{32}P]$-orthophosphate (30µCi/ml). In order to enhance the possibility of observing agonist-induced changes in PIP and PIP_2 it was necessary to prevent the specific activity of the phosphoinositides from increasing. This was achieved by adopting a pulse chase protocol, in which the $[^{32}P]$-

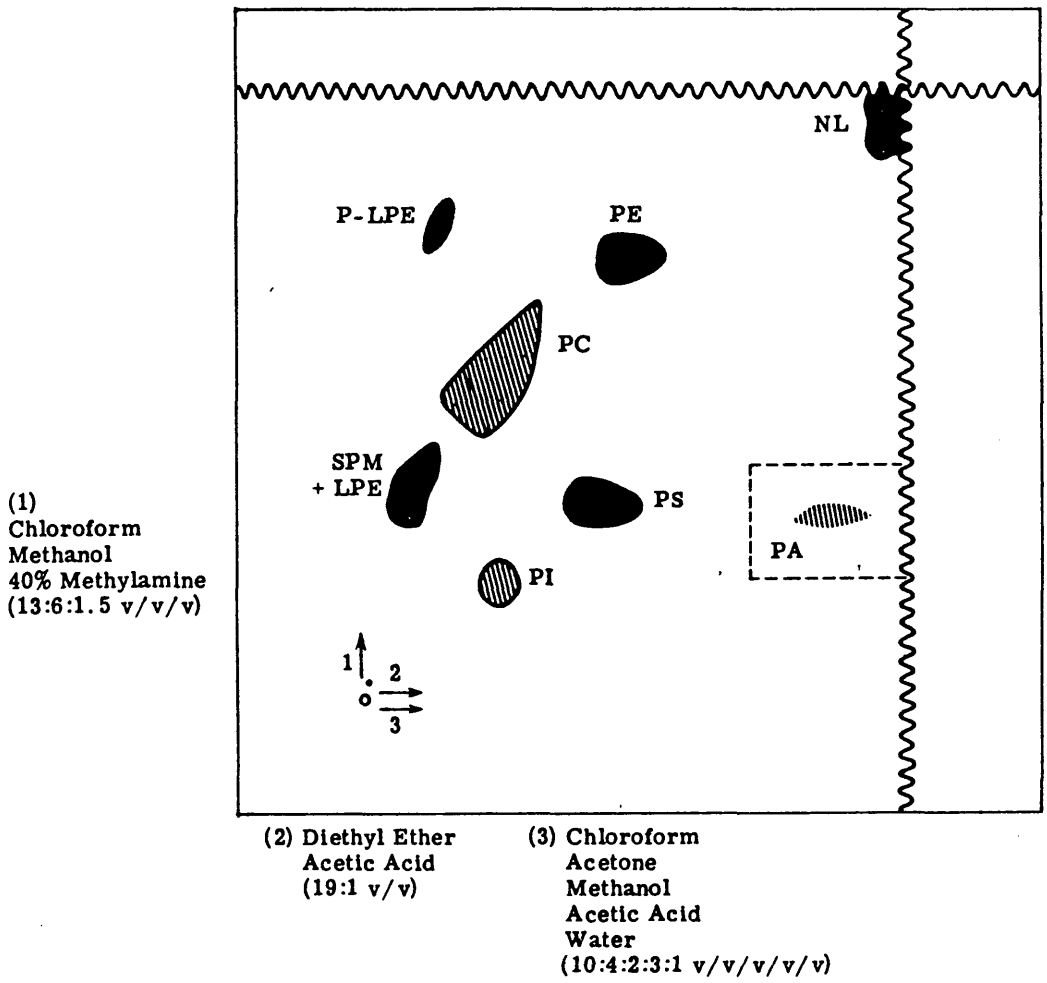


Figure 15.

Diagrammatic representation of phospholipid separation by two-dimensional thin layer chromatography by method of Yavin and Zutra (1977).

Phospholipids identified by iodine staining are indicated and the hatched spots represent those phospholipids which are significantly labelled following preincubation with [^{32}P]-orthophosphate. O-origin; PI-phosphatidylinositol; PS-phosphatidylserine; PA-phosphatidic acid; LPE-lyso phosphatidylethanolamine; SPM-sphingomyelin; PC-phosphatidylcholine; PE-phosphatidylethanolamine; P-LPE-plasmalogen lyso phosphatidylethanolamine; NL-neutral lipids.

labelled cells were pelleted by centrifugation (850g; 3 min; 20°C), resuspended in fresh buffer B containing KH_2PO_4 (100μM) and incubated for 45 min at 37°C. This had the effect of 'chasing' the label from the ATP pool, effectively diminishing the specific activity of neutrophil $[^{32}P]$-ATP, with a resultant decrease in specific activity of PtdIns (Pollock et al., 1984).

The cells were then pelleted and resuspended (2.5×10^7 cells/ml) in fresh, albumin-free, buffer B and 0.4ml aliquots were dispersed and allowed to equilibrate for 10 min before being challenged with agonists. At the appropriate time the reactions were terminated by transferring the entire sample to 1.2ml of chloroform:methanol (1:2, v/v) and thoroughly vortexed. The phospholipids were extracted by the addition of 2.4 N HCl (0.4ml) and chloroform (0.4ml) and then vortexed. The sample was partitioned into two distinct phases separated by a cell fragment interface by centrifugation (1,000g; 3 min). The lower organic phase was removed and stored at 4°C. The remaining aqueous phase was washed by mixing with chloroform (0.8ml) and centrifuged (1,000g; 3 min) to separate the phases. The aqueous phase was discarded and the two organic phases combined. The organic phases were washed by addition of 1.6ml methanol:1 N HCl (1:1), centrifuged (1,000g; 3 min), and the lower phase removed, dried at 40°C under N_2 and stored at -20°C (Schacht, 1981).

The phospholipids were resuspended in 100ul of chloroform: methanol:water (75:25:2, v/v/v) and spotted as 1.5cm bands (1.5cm from the bottom edge) on high performance t.l.c. plates for one dimensional separation of PtdIns, PIP and PIP_2 (Jolles et al., 1981). Prior to spotting, the plates were impregnated with potassium oxalate (1% in methanol:water; 2:3, v/v) and 'activated' by placing in an

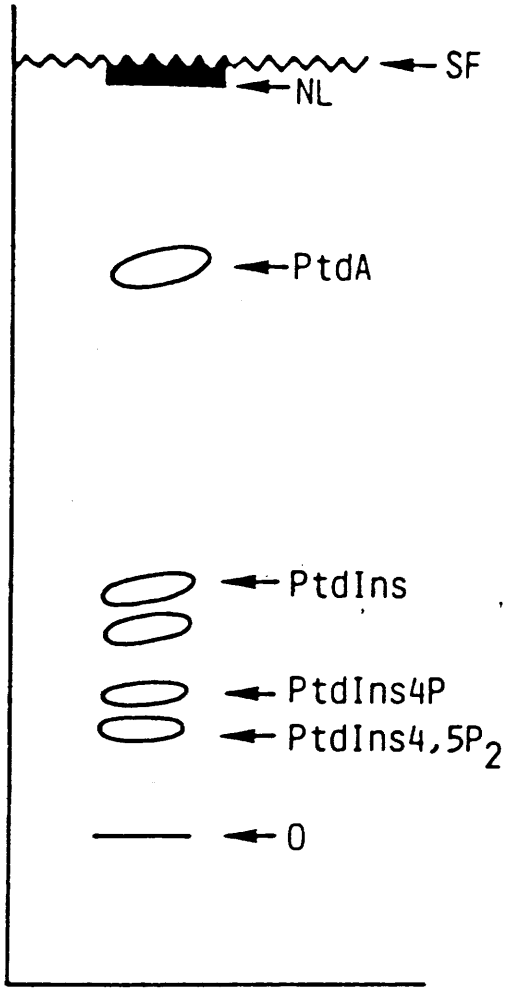


Figure 16.

Diagrammatic representation of phospholipid separation by one-dimensional thin layer chromatography by method of Jolles et al. (1981).

Phospholipids identified by iodine staining are indicated. O -origin; PIP_2 -phosphatidylinositol-4,5 bisphosphate, PIP -phosphatidylinositol-4 phosphate; PtdIns -phosphatidylinositol; PtdA -phosphatidic acid; NL -neutral lipids; SF -solvent front.

oven for 15 min at 110°C. The plates were developed in chloroform: acetone:methanol:glacial acetic acid:water (40:15:13:12:8, v/v/v/v/v) until the solvent front had reached 1cm from the top.

The individual phospholipids were visualised by iodine staining and autoradiography (figure 16) and identified by comparison with reference standards. Spots corresponding to the relevant phospholipids were scraped into vials and counted for radioactivity in a liquid scintillation counter.

9. $[Ca^{2+}]i$ MEASUREMENT.

Neutrophil $[Ca^{2+}]i$ was monitored using the fluorescent quinoline dye, quin2, which displays high affinity for Ca^{2+} and undergoes fluorescent changes as a result of Ca^{2+} binding (Tsien et al., 1982a; Rink & Pozzan, 1985). Neutrophils (10^8cells/ml) in buffer A were incubated (37°C; 10 min) with quin2 acetoxymethyl ester (100μM final concentration), which is membrane permeant and crosses the plasma membrane where it is rapidly hydrolysed by cytosolic esterases to form the hydrophilic polycarboxylate anion that is quin2. Samples were then diluted ten fold with thermally equilibrated buffer and incubated for a further 20 min at 37°C. At the end of the incubation period the cells were centrifuged (850g: 3 min) to remove extraneous dye and resuspended in the buffer at 10^7cell/ml (White et al., 1983b). Quin2 is thus trapped within the cytosol of the neutrophil and consequently the resting $[Ca^{2+}]i$ and the changes in $[Ca^{2+}]i$ which result from exposure to agonists can be determined. Neutrophils (2ml) were placed in 1cm square quartz cuvettes and changes in fluorescence were monitored in a Perkin LS3 Fluorescence Spectrometer at 37°C with standard monochromator settings of excitation at 339nm and emission at 492nm.

The $[Ca^{2+}]i$ was calculated according to the following equation:

$$[Ca^{2+}]i = Kd \times \frac{(F - Fmin)}{(Fmax - F)}$$

Where Kd represents the apparent dissociation constant of quin2 for calcium (Kd=115nM under these conditions); F is the fluorescence signal in arbitrary units and Fmax and Fmin are fluorescence at very high and low calcium respectively (Tsien et al., 1982 a;b). Fmax was obtained by the addition of 50μM digitonin to lyse the cell and expose all of the quin2 to the extracellular Ca^{2+} and to liberated intracellular Ca^{2+} and Fmin was obtained by the later addition of EGTA (20mM) to chelate all of the Ca^{2+} (Rink & Pozzan, 1985).

10. QUANTIFICATION OF RADIOACTIVITY.

All radioactive samples to be analysed were added to 5-10ml of either Scintillator-299 (Packard) or Ecoscint (National Diagnostics) in polythene vials and counted (1-5 min) for their radioactive content in a liquid scintillation counter (Packard Tri Carb or an LKB Wallac 1218 Rackbeta).

11. CALCULATION OF RESULTS.

Results are expressed as mean values ± standard error of mean (SEM) where individual points in each experiment were normally performed in triplicate. Data were compared statistically using the student's t-test and only values of probability (P) which were less than 0.05 were taken to indicate significance. The precise level of significance is indicated at each experiment where probability (P) values * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$.

In the phospholipid experiments; vehicle controls were carried out at the beginning and the end of each experiment and the test values were expressed as the percentage of these controls.

The term EC_{50} represents the concentration of agonist that elicits 50% of the maximum response of that agonist. The term IC_{50} represents the concentration of antagonist (inhibitor) that inhibits the response to an agonist by 50%. Both parameters were calculated by extrapolation from either the stimulatory or the inhibitory concentration response curves.

12. MATERIALS.

(a) Drugs and Reagents.

The drugs and reagents used in the course of this project are listed below and the sources indicated. Unless otherwise stated, all drugs were dissolved in glass-distilled water with subsequent dilutions in 0.9% saline.

LeukotrieneB_4 (LTB_4) was synthesised by Dr. Y. K. Yee (Stuart Pharmaceuticals, Division of ICI Americas, Wilmington, Delaware, U.S.A.) and dissolved in methanol.

The stock (1.8mM) platelet-activating factor (PAF, Bachem, Babbendorf, Switzerland) was dissolved in iso-osmotic saline containing bovine serum albumin (0.25%). Subsequent dilutions of this stock were made up in saline.

Formylmethionylleucylphenylalanine (FMLP, Sigma U.K.) was dissolved in dimethylsulphoxide (DMSO, Sigma).

Quin2-acetoxymethylester (quin2-AM) was obtained from Lancaster Synthesis (Morecambe, England) and was dissolved in DMSO.

Lysozyme Reagent Set was purchased from Worthington Diagnostics,

Freehold, New Jersey, U.S.A.

Ionomycin, purchased from Calbiochem, U.K., was initially dissolved in DMSO.

Phorbol myristate acetate dissolved in DMSO, cytochalasin B dissolved in DMSO, Bovine gamma globulins, Bovine serum albumin, HEPES, EDTA, EGTA, gelatin, TritonX-100, glutaraldehyde, sodium cacodylate, osmium tetroxide, indomethacin dissolved in DMSO, nordihydroguaiaretic acid, NDGA dissolved in DMSO; were all purchased from Sigma, U.K.

Pertussis toxin was a generous gift from Dr. Rodger Parton, Department Microbiology, Glasgow University.

LTB_4-antisera was prepared by Mr. R. Forder (ICI, U.K.)

Kadsurenone (2-(3,4-dimethoxyphenyl)-2,3-dihydro-3a-methoxy-3-methyl-5-(allyl)-6-2H-oxobenzofuran) was purchased from Merck, Sharpe & Dohme, New Jersey, U.S.A. and was dissolved in DMSO.

Revlon 5901 (α-pentyl-3-(2-quinolinylmethoxy)-benzene-methanol) was purchased from Revlon Health Care Group, New York, U.S.A. and dissolved in DMSO.

AA861 (2-(12-hydroxydodeca-5,10-dinyl)-3,5,6-trimethyl-1,4-benzoquinone), BW755C (3-amino-i- m-trifluoromethyl)-phenyl -2-pyrazoline HCl) and Nafazatrom (2,4-dihydro-5-methyl-2- 2-(napthyloxy ethyl-3H-pyrazol-3-one) were synthesized in the Chemistry Department at ICI Pharmaceuticals Divisions, U.K. (all dissolved in DMSO).

(b) Phospholipid Standards.

Phosphatidic acid (Sigma) was dissolved in chloroform:methanol (2:1), phosphatidylinositol (Sigma), phosphatidylinositol 4 phosphate (Sigma) and phosphatidylinositol 4,5 bisphosphate (Sigma) were dissolved in chloroform:methanol (1:1) and phosphatidylcholine

(Sigma) was dissolved in chloroform.

(c) T.L.C. Plates.

Merck Silica Gel 60 high performance t.l.c. plates (10cm x 20cm) and Mecherey-Nagel precoated t.l.c. plates SIL G-25 (20cm x 20cm) were purchased from MacFarlane Robson Ltd., Glasgow. The latter were cut into 4 smaller plates (10cm x 10cm) prior to use.

(d) Radiochemicals.

Carrier-free $[^{32}P]$-orthophosphate was provided by the Regional Isotopes Dispensary (Western Infirmary, Glasgow).

$[^{3}H]$-LTB_4, $[^{3}H]$-5HETE and $[^{14}C]$-arachidonic acid were purchased from Amersham International and $[^{3}H]$-LTB_4 metabolites were prepared by Dr. R. M. McMillan (ICI, England).

RESULTS AND DISCUSSION

CHAPTER 5: AGONIST-INDUCED HUMAN NEUTROPHIL ACTIVATION.

1. Electron Microscopy Studies.

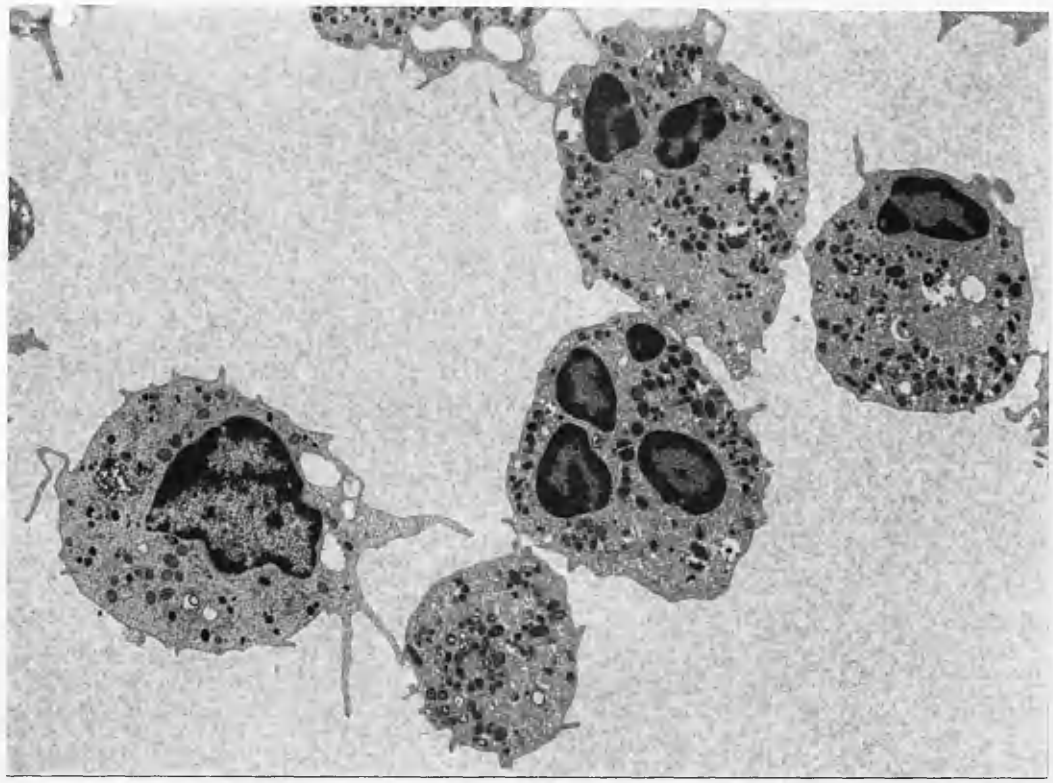
The general morphology of the unstimulated human neutrophil is described in Chapter 1. They are usually rounded, with little plasma membrane ruffling and with the occasional extrusion of fine projections which may or may not make cell to cell contact (figures 1 and 17a). Exposure of neutrophils to a variety of stimuli produce a number of cellular and biochemical responses which can vary depending on the type of agonist used. In this investigation I compared the morphological changes produced by the arachidonic acid metabolite, LTB_4; the ether lipid, PAF; and the chemotactic tripeptide, FMLP, using transmission electron microscopy.

1.1. Effect of LTB_4

Ultrastructural changes consistent with migrating activity (Schiffmann & Gallin, 1979) were observed after exposure of the cells to LTB_4. Unlike that seen in control preparations, after 10 seconds exposure to LTB_4 (30nM), there was marked plasma membrane ruffling affecting the whole surface with a strongly crenated or stellate profile (figure 17b). By 30 seconds the cells had become polarized with the formation of large lamellipodium (figure 18a) or in many cases knob-like, frilly uropodia or "tails" could be seen, which persisted for at least 5 minutes. These structures contained skeins of microfilaments and clusters of ribosomes but from which granules were excluded. No microtubules were detected in this area of the cell.

Stirring of the cells in the presence of an appropriate agonist

a



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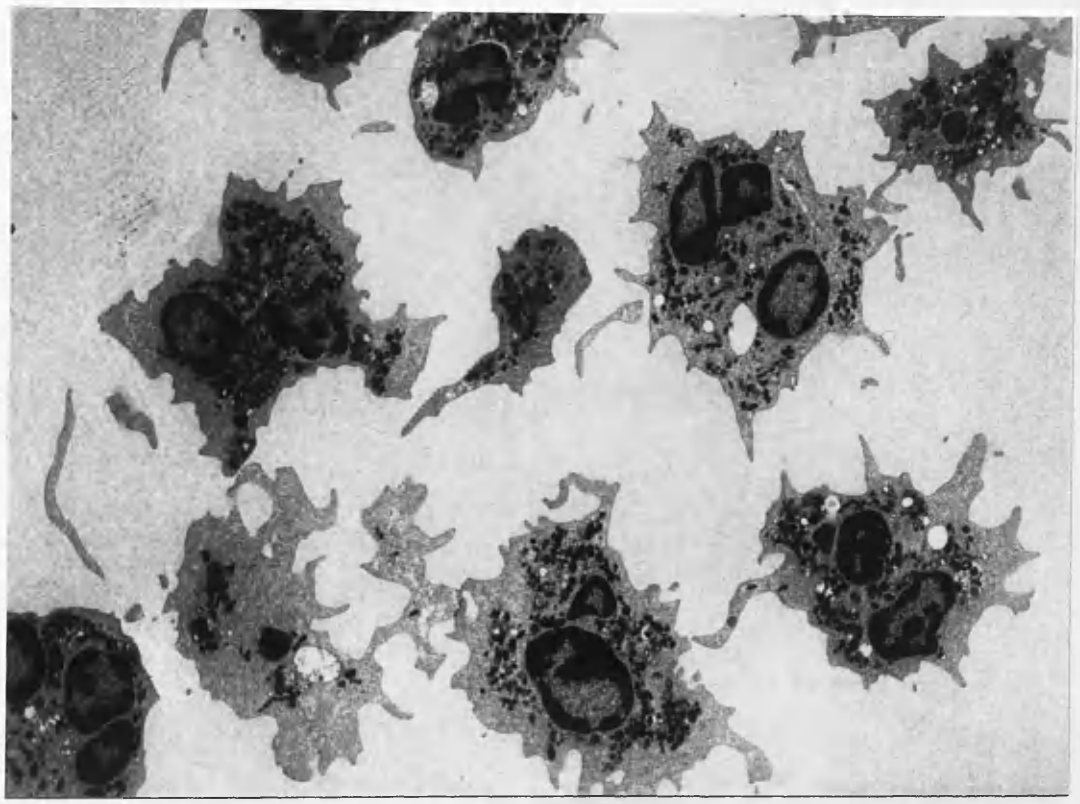
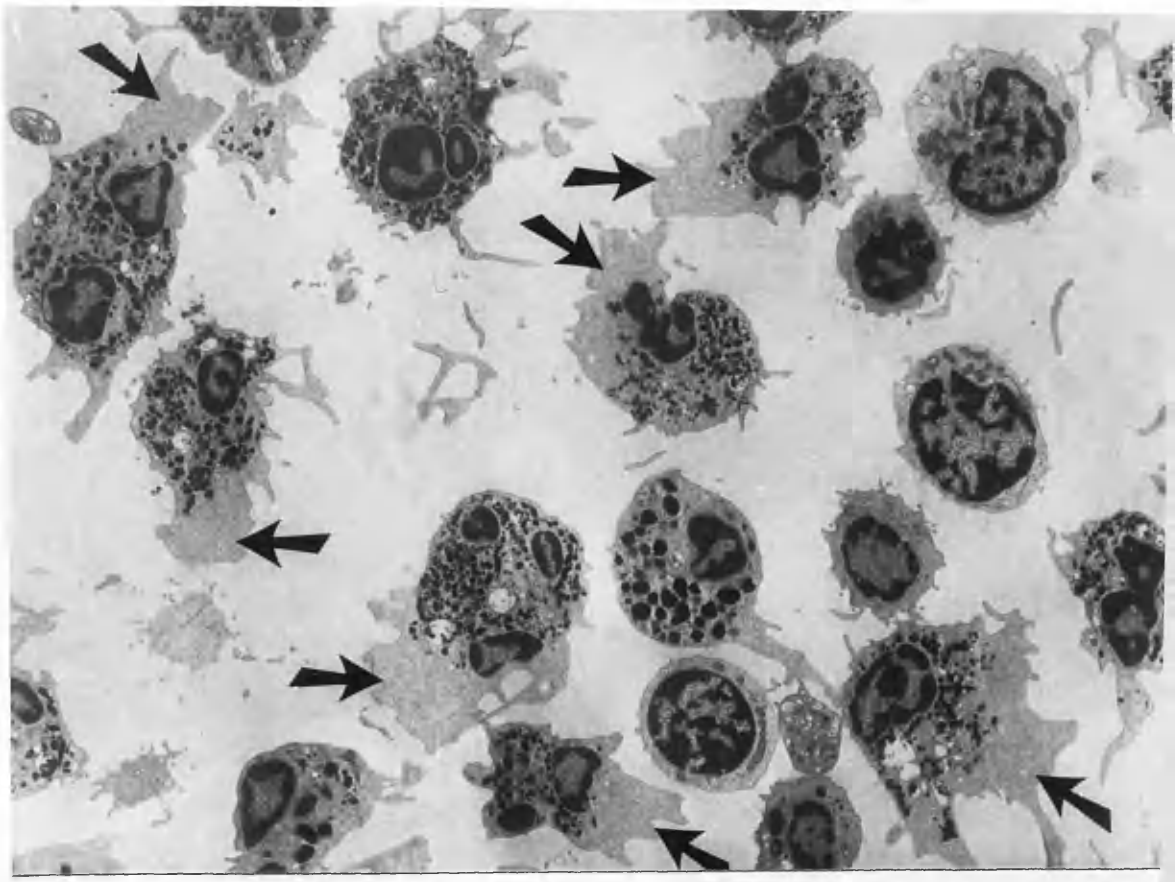


Figure 17.

(a) Control suspension of neutrophils. Generally rounded with smooth contours and the occasional extension of fine projections (EM x 3,800).

(b) Unstirred neutrophils exposed to LTB_4 (30nM) for 10 seconds showing marked plasma membrane ruffling over their entire surface (EM x 3,800).

a



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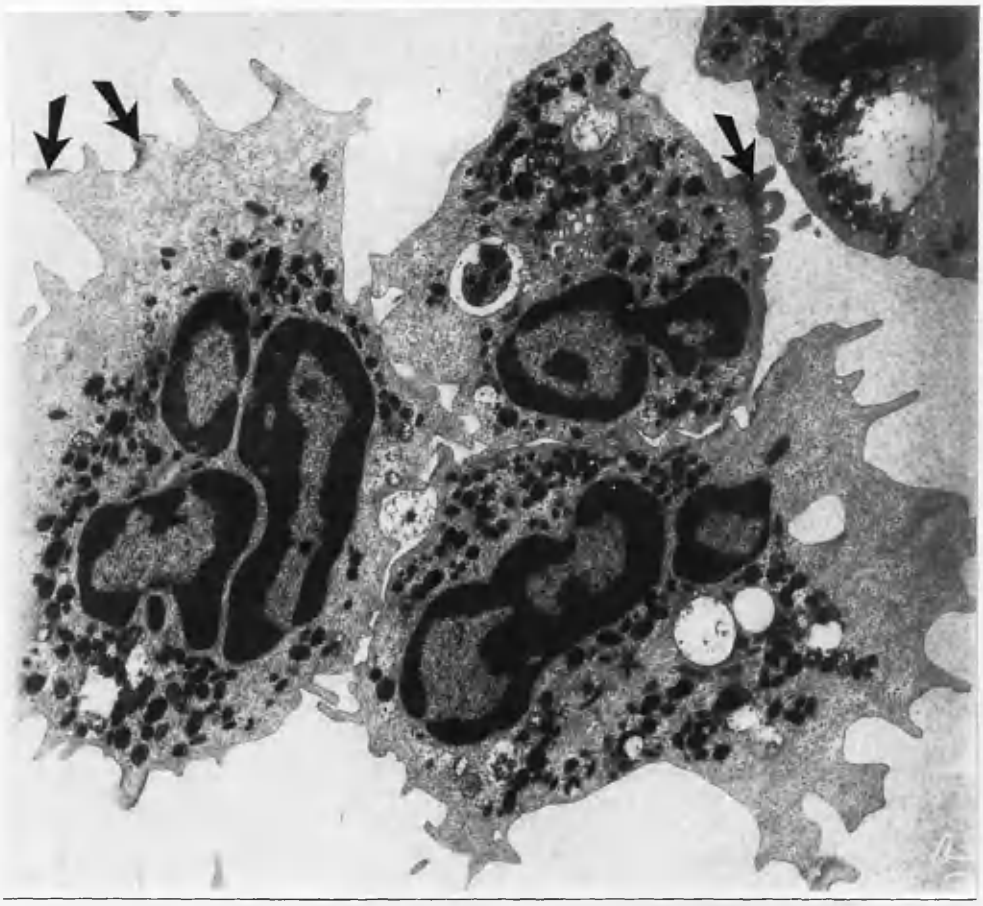


Figure 18.
(a) Unstirred neutrophils exposed to LTB_4 (30nM) for 30 seconds showing well developed lamellipodia (EM x 2,850).
(b) Aggregated neutrophils after 25 seconds exposure to LTB_4 (30nM) in stirred suspensions, with well developed lamellipodia. Sub-plasmalemmal microfilaments can be observed (arrows) (EM x 8,750).

causes cell to cell contact and aggregation. Figure 18b shows the effect of LTB_4 (30nM) at 30 seconds at which time the cells had aggregated into small clumps. Many of the neutrophils within the aggregate were polarized with the formation of a large, agranular lamellipodium extending outward from the aggregate. Plasma membranes were often closely aligned and bands of microfilaments could be seen parallel to, and just subjacent to, the cell surface, especially where the contours were smooth and round. In all cases individual cells could be distinguished; cell fusion had not occurred.

1.2. Effect of PAF

A similar ultrastructural profile was observed when PAF was examined. By 10 seconds (figure 19a) there was a generalised plasma membrane ruffling and by 30 seconds (figure 19b) the polarization of the cell and extension of the lamellipodium had occurred on exposure to 180nM PAF.

1.3. Effect of FMLP

The chemotactic tripeptide was not dissimilar to LTB_4 or PAF. In the presence of 100nM FMLP the neutrophils looked extremely "agitated" with pronounced membrane ruffling by 10 seconds (figure 20a) and the characteristic polarization and lamellipodium formation was evident by 30 seconds (figure 20b).

1.4. Effect of Cytochalasin B

The fungal metabolite, cytochalasin B, is extensively used as a pharmacological agent to promote overt secretion of granular contents thereby rendering the contents detectable in the external medium (see

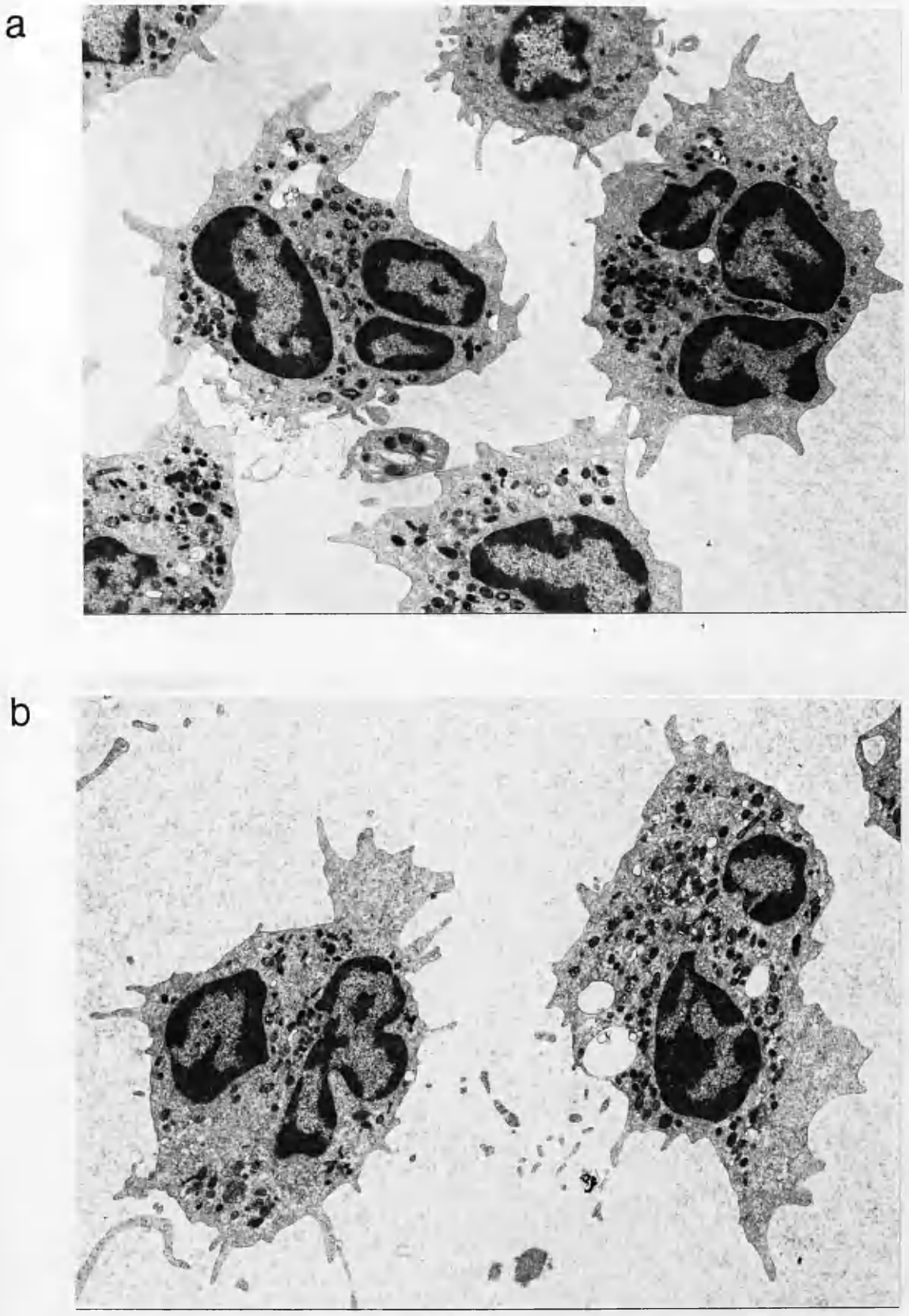
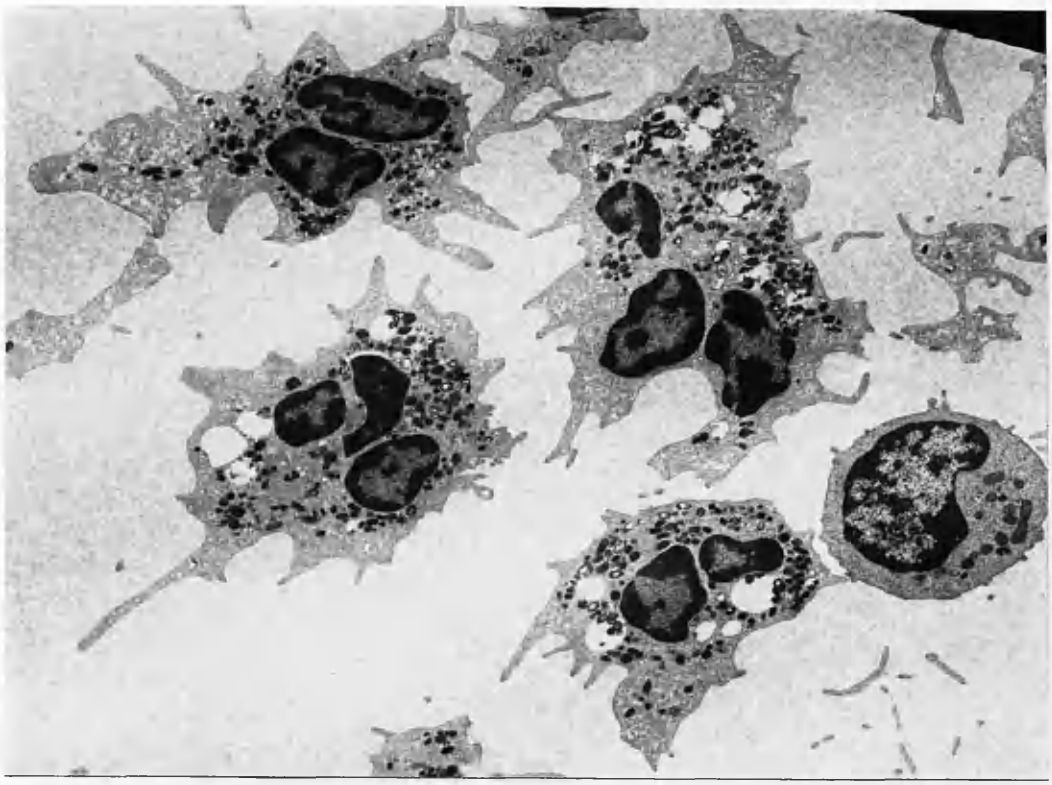


Figure 19.

(a) Unstirred neutrophils exposed to PAF (180nM) for 10 seconds showing marked plasma membrane ruffling over their entire surface (EM x 4,375).

(b) Unstirred neutrophils exposed to PAF (180nM) for 30 seconds showing well developed lamellipodia (EM x 6,125).

a



b

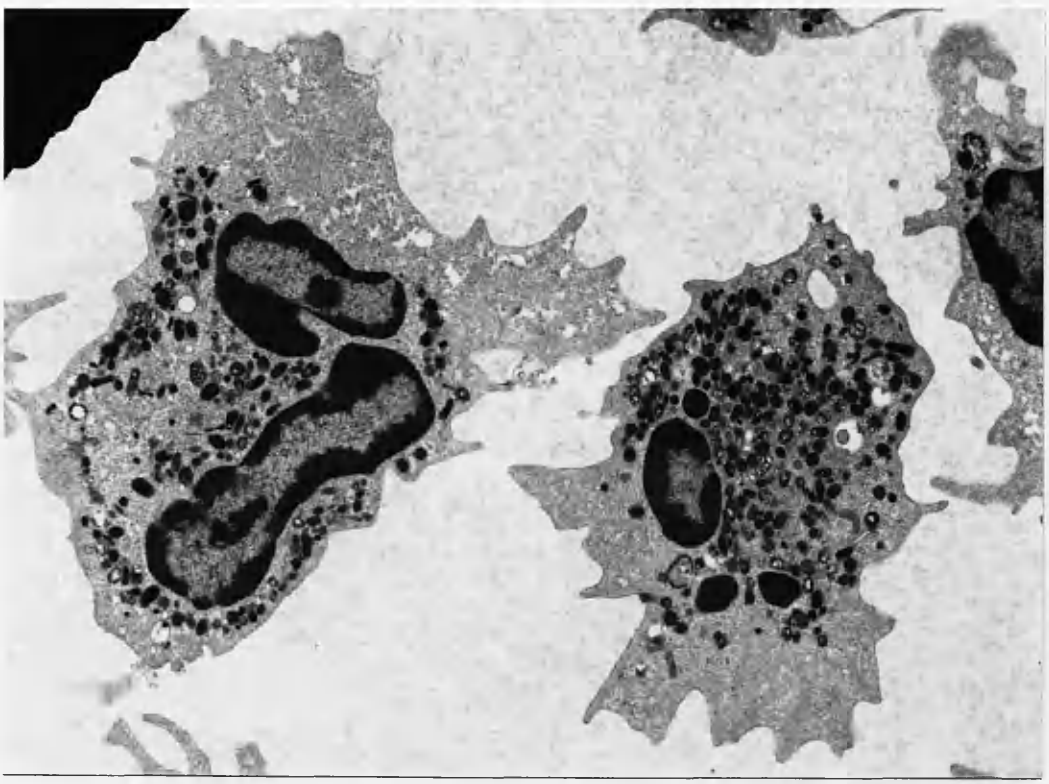


Figure 20.

(a) Unstirred neutrophils exposed to FMLP (100nM) for 10 seconds showing marked plasma membrane ruffling over their entire surface (EM x 4,375).

(b) Unstirred neutrophils exposed to FMLP (100nM) for 30 seconds showing well developed lamellipodia (EM x 6,125).

Chapter 1). However, caution must be taken when using this agent because it produces many other biochemical and physical effects within the cell. Pretreatment of human neutrophil suspensions with 5μg/ml for 10 min cytochalasin B produced dramatic alterations in cell ultrastructure (figure 21). The cells did not appear activated since no membrane ruffling was evident however there were large vacuoles present in the cytoplasm not usually found in untreated control cells. Addition of the agonists after cytochalasin B pretreatment (5μg/ml; 10 min) resulted in the loss of the cell's spherical appearance exhibiting marked blebbing and forming grotesque profiles. Figure 22 shows the effect of 180nM PAF (a) and 100nM FMLP (b) after 60 seconds in the presence of cytochalasin B. There was little polarization of the granules and they were not restricted to the inner regions of the cell but were present in the blebs and extrusions. Marked vacuolation was still evident especially beneath the uropod which frequently showed bleb formation with or without the presence of granules. The formation of clumps was not especially evident in the electron microscope, although some instances of cell to cell contact were observed.

2. Agonist-induced neutrophil aggregation.

Suspensions of stirred human neutrophils when exposed to a variety of stimuli become adhesive and form cell-cell aggregates (O'Flaherty et al., 1979; Ford-Hutchinson, 1983; Rossi et al., 1987b). Such neutrophil aggregation can be monitored as changes in light transmission through a stirred suspension of neutrophils using a standard platelet aggregometer as described in Chapter 4. The aim of this particular study was to investigate the nature and extent of the aggregatory response and to compare the cumulated

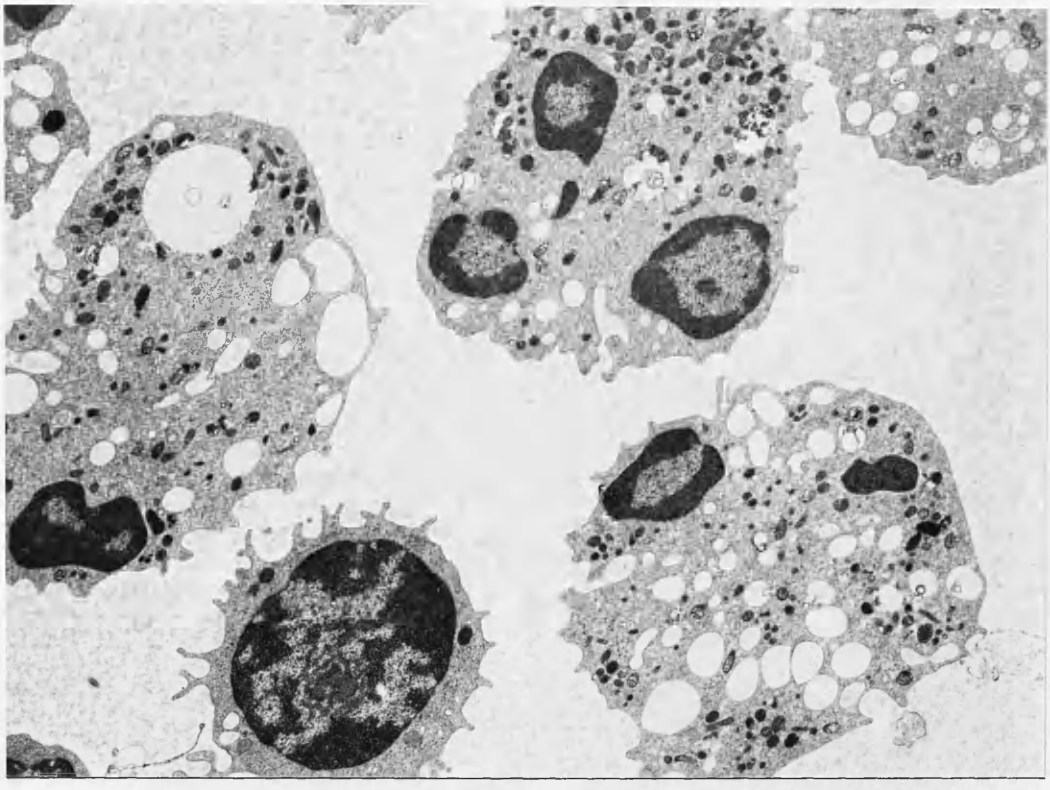
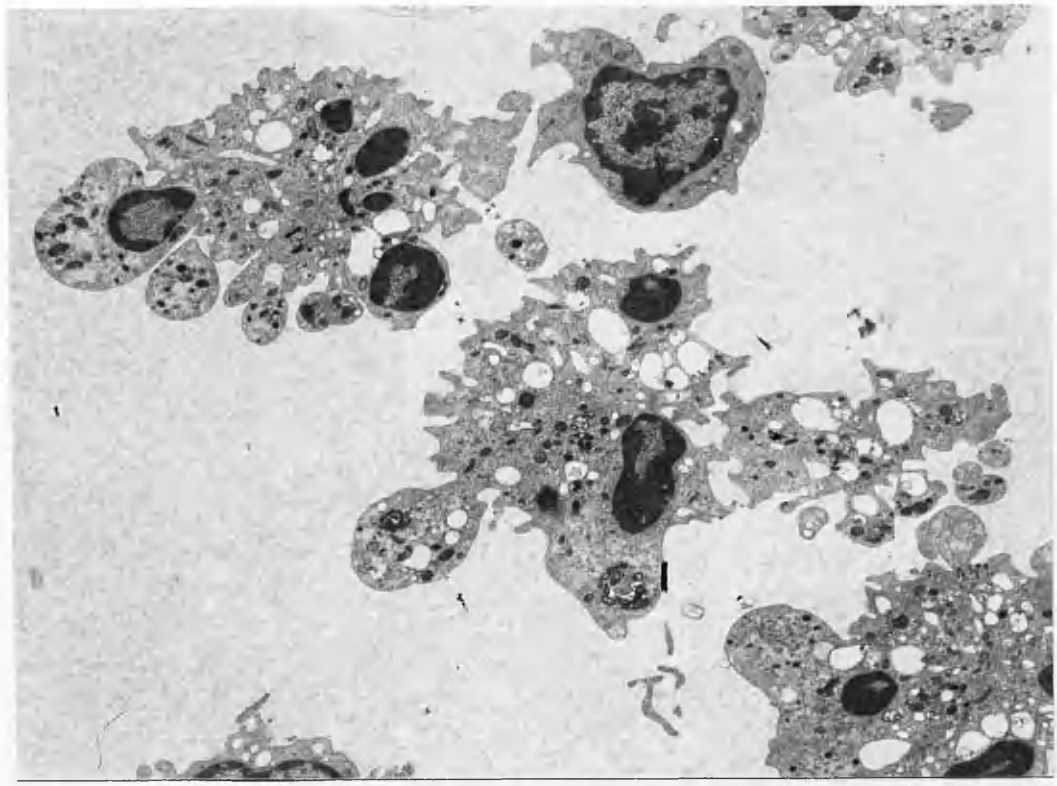


Figure 21.

Neutrophils pretreated with cytochalasin B (5µg/ml; 10 minutes) showing cytoplasm containing many vacuoles (EM x 5,250).

a



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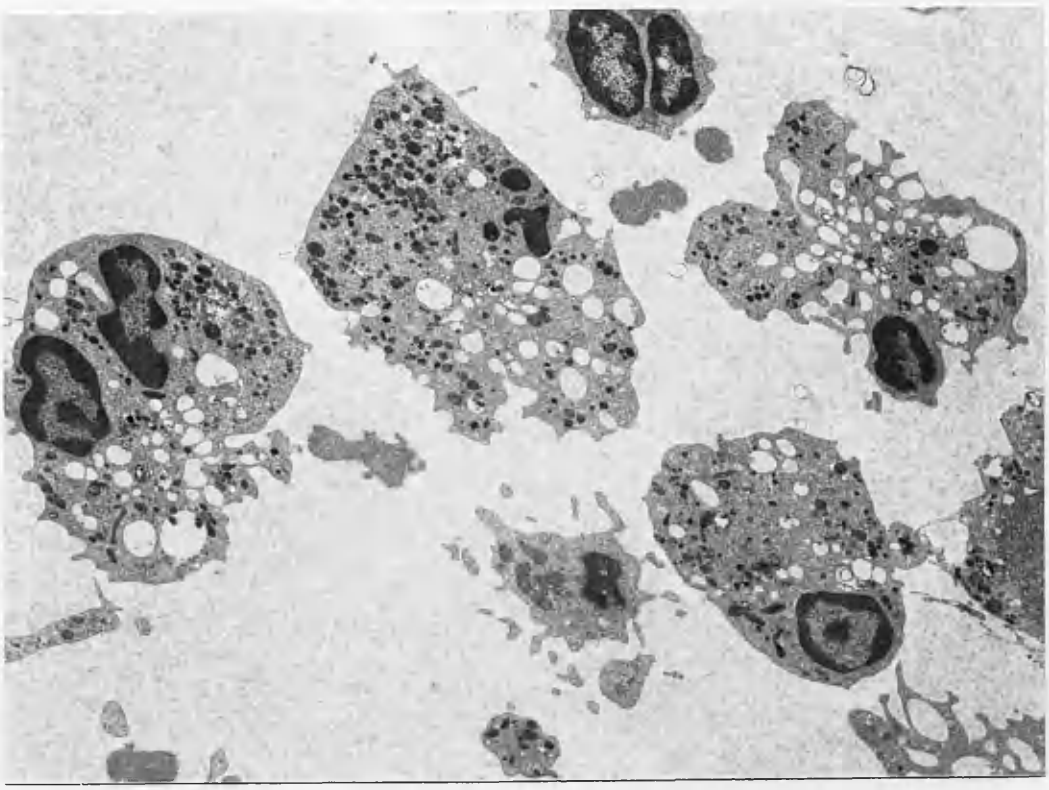


Figure 22.

(a) Neutrophils pretreated with cytochalasin B (5µg/ml; 10 minutes) followed by exposure of PAF (180nM) for 60 seconds showing vacuolation and bleb formation (EM x 5,250).

(b) Neutrophils pretreated with cytochalasin B (5µg/ml; 10 minutes) followed by exposure of FMLP (100nM) for 60 seconds showing vacuolation and bleb formation (EM x 5,000)

concentration-response curves induced by the various agonists.

2.1. Typical Aggregation Traces.

Aggregation traces induced by LTB_4, PAF and FMLP are shown in figure 23. These receptor directed agonists all caused a concentration-dependent aggregation; the effects observed were rapid in onset, maximal within two minutes of agonist addition, and reversible.

Aggregation when monitored photometrically is quantified in arbitrary units of light transmission, consequently it is difficult to compare the extent of aggregation evoked by different agonists when tested in different experiments. However, when examined in the same experiment, the extent of aggregation induced by FMLP and PAF was similar whereas LTB_4 was much less efficacious. Indeed it is not uncommon that the response elicited by LTB_4 is even less than that shown in figure 23, the maximum response induced by LTB_4 may only reach one-third or less than the responses elicited by FMLP and PAF.

When examining the aggregation traces induced by the phorbol ester, PMA, and the calcium ionophore, ionomycin, a different picture emerged (figure 24). Although the responses were concentration-dependent, they were irreversible, up to at least 10 min, and maximal aggregation was usually slower in onset, especially with lower concentrations of these stimuli, when compared to FMLP, PAF and LTB_4.

2.2. Concentration-Response Curves for Agonist-Induced Aggregation.

The next five figures show the cumulated concentration-response curves for the various agonists. FMLP induced aggregation in the

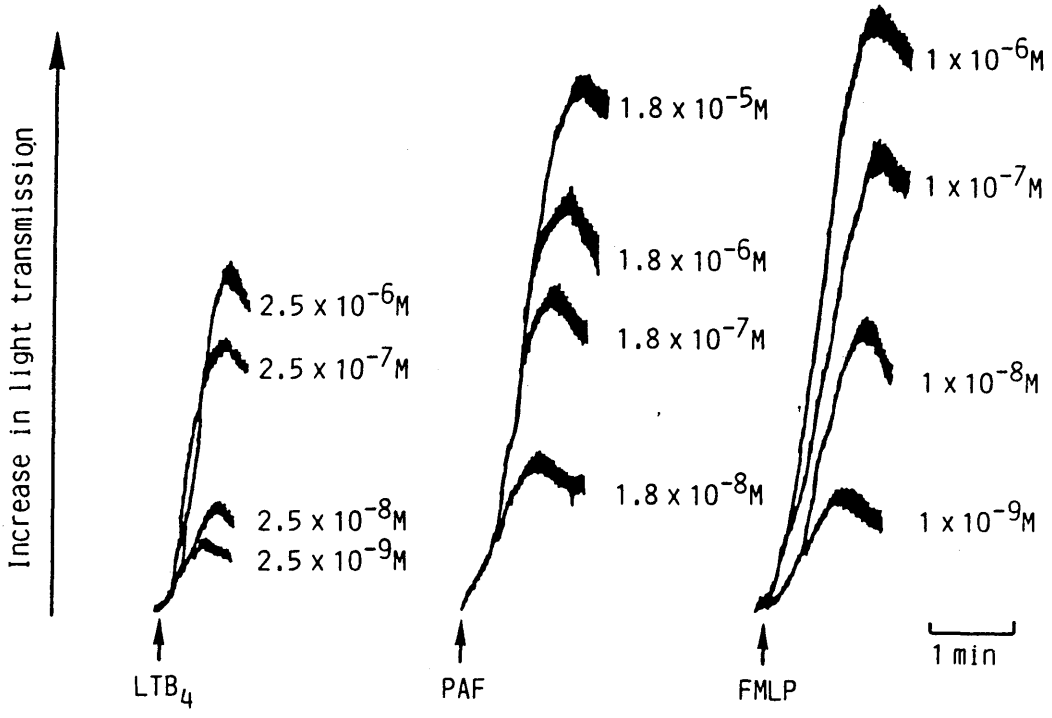


Figure 23.

Aggregation traces induced by LTB_4, PAF & FMLP in human neutrophils.

0.6 ml samples of neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to an agonist at the concentrations shown and the changes in light transmission recorded.

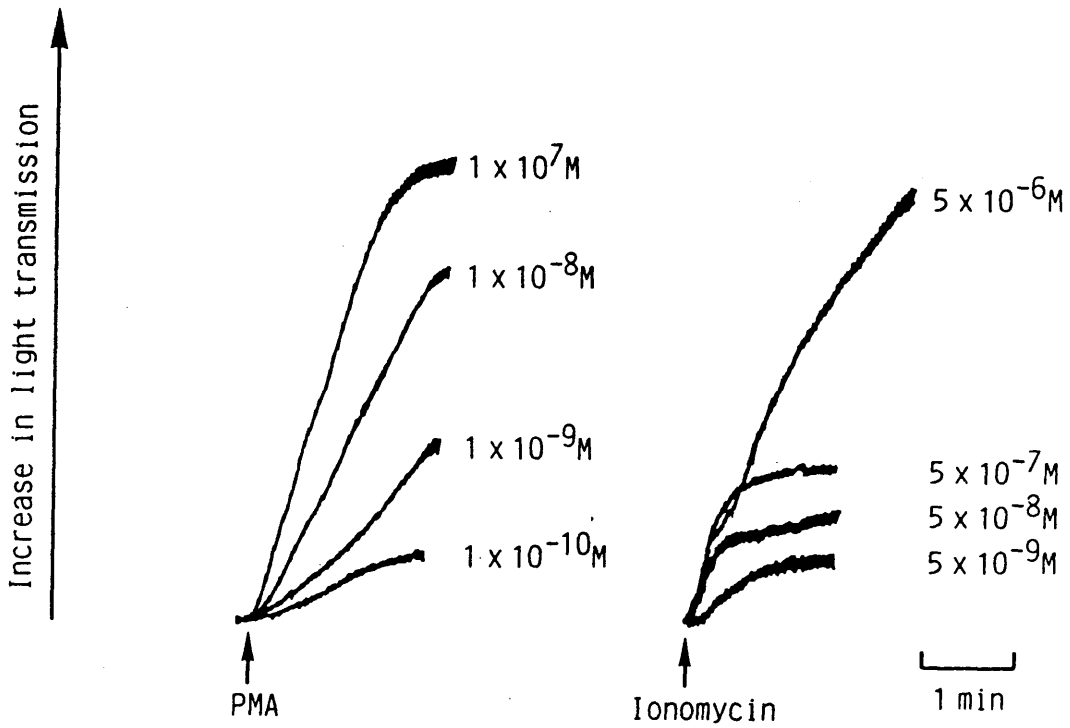


Figure 24.

Aggregation traces induced by PMA & ionomycin in human neutrophils.

0.6ml samples of neutrophils (2.5 x 10^7cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to an agonist at the concentrations shown and the changes in light transmission recorded.

concentration range of 0.1nM-1μM with an EC_{50} value, i.e. concentration producing 50% of the maximum response elicited by each particular agonist, of≈ 30nM (figure 25). Similarly, the other stimuli produced concentration-dependent aggregation; PAF in the range of 1nM-10μM; estimated EC_{50}≈ 1μM (figure 26), LTB_4 in the range of 0.25nM-2.5μM; EC_{50}≈ 50nM (figure 27), PMA in the range of 0.01nM-0.1μM; EC_{50}≈ 1nM (figure 28) and ionomycin in the range of 50nM-10μM; EC_{50}≈ 1μM (figure 29).

An overall comparison for each agonist is shown in figure 30. The rank order of potency of the five agonists in terms of induction of neutrophil aggregation is PMA > FMLP≈LTB_4 > PAF≈ionomycin.

3. Agonist-induced neutrophil degranulation.

In order for neutrophils to efficiently perform their function of destroying invading organisms and digestion of particulate matter, they have to be capable of releasing their battery of stored enzymes into the phagocytic vacuole or into the surrounding medium (Weissmann , 1982; Baggiolini & Dewald, 1985). The release of two enzymes was examined; B-N acetyl-glucosaminidase (NAG), found in the azurophil granules, and lysozyme located in the azurophil and specific granules.

3.1. Requirement of Cytochalasin B

Initial studies were performed to examine the dependence of each agonist, to release both enzymes, in the presence or absence of cytochalasin B. Neutrophils were pretreated, for 10 minutes, with either 5μg/ml cytochalasin B or with DMSO (vehicle) and then challenged

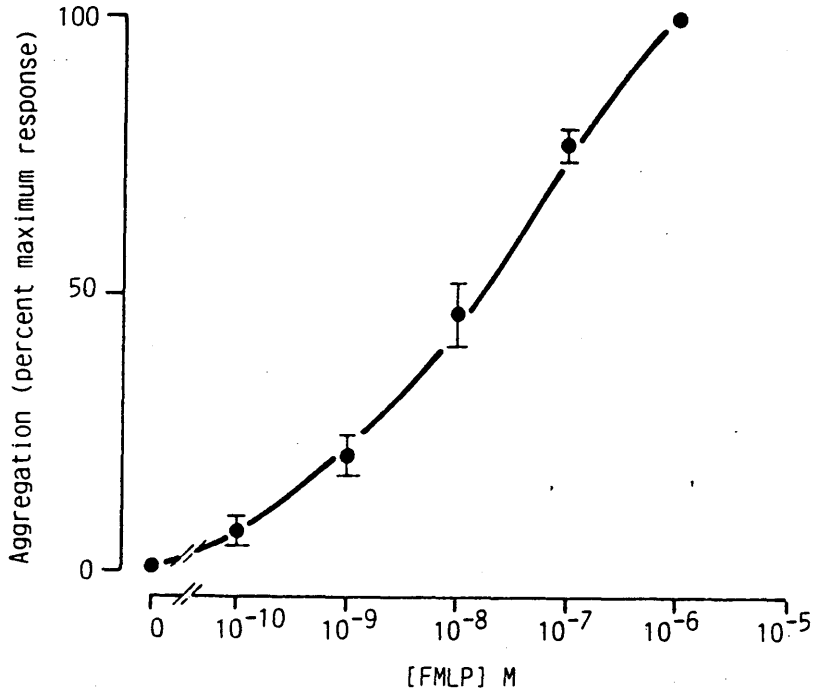


Figure 25.

Concentration-response relationship for FMLP-induced aggregation of human neutrophils.

0.6ml samples of neutrophils (2.5×10^7cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm The cells were exposed to FMLP at the concentrations shown and the changes in light transmission recorded. The results are expressed as a percentage of the maximum response and are mean values $\pm$ SEM for six experiments, each performed in triplicate.

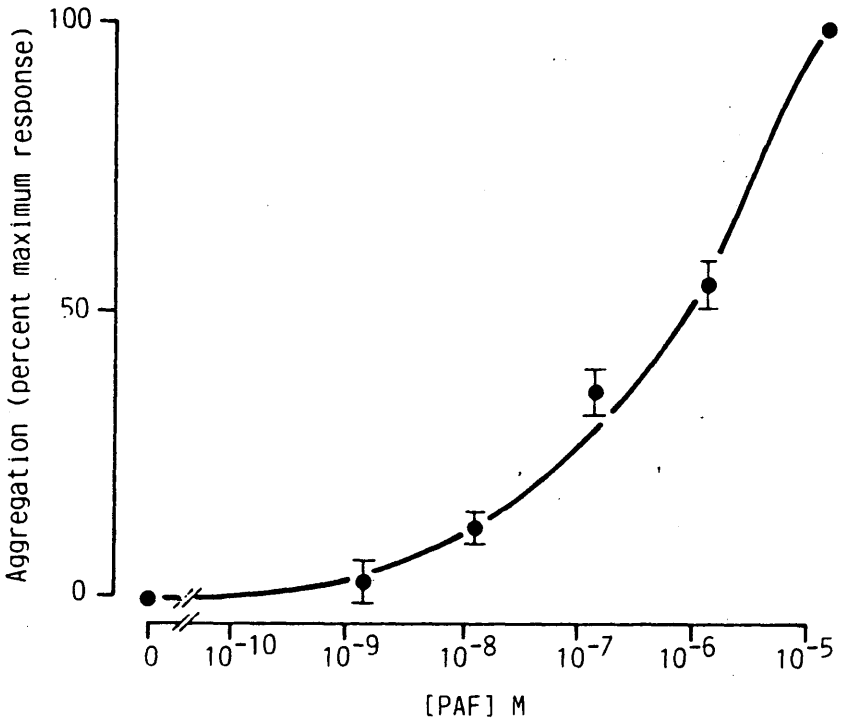


Figure 26.

Concentration-response relationship for PAF-induced aggregation of human neutrophils.

0.6ml samples of neutrophils (2.5×10^7cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to PAF at the concentrations shown and the changes in light transmission recorded. The results are expressed as a percentage of the maximum response and are mean values ± SEM for three experiments, each performed in triplicate.

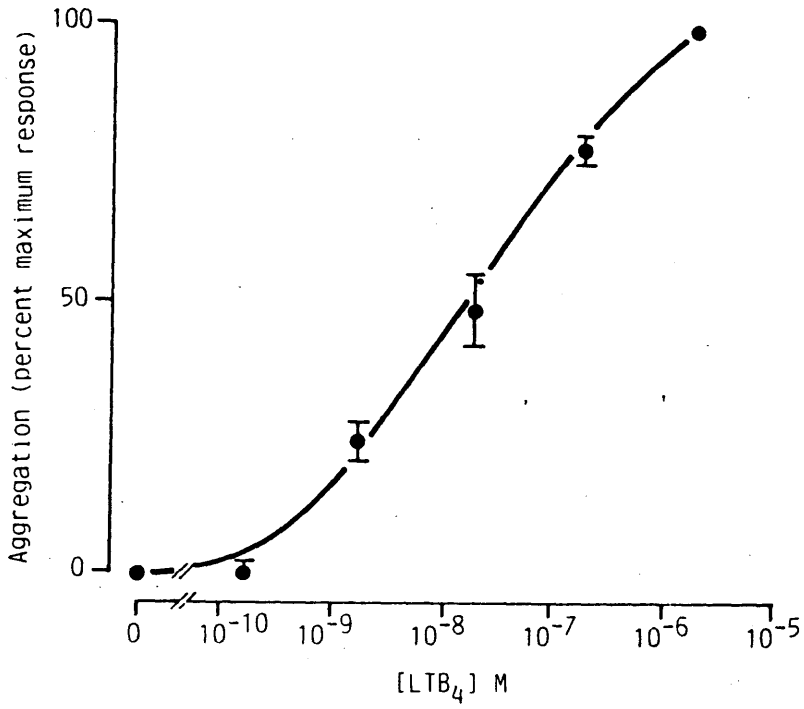


Figure 27.

Concentration-response relationship for LTB_4-induced aggregation of human neutrophils.

0.6ml samples of neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to LTB_4 at the concentrations shown and the changes in light transmission recorded. The results are expressed as a percentage of the maximum response and are mean values ± SEM for three experiments, each performed in triplicate.

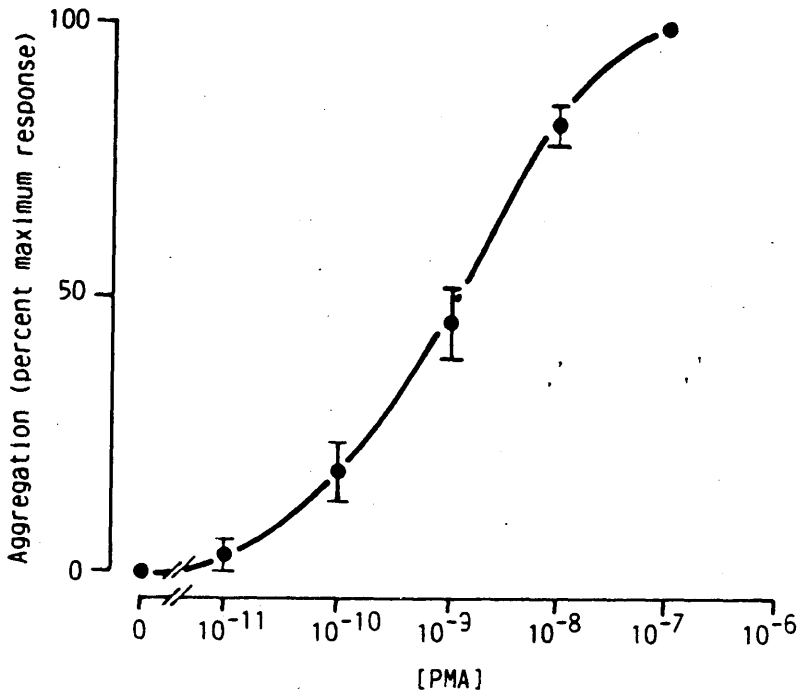


Figure 28.

Concentration-response relationship for PMA-induced aggregation of human neutrophils.

0.6ml samples of neutrophils (2.5×10^7cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm The cells were exposed to PMA at the concentrations shown and the changes in light transmission recorded. The results are expressed as a percentage of the maximum response and are mean values $\pm$ SEM for four experiments, each performed in triplicate.

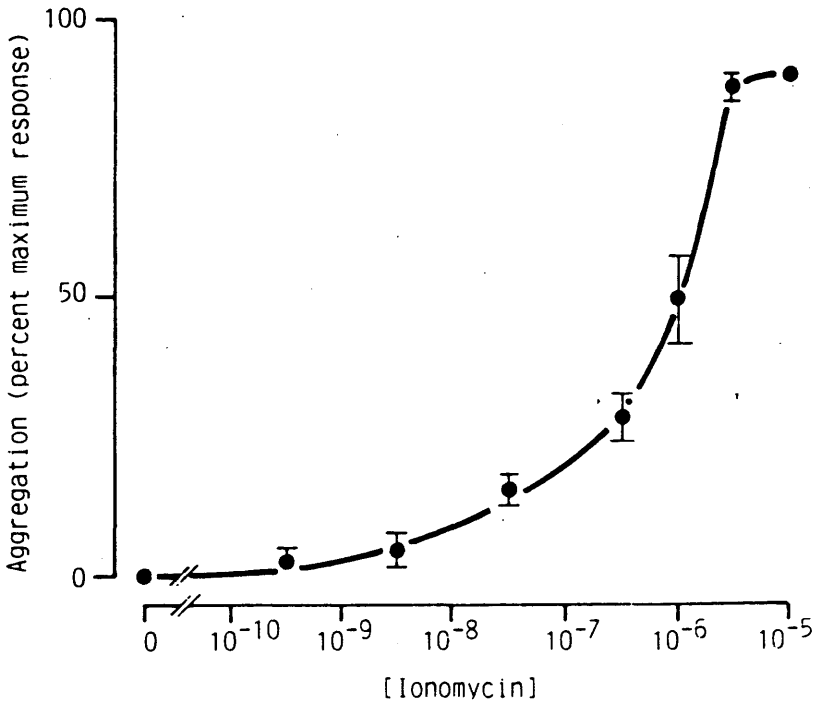


Figure 29.

Concentration-response relationship for ionomycin-induced aggregation of human neutrophils.

0.6ml samples of neutrophils (2.5 x 10^7cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to ionomycin at the concentrations shown and the changes in light transmission recorded. The results are expressed as a percentage of the maximum response and are mean values $\pm$ SEM for five experiments, each performed in triplicate.

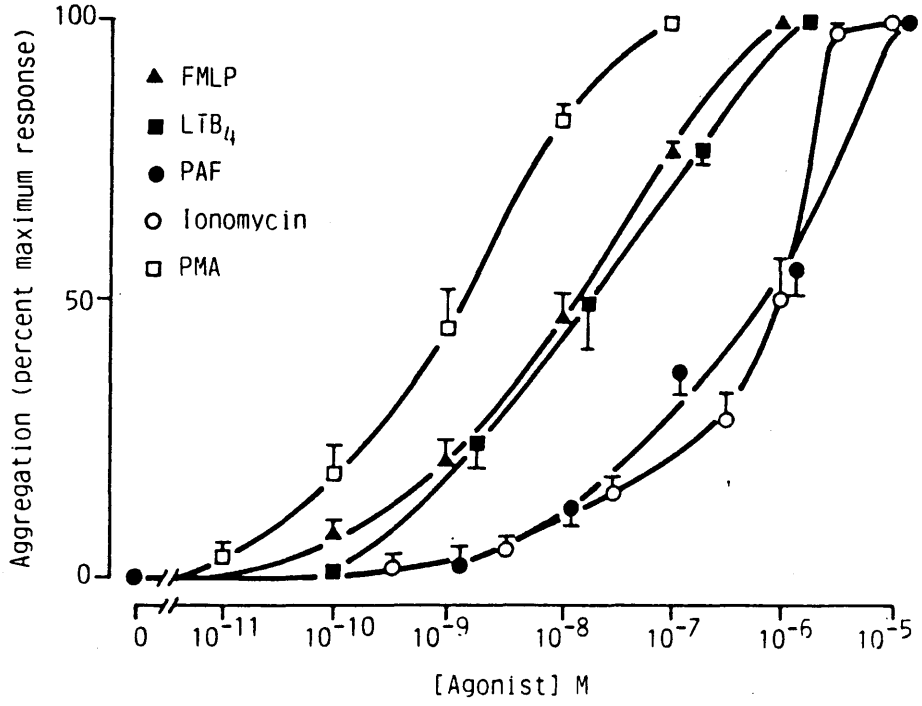


Figure 30.

Cumulated concentration-response curves for agonist-induced aggregation of human neutrophils.

0.6ml samples of neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to the agonists at the concentrations shown and the changes in light transmission recorded. The results are expressed as a percentage of the maximum response and are mean values + SEM for between three and six experiments, each performed in triplicate.

with high concentrations of agonists for a further 10 minutes and the enzyme release determined.

Figure 31 shows the effect of cytochalasin B on agonist-induced NAG release expressed as a percentage of the total enzyme content. The histogram clearly shows that cytochalasin B alone produces no significant release of NAG and that FMLP, PAF, LTB_4 and PMA all require the presence of cytochalasin B to release this enzyme into the external milieu. The calcium ionophore could release NAG both in the presence and absence of this compound.

The effect of cytochalasin B on agonist induced release of lysozyme is depicted by the histogram in figure 32. Again FMLP, PAF and LTB_4 require the presence of cytochalasin B, however PMA, as well as ionomycin, can release lysozyme in the absence of the fungal metabolite.

3.2. Agonist-Induced Enzyme Release.

The next stage of the investigations was to construct concentration-response curves for the release of both enzymes for all five agonists. Preincubation for 10 minutes with 5µg/ml cytochalasin B was used for all subsequent measurements of enzyme release.

a) NAG Release.

The next five graphs show the concentration-response curves for agonist-induced release of NAG (expressed as a percentage of the total NAG content). NAG release induced by all agonists was within the approximate concentration range of 1nM-10µM. FMLP ($EC_{50} \approx 50nM$; figure 33), PAF ($EC_{50} \approx 70nM$; figure 34), LTB_4 ($EC_{50} \approx 50nM$; figure 35), PMA ($EC_{50} \approx 60nM$; figure 36) and ionomycin ($EC_{50} \approx 500nM$; figure 37) induced

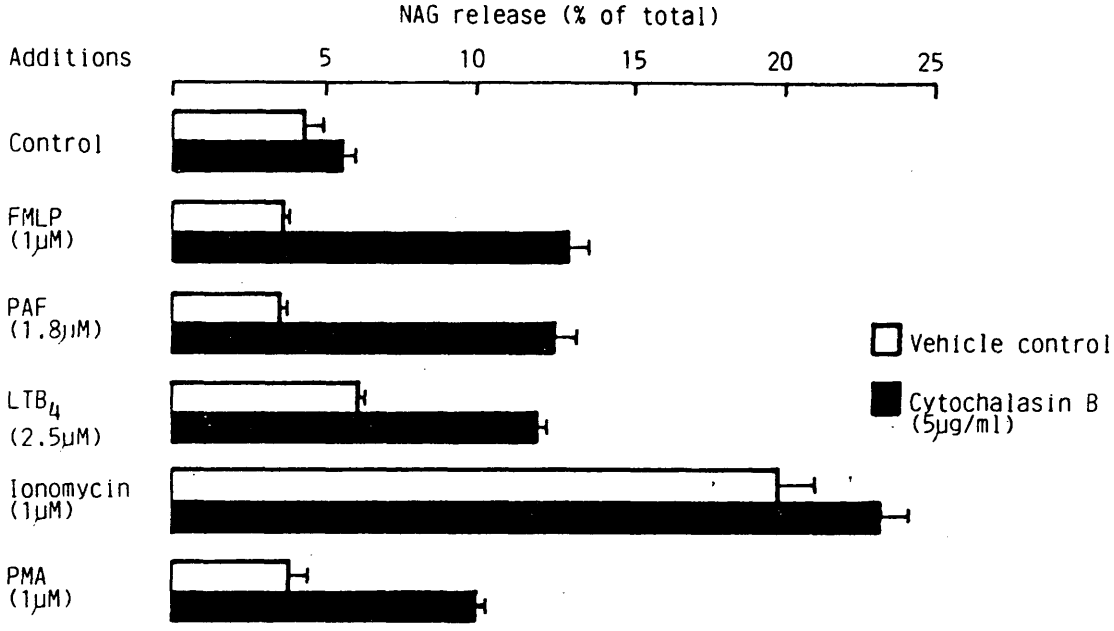


Figure 31.

The effect of cytochalasin B on agonist-induced NAG release from human neutrophils.

Neutrophils (1×10^7 cells/ml) were pretreated with cytochalasin B (5μg/ml), or vehicle control, for 10 minutes. The neutrophils were then challenged with agonist for a further 10 minutes at the concentrations indicated and the release of NAG determined. The results are mean values ± SEM for between three and four experiments, each performed in triplicate.

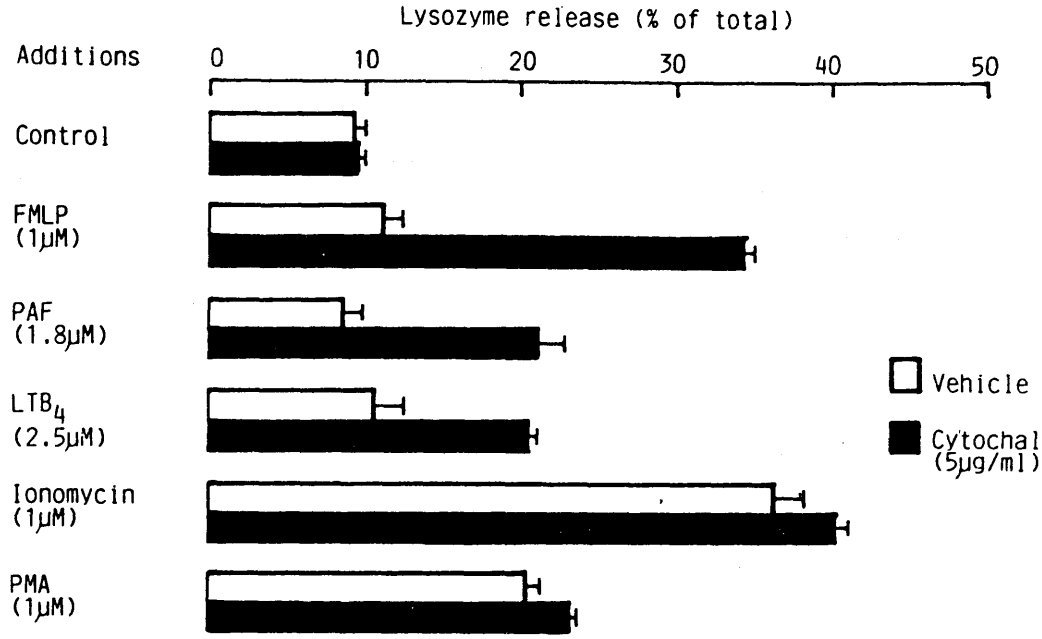


Figure 32.

The effect of cytochalasin B on agonist-induced lysozyme release from human neutrophils.

Neutrophils (1×10^7 cells/ml) were pretreated with cytochalasin B (5μg/ml), or vehicle control, for 10 minutes. The neutrophils were then challenged with agonist for a further 10 minutes at the concentrations indicated and the release of lysozyme determined. The results are mean values ± SEM for between three and four experiments, each performed in triplicate.

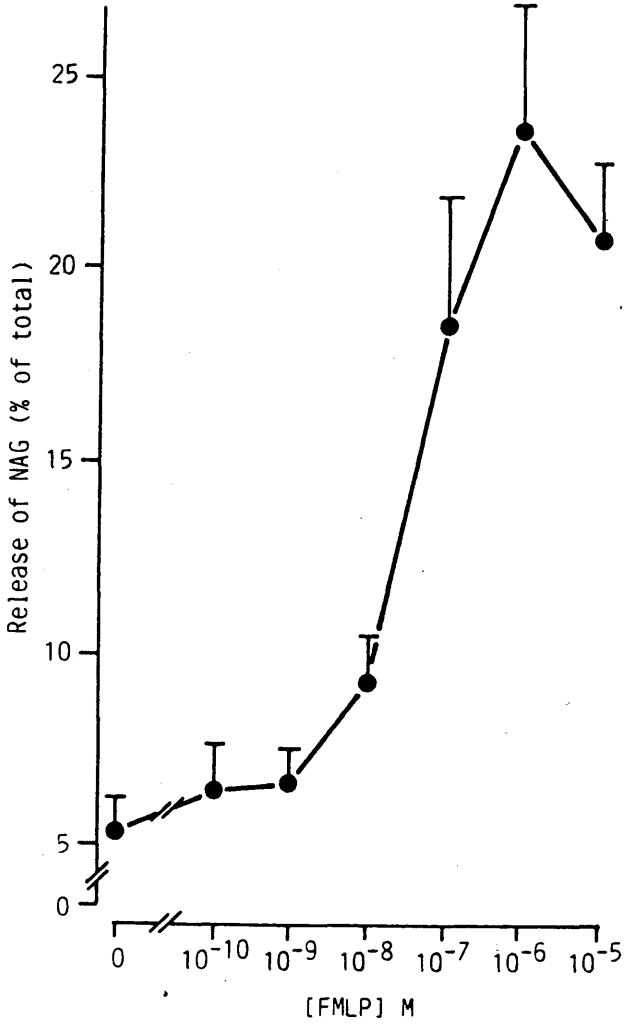


Figure 33.

Concentration-response relationship for FMLP-induced NAG release from human neutrophils.

0.6 ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5μg/ml) and then challenged with FMLP for a further 10 minutes at the concentrations indicated and the release of NAG determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for five experiments, each performed in triplicate.

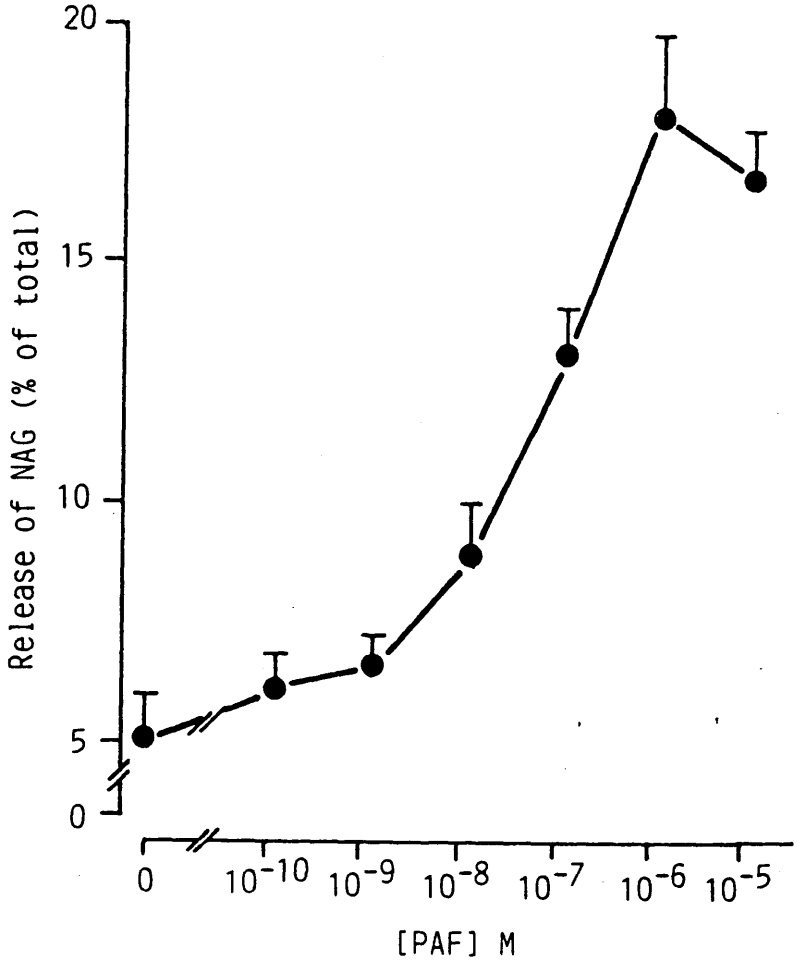


Figure 34.

Concentration-response relationship for PAF-induced NAG release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with PAF for a further 10 minutes at the concentrations indicated and the release of NAG determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for six experiments, each performed in triplicate.

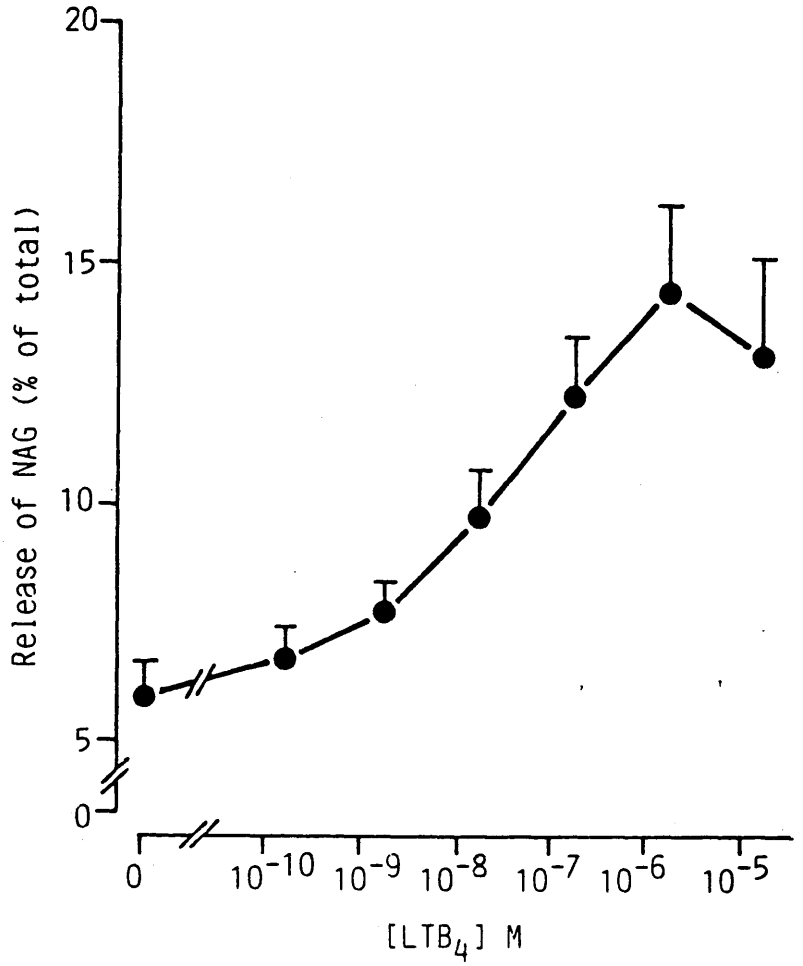


Figure 35.

Concentration-response relationship for LTB_4-induced NAG release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with LTB_4 for a further 10 minutes at the concentrations indicated and the release of NAG determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for four experiments, each performed in triplicate.

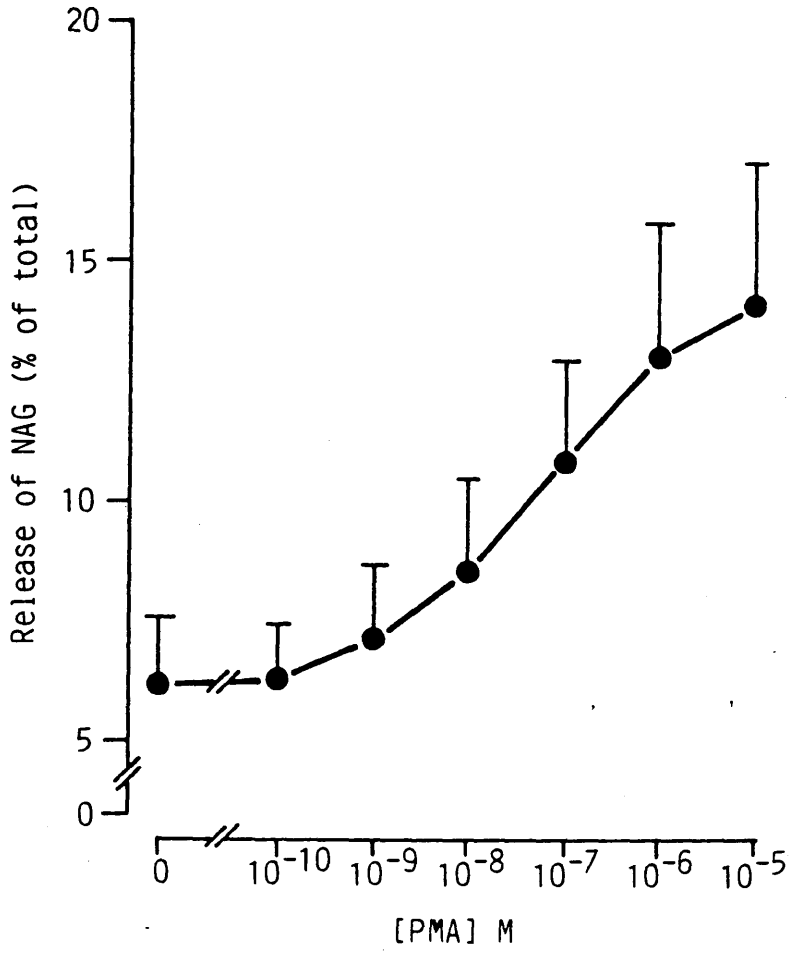


Figure 36.

Concentration-response relationship for PMA-induced NAG release from human neutrophils.

0.6 ml samples of neutrophils (1×10^7 cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with PMA for a further 10 minutes at the concentrations indicated and the release of NAG determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for four experiments, each performed in triplicate.

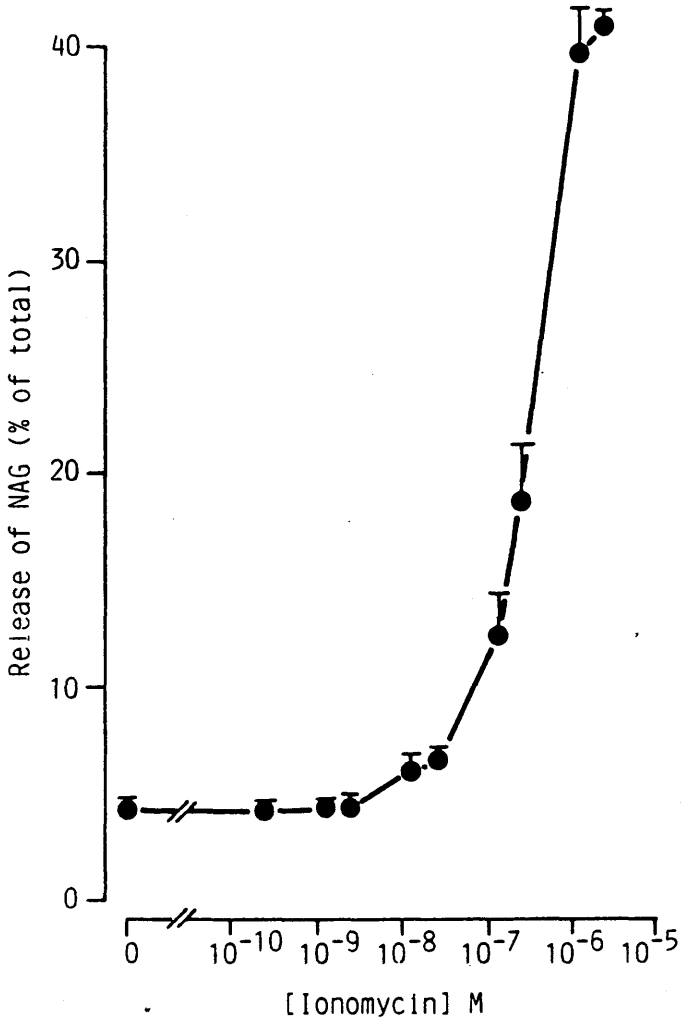


Figure 37.

Concentration-response relationship for ionomycin-induced NAG release from human neutrophils.

0.6ml samples of neutrophils (1×10^7 cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with ionomycin for a further 10 minutes at the concentrations indicated and the release of NAG determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for between three and six experiments, each performed in triplicate.

maximal release (basal subtracted) of approximately 20%, 14%, 11%, 7% and 37% respectively.

b) Lysozyme Release.

The concentration-response curves for agonist-induced lysozyme release (expressed as a percentage of the total lysozyme content) are shown in the five following figures. Again the agonists stimulated lysozyme release within the approximate concentration range of 1nM-10μM. FMLP ($EC_{50}\approx 50nM$; figure 38), PAF ($EC_{50}\approx 80nM$; figure 39), LTB_4 ($EC_{50}\approx 30nM$; figure 40), PMA ($EC_{50}\approx 90nM$; figure 41) and ionomycin ($EC_{50}\approx 400nM$; figure 42) induced maximal release (basal subtracted) of approximately 30%, 20%, 18%, 16% and 55% respectively.

4. Agonist-Induced LTB_4 Generation.

When human neutrophils become activated they have the capacity to hydrolyse membrane phospholipids by a number of enzymatic pathways to mobilise arachidonic acid; the precursor for the biosynthesis of leukotrienes, including LTB_4 (O'Flaherty, 1987). Most previous studies have examined the effects of the calcium ionophore, A23187, on LTB_4 biosynthesis in polymorphonuclear leucocytes. Reports on LTB_4 formation in response to more physiological stimuli, e.g. C5a, FMLP, PAF and immune complexes, are fewer, often conflicting, and the levels of LTB_4 produced are much lower than those produced by the calcium ionophore (Ham et al., 1983; Palmer & Salmon, 1983; McMillan et al., 1985; Haines et al., 1987).

4.1. Measurement by Specific Radioimmunoassay (RIA).

The aim of the following experiments was to examine and compare agonist-induced generation of LTB_4, monitored by specific RIA.

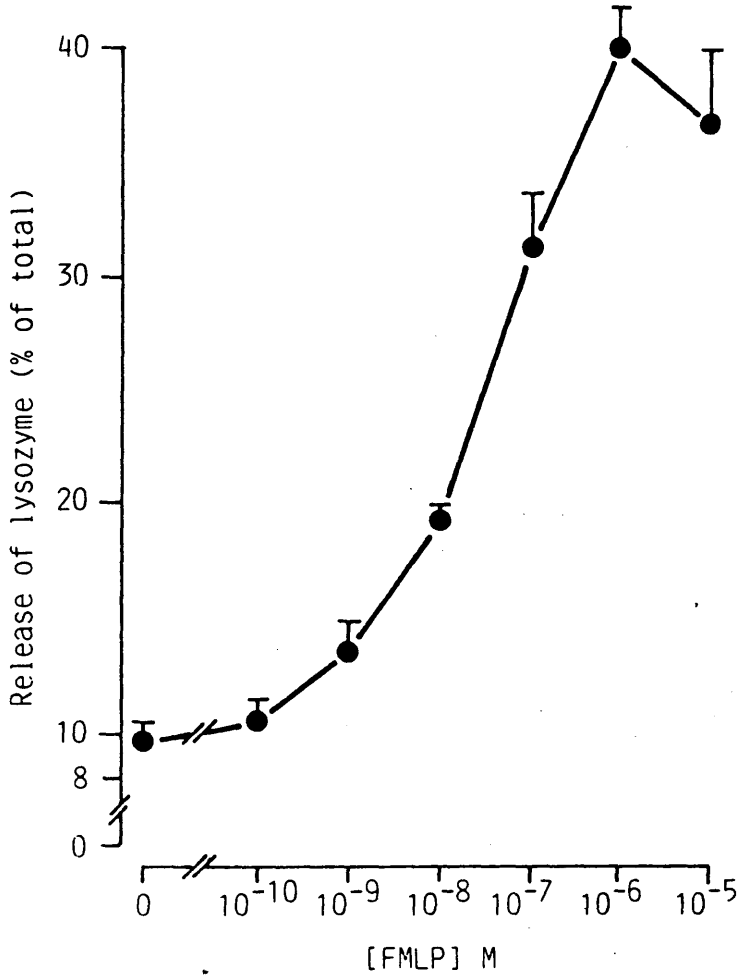


Figure 38.

Concentration-response relationship for FMLP-induced lysozyme release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with FMLP for a further 10 minutes at the concentrations indicated and the release of lysozyme determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for five experiments, each performed in triplicate.

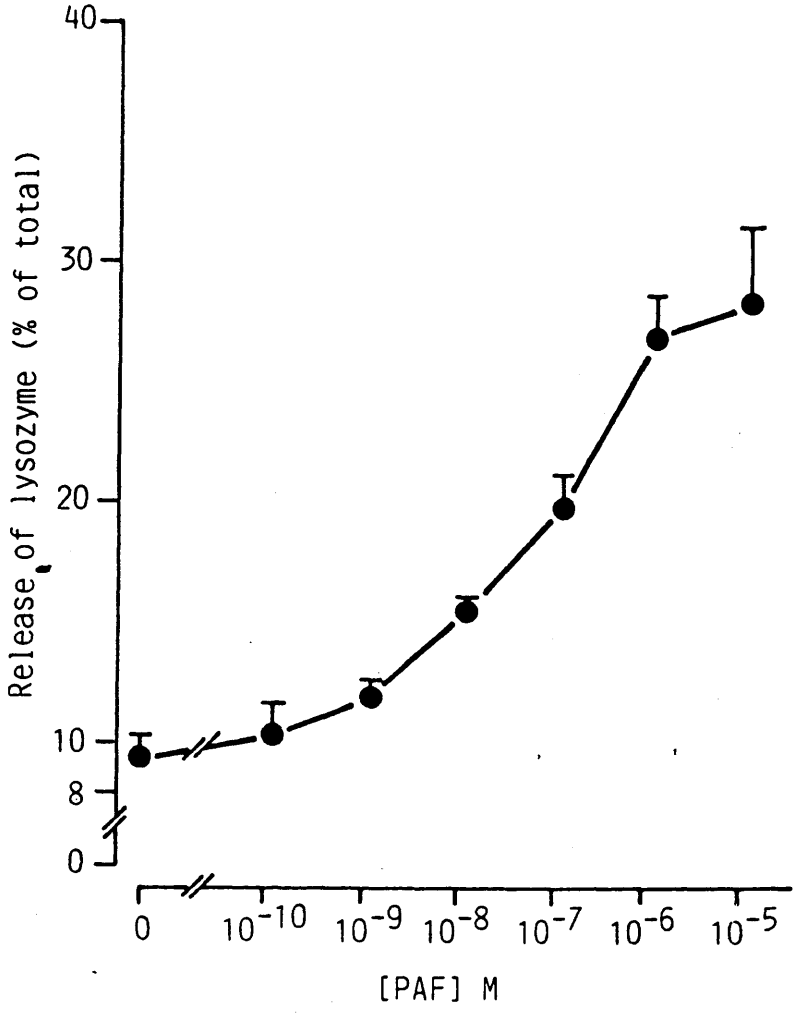


Figure 39.

Concentration-response relationship for PAF-induced lysozyme release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with PAF for a further 10 minutes at the concentrations indicated and the release of lysozyme determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for six experiments, each performed in triplicate.

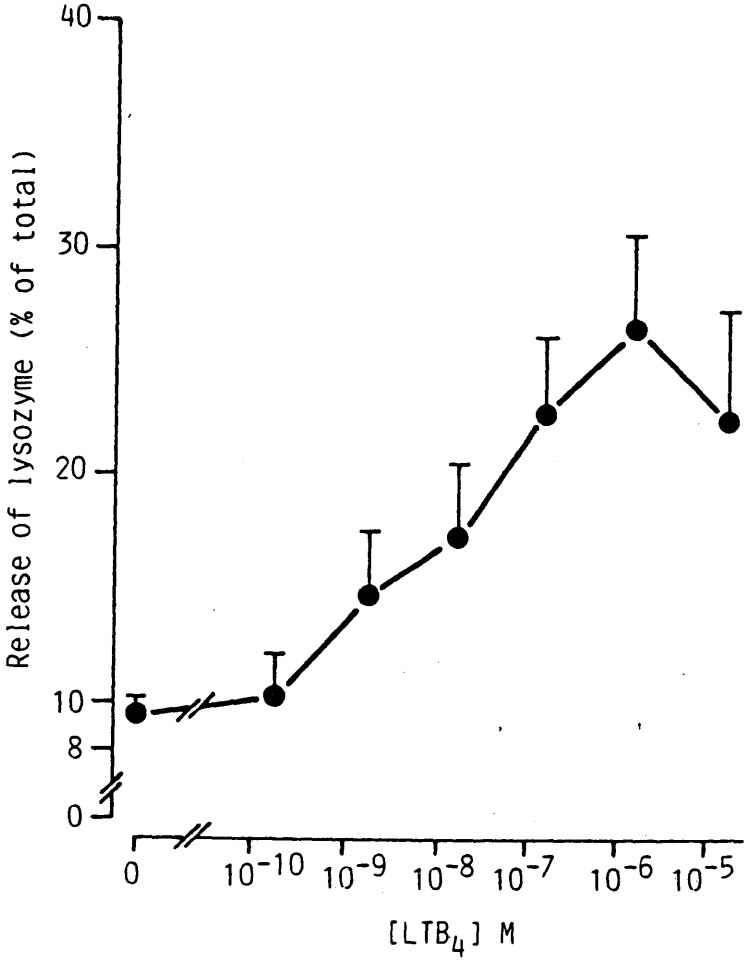


Figure 40.

Concentration-response relationship for LTB_4-induced lysozyme release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with LTB_4 for a further 10 minutes at the concentrations indicated and the release of lysozyme determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for four experiments, each performed in triplicate.

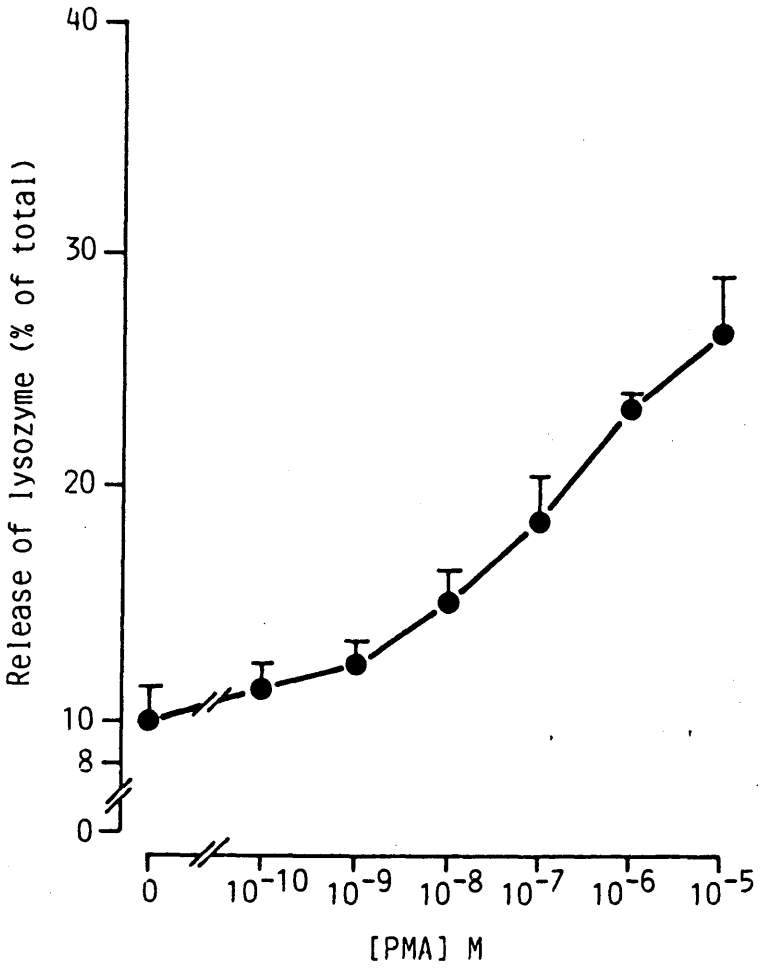


Figure 41.

Concentration-response relationship for PMA-induced lysozyme release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and then challenged with PMA for a further 10 minutes at the concentrations indicated and the release of lysozyme determined. The results are expressed as the percentage release of the total enzyme content and are mean values ± SEM for four experiments, each performed in triplicate.

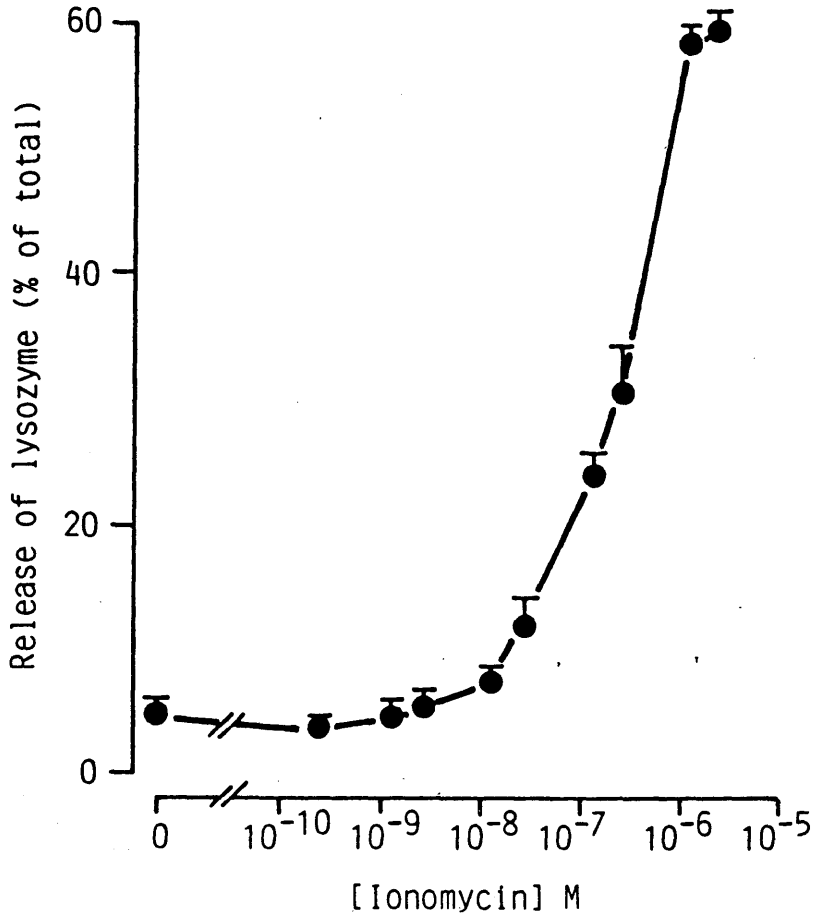


Figure 42.

Concentration-response relationship for ionomycin-induced lysozyme release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5μg/ml) and then challenged with ionomycin for a further 10 minutes at the concentrations indicated and the release of lysozyme determined. The results are expressed as the percentage release of the total enzyme content and are mean values + SEM for between three and six experiments, each performed in triplicate.

Initially, I examined the effect of ionomycin (figure 43) on LTB_4 generation; neutrophils were challenged with the calcium ionophore for 10 minutes, the reaction stopped and the levels of LTB_4 determined. The 10 minute time point was chosen since preliminary time course experiments established that at this time LTB_4 formation was maximal. Ionomycin induced a concentration-dependent generation of LTB_4 with an EC_{50} value of approximately 30nM. Maximal levels of LTB_4 were produced at concentrations of ionomycin above 100nM, reaching values of between 50-70ng/10^7cells.

The effects of FMLP and PAF on LTB_4 generation were next examined (figure 44). Both receptor directed agonists evoked significant production of LTB_4 at high concentrations. However, in comparison to ionomycin, the extents of LTB_4 formation were greatly reduced. Maximal levels produced by FMLP at 10μM were 3.5ng/10^7 cells and PAF at 18uM was only just significant at 1ng/10^7cells. No significant production of LTB_4 was produced by the phorbol ester, PMA (data not shown).

4.2. Authentication of Immuno-reactive LTB_4 by Reverse Phase HPLC.

When determining LTB_4 levels by RIA, it is best to check the authenticity of the stimulated LTB_4 by making sure that there is no cross-reactivity with other possible arachidonic acid derived products. The internal standard after separation by reverse phase HPLC and collection of fractions at various time points showed four major peaks occurring at elution times of 6.5, 15, 19.5 and 23 minutes corresponding to LTB_4 metabolites, LTB_4, 5HETE and arachidonic acid respectively (figure 45; upper panel).

An ionomycin (1μM) stimulated test sample was processed by HPLC and the fractions collected at all time points. When the fractions

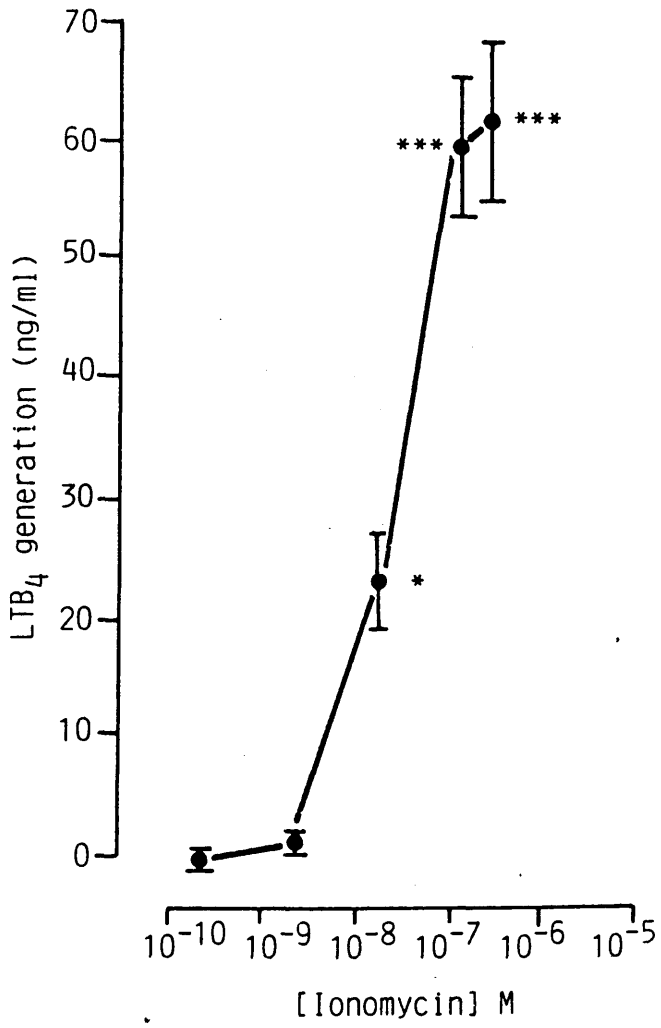


Figure 43.

Concentration-response relationship for ionomycin-induced LTB_4 generation by human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated for 10 minutes with cytochalasin B (5µg/ml) and then challenged with ionomycin for a further 10 minutes at the concentrations indicated and the production of LTB_4 determined. The results are expressed as the amount of LTB_4 produced (ng/ml) above basal and are mean values + SEM for between three and five experiments, each performed in triplicate. Analysis of data: *$P < 0.05$; ***$P < 0.001$.

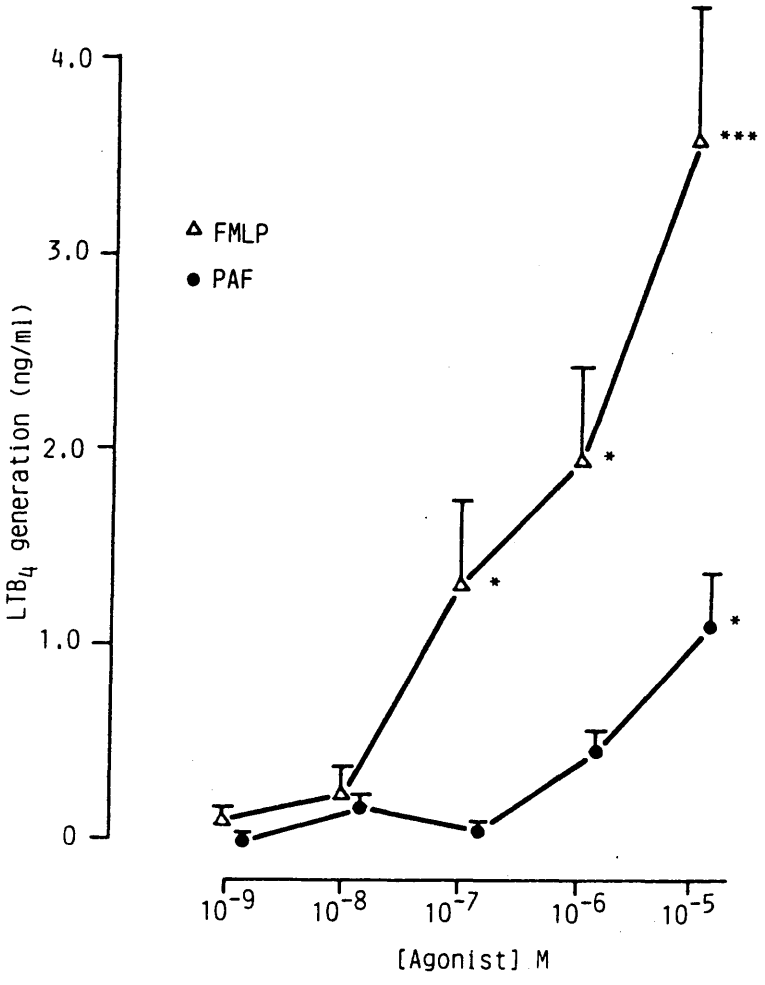


Figure 44.

Concentration-response relationship for agonist-induced LTB_4 generation by human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and then challenged with an agonist for a further 10 minutes at the concentrations indicated and the production of LTB_4 determined. The results are expressed as the amount of LTB_4 produced (ng/ml) above basal and are mean values + SEM for between three and five experiments, each performed in triplicate. Analysis of data: *$P < 0.05$; ***$P < 0.001$.

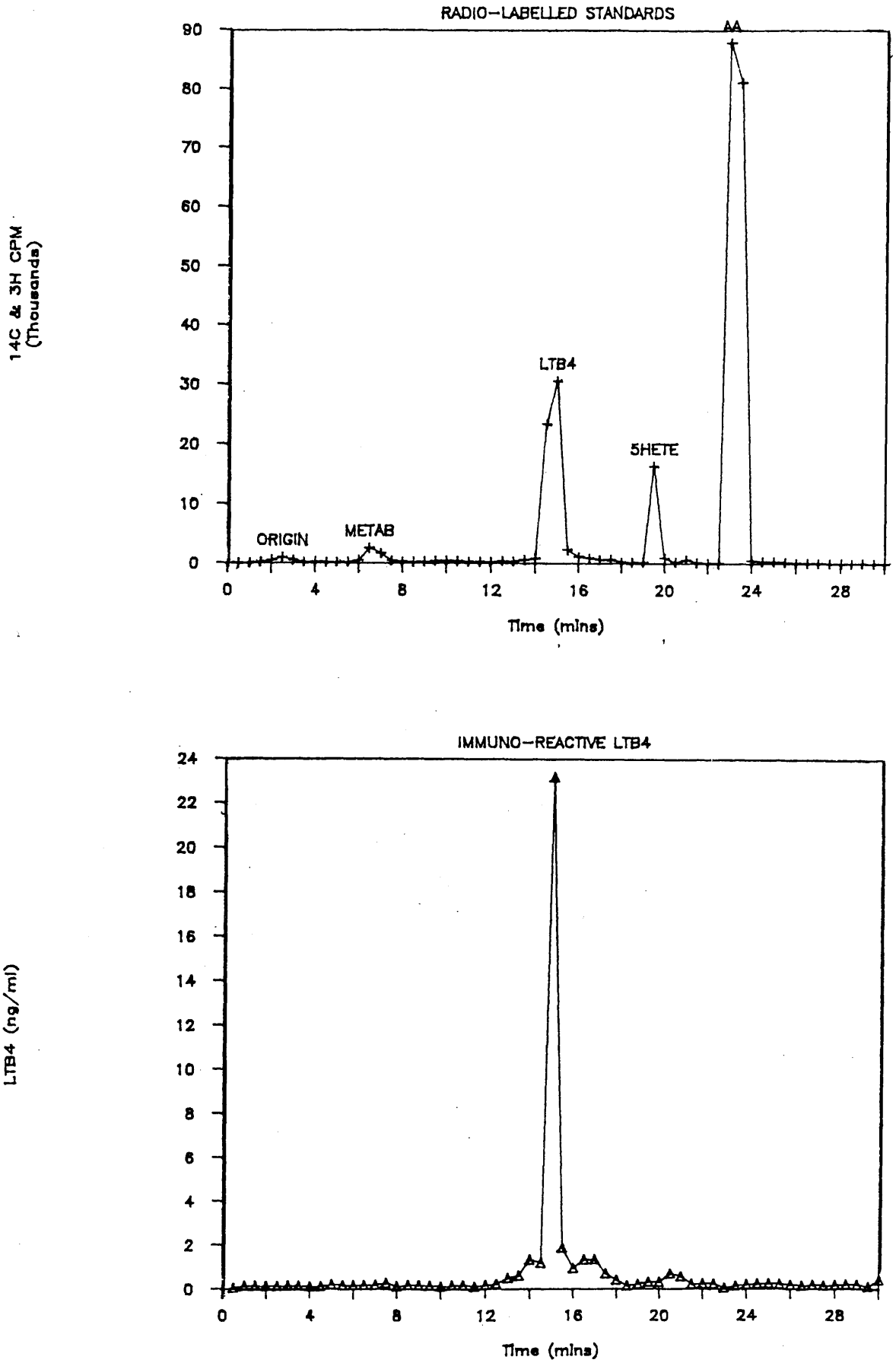


Figure 45.

Authentication of Immuno-reactive LTB_4 by Reverse Phase HPLC.

The upper panel shows radiolabelled internal standard, following fractionation using reverse phase HPLC and liquid scintillation counting.
The lower panel shows the LTB_4 immuno-reactivity of a fractionated human neutrophil sample stimulated with ionomycin (1μM) for 10 minutes.

(60 in total) were tested with RIA only one fraction, which eluted after 15 minutes, had significant immuno-reactivity. This fraction co-chromatographs with authentic LTB_4, suggesting that the eicosanoid generated by the ionophore is indeed LTB_4 (figure 45; lower panel).

5. Agonist-Induced Changes in $[Ca^{2+}]i$.

I next examined the correlation between agonist-induced aggregation and degranulation with changes in cytosolic-free calcium in quin2-loaded cells. When certain agonists are added to neutrophils preincubated with quin2 they elicit a concentration-dependent increase in fluorescence which can be calibrated to report $[Ca^{2+}]i$ and increases in $[Ca^{2+}]i$ (White et al., 1983b; Westwick & Poll, 1986).

5.1. Typical Fluorescence Records in Quin2-loaded Cells.

Recordings from a single experiment showing the effect of the receptor directed agonist are depicted in figure 46. The resting $[Ca^{2+}]i$ in human neutrophils is 135 $\pm$ 3nM (mean $\pm$ SEM) calculated from at least 50 determinations. The agonists all caused a rapid concentration-dependent elevation of fluorescence (and so $[Ca^{2+}]i$) until a maximal value (300-500nM in different experiments) was attained. Thereafter the fluorescence declined towards the resting value. Figure 47 shows the effect of ionomycin and PMA on neutrophil $[Ca^{2+}]i$. Ionomycin elicits a similar trace as FMLP, PAF and LTB_4 however the extent of elevation of $[Ca^{2+}]i$ reaches a greater maximum of between 1200-1500nM. In accordance with other workers (Sha'afi et al., 1983) PMA, at all concentrations tested, failed to elicit $[Ca^{2+}]i$ elevation.

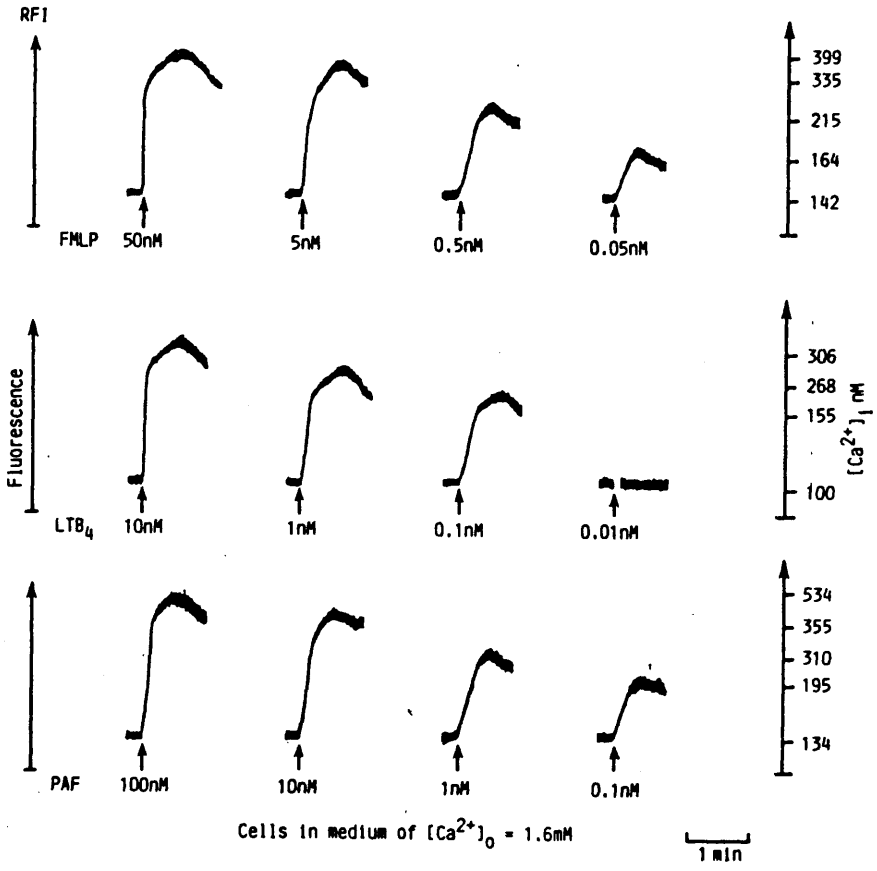


Figure 46.

Representative experimental traces of fluorescence changes in quin2 labelled neutrophils.

2.0ml samples of quin2 labelled neutrophils (1×10^7 cells/ml), in the presence of 1.6mM external Ca^{2+}, were exposed to agonist at the concentrations indicated. Fluorescence responses were monitored after agonist addition and the appropriate $[Ca^{2+}]i$ calibration scale is shown on the right of the fluorescence record.

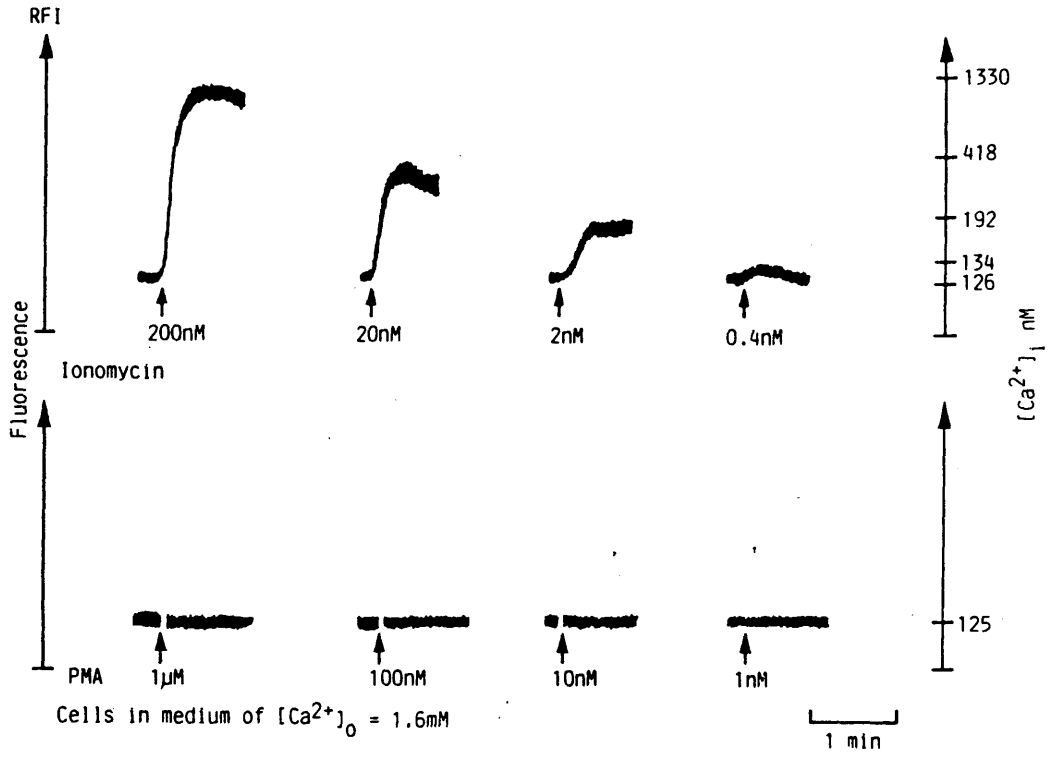


Figure 47.

Representative experimental traces of fluorescence changes in quin2 labelled neutrophils.

2.0ml samples of quin 2 labelled neutrophils (1×10^7 cells/ml), in the presence of 1.6mM external Ca^{2+}, were exposed to agonist at the concentrations indicated. Fluorescence responses were monitored after agonist addition and the appropriate $[Ca^{2+}]_i$ calibration scale is shown on the right of the fluorescence record.

5.2. Concentration-Response Curves for Agonist-Induced Elevation of $[Ca^{2+}]i$.

The extent of the elevation of $[Ca^{2+}]i$ induced by the different stimuli varied between experiments using cells isolated from different donors. The cumulated concentration-response curves for FMLP (EC_{50} value $\approx$ 1nM), PAF (EC_{50} value $\approx$ 5nM), LTB_4 (EC_{50} value $\approx$ 0.6nM) and ionomycin (EC_{50} value $\approx$ 80nM) are shown in figures 48, 49, 50 and 51 respectively.

The three receptor directed agonists were active in the concentration range of 0.1-100nM and the ionophore was active in the range of 1nM-1μM. The maximal increment in $[Ca^{2+}]i$ above basal was comparable for FMLP, PAF and LTB_4 (between 150-250nM) whereas ionomycin was more effective (maximum increment approximately 1400nM).

5.3. The effect of External Calcium on Agonist-induced $[Ca^{2+}]i$ Elevation.

In order to examine the possible sources of this agonist-induced elevation of $[Ca^{2+}]i$, I compared the rise in $[Ca^{2+}]i$ in cells suspended in the presence and absence of external calcium. Figure 52 depicts one such experiment; cells were either suspended in buffer containing 1.6mM Ca^{2+} or in the absence of Ca^{2+} but in the presence of the calcium chelator, EGTA (20mM). When the cells were challenged with agonists in the absence of external Ca^{2+} the response was dramatically reduced. Over several experiments using different concentrations of agonists it was estimated that approximately 80% of the signal arises via influx of external Ca^{2+} and that the remaining 20% presumably arises via mobilisation of internal Ca^{2+}.

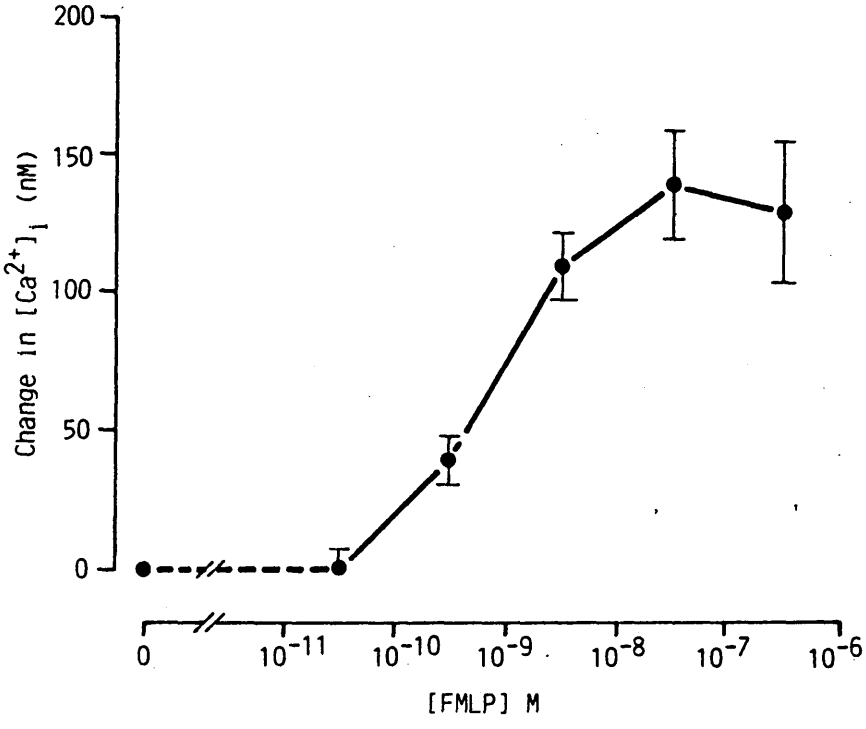


Figure 48.

Concentration-response relationship for FMLP-induced changes in $[Ca^{2+}]i$ in human neutrophils.

2.0ml samples of quin2 labelled neutrophils (1×10^7cells/ml), in the presence of 1.6mM external Ca^{2+}, were exposed to FMLP at the concentrations indicated. The ordinate shows the change in $[Ca^{2+}]i$ in nM above resting value (135 $\pm$ 3nM: n=50) and the results are mean values $\pm$ SEM from nine separate experiments using neutrophils isolated from different donors.

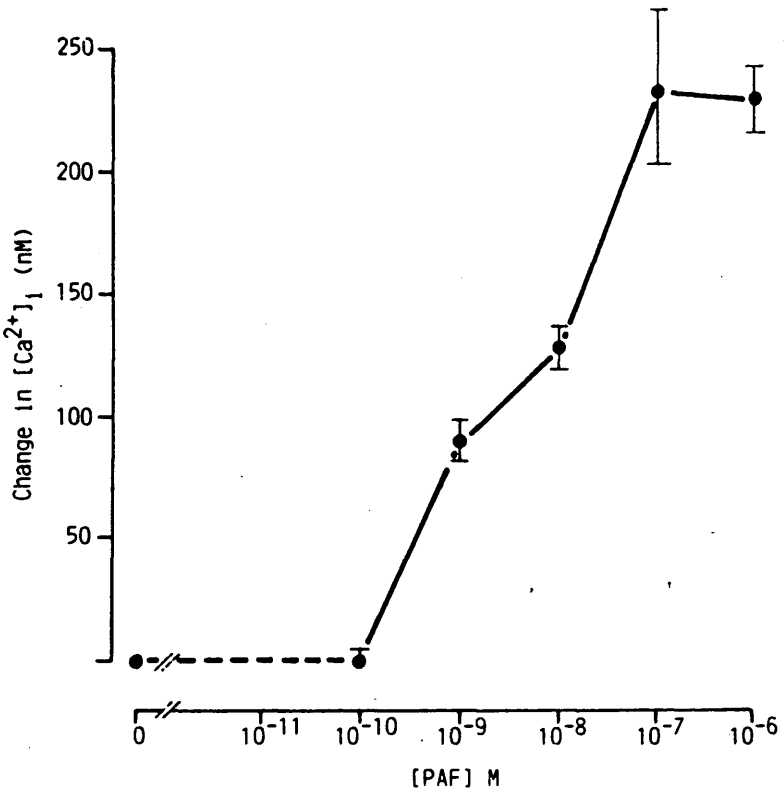


Figure 49.

Concentration-response relationship for PAF-induced changes in $[Ca^{2+}]_i$ in human neutrophils.

2.0ml samples of quin2 labelled neutrophils (1 x 10^7cells/ml), in the presence of 1.6mM external Ca^{2+}, were exposed to PAF at the concentrations indicated. The ordinate shows the changes in $[Ca^{2+}]_i$ in nM above resting value (135 $\pm$ 3nM: n=50) and the results are mean values $\pm$ SEM from five separate experiments using neutrophils isolated from different donors.

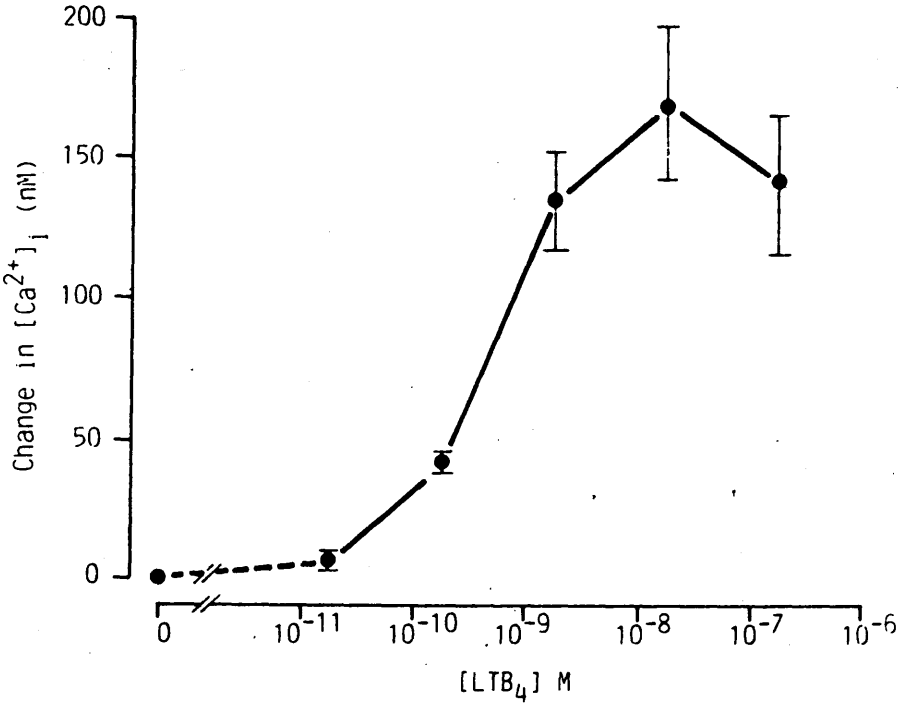


Figure 50.

Concentration-response relationship for LTB_4-induced changes in $[Ca^{2+}]i$ in human neutrophils.

2.0ml samples of quin2 labelled neutrophils (1 x 10^7cells/ml), in the presence of 1.6mM external Ca^{2+}, were exposed to LTB_4 at the concentrations indicated. The ordinate shows the change in $[Ca^{2+}]i$ in nM above resting value (135 $\pm$ 3nM: n=50) and the results are mean values $\pm$ SEM from five separate experiments using neutrophils isolated from different donors.

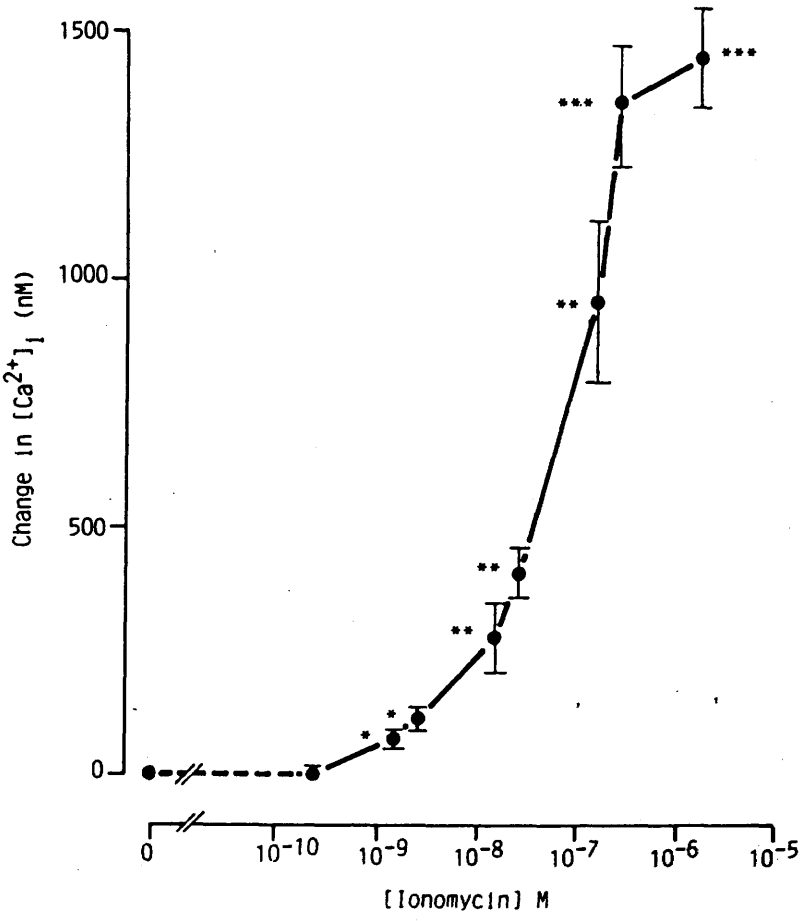


Figure 51.

Concentration-response relationship for ionomycin-induced changes in $[Ca^{2+}]_i$ in human neutrophils.

2.0ml samples of quin2 labelled neutrophils (1×10^7 cells/ml), in the presence of 1.6mM external Ca^{2+}, were exposed to ionomycin at the concentrations indicated. The ordinate shows the change in $[Ca^{2+}]i$ in nM above resting value (135 ± 3nM: n=50) and the results are mean values ± SEM from four separate experiments using neutrophils isolated from different donors. Analysis of data: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

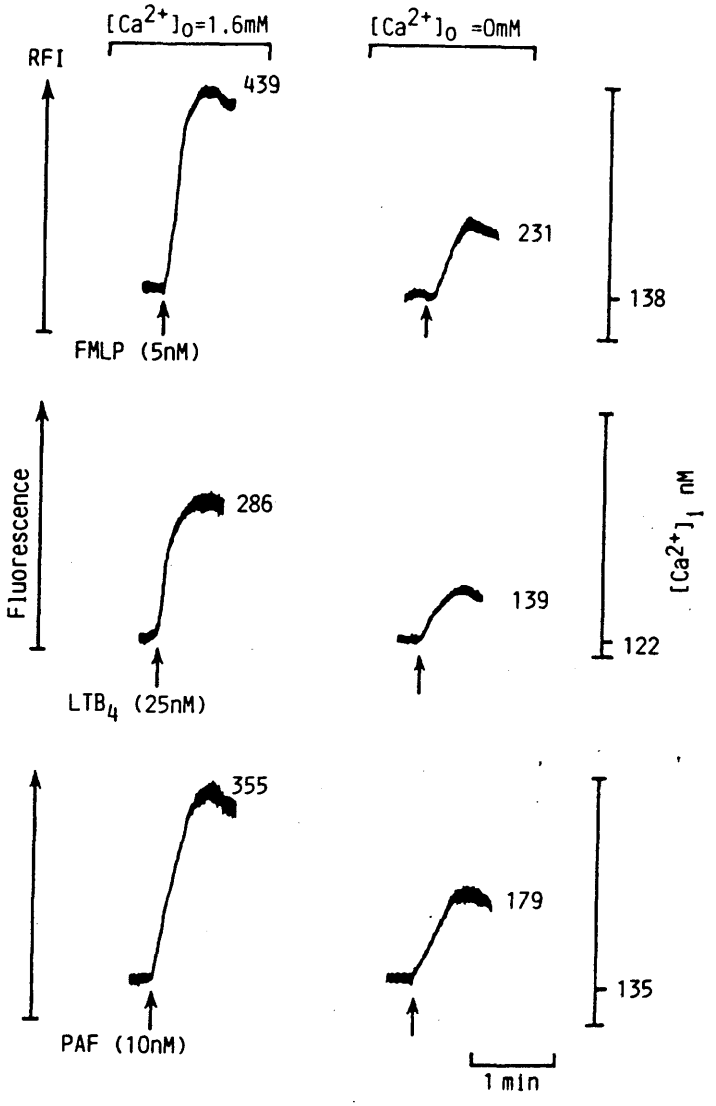


Figure 52.

Representative traces of fluorescence changes in quin2 labelled neutrophils demonstrating the effect of external calcium on agonist-induced $[Ca^{2+}]i$ elevation.

2.0ml samples of quin2 labelled neutrophils (1×10^7 cells/ml), were suspended in medium containing either 1.6mM or 0 (in the presence of 20mM EGTA) external calcium. The cells were then exposed to agonists at the concentrations indicated and changes in fluorescence were monitored. The $[Ca^{2+}]i$ calibration scale is shown on the right of the fluorescence record.

6. Agonist-Induced Phosphoinositide Hydrolysis.

Although previous studies have examined the effects of various agonists, especially FMLP, on phosphoinositide metabolism in neutrophils of other species (Rubin et al., 1981), characterisation of these effects on human neutrophils (Dougherty et al., 1984) is by no means complete. Whilst there is some evidence that receptors for PAF and LTB_4 on neutrophils are coupled to a similar transduction process (namely phosphoinositide hydrolysis and Ca^{2+} flux) this matter remains to be fully established (Bradford & Rubin, 1985; Naccache et al., 1985a). Indeed Volpi et al., (1984) reported that LTB_4 could mobilise calcium without the breakdown of phosphoinositides and the production of PtdA in rabbit neutrophils.

Accordingly, this section is concerned with investigating the effects of FMLP, PAF, LTB_4, ionomycin and PMA on PtdA, PtdIns, PIP and PIP_2 turnover to ascertain whether phosphoinositide hydrolysis serves as a/the transduction process by which these agonists generate second messenger molecules to influence human neutrophil function.

I began by examining the time course of PtdA formation using $[^{32}P]$-orthophosphate prelabelled neutrophils and concentrations of agonists that were maximally effective at eliciting aggregation, degranulation and elevation of $[Ca^{2+}]i$.

Preliminary studies established that neutrophils prepared under conditions of the radio-labelling procedures retained responsiveness in terms of agonist-induced cellular responsiveness. PtdA is the phosphorylated product of 1,2-diacylglycerol (DAG) hence this lipid acts as an indirect measure of phosphoinositide metabolism. After having established the optimal time for $[^{32}P]$-PtdA production, I examined the concentration-response curves for each agonist. For,

perhaps, a more direct index of phosphoinositide hydrolysis, I looked at the effects of various agonists on $[^{32}P]$-PtdIns, $[^{32}P]$-PIP and $[^{32}P]$-PIP_2 levels, again in neutrophils prelabelled with $[^{32}P]$-orthophosphate.

6.1. Effect of FMLP on neutrophil phosphoinositide metabolism.

Figure 53 depicts the time course of FMLP (1μM)-induced $[^{32}P]$-PtdA formation in human neutrophils. In all cases the levels of $[^{32}P]$-PtdA did not change significantly over the duration of the experiment in unstimulated neutrophils. FMLP (1μM) produced peak stimulation of $[^{32}P]$-PtdA formation within 2 minutes of agonist addition, reaching approximately a 2 fold increase above basal, which then begins to decrease towards unstimulated levels. Having established that peak stimulation of $[^{32}P]$-PtdA formation occurs at 2 minutes, I began examining the concentration-response curve for the chemotactic tripeptide at this time point (figure 54). Stimulation of $[^{32}P]$-PtdA formation was observed at a threshold concentration of 1-10nM, with maximum stimulation at 100nM-1μM. From the cumulated data maximum stimulation of $[^{32}P]$-PtdA formation was approximately 200% of vehicle control with an EC_{50} value of $\approx$50nM.

The final index of FMLP-induced phosphoinositide metabolism monitored, was the effect of the agonist (1μM) on $[^{32}P]$-PtdIns, $[^{32}P]$-PIP and $[^{32}P]$-PIP_2 levels; the results are shown on figure 55. There was a transient decrease in $[^{32}P]$-PtdIns (between 0-120 seconds) and $[^{32}P]$-PIP (between 0-60 seconds) levels and a more persistent decrease in $[^{32}P]$-PIP_2 (the time course for $[^{32}P]$-PtdA is included for comparison).

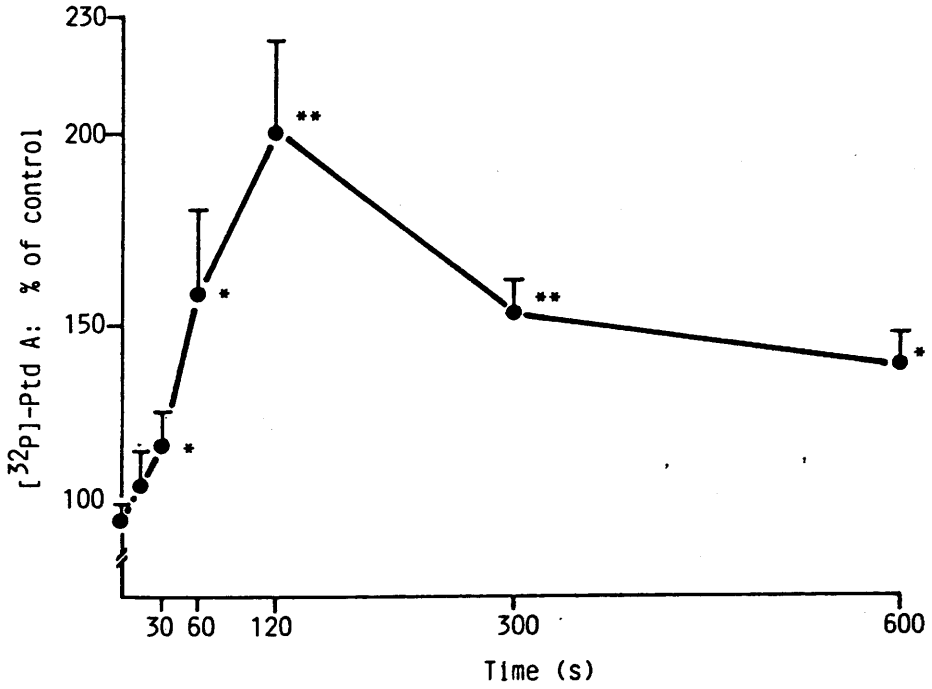


Figure 53.

Time course of FMLP (1μM)-induced $[^{32}P]$-PtdA formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1μM FMLP and the levels of $[^{32}P]$-PtdA measured at the times indicated. The results are the mean values ± SEM for three experiments, each performed in triplicate. Analysis of data: $*P < 0.05$; $**P < 0.01$.

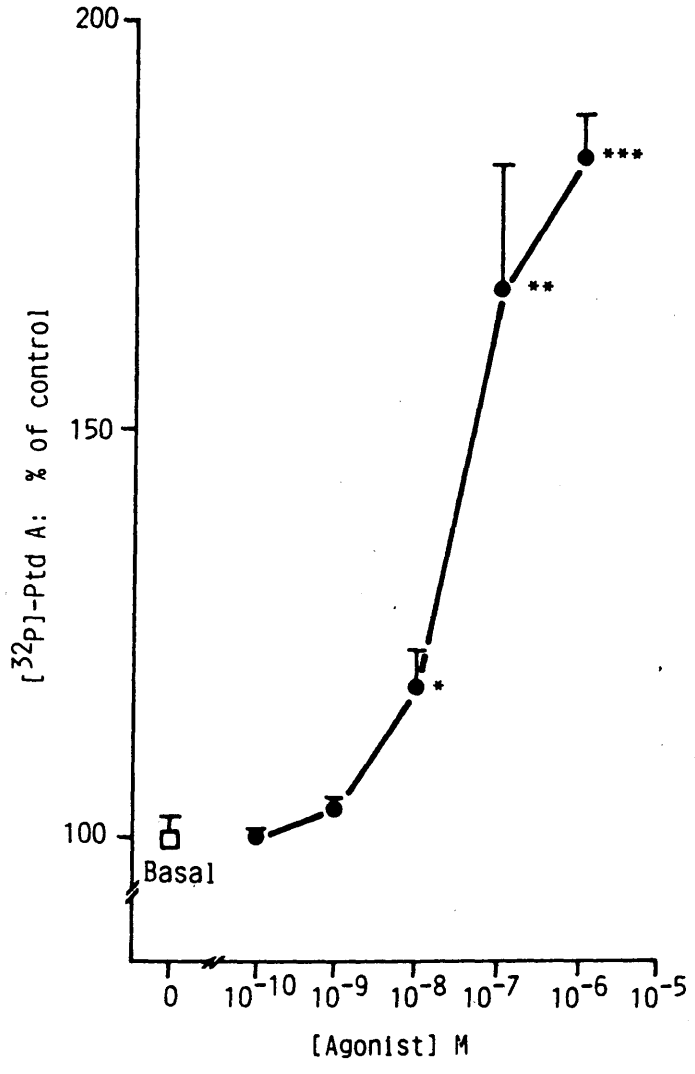


Figure 54.

Concentration-response curve for FMLP-induced $[^{32}P]$- formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to FMLP at the concentrations indicated. $[^{32}P]$-PtdA levels were measured 120 seconds after agonist addition. The results are mean values ± SEM for between three and eight experiments, each performed in triplicate. Analysis of data: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

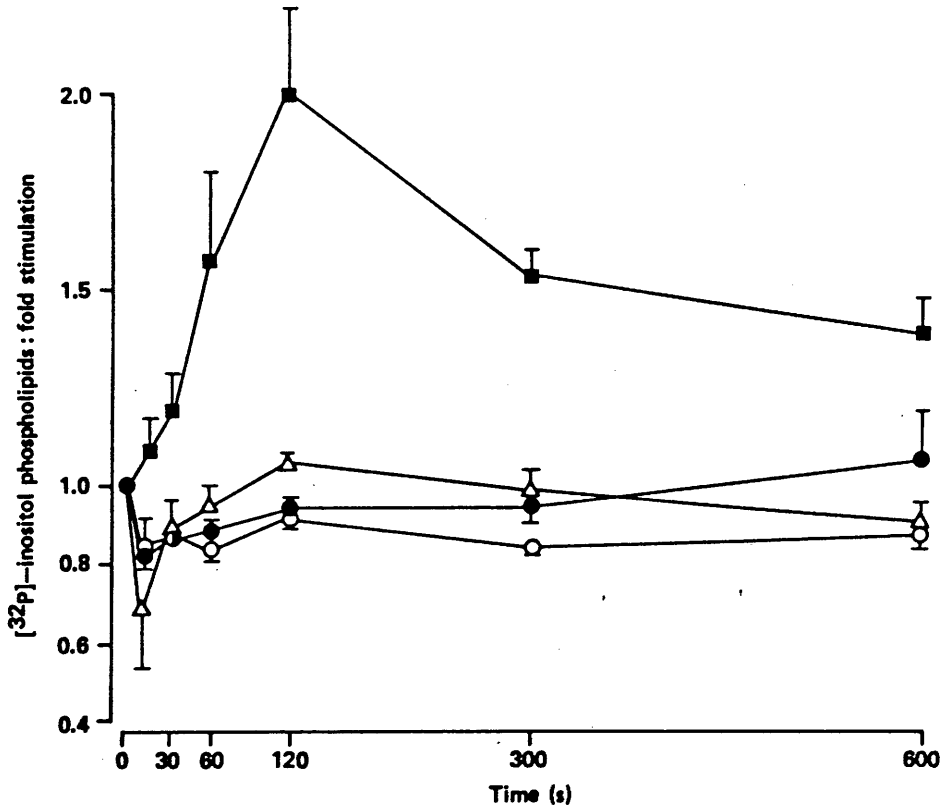


Figure 55.

Time course of FMLP (1uM)-induced changes in the levels of $[^{32}P]$-phospholipids in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1uM FMLP and the levels of $[^{32}P]$-PtdA (■), $[^{32}P]$-PtdIns (●), $[^{32}P]$-PIP (△) and $[^{32}P]$-PIP_2 (○) measured at the times indicated. The results are the mean values ± SEM for between two and five experiments, each performed in triplicate.

6.2. Effect of PAF on neutrophil phosphoinositide metabolism.

The time course for $[^{32}P]$-PtdA formation induced by PAF (1.8μM) (figure 56) is similar to the one observed with FMLP, in that maximal $[^{32}P]$-PtdA was reached at 2 minutes although it was only about a 60% increase above basal.

Looking at the concentration-response curve (figure 57), stimulation of $[^{32}P]$-PtdA formation was observed at a threshold concentration of 1.8-18nM, with maximum stimulation (160% of saline control) at 1.8-18μM with an EC_{50} value of $\approx$500nM.

Finally, there was a transient but non-significant decrease in $[^{32}P]$-PIP and $[^{32}P]$-PIP_2 levels (both at 15 seconds) with no change in $[^{32}P]$-PtdIns levels using a PAF concentration of 1.8μM (figure 58).

6.3. Effect of LTB_4 on neutrophil phosphoinositide metabolism.

When investigating the effect of LTB_4 on phosphoinositide hydrolysis a completely different picture was observed. I began by examining the time course for $[^{32}P]$-PtdA formation using a high concentration of LTB_4 (2.5μM) and could not observe any change in the levels of $[^{32}P]$-PtdA (figure 59; lower panel). I then looked at the lower concentration of 1.25nM and still no significant change in $[^{32}P]$-PtdA levels were detected (figure 59; upper panel). In order to check the viability of the cells, when examining LTB_4, I used FMLP to prove that the neutrophils were indeed responsive.

This phenomenon was investigated further by examining the concentration-response curve for this arachidonic acid metabolite (figure 60). LTB_4 was ineffective at eliciting $[^{32}P]$-PtdA at any concentration up to 2.5μM. Likewise, LTB_4 (1.5μM) failed to evoke

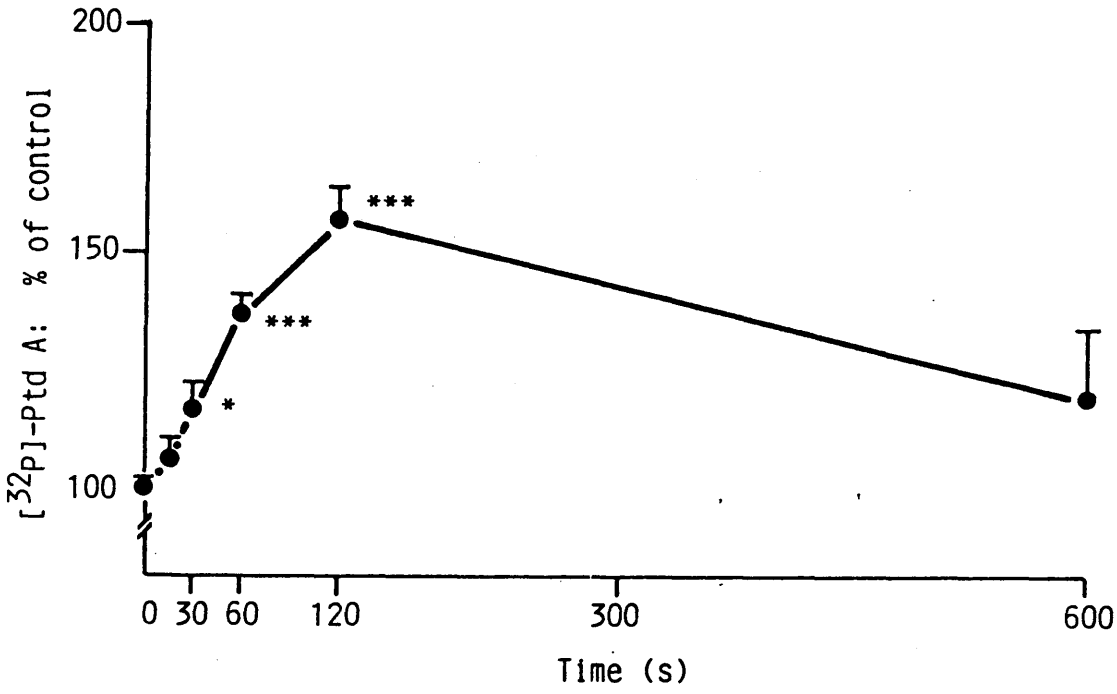


Figure 56.

Time course of PAF (1.8μM)-induced $[^{32}P]$-PtdA formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1.8μM PAF and the levels of $[^{32}P]$-PtdA measured at the times indicated. The results are the mean values ± SEM for four experiments, each performed in triplicate. Analysis of data: $*P < 0.05$; $***P < 0.001$.

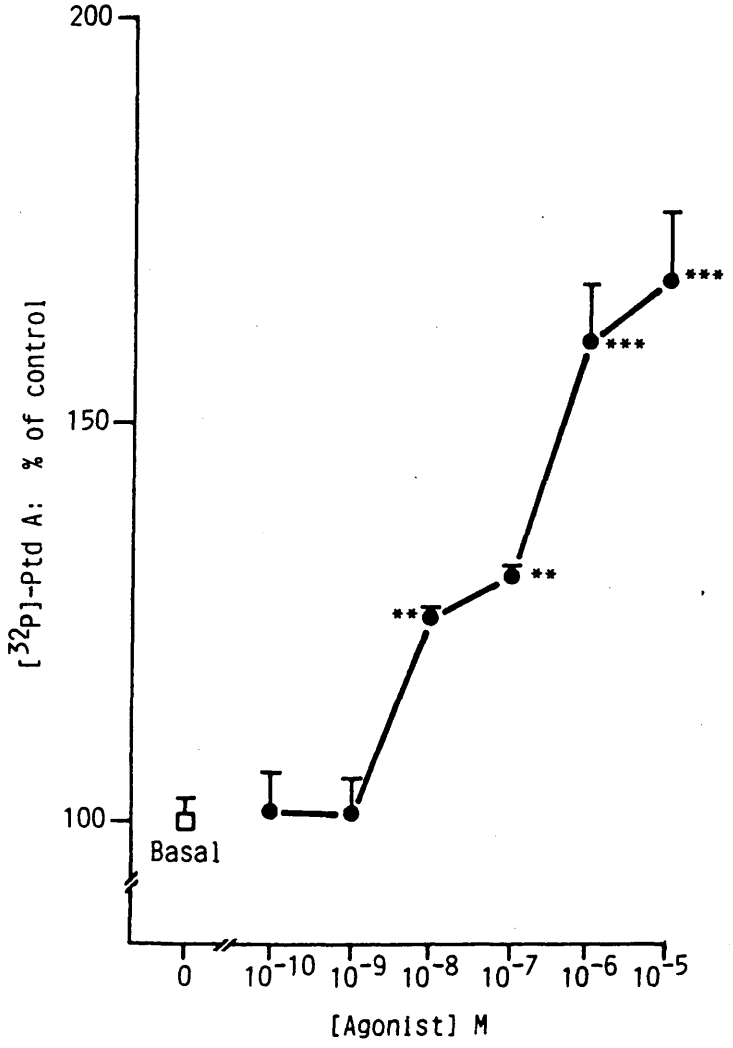


Figure 57.

Concentration-response curve for PAF-induced $[^{32}P]$-formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to PAF at the concentrations indicated. $[^{32}P]$-PtdA levels were measured 120 seconds after agonist addition. The results are mean values ± SEM for between three and five experiments, each performed in triplicate. Analysis of data: $^{**}P < 0.01$; $^{***}P < 0.001$.

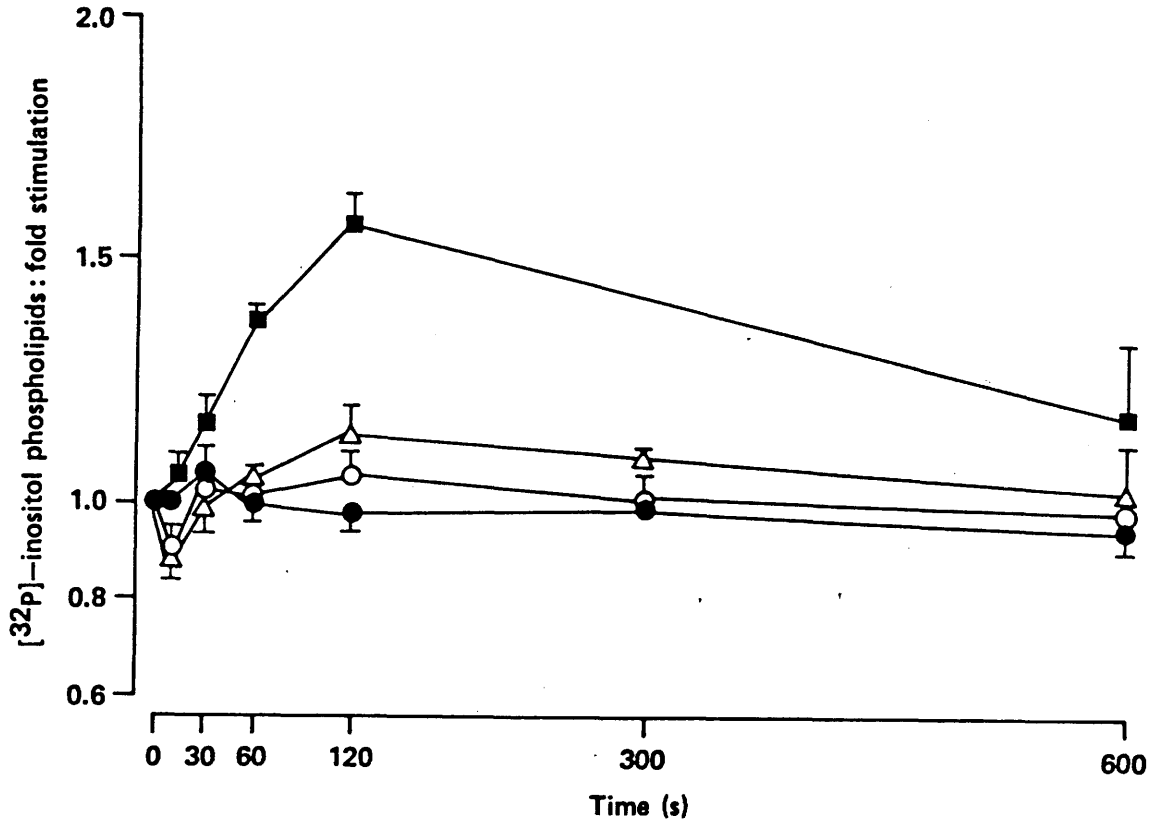


Figure 58.

Time course of PAF (1.8uM)-induced changes in the levels of $[^{32}P]$-phospholipids in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1.8uM PAF and the levels of $[^{32}P]$-PtdA (■), $[^{32}P]$-PtdIns (●), $[^{32}P]$-PIP (△) and $[^{32}P]$-PIP_2 (○) measured at the times indicated. The results are the mean values ± SEM for between two and five experiments, each performed in triplicate.

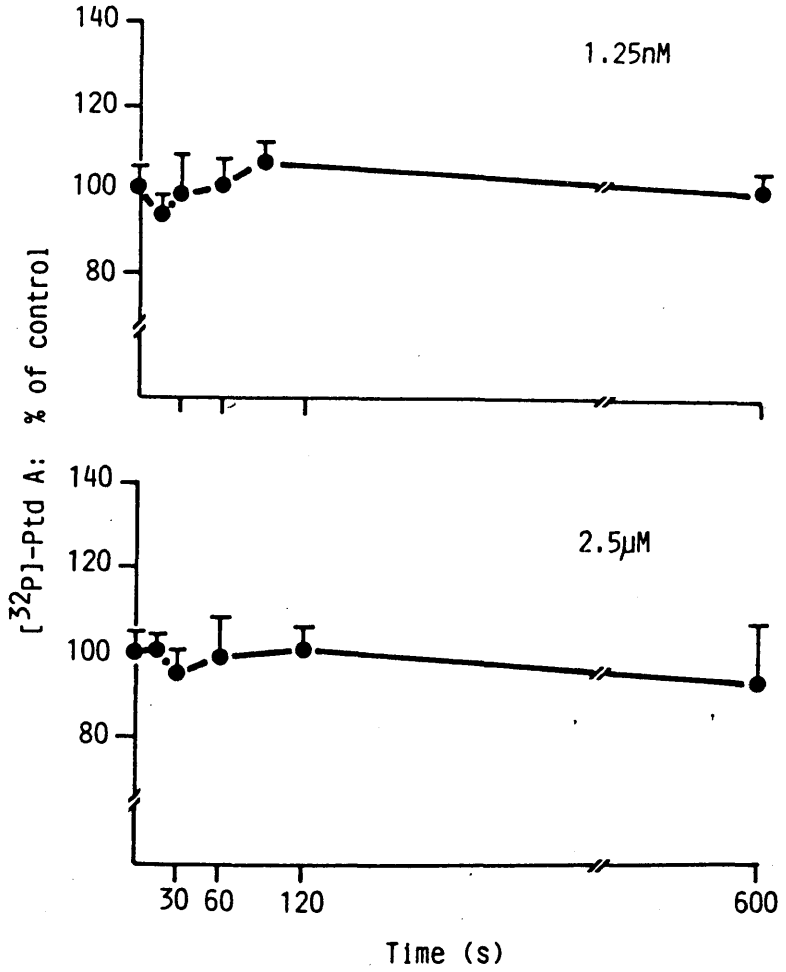


Figure 59.

Time course of LTB_4-induced $[^{32}P]$-PtdA formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1.25nM or 2.5μM LTB_4 and the levels of $[^{32}P]$-PtdA measured at the times indicated. The results are the mean values ± SEM of triplicate determinations. The experiment shown is typical of three similar experiments. All experiments examining the effect of LTB_4 were responsive to FMLP (1μM).

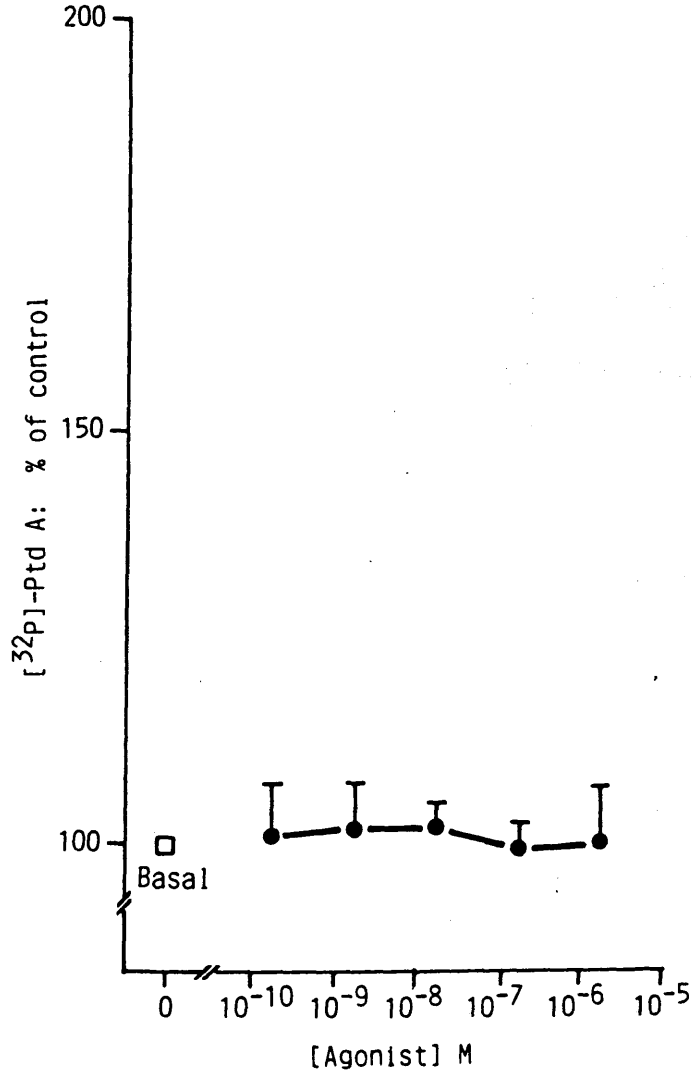


Figure 60.

Concentration-response curve for LTB_4-induced $[^{32}P]$-formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to LTB_4 at the concentrations indicated. $[^{32}P]$-PtdA levels were measured 120 seconds after agonist addition. The results are mean values $\pm$ SEM for three experiments, each performed in triplicate. All experiments examining the effect of LTB_4 were responsive to FMLP (1μM).

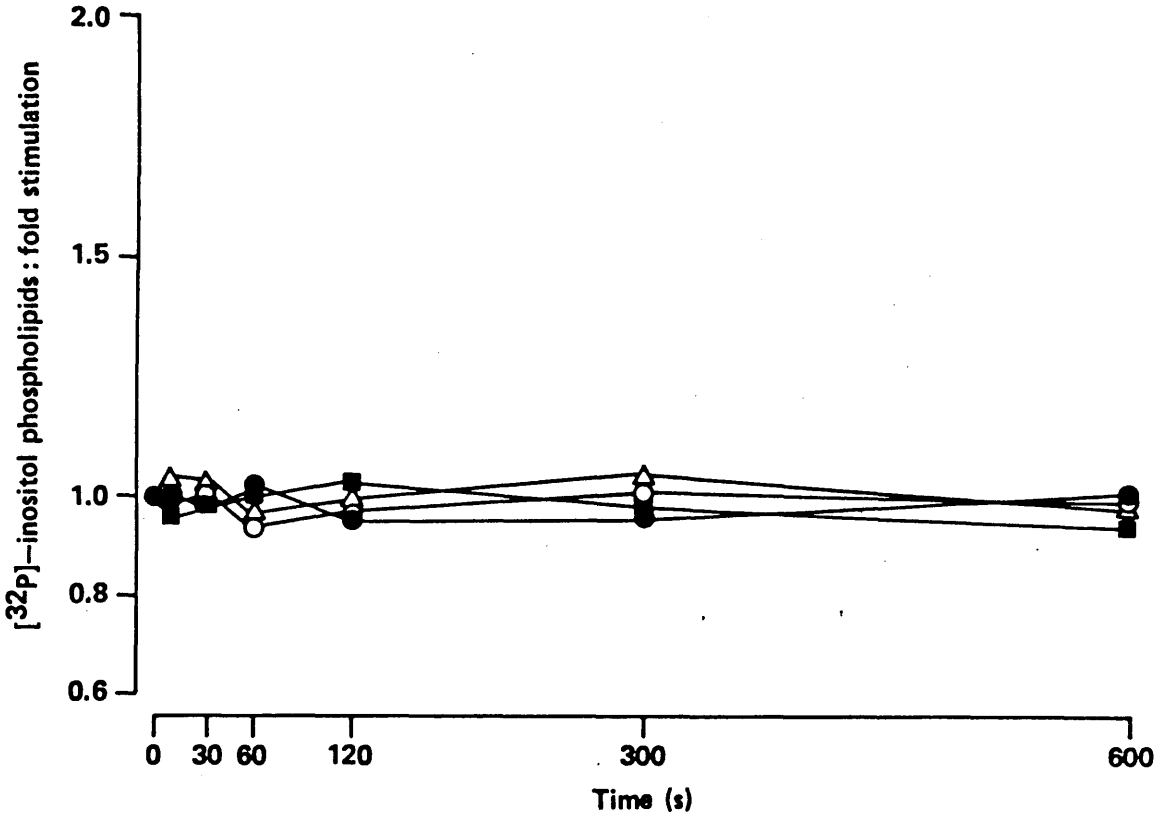


Figure 61.

Time course of LTB_4 (1.5μM)-induced changes in the levels of $[^{32}P]$-phospholipids in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1.5μM LTB_4 and the levels of $[^{32}P]$-PtdA (■), $[^{32}P]$-PtdIns (●), $[^{32}P]$-PIP (△) and $[^{32}P]$-PIP_2 (○) measured at the times indicated. The results are the mean values ± SEM for between two and five experiments, each performed in triplicate.

changes in the levels of $[^{32}P]$-PtdA, $[^{32}P]$-PtdIns, $[^{32}P]$-PIP and $[^{32}P]$-PIP_2 when examined up to 10 minutes following agonist addition (figure 61).

6.4. Effect of ionomycin on neutrophil phosphoinositide metabolism.

The literature is somewhat confusing and contradictory as to whether or not calcium ionophores can elicit phosphoinositide metabolism in neutrophilic leucocytes (Cockcroft et al., 1980). However when I examined the time course for ionomycin (1μM)-induced PtdA formation (figure 62) the ionophore did augment $[^{32}P]$-PtdA levels, although the pattern is different from that observed with FMLP and PAF. Maximal levels of PtdA formation (600% of control) was reached at later time points of 5-10 minutes. In order to make a direct comparison with the other agonist, a time point of 2 minutes was chosen to perform the concentration-response curve (figure 63) for ionomycin-induced $[^{32}P]$-PtdA formation. Maximal production of $[^{32}P]$-PtdA (approximately 380% of control) at 2 minutes was induced by 10-50μM ionomycin with an EC_{50} of $\approx$ 1μM. There was a transient reduction in $[^{32}P]$-PIP (not significant) and a more prolonged decrease in the levels of $[^{32}P]$-PtdIns (not significant) and $[^{32}P]$-PIP_2 (only significant at later time points) (figure 64).

6.5. Effect of PMA on neutrophil phosphoinositide metabolism.

The phorbol ester class of compound has been shown to stimulate human neutrophils by direct activation of protein kinase C and therefore should bypass receptor mediated phosphoinositide hydrolysis. The phorbol ester, PMA, when examined (1μM) had no significant effect, at all time points, on $[^{32}P]$-PtdA, $[^{32}P]$-PtdIns, and $[^{32}P]$-PIP_2 levels (figure 65). This suggests that, as expected, PMA does

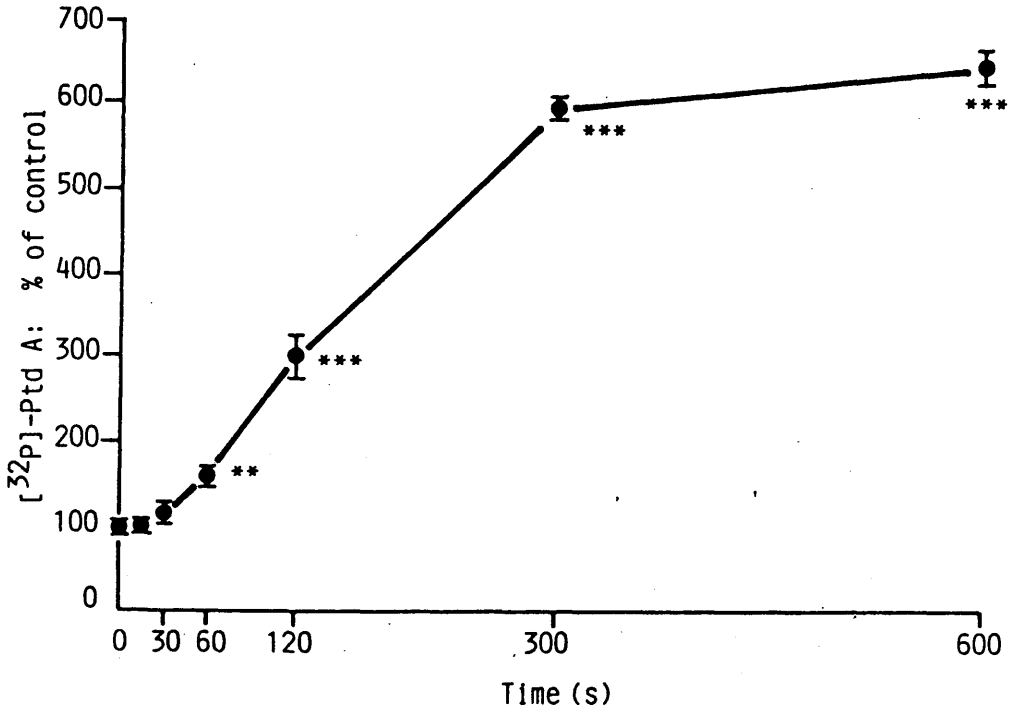


Figure 62.

Time course of ionomycin (1μM)-induced $[^{32}P]$-PtdA formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1μM ionomycin and the levels of $[^{32}P]$-PtdA measured at the times indicated. The results are the mean values + SEM for three experiments, each performed in triplicate. Analysis of data: $^{**}P < 0.01$; $^{***}P < 0.001$.

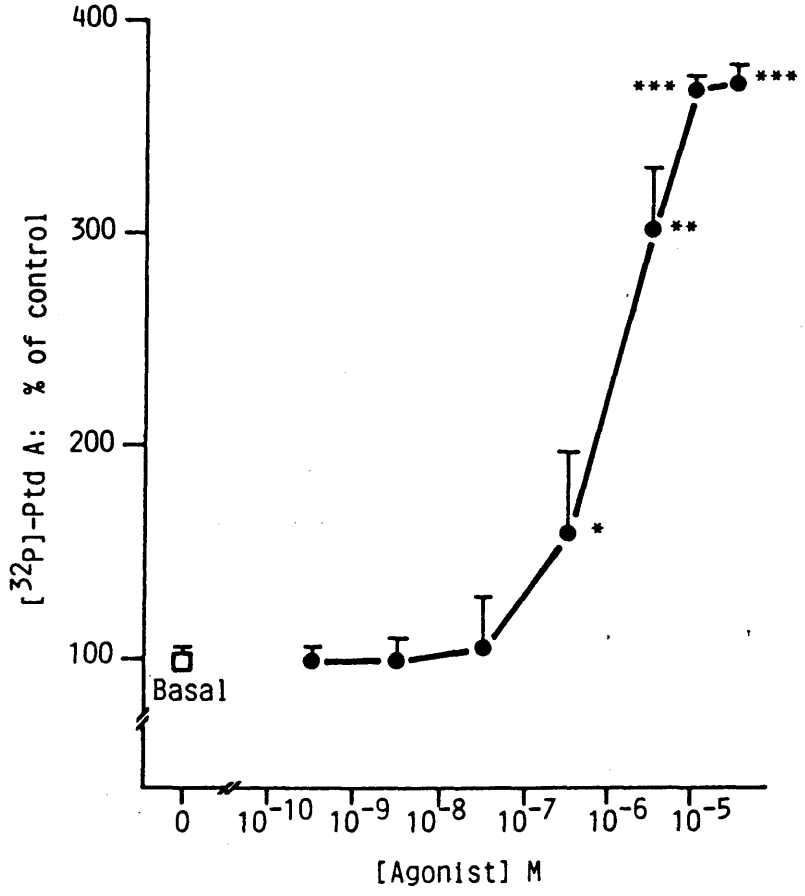


Figure 63.

Concentration-response curve for ionomycin-induced $[^{32}P]$-formation in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to ionomycin at the concentrations indicated. $[^{32}P]$-PtdA levels were measured 120 seconds after agonist addition. The results are mean values + SEM for three experiments, each performed in triplicate. Analysis of data: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

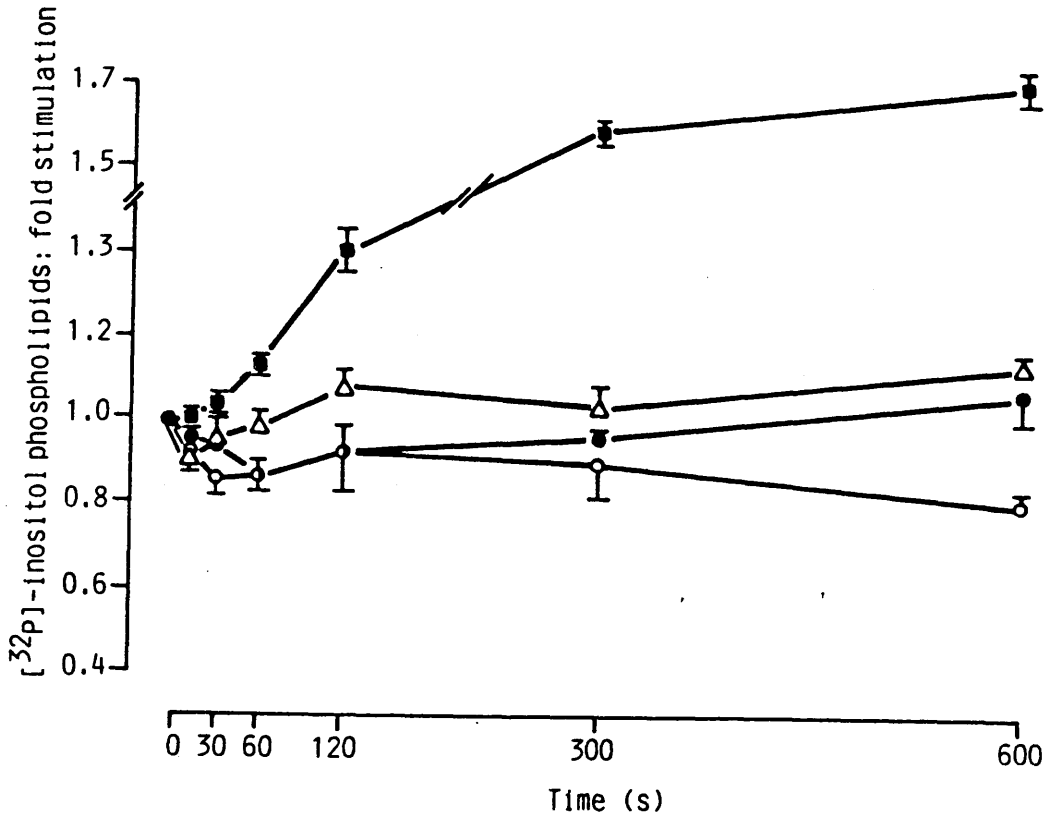


Figure 64.

Time course of ionomycin (1μM)-induced changes in the levels of $[^{32}P]$-phospholipids in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1μM ionomycin and the levels of $[^{32}P]$-PtdA (■), $[^{32}P]$-PtdIns (●), $[^{32}P]$-PIP (△) and $[^{32}P]$-PIP_2 (○) measured at the times indicated. The results are the mean values ± SEM for between two and five experiments, each performed in triplicate.

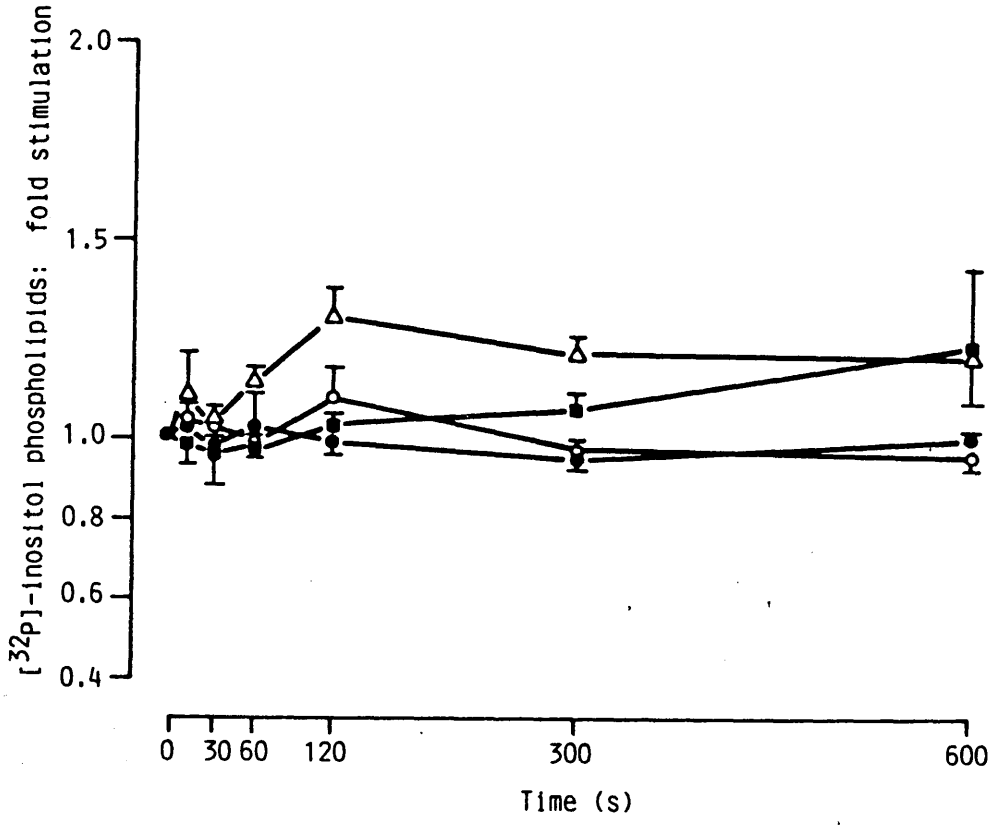


Figure 65.

Time course of PMA (1μM)-induced changes in the levels of $[^{32}P]$-phospholipids in human neutrophils.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were exposed to 1μM PMA and the levels of $[^{32}P]$-PtdA (■), $[^{32}P]$-PtdIns (●), $[^{32}P]$-PIP (△) and $[^{32}P]$-PIP_2 (○) measured at the times indicated. The results are the mean values + SEM for between two and five experiments, each performed in triplicate.

not stimulate phospholipase C activity, however, since PMA does cause a small rise in $[^{32}P]$-PIP, this may be indicative of some effect by PMA on phosphoinositide metabolism.

6.6. Effect of External Calcium on Agonist-Induced $[^{32}P]$-PtdA formation.

All my previous studies on $[^{32}P]$-PtdA formation have been performed with cells suspended in buffer containing 1.6mM Ca^{2+}. It was therefore interesting to examine the role this external calcium had on agonist-induced $[^{32}P]$-PtdA formation. The histogram (figure 66) shows the effect of 1μM FMLP, 1.8μM PAF and 1μM ionomycin on PtdA formation in the presence (1.6mM $[Ca^{2+}]o$) and absence (no $[Ca^{2+}]o$ and in the presence of 20mM EGTA) of external calcium. There was no significant difference in levels of $[^{32}P]$-PtdA formation elicited by FMLP (160-180% of control) and PAF (160-180% of control) in the presence and absence of external calcium. $[^{32}P]$-PtdA production induced by ionomycin in 1.6mM Ca^{2+} was almost reduced to basal level in the presence of the calcium chelator.

This therefore suggests that receptor directed phospholipase C activation occurs independently of the presence of external calcium. However, the data from ionomycin suggests that PtdA formation induced by the calcium ionophore requires external calcium possibly via activation of a Ca^{2+}-dependent phospholipase C (Cockcroft et al., 1981) or perhaps indicative of another molecular mechanism for PtdA production.

7. Cumulated Concentration-Response Curves.

When the data for each agonist are scaled to the same maximum response, a comparison of the concentration-dependence of the agonist

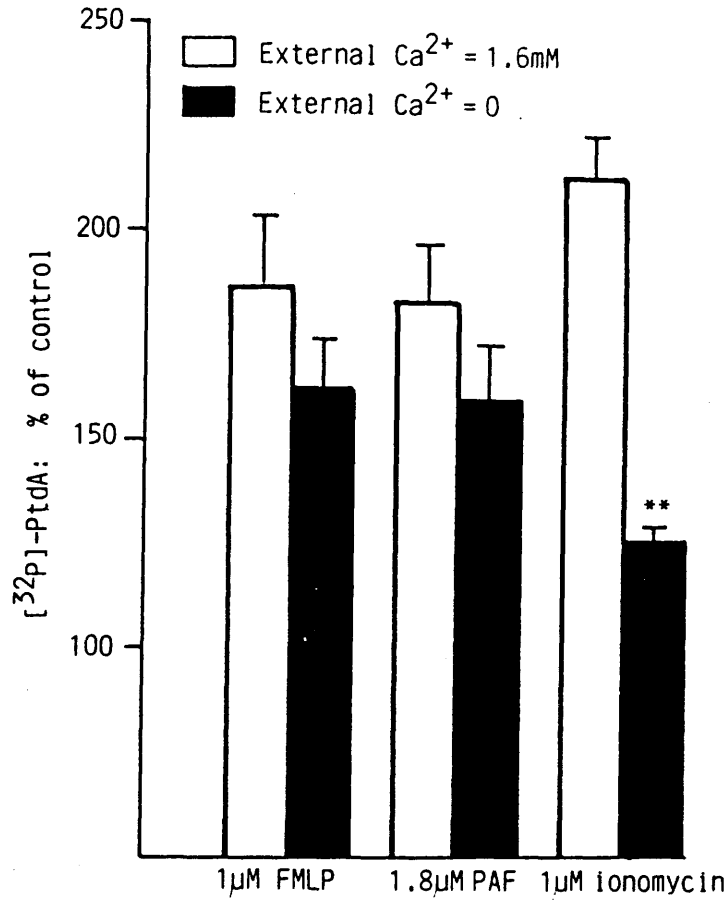


Figure 66.

<u>The effect of external calcium on agonist-induced $[^{32}P]$-PtdA formation.</u>

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were suspended in medium containing either 1.6mM or 0 (in the presence of 20mM EGTA) external calcium. The cells were then exposed to an agonist for 120 seconds, at which time $[^{32}P]$-PtdA levels were measured. The results are mean values + SEM for between three and four experiments, each performed in triplicate. Analysis of data: $^{**}P < 0.01$.

-induced transduction processes and functional responses can yield useful information on possible cause and effect relationships between the events.

7.1. FMLP.

Figure 67 gives the cumulated data for FMLP-induced human neutrophil activation. Each neutrophil function is expressed as a percentage of the maximal response induced by the chemotactic tripeptide plotted against increasing concentrations of agonist. The EC_{50} values for FMLP-induced elevation of $[Ca^{2+}]i$, aggregation, NAG release, lysozyme release and $[^{32}P]$-PtdA formation were approximately 1nM, 30nM, 50nM, 50nM and 50nM respectively.

FMLP evoked an elevation of neutrophil cytoplasmic-free calcium at concentrations significantly lower than were required to elicit aggregation, enzyme release and $[^{32}P]$-PtdA formation.

7.2. PAF.

A similar pattern was observed when PAF was examined (figure 68). The EC_{50} values for PAF-induced elevation of $[Ca^{2+}]i$, aggregation, NAG release, lysozyme release and $[^{32}P]$-PtdA formation were approximately 5nM, 1µM, 70nM, 80nM and 500nM respectively. All PAF-induced responses, including $[^{32}P]$-PtdA formation, lie to the right of the curve for PAF-induced elevation of $[Ca^{2+}]i$. Once again, $[^{32}P]$-PtdA formation is produced in a similar concentration range as the cellular responses, although aggregation seems to lie marginally further to the right.

7.3. LTB_4.

Figure 69 shows the concentration-dependence of LTB_4-induced

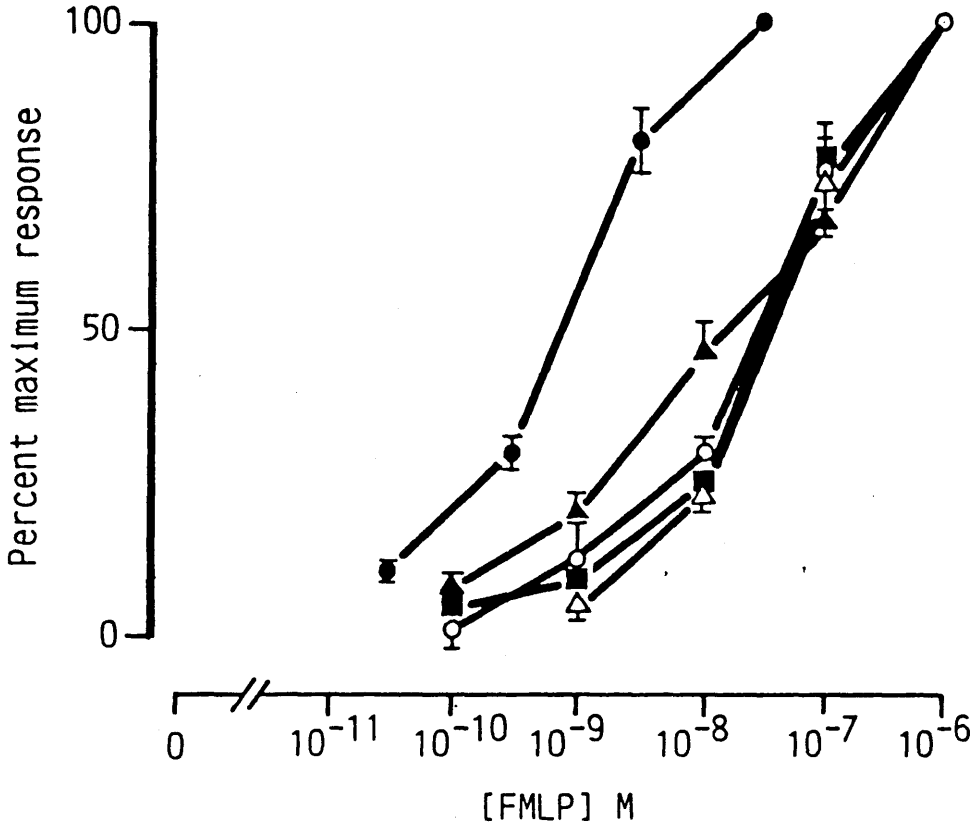


Figure 67.

Cumulated concentration-response curves for FMLP-induced human neutrophil activation.

Each neutrophil function; $[Ca^{2+}]i$ elevation (●), aggregation (▲), NAG release (■), lysozyme release (○) and $[^{32}P]$-PtdA formation (△) is expressed as a percentage of the maximal response elicited by FMLP.

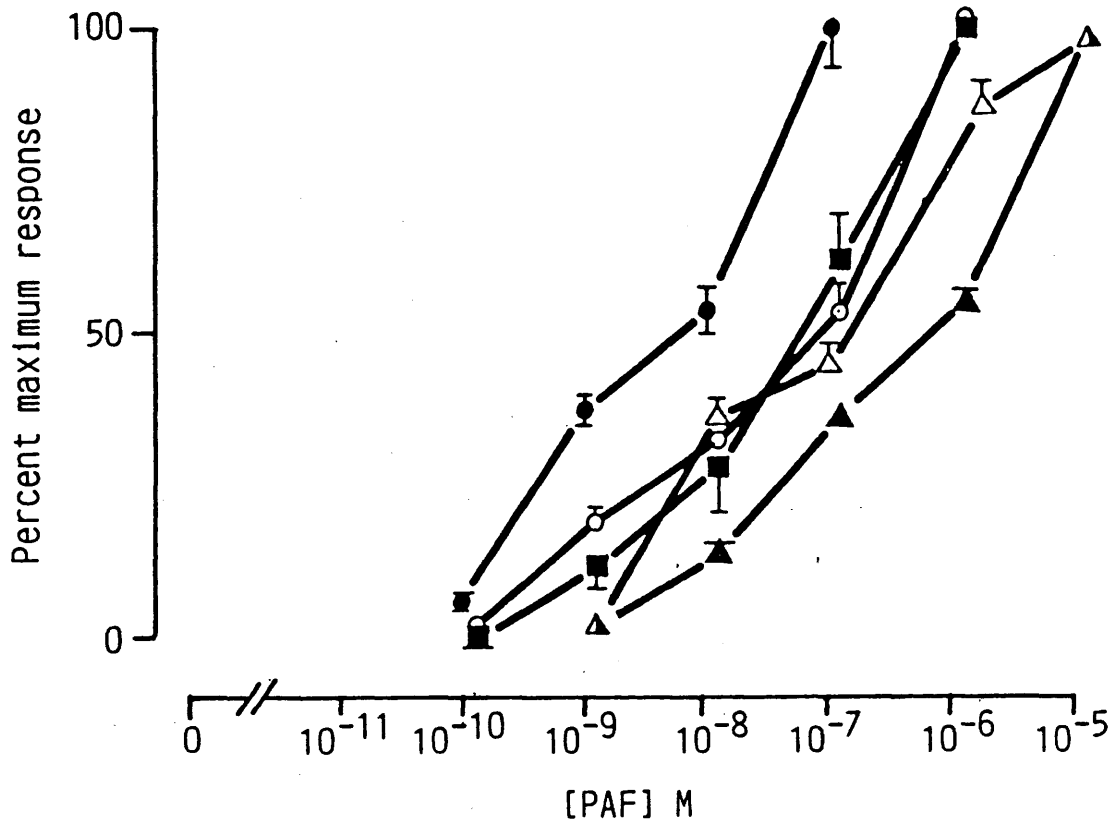


Figure 68.

Cumulated concentration-response curves for PAF-induced human neutrophil activation.

Each neutrophil function; $[Ca^{2+}]i$ elevation (●), aggregation (▲), NAG release (■), lysozyme release (○) and $[^{32}P]$-PtdA formation (△) is expressed as a percentage of the maximal response elicited by PAF.

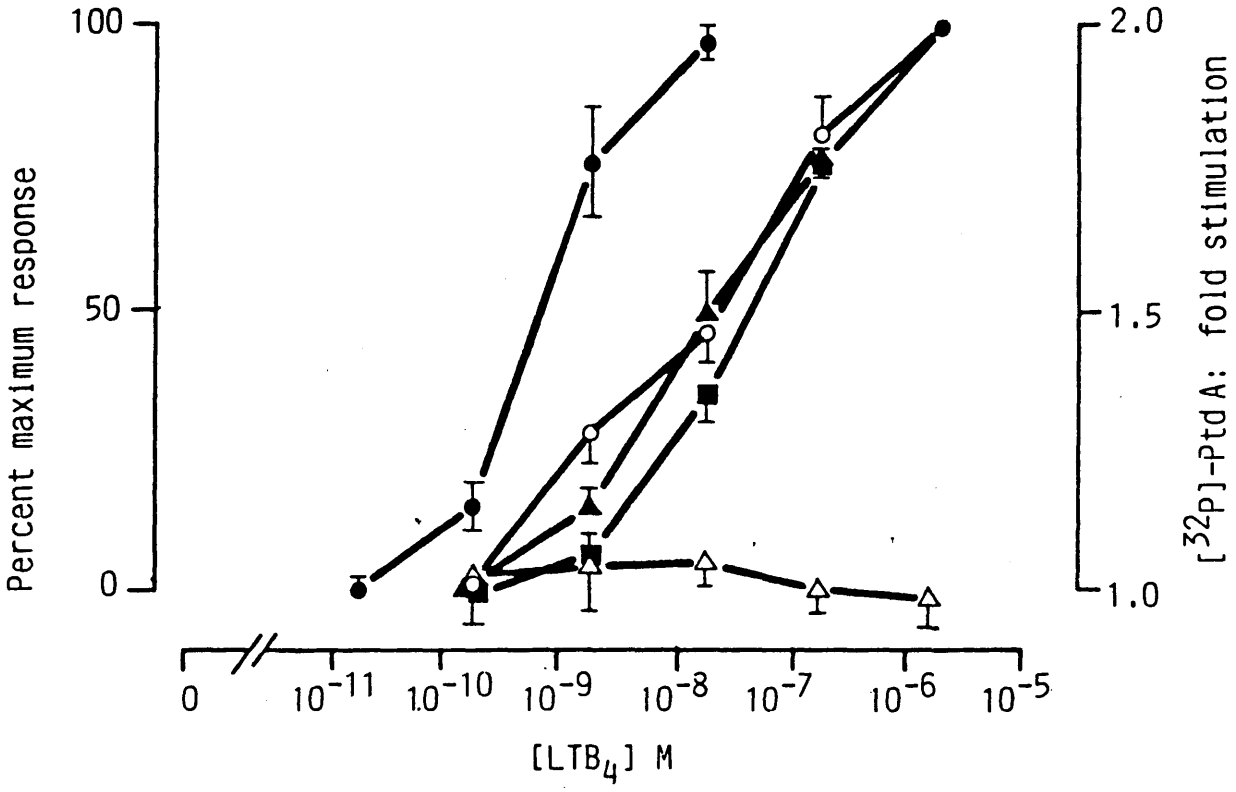


Figure 69.

Cumulated concentration-response curves for LTB_4-induced human neutrophil activation.

Each neutrophil function; $[Ca^{2+}]i$ elevation (●), aggregation (▲), NAG release (■) and lysozyme release (○) is expressed as a percentage of the maximal response elicited by LTB_4.
$[^{32}P]$-PtdA formation (△) is expressed as the fold stimulation above basal.

neutrophil responsiveness. Again, each neutrophil function is expressed as a percentage of the maximal response elicited by LTB_4. However, since it is impossible to express $[^{32}P]$-PtdA formation, in this case as a percentage of the maximum, $[^{32}P]$-PtdA formation is expressed as the fold stimulation above basal. The EC_{50} values for LTB_4-induced elevation of $[Ca^{2+}]i$, aggregation, NAG release and lysozyme release were approximately 0.6nM, 20nM, 50nM and 30nM respectively. LTB_4 is similar to FMLP and PAF in relation to the fact that LTB_4 caused an elevation of $[Ca^{2+}]i$ at concentrations lower than were required to elicit aggregation and enzyme release. It is also clear that in contrast to FMLP and PAF, LTB_4 caused no significant change in $[^{32}P]$-PtdA levels.

7.4. Ionomycin.

The cumulated effect of ionomycin on human neutrophil activation is depicted in figure 70. The results are expressed as a percentage of the maximum response elicited by the ionophore and the EC_{50} values for elevation of $[Ca^{2+}]i$, LTB_4 generation, aggregation, NAG release, lysozyme release and $[^{32}P]$-PtdA formation were approximately 80nM, 20nM, 1μM, 500nM, 400nM and 1μM respectively.

Also included on the right of the graph are the actual levels of $[Ca^{2+}]i$ elevation elicited by the ionophore. This enables the estimation of the approximate $[Ca^{2+}]i$ threshold, defined as the concentration at which a significant increase in cellular/biochemical response was evident. The $[Ca^{2+}]i$ thresholds were calculated to be ~600nM for aggregation, ~500nM for NAG release, ~600nM for lysozyme release, ~800nM for $[^{32}P]$-PtdA formation and ~200nM for LTB_4 biosynthesis.

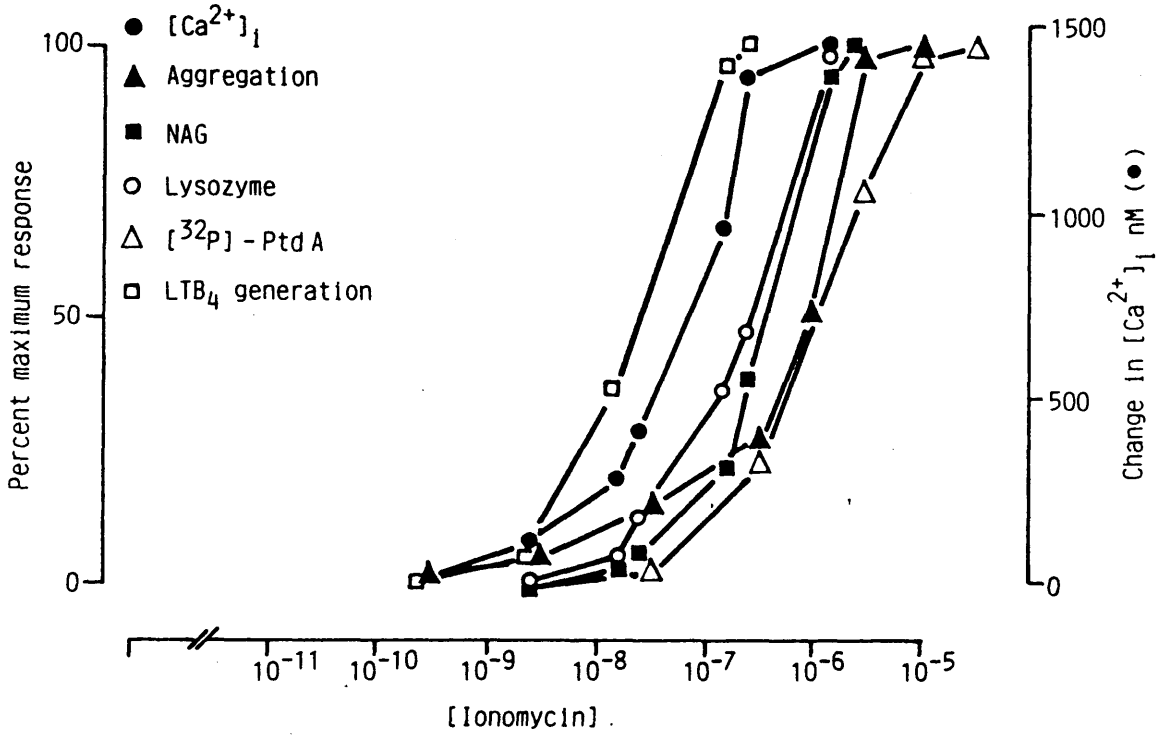


Figure 70.

Cumulated concentration-response curves for ionomycin-induced human neutrophil activation.

Each neutrophil function; $[Ca^{2+}]i$ elevation (●), LTB_4 generation (□), aggregation (▲), NAG release (■), lysozyme release (○) and $[^{32}P]$-PtdA formation (△) is expressed as a percentage of the maximal response elicited by ionomycin. In addition, the right axis gives the $[Ca^{2+}]i$ elevation (●) in nM above basal.

8. Discussion.

The major objective of this particular section was to investigate the relationship between the ultrastructural, functional (aggregation, degranulation and LTB_4 generation) and the more biochemical ($[Ca^{2+}]_i$ elevation and phosphoinositide hydrolysis) responses induced by LTB_4 and PAF in comparison with other neutrophil stimuli. Such an investigation may help elucidate the mechanism(s) of action of LTB_4 and PAF and assist in the understanding of the molecular transduction mechanisms involved in human neutrophil activation.

LTB_4 and PAF are potent lipid activators of human neutrophils and produce a similar but not identical array of responses to those produced by the chemotactic tripeptide, FMLP; the calcium ionophore, ionomycin, and the phorbol ester, PMA. The ultrastructural changes induced by LTB_4 and PAF are broadly similar to those produced by FMLP, with an initial period of general ruffling followed by polarization and lamellipodia formation. There is evidence of increased actin polymerization after neutrophils are exposed to FMLP with skeins of fine filaments traversing lamellipodia and forming sub-plasma bands (Rao & Varani, 1982; White et al., 1982; Howard & Meyer, 1984). Microtubule quantity has variously been described as increased (Goldstein et al., 1973; Boxer et al., 1979; Hoffstein, 1980) or unchanged but with alterations in their length and distribution (Anderson et al., 1982). The absence of microtubules in the lamellipodia is in agreement with previous observations (Davis et al., 1982; Oliver & Berlin, 1982). The polarization seen in response to LTB_4, PAF and FMLP is characteristic of migrating cells and, since the neutrophils were randomly orientated and the cells were not in a chemotactic gradient, the ultrastructural changes observed in unstirred samples probably reflects chemokinesis. A similar pattern

of morphological changes was observed in stirred suspensions of activated neutrophils except that small clumps containing aggregated neutrophils were evident. The clumps contained polarized cells with large agranular lamellipodia generally extending outward from the aggregate. Hoffstein et al., (1982) have reported similar ultrastructural changes with FMLP-induced aggregation of human neutrophils. The remarkable similarity in response in unstirred and stirred neutrophils may indicate that chemokinesis and aggregation are mediated by the same contractile mechanism.

A rapid, reversible and concentration-dependent aggregation of human neutrophils was observed with LTB_4, PAF and FMLP. The arachidonic acid metabolite was by far the least efficacious agonist. However, it was approximately equi-potent with FMLP and both were almost 10 fold more potent than the ether lipid. These data are in accordance with previous investigations (Ford-Hutchinson, 1983; O'Flaherty et al., 1981a). The aggregatory responses elicited by PMA and ionomycin were also concentration-dependent but not reversible. This probably reflects the fact that these agents are not acting upon specific cell surface receptors but causing neutrophil activation by stimulating protein kinase C and directly elevating $\left[Ca^{2+}\right]i$ respectively.

The concentration-dependent release of both NAG (an azurophil granule marker) and lysozyme (an azurophil and specific granule marker) induced by the receptor directed agonists all required the presence of cytochalasin B. Although all three agonists have a similar potency, FMLP releases greater amounts of both enzymes. The degranulation response of ionomycin and PMA are somewhat different from the other stimuli. Ionomycin stimulates the release of both enzymes in the absence of cytochalasin B. PMA is extremely weak at releasing NAG from the azurophil granules and any release is cytochalasin B dependent. However, the phorbol ester is capable of inducing lysozyme secretion in the presence or absence of this fungal metabolite. Other workers have found similar results demonstrating

that PMA is capable of releasing lysozyme but not β-glucuronidase, another azurophil enzyme, from human neutrophils (Estensen et al., 1974; Wright et al., 1977). The reason for this apparent selective release and differential mobilisation of human azurophil and specific granules remains unclear.

Caution, however, must be taken whilst interpreting data using cytochalasin B treated neutrophils. Ultrastructural studies reveal that cytochalasin B causes a distinctive vacuolation within the cytoplasm that is not present in untreated control cells. The addition of agonist to cytochalasin B treated neutrophils causes the formation of a bizarre profile with marked blebbing and little polarization of the granules. Thus, despite claims to the contrary (Chandler et al., 1983; Showell et al., 1982), cytochalasin B has been shown both here and in previous investigations to have a dramatic effect on cell ultrastructure (Zigmond & Hirsch, 1972; Hoffstein, 1981). It should also be recognised that this pharmacological tool produces a wide spectrum of other biological responses which may interfere with the normal functioning of the neutrophil (Chapter 1).

The release of arachidonic acid from membrane phospholipids, principally via activation of phospholipase A_2, is a phenomenon common to a variety of cell types. In human neutrophils arachidonic acid is predominantly acted upon by a calcium-dependent enzyme located in the cytoplasm known as 5-lipoxygenase. The resultant 5HPETE intermediate is sequentially acted upon by a dehydrase followed by a hydrolase enzyme to yield the biologically active LTB_4 (Chapter 2; figure 4). My results show that LTB_4 generation is produced in a concentration-dependent manner by ionomycin and that FMLP and PAF only produced a fraction of the amount of LTB_4 generated by the calcium ionophore.

Indeed, very high concentrations of PAF (18μM) are required for significant production of the arachidonic metabolite. Similar observations have been made by other workers using the calcium ionophore, A23187 (Palmer & Salmon, 1983; McMillan et al., 1985). However, data with more physiological agents are fewer and often contradictory.

Palmer & Salmon, (1983) using human neutrophils reported that serum-treated zymosan or FMLP produced low or barely detectable amounts of LTB_4 which could be potentiated by simultaneous addition of arachidonic acid or only marginally increased by pretreatment of cells with cytochalasin B. Haines et al., (1987) demonstrated that FMLP, heat-aggregated IgG and serum-treated zymosan produce low levels of LTB_4. This arachidonic acid metabolite was only produced in significant amounts when cells are loaded with arachidonic acid in quantities (50uM) that stimulate superoxide production and activate protein kinase C (Badwey et al., 1981; McPhail et al., 1984). Other workers have reported LTB_4 biosynthesis by neutrophils exposed to FMLP, serum-treated zymosan, C5a and monosodium urate (Jubiz et al., 1982; Claesson et al., 1981; Clancy et al., 1983; Ham et al., 1983; Serhan et al., 1984; Williams et al., 1985). Some of these studies, including this investigation, have many different factors or variables which may confuse interpretation of data. For example: contamination of neutrophil preparations with monocytes and other cells; use of high concentrations of neutrophils, arachidonic acid or agonists; reliability of detection techniques, etcetera.

One common feature that all the above studies share is that neutrophils produce large amounts of LTB_4 in response to calcium ionophores and that other inflammatory ligands produce low or barely detectable levels of this arachidonic acid metabolite. An interesting

observation made by Williams et al., (1985) is that there is an intracellular retention of LTB_4 by human neutrophils activated with unopsonized zymosan suggesting that the release of 5-lipoxygenase metabolites may be an event that is regulated separately from their generation. Whether there is a similar intracellular retention of 5 lipoxygenase products by neutrophils produced by other agents is unknown. Therefore, the precise reason for the differences between stimuli remains to be established.

All the receptor directed agonists caused[a] rapid concentration-dependent elevation of neutrophil $\left[Ca^{2+}\right]i$. The calcium ionophore was much more effective than FMLP, PAF and LTB_4 at elevating $\left[Ca^{2+}\right]i$. This may explain why ionomycin can elicit a massive production of LTB_4 whereas FMLP and PAF produce only barely detectable levels. The notable exception was PMA which did not cause a rise in $\left[Ca^{2+}\right]i$ at all concentrations tested; this is in agreement with previous findings (Sha'afi et al., 1983) and consistent with the hypothesis that phorbol esters cause the activation of cells by directly stimulating protein kinase C (Castagna et al., 1982). Studies with the calcium chelator, EGTA, demonstrated that the elevation of $\left[Ca^{2+}\right]i$ induced by FMLP, PAF and LTB_4 derives predominantly, but not exclusively, via influx of external calcium through channels that open as a consequence of receptor occupancy (White et al., 1983b; Naccache et al., 1985b).

The chemotactic tripeptide caused the formation of PtdA and the transient breakdown of PtdIns, PIP and PIP_2. These observed effects of FMLP are most readily explained via receptor linked hydrolysis of phosphoinositides catalysed by phospholipase C. Indeed, that this is so, has been shown both in intact cells (Dougherty et al., 1984) and membrane preparations (Smith et al., 1985). In the case of

PAF, only changes in PtdA are easily demonstrable. This may reflect the action of phospholipase C on phosphoinositides, but equally could be explained either by phospholipase D action on these phospholipids to yield PtdA directly (Kater et al., 1976) or by triglyceride lipase action on triglyceride to yield DAG (Allan & Michell, 1977) which subsequently would be phosphorylated by DAG kinase to produce PtdA. The present studies do not allow us to discriminate between these possibilities. Other investigations have shown that PAF causes phosphoinositide hydrolysis in a number of cell systems including rabbit neutrophil (Naccache et al., 1986) and human platelets (MacIntyre & Pollock, 1983). The breakdown of phosphoinositides and production of PtdA that occurs in neutrophils with calcium ionophores is somewhat confusing (this investigation; Cockcroft et al., 1981; Rubin et al., 1981; Cockcroft, 1984). Such an observation may be explained by the possibility that there exists a Ca^{2+}-dependent phospholipase C in the neutrophil which is activated by calcium ionophores. The fact that FMLP- and PAF-induced PtdA formation is not attenuated in the absence of external Ca^{2+} whereas the same response elicited by ionomycin was almost abolished may suggest that the calcium ionophore is doing something additional in neutrophils. Since the time course for PtdA formation is much slower to peak than FMLP and PAF, it cannot be ruled out that ionomycin could be producing an additional endogenous mediator of neutrophil activation that may stimulate phosphoinositide hydrolysis; for example PAF. PMA did not cause an elevation of PtdA, an observation consistent with the fact that phorbol esters bypass phosphoinositide hydrolysis by substituting for endogenous DAG to activate protein kinase C. The phorbol ester, however, did cause a late and small increase in PIP. Similar observations have been made in human platelets (McNicol, 1986; Halenda & Feinstein, 1984) and in lymphocytes (Boon et al., 1985)

where PMA stimulates the incorporation into PIP and PIP_2. Human neutrophils have been reported to "remodel" membrane phospholipids upon stimulation with PMA. Serhan et al., (1982) showed early (15 seconds) changes in phosphatidylcholine, phosphatidylserine and phosphatidylethanolamine and later (60 seconds) changes in PtdIns in response to the phorbol ester.

At the start of my investigations very little information concerning the possible stimulus-response coupling mechanisms for LTB_4 was available in the literature. Serhan et al., (1984) had proposed that LTB_4 may exert its effect by acting as a Ca^{2+} ionophore and Volpi et al., (1984) had published the first evidence to suggest that LTB_4 could mobilise Ca^{2+} without the breakdown of polyphosphoinositides and the formation of PtdA in rabbit neutrophils. The latter group demonstrated that the addition of FMLP and LTB_4 caused a concentration-dependent rise in $\left[Ca^{2+}\right]i$ as measured by quin2. In addition, they showed that $\left[^{32}P\right]$-prelabelled rabbit neutrophils exposed to the arachidonic acid metabolite at concentrations up to 70nM did not cause any significant breakdown of any of the phosphoinositides or generation of PtdA, arachidonic acid or DAG. Furthermore, the concentration-response curves for FMLP-induced generation of PtdA is shifted to the right when compared with that for calcium mobilisation. They concluded that there was an important qualitative difference between the mechanism of these two chemotactic agents. Bearing in mind these findings, we repeated these investigations using human neutrophils. Our results show that there is a lack of effect of LTB_4, up to concentrations of 2.5μM, on PtdA formation and on the levels of the phosphoinositides. This could indicate, as is the case in rabbit neutrophil, that receptors for this eicosanoid in the human neutrophil are not coupled to inositol lipid hydrolysis.

Two recent reports have demonstrated that LTB_4 can evoke phosphoinositide hydrolysis in guinea pig alveolar macrophages (Holian, 1986) and in human leukaemic HL60 cells differentiated with retinoic acid but not with DMSO (Andersson et al., 1986). This may indicate the existence of sub-types of LTB_4 receptors that could be differentiated according to the transduction process to which they are coupled. It should be noted that α_1 and α_2 adrenoreceptors can be differentiated by such criteria (Berridge, 1985). Alternatively, the ineffectiveness of LTB_4 on the various aspects of phosphoinositide hydrolysis in human (this study) and rabbit (Volpi et al., 1984; Sha'afi et al., 1985) may merely reflect the insensitivity of the assays involved. Indeed, various workers using rabbit neutrophils (Bradford & Rubin, 1985), rat peritoneal polymorphonuclear leucocytes (Mong et al., 1986) and, more recently, human neutrophils (Lew et al., 1987; Smith et al., 1987) prelabelled with $[^3H]$-inositol showed that LTB_4 promotes a rapid accumulation of $Ins(1,4,5)P_3$ suggesting that phosphoinositide hydrolysis may be occurring in these cells. A recent publication by Omann et al., (1987b)using human neutrophils showed that the N-formyl hexapeptide (10nM) caused an elevation of PtdA in a similar manner to my investigations. They also showed, however, that 40nM LTB_4 produced a small transient rise in PtdA, peaking at about 15 seconds which rapidly declines towards basal. The reason for these discrepancies is unknown and whether LTB_4 is actually causing a significant phosphoinositide turnover in neutrophils has not been fully established and awaits clarification. What is certain, however, is that there is a qualitative and quantitative difference between the responses of PAF and FMLP.

When the data for each neutrophil stimulus is scaled to the same maximum response, a comparison of the concentration-dependence of

agonist-induced transduction processes and cellular responses can yield valuable information on the possible cause and effect relationships between events. The concentration-response curves for aggregation and degranulation induced by all three receptor directed agonists, and for PtdA formation induced by FMLP and PAF, lie to the right of the corresponding curves of agonist-induced elevation of $[Ca^{2+}]i$. A similar relationship between FMLP (Rossi et al., 1985) and PAF (Naccache et al., 1986)-induced Ca^{2+} flux and functional responses has also been reported. As the agonist-induced functional responses (i.e. aggregation and degranulation) occur at concentrations of LTB_4, PAF and FMLP greater than those required to evoke maximal elevation of $[Ca^{2+}]i$, it is unlikely that the rise in $[Ca^{2+}]i$ per se mediates these neutrophil reactions. Consequently, if $[Ca^{2+}]i$ is the sole trigger for neutrophil activation, it must stimulate an intracellular amplification sequence that results in functional responses. Alternatively, one must invoke other mediators which act alone or in concert with elevated $[Ca^{2+}]i$ to evoke neutrophil activation. DAG, one of the immediate products of phosphoinositide hydrolysis, would be a prime candidate as such a synergistic or independent mediator. However, as FMLP and PAF induce phosphoinositide hydrolysis at higher concentrations than $[Ca^{2+}]i$ elevation and that LTB_4 can induce neutrophil activation apparently independently of phosphoinositide hydrolysis, there must exist products other than DAG which interact with elevated $[Ca^{2+}]i$ to elicit neutrophil responsiveness.

From the cumulated concentration-response curve for ionomycin-induced neutrophil activation, an estimation of the quantitative relationship between $[Ca^{2+}]i$ and cellular responsiveness may be determined. Rink et al., (1982) using quin2 loaded platelets stimulated with ionomycin revealed apparent Ca^{2+} thresholds (defined as the $[Ca^{2+}]i$ concentration at which a significant response was evident) for shape-change, 5HT release

and aggregation of approximately 500nM, 800nM and 2μM respectively. A similar analysis has not been made with human neutrophils although Lew et al., (1986) have shown that the $[Ca^{2+}]i$ threshold for degranulation (release of enzymes from azurophil, specific and small secretory vesicles) was approximately 200-300nM. From our results $[Ca^{2+}]i$ thresholds were estimated to be~600nM for aggregation,~600nM for NAG release,~500nM for lysozyme release,~800nM for PtdA formation and~200nM for LTB_4 production. Aggregation and degranulation were triggered by an approximate 400% increase in resting $[Ca^{2+}]i$ whereas PtdA formation required a 600% increase. Of the various indices of neutrophil activation, LTB_4 was the most sensitive to changes in $[Ca^{2+}]i$ and was initiated by only a 50% increase in basal $[Ca^{2+}]i$ (Rossi et al., 1987a). The extreme sensitivity to changes in $[Ca^{2+}]i$ could possibly be exploited to impose specific impairment of neutrophil LTB_4 biosynthesis via modulation of $[Ca^{2+}]i$. Whether these $[Ca^{2+}]i$ thresholds reported by quin2 are confirmed, using the newer generation of $[Ca^{2+}]i$ indicators such as fura-2 and indo-1, awaits further investigation.

CHAPTER 6: REGULATION OF NEUTROPHIL ACTIVATION BY G PROTEINS AND PROTEIN KINASE C.

1. The Role of G Proteins in Human Neutrophil Activation.

Although the role of guanine nucleotide binding regulatory proteins, G proteins, in the adenylate cyclase system is well established, their potential role in the modulation of cellular reactivity by agonists that operate through different transduction mechanisms is now emerging. In the subsequent investigations I utilised the bacterial toxin, pertussis toxin, which inhibits N_I or other similar G proteins, by NAD-ribosylation (Chapter 3). Studies using this pharmacological tool may help resolve whether a G protein is involved, either directly or indirectly, in agonist-induced human neutrophil responsiveness.

1.1. Effect of Pertussis Toxin on Agonist-induced Aggregation.

Under conditions known to promote NAD-ribosylation of GTP binding proteins, neutrophils were pretreated with pertussis toxin (15μg/ml; 2 hours) or with saline (as a control) and the aggregatory response elicited by the various agonists investigated. The response induced by all concentrations of FMLP, PAF and LTB_4 was attenuated by the toxin. Figures 71, 72 and 73 show the effect of pertussis toxin on FMLP (2.5μM), PAF (9μM) and LTB_4 (1.5μM) respectively and, even at these high agonist concentrations, the toxin significantly reduced the extent of the aggregatory response. Pertussis toxin, on the other hand, did not affect the aggregation induced by the calcium ionophore, ionomycin, (figure 74) or by the phorbol ester, PMA, (figure 75).

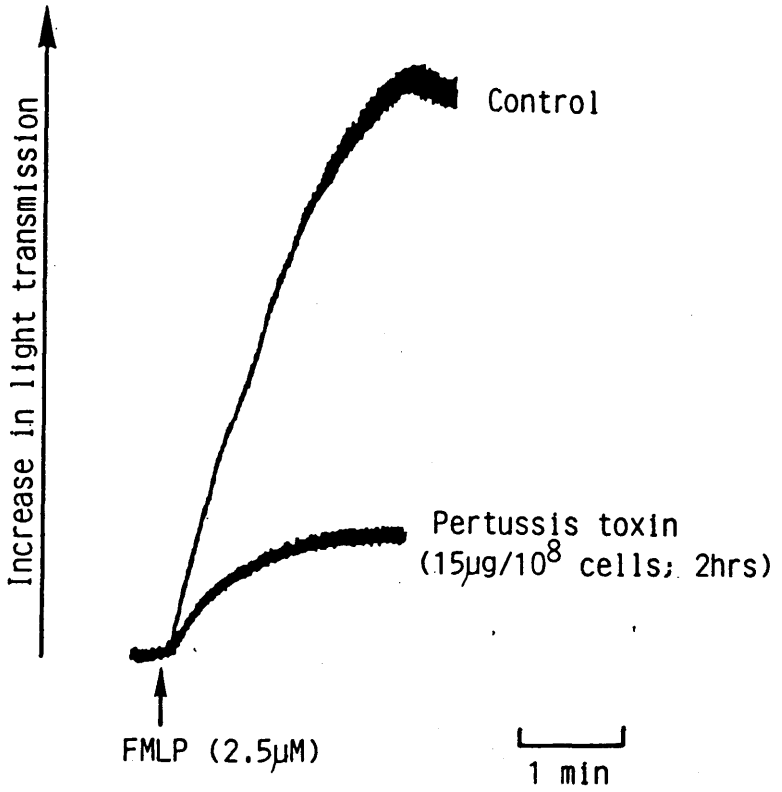


Figure 71.

The effect of pertussis toxin on FMLP-induced neutrophil aggregation.

Neutrophils (1 x 10^8cells/ml) were pretreated with pertussis toxin (15µg/ml), or saline control, for 2 hours. 0.6ml samples of washed neutrophils (2.5 x 10^7cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to FMLP (2.5µM) and the changes in light transmission recorded. The experiment is typical of three similar experiments, each performed in triplicate.

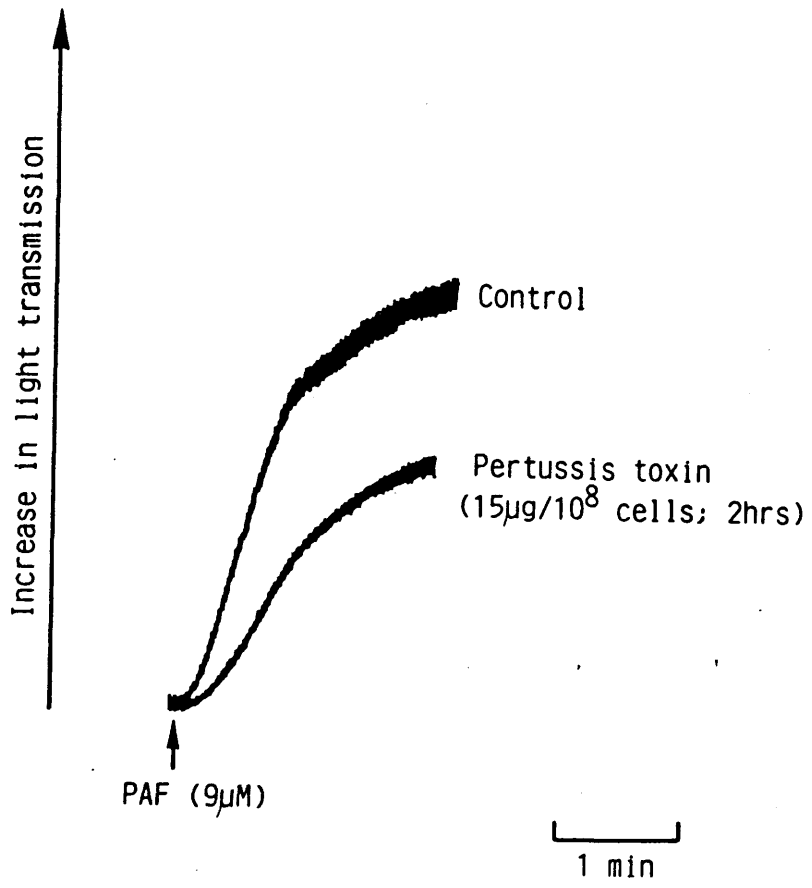


Figure 72.

The effect of pertussis toxin on PAF-induced neutrophil aggregation.

Neutrophils (1×10^8 cells/ml) were pretreated with pertussis toxin (15μg/ml), or saline control, for 2 hours. 0.6ml samples of washed neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to PAF (9μM) and the changes in light transmission recorded. The experiment is typical of three similar experiments, each performed in triplicate.

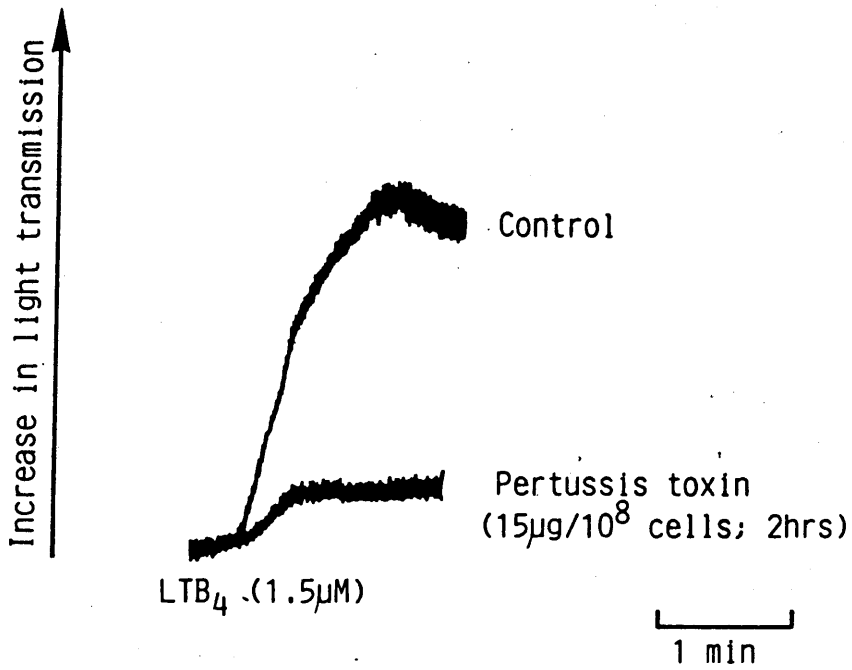


Figure 73.

The effect of pertussis toxin on LTB_4-induced neutrophil aggregation.

Neutrophils (1×10^8 cells/ml) were pretreated with pertussis toxin (15µg/ml), or saline control, for 2 hours. 0.6ml samples of washed neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to LTB_4 (2.5µM) and the changes in light transmission recorded. The experiment is typical of three similar experiments, each performed in triplicate.

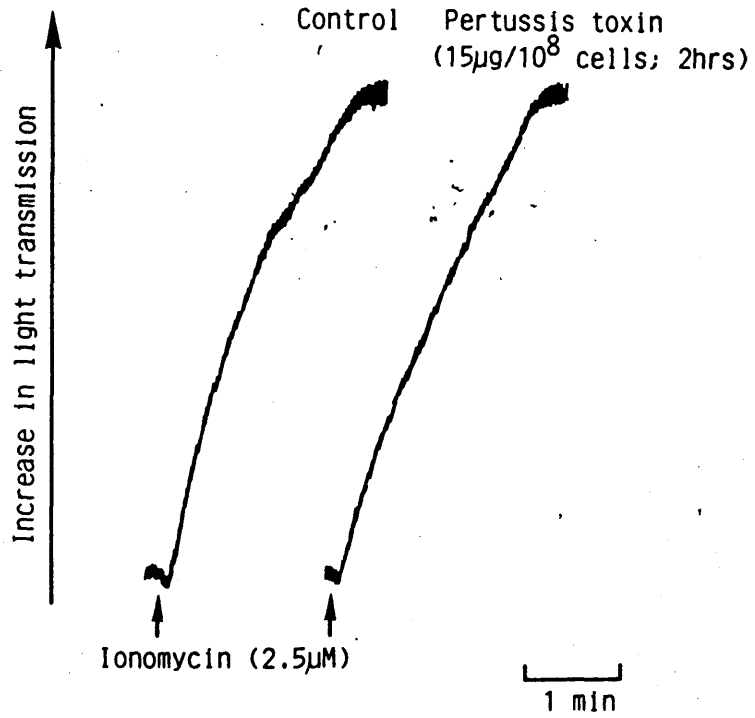


Figure 74.

The effect of pertussis toxin on ionomycin-induced neutrophil aggregation.

Neutrophils (1 x 10^8cells/ml) were pretreated with pertussis toxin (15µg/ml), or saline control, for 2 hours. 0.6ml samples of washed neutrophils (2.5 x 10^7cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to ionomycin (2.5µM) and the changes in light transmission recorded. The experiment is typical of three similar experiments, each performed in triplicate.

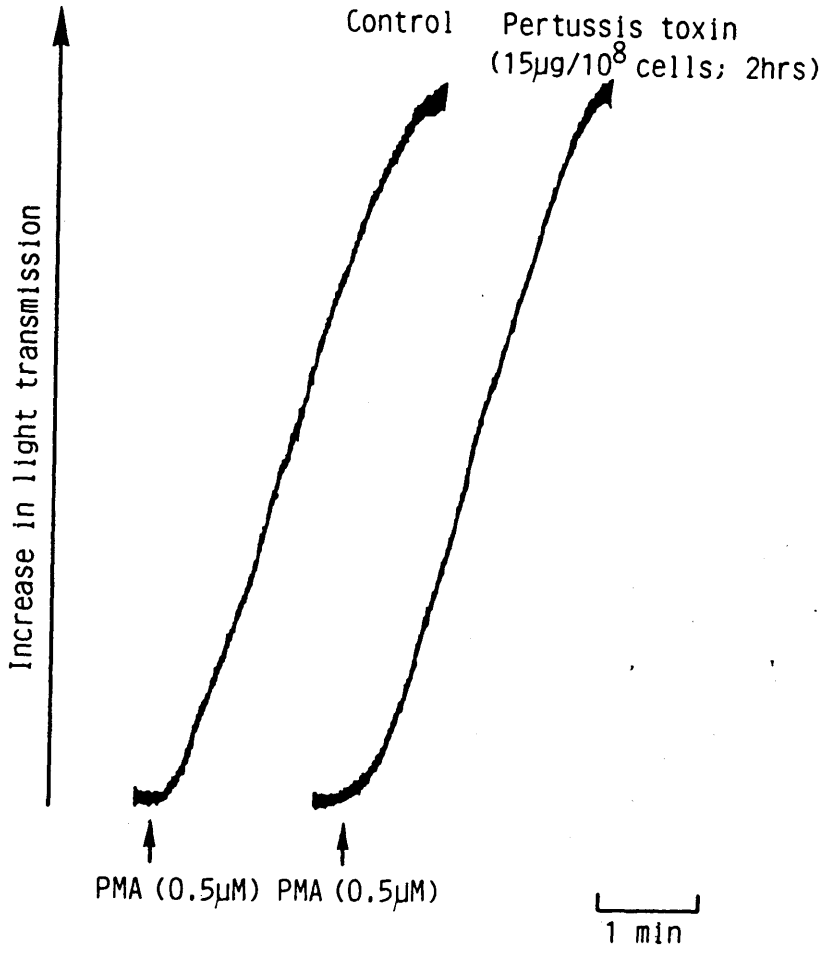


Figure 75.

The effect of pertussis toxin on PMA-induced neutrophil aggregation.

Neutrophils (1×10^8 cells/ml) were pretreated with pertussis toxin (15µg/ml), or saline control, for 2 hours. 0.6ml samples of washed neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were exposed to PMA (0.5µM) and the changes in light transmission recorded. The experiment is typical of three similar experiments, each performed in triplicate.

These observations suggest that the effect of the toxin is on receptor-directed activation and not simply via non-specific effects or by a general reduction in neutrophil responsiveness.

1.2. Effect of Pertussis Toxin on Agonist-induced Degranulation.

The effect of pertussis toxin on agonist-induced release of NAG (figure 76) and lysozyme (figure 77) was next examined. Both histograms clearly show that the release of NAG and lysozyme induced by FMLP (2.5μM), PAF (9μM) and LTB_4 (1.5μM) were severely abrogated (by approximately 70%) by pertussis toxin pretreatment whereas enzyme release induced by ionomycin and PMA was unaffected.

1.3. Effect of Pertussis Toxin on Agonist-induced Elevation of $[Ca^{2+}]i$.

Preincubation with pertussis toxin had no significant effect on the resting level of $[Ca^{2+}]i$. However, the elevation induced by all concentrations of the receptor-directed agonists was attenuated by pertussis toxin pretreatment. Figure 78 shows representative experimental traces of the responses induced by FMLP (2nM), PAF (0.9nM), LTB_4 (30nM) and ionomycin (100nM). Again, the response elicited by the ionophore in the presence of pertussis toxin was the same as the control ionomycin response.

1.4. Effect of Pertussis Toxin on Agonist-induced $[^{32}P]$-PtdA formation.

A similar profile was observed when agonist-induced $[^{32}P]$-PtdA

Figure 76.

The effect of pertussis toxin on agonist-induced NAG release from human neutrophils.

Neutrophils (1×10^8 cells/ml) were pretreated with pertussis toxin (15μg/ml), or saline control, for 2 hours. The cells were then washed and aliquoted into 0.6ml samples of 1×10^7 cells/ml and exposed to cytochalasin B (5μg/ml; 10 minutes). The neutrophils were finally challenged with an agonist for a further 10 minutes and NAG was measured. The results are mean values ± SEM for three experiments, each performed in triplicate. Analysis of data: $^{**}P < 0.01$; $^{***}P < 0.001$.

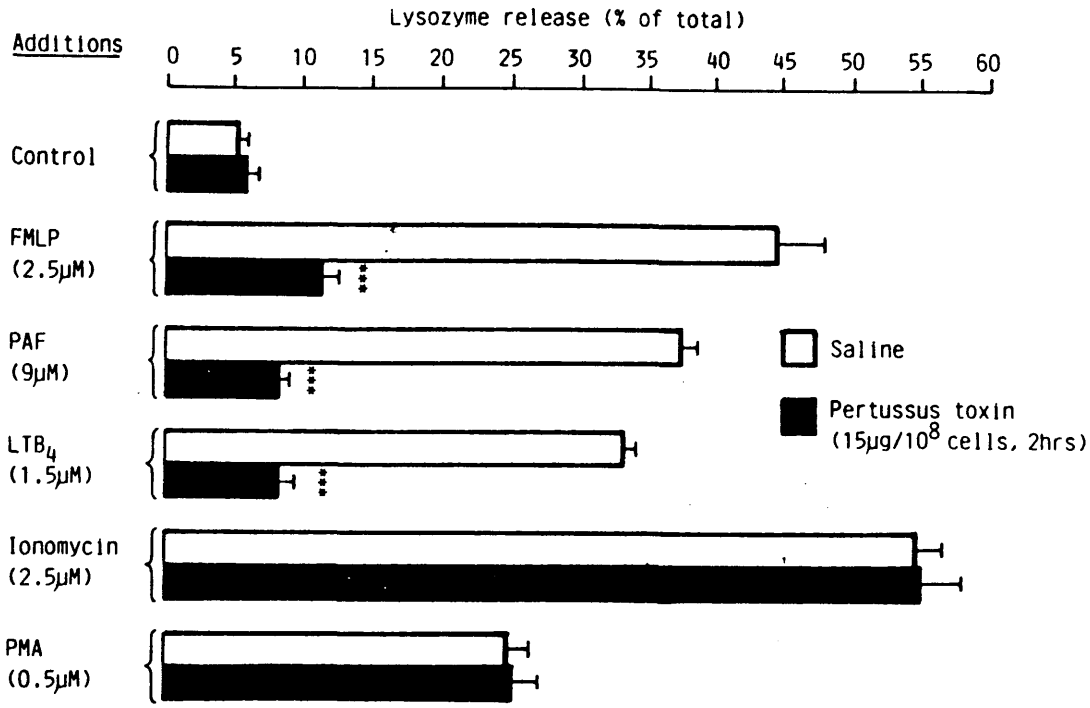


Figure 77.

The effect of pertussis toxin on agonist-induced lysozyme release from human neutrophils.

Neutrophils (1×10^8 cells/ml) were pretreated with pertussis toxin (15μg/ml), or saline control, for 2 hours. The cells were then washed and aliquoted into 0.6ml samples of 1×10^7 cells/ml and exposed to cytochalasin B (5μg/ml; 10 minutes). The neutrophils were finally challenged with an agonist for a further 10 minutes and lysozyme was measured. The results are mean values ± SEM for three experiments, each performed in triplicate. Analysis of data: ***$P < 0.001$.

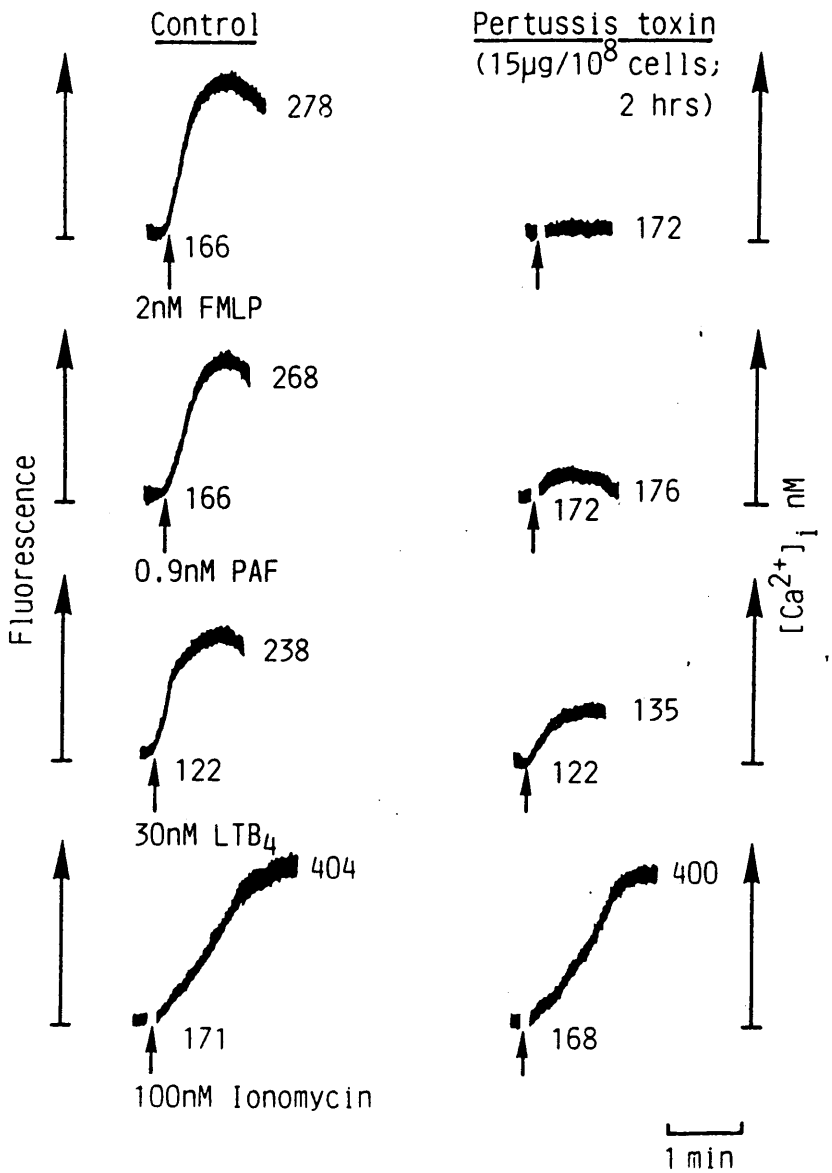


Figure 78.

The effect of pertussis toxin on agonist-induced changes in $[Ca^{2+}]i$ in human neutrophils.

Representative experimental traces of fluorescence changes in quin2 labelled neutrophils pretreated with pertussis toxin (15µg/ml), or saline control, for 2 hours. 2.0ml samples of quin2 labelled neutrophils (1 x 10^7cells/ml), in the presence of 1.6mM external Ca^{2+}, were exposed to agonist at the concentrations indicated. Fluorescence responses were monitored after agonist addition and the appropriate $[Ca^{2+}]i$ calibration scale is shown on the right of the fluorescence record. The experiment is typical of eight similar experiments.

was investigated (figure 79). Both the responses elicited by FMLP (1μM; 172 ± 15% of control) and PAF (9μM; 175 ± 16% of control) were inhibited to 117 ± 9% and 137 ± 8% respectively. $[^{32}P]$-PtdA formation induced by ionomycin (1μM; 165 ± 17% of control) was unaffected with toxin pretreatment.

2. The Role of Protein Kinase C in Human Neutrophil Activation.

Hydrolysis of inositol phospholipids, catalysed by phospholipase C yields two second messenger molecules namely $[Ca^{2+}]i$ and 1,2-diacyl glycerol (DAG). The former acts allegedly via stimulation of Ca^{2+}-calmodulin dependent protein kinase(s) and the latter via stimulation of protein kinase C. The effects of endogenous DAG on protein kinase C and on cellular reactivity can be mimicked by tumour-promoting phorbol esters, e.g. phorbol-12-myristate-13-acetate; PMA (Castagna et al., 1982). There is evidence emerging in a variety of cell systems to suggest that protein kinase C activation not only directly stimulates cellular activation but may also have a regulatory or inhibitory effect on agonist-induced cellular responsiveness. (Nishizuka, 1986; see Chapter 3). In order to investigate the possibility that similar regulatory mechanisms operate in the human neutrophil, I examined the effect of PMA on the potential transduction processes, namely $[Ca^{2+}]i$ elevation and phosphoinositide hydrolysis, involved in the neutrophil response to stimulatory agonists.

2.1. Effect of PMA on Agonist-induced Elevation of $[Ca^{2+}]i$.

Using quin2 labelled neutrophils FMLP (5nM), LTB_4 (10nM) and

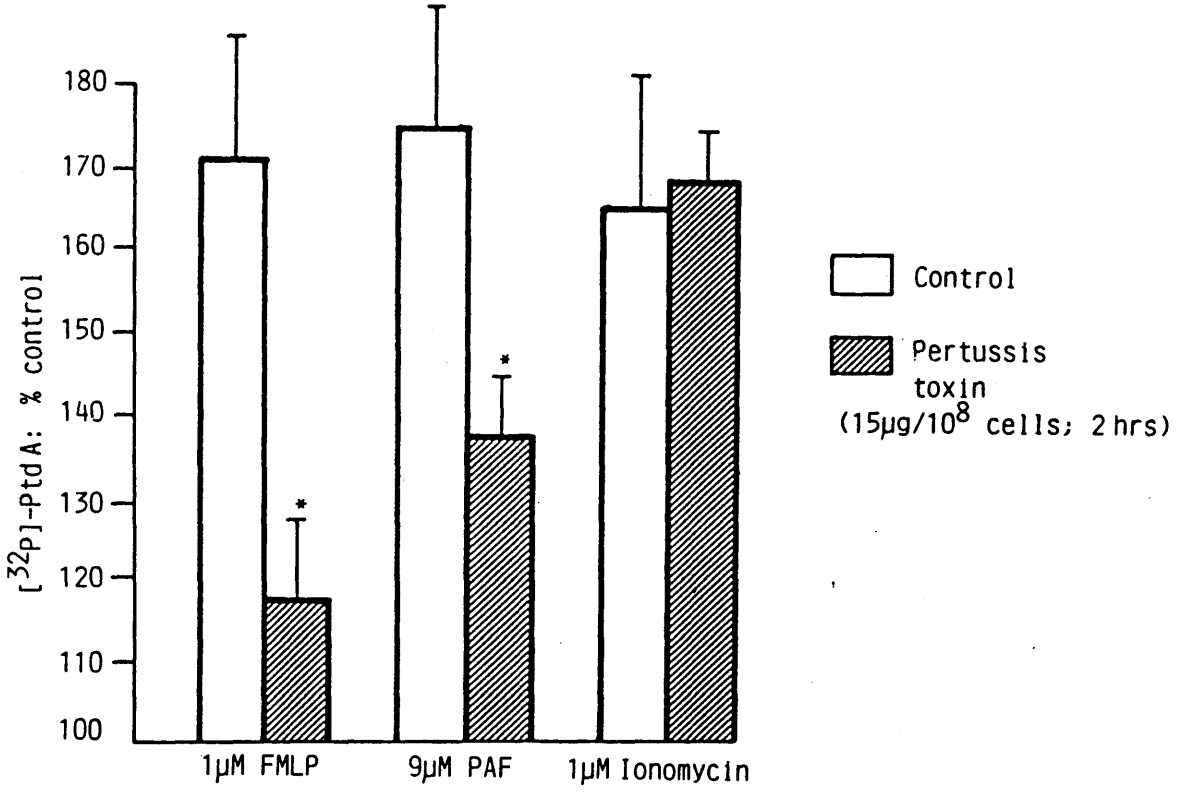


Figure 79.

The effect of pertussis toxin on agonist-induced $[^{32}P]$-PtdA formation in human neutrophils.

Neutrophils (1×10^8 cells/ml) were pretreated with pertussis toxin (15μg/ml), or saline control, and $[^{32}P]$-orthophosphate, for 2 hours. The cells were then washed and aliquoted into 0.4ml samples of 2.5×10^7 cells/ml and challenged with the agonist for 120 seconds, at which time $[^{32}P]$-PtdA levels were measured. The results are mean values + SEM for five experiments, each performed in triplicate. Analysis of data: $*P < 0.05$.

PAF (10nM) caused an elevation of resting value of 133nM to a maximum of between 280-290nM. When the cells were incubated for 120 seconds with PMA or DMSO (control) neither altered resting $[Ca^{2+}]i$. However, PMA (1-100nM) inhibited, in a concentration-dependent manner, the elevation of $[Ca^{2+}]i$ induced by all three agonists (figure 80).

2.2. Effect of PMA on Agonist-induced $[^{32}P]$-PtdA formation.

PMA (1μM; 120 seconds) had no effect on neutrophil $[^{32}P]$-PtdA levels when compared to its vehicle control. Figure 81 depicts the effect of PMA on FMLP-induced $[^{32}P]$-PtdA formation. In these experiments (n=3-4) control FMLP (1μM) elicited a 155 ± 6% response. This was attenuated, in a concentration-dependent manner, by preincubation with PMA (1nM-1μM) with an IC_{50} value (i.e. concentration producing 50% inhibition of the response elicited by a particular agonist) of ~ 1nM. Preincubation with PMA (1nM-1μM) also suppressed the response to PAF (1.8μM) which was 182 ± 12% of control (n=3-4) with an IC_{50} value of ~ 50nM (figure 82). Ionomycin (1μM) induced 168 ± 8% of the control level of $[^{32}P]$-PtdA (n=4-5): this stimulation was unaffected by the same concentration range of the phorbol ester (figure 83).

3. Discussion.

3.1. The role of G Proteins.

Over the last few years it has become apparent that G proteins

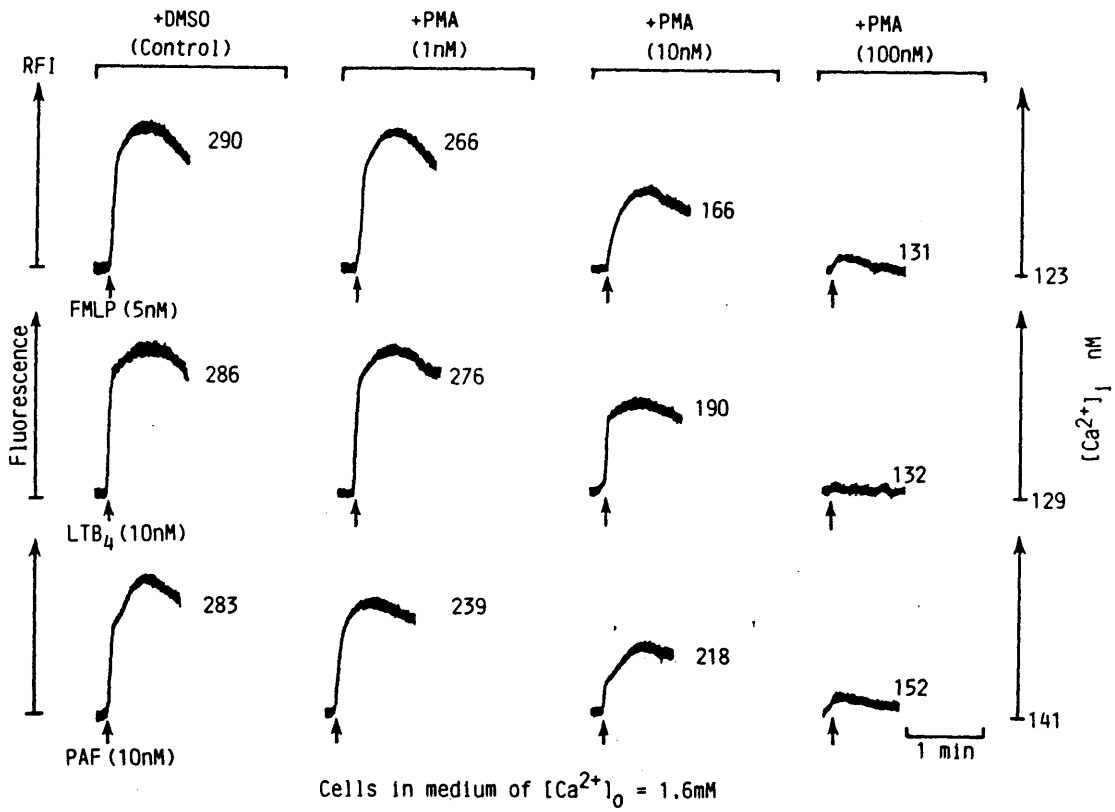


Figure 80.

Representative traces of fluorescence changes in quin2 labelled neutrophils demonstrating the effect of PMA on agonist-induced $[Ca^{2+}]i$ elevation.

2.0ml samples of quin2 labelled neutrophils (1×10^7 cells/ml), were suspended in medium containing 1.6mM external calcium and were preincubated for 120 seconds with PMA at the concentrations indicated. The cells were then exposed to the agonists and changes in fluorescence were monitored. The $[Ca^{2+}]i$ calibration scale is shown on the right of the fluorescence record.

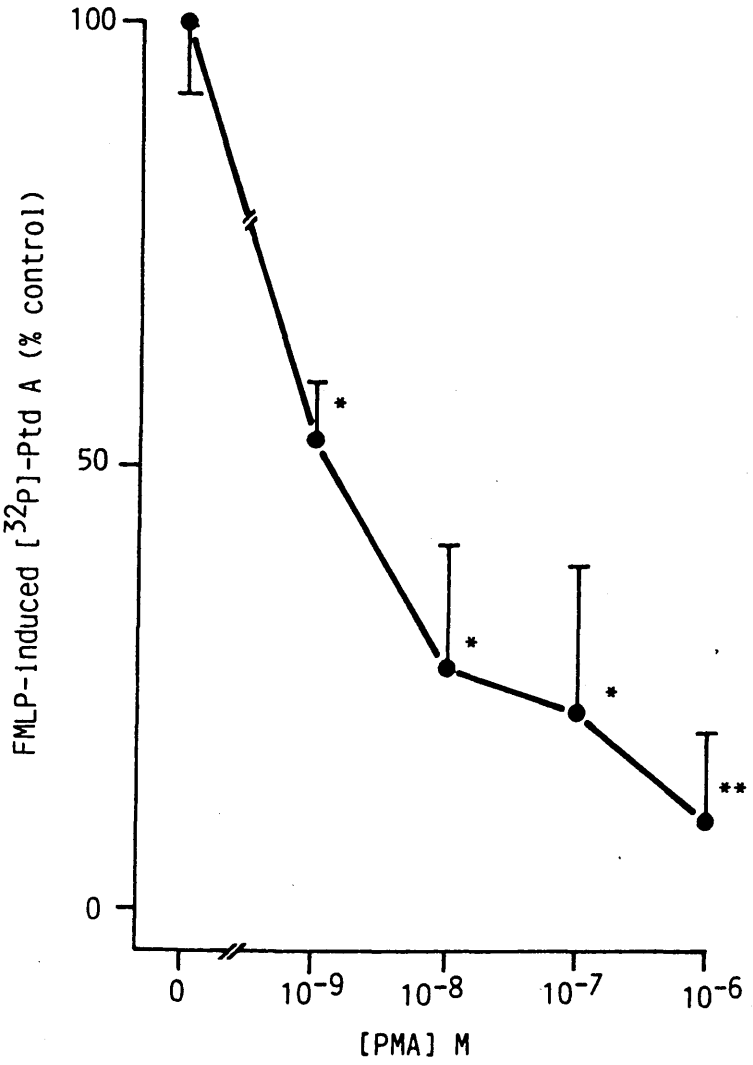


Figure 81.

The effect of PMA on FMLP (1μM)-induced $[^{32}P]$-PtdA formation.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were preincubated for 120 seconds with PMA at the concentrations indicated. The cells were then exposed to FMLP (1μM) for a further 120 seconds, at which time $[^{32}P]$-PtdA levels were measured. The data are expressed as a percentage of the control formation of $[^{32}P]$-PtdA induced by FMLP in the presence of the PMA vehicle, DMSO (0.1%).
The results are mean values + SEM for three experiments, each performed in triplicate. Analysis of data: $*P < 0.05$; $**P < 0.01$.

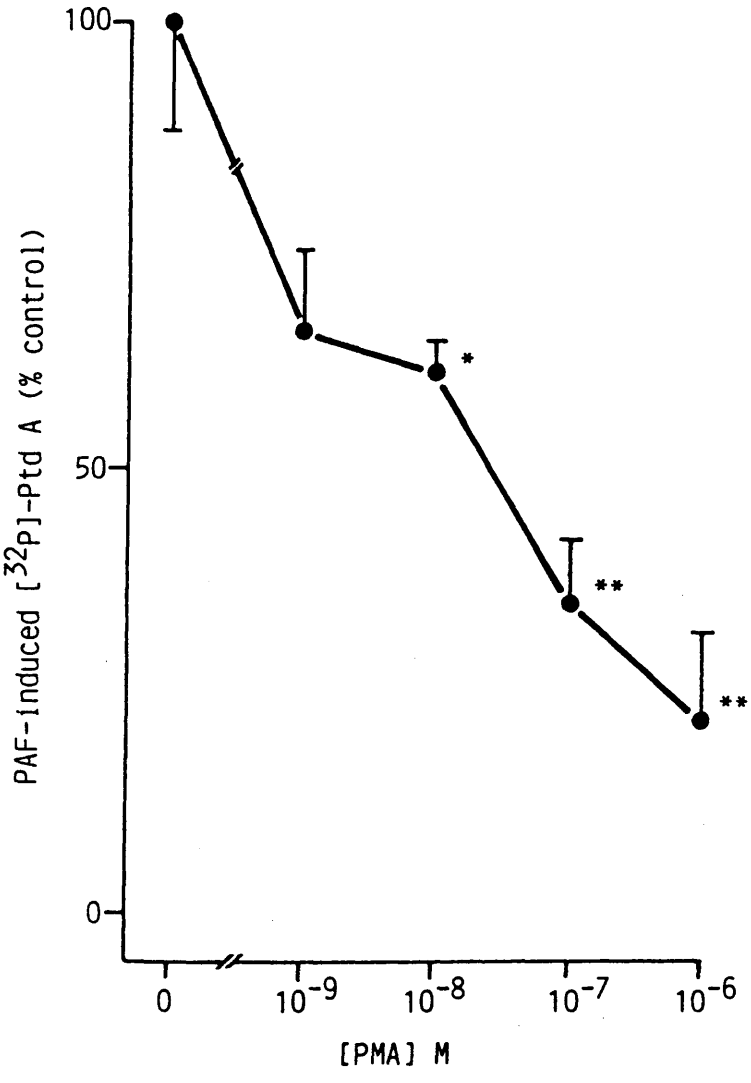


Figure 82.

The effect of PMA on PAF (1.8μM)-induced $[^{32}P]$-PtdA formation.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were preincubated for 120 seconds with PMA at the concentrations indicated. The cells were then exposed to PAF (1.8μM) for a further 120 seconds, at which time $[^{32}P]$-PtdA levels were measured. The data are expressed as a percentage of the control formation of $[^{32}P]$-PtdA induced by PAF in the presence of the PMA vehicle, DMSO (0.1%).
The results are mean values ± SEM for three experiments, each performed in triplicate. Analysis of data: $*P < 0.05$; $**P < 0.01$.

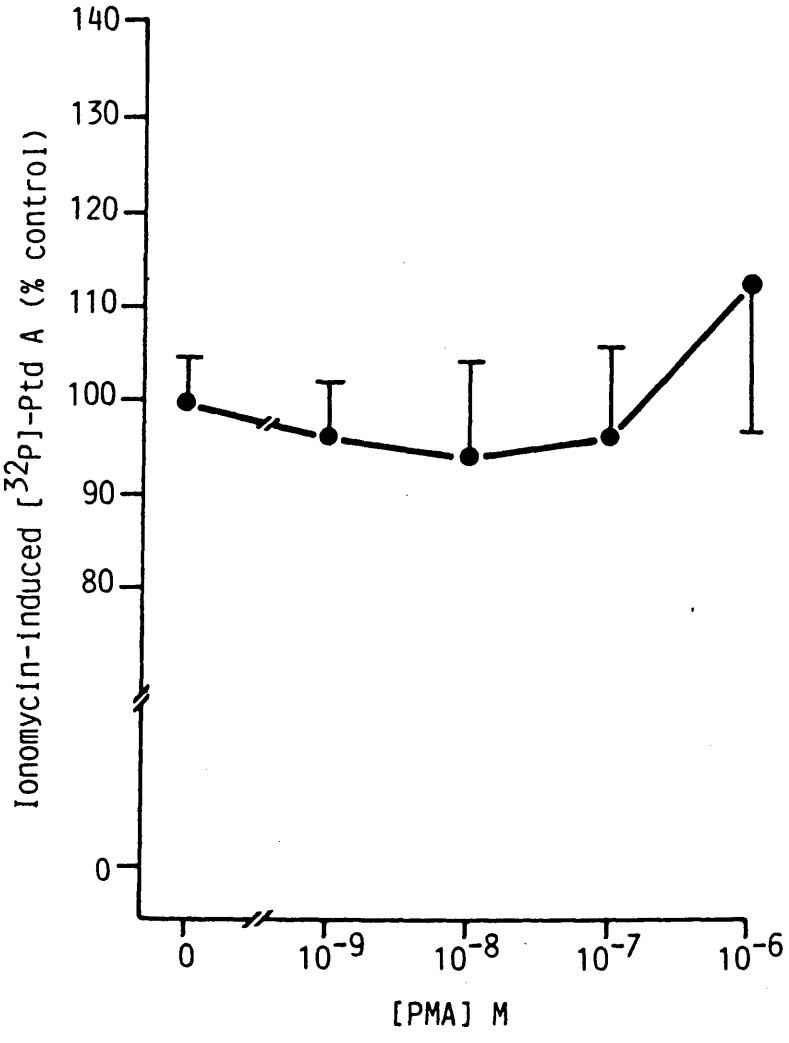


Figure 83.

The effect of PMA on ionomycin (1μM)-induced $[^{32}P]$-PtdA formation.

0.4ml samples of $[^{32}P]$-orthophosphate prelabelled neutrophils (2.5×10^7 cells/ml) were preincubated for 120 seconds with PMA at the concentrations indicated. The cells were then exposed to ionomycin (1μM) for a further 120 seconds, at which time $[^{32}P]$-PtdA levels were measured. The data are expressed as a percentage of the control formation of $[^{32}P]$-PtdA induced by ionomycin in the presence of the PMA vehicle, DMSO (0.1%).
The results are mean values ± SEM for three experiments, each performed in triplicate.

play an important role not only in the adenylate cyclase system (Gilman, 1984) or in photoreceptor transduction processes (Fung et al., 1981) but also in other second messenger systems such as phospholipase C catalysed phosphoinositide hydrolysis (Joseph, 1985). Important pharmacological agents which have contributed to the abundant literature concerning G proteins are two bacterial products, cholera toxin and pertussis toxin. In these investigations I utilised pertussis toxin which causes an ADP-ribosylation of Ni or other related G proteins (Chapter 3). It is clear that pertussis toxin inhibited FMLP, PAF and LTB_4-induced aggregation, degranulation and elevation of $[Ca^{2+}]i$ and also inhibited FMLP and PAF-induced PtdA formation. Since pertussis toxin did not attenuate neutrophil activation elicited by the calcium ionophore, ionomycin; or by the phorbol ester, PMA; the toxin is probably acting upon receptor-coupling mechanisms and not simply via a non-specific mechanism or by a general reduction in neutrophil responsiveness.

As outlined in Chapter 3, in accordance with my observations, other workers have demonstrated that pretreatment of neutrophils with pertussis toxin inhibited a number of FMLP-induced responses including aggregation, enzyme release, phosphoinositide hydrolysis and $[Ca^{2+}]i$ elevation (Omann et al., 1987a). There are some conflicting results concerning pertussis toxin inhibition of PAF-induced neutrophil activation. Lad et al., (1985a) using human neutrophils, showed that pertussis toxin inhibited PAF-induced chemotaxis, aggregation, superoxide generation and lysozyme release. In further investigations they demonstrated that this bacterial toxin diminished PAF-evoked elevation of $[Ca^{2+}]i$ in quin2 loaded human neutrophils (Lad et al., 1985b). More recently this has been confirmed by Verghese et al., (1987). Using rabbit neutrophils Naccache et al., (1985c; 1986)

showed that pertussis toxin inhibited PAF-induced degranulation and phosphoinositide hydrolysis. They also showed that PAF causes an elevation of neutrophil $[Ca^{2+}]i$ at concentrations higher than phosphoinositide hydrolysis and that this elevation of $[Ca^{2+}]i$ was not affected by pertussis toxin. This led to the conclusion that there may exist two distinct populations of PAF receptors, at least in the rabbit neutrophil. The explanation for this apparent species difference between rabbit and human neutrophil is unknown. It remains possible that in the rabbit neutrophil experiments, the toxin pretreatment (0.5μg/ml; 1 hour) may have been incomplete or that resistance to pertussis toxin could be attributed to a small residual pool of unribosylated G protein that remains coupled to this receptor.

In my investigations elevation of $[Ca^{2+}]i$ induced by PAF (all concentrations tested) was attenuated by pertussis toxin and that PAF-induced $[Ca^{2+}]i$ elevation occurs at lower concentrations of agonist than does PtdA formation (Chapter 5). Whether there exist two PAF receptors in the human neutrophil still remains to be established. There exists the possibility that there is a receptor reserve in the neutrophil for both FMLP and PAF, i.e. occupancy of only a fraction of the receptor pool may stimulate significant phosphoinositide turnover (below the detection limits of the PtdA assay employed in this study) enough to elicit maximal elevation of $[Ca^{2+}]i$. However, the precise relationship between phosphoinositide hydrolysis and changes in $[Ca^{2+}]i$ is still unclear.

Most data suggests that only receptors coupled to phospholipase C in haematopoetic cells are sensitive to pertussis toxin inhibition. For example, in human leukaemic (HL-60) cells (Brandt et al., 1985) and mast cells, (Nakamura and Ui, 1985) receptor-activated phosphoinositide hydrolysis is attenuated by pertussis toxin pretreatment. It remains possible that in other cell systems where pertussis toxin is not

effective at inhibiting phosphoinositide hydrolysis that there still exists pertussis toxin-insensitive phospholipase C coupled G proteins.

The data concerning LTB_4 indicates that neutrophil activation induced by this arachidonic acid metabolite is also coupled to a G protein. Results from Chapter 5 suggest that LTB_4 may produce its response independently of phosphoinositide hydrolysis (at least as monitored by changes in PtdA formation). Since pertussis toxin inhibited LTB_4-induced aggregation, enzyme release and $[Ca^{2+}]_i$ elevation, G proteins may be involved in processes other than phospholipase C activity. Indeed, recent evidence suggests that G proteins are involved directly in voltage-dependent calcium channels in neurones (Scott & Dolphin, 1986; Holz et al., 1986; Hescheler et al., 1987) and in muscarinic stimulation of K^+ channels in atrial cells. Whether G proteins exert their effects by interacting directly with chemotactic receptors in the human neutrophil remains to be established.

The identity of the pertussis toxin-sensitive G protein is unknown. However, Gierschik et al., (1986) have proposed that there exists a novel and distinct pertussis toxin substrate in the human neutrophil. They provided evidence showing that the predominant pertussis toxin substrate is immunochemically distinct from previously-identified substrates; namely Ni, Np and transducin.

In summary, it appears that agonist-induced neutrophil activation apparently involves a pertussis toxin-sensitive process, presumably (a) guanine nucleotide binding regulatory protein(s), that has (have) yet to be fully identified. With the development of new and improved techniques, a more accurate understanding of these membrane transducing components will be attained.

3.2. The role of protein kinase C.

Cellular activation may be regulated by at least two distinct

molecules; Ca^{2+} and DAG. Agonist receptor interaction can lead to the hydrolysis of phosphoinositide hydrolysis resulting in the formation of these two second messengers (Abdel-Latif, 1986). DAG is thought to stimulate protein kinase C which acts to elicit its cellular responses independently or synergistically with elevated levels of $[Ca^{2+}]i$. There is evidence to suggest that the tumour-promoting phorbol esters such as PMA can mimic the stimulatory effect of endogenous DAG on protein kinase C (Castagna et al., 1982).

The results clearly show that PMA is a potent activator of human neutrophils (Chapter 5). Although the phorbol ester induces aggregation and enzyme release (principally from specific granules) it does not cause any significant elevation of $[Ca^{2+}]i$ or PtdA formation. Other workers have obtained similar observations (Sha'afi et al., 1983; Naccache et al., 1984; White et al., 1984) and they have also shown that PMA is a potent stimulator of the respiratory burst (Fujita et al., 1984). Moreover, in neutrophils it has been shown that PMA causes protein phosphorylation in cells pre-labelled with $[^{32}P]$—orthophosphate (Ohtsuka et al., 1986; Pontremoli et al., 1986) and promotes translocation of protein kinase C from the cytosol to the membrane (Horn & Karnovski, 1986). Several investigations have shown that sub-threshold concentrations of PMA or OAG and calcium ionophore can synergize to cause neutrophil activation (Penfield & Dale, 1984; Robinson et al., 1984; O'Flaherty et al., 1984). In addition to the observed independent or synergistic stimulatory role, there is a great deal of evidence emerging to suggest that protein kinase C activation can also inhibit or modulate cellular activation in a wide variety of cellular systems including neutrophils (Nishizuka et al., 1984; 1986; Drummond & MacIntyre, 1985).

This study demonstrated that preincubation of human neutrophils

with PMA had a pronounced concentration-dependent inhibition on agonist-induced $[Ca^{2+}]i$ elevation. Similar observations were made by Naccache et al., (1984; 1985a)who showed that PMA inhibited FMLP, PAF and LTB_4-induced elevation of $[Ca^{2+}]i$ and enzyme secretion in rabbit neutrophils. Inhibition of agonist-induced elevation of $[Ca^{2+}]i$ by phorbol esters has subsequently been shown in a large variety of other isolated cells including human platelets (MacIntyre et al., 1985a), rat basophilic leukaemia cells (Sagi-Eisenberg et al., 1985), GH_3 pituitary cells (Drummond, 1985), hepatocytes (Cooper et al., 1985) astrocytoma cells (Orellana et al., 1985), vascular smooth muscle (Brock et al., 1986) and adrenal glomerulosa cells (Kojima et al., 1986).

Attenuation of agonist-induced elevation of $[Ca^{2+}]i$ may be attributed to an activation of a Ca^{2+} extrusion process. Indeed, Mottola & Romeo, (1982) showed that neutrophils exposed to PMA cause an extrusion of cytoplasmic Ca^{2+} to the extracellular milieu. It was subsequently demonstrated that an ATP-dependent calcium pump stimulated by PMA inhibited a rise in $[Ca^{2+}]i$ elicited by FMLP (Lagast et al., 1984). If the assumption is made that the elevation of neutrophil $[Ca^{2+}]i$ is produced as a consequence of inositol phosphate formation, one might expect that PMA may also inhibit agonist-induced phosphoinositide hydrolysis. Clearly our results demonstrate that PMA inhibits PtdA formation induced by FMLP and PAF. Since measurement of PtdA is an indirect index of phosphoinositide hydrolysis, this inhibitory effect of PMA may reflect a reduction of DAG kinase activity and not an effect on phospholipase C activity. Other workers have subsequently shown that PMA does have a direct action on phosphoinositide hydrolysis in the neutrophil (Kato et al., 1986; Smith et al., 1987). Similarly, phorbol ester produces inhibitory effects on agonist-induced phosphoinositide hydrolysis in a host of other cell systems. Some of

these include platelets (MacIntyre et al., 1985a; Watson & Lapetina., 1985), mast cells (Okano et al., 1985), lymphocytes (Mellors et al., 1985), vascular smooth muscle, (Brock et al., 1985; McMillan et al., 1986), hepatocytes (Corvera et al., 1986) and glomerulosa cells (Kojima et al., 1986).

It is possible that protein kinase C activation could phosphorylate and stimulate $Ins(1,4,5)P_3$ phosphatase (Molina y Vedia & Lapetina, 1986; Connolly et al., 1986) resulting in the removal of $Ins(1,4,5)_3$ and consequently decreasing the ability of the cell to mobilise calcium. This may account for part of the inhibitory effect of PMA. However, since agonist-induced PIP_2 hydrolysis and diacylglycerol formation are also inhibited by phorbol ester pretreatment (Brock et al., 1985; Zavoico et al., 1985), inhibition of phospholipase C activity must also occur.

If PMA is inhibiting the formation of inositol phosphates and also attenuates the elevation of $\left[Ca^{2+}\right]i$ induced by LTB_4, this may suggest that the mechanism of action of this lipid is indeed via phosphoinositide hydrolysis. In an elegant investigation it was shown that protein kinase activators (PMA, mezerein and rac-1-O-myristoyl-2-acetylglycerol) inhibited the binding of LTB_4 in human neutrophils by reducing the number of high affinity receptors to this eicosanoid (O'Flaherty et al., 1986a). Therefore, an inhibitory effect produced by PMA is not conclusive evidence for phosphoinositide hydrolysis involvement but may simply reflect a down regulation of receptors produced by protein kinase C activation.

The observed differential effects of PMA on agonist-induced PtdA formation is of interest. As indicated by the IC_{50} value for FMLP (1nM) and for PAF (50nM), the chemotactic tripeptide seems to be more susceptible than the ether lipid to inhibition by PMA. This

difference may be attributed to effects of protein kinase C on other components of the transduction process such as regulation of receptor number and receptor affinity. For example, phorbol esters can phosphorylate alpha$_1$ adrenoreceptors in smooth muscle (Leeb-Lundberg et al., 198) and reduce their affinity in hepatocytes (Corvera et al., 1986). The insulin receptor is also phosphorylated by protein kinase C activation (Jacobs et al., 1983). Neuroblastoma muscarinic acetylcholine receptors are rapidly internalised in response to phorbol esters (Liles et al., 1986) and PMA or OAG both produce a desensitisation to β-adrenergic agonist activation of the adenylate cyclase system of rat reticulocytes, an effect which is antagonised by the proposed protein kinase C inhibitor, H7 (1-5-isoquinolinesulphonyl-2-methylpiperazine)(Yamashita et al., 1986). Therefore, a similar effect at the FMLP receptor may explain why PtdA formation produced by the chemotactic tripeptide is inhibited with lower concentrations of PMA than required to attenuate PtdA production induced by the ether lipid.

It has also been reported that protein kinase C may produce its negative feedback effect by interfering with other important cellular processes, for example nucleotide cyclases (Bushfield et al., 1987), ion channels (Farley & Auerbach, 1986), contractile elements (Inagaki et al., 1984) and G proteins (Halenda et al., 1986). Indeed, Smith et al., (1987) have recently shown that phorbol esters disrupt the coupling of FMLP-activated G protein to phospholipase C.

Hence, there is abundant evidence to suggest that protein kinase C may serve as a bidirectional regulator of cellular reactivity; DAG not only stimulates the cell but also may inhibit or modulate the stimulatory transduction processes. The precise mechanism or site of action and physiological relevance of this protein kinase C-induced dampening of cellular activity still remains to be elucidated.

CHAPTER 7: INTERACTION BETWEEN LIPID MEDIATORS OF NEUTROPHIL ACTIVATION.

LTB_4 and PAF when added exogenously are potent activators of human neutrophils. Stimulation of neutrophils by agents such as calcium ionophores will produce numerous lipid products including both LTB_4 and PAF. These lipids produced endogenously may combine with specific receptors located in/on the cell surface membrane to mediate directly the evoked response or they may function to amplify neutrophil responsiveness elicited by other (exogenous) agonists. The major aim of the next part of my investigations was to examine the role of LTB_4 and PAF production in mediating cellular activation to exogenous agonists. This was attempted by (1) employing selective inhibitors of 5-lipoxygenase to prevent endogenous LTB_4 biosynthesis and (2) utilising the specific PAF receptor antagonist, kadsurenone, to abolish the effect of any endogenous PAF production.

I investigated the effect of lipoxygenase inhibition and kadsurenone on ionomycin-induced LTB_4 generation and on agonist-induced neutrophil aggregation and degranulation.

1. Effect of Lipoxygenase Inhibitors:-

1.1. On ionomycin-induced LTB_4 Generation.

The effect of a wide variety of agents that interfere with eicosanoid production was examined on ionomycin (1μM)-induced LTB_4 generation in human neutrophils. The response elicited by 1μM ionomycin (approximately 60ng/10^7cells) was taken as the control and the effect of the inhibitors (15 minute preincubation at 37°C), in the concentration range of 10nM-100μM, was expressed as a percentage of that control.

The compounds investigated were Revlon 5901, Indomethacin (a

relatively selective cyclooxygenase inhibitor), BW755C, NDGA, ICI 198143, AA861 and Nafazatrom. Their IC_{50} values were ~2μM, ~80μM, ~1μM, ~2μM, ~50μM, ~500nM and ~10μM respectively (figures 84 and 85). The potency of the various inhibitors was AA861 > BW755C > Revlon 5901 ≈ NDGA > Nafazatrom > ICI > Indomethacin.

1.2. On agonist-induced degranulation.

Samples from the above experiments were simultaneously taken to investigate the effects of these agents on ionomycin-induced release of NAG (figures 86 and 87) and lysozyme (figures 88 and 89). The compounds Revlon 5901, indomethacin, ICI 198143, and AA861 at all concentrations tested (10nM-100μM) had no significant effect on ionomycin release of both NAG and lysozyme. However, the compounds BW755C, NDGA and Nafazatrom at the high concentrations of 10-100μM did inhibit enzyme release (NAG and lysozyme) to some extent (maximal inhibition produced by any of these agents was by NDGA which attenuated enzyme release by approximately 35-45%).

To assess the role of endogenous LTB_4 on degranulation induced by receptor directed stimuli, I investigated the effects of the specific 5-lipoxygenase inhibitor, Revlon 5901, on this response. Revlon 5901 (15 minute preincubation) was examined on FMLP (1μM; figures 90a and 90b), PAF (1.8μM; figures 90c and 90d) and LTB_4 (1.5μM; figures 90e and 90f) induced release of both NAG and lysozyme. The amount of enzyme release induced by the various agonists in the absence of Revlon 5901 was taken as the control values and the effect of this compound was expressed as a percentage of that control. Revlon 5901 had no significant effect on the release of both enzymes induced by all three agonists.

1.3. On agonist-induced aggregation.

Aggregation, another marker of neutrophil activation, was examined in

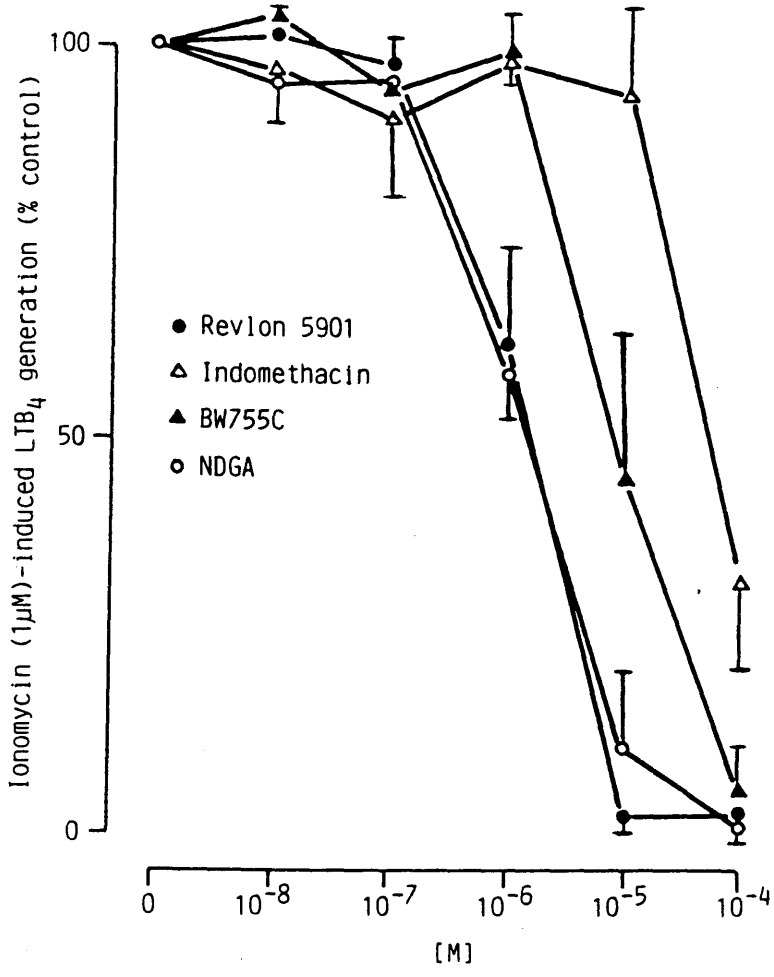


Figure 84.

The effects of agents that interfere with eicosanoid production on ionomycin-induced LTB_4 generation by human neutrophils.

0.6ml samples of neutrophils (1×10^7 cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and for 15 minutes with the various drugs at the concentrations indicated. The cells were then challenged with ionomycin (1μM) for a further 10 minutes and the production of LTB_4 determined. The results are expressed as the percentage of the amount of LTB_4 produced by ionomycin alone (approximately 60ng/ml) and are mean values ± SEM for four experiments, each performed in triplicate.

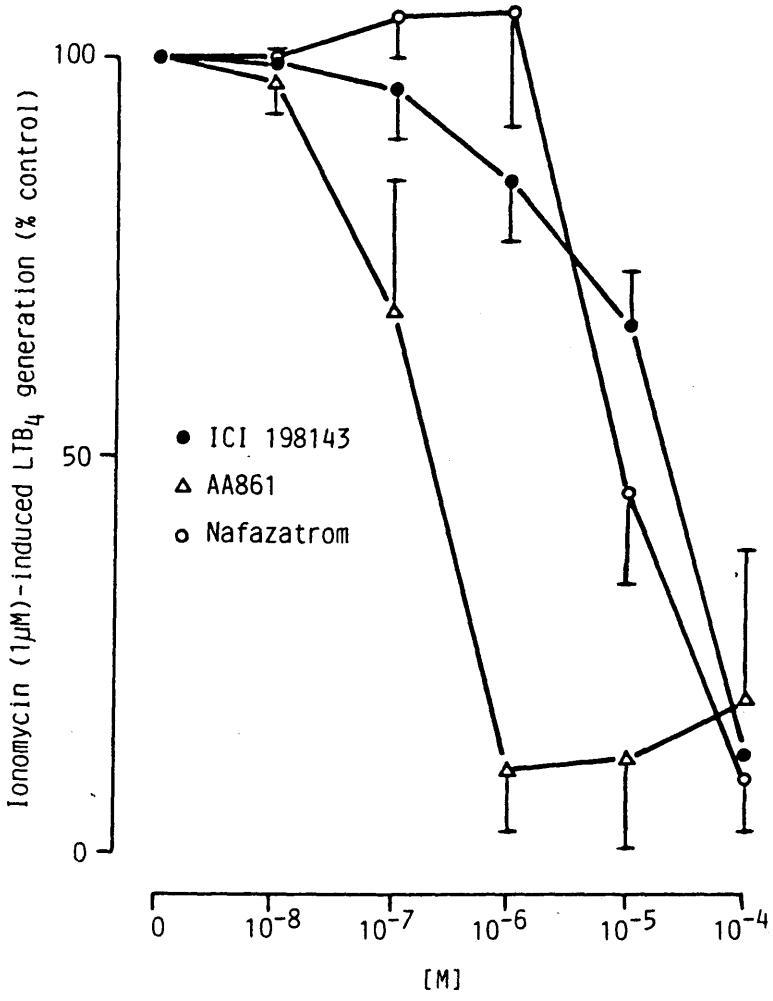


Figure 85.

The effect of agents that interfere with eicosanoid production on ionomycin-induced LTB_4 generation by human neutrophils.

0.6ml samples of neutrophils (1×10^7 cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and for 15 minutes with the various drugs at the concentrations indicated. The cells were then challenged with ionomycin (1μM) for a further 10 minutes and the production of LTB_4 determined. The results are expressed as the percentage of the amount of LTB_4 produced by ionomycin alone (approximately 60ng/ml) and are mean values ± SEM for four experiments, each performed in triplicate.

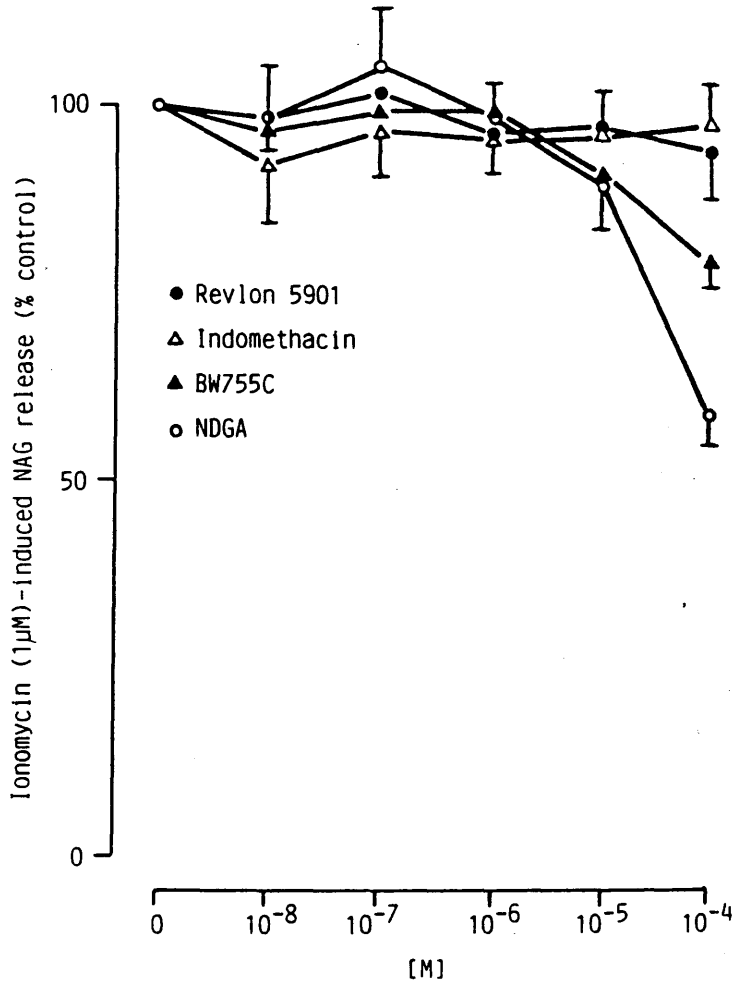


Figure 86.

The effect of agents that interfere with eicosanoid production on ionomycin-induced NAG release by human neutrophils.

0.6ml samples of neutrophils (1×10^7 cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and for 15 minutes with the various drugs at the concentrations indicated. The cells were then challenged with ionomycin (1μM) for a further 10 minutes and the release of NAG determined. The results are expressed as the percentage release of NAG induced by ionomycin alone and are mean values ± SEM for four experiments, each performed in triplicate.

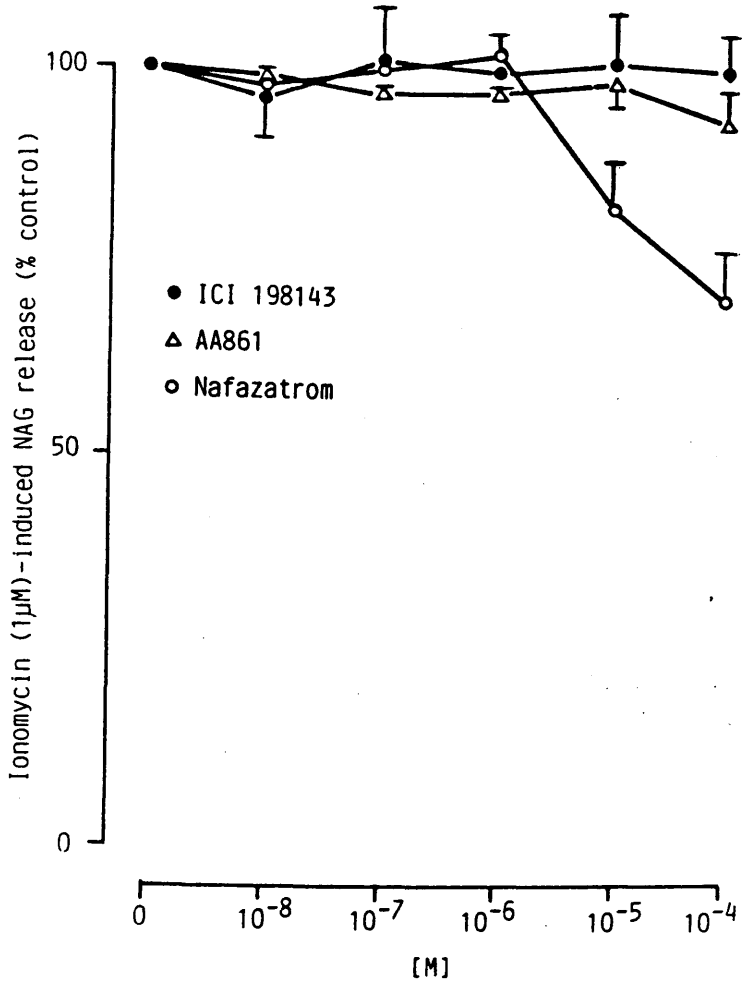


Figure 87.

The effect of agents that interfere with eicosanoid production on ionomycin-induced NAG release by human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and for 15 minutes with the various drugs at the concentrations indicated. The cells were then challenged with ionomycin (1μM) for a further 10 minutes and the release of NAG determined. The results are expressed as the percentage release of NAG induced by ionomycin alone and are mean values ± SEM for four experiments, each performed in triplicate.

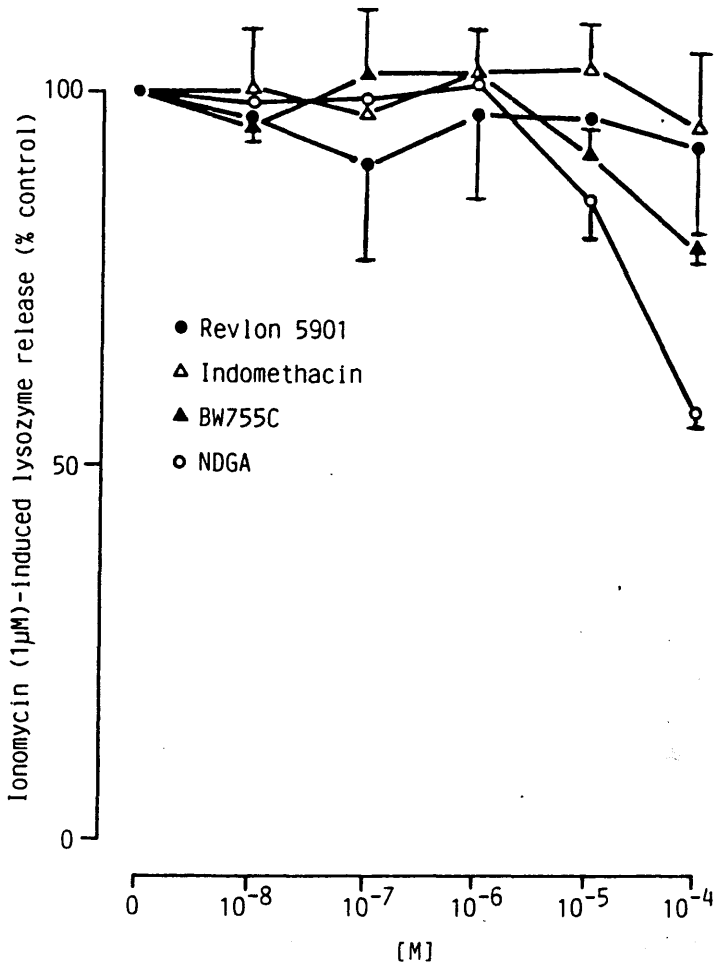


Figure 88.

<u>The effect of agents that interfere with eicosanoid production on ionomycin-induced lysozyme release by human neutrophils.</u>

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and for 15 minutes with the various drugs at the concentrations indicated. The cells were then challenged with ionomycin (1μM) for a further 10 minutes and the release of lysozyme determined. The results are expressed as the percentage release of lysozyme induced by ionomycin alone and are mean values ± SEM for four experiments, each performed in triplicate.

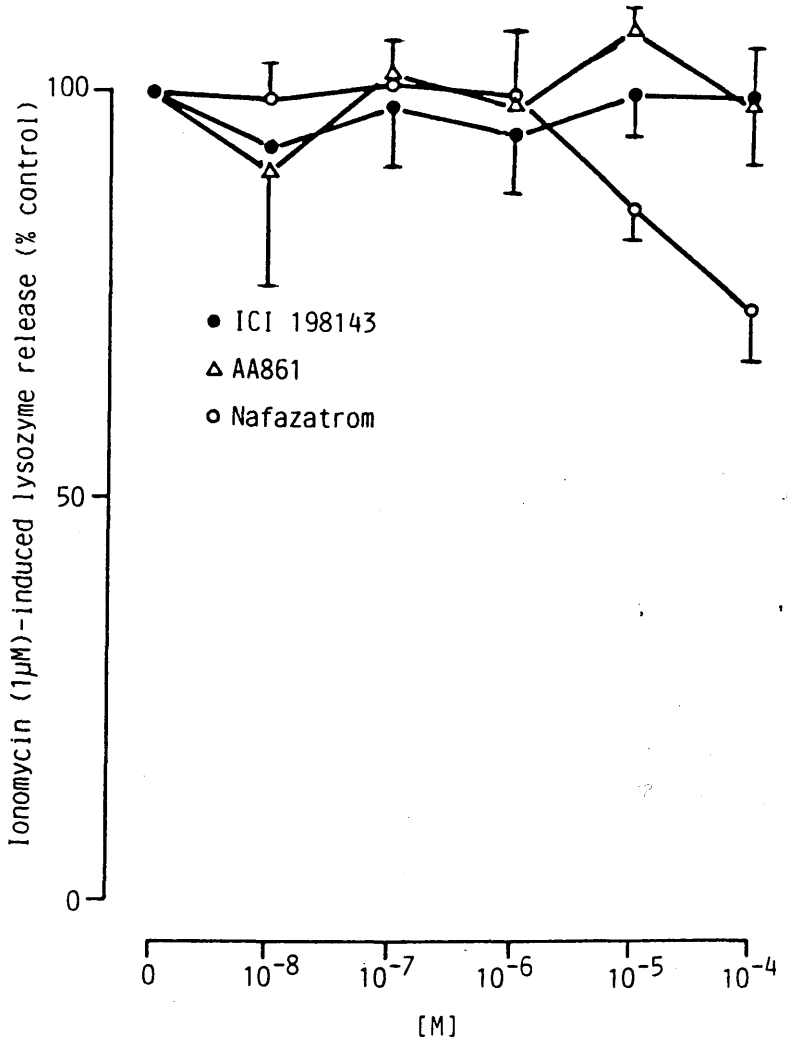


Figure 89.

The effect of agents that interfere with eicosanoid production on ionomycin-induced lysozyme release by human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and for 15 minutes with the various drugs at the concentrations indicated. The cells were then challenged with ionomycin (1μM) for a further 10 minutes and the release of lysozyme determined. The results are expressed as the percentage release of lysozyme induced by ionomycin alone and are mean values ± SEM for four experiments, each performed in triplicate.

Figure 90.

The effect of Revlon 5901 on agonist-induced enzyme release from human neutrophils.

0.6ml samples of neutrophils (1×10^7 cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5μg/ml) and for 15 minutes with Revlon 5901 at the concentrations indicated. The cells were then challenged with an agonist for a further 10 minutes and the enzyme release determined. The results are expressed as the percentage of the total enzyme release induced by the agonist alone.

(a) FMLP (1μM; 27.2% of total NAG).

(b) FMLP (1μM; 38.9% of total lysozyme).

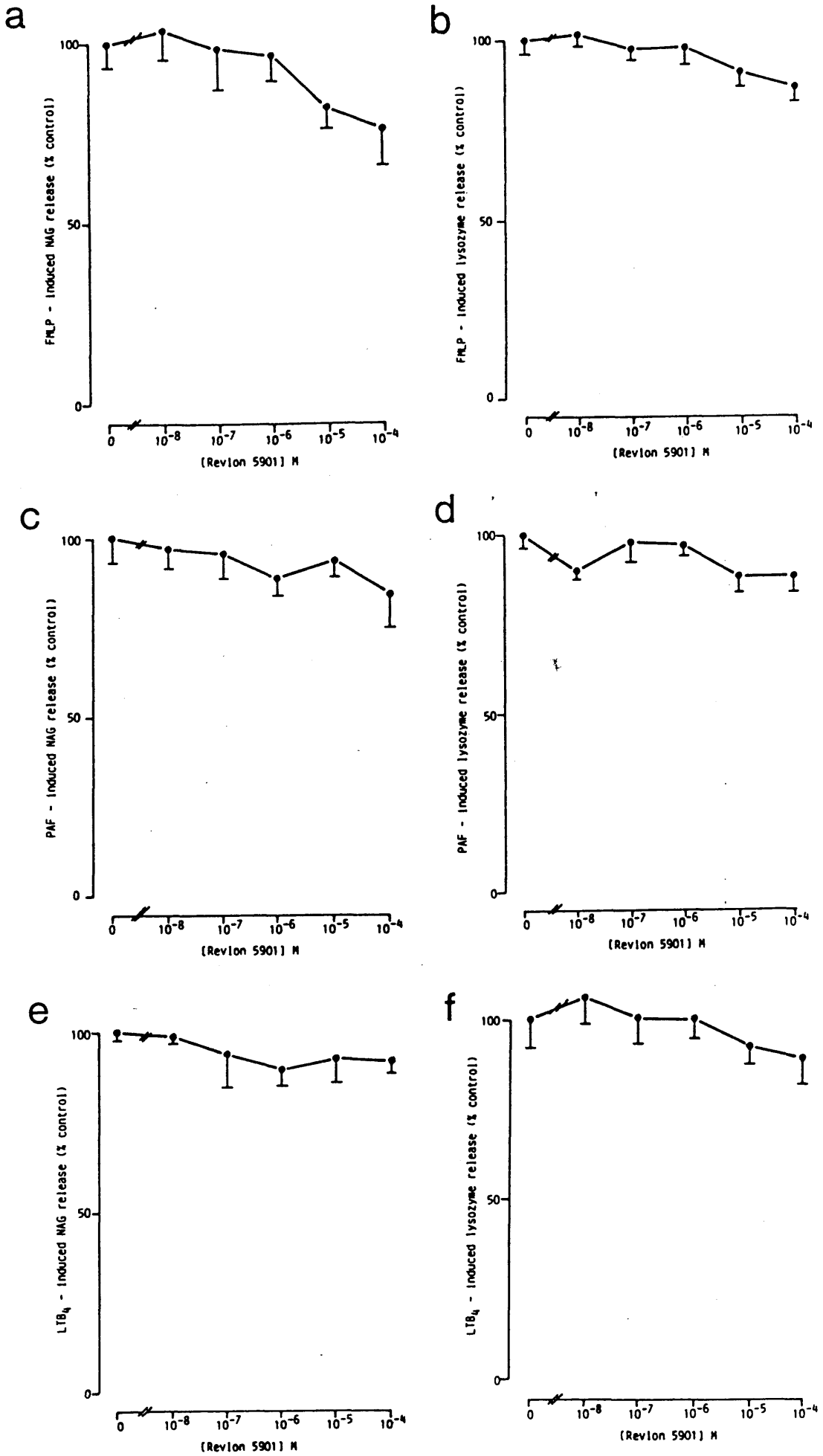
(c) PAF (1.8μM; 21.2% of total NAG).

(d) PAF (1.8μM; 32.0% of total lysozyme).

(e) LTB_4 (1.5μM; 21.4% of total NAG).

(f) LTB_4 (1.5μM; 30.3% of total lysozyme).

For each individual agonist the results are mean values ± SEM for three experiments, each performed in triplicate.


a
FMLP - induced NAG release (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
b
FMLP - induced lysozyme release (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
c
PAF - induced NAG release (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
d
PAF - induced lysozyme release (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
e
LTB4 - induced NAG release (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
f
LTB4 - induced lysozyme release (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M

the presence of varying concentrations of Revlon 5901. The effect of Revlon 5901 on control concentrations of ionomycin (1μM), FMLP (1μM), PAF (1.8μM) and LTB_4 (1.5μM) is shown in figures 91a,b,c and d respectively. This specific lipoxygenase inhibitor did not significantly affect the aggregatory response elicited by any of the stimuli.

2. Effect of the PAF receptor antagonist, Kadsurenone:-

2.1. On agonist-induced aggregation.

The effect of kadsurenone on agonist-induced aggregation was investigated. Samples were either preincubated with varying concentrations of the PAF receptor antagonist or with DMSO (as control) for 10 minutes at 37°C and aggregation was induced by PAF (1.8μM), FMLP (1μM) and LTB_4 (1.5μM). Kadsurenone inhibited PAF-induced aggregation in a concentration-dependent manner with an IC_{50} value of ~800nM (figure 92). The aggregatory responses elicited by FMLP (figure 93a) and LTB_4 (figure 93a) were unaffected by kadsurenone preincubation.

2.2. On agonist-induced degranulation.

Once again kadsurenone inhibited PAF (1.8μM)-induced release of both NAG (figure 94) and lysozyme (figure 95) with IC_{50} values of ~40nM and ~10nM respectively. Degranulation induced by FMLP (figures 96a and 96b) and LTB_4 (figures 96c and 96d) was virtually unaffected by this specific PAF receptor antagonist. However, the high concentration of 10μM kadsurenone exerted only a marginal, but significant, inhibitory effect on LTB_4-induced secretion of lysozyme.

2.3. On ionomycin-induced LTB_4 generation.

The effect of kadsurenone on ionomycin (1μM)-induced LTB_4

Figure 91.

The effect of Revlon 5901 on agonist-induced aggregation in human neutrophils.

0.6ml samples of neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were pretreated with Revlon 5901 (15 minutes) at the concentrations indicated. The cells were then challenged with an agonist and the changes in light transmission recorded. The results are expressed as the percentage of the response induced by the agonist alone.

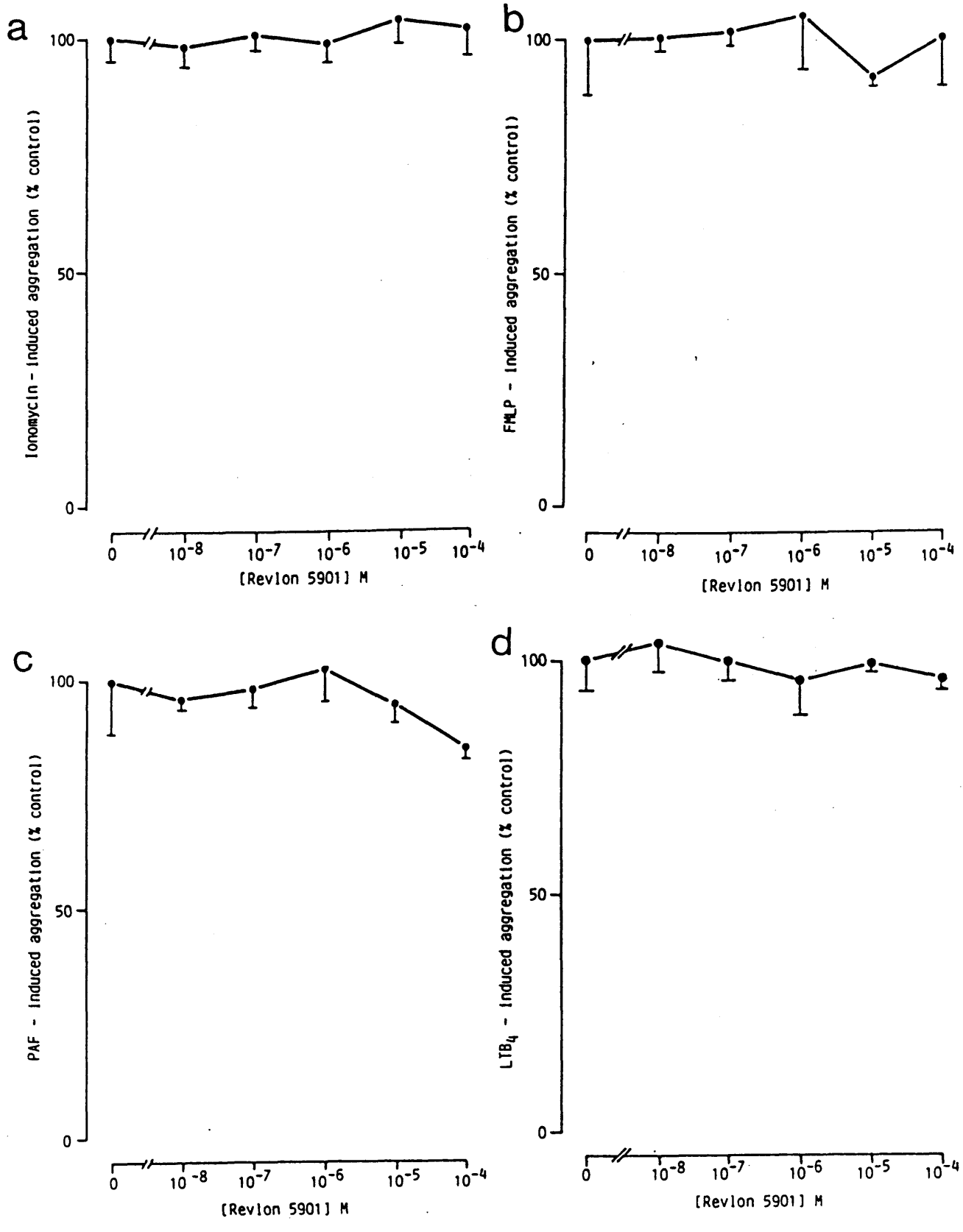
(a) Ionomycin (1μM).

(b) FMLP (1μM).

(c) PAF (1.8μM).

(d) LTB_4 (1.5μM).

For each individual agonist the results are mean values ± SEM for three experiments, each performed in triplicate.


a
Ionomycin - induced aggregation (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
b
FMLP - induced aggregation (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
c
PAF - induced aggregation (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M
d
LTB4 - induced aggregation (% control)
100
50
0
0
10-8
10-7
10-6
10-5
10-4
[Revlon 5901] M

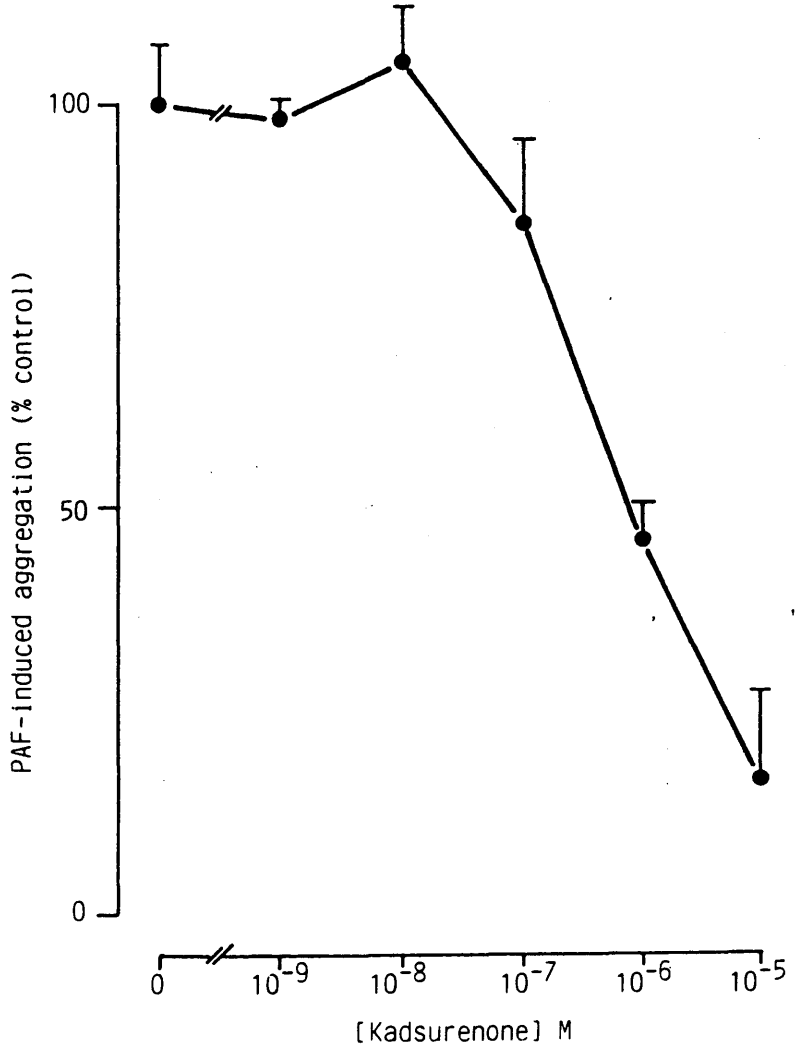


Figure 92.

The effect of kadsurenone on PAF-induced aggregation in human neutrophils.

0.6ml samples of neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were pretreated with kadsurenone for 10 minutes at the concentrations indicated. The cells were then challenged with PAF (1.8μM) and the changes in light transmission recorded. The results are expressed as the percentage of the response induced by PAF alone. The results are mean values ± SEM for three experiments, each performed in triplicate.

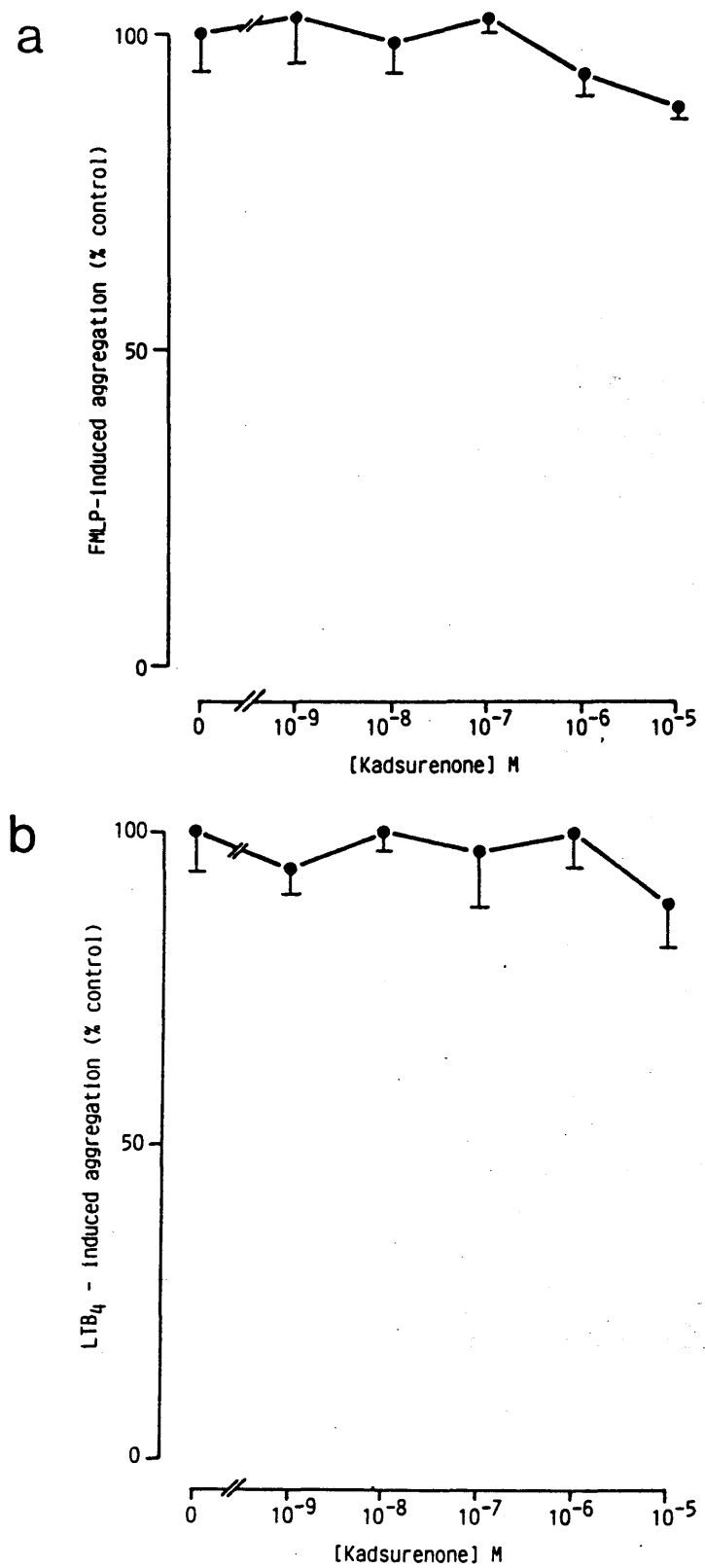


Figure 93.
The effect of kadsurenone on agonist-induced aggregation in human neutrophils.
0.6ml samples of neutrophils (2.5×10^7 cells/ml) were added to a cuvette containing a teflon-coated stirring bar revolving at 600rpm. The cells were pretreated with kadsurenone for 10 minutes at the concentrations indicated. The cells were then challenged with an agonist and the changes in light transmission recorded. The results are expressed as the percentage of the response induced by agonist alone.
(a) FMLP (1μM).
(b) LTB_4 (1.5μM).
For each individual agonist the results are mean values ± SEM for three experiments, each performed in triplicate.

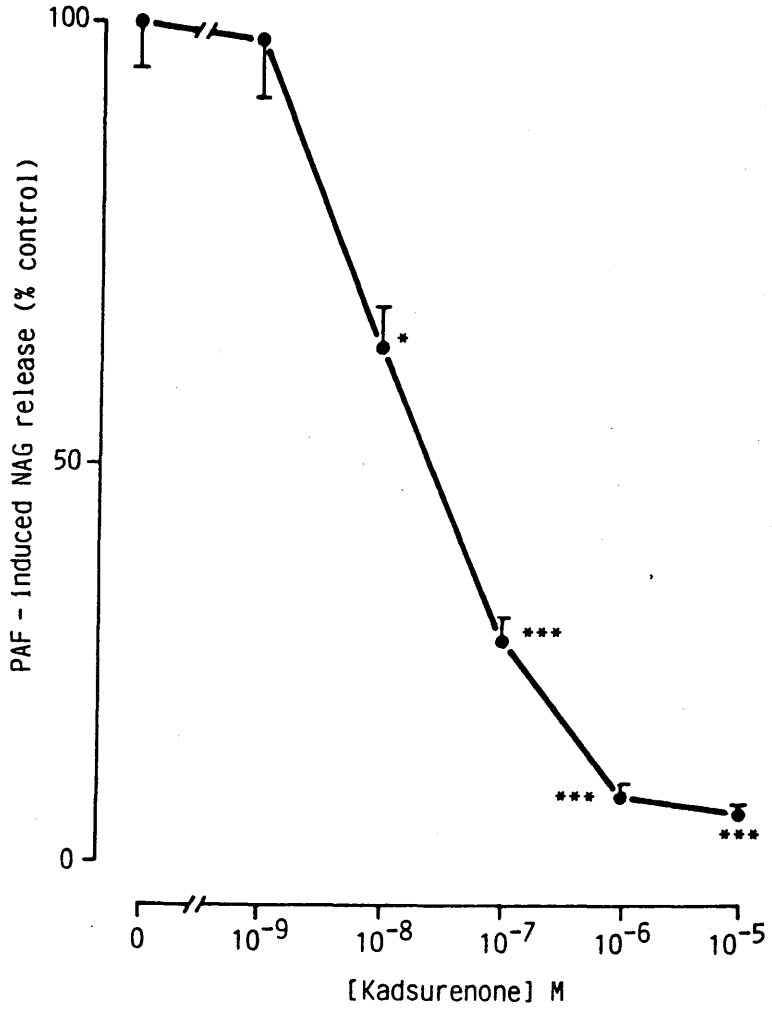


Figure 94.

The effect of kadsurenone on PAF-induced NAG release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated for 10 minutes with cytochalasin B (5µg/ml) and kadsurenone at the concentrations indicated. The cells were then challenged with PAF (1.8µM) for a further 10 minutes and the release of NAG determined. The results are expressed as the percentage of the release induced by PAF alone (16.9% of total NAG). The results are mean values ± SEM for three experiments, each performed in triplicate. Analysis of results: *$P < 0.05$; ***$P < 0.001$.

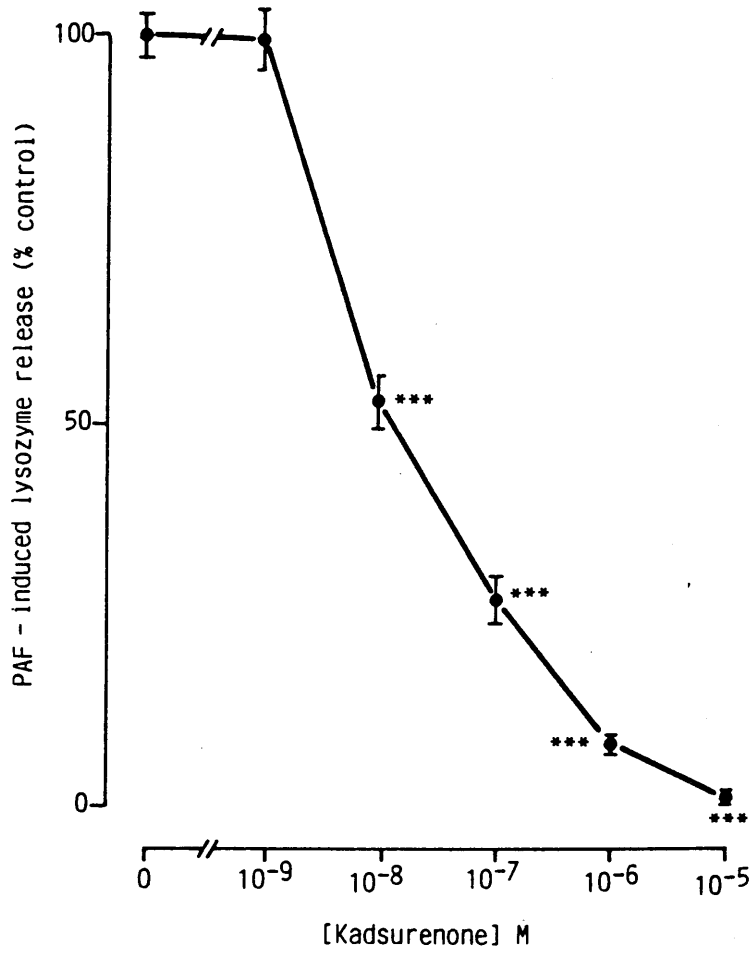


Figure 95.

The effect of kadsurenone on PAF-induced lysozyme release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated for 10 minutes with cytochalasin B (5μg/ml) and kadsurenone at the concentrations indicated. The cells were then challenged with PAF (1.8μM) for a further 10 minutes and the release of lysozyme determined. The results are expressed as the percentage of the release induced by PAF alone (28.6% of total lysozyme). The results are mean values ± SEM for three experiments, each performed in triplicate. Analysis of data: ***$P < 0.001$.

Figure 96.

The effect of kadsurenone on agonist-induced enzyme release from human neutrophils.

0.6ml samples of neutrophils (1 x 10^7cells/ml) were pretreated, for 10 minutes, with cytochalasin B (5µg/ml) and kadsurenone at the concentrations indicated. The cells were then challenged with an agonist for a further 10 minutes and the enzyme release determined. The results are expressed as the percentage of the total enzyme release induced by the agonist alone.

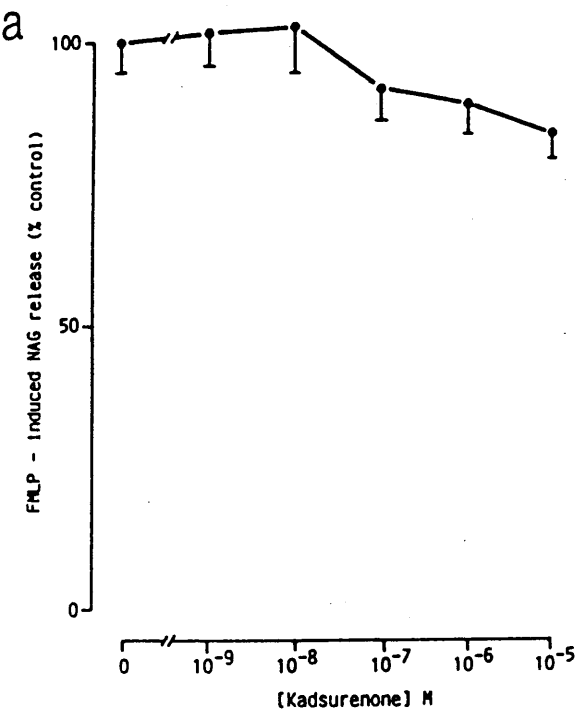
(a) FMLP (1µM; 21.2% of total NAG).

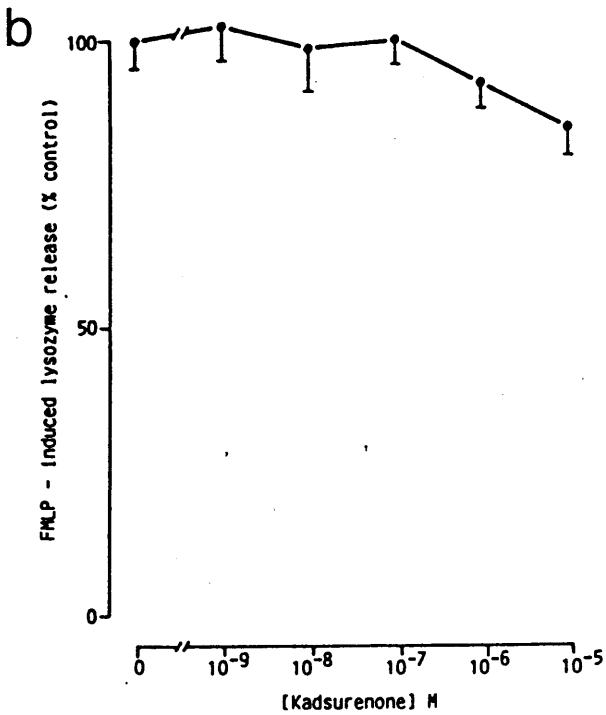
(b) FMLP (1µM: 32.9% of total lysozyme).

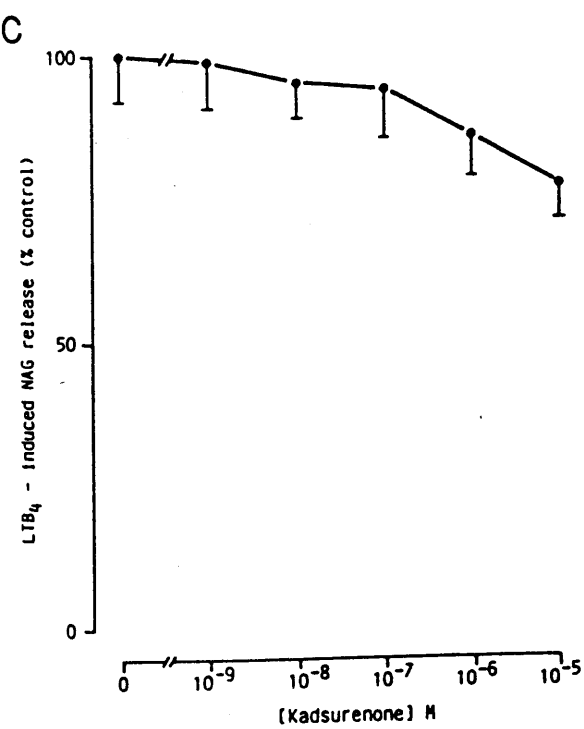
(c) LTB_4 (1.5µM; 16.4% of total NAG).

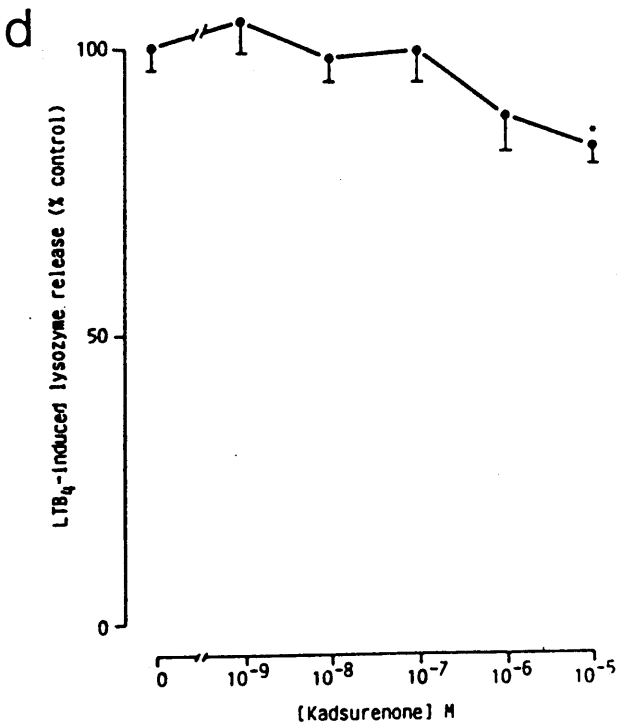
(d) LTB_4 (1.5µM; 26.5% of total lysozyme).

For each individual agonist the results are mean values ± SEM for three experiments, each performed in triplicate. Analysis of data: $P^* < 0.05$.


a
100
50
0
FMLP - Induced NAG release (% control)
0
10-9
10-8
10-7
10-6
10-5
[Kadsurenone] M


b
100
50
0
FMLP - Induced lysozyme release (% control)
0
10-9
10-8
10-7
10-6
10-5
[Kadsurenone] M


c
100
50
0
LTB4 - Induced NAG release (% control)
0
10-9
10-8
10-7
10-6
10-5
[Kadsurenone] M


d
100
50
0
LTB4-induced lysozyme release (% control)
0
10-9
10-8
10-7
10-6
10-5
[Kadsurenone] M

generation in human neutrophils was also examined (figure 97). The response elicited by 1μM ionomycin (approximately 60ng/ml) was taken as the control and the effect of kadsurenone (10 minute preincubation at 37°C), in the concentration range of 1nM-10μM, was expressed as a percentage of that control. There was no significant reduction in the generation of LTB_4 in the presence of this inhibitor.

3. Effect of LTB_4 on agonist-induced $[^{32}P]$-PtdA formation.

LTB_4, being a potent independent activator of human neutrophils, is also capable of potentiating the neutrophil functional responses evoked by other agonists. Since LTB_4 did not elicit a significant production of $[^{32}P]$-PtdA *per se*, there exists the possibility that this arachidonic acid metabolite may augment phosphoinositide metabolism induced by other agonists. Consequently, I investigated whether LTB_4 had an effect on agonist-induced $[^{32}P]$-PtdA formation.

Submaximal concentrations of FMLP (0.1μM) and PAF (0.9μM) that induce 143 ± 9 (figure 98) and 145 ± 19% (figure 99) $[^{32}P]$-PtdA formation respectively were used as positive controls. A preincubation of 2 minutes with LTB_4 (0.1nM-10μM) did not augment or attenuate $[^{32}P]$-PtdA formation evoked by other agonists.

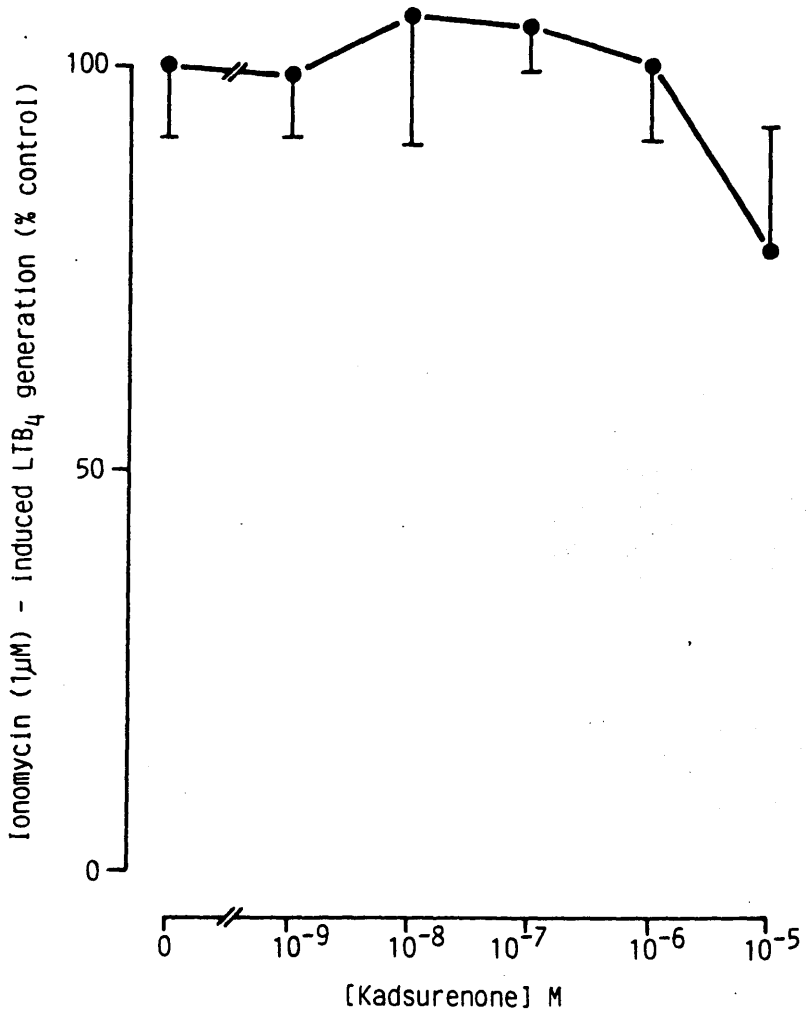


Figure 97.

The effect of kadsurenone on ionomycin-induced LTB_4 generation by human neutrophils.

0.6ml samples of neutrophils (1 x 10^7 cells/ml) were pretreated for 10 minutes with kadsurenone at the concentrations indicated and then challenged with ionomycin (1μM) for a further 10 minutes and the production of LTB_4 determined. The results are expressed as the percentage of the amount of LTB_4 produced by ionomycin alone (approximately 45ng/ml) and are mean values ± SEM for three experiments, each performed in triplicate.

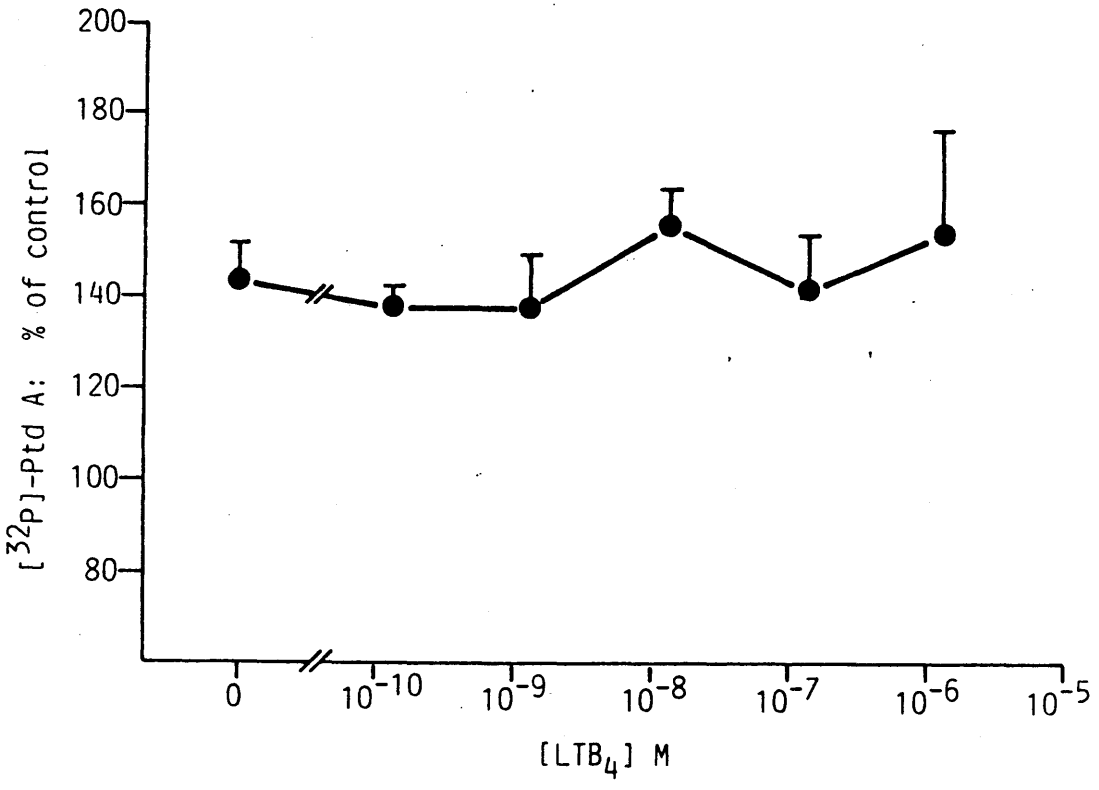


Figure 98.

The effect of LTB_4 on FMLP-induced $[^{32}P]$-PtdA formation.

0.4ml samples of neutrophils (2.5×10^7 cells/ml) were pretreated with LTB_4 for 2 minutes at the concentrations indicated. The cells were then challenged with FMLP (0.1μM) for a further 2 minutes and the production of $[^{32}P]$-PtdA determined. The results are mean values ± SEM for four experiments, each performed in triplicate.

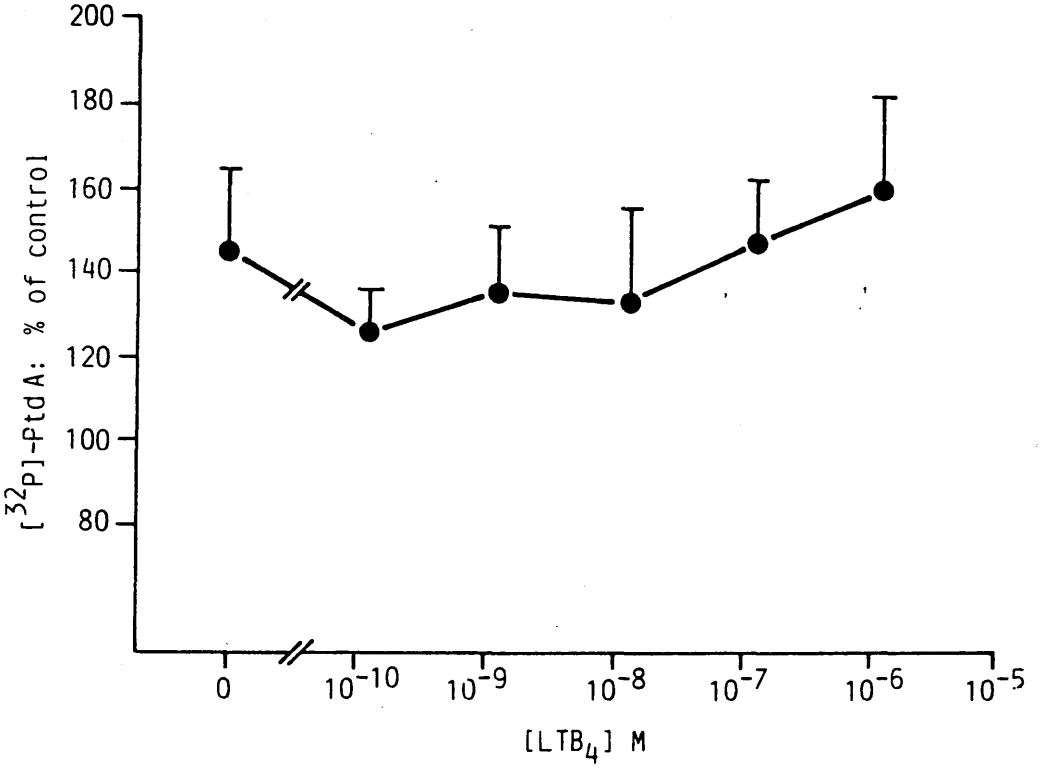


Figure 99.

The effect of LTB_4 on PAF-induced $[^{32}P]$-PtdA formation.

0.4ml samples of neutrophils (2.5×10^7 cells/ml) were pretreated with LTB_4 for two minutes at the concentrations indicated. The cells were then challenged with PAF (0.9uM) for a further 2 minutes and the production of $[^{32}P]$-PtdA determined. The results are mean values $\pm$ SEM for four experiments, each performed in triplicate.

4. Discussion.

Products derived from membrane phospholipids via activation of phospholipase A_2, namely LTB_4 and PAF, appear to be the most potent known stimulators of human neutrophils eliciting a whole repertoire of neutrophil functional responses including aggregation and degranulation. Many agents that activate neutrophils are also known to enhance the production of LTB_4 and PAF. However, to what extent these products contribute to the activation of neutrophils induced by other stimuli has not been clearly elucidated. Research into this area has been hampered by the lack of selective biosynthesis inhibitors and specific receptor antagonists.

Attenuation of the intracellular levels of 5-lipoxygenase products by 5-lipoxygenase inhibitors may determine the role of arachidonic acid metabolites in neutrophil function. In this study I investigated a number of agents known to interfere with eicosanoid production on ionomycin-induced LTB_4 biosynthesis. All the agents used, with varying degrees of potency and efficacy, inhibited production of LTB_4 induced by the calcium ionophore (figures 84 and 85). The same seven inhibitors were used to determine the potential contribution of endogenous LTB_4 to ionomycin-induced enzyme release. Of all the agents tested only NDGA (Showell et al., 1980), BW755C (Higgs et al., 1979) and nafazatrom (Honn & Dunn., 1982), at very high concentrations, inhibited enzyme release. At these concentrations (100μM), LTB_4 biosynthesis was almost completely inhibited whereas enzyme release was only partly attenuated. The cyclooxygenase inhibitor, indomethacin; and the putative 5-lipoxygenase inhibitors, Revlon 5901 (Coutts et al., 1985), ICI 198143 and AA861 (Ashida et al., 1983) did not inhibit either ionomycin-induced NAG or lysozyme release. The fact that the inhibitory effects of certain agents on neutrophil degranulation did not correlate closely with the degree of

inhibition of LTB_4 biosynthesis and that other more selective 5-lipoxygenase inhibitors did not attenuate neutrophil activation clearly suggests that products of 5-lipoxygenase are not be involved in ionomycin-induced enzyme release.

In order to investigate whether a similar conclusion could be made with neutrophil activation induced by the receptor directed agonists, I employed the more selective 5-lipoxygenase inhibitor, Revlon 5901, and studied its effect on agonist-induced degranulation and aggregation. This compound in the concentration range of 10nM-100μM did not significantly inhibit FMLP-, PAF- and LTB_4-induced enzyme release or aggregation, suggesting that neutrophil responsiveness elicited by these stimuli must occur independently of LTB_4 synthesis.

There are several reports in the literature suggesting that products of 5-lipoxygenase are indeed mediating neutrophil responsiveness elicited by other (exogenous) agonists. For example, Smolen & Weissmann, (1980) using human neutrophils demonstrated that the acetylenic derivative of arachidonic acid, 5,8,11,14-eicosatetraynoic acid (ETYA) inhibited A23187, FMLP and zymosan induced lysosomal enzyme release. Similar observations were made with the putative selective inhibitor of lipoxygenase, 5,8,11, Eicosatriynoic acid (ETI) (Marone et al., 1983). These authors concluded that products of arachidonic acid metabolism play an essential role in neutrophil activation and secretion. Caution must be taken when interpreting data using the above agents since they are relatively unselective and exert many other effects within the cell. Smith et al., (1986) demonstrated that aggregated IgG caused a concentration-dependent release of both lysozyme and myeloperoxidase that was inhibited by U-60,257 (piriprost potassium),another reported lipoxygenase inhibitor. These results indicate that a lipoxygenase product may mediate aggregated IgG enzyme release in human neutrophils.

Contrasting observations were made by Palmer & Salmon, (1985) who

showed that although BW755C inhibited LTB_4 biosynthesis in human neutrophils, it did not affect A23187-induced degranulation and was only a weak inhibitor of FMLP-induced degranulation. A recent investigation by Ozaki et al., (1986) compared a variety of inhibitors of the lipoxygenase pathway on a number of neutrophil parameters. They showed that U-60,257 had virtually no effect on superoxide production and degranulation and chemotaxis was only marginally suppressed. They also demonstrated that compounds used previously to implicate LTB_4 involvement exert other effects within the cell. For example, NDGA and esculitin inhibited NADPH oxidase, the enzyme complex involved in oxygen radical production. Thus interpretation of data generated using such compounds is not straightforward.

Care must be taken whilst using putative 5-lipoxygenase inhibitors since they may not be selective enough or they may interfere with a particular neutrophil response elicited by a particular agonist. However, my investigations suggest that LTB_4 may not be involved in aggregation and degranulation induced by other agonists.

The inhibition of PAF production may also help determine whether this ether lipid is involved in neutrophil activation elicited by other stimuli. This again has been marred by the lack of any specific PAF inhibitors. One indirect approach, that various workers have adopted, is to inhibit phospholipase A_2. Such inhibition would prevent the formation of PAF by preventing the breakdown of the alkyl-acyl-PAF to lyso-PAF, the immediate precursor of PAF. One must bear in mind that the use of phospholipase A_2 inhibitors may equally well be associated with the blockade of the production of arachidonic acid metabolites and other lyso-derivatives. One of the more specific phospholipase A_2 inhibitors is p-bromophenyacyl bromide

which exerts its effect by acetylation of a histidine located on the active site on the enzyme (Volwerk et al.,1974). This compound has been shown to inhibit FMLP, A23187 and serum-treated zymosan induced degranulation in human neutrophils (Smolen & Weissmann, 1980; Marone et al., 1983). Perhaps a more direct way to prevent the effects of any PAF production induced by other agonists would be to employ selective PAF receptor antagonists. There are a number of such receptor antagonists with varying degrees of specificity (Braquet & Godfroid, 1986).

One such compound, that I examined in this study, is the neolignan isolated from a Chinese herb (Caulis piperis futokadsurae) which has considerable antirheumatic and antiallergic properties. This drug, kadsurenone as it is known, was first discovered as a potent inhibitor of the binding of $[^3H]$-PAF to rabbit platelet membrane preparations where it displayed an IC_{50} of 0.1μM and a Kd of 58nM (Shen et al., 1985). Kadsurenone has also been shown to inhibit PAF-induced aggregation of platelets (Hwang et al., 1985) and PAF-induced aggregation and degranulation of human neutrophils (Shen et al., 1985). The effect of this specific PAF receptor antagonist on neutrophil activation induced by PAF, FMLP, LTB_4 and ionomycin was investigated. Kadsurenone caused a concentration-dependent inhibition of PAF-induced aggregation, NAG release and lysozyme release with virtually no effect on these responses elicited by the other stimuli. In order to check that this compound did not interfere with LTB_4 biosynthesis, by for example inhibiting phospholipase A_2 and/or 5-lipoxygenase, kadsurenone was tested against ionomycin-induced LTB_4. Kadsurenone did not attenuate LTB_4 generation.

From the above evidence, albeit circumstantial, it is suggested that LTB_4 and PAF are not mediating neutrophil activation elicited by

other stimuli. Whether aggregation and degranulation are the best indicators of neutrophil responsiveness is a matter of debate but, with the development of selective biosynthesis inhibitors and specific receptor antagonists, a more complete picture may be forthcoming.

CHAPTER 8. GENERAL CONCLUSIONS: LIPID MEDIATORS OF HUMAN NEUTROPHIL ACTIVATION.

The neutrophil is the predominant leucocyte in human blood and its main functions are to protect the body against invading organisms and to promote general tissue repair and maintenance. Hyperactivity of neutrophils resulting in the over-exuberant release of lysosomal enzymes, lipid autacoids and toxic oxygen metabolites may be one of the most important factors underlying chronic inflammatory conditions such as rheumatoid arthritis. Investigation of the control and regulatory mechanisms mediating the activation of isolated neutrophils may assist in the understanding of the *in vivo* function of these cells. Exposure of human neutrophils to a variety of particulate (e.g. immune complexes and zymosan) and soluble (e.g. C5a, FMLP, PAF and LTB_4) stimuli evoke a series of cellular responses including chemotaxis, aggregation, degranulation and superoxide generation. Also formed are numerous lipid products derived initially from acyl hydrolase action on membrane phospholipids. Such acyl hydrolase activation results in:

(i) The liberation of arachidonic acid and its subsequent conversion by the 5-lipoxygenase pathway to yield LTB_4 and 5-HETE.

(ii) The hydrolysis of 1-O-alkyl-2-acyl-sn-glyceryl-3-phosphorylcholine to yield free fatty acids (including arachidonic acid) and lyso PAF which can be converted to PAF by an acetyl transferase enzyme.

LTB_4 and PAF may thus be formed concomitantly in activated neutrophils from a common precursor. Since these lipids are potent independent activators of human neutrophils, they may also act as a positive feedback mechanism promoting further neutrophil activation. For example, formation of C5a at sites of inflammation or the release of bacterial metabolites at the site of invasion evokes the accumulation of neutrophils. These activated neutrophils, by releasing LTB_4 and PAF, trigger a second wave of neutrophil accumulation and activation. Alternatively, both lipids may be intracellular mediators or amplifiers of responses induced by other neutrophil activators such as C5a and FMLP.

Ultrastructural changes, aggregation and the release of both NAG and lysozyme induced by LTB_4 and PAF do not appear to differ markedly from the same responses evoked by the other receptor-directed agonist, namely FMLP (Chapter 5). The changes in $\left[Ca^{2+}\right]i$ and PtdA levels induced by PAF may well indicate that phosphoinositide hydrolysis is mediating neutrophil activation elicited by this ether lipid. As these events ($\left[Ca^{2+}\right]i$ elevation and PtdA formation) are

pertussis toxin sensitive and are inhibited by protein kinase C activators, the normal agonist-induced physiological response may be regulated by a G-protein and protein kinase C. The lack of effect of LTB_4 on PtdA formation or on PtdIns, PIP and PIP_2 breakdown suggests either that this lipid stimulates cellular responsiveness independently of phosphoinositide hydrolysis or that the technique of prelabelling with $[^{32}P]$-orthophosphate is too insensitive to detect small changes in inositol phospholipid degradation. Whichever the case, the results suggest that there is both a quantitative and qualitative difference between the changes in the levels of the inositol phospholipids and PtdA induced by the ether lipid and the chemotactic tripeptide and the lack of response observed with the arachidonic acid metabolite. Similar observations have been made in the platelet where ADP causes an elevation of $[Ca^{2+}]i$ without an apparent phosphoinositide hydrolysis (MacIntyre et al., 1986; Fisher et al., 1985). Another intriguing observation is that neutrophil activation elicited by LTB_4 is also inhibited by pertussis toxin and by preincubation with PMA (ergo stimulation of a G protein and protein kinase C respectively; discussed in Chapter 6). Therefore, the precise mechanism of action of this lipid still remains to be established. The fact that the concentration-response curve for the elevation of $[Ca^{2+}]i$ lies to the left of the curves for the other indices of neutrophil activation induced by all receptor-directed agonists may suggest that it is unlikely that the rise in

$[Ca^{2+}]i$ *per se* mediates these responses. Consequently $[Ca^{2+}]i$ elevation may trigger an intracellular amplification sequence or invoke formation of other mediators which act alone or in concert with this divalent cation.

As chemoattractants undoubtably are present in low concentrations at locations distal to the initial site of inflammation, an attractive *in vivo* application of these latter observations may be that chemotaxis is mediated by an elevation of $[Ca^{2+}]i$. Agonist-induced neutrophil locomotion and rises in $[Ca^{2+}]i$ are usually produced by lower concentrations of stimuli than the other cellular responses. As the cells move closer to the site of injury, the number of agonist/receptor interactions will increase with the increasing concentration of agonist. This, in turn, may trigger the other responses such as degranulation and release of toxic oxygen metabolites mediated, perhaps, by protein kinase C activation produced as a consequence of phosphoinositide hydrolysis or by other as yet unidentified stimulus-response coupling mechanisms.

The results presented in Chapter 5 also show that neutrophils are capable of producing large amounts of LTB_4 stimulated by the non-physiological stimulus, the calcium ionophore; ionomycin. However, other more physiological stimuli (FMLP and PAF) failed to generate similar levels of this eicosanoid. The observations made with the specific PAF receptor antagonist, kadsurenone, and the selective 5-lipoxygenase inhibitor, Revlon

5901, indicate that release of PAF and LTB_4 is unlikely to contribute to human neutrophil activation induced by other stimuli (Chapter 7).

Whether the data reported with lipid activation of isolated neutrophils can be extrapolated to a more *in vivo* interpretation awaits further investigation, the outcome of which may help in the understanding of many acute and chronic inflammatory conditions.

LITERATURE CITED

1. ALPHABETICAL INDEX OF REFERENCES.

ABDEL-LATIF A.A. (1986)
Pharmacol. Rev., 38: 227-272

ALLAN D. & MICHELL R.H. (1977)
Biochem. J., 164: 389-397

ALLISON F. & LANCASTER M.G. (1964)
Ann. NY. Acad. Sci., 116: 936-944

ALONSO F., GIL M.G., SANCHEZ-CRESPO M. & MATO J.M. (1982)
J. Biol. Chem., 275: 3376-3378

ANDERSON D.C., WIBLE L.J., HUGHES B.J., SMITH C.W. & BRINKLEY B.R. (1982)
Cell., 31: 719-729

ANDERSSON T., SCHLEGEL W., MONOD A., KRAUSE K-H., STENDAHL O. & LEW D.P. (1986)
Biochem. J., 240: 333-340

APPLEMAN M.M., ARIANO M.A., TAKEMOTO D.L. & WHITSON R.H. (1982)
Handbook Exp. Pharmacol., 58: 261-300

ASHIDA Y., SAIJO T., KURIKI H., MAKINO H., TERAO S. & MAKI Y. (1983)
Prostaglandins, 26: 955-972

ATHENS J.W. (1970)
In "Regulation of Haematopoesis". Vol. 2., Appleton-Century Crofts, New York. pp. 1143-1166

BABOIR B.M., KIPNES R.S. & CURNETTE J.T. (1973)
J. Clin. Invest., 52: 741-744

BADWEY J.A., CURNETTE J.T. & KARNOVSKY M.L. (1981)
J. Biol. Chem., 256: 12640-12643

BAGGIOLINI M. & DEWALD B. (1985)
Int. Archs. Allergy. Appl. Immun., 76 (suppl): 13-20

BAGGIOLINI M. (1980)
In "Handbook Inflam" Vol. 2; Chapter 6; pp. 163-188

BAGGIOLINI M., HIRSCH J.G., DE DUVE C. (1970)
J. Cell Biol., 45: 586-597

BAGGIOLINI M., BRETZ U. & DEWALD (1978)
In "Neutral Proteases of Human PMN".
Baltimore-Munich., pp. 3-17

BAINTON D.F. (1980)
In "Handbook Inflam" Vol. 2; Chapter 1: 1-26

BARBARO J.F. & ZVAIFLER N.J. (1966)
Proc. Exp. Biol. Med., 122: 1245-1247

BATTY I.R., NAHORSKI S.R. & IRVINE R.F. (1985)
Biochem. J., 232: 211-215

BECKER E.L. & SHOWELL H.J. (1972)
Z. Immunitaetsforsch. Exp. Ther., 143: 466-476

BENNETT J.P., COCKCROFT S. & GOMPERTS B.D. (1980)
Biochim. Biophys. Acta., 601: 584-591

BENVENISTE J. & ARNOUX B. (1983)
In "Platelet-activating factor"
INSERM Symp. 23, Elsevier Science Publications

BENVENISTE J. & VARGAFTIG B.B. (1983)
Trends Pharmacol. Sci. 4: 341-343

BENVENISTE J., HENSON P.M. & COCHRANE C.G. (1972)
J. Exp. Med., 136: 1356-1377

BENVENISTE J., TENCE M., VARENNE P., BIDAULT J., BOULLET C.
& POLONSKY J. (1979)
C.R. Acad. Sci. (Paris)., 289: 1037-1040

BERGMAN M.J., GUERRANT R.L., MURAD F., RICHARDSON S.H.,
WEAVER D. & MANDELL G.L. (1978)
J. Clin. Invest., 61: 227-234

BERRIDGE M.J. (1983)
Biochem J., 212: 849-858

BERRIDGE M.J. (1985)
Scientific American., 253: 142-152

BETZ S.J. & HENSON P.M. (1980)
J. Immunol., 125: 2756-2763

BEVAN S. & WOOD J.N. (1987)
Nature., 328: 20

BLANK M.L., LEE T.C., CRESS F.A., MALONE B., FITZGERALD V. & SNYDER F. (1984)
Biochem. Biophys. Res. Commun., 124: 156-163

BOKOCH G.M. & GILMAN A.G. (1984)
Cell., 39: 301-308

BOLTON T.B. (1979)
Physiol. Rev., 59: 606-718

BOON A.M., BERESFORD B.J. & MELLORS A. (1985)
Biochem. Biophys. Res. Commun., 129: 431-438

BORGEAT P. & SAMUELSSON B. (1979a)
J. Biol. Chem., 254: 2643-2646

BORGEAT P. & SAMUELSSON B. (1979b)
J. Biol. Chem., 254: 7865-7869

BORGEAT P. & SAMUELSSON B. (1979c)
Proc. Natl. Acad. Sci. USA., 76: 2148-2152

BOUCEK M. M. & SNYDERMAN R. (1976)
Science, 193: 905-907

BOXER L.A., YODER M., BONSIB S., SCHMIDT M., HO P., JERSILD R. & BAEHNER R.L. (1979)
J. Lab. Clin. Med., 93: 506-514

BOYDEN S. (1962)
J. Exp. Med., 115: 453-466

BRADFORD P.G. & RUBIN R.P. (1985)
Mol. Pharmacol., 27: 74-78

BRANDT S.J., DOUGHERTY R.W., LAPETINA E.G. & NEIDEL J.E. (1985)
Cell. Biol., 82: 3277-3280

BRAQUET P. & GODFROID J.J. (1986)
Trends. Pharmacol. Sci., 7: 397-403

BRASS L.F. & JOSEPH K.J. (1985)
J. Biol. Chem., 260: 15172-15179

BRAY M. (1983)
Brit. Med. Bull., 39: 249-254

BROCK T. A., RITTENHOUSE S. E., POWERS C. W., EKSTEIN L. S., GIMBRONE M. A. & ALEXANDER R. W. (1985)
J. Biol. Chem., 260: 14158-14162

BROEKMAN M.J. (1984)
Biochem. Biophys. Res. Commun., 120: 226-231

BRYANT R.E., DES PREZ R.M., VAN WAY M. & ROGERS D.E. (1966)
J. Exp. Med., 124: 483-499

BUCKLEY J.T. & HAWTHORN J.N. (1972)
J. Biol. Chem., 247: 7218-7223

BURGESS G.M., GODFREY P.P., McKINNEY J.S., BERRIDGE M.J., IRVINE R.F. & PUTNEY J.W. (1984a)
Nature, 309: 63-66

BURGESS G.M., McKINNEY J.S., IRVINE R.F., BERRIDGE M.J., HOYLE P.C. & PUTNEY J.W. jr. (1984b)
FEBS. Letters., 176: 193-196

BURGESS G.M., McKINNEY J.S., IRVINE R.F. & PUTNEY J.W. (1985)
Biochem. J., 232: 237-243

BUSHFIELD M., McNICOL A. & MacINTYRE D.E. (1985)
Biochem. J., 232: 267-271

BUSHFIELD M., McNICOL A. & MacINTYRE D.E. (1987)
Biochem. J., 241: 671-676

CAMP R.D.R., COUTTS A.A., GREAVES M.W., KAY A.B. & WALPORT M.J. (1983)
Br. J. Pharmacol., 80: 497-502

CAMPBELL A.K. & HALLETT M.B. (1983)
J. Physiol., 338: 537-550

CAMUSSI G., AGLIETTA M., CODA R., BUSSOLINO F., PIACIBELLO W. & TETTA C. (1981)
Immunol., 42: 191-199

CANTAU B., KEPPENS S., DeWULF H.D. & JARD S. (1980)
J. Receptor Res., 1: 137-168

CAREY F. & FORDER R.A. (1986)
Prostaglandins Leukotrienes Med. 22: 57-70

CARRUTHERS B.M. (1967)
Can. J. Physiol. Pharmacol., 45: 269-280

CASSEL D. & SELINGER Z. (1976)
Biochim. Biophys. Acta., 452: 538-551

CASTAGNA M., TAKAI Y., KAIBUCHI K., SANO K., KIKKAWA U. & NISHIZUKA Y. (1982)
J. Biol. Chem., 257: 7847-7851

CASWELL A.H. (1979)
Int. Rev. Cytol., 56: 145-181

CAUVIN C., LOUTZENHISER R. & VAN-BREEMEN C. (1983)
Ann. Rev. Pharmacol. Toxicol., 23: 373-396

CHANDLER D.E., BENNETT J.P. & GOMPERTS B. (1983)
J. Ultrastruct. Res., 82: 221-232

CHENOWETH D.E. & HUGLI T.E. (1978)
Proc. Natl. Acad. Sci. USA., 75: 3943-3947

CHENOWETH D.E., ERICKSON B.W. & HUGLI T.E. (1979)
Biochem. Biophys. Res. Commun., 86: 227-234

CLAESSON H-E. (1982)
FEBS. Letters., 139: 305-308

CLAESSON H-E., LUNDBERG U. & MALMSTEN C. (1981)
Biochem. Biophys. Res. Commun., 99: 1230-1237

CLANCY R.M., DAHINDEN C.A. & HUGLI T.E. (1983)
Proc. Natl. Acad. Sci. USA., 80: 7200-7204

COBBOLD P.H., CUTHBERTSON K.S.R., COYNS M.H. & RICE V. (1983)
J. Cell. Sci., 61: 123-136

COCHRANE C.G. & JANOFF A. (1974)
In "The Inflam. Resp." III; Acad. Press., New York:
pp. 85-162

COCHRANE C.G., UNANUE E.R. & DIXON F.J. (1965)
J. Exp. Med., 122: 99-114

COCKCROFT S. & GOMPERTS B.D. (1985)
Nature., 314: 534-536

COCKCROFT S. (1984)
Biochim. Biophys. Acad. 795: 37-46

COCKCROFT S., BENNETT J.P. & GOMPERTS B.D. (1980a)
FEBS. Letters., 110: 115-118

COCKCROFT S., BENNETT J.P. & GOMPERTS B.D. (1980b)
Nature., 288: 275-277

COCKCROFT S., BENNETT J.P. & GOMPERTS (1981)
Biochem. J., 200: 501-508

CODINA J., HILDEBRANDT J.D., SEKURA R.D., BIRNBAUMER M., BRYAN J., MANCLARK C.R., IYENGAR R. & BIRNBAUMER (1984)
J. Biol. Chem., 259: 5871-5886

COHEN H.J. & CHOVANIEC M.E. (1978a)
J. Clin. Invest., 61: 1081-1087

COHEN H.J. & CHOVANIEC M.E. (1978b)
J. Clin. Invest., 61: 1088-1096

CONNELLY T.M. & MAJERUS P.W. (1986)
Calcium Metabolism., pp. 1872: abst. 2285

CONNOLLY T.M., LAWING W.J. & MAJERUS P.W. (1986)
Cell., 46: 951-958

COOPER R.H., COLL K.E. & WILLIAMSON J.R. (1985)
J. Biol. Chem., 260: 3281-3288

CORVERA S., SCHWARZ K.R., GRAHAM R.M. & GARCIA-SAINZ J.A. (1986)
J. Biol. Chem., 261: 520-526

COUTTS S.M., KHANDWALA A., VAN INWEGEN R., CHAKRABORTY U., MUSSER J., BRUENS J., JARIWALA N., DALLY-MEADE V., INGRAM R., PRUSS T., JONES H., NEISS E. & WEINRYB I. (1985)
In "Prostaglandins, Leukotrienes and Lipoxins".
pp. 627-637

CRAWFORD J.P., MOVAT H.Z., MINTA J.O. & OPAS M. (1985)
Exp. Mol. Pathol., 42: 175-193

CREYER P.E., JARRET L. & KIPNIS D. (1969)
Biochim. Biophys. Acta., 177: 586-590

CRONKITE E.P. & VINCENT P.C. (1969)
Ser. Haematol., 2: 3-43

CROSS A.R., JONES O.T.G., GARCIA R. & SEGAL A.W. (1982)
Biochem. J., 208: 759-763

CZARNETZKI B.M. & BENVENISTE J. (1981)
Chem. Phys. Lipids., 29: 317-326

DANCEY J.T., DEUBELBEISS K.A., HARKER L.A. & FINCH C.A. (1976)
J. Clin. Invest., 58: 705-715

DAVIS B.H., WALTER R.J., PEARSON C.B., BECKER E.L. & OLIVER J.M. (1982)
Am. J. Path., 108: 206-216

DE SHAZO C.V., HENSON P.M. & COCHRANE C.G. (1972)
J. Clin. Invest., 51: 50-57

DE TOGNI P., CABRINI G. & DI VIRGILIO F. (1984)
Biochem J., 224: 629-635

DELLA BIANCA V., DE TOGNI P., GRZESKOWIAK M., VICENTINI L.M. & DI VIRGILIO F. (1986)
Biochim. Biophys. Acta., 886: 441-447

DEMOPOULOS C.A., PINCKARD R.N. & HANANAN D.J. (1979)
J. Biol. Chem., 254: 9355-9358

DEWALD B., BRETZ U. & BAGGIOLINI M. (1982)
J. Clin. Invest., 70: 518-525

DiROSA M., FLOWER R.J., HIRATA F., PARENTE L. & RUSSO-MARIE F. (1984)
Prostaglandins, 28: 441-442

DiVIRGILIO F., LEW D.P. & POZZAN T. (1984)
Nature., 310: 691-693

DiVIRGILIO F., VICENTINI L.M., TREVES S., RIZ G. & POZZAN T. (1985)
Biochem. J., 229: 361-367

DOLMATCH B. & NIEDEL J. (1983)
J. Biol. Chem., 258: 7570-7577

DOUGHERTY R.W., GODFREY P.P., HOYLE P.C., PUTNEY J.W. & FREER R.J. (1984)
Biochem J., 222: 307 -314

DOUGLAS W.W. & RUBIN R.P. (1961)
J. Physiol., 159: 40-57

DRUMMOND A.H. (1985)
Nature., 316: 752-755

DRUMMOND A.H. & MacINTYRE D.E. (1985)
Trends Pharmacol. Sci., 6: 233-234

DRUMMOND A.H. (1987)
Trends Pharmacol. Sci., 8: 129-133

DRUMMOND A.H., BUSHFIELD M. & MacPHEE C.H. (1984)
Mol. Pharmacol., 25: 201-208

ELFERINK J.G.R. & DEIERKAUF M. (1985)
Biochim. Biophys. Acta., 846: 364-369

ESTENSEN R.D., REUSCH M.E., EPSTEIN M.L. & HILL H.R. (1976)
Infect. Immunol., 13: 146-151

ESTENSEN R.D., WHITE J.G. & HOLMES B. (1974)
Nature, 248: 347-348

FAIN J.N. (1982)
Horz. Biochem. Biophys., 6: 237-276

FARLEY J. & AUERBACH S. (1986)
Nature, 319: 220-223

FECHEIMER M. & ZIGMOND S.H. (1983)
Cell. Motility, 3: 349-361

FEINSTEIN M.B., EGAN J.J., SHA'AFI R.I. & WHITE J. (1983)
Biochem. Biophys. Res. Commun., 113: 598-604

FERNANDEZ H. & HUGLI T.E. (1976)
J. Immunol., 117: 1688-1694

FERNANDEZ H.N., HENSON P.M., OTANI A. & HUGLI T.E. (1978)
J. Immunol., 120: 109-115

FISHER G.J., BAKSHIAN S. & BALDASSARE J.J. (1985)
Biochem. Biophys. Res. Commun., 129: 958-964

FLEMING A. & ALLISON V.D. (1922)
Proc. Roy. Soc. SerB., 94: 142

FLETCHER D.S., OSINGA D. & BONNEY R.J. (1986)
Biochem. Pharmacol., 35: 2601-2606

FLETCHER M.P. & GALLIN J.I. (1983)
Blood, 62: 792-799

FLOCKHART D.A. & CORBIN J.D. (1982)
CRC Crit. Rev. Biochem., 9: 133-186

FLORIO V.A. & STERNWEIS P.C. (1985)
J. Biol. Chem., 260: 3477-3483

FLOWER R.J. & BLACKWELL G.J. (1979)
Nature, 278: 456-459

FORD-HUTCHINSON A.W. (1983)
Br. J. Immunopharmacol., 5: 17-21

FORD-HUTCHINSON A.W., BRAY M.A., DOIG M.V., SHIPLEY M.E. &
SMITH M.J.H. (1980)
Nature, 286: 264-265

FOREMAN J.C., HALLETT M.B. & MONGAR J.L. (1977)
J. Physiol., 271: 193-214

FUJITA I., IRATA K., TAKESHIGE K. & MINAKAMI S. (1984)
Biochem. Biophys. Res. Comm., 120: 318-324

FUNG B.K.K., HURLEY J.B. & STRYER L. (1981)
Proc. Natl. Acad. Sci. USA., 78: 152-156

GABIG T.G. (1983)
J. Biol. Chem., 258: 6352-6366

GAINS N. (1980)
Eur. J. Biochem., 111: 199-202

GALLIN J.I. & ROSENTHAL A.S. (1974)
J. Cell. Biol., 62: 594-609

GALLIN J.I. & SELIGMANN B.E. (1984)
Fed. Proc., 43: 2732-2737

GIERSCHIK P., FALLOON J., MILLIGAN G., PINES M., GALLIN J.I. & SPIEGEL A. (1986)
J. Biol. Chem., 261: 8058-8062

GILMAN A.G. (1984)
Cell., 36: 577-579

GOLDMAN D.W. & GOETZL E.J. (1982)
J. Immunol., 129: 1600-1604

GOLDMAN D.W. & GOETZL E.J. (1984)
J. Exp. Med., 159: 1027-1041

GOLDSTEIN I.M., HOFFSTEIN S.T. & WEISSMANN G. (1975)
J. Cell. Biol., 66: 647-652

GOLDSTEIN I.M., HOFFSTEIN S.T., GALLIN J., WEISSMANN G. (1973)
Proc. Natl. Acad. Sci. USA., 70: 2916-2922

GOLDSTEIN I.M., HORN J.K., KAPLAN H.B. & WEISSMANN G. (1974)
Biochem. Biophys. Res. Commun., 60: 807-812

GOMPERTS B.D. (1983)
Nature, 306: 64-66

GOODHART M., FERRY W., GEYNET P. & HANOUNE J. (1982)
J. Biol. Chem., 257: 11577-11583

GORDON J.L. (1975)
In "Lysosomes in Biol. Pathol." Vol. 4;
Amsterdam:North-Holland., pp. 3-31

GRADY P.G. & THOMAS L.L. (1986)
Biochim. Biophys. Acta., 885: 282-293

GRAHAM R.C. & KARNOVSKI M.J. (1966)
J. Histochem. Cytochem., 14: 291-302

GRYNKIEWICZ G., POENIE M. & TSIEN R.Y. (1985)
J. Biol. Chem., 260: 3440-3450

HAINES K.A., GIEDD K.N., RICH A.M., KORCHAK H.M. & WEISSMANN G. (1987)
Biochem. J., 241: 55-62

HALENDA S.P. & FEINSTEIN M.B. (1984)
Biochem. Biophys. Res. Commun., 124: 507-513

HALENDA S.P., VOLPI M., ZAVOICO G.B., SHA'AFI R.I. & FEINSTEIN M.B. (1986)
FEBS. Letters., 204: 341-346

HALLCHER L.M. & SHERMAN W.R. (1980)
J. Biol. Chem., 255: 10896-10901

HALLETT M.B. & CAMPBELL A.K. (1982a)
In "Clinical and Biochemical Luminescence." pp. 87-133

HALLETT M.B. & CAMPBELL A.K. (1982b)
Nature, 295: 155-158

HALLETT M.B. & CAMPBELL A.K. (1983)
Immunol., 50: 487-495

HALLETT M.B., LUZIO J.P. & CAMPBELL A.K. (1981)
Immunol., 44: 509-576

HAM E.D., SODERMAN D.D., ZANETTI M.E., DOUGHERTY H.W., McCAULEY E. & KUEHL F.A. (1983)
Proc. Natl. Acad. Sci. USA., 80: 4349-4353

HAMBERG M. & SAMUELSSON B. (1974)
Proc. Natl. Acad. Sci. USA., 71: 3400-3404

HANSSON G., LINDGREN J.A., DAHLEN S.E., HEDQVIST P. & SAMUELSSON B. (1981)
FEBS. Letters., 130: 107-112

HASLAM R.J. & DAVIDSON M.M.L. (1984)
FEBS. Letters., 174: 90-95

HEILBRUNN L.V. (1940)
Physiol. Zool., 13: 88-94

HENSON P.M. (1971)
J. Immunol., 107: 1535-1546

HENSON P.M. (1981)
Pharmacol. Inflammation., 100: 63-81

HERLIN T., PETERSEN C.S. & ESMANN V. (1978)
Biochim. Biophys. Acta., 542: 63-76

HESCHELER J., ROSENTHAL W., TRAUTWEIN W. & SCHULTZ G. (1987)
Nature, 325: 445-447

HESLOP J.P., IRVINE R.F., TASHJIAN A. & BERRIDGE M.J. (1985)
J. Exp. Biol., 119: 395-401

HEYMANS F., MICHEL E., BORREL M.C., WICHROWSKI B., GODFROID J.J., CONVERT O., COEFFIER E., TENCE M. & BENVENISTE J. (1981)
Biochim. Biophys. Acta., 666: 230-237

HIGGS G.A., SALMON J.A. & SPAYNE J.A. (1981)
Br. J. Pharmacol., 74: 429-433

HIGGS G.A., FLOWER R.J. & VANE J.R. (1979)
Biochem. Pharmacol., 28: 1959-1961

HILDEBRANDT J.D., CODINA J., ROSENTHAL W., SUNYER T., IYENGAR R. & BIRNBAUMER L. (1985)
Adv. Cyc. Nucl. Prot. Res., 19: 87-101

HIRASAWA K. & NISHIZUKA Y. (1985)
Ann. Rev. Pharmacol. Toxicol., 25: 147-170

HIRATA M., SUEMATSU E., HASHIMOTO T., HAMICHI T. & KOGA T. (1984)
Biochem. J., 223: 229-236

HOFFSTEIN S.T. (1980)
In "The Cell Biology of Inflammation"., pp. 387-430

HOFFSTEIN S.T. (1981)
Meth. Cell. Biol., 23: 259-282

HOFFSTEIN S.T., FREIDMAN R.S. & WEISSMANN G. (1982)
J. Cell. Biol., 95: 234-241

HOKIN L.E. (1985)
Annu. Rev. Biochem., 54: 205-235

HOKIN M.R. & HOKIN L.E. (1953)
J. Biol. Chem., 203: 967-977

HOLIAN A. (1986)
FEBS. Letters., 201: 15-19

HOLMES R.P. & YOSS N.L. (1983)
Nature., 305: 637-638

HOLZ G.G., RANE S.G. & DUNLAP K. (1986)
Nature., 319: 670-672

HONN K.V. & DUNN J.R. (1982)
FEBS. Letters., 139: 65-68

HOPKINS N.K. LIN A.H. & GORMAN R.R. (1983)
Biochim. Biophys. Acta., 763: 276-283

HORN W. & KARNOVSKY M.L. (1986)
Biochem. Biophys. Res. Comm., 139: 1169-1175

HOUSLEY M.D. (1984)
Trends. Biochem. Sci., 9: 39-40

HOWARD T.H. & MEYER W.H. (1984)
J. Cell. Biol., 98: 1265-1271

HUANG E.M. & DETWILER T.C. (1986)
Biochem. J., 236: 895-901

HUEY R. & HUGLI T.E. (1985)
J. Immunol., 135: 2063-2068

HUMPHREY J.H. (1955)
Br. J. Exp. Pathol., 36: 268-282

HWANG S-B., LAM M-H. & CHANG M.N. (1986a)
J. Biol. Chem., 261: 13720-13726

HWANG S-B., LAM M-H. & PONG S-S. (1986b)
J. Biol. Chem., 261: 532-537

HWANG S-B., LAM M-H. & SHEN T.Y. (1985)
Biochem. Biophys. Res. Comm., 128: 972-979

HWANG S-B., LEE C.S., CHEAH M.J. & SHEN T.Y. (1983)
Biochem., 22: 4756-4763

HYSLOP P.A., OADES Z.G., JESAITIS A.J., PAINTER R.G., COCHRANE C.G. & SKLAR L.A. (1984)
FEBS. Letters., 166: 165-169

INAGAKI M., KAWAMOTO S. & HIDAKA H. (1984)
J. Biol. Chem., 259: 14321-14323

IRVINE R.F., ANGARD E.E., LETCHER A.J. & DOWNES C.P. (1985)
Biochem. J., 229: 505-511

IRVINE R.F., BROWN K.D. & BERRIDGE M.J. (1984)
Biochem. J., 221: 269-272

IRVINE R.F., LETCHER A.J., HESLOP J.P. & BERRIDGE M.J. (1986)
Nature., 320: 631-634

IRVINE R.I. & MOOR R.M. (1986)
Biochem. J., 240: 917-920

JACOBS S., SAYHAUN N.E., SALTIEL A.R. & CAUTRECASAS P. (1983)
Proc. Natl. Acad. Sci. USA., 80: 6211-6213

JAKOBS K.H. & AKTORIES K. (1983)
Biochim. Biophys. Acta., 732: 352-358

JAKOBS K.H., AKTORIES K. & SCHULTZ G. (1984)
Eur. J. Biochem., 140: 177-181

JENSEN J.A., SNYDERMAN R. & MERGENHAGEN S.E. (1969)
Proc. 3rd. Int. Symp. Cellular & Humoral Mech. Anaphylaxis & Allergy
Basel/New York 265

JESAITIS A.J., NAEMURA J.R., SKLAR L.A., COCHRANE C.G. & PAINTER R.G. (1984)
J. Cell. Biol., 98: 1378-1387

JOHNSON K.J. & WARD P.A. (1981)
J. Immunol., 126: 2365-2369

JOHNSON P.C., WARE J.A., CLIVEDEN P.B., SMITH M., DVORAK A.M. & SALZMAN E.W. (1985)
J. Biol. Chem., 260: 2069-2076

JOHNSON R.J. & CHENOWETH D.E. (1985)
J. Biol. Chem., 260: 7161-7164

JOHNSON M., CAREY F. & McMILLAN R.M. (1983)
Essays Biochem., 19: 40-141

JOLLES J., ZWIERS H., DEKKER A., WIRTZ K.W.A. & GISPEN W.H. (1981)
Biochem. J., 194: 283-291

JOSEPH S.K. (1985)
Trends. Biochem. Sci., 10: 297-298

JUBIZ W., RADMARK O., MALMSTED C., HANSSON G., LINDGRED J.A., PALMBLAD J., UDEN A-M & SAMUELSSON B. (1982)
J. Biol. Chem., 257: 6106-6110

KACZOROWSKI G.J., BARROS F., DETHMERS J.K. & TRUMBLE M.J. (1985)
Biochem., 24: 1394-1402

KARNOVSKY M.L. & WALLACH D.F.H. (1961)
J. Biol. Chem., 236: 1895-1901

KATER L.A., GOETZL E.J., AUSTEN K.F. (1976)
J. Clin. Invest., 57: 1173-1180

KATO H., ISHITOYA J. & TAKENAWA T. (1986)
Biochem. Biophys. Res. Comm., 139: 1272-1278

KATZ B. (1969)
In "The Release of Neural Transmitter Substances".
pp. 1-60

KISHIMOTO A., TAKAI Y., MORI T., KIKKAWA U. & NISHIZUKA Y. (1980)
J. Biol. Chem., 255: 2273-2276

KLEBANOFF S.J. & CLARK R.A. (1978)
In "The neutrophil function and clinical disorder",
Elsevier, North Holland Biomedical Press, Amsterdam.
225

KLEMPNER M.S. (1985)
J. Clin. Invest., 76: 303-310

KNIKER W.T. & COCHRANE C.G. (1965)
J. Exp. Med., 122: 83-98

KOJIMA I., SHIBATA H. & OGATA E. (1986)
Biochem. J., 237: 253-258

KOO C., LEFKOWITZ R.J. & SNYDERMAN R. (1983)
J. Clin. Invest., 72: 748-753

KORCHAK H.M. & WEISSMANN G. (1980)
Biochim. Biophys. Acta., 601: 180-194

KORCHAK K.M. & WEISSMANN G. (1978)
Proc. Natl. Acad. Sci. USA., 75: 3818-3822

KOSA E.P., WRIGHT J.E. & BECKER E.L. (1975)
Proc. Soc. Exp. Biol., 149: 476-479

KREBS E.G. (1984)
Biochem. Soc. Trans., 13: 813-820

KREISLE R.A. & PARKER C.W. (1983)
J. Exp. Med., 157: 628-641

KREISLE R.A., PARKER C.W., GRIFFIN G.L. SENOIR R.M. &
STENSON W.F. (1985)
J. Immunol., 134: 3356-3363

KUNO M. & GARDNER Ph. (1987)
Nature, 326: 301-304

LACKIE J.M. (1986)
In "Cell Movement & Cell Behaviour". Allen & Unwin Ltd.,
London.

LAD P.M., GOLDBERG B.J., SMILEY P.A. & OLSON C.V. (1985a)
Biochim. Biophys. Acta., 846: 286-295

LAD P.M., OLSON C.V. & GREWAL I.S. (1985b)
Biochem. Biophys. Res. Comm., 129: 632-638

LAD P.M., OLSON C.V., GREWAL I.S. & SCOTT S.J. (1985c)
Proc. Natl. Acad. Sci. USA., 82: 8643-8647

LAGAST H., POZZAN T., WALDVOGEL F.A. & LEW D.P. (1984)
J. Clin. Invest., 73: 878-883

LAMBRECHT G. & PARNHAM M.J. (1986)
Br. J. Pharmacol., 87: 287-289

LANE B. & SNYDERMAN R. (1984)
Fed. Proc., 43: 1417

LAPETINA E.G., SCHMITGES C.J., CHANRABOSE K. & CUATRECASAS P. (1977)
Biochem. Biophys. Res. Commun., 76: 828-835

LEEB-LUNDBERG L.M.F., COTECCHIA S., LOMASNEY J.W., DeBERNARDIS J.F., LEFKOWITZ R.J. & CARON M.G. (1985)
Proc. Natl. Acad. Sci. USA., 82: 5651-5655

LEKKA M., TSELEPIS A.D. & TSOUKATOS D. (1986)
FEBS. Letters., 208: 52-55

LEW D.P., DAYER J-M., WOLLHEIM C.B. & POZZAN T. (1984a)
FEBS. Letters., 166: 44-48

LEW P.D., MONOD A., WALDVOGEL F.A. & POZZAN T. (1987)
Eur. J. Biochem., 162: 161-168

LEW P.D., MONOD A., WALDVOGEL F.A., DEWALD B., BAGGIOLINI M. & POZZAN T. (1986)
J. Cell. Biol., 102: 2197-2204

LEW P.D., WOLLHEIM C.B., WALDVOGEL F.A. & POZZAN T. (1984b)
J. Cell. Biol., 99: 1212-1220

LEWIS R.A. & AUSTEN K.F. (1984)
J. Clin. Invest., 73: 889-897

LILES W.C., HUNTER D.D. MEIER K.E. & NATHANSON N.M. (1986)
J. Biol. Chem., 261: 5307-5313

LITOSCH I. & FAIN J.N. (1986)
Life Sci., 39: 187-194

LITOSCH I., WALLIS C. & FAIN J.N. (1985)
J. Biol. Chem., 260: 5464-5471

LLOYD J.V., NISHIZAWA E.E., HALDER J. & MUSTARD J.F. (1972)
Br. J. Haematol., 23: 571-585

LOTZ M. & HARRIS H. (1956)
Br. J. Exp. Pathol., 37: 477-480

MacINTYRE D.E. & ROSSI A.G. (1986)
Br. J. Pharmacol., 88: 654P

MacINTYRE D.E. & POLLOCK W.K. (1983)
Biochem. J., 212: 433-437

MacINTYRE D.E., BUSHFIELD M., MacMILLAN L.J., MOFFAT F.A., MURDOCH F.A., THOMSON A., ROSSI A.G. & McNICOL A. (1986)
Agents & Actions., 20: (Suppl)., 45-62

MacINTYRE D.E., McNICOL A. & DRUMMOND A.H. (1985a)
FEBS. Letters., 180: 160-164

MacINTYRE D.E., SHAW A.M., BUSHFIELD M., MacMILLAN L.J., McNICOL A. & POLLOCK W.K. (1985b)
Nouv. Rev. Fr. Hematol., 27: 258-292

MALECH H.L., GARDNER J.P., HEIMAN D.F. & ROSENZWEIG S.A. (1985)
J. Biol. Chem., 260: 2509-2514

MARASCO W.A., PHAN S.H., KRUTZSCH H., SHOWELL H.J., FELTNER D.E., NAIRN R., BECKER E.L. & WARD P.A. (1984)
J. Biol. Chem., 259: 5430-5439

MARCUS A.J., ULLMAN H.J. & SAFIER L.B. (1969)
J. Lipid Res., 10: 108-114

MARONE G., FIMIANI B., TORELLA G., POTO S., BIANCO P. & CONDORELLI M. (1983)
J. Clin. Immunol., 12: 111-116

MARONE G., THOMAS L.L. & LICHTENSTEIN L.M. (1980)
J. Immunol., 125: 2277-2283

MARTIN T.F.J. (1983)
J. Biol. Chem., 258: 14816-14822

MARTIN T.F.J., LUCAS D.O., BAJJALIEH S.M. & KOWALCHYK J.A. (1986)
J. Biol. Chem., 261: 2918-2927

MASSON P.L., HEREMANS J.F. & SCHONNES E. (1969)
J. Exp. Med., 130: 643

McMILLAN M., CHERNOW B. & ROTH B.L. (1986)
Biochem. Biophys. Res. Commun., 134: 970-974

McMILLAN R.M., MASTERS D.J., STERLING W.W. & BERNSTEIN P.R. (1985)
In "Prostaglandins, leukotrienes & lipoxins". pp.655-668

McNICOL A. (1986)
PhD Thesis. University of Glasgow.

McPHAIL L.C. & SNYDERMAN R. (1983)
J. Clin. Invest., 72: 192-200

McPHAIL L.C., CLAYTON C.C. & SNYDERMAN R. (1984)
J. Biol. Chem., 259: 5768-5775

MELLORS A., STAIMACH M.E. & COHEN A. (1985)
Biochim. Biophys. Acta., 833: 181-188

MELODELESI J., BORGESE N., DeCAMILLI P. & CECCARELLI B. (1978)
In "Cell Surface Rev." Vol. 1 Elsevier, North-Holland, New York., pp. 509-627

METCHNIKOFF E. (1887)
Ann. Inst. Pasteur., 1: 321

MICHELL B. (1983)
Trends. Biochem. Sci., 92: 263-265

MILLMAN M.S., CASWELL A.H. & HAYNES D.H. (1980)
Memb. Biochem., 3: 291-315

MOLINA y VEDIA L.M. & LAPETINA E.G. (1986)
J. Biol. Chem., 261: 10493-10495

MOLSKI T.F.P., NACCACHE P.H., MARSH M.L., KERMODE J. & SHA'AFI R.I. (1984)
Biochem. Biophys. Res. Commun., 124: 644-650

MONG S., CHI-ROSSO G., MILLER J., HOFFMAN K., RAZGAITIS K.A., BENDER P. & CROOK S.T. (1986)
Mol. Pharmacol., 30: 235-242

MORGAN J.P. & MORGAN K.G. (1982)
Pflugers Archiv., 395: 75-77

MOTTOLA C. & ROMEO D. (1982)
J. Cell. Biol., 93: 129-134

MUELLER H.W., O'FLAHERTY J.T. & WYKLE R.L. (1983)
J. Biol. Chem., 258: 6213-6218

MURPHY G., BRETZ U., BAGGIOLINI M. & REYNOLDS J.J. (1980)
Biochem. J., 192: 517-525

MURPHY P. (1976)
In "The Neutrophil". Plenum Publishing Corp., New York.

NACCACHE P.H., MOLSKI M.M., VOLPI M., SHEFCYK J., MOLSKI T.F.P., LOEW L., BECKER E.L. & SHA'AFI R.I. (1986)
J. Leukocyte Biol., 40: 533-548

NACCACHE P.H., MOLSKI T.F.P., BORGEAT P. & SHA'AFI R.I. (1984)
J. Cell. Physiol., 118: 13-18

NACCACHE P.H., MOLSKI T.F.P., BORGEAT P. & SHA'AFI R.I. (1985b)
J. Cell. Physiol., 122: 273-280

NACCACHE P.H., MOLSKI T.F.P., BORGEAT P., WHITE J.R. & SHA'AFI R.I. (1985a)
J. Biol. Chem., 260: 2125-2131

NACCACHE P.H., MOLSKI T.F.P., MODAIDI T., BECKER E.L., SHOWELL H.J. & SHA'AFI R.I. (1980)
Biochem. Biophys. Res. Commun., 97: 62-68

NACCACHE P.H., MOLSKI T.F.P., VOLPI M., BECKER E.L. & SHA'AFI R.I. (1985c)
Biochem. Biophys. Res. Commun., 130: 677-684

NACCACHE P.H., SHOWELL H.J., BECKER E.L. & SHA'AFI R.I. (1977)
J. Cell. Biol., 73: 428-444

NACCACHE P.H., SHOWELL H.J., BECKER E.L. & SHA'AFI R.I. (1979a)
J. Cell. Biol., 83: 179-186

NACCACHE P.H., VOLPI M., SHOWELL H.J., BECKER E.L. & SHA'AFI R.I. (1979b)
Sci., 203: 461-463

NAHAS G.G., TANNIERES M.L. & LENNON J.F. (1971)
Proc. Soc. Exp. Biol. Med., 138: 350-352

NAKAMURA T. & UI M. (1985)
J. Biol. Chem., 260: 3584-3593

NELSON R.D., McCORMACK R.T. & FIEGEL V.D. (1978)
In "Leukocyte Chemotaxis, Methods, Physiology & Clinical Applications". Ravens Press, New York.

NIEDEL J., DAVIS J. & CUATRECASAS P. (1980)
J. Biol. Chem., 255: 7063-7066

NIEDEL J., WILKINSON S. & CUATRECASAS P. (1979)
J. Biol. Chem., 254: 10700-10706

NISHIZUKA Y. (1984)
Nature., 308: 693-698

NISHIZUKA Y. (1986)
J. N. Cl., 76: 363-370

NUGTEREN D.H. (1975)
Biochim. Biophys. Acta., 380: 299-307

O'FLAHERTY J.T. (1987)
Biochem. Pharmacol., 36: 407-412

O'FLAHERTY J.T., REDMAN J.F. & JACOBSON D.P. (1986a)
FEBS. Letters., 206: 279-282

O'FLAHERTY J.T., SCHMITT J.D., McCALL C.E. & WYKLE R.L. (1984)
Biochem. Biophys. Res. Commun., 123: 64-70

O'FLAHERTY J.T., SHOWELL H.J., WARD P.A. & BECKER E.L. (1979)
Am. J. Pathol., 96: 799-809

O'FLAHERTY J.T., MILLER C.H., LEWIS J.C., WYKLE R.L., BASS D.A., McCALL C.E., WAITE M. & DeCHATELET L.R. (1981a)
Inflammation, 5: 193-201

O'FLAHERTY J.T., SHOWELL H.J. & WARD P.A. (1978b)
Immunol. Commun., 7: 495-502

O'FLAHERTY J.T., SHOWELL H.J., BECKER E.L. & WARD P.A. (1978a)
Am. J. Pathol., 92: 155-166

O'FLAHERTY J.T., SURLES J.R., REDMAN J., JACOBSON D., PIANTADOSI C. & WYKLE R.L. (1986b)
J. Clin. Invest., 78: 381-388

O'FLAHERTY J.T., WYKLE R.L., McCALL C.E., SHEWMAKE T.B., LEES C.L. & THOMAS M. (1981b)
Biochem. Biophys. Res. Commun., 101: 1290-1296

O'ROURKE F.A., HALENDA S.P., ZAVOICO G.B. & FEINSTEIN M.B. (1985)
J. Biol. Chem., 260: 956-962

OCHS D.C. & REED P.W. (1983)
J. Biol. Chem., 258: 10116-10122

OHTSUKA T., OKAMURA N. & ISHIBASHI S. (1986)
Biochim. Biophys. Acta., 888: 332-337

OKANO Y., TAKAGI H., NAKASHIMA S., TOHMATSU T. & NOZAWA Y. (1985)
Biochem. Biophys. Res. Commun., 132: 110-117

OLIVER J.M. & BERLIN R.D. (1982)
Adv. Exp. Med. Biol., 155: 113-131

OMANN G.M., ALLEN R.A., BOKOCH G.M., PAINTER R.G., TRAYNOR A.E. & SKLAR L.A. (1987a)
Physiol. Rev., 67: 285-322

OMANN G.M., TRAYNOR A.E., HARRIS A.L. & SKLAR L.A. (1987b)
J. Immunol., 138: 2626-2632

ORELLANA S.A., SOLSKI P.A. & HELLER-BROWN J. (1985)
J. Biol. Chem., 260: 5236-5239

OZAKI Y., OHASHI T. & NIWA Y. (1986)
Biochem. Pharmacol., 35: 3481-3488

PAINTER R.G., SCHMITT M., JESAITIS A.J., SKLAR L.A., PREISSNER K. & COCHRANE C.G. (1982)
J. Cell. Biochem., 20: 203-214

PALMER R.M.J. & SALMON J.A. (1983)
Immunol., 50: 65-73

PALMER R.M.J. & SALMON J.A. (1985)
Biochem. Pharmacol., 34: 1485-1490

PALMER R.M.J., STEPNEY R.J., HIGGS A. & EAKINS K.E. (1980)
Prostaglandins., 20: 411-418

PARISH W.E. (1969)
Br. J. Dermatol., 81: 28-35

PAYNE C.M. & SATTERFIELD V.F. (1980)
J. Clin. Pathol., 33: 505-508

PENFIELD A. & DALE M.M. (1984)
Biochem. Biophys. Res. Commun., 125: 332-336

PFEUFFER E., DREHER R.M., METZEGER H. & PFEUFFER T. (1985)
Proc. Natl. Acad. Sci. USA., 82: 3086-3090

PFEUFFER T.J. (1977)
J. Biol. Chem., 252: 7224-7234

PFEUFFER T.J. (1979)
FEBS. Lett., 101: 85-89

PIOMELLI D., VOLTERRA A., DALE N., SIEGELBAUM S.A., KANDEL E.R., SCHWARTZ S.A. & BELARDETTI F. (1987)
Nature, 382: 38-43

PIPER P. (1983)
Br. Med. Bull., 39: 255-259

PIPER P. (1984)
Physiol. Rev., 64: 744-761

PIROTZKI E., MISUMI J., BOULLET C. & BENVENISTE J. (1980)
C.R. Acad. Sci. Paris., 290: 1079-1082

PIROTZKY E., PFISTER A. & BENVENISTE J. (1985)
Br. J. Dermatol.,113: Suppl., 28: 91-94

POLLOCK W.K. (1984)
Ph.D Thesis. University of Glasgow.

PONTREMOLI S., MELLONI E., SALAMINO F., SPARATORE B., MICHETTI M., SACCO O. & HORECKER B.L. (1986)
Arch. Biochem. Biophys., 250: 23-29

POZZAN T., LEW D.P., WOLLHEIM C.B. & TSIEN R.Y. (1983)
Sci., 221: 1413-1415

PRENTKI M., WOLLHEIM C.B. & LEW P.D. (1984)
J. Biol. Chem., 259: 13777-13782

PRYZWANSKY K.B., STEINER A.L., SPITZNAGEL J.K. & KAPOOR C.L. (1981)
Sci., 211: 407-411

PUTNEY J.W. (1986)
Cell Calcium., 7: 1-12

RAO K.M.K. & VARANI J. (1982)
J. Immunol., 129: 1605-1607

RENOOIJ W. & SNYDER F. (1981)
Biochim. Biophys. Acta., 663: 545-556

REVAK S.D., RICE C.L., SCHRAUFSTATTER I.U., HALSEY W.A., BOHL B.P., CLANCY R.M. & COCHRANE C.G. (1985)
J. Clin. Invest., 76: 1182-1192

RICKARD J.E. & SHETERLINE P. (1985)
Biochem. J., 231: 623-628

RINGER S. (1883)
J. Physiol., 4: 29-42

RINK T.J. & POZZAN T. (1985)
Cell Calcium., 6: 133-144

RINK T.J., SMITH S.W. & TSIEN R.Y. (1982)
FEBS. Letters., 148: 21-26

RIVKIN I., ROSENBLATT J. & BECKER E.L. (1975)
J. Immunol., 115: 1126-1134

ROBINSON J.M., BADWEY J.A., KARNOVSKY M.L. & KARNOVSKI M.J. (1984)
Biochem. Biophys. Res. Commun., 122: 734-739

ROLLINS T.E. & SPRINGER M.S. (1985)
J. Biol. Chem., 260: 7157-7160

ROMEO D., ZABUCCHI C., MIANI N. & ROSSI F. (1975)
Nature, 253: 542-544

ROSS E.M. & GILMAN A.G. (1980)
Ann. Rev. Biochem., 49: 553-564

ROSSI A.G. & MacINTYRE D.E. (1986)
Adv. PG. Tx. LTs. Research., (in press)

ROSSI A.G., MacINTYRE, D.E. & McMILLAN R.M. (1987a)
Biochem. Soc. Trans., (in press)

ROSSI A.G., McMILLAN R.M. & MacINTYRE D.E. (1987b)
Agents & Actions., (in press)

ROSSI A.G., McMILLAN R.M. & MacINTYRE D.E. (1987c)
Br. J. Pharmacol., (in press)

ROSSI F., DELLA BIANCA V., GREZESKOWIAK M., DE TOGNI P. & CABRINI G. (1985)
FEBS. Letters., 181: 253-258

RUBIN R.P., SINK L.E. & FREER R.J. (1981)
Mol. Pharmacol., 19: 31-37

SAGI-EISENBERG R., LIEMAN H. & PECHT J. (1985)
Nature., 313: 59-60

SALMON J.A., SIMMONS P.A. & PALMER R.M.J. (1982)
Prostaglandins., 24: 225-235

SAMUELSSON & HAMMARSTROM S. (1980)
Prostaglandins., 19: 645-648

SAMUELSSON B., BORGEAT P., HAMMERSTROM S. & MURPHY R.C. (1979)
Prostaglandins, 17: 785-787

SANDOW A. (1952)
Yale J. Biol. Med., 25: 176-201

SASGURI T., HIRATA M. & KURIYAMA H. (1985)
Biochem. J., 231: 497-503

SCHACHT J. (1981)
Meth. Enzymol., 72: 626-631

SCHIFFMAN B., CORCORAN B.A. & WAHL S.M. (1975)
Proc. Natl. Acad. Sci. USA., 72: 1059-1062

SCHIFFMANN E. & GALLIN J.I. (1979)
Curr. Top. Cell. Reg., 15: 203-257

SCHMITT M., PAINTER R.G., JESAITIS A.J., PREISSNER K., SKLAR L.A. & COCHRANE C.G. (1983)
J. Biol. Chem., 258: 649-654

SCHRAMM M. & SELINGER Z. (1984)
Sci., 225: 1350-1356

SCOTT R.H. & DOLPHIN A.C. (1986)
Neurosci. Lett., 69: 59-64

SEKURA R.D. (1985)
Meth. Enzymol., 109: 558-566

SELAK M.A., CHIGNARD M. & SMITH J.B. (1986)
Agents & Actions., 20: 99-107

SELIGMANN B. & GALLIN J.I. (1980)
Mol. Immunol., 17: 191-200

SELIGMANN B., CHUSED T.M. & GALLIN J.I. (1981a)
Clin. Res., 29: 522

SELIGMANN B., CHUSED T.M. & GALLIN J.I. (1981b)
J. Clin. Invest., 68: 1225-1231

SELIGMANN B., GALLIN E., MARTIN D., SHAIN W. & GALLIN J.I. (1980)
J. Mem. Biol., 52: 257-272

SERHAN C.N., FRIDOVICH J., GOETZL E.J., DUNHAM P.B. &
WEISSMANN G. (1984)
J. Biol. Chem., 257: 4746-4752

SERHAN C.N., RADIN A., SMOLEN J.E., KORCHAK H., SAMUELSSON B.
& WEISSMANN G. (1982)
Biochem. Biophys. Res. Commun., 107: 1006-1012

SHA'AFI R.I. & NACCACHE P.H. (1983)
In 'Calcium in Biological Systems'. pp. 137-145

SHA'AFI R.I., VOLPI M. & NACCACHE P.H. (1985)
In "Prostaglandins, leukotrienes & lipoxins". pp. 241-250

SHA'AFI R.I., WHITE J.R., MOLSKI T.F.P., SHEFEYK J., VOLPI M.
& NACCACHE P.H. & FEINSTEIN M.B. (1983)
Biochem. Biophys. Res. Commun., 114: 638-645

SHEN T.Y., HWANG S-B., CHANG M.N., DOEBBER T.W., LAM M-H.T.,
WU M.S., WANG X., QUI HAN G. & ZHI LI R. (1985)
Proc. Natl. Acad. Sci. USA., 82: 672-676

SHIN H.S., SNYDERMAN R., FREIDMAN E., MELLORS A. & MAYER M.M.
(1968)
Sci., 162: 361-363

SHOWELL H.J. & BECKER E.L. (1976)
J. Immunol., 116: 99-105

SHOWELL H.J., GLOVSKI M.M. & WARD P. (1982)
Int. Arch. Allergy Appl. Immunol., 69: 62-67

SHOWELL H.J., NACCACHE P.H., SHA'AFI R.I. & BECKER E.L. (1980)
Life Sci., 27: 421-426

SHOWELL H.J., NACCACHE P.H., SHA'AFI R.I. & BECKER E.L. (1977)
J. Immunol., 119: 804-811

SIFFERT W. & AKKERMAN J.W.N. (1987)
Nature., 325: 456-458

SIFFERT W., SIFFERT G., SCHEID P., RIEMENS T., GORTER G. &
AKKERMAN J.W.N. (1987)
FEBS. Letters., 212: 123-126

SIMCHOWITZ L. & ROOS A. (1985)
J. Gen. Physiol., 85: 443-470

SIMCHOWITZ L., FISCHBEIN L.C., SPILBERG I. & ATKINSON J.P. (1980)
J. Immunol., 124: 1482-1491

SIMCHOWITZ L., SPILBERG I. & ATKINSON J.P. (1983)
J. Cycl. Nucl. Prot. Phos. Res., 9: 35-47

SIRAGANIAN R.P. & OSLER A.G. (1971)
J. Immunol., 106: 1244-1251

SKLAR L.A., FINNEY D.A., OADES Z.A., JESAITIS A.J., PAINTER R.G. &
COCHRANE C.G. (1984)
J. Biol. Chem., 259: 5661-5669

SMIGEL M., KATADA T., NORTHUP J.K., BOKOCH G.M., UI M. & GILMAN A.G.
(1984)
Adv. Cyc. Nucl. Protein Phosph. Res., 17: 1-18

SMITH C.D., UHING R.J. & SNYDERMAN R. (1987)
J. Biol. Chem., 262: 6121-6127

SMITH C.D., LANE B.C., KUSAKA I., VERGHESE M.W. & SNYDERMAN R.
(1985)
J. Biol. Chem., 260: 5875-5878

SMITH G.P. & PETERS T.J. (1980)
Biochim. Biophys. Acta., 673: 234-242

SMITH G.P. & PETERS T.J. (1981)
Eur. J. Clin. Invest., 10: 475-480

SMITH M.J.H., FORD-HUTCHINSON A.W. & BRAY M.A. (1980)
J. Pharm. Pharmacol., 32: 517-518

SMITH R.J., YEIN F.S., SPEZIALE S.C. & BOWMAN B.J. (1986)
Biochem. Biophys. Res. Commun., 136: 310-315

SMOLEN J. & WEISSMANN G. (1982)
Biochim. Biophys. Acta., 720: 172-180

SMOLEN J.E. & WEISSMANN G. (1980)
Biochem. Pharmacol., 29: 533-538

SMOLEN J.E. & WEISSMANN G. (1981)
Biochim. Biophys. Acta., 672: 197-206

SMOLEN J.E., KORCHAK H.M. & WEISSMANN G. (1980)
J. Clin. Invest., 65: 1077-1085

SMOLEN J.E., KORCHAK H.M. & WEISSMANN G. (1981)
Biochim. Biophys. Acta., 677: 512-520

SNYDERMAN R. & PIKE M.C. (1984)
Ann. Rev. Immunol., 2: 257-281

SNYDERMAN R., PHILLIPS J.K. & MERGENHAGAN S.E. (1971)
J. Exp. Med., 134: 1131-1343

SNYDERMAN R., PIKE M.C., EDGE S. & LANE B. (1984)
J. Cell. Biol., 989: 444-448

SNYDERMAN R., SHIN H.S., PHILLIPS J.K., GEWURZ H. &
MERGENHAGEN S.E. (1969)
J. Immunol., 103: 413-422

SOMLYO A.V., BOND M., SOMLYO A.P. & SCARPA A. (1985)
Proc. Natl. Acta. Sci. USA., 82: 5231-5235

SOPATA I. & DANCEWICZ A.M. (1974)
Biochim. Biophys. Acta., 370: 510-523

SPAT A., BRADFORD P.G., McKINNEY J.S., RUBIN R.P. & PUTNEY J.W. (1986)
Nature., 319: 514-516

STENDAHL O.I., HARTWIG J.H., BROTSCH E.A. & STOSSEL T.P. (1980)
J. Cell. Biol., 84: 215-224

STEPHENS C.G. & SNYDERMAN R. (1982)
J. Immunol., 128: 1192-1197

STREB H., IRVINE R.F., BERRIDGE M.J. & SCHULZ I. (1983)
Nature., 306: 67-69

SUTHERLAND E.W. & RALL T.W. (1960)
Pharmacol Rev., 12: 265-299

SUTHERLAND P.J., STEPHENSON D.G. & WENDT I.R. (1980)
Proc. Aust. Phys. Pharm. Soc., 11: 160

SWENDSEN C.L., ELLIS J.M., CHILTON F.H., O'FLAHERTY J.T. & WYKLE R.L. (1983)
Biochem. Biophys. Res. Commun., 113: 72-79

TAKENAWA T., ISHITOYA J. & NAGAI Y. (1986)
J. Biol. Chem., 261: 1092-1098

TAKESHINGE K., NABI Z.F., TATSCHECK B. & MINATHAMI S. (1980)
Biochem. Biophys. Res. Commun., 95: 410-415

TANNENBAUM S.W. (1978)
In "Cytochalasins: biochemical and cell biological aspects".
Oxford, North Holland Publ. Co.

TAYLOR C.W. & MERRIT J.E. (1986)
Trends. Pharmacol. Sci., 7: 239-242

TILL G.O., JOHNSON K.J., KUNKEL R. & WARD P.A. (1982)
J. Clin. Invest., 69: 1126-1135

TOUQUI L., ROTHHUT B., SHAW A.M., FRADIN A. VARGAFTIG B.B. & RUSSO-MARIE F. (1986)
Nature., 312: 177-180

TRIGGLE D.J. (1982)
In "Calcium blockers, Mechanisms of Actions & Clin. Appl."
pp. 121-134

TSCHARNER V., PROD'HOM B., BAGGIOLINI M. & REUTER H. (1986)
Nature., 324: 369-372

TSIEN R.Y. (1980)
Biochem., 19: 2396-2404

TSIEN R.Y. (1981)
Nature, 290: 527-528

TSIEN R.Y., POZZAN T. & RINK T.J. (1982a)
J. Cell. Biol., 94: 325-334

TSIEN R.Y., POZZAN T. & RINK T.J. (1982b)
Nature, 295: 68-71

TSIEN R.Y., RINK T.J. & POENIE M. (1985)
Cell Calcium., 6: 145-157

TURNER S.R., TAINER J.A. & LYNN W.S. (1975)
Nature, 257: 680-681

TYSON C.A., ZANDE H.V. & GEEN D.E. (1976)
J. Biol. Chem., 251: 1326-1332

UI M. (1984)
Trends. Pharmacol. Sci., 5: 277-279

VALONE F.H. & GOETZL E.J. (1983)
Immunol., 48: 141-149

VALONE F.H., COLES E., REINHOLD V.R. & GOETZL E.J. (1982)
J. Immunol., 129: 1637-1641

VERGARA J., TSIEN R.Y. & DELAY M. (1985)
Proc. Natl. Acad. Sci. USA., 82: 6352-6356

VERGHESE M.W., CHARLES L., JAKOI L., DILLON S.B. &
SNYDERMAN R. (1987)
J. Immunol., 138: 4374-4380

VERGHESE M.W., FOX K., McPHAIL L.C. & SNYDERMAN R. (1985)
J. Biol. Chem., 260: 6769-6775

VICENTINI L.M., DiVIRGILIO F., AMBROSINI A., POZZAN T. &
MELDOLESI J. (1985)
Biochem. Biophys. Res. Commun., 127: 310-317

VOLPI M., NACCACHE P.H. & SHA'AFI R.I. (1983)
J. Biol. Chem., 258: 4153-4158

VOLPI M., YASSIN R., TAO W., MOLSKI T.F.P., NACCACHE P.H. & SHA'AFI R.I. (1984)
Proc. Natl. Acad. Sci. USA., 81: 5966-5969

VOLWERK J.J., PIETERSON W.A. & DeHAAS G.H. (1974)
Biochem., 13: 1446-1454

VULLIET P.R., WOODGETT J.R., FERRARI S. & HARDIE D.G. (1985)
FEBS. Lett., 182: 335-339

WALLACE M.A. & FAIN J.N. (1985)
J. Biol. Chem., 260: 9527-9530

WARD P.A. & NEWMAN L.J. (1969)
J. Immunol., 102: 93-99

WARD P.A. & ZVAIFLER N.J. (1971)
J. Clin. Invest., 50: 606-616

WATSON S.P. & LAPETINA E.G. (1985)
Proc. Natl. Acad. Sci. USA., 82: 2623-2626

WATSON S.P., McCONNELL R.T. & LAPETINA E.G. (1984)
J. Biol. Chem., 259: 13199-13203

WEDMORE C.V. & WILLIAMS T.J. (1981)
Nature, 289: 646-650

WEISSMANN G. (1982)
J. Lab. Clin. Med., 100: 322-333

WELLS J.N. & HARDMAN J.G. (1977)
Adv. Cycl. Nucl. Res., 8: 119-143

WESTWICK J. & POLL C. (1986)
Agents & Actions., 19: 80-86

WHITE J.R., NACCACHE P.H. & SHA'AFI R.I. (1982)
Biochem. Biophys. Res. Commun., 108: 1144-1149

WHITE J.R., NACCACHE P.H. & SHA'AFI R.I. (1983a)
J. Biol. Chem., 258: 14041-14047

WHITE J.R., HUANG C-K., HILL J.M., NACCACHE P.H., BECKER E.L. & SHA'AFI R.I. (1984)
J. Biol. Chem., 259: 8605-8611

WHITE J.R., NACCACHE P.H., MOLSKI T.F.P., BORGEAT P. & SHA'AFI R.I. (1983b)
Biochem. Biophys. Res. Commun., 113: 44-50

WHITIN J.C., CLARK R.A., SIMONS E.R. & COHEN H.J. (1981)
J. Biol. Chem., 256: 8904-8906

WILKINSON P.C. & LACKIE J.M. (1979)
In "The inflammatory reaction". pp. 47-48

WILKINSON P.C. (1975)
Exp. Cell. Res., 93: 420-426

WILKINSON P.C. (1982)
In "Chemotaxis & Inflam.", Churchill Livingston, Edinburgh.

WILLIAMS J.D., LEE T.H., LEWIS R.A. & AUSTEN F. (1985)
J. Immunol., 134: 2624-2630

WILLIAMS T.J., HELLEWELL P.G. & JOSE P.J. (1986)
Agents & Actions., 19: 66-72

WILSON D.B., BROSS T.E., SHERMAN W.R., BERGER R.A. & MAJERUS P.W. (1985a)
Proc. Natl. Acad. Sci. USA., 82: 4013-4017

WILSON D.B., CONNOLLY T.M., BROSS T.E., MAJERUS P.W., SHERMAN W.R., TYLER A.N., RUBIN L.J. & BROWN J.E. (1985b)
J. Biol. Chem., 260: 13496-13501

WITTERS L.A., VATER C.A. & LIENHARD G.E. (1985)
Nature, 315: 777-778

WRIGHT D.G., BRALOVE D.A. & GALLIN J.I. (1977)
Am. J. Path., 87: 273-283

WYKLE R.L., MILLER C.H., LEWIS J.C., SCHMITT J.D., SMITH J.A., SURLES J.R., PIATADOSI C. & O'FLAHERTY J.T. (1981)
Biochem. Biophys. Res. Commun., 4: 1651-1658

YAMASHITA A., KUROKAWA T., DAN'URA T., HIGASHI K. & ISHIBASHI S. (1986)
Biochem. Biophys. Res. Commun., 138: 125-130

YAVIN E. & ZUTRA A. (1977)
Anal. Biochem., 80: 430-437

ZAVOICO G.B. & FEINSTEIN M.B. (1984)
Biochem. Biophys. Res. Commun., 120: 579-585

ZAVOICO G.B., HALENDA S.P., SHA'AFI R.I. & FEINSTEIN M.B. (1985)
Proc. Natl. Acad. Sci. USA., 82: 3859-3862

ZIGMOND S.H. & HIRSCH J.G. (1972)
Exp. Cell. Res., 73: 383-393

ZIGMOND S.H. & HIRSCH J.G. (1973)
J. Exp. Med., 137: 387-410

ZIGMOND S.H. (1974)
Nature., 249: 450-452

ZIGMOND S.H. (1978)
J. Cell Biol., 77: 269-287

ZIGMOND S.H., SULLIVAN S.J. & LAUFFENBURGER D.A. (1982)
J. Cell. Biol., 92: 34-43