A STUDY OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON HUMAN NEUTROPHIL TOXIC OXYGEN METABOLITE PRODUCTION

A Thesis submitted by

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for the degree of Doctor of Philosophy

in the University of London

1990

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For Mam and Dad,

whose love and unfaltering support I so appreciate

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ABSTRACT

A study was carried out on the effect of a wide range of non-steroidal anti-inflammatory drugs (NSAIDs) on the receptor- and post-receptor-mediated respiratory burst in human neutrophils. Superoxide (O_2^-) - which can give rise to toxic oxygen metabolites known to cause tissue damage - was measured spectrophotometrically by the reduction of ferricytochrome C. It was found that the NSAIDs fell into three categories: 1) those that increased O_2^- production, 2) those that had no effect and 3) those that decreased O_2^- production. These findings could have clinical relevance for the therapy of chronic inflammatory diseases such as rheumatoid arthritis.

In order to investigate the mode of action of those drugs which potentiated O_2^- release, a pharmacological study of the transduction mechanisms of the respiratory burst was undertaken. A number of cyclooxygenase and 5-lipoxygenase inhibitors had no effect on the stimulated O_2^- response, implying that the enhancing effect of the NSAIDs was independent of their effect on arachidonic metabolism. Putative "specific" inhibitors of the diacylglycerol (DAG) metabolizing enzyme, DAG kinase, gave similar results to those obtained with the potentiating NSAIDs (with the exception of PAF), indicating that the NSAID effect could involve increased DAG/protein kinase C (PKC) activity.

The role of the DAG/PKC pathway in the respiratory burst was addressed using a novel series of potent PKC inhibitors reported to be selective for PKC over other protein kinases. It was found that these agents inhibited O_2^- production induced by both receptor and post-receptor stimuli, and also in activation sequences that are reported to be Ca^{2+}-independent. Differences in the order of potency of the PKC inhibitors within the range of stimuli used indicated that there might be different PKC isoenzymes, differentially affected by the inhibitors. The potentiating NSAIDs were tested for an effect on isolated PKC, but none were found to directly activate the enzyme.

Inhibition by wortmannin of the O_2^- response to some stimuli may suggest a role for the phospholipase D pathway of DAG generation.

This study supports a role for the DAG/PKC pathway in signal-transduction for the neutrophil respiratory burst.

ACKNOWLEDGEMENTS

I am deeply indebted to my supervisor, Dr. Maureen M. Dale, for firstly accepting me as a PhD student and then providing constant help and guidance throughout the project, both in the experimental and writing phases. I am extremely grateful for the flood of ideas and the listening ear when there were problems.

I would like to thank Bob Muid for excellent technical assistance and both Stuart Hirst and Bob Muid for their friendship and the constant banter around the lab. that made life so pleasant. I wish to extend special thanks to Joyce Mancini for her constant help, support and friendship. I have also appreciated the help and friendship of many other staff and students of the Pharmacology department, who have made my time at UCL so pleasant and memorable and in particular, Adrienne Penfield, Ashwani Bahl and Karina Clay to mention but a few.

Special thanks go to other laboratories that have allowed me to use their facilities and provided some helpful advice, namely Dr. David Allan of Experimental Pathology, UCH and Dr. Laurie Garland of Wellcome Research Laboratories. I am grateful for having been made so welcome.

I appreciate the work of both Indira Kapadia and Vivien Grant for the excellent typing of this thesis, of Steve Richards for preparing the diagrams and of Stuart Hirst for help with the proof reading.

Finally, this work would not have been possible without the many members and students of Pharmacology department who so bravely donated blood during the course of this project – to all of you my most sincere thanks.

This project was funded by the Arthritic and Rheumatoid Council.

PUBLICATIONS

Some of the data presented in this thesis have been published in the following papers:

(1) MUID, R.E., TWOMEY, B. & DALE, M.M. (1988) The effect of inhibition of both diacylglycerol metabolism and phospholipase A_2 activity on superoxide generation by human neutrophils. *FEBS Lett.*, **234**, 235-240.

(2) TWOMEY, B., MUID, R.E. & DALE, M.M. (1989) The potentiation of human neutrophil superoxide generation by sodium meclofenamate. *Br. J. Pharmacol.*, **98**, 671P.

(3) TWOMEY, B., MUID, R.E. & DALE, M.M. (1990) The effect of putative protein kinase C inhibitors, K252a and staurosporine, on the human neutrophil respiratory burst activated by both receptor stimulation and post-receptor mechanisms. *Br. J. Pharmacol.*, **100**, 819-825.

(4) TWOMEY, B., MUID, R.E., NIXON, J.S., SEDGWICK, A.D., WILKINSON, S.E. & DALE M.M. (1990) The effect of new potent selective inhibitors of protein kinase C on the neutrophil respiratory burst. *Biochem. Biophys. Res. Commun.* (in press).

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(6) DALE, M.M. & TWOMEY, B. (1991) Chapter 3 - The neutrophil leucocyte (in preparation) for the 3rd edition of *Textbook of Immunopharmacology* (Dale, Foreman & Fan, eds.).

CONTENTS

ABBREVIATIONS

AA	Arachidonic acid
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BPB	p-Bromophenacyl bromide
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular calcium concentration
CANP	Calcium-activated neutral protease
CB	Cytochalasin B
CGD	Chronic granulomatous disease
CTC	Chlortetracycline
DAG	1,2-Diacyl-sn-glycerol
DH	1,2-Dihexanoyl-sn-glycerol
DiC_8	1,2-Dioctanoyl-sn-glycerol
DMARD	Disease-modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
DOEG	1,2-Dioctanoylethylene glycol
DTPA	Diethylenetriaminepentaacetic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (amino-ethylether) tetraacetic acid
FMLP	Formylmethionyl-leucyl-phenylalanine
G-protein	Guanine nucleotide-binding regulatory protein
GMP	Guanosine monophosphate
GSH	Glutathione
HBSS	Hanks balanced salt solution
γ-HCCH	γ-hexachlorocyclohexane
HEPES	4-(-2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
HETE	Hydroxyeicosatetraenoic acid
HOCl	Hypochlorous acid
HPETE	Hydroperoxyeicosatetraenoic acid
HWT	17-hydroxywortmannin
IL-1/2/4/6	Interleukin-1/2/4/6
IP_3	Inositol 1,4,5-trisphosphate
LTB_4	Leukotriene B_4
MLCK	Myosin light chain kinase
MOG	Monooleoylglycerol

NSAID	Non-steroidal anti-inflammatory drug
O_2^-	Superoxide anion
$^1O_2$	Singlet oxygen
OAG	1-Oleoyl, 2-acetylglycerol
OZ	Opsonized zymosan
PA	Phosphatidic acid
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PC/PE/PS	Phosphatidylcholine/ phosphatidylethanolamine/ phosphatidylserine
PDBu	Phorbol dibutyrate
PGs	Prostaglandins
PGE_1/I_1	Prostaglandin E_1/I_1
PIP_2/PI	Phosphatidyl 4,5-bisphosphate/ phosphatidylinositol
PKA	Protein kinase A or cyclic AMP-dependent protein kinase
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leucocyte
RA	Rheumatoid arthritis
RIA	Radioimmunoassay
SDS	Sodium dodecylsulphate
SOD	Superoxide dismutase
TNF	Tumour necrosis factor

CHAPTER ONE

THE INTRODUCTION

1.1 The Polymorphonuclear Leucocyte

Polymorphonuclear leucocytes (PMNs) or neutrophils are the predominant white cells in human blood. The mature leucocyte, of diameter 12-15μM, has a multilobed nucleus and numerous granules in its cytoplasm. It contains little else in the way of intracellular organelles. Endoplasmic reticulum is scanty and polyribosomes few, so PMNs are capable of only very low levels of protein synthesis; they are end cells, lacking the capacity for cell division and have a relatively short half life in the circulation (6-16 hours).

Microtubules form a framework within these cells and may be of importance in neutrophil activities such as directional movement and phagocytosis. The neutrophil is highly motile, being the first cell to appear at sites of acute inflammation to provide defense against many bacteria, which it engulfs, kills and then digests. This is usually regarded as its main activity in the body but it can also function as a secretory cell releasing extracellularly its granule contents, generating large quantities of toxic oxygen radicals and other inflammatory mediators such as leukotriene B_4 (LTB_4) and platelet-activating factor (PAF). Thus, although structurally primitive, this cell can be considered as functionally powerful, being capable of effecting the extracellular killing of large multicellular pathogens as well as other tissue cells.

The neutrophil membrane has receptors for numerous chemotaxins and opsonins which mediate its activation. On infection by an invading pathogen, neutrophils are caused to leave the circulation and migrate towards the inflamed area, under the influence of chemotaxins released from the inflammatory site. Accumulating in large numbers at the site of injury, neutrophils phagocytose the offending micro-organisms. The latter are rendered ingestible by opsonizing either with IgG antibody or the C3b fragment of complement, enabling them to be recognized by specific PMN receptors. The microfilament system facilitates the enclosure of the particle, so destruction can now begin.

Neutrophil-mediated killing involves two main mechanisms: 1) lysis by the granule enzymes and 2) damage exerted by toxic oxygen radicals. Killing and digestion of micro-organisms is accomplished by the concerted action of both mechanisms.

The cytoplasmic granules are of two main types:

1) Azurophil or primary granules containing: neutral proteases e.g. elastase and cathepsin G, acid phosphatases, lysozyme, myeloperoxidase

2) Specific or secondary granules containing: collagenase, lactoferrin, vitamin B_{12}-binding protein, lysozyme.

The enzymes, particularly those of the azurophil granules, participate in bacterial killing and degradation. The specific granules are believed to function mainly as a source of "cryptic receptors", fusing with the advancing edge of the neutrophil during chemotaxis, thereby providing receptors to replace those that are internalized during phagocytosis. There is also evidence that a third type of granule may be present, the tertiary or secretory vesicle, containing gelatinase and cytochrome b (a putative component of the respiratory burst oxidase).

The respiratory burst will be the subject of the remainder of this chapter as this is the main area of the project.

1.2 The Respiratory Burst

This term refers to a coordinated series of biochemical reactions, that conclude with the production of a group of highly reactive microbicidal agents by the partial reduction of oxygen. The events include:

1) increased consumption of oxygen
2) increased flow of glucose through the hexose monophosphate shunt
3) production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2).

The respiratory burst can be activated by a variety of particulate (e.g. opsonized zymosan, latex beads, IgG aggregates) and soluble (e.g. fMet-Leu-Phe, PAF, complement peptide C5a) stimuli. Although all the physiologically relevant activators act at specific receptors, the respiratory burst can also be activated *in vitro* by;

1) aggregation of surface molecules with antibodies and lectins
2) degradation of membrane phospholipids with exogenous

phospholipase C

3) elevation of cytosolic free calcium by ionophores
4) stimulation of protein kinase C by phorbol diesters and diacylglycerol analogues
5) dissociation of G-protein subunits by fluoride
6) fatty acids and detergents

These stimuli, in stimulating the respiratory burst, lead ultimately to the activation of a single enzyme system, namely *NADPH oxidase*. This enzyme complex catalyzes the one-electron reduction of oxygen, using NADPH preferentially as the electron donor (Km = 30μM), to produce superoxide or O_2^- (Babior, 1987). It is localized in the plasma membrane, or after phagocytosis in the phagosomal membranes of the cell, with its NADPH binding site projecting into the cytosol and O_2^- molecules being released extracellularly (Briggs *et al.*, 1975; Dewald *et al.*, 1979). The molecular identity of NADPH oxidase is still a matter of controversy but recorded molecular weights of the enzyme range from 150 000 to 1 000 000. The enzyme complex is thought to consist of various components arranged as an electron transport chain, with the available data favouring the following model of transport of reducing equivalents from NADPH to O_2:

$$\text{NADPH} \longrightarrow \text{flavoprotein} \longrightarrow \text{cytochrome b} \longrightarrow O_2^-$$

It is known that a flavoprotein is a component of the NADPH oxidase (Babior & Kipnes, 1977; Cross *et al.*, 1984). Also shown to be involved is a b-like cytochrome (Segal & Jones, 1979; Cross *et al.*, 1982) which has been characterized as having a peak of absorbance in the reduced state at 558nm and a low mid-point potential of -245mV (Cross *et al.*, 1981). However, the involvement of quinones is still unclear, with some reports suggesting that they mediate electron transfer from a flavoprotein to cytochrome b (Crawford & Schneider, 1982; Cunningham *et al.*, 1982) and other reports indicating the contrary (Cross *et al.*, 1983; Lutter *et al.*, 1984). During activation, translocation of oxidase components to the plasma or phagosomal membrane has been proposed, the components possibly originating from the granules. Complete understanding of the NADPH oxidase structure requires purification of oxidase components and the reconstitution of an active oxidase system, a task that has been hampered up to now by the extreme lability of the enzyme constituents on detergent extraction and low recovery of enzymatic activity.

1.3 Oxygen-derived metabolite production by PMNs

The primary oxygen-derived species generated by NADPH oxidase from the oxidation of NADPH and the one electron reduction of oxygen is O_2^- or superoxide anion, by the following reaction:

$$2O_2 + NADPH \longrightarrow 2O_2^- + NADP^+ + H^+$$

By definition, O_2^- is a free radical (i.e. $O_2^-\cdot$), since it contains an unpaired electron. Because of the general reactivity of free radicals, $O_2^-\cdot$ was considered a likely mediator of phagocyte-dependent cytotoxicity. In recent years, it has become clear that $O_2^-\cdot$ serves as a starting material for the production of many more reactive oxygen species. In the aqueous medium of biological systems, $O_2^-\cdot$ undergoes spontaneous dismutation or dismutation accelerated by a group of enzymes called superoxide dismutases (Fridovich, 1978) to form hydrogen peroxide (H_2O_2) (see Table 1.1). This reaction is greatly speeded by a decrease in pH to 4.8, where $O_2^-\cdot$ and $HO_2\cdot$ or perhydroxyl radical (the protonated form of $O_2^-\cdot$) concentrations are equal. In contrast to $O_2^-\cdot$, H_2O_2 is a relatively potent oxidant and about 80% of the H_2O_2 generated by human neutrophils arises from the dismutation of $O_2^-\cdot$ (Root & Metcalf, 1977). H_2O_2 can also participate in several chemical reactions resulting in the formation of additional reactive metabolites (see Table 1.1).

TABLE 1.1
Reactions resulting in formation of oxygen-derived metabolites

1. Reduction of molecular oxygen
   $O_2 + e^- \longrightarrow O_2^-\cdot$ (superoxide anion)

2. Dismutation of superoxide anion
   $2O_2^-\cdot + 2H^+ \longrightarrow H_2O_2 + O_2$ (or $^1O_2$) (singlet oxygen)
   $O_2^-\cdot + HO_2\cdot$ (perhydroxyl radical) $\longrightarrow H_2O_2 + O_2$

3. Haber-Weiss reaction
   $O_2^-\cdot + H_2O_2 \longrightarrow O_2$ (or $^1O_2$) $+ HO^- + HO\cdot$ (hydroxyl radical)

4. Fenton Reaction
   $O_2^-\cdot + Fe^{3+} \longrightarrow Fe^{2+} + O_2$
   $Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO\cdot$
   $O_2^-\cdot + H_2O_2 \longrightarrow O_2 + HO^- + HO\cdot$

5. Myeloperoxidase(MPO) – hydrogen peroxide – halide system
   $H_2O_2 + Cl^- \xrightarrow{MPO} H_2O + HOCl$ (hypochlorous acid)

The reactive species, singlet oxygen ($^1O_2$) is formed when one of the

two unpaired orbital electrons in molecular oxygen absorbs enough energy to provoke spin inversion. This electronically excited state of oxygen emitts light (chemiluminescence) as it reverts to the triplet ground state. Singlet oxygen may be formed as an additional product of some of the above depicted interconversions and is very reactive towards molecules that are electron-rich or contain unsaturated double bonds.

The hydroxyl radical (OH·) is a potent oxidizing agent. It was proposed as a phagocyte-derived oxygen metabolite to account for the experimentally observed potent oxidant powers of O_2^- metabolites in solution, that could neither be attributed to O_2^- or H_2O_2 alone. It was initially suggested that O_2^- and H_2O_2 reacted via the Haber-Weiss reaction (Table 1.1) to form OH·, but later the Fenton reaction was invoked when it became apparent that the Haber-Weiss reaction occurred too slowly to account for OH·-like oxidant production in cell-free systems (Halliwell & Gutteridge, 1981). The Fenton reaction requires the presence of a transition metal, with iron being the favoured candidate. Iron, in conjunction with several biological compounds (e.g. ferritin, transferrin or lactoferrin) can catalyze the formation of OH·, by first itself being reduced by O_2^- and then oxidized by H_2O_2 (Table 1.1).

A number of stimuli cause the release of myeloperoxidase into phagocytic vacuoles or the extracellular surroundings, and this enzyme can catalyze the oxidation of halides by H_2O_2, resulting in the generation of hypohalous acids. The high plasma concentration of Cl^- probably favours the use of this halide in the H_2O_2-myeloperoxidase system *in vivo*, giving rise to hypochlorous acid (HOCl). The extremely high reactivity of HOCl means that it does not accumulate in biological systems. It can either oxidize biological molecules, react with H_2O_2 to form singlet oxygen ($^1O_2$) or combine with the primary or secondary amines to form the longer-lived monochloramine and dichloramine compounds. The latter reaction allows the generation of a complex family of nitrogen-chlorine derivatives (Weiss *et al.*, 1983; Grisham *et al.*, 1984a):

$$R^1RNH + HOCl \longrightarrow R^1RNCl + H_2O$$

Chloramine oxidants can be recovered from human neutrophils up to 16 hours after the cells have been triggered (Test *et al.*, 1984).

Thus, a large armamentarium of oxygen-derived metabolites can be produced by neutrophils and other inflammatory cells. However, OH· and HOCl appear to be the most biologically reactive compounds, with the

greatest cytotoxic activity on a molar basis. In turn, there are several well characterized anti-oxidant defense systems which protect the host from the untoward effects of oxidants.

1.4 Host anti-oxidant defense mechanisms

Physiological anti-oxidant defense systems can be divided into two categories: intracellular enzymes and extracellular scavengers of oxygen radical electrons. Superoxide dismutase (SOD) (Roos *et al.*, 1980a) and catalase (Roos *et al.*, 1980b) catalyze the degradation of O_2^- and H_2O_2 respectively, both systems implicated as playing a protective role in maintaining the integrity of the host cell. Glutathione (GSH) peroxidase and reductase are complementary enzymes that function to maintain stable levels of GSH within the cytosol, which is important as a decrease in GSH levels results in increased susceptibility of the cells to oxidant injury (Fridovich, 1976). Since these anti-oxidant enzymes are cytosolic, they have limited ability to protect cells against extracellular oxygen-derived metabolites. Substances demonstrated to have oxygen radical scavenging potential in the extracellular milieu include ascorbic acid, vitamin E (α-tocopherol) (Tsen & Collier, 1972) and ceruloplasmin.

The physiological importance of an intact NADPH oxidase as a defense against bacterial infection is demonstrated in patients who have chronic granulomatous disease (CGD) of childhood. Patients with CGD have a genetically defective oxidase that is incapable of generating significant amounts of O_2^- and H_2O_2, and have an increased frequency and severity of deep-tissue infections with bacteria and fungi (Gallin *et al.*, 1983; Tauber *et al.*, 1983). There are two main types of this inherited disorder 1) an X-chromosome linked form where neutrophils are deficient in cytochrome b_{558} (Royer-Pokora *et al.*, 1986) and 2) an autosomal recessive type where the genetic basis is unknown but suggested to be due to a defective cytosolic factor (Malech & Gallin, 1987).

1.5 Phagocyte-derived oxygen metabolite-mediated cell and tissue injury

Toxic oxygen free radicals have been implicated as important pathological mediators of tissue-destructive events in many diseases including rheumatoid arthritis, myocardial reperfusion injury, respiratory distress syndrome, blistering skin disorders and ulcerative colitis (Cross *et al.*, 1987; Henson & Johnston, 1987). This review will

concentrate on oxygen radical-induced injury underlying rheumatoid arthritis, as the project has been closely concerned with this condition. Neutrophil-derived oxygen metabolites mediate their damage in 3 ways – by a direct effect on cellular molecules, by assisting the granule enzyme system to degrade tissue components and by mediating systemic inflammatory effects.

(i) Effects on cells

Oxygen metabolites can react with unsaturated lipids in cell membranes to initiate a series of lipid peroxidation reactions (Mead, 1976; Tappel & Dillard, 1981). The reactions can be initiated by OH·, alkoxy radicals (RO·), peroxy radicals (ROO·) or singlet oxygen and can lead to a self-perpetuating process since peroxy radicals are both reaction initiators and products of lipid peroxidation. This process causes a decrease in membrane fluidity and integrity, leading to increased permeability to cations such as Ca^{2+} and K^{+}. Accumulation of intracellular Ca^{2+} can activate specific Ca^{2+}-dependent phospholipases and protein kinases and has been proposed as a mechanism for the initiation of cell injury and death (Farber, 1982) and loss of K^{+} from cells may also induce pathological effects (Maridonneau *et al.*, 1983). The many products of lipid peroxidation such as hydroperoxides or their aldehyde derivatives can inhibit protein synthesis, block macrophage function and alter chemotactic and enzymatic activity (Blake *et al.*, 1987).

Since the most reactive oxidants, $^1O_2$, OH· and HOCl, are short-lived compounds that react with the nearest target molecule, the cell membrane was believed to be the primary site of injury by PMN-derived oxygen metabolites. However, others can permeate cell membranes, O_2^- via anion channels and H_2O_2 by diffusion, to initiate cell injury by reaction with intracellular molecules (Weiss, 1982). Also, the less reactive more lipophilic chloramines can diffuse across the plasma membrane to attack cytosolic components (Grisham *et al.*, 1984b).

Oxygen metabolic products are capable of reacting with DNA in target cells, causing sister chromatid exchange in Chinese hamster ovary cells (Weitberg *et al.*, 1983) and mediating DNA fragmentation in human leucocytes (Birnboim & Kanabus-Kaminska, 1985); it has been suggested that these reactions may be a cause of neutrophil-mediated cell death and the induction of "auto-immune" processes. A recent study has shown that activated neutrophils can induce prolonged DNA damage in neighbouring cells, that was coincident with the oxidative burst and

dependent on the ratio of neutrophil to target cells (Shacter *et al.*, 1988).

Certain reactive groups (e.g. sulphydryl groups) on amino acids, proteins or carbohydrates are susceptible to oxidation or reduction by oxygen radicals, thereby potentially altering the physical and biological properties of specific molecules. Therefore, depending on the target cell and the concentration and duration of exposure, PMN-derived oxygen metabolic products are capable of initiating cytotoxic injury in both prokaryotic (see section 1.1) and eukaryotic cells via reaction with either cell membrane-associated and/or intracellular target molecules.

(ii) Effects on extracellular tissue components and enzymes

Oxygen metabolites are also implicated in mediating **tissue injury** *in vivo* by altering the biochemical and biophysical properties of the structural proteins of tissues including elastin, collagen and mucopolysaccharides.

(*a*) The serum inhibitor of neutrophil elastase, α1-antiprotease can be inactivated by oxidizing the methionine residues at the active site (Carp & Janoff, 1979; Matheson *et al.*, 1979), thus allowing neutrophil elastase activity to continue unabated. One study has demonstrated how PMN-derived oxidants could directly enhance elastase-mediated proteolysis of extracellular matrix, which contains elastin as a major component (McGowan & Murray, 1987).

(*b*) Hyaluronic acid is altered in the presence of an O_2^- and H_2O_2 generating system, resulting in decreased viscosity and increased digestion by *N*-acetyl-β-D-glucosamidase (Greenwald & Moy, 1980). Degradation of hyaluronic acid has been suggested as a mechanism for the decreased viscosity and function of synovial fluid observed in patients with rheumatoid arthritis.

(c) In a series of studies it was revealed that endogenously generated HOCl or HO· by neutrophils was capable of directly activating two collagen-degrading metalloproteinases, collagenase (Weiss *et al.*, 1985; Burkhardt *et al.*, 1986) and gelatinase (Peppin & Weiss, 1986), both of which have strategic roles in the arsenal of the neutrophil. The two antiproteases, α_2-macroglobulin and tissue inhibitor of metalloproteinases (TIMP), are not very effective in controlling this enzyme activity as they can be inactivated on neutrophil stimulation (Weiss & Peppin, 1986; Okada *et al.*, 1988). Indeed, active neutrophil elastase, collagenase and myeloperoxidase as well as oxidatively inactivated α_1-proteinase inhibitor

can be detected in fluids from areas of inflammation in rheumatoid arthritis and other inflammatory disorders (Velvart & Fehr, 1987; Weiland *et al.*, 1987; Edwards *et al.*, 1988).

(*d*) It is known that neutrophils aid the damage to articular cartilage by degrading matrix components, mainly proteoglycan and inhibiting its synthesis (Bates *et al.*, 1984). In a recent study, it was found that both proteoglycan degradation and synthesis inhibition are largely attributable to elastase and secondarily to HOCl, whereas H_2O_2 impairs only proteoglycan synthesis (Kowanko *et al.*, 1989).

(iii) Systemic inflammatory effects

Oxygen metabolites have been shown to mediate pro-inflammatory events by the generation of a powerful chemotactic lipid from arachidonic acid (Perez *et al.*, 1980; Petrone *et al.*, 1980). This oxidant-derived lipid is distinct from arachidonic acid and is active at nanomolar concentrations.

Several authors, using oxygen metabolite scavengers and inhibitors of hydroxyl radical formation, have demonstrated that experimental tissue injury in *in vivo* animal models can be dependent on oxygen metabolite generation. Intravenous infusion of catalase or desferri-oxamine (an iron chelator and inhibitor of OH· formation) blocks neutrophil-dependent lung injury associated with both intravascular complement activation and extensive burn injury to the skin (Till *et al.*, 1982; Ward *et al.*, 1983). Likewise, oxygen metabolite scavengers have been shown to be effective in diminishing quantifiable tissue damage in a number of other *in vivo* inflammatory models (reviewed in Warren *et al.*, 1988). Thus, it seems conceivable that oxygen metabolite scavengers could be effective in modulating the extent of tissue injury observed in auto-immune diseases, and this has been shown to be the case for both *in vivo* and experimental models of rheumatoid arthritic disease (Menander-Huber & Huber, 1977; Hirschelmann & Bekemeier, 1981; Dillard *et al.*, 1982). Moreover, large numbers of active PMNs have been localized around vessels and in the synovium of affected joints in rheumatoid arthritic patients (Palmer *et al.*, 1986; Bender *et al.*, 1986).

A role for free radicals in the joint injury observed in rheumatoid arthritis is supported by the finding of increased levels of conjugated dienes and malonyldialdehyde (products of lipid peroxidation reactions) in both synovial fluid and blood plasma of patients with active disease (Lunec *et al.*, 1981; Muus *et al.*, 1979).

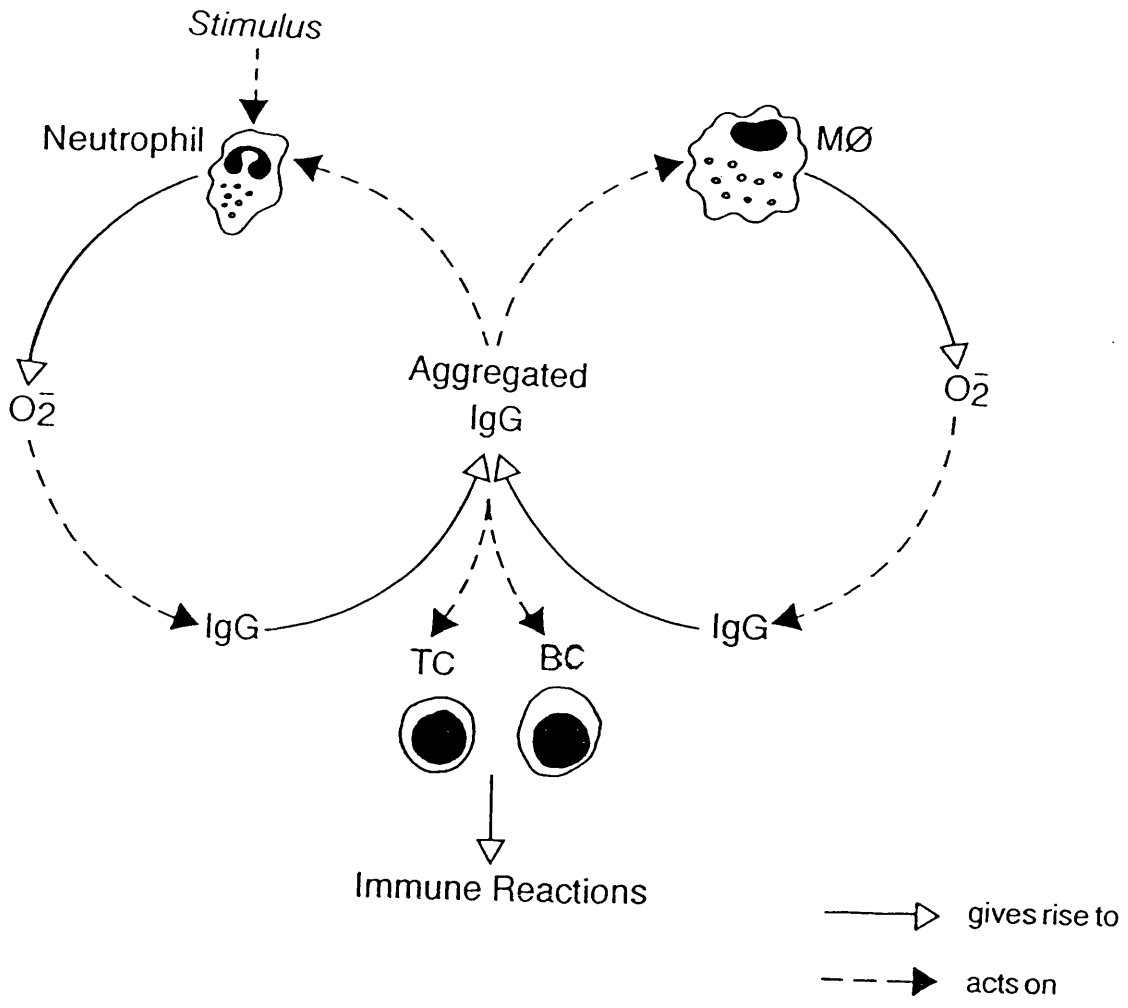


Figure 1.1: The auto-catalytic cycling of inflammatory reactions that is suggested to occur in an inflamed joint, represented schematically. Production of O_2^- from activated neutrophils can damage IgG molecules causing them to aggregate. These IgG aggregates can induce further activation of both neutrophils and macrophages to produce more O_2^-. In addition, the damaged IgG aggregates may be recognized by T cells and B cells as foreign, thereby initiating a series of auto-immune reactions.

An important study by Lunec *et al.* (1985) has shown that the production of O_2^- from activated PMNs can modify IgG molecules, causing them to aggregate and fluoresce, facilitating their detection. The O_2^--mediated modification is due to oxidation of aromatic amino acids to kynurenines, thus disrupting their 3-dimensional conformation and initiating aggregation. These aggregates are then capable of activating resting PMNs and macrophages to produce more O_2^-, thus giving rise to a self-perpetuating cycle of free radical production. In addition, these aggregates can function as auto-antigens for T cells and B cells, thus recruiting the more specific immune response. These events are summarized in fig. 1.1. Free radical damage to IgG provides a possible mechanism for *1)* the presence of rheumatoid factor (antibody-complexes of various immunoglobulin subclasses directed against self-IgG) and *2)* the activation of complement which might further amplify this destructive mechanism. Isolation of identical fluorescent IgG-complexes from fresh rheumatoid sera and synovial fluid indicates that this system has clinical relevance.

In summary, powerful oxidants are by nature short-lived and non-specific, and proteinases are held in check by either their own latency or a highly effective antiprotease screen. When both mechanisms are activated simultaneously, the neutrophil could subvert all the intrinsic barriers and thus contribute substantially to the joint damage that characterizes those chronic inflammatory states such as rheumatoid arthritis (reviewed in Weiss, 1989). The development of synthetic inhibitors of the oxidative burst, inhibitors directed against the proteolytic and hydrolytic enzymes and the synthesis of recombinant antiproteases is a priority of many drug companies today.

1.6 Pathogenesis of Rheumatoid Arthritis

The articular inflammation and joint destruction that characterizes rheumatoid arthritis (RA) is the result of a complex interaction between cellular elements - inflammatory, immunological and synovial lining cells and their soluble products (Harris, 1986; Zvaifler, 1983; Fassbender, 1984). The identity of the inciting agent in this condition is unknown. The subsequent pathogenesis of RA will be arbitrarily described under four loosely defined stages:

(1) Early synovial inflammation

- vascular proliferation in response to macrophage-derived angiogenesis factors e.g. tumour necrosis factor (TNF)
- lymphocyte diapedesis and activation (Freemont *et al.*, 1983)
- neutrophil and mononuclear cell infiltration
- synovial lining cells hypertrophy

(2) Synovial Immune Response

- generalized activation of accumulated cell population
- macrophages and/or stellate cells interdigitate to contact lymphocytes, presumably for "antigen" presentation (Young *et al.*, 1984); macrophages produce interleukin-1 (IL-1) and TNF to stimulate T lymphocytes
- T lymphocytes undergo clonal proliferation and produce inflammatory mediators such as IL-2, IL-4, IL-6, interferon-γ and B cell growth and differentiation factors
- plasma cells produce large amounts of IgG and IgM
- IgG aggregates activate the complement system
- vascular permeability increases
- immune complexes and activated complement are chemotactic for both synovial cells and PMNs

(3) Amplification of inflammation

- PMNs serve as amplifiers releasing lysosomal enzymes, toxic oxygen radicals and arachidonic acid metabolic products such as LTB_4
- macrophages are involved in ongoing inflammation mediating their effects in a similar manner to PMNs
- macrophages also release IL-1, which activates fibroblasts and chondrocytes, and TNF-α
- activation of the clotting system and generation of bradykinin and plasmin

(4) Synovial proliferation and destruction

- proteoglycan of cartilage is degraded by lytic enzymes derived from phagocytic synoviocytes and PMNs (Barrett, 1978)
- collagen of cartilage is cleaved by phagocyte-derived collagenases (Harris *et al.*, 1977)
- synovial cells proliferate and infiltrate the cartilage, simultaneously digesting enzymatically the tissue (Fassbender, 1984)
- fibroblasts and chondrocytes proliferate and release destructive enzymes

1.7 Non-steroidal anti-inflammatory drug therapy in RA

In no other kind of inflammation is there such heterogeneity as in rheumatoid synovitis (Harris, 1986), which thus presents a major challenge to rheumatologists. There is a dilemma, as no suitable disease-modifying agent exists at present. Lack of knowledge about the aetiology also limits the ability to treat the disease. RA is treated at

present with a mixture of (i) non-steroidal anti-inflammatory drugs (NSAIDs), (ii) disease-modifying anti-rheumatic drugs (DMARDs) and (iii) corticosteroids. This project is concerned with the first group, namely NSAIDs, which are used extensively for providing temporary relief of symptoms and signs despite their inability to affect the progress of the disease.

NSAIDs have, for many years, been widely and extensively used as anti-inflammatory and analgesic drugs. However, it is clear that they have several drawbacks. They can not only have quite serious side-effects (Rainsford, 1987) but they can also have pro-inflammatory actions. One aspect of this pro-inflammatory action relates to their inhibitory effect on prostanoid synthesis. Prostaglandins were first proposed to act as inflammatory mediators in 1970 (Willis, 1970) and the discovery that NSAIDs inhibit their synthesis *in vitro* and *in vivo* provided evidence in favour of an active role for prostaglandins in acute inflammatory conditions. However, it now appears that prostaglandins can also have a modulatory or homeostatic role in inflammation (Bonta & Parnham, 1978). NSAIDs by inhibiting this modulatory effect could be contributing a pro-inflammatory effect (this is discussed in Chapter 4).

They may also have a pro-inflammatory effect by an action on the oxidative burst of phagocytic cells separate from their effect on prostaglandin synthesis. Superoxide generation in neutrophils stimulated by two post-receptor stimuli was shown to be enhanced by indomethacin (Dale & Penfield, 1985) and by drugs from other NSAID classes (Penfield, 1988) but not by aspirin. This data confirmed previous studies by Gay *et al.* (1984) and by Bromberg & Pick (1983). These reports on the NSAID-mediated enhancement of the oxidative burst contrasted with the many studies reporting NSAID inhibition of inflammatory cell activation (reviewed in Chapter 4). As explained above, toxic oxygen metabolites can cause tissue damage and have the potential to generate auto-antigens from normal IgG (Lunec *et al.*, 1985). If some NSAIDs actually increase these actions they could exacerbate the progress of the underlying auto-immune/inflammatory reactions in RA even while having inhibitory effects on other aspects of cell activation.

The aim of the present project was firstly, to carry out a study of the effect of a wide range of NSAIDs from each of the main chemical groups on stimulated O_2^- generation and secondly, to investigate the mode of action of those drugs which potentiate O_2^- release. For this

latter purpose it was necessary to examine the transduction sequences involved in respiratory burst activation in order to "dissect out" the key enzymes/pathways/mediators involved in NADPH oxidase activation. Since it was not possible to study the effects of the NSAIDs directly on these pathways in the living cell without disrupting and distorting the pathways themselves, the approach was, in part, to look for a correlation between the effects of NSAIDs and the effects of "specific" inhibitors of key enzymes in the transduction pathways. The first product of the NADPH oxidase enzyme, namely O_2^-, was measured as an index of toxic oxygen metabolite production throughout the study. Before describing the results it is necessary to consider the intracellular transduction mechanisms believed to be involved in the activation of the respiratory burst oxidase.

1.8 PMN transduction mechanisms leading to respiratory burst activation

Due to the overwhelming flood of information during the course of this project it is not possible to give here a comprehensive review of the current ideas on this topic. The main events believed to occur from contact of agonist with the neutrophil to NADPH oxidase activation will therefore be summarized, with particular emphasis on the intracellular transduction pathways that were under investigation in this study.

PMN O_2^- generation can be initiated by a variety of stimuli acting at different cellular points, but regardless of the stimulus the same NADPH oxidase enzyme is utilized. It is thought that distinct activation pathways and regulatory networks are utilized by the various stimuli *en route* to NADPH oxidase activation (McPhail & Snyderman, 1983; Maridonneau-Parini *et al.*, 1986). The process of activation of NADPH oxidase can be divided into three phases: (1) the interaction between the stimulus and the neutrophil (recognition); (2) the molecular and functional modifications of the plasma membrane and intracellular constituents (transduction); (3) the final reaction(s) responsible for the transition of the respiratory burst enzyme from the quiescent to the active state.

Recognition: This involves binding of the stimulus to a specific membrane receptor as in the case of fMet-Leu-Phe (fMLP), PAF, LTB_4, immune complexes (Fc fragment receptor), opsonized particles (Fc or C3b receptor) etc. For a range of receptor stimuli, respiratory burst

activity, as measured by chemiluminescence, showed that the onset time and the maximal rates of NADPH oxidase activation were very similar or identical despite major differences in the duration of the response and the overall yield of H_2O_2 (Wymann *et al.*, 1987). It was proposed that these kinetic similarities relate to similar intracellular transduction signals for the receptor stimuli. However, considerably longer onset times and markedly lower rates were observed when NADPH oxidase activation was triggered by phorbol 12-myristate 13-acetate (PMA), Ca^{+2} ionophore or by both in combination (Wymann *et al.*, 1987; McPhail & Snyderman, 1983). The different patterns of activation were attributed to unique transductional pathways for these stimuli.

On ligand-receptor contact the activation of the oxidative burst is regulated by the occupancy, the rate of occupancy, the affinity of receptors and the number of receptors. The ligand-receptor complexes are short-lived, so continuous new interactions between the two are required for maintenance of the NADPH oxidase in the activated state or more specifically for continuous activation of the transduction reactions. Previous interaction with a stimulus generates a state of unresponsiveness to activation by the same stimulus which could be due to a decrease in the number of available receptors (Rossi *et al.*, 1983; English *et al.*, 1981). But the use of two stimuli added either together (referred to as synergism) or in sequence (referred to as "priming") can lead to an enhanced response over and above that produced by each stimulus alone. Virtually all combinations of activators have been tested (McPhail *et al.*, 1984a; Robinson *et al.*, 1984; Dale and Penfield, 1984; Bass *et al.*, 1987). Typically, the concentration of a stimulus used for priming is about ten-fold lower than that required for the same stimulus to activate (McPhail *et al.*, 1984a) and responses are characterized by a lower concentration requirement for the second stimulus (Dougherty & Niedel, 1986), a shortening of the lag period (Wymann *et al.*, 1987) and an increase in the maximal O_2^- response with the second stimulus (Della Bianca *et al.*, 1986; Grzeskowiak *et al.*, 1986). The effect is thought to be due to a priming of the transduction reactions and will be discussed more fully, in conjunction with data obtained as part of this study, in Chapter 6.

Transduction: Agonist-receptor binding orchestrates a whole series of enzymatic processes, resulting in the generation of many potential second messengers. In the first part of this section, the particular

enzymes activated and subsequent products will be described and then, those particular secondary mediators, namely diacylglycerol and Ca^{2+} that most closely correlate with O_2^- production will be examined.

One of the most widely documented reactions in transmembrane signalling is the activation of a membrane-bound phospholipase C (PLC) that hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) (Dougherty *et al.*, 1984; Di Virgilio *et al.*, 1985) with the formation of inositol 1,4,5-trisphosphate (IP_3) responsible for the mobilization of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Berridge & Irvine, 1984; Prentki *et al.*, 1984), and of diacylglycerol (DAG) that can stimulate a Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C or PKC) (Nishizuka, 1984a). It is proposed that the stimulation of the PIP_2-dependent PLC is probably the first transduction step for many of the functional responses of neutrophils (reviewed by Rossi, 1986) and would be followed by activation of other phospholipases (C, D, A_2) which are active on phosphatidylinositol (PI), phosphatidylcholine (PC) or other phospholipids, with the formation of further DAG, phosphatidate (PA) and fatty acids. It is likely that these phospholipases are activated by the increase in $[Ca^{2+}]_i$ caused by the initial activation steps (Cockcroft *et al.*, 1984; Ohta *et al.*, 1985; Bradford & Rubin, 1986; Truett *et al.*, 1988). The intracellular rise in Ca^{2+} levels on stimulation is postulated to be biphasic (Lew, 1989), consisting of an initial IP_3-mediated release of Ca^{2+} from an intracellular storage site, possibly the calciosome (Krause *et al.*, 1989), and a more delayed influx of Ca^{2+} across the plasma membrane. The influx of extracellular Ca^{2+} has been proposed to involve non-selective cation channels in the plasma membrane that are activated by the initial rise in Ca^{2+} levels (Von Tscharner *et al.*, 1986), but there is controversy on this point (Nasmith & Grinstein, 1987). The possibility that plasma membrane channels are opened by inositol 1,3,4,5,-tetrakis-phosphate has also been offered (Irvine & Moor, 1986). Most of the stimuli that activate the respiratory burst, initiate a rapid hydrolysis of phosphoinositides (discussed in more detail under the different stimuli in Chapter 3) with associated increases in DAG and Ca^{2+} levels (Dougherty *et al.*, 1984; Korchak *et al.*, 1988b; Naccache *et al.*, 1985a) that are sustained for roughly the duration of the burst. These studies support a role for phosphoinositide hydrolysis in the activation of the respiratory burst.

Burst O_2^- generation and phosphoinositide turnover are blocked by pretreatment of cells with pertussis toxin (Smith C.D. *et al.*, 1985; Brandt

et al., 1985; Naccache *et al.*, 1985d; Shirato *et al.*, 1988; Okajima & Ui, 1984), a compound which inactivates the guanine nucleotide regulatory protein (G-protein) of the G_i type by an ADP-ribosylation reaction. Also, non-hydrolysable GTP analogues potentiate the hydrolysis of PIP_2 produced by fMLP (Verghese *et al.*, 1985) and increase stimulated O_2^- production (Nasmith *et al.*, 1989). The activation of G-proteins by chemotactic stimuli is also observed in neutrophil membrane preparations as enhanced GTP binding and GTPase activity (Okajima *et al.*, 1985). Moreover, GTP analogues affect the binding of formyl peptides to neutrophil membranes suggesting a modification of the receptor upon interaction with a G-protein (Snyderman, 1984). These findings implicate a role for a G-protein in the coupling of receptor occupancy with PLC activation and production of second messengers.

PLC may use *alternative phospholipid substrates*, and phospholipases that do not act on phosphoinositides have been isolated (Wolf & Gross, 1985). Breakdown of PC to generate DAG has been noted in neutrophils in response to phorbol esters and receptor agonists (Besterman *et al.*, 1986a; Truett *et al.*, 1989). Also, radiolabelled 1-O-alkyl-2-acyl-glycero-phosphocholine is degraded to 1-O-alkyl-2-acylglycerol upon stimulation with fMLP and phorbol ester (Agwu *et al.*, 1989; Rider *et al.*, 1988).

Phospholipase D (PLD) which hydrolyzes phospholipids to produce PA, and then DAG by a PA phosphohydrolase has been proposed as a likely source of intracellular DAG in fMLP-stimulated neutrophils (Gelas *et al.*, 1989; Cockcroft & Allan, 1984). Apparently, the principal pathway for PI hydrolysis is PLD (Cockcroft *et al.*, 1985) and the hydrolysis of [^{3}H]alkyl-PC by PLD has also been demonstrated (Pai *et al.*, 1988). The relative contribution of PLD versus PLC pathways to DAG generation is not clear. *PA* itself, as a second messenger for the oxidative burst does not show good correlation with O_2^- production (Lambeth, 1988) but has been implicated as a regulator of $[Ca^{2+}]_i$ (Serhan *et al.*, 1981).

Phospholipase A_2 (PLA_2) pathways which generate arachidonate (AA) from a variety of diacyl- and 1-O-alkyl-2-acyl-phospholipids have also been implicated in stimulus-response coupling in the neutrophil. The possible candidates include not only AA but also its oxidation products whether generated via the cyclooxygenase or lipoxygenase pathways of metabolism. The subject will be reviewed in Chapter 7.

The picture that emerges from the plethora of studies on the transduction signals leading to respiratory burst activation, is that Ca^{2+} (via IP_3) and DAG (whatever the phospholipid or enzyme source) are the

predominant potential second messengers. Do they act in concert or can one or the other mediate the response independently? It has been proposed for many cell types that an increase in both Ca^{2+} and DAG may be required for signal transduction and that the two "pathways" function synergistically (Nishizuka, 1984a). Evidence has been put forward which suggests that this synergistic activation of the two pathways, by the Ca^{2+} ionophore, A23187, and the PKC activators, PMA and 1-oleoyl, 2-acetylglycerol (OAG), could be involved in neutrophil O_2^- generation (Dale & Penfield, 1984; Penfield & Dale, 1984; Di Virgilio *et al.*, 1984; Robinson *et al.*, 1984). How does this situation correlate with physiological receptor activation?

First let us look at *calcium*. A number of soluble and particulate stimuli initiate the production of O_2^- with an associated rise in $[Ca^{2+}]_i$ (Korchak *et al.*, 1988a; Naccache *et al.*, 1984; Lew *et al.*, 1984b; Gennaro *et al.*, 1984; Al-Mohanna & Hallett, 1988). The rapid changes in Ca^{2+}, plus the finding that high concentrations of Ca^{2+} ionophores activate O_2^- generation, have provoked considerable interest in this cation as a potential messenger. It was originally proposed that the oxidative burst can be activated by two distinct mechanisms, depending on the stimulus, one mechanism triggered by a rise in intracellular Ca^{2+} (with fMLP, C5a, LTB_4, concanavalin A and Ca^{2+} ionophores) and the other mechanism independent of intracellular Ca^{2+} (with PMA and latex beads) (Hallett & Campbell, 1984). Evidence that a rise in $[Ca^{2+}]_i$ is closely involved in this response comes from studies where the respiratory burst by receptor stimuli was prevented when the $[Ca^{2+}]_i$ was buffered by EGTA (Campbell & Hallett, 1983), and chelating extracellular Ca^{2+} by EGTA also reduces O_2^- generation by most stimuli. However, the elevation of $[Ca^{2+}]_i$ stimulated by fMLP was associated with a much greater degree of superoxide generation than that elicited by a Ca^{2+} ionophore, even though treatment with this latter stimulus caused a much greater increase in $[Ca^{2+}]_i$ (Pozzan *et al.*, 1983). Also, a monoclonal antibody to a neutrophil cell surface component produces an increase in $[Ca^{+2}]_i$ but does not stimulate O_2^- generation (Apfeldorf *et al.*, 1985). Interpretations are complicated by the subtle enzymatic requirements for basal $[Ca^{2+}]_i$, as well as the artifactual activation of some enzymes at high Ca^{2+} concentrations.

It is now generally agreed that a rise in $[Ca^{2+}]_i$ is likely to be necessary for optimal stimulation of the oxidative burst, but in itself is not sufficient (Rossi, 1986; Sha'afi & Molski, 1988). It may be that

receptor-activated increases in $[Ca^{2+}]_i$ are necessary for other functional responses such as degranulation or chemotaxis. This subject will be discussed further in Chapter 6.

Attention is therefore focussed on DAG and PKC, its endogenous "receptor". PMA and synthetic DAGs activate PKC and also stimulate the respiratory burst (Niedel *et al.*, 1983; Fujita *et al.*, 1984; Cox *et al.*, 1986) but the big question is the involvement of endogenously generated DAG in the PMN respiratory burst. Following receptor stimulation by fMLP a rapid appearance of DAG is detected using mass measurement (Preiss *et al.*, 1987; Rider & Niedel, 1987) consistent with a signalling role. Cytochalasin B, which augments fMLP-activated O_2^- release, caused a corresponding increase in the magnitude and duration of fMLP-stimulated DAG generation (Honeycutt & Niedel, 1986). The DAG increases occurred in parallel with the rise in O_2^- production, supporting a role for DAG in the respiratory burst. Similar results were also seen using other agonists.

Also consistent with a messenger role for DAG, an inhibitor of DAG kinase (R59022) that would elevate cellular DAG, enhanced O_2^- generation by a variety of receptor agonists (Muid *et al.*, 1987; Gomez-Cambronero *et al.*, 1987). The role of DAG inevitably implicates PKC in the transduction sequence to NADPH oxidase activation.

Protein Kinase C is considered to be the major intracellular recipient for synthetic DAGs which compete for all the [^{3}H]phorbol dibutyrate binding sites in intact PMNs and also cause activation of the respiratory burst (Cox *et al.*, 1986; Tauber, 1987). Also, addition of the catalytic fragment of PKC to resting neutrophil membranes activates NADPH oxidase, without the presence of any activating cofactors (Tauber *et al.*, 1989), as does the PKC holoenzyme plus PMA and Ca^{2+} (Cox *et al.*, 1985). From these and other studies there is little doubt that PKC mediates the respiratory burst in response to the direct PKC activators but there is still much controversy regarding its role in receptor-stimulated events. This subject is reviewed extensively in Chapter 5 together with the presentation of new evidence which could imply a focal position for PKC in both receptor and post-receptor stimulated O_2^- release.

According to some but not all workers in the field, PKC plays a central role in the PMN signalling mechanisms, although modulatory roles may be mediated by other second messenger systems generated on cell activation (Ca^{2+}, AA, etc.) which may affect the functioning of PKC. Modulation of PKC may involve the metabolism of sphingolipids to

generate the long-chain bases, sphingosine and sphinganine, which are potent inhibitors of PKC as has been demonstrated in GH_3 pituitary cells (Kolesnick & Clegg, 1988). Neutrophils contain significant quantities of endogenous sphingosine, the level of which changes with agonist treatment (Wilson *et al.*, 1988). DAG shows the best correlation with activation of PKC. The substrate protein(s) for PKC that could be responsible for activation of NADPH oxidase are reviewed elsewhere (Rossi *et al.*, 1986a; Babior, 1988), but a brief account will be presented below. It is not yet clear how specific phosphorylations are coupled to activation of the oxidase, nor whether kinases other than PKC may also participate in the activation sequence.

Activation of the oxidase: The molecular basis of NADPH oxidase activation is unknown but based on indirect evidence could involve (1) a covalent, post-translational modification of NADPH oxidase components by phosphorylation, acylation or methylation reactions; (2) a modification of the lipid environment in which the oxidase is embedded; and/or (3) the assembly of components of the electron-transfer chain, which can be either directly dependent on cross-linking or secondary to the above-mentioned modification of proteins or lipids.

Evidence accumulated during the past few years seems to suggest that phosphorylation reactions are directly or indirectly linked to activation of the NADPH oxidase. Many groups have demonstrated that endogenous proteins, either cytosolic or membrane-bound, are phosphorylated in intact leukocytes after treatment with different stimuli. Neutrophils activated with arachidonate and OAG showed an increase in the phosphorylation of a 46kDa protein in accordance with O_2^- production (Ohtsuka *et al.*, 1988a) and activation with PMA, diC_8 or chemoattractants caused the phosphorylation of two proteins, 47 and 49kDa, which accompanied the release of O_2^- (Badwey *et al.*, 1989a). Both authors concluded that the protein phosphorylation was likely to be a pre-requisite or regulatory to the stimulation of O_2^- release, as the dose-dependency and kinetics of the two responses were closely correlated. It has recently been demonstrated that both autosomal recessive (Segal *et al.*, 1985; Kramer *et al.*, 1988) and X-linked (Hayakawa *et al.*, 1986; Babior, 1988) CGD patients lack phosphorylation of proteins 44 to 48kDa; although X-linked CGD has been associated with a defective or absent cytochrome b_{558} (see above), the molecular basis of autosomal CGD had not previously been established. In another study with 94 CGD

patients it was shown that of the one third belonging to the autosomal form, 88% lacked a 47kDa protein and 12% lacked a 67kDa protein. In neutrophils from normal individuals, the 47 and 67kDa proteins, which have been shown to be phosphorylated upon cell stimulation, were found to be translocated to the plasma membrane (Clark *et al.*, 1990). No such translocation was observed in cells with X-linked CGD, although normal levels of phosphoprotein were present, suggesting that cytochrome b_{558} may function as the acceptor (Heyworth *et al.*, 1989). Papini *et al.* (1985) working with pig neutrophils found several phosphorylated products, but a 31.5kDa protein that co-purifies with the oxidase was prominently phosphorylated by a mechanism apparently mediated by PKC upon cell stimulation and it was suggested that this product was a component of the previously characterized oxidase (Serra *et al.*, 1984). In conclusion, it can be cautiously stated that activation of the respiratory burst is accompanied by phosphorylation reactions, but the real significance of these reactions for the cell response is still unclear and will not become so until the oxidase components are defined more precisely.

There has been speculation that the process of phosphorylation could involve some components of the oxidase or some other regulatory proteins, so allowing the flux of electrons from NADPH to O_2 by inducing the coupling of components of the oxidase or by changing the conformation of some components (Rossi, 1986). Studies in favour of the assembly of the electron-transport chain at the moment of stimulation have come from the use of cross-linking agents, like glutaraldehyde and N-hydroxysuccinimide, which have been shown to inhibit the activation of the burst when added *before* the stimulus but not after (Romeo *et al.*, 1977; Aviram *et al.*, 1984). Significantly, cleavage of the cross-linkages restores the neutrophil's response, indicating that aggregation of surface molecules plays a role in stimulation of the respiratory burst. However, it is not known whether the transmembrane signalling or the assembly of oxidase components is affected by inhibition of the lateral diffusion of surface molecules.

Evidence has been presented that cytochrome b_{558} translocates from granules to the plasma membrane during activation of the respiratory burst (Borregaard *et al.*, 1983; Ohno *et al.*, 1985), as also does a NADPH-binding component which originates from the cytoplasm (Smith R.M. *et al.*, 1989). There is evidence for other cytosolic components in NADPH oxidase activation (Curnutte *et al.*, 1989). These results also favour the

assembly model of oxidase activation.

It cannot be ruled out that protein phosphorylation is a necessary but not sufficient event for activation of the oxidase. If this is the case, protein phosphorylation could be relevant for the transformation of the oxidase from resting to a primed state, and full activation might require some other mechanism.

It is also possible that, in some cases, stimulation of the oxidase can occur independent of protein phosphorylation. In this context it is intriguing to note that NADPH oxidase can be activated *in vitro* in a cell free system, by adding arachidonate (McPhail *et al.*, 1985; Curnutte, 1985; Bromberg & Pick, 1984), oleic acid (Heyneman & Vercauteren, 1984), phosphatidic acid (Bellavite *et al.*, 1988), PMA (Cox *et al.*, 1985), sodium dodecyl sulfate (Cox *et al.*, 1987) or guanine nucleotides (Gabig *et al.*, 1987) to the membrane-bound oxidase. In these studies an additional soluble factor was required; in only one study was this identified as protein kinase C (Cox *et al.*, 1985) - for all the other studies the cytosolic cofactor was proposed to be PKC-independent. This activation mechanism could be linked to a rearrangement of components of the oxidase or to a perturbation of the lipid environment of the plasma membrane, in which the NADPH oxidase is embedded. A change in the membrane lipids could cause conformational modification of the oxidase which mimics some more subtle modification operative in the cellular response to physiological stimuli.

The particular transduction questions addressed in this project pertained to the involvement of the DAG/PKC pathway (in both PIP_2-dependent and independent modes of cell activation), the IP_3/Ca^{+2} pathway and the arachidonate-linked pathways in the stimulated respiratory burst, by both receptor and post-receptor stimuli. A study of this nature was deemed necessary in investigating the mode of action of the potentiating NSAIDs. Individual chapters have been assigned to deal with each of these topics independently, and the intricate inter-relationships between the transduction pathways will be discussed in Chapter 9.

CHAPTER TWO

METHODS AND MATERIALS

2.1 Preparation of human neutrophils

For each experiment 120ml of human blood was collected by venipuncture from healthy volunteers in the Pharmacology Department UCL, having firstly ascertained that the donor had not taken any drugs or medication for at least 24 hours prior to the donation. The blood was mixed with 120ml 0.9% sterile saline in a plastic flask containing 4.8ml of 0.1M ethylenediaminetetraacetic acid (EDTA), to chelate Ca^{2+} ions and thereby prevent clotting. The subsequent steps leading to the preparation of purified neutrophils were based on a method by Boyüm (1974) and comprised a two-stage process, the first being concerned with removing the monocyte fraction and the second stage involving the removal of erythrocytes.

2.1.1 Removal of monocytes

Removal of the monocyte layer entailed centrifugation of the whole blood-saline mixture over a density gradient of Ficoll-Isopaque. Ficoll is a sucrose polymer which enhances agglutination of erythrocytes, while Isopaque provides a high density solution without being too hypertonic. This separation medium was prepared by adding approximately 58ml 9% Ficoll solution to 24ml 34% Isopaque solution (which was stored in the dark due to its light sensitivity) and adjusting the specific gravity of the resulting mixture to 1.080g/ml at room temperature.

This mixture was then dispensed, in 10ml aliquots, into each of eight 50ml round-bottomed, plastic centrifuge tubes and the blood-saline-EDTA mixture was layered slowly, in approximately equal volume, over the Ficoll-Isopaque. The tubes were centrifuged at 300g for 30 minutes at room temperature in a Gallenkamp Labspin centrifuge. This first centrifuge resulted in the layering of the blood into its constituent parts in the following way: a red blood cell (RBC)-granulocyte "sludge" at the bottom of each tube, then the Ficoll-Isopaque layer with a buffy coat of monocytes at the lower side, and finally the larger volume of

plasma and saline. In each tube the supernatant was aspirated leaving only the RBC and granulocyte mixture, the starting point for the second stage of the purification.

2.1.2 Removal of erythrocytes/purification of granulocytes

The method used for removing the RBCs was a series of hypotonic lyses in ice-cold sterile distilled water. 15ml aliquots were added to each tube for one to two minute periods, and shaken well to resuspend the pellet. Then, an equal volume of cold, double-strength (× 2) Ca^{2+}- and Mg^{2+}-free HBSS was added, to restore tonicity. The tubes were inverted, to ensure thorough mixing, centrifuged for 10 minutes at 300g (at 4°C) in a MSE Chilspin centrifuge and the supernatants then aspirated. This cycle of lysis and centrifugation was repeated, gradually combining the cell pellets, until a single composite white cell pellet, without RBC contamination, resulted. The white cells were then suspended in a known volume of cold Ca^{2+}-free Tyrode solution (see section 2.7a). This method of preparation produced a population of neutrophils generally 98% pure.

2.1.3 Cell counting

After the cell preparation, a 0.1ml aliquot of cell-Tyrode suspension was removed and added to 0.9ml of a 0.4% solution of Trypan Blue. This stain is taken up by the dead and dying cells but is excluded by viable cells, thus serving the useful role of enabling both a cell count and a viability test to be performed. The cells were counted using an Improved Neubauer haemocytometer. The neutrophils were generally 100% viable and the average yield from 120ml blood was between 300 - 400 × 10^6 neutrophils, varying greatly between donors.

2.2 The assay for superoxide release

2.2.1 Theory

The O_2^- radical reduces ferricytochrome C to ferrocytochrome C, which can be followed spectrophotometrically at 550nm. Superoxide dismutase (SOD) can be used to distinguish between reactions caused by O_2^- and those that may be occurring due to the presence of other agents in the reaction mixture. Thus, SOD will, by intercepting O_2^-, have the effect of specifically inhibiting the reduction reactions involving this radical. The ferricytochrome C reduction reaction can be carried out both in the presence and absence of this enzyme, and the difference

between the two treatments will represent the reduction caused by O_2^- alone, so the system should be truly indicative of O_2^- release.

2.2.2 The method

The neutrophil suspension, having been prepared as described above, was diluted to 4 x 10^6 cells/ml in Ca^{2+}-free Tyrode solution (see section 2.7a) and then equilibrated at 37°C for 20 minutes. The next procedure was dependent on the nature of the stimulus and drug to be used. The cell suspension was divided into different aliquots and pre-incubated at 37°C in the presence or absence of inhibitor (i.e. drug under investigation), usually for 20 minutes; exceptions to this were the protein kinase C inhibitor studies to be specified at the end of this section. After 15 minutes of this pre-incubation period, cytochalasin B (5μg/ml) was added to those cells which were to be stimulated with fMLP, PAF or A23187, and incubation continued for another 5 minutes. Following this, the reaction was initiated when 500μl of the cell suspension (2 x 10^6 cells) was dispensed into 2.5ml tubes (Sterilin NA25 or Sterilin LP2) to which had been added 0.25ml of a solution of ferricytochrome C (made up at 4mg/ml), 100μl of the appropriate stimulus [or 100μl Tyrode in cells only tubes], 25μl of the appropriate concentration of "drug" [or 25μl of Tyrode in control tubes] and 25μl SOD (75 units) [or Tyrode solution in sample tubes]. The DMSO vehicle (although rarely exceeding 0.1%) was controlled for as appropriate in all experiments. The tubes were then whirlimixed and returned to the incubator at 37°C for 30 minutes, during which time the tubes were periodically shaken. The reaction was stopped by the addition of 500μl (1mM) N-ethylmaleimide, a sulfhydryl reagent which has been shown to completely inhibit O_2^- production at 10^{-4}M.

Following centrifugation at 1400g for 10 minutes at 4°C, the absorbance of the supernatant was read at 550nm in a Beckman DU-50 spectrophotometer.

All drugs and reagents were made up in a Ca^{2+}-free Tyrode, with the exception of ferricytochrome C, which was made up in a Ca^{2+}-containing Tyrode solution, that would give a final Ca^{2+} concentration of 3mM in all samples. All determinations were carried out in duplicate.

For the protein kinase C inhibitor studies, equilibrated cells (2 x 10^6) were added directly to the reaction tubes which contained 1mg ferricytochrome C, the appropriate inhibitor or Tyrode and either SOD or Tyrode; cytochalasin B was added to the appropriate cells immediately

prior to this addition. Incubation was continued for another 5 minutes before beginning the reaction with the desired stimulus.

Opsonized zymosan (OZ) and concanavalin A-zymosan (ConA-zymosan) were prepared as follows. Zymosan was suspended in saline, boiled for 30 minutes, centrifuged in a Gallenkamp Labspin centrifuge at 300g for 10 minutes and then the supernatant was aspirated. The subsequent pellet was washed twice in saline and the final suspension of the pellet at 10mg/ml was made in either autologous fresh human serum for OZ, or Ca^{2+}-containing HBSS with ConA at 0.5mg/ml for ConA-zymosan. Both the serum-zymosan and ConA-zymosan suspensions were incubated at 37°C for 30 minutes. Both mixtures were then centrifuged at 300g and the resulting pellet resuspended in saline (for OZ) and Ca^{2+}- and Mg^{2+}-free HBSS (for ConA-zymosan). The pellets were subsequently washed twice in their respective media and the final resuspension was carried out in Tyrode.

In the case of the stimulus sodium fluoride (NaF), a Dulbecco's PBS (see section 2.7c) was used for all experiments in which the concentration of NaCl was reduced so that the final salt concentration was physiological after the addition of NaF. Also, the calcium concentration was reduced with this stimulus to 0.31mM, to avoid precipitation of calcium phosphate.

2.2.3 The Calculation

Having measured the absorbance at 550nm of all the samples (with and without SOD), the absorbance was converted to nanomoles O_2^- per 5×10^6 neutrophils. The calculation will be described here but in practice the spectrophotometer was pre-programmed to carry out the manipulation. It is based on the Beer-Lambert law, which relates the absorbance of a substance to its concentration:

$$\Delta A = \varepsilon C l$$

where ΔA is the difference in absorbance between the tubes with and without SOD, C is the concentration of the absorbing substance, l is the length of the light path (1cm) and ε is the extinction coefficient of the absorbing substance – ε at 550nm = 15 mM^{-1} cm^{-1} (Margoliash & Frohwirt, 1959).

Therefore, $\frac{\Delta A}{15.5 \times 1}$ = mmol O_2^-/l generated per tube (i.e. 2×10^6 neutrophils).

The final volume in the spectrophotometric cuvette = 1.5ml

$$\text{Therefore,} \quad \frac{\Delta A \times 1.5 \times 10^6}{15.1 \times 1 \times 10^3} = \text{nmol } O_2^-/5 \times 10^6 \text{ neutrophils}$$

$$\frac{\Delta A \times 1.5 \times 10^3}{15.5} = \text{nmol } O_2^-/5 \times 10^6 \text{ neutrophils}$$

2.3 The Assay for LTB_4 production

2.3.1 Production of LTB_4 from PMNs

PMNs, isolated as described above, were suspended at 50 × 10^6 cells/ml in Tyrode and then dispensed, in 200μl aliquots (10 × 10^6 cells/ tube), into polystyrene 2.5ml tubes (Sterilin NA25). Cells were equilibrated at 37°C for 20 minutes. Then, 100μl of Ca^{2+}-Tyrode plus the drug under investigation (or Ca^{2+}-Tyrode only to the control tubes) were added simultaneously to give a final Ca^{2+} concentration of 3mM and the appropriate final drug concentration. Incubation was continued for another 5 minutes at 37°C. Then the stimulus, which was usually OZ (prepared as described in section 2.2.2), at × 2.5 the desired final concentration was added in 200μl aliquots and incubation continued for 30 minutes. The reaction was terminated by placing the tubes in a freezer for 5 minutes and the tubes were then centrifuged in a MSE Chilspin centrifuge for 10 minutes. The supernatants were recovered and decanted into another set of 2.5ml Eppendorf tubes, to be stored frozen at -20°C for subsequent determination of LTB_4.

2.3.2 LTB_4 determination by Radioimmunoassay (RIA)

Samples containing LTB_4 were thawed and mixed thoroughly. 100μl of each sample was assayed in duplicate for LTB_4 based on the method described by Salmon *et al.* (1982). The assay was performed in glass 12 × 75mm disposable culture tubes. All reagents were made up in 50mM Tris buffer (pH 8.6), containing 0.1% gelatin. Each assay tube contained the following:-

0.1ml buffer

0.1ml standard LTB_4 or unknown sample

0.1ml [^{3}H]LTB_4 (≡ 3000 cpm) [5μCi or 250μl per 15ml buffer]

0.1ml Rabbit anti-LTB_4 antibody

The anti-LTB_4 antibody was diluted to give approximately 50% binding of [^{3}H]LTB_4 in the absence of any added LTB_4 i.e. 400μl of diluted stock (1

in 10) per 15ml buffer. With each assay a standard curve containing 0, 5, 10, 20, 50, 100, 200, 500, 1000, 5000 pg LTB_4 was included. Also two additional tubes were included which would contain the minimum and maximum counts obtainable - a non-specific-binding (NSB) and a total counts (TC) tube, each composed of 0.1ml [^{3}H]LTB_4 and 0.3ml buffer. All standards and unknowns were assayed in duplicate.

The tubes were prepared as above and allowed to stand overnight at 4°C or for 1-4 hours at room temperature. This is the time necessary for the total LTB_4 in the tubes to come to equilibrium with the anti-LTB_4 antibody.

At the end of the incubation the tubes were transferred to ice. The next part of the procedure involved the separation of bound and free LTB_4, which was achieved using a charcoal-dextran suspension (prepared by adding 1.2g charcoal and 240mg dextran to 60ml water). This suspension was kept on ice and stirred continuously. To every tube, except TC tubes, 0.2ml of the dextran coated charcoal suspension was added. Tubes were whirlimixed immediately, left on ice for 10 minutes and then centrifuged at 1000g for 5 minutes at 4°C. The clear supernatant was then decanted into scintillation vials. The TC tubes received 0.2ml of water and went straight into scintillation vials. To each vial, 5ml of picofluor scintillant was added and the vials were counted in a Beckman LS 1801 for 4 minutes or 1000 counts. One important point to be noted in the separation procedure is that charcoal should only be added to the number of tubes that can be centrifuged in the one run. Samples left sitting in charcoal for longer than 10 minutes undergo a disruption of the bound to free equilibrium as charcoal begins to strip the [^{3}H]LTB_4 already "bound".

A standard curve was then constructed plotting cpm [^{3}H] against the standard LTB_4 concentrations (given in pg LTB_4) from which the LTB_4 content of the unknown samples could be read as pg LTB_4 produced/10 × 10^6 neutrophils. In practice, this manipulation was performed using a computer program.

2.4 Diacylglycerol Measurements

2.4.1 Radiolabelling studies

Neutrophil lipid was labelled by incubating resting cells with a number of radiolabelled lipid compounds, namely [^{3}H]glycerol, [^{3}H]palmitate and [^{14}C]stearate until equilibrium labelling was established. Diacylglycerol (DAG) production was determined by

measuring the increase in labelled DAG on cell stimulation over basal DAG in unstimulated cells.

a) 1(3)[^{3}H]glycerol labelling

Neutrophils, prepared as described in section 2.1, were suspended in Tyrode at 20 x 10^6 cells/ml (approximately 8ml of cells per experiment) and equilibrated at 37°C for 15 minutes. They were then incubated at 37°C for 60 minutes with 250μCi 1(3)[^{3}H]glycerol, after which the suspension was centrifuged at 300g for 10 minutes in a Gallenkamp Labspin. The labelled glycerol (free in the cell cytoplasm) was then chased from the cells by resuspending the above cell pellet in a 10mM glycerol-Tyrode solution, incubating at 37°C for 5 minutes and centrifuging for 5 minutes. This cycle was again repeated and the resulting cell pellet suspended at 40 x 10^6 cells/ml in Tyrode. The cell suspension was then equilibrated at 37°C for 15 minutes, before pre-incubating with drug or DMSO control for 20 minutes. Cell activation was initiated when 500μl of the cell suspension (or 20 x 10^6 cells) was aliquoted into pre-incubated test tubes containing Ca^{2+} (3mM for fMLP/A23187 or 0.31mM for fluoride) and stimulus, to give a final reaction volume of 1ml. At the indicated times (if a time-course experiment was being carried out) or after 30 minutes (if the reaction was being run to completion) the reaction was stopped by the addition of 3.75ml chloroform/methanol (1:2).

In those experiments where fluoride was the cell stimulus, a Dulbecco's PBS buffer (see Section 2.7c) was employed, taking similar precautions as in the superoxide assay (section 2.2), to ensure that the final salt concentration was physiological after the addition of sodium fluoride.

Then 1.25ml chloroform and 1.25ml 0.15M NaCl was added to each tube, whirlimixed for 5 seconds and centrifuged for 5 minutes to separate the two phases. The lower hydrophobic phase was decanted and placed in a desiccater for 1 hour to dry off the solvent. The dried lipids were then resuspended in 50μl chloroform and spotted onto thin layer chromatography (tlc) plates. Each sample was loaded onto a neutral and a phospholipid plate as two different solvent systems are required for their respective separations. The samples were divided between the neutral lipid and the phospholipid plates in the ratio 2:1. The neutral lipid solvent system contained 90ml benzene, 10ml diethyl-ether and 0.1ml glacial acetic acid, while the phospholipid system

contained 75ml chloroform, 45ml methanol, 3ml glacial acetic acid and 1ml water. When the plates were developed they were dried in a fumehood, placed in iodine tanks to highlight the spots which were then scrapped into plastic scintillation vials containing 8ml PCS scintillation fluid and 1ml methanol/water mixture (1:1). They were counted in a Searle scintillation counter for 10 minutes. Results were then expressed as counts in dpm in terms of the dpm counts of a constant lipid in the experiment (i.e. the origin), which could be regarded as an internal control and corrected for discrepancies in sample loading to the tlc plates.

b) *Double labelling with [^{14}C]stearate and [^{3}H]palmitate*

The first experiment using this double labelling procedure involved a study of the time course of label incorporation into the different cellular lipids. Neutrophils, 20 x 10^6 cells/ml in Dulbecco's PBS, were incubated with 10μCi [^{14}C]stearate and 20μCi [^{3}H]palmitate for varying time periods from 0 to 150 minutes; label incorporation was stopped by the addition of 3.75ml chloroform/methanol (2:1) and the lipid isolation procedure carried out as for the glycerol labelling experiments. The palmitate label was incorporated predominantly into triglyceride and phosphatidylcholine (PC) whereas the stearate label was taken up by PC, phosphatidylethanolamine (PE), phosphatidylinositol (PI) and by triglyceride to a lesser extent.

Fluoride-induced DAG generation was then examined using the double labelling procedure; cells were incubated with 10μCi [^{14}C]stearate and 20μCi [^{3}H]palmitate for 1 hour. Cell activation with fluoride and lipid extraction were carried out as in the glycerol labelling experiments.

2.4.2 DAG mass measurement using the *E. coli* DAG kinase assay

This assay was first described by Preiss *et al.* (1987) and is based on the following principle. DAG present in a crude lipid extract is quantitatively converted to [^{32}P]phosphatidic acid (PA) by *E. coli* DAG kinase. The mass of [^{32}P]PA produced measures the mass of DAG in the original lipid extract.

In the current study, it was first considered necessary to test the effect of the putative DAG kinase inhibitors (R59022 & DOEG) and the DAG lipase inhibitor (RHC80267) on the *E. coli* DAG kinase enzyme employed in the assay. This was achieved using the substrate, 1-stearoyl, 2-arachidonyl-sn-glycerol in place of cellular DAG (or more specifically diradylglycerols) for the reaction, and examining the effect

of the above drugs on the [^{32}P]PA mass produced.

Mixed lipid micelles were prepared by adding 5mM cardiolipin, stearoyl-arachidonylglycerol, with and without 100μM of the drug under investigation, to a stoppered glass tube in proportion to make a 50μl micelle mixture. This mixture was evaporated to dryness under N_2 and redissolved with brief sonication in a 255mM octyl-β-D-glucoside/1mM diethylenetriaminepentaacetic acid (DTPA) solution (pH 6.6). 20μl of this micelle mixture was used per assay tube. The other assay constituents were:

1. 50μl reaction buffer - composed of 100mM Imidazole base, 100mM NaCl, 25mM $MgCl_2$ and 2mM EGTA (pH 6.6)
2. 10μl of 20mM dithiothreitol - made up in 1mM DTPA
3. 10μl *E. coli* membrane preparation containing DAG kinase (specific activity 8μmol/min/mg protein)
4. 10μl of 10mM ATP ([γ-^{32}P]ATP at 100μCi/μmole ATP) plus 100mM imidazole - made up in 1mM DTPA.

The reaction was initiated by the addition of the latter ATP component and was terminated after 30 minutes by adding 0.9ml of 1% perchloric acid (w/v). Then 2.4ml of chloroform/methanol (1:1 v/v) was added, the tubes vortexed and centrifuged so that the mixture could be separated into two phases. The upper aqueous layer was removed by aspiration and the lower chloroform layer was washed twice by adding 2.1ml of theoretical upper phase (formed by mixing chloroform/methanol (1:1 v/v) with 1% perchloric acid in the ratios 12:12:10 (v/v/v) respectively). The tubes were again centrifuged and the lower chloroform layer was then aspirated into glass scintillation vials. The chloroform was allowed to evaporate, and then 5ml of scintillation fluid was added to each vial. The vials were then counted in a Beckman LS 1801 liquid scintillation counter.

Using this assay, the DAG metabolic inhibitors each caused a greater than 50% inhibition of the conversion of stearoyl-arachidonylglycerol to [^{32}P]phosphatidic acid in a single experiment. However, in another experiment the inhibitors had no significant effect on the assay. The technique was not subsequently used.

2.5 Intracellular Ca^{2+} measurements

2.5.1 Theory

Cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) was determined using a chemical derivative of EGTA, namely Quin 2 (see section 2.8). Quin 2 is a

fluorescent, highly selective Ca^{2+} indicator that, when trapped inside cells, can be used to measure and manipulate $[Ca^{2+}]_i$. Quin 2 is a tetra-carboxylic acid which binds Ca^{2+} with 1:1 stoichiometry and has an effective dissociation constant of 115nM in a cationic background mimicking cytoplasm. Its fluorescence signal (excitation 339nm, emission 492nm) increases about 5-fold going from Ca-free to Ca-saturated forms. The acetoxymethyl ester of Quin 2, designated as Quin 2/AM, is a membrane permeable derivative. Once Quin 2/AM penetrates the cell membrane it is cleaved by cytoplasmic esterases to yield Quin 2, which remains trapped in the cell.

Intracellular Quin 2, even at high millimolar concentrations, does not influence the $[Ca^{2+}]_i$ level (Tsien *et al.*, 1982). Because it is a high affinity chelator of Ca^{2+} and is generated in a Ca-free form inside the cells, it does bind $[Ca^{2+}]_i$ substantially. It is thus postulated that the plasma membrane maintains homeostasis of $[Ca^{2+}]_i$ by allowing net entry of Ca^{2+}. However, in the case of an external deficiency of Ca^{2+}, Quin 2 will cause a sizeable lowering of $[Ca^{2+}]_i$ as the chelated internal Ca^{2+} cannot be resupplied from the outside (Tsien *et al.*, 1982).

2.5.2 Experimental Procedure

In this study, Quin 2/AM was employed to measure the resting $[Ca^{2+}]_i$ in unstimulated neutrophils, and in conjunction with EGTA to deplete $[Ca^{2+}]_i$ in another population of cells; the Quin 2 signal in these latter cells would also indicate whether the $[Ca^{2+}]_i$ was significantly decreased. The procedure described by Rossi *et al.* (1989) and adopted from Tsien *et al.* (1982) was used.

Normal neutrophils (2.5×10^7/ml) were loaded with fluorophore by incubation with 10μM Quin 2/AM in HBSS containing 0.5mM Ca^{2+} and 1mM Mg^{2+} at 37°C. After 15 minutes the cell suspension was diluted to 5×10^6 cells/ml and the incubation was continued for 45 minutes. Another aliquot of cells were incubated under the same conditions without Quin 2/AM to check the autofluorescence due to cells and reagents. After loading, the cell suspension was washed twice and resuspended at 5×10^6 neutrophils/ml in Tyrode containing 0.5mM Ca^{2+} and 1mM Mg^{2+}. To deplete cells of Ca^{2+}, neutrophils were suspended at 5×10^7 cells/ml in Ca^{2+}-free HBSS containing 1mM EGTA and 40μM Quin 2/AM and incubated at 37°C for 60 minutes. The cell suspension was again washed twice in Ca^{2+}-free HBSS containing 1mM EGTA and the cells resuspended in EGTA-Tyrode at 5×10^6 cells/ml.

Each of the above three cell suspensions was divided in two, one set to be used for fluorescence readings and the other for obtaining minimum and maximum fluorescence readings. The latter procedure involved the addition of a 0.1% Triton X-100 solution to the cell suspensions to release the cytosolic Quin 2. Fluorescence was measured in a Perkin-Elmer 300 spectrofluorimeter at excitation and emission wavelengths of 339nm and 492nm respectively, using a stirring cuvette for all readings. The values obtained from those cells not loaded with Quin 2 were subtracted from the readings obtained with Quin 2-loaded cells, and $[Ca^{2+}]_i$ calculated using the equation:

$$[Ca] = \frac{Kd\ (F - F_{min})}{(F_{MAX} - F)}$$

where Kd (the dissociation constant of Ca^{2+} binding to Quin 2) = 115nM

F = fluorescence reading

F_{MAX} = fluorescence reading of normal cells treated with Triton X-100.

F_{min} = fluorescence reading of Ca^{2+}-depleted cells with Triton X-100.

2.6 Protein Kinase C activation assay

2.6.1 Partial purification of protein kinase C

Protein kinase C (PKC) was partially purified from rat brains by modification of the extraction procedure reported by Niedel *et al.* (1983). The isolation procedure was carried out by people at the Wellcome Research Laboratories. Briefly, the tissue was homogenized in the presence of Ca^{2+} so the PKC translocated to the membrane fraction, and was isolated on centrifugation. This fraction was then treated with EGTA to release PKC into the soluble phase, which was then separated out and treated with the specific ammonium chloride concentration to effect its precipitation. The PKC fraction was again solubilized and further purified by chromatographing on a DE-52 column.

PKC containing fractions were identified by measuring their histone-phosphorylating activity. The fractions were stored frozen at -80°C in the presence of 0.05% Triton X-100; under these conditions the protein was stable for several weeks.

2.6.2 Mixed micellar assay for PKC activity

The assay is based on the method described by Hannun *et al.* (1985) and measures histone phosphorylation using Triton X-100 mixed lipid micelles. The required amounts of phosphatidylserine, dihexanoylglycerol and drug (if required) in chloroform/methanol were aliquoted to lipid bottles, dried under a stream of nitrogen and solubilized in 3% Triton X-100 by sonication. In all experiments the micellar concentration of phosphatidylserine was fixed at 20mole% and taken from a stock 10mg/ml in chloroform/methanol (95:5). The dihexanoylglycerol concentration was varied between 0.001-10mole% and taken from a stock 1mg/ml in chloroform. The drugs were dissolved in methanol at 1mg/ml and aliquoted to the micellar mixture at their desired mole% concentration. The Triton X-100 component of the micelle remained constant at 125μl of a 3% Triton solution or 5.5625μmoles but varied in mole% terms according to the dihexanoylglycerol and drug concentrations introduced to the micelle.

The assay was carried out in glass 12 × 75 mm disposable culture tubes. A Tris/EGTA buffer was employed containing 25mM Tris base and 6.25mM EGTA, pH 7.5. The assay constituents were added to the reaction tubes in the following order:

1) 25μl histone III at 1mg/ml (final concentration) made up in Tris/EGTA buffer.
2) 25μl Triton mixed micelles (with varying components).
3) 25μl PKC enzyme at 1-10μg protein (final concentration).
4) 175μl of ATP-Ca-Mg solution at 100μM [γ-^{32}P]ATP (1.2μCi/ml), 100μM Ca^{2+} and 10μM Mg^{2+} (final concentrations) in Tris/EGTA buffer.

The reaction was initiated after addition of the latter ATP solution and terminated after 10 minutes at 22°C by addition of 1ml of ice-cold 25% trichloroacetic acid (w/v) and 1ml of ice-cold bovine serum albumin (500μg/ml). The resulting suspension was filtered through a GF/C filter and the precipitate then washed twice with 5ml ice-cold 25% trichloroacetic acid. Radioactivity retained on the filter was determined by liquid scintillation counting.

2.7 Media in the various assays

a) Tyrode Solution

The medium in which the cells were suspended during the course of this project was Tyrode solution. This contained: 137mM NaCl, 2.7mM KCl, 1mM $MgCl_2$, 1mg/ml glucose, 1mg/ml bovine serum albumin (BSA) and 20mM HEPES (4-(-2-hydroxyethyl)-1-piperazine ethanesulfonic acid), pH 7.4. Ca^{2+} (3mM) was added immediately prior to the beginning of the experiment, to avoid cell aggregation. The cells were thus suspended in Ca^{2+}-free medium, until the reaction was initiated.

b) Hanks Balanced Salt Solution (HBSS)

The medium used during the cell preparation was a Ca^{2+}- and Mg^{2+}-free HBSS, diluted down from the concentrated (× 10) stock solution with sterile distilled water to the required strength (× 2 - the use of which will be described in section 2.1.2) and buffered with CO_2-saturated sodium bicarbonate.

c) Phosphate-Buffered Saline (PBS)

A Dulbecco's PBS was employed for those experiments where sodium fluoride was used as a cell stimulant. This contained: 136.9mM NaCl. 2.7mM KCl, 8.1mM Na_2HPO_4, 1.5mM KH_2PO_4 and 0.5mM $MgCl_2$. Calcium concentration was reduced to 0.31mM to avoid precipitation with phosphate, and again (like Tyrode) was added immediately prior to the reaction.

and 1mg/ml glucose, 1mg/ml BSA

d) Tris Buffer

A Tris buffer was used for the LTB_4 radioimmunoassay (RIA) and contained: 50mM Tris and 0.1% gelatin, pH 8.6.

e) Imidazole Buffer

This buffer was employed for the DAG mass measurement assay (using *E. coli* DAG kinase) and was composed of: 100mM Imidazole base, 100mM NaCl, 25mM $MgCl_2$ and 2mM EGTA, pH 6.6.

f) Tris/EGTA Buffer

This buffer was used in the protein kinase C mixed micellar assay and contained: 25mM Tris and 6.25mM EGTA, pH 7.5

2.8 Materials

Acetylsalicylic acid (aspirin)	Sigma Chemical Co.,Poole UK
[γ-^{32}P]ATP	Amersham International plc,Bucks UK
Benoxaprofen	Gift from Wellcome Research Labs.
Bovine serum albumin (BSA)	Miles Laboratories Ltd.
BW A4C [a]	Gift from Wellcome Research Labs.
Cardiolipin	Sigma Chemical Co.,Poole UK
Charcoal	BDH Chemicals Ltd.,Poole UK
Compound I (chloracysine) & Compound II [b]	Gift from Beecham Research Labs.
Concanavalin A (ConA)	Sigma Chemical Co.,Poole UK
Cytochalasin B	Sigma Chemical Co.,Poole UK
Dextran	Sigma Chemical Co.,Poole UK
Diclofenac	Sigma Chemical Co.,Poole UK
Diethylenetriaminepentaacetic acid (DTPA)	Sigma Chemical Co.,Poole UK
1,2-Dihexanoyl-sn-glycerol (DH)	Sigma Chemical Co.,Poole UK
1,2-Dioctanoylethylene glycol (DOEG)	Sigma Chemical Co.,Poole UK
1,2-Dioctanoyl-sn-glycerol (diC_8)	Sigma Chemical Co.,Poole UK
Dimethyl sulfoxide (DMSO)	Sigma Chemical Co.,Poole UK
Dithiothreitol (DTT)	Sigma Chemical Co.,Poole UK
EDTA	BDH Chemicals Ltd.,Poole UK
EGTA	Sigma Chemical Co.,Poole UK
N-ethylmaleimide	Sigma Chemical Co.,Poole UK
Ferricytochrome C (horse heart,type III)	Sigma Chemical Co.,Poole UK
Ficoll	Pharmacia Fine Chemicals, Uppsala
Formylmethionyl-leucyl-phenylalanine (fMLP)	Sigma Chemical Co.,Poole UK
Glucose	BDH Chemicals Ltd.,Poole UK
[^{3}H]glycerol	Amersham International plc,Bucks UK
Hanks balanced salt solution	Gibco Ltd., Paisley
HEPES [c]	Gibco Ltd., Paisley
Histone III	Sigma Chemical Co.,Poole UK
Ibuprofen	Sigma Chemical Co.,Poole UK
Indomethacin	Sigma Chemical Co.,Poole UK
Ionophore A23187	Sigma Chemical Co.,Poole UK
Isopaque	Nyegaard & Co.,AS,Oslo Norway
Ketoprofen	Sigma Chemical Co.,Poole UK
[^{3}H]LTB_4	Amersham International plc,Bucks UK
Anti-LTB_4 antibody	Gift from Wellcome Research Labs.

K252a [d]	Gift from Dr Yamada of Kyowa Hakko Kogyo Co.Ltd.,Tokyo 194,Japan
Mefenamic acid	Sigma Chemical Co.,Poole UK
1-Monooleoylglycerol(MOG)	Sigma Chemical Co.,Poole UK
Naproxen [e]	Sigma Chemical Co.,Poole UK
Octyl-β-D-glucoside	Sigma Chemical Co.,Poole UK
[^{3}H]palmitate	Amersham International plc,Bucks UK
Phenylbutazone	Sigma Chemical Co.,Poole UK
Phorbol 12-myristate 13-acetate (PMA)	Sigma Chemical Co.,Poole UK
Phosphatidylserine (PS)	Gift from Wellcome Research Labs.
Piriprost (U60,257) [f]	Upjohn Co.,Kalamazoo,USA
Piroxicam	Sigma Chemical Co.,Poole UK
Quin 2/AM [g]	Novabiochem, Nottingham UK
R59022 [h]	Janssen Pharmaceutica NV,Beerse, Belgium
Rev 5901 [i]	Gift from Dr C.A.Sutherland Revlon Health Care, NY,USA
RHC80267 [j]	Gift from Dr C.A.Sutherland Revlon Health Care, NY,USA
Sodium fluoride	BDH Chemicals Ltd.,Poole UK
Sodium meclofenamate	Parke Davis & Co.,Pontypool,Gwent
Staurosporine	Gift from Dr Yamada of Kyowa Hakko Kogyo Co.Ltd,Tokyo 194,Japan
[^{14}C]stearate	Amersham International plc,Bucks UK
1-Stearoyl,2-arachidonyl -sn-glycerol	Gift from Wellcome Research Labs.
Sulindac	Merck Sharp & Dohme Research Labs. Hoddesdon, Herts UK
Superoxide dismutase (SOD)	Sigma Chemical Co.,Poole UK
Triton X-100	Koch Light Labs.,Coinbrook
Trypan blue	Gibco Ltd., Paisley
Wortmannin	Gift from Dr. Baggiolini, University of Bern, Switz.
Zymosan	Sigma Chemical Co.,Poole UK

a BW A4C = N-(3-phenoxycinnamyl)-acetohydroxamic acid

b Compound I (chloracysine) = 2-Chloro-10-(3-diethylamino-1-oxypropyl)-10H-phenothiazine

Compound II = 2-Chloro-10-(3[4methyl-1-piperazinyl]-1-oxypropyl)-10H-phenothiazine

c HEPES = 4(-2-hydroxyethyl)-1-piperazine ethanesulfonic acid

d K252a = (8R*,9S*,11S*)-(-)-9-hydroxy-9-methoxycarbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cycloocta[cde]trinden-1-one

e Naproxen = [+]-6-methoxy-α-methyl-2-naphthaleneacetic acid

f Piriprost (U60,257) = 6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1

g Quin 2/AM = 2-{[-bis-(carboxymethyl)-amino-5-methyl-phenoxy]-methyl}-6-methoxy-8-bis-(carboxy-methyl)-amino-quinoline tetrakis-(acetoxymethyl) ester

h R59022 = 6-[2-[4-[(4-fluorophenyl) phenylmethylene)-1-piperidinyl]ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidin-5-one

i Rev 5901 = α-pentyl-3-(2-quinolinylmethoxy)benzene methanol

j RHC80267 = 1,6-di(O-(carbamoyl)cyclohexanone oxime) hexane

CHAPTER THREE

STIMULI AND TECHNICAL ASPECTS

SUMMARY:

The aims of this chapter are two-fold - firstly, to introduce the various stimuli employed in this project as respiratory burst activators, together with a little background information about each and secondly, to highlight some of the technical aspects encountered with these stimuli during the study as a whole. In addition, the subject of inter-experimental variation is broached and discussed very briefly.

Activators of the respiratory burst were chosen to include a range that acted on the neutrophil at various points from the receptor to intracellular loci. Using this approach it was hoped to discern the main transduction pathways utilized under normal physiological conditions, and in an attempt to elucidate the mode/point of action of some of the drugs under investigation. The stimuli employed in this study were:

Those Acting at the Receptor

- platelet-activating factor (PAF) (Fig. 3.1a and b)
- fMet-Leu-Phe (fMLP) (fig. 3.1c)
- concanavalin A-zymosan (ConA-zymosan) (fig. 3.2a)
- opsonized zymosan (OZ) (fig. 3.2b)

Those Acting at the G-protein

- sodium fluoride (fig. 3.3a)

Those increasing intracellular calcium

- A23187 (fig. 3.3b).

Those Acting directly on protein kinase C

- phorbol 12-myristate 13-acetate (PMA) (fig. 3.3c)
- 1,2-dioctanoyl-sn-glycerol (diC_8) (fig. 3.3d)

During the course of the project, numerous concentration-response curves for the various stimuli were obtained and a representative example of each is presented in figs.3.1-3.3

3.1 PAF

Platelet-activating factor (PAF) was originally characterized in 1972 as a material, released from IgE-activated rabbit basophils, that aggregated platelets and caused the subsequent release of histamine (Benveniste *et al.*, 1972); hence it was implicated as a mediator of type I hyper-reactivity and allergic reactions (Henson & Pinckard, 1977). Since then PAF has been shown to be a mediator of a number of responses, apart from platelet activation, which include regulation of cellular immune responses and stimulation of acute inflammatory processes (Braquet & Rola-Pleszczynski, 1987). The structural elucidation of the compound demonstrated PAF to be 1-alkyl-2(R)-acetyl-glycero-3-phosphocholine (Demopoulos *et al.*, 1979). It is synthesized from precursor phospholipids through the concerted actions of phospholipase A_2 and acetyl-CoA acetyltransferase.

This multifunctional stimulus is an activator of neutrophils, stimulating degranulation, aggregation, oxidative metabolism and chemotaxis (Shaw *et al.*, 1981; Jouvin-Marche *et al.*, 1982). In addition to having direct effects on neutrophils, PAF has been shown to modulate cellular responses. Miniscule quantities of PAF have been shown to "prime" neutrophils for a markedly enhanced response on subsequent activation by the stimuli, fMLP and PMA (Vercellotti *et al.*, 1988; Ingraham *et al.*, 1987; Gay *et al.*, 1986). Responses which can be primed by PAF include superoxide generation, elastase release and aggregation, and are associated with enhanced expression of an adhesive membrane glycoprotein and an enhanced rise in intracellular calcium levels in response to the second agonist (Vercellotti *et al.*, 1988). These results indicate the presence of a PAF-mediated positive-feedback loop, which may serve to amplify neutrophil oxidative responses at sites of inflammation.

From [^{3}H]PAF binding studies to human neutrophils, it was reported that there exists two different kinds of membrane receptors, distinguished by their dissociation constant values and thus referred to as high and low affinity binding sites (O'Flaherty *et al.*, 1986b). On binding to the receptor, the ligand is deactivated by deacylation and the ligand-receptor complex is then internalized. It has been reported that protein kinase C activators can reduce the availability of high affinity PAF receptors and can also inhibit the subsequent PAF-induced Ca^{2+} transients and degranulation, the latter only occurring at high PKC activator concentrations (Molski *et al.*, 1988; O'Flaherty *et al.*, 1989).

PAF by itself is a weak activator of the oxidative burst, with a rapid onset of O_2^- release being followed by a rapid termination (Smith R.J. *et al.*, 1984). The biochemical events associated with the stimulation of neutrophils by PAF are interpreted in terms of the possible existence of two functionally distinct populations of receptors (Naccache *et al.*, 1986). The occupation of one set induces an increase in permeability to Ca^{2+}, in a manner that is sensitive to PKC activation, but not to treatment with pertussis toxin. The activation of the other set of receptors, at high PAF concentrations, stimulates the phosphoinositide-specific PLC and induces the attendant biochemical responses. The latter events, which are G-protein dependent, are believed to be involved in the generation of superoxide.

PAF was used extensively in this project as a clinically relevant stimulus for investigating the mechanisms of neutrophil activation; it was used in conjunction with cytochalasin B (CB). Some inter-subject variation in response to PAF was observed, with some individuals' neutrophils producing a very powerful oxidative burst (in the minority) while others generated only small amounts of O_2^- (the majority). If the two sets of responses were normalized the shape of the curves were identical. A representative example of the powerful and weak PAF-induced O_2^- response is shown in fig. 3.1a and b respectively.

3.2 FMet-Leu-Phe

Formyl-methionyl-leucyl-phenylalanine (fMLP), a tripeptide analogue of some bacterial proteins, is chemotactic to neutrophils at low concentrations (Schiffman *et al.*, 1975) while at high concentrations it initiates a brief (1-2min) burst of O_2^- release. FMLP is one member of a class of formyl-methionine-peptides that mediate their cellular effects by binding specific N-formyl peptide receptors, which were defined by measuring the direct binding of radiolabelled N-formyl peptides to human PMNs (Williams *et al.*, 1977). The binding of fMet-Leu-[^{3}H]Phe to PMNs was found to be saturable and the number of binding sites per cell ranged from 40 000-60 000. Computer analysis of binding studies revealed that, on the human PMN, the fMLP receptor is present in two different affinity states - a high and a low affinity state, the proportion of which are modulated by guanine nucleotides (Snyderman, 1984) or by prior exposure to agonist (Koo & Snyderman, 1983).

Binding of fMLP to its specific PMN receptor initiates a series of cellular responses including chemotaxis, O_2^- generation, aggregation and

degranulation; each of these processes is mediated via a pertussis toxin-inhibitable G-protein (Goldman *et al.*, 1985; Bradford & Rubin, 1985; Lad *et al.*, 1985). These responses are generally short-lived, possibly due to internalization of bound ligand, with down-regulation of receptor expression (Sullivan & Zigmond, 1980; Niedel *et al.*, 1979). FMLP-receptor interaction gives rise to a number of intracellular events - enhancement of phosphoinositide turnover with a decrease in PIP_2 and PI levels, an increase in PA and IP_3, mobilization of intracellular Ca^{2+} and DAG generation, which correlate with their biological responses (Serhan *et al.*, 1983; Dougherty *et al.*, 1984; Korchak *et al.*, 1988; Burnham *et al.*, 1989).

FMLP was employed only as a minor stimulus in this project (see fig. 3.1c for representative example of dose-response curve) and was always used in conjunction with CB. It was deemed not to be very relevant pathologically for the auto-immune inflammatory condition.

Cytochalasin B (CB) is a fungal metabolite, universally employed in studies involving lysosomal enzyme release and O_2^- production in PMNs, to bring about an enhancement of these two PMN responses. The rationale for the use of this agent is its microfilament inhibitory activity but little is understood how this effect translates into an increase in cellular response. Although CB was routinely used in our laboratory to increase the fMLP-, PAF- and A23187-mediated responses it was not necessary for other receptor stimuli, namely OZ, ConA-zymosan or IgG, or for the post-receptor PKC activators, PMA and diC_8.

There has been a degree of confusion in the literature about the actions of this agent despite its widespread usage. In an attempt to understand the mechanism of action of CB in the neutrophil several groups have looked at its effect on the intracellular biochemical events occurring on cell stimulation, reporting that CB caused both an elevation of intracellular Ca^{2+} (Naccache *et al.*, 1977; Steiner *et al.*, 1984) and DAG (Honeycutt & Niedel, 1986) by some unknown mechanism. It could be argued that inhibition of microfilament activities and/or actin polymerization might affect the stimulus-response coupling mechanism at the membrane to increase the generation of second messengers, like Ca^{2+} or DAG, which would in turn result in potentiation of the O_2^- response. The situation is still ambiguous and too complex to be dealt with here - besides an excellent review by Penfield (1988) describes the current status of this compound.

3.3 Zymosan, opsonized zymosan and concanavalin A-zymosan

Opsonized zymosan (OZ) is a phagocytic stimulus which initiates sustained O_2^- generation after a lag of 90 seconds. Zymosan alone does not produce a significant response (see fig. 3.2a), indicating that serum components must participate. Those serum factors that adhere to zymosan include immunoglobulin G (IgG) acting via Fc receptors, and the complement component C3b which binds to a specific C3b receptor. The interaction of zymosan-bound IgG and C3b with different receptors on the cell has been judged according to the specific inhibition by anti-IgG of IgG-coated zymosan (IGZ)-mediated reactions and by anti-C3b of the C3b-coated zymosan (C3Z)-mediated events (Roos *et al.*, 1981). The efficiency of IGZ and C3Z, however, for inducing O_2^- production was much less than that of OZ (Murata *et al.*, 1987). Also, IgG and C3b alone do not activate significantly suggesting that their mode of presentation is important.

The receptor distribution and structure of human neutrophil Fc receptors has been extensively studied using monoclonal antibodies and the number of receptor sites been given as 135 000 per neutrophil (Fleit *et al.*, 1982). Complement-receptor type 1 which recognizes C3b has been well characterized in human PMNs and has been shown to undergo internalization on activation, with the subsequent recycling of intact ligand-receptor complexes (Malbran *et al.*, 1988). The modulation of Fc receptors on the PMN surface has also been studied under different conditions (Patrone *et al.*, 1983).

OZ stimulates a substantial O_2^- burst (see fig. 3.2b) that is neither dependent on phagocytosis or lysosomal degranulation as shown from studies with CB-treated cells (Goldstein *et al.*, 1975). It has been shown in our laboratory that CB inhibits OZ-stimulated O_2^- release (Muid & Dale, unpublished observation), the significance of which is unclear.

Stimulation of neutrophils with OZ has been shown to be accompanied by phospholipase C-mediated PIP_2 breakdown, with a resultant increase in DAG and IP_3 (Meshulam *et al.*, 1988; Burnham *et al.*, 1989); the magnitude and duration of DAG and IP_3 generation as well as the breakdown of PIP_2 was considerably greater with OZ than with fMLP. This corresponds with the larger magnitude and longer duration of the OZ-stimulated oxidative burst, as compared to the chemotactic receptor stimuli. Also, phagocytosis of C3Z or IGZ was found to be associated with increased DAG and IP_3 production (Fallman *et al.*, 1989), which was only marginally affected by pertussis toxin, negating the participation of

a G-protein in the stimulus-response coupling with this stimulus.

Concanavalin A (ConA), a lectin with bivalent binding properties, may link and immobilize cell surface components by virtue of its bipartite structure and thus bring about activation. Given alone it produces a very weak respiratory burst but when used for coating zymosan it is transformed into a powerful stimulant (see fig. 3.2a). ConA-zymosan promotes both phagocytosis and respiratory burst activation but a recent study has shown that neither the activation of phosphoinositide turnover or the increase in intracellular Ca^{2+}, which accompany activation, are necessary processes for triggering either event (Rossi *et al.*, 1989). This report was considered interesting and merited inclusion in this investigation. The unique PIP_2-independent transduction mechanisms are discussed in Chapter 6.

3.4 Fluoride

Fluoride induces a respiratory burst (see fig. 3.3a) and degranulation of primary and specific granules in PMNs. Both responses are characterized by an unusually long lag time (Ξ 10minutes) but the response is sustained for up to 30 minutes (Curnutte *et al.*, 1979; Gabler *et al.*, 1989). The effect of fluoride on the respiratory burst was reversible i.e. fluoride-stimulated cells producing O_2^- could be washed, returning them to the resting state, and then restimulated to induce O_2^- release in amounts equivalent to those originally generated (Curnutte *et al.*, 1979). This rapid reversibility is atypical of fluoride's effects on G-proteins, which serve as intermediary units between the receptor and the phospholipases (English *et al.*, 1987). Fluoride is proposed to activate G-proteins by virtue of an aluminium-fluoride complex (AlF_4^-), which mimics the role of the γ-phosphate of GTP on binding an already inactive GDP-containing G-protein, to transform it to the active state (Bigay *et al.*, 1985; Cockcroft & Taylor, 1987).

Fluoride activation of neutrophils was found to be associated with PIP_2 hydrolysis, IP_3 accumulation and an increase in intracellular free Ca^{2+} levels, all of which preceded respiratory burst activation (English *et al.*, 1987; Strnad *et al.*, 1986). There is some ambiguity as to the sensitivity of fluoride-mediated responses to pertussis toxin, one group reporting no effect of this compound on PIP_2 turnover or Ca^{2+} mobilization (Strnad & Wong, 1985; Strnad *et al.*, 1986), whereas another group report an inhibition of O_2^- production and degranulation in its presence (Gabler *et al.*, 1989). This may indicate that fluoride-mediated cell

responses are PIP_2-independent.

In the present study, it was found that fluoride induced between 3- and 10-fold increases in labelled DAG levels relative to unstimulated cells over a 30 minute time period (see fig. 3.4a for a representative experiment). This correlated very closely with the time course of fluoride-stimulated O_2^- generation (fig. 3.4b). However, the order of increase in the DAG levels was too small to be matched with similar decreases in the phospholipids, taking into account the substantial labelling of the latter.

3.5 PMA and DiC_8

Tumour-promoting phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) have been shown to bind protein kinase C (PKC) intracellularly and thus, by virtue of the diacylglycerol (DAG)-like structure in the molecule, are able to substitute for diacylglycerol at extremely low concentrations (Nishizuka, 1984a; Yaminishi *et al.*, 1983). Like diacylglycerol, PMA dramatically increases the affinity of PKC for Ca^{2+}, rendering it fully active at physiological Ca^{2+} concentrations.

However phorbol esters are, in many ways, unsuitable for studies of the physiological activation of PKC as they have also been found to exert many other cellular effects. More importantly, endogenous diacylglycerol is present only transiently in membranes, while PMA is hardly degraded. A much closer approximation to physiological PKC activation can be achieved using the synthetic DAG, 1,2-dioctanoyl-sn-glycerol (diC_8).

Diacylglycerols derived from receptor-mediated hydrolysis of phosphoinositides have long saturated and unsaturated fatty acids attached to the glycerol backbone, with 1-stearoyl, 2-arachidonylglycerol being one of the main naturally occurring species. However, these compounds do not readily intercalate into intact cell membranes and thus cannot be used to activate cells *in vitro*. On the other hand, DAGs having unusually short fatty acid side chains such as diC_8 are very suitable for PKC activation in PMNs, and thus for investigating PKC-mediated cellular responses, for the following four reasons: (1) diC_8 showed similar activity (as judged by comparable V_{MAX} values) to the endogenous DAG, 1-stearoyl, 2-arachidonylglycerol, in activating PKC in an isolated PKC phosphorylation assay (Go *et al.*, 1987), (2) the ability of diC_8 to compete for essentially all [^{3}H]phorbol dibutyrate (PDBu) binding sites in intact PMNs demonstrated that this DAG can readily permeate into the cell membrane and gain access to PKC (Cox *et al.*,

1986), (3) when several synthetic DAGs were tested as stimulants of the respiratory burst, diC_8 was the most potent (Cox *et al.*, 1986) and (4) diC_8 showed a similar profile with respect to metabolism as endogenous DAG, namely both were handled almost exclusively by the DAG kinase pathway (Muid *et al.*, 1987; see also Chapter 5).

DiC_8 stimulates a very powerful respiratory burst after a lag time of 31 seconds (Cox *et al.*, 1986) and induces a sustained production of O_2^- beyond 15 minutes. In fact in terms of nmol O_2^- produced over a standard 30 minute period, diC_8 was the most powerful stimulant used in this study.

Some technical problems were incurred with the use of diC_8 as a stimulus. Concentrated diC_8 had to be dissolved in dry DMSO (molecular sieve pellets were added to the stock DMSO bottle for this purpose) and then frozen as stock diC_8. For experiments diC_8 was diluted from this stock into aqueous solution immediately before addition to the cells, to ensure that the active 1,2-sn-diC_8 did not racemize into its inactive stereospecific isomer, 2,3-sn-diC_8. A considerable amount of inter-batch variation was observed with diC_8 (unusually, only in the last 6 months of the project) in that the concentration giving the half maximum O_2^- response changed about a quarter of a log unit between batches, which produced noticeable shifts of the concentration-response curve, owing to the very steep nature of the curve. The O_2^- dose-response curve occurs over a range of diC_8 concentrations from 5-25μM. Representative examples of the O_2^- dose-response curves stimulated by both PMA and diC_8 are presented in fig. 3.3c & d.

3.6 A23187

A23187 is a carboxylic acid ionophore, which complexes and subsequently shunts into the cell divalent (Ca^{2+} and Mg^{2+}) but not monovalent cations from an aqueous medium, and thus has proved a useful tool for probing the effect of Ca^{2+} in various cell types. A23187 has been shown to produce an increase in oxygen consumption from human PMNs, requiring the presence of external Ca^{2+} (Zabucchi & Romeo, 1976) and furthermore, in the presence of CB, to induce O_2^- generation from rabbit PMNs (Becker *et al.*, 1979).

A23187 is a relatively poor stimulus for O_2^- production in human PMNs (fig. 3.3b), even in the presence of CB, and was only used in this study as a minor stimulus.

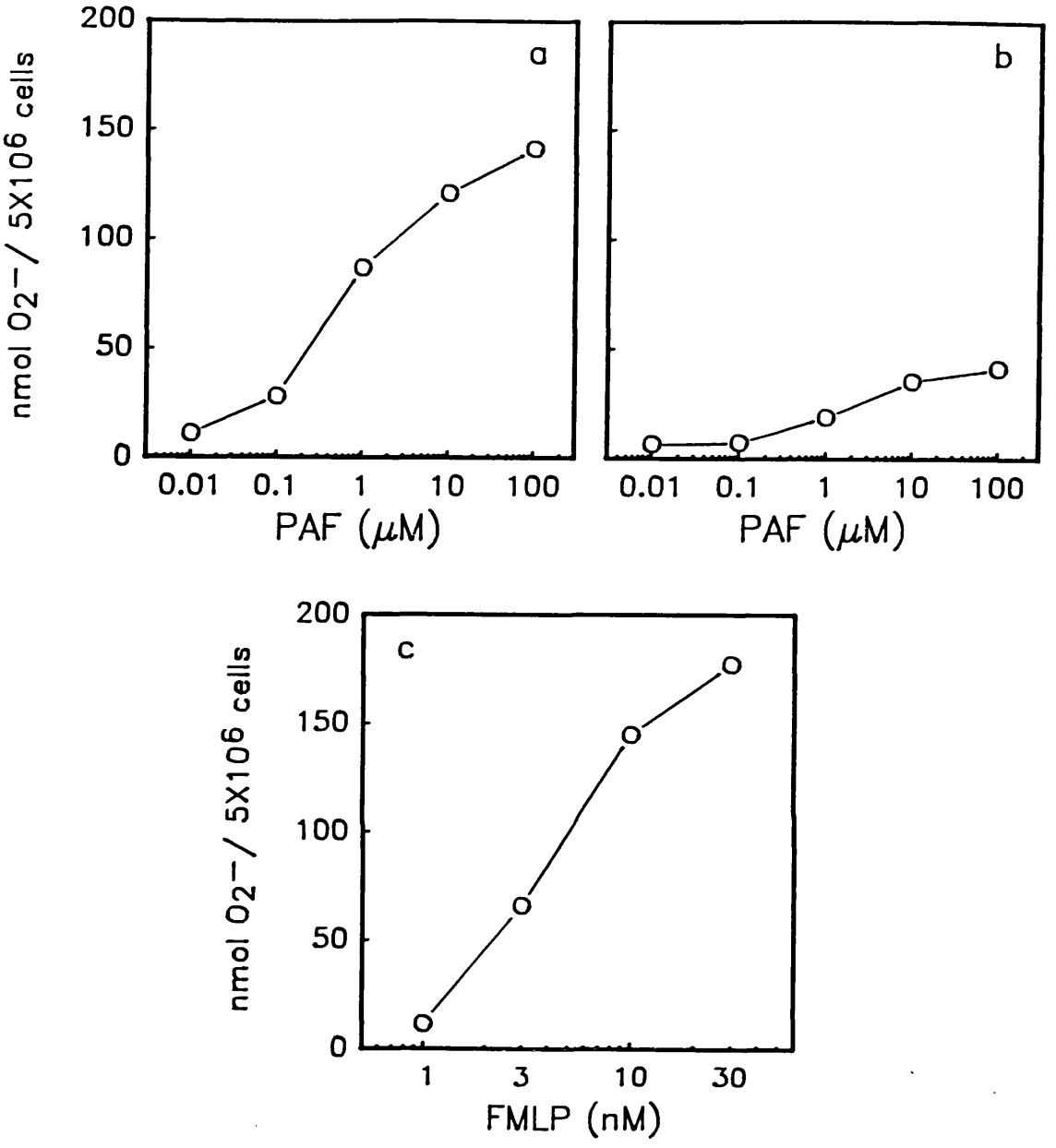


Figure 3.1: a) & b) Representative examples of PAF-induced O_2^- dose -response curves showing potent and weak stimulation respectively.
c) A representative example of an fMLP-induced O_2^- dose-response curve. Data points are the mean of sample duplicates ± range of the duplicates. Error bars were included but are too small to appear.

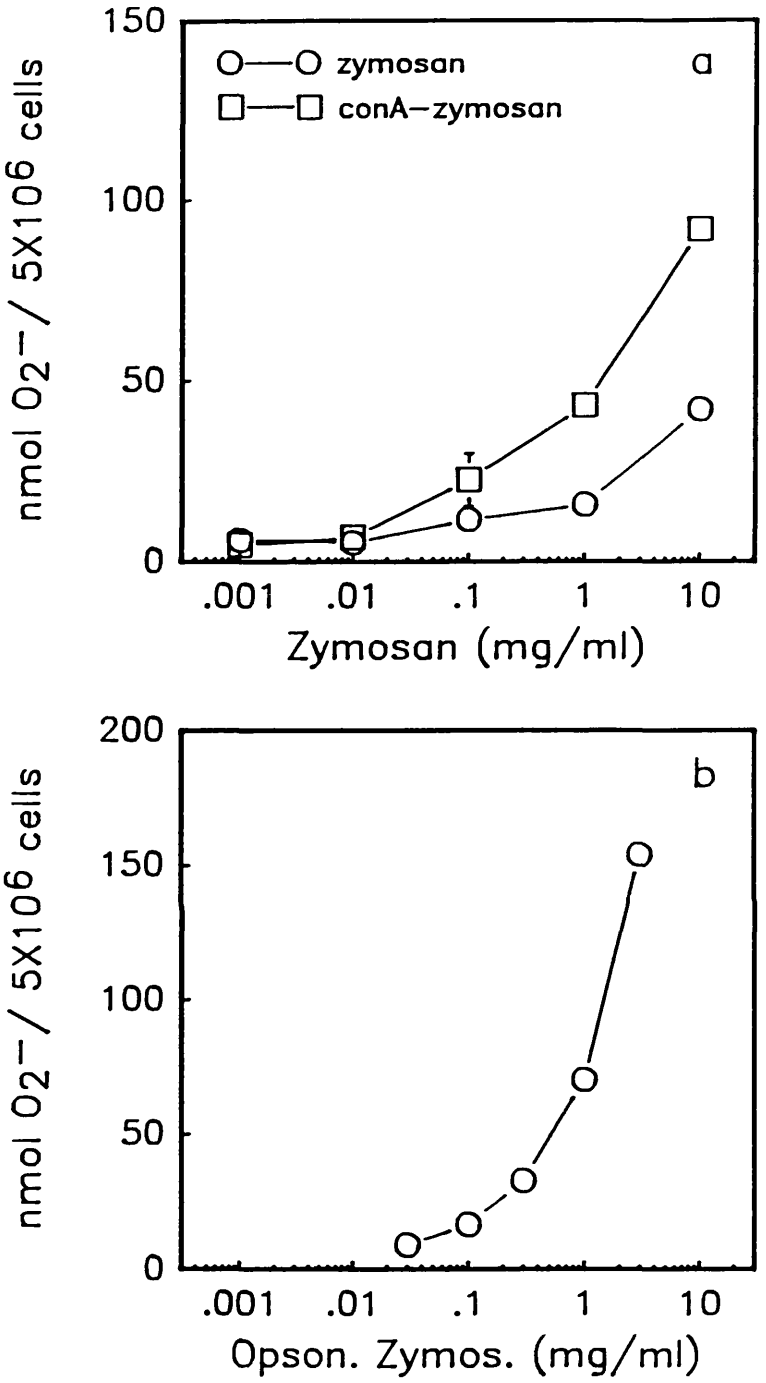


Figure 3.2: a) Representative examples of zymosan- and ConA-zymosan-induced O_2^- dose-response curves. b) A representative experiment showing an OZ-induced O_2^- dose-response curve. Data points are the mean of sample duplicates ± range of the duplicates. Error bars were included but are too small to appear.

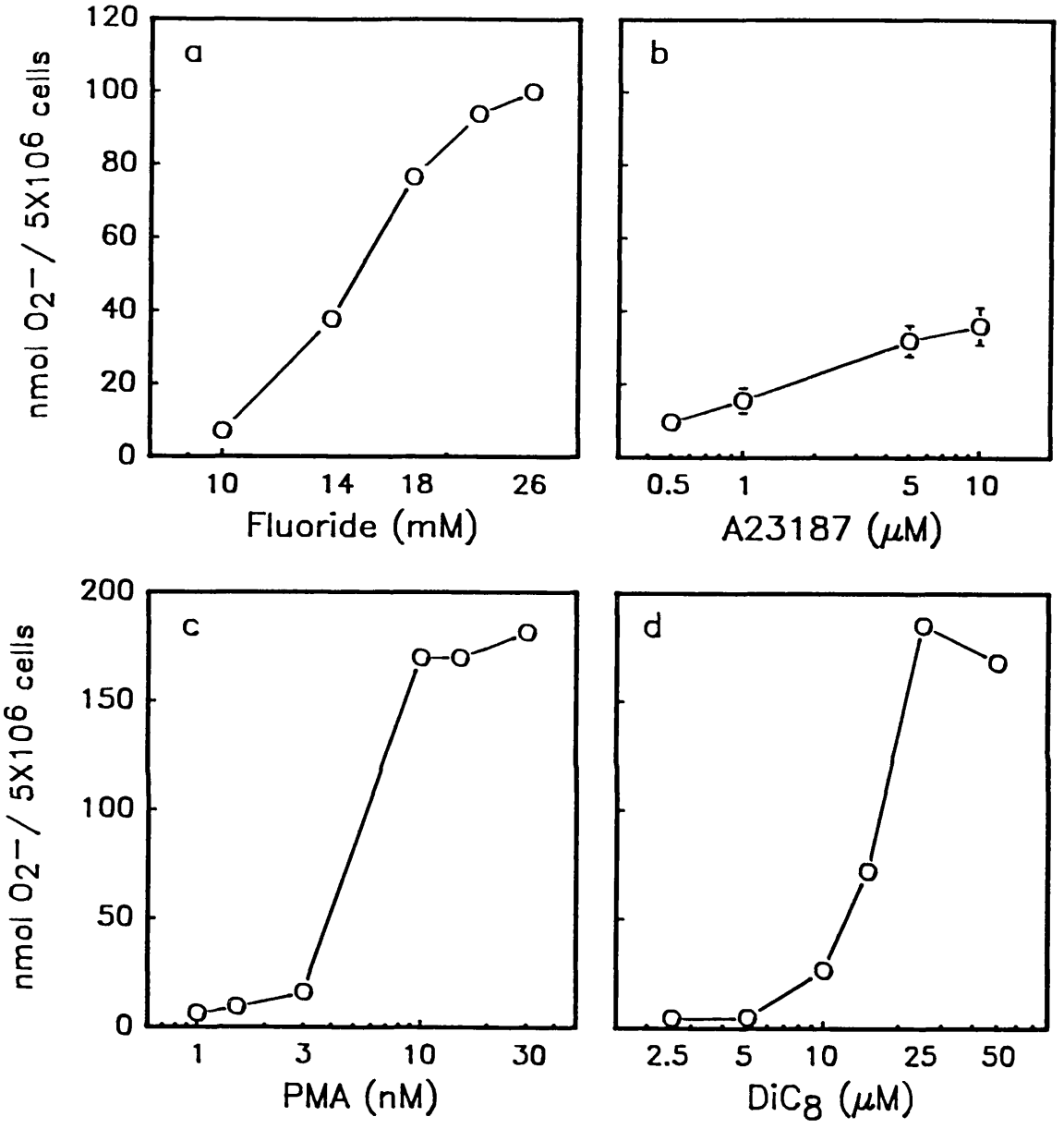


Figure 3.3: Representative examples of a) fluoride-, b) A23187-, c) PMA- and d) diC_8-induced O_2^- dose-response curves. Data points are the mean of sample duplicates ± range of the duplicates. Error bars were included but are too small to be shown.

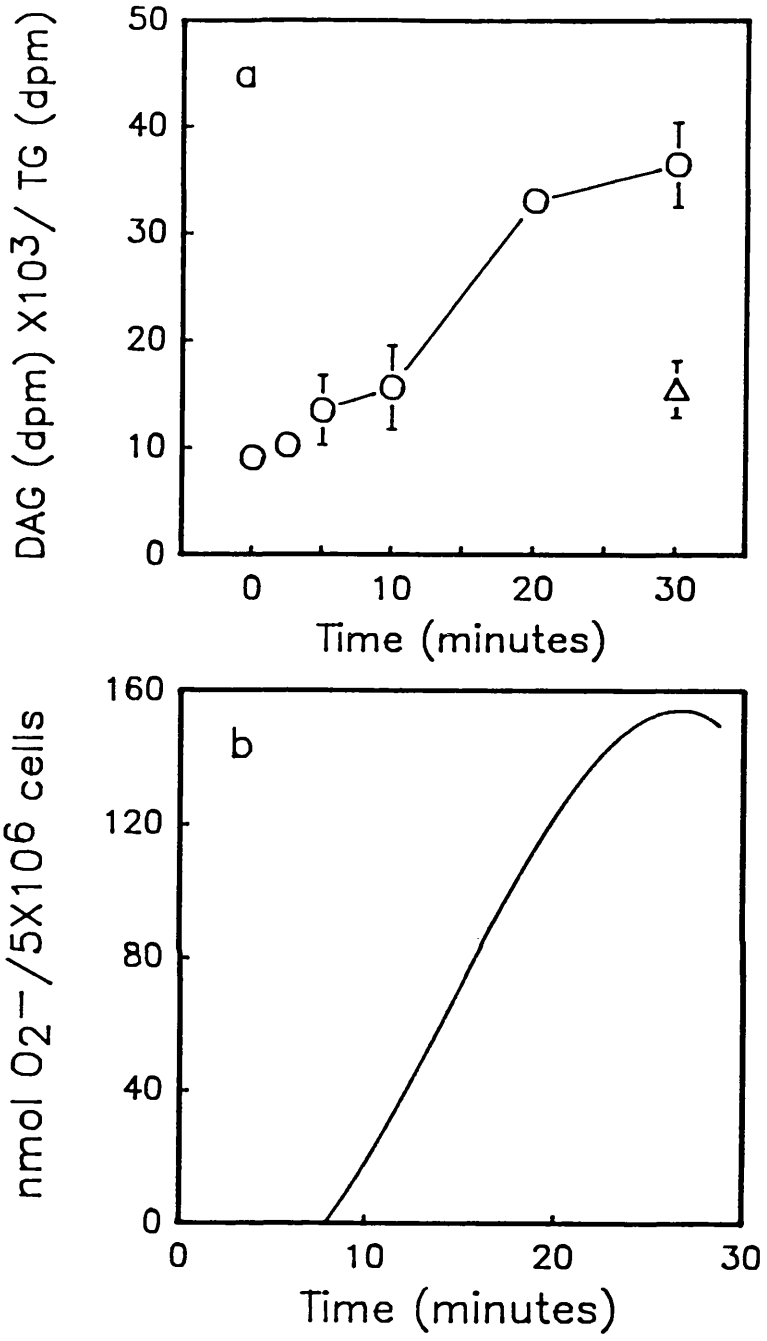


Figure 3.4: a) A time course of fluoride-induced [^{3}H]DAG generation from human PMNs prelabelled with [^{3}H]glycerol. Measurements were obtained from cells alone (Δ) and in the presence of 22mM fluoride (O). Data points represent the mean of sample duplicates $\pm$ range of the duplicates. b) A time course of fluoride(26mM)-stimulated O_2^- production. The data were obtained from a single representative experiment by continuous recording in a spectrophotometer; it was then digitized using Sigma Scan (Jandel Scientific, USA) and the curve fitted using Sigma Plot (Jandel Scientific, USA) by 3rd order polynomial regression.

3.7 The solvents

Most of the agents – stimuli and drugs alike – used in this study were hydrophobic and necessitated the use of an organic solvent. The solvent chosen in this project was dimethyl sulfoxide (DMSO). In the majority of experiments the concentration of DMSO never exceeded 0.1% and for the purposes of controlling for this factor in the drug studies, the same concentration was added to the control cells i.e. those without drug. In one series of experiments where the effect of a combination of three drugs on stimulated O_2^- release was being examined, the DMSO concentration reached 0.3%, so this "high" DMSO concentration was tested for an effect on the O_2^- response. It was found that 0.3% DMSO produced a small decrease in fluoride-stimulated O_2^- generation in some but not all experiments. The "high" DMSO concentration was also examined for its effect on cell viability using the trypan blue exclusion assay and was found to have no effect.

Ideally it is not advisable to introduce such a solvent into a biological system, and whenever possible saline was used, but the need to solubilize lipophilic compounds provided little other choice.

3.8 Sources of variation

One major problem encountered throughout this project was the large degree of inter-experimental variation. Ideally, the drug/stimulus concentration would have been the only variable but in these experiments an additional factor was the inevitable variation in response between blood donors' neutrophils. Moreover, even the same donor, used on different occasions, rarely produced the same quantitative response.

Working with human tissues is always a problem as there is no such thing as a standard population of humans. The inherent variations in donors' neutrophils, fluctuations in the circulating steroid hormone levels, transient minor infections, the fitness status of the donor may all be contributory factors to this inter-experimental variation. Even whether they cycled to work or came by tube could affect the results since the leucocytosis of exercise could cause marked fluctuations of neutrophils (McCarthy & Dale, 1988). Returning to the point about circulating hormone levels females, of course, are more susceptible to such fluctuations, due to their menstrual cycles and, in some cases, the taking of oral contraceptives. Unfortunately, the "pool" of donors was not sufficiently large to enable exclusive use of the males in the

department.

In spite of this inter-experimental variation, consistent concentration-response curves were obtained for all the stimuli in terms of shape and stimulus concentration, even though the quantitative release of O_2^- might vary. The problem was partly circumvented by normalizing the data in all experiments before calculating the mean and standard error, which tended to "iron out" some of those quantitative differences. However, there was also the problem, particularly with the NSAIDs, that different individuals responded with widely different quantitative effects (usually increases) to the same drug at the same concentration. In some cases, a consistent difference between the control and test dose-response curves, seen with all donors, appeared to be non-significant when the results were averaged i.e. what might have been an actual biological difference between test and control was lost in the inter-subject variation. Generally, the effect of a particular drug was qualitatively consistent between donors, though in some cases a drug that produced very significant increases with some donors' cells registered a "no effect" result with others, both sets measured under identical conditions. This situation necessitated large numbers of experiments being carried out to decipher some kind of pattern of effects. Even though the mean normalized data is presented for all drugs (sometimes with quite sizeable standard error bars), also included is a representative experiment to clarify the effect profile of the drug that was often masked in the mean data. It should be pointed out that the inter-subject variation in response to NSAIDs found in the present study is perhaps rather similar to the large variation in patients' responses to these drugs reported by clinicians.

Another source of variability comes from inter-batch variation in stimuli such as diC_8. There were differences in activity between batches which produced quite sizeable differences in the control concentration-response curves due to the steep nature of the curve.

Furthermore, the use of Gilson pipettes may have contributed to variability, both in the making up of stock solutions and in making the addition of reagents to the incubation mixtures during experiments. The pipettes were periodically checked and re-calibrated, but the use of these pipettes is still not ideal. Samples were carried out in duplicate, and whenever possible in triplicate, to try and obtain a representative mean value in each experiment.

CHAPTER FOUR

EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS ON TOXIC OXYGEN RADICAL PRODUCTION.

SUMMARY:

— A range of twelve non-steroidal anti-inflammatory drugs (NSAIDs), including members from each of the main chemical groups, were examined for their effects on the oxidative burst induced by the receptor stimulus, PAF, and the two post-receptor stimuli, fluoride and diC_8.

— It was found that the NSAID effects fell into three categories, 1) those that increased the stimulated O_2^- response, 2) those that had no effect and 3) those that decreased O_2^- production. All the drugs were without effect in unstimulated neutrophils. The clinical implications of these results are discussed.

— The mode of action of those NSAIDs that caused enhancement of the stimulated O_2^- response is discussed in relation to other data presented in this project. An effect of the NSAIDs on either the cyclooxygenase or 5-lipoxygenase (5-LO) pathways of arachidonate metabolism is unlikely to account for the potentiation because not all NSAIDs caused enhancement and 3 specific 5-LO inhibitors had no significant effect on the O_2^- response. The NSAIDs had no effect on an isolated PKC enzyme when tested under a number of activating conditions. It was proposed that the drugs could be inhibiting the DAG metabolizing enzyme, DAG kinase, thus prolonging the DAG signal and increasing the activation of PKC. This hypothesis was based on the close correlation of effects obtained between the enhancing NSAIDs and an inhibitor of DAG kinase, R59022, on both fluoride- and diC_8-induced O_2^- responses. R59022 did not cause an increase of PAF-stimulated O_2^- production, unlike the NSAIDs, and possible reasons for this apparent discrepancy are discussed. Other possible mechanisms that would explain the NSAID-mediated enhancement are also considered.

— The mechanism of action of those NSAIDs that inhibit the respiratory burst are also discussed.

4.1 INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are a wide and diverse group of compounds which are chemically heterogeneous, although most are carboxylic or enolic acid derivatives of several different classes, summarized in fig. 4.1. As a group they are weakly acidic, highly protein bound, highly hydrophobic planar molecules. There are also a smaller group of non-acidic compounds which have more limited usage. Many of them have been developed in the past two decades for the treatment of inflammation and the pain associated with arthritis and other inflammatory conditions. They have become one of the most widely used group of drugs in the history of medicine. It has been estimated that more than 30 million people take NSAIDs daily for relief of symptoms of rheumatoid arthritis (RA), osteoarthritis and other arthritides, for acute musculoskeletal conditions or miscellaneous injuries, and for dysmenorrhea or postoperative pain. Indeed, the total worldwide sales for these drugs, estimated to be $17 million in 1960, is expected to reach $1 billion per annum by 1990.

NSAIDs are generally characterized as anti-inflammatory, anti-pyretic and peripheral analgesic agents for the symptomatic relief of inflammation and pain. In chronic arthritis they provide relief from pain, stiffness and swelling, with consequent improvement in joint function; although continuous administration of large doses of drug are required for the relief to be maintained. It should be stressed that NSAID therapy has little effect in suppressing or retarding the underlying disease process particularly in states such as RA, where chronicity of the disease is evident by the progression of erosions, deformities or restriction of joint movements. Thus, NSAIDs serve as symptom-suppressing drugs while having little effect as curative or disease-modifying agents.

What is the mode of action of this diverse class of drugs? Although the NSAIDs have been known to inhibit a wide variety of reactions *in vitro*, no convincing relationship has been established which can explain *all* their known anti-inflammatory, anti-pyretic and analgesic effects. Many biochemical mechanisms of action were originally proposed, from uncoupling oxidative phosphorylation, protein denaturation, inhibition of proteases to stabilization of lysosomal and cellular membranes. Of great importance in understanding how NSAIDs work was the discovery that low concentrations of aspirin and indomethacin inhibited the enzymatic production of prostaglandins (PGs) (Vane, 1971). Since at that time,

there was some evidence that PGs participated in the pathogenesis of inflammation and fever, an inhibition of the biosynthesis of these autocoids could explain a considerable number of the clinical actions of the drugs. Subsequently, it was established that all NSAIDs inhibit cyclooxygenase (the first enzyme in the arachidonic acid cascade leading to the PGs) at their physiological concentrations (μM range), with a structure-activity relationship and chiral specificity that parallel their *in vivo* anti-inflammatory actions. These latter criteria were not satisfied by the earlier proposed modes of action.

PGs are always released when cells are damaged, and have been detected in increased concentrations in inflammatory exudates. The concentration of a PGE_2-like substance is about 20ng/ml in the synovial fluid of patients with RA. This decreases to zero in patients taking aspirin, demonstrating its effect on *in vivo* PG synthesis (Higgs *et al.*, 1974). In many different models of inflammation, NSAIDs have been found to eliminate the increase of endogenous PGs, to limit the extent of the inflammatory reaction and to change its time course. One such example is carageenin-induced inflammation in the rat paw (Moncada *et al.*, 1973).

The exact mode of action of most NSAIDs is still not entirely understood. While the current consensus favours inhibition of prostaglandin synthesis in inflamed tissues, thereby preventing both the sensitization of pain receptors to inflammatory mediators (e.g. histamine, 5-hydroxytryptamine, kinins) which play variable roles in different types of inflammation, and also preventing the vasodilatation caused by the vasodilator PGs, this is not the whole story. True, all the presently available NSAIDs share, as at least one of their effects, the ability to inhibit prostaglandin biosynthesis, but this action alone does not explain all of their observed effects in various inflammatory models. There is quite extensive variability in the effectiveness of NSAIDs to inhibit cyclooxygenase, with IC_{50} values varying over 1000-fold. Those drugs that are only weak inhibitors (i.e. isoxicam) are equipotent in their analgesic and anti-inflammatory effect with those that are strong inhibitors (e.g. indomethacin) (Eberl, 1982). NSAIDs also appear to be effective in animals that cannot synthesize PGs as a result of diets deficient in essential fatty acids (Bonta *et al.*, 1976). A comparison of the effects of aspirin and salicylate further highlights an inconsistency with the cyclooxygenase story. Compared with aspirin, sodium salicylate is a very weak inhibitor of cyclooxgenase *in vitro* but both drugs are

equally effective in terms of experimental and clinical anti-rheumatic effects (Vargaftig & Lefort, 1977; Humes *et al.,* 1981). A further inconsistency is that many NSAIDs are reported to decrease cell accumulation in experimental inflammation (Tanaka *et al.,* 1984; Perianin *et al.,* 1984), a phenomenon dependent on lipoxygenase rather than cyclooxygenase activity.

Finally, whereas low doses of aspirin and other NSAIDs markedly inhibit the synthesis of PGs *in vitro* (Vane, 1971) and *in vivo* (Pederson & Fitzgerald, 1984), higher doses are required for an anti-inflammatory effect *in vivo* (Higgs *et al.,* 1976; Flower *et al.,* 1980). It is possible that at higher concentrations, NSAIDs exert an anti-inflammatory effect independent of cyclooxygenase inhibition. These drugs have been described as inhibitors of neutrophil activation by a PG-independent mechanism, hence contributing to their anti-inflammatory effect (reviewed by Abramson & Weissmann, 1989). In many *in vitro* studies NSAIDs have been found to be inhibitory for numerous neutrophil functions (aggregation, chemotaxis, lysosomal enzyme release, O_2^- generation, etc.), which are summarized in Table 4.1. In general, fMLP-stimulated responses were inhibited with varying orders of potency, depending on the NSAID. Neutrophil responses induced by concanavalin A (ConA) and PMA were unaffected by all the NSAIDs except piroxicam, whereas opsonized zymosan (OZ)-, C5a-, and immune complex-stimulated responses required much higher concentrations of NSAID for inhibition or were unaffected by drug, excepting piroxicam again. The case of piroxicam is unique and will be dealt with more extensively in the discussion.

An explanation for the fMLP-mediated inhibition of neutrophil activation may be provided by the NSAID-mediated inhibition of [^{3}H]fMLP binding to the receptor measured by many groups; diclofenac and tolfenamic acid (Friman *et al.,* 1986), meclofenamate, ibuprofen and tolmetin (Shelly & Hoff, 1989), piroxicam (Edelson *et al.*, 1982; Abramson *et al.*, 1984), indomethacin (Abita, 1981; Perianin *et al.*, 1988; Cost *et al.,* 1981; Palmer, 1983) and finally phenylbutazone (Nelson *et al.*, 1981; Palmer, 1983). As very hydrophobic molecules, it is not surprising that the NSAIDs interfere with the binding of hydrophobic mediators, such as fMLP to their membrane receptors. On the other hand, phenylbutazone had no effect on [^{125}I]C5a receptor binding (Nelson *et al.*, 1981), and piroxicam was without effect on [^{3}H]ConA binding to its receptor on human neutrophils (Edelson *et al.*, 1982), which could explain why the

Table 4.1
Inhibitory Effects of NSAIDs on neutrophil responses

Stim.	Drug	Aggregation	Lys.Enz. Release	O_2^- generation	Chemotaxis	Reference
fMLP	indo.			↓		Taniguchi *et al.*,1988
fMLP	indo.			↓		Friman *et al.*, 1986
"	tol.a.			↓		
"	diclo.			↓		
fMLP	PBut.			↓		Perianin *et al.*,1983
PMA	"			↓		
OZ	"			(-)		
fMLP	meclo.		↓	↓	↓	Shelly & Hoff, 1989
"	tol.		↓	↓		
fMLP	indo.		↓	↓		Smolen &
OZ	"		(-)			Weissmann, 1980
PMA	pirox.			↓		Biemond *et al.*, 1986
OZ	"			↓		
fMLP	ibup.		↓	↓	↓	Nielsen &
C5a	"		↓	↓	↓	Webster, 1987
OZ	"		↓	↓		
PMA	"		(-)	(-)		
fMLP	ibup.	↓			↓	Maderazo *et al.*,1984
fMLP	indo.		↓			Kaplan *et al.*, 1984
"	aspir.	↓	↓	↓		
"	ibup.	↓	↓			
"	pirox.	↓	↓	↓		
fMLP	pirox.	↓	↓	↓		Edelson *et al.*, 1982
ConA	"	(-)	(-)	↓		
PMA	"	(-)	(-)	↓		
fMLP	pirox.		↓	↓		Tanaka *et al.*, 1984
ConA	"			(-)		
OZ	"			(-)		
A23187	"			(-)		
fMLP	PBut.		↓	↓		Neal *et al.*, 1987
"	indo.		↓	↓		
"	pirox.		↓	↓		
"	mef. a.		↓	↓		
fMLP	pirox.	↓	↓	↓		Abramson *et al.*, 1984
"	ibup.	↓	↓	(-)		

Abbreviations: indo., indomethacin: tol.a., tolfenamic acid; diclo., diclofenac; PBut., phenylbutazone; meclo., meclofenamate; tol.,tolmetin; pirox.,piroxicam; ibup.,ibuprofen; aspir.,aspirin; mef.a.,mefenamic acid
↓ and (-) represent inhibition and no effect respectively.

responses induced by these stimuli were less susceptible to inhibition by the NSAIDs.

The inhibition of neutrophil function by NSAIDs is assumed to be cyclooxygenase-independent for many reasons: 1) Sodium salicylate, an ineffective cyclooxygenase inhibitor, is as effective as aspirin (Abramson *et al.*, 1985). 2) The different NSAIDs exhibit very diverse effects that are both stimulus- and response-dependent. 3) Significantly higher concentrations of NSAIDs are required to inhibit some neutrophil responses than to inhibit PG synthesis. 4) Some NSAIDs (indomethacin, piroxicam) inhibit activation by C5-derived peptides and LTB_4 even when PGs are present (Abramson *et al.*, 1985). Rather than exerting antagonistic effects, as might be expected from the Vane hypothesis, PGE_1 and piroxicam appear to have additive effects in inhibiting PMN O_2^- generation in response to fMLP (Abramson *et al.*, 1984). 5) Salicylate, piroxicam and indomethacin have been reported to inhibit fMLP-induced ^{45}Ca movements and to enhance the fMLP-stimulated increase in cyclic AMP (Abramson *et al.*, 1985). In addition, indomethacin is reported to enhance the cyclic AMP increase induced by PAF (Hopkins *et al.*, 1983). It is relevant to note that cyclic AMP is reported to be an inhibitory messenger for neutrophil activation (see discussion). Thus these results with NSAIDs on neutrophil activity suggest that they can have a multiplicity of actions. Some of these actions (particularly those seen at clinically relevant doses) may contribute to the anti-inflammatory effects of the NSAIDs *in vivo* and may account for the clinical variability in responsiveness to different NSAIDs.

Until recently, most workers studying the *in vitro* actions of NSAIDs spoke in terms of their additional anti-inflammatory effects. However, studying the effects of NSAIDs on O_2^- release from human neutrophils has led us to focus on those effects of NSAIDs (i.e. cyclooxygenase-independent effects) which could **increase** inflammatory mechanisms. It was observed initially in this laboratory that indomethacin increased O_2^- production in neutrophils when activated with the post-receptor stimuli, 1-oleoyl, 2-acetylglycerol (OAG) and A23187 (Dale and Penfield, 1985). Consulting the literature, there were a few other studies reporting qualitatively similar observations. Gay *et al.* (1984) reported that indomethacin augmented the superoxide response stimulated by OZ, had no effect when fluoride or PMA were employed as oxidative stimuli but decreased the response to fMLP. They reported only inhibitory effects on O_2^- release with the other two NSAIDs included in the study, namely

sulphinpyrazone and phenylbutazone. These authors went on to investigate this phenomenon further, finding OZ-stimulated chemiluminescence to be increased by exposure of the cells to indomethacin; phagocytosis of radiolabelled *Staphylococcus aureus* by PMNs incubated with indomethacin was also enhanced, but to a relatively small degree (Gay *et al.*, 1985). Another study found indomethacin (but not piroxicam or ibuprofen) to enhance zymosan-mediated O_2^- production in rat PMNs (Ward *et al.*, 1984). Additionally, indomethacin has been found to enhance the O_2^- formation in chemically elicited guinea pig macrophages activated with a range of different stimuli, as well as provoking an oxidative burst in its own right in the absence of any stimulation (Bromberg and Pick, 1983).

These observations may be clinically significant in light of the possible *in vivo* effects of oxygen radicals. It has been demonstrated by numerous workers that the production of oxygen radicals can potentially bring about extensive tissue damage (discussed in Chapter 1). Furthermore, there is evidence that O_2^- produced by neutrophils can modify IgG molecules causing them to aggregate, these aggregates in turn acting as stimuli for further oxygen radical generation (Lunec *et al.*, 1985). This could constitute a self-perpetuating mechanism for the production of tissue-damaging oxygen radicals, discussed in Chapter 1 with an illustrative schematic diagram (fig. 1.1). Superoxide production by activated phagocytic cells could well be a key event in the pathogenesis of chronic inflammatory conditions such as RA. There is evidence that O_2^- can stimulate the production of an interleukin 1 (IL-1)-like agent from human neutrophils (Kasama *et al.*, 1989); IL-1 is another important potential mediator of inflammatory joint damage.

NSAIDs have been extensively used for their symptom-suppressing effects in the treatment of RA. Clinicians agree that this group of drugs does not slow the progress of the disease in the long-term. In light of the observations of Penfield (1988), where drugs from at least two of the main classes of NSAIDs were shown to cause a marked potentiation of oxygen radical production, activated by two post-receptor stimuli, an important question arose. *What effect will these NSAIDs have on the production of toxic oxygen metabolites if they are generated with a clinically relevant receptor stimulus?* The two NSAID classes under scrutiny are extensively used clinically and it is extremely likely that while providing relief from pain and morning stiffness, these drugs could be exacerbating the progress of the underlying auto-

immune/inflammatory condition through an effect on the oxidative burst.

This section of work aimed to carry out a careful quantitative study of the potential of various NSAIDs for exacerbating the production of tissue-damaging toxic oxygen metabolites in order to delineate those which might not be safe to use. A range of NSAIDs, from each of the main chemical groups (see fig. 4.1), were systematically analysed for their effects on O_2^- generation by PAF (a clinically relevant receptor stimulus) and compared to their effects with two post-receptor stimuli - fluoride (a G-protein activator) and diC_8 (a DAG analogue).

The second part of this study aimed to investigate the mechanism of the potentiating effect of the NSAIDs on the O_2^- response. In this chapter this mainly takes the form of a discussion drawing results from other chapters and comparing the NSAID profile of effects with that of reportedly specific enzyme inhibitors. Evidence is discussed whereby the potentiating effect of NSAIDs on O_2^- generation may be explained in terms of an inhibition of DAG metabolism, discounting some of the other possible modes of an enhancing signal being mediated by the oxidative pathways of arachidonate metabolism.

It was reported by Lukey *et al.* (1988) that the NSAID benoxaprofen caused a dose-related (50-200μM) activation of the neutrophil respiratory burst, as measured by lucigenin-enhanced chemiluminescence. The benoxaprofen-mediated activation of O_2^- generation was linear up to 3 minutes, peaked at 6 minutes and subsided thereafter; the response was shown to be eliminated in the presence of superoxide dismutase. The authors proposed that the direct stimulatory effect of benoxaprofen on the oxidative burst was mediated by protein kinase C (PKC), as it was also demonstrated to cause activation of purified PKC derived from both rat brain and human platelets. Benoxaprofen caused a dose-related (0.33-33μM) stimulation of PKC in the presence of all three physiological activators - Ca^{2+}, phosphatidylserine (PS) and 1,2-diolein. Benoxaprofen also stimulated PKC in the absence of PS and it was suggested that the NSAID may activate by substituting for PS. In view of this report it was deemed necessary to screen the NSAIDs, particularly "the enhancers" through a PKC-activation assay. Data is presented (section 4.2.15) showing the effects of a range of NSAIDs on isolated rat brain PKC phosphorylation, using a sensitive Triton mixed micellar assay (Hannun *et al.*, 1985). Also tested for an effect on isolated PKC for comparison were the DAG metabolic inhibitors.

4.2 RESULTS

4.2.1 The effect of NSAIDs on O_2^- generation induced by fluoride, diC_8 and PAF

In the overall study, it was found that the general effects of the NSAIDs on stimulated O_2^- generation fell into three categories (Table 4.2), 1) those that increase O_2^- production, 2) those that have no effect and 3) those that actually decrease O_2^- production. As there were some variations with regards the nature of the effects observed with the three stimuli, the effect of the drugs on PAF-stimulated O_2^- generation was chosen to classify the NSAIDs into one of the above three categories.

TABLE 4.2

NSAID increasers	benoxaprofen indomethacin sodium meclofenamate mefenamic acid
No Effects	diclofenac ketoprofen sulindac ibuprofen naproxen aspirin
NSAID decreasers	phenylbutazone piroxicam

A more detailed summary of the NSAID effects observed with all 3 stimuli is presented in Table 4.4, where it can be seen that there is a degree of variation as regards the drug effects with the individual stimuli. There are 2 general points to be noted from the results of this study. Firstly, the number of drugs that effected a potentiation of the post-receptor simulated responses was greater than the number increasing the respiratory burst induced by the receptor stimulus, PAF - 8 in the case of both fluoride and diC_8 whereas only 4 in the case of PAF. Secondly, the order of increase obtained with the post-receptor stimuli was very much larger than that seen with PAF. As a representative example, when the potentiating effect of meclofenamate was compared with the 3 stimuli, the fluoride-stimulated O_2^- response was enhanced over 7-fold, the diC_8 response nearly 4-fold whereas PAF-stimulated O_2^- generation was increased only 2-fold. This is not surprising in light of

the very stringent cellular controls and receptor negative-feedback mechanisms employed to tailor the cellular response to a specific physiological (or pathological) demand. Thus, the fluoride and diC_8 mechanisms for NADPH oxidase activation can continue unabated under the influence of a potentiating drug whereas the effect of drug on PAF-mediated activation is curtailed. This is extremely important when extrapolating *in vitro* effects to the *in vivo* performance of neutrophils in inflammatory surrounds.

In the remainder of this Results section each NSAID will be presented separately, in alphabetical order, with regards to its specific effect on the O_2^- response stimulated by the 3 stimuli, describing in detail the results obtained and any peculiarities specific to the drug. In order to eliminate the quantitative variation between experiments, the data from each experiment were normalized by expressing the amount of O_2^- generated, in the presence and absence of drug, as a percentage of the maximum *control* response, i.e. with each particular stimulus alone. The mean of these normalized values, together with their standard errors, were then calculated and the dose-response curves plotted, with and without drug.

One problem in assessing the results in this section is that with some drugs there appeared to be a particular type of variability between donors. In some experiments the effects of a drug seemed to fall into two quite distinct categories when the drug was being tested under identical experimental conditions - the drug having no effect at all on the cells of some donors, but marked effects (sometimes very marked effects) on others. In these experiments, presenting the results only in terms of the mean normalized data tended to obscure this phenomenon; therefore, in addition to the mean data, examples are included of those experiments in which the drugs had a marked effect on some donors' cells. It is possible that this phenomenon indicates a subpopulation of donors with an actual biological difference in response to the drugs in question. If this is so it could have clinical relevance. This point is developed in the discussion.

4.2.2 The effect of aspirin on stimulated O_2^- generation

Aspirin, at 1-100μM, had no effect on the stimulated O_2^- response with either fluoride (n = 10) or diC_8 (n = 5). For clarity, only the effect of the highest concentration of aspirin, i.e. 100μM, on the fluoride- and diC_8-stimulated response is presented in fig. 4.2a and b

respectively. Aspirin, at 100μM, had no effect on PAF-stimulated O_2^- production at the lower end of the dose-response curve (n = 6). In some experiments (2 out of 6) there was a marked inhibition at high PAF concentrations, but in the other four experiments aspirin had no effect on the response. The mean normalized dose response curve to PAF (n = 6), in the presence and absence of aspirin (100μM), is presented in fig. 4.2c. The mean value of the 6 experiments with aspirin was not statistically different from control at the top end of the dose-response so the overall result was interpreted as no effect for the purposes of this study. However, the possibility that the reducing effect of aspirin at high PAF concentrations is a real effect in some individuals cannot be ruled out.

4.2.3 The effect of benoxaprofen on stimulated O_2^- generation

Benoxaprofen (100μM) caused a marked potentiation of the mean normalized concentration-response curve for fluoride, shifting the curve to the left and causing a large increase in the maximum control response (n = 4) (fig. 4.3a).

Benoxaprofen potentiated the diC_8-stimulated O_2^- response – causing sinistral displacement of the concentration-response curve (n = 3) (fig. 4.3b). This effect was most marked at sub-maximal concentrations of diC_8 and unlike with fluoride, benoxaprofen did not influence the maximum control response.

The PAF O_2^- concentration-response curve was also enhanced by benoxaprofen (100μM), the enhancing effect being very consistent from low to high PAF concentrations (n = 6). Thus, the concentration-response curve was shifted significantly leftwards and the maximum control response significantly increased (fig. 4.3c).

4.2.4 The effect of diclofenac on stimulated O_2^- production

Diclofenac (100μM) appeared to displace the mean fluoride dose-response curve (n = 5) to the left but the standard error bars were very large and at none of the points was the drug effect statistically significant at $p < 0.05$ (fig. 4.4a). However, these fluoride experiments demonstrate a phenomenon (mentioned above) and discussed in Chapter 2 - donor variability. Diclofenac caused a very marked left-shift of the fluoride dose-response curve and an increase in the maximum response in 3 out of 5 experiments but had a minimum effect in the remaining 2. An example of the marked potentiation with one donors' cells is shown in

fig. 4.4b.

Diclofenac (100μM) displaced the mean normalized diC_8 concentration-effect curve to the left, with little effect on the maximum control response (n = 4) (fig. 4.4c). On the other hand, in 4 experiments, diclofenac failed to produce a significant increase in the PAF-stimulated response, as represented by the mean normalized dose-response curve in fig. 4.4d.

4.2.5 The effect of ibuprofen on stimulated O_2^- generation

In 5 experiments, ibuprofen (100μM) did not produce a significant effect on fluoride-stimulated O_2^- generation, the mean normalized data from which are plotted in fig. 4.5a. In 3 out of the 5 experiments a large potentiation by ibuprofen was observed, whereas it caused very little effect in the remaining 2 experiments. Thus, a single normalized experiment, representative of 3, is presented in fig. 4.5b, showing a marked sinistral displacement of the fluoride dose-response curve in the presence of ibuprofen.

Ibuprofen (100μM) effected a significant potentiation of the diC_8-induced O_2^- response (n = 3) (fig. 4.5c); it caused a marked leftward shift of the dose-response curve, with no change in the maximum response.

The effect of ibuprofen (100μM) on the PAF response showed a marginal potentiation in 2 experiments and no effect in a third experiment. The combined mean data, presented in fig. 4.5d, show that ibuprofen had no significant effect on the PAF O_2^- dose-response curve.

4.2.6 The effect of indomethacin on the stimulated O_2^- response

Indomethacin (100μM) caused a marked potentiation of the fluoride-induced O_2^- response, causing a leftward shift of the concentration-effect curve and an increase in the maximum control response (n = 9) (fig. 4.6a). It also produced a potentiation of the diC_8 response, causing a leftward displacement of the concentration-response curve (n = 4) (fig. 4.6b).

In 5 experiments indomethacin had no significant effect on the mean normalized concentration-response curve induced by PAF (fig. 4.6c). However, in 4 of the 5 experiments there was a consistent left-shift of the control curve as shown by a representative experiment in fig. 4.6d.

4.2.7 The effect of ketoprofen on the stimulated O_2^- response

Ketoprofen (100μM) had no significant effect on either the fluoride (n = 5) (fig. 4.7a) or PAF (n = 3) (fig. 4.7d) O_2^- dose-response curves.

Ketoprofen's effect on diC_8-stimulated O_2^- generation is less clear-cut. In 3 experiments, ketoprofen caused a definite potentiation of the diC_8 O_2^- response, exemplified by a representative normalized experiment in fig. 4.7c, whereas the effect of this agent on the mean normalized dose-response curve was not significant (fig. 4.7b). The reason for the variation masking the potentiation in this series of experiments lies with the control diC_8 curve, which showed a degree of inter-experimental variation, possibly due to inter-batch variation of the diC_8 samples supplied (see section 3.8).

4.2.8 The effect of sodium meclofenamate on stimulated O_2^- generation

Sodium meclofenamate (100μM) potentiated the response to all three stimuli. In the case of diC_8, it caused a very marked left-shift of the mean normalized concentration-effect curve but no significant change in the maximum (n = 4) (fig. 4.8a). In the case of the PAF dose-response curve, the drug effected both a left-shift and an increase in maximum (n = 4) (fig. 4.8b), which in some experiments was over 200%.

With fluoride, the effect of meclofenamate (100μM) was to cause a massive potentiation of the response with a very marked left-shift of the curve and a 7- to 8-fold increase of the O_2^- response at 6 and 10mM fluoride (fig. 4.9a). Above 10mM fluoride, meclofenamate effected an *inhibition* of the fluoride response back to control levels. The basis for this interesting effect is not known for sure, but it occurred in all 5 experiments. Since the potentiation observed at 10mM fluoride caused the highest level of superoxide generation that was seen with fluoride during the whole project, it is possible that activation at higher concentrations could have triggered a protective turn-off mechanism in the cell. (The possible existence of a negative-feedback mechanism mediating a turn-off of the fluoride response, together with other possibilities, will be discussed in more detail in Chapter 5). In light of these results lower concentrations of meclofenamate, 1μM and 10μM, were tested on the fluoride response. Meclofenamate (10μM) caused both a left-shift of the control dose-response curve and an increase in maximum (n = 5) (fig. 4.9b). The mean normalized results with 1μM meclofenamate were not significantly different from control (fig. 4.9c), although a small potentiation was seen in all 3 individual experiments.

4.2.9 The effect of mefenamic acid on stimulated O_2^- generation

Mefenamic acid (100μM), in 4 experiments, had a similar very powerful potentiating effect on the fluoride O_2^- response as meclofenamate. At low fluoride concentrations, namely 10 and 14mM, it caused a very marked left-shift of the control dose-response curve and a 2- to 3-fold increase in O_2^- release (fig. 4.10a). Mefenamic acid (100μM) showed an inhibitory effect at higher fluoride concentrations, reducing the O_2^- response back to control levels and even lower at 22mM fluoride (fig. 4.10a). Mefenamic acid (10μM) caused a consistent leftward shift of the control curve and an increase in maximum in 4 experiments, but the effect was not significant in the mean normalized data (fig. 4.10a). An apparent contradiction was observed when the effect of mefenamic acid (100μM) was re-examined on the fluoride O_2^- response one year after the above series of experiments were carried out. Here mefenamic acid produced a significant potentiation of the O_2^- response (n = 3) (fig. 4.10b) but no way near as great as that presented in fig. 4.10a; also the biphasic effect of the drug was not observed. The only explanation for this anomaly is that the drug had lost activity in the second series of experiments.

Mefenamic acid (100μM) caused a marked enhancement of the O_2^- response induced by diC_8, shifting the concentration-response curve very significantly leftwards while causing little change in the maximum response (n = 5) (fig. 4.11a). In 2 experiments mefenamic acid (10μM) effected a slight enhancement of the diC_8 O_2^- response – one of which is presented in fig. 4.11b.

Mefenamic acid (100μM) failed to produce a significant potentiation of the mean PAF response from 4 experiments, even though in each experiment there was a small drug-induced increase. Therefore, the mean normalized dose-response curve (fig. 4.11c) and a representative normalized experiment (fig. 4.11d) are presented to show the effect.

4.2.10 The effect of naproxen on the stimulated O_2^- response

Naproxen (100μM) caused a significant leftward shift of the control dose-response curves induced by both fluoride (n = 3) (fig. 4.12a) and diC_8 (n = 3) (fig. 4.12b), while having very little effect on the maximum control response in both cases. On the other hand, naproxen (100μM) caused no significant effect on the PAF O_2^- dose-response curve (n = 4) (fig. 4.12c).

4.2.11 The effect of phenylbutazone on stimulated O_2^- production

Phenylbutazone (100μM) produced a very significant decrease of the fluoride O_2^- response causing a marked right shift of the concentration-response curve with an apparent reduction of the maximum control response (n = 3) (fig. 4.13a). This agent had no significant effect on the mean normalized diC_8 curve (fig. 4.13b), even though a slight inhibition was observed in all 5 experiments. Phenylbutazone (100μM) did not significantly affect the PAF O_2^- dose-response curve when the normalized data from 3 experiments were pooled (fig. 4.13c). However, in each experiment this drug caused a substantial decrease of the O_2^- response as is exemplified from the representative experiment in fig. 4.13d, an effect that appeared to be lost in the averaging of experiments.

4.2.12 The effect of piroxicam on stimulated O_2^- generation

Piroxicam (100μM) had very little effect on the O_2^- responses induced by diC_8 (n = 6) (fig. 4.14b). The effect of piroxicam on the mean control dose-response curve induced by fluoride was to cause a rightward shift (n = 5) (fig. 4.14a) but a statistically significant inhibition was recorded at only one fluoride concentration, as given on the graph.

The effect of piroxicam (100μM) on the PAF response was inhibitory, with a rightward shift of the dose-response curve and a reduction in the maximum control response (n = 3) (fig. 4.14c).

4.2.13 The effect of sulindac on the stimulated O_2^- response

The effect of sulindac on fluoride-mediated O_2^- release was one of marked potentiation showing a dose-related increase at 10 and 100μM drug, where the enhancing effect was significant at both concentrations. The mean normalized data (n = 4) is plotted in fig. 4.15a. This result was somewhat surprising in view of the observation that this drug had no effect on the PAF response, the criterion for classifying a drug as "an enhancer", and also a negative result was obtained with the diC_8 response. The mean normalized concentration-response curves of diC_8 (n = 4) and PAF (n = 5), with and without sulindac, showing the "no effect" result are presented in fig. 4.15b & c respectively.

4.2.14 Do NSAIDs activate the oxidative burst in their own right?

Lukey *et al.* (1988) reported a benoxaprofen-mediated oxidative burst, as measured by chemiluminescence in neutrophils. As this NSAID

was categorized in the current study as one of the more potent "enhancing drugs" it was important to assess whether this direct stimulatory effect occurred in our system. Another study mentioned earlier, reported that indomethacin, by itself, was capable of evoking O_2^- generation from chemically elicited guinea pig macrophages (Bromberg & Pick, 1983). In all the NSAID experiments the effect of drug was routinely examined in unstimulated cells but the result was always negative. However, because our assay involved a 20 minute pre-incubation period of the cells with drug before stimulation and before coming into contact with the ferricytochrome C, any superoxide generated in this period would go undetected. Experiments were subsequently carried out where drug was added directly to the reaction mixture comprising of cells and ferricytochrome C, and incubation was continued for periods between 30 and 90 minutes. The results revealed that the very low basal O_2^- production recorded, of the order 4-6nmol/ 5×10^6 cells, was increased slightly as shown in fig. 4.16 in the presence of benoxaprofen and meclofenamate, but the increase was not statistically significant at $p < 0.05$ in an unpaired t-test. The same negative result was recorded for the other enhancing NSAIDs tested.

The macrophages used by Bromberg & Pick (1983) would have already been partially activated by their elicitation procedure and this may explain the fact that subsequent exposure to indomethacin stimulated the oxidative burst. The reason for the difference between our results and those of Lukey *et al.* (1988) could be that neutrophils too might require to be partially activated (i.e. primed) before an NSAID could induce an oxidative burst. It is possible that the isolation procedure used by Lukey *et al.* could have resulted in "primed" neutrophils. It should also be pointed out that a ferricytochrome C reduction assay was used in the current study for superoxide measurement while Lukey *et al.* (1988) used lucigenin-enhanced chemiluminescence.

4.2.15 The effect of the enhancing NSAIDs and DAG metabolizing inhibitors on an isolated PKC enzyme assay

Three NSAIDs which caused a potentiation of the O_2^- response – benoxaprofen, meclofenamate and indomethacin – at concentrations ranging from 0.001-10 mol% that corresponds in molar terms to 0.06-600μM, failed to cause an increase of PKC-induced histone III phosphorylation over and above that recorded in the presence of phosphatidylserine (PS) and dihexanoyl (DH). Aspirin, which had no

effect on the stimulated O_2^- response and was thus employed as a negative control, had no effect in the PKC-phosphorylation assay. These drugs were also tested for their effects on PKC-phosphorylation induced by enzyme alone, in the presence of only PS and in the presence of only DH; under these activating conditions the NSAIDs again did not cause any change from control in the phosphorylation pattern. Benoxaprofen also failed to affect histone III phosphorylation when Ca^{2+} concentrations were varied or when the drug was introduced to the assay system in the aqueous phase rather than in the Triton mixed micelle or lipid phase (see Chapter 2). The effect of meclofenamate, 0.001-10 mol%, was examined on the full DH-stimulated phosphorylation dose-response curve and was found to have no significant effect (fig. 4.17, with only 0.01 & 0.1 mol% meclofenamate included for clarity).

The DMARDs, auranofin and penicillamine, and the DAG metabolic inhibitors, R59022, RHC80267 and DOEG, had no significant effect on PKC-mediated phosphorylation when tested in the presence of PS and DH.

The above findings are summarized in Table 4.3.

Table 4.3

The Effect of some NSAIDs and DAG metabolic inhibitors on PKC-mediated Histone III Phosphorylation

Drug	Effect in the presence of PS (20mol%) and DH (0.01-0.1mol%)
Benoxaprofen	No Effect (n = 4)
Meclofenamate	No Effect (n = 6)
Indomethacin	No Effect (n = 2)
Aspirin	No Effect (n = 1)
Auranofin	No Effect (n = 1)
Penicillamine	No Effect (n = 1)
R59022	No Effect (n = 4)
DOEG	No Effect (n = 2)
RHC80267	No Effect (n = 3)

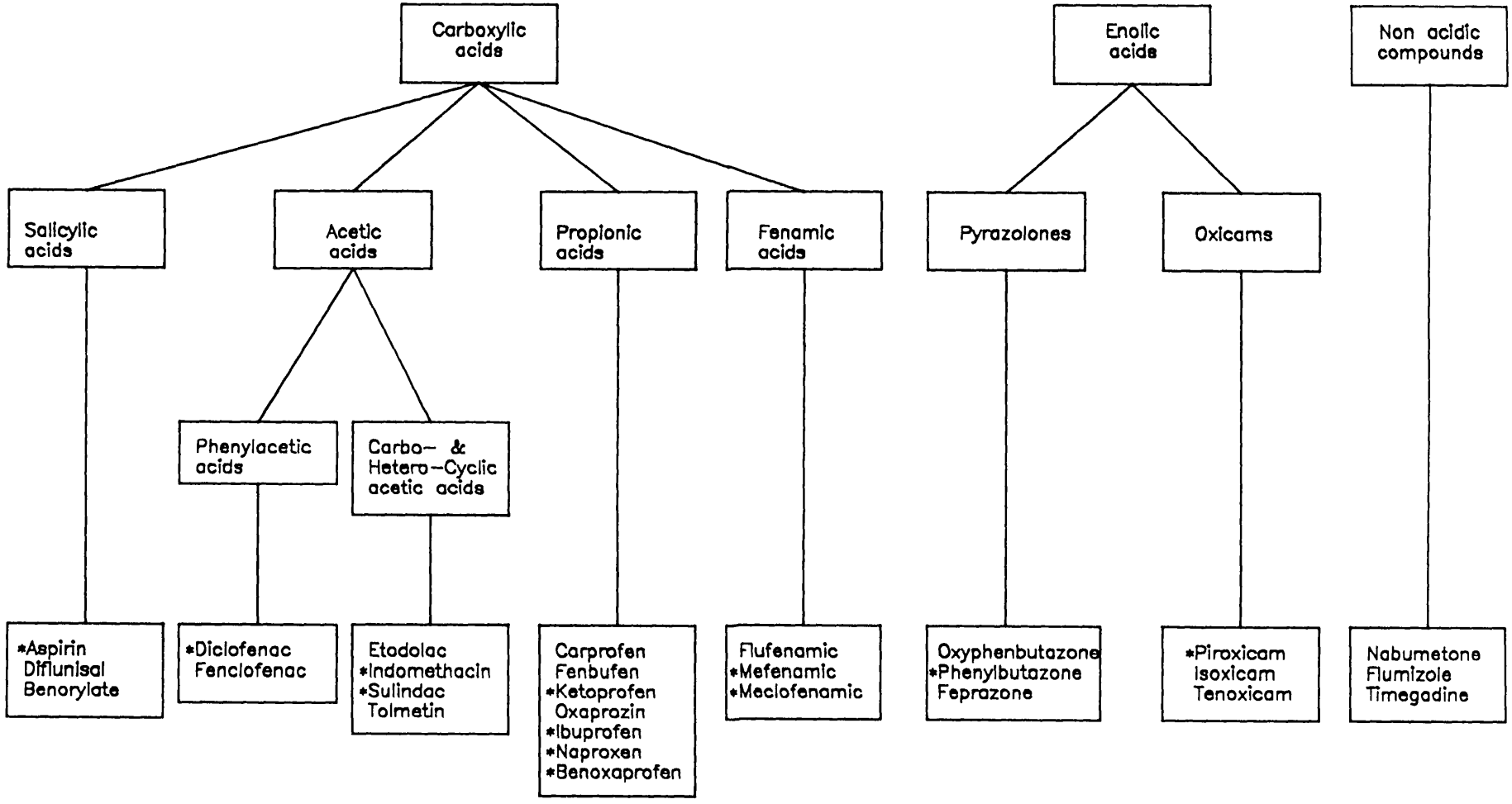


Figure 4.1: Major classes of non-steroidal anti-inflammatory drugs (NSAIDs) with representative examples (* denotes those used in this study).

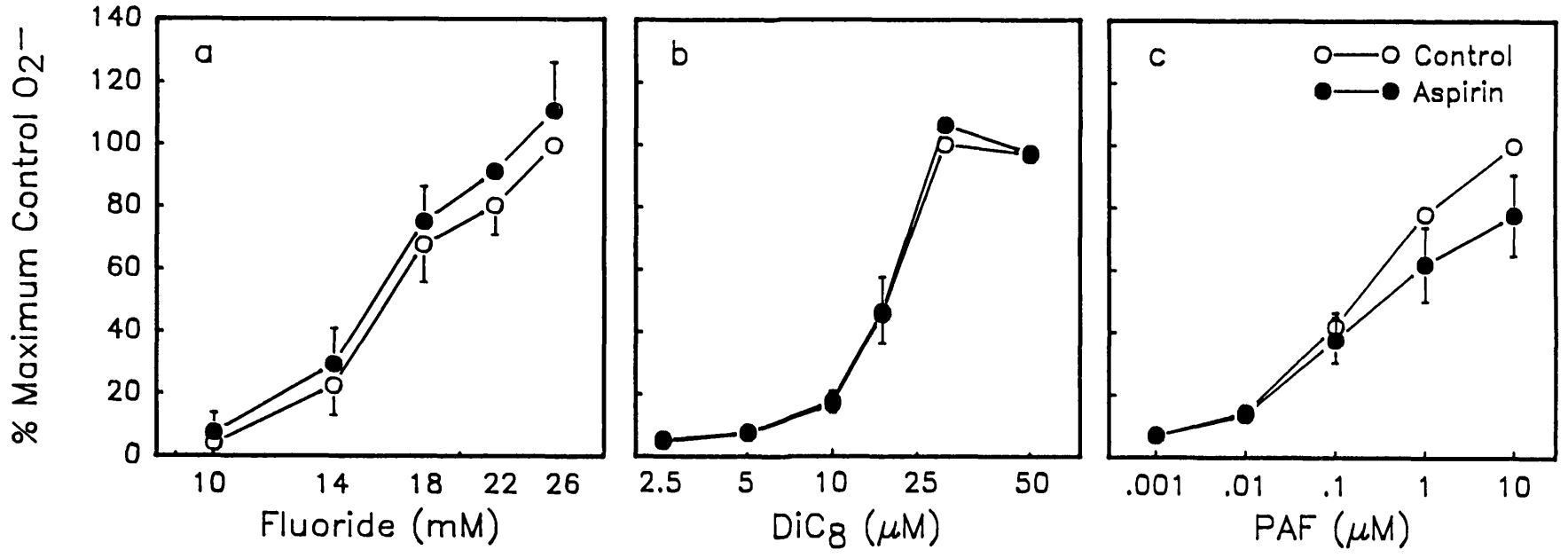


Figure 4.2: Effect of aspirin on O_2^- production induced by, a) fluoride (n = 10), b) diC_8 (n = 5) and c) PAF (n = 6). Responses were obtained either alone (O) or in the presence of 100μM aspirin (●). Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 76.94 ± 7.83 for fluoride, 147.02 ± 10.67 for diC_8 and 60.41 ± 10.55 for PAF. Error bars represent standard errors.

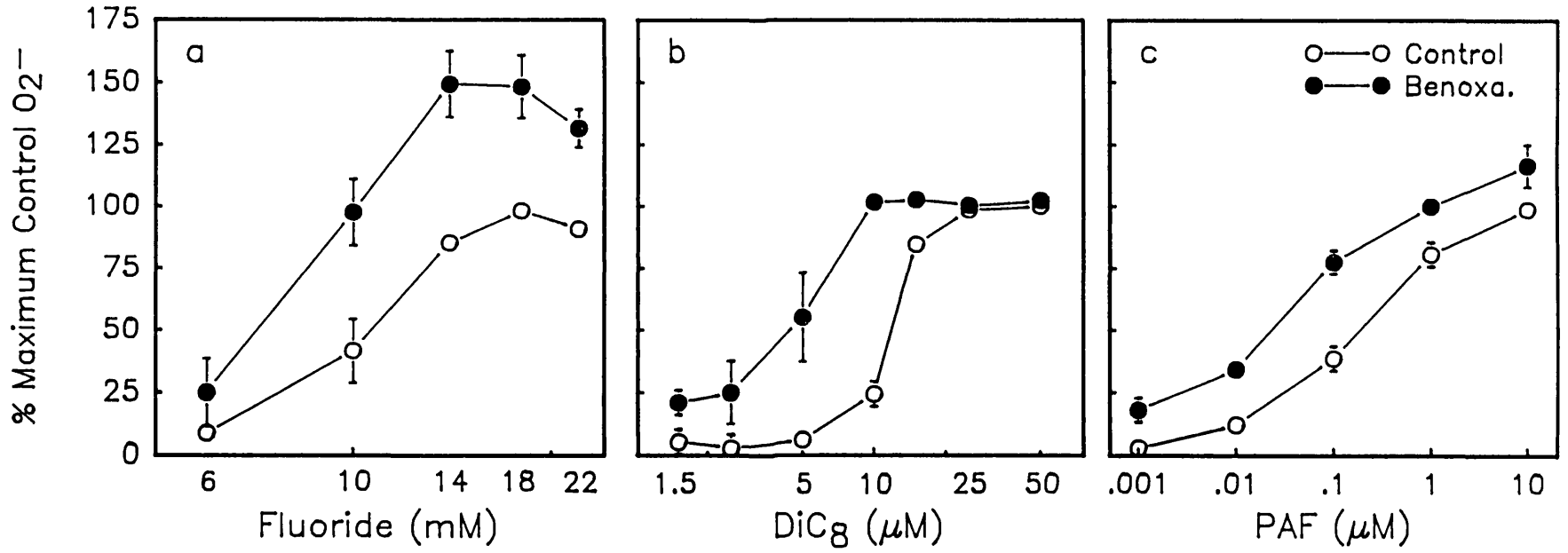


Figure 4.3: Effect of benoxaprofen on O_2^- production induced by, a) fluoride (n = 4), b) diC_8 (n = 3) and c) PAF (n = 6). Responses were obtained either alone (O) or in the presence of 100μM benoxaprofen (●). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 77.15 ± 19.22 for fluoride, 166.89 ± 10.46 for diC_8 and 60.49 ± 10.66 for PAF. Error bars represent standard errors.

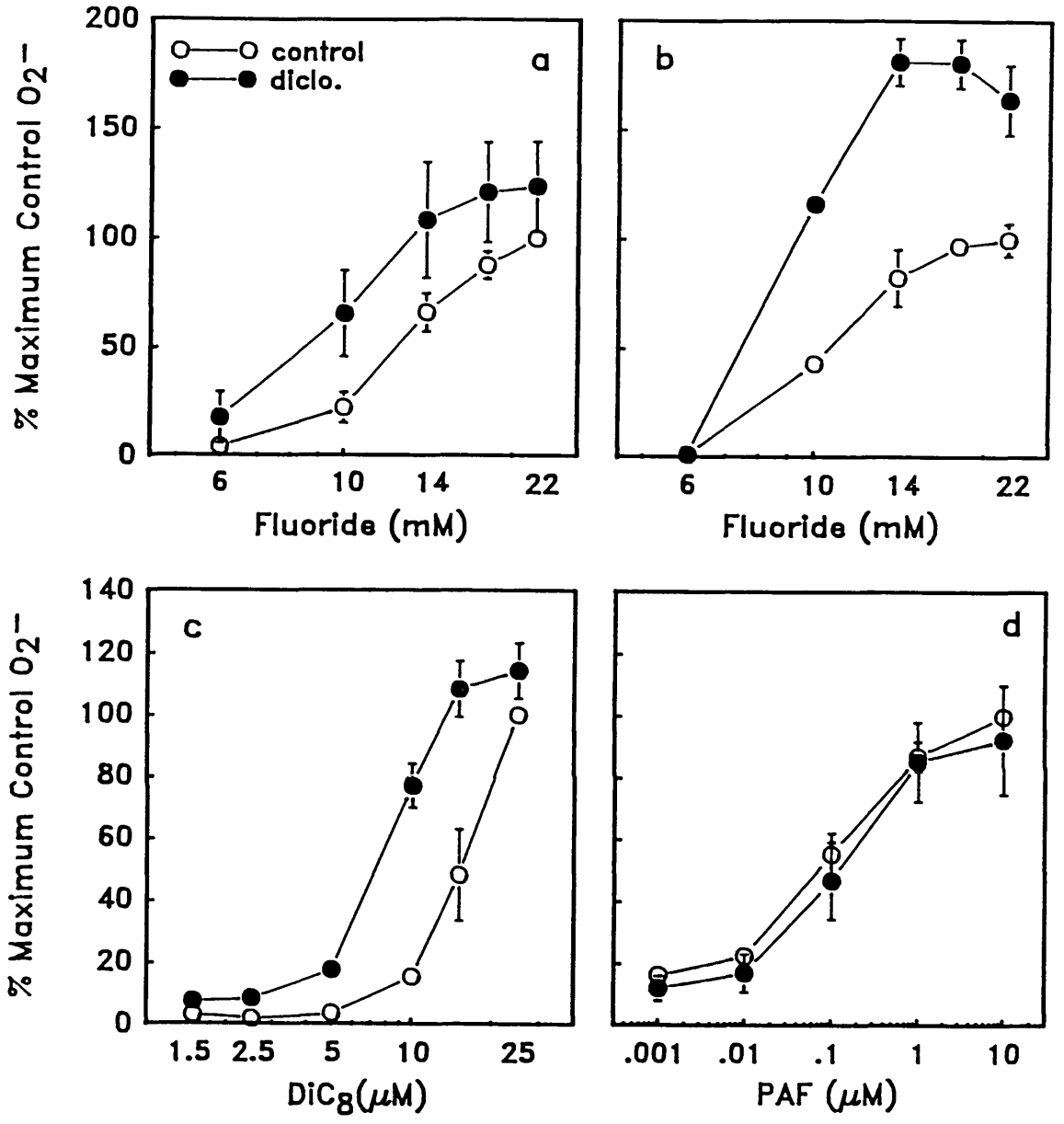


Figure 4.4: Effect of diclofenac on O_2^- production induced by a) fluoride (n = 5), c) diC_8 (n = 4) and d) PAF (n = 4). Stimulus alone (O) and in the presence of 100μM diclofenac (●). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 73.65 ± 8.71 for fluoride, 158.30 ± 12.29 for diC_8 and 57.31 ± 12.51 for PAF. Error bars represent standard errors. b) Fluoride, a single representative of the 3 experiments which showed a marked potentiation. The maximum control O_2^- release, as a mean of sample duplicates, was 67.26 ± 5.08 nmol/5 × 10^6 neutrophils and error bars represent the range of the duplicates.

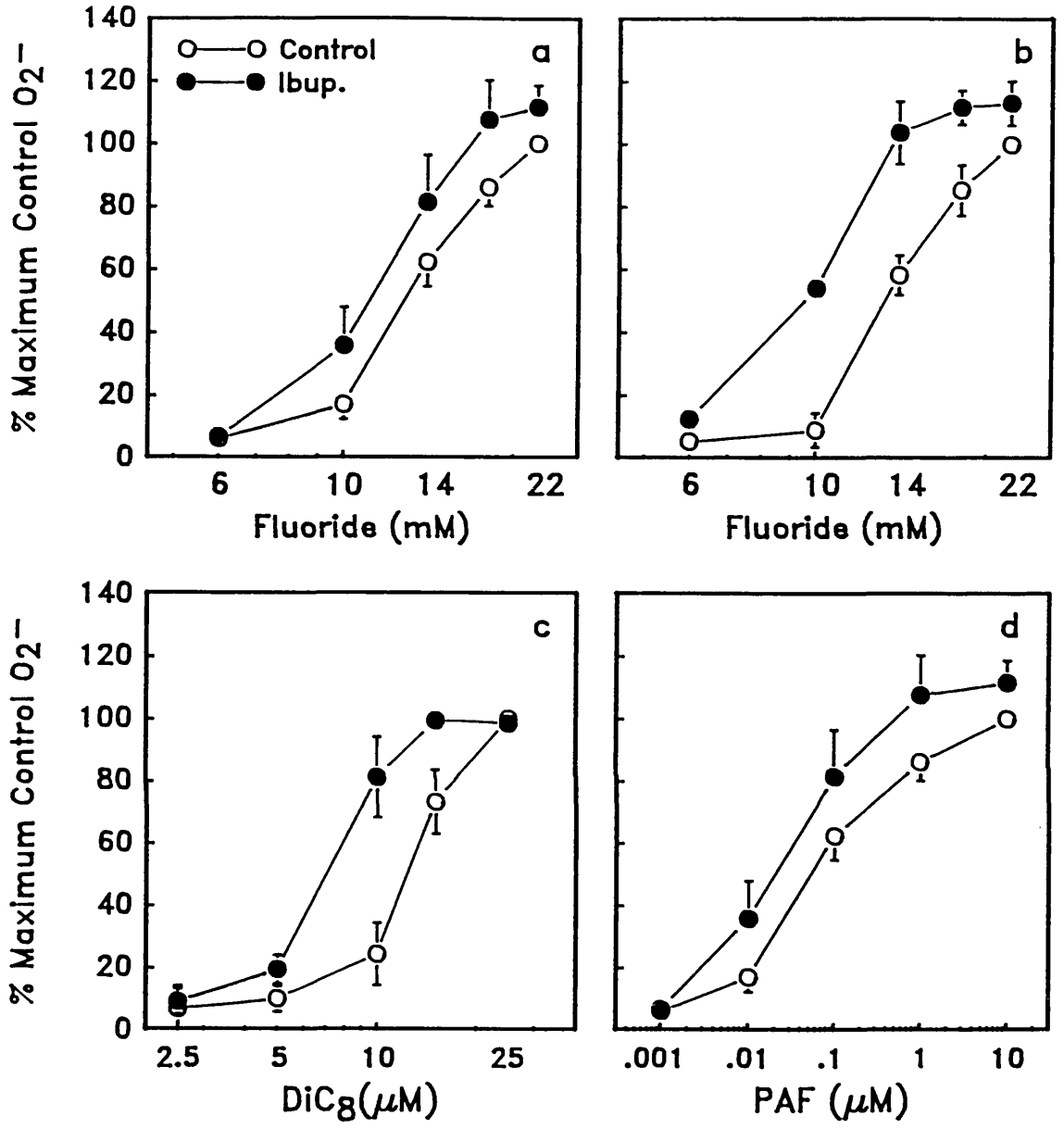


Figure 4.5: Effect of ibuprofen on O_2^- production induced by a) fluoride (n = 5), c) diC_8 (n = 3) and d) PAF (n = 3). Responses were obtained either alone (O) or in the presence of 100μM ibuprofen (●). Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 cells was 77.16 ± 8.77 for fluoride, 163.56 ± 11.74 for diC_8 and 53.83 ± 14.22 for PAF. Error bars represent standard errors. b) Fluoride, a single representative of the 3 experiments which showed a marked potentiation. The maximum control O_2^- release, as a mean of sample duplicates, was 69.80 ± 1.81 nmol O_2^-/5 x 10^6 neutrophils and error bars represent the range of the duplicates.

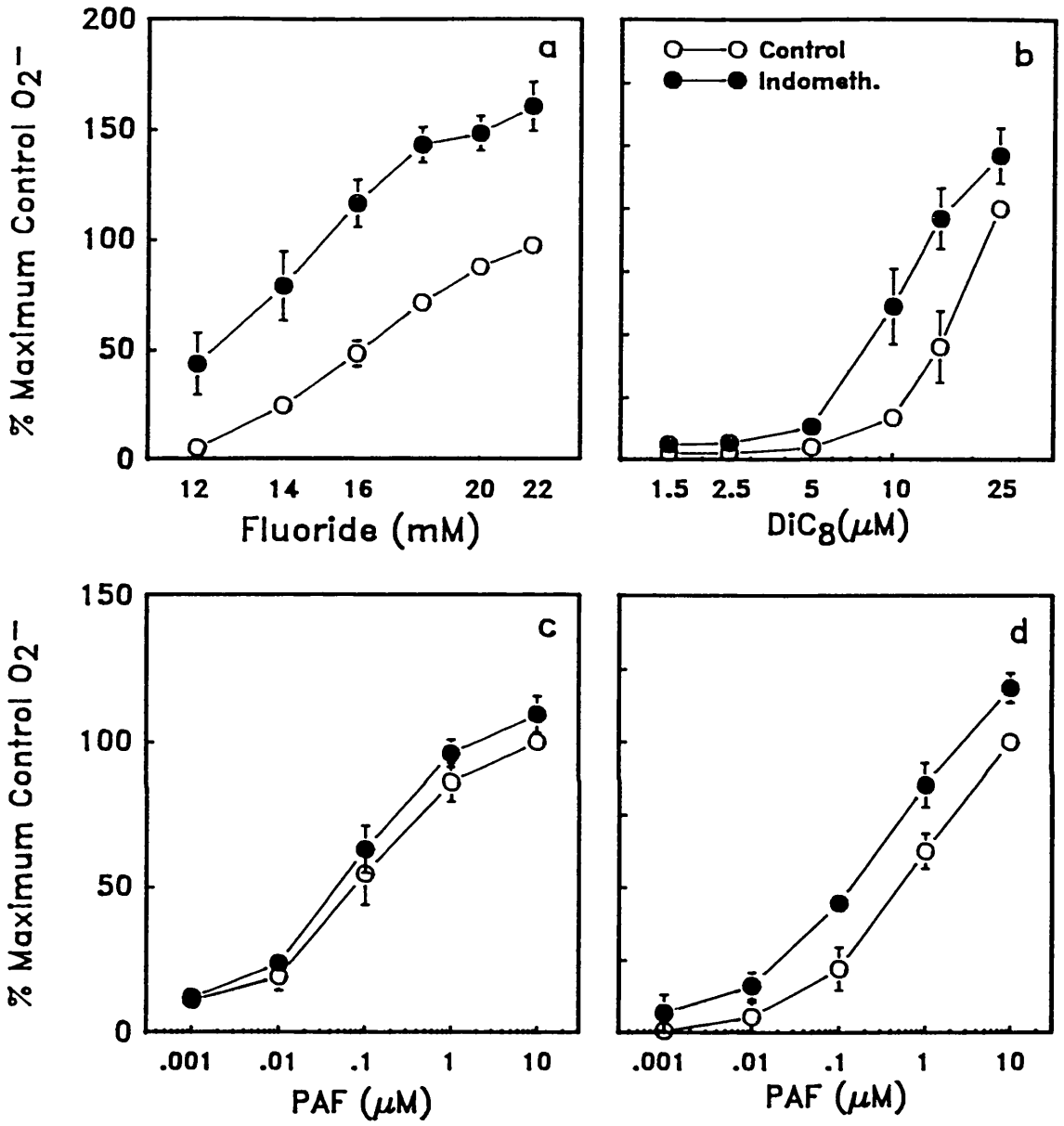


Figure 4.6: Effect of indomethacin on O_2^- production induced by a) fluoride (n = 9), b) diC_8 (n = 4) and c) PAF (n = 5). Stimulus alone (O) and in the presence of 100μM indomethacin (●). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 96.30 ± 8.01 for fluoride, 153.40 ± 10.59 for diC_8 and 66.00 ± 11.37 for PAF. Error bars represent standard errors. d) PAF, a single representative of the 4 experiments which showed a potentiation. The maximum control O_2^- release, as a mean of sample duplicates, was 37.74 ± 1.21 nmol O_2^-/5 × 10^6 neutrophils and error bars represent the range of the duplicates.

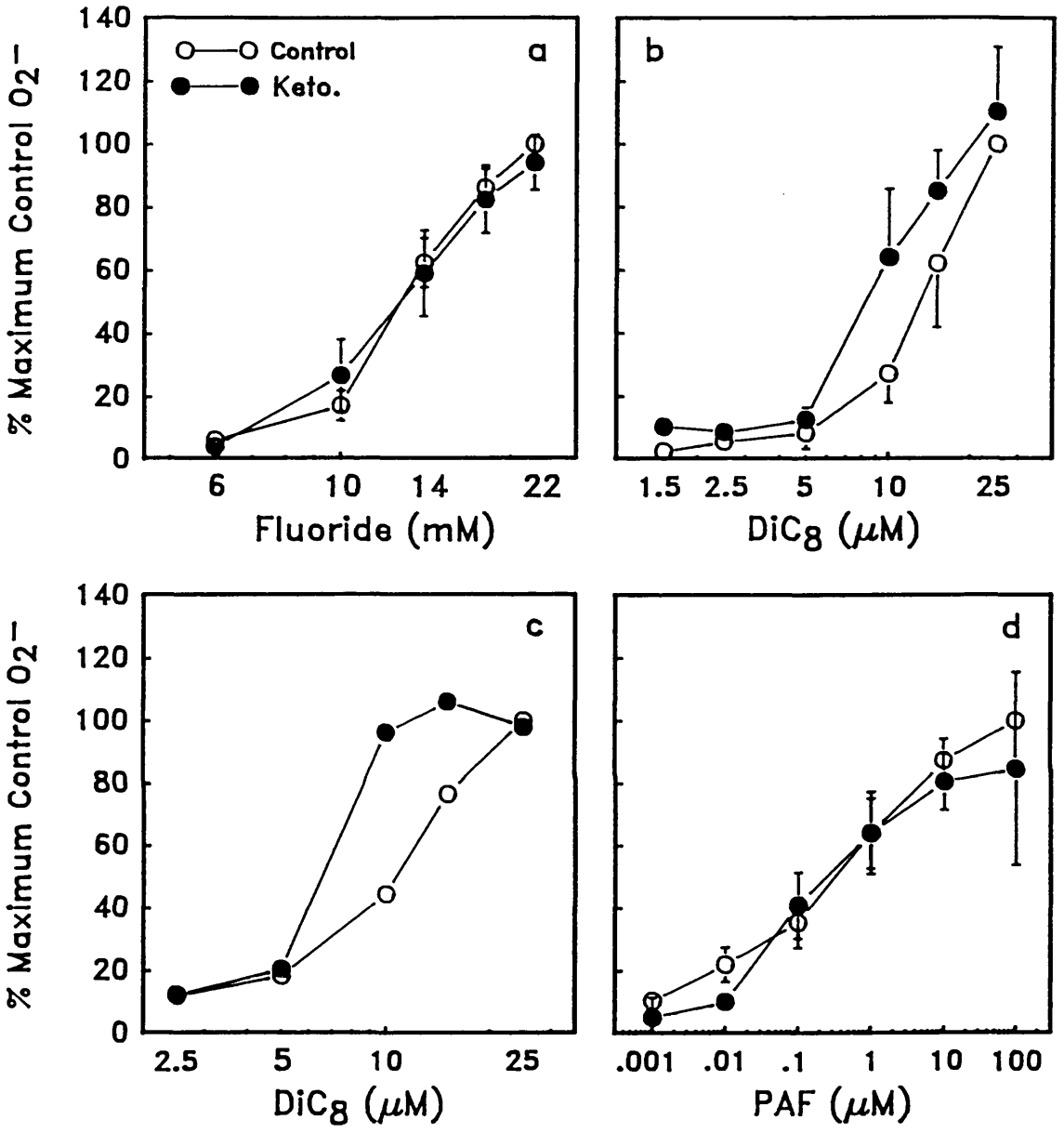


Figure 4.7: Effect of ketoprofen on O_2^- production induced by a) fluoride (n = 5), b) diC_8 (n = 3) and d) PAF (n = 3). Stimulus alone (O) and in the presence of 100μM ketoprofen (●). Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 77.16 ± 8.77 for fluoride, 161.63 ± 16.52 for diC_8 and 50.41 ± 14.76 for PAF. Error bars represent standard errors. c) DiC_8, a single representative of the 3 experiments in b. The maximum control O_2^- release, as a mean of sample duplicates, was 169.37 ± 4.35 nmol O_2^-/5 x 10^6 neutrophils and error bars represent the range of the duplicates.

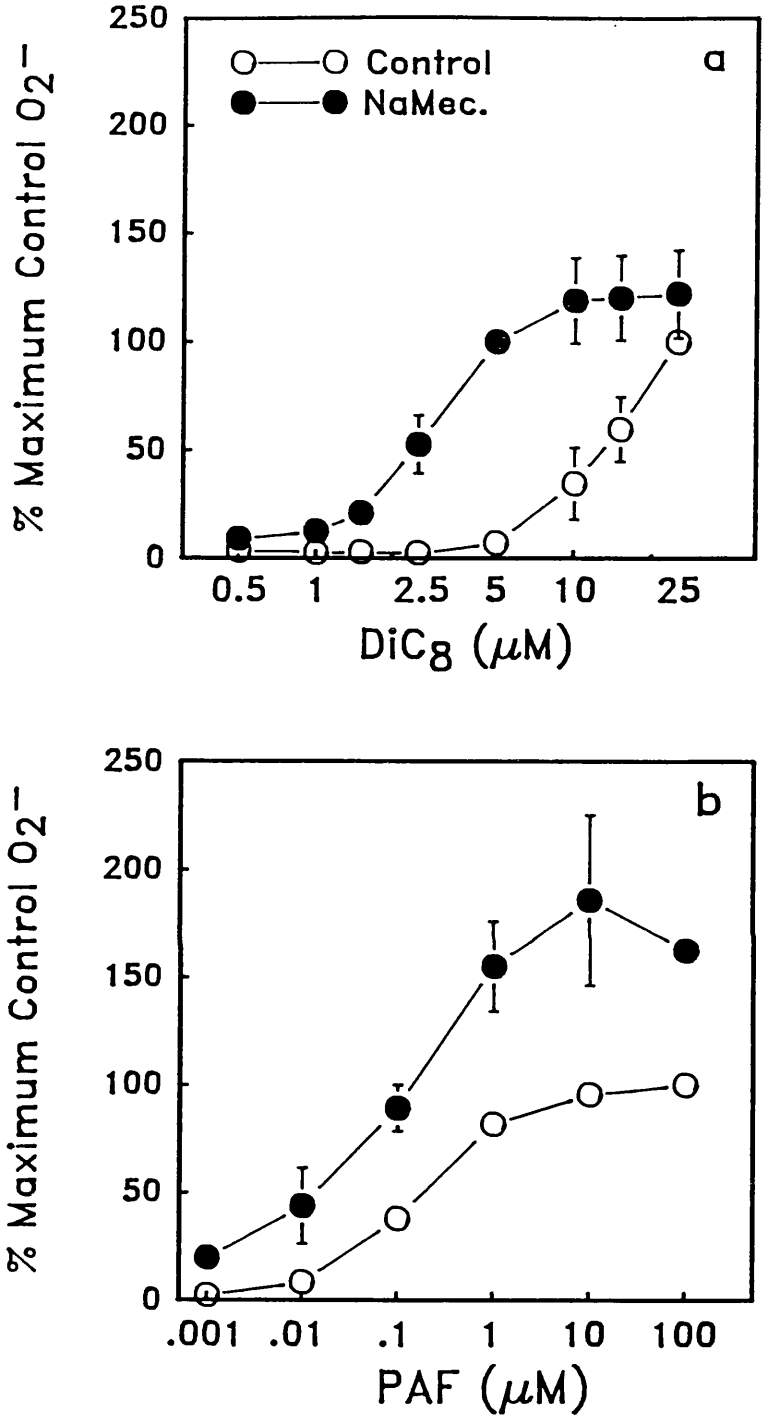


Figure 4.8: The effect of sodium meclofenamate on O_2^- production induced by a) diC_8 (n = 4) and b) PAF (n = 4). Responses were obtained either alone (O) or in the presence of 100μM sodium meclofenamate (●). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 157.21 ± 20.50 for diC_8 and 49.78 ± 10.85 for PAF. Error bars represent standard errors.

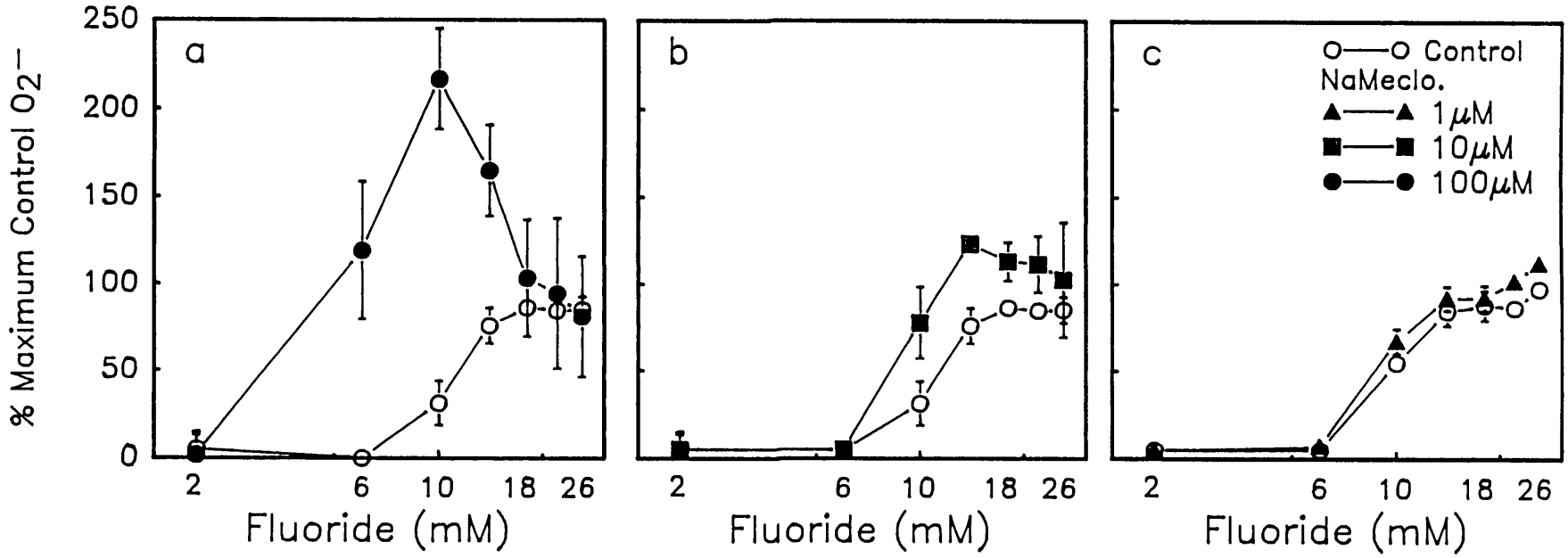


Figure 4.9: The effect of sodium meclofenamate on fluoride-induced O_2^- production, a) at 100μM (n = 5), b) at 10μM (n = 5) and c) at 1μM (n = 3). Responses were obtained either alone (O) or in the presence of sodium meclofenamate at 100μM (●), at 10μM (■) and at 1μM (▲). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 cells was 55.60 ± 16.76 for fluoride in a and b, and 70.33 ± 23.17 for fluoride in c. Error bars represent standard errors.

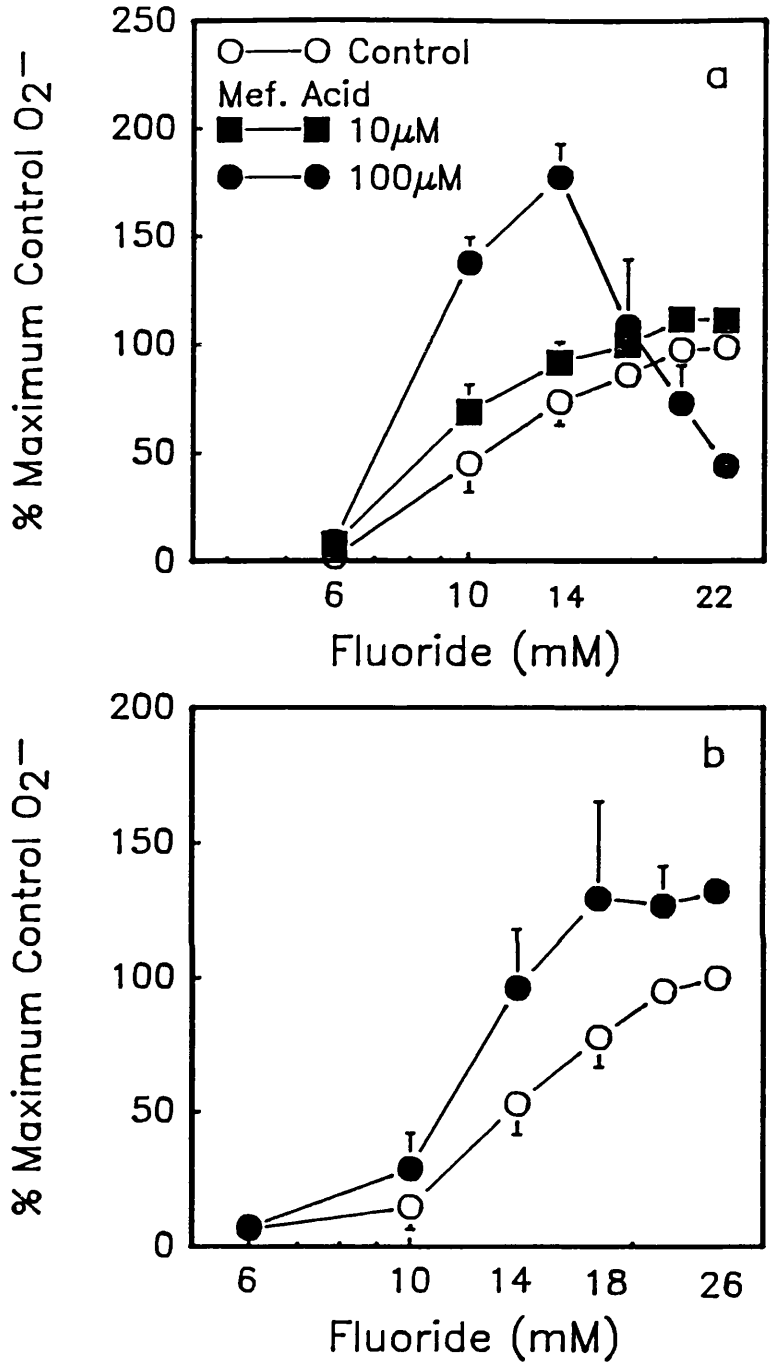


Figure 4.10: The effect of mefenamic acid on fluoride-induced O_2^- production in two separate studies; the result shown in a) (n = 4) was carried out one year previous to that shown in b) (n = 3). Responses were obtained either alone (O) or in the presence of mefenamic acid at 100μM (●) and at 10μM (■). The mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 65.33 ± 1.04 in a and 66.01 ± 8.59 in b. Error bars represent standard errors.

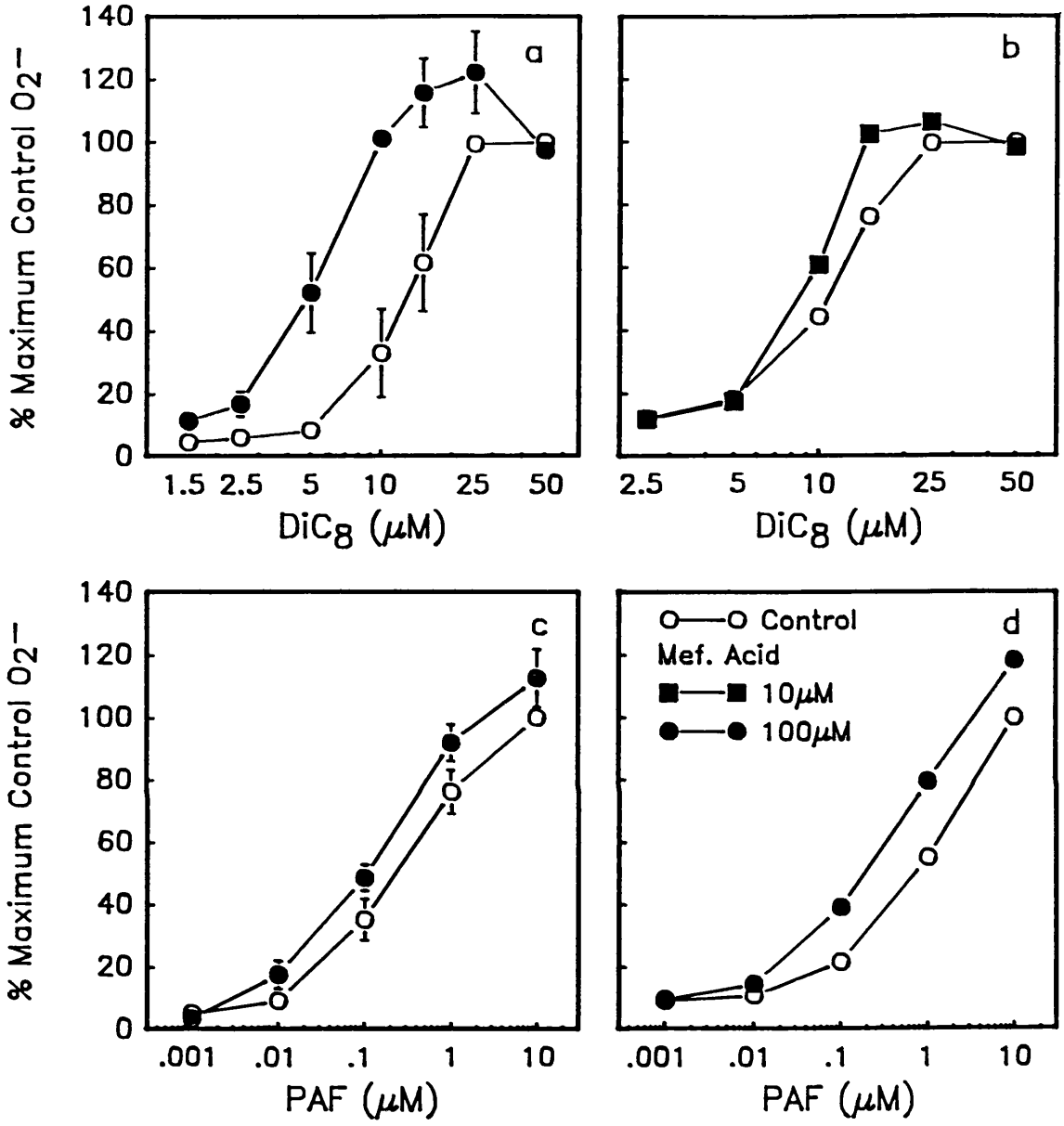


Figure 4.11: a) and b) The effect of mefenamic acid on diC_8-induced O_2^- production at 100μM (n = 5) and at 10μM (a single experiment representative of two) respectively. c) The effect of mefenamic acid on PAF-induced O_2^- production (n = 4) and d) a single representative of the 4 experiments in **c**. Stimulus alone (O) and in the presence of mefenamic acid at 100μM (●) or 10μM (■). Mean maximum control O_2^- release, as nmol/5×10^6 cells, for diC_8 was 157.27 ± 14.19 in **a** and 169.37 ± 4.35 (mean of sample duplicates) in **b** and for PAF was 57.64 ± 13.24 in **c** and 49.96 ± 1.33 (mean of sample duplicates) in **d**. Error bars in **a** & **c** represent SE and in **b** & **d** the range of sample duplicates.

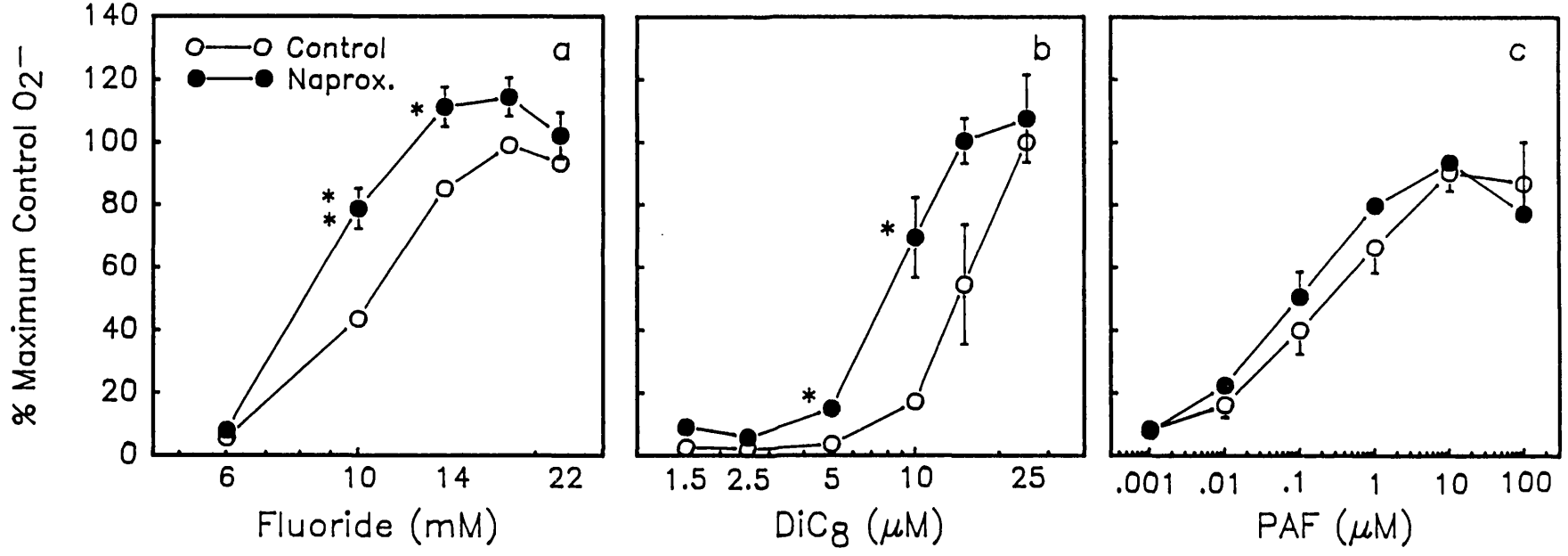


Figure 4.12: The effect of naproxen on O_2^- production induced by, a) fluoride (n = 3), b) diC_8 (n = 3) and c) PAF (n = 4). Stimulus alone (O) and in the presence of 100μM naproxen (●). Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 67.95 ± 11.21 for fluoride, 161.95 ± 16.60 for diC_8 and 54.26 ± 10.07 for PAF. Unpaired t-tests were performed on the data, * $P < 0.05$, ** $P < 0.01$. Error bars represent standard errors.

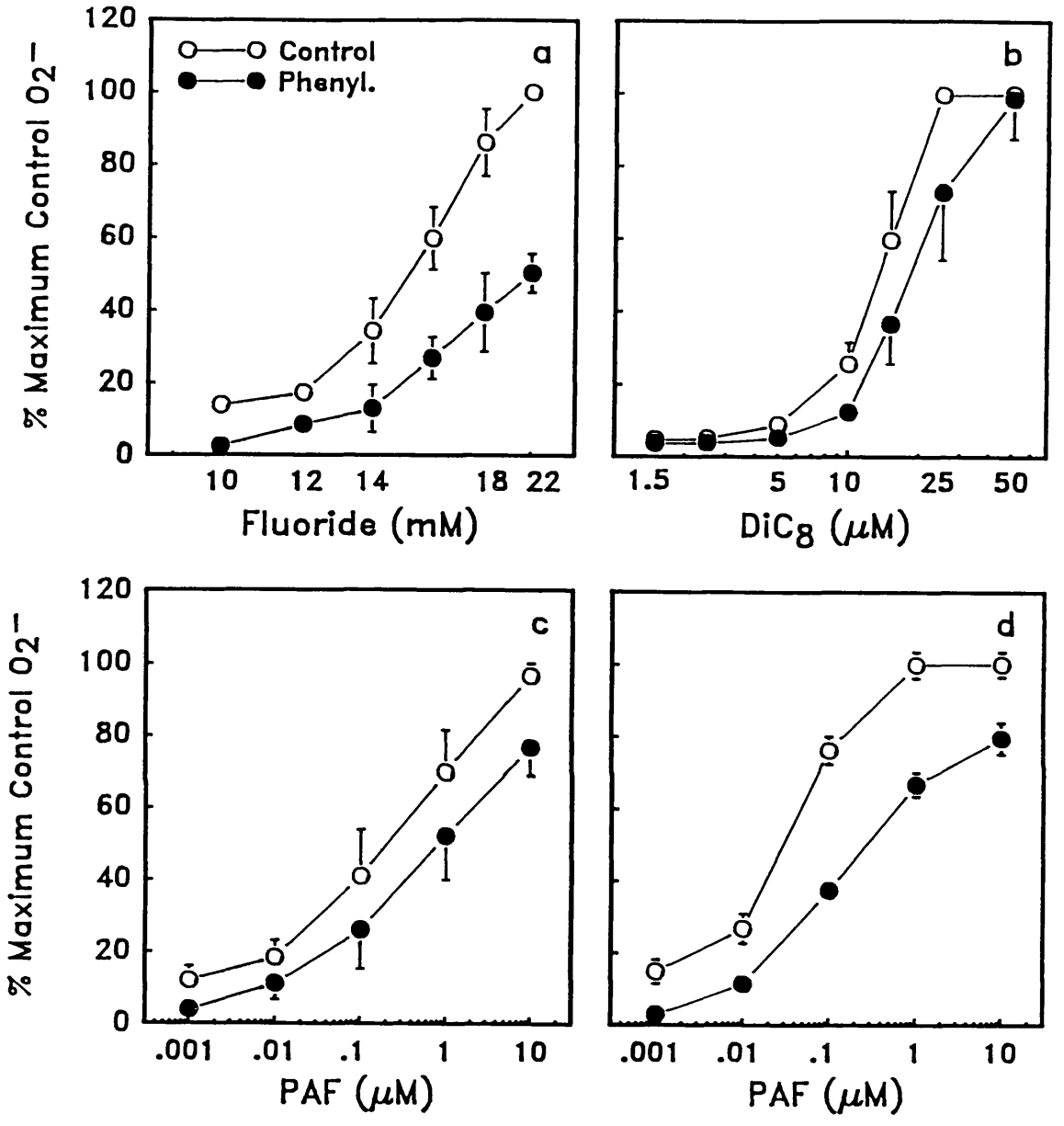


Figure 4.13: The effect of phenylbutazone on O_2^- production induced by a) fluoride (n = 3), b) diC_8 (n = 5) and c) PAF (n = 3). Responses were obtained either alone (O) or in the presence of 100μM phenylbutazone (●). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 99.33 ± 20.39 for fluoride, 146.88 ± 13.17 for diC_8 and 53.32 ± 9.92 for PAF. Error bars represent standard errors.
d) PAF, a single representative of the 3 experiments in c. Maximum control O_2^- release, as a mean of sample duplicates, was 78.03 ± 2.88 nmol/5 × 10^6 neutrophils and error bars represent the range of the duplicates.

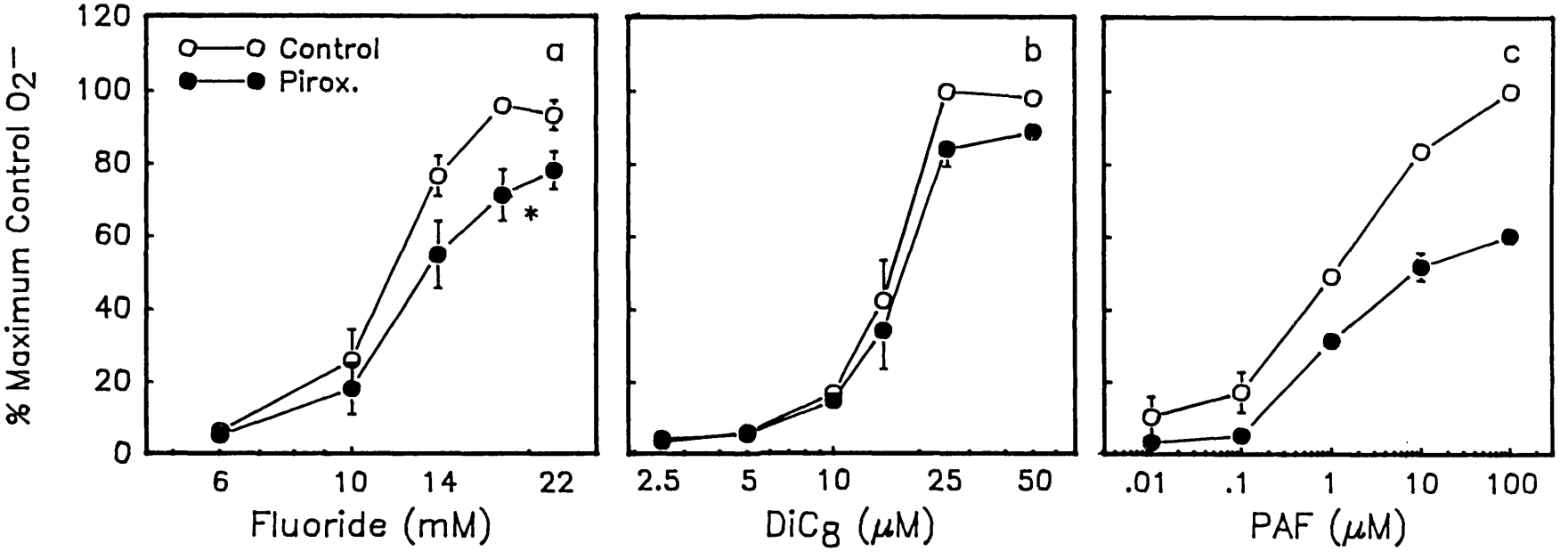


Figure 4.14: The effect of piroxicam on O_2^- production induced by, a) fluoride (n = 5), b) diC_8 (n = 6) and c) PAF (n = 3). Stimulus alone (O) and in the presence of 100μM piroxicam (●). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 95.47 ± 15.99 for fluoride, 133.37 ± 9.57 for diC_8 and 58.79 ± 18.88 for PAF. Unpaired t-tests were performed on the data, * $P < 0.05$. Error bars represent standard errors.

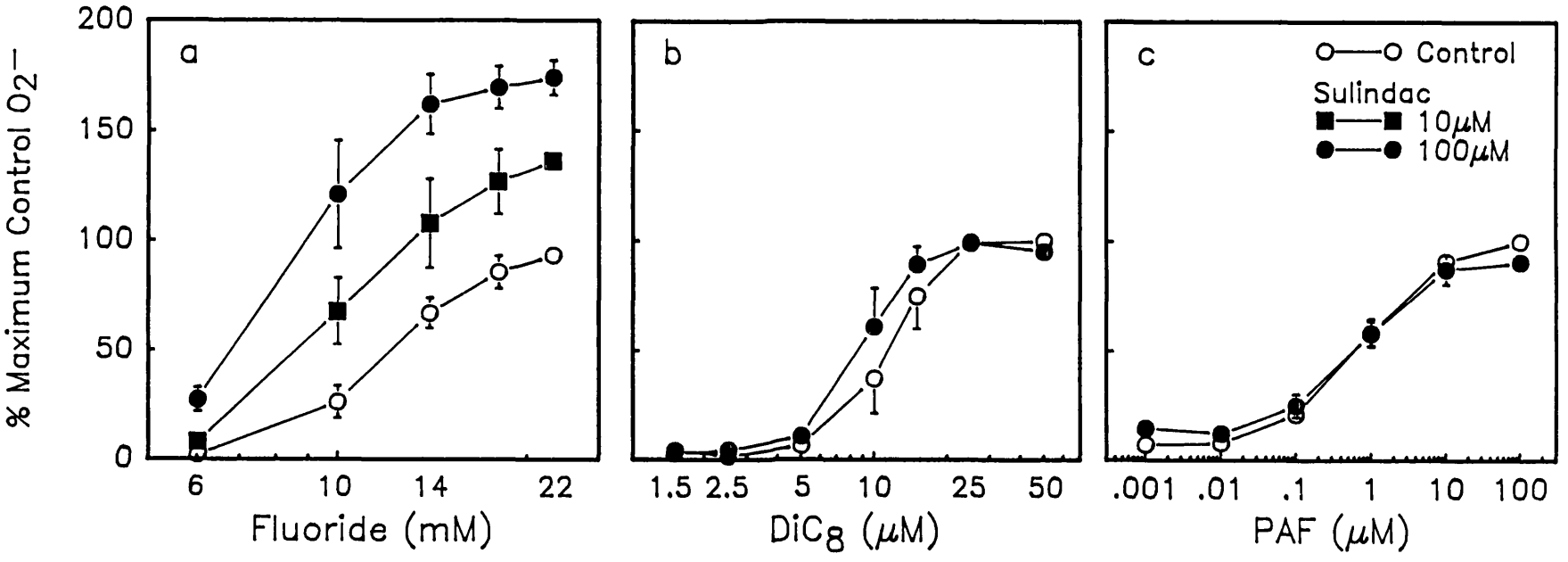


Figure 4.15: The effect of sulindac on O_2^- production induced by, a) fluoride (n = 4), b) diC_8 (n = 4) and c) PAF (n = 5). Stimulus alone (O) and in the presence of sulindac at 100μM (●) or at 10μM (■). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 60.94 ± 5.33 for fluoride, 152.01 ± 15.65 for diC_8 and 63.61 ± 19.57 for PAF. Error bars represent standard errors.

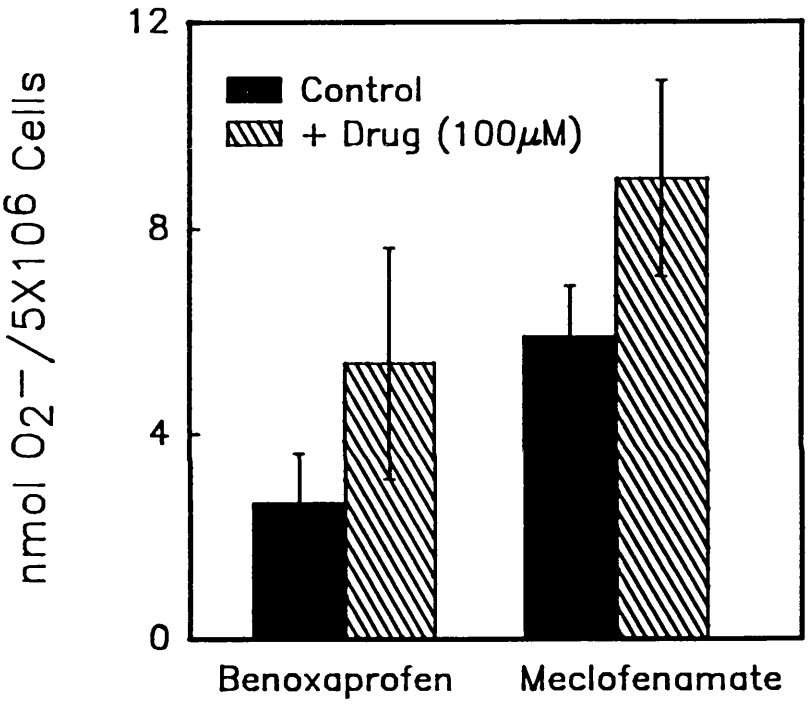


Figure 4.16: The effect of benoxaprofen and sodium meclofenamate on basal O_2^- production i.e. from unstimulated neutrophils; benoxaprofen (n = 11) and meclofenamate (n = 9).

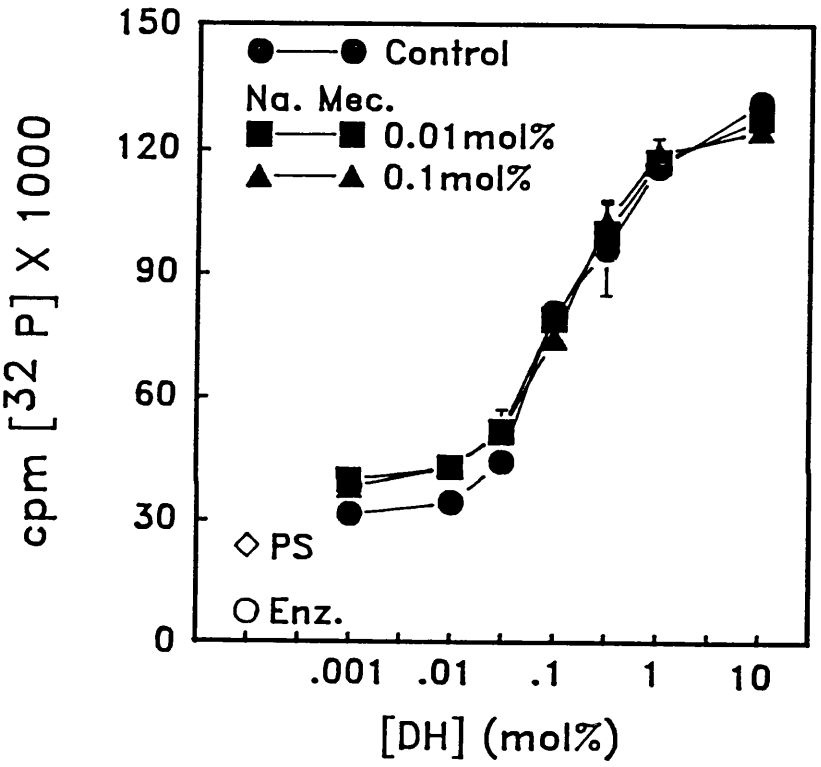


Figure 4.17: The effect of sodium meclofenamate on dihexanoyl (DH) -induced protein kinase C (PKC) activation. Phosphorylation was measured with PKC enzyme alone (○), plus phosphatidylserine (PS) (◇) and plus DH alone (●) or DH in the presence of meclofenamate at 0.01mol% (■) and 0.1mol% (▲). The graph shows the result of a single experiment representative of 2 independent experiments. Data points are the mean of sample duplicates ± range of the duplicates.

TABLE 4.4

Effect of NSAIDs on the stimulated O_2^- response

NSAID	Stimulus		
	Fluoride	DiC_8	PAF
Aspirin	(-)	(-)	(-)
Benoxaprofen	↑↑↑	↑↑↑	↑↑
Diclofenac	↑↑	↑↑	(-)
Ibuprofen	↑	↑	(-)
Indomethacin	↑	↑	↑
Ketoprofen	(-)	↑	(-)
Meclofenamate	↑↑↑	↑↑↑	↑↑
Mefenamic Acid	↑↑	↑↑↑	↑
Naproxen	↑	↑	(-)
Phenylbutazone	↓	(-)	↓
Piroxicam	↓	(-)	↓
Sulindac	↑↑	(-)	(-)

Key: (-) = no effect
↑ = slight increase in response
↑↑ = greater increase in response
↑↑↑ = markedly increased response
↓ = decrease in response

4.3 DISCUSSION

When a representative range of 12 NSAIDs were tested for their effect on stimulated O_2^- release, it was found that 4 out of the 12 exerted an enhancing action on O_2^- production induced by PAF and 8 out of the 12 showed an increase in the O_2^- response stimulated with the post-receptor stimuli, diC_8 and fluoride. It has also been found that some of these enhancing NSAIDs augmented O_2^- production in response to aggregated IgG and OZ, two relevant stimuli for the *in vivo* situation (Dale & Muid, unpublished results). Since neutrophil activation in an area of chronic inflammation will certainly involve a multiplicity of inflammatory stimuli which initiate the generation of toxic oxygen metabolites, the addition of a NSAID that could augment this response further, must surely present a potentially disastrous situation. Thus while providing temporary relief from the joint pain and swelling associated with a chronic inflammatory condition like RA, some NSAIDs *might* be exacerbating the underlying tissue damage.

It is obvious from the powerful enhancing effect of some of the NSAIDs with the post-receptor intracellular stimuli, that part if not all of their effect is mediated at some intracellular locus. What mode of action could explain the potentiating effect of these drugs on all the stimuli tested? Could the NSAID effects observed in this study occur with the recorded levels of drug found *in vivo*, and if so what are the clinical implications? Furthermore, could the effect of "the decreasers" be an intracellular phenomenon and what is the implication of this effect for the *in vivo* situation?

In an attempt to understand the mode of action of the enhancing NSAIDs, some intracellular transduction pathways employed by the various stimuli were investigated and these studies form the remainder of this thesis. Information is thus abstracted from later chapters and used in the first part of this discussion concerning the mode of action of those "enhancing" NSAIDs. Possible explanations for the "decreasing" effect of some NSAIDs is also discussed. The latter part of the discussion will deal with the questions of dose relevance, NSAID distribution, clinical implications and the possible inference for NSAID therapy.

4.3.1 What are the possible modes of action of the enhancing NSAIDs?

The possibility that the potentiating effects of the NSAIDs could be explained in terms of their inhibition of cyclooxygenase was first addressed. Prostaglandins (PGs) are reported to have a predominantly

inhibitory role in inflammatory cells. PGs of the E and I series have been shown to inhibit superoxide production by fMLP (Fantone & Kinnes, 1983; Fantone *et al.,* 1984), the inhibition correlating with their ability to increase intracellular cyclic adenosine monophosphate (cyclic AMP) levels (De Togni *et al.*, 1984; Smolen *et al.,* 1980). Cyclic AMP activates a cyclic AMP-dependent protein kinase – an enzyme which inhibits neutrophil activation. Inhibition by NSAIDs of this inhibitory mechanism could thus increase cell activation. The negative results obtained with aspirin on all three stimuli and the variable effects recorded with other NSAIDs depending on the stimulus, suggests that cyclooxygenase inhibition is not of importance in mediating the action of "the increasers".

In support of this view, the importance of the cyclooxygenase pathway in the neutrophil seems to be controversial. The indomethacin-inhibitable release of PGs E and F (Zurier & Sayadoff, 1975) and thromboxane B_2 (Goldstein *et al.*, 1978) to the surrounding medium on exposure to zymosan has been reported. However, Gordon *et al.* (1976) examined prostaglandin production by different inflammatory cells and found PMNs to produce very low amounts of prostaglandin-like activity, as opposed to macrophages which were demonstrated to sustain substantial prostaglandin production. Moreover, it has been reported by two independent groups that the major pathway of arachidonic acid (AA) metabolism in neutrophils involves 5-lipoxygenase, leading to the formation of leukotrienes and other hydroxy acids (Walsh *et al.*, 1981; Marcus *et al.*, 1984). The latter group went on to state that neutrophils do not appear to possess a measurable cyclooxygenase pathway.

A number of reports have described the potentiation by some NSAIDs of 5-lipoxygenase (5-LO) product generation in stimulated neutrophils; indomethacin increased LTB_4 production in A23187-stimulated PMNs (Docherty & Wilson, 1987), indomethacin, naproxen and ibuprofen stimulated AA metabolism *via* the lipoxygenase route in rat neutrophils from a reverse passive Arthus reaction (Myers & Siegel, 1983) and indomethacin markedly increased 5-LO product accumulation in rat leukocytes incubated with AA, whereas naproxen, ibuprofen and piroxicam each caused a smaller increase (Tavares *et al.*, 1985). In most of these cases, the potentiation has been attributed to diversion of AA substrate down the lipoxygenase pathway on inhibition of the cyclooxygenase route of AA metabolism, thereby increasing the concentration of lipoxygenase-derived products. It has been proposed that 5-LO metabolites may play a role in signal transduction. LTB_4, lipoxins and

other lipoxygenase-derived eicosanoids have been described as activators of both the oxidative burst (Serhan *et al.*, 1982; Serhan *et al.*, 1984; O'Flaherty *et al.*, 1985) and isolated PKC enzyme (Hansson *et al.*, 1986; Shearman *et al.*, 1989), the latter effect being offered as an explanation for the former. However, in this study diversion of AA metabolism down the 5-LO pathway as an explanation for the NSAID enhancing effects is unlikely, since aspirin at a concentration that would totally inhibit cyclooxygenase had no effect on the stimulated O_2^- response. The role of the 5-LO pathway in mediating the O_2^- response was addressed by inhibiting this pathway using three reportedly specific 5-LO inhibitors - BW A4C, Rev 5901 and piriprost (U60, 257) (see Chapter 7). It was found that these compounds had no effect on stimulated O_2^- production; the same result was obtained whether the studies were carried out in the presence or absence of aspirin. Thus, it was concluded that the 5-LO pathway did not play an important part in the transduction sequences leading to respiratory burst activation and was unlikely to be involved in mediating the NSAID effects.

Coincidently, the most potent enhancers of O_2^- production also happen to be potent inhibitors of isolated phospholipase A_2 (PLA_2) from many sources: indomethacin and mefenamic acid inhibit endometrial PLA_2 (Bonney *et al.*, 1988), indomethacin, meclofenamate and ibuprofen inhibit synovial PLA_2 (Franson & Weir, 1983), indomethacin inhibits rabbit PMN PLA_2 (Kaplan *et al.*, 1978; Taniguchi *et al.*, 1988), meclofenamate inhibits rabbit PMN PLA_2 (Franson *et al.*, 1980) and sulindac inhibits rabbit PMN PLA_2 (Kaplan-Harris & Elsbach, 1980). In general, the inhibition of PLA_2 by the NSAIDs was reported to be less sensitive than that of cyclooxygenase but is very dependent on Ca^{2+} concentrations and can be detected at drug concentrations as low as nM and μM at low (sub-millimolar) concentrations of Ca^{2+}. If one takes into account the current ideas on transduction which implicate PLA_2 activation as one of the signal transduction mechanisms in the neutrophil, an *inhibition* of PLA_2 is not reconcilable with *increased* neutrophil activation. Data will be presented in Chapter 7 showing the effect of two reported PLA_2 inhibitors, chloracysine (compound I) and compound II, on stimulated O_2^- generation; both agents were found either to produce an inhibition or to have no effect but there was no recorded increases. Thus, inhibition of PLA_2 by the NSAIDs was unlikely to be the explanation for their potentiating effects on O_2^- generation.

In addition, phospholipase C (PLC) activity in human PMNs has been

demonstrated to be inhibited by indomethacin at clinically significant levels (Shakir *et al.,* 1989) and aspirin has been shown to induce a PLC inhibitory protein in human monocytes (Bomalaski *et al.,* 1986). These effects may be relevant in explaining some of the inhibitory actions of NSAIDs on neutrophil activation but not the potentiating actions.

Evidence was presented by Lukey *et al.* (1988) to show that activation of the neutrophil oxidative burst by benoxaprofen could be due to apparent direct activation of PKC. In the present study, none of the NSAIDs used alone caused any activation of O_2^- release, in contrast to the above report. This was the first report of a direct NSAID-mediated activation of a neutrophil response and was considered important in light of the proffered hypothesis to explain the mechanism of activation. It was conceivable that the NSAIDs had a potentiating effect on O_2^- release by virtue of a direct effect on PKC, acting perhaps as allosteric activators. This possibility was investigated by screening the NSAIDs through a PKC-activation assay. It was found that neither benoxaprofen nor any of the other enhancing NSAIDs had any potentiating effect on PKC activity. The difference between these results and those of Lukey *et al.* is unexplained.

The potentiating effects of indomethacin on OAG- and A23187-stimulated O_2^- generation were proposed to involve an inhibition of diacylglycerol (DAG) metabolism, thus increasing DAG levels and the resultant activation of PKC which can be a powerful stimulant for O_2^- production (Dale & Penfield, 1985). This proposal was based on the experimental findings that specific inhibitors of both DAG kinase (R59022) and DAG lipase (RHC80267) augmented the OAG-stimulated respiratory burst in a qualitatively similar manner when used separately; also when tested in combination, R59022 and RHC80267 further augmented the O_2^- response. On the other hand, only R59022, the DAG kinase inhibitor, enhanced the A23187 response (Dale & Penfield, 1987). It was thus proposed that the DAG kinase metabolizing enzyme, whose inhibition produced a consistent O_2^- increase, was the main locus of action for the potentiating effect of indomethacin; though an action on the DAG lipase or other enzymes could not be ruled out. One other report described an effect of indomethacin on the accumulation of DAG in thrombin-stimulated platelets (Rittenhouse-Simmons, 1980). This author reported that indomethacin led to inhibition of DAG lipase, in preparations of broken platelets, and concluded that the DAG accumulation in whole cells was due to the impaired metabolism of DAG by DAG

lipase.

Following on from these studies the effect of R59022, the DAG kinase inhibitor, and RHC80267, the DAG lipase inhibitor, were examined on the O_2^- response mediated by the three stimuli employed in this study, namely fluoride, diC_8 and PAF. Also two other reportedly specific inhibitors of DAG kinase, dioctanoylethylene glycol (DOEG) and monooleoylglycerol (MOG) were included in the study. These results are presented in Chapter 5. To summarize, it was found that only inhibitors of DAG kinase consistently enhanced superoxide generation activated by the post-receptor stimuli, fluoride and diC_8. In addition, the profile of effects obtained with the DAG kinase inhibitors and the "enhancing" NSAIDs on the fluoride- and diC_8-stimulated responses were very similar. Both the DAG kinase inhibitors and the enhancing NSAIDs produced a very marked leftward shift of the fluoride dose-response curve and a marked increase in the maximum control response; on the other hand the diC_8 O_2^- dose-response curve was markedly leftward shifted in the presence of the DAG kinase inhibitors and the NSAIDs, while the maximum control response remained unchanged. Also, the DAG kinase inhibitors like the NSAIDs produced no neutrophil activation in their own right in unstimulated neutrophils. These results imply, without proving, that the enhancing NSAIDs could mediate their enhancing effects on the fluoride and diC_8 responses by inhibiting the DAG metabolizing enzyme, DAG kinase, thus prolonging the DAG signal, increasing PKC activity and the resultant O_2^- response. However, this proposal is based purely on the use of "specific" inhibitors and the blind interpretation of the whole cell responses so obtained, with little insight into the biochemical "goings on" at the intracellular level. Therefore, further studies measuring intracellular DAG levels on cell stimulation in the presence and absence of drug were carried out using two different techniques, in an attempt to subject the hypothesis to more rigorous testing. Unfortunately these studies were fraught with technical difficulties and it was not possible to obtain useful concrete data. Thus, circumstantial evidence is provided that the enhancing effects of the NSAIDs involve an inhibition of the DAG kinase metabolizing enzyme and hence promote the O_2^- response through increased activation of PKC. It should be noted that results with a range of specific PKC inhibitors (presented in Chapter 5) are consistent with an important role for the DAG/PKC pathway in the transduction sequences leading to the O_2^- response induced by both receptor and

post-receptor stimuli.

Although the correlation in effects between the enhancing NSAIDs and the effects of "specific" inhibitors of the DAG kinase enzyme are closely matched and fit very well to the DAG kinase locus of action mediating the NSAID effect, they fit only the fluoride and diC_8 data. The PAF-mediated O_2^- response was unaffected by inhibiting either the DAG kinase by R59022, DAG lipase by RHC80267 or both enzymes together using R59022 and RHC80267 in combination. In fact, R59022 caused a significant inhibition of the PAF O_2^- response. It is worth commenting that this effect of the DAG kinase inhibitor, R59022, on the PAF-stimulated O_2^- response is unique in that a number of other receptor stimuli, namely IgG, heat-aggregated IgG, OZ and fMLP, exhibited a consistently enhanced superoxide response with R59022 (Muid *et al.*, 1987). A lack of effect with the DAG lipase inhibitor, RHC80267, on receptor-stimulated O_2^- production was also reported in the study with the above four stimuli. Some of the enhancing NSAIDs produced a marked potentiation of O_2^- generation stimulated with IgG, heat-aggregated IgG and OZ (Dale & Muid, unpublished observations) which could be reconcilable with a DAG kinase inhibitory mode of action.

What is the mechanism of the potentiating effect of the NSAIDs on PAF-mediated O_2^- generation? Does the lack of effect of the DAG kinase inhibitor on the PAF O_2^- response undermine the postulated mechanism of action proposed for the other stimuli? It could suggest that the enhancing effect of the NSAIDs is a complex one, consisting of many elements, of which a DAG kinase inhibitory effect could be but one. Alternatively, R59022 has been demonstrated to act as an antagonist at a number of receptors (as detailed in chapter 5) and thus could be behaving as an antagonist at the PAF receptor, masking the R59022-induced increases in intracellular DAG and the concomitant enhancement of the O_2^- response. If one takes this view, a DAG kinase inhibitory action might still account for the NSAID-mediated potentiating effect of the PAF-induced O_2^- response.

It can also be suggested that the direct O_2^- stimulatory effect of indomethacin on elicited guinea pig macrophages (Bromberg & Pick, 1983) could be explained by the above hypothesis. In macrophages there is constant and extensive remodelling of the membrane [Steinman *et al.* (1976) showed that unstimulated macrophages interiorize an area of plasma membrane equivalent to their surface area every 35 minutes]. This interiorization could involve turnover of membrane phospholipids

with the generation of DAG or alternatively elicited macrophages may spontaneously generate DAG, which on exposure to indomethacin could have its metabolism inhibited, leading to increased accumulation of DAG and the initiation of O_2^- generation.

Another tentative possibility to explain the enhancing NSAID effect might be a direct stimulatory effect of these compounds on the NADPH oxidase enzyme, the key enzyme in O_2^- radical generation. With the NSAIDs being planar hydrophobic molecules capable of intercalating or binding to hydrophobic pockets in a recipient protein, the possibility of their binding such a specific target as NADPH oxidase or any other as yet unknown oxidative burst activating protein becomes conceivable. It has been reported that direct activation of isolated human neutrophil NADPH oxidase can be initiated by AA (Curnutte, 1985; Bromberg & Pick, 1984), oleic acid (Heyneman & Vercauteren, 1984) and the detergent SDS (Cox *et al.,* 1987), provided an additional cytosolic component was also present. Controversy exists with regards to the identity of this cytosolic component, some studies showing that it could be PKC (Cox *et al.,* 1985) and other reports demonstrating the activation to be PKC-independent (Curnutte *et al.*, 1987; Cox *et al.*, 1987; Traynor *et al.*, 1989). A NSAID that can competitively inhibit AA at the cyclooxygenase active site could be considered as a potential candidate for substituting non-specifically for AA at another protein activating site, particularly when the activation by AA at this site is not exclusive for AA and can occur with other fatty acids such as oleic acid or the detergent SDS. The activation by a NSAID at this site would have to be of an allosteric nature, requiring the simultaneous presence of another activator (possibly PKC) or some other intracellular messenger, since the NSAIDs on their own do not generate O_2^-. There is at present no documented evidence for any drugs behaving in this respect, so the idea is proffered only very cautiously.

An interesting phenomenon which merits inclusion here, is the discovery of high affinity binding sites (5nM) for indomethacin on human platelets (Magous *et al.*, 1985). These authors tested a wide range of NSAIDs for their ability to compete for the [^{3}H]indomethacin binding sites. Their results, when considered alongside the present results, bear reasonable correlations with the NSAID effects on O_2^- release. At the high affinity end of the range, very close in affinity to indomethacin was diclofenac and mefenamic acid; ibuprofen and naproxen appear approximately half-way down the list (with IC_{50}s 300nM and

750nM respectively). At the low affinity end of the spectrum came phenylbutazone and aspirin, with IC_{50}s for binding > 100 000nM. The sequence of affinities tentatively corresponds with the ability of these NSAIDs to enhance stimulated O_2^- generation; it would obviously be important to look for the existence of such binding sites in the neutrophil. Could this binding site be the DAG kinase metabolizing enzyme? Could the NSAID "receptor" be NADPH oxidase, or a similar protein that might be present in platelets (as there is no evidence that these cells are capable of O_2^- production)? Whatever the explanation, it seems possible that there could be a relationship between the two sets of results. A possible solution to the whole enigma might be found if the range of NSAIDs were tested for their effects on an isolated DAG kinase preparation or were screened through an isolated NADPH oxidase assay system such as that described by Cross & Jones (1986).

4.3.2 What is the mechanism of the decreasing effect of phenylbutazone and piroxicam on the O_2^- response?

These two NSAIDs produced similar results - a markedly depressed PAF and fluoride response but very little change of the diC_8 response.

Cyclooxygenase inhibition is eliminated as a mode of action because of the lack of effect seen with aspirin. Phenylbutazone has been described as a free-radical scavenger (Shen, 1984); this property would interfere with the O_2^- detection system, "mopping up" the generated O_2^- anions before they were able to reduce the ferricytochrome C. It is doubtful that this property wholly explains the inhibitory effect of either drug on the PAF or the fluoride response because these drugs had only a limited effect on the diC_8-stimulated O_2^- response. If there is a component of free radical scavenging in the inhibition of the O_2^- response induced by PAF and fluoride, particularly in the case of phenylbutazone where there was a small decrease in O_2^- production at all diC_8 concentrations, it only partly contributes to the overall inhibition.

The binding of [^{3}H]fMLP to its receptor on human neutrophils has been found to be markedly inhibited by both phenylbutazone (Dahinden & Fehr, 1980; Nelson *et al.*, 1981) and piroxicam (Abramson *et al.*, 1984; Kaplan *et al.*, 1984; Edelson *et al.*, 1982). This receptor antagonism was found to be specific for fMLP, as no inhibition of complement-derived chemoattractant-receptor binding by phenylbutazone (Nelson *et al.*, 1981) or of [^{3}H]ConA-receptor binding by piroxicam (Edelson *et al.*, 1982; Kaplan *et al.*, 1984) was reported. Both drugs failed to inhibit the

binding of PAF to its specific receptor (Shen *et al.*, 1983). It thus appears that antagonism of receptor binding was an unlikely explanation for the NSAID-mediated decrease of the PAF O_2^- response. In addition, both phenylbutazone and piroxicam inhibited fluoride-mediated O_2^- generation, and as it is believed that fluoride activates a G-protein, thereby by-passing the receptor, it renders receptor antagonism irrelevant. Thus, it can be concluded that the action of those "decreasing" drugs is likely to be at some intracellular point.

Phenylbutazone has been described in the literature as having an inhibitory effect on the oxidative burst, with the stimulus in most of these cases being fMLP (Simchowitz *et al.*, 1979; Neal *et al.*, 1987; Perianin *et al.*, 1983; Gay *et al.*, 1984). The PMA-induced oxidative response was demonstrated to be inhibited by phenylbutazone, albeit at higher concentrations than those shown to inhibit fMLP (Perianin *et al.*, 1983; Gay *et al.*, 1984). Also Perianin *et al.* (1983) reported no change in OZ-stimulated H_2O_2 production with phenylbutazone; whereas Gay *et al.* (1984) observed an inhibition of O_2^- production induced by both OZ and fluoride. It is likely that the inhibitory effect of phenylbutazone with fMLP involves a component of receptor binding antagonism but this does not explain its effects with the other stimuli. Strauss *et al.* (1968) working with guinea pig PMNs found phenylbutazone to inhibit both uptake and killing of *E. coli*, glycolysis and hexose monophosphate shunt activity; this latter action was also observed by other groups (Dahinden & Fehr, 1980; Nelson *et al.*, 1981). Perianin *et al.* (1983), however, found that phenylbutazone did not cause inhibition of particle ingestion. The inhibition of cellular metabolism may or may not contribute a role in the phenylbutazone-mediated decreased superoxide response observed in this study with PAF and fluoride.

Piroxicam has been reported by many groups to cause an inhibition of the fMLP-mediated oxidative burst (Biemond *et al.*, 1986; Kaplan *et al.*, 1984; Edelson *et al.*, 1982; Tanaka *et al.*, 1984). Inhibitory effects were also described when piroxicam was examined for an effect on the respiratory burst induced by a number of other stimuli – ConA, zymosan-treated serum and A23187 (Tanaka *et al.*, 1984), ConA and PMA (Kaplan *et al.*, 1984; Edelson *et al.*, 1982; Abramson *et al.*, 1984) and PMA and OZ (Biemond *et al.*, 1986). Strangely, piroxicam had *no effect* on ConA- or PMA-elicited lysosomal enzyme release or PMN aggregation (Abramson *et al.*, 1984; Edelson *et al.*, 1982; Kaplan *et al.*, 1984) and *no effect* on ConA-, zymosan treated serum- or A23187-induced lysosomal enzyme

release or chemotaxis (Tanaka *et al.*, 1984). Thus it seemed that the inhibitory effect of piroxicam on cell activation was limited to superoxide anion generation. Tanaka *et al.* (1984) present evidence to show that the inhibitory effect of piroxicam on fMLP-induced O_2^- generation could be reversed by increasing the concentration of extracellular Ca^{2+} and conclude that the inhibitory effect of piroxicam may be related to its capacity for modulating the association of Ca^{2+} with these cells. Similarly, Edelson *et al.* (1982) found piroxicam to inhibit the fMLP-, PMA- and ConA-induced decrease in chlortetracycline (CTC) fluorescence. The response to Ca^{2+}-mobilizing stimuli would be to cause a decrease in CTC fluorescence, concomitant with the release of membrane-associated Ca^{2+}.

Biemond *et al.* (1986) found that piroxicam, given to patients with RA and osteoarthritis, inhibited O_2^- generation in their isolated PMNs when stimulated with OZ or PMA. Subsequent *in vitro* experiments by these authors using membrane fragments containing NADPH oxidase isolated from activated PMNs demonstrated that diminished O_2^- production was only observed if piroxicam was present during cell activation and had no effect on O_2^- generation by the isolated membrane fragments. This led to the conclusion that piroxicam inhibited granulocyte O_2^- production by blocking the activation of NADPH oxidase. This proposal seems an unlikely explanation for the current results, as no effect on diC_8-induced O_2^- generation was observed; unless the inhibitory effect of piroxicam on NADPH oxidase activation described by the above authors was upstream from PKC.

A recent study has shown that piroxicam but not salicylate can inhibit fMLP-induced DAG generation in human neutrophils (Abramson *et al.*, 1990). This would explain the inhibition by piroxicam of the PAF and fluoride O_2^- responses and the lack of effect of this drug against diC_8-stimulated O_2^- generation; also aspirin had no effect on the O_2^- response stimulated by all these stimuli. This action of piroxicam would also explain its inhibitory effect against the oxidative burst in many of the above studies.

The mechanism of inhibition of O_2^- formation in the presence of phenylbutazone and piroxicam is still not entirely clear, but could be exploited in drug therapy where these NSAIDs administered long term may result in diminished tissue destruction by oxygen free radicals in inflammatory diseases and hence may show some degree of disease-modifying action.

4.3.3 How do the drug concentrations in this study correlate with recorded levels in patients under treatment?

In order to assess the clinical implications of the data obtained here it is necessary to know the relationship between the concentrations used *in vitro* and therapeutic plasma and synovial fluid concentrations. Unfortunately, the number of studies of NSAID levels in drug-treated patients are few and far between, and are generally only limited to aspirin and indomethacin. In one such study where 8 rheumatoid arthritic patients were given a single 50mg oral dose of indomethacin, serum drug levels peaked at one hour and were of the order 1-8μM, while the peak synovial fluid concentrations, occurring one hour later, were only 25% as great (0.3-1.7μM) (Emori *et al.*, 1973). After equilibration between plasma and synovium had been established, the half-time for disappearance was about 9 hours although at this time the drug concentration in the synovial fluid was slightly higher and persisted for longer than that in the serum. Another study reported indomethacin plasma concentrations, in patients on continuous treatment, of 1.5-8.5μM at 4-5 hours immediately after the last 25mg dose of drug (Hvidberg *et al.*, 1972). These levels are of the same order and display similar kinetics to those in the previous study, but in this study only half the dose of drug was used with patients on ongoing treatment. According to the ABPI Data Sheet Compendium doses as high as 150-200mg daily are recommended for patients with RA or gouty arthritis. Thus it is conceivable that at these higher dose regimens, significantly higher serum and synovial fluid concentrations may well be achieved. Also, the extreme variation in responsiveness to drugs between patients requires flexibility in the administration of NSAIDs and has led to the doctrine that there is no such thing as a fixed anti-inflammatory dose. The current practice in NSAID therapy (according to Nuki, 1983) is to administer a series of drugs to discover the "preferred" drug for the individual patient, and then to increase the dosage until there is a satisfactory response, provided no toxicity is evident.

In determining the clinical efficacy of a drug or the side effects it is not plasma levels that are important but drug concentration in the target tissue. In RA, the main target for NSAIDs is undoubtedly the joint so the subject of synovial NSAID levels needs to be addressed. The NSAID concentration in synovial fluid is dependent upon the nature of the exchanges occurring between the synovial fluid and the plasma through the anatomical and physiological barriers comprising the

synovium and the small vessel walls. These exchanges depend upon (i) the ability of free, unbound drug to diffuse freely back and forth across the synovium and (ii) how readily the plasma proteins, mainly albumin, with highly bound NSAID (varying from 75-99% depending on the drug) can cross the microvessel walls. Both of these factors can vary during inflammation. If the synovial fluid pH is reduced, as it is during inflammation, the acidic NSAIDs exist mainly in the undissociated form which are more lipophilic and diffuse more easily across the synovium and thus these drugs can become concentrated at inflammatory sites. This was shown in rat carageenin-induced inflammation where the concentrations of indomethacin, phenylbutazone and aspirin were three-, two- and one half-fold greater respectively, in the inflamed paw compared to control (Brune *et al.,* 1980). Quantitative changes in albumin levels have been described in rheumatoid patients, where the albumin content of both the intravascular and extravascular pools was shown to be decreased, due to increased catabolism (Ballantyne *et al.,* 1971). Reduced binding of drug to albumin increases the free unbound drug levels. Clinical studies comparing plasma and synovial fluid pharmacokinetics (reviewed in Famaey, 1987), in general, reveal that the time required to reach the peak synovial concentration is longer and the peak concentration considerably lower (40-80%) than that relating to peak plasma concentration. However, the disappearance time is much longer in the synovial fluid than in the plasma, so that once equilibration is established the synovial fluid concentration quickly exceeds the plasma concentration. It is also suggested that reduced protein-binding could give rise to a greater free, diffusible fraction and thus a greater active drug fraction in the synovium than in the plasma.

Another point to be noted is that both benzoic acid-like drugs (Raghoebar *et al.,* 1988) and indomethacin (Raghoebar *et al.,* 1989) have been shown to associate with human PMNs *in vitro,* and that environmental factors such as pH, lysed cells, inflammatory stimuli and metabolites may determine the degree of association *in vivo.* Also, it has been demonstrated that the concentration of NSAIDs in canine knee cartilage removed and studied ex *vivo* exceeded that in the surrounding medium (Palmoski & Brandt, 1985). These findings indicate that NSAIDs may accumulate in inflammatory cells and articular tissue.

Because of the many variables affecting the local concentration of NSAIDs in synovial fluid and the many more variables determining the drug and dosage pattern in individual patients it is near impossible to

predict the order of drug levels in the joint space, without good clinical drug measurement studies which are at present sadly lacking. However, it is clear from the above discussion that there is a complex interplay of factors, some associated with the inflammatory condition itself, that may serve to raise synovial fluid drug levels above that normally expected.

4.3.4 What are the clinical implications of the present study in the context of NSAID therapy?

For the purposes of this discussion NSAID levels in the synovium will be taken, as mentioned above, in the low μM range. Although the enhancing effects of the NSAIDs on O_2^- production presented in this study were at the higher concentration of 100μM, this concentration was selected in order to produce as definite an effect as possible for subsequent analysis. The potentiating effect described particularly for the more powerful enhancing NSAIDs (i.e. meclofenamate, mefenamic acid, benoxaprofen), was found to be dose-related over the range 1-100μM, although the enhancement seen at the lower end of this concentration range was only marginal. However, the potentiation by the potent "enhancers" was consistently present at the lower μM concentrations. When this slight enhancement is viewed in the context of the work by Lunec *et al.* (1985), a clinical relevance for this phenomenon becomes, at the very least, a possibility. These authors describe a mechanism that would give rise to self-replicating cycles of both O_2^- production and lymphocyte activation (see Chapter 1), thus triggering the kind of auto-immune reactions believed to underlie chronic inflammatory conditions such as RA. Even a very small enhancement of O_2^- generation (such as that seen at clinically relevant NSAID concentrations) with each successive activating cycle, could eventually give rise to a substantial amplified response and also an incremental increase in the accompanying tissue damage associated with the production of these toxic oxygen radicals (reviewed in Chapter 1). The IgG aggregates, which are formed in the presence of O_2^-, have a characteristic autofluorescence and aggregates with identical autofluorescence have been isolated from the serum and synovial fluid of rheumatoid patients.

Taking all these factors into account it is not inconceivable that treatment of rheumatoid arthritic patients with certain NSAIDs, particularly the more potent "enhancers", could lead to an exacerbation of the underlying disease process, despite temporary symptomatic relief. In this study *in vitro* experimental evidence was provided demonstrating

an enhancing effect by some NSAIDs of toxic oxygen radical generation induced by PAF – a clinically relevant stimulus. To test the relevance of such observations *in vivo*, a carefully controlled clinical study would have to be undertaken with the help of clinicians, where the PMN O_2^- response would be compared between two populations of patients, one population taking a non-enhancing NSAID, like aspirin, and the other taking one of the enhancing kind, like mefenamic acid. The stimulated O_2^- responses of PMNs from these patients would first have to be measured during a drug-free period, to establish a baseline, because of the large degree of inter-individual variation. This kind of study would assess whether similar drug-induced increases in the levels of stimulated O_2^- production occur *in vivo*, and would thus enable a definite statement on the clinical implications of our results to be reported. If our results were to be confirmed in patients, it would mean that clinicians may need to be more selective in the NSAIDs prescribed for treatment of chronic inflammatory conditions; those drugs that did not increase O_2^- production may constitute safe drugs useful in symptomatic treatment or preferably, those shown to decrease the response could provide some degree of disease-modifying action.

4.3.5 Cyclooxygenase-dependent pro-inflammatory effects of NSAIDs

Some NSAIDs which may give rise to debilitating side effects, mediated by a cyclooxygenase-independent mechanism, have been discussed above. However, the reduction of prostanoid synthesis itself may have some pro-inflammatory effects. Stable PGs are generally considered to be anti-inflammatory, inhibiting not only the activation of inflammatory cells *in vitro* (Zurier *et al.*, 1974; Henney *et al.*, 1971; Lehmeyer & Johnston, 1978) but also ameliorating experimental adjuvant arthritis (Zurier & Quagliata, 1971) and immune-complex nephritis *in vivo*. The effect of PGs on inflammatory cells has been demonstrated, as mentioned above, to be mediated by increased cyclic AMP levels. PGE_2 has been shown to suppress LTC_4-induced β-glucuronidase release from rat peritoneal macrophages while cyclooxygenase inhibitors promote the release (Schenkelaars & Bonta, 1986). Furthermore, E-type PGs derived from macrophages have been shown to modify the secretion of lymphokines by lymphocytes in response to antigen (Gordon *et al.*, 1976) and may provide a negative-feedback mechanism for regulating the extent and duration of cellular immune reactions. Thus, inhibition of the modulatory effect of PGs could give rise to increased inflammatory

effects. In one study, the enhancement of lymphocyte-mediated effects brought about by NSAIDs has been shown to be the result of inhibition of a prostaglandin negative-feedback mechanism (Lewis & Barrett, 1986). These authors discuss the possibility that NSAIDs, administered in large doses to patients suffering slight joint pain, might well exacerbate, perpetuate or even initiate a chronic arthritic condition.

There is also an indication that NSAIDs can accelerate the progression of osteoarthritis by reducing synthesis of the vasodilator PGs, thereby diminishing joint perfusion (Rashad *et al.*, 1989). In a controlled study, two treatment groups were administered with a strong (indomethacin) and a weak (azopropazone) prostaglandin synthesis inhibitor and it was revealed that the indomethacin group took a shorter time to reach the arthroplasty end-point and had lower concentrations of synovial PGs than the azopropazone group. In another recent report, the effects of indomethacin on antigen-induced arthritis in rabbits was investigated (Pettipher *et al.*, 1989). Although indomethacin reduced joint swelling and PGE_2 concentrations in synovial fluid, it was also shown to increase the loss of proteoglycan from articular cartilage and the number of lymphocytes in the inflamed synovial lining.

In conclusion, NSAIDs may have significant pro-inflammatory effects by inhibiting the beneficiary role of PGs in modulating inflammation as well as, possibly, potentiating toxic oxygen radical-mediated tissue injury, by a cyclooxygenase-independent mechanism. The symptomatic benefits of these drugs may be achieved at the expense of significant adverse effects on joint tissues, and may even call into question their widespread usage in the arthritic condition.

CHAPTER FIVE

THE DIACYLGLYCEROL/PROTEIN KINASE C PATHWAY

SUMMARY:

— The role of the diacylglycerol (DAG)/protein kinase C (PKC) pathway in the signal transduction sequence(s) leading to respiratory burst activation was assessed using the PKC inhibitors, K252a and staurosporine, which are reported to be potent for PKC but not selective, and a series of novel bis-indolyl maleimide compounds, which potently inhibit PKC (rat brain and human PMN) with a high degree of selectivity over both cyclic AMP-dependent protein kinase and Ca^{2+}/calmodulin-dependent kinase.

— K252a and staurosporine markedly inhibited the respiratory burst induced by PMA, diC_8, fluoride, fMLP, PAF and A23187, but the order of stimulus sensitivity to inhibition was very different with the two inhibitors. Staurosporine at low concentrations increased the fMLP- and PAF-stimulated O_2^- responses.

— The bis-indolyl maleimide compounds potently inhibited respiratory burst activity stimulated by diC_8, fluoride and fMLP.

— These findings support a role for PKC in stimulus-activation coupling for the respiratory burst induced by direct PKC activation, G-protein activation, receptor stimulation or by raising intracellular Ca^{2+}.

— Inhibitors of the DAG metabolizing enzyme, DAG kinase (R59022 & DOEG) consistently enhanced superoxide generation activated by the post-receptor stimuli - fluoride and diC_8. An inhibitor of DAG lipase (RHC80267) had no consistent effect. These results reinforce previous findings (Muid *et al.*, 1987) and may indicate that both exogenous diC_8 and DAG generated endogenously are metabolized by DAG kinase and thus converted to phosphatidate. This is further evidence supporting a role for PKC in the activation of the respiratory burst. However, inhibition of DAG metabolism failed to enhance PAF-induced O_2^- generation and this unique finding is discussed in relation to PAF stimulation.

5.1 INTRODUCTION

Protein kinase C (PKC) is a ubiquitous serine and threonine specific protein kinase found in several cells, tissues and organs (Nishizuka, 1984a). It requires both calcium and phospholipid for activity and has also been termed a phospholipid- and Ca^{2+}-dependent protein kinase. The kinase is activated by 1,2-diacylglycerol (DAG) transiently generated as a consequence of receptor-mediated hydrolysis of inositol-containing phospholipids by phosphodiesterase (phospholipase C (PLC)) attack, and/or by DAG generated from other phospholipids (e.g. phosphatidylcholine (PC)) by other enzymes (e.g. PC-dependent PLC or PLD). It is generally accepted that DAG stabilizes the quaternary complex between the enzyme, Ca^{2+} and membrane phospholipid. In addition to DAG the physiological stimulus for PKC, synthetic DAGs, such as 1-oleoyl, 2-acetylglycerol (OAG), 1,2-dioctanoylglycerol (diC_8) and 1,2-didecanoyl-glycerol (diC_{10}) are able to activate PKC in intact cells (Go *et al.*, 1987): arachidonate (AA) can also stimulate PKC (Hansson *et al.*, 1986). It should be noted that PKC is the intracellular target receptor for the tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (reviewed by Nishizuka, 1986).

The 80-82kDa protein kinase C from rat brain can be degraded by trypsin into 50 and 32kDa fragments (Lee & Bell, 1986). The 50kDa fragment, the catalytic domain, can be further degraded by trypsin whereas the 32kDa fragment cannot. [^{3}H]Phorbol dibutyrate (PDBu) binds to the 32kDa fragment and the binding is dependent on Ca^{2+} and phosphatidylserine (PS) and is of high affinity (Kd = 2.8nM). This smaller fragment represents the lipid binding, regulatory domain of PKC (Lee & Bell, 1986). PKC from a PMN source has also been isolated and characterized (Smith R.J. *et al.*, 1987).

It's now clear that there is more than one species of PKC molecule, and several discrete isoenzymes have been defined. These isoenzyme species show subtle differences in their mode of activation, sensitivity to Ca^{2+} and catalytic activity towards endogenous substrates (reviewed in Kikkawa *et al.*, 1989). The PKC family consists of six unique genes that give rise to at least seven polypeptides (Parker *et al.*, 1989), referred to as PKC-α, -βI, -βII, -γ, -δ, -ε and -ζ. Biochemical and immunocytochemical studies with isoenzyme-specific antibodies suggest that the PKC subspecies may be differently located in particular cell types and at limited intracellular locations, and may indicate that each isoenzyme has a unique role in signal transduction. Indeed two

isoenzymes of PKC found in HL-60 cells showed different sensitivities to activation by PMA (Beh *et al.*, 1989), and a PKC-ε isolated from murine brain showed a distinct substrate specificity and its activation was independent of Ca^{2+} (Schaap *et al.*, 1989).

In neutrophils, as in most other cells, the intracellular distribution of PKC is to some extent dependent on the state of activation of the cell. PKC was found to be localized mainly in the cytosol in unstimulated cells whereas significant translocation to fractions containing the plasma membrane was observable after stimulation (Wolfson *et al.*, 1985; Wolf *et al.*, 1985). In neutrophils, it is not clear if stimulation by chemoattractants requires the translocation of PKC from the cytosol to the plasma membrane (Cochet *et al.*, 1986). PKC translocation was accompanied by that of a calcium-activated neutral proteinase (CANP) which promotes proteolytic conversion of PKC in the presence of Ca^{2+} (Melloni *et al.*, 1985; Suzuki *et al.*, 1987). Activated PKC is then released to the cytosol which is fully active in the absence of Ca^{2+} and phospholipids (Nishizuka, 1986; Parker *et al.*, 1986). Once released from the membrane the active subunit of PKC reaches most of the other cell compartments, where phosphorylation of target substrates can occur. The physiological significance of the Ca^{2+}/phospholipid-independent protein kinase has not yet been fully established.

It is now well recognized that the synergistic interaction between PKC and Ca^{2+} pathways underlies a variety of cell responses (Nishizuka, 1984a; Berridge & Irvine, 1984), including the PMN respiratory burst (Robinson *et al.*, 1984; Dale & Penfield, 1984). A large body of evidence has accumulated to indicate that PKC has a dual action, providing positive-forward control as well as negative-feedback control over various steps of cell signalling processes (Nishizuka, 1986; Kikkawa & Nishizuka, 1986; Nishizuka, 1988). The positive-forward action of PKC seems to be important in mediating particular cell responses, while the negative-feedback mechanism involves the termination of the activating signal. A number of reports have suggested that in various cell types, PKC can activate the Ca^{2+}-transport ATPase and the Na^{+}/Ca^{2+} exchange protein both of which remove Ca^{2+} from the cytosol (Nishizuka, 1986). PKC can also inhibit the receptor-mediated hydrolysis of inositol phospholipids, thereby blocking the activation of the Ca^{2+}-signalling pathway (reviewed in Williamson & Hansen, 1987). The dual action of PKC provides a versatile regulatory system that is finely tuned by the transient generation of second messengers such as DAG. Furthermore,

the persistent action of PMA which prolongs the association of PKC with the membrane, can initiate the degradation of the PKC enzyme and its sustained disappearance from the cell, often referred to as down-regulation. Another level of regulatory complexity could involve the phenomenon of PKC-autophosphorylation (Huang *et al.*, 1986). It is not known whether the phosphorylation could be attributed to the action of one isoenzyme on itself or on another isoform to modulate *its* action.

5.1.1 The role of PKC in the respiratory burst

The role of PKC in mediating the activation of NADPH oxidase is addressed in this chapter. It has been shown that virtually all of the stimuli which cause O_2^- generation from PMNs can cause hydrolysis of the phosphoinositides which would result in the generation of DAG, the endogenous activator of PKC, and IP_3, which mediates the mobilization of intracellular Ca^{2+} (reviewed in Chapter 3 under the individual stimuli). The experimental evidence in favour of an indirect role for PKC in the neutrophil O_2^- response is overwhelming. However, a direct correlation between PKC activation and O_2^- generation under stimulatory conditions has not yet been clearly established. The available information will be discussed here. The involvement of Ca^{2+} in the respiratory burst will be reviewed in Chapter 6.

Activation of the respiratory burst by phorbol esters and exogenous DAGs implicates PKC directly in at least one mode of activation. Phorbol esters that activate PKC are known to release large quantities of O_2^- - more effectively than any other agents. For a series of phorbol esters, O_2^- generation from neutrophils paralleled activation of the isolated kinase (Robinson *et al.*, 1985); the activation by PDBu showed the same concentration dependence as that for binding of [^{3}H]PDBu to its cellular receptor, presumably PKC (Tauber *et al.*, 1982) and synthetic DAGs that stimulated O_2^- production competed for essentially all the [^{3}H]PDBu receptor binding sites (Cox *et al.*, 1986). Furthermore, dormant membrane fractions containing NADPH oxidase were activated by PMA, in the presence of cytosol or purified PKC plus factors required to activate PKC (including PS, Ca^{2+}, ATP), to generate O_2^- (Cox *et al.*, 1985). Another study demonstrated that the catalytic fragment of PKC activated resting membrane-associated NADPH oxidase in the presence of PS, ATP and Mg^{2+}; in this system neither PMA nor Ca^{2+} were required (Tauber *et al.*, 1989). Also, both PMA and synthetic DAGs, including OAG, can synergize with the calcium ionophore, A23187, to generate O_2^- (Dale &

Penfield, 1984; Penfield & Dale, 1984; Robinson *et al.*, 1984).

Although there seems to be little doubt that PKC mediates the respiratory burst in response to phorbol esters and exogenous DAGs, there is debate as regards its role when other agonists are used. The experimental approaches to this question are diverse and will be briefly outlined.

Attention is focussed on the incremental increase in intracellular DAG which follows cellular activation, using either mass measurement of endogenous DAGs or monitoring an increase in radiolabelled DAG from prelabelled cells. Using mass measurement a rapid appearance of DAG was detected following stimulation by a number of agonists, where large sustained DAG increases (260-2000% of basal levels) occurred in parallel with the generation of superoxide anion (Rider & Niedel, 1987; Burnham *et al.*, 1989). Cytochalasin B which augments the rate and extent of fMLP-activated O_2^- release caused a corresponding increase in the magnitude and duration of fMLP-stimulated DAG generation (Honeycutt & Niedel, 1986). Interestingly, a recent study has reported that fMLP induces an initial rise in DAG and $[Ca^{2+}]_i$, followed by a second sustained rise in DAG which was suggested to be triggered by an fMLP-induced Ca^{2+} influx (Truett *et al.*, 1988). However, in some of these studies an early increment of DAG within the first few seconds after fMLP stimulation, with kinetics identical to those for IP_3 formation, was not detected. It was concluded that the early DAG increment that *precedes* the O_2^- response is of very small magnitude compared to the large subsequent increase in DAG mass, but may be sufficient to initiate O_2^- production.

In another study, Preiss *et al.* (1987) have shown that fMLP-activated HL-60 cells cause DAG generation which begins 30 seconds after stimulation, peaks at 4 minutes and is still evident at 15 minutes. The authors suggest that the source of DAG is unlikely to be PIP_2 as IP_3 production demonstrates no lag period and is complete by 30 seconds (Dougherty *et al.*, 1984); it was proposed that PI may serve as the phospholipid source. This study also makes the point that fMLP-stimulated O_2^- production begins after a 15-30 second lag, reaches maximal rate at 1 minute and shuts off at 3-5 minutes; thus it is the termination of O_2^- production that coincides with the 4 minute DAG peak. It was reported by another group that, under certain activating conditions, there was no correlation between DAG accumulation and respiratory burst activation (Koenderman *et al.*, 1989). The observations that led to

the latter conclusion were: 1) both PAF and fMLP produced a comparable rise in DAG levels but only fMLP was able to induce O_2 uptake, indicative of respiratory burst activation 2) Opsonized zymosan (OZ)-stimulated respiratory burst activity reached 70% of its maximal value at 1 minute, whereas at this time no net accumulation of DAG was detected; after 1 minute DAG levels began to rise until a 3-fold increase was obtained at 5 minutes. These discrepancies have not yet been clarified or any indications given as to which second messengers other than DAG may be responsible for the NADPH oxidase activation under the above circumstances. (in [3H]arachidonate or [14C]glycerol prelabelled cells)

Using the labelling technique, it was shown that both fMLP and ConA triggered a rapid biphasic increase in levels of labelled DAG which correlated with ligand-induced O_2^- generation (Korchak *et al.*, 1988a), even though Ca^{2+} and phosphatidate (PA) changes were also detected in parallel.

Evidence which is consistent with a mediator role for DAG and hence PKC activation, comes from those studies where the DAG kinase metabolizing enzyme, DAG kinase, was inhibited (by R59022), thus increasing DAG levels, and an enhanced O_2^- response was observed with a number of receptor stimuli (Muid *et al.*, 1987; Gomez-Cambronero *et al.*, 1987).

Assays of PKC activity in intact neutrophils have either focused on intracellular phosphorylation of specific proteins or on the translocation of PKC from the cytosol to the plasma membrane (measured by using subcellular fractionation techniques), both of which have been taken as indirect evidence for PKC activation. The former phosphorylation approach has revealed much data (discussed in Chapter 1) but the findings show inconsistencies and the technique has disadvantages (reviewed in Tauber, 1987). In addition, phosphorylation of cellular proteins is only indicative of protein kinase activation, and does not specifically implicate PKC.

Translocation data revealed that phorbol esters caused a rapid migration of PKC (within seconds) to the plasma membrane in neutrophils or cytoplasts that correlated with O_2^- generation (Christiansen, 1988; Gennaro *et al.*, 1986). Exogenous DAGs and Ca^{2+} ionophores also caused translocation (Nishihira *et al.*, 1986; Christiansen, 1988), as did fluoride – the delayed time course of which paralleled the delayed activation of the O_2^- response (Strnad *et al.*, 1986). OZ and bacteria have also been shown to cause translocation (Christiansen *et al.*, 1987, 1988). In contrast to these reports, no mobilization of the kinase enzyme was

detected in PMNs challenged with ConA or PAF, and although C5a and fMLP produced a translocation in cytochalasin B-pretreated cells, none was detected in cells that were not so pretreated (Nishihira *et al.*, 1986; Pike *et al.*, 1986; Christiansen, 1988). However, as these latter stimuli are weak activators of the respiratory burst it may have proved difficult to observe the appearance of the membrane-associated kinase, or else the kinase may have been immediately degraded by CANP in the presence of Ca^{2+}. In any case the translocation assay was not proving fruitful as a key indicator of PKC participation in the O_2^- response, particularly as another study reported a lack of correlation between biological response and PKC translocation for a series of PMA-mediated events in a number of cell types (Bosca *et al.*, 1989).

There still exists a lot of controversy as to the participation of PKC in receptor-stimulated O_2^- generation as a result of a number of PKC inhibitor studies. Some compounds (chlorpromazine, trifluoperazine and verapamil) which were originally characterized as inhibitors of Ca^{2+}-linked pathways, but which also inhibit PKC, were shown to block the fMLP- and PMA-activated respiratory burst (Robinson *et al.*, 1985; Korchak *et al.*, 1984). Since then a plethora of reportedly "specific" inhibitors of PKC have been tested in the respiratory burst of human neutrophils, giving rise to widely divergent effects - some of which can be attributable to the side-effects (i.e. PKC-independent effects) demonstrated by these compounds. For example PKC inhibitors, such as H-7 and C-I, which competitively inhibit ATP binding to PKC, inhibited the respiratory burst stimulated by PMA but had no effect if fMLP or C5a was the stimulus (Wright & Hoffman, 1986; Gerard *et al.*, 1986). In contrast, the long-chain sphingoid bases which were demonstrated to competitively inhibit the binding of DAG and phorbol ester to PKC, blocked activation of the respiratory burst by all stimuli tested - PMA, PAF, fMLP, OZ and A23187 (Wilson *et al.*, 1986; Lambeth *et al.*, 1988). The problem of the anomalies with the PKC inhibitory studies will be broached again more extensively in the discussion. Suffice to say here, that there are some apparently contradictory data which remains to be reconciled.

The role of the DAG/PKC pathway was addressed in this study using two different approaches. Firstly, the effects of inhibitors of the DAG metabolizing enzymes were tested for their effects on the respiratory burst in an attempt to ascertain the involvement of PKC and also the

importance of DAG-stimulated PKC activity in this response. Secondly, the effects of a new series of compounds, developed as specific PKC inhibitors, were examined on the stimulated O_2^- response by a large range of stimuli. Clearly, it is of importance to determine whether the DAG/PKC pathway is involved in the transduction mechanism for O_2^- generation.

5.1.2 Inhibition of the DAG metabolizing routes

There is a large body of evidence demonstrating that DAG is generated on receptor activation of the neutrophil (see above). Clearly, there must be mechanisms for terminating the action of this potent intracellular messenger. The main mechanisms are thought to be phosphorylation by DAG kinase (to give PA) and deacylation by DAG lipase (fig. 5.1). Certainly there is evidence that DAG kinase is in fact translocated from the cytosol to the membrane after stimulation of the neutrophil with chemotactic peptide (Ishitoya *et al.*, 1987; Besterman *et al.*, 1986b). It has previously been suggested that the DAG formed after receptor stimulation is metabolized by the kinase rather than the lipase route (Muid *et al.*, 1987; Mege *et al.*, 1988), the receptor stimulants used being fMLP, IgG, heat-aggregated IgG and OZ. It has also been suggested that A23187-stimulated DAG generation is handled mainly by the kinase enzyme whereas the synthetic DAG, OAG, is metabolized by both the DAG kinase and DAG lipase pathways (Dale & Penfield, 1987). These suggestions were based on the use of two compounds: R59022, which is reported to inhibit DAG kinase in intact platelets, and to lead to increased PKC activity, the IC_{50} value for DAG kinase being 3.8μM, with 80% inhibition of the enzyme being obtained at 10μM (De Chaffoy de Courcelles *et al.*, 1985), and RHC80267 which was reported to be a potent and selective inhibitor of DAG lipase in platelets, with an IC_{50} value of 4μM (Sutherland & Amin, 1982).

The DAG kinase inhibitor, R59022, potentiated secretion and aggregation responses in human platelets challenged with thrombin (Nunn & Watson, 1987) and serotonin secretion induced by both thrombin and OAG (Tohmatsu *et al.*, 1987). The potentiation was correlated with increased formation of DAG, increased phosphorylation of a 40kDa protein (substrate for PKC) and decreased formation of PA. The mobilization of Ca^{2+} was either unaffected (Nunn & Watson, 1987) or decreased (Tohmatsu *et al.*, 1987) by R59022 and the formation of inositol phosphates was unaffected. These data were proffered to support a

role for the DAG/PKC pathway in platelet aggregation and secretion.

In the present study the investigation of a role for DAG in the respiratory burst was further extended to include the stimuli – fluoride, PAF and diC_8. The effect of R59022 and RHC90267, the DAG kinase and DAG lipase inhibitors respectively, was tested on stimulated O_2^- generation. There are reports highlighting some non-specific effects of R59022 (described in the discussion), consequently other available DAG kinase inhibitors were also included in the study, namely dioctanoylethylene glycol (DOEG) and monooleoylglycerol (MOG), for comparison with R59022. DOEG and MOG were found to be potent inhibitors of partially purified DAG kinase from pig brain with IC_{50}s 59μM and 91μM respectively (Bishop *et al.*, 1986; Bishop & Bell, 1986). In the latter study, DOEG inhibited DAG phosphorylation in thrombin-stimulated human platelets (70-100% at 100μM), leading to a prolonged DAG signal. MOG treatment (400μM) elevated DAG levels up to 10-fold in thrombin-stimulated platelets, although it was postulated that part of this effect may be due to inhibition of DAG lipase.

However, it should be stressed that there is controversy over the specificity of R59022 and DOEG for the DAG kinase; this is considered in detail in the discussion of the results.

5.1.3 Inhibition of PKC by specific inhibitors

We chose to address the question of the role of PKC in the respiratory burst using two new compounds which were reported to be potent inhibitors of this enzyme - namely the microbial alkaloids, K252a (Kase *et al.*, 1987) and staurosporine (Tamaoki *et al.*, 1986). K252a and staurosporine have been reported to act by competitively inhibiting ATP substrate binding to the enzyme. The IC_{50} values against isolated PKC enzyme for both these compounds varies somewhat depending on the source of the PKC, the method of isolation, the assay procedure for PKC activity and the concentration of ATP employed in this assay, so the available data is summarized in Table 5.1. Both K252a and staurosporine were tested for their effects on O_2^- production induced by six different burst activators - PMA, diC_8, A23187, fMLP, PAF and fluoride.

Although both K252a and staurosporine are potent PKC inhibitors they are not specific for PKC; they both have effects on other protein kinases. K252a is almost equipotent for PKC and both cyclic AMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent kinase, whereas staurosporine has some degree of selectivity for PKC over the

latter two kinases (see Table 5.1). The lack of selectivity of both these compounds, exemplified by a number of actions on other transduction enzymes, is summarized in Table 5.4 (in discussion) and reviewed in Ruegg & Burgess (1989).

Fortuitously, Roche Products produced a novel series of potent PKC inhibitors, namely bis-indolyl maleimides, which are related structurally to K252a and staurosporine (Davis *et al.*, 1989). These novel PKC inhibitors, totalling six in all, were shown to potently inhibit isolated PKC enzyme from both rat brain and human PMN (IC_{50} data given in Table 5.1). In fact the compounds showed very similar potency against the PKC isolated from either source. These compounds bind to PKC competitively with ATP, a property shared with K252a and staurosporine. An analogue of the above inhibitors, compound Ro31-6045, possessed no PKC inhibitory activity (see Table 5.1) and was used throughout the study as a negative control. The Roche compounds displayed a much lower potency against both isolated PKA (from bovine heart and human PMN) and Ca^{2+}/calmodulin-dependent protein kinase (from rat brain) (Davis *et al.*, 1989). Thus these compounds appeared to be both potent and selective inhibitors for PKC and in addition have been shown to block enzyme activity in an intracellular environment, namely inhibition of the PMA-induced phosphorylation of a 47kDa protein in intact human platelets (Davis *et al.*, 1989).

The bis-indolyl maleimide compounds appeared to be valuable tools in assessing the physiological importance of PKC in the human neutrophil respiratory burst, and were tested for their effects on the stimulated O_2^- response induced by diC_8 and fluoride. Also included for comparison is the effect of the Roche compounds on the fMLP-stimulated O_2^- response.

TABLE 5.1

Inhibition IC_{50} values (μM) against isolated protein kinase

	Rat brain PKC	Bovine heart PKA	Human PMN PKC	Human PKA	Ca/CAM PK
K252a	0.47[a] 0.025[b]	0.20[a]	0.27[e] 0.58[d]	0.16[e]	0.30[a]
Stauro	0.01[a] 0.003[c]	0.12[a]	0.002[e]	0.02[e]	0.04[a]
Ro31-7549	0.08[a]	5.1[a]	0.05[e]	4.20[e]	15[a]
Ro31-8161	0.03[a]	3.3[a]	0.02[e]	ND	14[a]
Ro31-8220	0.01[a]	1.5[a]	0.008[e]	ND	17[a]
Ro31-8425	0.008[f]	3.6[f]	ND	ND	19[f]
Ro31-8288	0.008[f]	7.4[f]	ND	ND	>100[f]
Ro31-8657	0.047[f]	95[f]	ND	ND	40[f]
Ro31-6045	>100[e]	100[e]	ND	ND	>100[e]

ND = not determined

a. Davis *et al.* (1989)
b. Kase *et al.* (1987)
c. Tamaoki *et al.* (1986)
d. Smith, R.J. *et al.* (1988).
f. Nixon *et al.* (unpublished observation)
e. Twomey *et al.* (1990c)

5.2 RESULTS

5.2.1 The effect of DAG kinase inhibitors (R59022, DOEG and MOG) and a DAG lipase inhibitor (RHC80267) on the stimulated respiratory burst

(i) Fluoride

DOEG, the DAG kinase inhibitor, at 10μM and 100μM, caused a marked dose-related left-shift of the fluoride concentration-response curve and also increased the mean maximum response (n = 3), 10μM increasing it by 25% and 100μM increasing it by more than 60% (fig. 5.2a). The increase in the fluoride response produced by 10μM DOEG, was statistically significant at the level $p < 0.05$ for all concentrations above that giving a threshold response. DOEG at 300μM had very interesting effects; it either produced a total inhibition, obliterating the fluoride concentration-response curve (result not shown), or it produced a massive increase of the fluoride O_2^- response at low fluoride concentrations, which turned to inhibition (even below control values) approaching higher fluoride concentrations. A single representative normalized experiment is plotted in fig. 5.3, comparing the effect of 300μM and 100μM DOEG on the fluoride O_2^- response. Incidentally, 300μM DOEG was shown to be non-toxic by the trypan blue exclusion method.

MOG proved to be a very unstable compound, losing activity on exposure to both light and air, so it was not extensively used in this study. It was found that both 10μM and 100μM MOG produced a substantial enhancement of the control fluoride response, given in Table 5.2.

TABLE 5.2

Effect of MOG on fluoride-stimulated O_2^- release from human neutrophils

	% Maximum Control (n = 2)		
Fluoride mM	Control	10μM MOG	100μM MOG
10	3.1	14.8	43.3
14	31.0	55.8	92.5
18	68.2	86.3	108.6
22	86.2	100.6	113.6
26	100.0	107.1	116.0

In 7 experiments, R59022 (10μM) - the DAG kinase inhibitor, markedly left-shifted the fluoride concentration-response curve, and markedly increased the mean maximum response (fig. 5.2b). The increase produced by R59022 was statistically significant at $p < 0.01$ at all concentrations of fluoride tested, except that giving a threshold response.

The DAG lipase inhibitor, RHC80267 (10μM), had variable effects on fluoride-stimulated O_2^- production. Out of a total of 6 experiments, 3 experiments showed a marked left-shift in the presence of this compound while in 3 others there was no significant effect. The mean concentration-effect curve showed a difference at the upper end of the curve which was statistically significant at the indicated p values (fig. 5.2b).

(ii) $\underline{DiC_8}$

DOEG, at 100μM, consistently caused a marked left-shift of the diC_8 concentration-effect curve, with little change in the maximum ($n = 6$). A representative experiment is presented in fig. 5.4a, as there was quite a lot of variation in the control curve between experiments which masked the extent of the DOEG enhancing effect. The other DAG kinase inhibitor, R59022 at 10μM, also caused a marked left-shift of the curve with no increase in the maximum response (n = 4) and is presented in fig. 5.4b.

The DAG lipase inhibitor, RHC80267 at 10μM, had no consistent effect. In 3 out of 4 experiments there was a slight left-shift of the curve in the presence of RHC80267, but the mean curve was not significantly different from the control curve, as is presented in fig. 5.4b.

(iii) PAF

The effect of both R59022 and RHC80267 on the PAF-stimulated oxidative response was somewhat surprising in that neither compound caused an enhanced O_2^- response. In 8 experiments, R59022 (10μM) not only failed to increase the response but caused a pronounced inhibition of the PAF concentration-response curve that was statistically significant at $p < 0.05$ for all concentrations of PAF, excepting the maximum. The mean PAF concentration-response curve in the presence and absence of R59022 is shown in fig. 5.5a. RHC80267 (10μM), the DAG lipase inhibitor, also caused a reduction of the PAF-induced O_2^- response, albeit much less pronounced than that of R59022; the differences were not statistically significant. The effect of RHC80267 on the

PAF dose-response curve is given in fig. 5.5b (n = 3).

5.2.2 The effect of the microbial products, K252a and staurosporine, on the stimulated respiratory burst

Both K252a and staurosporine inhibited the respiratory burst stimulated with the five stimuli – PMA, diC_8, fluoride, fMLP and PAF. A range of inhibitor concentrations were tested for an effect on two concentrations of stimulus (selected from those that produced between 20 and 100% response on the O_2^- dose-response curve), the results normalized, the means with standard errors from several experiments were calculated from the normalized data and graphically displayed as dose-inhibition curves. For each drug-stimulus combination an IC_{50} value ± % error was obtained by using Graphpad non-linear regression to fit a sigmoidal curve to the dose-inhibition data. The IC_{50} values were identical or very similar whether they were calculated at high or low stimulus concentration, so the high concentration was selected for all IC_{50} determinations. The IC_{50} values obtained for K252a and staurosporine with the range of stimuli are presented in Table 5.3, where they are compared with those obtained with the novel Roche PKC inhibitors.

The order of susceptibility to inhibition by K252a and staurosporine was found to be very different for the five stimuli examined, as can be seen from the IC_{50} data and will be addressed in the discussion.

Staurosporine caused dose-dependent inhibition of O_2^- generation induced by the post-receptor stimuli PMA, diC_8 and fluoride, as represented by their dose-inhibition curves in fig. 5.6a, b & c respectively. Interestingly, for the three post-receptor stimuli the IC_{50} values in the respiratory burst are quite close to the values deduced against the isolated enzyme (Table 5.1 and Table 5.3). Staurosporine was clearly more potent in inhibiting the fluoride-stimulated than either the PMA- or diC_8-stimulated respiratory burst. When this inhibitor was examined for its effect on the full dose-response curves of PMA and fluoride, it caused a dextral shift and a reduction in maximum in both cases; no parallel shifts were observed, (fig. 5.7a & c). In the case of diC_8, staurosporine caused a dextral parallel shift of the dose-response curve with near restoration of the maximum response (fig. 5.7b).

Staurosporine inhibited receptor-stimulated O_2^- generation by fMLP and PAF at IC_{50} values 40- to 70–fold greater than its IC_{50} value against the isolated enzyme (Table 5.1 & Table 5.3). The effect of staurosporine on both the fMLP and PAF O_2^- response was particularly

interesting in that low staurosporine concentrations produced a dose-dependent potentiation of superoxide generation while at high concentrations there was a dose-dependent inhibition, as presented in fig. 5.8a & b. In addition, low concentrations of staurosporine caused a leftward shift of the fMLP dose-response curve whereas high concentrations produced near total inhibition (fig. 5.9). The effect of staurosporine on the PAF dose-response curve was not determined.

K252a produced a marked inhibition of the PMA-, diC_8- and fluoride-stimulated O_2^- response, the dose-inhibition graphs for which are given in fig. 5.10a, b & c respectively, with fluoride again being the most susceptible to inhibition. K252a caused a parallel right-shift of the dose-response curves induced by PMA and diC_8 (seen in fig. 5.11a & b) in contrast to the effect of staurosporine which only caused a parallel right shift of the diC_8 O_2^- dose-response curve.

K252a also potently inhibited the oxidative burst stimulated with the receptor stimuli, fMLP and PAF, as can be seen from their respective dose-inhibition curves in fig. 5.12a & b. The effect of K252a on the fMLP, PAF and fluoride O_2^- dose-response curves was not determined. K252a markedly inhibited, in a dose-dependent fashion, the dose-response curve induced by the Ca^{2+} ionophore, A23187, as presented in fig. 5.11c.

5.2.3 The effect of novel selective bis-indolyl maleimide PKC inhibitors on the stimulated respiratory burst

All six of the novel selective PKC inhibitors, namely the bis-indolyl maleimides, potently inhibited the oxidative burst in response to diC_8 and fluoride, whereas the inactive compound Ro31-6045, which failed to inhibit the isolated PKC, had no effect on the stimulated O_2^- response. The IC_{50} values for each of the PKC inhibitors (Ro31-8425, Ro31-8220, Ro31-8288, Ro31-8161, Ro31-7549, Ro31-8657 and Ro31-6045) in the diC_8- and fluoride-stimulated respiratory burst are presented in Table 5.3 and compared with the IC_{50} values obtained in the fMLP-stimulated response (Muid & Dale, unpublished observation). IC_{50} values for the bis-indolyl maleimide inhibitors were obtained by a similar method as those for K252a and staurosporine. Then for each inhibitor, two doses (taken just above and just below its IC_{50} value) were examined for effect on the full stimulus dose-response curve (n = 1) to study the nature of the inhibition.

These novel selective PKC inhibitors potently inhibited **diC_8-stimulated O_2^- production.** The dose-inhibition data for the O_2^- response induced by 10μM and 25μM diC_8 with Ro31-8425, Ro31-8220 and Ro31-8288 are presented in fig. 5.13a, b & c respectively and with Ro31-8161, Ro31-7549 and Ro31-8657 in fig. 5.14a, b & c respectively. The IC_{50} values calculated from this data show a rank order of inhibitory potency (Table 5.3) that correlates very well with the rank order in the isolated rat brain PKC assay (Table 5.1). In addition, the fMLP-stimulated oxidative burst was inhibited at similar PKC inhibitor concentrations and exhibited a very similar rank order of potency with the range of inhibitors (Table 5.3). The control compound Ro31-6045 was without effect in both the diC_8- and fMLP-induced O_2^- response (Table 5.3).

The inhibitors were also examined for their effects on the diC_8 O_2^- dose-response curve; it was found that Ro31-7549, Ro31-8425 and Ro31-8161 caused a near parallel rightward shift of the curve with near restoration of the maximum response at high diC_8 concentrations - this data is presented in fig. 5.15a, b & c respectively. On the other hand, Ro31-8288 and Ro31-8220 produced a rightward shift of the control dose-response curve and a significant reduction of the maximum control response, as can be seen in fig. 5.16a & b. The compound Ro31-8657, although potent against PKC in the isolated enzyme assay (Table 5.1), did not cause a significant reduction of diC_8-stimulated O_2^- generation as can be clearly seen in fig. 5.16c. This is in agreement with the lack of inhibition also seen with this compound against the fMLP- and fluoride-induced responses (Table 5.3), an observation that was attributed to poor access of Ro31-8657 into the whole cell.

When the bis-indolyl maleimide inhibitors were tested on the **fluoride-mediated O_2^- burst** they were found to be 20- to 200-fold more potent in inhibiting this response as compared to that of diC_8 and fMLP (Table 5.3). The low and high fluoride concentrations for O_2^- generation were taken at 10mM and 18mM fluoride respectively and the effect of varying concentrations of Ro31-8425, Ro31-7549 and Ro31-8220 on the stimulated O_2^- response are presented in fig. 5.17a, b & c respectively. The fluoride dose-inhibition curves with Ro31-8161, Ro31-8288 and Ro31-8657 are given in fig. 5.18a, b & c respectively.

The pronounced inhibitory effect of these PKC inhibitors on fluoride-mediated O_2^- generation was also observed with K252a and staurosporine, which also showed much lower IC_{50} values against the

fluoride response than against that of the other stimuli (Table 5.3).

The range of PKC inhibitors, when tested on the fluoride dose-response curve, produced very marked rightward shifts and large reductions in the maximum control response that could not be restored by increasing the concentration of fluoride. The effect of the more potent compounds - Ro31-8425, Ro31-7549 and Ro31-8220 on the fluoride O_2^- dose-response curve are presented in fig. 5.19a, b & c respectively and compounds Ro31-8161, Ro31-8288 and Ro31-8657 in fig. 5.20a, b & c respectively.

As with the other stimuli, the inactive compound Ro31-6045 had little effect on fluoride O_2^- release (Table 5.3).

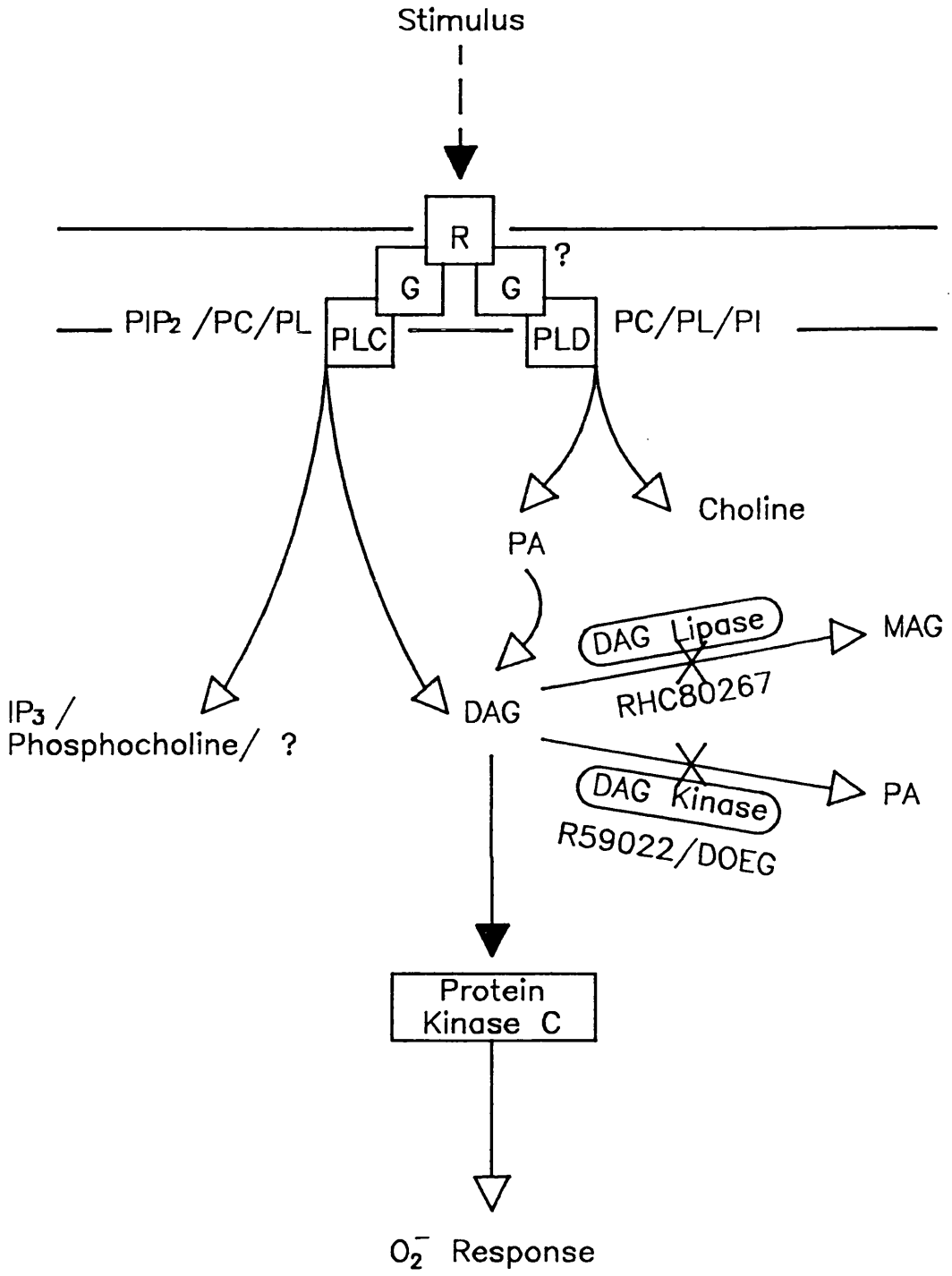


Figure 5.1: The DAG metabolic pathways. DAG can be derived by a phospholipase C (PLC) action on phosphatidylinositol bisphosphate (PIP_2), phosphatidylcholine (PC) or another phospholipid (PL) or by a phospholipase D (PLD) action on phosphatidylinositol phosphate (PI), PC or another PL. The DAG signal is terminated by either the action of a DAG lipase, which gives rise to monoacylglycerol (MAG), or by the action of a DAG kinase, to form phosphatidic acid (PA).

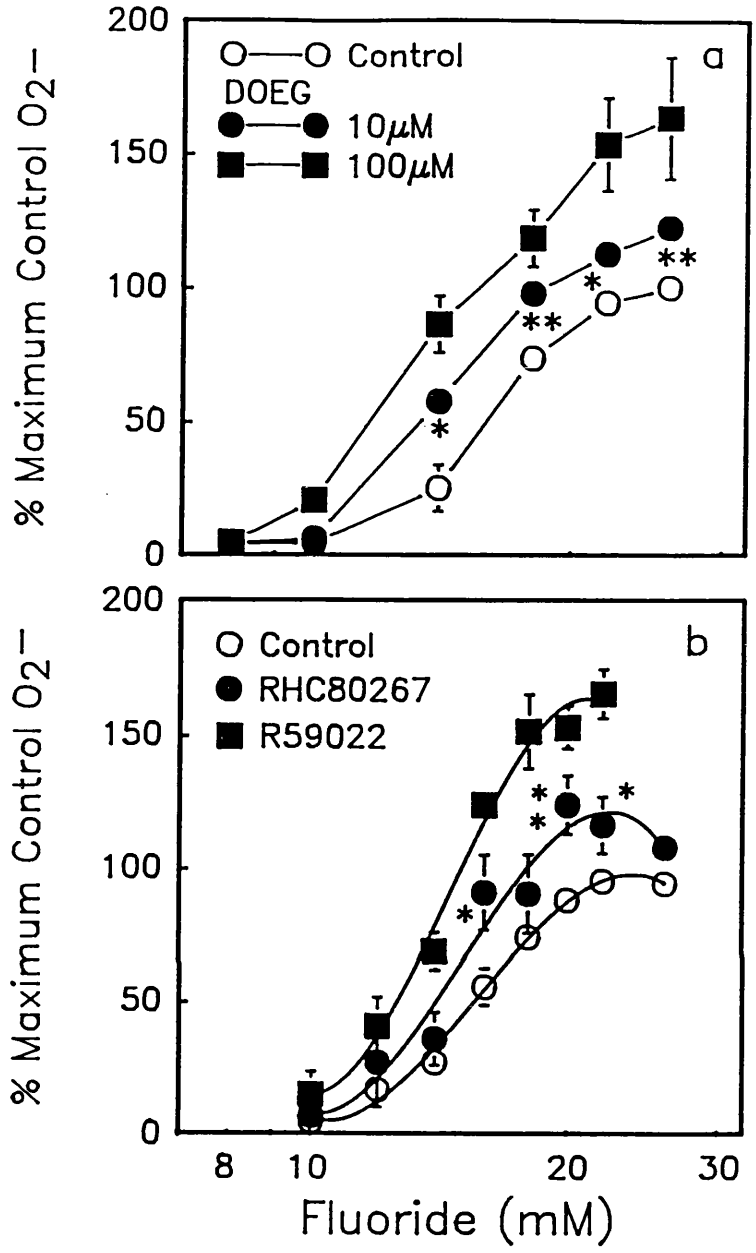


Figure 5.2: The effect of inhibitors of diacylglycerol (DAG) metabolizing enzymes on fluoride-stimulated O_2^- production.

a) Effect of the DAG kinase inhibitor, DOEG, (n = 3); fluoride alone (O), with 10μM DOEG (●) or with 100μM DOEG (■). Mean maximum control O_2^- release was 99.92 ± 22.36 nmol O_2^-/5 × 10^6 neutrophils.

b) Effect of the DAG kinase inhibitor, R59022, and the DAG lipase inhibitor, RHC80267; fluoride alone (n = 13) (O), with 10μM R59022 (n = 7) (■) or with 10μM RHC80267 (n = 6) (●). Mean maximum control O_2^- release was 96.24 ± 6.26 nmol/5 × 10^6 neutrophils. The curves were fitted using Sigma plot (Jandel Scientific, USA).

All data points represent mean ± standard error. Unpaired t-tests were performed on the data, * $P < 0.05$, ** $P < 0.01$.

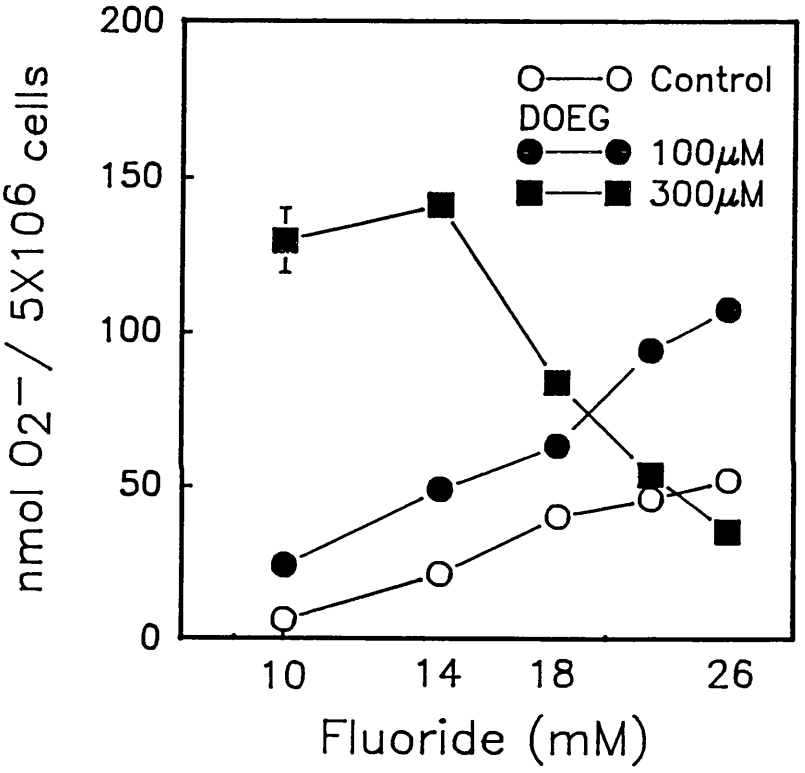


Figure 5.3: The effect of high concentrations of the DAG kinase inhibitor, DOEG, on fluoride-stimulated O_2^- generation (n = 1). Stimulus alone (O) and in the presence of DOEG at 100μM (●) or at 300μM (■). Data points represent the mean of sample duplicates and error bars the range of the duplicates.

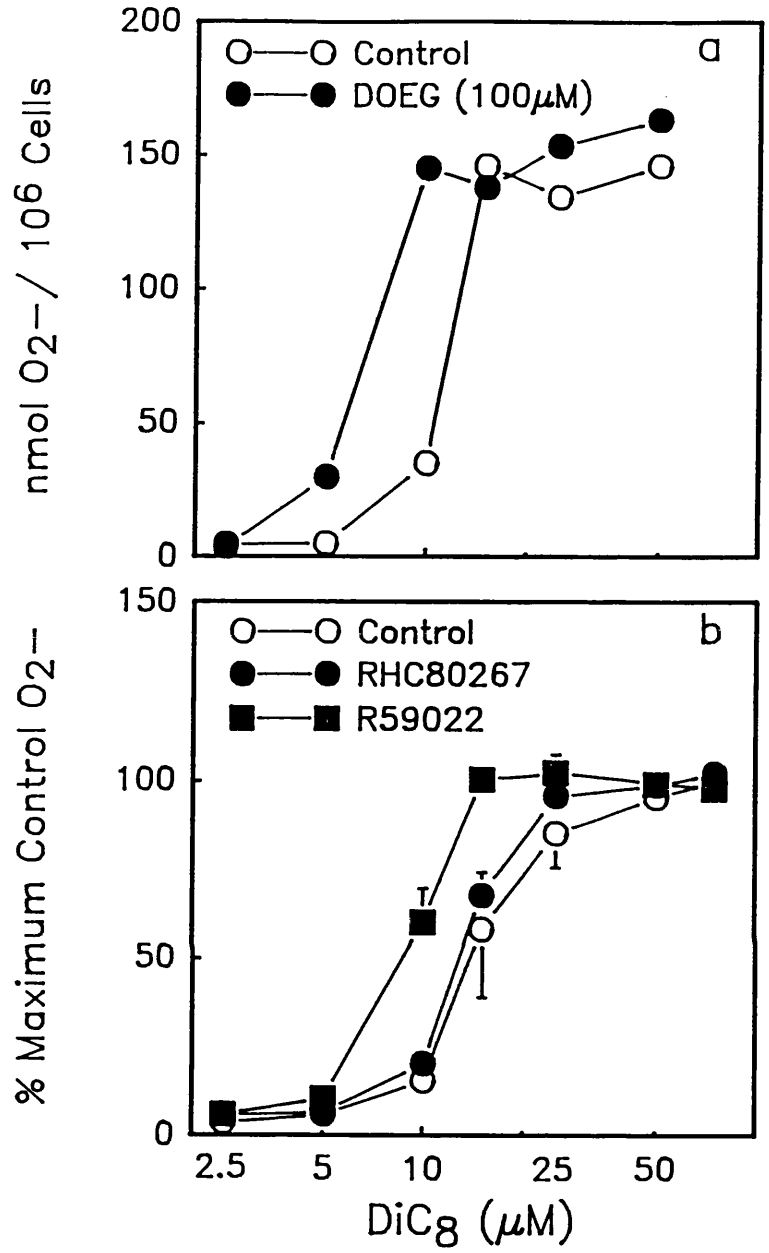


Figure 5.4: The effect of inhibitors of DAG kinase and DAG lipase on diC_8-induced O_2^- production.

a) Shows data from one experiment, but is representative of results obtained in six independent experiments; diC_8 alone (O), and with 100μM DOEG (●). Data points are the mean of sample duplicates and error bars the range of the duplicates.

b) The effect of the DAG kinase inhibitor, R59022, and the DAG lipase inhibitor, RHC80267, (n = 4); diC_8 alone (O), with 10μM R59022 (■) and with 10μM RHC80267 (●). Mean maximum control O_2^- release was 164.94 ± 7.51 nmol/5 × 10^6 neutrophils. Error bars represent standard errors.

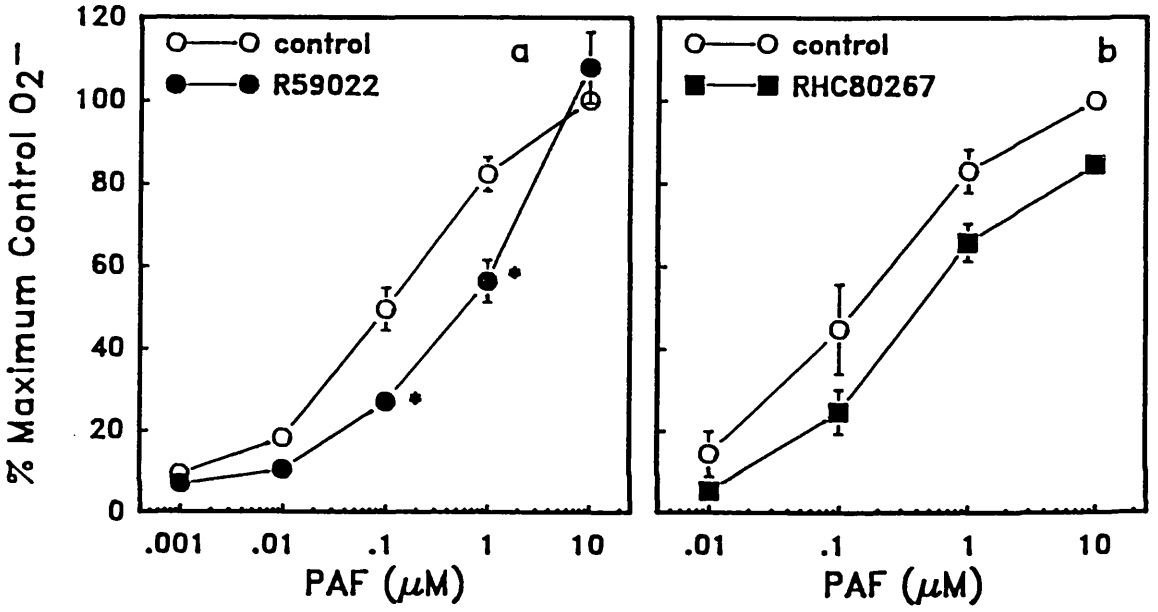


Figure 5.5: The effect of the DAG kinase inhibitor, R59022, and the DAG lipase inhibitor, RHC80267, on the PAF-mediated O_2^- response.
a) PAF alone (O) or with 10μM R59022 (●) (n = 8). Mean maximum control O_2^- release was 68.43 ± 9.12 nmol/5 × 10^6 neutrophils.
b) PAF alone (O) or with 10μM RHC80267 (■) (n = 3). Mean maximum control O_2^- release was 60.77 ± 20.70 nmol/5 × 10^6 neutrophils.
Unpaired t-tests were performed on the data, * $P < 0.05$. Error bars represent standard errors.

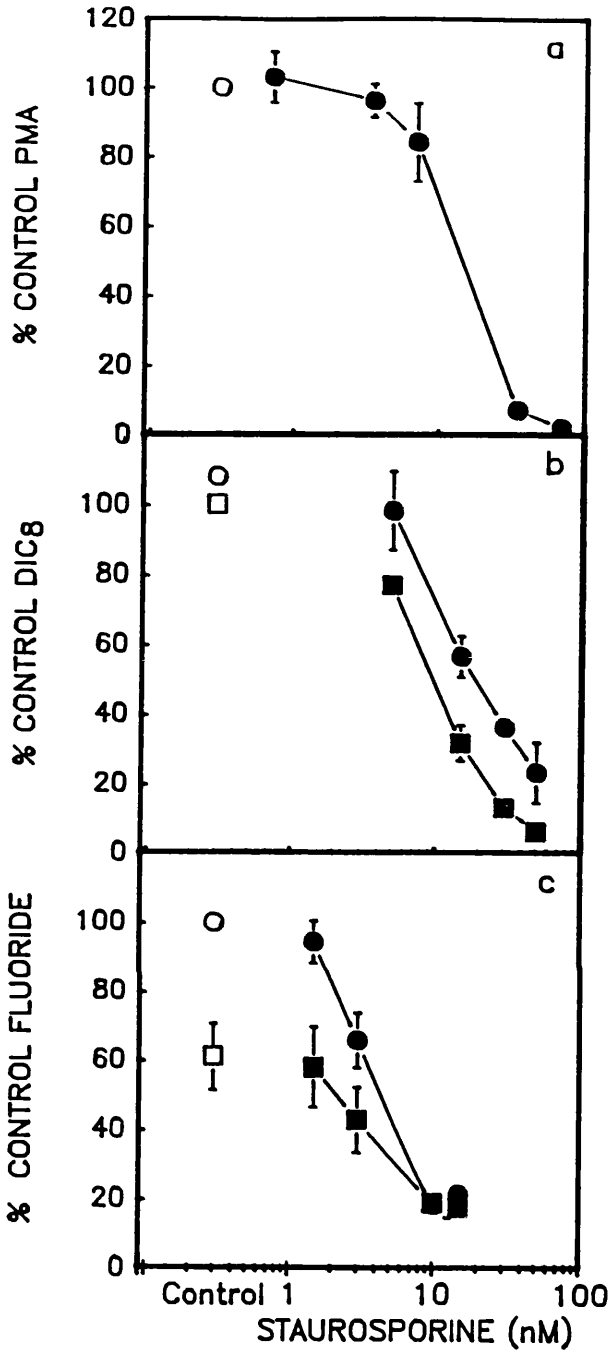


Figure 5.6: The effect of a range of staurosporine concentrations on the O_2^- response generated by 3 post-receptor stimuli.
a) PMA (5nM) alone (O) and with staurosporine (●)(n = 5). b) DiC_8 (75μM) alone (O) and with staurosporine (●), diC_8 (25μM) alone (□) and with staurosporine (■) (n = 3). c) Fluoride (22mM) alone (O) and with staurosporine (●), fluoride (10mM) alone (□) and with staurosporine (■)(n = 4). Mean maximum control O_2^- release, as nmol/5x10^6 cells was 154.64 ± 6.23 for PMA, 132.99 ± 14.31 for diC_8 and 74.64 ± 15.34 for fluoride. Error bars represent SE.

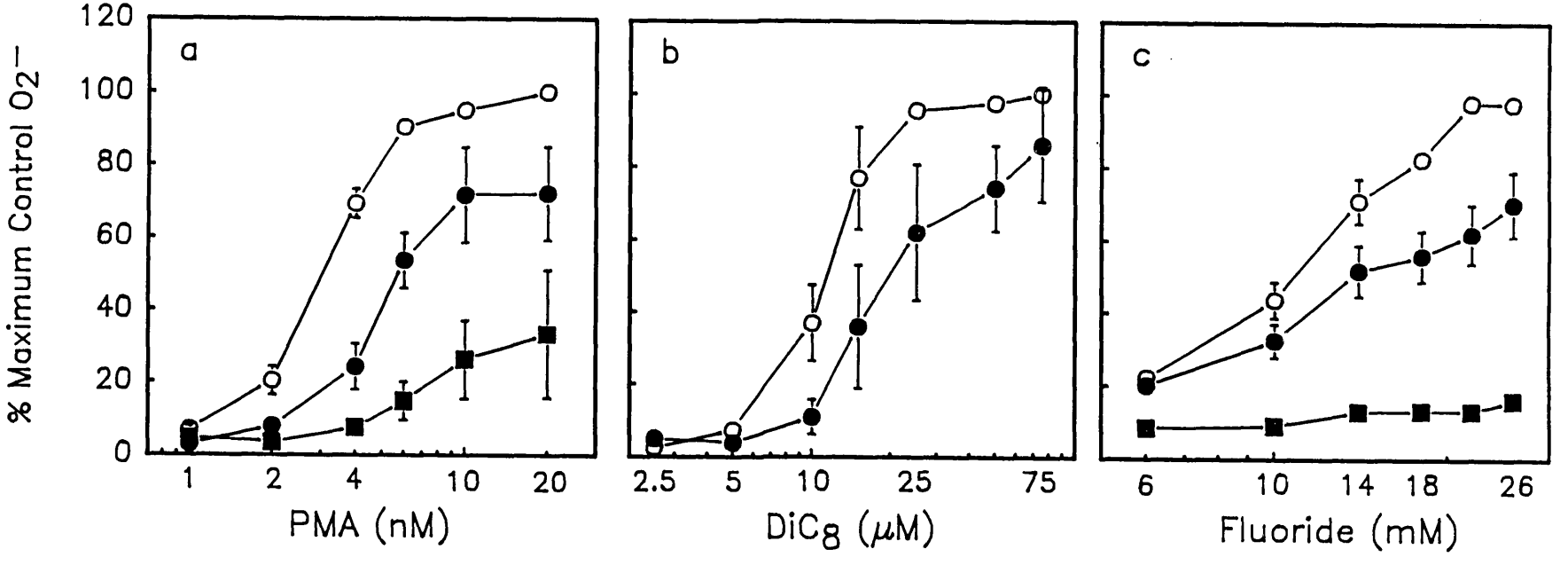


Figure 5.7: The effect of staurosporine on the O_2^- dose-response curves of three post-receptor stimuli.
a) PMA, alone (O) and in the presence of staurosporine at 7nM (●) or at 20nM (■) (n = 4).
b) DiC_8, alone (O) and in the presence of staurosporine at 15nM (●) (n = 3).
c) Fluoride, alone (O) and in the presence of staurosporine at 3nM (●) or at 10nM (■) (n = 5).
The mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 163.35 ± 8.46 for PMA, 177.51 ± 2.41 for diC_8 and 82.19 ± 11.16 for fluoride. Error bars represent standard errors.

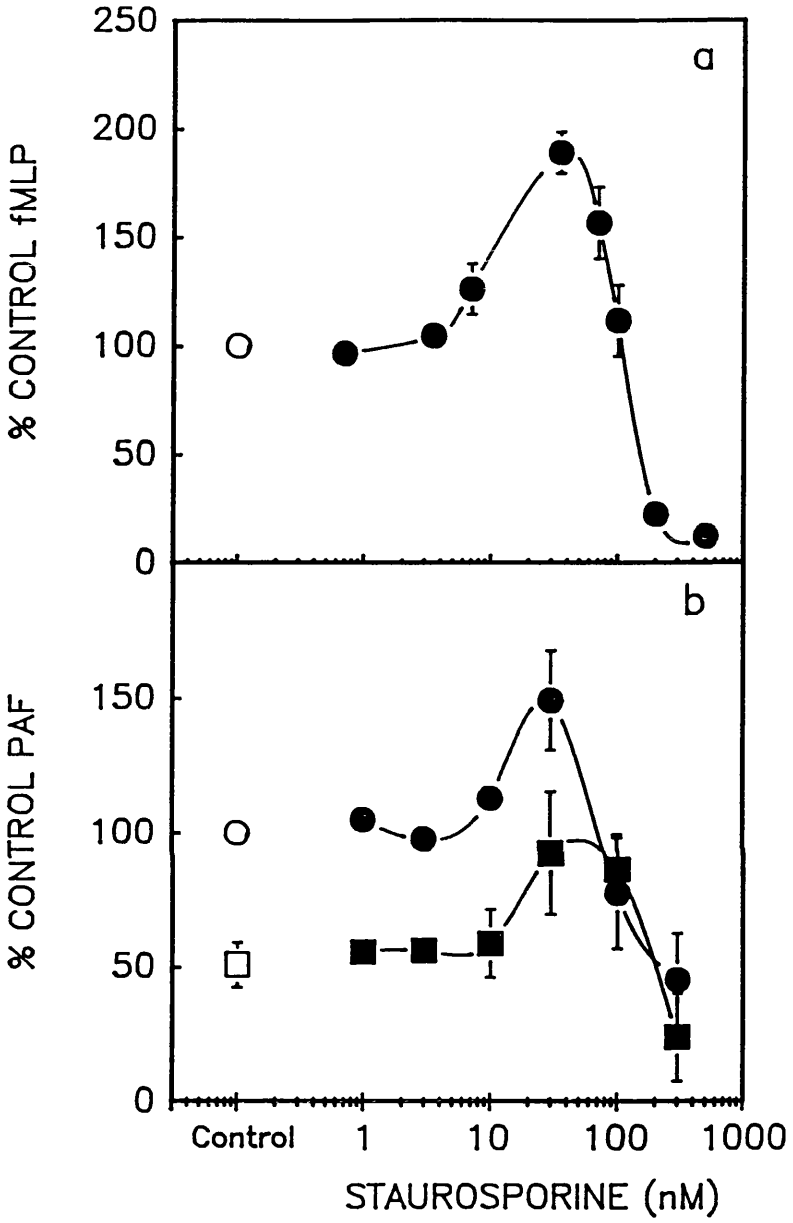


Figure 5.8: The effect of a range of staurosporine concentrations on the O_2^- response induced by fMLP and PAF.
a) FMLP (10nM) alone (O) and in the presence of staurosporine (●) (*n* = 5). b) PAF (10μM) alone (O) and in the presence of staurosporine (●), PAF (1μM) alone (□) and in the presence of staurosporine (■) (*n* = 3). Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 61.81 ± 3.46 for fMLP and 31.13 ± 4.33 for PAF. Error bars represent standard errors.

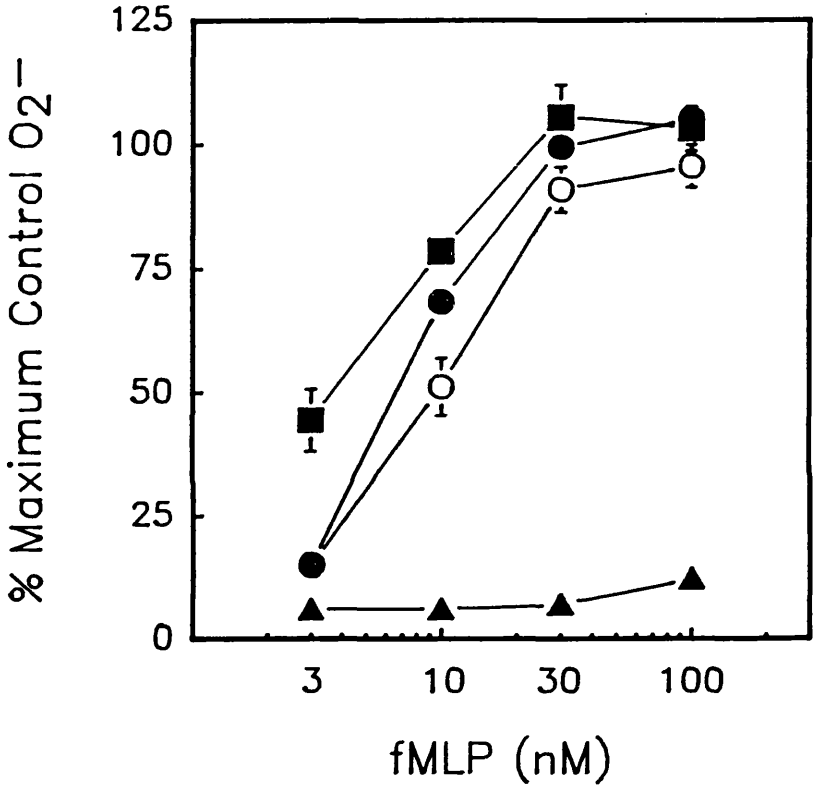


Figure 5.9: The effect of staurosporine on the fMLP-stimulated O_2^- dose-response curve. FMLP alone (O) and in the presence of staurosporine at 7nM (●), 35nM (■) or 200nM (▲) (n = 3). The mean maximum control O_2^- release was 137.47 ± 13.79 nmol/5 x 10^6 neutrophils. Error bars represent standard errors.

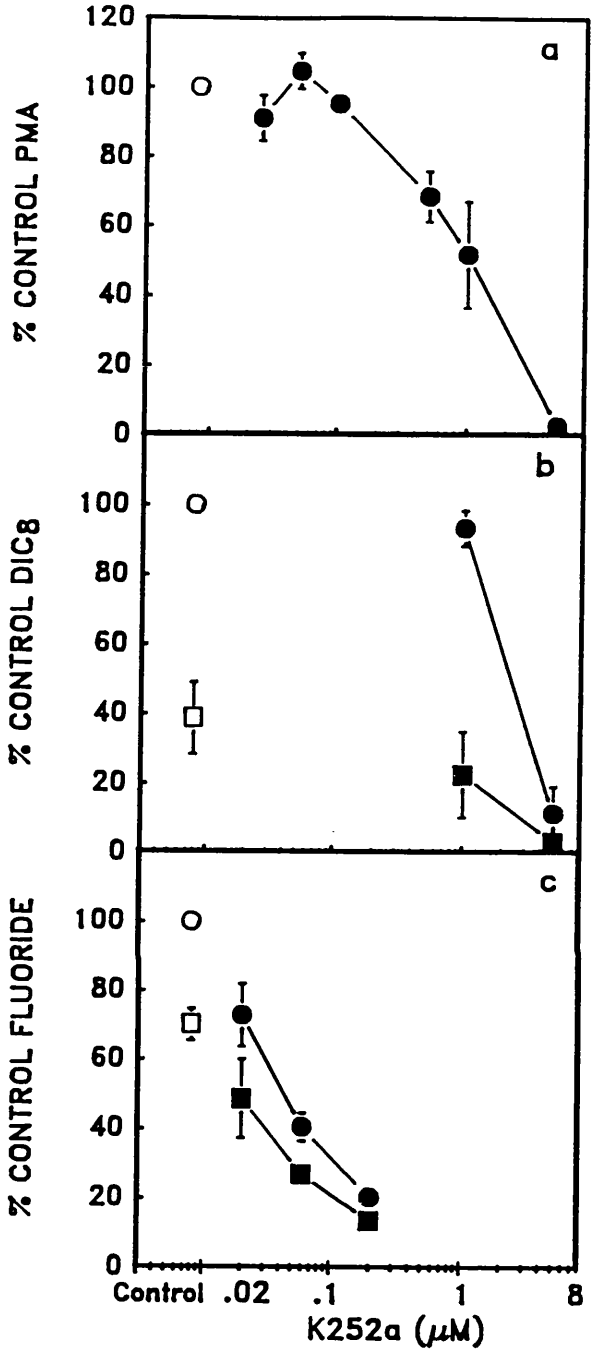


Figure 5.10: The effect of a range of K252a concentrations on the O_2^- response generated by 3 post-receptor stimuli.

a) PMA (5nM) alone (O) and in the presence of K252a (●) (n = 4).

b) DiC_8 (25μM) alone (O) and in the presence of K252a (●), diC_8 (10μM) alone (□) and in the presence of K252a (■) (n = 3). c) Fluoride (22mM) alone (O) and in the presence of K252a (●), fluoride (10mM) alone (□) and in the presence of K252a (■) (n = 3). Mean maximum control O_2^- release, as nmol/5×10^6 cells was 150.07 ± 6.21 for PMA, 169.41 ± 3.71 for diC_8 and 59.20 ± 8.26 for fluoride. Error bars represent SE.

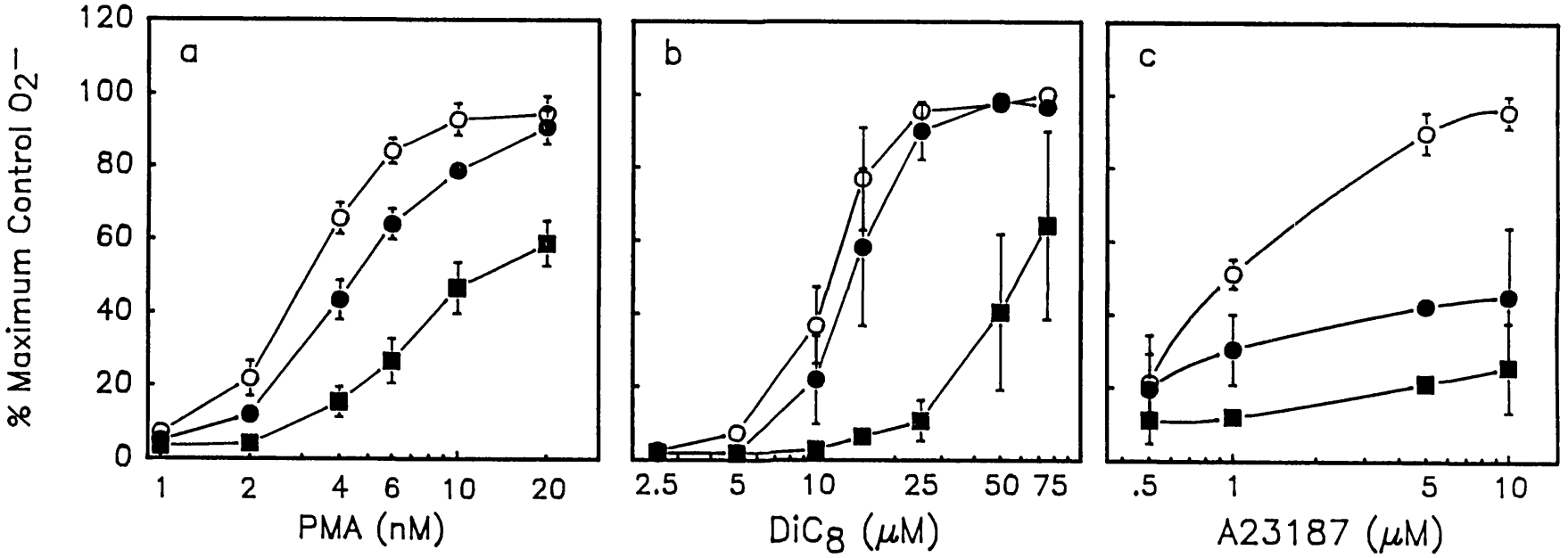


Figure 5.11: The effect of K252a on the O_2^- dose-response curves of three post-receptor stimuli.

a) PMA, control (O) and with K252a at 0.3μM (●) or 1.0μM (■) (n = 4).

b) DiC_8, control (O) and with K252a at 1μM (●) or 5μM (■) (n = 3).

c) A23187, control (O) and with K252a at 0.2μM (●) or 1.0μM (■) (n = 3)

Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 160.17 ± 5.58 for PMA, 177.51 ± 2.41 for diC_8 and 23.87 ± 6.16 for A23187. Error bars represent standard errors.

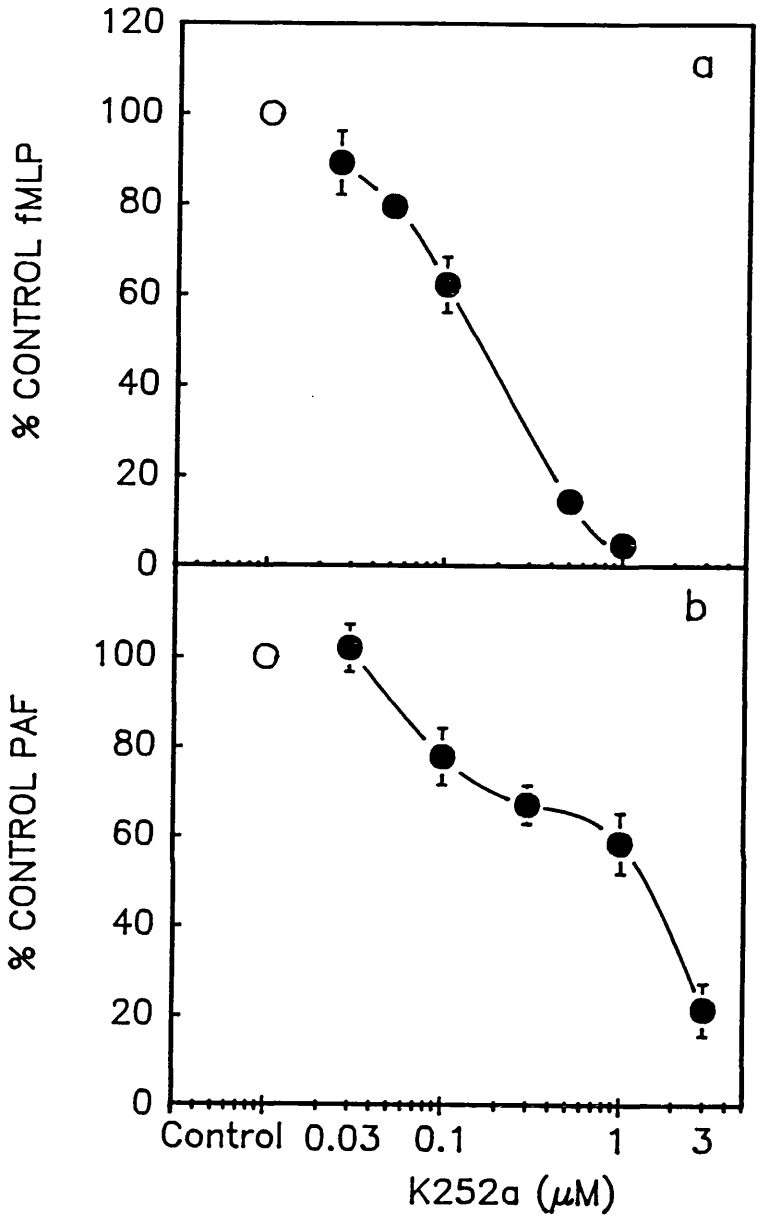


Figure 5.12: The effect of varying K252a concentrations on receptor-stimulated O_2^- generation.

a) FMLP (10nM) alone (O) and with K252a (●) (n = 3).

b) PAF (10μM) alone (O) and with K252a (●) (n = 3).

Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 85.35 ± 23.41 for fMLP and 29.07 ± 3.79 for PAF. Error bars represent standard errors.

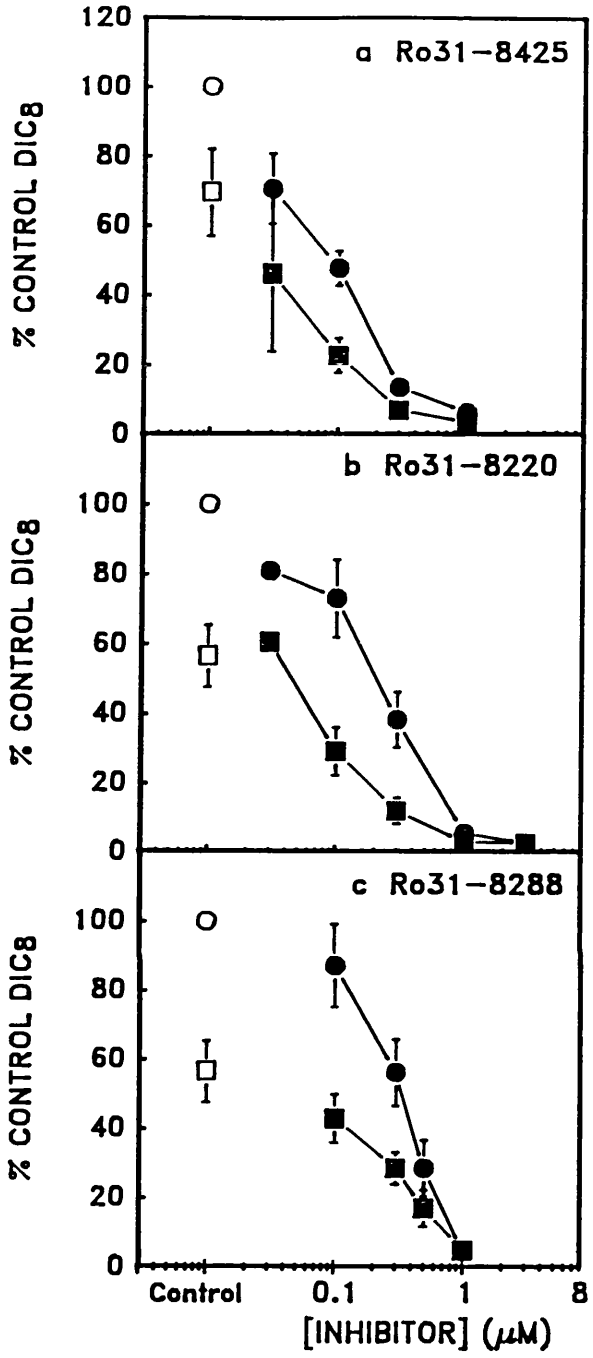


Figure 5.13: The effect on diC_8-induced O_2^- production of varying concentrations of three novel protein kinase C inhibitors - namely a) Ro31-8425 (n = 4), b) Ro31-8220 (n = 5) and c) Ro31-8288 (n = 4). Responses were obtained with diC_8 (25μM) alone (O) and in the presence of inhibitor (●), or with diC_8 (10μM) alone (□) and in the presence of inhibitor (■). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 140.72 ± 14.56 in a, 148.10 ± 18.13 in b and 144.75 ± 23.00 in c. Error bars represent standard errors.

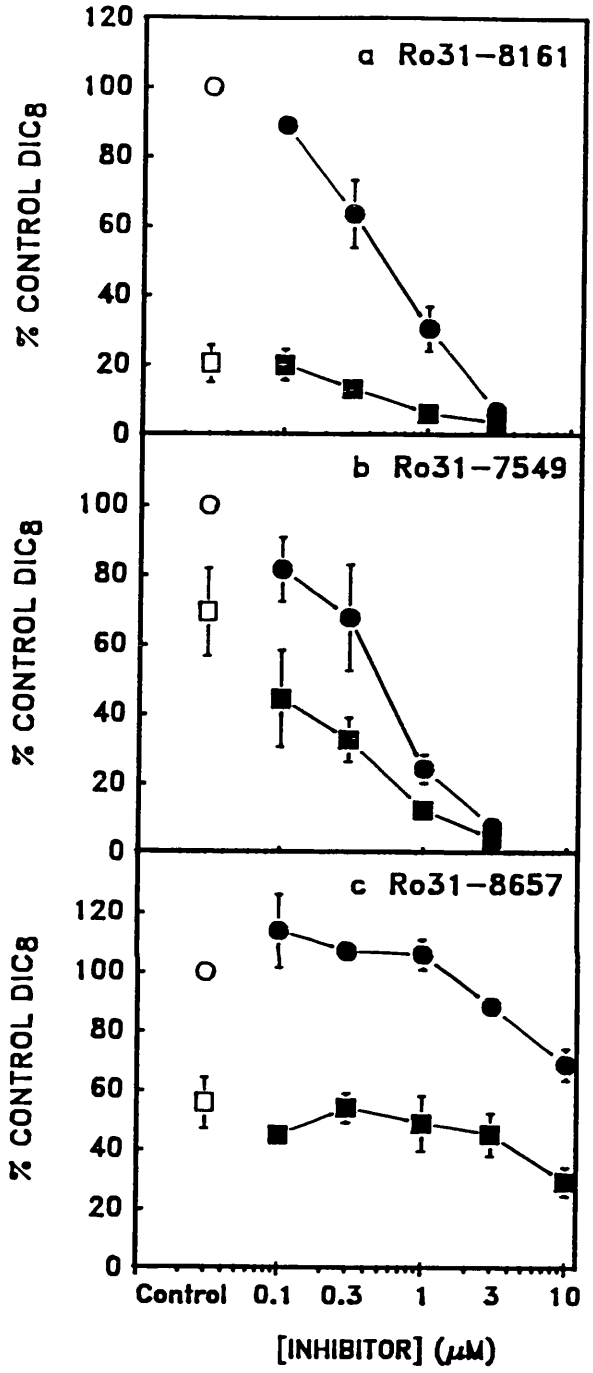


Figure 5.14: The effect on diC_8-induced O_2^- production of varying concentrations of three novel protein kinase C inhibitors - namely a) Ro31-8161 (n = 4), b) Ro31-7549 (n = 4) and c) Ro31-8657 (n = 5). Responses were obtained with diC_8 (25μM) alone (O) and in the presence of inhibitor (●) or with diC_8 (10μM) alone (□) and in the presence of inhibitor (■). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 148.04 ± 15.69 in a, 140.72 ± 14.56 in b and 140.94 ± 15.51 in c. Error bars represent standard errors.

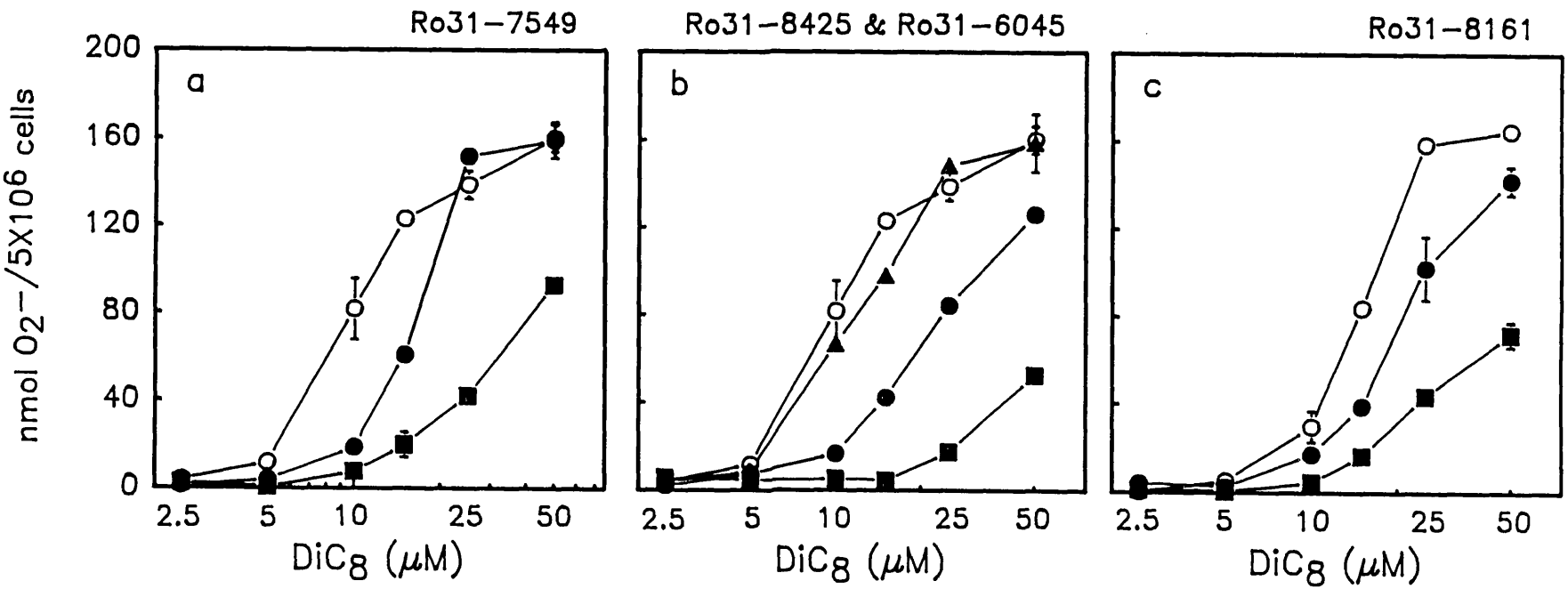


Figure 5.15: The effect of 3 novel protein kinase C inhibitors on the diC_8-induced O_2^- dose-response curve.

a) DiC_8 alone (○) and in the presence of Ro31-7549 at 0.3μM (●) or 1.0μM (■).

b) DiC_8 alone (○) and in the presence of Ro31-8425 at 0.1μM (●) or 0.3μM (■), and Ro31-6045 at 3.0μM ( ).

c) DiC_8 alone (○) and in the presence of Ro31-8161 at 0.3μM (●) or 1.0μM (■).

The graphs show the result of single experiments and all data points represent the mean of sample duplicates ± range of the duplicates.

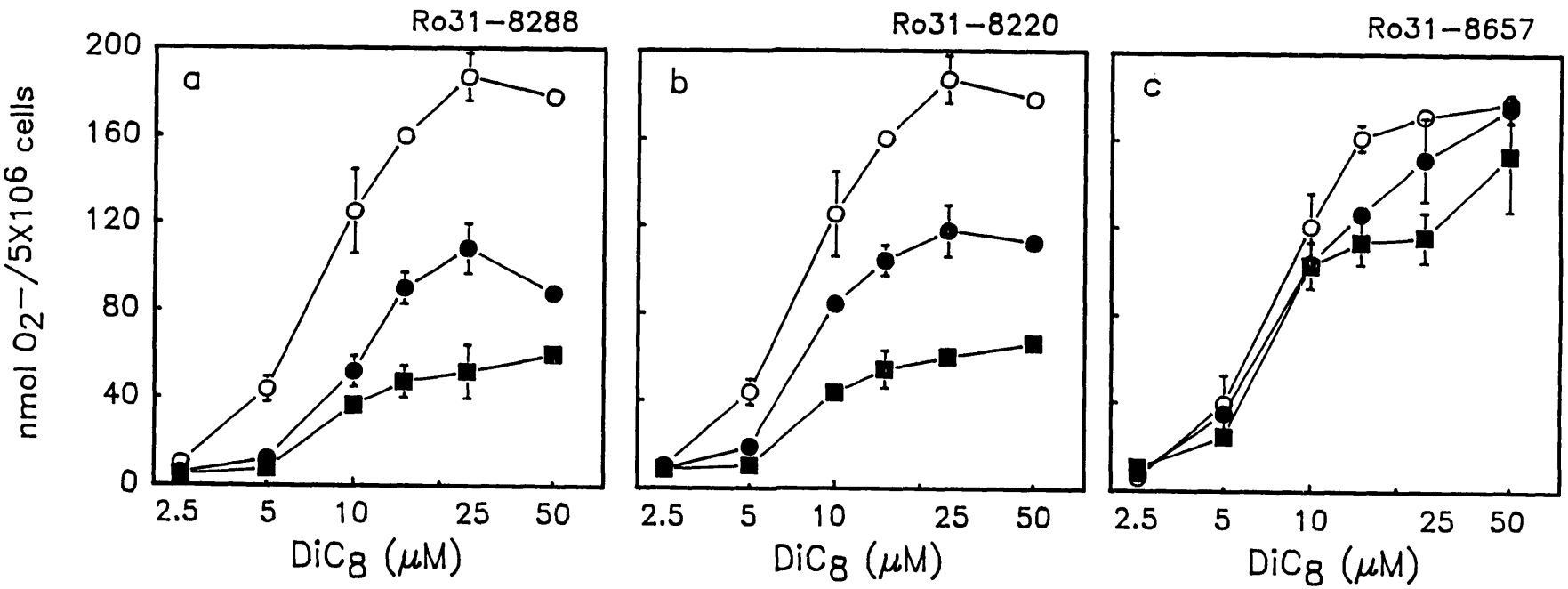


Figure 5.16: The effect of 3 novel protein kinase C inhibitors on the diC_8-induced O_2^- dose-response curve.

a) DiC_8 alone (O) and in the presence of Ro31-8288 at 0.3μM (●) or 0.5μM (■).

b) DiC_8 alone (O) and in the presence of Ro31-8220 at 0.1μM (●) or 0.3μM (■).

c) DiC_8 alone (O) and in the presence of Ro31-8657 at 1μM (●) or 10μM (■).

The graphs show the result of single experiments and all data points represent the mean of sample duplicates ± range of the duplicates.

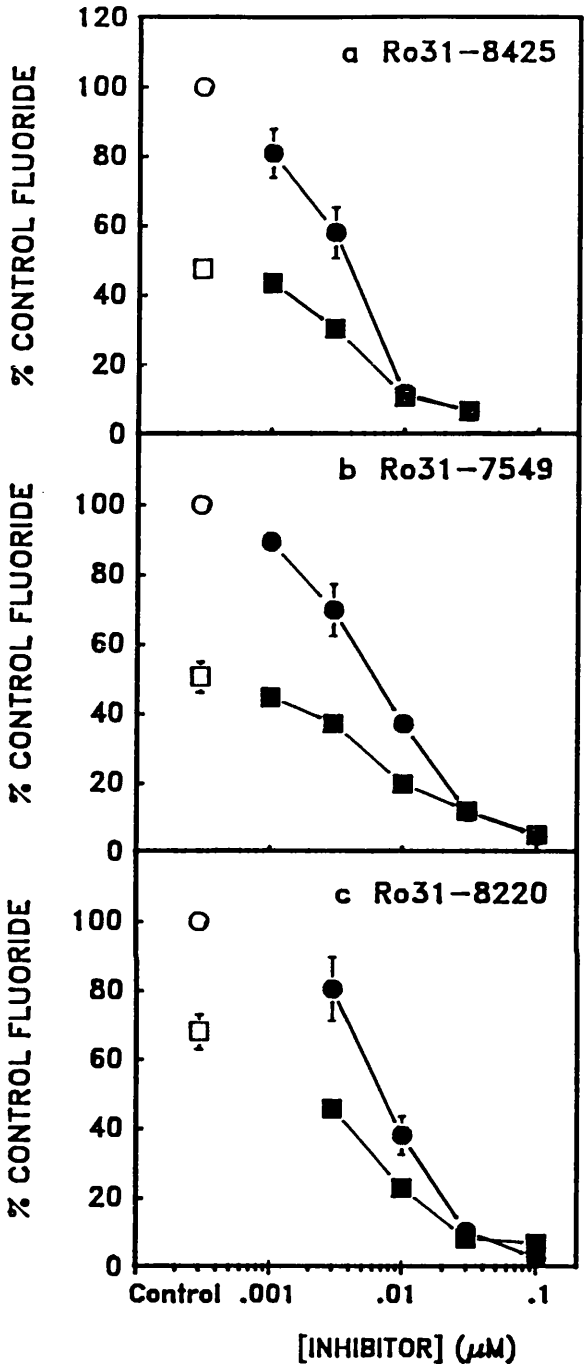


Figure 5.17: The effect on fluoride-induced O_2^- production of varying concentrations of three novel protein kinase C inhibitors - namely a) Ro31-8425 (n = 4), b) Ro31-7549 (n = 4) and c) Ro31-8220 (n = 4). Responses were obtained with fluoride (18mM) alone (O) and in the presence of inhibitor (●) or with fluoride (10mM) alone (□) and in the presence of inhibitor (■). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 71.59 ± 9.88 in a, 78.61 ± 8.60 in b and 66.81 ± 9.69 in c. Error bars represent standard errors.

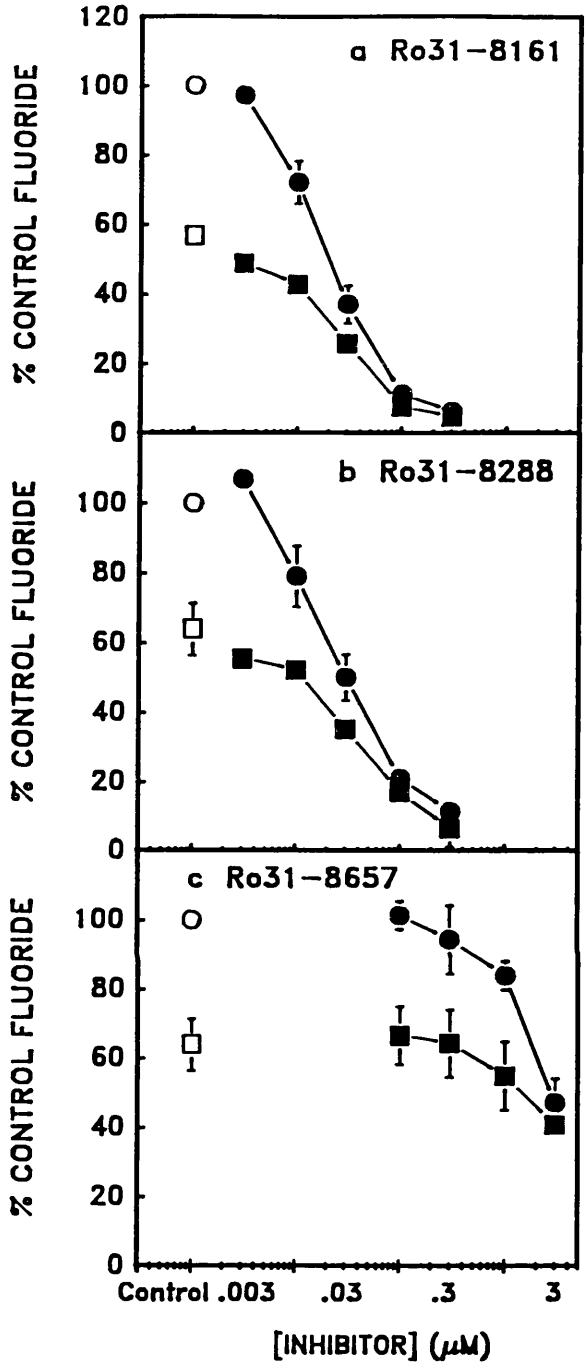


Figure 5.18: The effect on fluoride-induced O_2^- production of varying concentrations of three novel protein kinase C inhibitors - namely a) Ro31-8161 (n = 4), b) Ro31-8288 (n = 4) and c) Ro31-8657 (n = 4). Responses were obtained with fluoride (18mM) alone (O) and in the presence of inhibitor (●) or with fluoride (10mM) alone (□) and in the presence of inhibitor (■). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 76.74 ± 8.73 in a and 66.00 ± 9.58 in b and c. Error bars represent standard errors.

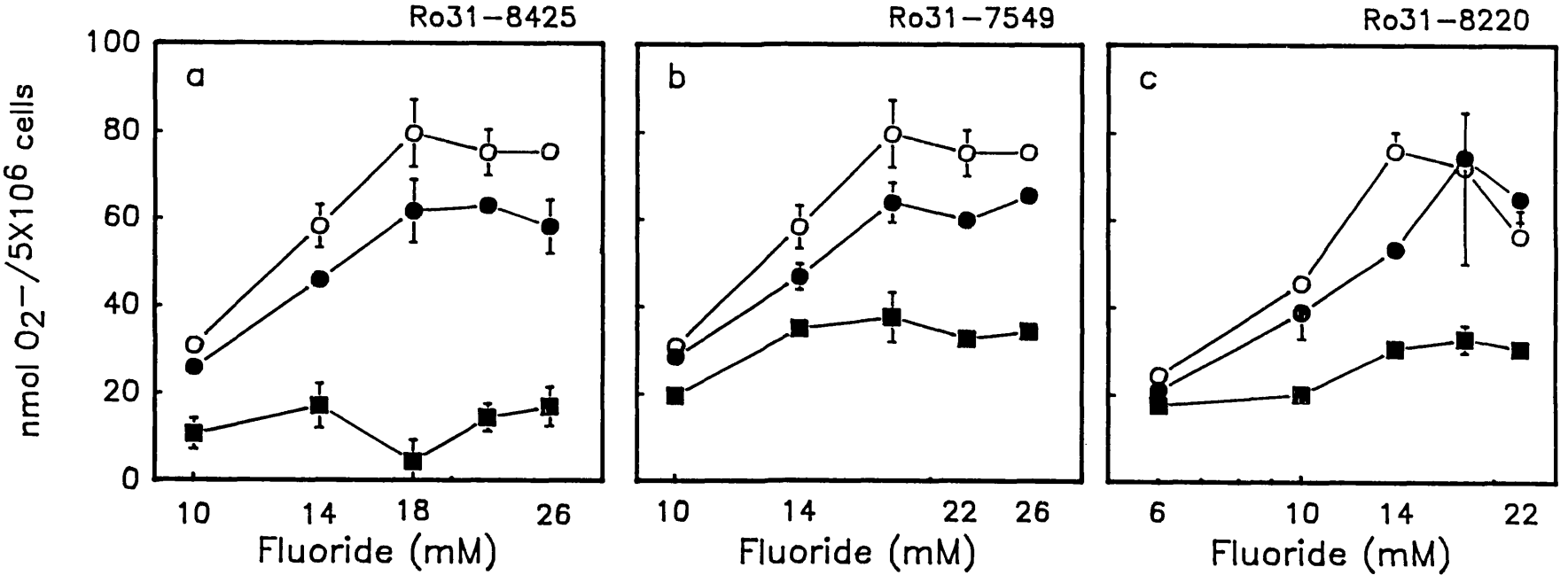


Figure 5.19: The effect of 3 novel protein kinase C inhibitors on the fluoride-induced O_2^- dose-response curve.

a) Fluoride alone (○) and in the presence of Ro31-8425 at 0.003μM (●) or 0.01μM (■).

b) Fluoride alone (○) and in the presence of Ro31-7549 at 0.003μM (●) or 0.01μM (■).

c) Fluoride alone (○) and in the presence of Ro31-8220 at 0.003μM (●) or 0.01μM (■).

The graphs show the result of single experiments and all data points represent the mean of sample duplicates ± range of the duplicates.

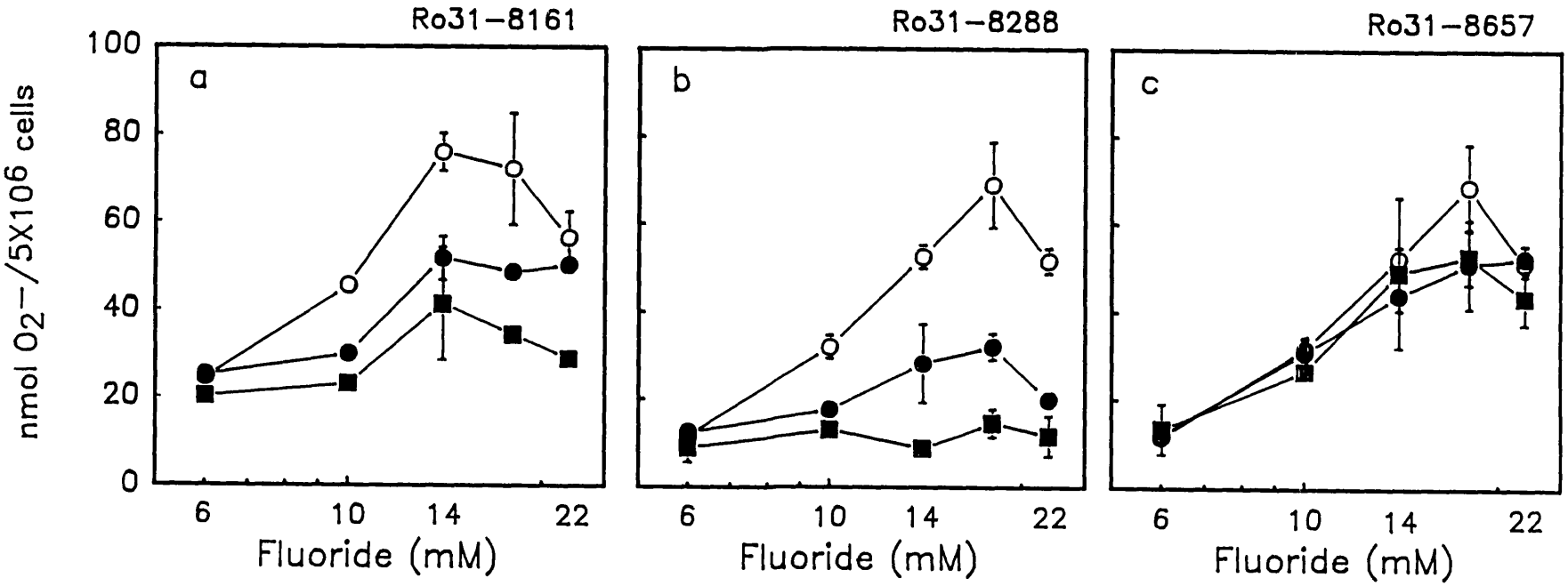


Figure 5.20: The effect of 3 novel protein kinase C inhibitors on the fluoride-induced O_2^- dose-response curve.

a) Fluoride alone (○) and in the presence of Ro31-8161 at 0.01μM (●) or 0.03μM (■).

b) Fluoride alone (○) and in the presence of Ro31-8288 at 0.03μM (●) or 0.1μM (■).

c) Fluoride alone (○) and in the presence of Ro31-8657 at 0.3μM (●) or 1.0μM (■).

The graphs show the result of single experiments and all data points represent the mean of sample duplicates ± range of the duplicates.

TABLE 5.3

Inhibitor IC_{50} values (μM) with stimulated O_2^- generation

	PMA	DiC_8	fluoride	FMLP	PAF
K252a	0.850 ± 0.102 0.680[a]	2.62 ± 0.03 2.2[a]	0.078 ± 0.008	0.176 ± 0.007 0.36[a]	0.920 ± 0.109 0.20[a]
Stauro	0.0136 ± 0.0001 0.004[b] 0.017[c]	0.014 ± 0.001	0.0054 ± 0.0002	0.180 ± 0.108 no effect[b] 0.024[c]	0.260 ± 0.037
Ro31-7549	ND	0.671 ± 0.101	0.0079 ± 0.0001	1.969 ± 0.049	ND
Ro31-8161	ND	0.633 ± 0.125	0.0220 ± 0.0004	1.500 ± 0.045	ND
Ro31-8220	ND	0.285 ± 0.002	0.010 ± 0.002	0.95 ± 0.01	ND
Ro31-8425	ND	0.148 ± 0.006	0.0046 ± 0.0007	0.60 ± 0.05	ND
Ro31-8288	ND	0.379 ± 0.008	0.051 ± 0.003	0.767 ± 0.050	ND
Ro31-8657	ND	15.25 ± 0.81	2.75 ± 0.14	4.80 ± 0.32	ND
Ro31-6045	ND	>10	>3.0	>10.0	ND

ND = not determined

a. Smith R.J. *et al.* (1988)

b. Sako *et al.* (1988)

c. Dewald *et al.* (1989)

5.3 DISCUSSION

5.3.1 Involvement of the DAG pathway in the respiratory burst

It is necessary to make the point that the inhibitors of DAG metabolism used in this study may not be entirely specific, and that the conclusions set out below are only valid to the extent that the compounds used are selective for the DAG metabolizing enzymes (discussed later).

(i) Stimulation with fluoride

The results presented in this study indicate that inhibition of DAG metabolism consistently increased fluoride-stimulated O_2^- production. But the fluoride O_2^- response was affected by the DAG metabolic inhibitors in a slightly different manner to that of receptor-stimulated O_2^- release, in that it was consistently increased by the putative DAG kinase inhibitors (R59022 and DOEG), while the DAG lipase inhibitor (RHC80267) had variable effects; the receptor-stimulated O_2^- responses were enhanced *only* by DAG kinase inhibition (using R59022) (Muid *et al.*, 1987). In some experiments with fluoride the DAG lipase metabolizing route appeared to play a role as well as the DAG kinase, while in others the results indicated that the DAG kinase was the sole DAG metabolizing pathway. It is possible that DAG kinase is closely associated with the fMLP and Fc/C3b receptors while the DAG lipase is not; on the other hand G-proteins, on which fluoride acts, may be free in the plasma membrane and the DAG generated on phospholipase C activation may therefore be accessible to membrane DAG lipase on some but not all occasions.

In conclusion, it can be proposed that the DAG generated on stimulation at the receptor, namely the fMLP, C3b and Fc receptors (Muid *et al.*, 1987; Mege *et al.*, 1988), on G-protein activation with fluoride (results of this study), on stimulation with the Ca^{2+} ionophore, A23187 (Dale & Penfield, 1987), and by the membrane perturbant, γ-HCCH (Muid & Dale, unpublished observation), is metabolized predominantly by the DAG kinase enzyme; inhibition of this metabolism by putatively specific inhibitors produced a marked enhancement of the stimulated O_2^- response. This lends support to the role of DAG, and hence the DAG/PKC pathway, in the signal transduction sequence leading to NADPH oxidase activation.

Another notable point from these results is the effect of high concentrations of DOEG on the fluoride O_2^- response. If the biphasic

effect of DOEG, a very marked potentiation at low fluoride concentrations and inhibition at high fluoride concentrations, can be interpreted in terms of DAG inhibition it lends credence to the hypothesis that potent PKC activation can lead to a negative-feedback mechanism. It is interesting to note that the most potent enhancing NSAIDs, namely sodium meclofenamate and mefenamic acid, also showed this same profile of effects at low and high fluoride concentrations. This supports the proposal that they too are potently inhibiting DAG kinase, thus increasing DAG levels and bringing about marked PKC activation. The locus of action of the PKC-mediated "turn-off" mechanisms has been investigated in a number of cell types by exposure to prolonged PMA treatment, and the number of resulting cellular effects observed was quite diverse. These are discussed extensively in Chapter 9 (general discussion).

(ii) Metabolism of exogenous diC_8

Previous studies in platelets have shown that the primary metabolic fate of cell permeable, exogenous DAGs is conversion to PA by DAG kinase (Bishop & Bell, 1986). In the present study we report that the exogenous diC_8 also appeared to be metabolized mainly by DAG kinase as indicated by the increased respiratory burst in the presence of both putative DAG kinase inhibitors, R59022 and DOEG. The DAG lipase inhibitor, RHC80267, had no significant effect on diC_8-induced O_2^- generation. In contrast it has been reported that when another exogenous DAG, OAG, was used as a stimulus, O_2^- production was consistently enhanced by both R59022 and RHC80267 (Dale & Penfield, 1987); also both inhibitors in combination caused a further enhancement of the OAG O_2^- response (Penfield, 1988). The implication of these two sets of results is that the metabolism of diC_8 resembles the metabolism of endogenous DAG, generated by both receptor and post-receptor mechanisms, more closely than does the metabolism of OAG. Thus diC_8 may be a more physiological stimulus for studying PKC activation than OAG, or for that matter PMA which is known to persist in membranes for long periods (Nishizuka, 1984a); the metabolism of PMA has, however, been demonstrated to be metabolized in part by DAG lipase (Cabot, 1984).

(iii) Stimulation with PAF

The effect of both the DAG kinase and lipase inhibitors on the PAF-induced oxidative burst was in marked contrast to that observed with all other stimuli investigated in that both R59022 and RHC80267

produced a decrease of the O_2^- response, as opposed to the widely recorded R59022-induced potentiation of the stimulated oxidative burst. It should be noted that only the R59022-mediated decrease of PAF-stimulated O_2^- production was statistically significant. An increase of the PAF O_2^- response by R59022 has however been reported by another group (Mege *et al.*, 1988). In general, the putative DAG lipase inhibitor, RHC80267, had no consistent effect with most stimuli, even though a decrease of the O_2^- response induced by the receptor stimuli fMLP and heat-aggregated IgG was reported; but the decreases did not reach statistical significance (Muid et al., 1987).

Does the significant R59022-mediated and the less pronounced RHC80267-mediated decrease of the PAF O_2^- response exclude the participation of the DAG pathway in the stimulus-response coupling with this agonist? This fact cannot be ruled out given the available data. However, in the original characterization studies it was suggested that a degree of caution should be exercised with the application of R59022 to cellular systems together with receptor agonists in view of the fact that this compound is a weak dopamine D_2, adrenaline α_1 and histamine H_1 receptor antagonist (K_i = 10^{-8} - 10^{-7}M) and a potent serotonin S_2 antagonist (K_i < 10^{-9}M) (De Chaffoy de Courcelles *et al.*, 1985). Also, R59022 has been found to possess antimuscarinic properties in inhibiting the muscarinic agonist-mediated [^{3}H]cyclic GMP response and antagonist binding in mouse neuroblastoma cells (Lai & El-Fakahany, 1990). It is possible that R59022 could also have antagonist effects at the PAF receptor. This would explain the decrease of the PAF oxidative response in the presence of R59022, which could very well mask the R59022-induced increase of intracellular DAG levels and the concomitant enhancement of the O_2^- response.

(iv) <u>Are the putative DAG metabolic inhibitors specific?</u>

Most of the biochemical measurements carried out in intact platelets with R59022 deduced that this compound markedly inhibited DAG kinase (see introduction) at concentrations between 1-10μM, having negligible effects, under basal conditions, on ^{32}P levels in lipids and proteins at concentrations $\lesssim$ 10μM (De Chaffoy de Courcelles *et al.*, 1985). In this latter study, when longer incubation times were used, 10μM R59022 was shown to have significant effects in increasing ^{32}P incorporation into PA, PIP and PC. The increase in [^{32}P]PA labelling implied that there could be an increase in PA levels that does not stem from a DAG kinase

action. In another study, R59022 at 10μM was reported to potentiate [^{3}H]PA formation in human PMNs stimulated with fMLP (Mahadevappa, 1988). When used at 50μM, R59022 was reported to *directly* cause a significant hydrolysis (6-7%) of [^{3}H]PI which resulted in an accumulation of [^{3}H]DAG and [^{3}H]PA. PLC and/or D activation was given as the most likely route of production of the latter messengers rather than inhibition of DAG kinase (Mahadevappa, 1988). Another conflicting study reports that 10μM R59022 caused a reduction of fMLP-stimulated [^{32}P]PA generation, that was consistent with an inhibition of the DAG kinase enzyme (Mege *et al.*, 1988), whilst showing a slight increase in basal [^{32}P]PA levels in R59022-treated unstimulated cells.

There is also a certain degree of controversy regarding the effect of both R59022 and DOEG on $[Ca^{2+}]_i$ levels in human PMNs. Mege *et al.* (1988) reported that the basal $[Ca^{2+}]_i$, but not the rise in $[Ca^{2+}]_i$ produced by fMLP or PAF, is elevated by R59022. This increase of $[Ca^{2+}]_i$ induced by R59022 in unstimulated PMNs has been confirmed and also reported to occur with DOEG (Nasmith & Grinstein, 1989). The latter group went on to report that neither R59022 or DOEG enhanced fMLP-stimulated O_2 consumption (indicative of respiratory burst activation) in electropermeabilized PMNs, under conditions where $[Ca^{2+}]_i$ was held constant using EGTA. They concluded that the R59022- and DOEG-mediated enhancement of the respiratory burst was a result of the rise in $[Ca^{2+}]_i$, rather than an inhibition of DAG kinase. However, this explanation does not take account of the fact that R59022 does not raise $[Ca^{2+}]_i$ levels over and above that recorded in fMLP- or PAF-stimulated cells (Mege *et al.*, 1988).

In answer to the proposal that R59022 and DOEG are not specific for DAG kinase in that they also increase PA and $[Ca^{2+}]_i$ levels, it should be stressed that neither inhibitor produced activation of the oxidative burst in unstimulated PMNs (results not shown). It is possible that there is synergistic interaction between the R59022-induced $[Ca^{2+}]_i$ and/or PA increases and the agonist-stimulated DAG and/or $[Ca^{2+}]_i$ increases, which could explain the R59022-mediated enhancement of the stimulated respiratory burst – a mechanism that would in fact be partially DAG kinase-independent.

It is obvious from the conflicting data that the mechanism of action of R59022 and DOEG may be rather complex but it is clear that they potently inhibit DAG kinase. In so far as the data in this study were obtained at low inhibitor concentrations at short time periods it is likely

that the enhancement of the respiratory burst is attributable to DAG kinase inhibition. To the extent that it might increase PA, R59022 could contribute to increased DAG levels (through the action of a PA phosphohydrolase) - an increase that would be augmented by its additional effect on DAG kinase. In any case, it is proposed that these results support a focal role for DAG in the transduction mechanisms leading to respiratory burst activation.

(v) The DAG kinase protein

A number of general points were raised in a recent review concerning the DAG kinase protein (Kanoh *et al.,* 1990). Several immunologically distinct DAG kinase isoenzymes have been identified to date. Only two DAG kinase isoenzymes, derived from pig thymus cytosol, have been highly purified – a 80kDa and a 150kDa species. Interestingly, R59022 was found to inhibit only the 80kDa DAG kinase isoenzyme (IC_{50} = 10μM), whereas the 150kDa species was virtually unaffected by the inhibitor (Sakane *et al.,* 1989). The 80kDa, R59022-sensitive DAG kinase isoenzyme has not been found in platelets (Yamada & Kanoh, 1988) or neutrophils (Yamada *et al.,* 1989) so it remains to be confirmed whether these cells possess a novel DAG kinase species that is also inhibited by R59022. The two DAG kinase isoenzymes were also found to be differentially regulated; the 80kDa isoenzyme was relatively inactive without added activators whereas the 150kDa isoenzyme was fully active in the absence of activators (Sakane *et al.,* 1989). Furthermore, the occurrence of DAG kinase isoforms has also been found in murine fibroblasts (MacDonald *et al.,* 1988) and in rat brain (Maroney & Macara, 1989). In the former cell type, enzyme species were found in distinct subcellular compartments and with different specificities towards molecular species of DAG. It is conceivable that DAG kinase isoenzymes may possess distinct enzymological properties with effects on cellular modulation, analogous to the emerging status of PKC isoenzymes.

Another observation that suggests a functional inter-relationship between DAG kinase and PKC is the ability of PKC *in vitro* to actively phosphorylate the 80kDa kinase (Kanoh *et al.,* 1989); although the phosphorylation did not significantly affect the catalytic properties of DAG kinase, the phosphorylated enzyme was recovered bound to PS vesicles, whereas the non-phosphorylated form was found in the soluble phase. As neutrophil DAG kinase has been shown to translocate from cytosol to membranes on stimulation with fMLP, PMA or OAG (Ishitoya *et al.,* 1987;

Besterman *et al.*, 1986b), this finding may provide a clue as to the mode of DAG kinase activation on cell stimulation. Also, if extrapolated to the intact cell, it provides a means for compartmentalizing both PKC and DAG kinase in the membrane to compete for DAG.

5.3.2 Can the PKC inhibitor studies be reconciled with a role for PKC in the respiratory burst?

The main controversy of late involving the use of PKC inhibitors to investigate the oxidative response in neutrophils stems from studies carried out using dual inhibitors of both PKC and cyclic AMP-dependent protein kinase (PKA). PKA, which is activated by increased cyclic AMP, is a negative regulator of the oxidative burst (Smolen *et al.*, 1980; Fantone, *et al.*, 1984; De Togni *et al.*, 1984). Inhibiting both the positive PKC effects and the negative PKA effects could result in the "no effect" situation recorded on receptor stimulation with the non-specific inhibitors, H-7 (Wright & Hoffman, 1986; Seifert & Schachtele, 1988) and C-I (Gerard *et al.*, 1986). These authors reported inhibition of the PMA-triggered respiratory burst, so it was concluded that PKC mediated the response to the direct PKC activators but was not involved in the receptor-stimulated response. In contrast, other conflicting reports show a *total* inhibition of the burst stimulated by both fMLP and PMA in the presence of H-7 and C-I (Fujita *et al.*, 1986; Nath *et al.*, 1989).

As mentioned in the introduction the more selective PKC inhibitors, the sphingoid bases (Hannun *et al.*, 1986), are effective inhibitors of the burst whether activated by receptor stimulation or by direct PKC activation (Wilson *et al.*, 1986; Lambeth *et al.*, 1988). However, it should be noted that there are reports of non-selective effects of sphinganine on $[Ca^{2+}]_i$ levels (Pittet *et al.*, 1987) and of sphingosine inhibiting calmodulin-dependent enzymes (Jefferson & Schulman, 1988). Sphingosine has recently been shown to inhibit the 150kDa DAG kinase isoenzyme, while causing activation of the 80kDa isoform (Sakane *et al.*, 1989); these isoenzymes were derived from pig thymus but, since the neutrophil DAG kinase enzyme(s) have not yet been isolated, it is not known whether sphingosine has these effects in the neutrophil. Other PKC inhibitors which have been demonstrated to block neutrophil activation under some circumstances are polymixin B (Naccache *et al.*, 1985c) and retinal (Cooke & Hallett, 1985; Seifert & Schachtele, 1988) but both compounds also show a profile of effects that relate more closely to PKC activation than inhibition (Kiss *et al.*, 1987; Lochner *et al.*, 1986;

Seifert & Schachtele, 1988).

Data presented in this chapter show that the two potent, and reportedly specific inhibitors, K252a and staurosporine, inhibit the respiratory burst when activated not only by the direct PKC activators, PMA and diC_8, but also by the G-protein activator, fluoride, the Ca^{2+} ionophore, A23187, and the receptor stimuli, fMLP and PAF. These results are in agreement with previous reports where K252a has been shown to block fMLP-, PMA- and diC_8-stimulated O_2^- generation (Smith, R.J. *et al.*, 1988), and staurosporine has been shown to inhibit both the PMA- and fMLP-induced respiratory burst (Dewald *et al.*, 1989). In contrast, Sako *et al.* (1988) reported staurosporine to be only an inhibitor of the PMA O_2^- response with no effect on that induced by fMLP. These findings are also included in Table 5.3 for comparison. Also in contrast to the findings presented here, optimal stimulation of O_2^- release in guinea pig neutrophils by diC_8 was shown to be only approximately 25% inhibited in the presence of 150nM staurosporine, whereas the response stimulated with suboptimal diC_8 was greater than 70% inhibited at the same staurosporine concentration (Badwey *et al.*, 1989b). This study also reported an inhibition of the phosphorylation of the 47 and 49kDa proteins by staurosporine when either amount of diC_8 was utilized and concluded that diC_8 may function, under certain circumstances, in a stimulatory pathway for O_2^- release that is independent of PKC. However, the data presented in this current study show that responses obtained at high and low concentrations of diC_8 are totally inhibited at 60nM staurosporine.

The inhibition by both K252a and staurosporine of OZ- and heat-aggregated IgG-stimulated O_2^- generation has also been demonstrated (Twomey *et al.*, 1990b). However, the report by Koenderman *et al.* (1989) mentioned previously in the introduction, shows that the early period of OZ-induced respiratory burst activity, which occurred without accumulation of DAG, was insensitive to staurosporine, but the second phase of the OZ O_2^- response, which *was* accompanied by a rise in DAG, was markedly inhibited by staurosporine.

Clearly there is still some conflict. K252a and staurosporine are both potent inhibitors of the isolated PKC enzyme but, as can be seen from Table 5.1, only staurosporine has a degree of selectivity for PKC over PKA; K252a is approximately equipotent with respect to inhibition of either enzyme. Both compounds inhibit the Ca^{2+}/calmodulin-dependent kinase at concentrations similar to those for PKC inhibition (Table 5.1;

TABLE 5.4

Cellular effects of K252a and Staurosporine, independent of PKC inhibition

Inhibitor	Effect	Reference
K252a	inhibits MLCK (smooth muscle)	Nakanishi et al., 1988
K252a	inhibits MLCK (platelet)	Yamada et al., 1988
K252a	*activation of 76kDa protein kinase from porcine spleen	Gschwendt et al., 1989
K252a	inhibits calmodulin-dependent phosphodiesterase	Matsuda et al., 1988
Staurosporine	inhibits MLCK (platelet)	Watson et al., 1988
Staurosporine	inhibits mit-ogenesis induced by tyrosine kinase	Smith,C.D. et al., 1988
Staurosporine	cytotoxic to HeLa S3 cells ($IC_{50} = 4 \times 10^{-12}\mu$)	Tamaoki et al., 1986
Staurosporine	enhances HL-60 differentiation	Okazaki et al., 1988
Staurosporine	*induced changes on morphology and differentiation in mouse epidermal cells	Sako et al., 1988
Staurosporine	*induced association of PKC with erythrocyte membranes	Wolf & Baggiolini, 1988
Staurosporine	*induced the release of gelatinase and Vit-B_{12}-binding protein from human PMNs	Dewald et al., 1989

* denoting phorbol ester like effects of staurosporine and K252a.

Davis *et al.*, 1989). K252a has also been described as an inhibitor of the cyclic GMP-dependent kinase (Kase *et al.*, 1987) which like PKA could inhibit cell activation (Nishizuka, 1984b). In fact, both of these inhibitors have been described as having many cellular effects that could not be attributed to PKC, summarized in Table 5.4.

In view of these actions, what interpretation can be put on the inhibitory effect of these two compounds on the oxidative burst in this study? Clearly the inhibition of the response by all six stimuli could not be attributed to an effect of these compounds on PKA or cyclic GMP-dependent kinase since this would potentiate rather than decrease the burst - though some modulating actions through these enzymes cannot be ruled out. It is generally agreed that the calcium pathway is not of primary importance in respiratory burst activation (Lambeth, 1988) so an inhibition mediated through the Ca^{2+}/calmodulin-dependent kinase seems unlikely. Our results are unlikely to be due to the compounds inhibiting myosin light chain kinase (MLCK) since the motor appears not implicated in the oxidative burst; this is evidenced by the fact that cytoplasts which lack all the main elements of the motor are still capable of O_2^- generation (Roos *et al.*, 1983; Korchak *et al.*, 1983). Tyrosine kinases are mainly involved in those systems concerned with cell growth and metabolism (Hunter & Cooper, 1985) and rarely implicated in the signal transduction of short-term responses. That the inhibitory effects of these compounds was due to non-selective toxicity could not be totally ruled out but cell viability, as determined by trypan blue exclusion, was greater than 99% at the end of the experiment. Indeed, IC_{50} values obtained from the respiratory burst assay are only up to 10 times greater than those recorded against the isolated PMN PKC enzyme (with exception of the IC_{50} values for staurosporine with fMLP and PAF). This represents quite good agreement, considering the higher intracellular ATP levels in whole cells (of the order 1mM) as opposed to the lower concentrations ($\simeq$ 10μM) employed in the isolated PKC enzyme assays. Therefore it is proposed (but not finally proven) that the inhibition of the oxidative burst in this study is due mainly to inhibition of PKC. If one takes this view, our results with K252a and staurosporine lend credence to the hypothesis that PKC *does* have a role in the oxidative burst not only when stimulated directly by PKC activators, but also when stimulated by agents acting on receptors and at other points prior to the DAG/PKC pathway.

Some aspects of our results merit further consideration, namely both

inhibitors showed very different IC_{50} values with the various stimuli employed, and the relative rank order of inhibitory potency with the range of stimuli was very different for the two inhibitors. For K252a-induced inhibition of O_2^- release, the order of potency was:

fluoride > fMLP > PMA > PAF > diC_8.

For staurosporine-induced inhibition, the order of potency changed to:

fluoride > diC_8, PMA > fMLP > PAF.

In contrast, the PKC inhibitor sphinganine blocked superoxide generation stimulated with fMLP, DAG, PMA, OZ and AA at the same inhibitor concentration (Wilson *et al.*, 1986), the authors concluding that all the stimuli shared a common inhibited step. The results with K252a and staurosporine were surprising since both inhibitors are reported to inhibit competitively at the ATP substrate binding site and thus should have a similar inhibitory effect on all the stimuli, or at least should result in the stimuli being placed in the same order as regards susceptibility to inhibition. There are at least two explanations to account for the results:

(1) The stimuli employed could have different modes of cell activation and could be recruiting different PKC isoenzymes (see introduction of this chapter). The specific recruitment of distinct PKC isoenzymes under different activating conditions has not yet been verified but differences in sensitivity of these isoenzymes to K252a and staurosporine could account for the variation in the IC_{50} data.

(2) The role of PKA, which is known to modulate the oxidative burst (as detailed above), may vary quantitatively with different stimuli. K252a and staurosporine have different selectivity constants for inhibition of PKC over PKA (1.7 and 0.1 respectively) and this could explain the differing order of potency with the 6 different stimuli used. Different effects on other enzymes, such as the cGMP-dependent kinase, may also contribute.

The potentiating effect at low concentrations of staurosporine on both the fMLP- and PAF-stimulated O_2^- responses was unexpected and occurred over a very small concentration range. Staurosporine has been used as a PKC inhibitor in numerous studies on a variety of cell types, and a biphasic effect such as found in this study has not, to our knowledge, been reported. This potentiating effect may or may not be PKC-mediated. It is interesting to note that staurosporine has been described in a few systems as having PKC activating effects, similar to phorbol esters (see Table 5.4). Thus the staurosporine-mediated

potentiation of O_2^- generation may be due to a positive effect on PKC (or a specific PKC isoenzyme). Interestingly, a recent report by Reinhold *et al.* (1990) shows that staurosporine can lead to a marked, dose-dependent enhancement of phosphatidylethanol formation (indicative of PLD activation) in fMLP-stimulated human neutrophils. Even though the staurosporine concentrations showing this PLD-potentiating phenomenon were very much higher than the concentrations seen in the current study to potentiate the O_2^- response, it is intriguing to speculate that the latter effect may be in some way due to a PLD action. The enhancement of PLD activity could be mediated directly by staurosporine or, as purported by the above authors, PKC may normally exert an inhibitory effect on this enzyme which is blocked in the presence of staurosporine. Another possibility for staurosporine's effect on the O_2^- response could be that as in smooth muscle cells, PKC isoenzymes with both stimulant and inhibitory roles in transduction may exist (Kariya *et al.*, 1989) which could be differentially activated by different stimuli. The inhibition by staurosporine of two such isoenzymes at different concentrations could explain the biphasic effect.

The potentiating effect of staurosporine at low concentrations provides an explanation for the high IC_{50} values needed for inhibition of both fMLP- and PAF-stimulated O_2^- generation, in that the dose-inhibition curves could represent the resultant between the potentiating and inhibiting effects of staurosporine.

In view of the effects of both K252a and staurosporine on all the other protein kinases, in addition to PKC, it was deemed extremely important to study the effect of potent and more selective PKC inhibitors on the respiratory burst. The novel bis-indolyl maleimide inhibitors used in this study were found to be much more selective for PKC than for both PKA or Ca^{2+}/calmodulin-dependent kinase. This is particularly important when investigating a cellular response such as the respiratory burst which has been shown to be negatively modulated by PKA (De Togni *et al.*, 1984) and has been proposed to be mediated by a PKC-independent pathway, involving possibly a calmodulin-dependent kinase (Wright & Hoffman, 1986; Cooke & Hallett, 1985; Takeshige & Minakami, 1981). These novel compounds potently inhibited the respiratory burst stimulated by the PKC activator, diC_8, the G-protein activator, fluoride, and the receptor stimulus, fMLP. Thus, this data strengthens the conclusion from the K252a and staurosporine data,

where the reported inhibitions could not be definitively attributed to a PKC effect.

The relative rank order of inhibitory potency of the bis-indolyl maleimides in the isolated rat brain PKC assay:
Ro31-8425, Ro31-8288 > Ro31-8220 > Ro31-8161 > Ro31-8657 > Ro31-7549
correlated very closely to the order in both the diC_8-stimulated respiratory burst:
Ro31-8425 > Ro31-8220 > Ro31-8288 > Ro31-8161 > Ro31-7549 > Ro31-8657
and the fMLP-induced O_2^- burst:
Ro31-8425 > Ro31-8288 > Ro31-8220 > Ro31-8161 > Ro31-7549 > Ro31-8657.
It appears that intracellular PKC is likely to be the locus of action for these compounds in inhibiting the O_2^- response. However, approximately 100-fold greater concentrations were required for inhibition in the intact cell, which was again attributed to the higher intracellular ATP concentrations as opposed to those used in the isolated enzyme assay. This reduced potency at an intracellular site tallies with the observation that higher inhibitor concentrations were also required to antagonize the PMA-induced phosphorylation of a 47kDa protein in intact human platelets (Davis *et al.*, 1989).

Fluoride-mediated O_2^- release was much more susceptible to inhibition by K252a, staurosporine and the bis-indolyl maleimides compared to diC_8 and fMLP. One explanation for this phenomenon is that fluoride can cause a decrease in the intracellular ATP concentrations (Svec, 1985) such that the inhibitor concentration required to block PKC activity would be much reduced. Alternatively, fluoride may mobilize a different species of DAG (with different fatty acid side chains) which activates a particular PKC isoenzyme that is more sensitive to all these PKC inhibitors.

The bis-indolyl maleimide inhibitors were found to effect a marked rightward shift of the O_2^- dose-response curve stimulated by fluoride with a significant reduction of the maximum control response that could not be restored by increasing the fluoride concentration. On the other hand, only two compounds, Ro31-8288 and Ro31-8220, caused this pattern of inhibition of the diC_8-stimulated O_2^- dose-response curve. In these circumstances, it appeared that inhibiting PKC blocked the pathway for optimal NADPH oxidase activation, that could not be overcome by raising the stimulus concentration. In contrast, the diC_8-induced O_2^- dose-response was shifted rightwards in a parallel fashion in the presence of Ro31-8425, Ro31-7549 and Ro31-8161.

It should also be noted that another novel PKC inhibitor, 1-O-alkyl-2-O-methylglycerol (AMG), which is selective for PKC over both PKA and Ca^{2+}/calmodulin-dependent kinase, has been shown by Kramer *et al.* (1989) to inhibit the respiratory burst induced by both PDBu and fMLP. AMG-C_{16} (with an hexadecyl chain at the sn-1 position) was found to inhibit the O_2^- response induced by suboptimal concentrations of PDBu, but respiratory burst activity could be restored by the subsequent addition of a supraoptimal dose of PMA - thus indicating that only the activation of the NADPH oxidase via PKC was inhibited and not the enzyme itself. Inhibition of the fMLP-stimulated oxidative response by AMG-C_{16} was also dependent on the concentration of fMLP. Interestingly, this study also focused on inhibition by AMG-C_{16} of 47kDa protein phosphorylation which is is believed to be functionally associated with NADPH oxidase. AMG-C_{16} inhibited the phosphorylation of the 47kDa protein, at suboptimal PDBu concentrations; recovery of protein phosphorylation in parallel with that of the respiratory response was obtained by increasing the PDBu concentration. FMLP-induced 47kDa protein phosphorylation, which was also inhibited by AMG-C_{16}, could only be recovered at high concentrations of fMLP, even though respiratory burst activity was restored at lower concentrations - possibly indicating the involvement of a second signal. However, these data support a role for PKC in the activation of the respiratory burst by phorbol esters and fMLP, and are consistent with the data presented in this current study.

In conclusion, the neutrophil respiratory burst induced by diC_8, fluoride and fMLP is markedly inhibited by six novel selective PKC inhibitors; the oxidative burst induced by PMA, diC_8, fluoride, fMLP, PAF and A23187 is also markedly inhibited by the potent but less selective K252a and staurosporine. Taken together with the data that the respiratory burst stimulated with diC_8 and fluoride is increased when the metabolism of DAG is inhibited as also is the oxidative burst induced by fMLP, OZ, IgG and heat-aggregated IgG (Muid *et al.*, 1987), OAG and A23187 (Dale & Penfield, 1987) and γ-HCCH (Muid & Dale, unpublished observation), these results imply that the DAG/PKC pathway has a role in the neutrophil respiratory burst stimulated by both receptor and post-receptor mechanisms.

CHAPTER SIX

PROTEIN KINASE C INVOLVEMENT IN THE IP_3/Ca^{2+}-INDEPENDENT PATHWAY

SUMMARY:

— An activation sequence had been described whereby pretreatment of neutrophils with non-stimulatory doses of PMA (PMA priming) before subsequent stimulation with fMLP resulted in a potentiated respiratory burst that was PIP_2- and IP_3/Ca^{2+}-independent (Della Bianca *et al.*, 1986).

— It was found that fluoride also gave a potentiated burst after PMA priming, but the opsonized zymosan O_2^- response was unaffected by this treatment.

— The role of PKC in this PIP_2-independent pathway, stimulated by fMLP and fluoride, was addressed using the potent PKC inhibitor, K252a. K252a was also examined for an effect on the respiratory burst induced by ConA-zymosan in both normal and Ca^{2+}-depleted neutrophils; stimulation in the latter cells has been proposed to occur in the absence of PIP_2 hydrolysis (Rossi *et al.*, 1989).

— It was found that K252a inhibited the oxidative burst stimulated by fMLP and fluoride in both normal and PMA-primed cells. In addition, it inhibited O_2^- generation induced by ConA-zymosan in both normal and Ca^{2+}-depleted cells. These results are consistent with a role for PKC in both the PIP_2-dependent and independent transduction pathways initiated by the above stimuli.

— The PKC-dependent, PIP_2-independent transduction pathways that may be involved on stimulation of both the PMA-primed and Ca^{2+}-depleted neutrophils are discussed in relation to other routes of DAG generation and the possible participation of arachidonate.

6.1 INTRODUCTION

It has long been known that neutrophils challenged with two stimuli, administered either together or sequentially, give rise to much enhanced responses. Under priming conditions, the respiratory burst is characterized by lower concentration requirements for both stimuli, and an increase in the rate and duration of the O_2^- response (Dewald *et al.*, 1984; Dewald & Baggiolini, 1985; McPhail *et al.*, 1984a; Della Bianca *et al.*, 1986; Bass *et al.*, 1989).

There is good evidence to suggest that under certain of these priming activation sequences, the respiratory burst can occur with decreased or absent phosphoinositide turnover and little or no increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). Some activation sequences involve an initial minimal or threshold stimulation of PKC with PMA, followed by:

(i) fMLP stimulation in normal neutrophils, resulting in a greatly enhanced oxidative burst over and above that given by fMLP alone (Della Bianca *et al.*, 1986).

(ii) fMLP stimulation and response in Ca^{2+}-depleted neutrophils which would otherwise not respond to fMLP at all (Grzeskowiak *et al.*, 1986).

(iii) fluoride stimulation and response in Ca^{2+}-depleted neutrophils which would otherwise not respond to fluoride (Della Bianca *et al.*, 1988).

In these circumstances the potentiated respiratory burst, or the very existence of a burst in the case of the Ca^{2+}-depleted neutrophils, was demonstrated to occur in the absence of or with much reduced activation of 1) phosphoinositide breakdown, 2) phosphatidate (PA) accumulation, 3) generation of inositol phosphates (IP_1, IP_2 and IP_3) and 4) Ca^{2+} mobilization in normal cells (Grzeskowiak *et al.*, 1986; Della Bianca *et al.*, 1986). Activation with fMLP, both in the primed and unprimed state, was sensitive to pertussis toxin (Della Bianca *et al.*, 1986), indicative of G-protein participation. Furthermore, fluoride-induced translocation of PKC from the cytosol to the plasma membrane, observed under normal activating conditions, could not be reproduced in the primed activation sequence, which consisted of subthreshold exposure to PMA followed by fluoride stimulation in Ca^{2+}-depleted cells (Della Bianca *et al.*, 1988).

The previous two authors concluded that under certain priming conditions, activation at the receptor (by fMLP) or at the G-protein (by

fluoride) could trigger a sequence of transduction reactions for the activation of NADPH oxidase different from those involving the dual activation pathway, namely IP_3/Ca^{2+} and DAG/PKC. PKC activation must be involved in the priming stage because of the requirement for PMA, which then presumably "switches off" the phosphoinositide pathway either at the level of the receptor, the G-protein or the phospholipase. Subsequent stimulation by fMLP or by fluoride must involve an alternative G-protein-dependent pathway of NADPH oxidase activation. This alternative transduction pathway is as efficient or maybe even more efficient that the classical IP_3 and DAG dual activation pathway, as indicated by the potentiated respiratory burst. The involvement of DAG and PKC in this alternative pathway had been questioned by the above authors and was addressed in the current study using a potent and reportedly specific PKC inhibitor, K252a.

Another novel activation mechanism has recently been described (Rossi *et al.*, 1989). ConA-mediated phagocytosis of yeast and the accompanying respiratory burst were reported to be associated with phosphatidylinositol bisphosphate (PIP_2) turnover, inositol phosphate generation, a rise in $[Ca^{2+}]_i$ levels, formation of PA and the release of arachidonate (AA). But Ca^{2+}-depleted neutrophils, though still maintaining the capacity for both ConA-mediated phagocytosis of yeast cells and the associated respiratory burst, did not cause any turnover of phosphoinositides, generation of PA, release of AA or increase in $[Ca^{2+}]_i$. It was also reported that the phagocytosis and respiratory burst mediated by ConA-yeast, both in normal and Ca^{2+}-depleted cells, were not affected by H-7, a putative inhibitor of PKC. It was concluded that the activation of the above transduction pathways were unnecessary for both the ingestion of ConA-coated yeast particles and the associated respiratory burst, and were perhaps secondary events required for other cellular responses. The role of PKC in mediating the respiratory burst induced by ConA-coated zymosan (ConA-zymosan), in both normal and Ca^{2+}-depleted cells, was investigated in the current study with K252a, a potent putative inhibitor of this enzyme.

6.2 RESULTS

6.2.1 The effect of PMA priming on the O_2^- response to fMLP, fluoride and opsonized zymosan

The present study began with an investigation to see if subthreshold exposure to PMA before stimulation with other stimuli besides fMLP, namely fluoride and opsonized zymosan (OZ), could also result in a potentiated oxidative response. It was found that exposure to increasing non-stimulatory doses of PMA resulted in a dose-dependent potentiation of both the fMLP-stimulated O_2^- dose-response curve (confirming the previous report) and that stimulated by fluoride. The control concentration-response curves with both fMLP and fluoride under PMA priming conditions were displaced to the left and the maximum responses increased (see fig. 6.1a & b). In contrast, the OZ-induced O_2^- response was unaffected by PMA pretreatment. In fact, concentrations of PMA even higher than threshold (i.e. 1.5ng/ml which by itself elicits a minimal response) caused only a quantitatively similar increase of the OZ O_2^- dose-response curve (fig. 6.1c).

Thus, it can be concluded that the respiratory burst to both fMLP and fluoride are markedly potentiated by pretreatment with non-activating doses of PMA; this activation sequence has been reported to occur independently of IP_3/Ca^{2+} production. However, the respiratory burst induced by OZ appeared not to be potentiated in this manner. These data imply that under certain circumstances the IP_3/Ca^{2+} pathway and PIP_2 hydrolysis are not essential for the respiratory burst. Using K252a, we have attempted to address the role of PKC in these circumstances.

6.2.2 The effect of K252a on the PMA-primed responses to fMLP and fluoride

K252a, at 1μM, resulted in virtual obliteration of both the fMLP control and PMA-primed dose-response curves, as represented in fig. 6.2a (n = 3). Similar findings were observed with fluoride where K252a, at the lower concentration of 0.2μM, maximally inhibited both the control and PMA-potentiated dose-response curves (n = 3), as shown in fig. 6.2b. It is important to note here that the K252a was added to the cells after the PMA priming treatment and before stimulus activation, so it is the *secondary* putative PKC activation that is being inhibited in these experiments.

6.2.3 The effect of K252a on the ConA-zymosan-stimulated O_2^- response

The respiratory burst induced by ConA-zymosan in normal cells was inhibited markedly by 1μM K252a and the diminished O_2^- response induced by ConA-zymosan in Ca^{2+}-depleted cells was also inhibited by 1μM K252a, shifting the concentration-response curves to the right and depressing the maximum response. The mean normalized data from 4 experiments are presented in fig. 6.3. Furthermore, in collaborative experiments K252a has been demonstrated to effect a dose-related inhibition of ConA-zymosan-stimulated O_2^- release, in both normal and Ca^{2+}-depleted neutrophils (Twomey *et al.*, 1990a).

The mean resting $[Ca^{2+}]_i$ concentration for normal neutrophils, recorded by Quin 2 fluorescence was 125.09 $\pm$ 3.97nM (n = 3), whereas in Ca^{2+}-depleted cells the mean resting Ca^{2+} concentration was reduced to 20.86 $\pm$ 4.04nM (n = 3) (Twomey *et al.*, 1990a).

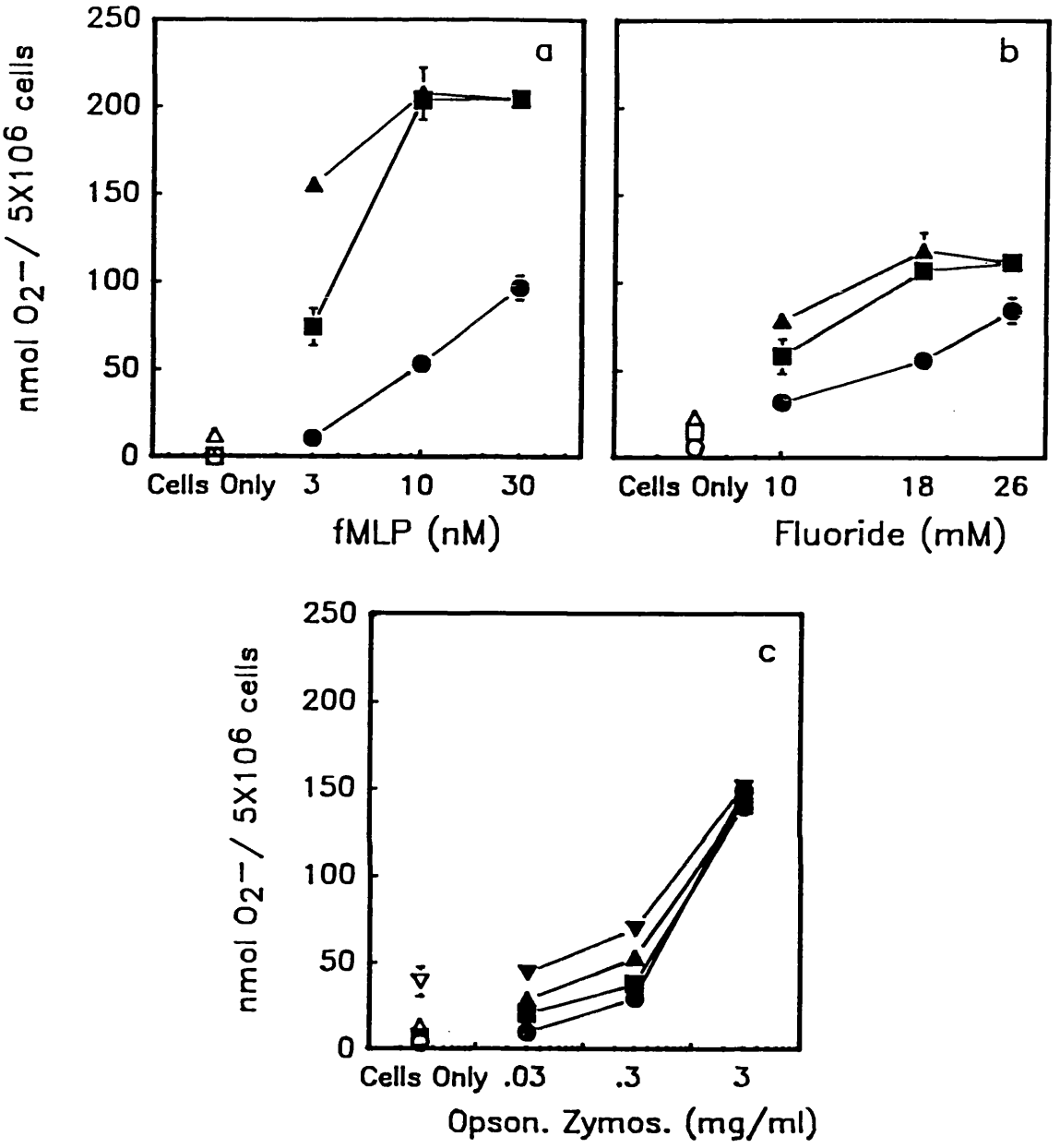


Figure 6.1: The dose-dependent effect of PMA pretreatment on subsequent O_2^- production stimulated by a) fMLP, b) fluoride and c) OZ. Cells alone (○) and in the presence of PMA at 0.5ng/ml (□), 1.0ng/ml (△) and 1.5ng/ml (▽). Stimulated responses were obtained either with control cells (●) or with cells that had been pretreated with PMA at 0.5ng/ml (■), 1.0ng/ml (▲) and 1.5ng/ml (▼). The graphs in a,b & c show the result of single experiments, but are representative of 4, 2 & 6 independent experiments respectively. All data points are the mean of sample duplicates ± range of the duplicates.

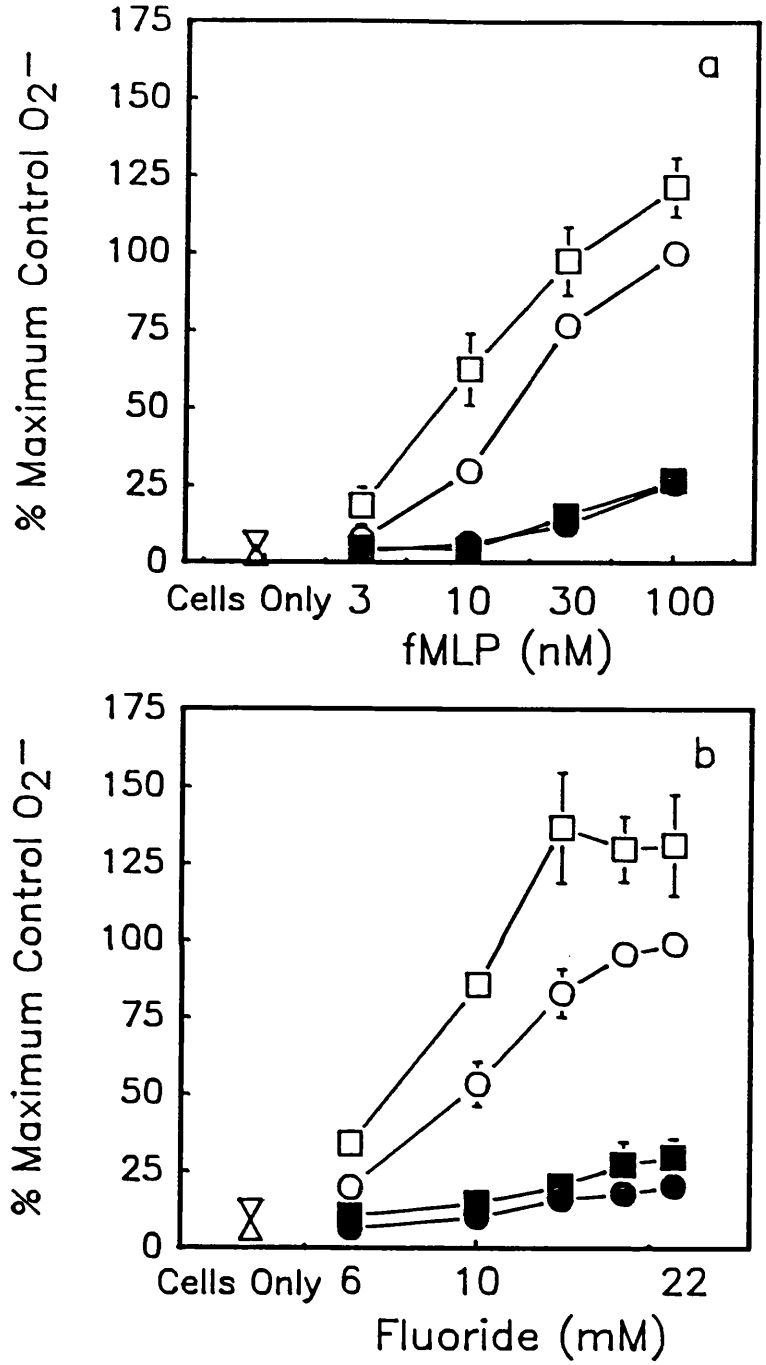


Figure 6.2: The effect of the PKC inhibitor, K252a, on a) fMLP- and b) fluoride-induced O_2^- production in both control and PMA-primed neutrophils. Responses were obtained in control cells with stimulus alone (O) and in the presence of K252a (●) or in PMA (0.5ng/ml)-primed cells with stimulus alone (□) and in the presence of K252a (■); K252a was 1μM in a and 0.2μM in b. (△) represents cells alone and (▽) cells in the presence of 0.5ng/ml PMA. The mean maximum control O_2^- release, as nmol/5×10^6 cells was 141.10 ± 15.75 for fMLP (n = 3) and 97.02 ± 11.76 for fluoride (n = 3). Error bars represent SE.

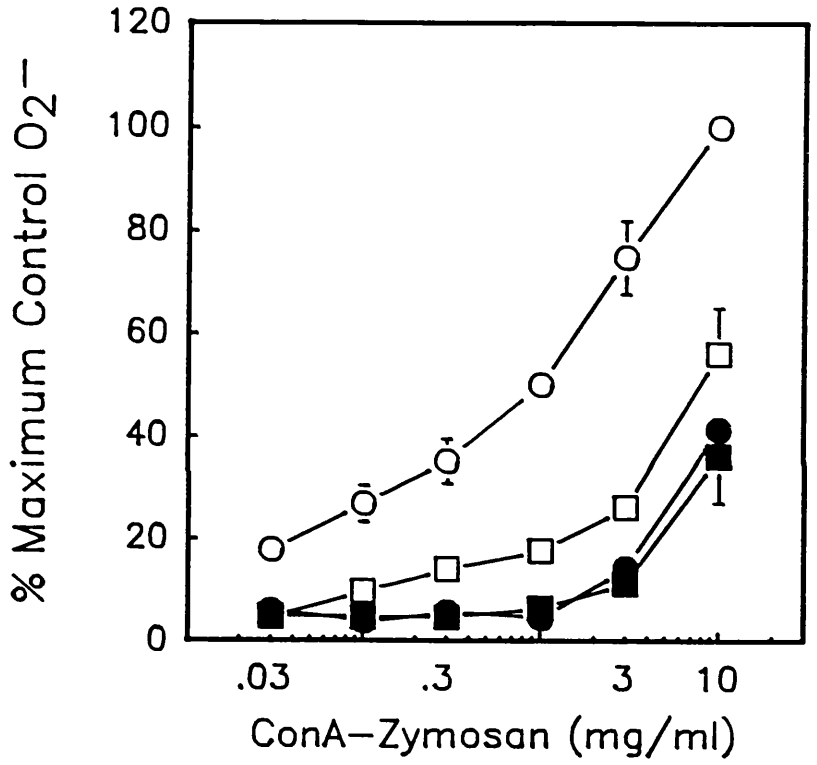
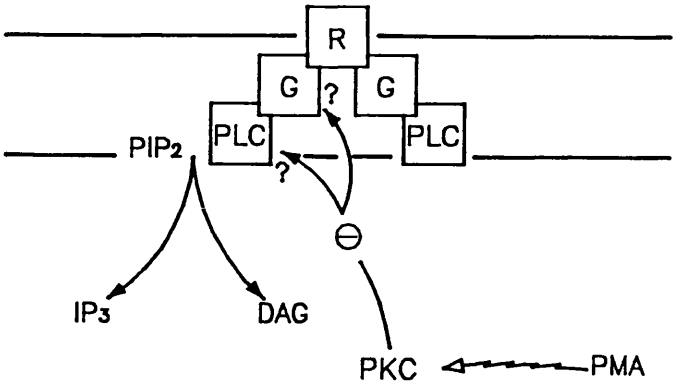


Figure 6.3: The effect of K252a on ConA-zymosan-induced O_2^- production in both control and Ca^{2+}-depleted cells. Responses were obtained in control cells with ConA-zymosan alone (O) and in the presence of 1μM K252a (●) or in Ca^{2+}-depleted cells with ConA-zymosan alone (□) and in the presence of 1μM K252a (■) (n = 3). Mean maximum control O_2^- release was 68.74 ± 8.49 nmol/5×10^6 cells. Error bars represent SE.

a) PMA Priming



b) Three possibilities for subsequent stimulation

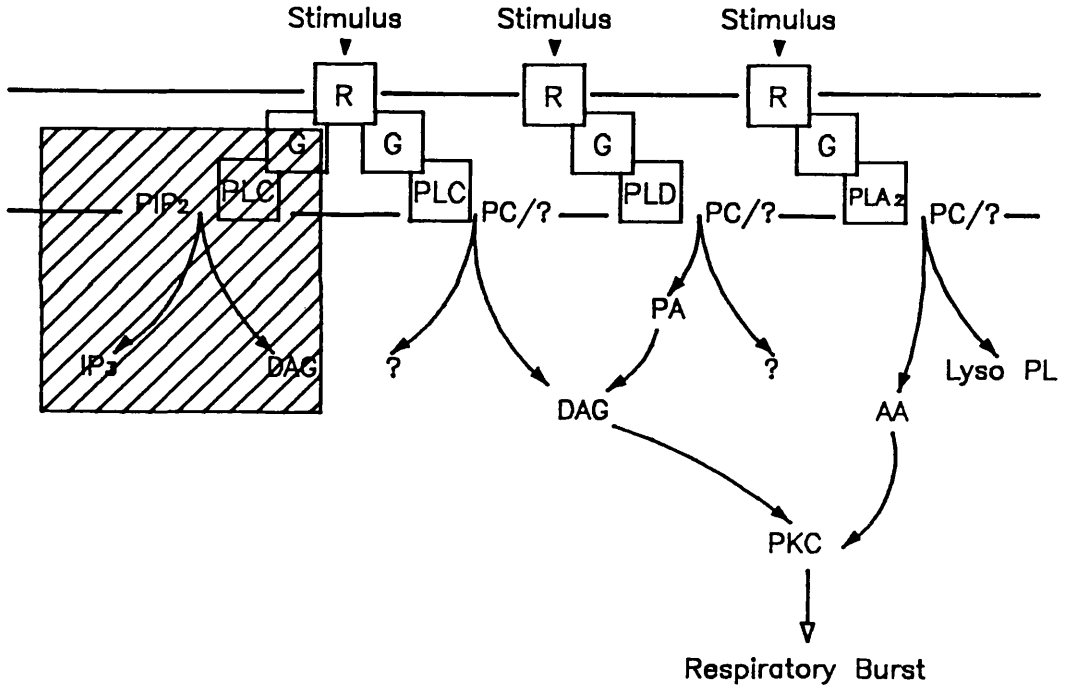


Figure 6.4: The sequence of transduction events postulated to occur on PMA priming of neutrophils and subsequent stimulation. a) The proposed outcome of PMA priming whereby PKC stimulation may switch off the PIP_2 pathway as shown. b) 3 possible options for mediating the respiratory burst (involving PKC activation) on receptor or G-protein stimulation:- breakdown of PC or any phospholipid (PL) by 1) a PLC action or 2) a PLD action giving rise to DAG either directly or via a PA route, 3) generation of AA, which might be the PKC activator, by a PLA_2-mediated hydrolysis of PC or any arachidonate-containing PL.

6.3 DISCUSSION

For neutrophil respiratory burst activation, a number of distinct activating sequences have been described that are PIP_2- and IP_3/Ca^{2+}-independent. These observations are seemingly in contrast with current opinion that the dual IP_3 and DAG activating pathway is responsible for mediating the oxidative burst, with the phosphoinositides being generally accepted as the main source of both second messengers. In this study, the role of PKC was addressed in two such reportedly PIP_2-independent transduction sequences - 1) the potentiated respiratory burst induced by fMLP or fluoride in PMA-primed cells and 2) the ConA-zymosan-induced O_2^- response in Ca^{2+}-depleted neutrophils. It was found that K252a, a potent PKC inhibitor, markedly inhibited the oxidative burst induced by both of these activation mechanisms. The activation of normal cells by fMLP, fluoride and ConA-zymosan i.e. reported to occur in association with phosphoinositide turnover (carried out in parallel with the PIP_2-independent activation methods) was inhibited to the same degree by K252a.

To the extent that K252a is selective for PKC, these results are consistent with the involvement of PKC both in the PIP_2/Ca^{2+}-dependent and independent transduction pathways leading to respiratory burst activation with fMLP, fluoride and ConA-zymosan.

6.3.1 What is the PKC-dependent, PIP_2-independent pathway?

In the novel activation mechanism which involves PMA priming before cell stimulation with fMLP or fluoride, PKC is certainly involved for the priming step, which probably results in a "turn-off" of the PIP_2 route of second messenger generation. Previous studies suggest that PKC activation can cause feedback inhibition of further hydrolysis of inositol phospholipids (Smith C.D. *et al.*, 1987; Kikuchi *et al.*, 1987). In the above experiments, K252a was shown to inhibit the subsequent O_2^- response after PMA primin g. This is consistent with PKC being involved in the potentiated response on stimulus activation. The question was - what was the activating pathway recruited on subsequent administration of fluoride or fMLP? It has been shown in neutrophils that priming with PMA before fMLP stimulation augmented the generation of DAG about 2-fold over and above that induced by fMLP alone, the level of which correlated with the potentiated O_2^- response (Tyagi *et al.*, 1988). This latter study also suggested that the enhancement of DAG generation was concomitant with switching the source of DAG from PIP_2 to an

alternative phospholipid, and reported that the sequence of events was blocked by the specific PKC inhibitor, sphinganine. Importantly, the study by Tyagi *et al.* (1988) also showed that PA levels were increased at early time points by stimulation of PMA-primed cells. Possible sources of the observed DAG increase include hydrolysis of other phospholipids or triglyceride, or even *de novo* synthesis.

Breakdown of phosphatidylcholine (PC) to generate DAG has been noted in several tissues in response to phorbol esters and PKC-linked agonists (Irving & Exton, 1987; Besterman *et al.*, 1986a; Daniel *et al.*, 1986; Guy & Murray, 1982; Rider *et al.*, 1988). In platelets, a number of phospholipids - PC, phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and 1-alkenyl-2-acyl-PE were depleted in response to agonists (Takamura *et al*, 1987). Additional evidence that PC can serve as a source of DAG comes from the identification of a PC-specific phospholipase C (PLC) (Wolf & Gross, 1985). There are at least 3 different ways in which PC degradation could give rise to DAG production:- 1) by a PLC-catalyzed degradation of PC with the production of phosphocholine and DAG, 2) by the sequential action of PLD, which would give rise to PA, and PA phosphohydrolase, which can further metabolize the PA to DAG (see Pappu & Hauser, 1983) and 3) by a PKC-mediated breakdown of PC involving an, as yet, unknown mechanism (Pelech & Vance, 1989).

It has been reported that phorbol diesters, serum and platelet-derived growth factor can activate the hydrolysis of PC within seconds in the pre-adipocytic cell line 3T3-L1 by a PLC-mediated mechanism (Besterman *et al.*, 1986a), and that PLC treatment of Chinese hamster ovary and HeLa cells increased rates of PC hydrolysis with the production of DAG and the incorporation of choline into PC (Kent *et al.*, 1986). Interestingly, activation of neutrophils by PMA, ionophore or fMLP has been shown to lead to a sustained DAG accumulation accompanied by the disappearance of PC (Truett *et al.*, 1989). The authors proposed a PC-specific PLD as the plausible mechanism for the hydrolytic reactions. Moreover, a recent study has suggested that PLD-mediated diradylglycerol formation correlates with superoxide production in fMLP-stimulated human neutrophils (Bonser *et al.*, 1989). There is also evidence that phorbol diesters can activate the PLD pathway of DAG generation from PC in many cell types, thus implicating the involvement of PKC (Cabot *et al.*, 1989; Kinsky *et al.*, 1989; Hii *et al.*, 1989). Another study reports that dioctanoylglycerol as well as phorbol diesters can

stimulate the accumulation of both diacylglycerols and alkylacylglycerols in human neutrophils, the accumulation being sustained for long time periods (Rider *et al.*, 1988).

Neutrophils contain large amounts of 1-O-alkyl-2-acylglycero-phosphocholine (1-O-alky-2-acyl-GPC), constituting almost half the total diradyl-GPC fraction (Mueller *et al.*, 1984), which has been demonstrated to be hydrolyzed via a PLD mechanism by both receptor agonists (fMLP) and phorbol esters to yield 1-O-alkyl-2-acyl-PA and 1-O-alkyl-2-acyl-glycerol (Agwu *et al.*, 1989; Pai *et al.*, 1988; Gelas *et al.*, 1989). However, the importance of 1-O-alkyl-2-acylglycerols in cellular responses is not yet clear.

Therefore, subsequent to PMA priming of neutrophils, receptor stimulation (as with fMLP) or G-protein stimulation (as with fluoride) could trigger DAG formation by a PC-specific PLC or (as seems more likely from the above reports) a PC-specific PLD mechanism. It has been demonstrated that PC breakdown in endothelial cells via the activation of PLD is coupled to a guanine nucleotide-binding protein (Martin & Michaelis, 1989), which lends further support to the above possibility. A receptor or a G-protein coupled to a PC-specific PLD could provide a source of non-PIP_2 DAG in the fMLP- or fluoride-stimulated PMA-primed cells that would give rise to the PKC-dependent respiratory burst. The DAG thus generated, or alternatively the priming PMA, may activate PKC to stimulate breakdown of further choline-linked phospholipids, as inferred from some of the studies mentioned above in which phorbol esters were reported to stimulate PC turnover in many cell types, by a PKC-directed mechanism of PLD activation. Therefore, a positive-feedback system that enhances or prolongs the stimulus-evoked production of DAG may exist, opposing the negative-feedback system that terminates the phosphoinositide pathway of DAG and IP_3 generation. This positive-feedback may contribute to the potentiated respiratory burst in PMA-primed cells.

However, the possibility that AA may play a part cannot be ruled out. Receptor stimulation can activate phospholipase A_2 (PLA_2) which gives rise to AA (Dahinden *et al.*, 1988) and there is evidence that this may be coupled via a distinct G-protein from that which regulates the PIP_2-dependent PLC (Axelrod *et al.*, 1988; Burgoyne *et al.*, 1987; Cockcroft & Stutchfield, 1989). AA and some of its metabolic products are known to be PKC activators (Hansson *et al.*, 1986; O'Brian *et al.*, 1988). Thus, fMLP- or fluoride-induced activation in PMA-primed cells

could involve a PLA_2-linked pathway. The above three possibilities for PIP_2-independent transduction are outlined in fig. 6.4.

The other novel activation mechanism, namely the ConA-zymosan-mediated oxidative burst in Ca^{2+}-depleted cells, has been reported to occur not only in the absence of PIP_2 hydrolysis but also of PA generation and AA release (Rossi *et al*, 1989). This transduction pathway was also described as PKC-independent from studies carried out using the non-specific PKC inhibitor, H-7. In the present study K252a, which is reported to be a more potent and more specific PKC inhibitor, produced a dose-related inhibition of the ConA-zymosan-mediated oxidative burst in both normal and Ca^{2+}-depleted neutrophils. This suggests that PKC activation *is* involved in burst activation with this stimulus. The endogenous PKC activator, however, is unlikely to be PLD-derived DAG, as suggested for the above PIP_2-independent activation sequence, since there was no increase in PA. Rossi *et al.* (1989) also reported that the ConA-zymosan-induced burst, in both normal and Ca^{2+}-depleted neutrophils, was insensitive to pertussis toxin but was blocked by cytochalasin B. It is therefore possible that DAG could be generated either by a non-G-protein-sensitive PLC or by a pertussis toxin-insensitive G-protein coupled to PLC, possibly acting on PC, which is inhibited by cytochalasin B. One could hypothesize that this DAG would be metabolized not by the DAG kinase pathway, which leads to PA, but by DAG lipase.

Whichever pathway(s) is involved in PIP_2-independent activation, our results with K252a in the present study support a role for PKC in the transduction pathways leading to O_2^- generation.

Taken together with the results from the previous chapter, where the O_2^- response induced by a number of receptor and post-receptor stimuli was shown to be inhibited by an array of specific PKC inhibitors, it is proposed that PKC plays a crucial role, both in the PIP_2-dependent (physiological activation) and independent pathways of respiratory burst activation.

6.3.2 Is the stimulated respiratory burst dependent on basal $[Ca^{2+}]_i$, a Ca^{2+} flux or neither?

PMA-primed neutrophils show a reduced Ca^{2+} flux on activation with fMLP and fluoride, when compared to normal neutrophils, despite a much potentiated respiratory burst. This could be interpreted in two ways

1) the alternative transduction pathway activated by fMLP or fluoride in the PMA-primed cells gives rise to a potent intracellular messenger(s) that more than compensates for the reduced Ca^{2+} levels or 2) the mobilization of Ca^{2+} that occurs in normal cell activation is not necessary for mediating the respiratory burst. However, the transduction sequence that operates on stimulation of PMA-primed cells may not come into play during *normal* burst activation, which may still necessitate a Ca^{2+} flux. It can be concluded that the report of Della Bianca *et al.* (1986) indicates that only the increased Ca^{2+} signal was diminished on activation of the PMA-primed cells; basal $[Ca^{2+}]_i$ levels were not affected.

Data is presented in the present chapter in agreement with a previous report (Rossi *et al.*, 1989) to show that O_2^- generation can be induced by ConA-zymosan in neutrophils that are depleted of $[Ca^{2+}]_i$ (i.e. levels of 20nM as opposed to normal resting levels of 125nM). This indicates that, under certain unique conditions, activation of NADPH oxidase occurs not only in the absence of a Ca^{2+} flux but also when resting $[Ca^{2+}]_i$ levels are diminished. Also, O_2^- generation can be induced in Ca^{2+}-depleted cells by either fMLP or fluoride, provided there is prior subthreshold exposure to PMA (Grzeskowiak *et al.*, 1986; Della Bianca *et al.*, 1988). In another study, it has been shown that double stimulation with fMLP and ConA, given in sequence or simultaneously, restores the activation of the respiratory burst in Ca^{2+}-depleted cells that would not occur with either stimulus alone (Rossi *et al.*, 1986b). Thus it is reasonable to infer that the respiratory burst can be activated (albeit not optimally in some cases) by mechanisms virtually independent of the presence of Ca^{2+}. However, it needs to be stressed that the measurements of $[Ca^{2+}]_i$ in Ca^{2+}-depleted cells may give an inaccurate indication of the true intracellular situation, in that the Ca^{2+} necessary for signal transduction could be sequestered in specific areas (e.g. calciosome) and may not be depleted in line with depletion of general cytosolic Ca^{2+}.

Taken together these results indicate that NADPH oxidase activation is not dependent on a Ca^{2+} flux or on resting $[Ca^{2+}]_i$ levels under certain activating conditions, and may reflect the fact that the oxidative burst is not necessarily dependent on this second messenger under normal physiological conditions. Conflicting results have been reported regarding the effect of Ca^{2+} on the catalytic activity of isolated membrane-associated NADPH oxidase from activated leukocytes.

According to some reports optimal activity of the enzyme requires Ca^{2+} (Suzuki *et al.*, 1985; Green *et al.*, 1983) while according to others the oxidase is unaffected by Ca^{2+} (Yamaguchi *et al.*, 1983). Activation of isolated NADPH oxidase from unstimulated cells by AA and a cytosolic component has been demonstrated to require Mg^{2+} but not Ca^{2+} (Maridonneau-Parini & Tauber, 1986; Clark *et al.*, 1987).

In this context, it is relevant to mention that O_2^- release can be elicited maximally by certain stimuli such as PMA and synthetic DAGs without a rise in $[Ca^{2+}]_i$ (Sha'afi *et al.*, 1983) and also in Ca^{2+}-depleted neutrophils (Grzeskowiak *et al.*, 1986; Di Virgilio *et al.*, 1984). Furthermore, a series of Ca^{2+}-linked stimuli (fMLP, LTB_4 and PAF) produced similar kinetics and magnitudes of $[Ca^{2+}]_i$ elevation, but resulted in widely different rates of O_2^- production (Korchak *et al.*, 1988b)

Nevertheless, there is much evidence that A23187 and either PMA or OAG can synergize to activate the respiratory burst (Dale and Penfield, 1984; Di Virgilio *et al.*, 1984). Also, low concentrations of the Ca^{2+}-linked stimuli fMLP, 5-HETE and Ca^{2+} ionophore can prime neutrophils for activation by PMA and DAG (Badwey *et al.*, 1988; Bass *et al.*, 1987; Dewald *et al.*, 1984; O'Flaherty & Nishihira, 1987). Priming by Ca^{2+}-linked stimuli has been correlated with enhanced binding of [^{3}H]PDBu to its PKC receptor (French *et al.*, 1987; Dougherty & Niedel, 1986) and furthermore, increased $[Ca^{2+}]_i$ leads to translocation of PKC to the membrane fraction (O'Flaherty & Nishihira, 1987). Both these observations would support the argument presented in Chapter 1, that a Ca^{2+} flux alone is not a sufficient signal for activation of the respiratory burst, although it can augment or accelerate the response.

In conclusion, it appears that the NADPH oxidase itself can function independently of Ca^{2+} and that a Ca^{2+} flux is not sufficient *per se* for activation of the respiratory burst but may augment the response in conjunction with other messengers. In addition, basal $[Ca^{2+}]_i$ may be required for optimal activation of those enzymes involved in the transduction sequences leading to the activation of NADPH oxidase.

CHAPTER SEVEN

ARACHIDONATE AND ITS METABOLITES

SUMMARY:

— The participation of endogenously released arachidonate (AA) and its subsequent metabolism by the 5-lipoxygenase (5-LO) pathway in mediating the respiratory burst was addressed in this chapter.

— When a series of 5-LO inhibitors, namely BW A4C, Rev 5901 and piriprost (U60,257) were examined for an effect on O_2^- generation, induced by opsonized zymosan (OZ), fMLP, fluoride and diC_8, it was found that these agents had no significant effect on the response at or near their IC_{50} concentrations. Thus, it appears that 5-LO metabolites do not play a predominant role in the O_2^- response.

— The involvement of phospholipase A_2 (PLA_2) activation in the O_2^- response was investigated using two reported PLA_2 inhibitors, compound I (chloracysine) and compound II. It was found that these compounds potently inhibited O_2^- release induced by fMLP, OZ and fluoride, and had little effect on the response stimulated by PAF, A23187 or diC_8. Although compound I and II have been shown in this study to inhibit OZ-induced LTB_4 release, the selectivity of the compounds for PLA_2 is questioned and the results interpreted with caution.

— The participation of endogenously released AA was also examined using a combination of inhibitors, namely a DAG kinase inhibitor (R59022), a DAG lipase inhibitor (RHC80267) and an inhibitor of PLA_2 (either indomethacin or sodium meclofenamate) which should block the main routes of AA release. This drug combination was shown to cause a marked enhancement of O_2^- production stimulated with the post-receptor stimuli, fluoride and γ-hexachlorocyclohexane (γ-HCCH), while that induced by the receptor stimuli, OZ and IgG, was decreased (Muid *et al.*, 1988). On one interpretation these results call into question the role of AA in the transduction of O_2^- generation by post-receptor stimuli, but support a role for AA in receptor-mediated transduction. Other possible explanations for the drug effects are also offered.

7.1 INTRODUCTION

Arachidonate acid (AA) is a 20 carbon, unsaturated fatty acid which is stored esterified at the sn-2 position in glycerophospholipids of the plasma membrane. It can be released following cell stimulation by one or more of the following pathways (reviewed in Irvine, 1982; Nakashima *et al.*, 1988). Firstly, AA can be cleaved from phospholipids or phosphatidic acid (PA) by a phospholipase A_2 (PLA_2) action. Secondly, diacylglycerol lipase can cleave AA from diacylglycerol (DAG), formed on hydrolysis of the phosphoinositides by phospholipase C (PLC). (Note that DAG could also be derived from other phospholipids via the action of a PLC or a PLD). In terms of mass, the first pathway is more important than the second. PLA_2 can act on a variety of diacyl-containing phospholipids - phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Nielsen *et al.*, 1988; Nakashima *et al.*, 1988), PA as well as 1-O-alkyl-2-acyl-glycerophospholipids (Chilton, 1989). When the substrate is alkyl-PC the products are not only AA, but also lyso-alkyl-PC which can be converted by an acetyltransferase into 1-O-alkyl-2-acetyl-PC or PAF (Chilton *et al.*, 1984; Vargaftig & Benveniste, 1983).

There are a number of reports suggesting different mechanisms by which the activation of PLA_2 is regulated. PLA_2 can be activated *in vitro* by unphysiologically high levels of Ca^{2+} (Jesse & Franson, 1979) and it is generally agreed that Ca^{2+} is necessary for the activity of this enzyme *in vivo* (Sha'afi & Molski, 1988). However, recent evidence suggests that exogenously added DAGs (Kramer *et al.*, 1987) and PMA (McColl *et al.*, 1986; McIntyre *et al.*, 1987) can synergize with Ca^{2+} in activating cellular PLA_2. Thus, phosphoinositide hydrolysis by PLC may contribute to the regulation of PLA_2, via Ca^{2+} and DAG increases, consistent with the finding that activation of PLC precedes that of PLA_2 in stimulated guinea pig neutrophils (Takenawa *et al.*, 1983). Also, PLA_2 activation in fMLP-stimulated HL-60 cells has been shown to involve co-operative interactions between DAG formed endogenously and Ca^{2+} (Billah & Siegel, 1987). Other studies report that GTP analogues induced the release of [^{3}H]AA from prelabelled permeabilized rabbit neutrophils (Nakashima *et al.*, 1988) and that pertussis toxin inhibited the PAF-induced [^{3}H]AA release but not the rise in $[Ca^{2+}]_i$ in these same cells (Tao *et al.*, 1989). These effects were also observed in platelets (Nakashima *et al.*, 1987; Fuse & Tai, 1987) and the authors concluded that receptor stimulation linked to PLA_2 activation is coupled by a

G-protein distinct from that which links the receptor to PLC. Evidence is also presented by Cockcroft & Stutchfield (1989) showing that PLA_2 is receptor-coupled in HL-60 cells and human neutrophils, independently of PLC, via a distinct pertussis toxin-sensitive G-protein. Furthermore, there is evidence of receptor-mediated activation of PLA_2 via GTP-binding proteins distinct from those that regulate the phosphoinositide pathway in other cell types (reviewed in Axelrod *et al.*, 1988; Burgoyne *et al.*, 1987).

It has been shown that there are two major pathways of metabolism of free AA, the cyclooxygenase pathway to form prostaglandins (e.g. PGE_2, PGI_2) and thromboxanes (e.g. TXA_2, TXB_2), and the lipoxygenase pathway to generate a series of hydroperoxy and hydroxy fatty acids (e.g. HPETEs, HETES and leukotrienes). The preferential pathway of AA metabolism in PMNs is via the lipoxygenase (Samuelsson *et al.*, 1980; Walsh *et al.*, 1981; Marcus *et al.*, 1984) and more specifically the 5- and 15-lipoxygenase. AA is converted, via the 5-lipoxygenase (5-LO) pathway to 5-HPETE, which may be further transformed into leukotrienes (see fig. 7.1). Leukotriene B_4 (LTB_4) is thought to be an extremely potent endogenous mediator of inflammation (Ford-Hutchinson *et al.*, 1980; Samuelsson, 1983; Lewis & Austin, 1984). It can cause PMNs to move to a site of inflammation, to degranulate and to generate toxic oxygen radicals (Bray *et al.*, 1980; Serhan *et al.*, 1982; Omann *et al.*, 1987).

Another series of oxygenated products of AA can be derived through the action of 5-LO on 15-HPETE leading to the formation of lipoxins A and B (see fig. 7.1) (Serhan *et al.*, 1984). Lipoxin A has been shown to be a potent activator of both O_2^- production and lysosomal enzyme release in PMNs (Serhan *et al.*, 1984).

PLA_2-associated pathways in the neutrophil give rise not only to AA, but also to PAF, prostaglandins, thromboxanes, leukotrienes and lipoxins. The flux through these pathways is determined in part by the activity of PLA_2, which limits the availability of AA (Jouvin-Marche *et al.*, 1984; McIntyre *et al.*, 1987; Sun and McGuire, 1984). However, additional regulation at the level of the other enzymes involved may also dictate the distribution of products. Modulation induced by inflammatory agents at the 5-LO has been implied (Clancy *et al.*, 1983). AA has been shown to be used not only as a substrate, but also an activator of the 5-LO in a pertussis toxin-sensitive manner (McColl *et al.*, 1989). While PMA alone failed to induce LTB_4 release, it enhanced the release of LTB_4 and other

leukotrienes in conjunction with A23187 and the receptor stimuli - fMLP and OZ - implicating a role for PKC in modulating 5-LO activity in stimulated human neutrophils (Liles *et al.*, 1987; Raulf & Konig, 1988). Additional regulation at the level of other metabolic enzymes, such as PAF acetyltransferase has also been demonstrated (Alonso *et al.*, 1982).

The most potent stimulus for activation of the PLA_2-linked pathway is A23187 which has been shown in numerous studies to cause marked release of [^{3}H]AA from prelabelled neutrophils (Walsh *et al.*, 1981; Godfrey *et al.*, 1987; Foster & Rush, 1986; Bokoch & Gilman, 1984), as well as production of PAF, LTB_4 and HETEs (Borgeat & Samuelsson, 1979; Palmer & Salmon, 1983). In contrast, fMLP is a poor stimulant of AA release from human PMNs (Mahadevappa & Powell, 1989). Another study has shown that fMLP does not induce the mobilization of any endogenous AA from rabbit PMNs, unless a second signal (e.g. A23187) is also present (Meade *et al.*, 1986); nor does it cause production of LTB_4 or other lipoxygenase metabolites from human PMNs unless a high exogenous source of AA is provided (Haines *et al.*, 1987). On the contrary, there is a report in which rabbit neutrophils stimulated with either fMLP or PAF have been demonstrated to release [^{3}H]AA (Tao *et al.*, 1989), and guinea pig neutrophils were also found to release [^{3}H]AA in response to fMLP (Bokoch & Gilman, 1984). On the other hand, the release of AA, albeit of smaller magnitude and more variable than that induced by A23187, can be effected by OZ (Walsh *et al.*, 1981; Maridonneau-Parini *et al.*, 1986), immune complexes (Godfrey *et al.*, 1987) and fluoride (Bokoch & Gilman, 1984). Metabolism to other products was also demonstrated with these latter stimuli (this subject will be readdressed in the discussion). Interestingly, the mobilization of AA was pertussis toxin-resistant in the case of A23187 but sensitive to this treatment with fMLP, fluoride (Bokoch & Gilman, 1984; Ohta *et al.*, 1985) and PAF (Tao *et al.*, 1989). Another study reports that fluoride stimulated the generation of leukotrienes from human PMNs but their release was not influenced by pertussis or cholera toxin (Brom *et al.*, 1989). In this same study, stimulation with fluoride caused an enhanced exchange rate for GDP with GTP, indicating that a direct activation of G-proteins could be involved in generation of the lipoxygenase products.

The possible participation of AA or one of its oxygenated metabolites in the activation of the respiratory burst has received much attention of late. Evidence in favour of this idea comes from the following observations:

(1) Exogenous AA as well as other cis-unsaturated fatty acids have been found to stimulate O_2^- production in neutrophils and macrophages (Curnutte *et al.*, 1984; Badwey *et al.*, 1981; Morimoto *et al.*, 1986; Bromberg & Pick, 1983). LTB_4 alone can induce a very short oxidative burst (Omann *et al.*, 1987) and can prime cells for an increased response to other stimuli such as fMLP (Dewald & Baggiolini, 1985). 5-HETE can also prime cells in response to phorbol ester and DAG (Badwey *et al.*, 1988).

(2) AA, myristic acid, linolenic acid and other unsaturated long-chain fatty acids as well as lipoxins A and B and hydroperoxy derivatives of the unsaturated fatty acids were found to activate PKC isolated from human neutrophils and other sources (McPhail *et al.*, 1984b; Morimoto *et al.*, 1988; Hansson *et al.*, 1986; O'Brian *et al.*, 1988; Murakami & Routtenberg, 1985). In most of these studies, AA or its metabolites substituted for phospholipid in the PKC assay, and in some cases the activation was independent of Ca^{2+}.

(3) The addition of AA to rabbit, guinea pig and human neutrophils has been reported to cause a rapid and dose-dependent rise in the level of $[Ca^{2+}]_i$ (Volpi *et al.*, 1984; Morimoto *et al.*, 1986; Beaumier *et al.*, 1987); the activity of AA appeared to be due to the fatty acid itself and not to one of its metabolites. In a more recent study, data was presented to indicate that the mechanism of mobilization of Ca^{2+} by AA in human neutrophils was complex, involving two temporally distinct phases and specific activation pathways (Naccache *et al.*, 1989). LTB_4 also causes a rise in $[Ca^{2+}]_i$ without the breakdown of polyphosphoinositides and the production of PA (Lew *et al.*, 1984a; Volpi *et al.*, 1984).

(4) Both cis- and trans-unsaturated fatty acids can activate isolated NADPH oxidase from plasma membranes of human neutrophils in the presence of a neutrophil cytosolic factor (McPhail *et al.*, 1985; Seifert & Schultz, 1987); this mechanism of activation has been demonstrated to be independent of PKC activation (Cox *et al.*, 1987; Seifert & Schultz, 1987); although, in one study the cytosolic factor was identified as PKC (Cox *et al.*, 1985).

(5) As stated above, activation of neutrophils by a number of stimuli has been shown to cause AA release, possibly through the involvement of PLA_2. Mepacrine and p-bromophenacyl bromide (BPB), inhibitors of PLA_2 (Chang *et al.*, 1987), have been reported to inhibit both the release of [^{3}H]AA and the generation of O_2^- stimulated by fMLP and OZ (Foster & Rush, 1986; Smolen & Weissmann, 1980; Maridonneau-Parini *et al.*, 1986). O_2^- production in response to fMLP and ConA in guinea pig macrophages was also inhibited by BPB (Bromberg & Pick, 1983). However, interpretation of results is complicated by the lack of specificity of these inhibitors. For example, Hoffman *et al.* (1982) found that both mepacrine and BPB also inhibited the PI-specific PLC and furthermore, that BPB inhibited DAG lipase. In addition, Lanni & Becker (1985) found BPB to exert no inhibitory effect on PLA_2 in intact neutrophils. In contrast, Duque *et al.* (1986) found a significant inhibition of a membranous fraction of fMLP-stimulated PLA_2 activity by BPB in rat neutrophils, although total cellular PLA_2 was only minimally affected. This study also reported an inhibition of both fMLP- and PMA-induced O_2^- generation and lysosomal enzyme release by BPB but in addition, it caused an inhibition of the change in transmembrane potential associated with stimulus-response coupling, which could explain the inhibition of the cellular responses. A more recent study reports that the stimulation of human neutrophil NADPH oxidase by PMA was inhibited by a variety of PLA_2 inhibitors, including BPB but also a number of newly developed compounds, namely Ro31-4639, and Beecham compounds I and II (Henderson *et al.*, 1989). The authors concluded that AA released by PLA_2 is necessary for both the activation and the maintenance of O_2^- generation by NADPH oxidase.

In this present study, the above compound I (chloracysine, 2-chloro -10-(3-Diethylamino-1-oxypropyl)-10H-phenothiazine) and compound II (2-chloro-10-(3[4methyl-1-piperazinyl]-1-oxypropyl-10H-phenothiazine) were tested for their effect on stimulated O_2^- production induced by fluoride, fMLP, PAF, A23187 and diC_8. It should be noted that although both compounds I and II have been described as putative PLA_2 inhibitors, there is very little information as yet to support such a mechanism of action. Chloracysine has been shown to inhibit the rise in rat gastric mucosal 6-keto-$PGF_{1\alpha}$ production induced by PLA_2 (from *Naja*

naja) (Melarange & Gillett, 1988). In some preliminary studies the stimulated release of preincorporated [^{14}C]AA from human leukocytes was also inhibited by chloracysine (R. Melarange, personal communication). The action of these two postulated PLA_2 inhibitors, together with their effects on the stimulated respiratory burst, will be discussed more extensively in the discussion.

From the observations outlined above it appears that exogenous AA or one its metabolites could be involved in stimulus-activation coupling for the neutrophil respiratory burst. However, whether endogenously released AA or another metabolic product of AA is implicated in neutrophil transduction mechanisms is still unclear. A number of questions pertaining to the possible involvement of AA and/or products of the 5-LO pathway in NADPH oxidase activation were addressed in the current study.

Firstly, was LTB_4 produced on activation of the PMN with the stimuli employed in this study, namely OZ, fMLP and fluoride? This was determined using a specific radioimmunoassay (RIA) for LTB_4.

Secondly, what effect does inhibiting the 5-LO pathway of AA metabolism have on the stimulated oxidative burst? A selection of reportedly specific inhibitors of 5-LO were tested for their effect on the fluoride-, fMLP-, OZ- and diC_8-stimulated O_2^- responses. The 5-LO inhibitors employed were:

1) BW A4C or N-(3-phenoxycinnamyl)-acetohydroxamic acid - which has been described as a potent inhibitor of the synthesis of LTB_4 and [^{14}C]5-HETE from AA, and of the synthesis of LTB_4 from 5-HPETE (Tateson *et al.*, 1988). This implies an inhibition of both 5-LO and LTA_4 synthase. In the above study, BW A4C showed considerable selectivity for inhibition of 5-LO over cyclooxygenase (IC_{50} values of 0.1 $\pm$ 0.03μM and 3.2 $\pm$ 0.8μM respectively) and over 15-lipoxygenase (IC_{50} value for 15-LO was 20 times higher than that for 5-LO). In this current study, BW A4C was used at 1μM, a concentration shown to cause 90% inhibition of 5-LO and 10% inhibition of cyclooxygenase.

2) Rev 5901 or α-pentyl-3-(2-quinolinylmethoxybenzene)methanol- which has been reported to inhibit human PMN 5-LO with an IC_{50} value of 6μM. It has also been shown to inhibit dose-dependently (from 1-10μM) both antigen- and A23187-induced leukotriene release from human lung tissue (Tennant *et al.*, 1987). The leukotrienes determined in the latter assay were the

sulphidopeptide leukotrienes – LTC_4, LTD_4 and LTE_4.

3) Piriprost (U-60,257) or 6,9-deepoxy-6,9-(phenylimino)-$\Delta^{6,8}$-prostaglandin I_1 has been demonstrated to inhibit A23187-induced formation of leukotrienes C and D in rat mononuclear cells with an IC_{50} of 4.6μM (Bach *et al.*, 1982, 1983). It was also shown not to inhibit thromboxane B_2 formation, indicating a selectivity for the lipoxygenase over the cyclooxygenase route of AA metabolism. However, piriprost did inhibit glutathione S-transferase (mediating the conversion of LTA_4 to sulphidopeptide leukotrienes, see fig. 7.1) of rat basophil leukemia cells (IC_{50} = 37μM).

Thirdly, the question of the participation of endogenous AA in the stimulation of O_2^- was tackled using two different approaches: (1) As mentioned above, chloracysine (compound I) and a closely related analogue, compound II, were examined for their effect on the respiratory burst stimulated by a variety of agonists. (2) The main potential routes of AA generation which are thought to be PC, PE, PI or PA via a PLA_2 action, or the phosphoinositides by the sequential action of PLC and DAG lipase (see fig. 7.1) were simultaneously inhibited by a cocktail of inhibitors comprising - RHC80267, a DAG lipase inhibitor (Sutherland & Amin, 1982), R59022, a DAG kinase inhibitor, thereby blocking the generation of PA (De Chaffoy de Courcelles *et al.*, 1985), and either indomethacin or meclofenamate. It had previously been demonstrated that neutrophil PLA_2 can be inhibited by indomethacin (Kaplan *et al.*, 1978) and meclofenamate (Franson *et al.*, 1980). Thus, a combination of either of these latter two compounds with the inhibitor of DAG lipase plus the inhibitor of DAG kinase should prevent release of AA from all the sources specified above. In this study, the effect of the inhibitory cocktail was examined on O_2^- release induced by the G-protein activator, fluoride, and compared with results obtained with the post-receptor stimulus, γ-HCCH and the receptor stimuli, OZ and IgG. γ-HCCH is known to activate the oxidative burst (English *et al.*, 1986) and cause PIP_2 breakdown possibly by perturbation of membrane phospholipids for PLC action (Parries & Hokin-Neaverson, 1985).

7.2 RESULTS

7.2.1 LTB_4 generation

OZ effected a dose-dependent generation of LTB_4 as measured by a RIA assay, over the same concentration range that gave rise to the O_2^- dose-response curve. One feature of the OZ-induced LTB_4 generation that deserves a mention was the huge variation observed in LTB_4 mass (expressed as pg $LTB_4/10^7$ cells) produced between experiments, but in all cases a dose-dependent production of LTB_4 was obtained (n = 14). A representative experiment is presented in fig. 7.2a, illustrating the OZ-induced LTB_4 dose-response curve.

A number of the enzyme inhibitors which were to be employed subsequently in the O_2^- study were tested for their effect on OZ-stimulated LTB_4 generation. The 5-LO inhibitor, BW A4C at 0.1μM caused a very marked inhibition of the LTB_4 dose-response curve (n = 3), with 1μM BW A4C effecting maximal inhibition (n = 9). A single representative experiment is presented in fig. 7.2b, showing the effect of BW A4C at 0.1μM. The other 5-LO inhibitor, Rev 5901, at 6 and 60μM caused a dose-dependent reduction of OZ-induced LTB_4 release (n = 1-3), with the 60μM dose totally inhibiting the response; a single representative experiment is presented in fig. 7.2c.

The putative PLA_2 inhibitors, compound I (chloracysine) and compound II, at 100μM caused approximately 95% and 90% inhibition respectively, of LTB_4 generation induced by OZ in a single experiment which is presented in fig. 7.2d.

Additionally, in a single experiment fluoride was shown to induce LTB_4 generation, dose-dependently, over the same concentration range that also gave rise to the O_2^- dose-response curve. The fluoride-induced LTB_4 release, like that with OZ, was maximally inhibited in the presence of 1μM BW A4C and 80% inhibited by 6μM Rev 5901, as is shown in fig. 7.3. FMLP was tested for its ability to generate LTB_4, but the result proved negative at all concentrations tested (3nM-100nM) (result not shown). Also, unstimulated cells failed to sustain any basal production of LTB_4 (result not shown).

7.2.2 The effect of 5-lipoxygenase inhibition on stimulated O_2^- production

(1) BW A4C

BW A4C, at 1μM had no effect on the O_2^- response induced by diC_8 (n = 1), fluoride (n = 4), fMLP (n = 6) and OZ (n = 5). The mean normalized data showing the effect of BW A4C on diC_8-, fluoride-, fMLP-

and OZ-induced O_2^- generation is presented in fig. 7.4 a, b, c, & d respectively.

(2) Rev 5901

Rev 5901, at 6μM had no significant effect on O_2^- production induced by fluoride (n = 3), fMLP (n = 3) or OZ (n = 4). Fluoride-induced O_2^- production was increased by 6μM Rev 5901 in some experiments, but the difference was not statistically significant as determined by an unpaired Student's t-test. On the other hand, 6μM Rev 5901 caused a non-significant decrease of fMLP-stimulated O_2^- generation. The effect of 6μM Rev 5901 on the mean normalized O_2^- dose-response curves obtained with fluoride, fMLP and OZ is shown in fig. 7.5a, b & c, respectively.

(3) Piriprost (U60,257)

Piriprost, at 100μM showed different effects with both OZ- and fluoride-stimulated O_2^- production. Piriprost at 100μM, had no effect on the OZ O_2^- dose-response curve in 2 experiments and this result is plotted graphically in fig. 7.6a. In contrast, piriprost caused a marked potentiation of fluoride-stimulated O_2^- generation. The effect of piriprost on the fluoride O_2^- dose-response curve (n = 2), as presented in fig. 7.6b, was very similar to the effect of the enhancing NSAIDs (presented in Chapter 4) in that a marked left-shift of the dose-response curve plus a large increase of the maximum control response was observed. The variable effects of piriprost on the fluoride- and OZ-stimulated O_2^- response will be compared with the effects of piriprost on the response induced by other stimuli in the discussion.

7.2.3 The role of endogenously released AA in O_2^- production

(1) The effect of compound I (chloracysine) and compound II on the stimulated O_2^- response

The effect of these two putative PLA_2 inhibitors, at a range of concentrations, were examined on two concentrations of stimulus. The results obtained were normalized and averaged, and the data plotted as dose-inhibition curves.

Compound I and II both effected a very marked dose-related inhibition of fluoride-induced O_2^- production (n = 3), with IC_{50} values of 25 and 20μM respectively, obtained from the dose-inhibition data plotted in fig. 7.7a & b respectively. Similarly, both compounds I and II caused an inhibition of the fMLP-induced O_2^- response (n = 3), with IC_{50} values

of 30μM recorded for both inhibitors alike and the result is graphically displayed in fig. 7.8a & b respectively. A pilot study has shown that compound I at 100μM caused 60% inhibition of OZ-stimulated O_2^- generation (Muid & Dale, unpublished results). In contrast, the PLA_2 inhibitors had very little effect on the PAF O_2^- response. Both compounds I and II only marginally inhibited the O_2^- response at 100μM PAF (n = 4), with an IC_{50} value > 100μM, and likewise caused a variable but non-significant inhibition of the response at 1μM PAF (n = 2); the dose-inhibition curves relating to this result are presented in fig. 7.7c & d respectively.

Compounds I and II were also tested for their effect on the O_2^- response induced by the intracellular activators, A23187 and diC_8. In 3 experiments, compound I inhibited A23187-stimulated O_2^- production (IC_{50} = 100μM). Compound II caused a less pronounced inhibition of the A23187 response in 3 experiments (IC_{50} > 100μM). The dose-inhibition data for compounds I and II with the A23187 response are presented in fig. 7.8c & d respectively. Both compounds I and II, at 100μM, had no effect on diC_8-stimulated O_2^- generation, when tested on the full dose-response curve (n = 3); this result is presented graphically in fig. 7.9.

(*2*) <u>The effect of inhibiting all the potential sources of AA release on O_2^- production</u>

A combination of the DAG kinase inhibitor, R59022 at 10μM, the DAG lipase inhibitor, RHC80267 at 10μM, and a PLA_2 inhibitor, indomethacin at 100μM, markedly left-shifted the fluoride concentration-response curve (n = 5) (fig. 7.10a). The mean maximum response was also markedly increased with the drug combination. Similar results were obtained when meclofenamate was substituted for indomethacin and this result (n = 3) is plotted in fig. 7.10b.

The same two cocktails of reagents caused marked potentiation of the concentration-response curve induced by γ-HCCH (Muid *et al.*, 1988). When used with receptor stimuli (OZ and IgG), the combination of drugs - RHC80267, R59022 and meclofenamate - produced the opposite effect. A consistent right-shift of the concentration-response curve to OZ and an inhibition of the O_2^- response induced by a single concentration of IgG was recorded (Muid *et al.*, 1988). These findings are considered with relation to the above results in the discussion.

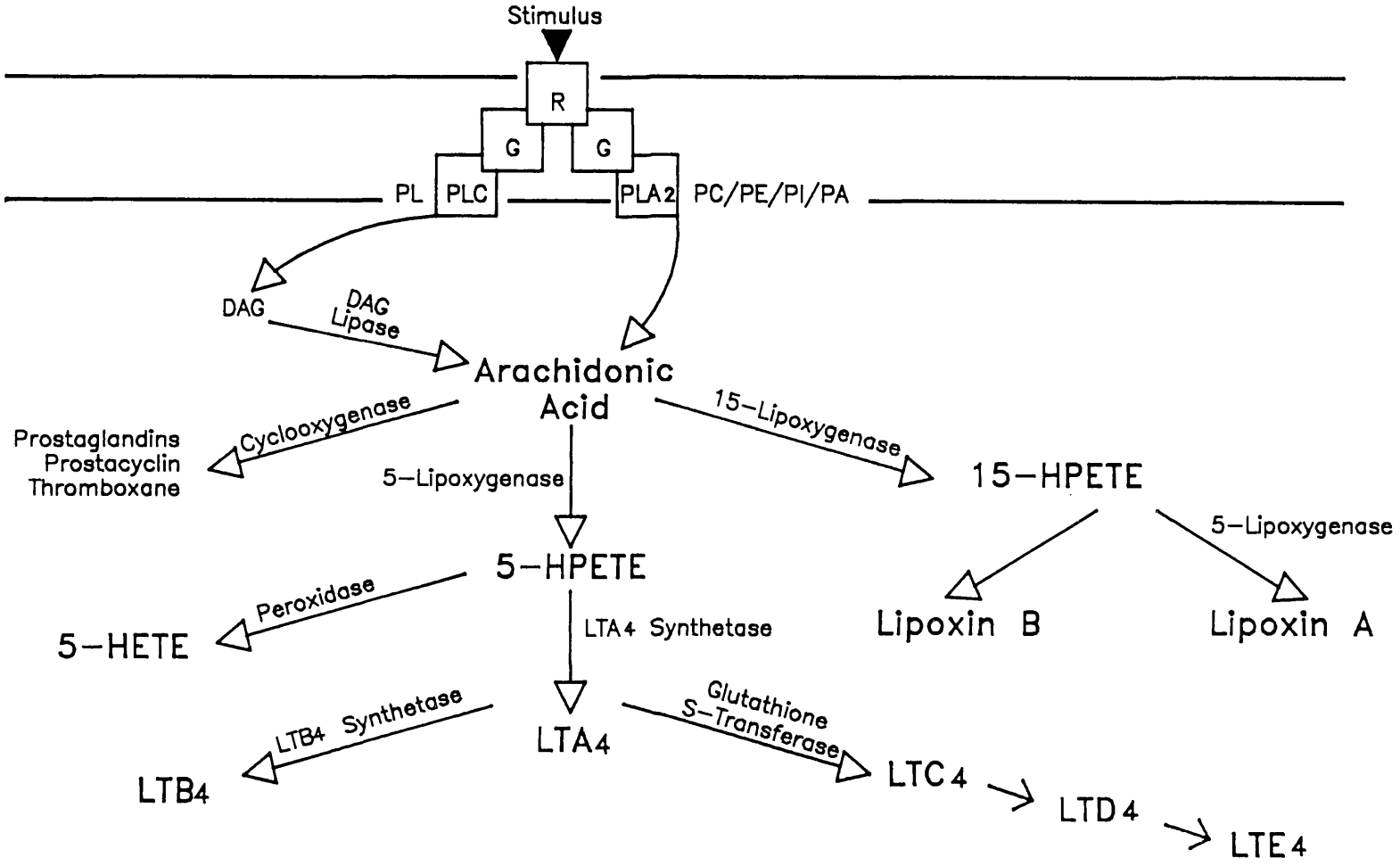


Figure 7.1: Generation and metabolism of arachidonic acid (AA). AA can be derived from PC, PA or any AA-containing PL by a PLA_2 action. The PLA_2 may be activated by a receptor-coupled G-protein; or by high Ca^{+2} concentrations, by a PKC action or by both in synergy. Alternatively, AA may be cleaved from DAG by a DAG lipase. AA is metabolized by either the cyclooxygenase, the 5- or 15-lipoxygenase pathways giving rise to the above indicated products.

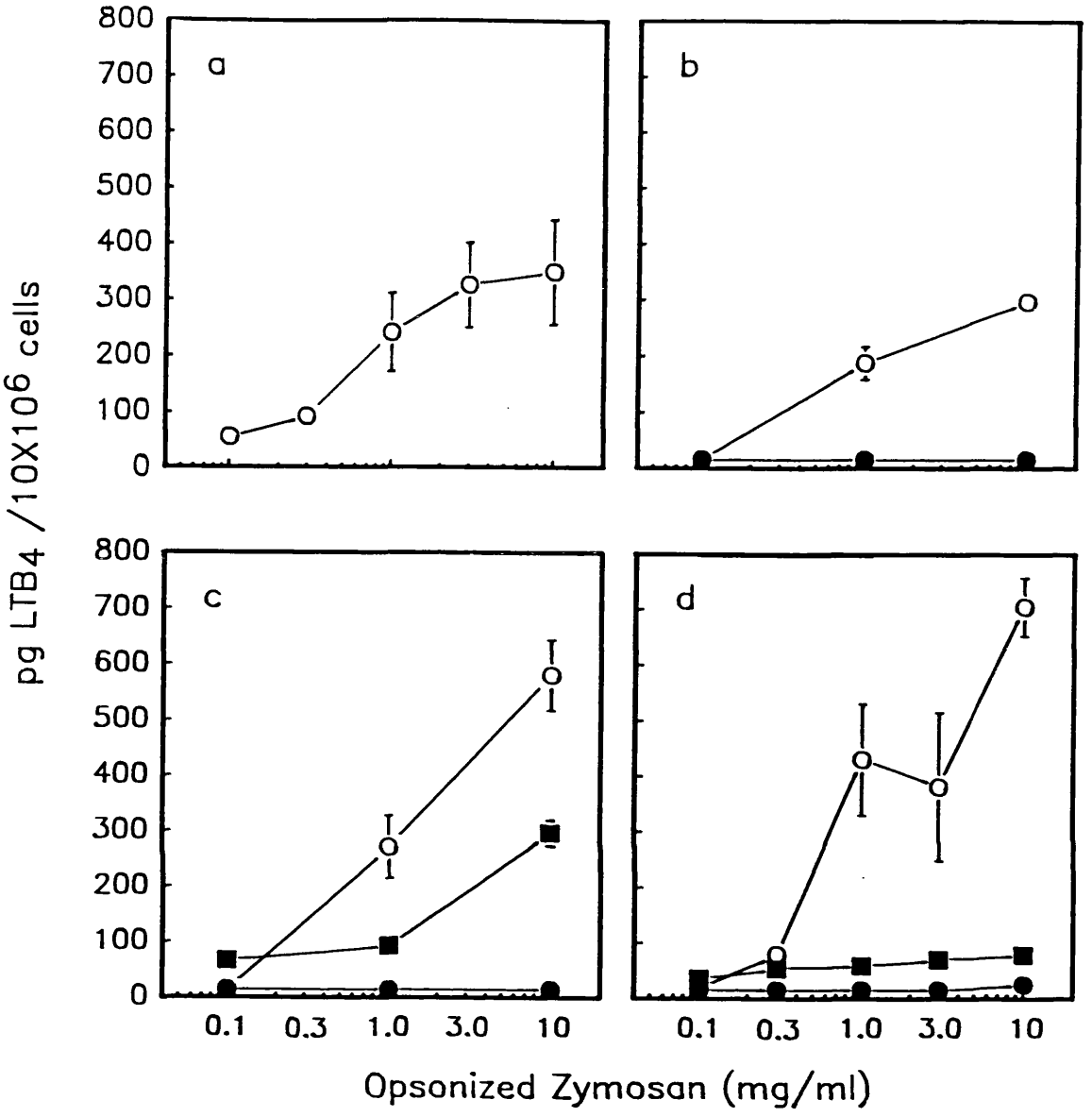


Figure 7.2: Opsonized zymosan (OZ)-stimulated LTB_4 production.

a) A representative experiment, OZ alone (O) (n = 1).

b) The effect of BW A4C; OZ alone (O) and in the presence of BW A4C at 0.1μM (●) (n = 1).

c) The effect of Rev 5901; OZ alone (O) and in the presence of Rev 5901 at 6μM (■) or at 60μM (●) (n = 1).

d) The effect of compound I and II; OZ alone (O) and in the presence of 100μM compound I (●) or 100μM compound II (■) (n = 1).

The experiments shown in b and c are each representative of 3 individual experiments. All data points represent the mean of sample duplicates ± range of the duplicates.

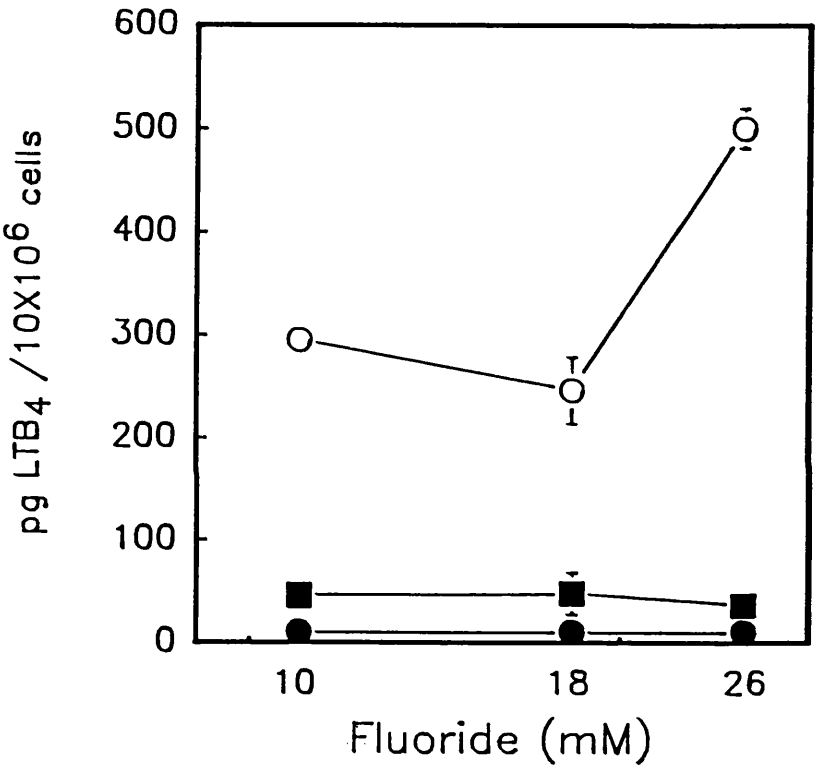


Figure 7.3: The effect of 5-lipoxygenase inhibitors on fluoride-induced LTB_4 production. Fluoride alone (O) and in the presence of 1μM BW A4C (●) or 6μM Rev 5901 (■) (n = 1). Data points represent the mean of sample duplicates ± range of the duplicates.

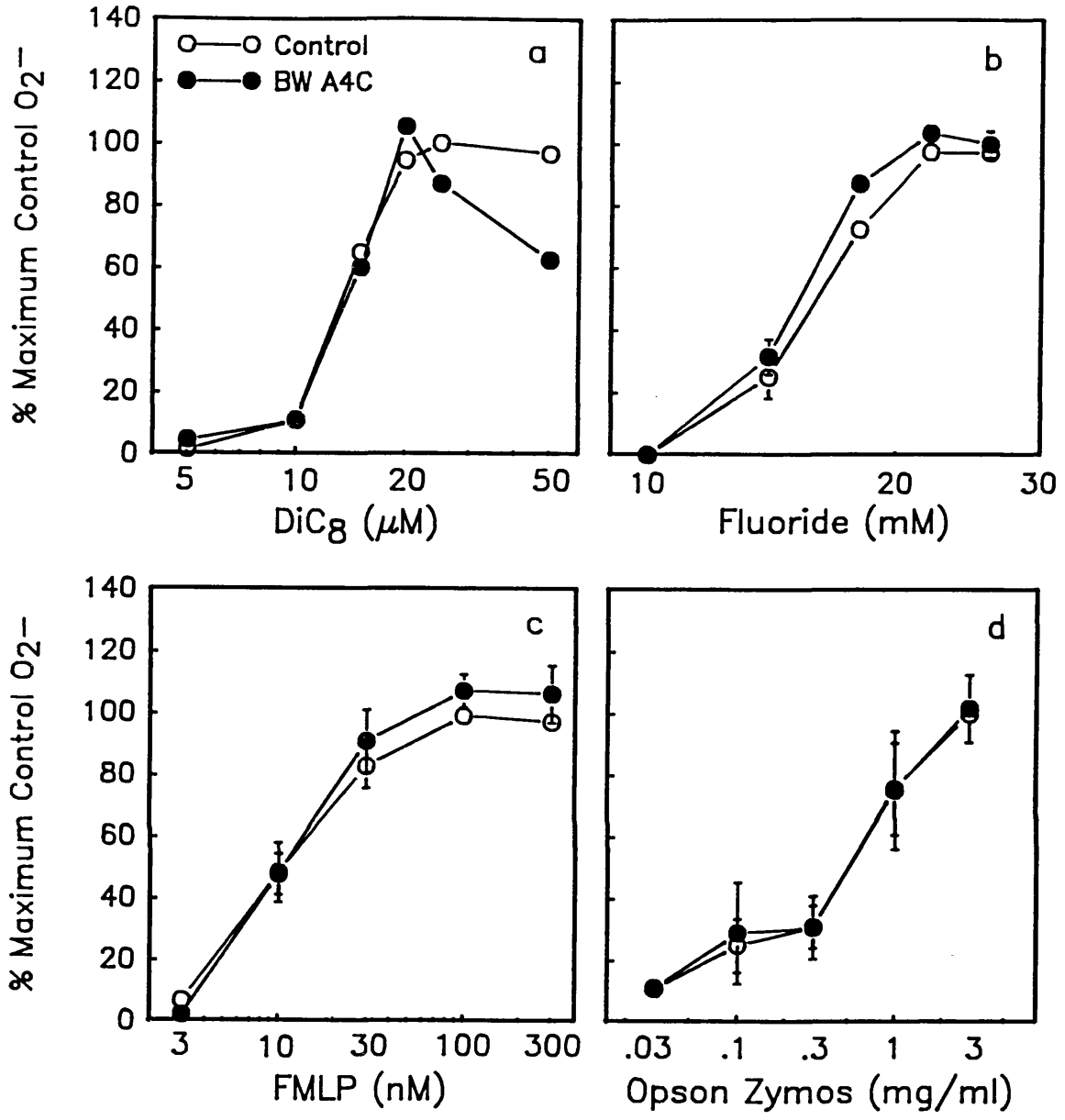


Figure 7.4: The effect of BW A4C on O_2^- production induced by a) diC_8 (n = 1), b) fluoride (n = 4), c) fMLP (n = 6) and d) OZ (n = 5). Responses were obtained either alone (O) or in the presence of 1μM BW A4C (●). The mean of the sample duplicates for the diC_8 maximum control response was 144.45 ± 1.94 nmol O_2^-/5 × 10^6 neutrophils. Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils, was 105.02 ± 8.79 for fluoride, 109.49 ± 13.44 for fMLP and 136.75 ± 5.44 for OZ. Error bars in a represent the range of sample duplicates and in b, c & d , the standard errors.

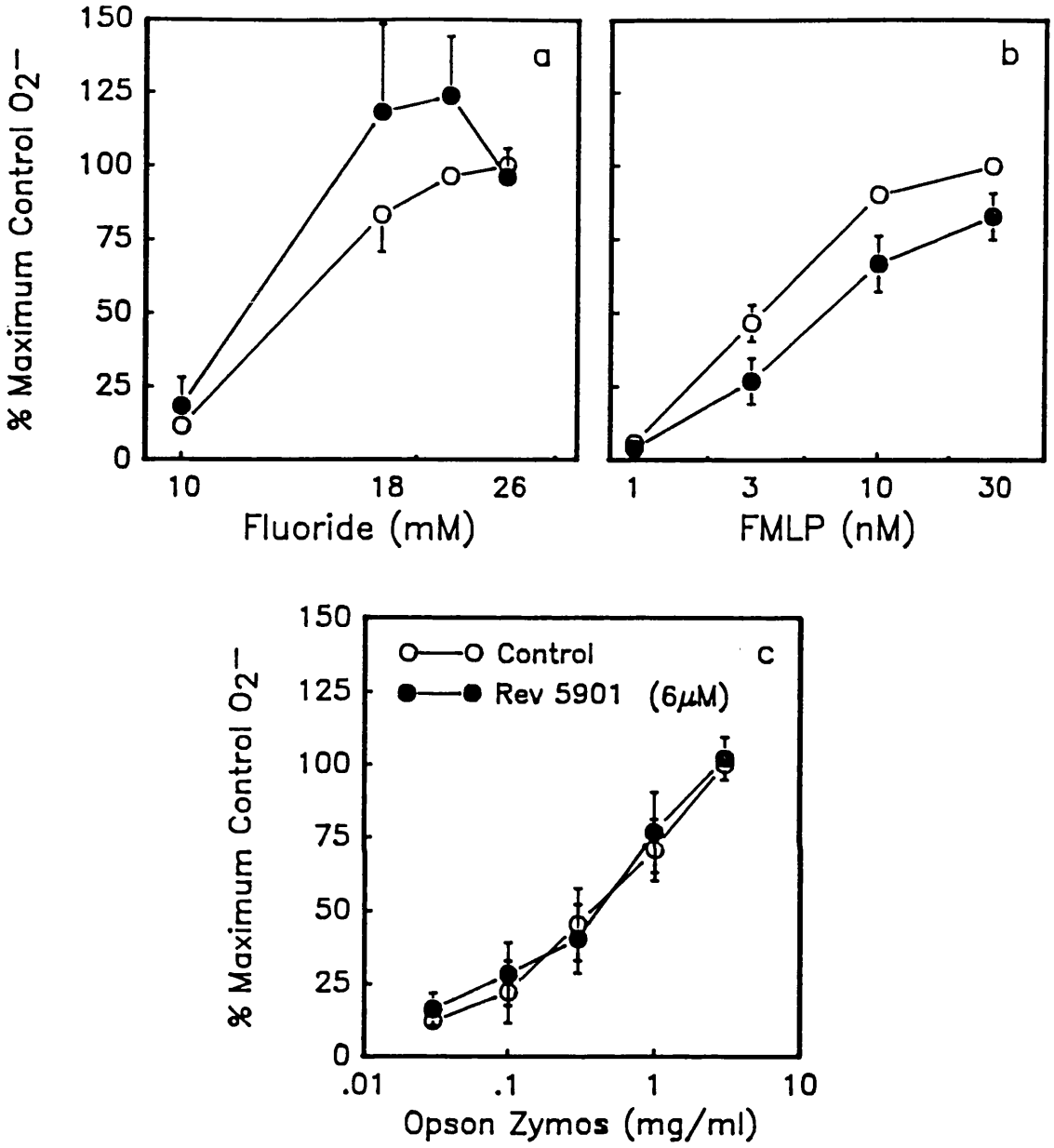


Figure 7.5: The effect of Rev 5901 on O_2^- production induced by a) fluoride (n = 3), b) fMLP (n = 3) and c) OZ (n = 4). Responses were obtained either alone (O) or in the presence of 6μM Rev 5901 (●). The mean maximum control O_2^- response, expressed as nmol/5 × 10^6 neutrophils was 72.34 ± 6.14 for fluoride, 139.04 ± 27.00 for fMLP and 164.81 ± 3.86 for OZ. Error bars represent standard errors.

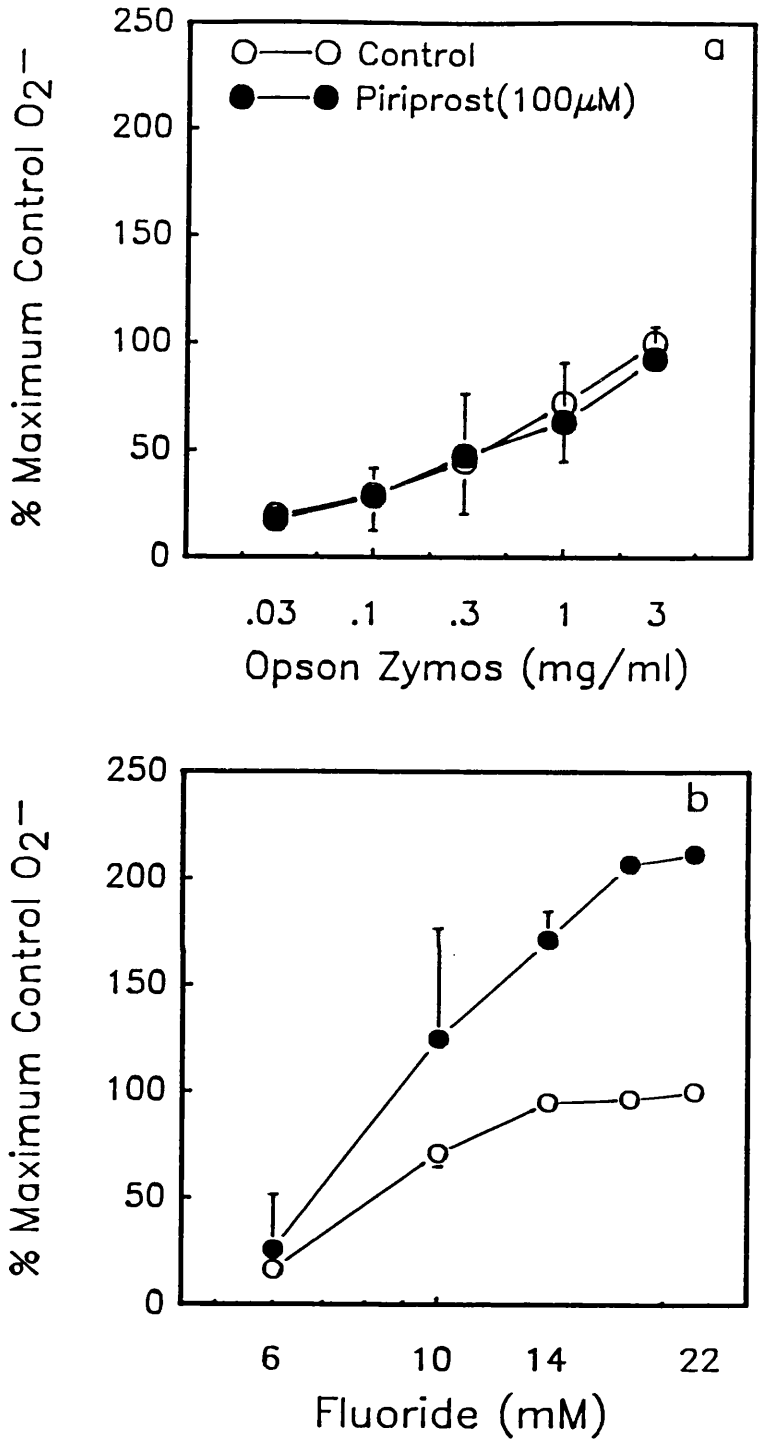


Figure 7.6: The effect of piriprost on O_2^- production induced by a) OZ (n = 2) and b) fluoride (n = 2). Responses were obtained either alone (O) or in the presence of 100μM piriprost (●). Mean maximum control O_2^- release, expressed as nmol/5 × 10^6 neutrophils was 72.28 ± 10.04 for fluoride and 155.52 ± 55.77 for OZ. Error bars represent the range of mean data.

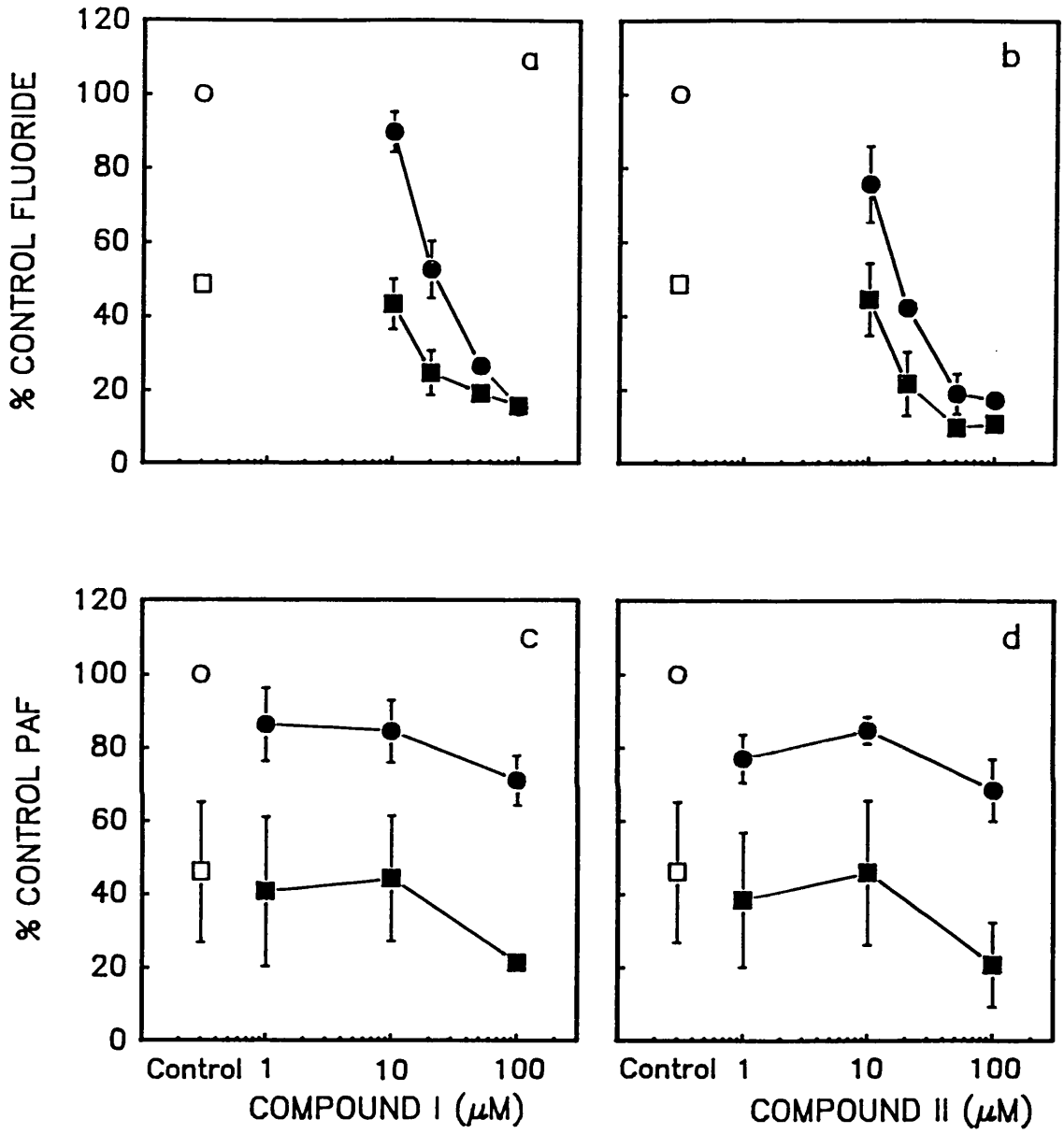


Figure 7.7: a) & b) The effect of varying concentrations of compound I & II respectively, on fluoride-stimulated O_2^- generation (n = 3). Fluoride (22mM) alone (O) and with compound I/II (●), fluoride (10mM) alone (□) and with compound I/II (■). Mean maximum control O_2^- release was 49.68 ± 10.62 nmol/5 × 10^6 neutrophils.

c) & d) The effect of varying concentrations of compound I & II respectively, on PAF-stimulated O_2^- generation. PAF (100μM) alone (O) and with compound I/II (●) (n = 4), PAF (1μM) alone (□) and with compound I/II (■) (n = 2). Mean maximum control O_2^- release was 44.64 ± 4.41 nmol/5 × 10^6 neutrophils. Error bars represent standard errors.

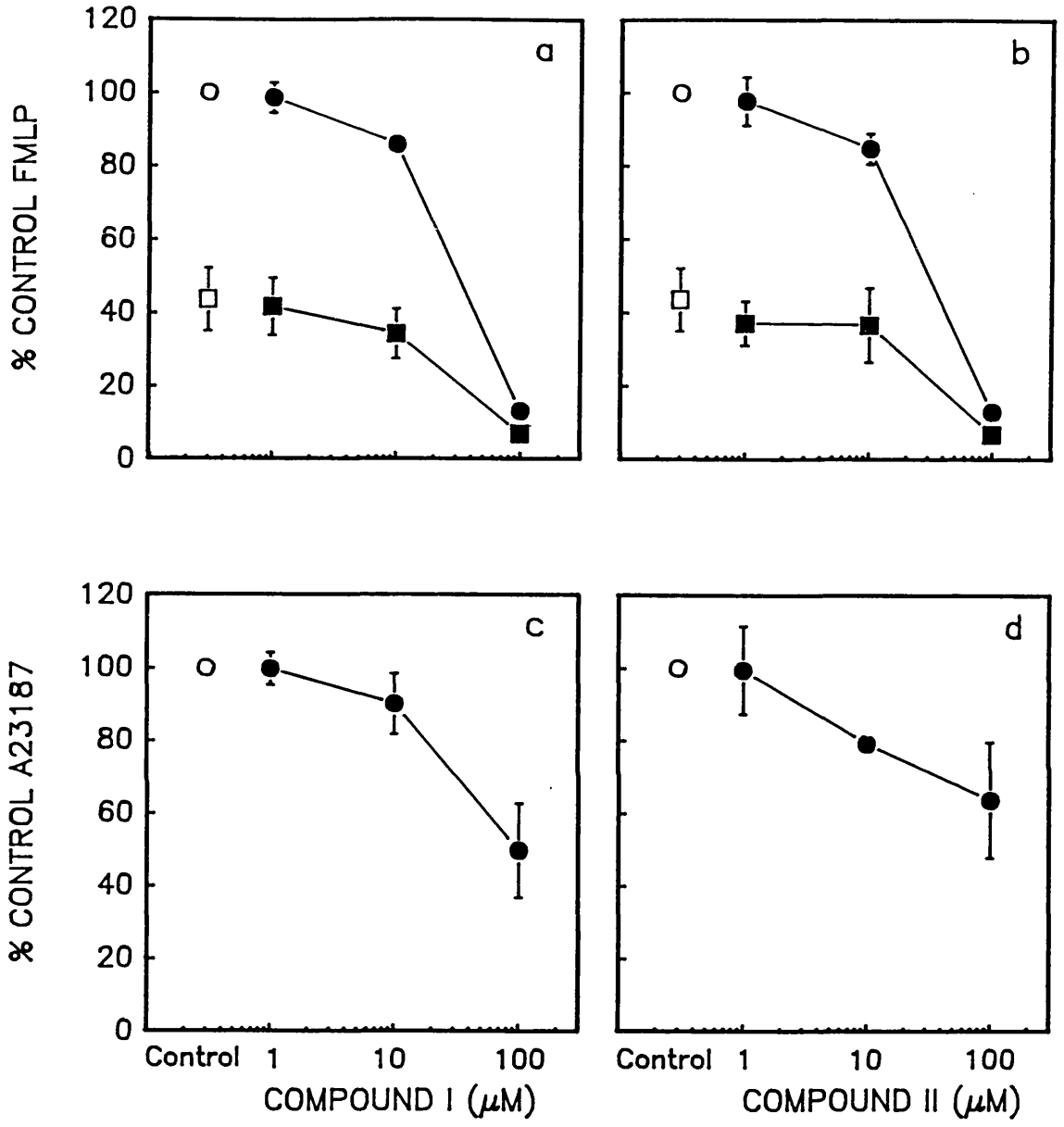


Figure 7.8: a) & b) The effect of varying concentrations of compound I & II respectively, on fMLP-stimulated O_2^- generation (n = 3). FMLP (30nM) alone (O) and with compound I/II (●), fMLP (10nM) alone (□) and with compound I/II (■). Mean maximum control O_2^- release was 82.42 ± 18.76 nmol/5 × 10^6 neutrophils.

c) & d) The effect of varying concentrations of compound I & II respectively, on A23187-stimulated O_2^- generation (n = 3). A23187 (10μM) alone (O) and with compound I/II (●). Mean maximum control O_2^- release was 28.91 ± 2.38 nmol/5 × 10^6 neutrophils.

Error bars represent standard errors.

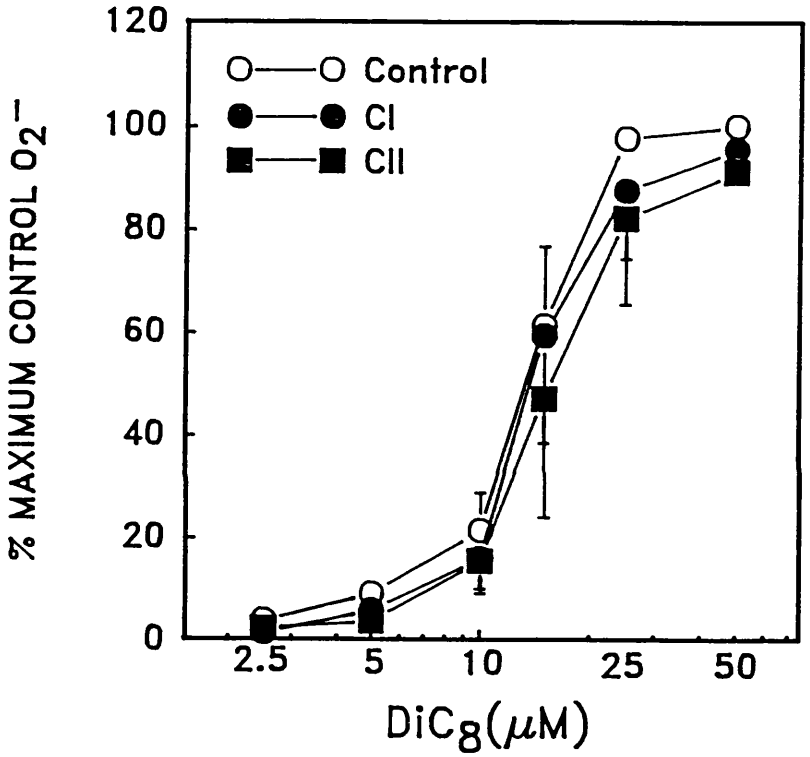


Figure 7.9: The effect of compound I & II on the diC_8-stimulated O_2^- dose-response curve (n = 3). DiC_8 alone (O) and with 100μM compound I (●) or 100μM compound II (■). Mean maximum control O_2^- release was 174.53 $\pm$ 5.43 nmol/5 $\times$ 10^6 neutrophils. Error bars represent standard errors.

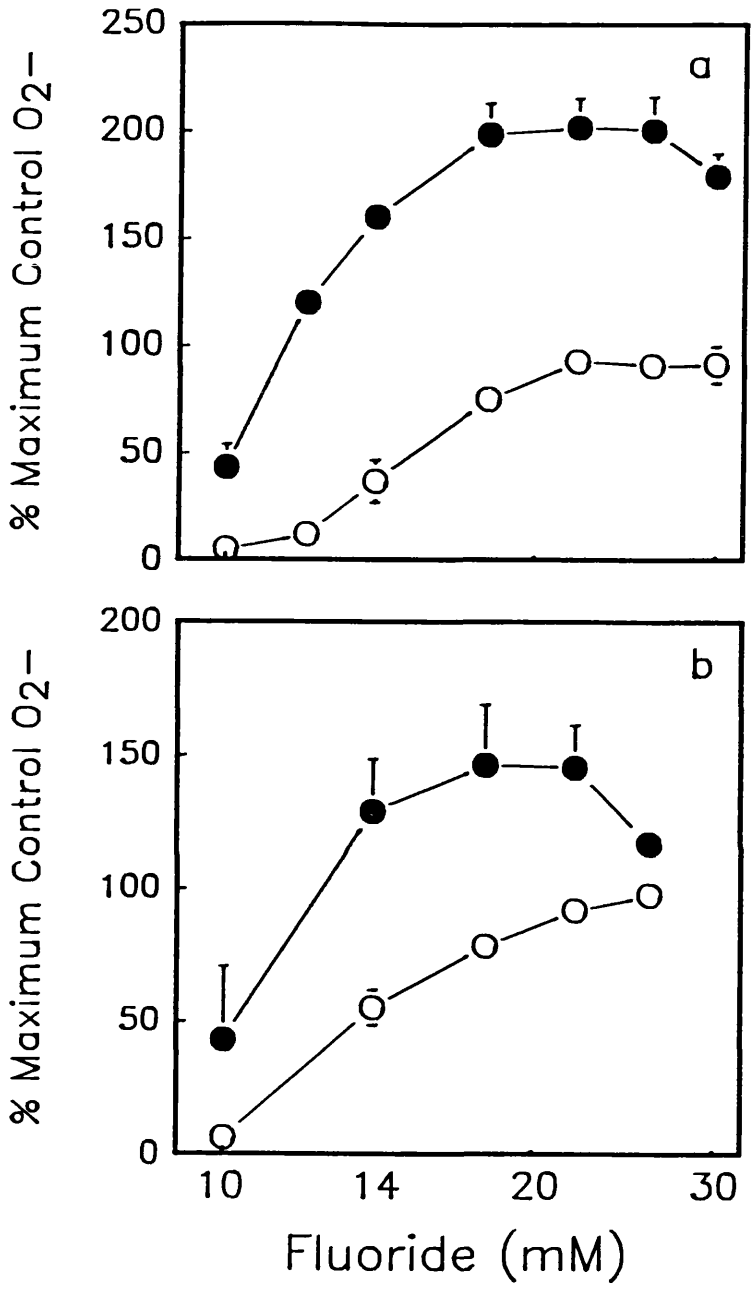


Figure 7.10: The effect of a combination of inhibitors of arachidonate generation on fluoride-induced O_2^- production. a) Fluoride alone (O) or in the presence of the DAG kinase inhibitor, R59022 (10μM), the DAG lipase inhibitor, RHC80267 (10μM), and a PLA_2 inhibitor, indomethacin (100μM) (●) (n = 5). b) Fluoride alone (O) or in the presence of R59022 (10μM), RHC80267 (10μM) and a PLA_2 inhibitor, sodium meclofenamate (100μM) (●) (n = 3). Mean maximum control O_2^- release, expressed as nmol/5×10^6 neutrophils was 83.02 ± 9.61 in **a** and 103.46 ± 10.90 in **b**.

5-LO product that has been implicated as a neutrophil activator. Lipoxins A and B, the 5-HETEs and the 5-HPETEs have also received much attention in this respect (see introduction) and thus could serve as possible second messenger candidates *en route* to burst activation. The results obtained with the two selective 5-LO inhibitors, BW A4C and Rev 5901, at or just above their IC_{50} values, on the stimulated O_2^- response induced by fluoride, fMLP and OZ, were negative. In so far as these compounds were acting as 5-LO inhibitors, inhibition of the 5-LO pathway had no effect on the respiratory burst induced by the above stimuli. The diC_8-stimulated oxidative burst was also unaffected by BW A4C and was not tested with Rev 5901. Both 5-LO inhibitors were shown in this study to totally inhibit OZ-induced LTB_4 generation so the 5-LO pathway was assumed to be blocked in the presence of these compounds. It is also necessary to include here a conflicting result with regards the effect of BW A4C on the fMLP-induced O_2^- response recorded by Penfield (1988). In the latter study, BW A4C at 1μM was shown to produce a consistent 20% decrease of the control fMLP dose-response curve, as opposed to the "no effect" situation recorded here. This discrepancy remains, at present, unexplained.

Piriprost (100μM) in this study had no effect on OZ-stimulated O_2^- production, which is in agreement with another report (Flament *et al.*, 1988). This 5-LO inhibitor has been reported to cause a rightward shift of the fMLP O_2^- dose-response curve (Penfield, 1988; Mehta *et al.*, 1987; Flament *et al.*, 1988). The latter group investigating this phenomenon, reported that exogenously added LTB_4 was unable to reverse the inhibitory effect of piriprost on the fMLP response. They concluded that piriprost was behaving as a specific competitive antagonist of fMLP, an action that did not involve lipoxygenase inhibition, and might be exerted at the level of the fMLP receptor or its associated mechanisms of transduction.

The effect of piriprost on the post-receptor stimuli is somewhat reminiscent of the enhancing NSAID effects, reported in Chapter 4. In this study piriprost was found to potentiate markedly the fluoride O_2^- response, and Penfield (1988) reported that it markedly enhanced O_2^- release induced by both OAG and A23187. These increased responses are unlikely to be due to an inhibition of 5-LO, considering that neither of the more specific inhibitors, BW A4C or Rev 5901, exerted such an action on O_2^- generation. Piriprost may thus have some non-specific intracellular action that would enhance cellular responses induced by

post-receptor stimuli and have very little effect or reduce those stimulated at the receptor. The compound may have been affecting the DAG metabolic enzymes in the same way as that proposed for the enhancing NSAIDs in Chapter 4, or it may have another action altogether.

To summarize, the current results with the 5-LO inhibitors suggest that metabolic products of this pathway do not appear to play a significant role in the transduction mechanisms leading to NADPH oxidase activation, whether activated at the receptor or beyond. In another study, Ozaki *et al.* (1986) using a different series of lipoxygenase inhibitors also observed very little correlation between inhibition of the lipoxygenase pathway or LTB_4 synthesis and the respiratory burst. Thus, these data appear to exclude an intracellular second messenger role for the potent inflammatory mediator - LTB_4 as well as the 5-HETEs, the 5-HPETEs and the 15-HPETE metabolite, lipoxin A; conversion of 15-HPETE to lipoxin A requires the action of 5-LO (Ueda *et al.*, 1987). These data, taken with the results in Chapter 4 where inhibiting the cyclooxygenase pathway with some NSAIDs did not significantly affect the stimulated respiratory burst, infers that the AA metabolic pathways are unlikely to be involved in the activation of NADPH oxidase.

The role of endogenous AA as an intracellular mediator for O_2^- generation was then addressed in this study. Initiation of the AA cascade by PLA_2 *in vivo* is thought to be carefully controlled by endogenous modulators - the lipocortins. Lipocortin-like proteins are a family of steroid-induced agents reported to be inhibitors of PLA_2 activity, and believed to have potential anti-inflammatory activity. The action of the glucocorticoids has been attributed to the induction of these lipocortin proteins (Flower & Blackwell, 1979), and related proteins have been isolated from a variety of tissues including monocytes (Flower & Blackwell, 1979) and neutrophils (Hirata *et al.*, 1980). The best characterized form is a protein of relative molecular mass 40kDa, which is phosphorylated *in vivo* by protein tyrosine kinases and by protein serine-threonine kinases such as PKC (Pepinsky & Sinclair, 1986). When the cell is in a resting state, lipocortin is believed to be "active" in inhibiting the PLA_2 enzyme (reviewed in Brugge, 1986), thereby preventing the production of leukotrienes and prostaglandins; a conflicting study reports that lipocortin-mediated inhibition occurs by sequestering the phospholipid substrate (Davidson *et al.*, 1987). Following cell

activation it is proposed that lipocortin is phosphorylated, possibly by PKC in PMNs, which reverses the PLA_2 inhibition (Hirata, 1981; Stoehr *et al.,* 1990), thus allowing the generation of inflammatory mediators (reviewed in Geisow *et al.,* 1987). The phosphorylation of lipocortin in PMNs has been found to parallel the release of AA during stimulation by chemoattractant (Hirata, 1981). This mechanism provides an explanation for the modulation of PLA_2-linked pathways by PMA and other PKC activators given in the introduction, and also suggests an important role for PLA_2 in cell activation.

The participation of the PLA_2 pathway of AA generation in the respiratory burst was assessed using two recently developed PLA_2 inhibitors, namely compound I (chloracysine) and compound II. In the presence of these inhibitors, fMLP-, OZ- and fluoride-stimulated O_2^- generation was markedly inhibited in a concentration-dependent manner; O_2^- generation stimulated by PAF and A23187 was only marginally affected whereas the diC_8-induced response was unchanged by the compounds. There are a number of points to be noted given the above profile of effects. Firstly, the absence of inhibition of the A23187-stimulated response was somewhat surprising given that A23187 is a good stimulus for activation of PLA_2-linked pathways, as compared to the soluble stimuli, fMLP and PAF. The O_2^- response induced by fMLP (a stimulus which is believed by some not to activate the PLA_2 pathway significantly) was markedly inhibited by these two reported PLA_2 inhibitors. This seems to indicate that lipases other than PLA_2 are also being affected by compounds I and II, but as outlined in the introduction very few characterization studies have yet been carried out, so the possible non-specific sites of action of these compounds remain obscure. A tentative proposal that the compounds could be inhibiting phospholipase C or D (PLC or D) is, however, offered. It should be noted that the two PLA_2 inhibitors potently inhibited OZ-induced LTB_4 production indicating that the compounds do in fact inhibit PLA_2.

Secondly, the lack of effect of both compounds I and II in inhibiting the O_2^- response induced by the PKC activator, diC_8, is in contrast to the total inhibition of the O_2^- response induced by another PKC activator, PMA, reported by Henderson *et al.* (1989). The latter group also reported that O_2^- generation could be restored by the addition of AA or sodium dodecylsulphate in the presence of the PLA_2 inhibitors. This result is somewhat surprising and although not tested experimentally a

number of possible explanations are offered. PMA, used alone, has been shown to be ineffective in stimulating [^{3}H]AA release from human PMNs (McColl *et al.*, 1986) and HL-60 cells (Billah & Siegel, 1987) and also failed to initiate the synthesis of LTB_4 and other 5-LO metabolites in human PMNs (Liles *et al.*, 1987; Raulf & Konig, 1988); but it does augment these responses when used in conjunction with A23187. These studies thus infer that PMA on its own, does *not* significantly activate PLA_2 and hence exclude the involvement of PLA_2-derived AA as a second messenger in PMA-stimulated O_2^- generation. However, there are a number of studies reporting that PMA acting through PKC can stimulate the production of DAG and alkylacylglycerols via a PLC and/or D action (see Chapter 6). A recent report has found that PMA-induced O_2^- production is not always related to a decrease in cytosolic PKC activity (Gaudry *et al.*, 1990) and may thus involve other PMA-activated phospholipases. The ablation of the PMA-induced O_2^- response by a series of PLA_2 inhibitors (including compounds I and II) might be due to an effect of these compounds on those membrane phospholipases that PMA can activate directly, namely PLC and/or PLD. The restoration of the O_2^- response by exogenous AA could be due to the direct O_2^- activating effect of AA mediated via PKC, to its Ca^{2+} ionophore properties or to its effects on the NADPH oxidase system itself (see introduction). On the other hand, the diC_8-stimulated O_2^- response, which is believed to be mediated predominantly by PKC, is unaffected by the postulated PLA_2/PLD/PLC inhibitors.

Alternatively, O_2^- production induced by PMA may be due in part to a membrane perturbant property of PMA, as has been reported elsewhere (Dawson *et al.*, 1984; Tao *et al.*, 1989), which may give rise to PLA_2 activation. In this circumstance, the O_2^- response would be susceptible to the PLA_2 inhibitors.

In conclusion, the recently described PLA_2 inhibitors, compounds I and II, have been demonstrated to potently inhibit O_2^- release induced by fMLP, OZ and fluoride and to have little effect on the response induced by PAF, A23187 and diC_8, although the result must be interpreted with caution. AA may be involved as a second messenger in the transduction sequences stimulated by both fMLP, OZ and fluoride, but the possibility that the inhibition might not be due to an effect on PLA_2 cannot be overlooked.

The participation of endogenously generated AA in the O_2^- response was also investigated by measuring the effect of a mixture of agents

which are postulated to inhibit the main pathways involved in AA release. The results show that the cocktail of inhibitors not only failed to decrease O_2^- generation by stimuli acting at non-receptor sites, namely fluoride and γ-HCCH, but they actually increased it. However, the cocktail of inhibitors marginally decreased OZ- and IgG-stimulated O_2^- production. One interpretation of these results must take cognisance of the possibility that both indomethacin and meclofenamate, in addition to their known effects on cyclooxygenase and PLA_2, may also be inhibitors of DAG metabolism (see Chapter 4). Taking this possibility into account, an explanation for the increased O_2^- generation with the post-receptor stimuli is that, by inhibiting DAG metabolism and increasing DAG levels, the resultant increased PKC activation could have more than compensated for the elimination of the putative AA pathway. In addition, the decreased response observed with OZ and IgG could be interpreted in terms of the increased PKC activity mediating a negative-feedback mechanism at the receptor or the G-protein (see Chapter 5) but prior to the locus of action of the fluoride stimulus. The post-receptor stimuli may not have been susceptible to such a feedback effect.

Another possible explanation for the different results obtained with the mixture of inhibitors on the receptor- and post-receptor-mediated responses is that the oxidative burst which follows receptor stimulation might, in addition to PIP_2 degradation, require activation of the additional PLA_2 pathway. Receptor stimulation coupled to PLA_2 activation by a distinct G-protein from that reported to be involved with the PIP_2 pathway has been described (Nakashima *et al.*, 1988; Tao *et al.*, 1989; Fuse & Tai, 1987; Cockcroft & Stutchfield, 1989). If arachidonate generation was a necessary signal for receptor-mediated O_2^- release, inhibition of such generation might well give the recorded inhibition. Support for the possibility that an AA pathway could be implicated in the receptor-mediated oxidative burst is provided by a report which showed that lipocortin, known to inhibit the release of AA, decreased receptor-mediated but not PMA-mediated H_2O_2 production (Stevens *et al.*, 1988).

In summary, the two approaches used to assess the role of endogenous AA in O_2^- generation have yielded inconclusive results. In one approach - i.e. using PLA_2 inhibitors - the fluoride, fMLP and OZ responses were inhibited, while in the second approach - using a technique aimed at preventing AA release - only OZ- and IgG-stimulated responses were inhibited. FMLP was not included in the latter study as

indomethacin and possibly meclofenamate are thought to competitively inhibit fMLP binding to its receptor (Palmer, 1983).

Other groups investigating the role of endogenous AA in the transduction process have used radioactive AA, measuring its accumulation and loss from phospholipids on stimulation of prelabelled cells. This technique has serious drawbacks (reviewed in Irvine, 1982) because several distinct pools of AA may exist, with continuous, rapid acylation and reacylation of the phospholipids in these pools, which leads to difficulties in quantifying stimulus-mediated AA liberation from measurements of radioactivity alone. Reacylation of deacylated phospholipids may obscure the AA signal and phospholipids that take up radiolabelled AA at different rates further confuse the results. However, using this technique in A23187-, IgG- and OZ-stimulated neutrophils Godfrey *et al.* (1987) found no correlation between O_2^- generation and PLA_2-mediated AA generation or its subsequent metabolism.

Much more work combined with more specific approaches are necessary in this area to ascertain the role of endogenously released AA. As stated in the introduction, unsaturated fatty acids have been shown in some studies to activate PKC in a Ca^{2+}-independent manner, although there is controversy on this point. It is interesting to note that recent studies have verified that it is only the γ-isoform of PKC that is sensitive to the low levels of AA (e.g. 25μM) which could be plausibly produced during receptor activation (Shearman *et al.*, 1989). Lipoxin A was also demonstrated to be a potent, selective activator of the γ-subspecies of PKC, and activation occurred in the absence of phospholipids, DAG and Ca^{2+}. Other interesting ideas pertaining to the involvement of AA in neutrophil activation include the following: (1) PKC can be activated in parallel with DAG and the AA metabolite, 5-HETE, to stimulate degranulation and O_2^- generation in human neutrophils (O'Flaherty *et al.*, 1985; Badwey *et al.*, 1988). (2) It has been suggested that phosphorylation of 46kDa protein(s), indicative of PKC activation, and the release of AA can synergistically induce O_2^- production in guinea pig PMNs (Ohtsuka *et al.*, 1988b). It must be emphasized that there is as yet no evidence from intact cells that AA generated endogenously can act as it does when administered exogenously or that the effect of AA on isolated enzyme systems is relevant in physiological cell activation; thus further studies are needed in this area.

CHAPTER EIGHT

WORTMANNIN-SENSITIVE TRANSDUCTION MECHANISMS

SUMMARY:

— The sterol fungal metabolite, wortmannin, has been described as an inhibitor of the transduction sequence(s) leading to respiratory burst activation, without affecting receptor function, phosphoinositide metabolism, Ca^{2+} mobilization or PKC activity. In this study, the effect of wortmannin on the stimulated O_2^- response induced by a number of stimuli acting at different cellular points was investigated.

— It was found that the oxidative burst induced by the soluble receptor stimulus, PAF, was potently inhibited by wortmannin. Opsonized zymosan (OZ)-stimulated O_2^- release was less sensitive to inhibition, and high concentrations of wortmannin were required for inhibition of the burst induced by the post-receptor stimuli, A23187 and fluoride. The O_2^- response stimulated by the direct PKC activators, PMA and diC_8, was unaffected by wortmannin.

— A recent study by Reinhold *et al.* (1990) reported that fMLP-induced phospholipase D (PLD) activity was inhibited by wortmannin, but not PLD activity stimulated by A23187 or PMA; thus wortmannin did not directly inhibit PLD but only receptor-activated phospholipase activity. On the basis of this study, an interpretation of the above results is proffered in terms of an inhibition by wortmannin of a distinct G-protein coupled to the PLD. It is postulated that the PAF-stimulated O_2^- response may involve a G-protein–coupled PLD route of DAG generation, that the OZ-stimulated O_2^- response maybe less dependent on this PLD pathway while O_2^- generation stimulated by the post-receptor stimuli may involve a more direct, G-protein–independent, activation mechanism.

8.1 INTRODUCTION

A sterol-like fungal metabolite, wortmannin, and a number of natural and chemically-derived analogues have been reported to block the respiratory burst during phagocytosis of zymosan and opsonized zymosan (OZ) (Baggiolini *et al.*, 1987). The oxidative burst in both neutrophils and mononuclear phagocytes was inhibited at nanomolar concentrations. The wortmannins at these concentrations were not cytotoxic, as determined by lactate dehydrogenase release. The activity of a NADPH oxidase preparation, isolated from OZ-stimulated neutrophils, was unaffected by 17-hydroxywortmannin (HWT) at concentrations 1000-fold higher than its IC_{50} for the OZ-stimulated O_2^- response in whole cells. Thus, wortmannins do not appear to directly inhibit the respiratory burst enzyme and do not interfere with the detection of superoxide.

Superoxide production induced in human neutrophils by the chemotactic agonists - fMLP, C5a, PAF and LTB_4 was inhibited by HWT with very similar potencies (Dewald *et al.*, 1988); IC_{50} values for the different agonists were within the range 4.6-8.0 nM. In contrast, no effect was observed when the cells were challenged with the direct PKC activators, PMA or OAG, suggesting that the wortmannins were not acting as PKC inhibitors. It was also demonstrated that HWT and other wortmannins did not interfere with the binding of labelled fMLP to its receptor. In addition, HWT did not influence fMLP-induced $[Ca^{2+}]_i$ changes, suggesting that this compound was not affecting either PIP_2-dependent phospholipase C (PLC) activity or its coupled G-protein function.

Thus, the wortmannins can interfere with the signal transduction sequence initiated by both particulate and soluble receptor stimuli leading to activation of the respiratory burst oxidase, although the affected component(s) have not yet been identified. The wortmannins apparently do not inhibit receptor function, G-proteins coupled to PIP_2-dependent PLC, phosphoinositide metabolism, the increase in $[Ca^{2+}]_i$ or the catalytic activity of PKC (Dewald *et al.*, 1988).

It is intriguing to note that on the basis of their work with HWT, Dewald *et al.* (1988) have suggested that receptor agonists initiate two signal transduction sequences. One sequence was Ca^{2+}-dependent, HWT-insensitive and probably involves PIP_2 turnover with the generation of IP_3 and DAG. The other sequence was independent of Ca^{2+} and *was* inhibited by HWT. Both sequences were inhibited by treatment of the cells with pertussis toxin, indicative that functional G-proteins were

required and the results also suggest that PKC was involved in the two activation sequences. It was proposed that **both** sequences were necessary for the receptor-mediated respiratory burst.

The wortmannins also inhibited exocytosis of both the specific and azurophil granules stimulated by fMLP, PAF or LTB_4 (Dewald *et al.*, 1988), although higher HWT concentrations were required for inhibition of exocytosis than inhibition of O_2^- generation. On the other hand, under conditions were fMLP-induced O_2^- from human PMNs was totally inhibited, fMLP-induced aggregation was unaffected by wortmannin, and adherence to a gelatin matrix was reduced by only 30% (Reinhold *et al.*, 1990). Also, the wortmannins did not inhibit phagocytosis (Baggiolini *et al.*, 1987). Thus, it appears that the wortmannin-sensitive transduction pathway plays a predominant role in the neutrophil O_2^- response, is involved to a lesser extent in exocytosis and may not be very significant in other neutrophil responses such as aggregation, adhesion or phagocytosis.

In the current study, the effect of wortmannin was examined on the neutrophil respiratory burst stimulated by PAF, fluoride, OZ, diC_8, PMA and A23187 in an attempt to localize the site in the transduction scheme at which the compound may be acting. Wortmannin was found to be only half as potent as HWT in inhibiting zymosan-induced H_2O_2 production in human neutrophils, but more potent than the other 11 derivatives tested (Baggiolini *et al.*, 1987).

8.2 RESULTS

Dose-inhibition curves were obtained for wortmannin with the different stimuli as for the PKC inhibitors in Chapter 5. It should be noted that wortmannin was pre-incubated with neutrophils for 5 minutes before stimulation with agonist.

Wortmannin dose-dependently inhibited PAF-stimulated O_2^- generation (n = 4), with an IC_{50} value of 0.005 μM estimated from its dose-inhibition curve which is presented fig. 8.1a. OZ-stimulated O_2^- release was inhibited in a single experiment, represented in fig. 8.1b, with an IC_{50} value of 0.02μM. Fluoride-stimulated O_2^- generation was also inhibited but the response was a 1000-fold less sensitive to wortmannin than PAF. The mean dose-inhibition curve (n = 4) is presented in fig. 8.1c, and an IC_{50} value of 4μM was noted for the inhibition. In agreement with earlier studies, the PMA-stimulated O_2^- response was not very susceptible to inhibition by wortmannin. A range of concentrations

up to 10μM wortmannin inhibited the PMA response by less than 50%. The dose-inhibition data (n = 3) for PMA is shown in fig. 8.2a. The other direct PKC activator, diC_8, showed a similar pattern in that 10μM wortmannin did not achieve 50% inhibition of the diC_8 O_2^- response (n = 3), as is shown in fig. 8.2b.

The A23187-stimulated O_2^- response was dose-dependently inhibited by wortmannin, although there was a large degree of inter-experimental variation. A plot of the dose-inhibition curve (n = 2) is given in fig. 8.2c and an IC_{50} value of approximately 0.45μM was estimated.

8.3 DISCUSSION

In summary, wortmannin inhibited the respiratory burst induced by both soluble, PAF, and phagocytic, OZ, receptor stimuli, with the latter requiring slightly higher concentrations (IC_{50} value for OZ is 4-fold higher than that estimated for PAF). This is in agreement with previous reports. In contrast, the oxidative response elicited by post-receptor stimuli in general was much less sensitive to inhibition. The A23187-stimulated O_2^- response was inhibited by wortmannin, albeit at higher concentrations than for the receptor stimuli, and the fluoride O_2^- response was even less susceptible to inhibition than that of A23187. Both PMA- and diC_8-stimulated O_2^- production were not significantly affected by wortmannin.

Wortmannin and its analogues were originally described as compounds that could interfere with the transduction pathways leading to neutrophil activation, and particularly to activation of the respiratory burst oxidase. A recent report has presented evidence that wortmannin can inhibit fMLP-induced PLD activation, as determined by an inhibition of phosphatidylethanol release (Reinhold *et al.*, 1990). However, these authors report that PLD activity itself was not inhibited, as phosphatidylethanol formation stimulated by either A23187 or PMA was unaffected by wortmannin. It was therefore concluded that the site of action of wortmannin is proximal to receptor-stimulated PLD activation. A scheme representing three separate routes by which receptor activation could lead to stimulation of PLD was presented by Reinhold *et al.* (1990) - (1) a Ca^{2+}-dependent route that could be directly activated by increasing $[Ca^{2+}]_i$ with A23187, (2) a PKC-dependent route that could be effected independently by PMA, and (3) a wortmannin-inhibitable route whose components are as yet undefined. It should be noted that the existence of two independent PLD activities with different pH optima and

intracellular location has been demonstrated in human neutrophils (Balsinde *et al.*, 1989).

Receptor-stimulated PLD activation has been described in many cell types (see discussion in Chapter 6). Moreover, recent studies with hepatocytes (Bocckino *et al.*, 1987) and endothelial cells (Martin & Michaelis, 1989) indicate that a receptor-guanine nucleotide binding protein–PLD sequence is involved in activation of agonist-induced phosphatidate (PA) accumulation. In both studies, GTPγS (a non-hydrolysable GTP analogue) caused a time-dependent increase in PA that correlated with a degradation of phosphatidylcholine, thus giving rise to a similar pattern of activation as was recorded on receptor stimulation. Also, GTPγS has been observed to activate a PLD enzyme in HL-60 cell lysates (Tettenborn & Mueller, 1988), implicating the involvement of a G-protein.

Could a unique G-protein that mediates receptor-stimulated PLD activation be the wortmannin-sensitive site? This possibility is purely speculative but very interesting. It may indicate the dependence of the different routes of respiratory burst activation on the G-protein-PLD transduction pathway. The O_2^- response induced by chemotactic agonists such as PAF may involve a DAG signal that is derived largely by a receptor-coupled PLD route linked with PA phosphohydrolase. The OZ O_2^- response may be less dependent on the receptor-coupled PLD metabolic pathway while the oxidative response induced by post-receptor mechanisms, namely raising $[Ca^{2+}]_i$ with A23187 or activating PKC by PMA or diC_8, may not involve G-protein-induced PLD activity. The results do not necessarily exclude the involvement of PLD, as the wortmannins do not directly affect the enzyme activity (Reinhold *et al.*, 1990). The G-protein activated by fluoride *en route* to NADPH oxidase activation may not be closely linked to PLD as wortmannin was relatively ineffective in inhibiting fluoride-induced O_2^- generation.

In agreement with the above hypothesis, a study by Bonser *et al.* (1989) indicated that a receptor-linked PLD route provides an important source of diradylglycerol in fMLP-stimulated neutrophils, and that this pathway appeared to be involved in fMLP-stimulated O_2^- generation. Furthermore, evidence was presented to show that the PLD pathway may be less important in the OZ-invoked response.

As was outlined in the introduction to this chapter, Dewald *et al.* (1988) have described a distinct HWT-sensitive transduction sequence that is activated by chemotactic receptor agonists in human neutrophils.

They have defined this transduction sequence as Ca^{2+}- and PIP_2-independent but pertussis toxin-sensitive, implying that it does involve a G-protein. It is intriguing to speculate that this transduction sequence may involve a wortmannin sensitive G-protein that is coupled to PLD. Dewald *et al.* (1988) proposed that this wortmannin-sensitive transduction sequence is activated concurrently with the PIP_2-dependent, wortmannin-insensitive classical transduction pathway; both are believed to be necessary for optimal respiratory burst activation by receptor agonists.

The order of susceptibility of neutrophil cellular responses to the wortmannins — with the O_2^- response being the most susceptible, exocytosis being less sensitive, and aggregation, adhesion and phagocytosis not significantly affected — may indicate the degree of involvement of the G-protein–PLD pathway in the stimulus-activation coupling sequence for these cell responses.

The effect of the wortmannins on cellular responses may equally likely be due to other actions of these compounds that have not yet been unveiled.

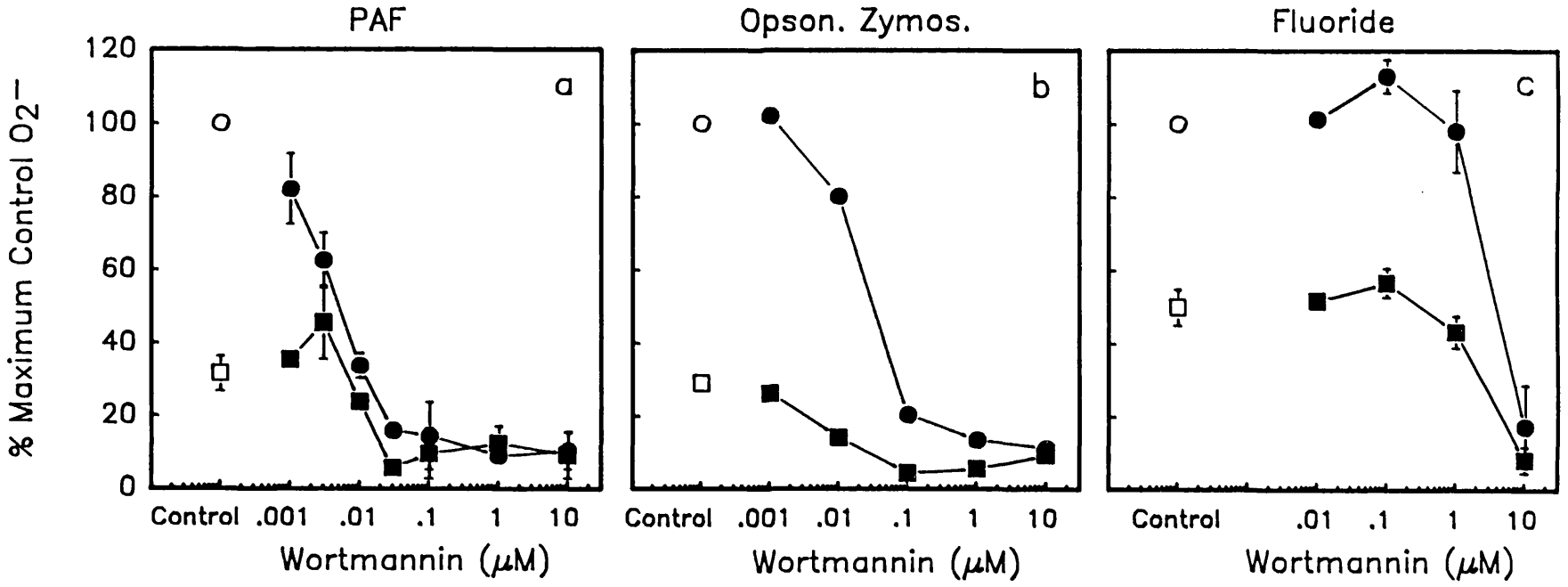


Figure 8.1: The effect of varying wortmannin concentrations on the O_2^- response induced by PAF, OZ and fluoride. a) PAF (100μM) alone (○) and in the presence of wortmannin (●), PAF (10μM) alone (□) and in the presence of wortmannin (■); n = 4. b) OZ (3mg/ml) alone (○) and in the presence of wortmannin (●), OZ (0.3mg/ml) alone (□) and in the presence of wortmannin (■); n = 1. c) Fluoride (18mM) alone (○) and in the presence of wortmannin (●), fluoride (10mM) alone (□) and in the presence of wortmannin(■); n = 4.

Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 35.69 ± 5.36 for PAF and 66.08 ± 10.56 for fluoride. The maximum control response for OZ, as a mean of sample duplicates, was 91.58 ± 4.48 nmol O_2^-/5 x 10^6 cells. Error bars in a & c represent standard errors, while in b they denote the range of sample duplicates.

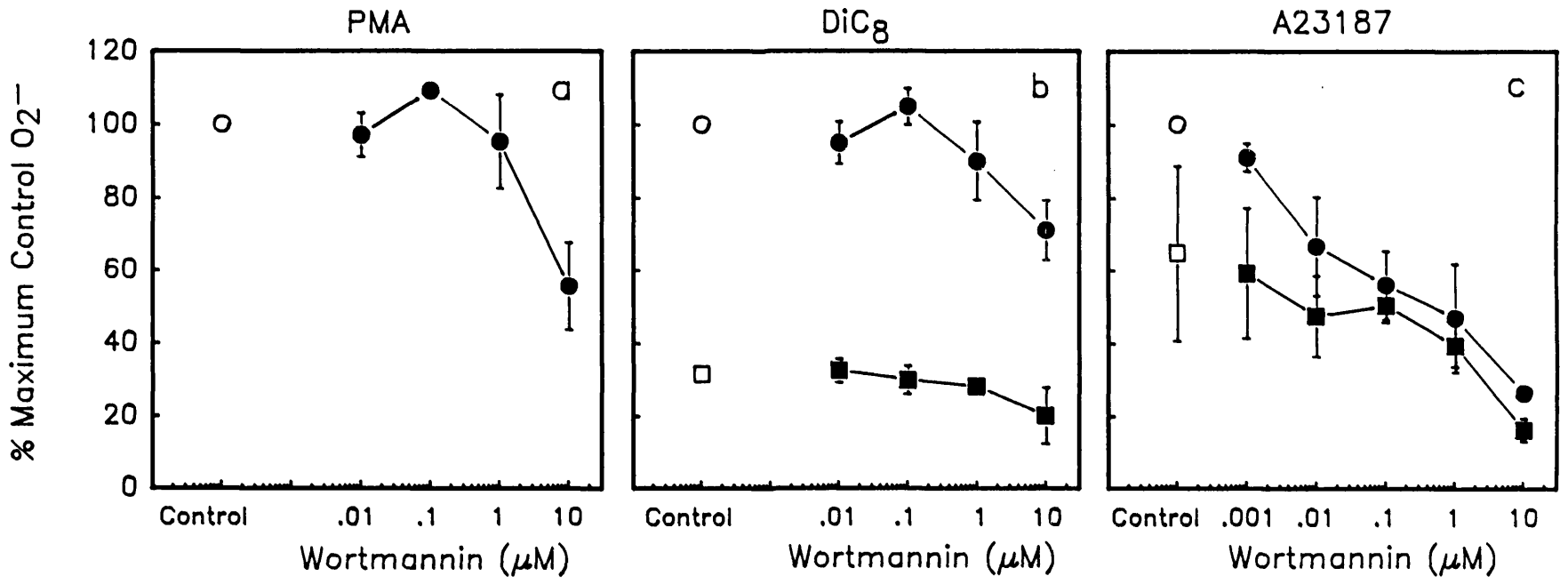


Figure 8.2: The effect of varying wortmannin concentrations on the O_2^- response induced by PMA, diC_8 and A23187. a) PMA (5nM) alone (○) and in the presence of wortmannin (●); *n* = 3. b) DIC_8 (25μM) alone (○) and in the presence of wortmannin (●), diC_8 (15μM) alone (□) and in the presence of wortmannin (■); *n* = 3. c) A23187 (10μM) alone (○) and in the presence of wortmannin (●), A23187 (1μM) alone (□) and in the presence of wortmannin (■); *n* = 2.

Mean maximum control O_2^- release, expressed as nmol/5 x 10^6 neutrophils was 125.29 ± 26.80 for PMA, 127.23 ± 31.91 for diC_8 and 51.60 ± 13.19 for A23187. Error bars in **a** & **b** represent standard errors while in **c** they denote the range of mean data.

CHAPTER NINE

GENERAL DISCUSSION

The aims of this chapter are two-fold; firstly, to summarize briefly the main experimental findings of this project and secondly, to present the current picture of the stimulus-response coupling events, introduced in Chapter 1, believed to be involved in respiratory burst activation.

9.1 General Findings and Implications:

A study of the effect of a wide range of clinically used NSAIDs on the production of oxygen metabolites revealed that some caused a marked potentiation of the stimulated response induced by both receptor and post-receptor stimuli. These findings could have clinical implications for the therapy of rheumatoid arthritis (RA) and other inflammatory disorders since toxic oxygen metabolites are known to produce tissue damage and could be involved in the self-perpetuating mechanism of joint damage, underlying RA. The NSAID-mediated enhancement of the O_2^- response is discussed extensively with relation to its potential *in vivo* capacity for exacerbation of inflammatory tissue damage in Chapter 4. The fact that the NSAIDs could be divided into three categories - those that enhance, those that have no effect and those that decrease O_2^- production offers a means of avoiding these toxic effects by selective prescribing on the part of clinicians. However, as stated in the above chapter, any definitive statements regarding the clinical effects of the enhancing NSAIDs would first necessitate a carefully controlled clinical trial with, for example, arthritic patients during both a drug-free and a NSAID-dosing period; to determine if the drug-induced enhancement of the level of neutrophil-derived O_2^- release occurs in *ex vivo* measurements.

The powerful potentiating effect of the NSAIDs on the O_2^- response was considered very interesting. The second part of the project comprised a pharmacological study of the transduction mechanisms involved in mediating the respiratory burst, but a mechanism of action that would explain this NSAID effect was kept in sight.

There had previously been evidence, with post-receptor stimuli, that the NSAID-mediated increase in O_2^- production was not necessarily due to an effect on cyclooxygenase *per se* (Penfield, 1988). This was confirmed in this study with both receptor and post-receptor stimuli. It was thus concluded that the enhancing effect of the NSAIDs was independent of their inhibition of cyclooxygenase.

The 5-lipoxygenase (5-LO) pathway of AA metabolism appeared not to be associated with respiratory burst activation as a series of 5-LO inhibitors had no significant effect on the O_2^- response. This same result was obtained in the presence of a cyclooxygenase inhibitor. Thus, the possibility that diversion of AA metabolism down the 5-LO pathway (on inhibition of the cyclooxygenase pathway) was responsible for mediating the increased cell response, in the presence of those enhancing NSAIDs, was considered as highly unlikely. Less clearcut conclusions could be drawn from the studies involving the PLA_2 pathway or endogenously generated AA in the O_2^- response. Some agonist-stimulated responses were inhibited by two reported PLA_2 inhibitors, though the specificity of the compounds is uncertain. Further work is necessary to clarify this question.

Attention was then focussed on the DAG/PKC transduction pathway, which was postulated to be of primary importance in NADPH oxidase activation. It was found from studies with DAG metabolizing inhibitors that DAG was an important second messenger in mediating the respiratory burst, whether stimulated at the receptor or beyond by a number of post-receptor mechanisms. A correlation was found between the effects of "specific" inhibitors of the DAG metabolizing enzyme - DAG kinase - and the NSAIDs, which led to the suggestion that the enhancing actions of these latter agents could be due to an inhibition of DAG kinase; this would give rise to a longer-lived activating signal and a potentiation of the O_2^- response. It was also concluded, from experimental evidence, that this mode of action was unlikely to be the full explanation. Another locus of action for the NSAIDs that was proposed was the PKC enzyme itself, but this was subsequently refuted when a range of the enhancing NSAIDs failed to potentiate the activation of isolated PKC, either alone or in combination with a DAG activator, dihexanoyl.

It was then deemed necessary to address the possible involvement of PKC in the oxidative burst, if the above hypothesis of DAG kinase inhibition by the NSAIDs was to be considered. There has been much controversy over the involvement of PKC in receptor-mediated O_2^-

production, stemming from studies carried out using poorly-specific PKC inhibitors. In the present project it was found that K252a and staurosporine, two recently described potent but not selective PKC inhibitors, and six novel bis-indolyl maleimide compounds (structurally derived from K252a and staurosporine), characterized as potent and selective inhibitors of PKC, all potently inhibited O_2^- production induced by both receptor and post-receptor mechanisms. Differences in the order of potency of the eight compounds with the range of stimuli indicated that there might be distinct PKC isoenzymes employed by the individual stimuli, differentially affected by the inhibitors. This is an intriguing phenomenon when considering PKC as a plausible target for potential pharmacological intervention.

The results obtained in this study strongly support an important role for PKC in the signal transduction sequences leading to generation of O_2^- and hence to toxic oxygen radicals. Evidence from the literature was presented in Chapter 1, pertaining to the involvement and the mode of action of these oxygen metabolites in mediating the tissue damage associated with inflammatory disorders such as RA. It is also becoming increasingly recognized that neutrophils and their oxygen free radical-derived metabolites contribute to ischemia-induced myocardial injury and, in particular, reperfusion-induced damage (Lucchesi & Mullane, 1986; Engler, 1989). The use of free radical scavengers has been proposed and is being tested experimentally as a means of reducing or arresting the oxygen radical-mediated tissue injury associated with the above conditions, and other disease states (Halliwell, 1987). Another approach in the rational design of drugs would be to target PKC, an enzyme which has been suggested in the present project to be crucial in mediating the O_2^- response. However, the ubiquity of the enzyme has raised doubts that inhibition of PKC would allow any degree of specificity. The recent identification of the heterogeneity of PKC suggests that differential modulation of specific processes mediated by particular PKC isoenzymes may be an achievable goal. At present seven subspecies of PKC have been described (see Chapter 5), with differential tissue distribution and functional properties. Some forms of PKC may require a lipid-Ca^{2+} complex for activation whereas others are stimulated directly by cis-unsaturated free fatty acid or lipoxin A (Coussens *et al.*, 1986). Thus, different lipids may be responsible for mediating the activation of individual PKC isoenzymes which in turn may give rise to specific cell responses. If these PKC isoenzymes possess differential

sensitivities to various PKC inhibitors (as is suggested from the data presented in this study), then there might well be potential for modulation of specific PKC-mediated responses such as the respiratory burst.

It is relevant here to mention a study in which oral administration of K252a showed a dose-dependent inhibition of cutaneous anaphylaxis in rats and anaphylactic broncho-constriction in passively sensitized guinea pigs (Ohmori *et al.*, 1988). In addition, K252a, as well as dexamethasone, showed remarkable inhibitory effects on both rat paw and rat ear oedema induced by variety of agents. It was concluded from this study that K252, by oral administration, has anti-allergic and anti-inflammatory effects (though it should be stated that the effect of K252a on broncho-constriction could have been due to its inhibitory effect on myosin light chain kinase - see Table 5.4). In another study, Mahoney *et al.* (1989) disclosed that auranofin and aurothioglucose, two of the disease-modifying anti-rheumatic drugs (DMARDs), inhibited isolated PKC from bovine brain and human neutrophil and concluded that this effect may be jointly responsible for the therapeutic action of these drugs. However, a pilot study in the present project showed that the DMARDs, auranofin and penicillamine, had no effect on isolated rat brain PKC.

In conclusion, it appears that PKC may have a focal role in mediating not only the neutrophil respiratory burst but also other inflammatory responses, a property that could be potentially exploited for the development of novel anti-inflammatory drugs.

9.2 PKC as a multidirectional regulator of neutrophil function

The exact mode of action of PKC in the activation of the neutrophil respiratory burst is not clearly understood but a model has been proposed by Sha'afi & Molski (1988) which explains some of the available information. Activation of the neutrophil by stimuli (such as phorbol esters) that do not cause a significant rise in $[Ca^{2+}]_i$ translocates PKC from the cytosol to the plasma membrane. Since the $[Ca^{2+}]_i$ remains low, little cleavage of the membrane PKC by calcium-activated neutral protease (CANP) occurs. The activated membrane-associated PKC then phosphorylates one or more proteins leading to the activation of NADPH oxidase. On the other hand, activation of the neutrophil by stimuli that raise $[Ca^{2+}]_i$, such as chemotactic agonists, causes the translocation of some of the PKC to the membrane and also the cleavage of most of the membrane-associated PKC by CANP into the regulatory unit which remains in the membrane and the catalytic unit which is released to the

cytosol. The released Ca^{2+}/phospholipid-independent protein kinase can phosphorylate both the granule proteins that would initiate degranulation, as well as the proteins that lead to respiratory burst activation. Those proteins that are phosphorylated by PKC on neutrophil activation are described in Chapter 1, and for some the phosphorylation reactions have been demonstrated to closely correlate NADPH oxidase activation. However, it is not yet clear whether phosphorylation reactions *alone* are sufficient for activation of NADPH oxidase. Other possibilities by which this enzyme complex could be stimulated are also outlined in Chapter 1.

There is evidence to show that the Na^+/H^+ antiporter, which mediates the efflux of H^+ in exchange for Na^+, is set into motion via PKC-mediated phosphorylation (Rosoff *et al.*, 1984; Besterman *et al.*, 1985) and that the secretion of protons is linked to activation of the respiratory burst (Borregaard *et al.*, 1984, Wright *et al.*, 1986). The precise role of Na^+/H^+ exchange in the activation mechanism of NADPH oxidase has yet to be defined.

PKC has been found in platelets to increase activation of the PI and PIP kinases, providing positive-feedback for agonist stimulation, since these enzymes are primarily responsible for maintaining PIP_2 substrate levels for the generation of phospholipase C-derived DAG and IP_3 (De Chaffoy de Courcelles *et al.*, 1984; Halenda & Feinstein, 1984).

The phospholipase A_2-inhibitory protein, lipocortin, has also been postulated as a substrate for PKC-induced phosphorylation in both platelets (Khanna *et al.*, 1986; Mobley & Taai, 1985) and neutrophils (Hirata, 1981; Stoehr *et al.*, 1990). However, from the data and arguments presented in Chapter 7 the role of endogenously released AA in activating the respiratory burst oxidase still remains obscure.

Outlined above are a number of PKC-mediated phosphorylation reactions that could potentially lead to activation of the respiratory burst or other cell responses. A number of studies have indicated that PKC could *also* provide an elegant and comprehensive mechanism of auto-regulation by the phosphorylation of other transduction enzymes and/or proteins. This modulation could take the form of either positive- or negative-feedback control, depending on the state of activation of the cell. Listed above are a number of possible mechanisms whereby an initial activation of PKC could mediate or further amplify the activating signal, and thus may also be termed positive-feedback. More importantly, it has been recently demonstrated in many cell types that PKC activation can itself either initiate or accelerate the hydrolysis of

phosphatidylcholine (PC) by the activation of a phospholipase D (PLD) or a PC-specific phospholipase C (PC-PLC) (see discussion in Chapter 6) (Cabot *et al.*, 1989; Guy & Murray, 1982; Hii *et al.*, 1989; Kinsky *et al.*, 1989; Besterman *et al.*, 1986a; Truett *et al.*, 1989; Tettenborn & Mueller, 1988; Daniel *et al.*, 1986; Agwu *et al.*, 1989; Gelas *et al.*, 1989).

On the other hand, PKC has been implicated in a number of shut-off mechanisms that appear to oppose the positive-feedback actions of the enzyme. Activation of PKC by phorbol esters and DAG has been shown to reduce agonist-evoked PIP_2 hydrolysis and Ca^{2+} flux in various tissues. Neutrophils pre-exposed to phorbol esters for short periods of time (1-5 minutes) were found to show a substantially decreased responsiveness (i.e. exocytosis) to subsequent stimulation by a variety of Ca^{2+}-mobilizing stimuli (Lagast *et al.*, 1984; Rickard & Sheterline, 1985; Naccache *et al.*, 1985b & d). This PKC-mediated effect may play a crucial role in negative-feedback regulation of the Ca^{2+}-mobilizing or PIP_2-dependent transduction pathway and is probably due to a variety of PKC actions. Phosphorylation of the receptor itself with inhibition of agonist binding or uncoupling of the receptor from its G-protein may provide one possible mechanism. PMA has been demonstrated to cause phosphorylation of the α_1-receptor, coincident with a decreased ability of α_1-adrenergic agonists to stimulate phosphoinositide turnover (Leeb-Lundberg *et al.*, 1985), and the regulation of the LTB_4 receptor by phosphorylation in human neutrophils has also been demonstrated (O'Flaherty *et al.*, 1986a). A second mechanism could involve a direct phosphorylation of the α-subunit of the G-protein, rendering it inactive in stimulating PLC, possibly by increasing its GTPase activity and the reassociation with $\beta\gamma$ subunits, or alternatively by preventing its activation by GTP. Phosphorylation of the α-subunit of G_i by PKC has been observed in platelet membranes, as well as with the purified α-subunit of G_i (Katada *et al.*, 1985). In another study, treatment of rabbit neutrophils with phorbol esters was found to cause the increased ADP-ribosylation of a 41kDa protein catalyzed by pertussis toxin and to inhibit the activation of GTPase stimulated by fMLP (Matsumoto *et al.*, 1986). This 41kDa protein which was phosphorylated by PKC was postulated to be the α-subunit of a G-protein, and was proposed to be functionally less active than its dephosphorylated counterpart. Furthermore, phorbol esters have been shown to disrupt coupling of the activated G-protein to PLC (Smith C.D. *et al.*, 1987; Kikuchi *et al.*, 1987).

A study by Geny *et al.* (1989) has shown that PMA pretreatment

potently inhibited PLC activity in permeabilized HL-60 cells, whether activated by Ca^{2+} or by stimulating the G-protein with either fluoride or GTPγS (the non-hydrolysable analogue of GTP). Phorbol esters and OAG have been shown to diminish both inositol phosphate and DAG levels in proliferating HL-60 cells (Geny *et al.*, 1988). It was concluded by the authors that the PIP_2-dependent PLC (PIP_2-PLC) was the target for phosphorylation by PKC, leading to inhibition of its catalytic function.

PKC has been shown to activate IP_3-5-phosphatase, thereby removing the second messenger for Ca^{2+} mobilization (Connolly *et al.*, 1986). Lagast *et al.* (1984) reported that the Ca^{2+}-ATPase in the neutrophil plasma membrane, which is largely responsible for restoring Ca^{2+} homeostasis following cell activation, was activated by PKC; this action may also contribute to the mechanism of feedback-inhibition.

In summary, PKC in addition to directly mediating cell responsiveness, may also act as a bidirectional regulator, involving both positive- and negative-feedback mechanisms. This is an intriguing phenomenon when considering an overview of stimulus-response coupling in cell activation, as was presented by Loffelholz (1989). Several Ca^{2+}-mobilizing agents stimulate the hydrolysis of choline and inositol phospholipids thereby producing DAG from different sources. If the DAG (from either source) stimulates PKC, the breakdown of choline phospholipids could be facilitated while phosphoinositide hydrolysis could be inhibited - both PKC-mediated mechanisms opposing each other and occurring simultaneously. The PKC-mediated breakdown of PC could be due to an action on a PLD, as demonstrated in HeLa cells (Hii *et al.*, 1989) and rat fibroblasts (Cabot *et al.*, 1989) or on a PC-specific PLC, as demonstrated by Besterman *et al.* (1986a) in a variety of cell types. Inhibition of the PIP_2-dependent pathway by PKC could involve any of the number of negative-feedback mechanisms outlined above. Figure 9.1 schematically illustrates the postulated bidirectional actions of PKC in respiratory burst activation: a positive system that enhances or prolongs the agonist-evoked production of DAG from PC opposes a negative system that blocks the PIP_2 transduction pathway, and hence the generation of PIP_2-derived DAG and IP_3. Evidence which also supports such a scheme of events comes from the study in which PMA pretreatment of human neutrophils was shown to potentiate subsequent fMLP-stimulated DAG generation (as well as O_2^- generation) concomitant with switching the source of DAG from PIP_2 to an alternative lipid (maybe PC) (Tyagi *et al.*, 1988).

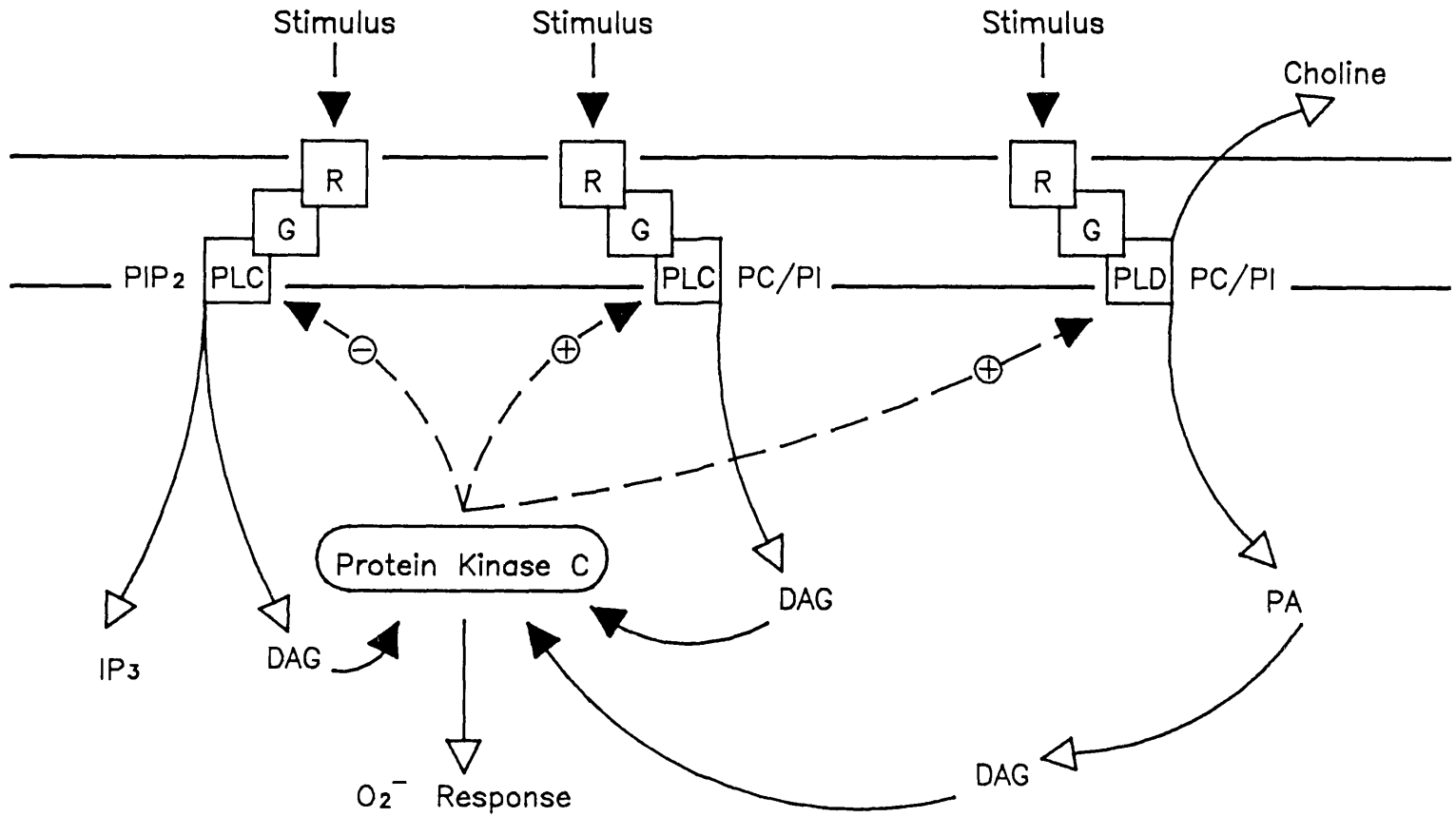


Figure 9.1: A schematic diagram of the stimulus-response coupling that may be involved in the respiratory burst, showing possible feedback regulation. DAG may be generated via PLC action on PIP_2, PC (or any other PL) or via PLD action on PC, PI (or any other PL). The resulting PKC activation, as well as initiating a respiratory burst, may inhibit subsequent PIP_2 hydrolysis (denoted by −), while possibly causing activation of a PLC or a PLD which would generate a second, more prolonged phase of DAG (denoted by +), maintaining PKC activation and the O_2^- response.

9.3 The respiratory burst of neutrophils: stimulus-response coupling

Whether the above modulatory sequence operates under normal physiological conditions in receptor-activated neutrophil responses is still unclear but opens many avenues for future research. It would be important to determine whether PC hydrolysis occurs, and if so whether it is through the action of a PLD or a PC-dependent PLC pathway. The activation of the PLD pathway in human neutrophils by fMLP, PMA and ionophore has already been demonstrated (Truett *et al.*, 1989; Reinhold *et al.*, 1990). Furthermore, the fMLP-stimulated respiratory burst has been more closely correlated with PLD activation than PIP_2 hydrolysis in another study in human neutrophils (Bonser *et al.*, 1989). It is relevant to note that the neutrophil respiratory burst was shown in the current study to occur under conditions where PIP_2 turnover was proposed to be absent and also in Ca^{2+}-depleted cells; this result was also reported by others (Della Bianca *et al.*, 1986; Grzeskowiak *et al.*, 1986; Rossi *et al.*, 1989). In addition, the inhibitory effect of wortmannin on receptor-stimulated O_2^- generation presented in the current study and others (Reinhold *et al.*, 1990; Dewald *et al.*, 1988) could, on one interpretation, support a role for PLD activation in respiratory burst transduction. Thus, the stimulation of PLD, either via a receptor-coupled mechanism or mediated through PKC activation, may play a significant role in the stimulus-response coupling for the neutrophil as well as in many other cell types.

It is proposed that sustained activation of the DAG/PKC pathway is necessary for mediating some cell responses (Rasmussen *et al.*, 1986; reviewed by Loffelholz, 1989). Superoxide production was initially thought to require two phases of DAG generation - an initial transient increase due to PIP_2 breakdown, and a second more prolonged increase possibly due to Ca^{2+}-activated PI/PIP-dependent PLC. The proposal of such a sequence came from studies where PLC-mediated PIP_2 turnover was reported to take place without an initial rise in Ca^{2+} (Dougherty *et al.*, 1984; Smith C.D. *et al.*, 1986) and the finding that PI and PIP turnover were dependent on a rise in Ca^{2+} (Cockcroft, 1982; Cockcroft, 1984; Cockcroft *et al.*, 1984). It has been shown, more recently, that sequential metabolic pathways may activate the respiratory burst in PMNs stimulated by chemoattractants (Truett *et al.*, 1988). According to these authors, the response is initiated by PIP_2 hydrolysis which results in rapid Ca^{2+} mobilization (< 5 seconds) and DAG release (peak at 30 seconds); to fully activate the respiratory burst a further

sustained Ca^{2+} influx must be triggered which supports a prolonged new phase of DAG production that is independent of PIP_2 hydrolysis. This second phase of DAG production could be mediated by PKC activation and could be derived via a PLD or a PC-dependent PLC mechanism, as summarized in fig. 9.1; but the question still remains speculative.

It is interesting to note that angiotensin II stimulation of vascular smooth muscle cells also caused a biphasic formation of DAG, and only the first peak was accompanied by a decrease in PIP_2 (Griendling *et al.*, 1988). The rapid PIP_2-dependent phase of DAG production could be blocked by phorbol ester, while the sustained phase of DAG formation remained unchanged.

Thus, it appears that a complex interplay of transduction enzymes appear to govern the initiation and maintenance of the neutrophil respiratory burst, with PKC appearing to occupy a crucial central position. Furthermore, modulation of the activating DAG/PKC pathway has been proposed by the physiological inhibitors of PKC, namely the sphingolipids when it was demonstrated that 1,2-DAGs can activate a sphingomyelinase in GH_3 pituitary cells (Kolesnick, 1987; Kolesnick & Clegg, 1988).

When considering the focal position of PKC in oxidative burst activation, the role of Ca^{2+} must not be overlooked. Elegant synergism experiments between the Ca^{2+} and PKC pathways have demonstrated an important mechanism of activation of this response (Dale & Penfield, 1984; Robinson *et al.*, 1984). Signal-induced elevations of $[Ca^{2+}]_i$ increase the binding affinity of PKC for phorbol esters/DAGs in HL-60 cells (Dougherty & Niedel, 1986) and in human PMNs (French *et al.*, 1987). Such an effect would allow for full activation of the enzyme under conditions where the concentration of phorbol esters/DAGs would be suboptimal. Formulation of a model for the activation of PKC takes account of the fact that attachment of 1,2-DAG to a surface-bound PKC-PS-Ca^{2+} complex stabilizes this quaternary structure and causes activation (Ganong *et al.*, 1986). An extensive discussion on the role of Ca^{2+} in the stimulated respiratory burst of neutrophils appears in Chapter 6; it was concluded that while a Ca^{2+} flux appears to be superfluous, a permissive role for Ca^{2+} may be required for optimal activation.

The object of this study was, in part, to investigate the activation mechanisms for the neutrophil oxidative burst, and it is hoped that the results obtained have made some contribution to this end. However, it is clear that the complex interplay of transduction pathways involved

need further clarification. Interconnections between PLC-, PLD- and PLA_2-mediated pathways, as well as possible links to sphingolipid pathways, provide the basis for a complex network of regulatory machinery. The relative contributions and interactions among these pathways remain a challenge for further studies.

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