Membrane Potential and Intracellular Cyclic AMP as Regulators of Calcium Homeostasis in Formyl Peptide-Activated Human Neutrophils: Lessons From Chronic Granulomatous Disease

by

Gregory Ronald Tintinger

MBBCh (Witwatersrand)

MMed (Pretoria)

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Summary

Neutrophils play a key role in the systemic inflammatory response which may lead to serious tissue injury and multiple organ dysfunction. In this setting, activated neutrophils, largely in response to tumour necrosis factor-alpha (TNF-α), secrete reactive oxidants, granule proteases and bioactive lipids, as well as pro-inflammatory cytokines, emphasising the importance of these cells as targets for anti-inflammatory therapies. There are, however, only a few currently available agents that directly modulate neutrophil pro-inflammatory responses in clinical practice, with corticosteroids being relatively ineffective against these cells. Although, the anti-inflammatory potential of cAMP-elevating agents has been recognised, the exact molecular/biochemical mechanisms which underlie the anti-inflammatory actions of epinephrine and related β-agonists with neutrophils, have not been established. Epinephrine treatment of neutrophils resulted in increased intracellular cAMP and dose-related inhibition of both superoxide production and elastase release, which was potentiated by the type 4 phosphodiesterase inhibitor, rolipram, further supporting a cAMP-mediated effect. Although epinephrine did not affect the release of Ca^{2+} from neutrophil intracellular stores, the rate of clearance of cytosolic Ca^{2+} was accelerated by this agent. In the setting of decreased efflux and a reduction in store-operated influx of Ca^{2+}, these effects of epinephrine are compatible with enhancement of the cAMP-dependent Ca^{2+} sequestering/resequestering endo-membrane Ca^{2+}-ATPase. Epinephrine therefore down-regulates the pro-inflammatory activation of neutrophils by cAMP-mediated enhancement of the clearance of cytosolic Ca^{2+}. Comparison of the effects of 4 selective (fenoterol, formoterol, salbutamol and salmeterol) and 3 non-selective (epinephrine, norepinephrine and isoproterenol) β-adrenoreceptor agonists, on the pro-inflammatory activities of human neutrophils, demonstrated that the agents tested clearly differ with respect to anti-inflammatory potential. Epinephrine, isoproterenol, fenoterol and formoterol significantly increased intracellular concentrations of cAMP in neutrophils, an activity which was paralleled by inhibition of the production of reactive oxidants and release of elastase from FMLP-activated cells. Salbutamol and salmeterol on the other hand, did not cause significant

suppression of the pro-inflammatory activities of these cells. The effect of norepinephrine was intermediate between these two groups. The inhibitory effects of β-agonists are mediated via β_2-adrenergic receptors on the neutrophil membrane.

The relationship between activation of NADPH oxidase, alterations in membrane potential and triggering of Ca^{2+} fluxes in human phagocytes has been investigated using neutrophils from 4 subjects with chronic granulomatous disease (CGD). Activation of CGD neutrophils was accompanied by a prolonged increase in cytosolic Ca^{2+}, occurring in the setting of trivial membrane depolarisation and accelerated influx of Ca^{2+}. This was associated with hyperactivity of the cells with excessive elastase release, which was attenuated by the type 4 phosphodiesterase inhibitor, rolipram. These findings support the involvement of NADPH oxidase in regulating membrane potential and Ca^{2+} influx in activated neutrophils, and may explain the disordered inflammatory responses, and granuloma formation, which are characteristic of CGD.

Store-operated influx of Ca^{2+} into activated neutrophils is stringently regulated, presumably to prevent hyperactivation of the cells. The major contributors to this physiologic, anti-inflammatory process are NADPH oxidase which, by its membrane depolarising actions excludes extracellular Ca^{2+}, and the plasma membrane and endomembrane Ca^{2+}-ATPases, which mediate clearance of store-derived cation. Subsequent influx of the cation, through store-operated Ca^{2+} channels is controlled by the relatively slow, restraining, membrane repolarising action of the Na^+/Ca^{2+} exchanger, enabling efficient diversion of incoming cation into stores.

Keywords: Neutrophils; epinephrine; calcium; chemoattractants; elastase; chronic granulomatous disease; membrane depolarisation/repolarisation; Na^+/Ca^{2+} exchanger; store-operated calcium channels.

Samevatting

Neutrofiele speel 'n sleutelrol in die sistemiese ontstekingsreaksie wat mag lei tot ernstige weefselskade en veelvuldige orgaanversaking. In hierdie milieu skei geaktiveerde neutrofiele, hoofsaaklik in reaksie op tumornekrosefaktor-α (TNF-α), reagerende oksidante, granulêre proteases en bio-aktiewe lipiede, sowel as proïnflammatoriese sitokiene, wat die belang van hierdie selle beklemtoon as 'n teiken vir antiïnflammatoriese behandelings. Daar is egter tans slegs 'n paar beskikbare middels wat neutrofiel proïnflammatoriese reaksies in die kliniese opset direk reguleer, met kortikosteroïde wat redelik oneffektief is teen hierdie selle. Alhoewel die antiïnflammatoriese potensiaal van cAMP-verhogende middels besef word, is die presiese molekulêre/biochemiese meganismes wat die grondslag vorm vir die antiïnflammatoriese aksies van epinefrien en verwante β-agoniste met neutrofiele, nog nie vasgestel nie. Behandeling van neutrofiele met epinefrien het aanleiding gegee tot verhoogde intrasellulêre cAMP en dosis-verwante inhibisie van beide superoksiedproduksie en elastase vrystelling, soos bemiddel deur die tipe 4 fosfodiësterase remmer rolipram, wat verder 'n cAMP-bemiddelde effek ondersteun. Hoewel epinefrien nie die vrystelling van Ca^{2+} uit instrasellulêre neutrofielstore beïnvloed het nie, was die opruimingstempo van sitosoliese Ca^{2+} versnel deur hierdie agent. In die raamwerk van 'n afname in uitvloei en verminderde stoor-beheerde invloei van Ca^{2+}, is hierdie effekte van epinefrien verenigbaar met 'n versterking van die cAMP-afhanklike Ca^{2+}-opnemende/heropnemende endomembraan Ca^{2+}-ATPase. Epinefrien onderdruk dus die proïnflammatoriese aktivering van neutrofiele, deur middel van 'n cAMP-bemiddelde verbetering in die opruiming van sitosoliese Ca^{2+}. Vergelyking van die effekte van 4 selektiewe (fenoterol, formoterol, salbutamol en salmeterol) en 3 nie-selektiewe (epinefrien, norepinefrien en isoproterenol) β-adrenoreseptor agoniste op die proïnflammatoriese aktiwiteit van menslike neutrofiele, toon dat die agente wat getoets is, duidelik verskil met betrekking tot antiïnflammatoriese potensiaal. Epinefrien, isoproterenol, fenoterol en formoterol het die intrasellulêre konsentrasies van cAMP in neutrofiele betekenisvol verhoog, 'n effek vergelykbaar met die onderdrukking van die

produksie van reagerende oksidante en vrystelling van elastase uit FMLP-geaktiveerde selle. In teenstelling hiermee het salbutamol en salmeterol nie betekenisvolle onderdrukking van die proïnflammatoriese werking van hierdie selle veroorsaak nie. Die effek van norepinefrien was geleë tussen hierdie twee groepe. Die onderdrukkende effekte van β-agoniste word bemiddel via β_2-adrenerge reseptore op die neutrofielmembraan.

Die verwantskap tussen aktivering van NADPH-oksidase, verandering in membraanpotensiaal en die inisiëring van Ca^{2+}-vloei in menslike fagosiete, is ondersoek met behulp van neutrofiele van 4 proefpersone met chroniese granulomateuse siekte (CGS). Aktivering van CGS-neutrofiele het gepaard gegaan met 'n verlengde toename in sitosoliese Ca^{2+}, wat plaasgevind het in die teenwoordigheid van onbeduidende membraandepolarisasie en versnelde Ca^{2+}-invloei. Dit was geassosieer met hiperaktiwiteit van die selle met oormatige elastase-vrystelling, wat verminder is deur die tipe 4 fosfodiësterases remmer rolipram. Hierdie bevindings ondersteun die betrokkenheid van NADPH-oksidase in die regulering van membraanpotensiaal en Ca^{2+}-invloei in geaktiveerde neutrofiele in, en mag die verwarrende ontstekingsreaksies en granuloomvorming wat kenmerkend is van CGS, verklaar.

Stoor-beheerde invloei van Ca^{2+} in geaktiveerde neutrofiele word streng beheer, waarskynlik om oormatige aktivering van hierdie selle te voorkom. Die hoof bydraers tot hierdie fisiologiese, antiïnflammatoriese proses is NADPH-oksidase, wat deur middel van sy membraan-depolariserende werking ekstrasellulêre Ca^{2+} uitsluit, en die plasmamembraan en endomembraan Ca^{2+}-ATPases, wat die opruiming van die stoor-afkomstige katioon bemiddel. Daaropvolgende invloei van die katioon deur stoor-beheerde Ca^{2+}-kanale word beheer deur die betreklik stadige, beheersde, membraan-herpolariserende werking van die Na^+/Ca^{2+}-uitruiler, wat doeltreffende omleiding van inkomende katioon na store bewerkstellig.

Sleutelwoorde: Neutrofiele; epinefrien; kalsium; chroniese granulomateuse siekte; elastase; membraan-depolarisasie/herpolarisasie; Na^{+}/Ca^{2+}-uitruiler; stoor-beheerde kalsiumkanale.

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β-AR	- Beta-adrenergic receptor
ARDS	- Acute respiratory distress syndrome
ATP	- Adenosine triphosphate
cAMP	- Adenosine 3,5' cyclic monophosphate
CB	- Cytochalasin B
CGD	- Chronic granulomatous disease
DAG	- Diacylglycerol
DMSO	- Dimethylsulfoxide
EGTA	- Ethylene glycol-bis(beta-amino-ethyl-ether)-N,N,N',N'-tetraacetic acid
FMLP	- N-formyl-L-methionyl-L-leucyl-L-phenylalanine
G-CSF	- Granulocyte colony stimulating factor
GM-CSF	- Granulocyte-macrophage colony stimulating factor
HBSS	- Hanks' balanced salt solution
IFN	- Interferon
IL	- Interleukin
LECL	- Lucigenin enhanced chemiluminescence
NADPH	- Nicotinamide adenine dinucleotide phosphate
PAF	- Platelet activating factor
PDE	- Phosphodiesterase
PLA_2	- Phospholipase A_2
PLC	- Phospholipase C
PMA	- Phorbol myristate acetate
RMP	- Resting membrane potential
TNFα	- Tumour necrosis factor-alpha

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

The modulation of neutrophil-mediated tissue injury remains an important therapeutic goal in clinical medicine. Achievement of this goal, however, relies on a detailed understanding of the intracellular mechanisms regulating the pro-inflammatory activities of activated neutrophils. In this regard, calcium is considered a key intracellular second messenger controlling vital functional responses in neutrophils. Calcium is required for optimal neutrophil activity, with the magnitude and duration of intracellular Ca^{2+} signals contributing significantly to activation of the superoxide-generating NADPH oxidase, concomitant with the mobilization of cytosolic granules. Although calcium is essential for optimal activation of numerous intracellular enzymes, excessive cytosolic concentrations may lead to cytotoxicity or aberrant functional responses. Therefore, cytosolic calcium is tightly regulated in neutrophils. The modulation of intracellular calcium in activated neutrophils, may thus represent an important anti-inflammatory therapeutic strategy. Cyclic AMP-elevating agents, including catecholamines, have been reported to down-regulate neutrophil pro-inflammatory responses to calcium-mobilizing stimuli, suggesting an effect on calcium metabolism.

Alterations in the membrane potential of activated neutrophils have been suggested to play an important role in regulating calcium homeostasis, although the mechanisms which underlie this interaction have not been conclusively established.

The laboratory research, the results of which are presented in this thesis, was undertaken to investigate the following:

1. The role of cAMP-elevating agents in the restoration of calcium homeostasis in activated neutrophils. Epinephrine, an endogenous anti-

inflammatory mediator, and non-specific beta-adrenergic agonist, was selected for these studies. The effects of epinephrine on the pro-inflammatory activities of human neutrophils *in vitro*, were compared to those of various selective and non-selective beta-adrenoreceptor agonists.

2. The relationship between alterations in membrane potential and calcium fluxes in activated neutrophils. Neutrophils from normal subjects and patients with chronic granulomatous disease (CGD) were used for this purpose as the absence of significant membrane depolarisation responses in CGD cells provides an ideal system for investigating the relationship between alterations in membrane potential and calcium fluxes in neutrophils.

3. The mechanisms responsible for maintaining the resting membrane potential of human neutrophils, as well as those mediating membrane depolarisation and repolarisation responses to activating stimuli.

The thesis consists of a literature review focusing on neutrophil-mediated tissue injury with emphasis on the role of neutrophil-derived reactive oxygen species in this process. The interactions between beta-adrenergic agonists and activated neutrophils are discussed, as well as the signal transduction pathways leading to activation of NADPH oxidase. Neutrophil responses to cAMP-elevating agents are reviewed prior to the section on calcium fluxes in activated neutrophils. This is followed by a consideration of current knowledge about the mechanisms responsible for maintaining the resting membrane potential of human neutrophils and those mediating membrane depolarisation and repolarisation responses in activated cells. The literature review concludes with a discussion of the role of alterations in membrane potential in regulating calcium homeostasis in activated neutrophils. The materials and methods used are described in each of the four chapters devoted to presentation of results.

The thesis concludes with an integrated discussion on the significance of the experimental findings and the clinical relevance thereof.

1.2 Neutrophil-mediated tissue injury

1.2.1 Neutrophils and host defense

Neutrophils constitute a vital component of the host's defence mechanisms and are immediately available for antigen-non-specific immune responses (Claman, 1992). These cells are essential for providing protection against invading bacteria and fungi and form part of a sophisticated network of inflammatory cells that ultimately ensure survival of the host in a hostile environment. The role of neutrophils has been aptly described thus, "Leukocytes constitute a silent army, entrusted to defend our borders against the agents of everyday germ warfare. Guided by sensors that can detect the faintest molecular traces of trouble, leukocytes are highly mobile and fully equipped to battle microbial transgressors." (Lehrer *et al,* 1988).

Polymorphonuclear leukocytes are produced by the bone marrow, where 60% of bone marrow capacity is devoted to ensuring a continual storage and circulating pool of these important cells. Circulating neutrophils account for only 2 – 3% of the total population, with 7 – 8% residing in the tissues (Holland and Gallin, 1998). The fifty billion neutrophils present in the bloodstream at any one time, circulate for approximately 10 hours before migrating into the tissues to complete their short lives of 1 – 2 days (Bainton, 1992).

Neutrophils are extremely well equipped to destroy micro-organisms. In order for them to carry out this function effectively, they must be able to perform each stage of a multistep process successfully to achieve the final goal of intracellular

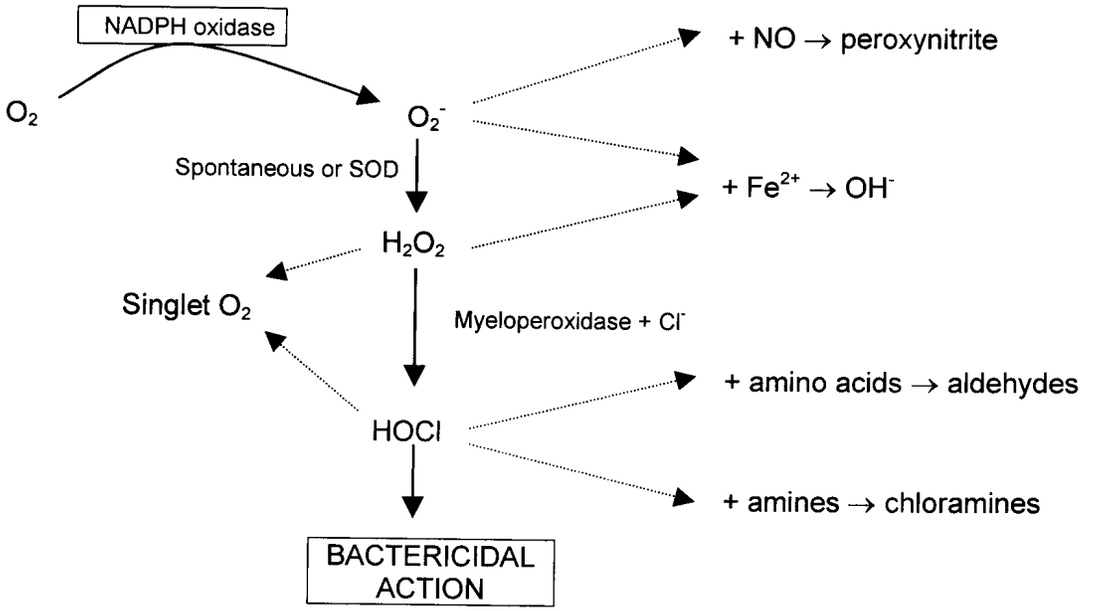
killing of bacteria (Matzner, 1997). The initial stage of movement to the site of inflammation or infection (chemotaxis) occurs in response to numerous stimuli such as tumour necrosis factor alpha (TNF-α), interleukin-8 (IL-8), endotoxin and leukotriene B_4. Neutrophil-endothelial interactions are regulated by a sequential cascade of molecular events charactersied functionally by i) rolling or tethering, ii) triggering or activation, iii) firm adhesion and iv) motility and migration (Adams and Shaw, 1994; Alessandro *et al.,* 1997). Rolling of neutrophils along post-capillary venules is mediated by the interaction of neutrophil L-selectin with endothelial E-selectin. Neutrophils may then be activated by numerous molecules such as IL-8 and platelet activating factor which upregulate neutrophil integrins, especially CD11b-CD18 (Adams and Shaw, 1994). Activated CD11b-CD18 molecules on the neutrophil membrane mediate firm adhesion by binding strongly to endothelial intercellular adhesion molecule (IACM-1) (Witko-Sarsat *et al.,* 2000). Following adherence, neutrophils migrate through the endothelial barrier towards higher concentrations of chemoattractants at the site of inflammation (Dallegri and Ottonello, 1997). Neutrophil migration through the extracellular matrix is facilitated by neutrophil proteolytic enzymes which digest matrix proteins. At the source of infection, ingestion of foreign micro-organisms (phagocytosis) by neutrophils occurs prior to the final stage of intracellular killing and digestion of bacteria (Witko-Sarsat *et al*, 2000).

1.2.2 Reactive oxygen species

Activated neutrophils produce a wide array of mediators that are able to destroy micro-organisms. These bactericidal mediators include reactive oxygen metabolites such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and the superoxide anion (O_2^-), as well as numerous proteolytic and other enzymes such as elastase, cathepsin G, metalloproteinases and myeloperoxidase. (Fujishima and Aikawa, 1995). The most potent antimicrobial system of the neutrophil is that which produces reactive oxidants. The formation of reactive oxidant species in neutrophils is represented schematically in Figure 1.1 (page 6).

Figure 1.1

Formation of neutrophil reactive oxidant species



This system consisting of the assembled, membrane-bound NADPH oxidase generates toxic oxidants when activated, which are released into phagolysosomes. NADPH oxidase transports electrons from the electron donor NADPH to molecular oxygen dissolved in the extracellular fluid or inside the phagosome according to the equation,

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

The superoxide anion (O_2^-) thus formed, undergoes rapid spontaneous dismutation to H_2O_2 and O_2. This reaction can also be catalysed by the enzyme, superoxide dismutase.

Most of the H_2O_2 generated from O_2^- is rapidly converted to hypochlorous acid (HOCl), a reaction catalysed by myeloperoxidase as follows (Weiss, 1989; Hampton *et al*, 1998):

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$$

Hypochlorous acid is itself a potent oxidant and may react with amine molecules to form chloramines, another class of oxidant molecule. Hypochlorous acid may

in addition react with amino acids to form reactive aldehydes or with H_2O_2 to form singlet oxygen.
O_2^- and H_2O_2 , in the presence of iron as the catalyst, may generate hydroxyl radicals (OH^-) which are able to form secondary radicals and establish a free radical chain reaction (Babior, 2000). Activated neutrophils release nitric oxide (NO) which forms peroxynitrite in the presence of O_2^-. Peroxynitrite may lead to peroxidation of membrane lipids (Ali *et al*, 1997; Babior, 2000).

Reactive oxygen species are potent oxidants which induce severe oxidative stress and cellular damage. The degree of tissue injury is dependent on the site of oxidant production as well as the integrity of local antioxidant defenses (Cross *et al*, 1994). Reactive oxidants are able to damage DNA molecules (Halliwell and Aruoma, 1991), alter cellular calcium homeostasis with increased intracellular calcium concentrations and deplete cells of their stores of ATP and NAD as well as altering surface receptor structure by modifying critical thiol and disulphide groups (Cross *et al*, 1994). Hypochlorous acid, a powerful oxidant, exerts direct cytolytic effects by damaging cell membranes (Weiss, 1989).

Oxidants may react with unsaturated fatty acids forming lipid hydroperoxides and aldehydes which activate phospholipases and protein kinases. This in turn enhances release of inflammatory mediators such as PAF and LTB_4 (Cross *et al*, 1994).

Oxidant stress also plays an important role in carcinogenesis with damage to DNA leading to various gene mutations (Kerr *et al*, 1996). Hydrocarbons may be oxidised into alkylating agents which results in alkylated DNA. These effects may be important in chronic inflammation which is associated with the development of neoplasia (Babior, 2000).

Nitric oxide produced by inflammatory cells has numerous biologic actions including vasodilatation and increased capillary permeability (Änggård, 1994).

The cytotoxic properties of nitric oxide are enhanced in the presence of oxidants with formation of peroxynitrite which may lead to lipid peroxidation. Nitric oxide produced by neutrophils may increase TNF production and further amplify the immune response (Ali *et al*, 1997). Tissues damaged by oxidants release pro-inflammatory cytokines with a positive feedback response as more inflammatory cells are recruited and activated.

Endogenous antioxidant defenses are therefore important in controlling or preventing oxidative damage. The mechanisms for neutralising oxidants include enzyme systems and antioxidant molecules present inside cells or in the extracellular fluid. The enzyme catalase converts H_2O_2 into O_2 and water and may act to scavenge HOCl, while the enzyme glutathione peroxidase utilises reduced glutathione to catalyse the detoxification of the highly membrane permeable oxidant, H_2O_2 (Jackson and Cochrane, 1988). Glutathione is an important endogenous antioxidant which is oxidised during reactions with oxidants. Reduced glutathione is regenerated by glutathione reductase (Whitin and Cohen, 1988).

A number of antioxidants are found in body fluids such as respiratory secretions and include surfactant, mucin, ascorbic acid, metal binding proteins and alpha-tocopherol (Cross *et al*, 1994). When the host's antioxidant systems are overwhelmed, oxidant-mediated tissue damage results.

Neutrophils protect themselves from oxidative damage by means of catalase, superoxide dismutase and reduced glutathione (Whitin and Cohen, 1988). Hypochlorous acid reacts with taurine inside neutrophils to form the non-toxic taurine chloramine (Babior, 2000). Myeloperoxidase can attenuate the inflammatory response by inactivating chemoattractants and reducing the mobility of phagocytic cells (Holland and Gallin, 1998), while nitric oxide may down-regulate neutrophil adhesion (Dallegri and Ottonello, 1997).

The powerful destructive capability of human neutrophils incorporating diverse reactive oxidants as well as bactericidal proteins and enzymes, is clearly beneficial to the host and when deficient or absent, as exemplified in patients with chronic granulomatous disease, results in recurrent, often life-threatening infections.

Neutrophils function not only as effector cells eliminating micro-organisms, but also as important immunoregulatory cells. Neutrophils are able to participate in both the amplification and down-regulation of the immune response by their ability to produce pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, IL-3, interferon-α, granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage CSF (Witko-Sarsat *et al*, 2000) as well as the anti-inflammatory cytokines IL-1 receptor antagonist and transforming growth factor-β (TGF-β) (Fujishima and Aikawa, 1995; Matzner, 1997).

1.2.3 Neutrophil-induced tissue damage

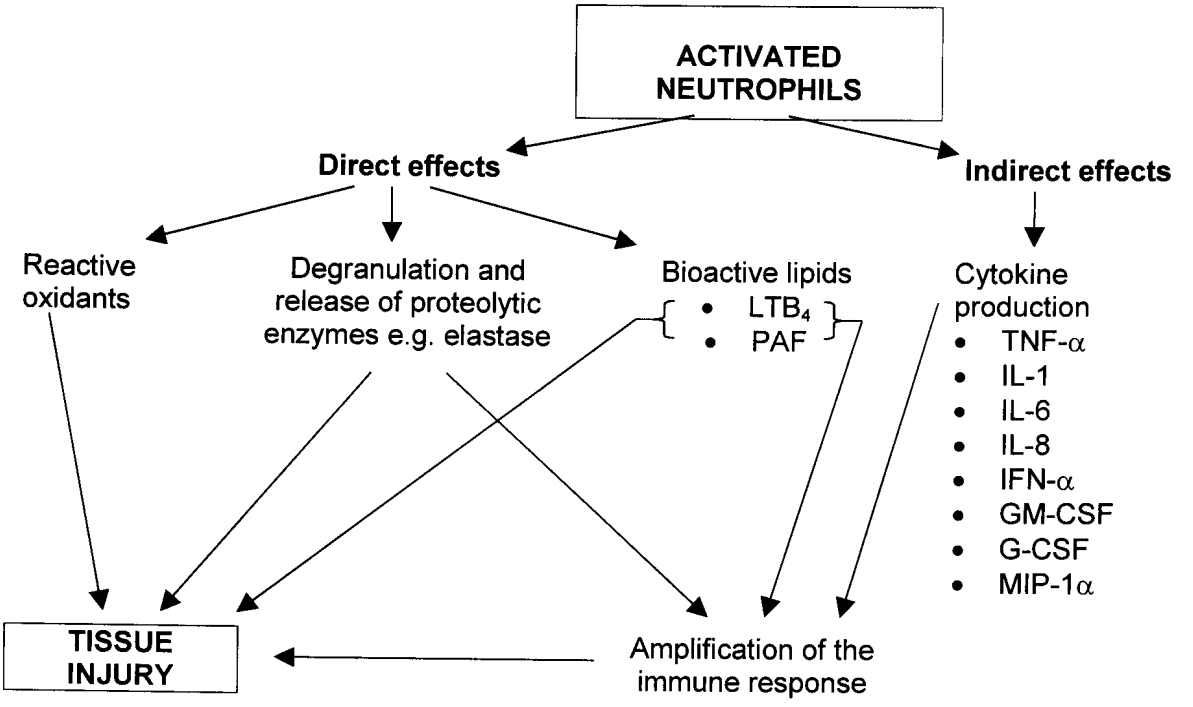
Activated neutrophils, in response to various stimuli, release an array of toxic reactive oxidants and powerful enzymes, which are able to combat and destroy invading microbes. Significantly, these destructive mediators may also damage normal host tissues during the inflammatory response and neutrophils have been implicated in the pathogenesis of a number of diseases. Conditions in which neutrophils may contribute to serious tissue injury include chronic obstructive pulmonary disease, acute respiratory distress syndrome, immune-complex alveolitis, glomerulonephritis, rheumatoid arthritis, gouty arthritis, ulcerative colitis, vasculitis, psoriasis, cystic fibrosis, sepsis and multi-organ failure, myocardial reperfusion injury, bronchiectasis, familial Mediterranean fever, Sweet's syndrome and Behçet's disease (Weiss, 1989; Hansen, 1995; Dallegri and Ottonello 1997; Matzner, 1997; Witko-Sarsat *et al*, 2000).

Neutrophils play a significant role in the pathogenesis and pathology of chronic obstructive pulmonary disease (COPD) and certain types of asthma. Neutrophils are recruited to the airways of patients with COPD and samples of induced sputum from these patients show increased numbers of neutrophils and elevated concentrations of IL-8 (Repine *et al.,* 1997). Activated neutrophils release reactive oxygen species which directly damage lung tissue, as well as inactivating antioxidant and antiprotease defences (Rahman and MacNee, 1996). Loss of antiprotease enzymes facilitates a protease/antiprotease imbalance within lung tissue that contributes to the proteolytic destruction of small airways, alveolar ducts and alveoli (Jeffrey, 1998). Neutrophil numbers are increased in the airways of patients with near-fatal asthma (Lamblin *et al.,* 1998) and may therefore be important cells in thepathogenesis of this form of asthma.

Neutrophil granules contain more than 50 hydrolytic enzymes and toxic molecules (Weiss, 1989) which, together with potent reactive oxidants, cannot discriminate between invading pathogens and normal tissues. This lack of discriminatory power renders normal host cells susceptible to attack during neutrophilic inflammation. The mechanisms responsible for activated neutrophils damaging normal host tissues may be due to direct or indirect effects. The direct effects are mediated by the products of neutrophil activation which include toxic oxygen metabolites, granule proteolytic enzymes and bioactive lipids. Indirectly, cytokines and related molecules released by neutrophils, contribute to the amplification of the immune response by attracting and stimulating more neutrophils and other inflammatory cell types. These events are summarised in Figure 1.2 (page 11).

Figure 1.2

Mechanisms of Tissue Injury



MIP-1α (Macrophage inhibitory protein-1α)
LTB_4 (Leukotriene B_4)
PAF (Platelet activating factor)

Micro-organisms are usually phagocytosed and subsequently killed inside the phagosome, which minimises cell damage to surrounding tissues. However, toxic substances may be concomitantly released into the extra-cellular space, so-called "regurgitation during feeding", with resultant damage and destruction of normal host tissues. The "regurgitation during feeding" phenomenon may occur when the target such as an immune complex or antibody coated cell is too large for phagocytosis, or in the presence of an overwhelming number of activating stimuli (Dallegri and Ottonello, 1997), or when the activating stimulus persists and cannot be removed (Ali *et al*, 1997).

Tissue damage to the host is also a feature of autoimmune diseases with the immune response directed at self-antigens. Another factor which may perpetuate damage to host tissues is the failure to adequately down regulate the immune response (Ali *et al*, 1997).

This mechanism may be particularly important during sepsis (Bone *et al.*, 1997). Sepsis is the systemic response to severe infection and consists of a complex immuno-inflammatory cascade (Bone *et al* , 1997). Sepsis is a common clinical problem, and according to the Center for Disease Control and Prevention in the USA is now considered the third most common infectious cause of death (Bone *et al*, 1997). The incidence of sepsis has continued to rise and septic shock is the leading cause of death in medical and surgical intensive care units (Astiz and Rackow, 1998). The number of patients with sepsis who develop septic shock has been estimated at 40% overall (Herdegen and Bone, 1992) and septic shock is associated with a mortality rate of 40 – 60% (Rackow and Astiz, 1991), despite intensive use of antibiotic therapy (Fagan and Singer, 1995). The systemic inflammatory response consists of an initial pro-inflammatory cascade involving activation of numerous inflammatory cells, including neutrophils, with release of pro-inflammatory cytokines such as interleukin-1, tumour necrosis factor and interleukin-6 (Rackow and Astiz, 1991).
This initial cytokine response is later modulated by a compensatory anti-inflammatory cascade that down-regulates the production of pro-inflammatory mediators (Bone *et al*, 1997). Loss of regulation of the pro-inflammatory response may lead to a massive systemic reaction with septic shock, multi-organ failure and death (Bone *et al*, 1997; Dianello, 1997). The significance of an ongoing, excessive pro-inflammatory response has led to a search for agents that mediate down-regulation of the pro-inflammatory cascade.

Neutrophils also play an important role in the pathogenesis of the acute respiratory distress syndrome (ARDS) which occurs in 30 – 80% of patients with septic shock (Rackow and Astiz, 1991). During acute lung injury, large numbers

of activated polymorphonuclear leukocytes are sequestered to the pulmonary microvasculature. Activated neutrophils adhere to capillary endothelium and migrate into the pulmonary interstitium and alveolar spaces (Sessler *et al*, 1996; Hasleton and Roberts, 1999). Toxic mediators released by neutrophils at these sites produce the severe endothelial and epithelial damage characteristic of ARDS (Gadek and Pacht, 1996).

1.2.4 Proteolytic enzymes

The most destructive proteolytic enzymes released following neutrophil degranulation are elastase, gelatinase and collagenase, which attack and digest extracellular matrix proteins (Weiss, 1989). Elastase has the most destructive potential with large quantities (up to 3 pg/cell) stored in azurophil granules (Janoff, 1985). Elastase is able to destroy numerous proteins and extracellular matrix components including elastin, collagen types I, II, III, IV and VI, laminin, fibronectin, proteoglycans and fibrinogen (Fujishima and Aikawa, 1995; Janoff, 1985). The extracellular matrix functions not only as a surrounding supportive substance, but also regulates important cell functions including cellular migration, growth and differentiation, as well as facilitating the orderly repair of injured tissues (Weiss, 1989). Therefore, destruction of the extracellular matrix potentiates tissue damage.

In order to protect matrix proteins, the extracellular fluid and plasma contain significant concentrations of antiproteinases that are able to rapidly and irreversibly inhibit neutrophil-derived proteinases. These antiproteinases include α_1-proteinase inhibitor, α_2-macroglobulin and secretory leukoproteinase inhibitor (Weiss, 1989).

Oxidants, produced by neutrophils, can inactivate these antiproteinases and therefore render host tissues more susceptible to proteolytic destruction (Rahman and MacNee, 1996). Elastase itself is resistant to oxidative degradation

at the concentrations produced by human neutrophils (Vissers and Winterbourn, 1987). In addition to its effects on matrix proteins, elastase can promote changes in microvascular permeability, increase production of kinins, as well as activating complement components (Dallegri and Ottonello, 1997). Elastase increases IL-8 production from epithelial cells which further recruits and activates neutrophils (Dallegri and Ottonello, 1997). These effects exacerbate tissue injury.

1.2.5 Bioactive lipids

Phospholipase A_2 is activated following neutrophil stimulation and generates lyso-PAF and arachidonic acid from membrane phospholipids. Lyso-PAF is converted to PAF and leukotriene B_4 is generated from arachidonic acid by the enzymes acetyltransferase and lipoxygenase respectively (Zimmerman *et al,* 1992). PAF and LTB_4 are potent chemoattractants and therefore serve to amplify the immune response. In addition, PAF may cause direct cellular damage (Fujishima and Aikawa, 1995), while both PAF and LTB_4 potentiate oxidant production by NADPH oxidase.

1.2.6 Modulation of neutrophil-mediated tissue injury

In light of the marked potential for activated neutrophils to mediate serious tissue damage and their associated implication in the pathogenesis of an array of human diseases, it is not surprising that numerous agents and strategies have been evaluated for their ability to attenuate neutrophil proinflammatory responses.

In order to achieve this goal, therapeutic strategies have been targeted at each stage of the activation process. Inhibitors of chemotaxis and adhesion, as well as multiple receptor antagonists have been tested with limited success (Dallegri and

Ottonello, 1997). Numerous substances ranging from enzyme inhibitors to calcium channel blockers and glucocorticoids have also been investigated.

The apparent lack of efficacy of these agents in controlling neutrophil activation has been attributed to poor specificity, a complex, interacting network of activating cells and cytokines that prohibits targeted down-regulation of inflammatory cells, an incomplete understanding of the molecular mechanisms responsible for neutrophil activation and effector responses, as well as unacceptable side effects on other organs or tissues (Fujishima and Aikawa, 1995).

An agent that effectively down-regulates neutrophil pro-inflammatory responses must have a predictable, repeatable and specific effect that attenuates both the direct and indirect mechanisms responsible for cellular damage. Research should be aimed at acquiring an in-depth understanding of the molecular pathways involved in all aspects of neutrophil activation leading to oxidant production and degranulation, and then applying this information to the search for agents that modulate neutrophil-mediated tissue injury. These have been the goals of this research project.

1.3 Neutrophils and adrenergic agonists

1.3.1 Interaction between beta-adrenergic agonists and activated neutrophils

The obvious necessity for effectively modulating the pro-inflammatory responses of stimulated neutrophils has prompted investigation of the effects of numerous agents, including catecholamines, on neutrophil functions. Epinephrine is an important endogenous regulatory hormone and plasma levels are markedly elevated during conditions of stress. Resting epinephrine levels vary from 10 – 70

pg/ml and may rise to > 400 pg/ml during heavy exercise, while following an acute myocardial infarction, these may reach 1000 pg/ml (Cryer, 1980).
It is now well recognised that the neuroendocrine system is able to interact with and exerts significant modulatory effects on the immune system. The adrenal gland produces catecholamines and cortisol which alter leucocyte numbers and function. With regard to neutrophils, conflicting reports exist on the responses of these cells to adrenergic agonists.

Qualliotine *et al* (1972), found that the catecholamines, epinephrine and norepinephrine stimulated oxidative metabolism with increased oxygen consumption by neutrophils, as well as enhanced activity of the hexose monophosphate shunt. Busse and Sosman (1984), investigated the effects of catecholamines on neutrophil responses following stimulation with opsonised zymosan. Their results, using luminol-enhanced chemiluminescence to measure oxidant production, showed inhibition of the respiratory burst by isoproterenol, epinephrine and norepinephrine. The inhibitory effects of these agents were noted only in the presence of theophylline. Hetherington and Quie (1985), used opsonised zymosan and phorbol myristate acetate (PMA) to stimulate neutrophils pre-incubated with epinephrine. No significant effect on luminol-enhanced chemiluminescence was observed.
In contrast to the above, Tecoma *et al* (1986), described marked inhibition of oxidant production by neutrophils stimulated with a formyl peptide when either isoproterenol, epinephrine or norepinephrine were added together with the stimulant. These investigators also showed that the inhibitory effects of isoproterenol could be largely blocked by the beta-adrenergic antagonist, propranolol.

Markiewicz *et al* (1989), again using luminol-enhanced chemiluminescence to measure the oxidative burst of neutrophils activated with opsonised zymosan, found a decreased response following incubation with epinephrine. Similar inhibitory effects of epinephrine were reported by Bazzoni *et al* (1991), with the

formyl peptide (N-formyl-methionyl-leucyl-phenylalanine or FMLP) added as the stimulant. More recently, Weiss *et al* (1996), investigated the effects of epinephrine, dopamine and dobutamine on luminol-enhanced chemiluminescence in FMLP-activated neutrophils. Epinephrine significantly inhibited the chemiluminescence response. Burns *et al* (1997), used human volunteers who received intravenous infusions of epinephrine, dobutamine or dopexamine. Blood was drawn at intervals during and after infusion of the drug. Neutrophil responses to FMLP and opsonised zymosan were subsequently measured by means of luminol-enhanced chemiluminescence. Neutrophils from subjects infused with epinephrine showed an exaggerated chemiluminescence response in contrast to most previous studies. The fact that purified neutrophils were not used in this study may have contributed to enhanced activation of the cells.

The above studies, although interesting, suffer from several limitations. Firstly, the use of luminol-enhanced chemiluminescence procedures to detect superoxide generation in activated neutrophils may complicate the interpretation of results for various reasons, i) luminol may in certain situations inhibit the release of superoxide, ii) luminol may traverse biological membranes and react with intracellular oxidants and, iii) luminol lacks specificity and may emit light in response to myeloperoxidase metabolites and superoxide anions (Dahlgren and Karlsson, 1999). In contrast, lucigenin is highly sensitive to and more specific for the superoxide anion (Minkenberg and Ferber, 1984). Currently, determination of oxidant production using a lucigenin-enhanced chemiluminescence assay is more appropriate. Secondly, none of these investigators attempted to identify comprehensively the molecular and biochemical mechanisms which mediate the anti-inflammatory properties of epinephrine.

With regard to neutrophil degranulation responses, Busse and Sosman (1984), observed inhibition of lysosomal beta-glucuronidase release in the presence of isoproterenol and theophylline. Bazzoni *et al* (1991), obtained similar results with

epinephrine (10 μM) causing ~ 40% inhibition of lysozyme and β-glucuronidase release from neutrophils activated with FMLP. Degranulation responses to FMLP in neutrophils from patients with cystic fibrosis, were significantly inhibited following pre-incubation for 5 min with epinephrine (1 μM) (Suter *et al*, 1989). In this study, primary granule β-glucuronidase release was reduced by 28 – 32% and vitamin B_{12}-binding protein released from secondary granules was inhibited by 15% compared to control cells. De Togni *et al* (1984), in evaluating neutrophil responses to cyclic AMP, found that release of β-glucuronidase and vitamin B_{12}-binding protein were significantly inhibited by addition of the cell permeable, dibutyryl cAMP.

1.3.2 Beta-adrenergic drugs and the beta-adrenergic receptor

In order to adequately elucidate the molecular mechanisms whereby epinephrine is able to attenuate superoxide production by and elastase release from activated neutrophils, it is necessary to understand the intracellular events associated with binding of adrenergic agonists to their receptors. Endogenous catecholamines such as epinephrine and norepinephrine act non-selectively at alpha (α), beta-1 (β_1) and beta-2 (β_2) receptors. The β-adrenergic agonists consist structurally of a catechol ring which determines primarily the potency of the molecule and an ethanolamine side chain which confers selectivity to the molecule (Popa, 1986). Various catecholamine derivatives act selectively at β_2- receptors.

Bazzoni *et al* (1991), demonstrated that while the non-selective beta-adrenergic antagonist, propranolol, reversed the inhibitory effect of epinephrine on neutrophil superoxide production, the alpha-receptor antagonist, phenylephrine, was ineffective. The inhibitory effect of isoprenaline was also markedly attenuated by propranolol (Tecoma *et al*, 1986). These results implicate agonist binding to β-adrenergic receptors (β-AR) on the neutrophil membrane as the initial step mediating down-regulation of pro-inflammatory responses. Weiss *et al* (1996), were unable to document any attenuation of epinephrine's inhibitory effect using

a potent β_1-AR antagonist. The inability of a selective β_1-antgonist to alter epinephrine's inhibitory effects on neutrophil responses, supports a β_2-receptor mediated mechanism.

The human β-adrenergic receptor is composed of 413 amino acid residues with seven transmembrane segments, characteristic of G-protein-coupled receptors (Barnes, 1995). Following binding of a beta-agonist to the receptor, a conformational change takes place in the alpha subunit of the G-protein which results in activation of membrane-bound adenylyl cyclase. Adenylyl cyclase in turn, converts ATP to the important intracellular messenger cyclic AMP, which activates protein kinase A (PKA). PKA is able to phosphorylate intracellular proteins and thus regulate key cellular responses (Barnes, 1995).

Binding of β-agonists to the β-AR is rapid, reversible and activation of the receptor is dependent on continuous occupancy by the agonist. Neutrophil membranes contain approximately 1000 receptors per cell which are fully saturated with isoprenaline at a concentration of 1 μM (Mueller, 1988). Agonist binding stabilises the receptor in a high affinity state which facilitates coupling to the G-protein.

1.3.3 Selective and non-selective β-adrenergic agonists

Beta-adrenergic agonists may bind to two classes of β-receptors, namely β_1 and β_2. The non-selective agonists isoproterenol, epinephrine and norepinephrine interact with β_1 and β_2, while selective agonists including salbutamol, fenoterol, formoterol and salmeterol exert their effects at β_2-receptors only (Popa, 1986). Agonist β-receptor selectivity is dependent on the structure of the ethanolamine side chain within the molecule (Popa,1986).

In addition to differences in selectivity of binding, β-adrenergic agonists also differ with respect to agonist potency and are classified as full or partial agonists (Bremner *et al*, 1996). Full agonists induce maximal biological responses, while partial agonists, at similar concentrations, produce less than maximal responses (Bremner *et al*, 1996). The potency of an agonist for inducing a biological response is dependent on its affinity for the receptor, as well as its intrinsic activity at the receptor (Moore *et al*, 1998). The intrinsic efficacy of β-agonists bound to β-receptors correlates with the resultant increase in intracellular cAMP that occurs with activation of the β-adrenergic receptor (Bremner *et al*, 1996). In studies using isolated guinea pig tracheae to compare the intrinsic activities of various β-agonists, the relative ranking in magnitude of response was isoproterenol > formoterol > fenoterol = salbutamol > salmeterol (Lemoine *et al*, 1992; Lindén *et al*, 1993). Similar studies comparing the rank order of potency of selective and non-selective β-agonists in neutrophils, have not been performed.

1.4 Neutrophil activation and signal transduction pathways

1.4.1 Membrane receptors

Neutrophils respond to environmental stimuli with a number of effector responses including chemotaxis and adherence, phagocytosis, oxidant production and degranulation mediated via specific receptors at the cell surface. A number of different types of receptor are found on the outer membrane and include adherence receptors, chemotaxic receptors, phagocytic receptors, cytokine-sensitive receptors and receptors performing unknown functions (Lew, 1990).
Receptors may also be grouped according to their structure as G-protein linked, membrane tyrosine kinases, tyrosine kinase-linked, glycosylphosphatidylinositol-linked, adhesion molecules and ceramide-linked (TNF receptor) (Downey *et al*, 1995). G-protein linked receptors include those for FMLP, complement

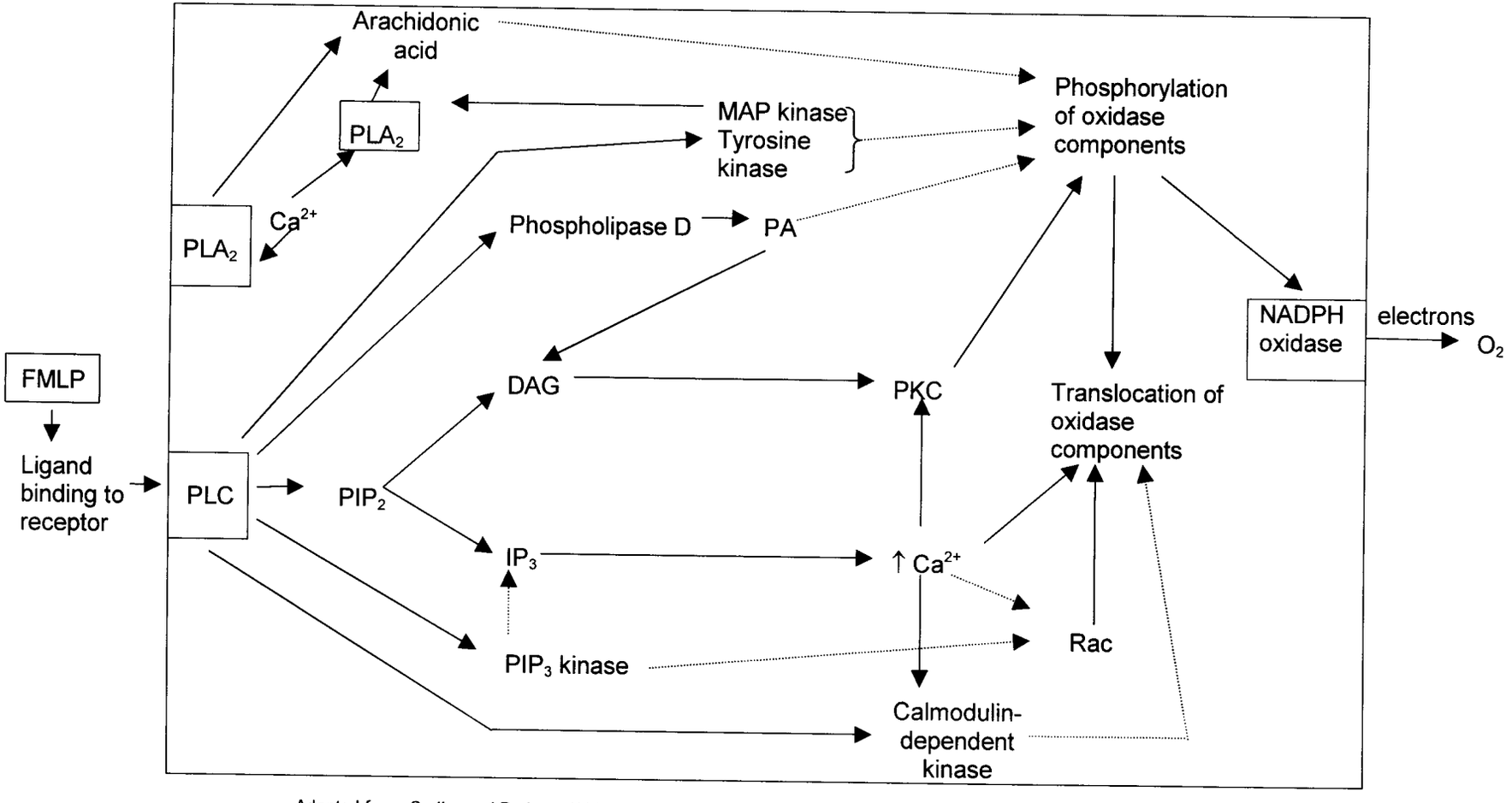
component (C5a), PAF, LTB_4, IL-8 and various chemokines (Downey *et al*, 1995).

Formyl peptides derived from bacterial wall products (including FMLP), are potent neutrophil chemotactic and activating stimuli. The outer membrane of human neutrophils contains approximately 55 000 FMLP receptors (Lew, 1990). These important receptors show strict structural specificity with kinetic properties of saturable, rapid binding (50% of receptors within 30 seconds) and rapid internalisation (Weinbaum et al, 1982; Sandborg and Smolen, 1988). The equilibrium dissociation constant is in the range 10 – 20 nM (Weinbaum *et al*, 1982; Lew, 1990). An optimal response can be evoked with only 10 – 20% of receptors occupied by the agonist. FMLP receptors are also present in the membranes of specific granules (Sandborg and Smolen, 1988). FMLP receptors are coupled to a membrane G-protein consisting of G_α, G_β and G_γ subunits. Following ligand binding to this receptor, phospholipase C is activated by the $G_{\beta\gamma}$ subunit (Condliffe *et al*, 1998).

1.4.2 Signal transduction pathways leading to activation of NADPH oxidase

Signal transduction pathways serve to transmit activating signals from the plasma membrane or cytosol to the target enzyme and produce marked amplification of the inciting stimulus utilising intracellular second messengers. Those activated by FMLP are shown in Figure 1.3 (page 22). FMLP is an extremely important activator of neutrophils in experimental systems and *in vivo*. FMLP-mediated activation occurs predominantly via phospholipase C with generation of inositol-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a potent stimulus for calcium release from intracellular stores while DAG activates protein kinase C, an event greatly potentiated by cytosolic calcium (Sadler and Badwey, 1988; Condliffe *et al*, 1998).

Figure 1.3 FMLP-mediated Signal Transduction Pathways Activating NADPH Oxidase



Adapted from: Sadler and Badwey (1988); Downey *et al* (1995); Hallett and Lloyds (1995); Condliffe *et al* (1998)

DAG = diacylglycerol; IP_3= inositol (1,4,5)triphosphate; MAP kinase = mitogen-activated protein kinase; PIP_2 = phosphatidylinositol 4,5-bis-phosphate; PIP_3 = phosphatidylinositol 3,4,5-triphosphate; PLA_2 = phospholipase A_2; PLC = phospholipase C; PA = phosphatidic acid; Rac = GTP binding protein.

In addition to the above pathway, FMLP binding to its membrane receptor may lead to NADPH oxidase activation via alternative routes including phospholipase D and phosphatidic acid, mitogen-activated protein (MAP) kinase, tyrosine kinases, phosphatidylinositol 3,4,5-triphosphate (PIP_3) kinases and other unknown mediators (Condliffe *et al*, 1998).

Stimuli other than FMLP, such as phorbol myristate acetate (PMA), opsonised zymosan and the calcium ionophore (A23187) also activate neutrophil oxidant production. PMA has high affinity for protein kinase C which is directly activated in a calcium-independent manner. PMA may also increase intracellular DAG and phosphatidic acid (Walker and Ward, 1992). A23187 induces flooding of the cytosol with Ca^{2+} which activates Ca^{2+}-dependent PKC.

1.5 NADPH oxidase: assembly and function

Neutrophils, as professional phagocytes, generate toxic reactive oxygen species when activated, in order to facilitate intracellular killing of microbes. The assembly and activation of NADPH oxidase components is the key event that results in transmembrane transport of electrons from NADPH to molecular oxygen, according to the equation:

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

O_2^- undergoes spontaneous and enzymatic dismutation to H_2O_2, from which HOCl (the most bactericidal oxidant) is generated by myeloperoxidase (Hampton *et al*, 1998). NADPH oxidase consists of membrane and cytosolic components. The membrane components include cytochrome b_{558} and Rap1A, with cytochrome b_{558} consisting of 2 subunits, $gp91^{phox}$ and $p22^{phox}$. Three proteins, $p47^{phox}$, $p67^{phox}$ and rac-2 (guanosine triphosphatase) provide the cytosolic components necessary for full activation of the oxidase (Clark, 1999). Following an appropriate stimulus, the cytosolic components translocate to the cytochrome b_{558} docking site on the inner surface of the cytoplasmic membrane. The

translocation of $p67^{phox}$ is entirely dependent on the presence of $p47^{phox}$ (Clark, 1999) with rac-2 serving as a regulatory element (Malech and Nauseef, 1997).

The $gp91^{phox}$ subunit of cytochrome b_{558} contains the NADPH, flavin and heme binding domains, but is neither stable nor active alone (Malech and Nauseef, 1997). The role of $p22^{phox}$ is to stabilise $gp91^{phox}$, coordinate heme binding to $gp91^{phox}$ and to provide docking sites for the cytoplasmic components (Malech and Nauseef, 1997). The heme and flavin groups bind to $gp91^{phox}$ and participate in electron transfer (Segal *et al*, 2000). Rap 1A, a member of the Ras family, also binds to cytochrome b_{558} and is important for the activity of the oxidase (Segal *et al*, 2000). Once all the components of the oxidase are fully assembled on the inner surface of the membrane, electrons begin to flow from NADPH to FAD, then to heme centers and finally to molecular O_2 (Clark, 1999). Importantly, the flow of electrons is vectorially oriented across the membrane from inside the membrane towards its outer surface. This unidirectional flow of electrons leads to depolarisation of the membrane potential (Schrenzel *et al*, 1998).

The initial step in the activation process of NADPH oxidase is phosphorylation of the cytosolic component, $p47^{phox}$. Following phosphorylation of $p47^{phox}$, translocation of the cytosolic components occurs. Cytochrome b_{558} is found exclusively within membranes of mostly specific or gelatinase-containing granules (85%) or within the plasma membrane (~ 15%) (Segal *et al*, 2000). These specific locations of cytochrome b_{558} facilitate generation of reactive oxidants inside phagolysosomes following the mobilisation of cytoplasmic granules which fuse with the plasma membrane and discharge their contents into phagolysosomes (Segal *et al*, 2000). This tight regulation of superoxide production and degranulation serves to limit damage to normal bystander cells and tissues. Other regulatory mechanisms include the complex phosphorylation and translocation processes which prevent inadvertent activation of the oxidase, a number of signal transduction pathways linking membrane receptors to oxidase activation (Hopkins *et al*, 1992), as well as endogenous down-regulators inside

the neutrophil. Endogenous down-regulators include PR39 (a proline-rich antibacterial peptide), defensin HNP-1, proteinase 3 and nitric oxide (Segal *et al*, 2000). The cytosolic protein, $p40^{phox}$, may also negatively regulate oxidase activity (Nauseef, 1999).

Activation of NADPH oxidase is an energy dependent, reversible process which reaches a steady state and can function at oxygen concentrations as low as 1% over a broad pH range (7.0 – 7.5) (Babior and Woodman, 1990). The trigger for NADPH oxidase activation is phosphorylation of cytosolic $p47^{phox}$. This key phosphorylation step may be catalysed by several kinases, of which protein kinase C is the most important. Other relevant kinases include phosphatidic acid-activated kinase, p21-activated kinase, p38 and calmodulin-dependent kinase (Nauseef, 1999; Tauber, 1987). Arachidonic acid, in the presence of calcium, is also able to activate the oxidase (Tauber, 1987). Arachidonic acid may act synergistically with protein kinases during activation of NADPH oxidase (Shiose and Sumimoto, 2000).

1.6 Neutrophil degranulation

Human neutrophils contain numerous granules in their cytosol which serve as storage sites for a wide range of important substances including enzymes and receptors. These cytoplasmic granules can be classified into 4 main types, azurophil (primary), specific (secondary), gelatinase (tertiary) granules and secretory vesicles, on the basis of their contents (Borregaard *et al*, 1993). These are summarised in Table 1.1 (page 26).

The physiologic significance of these granules relates to the many functions mediated by granular contents including microbial destruction and amplification of the immune response. Neutrophil granules are mobilised following activation of

the cell and discharge their contents into phagolysosomes which facilitate destruction of microbes or other ingested particles.

Table 1.1 Summary of the most important contents of human neutrophil granules (Borregaard *et al*, 1993)

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Elastase	Collagenase	Gelatinase	Plasma proteins
Myeloperoxidase	Gelatinase	Acetyl transferase	Cytochrome b_{558}
Lysozyme	Histaminase	CD11b	CD11b
β-glucuronidase	Heparanase	FMLP-receptors	FMLP-receptor
Cathepsins	Lactoferrin		Alkaline phosphatase
Defensins	Lysozyme		Uroplasminogen activator-receptor
Proteinase-3	Vit B_{12}-binding protein		
α_1-antitrypsin	Plasminogen activator		
Acid muco-polysaccharide	β_2-microglobulin		
Acid β-glycero-phosphatase	Cytochrome b_{558}		
Azurocidin	FMLP-receptors		
Sialidase	CD11b		
Bactericidal permeability increasing protein	TNF-receptor		
	Fibronectin receptor		

Neutrophil oxidant-independent mechanisms account for approximately 20% of microbial killing (Hampton *et al*, 1998). The most abundant tissue destroying protease released by neutrophils is elastase. Elastase facilitates the penetration of neutrophils through extracellular matrix in order to reach the source of chemotactic cytokines and elastase is able to digest collagen, elastin and

proteoglycans (Dallegri and Ottonello, 1997). It has also been reported that in the setting of lung inflammation, neutrophil migration from capillaries to the alveoli is faciliated as they displace interstitial fibroblasts and move through pre-existing holes in the basal lamina (Walker *et al,* 1995). Elastase may promote endothelial cell damage and enhance IL-8 production by epithelial cells (Dallegri and Ottonello, 1997), both of which contribute to amplification of the immune response. In addition, the membrane of specific granules contains cytochrome b_{558}, FMLP receptors and the adhesion molecule CD11b, all of which augment pro-inflammatory responses.

Degranulation may be triggered by multiple stimuli including FMLP, PAF, LTB_4, thromboxane B_2, DAG and intracellular Ca^{2+}. Secretion from granules is enhanced by cytochalasin B and occurs rapidly with a lag period of < 10 sec and is usually complete by 2 minutes (Boxer and Smolen, 1988). PMA induces only partial and selective degranulation releasing the contents of specific, but not azurophil granules (Lehrer, 1982). With cytosolic Ca^{2+} being an important trigger for degranulation, it is important to note that the mobilisation of granules is hierarchic with differential responses and sensitivity to rising cytosolic Ca^{2+} concentrations (Sengeløv, 1996). Gelatinase granules are mobilised first in response to small increments in cytosolic Ca^{2+} concentration. A rise in the cytosolic calcium concentration of only 100 nM leads to granule release from 20% of gelatinase granules with no translocation of specific or azurophil granules. With increments of 300 nM, specific granules are also mobilised with gelatinase granules, while azurophil granules are mobilised last when cytosolic calcium is elevated by 800 nM (Borregaard *et al*, 1993; Sengeløv, 1996).

1.7 Neutrophil responses to cAMP-elevating agents

Cyclic AMP is an important intracellular second messenger which regulates cellular responses by activating protein kinase A. Protein kinase A, in turn,

phosphorylates enzymes or proteins and thereby regulates various cellular functions (Sandborg and Smolen, 1988). It is well recognised that elevation of intracellular cAMP levels in neutrophils attenuates granule enzyme release and oxidant production following stimulation of the cells (Suter *et al*, 1989; Moore and Willoughby, 1995; Bloemen *et al*, 1997). cAMP may therefore act as an endogenous mechanism for down-regulating pro-inflammatory responses in inflammatory cells (Moore and Willoughby, 1995). Human neutrophils are able to synthesise and store catecholamines, which may act as endogenous, autoregulatory hormones, down-regulating the pro-inflammatory responses of these cells (Cosentino *et al*, 1999).

Following stimulation with FMLP, cAMP levels inside neutrophils rise rapidly to 2 – 3 times basal levels (Weiss *et al*, 1996). This 'paradoxic' increase in cAMP levels peaks at 10 – 15 seconds and returns to baseline after 2 – 5 min (Bleich, 1980) and may constitute a physiologic mechanism to counter excessive activation of neutrophils. The mechanism for this increase in cAMP has been attributed to an endogenous adenosine-mediated potentiation of adenylyl cyclase acting via A_{2A} receptors, coupled to the enzyme (Iannone *et al*, 1989).
The amplitude and duration of the cAMP response are regulated by intracellular phosphodiesterase enzymes (PDEs) which degrade cAMP (Beavo, 1995). The most abundant subtype found in neutrophils is the phosphodiesterase 4B2 (cAMP specific) isoenzyme (Wang *et al*, 1999; Ortiz *et al*, 2000) with very little PDE 5 (Torphy, 1998).

Inhibition of PDE 4, in the presence of endogenous activators of adenylyl cyclase, potentiates the effects on intracellular cAMP (Torphy, 1998). This synergistic effect is operative when neutrophils are activated in the presence of a β-adrenergic agonist and a PDE inhibitor (Bazzoni *et al*, 1991; Moore and Willoughby, 1995). Significantly, the inhibitory effect of cAMP-elevating agents is absent during PMA-stimulated responses in neutrophils (De Togni *et al*, 1984; Nagata *et al*, 1992). This observation implicates alterations in Ca^{2+} kinetics as the

mechanism responsible for the inhibition of superoxide production and degranulation in FMLP-stimulated neutrophils. A number of mechanisms have been proposed for this cAMP-mediated alteration in Ca^{2+} kinetics. These include inhibition of phospholipase C activity with reduced production of inositol triphosphate and diacylglycerol (De Togni *et al*, 1984; Suter *et al,* 1989; Nagata *et al*, 1992), inhibition of Ca^{2+} mobilisation from intracellular stores (De Togni *et al*, 1984; Nagata *et al*, 1992) or via an accelerated rate of Ca^{2+} re-uptake into storage vesicles (De Togni *et al*, 1984; Nagata *et al*, 1992; Anderson *et al*, 1998).

The effect of epinephrine on cAMP levels in resting and FMLP-activated neutrophils is important and has been variously reported by previous investigators. Bazzoni et al (1991), observed a slight effect on resting cells with cAMP levels rising from 7.9 ± 0.5 pmol/10^7 cells to 9.1 ± 0.5 pmol/10^7 cells in the presence of epinephrine. For FMLP-activated cells, cAMP levels (1 min after stimulation) reached 14.7 ± 0.9 pmol/10^7 cells without epinephrine and 27.4 ± 1.4 pmol/10^7 cells with epinephrine (1 μM). A similar potentiation of the FMLP response in the presence of isoproterenol was reported by Tecoma *et al* (1986). This amplification of the FMLP-induced increase in cAMP levels has been attributed to enhanced basal activity of adenylate cyclase in the presence of a β-adrenergic agonist prior to addition of the stimulant (O'Dowd and Newsholme, 1997).

The cAMP response to the full agonist, isoproterenol, may be greater than that observed with epinephrine, and correlates with the degree of inhibition of superoxide production (Nagata *et al*, 1992). Amplification of the cAMP response to β-adrenergic agonists may also be achieved by addition of PDE 4 inhibitors, such as Ro 20-1724 (Bazzoni *et al*, 1991). The above observations are consistent with a cAMP-mediated inhibition of the pro-inflammatory responses of activated neutrophils which is concentration dependent.

1.8 Calcium kinetics in activated human neutrophils

Alterations in the concentration of cytosolic calcium regulates many key intracellular processes and the cation serves as an important intracellular second messenger relaying information from the cell membrane to the interior of the cell (Parekh and Penner, 1997; Barritt, 1999). The magnitude, location and duration of changes in cytosolic free calcium convey the Ca^{2+} signal to target enzymes and proteins inside the cell (Barritt, 1999). Calcium mediated signals are important for neutrophil superoxide production, degranulation and bioactive lipid formation following binding of appropriate ligands to membrane receptors. (Schwab *et al*, 1992). Modulation of Ca^{2+} kinetics may therefore constitute an important strategy for anti-inflammatory therapy.

The cytosolic Ca^{2+} concentration in resting neutrophils is maintained at 100 nM (Westwick and Poll, 1986; Lew, 1989). The neutrophil cytoplasm contains numerous calcium storage vesicles or calciosomes which release their Ca^{2+} contents in response to an appropriate signal, usually inositol triphosphate (Lew, 1989).

Receptor bound inositol triphosphate induces a biphasic calcium response with an initial rapid rise within 6.5 $\pm$ 2.5 seconds (Theler *et al*, 1995) to reach peak concentrations in the micromolar range (Westwick and Poll, 1986; Lew 1989). This is followed by an initial rapid, then gradual decline over minutes towards baseline values (Lew, 1989). The initial rapid decline from peak levels is essential to protect the cell from toxic flooding of the cytosol by Ca^{2+} and is achieved by means of two main mechanisms. The initial release of Ca^{2+} from calciosomes is accompanied by an almost simultaneous efflux of Ca^{2+} across the outer membrane into the extracellular fluid (Westwick and Poll, 1986; Barritt, 1999). Calcium efflux across the plasma membrane is mediated by a Ca^{2+}-ATPase activated by Ca^{2+}/calmodulin (Westwick and Poll, 1986; Lew 1989; Carafoli *et al*, 1992). The second mechanism whereby Ca^{2+} is removed from the cytosol involves active pumping of Ca^{2+} ions back into calciosomes. This resequestration

of Ca^{2+} into storage vesicles is dependent on the activity of an endomembrane Ca^{2+}-ATPase (Lew, 1989; Favre *et al*, 1996).

Efflux of Ca^{2+} across the plasma membrane is complete at about 30 sec following activation and is followed by influx of Ca^{2+} across the outer membrane. This Ca^{2+} influx occurs in order to replenish intracellular stores, with incoming Ca^{2+} diverted directly to the calciosomes with little effect on cytosolic concentrations (Barritt, 1999). Calcium efflux and influx are chronologically distinct events with onset of influx 30 – 60 sec after activation of the cells and completion of influx at 5 min (Anderson and Goolam Mahomed, 1997). Calcium influx occurs via store-operated channels which open in response to emptying of intracellular calciosomes. These store-operated channels are sensitive to changes in membrane potential and allow entry of Mn^{2+} as well as Ca^{2+} (Alonso-Torre *et al*, 1993; Barritt, 1999). Calciosomes have emptied their calcium stores within 10 – 15 sec (Alonso-Torre *et al*, 1993), but influx of Ca^{2+} is delayed until 1 – 2min following stimulation (Montero *et al*, 1994). The mechanism for delayed calcium influx is presumably due to inhibition of influx of the cation during membrane depolarisation (Di Virgilio *et al*, 1987; Geiszt *et al*, 1997; Parekh and Penner, 1997). The message from the Ca^{2+} stores to open membrane store-operated channels is not known, but may be due to protein phosphorylation (Montero *et al*, 1994), contact activation by Ca^{2+}, translocation of Ca^{2+}-containing vesicles or transfer of soluble factors such as calcium influx factor, cGMP, kinase enzymes or G-proteins (Favre *et al*, 1996). Irrespective of what the exact 'message' may be, it is well established that the filling state of intracellular calcium stores regulates Ca^{2+} influx (Parekh and Penner, 1997).

1.9 Regulation of the membrane potential in resting and activated neutrophils

The plasma membrane of human neutrophils allows these specialised cells to react to multiple environmental stimuli with membrane receptors playing a key

role in ligand recognition and initiation of signal transduction pathways, leading to effector responses typical of activated neutrophils. An equally important function of the plasma membrane is to maintain ionic concentration gradients. That ionic gradients are maintained across the neutrophil outer membrane is evident from the marked differences in the intracellular and extracellular concentrations of potassium (K^+), sodium (Na^+) and chloride (Cl^-) ions. The intracellular K^+ concentration is approximately 120 – 138 mM (Seligmann *et al*, 1980; Simchowitz *et al*, 1982; Krause *et al*, 1991; Jankowski and Grinstein, 1999), with an extracellular concentration of 5 mM (Krause *et al*, 1991). The corresponding intra- and extracellular concentrations of Na^+ are 20 mM and 140 mM, with chloride concentrations maintained at 80 mM and 140 mM, respectively (Krause *et al*, 1991). At these concentrations, the equilibrium potential calculated for potassium ions (according to the Nernst equation) (Seligmann *et al*, 1980) is ~ -85 mV. This is close to the resting membrane potential (RMP) of neutrophils which has been variously reported as -53 mV (Simchowitz, 1988), -60mV (Demaurex *et al*, 1993b), -67 mV (Mottola and Romeo, 1982), -75 mV (Majander and Wikström, 1989) and -100 mV (Henderson *et al*, 1987). A number of mechanisms for maintaining this electrical potential difference have been suggested. These include voltage-activated K^+ channels (Krause *et al*, 1991; Demaurex *et al*, 1993b) and the membrane-associated, electrogenic Na^+/K^+-ATPase exchanger. The relative contribution from the Na^+/K^+-ATPase may vary from none at all (Seligmann *et al*, 1980), to 20% of the RMP (Majander and Wikström, 1989), to being the sole contributor maintaining the resting membrane potential (Bashford and Pasternak, 1985). The potassium ion concentration gradient, as a function of membrane potassium permeability and conductance, has also been suggested as the primary mechanism responsible for generating the RMP (Seligmann *et al,* 1980; Mottola *et al*, 1982; Myers *et al*, 1990). In addition, an inwardly rectifying potassium channel, sensitive to tetraethylammonium chloride (a non-specific K^+-channel inhibitor), has been reported to set the RMP of eosinophils close to the equilibrium potential for potassium (Banfi *et al*, 1999).

Membrane depolarisation is a critical event in the normal functioning of excitable tissues such as nerve and muscle and also occurs in non-excitable cells including eosinophils and neutrophils. Numerous mechanisms have been proposed for mediating neutrophil membrane depolarisation, but the precise mechanisms have not been established. A number of ions and ion fluxes have been implicated. Sodium influx has been considered important by some investigators (Luscinskas *et al*, 1988; Majander and Wikström, 1989) and refuted by others (Kuroki *et al*, 1982; Myers *et al*, 1990). Influx of calcium ions has also been advocated as a contributory mechanism (Scharff and Foder, 1996). A role for chloride ion efflux has also been suggested (Myers *et al*, 1990; Krause and Welsh, 1990; Menegazzi *et al*, 1996). The intracellular chloride concentration of resting neutrophils is relatively high compared to other cells at approximately 80 to 90 mM (Menegazzi *et al*, 1999), which must be actively maintained (Shimizu *et al*, 1993) with 20% of steady state chloride fluxes involving an active transport mechanism against the concentration gradient (Simchowitz and De Weer, 1986). Chloride efflux apparently accompanies activation of the cells with FMLP and phorbol esters (Myers *et al*, 1990; Shimizu *et al*, 1993), although the timing of this event is controversial. Efflux of Cl^- ions reportedly precedes superoxide production triggered by β_2-integrin cross-linking (Menegazzi *et al*, 1999), with chloride channels possibly playing an important role in regulating superoxide production by activated eosinophils (Schwingshackl *et al*, 2000). Chloride efflux may occur rapidly with an immediate decline in the intracellular concentration of chloride in PMA-stimulated neutrophils (Myers *et al*, 1990), and is evident at 5 min after addition of TNFα (Menegazzi *et al*, 1996). The chloride conductance channel is activated by calcium (Krause and Welsh, 1990) and inhibited by ethacrynic acid (Menegazzi *et al*, 1996) and may directly contribute to the membrane depolarisation response (Myers *et al*, 1990; Grinstein *et al*, 1992). Currently however, the mechanism mediating membrane depolarisation in activated neutrophils, is largely attributed to the vectorial flux of electrons across the outer membrane (Demaurex *et al*, 1993b; Schrenzel *et al*, 1998). This process is dependent on the activity of NADPH oxidase, which transports

electrons from NADPH to molecular oxygen, creating a depolarising electron current. The resting membrane potential of -60mV rapidly depolarises to ~ 0mV, following stimulation of neutrophils (Demaurex *et al*, 1993b).

Membrane depolarisation is complete at about 1 min after activation of the cells, and is followed by a slower phase of repolarisation over several minutes (Henderson *et al*, 1987). Proposed mechanisms mediating neutrophil membrane repolarisation have included altered permeability to potassium ions (Seligmann *et al*, 1980) and activation of the Na^+/K^+-ATPase exchange mechanism (Majander and Wikström, 1989). Although plausible, the role of activation of membrane-associated Na^+/K^+-ATPase during repolarisation of the membrane potential has not been confirmed. Instead, much attention has focused on proton conductance as an acid extrusion mechanism which also leads to concomitant repolarisation of the cell membrane (Banfi *et al*, 2000).

The respiratory burst of activated neutrophils generates protons (~ 50 nmol $H^+/10^6$ cells) which imposes a considerable acid load on the cell and could potentially decrease cytosolic pH to 1.6 (Nanda and Grinstein, 1991). In order to maintain intracellular pH > 6.4 (Nanda and Grinstein, 1991), protons are transported out of the cell (Henderson *et al*, 1987). This proton conductance mechanism is activated at depolarising potentials (Schrenzel *et al*, 1998) and rapidly translocates protons across the cell membrane. This flux of protons to the exterior of the cell is electrogenic, thereby facilitating repolarisation of the membrane potential (Demaurex *et al*, 1993b). The proton conductance pathway is highly specific for H^+ ions (Demaurex *et al*, 1993b; Banfi *et al*, 2000) and is inhibited by Zn^{2+} or Cd^{2+}, a characteristic common to other membrane proton transporters (Kapus *et al*, 1992; Banfi *et al*, 2000). The precise membrane channel involved in this process is not known with certainty, but an assembled (Nanda *et al*, 1994a) or functional NADPH oxidase (Nanda *et al.*, 1993; Banfi *et al*, 1999) is required. The $gp91^{phox}$ membrane component of NADPH oxidase may provide the conductance pathway (Henderson *et al*, 1997; Segal *et al.*,

2000). Cells from patients with X-linked CGD, lacking the normal $gp91^{phox}$ component, are however still able to transport protons out of the cell by means of an endogenous H^+ conductance distinct from $gp91^{phox}$ (Banfi *et al*, 1999). A number of these studies have been conducted with eosinophils (Schrenzel *et al*, 1998; Banfi *et al*, 1999), given the technical difficulties inherent in patch-clamping procedures with neutrophils. Neutrophils are not ideally suited for patch-clamping techniques as they are short-lived, small, and highly motile cells (Nanda *et al*, 1994b). Nevertheless, some investigators have used this procedure to study neutrophil proton conductance and ion fluxes (Demaurex *et al*, 1993a), with similar results to those obtained with eosinophils.

Two further mechanisms exist for extruding protons from activated neutrophils, incorporating an electrically silent, amiloride sensitive, Na^+-H^+ antiporter (Simchowitz, 1985) and active transport via the vacuolar-type (V-type) ATPases (Nanda and Grinstein, 1995). The non-electrogenic Na^+-H^+ exchange mechanism is unlikely to participate in restoring the membrane potential during repolarisation. The V-type ATPase pathway for H^+ extrusion in FMLP-stimulated neutrophils has been reported to be activated after a lag period of 1 – 2 min. This bafilomycin-sensitive, electrogenic, H^+ conductance may therefore contribute to pH regulation (Nanda and Grinstein, 1995), and membrane repolarisation in activated neutrophils, although this is not considered to be the major mechanism for proton extrusion (Henderson *et al*, 1987).

1.10 The role of membrane depolarisation in regulating neutrophil pro-inflammatory responses: Lessons from chronic granulomatous disease

Recent reports have highlighted the close link between depolarisation and electron transfer during the respiratory burst of activated neutrophils with cells from patients with chronic granulomatous disease being unable to generate oxidants and therefore lacking any significant depolarisation response (Schrenzel

et al, 1998). The functional significance of neutrophil membrane depolarisation has not been unequivocally established, but some available evidence supports the contention that at depolarising potentials, calcium influx is abolished, protecting the cell interior from Ca^{2+} overload (Di Virgilio *et al*, 1987; Jankowski and Grinstein, 1999). This negative feedback effect following NADPH oxidase activation may serve as an endogenous down-regulatory mechanism on calcium-mediated pro-inflammatory responses.

Chronic granulomatous disease (CGD) encompasses a group of inherited disorders, characterised by defects in the oxidant generating NADPH-oxidase complex of phagocytes. Abnormal phagocytic function is manifest clinically as recurrent, often life-threatening bacterial and fungal infections (Dinauer, 1992). Inheritance of CGD may be X-linked or autosomal recessive with genetic mutations resulting in abnormal function of one of the membrane or cytosolic components (Gallin *et al*, 1983). X-linked defects in $gp91^{phox}$ account for $^{2}/_{3}$ of cases while the autosomal recessive form is associated with abnormal $p47^{phox}$ (2,5%), $p22^{phox}$ (5%) or $p67^{phox}$ (5%) (Segal *et al*, 2000). Oxidant production in all forms of CGD is either trivial or absent with markedly diminished membrane depolarisation responses (Åhlin *et al*, 1995). As depolarisation may play an important role in regulating calcium influx into activated neutrophils, it follows that cells from CGD patients are ideal for studying the functional relationship between alterations in membrane potential and calcium kinetics. Disordered calcium homeostasis has been reported in CGD neutrophils as an almost instantaneous influx of calcium following activation with FMLP, in contrast to the delayed influx observed with normal neutrophils (Geiszt *et al*, 1997).

The functional significance of this abnormal response is not known, but in addition to recurrent infections, CGD patients display aberrant inflammatory responses with extensive granuloma formation in various organs (Jackson *et al*, 1995). These inflammatory granulomas may occur in the lungs, liver, skin, lymph nodes, gastrointestinal tract or urogenital tract and represent a paradoxically

exaggerated inflammatory response with apparent sensitivity to corticosteroids (Morgenstern *et al*, 1997).

An understanding of the molecular / biochemical mechanisms involved in this aberrant, excessive inflammatory response in CGD may promote the development of novel anti-inflammatory and immunoregulatory strategies (Morgenstern *et al*, 1997), applicable to normal neutrophils.

1.11 Objectives of this thesis

The primary goals of this study were to investigate the following:

1. The effects of intracellular cyclic AMP on the regulation of calcium homeostasis and pro-inflammatory responses of activated human neutrophils *in vitro*. Epinephrine and related beta-adrenergic agonists, as well as a phosphodiesterase inhibitor, were used as intracellular probes to manipulate cyclic AMP levels in neutrophils. The effects of altering intracellular cAMP concentrations on the functional responses of activated neutrophils were determined by measuring superoxide production by and elastase release from stimulated cells. Alterations in oxidant generation and degranulation in FMLP-activated neutrophils were compared with the effects of cAMP-elevating agents on calcium fluxes, assessed by means of fura-2 fluorescence procedures and radiometric assay with radiolabelled calcium.

2. The relationship between alterations in membrane potential and the regulation of calcium homeostasis in activated neutrophils. Calcium is rapidly cleared from the cytosol of activated neutrophils, followed by the carefully regulated influx of extracellular cation. The role of membrane

potential changes in regulating calcium influx was investigated, as well as the functional consequences of disordered calcium homeostasis.

3. The mechanisms responsible for maintaining the resting membrane potential of human neutrophils and those mediating membrane depolarisation and repolarisation responses following activation of the cells. The underlying mechanisms leading to alterations in membrane potential were correlated with calcium fluxes in order to elucidate the mechanistic interdependence of these events.

CHAPTER 2

COMPARISON OF THE EFFECTS OF SELECTIVE AND NON-SELECTIVE BETA-ADRENERGIC RECEPTOR AGONISTS ON THE PRO-INFLAMMATORY RESPONSES OF HUMAN NEUTROPHILS *IN VITRO*

2.1 Introduction

Although the broad-spectrum, anti-inflammatory potential of cAMP-elevating agents has long been recognised (reviewed by Moore and Willoughby, 1995), the development of clinically useful cAMP-based, anti-inflammatory chemotherapeutic agents has enjoyed limited success due to lack of selectivity for immune and inflammatory cells. Therefore, agents such as β_2-adrenoreceptor agonists, which can be delivered directly to inflamed airways by inhalation, minimising systemic side effects, should prove potentially useful anti-inflammatory agents for diseases such as bronchial asthma and chronic obstructive pulmonary disease. Surprisingly however, inhaled β_2-adrenoceptor agonists are considered to have negligible anti-inflammatory activity (Barnes, 1995; Barnes, 1998). This has been attributed to both low numbers and rapid desensitisation of β_2-adrenoceptors on inflammatory cells (Barnes, 1995). Although plausible, these proposed mechanisms appear to be based largely on experience with salmeterol (Barnes, 1995) and discount the well-documented β-adrenoceptor/cAMP-mediated anti-inflammatory interactions of epinephrine, isoproterenol and formoterol with human granulocytes *in vitro* (Busse and Sosman, 1984; Bowden *et al*, 1994; Weiss *et al*, 1996), suggesting that anti-inflammatory activity may not be a common property of β-agonists.

To resolve this issue, the effects of seven different β-agonists, (which vary with respect to both receptor specificity and agonist activity), on the pro-inflammatory activities of human neutrophils *in vitro* have been investigated. These effects on neutrophil pro-inflammatory activity have been correlated with changes in intracellular cAMP. To my knowledge there are no previous studies in which the effects of selective and non-selective β-agonists on neutrophil pro-inflammatory responses have been investigated. The agents selected for comparison are epinephrine, norepinephrine, isoproterenol, fenoterol, formoterol, salbutamol and salmeterol.

2.2 Materials and methods

2.2.1 Pharmacologic Agents

Epinephrine, norepinephrine and isoproterenol were purchased from the Sigma Chemical Co., St Louis, MO, while fenoterol and formoterol were provided by Boehringer Ingelheim KG, Germany and Novartis, Basel, Switzerland respectively and salbutamol and salmeterol by the Glaxo Wellcome Medicines Research Centre, Stevenage, Herts, UK. These agents were dissolved in 0.05 M HCl to give stock concentrations of 10 mM and diluted thereafter in Hanks balanced salt solution (HBSS, pH 7.4) and used in the assays described below at a fixed, final concentration of 1 μM. Rolipram, a selective inhibitor of type 4 phosphodiesterase (PDE), the predominant type present in human neutrophils, was also obtained from Glaxo Wellcome and dissolved to 10 mM in dimethylsulfoxide (DMSO). The β-adrenoreceptor antagonists atenolol (β_1-selective) and propranolol (non-selective) were provided by Astra Zeneca, Macclesfield, Cheshire, UK and dissolved to 10 mM in HBSS, while the α-adrenoreceptor antagonists, 2-[(4-phenyl-piperazine-1-yl)methyl]-2,3-dihydro-imidazo[1,2c]quinazolin-5(6H)-one (α_1-selective) and RS79948 (α_2-selective) were obtained from Tocris Cookson Ltd, Bristol, UK and dissolved to 10 mM in DMSO. Unless indicated, all other chemicals and reagents were purchased from the Sigma Chemical Co.

2.2.2 Neutrophils

Purified human neutrophils were prepared from heparinised venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Neutrophils were separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) before sedimentation with 3% gelatin in order to remove

most of the erythrocytes. Following centrifugation (280 g at 10°C for 10 minutes), erythrocytes were removed by selective lysis with 0.83% NH_4CL at 4°C for 10 minutes. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to $1x10^7$/ml in PBS and held on ice until used.

Circulating neutrophils are not pnehotypically identical and this may result in heterogeneous functional responses from individual cells to activating stimuli. Therefore, results of experiments using pruified neutrophil suspensions, reflect the sum of responses of phenotypically variable populations. In addition, it should be acknowledged that the purification procedures used to separate neutrophils, may lead to alterations in the functions of these cells (Van Eeden *et al.*, 1999). I have therefore used fully standardised purification procedures throughout the period of this study.

2.2.3 Superoxide Generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg and Ferber, 1984). Neutrophils were pre-incubated for 15 min at room temperature and thereafter for 15 min at 37°C in 900 µl HBSS containing 0.2 mM lucigenin in the presence and absence of the test β-agonists at 1 µM (final). Where appropriate, neutrophils were pre-incubated with either rolipram (0.05 - 0.1 µM) atenolol, or propranolol, or an α_1- or α_2-adrenoreceptor antagonist (all at 2 µM) for 10 min at 37°C followed by addition of the β-agonists. The cells were then incubated for a further 5 min at 37°C before addition of the stimulant N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µM). Spontaneous and FMLP-activated LECL responses were then recorded in an LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of the stimulant (100 µl). LECL readings were integrated for 5 sec intervals and recorded as millivolts x $seconds^{-1}$ (mVs^{-1}). Additional experiments were performed to investigate the superoxide scavenging

potential of the test agents at 1 µM using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits/ml) superoxide-generating system.

2.2.4 Elastase Release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils were incubated at a concentration of $1x10^7$/ml in HBSS in the presence or absence of the test β-agonists at 1 µM (final) for 10 min at 37°C. In those experiments where neutrophils were pre-incubated with rolipram (0.05 and 0.1 µM), atenolol or propranolol, or an α_1- or α_2 -adrenoreceptor antagonist (all at 2 µM), the β-agonists were added to the cells 5 min after these agents followed by a further 5 min of preincubation at 37°C. The stimulant FMLP (0.1 µM) in combination with cytochalasin B (0.5 µM) was then added to the cells which were incubated for 15 min at 37°C. The tubes were then transferred to an ice-bath, followed by centrifugation at 400 g for 5 min to pellet the cells. The neutrophil-free supernatants were decanted and assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al,* 1982). Briefly, 125 µl of supernatant was added to 125 µl of the elastase substrate N-succinyl-L-alanyl-alanine-p-nitroanilide (3 mM in DMSO) in 0.05 M Tris-HCl (pH 8.0). Elastase activity was then assayed spectrophotometrically at a wavelength of 405 nm. The results are expressed as the mean percentage of the enzyme released by the corresponding FMLP/CB-activated, drug-free control systems.

2.2.5 Intracellular cAMP

Neutrophils at a concentration of $1x10^7$/ml in HBSS were incubated for 15 min at 37°C with and without the β-agonists at 1 µM. Following incubation, the reactions were terminated and the cAMP extracted by the addition of ice-cold ethanol (65% v/v). The resultant precipitates were washed twice with ice-cold ethanol and the supernatants pooled and centrifuged at 2000 g for 15 min at 4°C.

The supernatants were then transferred to fresh tubes and evaporated at 60°C under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP [^{125}I] scintillation proximity assay system (Amersham International plc, Buckinghamshire, UK), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmoles cAMP/10^7 neutrophils.

2.2.6 Statistical analysis

The results of each series of experiments are expressed as the mean ± standard error of the mean (SEM). Levels of statistical significance were calculated using the Students *t*-test when 2 groups were compared, or by analysis of variance with a subsequent Tukey-Kramer multiple comparisons test for multiple groups. .

2.3 Effects of selective and non-selective β-adrenoreceptor agonists on neutrophil superoxide generation

The effects of epinephrine, norepinephrine, isoproterenol, fenoterol, formoterol, salbutamol and salmeterol on the production of superoxide by neutrophils activated with FMLP are shown in Figure 2.1 (page 45).

Epinephrine, isoproterenol, fenoterol and formoterol significantly inhibited superoxide production by 25 – 30% ($p < 0.05$). The inhibitory effects of salbutamol and salmeterol were slight (< 10%), with an intermediate response to norepinephrine (19%).

The effects of rolipram (0.05 and 0.1 μM) in combination with epinephrine, isoproterenol, norepinephrine and salbutamol on superoxide production by FMLP-activated neutrophils are shown in Table 2.1 (page 46).

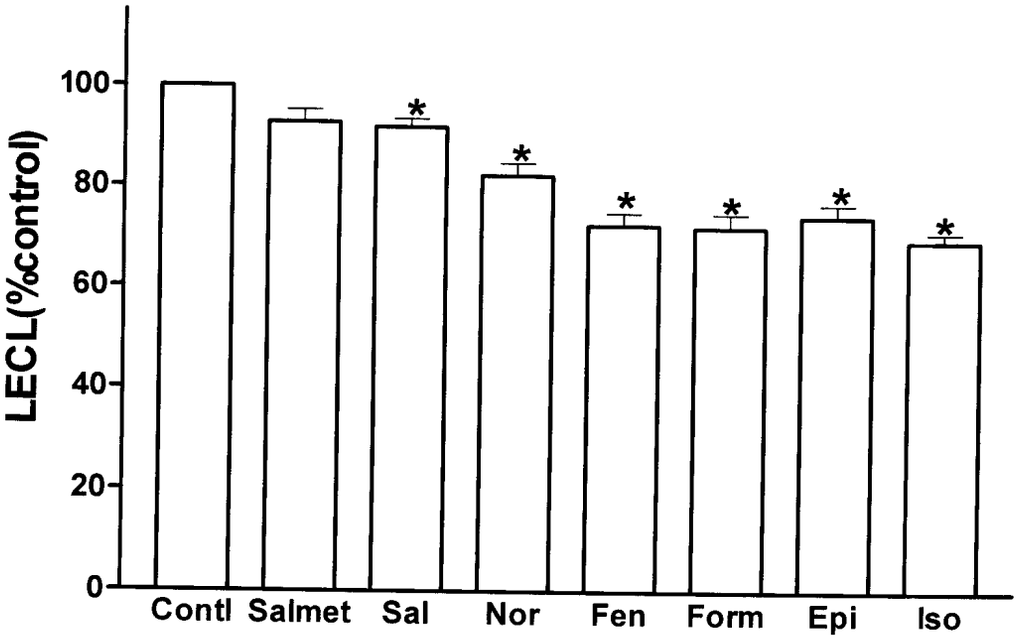


Figure 2.1: The effects of the β-agonists on superoxide production by FMLP-activated neutrophils. The results of 20-30 experiments are expressed as the mean percentage ± SEM of the corresponding drug-free control system. The absolute mean peak LECL value for the control system was 1273 ± 53 $mV.s^{-1}$. (Contl = control; salmet = salmeterol; sal = salbutamol; nor = norepinephrine; fen = fenoterol; form = formoterol; epi = epinephrine; iso = isoproterenol). *$p<0.05$

The inhibitory effects of epinephrine, isoproterenol and norepinephrine were potentiated by rolipram in a dose-dependent manner. Salbutamol, when used alone, caused no significant inhibition of superoxide production, but in combination with rolipram, significant inhibition was observed. The magnitude of the inhibitory response with salbutamol plus rolipram, was greater than that observed with rolipram alone.

Table 2.1. Effects of epinephrine, isoproterenol, norepinephrine and salbutamol in combination with rolipram, on superoxide production by FMLP-activated neutrophils.

Agent	Superoxide Production
Rolipram 0.05 μM	84 ± 7
Rolipram 0.1 μM	66 ± 4*
Epinephrine 1 μM	73 ± 4*
Epinephrine + Rolipram 0.05 μM	58 ± 4*
Epinephrine + Rolipram 0.1 μM	48 ± 7*
Isoproterenol 1 μM	68 ± 3*
Isoproterenol + Rolipram 0.05 μM	59 ± 4*
Isoproterenol + Rolipram 0.1 μM	44± 2*
Norepinephrine 1 μM	75 ± 2*
Norepinephine + Rolipram 0.05 μM	68 ± 5*
Norepinephine + Rolipram 0.1 μM	47 ± 3*
Salbutamol 1 μM	102 ± 4
Salbutamol + Rolipram 0.05 μM	71 ± 3*
Salbutamol + Rolipram 0.1 μM	54 ± 4*

The results of 6 experiments are expressed as the mean percentage of the drug-free, FMLP-activated control ± SEM. The absolute peak value for superoxide production by FMLP-activated neutrophils was 743 ± 41 $mV.s^{-1}$. *$p < 0.005$ for comparison with the drug-free control system.

In order to determine which type of receptor mediates the inhibitory effects of selective and non-selective β-adrenoreceptor agonists, neutrophils were pre-incubated with various receptor antagonists. An α_1-receptor antagonist, 2-[(4-phenyl-piperazine-1-yl)methyl]-2,3-dihydroimidazo[1,2c]quinazolin-5(6H)-one and an α_2-receptor antagonist (RS79948) as well as a non-selective β_1- and β_2-receptor antagonist (propranolol) and a specific β_1-antagonist (atenolol) were selected. This combination of receptor antagonists allows inhibition of all adrenergic receptors (α and β) that may contribute to the effects of β-agonists on neutrophils.

The effects of propranolol and atenolol on epinephrine-, isoproterenol-, and formoterol-mediated inhibition of superoxide production by FMLP-stimulated neutrophils, as well as the effects of an α_1- and α_2-receptor antagonist on epinephrine-mediated inhibition of superoxide production by FMLP-activated neutrophils, are shown in Table 2.2.

Table 2.2. The effects of propranolol and atenolol on the inhibition of superoxide production mediated by epinephrine, isoproterenol and formoterol, as well as the effects of an α_1- and α_2-antagonist on epinephrine-mediated inhibition of superoxide production.

Agent	**Superoxide Production (LECL)**
Propranolol 2 μM	103 ± 2
Epinephrine 1 μM	63 ± 3*
Propranolol + Epinephrine	95 ± 3
Isoproterenol 1μM	51 ± 3*
Propranolol + Isoproterenol	112 ± 6
Formoterol 1 μM	75 ± 1*
Propranolol + Formoterol	95 ± 4
Atenolol 2 μM	94 ± 3
Atenolol + Epinephrine	62 ± 3*
Atenolol + Isoproterenol	58 ± 3*
Atenolol + Formoterol	74 ± 2*
α_1-antagonist 2 μM	95 ± 4
α_1-antagonist + Epinephrine	70 ± 3*
α_2-antagonist 2 μM	97 ± 5
α_2-antagonist + Epinephrine	62 ± 5*

The results of 6 - 13 experiments are expressed as the mean percentage of control ± SEM. The absolute peak values for superoxide production by unstimulated and FMLP-activated neutrophils were 224 ± 19 and 1184 ± 72 mVs^{-1} respectively. *$p < 0.005$ for comparison with the drug-free control system.

Propranolol, a β_1- and β_2-receptor antagonist, completely attenuated the inhibitory effects of epinephrine, isoproterenol and formoterol on FMLP-activated superoxide production, while atenolol was ineffective with all of these agents. The α_1 and α_2-receptor antagonists did not attenuate the inhibitory effects of epinephrine on superoxide production by FMLP-stimulated neutrophils.

Previous investigators (Weiss *et al*, 1996) reported that the inhibitory effect of epinephrine on neutrophil superoxide production was partly attributable to the ability of this agent to scavenge free radicals. In order to test the hypothesis that β-agonists may scavenge free radicals, the oxidant-scavenging potential of epinephrine, isoproterenol, norepinephrine and salbutamol was evaluated in a cell-free, xanthine-xanthine oxidase superoxide generating system (Table 2.3).

Table 2.3. Effect of epinephrine, isoproterenol, norepinephrine and salbutamol on superoxide generation in a cell-free xanthine (1 mM)-xanthine oxidase system.

System	Superoxide Production
Epinephrine 1 μM	104 ± 4
Isoproterenol 1 μM	102 ± 3
Norepinephrine 1 μM	98 ± 5
Salbutamol 1 μM	100 ± 4

The results of 8 – 10 experiments are expressed as the mean percentage of control ± SEM. The absolute peak value for the control drug-free system was 1370 ± 37 $mV.s^{-1}$.

None of the agents at the concentration tested, demonstrated any oxidant scavenging properties.

2.4 Effects of selective and non-selective β-agonists on elastase release from activated neutrophils

The effects of the test agents on elastase release from FMLP/CB-activated neutrophils are shown in Figure 2.2 (page 49).

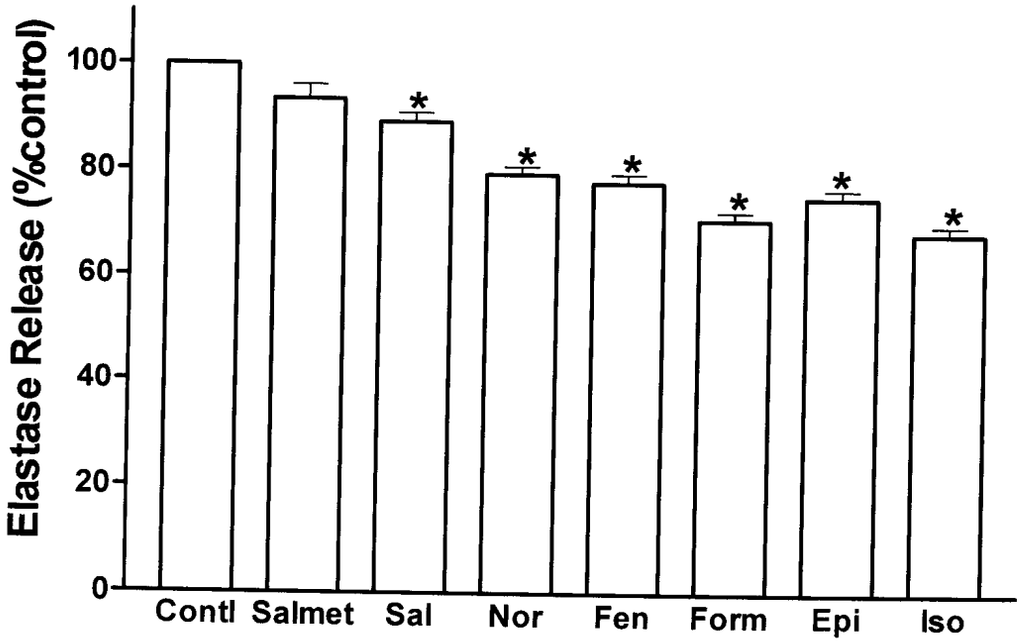


Figure 2.2: The effects of the β-agonists on elastase release from FMLP/CB-activated neutrophils. The results of 24 experiments are expressed as the mean percentage ± SEM of the corresponding drug-free control system. The absolute values for elastase release from unstimulated and FMLP/CB-activated neutrophils were 36 ± 4 and 1340 ± 52 milliunits of enzyme/10^7 cells.
*$p<0.05$

In these experiments, the most pronounced inhibition of elastase release was observed with isoproterenol and formoterol (32% and 29% reduction in elastase release respectively). Epinephrine, fenoterol and norepinephrine caused slightly less, but still significant inhibition of elastase release (25%, 22% and 21% respectively). The effects of salbutamol and salmeterol were minimal in comparison to the other agents tested (11% and 7% inhibition of elastase release respectively). The effects of pre-treatment with propranolol and atenolol on the inhibition of elastase release from FMLP/CB-stimulated neutrophils mediated by epinephrine, isoproterenol and formoterol are shown in Table 2.4 (page 50) as well as the response to pre-incubation with an α_1- and α_2-antagonist on epinephrine- mediated inhibition of elastase release from activated cells.

Propranolol neutralised the inhibitory effects of all the β-agonists tested on the release of elastase from FMLP/CB-activated neutrophils, while atenolol caused

trivial antagonism. Pre-treatment of neutrophils with a selective α_1- or α_2-adrenoreceptor antagonist did not affect the epinephrine-mediated inhibition.

The effects of combining epinephrine, isoproterenol, norepinephrine or salbutamol with rolipram on the release of elastase from FMLP/CB-activated neutrophils are shown in Table 2.5 (page 51).

Table 2.4. Effects of propranolol and atenolol on the inhibition of elastase release mediated by epinephrine, isoproterenol and formoterol as well as the effects of an α_1- and α_2-antagonist on epinephrine-mediated inhibition of elastase release.

System	Elastase Release
Propranolol 2 μM	102 ± 4
Epinephrine 1 μM	71 ± 2**
Propranolol + Epinephrine	98 ± 1
Isoproterenol 1 μM	66 ± 1**
Propranolol + Isoproterenol	109 ± 3
Formoterol 1 μM	80 ± 3**
Propranolol + Formoterol	123 ± 5
Atenolol 2μM	100± 3
Atenolol + Epinephrine	83 ± 2**
Atenolol + Isoproterenol	78 ± 2**
Atenolol + Formoterol	82 ± 2**
α_1-antagonist 2 μM	99 ± 2
α_1-antagonist + Epinephrine	74 ± 2**
α_2-antagonist 2 μM	106 ± 4
α_2-antagonist + Epinephrine	68 ± 2**

The results of 6 – 12 experiments are expressed as the mean percentage of the drug-free control system ± SEM. The absolute value for elastase release from unstimulated and FMLP/CB-activated neutrophils were 94 ± 12 and 1376 ± 12 milliunits enzyme/10^7 cells. *$p < 0.05$; **$p < 0.005$ for comparison with the drug-free control.

Table 2.5. Effects of epinephrine, isoproterenol, norepinephrine and salbutamol in combination with rolipram on the release of elastase from FMLP/CB-activated neutrophils.

Agent	Elastase release (% control)
Rolipram 0.05 μM	78 ± 3*
Rolipram 0.1 μM	64 ± 6*
Epinephrine 1 μM	70 ± 5*
Epinephrine + Rolipram 0.05 μM	46 ± 2*
Epinephrine + Rolipram 0.1 μM	42 ± 3*
Isoproterenol 1 μM	61± 4*
Isoproterenol + Rolipram 0.05 μM	44 ± 2*
Isoproterenol + Rolipram 0.1 μM	41 ± 4*
Norepinephrine 1 μM	77 ± 3*
Norepinephrine + Rolipram 0.05 μM	54 ± 2*
Norepinephrine + Rolipram 0.1μM	45 ± 5*
Salbutamol 1 μM	88 ± 2*
Salbutamol + Rolipram 0.05 μM	62 ± 3*
Salbutamol + Rolipram 0.1 μM	52 ± 4*

The results of 6 experiments are expressed as the mean percentage of enzyme released by control, drug-free FMLP/CB-activated neutrophils. *$p < 0.005$ for comparison with the control, drug-free system. The absolute values for elastase release from unstimulated and FMLP/CB-activated neutrophils were 35 ± 6 and 1488 ± 53 milliunits of enzyme/10^7 cells respectively.

Combinations of the β-agonists with the selective PDE 4 inhibitor, rolipram, resulted in levels of inhibition of elastase release which were greater than those observed with the individual agent.

2.5. The effects of the β-agonists on neutrophil cAMP levels

The effects of the various β-agonists on neutrophil cAMP levels are shown in Figure 2.3 (page 52).

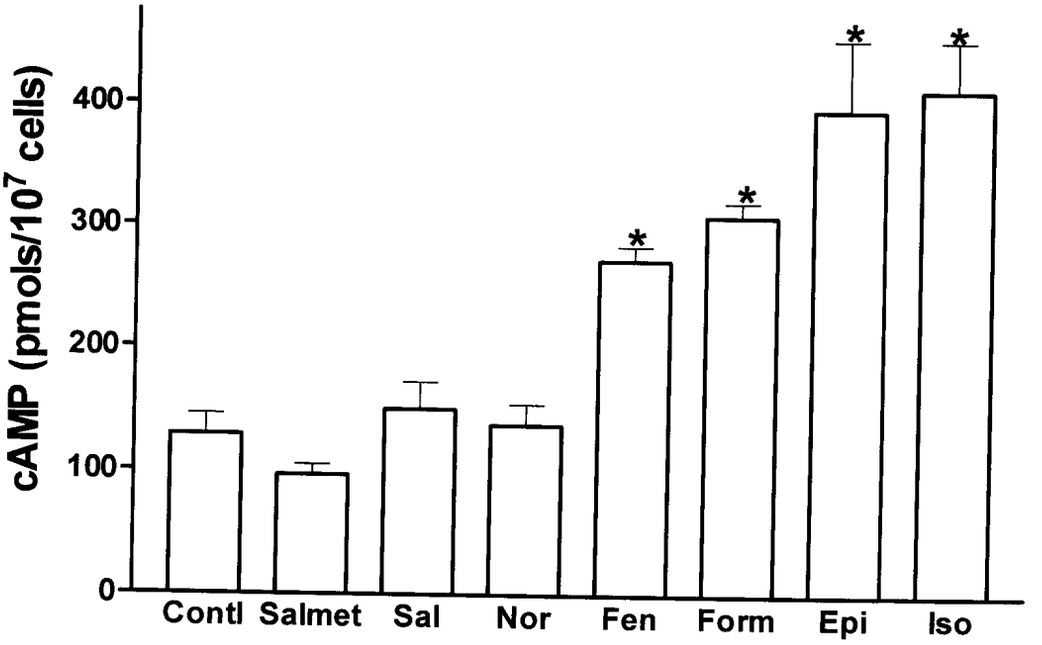


Figure 2.3: The effects of the β-agonists on neutrophil cAMP levels. The results of 4 experiments are expressed as the mean intracellular cAMP concentration ± SEM as pmols/10^7 cells.
*$p<0.05$

Of the seven agents tested, epinephrine, isoproterenol, fenoterol and formoterol caused a substantial and significant ($p < 0.05$ for all four agents) increase in intracellular cAMP levels, while these were not significantly altered in neutrophils treated with norepinephrine, salbutamol and salmeterol.

2.6 Discussion

The initial goal of this study was to compare the effects of selective and non-selective β-adrenergic agonists on the pro-inflammatory responses of activated human neutrophils *in vitro*, which to my knowledge has not been previously investigated. Comparison of various selective and non-selective β-agonists, demonstrated that these agents clearly differ with respect to anti-inflammatory potential. Epinephrine, isoproterenol, fenoterol and formoterol significantly increased intracellular concentrations of cAMP in neutrophils, an activity which was paralleled by inhibition of the production of reactive oxidants and release of elastase following activation of these cells with FMLP. Salbutamol and salmeterol on the other hand, did not detectably alter cAMP levels in neutrophils, nor did they cause significant suppression of the pro-inflammatory activities of these cells. The effect of norepinephrine on superoxide production by, and elastase release from FMLP-activated neutrophils was intermediate between these two groups, although the increase in intracellular cAMP was similar to that observed with salbutamol and salmeterol. Because of its intermediate potency as a β_2-agonist, the effects of this agent on intracellular cAMP may subside rapidly and be difficult to detect.

Alternative anti-inflammatory mechanisms such as oxidant-scavenging were not detected in the present study and are only operative at concentrations of the β-agonists in considerable excess of 1 μM (Gillisen *et al*, 1994; Gillisen *et al*, 1995). Fenoterol, formoterol and salbutamol at concentrations of up to 50 μM do not possess membrane-stabilising properties in vitro (Anderson *et al*, 1998), as is the case with epinephrine, norepinephrine and isoproterenol.

Pre-treatment of neutrophils with rolipram potentiated the inhibitory effects of the β-agonists on superoxide production and elastase release, demonstrating that intracellular PDE 4 activity is also a determinant of the anti-inflammatory efficacy of these agents mediated via cAMP. The involvement of cAMP in the anti-

inflammatory interactions of epinephrine, isoproterenol and formoterol with neutrophils is strengthened by the observation that propranolol, but not atenolol or the α-adrenoreceptor antagonists, neutralised the inhibitory effects of these β-agonists on superoxide production. Similar results were obtained with elastase release, although slight attenuation of the inhibitory effects of all 3 β-agonists was observed with atenolol suggesting a lack of absolute specificity of atenolol, for β_1-adrenoreceptors (Hoffman and Lefkowitz, 1992).

The differential responses of human neutrophils to the cAMP-elevating effects and anti-inflammatory actions of the various β-agonists used in the present study are probably related to the density of β_2-adrenoreceptors on the neutrophil membrane (Whaley *et al*, 1994), as well as the concentration, receptor affinity and intrinsic activity at the receptor of the agents tested (Dickey *et al*, 1996; Moore *et al*, 1998). The β_2-receptor density on human neutrophils is relatively low at approximately 1000 receptors per cell (Mueller *et al*, 1988) in comparison with the 50 000 FMLP-receptors per cell (Tecoma *et al*, 1986). This low receptor density with little receptor reserve, means that submaximal responses may be seen with the partial agonists salbutamol and salmeterol, as opposed to those observed with full agonists (epinephrine, isoproterenol, formoterol) or those with intermediate potency (Dickey *et al*, 1996). The various β-agonists were assessed for anti-inflammatory potential at a fixed final concentration of 1 μM. In the case of isoproterenol, neutrophil β-receptors are fully saturated at this concentration of the agonist (Mueller *et al*, 1988). Although the relative affinities of the various test agents at β_2-receptors were not measured directly in the present study, the magnitude of the increases in intracellular cAMP in response to the β-agonists and the resultant anti-inflammatory effects are dependent on both receptor affinity and intrinsic activity (Johnson, 1998). The combined properties of receptor affinity for, and intrinsic activity at the β-receptor were therefore indirectly determined by means of the intracellular cAMP levels following 15 min incubation of neutrophils with the β-agonists. The relative potencies of these agents at the

neutrophil membrane β-adrenoreceptors were isoproterenol > epinephrine > formoterol > fenoterol > norepinephrine > salbutamol > salmeterol.

Interestingly, the magnitude of potentiation of the inhibitory effects of isoproterenol, epinephrine, norepinephrine and salbutamol on superoxide production by and elastase release from activated neutrophils, in the presence of rolipram, is inversely related to their potency as agonists. This suggests that the slower rate of cAMP generation with partial agonists allows more rapid hydrolysis of cAMP by the intracellular phosphodiesterase.

These observations suggest that the anti-inflammatory effects of β_2-agonists, if operative *in vivo*, may be optimised by combining these agents with a selective PDE 4 inhibitor.

CHAPTER 3

THE EFFECTS OF EPINEPHRINE ON Ca^{2+} FLUXES AND ON THE PRO-INFLAMMATORY RESPONSES OF ACTIVATED HUMAN NEUTROPHILS *IN VITRO*

3.1 Introduction

Considering the large number of serious diseases in which neutrophils play a pathogenic role, it is hardly surprising that modulation of neutrophil-mediated tissue injury remains such an important therapeutic goal (Fujishima and Aikawa, 1995). In this regard, agents such as corticosteroids have shown minimal efficacy (Cox, 1995; Barnes, 1998), while others, including cAMP-elevating agents, have received increased interest (Moore and Willoughby, 1995). The focus on cAMP-elevating agents has drawn attention to the role of β-adrenergic agonists as modulators of neutrophil pro-inflammatory responses. The anti-inflammatory interactions of epinephrine with human neutrophils have been alluded to in several previous studies, with varying results obtained by different investigators (Busse and Sosman, 1984; Hetherington and Quie, 1985; Bazzoni *et al*, 1991; Weiss *et al*, 1996 and Burns *et al*, 1997). Reactive oxidant production in previous reports was measured by means of a luminol-enhanced chemiluminescence procedure which is a composite assay lacking in specificity. Previous investigators focused on the neutrophil enzymes lysozyme and glucuronidase, but not elastase, which is the enzyme of particular clinical relevance to this study, with elastase specifically implicated as one of the most important enzymes responsible for neutrophil-mediated tissue damage (Dallegri and Ottonello, 1997). Despite apparent interest, the exact molecular / biochemical mechanisms which underlie the anti-inflammatory interactions of cAMP-elevating agents with neutrophils have not been established.

3.2 Materials and Methods

Epinephrine, purchased from the Sigma Chemical Co. and formoterol provided by Novartis, Basel, Switzerland, were dissolved in 0.05 M HCl to give a stock concentration of 10 mM, diluted thereafter in Hanks balanced salt solution (HBSS, pH 7.4) and used in the assays described below at final concentration range of 0.01 μM–1 μM (epinephrine) and 1 μM (formoterol). Rolipram, a

selective inhibitor of type 4 phosphodiesterase (PDE), the predominant type present in human neutrophils (Wang *et al.,* 1999), was obtained from Glaxo Wellcome plc, and dissolved to 10 mM in dimethylsulfoxide (DMSO), while the β-adrenoceptor antagonists atenolol (β_1-selective) and propranolol (non-selective) were provided by Astra Zeneca, and dissolved to 10 mM in HBSS, while the α-adrenoceptor antagonists, 2-[(4-phenyl-piperazine-1-yl)methyl]-2,3-dihydroimi-dazo-[1,2c] quinazolin-5(6H)-one (α_1-selective) and RS79948 (α_2-selective) were obtained from Tocris Cookson Ltd, and dissolved to 10mM in DMSO.

3.2.1 Neutrophils

Purified human neutrophils were prepared from heparinised venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Neutrophils were separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) before sedimentation with 3% gelatine in order to remove most of the erythrocytes. Following centrifugation (280 g at 10°C for 10 minutes), residual erythrocytes were removed by selective lysis with 0,83% ammonium chloride at 4°C for 10 minutes. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to $1x10^7$/ml in PBS and held on ice until used.

3.2.2 *Superoxide generation*

This was measured using lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) (Minkenberg and Ferber, 1984). Neutrophils were pre-incubated for 15 min at 37°C in 900 µl HBSS containing 0.2 mM lucigenin in the presence and absence of epinephrine (final concentration 0.1 µM -1 µM). Spontaneous and phorbol myristate acetate (PMA) (25 ng/ml), opsonised zymosan (500 µg/ml), calcium ionophore (A23187)(1 µM) as well as N-formyl-

methionyl-leucyl-phenylalanine (FMLP) (1 μM) -activated LECL responses were then recorded using an LKB Wallac 1251 chemiluminometer after the addition of the stimulant. LECL readings were integrated for 5 second intervals and recorded as mV x seconds^{-1} (mVs^{-1}).

Additional experiments were undertaken to investigate the following: i) the effects of rolipram (0.05 μM and 0.1 μM final) on epinephrine (1 μM)-mediated modulation of superoxide production by PMA, opsonised zymosan, A23187 and FMLP-activated neutrophils; rolipram was present with epinephrine throughout the pre-incubation period, ii) the effects of adding epinephrine (0.01 μM - 1 μM) 30 sec prior to FMLP on superoxide production by neutrophils in comparison to systems in which the adrenoreceptor agonist was present with the cells throughout the 15 min preincubation period, and iii) the superoxide-scavenging potential of epinephrine (0.01 - 2 μM) using a cell-free xanthine (1 mM)-xanthine oxidase (70 milliunits/ml) superoxide generating system.

3.2.3 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived protease, elastase. Neutrophils were incubated at a concentration of 1x10^7/ml in HBSS in the presence and absence of epinephrine (0.1 μM - 1 μM) for 10 min at 37°C. The stimulant FMLP (0.1 μM) in combination with cytochalasin B (1 μM) was then added and the reaction mixtures incubated for 15 min at 37°C. The tubes were then transferred to an ice-bath, followed by centrifugation at 400 *g* for 5 min to pellet the cells. The neutrophil free supernatants were then decanted and assayed for elastase activity using a micro-modification of a standard spectrophotometric procedure (Beatty *et al.*, 1982). Briefly, 125 μl of supernatant was added to 125 μl of the elastase substrate N-succinyl-L-alanyl-alanine-p-nitroanilide, 3 mM in 0,3% dimethyl sulfoxide in 0,05 M Tris-HCl (pH 8,0). Elastase activity was then assayed at a wavelength of 405 nm.

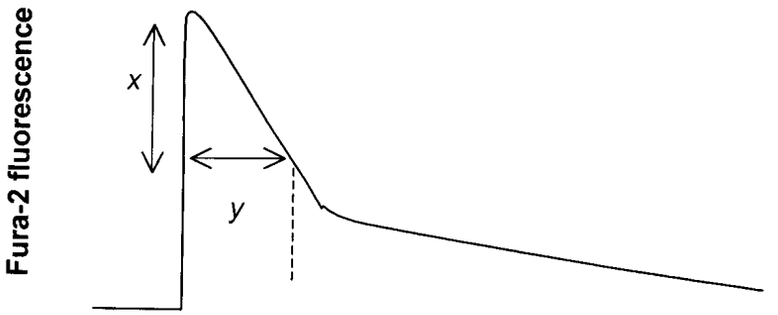
Additional experiments were undertaken to investigate the following: i) the effects of rolipram (0.05 µM and 0.1 µM) on epinephrine (1 µM)-mediated modulation of elastase release by FMLP/CB-activated neutrophils, and ii) the effects of adding epinephrine (0.01 – 1 µM) 30 sec prior to FMLP on elastase release from neutrophils in comparison to systems in which the epinephrine was present with the cells throughout the 15 min preincubation period.

3.2.4 Intracellular calcium fluxes

These were measured spectrofluorimetrically using fura-2/AM (Calbiochem Corp.) as the calcium-sensitive indicator of cytoplasmic Ca^{2+} (Grynkiewicz *et al,* 1985). Neutrophils ($1x10^7$/ml) were preloaded with fura-2 (2 µM) for 30 min at 37°C in PBS, washed twice, resuspended in PBS at 1 x 10^7/ml and held on ice until use. For measurement of intracellular Ca^{2+} fluxes the neutrophils were transferred to indicator-free, Hanks' balanced salt solution (HBSS; pH7.4) containing 1.25 mM $CaCl_2$. This medium is referred to hereafter as Ca^{2+}-replete HBSS. The fura-2-loaded cells were then preincubated in the presence and absence of epinephrine (0.1 µM–1 µM) for 8 minutes at 37°C followed by transfer to a disposable reaction cuvette, which was maintained at 37°C in a Hitachi 650–10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm respectively. After a stable base-line was obtained (1 min), the neutrophils were activated with FMLP (1 µM) and the subsequent increase in fura-2 fluorescence intensity was monitored over a 5 min period. The final volume in each cuvette was 3 ml, containing a total of $6x10^6$ neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously (Grynkiewicz *et al,* 1985).

These effects can be quantified by measuring the time taken for the fluorescence trace to return to half peak values (t½), which is dependent on the rate of clearance of free Ca^{2+} from the neutrophil cytoplasm. Assuming a near linear

relationship from the peak to the corresponding fluorescence intensity at t½, the clearance rate of cytosolic free Ca^{2+} can be calculated and expressed in pmol/min.



x = 50% of the peak increment in cytosolic Ca^{2+} following addition of FMLP

y = time taken to decline to half peak values

x/y = clearance rate of free cytosolic Ca^{2+} (pmol/min)

The effects of rolipram (0.05 µM and 0.1 µM), as well as those of propranolol (2 µM), on epinephrine (1 µM)-mediated modulation of Ca^{2+} fluxes in FMLP-activated neutrophils were also investigated, as well as the effects of adding epinephrine (0.01 – 1 µM) 30 sec prior to FMLP on fura-2 fluorescence responses in comparison to systems in which the adrenoreceptor agonist was present with the cells throughout the 15 min pre-incubation period. In addition, the effects of formoterol (1 µM) on fura-2 fluorescence responses in FMLP-activated neutrophils were investigated.

3.2.5 *Radiometric assessment of Ca^{2+} fluxes*

$^{45}Ca^{2+}$ (Calcium-45 chloride, specific activity 18.53 mCi/mg, Du Pont NEN Research Products) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and activated neutrophils. In the assays of Ca^{2+} efflux and influx described below, the radiolabelled cation was always used at a fixed, final concentration of 2 μCi/ml, containing 50 nmol cold carrier Ca^{2+} (as $CaCl_2$). The final assay volumes were always 5 ml containing a total of $1x10^7$ neutrophils. The standardisation of the procedures used to load the cells with $^{45}Ca^{2+}$, as well as a comparison with silicone oil-based methods for the separation of labelled neutrophils from unbound isotope, have been described (Anderson and Goolam Mahomed, 1997).

3.2.6 *Efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils*

Neutrophils ($1x10^7$/ml) were loaded with $^{45}Ca^{2+}$ (2 μCi/ml) for 30 min at 37°C in HBSS which was free of unlabelled Ca^{2+}. The cells were then pelleted by centrifugation, washed once with, and resuspended in ice-cold Ca^{2+}-replete HBSS and held on ice until use, which was always within 10 min of completion of loading with $^{45}Ca^{2+}$. By use of this procedure, the FMLP-activated fura-2 responses of neutrophils, similarly processed in HBSS containing 1 μM cold $CaCl_2$ followed by washing with and suspension in Ca^{2+}-replete HBSS did not differ from those of cells which had been maintained in Ca^{2+}-replete HBSS throughout, indicating that at the time of measurement of efflux in the $^{45}Ca^{2+}$ system there is no detectable depletion of intracellular Ca^{2+} (Anderson and Goolam Mahomed, 1997). The $^{45}Ca^{2+}$-loaded neutrophils ($2x10^6$/ml) were then preincubated for 10 min at 37°C in Ca^{2+}-replete HBSS, in the presence and absence of epinephrine (1 μM) and formoterol (1μM), followed by activation with FMLP (1 μM) and measurement of the efflux of $^{45}Ca^{2+}$ over 60 sec, after which efflux is complete. The reactions were terminated by the addition of 10 ml ice-

cold, Ca^{2+}-replete HBSS to the tubes which were then transferred to an ice-bath. The cells were then pelleted by centrifugation at 400 *g* for 5 min followed by washing with 15 ml ice-cold, Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml of 0.5% triton X-100/0.1 M NaOH and the radioactivity assessed in a liquid scintillation spectrometer. Control, cell-free systems (HBSS and $^{45}Ca^{2+}$ only) were included for each experiment and these values were subtracted from the relevant neutrophil-containing systems. These results are presented as the amount of cell-associated radiolabelled cation (pmol $^{45}Ca^{2+}$ per 10^7 cells).

In an additional series of experiments, the effects of thapsigargin, a highly specific inhibitor of the endo-membrane Ca^{2+}-ATPase (Lytton *et al,* 1997), on epinephrine (1 μM)-mediated modulation of FMLP-activated efflux of $^{45}Ca^{2+}$ from neutrophils were investigated over a 60 sec time course. Thapsigargin was used at a final, predetermined concentration of 1 μM and was added simultaneously with FMLP to $^{45}Ca^{2+}$-loaded neutrophils which had been pre-incubated for 10 min with epinephrine.

3.2.7 Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils

To measure the net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were loaded with cold, Ca^{2+}-replete HBSS for 30 min at 37°C, after which the cells were pelleted by centrifugation, then washed once with, and resuspended in ice-cold Ca^{2+}-free HBSS and held on ice until used. Pre-loading with cold Ca^{2+} was undertaken to minimise spontaneous uptake of $^{45}Ca^{2+}$ (unrelated to FMLP activation) in the influx assay. The efficiency of this loading procedure was demonstrated by measurement of the FMLP-activated fura-2 responses of the Ca^{2+}-loaded neutrophils, which did not differ from those of neutrophils maintained in Ca^{2+}-replete HBSS (Anderson and Goolam Mahomed, 1997). The Ca^{2+}-loaded neutrophils ($2x10^6$/ml), were then incubated for 10 min in the presence and

absence of epinephrine (1 μM) and formoterol (1 μM) at 37°C in Ca^{2+}-free HBSS followed by simultaneous addition of FMLP and $^{45}Ca^{2+}$ (2 μCi/ml), or $^{45}Ca^{2+}$ only to control, unstimulated systems. Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils was then monitored over a 5 min period, after which influx is complete (Anderson and Goolam Mahomed, 1997) and compared with the uptake of the radiolabelled cation by identically-processed, unstimulated cells.

3.2.8 Measurement of intracellular cAMP

Neutrophils at a concentration of 1 x 10^7/ml in HBSS were preincubated for 10 min at 37°C with and without epinephrine (1 μM). Following preincubation, the cells were treated with 1 μM FMLP (stimulated cells), or an equal volume of HBSS (unstimulated cells), in a final volume of 1 ml, and the reactions terminated and the cAMP extracted by the addition of ice-cold ethanol (65% v/v) at 1 min after addition of the stimulant. The resultant precipitates were washed twice (2000 g for 15 min at 4°C) with ice-cold ethanol and the supernatants pooled and evaporated at 60°C under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP [^{125}I] scintillation proximity assay system (Amersham International plc), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmoles cAMP/10^7 neutrophils. Because cAMP is rapidly hydrolysed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μM rolipram.

3.2.9 *Membrane potential*

The potential sensitive fluorescent dye dipentyloxacarbocyanine [di-O-C_5(3)] was used to measure changes in membrane potential in activated neutrophils. The cells (1 x 10^6/ml) were pre-incubated for 10 min at 37°C in HBSS containing 80 nM (final) di-O-C_5(3) after which they were transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence

spectrophotometer with excitation and emission wavelengths set at 460 nm and 510 nm respectively. The neutrophils were then activated with FMLP (1 μM) and the subsequent alterations in fluorescence intensity monitored over a 5 – 10 min period. Where appropriate, cells were pre-incubated with epinephrine (1 μM) for 8 minutes prior to addition of the stimulant. The final volume in each cuvette was 3 ml containing a total of 3×10^6 neutrophils.

3.2.10 Statistical analysis

The results of each series of experiments are expressed as the mean ± standard error of the mean (SEM). Levels of statistical significance were calculated using the Students *t*-test when 2 groups were compared, or by analysis of variance with a subsequent Tukey-Kramer multiple comparisons test for multiple groups. Correlations between parameters were calculated using Pearson's correlation coefficient.

3.3 Effects of Epinephrine on Superoxide Production by Activated Neutrophils

For these studies, various stimuli which activate different signal transduction pathways were utilised. Three agents which elevate the cytosolic calcium concentration $[Ca^{2+}]i$, opsonised zymosan, FMLP and A23187 (calcium ionophore) were used and one stimulant, PMA, which has no effect on $[Ca^{2+}]i$ (Tauber, 1987). Opsonised zymosan and FMLP activate physiological transduction pathways, while the calcium ionophore, A23187, leads to artificial flooding of the cytosol with Ca^{2+} (Simchowitz *et al*, 1980). In addition, the PDE 4 inhibitor, rolipram, was included in these experiments to prevent hydrolysis of cAMP-mediated responses. The effects of epinephrine on superoxide production by activated neutrophils are shown in Table 3.1 (page 66).

Superoxide production was inhibited in a dose-dependent manner by epinephrine in those systems activated with opsonised zymosan, A23187 and FMLP. Statistically significant inhibition was observed at the lowest concentration of epinephrine (0.1 μM). The magnitude of the inhibitory effect of epinephrine was greatest in FMLP-activated neutrophils, with 25% inhibition compared to the control system, at an epinephrine concentration of 1 μM. No inhibition was evident with PMA-activated neutrophils at all concentrations of epinephrine tested.

Table 3.1. Effects of epinephrine (0.1 – 1 μM) on superoxide production by PMA-, opsonised zymosan-, A23187- and FMLP-activated neutrophils.

	Peak LECL responses of neutrophils activated with:			
System	**PMA**	**Opsonised zymosan**	**A23187**	**FMLP**
Epinephrine 0.1 μM	97 ± 3	95 ± 2*	91 ± 3*	93 ± 3*
Epinephrine 0.5 μM	97 ± 2	93 ± 3*	82 ± 3**	79 ± 4**
Epinephrine 1.0 μM	97 ± 2	88 ± 3**	80 ± 3**	75 ± 3**

The results of 7 – 12 experiments are expressed as the mean percentage of the epinephrine-free control ± SEM. The absolute peak values for superoxide production by PMA-, opsonised zymosan-, A23187- and FMLP-activated neutrophils were 3546 ± 164 $mV.s^{-1}$, 2213 ± 214 $mV.s^{-1}$, 1533 ± 188 $mV.s^{-1}$ and 1659 ± 135 $mV.s^{-1}$, respectively. The corresponding absolute peak values for unstimulated neutrophils were 210 ± 17 $mV.s^{-1}$, 223 ± 21 $mV.s^{-1}$, 318 ± 29 $mV.s^{-1}$ and 233 ± 22 $mV.s^{-1}$. *$p < 0.05$; **$p < 0.005$ for comparison with the epinephrine-free control system.

The effects of epinephrine (1 μM) with and without the phosphodiesterase 4 inhibitor, rolipram (0.05 and 0.1 μM) on superoxide production by activated neutrophils, are shown in Table 3.2 (page 67).

The addition of rolipram potentiated the inhibitory effects of epinephrine on superoxide production in neutrophils activated with opsonized zymosan, A23187 and FMLP, but not PMA. In the presence of rolipram only, at 0.1 μM, statistically significant inhibition of superoxide generation was observed in cells stimulated with A23187 and FMLP. The magnitude of this inhibitory effect (50%) on neutrophil superoxide production was greatest in FMLP-activated cells pre-incubated with epinephrine (1 μM) and rolipram (0.1 μM).

The results shown in Tables 3.1 and 3.2 (pages 66 and 67 respectively), confirm the significant inhibitory effects of epinephrine on superoxide production by neutrophils activated with calcium-mobilising stimuli.

Table 3.2. Effects of epinephrine with and without rolipram (0.05 – 0.1 μM) on superoxide production by PMA-, opsonised zymosan-, A23187- and FMLP-activated neutrophils.

	Peak LECL responses of neutrophils activated with:			
System	**PMA**	**Opsonised zymosan**	**A23187**	**FMLP**
Epinephrine 1 μM	106 ± 3	91 ± 1**	83 ± 5*	77 ± 4**
Rolipram 0.05 μM only	110 ± 2	102 ± 2	90 ± 7	93 ± 6
Epinephrine + Rolipram 0.05 μM	104 ± 3	90 ± 3*	67 ± 8*	65 ± 3**
Rolipram 0.1 μM only	107 ± 4	93 ± 6	75 ± 5**	76 ± 4**
Epinephrine + Rolipram 0.1 μM	103 ± 3	88 ± 1**	61 ± 6**	50 ± 3**

The results of 4 – 8 experiments are expressed as the mean percentage of the epinephrine-free control ± SEM. The absolute peak values for superoxide production by PMA-, opsonised zymosan-, A23187- and FMLP-activated neutrophils were 3863 ± 113 $mV.s^{-1}$, 1978 ± 22 $mV.s^{-1}$, 1292 ± 165 $mV.s^{-1}$ and 777 ± 74 $mV.s^{-1}$ respectively. *$p < 0.05$; **$p < 0.005$ for comparison with the epinephrine-free control system.

3.4 Effects of epinephrine on elastase release from activated neutrophils

The effects of epinephrine, with and without rolipram, on elastase release from FMLP/CB-activated neutrophils are shown in Table 3.3.

Epinephrine inhibited elastase release in a dose-dependent fashion with statistically significant inhibition observed at the lowest concentration of 0.1 μM. Addition of the phosphodiesterase 4 inhibitor, rolipram, markedly potentiated the inhibitory effects of epinephrine with 54% inhibition in the presence of epinephrine 1 μM and rolipram 0.1 μM, relative to 31% with epinephrine alone. Potentiation of the inhibitory effects of epinephrine by a PDE inhibitor on both oxidant production and elastase degranulation, implicates cAMP as a possible mediator of these interactions.

Table 3.3. Effects of epinephrine (0.1 – 1 μM) with and without rolipram on elastase release by FMLP/CB-activated neutrophils.

System	Elastase Release
Epinephrine 0.1 μM	89 ± 4*
Epinephrine 0.5 μM	75 ± 3**
Epinephrine 1 μM	69 ± 2**
Rolipram 0.05 μM	91 ± 8
Epinephrine 1 μM + Rolipram 0.05 μM	52 ± 4**
Rolipram 0.1 μM	59 ± 5**
Epinephrine 1 μM + Rolipram 0.1 μM	46 ± 4**

The results of 6 – 7 experiments are expressed as the mean percentage of the epinephrine-free, FMLP/CB control ± SEM. The amount of elastase released from FMLP/CB-activated neutrophils was 1392 ± 48 milliunits enzyme/10^7 cells. *$p < 0.05$; **$p < 0.005$ for comparison with the epinephrine-free control system.

3.5 Effects of epinephrine with and without alpha- and beta-receptor antagonists on superoxide production by and elastase release from activated neutrophils

The effects of epinephrine with and without propranolol, atenolol, an α_1-antagonist or α_2-antagonist on superoxide production by, and elastase release from activated neutrophils, are shown in Chapter 2 (Tables 2.2 and 2.4, pages 47 and 50, respectively.)

Propranolol, a β_1- and β_2-receptor antagonist, completely attenuated the inhibitory effects of epinephrine on both superoxide production and elastase release, while the other receptor antagonists were ineffective. These results suggest that epinephrine exerts its inhibitory effects via β_2-adrenergic receptors. This is in keeping with previous studies (Weiss *et al*, 1996), although these investigators concluded that part of epinephrine's inhibitory effect was due to the ability of the drug to scavenge free radicals. The oxidant-scavenging potential of epinephrine (0.1 – 2 μM) was evaluated in a cell-free, xanthine-xanthine oxidase superoxide generating system (Table 3.4).

Table 3.4. Effect of epinephrine (0.1 – 2 μM) on superoxide generation by a cell-free xanthine–xanthine oxidase system.

System	Superoxide production
Epinephrine 0.1 μM	99 ± 6
Epinephrine 0.5 μM	98 ± 4
Epinephrine 1 μM	104 ± 4
Epinephrine 2 μM	98 ± 4

The results of 8 – 10 experiments are expressed as the mean percentage of control ± SEM. The absolute peak value for the control drug-free system was 1370 ± 37 $mV.s^{-1}$.

Epinephrine at all concentrations tested, did not possess superoxide-scavenging properties. The peak LECL responses for the systems containing epinephrine at 0.1, 0.5, 1.0 and 2.0 μM were 1352 ± 77, 1336 ± 60, 1421 ± 62 and 1339 ± 62 $mV.s^{-1}$ respectively.

3.6 Effects of epinephrine when added 30 sec or 15 minutes prior to the stimulant on neutrophil superoxide production and elastase release

Previous investigators (Tecoma *et al*, 1986), described a greater magnitude of inhibition of superoxide production by activated neutrophils when isoproterenol was added 30 sec prior to the stimulant, compared with incubation times of 4 – 6 min preceding activation of the cells. This relationship was observed at all concentrations of isoproterenol tested and could not be attributed to the binding of isoproterenol to formyl peptide receptors. The effects on superoxide generation by and elastase release from activated neutrophils pre-incubated with epinephrine for 30 sec versus a longer incubation time of 15 min , prior to addition of FMLP, are shown in Table 3.5 (page 71) and Table 3.6 (page 72), respectively.

These results are important in that epinephrine at concentrations as low as 0.01 μM significantly inhibited neutrophil superoxide production when added 30 sec prior to FMLP. At each concentration of epinephrine tested, statistically significant potentiation of the inhibitory response on superoxide generation was observed with the short incubation time (30 sec) compared to the longer incubation time (15 min). The p values for each concentration of epinephrine, 0.01, 0.05, 0.1 and 1 μM, comparing the 30 sec to the 15 min incubation times were < 0.02, < 0.0005, < 0.0001 and < 0.0001 respectively.

Although superoxide production appears exquisitely sensitive to a 30 sec incubation time with epinephrine, less striking results were noted with elastase release (Table 3.6, page 72). Pre-incubation of neutrophils with epinephrine (0.1 μM and 1 μM) for 30 sec, compared to a 15 min incubation time prior to addition of the stimulant, significantly enhanced the inhibitory effect of epinephrine on elastase release from FMLP/CB-activated cells. This potentiation of the inhibitory effect of epinephrine was not observed at concentrations of 0.01 μM and 0.05 μM, although significant inhibition of 12% ($p < 0.02$), was already present following 15 minutes pre-incubation with epinephrine at 0.01 μM.

Table 3.5. Effects of epinephrine when added 30 sec or 15 min prior to FMLP on neutrophil superoxide production.

System	**Superoxide production when epinephrine added:**	
	15 min before FMLP	30 sec before FMLP
Epinephrine 0.01 μM	98 ± 3	83 ± 1*
Epinephrine 0.05 μM	93 ± 4	74 ± 2*
Epinephrine 0.10 μM	91 ± 2*	67 ± 2*
Epinephrine 1 μM	77 ± 1*	56 ± 2*

The results of 6 – 26 experiments are expressed as the mean percentage ± SEM of the epinephrine-free, FMLP-activated control system for which the absolute value was 1322 ± 103 $mV.s^{-1}$. *$p < 0.005$ for comparison with the epinephrine-free control system.

3.7 Effects of epinephrine on cytosolic calcium fluxes in activated human neutrophils

Neutrophils activated with PMA were insensitive to the inhibitory effects of epinephrine on superoxide production by these cells. As calcium mobilisation does not occur following neutrophil stimulation with PMA, this suggests that

alterations in calcium fluxes may explain the inhibitory effects of epinephrine. The fura-2 fluorescence response is a sensitive indicator of cytosolic calcium concentration changes over time and the response to epinephrine from 3 typical experiments is shown in Figure 3.1 (page 74).

Table 3.6. Effects of epinephrine when added 30 sec or 15 min prior to FMLP/CB on neutrophil elastase release.

System	**Elastase release when epinephrine added:**	
	15 min before FMLP/ CB	30 sec before FMLP/CB
Epinephrine 0.01 μM	88 ± 3*	92 ± 1**
Epinephrine 0.05 μM	81 ± 2**	80 ± 1**
Epinephrine 0.10 μM	79 ± 2**	75 ± 1**
Epinephrine 1 μM	51 ± 1**	40 ± 1**

The results of 6 – 9 experiments are expressed as the mean percentage ± SEM of the epinephrine-free, FMLP/CB-activated control system for which the absolute value was 1011 ± 10 milliunits enzyme/10^7 cells. *$p < 0.05$; **$p < 0.005$ for comparison with the epinephrine-free control system.

Activation of neutrophils with FMLP resulted in an abrupt increase in fura-2 fluorescence intensity which coincided with the rise in cytosolic Ca^{2+} concentrations, and quickly subsided, returning to base-line values after several minutes. Epinephrine (1 μM), significantly accelerated the decline in peak fluorescence without altering the intensity of this rapidly occurring peak fluorescence response.

The effects of epinephrine (0.1 – 1 μM) in a larger series of experiments on the peak cytosolic Ca^{2+} concentrations ($[Ca^{2+}]i$), the time taken for fluorescence

intensity to decline to half peak values (t½), as well as the clearance rates of free cytosolic Ca^{2+} following activation with FMLP, are shown in Table 3.7.

Table 3.7. Effects of epinephrine on the peak intracellular calcium concentrations $[Ca^{2+}]i$ and time taken for these to decline to half peak values together with the clearance rates of free calcium from the cytoplasm of FMLP-activated neutrophils.

System	Peak $[Ca^{2+}]i$ values (nM)	Time taken to decline to half peak values (min)	Clearance rate of free Ca^{2+} (pmol/min)
Control	365 ± 31	0.97 ± 0.05	139 ± 20
Epinephrine 0.1 μM	349 ± 33	0.86 ± 0.05	145 ± 19
Epinephrine 0.5 μM	399 ± 50	0.83 ± 0.03	174 ± 22*
Epinephrine 1 μM	365 ± 46	0.75 ± 0.03*	178 ± 25**

The results of 9 experiments are expressed as the mean values ± SEM. The $[Ca^{2+}]i$ value for unstimulated neutrophils was 109 ± 8 nM. *$p < 0.05$; **$p < 0.005$ relative to the epinephrine-free control system.

The magnitude of the abrupt rise in cytosolic free Ca^{2+} was not significantly altered, but the time taken for the fura-2 fluorescence to reach half its peak value was reduced and this correlated well with the calculated progressive increase in the clearance rates of free cytosolic Ca^{2+}, reaching statistical significance at an epinephrine concentration of 0.5 μM ($p < 0.05$).

The effects of epinephrine (1 μM) and rolipram (0.05 μM and 0.1 μM) individually and in combination, on the peak $[Ca^{2+}]i$, time taken for fluorescence intensity to decline to half peak values, as well as the clearance rates of free cytosolic Ca^{2+} for FMLP-activated neutrophils, are shown in Table 3.8 (page 75).

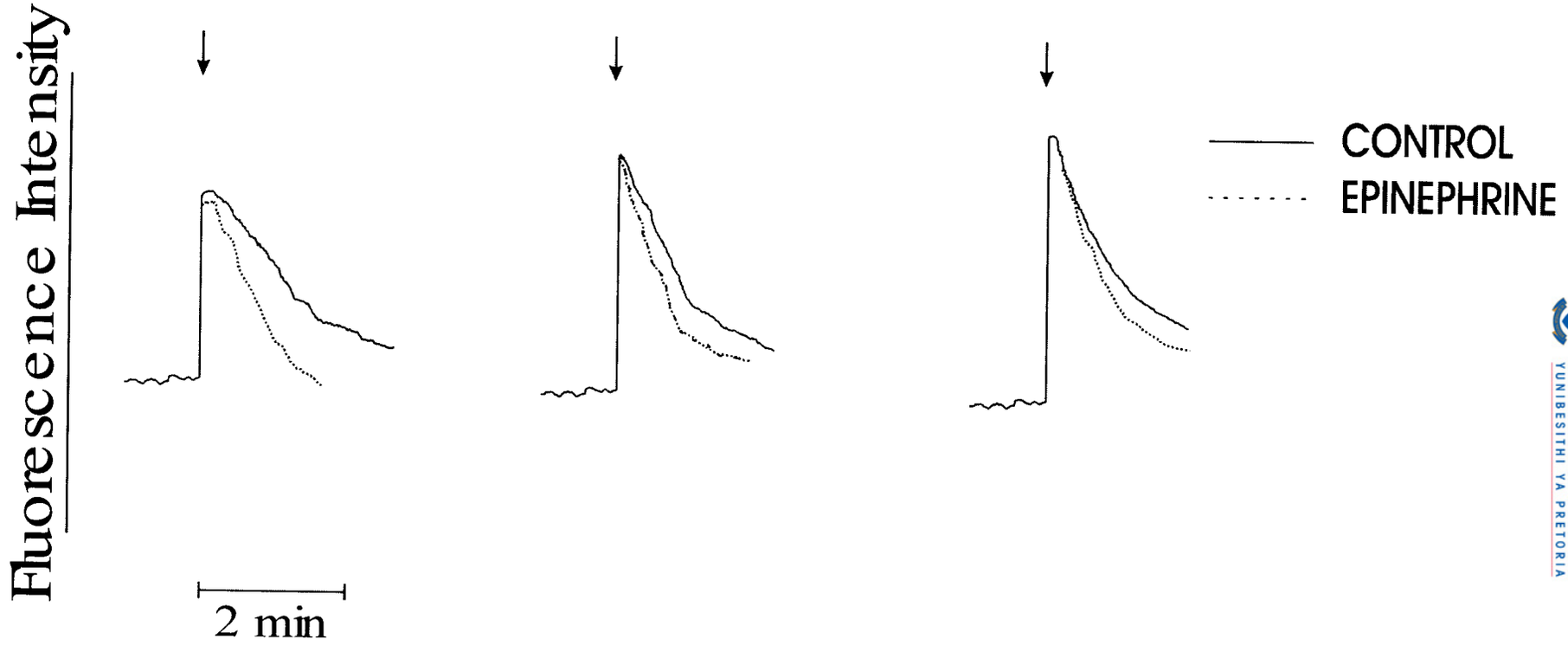


Figure 3.1: The effects of epinephrine (1 μM) on the time course of the fura-2 fluorescence response of FMLP-activated neutrophils from 3 different subjects. FMLP was added as indicated (↓) when a stable base-line was obtained (± 1 min).

Table 3.8. Effects of epinephrine and rolipram individually and in combination on the peak intracellular calcium concentrations $[Ca^{2+}]i$ and time taken for these to decline to half peak values together with the clearance rates of free calcium from the cytoplasm in FMLP-activated neutrophils.

System	Peak $[Ca^{2+}]i$ values (nM)	Time taken to decline to half peak values (min)	Clearance rate of free Ca^{2+} (pmol/min)
Control	268 ± 23	0.97 ± 0.08	92 ± 3
Epinephrine 1 μM	256 ± 20	0.76 ± 0.03*	109 ± 4**
Rolipram 0.05 μM	259 ± 25	0.78 ± 0.07**	109 ± 4**
Rolipram 0.1 μM	267 ± 25	0.71 ± 0.04**	124 ± 3**
Epinephrine + Rolipram 0.05 μM	252 ± 18	0.6 ± 0.06**	133 ± 4**
Epinephrine + Rolipram 0.1 μM	260 ± 21	0.57 ± 0.01**	150 ± 8**

The results of 8 experiments are expressed as the mean values ± SEM. The $[Ca^{2+}]i$ value for unstimulated neutrophils was 91 ± 14 nM. *$p < 0.05$; **$p < 0.005$ relative to the control untreated system.

Epinephrine and rolipram in combination, had no effect on peak fluorescence intensity, but the time taken to reach half peak values was significantly less ($p < 0.005$) for combinations of these agents relative to the effects observed with the individual agents, and correlated closely with the enhanced clearance rates of free cytosolic Ca^{2+} (correlation coefficient r -0.96; $p<0.005$).These results confirm the interactive effects of epinephrine and rolipram when used in combination and demonstrates the effect of cAMP-elevating agents on calcium kinetics in activated neutrophils.

The effects of pre-incubation of the cells with propranolol on the epinephrine-mediated hastening of the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils are shown in Table 3.9 (page 76).

Table 3.9. Effects of epinephrine with and without propranolol on the peak intracellular calcium concentrations $[Ca^{2+}]i$ and time taken for these to decline to half peak values together with the clearance rates of free calcium from the cytoplasm in FMLP-activated neutrophils.

System	Peak $[Ca^{2+}]i$ values (nM)	Time taken to decline to half peak values (min)	Clearance rate of free Ca^{2+} (pmol/min)
Control	421 ± 17	1.35 ± 0.09	129 ± 4
Propranolol 2 μM	399 ± 24	1.28 ± 0.13	128 ± 3
Epinephrine 1 μM	412 ± 17	1.00 ± 0.07*	170 ± 5*
Propranolol + Epinephrine	400 ± 14	1.38 ± 0.15	121 ± 8

The results of 4 experiments are expressed as the mean values ± SEM. *$p < 0.005$ for comparison with the control drug-free system.

Propranolol completely prevented the epinephrine-mediated response, in keeping with previous results on superoxide production and elastase degranulation, confirming a β_2-adrenergic receptor-mediated effect on calcium kinetics in activated neutrophils.

To evaluate whether the differential effects on superoxide production and elastase release observed with epinephrine pre-incubation times of 30 sec versus 15 min are also related to altered Ca^{2+} fluxes, the effects of a shorter epinephrine incubation time (30 sec versus 15 min) on the fura-2 fluorescence responses of FMLP-activated neutrophils were measured and these results are shown in Table 3.10 (page 77).

Table 3.10. Effects of epinephrine when added 30 sec or 15 min prior to FMLP on the peak intracellular calcium concentrations $[Ca^{2+}]i$ and time taken for these to decline to half peak values together with the clearance rates of free calcium from the cytoplasm in FMLP-activated neutrophils.

System	Peak $[Ca^{2+}]i$ values (nM)	Time taken to decline to half peak values (min)	Clearance rate of free Ca^{2+} (pmol/min)
Control	381 ± 14	1.21 ± 0.07	124 ± 6
15 min before FMLP:			
Epinephrine 0.01 μM	334 ± 25	1.10 ± 0.08	122 ± 7
Epinephrine 0.05 μM	313 ± 11	0.98 ± 0.05	129 ± 7
Epinephrine 0.1 μM	389 ± 8	1.10 ± 0.03	140 ± 5*
Epinephrine 1 μM	375 ± 15	1.02 ± 0.08	147 ± 8*
30 sec before FMLP:			
Epinephrine 0.01 μM	357 ± 14	1.10 ± 0.05	131 ± 9
Epinephrine 0.05 μM	335 ± 35	1.01 ± 0.11	133 ± 1.4
Epinephrine 0.1 μM	374 ± 14	0.94 ± 0.03*	158 ± 5*
Epinephrine 1 μM	357 ± 18	0.86 ± 0.05*	168 ± 10**

The results of 5 – 9 experiments are expressed as the mean values ± SEM. *$p < 0.05$; **$p < 0.005$ for comparison with the control epinephrine-free system.

The magnitude of the peak fluorescence intensity was not altered, while the time taken to decline to half peak values, and the clearance rate of Ca^{2+} from the cytosol were accelerated for both incubation times at epinephrine concentrations of 0.1 and 1 μM, relative to the control drug-free system. Pre-incubation of epinephrine for 30 sec prior to addition of FMLP did not significantly alter the clearance rates of cytosolic Ca^{2+} at epinephrine concentrations of 0.01 μM and 0.05 μM.

The clearance rates of cytosolic free Ca^{2+} following a 30 sec incubation time with epinephrine prior to addition of FMLP, were significantly faster than those observed following the 15 min incubation time at epinephrine concentrations of 0.1 μM ($p < 0.02$) and 1 μM ($p < 0.03$). Similar results were obtained with the time taken for fluorescence intensity to decline to half the peak value, with statistically significant differences at epinephrine concentrations of 0.1 μM ($p < 0.0001$) and 1 μM ($p < 0.03$), for comparison of the 30 sec and 15 min incubation times.
These results indicate that the greater magnitude of inhibitory response to a 30 sec epinephrine pre-incubation time compared to 15 minutes of pre-incubation, is reflected not only in superoxide production by and elastase release from FMLP-activated neutrophils, but also in the clearance rate of cytosolic free Ca^{2+}. This supports the proposed role of accelerated cytosolic Ca^{2+} clearance in mediating the inhibitory response to epinephrine.

3.8 Effects of formoterol on calcium fluxes in activated neutrophils

Formoterol, a long-acting β_2-agonist, significantly inhibited superoxide production by and elastase release from activated neutrophils (Chapter 2, Figure 2.1, page 43 and Figure 2.2, page 48) and was the most active of all selective, pharmacologic β_2-agonists tested with respect to anti-inflammatory activity. Limited additional experiments were undertaken to evaluate the effects of formoterol on intracellular calcium fluxes in activated neutrophils and to compare these with the effects of epinephrine, a physiologic non-selective β-agonist, on calcium fluxes in these cells.

The effects of formoterol (1 μM) on the peak intracellular calcium concentration, time taken for these to decline to half peak values and clearance rates of free calcium from the cytosol of FMLP-activated neutrophils were evaluated in a

series of 5 different experiments. The magnitude of the peak fluorescence intensity following Ca^{2+} release from storage vesicles was unaffected by formoterol, but the time taken to decline to half peak values was significantly reduced from 1.70 ± 0.03 min to 1.00 ± 0.03 min in the presence of formoterol ($p < 0.005$). The corresponding clearance rate of free Ca^{2+} was significantly accelerated from 99 ± 6 pmol/min (control cells) to 160 ± 8 pmol/min (formoterol-treated cells) ($p < 0.05$). This represents a 60% increase in the clearance rate of free cytosolic Ca^{2+} compared to an increase of 26% (mean value from Tables 3.7, 3.9 and 3.10; pages 72, 75 and 76 respectively), observed with epinephrine.

3.9 Effect of epinephrine and formoterol on $^{45}Ca^{2+}$ fluxes in activated neutrophils

The fura-2 fluorescence results indicated that epinephrine and formoterol hasten the clearance rate of cytosolic Ca^{2+} following neutrophil activation. Accelerated clearance can be achieved either by enhancement of the immediate efflux of Ca^{2+} from the cytosol, or by reducing the delayed store-operated influx of Ca^{2+}, or accelerating Ca^{2+} resequestration into calciosomes. Combinations of these mechanisms may also be operative.

To elucidate which mechanism(s) is/are responsible for the observed accelerated Ca^{2+} clearance from the cytosol, Ca^{2+} fluxes in resting and activated neutrophils were measured, using $^{45}Ca^{2+}$ as tracer to label the intracellular Ca^{2+} pool. In order to determine the magnitude of $^{45}Ca^{2+}$ efflux from and influx into activated neutrophils, with and without epinephrine and formoterol, radiometric efflux and influx experiments were performed to enable distinction between net efflux and net influx of the cation.

3.10 Efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils

For these experiments, neutrophils were pre-loaded with $^{45}Ca^{2+}$, then washed and transferred to Ca^{2+}-replete HBSS (to minimise re-uptake of radiolabelled cation) followed by activation with FMLP and measurement of the amount of remaining cell-associated $^{45}Ca^{2+}$ 60 sec after addition of FMLP, at which time efflux is complete (Anderson and Goolam Mahomed, 1997). The effects of epinephrine, with and without thapsigargin, on the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils are shown in Table 3.11.

Table 3.11. Effects of epinephrine, with and without thapsigargin, on the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils.

Agent	**Amount of $^{45}Ca^{2+}$ released from neutrophils 60 sec after the addition of FMLP (pmol/10^7 cells)**
FMLP only	152 ± 6
Epinephrine 1 μM	115 ± 7*
Thapsigargin 1 μM	153 ± 18
Epinephrine + Thapsigargin	134 ± 15

The results of 4 - 12 experiments are expressed as the mean value ± SEM. *$p < 0.005$ for comparison of $^{45}Ca^{2+}$ efflux from control, epinephrine- and thapsigargin-treated cells.

Exposure of control neutrophils to FMLP resulted in efflux of the radiolabelled cation from the neutrophils which corresponded to loss of approximately 44% of cell-associated cation over the 60 sec time-course of the experiment. No loss of $^{45}Ca^{2+}$ was observed in the control, unstimulated neutrophils over the 60 sec incubation period during which efflux was measured (not shown). Pre-treatment

of neutrophils with epinephrine, significantly reduced the extent of efflux of $^{45}Ca^{2+}$ from the cells compared to the control system (115 ± 7 pmol/10^7 cells versus 152 ± 6 pmol/10^7 cells, respectively) ($p < 0.005$). Treatment of neutrophils with thapsigargin (added simultaneously with FMLP) markedly attenuated the epinephrine-related reduction in efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils (Table 3.11, page 80).

Formoterol (results not shown) significantly reduced the magnitude of efflux of cell-associated $^{45}Ca^{2+}$ from 152 ± 6 pmol/10^7 cells for the control system to 110 ± 7pmol/10^7 cells for the formoterol-treated cells. This represents a 28% reduction in efflux of cell-associated $^{45}Ca^{2+}$ compared to the 24% reduction for epinephrine (also at 1 μM).

3.11 Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils

The effects of epinephrine and formoterol on the influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils are shown in Table 3.12.

Table 3.12. Effects of epinephrine and formoterol on the influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils.

Agent	**Influx of $^{45}Ca^{2+}$ into neutrophils 5 min after the addition of FMLP (pmol/10^7 cells)**
FMLP only	155 ± 12
Epinephrine 1 μM	99 ± 6*
Formoterol 1 μM	78 ± 7*

The results of 11 experiments are expressed as the mean value ± SEM. *$p < 0.005$ for comparison of $^{45}Ca^{2+}$ uptake by control and epinephrine- or formoterol-treated cells.

The net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils was measured over a fixed 5 min time-course during which activation of control neutrophils with FMLP resulted in a substantial influx of $^{45}Ca^{2+}$ (155 ± 12 pmol/10^7 cells), while there was only trivial influx of the radiolabelled cation into control, identically processed neutrophils not exposed to FMLP (28 ± 2 pmol/10^7 cells). Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils pretreated with epinephrine, was significantly reduced by 36% to 99 ± 6 pmol/10^7 cells ($p < 0.005$), with negligible influx into resting epinephrine-treated cells (21 ± 3 pmol/10^7 cells). Formoterol significantly attenuated the magnitude of $^{45}Ca^{2+}$ influx from 155 ± 12 pmol/10^7 cells (control system) to 78 ± 7 pmol/10^7 cells (formoterol treated) ($p < 0.005$). $^{45}Ca^{2+}$ influx was reduced by 50% with formoterol compared to 36% with epinephrine.

3.12 Effect of epinephrine on intracellular cAMP levels

Beta-adrenergic agonists, including epinephrine, are known to elevate intracellular cAMP levels via a G-protein-coupled activation of adenyl cyclase (Barnes, 1995). The effect of β-adrenergic agonists on cAMP levels in resting and FMLP-activated neutrophils has been variously reported by previous investigators, with some documenting an amplification of the FMLP-induced increase in cAMP levels in the presence of isoproterenol or epinephrine (Tecoma *et al*, 1986 and Bazzoni *et al*, 1991). The results of experiments to measure intracellular cAMP levels in resting and FMLP-activated neutrophils pre-incubated with epinephrine are shown in Table 3.13 (page 83). Because cAMP is rapidly hydrolysed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μM rolipram.

Table 3.13. Effects of epinephrine, in the presence of rolipram (1μM), on the intracellular cAMP concentrations in unstimulated and FMLP-activated neutrophils at 1 min after addition of the stimulant.

System	Intracellular cAMP concentration (pmol/10^7 cells)	
	Without FMLP	With FMLP
Rolipram	95 ± 33	355 ± 156
Rolipram + Epinephrine 1μM	113 ± 21	667 ± 13

The results of 3 – 7 experiments are expressed as the mean value ± SEM. Statistical significance was not observed due to the small number of experiments.

The intracellular cAMP concentration increased slightly in unstimulated neutrophils in the presence of epinephrine. However, addition of epinephrine to FMLP-stimulated neutrophils markedly enhanced the elevation of intracellular cAMP levels, compared to control FMLP-activated cells.

3.13 Effect of epinephrine on membrane depolarisation

Pre-incubation of neutrophils with epinephrine (1 μM), slightly reduced the magnitude of the membrane depolarisation response following activation of the cells with FMLP. In 11 separate experiments, the magnitude of membrane depolarisation in the presence of epinephrine was reduced by 11% compared to that of control cells (66 ± 4 mV and 74 ± 4 mV respectively, $p < 0.004$).

3.14 Discussion

Epinephrine, an endogenous anti-inflammatory mediator (Dallegri and Ottonello, 1997), and an important therapeutic agent in septic shock (Ognibene, 1997), was

selected for further experiments to evaluate the exact molecular/biochemical mechanisms which underlie the anti-inflammatory interactions of β-adrenoreceptor agonists with neutrophils. Treatment of neutrophils with epinephrine, resulted in dose-related inhibition of superoxide production by these cells, consistent with a receptor-mediated effect (Weiss *et al*, 1996). Significant inhibition was observed at an epinephrine concentration of 0.01 μM, with maximum inhibition at 1 μM. Epinephrine concentrations of 1 μM have been shown to inhibit endotoxin-induced release of TNF by inflammatory cells *in vitro* (Severn *et al*, 1992; Van der Poll *et al*, 1996). Importantly endogenous adenosine released by damaged cells, potentiates the inhibitory effects of epinephrine, even at physiological concentrations of epinephrine (0.48×10^{-9} mol/l) (Bazzoni *et al*, 1991). Plasma epinephrine levels may reach 1000 pg/ml and higher following myocardial infarction (Cryer, 1980), while those measured *in vivo* during a constant epinephrine infusion at therapeutic doses were 1037 ± 179 pg/ml (Van der Poll *et al*, 1996). These concentrations of epinephrine *in vivo* are equivalent to 0.01 μM in the experimental setting, which means that the effects of epinephrine observed *in vitro* may be operative *in vivo* under conditions of stress and during therapeutic infusions.

The inhibitory effects of epinephrine on superoxide production were consistent and repeatable with highly statistically significant inhibition at a concentration of 1 μM in FMLP-activated cells. These observations are essentially in agreement with previous reports describing an inhibitory effect of epinephrine on oxidant production by neutrophils stimulated with formyl peptides *in vitro* (Tecoma *et al*, 1986; Bazzoni *et al*, 1991; Weiss *et al*, 1996), but contrast with those of other investigators, who were unable to document inhibitory effects of epinephrine on neutrophil membrane-associated oxidative metabolism (Hetherington and Quie, 1985; Burns *et al*, 1997). In the study by Hetherington and Quie, the precise concentration of epinephrine in blood samples was not measured and epinephrine was used primarily to induce margination of polymorphonuclear

leukocytes. Burns *et al* (1997), did not use purified neutrophils which may have complicated the interpretation of these results.

The inhibitory effect of epinephrine on neutrophil superoxide production did not occur in isolation as elastase release from the cells was also significantly attenuated at all concentrations tested (0.01 – 1 μM), in a dose-related fashion. Although epinephrine has been reported to reduce lysozyme and β-glucuronidase release from FMLP-stimulated neutrophils (Busse and Sosman, 1984; Bazzoni *et al*, 1991), the attenuation of elastase release observed in this study, greatly enhances the clinical relevance of this effect, given the powerful destructive capability of this proteolytic enzyme (Vender, 1996). The inhibitory effect of epinephrine has not been reported in any system using PMA-activated neutrophils, regardless of whether superoxide production (Tecoma *et al*, 1986; Nagata *et al*, 1992) or release of the granule polypeptides lactoferrin and β-glucuronidase (Hetherington and Quie, 1985), were used as markers of neutrophil activation. Neutrophils activated with PMA were also insensitive to the effects of epinephrine in the present study with respect to both oxidant production and elastase release. These observations reflect the insensitivity of neutrophil PMA-activated responses to cAMP-elevating agents (Nagata *et al*, 1992; Weiss *et al*, 1996), and suggest that epinephrine operates at an intracellular level upstream of protein kinase C (Weiss *et al*, 1996).

Events upstream of protein kinase C are important in the signal transduction pathway leading to PKC and ultimately, NADPH oxidase activation. The observation that a β-adrenergic agonist binding to ~ 1000 available receptors is able to exert an inhibitory influence on FMLP-responses mediated via ~ 50 000 receptors, implies a significant amplification of the β-adrenergic receptor-mediated signal (Mueller *et al*, 1988). The most proximal event is the association of agonist with its receptor, followed by activation of adenylate cyclase (Barnes, 1995). The involvement of β-adrenergic receptors has been confirmed in experiments with various adrenergic receptor antagonists. The anti-inflammatory

interactions of epinephrine were attenuated by the non-specific β_1- and β_2-receptor antagonist, propranolol. Complete attenuation of epinephrine's effects were evident for both superoxide production by and elastase release from activated neutrophils, pre-incubated with propranolol. The selective α_1- and α_2-adrenoreceptor antagonists did not attenuate epinephrine's inhibitory properties, excluding any contribution via α-adrenoreceptors. Atenolol, a selective antagonist of β_1-adrenoreceptors, did not prevent epinephrine-mediated inhibition of superoxide production by FMLP-activated neutrophils, but did cause modest attenuation of the inhibitory effect of epinephrine on elastase release from these cells, possibly as a result of a lack of absolute specificity of this agent for β_1-adrenoreceptors (Hoffman and Lefkowitz, 1992). These observations support the involvement of β_2-adrenoreceptors in the anti-inflammatory interactions of epinephrine with neutrophils. The β-receptors on neutrophil outer membranes are fully saturated during incubation with isoproterenol at a concentration of 1 μM (Mueller *et al*, 1988), which is likely to optimally activate the β-receptor-coupled signal transduction pathway.

Interestingly, the magnitude of the epinephrine-mediated inhibition of superoxide production by FMLP-activated neutrophils was greater when the adrenoreceptor agonist was added to the cells 30 sec prior to the stimulant in comparison with systems in which a 15 min exposure time was used. Using the brief exposure time, the inhibitory effects of epinephrine could be detected at concentrations of 0,01 μM and upwards. This relationship between short duration of exposure and magnitude of inhibition of superoxide production by activated neutrophils has previously been described for isoproterenol (Tecoma *et al*, 1986). The enhanced inhibitory effect of epinephrine on superoxide production by activated neutrophils following a 30 sec incubation time, although evident, was less pronounced in systems measuring elastase release from the cells. This may be due to the fact that statistically significant inhibition of elastase release from activated neutrophils was already apparent at an epinephrine concentration of 0.01 μM in

cells pre-incubated for 15 min, suggesting a higher level of sensitivity of the degranulation response to cAMP-elevating agents, although in neutrophils activated with C5a, superoxide production was found to be more sensitive to regulation by cAMP-elevating agents than lysozyme release (Nagata *et al*, 1992).

The mechanism proposed by Tecoma *et al* (1986) for the differential response to isoproterenol following shorter incubation times was one of rapid desensitisation of β_2-adrenoreceptors. Desensitisation of β-receptors in the presence of agonist is a well recognised phenomenon (Feldman *et al*, 1983; Barnes, 1999; Liggett, 1999). This loss of responsiveness to the β-agonist must occur rapidly, as differences are already apparent at 2 – 5 min of exposure to the agonist (Tecoma *et al*, 1986). This rapid desensitisation suggests functional uncoupling of the β-adrenoreceptor from the stimulatory G_s-protein (Barnes, 1999). Uncoupling of the β-receptor from the G_s-protein occurs following phosphorylation of occupied receptors, mediated by kinase enzymes (Liggett, 1999). The low β-receptor reserve on neutrophils, ~ 1000 receptors per cell (Mueller et al, 1988), leads to a reduced functional response when β-receptors are phosphorylated (Barnes, 1999). Phosphorylated β-receptors are rapidly internalised by endocytosis (plateauing at 5 min), and may be recycled to the membrane with a recycling time (t½) of 7.9 min if the agonist is withdrawn (Morrison *et al*, 1996). In the continued presence of an agonist, recycling reaches a steady-state dynamic equilibrium after about 10 min (Morrison *et al*, 1996). An alternative mechanism for the rapid loss of responsiveness to β-agonists, lies in the up-regulation of phosphodiesterases with enhanced degradation of cAMP. Rapid activation of phosphodiesterases through cAMP and protein kinase A-mediated phosphorylation, leading to augmented function of the phosphodiesterse enzyme, has been reported to occur within 10 – 15 min of agonist exposure (Giembycz, 1996). Further evidence in support of this mechanism, may be derived from studies investigating the effects of isoproterenol with and without a PDE 4 inhibitor (rolipram), on neutrophil intracellular cAMP levels (Galand and Britt, 1984). Incubation of neutrophils with isoproterenol alone increased intracellular

cAMP levels to a peak at 15 – 30 sec. In the presence of isoproterenol and rolipram, the magnitude of the peak was doubled and persisted for 120 sec before declining towards basal levels (Galant and Britt, 1984). This suggests that at least part of the loss of responsiveness observed with β-agonists may be due to activation of PDE 4. It seems likely that both mechanisms are operative concurrently in neutrophils, but together translate into a reduced functional response to epinephrine of ~ 20% for superoxide inhibition and less for elastase degranulation (~ 5 – 10%) (observations from the results of Tables 3.5 and 3.6, pages 70 and 71 respectively).

The clinical relevance of β-receptor desensitisation in neutrophils may have been over-estimated as β-agonists dissociate rapidly from the receptor (< 30 sec) with maximal inhibition requiring occupancy of only 40 – 50% of available receptors (Mueller *et al*, 1988). In addition, the development of second generation type 4 phosphodiesterase inhibitors may enable these problems to be overcome (Torphy, 1998). Rolipram in combination with epinephrine (Tables 3.2 and 3.3, pages 66 and 67 respectively), potentiated the inhibitory effects of epinephrine alone. This was observed at all concentrations of epinephrine tested and was evident for both the superoxide generation and degranulation responses of activated neutrophils. These observations are in keeping with previous studies documenting an enhanced response to β-adrenergic agonists in combination with a PDE inhibitor (Galant and Britt, 1983; Bazzoni *et al*, 1991; Torphy, 1998).

Oxidant-scavenging by epinephrine has been suggested as an anti-inflammatory mechanism by previous investigators (Weiss *et al*, 1996). Experiments using a cell-free xanthine-xanthine oxidase superoxide generating system excluded this possibility at all concentrations of epinephrine tested.

The important observation that PMA-activated neutrophils were insensitive to the inhibitory effects of epinephrine suggested not only that the target must lie upstream of PKC, but also that alterations in calcium fluxes following neutrophil

activation may play an important role in mediating the responses to epinephrine. The importance of changes in the cytosolic Ca^{2+} concentration during activation of PKC and phospholipase A_2, as well as granule enzyme release are well documented (Sadler and Badwey, 1988; Sengeløv, 1996; Condliffe *et al*, 1998). Transient increases in cytosolic free Ca^{2+} precede and are a pre-requisite for receptor-mediated activation of NADPH oxidase and degranulation (Borregaard *et al*, 1993; Pettit and Hallet, 1998).

The relationship between epinephrine-mediated anti-inflammatory effects on neutrophils and modulation of intracellular Ca^{2+} metabolism has not been investigated in previous studies. The effects of epinephrine on cytosolic Ca^{2+} fluxes were investigated in the current study using fura-2 spectrofluorimetry. Epinephrine, at concentrations that suppressed the pro-inflammatory activities of these cells, had no effect on the abrupt increase in cytosolic Ca^{2+} which accompanied activation with FMLP. This observation demonstrates that epinephrine does not affect the FMLP-mediated activation of phospholipase C or the subsequent interaction of inositol triphosphate with Ca^{2+} mobilising receptors on intracellular Ca^{2+} stores. However, the subsequent progressive decline in peak fura-2 fluorescence was accelerated in epinephrine-treated neutrophils, indicative of hastened clearance of Ca^{2+} from the cytosol and an associated attenuation of Ca^{2+} influx.

The accelerated decline in fura-2 fluorescence mediated by epinephrine was dose-dependent and was attenuated by treatment of the cells with propranolol, supporting a β_2-adrenoreceptor mediated effect. The addition of rolipram further potentiated the rate of clearance of Ca^{2+} from the cytosol, suggesting the involvement of cAMP in mediating the responses to epinephrine. In keeping with the effects on superoxide production and elastase release, a shorter time of exposure of neutrophils to epinephrine prior to activation with FMLP, further enhanced the rate of cytosolic calcium clearance, compared to longer incubation times.

The accelerated rate of clearance of Ca^{2+} from the cytosol may result from reduced store-operated Ca^{2+} influx, increased efflux across the outer membrane or accelerated resequestration into calciosomes. One proposed mechanism mediating the effects of cAMP is inhibition of phosphatidylinositol turnover and/or production of inositol triphosphate and diacylglycerol (Suter *et al*, 1989; Nagata *et al*, 1992). This is unlikely as Ca^{2+} release from storage vesicles (indicated by the peak fura-2 fluorescence) is not altered by epinephrine. Cyclic AMP-mediated inhibition of Ca^{2+} influx via receptor-operated Ca^{2+} channels suggested by previous investigators (Ahmed *et al*, 1995) is improbable as Ca^{2+} re-uptake into neutrophils is store-operated and therefore dependent on the filling state of calcium storage vesicles (Alonso-Torre *et al*, 1993). The experiments by Ahmed *et al* (1995), in which the magnitude of Ca^{2+} influx, in the presence and absence of cAMP, was measured as the fura-2 fluorescence response to addition of Ca^{2+} following prior FMLP-activation of neutrophils in Ca^{2+}-free medium, do not reflect the true influx of Ca^{2+}. The attenuated rise in cytosolic Ca^{2+}, observed in the presence of cAMP, following replenishment of the Ca^{2+} content of the extra-cellular fluid, may have been due to accelerated Ca^{2+} resequestration into calciosomes and therefore not indicative of primary inhibition of Ca^{2+} influx. It is accepted that changes in endomembrane Ca^{2+}-ATPase activity may result in erroneous impressions of Ca^{2+} influx (Parekh and Penner, 1997), with refilling of intracellular stores occurring via a relatively privileged uptake pathway that largely bypasses the cytoplasm (Tsien, 1990).

The same authors (Ahmed *et al*, 1995), in similar experiments with neutrophils in Ca^{2+}-free medium followed by Ca^{2+} replenishment, observed the failure of a cAMP-increasing agent to inhibit thapsigargin-induced Ca^{2+} influx into the cells. Based on this observation, they suggested that cAMP does not affect store-operated Ca^{2+} influx. The alternative explanation for these results is that cAMP will not be able to exert its effect on endomembrane Ca^{2+}-ATPase enzymes already inhibited by thapsigargin. Accelerated efflux of Ca^{2+} from the cytosol (De

Togni *et al*, 1984) and enhanced reuptake by storage vesicles (De Togni *et al*, 1984; Nagata *et al*, 1992), have also been suggested, but not confirmed, as possible mechanisms mediating the effects of cAMP on cytosolic Ca^{2+} concentrations in activated neutrophils.

In order to resolve these issues, radiometric procedures, which can distinguish between net efflux and net influx of Ca^{2+}, were used. These procedures allow identification of the mechanisms (enhancement of efflux and/or inhibition of influx) of the epinephrine-mediated accelerated clearance of calcium from the cytosol of FMLP-activated neutrophils.

As previously reported (Anderson *et al*, 1998; Barritt, 1999), addition of FMLP to $^{45}Ca^{2+}$-loaded neutrophils resulted in an immediate efflux of the radiolabelled cation, coincident with the increase in cytosolic Ca^{2+}, which terminates at 30 – 60 sec after addition of the stimulus. Addition of FMLP to the neutrophils in this study elicited an efflux of Ca^{2+} which corresponded on average to 50% of the cell-associated cation, suggesting that only part of the intracellular Ca^{2+} pool is mobilised following activation of the neutrophils, or that much of the cytosolic Ca^{2+} is resequestered by the endo-membrane Ca^{2+}-ATPase. Pretreatment of neutrophils with epinephrine did not potentiate, but rather suppressed the FMLP-activated efflux of Ca^{2+} by 24% compared to control cells.

Net influx of Ca^{2+}into FMLP-activated neutrophils has been reported to occur at around 1 – 2 min after addition of the stimulus and to be complete at about 5 min (Geiszt *et al*, 1997; Anderson *et al*, 1998). This delayed influx of Ca^{2+} is characteristic of store-operated influx which is operative in neutrophils and is required for refilling of intracellular stores (Favre *et al*, 1996). Treatment of neutrophils with epinephrine significantly decreased the amount of Ca^{2+} which entered FMLP-activated neutrophils during store-operated influx of the cation. Together with the results of the fura-2 experiments, the observation that epinephrine decreases both FMLP-activated efflux and store-operated influx of

Ca^{2+} suggests that this adrenoreceptor agonist up-regulates the activity of the cAMP-dependent protein kinase-activatable endo-membrane Ca^{2+}-ATPase (Schatzmann, 1989). This may explain the decreased efflux of Ca^{2+} as a consequence of competition between the up-regulated endo-membrane and plasma membrane Ca^{2+}-ATPases for cytosolic Ca^{2+}. Up-regulation of the endo-membrane Ca^{2+}-ATPase would result in enhancement of resequestration of cytosolic Ca^{2+} and increased refilling of stores with endogenous cation, and a consequent reduction in the magnitude of the store-operated influx of extracellular Ca^{2+}. This contention is supported by the observation that thapsigargin, a selective inhibitor of the endomembrane Ca^{2+}-ATPase (Lytton *et al*, 1997) antagonised the epinephrine-mediated reduction in the efflux of Ca^{2+} from FMLP-activated neutrophils. In addition, cAMP increases the intracellular production of inositol 1,3,4,5-tetrakisphosphate (IP_4) (Tsien, 1990), which in turn enhances the activity of the endomembrane Ca^{2+}-ATPase (Lew, 1989).

It is unlikely that the reduced Ca^{2+} efflux observed with the radiometric procedure is due to inhibition of the plasma membrane Ca^{2+}-ATPase, as this would result in a sustained elevation of cytosolic calcium which is not evident from the fura-2 fluorescence tracings. In addition, unlike the endomembrane ATPase, the plasma membrane Ca^{2+}-ATPase is modulated by calmodulin, as opposed to cAMP, which shifts the pump to a higher affinity state for Ca^{2+}, resulting in enhanced maximal velocity (Carafoli *et al*, 1992).

Formoterol, a long-acting β_2-agonist, significantly accelerated the rate of clearance of cytosolic calcium in FMLP-activated neutrophils, in keeping with a cAMP-mediated accelerated resequestration of Ca^{2+} into storage vesicles. Formoterol attenuated $^{45}Ca^{2+}$ efflux from FMLP-activated neutrophils by 28% compared to control cells, with a 50% reduction in the true influx of $^{45}Ca^{2+}$ into these cells, compatible with activation of the endomembrane Ca^{2+}-ATPase by cAMP. The similarity of these formoterol-mediated alterations in Ca^{2+} fluxes to

those observed with epinephrine, further strengthens the key role for cAMP in suppressing the pro-inflammatory activities of human neutrophils.

The important role for cAMP in mediating the observed alterations in Ca^{2+} homeostasis is suggested by a number of lines of evidence. These include the lack of sensitivity of PMA-activated cells to cAMP-elevating agents (De Togni *et al*, 1984; Nagata *et al*, 1992), the well-recognised activation of adenylate cyclase by β-adrenergic agonists (Barnes, 1995), as well as the synergy that exists between cAMP-elevating agents and PDE inhibitors (Moore and Willoughby, 1995). The PDE 4 inhibitor, rolipram, potentiated the increase in intracellular cAMP levels induced by epinephrine in FMLP-activated neutrophils, supporting a role for cAMP in mediating the effects of epinephrine and rolipram on oxidant production, elastase degranulation and calcium kinetics in activated neutrophils. Pre-incubation of neutrophils with epinephrine resulted in increased intracellular cAMP concentrations, especially following activation of the cells with FMLP, an observation which strengthens the relationship between occupation of β_2-adrenoreceptors, activation of adenylate cyclase and suppression of neutrophil reactivity. The amplifying effect of epinephrine on the cAMP response to FMLP is interesting and has been observed by other investigators (Tecoma *et al*, 1986; Bazzoni *et al*, 1991), compatible with sensitisation of basal adenylate cyclase activity by epinephrine (O'Dowd and Newsholme, 1997). Addition of epinephrine to FMLP-activated cells in the presence of rolipram, potentiated the elevation in intracellular cAMP levels by preventing the rapid PDE 4-mediated hydrolysis of cAMP (Torphy, 1998).

Cyclic AMP-elevating agents significantly suppress neutrophil pro-inflammatory activity, and it may therefore be postulated that use of these agents in a clinical setting, may predispose patients to infection. Although plausible, this seems unlikely as the capacity of neutrophils to produce oxygen radicals far exceeds the minimum requirements for microbial activity. Evidence for this is derived from persons who are carriers of the gene for the X-linked form of CGD. Despite only

10% of the normal capcity for oxidant generation, they do not appear unduly prone to infection (Segal *et al,* 2000). In addition, inhaled agents delivered directly to the airways, may circumvent the risk of systemic immunosuppression.

The physiological function of membrane depolarisation in neutrophils has not been conclusively established, but may contribute to the restoration of calcium homeostasis by limiting the influx of extracellular calcium (Di Virgilio *et al*, 1987). It is therefore plausible that epinephrine, which attenuates the membrane depolarisation response to FMLP, may promote earlier or accelerated influx of extracellular Ca^{2+} and diversion of the cation into stores. This was difficult to demonstrate during radiometric Ca^{2+} influx experiments, probably as a result of the slight reduction in the magnitude of membrane depolarisation observed with epinephrine-treated cells, and due to the activity of the plasma membrane Ca^{2+}-ATPase efflux pump during the initial 30 sec subsequent to activation of the cells. To further investigate the relationship between membrane depolarisation and calcium influx in a physiological setting, experiments were performed with cells from patients with chronic granulomatous disease that lack any significant membrane depolarisation response to calcium-mobilising stimuli, and these are described in the following chapter.

In conclusion, epinephrine has been shown in the current study to modulate the pro-inflammatory activities of human neutrophils *in vitro*, apparently by cAMP-dependent acceleration of restoration of Ca^{2+} homeostasis in these cells.

CHAPTER 4

THE ROLE OF MEMBRANE DEPOLARISATION IN REGULATING CALCIUM INFLUX IN ACTIVATED HUMAN NEUTROPHILS: LESSONS FROM CHRONIC GRANULOMATOUS DISEASE

4.1 Introduction

The resting membrane potential of human neutrophils is maintained at –60 to –75 mV (Majander and Wikström, 1989; Demaurex *et al*, 1993b). Following activation of the cells, the membrane potential depolarises to values in excess of 0 mV (Demaurex *et al*, 1993b). The mechanism mediating membrane depolarisation in neutrophils is currently attributed to the vectorial flow of electrons across the plasma membrane (Henderson *et al*, 1987; Schrenzel *et al*, 1998). Neutrophils may generate 10^8 electrons/sec/cell which can produce an electron current of 16 pA (Demaurex *et al*, 1993b). NADPH oxidase plays a critical role in mediating electron flow and membrane depolarisation, with cells from patients with chronic granulomatous disease (CGD) unable to generate reactive oxidants or depolarise their membranes (Matsuura *et al*, 1984). This is supported by experiments using the specific NADPH oxidase inhibitor, diphenylene iodonium sulphate (DPI), which markedly attenuates oxidant production and consequently membrane depolarisation (Henderson *et al*, 1987).

Membrane depolarisation has been reported to play an important role in regulating neutrophil calcium homeostasis by inhibiting store-operated calcium influx which occurs at 30 – 60 sec following activation with FMLP (Di Virgilio *et al*, 1987). This suggests that cells lacking a membrane depolarisation response will be unable to effectively regulate calcium homeostasis which may result in flooding of the cytosol by Ca^{2+}, leading to hyperactivation of the cells. In the current study I have investigated the relationship between NADPH oxidase, membrane potential, Ca^{2+} fluxes and pro-inflammatory activities of neutrophils from healthy control subjects and patients with CGD.

4.2 Materials and Methods

Unless indicated all chemicals and reagents were purchased from the Sigma Chemical Co, St Louis, MO, USA.

4.2.1 Neutrophils

Purified neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of CGD subjects and healthy adult volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatin to remove most of the erythrocytes. After centrifugation, erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4°C for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1 x 10^7/ml in PBS and held on ice until used.

4.2.2 Superoxide production

This was measured only on the initial visit of each CGD patient using lucigenin (bis-N-methylacridinium nitrate) chemiluminescence (LECL) (Minkenberg and Ferber, 1984). Neutrophils (1x10^6) were preincubated for 15 min at 37°C in 900 µl of indicator-free Hanks' balanced salt solution (HBSS, pH 7.4, 1.25 mM $CaCl_2$) containing 0.2 mM lucigenin after which they were activated with the synthetic chemotactic tripeptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µM final) or phorbol 12-myristate 13-acetate (PMA, 20 ng/ml final). Spontaneous and stimulated LECL responses were then monitored with an LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of the stimulant (100 µl). LECL readings were integrated for 5 sec intervals and recorded as millivolts/ sec

(mVs^{-1}). Additional experiments were performed to investigate the effects of staurosporine (200 nM) (preincubated with cells from normal subjects for 15 min at 37°C) on FMLP-activated LECL responses.

4.2.3 Membrane potential

The potential sensitive fluorescent dye dipentyloxacarbocyanine [di-0-C_5 (3)] was used to measure changes in membrane potential in activated neutrophils (Seligmann and Gallin, 1980). The cells ($1x10^6$/ml) were preincubated for 10 min at 37°C in HBSS containing 80 nM (final) di-0-C_5(3) after which they were transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 460 nm and 510 nm respectively. Where appropriate, the cells were pre-incubated with staurosporine (200 nM) for 10 min at 37°C. The neutrophils were then activated with FMLP (1 µM) and the subsequent alterations in fluorescence intensity monitored over a 5-10 min period. The final volume in each cuvette was 3 ml containing a total of $3x10^6$ neutrophils.

4.2.4 Spectrofluorimetric measurement of Ca^{2+} fluxes

Fura-2/AM (Calbiochem Corp., La Jolla, CA) was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments (Grynkiewicz *et al.*, 1985). Neutrophils (1×10^7/ml) were pre-loaded with fura-2 (2 µM) for 30 min at 37°C in phosphate-buffered saline (PBS, 0.15 M, pH 7.4), washed twice and resuspended in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4) containing 1.25 mM $CaCl_2$, referred to hereafter as Ca^{2+}-replete HBSS. The fura-2-loaded cells (2×10^6/ml) were then pre-incubated for 10 min at 37°C after which they were transferred to disposable reaction cuvettes, which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm respectively. After a stable base-line was obtained (1 min), the neutrophils were activated by addition of FMLP (1 µM)

and the subsequent increase in fura-2 fluorescence intensity monitored over a 5 min period. The final volume in each cuvette was 3 ml containing a total of 6 x 10^6 neutrophils.

The data from these experiments were used to compare the following in control and CGD neutrophils: i) basal concentrations of Ca^{2+} in unstimulated cells ii) peak increments in cytosolic Ca^{2+} concentrations and the duration of these in stimulated cells iii) the rate of clearance of Ca^{2+} from the cytosol of FMLP-activated cells.

In an additional series of experiments, the effects of the cyclic AMP-elevating agent rolipram (1 µM final), an inhibitor of type 4 phosphodiesterase, the predominant type found in human neutrophils (Torphy, 1998), on FMLP-activated Ca^{2+} fluxes in CGD neutrophils were investigated. Rolipram was present with the neutrophils throughout the 10 min preincubation period prior to addition of FMLP. The effects of staurosporine (200 nM) on the fura-2 fluorescence responses in FMLP-activated neutrophils from normal subjects, were also investigated. The cells were pre-incubated with staurosporine for 8 min prior to addition of the stimulant.

4.2.5 Mn^{2+} quenching of fura-2 fluorescence

Cells loaded with fura-2/AM as described above were activated with FMLP (1 µM) in the presence of 300 µM $MnCl_2$ (added 5 min prior to FMLP) and fluorescence quenching as a measure of Ca^{2+} influx was determined at an excitation wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm (Geiszt *et al.,* 1997). These experiments were performed using cells from two control subjects and two CGD patients (DT and RS).

4.2.6 *Radiometric assessment of Ca^{2+} fluxes*

$^{45}Ca^{2+}$ (Calcium-45 chloride, specific activity 18.53 mCi/mg, Du Pont NEN Research Products, Boston, MA) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and activated neutrophils. In the assays of net efflux and influx of Ca^{2+} described below, the radiolabelled cation was always used at a fixed, final concentration of 2 µCi/ml, containing 50 nmoles cold carrier Ca^{2+}. The final assay volumes were always 5 ml containing a total of 1 x 10^7 neutrophils. The standardisation of the procedures used to load the cells with $^{45}Ca^{2+}$, as well as a comparison with silicone oil-based methods for the separation of labelled neutrophils from unbound isotope, have been described elsewhere (Anderson and Goolam Mahomed, 1997).

4.2.7 *Efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils*

To measure net efflux of $^{45}Ca^{2+}$ from neutrophils uncomplicated by concomitant influx of the radiolabelled cation, the cells (1 x 10^7/ml) were loaded with $^{45}Ca^{2+}$ (2 µCi/ml) for 30 min at 37°C in HBSS. The neutrophils were then pelleted by centrifugation, washed once with, and resuspended in ice-cold Ca^{2+}-replete HBSS and held on ice until use, which was always within 10 min of completion of loading with $^{45}Ca^{2+}$. Using this procedure, the FMLP-activated fura-2 responses of neutrophils, similarly processed in HBSS containing 1 µM cold $CaCl_2$ followed by washing with, and suspension in Ca^{2+}-replete HBSS, did not differ from those of cells which had been maintained in Ca^{2+}-replete HBSS throughout, indicating that at the time of measurement of efflux in the $^{45}Ca^{2+}$ system there is no meaningful depletion of intracellular Ca^{2+} (Anderson and Goolam Mahomed, 1997). The $^{45}Ca^{2+}$-loaded neutrophils (2 x 10^6/ml) were then preincubated for 10 min at 37°C in Ca^{2+}-replete HBSS, followed by activation with FMLP (1 µM) and measurement of the kinetics (10, 30 and 60 sec) of net efflux of $^{45}Ca^{2+}$. FMLP was omitted from the corresponding control systems.

Reactions were stopped by the addition of 10 ml Ca^{2+}-replete HBSS to the tubes which were transferred immediately to an ice-bath. The cells were then pelleted by centrifugation at 400 g for 5 min followed by washing with 15 ml ice-cold Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml of triton X-100/0.1 M NaOH and the radioactivity assessed in a liquid scintillation spectrometer. Control, cell-free systems (HBSS and $^{45}Ca^{2+}$ only) were included for each experiment and these values were subtracted from the relevant neutrophil-containing systems. These results are presented as the amount of Ca^{2+} released from the cells during efflux (pmoles/10^7 cells).

4.2.8 Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils

To measure the net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were loaded with cold, Ca^{2+}-replete HBSS for 30 min at 37°C after which they were pelleted by centrifugation, then washed once with, and resuspended in ice-cold Ca^{2+}-free HBSS and held on ice until used. Pre-loading with cold Ca^{2+} was undertaken to minimise spontaneous uptake of $^{45}Ca^{2+}$ (unrelated to FMLP activation) in the influx assay. The efficiency of this loading procedure was demonstrated by measurement of the FMLP-activated fura-2 responses of the Ca^{2+}-loaded neutrophils, which were similar to those of neutrophils maintained in Ca^{2+}-replete HBSS. The Ca^{2+}-loaded neutrophils (2 x 10^6/ml) were then incubated for 10 min at 37°C in Ca^{2+}-free HBSS followed by simultaneous addition of FMLP and $^{45}Ca^{2+}$ (2 µCi/ml), or $^{45}Ca^{2+}$ only to control, unstimulated systems. The kinetics of influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils were then monitored over a 5 min period (at 10, 20, 30 and 60 sec and 2, 3 and 5 min after the addition of FMLP) and compared with those of influx of the radiolabelled cation into the identically-processed, unstimulated cells.

4.2.9 Elastase release

Neutrophil degranulation was measured according to the extent of release of the primary granule-derived enzyme, elastase. Neutrophils were preincubated at a concentration of 1 x 10^7/ml in HBSS for 10 min at 37°C. FMLP (0.1 µM) in combination with a sub-maximal concentration of cytochalasin B (1 µM) was then added to the cells, which were incubated for 15 min at 37°C. The tubes were then transferred to an ice bath, followed by centrifugation at 400g for 5 min to pellet the cells. The neutrophil-free supernatants were then decanted and assayed for elastase activity using a micro-modification of a standard colorimetric procedure (Beatty *et al,* 1982). Briefly, 125 µl of supernatant was added to the elastase substrate N-succinyl-L-alanyl-L-analyl-L-alanine-p-nitroanilide (3 mM in DMSO) in 0.05 M Tris-HCl (pH 8.0) and elastase activity monitored at a wavelength of 405 nm.

In an additional series of experiments the following were also investigated i) the effects of rolipram (1 µM final) on the release of elastase from control and CGD neutrophils and ii) a comparison of elastase release from control and CGD neutrophils using sub-maximal and maximal combinations of FMLP with CB (0.1 µM/1 µM and 1 µM/10 µM).

Because elastase has been reported to be functionally inactivated by very high concentrations of reactive oxidants (Vissers and Winterbourn, 1987) additional experiments were performed to control for possible over-estimation of elastase activity in supernatant fluids from stimulated CGD neutrophils. Hydrogen peroxide at final concentrations of 50, 100, 500 and 1000 µM or an equal volume of HBSS (50 µl) were added to 950 µl of supernatant fluid from FMLP-activated control and CGD (DT and RS) neutrophils which was then assayed for elastase activity following 15 min of incubation at 37°C. Maximally stimulated neutrophils have been reported to generate 100 nanomoles $H_2O_2/10^6$ cells over a 30 min incubation period (Test and Weiss, 1984).

4.2.10 Phospholipase A_2 activity

This was measured using a radiometric thin layer chromatography procedure (Bradova *et al.*, 1990). Neutrophils ($1x10^7$/ml) were coincubated with 5 µCi/ml radiolabelled arachidonate [5,6,8,9,11,12,14,15-^{3}H(N), 185 Ci/mmol, Du Pont NEN] for 15 min at 37°C in Ca^{2+}-free HBSS containing 5 µM indomethacin, to allow incorporation of radiolabelled arachidonate into membrane phospholipids. The cells were then washed twice and resuspended to $1x10^7$/ml in Ca^{2+}-replete HBSS. The cells ($2.5x10^6$/ml) were then preincubated for 10 min at 37°C prior to the addition of FMLP (1 µM final) in a final volume of 2 ml. This was followed by a 3 min incubation at 37°C (predetermined in preliminary kinetics experiments) after which the reactions were terminated and ^{3}H-arachidonate extracted by the addition of 5 ml n-hexane/ isopropanol/HCl (300:200:4 vol/vol/vol) and thorough mixing. The upper organic phase was removed and evaporated to dryness under a nitrogen stream. The lipids were reconstituted in 40 µl hexane/isopropanol and spotted onto silica gel pre-coated TLC plates (Merck, Darmstadt, Germany) together with 2 µM unlabeled arachidonate standard to facilitate detection. The plates were developed in chloroform/acetone (96:4 vol/vol) and then exposed to iodine vapours. The arachidonate spots were localised, excised and assayed for radioactivity.

CGD Patients

Two related patients (first cousins) aged 12 (JS) and 15 years (DT) at the time of the first visit respectively, with X-linked CGD (C-668->T mutation resulting in an Arg-226-Stop in $gp91^{phox}$) were each investigated on three different occasions over a 16 month period. In the case of Patient JS the values for serum C-reactive protein (CRP) on the three consecutive visits were 8.0, 7.5 and 11.7 µg/ml, while for Patient DT these were 40.0, < 3.0 and < 3.0 µg/ml. Two additional patients, brother (DE) and sister (RS) aged 24 and 28 years respectively, with the autosomal recessive form of CGD, were investigated on one and four occasions

respectively, on each of which the CRP values were < 3.0 μg/ml. The molecular abnormality of Patient RS was characterised as a GT deletion in Exon 2 (deficiency of $p47^{phox}$).

4.2.11 Statistical analysis

The results of each series of experiments are expressed as the mean ± standard error of the mean (SEM). Levels of statistical significance were calculated using the Students *t*-test when 2 groups were compared, or by analysis of variance with a subsequent Tukey-Kramer multiple comparisons test for multiple groups.

4.3 Superoxide production

Superoxide production by activated neutrophils from control and CGD subjects is shown in Table 4.1. The lucigenin-enhanced chemiluminescence responses of FMLP- and PMA-activated CGD neutrophils were virtually undetectable in comparison to those of cells from control subjects.

Table 4.1. FMLP- and PMA-activated lucigenin-enhanced chemiluminescence (LECL) responses of neutrophils from control and CGD subjects.

	LECL responses of neutrophils			
	Without FMLP	**With FMLP**	**Without PMA**	**With PMA**
Control (n = 4)	578 ± 55	2129 ± 409	623 ± 94	4505 ± 488
CGD (n = 4)	15 ± 2	38 ± 14	19 ± 4	38 ± 14

The results are expressed as the mean peak LECL values in $mV.s^{-1}$ ± SEM.

4.4 Membrane potential

FMLP-activated alterations in membrane potential in control and CGD neutrophils are shown in Figure 4.1 (page 106).

Exposure of control neutrophils to FMLP resulted in rapid membrane depolarisation (84 ± 4 mV) which terminated at around 30 sec and was followed 60 – 90 sec later by membrane repolarisation which was complete at 5 min, but which did not recover to pre-activation values. In contrast, the FMLP-activated decrease in membrane potential in CGD neutrophils was trivial (8 ± 2 mV; average of 9 – 10% of the control response), with variable time taken for complete repolarisation.

4.5 Intracellular calcium fluxes

The results shown in Figure 4.2 (page 107) are typical traces of the FMLP-activated fura-2 responses of control and CGD neutrophils. Addition of FMLP to neutrophils was accompanied by the characteristic, abrupt increase in fura-2 fluorescence due to an increase in the cytosolic concentrations of Ca^{2+}, the magnitude of which was similar in control and CGD neutrophils. In the case of control neutrophils, attainment of peak fluorescence was accompanied by a rapid decline in fluorescence intensity which was delayed in CGD neutrophils, indicative of impaired clearance of Ca^{2+} from the cytosol.

Peak increments in cytosolic Ca^{2+} concentrations following addition of FMLP to control and CGD neutrophils, time taken to initiation of the abrupt linear decline in fluorescence intensity and the rate of Ca^{2+} clearance during this phase, are shown in Table 4.2 (page 108) for a larger series of experiments including repeated measurements on Patients JS, DT and RS.

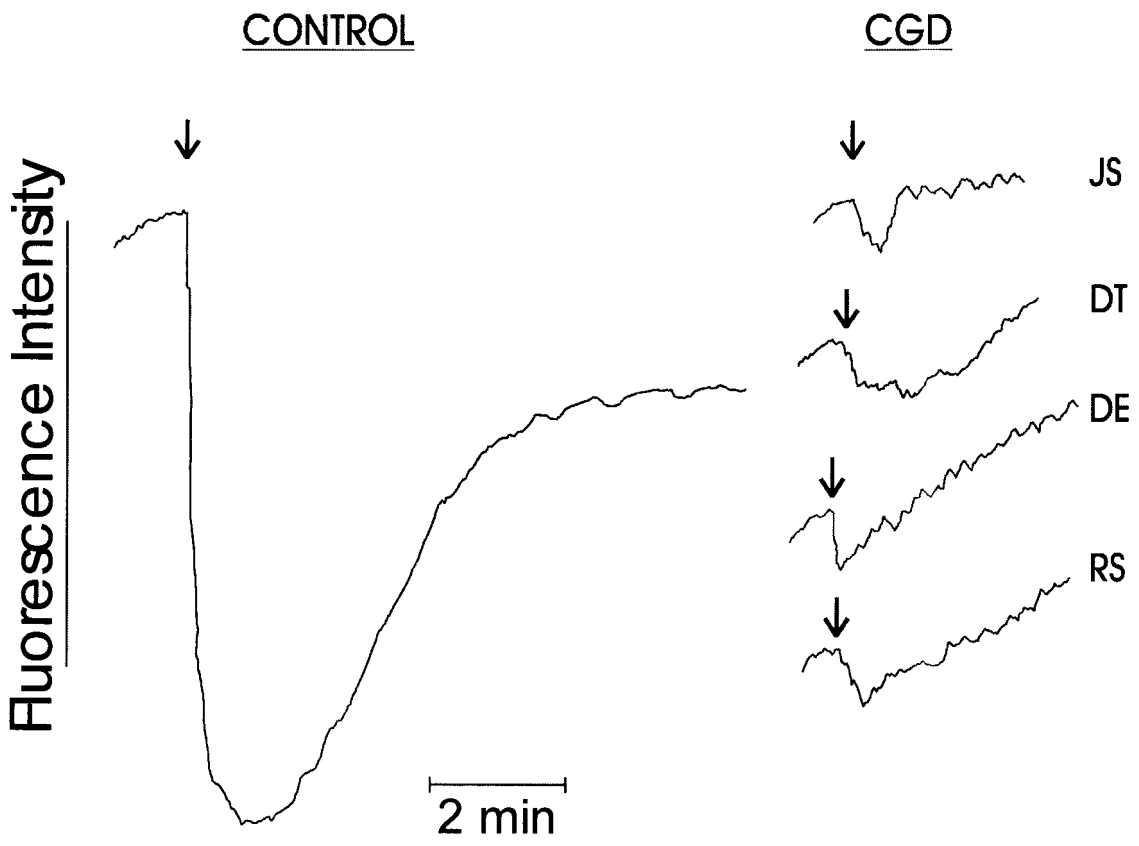


Figure 4.1: FMLP (1 μM) activated alterations in the membrane potential of neutrophils from one typical control subject and from 4 different patients with CGD (JS, DT, DE, RS). FMLP was added as indicated (↓) approximately 1 min after transfer of neutrophils to the reaction cuvettes. The assays were repeated on 3 and 2 visits several months apart on patients JS and RS respectively and similar results were obtained on each occasion. All traces are on the same scale.

There were no significant differences between control and CGD neutrophils with respect to basal and peak concentrations of cytosolic Ca^{2+}, as well as the rate of clearance of cytosolic cation. However, the time taken to initiation of the abrupt decline in peak fluorescence intensity was significantly ($p < 0.0001$) longer in CGD neutrophils.

The effects of rolipram on the fura-2 responses of control and CGD neutrophils are also shown in Figure 4.2 (page 107).

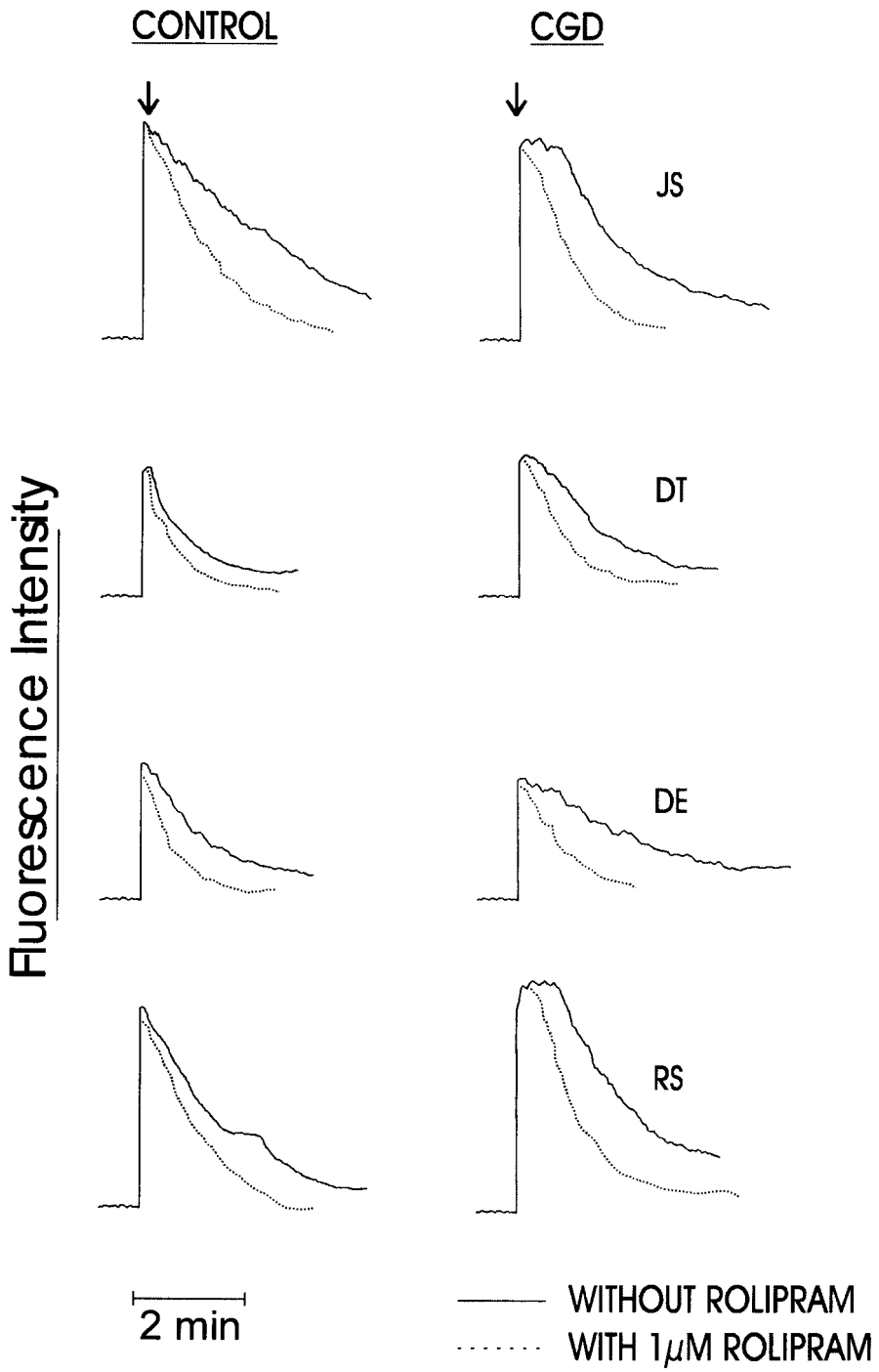


Figure 4.2: FMLP (1 μM)-activated fura-2 fluorescence responses of neutrophils from 4 different control and CGD subjects (JS, DT, DE, RS). Neutrophils from each control subject were paired with those of a CGD patient as shown. FMLP was added when a stable base-line was obtained (± 1 min). The assays were repeated on 3, 3 and 4 visits several months apart on patients JS, DT and RS respectively and similar results were obtained on each occasion.

Table 4.2. Peak increments in cytosolic Ca^{2+} concentrations, time taken to onset of clearance and rates of clearance of the cation in FMLP-activated control and CGD neutrophils.

	Peak increments in cytosolic Ca^{2+} (pmol) ◆	**Time taken (sec) to onset of clearance of cytosolic Ca^{2+}**	**Clearance rate of cytosolic Ca^{2+} (pmol/min)**
Control neutrophils (n = 9)	282 ± 28	7 ± 1	93 ± 9
CGD neutrophils (n = 14)	276 ± 21	43 ± 2*	86 ± 6

The results are expressed as the mean values ± SEM. The basal cytosolic concentrations for unstimulated control and CGD neutrophils were 105 ± 62 and 89 ± 31 pmol/6 x 10^6 cells respectively. ◆Peak increments in cytosolic Ca^{2+} were adjusted for basal values. *$p < 0.001$ for comparison between control and CGD neutrophils.

Addition of this agent to both normal and CGD cells did not affect either the basal levels of fluorescence in resting neutrophils or the peak fluorescence intensity following addition of FMLP. However, the rates of clearance of cytosolic Ca^{2+} were significantly faster in rolipram-treated cells. In normal cells the rates of Ca^{2+} clearance in the absence and presence of rolipram were 93.4 ± 8.8 and 156.6 ± 12.1 pmol/min ($p < 0.001$) respectively, while the corresponding values for CGD cells were 86.3 ± 5.6 and 160.6 ± 5.3 pmol/min ($p < 0.001$). Rolipram also significantly decreased the time taken to initiation of the abrupt linear decline in peak fluorescence intensity in FMLP-activated CGD cells (43 ± 2 sec versus 6.6 ± 1 sec, $p < 0.001$, in the absence and presence of rolipram), the value for rolipram-treated CGD cells being similar to that for stimulated control cells in the absence of rolipram (7 ± 1 sec).

4.6 Influx of Ca^{2+} using Mn^{2+} quenching of fura-2 fluorescence

The results of the indirect measurement of Ca^{2+} influx into FMLP-activated control and CGD neutrophils using Mn^{2+} quenching of fura-2 fluorescence are shown in Figure 4.3 (page 110).

In control cells the decrease in fluorescence intensity was delayed for about 30 sec after addition of FMLP, followed by an almost linear decrease over 3 – 4 min. In the case of CGD cells, the decrease in fluorescence intensity occurred at around 30 sec after the addition of FMLP, but proceeded at almost double the rate to that observed in control cells ($p < 0.001$) over the initial 2 min period of the time course (data from 6 repeat measurements on control cells and cells from Patient DT). Similar differences were observed using cells from Patient RS (not shown).

4.7 Efflux of $^{45}Ca^{2+}$

In these experiments, control and CGD neutrophils which had been preloaded with $^{45}Ca^{2+}$ and then washed and transferred to Ca^{2+}-replete HBSS (to minimise re-uptake of radiolabelled cation), were activated with FMLP followed by measurement of the amount of cell-associated $^{45}Ca^{2+}$. Exposure of both control and CGD neutrophils to FMLP resulted in an abrupt efflux of the radiolabelled cation which terminated approximately 30 sec after addition of the stimulant, resulting in the loss of about 40% of cell-associated $^{45}Ca^{2+}$. The rates and extent of efflux of $^{45}Ca^{2+}$ from FMLP-activated control and CGD neutrophils were not significantly different. The amounts of cation discharged from FMLP-activated control and CGD neutrophils 30 sec after activation were 157 ± 17 and 160 ± 14 pmol $Ca^{2+}/10^7$ cells respectively.

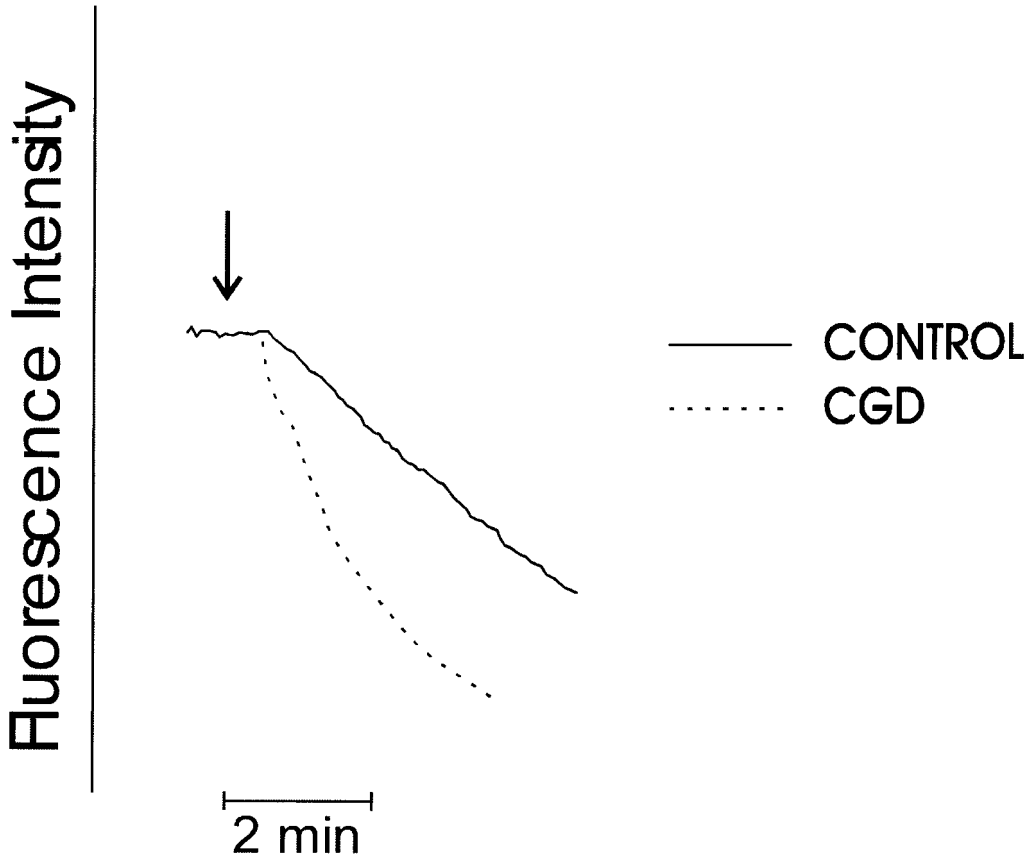


Figure 4.3: FMLP (1 μM)-activated Mn^{2+} quenching of the fura-2 responses of control and CGD (DT) neutrophils. FMLP was added as indicated (↓) and the results shown are typical traces of 6 replicates.

4.8 Influx of $^{45}Ca^{2+}$

For these experiments control and CGD neutrophils were preloaded with cold Ca^{2+}, then transferred to Ca^{2+}-free HBSS prior to activation with FMLP, which was added simultaneously with $^{45}Ca^{2+}$. This step (loading with cold Ca^{2+}) was undertaken to minimise spontaneous uptake of $^{45}Ca^{2+}$ by neutrophils (Anderson *et al*, 1998). The results of these experiments, which were designed to measure net influx of $^{45}Ca^{2+}$ into FMLP-activated control and CGD neutrophils, are shown in Table 4.3 (page 111).

Activation of control neutrophils with FMLP under these experimental conditions resulted in a delayed uptake of $^{45}Ca^{2+}$ which was detectable after a lag phase of about 30 – 60 sec and continued to 5 min after addition of the stimulus. The mean time taken for detection of influx of $^{45}Ca^{2+}$ was shorter, but not significantly so, for FMLP-activated CGD cells relative to control cells (20 sec and 30 sec respectively). However, in the case of FMLP-activated CGD neutrophils, influx proceeded at a significantly faster rate over the first 3 min of the time course of the experiment and was complete at 3 min after addition of the stimulus, in comparison with 5 min, in the case of control cells. Influx of $^{45}Ca^{2+}$ was a true consequence of activation of neutrophils with FMLP, since there was only trivial influx of the radiolabelled cation over the same time course into control identically processed neutrophils which had not been exposed to FMLP.

Table 4.3. Kinetics of influx of $^{45}Ca^{2+}$ into FMLP-activated control and CGD neutrophils.

	Influx of Ca^{2+} into FMLP-activated neutrophils (pmol cell-associated $Ca^{2+}/10^7$ neutrophils)	
Time after addition	**Control neutrophils (n = 6)**	**CGD neutrophils (n = 6)**
10 sec	0	1 ± 1
20 sec	0	18 ± 10
30 sec	4 ± 4	34 ± 16
60 sec	16 ± 8	54 ± 9*
2 min	56 ± 9	107 ± 18*
3 min	109 ± 13	166 ± 13*
5 min	156 ± 16	167 ± 15

The results are expressed as the mean values for 6 different control subjects and the 4 different CGD patients with repeat evaluations performed on DT and RS. *$p < 0.05$ – $p < 0.001$ for comparison between the uptake of $^{45}Ca^{2+}$ by control and CGD cells at the corresponding times. The amount of influx of Ca^{2+} into unstimulated control and CGD neutrophils at 5 min was 17 ± 6 and 27 ± 4 pmol/10^7 cells respectively.

4.9 Elastase release

Elastase release from FMLP/CB-activated control and CGD neutrophils is shown in Figure 4.4 (page 113).

Activation of neutrophils from all 4 CGD patients on each occasion tested, resulted in a significantly higher release of elastase than that observed with control neutrophils. In a single experiment designed to investigate the effects of increasing the potency of the activator of degranulation, the differential release of elastase from CGD neutrophils from a single subject (Patient JS), relative to that of control cells, appeared to be independent of the concentration of the stimulus. The release of elastase from control and CGD neutrophils activated with 0.1 μM FMLP/1 μM CB was 407 ± 8 and 552 ± 21 milliunits elastase/10^7 cells respectively ($p < 0.001$), while the corresponding values for cells activated with 1 μM FMLP/10 μM CB were 1282 ± 38 and 1988 ± 58 milliunits elastase ($p < 0.001$). The background values for unstimulated control and CGD neutrophils were 15 ± 1 and 34 ± 3 milliunits elastase respectively.

The effects of rolipram on the release of elastase from FMLP/CB activated control and CGD neutrophils are shown in Table 4.4 (page 114). The PDE 4 inhibitor significantly reduced the release of elastase from both control and CGD neutrophils

Coincubation of the cell-free supernatants from FMLP/CB activated control and CGD neutrophils with added H_2O_2 (50 – 1000 μM) for 15 min at 37°C did not inhibit, but rather modestly increased, the functional reactivity of elastase. In systems exposed to H_2O_2 the activities of elastase (milliunits/10^7 cells) in the supernatant of control neutrophils without and with H_2O_2, (1000 μM) were 173 ± 10 and 216 ± 2 respectively, while the corresponding values for CGD cells

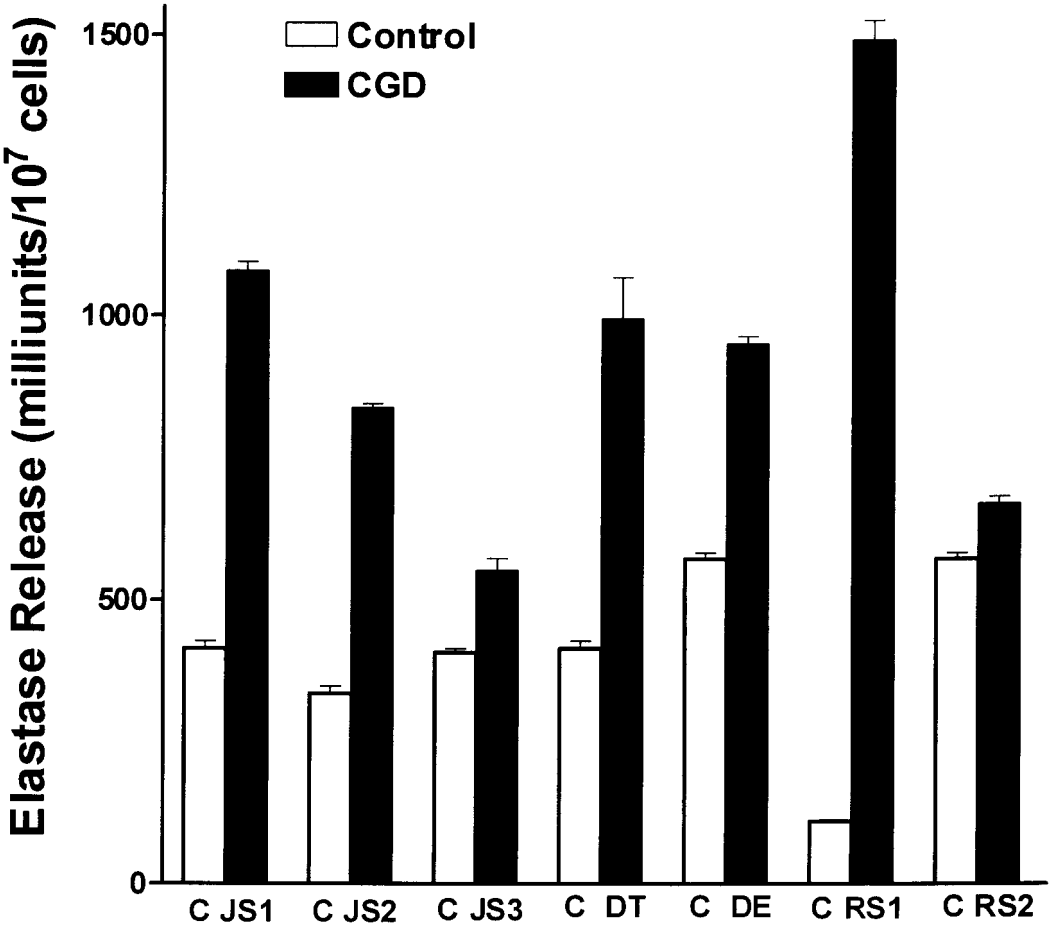


Figure 4.4: FMLP/CB (0.1 μM/1 μM)-activated release of elastase from control and CGD (JS, DT, DE, RS) neutrophils. The paired responses of control and CGD cells are expressed as the mean amount of elastase released in milliunits enzyme/10^7 cells with 3-6 replicates for each value. CGD patient JS was evaluated on 3 separate occasions several months apart (JS1, JS2, JS3), patient RS on two occasions (RS1, RS2) and the others (DT, DE) on one occasion. The range of values for the spontaneous release of elastase from unstimulated neutrophils from control and CGD subjects (all subjects on all occasions tested) was 15-36 ($\bar{x}$ = 29) and 31-41 ($\bar{x}$ = 35) milliunits elastase/10^7 cells respectively.
The level of statistical significance for comparison of combined values for elastase release from FMLP/CB-activated control and CGD neutrophils was $p<0.0001$.

(Patient DT) were 388 ± 10 and 469 ± 10. In a second experiment the corresponding values for control neutrophils were 174 ± 10 and 227 ± 3, while those for CGD cells (Patient RS) were 296 ± 2 and 322 ± 3. In a series of control experiments it was found that H_2O_2 (1000 μM) did not affect the assay system for detection of elastase activity.

Table 4.4. Effects of rolipram on the release of elastase from FMLP/CB-activated control and CGD neutrophils.

	Release of elastase from FMLP/CB-activated neutrophils	
	Without rolipram	**With rolipram (1 μM)**
Control neutrophils (n = 3)	1076 ± 228	536 ± 104*
CGD neutrophils (n = 4)	2828 ± 196	352 ± 4**

The results are expressed as the mean values ± SEM in milliunits enzyme/10^7 cells and the assay was performed in triplicate for each subject. The values for unstimulated neutrophils from control and CGD subjects were 39 ± 4 and 41 ± 5 milliunits enzyme/10^7 cells respectively.
*$p < 0.002$; **$p < 0.0002$

4.10 Effects of staurosporine on superoxide production and membrane depolarisation in activated neutrophils

The significant alterations in Ca^{2+} homeostasis in CGD neutrophils observed in the above experiments, highlight the important role of membrane depolarisation in regulating Ca^{2+} influx. If these alterations in Ca^{2+} homeostasis could be repeated in other experimental systems which mimic CGD, this would further support the interdependence of alterations in membrane potential and calcium influx in controlling the pro-inflammatory activities of neutrophils.

Additional experiments were performed using normal neutrophils treated with staurosporine (200 nM), a protein kinase C inhibitor (Nigam *et al*, 1995), to mimic the CGD phenotype. Protein kinase C is an important enzyme in the signal transduction pathways leading to NADPH oxidase activation (Arcaro and Wymann, 1993). Diphenylene iodonium sulphate (DPI) was not used as this agent has been shown to exert other non-specific effects on membrane ion channels in addition to inhibition of NADPH oxidase (Wyatt *et al*, 1994).

The results of experiments to assess the effects of staurosporine on superoxide generation by FMLP-activated neutrophils are shown in Table 4.5 (page 116), together with the effects of staurosporine on membrane depolarisation in FMLP-activated neutrophils.

Staurosporine significantly inhibited superoxide generation by FMLP-activated neutrophils with a corresponding attenuation of the membrane depolarisation response, supporting the inter-dependence of these two events.

The results shown in Figure 4.5 (page 116) are traces from typical experiments using cells from different donors, depicting the effect of staurosporine (200 nM) on the fura-2 fluorescence responses of FMLP-activated neutrophils.

Pre-incubation of neutrophils with staurosporine did not affect the initial abrupt increase in fura-2 fluorescence, which accompanies activation of the cells with FMLP. This was followed by a rapid decline in fluorescence intensity in control neutrophils, which was significantly delayed in staurosporine-treated cells.

Table 4.5. Effects of staurosporine (200 nM) on superoxide production and membrane depolarisation in FMLP-activated neutrophils.

	Superoxide production (% control)	**Membrane depolarisation (% control)**
Staurosporine	4 ± 0.4*	52 ± 9*

The results of 7 – 14 experiments are expressed as the mean percentage of the inhibitor-free, FMLP-activated control system ± SEM. The absolute peak value for superoxide production by FMLP-activated neutrophils was 870 ± 125 mV.s-1. The magnitude of membrane depolarisation for control cells was 40 ± 3 mV. *p < 0.05 for comparison with the inhibitor-free control system.

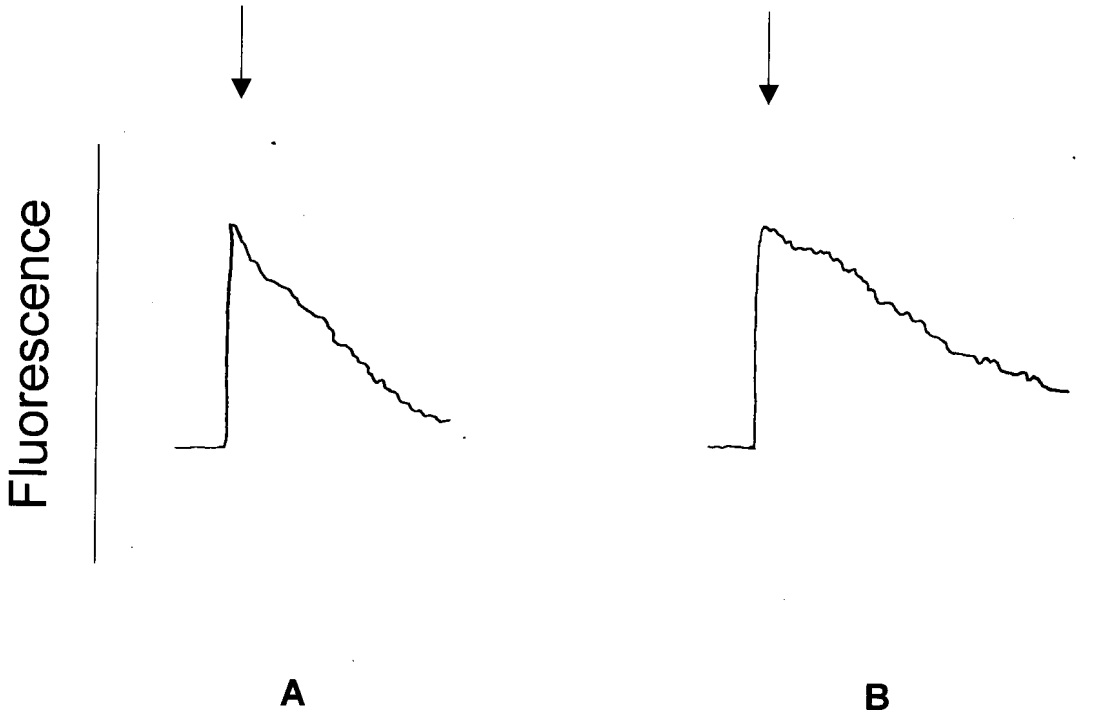


Figure 4.5: The effects of staurosporine (200 nM) on the time course of the fura-2 fluorescence responses of FMLP-activated neutrophils. Typical tracings from 6 experiments with control (A) and staurosporine-treated (B) neutrophils are shown. FMLP was added as indicated (↓) when a stable base-line was obtained (± 1 min).

The effects of staurosporine on the peak cytosolic Ca^{2+} concentrations $[Ca^{2+}]_i$, the time taken for fluorescence intensity to decline to half peak values (t½), as well as the clearance rates of free cytosolic Ca^{2+} following activation with FMLP, are shown in Table 4.6.

Table 4.6. Effects of staurosporine on the peak cytosolic $[Ca^{2+}]i$, time taken for the cytosolic $[Ca^{2+}]i$ to reach half peak values (t½), rate of clearance of cytosolic Ca^{2+} and release of elastase from activated neutrophils.

FMLP-mediated responses in the presence of staurosporine (200 nM)	**Control**	**Staurosporine**
Peak cytosolic $[Ca^{2+}]i$ (nM)	335 ± 6	335 ± 6
Time taken to reach half peak values (min)	1.9 ± 0.1	3.0 ± 0.2*
Clearance rate of cytosolic Ca^{2+} (pmol/min)	91 ± 2	56 ± 4*
Elastase release (milliunits enzyme/10^7 cells)	1812 ± 116	2415 ± 31*

The results of 4 – 14 experiments are expressed as the mean value ± SEM. *$p < 0.005$ for comparison with control untreated cells.

Staurosporine did not alter the peak fura-2 fluorescence intensity, but significantly increased the time taken to decline to half peak values, with an associated marked retardation of the rate of Ca^{2+} clearance from the cytosol, paralleling the alterations observed in CGD neutrophils. Elastase degranulation was significantly potentiated (133% of control cells), in response to Ca^{2+} flooding and hyperactivation of the cells analogous to the responses observed with CGD.
Although the use of staurosporine does not perfectly replicate the CGD phenotype, the striking similarities observed with staurosporine support the deductions made in respect of the functional inter-dependence of NADPH oxidase activity, membrane depolarisation and Ca^{2+} influx in neutrophils.

4.11 Discussion

Although the relationship between activation of granulocyte membrane-associated oxidative metabolism and alterations in membrane potential is well-recognised, the functional consequences of attenuated depolarisation secondary to the absence of NADPH-oxidase in phagocytes from CGD subjects remain unknown (Matsuura *et al*, 1984). Importantly, the abruptly-occurring depolarisation which accompanies activation of various types of inflammatory cell, including basophils, mast cells and neutrophils, has been shown to limit the influx of extracellular Ca^{2+} (Di Virgilio *et al*, 1987; Mohr and Fewtrell, 1987; Penner *et al*, 1988). It has been proposed that when the cells are depolarised, the driving force for entry of Ca^{2+} is markedly reduced because the electrical component of the electrochemical gradient for Ca^{2+} is abolished (Mohr and Fewtrell, 1987; Penner *et al*, 1988). Carefully regulated influx of Ca^{2+} during recovery of membrane potential may facilitate diversion of incoming cation into stores, thereby preventing flooding of the cytosol with Ca^{2+} and possible hyperactivation of the cells. In the current study, the relationship between NADPH-oxidase dependent alterations in membrane potential and maintenance of Ca^{2+} homeostasis has been investigated using FMLP-activated neutrophils from control and CGD subjects. Neutrophils from all four CGD subjects (two with X-linked and two with autosomal recessive CGD) demonstrated markedly blunted, but not absent, membrane depolarisation responses following stimulation with FMLP and PMA.

Calcium fluxes in FMLP-activated control and CGD neutrophils were measured and compared using fura-2 spectrofluorimetry, together with radiometric procedures, allowing distinction between efflux and influx of Ca^{2+} and identification of the origins (extracellular or intracellular) of cytosolic Ca^{2+} (Anderson *et al*, 1997). Exposure of fura-2-loaded CGD neutrophils to FMLP was accompanied by an immediate increase in fluorescence intensity, which was of

similar magnitude to that observed in control cells, confirming that PLC/ITP_3-mediated mobilisation of intracellular Ca^{2+} is normal in CGD neutrophils (Lew *et al*, 1984; Geiszt *et al*, 1997). However, the decline in peak fluorescence intensity was delayed by up to 40 – 50 sec in CGD cells, indicative of impairment of the clearance of Ca^{2+} from the cytosol. This could not be attributed to defective efflux of Ca^{2+}, because the rate and extent of extrusion of the cation, as has been reported previously (Herlin and Borregaard, 1983), were similar in control and CGD cells, demonstrating that, although coincident, Ca^{2+} efflux and membrane depolarisation are not inter-dependent events in FMLP-activated neutrophils.

The observation that the early occurring efflux of Ca^{2+} is unimpaired in FMLP-activated CGD neutrophils, suggested that uncontrolled influx may be responsible for the sustained elevation of cytosolic Ca^{2+}. This contention was supported by data from the radiometric procedure which demonstrated accelerated influx of Ca^{2+} into FMLP-activated CGD neutrophils. Influx of the cation was detected earlier, proceeded at a faster rate, and terminated earlier in CGD cells, in comparison to control cells, which demonstrated the typical delayed, store-operated influx of the cation (Montero *et al*, 1991; Favre *et al*, 1996). The altered fura-2 responses observed in neutrophils from all four CGD subjects in the present study, differ from those reported by other authors who used either fura-2 (Geiszt *et al*, 1997), or quin-2 (Lew *et al*, 1984) as the Ca^{2+}-sensitive intracellular fluorescent dyes. Although Geiszt and colleagues, were unable to detect alterations in the fura-2 responses of FMLP-activated neutrophils, relative to those of control cells, they did, however, observe an immediate influx of Ca^{2+} into CGD cells, while uptake of the cation by control cells was detectable only after a lag period of 2 min. This differential influx of Ca^{2+} into control and CGD neutrophils was detected using an indirect Mn^{2+}/fura-2 fluorescence quenching procedure (Geiszt *et al*, 1997). The applicability of this procedure to the measurement of Ca^{2+} influx through store-operated Ca^{2+} channels has been questioned, largely because Mn^{2+} can enter cells via other channels and may itself interfere with store-operated regulatory mechanisms (Parekh and Penner, 1997). Nevertheless, the observation made by Geiszt *et al*

of accelerated influx of Ca^{2+}, in the setting of trivial membrane depolarisation in FMLP-activated CGD cells is clearly supported by the data from the present study, in which both radiometric and Mn^{2+}/fura-2 fluorescence quenching procedures were used to detect influx of Ca^{2+} into neutrophils. Geiszt and colleagues also excluded the alterations in intracellular pH which accompany activation in normal neutrophils, but which are absent in CGD cells (Segal *et al*, 2000), as being involved in the regulation of Ca^{2+} movements. Moreover, the fluorescence intensity of fura-2 is stable to pH 6,75 (Grynkiewicz *et al*, 1985).

Measurement of the release of elastase from activated CGD neutrophils, a Ca^{2+} dependent function (Sengeløv, 1996), suggested that dysregulation of Ca^{2+} influx results in altered pro-inflammatory functions of these cells. Increased activity of elastase, which could not be attributed to oxidative inactivation of the protease by H_2O_2, following exposure of CGD neutrophils to FMLP/CB, suggests that hyperactivity of this oxygen-independent function is consequent to disordered Ca^{2+} homeostasis in CGD neutrophils. A similar increased spontaneous and FMLP-stimulated PLA_2 activity and arachidonic acid release was also observed from neutrophils of these CGD subjects (Experiments by Dr HC Steel). In addition to the inability of CGD phagocytes to oxidatively inactivate mediators of inflammation including leukotrienes and chemoattractants (Segal *et al*, 2000), the mechanisms described in the current study may also contribute to poorly controlled inflammatory responses and granuloma formation in this disease. It is noteworthy in this respect, that neutrophil primary granules contain, in addition to elastase, chemoattractants for monocytes, as well as for $CD4^+$ and $CD8^+$ T-lymphocytes (Chertov *et al*, 1996; Taub *et al*, 1996). Alternatively, increased release of primary granule enzymes and enhanced phospholipase A_2 activity may partially compensate for the absence of oxidant-mediated antimicrobial activity in these cells (Kondo and Kanai, 1985; Belaauoaj *et al*, 1998). In several previous studies employing particulate stimuli and different markers of neutrophil granule release to those used in the present study, no enhancement of degranulation was reported (Baehner *et al*, 1969; Mandell and Hook, 1969), while

Voetman *et al*, (1981) reported that release of granule enzymes from activated CGD neutrophils was 2 – 3 fold greater than that from normal neutrophils.

The proposed relationship between sustained elevation of cytosolic Ca^{2+} and increased release of the primary granule enzyme elastase in activated CGD neutrophils was further investigated using the type 4 PDE inhibitor, rolipram. Rolipram accelerates the clearance of cytosolic Ca^{2+} by enhancing the activity of the endo-membrane Ca^{2+}ATPase, which down-regulates the pro-inflammatory activity of these cells (Anderson *et al*, 1998). In the current study, coincubation of CGD neutrophils, as well as control neutrophils with rolipram, resulted in accelerated clearance of Ca^{2+} from the cytosol following activation with FMLP, which was associated with a marked reduction in the release of elastase from these cells. Importantly, treatment with rolipram converted CGD neutrophils to a normal phenotype with respect to both Ca^{2+} clearance and degranulation. These observations suggest that rolipram, presumably by causing cAMP-dependent up-regulation of the activity of the endo-membrane Ca^{2+}-ATPase, can restore, albeit indirectly, Ca^{2+} homeostasis in activated CGD neutrophils. Second generation type 4 PDE inhibitors, which retain efficient PDE 4 inhibitory activity in the setting of attenuation of side effects (Torphy, 1998), may therefore be useful in the treatment of disordered inflammatory responses in CGD.

Two additional lines of evidence support the contention that altered Ca^{2+} influx and hyperactivation of the oxygen-independent pro-inflammatory functions of stimulated neutrophils occur in CGD. Firstly, the observations that treatment of normal neutrophils with the protein kinase inhibitor staurosporine (200 nM) converts normal neutrophils to a CGD-like phenotype characterised by decreased FMLP-activated superoxide production and membrane depolarisation, in the setting of prolonged Ca^{2+} transients and increased release of elastase, is in keeping with observations by previous investigators (Dewald *et al*, 1989; Wong *et al*, 1992). Ideally, one would have preferred to use diphenylene iodonium, a direct inhibitor of NADPH oxidase (Cross, 1990). However, it was found that this

agent, at concentrations which inhibit the phagocyte oxidase, also inhibits phospholipase A_2, as well as release of Ca^{2+} from intracellular stores in activated neutrophils (unpublished observations). Secondly, to exclude the possibility that the inability of CGD neutrophils to oxidatively inactivate FMLP (Clark *et al*, 1980), may explain the prolonged peak Ca^{2+} transients in activated neutrophils, the fura-2 responses of normal neutrophils activated with N-formyl-norleucyl-leucyl-phenylalanine (1 μM), an oxidation-insensitive chemotactic peptide (Clark *et al*, 1980), were measured. The fura-2 responses of normal neutrophils activated with this oxidation-insensitive chemotactic peptide were similar to those observed with FMLP, demonstrating that differences in the fura-2 responses of normal and CGD neutrophils are not attributable to differences in the abilities of these cells to inactivate FMLP.

In conclusion, failure of depolarisation in CGD neutrophils is associated with Ca^{2+} overload due to accelerated influx of the cation and hyperactivity of several pro-inflammatory activities of these cells.

CHAPTER 5

MECHANISMS MEDIATING ALTERATIONS IN THE MEMBRANE POTENTIAL OF HUMAN NEUTROPHILS AND THE RELATIONSHIP BETWEEN THESE AND THE REGULATION OF CALCIUM HOMEOSTASIS

5.1 Introduction

The plasma membrane of human neutrophils allows these cells to interact with other cells and respond to numerous external stimuli. Membrane receptors bind specific ligands leading to the activation of signal transduction pathways which regulate effector responses such as oxidant production and degranulation. In this regard, calcium is considered a key intracellular second messenger and activator of the pro-inflammatory activities of neutrophils, with the magnitude and duration of intracellular Ca^{2+} signals contributing significantly to activation of the superoxide-generating NADPH oxidase, concomitant with the mobilisation of cytosolic granules. Therefore, cytosolic calcium concentrations are tightly regulated in resting and activated neutrophils with stringent control mechanisms preventing Ca^{2+} overload and hyperactivation of the cells.

The reported mechanisms responsible for maintaining the resting membrane potential (RMP) of human neutrophils include voltage-activated K^+ channels (Krause *et al*, 1991), K^+ conductance and permeability across the membrane (Seligmann *et al,* 1980; Mottola *et al*, 1982; Myers *et al*, 1990), as well as the electrogenic membrane-associated Na^+/K^+-ATPase (Bashford and Pasternak, 1985). Passive proton conductance across the plasma membrane and down the electrochemical gradient (Banfi *et al*, 1999; Demaurex, 1993a) may theoretically contribute to the RMP as positively charged ions enter the cell. The membrane component (V_o) of the V-type ATPase may function as a proton channel in resting cells and is inhibited by bafilomycin (Zhang, 1994).

Additional theoretical, but unreported mechanisms which may contribute to maintaining the RMP include Cl^- ion fluxes and the activity of the electrogenic Na^+/Ca^{2+} exchanger at the outer membrane. Non-specific K^+ channels sensitive to the inhibitor tetraethylammonium (TEA) have been reported to contribute to the RMP across eosinophil cell membranes (Banfi *et al*, 1999) and may be operative in neutrophils.

Activation of NADPH oxidase causes an abrupt membrane depolarisation, followed after about 1 minute, by a slower phase of repolarisation. The mechanism mediating membrane depolarisation in activated neutrophils has been largely attributed to the vectorial flux of electrons across the outer membrane (Demaurex *et al*, 1993b), although transmembrane fluxes of Na^+, K^+, Ca^{2+} and Cl^- ions may also play a role during the depolarisation response. Proposed mechanisms mediating neutrophil membrane repolarisation have included altered membrane permeability to potassium ions (Seligmann *et al*, 1980) and activation of the Na^+/K^+-ATPase exchange mechanism (Majander and Wikström, 1989). Recently, a slowly-activatable H^+conductance that allows only H^+ extrusion, has received much attention as a mechanism mediating membrane repolarisation in eosinophils (Banfi *et al*, 2000). Alterations in the membrane potential of activated neutrophils may play an important role in regulating Ca^{2+} homeostasis. It has been reported that at depolarising potentials Ca^{2+} influx is abolished as the electrical component of the electrochemical gradient is unfavourable for Ca^{2+} influx (Di Virgilio *et al*, 1987; Parekh and Penner, 1997). Although the apparent involvement of plasma membrane depolarisation/ repolarisation is an attractive regulatory mechanism to control Ca^{2+} entry across the neutrophil plasma membrane, its exact involvement in the maintenance and/or restoration of Ca^{2+} homeostasis in these cells has not been conclusively established.

The aim of the current study was to determine the relationship between membrane potential and the regulation of calcium homeostasis in resting and activated neutrophils. I have also investigated the mechanistic relationships which may exist between alterations in membrane potential and cation (Ca^{2+}, K^+, Na^+) fluxes in chemoattractant-activated neutrophils.

5.2 Materials and Methods

5.2.1 Materials

KB-R7943 {2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothioureamethane sulfonate}, a selective inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger (Ca^{2+} influx, Na^+ efflux) was kindly provided by Dr K Yokata, Nippon Organon K.K., Japan, while all radiochemicals were purchased from DuPont NEN. Unless indicated all other chemicals and reagents were purchased from Sigma.

5.2.2 Neutrophils

Purified human neutrophils were prepared from heparinised venous blood (5 units of preservative-free heparin per ml of blood) from healthy adult volunteers. Neutrophils were separated from mononuclear leukocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) before sedimentation with 3% gelatin in order to remove most of the erythrocytes. Following centrifugation (280 g at 10°C for 10 min), residual erythrocytes were removed by selective lysis with 0.83% ammonium chloride at 4°C for 10 minutes. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to $1x10^7$/ml in PBS and held on ice until used.

A limited number of experiments were performed using neutrophils from two individuals, brother and sister ages 25 and 29 years respectively, with the autosomal recessive form of chronic granulomatous disease (CGD) (deficiency of $p47^{phox}$, GT deletion in exon 2).

5.2.3 *Membrane potential*

The potential sensitive fluorescent dye dipentyloxacarbocyanine (di-O-C_5(3)) was used to measure changes in membrane potential in activated neutrophils. The cells ($1x10^6$/ml) were pre-incubated for 10 min at 37°C in indicator-free Hanks balanced salt solution (HBSS, pH 7.4) containing 80 nM (final) di-O-C_5(3), after which they were transferred to disposable reaction cuvettes and held at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 460 nm and 510 nm, respectively. The neutrophils were then activated with the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 µM final) and the subsequent alterations in fluorescence intensity monitored over a 5-10 min period. The final volume in each cuvette was 3 ml containing a total of $3x10^6$ neutrophils. This procedure was used to determine the effects of the agents shown in Table 5.1 on the membrane potential of resting and FMLP-activated neutrophils.

Table 5.1 Final concentrations, supplier, solubility and mode of action of the agents used in experiments designed to investigate the mechanisms which regulate membrane potential in resting and FMLP-activated neutrophils.

Agent	Supplier	Solubility	Mode of Action
Ouabain (50 – 100 µM)	Sigma	DMSO	Selective Na^+/K^+-ATPase inhibitor
*[1]KB-R7943 (2.5 – 10 µM)	Nippon Organon K.K.	DMSO	Selective inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger
*[2]Benzamil (200 µM)	Sigma	H_2O	Non-selective inhibitor of the Na^+/Ca^{2+} exchanger
Potassium chloride (25 – 100 mM)	MERCK	H_2O	Increases the extracellular $[K^+]$

Bafilomycin (100 nM)	Sigma	DMSO	Selective inhibitor of the vacuolar-type ATPases
Diethylpyrocarbonate (DEPC) (12 μM)	Sigma	DMSO	Selective inhibitor of the vacuolar-type ATPases
Valinomycin (10 μM)	ICN	DMSO	Potassium ionophore
Staurosporine (200 nM)	Sigma	DMSO	Selective protein kinase C inhibitor
Diphenylene iodonium (DPI) (10 μM)	Tocris	DMSO	Selective NADPH oxidase inhibitor
Wortmannin (100 nM)	Sigma	DMSO	Selective inhibitor of phosphatidylinositol 3-kinase
Ethacrynic acid (100 – 200 μM)	ICN	DMSO	Chloride channel blocker
5-nitro-2-(3phenylpropylamino)benzoic acid (NPPB) (3 μM)	Tocris	DMSO	Chloride channel blocker
Herbimycin (2 μM)	Sigma	DMSO	Inhibitor of tyrosine kinases
Iberiotoxin (1 μg/ml)	Tocris	H_2O	Inhibitor of Ca^{2+}-activated K^+ channels
Glibenclamide (3 μM)	Tocris	Ethanol	Inhibitor of ATP-dependent K^+ channels
Amiloride (1 μM)	Sigma	H_2O	Inhibitor of Na^+/H^+ exchange
*[3]SK&F96365 (10 μM)	Sigma	DMSO	Selective inhibitor of store-regulated Ca^{2+} channels
Ethylene glycol-bis(beta-amino-ethyl-ether)-N,N,N',N'-tetraacetic acid (EGTA) (100 μM – 10 mM)	Sigma	H_2O	Extracellular Ca^{2+}-chelating agent
Zinc chloride (100 – 250 μM)	MERCK	H_2O	Inhibitor of membrane proton conductance
Platelet activating factor (PAF) (0.01 μg/ml)	Sigma	DMSO	Biologically active phospholipid
Tetra-ethylammonium chloride (10 mM)	Sigma	H_2O	Non-selective inhibitor of potassium channels

*[1]: {2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea methanesulfonate}

*[2]: (N-[benzylamidino]-3,5-diamino-6-chloropyrazinecarboxamide

*[3]: 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1-H-imidazole hydrochloride

In a further series of experiments, the effects of a lower concentration of EGTA (100 μM) on the membrane potential of resting and FMLP-stimulated cells suspended in nominally Ca^{2+}-free (50 μM) HBSS were assessed with and without added $CaCl_2$ (1 mM, final) which was added to the cuvettes 5 min after FMLP during the maximal depolarisation phase.

Additional experiments were performed to investigate the effects of platelet activating factor (PAF) (0.01 μg/ml), with and without SK&F96365 (10 μM), on the membrane potential of resting neutrophils.

The plasma membrane plays an important role in the generation of ionic concentration gradients between the cell interior and the extracellular fluid. Significant concentration gradients exist for potassium (K^+), sodium (Na^+), chloride (Cl^-) and calcium (Ca^{2+}) ions. The intracellular Na^+ concentration $[Na^+]_i$ of human neutrophils has been variously reported as 13.5 mM (Jankowski and Grinstein, 1999), 20 mM (Krause *et al*, 1991), 25 mM (Simchowitz *et al*, 1982) and 26 mM (Ince, 1987). The neutrophil intracellular K^+ concentration $[K^+]_i$ may also vary from 120 mM (Krause *et al*, 1991; Simchowitz *et al*, 1982), 125 mM (Ince, 1987) or 138 mM (Jankowski and Grinstein, 1999). The chloride concentration inside the neutrophil $[Cl^-]_i$ is greater than most other cells at ~ 80 mM (Simchowitz and De Weer, 1986).

The extracellular fluid ion concentrations in Hanks Balanced Salt Solution are stable at 141 mM for Na^+ ions $[Na^+]_o$, 5.7 mM for K^+ ions $[K^+]_o$, 145 mM for Cl^- ions $[Cl^-]_o$ with a calcium ion concentration of 1.26 mM. It is thus evident that significant ion concentration gradients are maintained across the outer membrane of the cell. These ionic gradients in turn lead to the formation of a resting electrical potential difference across the plasma membrane according to the Goldmann constant field equation (Simchowitz and De Weer, 1986). Using this equation, the resting membrane potential (RMP) across the neutrophil plasma membrane can be calculated as follows:

$$RMP = -61\ \ell n\ \frac{K_P[K^+]_o + K_{Na}[Na^+]_o + Kcl[Cl^-]_i}{K_P[K^+]_i + K_{Na}[Na]_i + Kcl[Cl^-]_o}$$

Where

K_P (permeability coefficient of potassium) = 4×10^{-8} cm/s
K_{Na} (permeability coefficient of sodium) = 5×10^{-9} cm/s
K_{Cl} (permeability coefficient of chloride) = 4×10^{-9} cm/s
(Simchowitz and De Weer, 1986)

Assuming intracellular concentrations of K^+, Na^+ and Cl^- ions of 130 mM, 20 mM and 80 mM respectively, and with measured ion concentrations of K^+, Na^+ and Cl^- in Hank's balanced salt solution (HBSS) of 5.7 mM, 141 mM and 145 mM respectively, the resting membrane potential for neutrophils in HBSS can be calculated as –45 mV.

If the external $[K^+]$ is varied by adding potassium chloride to achieve final extracellular K^+ concentrations of 31 mM, 55.7 mM, 80.7 mM and 105.7 mM, then the resultant RMP for each concentration of extracellular K^+ can be calculated and used to calibrate the change in RMP that occurs under experimental conditions, expressed as mV/cm. Neutrophils treated with variable concentrations of potassium chloride in three separate experiments were used to calibrate a mean value of about 10mV/cm for alterations in membrane potential.

5.2.4 Superoxide generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg and Ferber, 1984). Neutrophils were pre-incubated for 15 min at room temperature and thereafter for 15 min at 37°C in 900 µl HBSS containing 0.2 mM lucigenin in the presence and absence of staurosporine (200 nM), diphenylene iodonium chloride (DPI)(10 µM),

wortmannin (100 nM), ethacrynic acid (100-200 µM), ouabain (50 µM) and herbimycin (2 µM). Spontaneous and FMLP (1 µM)-activated LECL responses were then recorded in an LKB Wallac 1251 chemiluminometer (Turku, Findland) after the addition of the stimulant (100 µl). LECL readings were integrated for 5 sec intervals and recorded as millivolts seconds^{-1} (mVs^{-1}).

5.2.5 *Spectrofluorimetric measurement of cytosolic Ca^{2+}*

Fura-2/AM was used as the fluorescent Ca^{2+}-sensitive indicator for these experiments. Neutrophils (1x10^7/ml) were preloaded with fura-2/AM (2 µM) for 30 min at 37°C in PBS, washed twice and resuspended in HBSS. The fura-2-loaded cells were then preincubated for 10 min at 37°C after which they were transferred to disposable reaction cuvettes which were maintained at 37°C in a Hitachi 650 10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 nm and 500 nm respectively. After a stable base line was obtained (1 min), the neutrophils were activated by addition of FMLP (1 µM) and the subsequent increase in fura-2 fluorescence intensity was monitored over a 5 min period. The final volume in each cuvette was 3 ml containing 6x10^6 neutrophils and the data was used to calculate cytosolic Ca^{2+} concentrations, as previously described (Grynkiewicz *et al*, 1985). This procedure was used to investigate fluctuations in cytosolic Ca^{2+} concentrations following activation of neutrophils with FMLP in the presence and absence of KCl (25-100 mM), KB-R7943 (2.5-10 µM), or SKF 96365 (10 µM), as well as the effects of PAF (0.01 µg/ml) on cytosolic Ca^{2+} concentrations in resting cells.

5.2.6 *Mn^{2+} quenching of fura-2 fluorescence*

Cells loaded with fura-2 as described above were activated with FMLP (1 µM) in HBSS containing 300 µM $MnCl_2$ (added 5 min prior to FMLP) and fluorescence quenching as a measure of Ca^{2+} influx was monitored at an excitation wavelength of 360 nm, which is an isosbestic wavelength, and at an emission

wavelength of 500 nm (Geiszt *et al*, 1997). This procedure was used to investigate the effects of KB-R7943 (2.5-10 μM) and SK&F96365 (10 μM) on FMLP-activated influx of Ca^{2+}. The effect of PAF (0.01 μg/ml) on Ca^{2+} influx was also investigated using this procedure, as well as the effect of KB-R7943 (5 μM) on FMLP-activated Ca^{2+} influx into CGD neutrophils.

5.2.7 Radiometric assessment of Ca^{2+} fluxes

$^{45}Ca^{2+}$ (calcium-45 chloride, specific activity 28.81 mCi/mg) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and FMLP-activated neutrophils. The standardisation of the procedures used to load the cells with $^{45}Ca^{2+}$, as well as a comparison with silicone oil-based methods for the separation of labelled neutrophils from unbound isotope, has been described elsewhere (Anderson and Goolam Mahomed, 1997).

In the first series of experiments, designed to compare transmembrane fluxes of $^{45}Ca^{2+}$ with the fura-2 responses and alterations in membrane potential of FMLP-activated neutrophils from 6 different healthy, adult control subjects, neutrophils ($2x10^6$/ml) were resuspended and equilibrated for 15 min at 37°C in HBSS (final volume, 5 ml) containing $^{45}Ca^{2+}$ (2 μCi/ml) and 50 nmol cold carrier $CaCl_2$ as the sole source of Ca^{2+}. The amount of cell-associated $^{45}Ca^{2+}$ was then measured immediately prior to, and at 10, 20 and 30 sec, as well as 1, 1.5, 2, 3 and 5 min after the addition of FMLP (1 μM). Reactions were stopped by the addition of 10 ml ice-cold Ca^{2+}-replete HBSS to the tubes, which were transferred to an ice bath. The cells were then pelleted by centrifugation, followed by washing with 15 ml ice-cold HBSS, and the cell pellets finally dissolved in 0.5 ml of 0.5% triton X-100/0.1 M NaOH and the radioactivity was assessed in a liquid scintillation spectrometer. These results are presented as the amount of cell-associated radiolabelled cation (pmoles of $^{45}Ca^{2+}$).

5.2.8 *Efflux of $^{45}Ca^{2+}$ from radiolabelled neutrophils*

To measure net efflux of $^{45}Ca^{2+}$ from neutrophils uncomplicated by concomitant influx of the radiolabelled cation, the cells (10^7/ml) were loaded with $^{45}Ca^{2+}$ (2 µCi/ml) for 20 min at 37°C in Ca^{2+}-free HBSS. The neutrophils were then pelleted by centrifugation, washed once with and resuspended in Ca^{2+}-replete HBSS, and held on ice until use, which was within 15-20 min of loading with $^{45}Ca^{2+}$. The $^{45}Ca^{2+}$-loaded neutrophils were then preincubated for 10 min at 37°C in Ca^{2+}-replete HBSS followed by activation with FMLP (1 µM) and measurement of the efflux of $^{45}Ca^{2+}$ over 60 sec, after which efflux is complete. FMLP was omitted from the corresponding control systems. The reactions were terminated by the addition of 10 ml of ice-cold Ca^{2+}-replete HBSS to the tubes, and the cells were processed as above. This procedure was used to investigate the effects of KCl (25-100 mM), KB-R7943 (2.5-10 µM) and SK&F96365 (10 µM) on the efflux of Ca^{2+} from FMLP-activated neutrophils.

5.2.9 *Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils*

To measure net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were loaded with cold Ca^{2+} by preincubation for 15 min at 37°C in Ca^{2+}-replete (1.25 mM $CaCl_2$) HBSS, after which they were pelleted by centrifugation, then washed once with, and resuspended in ice-cold Ca^{2+}-free HBSS and held on ice until used. Pre-loading with cold Ca^{2+} was undertaken to minimise spontaneous uptake of radiolabelled cation (unrelated to FMLP activation) in the influx assay (Anderson and Goolam Mahomed, 1997). The Ca^{2+}-loaded neutrophils ($2x10^6$/ml) were then incubated for 10 min at 37°C in Ca^{2+}-free HBSS followed by simultaneous addition of FMLP (1 µM) and $^{45}Ca^{2+}$ (2 µCi/ml containing 50 nmol cold carrier Ca^{2+}) or $^{45}Ca^{2+}$ only to control, unstimulated systems. The final volume in each tube was 5 ml containing 10^7 cells. Influx of $^{45}Ca^{2+}$ into FMLP-activated and control neutrophils in the presence or absence of KCl (25-100 mM, final), KB-R7943 (2.5-10 µM), or

SK&F96365 (10 μM) was then monitored over a 5 min period, after which influx is complete.

In an additional series of experiments, the influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils from two individuals with chronic granulomatous disease, in the presence and absence of KB-R7943 (5 μM), was monitored at 5 min after the addition of FMLP.

5.2.10 Efflux of $^{22}Na^{+}$ from FMLP-activated neutrophils

To measure net efflux of $^{22}Na^{+}$ from FMLP-activated neutrophils, uncomplicated by concomitant uptake of the radiolabelled cation, the cells ($2x10^6$/ml) were washed with and resuspended in 50 mM Hepes-Tris buffer (pH 7.4) supplemented with 135 mM choline chloride, 1.1 mM glucose, 1.25 mM $CaCl_2$, 0.8 mM $MgSO_4$, 5 mM KCl, and 1 mM KH_2PO_4 containing 5 μCi/ml $^{22}Na^{+}$ (sodium-22, specific activity 399 mCi/mg). The cell suspension was then incubated for 20 min at 37°C to allow uptake of $^{22}Na^{+}$ after which the cells were washed once with and resuspended in HBSS to $1x10^7$/ml. The $^{22}Na^{+}$-loaded neutrophils were then preincubated for 10 min at 37°C with and without EGTA (5 and 10 mM), KB-R7943 (2.5-10 μM), or SK&F96365 (10 μM) and the extent of efflux of $^{22}Na^{+}$ monitored over a 5 min time course following the addition of FMLP (1 μM, final) or an equal volume of HBSS to unstimulated systems. The final volume in each tube was 5 ml containing a total of 10^7 neutrophils. Reactions were terminated by the addition of ice-cold HBSS and the cells processed as above for $^{45}Ca^{2+}$ influx experiments. The amount of cell-associated $^{22}Na^{+}$ was determined using a LKB Wallac 1261 Multigamma counter following lysis of the cells with 0.5 ml of 0.5% triton X-100/0.05 M NaOH. Appropriate background systems consisting of identically processed, $^{22}Na^{+}$-exposed cells which had been maintained at 4°C throughout the entire time course of the experiment were included and the values for these were subtracted from the corresponding experimental systems.

Importantly, the FMLP-activated fura-2 fluorescence and membrane depolarisation/repolarisation responses of neutrophils which had been subjected to the $^{22}Na^{+}$-loading procedure followed by transfer to HBSS did not differ from those of cells which were maintained in HBSS throughout, but were otherwise identically processed (data not included).

The validity of the neutrophil $^{22}Na^{+}$-loading procedure was evaluated by measurement of the extent of uptake of the radiolabelled cation in the presence and absence of the Na^{+}, K^{+}-ATPase inhibitor, ouabain (50 µM, final). Inclusion of the inhibitor almost doubled the intracellular Na^{+} concentration (data not shown).

5.2.11 Influx of $^{22}Na^{+}$ into FMLP-activated neutrophils

To measure the net influx of $^{22}Na^{+}$ into FMLP-activated neutrophils the cells ($2x10^{6}$/ml) were washed with and resuspended in Hepes-Tris/choline chloride buffer and preincubated for 10 min at 37°C followed by simultaneous addition of FMLP (1 µM) and $^{22}Na^{+}$ (5 µCi/ml) or $^{22}Na^{+}$ to control, unstimulated systems. The amount of cell-associated $^{22}Na^{+}$ was then measured as described above at 10, 20, 30 and 60 sec after the addition of FMLP to the neutrophils, this being the time course of efflux of Ca^{2+} from FMLP-activated neutrophils.

5.2.12 Assay of transmembrane fluxes of K^{+}

$^{86}Rb^{+}$ was used as a tracer for measuring K^{+} uptake and efflux (Simchowitz and De Weer, 1986). For uptake studies, neutrophils ($2x10^{6}$/ml) were suspended in isotonic Tris buffer (20 mM Tris, 122 mM NaCl, 4 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 5 mM glucose, pH 7.4) containing 2 uCi/ml of $^{86}Rb^{+}$ (Rubidium-86 chloride, specific activity 1.48 mCi/mg) for 15 min at 37°C followed by addition of FMLP (1 µM) or an equal volume of buffer to unstimulated systems (final volume in each tube of 5 ml). The kinetics of uptake of $^{86}Rb^{+}$ by control and FMLP-

activated neutrophils were then monitored over a 10 min time course (10, 20 and 30 sec, 1, 2, 3, 5 and 10 min). Reactions were terminated by the addition of ice-cold Tris buffer and the cells washed twice, followed by lysis of the cell pellets with 0.5 ml of 0.5% triton X-100/0.05 M NaOH and measurement of radioactivity in the lysates by liquid scintillation spectrometry. Na^+, K^+-ATPase activity was taken as the difference in $^{86}Rb^+$ uptake in the presence and absence of 50 µM ouabain.

For efflux experiments, neutrophils were preloaded with $^{86}Rb^+$ by incubating the cells (10^7 cells/ml) with $^{86}Rb^+$ (5 µCi/ml) for 20 min in isotonic Tris buffer. The cells were then washed twice with and resuspended in ice-cold Tris buffer at $2x10^6$/ml. The rates of efflux of the radiolabelled cation from control and FMLP-activated neutrophils were then monitored over a 60 sec time course (10, 20, 30 and 60 sec).

5.2.13 Statistical analysis

The results of each series of experiments are expressed as the mean ± standard error of the mean (SEM). Levels of statistical significance were calculated using the Students *t*-test when 2 groups were compared, or by analysis of variance with a subsequent Tukey-Kramer multiple comparisons test for multiple groups. Correlations between parameters were calculated using Pearson's correlation coefficient.

5.3 Mechanisms responsible for maintaining the RMP of human neutrophils

To determine the mechanisms responsible for maintaining the RMP of neutrophils, experiments were performed to assess the effects of potassium channel inhibitors (iberiotoxin, glibenclamide and TEA), potassium chloride at

variable concentrations added to the cell suspension, valinomycin (potassium ionophore), ouabain (a specific inhibitor of Na^+/K^+ ATPase), bafilomycin and DEPC (specific inhibitors of the vacuolar-type ATPases), ethacrynic acid and NPPB (chloride channel inhibitors), as well as KB-R7943 (a selective inhibitor of the reverse mode of the Na^+/Ca^{2+} exchanger), on the resting membrane potential of human neutrophils *in vitro*. The effects of these agents on the RMP of neutrophils are shown in Figure 5.1 (page 138).

Addition of potassium chloride (KCl) (25 – 100 mM), rapidly depolarised the membrane potential in a concentration-dependent manner. Ouabain (50 and 100 μM), induced an almost immediate depolarisation of the resting membrane potential, similar in magnitude to KCl (25 mM), which then stabilised at a new lower level. The potent and specific Na^+/Ca^{2+} channel inhibitor, KB-R7943 (2.5 – 10 μM), rapidly depolarised the RMP, in a similar fashion to that observed with KCl. Bafilomycin (100 nM), DEPC (12 μM) and valinomycin (10μM) all led to rapid hyperpolarisation of the RMP, which plateaued at a higher level. No further change in the RMP could be detected even after 10 minutes incubation with these agents.

Using the calibration value for alterations in membrane potential of 10 mV/cm, the magnitudes of the changes in RMP (mV) observed with each of the agents tested were calculated and are shown in Table 5.2 (page 139).

The other agents tested, iberiotoxin, glibenclamide, TEA, ethacrynic acid and NPPB did not significantly alter the RMP (not shown).

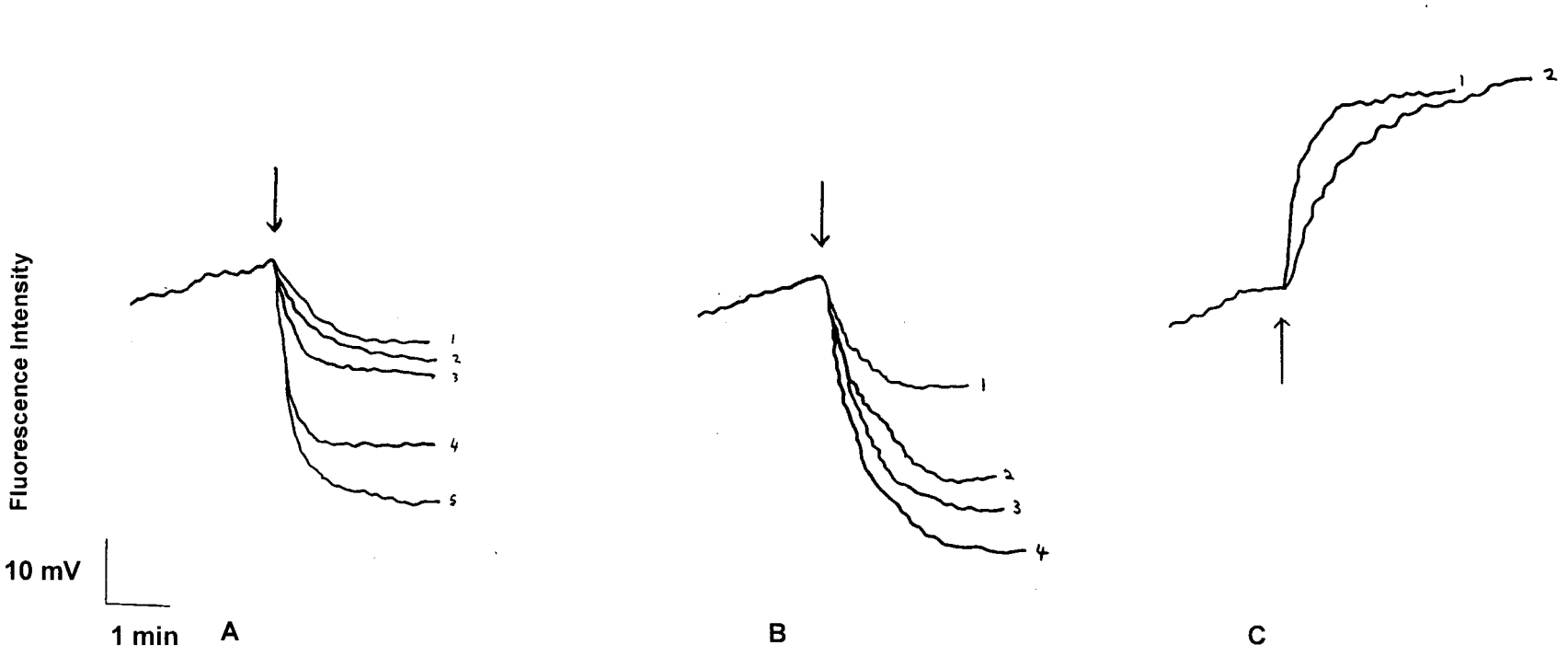


Figure 5.1. The effects of ouabain (50 and 100 μM), KB-R7943 (2.5, 5 and 10 μM), KCl (25, 50, 75 and 100 mM), bafilomycin (100 nM), valinomycin (10 μM) and DEPC (12 μM) on the resting membrane potential of human neutrophils.

Key:

A 1 = ouabain 50 μM

2 = ouabain 100 μM

3 = KB-R7943 2.5 μM

4 = KB-R7943 5 μM

5 = KB-R7943 10 μM

B 1 = KCl 25 mM

2 = KCl 50 mM

3 = KCl 75 mM

4 = KCl 100 mM

C 1 = bafilomycin 100 nM and valinomycin 10 μM

2 = DEPC 12 μM

Table 5.2: Effects of KCl, ouabain, KB-R7943, bafilomycin, DEPC and valinomycin on the resting membrane potential.

Agent	**Alteration in RMP (mV)**
KCl (25 mM)	+ 19 ± 0.7
KCl (50 mM)	+ 32 ± 1.6
KCl (75 mM)	+ 36 ± 0.6
KCl (100 mM)	+ 46 ± 1.2
Ouabain (50 μM)	+ 12 ± 0.5
Ouabain (100 μM)	+ 15 ± 0.9
KB-R7943 (2.5 μM)	+ 18 ± 1.4
KB-R7943 (5 μM)	+ 29 ± 1.5
KB-R7943 (10 μM)	+ 41 ± 4
Bafilomycin (100 nM)	- 22 ± 1.7
DEPC (12 μM)	- 25 ± 3
Valinomycin (10 μM)	- 24 ± 2.5

The results of 4 – 10 experiments are expressed as the absolute change in RMP (mV) following addition of each agent.

5.4 Mechanisms mediating membrane depolarisation in neutrophils

Although the mechanism mediating membrane depolarisation in human neutrophils has been attributed almost exclusively to electron flux across the plasma membrane during oxidant generation by NADPH oxidase, the slight reduction of the magnitude of the membrane depolarisation response (11%) observed with epinephrine-treated cells (Chapter 3, page 82) is significantly less than the degree of inhibition of superoxide production (25%) in the presence of epinephrine (Chapter 3, page 65). If membrane depolarisation is mediated solely by oxidant production, the magnitude of epinephrine-mediated inhibition of both superoxide production and membrane depolarisation would be expected to be equivalent. The greater magnitude of the epinephrine-mediated inhibition of superoxide production compared to that on membrane depolarisation observed with FMLP-activated neutrophils, suggested that another mechanism, in addition to electron flux across the outer membrane, may be operative during the abruptly occurring membrane depolarisation. This is further supported by the observation that in CGD neutrophils activated with FMLP, residual membrane depolarisation occurred despite almost undetectable superoxide production (Chapter 4, page 105). Additional mechanisms which may theoretically mediate membrane depolarisation in human neutrophils include ion fluxes (Na^+, K^+ or Cl^-), release of Ca^{2+} from cytosolic storage vesicles and Ca^{2+} influx across the outer membrane of the cell from extracellular reservoirs. Experiments to evaluate the effects of staurosporine (200 nM), DPI (10 μM), wortmannin (100 nM), ethacrynic acid (100 – 200 μM), ouabain (50 μM), herbimycin (2 μM), NPPB (3 μM), iberiotoxin (1 μg/ml), glibenclamide (3 μM) and amiloride (1 μM) on membrane depolarisation and superoxide production in FMLP activated neutrophils are shown in Table 5.3 (page 141).

Table 5.3. Effects of staurosporine, diphenylene iodonium chloride (DPI), wortmannin, ethacrynic acid, ouabain and herbimycin on membrane depolarisation and superoxide production as well as the effects of NPPB, iberiotoxin, glibenclamide and amiloride on the magnitude of membrane depolarisation in FMLP-activated neutrophils.

Agent	**Membrane depolarisation (mV)**	**Superoxide production (% Control)**
Staurosporine (200 nM)	38 ± 7*	4 ± 0.4*
DPI (10 μM)	29 ± 1*	3 ± 0.5*
Staurosporine + DPI	25 ± 5*	
Wortmannin (100 nM)	32 ± 3*	20 ± 2*
Ethacrynic acid (100 μM)	61 ± 4*	64 ± 1*
Ethacrynic acid (200 μM)	59 ± 4*	47 ± 3*
Ouabain (50 μM)	66 ± 2*	90 ± 2*
Herbimycin (2 μM)	72 ± 1	71 ± 6*
NPPB (3 μM)	67 ± 6	
NPPB + DPI	24 ± 2*	
Iberiotoxin (1 μg/ml)	72 ± 2	
Glibenclamide (3 μM)	77 ± 12	
Amiloride (1 μM)	70 ± 2	

The results of 5 – 20 experiments are expressed as the mean percentage of the inhibitor-free, control system ± SEM. The absolute peak value for superoxide production by untreated FMLP-activated neutrophils was 870 ± 125 mV.s-1. The magnitude of membrane depolarisation for control cells was 73 ± 2 mV. *$p < 0.05$ for comparison with the control system.

Staurosporine, DPI, wortmannin and ethacrynic acid attenuated both superoxide production by activated neutrophils as well as the magnitude of the membrane depolarisation response to FMLP, although the degree of inhibition was not equivalent, with greater inhibition of superoxide generation. In the presence of ouabain, both superoxide production by FMLP-activated neutrophils and the magnitude of membrane depolarisation were modestly inhibited. Herbimycin

significantly attenuated superoxide production without an effect on membrane depolarisation. NPPB, iberiotoxin, glibenclamide and amiloride did not alter membrane depolarisation in FMLP-activated neutrophils, which suggests that membrane depolarisation of human neutrophils is not mediated by Cl^- and K^+ ion fluxes and that Na^+/H^+ ion exchange plays no role during alterations in membrane potential. The membrane depolarisation response to FMLP could not be abolished even with combinations of the agents tested. The mean magnitude of inhibition of membrane depolarisation in FMLP-activated neutrophils treated with staurosporine, DPI, wortmannin, ethacrynic acid, ouabain and herbimycin was 30 ± 9%. In comparison, the mean magnitude of inhibition of superoxide production by FMLP-activated neutrophils treated with the same agents was almost double that on membrane depolarisation at 57 ± 13%.

The possible contributions of Ca^{2+} release from calciosomes, or Ca^{2+} influx from the extracellular fluid to membrane depolarisation in neutrophils, was investigated using platelet-activating factor (PAF) (0.01 μg/ml) that mobilises stored Ca^{2+} with concomitant activation of store-operated Ca^{2+} influx, but without any detectable superoxide generation at this PAF concentration (determined during preliminary experiments, results not shown).

Stimulation of neutrophils with PAF rapidly depolarised the RMP; the magnitude of this depolarisation response was 15 ± 1 mV. An abrupt increase in fura-2 fluorescence accompanied Ca^{2+} release from storage vesicles. The peak cytosolic Ca^{2+} concentration in PAF-activated cells was 362 ± 20 nM. Calcium reuptake from extracellular reservoirs could be detected at about 10 to 20 sec following addition of PAF as an abrupt alteration in the rate of decline of the Fura-2 fluorescence intensity. In the presence of SK&F96365 (10 μM), a selective inhibitor of store operated calcium influx channels, the PAF-induced release of Ca^{2+} from storage vesicles was unaltered, while store-operated Ca^{2+} influx was prevented (as determined by Mn^{2+} quenching of Fura-2 fluorescence, results not shown). The membrane depolarisation response to PAF in the presence of

SK&F96365 was intact, despite inhibition of store-operated Ca^{2+} influx (results not shown).

5.5 Apparent involvement of Na^+/Ca^{2+} exchange in membrane repolarisation and store-operated uptake of extracellular Ca^{2+} by chemoattractant-activated human neutrophils

5.5.1 FMLP-activated neutrophil depolarisation/repolarisation, fura-2 fluorescence and transmembrane Ca^{2+} fluxes

A comparison of these responses was performed using neutrophils from 6 different individuals and a typical set of responses from a single individual is shown in Figure 5.2 (page 144). Exposure of the cells to FMLP was accompanied by the characteristic abrupt increase in fura-2 fluorescence followed by a rapid subsidence in fluorescence intensity, coincident with the release of Ca^{2+} from intracellular stores and clearance of cytosolic cation respectively. These events coincided with a dramatic decrease in membrane potential and efflux of $^{45}Ca^{2+}$, both of which levelled off at around 30 sec after the addition of FMLP. Repolarisation was evident at 1-2 min after addition of FMLP and was associated with influx of extracellular Ca^{2+} which was detected indirectly as an alteration (reduction) in the rate of decline in fura-2 fluorescence intensity, as well as directly according to the uptake of radiolabelled cation by the cells. The kinetics of repolarisation of FMLP-activated neutrophils were similar to those of uptake of Ca^{2+}.

The average times taken to onset of repolarisation and influx of Ca^{2+} using both the fura-2 fluorescence and radiometric procedures in FMLP-activated neutrophils from 6 different individuals were 1.80 ± 0.2, 1.85 ± 0.2 and 1.75 ± 0.1 min respectively. The correlation coefficients between the times taken after

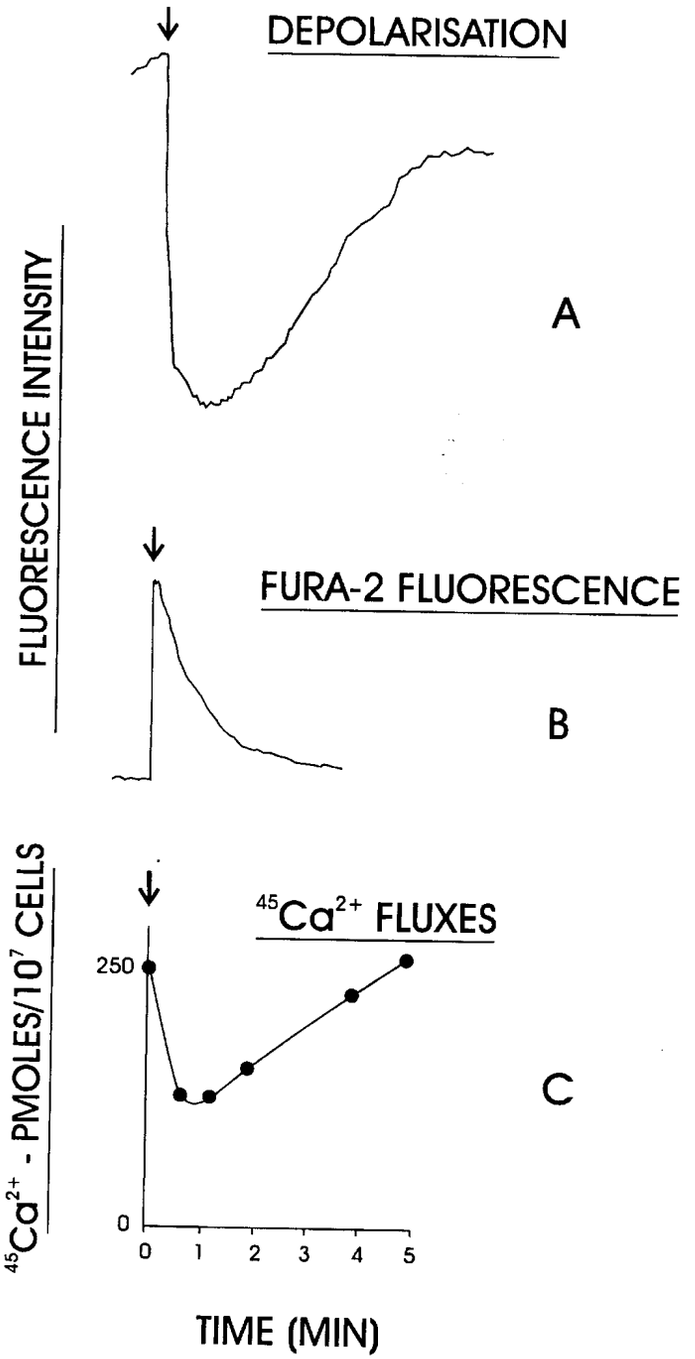


Figure 5.2: Investigation and comparison of the alterations in membrane potential (depolarisation), cytosolic free Ca^{2+} concentrations (fura-2 fluorescence) and efflux and influx of Ca^{2+} ($^{45}Ca^{2+}$ fluxes) which accompany activation of human neutrophils with the N-formylated chemotactic tripeptide, FMLP. The data shown are those for neutrophils from a single individual and representative of the data obtained using cells from 6 different individuals. Addition of FMLP (1 μM, final) is denoted by the arrow (↓).

activation of neutrophils with FMLP to initiation of repolarisation and influx of Ca^{2+} using the fura-2 fluorescence and radiometric procedures were r=0.98 (p=0.0005) and r=0.88 (p=0.02) respectively, while that for time taken to influx of Ca^{2+} using the fura-2 fluorescence and radiometric procedures was r=0.82 (p=0.04).

5.5.2 *Effects of KCl, KB-R7943, SKF 96365, EGTA and $ZnCl_2$ on membrane potential*

The effects of KCl (25-100 mM) on neutrophil resting membrane potential and FMLP-mediated alterations in this are shown in Figure 5.3 (page 146). Addition of KCl to the cell-suspending medium resulted in the well-recognised, dose-related decrease in membrane potential which declined further on addition of FMLP, followed by repolarisation to almost pre-activation levels, but remaining at all times below the corresponding values for control cells.

The effects of the selective Na^+/Ca^{2+}-exchanger inhibitor, KB-R7943, on membrane potential, are shown in Figure 5.4 (page 147). Addition of KB-R7943 (5 µM), resulted in a rapid, but small decline in the resting fluorescence intensity, which stabilised at a new lower value within 30-60 s. Following stabilisation of the resting membrane potential, activation with FMLP produced a rapid membrane depolarisation, the magnitude of which was slightly greater than that observed with control cells. In the presence of KB-R7943, however, marked attenuation of the rate and extent of membrane repolarisation was observed, which persisted for the entire 10 minute test period. Similar results (not shown) were obtained with the non-selective Na^+/Ca^{2+} exchange inhibitor benzamil (200 µM). Neither the FMLP-activated membrane depolarisation nor the subsequent repolarisation responses of neutrophils were affected by SKF 96365 (not shown).

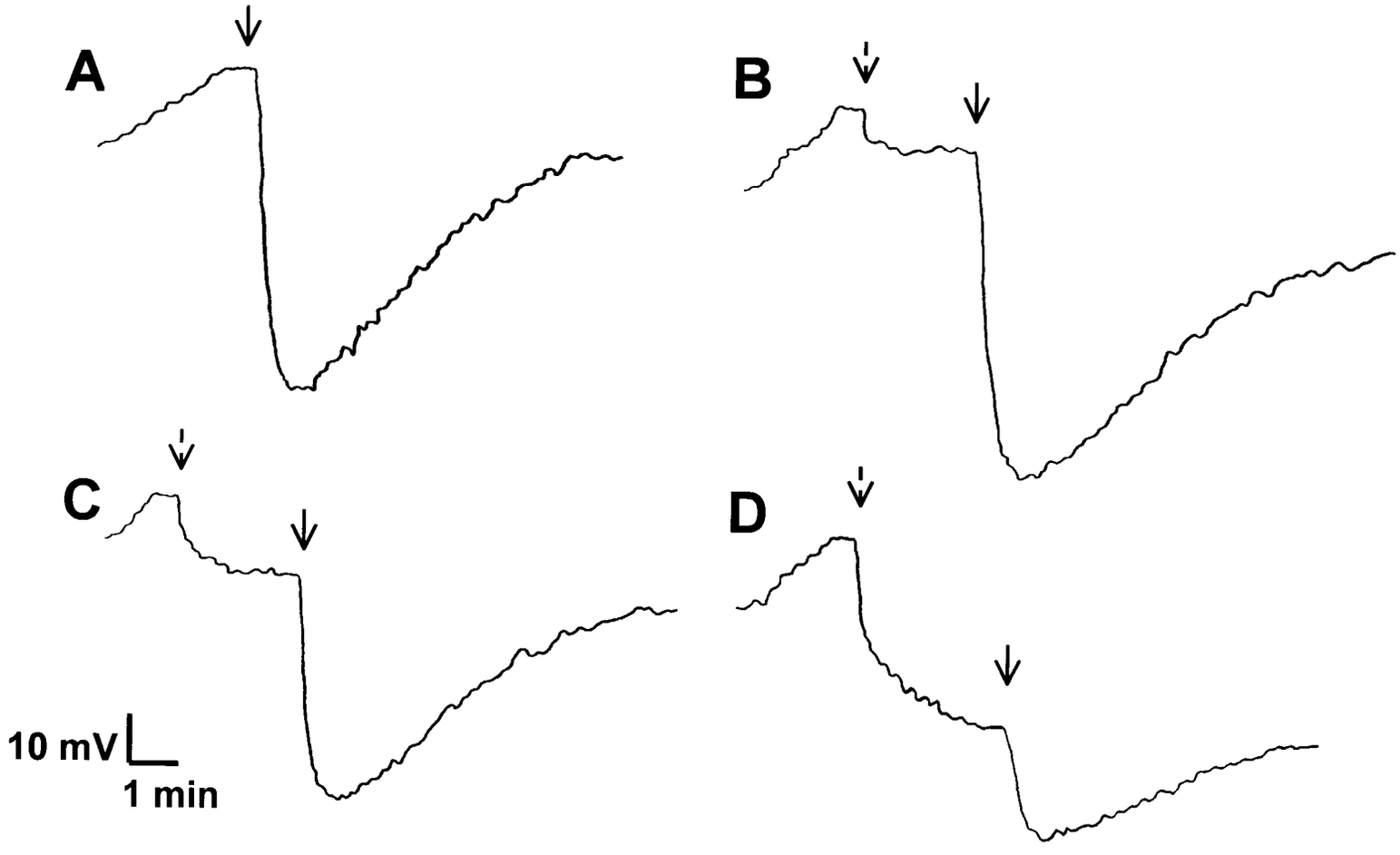


Figure 5.3: Investigation of the effects of addition of KCl (25-100 mM) on the membrane potential of resting and FMLP-activated neutrophils. The traces shown are those from a single representative experiments (4 in the series). The trace on the upper left side of the figure (A) shows the alterations in neutrophil membrane potential following the addition of FMLP (↓), while the effects of KCl at 25, 50 and 100 mM added as indicated (↓) on resting membrane potential, as well as on subsequent alterations in this following addition of FMLP (↓) are shown in traces B, C and D respectively.

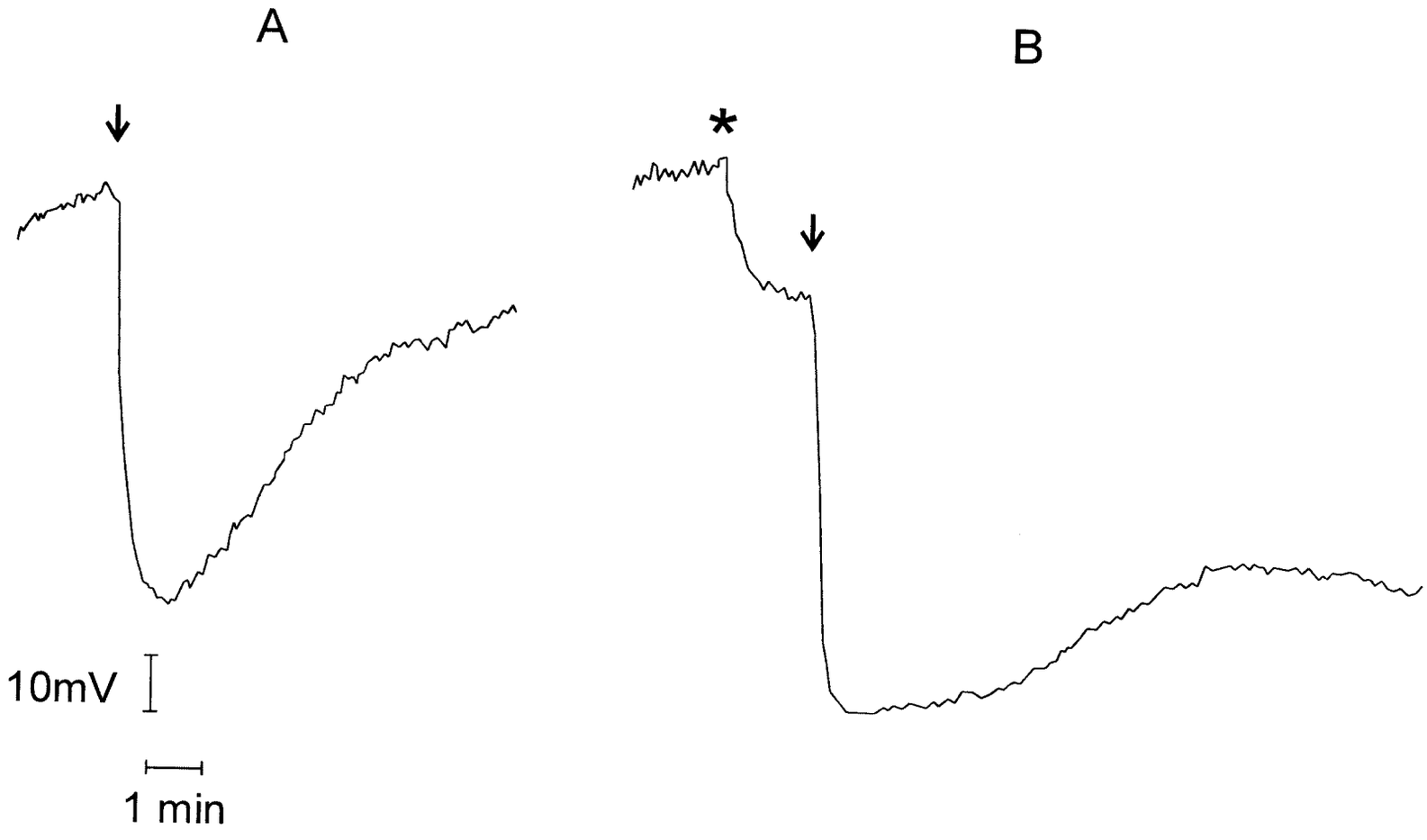


Figure 5.4: Investigation of the effects of KB-R7943 on the membrane potential of resting and FMLP-activated neutrophils. The traces shown are those from a single representative experiment (4 in the series). The trace on the left side of the figure (A) shows the alterations in neutrophil membrane potential following the addition of FMLP (↓), while the effects of KB-R7943 (5 μM) added as indicated (*) on resting membrane potential, as well as on subsequent alterations in this following addition of FMLP (↓) are shown on the right (B).

The effects of addition of EGTA (10 mM) to the cell-suspending medium on FMLP-activated alterations in neutrophil membrane potential are shown in Figure 5.5 (page 149). The addition of EGTA had no effect on the resting potential over a 10 min test period (not shown) and did not alter the magnitude of the depolarisation response after addition of FMLP. However, inclusion of EGTA almost completely abolished the membrane repolarisation response. The corresponding responses of neutrophils suspended in HBSS containing 50 µM $CaCl_2$ in the presence and absence of a lower concentration of EGTA (100 µM) are shown in Figure 5.5 (page 149). The results were similar to those observed in Ca^{2+}-replete HBSS ± EGTA at 10 mM. Replenishment of extracellular Ca^{2+} during the phase of EGTA-mediated suppression of membrane repolarisation, resulted in a recovery of membrane potential at a rate and of a magnitude similar to that observed with control cells.

Neutrophil resting membrane potential, as well as FMLP-mediated depolarisation and repolarisation, were not affected by treatment of the cells with $ZnCl_2$ at concentrations of up to 250 µM (data not shown).

5.5.3 *Effects of KCl, KB-R7943 and SKF 96365 on transmembrane Ca^{2+} fluxes*

The effects of KCl, KB-R7943 and SKF 96365 on peak cytosolic Ca^{2+} concentrations, as well as on the efflux of the cation from FMLP-activated neutrophils are shown in Table 5.4 (page 150). KCl, KB-R7943 and SKF 96365 at concentrations of up to 100 mM, 5 µM and 10 µM respectively had no effects on the release of Ca^{2+} from neutrophil intracellular stores or on the subsequent efflux of the cation.

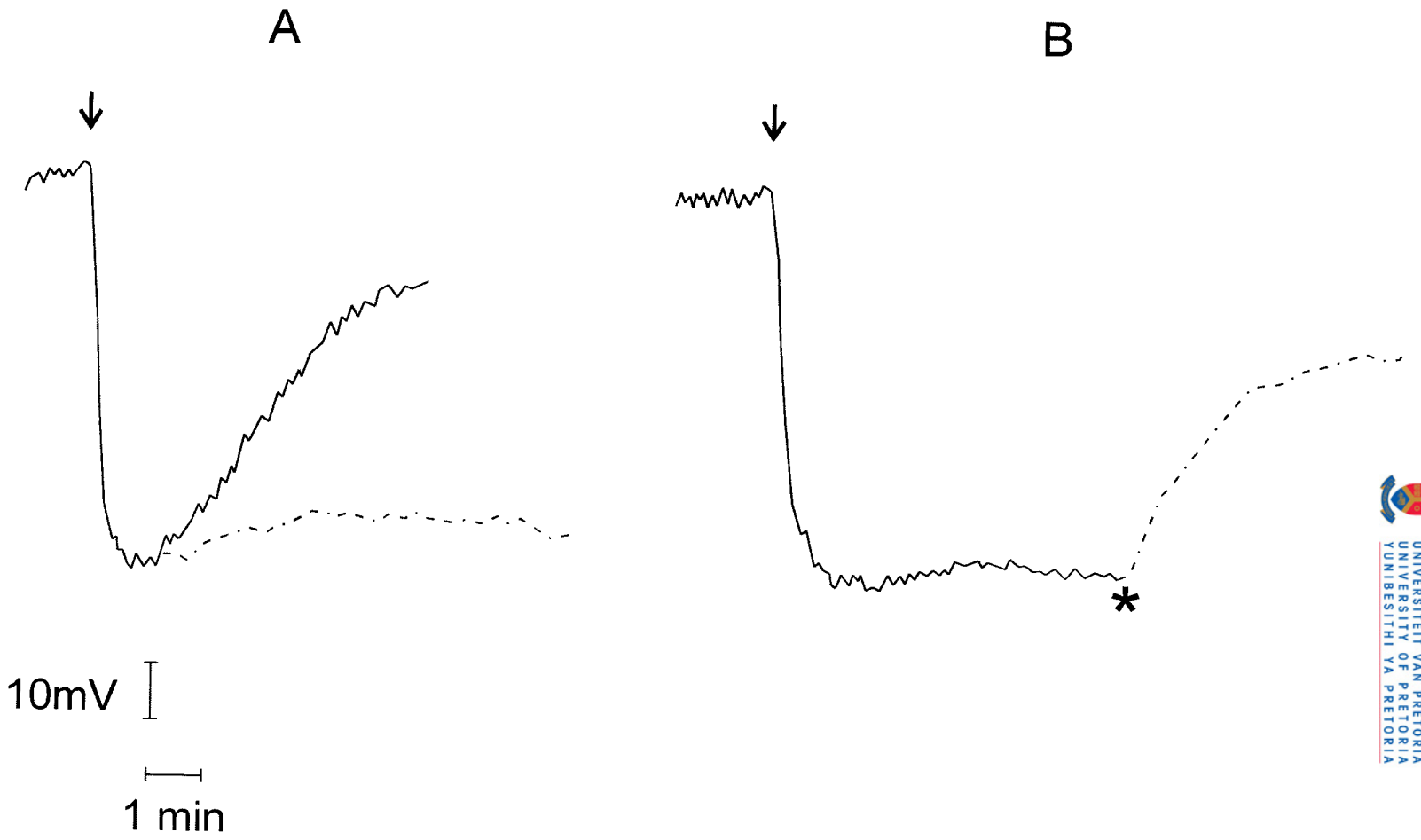


Figure 5.5: Investigation of the effects of EGTA on FMLP-activated alterations in neutrophil membrane potential. The traces shown are those from a single representative experiment (4 in the series). The trace on the left side of the figure (A) shows the alterations in neutrophil membrane potential following the addition of FMLP (↓) to cells suspended in Ca^{2+}-replete HBSS in the absence (———) and presence of 10 mM EGTA (- - - - -), while the corresponding responses of neutrophils suspended in nominally Ca^{2+}-free HBSS containing 100 µM EGTA, to which $CaCl_2$ (1 mM) was added 5 min after FMLP (*), are shown on the right (B).

Table 5.4: Effects of KCl (25-100 mM), KB-R7943 (5 μM) and SKF 96365 (10 μM) on the peak intracellular calcium concentrations $[Ca^{2+}]i$ and efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils

System	Peak $[Ca^{2+}]i$ values (nM)	Amount of $^{45}Ca^{2+}$ released from neutrophils 60 s after the addition of FMLP (pmol/10^7 cells)
FMLP only	369 ± 16	151 ± 12
KCl 25 mM	358 ± 18	144 ± 10
KCl 50 mM	349 ± 14	163 ± 20
KCl 100 mM	350 ± 16	171 ± 19
KB-R7943 5 μM	351 ± 7	183 ± 13
SKF 96365 (10 μM)	352 ± 14	146 ± 5

The results of 4-9 experiments are expressed as the mean ± SEM. The mean basal cytosolic Ca^{2+} concentration in resting cells was 114 ± 10 nM.

The effects of KCl (25-100 mM), KB-R7943 (2.5-10 μM) and SKF 96365 (10 μM) on the magnitude of both the spontaneous and the FMLP-activated influx of Ca^{2+} into FMLP-activated neutrophils are shown in Figures 5.6, 5.7 and 5.8 (pages 151, 152 and 153) respectively. KCl at all concentrations tested attenuated the FMLP-activated, but not the spontaneous influx of $^{45}Ca^{2+}$ into neutrophils, while KB-R7943 caused significant dose-related suppression of the influx of $^{45}Ca^{2+}$ into FMLP-activated cells. SKF 96365 attenuated the spontaneous uptake of $^{45}Ca^{2+}$ into resting neutrophils with almost complete inhibition of $^{45}Ca^{2+}$ influx into FMLP-activated cells. The inhibitory effects of KB-R7943, and SKF 96365 were confirmed using the Mn^{2+} quenching of Fura-2 fluorescence assay (data not shown). The influx of $^{45}Ca^{2+}$ into FMLP-activated CGD neutrophils in the presence of KB-R7943 (5 μM) was similar to that observed in control, untreated CGD neutrophils being 150 ± 15 pmol/10^7 cells and 148 ± 16 pmol/10^7 cells, respectively. The apparent inability of KB-R7943 to attenuate Ca^{2+} influx into FMLP-activated CGD neutrophils was confirmed using the Mn^{2+} quenching of fura-2 fluorescence assay (data not shown).

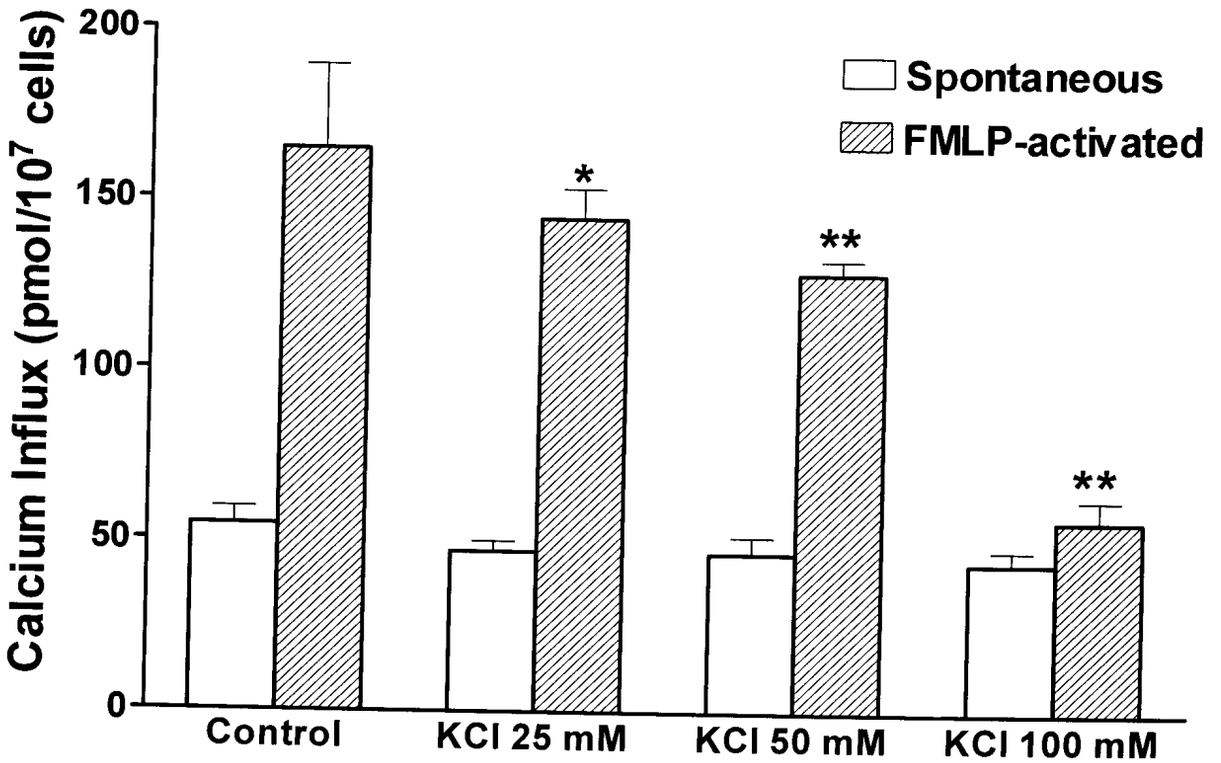


Figure 5.6: Investigation of the effects of KCl (25-100 mM) on the magnitude of both the spontaneous and FMLP-activated influx of $^{45}Ca^{2+}$ into neutrophils. The results of 3-12 experiments are expressed as the mean uptake of $^{45}Ca^{2+}$ in pmol/10^7 cells for unstimulated and FMLP-activated systems at 5 min after addition of the chemoattractant; vertical lines show SEM. *$p<0.05$; **$p<0.005$ for comparison with the FMLP-activated control system.

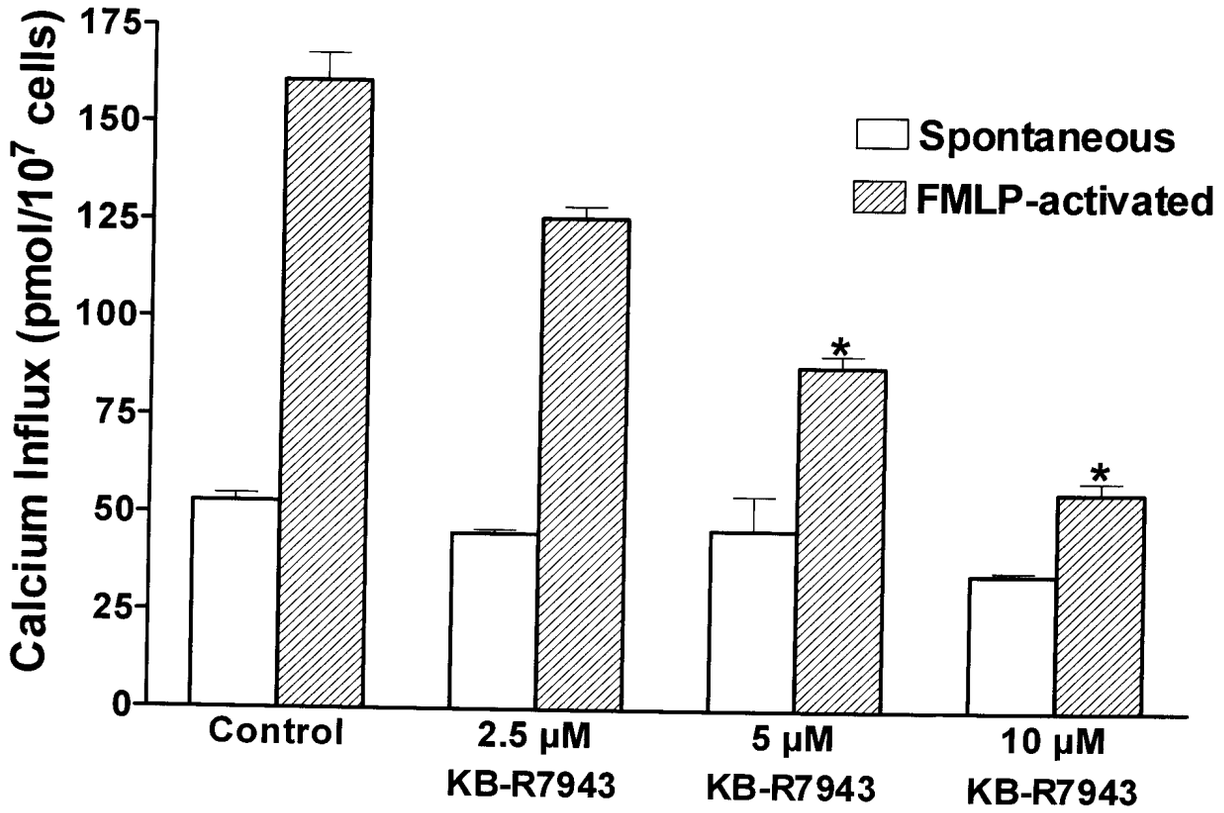


Figure 5.7: Investigation of the effects of KB-R7943 (2.5-10 µM) on the magnitude of both the spontaneous and FMLP-activated influx of $^{45}Ca^{2+}$ into neutrophils. The results of 6-21 experiments are expressed as the mean uptake of $^{45}Ca^{2+}$ in pmol/10^7 cells for unstimulated and FMLP-activated systems at 5 min after the addition of the chemoattractant; vertical lines show SEM.

*$p<0.001$ for comparison with the FMLP-activated control system.

$^{45}Ca^{2+}$ uptake by resting and FMLP-activated control cells at 5 min was 53 ± 2 pmol/10^7 cells and 161 ± 7 pmol/10^7 cells respectively.

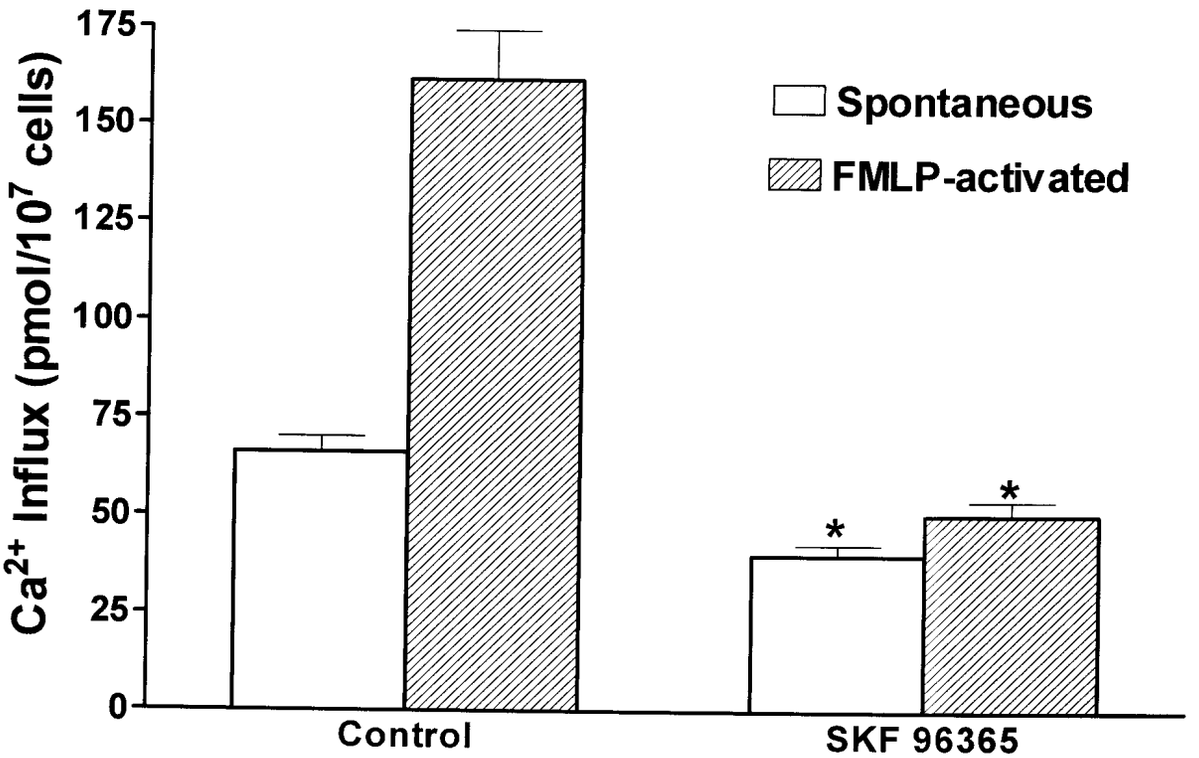


Figure 5.8: Investigation of the effects of SKF 96365 (10 μM) on the magnitude of both spontaneous and FMLP-activated influx of $^{45}Ca^{2+}$ into neutrophils. The results of 5-11 experiments are expressed as the mean uptake of $^{45}Ca^{2+}$ in pmol/10^7 cells for unstimulated and FMLP-activated systems at 5 min after addition of the chemoattractant; vertical lines show SEM. *$p<0.05$ for comparison with the corresponding untreated control systems with and without FMLP.

5.5.4 *Transmembrane fluxes of Na^+*

Activation of neutrophils with FMLP did not result in influx of extracellular $^{22}Na^+$ at any of the times investigated (10, 20, 30 and 60 sec). The values at 60 sec for control and FMLP-activated neutrophils were 428 ± 31 and 412 ± 24 cpm respectively.

Exposure of neutrophils to FMLP was, however, accompanied by efflux of the cation from the cells. The kinetics of efflux of $^{22}Na^+$ from resting and FMLP-activated neutrophils are shown in Figure 5.9 (page 155). Exposure of neutrophils to FMLP resulted in an efflux of $^{22}Na^+$ which was detectable at 1 min and maximal after 5 min, resulting in an average loss of 65% of cell-associated cation. The effects of KB-R7943 (2.5-10 µM) on $^{22}Na^+$ efflux from FMLP-activated neutrophils are shown in Figure 5.10 (page 156). Inclusion of KB-R7943, inhibited the efflux of $^{22}Na^+$ from FMLP-activated neutrophils in a dose-dependent manner.

The efflux of $^{22}Na^+$ from FMLP-activated neutrophils at 5 min was not significantly altered in the presence of EGTA (5 and 10 mM), being 44 ± 2% and 57 ± 3% of cell-associated $^{22}Na^+$ respectively, compared to 47 ± 8% from control cells.

The inclusion of SKF 96365 (10 µM), did not significantly alter the magnitude of $^{22}Na^+$ efflux from FMLP-activated neutrophils 5 min after addition of the stimulant, being 49 ± 7% and 43 ± 8% of cell associated $^{22}Na^+$ from control and SKF 96365-treated cells respectively.

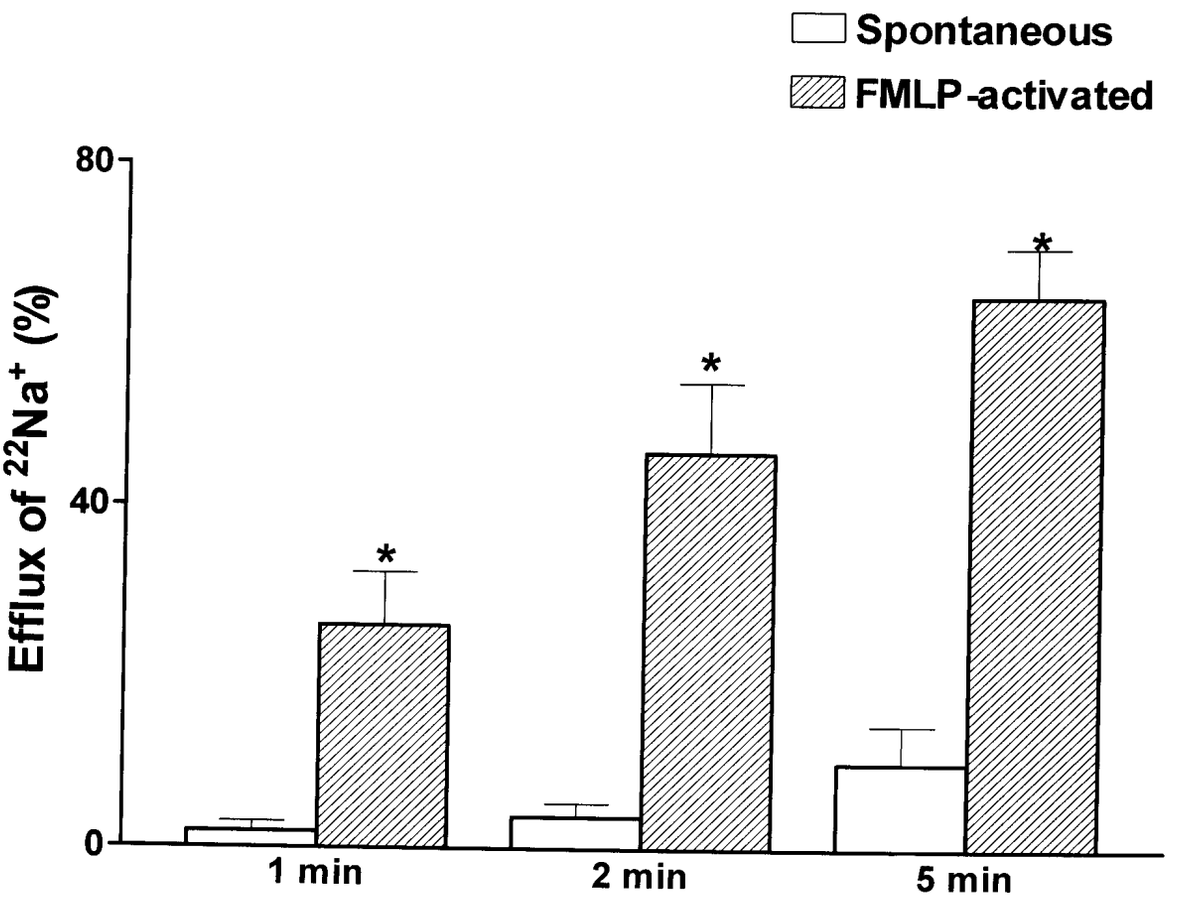


Figure 5.9: Measurement of the kinetics of efflux of $^{22}Na^{+}$ from resting and FMLP-activated neutrophils. The results of 6 experiments are expressed as the mean percentage ± SEM of $^{22}Na^{+}$ discharged from the cells over a 5 min time course following the addition of FMLP.

*$p<0.05$ for comparison with the corresponding value for unstimulated cells.

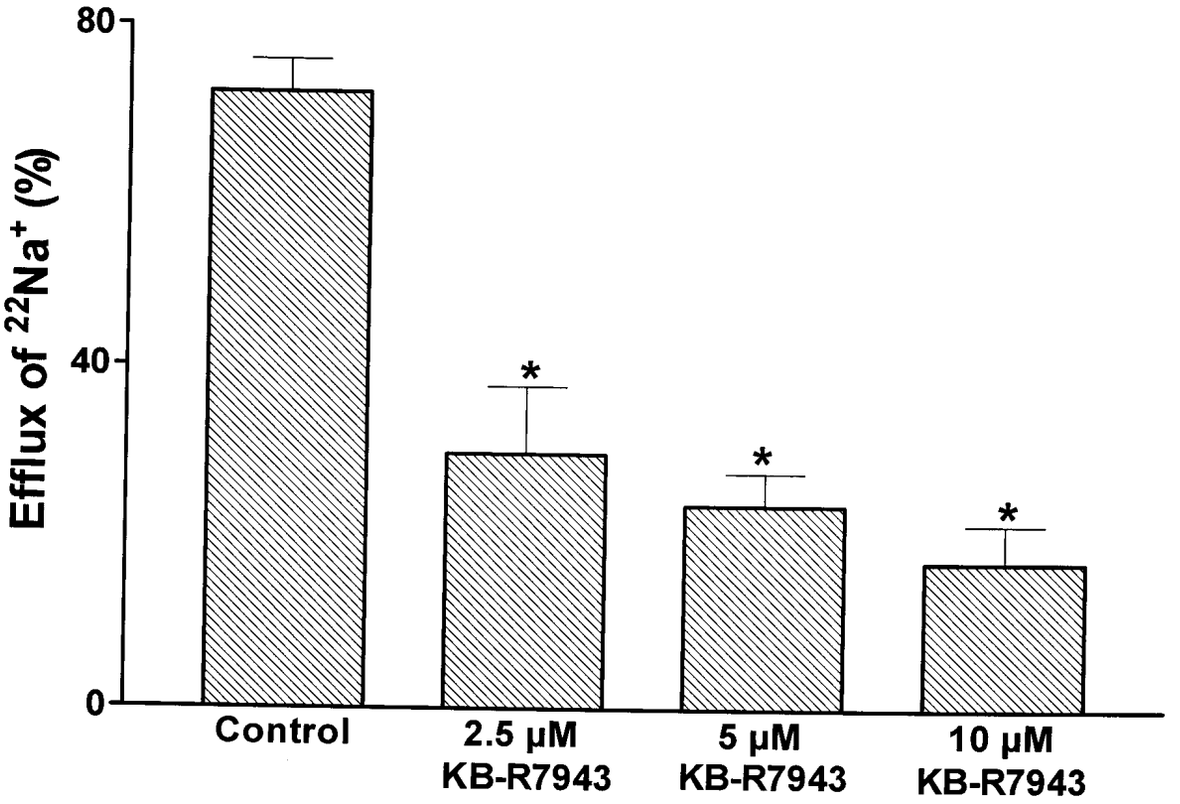


Figure 5.10: Investigation of the effects of KB-R7943 (2.5-10 µM) on $^{22}Na^+$ efflux from FMLP-activated neutrophils. The results of 6-18 experiments are expressed as the mean percentage ± SEM of $^{22}Na^+$ discharged from the cells over a 5 min time course following the addition of FMLP. *p<0.001 for comparison with control, untreated cells.

5.5.5 Transmembrane fluxes of K^+

Relative to untreated control cells, addition of FMLP to neutrophils did not cause detectable efflux of $^{86}Rb^+$. At 60 sec after addition of the chemoattractant, the amounts of $^{86}Rb^+$ associated with unstimulated and FMLP-activated cells were 26145 ± 394 and 27286 ± 903 cpm respectively. For influx experiments the amounts of ouabain-inhibitable, cell-associated $^{86}Rb^+$ at 1, 2, 3 and 5 min after addition of FMLP were 2593 ± 330, 2541 ± 322, 2254 ± 314 and 3304 ± 405 cpm respectively, while the corresponding values for unstimulated cells were 2485 ± 140, 2877 ± 209, 2342 ± 248 and 3109 ± 281 cpm.

5.6 Discussion

The resting membrane potential (RMP) of human neutrophils *in vitro* is determined by numerous factors. These include the potassium concentration gradient across the outer membrane as demonstrated by the significant depolarising effect observed when potassium chloride was added to the neutrophil suspensions. Valinomycin, which abolishes the potassium concentration gradient, hyperpolarised the cells as intracellular potassium leaked out into the extracellular fluid. The alterations in RMP associated with increasing extracellular potassium concentrations are in keeping with previous reports (Seligmann *et al* , 1980; Mottola *et al*, 1982; Myers *et al*, 1990). The role of the membrane-associated Na^+/K^+-ATPase was confirmed as ouabain depolarised the cells, albeit to a modest extent. The contribution from Na^+/K^+-ATPase activity appears to be slight and is not the major contributor as proposed by some investigators (Bashford and Pasternak, 1985).

Additional mechanisms maintaining the RMP of human neutrophils which have not, to my knowledge, been previously reported, include the activity of the Na^+/Ca^{2+} exchanger, as well as an inwardly rectifying proton current. The Na^+/Ca^{2+} exchanger present on the plasma membrane is operative in reverse mode in resting cells, leading to the electrogenic efflux of 3 intracellular Na^+ ions for each Ca^{2+} ion entering the cell. Addition of the selective Na^+/Ca^{2+} exchange inhibitor, KB-R7943 to resting neutrophils, significantly reduced the RMP. KB-R7943 inhibits the reverse mode (Na^+ efflux/Ca^{2+} influx) of the exchanger (Blaustein and Lederer, 1999) and in keeping with this, the decay in RMP in the presence of KB-R7943 was associated with the accumulation of radiolabelled Na^+ in a concentration-dependent manner. It is therefore likely that basal activity of the Na^+/Ca^{2+} exchanger operating in reverse mode contributes to maintenance of the RMP in neutrophils by extruding Na^+ ions in a similar fashion to Na^+/K^+-ATPase. In addition, this provides evidence that the regulation of calcium

homeostasis in resting neutrophils is coupled to the maintenance of the RMP by at least one mechanism, namely Na^+/Ca^{2+} exchange.

The addition of bafilomycin hyperpolarised the RMP of neutrophils, suggesting that proton fluxes may occur in resting cells and contribute to the maintenance of the RMP. The negative resting potential may favour the inward movement of protons down the electrical gradient (Demaurex, 1993a; Banfi *et al*, 1999) with the membrane-component (V_o) of the V-type ATPase acting as a proton conducting channel (Zhang, 1994). Therefore, bafilomycin, which binds to and inhibits the membrane (V_o) component of the V-type ATPase, may hyperpolarise the outer membrane by attenuating passive proton influx currents in resting cells. These results were further supported by the effects of DEPC (an inhibitor of inwardly rectifying H^+ channels in eosinophils) (Banfi *et al*, 1999), which also hyperpolarised the membrane potential.

The RMP of human neutrophils is not dependent on the activity of potassium channels, as neither the non-specific K^+-channel inhibitor (TEA), nor inhibitors of specific types of K^+-channels had any effect on the RMP. Ethacrynic acid and NPPB (chloride channel inhibitors) were also ineffective in altering the RMP, suggesting that chloride fluxes in resting cells are not involved in maintaining the RMP.

Membrane depolarisation in human neutrophils is dependent on the activity of the assembled NADPH complex which transfers electrons to molecular oxygen during oxidant production (Schrenzel *et al*, 1998). Cells lacking a functional NADPH oxidase enzyme complex, such as those from patients with CGD, are unable to generate reactive oxidants, resulting in a significantly attenuated membrane depolarisation response. While some investigators have reported the complete absence of a membrane depolarisation response in PMA-activated neutrophils from patients with chronic granulomatous disease (Seligmann and Gallin, 1980; Gallin *et al*, 1983), others have observed a very small depolarisation

response in CGD neutrophils (Whitin *et al*, 1980; Lew *et al*, 1984). Repeated experiments using cells from the four CGD patients in this study, confirmed a consistent, but trivial membrane depolarisation response. This residual FMLP-associated depolarisation response in CGD neutrophils could not be inhibited by the selective NADPH oxidase inhibitor, diphenylene iodonium (determined during preliminary experiments, results not shown), suggesting that superoxide production may not be the only mechanism mediating membrane depolarisation in these cells. Similarly, membrane depolarisation persisted in FMLP-activated neutrophils from normal subjects pre-incubated with DPI and/or staurosporine, despite only trivial oxidant production in the presence of these agents, supporting the contention that alternative mechanisms may contribute to the membrane depolarisation response.

Alternative, but unreported mechanisms which may theoretically contribute to membrane depolarisation in FMLP-activated neutrophils, include Ca^{2+} influx across the plasma membrane, Ca^{2+} release from storage vesicles, efflux of Cl^- ions and influx of K^+ ions. Influx of extracellular Ca^{2+} across the plasma membrane may depolarise the membrane potential as has been reported in experiments with thapsigargin, which induces Ca^{2+} influx by depleting intracellular Ca^{2+} stores (Scharff and Foder, 1996). Platelet activating factor (PAF), at a concentration of 0.01 μg/ml, mediates Ca^{2+} release from stores with associated re-uptake of extracellular Ca^{2+} without detectable superoxide production. Therefore, PAF (0.01 μg/ml) was used to evaluate the contribution of Ca^{2+} influx and Ca^{2+} release from stores to membrane depolarisation in human neutrophils. In the present study, a role for Ca^{2+} influx as a mediator of membrane depolarisation could not be confirmed, as the membrane depolarisation response to PAF was not altered in the presence of SK&F96365, a selective inhibitor of store-operated Ca^{2+} channels (Merritt *et al*, 1990). As no oxidant production could be detected in neutrophils activated with PAF, the depolarisation response observed with this agent may therefore be attributable to Ca^{2+} release from intracellular calciosomes. Rapid increases in cytosolic Ca^{2+}

concentrations adjacent to the plasma membrane may alter the electrical gradient across the outer membrane, thereby contributing to the membrane depolarisation responses observed with PAF and FMLP. This mechanism may also mediate the residual membrane depolarisation observed in FMLP-activated CGD neutrophils. Although efflux of chloride (Cl^-) ions may supposedly contribute to the depolarisation response in neutrophils activated with Ca^{2+}-mobilising stimuli (Krause and Welsh, 1990), my observations that the chloride channel inhibitor 5-nitro-2(3-phenylpropylamino)-benzoic acid (NPPB) (Phipps *et al*, 1996), did not attenuate membrane depolarisation in neutrophils, makes this an unlikely mechanism to account for residual depolarisation in human neutrophils. Similarly, potassium channels do not appear to play any significant role in mediating membrane depolarisation.

Following receptor-mediated activation of neutrophils by N-formyl peptides, there is a rapid and transient increase in the concentration of cytosolic free Ca^{2+}, an event which precedes and is a prerequisite for activation of neutrophil pro-inflammatory functions (Pettit and Hallett, 1998). Restoration of Ca^{2+} homeostasis in activated neutrophils is essential to prevent Ca^{2+} overload with resultant hyperactivation of these cells, which may contribute to exaggerated inflammatory responses. Efficient clearance of Ca^{2+} from the cytosol is facilitated by delayed uptake of the cation from extracellular reservoirs, characteristic of calcium influx via store-operated channels (Parekh and Penner, 1997).

The precise molecular mechanisms that regulate store-operated calcium influx are not currently known, but the alterations in membrane potential which accompany NADPH oxidase activation may play an important role in limiting the influx of extracellular Ca^{2+}. It has been proposed that when the cells are depolarised, the driving force for Ca^{2+} entry is abolished as the electrical component of the electrochemical gradient is not favourable for Ca^{2+} entry (Di Virgilio *et al,* 1987). Following depolarisation, the membrane potential is gradually restored towards its resting level over a 5-10 minute time course.

Interestingly, as demonstrated in the current study, the kinetics of influx of extracellular Ca^{2+} into FMLP-activated human neutrophils are superimposable on those of membrane repolarisation, supporting, albeit indirectly, a mechanistic relationship between these events. This contention was supported by data from experiments in which the RMP of neutrophils was manipulated by increasing the KCl concentration of the cell-suspending medium. This resulted in dose-related spontaneous membrane depolarisation and attenuation of the delayed, store-operated uptake of extracellular Ca^{2+} on subsequent activation of the cells with FMLP. As discussed below, this proposed relationship between membrane repolarisation and calcium influx was reinforced by data from experiments using KB-R7943.

There are several possible mechanisms which may promote membrane repolarisation in chemoattractant-activated neutrophils. In the case of activated eosinophils, for example, much attention has focused on proton conductance as an acid extrusion mechanism which, being electrogenic, results in repolarisation of the cell membrane (Banfi *et al,* 1999). However, as observed in the current study, the involvement of this mechanism in neutrophils is improbable due to the failure of Zn^{2+}, an inhibitor of the proton conductance pathway used at concentrations equal to and greater than those used in previous studies (Nanda and Grinstein, 1995; Banfi *et al.,* 2000), to alter the rate and magnitude of membrane repolarisation in FMLP-activated neutrophils. Although we observed outward movement of Na^+ coincident with membrane repolarisation in activated neutrophils, this was not coupled to detectable increases in uptake of K^+ ($^{86}Rb^+$), excluding possible involvement of Na, K^+-ATPase in these events. This observation did, however, implicate the Na^+/Ca^{2+} exchanger, operating in reverse mode, in mediating membrane repolarisation. The Na^+/Ca^{2+} exchanger is electrogenic, mediating the net movement of a positive charge when 3 Na^+ are exchanged for 1 Ca^{2+} and can operate in forward or reverse mode, promoting either efflux or influx of Ca^{2+} respectively (Blaustein and Lederer, 1999). The

existence of a Na^+/Ca^{2+} exchange mechanism on the plasma membrane of neutrophils (Simchowitz and Cragoe, 1988), and lymphocytes (Balasubramanyan *et al,* 1994) has been described previously. Its activity is upregulated by binding of Ca^{2+} to a high-affinity regulatory site on the cytosolic side of the exchanger, promoting sodium efflux and calcium influx following Ca^{2+} release from storage vesicles (Blaustein and Lederer, 1999). This sensitivity of the plasma membrane Na^+/Ca^{2+} exchanger to increases in cytosolic calcium concentrations, although paradoxical, may facilitate Ca^{2+} influx following emptying of intracellular calcium stores (Blaustein and Lederer, 1999), while concomitantly mediating the electrogenic recovery of the membrane potential towards resting levels.

Additional evidence in support of the involvement of the Na^+/Ca^{2+} exchanger in mediating membrane repolarisation in neutrophils, as well as dependence of Ca^{2+} influx on these events, was derived from experiments using KB-R7943, an inhibitor of the reverse mode of the exchanger (Iwamato *et al,* 1996), and EGTA, a Ca^{2+} chelator. Treatment of neutrophils with KB-R7943, at concentrations which did not affect release of Ca^{2+} from intracellular stores, or subsequent efflux of the cation, caused dose-related attenuation of the membrane repolarisation response of FMLP-activated neutrophils in the setting of decreased efflux and influx of Na^+ and Ca^{2+} respectively. Inclusion of EGTA in the cell-suspending medium also attenuated the membrane repolarisation response of FMLP-activated neutrophils, which was reversed by subsequent addition of excess Ca^{2+}. Interestingly, Na^+ efflux from FMLP-activated neutrophils was unaffected by EGTA, which although surprising considering the effects of the chelator on membrane repolarisation, can be explained on the basis that the Na^+/Ca^{2+} exchanger can revert to Na^+/Na^+ exchange mode (non-electrogenic) in the absence of extracellular Ca^{2+} (Blaustein amd Lederer, 1999). Taken together these data (efflux of Na^+, inhibitory actions of KB-R7943 and EGTA) support the involvement of the Na^+/Ca^{2+} exchanger operating in reverse mode in promoting membrane repolarisation in activated neutrophils.

Interestingly, and in agreement with previous reports (Simchowitz and Cragoe, 1988), we were unable to detect influx of Na^+ into FMLP-activated neutrophils during the early efflux of Ca^{2+} from the cytosol of FMLP-activated neutrophils. These observations demonstrate that the Na^+/Ca^+ exchanger has no major involvement, if any, in Ca^{2+} efflux from chemoattractant-activated neutrophils, its primary role being to effect membrane repolarisation.

To address the question of the possible role of the Na^+/Ca^{2+} exchanger as a primary transporter of extracellular Ca^{2+} in store refilling, we investigated the effects of SKF 96365, an inhibitor of uptake of Ca^{2+} via store-operated Ca^{2+} channels (Merritt *et al,* 1990), on influx and efflux of Ca^{2+} and Na^+ respectively, as well as on the membrane repolarisation responses of FMLP-activated neutrophils. At a concentration which did not affect release of Ca^{2+} from intracellular stores, or its subsequent efflux, SKF 96365 almost completely inhibited the store-operated influx of Ca^{2+}, but had no effects on efflux of Na^+ or membrane repolarisation. These observations do not support a primary role for the Na^+/Ca^{2+} exchanger in promoting meaningful uptake of extracellular Ca^{2+} for refilling of stores in chemoattractant-activated neutrophils, the probable function of the exchanger being to facilitate uptake of the cation by mediating membrane repolarisation. Influx of Ca^{2+} appears to occur primarily, if not almost exclusively, through store-operated Ca^{2+} channels, which is nevertheless dependent on membrane repolarisation.

This contention is supported by data using CGD neutrophils. Previous investigators have reported that influx of extracellular Ca^{2+} into chemoattractant-activated CGD neutrophils is accelerated as a consequence of the failure of these cells to undergo NADPH oxidase-mediated membrane depolarisation (Geiszt *et al,* 1997). In the current study, we have demonstrated that the accelerated uptake of Ca^{2+} by FMLP-activated CGD neutrophils is prevented by treatment of the cells with SKF 96365 (data not included), while, unlike normal neutrophils, influx of the cation into CGD cells is insensitive to KB-R7943. These

observations support the proposed primary role for the Na^+/Ca^{2+} exchanger in mediating membrane repolarisation, which in turn regulates the uptake of Ca^{2+} by store-operated Ca^{2+} channels. The Na^+/Ca^{2+} exchanger may therefore represent a novel therapeutic target for the pharmacological modulation of calcium fluxes and pro-inflammatory responses in activated human neutrophils.

In conclusion, the store-operated influx of Ca^{2+} into activated neutrophils is stringently regulated, presumably to prevent hyperactivation of the cells. The major contributors to this physiologic, anti-inflammatory process are NADPH oxidase which, by its membrane depolarising actions excludes extracellular Ca^{2+}, and the plasma membrane and endomembrane Ca^{2+}-ATPases which mediate clearance of store-derived cation. Subsequent influx of the cation, through store-operated Ca^{2+} channels is controlled by the relatively slow, restraining, membrane repolarising action of the Na^+/Ca^{2+} exchanger, enabling efficient diversion of incoming cation into stores.

CHAPTER 6

CLINICAL RELEVANCE AND CONCLUSION

6.1 The clinical relevance and therapeutic potential of cAMP-elevating agents

The lack of effective therapeutic approaches for modulating tissue injury in neutrophilic inflammation (Dallegri and Ottonello, 1997), and the apparent insensitivity of neutrophils to the anti-inflammatory actions of corticosteroids (Cox, 1995), underscores the requirement for novel anti-inflammatory chemotherapeutics which can effectively control the harmful activities of these cells. In this regard, the findings of the current study are clinically relevant and contribute significantly to the search for agents which modulate neutrophil-mediated tissue injury in a clinical setting.

The current study demonstrates the anti-inflammatory properties of selective and non-selective β-adrenoceptor agonists that suppress the pro-inflammatory activities of activated neutrophils. The anti-inflammatory potential of the various β-agonists clearly differ, with the relative potencies of these agents, as determined in this study, being isoproterenol > formoterol > epinephrine > fenoterol > norepinephrine > salbutamol > salmeterol. This suggests that, of the selective β-agonists investigated, formoterol may have the greatest anti-inflammatory potential. Numerous studies with ß-agonists *in vivo* support the contention that these agents may possess clinically significant anti-inflammatory properties. The long-acting β-agonist formeterol has been reported to reduce the number of inflammatory cells (mast cells and eosinophils) within the submucosa of asthmatic patients (Wallin *et al,* 1998) and reduces neutrophil adhesion to tracheal venules in the rat model (Bowden *et al,* 1994). Recent clinical trials have shown that the addition of inhaled formoterol to low or high doses of inhaled budesonide in patients with chronic asthma, significantly reduced the rates of acute exacerbations, as well as improving symptoms and lung function parameters over a period of one year (Pauwels *et al,* 1997). Salmeterol and formoterol (long-acting beta agonists) provide significant improvements in clinical

parameters in patients with chronic obstructive pulmonary disease (Johnson and Rennard, 2001). Both of these agents reduce neutrophil adhesion to endothelial and airway epithelial cells (Johnson and Rennard, 2001). Salmeterol has been reported to inhibit TNF-induced release of IL-8 from human airway smooth muscle cells *in vitro* and may interfere with the synthesis of platelet-activating factor (Johnson and Rennard, 2001).

Epinephrine has been shown in the current study to significantly modulate the pro-inflammatory activities of human neutrophils *in vitro*, apparently by cAMP-dependent acceleration of the restoration of Ca^{2+} homeostasis in these cells. The anti-inflammatory interactions of epinephrine with human neutrophils were observed at physiological concentrations of epinephrine. Neutrophils are down-regulated by endogenous anti-inflammatory mediators, including epinephrine and adenosine, generated at sites of inflammation (Dallegri and Ottonello, 1997). The complex interaction between stress hormones and inflammatory cells allows TNF-α and IL-1 to induce the release of stress hormones such as epinephrine and norepinephrine, which in turn may down-regulate the inflammatory response by inhibiting endotoxin-stimulated TNF-α production by mononuclear cells (Van der Poll *et al,* 1996). Epinephrine may also contribute to anti-inflammatory processes by potentiating lipopolysaccharide-induced IL-10 release from mononuclear cells (Van der Poll *et al,* 1996).

The results of the current study underscore the relationship between epinephrine-mediated anti-inflammatory effects on neutrophils and the modulation of calcium fluxes following activation of these cells. Epinephrine has been shown to hasten the clearance of cytosolic Ca^{2+} in formyl peptide activated neutrophils consequent to upregulation of the activity of the endomembrane Ca^{2+}-ATPase, which resequesters cytosolic Ca^{2+}. Epinephrine and related β-agonists down regulate neutrophil pro-inflammatory responses by elevating intracellular cAMP, with resultant activation of protein kinase A, which in turn facilitates the clearance of cytosolic Ca^{2+} in activated neutrophils.

Evidence for the anti-inflammatory properties of cyclic AMP-elevating agents *in vivo* has been reported recently. The beneficial effects of supranormal oxygen delivery to critically ill surgical patients achieved by means of catecholamine infusions, has been recognised, although the mechanism responsible for improved outcome in these patients, has been questioned (Uusaro and Russell, 2000). The anti-inflammatory properties of catecholamines, unrelated to improved oxygen delivery, have been suggested as a possible mechanism for improved survival in these patients (Uusaro and Russell, 2000). Importantly, as a result of their anti-inflammatory properties, beta agonists may enhance resolution during acute lung injury (Ware and Matthay, 2000) and have been reported to accelerate the clearance of fluid from alveoli, thus promoting healing in ARDS (Berthiaume *et al,* 1999). Beta-agonists may also activate endogenous anti-inflammatory pathways inside endothelial cells, thereby modifying pro-inflammatory responses during sepsis (Tighe *et al,* 1996). In addition, beta-2 agonists have been reported to attenuate the release of eosinophil-activating cytokines from human airway smooth muscle cells stimulated with IL-1 and TNF-α (Hallsworth *et al,* 2001). Neutrophil reactive oxidants are also primary activators of the pro-inflammatory transcription factor, nuclear factor-κB (NFκB) which may amplify immune and inflammatory responses (Barnes and Karin, 1997). Therefore, an additional anti-inflammatory property of epinephrine and related β-agonists, may reside in their ability to reduce the oxidant stress within neutrophils and in so doing, inhibit NFκB activation.

The enormous potential of cAMP-elevating agents, including epinephrine, to down-regulate the pro-inflammatory activities of human neutrophils, confirms the therapeutic and clinical relevance of these agents as modulators of neutrophil-mediated tissue injury.

Human neutrophil elastase has been strongly implicated in numerous respiratory diseases, including cystic fibrosis, emphysema, chronic bronchitis and ARDS. Elastase is able to destroy all structural components of the lung extracellular

matrix, as well as inducing mucus hypersecretion and impaction, increasing capillary permeability and enhancing IL-8 release from epithelial cells (Vender, 1996). Elastase may also promote complement activation by digesting C1-inhibitor (Dallegri and Ottonello, 1997). Therefore, agents which inhibit elastase directly, or reduce elastase release from activated neutrophils, should be of potential therapeutic benefit (Lee and Downey, 2001). Epinephrine and other β-agonists investigated in the current study are able to significantly attenuate neutrophil elastase release. Degranulation in neutrophils is associated with the release of potent chemoattractants for monocytes, T lymphocytes and B lymphocytes (Chertov *et al,* 1996; Taub *et al,* 1996), underscoring the anti-inflammatory potential of agents that modulate degranulation responses.

The reported synergy that exists between β-agonists and phosphodiesterase inhibitors (Torphy, 1998), has been confirmed in this study, and may allow optimisation of the anti-inflammatory efficacy of β-agonists by combining these agents with a selective PDE 4 inhibitor. Selective PDE 4 inhibitors are reported to suppress acute lung injury in mice (Miotla *et al,* 1998) and attenuate airway inflammation in experimental asthma in guinea pigs (Underwood *et al,* 1998). In addition, isoproterenol in combination with the PDE inhibitor IBMX significantly inhibited neutrophil adhesion to human bronchial epithelial cells (Bloemen *et al,* 1997).

The marked abnormalities in calcium homeostasis in activated neutrophils from patients with chronic granulomatous disease have been demonstrated in this study. The hyperactivation of CGD neutrophils secondary to dysregulation of Ca^{2+} homeostasis and accelerated Ca^{2+} influx following stimulation, leads to enhancement of several pro-inflammatory responses. The failure of membrane depolarisation in CGD neutrophils is associated with Ca^{2+} overload due to accelerated influx of the cation. This suggests that the depolarisation response observed in activated neutrophils from normal subjects may down-regulate the pro-inflammatory activity of these cells. Phosphodiesterase 4 inhibitors have

been shown in the current study to reverse the abnormal calcium homeostasis observed in CGD neutrophils. The 'paradoxical' granulomatous inflammation that may occur in patients with CGD, highlights the relationship between disordered Ca^{2+} regulation and the pro-inflammatory activity of neutrophils. This abnormal granulomatous inflammatory response may be ideally suited to treatment with phosphodiesterase inhibitors which reverse the intrinsic abnormality.

An additional original and potentially important observation is that membrane repolarisation in FMLP-activated neutrophils is apparently mediated by the plasma membrane Na^+/Ca^{2+} exchanger operating in reverse mode. Sodium ions are extruded from the cell in exchange for Ca^{2+} ions, an electrogenic process mediating membrane repolarisation and facilitating the controlled uptake of Ca^{2+} into neutrophils. Membrane repolarisation is initiated by the Na^+/Ca^{2+} exchanger with calcium uptake from extracellular reservoirs occurring predominantly via store-operated mechanisms with a trivial contribution from the Na^+/Ca^{2+} exchange mechanism.

The results of this study have enabled me to propose an integrated model of restoration of calcium homeostasis in formyl peptide-activated human neutrophils, which is represented schematically in the accompanying figure (Figure 6.1, page 171).

Following receptor-mediated activation of human neutrophils, there is an abrupt and short-lived increase in the concentration of cytosolic free Ca^{2+}, an event which precedes and is a prerequisite for initiation of the pro-inflammatory activities of these cells. The peak increase in cytosolic Ca^{2+} subsides rapidly thereafter reaching base-line values within 3-5 minutes. Restoration of Ca^{2+} homeostasis in activated neutrophils is essential to prevent Ca^{2+} overload and hyperactivity of these cells, as demonstrated in neutrophils from patients with CGD. This is dependent on the efficient clearance of Ca^{2+} from the cytosol of the cells and is accomplished through the action of the plasma membrane Ca^{2+}-

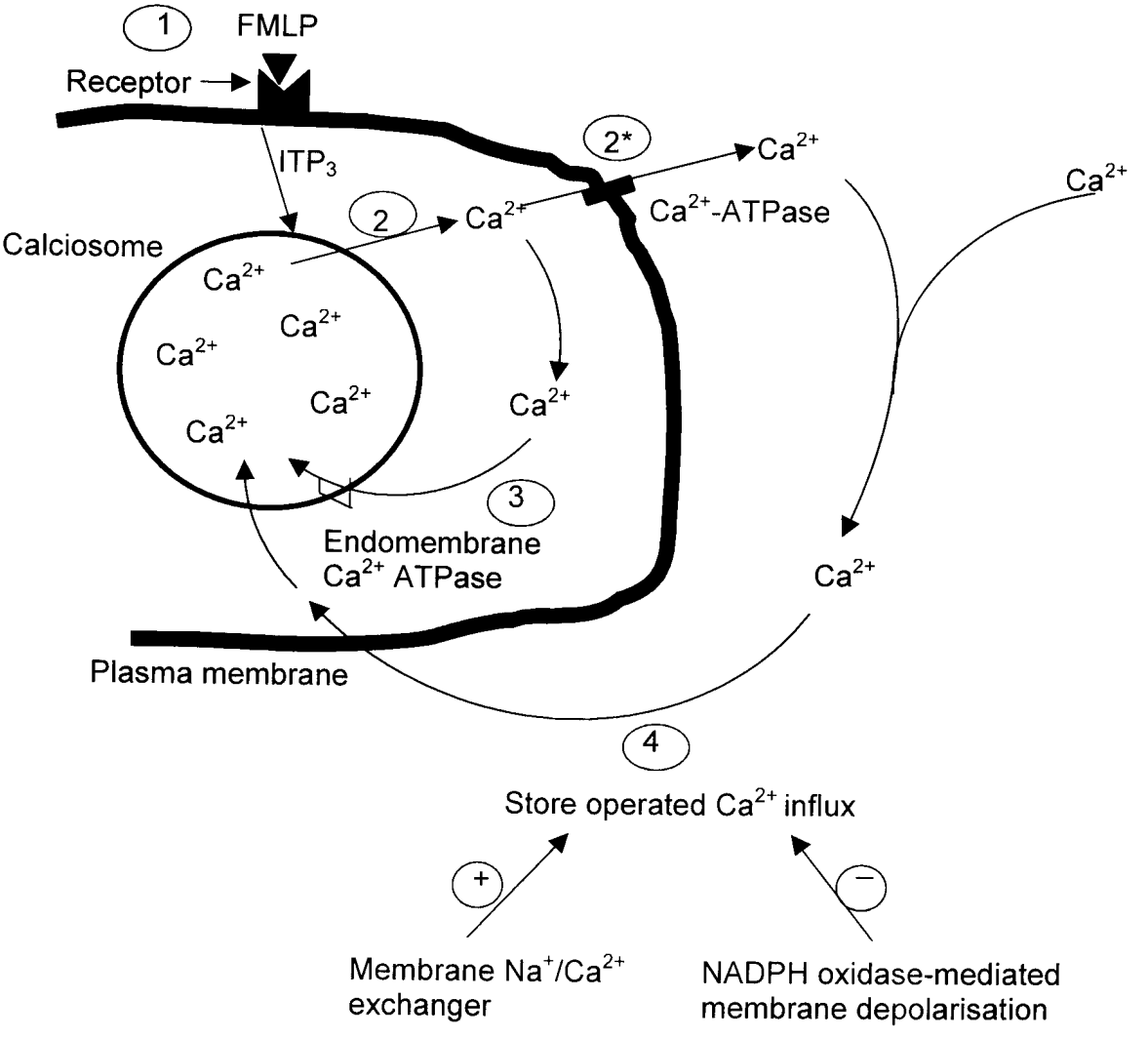


Figure 6.1: Mechanisms of release of Ca^{2+} from intracellular stores and clearance of cytosolic Ca^{2+} in FMLP-activated neutrophils: 1) neutrophil activation following FMLP binding to its receptor and generation of inositol triphosphate (ITP_3) 2) immediate release of Ca^{2+} from calciosomes into the cytosol 2*) concomitant efflux of Ca^{2+} across the plasma membrane (Ca^{2+}-ATPase mediated) 3) early re-uptake of Ca^{2+} into calciosomes mediated by the endomembrane Ca^{2+}-ATPase 4) delayed (60 sec – 5 min) store-operated influx of extracellular Ca^{2+} to refill intracellular stores.

ATPase, a Ca^{2+}-efflux pump, and the endo-membrane Ca^{2+}-ATPase which resequesters the cation back into intracellular stores. Operating in unison, these two Ca^{2+} pumps are the major effectors of clearance of the cation from the cytosol. The endomembrane Ca^{2+}-ATPase may be upregulated by cAMP-dependent activation of protein kinase A which accelerates Ca^{2+} resequestration into storage vesicles. The plasma membrane Ca^{2+}-ATPase is modulated by calmodulin which shifts the pump to a higher affinity state for Ca^{2+}, resulting in enhanced maximal velocity. A dramatic and transient (over a 30 second time course) efflux of Ca^{2+} immediately follows release of the cation from stores in FMLP-activated neutrophils which results in discharge of about 50% of cell-associated cation.

The subsequent influx of extracellular cation, primarily for refilling of stores, is delayed and occurs at around 1 minute after the addition of FMLP (when peak pro-inflammatory activity has subsided), terminating at around 5 minutes. This type of calcium uptake is characteristic of a store-operated Ca^{2+} influx. Delayed Ca^{2+} influx allows efficient clearance of store-derived cytosolic Ca^{2+}, unhampered by concomitant influx of extracellular cation. The precise molecular mechanisms that regulate store-operated calcium influx are not currently known, but the alterations in membrane potential which accompany NADPH oxidase activation play an important role in limiting the influx of extracellular Ca^{2+}. When the cells are depolarised, the driving force for Ca^{2+} entry is abolished as the electrical component of the electrochemical gradient is not favourable for Ca^{2+} entry.

Membrane repolarisation in FMLP-activated neutrophils becomes evident at around 1 minute after exposure to the chemoattractant and proceeds gradually over a 5-10 minute time course with the kinetics of membrane repolarisation superimposable on those of influx of extracellular calcium. Gradual repolarisation and consequent carefully regulated influx of Ca^{2+} ensure efficient diversion of incoming cation into stores by the endo-membrane Ca^{2+}-ATPase, thereby preventing flooding of the cytosol with Ca^{2+}. Membrane repolarisation is

mediated by the electrogenic Na^+/Ca^{2+} exchanger operating in reverse mode, which facilitates the net movement of a positive charge when 3 Na^+ are exchanged for 1 Ca^{2+}. Its activity is upregulated by binding of Ca^{2+} to a high-affinity regulatory site on the cytosolic side of the exchanger, promoting sodium efflux and calcium influx following Ca^{2+} release from storage vesicles. This sensitivity of the plasma membrane Na^+/Ca^{2+} exchanger to increases in cytosolic calcium concentrations, although paradoxical, may facilitate Ca^{2+} influx following emptying of intracellular calcium stores, while concomitantly mediating the electrogenic recovery of the membrane potential towards resting levels. The major function of the exchanger is to facilitate Ca^{2+} uptake, with influx of Ca^{2+} occurring primarily, if not exclusively, through store-operated Ca^{2+} channels. Apparently, an important physiologic function of NADPH oxidase, in addition to the generation of reactive oxidants, is to indirectly regulate store-operated calcium influx through membrane depolarisation, thereby down-regulating the pro-inflammatory activity of activated neutrophils.

In conclusion these novel insights into the mechanisms utilised by chemoattractant-activated human neutrophils to restore Ca^{2+} homeostasis, have identified several potential targets for neutrophil-directed anti-inflammatory chemotherapy. These are the endo-membrane Ca^{2+}-ATPase, the Na^+/Ca^{2+} exchanger and store-operated Ca^{2+} channels. While selective targeting (up-regulation) of the endo-membrane Ca^{2+}-ATPase by cAMP-elevating, pharmacologic agents may be possible, selective inhibition of the Na^+/Ca^{2+} exchanger and store-operated Ca^{2+} channels in neutrophils may be more difficult.

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