9. An International Classification of Retinopathy of Prematurity. Committee for the Classification of Retinopathy of Prematurity. *Arch Ophthalmol* 1984; **102:** 1130-1134.
9. Phelps DL. Retinopathy of prematurity: An estimate of vision loss in the United States — 1979. *Pediatrics* 1981; **67:** 924-926.
10. Palmer EA, Flynn JT, Hardy RJ, *et al.* Incidence and early course of retinopathy of prematurity. *Ophthalmology* 1991; **98:** 1628-1640.
11. Saunders RA, Donahue ML, Christman LM, *et al.* Racial variation in retinopathy of prematurity. *Arch Ophthalmol* 1997; **115:** 604-608.
12. Kirsten GF, van Zyl JI, le Grange M, Ancker E, van Zyl F. The outcome at 12 months of very-low-birth-weight infants ventilated at Tygerberg Hospital. *S Afr Med J* 1995; **85:** 649-654.
13. O'Sullivan J, Gilbert C, Foster A. The causes of childhood blindness in South Africa. *S Afr Med J* 1997; **87:** 1691-1695.
14. Ballard JL, Khoury JC, Wedig K, *et al.*. New Ballard Score, expanded to include extreme premature infants. *J Pediatr* 1991; **119:** 417-423.
15. Fletcher MA, ed. *Physical Diagnosis in Neonatology*. Philadelphia: Lippincott-Raven Press, 1998: 57.
16. Reynolds JD, Hardy RJ, Kennedy KA, *et al.* Lack of efficacy of light reduction in preventing retinopathy of prematurity. Light Reduction in Retinopathy of Prematurity (LIGHT-ROP) Cooperative Group. *N Eng J Med* 1998; **338:** 1572-1576.
17. Cryotherapy for Retinopathy of Prematurity Cooperative Group. Multicenter trial of cryotherapy for retinopathy of prematurity: three month outcome. *Arch Ophthalmol* 1990; **108:** 195-204.
18. Friling R, Rosen SD, Monos T, Karplus M, Yassur Y. Retinopathy of prematurity in multiple-gestation, very low birth weight infants. *J Pediatr Ophthalmol Strabismus* 1997; **34:** 96-100.
19. Brown BA, Thach AB, Song JC, Marx JL, Kwun RC, Frambach DA. Retinopathy of prematurity: evaluation of risk factors. *Int Ophthalmol* 1998; **22:** 279-283.
20. Fellows RR, McGregor ML, Bremer DL, Rogers GL, Miller D. Retinopathy of prematurity in discordant twins. *J Pediatr Ophthalmol Strabismus* 1995: **32:** 86-88.

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REGULATION OF CALCIUM HOMEOSTASIS IN ACTIVATED HUMAN NEUTROPHILS — POTENTIAL TARGETS FOR ANTI-INFLAMMATORY THERAPEUTIC STRATEGIES

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Objectives. The objectives of the current study were to: *(i)* present an integrated model for the restoration of calcium homeostasis in activated human neutrophils based on current knowledge and recent research; and *(ii)* identify potential targets for the modulation of calcium fluxes in activated neutrophils based on this model and to investigate the effects of intracellular probes which target key processes involved in calcium homeostasis and pro-inflammatory activity in these cells.

Design and setting. Laboratory-based experimental research using purified human neutrophils from healthy, adult human volunteers.

Outcome measures. Calcium metabolism and pro-inflammatory activity of neutrophils.

Results. Modulation of calcium fluxes in activated human neutrophils can be achieved by cAMP-dependent upregulation of the activity of the endomembrane Ca^{2+}-ATPase which resequesters cytosolic Ca^{2+}. Formoterol, a long-acting β_2-agonist, elevates intracellular cAMP levels, accelerates Ca^{2+} restoration in activated neutrophils and downregulates the pro-inflammatory responses of these cells. Alterations in the membrane potential of activated neutrophils may play a role in regulating calcium reuptake into the cells as attenuation of the membrane depolarisation response is associated with accelerated calcium influx.

Conclusions. Modulation of the activity of the endomembrane Ca^{2+}-ATPase in human neutrophils represents an important target for anti-inflammatory

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therapeutic strategies, while new insights into the role played by membrane depolarisation in regulating calcium fluxes in these cells may also facilitate development of novel anti-inflammatory agents directed against neutrophils.

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Neutrophils, as professional phagocytes, constitute the most abundant component of the human leucocyte population and although essential for host defence against invading microbes, may also damage normal host cells and tissues during the inflammatory response. Activated neutrophils secrete a variety of toxic molecules including reactive oxidants, proteolytic enzymes such as elastase, as well as bioactive lipids, all of which may mediate significant tissue injury when the inflammatory response is inadequately downregulated.[1] Neutrophils have been implicated in the pathogenesis of diverse diseases including chronic obstructive pulmonary disease (COPD), acute respiratory distress syndrome (ARDS), rheumatoid arthritis, vasculitis, sepsis, multi-organ failure and myocardial reperfusion injury.[2]

Calcium plays a vital role as an intracellular second messenger leading to optimal activation of neutrophils in response to numerous activating stimuli such as bacterial N-formylated polypeptide chemoattractants. Alterations in the cytosolic calcium concentration of neutrophils regulate the activities of key intracellular proteins and enzymes, which ultimately determine the magnitude of the pro-inflammatory responses of these cells to activating stimuli.[3] Termination of pro-inflammatory activity is dependent on the restoration of cytosolic calcium towards basal levels. Therefore, dysregulation of calcium metabolism in activated neutrophils may potentiate tissue injury during the systemic inflammatory response. In contrast, appropriate modulation of calcium fluxes in activated neutrophils may downregulate pro-inflammatory activity.

Unlike other immune and inflammatory cells, the mature human neutrophil is relatively resistant to the direct anti-inflammatory actions of corticosteroids. Various mechanisms, including paucity of mitochondria (confers resistance to steroid-induced apoptosis) and high cytosolic concentrations of the inactive β-isoform of the glucocorticoid receptor, may account for the relative insensitivity of these cells to corticosteroids,[4,5] and may underpin the often unsatisfactory therapeutic response to these agents in neutrophil-mediated inflammatory disorders such as ARDS, cystic fibrosis, COPD, bronchiectasis and certain types of bronchial asthma. This relative insensitivity of neutrophils to corticosteroids has stimulated the search for alternative agents which may effectively modulate neutrophil effector responses.

REGULATION OF CALCIUM FLUXES IN ACTIVATED HUMAN NEUTROPHILS

An integrated model for the restoration of calcium homeostasis in chemoattractant-activated human neutrophils, represented schematically in Fig. 1, is discussed below.

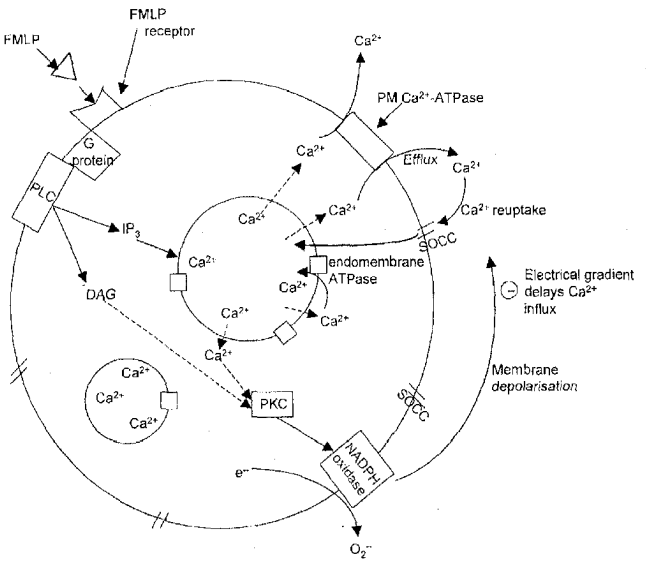

Fig. 1. Calcium metabolism in FMLP-activated human neutrophils. Following phospholipase C (PLC) activation by FMLP, inositol triphosphate (IP_3) is generated which mediates Ca^{2+} mobilisation from intracellular stores. The activity of the plasma membrane Ca^{2+}-ATPase efflux pump is upregulated almost immediately and operating in unison with the Ca^{2+}-resequestering endomembrane ATPase, restores cytosolic Ca^{2+} towards basal levels. Calcium influx from the extracellular fluid via store-operated calcium channels (SOCCs) is delayed by plasma membrane depolarisation which accompanies protein kinase C (PKC)-mediated activation of NADPH-oxidase.

The cytosolic calcium concentration in unstimulated neutrophils is maintained at an extremely low level of ~ 100 nM, in contrast to the extracellular calcium concentration in the millimolar range.[6] Low basal cytosolic calcium concentrations in resting cells are achieved by storing calcium inside specialised vesicles (calciosomes),[6] while calcium pumps located on the plasma membrane (plasma membrane Ca^{2+}-ATPases) extrude excess cytosolic Ca^{2+}.[7] Activation of neutrophils by Ca^{2+}-mobilising stimuli (formyl peptides, leukotriene B_4, interleukin-8 (IL-8), platelet-activating factor (PAF) and complement fragment C5a), generates inositol triphosphate (IP_3) and diacylglycerol (DAG) from hydrolysis of membrane phospholipids via G-protein coupled activation of phospholipase C. Inositol triphosphate rapidly mobilises Ca^{2+} from intracellular storage sites with an associated abrupt increase in the cytosolic Ca^{2+} concentration which peaks in 10 - 15 seconds.[8] Following this rapid peak, the cytosolic calcium concentration is gradually restored towards basal levels (Fig. 2).

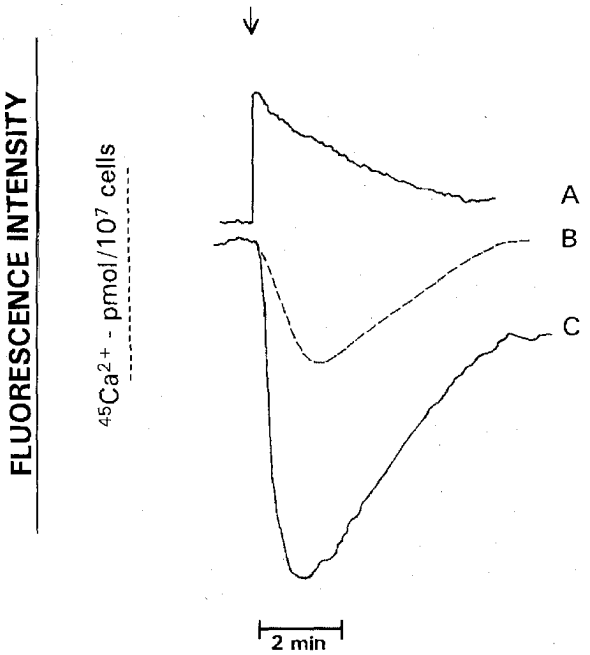

Fig. 2. Comparative time course of the fura-2 fluorescence response (A), radiometric $^{45}Ca^{2+}$ efflux and influx (- - - B) and alterations in membrane potential (C), which accompany FMLP-mediated activation (↓) of human neutrophils (traces show responses of neutrophils from the same subject).

This restoration of calcium homeostasis is essential for preventing Ca^{2+} flooding of the cytosol and allowing refilling of storage vesicles with Ca^{2+}. Calcium reuptake into storage vesicles is mediated by the endomembrane Ca^{2+}-ATPase which is sensitive to upregulation by cAMP-dependent protein kinase.[9] In addition to calcium reuptake and resequestration into calciosomes, other mechanisms exist for the clearance of cytosolic Ca^{2+} following receptor-mediated activation of neutrophils. These include the Ca^{2+} efflux pump (plasma membrane Ca^{2+}-ATPase), the activity of which is upregulated by rising cytosolic calcium concentrations, mediating rapid Ca^{2+} efflux from the cell,[6] as well as membrane depolarisation, an event which accompanies activation of neutrophils.

Elevated cytosolic Ca^{2+} levels are required for optimal DAG-mediated activation of protein kinase C[10] with consequent assembly and activation of membrane-associated NADPH oxidase which generates reactive oxygen species as electrons are transferred vectorially across the plasma membrane to molecular oxygen.[11] This vectorial flux of electrons leads to a significant membrane depolarisation response from a resting potential of ~ -60 mV to greater than 0 mV (Fig. 2, C). Depolarisation of the plasma membrane creates an electrical gradient unfavourable for Ca^{2+} entry,[12] thereby excluding influx of Ca^{2+} from extracellular reservoirs. Calcium influx from extracellular pools is delayed and occurs via plasma membrane store-operated calcium channels (SOCCs) which open in response to the discharge of Ca^{2+} from storage vesicles.[13] This characteristic delayed reuptake of extruded Ca^{2+} via store-operated channels begins only with the onset of membrane repolarisation (~ 1 minute after cellular activation). Once initiated, the kinetics of Ca^{2+} influx parallel those of membrane repolarisation suggesting a mechanistic interdependence of these events (Fig. 2). Membrane repolarisation in N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-activated neutrophils becomes evident at around 1 minute after exposure to the chemoattractant and proceeds gradually over a 5 - 10-minute time course. Gradual repolarisation and consequent carefully regulated influx of Ca^{2+} ensure efficient diversion of incoming cation into stores by the endomembrane Ca^{2+}-ATPase, thereby preventing flooding of the cytosol with Ca^{2+}.

POTENTIAL TARGETS FOR ANTI-INFLAMMATORY THERAPEUTIC STRATEGIES

Based on the model for regulation of calcium fluxes outlined above, restoration of calcium homeostasis in activated neutrophils is achieved primarily by means of three important mechanisms. Firstly, the Ca^{2+}/calmodulin-dependent plasma membrane Ca^{2+}-ATPase efflux mechanism which pumps cytosolic Ca^{2+} out of the cell. Secondly, the endomembrane Ca^{2+}-ATPase resequesters cytosolic Ca^{2+} back into storage vesicles. Thirdly, the reuptake of Ca^{2+} via store-operated calcium channels is delayed by the electrical gradient created by membrane depolarisation, thereby facilitating efficient clearance of cytosolic Ca^{2+}. Each of these mechanisms may be manipulated pharmacologically in order to determine their relative importance and contribution towards restoring calcium homeostasis in activated neutrophils.

The activity of the endomembrane ATPase may be altered pharmacologically by β-adrenergic agonists. A previous study from our laboratory has demonstrated that, of a range of selective β_2-agonists tested, the agent that caused greatest increase in neutrophil intracellular cAMP levels, was formoterol.[14] Formoterol, a long-acting, selective β_2-agonist, elevates intracellular cAMP concentrations[14] with consequent modulation of the activity of the endomembrane ATPase which is upregulated by cAMP-dependent protein kinase. The intracellular concentration of cAMP rises briefly following activation of neutrophils with formyl peptides,[15] with cAMP rapidly hydrolysed by intracellular phosphodiesterases. This physiological elevation of intracellular cAMP levels may be potentiated by β-adrenergic agonists, such as formoterol, acting via G-protein-coupled receptors. Salmeterol is another long-acting selective β_2-agonist. However, this agent was not used in this study, as its effects are complicated by membrane-stabilising properties.

The membrane depolarisation response observed in FMLP-activated neutrophils may be indirectly manipulated by inhibiting superoxide production with an agent such as wortmannin. Wortmannin inhibits phosphatidylinositol 3-

kinase,[16] an important enzyme in the signal transduction pathways leading to activation of NADPH oxidase. Inhibition of the oxidase markedly attenuates oxidant generation and therefore the magnitude of membrane depolarisation.

Investigation of the effects of modulation of the activity of the endomembrane Ca^{2+}-ATPase and the magnitude of membrane depolarisation, provide insights into the roles played by the endomembrane ATPase and membrane potential during the restoration of calcium homeostasis in activated neutrophils. In addition, the effects of modulation of calcium fluxes on neutrophil pro-inflammatory responses (superoxide production and elastase release) can be determined. For these investigations, we have selected formoterol and wortmannin as appropriate intracellular probes. The effects of formoterol (1 μM) and wortmannin (50 - 100 nM) on neutrophil superoxide production by FMLP-activated neutrophils, as well as the effects of formoterol on elastase release from FMLP/cytochalasin B (CB)-activated neutrophils were investigated by means of spectrofluorimetric and colourimetric procedures respectively.

Formoterol significantly inhibited superoxide production by FMLP-activated neutrophils with similar effects on elastase release from FMLP/CB-activated cells (28% and 26% inhibition respectively, $P < 0.05$). Superoxide production by activated neutrophils in the presence of wortmannin was markedly reduced to 20% of that observed in control cells ($P < 0.005$).

Neutrophils activated with stimuli, such as phorbol myristate acetate, which do not mobilise stored calcium, are insensitive to the inhibitory effects of cAMP-elevating agents such as formoterol. This suggests that alterations in calcium fluxes may explain the inhibitory effects of formoterol on cells activated with Ca^{2+}-mobilising stimuli.

The fura-2 fluorescence response is a sensitive indicator of cytosolic calcium concentration changes over time and the responses of human neutrophils to FMLP and PAF, in the presence and absence of formoterol, are shown in Fig. 3.

Activation of neutrophils with FMLP resulted in an abrupt increase in fura-2 fluorescence intensity that coincided with the release of Ca^{2+} from intracellular stores into the cytosol, and quickly subsided returning to baseline values after several minutes. The magnitude of the peak fluorescence intensity following Ca^{2+} release from storage vesicles was unaffected by formoterol (1 μM), but the decline in peak fluorescence intensity was significantly accelerated, indicating more rapid clearance of cytosolic Ca^{2+} than that observed in control cells. The time taken for the peak fluorescence intensity to decline to half peak values was significantly reduced from 1.70 ± 0.03 minutes (control cells) to 1.00 ± 0.03 minutes in the presence of formoterol ($P < 0.005$). The corresponding clearance rate of free cytosolic Ca^{2+} was significantly accelerated from 99 ± 6 pmol/min (control cells) to 160 ± 8 pmol/min (formoterol-treated cells) ($P < 0.05$), representing a 60% increase in the clearance rate of Ca^{2+}.

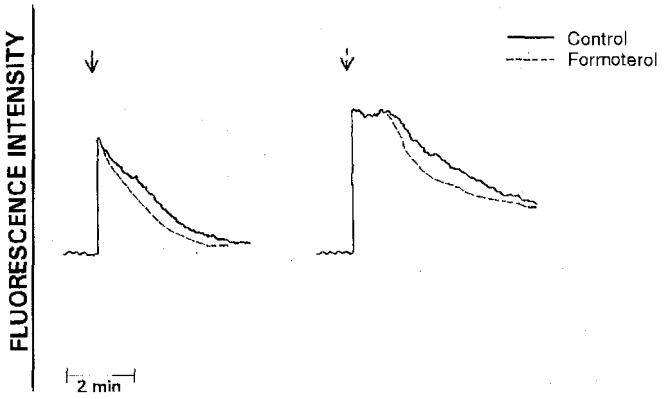

Fig. 3. The effects of formoterol (1 μM) on the time course of the fura-2 fluorescence response of FMLP-(A) and PAF-activated (B) neutrophils from two different subjects. FMLP (↓) and PAF (↓) were added as indicated when a stable baseline was obtained (± 1 minute).

Activation of neutrophils with PAF was also associated with an immediate rise in fura-2 fluorescence intensity, followed by a plateau phase of ~ 1 minute duration. Subsequent to this plateau, fura-2 fluorescence intensity declined gradually towards basal levels over ~ 3 minutes. Pre-incubation of neutrophils with formoterol did not alter peak fluorescence intensity, but significantly accelerated the rate of decline to pre-activation levels. The absolute decrement in the concentration of cytosolic Ca^{2+} 2 minutes after addition of PAF was significantly greater in formoterol-treated cells, at 61 ± 4 pmol/6 x 10^6 cells versus 41 ± 4 pmol/6 x 10^6 cells (control system), indicative of hastened clearance of cytosolic Ca^{2+} ($P < 0.05$).

Accelerated Ca^{2+} clearance following neutrophil activation can be achieved by: *(i)* enhancement of the immediate efflux of Ca^{2+} from the cytosol; *(ii)* reducing the magnitude of the delayed store-operated influx of Ca^{2+}; or *(iii)* acceleration of 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 in formoterol-treated cells, Ca^{2+} fluxes in resting and activated neutrophils were measured, using $^{45}Ca^{2+}$ as tracer to label the intracellular Ca^{2+} pool to determine the magnitude of $^{45}Ca^{2+}$ efflux from and influx into activated neutrophils, with and without formoterol.

For efflux experiments, neutrophils were pre-loaded with $^{45}Ca^{2+}$, then washed and transferred to Ca^{2+}-replete Hanks balanced salt solution (HBSS) (to minimise reuptake of radiolabelled cation) followed by activation with FMLP and measurement of the amount of remaining cell-associated $^{45}Ca^{2+}$ 60 seconds after addition of FMLP, at which time efflux is complete.

Formoterol 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 ± 7 pmol/10^7 cells for formoterol-treated cells ($P < 0.005$, $N = 6$). This represents a 28% reduction in efflux of cell-associated $^{45}Ca^{2+}$.

Measurement of the net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils, measured over a fixed 5-minute time-course, revealed a substantial influx of $^{45}Ca^{2+}$, while there was only trivial influx of the radiolabelled cation into identically processed neutrophils not exposed to FMLP (28 ± 2 pmol/10^7 cells). Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils pretreated with formoterol, was significantly reduced from 155 ± 12 pmol/10^7 cells (untreated neutrophils) to 78 ± 7 pmol/10^7 cells (formoterol-treated neutrophils) ($P < 0.005$, $N = 11$).

Membrane depolarisation has been reported to play an important role in regulating neutrophil calcium homeostasis by inhibiting and delaying store-operated calcium influx. This suggests that the opposite may also occur when the membrane depolarisation response is significantly attenuated in activated neutrophils, predisposing to flooding of the cytosol by Ca^{2+}. Wortmannin, which inhibits superoxide production in FMLP-activated neutrophils has been used to investigate the relationship between NADPH oxidase, membrane potential and Ca^{2+} fluxes in these cells.

The significant inhibition of superoxide generation by FMLP-activated neutrophils in the presence of wortmannin, was associated with a corresponding attenuation of the magnitude of the membrane depolarisation response from 80 ± 6 mV for control cells to 36 ± 4 mV for wortmannin-treated cells ($P < 0.005$, $N = 6$), supporting the interdependence of these two events.

Pre-incubation of neutrophils with wortmannin (50 nM) did not affect the initial abrupt increase in fura-2 fluorescence, which accompanies activation of the cells with FMLP. However, the subsequent steady decline in fura-2 fluorescence intensity observed in untreated neutrophils was delayed for about 1 minute in wortmannin-treated cells (Fig. 4).

The effect of wortmannin on the rate of Ca^{2+} influx into FMLP-activated neutrophils was determined using radiometric procedures. The magnitude of $^{45}Ca^{2+}$ influx into control neutrophils 2 minutes after activation with FMLP was 56 ± 1 pmol/10^7 cells and 83 ± 1 pmol/10^7 cells into neutrophils pre-incubated with wortmannin ($P < 0.05$). The corresponding values 3 minutes after addition of FMLP were 83 ± 4 pmol/10^7 and 99 ± 2 pmol/10^7 cells for control and wortmannin-treated neutrophils, respectively ($P < 0.05$). These results were confirmed by means of the Mn^{2+} quenching of fura-2 fluorescence assay (Fig. 4). Following a delay of about 30 seconds after addition of FMLP, fura-2 fluorescence intensity declines as Mn^{2+} enters the cell via store-operated calcium channels. In the presence of wortmannin, the decline in fura-2 fluorescence intensity (and therefore Ca^{2+} influx) occurred earlier and at a significantly greater rate than that observed in untreated neutrophils. The absolute change in fluorescence intensity measured 2 minutes following addition of FMLP was

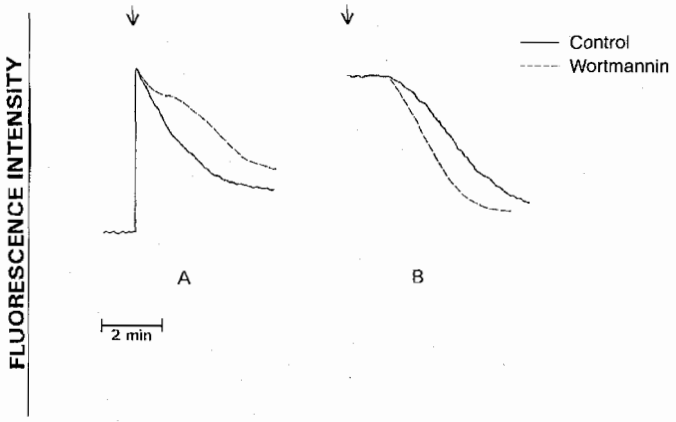

Fig. 4. The effects of wortmannin (50 nM) on the fura-2 fluorescence response (A) and on the Mn^{2+} quenching of fura-2 fluorescence (B), in FMLP-activated neutrophils. Typical tracings from the same subject are shown with FMLP added as indicated (↓) when a stable baseline was obtained (± 1 minute).

2.1 ± 0.03 cm (control cells) and 2.8 ± 0.05 cm (wortmannin-treated cells) ($P < 0.05$), while those measured 3 minutes after addition of FMLP were 3.1 ± 0.1 cm and 4.1 ± 0.08 cm ($P < 0.05$) in the absence and presence of wortmannin respectively ($N = 6$).

Discussion

Treatment of neutrophils with formoterol inhibited neutrophil pro-inflammatory activity (superoxide production and elastase release), without any effect on the abrupt increase in cytosolic Ca^{2+}, which accompanies activation with FMLP. This observation demonstrates that formoterol 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 formoterol-treated neutrophils, indicative of hastened clearance of Ca^{2+} from the cytosol. Accelerated clearance of Ca^{2+} is not due to enhancement of efflux, but occurs in the setting of decreased Ca^{2+} efflux observed using radiometric procedures. In addition, the amount of Ca^{2+} which entered FMLP-activated neutrophils during store-operated influx of the cation was significantly reduced in formoterol-treated cells. Together with the results of the fura-2 experiments, the observations that formoterol attenuates both FMLP-activated efflux and store-operated influx of Ca^{2+} suggests that this agent upregulates the activity of the cAMP-dependent protein kinase-activatable endomembrane Ca^{2+}-ATPase. This may explain the decreased efflux of Ca^{2+} as the upregulated endomembrane Ca^{2+}-ATPase and the plasma membrane Ca^{2+}-ATPase compete for cytosolic Ca^{2+}. Enhanced activity of the endomembrane ATPase results in rapid refilling of stores due to enhanced resequestration of cytosolic Ca^{2+},

leading to a reduction in the magnitude of store-operated Ca^{2+} influx required for refilling of storage vesicles.

The effects of formoterol on the fura-2 fluorescence responses in PAF-activated neutrophils are similar to those observed in FMLP-activated cells, again indicative of accelerated resequestration of Ca^{2+} into calciosomes by the upregulated endomembrane ATPase.

The relationship between NADPH-oxidase dependent alterations in membrane potential and maintenance of calcium homeostasis was investigated using FMLP-activated neutrophils from control and wortmannin-treated cells. Wortmannin markedly reduced the membrane-depolarisation response in FMLP-activated cells with an associated alteration in cytosolic Ca^{2+} transients. The delayed early clearance of cytosolic Ca^{2+} in FMLP-activated wortmannin-treated cells occurs in the setting of attenuation of the electrical gradient across the plasma membrane and accelerated Ca^{2+} influx. These observations suggest that reduction in the magnitude of membrane depolarisation in FMLP-activated, wortmannin-treated neutrophils, facilitates accelerated Ca^{2+} influx from extracellular reservoirs with concomitant prolongation of Ca^{2+} transients.

CLINICAL RELEVANCE

Neutrophil recruitment to sites of inflammation or infection may be followed by activation of these cells with release of an array of toxic molecules that cannot discriminate between invading pathogens and normal tissues. This lack of discriminatory power renders normal host cells susceptible to attack during neutrophilic inflammation.

Therefore, modulation of neutrophil-mediated tissue injury remains an important therapeutic goal. In this regard, cAMP-elevating agents possess significant anti-inflammatory properties[17] consequent to the ability of these agents to modulate calcium fluxes effectively in activated neutrophils. Evidence for the anti-inflammatory properties of cAMP-elevating agents continues to accumulate. 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.[18] The anti-inflammatory properties of catecholamines, unrelated to improved oxygen delivery, have been suggested as a possible mechanism for improved survival in these patients.[18] Beta-agonists may also activate endogenous anti-inflammatory pathways inside endothelial cells, thereby modifying pro-inflammatory responses during sepsis.[19]

Neutrophil reactive oxidants are also primary activators of the pro-inflammatory transcription factor, nuclear factor-κB (NFκB) which may amplify immune and inflammatory responses.[20] Therefore, an additional anti-inflammatory property of cAMP-elevating agents may reside in their ability to reduce the oxidant stress within neutrophils, and in so doing inhibit NFκB activation. Rolipram, a phosphodiesterase 4 inhibitor that elevates intracellular cAMP levels, has been reported to inhibit tumour necrosis factor-α (TNF-α)-induced NFκB activation in T-lymphocytes.[21]

Formoterol, a long-acting β_2-agonist, alters calcium fluxes in activated human neutrophils with associated downregulation of the pro-inflammatory responses of these cells *in vitro* (as demonstrated in the current study). The anti-inflammatory properties of long-acting β_2-agonists may also extend to other important cell types. Formoterol potentiates the inhibitory effects of budesonide on the expression of adhesion molecules and release of granulocyte macrophage colony-stimulating factor (GM-CSF) by lung fibroblasts stimulated with IL-8,[22] and similarly inhibits GM-CSF production by human bronchial epithelial cells in the presence of TNF-α.[23] Formoterol also enhances the clearance of alveolar oedema fluid in a murine model of acute lung injury.[24] 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.[25]

The anti-inflammatory interactions of long-acting β_2-agonists with inflammatory cells appear to be operative *in vivo* in patients with inflammatory airway disorders such as bronchial asthma and COPD. Formoterol inhalation therapy, when administered to asthmatic patients, reduces the number of mast cells and eosinophils within the submucosa.[26,27] Recent clinical trials have demonstrated reduced rates of acute exacerbations and fewer symptoms in patients with chronic asthma, as well as improved lung function parameters over a period of 1 year, when inhaled formoterol is combined with low or high doses of inhaled corticosteroids.[28,29] In this setting, salmeterol and formoterol improve the quality of life of asthmatic patients[30] and their clinical efficacy is characterised by few significant side-effects and good tolerability.[31]

The role of inhaled long-acting β_2-agonists in the treatment of COPD is becoming increasingly important[32] with these agents able to alleviate symptoms significantly in COPD patients.[25] Possible mechanisms for the beneficial effects of these agents, in addition to bronchodilatation, include inhibition of neutrophil adhesion to endothelial cells, decreased neutrophil activation, as well as induction of neutrophil apoptosis.[25] Salmeterol possesses intrinsic membrane stabilising properties that may confer additional therapeutic benefits.[33] Formoterol may be more effective and better tolerated than theophylline in stable COPD[32,34] and is gaining acceptance as first-line bronchodilator therapy for this disease.[32] It should be emphasised, however, that in patients with asthma, long-acting β_2-agonists should be added to low or moderate doses of inhaled corticosteroids which form the mainstay of asthma management.

Although downregulation of β-adrenergic receptors may theoretically limit the anti-inflammatory potential of these

agents, this does not appear to be of clinical significance as formoterol retained its efficacy throughout the Formoterol and Corticosteroids Establishing Therapy (FACET) trial.[27] In addition, newer generation phosphodiesterase 4 inhibitors, with enhanced inflammatory cell selectivity, if used in combination with β_2-agonists, may potentiate the anti-inflammatory activity of cAMP-elevating agents.[35]

Modulation of the membrane depolarisation response in activated neutrophils leads to accelerated calcium influx into wortmannin-treated neutrophils *in vitro*. Recent studies of neutrophils from patients with chronic granulomatous disease (CGD), have provided confirmatory evidence for the role of membrane depolarisation in regulating calcium influx into activated neutrophils.[36] Accelerated calcium reuptake by CGD neutrophils in the setting of marked attenuation of membrane depolarisation responses (resembling those observed in wortmannin-treated cells), results in exaggerated release of proteolytic enzymes and excessive production of bioactive phospholipids.[36] *In vivo*, patients with CGD exhibit abnormal granulomatous reactions. These may result from hyperactivation of phagocytes as demonstrated *in vitro*, and are exquisitely sensitive to corticosteroids. Importantly, neutrophil primary granules also contain chemoattractants for T-lymphocytes and monocytes,[37] which if released during enhancement of degranulation, may contribute to aberrant immune responses in CGD.

CONCLUSION

Cyclic AMP-elevating agents, including formoterol, accelerate restoration of calcium homeostasis in FMLP-activated neutrophils with associated downregulation of the pro-inflammatory activities of these cells. The importance of the endomembrane Ca^{2+}-ATPase as a therapeutic target in neutrophil-mediated diseases is underscored by the apparent clinical efficacy of formoterol in inflammatory airway disorders.

Membrane depolarisation plays a critical role in regulating Ca^{2+} influx in FMLP-activated neutrophils, thereby facilitating cytosolic clearance of the cation. Therefore, new insights into our understanding of the relationship between membrane depolarisation and calcium metabolism in activated human neutrophils, have identified another potential target which may be exploited in the development of novel anti-inflammatory therapeutic agents.

References

1. Dallegri F, Ottonello L. Tissue injury in neutrophilic inflammation. *Inflamm Res* 1997; **46:** 382-391.
2. Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mecarelli L. Neutrophils: molecules, functions and pathophysiological aspects. *Lab Invest* 2000; **80:** 617-653.
3. Barritt G. Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signaling requirements. *Biochem J* 1999; **337:** 153-169.
4. Peachman KK, Lyles DS, Bass DA. Mitochondrial differences in glucocorticoid treated eosinophils and neutrophils. *Am J Respir Crit Care Med* 2000; **161:** A899.
5. Strickland I, Kisich K, Hauk PJ, Chrousos GP, Klemm DJ, Leung DYM. High constitutive glucocorticoid receptor beta in human neutrophils enables them to reduce their spontaneous rate of cell death in response to corticosteroids. *J Exp Med* 2001; **193:** 585-593.
6. Lew DP. Receptor signalling and intracellular calcium in neutrophil activation. *Eur J Clin Invest* 1989; **19:** 338-346.
7. Westwick J, Poll C. Mechanisms of calcium homeostasis in the polymorphonuclear leukocyte. *Agents Actions* 1986; **9:** 80-86.
8. Alonso-Torre SR, Alvarez J, Montero M, Sanchez A, Garcia-Sancho J. Control of Ca^{2+} entry into HL60 and U937 human leukaemia cells by the filling state of the intracellular Ca^{2+} stores. *J Biol Chem* 1993; **268:** 13055-13061.
9. Schatzman HJ. The calcium pump of the surface membrane and of the sarcoplasmic reticulum. *Ann Rev Physiol* 1989; **51:** 473-485.
10. Condliffe AM, Kitchen E, Chilvers ER. Neutrophil priming: pathophysiological consequences and underlying mechanisms. *Clin Sci* 1998; **94:** 461-467.
11. Schrenzel J, Serrander L, Bànfi B, *et al*. Electron currents generated by the human phagocyte NADPH oxidase. *Nature* 1998; **392:** 734-737.
12. Di Virgilio F, Lew DP, Andersson T, Pozzan T. Plasma membrane potential modulates chemotactic-peptide stimulated cytosolic free Ca^{2+} changes in human neutrophils. *J Biol Chem* 1987; **262:** 4574-4579.
13. Parekh A, Penner R. Store depletion and calcium influx. *Physiol Rev* 1997; **77:** 901-930.
14. Tintinger GR, Anderson R, Theron AJ, Ramafi G, Ker JA. Comparison of the effects of selective and non-selective beta-adrenergic agonists on the pro-inflammatory activities of human neutrophils *in vitro*. *Inflammation* 2000; **24:** 239-249.
15. Weiss M, Schneider EM, Tarnow J, *et al*. Is inhibition of oxygen radical production of neutrophils by sympathomimetics mediated via beta-2 adrenoceptors? *J Pharmacol Exp Ther* 1996; **278:** 1105-1113.
16. Arcaro A, Wymann P. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-triphosphate in neutrophil responses. *Biochem J* 1993; **296:** 297-301.
17. Moore AR, Willoughby DA. The role of cAMP regulation in controlling inflammation. *Clin Exp Immunol* 1995; **101:** 387-389.
18. Uusaro A, Russel JA. Could anti-inflammatory actions of catecholamines explain the possible beneficial effects of supranormal oxygen delivery in critically ill surgical patients? *Intensive Care Med* 2000; **26:** 299-304.
19. Tighe D, Moss R, Bennett D. Cell surface adrenergic receptor stimulation modifies the endothelial response to systemic inflammatory response syndrome. *New Horizons* 1996; **4:** 426-442.
20. Barnes PJ, Karin M. Nuclear factor-κB - a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; **336:** 1066-1071.
21. Jimenez JL, Punzon C, Navarro J, Mundoz-Fernandez MA, Fresno M. Phosphodiesterase 4 inhibitors prevent cytokine secretion by T lymphocytes by inhibiting nuclear factor-kappa B and nuclear factor of activated T cells. *J Pharmacol Exp Ther* 2001; **299:** 753-759.
22. Spoelstra RM, Postma DS, Hovenga H, Noordhoek JA, Kauffman HF. Additive anti-inflammatory effect of formoterol and budesonide on human lung fibroblasts. *Thorax* 2002; **57:** 237-241.
23. Korn SH, Jerre A, Brattsand R. Effects of formoterol and budesonide on GM-CSF and IL-8 secretion by triggered human bronchial epithelial cells. *Eur Respir J* 2001; **17:** 1070-1077.
24. McGraw DW, Fukuda N, James PF, *et al*. Targeted transgenic expression of beta(2)-adrenergic receptors to type II cells increases alveolar fluid clearance. *Am J Physiol* 2001; **281:** L895-903.
25. Johnson M, Rennard S. Alternative mechanisms for long-acting β_2-adrenergic agonists in COPD. *Chest* 2001; **120:** 158-270.
26. Wallin A, Sandström T, Söderberg M, *et al*. The effects of regular inhaled formoterol, budesonide and placebo on mucosal inflammation and clinical indices in mild asthma. *Am J Respir Crit Care Med* 1998; **158:** 79-86.
27. Wilson SJ, Wallin A, Della-Cioppa G, Sandstrom S, Holgate ST. Effects of budesonide and formoterol on NF-kappa B, adhesion molecules, and cytokines in asthma. *Am J Respir Crit Care Med* 2001; **164:** 1047-1052.
28. Pauwels RA, Löfdahl C-G, Postma DS, *et al*. Effect of inhaled formoterol and budesonide on exacerbations of asthma. *N Engl J Med* 1997; **337:** 1405-1411.
29. O'Byrne PM, Barnes PJ, Rodriguez-Roison R, *et al*. Low dose inhaled budesonide and formoterol in mild persistent asthma: the OPTIMA randomized trial. *Am J Respir Crit Care Med* 2001; **164:** 1392-1397.
30. Barnes PJ. Clinical outcome of adding long-acting beta-agonists to inhaled corticosteroids. *Respir Med* 2001; **95:** S12-16.
31. Zetterstrom O, Bahl R, Medlen H, *et al*. Improved asthma control with budesonide/formoterol in a single inhaler, compared with budesonide alone. *Eur Respir J* 2001; **18:** 262-268.
32. Cazzola M, Donner DF. Long-acting beta 2 agonists in the management of stable chronic obstructive pulmonary disease. *Drugs* 2000; **60:** 307-320.
33. Anderson R, Feldman C, Theron AJ, *et al*. Anti-inflammatory membrane-stabilizing interactions of salmeterol with human neutrophils *in vitro*. *Br J Pharmacol* 1996; **117:** 1387-1394.
34. Rossi A, Kristafek P, Levine BE, *et al*. Comparison of the efficacy, tolerability, and safety of formoterol dry powder and oral, slow-release theophylline in the treatment of COPD. *Chest* 2002; **121:** 1058-1069.
35. Torphy TJ. Phosphodiesterase enzymes: molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med* 1998; **157:** 351-370.
36. Tintinger GR, Theron AJ, Steel HC, Anderson R. Accelerated calcium influx and hyperactivation of neutrophils in chronic granulomatous disease. *Clin Exp Immunol* 2001; **123:** 254-263.
37. Taub DD, Anver M, Oppenheim JJ, Longo DL, Murphy WJ. T lymphocyte recruitment by interleukin-8 (IL-8): IL-8-induced degranulation of neutrophils releases potent chemoattractants for human T lymphocytes both *in vitro* and *in vivo*. *J Clin Invest* 1996; **97:** 1931-1941.

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