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The anti-inflammatory interactions of epinephrine with human neutrophils *in vitro* are achieved by cyclic AMP-mediated accelerated resequestration of cytosolic calcium

Gregory R. Tintinger[a,*], Annette J. Theron[b], Ronald Anderson[b], James A. Ker[a]

[a]*Department of Internal Medicine, University of Pretoria, South Africa*
[b]*Medical Research Council Unit for Inflammation and Immunity, Department of Immunology, Institute for Pathology, Faculty of Medicine, University of Pretoria, South Africa*

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Abstract

This study was designed to evaluate the effects of epinephrine (0.01–1 μM) on superoxide production by, and release of elastase from human neutrophils activated with the chemotactic tripeptide, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) (1 μM) *in vitro*, and to relate alterations in these responses to changes in adenosine 3,5′ cyclic monophosphate (cAMP) and cytosolic free Ca^{2+}. Cyclic AMP, superoxide production and elastase release were measured by radioimmunoassay, lucigenin-enhanced chemiluminescence, and a colorimetric procedure respectively. Cytosolic Ca^{2+} fluxes were measured by fura-2 spectrofluorimetry in combination with radiometric procedures that enable distinction between net efflux and influx of the cation. Epinephrine treatment of neutrophils resulted in increased cAMP and dose-related inhibition of both superoxide production and elastase release, which was potentiated by the type 4 phosphodiesterase inhibitor, rolipram, and attenuated by propranolol, but not by selective β_1-, α_1- or α_2-adrenoreceptor antagonists. Although epinephrine did not affect the FMLP-activated abruptly-occurring increase in fura-2 fluorescence intensity, indicating no effects on the release of Ca^{2+} from neutrophil intracellular stores, this agent accelerated the rate of decline in fluorescence in the setting of decreased efflux and a reduction in store-operated influx of Ca^{2+}. These effects of epinephrine on the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils were attenuated by propranolol, and are compatible with enhancement of the activity of the cAMP-dependent Ca^{2+} sequestering/resequestering endo-membrane Ca^{2+}-ATPase. We conclude that epinephrine down-regulates the pro-inflammatory activities of neutrophils by cAMP-mediated enhancement of the clearance of cytosolic Ca^{2+}.

Keywords: Epinephrine; Neutrophils; Calcium

1. Introduction

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 tumor necrosis factor-alpha (TNF-α), secrete a series of indiscriminate cytotoxins, such as reactive oxidants, granule proteases and bioactive lipids, as well as pro-inflammatory cytokines [1,2], emphasizing 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 [3–5].

Although the primary therapeutic uses of epinephrine at present range from the treatment of acute anaphylactic reactions to hemodynamic support in septic shock, this agent also has the potential to suppress the pro-inflammatory actions of activated neutrophils. The anti-inflammatory properties of epinephrine result from the interactions of this agent with β_2-adrenoreceptors on mononuclear leukocytes and consequent elevation of intracellular cAMP, leading to suppression of generation of TNF-α, which recruits and activates neutrophils [6,7]. Epinephrine has also been re-

* Corresponding author. Tel.: +27-12-319-2425; fax: +27-12-323-0732.

Abbreviations: cAMP, adenosine 3,5′ cyclic monophosphate; CB, cytochalasin B; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks balanced salt solution; LECL, lucigenin-enhanced chemiluminescence; PDE4, phosphodiesterase isoenzyme 4; PMA, phorbol myristate acetate; TNF-α, tumor necrosis factor-alpha.

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ported to interact directly with neutrophils, resulting in cAMP-mediated attenuation of the responses of these cells to various pro-inflammatory stimuli [8]. Importantly, as a result of their anti-inflammatory properties, beta-agonists may also enhance resolution during acute lung injury [9].

The direct anti-inflammatory interactions of epinephrine with human neutrophils have been alluded to in several previous studies [10,11]. However, the exact molecular/biochemical mechanisms which underlie the anti-inflammatory actions of this agent with neutrophils have not been established. Interestingly, other cAMP-elevating agents such as the type 4 phosphodiesterase inhibitor, rolipram, as well as dibutyryl cAMP, have been reported to accelerate the clearance of Ca^{2+} from the cytosol of activated neutrophils, resulting in down-regulation of the pro-inflammatory activities of these cells [12,13]. These anti-inflammatory effects of rolipram and dibutyryl cAMP appear to be mediated by cAMP-dependent enhancement of the activity of the Ca^{2+} sequestering/re-sequestering endo-membrane Ca^{2+}-ATPase [13].

In the current study, we have evaluated the effects of epinephrine on superoxide production by and elastase release from activated neutrophils *in vitro*, and related alterations in these responses to changes in intracellular cAMP and cytosolic free Ca^{2+}.

2. Materials and methods

2.1. Pharmacologic agents

Epinephrine, purchased from the Sigma Chemical Co., was 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 a, final concentration range of 0.01 μM-1 μM. Rolipram, a selective inhibitor of type 4 phosphodiesterase (PDE), the predominant type present in human neutrophils [14,15], 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-dihydroimidazo-[1,2c]quinazolin-5(6H)-one (α_1-selective) and RS79948 (α_2-selective) were obtained from Tocris Cookson Ltd, and dissolved to 10 mM in DMSO.

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. After centrifugation (280 *g* at 10° for 10 min), residual 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 1×10^7/mL in PBS and held on ice until used.

2.3. Superoxide generation

This was measured using lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) [16]. Neutrophils were pre-incubated for 15 min at 37° in 900 μL HBSS containing 0.2 mM lucigenin in the presence and absence of epinephrine (final concentration 0.01 μM − 1 μM). Spontaneous and 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 (100 μL). LECL readings were integrated for 5 second intervals and recorded as mV $\times$ 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), atenolol and propranolol, as well as those of the α_1- or α_2-adrenoreceptor antagonists (all at 2 μM final, the maximal concentration of antagonists, which by themselves had no effect on the neutrophil functions under investigation, as established in a series of preliminary experiments) on epinephrine (1 μM)-mediated modulation of superoxide production by FMLP-activated neutrophils; rolipram was present with epinephrine throughout the pre-incubation period, while the α/β-receptor antagonists were added to the cells 5 min before epinephrine ii) the effects of adding epinephrine (0.01–1 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 (1 μM) using a cell-free xanthine (1 mM)-xanthine oxidase (70 milliunits/ml) superoxide generating system.

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 2×10^6/mL in HBSS in the presence and absence of epinephrine (0.1–1 μM) for 10 min at 37°. 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°. 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. 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% dimethylsulfoxide in 0,05 M Tris-HCl (pH 8,0). Elastase activity was then assayed at a wavelength of 405 nm.

In an additional series of experiments the effects of rolipram (0.05 μM and 0.1 μM), as well as those of the various α- and β-receptor antagonists (all at 2 μM) on epinephrine (1 μM)-mediated modulation of elastase release by FMLP/CB-activated neutrophils were investigated.

2.5. Intracellular calcium fluxes

These were measured spectrofluorimetrically using fura-2/AM (Calbiochem Corp.) as the calcium-sensitive indicator of cytoplasmic Ca^{2+} [17]. Neutrophils (1×10^7/mL) were preloaded with fura-2 (2 μM) for 30 min at 37°C in PBS, washed twice, resuspended in PBS at 1×10^7/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 pre-incubated in the presence and absence of epinephrine (0.1–1 μM) for 8 minutes at 37° followed by transfer to a disposable reaction cuvette, which was maintained at 37° 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 6×10^6 neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously [17].

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.

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) 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 radiolabeled 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 1×10^7 neutrophils. The standardization 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 labeled neutrophils from unbound isotope, have been described [18].

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

Neutrophils (1×10^7/mL) were loaded with $^{45}Ca^{2+}$ (2 μCi/ml) for 30 min at 37° in HBSS which was free of unlabeled 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+} [18]. The $^{45}Ca^{2+}$-loaded neutrophils (2×10^6/mL) were then preincubated for 10 min at 37° in Ca^{2+}-replete HBSS, in the presence and absence of epinephrine (1 μM), followed by activation with FMLP (1 μM) and measurement of the efflux of $^{45}Ca^{2+}$ over 60 sec, after which efflux is complete [18]. 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 radiolabeled 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 [19] 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.

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 radiolabeled cation, the cells were loaded with cold, Ca^{2+}-replete HBSS for 30 min at 37°, 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 minimize 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,

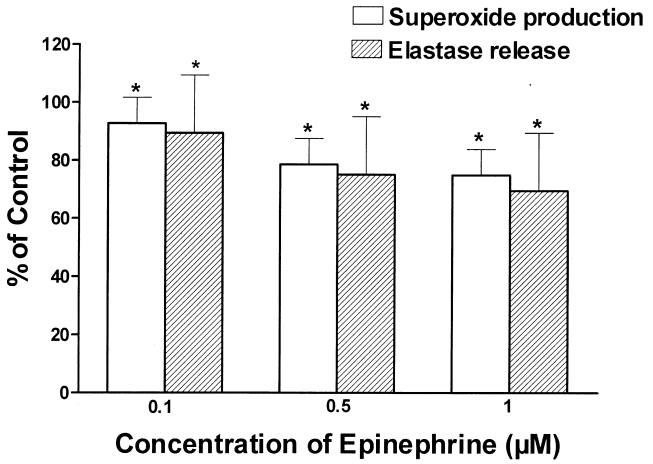

Fig. 1. The effects of epinephrine on superoxide production by and elastase release from FMLP-activated neutrophils. The results of 7–11 experiments are expressed as the mean percentage ± SEM of the corresponding drug-free control system. The absolute mean peak values for superoxide production by unstimulated and FMLP-activated neutrophils were 213 ± 29 and 1081 ± 122 mV.s^{-1} respectively. The corresponding values for elastase release were 31 ± 6 and 250 ± 43 milliunits enzyme/10^7 cells. *$P <$ 0.05 for comparison with the epinephrine-free control system.

which did not differ from those of neutrophils maintained in Ca^{2+}-replete HBSS [18]. The Ca^{2+}-loaded neutrophils (2×10^6/mL), were then incubated for 10 min in the presence and absence of epinephrine (1 μM) at 37° 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 [18] and compared with the uptake of the radiolabeled cation by identically-processed, unstimulated cells.

2.9. Measurement of intracellular cAMP

Neutrophils at a concentration of 1×10^7/mL in HBSS were preincubated for 10 min at 37° 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°) with ice-cold ethanol and the supernatants pooled and evaporated at 60° 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 hydrolyzed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μM rolipram.

2.10. Statistical analysis

The results of each series of experiments are expressed as the mean values ± the standard error of the mean (SEM). Where appropriate, levels of statistical significance were calculated using a paired Student's *t*-test.

3. Results

3.1. Superoxide generation

The effects of epinephrine on superoxide production by neutrophils activated with FMLP, are shown in Fig. 1. Epinephrine at concentrations of 0.1–1 μM caused statistically significant, dose-related inhibition of oxidant production in neutrophils activated with FMLP ($P < 0.05$). The results of experiments designed to investigate the effects of epinephrine in combination with rolipram are shown in Table 1. The magnitude of the inhibition of superoxide production by FMLP-activated neutrophils was considerably higher in the presence of combinations of rolipram (0.05 μM and 0.1 μM) and epinephrine than that observed with the individual agents.

The results of experiments designed to investigate the effects of propranolol, atenolol, an α_1-antagonist or α_2-antagonist (all at 2 μM) on epinephrine (1 μM)-mediated inhibition of superoxide production by FMLP-activated neutrophils, are shown in Table 2. Propranolol markedly attenuated the inhibitory effect of epinephrine on neutrophil oxidant generation, while atenolol and the α_1- or α_2-antagonists were ineffective.

The effects of epinephrine (0.01–1 μM) on superoxide production when added to neutrophils 30 sec before FMLP, as opposed to being present with the cells throughout the 15 min preincubation period, are shown in Table 3. The inhibitory effects of epinephrine on the production of superoxide by FMLP-activated neutrophils were more pronounced

Table 1
Effects of epinephrine with and without rolipram on superoxide production by FMLP-activated neutrophils and elastase release from FMLP/CB-activated neutrophils

System	Superoxide production	Elastase release
Epinephrine 1 μM only	77 ± 4*	67 ± 2*
Rolipram 0.05 μM only	93 ± 6	91 ± 8
Rolipram 0.1 μM only	76 ± 4*	59 ± 5*
Epinephrine + Rolipram 0.05 μM	65 ± 3*	52 ± 4*
Epinephrine + Rolipram 0.1 μM	50 ± 3*	46 ± 4*

The results of 6 experiments are expressed as the mean percentage of the epinephrine-free, FMLP control ± SEM. The absolute peak value for superoxide production by FMLP-activated neutrophils was 777 ± 74 mV.s^{-1}. The corresponding value for elastase release was 437 ± 61 milliunits enzyme/10^7 cells.

* $P < 0.05$ for comparison with the epinephrine-free control system.

Table 2
Effects of epinephrine with and without propranolol, atenolol, an alpha$_1$-antagonist or alpha$_2$-antagonist on superoxide generation by FMLP-activated neutrophils and elastase release from FMLP/CB-activated neutrophils

System	Superoxide production	Elastase release
Epinephrine 1 μM only	63 ± 3*	71 ± 1*
Propranolol 2 μM only	96 ± 3	106 ± 2
Epinephrine + Propranolol	95 ± 3	103 ± 2
Atenolol 2 μM only	93 ± 5	104 ± 2
Epinephrine + Atenolol	62 ± 3*	81 ± 2*
α_1-antagonist 2 μM only	95 ± 4	99 ± 2
Epinephrine + α_1-antagonist	70 ± 3*	74 ± 2*
α_2-antagonist 2 μM only	97 ± 5	106 ± 4
Epinephrine + α_2-antagonist	62 ± 5*	68 ± 2*

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 199 ± 19 and 1047 ± 69 mV.s^{-1} respectively. The corresponding values for elastase release were 53 ± 8 and 267 ± 23 milliunits enzyme/10^7 cells.

* $P < 0.05$ for comparison with the epinephrine-free control system.

when added 30 sec before FMLP, with statistically significant ($p < 0.05$) inhibition observed at concentrations of 0.01 μM and upwards.

Using the cell-free xanthine/xanthine oxidase superoxide-generating system, epinephrine, at all concentrations tested, did not possess superoxide-scavenging properties. The peak LECL responses for the control system and systems containing epinephrine at 0.1, 0.5, and 1 μM were 1370 ± 37, 1352 ± 77, 1336 ± 60, and 1421 ± 62, respectively (data from 8 experiments).

3.2. Elastase release

The effects of epinephrine on elastase release from FMLP/CB-activated neutrophils, are shown in Fig. 1. Elastase release from FMLP/CB-activated neutrophils was significantly ($P < 0.05$) inhibited by epinephrine in a dose-dependent manner with 11%, 25%, and 30% inhibition at concentrations of 0.1 μM, 0.5 μM, and 1 μM, respectively. Rolipram at 0.05 μM and 0.1 μM significantly ($P < 0.05$) potentiated the inhibitory effects of epinephrine (Table 1).

The effects of pre-incubation of neutrophils with propranolol, atenolol, an α_1- or α_2-antagonist (all at 2 μM), prior to addition of epinephrine on elastase release from FMLP/CB-activated neutrophils are shown in Table 2. Pre-incubation of neutrophils with propranolol completely abolished this response, while the α- and $\beta_1$receptor antagonists were ineffective. Significant, but incomplete attenuation of the inhibitory effect of epinephrine was observed with atenolol.

3.3. Fura-2 responses of FMLP-activated neutrophils

The results shown in Fig. 2 are traces from 3 typical experiments using cells from different donors depicting the effect of epinephrine (1 μM) on the fura-2 responses of FMLP-activated neutrophils. 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. Pre-incubation of neutrophils with epinephrine did not affect the initial rapid rise in fura-2 fluorescence following activation of the cells with FMLP. The addition of epinephrine did, however, result in an accelerated dose-dependent decline in fura-2 fluorescence towards the baseline. The time taken for the fluorescence intensity to return to half the peak value (T½), is dependent on the rate of clearance of free Ca^{2+} from the neutrophil cytoplasm and 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 pmols/min.

The effect 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 for free cytosolic Ca^{2+} following activation with FMLP, are shown in Table 4. The magnitude of the abrupt rise in cytosolic free Ca^{2+} was not significantly altered as mentioned above, but the time taken for the fura-2 fluorescence response to reach half its peak value was reduced and this correlated well with the calculated progressive increase in the clearance rates of free cytosolic calcium, reaching statistical significance at an epinephrine concentration of 0.5 μM ($P < 0.05$).

The epinephrine (1 μM)-mediated hastening of the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils was completely prevented by pre-incubation of the cells with propranolol (results not shown).

The effects of epinephrine (1 μM) and rolipram (0.05 μM and 0.1 μM) individually and in combination, on the peak cytosolic Ca^{2+} concentrations, time taken for fluorescence intensity to decline to half peak values, as well as the clearance rates of free cytosolic Ca^{2+} for

Table 3
Effects of epinephrine when added 30 sec or 15 min before 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}.

*$P < 0.05$ for comparison with the epinephrine-free control system.

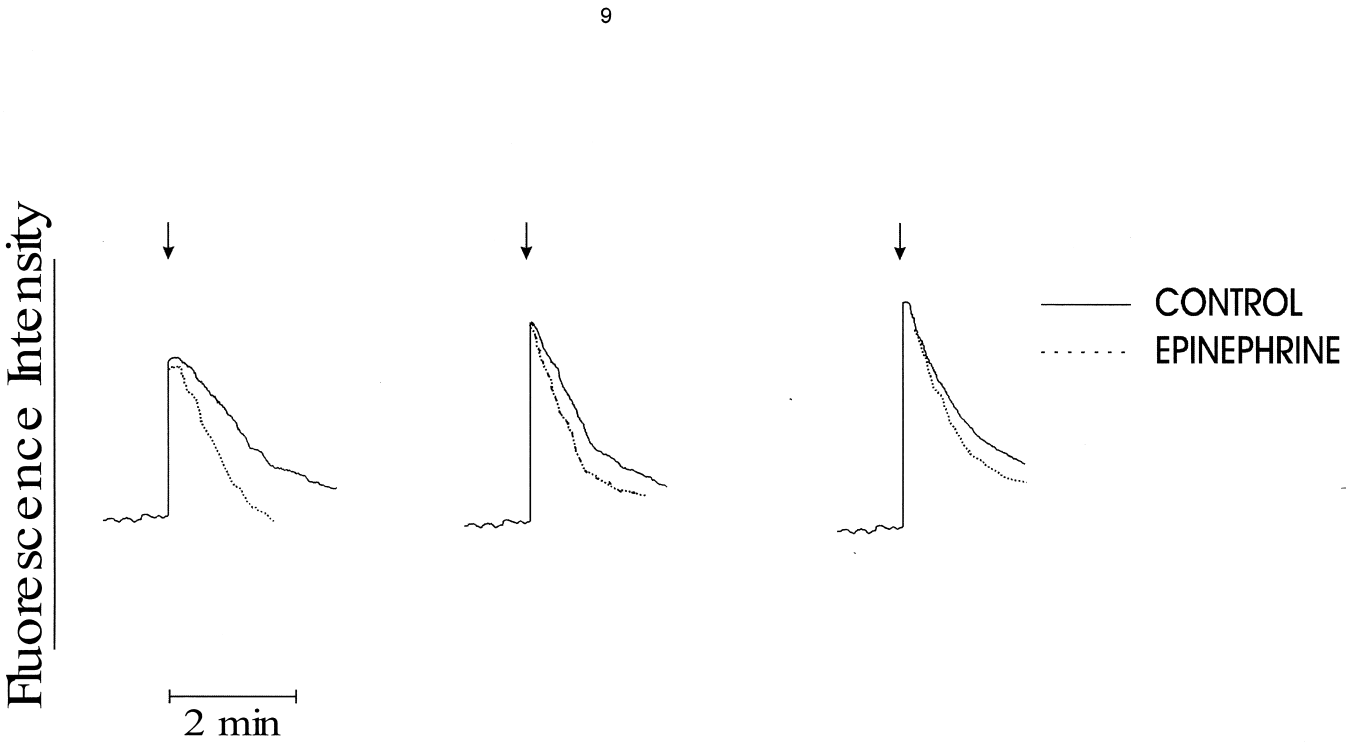

Fig. 2. The effects of epinephrine (1 μM) on the time course of the fura-2 fluorescence response of FMLP-activated neutrophils from 3 different subjects. FMLP was added as indicated ($\downarrow$) when a stable base-line was obtained ($\pm$1 min).

FMLP-activated neutrophils are shown in Table 5. Epinephrine and rolipram in combination, had no effect on peak fluorescence intensity, but the time taken to reach half peak values was significantly less ($P < 0.05$) 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+}.

3.4. 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 minimize re-uptake of radiolabelled cation) followed by activation with FMLP and by measurement of the amount of remaining cell-associated $^{45}Ca^{2+}$ 60 sec after addition of FMLP at which time efflux is complete [18]. Exposure of the drug-free, control neutrophils to FMLP resulted in efflux of the radiolabelled cation from the neutrophils, which corresponded to loss of approximately 56% 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. Pretreatment of the neutrophils with epinephrine, significantly reduced the extent of efflux of $^{45}Ca^{2+}$ from the cells compared to the control system (113 $\pm$ 3 pmol/10^7 cells and 148 $\pm$ 6 pmol/10^7 cells, respectively) ($P < 0.05$). 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 6).

Table 4
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+} (pmols/min)
Control	365 $\pm$ 31	0.97 $\pm$ 0.05	139 $\pm$ 20
Epinephrine 0.1 μM	349 $\pm$ 33	0.86 $\pm$ 0.05	145 $\pm$ 19
Epinephrine 0.5 μM	399 $\pm$ 50	0.83 $\pm$ 0.03	174 $\pm$ 22*
Epinephrine 1 μM	365 $\pm$ 46	0.75 $\pm$ 0.03*	178 $\pm$ 25*

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

Table 5
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+} (pmols/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.
*$P < 0.05$.

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

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 $^{45}Ca^{2+}$ (122 ± 6 pmol/10^7 cells), while there was only trivial influx of the radiolabeled cation into control, identically processed neutrophils not exposed to FMLP (24 ± 3 pmol/10^7 cells). Influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils pretreated with epinephrine (1 μM) was significantly reduced (34%) to 80 ± 4 pmol/10^7 cells ($P < 0.05$), with neglible influx into resting epinephrine treated cells (15 ± 3 pmol/10^7 cells).

3.6. Intracellular cAMP levels

The intracellular cAMP concentration in unstimulated neutrophils was 95 ± 33 pmol/10^7 cells which increased slightly in the presence of epinephrine (1 μM) to 113 ± 21 pmol/10^7 cells. However, addition of epinephrine to FMLP-stimulated neutrophils markedly enhanced the elevation of intracellular cAMP levels from 355 ± 156 pmol/10^7 cells for control FMLP-activated cells to 667 ± 13 pmol/10^7 for neutrophils pretreated with epinephrine prior to addition of FMLP.

Table 6
Effects of epinephrine with and without thapsigargin on the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils

System	Amount of $^{45}Ca^{2+}$ released from neutrophils 60 s after the addition of FMLP (pmol/10^7 cells)
FMLP only	150 ± 18
Epinephrine 1 μM	74 ± 15*
Thapsigargin 1 μM	153 ± 18
Epinephrine + Thapsigargin	135 ± 15

The results of 4 separate experiments are expressed as the mean values ± SEM.
*$P < 0.05$ for comparison with the control system.

4. Discussion

In the current study, treatment of neutrophils with the non-specific adrenoreceptor agonist, epinephrine, resulted in dose-related inhibition of the production of superoxide by, and release of elastase from, these cells. The anti-inflammatory interactions of epinephrine were attenuated by propranolol, but not by selective antagonists of α_1- or α_2-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 [20]. These observations implicate β_2-adrenoreceptors in the anti-inflammatory interactions of epinephrine with neutrophils. Neutrophil outer membranes contain approximately 1000 β-receptors per cell [21] which are fully saturated during incubation with the non-selective adrenoreceptor agonist, isoproterenol at a concentration of 1 μM [21]. Beta-receptors are coupled to a regulatory G_s-protein and agonist binding results in activation of adenylate cyclase [22].

Our observations that epinephrine inhibits the pro-inflammatory activities of neutrophils are essentially in agreement with several previous reports which have described the inhibitory effects of epinephrine on oxidant production by neutrophils stimulated with formyl peptides *in vitro* [10,23,24]. A reduction in the oxidative burst measured by luminol-enhanced chemiluminescence in neutrophils stimulated with opsonized zymosan in the presence of epinephrine has also been documented [25,26]. These findings contrast with those of other investigators, who were unable to document inhibitory effects of epinephrine on neutrophil membrane-associated oxidative metabolism [27,28]. Epinephrine has also been reported to reduce lysozyme and β-glucuronidase release from FMLP-stimulated neutrophils [24], but had no significant effect on the release of lactoferrin and β-glucuronidase following stimulation with opsonized zymosan or PMA [27]. This latter observation is not entirely unexpected since the insensitivity of the PMA-activated responses of

human neutrophils to cAMP-elevating agents is well-recognized.

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 before 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 and was attributed to rapid desensitization of β_2-adrenoreceptors [23]. Alternatively, up-regulation of phosphodiesterases and enhanced degradation of cAMP during prolonged exposure of neutrophils to β_2-agonists [29] may also explain the time-dependent decline in the anti-inflammatory potency of epinephrine. In keeping with the effects on superoxide production a shorter exposure time of neutrophils to epinephrine further enhanced the rate of cytosolic calcium clearance compared to longer incubation times (results not shown).

Coincubation of neutrophils with epinephrine resulted in increased intracellular cAMP, 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. That addition of epinephrine was able to markedly amplify the cAMP response induced by FMLP is interesting and has been observed by other investigators [23,24] compatible with sensitization of basal adenylate cyclase activity by epinephrine. The involvement of cAMP in the anti-inflammatory action of epinephrine is also supported by observations made in the present study that the selective PDE4 inhibitor, rolipram, potentiates both the cAMP-elevating and anti-inflammatory interactions of epinephrine with neutrophils.

Epinephrine, at the concentrations used in the current study, operating via β_2-adrenoreceptors, adenylate cyclase and cAMP, has also been reported by others to suppress the pro-inflammatory functions of human neutrophils *in vitro* [10,23,24]. However, in these previous studies the relationship between epinephrine-mediated anti-inflammatory effects on neutrophils and modulation of intracellular Ca^{2+} metabolism was not investigated. Transient increases in cytosolic free Ca^{2+} precede and are a prerequisite for receptor-mediated activation of NADPH-oxidase and degranulation [30–32].

In the current study, using fura-2 spectrofluorimetry, treatment of neutrophils with epinephrine, at concentrations which suppressed the pro-inflammatory activities of these cells, had no effects 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+} mobilizing receptors on intracellular 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 was attenuated by treatment of the cells with propranolol and potentiated by rolipram, suggesting the involvement of cAMP.

Radiometric procedures which can distinguish between net efflux and net influx of Ca^{2+} were used to identify the mechanisms (e.g. enhancement of efflux and/or inhibition of influx) of epinephrine-mediated accelerated clearance of the cation from the cytosol of FMLP-activated neutrophils. Addition of FMLP to $^{45}Ca^{2+}$-loaded neutrophils has previously been reported to result in an immediate efflux of the radiolabeled cation, coincident with the increase in cytosolic Ca^{2+}, which terminates at 30–60 sec after addition of the stimulus [18]. In the current study, addition of FMLP to the neutrophils elicited an efflux of Ca^{2+} which corresponded on average to 56% of the cell-associated cation. This observation demonstrates that only part of the intracellular Ca^{2+} pool is mobilized following exposure of neutrophils, or that much of the cytosolic Ca^{2+} is re-sequestered 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+}.

Net influx of Ca^{2+} into FMLP-activated neutrophils has been reported to occur at around 1–2 min after the addition of the stimulus and to be complete at about 5 min [18,33,34]. This delayed influx of Ca^{2+} is characteristic of a store-operated influx which is operative in a large variety of cell types, including neutrophils, and is required for refilling of intracellular stores [35]. In the current study, 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 [36,37]. 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+}-ATPase for cytosolic Ca^{2+}. Up-regulation of the endo-membrane Ca^{2+}-ATPase would also result in enhancement of re-sequestration of cytosolic Ca^{2+} and increased re-filling of stores with endogenous cation, and a consequent reduction in the magnitude of the subsequent store-operated influx of extracellular Ca^{2+}. This contention is supported by the observation that thapsigargin, a selective inhibitor of the endo-membrane Ca^{2+}-ATPase [19] antagonized the epinephrine-mediated reduction in the efflux of Ca^{2+} from FMLP-activated neutrophils.

Unlike the endo-membrane Ca^{2+}-ATPase, the plasma membrane Ca^{2+}-ATPase appears to be modulated by cal-

modulin, as opposed to cAMP, which shifts the pump to a higher affinity state for Ca^{2+}, resulting in enhanced maximal velocity [38]. Accelerated clearance of Ca^{2+} from the cytosol of epinephrine-treated, FMLP-activated neutrophils is probably achieved through the action of the up-regulated endo-membrane Ca^{2+}-ATPase operating in unison with the plasma membrane Ca^{2+}-efflux pump.

The pharmacological manipulation of neutrophil function in clinical practice is extremely relevant in view of the number of serious disorders in which neutrophil-mediated tissue injury plays a key pathogenetic role. However, few pharmacological agents in widespread clinical use are able to exert predictable suppression of the potentially harmful responses of activated neutrophils, including oxidant production and degranulation [5]. The clinical potential of cAMP-elevating agents as inhibitors of neutrophil pro-inflammatory activities, is supported by the inhibitory effects of isoproterenol on neutrophil adhesion to bronchial epithelium [39], the anti-inflammatory activity of the second generation PDE 4 inhibitor, SB 207499, in experimental asthma in guinea pigs [40], as well as the suppression of endotoxin-induced acute lung injury in mice by rolipram [41]. Moreover, the inhibitory effects of epinephrine on TNF-α production by activated inflammatory cells *in vivo* [8], underscore the anti-inflammatory potential of this agent.

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. Given the insensitivity of neutrophils to corticosteroids [3–5], these anti-inflammatory actions of epinephrine, if operative in the clinical setting, may represent a useful, albeit secondary, additional property of this agent.

References

[1] Fujishima S, Aikawa N. Neutrophil-mediated tissue injury and its modulation. Intensive Care Med 1995;21:277–85.

[2] Dallegri F, Ottonello L. Tissue injury in neutrophilic inflammation. Inflamm Res 1997;46:382–91.

[3] Cox G. Glucocorticoid treatment inhibits apoptosis in human neutrophils. J Immunol 1995;154:4719–25.

[4] Meagher LC, Cousin JC, Seckl JR, Haslett C. Opposing effects of glucocorticoids on the rate of apoptosis in neutrophilic and eosinophilic granulocytes. J Immunol 1996;156:4422–8.

[5] Barnes PJ. New therapies for chronic obstructive pulmonary disease. Thorax 1998;53:137–47.

[6] Pettipher ER, Labasi JM, Salter ED, Stam EJ, Cheng JB, Griffiths RJ. Regulation of tumour necrosis factor production by adrenal hormones *in vivo*: insights into the anti-inflammatory activity of rolipram. Br J Pharmacology 1996;117:1530–44.

[7] Van der Poll T, Coyle SM, Barbosa K, Braxton CC, Lowrey SF. Epinephrine inhibits tumor necrosis factor-α and potentiates interleukin 10 production during human endotoxemia. J Clin Invest 1996;97:713–19.

[8] Moore AR, Willoughby DA. The role of cAMP regulation in controlling inflammation. Clin Exp Immunol 1995;101:387–9.

[9] Ware LB, Matthay MA. The Acute Respiratory Distress Syndrome. New Engl J Med 2000;342:1334–49.

[10] Weiss M, Schneider EM, Tarnow J, Mettler S, Krone M, Teschemacher A, Lemoine H. Is inhibition of oxygen radical production of neutrophils by sympathomimetics mediated via beta-2 adrenoceptors? J Pharmacol Exp Ther 1996;278:1105–13.

[11] Garcia C, Pithon-Curie TC, Firmano MD, De Melo MP, Newsholme P, Curi R. Effects of adrenaline on glucose and glutamine metabolism and superoxide production by rat neutrophils. Clin Sci 1999;96:549–55.

[12] Villagrasa V, Navarette C, Sanz C, Berto L, Perpina M, Cortijo J, Morcillo EJ. Inhibition of phosphodiesterase IV and intracellular calcium levels in human polymorphonuclear leukocytes. Methods Find Exp Clin Pharmacol 1996;18:239–45.

[13] Anderson R, Goolam MA, Theron AJ, Ramafi G, Feldman C. Effect of rolipram and dibutyryl cyclic AMP on resequestration of cytosolic calcium in FMLP-activated human neutrophils. Br J Pharmacol 1996;124:547–55.

[14] Torphy TJ. Phosphodiesterase isoenzymes: molecular targets for novel antiasthma agents. Am J Respir Crit Care Med 1998;157:351–70.

[15] Wang P, Wu P, Ohleth KM, Egan RW, Billah MM. Phosphodiesterase 4B2 is the predominant phosphodiesterase species and undergoes differential regulation of gene expression in human monocytes and neutrophils. Mol Pharmacol 1998;56:170–4.

[16] Minkenberg I, Ferber E. Lucigenin-dependent chemiluminescence as a new assay for NADPH-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. J Immunol Meth 1984;71:61–7.

[17] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.

[18] Anderson R, Goolam Mahomed. Calcium efflux and influx in FMLP-activated human neutrophils are chronologically distinct events. Clin Exp Immunol 1997;110:132–8.

[19] Lytton J, Wiestlin M, Hanley MR. Thapsigargin inhibits the sarcoplasmic or endoplasmic reticulum Ca-ATPase family of calcium pumps. J Biol Chem 1997;266:17067–71.

[20] Hoffman BB, Lefkowitz RJ. Adrenergic Receptor Antagonists. In: Goodman AG, Rall TW, Nies AS, Taylor P, editors The Pharmacological Basis of Therapeutics. Singapore: McGraw-Hill, 1992, p. 221–43.

[21] Mueller H, Motulsky HJ, Sklar LA. The potency and kinetics of the β-adrenergic receptors on human neutrophils. Mol Pharmacol 1997;34:347–53.

[22] Barnes PJ. State of the art: beta-adrenergic receptors and their regulation. Am J Respir Crit Care Med 1995;152:838–60.

[23] Tecoma ES, Motulsky HJ, Traynor AE, Omann GM, Muller H, Sklar LA. Transient catecholamine modulation of neutrophil activation: kinetic and intracellular aspects of isoproterenol action. J Leukocyte Biol 1986;40:629–44.

[24] Bazzoni G, Dejana E, Del Maschio A. Adrenergic modulation of human polymorphonuclear leucocyte activation. Potentiating effect of adenosine. Blood 1991;77:2042–8.

[25] Busse WW, Sosman JM. Isoproterenol inhibition of isolated human neutrophil function. J Allergy Clin Immunol 1984;73:404–10.

[26] Markiewicz K, Kantorski J, Malec P, Tchórzewski H. Effect of epinephrine, propranolol and verapamil on the chemiluminescence of neutrophils in duodenal ulcer patients. Archs Immunol Ther Experiment 1989;37:141–7.

[27] Hetherington SV, Quie PG. Human polymorphonuclear leukocytes of the bone marrow, circulation and marginated pool: function and granule protein content. Am J Hematol 1985;20:235–46.

[28] Burns AM, Keogan A, Donaldson M, Brown DL, Park GR. Effects of inotropes on human leucocyte numbers, neutrophil function and lymphocyte subtypes. Br J Anaesth 1997;78:530–5.

[29] Giembycz MA. Phosphodiesterase 4 and tolerance to β_2-adrenoreceptor agonists in asthma. Trends Pharmacol Sci 1996;17:331–6.

[30] Pettit EJ, Hallet MB. Two distinct Ca^{2+} storage and release sites in human neutrophils. J Leuk Biol 1998;63:225–32.
[31] Krause K-H, Clark RA, Wymann MP. European workshop on the cell biology of phagocytes. J Leuk Biol 1997;61:1–5.
[32] Borregaard N, Cowland JB. Granules of the human neutrophilic polymorphonuclear leukocyte. Blood 1997;89:3503–52.
[33] Montero M, Alvarez J, Garcia-Sanchez J. Agonist-induced Ca^{2+} influx in human neutrophils is secondary to the emptying of intracellular calcium stores. Biochem J 1991;289:761–6.
[34] Geiszt M, Kapus A, Nemet K, Farkos L, Ligeti E. Regulation of capacitative Ca^{2+} influx in human neutrophil granulocytes: alterations in chronic granulomatous disease. J Biol Chem 1997;272: 26471–78.
[35] Favre CJ, Nüsse O, Lew DP, Krause K-H. Store-operated Ca^{2+} influx: What is the message from the stores to the membrane? J Lab Clin Med 1996;128:19–26.
[36] Schatzmann HJ. The calcium pump of the surface membrane and of the sarcoplasmic reticulum. Ann Rev Physiol 1989;51:473–85.
[37] Tao J, Johannson JS, Haynes DH. Stimulation of dense tubular Ca^{2+} uptake in human platelets by cAMP. Biochim Biophys Acta 1992; 1105:29–39.
[38] Lagast H, Lew DP, Waldvogel FA. Adenosine triphosphatase-dependent calcium pump in the plasma membrane of guinea pig and human neutrophils. J Clin Invest 1984;73:107–415.
[39] Bloemen PGM, Van Den Tweel MC, Hendricks PAJ, Engels F, Kester MHA, Van De Loo PGF, Blomjous FJ, Nijkamp FP. Increased cAMP levels in stimulated neutrophils inhibit their adhesion to bronchial epithelial cells. Am J Physiol 1997;272:L580–7.
[40] Underwood DC, Bochnowicz S, Osborn RR, Katzer CJ, Luttmann MA, Hay DWP, Gorycki PD, Christensen SB, Torphy TJ. Antiasthmatic activity of the second-generation phosphodiesterase 4 (PDE 4) inhibitor SB207499 (Ariflo) in the guinea pig. J Pharmacol Exp Ther 1998;287:988–95.
[41] Miotla JA, Teixeira MM, Hellewell PG. Suppression of acute lung injury in mice by an inhibitor of phosphodiesterase type 4. Am J Respir Cell Mol Biol 1998;18:411–20.