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Effect of rolipram and dibutyryl cyclic AMP on resequestration of cytosolic calcium in FMLP-activated human neutrophils

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1 We have investigated the effects of the selective phosphodiesterase (PDE) type 4 inhibitor, rolipram (0.01–1 μM) on cytosolic Ca^{2+} fluxes in FMLP-activated human neutrophils, as well as on superoxide production by, and release of elastase from, these cells.

2 Cytosolic Ca^{2+} fluxes were measured by use of fura-2 spectrofluorimetry in combination with a radiometric procedure that enables distinction between net efflux and influx of the cation. Superoxide production and elastase release were measured by lucigenin-enhanced chemiluminescence and a colorimetric procedure, respectively.

3 Pretreatment of neutrophils with rolipram did not affect the FMLP-activated release of Ca^{2+} from intracellular stores, but was associated with dose-related acceleration of the rate of decline in fura-2 fluorescence and with decreased efflux, as well as store-operated influx of $^{45}Ca^{2+}$, indicative of enhancement of resequestration of the cation by the endo-membrane Ca^{2+}-ATPase.

4 Inhibition of superoxide production and elastase release was observed at concentrations of rolipram which accelerated the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils.

5 These effects of rolipram on FMLP-activated Ca^{2+} fluxes, superoxide generation and elastase release were mimicked by pretreatment of neutrophils with dibutyryl cyclic AMP (0.5–4 mM), while theophylline (10–150 μM), a non-specific PDE inhibitor, as well as the β_2-agonist, salbutamol, were less effective.

6 We conclude that rolipram deactivates FMLP-stimulated human neutrophils by enhancement of cyclic AMP-dependent resequestration of cytosolic Ca^{2+}.

Keywords: Rolipram; dibutyryl cyclic AMP; neutrophils; calcium efflux; calcium influx; superoxide; elastase

Introduction

Receptor-coupled activation of adenylate cyclase and/or inhibition of phosphodiesterases (PDEs) results in increased intracellular concentrations of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and inhibition of the pro-inflammatory activities of neutrophils, eosinophils, monocytes, macrophages, lymphocytes and mast cells (reviewed by Moore & Willoughby, 1995). Although the exact molecular/biochemical mechanisms of cyclic AMP-mediated anti-inflammatory activity have not been established, the susceptibility of these different cell types suggests that a common pathway involved in cell activation and mediator release may be affected.

Since increased concentrations of cytosolic Ca^{2+} precede, and are a pre-requisite, for activation of inflammatory cells, cellular Ca^{2+} metabolism, which is modulated in activated platelets by cyclic AMP-elevating agents (Brace *et al*., 1985; Johansson *et al*., 1992; Tao *et al*., 1992), is a potential target for cyclic nucleotide-mediated modulation. In the case of human neutrophils, exposure of these cells to cyclic AMP-elevating agents has been shown to inhibit the release of Ca^{2+} from intracellular stores (Nielson *et al*., 1988), or to cause accelerated efflux (De Togni *et al*., 1985; Villagrasa *et al*., 1996) and/or decreased influx of the cation (De Togni *et al*., 1985; Schudt *et al*., 1991; Ahmed *et al*., 1995; Villagrasa *et al*., 1996). Although these data demonstrate that cyclic AMP-elevating agents cause altered Ca^{2+} metabolism in activated neutrophils, the spectrofluorimetric procedures used in all of these studies cannot distinguish between efflux and influx of the cation, which clearly complicates accurate interpretation of the data.

In the present study, we have used the combination of a fura 2-based spectrofluorimetric method (Grynkiewicz *et al*., 1985) and a radioassay (Anderson & Goolam Mahomed, 1997), which together facilitate distinction between Ca^{2+} efflux and influx, to investigate the effects of rolipram, a selective inhibitor of phosphodiesterase 4 (PDE 4; Schwabe *et al*., 1976; Nielson *et al*., 1990), theophylline, salbutamol and dibutyryl cyclic AMP on Ca^{2+}-fluxes and Ca^{2+}-dependent functions in formyl methionyl leucylphenylalanine (FMLP)-activated neutrophils.

Methods

Neutrophils

Purified neutrophils were prepared from heparin-treated (5 units of preservative-free heparin ml^{-1}) venous blood of healthy adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque-1077 (Sigma Diagnostics, St Louis, MO, U.S.A.) 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 0.84% 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^{-1} in PBS and held on ice until used.

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Spectrofluorimetric measurement of Ca^{2+} fluxes

Fura-2/AM (Calbiochem Corp, La Jolla, California, U.S.A.) was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments. Neutrophils (1×10^7 ml^{-1}) 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 Hank's 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^{-1}) were then pre-incubated with rolipram (0.01–1 μM), theophylline (10–150 μM), salbutamol (0.1 and 1 μM) or dibutyryl cyclic AMP (0.5–4 mM) for 5–10 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 the synthetic, chemotactic tripeptide, N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, used at a final concentration of 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 6×10^6 neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously (Grynkiewicz *et al*., 1985).

Additional experiments were performed to investigate the effects of salbutamol (0.1 and 1 μM) in combination with a low concentration of rolipram (0.005 μM) on the fura-2 fluorescence responses of FMLP-activated neutrophils.

Radiometric assessment of Ca^{2+} fluxes

$^{45}Ca^{2+}$ (Calcium-45 chloride, specific activity 18.53 mCi mg^{-1}, Du Pont NEN Research Products, Boston, MA, U.S.A.) 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^{-1}, which is equivalent to 1 μM $CaCl_2$. 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 labelled neutrophils from unbound isotope, have been described elsewhere (Anderson & Goolam Mahomed, 1997).

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

Neutrophils (1×10^7 ml^{-1}) were loaded with $^{45}Ca^{2+}$ (2 μCi ml^{-1}) 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 & Goolam Mahomed, 1997). The $^{45}Ca^{2+}$-loaded neutrophils (2×10^6 ml^{-1}) were then preincubated for 10 min at 37°C in Ca^{2+}-replete HBSS, in the presence and absence of rolipram (0.1–1 μM), theophylline (10–150 μM), salbutamol (0.1 and 1 μM) and dibutyryl cyclic AMP (0.5–1 mM), followed by activation with FMLP (1 μM) and measurement of the kinetics (10, 20, 30 and 60 s) of efflux of $^{45}Ca^{2+}$. 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 (Anderson & Goolam Mahomed, 1997). 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 spectrophotometer. 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*., 1991) on dibutyryl cyclic AMP (1 mM)- and rolipram (1 μM)-mediated modulation of FMLP-activated efflux of $^{45}Ca^{2+}$ from neutrophils were investigated over a 60 s 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 dibutyryl cyclic AMP or rolipram.

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 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, which did not differ from those of neutrophils maintained in Ca^{2+}-replete HBSS (Anderson & Goolam Mahomed, 1997). The Ca^{2+}-loaded neutrophils (2×10^6 ml^{-1}), were then incubated for 10 min in the presence and absence of rolipram, theophylline, salbutamol and dibutyryl cyclic AMP at 37°C in Ca^{2+}-free HBSS followed by simultaneous addition of FMLP and $^{45}Ca^{2+}$ (2 μCi ml^{-1}), 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 and compared with those of influx of the radiolabelled cation into the identically-processed, unstimulated cells.

Superoxide generation

This was measured by use of a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils were pre-incubated for 15 min in 900 μl HBSS containing 0.2 mM lucigenin in the presence and absence of rolipram, theophylline, salbutamol or dibutyryl cyclic AMP. Spontaneous and FMLP (1 μM)-activated LECL responses were then recorded with a LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of the stimulant (100 μl). LECL readings were integrated for 5 s intervals and recorded as mV seconds^{-1} (mVs^{-1}). Additional experiments were performed to investigate the following: (i) the effects of salbutamol (1 μM) in combination with rolipram (0.005 μM) on the LECL responses of FMLP-activated neutrophils and (ii) the superoxide-

scavenging activity of rolipram, theophylline, salbutamol and dibutyryl cyclic AMP with a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 mu ml^{-1}) superoxide-generating system.

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 $1 \times 10^7 ml^{-1}$ in HBSS in the presence or absence of rolipram (0.01 – 1 μM), theophylline (10 – 150 μM), salbutamol (0.1 and 1 μM) or dibutyryl cyclic AMP (0.5 and 1 mM) for 10 min at 37°C. The stimulant FMLP (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-L-alanyl-L-alanine-p-nitroanilide, 3 mM in 0.3% dimethyl sulphoxide (DMSO) in 0.05 M Tris-HCl (pH 8.0). Elastase activity was assayed at a wavelength of 405 nm. These results are expressed as the mean percentage of the amount of enzyme released by the corresponding FMLP/CB-activated, drug-free control systems.

Drugs and reagents

The selective PDE 4 inhibitor rolipram and the β_2-agonist, salbutamol were kindly provided by Dr Malcolm Johnson, GlaxoWellcome plc, Stockley Park West, London, while the non-selective PDE inhibitor, theophylline, and the cell-permeable cyclic AMP analogue, dibutyryl cyclic AMP were purchased from the Sigma Chemical Co, St Louis, MO, U.S.A. Rolipram was used because of its potent effects on PDE 4, the predominant type present in human neutrophils (Schwabe *et al*., 1976; Nielson *et al*., 1990), while salbutamol was preferred to other short- and long-acting β_2-agonists because its interactions with human neutrophils are uncomplicated by other activities such as superoxide-scavenging or membrane-stabilizing properties (Anderson *et al*,, 1996). Theophylline was selected for investigation because of its usefulness in the treatment of bronchial asthma and possible anti-inflammatory activity (Barnes & Pauwels, 1994). Rolipram was dissolved in DMSO to give a stock concentration of 10 mM and diluted in the same solvent. The final concentration of DMSO in all assay systems in which rolipram was used was 0.5% or less and appropriate solvent systems were included. Theophylline, salbutamol and dibutyryl cyclic AMP were dissolved in distilled water, 0.05 N HCl and HBSS respectively to give stock solutions of 10 mM, 10 mM and 80 mM and diluted thereafter in HBSS. Unless indicated, all other chemicals and reagents were purchased from the Sigma Chemical Co.

Statistical analysis

The results of each series of experiments are expressed as the mean values ± s.e. mean. Levels of statistical significance were calculated by paired Student's *t* test when comparing two groups, or by analysis of variance (ANOVA) with subsequent Tukey-Kramer multiple comparisons test for multiple groups.

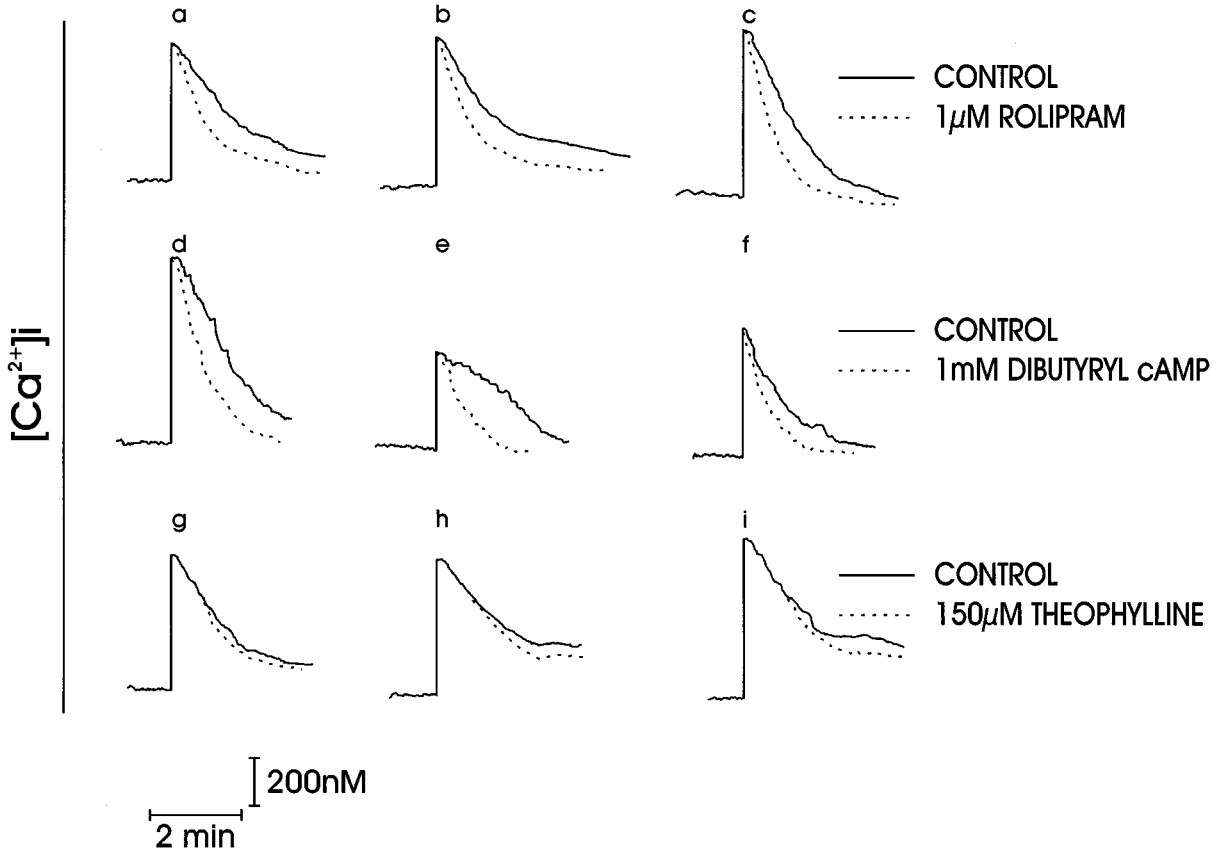

Figure 1 FMLP-activated fura 2 fluorescence responses of control and drug-treated neutrophils. FMLP was added when a stable base-line was obtained (±1 min). Traces (a – c), (d – f) and (g – i) are derived from 9 different experiments and show the effects of 1 μM rolipram, 1 mM dibutyryl cyclic AMP and 150 μM theophylline, respectively.

A computer-based software system (Instat II) was used for analysis. Significance levels were taken at a P value of <0.05.

Results

Fura-2 responses of FMLP-activated neutrophils

The results shown in Figure 1 are traces from 9 typical experiments, which depict the effects of 1 μM rolipram, 1 mM dibutyryl cyclic AMP and 150 μM theophylline on the fura-2 responses of FMLP-activated neutrophils. Addition of FMLP to neutrophils was accompanied by the characteristic, abrupt increase in fura-2 fluorescence due to an increase in the cytosolic concentration of Ca^{2+}. Rolipram, theophylline and dibutyryl cyclic AMP did not affect this abrupt increase in peak fluorescence intensity. However, all 3 agents hastened the rate of the subsequent decline in fluorescence intensity, indicative of accelerated clearance of Ca^{2+} from the cytosol. Relative to rolipram and dibutyryl cyclic AMP, the effects of theophylline on the rate of decline in fluorescence intensity were delayed and less impressive. Salbutamol (0.1 and 1 μM) did not affect either the peak fluorescence intensity or the rate of decline of fluorescence in fura-2-loaded, FMLP-activated neutrophils (not shown).

The results shown in Table 1 are those from a larger series of experiments and show peak cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$), as well as the time taken for fluorescence intensity to decline to half peak ($t_{½}$) values, for neutrophils activated with FMLP in the presence and absence of varying concentrations of rolipram, theophylline, salbutamol and dibutyryl cyclic AMP. As indicated above none of the test agents affected the abruptly occurring increase in $[Ca^{2+}]_i$ following activation of neutrophils with FMLP. Rolipram and dibutyryl cyclic AMP at concentrations of 0.01 μM and 0.5 mM and upwards respectively, significantly decreased the time taken for fluorescence intensity to decline to half peak values, while theophylline was less impressive and salbutamol was without detectable effects. There were no significant differences between 0.5 mM and 1 mM dibutyryl cyclic AMP or between concentrations of 1 mM, 2 mM and 4 mM of this agent, while 2 mM and 4 mM were significantly different ($P<0.05$ and $P<0.001$, respectively) from 0.5 mM. In the case of rolipram, there were no detectable differences between the effects of 0.01 μM and 0.025 μM on the rate of decline in fluorescence intensity, while all higher concentrations (0.05–1 μM) were significantly different ($P<0.05$–$P<0.0001$) from those of 0.01 μM rolipram, and the effects of this agent at 0.05 μM were significantly different ($P<0.05$) from those of 1 μM. There were no significant differences between concentrations of 0.1, 0.25, 0.5 and 1 μM rolipram.

The effects of salbutamol (1 μM) and rolipram (0.005 μM) in combination, on the peak cytosolic Ca^{2+} concentrations of FMLP-activated neutrophils, as well as on the time taken for fluorescence intensity to decline to half peak values are shown in Table 2. Rolipram and salbutamol in combination had no effects on peak fluorescence intensity, but the time taken to reach half peak values was significantly less for the combination than for the individual agents.

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

In these experiments, neutrophils which had been pre-loaded with $^{45}Ca^{2+}$ and then washed and transferred to Ca^{2+}-replete

Table 1 Peak intracellular calcium concentrations $[Ca^{2+}]_i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils treated with dibutyryl cyclic AMP, rolipram, theophylline and salbutamol

Agent	*Peak $[Ca^{2+}]_i$ values* (nM)	*Time taken to decline to half peak values* (min)
Control	648±38	1.30±0.03
Dibutyryl cAMP 0.5 mM	651±44	1.04±0.06*
Dibutyryl cAMP 1 mM	649±41	0.92±0.04*
Dibutyryl cAMP 2 mM	649±35	0.85±0.03*
Dibutyryl cAMP 4 mM	640±47	0.76±0.02*
Rolipram 0.01 μM	634±53	1.11±0.03*
Rolipram 0.025 μM	628±37	1.01±0.03*
Rolipram 0.05 μM	676±52	0.91±0.01*
Rolipram 0.1 μM	651±46	0.89±0.05*
Rolipram 0.5 μM	602±47	0.77±0.03*
Rolipram 1 μM	622±47	0.72±0.03*
Theophylline 150 μM	609±41	1.09±0.03*
Salbutamol 1 μM	620±50	1.25±0.04

The results of 7 experiments are expressed as the mean values±s.e.mean. The $[Ca^{2+}]_i$ value for resting, unstimulated neutrophils was 146±28 nM. *$P<0.05$.

Table 2 Peak intracellular calcium concentrations $[Ca^{2+}]_i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils treated with rolipram and salbutamol individually and in combination

System	*Peak $[Ca^{2+}]_i$ values* (nM)	*Time taken for peak $[Ca^{2+}]_i$ to decline to half peak values* (min)
Control	742±18	1.3±0.10
Rolipram 0.005 μM	806±17	1.1±0.10
Salbutamol 1 μM	762±28	1.4±0.13
Rolipram+salbutamol	778±33	0.9±0.10*

The results of 6 experiments are presented as the mean values±s.e.mean. *$P<0.001$, $P<0.05$ and $P<0.001$, respectively, for comparison of the system containing rolipram and salbutamol with the control system and for systems containing rolipram or salbutamol only.

HBSS (to minimize re-uptake of radiolabelled cation) were activated with FMLP in the presence and absence of rolipram (1 μM), theophylline (150 μM), salbutamol (1 μM) and dibutyryl cyclic AMP (1 mM) followed by measurement of the amount of cell-associated $^{45}Ca^{2+}$. Exposure of the drug-free, control neutrophils to FMLP resulted in an abrupt efflux of the radiolabelled cation from the neutrophils, which terminated approximately 30 s after the addition of the stimulant, resulting in the loss of about 50% of cell-associated $^{45}Ca^{2+}$. Importantly, there was no loss of cell-associated $^{45}Ca^{2+}$ from control, unstimulated neutrophils over the brief 60 s incubation period during which efflux was measured (Figure 2). Pre-incubation of neutrophils with salbutamol did not affect the rate or extent of efflux of $^{45}Ca^{2+}$ (not shown), while these were slightly, but not significantly, increased by theophylline. However, a significant reduction in efflux of $^{45}Ca^{2+}$ at 60 s was observed in FMLP-activated neutrophils which had been exposed to rolipram ($P<0.005$) or dibutyryl cyclic AMP ($P<0.01$). The results of a series of experiments in which the effects of rolipram at concentrations of 0.1, 0.5 and 1 μM and dibutyryl cyclic AMP at 0.5 and 1 mM on the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils using a fixed 60 s incubation period are shown in Table 3. Both agents caused dose-related inhibition of the efflux of $^{45}Ca^{2+}$ from activated neutrophils. Although these differed significantly from the control system, there were no significant differences between the effects of 0.5 mM and 1 mM dibutyryl cyclic AMP, or between those of 0.1, 0.5 and 1 μM rolipram.

The effects of the endo-membrane Ca^{2+}-ATPase inhibitor, thapsigargin on dibutyryl cyclic AMP- and rolipram-mediated modulation of efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils are shown in Figure 3. In the presence of thapsigargin neither dibutyryl cyclic AMP nor rolipram affected the efflux of the radiolabelled cation from the FMLP-stimulated cells.

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

For these experiments neutrophils were pre-loaded 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 minimize spontaneous uptake of $^{45}Ca^{2+}$ by neutrophils (Anderson & Goolam Mahomed, 1997). The results of these experiments designed to measure net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils are shown in Figure 4. Activation of control, drug-free neutrophils with FMLP under these experimental conditions resulted in a delayed uptake of $^{45}Ca^{2+}$, which occurred after a lag phase of 30–60 s. Influx of $^{45}Ca^{2+}$ appeared to be 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,

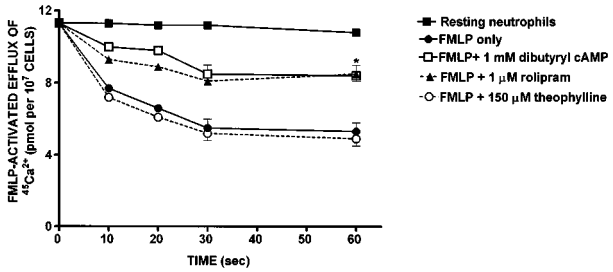

Figure 2 Kinetics of efflux of $^{45}Ca^{2+}$ from resting and FMLP-activated control neutrophils, and from FMLP-activated neutrophils treated with 1 μM rolipram, 1 mM dibutyryl cyclic AMP and 150 μM theophylline. The results of 4–5 different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol/10^7 cells) with vertical lines showing s.e.mean; $*P<0.01$ and $*P<0.005$ for the reduction in efflux at 60 s observed with FMLP-activated neutrophils in the presence of dibutyryl cyclic AMP and rolipram, respectively, by comparison with control cells activated by FMLP in the absence of these agents.

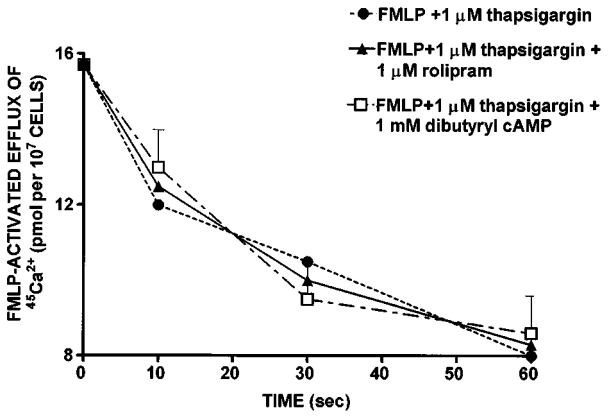

Figure 3 Kinetics of efflux of $^{45}Ca^{2+}$ from thapsigargin (1 μM)-treated, FMLP-activated control neutrophils and from similarly treated neutrophils which had been pre-incubated with 1 μM rolipram or 1 mM dibutyryl cyclic AMP. The results of 3 different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol/10^7 cells) and vertical lines show s.e.mean.

Table 3 Effects of dibutyryl cyclic AMP and rolipram on the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils

Agent	*Amount of $^{45}Ca^{2+}$ released from neutrophils 60 s after the addition of FMLP* (pmol/10^7 cells)
FMLP only	6.3±1.5
FMLP+0.5 mM dibutyryl cAMP	3.0±1*
FMLP+1 mM dibutyryl cAMP	2.3±0.6*
FMLP+0.1 μM rolipram	3.8±1*
FMLP+0.5 μM rolipram	2.3±0.5*
FMLP+1 μM rolipram	2.5±0.5*

The results of 5–6 experiments are expressed as the mean values±s.e.mean. $*P<0.05$ for comparison with the control system.

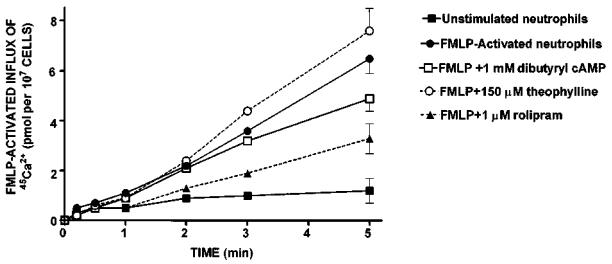

Figure 4 Kinetics of influx of $^{45}Ca^{2+}$ into resting (unstimulated) and FMLP-activated control neutrophils, and into FMLP-activated neutrophils treated with 1 μM rolipram, 1 mM dibutyryl cyclic AMP and 150 μM theophylline. The results of 3–5 experiments are expressed as the mean uptake of $^{45}Ca^{2+}$ in pmol/10^7 cells; vertical lines show s.e.mean.

identically-processed neutrophils, which had not been exposed to FMLP. Pretreatment of neutrophils with salbutamol (0.1 and 1 μM) did not detectably alter either the rate or the extent of influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils (not shown), while theophylline (150 μM) caused a slight, but insignificant increase in the extent of influx of the radiolabelled cation after 5 min. Rolipram and dibutyryl cyclic AMP, however, significantly reduced the influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils. Five minutes after the addition of FMLP to neutrophils the respective P values for inhibition of influx in the presence of rolipram and dibutyryl cyclic AMP were <0.0001 and <0.01.

The effects of varying concentrations of rolipram (0.1, 0.25, 0.5 and 1 μM) and dibutyryl cyclic AMP (0.5 and 1 mM) on the influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils after a fixed 5 min incubation period are shown in Table 4. Both agents caused dose-related inhibition of uptake of the radiolabelled cation, which achieved statistical significance for both concentrations of dibutyryl cyclic AMP and for 0.25, 0.5 and 1 μM rolipram. However, there were no significant differences between the effects of 0.5 mM and 1 mM dibutyryl cyclic AMP or between those of the various rolipram concentrations.

Superoxide generation and elastase release

The effects of rolipram, theophylline, salbutamol and dibutyryl cyclic AMP on the production of superoxide by, and release of elastase from, neutrophils activated with FMLP and FMLP/CB respectively are shown in Table 5. Rolipram and dibutyryl cyclic AMP, at concentrations which affected Ca^{2+} metabolism in activated neutrophils, caused statistically significant dose-related inhibition of both superoxide generation and elastase release. However, there were no statistically significant differences between concentrations of rolipram or those of dibutyryl cyclic AMP. Theophylline caused modest inhibition of superoxide generation without affecting elastase release, while salbutamol had no effects on either of these. The interactive effects of a low concentration of rolipram (0.005 μM) in combination with salbutamol (1 μM) on FMLP-activated superoxide production by neutrophils are shown in Table 6. The combination of salbutamol and rolipram was more effective in inhibiting superoxide generation than either agent alone.

None of the test agents at the concentrations used was found to possess superoxide-scavenging activity using the cell-free hypoxanthine-xanthine oxidase system (not shown).

Table 4 Effects of dibutyryl cyclic AMP and rolipram 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	5.6±1.3
FMLP+0.5 mM dibutyryl cAMP	4.1±0.3*
FMLP+1 mM dibutyryl cAMP	3.4±0.5*
FMLP+0.1 μM rolipram	3.7±0.5
FMLP+0.25 μM rolipram	2.9±0.3*
FMLP+0.5 μM rolipram	2.7±0.3*
FMLP+1 μM rolipram	2.7±0.4*

The results of 5 experiments are expressed as the mean values±s.e.mean. For these experiments the relevant background values for unstimulated neutrophils in the absence of FMLP were subtracted from the values for FMLP-activated systems. *$P<0.01$, for comparison with the control system.

Table 5 Effects of dibutyryl cyclic AMP, rolipram, theophylline and salbutamol on the production of superoxide (LECL) and release of elastase by FMLP-activated neutrophils

Agent	*LECL* (% control)	*Elastase release* (% control)
Dibutyryl cAMP 0.5 mM	42±5*	71±6*
Dibutyryl cAMP 1 mM	32±3*	65±8*
Rolipram 0.01 μM	65±6*	67±3*
Rolipram 0.1 μM	48±7*	47±2*
Rolipram 0.5 μM	37±5*	50±3*
Rolipram 1 μM	35±5*	50±3*
Theophylline 37.5 μM	111±4	128±9
Theophylline 75 μM	79±9	127±13
Theophylline 150 μM	74±4	110±10
Salbutamol 1 μM	96±3	114±5

The results of 8 experiments are expressed, in the case of superoxide generation, as the mean percentage±s.e.mean of the peak LECL values of the corresponding drug-free, FMLP-activated control system (absolute value 904±60 mV s^{-1}), or in the case of elastase release as the mean percentage of enzyme released by FMLP/CB-activated control neutrophils (absolute values of 0.01±0.002 and 0.63±0.05 units of enzyme per 10^7 unstimulated and stimulated cells, respectively). *$P<0.05$.

Table 6 The effects of rolipram and salbutamol individually and in combination on superoxide generation (LECL) by FMLP-activated neutrophils

Agent	*LECL* (% control)
Rolipram 0.005 μM	85±4
Salbutamol 1 μM	92±3
Rolipram+salbutamol	62±5*

The results of 6 experiments are expressed as the mean percentage±s.e.mean of the peak LECL values of the corresponding drug-free, FMLP-activated control system (absolute value 696±117 mV s^{-1}). *$P<0.001$, $P<0.05$ and $P<0.01$, respectively, for comparison of the combination with the control system and for systems containing rolipram or salbutamol alone.

Discussion

Exposure of neutrophils to chemoattractants, including FMLP, has been shown to cause a transient increase in cyclic AMP concentrations (Anderson *et al*., 1976; Iannone *et al*., 1989), which is achieved by direct (Tsu *et al*., 1995) and indirect (Verghese *et al*., 1985; Iannone *et al*., 1989) mechanisms. Of the cyclic AMP-elevating, pharmacological agents used in the present study, the PDE inhibitors, particularly rolipram, have been shown to cause pronounced and sustained increases in cyclic AMP concentrations in FMLP-activated neutrophils (Nielson *et al*., 1988; Iannone *et al*., 1989; Schudt *et al*., 1991). However, β_2-agonists such as salbutamol are apparently ineffective stimulators of cyclic AMP synthesis in neutrophils (Marone *et al*., 1980) and only when used in combination with PDE inhibitors do they cause significant inhibition of neutrophil activation (Nielson, 1987).

With the fura-2 spectrofluorimetric procedure, none of the three cyclic AMP-elevating, pharmacological agents used in the present study, as well as dibutyryl cyclic AMP, affected the immediate increase in the concentration of cytosolic Ca^{2+} in FMLP-activated neutrophils. Since the radiometric procedure demonstrated no detectable influx of extracellular Ca^{2+}

coincident with peak fluorescence intensity in FMLP-activated neutrophils, the increase in the cytosolic concentration of the cation appears to result exclusively from its release from intracellular storage pools. These observations suggest that cyclic AMP-elevating agents do not affect the activation of phospholipase C and generation of inositol triphosphate in FMLP-activated neutrophils, nor do they interfere with the interaction of this second messenger with Ca^{2+}-mobilizing receptors on calciosomes or the subsequent discharge of the cation from these stores (Prentki *et al*., 1984; Krause *et al*., 1989).

Although none of the test agents affected the release of Ca^{2+} from intracellular storage pools, the rate of decline of peak fura-2 fluorescence in FMLP-activated neutrophils was hastened by dibutyryl cyclic AMP, rolipram and, to a lesser extent, theophylline indicative of accelerated clearance of Ca^{2+} from the cytosol. Relative to rolipram and dibutyryl cyclic AMP, the effects of theophylline on the rate of decline of peak fura-2 fluorescence were delayed and less impressive. In apparent confirmation of the relative ineffectiveness of β_2-agonists *per se* in modulating neutrophil activation (Marone *et al*., 1980), salbutamol only caused significant acceleration of the rate of decline in the peak fura-2 fluorescence responses of FMLP-activated neutrophils when used in combination with a low dose of rolipram (0.005 μM). These observations with the fura-2 system are in agreement with previous findings that rolipram (Villagrasa *et al*., 1996), and theophylline in combination with dibutyryl cyclic AMP (De Togni *et al*., 1984) accelerate the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils. However, the spectrofluorimetric procedures used by us and these other investigators (De Togni *et al*., 1984; Villagrasa *et al*., 1996) cannot, in isolation, be used to identify the mechanism (accelerated efflux and/or re-sequestration of the cation, or inhibition of influx) by which cyclic AMP-elevating agents accelerate the removal of Ca^{2+} from the cytosol of FMLP-activated neutrophils.

In an attempt to address this problem, we combined radiometric procedures with the fura-2 spectrofluorimetric method. The radiometric procedures, unlike the fura-2 fluorescence system, can distinguish between net efflux and influx of Ca^{2+} following activation of neutrophils with FMLP (Anderson & Goolam Mahomed, 1997). By use of these procedures, exposure of neutrophils to FMLP was accompanied by an abrupt efflux of $^{45}Ca^{2+}$, which terminated at about 30 s after addition of the stimulant, resulting in the loss of about 50% of cell-associated cation. This observation suggests that not all of the intracellular Ca^{2+} pool is discharged during exposure of neutrophils to the chemoattractant, or that rapid re-sequestration of mobilized cation, as a consequence of activation of the endo-membrane Ca^{2+}-ATPase (Schatzman, 1989), also contributes to clearance of the cation from the cytosol.

Interestingly, during the period of efflux there was no detectable influx of $^{45}Ca^{2+}$ into FMLP-stimulated neutrophils. Net influx of the cation occurred only after efflux had terminated, being detected at 30–60 s after addition of FMLP, and terminating, as reported previously (Anderson & Goolam Mahomed, 1997), at around 5 min after addition of the stimulant. This delayed influx of Ca^{2+} is characteristic of a store-operated influx, which is operative in a large variety of cell types (Favre *et al*., 1996), including neutrophils (Montero *et al*., 1991; Anderson & Goolam Mahomed, 1997), and those cells that do not appear to have it are considered to be rather exceptional. A close negative relationship between membrane depolarization and store-operated Ca^{2+} influx has recently been described in FMLP-activated human neutrophils (Geiszt *et al*., 1997).

Pretreatment of neutrophils with dibutyryl cyclic AMP and rolipram, but not with theophylline, significantly reduced the amount of Ca^{2+} released from FMLP-activated neutrophils, as well as the magnitude of the subsequent store-operated influx of the cation. In conjunction with the results of the fura-2 experiments, these observations suggest that rolipram and dibutyryl cyclic AMP up-regulate the activity of the cyclic AMP-dependent protein kinase (PKA)-activatable endo-membrane Ca^{2+}-ATPase (Schatzmann, 1989; Tao *et al*., 1992). This would result in 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 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+}.

While these results are compatible with a primary, up-regulatory effect of dibutyryl cyclic AMP and rolipram on the endo-membrane Ca^{2+}-ATPase of activated neutrophils, the effects, if any, of these intracellular cyclic AMP-elevating agents on the activity of the plasma membrane Ca^{2+}-efflux pump were more difficult to detect. In neutrophils the activity of the plasma membrane Ca^{2+}-ATPase appears to be modulated by calmodulin which shifts the pump to a higher affinity state for Ca^{2+}, resulting in enhanced maximal velocity (Lagast *et al*., 1984). In an attempt to unravel possible effects of dibutyryl cyclic AMP and rolipram on the neutrophil plasma membrane Ca^{2+}-efflux pump, we investigated the effects of these agents on the efflux of Ca^{2+} from FMLP-activated neutrophils which had been treated with thapsigargin, a selective inhibitor of the endo-membrane Ca^{2+}-ATPase (Lytton *et al*., 1991). This strategy was used to eliminate the possible complicating effects of up-regulation of this Ca^{2+}-storage pump by dibutyryl cyclic AMP and rolipram. Treatment of neutrophils with thapsigargin abolished the dibutyryl cyclic AMP- and rolipram-mediated reduction in efflux of Ca^{2+} from FMLP-activated neutrophils, demonstrating that these agents have no inhibitory or stimulatory effects on the activity of the plasma membrane Ca^{2+}-ATPase. Accelerated clearance of Ca^{2+} from the cytosol of dibutyryl cyclic AMP- and rolipram-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.

Theophylline at the concentrations used (up to 150 μM) did not significantly affect either the efflux from, or the influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils. This is in agreement with the fura-2 fluorescence results, which demonstrated that the effects of theophylline on clearance of cytosolic Ca^{2+} in FMLP-activated neutrophils were delayed and of lesser magnitude than those of rolipram and dibutyryl cyclic AMP, possibly as a consequence of the modest inhibitory effects of this agent on PDE (Polson *et al*., 1978). Alternatively, theophylline by acting as an antagonist of type A_2 receptors (Dalziel & Westfall, 1994) may interfere with FMLP-stimulated synthesis of cyclic AMP, which is apparently achieved by an indirect mechanism involving adenosine-mediated activation of adenylate cyclase (Iannone *et al*., 1989).

The relationship between the observed enhancement of clearance of cytosolic Ca^{2+} in FMLP-activated neutrophils by the cyclic AMP-elevating agents and alterations in neutrophil functions were also investigated. It is well-recognized and

accepted that oxidant generation and granule enzyme release by stimuli which cause mobilization of Ca^{2+} in neutrophils are sensitive to inhibition by cyclic AMP-elevating agents, including rolipram and dibutyryl cyclic AMP (Ladd *et al*., 1985; Nielson *et al*., 1990; Moore & Willoughby, 1995). Interestingly, in our study rolipram and dibutyryl cyclic AMP caused inhibition of FMLP-activated superoxide generation and elastase release at the same concentrations (and with the same relative potencies) as those which accelerated the clearance of cytosolic Ca^{2+} from stimulated neutrophils, implying a mechanistic relationship between the two. Theophylline at 75 μM and 150 μM caused weak, but insignificant inhibition of superoxide generation, and had no effects on FMLP/CB-activated release of elastase from neutrophils, possibly as a result of the potency of this stimulus relative to FMLP alone.

In conclusion, the results presented here demonstrate that cyclic AMP-elevating agents, particularly rolipram, accelerate the re-sequestration of cytosolic Ca^{2+} in FMLP-activated neutrophils, probably by up-regulation of the activity of the endo-membrane Ca^{2+}-ATPase, leading to inhibition of Ca^{2+}-dependent neutrophil functions. This mechanism is likely to account for the anti-inflammatory properties of these agents, at least in the case of neutrophils.

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