British Journal of Pharmacology (2000) 130, 717–724

www.nature.com/bjp

Accelerated resequestration of cytosolic calcium and suppression of the pro-inflammatory activities of human neutrophils by CGS 21680 *in vitro*

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1 We have investigated the effects of the adenosine A_{2A} receptor agonist CGS 21680 (0.01–1 μM) on reactive oxidant production by, and elastase release from FMLP-activated human neutrophils, as well as on cytosolic Ca^{2+} fluxes and intracellular concentrations of cyclic AMP.

2 Oxidant production, elastase release and cyclic AMP were assayed using lucigenin-enhanced chemiluminescence, colourimetric and radioimmunoassay procedures respectively, while cytosolic Ca^{2+} fluxes were measured by fura-2 spectrofluorimetry in combination with radiometric procedures which distinguish between net efflux and influx of the cation.

3 Treatment of neutrophils with CGS 21680 did not affect the FMLP-activated release of Ca^{2+} from intracellular stores, but resulted in dose-related acceleration of the rate of decline in fura-2 fluorescence, as well as decreases in both efflux and store-operated influx of Ca^{2+}, compatible with enhancement of resequestration of the cation by the endo-membrane Ca^{2+}-ATPase. These effects on neutrophil Ca^{2+} handling were associated with increased intracellular cyclic AMP and with inhibition of oxidant production and release of elastase.

4 In contrast, treatment of neutrophils with the selective A_{2A} receptor antagonist, ZM 241385 (2.5 μM), prevented the transient increase in cyclic AMP in FMLP-activated neutrophils which was associated with delayed sequestration of incoming Ca^{2+} during store-operated influx.

5 The CGS 21680-mediated reduction of Ca^{2+} efflux from FMLP-activated neutrophils was also antagonized by pretreatment of the cells with ZM 241385 (2.5 μM), as well as by thapsigargin (1 μM), an inhibitor of the endo-membrane Ca^{2+}-ATPase. ZM 241385 also neutralized the cyclic AMP-elevating and anti-inflammatory interactions of CGS 21680 with neutrophils.

6 We conclude that A_{2A} receptors regulate the pro-inflammatory activities of human neutrophils by promoting cyclic AMP-dependent sequestration of cytosolic Ca^{2+}.

British Journal of Pharmacology (2000) **130,** 717–724

Keywords: CGS 21680; adenosine; A_{2A} receptors; cyclic AMP; calcium efflux; calcium influx; neutrophils; elastase; superoxide; ZM 241385

Abbreviations: AR, adenosine receptor; cyclic AMP, adenosine 3′:5′ cyclic monophosphate; CB, cytochalasin B; CGS 21680, 2(4-[(2-carboxyethyl)phenyl]ethylamino)-5′-N-ethylcarboxamido adenosine; DMSO, dimethyl sulphoxide; FMLP, N-formyl-L-methionyl-L-leucyl-L-phenylalanine; HBSS, Hanks balanced salt solution; LECL, lucigenin-enhanced chemiluminescence; PBS, phosphate-buffered saline; ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4)triazolo[2,3-a][1,3,5]triazin-5-yl aminoethyl) phenol

Introduction

In spite of their undisputed clinical efficacy, concerns about the long-term safety of inhaled corticosteroids in patients with bronchial asthma, as well as the apparent insensitivity of neutrophils to the anti-inflammatory effects of these agents, has underscored the requirement for novel anti-inflammatory chemotherapies (Cox, 1995; Meagher *et al*., 1996; McFadden, 1998). The anti-inflammatory potential of physiologic and pharmacologic cyclic AMP-elevating agents, which spans many different types of immune and inflammatory cells, including neutrophils, has been recognized for more than two decades (Moore & Willoughby, 1995). However, the development of clinically useful cyclic AMP-based, anti-inflammatory chemotherapeutic agents has, until recently, enjoyed limited success due to lack of selectivity of these for immune and inflammatory cells. Recent innovations include the second generation type 4 phosphodiesterase (PDE) inhibitors (Torphy, 1998; Underwood *et al*., 1998) and adenosine receptor (AR) agonists operative at the A_{2A} receptor subtype (Ongini & Fredholm, 1996).

Novel second generation inhibitors of type 4 PDE, the predominant type found in human neutrophils being PDE 4B2 (Wang *et al*., 1999), have been designed to maximize anti-inflammatory efficacy in the setting of decreased gastrointestinal toxicity (Torphy, 1998). The anti-inflammatory effects of type 4 PDE inhibitors are mediated by cyclic AMP-dependent mechanisms (Underwood *et al*., 1998), which, in the case of neutrophils, involve accelerated clearance of cytosolic Ca^{2+} by apparent enhancement of the activity of the endo-membrane Ca^{2+}-ATPase (Anderson *et al*., 1998).

Subtype A_{2A} receptors have recently been demonstrated on human neutrophils (Varani *et al*., 1998). Occupation of these by adenosine or adenosine agonists has been reported to suppress the pro-inflammatory activities of human neutrophils, which in some (Hannon *et al*., 1998; Sullivan & Linden, 1998; Varani *et al*., 1998), but not all (Cronstein *et al*., 1985;

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Iannone *et al*., 1989) studies has been attributed to a cyclic AMP-dependent mechanism.

In the present study we have investigated the effects of the prototype $A_{2A}R$ agonist, CGS 21680 (Phillis *et al*., 1990), as well as those of the highly selective $A_{2A}R$ antagonist, ZM 241385 (Poucher *et al*., 1995), on the pro-inflammatory activities of FMLP-activated human neutrophils *in vitro*, and related changes in these to alterations in Ca^{2+} fluxes and intracellular cyclic AMP.

Methods

Neutrophils

Purified neutrophils were prepared from heparinized (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 $400 \times g$ 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.

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 Hanks' balanced salt solution (HBSS, pH 7.4) containing 1.25 mM $CaCl_2$, referred to hereafter as Ca^{2+}-replete HBSS. The fura-2-loaded cells (2×10^6 ml^{-1}) were then pre-incubated with CGS 21680 (0.01 – 1 μM) at 37°C for 10 min 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 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. At this concentration (1 μM) FMLP activates secretion and superoxide production by neutrophils and has maximal effects on the release of Ca^{2+} from intracellular stores (Snyderman & Uhing, 1992). 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 pretreatment with the selective A_{2A} receptor antagonist ZM 241385 (Poucher *et al*., 1995) at 2.5 μM on CGS 21680-mediated alterations in the fura-2 fluorescence responses of FMLP-activated neutrophils. ZM 241385 was added during preincubation of the cells at 37°C, 5 min prior to CGS 21680.

Radiometric assessment of Ca^{2+} fluxes

$^{45}Ca^{2+}$ (Calcium-45 chloride, specific activity 25.4 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} 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 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 $^{45}Ca^{2+}$. 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 1 μM CGS 21680, followed by activation with FMLP (1 μM) and measurement of the kinetics (10, 20, 30 and 60 s) of efflux of $^{45}Ca^{2+}$. A fixed incubation time of 60 s was used for dose-response experiments. 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 $400 \times g$ for 5 min followed by washing with 15 ml ice-cold, Ca^{2+}-replete HBSS and the cell pellets finally dissolved in 0.5 ml of Triton X-100/0.1 M NaOH and the radioactivity assessed in a liquid scintillation spectrometer. Control, cell-free systems (HBSS and $^{45}Ca^{2+}$ only) were included for each experiment and these values were subtracted from the relevant neutrophil-containing systems. The results are presented as the amount of cell-associated radiolabelled cation (pmol $^{45}Ca^{2+}.10^7$ $cells^{-1}$).

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), as well as those of ZM 241385 on CGS 21680 (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 CGS 21680, while ZM 241385 (2.5 μM) was added to the cells 5 min before the adenosine receptor agonist.

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

To measure the net influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils, uncomplicated by concomitant efflux of the radiolabelled cation, the cells were loaded with cold, Ca^{2+}-replete HBSS for 30 min at 37°C after which they were pelleted by centrifugation, then washed once with, and resuspended in ice-cold Ca^{2+}-free HBSS and held on ice until used. Pre-loading with cold Ca^{2+} was undertaken to minimize spontaneous uptake of $^{45}Ca^{2+}$ (unrelated to FMLP activation) in the influx assay. The Ca^{2+}-loaded neutrophils (2×10^6 ml^{-1}), were then incubated for 10 min in the presence and absence of CGS 21680 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.

A fixed time interval (5 min) was used for experiments in which the effects of varying concentrations of CGS 21680 (0.1 and 1 μM) on the influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils were investigated.

Oxidant generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils (1×10^6 ml^{-1}, final) were pre-incubated for 15 min in 900 μl HBSS containing 0.2 mM lucigenin in the presence and absence of CGS 21680 (0.01 – 1 μM). Spontaneous and FMLP (1 μM)-activated LECL responses were then recorded using an 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 s^{-1}. Additional experiments were performed to investigate the following: (i) the effects of ZM 241385 (2.5 μM), and thapsigargin (1 μM) on the CGS 21680 (1 μM)-mediated inhibition of the LECL responses of FMLP-activated neutrophils; and (ii) the superoxide-scavenging potential of CGS 21680, thapsigargin and ZM 241385 using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits 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 1×10^7 ml^{-1} in HBSS in the presence or absence of CGS 21680 (0.01 – 1 μM) with and without ZM 241385 (2.5 μM) or thapsigargin (1 μM) for 10 min at 37°C. The stimulant FMLP (0.1 μM) in combination with cytochalasin B (1 μM) was then added and the reaction mixtures incubated for 10 min at 37°C. The tubes were then transferred to an ice-bath, followed by centrifugation at $400 \times g$ 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 and the results expressed as the mean percentage of the amount of enzyme released by the corresponding FMLP/CB-activated, drug-free control systems.

Intracellular cyclic AMP levels

Neutrophils at a concentration of 1×10^7 ml^{-1} in HBSS were preincubated for 10 min at 37°C with CGS 21680 (1 μM) with and without ZM 241385 (2.5 μM). Following preincubation, the cells were activated with 1 μM FMLP (stimulated cells), or an equal volume of HBSS (unstimulated cells), in a final volume of 1 ml, after which the reactions were terminated and the cyclic AMP extracted by the addition of ice-cold ethanol (65% v v^{-1}) at 20 s, 1, 3 and 5 min after addition of the stimulant. The resultant precipitates were washed twice with ice-cold ethanol and the supernatants pooled and centrifuged at $2000 \times g$ for 15 min at 4°C. The supernatants were then transferred to fresh tubes and evaporated at 60°C under a stream of air. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cyclic AMP using the Biotrak cAMP [^{125}I] scintillation proximity assay system (Amersham International plc, Buckinghamshire, U.K.), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmol cyclic AMP 10^7 neutrophils^{-1}. Because cyclic AMP is rapidly hydrolyzed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μM rolipram, a selective inhibitor of type 4 phosphodiesterase, the predominant type present in human neutrophils (Torphy, 1998).

Drugs and reagents

CGS 21680 and rolipram were kindly provided by Dr Malcolm Johnson, (GlaxoWellcome plc, Stockley Park West, London, U.K.) while thapsigargin and ZM 241385 were purchased from the Sigma Chemical Co. and Tocris Cookson Ltd, (Bristol, U.K.) respectively. Rolipram and thapsigargin were dissolved in DMSO to give a stock concentration of 10 mM for each 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. CGS 21680 and ZM 241385 were dissolved in 0.1 N NaOH to give stock solutions of 10 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. 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 fluorescence responses of FMLP-activated neutrophils

The results shown in Figure 1 are traces from three different experiments which depict the effects of 1 μM CGS 21680 on the fura-2 responses of FMLP-activated neutrophils. Addition of FMLP to neutrophils in each experiment was accompanied by the characteristic, abrupt increases in fura-2 fluorescence due to elevated concentrations of cytosolic Ca^{2+}. CGS 21680 did

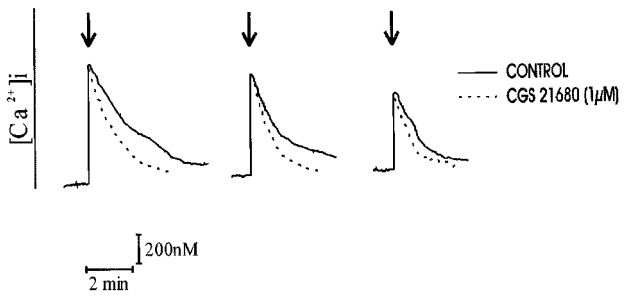

Figure 1 FMLP-activated fura-2 fluorescence responses of control and CGS 21680 (1 μM)-treated neutrophils. FMLP (1 μM) was added as indicated (↓) when a stable base-line was obtained (±1 min). The traces shown are from three different experiments.

not alter this abrupt increase in fluorescence intensity, demonstrating that this agent does not affect the release of Ca^{2+} from cellular stores. However, treatment of the cells with the AR agonist hastened the rate of the subsequent decline in fluorescence intensity, indicative of accelerated clearance of Ca^{2+} from the cytosol.

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 CGS 21680. As indicated above, CGS 21680 did not affect the abruptly occurring increase in $[Ca^{2+}]_i$ following activation of the cells with FMLP. However, the $A_{2A}R$ agonist caused dose-related acceleration in the rate of decline in peak fluorescence intensity, which at all four concentrations tested differed significantly from the drug-free control system.

The effects of ZM 241385 (2.5 μM) alone on the fura-2 responses of FMLP-activated control neutrophils are shown in Figure 2. ZM 241385 had no effects on either peak fluorescence intensity or the subsequent decline in fluorescence during the 2 min period following the addition of FMLP to the neutrophils. Thereafter however, there was a slight transient increase in fluorescence intensity in ZM 241385-treated cells, but not in control neutrophils, which coincided with the store-operated influx of extracellular Ca^{2+}. This lasted for 1–2 min after which fluorescence again subsided, but at a slower rate than that of the control cells. These effects of ZM 241385 on the fura-2 responses of FMLP-activated neutrophils were observed in 12 consecutive experiments using neutrophils from four different donors, and are compatible with interference with the sequestration of incoming Ca^{2+} into storage organelles.

Table 1 Effects of CGS 21680 on peak cytosolic calcium concentrations ($[Ca^{2+}]_i$) and rates of clearance (half peak values) in FMLP-activated neutrophils

Agent	*Peak $[Ca^{2+}]_i$ Values* (nM)	*Time taken to decline to half peak values* (min)
Control	474±53	1.1±0.1
CGS 21680 0.01 μM	418±24	0.87±0.1*
CGS 21680 0.1 μM	414±13	0.68±0.1*
CGS 21680 0.5 μM	415±17	0.65±0.1*
CGS 21680 1 μM	417±14	0.60±0.1*

The results of ten experiments are expressed as the mean values±s.e.mean. The $[Ca^{2+}]_i$ value for control, unstimulated neutrophils was 111±9 nM. *$P<0.05$ for comparison with the control system.

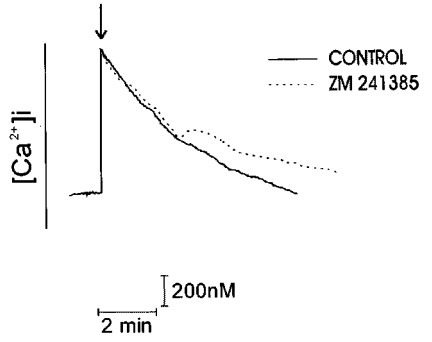

Figure 2 FMLP (1 μM)-activated fura-2 fluorescence responses of control and ZM 241385 (2.5 μM)-treated neutrophils. FMLP was added as indicated (↓) when a stable baseline was obtained (±1 min). This is a typical trace from 12 different experiments.

The effects of ZM 241385 (2.5 μM) alone and in combination with CGS 21680 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. Importantly, the half peak point of the decline in fura-2 fluorescence was reached before the effects of ZM 241385 *per se* on Ca^{2+} sequestration were evident. Pretreatment of neutrophils with ZM 241385 antagonized ($P<0.05$) CGS 21680-mediated acceleration of the clearance of cytosolic Ca^{2+} from activated neutrophils without affecting peak fluorescence intensity.

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 HBSS (to minimize re-uptake of radiolabelled cation) were activated with FMLP in the presence and absence of CGS 21680 (1 μM) followed by measurement of the amount of cell-associated $^{45}Ca^{2+}$. The results in Figure 3 show that 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 and resulted in the loss of 44% of cell-associated $^{45}Ca^{2+}$, while there was no detectable loss of the cation from

Table 2 Effects of CGS 21680±ZM 241385 on peak intracellular calcium concentrations $[Ca^{2+}]_i$ and rates of clearance in FMLP-activated neutrophils

System	*Peak $[Ca^{2+}]_i$ values* (nM)	*Time taken for peak $[Ca^{2+}]_i$ to decline to half peak values* (min)
Control	500±14	1.25±0.1
CGS 21680 1μM	495±18	0.83±0.1*
ZM 241385 2.5 μM	458±24	1.15±0.1
CGS 21680+ZM 241385	488±15	1.16±0.1**

The results of six experiments are expressed as the mean values±s.e.mean. *$P<0.05$ for comparison with the control system. **$P<0.05$ for comparison with CGS 21680 alone.

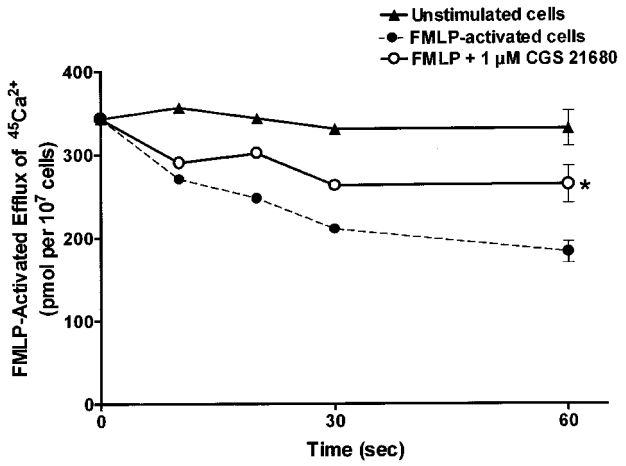

Figure 3 Kinetics of efflux of $^{45}Ca^{2+}$ out of unstimulated neutrophils and neutrophils activated with FMLP (1 μM) in the absence and presence of CGS 21680 (1 μM). The results of 11 different experiments are expressed as the mean amount of cell associated $^{45}Ca^{2+}$ (pmol 10^7 cells^{-1}) and vertical lines show s.e.mean. *$P<0.05$ for comparison with the FMLP-activated, CGS 21680-free control system.

unstimulated cells over the 60 s time course of the experiment. Treatment of neutrophils with CGS 21680 (1 μM) caused a significant decrease ($P<0.05$ at 60 s) in the efflux of $^{45}Ca^{2+}$ following activation of the cells with FMLP (Figure 3). The results of a series of experiments in which the effects of CGS 21680 at concentrations of 0.01, 0.1 and 1 μM on the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils using a fixed 60 s incubation period are shown in Table 3. The $A_{2A}R$ agonist caused dose-related inhibition of efflux of $^{45}Ca^{2+}$.

The effects of thapsigargin and ZM 241385 on the CGS 21680-mediated decrease in efflux of $^{45}Ca^{2+}$ out of FMLP-activated neutrophils are shown in Table 4. Thapsigargin (inhibitor of the endo-membrane Ca^{2+}-ATPase) and ZM 241385 attenuated the effects of CGS 21680 on efflux of $^{45}Ca^{2+}$. Over the short time-course of these experiments (60 s) the antagonists *per se* caused modest, statistically insignificant increases in the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils.

Influx of $^{45}Ca^{2+}$ 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 are shown in Figure 4. Activation of control, drug-free neutrophils with FMLP under these experimental conditions resulted in a delayed influx 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 the influx of the radiolabelled cation over the same time-course into control, identically-processed neutrophils, which received $^{45}Ca^{2+}$ only in the absence of FMLP, was considerably less. Pretreatment with CGS 21680 (1 μM) resulted in decreased influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils ($P<0.05$ for the 5 min value).

In dose-response experiments the net influx of $^{45}Ca^{2+}$ 5 min after exposure to FMLP was 143 ± 3, 124 ± 3 ($P<0.05$) and 104 ± 3 ($P<0.05$) pmol 10^7 cells^{-1} for control neutrophils and for cells pretreated with 0.1 and 1 μM CGS 21680 respectively ($n=7$).

ZM 241385 (2.5 μM) did not affect the store-operated influx of $^{45}Ca^{2+}$ into FMLP-activated neutrophils. The net influx of $^{45}Ca^{2+}$ 5 min after exposure to FMLP was 189 ± 6 and 179 ± 6 pmol 10^7 cells^{-1} for control and ZM 241385-treated neutrophils respectively ($n=6$).

Table 3 Effects of varying concentrations of CGS 21680 on the efflux of $^{45}Ca^{2+}$ out of FMLP-activated neutrophils

Agent	*Amount of Ca^{2+} released from neutrophils 60 s after the addition of FMLP* (pmol 10^7 cells^{-1})
FMLP only	162 ± 12
FMLP + CGS 21680 0.01 μM	132 ± 14
FMLP + CGS 21680 0.1 μM	83 ± 9
FMLP + CGS 21680 1 μM	65 ± 5

The results of three experiments are expressed as the mean values ± s.e.mean.

Table 4 Effects of thapsigargin and ZM 241385 on the CGS 21680-mediated reduction in efflux of $^{45}Ca^{2+}$ out of FMLP-activated neutrophils

System	*Amount of $^{45}Ca^{2+}$ released from neutrophils 60 s after the addition of FMLP* (pmol 10^7 cells^{-1})
FMLP only	159 ± 11
FMLP + CGS 21680 1 μM	76 ± 10*
FMLP + thapsigargin 1 μM	177 ± 10
FMLP + CGS 21680 + thapsigargin	152 ± 12
FMLP + ZM 241385 2.5μM	173 ± 11
FMLP + CGS 21680 + ZM 241385	163 ± 10

The results of six experiments are expressed as the mean values ± s.e.mean. *$P<0.05$ for comparison with the drug-free (FMLP only) control system.

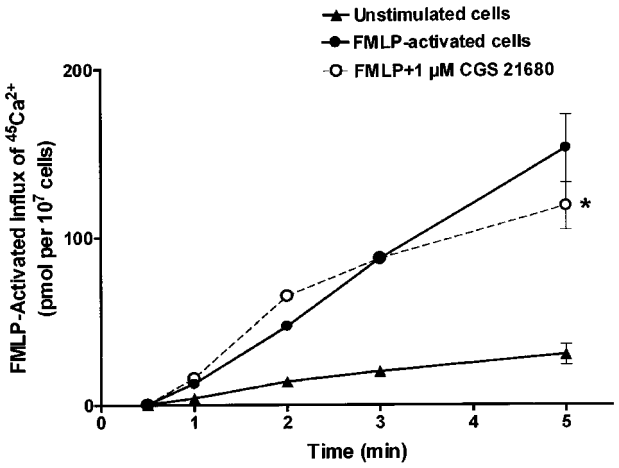

Figure 4 Kinetics of influx of $^{45}Ca^{2+}$ into unstimulated neutrophils activated with FMLP (1 μM) in the absence and presence of CGS 21680 (1 μM). The results of eight different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol 10^7 cells^{-1}) and vertical lines show s.e.mean. *$P<0.05$ for comparison with the FMLP-activated, CGS 21680-free control system.

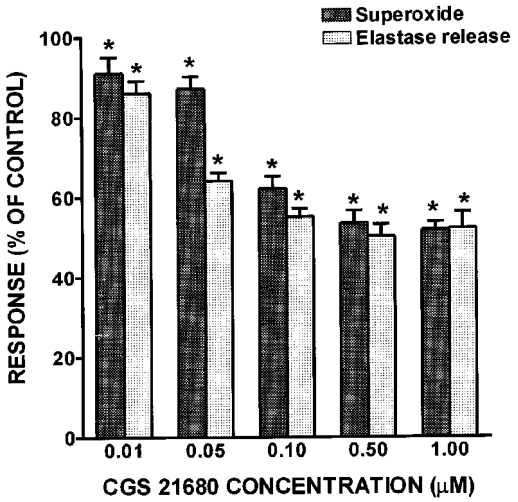

Figure 5 The effects of varying concentrations of CGS 21680 (0.01–1 μM) on superoxide production by FMLP (1 μM)-activated neutrophils and on elastase release from FMLP/CB-activated neutrophils. The results of eight (superoxide), measured by lucigenin-enhanced chemiluminescence (LECL) and ten (elastase) different experiments are presented as the mean percentages of the drug-free control systems and vertical lines show s.e.mean. In the case of superoxide production, the absolute values for resting and FMLP-activated neutrophils were 256 ± 23 and 911 ± 84 mV s^{-1} respectively. The corresponding values for elastase release were 40 ± 2 and 598 ± 70 milliunits enzyme per 10^7 cells respectively.

Superoxide generation and elastase release

The effects of CGS 21680 on oxidant production by, and release of elastase from, neutrophils activated with FMLP and FMLP/CB respectively are shown in Figure 5. The $A_{2A}R$ agonist caused dose-related inhibition of both oxidant production and elastase release which achieved statistical significance ($P<0.05$) at concentrations of 0.01 μM and upwards. In both cases the degrees of inhibition caused by 0.1 and 1 μM CGS 21680 did not differ significantly. The effects of ZM 241385 and thapsigargin on CGS 21680-mediated inhibition of oxidant production by, and elastase release from, activated neutrophils, are shown in Table 5. Both thapsigargin and to a lesser extent ZM 241385 potentiated the release of elastase from FMLP/CB-activated neutrophils, in the absence of CGS 21680, but caused no significant enhancement of superoxide production. Pretreatment of the cells with ZM 241385 completely antagonized the anti-inflammatory effects of the $A_{2A}R$ agonist, while thapsigargin was only slightly less effective.

Table 5 Effects of thapsigargin and ZM 241385 on CGS 21680-mediated inhibition of superoxide production by, and elastase release from activated neutrophils

System	*Superoxide Production (% control)*	*Elastase release (% control)*
CGS 21680 1 μM	56±3*	40±1*
Thapsigargin 1 μM	118±8	142±2*
ZM 241385 2.5 μM	108±8	126±3*
CGS 21680+thapsigargin	101±6	114±2
CGS 21680+ZM 241385	100±5	126±10

The results of six (superoxide) and 8–12 (elastase) different experiments are presented as the mean percentages of the drug-free control systems±s.e.mean. In the case of superoxide production, the absolute values for resting and FMLP-activated neutrophils were 282±28 and 1197±50 mv s^{-1} respectively. The correponding values for elastase release from FMLP/CB-activated neutrophils were 40±2 and 598±10 milliunits enzyme 10^7 $cells^{-1}$ respectively. *$P<0.05$ for comparison with the drug-free (FMLP or FMLP/CB only) control system.

Table 6 The effects of CGS 21680 and ZM 241385 individually and in combination on cylic AMP levels in unstimulated and FMLP-activated neutrophils

System	*Intracellular cyclic AMP* (pmol 10^7 $cells^{-1}$)
Unstimulated neutrophils	30±0.2
Unstimulated neutrophils+CGS 21680 1 μM	59±3*
Unstimulated neutrophils+ZM 241385 2.5 μM	12±1.2
Unstimulated neutrophils+CGS 21680+ZM 241385	14±1.2
FMLP-activated neutrophils	127±6*
FMLP-activated neutrophils+CGS 21680	240±15**
FMLP-activated neutrophils+ZM 241385	41±4
FMLP-activated neutrophils+CGS 21680+ZM 241385	38±0.4

The results of five different experiments are expressed as the mean intracellular concentration of cyclic AMP±s.e.mean measured at 1 min after the addition of the stimulant, FMLP. *$P<0.05$ for comparison with the unstimulated, CGS 21680-free control system and **$P<0.05$ for comparison with FMLP-activated neutrophils in the absence of CGS 21680.

At the highest concentration used (1 μM), CGS 21680, as well as thapsigargin (1 μM) and ZM 241385 (2.5 μM) did not possess superoxide-scavenging activity according to results obtained with the cell-free xanthine-xanthine oxidase system (not shown).

Intracellular cyclic AMP

The effects of CGS 21680 (1 μM) on cyclic AMP in unstimulated and FMLP-activated neutrophils in the presence and absence of ZM 241385 (2.5 μM) are shown in Table 6. Exposure of neutrophils to FMLP was accompanied by an increase in intracellular cyclic AMP which was inhibited by ZM 241385. Treatment of both stimulated and unstimulated neutrophils with CGS 21680 resulted in significant increases in intracellular cyclic AMP concentrations which were completely abolished by pretreatment of the cells with ZM 241385 prior to the addition of the $A_{2A}R$ agonist.

Discussion

Transient elevations in cytosolic Ca^{2+} precede, and are a prerequisite, for receptor-mediated activation of neutrophil adhesion to vascular endothelium, superoxide production and granule enzyme release (Lew *et al*., 1986; Thelen *et al*., 1993; Pettit & Hallett, 1996). Hyperactivation of neutrophils is prevented by the action of the calmodulin-dependent plasma membrane (Lagast *et al*., 1984) and cyclic AMP-dependent protein kinase (PKA)-activated endo-membrane Ca^{2+}-ATPases (Schatzmann, 1989; Tao *et al*., 1992), and possibly by a Na^+/Ca^{2+} exchanger (Simchowitz *et al*., 1990) operating in unison to promote rapid clearance of cytosolic Ca^{2+}. The transient elevation in cyclic AMP which accompanies the exposure of human neutrophils to chemoattractants (Anderson *et al*., 1976) has been reported to be mediated by endogenously-generated adenosine acting *via* adenylate-cyclase coupled $A_{2A}R$ and may be involved in the restoration of Ca^{2+} homeostasis in these cells and down-regulation of their pro-inflammatory activities (Iannone *et al*., 1989) by up-regulating the activity of the endo-membrane Ca^{2+}-ATPase.

This contention is supported by a previous study which reported that rolipram, the prototype selective PDE4 inhibitor, and dibutyryl cyclic AMP decrease the pro-inflammatory activities of human neutrophils *in vitro* by cyclic AMP-dependent enhancement of the activity of the endo-membrane Ca^{2+}-ATPase (Anderson *et al*., 1998). Rolipram- and dibutyryl cyclic AMP-mediated enhancement of the activity of this Ca^{2+} sequestering/re-sequestering pump resulted in accelerated clearance of the cation from the cytosol of FMLP-activated neutrophils and was accompanied by decreased pro-inflammatory activity of these cells.

Although the anti-inflammatory properties of adenosine and $A_{2A}R$ agonists are well recognized (Cronstein *et al*., 1985; Hannon *et al*., 1998; Sullivan & Linden, 1998; Varani *et al*., 1998), the exact molecular/biochemical mechanisms by which these effects are achieved, as well as the involvement of cyclic AMP, remain uncertain. In the present study, we investigated the effects of the $A_{2A}R$ agonist, CGS 21680, on intracellular cyclic AMP and Ca^{2+} handling by activated human neutrophils and compared these with alterations in pro-inflammatory activity.

Using the fura-2 spectrofluorimetric procedure, treatment of neutrophils with CGS 21680 did not affect the abruptly-occurring peak fluorescence responses of FMLP-activated neutrophils. Since no detectable influx of extracellular Ca^{2+}

coincident with peak fluorescence intensity in FMLP-activated neutrophils was detected with the radiometric procedure, the increase in the cytosolic concentration of the cation appears to originate through its release from intracellular stores. These observations demonstrate that CGS 21680 does not affect the activation of phospholipase C and generation of inositol triphosphate in FMLP-activated neutrophils, nor does it interfere with the interaction of this second messenger with Ca^{2+}-mobilizing receptors on calciosomes, or the subsequent release of the cation from these stores (Prentki *et al*., 1984; Krause *et al*., 1989).

Although CGS 21680 did not affect the release of Ca^{2+} from intracellular stores, this agent, at concentrations of 0.01–1 μM, hastened the rate of decline in peak fluorescence intensity, indicative of accelerated clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils. Accelerated clearance of cytosolic Ca^{2+} could result from several different mechanisms, including enhancement of efflux and/or re-sequestration of the cation, or inhibition of influx. To identify which of these was altered by CGS 21680 we used radiometric procedures, which facilitate distinction between net efflux and influx of the cation, in combination with the fura-2 fluorescence method (Anderson & Goolam Mahomed, 1997).

Using the radiometric procedures, exposure of neutrophils to FMLP was accompanied by an abrupt efflux of $^{45}Ca^{2+}$. This efflux coincided with the peak in fura-2 fluorescence intensity and terminated at about 30 s after the addition of the stimulant, resulting in extrusion of 44% of cell-associated cation. This observation suggests that not all of the intracellular Ca^{2+} pool is mobilized during exposure of neutrophils to the chemoattractant, or that rapid re-sequestration of cytosolic Ca^{2+}, as a result of activation of the Ca^{2+} sequestering/re-sequestering endo-membrane Ca^{2+}-ATPase also contributes to removal of the cation from the cytosol (Schatzman, 1989). As previously reported (Anderson & Goolam Mahomed, 1997; Anderson *et al*., 1998), 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 ceased, being detected at 30–60 s after addition of FMLP and terminating 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 many cell types, including neutrophils (Nontero *et al*., 1991; Favre *et al*., 1996; Anderson & Goolam Mahomed, 1997).

Treatment of neutrophils with CGS 21680 significantly reduced the amount of Ca^{2+} released from FMLP-activated neutrophils in the setting of a decrease in the magnitude of the subsequent store-operated influx of the cation. Together with the results of the fura-2 experiments, these observations suggest that CGS 21680 up-regulates the activity of the PKA-activatable endo-membrane Ca^{2+}-ATPase (Schatzmann, 1989; Tao *et al*., 1992), resulting in decreased efflux of Ca^{2+} as a consequence of competition between the up-regulated endo-membrane Ca^{2+}-ATPase and the plasma membrane Ca^{2+}-pump for cytosolic Ca^{2+}. Accelerated clearance of Ca^{2+} from the cytosol of CGS 21680-treated, FMLP-activated neutrophils is probably achieved through the action of these two Ca^{2+}-ATPases operating in unison.

Upregulation of the endo-membrane Ca^{2+}-ATPase would also explain the decreased store-operated influx of Ca^{2+} into CGS 21680-treated, FMLP-activated neutrophils. Accelerated activation and/or increased efficiency of this system would result in enhancement of re-sequestration of cytosolic Ca^{2+}. Utilization of endogenous Ca^{2+} for re-filling of the stores would in turn decrease the requirement for exogenous cation, with a consequent reduction in the magnitude of the subsequent store-operated influx.

This mechanism of CGS 21680-mediated acceleration of clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils is supported by experiments in which thapsigargin, a highly selective inhibitor of the endo-membrane-ATPase (Lytton *et al*., 1991), was used. Treatment of neutrophils with this agent abolished the CGS 21680-mediated reduction in the efflux of Ca^{2+} from FMLP-activated neutrophils. The observed restoration of efflux by thapsigargin in CGS-treated neutrophils also demonstrates that the AR agonist does not affect the calmodulin-dependent plasma membrane Ca^{2+}-ATPase.

The influence of CGS 21680 on superoxide production by, and release of elastase from stimulated neutrophils was also investigated and compared with the effects of this agent on the handling of cytosolic Ca^{2+} by these cells. At the same concentrations which accelerated the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils, CGS 21680 inhibited both superoxide generation and elastase release. As was the case with Ca^{2+} clearance, thapsigargin neutralized the inhibitory effects of CGS 21680 on superoxide production and elastase release, demonstrating a mechanistic link between these events.

The involvement of cyclic AMP in mediating the effects of CGS 21680 on Ca^{2+} homeostasis and on the production/release of inflammatory mediators by activated neutrophils was strengthened by the following observations: (i) intracellular concentrations of cyclic AMP were increased following exposure of the cells to CGS 21680; and (ii) the selective $A_{2A}R$ receptor antagonist ZM 241385 almost completely antagonized the effects of CGS 21680 on intracellular cyclic AMP, Ca^{2+} clearance, superoxide production and elastase release. ZM 241385-mediated antagonism of the cyclic AMP-elevating and anti-inflammatory interactions of CGS 21680 with neutrophils demonstrates that these effects are mediated *via* the adenylate cyclase-coupled $A_{2A}R$.

Interestingly, the effects of ZM 241385 *per se* on neutrophils were opposite to those of CGS 21680. At the single concentration tested, this agent attenuated the FMLP-activated transient increase in neutrophil cyclic AMP in the setting of apparent interference with the sequestration of incoming Ca^{2+} during store-operated influx of the cation. This latter contention is based on the observation that during the late stages of the fura-2 fluorescence response of FMLP-activated neutrophils, when fluorescence had subsided to close to baseline values, there was a transient increase in fluorescence intensity and a subsequent delay in the return to basal fluorescence in ZM 241385-treated cells. These events coincided with store-operated influx of Ca^{2+} and are compatible with interference with sequestration of incoming cation. In an additional series of experiments, which were not included in the current study, we observed that following the return to basal fluorescence, re-stimulation of fura-2-loaded, ZM 241385-treated neutrophils with FMLP resulted in immediate peak fura-2 fluorescence responses which were similar to those of control re-stimulated cells. These observations confirm that sequestration, albeit at a reduced level of efficiency, as opposed to an efflux mechanism, is responsible for the eventual return to baseline fluorescence in ZM 241385-treated, FMLP-activated neutrophils.

ZM 241385-mediated impairment of Ca^{2+} sequestration is the probable consequence of attenuated production of cyclic AMP by FMLP-activated neutrophils, resulting in failure to up-regulate the cyclic AMP/PKA-activatable, Ca^{2+}-sequestering endo-membrane Ca^{2+}-ATPase. Dysregulation of Ca^{2+} homeostasis may also explain the increased release of elastase from ZM 241385-treated FMLP/CB-activated neutrophils.

These observations support the contention that endogenously-generated adenosine mediates the FMLP-induced increase in cyclic AMP through autocrine interactions with $A_{2A}R$ (Iannone *et al*., 1989), which may result in restoration of Ca^{2+} homeostasis and down-regulation of the pro-inflammatory activities of these cells.

In conclusion, the results presented here demonstrate that CGS 21680, as is the case with other cyclic AMP-elevating agents such as rolipram (Anderson *et al*., 1998), accelerates the re-sequestration of cytosolic Ca^{2+} in FMLP-activated neutrophils, probably by up-regulation of the endo-membrane Ca^{2+}-ATPase, leading to inhibition of Ca^{2+}-dependent neutrophil functions, while ZM 241385 has opposite effects on these. If they can be selectively targeted onto immune and inflammatory cells, AR agonists operative at the level of $A_{2A}R$ represent a novel group of anti-inflammatory agents which may be useful in the treatment of those disorders involving hyperactivity of neutrophils.

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(Received October 28, 1999
Revised January 24, 2000
Accepted March 7, 2000)