Chapter 3

Apparent Involvement of the A_{2A} Subtype Adenosine Receptor in the Anti-Inflammatory Interactions of CGS 21680, Cyclopentyladenosine and IB-MECA with Human Neutrophils

3.1 Introduction

The wide-ranging, receptor-mediated, physiologic activities of adenosine involve interactions of this agent with at least four types of plasma membrane receptors, designated A_1, A_{2A}, A_{2B} and A_3. These vary with respect to ligand binding properties, tissue distribution and transductional mechanisms utilized in intracellular signalling (Stiles, 1992; Cronstein, 1994). Although adenosine is an important regulator of many physiologic processes, including immune and inflammatory responses, its chemotherapeutic potential is limited by an extremely short half life *in vivo* and by receptor promiscuity (Stiles, 1992; Cronstein, 1994). These problems have resulted in the development of synthetic agonists which are selective for the different types of AR.

The broad spectrum anti-inflammatory properties of adenosine and its analogues are well-recognized and span many different types of immune and inflammatory cells, including neutrophils (Cronstein, 1994; Hannon *et al.*, 1998) and eosinophils (Walker *et al.*, 1997; Ezeamuzi & Philips, 1999). There is compelling evidence for the presence of A_{2A} receptors on human neutrophils (Fredholm *et al.*, 1996; Varani *et al.*, 1998), while indirect evidence supports the existence of A_1 and A_3 receptors on these cells, as well as on eosinophils (Rose *et al.*, 1988; Bouma *et al.*, 1997; Fredholm, 1997; Walker *et al.*, 1997; Ezeamuzie & Philips, 1999). With respect to neutrophils, A_1 and A_{2A} receptors have been reported to exert opposing effects on the pro-inflammatory activities of these cells. Interaction of adenosine or its analogues with A_1 receptors on neutrophils has been reported to potentiate adherence to vascular endothelium and chemotaxis (Cronstein *et al.*, 1985; Cronstein *et al.*, 1992), while activation of A_{2A} receptors results in suppression of the production of reactive oxidants by these cells (Cronstein *et al.*, 1985; Hannon *et al.*, 1998), as well as decreased expression of ß2-integrins and adherence to vascular endothelium (Cronstein *et al.*, 1992; Nolte *et al.*, 1992). Neutrophil degranulation on the other hand has been reported to be either insensitive to adenosine (Cronstein *et al.*, 1985), or to be inhibited by mechanisms involving both A_2 and A_3 receptors (Bouma *et al.*, 1997). In the case of eosinophils, interaction of adenosine or its analogues with A_3

receptors appears to promote down-regulation of the pro-inflammatory activities of these cells (Walker *et al.*, 1997; Ezeamuzie & Philips, 1999).

Although adenosine and its analogues acting via A_{2A} receptors suppress some of the pro-inflammatory activities of activated neutrophils, there are several aspects of this relationship, including the involvement of cAMP and the apparent insensitivity of degranulation, which require clarification. With this in mind, the current study was undertaken to identify the AR types involved in regulating the reactive oxidant-generating and degranulation responses of activated human neutrophils, as well as the dependence of these anti-inflammatory activities on receptor-mediated increases in intracellular cAMP.

3.2 Materials and Methods

3.2.1 Adenosine receptor agonists

N^6-cyclopentyladenosine (CPA, A_1R agonist), 2(4-[(2-carboxyethyl)phenyl] ethylamino)-5′-N-ethylcarboxamido adenosine - CGS 21680, $A_{2A}R$ agonist) and N^6-(3-iodobenzyl)-5′N-methylcarbamoyladenosine (IB-MECA, A_3R agonist) and rolipram, a selective inhibitor of type 4 phosphodiesterase, the predominant type found in human neutrophils (Torphy, 1998), were kindly provided by Dr Malcolm Johnson, GlaxoWellcome plc, Stockley Park West, London, UK. These agents were dissolved to stock concentrations of 10 mM in 0.05 N HCl (CPA and IB-MECA), 0.1 N NaOH (CGS 21680) or dimethylsulfoxide (rolipram) and diluted thereafter in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4) and used in the various assays described below at a final concentration range of 0.01-1 µM. ZM 241385, a highly selective antagonist of A_{2A} receptors (Poucher *et al.*, 1995), was purchased from Tocris Cookson Ltd, Bristol, UK and dissolved to 10mM in 0.1 N NaOH and used at concentrations of 0.1- 2.5 µM. Unless indicated all other chemicals and reagents were purchased from the Sigma Chemical Co.

3.2.2 Neutrophils

Purified neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 g for 25 min at room temperature. The resultant pellet was suspended in phosphate-buffered saline (PBS, 0.15 M, pH 7.4) and sedimented with 3% gelatine 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 x 10^7/ml in PBS and held on ice until used.

3.2.3 Oxidant generation

This was measured using a lucigenin (bis-N-methylacridinium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils (1 x 10^6/ml, final) were pre-incubated for 15 min in 900 µl HBSS containing 0.2 mM lucigenin in the presence and absence of the AR agonists (0.01-1 µM) prior to activation with the synthetic chemotactic tripeptide FMLP (1 µM). Spontaneous and FMLP (1 µM)-activated LECL responses were then recorded using a LKB Wallac 1251 chemiluminometer after the addition of the stimulant (100 µl). LECL readings were integrated for 5s intervals and recorded as mV x seconds^{-1} (mVs^{-1}). Additional experiments were performed to investigate the following : i) the effects of ZM 241385 (2.5 µM) added during preincubation at 37°C, 5 min before the AR agonists on the CGS 21680, CPA and IB-MECA (1 µM)-mediated inhibition of the LECL responses of FMLP-activated neutrophils; adenosine (1 µM) was also included in these experiments to monitor the activity of ZM 241385, ii) the effects of low concentrations (0.1 and 0.25 µM) of ZM 241385 on the inhibition of FMLP-activated neutrophil superoxide production mediated by CGS 21680, CPA and IB-MECA (all at 1 µM) and iii) the superoxide-scavenging activity of CGS 21680, CPA, IB-MECA and ZM 241385 using a cell-free hypoxanthine (1 mM)-xanthine oxidase (17 milliunits/ml) superoxide-generating system.

3.2.4 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 x 10^6/ml in HBSS in the presence or absence of the AR agonists (0.01-1 μM) with and without ZM 241385 (0.1-2.5 μM) for 10 min at 37°C. The stimulant FMLP (0.1 μM) in combination with CB (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 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 sulfoxide 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.

3.2.5 Intracellular cAMP levels

Neutrophils at a concentration of 2 x 10^6/ml in HBSS were preincubated for 10 min at 37°C with CGS 21680, CPA or IB-MECA (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, and the reactions terminated and the cAMP extracted by the addition of ice-cold ethanol (65% v/v) at 20 sec, 1 min, 3 min 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 2000g for 15 min at 4°C. The supernatants were then transferred to fresh tubes and evaporated at 60°C under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP [^{125}I] scintillation proximity assay system (Amersham International plc.), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmoles cAMP/10^7 neutrophils. Because cAMP is rapidly hydrolysed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μM rolipram.

3.2.6 Spectrofluorimetric measurement of Ca^{2+} fluxes

Fura-2/AM (Calbiochem Corp), was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments. Neutrophils (1 x 10^7/ml) were pre-loaded with fura-2 (2 μM) for 30 min at 37°C in phosphate-buffered saline (PBS, 0.15 M, pH 7.4), washed twice and resuspended in HBSS. The fura-2-loaded cells (2 x 10^6/ml) were then pre-incubated with CPA, CGS 21680 or IB-MECA (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 nm and 500 nm respectively. After a stable base-line was obtained (1 min), the neutrophils were activated by addition of FMLP (1 μM) and the subsequent increase in fura-2 fluorescence intensity monitored over a 5 min period. The final volume in each cuvette was 3 ml containing a total of 6 x 10^6 neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously (Grynkiewicz *et al.*, 1985).

Additional experiments were performed to investigate the effects of pre-treatment with the selective A_{2A} receptor antagonist ZM 241385 at 2.5 μM on CGS 21680, CPA, and IB-MECA (1 μM)-mediated alterations in the fura-2 fluorescence responses of FMLP-activated neutrophils.

3.2.7 IL-8 Production

Neutrophils (1 x 10^6ml) were preincubated for 10 min with and without CGS (1 μM) in HBSS prior to the addition of the synthetic chemotactic tripeptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μM, final). FMLP-free control systems received an equal volume of HBSS. The final volume in each tube was 2 ml. Total and extracellular IL-8 were measured using antibody capture ELISA procedures (Roche Diagnostics Corp, Indianapolis) after 6 hours of incubation at 37°C following the addition of FMLP to the cells. Total IL-8 was measured in the lysates of neutrophils which had been treated with 0.01% lysophosphatidylcholine followed by centrifugation at 300 g for 5 min to remove cell debris, while extracellular cytokines

were measured in cell-free supernatants following the removal of the cells by centrifugation.

Calculations were performed by using the standards provided with the kit to prepare a six point calibration curve and a standard curve was plotted correlating the mean absorbance values of the standards (y-axis) to the analyte concentrations of the standard (x-axis). The lot-specific concentration of each standard is listed on its bottle label. Analyte concentrations were determined by locating the mean sample absorbance on the y-axis and reading from the x-axis the analyte concentration that corresponds to the specific absorbance value (h-Interleukin-8 ELISA, cat. No 1 967 932, Boehringer Mannheim).

3.2.8 Statistical analysis

The results of each series of experiments are expressed as the mean values ± SEM. 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.

3.3 Results

3.3.1 Oxidant production

The effects of CGS 21680, CPA and IB-MECA on superoxide production by neutrophils activated with FMLP are shown in Figure 3.1 (page 67). Superoxide production was inhibited by CGS 21680 at all concentrations tested (0.01-1 μM) with maximal inhibition observed at concentrations of 0.1-1 μM. IB-MECA was less effective, causing significant inhibition of superoxide production only at concentrations of 0.5 and 1 μM, while CPA was the least effective, causing inhibition only at 1 μM.

The effect of pre-treatment of neutrophils with ZM 241385 (2.5 μM) on the inhibition of the production of superoxide by FMLP-activated neutrophils mediated by 1 μM CGS 21680, CPA and IB-MECA are shown in Table 3.1 (page 68). The A_{2A} receptor antagonist *per se* slightly increased superoxide production, and also neutralized the inhibitory effects of all 3 AR agonists. ZM 241385 (2.5 μM) also inhibited the effects of adenosine (1 μM) on superoxide production by FMLP-activated neutrophils, with the responses of neutrophils exposed to adenosine only or to adenosine + ZM 241385 being 57 ± 4 % ($p<0.05$) and 109 ± 7 % of the corresponding drug-free control system respectively.

The effects of low concentrations of ZM 241385 (0.1 and 0.25 μM) on CGS 21680-, CPA- and IB-MECA (all at 1 μM)-mediated inhibition of superoxide production by FMLP-activated neutrophils are shown in Table 3.2 (page 68). The inhibitory effects of CPA and IB-MECA on neutrophil superoxide production were completely attenuated by ZM 241385 at both concentrations used, while those of CGS 21680 were completely neutralised only at 0.25 μM ZM 241385.

In experiments designed to evaluate the superoxide-scavenging potential of CGS 21680, CPA and IB-MECA, all 3 AR agonists at the highest concentration tested (1 μM), as well as ZM 241385 (2.5 μM) did not possess superoxide-scavenging properties. LECL values for the control system and for systems containing CGS 21680, CPA, IB-MECA and ZM 241385 were 1464 ± 51, 1419 ± 159, 1451 ± 75, 1412 ± 165 and 1443 ± 131 $mV.s^{-1}$ respectively (results of 12 experiments).

3.3.2 Elastase release

The effects of the 3 AR agonists on elastase release from FMLP/CB-activated neutrophils are shown in Figure 3.2 (page 70). CGS 21680 and IB-MECA caused dose-related inhibition of elastase release which was evident at 0.01 μM, while CPA exerted inhibitory effects at 1 μM.

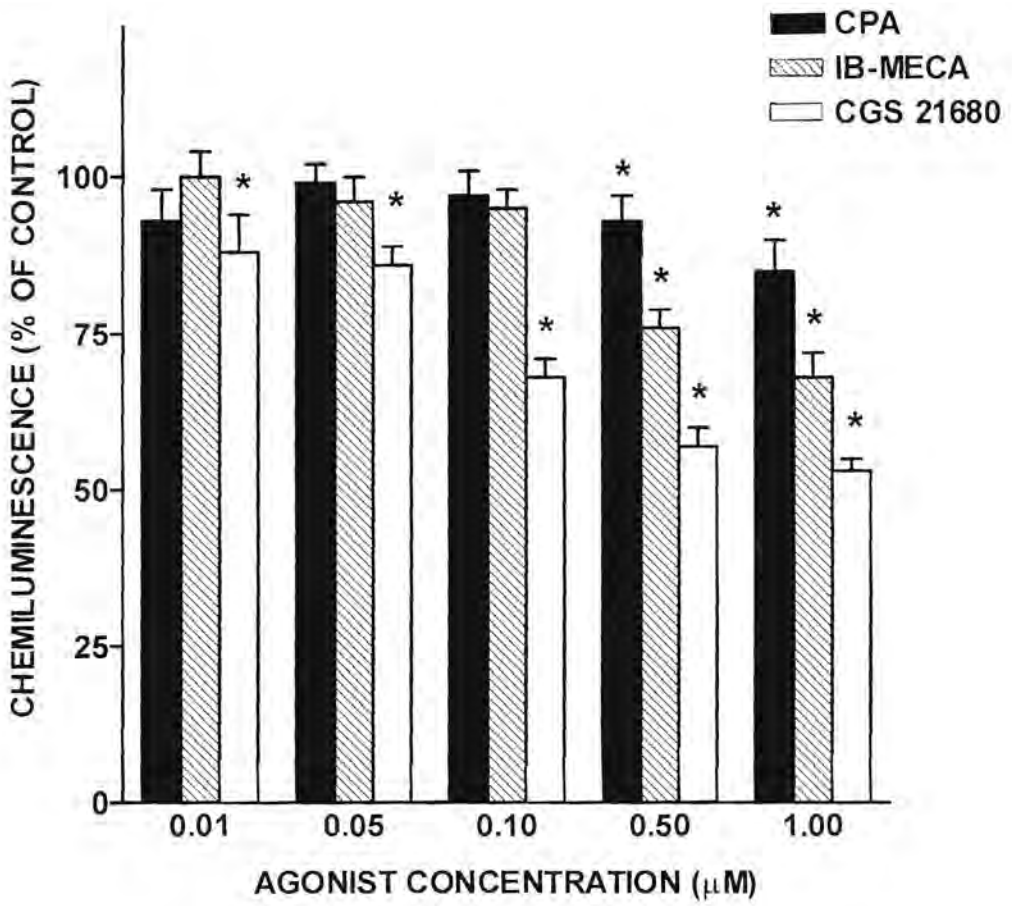

Figure 3.1: The effects of varying concentrations of CGS 21680, CPA, and IB-MECA on the production of superoxide by FMLP-activated neutrophils. The results of 6-23 experiments are presented as the mean percentages ± SEMs of the control systems. The absolute values for unstimulated and FMLP-activated control neutrophils were 390 ± 112 and 1064 ± 79 $mV.sec^{-1}$, respectively. *$p<0.05$.

Table 3.1 : The effects of ZM 241385 on CGS 21680-, CPA-, and IB-MECA-mediated inhibition of neutrophil superoxide generation and elastase release.

System	Superoxide production (% control)	Elastase release (% control)
ZM 241385 2.5 µM only	108 ± 8	126 ± 3
CGS 21680 1 µM only	56 ± 3	49 ± 4
ZM 241385 + CGS 21680	100 ± 5	126 ± 10
CPA 1 µM only	71 ± 11	70 ± 1
ZM 241385 + CPA	108 ± 8	127 ± 2
IB-MECA 1 µM only	63 ± 6	47 ± 1
ZM 241385 + IB-MECA	96 ± 7	102 ± 3

The results of 6-12 experiments are presented as the mean percentages ± SEMs of the corresponding control systems for which the absolute values were 1358 ± 57 $mV.s^{-1}$ and 600 milliunits enzyme/10^7 cells for superoxide production and elastase release respectively.

Table 3.2 : The effects of low concentrations (0.1 and 0.25 µM) of ZM 241385 on CGS 21680-, CPA-, and IB-MECA-mediated inhibition of neutrophil superoxide production.

System	Superoxide production (% control)
ZM 241385 0.1 µM only	104 ± 2
ZM 241385 0.25 µM only	103 ± 3
CGS 21680 1 µM only	59 ± 1
CGS 21680 + ZM 241385 0.1 µM	87 ± 2
CGS 21680 + ZM 241385 0.25 µM	100 ± 1
CPA 1 µM only	72 ± 2
CPA + 0.1 µM ZM 241385	108 ± 7
CPA + 0.25 µM ZM 241385	109 ± 3
IB-MECA 1 µM only	61 ± 3
IB-MECA + 0.1 µM ZM 241385	101 ± 5
IB-MECA + 0.25 µM ZM 241385	110 ± 3

Data from 6 experiments are presented as the mean percentages ± SEMs of the corresponding control system for which the absolute value was 1032 ± 55 $mV.s^{-1}$

The effects of ZM 241385 (2.5 μM) pre-treatment of neutrophils on the inhibition of FMLP/CB-activated release of elastase mediated by 1 μM CGS 21680, CPA and IB-MECA are shown in Table 3.1 (page 68). ZM 241385 *per se* potentiated the release of elastase from stimulated neutrophils and completely (CGS 21680 and CPA) or almost completely (IB-MECA) antagonised the inhibitory actions of the AR receptor agonists. Similar effects were observed when ZM 241385 was combined with lower concentrations of CGS 21680 and IB-MECA (data not shown).

3.3.3 cAMP

The effects of CGS 21680 and IB-MECA on intracellular cAMP in unstimulated and FMLP-stimulated neutrophils are shown in Table 3.3 (Page 70). CGS 21680 increased cAMP in resting cells, while IB-MECA had minimal effects. Activation of neutrophils with FMLP resulted in an increase in cAMP which was augmented to a similar extent by both CGS 21680 and IB-MECA at 1 μM. ZM 241385 caused a drop in cAMP levels in resting neutrophils in both the absence and presence of CGS 21680 and IB-MECA. The A_{2A} receptor antagonist also attenuated the increase in cAMP in FMLP-activated neutrophils in both the absence and presence of the AR agonists.

Treatment of neutrophils with CPA did not significantly affect intracellular cAMP in either unstimulated or stimulated neutrophils. In the case of resting neutrophils, concentrations of intracellular cAMP were 30 ± 2 and 28 ± 20 pmol/10^7 cells in the absence and presence of 1 μM CPA, while the corresponding values for FMLP-activated cells were 127 ± 6 and 85 ± 35 pmol/10^7 cells.

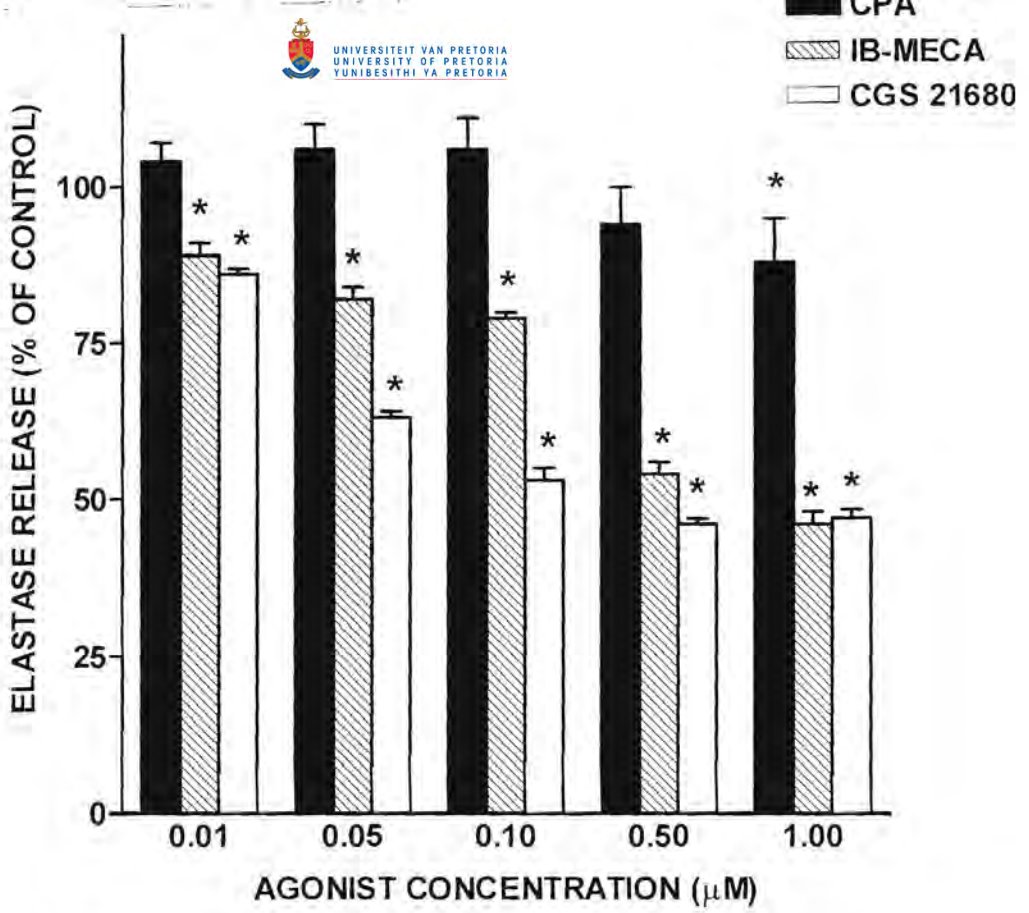

Figure 3.2: The effects of varying concentrations of CGS 21680, CPA, and IB-MECA on the release of elastase from FMLP/CB-activated neutrophils. The results of 15 experiments are presented as the mean percentages ± SEMs of the control systems. The absolute values for unstimulated and FMLP/CB-activated neutrophils were 3 ± 0.8 and 593 ± 4 milliunits enzyme/10^7 cells, respectively. *p<0.05.

Table 3.3 : The effects of CGS 21680 and IB-MECA individually and in combination with ZM 241385 on cAMP in unstimulated and FMLP-activated neutrophils

	Intracellular cAMP (pmol/10^7 cells) on :	
System	Unstimulated cells	FMLP-activated cells
Control	30 ± 2	127 ± 6
CGS 21680 1 μM	59 ± 3	240 ± 15
IB-MECA 1 μM	39 ± 6	264 ± 16
ZM 241385 2.5 μM	12 ± 1	41 ± 4
CGS 21680 + ZM 241385	14 ± 1	38 ± 4
IB-MECA + ZM 241385	11 ± 4	68 ± 4

The results of 5 different experiments are presented as the mean values ± SEMs measured at 1 min after the addition of FMLP.

3.3.4 Fura-2 fluorescence

The results shown in Figure 3.3 (page 72) are traces from a single representative experiment which depict the effects of the 3 AR agonists at 1 μM 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 the transient elevation in the concentration of cytosolic Ca^{2+}. This abrupt increase in fluorescence intensity was unaltered by the AR agonists, demonstrating that these agents do not affect the release of Ca^{2+} from cellular stores. However, treatment of neutrophils with CGS 21680 and IB-MECA at 1 μM, and to a lesser extent with CPA, 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 3.4 (page 73) 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 ($t½$) values for neutrophils activated with FMLP in the presence and absence of varying concentrations of CGS 21680, CPA and IB-MECA. As indicated above, none of the AR agonists affected the abruptly occurring increase in $[Ca^{2+}]i$ following activation of the cells with FMLP. However, CGS 21680 (0.01-1 μM) and IB-MECA (0.5-1 μM) caused dose-related acceleration in the rate of decline in peak fluorescence, while CPA was effective only at 1 μM.

The effects of ZM 241385 on CGS 21680-, CPA- and IB-MECA-mediated enhancement of the clearance of Ca^{2+} from the cytosol of FMLP-activated neutrophils, as well as on peak $[Ca^{2+}]i$ are shown in Table 3.5 (page 73) . The A_{2A} receptor antagonist prevented CGS 21680-, CPA- and IB-MECA-mediated acceleration of clearance of Ca^{2+} from the cytosol, but did not affect peak $[Ca^{2+}]i$.

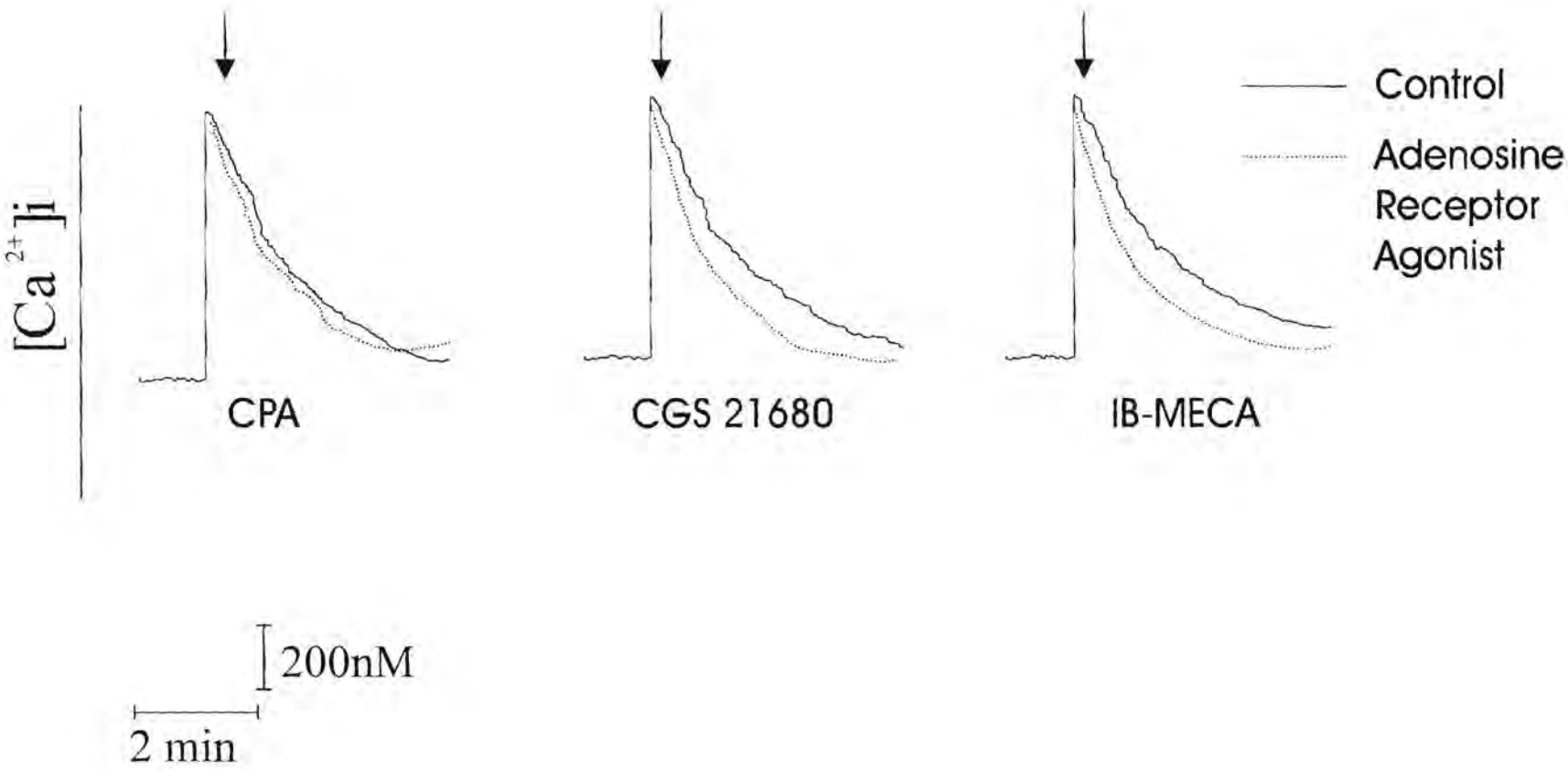

Figure 3.3: FMLP-activated fura-2 responses of neutrophils with and without CGS 21680, CPA, or IB-MECA (all at 1 μM). FMLP was added as indicated (↓) when a stable base line was obtained (± 1 min). Traces are from a single representative experiment.

Table 3.4 : Peak intracellular calcium concentrations $[Ca^{2+}]i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils treated with the adenosine receptor agonists.

System	Peak $[Ca^{2+}]i$ values (nM)	Time taken to decline to half peak values (min)
Control	497 ± 28	1.5 ± 0.1
CGS 21680 0.1 µM	442 ± 22	0.9 ± 0.1*
CGS 21680 0.5 µM	425 ± 28	0.9 ± 0.1*
CGS 21680 1 µM	456 ± 39	0.8 ± 0.1*
CPA 0.1 µM	480 ± 55	1.5 ± 0.1
CPA 0.5 µM	461 ± 27	1.4 ± 0.2
CPA 1 µM	486 ± 28	1.2 ± 0.1*
IB-MECA 0.1 µM	485 ± 55	1.3 ± 0.1
IB-MECA 0.5 µM	458 ± 27	1.1 ± 0.1
IB-MECA 1 µM	483 ± 25	1.0 ± 0.1*

The results of 5-12 experiments are presented as the mean values ± SEMs.
*$p<0.05$ for comparison with the control.

Table 3.5 : The effects of ZM 241385 on peak intracellular calcium concentrations $[Ca^{2+}]i$ and time taken for these to decline to half peak values in FMLP-activated neutrophils with and without the adenosine receptor agonists

System	Peak $[Ca^{2+}]i$ values (nM)	Time taken to decline to half peak values
Control	496 ± 9	1.5 ± 0.10
ZM 241385 2.5 µM only	497 ± 6	1.5 ± 0.07
CGS 21680 1 µM only	492 ± 18	1.1 ± 0.04*
ZM 241385 + CGS 21680	485 ± 15	1.5 ± 0.06
CPA 1 µM only	494 ± 6	1.2 ± 0.06*
ZM 241385 + CPA	464 ± 18	1.5 ± 0.07
IB-MECA 1 µM only	497 ± 9	1.1 ± 0.07*
ZM 241385 + IB-MECA	500 ± 9	1.5 ± 0.07

The results of 4 experiments are presented as the mean values ± SEMs.
*$p<0.05$ for comparison with the control.

3.3.5 IL-8 production

The effect of CGS (1 μM) on IL-8 production by unactivated and FMLP-activated neutrophils following 6 hours of incubation at 37°C are shown in Table 3.6. Activation of the cells with FMLP resulted in modest, but statistically significant increases in both extracellular ($p<0.0002$) and total ($p<0.008$) IL-8 (relative to the corresponding values for unstimulated cells). Inclusion of CGS 21680 did not significantly affect the synthesis of IL-8 by either unstimulated or FMLP-activated cells.

Table 3.6: The effect of CGS (1 μM) on IL-8 production by unactivated and FMLP-activated human neutrophils.

System	IL-8 production (pg/10^6 cells)		
	Mean	**SEM**	**N(number)**
T=0:			
Control (extracellular IL-8)	0	0	6
Control total (intra- & extracellular IL-8)	99.8	17.8	7
T=6h:			
Unactivated cells:			
Control (extracellular IL-8)	394.2	30.6	9
Control total (intra- + extracellular IL-8)	1099.1	83.4	11
CGS (extracellular IL-8)	361.7	70.3	8
CGS total (intra- + extracellular IL-8)	1153.0	152.5	8
T=6h:			
Activated cells:			
FMLP (extracellular IL-8)	861.5	66.1*	9
FMLP total (intra + extracellular IL-8)	1876.1	312.6**	6
FMLP CGS (extracellular IL-8)	667.1	110.2	8
FMLP CGS total (intra- + extracellular IL-8)	1492.7	298.5	6

The results of these experiments are expressed as the mean values ± SEMs.

*$P<0.0002$ for comparison with control (unactivated cells)

**$P<0.008$ for comparison with control total (unactivated cells)

3.4 Discussion

The results of the present study support the contention that subtype A_{2A}Rs down-regulate the pro-inflammatory activities of human neutrophils (Hannon *et al.*, 1998; Sullivan & Linden, 1998; Varani *et al.*, 1998). Although CPA and IB-MECA were found to suppress the oxidant-generating and degranulation responses of neutrophils, the inhibitory effects of these agents were less than those of CGS 21680, and, in the case of superoxide production, were observed only at concentrations at which receptor selectivity is diminished (Hannon *et al.*, 1998; Sullivan & Linden, 1998). While IB-MECA appeared to be more effective in suppressing elastase release, as opposed to superoxide production by activated neutrophils, apparently consistent with a role for A_3 receptors in regulating neutrophil degranulation (Bouma *et al.*, 1997), this differential sensitivity of the two responses was also observed with CGS 21680. The order of agonist potencies to inhibit the pro-inflammatory activities of neutrophils is the same as that reported by others (Hannon *et al.*, 1998), and also identical to the order of potency to inhibit the A_{2A} receptor defined by radioligand binding to rat and human brain (Jarvis *et al.*, 1989; Wan *et al.*, 1990). Moreover, the inhibitory effects of IB-MECA, as well as those of CGS 21680 and CPA, on both elastase release and superoxide production were neutralised by pre-treatment of the cells with the highly selective A_{2A}R antagonist, ZM 241385. These observations suggest that the concentration-dependent anti-inflammatory interactions of all three AR agonists with neutrophils are mediated through interactions with A_{2A}Rs. However, due to the absence of complete specificity of the various agonists for their respective AR subtypes (Hannon *et al.*, 1998), the data needs to be interpreted cautiously.

The interaction of ZM 241385 with adenosine receptors on human neutrophils was confirmed by the observation that the receptor antagonist completely attenuated the inhibitory effects of adenosine on FMLP-activated superoxide production by these cells. The selectivity of ZM 241385 for the A_{2A} receptor subtype was supported by the observation that at relatively low concentrations (0.1 and 0.25 μM), the receptor antagonist completely neutralised the anti-oxidative interactions of CPA and IB-

MECA (both at 1 μM) with neutrophils, while complete antagonism of CGS 21680 (1 μM) was only observed with 0.25 μM ZM 241385.

The transient increase in cAMP which accompanies exposure of neutrophils to FMLP is well-recognized (Anderson *et al.*, 1998). In the present study CGS 21680 and IB-MECA, but not CPA, increased cAMP in both unstimulated and FMLP-activated neutrophils, indicative of a relationship between elevated intracellular concentrations of this cyclic nucleotide and suppression of the pro-inflammatory activities of these cells. Failure to detect enhancement of cAMP by CPA may be related to the counteracting effects of this agent on adenylate cyclase, with inhibition and stimulation resulting from interactions with A_1 and A_{2A} receptors respectively (Cronstein, 1994; Hannon *et al.*, 1998). The enhancing effects of both CGS 21680 and IB-MECA on cAMP were attenuated by pre-treatment of neutrophils with ZM 241385, supporting the involvement of A_{2A} receptors.

Not only did ZM 241385 attenuate the increase in neutrophil cAMP mediated by CGS 21680 and IB-MECA, this selective A_{2A} receptor antagonist also decreased cAMP in AR agonist-free, unstimulated cells and abolished the transient increase in cAMP on exposure of these cells to FMLP. These observations support the contention that endogenously generated adenosine released from human neutrophils causes autocrine activation of adenylate cyclase by interacting with A_{2A} receptors, a process which is amplified in response to FMLP (Iannone *et al.*, 1989).

Researchers from my group and elsewhere have previously reported that the anti-inflammatory interactions of cAMP-elevating agents with human neutrophils are achieved through accelerated clearance of Ca^{2+} from the cytosol of activated neutrophils as a result of up-regulation of the activity of the cAMP-dependent protein kinase-modulated, Ca^{2+}-sequestering endo-membrane Ca^{2+}-ATPase (Villagrasa *et al.*, 1996; Anderson *et al.*, 1998). In the present study, CGS 21680, CPA and IB-MECA did not affect the immediately occurring elevation in cytosolic Ca^{2+} following activation of neutrophils with FMLP. However, all three AR agonists, at the same concentrations which inhibited superoxide production and elastase release, accelerated the clearance of Ca^{2+} from the cytosol of the cells. These effects of the AR agonists on neutrophil Ca^{2+} handling were antagonised by pre-treatment of the

cells with ZM 241385, consistent with the involvement of A_{2A} receptors and cAMP. Taken together, these observations are compatible with a mechanism of anti-inflammatory activity involving increased efficiency of the endo-membrane Ca^{2+}-ATPase.

Exposure of neutrophils to FMLP was accompanied by modest, but nevertheless significant increases in both total and extracellular IL-8 following 6 hours of incubation at 37°C. This observation with respect to the total concentration of IL-8 contrasts with the findings reported in Table 2.5 (Chapter 2) in which the corresponding increases in FMLP-activated cells did not achieve statistical significance. This difference may be attributable to the larger series of experiments performed and reported on in the current chapter. Irrespective of these differences, I was unable to detect any significant inhibition of spontaneous and FMLP-activated synthesis of IL-8 by neutrophils treated with CGS 21680. This may be a true effect of CGS 21680. Alternatively, exposure times in excess of 6 hours may be required to detect meaningful effects of CGS 21680 on IL-8 synthesis by neutrophils.

In conclusion, the results of the current study underscore the apparent role of A_{2A} receptors as opposed to A_1 or A_3 receptors, in down-regulating the pro-inflammatory activities of human neutrophils. If safe and highly selective pharmacologic agonists of A_{2A} receptors can be developed, these agents may prove to be particularly useful in the anti-inflammatory chemotherapy of corticosteroid-insensitive, neutrophil-mediated disorders, particularly chronic inflammatory diseases of the airways, including chronic obstructive pulmonary disease, cystic fibrosis, bronchiectasis and certain categories of asthmatics.

Chapter 4

Accelerated Re-sequestration of Cytosolic Calcium and Suppression of the Pro-Inflammatory Activities of Human Neutrophils by CGS 21680 *In Vitro*

4.1 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 cAMP-elevating agents, which spans many different types of immune and inflammatory cells, including neutrophils, has been recognised for more than two decades (Moore & Willoughby, 1995). However, the development of clinically useful cAMP-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 level of 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 maximise anti-inflammatory efficacy in the setting of decreased gastrointestinal toxicity (Torphy, 1998). The anti-inflammatory effects of type 4 PDE inhibitors are mediated by cAMP-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; Iannone *et al.*, 1989) studies has been attributed to a cAMP-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 cAMP.

4.2 Materials and methods

4.2.1 Drugs and reagents

CGS 21680 and rolipram were kindly provided by Dr Malcolm Johnson, (GlaxoWellcome plc, Stockley Park West, London, UK), while thapsigargin and ZM 241385 were purchased from the Sigma Chemical Co and Tocris Cookson Ltd, (Bristol, UK) 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.

4.2.2 Neutrophils

Purified neutrophils were prepared from heparinised (5 units of preservative-free heparin/ml) venous blood of healthy adult human volunteers and separated from mononuclear leucocytes by centrifugation on Histopague®-1077 (Sigma Diagnostics, St Louis, MO, USA) 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% gelatine 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 x 10^7/ml in PBS and held on ice until used.

4.2.3 Spectrofluorimetric measurement of Ca^{2+} fluxes

Fura-2/AM (Calbiochem Corp, La Jolla, California, USA) was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments. Neutrophils (1 x 10^7/ml) were pre-loaded with fura-2 (2 µM) for 30 min at 37°C in phosphate-buffered saline (PBS, 0.15 M, pH 7.4), washed twice and resuspended in indicator-free Hanks' balanced salt solution (HBSS, pH 7.4) containing 1.25 mM $CaCl_2$, referred to hereafter as Ca^{2+}-replete HBSS. The fura-2-loaded cells (2 x 10^6/ml) 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 wavelenghths 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 x 10^6 neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously (Grynkiewics *et al.*, 1985).

Additional experiments were performed to investigate the effects of pre-treatment 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.

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

In a limited series of experiments cells loaded with fura-2/AM as described above were activated with FMLP (1 µM) in the presence and absence of CGS 21680 (1 µM) in HBSS supplemented with 300 µM $MnCl_2$ (added 5 min prior to FMLP) and fluorescence quenching as a measure of Ca^{2+} influx was determined at an excitation

wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm (Geiszt *et al.*, 1997).

4.2.5 Radiometric assessment of Ca^{2+} fluxes

$^{45}Ca^{2+}$ (Calcium-45 chloride, specific activity 25.4 mCi/mg, Du Pont NEN Research Products, Boston, MA, USA.) was used as tracer to label the intracellular Ca^{2+} pool and to monitor Ca^{2+} fluxes in resting and activated neutrophils. In the assays of Ca^{2+} efflux and influx described below, the radiolabelled cation was always used at a fixed, final concentration of 2 µCi/ml containing 50 nmol cold carrier Ca^{2+} (as $CaCl_2$). The final assay volumes were always 5 ml containing a total of 1 x 10^7 neutrophils. The standardisation of the procedures used to load the cells with $^{45}Ca^{2+}$, as well as a comparison with silicone oil-based methods for the separation of labelled neutrophils from unbound isotope, have been described elsewhere (Anderson & Goolam Mahomed, 1997).

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

Neutrophils (1 x 10^7/ml) were loaded with $^{45}Ca^{2+}$ (2 µCi/ml) for 30 min at 37°C in HBSS which was free of unlabelled Ca^{2+}. The cells were then pelleted by centrifugation, washed once with, and resuspended in ice-cold Ca^{2+}-replete HBSS and held on ice until use, which was always within 10 min of completion of loading with $^{45}Ca^{2+}$. The $^{45}Ca^{2+}$-loaded neutrophils (2 x 10^6/ml) were then preincubated for 10 min at 37°C in Ca^{2+}-replete HBSS, 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 sec) of efflux of $^{45}Ca^{2+}$. A fixed incubation time of 60 sec 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 *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).

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

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

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

4.2.8 Oxidant generation

This was measured using a lucigenin (bis-N-methylacridnium nitrate)-enhanced chemiluminescence (LECL) method (Minkenberg & Ferber, 1984). Neutrophils (1 x

10^6/ml, 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 a LKB Wallac 1251 chemiluminometer (Turku, Finland) after the addition of the stimulant (100 µl). LECL readings were integrated for 5 sec intervals and recorded as mV x seconds^{-1} (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) superoxide-generating system.

4.2.9 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 x 10^7/ml 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 *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.

4.2.10 Intracellular cAMP levels

Neutrophils at a concentration of 1 x 10^7/ml 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 cAMP extracted by the addition of ice-cold ethanol (65% v/v) at 20 sec, 1 min, 3 min 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 *g* for 15 min at 4°C. The supernatants were then transferred to fresh tubes and evaporated at 60°C under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP [^{125}I] scintillation proximity assay system (Amersham International plc., Buckinghamshire, UK), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmol cAMP/10^7 neutrophils. Because cAMP is rapidly hydrolysed 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).

4.2.11 Statistical analysis

The results of each series of experiments are expressed as the mean values ± SEM. 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.

4.3. Results

4.3.1 Fura-2 fluorescence responses of FMLP-activated neutrophils

The results shown in Figure 4.1 (page 86) are traces from 3 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

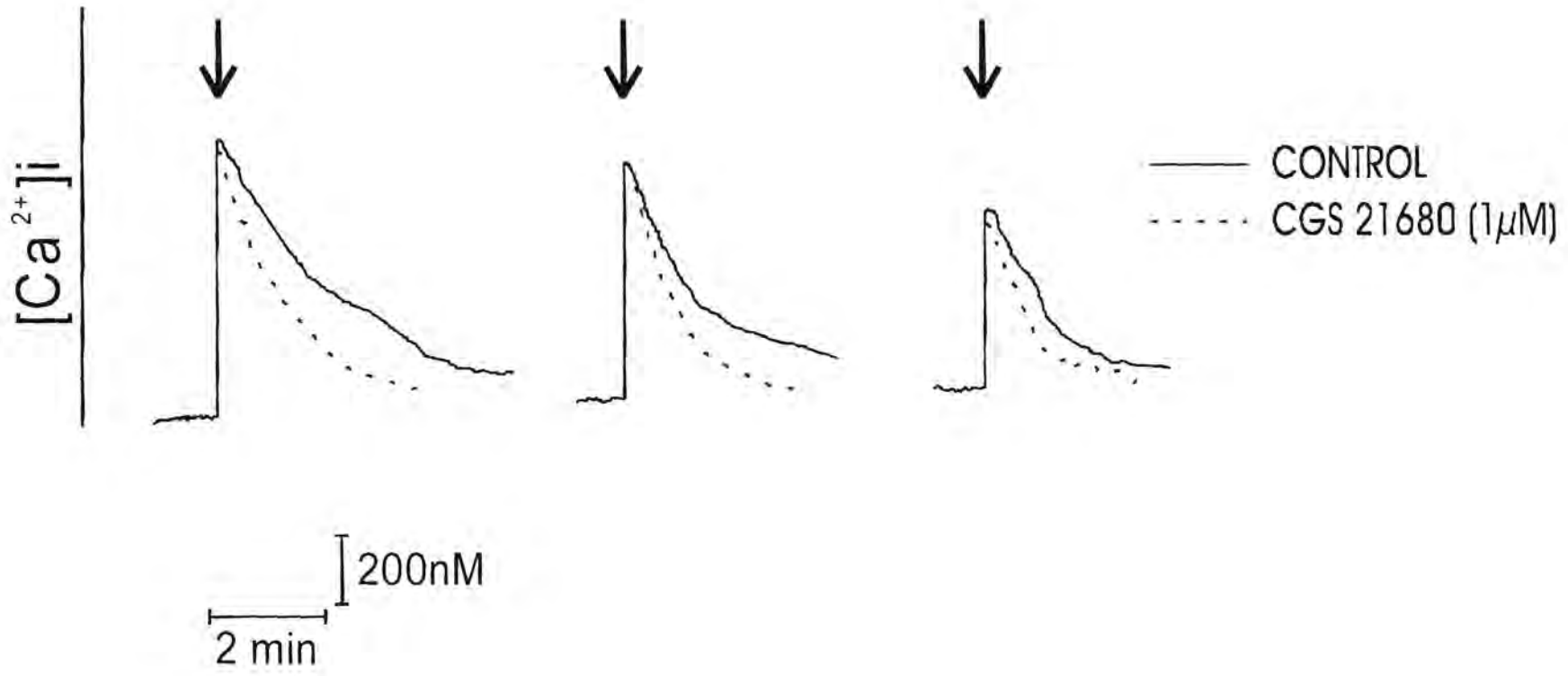

Figure 4.1: FMLP-activated fura-2 fluorescence responses of control and CGS 21680 (1 μM)-treated neutrophils. FMLP (1 μM) was added as indicated (↓) when a stable base-line was obtained (± 1 min). The traces shown are from 3 different experiments.

accompanied by the characteristic, abrupt increases in fura-2 fluorescence due to elevated concentrations of cytosolic Ca^{2+}. CGS 21680 did 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 4.1 (page 88) 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 ($t½$) 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} agonist caused dose-related acceleration in the rate of decline in peak fluorescence intensity, which at all 4 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 4.2 (page 89). 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 4 different donors, and are compatible with interference with the sequestration of incoming Ca^{2+} into storage organelles. 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 4.2 (page 88). Importantly, the half peak point of the decline of fura-2

fluorescence was reached before the effects of ZM 241385 *per se* on Ca^{2+} sequestration were evident. Pre-treatment of neutrophils with ZM 241385 antagonised ($p<0.05$) CGS 21680-mediated acceleration of the clearance of cytosolic Ca^{2+} from activated neutrophils without affecting peak fluorescence intensity.

Table 4.1 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 10 experiments are expressed as the mean values ± SEM. The $[Ca^{2+}]_i$ value for control, unstimulated neutrophils was 111 ± 9 nM.

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

Table 4.2 Effects of CGS 21680 ± ZM 241385 on peak intracellular calcium concentrations $[Ca^{2+}]_i$ and rates of clearance in FMLP-activated neutrophils

Systems	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 1 μM	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 6 experiments are expressed as the mean values ± SEM.

*$p<0.05$ for comparison with the control system.

** $p<0.05$ for comparison with CGS 21680 alone.

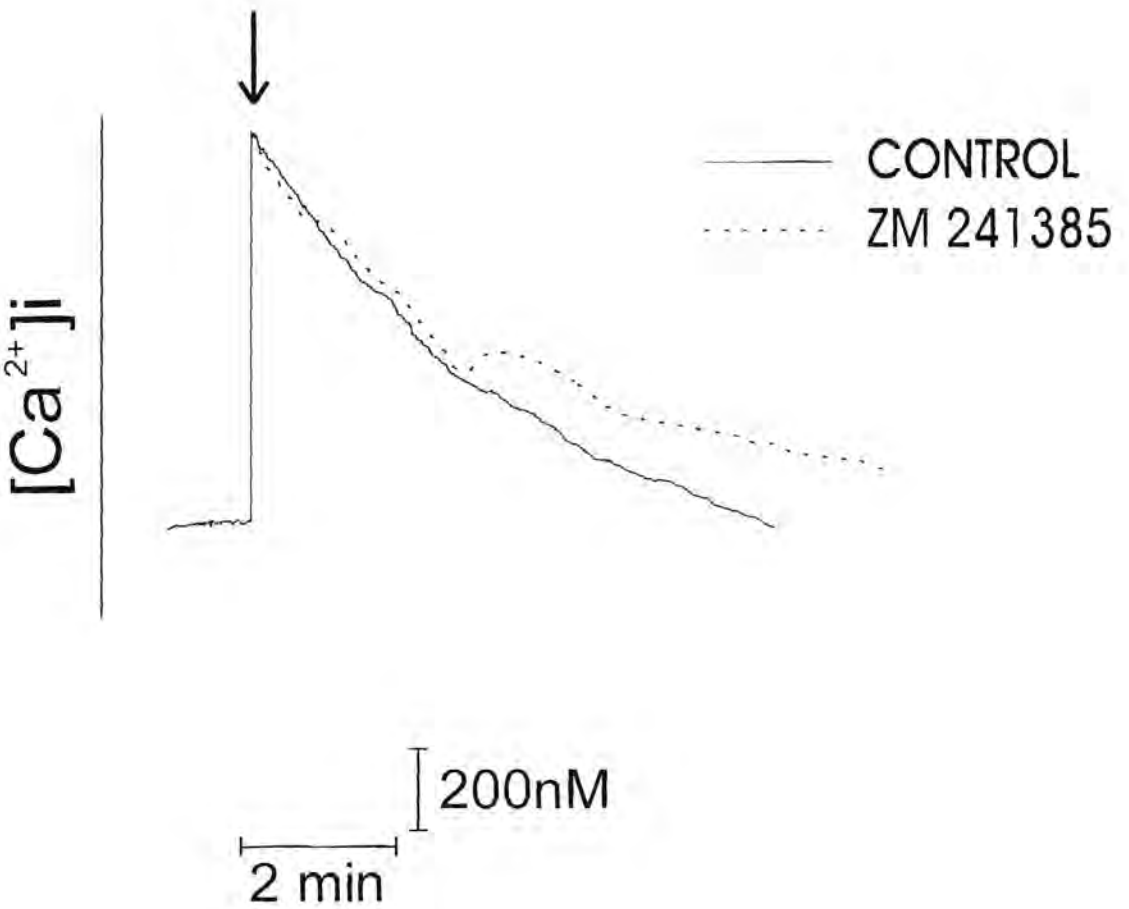

Figure 4.2: FMLP (1 μM)-activated fura-2 fluorescence responses of control and ZM 241385 (2.5 μM)-treated neutrophils. FMLP was added as indicated (↓) when a stable baseline was obtained (± 1 min). This is a typical trace from 12 different experiments.

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

The results of the indirect measurement of Ca^{2+} influx into FMLP-activated control and CGS 21680 (1 μM)-treated neutrophils are shown in Figure 4.3 (page 91). The traces shown are from a typical experiment (4 in each series) and clearly show a decrease in both the rate and extent of influx of Ca^{2+} into CGS 21680-treated neutrophils. Because of the reported limitations of this method (Favre *et al.*, 1996), measurement of influx of Ca^{2+} by radiometric procedures, as described below, was identified as the preferred procedure.

4.3.3 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 minimise 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 4.4 (page 92) 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 sec 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 unstimulated cells over the 60 sec time course of the experiment. Treatment of neutrophils with CGS 21680 (1 μM) caused a significant decrease ($p<0.05$ at 60 sec) in the efflux of $^{45}Ca^{2+}$ following activation of the cells with FMLP (Figure 4.4, page 92). 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 sec incubation period are shown in Table 4.3 (page 93). The A_{2A} agonist caused dose-related inhibition of efflux of $^{45}Ca^{2+}$

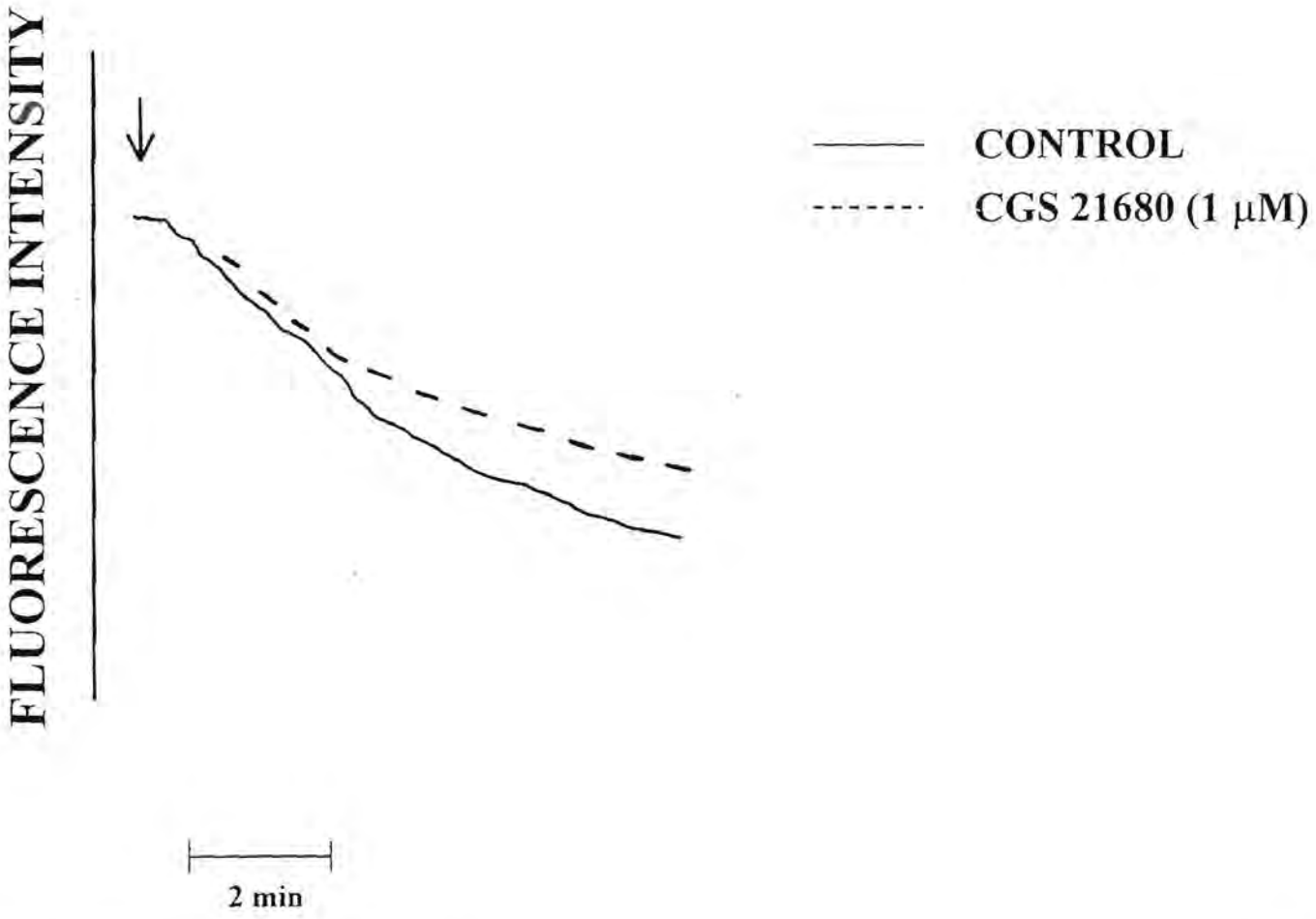

Figure 4.3: FMLP (1 μM)-activated Mn^{2+} quenching of the fura-2 responses of control and CGS 21680 (1 μM)-treated neutrophils. FMLP was added as indicated (↓) and the results shown are typical traces from 4 experiments. The decline in fluorescence intensity represents influx of Ca^{2+}.

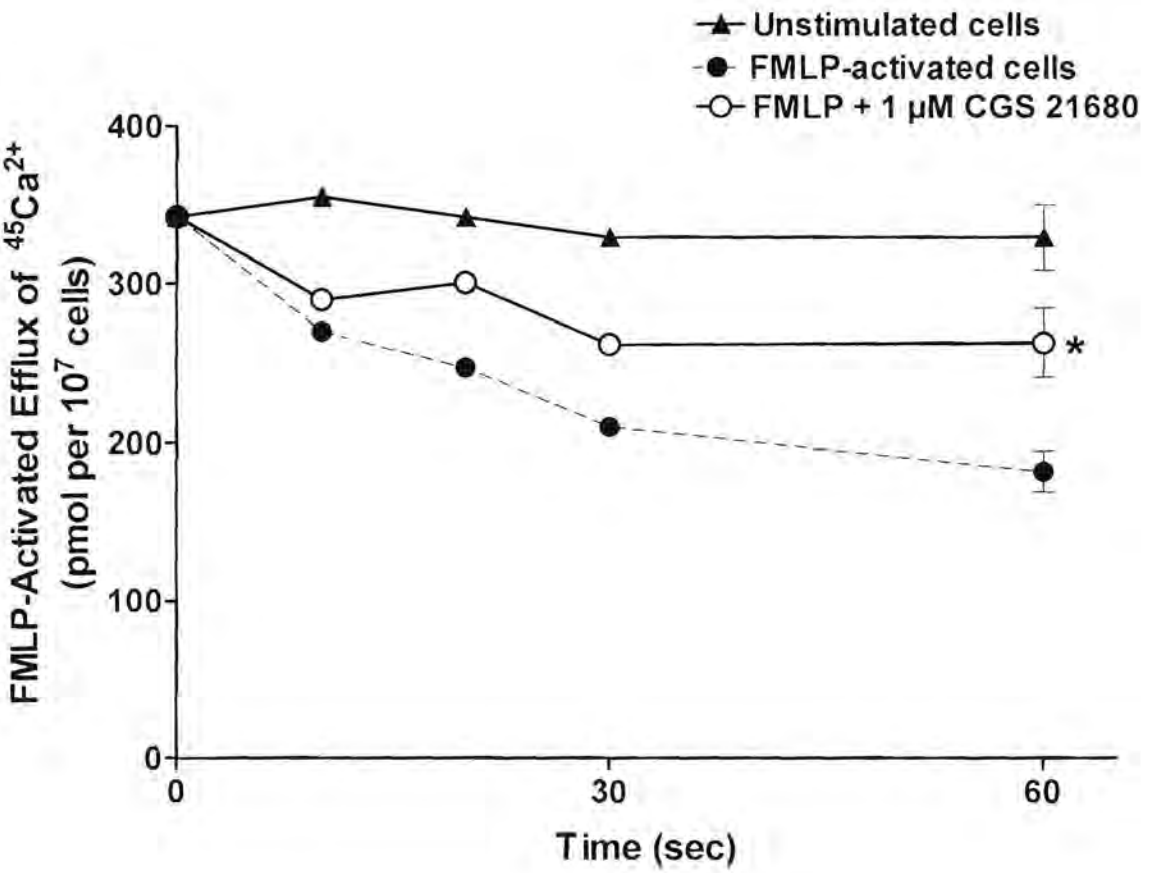

Figure 4.4: Kinetics of efflux of $^{45}Ca^{2+}$ from unstimulated neutrophils and neutrophils activated with FMLP (1 µM) in the absence and presence of CGS 21680 (1 µM). The results of 11 different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol per 10^7 cells) and vertical lines show SEM.

*$p<0.05$ for comparison with the FMLP-activated, CGS 21680-free control system.

Table 4.3: Effects of varying concentrations of CGS 21680 on the efflux of $^{45}Ca^{2+}$ out of FMLP-activated neutrophils

Agent	Amount of $^{45}Ca^{2+}$ released from neutrophils 60s after the addition of FMLP (pmol per 10^7 cells)
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 3 experiments are expressed as the mean values ± SEM.

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 4.4. 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 sec) the antagonist *per se* caused modest, statistically insignificant increases in the efflux of $^{45}Ca^{2+}$ from FMLP-activated neutrophils.

Table 4.4: 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 $[Ca^{2+}]_i$ released from neutrophils 60 sec after the addition of FMLP (pmol per 10^7 cells)
FMLP only	159 ± 11
FMLP + CGS 21680 1 μM	76 ± 10*
FMLP + thapsigargin 1 μM	177 ± 10
FMLP + CGS 21680 + thapsigargin 1 μM	152 ± 12
FMLP + ZM 241385 2.5μM	173 ± 11
FMLP + CGS 21680 + ZM 241385 2.5 μM	163 ± 10

The results of 6 experiments are expressed as the mean values ± SEM.* $P<0.05$ for comparison with the drug-free (FMLP only) control system.

4.3.4 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 minimise spontaneous uptake of $^{45}Ca^{2+}$ by neutrophils (Anderson & Goolam Mahomed, 1997). The results of these experiments are shown in Figure 4.5 (page 95). 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 sec. 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. Pre-treatment 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 per 10^7 cells for control neutrophils and for cells pre-treated 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 per 10^7 cells for control and ZM 241385-treated neutrophils respectively (n=6).

4.3.5 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 4.6 (page 96). 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 μM 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,

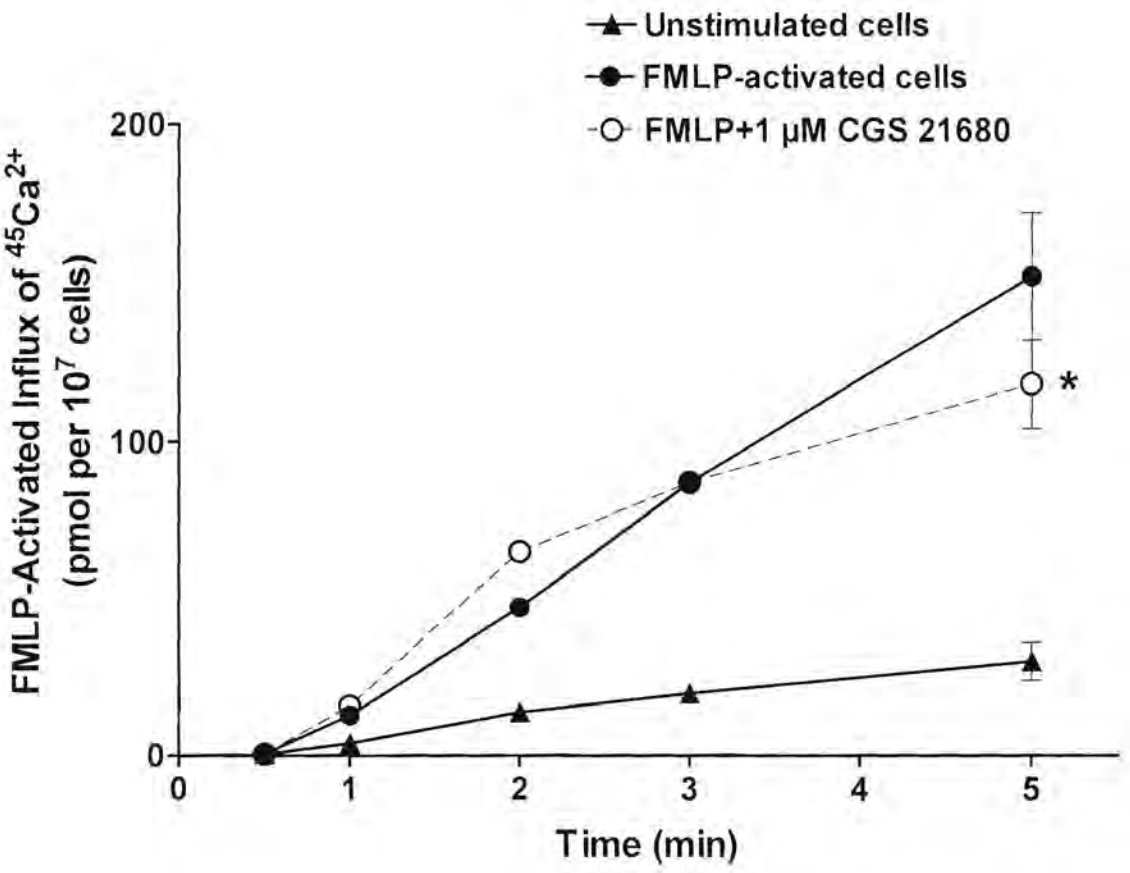

Figure 4.5: Kinetics of influx of $^{45}Ca^{2+}$ into unstimulated neutrophils and neutrophils activated with FMLP (1 µM) in the absence and presence of CGS 21680 (1 µM). The results of 8 different experiments are expressed as the mean amount of cell-associated $^{45}Ca^{2+}$ (pmol per 10^7 cells) and vertical lines show SEM.

*$p<0.05$ for comparison with the FMLP-activated, CGS 21680-free control system.

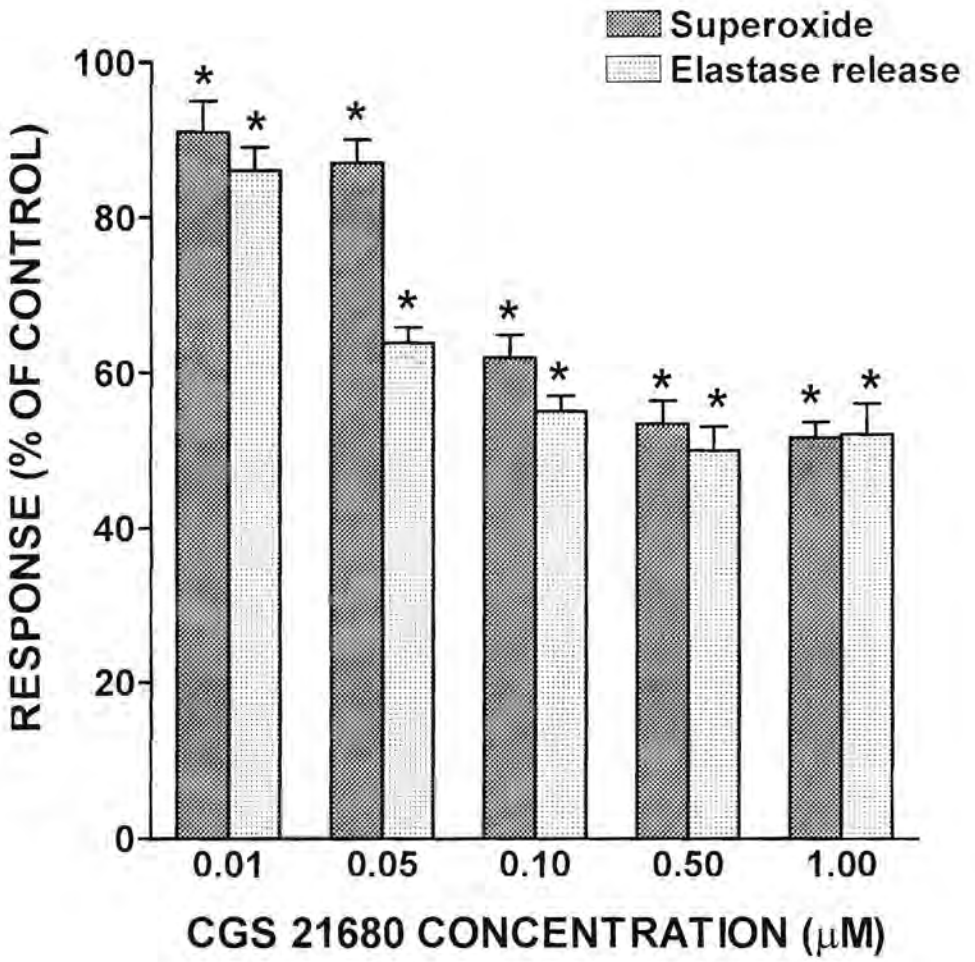

Figure 4.6: The effects of varying concentrations of CGS 21680 (0.01 –1 μM) on superoxide production by FMLP (0.1 μM)-activated neutrophils and on elastase release from FMLP/CB-activated neutrophils. The results of 8 (superoxide), measured by lucigenin-enhanced chemiluminescence (LECL) and 10 (elastase) different experiments are presented as the mean percentage of the drug-free control systems and vertical lines show SEM. In the case of superoxide production, the absolute values for resting and FMLP-activated neutrophils were 256 ± 23 and 911 ± 84 millivolts/sec respectively. The corresponding values for elastase release were 40 ± 2 and 598 ± 70 milliunits enzyme per 10^7 cells respectively.

and elastase release from, activated neutrophils, are shown in Table 4.5. 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. Pre-treatment of the cells with ZM 241385 completely antagonised the anti-inflammatory effects of the $A_{2A}R$ agonist, while thapsigargin was only slightly less effective.

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 hypoxanthine-xanthine oxidase system (not shown).

Table 4.5: 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 6 (superoxide) and 8-12 (elastase) different experiments are presented as the mean percentages of the drug-free control systems ± SEM. 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 corresponding values for elastase release from FMLP/CB-activated were 40 ± 2 and 598 ± 10 milliunits enzyme per 10^7 cells respectively.
*$p<0.05$ for comparison with the drug-free (FMLP or FMLP/CB only) control system.

4.3.6 Intracellular cAMP

The effects of CGS 21680 (1 μM) on cAMP in unstimulated and FMLP-activated neutrophils in the presence and absence of ZM 241385 (2.5 μM) are shown in Table 4.6. Exposure of neutrophils to FMLP was accompanied by an increase in intracellular cAMP which was inhibited by ZM 241385. Treatment of both stimulated and unstimulated neutrophils with CGS 21680 resulted in significant increases in intracellular cAMP concentrations which were completely abolished by pre-treatment of the cells with ZM 241385 prior to the addition of the $A_{2A}R$ agonist.

Table 4.6: The effects of CGS 21680 and ZM 241385 individually and in combination on cAMP levels in unstimulated and FMLP-activated neutrophils.

System	Intracellular cAMP (pmol per 10^7 cells)
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 ± 1.5**
FMLP-activated neutrophils + ZM 241385	41 ± 4
FMLP-activated neutrophils + CGS 21680 + ZM 241385	38 ± 0.4

The results of 5 different experiments are expressed as the mean intracellular concentration of cAMP ± SEM 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.

4.4. 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 cAMP-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 cAMP 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 cAMP decrease the pro-inflammatory activities of human neutrophils *in vitro* by cAMP-dependent enhancement of the activity of the endo-membrane Ca^{2+}-ATPase (Anderson *et al.*, 1998). Rolipram- and dibutyryl cAMP-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-recognised (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 cAMP, remain uncertain. In the present study, we investigated the effects of the $A_{2A}R$ agonist, CGS 21680, on intracellular cAMP 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 sec 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 mobilised 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 sec 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 (Montero *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. CGS 21680-mediated reduction in store-operated influx of Ca^{2+} in FMLP-activated neutrophils was also observed using the Mn^{2+} quenching of fura-2 fluorescence procedure. The usefulness of this method has, however, been questioned because store-operated channels are Ca^{2+}-selective with a small single channel conductance (Favre *et al.*, 1996). Together with the results of the conventional 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+}. Utilisation 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 neutralised the inhibitory effects of CGS 21680 on superoxide production and elastase release, demonstrating a mechanistic link between these events.

The involvement of cAMP 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 cAMP were increased following exposure of the cells to CGS 21680 and ii) the selective $A_{2A}R$ receptor antagonist ZM 241385 almost completely antagonised the effects of CGS 21680 on intracellular cAMP, Ca^{2+} clearance, superoxide production and elastase release. ZM 241385-mediated antagonism of the cAMP-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 cAMP 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 has 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 cAMP by FMLP-activated neutrophils, resulting in failure to up-regulate the cAMP-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 cAMP 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 cAMP-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.

Chapter 5

Concluding Comments

5.1 Concluding Comments

The inability of corticosteroids to directly affect the immediately/early-activatable pro-inflammatory functions of human neutrophils *in vitro*, has been confirmed in the current study (Chapter 2). Neither dexamethasone nor fluticasone had any meaningful inhibitory effects on superoxide production by and release of elastase from chemoattractant-activated neutrophils, while in some cases potentiation of these activities was evident. Moreover, prolonged exposure (for up to 6 hours) of neutrophils also failed to affect the synthesis by, and release of IL-8 from the cells. Due to the high level of spontaneous apoptosis which was evident at 6 hours (R. Cockeran, Department of Immunology, University of Pretoria), I was unable to reliably extend the time-course of my experiments beyond this, which may explain the difference between my results and those reported by Cox (1995). The latter author reported that treatment of neutrophils with dexamethasone for 24 hours resulted in a significant decrease in the synthesis of IL-8 by these cells.

The apparent insensitivity of neutrophils to corticosteroids may be explained by several co-existent and possibly interactive mechanisms. Firstly, neutrophils are pro-programmed to respond instantly to receptor-mediated activation with chemoattractants, cytokines and opsonized particles, independently of *de novo* protein synthesis. Secondly, neutrophils are insensitive to the apoptosis-inducing actions of corticosteroids (Cox, 1995; Meagher *et al.*, 1996), due possibly, but not necessarily limited to, the paucity of mitochondria in these cells (Peachman *et al.*, 2000). Thirdly, the relatively high ratio of GRß to GRα in the neutrophil results in a high frequency of GRα/GRß heterodimers which have only 15-20% of the transactivating activity of GRα homodimers (Strickland *et al.*, 2001). Indeed, GRß lacks the steroid binding domain which makes it unable to bind glucocorticoids, reduces its affinity for DNA recognition sites, abolishes its ability to transactivate glucocorticoid-sensitive genes and makes it function as a dominant inhibitor of GRα through the formation of antagonistic GRα/GRß heterodimers (Strickland *et al.*, 2001).

Taken together, all of this seems to suggest that neutrophils are not particularly responsive to the anti-inflammatory actions of corticosteroids. Nevertheless, important indirect regulatory effects of corticosteroids on neutrophils mediated via down-regulation of the synthesis, release and/or expression of neutrophils-targeted pro-inflammatory polypeptides (adhesion molecules, cytokines) cannot be excluded.

A glucocorticoid-induced decrease in L-selectin expression has been demonstrated on neutrophils in the maturation pool of the bone marrow and may constitute an important mechanism in the reduction of PMN recruitment into inflammatory sites (Nakagawa *et al.*, 1999). This decrease was detectable 8-12 hours after GC treatment.

The ineffectiveness of corticosteroids in controlling the pro-inflammatory activities of neutrophils set the scene for the subsequent, and indeed the most important aspects of my laboratory research, which were to identify pharmacologic agents which would effectively suppress neutrophil NADPH oxidase activity and granule enzyme release. Given the well-recognised anti-inflammatory actions of cAMP-elevating agents, which include inhibition of the early-activatable pro-inflammatory activities of neutrophils (Moore & Willoughby, 1995), in the setting of the recent development of pharmacologic agents such as second generation type 4 PDE inhibitors (Torphy, 1998; Wang *et al.*, 1999) and subtype-selective agonists of adenosine receptors (Rieger *et al.*, 2001; Sullivan *et al.*, 2001), my research, much of which is original, was directed at identifying the anti-inflammatory potential and biochemical mechanisms of action of the latter group of agents.

Interestingly, CGS 21680 was found to be the most potent of the three adenosine receptor subtype agonists tested, implicating the adenylate cyclase/cAMP-coupled $A_{2A}R$ subtype as being primarily involved in regulating neutrophils pro-inflammatory activity. The involvement of this adenosine receptor subtype, as well as that of cAMP, was underscored by the action of ZM 241385, which attenuated the enhancing effects of CGS 21680 on neutrophil cAMP and the inhibitory actions of the $A_{2A}R$ agonist on FMLP-activated superoxide production and elastase release. The effects of CGS 21680 on neutrophil cAMP and pro-inflammatory activities were associated with definite alterations in Ca^{2+} handling by the cells following exposure to

FMLP. These were characterised by accelerated clearance of Ca^{2+} from the cytosol by FMLP-activated neutrophils, which was compatible with a cAMP-dependent mechanism involving up-regulation of the endo-membrane Ca^{2+}-ATPase. I am not aware of previous reports documenting these cAMP-dependent enhancing effects of CGS 21680 on restoration of Ca^{2+} homeostasis to chemoattractant-activated neutrophils.

The effects of ZM 241385 *per se* (in the absence of CGS 21680) on neutrophils are noteworthy. Treatment of the cells with this agent completely attenuated the FMLP-mediated transient increase in intracellular cAMP in the setting of prolongation of the elevation in the cytosolic Ca^{2+} concentration, increased release of elastase and a modest increase in superoxide production. These observations are compatible with the involvement of neutrophil-derived adenosine in the autocrine restoration of Ca^{2+} homeostasis and down-regulation of the pro-inflammatory activities of these cells. Complete attenuation of the FMLP-activated increase in cAMP by ZM 241385 further suggests that endogenous adenosine operating via subtype $A_{2A}R$ and cAMP, as opposed to other potential endogenous regulators such as prostaglandin E_2 operating via EP_2 and EP_4 receptors (Talpain *et al.*, 1995), is primarily involved in the physiological restoration of Ca^{2+} homeostasis to activated neutrophils. Although adenosine has been proposed by others to function as a physiological anti-inflammatory agent (Cronstein, 1994), the mechanistic data reported here are, to my knowledge, original.

With respect to ongoing and future research on the anti-inflammatory potential of subtype $A_{2A}R$ agonists, particularly in controlling neutrophils, there are several aspects which merit brief comment. Considerable effort is being devoted to the development of $A_{2A}R$ agonists with improved selectivity (Rieger *et al.*, 2001; Sullivan *et al.*, 2001), and at least one of these is currently undergoing phase 1 clinical trials (Dr M Johnson, Glaxo Smith Kline, UK). In the case of neutrophil-mediated inflammation, selectivity may be improved and side-effects reduced by combining an $A_{2A}R$ agonist with a selective inhibitor of PDE 4B2, the predominant type found in human neutrophils (Wang *et al.*, 1999). Such a combination strategy may increase efficacy in the setting of reduced toxicity. I have recently been reliably informed that exposure of certain types of inflammatory cells (unspecified) to corticosteroids is

accompanied by upregulation of $A_{2A}R$ (Dr M Johnson, Glaxo Smith Kline, UK), which, bearing in mind the apparent insensitivity of neutrophils to corticosteroids, may, or may not, justify the combined use of $A_{2A}R$ agonists and corticosteroids, as is currently the case with long-acting $ß_2$-agonists and corticosteroids. Corticosteroids appear to activate transcription of the gene encoding the $A_{2A}R$, in the setting of deactivation of the gene encoding the A_3R. Finally, potential side-effects may also be minimised through the development of inhaled $A_{2A}R$ agonists for the treatment of inflammatory airway disorders (Dr M Johnson, Glaxo Smith Kline, UK).

Future research is required to establish *in vivo* anti-inflammatory activity of A_{2A} agonists, optimal routes of administration of these agents, as well as assessment of anti-inflammatory potency in combination with other classes of anti-inflammatory agents especially type 4 phosphodiesterase inhibitors and development of selective and more potent A_{2A} agonists.

In conclusion, the results presented in this thesis demonstrate that the $A_{2A}R$ agonist, CGS 21680, possesses neutrophil-directed anti-inflammatory activities which are dependent on activation of adenylate cyclase and up-regulation of the Ca^{2+} sequestering/re-sequestering endo-membrane Ca^{2+}-ATPase. Although promising, no such agent is currently available for clinical application and further research is required to optimise selectivity and anti-inflammatory activity in the setting of minimal toxicity.