Biochemical Pharmacology, Vol. 60, pp. 993–999, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00414-7

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

Susanna S. Visser, Annette J. Theron,† Grace Ramafi,† James A. Ker* and Ronald Anderson‡*

*Division of Pulmonology, Department of Internal Medicine and Medical Research Council Unit for Inflammation and Immunity and †Department of Immunology, Faculty of Medicine, University of Pretoria, Pretoria, South Africa

ABSTRACT. This study was undertaken to identify the adenosine receptor (AR) subtypes which down-regulate the proinflammatory activities of human neutrophils, as well as the involvement of adenosine 3′,5′-cyclic monophosphate (cAMP) and its relationship to cellular handling of Ca^{2+} in mediating these effects. Neutrophils were treated with varying concentrations (0.01–1 μM) of AR agonists operative at A_1 (N^6-cyclopentyladenosine, CPA), A_{2A} (2(4-[(2-carboxyethyl)phenyl]ethylamino)-5′-*N*-ethylcarboxamidoadenosine, CGS 21680), and A_3 (N^6-(3-iodobenzyl-5′-*N*-methylcarbamoyladenosine, IB-MECA) receptors, after which they were activated with the chemoattractant, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP, 1 μM). Intracellular cAMP, superoxide, and elastase were assayed using radioimmunoassay, lucigenin-enhanced chemiluminescence (LECL), and colorimetric procedures, respectively, while changes in the concentrations of cytosolic Ca^{2+} were monitored by fura-2-based spectrofluorimetry. CGS 21680, at all concentrations tested, inhibited superoxide production in a dose-related manner, while CPA and IB-MECA were effective only at the highest concentrations tested (0.5–1 μM). The release of elastase from activated neutrophils was also inhibited by all three AR agonists, but was more sensitive to CGS 21680 and IB-MECA than was superoxide production. The inhibitory effects of all 3 agonists on superoxide production and elastase release were associated with accelerated clearance of Ca^{2+} from the cytosol of activated neutrophils, and were effectively neutralized by pretreatment of the cells with the highly selective A_{2A}R antagonist, ZM 241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5yl amino]ethyl)phenol). Increased cAMP was detected in neutrophils treated with CGS 21680 and IB-MECA (1 μM). These data support the involvement of the A_{2A}R subtype in the suppression of superoxide production and degranulation by activated human neutrophils, probably by cAMP-mediated alterations in Ca^{2+} handling. BIOCHEM PHARMACOL **60**;7:993–999, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. adenosine; calcium; cyclic AMP; CGS 21680; elastase; neutrophils; superoxide; ZM 241385

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 signaling [1, 2]. 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 [1, 2]. 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 [1, 3] and eosinophils [4, 5]. There is compelling evidence for the presence of A_{2A} receptors on human neutrophils [6, 7], while indirect evidence supports the existence of A_1 and A_3 receptors on these cells, as well as on eosinophils [4, 5, 8–10]. With respect to neutrophils, A_1 and A_{2A} receptors have been reported to exert opposing effects on the proinflammatory 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 [11, 12], while activation of A_{2A} receptors results in suppression of the production of reactive oxidants by these cells [3, 12], as well

‡ Corresponding author: Dr. Ronald ANDERSON, Dept. of Immunology, University of Pretoria, P.O. Box 2034, Pretoria 0001, South Africa. Tel. +27 12 319 2425; FAX +27-12-323-0732; E-mail: randerso@postillion.up.ac.za

Received 2 November 1999; accepted 13 March 2000.

as decreased expression of β2-integrins and adherence to vascular endothelium [11, 13]. Neutrophil degranulation, on the other hand, has been reported to be either insensitive to adenosine [12] or to be inhibited by mechanisms involving both A_2 and A_3 receptors [9]. In the case of eosinophils, interaction of adenosine or its analogues with A_3 receptors appears to promote down-regulation of the proinflammatory activities of these cells [4, 5].

Although adenosine and its analogues acting via A_{2A} receptors suppress some of the proinflammatory 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.

MATERIALS AND METHODS

Adenosine Receptor Agonists

CPA (A_1R agonist), CGS 21680 (A_{2A}R agonist), IB-MECA (A_3R agonist), and rolipram, a selective inhibitor of type 4 phosphodiesterase, the predominant type found in human neutrophils [14], were kindly provided by Dr Malcolm Johnson, GlaxoWellcome plc. 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 dimethyl sulfoxide (rolipram) and diluted thereafter in indicator-free 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 [15], was purchased from Tocris Cookson Ltd. and dissolved to 10 mM 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.

Neutrophils

Purified neutrophils were prepared from heparinized (5 units of preservative-free heparin/mL) venous blood of healthy adult human volunteers and separated from mononuclear leukocytes by centrifugation on Histopaque®-1077 (Sigma Diagnostics) cushions at 400 *g* for 25 min at room temperature. The resultant pellet was suspended in 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° for 10 min. The neutrophils, which were routinely of high purity (>90%) and viability (>95%), were resuspended to 1×10^7/mL in PBS and held on ice until used.

Oxidant Generation

This was measured using an LECL (bis-*N*-methylacridinium nitrate) method [16]. Neutrophils (1×10^6/mL, final) were preincubated 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 an LKB Wallac 1251 chemiluminometer after the addition of the stimulant (100 μL). LECL readings were integrated for 5-sec intervals and recorded as mV × seconds^{-1} (mV.sec^{-1}). Additional experiments were performed to investigate the following: i) the effects of ZM 241385 (2.5 μM) added during preincubation at 37°, 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.

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 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°. 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°. 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 [17]. 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

* *Abbreviations:* AR, adenosine receptor; cAMP, adenosine 3′,5′-cyclic monophosphate; CB, cytochalasin B; CGS 21680, 2(4-[(2-carboxyethyl) phenyl] ethylamino)-5′-*N*-ethylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; FURA-2/AM, 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-N,N,N′,N′,-tetraacetic acid-acetoxy methylester oil; HBSS, Hanks' balanced salt solution; LECL, lucigenin-enhanced chemiluminescence; IB-MECA, N^6-(3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine; and ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4] triazolo[2,3-*a*][1,3,5]triazin-5yl amino]ethyl)phenol.

of enzyme released by the corresponding FMLP/CB-activated, drug-free control systems.

Intracellular cAMP Levels

Neutrophils at a concentration of 1×10^7/mL in HBSS were preincubated for 10 min at 37° 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 2000 *g* for 15 min at 4°. The supernatants were then transferred to fresh tubes and evaporated at 60° under a stream of nitrogen. The dried extracts were reconstituted in assay buffer (0.05 M acetate buffer, pH 5.8) and assayed for cAMP using the Biotrak cAMP ^{125}I scintillation proximity assay system (Amersham International plc), which is a competitive binding radioimmunoassay procedure. These results are expressed as pmol cAMP/10^7 neutrophils. Because cAMP is rapidly hydrolyzed in neutrophils by phosphodiesterases, these experiments were performed in the presence of 1 μM rolipram.

Spectrofluorimetric Measurement of Ca^{2+} Fluxes

Fura-2/AM (Calbiochem) was used as the fluorescent, Ca^{2+}-sensitive indicator for these experiments. Neutrophils (1×10^7/mL) were preloaded with fura-2 (2 μM) for 30 min at 37° in PBS (0.15 M, pH 7.4), washed twice, and resuspended in HBSS. The fura-2-loaded cells (2×10^6/mL) were then preincubated with CPA, CGS 21680, or IB-MECA (0.01–1 μM) at 37° for 10 min after which they were transferred to disposable reaction cuvettes, which were maintained at 37° in a Hitachi 650-10S fluorescence spectrophotometer with excitation and emission wavelengths set at 340 and 500 nm, respectively. After a stable baseline 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 6×10^6 neutrophils. Cytoplasmic Ca^{2+} concentrations were calculated as described previously [18].

Additional experiments were performed to investigate the effects of pretreatment 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.

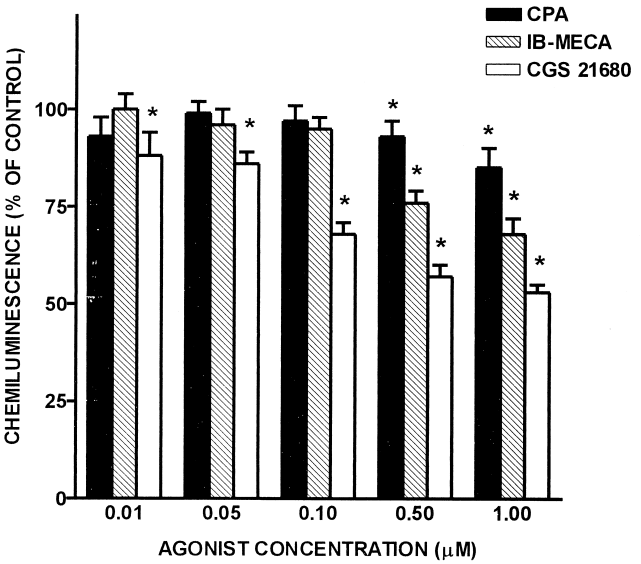

FIG. 1. The effects of varying concentrations of CGS 21680, CPA, and IB-MECA on the production of superoxide by FMLP-activated neutrophils. The results of 6–23 experiments are presented as the mean percentages ± SEMs of the control systems. The absolute values for unstimulated and FMLP-activated control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

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.

RESULTS

Oxidant Production

The effects of CGS 21680, CPA, and IB-MECA on superoxide production by neutrophils activated with FMLP are shown in Fig. 1. 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 and CPA were less effective, causing significant inhibition of superoxide production only at concentrations of 0.5 and 1 μM.

The effect of pretreatment 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 1. 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

TABLE 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 $mV.sec^{-1}$ and 600 milliunits enzyme/10^7 cells for superoxide production and elastase release, respectively.

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 2. 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 neutralized 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 $mV.sec^{-1}$, respectively (results of 12 experiments).

TABLE 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 $mV.sec^{-1}$.

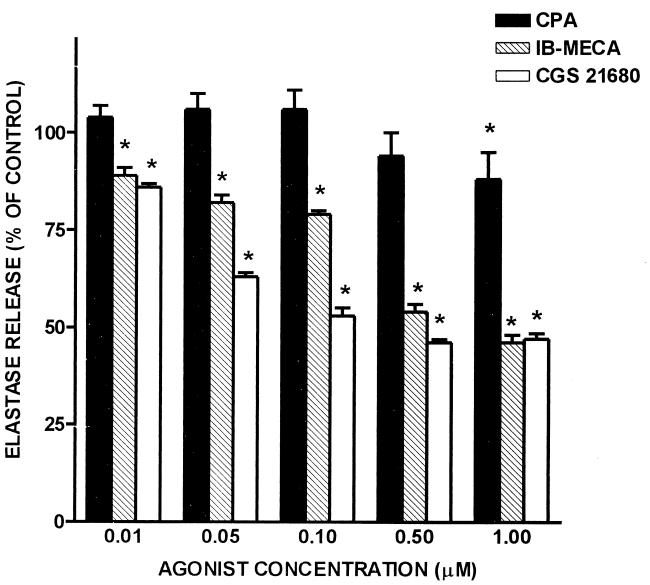

FIG. 2. The effects of varying concentrations of CGS 21680, CPA, and IB-MECA on the release of elastase from FMLP/CB-activated neutrophils. The results of 15 experiments are presented as the mean percentages ± SEMs of the control systems. The absolute values for unstimulated and FMLP/CB-activated neutrophils were 3 ± 0.8 and 593 ± 4 milliunits enzyme/10^7 cells, respectively. *$P < 0.05$.

Elastase Release

The effects of the 3 AR agonists on elastase release from FMLP/CB-activated neutrophils are shown in Fig. 2. 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. The effects of ZM 241385 (2.5 μM) pretreatment 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 1. ZM 241385 per se potentiated the release of elastase from stimulated neutrophils and completely (CGS 21680 and CPA) or almost completely (IB-MECA) antagonized 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).

cAMP

The effects of CGS 21680 and IB-MECA on intracellular cAMP in unstimulated and FMLP-stimulated neutrophils are shown in Table 3. 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.

TABLE 3. The effects of CGS 21680 and IB-MECA individually and in combination with ZM 241385 on cAMP in unstimulated and FMLP-activated neutrophils

System	Intracellular cAMP (pmol/10^7 cells)	
	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.

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.

Fura-2 Fluorescence

The results shown in Fig. 3 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 4 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 of the rate of decline in peak fluorescence, while CPA was effective only at 1 μM.

TABLE 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.

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 5. 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.

DISCUSSION

The results of the present study support the contention that subtype A_{2A}Rs down-regulate the proinflammatory activi-

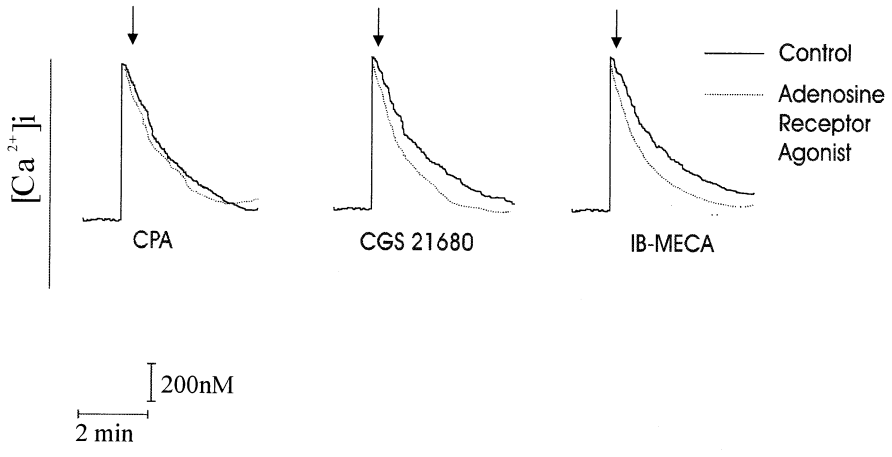

FIG. 3. FMLP-activated fura-2 responses of neutrophils with and without CGS 21680, CPA, or IB-MECA (all at 1 μM). FMLP was added as indicated (↓) when a stable base line was obtained (±1 min). Traces are from a single representative experiment.

TABLE 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.

ties of human neutrophils [3, 7, 19]. 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 [3, 19]. 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 [9], this differential sensitivity of the two responses was also observed with CGS 21680. The order of agonist potencies to inhibit the proinflammatory activities of neutrophils is the same as that reported by others [3] and also identical to the order of potency to inhibit the A_{2A} receptor defined by radioligand binding to rat and human brain [20, 21]. Moreover, the inhibitory effects of IB-MECA, as well as those of CGS 21680 and CPA, on both elastase release and superoxide production were neutralized by pretreatment of the cells with the highly selective A_{2A}R agonist, 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 [3], the data should 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 neutralized the antioxidative 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 [22]. 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 proinflammatory activities of these cells. Our 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 [1, 3]. The enhancing effects of both CGS 21680 and IB-MECA on cAMP were attenuated by pretreatment 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 [22].

We and others 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 apparent up-regulation of the activity of the cAMP-dependent protein kinase-modulated, Ca^{2+}-sequestering endomembrane Ca^{2+}-ATPase [23, 24]. 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 antagonized by pretreatment 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 endomembrane Ca^{2+}-ATPase.

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 proinflammatory 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.

References

1. Cronstein BN, Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol* **76:** 5–13, 1994.
2. Stiles GL, Adenosine receptors. *J Biol Chem* **267:** 6451–6454, 1992.
3. Hannon JP, Bray-French KM, Phillips RM and Fozard JR, Further pharmacological characterization of the adenosine receptor subtype mediating inhibition of the oxidative burst in human isolated neutrophils. *Drug Dev Res* **43:** 214–224, 1998.
4. Walker BA, Jacobson MA, Knight DA, Salvatore CA, Weir T, Zhou D and Bai TR, Adenosine A_3 receptor expression and function in eosinophils. *Am J Respir Cell Mol Biol* **16:** 531–537, 1997.
5. Ezeamuzie CI and Philips E, Adenosine A_3 receptors on human eosinophils mediate inhibition of degranulation and superoxide anion release. *Br J Pharmacol* **127:** 188–194, 1999.
6. Fredholm BB, Zhang Y and van der Ploeg I, Adenosine A_{2A} receptors mediate the inhibitory effect of adenosine on formyl-Met-Leu-Phe-stimulated respiratory burst in neutrophil leucocytes. *Naunyn Schmiedebergs Arch Pharmacol* **354:** 262–267, 1996.
7. Varani K, Gessi S, Dionisotti S, Ongini E and Borea PA, [^{3}H]-SCH58261 labelling of functional A_{2A} adenosine receptors on human neutrophils. *Br J Pharmacol* **123:** 1723–1731, 1998.
8. Rose FR, Hirschhorn R, Weissmann G and Cronstein BN, Adenosine promotes neutrophil chemotaxis. *J Exp Med* **167:** 1186–1194, 1988.
9. Bouma MG, Jeunhomme TM, Boyle DL, Dentener MA, Voitenok NN, van den Wildenberg FA and Buurman WA, Adenosine inhibits neutrophil degranulation in activated human whole blood: Involvement of A_2 and A_3 receptors. *J Immunol* **158:** 5400–5408, 1997.
10. Fredholm BB, Purines and neutrophil leucocytes. *Gen Pharmacol* **28:** 345–350, 1997.
11. Cronstein BN, Levin RI, Phillips K, Hirschhorn R, Abramson SB and Weissmann G, Neutrophil adherence to endothelium is enhanced via adenosine A_1 receptors. *J Immunol* **148:** 2201–2206, 1992.
12. Cronstein BN, Rosenstein ED, Kramer SB, Weissmann G and Hirschhorn R, Adenosine: A physiologic modulator of superoxide anion generation by human neutrophils. Adenosine acts via an A_2 receptor on human neutrophils. *J Immunol* **135:** 1366–1371, 1985.
13. Nolte D, Lorenzen A, Lehr HA, Bokoch GK and Sklar LA, Reduction of postischemic leukocycte–endothelium interaction by adenosine via A_2 receptor. *Naunyn Schmiedebergs Arch Pharmacol* **346:** 234–237, 1992.
14. Torphy TJ, Phosphodiesterase enzymes: Molecular targets for novel antiasthma agents. *Am J Respir Crit Care Med* **157:** 351–370, 1998.
15. Poucher SM, Keddie JR, Singh P, Stoggall SM, Caulkett PW, Jones G and Coll MG, The *in vitro* pharmacology of ZM 241385, a potent non-xanthine A_{2A} selective adenosine receptor antagonist. *Br J Pharmacol* **115:** 1096–1102, 1995.
16. Minkenberg I and Ferber E, Lucigenin-dependent chemiluminescence as a new assay for NADPH-oxidase activity in particulate fractions of human polymorphonuclear leukocytes. *J Immunol Methods* **71:** 61–67, 1984.
17. Beatty K, Robertie P, Senior RM and Travis J, Determination of oxidized alpha-1-proteinase inhibitor in serum. *J Lab Clin Med* **100:** 186–192, 1982.
18. Grynkiewicz G, Poenie M and Tsien RY, A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:** 3440–3450, 1985.
19. Sullivan GW and Linden J, Role of A(2A) adenosine receptors in inflammation. *Drug Dev Res* **45:** 103–112, 1998.
20. Jarvis MF, Schultz R, Hutchison AJ, Do UH, Sills MA and Williams M, [3H]CGS 21680, a selective A_2 adenosine receptor agonist directly labels A_2 receptors in rat brain. *J Pharmacol Exp Ther* **251:** 888–893, 1989.
21. Wan W, Sutherland GR and Geiger JD, Binding of the adenosine A_2 receptor ligand [^{3}H]CGS 21680 to human and rat brain: Evidence for multiple affinity sites. *J Neurochem* **55:** 1763–1771, 1990.
22. Iannone MA, Wolberg G and Zimmerman TP, Chemotactic peptide induces cAMP elevation in human neutrophils by amplification of the adenylate cyclase response to endogenously produced adenosine. *J Biol Chem* **264:** 20177–20180, 1989.
23. Anderson R, Goolam Mahomed A, Theron AJ, Ramafi G and Feldman C, Effect of rolipram and dibutyryl cyclic AMP on resequestration of cysolic calcium in FMLP-activated human neutrophils. *Br J Pharmacol* **124:** 547–555, 1998.
24. Villagrasa V, Navarette C, Sanz C, Berto L, Perpina M, Cortijo J and Morcillo EJ, Inhibition of phosphodiesterase IV and intracellular calcium levels in human polymorphonuclear leukocytes. *Methods Find Exp Clin Pharmacol* **18:** 239–245, 1996.