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Occupancy of adenosine receptors raises cyclic AMP alone and in synergy with occupancy of chemoattractant receptors and inhibits membrane depolarization

Bruce N. CRONSTEIN,* Sara B. KRAMER, Elliot D. ROSENSTEIN, Helen M. KORCHAK, Gerald WEISSMANN and Rochelle HIRSCHHORN

Department of Medicine, Divisions of Rheumatology and Medical Genetics, New York University Medical Center, 550 First Avenue, New York, NY 10016, U.S.A.

We have recently demonstrated that adenosine, acting via adenosine A_2 receptors, inhibits generation of superoxide anions (O_2^-) by stimulated neutrophils. To determine the mechanism(s) by which adenosine inhibits O_2^- generation stimulated by the chemoattractant *N*-formylmethionylleucylphenylalanine (FMLP), we examined cyclic AMP (cAMP) concentrations, stimulated membrane depolarization and Ca^{2+} movements. Neither adenosine nor 5'-*N*-ethylcarboxamidoadenosine (NECA), the most potent agonist at adenosine A_2 receptors, increases neutrophil cAMP content. However in the presence of the non-methylxanthine phosphodiesterase inhibitor, Ro-20-1724, both adenosine and NECA elicit a reversible increase in intracellular cAMP concentration. The chemoattractant FMLP also elicits an increment in the neutrophil cAMP content. NECA, in the presence of Ro-20-1724, synergistically enhances the increment in cAMP following stimulation by FMLP. However Ro-20-1724 does not potentiate the inhibition of O_2^- generation by NECA. Unlike other agents which increase neutrophil cAMP concentrations, NECA, even in the presence of a phosphodiesterase inhibitor, only trivially inhibits degranulation. We also found that adenosine markedly inhibits stimulated membrane depolarization but does not affect the stimulated increment in free ionized intracellular calcium. Moreover, inhibition by adenosine of O_2^- generation does not vary with the concentration of extracellular calcium. These results fulfil the last criterion for the demonstration of an A_2 receptor on human neutrophils, and indicate that adenosine occupies an A_2 receptor on neutrophils to raise intracellular cAMP in synergy with occupancy of the FMLP receptor. The results reported here also indicate that cAMP is not the second messenger for inhibition of O_2^- generation by adenosine and its analogues.

INTRODUCTION

We have recently demonstrated that adenosine and some of its analogues specifically inhibit superoxide anion (O_2^-) generation while only minimally inhibiting degranulation or aggregation by stimulated neutrophils (Cronstein *et al.*, 1983). We subsequently demonstrated that adenosine occupies a specific receptor on the surface of the neutrophil (Cronstein *et al.*, 1985). Moreover we and others have characterized neutrophil adenosine receptors as A_2 receptors by the following criteria: the IC_{50} of adenosine for inhibition of O_2^- generation, the order of potency of adenosine analogues, the ability of theophylline or other methylxanthines to antagonize the effects of adenosine and the characteristic binding of a labelled adenosine analogue to whole neutrophils (Cronstein *et al.*, 1985; Iannone *et al.*, 1985; Roberts *et al.*, 1985). In general, occupancy of adenosine A_2 receptors stimulates accumulation of intracellular cyclic AMP (cAMP), which acts as a second messenger to alter cellular function (van Calker *et al.*, 1979; Londos *et al.*, 1980; Daly *et al.*, 1981). However, we have not shown such effects of adenosine in our earlier studies and therefore the last criterion for demonstration of the presence of an A_2 receptor remained unfulfilled.

We report here the effect of adenosine receptor occupancy on three mechanisms of signal transduction in the neutrophil; cAMP, membrane depolarization and Ca^{2+} fluxes. In the presence of a non-methylxanthine phosphodiesterase inhibitor, both adenosine and 5'-*N*-ethylcarboxamidoadenosine (NECA), the most potent A_2 receptor agonist, elicit an increment in neutrophil cAMP content which rapidly returns to basal levels. Moreover occupation of adenosine receptors raises cAMP in synergy with occupancy of *N*-formylmethionyl-leucylphenylalanine (FMLP) receptors. But inhibition of phosphodiesterase does not potentiate the effect of NECA on O_2^- generation. Adenosine diminishes FMLP-stimulated membrane depolarization in the neutrophil, as reported by decrements in DiOC6 fluorescence. Inhibition by adenosine of O_2^- generation does not vary with extracellular calcium concentration ($[Ca^{2+}]_o$). Nor does adenosine interfere with the rise of free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) resulting from stimulation of the neutrophil by FMLP, as reported by quin2 fluorescence.

METHODS

Materials

Cytochalasin B was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Cytochrome *c* (type III)

Abbreviations used: cAMP, cyclic AMP; FMLP, *N*-formylmethionylleucylphenylalanine; NECA, 5'-*N*-ethylcarboxamidoadenosine; O_2^-, superoxide anion; $[Ca^{2+}]_i$, intracellular calcium; $[Ca^{2+}]_o$, extracellular calcium; PMN, polymorphonuclear leucocytes.

* To whom correspondence should be addressed.

adenosine and adenosine deaminase (type VIII) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Superoxide dismutase was obtained from Miles Laboratories (Elkhart, IN, U.S.A.). Quin2 was purchased from Lancaster Synthesis (Morecambe, Lancs., U.K.).

FMLP was purchased from Vega Biochemicals (Tucson, AZ, U.S.A.). NECA was purchased from Research Biochemials Inc. (Wayland, MA, U.S.A.). DiOC6 (3,3'-dihexyloxacarbocyanine iodide) was purchased from Eastman Kodak (Rochester, NY, U.S.A.). Kits used for determination of cAMP concentration were obtained from New England Nuclear (Boston, MA, U.S.A.). Ro-20-1724 was a generous gift of Dr. P. Sorter (Hoffman–La Roche, Inc., Nutley, NJ, U.S.A.).

Preparation of cell suspensions

Heparinized blood was obtained from normal volunteers. Purified preparations of neutrophils were isolated by means of Hypaque–Ficoll gradients followed by dextran sedimentation and hypotonic lysis of erythrocytes (Boyum, 1968). This procedure allowed studies of cell suspensions containing $98 \pm 2\%$ neutrophils with few contaminating erythrocytes or platelets. The cells were suspended in a buffered salt solution consisting of Na^+ (150 mM), K^+ (5 mM), Ca^{2+} (1.3 mM), Mg^{2+} (1.2 mM), Cl^- (155 mM) and Hepes (10 mM), pH 7.45. In some experiments the neutrophils were suspended in a medium consisting of Na^+ (150 mM), Cl^- (155 mM), Mg^{2+} (1.2 mM), K^+ (5 mM) and Hepes (10 mM), pH 7.45, without Ca^{2+} or with Ca^{2+} (2.6 mM).

O_2^- generation

O_2^- generation was monitored by determination of superoxide dismutase-inhibitable reduction of cytochrome *c* in the presence of cytochalasin B. Duplicate reaction mixtures containing 2×10^6 neutrophils, 75 nmol of cytochrome *c* and adenosine or NECA at designated concentrations in a final volume of 1 ml. The mixtures were incubated in the presence of cytochalasin B (5 μg/ml) at 37 °C for 5 min before exposure to stimuli (FMLP, 0.1 μM). After stimulation (5 min) cells were spun down at 4 °C and 1000 *g* in a Sorvall RC-3 centrifuge (Dupont, Newtown, CT, U.S.A.) and the supernatants were collected. Absorption at 550 nm was determined in a spectrophotometer (model 250) from Gilford Instruments Laboratories (Oberlin, OH, U.S.A.). The molar quantity of O_2^- generated was calculated from the difference in absorption at 550 nm between ferricyanide-oxidized and potassium thiosulphite-reduced cytochrome *c* (Goldstein *et al.*, 1975). The data were calculated as nmol of cytochrome *c* reduced/5 min per 10^6 neutrophils and expressed as nmol/10^6 cells.

Membrane depolarization

The carbocyanine-dye fluorescence response was measured by a modification of the method of Seligmann & Gallin (1980). Neutrophils (1.25×10^6/ml) were incubated with DiOC6 for 5 min at 37 °C in a 1 cm pathlength cuvette with a final volume of 2 ml. Fluorescence measurements were made in a Perkin–Elmer 650–10S spectrofluorimeter (emission wavelength, 510 nm, excitation wavelength, 470 nm). The stimulus was injected through an injection port on the spectrofluorimeter.

cAMP generation

Neutrophils (10^8/ml final concn.) were incubated in the presence and absence of FMLP (0.1 μM) and buffer, adenosine or NECA, at 37 °C. In some experiments Ro-20-1724 (50 nM) was added to cell suspensions for the duration of the incubations. The reaction was stopped at appropriate time intervals by the addition of iced 10% HCl/90% ethanol. Reaction mixtures were then subjected to rapid freeze-thawing using a solid CO_2/acetone bath and centrifuged at 3000 *g* in a Beckman model B microfuge for 5 min (Simchowitz *et al.*, 1980). Portions of the supernatants were removed, evaporated under a nitrogen atmosphere and the resultant residues were resuspended in 0.05 M-sodium acetate buffer (pH 6.2) and assayed at two different dilutions for cAMP content by radioimmunoassay as described by the manufacturers of the kit. This procedure permitted recovery of 80–85% of added cAMP, as determined by recovery of [^{3}H]cAMP.

Calculation of cAMP data

Standard values were fitted to a curve by means of the ALLFIT program (Dr. Carl Johnson, University of Cincinnati College of Medicine, Cincinnati, OH, U.S.A.) on an Apple IIe desktop computer, and unknown values were calculated by reference to the standard curve (DeLean *et al.*, 1978). The data were calculated as pmol of cAMP/10^7 neutrophils.

Measurement of degranulation

For determination of granule release, neutrophils (2×10^6/ml) are incubated with buffer or NECA in the presence of cytochalasin B (5 μg/ml) for 5 min at 37 °C. The cells are then stimulated with FMLP (0.1 μM) and incubated for 5 min at 37 °C. The cell suspensions are then centrifuged at 1000 *g* for 5 min at 4 °C and portions of the supernatants are then taken for standard determinations of β-glucuronidase activity (azurophil granule) and vitamin B_{12}-binding protein (specific granule). These methods have been described in detail elsewhere (Lau *et al.*, 1965; Brittinger *et al.*, 1968). Total activity and residual cell-associated activity are measured in selected reaction mixtures after cells are lysed by addition of Triton X-100 (0.2%). Results are calculated as the percentage of total cellular activity released into the supernatants.

Determination of movements in cytoplasmic calcium as measured by quin2 fluorescence

Movements in cytoplasmic Ca^{2+} were measured by using the method of Tsien *et al.* (1982). Neutrophils (100×10^6/ml) were incubated with quin2 acetoxymethyl ester (50 μM) at 37 °C for 30 min, diluted with Hepes buffer to 10×10^6/ml, incubated for 20 min at 37 °C, washed and resuspended in buffer. Cells (5×10^6/ml) were then placed in a cuvette and fluorescence was continuously monitored using a Perkin–Elmer fluorimeter with excitation at 339 nm and emission at 492 nm. Changes in fluorescence of the quin2-loaded neutrophils were monitored before and after stimulation with FMLP (0.1 μM) in the presence and absence of adenosine. Free cytosolic ionized calcium concentrations were estimated from changes in total calcium present and background

fluorescence in the presence of Triton X-100 and EGTA (pH 8.5) respectively. Calcium concentrations were estimated by using the following formula (Tsien *et al.*, 1982):

$$[Ca^{2+}] = 115(F - F_{min.})/(F_{max.} - F)$$

Statistical analysis of data

Unless stated otherwise all differences between means were analysed by use of the Student's *t* test.

Calculation of adenosine concentration giving 50% maximal inhibition (IC_{50}) of O_2^- generation

Inhibition data were fit to a curve by the ALLFIT program on an Apple IIe desktop computer and the IC_{50} was calculated by means of the computer program (DeLean *et al.*, 1978).

Statistical analysis of variance between inhibition curves

F values were derived by use of the following formula:

$$[(SS_a - SS_b)/(df_a - df_b)]/(SS_b/df_b)$$

in which SS_a is the sum of the squares of the differences when the curve is forced to conform to external constraints, SS_b is the sum of the squares of the differences of the unconstrained curve and df is the degrees of freedom (Cronstein *et al.*, 1985; DeLean *et al.*, 1978).

RESULTS

Adenosine alone does not increase neutrophil cAMP content

Since previous studies have demonstrated that occupancy of adenosine A_2 receptors leads to accumulation of intracellular cAMP in many different cell types (van Calker *et al.*, 1979; Londos *et al.*, 1980; Daly *et al.*, 1981), we studied the effects of adenosine and NECA on neutrophil cAMP content. Adenosine (100 μM) did not significantly alter the concentration of cAMP in unstimulated cells. After stimulation with FMLP (0.1 μM) neutrophils promptly increased their intracellular concentrations of cAMP with a maximum at 30 s after stimulation (25 ± 4 pmol/10^7 PMN). Adenosine (100 μM) neither increased nor decreased the maximum cAMP concentration achieved in FMLP-stimulated cells (21 ± 4 pmol/10^7 PMN, Fig. 1; incubation of neutrophils for 4 min did not alter the intracellular cAMP concentration of approx. 7 pmol/10^7 cells).

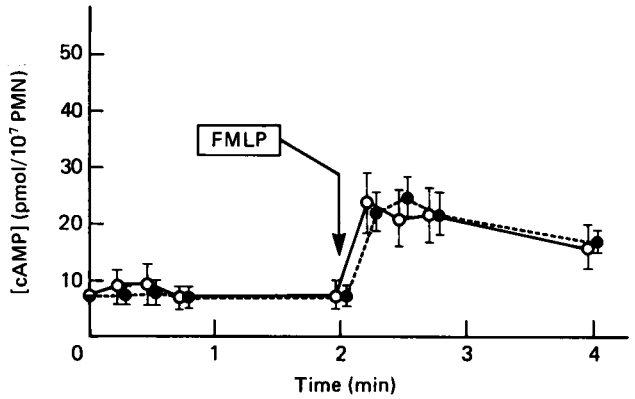

Fig. 1. Effect of adenosine on cAMP in resting and FMLP-stimulated neutrophils

Adenosine (●; 100 μM) or buffer (○) was added to suspensions of neutrophils (10×10^6/ml) at time 0 and FMLP (0.1 μM) was added after 2 min. The reactions were terminated by addition of ethanol/10% HCl at the indicated time points. cAMP concentrations in the lysates were determined by radioimmunoassay, as described in the text. These results represent the means ± S.E. of six to ten separate determinations using the cells of different donors.

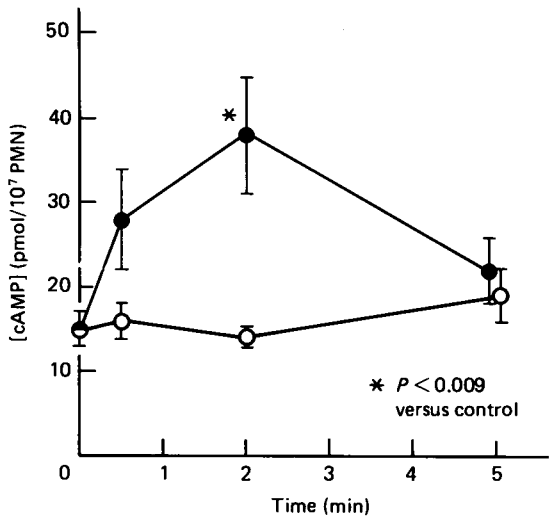

Fig. 2. Effect of adenosine on cAMP in resting neutrophils in the presence of RO-20-1724

Neutrophils (10×10^6) were incubated for the indicated periods of time in the presence of Ro-20-1724 (50 nM) and buffer (○) or adenosine (●; 100 μM). The reactions were terminated by addition of ethanol/10% HCl and the cAMP concentrations in the lysates were determined by radioimmunoassay. These results represent the means ± S.E. of seven separate determinations using the cells of different donors.

Adenosine increases neutrophil cAMP in the presence of a phosphodiesterase inhibitor

We next studied the effect of adenosine on neutrophil cAMP content in the presence of Ro-20-1724, a non-methylxanthine phosphodiesterase inhibitor. In the presence of the phosphodiesterase inhibitor the intracellular concentration of cAMP in resting cells doubled (approx. 15 pmol/10^7 PMN) but remained constant over the period of the incubation (Fig. 2). Addition of adenosine stimulated a 2–3-fold increase in intracellular cAMP concentration with a maximum at 2 min (38 ± 7 versus 14 ± 1 pmol of cAMP/10^7 PMN, Fig. 2) and a return to baseline by 5 min (22 ± 4 versus 19 ± 3 pmol of cAMP/10^7 PMN, Fig. 2).

NECA increases neutrophil cAMP

Metabolism of adenosine by neutrophils diminishes the concentration of adenosine remaining in the medium, which could diminish the effect of adenosine on cAMP concentrations in neutrophils. In contrast NECA, the most potent adenosine A_2 receptor agonist with respect to its ability to inhibit O_2^- generation, is only poorly metabolized. We therefore studied the effect of NECA

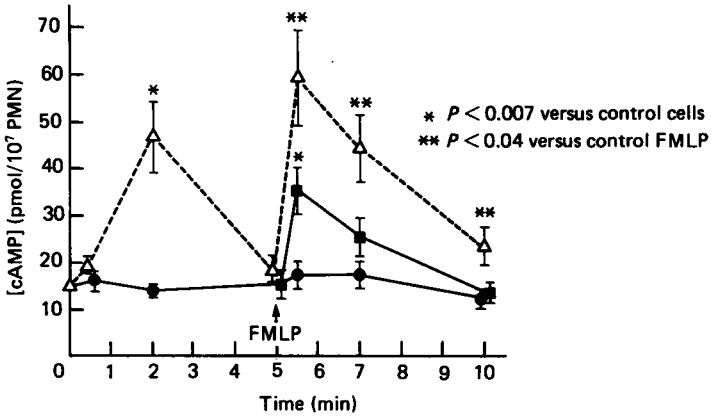

Fig. 3. Effect of NECA on cAMP in resting and FMLP-stimulated neutrophils in the presence of Ro-20-1724

Neutrophils (10×10^6) were incubated for the indicated periods of time in the presence of Ro-20-1724 (50 nM) and buffer (●), NECA (△; 50 nM), FMLP (■; 100 nM) or their combination. The reactions were terminated by addition of ethanol/10% HCl and the cAMP concentrations in the lysates were determined by radioimmunoassay. These results represent the means ± S.E.M. of five to 11 separate determinations, using the cells of different donors.

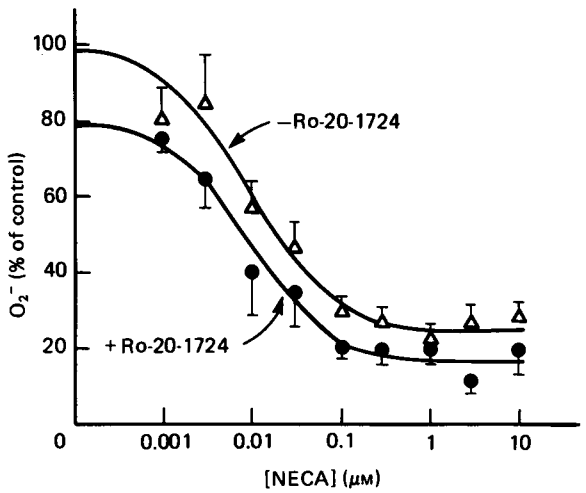

Fig. 4. Inhibition by NECA of FMLP-stimulated O_2^- generation in the absence or presence of Ro-20-1724

Neutrophils [$(1–2) \times 10^6$/ml] were incubated with buffer containing cytochalasin B (5 μg/ml) and NECA at varying concentrations in the presence (●) or absence (△) of Ro-20-1724 (50 nM) for 5 min before stimulation with FMLP (0.1 μM). The results are expressed as percentage of control O_2^- generation and each point represents the mean ± S.E.M. of four separate experiments using the cells of different donors. Control O_2^- generation in these experiments was 41 ± 8 nmol/5 min per 10^6 PMN.

on cAMP concentrations in neutrophils. Similarly with adenosine, NECA alone did not stimulate a detectable increase in cAMP content (16 ± 3 versus 16 ± 2 pmol/10^7 PMN after incubation for 2 min with 0.5 μM-NECA or buffer, respectively). In the presence of the phosphodiesterase inhibitor Ro-20-1724, NECA (0.10 and 0.50 μM) stimulated a 3-fold rise in cAMP (maximum cAMP 43 ± 8 and 47 ± 8 pmol/10^7 PMN, respectively, $n = 5$, $P < 0.003$ versus control). NECA, like adenosine, stimulated maximum cAMP accumulation after 2 min of incubation and, after stimulation by NECA, the cAMP concentration also returned to baseline by 5 min. These results are consistent with the presence of adenosine A_2 receptors on neutrophils and are similar to those previously reported by Iannone *et al.* (1985).

NECA and FMLP increase neutrophil cAMP synergistically

Stimulation of the neutrophil by FMLP elicits increased cAMP concentrations in the neutrophil. We studied the effect of NECA (0.5 μM) on the FMLP-induced increment in cAMP concentration to determine whether occupancy of adenosine receptors further affects the generation of this intracellular signal. The chemoattractant FMLP alone (in the presence of Ro-20-1724) elicited a 2-fold increment in cAMP concentration which was maximal by 30 s after stimulation but returned to basal concentrations by 5 min after stimulation (Fig. 3). While NECA alone (in the presence of Ro-20-1724) rapidly increased the cAMP concentration, cAMP returned to baseline after 5 min (Fig. 3). To test for synergy we added FMLP to cells previously incubated with NECA, but at a time when the cAMP concentration had fallen to baseline (5 min). The increase in cAMP induced by stimulation with FMLP was markedly increased in the presence of NECA (4-fold, Fig. 3). Additionally, NECA induced the stimulated neutrophil to maintain increased cAMP concentrations for as long as 5 min after addition of FMLP, whereas in the absence of NECA, there was no detectable increase after 5 min (Fig. 3). These findings are in accord with those of Elliott *et al.* (1986) who reported that adenosine enhances the FMLP-induced increment in cAMP in human monocytes.

Adenosine must be continually present to inhibit O_2^- generation

We have previously demonstrated that enzymic removal of adenosine by adenosine deaminase (ADA, 0.25 units/ml), as late as 1 min before stimulation with FMLP, completely reverses the effects of adenosine on neutrophils (Cronstein *et al.*, 1983). We next determined whether Ro-20-1724, by permitting greater or more prolonged adenosine-induced cAMP elevations in the neutrophil, could prolong the inhibitory effect of adenosine on O_2^- generation, despite removal of adenosine by ADA. Both Ro-20-1724 (50 nM) and adenosine (100 nM) inhibited O_2^- generation alone, and there was an additive increase in inhibition in the presence of these two agents together (33 ± 5, 36 ± 5 and 57 ± 10% inhibition respectively, $P < 0.008$ versus control, $n = 4$). Adenosine deaminase, by removing endogenously generated extracellular adenosine, enhances O_2^- generation both in the presence and absence of Ro-20-1724 (136 ± 17) and 154 ± 11% of control respectively, $n = 4$). Even in the presence of Ro-20-1724, adenosine deaminase reverses the effect of exogenous (0.1 μM) adenosine on O_2^- generation (134 ± 14% of control, $n = 4$) at a time when cAMP concentrations are maximal. Therefore even in the presence of a phosphodiesterase inhibitor adenosine must be continuously present to inhibit neutrophil function. Moreover, our data suggest that the initial rise

Table 1. NECA minimally inhibits degranulation by FMLP-stimulated neutrophils in the presence and absence of Ro-20-1724

Neutrophils (2×10^6/ml) were incubated in the presence of cytochalasin B (5 μg/ml) and buffer or NECA at the indicated concentrations for 5 min at 37 °C before stimulation with FMLP (0.1 μM). The supernatants were collected and β-glucuronidase activity and vitamin B_{12}-binding protein concentration were measured as described in the Materials and methods section. Mean release of β-glucuronidase and vitamin B_{12}-binding protein was 22 ± 1 and 23 ± 2% respectively, of the total cellular content (as determined in detergent lysates). The results presented here represent the means ± S.E.M of four separate experiments using cells from four different donors: $^*P < 0.005$; $^{**}P < 0.01$; $^{***}P < 0.05$, versus control.

NECA concentration (μM)	Inhibition (%)			
	β-Glucuronidase		Vitamin B_{12}-binding protein	
	+Ro-20-1724	−Ro-20-1724	+Ro-20-1724	−Ro-20-1724
10.00	18±5*	22±3**	3±4	9±3
1.00	21±2*	19±2**	3±2	6±1
0.50	25±4**	19±2**	6±4	6±2
0.10	14±7	21±5***	5±2	7±6
0.05	20±3**	16±3***	13±4	3±5
0	10±5	0	4±4	0

of cAMP elicited by adenosine does not, by itself, account for inhibition of O_2^- generation by stimulated neutrophils.

Inhibition of phosphodiesterase does not potentiate the effect of NECA

If cAMP is the second messenger for adenosine receptor-mediated inhibition of O_2^- generation, then Ro-20-1724 should increase the inhibitory potency of NECA (or other adenosine receptor agonists) by virtue of its ability to enhance the increment in cAMP induced by these agents. We therefore determined whether Ro-20-1724 was synergistic with NECA with respect to inhibition of O_2^- generation by stimulated neutrophils. There was 27 ± 7% inhibition of FMLP-stimulated O_2^- generation by Ro-20-1724 alone. Inhibition of O_2^- generation by NECA was dose-dependent in both the presence and absence of Ro-20-1724 (Fig. 4). Although inhibition of stimulated O_2^- generation by Ro-20-1724 was additive to that of NECA, Ro-20-1724 did not potentiate the effect of NECA. Thus, the IC_{50} of NECA for O_2^- generation in these experiments was not significantly different in the absence or presence of Ro-20-1724 (8 ± 2 versus 12 ± 5 nM, respectively). Therefore, while Ro-20-1724 enhances the effect of NECA on intracellular cAMP concentrations, Ro-20-1724 does not potentiate the effect of NECA on O_2^- generation. This data does not support the hypothesis that cAMP acts as the second messenger for inhibition of O_2^- generation by adenosine and its analogues.

Inhibition of phosphodiesterase does not increase the effect of NECA on degranulation

Agents that stimulate or enhance an increment in neutrophil cAMP content inhibit degranulation as well as O_2^- generation, e.g. prostaglandins (Zurier *et al.*, 1973; Smolen *et al.*, 1980; Lad *et al.*, 1985). However we have previously found that adenosine does not significantly inhibit release of lysozyme, an enzyme found in both specific and azurophil granules, and only moderately inhibits release of β-glucuronidase, a marker of azurophil granule release. We next determined whether NECA

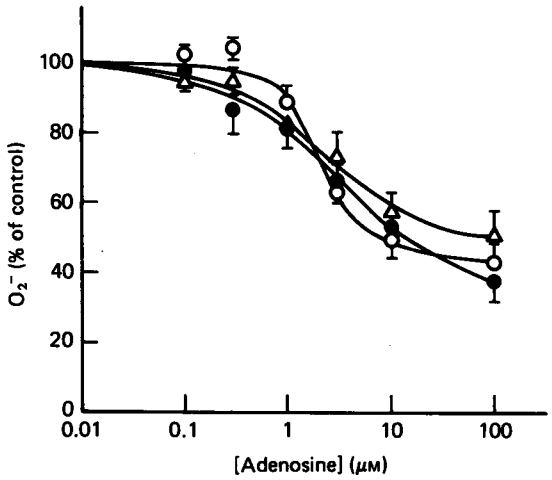

Fig. 5. Inhibition by adenosine of FMLP-stimulated O_2^- generation in the absence or presence of $[Ca^{2+}]_o$

Neutrophils were incubated with buffer containing 0 (△), 1.3 mM (●) or 2.6 mM (○) Ca^{2+}, cytochalasin B (5 μg/ml) and adenosine at varying concentrations for 5 min before stimulation with FMLP (0.1 μM). The results are expressed as percentage of control O_2^- generation and each point represents the mean ± S.E.M. of five separate experiments using the cells of different donors.

inhibited FMLP-stimulated release of β-glucuronidase and vitamin B_{12}-binding protein, the latter being a more specific marker of specific granule release than lysozyme, and whether Ro-20-1724, via its effects on cAMP metabolism, increased inhibition by NECA. We found that NECA, similarly with adenosine, modestly inhibited release of β-glucuronidase and did not affect vitamin B_{12}-binding protein release (Table 1). Ro-20-1724 neither inhibited degranulation nor potentiated the effect of NECA on degranulation stimulated by FMLP. These results are in accord with our previous findings (Cronstein *et al.*, 1983). Grinstein & Furuya (1986) have reported similar results and have demonstrated that adenosine

Table 2. Adenosine inhibits FMLP-stimulated membrane depolarization

Neutrophils (2.5×10^6) were incubated in the presence of cytochalasin B (5 μg/ml), DiOC6 (0.25 μM) and either adenosine, at the indicated concentrations, or buffer for 5 min at 37 °C. The cells were then stimulated with FMLP (0.1 μM) and the fluorescence was monitored continuously. The maximal decrement in DiOC6 fluorescence was determined for control and adenosine-treated cells and the data are expressed as the percentage inhibition of the maximal decrement of control cells. These results represent the means ± S.E.M. of four separate experiments using the neutrophils from different donors. *P* values were determined by using the Student's *t* test.

Adenosine concentration (μM)	Inhibition (%)	*P* (versus control)
100.0	60 ± 7	< 0.003
10.0	56 ± 5	< 0.002
1.0	48 ± 4	< 0.002
0.1	34 ± 10	< 0.02

does not alter the kinetics of degranulation as well. Therefore adenosine and its analogues are unlike other agents which elicit an increment in neutrophil cAMP since occupancy of adenosine receptors does not inhibit degranulation (Zurier *et al.*, 1973; Smolen *et al.*, 1980; Lad *et al.*, 1985). Moreover these results do not support a role for increments in cAMP as second messengers for modulation of neutrophil degranulation.

Inhibition of O_2^- by adenosine does not depend on the presence of extracellular Ca^{2+}

Pasini *et al.* (1985) have suggested that adenosine blocks the stimulated entry of $[Ca^{2+}]_o$ into the cell and thereby inhibits O_2^- generation by stimulated neutrophils. We therefore determined whether adenosine inhibits O_2^- generation stimulated by interaction of a ligand (FMLP) with its receptor on neutrophils in the absence of $[Ca^{2+}]_o$ or whether increasing $[Ca^{2+}]_o$ reverses inhibition by adenosine of FMLP-stimulated O_2^- generation. Neutrophils generated less O_2^- in the absence of $[Ca^{2+}]_o$ than they did in the presence of 1.3 or 2.6 mM $[Ca^{2+}]_o$ (12 ± 3 versus 22 ± 2 or 22 ± 3 nmol of O_2^-/10^6 PMN respectively, $P < 0.007$). Inhibition of O_2^- generation by adenosine did not vary with $[Ca^{2+}]_o$ (Fig. 5). We conclude that adenosine does not inhibit generation of O_2^- by preventing uptake of $[Ca^{2+}]_o$ by FMLP-stimulated neutrophils. Additionally an increase of $[Ca^{2+}]_o$ does not overcome inhibition by adenosine of O_2^- generation.

Neither adenosine nor NECA prevents the stimulated rise in $[Ca^{2+}]_i$

Stimulated rises in free cytosolic Ca^{2+} ($[Ca^{2+}]_i$) are thought to act as second messengers in neutrophils (cf. Korchak, 1985). Therefore we determined the effect of adenosine and NECA on stimulated increases in $[Ca^{2+}]_i$. In resting cells $[Ca^{2+}]_i$ did not differ in the presence or absence of adenosine (100 μM, 84 ± 11 versus 91 ± 13 nM respectively, $n = 5$). $[Ca^{2+}]_i$ increases rapidly, following FMLP stimulation (as reported by changes in quin2 fluorescence). Similar increments in $[Ca^{2+}]_i$, followed stimulation of neutrophils by FMLP (0.1 μM), in the absence and presence of adenosine (100 μM, 169 ± 29 and 156 ± 29 nM respectively, $n = 5$). Similarly, NECA (10 μM), the most potent A_2 receptor agonist, did not affect either basal $[Ca^{2+}]_i$ or FMLP-stimulated ($10^{-9} - 10^{-7}$ M) increments in $[Ca^{2+}]_i$ (results not shown).

Adenosine inhibits stimulated membrane depolarization

Changes in neutrophil membrane potential occur rapidly after stimulation. We next determined whether occupancy of adenosine receptors alters membrane depolarization as reported by decrements in DiOC6 fluorescence. Adenosine does not affect resting membrane potential, as reported by DiOC6 fluorescence (results not shown). In contrast, adenosine at concentrations ranging from 0.1 to 100 μM, inhibits membrane depolarization stimulated by FMLP (Table 2).

DISCUSSION

In the studies reported here we examined two mechanisms by which occupancy of adenosine receptors could inhibit O_2^- generation by stimulated neutrophils while interfering minimally, if at all, with degranulation: effects on $[Ca^{2+}]_i$ and cAMP concentrations. We did not find that occupancy of the adenosine receptor alters stimulated Ca^{2+} movements in the neutrophil. Nor did we find evidence to suggest that the second messenger for adenosine inhibition of O_2^- generation is cAMP.

Previous studies have suggested that cAMP acts as a second messenger for adenosine A_2 receptor modulation of cellular function. We found that both adenosine and NECA stimulate the accumulation of cAMP in the neutrophil, but only in the presence of the phosphodiesterase inhibitor Ro-20-1724. The demonstration that these nucleosides stimulate cAMP accumulation fulfils the final criterion that the adenosine receptor on neutrophils is of the A_2 type (van Calker *et al.*, 1979; Londos *et al.*, 1980; Cronstein *et al.*, 1985; Iannone *et al.*, 1985; Roberts *et al.*, 1985). Surprisingly, the increased cAMP concentrations found when Ro-20-1724 and NECA are added to cells simultaneously are not paralleled by greater inhibition of O_2^- generation. The results of the experiments reported here suggest that the increment in cAMP content elicited by adenosine and NECA is not sufficient to inhibit O_2^- generation by stimulated neutrophils. Moreover other agents that elicit a similar increment in cAMP differ from adenosine and NECA in that they are potent inhibitors of degranulation. In contrast NECA and FMLP synergistically increase neutrophil cAMP content in the presence of Ro-20-1724 but there is no corresponding inhibition of vitamin B_{12}-binding protein release and only minimal inhibition of β-glucuronidase release. Therefore our results do not support a role for cAMP as the intracellular messenger for adenosine inhibition of O_2^- generation. The effects of adenosine and its analogues on other steps in the activation pathway of the neutrophil remain to be explored. These include phospholipid turnover, generation of diacylglycerol or other lipid intermediates and protein phosphorylation, myristoylation or tyrosinylation.

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