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Effects of adenosine on polymorphonuclear leucocyte function, cyclic 3′ : 5′-adenosine monophosphate, and intracellular calcium

[1]Christopher P. Nielson & Robert E. Vestal

Clinical Pharmacology and Gerontology Research Unit, Veterans Administration Medical Center, Boise, Idaho and Division of Gerontology and Geriatric Medicine, Department of Medicine, University of Washington, Seattle, Washington, U.S.A.

1 Inhibition of human polymorphonuclear leucocyte (PMN) function by adenosine was studied with respect to effects of adenosine on intracellular cyclic AMP and calcium during the PMN respiratory burst.

2 The adenosine analogue 5′-N-ethylcarboxamide-adenosine (NECA) and L-N^6-phenyl-isopropyl-adenosine (L-PIA) inhibited PMN oxygen metabolite generation with relative potencies (NECA > adenosine > L-PIA) characteristic of an A_2 receptor.

3 The respiratory burst was inhibited by adenosine when PMN were activated by calcium ionophore or chemotactic peptide but not when cells where activated by oleoyl-acetyl-glycerol (OAG).

4 Adenosine increased intracellular cyclic AMP during the PMN respiratory burst regardless of whether cells were stimulated by ionophore, chemotactic peptide or OAG.

5 To determine whether the differences in cell inhibition by adenosine were related to differences in intracellular calcium mobilization by each activating agent, calcium was evaluated with the fluorescent probe, indo-1. Adenosine suppressed the increase in intracellular calcium following PMN activation by calcium ionophore or chemotactic peptide. In contrast, calcium did not increase in PMN activated by OAG and adenosine did not affect intracellular calcium changes following this stimulus.

6 These results demonstrate that physiological concentrations of adenosine inhibit the PMN respiratory burst in association with an increase in intracellular cyclic AMP and reduction of intracellular calcium.

Introduction

Adenosine is an important regulator substance with potent effects on cardiovascular, pulmonary, endocrine and neuronal tissues (Daly, 1982; Berne *et al.*, 1982). Although adenosine has been shown to inhibit polymorphonuclear leucocyte (PMN) function (Cronstein *et al.*, 1983; 1987), the mechanisms of PMN regulation by adenosine are not well understood. The PMN generates reactive oxygen metabolites which are critical in host defence but also highly toxic to human tissues (Klebanoff, 1980; Babior, 1984). Consequently, regulation of PMN function by adenosine may be of physiological importance.

Both A_1 and A_2 adenosine receptors may be present on human PMN (Cronstein *et al.*, 1985; Marone *et al.*, 1985). The A_2 receptor subtype mediates adenylate cyclase stimulation while the A_1 subtype causes adenylate cyclase inhibition (Londos *et al.*, 1980). Because adenosine 3′ : 5′-cyclic monophosphate (cyclic AMP) may inhibit PMN function (Ignarro & Columbo, 1973; Busse & Sosman, 1984), effects of adenosine mediated by cyclic AMP may be relevant to the mechanism of PMN inhibition by adenosine.

Two partially distinct biochemical pathways of stimulus-response coupling have been identified in the PMN (McPhail & Snyderman, 1983). One pathway requires intracellular calcium mobilization while the other is associated with diacylglycerol activation of protein kinase C and is relatively calcium independent (Takenawa *et al.*, 1983; Nishizuka, 1984). Because only the calcium-dependent pathways of stimulus-response coupling may be inhibited by

[1] Author for correspondence at Research Service (151) Veterans Administration Medical Center, 500 West Fort St., Boise, Idaho 83702, U.S.A.

elevation of cyclic AMP (Mack *et al.*, 1986; Nielson, 1987), effects of adenosine on the respiratory burst were evaluated with respect to the mechanisms of cell activation.

Methods

Isolation of PMN

Blood samples from heathy volunteers, age 20 to 45 years, were drawn between 8 h 00 min and 9 h 00 min. All subjects were asked to abstain from methylxanthine-containing foods for one day before the study. No subject was using any medication. PMN were isolated from venous blood anticoagulated with 10 units ml^{-1} heparin. To sediment the red bloods cells, 30 ml of blood was added to 30 ml of 3% dextran containing 0.1% bovine serum albumin (BSA) and 1 mg ml^{-1} ethylenediaminetetraacetic acid. After 20 min the leucocyte-rich plasma was layered onto 3 ml Ficoll-Hypaque (6.4 g% Ficoll, 9.72 g% Diatriazoate, 0.1% BSA, SpG 1.077) (Boyum, 1968). PMN were isolated by centrifugation (200 *g*, 20 min) and hypotonic (10 s, distilled water) lysis of erythrocytes. PMN were stored in plasma from the same donor at 4°C until utilized (30 min or less). Immediately before each experiment PMN were removed from plasma by centrifugation at 200 *g* for 10 min, washed and resuspended in phosphate buffer. The final preparation was over 95% PMN and of these 95% were viable. Adenosine or adenosine analogues were introduced immediately before cell activation with calcium ionophore, chemotactic peptide or diacylglycerol.

Luminescence detection of oxygen metabolities

After cell suspension in phosphate buffer, PMN were stimulated by addition of N-formyl-methionyl-leucine-phenylalanine (FMLP, 1 μM), ionomycin (0.2 μM), A23187 (0.2 μM) or oleoyl-acetyl-glycerol (OAG, 1 μM). Luminescence response was measured using either a Beckman LS-7500 scintillation counter set in the out-of-coincidence mode or a Packard Instrument Model 6500 Picolight Luminometer. The temporal characteristics of PMN activation were determined with 10^4 PMN ml^{-1} and 100 μM lucigenin. This low cell concentration minimized effects of mediators generated during PMN activation and provided the most reproducible results. Concentration-effects of adenosine and adenosine analogue were evaluated in a second set of experiments using 5×10^4 PMN ml^{-1} and 1 μM lucigenin. Lucigenin (1 μM or 100 μM) did not alter cell viability as assessed by trypan blue exclusion. Lucigenin-dependent luminescence was completely inhibited by superoxide dismutase (100 u ml^{-1}) and therefore appeared to correlate with superoxide anion generation as previously demonstrated (Chari-Bitron *et al.*, 1983; Stevens & Hong, 1984). The stimulating agents and adenosine did not alter lucigenin-dependent luminescence in studies using xanthine-xanthine oxidase generation of superoxide anion (in the presence or absence of unactivated PMN).

Intracellular calcium measurement

The fluorescent probe indo-1/AM was used to evaluate intracellular calcium as described by Tsien and associates (Grynkiewicz *et al.*, 1985). PMN were incubated with 1 μM indo-1/AM in the subject's plasma for 60 min. Cells were then washed with buffer (without calcium), centrifuged at 200 *g* for 10 min and resuspended at a concentration of 1×10^4 PMN ml^{-1} in phosphate buffer with 1 mM calcium. Fluorescence using excitation at 355 nM was measured with a fluorescence spectrophotometer (Model LS-5, Perkin-Elmer, Oak Brook, Illinois). The ratio of 410 nM fluorescence (calcium-bound indo-l) to 485 nM fluorescence (calcium-free indo-1) was used as an index of intracellular calcium concentration. Measurements were performed after PMN incubation, after addition of adenosine and at 30 s intervals after cell activation. Calcium-dependent changes in indo-1 acid fluorescence in a cell free test system were not altered by adenosine, FMLP, ionomycin or OAG. Changes in intracellular calcium were evaluated as the change in fluorescence compared with measurements immediately before PMN stimulation. The initial fluorescence was therefore standardized to zero and subsequent positive ratios reflected an increase, while negative results indicated a decrease in calcium following activation. Evaluation of fluorescence in the presence of a high ionomycin concentration (100 μM) indicated that a 0.1 increase in indo-1 ratio was associated with a 67 nM increase in calcium (calcium concentration range of 100 nM–5 μM).

Cyclic AMP measurement

PMN (6×10^6 ml^{-1}) were suspended in buffer and activated by 1 μM FMLP, 0.2 μM A23187 or 1 μM OAG. Appropriate adenosine dilutions were added immediately before PMN activation. Aliquots of PMN (1×10^6) were removed for baseline and cyclic AMP measurement at appropriate times. Cyclic AMP was extracted by sonication of PMN in acidified ethanol (5 s at 35% maximum energy, Model 300, Fisher Sonic Dismembrator). The specimen was then centrifuged at 1000 *g* for 10 min and the supernatant decanted from the pellets of denatured protein. The pellet was resuspended in 50% ethanol,

centrifuged at 1000 *g* for 10 min, and the supernatant again decanted. The supernatants were dried under nitrogen and stored at −70°C. The specimens were resuspended in 0.05 M Tris, pH 7.5 with 4 mM EDTA and cyclic AMP was measured by a competitive protein binding assay originally described by Gilman (1970). Preliminary studies demonstrated a 90% decrease in detectable cyclic AMP following incubation of specimens with bovine heart phosphodiesterase in phosphate buffer at 37°C for 60 min. Effects of adenosine and PMN activators on cyclic AMP generation were studied in paired experiments using cells from the same donor isolated in a single procedure.

Data analysis

Luminescence was evaluated either as a function of time or was integrated over the interval 2–4 min after PMN activation. PMN activation was standardized either as a percentage of simultaneous PMN response using a sample not exposed to adenosine (adenosine dose-response analysis) or as a percentage of maximal luminescence achieved during 15 min following activation (temporal analysis of cell activation). Comparisons of drug effects were paired using PMN from the same blood sample, with activation and analysis performed simultaneously under each experimental condition. Experiments were usually repeated five times using specimens from different donors. Dose-response curves were evaluated with computer assisted parametric curve fitting to the logistic equation (DeLean *et al.*, 1978) for estimation of slope, drug concentration causing 50% maximal response (EC_{50}) and maximal response.

Reagents

Dulbecco's phosphate buffered saline was prepared with 1 mg ml^{-1} glucose, 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The calcium ionophores A23187 (0.2 μM) and ionomycin (0.2 μM), the chemotactic peptide N-formyl-methionyl-leucine-phenylalanine (FMLP) (1 μM), and the synthetic diacylglycerol 1-oleoyl-2-acetyl-glycerol (OAG) (1 μM) were used to induce the PMN respiratory burst. A23187, FMLP and OAG were dissolved in dimethylsulphoxide and diluted in distilled water. Lucigenin (10,10′-dimethyl-bis-9,9′-biacridinium nitrate), a luminescent probe for superoxide anion, was dissolved in buffer. All diluents were included in all samples of any study at equal concentrations. The diluents had no effects on PMN function.

Indo-1/AM was obtained from Molecular Probes, Inc., Junction City, Oregon, U.S.A. A23187 was obtained from Behring-Calibiochem, La Jolla, CA, U.S.A. 5′-N-ethylcarboxamide-adenosine (NECA) was obtained from Research Biochemicals Inc., Wayland, MA, U.S.A. Materials used in cyclic AMP assays were obtained from Amersham Corporation, Arlington Heights, IL, U.S.A. All other materials were purchased from Sigma Chemical Co, St Louis, MO, U.S.A.

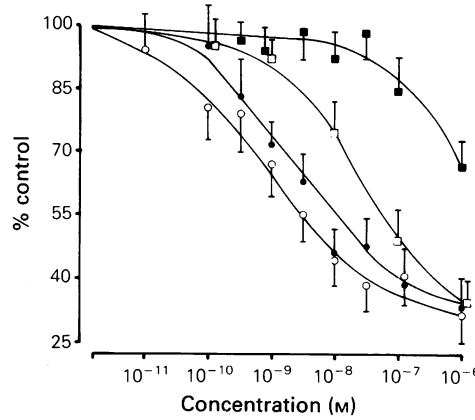

Figure 1 Effects of adenosine (●), 5′-N-ethylcarboxamide-adenosine (NECA, ○), L-N^6-phenylisopropyl-adenosine (L-PIA, □), and D-PIA (■) when introduced at the time of polymorphonuclear leucocyte (5×10^4 ml^{-1}) activation by 0.2 μM A23187. Lucigenin (1 μM)-dependent luminescence was integrated over 10 min following cell activation. The absolute magnitude of maximal luminescence was $103 \pm 23 \times 10^4$ c.p.m. Data represent means of specimens from 5 subjects; vertical lines show s.e.mean.

Results

Adenosine caused rapid, potent inhibition of PMN oxygen metabolite generation following stimulation with calcium ionophore (0.2 μM A23187) as measured using lucigenin (1 μM)-dependent luminescence (Figure 1). PMN stimulated by A23187 generated peak luminescence between 2 and 4 min following activation and the respiratory burst resolved over 20–30 min. Cells were inhibited by adenosine throughout the period of the respiratory burst. Adenosine inhibition was significant at concentrations as low as 1 nM (72% control, $P < 0.05$) and was maximal at concentrations over 100 nM.

To characterize the PMN receptor mediating adenosine effects, the adenosine analogues, 5′-N-ethylcarboxamide-adenosine (NECA) and N^6-phenyl-isopropyl-adenosine (PIA), were studied (Figure 1). The adenosine receptor which inhibits adenylate cyclase (A_1 receptor) is stimulated by adenosine analogues with relative affinities of L-

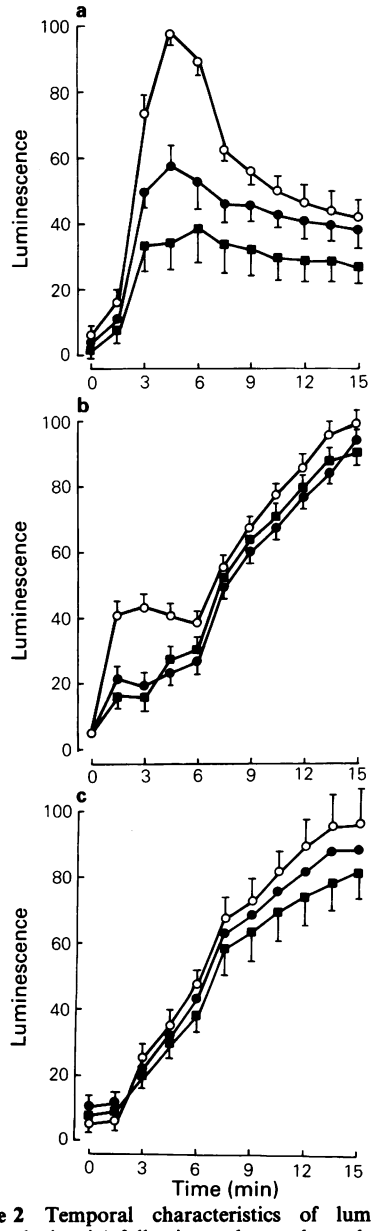

Figure 2 Temporal characteristics of luminescence (100 μM lucigenin) following polymorphonuclear leucocyte (PMN) (1×10^4 PMN per ml) activation by (a) 0.2 μM ionomycin, (b) N-formyl-methionyl-leucyl-phenylalanine 1 μM (FMLP), or (c) 1 μM oleoyl-acetyl-glycerol (OAG) in the presence of 1 nM adenosine (●), 100 nM adenosine (■) or no drug (○). PMN activation was standardized as % of maximal response achieved in control (activated without adenosine) specimen for each stimulus. The absolute magnitudes of the maximal response were 178 ± 35, 257 ± 58 and 134 ± 46 c.p.m. $\times 10^3$ using FMLP, A23187 and OAG, respectively. Data represent means of samples from 5 subjects; vertical lines show s.e.mean.

PIA > adenosine > NECA. Affinities of the analogues at the stimulatory (A_2) receptors are the reverse, NECA > adenosine > L-PIA. PMN inhibition by NECA was significant at 0.1 nM ($80 \pm 6\%$ control, $P < 0.05$) while effects of L-PIA were only significant at concentrations greater than 10 nM ($74 \pm 8\%$ control, $P < 0.05$). The concentrations of NECA, adenosine, and L-PIA required to cause 50% of maximal PMN inhibition were 1.0 ± 0.3 nM, 1.7 ± 0.2 nM and 26 ± 4 nM (means $\pm$ s.e.mean estimated from computer assisted curve fitting to the logistic equation) (DeLean *et al.*, 1978). Thus, the relative potencies of adenosine analogues were characteristic of an A_2 adenosine receptor response.

The respiratory burst may be induced by stimuli which elevate intracellular calcium (A23187 or ionomycin), activate phospholipase C (FMLP) or directly activate protein kinase C (OAG). To determine whether adenosine would cause similar inhibition of PMN stimulated through each biochemical pathway, the effects of adenosine were compared with cells activated by ionomycin, FMLP or OAG. Adenosine caused potent inhibition of PMN activated by ionomycin and inhibited the initial phase of the respiratory burst in PMN activated by chemotactic peptide (1 μM FMLP). In contrast, PMN activated by synthetic diacylglycerol (1 μM OAG) were minimally inhibited (Figure 2).

The A_2 adenosine receptor activates adenylate cyclase in many cell types (Daly, 1982). To determine whether cyclic AMP may mediate the effects of adenosine on PMN function, intracellular cyclic AMP was measured following adenosine exposure in both resting and activated PMN. Adenosine at concentrations of 1 nM and 100 nM rapidly (1 min) induced a modest cyclic AMP elevation in unactivated PMN and a marked increase in cyclic AMP during cell activation (Table 1). Although adenosine had a minimal effect on the respiratory burst when PMN were stimulated by OAG, cyclic AMP accumulation induced by adenosine was similar in PMN stimulated by either ionophore, FMLP or OAG. Additional measurements during the period of OAG-induced luminescence confirmed that an elevation of intracellular cyclic AMP was induced during this period despite the lack of inhibition. (Cyclic AMP was 1.70 ± 0.07 and 2.84 ± 0.03 pmol per 10^6 PMN in control specimens and PMN exposed to 100 nM adenosine respectively, at 8 min after OAG activation ($P < 0.05$)).

An elevation of intracellular calcium may be required for induction of the respiratory burst in PMN stimulated by calcium ionophore and FMLP (Simchowitz & Spilberg, 1979). Calcium mobilization may be less important in cells activated by agents which directly stimulate protein kinase C (Lehmeyer *et al.*, 1979). Because adenosine appeared to inhibit specifically PMN stimulation by agents which

Table 1 Cyclic AMP following cell exposure to adenosine

	PMN activating agent			
Adenosine	*None*	*A23187*	*FMLP*	*OAG*
0 nM	0.57 ± 0.03	1.21 ± 0.10	1.47 ± 0.05	0.83 ± 0.09
1 nM	0.97 ± 0.04*	2.67 ± 0.29*		
100 nM	1.56 ± 0.03*	3.22 ± 0.12*	2.06 ± 0.06*	2.33 ± 0.05*

Cyclic AMP (pmol per 10^6 polymorphonuclear leucocyte (PMN)) measured 1 min following addition of adenosine and/or cell activation by 0.2 μM A23187, 1 μM N-formyl-methionyl-leucyl-phenylalanine (FMLP), or 1 μM oleoyl-acetyl-glycerol (OAG). Data represent means ± s.e.mean using specimens from 5 subjects. *$P < 0.05$, in comparison with the sample not including adenosine.

induce calcium mobilization, intracellular calcium during cell activation was evaluated with the fluorescent probe indo-1 (Figure 3). Intracellular calcium increased in PMN activated by ionophore or FMLP but not cells activated by OAG. Adenosine blocked the increase in calcium induced by either ionophore or FMLP (Figure 3). In contrast, adenosine had an insignificant effect upon intracellular calcium in OAG-activated PMN.

Discussion

These results confirm previous findings that physiological concentrations of adenosine inhibit the PMN respiratory burst (Cronstein *et al.*, 1985; Schrier & Imre, 1986). Because adenosine concentrations of over 700 nM occur *in vivo* (Sanchez *et al.*, 1983), adenosine may have an important role in regulation of PMN function. Our studies further demonstrate that the effects of adenosine are dependent upon the biochemical pathway of PMN stimulus-response coupling.

The PMN respiratory burst may be induced by either protein kinase C stimulation or elevation of intracellular calcium (McPhail & Snyderman, 1983). Although the biochemical pathways are only partially distinct, induction of the respiratory burst by OAG is principally mediated by protein kinase C activation while effects of A23187 are mediated by elevation of intracellular calcium. The chemotactic peptide FMLP stimulates phospholipase C with resultant release of inositol tris-phosphate and diacylglycerol. Because inositol tris-phosphate mobilizes intracellular calcium and diacylglycerol activates protein kinase C, the respiratory burst when induced by FMLP is activated through both calcium and protein kinase C dependent pathways (Takenawa *et al.*, 1985). The two pathways of stimulus-response coupling may be important with respect to characteristics of cell response. The calcium-dependent pathway transduces rapid, transient responses while more prolonged responses are often initiated through the protein kinase C pathway (Rasmussen, 1986).

Our results suggest that adenosine selectively inhibits the calcium-dependent pathway of PMN stimulus-response coupling. This conclusion was supported both by the observation that adenosine inhibited PMN activated by A23187 or FMLP but not cells stimulated by OAG, and by studies with indo-1 which clearly demonstrated inhibition of calcium mobilization by adenosine. The biphasic respiratory burst in PMN stimulated by FMLP suggested that two pathways of stimulus-response coupling may have been activated. Although a second phase of the respiratory burst induced by FMLP has been related to cell surface contact (Dahinden *et al.*, 1983), the biochemical basis for a biphasic response is not well established. In our studies the initial phase of the respiratory burst was associated with an increase in intracellular calcium and was inhibited by adenosine. The second phase induced by FMLP was temporally similar to PMN activation following OAG stimulation and was minimally affected by adenosine. Because FMLP induces both calcium mobilization (inositol tris-phosphate) and protein kinase C activation (diacylglycerol), it is possible that the first phase of FMLP activation was induced by inositol tris-phosphate release and the second phase was induced by diacylglycerol release. Further studies of phosphatidylinositol hydrolysis and inositol tris-phosphate release would be required to confirm this hypothesis.

The studies of adenosine analogues demonstrated potencies of NECA, adenosine and PIA (NECA > adenosine > PIA) which were characteristic of an A_2 adenosine receptor (Londos *et al.*, 1980). The increase in PMN cyclic AMP induced by adenosine was consistent with adenylate cyclase stimulation by A_2 receptors as has been described in many cell types (Daly, 1982). Effects of cyclic AMP including reduction of intracellular calcium (Rasmussen, 1986) and inhibition of phosphatidylinositol hydrolysis (Della Bianca *et al.*, 1986) could be mechanisms of PMN inhibition by adenosine.

Because membrane transduction of FMLP stimulation requires phospholipase C (PLC) hydrolysis of phosphatidylinositol, an inhibitory effect of cyclic

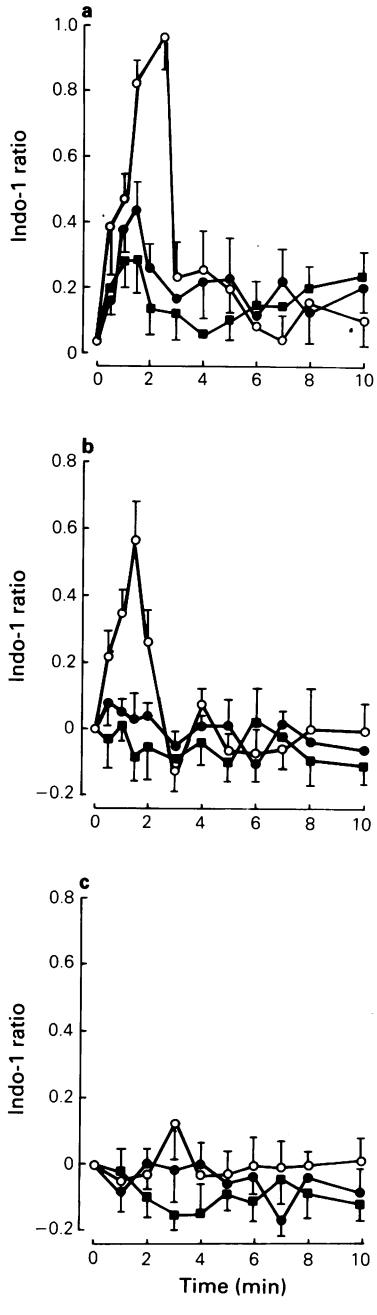

Figure 3 Change in intracellular calcium as indicated by indo-1 fluorescence ratio (410 nM/490 nM) following activation of 1×10^4 PMN ml^{-1} with (a) 0.2 μM ionomycin, (b) 1 μM FMLP or (c) 1 μM OAG in the presence of 100 nM adenosine (■), 1 nM adenosine (●) or no drug (○). Data represent means using samples from 5 subjects; vertical lines show s.e.mean. For abbreviations used see legend to Figure 2.

AMP on PLC could profoundly inhibit PMN stimulation by FMLP. However, inhibition of PLC would not provide a reason either for selective inhibition of the initial phase of FMLP activation or for a reduction in intracellular calcium in PMN stimulated by calcium ionophore. It is unlikely that adenosine or cyclic AMP would prevent calcium influx induced by ionophore, because calcium ionophore causes a passive diffusion of calcium across the plasma membrane. However, effects of cyclic AMP including calcium sequestration and stimulation of membrane channels which actively extrude calcium from the cell could reduce intracellular calcium (Rasmussen & Barrett, 1984). Such effects of cyclic AMP that reduce directly intracellular calcium would be consistent with our results showing (1) inhibition of PMN activated by calcium ionophore, (2) inhibition of an initial calcium-dependent phase of FMLP activation, and (3) a minimal effect of adenosine on PMN activated by OAG.

In summary adenosine inhibits the PMN respiratory burst, elevates intracellular cyclic AMP and reduces intracellular calcium mobilization. The importance of evaluation of each pathway of stimulus-response coupling in studies of PMN function is apparent, because the effects of adenosine were selective to PMN stimulated by chemotactic peptide or low concentrations of calcium ionophore. Our results are consistent with the hypothesis that adenosine inhibition of the PMN may be caused by cyclic AMP-mediated depression in intercellular calcium mobilization.

Note added in proof

Cronstein *et al.* (1988) recently reported less effect of adenosine on PMN cyclic AMP and calcium than we found. Many methods were different in the two studies. Potentially significant aspects of our methods include the use of EDTA in PMN isolation, cell resuspension in plasma, room temperature, PBS buffer and timing of adenosine addition.

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