The Adenosine/Neutrophil Paradox Resolved: Human Neutrophils Possess Both A_1 and A_2 Receptors that Promote Chemotaxis and Inhibit O_2^- Generation, Respectively

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Abstract

Occupancy of specific receptors on neutrophils by adenosine or its analogues diminishes the stimulated release of toxic oxygen metabolites from neutrophils, while paradoxically promoting chemotaxis. We now report evidence that two distinct adenosine receptors are found on neutrophils (presumably the A_1 and A_2 receptors of other cell types). These adenosine receptors modulate chemotaxis and O_2^- generation, respectively. N^6-Cyclopentyladenosine (CPA), a selective A_1 agonist, promoted neutrophil chemotaxis to the chemoattractant FMLP as well as or better than 5′*N*-ethylcarboxamidoadenosine (NECA). In contrast, CPA did not inhibit O_2^- generation stimulated by FMLP. Pertussis toxin completely abolished promotion of chemotaxis by CPA but enhanced inhibition by NECA of O_2^- generation. Disruption of microtubules by colchicine or vinblastine also abrogated the enhancement by NECA of chemotaxis whereas these agents did not markedly interfere with inhibition by NECA of O_2^- generation. FMLP receptors, once they have bound ligand, shift to a high affinity state and become associated with the cytoskeleton. NECA significantly increased association of [^{3}H]FMLP with cytoskeletal preparations as it inhibited O_2^-. Disruption of microtubules did not prevent NECA from increasing association of [^{3}H]FMLP with cytoskeletal preparations. Additionally, CPA (A_1 agonist) did not increase binding of [^{3}H]FMLP to the cytoskeleton as well as NECA (A_2 agonist). These studies indicate that occupancy of one class of adenosine receptors (A_1) promotes chemotaxis by a mechanism requiring intact microtubules and G proteins whereas engagement of a second class of receptors (A_2) inhibits O_2^- generation. Signalling via A_2 receptors is independent of microtubules, insensitive to pertussis toxin and is associated with binding of [^{3}H]FMLP to cytoskeletal preparations. (*J. Clin. Invest.* 1990. 85:1150–1157.) adenosine • adenosine receptor • neutrophil • cytoskeleton • chemotaxis

Introduction

Inflammation is the primary response to tissue injury or microbial invasion and is characterized by the local accumulation of neutrophils. While neutrophils are essential for limiting the spread of infection, stimulated neutrophils are capable of damaging injured tissues while en route to sites of infection or inflammation. We have recently discovered that release of adenosine is one mechanism by which normal cells may protect themselves from activated neutrophils (1). Extracellular adenosine and its analogues diminish generation of toxic oxygen products (e.g., O_2^- or H_2O_2) by activated neutrophils, yet promote neutrophil chemotaxis (2–4). No other known physiologic agent affects neutrophil function in such a paradoxical fashion.

We and others have previously reported that adenosine and its analogues inhibit O_2^- generation by occupying specific adenosine A_2 receptors on the neutrophil (2, 3, 5, 6). More recently we have reported that adenosine occupies a similar receptor to promote chemotaxis (2, 4). Thus, the order of potency of various adenosine analogues for inhibition of O_2^- generation is identical to the order of potency of these analogues for promotion of chemotaxis (5′*N*-ethylcarboxamidoadenosine [NECA])[1] > adenosine ≥ N^6-phenylisopropyladenosine [2, 4]). However, we have observed that NECA, the most potent inhibitor of O_2^- generation and promoter of chemotaxis described to date, promotes chemotaxis at concentrations much lower than those required for inhibition of O_2^- generation (EC_{50} 9 pM vs. IC_{50} 17 nM, respectively [2, 4]). The discrepancy between the potency of NECA for inhibition of O_2^- generation and promotion of chemotaxis suggests that either occupancy of fewer adenosine receptors is required to promote chemotaxis than inhibit O_2^- generation or that occupancy of separate adenosine receptors inhibits O_2^- generation or promotes chemotaxis.

We now report evidence that neutrophils possess two pharmacologically distinct adenosine receptors (consistent with A_1 and A_2 by the classification of van Calker et al. [7]) which, when occupied, modulate chemotaxis and O_2^- generation, respectively, in response to the chemotactic peptide *N*-formylmethionyl-leucyl-phenylalanine (FMLP). Adenosine, acting at A_1 receptors, promotes chemotaxis by a mechanism that requires intact G proteins and microtubules. In contrast, adenosine, acting at A_2 receptors, inhibits O_2^- generation by a G protein and microtubule independent mechanism associated with increased binding of FMLP to a cytoskeletal preparation from neutrophils.

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1. *Abbreviations used in this paper:* CGS-21680, 2-*para*-2-carboxyethylphenylamino-5′*N*-ethylcarboxamidoadenosine; CPA, N^6-cyclopentyladenosine; CV-1808, 2-phenylaminoadenosine; EC_{50}, the concentration at which half-maximal enhancement occurs; IC_{50}, the concentration at which half-maximal inhibition occurs; NECA, 5′*N*-ethylcarboxamidoadenosine; O_2^-, superoxide anion; PIA, N^6-phenylisopropyladenosine.

Methods

Materials. Cytochrome *c* (Type III), FMLP and superoxide dismutase were obtained from Sigma Biochemical Co. (St. Louis, MO). Cytochalasin B was purchased from Aldrich Biochemical Co. (Milwaukee, WI). N^6-cyclopentyladenosine, 5′*N*-ethylcarboxamidoadenosine, and CV-1808 were obtained from Research Biochemicals, Inc. (Natick, MA). CGS-21680 was synthesized and provided by Ciba Geigy Pharmaceuticals, Inc. [^{3}H]*N*-formyl-methionyl-leucyl-phenylalanine was obtained from NEN DuPont, Inc. (Wilmington, DE). All other reagents and chemicals were the highest grade obtainable.

Isolation of neutrophils. Human neutrophils were isolated from whole blood after centrifugation through Hypaque-Ficoll gradients, sedimentation through dextran (6% wt/vol), and hypotonic lysis of red blood cells. This procedure allowed study of populations that were 98±2% neutrophils with few contaminating erythrocytes or platelets. Neutrophils were suspended in Dulbecco's PBS supplemented with Mg^{2+} (1.2 mM) and Ca^{2+} (1.3 mM).

Superoxide anion generation. Superoxide anion generation was monitored by determination of superoxide dismutase inhibitable reduction of cytochrome *c*. Duplicate reaction mixtures containing 1–2 × 10^6 neutrophils, 75 nmol ferricytochrome *c* and various agents at desired concentrations in a final volume of 1 ml were incubated for 5 min at 37°C in the presence of cytochalasin B (5 μg/ml) before addition of the stimulus (FMLP, 0.1 μM). 5 min after stimulation the cells were centrifuged at 4°C at 1000 *g* for 5 min and the supernates collected. Absorption at 550 nmol was then measured and the nanomoles of superoxide anion generated calculated as previously described. The data are reported as nmol cytochrome *c* reduced/10^6 PMN/5 min and is expressed as nanomoles of O_2^- (8).

Chemotaxis. Chemotaxis was determined by the method of chemotaxis under agarose, essentially as described by Nelson et al. (9). Agarose was dissolved in water by boiling and then kept at 48°C. Medium (RPMI 1640) and human serum albumin (1% final concentration) were added to the agarose and the pH adjusted to 7.3 with $Na^+HCO_3^-$ (7%). The agarose solution was then poured onto Petri dishes and allowed to cool. Six series of three wells (3 mm in diameter and 5 mm apart) were then cut into the agarose using a template. The central well in each series was filled with 10 μl of a suspension of neutrophils (1–3 × 10^7/ml) while the outermost well was filled with FMLP (0.1 μM). The innermost well in each series was filled with buffer. The plates were incubated for 3–4 h at 37°C in a 5% CO_2 atmosphere. At the end of the incubation the cells were fixed with formaldehyde (3.7%) and stained with hematoxylin. The plates were analyzed by computerized digital image analysis in a modification of the method described by Fordham et al. (10). Essentially, the video image of the stained, migrating cells is reduced to shades of gray and the density of the image was analyzed by the computer with weighting of the intensity by a factor corresponding to the distance migrated from the well. A chemotactic index was calculated by subtracting the calculated density of cells that had migrated towards the well containing buffer (chemokinesis) from the calculated density of the cells which had migrated towards the chemoattractant (chemotaxis). The data are expressed as a percentage of control.

Association of chemoattractant receptors with the cytoskeleton. Neutrophils (5 × 10^6) were incubated with [^{3}H]FMLP (25 nM) in the presence and absence of adenosine receptor agonists (1 μM). The reaction was terminated by addition of a fourfold excess of ice-cold buffer. After washing with ice-cold buffer the cells were lysed with Triton X-100 (0.5% final) in an ice-water bath, layered over lysing buffer containing Triton X-100 (0.5%) and then the cytoskeletons were isolated by centrifugation in a microfuge for 1 min at 4°C. The cytoskeletal pellets were resuspended in scintillation fluid and the radioactivity quantitated. Replicate incubations with labeled FMLP were carried out in the presence of excess unlabeled FMLP (10 μM, nonspecific binding) and the specific binding was calculated as the difference between the total and nonspecific binding (11, 12). In preliminary studies we found that < 3% of [^{3}H]FMLP added to Triton X-100 lysates of neutrophils was recovered with the Triton-insoluble material (cytoskeletal preparations) and 5′*N*-ethylcarboxamidoadenosine (1 μM) did not directly increase association of [^{3}H]FMLP with the Triton-insoluble material.

Results

We studied the effect of four adenosine receptor agonists on stimulated neutrophil functions. These adenosine analogues differ greatly with respect to their potency as agonists at adenosine receptor subtypes (as determined by inhibition of ligand binding in rat brain preparations [13]). The agent that we have previously shown to be the most potent promoter of chemotaxis and inhibitor of O_2^- generation, NECA, is equally potent at A_1 and A_2 receptors. We have now studied the 600- to 700-fold A_1 specific agonist N^6-cyclopentyladenosine (CPA) and two relatively A_2 specific adenosine analogues: 2-phenylaminoadenosine (CV-1808, five- to sevenfold A_2-specific) and 2-*para*-2-carboxyethylphenylamino-5′*N*-ethylcarboxamidoadenosine (CGS-21680, 140-fold A_2 specific) (13). We first examined the effect of these adenosine receptor agonists on O_2^- generation by neutrophils stimulated with FMLP (0.1 μM). CV-1808 and CGS-21680 inhibited O_2^- generation to the same extent as NECA (Fig. 1 *a* and Table I). CV-1808, CGS-21680 and NECA promoted chemotaxis similarly (Fig. 1 *b* and Table I). In contrast, CPA, the selective A_1 agonist, was a very poor inhibitor of O_2^- generation (Fig. 1 *a*) but was a significantly more potent promoter of chemotaxis than the other agonists tested (Fig. 1 *b* and Table I). The observation that CPA did not inhibit O_2^- generation but was a potent promoter of chemotaxis strongly suggests that adenosine modulates chemotaxis by a different receptor than that which inhibits O_2^-. Moreover, the low concentrations (picomolar range) of all of the adenosine analogues that promote chemotaxis are consistent with occupancy of an A_1 receptor.

Adenosine A_1 and A_2 receptors also differ from each other by the mechanisms through which, when occupied, they influence intracellular events. Stimulus-response coupling at adenosine A_1 receptors proceeds via GTP binding (G) proteins which are inactivated after incubation with pertussis toxin (14–21). To further confirm that adenosine and its analogues promote chemotaxis by engaging A_1 receptors we studied the effect of CPA on chemotaxis in the presence or absence of pertussis toxin. We found that pertussis toxin completely abrogated the ability of CPA to promote chemotaxis (Fig. 2 *a*). In contrast, pertussis toxin potentiated, minimally, inhibition of O_2^- generation by NECA (Fig. 2 *b*).

We next explored the possibility that cytoskeletal elements are required for adenosine to either promote chemotaxis or inhibit O_2^- generation. We therefore determined whether disruption of neutrophil microtubules by colchicine or vinblastine, agents that disrupt neutrophil microtubules without markedly affecting neutrophil chemotaxis (22, 23), interfered with the effect of adenosine receptor agonists on chemotaxis or O_2^- generation. Both vinblastine and colchicine completely abrogated the effect of NECA on chemotaxis (Fig. 3 *a*). In contrast, neither vinblastine nor colchicine markedly interfered with the ability of NECA to inhibit O_2^- generation (Fig. 3 *b*). Identical results were obtained using the more selective A_2 agonist CGS-21680 (Table II). These experiments show that, unlike inhibition of O_2^- generation, adenosine A_2 receptor ago-

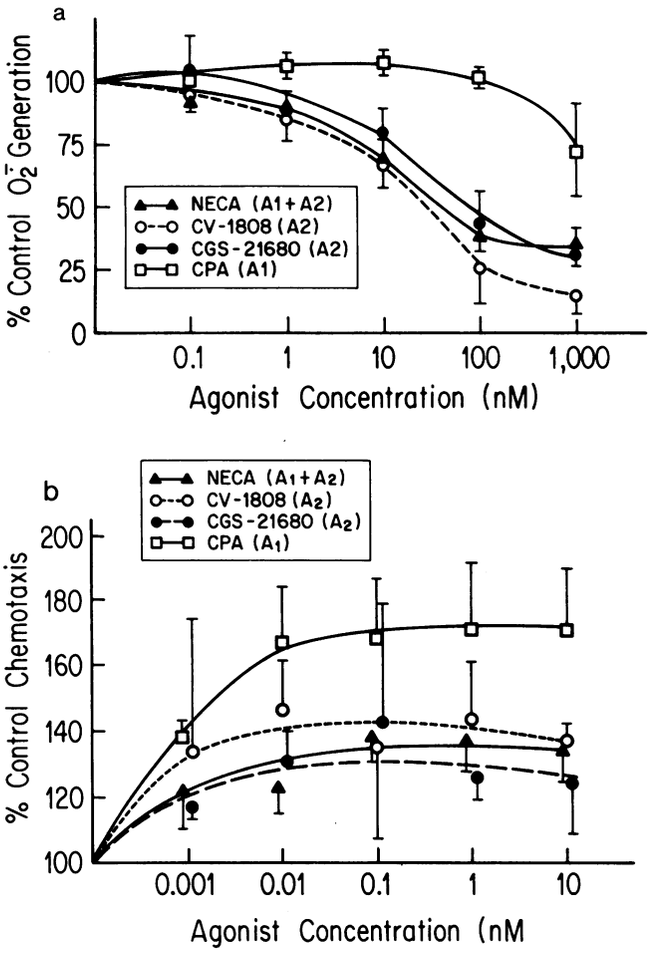

Figure 1. The effects of adenosine receptor agonists on superoxide anion (O_2^-) generation and chemotaxis stimulated by FMLP. (*a*) Neutrophils (2×10^6/ml) were incubated in the presence of buffer or adenosine analogues and cytochalasin B (5 μg/ml) for 5 min at 37°C before addition of FMLP (0.1 μM) and quantitation of O_2^- generation. The results shown for CPA, CGS-21680, and CV-1808 represent the means and standard errors of three separate experiments performed in duplicate. The results shown for inhibition of O_2^- by NECA represent the means of nine separate experiments performed in duplicate. Control O_2^- generation in these experiments was 35±2 nmol/10^6PMN per 5 min. Maximal inhibition of O_2^- generation by CV-1808, CGS-21680, and NECA was 83±5%, 83±5%, and 75±8% inhibition, respectively. (*b*) Neutrophils (2.4×10^5) were placed in wells cut into agarose, as described, in the presence of buffer or adenosine analogues at the described concentrations and incubated for 3.5–4 h or until the leading front was no more than 3.5 mm from the origin. Results are expressed as percent of control chemotaxis (chemotactic index) and represent the mean±SEM of 3 (CV-1808 and CGS-21680) to 9 (NECA and CPA) separate experiments performed in triplicate. Maximal promotion of chemotaxis by CV-1808, CGS-21680, NECA and CPA was 147±21%, 152±17%, 142±12%, and 163±6% of control, respectively. Promotion of chemotaxis by CPA was significantly greater than promotion by the other agents studied ($P < 0.001$, analysis of variance). Control chemotaxis in these experiments was 38,716±4,706 pixels.

nists promote chemotaxis by a pathway dependent upon G proteins and intact microtubules.

Activation of neutrophils by FMLP to generate O_2^- proceeds via activation of a GTP binding protein (24–30) with

Table I. Adenosine Agonists Inhibit Superoxide Anion Generation and Promote Chemotaxis

Agonist	IC_{50} for O_2^- generation	EC_{50} for chemotaxis
NECA	23,000±12,000 pM	9±12 pM
CV-1808	17,000±8,000 pM	28±8 pM
CGS-21680	55,000±28,000 pM	65±60 pM
CPA	>1,000,000 pM	2±5 pM

The data from which these figures are derived are shown in Fig. 1, *a* and *b*. The IC_{50} and EC_{50} are defined as the concentrations at which 50% of maximal inhibition or enhancement, respectively, are observed. The data were analyzed by means of the ALLFIT program on an Apple IIe desktop computer, as we have previously described (2).

resulting generation of a variety of intracellular messengers including fluxes in $[Ca^{2+}]_i$, alterations in intracellular pH, rises in cellular cAMP content and phosphorylation of proteins. Previous studies show that inhibition by adenosine of O_2^- generation does not depend upon adenosine-mediated alterations of Ca^{2+} transients, ion fluxes or cAMP (31–33) and experiments reported here suggest that adenosine receptor agonists inhibit O_2^- generation by a mechanism that does not depend upon intact G proteins (pertussis toxin independent). To determine whether inhibition of stimulated O_2^- generation results from interference by adenosine with an activation step distal to activation of G protein, we studied the effect of adenosine on O_2^- generation stimulated by sodium fluoride (Na^+F^-) since fluoride ion directly activates G proteins bypassing ligand-receptor binding (reviewed in 34–36). As compared with FMLP, fluoride is a poor stimulus of O_2^- generation (4±1 vs. 28±3 nmol/10^6 PMN per 5 min). NECA (1 μM) inhibits O_2^- generation stimulated by FMLP by 76±5% ($P < 0.0001$ vs. control, $n = 8$) but inhibits O_2^- generation stimulated by Na^+F^- by only 17±3% ($P < 0.0001$ vs. NECA, $P < 0.003$ vs. control, $n = 8$). These data indicate that the adenosine receptor agonist NECA inhibits O_2^- generation stimulated by FMLP significantly better than O_2^- generation stimulated by Na^+F^-. This observation suggests that adenosine receptor occupancy inhibits O_2^- generation by altering a step proximal to activation of G proteins, perhaps even the interaction of G protein with occupied chemoattractant receptors.

One mechanism by which adenosine could inhibit O_2^- generation is to "desensitize" the neutrophil to stimulation by chemoattractant. Recent studies by Jesaitis and co-workers have suggested that after activation of the neutrophil, chemoattractant-receptor complexes associate with a cytoskeletal fraction (12). Association of chemoattractant receptors with the cytoskeleton is linked to inability of the neutrophil to generate O_2^- in response to chemoattractant (37). We therefore studied the effect of adenosine receptor agonists on association of FMLP receptors with the "cytoskeleton" to determine whether adenosine receptor occupancy inhibits O_2^- generation stimulated by FMLP by desensitizing FMLP receptors. Labeled FMLP associated with the cytoskeleton in a time dependent fashion, as previously reported (12). The adenosine receptor agonist NECA significantly increased association of [^{3}H]FMLP with the cytoskeleton at all time points after 30 s (Fig. 4).

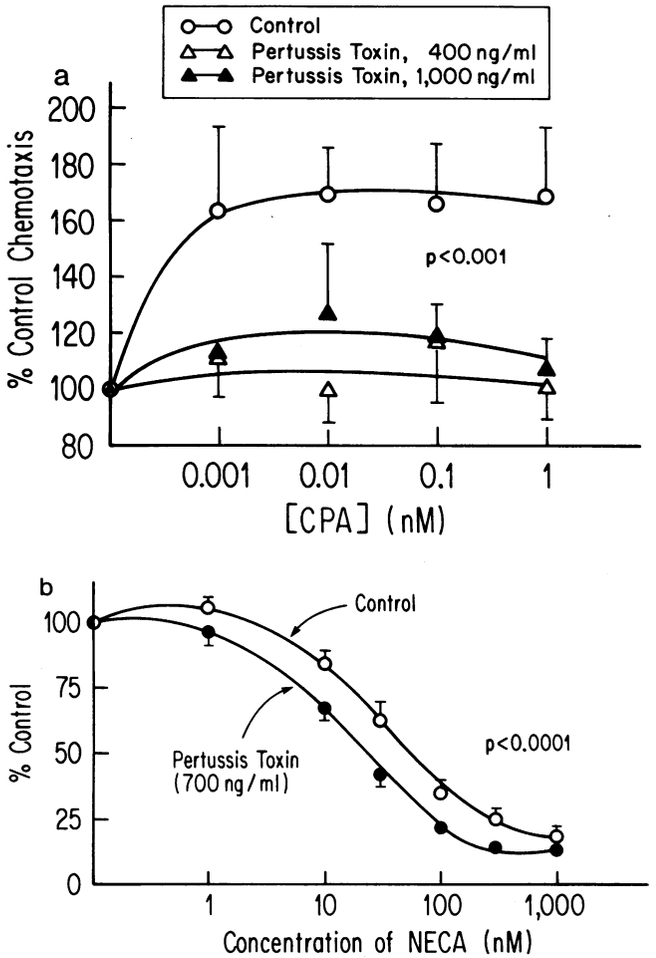

Figure 2. Adenosine receptor agonists promote chemotaxis and inhibit O_2^- generation: effects of pertussis toxin. (*a*) Chemotaxis to FMLP was studied, as described, in the presence or absence of NECA at the indicated concentrations of pertussis toxin (400 or 1,000 ng/ml). At the concentrations studied (400 and 1,000 ng/ml) pertussis toxin did not interfere with chemotaxis (105±8 and 105±8% of control, respectively). The results shown here are the mean±SEM of five experiments performed in triplicate. Pertussis toxin significantly reversed the effect of CPA on chemotaxis ($P < 0.007$, analysis of variance). Control chemotaxis in these experiments was 55,104±3,937 pixels. (*b*) Neutrophils were incubated with pertussis toxin (700 ng/ml) for 1 h at 37°C before determination of O_2^- generation, determined as described. The results shown are the mean±SEM of five separate experiments performed in duplicate. Incubation of neutrophils with pertussis toxin alone significantly reduced O_2^- generation in response to FMLP (36±14% inhibition, $P < 0.05$, Student's *t* test). Control O_2^- generation in these experiments was 27±2 nmol/10^6 PMN per 5 min. There was significantly greater inhibition of O_2^- generation by NECA in the presence of pertussis toxin ($P < 0.0001$, analysis of variance) than in its absence.

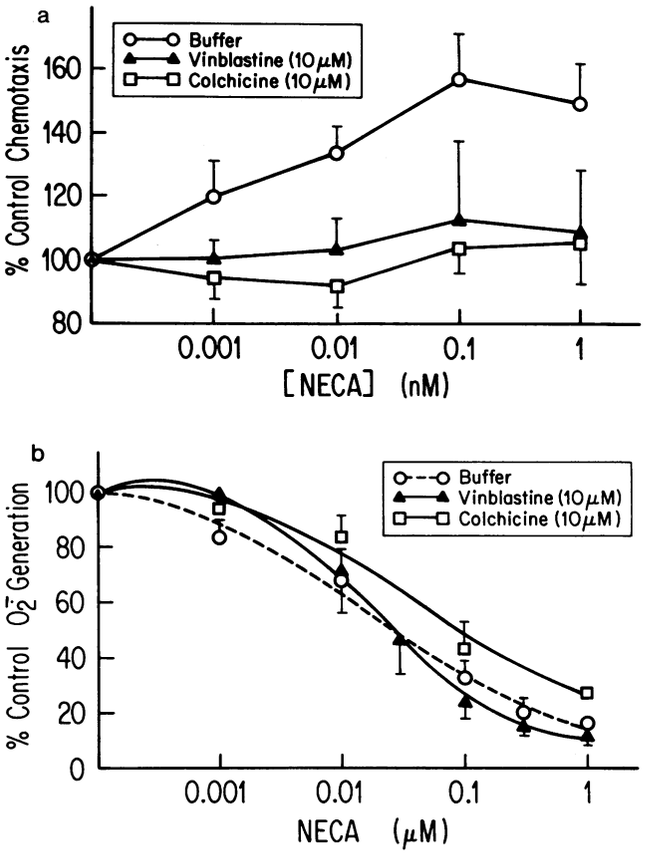

Figure 3. NECA promotes chemotaxis and inhibits O_2^- generation: effects of vinblastine and colchicine. (*a*) Chemotaxis to FMLP was studied in the presence or absence of colchicine (10 μM), vinblastine (10 μM), and NECA as described. Similar to previous reports (23), colchicine promoted chemotaxis (121±8% of control, $P < 0.05$). In contrast, vinblastine did not significantly affect chemotaxis (98±9% of control). Results shown are the mean±SEM of five separate determinations performed in triplicate. Colchicine and vinblastine significantly abrogated promotion of chemotaxis by CPA ($P < 0.001$, analysis of variance). Control chemotaxis in these experiments was 46648±2952 pixels. (*b*) After incubation of neutrophils with buffer, colchicine (10 μM) or vinblastine (10 μM) for 30 min at 37°C neutrophils were stimulated (FMLP, 0.1 μM) in the presence of NECA and O_2^- generation determined, as described. Neither vinblastine nor colchicine significantly affected O_2^- generation (103±3 and 88±4% of control, respectively). Inhibition of O_2^- generation by NECA was significantly less in the presence of colchicine ($P < 0.01$, ANOVA) but not vinblastine. The results shown are the mean±SEM of five different experiments performed in duplicate. Control O_2^- generation in these experiments was 26±2 nmol/10^6 PMN per 5 min.

We next sought to determine whether increased association of FMLP with the cytoskeleton is associated with inhibition of O_2^- generation and/or promotion of chemotaxis. We therefore compared binding of [^{3}H]FMLP to cytoskeletal preparations in the presence of NECA, a potent inhibitor of O_2^- generation and promoter of chemotaxis, to binding in the presence of CPA, a poor inhibitor of O_2^- generation but potent promoter of chemotaxis. NECA was a significantly more potent promoter of [^{3}H]FMLP binding to the cytoskeleton than CPA (Table III). Since we had shown that disruption of microtubules by vinblastine and colchicine abrogated the effect of adenosine receptor agonists on chemotaxis without affecting inhibition by adenosine receptor agonists of O_2^- we studied the effect of NECA on [^{3}H]FMLP binding to the cytoskeleton in the presence of colchicine and vinblastine. Vinblastine and colchicine interfered with neither the binding of [^{3}H]FMLP to the cytoskeleton nor the increment in [^{3}H]FMLP binding to cytoskeletal fractions due to NECA (Table IV). The results of these experiments demonstrate that adenosine receptor agonists promote association of chemoattractant-receptor complexes

Table II. CGS-21680 Inhibits O_2^- Generation by Pertussis Toxin-treated and Untreated Neutrophils

Concentration of CGS-21680	Inhibition	
	Control	+ Pertussis toxin
0.01 μM	43±8%	38±5%
0.10 μM	76±8	80±6
1.00 μM	75±7	75±9

Neutrophils were incubated with pertussis toxin (700 ng/ml) for 1 h at 37°C before determination of O_2^- generation, determined as described. The results shown are the means±SEM of five separate experiments performed in duplicate. Incubation of neutrophils with pertussis toxin alone significantly reduced O_2^- generation in response to FMLP (53±12% inhibition, $P < 0.02$, Student's t test). Control O_2^- generation in these experiments was 24±4 nmol/10^6 PMN per 5 min.

with the cytoskeleton in a manner that parallels inhibition of O_2^- generation but not promotion of chemotaxis.

Discussion

These data demonstrate the presence of two distinct adenosine receptors on neutrophils which, when occupied, inhibit stimulated O_2^- generation or promote chemotaxis. The receptor by which adenosine and its analogues modulate chemotaxis resembles previously described A_1 receptors since it possesses high affinity for adenosine (EC_{50} for chemotaxis in the picomolar range) and modulates neutrophil chemotaxis by a G protein–mediated mechanism. Additionally, A_1 receptor occupancy in the neutrophil promotes chemotaxis by a mechanism that requires intact microtubules. In contrast, the receptor by which adenosine and its analogues inhibit O_2^- generation is of relatively low affinity (IC_{50} for O_2^- generation in the nanomolar range) and does not depend upon functional G proteins or intact microtubules to inhibit stimulated O_2^- generation, an interaction consistent with occupancy of an A_2 receptor. Alternatively, the adenosine receptors present on neutrophils may represent one or two new classes of adenosine receptor since ligands that are A_2 specific (CGS-21680 and CV-1808) in other cells or species do not differentiate in their potency between inhibition of O_2^- generation and promotion of chemotaxis.

The mechanism by which adenosine receptor occupancy inhibits O_2^- generation has not been well understood. Previous studies have shown that adenosine receptor occupancy does not inhibit O_2^- generation by altering stimulated Ca^{2+} metabolism, Na^+-H^+ pumps or increasing intracellular levels of cAMP (31–33). Our results suggest one mechanism by which adenosine receptor occupancy inhibits O_2^- generation by activated

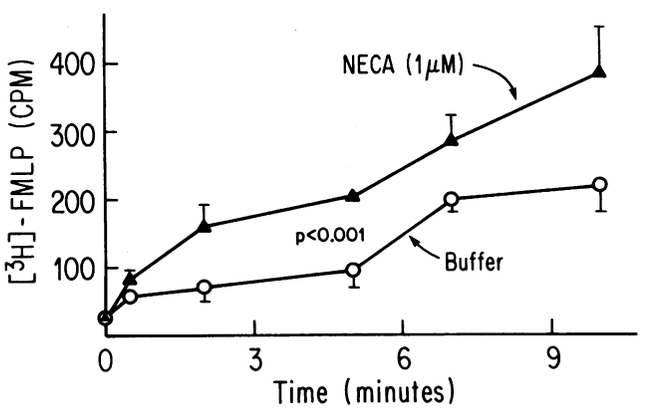

Figure 4. NECA promotes association of [^{3}H]FMLP with the cytoskeleton. Neutrophils (5 × 10^6/ml) were incubated with [^{3}H]FMLP (200 nM) in the presence (nonspecific) or absence (total) of unlabeled FMLP (20 μM) followed by incubation for the indicated time. The reaction was terminated by addition of a fourfold excess of iced buffer followed by washing at 4°C, lysis with Triton X-100 (0.5%) and centrifugation of insoluble material through an 8% sucrose gradient in a microcentrifuge (900 g for 5 min at 4°C). The radioactivity associated with the Triton X-100 insoluble material was then quantitated. The specific binding was calculated after subtraction of nonspecific binding from total binding. The results shown represent the mean±SEM of three to six separate experiments performed in triplicate.

Table III. Association of [^{3}H] FMLP with the Cytoskeleton: Effects of NECA and CPA

Condition	[^{3}H] FMLP binding	Increment in [^{3}H] FMLP binding
	cpm	
Buffer	1,727±249	
NECA (1 μM)	2,338±297*	643±177‡
CPA (1 μM)	2,017±253§	275±159

Neutrophils (10 × 10^6/ml) were incubated in the presence of buffer, NECA, or CPA at the indicated concentrations for 5 min at 37°C before addition of [^{3}H] FMLP in the presence or absence of unlabeled FMLP followed by further incubation for 5 min at 37°C. The radioactivity of Triton X-100-insoluble material was then quantitated, as above. The results shown are the means±SEM of five separate experiments performed in triplicate.

* $P < 0.03$ vs. control, Wilcoxon ranked pairs test.

‡ $P < 0.03$ vs. CPA, Wilcoxon ranked pairs test.

§ $P < 0.05$ vs. NECA, Wilcoxon ranked pairs test.

Table IV. NECA Promotes Association of [^{3}H]FMLP with the Cytoskeleton: Effects of Vinblastine and Colchicine

Condition	Control	+NECA	Mean increment
		cpm	
Buffer	957±242	1,356±148	657±206
Colchicine	883±329	1,713±192	776±246
Vinblastine	834±199	1,220±174	480±177

Neutrophils (10 × 10^6/ml) were incubated in the presence of buffer, colchicine (10 μM) or vinblastine (10 μM) for 30 min at 37°C before addition of NECA (1 μM) or buffer. After further incubation in the presence or absence of NECA for 5 min, [^{3}H]FMLP (200 nM) was added. The results shown are the means±SEM of five separate experiments performed in triplicate. Analysis of the data by two-way analysis of variance reveals that binding varies significantly with the presence of NECA ($P < 0.001$) but not colchicine or vinblastine.

neutrophils. It has previously been reported that chemoattractant peptides bind to receptors, which then undergo a marked increase in their affinity for the ligand and associate, transiently, with actin and fodrin rich cytoskeletal fractions (11, 12, 37–39). The association of high affinity chemoattractant receptors and bound ligand with the cytoskeleton appears to be involved in termination of O_2^- generation by neutrophils (37). Occupancy of adenosine A_2 receptors promotes association of chemoattractant-receptor complexes with the cytoskeleton in a steady-state process previously associated with termination of O_2^- generation in response to chemotactic peptides (37). The mechanism by which adenosine receptor occupancy promotes association of chemoattractant receptor-ligand complexes with the cytoskeleton does not require intact microfilaments since adenosine receptor occupancy inhibits O_2^- generation similarly in the presence and absence of cytochalasin B, an agent that disrupts microfilaments (3, 40). Similarly, intact microtubules are not required since we found that neither colchicine nor vinblastine interfere with either inhibition of O_2^- generation or binding of labeled FMLP to the cytoskeleton. Although the mechanism by which adenosine receptor occupancy promotes binding of chemoattractant receptor-ligand complexes to the cytoskeleton is not known our data suggest that adenosine receptor occupancy inhibits O_2^- generation by promoting the transient association of chemoattractant-receptor complexes with the cytoskeleton.

Adenosine A_1 receptors on neutrophils, similar to those on other cell types (14, 15, 17–21, 41–44), modulate neutrophil chemotaxis by a G protein–mediated mechanism. Activation by FMLP of the neutrophil also requires intact G proteins (24–30, 45–52). It is unlikely that the same G protein is linked to both adenosine A_1 and FMLP receptors since occupancy of adenosine A_1 receptors does not affect O_2^- generation either alone (data not shown) or in response to FMLP. Final resolution of this question will await isolation of the G proteins involved.

Intact microtubules are required for promotion of chemotaxis by adenosine A_1 receptor occupancy. The direct role of microtubules in chemotaxis is minimal since colchicine increases, whereas vinblastine does not affect chemotaxis; thus the role of microtubules in adenosine receptor–mediated promotion of chemotaxis is unclear. Intact microtubules may be required to maintain adenosine A_1 receptors in a functional state or may provide an auxiliary pathway for recycling of chemoattractant receptors to the surface, a process required for chemotaxis (53).

These studies suggest that adenosine plays a unique role in modulation of inflammation. The concentration of adenosine present in blood ($\sim$ 0.3 μM, Cronstein, B. N., and J. E. Slater, unpublished data, [54]) is sufficient to markedly inhibit generation of toxic oxygen compounds thereby protecting vascular endothelium from damage by activated neutrophils (1). At the low concentrations of adenosine that may be present in the relatively acellular extravascular tissues on the periphery of inflammatory sites adenosine occupies high affinity receptors on neutrophils to promote directed migration toward infected or inflammatory loci. At the higher concentrations of adenosine that are present at sites of injured (but not necrotic) tissues (55) occupancy of low affinity adenosine receptors inhibits the generation of toxic oxygen metabolites by activated neutrophils, thereby protecting injured tissues from further damage by activated neutrophils en route to sites of inflammation. Dead cells release their cytoplasmic enzymes including adenosine deaminase (unpublished observations, Marchetta, P., and B. N. Cronstein). This ubiquitous cytoplasmic enzyme, when released from dead or dying cells, can degrade adenosine to inosine, thereby permitting neutrophils to release greater quantities of bactericidal oxygen metabolites such as O_2^- and H_2O_2 (40, 56).

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