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Adenosine, a cytoprotective autocoid: effects of adenosine on neutrophil plasma membrane viscosity and chemoattractant receptor display

Bruce N. Cronstein, F. Roberta Rose and Catherine Pugliese

Division of Rheumatology, Department of Medicine, New York University Medical Center, New York, NY (U.S.A.)

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At inflammatory sites neutrophils are stimulated to produce a variety of toxic agents, yet rarely harm the endothelium across which they migrate. We have recently found that endothelium releases adenosine which, acting via receptors on the surface of human neutrophils, inhibits generation of toxic metabolites by stimulated neutrophils but, paradoxically, promotes chemotaxis. Agents which diminish plasma membrane viscosity affect neutrophil function similarly, possibly by modulating chemoattractant receptor number or affinity. We therefore determined whether adenosine receptor agonists modulate neutrophil function by decreasing membrane viscosity and/or changing the affinity of chemoattractant (*N*-fMet-Leu-Phe, FMLP) receptors. Surprisingly, 5′-(*N*-ethylcarboxamido)adenosine (NECA, 10 μM), the most potent agonist at neutrophil adenosine receptors, increased plasma membrane viscosity, as measured by fluorescence anisotropy of the plasma membrane specific probe 1-(4-trimethylaminophenyl)-6-diphenyl-1,3,5-hexatriene (TMA-DPH), in unstimulated neutrophils from a mean microviscosity of 1.67 ± 0.02 (S.E.) to 1.80 ± 0.02 ($p < 0.001$) while inosine (10 μM), a poor adenosine receptor agonist, had no effect (1.73 ± 0.04, p = n.s. vs. control, $p < 0.01$ vs. NECA). Adenosine receptor agonists increased plasma membrane viscosity in neutrophils with the same order of potency previously seen for inhibition of superoxide anion generation and enhancement of chemotaxis (NECA > adenosine = N^6-phenylisopropyladenosine). The adenosine receptor antagonist 8-(*p*-sulfophenyl)theophylline reversed the effect of NECA on plasma membrane viscosity. Unlike other agents which modulate plasma membrane viscosity, NECA (10 μM) did not significantly change the number or affinity of [^{3}H]FMLP binding sites on neutrophils. In contrast to the hypothesis of Yuli et al. these results indicate that occupancy of adenosine receptors on neutrophils increases plasma membrane viscosity without affecting chemoattractant receptor display.

Introduction

At sites of inflammation or infection circulating neutrophils adhere to endothelium, migrate out of the vasculature and follow a trail of chemoattractants to their source. However, the same chemoattractants which induce migration stimulate neutrophils to release substances toxic to the endothelium. It is therefore surprising that neutrophils only rarely injure the endothelium across which they migrate. We have recently demonstrated that one compound released by endothelium, adenosine, inhibits generation by neutrophils of toxic oxygen metabolites and protects vascular endothelium from damage by stimulated neutrophils [1–3]. Surprisingly adenosine *promotes* neutrophil chemotaxis [4]. Thus adenosine may protect the vasculature from damage by migrating neutrophils whilst hastening the arrival of neutrophils at sites of tissue damage or infection.

We and others have demonstrated that adenosine and its analogues modulate neutrophil function by occupying specific adenosine receptors on the surface of human neutrophils [4–7]. The mechanism(s) by which occupancy of adenosine receptors inhibits O_2^- generation and promotes chemotaxis is unclear. Indeed, occupancy of adenosine receptors does not inhibit O_2^- generation by altering intracellular cAMP concentrations, interfering with stimulated Ca^{2+} movements or modulating the function of the Na^+/H^+ antiport [8–10].

One mechanism by which adenosine could inhibit release of oxygen metabolites and enhance chemotaxis

Abbreviations: TMA-DPH, 1-(4-trimethylaminophenyl)-6-diphenyl-1,3,5-hexatriene; FMLP, *N*-formylmethionylleucylphenylalanine; NECA, 5′-(*N*-ethylcarboxamido)adenosine; PIA, N^6-phenylisopropyladenosine; O_2^-, superoxide anion.

Correspondence: B.N. Cronstein, Division of Rheumatology, Department of Medicine, New York University Medical Center, 550 First Avenue, New York, NY 10016, U.S.A.

was recently suggested by Yuli and co-workers. Yuli et al. [11] reported that agents which decrease the viscosity of the neutrophil plasma membrane alter display of chemoattractant receptors and thereby inhibit stimulated generation of toxic oxygen metabolites and promote chemotaxis. Therefore we sought to determine whether occupancy of adenosine receptors inhibits O_2^- generation and promotes chemotaxis by modulating the physical characteristics of the plasma membrane or altering chemoattractant receptor display.

We now report experiments which appear to negate the hypothesis of Yuli et al. Our data show that occupancy of adenosine receptors increases rather than decreases plasma membrane viscosity in the neutrophil. Moreover, 5′-(*N*-ethylcarboxamido)adenosine (NECA), the most potent agonist at neutrophil adenosine receptors, changes neither the number nor the affinity of *N*-formylmethionylleucylphenylalanine (FMLP) receptors.

Methods

Materials: 5′-(*N*-Ethylcarboxamido)adenosine (NECA), N^6-phenylisopropyladenosine (PIA) and 8-(*p*-sulfophenyl)theophylline (8-PST) were obtained from Research Biochemicals, Inc. (Wayland, MA). 1-(4-Trimethylaminophenyl)-6-diphenyl-1,3,5-hexatriene (TMA-DPH) was purchased from Molecular Probes, Inc. (Eugene, OR). *N*-Formylmethionylleucylphenylalanine (FMLP), inosine and adenosine were obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll-hypaque (Lymphoprep®) was purchased from Nygaard, Ab (Oslo, Norway). [^{3}H]FMLP was purchased from New England Nuclear (Boston, MA).

Isolation of neutrophils, peripheral blood mononuclear cells and erythrocytes Human neutrophils were isolated from whole blood after centrifugation through hypaque-Ficoll gradients, sedimentation through dextran (6%, w/v), and hypotonic lysis of red blood cells [12]. This procedure allows study of populations that are 98 ± 2% neutrophils with few contaminating erythrocytes or platelets. Peripheral blood mononuclear cells were harvested from the interface and washed three times with phosphate-buffered saline (PBS). Erythrocytes were obtained after dextran sedimentation and washed three times in PBS. For all experiments cells were suspended in phosphate buffered saline (PBS) supplemented with Mg^{2+} (0.5 mM) and Ca^{2+} (0.9 mM).

Binding of FMLP to neutrophils. Binding studies were performed using a modification of the method of Koo et al. [13]. In brief, neutrophils were suspended in medium at a final concentration of $10 \cdot 10^6$/ml in the absence (total binding) and presence (non-specific binding) of 10 μM FMLP and then incubated with varying concentrations of [^{3}H]-FMLP at 4°C or 22°C for 60 min. In preliminary studies binding was found to be at equilibrium under these conditions. The cells were then aspirated onto a glass fiber filter (GFB, Whatman) using a cell harvester and washed with buffer (1 ml in less than 5 s). The filters were dried, cut out and placed in scintillant for 5 days before the radioactivity was quantitated. The radioactivity of the pellets was determined for both total and non-specific binding. The difference between total and non-specific binding is defined as the specific binding. Modeling of the data was carried out using LIGAND, a nonlinear least-squares fitting computer program for receptor modeling [14], on a desktop computer (IBM-XT).

Determination of plasma membrane fluidity. Neutrophils, mononuclear cells or erythrocytes were fluorescently labeled by incubating $1 \cdot 10^6$ neutrophils or mononuclear cells with 1-(4-trimethylaminophenyl)-6-diphenyl-1,3,5-hexatriene(TMA-DPH, 1 μM) in PBS for 3 min [15,16]. Erythrocytes (1% v/v) were similarly incubated and adjusted so that the right-angle scatter was similar to that found for either neutrophils or mononuclear cells. Fluorescence polarization was determined by use of a microviscosimeter (Elscint, Israel). Cell suspensions were gently mixed during measurement in order to obtain even distribution of the cells and all measurements were made at 37°C. Fluorescence anisotropy values (microviscosity) were calculated as described by Shinitzky and Barenholz [17] and Shinitzky and Inbar [18] using published values for the r_0 of TMA-DPH [19]. In preliminary studies we found that butanol (2.5%) diminished neutrophil membrane viscosity and cooling (22°C) increased plasma membrane viscosity in neutrophils, as determined by the technique. In other preliminary experiments we were unable to detect nuclear fluorescence in neutrophils treated with TMA-DPH using fluorescence microscopy for periods as long as 15 min. In those experiments in which the effect of stimulation by FMLP was examined the chemoattractant was added in a small volume (20 μl to 2 ml) after an initial reading was taken. Under the conditions of the assay there was no detectable change in the right angle scatter. The data is reported as the microviscosity parameter which is dimensionless.

Statistical analysis. All data points are reported as the mean and standard error unless otherwise noted. Differences from control or between curves were assessed by means of the appropriate level of analysis of variance. Statistical tests were carried out on a desk-top computer (IBM-XT compatible) utilizing programs contained in PC ANOVA (Human Systems Dynamics, Northridge, CA).

Results

5′-(N-Ethylcarboxamido)adenosine (NECA, 10 μM) but not inosine (10 μM) increases plasma membrane viscosity

Using the technique of fluorescence anisotropy we studied the effect of the most potent agonist at neu-

TABLE I

5'-(N-Ethylcarboxamido)adenosine (NECA, 10 μM) modulates plasma membrane viscosity in neutrophils but not mononuclear cells (PBMs) or erythrocytes (RBCs)

Cells were loaded with TMA-DPH, as described, before incubation with buffer or NECA at the indicated concentrations. After recording baseline viscosity NECA was added in a small volume (20 μl/2 ml of sample, respectively) and viscosity was recorded. All determinations were performed in duplicate and determinations were made on cells from different donors.

Cells	Condition	Membrane viscosity (mean ± S.E.)
PMNs	Buffer	1.67 ± 0.02 $n = 14$
PMNs	NECA	1.80 ± 0.02 $n = 14$ *
PBMs	Buffer	1.76 ± 0.06 $n = 9$
PBMs	NECA	1.81 ± 0.03 $n = 9$
RBCs	Buffer	0.87 ± 0.08 $n = 8$
RBCs	NECA	0.91 ± 0.08 $n = 8$

* $p < 0.001$ vs. buffer, Student's t-test.

trophil adenosine receptors, 5'-(*N*-ethylcarboxamido)adenosine (NECA), on neutrophil plasma membrane viscosity. NECA (10 μM) increased plasma membrane viscosity (Table I). Inosine, a purine which is not an agonist at adenosine receptors, did not modulate plasma membrane viscosity (1.73 ± 0.04, p, n.s. vs. control, $p < 0.01$ vs. NECA, $n = 14$).

NECA does not alter the plasma membrane viscosity of either peripheral blood mononuclear cells or erythrocytes

To determine whether NECA increased the viscosity of neutrophil plasma membranes by insertion into the plasma membrane or by altering fluorescence of membrane-bound dye we next studied the effect of NECA on the plasma membrane viscosity of other cell types. NECA changed neither the plasma membrane viscosity of peripheral blood mononuclear cells nor of erythrocytes (Table I). These results demonstrate that NECA specifically alters the membrane viscosity of only neutrophils.

Adenosine increases plasma membrane viscosity by engaging adenosine A_2 receptors

Two different classes of adenosine receptors have been described, A_1 and A_2; these receptors can be differentiated by their characteristic order of agonist potency. We therefore compared the effect of NECA (the most potent A_2 agonist), adenosine and N^6-phenylisopropyladenosine (PIA) on plasma membrane viscosity in order to determine whether adenosine and its analogues increase membrane viscosity by engaging the same receptor which modulates neutrophil function. NECA was significantly more potent than either adenosine ($p < 0.01$) or PIA ($p < 0.01$) with respect to increasing plasma membrane viscosity (Fig. 1). The order of agonist potency for modulation of plasma mambrane viscosity is the same as that we have previously described for inhibition of O_2^- generation and chemotaxis.

The adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (8-PST) reverses the effect of NECA on plasma membrane viscosity

Methylxanthines such as theophylline or 8-PST, an analogue which is taken up poorly by cells, are antagonists at adenosine receptors. To confirm that adenosine and its analogues increase plasma membrane viscosity by occupying adenosine A_2 receptors we next studied the effect of 8-PST on neutrophil plasma membrane viscosity. The methylxanthine 8-PST did not significantly affect plasma membrane viscosity alone but completely reversed the effect of NECA on plasma membrane viscosity (Table II). The results of these experiments further support the hypothesis that occupancy of adenosine receptors increases plasma membrane viscosity.

Occupancy of adenosine receptors does not affect the affinity or number of neutrophil receptors for the chemoattractant FMLP

Alterations in plasma membrane viscosity have been associated with changes in the affinity state of neutrophil receptors for chemoattractants [11]. We therefore examined the effect of NECA (10 μM) on binding of [^{3}H]FMLP to whole neutrophils. When binding was studied at 4°C the NECA did not significantly alter the number of FMLP receptors ($18\,700 \pm 2\,500$ vs. $15\,000 \pm 3\,000$ receptors/cell in the absence and presence of NECA, respectively, $n = 4$). Nor did NECA signifi-

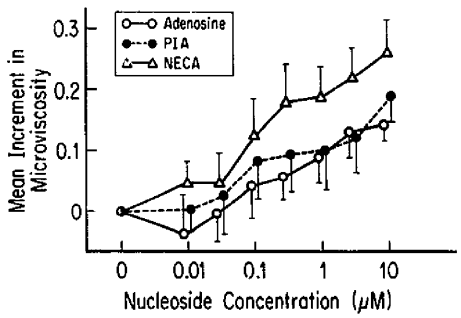

Fig. 1. Adenosine receptor occupancy increases plasma membrane viscosity. Neutrophils were loaded with TMA-DPH, as described, before incubation and the baseline viscosity read. Adenosine receptor agonists 5'-(*N*-ethylcarboxamido)adenosine (NECA, A_2 agonist), N^6-phenylisopropyladenosine (PIA, A_1 agonist) and adenosine were then added and the viscosity recorded. Plotted is the mean increment in microviscosity from baseline which occurred at the various concentrations of agonists. Each point represents the mean and standard error of 5–9 separate experiments performed in duplicate with a bsaseline viscosity of 1.55 ± 0.06.

TABLE II

8-(Sulfophenyl)theophylline (PST, 10 μM) abolishes the effect of 5'-(N-ethylcarboxamido)adenosine (NECA, 0.1 μM) on plasma membrane microviscosity

Neutrophils ($1 \cdot 10^6$/ml) were loaded with TMA-DPH, as described, before incubation with buffer, PST, NECA or their combination at the indicated concentrations. After recording baseline viscosity 8-PST, NECA or their combination was added in a small volume (20 μl/2 ml of sample) and viscosity was recorded. All determinations were performed in duplicate and determinations were made on neutrophils from different donors.

Condition	Plasma membrane viscosity (mean ± S.E.)
Buffer	1.55 ± 0.06 $n = 8$
PST	1.54 ± 0.06 $n = 8$
NECA	1.72 ± 0.03 $n = 8$ *
PST + NECA	1.54 ± 0.05 $n = 8$

* $p < 0.01$ vs. PST + NECA, Student's *t*-test.

cantly alter the affinity of FMLP receptors ($K_d = 41 \pm 15$ vs. 22 ± 6 nM in the absence and presence of NECA, respectively, $n = 4$). Similarly, when binding was studied at 22°C NECA altered neither the number (19 100 ± 4500 vs. 21 000 ± 12 100 receptors/cell in the absence and presence of NECA, respectively, $n = 4$) nor affinity ($K_d = 17.5 \pm 6.3$ vs. 13.5 ± 2.1 nM in the absence and presence of NECA, respectively, $n = 4$) of FMLP receptors. In all of the experiments performed at either 4°C or 22°C binding conformed best to a one-receptor model and in none of these experiments did binding in the presence of cells treated with NECA differ significantly from those incubated in buffer alone. Thus occupancy of adenosine receptors does not modulate stimulated neutrophil function by altering the display of chemoattractant receptors.

Discussion

The results of the experiments reported here demonstrate that occupancy of adenosine receptors induces a change in the plasma membrane of the neutrophil. The physical change in the neutrophil plasma membrane induced by adenosine receptor agonists is neither associated with a change in the number or affinity of FMLP receptors. Therefore our data indicate that modulation of neutrophil plasma membrane viscosity is not sufficient to alter either the number or affinity of chemoattractant receptors.

The findings reported here confirm those recently reported by Coates and co-workers on the chemotactic response to FMLP [20]. Coates et al. observed that, in neutrophils from normal humans, increased plasma membrane viscosity is directly correlated with enhanced chemotaxis. Our results also demonstrate a correlation between enhanced chemotaxis and increased plasma membrane viscosity in the presence of adenosine receptor agonists. One mechanism by which viscosity and chemotaxis could be related in normal neutrophils is via the effect of endogenously released adenosine on neutrophils. We and others have previously demonstrated that neutrophils release adenosine at concentrations sufficient to modulate neutrophil function [1,21,22]. Therefore the correlation of increased chemotaxis with increased plasma membrane viscosity in normal neutrophils may depend on variable rates of adenosine release from the cells of different donors.

In contrast to the association between increased plasma membrane viscosity and enhanced chemotaxis reported here and by Coates et al. [20], others have reported that high concentrations of aliphatic alcohols markedly reduce plasma membrane viscosity yet enhance chemotaxis [11]. Moreover treatment of neutrophils with aliphatic alcohols, like adenosine receptor agonists, inhibits O_2^- generation in response to FMLP. However, unlike adenosine receptor occupancy partial solubilization of neutrophil plasma membranes also modulates the affinity state of the FMLP receptor and may thereby modulate neutrophil function. The divergent effects of adenosine receptor agonists and aliphatic alcohols on neutrophil plasma membrane viscosity indicate that modulation of plasma membrane viscosity is not sufficient to alter chemoattractant receptor display.

By contrast with neutrophils, even high concentrations of NECA do not significantly affect the plasma membrane viscosity of red blood cells or mononuclear cells. While mononuclear cells and some T lymphocytes possess adenosine receptors [23–25] the receptor bearing cells (which might be expected to respond in a fashion similar to neutrophils) represent only a minority of peripheral blood mononuclear cells. Additionally, erythrocytes are not known to possess adenosine receptors. Thus, the observation that neither FMLP nor NECA change the plasma membrane viscosity of these two cell populations further confirms the hypothesis that both FMLP and adenosine receptor agonists modulate plasma membrane viscosity via occupancy of specific receptors on the surface of neutrophils. Moreover, the observation that the ligand used does not alter the plasma membrane viscosity of erythrocytes or peripheral blood mononuclear cells suggests that adenosine and its analogues do not interact directly with the lipid bilayer of the plasma membrane. Yuli and coworkers have proposed the hypothesis that changes in the physical state of the plasma membrane modulate chemoattractant receptor display and, thereby, enhance chemotaxis and inhibit O_2^- generation [11]. The data presented here indicate that changes in the physical state of the plasma membrane may be divorced from alterations of chemoattractant receptor display yet still significantly inhibit O_2^- generation and enhance chemotaxis.

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