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Adenosine A₂ Receptor Occupancy Regulates Stimulated Neutrophil Function via Activation of a Serine/Threonine Protein Phosphatase*

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Adenosine modulates generation of superoxide anion by neutrophils via occupancy of specific adenosine A₂A receptors. However, the intracellular signal transduction pathways by which occupancy of neutrophil adenosine A₂A receptors inhibits superoxide anion generation (O₂⁻) are not well understood. We, therefore, tested the hypothesis that signaling at polymorphonuclear leucocyte (PMN) adenosine receptors proceeds via activation of a serine/threonine protein phosphatase (pp). Both the specific pp1 inhibitor calyculin A (10 nm) and the pp2A inhibitor okadaic acid (10 μM) enhanced O₂⁻ generation (185 ± 24 and 189 ± 35% of control, respectively, p < 0.0001 for both, n = 8), as reported previously. Calyculin A, but not okadaic acid, completely reversed inhibition of stimulated O₂⁻ generation by the adenosine A₂ receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA; IC₅₀ = 30 nm; p < 0.0001, analysis of variance). Calyculin A also reversed the adenosine receptor-mediated desensitization of bound chemoattractant receptors in neutrophils. Treatment of PMNs with NECA increased the pp1 activity of crude membrane preparations in a time- and dose-dependent fashion (EC₅₀ = 40 nm; p < 0.001, analysis of variance, n = 5). NECA inhibited cytosolic protein phosphatase activity by 78 ± 12% (p < 0.003, n = 6) but did not shift pp1 catalytic subunit from cytosol to plasma membrane. Similar changes were observed in neutrophil cytoplasts depleted of organelles and nucleus. Moreover, the selective protein kinase A inhibitor KT5720 (10 μM) reversed the capacity of dibutylryl cAMP but not NECA to increase pp1 activity (p < 0.01, n = 5) in keeping with its effects on O₂⁻ generation. Western blot analysis of PMN subcellular fractions demonstrated the presence of pp1α and pp1γ, but not pp1β, isoforms in both cytosol and plasma membrane but not in azurophil or specific granules. We conclude from these studies that signal transduction by adenosine in PMN proceeds via a novel pathway: cAMP-independent activation of a serine/threonine protein phosphatase in the plasma membrane.

Adenosine, an autacoid released by many different cell types, regulates a variety of stimulated neutrophil functions including production of superoxide anion generation (1, 2), β₂-integrin- and l-selectin-mediated adhesion to endothelial cells (3, 4), and phagocytosis (5). Adenosine regulates these neutrophil functions by coupling with specific cell surface receptors (2) on the neutrophil (reviewed in Ref. 6).

Four different types of adenosine receptors (A₁, A₂A, A₂B, and A₃) have been described, both at the molecular level and by pharmacological analyses (7). Based on pharmacologic data, it appears that the A₂A receptor on neutrophils mediates the inhibition of neutrophil superoxide anion generation, adhesion, and phagocytosis (6). Before their resolution at the molecular level, adenosine A₂ receptors were believed to mediate cellular function via activation of adenyl cyclase, with CAMP as their intracellular messenger. As expected, occupancy of adenosine A₂ receptors stimulated the accumulation of CAMP in neutrophils, but unexpectedly, CAMP proved not to be the second messenger for inhibition of stimulated superoxide anion generation (8–10).

Because adenosine A₂ receptor occupancy had previously been shown to increase cytosolic protein phosphatase activity in bovine adrenal chromaffin (PC12) cells (11), we tested the hypothesis that occupancy of neutrophil adenosine receptors stimulates an increase in the serine/threonine protein phosphatase activity of the plasma membrane where it would be situated to modulate function of the neutrophil NADPH-oxidase. We found that the protein phosphatase 1 (pp1) inhibitor calyculin A completely reversed the effects of adenosine receptor occupancy on stimulated neutrophil generation of superoxide anion. Moreover, adenosine receptor occupancy stimulated an increase in plasma membrane-associated protein phosphatase activity, most likely pp1, and that activation of this phosphatase is independent of cAMP.

**EXPERIMENTAL PROCEDURES**

Materials—5'-N-Ethylcarboxamidoadenosine (NECA), N-formyl-methionyl-leucyl-phenylalanine (FMLP), ATP, phospholipase b, phosphorylase kinase, bovine serum albumin, Ficol, type 70, cytochalasin B, caffeine, cytochrome c, superoxide dismutase, and Brij were supplied by Sigma. KT5720 was obtained from Kamiya Biomedical, Inc. (Thousand Oaks, CA). Alkaline phosphatase-conjugated goat anti-rabbit IgG (H and L) and alkaline phosphatase substrate package were purchased from Life Technologies, Inc. Anti-human protein phosphatase-1 rabbit polyclonal antiserum (affinity purified) was supplied by Upstate Biotechnology, Inc. (Lake Placid, NY). Affinity-purified rabbit anti-protein phosphatase 1γ and anti-protein phosphatase 1γ were generous gifts from Dr. Edgar F. da Cruz e Silva (Rockefeller University, New York, NY). BCA protein assay kit was purchased from Pierce Chemical Co. [γ³²P]ATP was obtained from DuPont NEN.

Isolation of Neutrophils—Human neutrophils were isolated from whole blood after centrifugation through Hypaque-Ficol gradients, sedimentation through dextran (6% w/v), and hypotonic lysis of red blood cells. This procedure allows study of populations that are 98 ± 2% neutrophils with few contaminating erythrocytes or platelets. Neutro-

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Neutral phils were suspended in HEPES-buffered saline supplemented with Mg²⁺ (1.2 mM) and Ca²⁺ (1.3 mM) (12). Quantitation of Superoxide Anion Generation—Superoxide anion generation was monitored by determination of the superoxide dismutase inhibited reduction of ferricytochrome c. Neutrophils (20 × 10⁶/ml) were preincubated for 30 min (37°C) in the presence of okadaic acid (10 μM), calyculin A (10 μM), or medium. Duplicate cultures containing 2 × 10⁶ neutrophils and 75 nmol of ferricytochrome c, in the presence and absence of superoxide dismutase (1 μg/ml), cytochalasin B (5 μg/ml), and FMLP (0.1 μM) as a stimulus were then prepared. After incubation (10 min at 37°C) the cells were then centrifuged at 4°C (500 × g), the supernatants were collected, and radioactivity was quantitated in a scintillation counter. All assays were performed in duplicate, and replicates varied by less than 10%.

Preparation of Subcellular Fractions of Whole Neutrophils—Subcel- lular fractions were separated by a variation of the method of Fridberg et al. (20). Neutrophils suspensions were subjected to two hypotonic lysis steps, and then resuspended (1.0–1.5 × 10⁹) in ice-cold relaxation buffer with protease inhibitors (leupeptin, chymostatin, pepstatin A, and antipain at 10 μg/ml; 2 μg N-tosyl-L-lysine chloromethyl ketone, and 100 μl/kg inhibitor unit/ml aprotonin). The neutrophil suspension was pressurized with nitrogen for 20 min at 350 psi with constant stirring in a nitrogen bomb (Parr Instruments Co., Moline, IL) at 4°C. The catalyse was then collected dropwise into EGTA, pH 7.4, sufficient for a final concentration of 1.25 mM. Nuclei and unbroken cells were pelleted by centrifugation (500 × g for 10 min at 4°C). The supernatant was decanted and then loaded onto sucrose gradients (40% that had been preceded to 4°C. Cytosol, plasma membrane, azurophil granule, and specific granule fractions were isolated by ultracentrifugation (150,000 × g for 120 min at 4°C) in an SW41 rotor (Beckman Instruments). Membranes and granule fractions were subjected to seven cycles of freeze thawing in the presence of protease inhibitors, followed by centrifugation (200,000 × g for 10 min at 4°C) in a 100.3 rotor of a TL-100 ultracentrifuge (Beckman); pellets were resuspended in 200–300 μl of 20 mM Tris-HCl and protease inhibitors and then stored frozen at −70°C until ready for use. SDS-Polyacrylamide Gel Electrophoresis—Proteins were solubilized in a buffer consisting of 2% SDS, pH 6.7, in the presence of dithiothreitol (50 mM) and then electrophoresed through a 12.5% SDS-polyacrylamide gel with 5% stacking gel (21) in a “mini-gel” apparatus (Bio-Rad). Membranes and granule fractions were subjected to seven cycles of freeze-thawing in the presence of protease inhibitors, followed by centrifugation (200,000 × g for 10 min at 4°C) in a 100.3 rotor of a TL-100 ultracentrifuge (Beckman); pellets were resuspended in 200–300 μl of 20 mM Tris-HCl and protease inhibitors and then stored frozen at −70°C until ready for use. SDS-Polyacrylamide Gel Electrophoresis—Separated proteins were electrophoresed on nitrocellulose in Dunn carbonate buffer. Blots were blocked with 5% nonfat dry milk in phosphate-buffered saline with 0.3% Tween 20. Blots were probed with specific antisera, washed, and developed with alkaline phosphatase-linked antiserum (1:100–1:1000 dilutions), followed by visualization by standard technique (22). Rat brain extract (5 μg of protein) containing immunoreactive pp1 was used as a positive control in these experiments (data not shown).

RESULTS
Effect of Okadaic Acid (10 μM) and Calyculin A (10 nM) on Stimulated Superoxide Anion Generation and Its Regulation by an Adenosine A2 Receptor Agonist—To determine whether activation of serine/threonine protein phosphatases is relevant to inhibition of neutrophil function by adenosine receptor agonists, we examined the effect of okadaic acid (10 μM), a relatively specific inhibitor of protein phosphatase 2a (pp2a), or calyculin A (10 nM), a specific inhibitor of protein phosphatase 1 (pp1), on the capacity of the adenosine A2 receptor agonist NECA to inhibit superoxide anion generation by neutrophils. Both okadaic acid and calyculin A enhanced superoxide anion generation in response to the chemoattractant FMLP (189 ± 35 and 185 ± 24% of control, respectively; p < 0.0001 for both, n = 8), and as we and others have reported previously (6), NECA inhibited superoxide anion generation in response to FMLP (IC₅₀ = 30 nM; Fig. 1). However, NECA failed to inhibit superoxide anion generation in neutrophils preincubated with calyculin A (Fig. 1). In contrast, okadaic acid treatment of neutrophils did not affect the ability of NECA (1 μM) to inhibit superoxide anion generation (61 ± 4 versus 62 ± 4% inhibition in the absence and presence of okadaic acid; p = not significant, n = 7). These results are consistent with the hypothesis that adenosine receptor occupancy regulates superoxide anion generation via activation of a protein phosphatase, most likely pp1. Effect of NECA on Association of Bound Chemoattractant
Receptors to the Cytoskeleton—We have demonstrated previously that adenosine $A_2$ receptor occupancy inhibits stimulated neutrophil function by promoting more rapid and complete association of chemoattractant receptors with the cytoskeleton. This phenomenon is associated with desensitization of chemoattractant receptors (9, 10, 16). We, therefore, determined whether inhibition of protein phosphatase activity could reverse the effect of NECA on association of bound FMLP receptors with the cytoskeleton. As demonstrated previously (9, 16), NECA promoted association of chemoattractant, bound to its receptor, with the cytoskeleton (177 ± 21% of control association; $p < 0.001$, $n = 5$). Calyculin A alone induced a small but significant increase in association of chemoattractant receptors with the cytoskeleton (120 ± 15% of control association; $p < 0.05$, $n = 5$). However, calyculin A partially reversed the effect of NECA on association of chemoattractant receptors with the cytoskeleton in parallel with its effects on inhibition by NECA of superoxide anion generation (142 ± 6% of control association; $p < 0.05$ versus NECA alone, $n = 5$).

Effect of NECA on Protein Phosphatase Activity in Plasma Membrane and Cytosolic Fractions—We next examined protein phosphatase activity in soluble and particulate fractions of human neutrophils pretreated with NECA. In contrast to previous studies (11), we observed that occupancy of adenosine $A_2$ receptors by NECA (1 $\mu M$) significantly decreased soluble protein phosphatase activity but increased protein phosphatase activity in the particulate fraction of neutrophils (Table I). To determine whether release of proteolytic enzymes from neutrophil granules during preparation might have contributed to the decreased protein phosphatase activity present in the soluble fraction, we studied the effect of NECA on protein phosphatase activity in neutrophil cytoplasts (neutrophil fragments free of nuclei and granules but containing cytosol and filamentous structures). Like whole neutrophils, incubation of cytoplasts with NECA for 5 min led to an increase in plasma membrane-associated protein phosphatase activity and a decrease in cytosolic activity (Table I). The effect of NECA on serine/threonine phosphatase activity in the particulate fraction of whole neutrophils was dose-dependent and occurred at concentrations similar to those that inhibit superoxide anion generation ($EC_{50} = 40$ nM; Fig. 2). Incubation of isolated plasma membranes, cytosol, or their combination with NECA did not induce any increase in protein phosphatase activity (92 ± 5, 101 ± 8, or 107 ± 5% of control phosphatase activity, respectively; $p = $ not significant, $n = 6$).

Subcellular Localization of Protein Phosphatase 1 Isozymes—Since occupancy of adenosine receptors increased protein phosphatase activity in one cellular compartment (particulate fraction) but decreased protein phosphatase activity in a different cellular compartment (soluble fraction), we determined the subcellular localization of different protein phosphatase catalytic isozymes by Western blot analysis. Neutrophils, like other cell types tested (23, 24), express pp1$_{64}$ and pp1$_{70}$ but not pp1$_{2}$ isozymes (Fig. 3). pp1$_{64}$ was expressed in both cytosol and plasma membrane, but significant expression of pp1$_{64}$ was not detected in granule fractions. Proteolytic cleavage of a terminal hexapeptide most likely accounts for the altered mobility of cytosolic pp1$_{64}$ detected by Western blot. NECA did not

![FIG. 1. Calyculin A (10 nM) reverses the effect of the A$_2$ agonist NECA on superoxide anion generation.](Image)

![FIG. 2. NECA increases protein phosphatase activity in the particulate fraction of neutrophils in a dose-dependent fashion.](Image)

| TABLE I | Protein phosphatase activity in plasma membrane and cytosol fractions |
|----------------|--------------------------------------------------------------------|
|               | Plasma membrane (particulate fraction)                              | Cytosol fraction (supernate) |
| Neutrophils   | $213 \pm 35%^a$                                                     | $64 \pm 15%^b$               |
|               | $n = 4$                                                             | $n = 4$                      |
| Neutrophil cytoplasts | $171 \pm 25%^a$                                                   | $22 \pm 12%^c$              |
|               | $n = 6$                                                             | $n = 6$                      |

$^a p < 0.03$ versus control.  
$^b p < 0.01$ versus control.  
$^c p < 0.003$ versus control.

$^2$ E. F. da Cruz e Silva, personal communication.
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**Fig. 3. Subcellular localization of protein phosphatase isozymes.** Subcellular fractions of neutrophils were separated, and samples of plasma membrane (PM; 25 μg of protein), cytosol (Cyt; 100 μg of protein), specific granule (SG; 25 μg of protein), and azurophil granule (AG; 25 μg of protein) fractions were subjected to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted for phosphoprotein phosphatase isozymes, as described. Rat brain extracts (5 μg of protein) containing immunoreactive protein phosphatase 1 were used as a positive control in these experiments. Similar results were observed in at least one other experiment for each isozyme.

**Fig. 4. The adenosine A2 agonist NECA does not affect subcellular localization of pp1 enzymatic subunits.** After incubation with buffer or NECA, subcellular fractions of neutrophil cytoplasts were separated, and samples of plasma membrane (100 μg/lane) and cytosol (50 μg/lane) were subject to SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blotted for phosphoprotein phosphatase isozymes, as described. Similar results were observed in at least one other experiment for each isozyme in neutrophil cytoplasts and in three experiments with whole neutrophils.

**Fig. 5. The protein kinase A antagonist KT5720 (10 μM) reverses the effect of dibutyryl cAMP (dbcAMP) but not NECA on protein phosphatase activity in neutrophils.** Neutrophils were incubated in the presence or absence of KT5720 for 30 min (37°C), washed, and then incubated with medium, NECA, or dibutyryl cAMP for 5 min (37°C) before washing, lysis, and separation of particulate from soluble fractions. Phosphoprotein phosphatase activity was assayed as described under “Experimental Procedures.” These results represent the mean of from 3 to 6 separate experiments performed in duplicate bars, S.E.

**Table 1.**

| Subcellular Fraction | Protein Phosphatase 1α | Protein Phosphatase 1γ1 | Protein Phosphatase 1γ2 |
|----------------------|------------------------|------------------------|------------------------|
| Plasma Membrane (PM) | 36kD                   | 36kD                   | 36kD                   |
| Cytosol (Cyt)        | 36kD                   | 36kD                   | 36kD                   |
| Specific Granule (SG)| 36kD                   | 36kD                   | 36kD                   |
| Azurophil Granule (AG)| 36kD                  | 36kD                   | 36kD                   |

**Discussion**

We report here evidence that adenosine receptors on human neutrophils modulate stimulated superoxide anion generation via cAMP-independent activation of a plasma membrane-associated serine/threonine protein phosphatase. Evidence to support this hypothesis includes: 1) reversal of adenosine receptor-mediated inhibition of superoxide anion generation by the protein phosphatase 1 inhibitor calyculin A (but not the protein phosphatase 2A inhibitor okadaic acid); 2) increased serine/threonine protein phosphatase activity of particulate fractions from neutrophils treated with the adenosine A2 receptor agonist NECA; and 3) reversal by the protein kinase A inhibitor KT5720 of dibutyryl cAMP-mediated, but not adenosine receptor-mediated, inhibition of superoxide anion generation and stimulation of protein phosphatase activity.

Previous studies have shown, in nonhematopoietic cells, that adenosine receptors signal via activation of serine/threonine protein phosphatases. Mateo et al. (11) have demonstrated that adenosine A2 receptors activate a cytosolic protein phosphatase in neural cells, although the functional significance of this adenosine receptor-induced change is not clear. Gupta et al. (25) have reported that adenosine A2 receptor agonists diminish isoproterenol-induced increases in protein phosphatase inhibitor-1 (ppi-1) activity in cardiac muscle and would, therefore, be expected to increase cytosolic protein phosphatase 1 activity. The studies reported here contrast sharply with those previous studies; the A2 agonist NECA inhibited soluble (cytosolic) protein phosphatase activity but increased protein phosphatase activity in the particulate fraction. Moreover, the adenosine receptor-induced inhibition of superoxide anion generation correlates best with the enhanced protein phosphatase activity in the particulate fraction.

Signal transduction at adenosine receptors, like other members of the family of seven-transmembrane spanning receptors, is mediated by a family of heterotrimeric proteins which, in the active state, bind and hydrolyze GTP (G proteins). Adenosine
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Phosphorylation status of either pp1 isozymes or associated regulatory subunits of pp1 have yet to be investigated. By using immobilization techniques, we have been unable to demonstrate any change in phosphorylation status of either pp1 isozymes or associated proteins after treatment of neutrophils with adenosine receptor agonists and other posttranslational modifications of the enzymatic or regulatory subunits of pp1 have yet to be investigated. The simplest explanation for the disparate effects of adenosine receptor occupancy on particulate and cytosolic protein phosphatase activity is that protein phosphatase enzymatic subunit is translocated from the cytosol to the plasma membrane. However, we were unable to demonstrate any change in the subcellular distribution of protein phosphatase catalytic subunits after stimulation with NECA. Alternatively, adenosine receptor occupancy may signal two separate events: activation of a protein phosphatase in the particulate fraction, and inhibition of cytosolic protein phosphatase activity. Indeed, previous studies have suggested that Gi-linked receptors activate a cytosolic protein phosphatase inhibitor (ppl-1) via a cAMP-dependent mechanism (30), and similar events may proceed in the cytosol of adenosine-treated neutrophils.

Previous studies have demonstrated that agents that inhibit protein phosphorylations (okadaic acid and calyculin A) prevent termination of neutrophil superoxide anion generation and thereby increase the amount of superoxide anion generated (31–35), as found here. The effects of protein phosphatase inhibitors on stimulated neutrophil function correlate with inhibition of dephosphorylation of p47^phox, a protein involved in the assembly of the NADPH oxidase of the neutrophil; dephosphorylation of p47^phox and probably other proteins lead to termination of superoxide anion generation (32, 33, 35). Adenosine receptor occupancy leads to premature termination of superoxide anion generation (6) mediated, as shown here, via activation of a protein phosphatase. Adenosine receptor agonists have not previously been shown to increase the rate at which p47^phox is dephosphorylated, although careful kinetic studies have not been performed (9). It is tempting to speculate that adenosine receptor occupancy inhibits superoxide anion generation by activating a specific phosphatase that dephosphorylates p47^phox and thereby terminates the generation of superoxide anion by stimulated neutrophils. Alternatively, adenosine receptor-stimulated phosphatases may dephosphorylate other, as yet unidentified, upstream signaling proteins that promote the association of bound chemoattractant receptors with the cytoskeleton, thereby desensitizing the chemoattractant receptors and terminating the generation of superoxide anion.

Regardless of the molecular targets for adenosine receptor-stimulated protein phosphatases, the results presented here demonstrate, for the first time, that adenosine A2 receptors on human neutrophils signal via activation of a phosphoprotein phosphatase located in the particulate fraction (plasma membrane). Our results further suggest that the protein phosphatase activated is protein phosphatase 1 and that activation of this phosphatase is independent of cAMP and its downstream effector protein kinase A.

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