Stimulus–response uncoupling in the neutrophil

Adenosine A<sub>2</sub>-receptor occupancy inhibits the sustained, but not the early, events of stimulus transduction in human neutrophils by a mechanism independent of actin-filament formation

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Generation of superoxide anion (O<sub>2</sub><sup>-</sup>) in response to occupancy of neutrophil chemoattractant receptors requires both early events (‘triggering’) and sustained signals (‘activation’). We have previously demonstrated that occupancy of adenosine A<sub>2</sub> receptors inhibits O<sub>2</sub><sup>-</sup> generation by neutrophils. In parallel, adenosine-receptor occupancy promotes association of bound N-formylmethionyl-leucyl-phenylalanine (fMLP) receptors with the cytoskeleton, a process associated with termination of neutrophil activation (stimulus–response uncoupling). We undertook this study to determine whether inhibition of neutrophil function by adenosine-receptor occupancy requires intact actin filaments and to examine the effect of adenosine-receptor occupancy on the stimulated generation of intracellular signals involved in neutrophil triggering and activation. Occupancy of adenosine A<sub>2</sub> receptors by 5′-N-ethylcarboxamidoadenosine (NECA, 1 μM) significantly increased (130 ± 1% of control, P < 0.001, n = 3) association of [3H]fMLP with cytoskeletal preparations. Cytochalasin B (5 μg/ml), an agent which disrupts actin filaments, completely blocked association of [3H]fMLP with cytoskeletal preparations, as previously reported. However, NECA markedly increased association of [3H]fMLP with the cytoskeleton even in the presence of cytochalasin B (P < 0.0002). Moreover, NECA did not significantly affect either the early (30 s) or the late (5 min) formation of actin filaments after stimulation by chemoattractant (fMLP, 0.1–100 nM). Cytochalasin B markedly inhibited actin-filament formation by stimulated neutrophils, and NECA did not reverse the effect of cytochalasin B on actin-filament formation. Adenosine-receptor occupancy did not affect the rapid peak in diacylglycerol generation (< 15 s) from either [3H]arachidonate- or [14C]glycerol-labelled phospholipid pools. However, as would be predicted if occupancy of the adenosine receptor was a signal for early termination of cell activation, NECA (1 μM) markedly diminished the slow sustained generation of diacylglycerol. These results suggest that adenosine-A<sub>2</sub>-receptor occupancy does not affect triggering of the neutrophil, but that occupancy of adenosine receptors is an early signal for the termination of neutrophil activation, i.e. the ‘premature’ finish of signal transduction. Moreover, these data indicate that at least two pathways are available for increasing the association of ligated chemoattractant receptors with the cytoskeleton of neutrophils: F-actin-dependent and -independent.

INTRODUCTION

Chemoattractants such as N-formylmethionyl-leucyl-phenylalanine (fMLP) bind to specific receptors on neutrophils and initiate a series of events provoking neutrophil functions: superoxide anion (O<sub>2</sub><sup>-</sup>) generation, aggregation and degranulation. Despite the continued presence of chemoattractant, neutrophils in suspension terminate these responses within 5–10 min after stimulation. Termination of the neutrophil response to chemoattractants has been attributed to the association of bound receptors with the cytoskeleton and the segregation of these receptors to domains of the plasma membrane rich in actin and fodrin but depleted of the GTP-binding proteins required for further signal transduction (Fechheimer & Zigmond, 1983; Jesaitis et al., 1984, 1988, 1989; Zigmond & Tranquillo, 1986). Further evidence to support this hypothesis is provided by the observation that dihydrocytochalasin B, an agent that depolymerizes filamentous actin (F-actin), reverses the association of bound fMLP receptors with the cytoskeleton and amplifies the response of neutrophils to fMLP (Jesaitis et al., 1986).

We and others have demonstrated that neutrophils possess receptors for adenosine which, when occupied, inhibit generation of oxygen radicals (e.g. O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub>) (Cronstein et al., 1983, 1985, 1990; Roberts et al., 1985; Schrier & Imre, 1986; Schmeichel & Thomas, 1987). Studies from our laboratory have suggested that a novel mechanism by which adenosine-receptor occupancy inhibits fMLP-stimulated O<sub>2</sub><sup>-</sup> generation; adenosine-receptor occupancy promotes more rapid and complete association of occupied fMLP receptors with the cytoskeleton (Cronstein et al., 1990). However, occupancy of adenosine receptors inhibits O<sub>2</sub><sup>-</sup> generation provoked by fMLP even under conditions where F-actin formation is inhibited (in the presence of cytochalasin B) (Cronstein et al., 1983; Roberts et al., 1985).

We now report that occupancy of adenosine receptors promotes association of occupied chemoattractant receptors by a mechanism independent of F-actin formation: occupancy of adenosine A<sub>2</sub> receptors promotes association of occupied fMLP receptors with the cytoskeleton, even in the presence of cytochalasin B. These data are consistent with the hypothesis that adenosine-receptor occupancy promotes ‘premature’ termination of signal transduction. Moreover, occupancy of adenosine receptors inhibits the slow, sustained, phase of diacylglycerol generation (‘activation’), but not the early, transient, diacylglycerol generation which follows fMLP stimulation (‘triggering’).

Abbreviations used: fMLP, N-formylmethionyl-leucyl-phenylalanine; NECA, 5′-N-ethylcarboxamidoadenosine; F-actin, filamentous actin; NBD-phallicidin, nitrobenzoxadiazole-phallicidin.
MATERIALS AND METHODS

Materials

N-Formyl-L-methionyl-L-leucyl-L-[ring-3,4,5-3H(N)]phenylalanine ([3H]MLP, 57 Ci/mmol), [5,6,8,9,11,12,14,15-3H]arachidonic acid (76 Ci/mmol) and [U-14C]glycerol (153 mCi/mmol) were all purchased from NEN-Dupont (Wilmingon, DE, U.S.A.). 5'-N-Ethylcarboxamidoadenosine (NECA) was purchased from Research Biochemicals (Natick, MA, U.S.A.) and cytochalasin B was obtained from Aldrich Biochemical Co. (Milwaukee, WI, U.S.A.). Pyrogen-free dextran was supplied from Pharmacia Chemicals (Piscataway, NJ, U.S.A.). Nitrobenzoxadiazole (NBD)-phallacidin was obtained from Molecular Probes (Eugene, OR, U.S.A.). The chemotactic peptide FMLP, Triton X-100 and all other reagents and salts were obtained from Sigma Biochemical Co. (St. Louis, MO, U.S.A.).

Isolation of neutrophils

Human neutrophils were isolated from whole blood after centrifugation through Hypaque/Ficoll gradients, sedimentation through dextran (6.0%, w/v), and hypo-osmotic lysis of erythrocytes. This procedure allowed study of populations that were 98±2% neutrophils with few contaminating erythrocytes or platelets. Neutrophils were then suspended in Dulbecco's phosphate-buffered saline (PBS) supplemented with Mg2+ (0.9 mM) and Ca2+ (1.3 mM) (Boyum, 1968).

Association of chemoattractant receptors with cytoskeletal preparations

Neutrophils (5×106/ml) were incubated with and without cytochalasin B (5 μg/ml) and NECA (1 μM) in phosphate-buffered saline for 5 min at 37 °C before addition of [3H]MLP (25 nM). Cells were then incubated for 5 min at 37 °C before the reaction was terminated by addition of a fourfold excess of ice-cold buffer containing unlabelled FMLP (10 μM). After washing with ice-cold buffer, the cells were pelleted and lysed in Tris/HCl buffer (20 mM, pH 7.4) containing Mg2+ (3 mM) and Triton X-100 (0.5%, v/v). The lysates were layered over sucrose (8%, w/v) and the cytoskeletal fraction was isolated by centrifugation (9000 g) for 1 min at 4 °C. The cytoskeletal pellets were resuspended in scintillation fluid and the radioactivity was measured. Replicate incubations with [3H]MLP were carried out in the presence of excess unlabelled FMLP (10 μM, non-specific binding), and the specific binding was calculated as the difference between the total and non-specific binding (Jesaitis et al., 1984, 1988). The results shown are the means (± S.E.M.) of three separate experiments performed in triplicate. In preliminary studies we found that <3% of [3H]MLP added to Triton X-100 lysates of neutrophils was recovered with the Triton-insoluble material (cytoskeletal preparations), and NECA (1 μM) did not directly increase association of [3H]MLP with the Triton-insoluble material.

Quantification of F-actin content in stimulated neutrophils

F-actin content of FMLP-stimulated neutrophils was quantified by the methods of Howard & Meyer (1984) and Howard & Oresajo (1985). Briefly, neutrophils (1×106/ml) were incubated for 5 min at 37 °C in the presence of buffer or buffer containing NECA (1 μM). At the end of this incubation, buffer or buffer containing FMLP (0.1–100 nM) was added and the cells were incubated at 37 °C for the indicated period of time. Reactions were terminated by the addition of formalin (3.7%, v/v). Cells were fixed by incubation with formalin for 15 min at room temperature before addition of lysophosphatidylcholine (100 μg/ml) and NBD-phallacidin (0.165 μM). The relative fluorescence of the cells was analysed by cytofluorograph (Facscan; from Becton–Dickinson, or Ortho 50H Cytofluorograph, from Ortho Pharmaceuticals). The mean fluorescence of resting cells was determined and adjusted to a reading of 100 relative fluorescence units. The mean fluorescence of experimental samples was then recorded and the data were reported as percentages of resting mean fluorescence.

Diacylglycerol generation

Neutrophils (75×106/ml) were incubated with [3H]arachidonate (3 μCi/ml) and [14C]glycerol (7 μCi/ml) for 30 min at 37 °C and then washed twice to remove unincorporated label. The neutrophils (17×106/ml) were then incubated with cytochalasin B (5 μg/ml) and buffer or FMLP in the presence or absence of NECA (1 μM) for various periods of time, and the reaction was terminated by addition of chloroform/methanol (2:5, v/v). Samples were extracted by a modification of the technique of Bligh & Dyer (1959). Chlорофос phases were concentrated under nitrogen, and resuspended in 50 μl of chloroform/methanol (2:1, v/v). Samples were applied to heat-activated silica gel GF plates, and lipid fractions were separated by t.l.c. using the solvent hexane/ether/acetic acid (50:50:1, by vol.). Lipids were detected by iodine staining, scraped off the plate, and the radioactivity was quantified (Reibman et al., 1988). The data were expressed as percentages of label recovered as diacylglycerol in resting neutrophils.

Statistical analysis

Data were analysed by two-way analysis of variance (ANOVA). Post hoc analysis of differences between groups was performed by using Tukey's test for highest significant difference. All analyses were performed on an NEC Powermate 2 desktop computer by using the CSS statistics package software (Statsoft, Tulsa, OK, U.S.A.).

RESULTS

NECA increases association of bound chemoattractant receptors with cytoskeletal preparations even in the presence of cytochalasin B

We have previously reported that occupancy of adenosine A2 receptors increases association of bound FMLP receptors with cytoskeletal preparations (Cronstein et al., 1990). As previously, we found that NECA (1 μM), the most potent A2-adenosine-receptor agonist, increases association of [3H]MLP with the cytoskeleton (130±75% of control; P < 0.001, Table 1). We next determined the effect of cytochalasin B (5 μg/ml) on association

<table>
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<tr>
<th>Table 1. NECA promotes association of chemoattractant receptors with the cytoskeleton even in the presence of cytochalasin B</th>
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<tr>
<td>Control (c.p.m.)</td>
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<tr>
<td>Neutrophils (5×106/ml) were incubated for 5 min at 37°C in the presence of buffer alone, cytochalasin B (5 μg/ml), NECA (1 μM) or their combination before addition of [3H]MLP (50 nM). Cells were then incubated for 5 min at 37°C before isolation of cytoskeletal preparations, as described in the Materials and methods section. Two-way analysis of variance demonstrates that association of [3H]MLP with the cytoskeleton varies significantly owing to the presence of NECA (P &lt; 0.002, n = 3) and cytochalasin B (P &lt; 0.0002, n = 3). Results are means ± S.E.M.</td>
</tr>
<tr>
<td>Control</td>
</tr>
<tr>
<td>Cytochalasin B</td>
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Fig. 1. Effect of adenosine-receptor occupancy on F-actin formation

Neutrophils (1 × 10⁶/ml) were incubated for 5 min at 37 °C in the presence of buffer alone (■, control) or NECA (1 µM; □). Buffer alone or an equal volume of buffer containing fMLP to give the concentrations indicated were added and cells were incubated for 5 min at 37 °C before fixation of the cells and staining for F-actin content, as described in the Materials and methods section. Results shown are means ± S.E.M. of 3–5 separate determinations performed in duplicate.

Table 2. NECA does not reverse the effect of cytochalasin B on F-actin formation

<table>
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<tr>
<th>Condition</th>
<th>F-actin (% of resting value)</th>
<th>+ NECA (1 µM)</th>
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<tbody>
<tr>
<td>Resting</td>
<td>100</td>
<td>98 ± 6</td>
</tr>
<tr>
<td>Cytochalasin B</td>
<td>120 ± 7</td>
<td>111 ± 13</td>
</tr>
<tr>
<td>fMLP</td>
<td>244 ± 13*</td>
<td>270 ± 43*</td>
</tr>
<tr>
<td>fMLP + Cytochalasin B</td>
<td>148 ± 8**</td>
<td>160 ± 15**</td>
</tr>
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</table>

of [³H]fMLP with the cytoskeleton. Cytochalasin B completely inhibited association of bound fMLP receptors with the cytoskeleton (1 ± 1% of control; *P < 0.0001, Table 1). However, NECA almost completely reversed the effect of cytochalasin B on association of [³H]fMLP with the cytoskeleton (P < 0.0001, Table 1).

Adenosine-A₂-receptor occupancy does not affect formation of actin filaments by stimulated neutrophils

To determine whether adenosine-receptor occupancy increased association of bound fMLP receptors with the cytoskeleton by promoting actin-filament formation, we examined the effect of NECA on formation of F-actin in resting and fMLP-stimulated neutrophils. fMLP increased the quantity of detectable filamentous actin in neutrophils in a dose-dependent manner (Fig. 1), as previously shown (cf. Howard & Meyer, 1984; Howard & Oresajo, 1985). The cellular content of F-actin was maximal 30 s after stimulation and declined towards baseline by 5 min (data not shown). Occupancy of adenosine A₂ receptors by NECA did not significantly affect F-actin formation by fMLP-stimulated neutrophils after either 30 s or 5 min of incubation (108 ± 10% or 99 ± 5% of control respectively; Fig. 1). As previously reported (cf. White et al., 1983), cytochalasin B (5 µg/ml) markedly inhibited the assembly of F-actin, which occurs after stimulation with fMLP (Table 2). NECA did not reverse the effects of cytochalasin B on stimulated or unstimulated F-actin formation (Table 2).

Adenosine-A₂-receptor occupancy inhibits slow diacylglycerol formation, but not the rapid peak of diacylglycerol formation

We had previously demonstrated that occupancy of adenosine receptors does not affect the generation of the rapid intracellular signals generated in response to stimulation by chemoattractants...
(e.g. increments in intracellular [Ca$^{2+}$]; Cronstein et al., 1988). To establish further that occupancy of adenosine receptors inhibits activation but not triggering, we studied the effect of NECA (1 μM) on generation of diacylglycerol by neutrophils stimulated by fMLP (0.1 μM). NECA did not affect the generation of diacylglycerol from either [3H]arachidonic acid- or [3H]glycerol-labelled neutrophils exposed to fMLP for less than 30 s (‘triggering’; Figs. 2 and 3 respectively). In contrast, NECA significantly inhibited generation of diacylglycerol by neutrophils exposed to chemoattractant for 30 s to 5 min (‘activation’; Figs. 2 and 3). These observations are in accord with the hypothesis that adenosine-receptor occupancy prematurely hastens the inactivation of bound chemoattractant receptors to permit the stimulated generation of rapid intracellular messengers (triggering) while dampening the production of slowly generated intracellular messengers (activation).

**DISCUSSION**

We and others have previously demonstrated that occupancy of adenosine A$_2$ receptors on the surface of human neutrophils inhibits O$_{2}^{-}$ generation, but not aggregation or degranulation stimulated by the chemoattractant fMLP (Marone et al., 1980; Cronstein et al., 1983; Roberts et al., 1985; Grinstein & Furuya, 1986; Schrier & Imre, 1986; Schmeichel & Thomas, 1987). Moreover, we have provided evidence that adenosine-receptor occupancy promotes, in parallel with inhibition of O$_{2}^{-}$ generation, the association of bound chemoattractant receptors with cytoskeletal preparations (Cronstein et al., 1990), a phenomenon previously associated with termination of neutrophil responses (Fechheimer & Zigmond, 1983; Jesatis et al., 1984, 1986; Zigmond & Tranquillo, 1986; Sarndahl et al., 1989). We further show that occupancy of adenosine receptors promotes association of ligand–receptor complexes with the cytoskeleton despite inhibition by cytochalasin B of F-actin formation.

The results reported here further confirm the distinction between ‘triggering’ and ‘activation’ of the neutrophil. Previous studies have indicated that interaction of agonists with chemoattractant receptors on the neutrophil results in a series of rapid events (triggering) which include mobilization of intracellular Ca$^{2+}$ and generation of Ins$P_3$ and diacylglycerol (Table 3). Triggering of the neutrophil is essentially complete after interaction of agonists with receptors for as little as 10–15 s (Korchak et al., 1984; Sklar et al., 1985). Moreover, triggering correlates well with degranulation, since the neutrophil is fully committed to degranulation after occupancy of chemoattractant receptors for 10 s (Korchak et al., 1984). Adenosine-receptor occupancy does not affect mobilization of intracellular Ca$^{2+}$ (Cronstein et al., 1988; Skubitza et al., 1988; Ward et al., 1988), Ins$P_3$ generation (Walker et al., 1990) or, as shown here, the rapid wave of diacylglycerol generation. Thus, as would be predicted if degranulation and triggering are related, adenosine and its agonists do not affect neutrophil degranulation (Marone et al., 1980; Cronstein et al., 1983, 1988; Grinstein & Furuya, 1986).

We (Reibman et al., 1988; Haines et al., 1988b) and others (Truet et al., 1988) have demonstrated that occupation of the fMLP receptor provokes dual waves of diacylglycerol formation (rapid or ‘triggering’; slow or ‘activation’). If occupancy of adenosine receptors promotes ‘inactivation’ of chemoattractant receptors (via association with cytoskeleton), then adenosine and its agonists would be expected to inhibit the events associated with continuous chemoattractant-receptor occupancy (activation): the slow wave of diacylglycerol generation and O$_{2}^{-}$ generation (Table 3). Indeed, we have observed that adenosine-receptor occupancy does inhibit activation.

Occupancy of adenosine A$_2$ receptors is associated with accumulation of cyclic AMP in various cell types (cf. van Calker et al., 1979), and A$_2$ receptors on the neutrophil also promote cyclic AMP accumulation (Cronstein et al., 1988; Iannone et al., 1989; Nielson & Vestal, 1989). However, results of our previous studies and those of others are not consistent with the hypothesis that cyclic AMP is the intracellular messenger for modulation of neutrophil function by adenosine-receptor occupancy (Cronstein et al., 1988; Sullivan et al., 1990). Moreover, it is unlikely that the effects of adenosine-receptor occupancy on diacylglycerol generation are mediated via cyclic AMP, since isoprenaline, a β-adrenergic agent which promotes a similar increase in neutrophil cyclic AMP content, does not affect the late phase of diacylglycerol generation in stimulated neutrophils (Reibman et al., 1990).

Our results confirm the hypothesis that, in the neutrophil, association of bound chemoattractant receptors with the cytoskeleton dissociates bound receptors from the signal-transduction apparatus, thereby terminating the continued generation of intracellular signals required for neutrophil function. Thus cytochalasin B blocks association of bound fMLP receptors with the cytoskeleton, permits increased generation of critical intracellular signals, and thereby amplifies the functional responses of the neutrophil. Conversely, adenosine-receptor occupancy promotes the association of bound chemoattractant receptors with the cytoskeleton, inhibits generation of some intracellular signals, and thereby inhibits functional responses in the neutrophil. However, the effects of adenosine on intracellular signals and functions in the neutrophil do not permit identification of the signals which are critical for O$_{2}^{-}$ generation, since the slow wave of diacylglycerol generation is not required for the respiratory burst (Haines et al., 1988a). Uncoupling of bound chemoattractant receptors by adenosine and its analogues must inhibit some, as yet, unidentified, but sustained signal(s) for O$_{2}^{-}$ generation.

Whereas termination by adenosine of the stimulated response of neutrophils in suspension is independent of actin-filament formation, the mechanism by which adenosine inhibits the responses of adherent neutrophils to chemoattractants most likely depends upon stimulated F-actin formation, since cytochalasin B abrogates the effect of adenosine on adherent neutrophils (de la Harpe & Nathan, 1989). Similarly, priming of neutrophils with tumour necrosis factor may alter signal transduction in neutrophils, since occupancy of adenosine receptors inhibits Ca$^{2+}$ fluxes in primed, but not unprimed, neutrophils (Sullivan et al., 1990).
We conclude that adenosine, acting at A$_2$ receptors on the neutrophil, modulates neutrophil function by promoting the separation of bound stimulatory receptors from their transduction apparatus. The highly selective inhibition of neutrophil function by adenosine-receptor occupancy is most likely due to the inhibition of only those neutrophil responses which require sustained production of intracellular signals, O$_2^*$ generation and adherence. The specificity of inhibition by adenosine of neutrophil function is noteworthy, as the endogenous release of adenosine at sites of inflammation or infection could diminish the propensity of neutrophils to injure healthy tissues and cells inadvertently in their immediate vicinity but permit the activated neutrophil to deal effectively with pathogens.

We acknowledge the outstanding technical assistance provided by Melissa Dworkin, Xiaoyin Tang, Catherine Pugliese and Deborah Nicholls. We also thank Dr. Rochelle Hirschhorn and Dr. Gerald Weissmann for their helpful discussions and for reviewing this manuscript. This research was supported by grants from the Arthritis Foundation, New York Chapter (K.A.H. and B.N.C.), and the National Institutes of Health (HL17921 and AR11949 to Dr. Gerald Weissmann). This research was performed during B.N.C.'s tenure as a Clinical Investigator of the National Institutes of Health (AR01490) and as the Irene Duggan Arthritis Investigator of the Arthritis Foundation.

REFERENCES

van Calker, D., Muller, M. & Hampech, B. (1979) J. Neurochem. 33, 999–1005

Received 21 May 1991; accepted 22 August 1991