Inhibition by adenosine of reactive oxygen metabolite production by human polymorphonuclear leucocytes

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1. The stimulation of reactive oxygen metabolite production from human polymorphonuclear leucocytes by chemotactic peptide (fMet-Leu-Phe) was inhibited by adenosine with a $K_{0.5}$ of $0.6\ \mu\text{M}$. 2. Dipyridamole ($0.1\ \mu\text{M}$), an inhibitor of adenosine uptake, did not prevent the effect of adenosine. 3. Non-metabolizable analogues could substitute for adenosine in the potency order N -ethoxycarboxamide-adenosine > 2-chloroadenosine > adenosine > L- N^6 -(phenylisopropyl)adenosine = D- N^6 -(phenylisopropyl)adenosine, which is characteristic of an A_2 adenosine receptor. The effects of adenosine, 2-chloroadenosine and N -ethoxycarboxamideadenosine were reversed by 8-phenyltheophylline. 4. When endocytosis was inhibited with cytochalasin B, cells were still susceptible to adenosine receptor agonists. 5. 2-Chloroadenosine ($10\ \mu\text{M}$) reduced the activation of respiration in response to chemotactic peptide from 3.3-fold to 1.4-fold. 6. Activation of reactive oxygen metabolite production in response to latex beads was not reversed by adenosine or its analogues. 7. It was concluded that adenosine acts at an A_2 adenosine receptor to antagonize the activation of polymorphonuclear leucocytes by those stimuli, such as chemotactic peptide, which cause an increase in the intracellular free Ca^{2+} concentration.

Polymorphonuclear leucocytes activated by phagocytic and chemotactic stimuli undergo a respiratory burst (Baldrige & Gerald, 1933; Ado, 1933) leading to the formation of oxygen radicals and other reactive oxygen metabolites (Babior *et al.*, 1973; Weening *et al.*, 1975). We have recently reported two distinct mechanisms for activation of reactive oxygen metabolite generation in polymorphonuclear leucocytes (Hallett & Campbell, 1983). One class of primary stimuli, exemplified by the chemotactic peptide fMet-Leu-Phe, caused a rise in intracellular free Ca^{2+} concentration which was necessary for the activation. The other group of stimuli, exemplified by unopsonized latex beads, stimulated reactive oxygen metabolite production by a mechanism independent of a rise in intracellular free Ca^{2+} .

Whilst there are many reports of secondary regulation of secretion and chemotaxis by polymorphonuclear leucocytes (Goldberg *et al.*, 1974; Campbell, 1983) there have been few reports of

secondary regulation of reactive oxygen metabolite production, apart from those concerned with direct inhibition of enzymes of the pathways, or the effect of scavengers (Hallett & Campbell, 1983; Halliwell & Gutteridge, 1984; Campbell *et al.*, 1985). Recently, however, it has been reported that adenosine inhibits superoxide anion generation in human polymorphonuclear leucocytes (Cronstein *et al.*, 1983). Such effects of adenosine could be mediated by cell-surface receptors or through purine metabolism (Schwabe, 1981).

The aim of the work reported here was, therefore, to determine whether adenosine inhibition in polymorphonuclear leucocytes was a receptor-mediated phenomenon, and if so, to identify the class of adenosine receptor responsible. It was further intended to establish whether the effect of adenosine was selective for the calcium-dependent or calcium-independent stimuli.

Experimental

Chemicals

Percoll was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden, luminol from BDH

Abbreviations used: NECA, N -ethoxycarboxamide-adenosine; D- and L-PIA, D- and L- N^6 -(phenyl isopropyl)adenosine.

Chemicals, Poole, Dorset, U.K., and bovine serum albumin (fraction V) from Armour Pharmaceuticals Co., Eastbourne, Sussex, U.K. Latex beads (diameter $1.01\mu\text{m}$) were purchased from Dow Chemicals, Indianapolis, IN, U.S.A. and chemotactic peptide (fMet-Leu-Phe), adenosine and 2-chloroadenosine from Sigma Chemical Co., Poole, Dorset, U.K. D- and L- N^6 -(Phenylisopropyl)adenosine (PIA) were purchased from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. N-Ethylcarboxamideadenosine (NECA) was kindly given by Dr. D. N. Cusack, Department of Pharmacology, King's College, London WC2R 2LS, U.K.

All other chemicals were of AnalaR grade and were purchased from BDH.

Isolation of human peripheral polymorphonuclear leucocytes

Heparinized blood (10ml) was obtained from normal male volunteers and centrifuged at $500g$ for 5 min. The cell pellet was then injected underneath a discontinuous Percoll gradient consisting of 10ml each of densities 1.11 g/ml, 1.09 g/ml and 1.07 g/ml in 0.15M-NaCl. These were then centrifuged at $500g$ for 45 min. The polymorphonuclear leucocyte layer was then aspirated and diluted in Hepes-buffered Krebs medium containing 0.1% bovine serum albumin, 120mM-NaCl, 4.8mM-KCl, 1.2mM-MgSO₄, 1.2mM-KH₂PO₄, 1.3mM-CaCl₂ and 25mM-Hepes, adjusted to pH 7.4 with NaOH. The cells were centrifuged at $400g$ for 1 min, washed, centrifuged again and finally resuspended in 1 ml of the same buffer. Whole blood (10ml) yielded 2×10^7 cells which were 98% polymorphonuclear leucocytes.

Measurement of reactive oxygen metabolite production

Reactive oxygen metabolite production was measured in cell suspensions (5×10^5 cells/ml) using luminol-dependent chemiluminescence. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) in dimethyl sulphoxide was added to the cell suspensions to give a final concentration of $11\mu\text{M}$ -luminol and 0.1% (v/v) dimethyl sulphoxide. Luminescence was measured in the thermostatically controlled light-housing of a purpose built luminometer, as previously described (Campbell *et al.*, 1979; Hallett & Campbell, 1983). All experiments were repeated on two to four separate cell preparations.

Measurement of the respiratory burst

Oxygen uptake was measured polarographically by using a Clark type oxygen electrode at 37°C . All experiments were conducted on two separate cell preparations.

Results

Inhibition by adenosine of reactive oxygen metabolite production stimulated by chemotactic peptide

Adenosine inhibited reactive oxygen metabolite production induced by the chemotactic peptide, fMet-Leu-Phe as measured by luminol-dependent chemiluminescence (Fig. 1a). This inhibition was dose-dependent over the range $0.1\mu\text{M}$ – $100\mu\text{M}$ -adenosine, with a $K_{0.5}$ of $0.6\mu\text{M}$ for adenosine and a maximal inhibition of 82% (Fig. 1b). Inhibition by adenosine was also observed in the presence of

Fig. 1. Inhibition by adenosine of reactive oxygen metabolite production induced by chemotactic peptide (a) Polymorphonuclear leucocytes (5×10^5 cells/ml) were stimulated with chemotactic peptide ($1\mu\text{M}$) after preincubation for 2 min at 37°C (i) in the absence or (ii) in the presence of $10\mu\text{M}$ -adenosine. Luminescence was measured in the presence of $11\mu\text{M}$ -luminol and recorded on a chart recorder calibrated in counts/s (see the calibration marker). (b) Cells were preincubated for 2 min at 37°C with the concentration of adenosine shown, with ($\blacklozenge$) or without ($\diamond$) dipyrindamole ($0.1\mu\text{M}$). After the addition of chemotactic peptide ($1\mu\text{M}$) the peak height of the luminol-enhanced chemiluminescence response was measured.

0.1 μM -dipyridamole, an inhibitor of adenosine uptake (Mills *et al.*, 1972) (Fig. 1b).

Effect of 2-chloroadenosine on reactive oxygen metabolite production induced by chemotactic peptide

2-Chloroadenosine, a non-metabolizable analogue of adenosine, also inhibited reactive oxygen metabolite production induced by chemotactic peptide (Fig. 2a). Inhibition was dose-dependent in the range 0.1–100 μM with a $K_{0.5}$ of 0.25 μM -2-

Fig. 2. Inhibition by 2-chloroadenosine of activation of polymorphonuclear leucocytes by chemotactic peptide (a) Luminol-enhanced chemiluminescence. Cells ($5 \times 10^5/\text{ml}$) were preincubated for 2 min at 37°C with 2-chloroadenosine (0.1–100 μM) prior to the addition of chemotactic peptide (1 μM) and measurement of luminol-enhanced chemiluminescence. (b) Respiration. Cells ($5 \times 10^5/\text{ml}$) were preincubated at 37°C in the presence (○) or absence (●) of 2-chloroadenosine (10 μM). Chemotactic peptide (1 μM) was added as indicated by the arrow, and the resting and stimulated rates of oxygen consumption were measured with an oxygen electrode.

chloroadenosine. This compound offered advantages over adenosine in experiments using large numbers of cells, since it was not deaminated by adenosine deaminase, and thus was used in place of adenosine in the experiments described below.

When activation of polymorphonuclear leucocytes was detected by following the rate of oxygen consumption using an oxygen electrode, 2-chloroadenosine was found to inhibit the respiratory burst of cells activated by chemotactic peptide (Fig. 2b). The ratio of stimulated to unstimulated oxygen consumption was reduced from 3.33 to 1.4 in the presence of 2-chloroadenosine. This inhibition of the respiratory burst indicated that the effect of adenosine agonists was an inhibition of cell activation, rather than the scavenging of reactive oxygen species responsible for luminol-dependent chemiluminescence.

Inhibition of cell activation by other adenosine analogues

Adenosine effects can be mediated by A_1 or A_2 adenosine receptors which may be distinguished by differing affinities for synthetic analogues of adenosine. Activation of human polymorphonuclear leucocytes by chemotactic peptide was also inhibited by NECA, D-PIA and L-PIA. A comparison of dose responses to these compounds and 2-chloroadenosine, shown in Fig. 3, indicated that

Fig. 3. Dose-dependence of inhibition of chemotactic peptide induced reactive oxygen metabolite production by structural analogues of adenosine

Polymorphonuclear leucocytes ($5 \times 10^5/\text{ml}$) were preincubated for 2 min with different concentrations of 2-chloroadenosine (○), D-PIA (▲), L-PIA (△) or NECA (□) or in the absence of adenosine analogues at 37°C , prior to the addition of chemotactic peptide (1 μM). The peak height of the resulting luminescent response was measured and the percentage activity calculated as a ratio of activity in the presence of analogues to that in their absence.

NECA was the most potent inhibitor, having a $K_{0.5}$ of $0.04 \mu\text{M}$. D-PIA and L-PIA were approximately equipotent to each other, but were less potent than NECA or 2-chloroadenosine. This potency order is characteristic of an A_2 -adenosine receptor.

Reversal of adenosine inhibition by 8-phenyltheophylline

8-Phenyltheophylline is a specific antagonist at both A_1 and A_2 adenosine receptors (Smellie *et al.*, 1979). 8-Phenyltheophylline reversed the adenosine inhibition of the chemotactic peptide response (Table 1). Similarly, it reversed the effect of 2-chloroadenosine and NECA, confirming that the inhibition was receptor-mediated.

Effect of adenosine agonists on cells pretreated with cytochalasin B

In polymorphonuclear leucocytes preincubated with cytochalasin B both endocytosis and the production of reactive oxygen metabolites in response to latex beads are abolished (Hallett & Campbell, 1983). However, the production of reactive oxygen metabolites in response to chemotactic peptide is enhanced and biphasic. The effect of adenosine and of NECA on the chemotactic

peptide response of cytochalasin B-inhibited cells was therefore investigated to determine whether they still modulated reactive oxygen metabolite production in the absence of endocytosis, and to determine if the two phases of the response were inhibited to the same extent.

Adenosine and NECA inhibited chemotactic peptide-induced reactive oxygen metabolite production from polymorphonuclear leucocytes treated with cytochalasin B (Fig. 4). Both phases of the response were inhibited. However, the second phase was inhibited more than the first, $10 \mu\text{M}$ -adenosine inhibiting the first phase by $50\% \pm 3\%$ and the second phase by $71\% \pm 4\%$ ($n = 3$). NECA was a more potent inhibitor than adenosine, as was the case in cells not pretreated with cytochalasin B: $10 \mu\text{M}$ -NECA inhibited the first phase by $86\% \pm 4\%$ and the second phase by $88\% \pm 5\%$ ($n = 3$).

Effect of adenosine agonists on polymorphonuclear leucocytes activated by latex beads

Reactive oxygen metabolite production induced by latex beads was not inhibited by adenosine (Fig. 5a). Hence, whereas $100 \mu\text{M}$ -adenosine inhibited the chemotactic peptide-induced response by more than 80% , there was no detectable ($<2.5\%$) inhibition of the latex bead response. Likewise, addition of 2-chloroadenosine (0.1 – $100 \mu\text{M}$) to cells activated by latex beads did not inhibit reactive

Table 1. *Reversal of adenosine inhibition by 8-phenyltheophylline*

Cells ($5 \times 10^5/\text{ml}$) were preincubated for 2 min at 37°C , with different concentrations of adenosine, 2-chloroadenosine or NECA, in the presence or absence of 8-phenyltheophylline ($10 \mu\text{M}$). Cells were then stimulated by the addition of chemotactic peptide ($1 \mu\text{M}$) and the luminol-dependent chemiluminescence was measured. The peak height of the responses were measured, and the percentage activity calculated by using the uninhibited peak heights obtained in the presence and absence of 8-phenyltheophylline. The data shown are results from a representative of three experiments.

Agonist	Concn. (μM)	Activity (% of uninhibited activity)	
		Agonist only	Agonist + 8-phenyltheo- phylline
Adenosine	1	75	100
	10	60	67
	100	20	44
2-Chloroadenosine	1	70	100
	10	50	69
	100	25	28
NECA	0.1	37	100
	1	30	39
	10	25	28

Fig. 4. *Inhibition by 2-chloroadenosine or NECA of the luminol-dependent chemiluminescence response of cytochalasin B-treated cells activated by chemotactic peptide*
Cells ($5 \times 10^5/\text{ml}$) were preincubated for 3 min at 37°C with cytochalasin B ($5 \mu\text{g}/\text{ml}$). Then (i) Krebs-Hepes buffer, (ii) adenosine ($10 \mu\text{M}$), or (iii) NECA ($10 \mu\text{M}$) were added and the preincubation was continued for a further 2 min. Chemotactic peptide ($1 \mu\text{M}$) was then added and the luminol-dependent chemiluminescence response was measured.

Fig. 5. Effect of adenosine and 2-chloroadenosine on polymorphonuclear leucocyte activation by latex beads (a) Luminol-chemiluminescence. Cells ($5 \times 10^5/\text{ml}$) were stimulated with latex beads ($4 \times 10^9/\text{ml}$) after preincubation for 2 min (i) in the absence or (ii) in the presence of adenosine ($10 \mu\text{M}$). (b) Respiration. Cells ($5 \times 10^5/\text{ml}$) were incubated in the presence (○) or absence (●) of 2-chloroadenosine ($10 \mu\text{M}$) and the resting rate of oxygen consumption was measured. Latex beads ($4 \times 10^9/\text{ml}$) were then added, as indicated by the arrow, and the new rate of oxygen consumption was measured.

oxygen metabolite production as measured by luminol-dependent chemiluminescence.

When cell activation was monitored by using an oxygen electrode 2-chloroadenosine did not significantly inhibit the respiratory burst of cells activated by latex beads (Fig. 5b). The ratio of stimulated to unstimulated oxygen consumption was 3.69 in the absence and 3.71 in the presence of 2-chloroadenosine.

Discussion

Adenosine regulates the function of many mammalian cells (Berne *et al.*, 1984). Its spectrum of actions include regulation of blood flow, reversal of the effects of catabolic hormones, promotion of the action of anabolic hormones, inhibition of

secretion and neurotransmitter release and effects on cell growth and differentiation (Newby, 1984).

Most effects of adenosine are exerted through cell-surface receptors (Schwabe, 1981), although some effects, particularly those on cell growth and those observed at supraphysiological concentrations, result from interaction with purine metabolism. It is therefore important to discriminate between receptor-mediated and metabolic effects. This can be done by the application of the following criteria (Schwabe, 1981). Receptor-mediated effects are not prevented by inhibitors of adenosine uptake, they show characteristic structural and stereospecific requirements for synthetic analogues of adenosine, and they are antagonized by the methylxanthines, of which 8-phenyltheophylline is among the most potent and selective (Smellie *et al.*, 1979). In addition two classes of adenosine receptor have been defined on the basis of structure-activity relationships and on the coupling of the receptor to either activation or inhibition of adenylate cyclase (Van Calcar *et al.*, 1978, 1979).

Adenosine-mediated inhibition of chemotactic peptide-induced reactive oxygen metabolite production from polymorphonuclear leucocytes was not prevented by dipyrindamole (Fig. 1a), but was prevented by 8-phenyltheophylline (Table 1). Adenosine analogues showed the potency order $\text{NECA} > 2\text{-chloroadenosine} > \text{D-PIA} = \text{L-PIA}$ (Fig. 3), characteristic of an A_2 adenosine receptor. These receptors mediate activation of adenylate cyclase in many cell types (Londos *et al.*, 1980) and therefore it is likely, that their action on polymorphonuclear leucocytes is also mediated through elevation of intracellular cyclic AMP concentrations.

Adenosine inhibited the stimulation of reactive oxygen metabolite production by chemotactic peptide but not that by latex beads (Fig. 5). Since the response to chemotactic peptide but not latex beads is mediated by an increase in intracellular free Ca^{2+} concentration (Hallett & Campbell, 1983, 1984), this suggests that only the Ca^{2+} -mediated responses may be antagonized by adenosine. Similarly, Cronstein *et al.* (1983) showed that adenosine inhibited reactive oxygen metabolite production induced by the calcium ionophore A23187 but not by phorbol myristate, which in contrast does not elevate the intracellular Ca^{2+} concentration (Sha'afi *et al.*, 1983). Thus adenosine appears to be a secondary regulator of only those primary stimuli whose actions are dependent on a rise in intracellular free Ca^{2+} . It remains to be determined whether adenosine, presumably acting via cyclic AMP, alters directly the Ca^{2+} transient or changes the effectiveness of intracellular free Ca^{2+} in mediating the end-response.

The discovery of adenosine inhibition of reactive oxygen metabolite production raises the possibility that this regulatory pathway plays a physiological role in the inflammatory response. Adenosine, derived perhaps from damaged tissue, might act to limit further reactive oxygen metabolite-induced destruction and this has important implications for immune-based diseases. Furthermore, the identification of primary stimuli and secondary regulators of polymorphonuclear leucocytes provides a cell system for establishing whether reactive oxygen metabolite production can be ascribed to phenomena defined as threshold at the level of individual cells (Campbell, 1983; Edwards *et al.*, 1983).

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