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PROPENTOFYLLINE ENHANCEMENT OF THE ACTIONS OF ADENOSINE ON NEUTROPHIL LEUKOCYTES

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Abstract—In agreement with previous results, activation of adenosine A_2 receptors was found to inhibit the exocytotic release of elastase and the oxidative burst induced by formyl-MetLeuPhe (fMLP) in human neutrophils. The adenosine analogue 5′-*N*-ethylcarboxamidoadenosine (NECA) was more potent than adenosine (IC_{50} 14 vs 64 nM). The effects of adenosine and NECA were not influenced by the A_1-adenosine receptor selective antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; 300 nM), but were abolished by the non-selective adenosine receptor antagonist 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate (CGS 15943; 10 μM). Propentofylline *per se* caused a concentration-dependent inhibition of H_2O_2 production. At 100 μM, propentofylline significantly enhanced the effect of adenosine, but not that of NECA. This effect of propentofylline was shared by the known uptake inhibitor dipyridamole. Neither adenosine nor propentofylline altered fMLP-induced inositol-(1,4,5)-trisphosphate (IP_3) formation. The results demonstrate that propentofylline can counteract neutrophil activation, at least partly by enhancing the action of adenosine through blocking its removal, and that the effect is exerted at a step after the initial receptor events.

Key words: adenosine receptor agonists; adenosine receptor antagonists; adenosine transport inhibitors; propentofylline; neutrophil leukocytes

Neutrophils are the most abundant white cells and in mammals provide a first line defense against microbial infections. Circulating neutrophil leukocytes adhere to the endothelium at sites of inflammation via defined cell adhesion molecules belonging to the integrin family [1]. After this initial step they follow a gradient of chemotactic factors to reach the infectious agents and combat them by means of release of lytic enzymes and oxygen-derived free radicals. Ischemia provides another powerful stimulus for neutrophil activation [2]. There is now good evidence that neutrophil activation contributes to the reperfusion damage after ischemia [2, 3].

Ischemia is also a powerful stimulus for adenosine production [4]. Adenosine has been shown to reduce leukocyte adhesion to the endothelium [5, 6], reduce the expression integrins on the neutrophil surface [1] and reduce the release of free radicals [7]. The vascular endothelium may protect itself against damage from activated neutrophils by releasing adenosine [8, 9].

Propentofylline is a xanthine derivative that has been shown to protect against cerebral damage after ischemia [4, 10, 11]. The mechanism by which it produces its beneficial effects is not completely understood [12]. It has, however, been shown to increase adenosine levels and enhance the actions of adenosine probably because it acts as an inhibitor of adenosine uptake [13, 14]. We therefore examined whether propentofylline was able to enhance actions of adenosine on neutrophil leukocytes. Neutrophils were activated with the chemotactic formyl peptide fMLP§; the exocytosis response was measured by examining elastase release, and the respiratory burst by measuring H_2O_2 production using the luminol reaction [15].

MATERIALS AND METHODS

Chemicals. Adenosine was from Aldrich-Europe (Beerse, Belgium). Propentofylline and CGS 15943 were gifts from Hoechst (Frankfurt am Main, Germany) and Ciba Geigy (Summit, NJ, U.S.A.), respectively. D-myo-[2-^{3}H]inositol 1,4,5-trisphosphate ([^{3}H]IP_3 51.4 Ci/mmol) was from Amersham (Amersham, U.K.). Percoll was from Pharmacia Fine Chemicals (Uppsala, Sweden). S-2484 was obtained from KabiVitrum AB (Stockholm, Sweden). DPCPX was from Research Biochemicals Incorporated (Natick, MA, U.S.A.). Dipyridamole was from Boehringer Ingelheim (Mannheim, Germany). Hanks' balanced salt solution (HBSS), NECA, fMLP, cytochalasin B, luminol, sodium azide and

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§ Abbreviations: IP_3, inositol-(1,4,5)-trisphosphate; NECA, 5′-*N*-ethylcarboxamidoadenosine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; CGS 15943, 9-chloro-2-(2-furanyl)-5,6-dihydro-[1,2,4]-triazolo[1,5]quinazolin-5-imine monomethanesulfonate; fMLP, formyl-MetLeuPhe; S-2484, L-pyroglutamyl-L-prolyl-L-valine-*p*-nitroanilide; NBMPR, nitrobenzylthioinosine; PCA, para-chloroamphetamine.

peroxidase were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). fMLP, cytochalasin B, luminol, S-2484 and CGS 15943 were dissolved in DMSO. Dipyridamole was dissolved in ethanol. The other chemicals were dissolved in water.

Neutrophil preparation. Human neutrophils were isolated from 20 mL of buffy coat prepared from citrated whole blood supplied by the Karolinska Hospital Blood Center. The buffy was diluted 2:1 with PBS and neutrophils were separated by a one-step Percoll technique [16]. We employed high (74%) and low (55%) density Percoll. High density Percoll (15 mL) was layered in plastic centrifuge tubes and 15 mL of low density Percoll was then layered on with great care in order to have clearly separated layers. Diluted blood (20 mL) was then placed carefully on the Percoll layers. After centrifugation at 500 *g* at room temperature for 30 min, the neutrophil-rich band which layered over the red cell pellet was collected with a plastic pipette and washed three times with PBS. Contaminating erythrocytes were lysed by exposure to 2 mL of distilled water for 30 sec [17]. Osmolarity was restored by adding 40 mL of PBS. Cells were centrifuged at 200 *g* and then resuspended to the desired cell concentration in HBSS. The final preparation contained over 95% neutrophils as shown by Giemsa stain, 98% of which were assessed as viable by trypan blue exclusion.

Measurement of hydrogen peroxide production (H_2O_2). H_2O_2 production by neutrophils was determined using luminol amplified chemiluminescence as described by Wymann *et al.* [18]. This is a sensitive, but somewhat unselective, method to assess respiratory burst in neutrophils. The primary reaction is a reduction of molecular oxygen to superoxide; this is then rapidly converted to hydrogen peroxide by non-enzymatic dismutation in the medium. As a test solution, 250 μL each of 1 mM luminol, 10 mM sodium azide and 1000 U/mL horseradish peroxidase were diluted in HBSS to 25 mL. Neutrophils were suspended in HBSS at a concentration of 10×10^6/mL. Neutrophil suspension (100 μL) was added to 850 μL of the test solution. The mixture was incubated with either

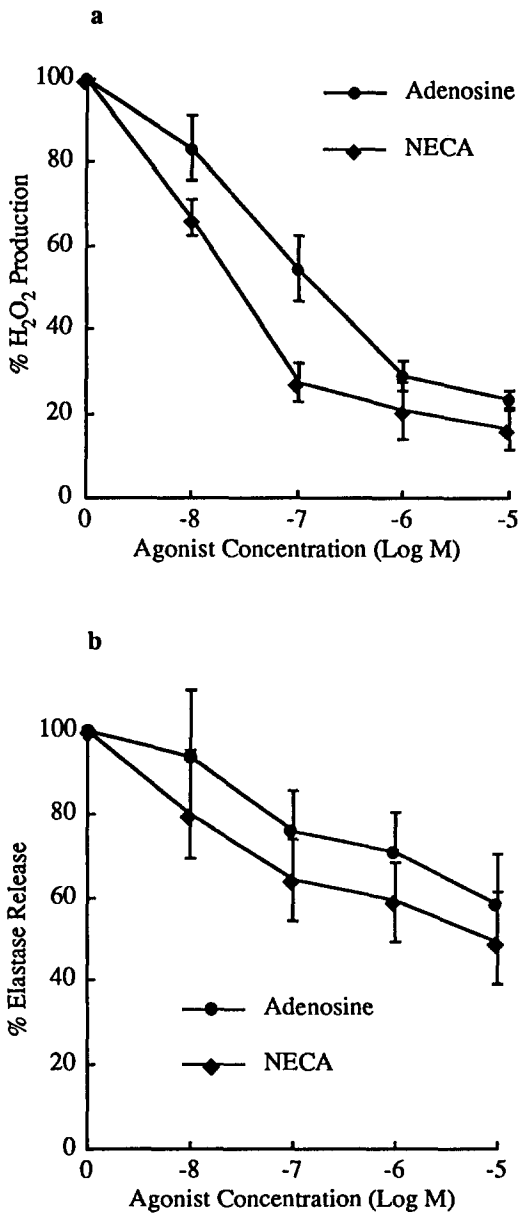

Fig. 1. The effects of adenosine receptor agonists on H_2O_2 production (a) and elastase release (b) induced by fMLP. Drugs were added 5 min before addition of fMLP (1 μM). The data are expressed as means and standard errors of four (NECA) and eight (panel a) or four (panel b) (adenosine) experiments using blood from different donors; % of control (arbitrary chemiluminescence units: control, 4890 ± 675 in panel a; absorbance units: control, 0.52 ± 0.08 in panel b).

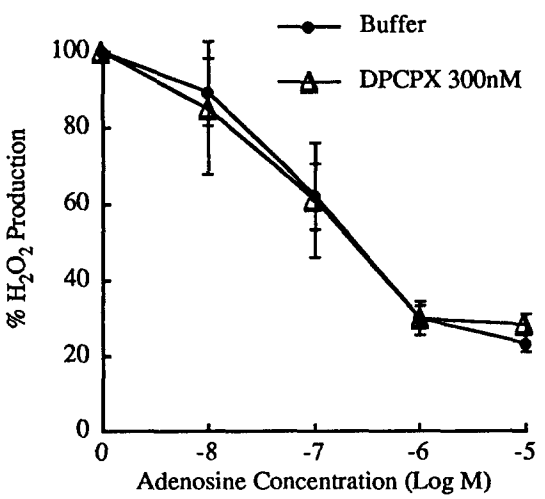

Fig. 2. The effect of adenosine on H_2O_2 production in the presence of DPCPX. DPCPX (300 nM) was added together with adenosine 5 min before fMLP. The data are the means and standard errors of four experiments using blood from different donors. Results are normalized as % of control values (4562 ± 520 and 4425 ± 666 arbitrary chemiluminescence units in the control and the DPCPX groups, respectively).

Table 1. Potency of adenosine and NECA, alone and in combination with propentofylline, as inhibitors of fMLP-induced respiratory burst (H_2O_2) and enzyme release (elastase)

Drug	H_2O_2 IC_{50} (nM)	Elastase
Adenosine	64 (36–117)	63 (6–630)
NECA	14 (11–19)	16 (3–94)
Adenosine + propentofylline 100 μM	14 (9–21)	51 (7–537)
NECA + propentofylline 100 μM	7 (5–12)	ND

Results are presented as IC_{50} values (mean and 95% confidence interval) from at least four replicate experiments.
ND, not determined.

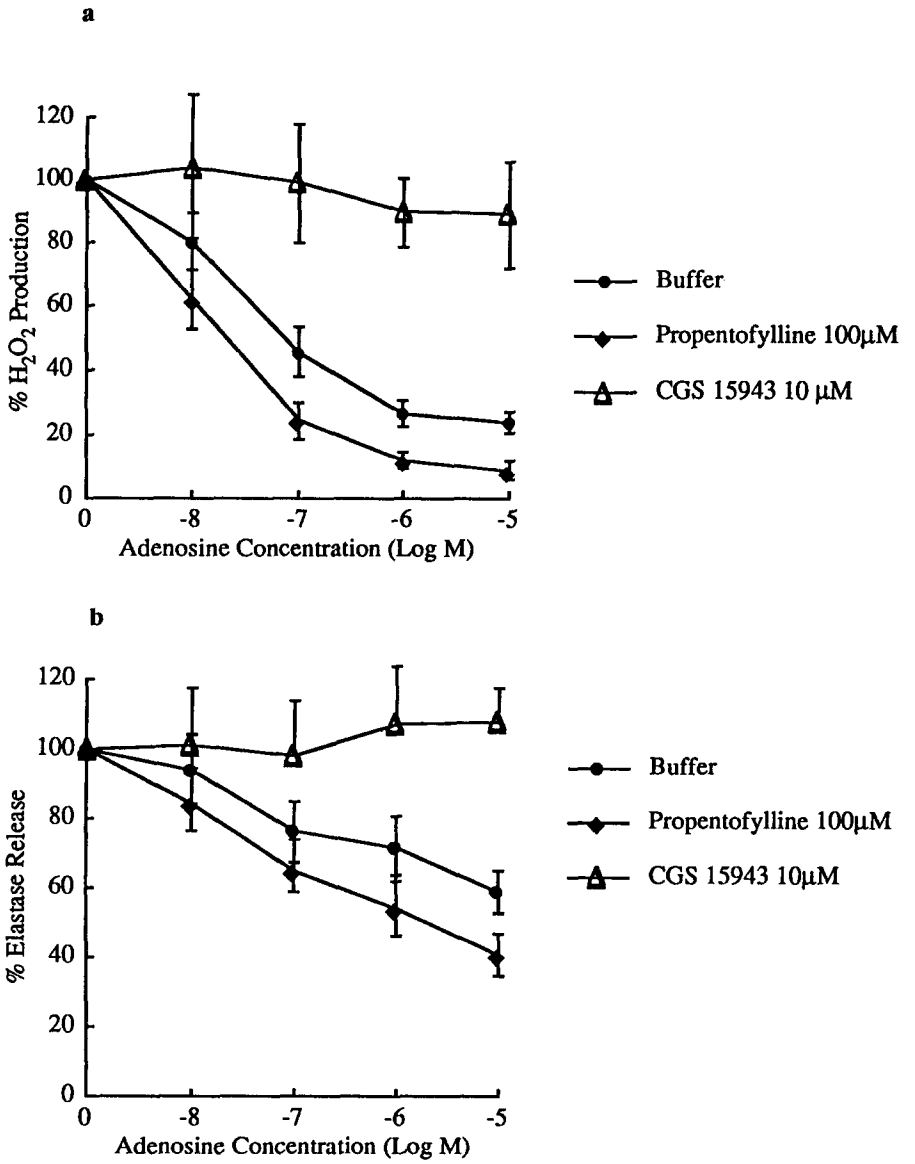

Fig. 3. The effects of adenosine on H_2O_2 production (a) and elastase release (b) in the presence or absence of propentofylline or CGS 15943. Propentofylline (100 μM) or CGS 15943 (10 μM) was added together with adenosine 5 min before fMLP (1 μM). The data are the means and standard errors of four (propentofylline, CGS 15943) and eight (adenosine) experiments using blood from different donors, normalized as % of control (arbitrary chemiluminescence units: buffer, 4834 ± 425; propentofylline, 3906 ± 618; CGS 15943, 3400 ± 565 in panel a; absorbance units: buffer, 0.52 ± 0.08; propentofylline, 0.48 ± 0.03, CGS 15943, 0.32 ± 0.06 in panel b).

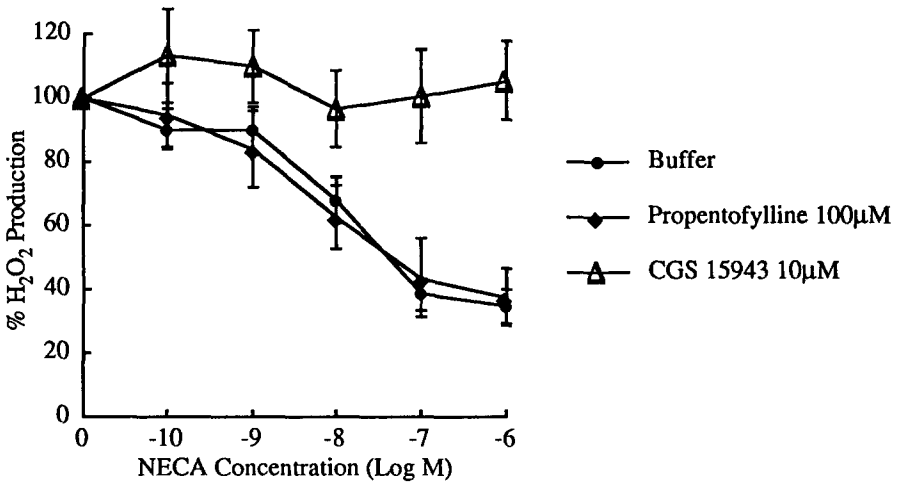

Fig. 4. The effects of NECA on H_2O_2 production in the presence or absence of propentofylline or CGS 15943. NECA was added with or without propentofylline (100 μM) or CGS 15943 (10 μM) for 5 min before fMLP (1 μM). The data are the means and standard errors of four experiments using blood from different donors, normalized as % of control (arbitrary chemiluminescence units: control, 7412 ± 401; propentofylline, 4716 ± 506; CGS 15943, 6593 ± 573).

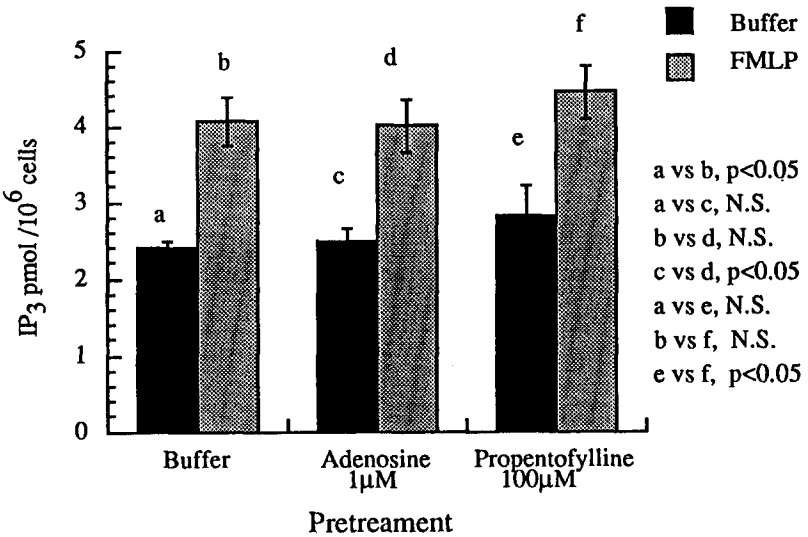

Fig. 5. The effects of adenosine or propentofylline on IP_3 production in fMLP-stimulated neutrophils. Peak IP_3 mass was determined at the peak 10 sec following the addition of fMLP. Data are means and standard errors from two experiments, each performed in triplicate. N.S., not significant (t-test).

control or agonists at different concentrations (40 μL) for 5 min in a plastic cuvette. Ten microlitres of 1 μM fMLP was added to the cuvette and luminol amplified chemiluminescence was measured by a luminometer (Chrono-log Corporation, U.S.A.). Activity was expressed as the percentage of the uninhibited peak height. Because DMSO (above 0.5%) *per se* inhibits H_2O_2 production, the concentration of DMSO in the suspension was kept below 0.1%.

Measurement of granulocyte elastase. Neutrophils (2×10^6/mL) were suspended in 1 mL of HBSS and preincubated at 37° in two successive steps of 5 min each, with 4 μg of cytochalasin B and varying concentrations of agonist. After 20 min of incubation at 37° with fMLP, the suspension was centrifuged for 10 min at 1000 g. The supernatant was carefully collected and used as the sample [19]. The sample (200 μL) was mixed with 200 μL of the buffer solution (Tris 100 mM, NaCl 960 mM, pH 8.3) and incubated at 37° for 3 min. The substrate solution (200 μL) (S-2484, 2 mM) was added to the mixture. After exactly

180 sec of incubation at 37°, the reaction was stopped by the addition of 200 μL of 20% acetic acid. The sample was transferred to a spectrophotometer (Beckman DU-64) and absorbance was read at 405 nM. Sample blanks were prepared by omitting the substrate or by adding acetic acid prior to substrate.

Measurement of IP_3. Neutrophil suspensions (8×10^6 cells in each tube) were preincubated for 20 min and then exposed to buffer, adenosine (1 μM) or propentofylline (100 μM) for 5 min, after which fMLP (1 μM) was added. The reaction was terminated by the addition of 50 μL cold 2.8 M PCA at 0, 10 or 30 sec. Samples were placed on ice for 60 min. After neutralization with 60 μL of buffer (2 mM KOH, 1 M Tris, 60 mM EDTA), samples were centrifuged for 10 min at 1000 *g* at 4°.

The binding assay was performed essentially as described by Gerwins [20]. Twenty-five microlitres of sample (or IP_3 standards), [^{3}H]IP_3 (3000–3500 cpm), binding protein (approximately 0.5 mg) and assay buffer (100 mM Tris–HCl, pH 9, 4 mM EDTA, 4 mM EGTA, 4 mg/mL bovine serum albumin) were added to 96 well micro titer plates with U-shaped well bottoms and incubated at 4° for 1 hr. Bound [^{3}H]IP_3 was separated from free by filtration using the Skatron semi automatic cell harvester 7019 (Skatron AS, Tranby, Norway). Filters were then transferred to scintillation vials. Three millilitres of scintillation fluid (Optiphase "HiSafe3", Pharmacia-LKB, Sweden) was added and the vials were left for 24 hr. Before counting, the vials were shaken. All assays were performed in triplicate.

Statistics. All results are given as means ± standard errors. Results were compared by one way analysis of variance or *t*-test using the Primer program on a Macintosh Computer. IC_{50} was analysed by means of the InPlot program on an IBM Computer.

RESULTS

Both adenosine and the adenosine analogue NECA inhibited H_2O_2 production in fMLP-stimulated neutrophils in a concentration-dependent manner over the range 0.01–10 μM. NECA was somewhat more potent and efficacious than adenosine. The IC_{50} was 14 and 64 nM, and maximal inhibition 83 and 76% for NECA and adenosine, respectively (Fig. 1a, Table 1).

Adenosine and NECA also inhibited fMLP-induced release of elastase in a concentration-dependent manner (Fig. 1b). The maximal inhibition afforded by NECA and adenosine was 51 and 42%, respectively. Both adenosine and NECA are rather unselective adenosine receptor agonists. The adenosine A_1-receptor selective antagonist DPCPX did not inhibit the effect of adenosine in a concentration of 0.3 μM (Fig. 2), a concentration 100–1000 times higher than its K_D at the A_1 receptor [21]. The non-selective A_1- and A_2-receptor antagonist CGS 15943, however, virtually abolished the effect of both adenosine (Fig. 3) and NECA (Fig. 4). Thus, in agreement with previous results [22], the effect appeared to be mediated via an A_2 receptor.

fMLP not only increased oxidative burst and elastase release, but also increased intracellular IP_3. In agreement with previous findings [23] adenosine did not affect this early step in the signalling cascade (Fig. 5).

Propentofylline *per se* produced a concentration-dependent inhibition of fMLP-induced oxidative burst (Fig. 6). This was not due to inhibition of an early step in fMLP-activation of the neutrophils since IP_3 formation was unaffected (Fig. 5). At a low concentration (1 μM) propentofylline did not alter the inhibitory effect of adenosine (data not shown). The inhibitory effects of adenosine on fMLP-activated neutrophil oxidative burst were markedly enhanced in the presence of a high concentration of propentofylline (100 μM). The maximal inhibition of fMLP-stimulated oxidative burst was increased from 76 to 91% (Fig. 3a). IC_{50} for adenosine was lowered from 64 to 14 nM (Table 1). The ability of adenosine to antagonize fMLP-induced elastase release was also enhanced by propentofylline. The maximal inhibition was increased from 42 to 58% and the IC_{50} was slightly reduced from 63 to 51 nM (Fig. 3b, Table 1). However, the ability of NECA to inhibit fMLP-induced oxidative burst was unaffected by propentofylline (Fig. 4). This is to be expected if propentofylline acts as an uptake inhibitor, because NECA is not eliminated by the transport system that removes adenosine. In order to test further whether uptake inhibition could potentiate the effect of adenosine on neutrophils, we examined the effect of a well established adenosine uptake blocker, dipyridamole [24]. As with propentofylline, dipyridamole *per se* reduced the effect of fMLP. The effect was minimal (less than 20%) up to 0.3 μM, but was pronounced thereafter, reaching more than 85% at 3 μM, essentially in agreement with the results of Suzuki *et al.* [25]. As seen in Fig. 7 dipyridamole, at a low concentration (0.3 μM) that

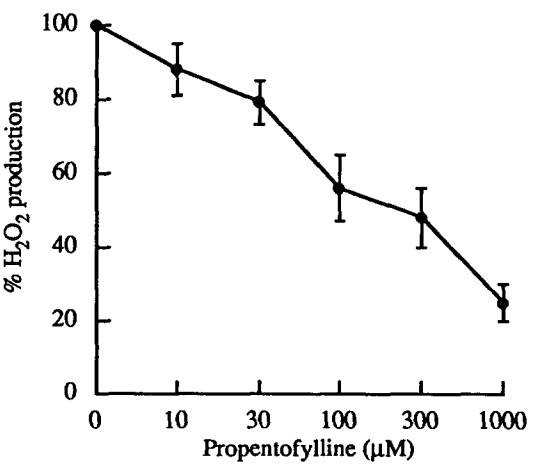

Fig. 6. The effects of propentofylline alone on H_2O_2 production. Propentofylline was added 5 min before fMLP (1 μM). The data are the means and standard errors of four experiments from different donors. Values represent % of control (which was 4243 ± 1219 arbitrary chemiluminescence units).

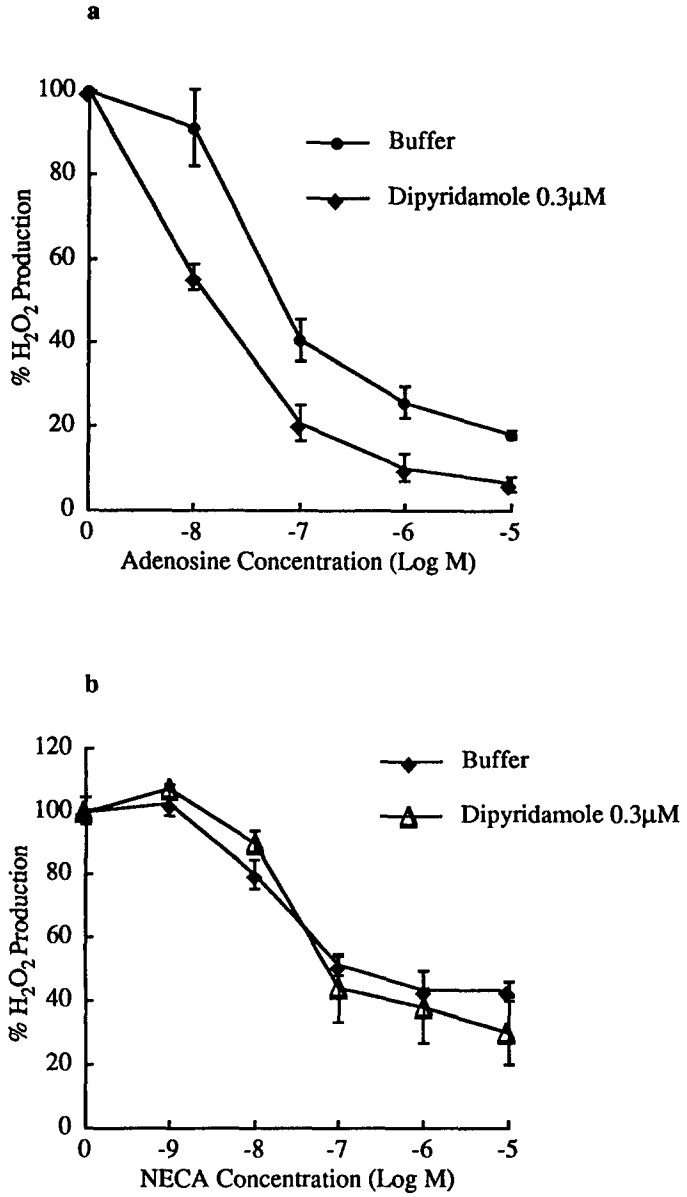

Fig. 7. The effects of adenosine (a) and NECA (b) on H_2O_2 production in the presence or absence of dipyridamole. Dipyridamole (0.3 μM) was added with adenosine or NECA 5 min before fMLP (1 μM). Control in adenosine group and dipyridamole group are buffer and dipyridamole (0.3 μM), respectively. Control values in panel a: buffer, 3675 ± 171; dipyridamole, 4750 ± 689 arbitrary chemiluminescence units; in panel b: buffer, 6950 ± 453; dipyridamole, 6530 ± 183.

per se affects neutrophils only slightly, enhanced the effects of adenosine (Fig. 7a). On the other hand, dipyridamole (0.3 μM) did not alter the inhibitory effect of NECA (Fig. 7b). However, another transport inhibitor, NBMPR, did not affect the response to adenosine in any of four separate experiments (results not shown).

DISCUSSION

In agreement with previous reports [26], we found that adenosine analogues inhibited fMLP-stimulated oxidative burst and exocytosis in neutrophils in a concentration-dependent fashion. Also in agreement with previous findings, the effect appeared to be due to activation of A_2 receptors [1, 22, 27]. Although neutrophils possess A_1 as well as A_2 receptors [22], DPCPX, a potent and selective antagonist of A_1 receptors, had no effect on fMLP responses. Since adenosine was already active in nanomolar concentrations, the receptor may be of the A_{2a} subtype [21, 28], which responds to adenosine analogues at much lower concentrations than does the A_{2b} receptor. The novel finding is that propentofylline could significantly enhance the inhibitory effect of adenosine. On the other hand,

it did not enhance the effect of the adenosine analogue NECA. This is similar to the situation in brain slices, where propentofylline enhanced adenosine- but not NECA-induced cAMP accumulation [13]. This strongly suggests that the effect of propentofylline is exerted prior to the activation of the adenosine receptor. The mechanism involved is probably the reported effect of propentofylline on adenosine uptake [13, 14, 29, 30]. This contention is supported by the finding that dipyridamole, a well-known inhibitor of adenosine transport [24], also enhanced the effect of adenosine, but not that of NECA. It was more surprising that NBMPR had no effect. NBMPR is a potent inhibitor of adenosine transport in many cells. The reason could well be that human neutrophils possess a transporter that is inhibited by dipyridamole, but not by NBMPR. Such transporters have previously been described, in the brain for example [31]. Both dipyridamole and propentofylline, when given at higher concentrations, *per se* inhibited oxidative burst in neutrophil leukocytes. The mechanism responsible for this was not examined specifically. However, it is known that both propentofylline and dipyridamole can act as cyclic AMP phosphodiesterase inhibitors [13, 24]. Conceivably this action could raise cyclic AMP to inhibitory levels.

It is tempting to speculate that the effect of propentofylline described here is related to its known protective effects in cerebral ischemia in animals and man [10, 11, 32]. The effect described here also shows some similarity to the ability of propentofylline to reduce free radical formation in microglial cells and macrophages [33]. Thus, a primary action on adenosine transport may influence several types of cells that are important in the development of ischemic cell damage.

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