Adenosine A_{2b} Receptors Evoke Interleukin-8 Secretion in Human Mast Cells

An Enprofylline-sensitive Mechanism with Implications for Asthma

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Abstract

Adenosine potentiates mast cell activation, but the receptor type and molecular mechanisms involved have not been defined. We, therefore, investigated the effects of adenosine on the human mast cell line HMC-1. Both the A_{2a} selective agonist CGS21680 and the A_{2a}/A_{2b} nonselective agonist 5'-*N*-ethylcarboxamidoadenosine (NECA) increased cAMP, but NECA was fourfold more efficacious and had a Hill coefficient of 0.55, suggesting the presence of both A_{2a} and A_{2b} receptors. NECA 10 μM evoked IL-8 release from HMC-1, but CGS21680 10 μM had no effect. In separate studies we found that enprofylline, an antiasthmatic previously thought to lack adenosine antagonistic properties, is as effective as theophylline as an antagonist of A_{2b} receptors at concentrations achieved clinically. Both theophylline and enprofylline 300 μM completely blocked the release of IL-8 by NECA. NECA, but not CGS21680, increases inositol phosphate formation and intracellular calcium mobilization through a cholera and pertussis toxin–insensitive mechanism. In conclusion, both A_{2a} and A_{2b} receptors are present in HMC-1 cells and are coupled to adenylate cyclase. In addition, A_{2b} receptors are coupled to phospholipase C and evoke IL-8 release. This effect is blocked by theophylline and enprofylline, raising the possibility that this mechanism contributes to their antiasthmatic effects. (*J. Clin. Invest.* 1995. 96:1979–1986.) Key words: adenosine • mast cells • phospholipase C • interleukin-8 • enprofylline

Introduction

It has long been recognized that adenosine interacts with mast cells (1). Adenosine does not produce direct activation of mast cells, but it potentiates mast cell activation induced by a variety of stimuli. The adenosine receptor type present in mast cells varies depending on the type of mast cell and species studied. A3 receptors potentiate activation of rat basophil leukemia cells (RBL-2H3), a cell line used as a model for rat mast cells (2). On the other hand, mRNA for both A_{2a} and A_{2b} receptors have been identified in mouse bone marrow–derived mast cells. Activation of these cells appears to be modulated by A_{2b} receptors (3). A_2 receptors appear to mediate activation of human mast cells (4), but the receptor subtype involved is not known.

The molecular mechanisms by which adenosine potentiates mast cell activation are not fully defined. It was initially proposed that cAMP is involved in this process (5), but, at least in rat mast cells, the effect of adenosine on mediator release seems to be independent of cAMP (6). More recently, it has been shown that adenosine activates protein kinase C in mouse bone marrow–derived mast cells. Furthermore, adenosine-induced potentiation of mediator release in these cells was mimicked by low concentrations of direct activators of protein kinase C. High concentrations of these activators, however, produced the opposite effect (7). Adenosine A3 receptors are also reported to activate phospholipase C via a pertussis toxin–sensitive mechanisms (8).

Although the functional relevance of adenosine actions on mast cells remains to be established, indirect evidence suggest that adenosine activates mast cells in the human lung, provoking bronchoconstriction (9, 10). It has been proposed, therefore, that adenosine plays a role in asthma. We believe it is important to determine the adenosine receptor type(s) present in human mast cells, given the disparity found among different species, and to investigate their molecular mechanism of action.

A major limitation in this area of research has been the difficulty in obtaining a pure preparation of human mast cells. Adenosine A_2 receptors have been reputed to potentiate activation of partially purified dispersed human lung mast cells (4). Inhibition of mast cell activation by adenosine has also been reported in human lung fragments (11), dispersed human lung mast cells (12), and purified lung mast cells (4), depending on the concentration of adenosine used, or the time of incubation. The interpretation of these results, however, can be confounded by the potential indirect effects of adenosine on cell types other than mast cells present in these preparations.

A human mast cell line (HMC-1),[1] derived from a patient with mast cell leukemia, has recently been described (13). Phenotypic characterization of HMC-1 cells revealed considerable similarities with normal human mast cells. HMC-1 cells contain tryptase but not chymase. On the basis of their neutral protease contents, therefore, HMC-1 cells resemble the MC_T type of human mast cells (14), which correspond to the human lung mast cell (15). We have used this cell line to study adenosine receptors and their intracellular signaling pathways.

A major challenge to the hypothesis that adenosine-induced bronchoconstriction plays a role in asthma is the fact that enpro-

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1. *Abbreviations used in this paper:* CGS, 2-[*p*-(carboxyethyl)-phenylethylamino]-5'-*N* ethylcarboxamidoadenosine hydrochloride; DPSPX, 1,3-dipropyl-8-sulfophenylxanthine; G-protein, guanine nucleotide-binding regulatory protein; HEL, human erythroleukemia; HMC-1, human mast cell line; NECA, 5'-*N* ethylcarboxamidoadenosine.

fylline, a theophylline analogue, is an effective antiasthmatic but is thought to lack adenosine antagonistic properties. However, the effect of enprofylline on A_{2b} receptors has not, to our knowledge, been previously studied. We, therefore, examined the effect of enprofylline on the actions of adenosine on human erythroleukemia cells known to be mediated by A_{2b} receptors (16).

Methods

Cells. Human erythroleukemia (HEL) cells were obtained from the American Type Culture Collection (TIB 180; Rockville, MD) and maintained in suspension culture at a density between 3 and 9×10^5 cells/ml by dilution with RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 10% (vol/vol) newborn calf serum, antibiotics, and 2 mM glutamine.

HMC-1 cells were generous gift from doctor J. H. Butterfield (Mayo Clinic, Rochester, MN) and maintained in suspension culture at a density between 3 and 9×10^5 cells/ml by dilution with Iscove's medium supplemented with 10% (vol/vol) FBS, 2 mM glutamine, antibiotics, and 1.2 mM α-thioglycerol. Cells were kept under humidified atmosphere of air/CO_2 (19:1) at 37°C.

Measurement of intracellular calcium. Cytosolic free calcium concentrations were determined by fluorescent dye technique. HMC-1 cells (2×10^6 cells/ml) were loaded with 1 μM FURA-2/acetoxymethyl ester in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM Hepes-NaOH, pH 7.4, and 0.35% BSA. After incubation for 30 min, cells were washed to remove excess of FURA-2 and were resuspended in the same buffer containing 0.25 U/ml adenosine deaminase. HMC-1 cells were suspended at a concentration of 10^5 cells/ml in the same buffer without BSA. Fluorescence was monitored at an emission wavelength of 510 mm and excitation wavelengths of 340 and 380 mm. Maximal fluorescence was determined by addition of 20 μl of 0.4% digitonin. Minimal fluorescence was determined by addition of 40 μl of 1 M EGTA. The intracellular calcium was calculated using previously described formulas (17), assuming a K_d of 224 nM. Fluorescence was measured with a spectrofluorimeter (Fluorolog 2; Spex Industries, Inc., Edison, NJ) in a thermostated cuvette (37°C).

Measurement of cAMP. Before each experiment, HMC-1 and HEL cells were harvested, washed by centrifugation (100 *g* for 10 min), and resuspended in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM Hepes-NaOH, pH 7.4, and 0.25 U/ml adenosine deaminase to a concentration of 3×10^6 cells/ml (HMC-1 cells) or 10^7 cells/ml (HEL cells). HMC-1 cells were preincubated for 3 min at 37°C in a total vol of 198 μl (178 μl for HEL cells) of buffer, containing the cAMP phosphodiesterase inhibitor papaverine (0.1 mM). cAMP accumulation in response to adenosine agonists was measured by the addition of the agonist (2 μl) to the cell suspension. The adenosine antagonists enprofylline and theophylline (20 μl), or buffer control, were added to HEL cells as indicated. Cells were then mixed with a vortex and the incubation allowed to proceed for 3 min (2 min for HEL cells) at 37°C. The reaction was stopped by addition of 50 μl of 25% TCA. TCA-treated extracts were washed five times with 10 vol of water-saturated ether. cAMP concentrations were determined by competition binding of tritium-labeled cAMP to a protein derived from bovine muscle which has high specificity for cAMP (18) (cAMP assay kit, TRK.432; Amersham Corp., Arlington Heights, IL).

Measurement of [^{3}H]inositol phosphate formation. Formation of inositol phosphate was determined using a modification of the procedure described by K. Seuwen et al. (19). HMC-1 cells, at the concentration 10^7 cells/ml, were labeled to equilibrium with *myo*-[^{3}H]inositol (2 μCi/ml, DuPont-NEN, Boston, MA) for 24 h in serum-free Iscove's medium containing 0.25 U/ml adenosine deaminase. The HMC-1 cells were then washed twice and resuspended in buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 5 g/liter D-glucose, 10 mM Hepes-NaOH, pH 7.4, 1 U/ml adenosine deaminase, and 20 mM LiCl to a concentration of 5×10^7 cells/ml. After preincubation at room temperature for 10 min, cells (178 μl) were added to tubes containing adenosine agonists (2 μl) and antagonists (20 μl) or their corresponding vehicles, and the incubation was allowed to proceed for 30 min at 37°C. Cells were collected by centrifugation and resuspended in 200 μl of ice-cold 10 mM formic acid (pH 3). After 30 min, this solution, containing the extracted inositol phosphates and inositol, was collected by centrifugation and diluted with 800 μl of 5 mM NH_3 solution (final pH, 8–9). This solution was then applied to a column containing 0.2 ml anion exchange resin (AG 1-X8, formate form, 200–400 mesh; Bio-Rad Laboratories, Richmond, CA). Free inositol and glycerophosphoinositol were eluted with 1.25 ml of H_2O and 1 ml of 40 mM ammonium formate/formic acid, pH 5, respectively. Total inositol phosphates were eluted in the single step with 1 ml of 2 M ammonium formate/formic acid, pH 5, and radioactivity was measured by liquid scintillation counting.

Determination of IL-8 secretion. HMC-1 cells were harvested and resuspended to a concentration of 10^6 cells/ml in serum-free Iscove's media, containing 0.25 U/ml adenosine deaminase. Cells were incubated for 18 h under humidified atmosphere of air/CO_2 (19:1) at 37°C with the reagents indicated in Results. At the end of this incubation period the culture media were collected by centrifugation at 100 *g* for 10 min. IL-8 concentrations were measured in the culture media using an ELISA method (Quantikine; R & D Systems, Minneapolis, MN).

Drugs. 1,3-dipropyl-8-sulfophenylxanthine (DPSPX), 5′-*N*-ethylcarboxamidoadenosine (NECA), 2-[*p*-(carboxyethyl)-phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS 21,680) and 3-*n*-propylxanthine (enprofylline) were purchased from Research Biochemicals, Inc. (Natick, MA). Papaverine, calcium ionophore A23187, PMA, and theophylline were obtained from Sigma Chemical Co. (St. Louis, MO). Forskolin was purchased from Calbiochem Corp. (La Jolla, CA).

Data analysis. Calculation of 50% effective concentration (EC_{50}) values from dose-response curves was performed by nonlinear regression analysis using InPlot 4.0 software (GraphPAD Software for Science, San Diego, CA) on a microcomputer. Statistical analysis was performed using InStat 2.0 software (GraphPAD Software). Unpaired Student's *t* test was used for single comparisons. The criterion for significance was $P < 0.05$. Results are presented as mean±standard error.

Results

Effect of enprofylline on adenosine A_{2b} receptors in human erythroleukemia cells. Increasing concentrations of enprofylline produced parallel rightward shifts of the dose-response curve for NECA-induced cAMP accumulation (Fig. 1 *A*). Schild regression analysis revealed slopes close to unity (0.9), indicating that enprofylline acts as a simple competitive antagonist of A_{2b} receptors. The intercept of this linear regression, which is used to estimate the K_i of antagonists, was 7 μM (Fig. 1 *B*). We compared the effects of enprofylline to those of theophylline, another antiasthmatic agent, and DPSPX. Schild analysis of these compounds yielded slopes of 0.9 and 1, and K_i of 13 μM and 141 nM, respectively.

Effect of adenosine agonists on IL-8 production in human mast cells. Incubation of HMC-1 cells with a combination of 50 ng/ml PMA and 200 nM calcium ionophore A23187 for 18 h increased IL-8 release from 12±4 to 1,785±86 pg/10^6 cells ($n = 5$, $P < 0.001$). The response to this combination of drugs was decreased by 22% in the presence of 300 μM enprofylline (to 1,391±74 pg/10^6 cells, $n = 5$, $P < 0.01$). Incubation with enprofylline alone had no significant effect on spontaneous release of IL-8 (to 8±6 pg/10^6 cells, $n = 5$, $P > 0.5$).

Incubation of HMC-1 cells with the nonselective A_{2a}/A_{2b}

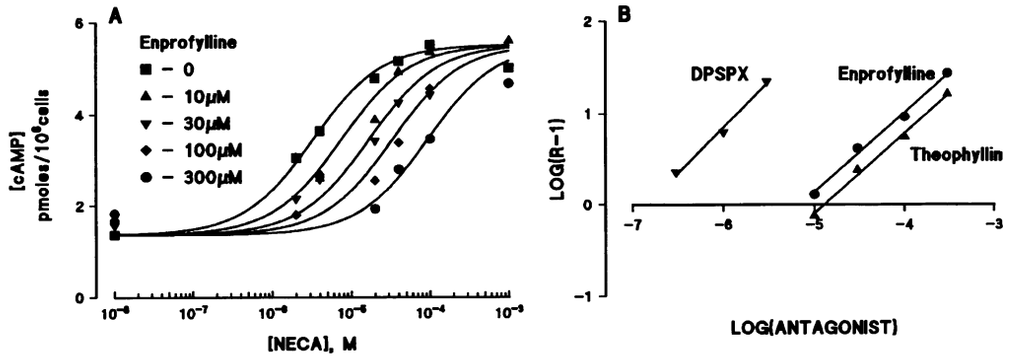

Figure 1. Antagonistic effects of methylxanthines on A_{2b} receptors in HEL cells. (*A*) Dose-response curves for accumulation of cAMP produced by NECA in HEL cells. Dose-response curves were repeated in the absence and in presence of increasing concentrations of enprofylline, which produced a progressive shift to the right. A representative experiment of four is shown. (*B*) Schild analysis of the data from (*A*) and data obtained in similar experiments with the adenosine receptor antagonists theophylline and DPSPX. Schild analysis revealed linear relationships for all compounds, implying competitive antagonism at A_{2b} receptors.

agonist NECA (10 μM) for 18 h resulted in a 26-fold increase in the release of IL-8 (from 12±4 to 306±23 pg/10^6 cells, n = 5, $P < 0.001$). The increase in IL-8 release produced by NECA was blocked if cells were incubated in the presence of 300 μM enprofylline (38±6 pg IL-8/10^6 cells, $n = 5$, $P < 0.001$ compared to NECA + vehicle) or 300 μM theophylline (57±10 pg IL-8/10^6 cells, n = 5, $P < 0.001$ compared to NECA + vehicle) (Fig. 2 *A*). In contrast to the stimulatory effects of NECA on IL-8 release, the selective A_{2a} agonist CGS 21680 (10 μM) produced only a marginal increase in IL-8 release (29±4 pg IL-8/10^6 cells, n = 5, P = 0.05) comparable to the effect produced by NECA in the presence of enprofylline.

We also determined if adenosine potentiated IL-8 production induced by an independent stimulus. PMA (0.6 ng/ml, 1 nM) stimulated IL-8 production to 525±20 pg/10^6 cells (n = 3). NECA (10 μM) increased IL-8 production to 356±20 pg/10^6 cells (n = 3) (Fig. 2 *B*). The combination of NECA and PMA stimulated IL-8 production to 2,594±122 pg/10^6 cells (n = 3). To determine if this potentiation could be due to activation of adenylate cyclase by NECA, we used forskolin and 8-Br-cAMP as controls. Neither forskolin (1, 10, and 100 μM, shown to stimulate adenylate cyclase in these cells) nor 8-Br-cAMP (1, 10, and 100 μM) induced IL-8 production. Likewise, these compounds had no effect on PMA-induced IL-8 production (data not shown).

Effect of adenosine receptor activation on cAMP in HMC-1 cells. The unstimulated level of cAMP in HMC-1 was 2.8±0.1 pmol/10^6 cells. Forskolin (100 μM) increased cAMP accumulation 14-fold, to 38.4±3.0 pmol/10^6 cells ($n = 6$, $P < 0.001$). Forskolin-stimulated cAMP accumulation was not affected by coincubation with 100 μM CGS 21680 (38.0±5.1 pmoles/10^6 cells, n = 3, $P > 0.05$ compared to forskolin + vehicle) but was greater in the presence of 100 μM NECA (to 56.37±5.1 pmol/10^6 cells, n = 3, $P < 0.001$ compared to forskolin + vehicle).

Adenosine agonists produced a dose-dependent accumulation of cAMP in HMC-1 in the absence of forskolin (Fig. 3). NECA was more efficacious than CGS 21680; at concentrations producing maximal effects (1 mM), NECA produced an eight-fold increase in cAMP (to 22.3±3.2 pmol/10^6 cells, n = 3),

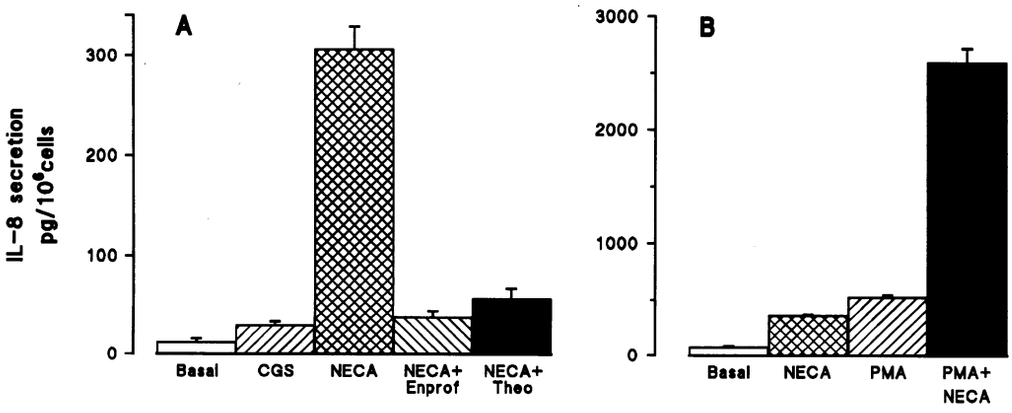

Figure 2. Release of IL-8 from HMC-1 cells. (*A*) Release of interleukin-8 into culture media by unstimulated HMC-1 cells (*Basal*), or by cells stimulated with 10 μM CGS 21,680 (*CGS*), and with 10 μM NECA in the absence (*NECA*) or in the presence of 300 μM enprofylline (*NECA + Enprof*), or 300 μM theophylline (*NECA + Theo*). Values are expressed as mean±standard error of five experiments. (*B*) Release of IL-8 into culture media by unstimulated HMC-1 cells (*Basal*), or by cells stimulated with 10 μM NECA, 1 nM PMA, or 10 μM NECA combined with 1 nM PMA. Values are expressed as mean±standard error of three experiments. Note the difference in the scale of the y-axis between panels *A* and *B*.

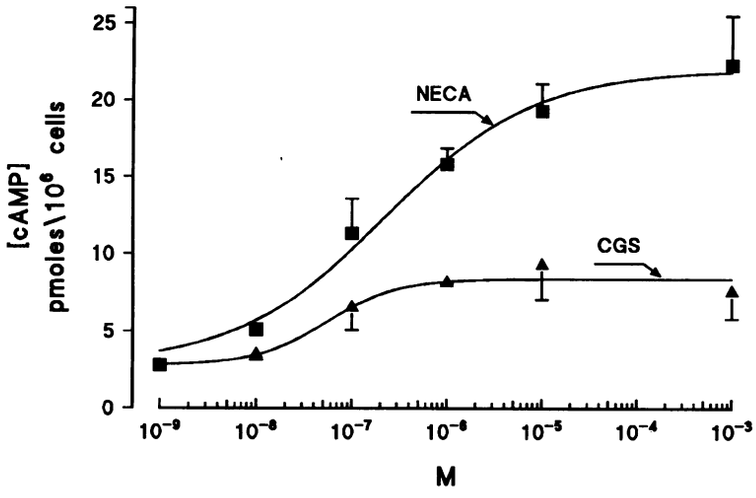

Figure 3. Effect of increasing concentrations of adenosine receptor agonists on cAMP accumulation in HMC-1 cells. Values are expressed as mean±standard error of three experiments.

but CGS 21680 produced only a twofold increase in cAMP (to 7.5±1.7 pmol/10^6 cells, $n = 3$). Nonlinear regression analysis of concentration-response curves revealed an EC_{50} of 225 nM for NECA and 54 nM for CGS 21680. The Hill coefficient for CGS 21680 was close to unity (1.1), consistent with stimulation of cAMP production through a single adenosine receptor subtype. On the other hand, the concentration-response relationship for NECA was characterized by a Hill coefficient of 0.55, suggesting the involvement of more than one adenosine receptor subtype on this effect.

Effect of adenosine receptor activation on intracellular Ca^{2+} in HMC-1 cells. Adenosine analogues produced a dose-dependent increase in intracellular calcium content in HMC-1 (Fig. 4). NECA was more efficacious than CGS 21680; at concentrations (100 μM) producing maximal effects, NECA and CGS 21680 increased Ca^{2+} by 210±6 nM and 75±6 nM, respectively. On the other hand, both agonists had similar potencies; the EC_{50} for NECA and CGS 21680, estimated by nonlinear analysis, were 334 and 296 nM, respectively. It is worth noting, however, that NECA produced a shallow concentration-response curve, with a Hill coefficient significantly lower than unity (0.6). This suggests that NECA stimulates intracellular Ca^{2+} through an interaction with more than one receptor site.

In ancillary studies we demonstrated that the NECA-induced increase in FURA-2 fluorescence could not be explained by leakage of the dye to the extracellular space, because we found that 100 μM NECA did not increase FURA-2 content in the supernatant (data not shown). The increase in intracellular Ca^{2+} produced by NECA, therefore, can be explained either by an increase in extracellular Ca^{2+} influx or by mobilization of internal stores. NECA (10 μM) increased intracellular Ca^{2+} even in cells incubated in the absence of extracellular Ca^{2+}, that is, in a calcium-free medium containing 1 mM EGTA (Fig. 5 *A*). This indicates that NECA increases intracellular Ca^{2+} by evoking Ca^{2+} mobilization. On the other hand, CGS 21680 had no effect on intracellular calcium under these conditions (Fig. 5 *A*). It should be noted that NECA induced a sustained elevation in Ca^{2+} levels in the presence of extracellular Ca^{2+}, but, in the absence of extracellular Ca^{2+}, it induced a transient rise in cytoplasmic Ca^{2+}.

We used an additional approach to determine if NECA can stimulate extracellular calcium influxes or intracellular calcium

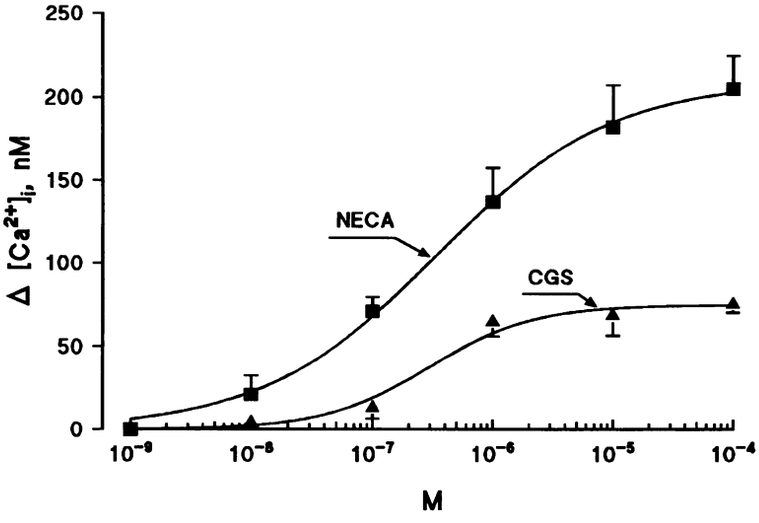

Figure 4. Effect of increasing concentrations of adenosine receptor agonists on free intracellular Ca^{2+} levels in HMC-1 cells. Experiments were performed in the presence of 1 mM $CaCl_2$ in the extracellular media. Values are expressed as mean±standard error of six experiments.

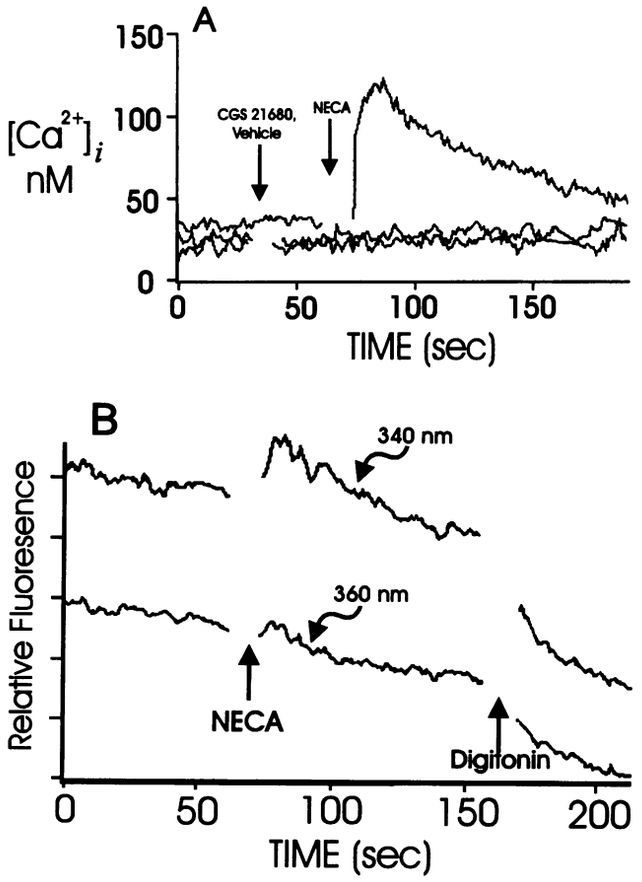

Figure 5. Effect of adenosine analogues on intracellular calcium in HMC-1 cells. (*A*) Effect of 10 μM NECA, 10 μM CGS 21,680, or vehicle on free intracellular Ca^{2+} level in HMC-1. Experiments were performed in the absence of extracellular Ca^{2+}. Cells were maintained in 1 mM $CaCl_2$ buffer and were resuspended immediately before each measurement in a calcium-free buffer with the addition of EGTA to a final concentration of 1 mM. (*B*) Effect of 100 μM NECA on calcium influx and mobilization from internal stores. FURA-2 fluorescence was monitored at two excitation wavelengths, 340 nm (to monitor calcium mobilization), and 360 nm (to monitor calcium influx) in the presence of $MnCl_2$ (20). Experiments were performed as described above. $MnCl_2$ was added instead of EGTA to a final concentration of 100 μM.

mobilization in HMC-1 cells. Mn^{2+} and Ca^{2+} have been shown to share the same channel for entry into cells. At an excitation wavelength of 360 nm and an emission wavelength of 500 nm, fluorescence is selectively quenched by influx of Mn^{2+} and is unaltered by changes in Ca^{2+}. On the other hand, mobilization of Ca^{2+} from internal stores can be assessed simultaneously by monitoring fluorescence at an excitation wavelength 340 nm in cells incubated in a calcium-free medium (20). For these studies Mn^{2+} was added into Ca^{2+}-free buffer just before each measurement, to a final concentration of 100 μM. As shown in Fig. 5 *B*, the addition of NECA to the incubation medium produced an initial increase in the Ca^{2+} signal, followed by slight quenching of the FURA 2 signal by Mn^{2+}. This suggests that NECA induces an initial mobilization of Ca^{2+} from intracellular stores and a subsequent (and probably calcium-mediated) cation influx from extracellular media.

We then determined the potential role of G-proteins in adenosine actions on intracellular Ca^{2+} in HMC-1 cells. Cholera

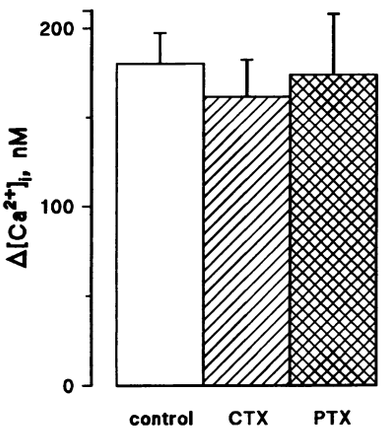

Figure 6. Rise in free intracellular Ca^{2+} levels produced by 10 μM NECA in HMC-1 cells incubated with vehicle (*control*), 100 ng/ml cholera toxin (*CTX*), or 500 ng/ml pertussis toxin (*PTX*) for 24 h. Values are expressed as mean±standard error of five experiments.

toxin was used as a way to increase the basal activity of the stimulatory guanine nucleotide-binding proteins (G_s) and pertussis toxin was used as a way to block the family of inhibitory guanine nucleotide-binding protein (G_i). Pretreatment of HMC-1 cells with 100 ng/ml cholera toxin or 500 ng/ml pertussis toxin for 24 h had no effect on basal or NECA-stimulated intracellular Ca^{2+} levels (Fig. 6). This effect, therefore, is not mediated by coupling to G_s or G_i proteins. To determine the potential role of cAMP we used forskolin. Forskolin, at concentrations of 10 and 100 μM, had no effect on basal Ca^{2+} or on the increase in Ca^{2+} produced by NECA (data not shown). Forskolin effectively stimulated adenylate cyclase at these concentrations. These results, therefore, suggest that NECA-induced rise in intracellular Ca^{2+} is not mediated by an increase cAMP levels in HMC-1.

Effect of adenosine receptor activation on inositol phosphate formation in HMC-1 cells. The major pathway of intracellular calcium mobilization involves phospholipase C activation with phosphoinositide hydrolysis. To determine the role of this pathway on adenosine actions, we measured the accumulation of total inositol phosphates in the presence of 20 mM LiCl. NECA 10 μM considerably increased the accumulation of inositol phosphates (from 2,402±48 dpm/tube to 3,906±99 dpm/tube, $n = 5$, $P < 0.001$, Fig. 7). In contrast, 10 μM CGS 21680 had no effect on levels of inositol phosphates. Enprofylline 300 μM and theophylline 300 μM blocked the increase in inositol phosphates produced by NECA in HMC-1 cells. Neither antagonist affected basal levels of inositol phosphates.

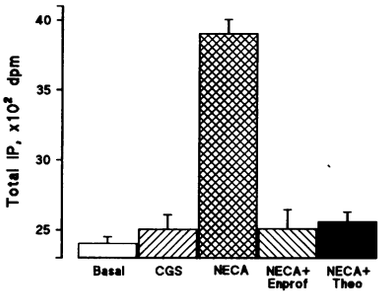

Figure 7. Accumulation of total inositol phosphates (*IP*) in unstimulated cells (*Basal*), and in cells stimulated with 10 μM CGS 21,680 (*CGS*), or 10 μM NECA in the absence (*NECA*), or in the presence of 300 μM enprofylline (*NECA + Enprof*), or 300 μM theophylline (*NECA + Theo*). Values are expressed as mean±standard error of five experiments.

Discussion

The hypothesis that adenosine plays a role in asthma was initially suggested by the recognition that theophylline blocks adenosine receptors at concentrations achieved clinically (21, 22). Even though methylxanthines may have other mechanisms of action in vitro, such as inhibition of phosphodiesterases or mobilization of intracellular calcium, it is believed that these effects require higher concentrations than those clinically achieved in vivo (21). The antiasthmatic effects of theophylline, therefore, could result from blockade of endogenous adenosine. This assumes, however, that adenosine is able to provoke asthma. In support of this assumption, administration by inhalation of adenosine, or of its precursor AMP, provokes bronchoconstriction in asthmatics but not in normal subjects (23). Adenosine-induced bronchoconstriction is most likely mediated by activation of mast cells because this effect is blocked not only by adenosine receptor antagonists (9) but also by selective histamine H1 blockers (24, 25) and cromolyn sodium (23, 26). These in vivo findings are in agreement with the observations made by Marquardt and colleagues demonstrating that adenosine activates mast cells in vitro (7, 27).

A major challenge to the hypothesis that adenosine contributes to asthma comes from the "enprofylline paradox." Enprofylline (3-*N*-propylxanthine) is as effective as theophylline (1, 3, 7, trimethylxanthine) in the treatment of asthma, but was, heretofore, believed not to block adenosine receptors (28, 29). This assertion, however, derives mostly from studies performed before the recognition of the existence of A_2 receptor subtypes. More recently, it has been shown that enprofylline does not compete for A_3 receptor binding (30). To the best of our knowledge, the possibility that enprofylline blocks A_{2b} receptor has been overlooked. Our studies found enprofylline to be as potent as theophylline as an A_{2b} receptor antagonist. Furthermore, the K_i of enprofylline (7 μM) is within its recommended therapeutic plasma levels (5–25 μM), indicating that plasma concentrations reached under clinical conditions are sufficient to block A_{2b} receptors.

It has been emphasized that A_{2b} receptors have, in general, significantly less affinity for adenosine agonists than do the other known adenosine receptor subtypes. This is indeed a criterion used to characterize A_{2b} receptors. Less recognized is the fact that A_{2b} receptors may have a similar or even greater affinity to some adenosine antagonists. For example, the affinity of DPSPX for A_{2b} receptors (K_i, 0.1 μM) is 10-fold higher than that for A_{2a} receptors (16). DPSPX is, to the best of our knowledge, the most potent antagonist of A_{2b} receptors known to date, but a systematic search for methylxanthine analogues with A_{2b} blocking properties has not been undertaken. It is possible that more potent antagonists exist or can be developed. Even though enprofylline is 100-fold less potent than DPSPX, it is the only known selective A_{2b} antagonist to date. Therefore, it can become a useful pharmacological tool to characterize A_{2b} receptors and their functional relevance.

It has been assumed that enprofylline alleviates asthma through mechanisms other than adenosine receptor antagonism. Intravenous enprofylline was found to be less effective than theophylline in blocking adenosine-induced bronchoconstriction (31). Plasma concentrations of enprofylline, however, were four times lower than theophylline in that study, whereas our results suggest that both methylxanthines are equipotent in blocking A_{2b} receptors. We believe, therefore, that our findings

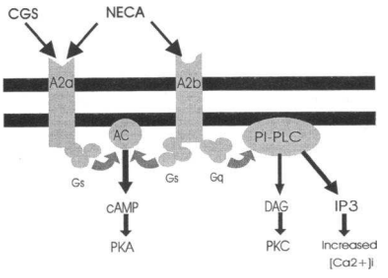

Figure 8. Proposed intracellular pathways for adenosine receptors in HMC-1 cells. See text for details. *CGS,* CGS 21680; *AC,* adenylate cyclase; *PI-PLC,* phosphatidylinositol specific phospholipase C; *PKA,* cAMP-dependent protein kinase; *DAG,* diacylglycerol; *IP3,* inositol trisphosphate; *PKC,* protein kinase C.

solve the enprofylline paradox and raise the possibility that its antiasthmatic effects are due, at least partially, to blockade of adenosine A_{2b} receptors. This postulate, however, assumes that A_{2b} receptors activate mast cells. It is this possibility that we wanted to examine in the human mast cell line HMC-1.

Our results indicate the presence of both A_{2a} and A_{2b} receptors in HMC-1 cells. Both receptor subtypes contribute to adenylate cyclase activation, most likely through coupling with G_s proteins, as previously described in other cell types (16). It is possible that A_{2a} receptors also contribute to the increase in intracellular calcium observed in HMC-1 cells. This would explain the small increase in intracellular calcium produced by CGS 21680 (Fig. 4). This effect, however, is not observed in cells incubated in the absence of extracellular calcium. We found no functional consequence of this phenomenon, since we have no evidence that A_{2a} receptors contribute to phospholipase C activation or IL-8 release in HMC-1 cells. For these reasons, we have not explored the molecular mechanisms of A_{2a}-mediated calcium rise. Because forskolin had no effect on intracellular calcium, it is unlikely that this effect is mediated by cAMP. It is possible that A_{2a} and A_{2b} receptors are directly coupled to a calcium channel through G_s proteins, as suggested for A_{2b} receptors (32) and other G_s-coupled receptors (33, 34).

One of the main differences between A_{2a} and A_{2b} receptors entails modulation of intracellular calcium. Whereas A_{2a} receptors generally inhibit intracellular calcium rises in most cell types (35, 36), activation of A_{2b} receptors generally potentiate intracellular calcium rises. The molecular mechanisms by which A_{2b} modulates intracellular calcium appear to be different among cell types. A_{2b} receptors potentiate a P-type calcium current in hippocampal neurons (37). In human erythroleukemia cells, A_{2b} receptors facilitate calcium influx through a G_s protein–coupled, but cAMP-independent process (32), most likely involving the opening of a calcium channel. Our results show that A_{2b} receptors increase intracellular calcium in HMC-1 cells through a cholera- and pertussis toxin–insensitive process. These findings suggest that a guanine nucleotide-binding protein of the G_q family is involved. The increase in phosphoinositide hydrolysis and intracellular calcium mobilization suggest that A_{2b} receptors activate phospholipase C, and presumably also protein kinase C (Fig. 8).

IL-8 secretion from HMC-1 cells requires increases in gene transcription and de novo protein synthesis (38), but the cellular events leading to this process have not been characterized. Our results do not support involvement of adenylate cyclase, since neither forskolin nor 8-Br-cAMP stimulated IL-8 production. It could be proposed that adenosine-induced IL-8 secretion is the

result of A_{2b}-mediated phospholipase C activation. This would lead to phosphoinositide hydrolysis, calcium mobilization, and protein kinase C activation. In support of this proposal, the most potent stimulus known to evoke IL-8 release from HMC-1 is activation of protein kinase C by phorbol ester (38). Furthermore, there is a striking similarity between the effects of adenosine agonists and antagonists on inositol phosphate formation and IL-8 release (compare Figs. 2 and 7). A direct causal relationship, however, has not been proven.

Marquardt and colleagues (7) have previously demonstrated that adenosine analogues induce translocation of protein kinase C activity in cell membranes of mouse bone marrow–derived mast cells, and suggested that this process contributes to adenosine-induced potentiation of mast cell activation. More recently, these investigators have reported that A_{2b} receptors mediate adenosine actions in these mast cells (3). Our results in human mast cells, therefore, correspond closely to those found by Marquardt and colleagues, in mouse bone marrow–derived mast cells. Two differences between their finding and ours are worth noting. First, adenosine actions on mouse bone marrow–derived mast cells were found to be pertussis toxin–sensitive (39) whereas we found no evidence of G_i coupling in these human mast cells. Second, adenosine does not activate bone marrow–derived mast cells directly; it rather potentiates mast cell activation. In contrast, adenosine alone produced significant IL-8 release in HMC-1 cells. Adenosine also greatly potentiated IL-8 production induced by PMA (Fig. 2 *B*). This effect appears to be synergistic rather than additive and is similar to that observed in mouse bone marrow–derived mast cells. Adenylate cyclase is not involved in this process, since potentiation of PMA-induced IL-8 production was not reproduced by 8-Br-cAMP. Further studies are required to define the mechanisms of adenosine-induced potentiation. It should be noted that inhaled adenosine does not require other stimulants to provoke bronchoconstriction in asthmatics. Whether mast cells are in a constant "preactivated" state in asthma is speculative.

It has been suggested that the recently recognized A3 receptor modulates mast cell activation (40). mRNA encoding A3 receptors is expressed in rat basophil leukemia cells (RBL-2H3), and these receptor types reportedly potentiate activation of these surrogate rat mast cells (2). However, A3 receptors, while prominent in rat mast cells, have not been shown to be functionally present in mast cells derived from other species. Also, the rat A3 receptor is generally insensitive to methylxanthines, including theophylline (41). Human (42) and sheep (30) A3 receptors are sensitive to the antagonistic effects of theophylline and other methylxanthines, but they have a low affinity to enprofylline (30). A3 receptors, therefore, are less likely to be involved in asthma, given the efficacy of enprofylline in the treatment of this disease process. Although we found no evidence for the functional expression of A3 receptors in HMC-1 cells, it remains possible that this receptor type is expressed in other human mast cells.

In summary, our results indicate that the human mast cell line HMC-1 functionally expresses A_{2b} receptors. Their activation leads to increases in phosphoinositide hydrolysis, intracellular calcium mobilization, and IL-8 secretion. Enprofylline is a competitive antagonist of A_{2b} receptors and inhibits adenosine-mediated IL-8 secretion in human mast cells. Taken together, these results support the hypothesis that A_{2b} receptors are involved in the putative role of adenosine in asthma. This conclusion, however, is based on the assumptions that antagonism of A_{2b} receptors accounts for the antiasthmatic effects of enprofylline, and that the HMC-1 cell line is an adequate model for adenosine receptors in human lung mast cells. The validity of these assumptions remains to be determined.

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