Adenosine A2b 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 A2a selective agonist CGS21680 and the A2a/A2b 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 A2a and A2b 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 A2b 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 intracellular calcium mobilization through a cholina- and pertussis toxin–insensitive mechanism. In conclusion, both A2a and A2b receptors are present in HMC-1 cells and are coupled to adenylate cyclase. In addition, A2a 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 A2a and A2b receptors have been identified in mouse bone marrow–derived mast cells. Activ-
fylline, a theophylline analogue, is an effective antiasthmatic but is thought to lack adenosine antagonistic properties. However, the effect of enprofylline on A2b 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 A2b 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 \times 10^8 cells/ml by dilution with RPMI 1640 medium supplemented with 10% (vol/vol) FBS, 1% (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 \times 10^5 cells/ml by dilution with Iscove’s medium supplemented with 10% (vol/vol) FBS, 2 mM glutamine, antibiotics, and 1.2 mM \( \alpha \)-thioglycerol. Cells were kept under humidified atmosphere of air/CO2 (19:1) at 37°C.

Measurement of intracellular calcium. Cytosolic free calcium concentrations were determined by fluorescent dye technique. HMC-1 cells (2 \times 10^3 cells/ml) were loaded with 1 \u03bcM FURA-2/acetoxymethylester in a buffer containing 150 mM NaCl, 2.7 mM KCl, 0.37 mM NaHPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 5 g/liter \( \alpha \)-glucose, 10 mM Hepes-NaOH, pH 7.4, and 0.35% BSA. After incubation for 30 min, cells were resuspended to remove excess 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 400 nm. Cells were then washed to remove excess Hepes-NaOH. Fluorescence of intracellular calcium was calculated using previously described formulas (17), assuming a Kₚ of 224 nM. Fluorescence was measured with a spectrofluorometer (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 NaHPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 5 g/liter \( \alpha \)-glucose, 10 mM Hepes-NaOH, pH 7.4, and 0.25 U/ml adenosine deaminase to a concentration of 3 \times 10^5 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 \mu l (178 \mu l for HEL cells) of buffer, containing the nonselective phosphodiesterase inhibitor papaverine (0.1 \mu M). cAMP accumulation in response to adenosine agonists was measured by the addition of the agonist (2 \mu M) to the cell suspension. The adenosine antagonists enprofylline and theophylline (20 \mu M), 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 \mu l 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 \([^3H]\)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 10^6 cells/ml, were labeled to equilibrium with myo-[\( ^3H \)]inositol (2 \muCi/ml, DuPont-NEC, 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 NaHPO₄, 1 mM MgSO₄, 1 mM CaCl₂, 5 g/liter \( \alpha \)-glucose, 10 mM Hepes-NaOH, pH 7.4, 1 U/ml adenosine deaminase, and 20 mM LiCl to a concentration of 5 \times 10^5 cells/ml. After preincubation at room temperature for 10 min, cells (178 \mu l) were added to tubes containing adenosine agonists (2 \mu M) and antagonists (20 \mu M) 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 \mu l 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 \mu l of 5 mM NH₃ 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 H₂O and 1 ml of 40 mM ammonium formate/formic acid, pH 5, respectively. Total inositol phosphates were eluted in the same 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/CO₂ (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'-\( \alpha \)-ethylcarboxamidoadenosine (NECA), 2-[\( \beta \)-carboxyl(ethyl)-\( \alpha \)-phenylethylamino]-5'-\( \alpha \)-ethylcarboxamidoadenosine hydrochloride (CGS 21,680) and 3-\( \alpha \)-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 (EC₅₀) 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 A₂b 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 A₂b receptors. The intercept of this linear regression, which is used to estimate the Kᵦ of antagonists, was 7 \mu M (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 Kᵦ of 13 \mu M and 141 \mu M, 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 mM 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 \mu M 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 A₂b/A₂b...
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/10⁶ 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-8/10⁶ cells, n = 5, P < 0.001 compared to NECA + vehicle) or 300 μM theophylline (57±10 pg IL-8/10⁶ 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 A₂b agonist CGS 21680 (10 μM) produced only a marginal increase in IL-8 release (29±4 pg IL-8/10⁶ 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/10⁶ cells (n = 3). NECA (10 μM) increased IL-8 production to 356±20 pg/10⁶ cells (n = 3) (Fig. 2 B). The combination of NECA and PMA stimulated IL-8 production to 2,594±122 pg/10⁶ 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/10⁶ cells. Forskolin (100 μM) increased cAMP accumulation 14-fold, to 38.4±3.0 pmol/10⁶ cells (n = 6, P < 0.001). Forskolin-stimulated CAMP accumulation was not affected by coincubation with 100 μM CGS 21680 (38.0±5.1 pmol/10⁶ cells, n = 3, P > 0.05 compared to forskolin + vehicle) but was greater in the presence of 100 μM NECA (to 56.3±7.5 pmol/10⁶ 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/10⁶ cells, n = 3),

Figure 1. Antagonistic effects of methylxanthines on A₂b 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 A₂b receptors.

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.
but CGS 21680 produced only a twofold increase in cAMP (to 7.5±1.7 pmol/10⁶ cells, n = 3). Nonlinear regression analysis of concentration-response curves revealed an EC₅₀ 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 Ca²⁺ 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 Ca²⁺ by 210±6 nM and 75±6 nM, respectively. On the other hand, both agonists had similar potencies; the EC₅₀ 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 Ca²⁺ 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 Ca²⁺ produced by NECA, therefore, can be explained either by an increase in extracellular Ca²⁺ influx or by mobilization of internal stores. NECA (10 μM) increased intracellular Ca²⁺ even in cells incubated in the absence of extracellular Ca²⁺, that is, in a calcium-free medium containing 1 mM EGTA (Fig. 5 A). This indicates that NECA increases intracellular Ca²⁺ by evoking Ca²⁺ 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 Ca²⁺ levels in the presence of extracellular Ca²⁺, but, in the absence of extracellular Ca²⁺, it induced a transient rise in cytoplasmic Ca²⁺.

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

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**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.

**Figure 4.** Effect of increasing concentrations of adenosine receptor agonists on free intracellular Ca²⁺ levels in HMC-1 cells. Experiments were performed in the presence of 1 mM CaCl₂ in the extracellular media. Values are expressed as mean±standard error of six experiments.
mobilization in HMC-1 cells. Mn2+ and Ca2+ 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 Mn2+ and is unaltered by changes in Ca2+. On the other hand, mobilization of Ca2+ 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 Mn2+ was added into Ca2+-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 Ca2+ signal, followed by slight quenching of the FURA 2 signal by Mn2+. This suggests that NECA induces an initial mobilization of Ca2+ 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 Ca2+ in HMC-1 cells. Cholera toxin was used as a way to increase the basal activity of the stimulatory guanine nucleotide-binding proteins (Gs) and pertussis toxin was used as a way to block the family of inhibitory guanine nucleotide-binding protein (Gi). 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 Ca2+ levels (Fig. 6). This effect, therefore, is not mediated by coupling to Gi or Gs proteins. To determine the potential role of cAMP we used forskolin. Forskolin, at concentrations of 10 and 100 μM, had no effect on basal Ca2+ or on the increase in Ca2+ produced by NECA (data not shown). Forskolin effectively stimulated adenylate cyclase at these concentrations. These results, therefore, suggest that NECA-induced rise in intracellular Ca2+ is not mediated by an increase in 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 2,40±248 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 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 Ca2+ level in HMC-1. Experiments were performed in the absence of extracellular Ca2+. Cells were maintained in 1 mM CaCl2 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 MnCl2 (20). Experiments were performed as described above. MnCl2 was added instead of EGTA to a final concentration of 100 μM.

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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, therefore, believed not to block adenosine receptors (28, 29). This assertion, however, derives mostly from studies performed before the recognition of the existence of A2 receptor subtypes. More recently, it has been shown that enprofylline does not compete for A1 receptor binding (30). To the best of our knowledge, the possibility that enprofylline blocks A3 receptors has been overlooked. Our studies found enprofylline to be as potent as theophylline as an A3 receptor antagonist. Furthermore, the Kᵢ 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 A3 receptors.

It has been emphasized that A3 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 A3 receptors. Less recognized is the fact that A3 receptors may have a similar or even greater affinity to some adenosine antagonists. For example, the affinity of DPSPX for A3 receptors (Kᵢ, 0.1 μM) is 10-fold higher than that for A2b receptors (16). DPSPX is, to the best of our knowledge, the most potent antagonist of A3 receptors known to date, but a systematic search for methylxanthine analogues with A3b 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 A3 receptor antagonist to date. Therefore, it can become a useful pharmacological tool to characterize A3 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 A3 receptors. We believe, therefore, that our findings solve the enprofylline paradox and raise the possibility that its antiasthmatic effects are due, at least partially, to blockade of adenosine A3 receptors. This postulate, however, assumes that A3 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 A2a and A2b receptors in HMC-1 cells. Both receptor subtypes contribute to adenylyl cyclase activation, most likely through coupling with G, proteins, as previously described in other cell types (16). It is possible that A2b 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 A3 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 A3-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 A2a and A2b receptors are directly coupled to a calcium channel through G, proteins, as suggested for A2b receptors (32) and other G,-coupled receptors (33, 34).

One of the main differences between A2a and A2b receptors entails modulation of intracellular calcium. Whereas A2b receptors generally inhibit intracellular calcium rises in most cell types (35, 36), activation of A2b receptors generally potentiate intracellular calcium rises. The molecular mechanisms by which A2b modulates intracellular calcium appear to be different among cell types. A2b receptors potentiate a P-type calcium current in hippocampal neurons (37). In human erythroblastic cells, A2b receptors facilitate calcium influx through a G, protein–coupled, but cAMP-independent process (32), most likely involving the opening of a calcium channel. Our results show that A2b 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, family is involved. The increase in phosphoinositide hydrolysis and intracellular calcium mobilization suggest that A2b 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 adenylyl cyclase, since neither forskolin nor 8-BrcAMP stimulated IL-8 production. It could be proposed that adenosine-induced IL-8 secretion is the
result of A2B-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 A2B 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 Gxi 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. 2B). 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 A3B receptors. Their activation leads to increases in phosphoinositide hydrolysis, intracellular calcium mobilization, and IL-8 secretion. Enprofylline is a competitive antagonist of A3B receptors and inhibits adenosine-mediated IL-8 secretion in human mast cells. Taken together, these results support the hypothesis that A3B receptors are involved in the putative role of adenosine in asthma. This conclusion, however, is based on the assumptions that antagonism of A2B 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.

Acknowledgments

The authors thank Drs. William Serafin and Jack Wells for helpful suggestions in the design of studies and interpretation of results, and Dr. J. H. Butterfield for providing HMC-1 cells. This work was supported by grants RR00095 (Clinical Research Center) and HL-14192 (Specialized Center of Research in Hypertension) from the National Institutes of Health. Dr. Feoktistov is a recipient of an American Lung Association research grant.

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