Inosine Binds to A_3 Adenosine Receptors and Stimulates Mast Cell Degranulation

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Abstract

We investigated the mechanism by which inosine, a metabolite of adenosine that accumulates to > 1 mM levels in ischemic tissues, triggers mast cell degranulation. Inosine was found to do the following: (*a*) compete for [^{125}I]*N*^{6}-aminobenzyladenosine binding to recombinant rat A_3 adenosine receptors (A_3AR) with an IC_{50} of 25±6 μM; (*b*) not bind to A_1 or A_{2A} ARs; (*c*) bind to newly identified A_3ARs in guinea pig lung (IC_{50} = 15±4 μM); (*d*) lower cyclic AMP in HEK-293 cells expressing rat A_3ARs (ED_{50} = 12±5 μM); (*e*) stimulate RBL-2H3 rat mast-like cell degranulation (ED_{50} = 2.3±0.9 μM); and (*f*) cause mast cell–dependent constriction of hamster cheek pouch arterioles that is attenuated by A_3AR blockade. Inosine differs from adenosine in not activating A_{2A}ARs that dilate vascular smooth muscle and inhibit mast cell degranulation. The A_3 selectivity of inosine may explain why it elicits a monophasic arteriolar constrictor response distinct from the multiphasic dilator/constrictor response to adenosine. Nucleoside accumulation and an increase in the ratio of inosine to adenosine may provide a physiologic stimulus for mast cell degranulation in ischemic or inflamed tissues. (*J. Clin Invest.* 1997. 100:2849–2857.) Key words: receptors • purinergic • xanthines • vasoconstriction • asthma

Introduction

Of the four adenosine receptor subtypes that have been cloned (A_1, A_{2A}, A_{2B}, and A_3), the A_3 receptor has recently been implicated as a facilitator of allergic and inflammatory responses (1, 2). Before this discovery, Church and Hughes (3) had noted that adenosine facilitates immunological stimulation of histamine release from rat peritoneal mast cells that is not blocked by xanthines (known to block A_1 and A_2 adenosine receptors), suggesting that facilitation of histamine release is mediated by a then unrecognized purinoceptor. It is now known that insensitivity to xanthine blockade is a characteristic of rat, but not human, A_3 adenosine receptors (4, 5). There also is an inhibitory component of adenosine action to diminish mast cell degranulation that is mediated by an A_2 receptor coupled to cyclic AMP accumulation (6–8).

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Mast cells are found in the lung where they release mediators that constrict bronchiolar smooth muscle. In addition, mast cells are present in other tissues where they reside on the adventitial surface of blood vessels. Degranulation of perivascular mast cells can trigger local microvascular vasoconstriction and systemic hypotension (9, 10). Consistent with its effects on isolated mast cells, adenosine acting at A_3 and A_{2A} adenosine receptors, respectively, has been found to enhance and inhibit degranulation of mast cells surrounding hamster cheek pouch arterioles (11). We have noted recently that like adenosine, inosine (10 μM) also elicits vasoconstriction of hamster cheek pouch arterioles as a result of mast cell degranulation (13). This response to inosine is interesting because there are no known receptors for inosine, and the nucleoside does not bind to A1 or A2 adenosine receptors (14). In this report we show that inosine binds to recombinant rat A_3 adenosine receptors, stimulates RBL-2H3 mast-like cell degranulation, binds to newly characterized A_3 receptors found in guinea pig lung, and constricts hamster cheek pouch arterioles by binding to A_3 receptors on perivascular mast cells. We discuss how the discovery of the receptor-mediated effects of adenosine and inosine on A_{2A} and A_3 receptors can account for previously unexplained responses of mast cells and blood vessels to inosine and inhibitors of adenosine deamination.

Methods

Materials. Tissue culture media and reagents were purchased from GIBCO BRL (Gaithersburg, MD). Adenosine deaminase was from Boehringer Mannheim Biochemicals (Indianapolis, IN); N^6 cyclopentyladenosine, 8-sulfophenyltheophylline (8-SPT),[1] and *R*-N^6-phenylisopropyladenosine, were from Research Biochemicals, Inc. (Natick, MA); C^8-(*N*-methylisopropyl)-amino-N^6-(5′-endohydroxy)-endonorboran-2-yl-9-methyladenine (WRC-0571) was from Dr. Pauline Martin (Discovery Therapeutics, Inc., Richmond, VA); 8-(4-carboxyethenylphenyl)-1,3-dipropylxanthine (BW-A1433), *N*^{6}-(4-amino-3-iodobenzyl)adenosine (ABA), I-ABA, and 3-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine (I-ABOPX) were from Dr. Susan Daluge (Glaxo-Wellcome, Research Triangle Park, NC); *N*^{6}-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide (IB-MECA) was from Dr. Saul Kadin (Pfizer Inc., Groton, CT); rat A_3 adenosine receptor cDNA was from Dr. Fereydoun Sajjadi (Gensia, Inc., La Jolla, CA); Rat A_1 and A_{2A} adenosine receptor cDNAs were from Dr. Steven Reppert (Harvard University, Boston, MA); and rat basophilic leukemia 2H3 clonal cells (RBL-2H3) were from Dr. R.P. Siraganian (National Institutes of Health, Bethesda, MD). ABA and 2-[2-(4-amino-3-iodophenyl) ethylamino]adenosine (APE) were radioiodinated and purified as described previously (15, 16).

1. *Abbreviations used in this paper:* 8-SPT, 8-sulfophenyltheophylline; ABA, *N*^{6}-(4-amino-3-iodobenzyl)adenosine; APE, 2-[2-(4-amino-3-iodo-phenyl)ethylamino]adenosine; BW-A1433, 8-(4-carboxyethenylphenyl)-1,3-dipropylxanthine; I-ABOPX, 3-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine; IB-MECA, *N*^{6}-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; RBL-2H3, rat basophilic leukemia 2H3 clonal cells; WRC-0571, C^8-(N-methylisopropyl)-amino-N^6-(5′-endohydroxy)-endonorbornan-2-yl-9-methyladenine.

Stable transfection of HEK 293 cells. For stable transfections, the cDNAs of rat A_1, A_{2A}, or A_3 receptors were subcloned into the expression plasmid CLDN10B, and introduced into HEK-293 cells by means of Lipofectin (GIBCO BRL; 17). Colonies were screened for adenosine receptor expression by radioligand binding using [^{125}I]ABA (A_1, A_3) or [^{125}I]APE (A_{2A}). Colonies were maintained in growth medium supplemented with 0.5 μg/ml G 418.

Membrane preparation. HEK-293 cell monolayer cultures were washed with PBS (GIBCO/BRL) and harvested in buffer A (10 mM Na-Hepes, 10 mM EDTA, pH 7.4) supplemented with protease inhibitors (10 μg/ml benzamidine, 100 μM PMSF, and 2 μg/ml each of aprotinin, pepstatin and leupeptin). The cells were homogenized, centrifuged at 30,000 *g* for 25 min, and washed twice with buffer HE (10 mM Na-Hepes, 1 mM EDTA, pH 7.4, plus protease inhibitors). Guinea pig brains or lungs were rinsed in ice-cold saline, transferred to buffer A, and homogenized for 30 s at setting 3 using a homogenizer (Brinkmann Instruments, Inc., Westbury, NY). Membranes were pelleted and washed twice by centrifugation. Final pellets were resuspended in HE, supplemented with 10% (wt/vol) sucrose and protease inhibitors, and frozen in aliquots at −80°C. Protein concentrations were measured by fluorescamine fluorescence using BSA standards (18).

Radioligand binding assays. Radioligands and membranes (10–50 μg membrane protein) were incubated in triplicate for 2.5 h at 21°C in 100 μl of buffer containing 10 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, and 1 U/ml adenosine deaminase. Nonspecific binding was measured in the presence of 10 μM of the nonradioactive forms of the radioligands. For some equilibrium binding assays, the specific activity of [^{125}I]ABA was reduced 10–20-fold with the corresponding nonradioactive compound. Binding assays were terminated by filtration over Whatman GF/C glass fiber filters using a Brandel cell harvester (Gaithersburg, MD). The filters were rinsed with three times with 4 ml ice-cold 10 mM Tris-Cl (pH 7.4) and 5 mM $MgCl_2$ at 4°C, and were counted in a Wallac γ counter. Competition binding assays were carried out using 0.5–1 nM [^{125}I]ABA or [^{125}I]APE and varying concentrations of competing ligands in a final volume 0.1 ml.

Analysis of radioligand binding. [^{125}I]ABA–specific binding to A_3 adenosine receptors was optimally fit (19) to a two-site binding model: $B = B_{max1} \times L/(K_{D1} + L) + B_{max2} \times L/(K_{D2} + L) + f \times L$ (where B = total binding, L = free radioligand, and f = fraction of L nonspecifically bound). Specific binding of [^{125}I]ABA to lung membranes was fit to a single site hyperbolic equation. To obtain IC_{50} values and Hill coefficients in competition binding assays, data were fit to a four-parameter logistic equation: $B = B_0[1-[I]^N/(IC_{50}^N + I^N)]$ (where B = specific binding, B_0 = specific binding in the absence of inhibitor, [I] = concentration of inhibitor and N = Hill coefficient).

Cyclic AMP assays. HEK-293 cells transfected to express rat A_3 adenosine receptors were removed from plates in ice-cold PBS supplemented with 5 mM EDTA, washed twice with PBS, resuspended in serum-free Hepes-buffered DMEM (20 mM Hepes, pH 7.2) and pipetted into test tubes (50,000 cells/0.2 ml) at 21°C. Suspended cells were incubated for 15 min with 1 U/ml adenosine deaminase, 1 μM isoproterenol,[2] 100 μM ascorbic acid, 20 μM Ro-20-1724, and various concentrations of other compounds as indicated. Isoproterenol-stimulated cyclic AMP concentrations ranged from 40–60 pmol/ml, and were maximally reduced by ~ 50% upon addition of 1 μM IB-MECA in cells transfected with rat A_3 receptors. IB-MECA and inosine had no effect on untransfected HEK-293 cells. Data were normalized according to the following ratio: $(R-R_{max})/(R_0-R_{max})$ where R represents [cyclic AMP], R_0 represents [cyclic AMP] with zero adenosine agonist, and R_{max} represents [cyclic AMP] with 1 μM IB-MECA. Assays were terminated by the addition of 0.5 ml of 0.15 N HCl. Cyclic AMP in the acid extract (0.5 ml) was acetylated and measured by automated radioimmunoassay (20).

Mast cell degranulation. The release of β-hexosaminidase (a granule-associated protein that parallels histamine release) was used as a measure of degranulation of RBL-2H3 cells by modification of the method of Schwartz et al. (21). Cells were primed to give near 100% occupancy of IgE receptors by overnight incubation with 0.5 μg/ml anti-DNP-albumin IgE. Mast cells were washed twice in Ca^{2+}/Mg^{2+}-free Tyrode's buffer (137 mM NaCl, 12 mM $NaHCO_3$, 5.6 mM glucose, 2.7 mM KCl, 0.4 mM NaH_2PO_4, 0.1 g/100 ml gelatin, 25 mM Hepes, pH 7.4) and then resuspended in complete Tyrode's buffer (1 mM $CaCl_2$ and 0.5 mM $MgCl_2$) at a density of 2×10^5 cells/ml. Cells were then transferred to a 96-well plate in 250-μl aliquots and prewarmed to 37°C for 15 min with a 1 ng/ml DNP-albumin. 50-μl aliquots of 5× agonists were added for 20 min at 37°C with shaking. Degranulation reactions were stopped by placing the plate on ice for 10 min and pelleting the cells by centrifugation at 200 *g* for 10 min (4°C). 200 μl of the supernatants were removed and added to 50 μl 5 mM *p*-nitrophenyl-*N*-acetyl-d-glucosaminide, 100 mM citric acid, pH 3.8, and incubated at 37°C for 2 h with shaking to measure β-hexosaminidase activity. The reactions were stopped by addition of 50 μl of 0.4 M $NaCO_3$. To measure total β-hexosaminidase content, 50 μl of lysis buffer (complete Tyrode's buffer plus 0.6% Triton X-100) was added to 250-μl aliquots of cells, and 20 μl was removed and analyzed. Absorbance was read at 405 nm using a Titertek Multiskan II plate reader (Flow Labs, McLean, VA). Experiments were performed in triplicate, and release of β-hexosaminidase is expressed as fraction of the total enzyme found in unstimulated cells.

Hamster cheek pouch arterioles. Hamster cheek pouches were everted and prepared for in vivo microscopy as previously described (13). Cheek pouches were superfused with a bicarbonate-buffered saline solution at 37°C that contained the following (in mM): 131.9 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, and 20.0 $NaHCO_3$. The superfusion solution was gassed with 5% CO_2/95% N_2. After 30 min of equilibration, microvessels were observed using a Optiphot microscope (Nikon Inc., Melville, NY) equipped with a Leitz (Michigan City, IN) 50× objective (numerical aperture = 0.60). Images were projected to a Dage-MTI video camera with a Newvicon tube, displayed on a video monitor, and recorded using an Omnivision II video cassette recorder (model NV-8950; Panasonic, Secaucas, NJ). Adenosine or inosine was diluted from a stock solution into an isotonic, buffered saline solution (mM composition: 131.9 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 10.0 3-[*N*-morpholino] propane-sulfonic acid; pH 7.4) and loaded into glass micropipettes (~ 5-mm tip diameter). Pipettes were placed 25 μm from the arteriolar wall, and nucleoside was ejected with a Picospritzer II (General Valve Corp., Fairfield, NJ) using 100% nitrogen at a pressure of 20 psi. The pulse duration varied according to the vessel diameter, but remained in the range of 10–100 msec. Local picospritzer application of the buffered saline vehicle had no vasomotor effect. BW-A1433 was prepared as a 100 mM stock solution in 500 mM NaOH, and was diluted 1:20 or 1:10 in 0.9% NaCl. The drug was delivered at a flow rate of 0.05 ml/min through a side port into the superfusate (flowing at 5 ml/min) using a syringe pump (model 355, Sage Instruments) to yield a final concentration on the cheek pouch of 50 or 100 μM.

Analysis of diameter changes. Changes in arteriolar diameter were measured from videotape using a video caliper (Colorado Video Inc., Boulder, CO). Two parameters were measured as previously described (13), the percent of tested vessels that constricted, and the magnitude of the constriction, defined as the difference be-

2. A_3 adenosine receptor agonists counteract the action of isoproterenol or forskolin to increase cyclic AMP in HEK-293 cells. The isoproterenol response is mediated by an endogenous β− adrenergic receptor. Forskolin was avoided in these studies because of the existence of an endogenous A_{2B} adenosine receptor in HEK-293 cells that is synergistically activated by forskolin. In the presence of isoproterenol, activation of A_{2B} adenosine receptors has little effect to increase cyclic AMP over the response elicited by adrenergic stimulation alone. Inosine has no effect on isoproterenol- or forskolin-stimulated cyclic AMP accumulation in untransfected HEK-293 cells.

tween the initial vessel diameter and the peak constriction expressed as a percent of initial diameter [(change in luminal diameter [mm]/initial diameter [mm]) × 100]. Arterioles that did not respond were given a value of 0%. Because of tachyphylaxis in the constrictor response, single sites along arterioles were tested only once. The data are expressed as number of arterioles, and represent the sum of five to six experimental preparations.

Statistical analysis. The fractional incidence of constriction was determined, and control groups were compared with the treated groups using a z test. The magnitude of the change in arteriolar diameter for control versus treated groups was analyzed using a Mann-Whitney rank sum test. Where multiple groups were compared, an ANOVA on ranks was performed. In all comparisons and tests, $P < 0.05$ was considered significant.

Results

Characterization of recombinant rat A_3 adenosine receptors by radioligand binding. In initial experiments we examined the ligand-binding properties of recombinant rat A_3 adenosine receptors. The cDNA for these receptors was subcloned into the CLDN10B expression vector and stably expressed in HEK-293 cells that have no detectable endogenous A_3 receptors. Saturation binding of [^{125}I]ABA to membranes containing rat A_3 receptors is shown in Fig. 1 *A*.[3] The B_{max} of recombinant rat A_3 receptors expressed in HEK-293 cells (> 12 pmol/mg protein) is over 50 times higher than the B_{max} reported previously in CHO cells (4). The IC_{50} values of competing compounds for recombinant rat A_3 receptors are summarized in Table I. We were able to confirm that xanthines are weak antagonists of rat A_3 receptors, but as illustrated in Fig. 1 *B* and summarized in Table I, the rat A_3 receptor can be blocked by > 10 μM concentrations of the acidic 8-phenylxanthines BW-A1433 or I-ABOPX.

Table I. Competition by Various Compounds for [^{125}I]ABA Binding to Recombinant Rat A_3 Adenosine Receptors

	IC_{50} (nM) or % inhibition by 1 mM
Adenosine analogs	
IB-MECA	9.51±2.70
R-PIA	16.0±5.5
CPA	250±42
8-phenylxanthine antagonists	
I-ABOPX	14700±4500
BW-A1433	23500±9100
8-SPT	21.1±4.7%
9-methyladenine antagonist	
WRC-0571	232000±14400
Adenosine metabolites	
Inosine	24900±6300
Hypoxanthine	21.2±5.6%
Xanthine	32.8±6.4%

Membranes were prepared from stably transfected HEK-293 cells. Data are means±SEM, n = 3–5.

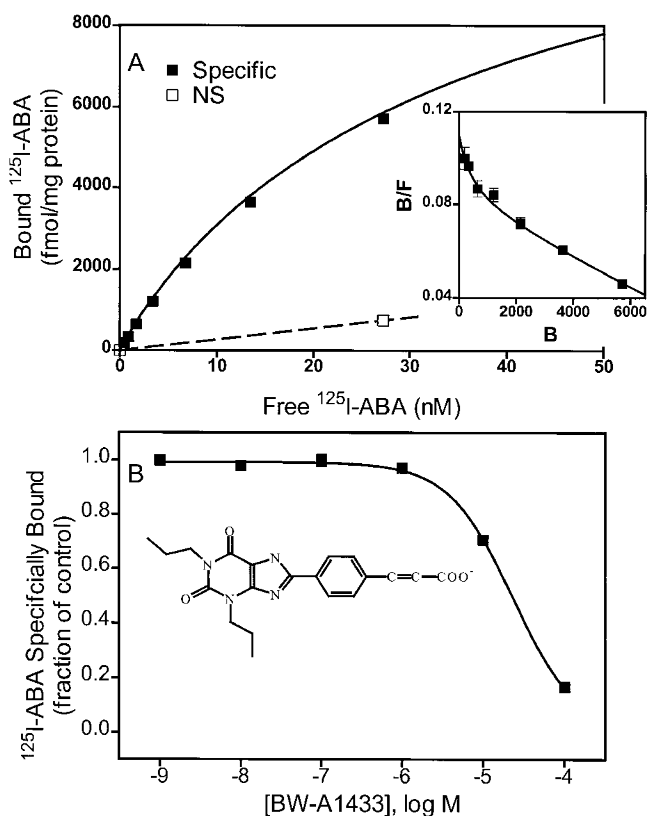

Figure 1. Ligand binding characteristics of recombinant rat A_3 adenosine receptors. (*A*) Specific (■) and nonspecific (□) binding of [^{125}I]ABA to membranes prepared from HEK-293 cells stably transfected with the rat A_3 adenosine receptor. The *inset* is a Scatchard plot of specific binding. Each tube contained 21 μg membrane protein. The specific activity of [^{125}I]ABA was diluted 10-fold with nonradioactive [I]ABA. Specific binding ranged between 1,300 and 42,000 cpm. The data were optimally fit to two binding sites: B_{max1} = 667 fmol/mg protein, K_{D1} = 5.8 nM; B_{max2} = 12,610 fmol/mg protein, K_{D2} = 37 nM. (*B*) Competition by BW-A1433 (structure shown) for [^{125}I]ABA binding. Each tube contained 1 nM [^{125}I]ABA and 15 μg membrane protein. Nonspecific binding was < 5% of total binding. Where omitted, standard error bars are smaller than the symbols. The IC_{50} of BW-A1433 is 23 μM. Results of triplicate experiments are summarized in Table I.

WRC-0571 Binding to A_1 and A_3 adenosine receptors. Since radioligands such as [^{125}I]ABA and [^{125}I]AB-MECA (22) bind to both A_1 and A_3 adenosine receptors, we sought to identify an optimal antagonist useful for selectively blocking A_1 receptors in cells or tissues that express both receptors. The most useful compound we identified for this purpose is WRC-0571. As shown in Fig. 2 *A*, WRC-0571 is over 100,000-fold selective for the A_1 over the A_3 adenosine receptor in the rat. At a concentration of 1 μM, WRC-0571 occupies > 99% of rat A_1 receptors and < 1% of rat A_3 receptors. We next measured [^{125}I]ABA binding to lung membranes of various species to determine if we could detect evidence of A_3 receptors, i.e., [^{125}I]ABA binding sites that are not blocked by WRC-0571. When added with 1 nM [^{125}I]ABA, specific binding (defined as binding not displaced by 10 μM I-ABA) to lung membranes of various species assessed in triplicate experiments was as follows: guinea pig , 85±6% > hamster, 19±3% = cat, 18±4% >

3. The curvilinear Scatchard plot (Fig. 1 *A*, inset) is due to [^{125}I]ABA binding to two affinity states of A_3 β-adenosine receptors. High and low affinity states represent G protein coupled and uncoupled receptors, respectively. The majority of overexpressed recombinant A_3 receptors are uncoupled (Jin and Linden, unpublished data).

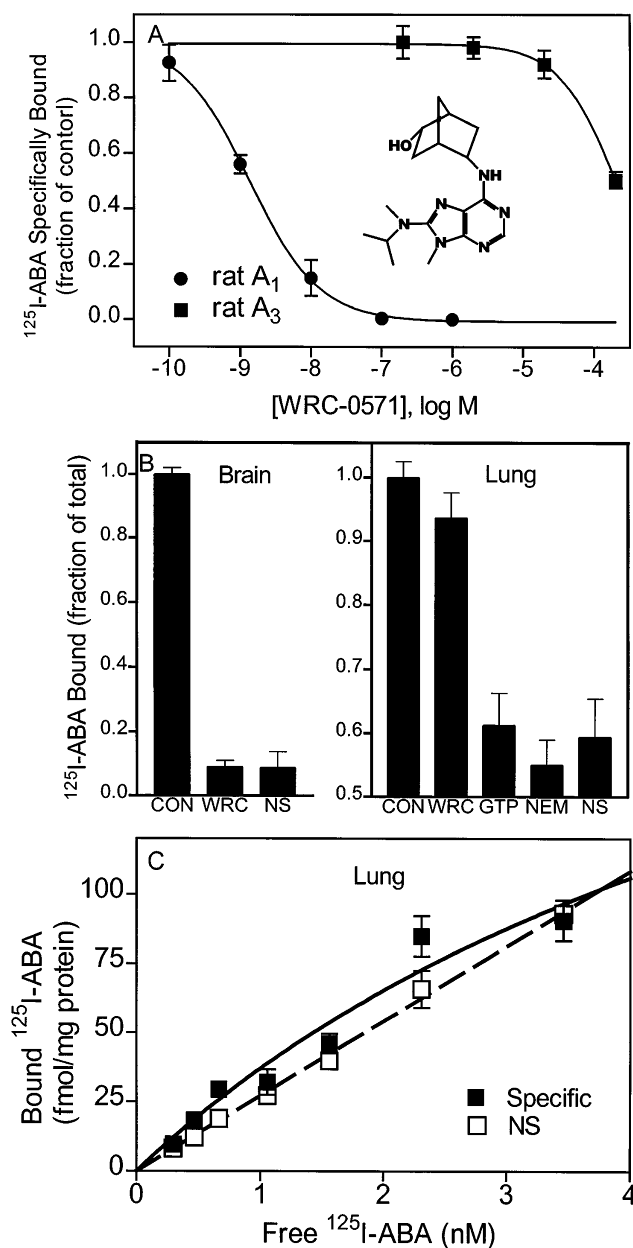

Figure 2. [^{125}I]ABA and WRC-0571 binding to recombinant rat adenosine receptors and guinea pig tissues. (*A*) Membranes derived from HEK-293 cells stably transfected with rat A_1 or A_3 receptors (25 μg membrane protein) were incubated with 1 nM [^{125}I]ABA and various concentration of WRC-0571 or 10 μM nonradioactive I-ABA to define nonspecific binding. Specific binding was > 85% of total binding. IC_{50} values and Hill coefficients are as follows: A_1, 1.35 nM, 0.88; A_3, 207 μM, 1.07. (*B*) Effects of 1 μM WRC-0571 (*WRC*), 10 μM I-ABA (*NS*), 100 μM GTPS (*GTP*), and 100 μM *N*-ethylmaleimide (*NEM*) on [^{125}I]ABA (1.2 nM) binding to guinea pig brain and lung membranes (50 μg membrane protein). (*C*) Binding isotherm of [^{125}I]ABA binding to guinea pig lung membranes. Single-site binding parameters are as follows: B_{max} = 285 fmol/mg protein; K_D = 6.9 nM. Each *bar* or *point* is the mean±SEM of triplicate determinations. The results are typical of triplicate experiments.

dog, rat (< 10%) > human (none detected). A_3 binding sites, defined as specific binding sites insensitive to blockade by 1 μM WRC-0571, represent 85, 70, and 65%, respectively, of the total specific binding sites in lung membranes of guinea pig, hamster, and cat. We also detected 46±5% specific binding of [^{125}I]ABA to human pineal membranes, and 70% of these sites are A_3 (insensitive to blockade by 1 μM WRC-0571). This finding is consistent with detection of high levels of A_3 transcript in the pineal (23). By virtue of relatively high specific binding compared with other species, radioligand binding to guinea pig lung membranes was further characterized. Because of possible species differences, we also evaluated [^{125}I]ABA binding± WRC-0571 to brain membranes since the brain is a rich source of A_1 adenosine receptors. Fig. 2 *B* shows that [^{125}I]ABA binds specifically to receptors in guinea pig brain and lung membranes. Specific binding of [^{125}I]ABA binding to guinea pig brain membranes is completely inhibited by 1 μM WRC-0571, whereas specific binding to lung membranes is minimally affected. These data suggest that [^{125}I]ABA binds predominantly to A_1 and A_3 receptors in guinea pig brain and lung membranes, respectively. WRC-0571–insensitive specific binding of

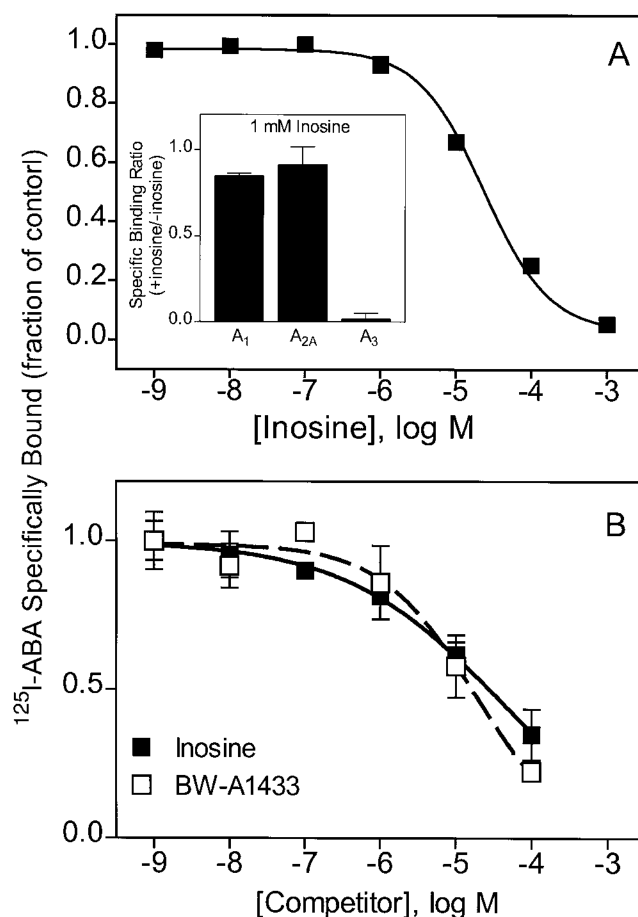

Figure 3. Binding of inosine to rat and guinea pig adenosine receptor subtypes. (*A*) Competition by inosine for specific [^{125}I]ABA binding to membranes of HEK-293 cells containing recombinant rat A_3 adenosine receptors. Each tube contained 20 μg membrane protein and 1 nM [^{125}I]ABA. The IC_{50} of inosine is 25 μM. The *inset* shows the fraction of specific radioligand binding remaining after addition of 1 mM inosine to recombinant rat A_1, A_{2A}, or A_3 receptors, mean±SEM, n = 3. (*B*) Competition by inosine and BW-A1433 for [^{125}I]ABA-specific binding to guinea pig lung membranes. IC_{50} values and Hill coefficients are as follows: inosine, 16 μM, 0.51; and BW-A1433, 23 μM, 0.87. The results are typical of triplicate experiments.

[^{125}I]ABA detected in guinea pig lung was abolished by guanosine 5′-O-(3-thiophosphate) (GTPγS) or *N*-ethylmalemide, providing evidence that [^{125}I]ABA binds to an A_3 receptor that couples to Gi/o G proteins in guinea pig lung (24). Although it was not possible to saturate [^{125}I]ABA binding sites on guinea pig lung homogenates because of high nonspecific binding at radioligand concentrations above 5 nM, binding isotherms such as the one shown in Fig. 2 *C* demonstrate what appears to be a saturable component of high-affinity [^{125}I]ABA binding with a B_{max} of 281±24 fmol/mg protein ($n = 3$).

Competition by inosine for [^{125}I]ABA binding to recombinant A_3 adenosine receptors and to guinea pig lung membranes. Since 10–100 μM inosine mimics the action of adenosine to constrict hamster cheek pouch arterioles by degranulating perivascular mast cells (13), we decided to investigate the possibility that inosine in this concentration range activates A_3 adenosine receptors. Inosine was found to compete for [^{125}I]ABA binding to recombinant rat A_3 receptors with an IC_{50} of 25 μM (Fig. 3 and Table I). The metabolites of inosine, hypoxanthine, and xanthine have lower affinity than inosine for rat A_3 adenosine receptors (Table I). To determine if inosine binds selectively to the A_3 subtype of adenosine receptors, 1 mM inosine was used in competition binding experiments with recombinant rat A_1, A_{2A}, and A_3 receptors. As shown in Fig. 3 *A* (*inset*), 1 mM inosine competes for [^{125}I]ABA-specific binding to rat A_3 receptors, but inosine competes minimally for radioligand binding to other adenosine receptor subtypes.

To determine if [^{125}I]ABA binding sites in the guinea pig lung have pharmacologic characteristics similar to recombinant rat A_3 receptors, we evaluated the potencies of inosine and BW-A1433 in competition binding assays (Fig. 3 *B*). Both inosine and BW-A1433 bind to recombinant rat A_3 adenosine receptors and putative A_3 receptors on guinea pig lung membranes with similar IC_{50} values in the range of 15–35 μM. Hill coefficients for inosine and BW-A1433 were 0.46±0.15 and 0.85±0.1, respectively. These data are consistent with the possibility BW-A1433 is an antagonist and inosine is an agonist that binds to more than one affinity state of A_3 receptors in the lung.

Functional effects of inosine on transfected HEK-293 cells and mast cells. In functional assays, inosine was found to inhibit isoproterenol-stimulated[2] cyclic AMP accumulation in HEK-293 cells transfected with recombinant A_3 receptors, and to stimulate degranulation of rat RBL-2H3 mast-like cells (Fig. 4, Table II). The ED_{50} of inosine to lower cyclic AMP and to facilitate IgE-stimulated degranulation are 12.2±5.3 μM and 2.3±0.9 μM, respectively. These data indicate that inosine is an agonist of rat A_3 adenosine receptors. The cyclic AMP–lowering effect of inosine was surmountably blocked by the addition of 50 μM BW-A1433, which increased the ED_{50} to 41 μM (Fig. 4 *A*). As expected, the synthetic A_3-selective compound, IB-MECA, is much more potent than inosine as an agonist of functional responses in these cell lines. The functional ED_{50} values of inosine and IB-MECA are similar in transfected HEK-293 cells and RBL-2H3 cells, and also are similar to IC_{50} values derived from radioligand binding assays to rat receptors and guinea pig lung membranes (Fig. 3, Tables I and II).

Vascular effects of adenosine and inosine. We have noted previously that both adenosine and inosine can elicit transient mast cell–dependent vasoconstriction of hamster cheek pouch arterioles. These constrictions can be blocked by the mast cell stabilizer cromolyn sodium (9, 11, 13). Application of adenosine can elicit constriction, dilation, or a multiphasic dilator/constrictor response such as the typical response shown in Fig. 5 *A*. In contrast to adenosine, picospritzer application of inosine to individual arterioles usually causes a monophasic constriction as illustrated in Fig. 5 *B*.

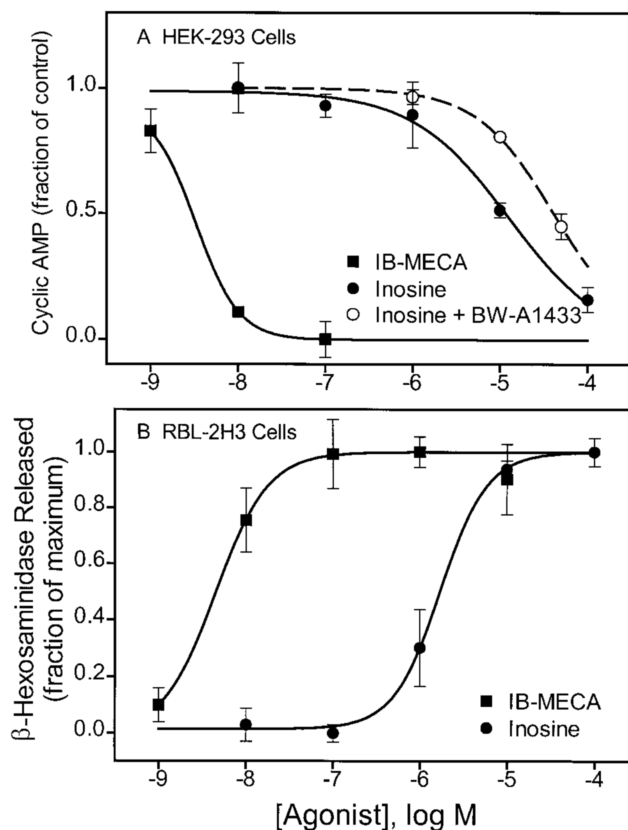

Figure 4. Functional effects of inosine and IB-MECA on cells transfected with A_3 adenosine receptors and RBL-2H3 rat mast-like cells. (*A*) Effects of inosine on isoproterenol-stimulated cyclic AMP accumulation in HEK 293 cells transfected with rat A_3 receptors. The basal cyclic AMP concentration, 1.5 pmol/ml, increased to 60 pmol/ml upon the addition of 10 μM isoproterenol. Maximal inhibition of the isoproterenol response, defined by addition of 1 μM IB-MECA, is 50%. ED_{50} values are as follows: IB-MECA, 3.3 nM; inosine, 12.8 μM; inosine + 50 μM BW-A1433, 41 μM. (*B*) IB-MECA- and inosine-induced β-hexosaminidase release from rat RBL-2H3 mast-like cells. Data points represent triplicate determinations of fractional (maximal-basal) release. The maximal and basal release are 30 and 5%, respectively, of the total cell content. ED_{50} values are as follows: IB-MECA, 4.2 nM; inosine, 1.7 μM. ED_{50} values from triplicate experiments are summarized in Table II.

The reason why adenosine produces a complex and inconsistent vascular response can be attributed to dilator A_2 adenosine receptors on vascular smooth muscle cells, and to A_{2A} receptors on mast cells that inhibit degranulation and counteract the action of A_3 receptors (8, 25). Also, when adenosine is added to the cheek pouch it is subject to transport into cells and conversion to inosine by the predominately intracellular enzyme adenosine deaminase that is particularly rich in vascular endothelial cells (26). The conversion over time of adenosine to inosine may contribute to the multiphasic vascular response. The monophasic vascular constrictor response to

Table II. ED_{50} Values (nM) of IB-MECA, NECA, and Inosine to Degranulate RBL-2H3 Mast-like Cells, and to Lower Cyclic AMP in HEK-293 Cells Stably Transfected with Rat A_3 Adenosine Receptors

	Mast cell degranulation*	HEK-293 cell cyclic AMP inhibition‡
IB-MECA	3.60±0.97	5.0±2.0
NECA	301±59	
Inosine	2280±850	12200±5300

Data are mean±SEM of ED_{50} values from three experiments. *β-hexosaminidase release. ‡Inhibition of isoproterenol-stimulated cyclic AMP accumulation.

inosine is consistent with the inability of inosine to bind to recombinant rat A_{2A} receptors (Fig. 3 *A*, *inset*). If the constrictor response to inosine is mediated by A_3 receptors, we reasoned that the antagonist BW-A1433 should block the response. Fig. 5 *C* tabulates the effects of 144 picospritzer applications of inosine to vessels superfused with 0, 50, or 100 μM BW-A1433. If it is assumed that the response to inosine is proportional to A_3 receptor occupancy, the expected reduction of a submaximal inosine response can be predicted by the equation $R = R_0$ [antagonist]/(IC_{50} + [antagonist]), where R_0 and R refer to inosine responses in the absence and presence of the competitive antagonist, respectively. Using 23 μM as the IC_{50} of BW-A1433, derived from binding to recombinant rat A_3 receptors and guinea pig lung membranes (Table I, Fig. 3 *B*) and assuming that the binding affinity of BW-A1433 is the same in the hamster, the observed and predicted reductions in the inosine constrictor response in the presence of 50 μM BW-A1433 are 68 and 68%, respectively (Fig. 5 *C*). The observed and predicted reductions in response in the presence of 100 μM BW-A1433 are 80 and 81%, respectively. Hence, the data are consistent with the hypothesis that inosine produces constriction by binding to A_3 adenosine receptors that are competitively blocked by BW-A1433 with a K_I of 23 μM.

Discussion

Adenosine exerts autocrine and paracrine effects in all tissues by signaling through four G protein–coupled receptors. Inosine, the product of adenosine deamination, has generally been thought to play little or no role in receptor-mediated cell signaling. In this study, however, we have shown that inosine is a selective agonist of the A_3 adenosine receptor. Inosine was shown in radioligand binding assays and in functional assays to bind to and activate A_3 adenosine receptors (*a*) on HEK-293 cells stably transfected with rat A_3 adenosine receptors; (*b*) on RBL-2H3 rat mast-like cells; (*c*) in guinea pig lung membranes; and (*d*) on perivascular mast cells of hamster cheek pouch arterioles. Inosine binds to or activates rodent (rat, guinea pig, and hamster) A_3 adenosine receptors in the range of 10–50 μM. The range of inosine concentrations over which binding and activation of A_3 adenosine receptors was observed also is similar to the range over which inosine induces ruthenium red staining of degranulating mast cells in the hamster cheek pouch (13). Adenosine analogs such as IB-MECA and [^{125}I]ABA bind to A_3 receptors with over 1,000 times higher affinity than does inosine. Nevertheless, sensitivity to inosine in the 10–50 μM concentration range is likely to be physiologically significant. Normal interstitial concentrations of inosine have been reported to be in the range of 100 μM, and during tissue ischemia, inosine, the predominant nucleoside that accumulates in the interstitial space, rises to > 1 mM (27, 28). Hence, although A_3 adenosine receptors do not have very high affinity for inosine, interstitial concentrations of the nucleoside may be high enough to activate the A_3 receptors, even in well-perfused tissues and certainly in ischemic tissues.

Pharmacological properties of rat A_3 adenosine receptors. We have previously characterized recombinant sheep and human A_3 adenosine receptors (5, 23). These receptors share the property of rat A_3 receptors of binding certain N^6-substituted adenosine analogs such as IB-MECA and I-ABA with high af-

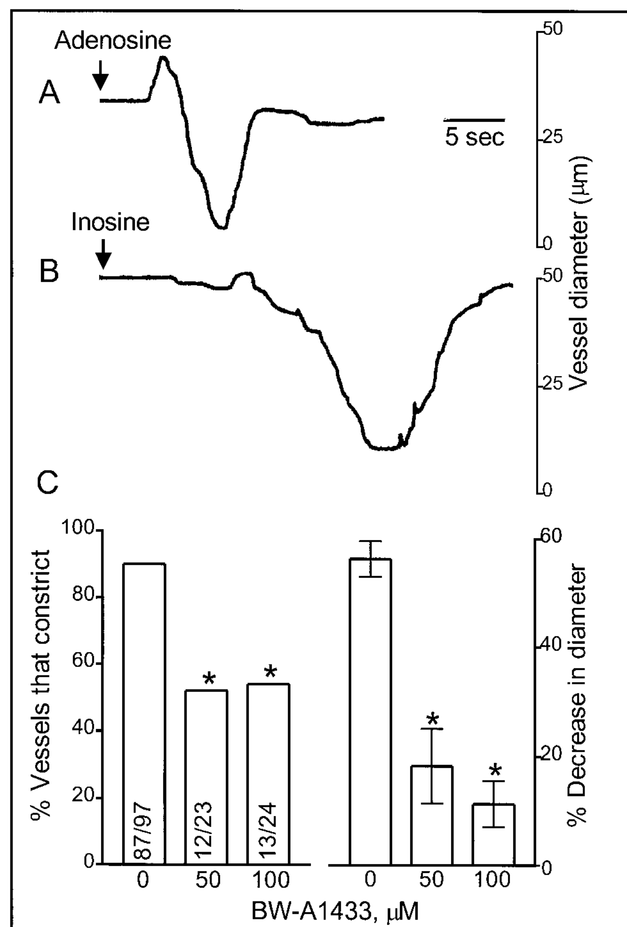

Figure 5. Vasomotor responses to adenosine and inosine in hamster cheek pouch arterioles. Typical responses of individual arterioles to the picospritzer application of (*A*) adenosine and (*B*) inosine. The concentration of nucleosides within micropipetts was 100 μM, and the compounds were applied for 30–50 msec. (*C*) Responses pooled from 144 vessels to picospritzer application of inosine during superfusion of cheek pouches with buffer containing the indicated concentrations of BW-A1433. The number of vessels that constricted/ total vessels tested are indicated within bars on the left. The % decrease in diameter is indicated on the right. Data are mean±SEM. *Indicates statistical significance at $P < 0.05$.

finity (Fig. 1 and Table I). There is, however, a large difference among species in the binding of xanthine antagonists. In initial reports, the rat A_3 receptor was described as being insensitive to blockade by xanthines (29). In this study we were able to very accurately measure the binding properties of rat A_3 receptors, in part because we achieved very high expression levels in HEK-293 cells (Fig. 1). We show that two xanthines, I-ABOPX and BW-A1433, do block rat A_3 receptors, but these compounds are 100–1,000 times less potent antagonists of rat A_3 receptors than of human or sheep A_3 receptors. The results shown in Figs. 3 and 5 indicate that this relatively low potency for BW-A1433 appears to be shared by the guinea pig and the hamster. Although I-ABOPX is somewhat more potent than BW-A1433, the latter compound was used as an antagonist of inosine-mediated responses in the hamster cheek pouch (Fig. 5) because it has much greater aqueous solubility and stays in solution in physiological buffers at a concentration of 100 μM. The results also indicate that 8-SPT is a very weak antagonist of the rat A_3 adenosine receptors (Table I). This result confirms the validity of the strategy of using 8-SPT to block all adenosine receptors except the A_3 receptor in the rat (10). Insensitivity to xanthines provides an explanation for the inability of these antagonists to prevent adenosine from facilitating rat mast cell degranulation (see below), but it is significant that this insensitivity to xanthines cannot be extrapolated to nonrodent species, including man.

Detection of A_3 adenosine receptors in lung membranes. To date, the pharmacological characterization of A_3 adenosine receptors has been based almost entirely on radioligand binding to recombinant receptors. There have been attempts to measure A_3 adenosine receptors in rat brain using the nonselective agonist [^{125}I]AB-MECA in combination with XAC, which blocks rodent A_1 and A_2 receptors more potently than A_3 receptors. As recently pointed out by Shearman and Weaver (22), however, A_3 receptor binding in rat brain, defined as [^{125}I]AB-MECA binding resistant to 1 μM XAC, is marginally detectable and comprises $< 5\%$ of the total specific binding sites. Here we demonstrate [^{125}I]ABA binding to what appears to be predominantly A_3 receptors in guinea pig lung membranes. To selectively block A_1 receptors without blocking A_3 receptors we have used WRC-0571 (30), which is extraordinarily A_1-selective (see Fig. 2 *A*). The [^{125}I]ABA binding sites in guinea pig lung represent the first example of a clearly defined population of A_3 adenosine receptors to be detected in a tissue based on the following criteria: (*a*) specific binding is insensitive to blockade by an A_1 selective antagonist, WRC-0571; (*b*) specific binding is nearly abolished by the addition of GTPγS or *N*-ethylmaleimide, consistent with the binding site being a receptor coupled to a Gi/o G protein; and (*c*) the lung binding site has affinities for [^{125}I]ABA, inosine, and BW-A1433 similar to recombinant rat A_3 receptors and inconsistent with binding to any other adenosine receptor subtypes.

Although there are mast cells resident in the lung, the abundance of the A_3 binding sites in guinea pig lung homogenates, > 200 fmol/mg protein (Fig. 3 *C*), suggests that the receptors are not limited just to the mast cells that comprise only a small fraction of the total number of cells in the lung. It is notable also that transcript for the A_3 adenosine receptor is much higher in the human lung than it is in the lungs of rodent species (1). The non-mast cell sites and function of putative A_3 receptors in the lung are unknown.

Vascular responses to inosine. Adenosine produces a direct dilatory effect on vascular smooth muscle that, in various blood vessels, is mediated by A_{2A} and/or A_{2B} receptors (31). The results of previous studies with hamster cheek pouch arterioles indicate that adenosine and inosine both produce mast cell–dependent vasoconstriction (9, 11–13). This study provides an explanation for these findings by demonstrating that inosine is an agonist of A_3 adenosine receptors that exist on perivascular mast cells. There are several lines of evidence that support this conclusion: (*a*) inosine binds to recombinant rat A_3 receptors; (*b*) inosine is a functional agonist of A_3 receptors in transfected HEK-293 cells; (*c*) inosine is a functional agonist of A_3 receptors on rat RBL-2H3 mast cells; (*d*) inosine binds to putative A_3 adenosine receptors on guinea pig lung membranes; (*e*) the potencies of inosine in all of these assay systems are similar; and (*f*) BW-A1433 was found to have similar potencies to competitively block inosine in some of these assay systems. Since our data indicate that inosine can produce vasoconstriction in hamster cheek pouch arterioles, we searched the literature for additional evidence of mast cell–dependent vasoconstriction in response to inosine. A report published by Sakai and Akima in 1978 (32) is particularly striking in this regard. In that study, the isolated hindlimb of the rat was perfused through the femoral artery. Single injections of adenosine (1–300 μg) induced a biphasic response, a long-lasting vasoconstriction preceded by a transient vasodilation. Inosine produced only vasoconstriction. Note that these responses to adenosine and inosine in the rat femoral artery are similar to the cheek pouch arteriole responses depicted in Fig. 5, *A* and *B*, respectively. More recent studies indicate that adenosine analogs that activate A_3 adenosine receptors produce mast cell–dependent vascular responses (10, 33). It has also been noted that reoxygenation after myocardial hypoxia produces degranulation of myocardial mast cells (34). The vascular and cardiac responses to nucleosides or hypoxia may be mediated in part by adenosine and inosine acting on A_3 receptors of tissue resident mast cells.

Interaction between adenosine and inosine in the regulation of mast cells. Previous studies with isolated human mast cells and basophils are consistent with the existence of A_3 adenosine receptors that facilitate IgE-dependent degranulation (6) and A_{2A} receptors that inhibit degranulation by a cyclic AMP–dependent mechanism (7). In human lung fragments adenosine inhibits histamine release via a site antagonized by XAC, but adenosine stimulates release via a mechanism that unexpectedly was found to be blocked by the adenosine uptake inhibitor NBTI (35). The authors concluded that adenosine needs to reach the interior of cells in order to facilitate mast cell degranulation. Similarly, in rat peritoneal mast cells, adenosine can inhibit and facilitate mast cell degranulation. Degranulation is inhibited via A_2 receptors, and is facilitated by a xanthine-insensitive mechanism that is blocked by NBTI (25, 36). Based on the results of this study, we propose that the reason adenosine needs to gain access to the cell interior to facilitate mast cell degranulation is because maximal degranulation triggering requires conversion of adenosine to inosine. This deamination takes place primarily within cells. This scheme, illustrated in Fig. 6, incorporates the idea that adenosine can both stimulate and inhibit mast cell degranulation by activating A_3 and A_{2A} receptors, respectively. We propose that activation of adenosine receptors is increased in ischemic tissues by adenosine and inosine accumulation. Furthermore, degran-

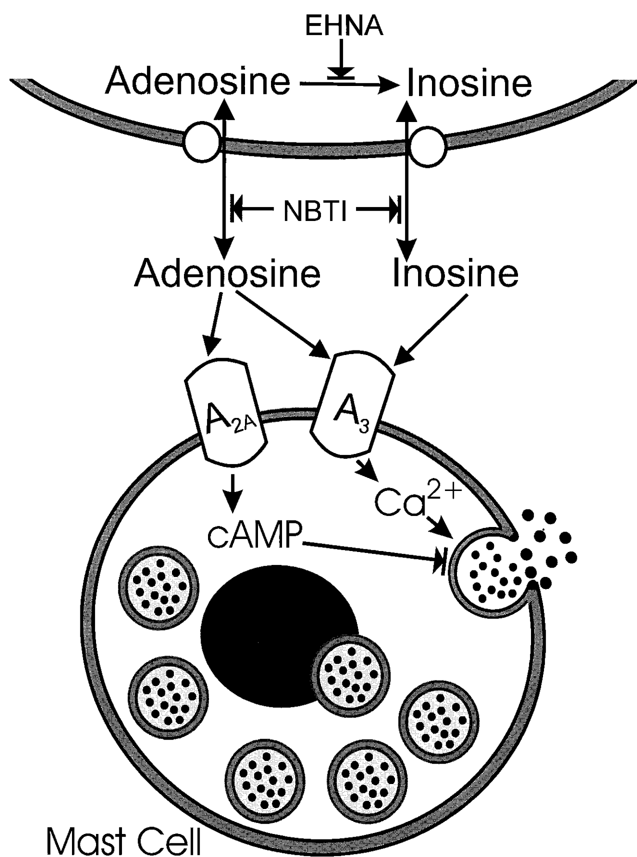

Figure 6. Diagram showing adenosine receptor–mediated mast cell responses. Degranulation of mast cells is facilitated by A_3 receptors coupled to Ca^{2+} mobilization and attenuated by A_{2A} receptors that elevate intracellular cyclic AMP. Adenosine is depicted as a nonselective agonist of A_{2A} and A_3 receptors, whereas inosine selectively activates A_3 adenosine receptors. Deamination of adenosine to inosine is catalyzed by the predominantly intracellular enzyme adenosine deaminase, which can be blocked by erythro-9-(2-hydroxy-3-nonyl)-adenine (*EHNA*). Adenosine uptake occurs by a facilitated nucleoside transporter (○) that can be blocked by S-(*p*-nitrobenzylthioinosine) (*NBTI*). The model predicts that maximal adenosine-mediated degranulation of mast cells requires its conversion to inosine.

ulation is favored by conversion of adenosine to inosine because adenosine is a nonselective agonist of A_{2A} and A_3 adenosine receptors, whereas inosine is a selective agonist of A_3 adenosine receptors. One prediction of this model is that inhibitors of adenosine deamination such as EHNA should prevent adenosine-mediated mast cell degranulation by preventing conversion of adenosine to inosine. Evidence of such inhibition by EHNA has in fact been observed (9). A second prediction of the model depicted in Fig. 6 is that mast cell degranulation in response to inosine should be attenuated by selective agonists of A_{2A} adenosine receptors. We have shown recently that addition of the selective A_{2A} agonist CGS21680 attenuates perivascular mast cell degranulation in response to inosine (Fenster, M., unpublished data). Hence, the scheme shown in Fig. 6 accounts for the previously puzzling finding that inhibitors of adenosine transport or deamination can block the action of adenosine to facilitate mast cell degranulation.

In conclusion, the results of this study indicate that physiologically significant concentrations of inosine selectively activate A_3 adenosine receptors. The conversion of adenosine to inosine may provide a stimulus for mast cell degranulation, and possibly other as yet unknown responses mediated by A_3 adenosine receptors, in part by removing an inhibitory input from adenosine mediated by other subtypes of adenosine receptors that are insensitive to inosine. The notions that inosine participates in receptor-mediated signaling, and that the conversion of adenosine to inosine can facilitate A_3-mediated responses, represent new paradigms for conceptualizing purinergic signaling mechanisms.

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