0026-895X/97/050846-15$3.00/0

MOLECULAR PHARMACOLOGY, **52:**846–860 (1997).

Canine Mast Cell Adenosine Receptors: Cloning and Expression of the A_3 Receptor and Evidence that Degranulation Is Mediated by the A_{2B} Receptor

JOHN A. AUCHAMPACH, XIAOWEI JIN, TINA C. WAN, GEORGE H. CAUGHEY, and JOEL LINDEN

Departments of Medicine (Cardiology), and Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, Virginia 22908 (J.A.A., X.J., T.C.W., J.L.), and Department of Medicine, Cardiovascular Research Institute, University of California at San Francisco, San Francisco, California 94143 (G.H.C.)

Received June 17, 1997; Accepted July 31, 1997

SUMMARY

We cloned and characterized the canine A_3 adenosine receptor (AR) and examined AR-induced degranulation of the BR line of canine mastocytoma cells. Canine A_3AR transcript is found predominantly in spleen, lung, liver, and testes and encodes a 314-amino acid heptahelical receptor. ^{125}I-N^6-Aminobenzyladenosine binds to two affinity states of canine A_3AR with K_D values of 0.7 ± 0.1 and 16 ± 0.8 nM, reflecting G protein-coupled and -uncoupled receptors, respectively. Xanthine antagonists bind with similar affinities to human, canine, and rabbit receptors but with 80–400-fold lower affinities to rat A_3AR. Although canine BR mastocytoma cells contain A_1AR, A_{2B}AR, and A_3AR, degranulation seems to be mediated primarily by A_{2B}ARs stimulated by the nonselective agonist 5′-*N*-ethylcarboxamidoadenosine (NECA) but not by the A_3-selective agonist N^6-(3-iodobenzyl)adenosine-5′-*N*-methylcarboxamide. NECA-stimulated degranulation is not prevented by pertussis toxin and is blocked by enprofylline (K_i = 7 μM), an antiasthmatic xanthine with low affinity (K_i > 100 μM) for A_1AR, A_{2A}AR, and A_3AR. NECA increases canine mastocytoma cell cAMP, Ca^{2+}, and inositol trisphosphate levels; these responses are antagonized half-maximally by 7–15 μM enprofylline. The results suggest that (i) the cloned canine A_3AR is structurally and pharmacologically more similar to human than to rat A_3AR; (ii) the A_{2B}AR, and not the A_1AR or A_3AR, is principally responsible for adenosine-mediated degranulation of canine BR mastocytoma cells; and (iii) the BR cell A_{2B}AR couples to both Ca^{2+} mobilization and cAMP accumulation. Although A_{2B} receptors play a major role in the regulation of BR mast cell degranulation, multiple AR subtypes and G proteins may influence mast cell functions.

Adenosine exerts numerous physiological effects that were originally thought to be mediated by three adenosine receptors, A_1, A_{2A}, and A_{2B}. In the early 1990s, a new adenosine receptor was cloned from rat tissues, first by Meyerhof *et al.* (1) and then by Zhou *et al.* (2), who named it A_3. More recently, human (3), sheep (4), and rabbit (5) A_3 adenosine receptors have been cloned and characterized. Functional expression of A_3 adenosine receptors from various species indicates that A_1 and A_3 receptors bind the radioligands [^{125}I]APNEA, [^{125}I]ABA, and [^{125}I]AB-MECA and are negatively coupled to adenylyl cyclase (2, 4, 6). One unusual property of A_3 adenosine receptors is a major difference among species in the binding affinity of xanthine antagonists. In particular, the rat receptor is resistant to blockade by xanthines, whereas sheep, human, and rabbit receptors bind certain xanthines with high affinity, although with distinct potency orders (3).

The addition of adenosine to rat basophilic leukemic cells (RBL 2H3 cells; a tumor cell line resembling mast cells) causes facilitation of the release of granules, which is mediated by A_3 adenosine receptors (7, 8). A_3 receptor activation also triggers the degranulation of mast cells surrounding hamster cheek pouch arterioles (9). Based on these results, the observation that the inhalation of adenosine produces histamine release and bronchoconstriction in asthmatics but

This work was supported by National Institutes of Health Grants RO1-HL37942 and T32-HL07284.

ABBREVIATIONS: [^{125}I]APNEA, N^6-2-(4-amino-3-[^{125}I]iodophenyl)adenosine; [^{125}I]ABA, N^6-(4-amino-3-[^{125}I]iodobenzyl)adenosine; [^{125}I]AB-MECA, N^6-(4-amino-3-[^{125}I]iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; IB-MECA, N^6-(3-iodobenzyl)-adenosine-5′-*N*-methylcarboxamide; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; XAC, 8-(4-[(2-aminoethyl)aminocarbonylmethyloxy]-phenyl)-1,3-dipropylxanthine; CPA, N^6-cyclopentyladenosine; AM, acetoxymethyl ester; (*R*)-PIA, (*R*)-N^6-phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; I-ABOPX, 3-(4-amino-3-iodobenzyl)-8-oxyacetate-1-propyl-xanthine; 8-SPT, 8-sulfophenyltheophylline; NBTI, nitrobenzylthioinosine; RT, reverse transcription; PCR, polymerase chain reaction; BSA, bovine serum albumin; SSC, standard saline citrate; SDS, sodium dodecyl sulfate; DMEM, Dulbecco's modified Eagle's medium; HEK, human embryonic kidney; $InsP_3$, inositol trisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;.

not in nonasthmatics (10, 11) and the discovery of high levels of A_3 adenosine receptor transcript in human and sheep lung, we proposed a role for the A_3 adenosine receptor in the pathophysiology of asthma (12). Because rodents may be poor animal models for the investigation of the role of A_3 receptors in human allergy and asthma, we decided to clone the canine A_3 adenosine receptor as a first step toward characterizing the role of A_3 adenosine receptors in canine models of asthma.

Here, we report the cloning, expression, and pharmacological characterization of an A_3 adenosine receptor cDNA isolated from BR cells [canine mastocytoma cells (13)]. Low levels of both A_1 and A_3 adenosine receptors are found on BR cells, but these are not primarily responsible for stimulating degranulation of this canine mastocytoma cell line. Rather, an A_{2B} adenosine receptor causes degranulation via a pertussis toxin-insensitive pathway that mobilizes mastocytoma cell Ca^{2+} and can be blocked by the antiasthmatic xanthine enprofylline (14).

Experimental Procedures

Materials. All chemicals were obtained from Sigma Chemical (St. Louis, MO) with the following exceptions. IB-MECA was from Dr. Saul Kadin (Pfizer, Groton, CT). I-ABA and I-ABOPX (also known as BW-A522) were from Dr. Susan Daluge (Glaxo-Wellcome, Research Triangle Park, NC). WRC-0571 [C^8-(*N*-methylisopropyl)-amino-N^6-(5′-endohydroxy)-endonorbornan-2-yl-9-methyladenine] was from Dr. Pauline Martin (Discovery Therapeutics, Richmond, VA). APNEA was from Dr. Ray Olsson (University of South Florida, Tampa, FL). RDC7 (dog A_1 adenosine receptor cDNA) was from Dr. Guy Vassart (Brussels, Belgium). Rat A_3 adenosine receptor cDNA was from Dr. Fereydoun Sajjadi (Gensia, La Jolla, CA). Human A_3 adenosine receptor cDNA was from Dr. Marlene Jacobson (Merck, West Point, PA). Rabbit A_3 adenosine receptor cDNA was from Dr. Scott Kennedy (Pfizer, Groton, CT). HMC-1 mast cells were from Dr. J. H. Butterfiled (Mayo Clinic, Rochester, MN). NECA, CGS 21680 (2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine), (*R*)-PIA, CPA, CPX, XAC, 8-SPT, theophylline, and enprofylline were purchased from Research Biochemicals (Natick, MA). Ro 20–1724 [4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone] was from BIOMOL Research Laboratories (Plymouth Meeting, PA). Adenosine deaminase was from Boehringer-Mannheim Biochemicals (Indianapolis, IN). Fura-2/AM was from Molecular Probes (Eugene, OR). *myo*-[^{3}H]Inositol was from Amersham Life Sciences (Arlington Heights, IL). Dowex AG 1- X8 was from BioRad (Richmond, CA). [^{125}I]ABA was synthesized as described previously (15). Cell culture media and supplies were from GIBCO BRL (Gaithersburg, MD).

Cell culture. COS-7 cells were grown in DMEM with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Canine BR mastocytoma cells were grown in low-glucose DMEM supplemented with 2% calf serum, 25 mM HEPES, 1.5 mM *l*-histidine, 100 units/ml penicillin G, and 100 μg/ml streptomycin. The medium was changed every 3 days, and the cells were replated weekly.

Molecular cloning. To obtain the full-length sequence of the canine A_3 adenosine receptor cDNA, a library prepared in λgt10 from BR cell poly(A)$^+$ RNA was screened using a probe generated by RT-PCR of total RNA isolated from dog tissues using the cDNA cycle kit (InVitrogen, La Jolla, CA). Primers for amplification were primer A (sense 132–152), 5′-GACCACCACCTTCTATTTCA-3′; and primer B (antisense 660–680), 5′-GTCTTGAACTCCCGA/TCC-3′. The primers correspond to conserved regions within the first and third intracellular loops of the human, sheep, and rat A_3 adenosine receptor cDNAs. Each reaction cycle consisted of incubations at 95° for 1 min, 55° for 2 min, and 72° for 3 min with 0.02 unit/ml of *Taq* polymerase (Promega, Madison, WI). PCR fragments were subcloned into the TA vector (InVitrogen) and sequenced with Sequenase (United States Biochemical, Cleveland, OH) using modifications for double-stranded sequencing. One fragment from lung RNA was found to be ~90% identical to the human, rat, and sheep A_3 receptor transcripts. This probe was labeled with [α-^{32}P]dCTP (Primit II; Stratagene, La Jolla, CA) and used to screen the BR cell cDNA library. Library screening was carried out by plaque-filter hybridization. Filters were hybridized at 65° overnight in 10% dextran sulfate, 1 M NaCl, 100 μg/ml herring sperm DNA, and 1×10^6 cpm/ml radiolabeled probe and then washed in 0.5× SSC/0.5% SDS at 65°. Recombinants hybridizing to the probe were plaque-purified and reprobed. Recombinant phage DNA isolated by the plaque lysate method were digested with *Eco*RI and electrophoresed through a 1% agarose gel to determine the insert sizes. Several clones were identified ranging in size from 0.9 to 3 kb. One clone ($cA_3$13.1), which was 1.6 kb long, was subcloned into the *Eco*RI site of the plasmid vector pGEM-7z(−) (Promega). Double-stranded DNA was isolated, and both strands were sequenced in full, first by using T7 and Sp6 primers to get nucleotide sequence information near the 5′ termini, and then with a series of synthetic oligonucleotide primers derived from sequences determined previously.

Radioligand binding studies. Membranes were prepared from COS-7 cells expressing the canine A_3 receptor ($cA_3$13.1) or the canine A_1 receptor (RDC7); HEK 293 cells stably expressing human, rabbit or rat A_3 adenosine receptors; or canine BR cells. The full coding region of the receptor cDNAs were subcloned into the expression vector CLDN10B and transiently expressed (60 hr) in COS cells by the DEAE-dextran method (16) or stably expressed in HEK 293 cells after transfection by the Ca^{2+} phosphate precipitation method (17) and selection in 2 mg/ml G-418. Transfected cells were washed in phosphate-buffered saline; homogenized in 10 mM EDTA, 10 mM Na-HEPES, pH 7.4, and 0.1 mM benzamidine; and centrifuged at $20,000 \times g$ for 20 min. Pellets were resuspended and washed in 10 mM Na-HEPES, 1 mM EDTA, pH 7.4, and 0.1 mM benzamidine (HE buffer) and resuspended in the same buffer with 10% (w/v) sucrose (sucrose buffer) at a membrane protein concentration of 1 mg/ml. Because [^{125}I]ABA bound poorly to crude BR cell membranes, plasma membranes were enriched by preparing P2 pellets. Cells were homogenized in sucrose buffer and centrifuged at $500 \times g$ for 10 min. The pellet was resuspended in sucrose buffer and centrifuged again at $500 \times g$. The pooled supernatants were diluted 3-fold, pelleted, and washed twice by centrifugation at $20,000 \times g$ for 20 min in HE buffer; resuspended; and frozen in sucrose buffer. Protein concentrations were determined using fluorescamine with BSA as standard. Membranes were frozen in aliquots and stored at −80°. For radioligand binding studies, cell membranes were incubated in 0.1 ml for 3 hr at 21° with 5 mM $MgCl_2$ and 5 units/ml adenosine deaminase. For equilibrium binding assays, 6–8 concentrations of [^{125}I]ABA were used in triplicate in tubes, each containing 10–60 μg of membrane protein, and the specific activity of [^{125}I]ABA was reduced 10–20-fold with the nonradioactive compound. Nonspecific binding was measured in the presence of 5 μM I-ABA. [^{125}I]ABA was found to have a higher ratio of specific to nonspecific binding than an alternative radioligand, [^{125}I]AB-MECA. For competition experiments, 0.5–1 nM [^{125}I]ABA was added to tubes, and competing ligands were added over a range of concentrations; the tubes contained 10–50 μg of membrane protein in a final volume of 0.1 ml.

Analysis of binding data. Specific [^{125}I]ABA binding to A_1 adenosine receptors was optimally fit to a single site binding model using Marquardt's nonlinear least-squares interpolation (18). [^{125}I]ABA was found to bind to two affinity states of the recombinant canine, human, and rat A_3 receptors. For two-site Scatchard transformation, the relationship between bound/free and bound can be shown to be described by a quadratic equation: bound/free $= A * X * X + B * X + C$, where A = bound; $X = K_{d1}/K_{d2}$; $B = K_{d1} * X + K_{d2} * X - B_{max1} * K_{d2} - B_{max2} * K_{d1}$; $C = X * X - B_{max1} * X - B_{max2} * X$. Optimal parameters for two-site Scatchard plots were generated by using the

binomial theorem to solve this equation within each iteration of nonlinear least-squares analysis.

IC_{50} values of compounds in competition experiments were fit to $SB_i = B_i - (B_i - NS)\,[I]/(IC_{50i} + [I])$ where i is the number of binding sites, SB is specific binding, and NS is nonspecific binding. K_i values were calculated from IC_{50}, B_{max}, the concentration of [^{125}I]ABA, and its K_d value, as described previously (19). For A_3 receptors, the determination of the K_i values of competing agonists for an agonist radioligand ([^{125}I]ABA), is complicated by the fact that both the radioligand the competing compounds bind to two affinity states. This is described by four equations: $LB = B_{max1} * (L/K_{d1})/(1 + L/K_{d1} + C/K_{i1}) + B_{max2} * (L/K_{d2})/(1 + L/K_{d2} + C/K_{i2}) + f * L$; $CB = B_{max1} * (C/K_{i1})/(1 + L/K_{d1} + C/K_{i1}) + B_{max2} * (C/K_{i2})/(1 + L/K_{d2} + C/K_{i2}) + f * C$; $LT = L + LB$; $CT = C + CB$; where LB is radioligand bound, CB is competitor bound, L is free radioligand, C is free competitor, and f is fraction of L or C nonspecifically bound (assumed to be equal). K_{d1}, K_{d2}, and the fraction of coupled receptors were derived from equilibrium radioligand binding in the absence of competitor. The other parameters were determined by simultaneously solving these four equations by interpolation within each iteration of nonlinear least-squares analysis. For the analysis of antagonist binding, K_{i1} and K_{i2} values were set to be equal based on the assumption that antagonists bind with similar affinities to G protein-coupled and -uncoupled receptors.

Northern blots. Northern analysis and RT-PCR were used to determine the tissue distribution of A_3 adenosine receptor transcript and to identify A_{2B}, A_1, and A_3 receptor transcripts in BR cells. Total RNA was extracted and poly(A)$^+$ RNA was selected using oligo(dt) cellulose. Five micrograms of poly(A)$^+$ RNA was electrophoresed through 1% agarose gels containing 1% formaldehyde and then transferred to nylon membranes (Genescreen Plus; DuPont). The membranes were hybridized in 10% dextran sulfate, 1 M NaCl, and 100 μg/ml herring sperm DNA with 1×10^6 cpm/ml random-labeled probe at 65° overnight. Filters were washed with 0.5× SSC/0.5% SDS at 65° and then exposed to Amersham Hyperfilm MP for 24–48 hr. The A_3 receptor probe consisted of a 600-bp PCR fragment of $cA_3$13.1, corresponding to approximately half of the carboxyl-terminal sequence and the 3′ noncoding region. The A_{2B} receptor probe consisted of a 500-bp PCR fragment generated by RT-PCR from BR cell RNA corresponding to transmembrane regions I–IV.

For RT-PCR, 1 μg of poly(A)$^+$-selected RNA was reverse-transcribed and amplified using primers A and B described above. The reactions were electrophoresed and transferred to nylon membranes (Hybond N$^+$; Amersham) using denaturing buffer (0.4 N NaOH) and hybridized with specific probe. Filters were washed under stringent conditions (0.1× SSC/0.5% SDS at 65° for 1 hr). Control reactions were included in which RT or RNA was excluded from the reactions. Some of the PCR fragments were subcloned into the TA vector and partially sequenced.

Mastocytoma cell degranulation. As an indicator of degranulation of BR cells, we measured the release of β-hexosaminidase (a granule-associated protein that parallels histamine release) using a modification of the method of Schwartz *et al.* (20). BR cells grown in suspension were washed twice in Ca^{2+}/Mg^{2+}-free Tyrode's buffer and then resuspended in complete Tyrode's at a density of 1.2×10^6 cells/ml. Cells were then transferred to a 96-well plate in 250-μl aliquots and prewarmed to 37° for 15 min. Cells were stimulated with agonists added in 50-μl aliquots for 20 min at 37° with shaking. The reactions were stopped by placing the plate on ice for 10 min and then pelleting the cells by centrifugation at $200 \times g$ for 10 min (4°). Two hundred microliters of the supernatant was removed and added to 50 μl of 5 mM *p*-nitrophenyl-*N*-acetyl-D-glucosaminide, and 100 mM citric acid, pH 3.8, and incubated at 37° for 2 hr with shaking before the addition of 50 μl of 0.4 M $NaCO_3$. Total cellular β-hexosaminidase was determined by adding 50 μl of lysis buffer (complete Tyrode's buffer plus 0.6% Triton A-100) to 250-μl aliquots of cells, and 20 μl was removed and assayed. Absorbance was read at 405 nm using a Titertech Multiskan II plate reader. Experiments were performed in triplicate, and release of β-hexosaminidase is expressed as percentage of the total content of unstimulated cells.

cAMP. BR cells were washed twice and resuspended in serum-free low-glucose DMEM containing 25 mM HEPES, 1 unit/ml adenosine deaminase, and 20 μM Ro 20–1724 and then transferred to polypropylene test tubes (1×10^6 cells/0.2 ml, 21°). Drugs were added in 50-μl aliquots, and the tubes were placed in a 37° shaking water bath for 20 min. Assays were terminated by the addition of 500 μl of 0.15 N HCl. cAMP in the acid extract (500 μl) was acetylated and quantified by automated radioimmunoassay.

Intracellular Ca^{2+}. BR cells were loaded with 1 μM Fura-2/AM in buffer containing 100 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM KH_2PO_4, 25 mM $NaHCO_3$, 0.5 mM $CaCl_2$, 2.7 g/liter D-glucose, 20 mM Na-HEPES, pH 7.4, and 0.25% BSA for 45 min. Cells were washed and resuspended in the same buffer without BSA, plus 1 unit/ml adenosine deaminase to a density of 1×10^6 cells/ml. Fluorescence was measured with an SLM spectrofluorimeter in a thermostable cuvette (37°).

$InsP_3$. BR cells were preincubated for 24 hr with 2.5 μCi/ml *myo*-[^{3}H]inositol in inositol-free low-glucose DMEM supplemented with 2% dialyzed fetal calf serum. The labeled cells were washed and resuspended in low-glucose DMEM with 25 mM HEPES, 1 unit/ml adenosine deaminase, and 100 mM LiCl and then transferred to polypropylene test tubes (4×10^5 cells/0.2 ml) at 37° in a shaking water bath and stimulated by 5× agonists added in 50-μl aliquots for 10 min. Assays were terminated by the addition of 400 μl stop solution (0.5 M $HCLO_4$, 5 mM EDTA, and 1 mM diethylenetriaminpentacetic acid) plus 1 mg/ml phytic acid and placed on ice for 30 min before the addition of 5 M K_2CO_3 to raise the pH to 8–9. After centrifugation, the supernatants were passed through a 0.2-μm filter, applied to 1-ml Dowex AG 1-X8 columns (200–400 mesh), and washed with 5 ml of H_2O and 5 ml of 40 mM HCl; then, $InsP_3$ was eluted with 5 ml of 170 mM HCl.

Results

Molecular cloning of the canine A_3 adenosine receptor. The screening of a canine mastocytoma cDNA library with an A_3 adenosine receptor probe generated by RT-PCR resulted in the identification of several positively hybridizing clones. A clone designated $cA_3$13.1 contains a 1.6-kb insert with an open reading frame corresponding to 314 amino acids and 181 and 480 bp of 5′ and 3′ untranslated sequence, respectively (Fig. 1). A hydrophilicity plot of the deduced amino acid sequence predicts seven transmembrane domains, which are indicated in Fig. 2A. Sites found to be conserved within all species of A_3 adenosine receptors cloned to date include a putative palmitoylation site at Cys305 of the consensus sequence and two putative *N*-linked glycosylation sites at Asn4 and Asn162. Several putative phosphorylation sites are conserved among the A_3 adenosine receptors, including four potential sites for phosphorylation by protein kinase C (Thr124, Thr125, Ser/Thr215, and Thr230); one potential site for phosphorylation by tyrosine kinases (Tyr120); and one potential site for phosphorylation by cAMP/cGMP-dependent protein kinases (Thr294). The carboxyl tail distal to the palmitoylation site contains several serine/threonine residues that are surrounded by acidic groups that may be sites for phosphorylation by G protein receptor kinases.

The deduced amino acid sequence of $cA_3$13.1 is 88%, 86%, 72%, and 77% identical to the human, sheep, rat, and rabbit receptors (Fig. 2B), respectively, and 52% and 47% identical to canine A_1 and A_{2A} receptors, suggesting that $cA_3$13.1 is the canine species homolog of the A_3 adenosine receptor. Be-

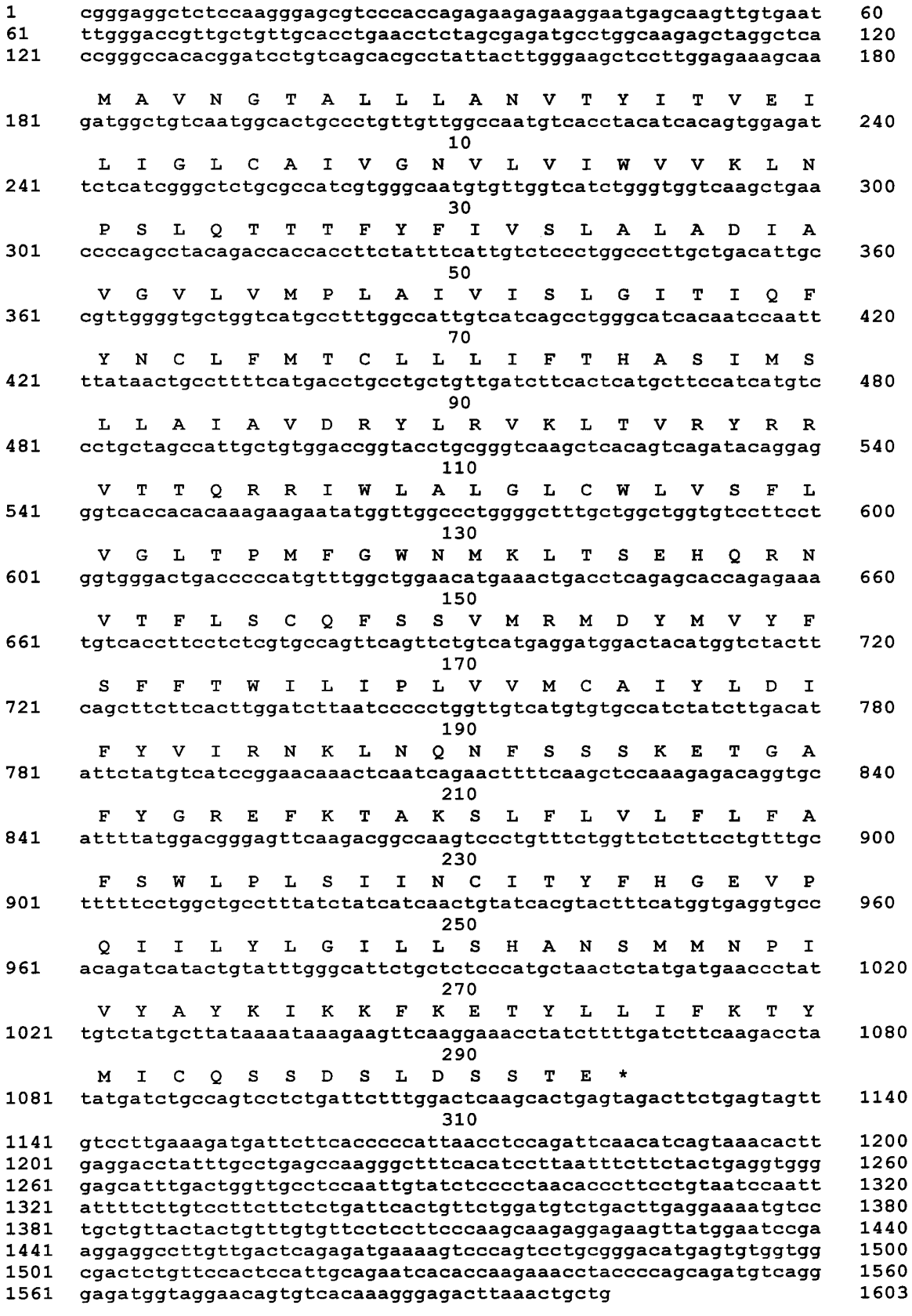


Fig. 1. Nucleotide and amino acid sequences of $cA_3$13.1, the canine A_3 adenosine receptor. The sequence has been deposited in GenBank (accession no. U54792).

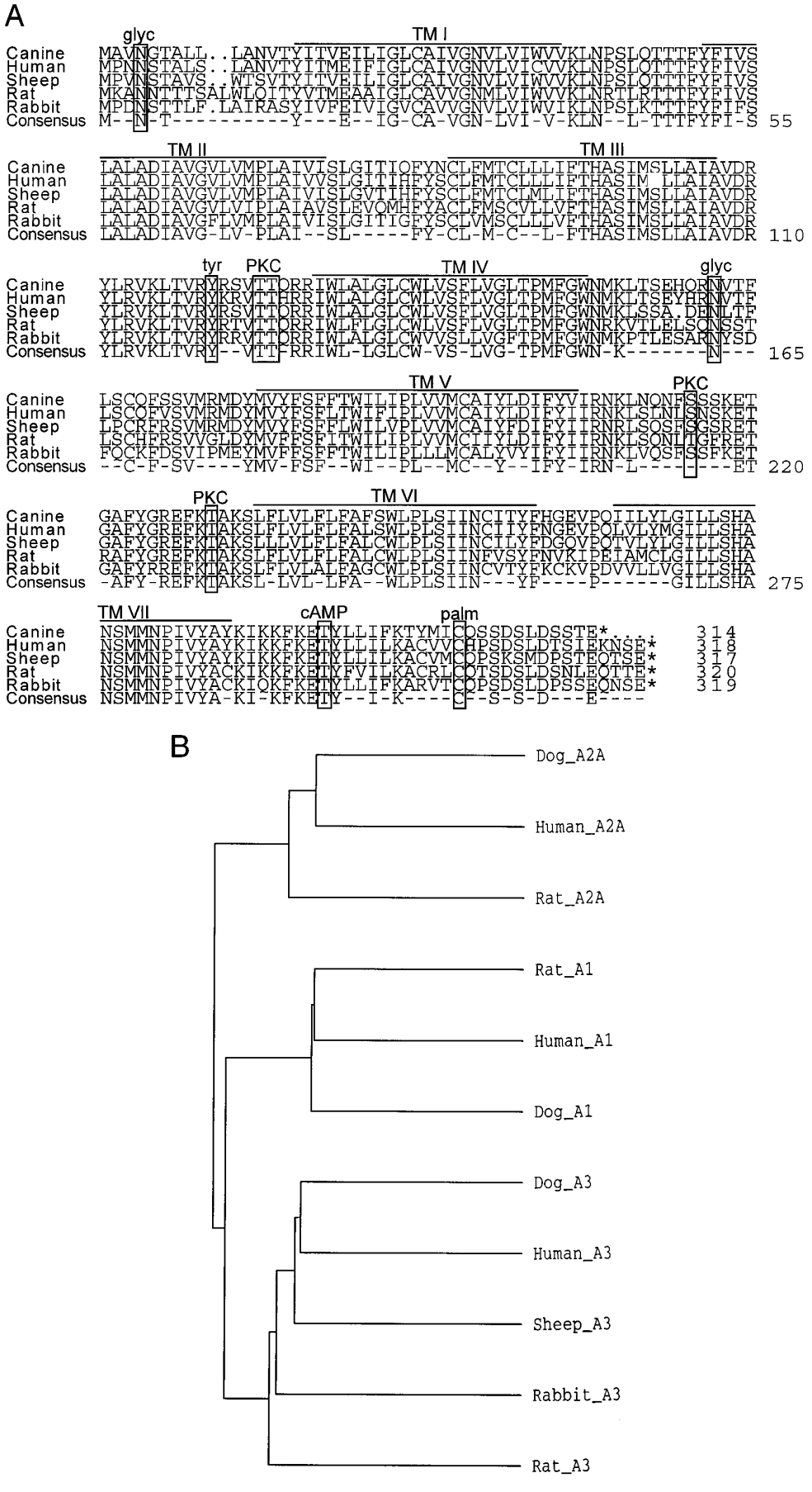


Fig. 2. Deduced amino acid sequence of $cA_3$13.1, the canine A_3 adenosine receptor. A, Alignment with human, sheep, rat, and rabbit A_3 adenosine receptor sequences. *Solid lines*, putative transmembrane (*TM*) domains with numbered designations (I–VII). *Dashes*, sequence gaps. Sites conserved among all species for possible *N*-linked glycosylation (*glyc*); phosphorylation by protein kinase C (*PKC*), cAMP-dependent protein kinase (*cAMP*), tyrosine kinases (*tyr*), and palmitoylation (*palm*) are designated. B, Dendogram illustrating species and subtype sequence differences among adenosine receptors. Distance along the horizontal axis is proportional to divergence of the amino acid sequences.

tween species, the greatest degree of homology lies within the transmembrane domains, and the least lies within the carboxyl tail. Of the five species of A_3 adenosine receptors cloned to date, the human receptor amino acid sequence is most similar to the canine and least similar to the rat. We noted previously that the human and rat A_3 adenosine receptors are unusually divergent for species homologs (12). In contrast, canine and human A_3 adenosine receptors show a degree of amino acid sequence homology that is similar to species differences among the other adenosine receptor subtypes (Fig. 2B).

Tissue distribution of A_3 mRNA. Northern blots probing for $cA_3$13.1 transcripts in several different canine tissues revealed two hybridizing bands of 1.9 and 2.7 kb (Fig. 3). Transcripts were most abundantly expressed in spleen, but high levels also were detected in lung and liver. Two major hybridizing bands were also observed in testes, but the sizes were 1.3 and 2.4 kb. Transcripts were not detected in heart or kidney by Northern analysis. Using the sensitive technique of RT-PCR, trace transcripts were observed in all six tissues studied (data not show). Transcripts for A_1, A_3, and A_{2B} adenosine receptors were detected by Northern blotting of BR cell poly(A)$^+$ mRNA (data not shown). The size of the A_3 transcripts, 1.9 and 2.7 kb, is the same as in dog spleen, lung, and liver. The A_{2B} transcript sizes are 1.6 and 1.8 kb and correspond to transcript sizes noted previously for A_{2B} receptor transcripts in mouse bone marrow-derived mast cells (21).

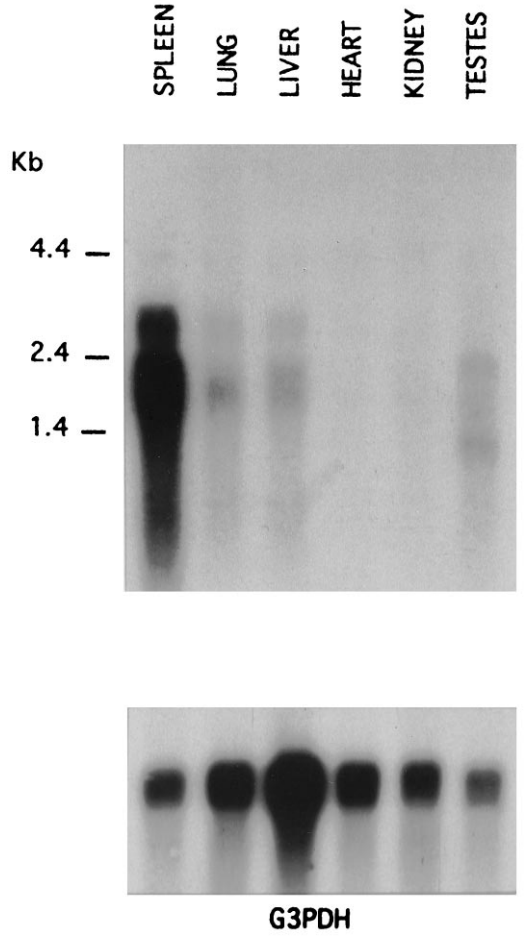


Fig. 3. Tissue localization of the canine A_3 adenosine receptor transcript. Northern blots of poly(A)$^+$ RNA (5 μg/lane) from six different dog tissues using a probe corresponding to the carboxyl-terminal tail and 3′ untranslated region of $cA_3$13.1. The same blot was stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase transcript (*G3PDH*). RNA size markers are indicated.

Pharmacological characterization of canine A_3 adenosine receptors. Binding of [^{125}I]ABA was measured to membranes prepared from COS-7 cells transfected with $cA_3$13.1 (Fig. 4). Specific binding was absent in untransfected cells and was abolished by 1 μM nonradioactive I-ABA. *N*-Ethylmaleimide has been reported to alkylate G proteins in the $G_{i/o}$ family and cause them to become uncoupled from receptors. GTPγS and *N*-ethylmaleimide both reduced specific binding of [^{125}I]ABA to canine A_3 adenosine receptors by ~60%, indicating that the radioligand is an agonist and that G_i/G_o proteins couple to the A_3 receptor (Fig. 4A). In equilibrium binding studies, [^{125}I]ABA specific binding was consistently found to fit significantly ($p < 0.01$) better to a two-site than to a one-site model (22). The respective high and low affinity K_d values of [^{125}I]ABA are 0.53 ± 0.13 and 16.4 ± 0.8 nM, and B_{max} values are 250 ± 9 and 768 ± 123 fmol/mg of membrane protein. In the presence of 50 μM GTPγS, [^{125}I]ABA binds only to the low affinity site, with a K_d value of 17.4 ± 0.1 nM and a B_{max} value of 768 ± 123.0 fmol/mg of total protein. The conversion of receptors from two affinity states to a single low affinity state on the addition of GTPγS is most clearly illustrated by Scatchard analysis (Fig. 4C). These results suggest that the high affinity site reflects binding to G protein-coupled receptors and the low affinity site reflects binding to uncoupled receptors. A similar analysis indicates that [^{125}I]ABA also binds to two affinity states of recombinant rabbit A_3 adenosine receptors with K_D values of 1.2 and 34 nM (not shown). In contrast, in filtration assays, [^{125}I]ABA detects only the high affinity state of canine A_1 receptors transiently expressed in COS-7 cells (K_d = 2.67 ± 0.50 nM, B_{max} = 1275 ± 52 fmol/mg protein; Fig. 5), and specific binding is almost completely abolished by the addition of GTPγS (data not shown).

We next compared the binding properties of recombinant canine A_1 and A_3 adenosine receptors. Fig. 6 shows the results of competition binding studies with compounds found to be the most A_3 selective, IB-MECA and I-ABOPX, and those most A_1 selective, CPA and WRC-0571. K_i values of these and other compounds are summarized in Tables 1 and 2. These tables also summarize the relative affinities of competing compounds for canine A_1 and A_3 receptors. For the A_3 receptor subtype, two dissociation constants for agonists and single dissociation constants for antagonists were calculated as described in Experimental Procedures. The potency order of agonists for canine A_3 receptors was IB-MECA ≥ [^{125}I]ABA > PIA > APNEA > CPA > NECA > CGS 21680. IB-MECA is 58-fold selective for the A_3 over the A_1 adenosine receptor (high affinity sites), and WRC-0571 and CPA are 36- and 12-fold selective, respectively, for the A_1 over the A_3 receptor. The canine A_3 adenosine receptor binds antagonists with the potency order of I-ABOPX > CPX > XAC > BWA 1433 > WRC 0571 > 8-SPT (Table 2). Theophylline and enprofylline bind very weakly to canine A_3 adenosine receptors; a 100 μM concentration of these compounds reduces specific binding by only ~40%. I-ABOPX is 16-fold selective for canine A_3 over A_1 adenosine receptors.

Table 3 shows a comparison of the binding affinities of four

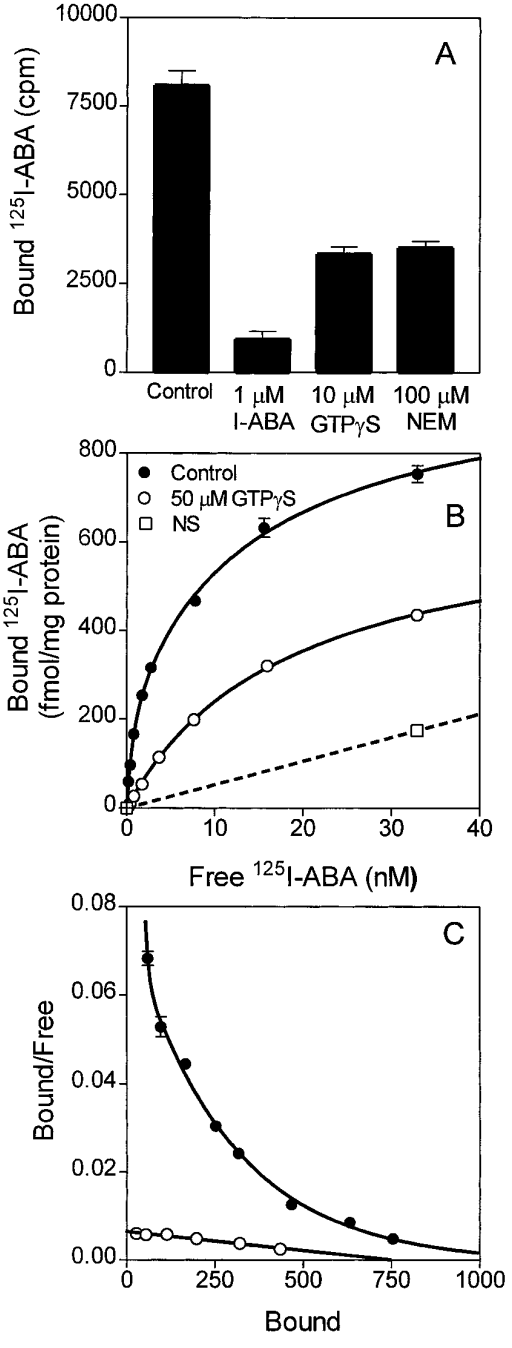


Fig. 4. Radioligand binding to recombinant canine A_3 adenosine receptors. A, Inhibition of [^{125}I]ABA (1.4 nM) binding to COS-7 cell membranes expressing $cA_3$13.1 by unradiolabeled I-ABA, GTPγS, and *N*-ethylmaleimide. B, Equilibrium specific and nonspecific (*NS*) binding of [^{125}I]ABA to transfected COS-7 cell membranes in the presence and absence of GTPγS. C, Scatchard transformation of the specific binding data shown in B. For B and C, control data were fit optimally to two-site equations as described in Experimental Procedures. Values are mean ± standard error of triplicate determinations (25 μg of membrane protein/tube); where omitted, standard error bars are smaller than the symbols. The results are typical of three experiments. Binding parameters are summarized in Table 1.

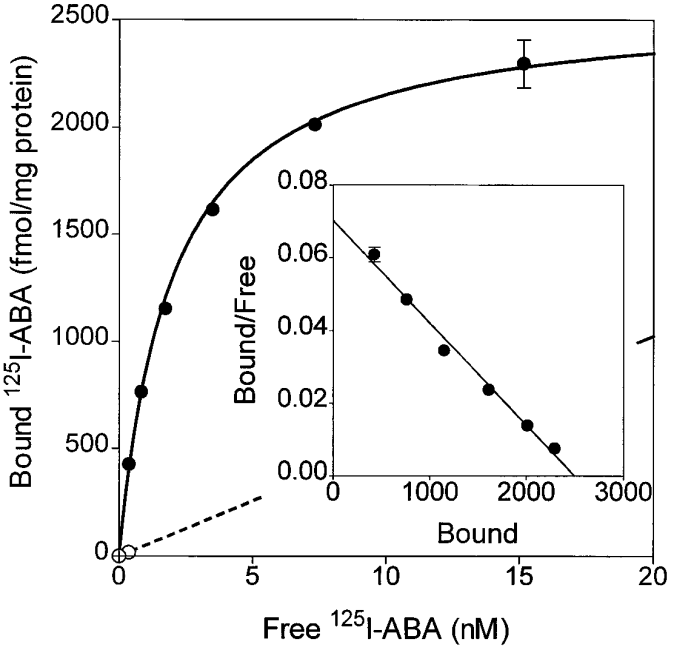


Fig. 5. Radioligand binding to recombinant canine A_1 adenosine receptors (RDC7). Equilibrium specific (●) and nonspecific binding (○) binding of [^{125}I]ABA to transfected COS-7 cell membranes is plotted. *Inset*, Scatchard transformation of the specific binding. Values are mean ± standard error of triplicate determinations (10 μg of membrane protein/tube); where omitted, standard error bars are smaller than the symbols. The results are typical of three experiments. Binding parameters are summarized in Table 1.

xanthines with those of recombinant human, canine, rabbit, and rat A_3 receptors and confirms that there are marked species differences in the binding affinities of xanthine antagonists. Of the four xanthines examined, the average ratio of binding affinities is 9.6 (canine/human), 5.0 (rabbit/human), and 212 (rat/human). The canine A_3 receptor binds CPX with higher affinity than any of the other species examined.

[^{125}I]ABA binding to canine BR mastocytoma cell membranes. Because both A_1 and A_3 adenosine receptor transcripts were detected in canine BR cells, we next determined whether [^{125}I]ABA binding to A_1 and/or A_3 receptors could be detected in membranes prepared from these cells. Little specific binding could be detected to crude membranes, but the binding of 0.4 nM radioligand to an enriched P2 membrane preparation was 80% specific (Fig. 7). Of the [^{125}I]ABA binding site on BR cell membranes, 24 ± 3% (three experiments) bind WRC-0571 with low affinity (IC_{50} = 22 ± 9 μM) characteristic of A_3 receptors; the remainder bind WRC-0571 with high affinity (IC_{50} = 114 ± 38 nM) characteristic of A_1 receptors (Fig. 7, Table 2). When added at 0.4 nM, [^{125}I]ABA labeled only 2.4 fmol/mg of protein of A_3 receptors in the P2 membranes of BR cells, suggesting the density of A_3 receptors on BR cells is low.

Characterization of the adenosine receptor that causes degranulation of canine mastocytoma cells. Adenosine agonists have been shown to enhance A23187 (calcimycin)-stimulated degranulation of several types of mast cells, including murine bone marrow-derived mast cells, human lung mast cells, RBL-2H3 cells, and rat peritoneal mast cells (23, 24). Fig. 8 shows the effect of increasing concentrations of Ca^{2+} ionophore to elicit β-hexosaminidase release from BR cells when administered alone or in combination with the nonselective adenosine receptor agonist NECA (10 μM). When administered alone, A23187 evokes a maximal release of ~15.6% of the total cellular content after

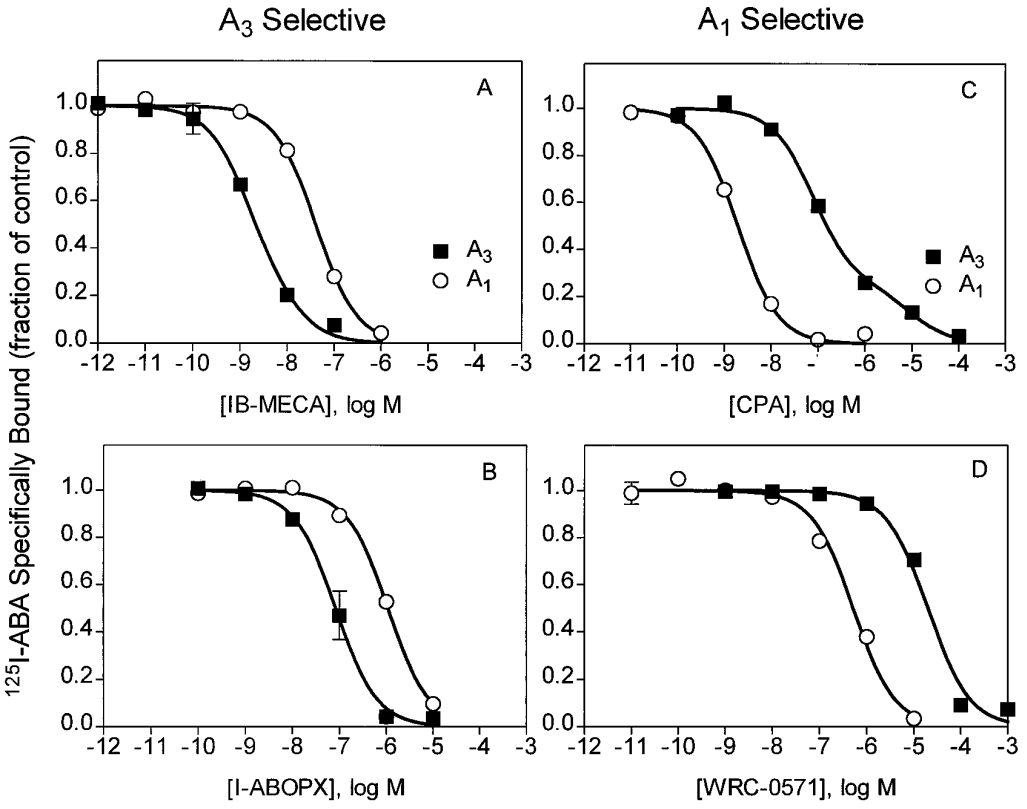


Fig. 6. Competition for [^{125}I]ABA binding to COS-7 cell membranes derived from cells transfected with canine A_3 or A_1 adenosine receptors. Binding is plotted as the fraction of control specific binding (>90% of total binding). Values are mean ± standard error of triplicate determinations from three experiments. Protein, 25 μg/tube (A_3) or 10 μg/tube (A_1), [^{125}I]ABA, 150,000–300,000 cpm/tube (0.4–0.8 nM). The binding of [^{125}I]ABA and competing ligands was fit to one or two binding sites as described in Experimental Procedures.

20 min of stimulation. NECA (10 μM) alone also stimulates β-hexosaminidase release (5.81 ± 0.59%), and costimulation with NECA and A23187 increases β-hexosaminidase release to 28.2 ± 1.8%. NECA decreases the EC_{50} for A23187 from 0.32 ± 0.06 to 0.13 ± 0.08 μM.

We next conducted experiments to determine whether NECA acts at receptor sites on the cell surface or at intracellular sites. To test the possibility that NECA may gain access into BR cells via facilitated uptake and influence the degranulation response via an intracellular mechanism, BR cells were preincubated for 15 min with NBTI (1 μM), an inhibitor of nucleoside transport. As shown in Fig. 8B, NBTI did not influence the concentration response of NECA to stimulate β-hexosaminidase release. As additional evidence that NECA acts at cell surface adenosine receptors, we added XAC, a nonselective antagonist of all subtypes of canine adenosine receptors, including A_3 (see Table 2). XAC completely abolished the stimulatory effect of NECA (Fig. 8B). Additional experiments were performed with NBTI and XAC during costimulation with a combination of A23187 and NECA. As in the absence of A23187, NBTI had no effect, and XAC abolished NECA-mediated degranulation responses (data not shown).

Pertussis toxin blocks the inhibition of cAMP accumulation in CHO-K1 cells that is mediated by recombinant rat A_3 adenosine receptors (2). Pertussis intoxication of rats also reduces a putative A_3 adenosine receptor-mediated hypotensive response (25). These data suggest that A_3 adenosine receptors functionally couple to G_i/G_o proteins; therefore, we examined the effect of pretreating BR cells with pertussis toxin on the ability of NECA to stimulate degranulation (Fig. 9). For these experiments, BR cells were cultured in serum-free medium with 0.3 or 1 μg/ml of pertussis toxin for 24 hr.[1] We found that cells cultured in serum-free medium released a greater amount of β-hexosaminidase in response to 1 μM A23187 (~25–35% without serum versus ~15% with serum). Pretreatment of cells with either concentration of pertussis toxin did not prevent NECA-stimulated degranulation. These data suggest that neither A_1 nor A_3 adenosine receptors are solely responsible for adenosine-mediated degranulation of BR cells.

We next determined the potency order of various adenosine analogs to stimulate β-hexosaminidase release from BR cells. In addition to NECA, we examined (*R*)-PIA (A_1 selective), CGS 21680 (A_{2A} selective), and IB-MECA (A_3 selective). Experiments were performed in the presence or absence of 1 μM A23187 (results are illustrated in Fig. 10 and summarized in Table 4). The potency order of agonists to stimulate canine mast cell degranulation, NECA > PIA > CGS-21680 > IB-MECA, differs from the potency order of these compounds for binding to canine A_3 adenosine receptors, IB-MECA > PIA > NECA > CGS-21680. IB-MECA has very little stimulatory effect on BR cell degranulation and, when added at 10 μM, IB-MECA inhibits degranulation.

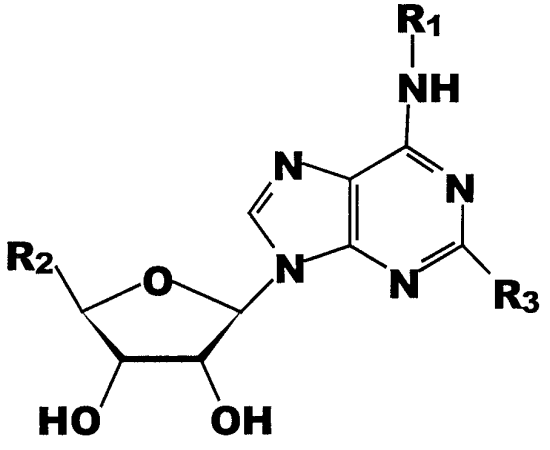
The observed potency order for adenosine analogs to stimulate BR mast degranulation is similar to the potency order reported by Brackett and Daly (26) for activating A_{2B} receptors on NIH 3T3 fibroblasts and the potency order for binding to recombinant human A_{2B} receptors (27). Furthermore, A_{2B} receptors in general have low affinity for agonists, similar to their low affinity for stimulating degranulation of BR cells

[1] Serum-free medium was used to avoid the potential for neutralization of pertussis toxin by anti-toxin antibodies that exist within some lots of serum.

TABLE 1
Dissociation constants of agonists for the canine A_3 (cA_3 13.1) and A_1 adenosine receptors determined in binding assays

R_1	R_2	R_3	A_3 receptor (cA_3 13.1)		A_1 receptor	A_1/A_3[b]	A_3/A_1[b]
			K_{I1}	K_{I2}	K_I		
					nM		
IB-MECA	3-Iodobenzyl	Methylcarboxamido	H	0.53 ± 0.13	89.5 ± 41.3	30.6 ± 2.10	57.7
I-ABA	3-Iodo-4-aminobenzyl	HO-CH_2	H	0.71 ± 0.09*	16.4 ± 0.80[a]	2.67 ± 0.50	3.76
APNEA	4-Aminophenylethyl	HO-CH_2	H	8.10 ± 0.13	250 ± 72	13.9 ± 2.12	1.72
(*R*)PIA	Phenylisopropyl	HO-CH_2	H	2.71 ± 0.47	472 ± 36	2.31 ± 0.10	1.17
NECA	H	Ethylcarboxamido	H	34.3 ± 4.9	10,300 ± 4,060	2.92 ± 0.30	11.8
CPA	Cyclopentyl	HO-CH_2	H	21.9 ± 4.2	3,840 ± 1,240	1.82 ± 0.39	12.0
CGS 21680	H	Ethylcarboxamido	(4-Carboxyethyl)-phenyethylamino	398 ± 16	70,600 ± 710		

K_I values (nM ± standard error of three to six experiments) obtained from competition experiments using [^{125}I]ABA as the radioligand; K_{I1} and K_{I2}, dissociation constant for the high and low affinity sites for the A_3 receptor, respectively; [a] K_D values for [^{125}I]ABA determined in equilibrium binding assays; [b] ratio of the K_{I1} values for the A_3 receptor and K_I value for the A_1 receptor. The procedures for deriving K_{I1} and K_{I2} are described in the text.



(Table 4). Because A_{2B} receptor transcript is found in BR cells, we postulated that A_{2B} adenosine receptors are expressed on BR cells and, when activated, stimulate degranulation. To test this hypothesis, we investigated the effects of enprofylline, which we recently identified as a selective antagonist of recombinant human A_{2B} receptors (27). Concentration-response curves for degranulation in response to NECA were generated in the presence of 10, 50, and 250 μM enprofylline. As shown in Fig. 11A, increasing concentrations of enprofylline produced parallel rightward shifts of the concentration-response curves for β-hexosaminidase release. Schild regression analysis revealed a slope close to unity (1.12 ± 0.45), suggesting that enprofylline acts as a competitive antagonist at a single receptor subtype. The K_D value of enprofylline was estimated to be 7.8 ± 3.3 μM. This value is almost identical to the K_I value of enprofylline for binding to human A_{2B} receptors and is well below the K_I value of enprofylline for canine A_1 or A_3 receptors. Interestingly, the K_i value of enprofylline for A_{2B} receptors lies within the therapeutic range of this compound as an antiasthmatic therapeutic agent (28). Because enprofylline also inhibits cAMP phosphodiesterase, we evaluated the effects of another phosphodiesterase inhibitor, Ro 20–1724, on NECA-induced β-hexosaminidase release. Ro 20–1724 is a nonxanthine that does not bind to adenosine receptors. As shown in Fig. 11B, Ro 20–1724 has no effect on NECA-induced degranulation. The data are consistent with the possibility that enprofylline blocks BR cell degranulation by binding to A_{2B} adenosine receptors.

Second messenger responses evoked by A_{2B} receptor activation in canine mastocytoma cells. We next measured second messenger responses to adenosine receptor activation in BR cells. Unlike A_3 adenosine receptors, which are inhibitory to adenylyl cyclase, A_{2B} adenosine receptors stimulate adenylyl cyclase (26). We measured changes in intracellular levels of cAMP, Ca^{2+}, and $InsP_3$ in response to NECA, CGS 21680, and IB-MECA. As shown in Fig. 12, NECA, but not CGS 21680 or IB-MECA, produces a concentration-dependent increase in intracellular levels of cAMP. The absence of cAMP accumulation in response to CGS-21680 indicates that accumulation of the cyclic nucleotide in response to NECA is not mediated by A_{2A} adenosine receptors. The calculated EC_{50} for NECA to increase cAMP in BR cells is 0.9 ± 0.2 μM, which is similar to the EC_{50} value for NECA to stimulate β-hexosaminidase release (0.6 ± 0.3 μM), suggesting that both responses are mediated by the same receptor subtype. NECA (1 μM) also increased intracellular levels of Ca^{2+} and $InsP_3$, whereas 1 μM CGS -21680 or 1 μM IB-MECA had little effect (Fig. 12). Small responses to CGS 21680 and IB-MECA to influence cAMP, Ca^{2+}, or $InsP_3$ suggest that selective activation of A_{2A} or A_3 receptors has little effect on cAMP or Ca^{2+} signaling in canine BR mastocytoma cells.

Enprofylline competitively antagonized the increases in intracellular levels of cAMP and Ca^{2+} produced by NECA (Fig. 13). By Schild regression analysis (slope = 0.81 ± 0.15), the K_D value of enprofylline was estimated to be 4.7 ± 3.2 μM from the cAMP response and 15 μM from the Ca^{2+} response,

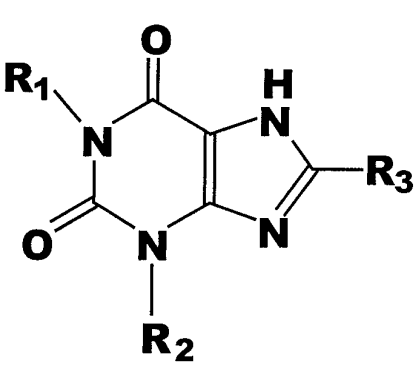
TABLE 2

Dissociation constants of antagonists for the canine A_3 (cA_3 13.1) and canine A_1 adenosine receptors determined in binding assays

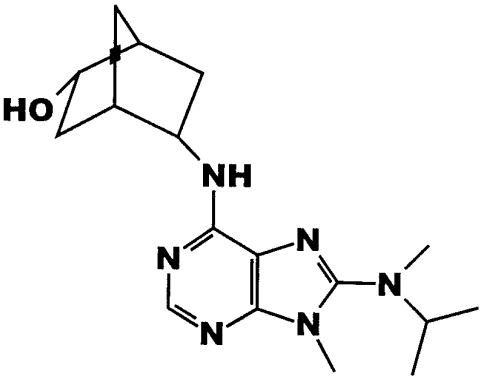
All structures except WRC-0571 are xanthines.

	Xanthine			K_i		A_1/A_3	A_3/A_1
	R_1	R_2	R_3	A_3	A_1		
				nM			
I-ABOPX	Propyl	3-Iodo-4-aminobenzyl	Phenyl-oxyacetate	37.5 ± 11	601 ± 99	16.0	
XAC	Propyl	Propyl	Phenyl-(2-aminoethyl)aminocarbonylmethyloxy	138 ± 22	159 ± 12	1.2	
8-SPT	Methyl	Methyl	Phenyl-sulfol	25,300 ± 4,200	6,460 ± 590		3.9
CPX	Propyl	Propyl	Cyclopentyl	115 ± 10	11.4 ± 2.1		10.1
BW-A1433	Propyl	Propyl	Phenyl-acrylate	1,880 ± 190	132 ± 11		14.2
WRC-0571	(Nonxanthine)			17,200 ± 2,100	484 ± 41		35.4
Theophylline	Methyl	Methyl	H	>100,000			
Enprofylline	H	Propyl	H	>100,000	>100,000		

K_i values obtained from competition experiments using [^{125}I]ABA as the radioligand; all values are reported as mean ± standard error of three to six experiments.



Xanthines



WRC-0571

TABLE 3

Species differences in the binding of xanthines to A_3 adenosine receptors

	K_i				K_i ratio (other species/human)		
	Human	Canine	Rabbit	Rat	Canine	Rabbit	Rat
	nM						
I-ABOPX	18	37.5	179	1,500	2.1	9.9	83
BW-A1433	55	1,880	384	15,000	34	7.0	272
XAC	71	138	106	29,000	1.9	1.5	408
CPX	509	115	708	43,000	0.23	1.4	84

K_i values were obtained from competition experiments using recombinant receptors from the indicated species with [^{125}I]ABA as the radioligand for three to six experiments. In all cases, the standard error was <30% of the mean.

values similar to the K_D value estimated for enprofylline to inhibit NECA-stimulated β-hexosaminidase release (7.8 μM). These results suggest that A_{2B} receptors in BR cells are positively coupled to adenylyl cyclase and phospholipase C.

Discussion

We cloned and characterized a canine A_3 adenosine receptor cDNA designated $cA_3$13.1 from canine BR mastocytoma cells. The clone is homologous to A_3 receptors cloned from other species. Transcripts for A_1 and A_{2B} adenosine receptors also were detected in BR cells, and evidence of A_1, A_{2B}, and A_3 receptor expression was found on the basis of radioligand binding or functional assays. The A_{2B} receptor predominates in the regulation of BR cell degranulation, as discussed in detail below.

The tissue distribution of A_3 adenosine receptor transcript in dog is similar to the human distribution (3), with highest levels expressed in spleen, followed by lung and liver. The tissue distribution is different from rat, in which transcript is much more abundant in testes than in other tissues (1). In terms of sequence homology and pharmacology, the canine A_3 adenosine receptor is more similar to the human than to the rat A_3 receptor.

The results of radioligand binding assays with the A_1/A_3 agonist [^{125}I]ABA indicate that the canine A_3 adenosine receptor binds to both G protein-coupled and -uncoupled receptors with K_D values that differ by ~30-fold. The potency order of agonists for the A_3 receptor, IB-MECA > R-PIA ≥ NECA > CPA, is consistent among species. In all species, IB-MECA and CPA are A_3 and A_1 selective, respectively. Specific binding of [^{125}I]ABA to the uncoupled conformation of the canine A_3 aden-

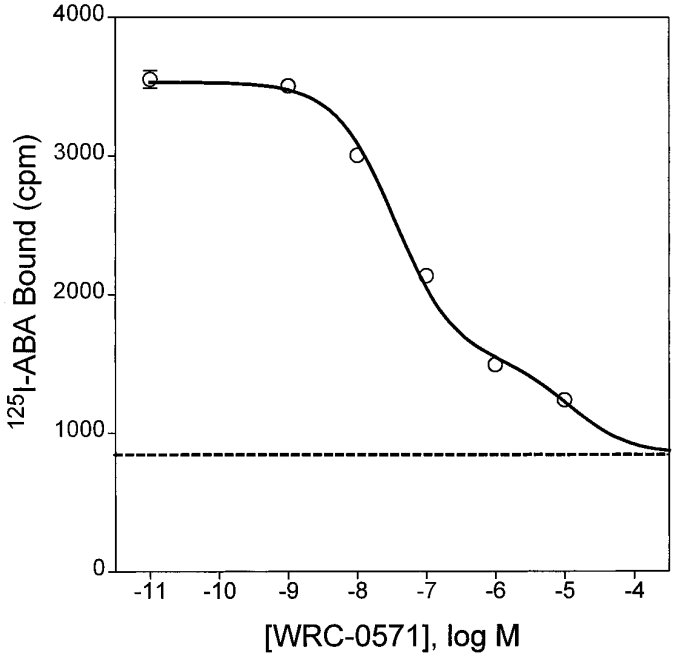


Fig. 7. Competition by WRC-0571 for [^{125}I]ABA binding to enriched plasma membranes derived from BR cells. P2 membranes were prepared as described in Radioligand Binding Studies. Binding was determined in triplicate to membranes containing 156,000 cpm (0.4 nM) [^{125}I]ABA, 60 μg of P2 membrane protein, and various concentrations of WRC-0571. Nonspecific binding (10 μM I-ABA) is 846 $\pm$ 18 cpm (*dashed line*). Standard error bars are smaller than the symbols. The data were fit to a two-site model probably reflecting WRC-0571 binding to A_1 and A_3 adenosine receptors. Binding parameters from triplicate experiments are summarized in the text.

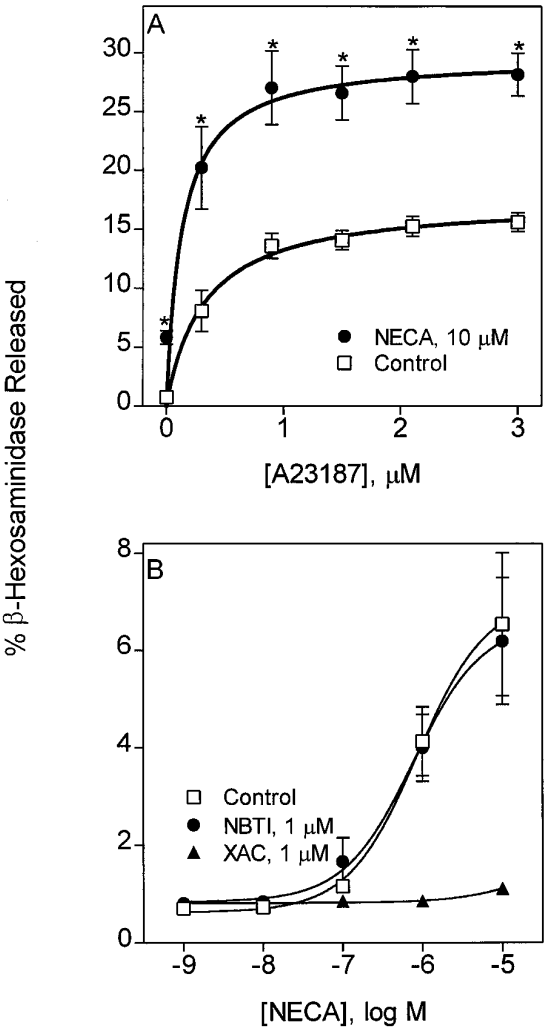


Fig. 8. Stimulation of β-hexosaminidase release from BR cells by A23187 and NECA. A, Cells (300,000/tube) were stimulated with increasing concentrations of A23187 in the presence or absence of 10 μM NECA for 20 min. β-Hexosaminidase released into the supernatant was measured as described in Experimental Procedures. B, Effects on NECA-stimulated β-hexosaminidase release of pretreatment of cells for 15 min with NBTI (1 μM) or XAC (1 μM). Data are pooled from three separate experiments, each assayed in triplicate. *, Different from control ($p < 0.05$).

osine receptor distinguishes the canine A_3 receptor from the uncoupled canine A_1 receptor, which has too low affinity for [^{125}I]ABA binding to be detected in filtration assays. However, [^{125}I]ABA binds with 10 times higher affinity to bovine than to canine A_1 receptors, and the radioligand can detect two affinity states of the bovine A_1 adenosine receptor [K_d = 0.09 and 10.4 nM (29)]. The detection in filtration assays of two affinity states of A_3 receptors complicates the analysis of competition binding assays because the radioligand binds with two affinities and competing agonists and antagonists bind with two or one affinities, respectively. To calculate the K_i values of competing compounds required the derivation of nonstandard analytical procedures (see Analysis of Binding Data). As summarized in Table 1, I-ABA and IB-MECA both bind with high affinity ($K_D < 1$ nM) to the G protein-coupled conformation of canine A_3 receptors. Failure to analytically resolve the two agonist affinity states in radioligand binding assays will result in underestimation of high affinity agonist dissociation constants as well as errors in the calculation of the dissociation constants of competing compounds based on the Cheng and Prusoff formula (30). It is notable that in the range of 0.1–1 μM, compounds that often are used as selective agonists of A_1 receptors (CPA) or A_{2A} receptors (CGS-21680) also will bind to canine A_3 receptors. Hence, caution must be taken in attributing functional responses of these compounds to particular adenosine receptor subtypes.

This study confirms and extends the observation that there are substantial species differences in the binding of xanthines to A_3 adenosine receptors. The rat A_3 adenosine receptor, the first A_3 adenosine receptor to be cloned, was originally reported not to bind xanthine antagonists (2). Subsequent studies have shown that xanthines bind weakly to the rat receptor. The most potent xanthine antagonist, I-ABOPX, binds to the rat A_3 receptor with a K_I value of 1.5 μM. In contrast, sheep, human, and canine A_3 receptors bind I-ABOPX with 80–500 times higher affinity (3, 4).

CPX is widely regarded as a selective antagonist of A_1 adenosine receptors. Although CPX is >250-fold selective for human A_1 over A_3 receptors (27), this selectivity drops to only 10-fold in the case of canine receptors. This is partly due to the fact that compared with human and sheep A_3 receptors, canine A_3 receptors bind CPX with relatively high affinity. In addition, canine A_1 adenosine receptors have lower affinity than other species for CPX. Consequently, CPX is not particularly useful for discriminating between A_1 and A_3 receptor-mediated responses in the dog. A preferable compound for this purpose is WRC-0571, an A_1-selective nonxanthine antagonist that is >4000-fold selective for human A_1 over A_3 receptors (31). Although WRC-0571 binds with much lower

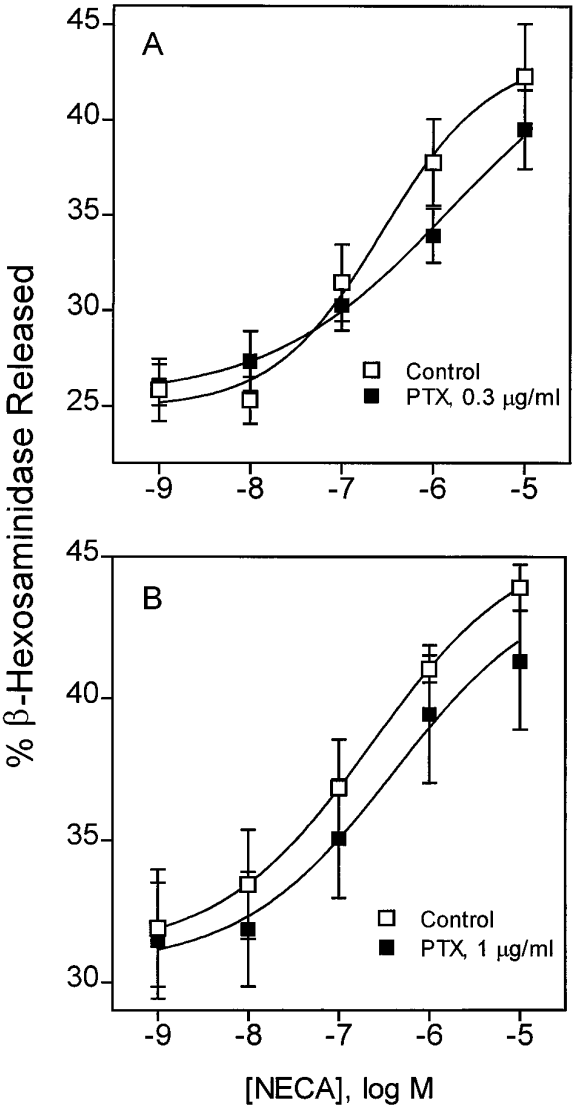


Fig. 9. Effect of pretreatment of BR cells with pertussis toxin (*PTX*) on NECA-stimulated degranulation. Cells were pretreated for 24 hr with 0.3 μg/ml (A) or 1 μg/ml (B) pertussis toxin and then stimulated for 20 min with 1 μM A23187 and various concentrations of NECA. Data are pooled from three separate experiments, each assayed in triplicate.

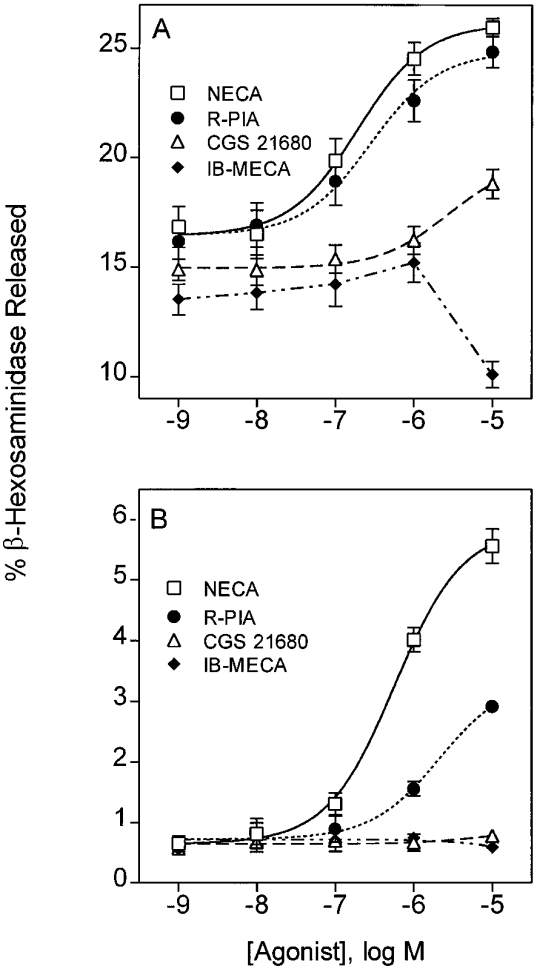


Fig. 10. β-Hexosaminidase release from BR cells in response to adenosine receptor agonists. A, Cells were stimulated with adenosine agonists and 1 μM A23187 for 20 min. B, Cells were stimulated with adenosine agonists alone. Values are mean ± standard error of data pooled from three separate experiments, each assayed in triplicate.

TABLE 4

Potency of adenosine analogs to stimulate β-hexosaminidase release from canine BR mastocytoma cells mean ± SEM, n = 3

	EC_{50}[a]	
	Without A23187	With 1 μM A23187
	μM	
NECA	0.58 ± 0.27	0.19 ± 0.03
(*R*)PIA	11.5 ± 2.5	0.47 ± 0.14
CGS 21680	[b]	32.4 ± 33.5
IB-MECA	[b]	[b]

[a] Data were pooled from three independent experiments.
[b] No β-hexosaminidase release was observed.
Values are mean ± standard error for three determinations.

affinity to canine A_1 receptors (K_I = 484 nM) than to human A_1 receptors (K_I = 3 nM), it still is 35-fold selective as an antagonist of canine A_1 over A_3 receptors. Species differences in binding affinity also are significant for BW-A1433 [8-(4-carboxyethenylphenyl)-1,3-dipropylxanthine], which is sometimes used as a A_3 receptor antagonist on the basis of its moderate affinity for sheep and human A_3 receptors (3, 4). BW-A1433 is a relatively weak and nonselective antagonist of canine A_3 receptors, binding with 10-fold lower affinity to canine than to human A_3 receptors.

Enprofylline, an antiasthmatic agent that has moderate affinity for human A_3 receptors [K_I = 156 ± 110 μM (27)], binds poorly to the canine A_1 and A_3 receptors (K_I > 100 μM). Because enprofylline binds to the human A_{2B} adenosine receptors with a K_I value of 7 μM (27), the compound was evaluated in this study to discriminate between canine A_{2B} and A_1 or A_3 adenosine receptor-mediated responses. Inasmuch as the canine A_3 receptor clone was isolated from a canine mastocytoma cDNA library, we anticipated that the A_3 receptor subtype would be responsible for stimulating the release of granule-associated mediators. However, A_{2B} and A_1 as well as A_3 transcript were found in BR cells, and low levels of A_1 and A_3 receptor binding sites could be detected on enriched plasma membranes prepared from the canine mastocytoma cells. Nevertheless, several lines of evidence indicate that BR cell degranulation requires activation of the A_{2B} but not the A_1 or A_3 adenosine receptor: (i) degranulation of BR cells is not prevented by pretreatment of cells with per-

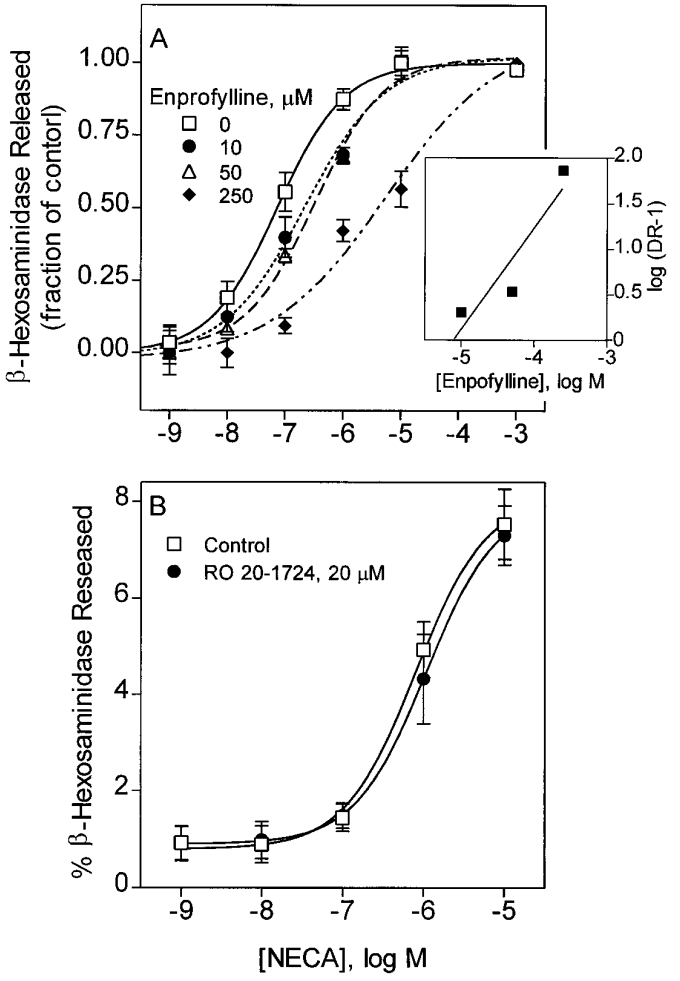


Fig. 11. Effects of enprofylline and Ro 20–1724 on β-hexosaminidase release from BR cells. A, Antagonistic effect of enprofylline on NECA-induced β-hexosaminidase release from BR cells costimulated with 1 μM A23187 and various concentrations of NECA. *Inset*, Schild plot; for enprofylline, $pA_2 = 5.1$. B, Cells were pretreated with or without 20 μM Ro 20–1724 for 15 min and then stimulated with NECA. Data are mean ± standard error of three determinations; similar results were observed in a replicate experiment.

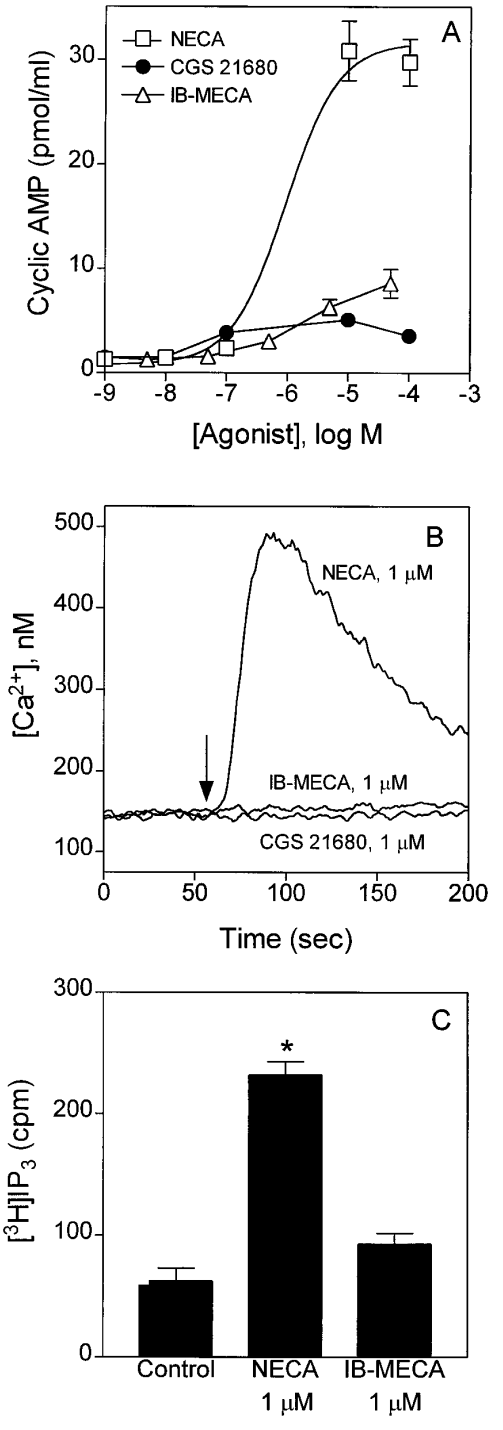


Fig. 12. Changes in intracellular levels of cAMP, Ca^{2+}, and $InsP_3$ in BR cells during stimulation with adenosine agonists. A, cAMP levels were measured in suspended BR cells incubated for 10 min with 20 μM Ro 20–1724. B, Ca^{2+} levels were measured in suspended cells loaded with FURA-2/AM. C, $InsP_3$ levels were measured in cells prelabeled with *myo*-[^{3}H]inositol. All incubations included 1 unit/ml adenosine deaminase. Data are mean ± standard error of three experiments. *, Greater than control and IB-MECA ($p < 0.01$).

tussis toxin; (ii) the response is blocked by enprofylline with a pA_2 value near 5, an affinity similar to that of human A_{2B} receptors and higher that the affinity of enprofylline for canine A_1 or A_3 receptors; (iii) the potency order of agonists to stimulate degranulation, NECA > PIA > CGS-21680 > IB-MECA, differs from the potency order of these compounds to bind to recombinant canine A_3 adenosine receptors; and (iv) NECA, but not CGS-21680, elevates cAMP, which is consistent with the existence of functional A_{2B} receptors on BR cells.

It was somewhat unexpected that A_{2B} adenosine receptors seem to couple to Ca^{2+} mobilization in canine mastocytoma cells inasmuch as A_{2B} adenosine receptors have been shown to couple to stimulation of cAMP accumulation (26). Apparent dual coupling to cAMP and Ca^{2+} has also been noted in HEK 293 cells stably transfected with recombinant human A_{2B} receptors,[2] and it is significant in this regard that the expression of recombinant rat A_{2B} receptors in *Xenopus laevis* oocytes results in the appearance of adenosine-mediated Ca^{2+}-dependent Cl^- current (32). Dual coupling of G protein coupled receptors to G_s and $G_{q/11}$ is not unprecedented. For example, the human prostacyclin receptor also displays such dual coupling (33). Coupling of A_{2B} adenosine receptors to a

[2] X. Jin and J. Linden, unpublished observations.

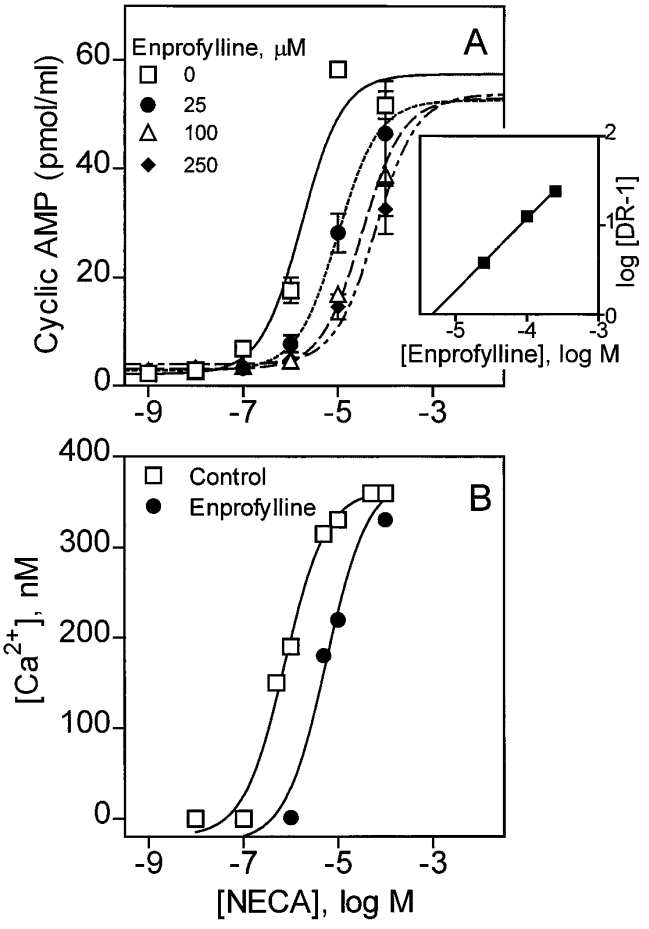


Fig. 13. Antagonistic effects of enprofylline on second messenger responses in canine BR cells. A, cAMP responses to NECA and enprofylline. *Inset*, Schild plot; for enprofylline, $pA_2 = 5.3$. B, Concentration-dependence of Ca^2 responses to NECA with and without enprofylline (100 μM); the EC_{50} values for NECA are 760 and 5725 nM in the absence and presence of enprofylline, respectively. The pA_2 value for enprofylline is estimated to be 4.8. Results are typical of three experiments.

Ca^{2+}-mobilizing G protein resistant to pertussis toxin ($G_{q/11}$) may be essential for triggering BR cell degranulation because agents that elevate cAMP in various kinds of mast cells, including agonists of A_{2A} adenosine receptors, either have no effect or are inhibitory to degranulation (34, 35).

The conclusions of previous studies have been inconsistent regarding the adenosine receptor subtype that mediates mast cell degranulation. Recent DNA antisense experiments suggest that activation of A_1 adenosine receptors may contribute to bronchoconstriction in a rabbit model of asthma (36). However, the low potency of various A_1-selective xanthines to block histamine release from asthmatic human lung fragments (10) and the low potency of enprofylline to block human A_1 adenosine receptors (27) are consistent with the participation of A_{2B} and/or A_3 receptors in human disease. The A_3 adenosine receptor has been implicated in the degranulation of RBL 2H3 rat mast cells and in triggering vascular responses[3] secondary to degranulation of mast cells in the hamster cheek pouch (9, 37) and the pithed rat (25). A_{2B} adenosine receptors seem to mediate the degranulation of murine bone marrow-derived mast cells (21), and although pretreatment of RBL 2H3 rat mast cells with pertussis toxin abolishes NECA-mediated degranulation, Ca^{2+} mobilization in these cells requires activation of G_{i3} or G_q (38). Moreover, activation of phosphoinositide breakdown in RBL 2H3 cells is not well correlated with the affinity of adenosine analogs for A_3 adenosine receptors (39). The treatment of murine bone marrow mast cells with pertussis toxin produces a decrease in the potency of adenosine to enhance degranulation in response to A23187, similar to the result in the current study with canine mastocytoma cells. Pretreatment of murine mast cells with pertussis toxin fails to reduce adenosine-mediated Ca^{2+} mobilization, which is consistent with an A_{2B}-mediated, but not an A_3-mediated, response (40). In the human HMC-1 mast cell leukemia line, the ability of 300 μM enprofylline to block NECA-stimulated interleukin-8 release was taken as evidence that this response also is mediated by A_{2B} adenosine receptors (41). The current study tends to substantiate previously published reports that suggest the receptor subtype primarily responsible for adenosine-mediated mast cell degranulation is variable. It is not yet clear whether this variability depends on species, tissue source, or environmental factors that affect signaling cascades and/or the phenotype of various mast cells. The results of this study indicate that A_{2B} receptors play a major role in the regulation of mast cell degranulation but are consistent with possible participation of multiple adenosine receptor subtypes and multiple G proteins.

The canine A_3 adenosine receptor described in this study is structurally and pharmacologically more similar to human A_3 receptors than are A_3 receptors from rodent species. This finding, along with the fact that it seems that murine bone marrow mast cells, canine mastocytoma, and human HMC-1 leukemic mast cells are regulated by adenosine, primarily via A_{2B} receptors, raises the possibility that canine models of asthma may be better predictors of human disease than rodent models. It will be important to determine which adenosine receptor subtype or subtypes are responsible for facilitating mast cell degranulation in the asthmatic human lung. Once the predominant receptor or receptors are identified, novel antagonists superior to theophylline and enprofylline for the treatment of asthma may be developed.

Acknowledgments

cAMP determinations were made at the University of Virginia Radioimmunoassay Core. We are grateful to Fereydoun Sajjadi (Gensia), Marlene Jacobson (Merck), and Scott Kennedy (Pfizer) for their gifts of A_3 adenosine receptor cDNAs and to J. H. Butterfield (Mayo Clinic, Cleveland, OH) for HMC-1 cells.

References

1. Meyerhof, W., R. Müller-Brechlin, and D. Richter. Molecular Cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* **284:**155–160 (1991).
2. Zhou, Q. Y., C. Li, M. E. Olah, G. L. Stiles, and O. Civelli. Molecular cloning and characterization of an adenosine receptor: the A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **89:**7432–7436 (1992).
3. Salvatore, C. A., M. A. Jacobson, H. E. Taylor, J. Linden, and R. G. Johnson. Molecular cloning and characterization of the human A_3 adenosine receptor. *Proc. Natl. Acad. Sci. USA* **90:**10365–10369 (1993).
4. Linden, J., H. E. Taylor, A. S. Robeva, A. L. Tucker, J. H. Stehle, S. A. Rivkees, J. S. Fink, and S. M. Reppert. Molecular cloning and functional expression of a sheep A_3 adenosine receptor with widespread tissue distribution. *Mol. Pharmacol.* **44:**524–532 (1993).
5. Hill, R. J., J. J. Oleynek, C. F. Hoth, M. A. R. Kiron, W. F. Weng, R. T.

[3] Mast cell mediators such as histamine can produce vasoconstriction or vasodilation. Microvascular vasoconstriction is mediated in part by histamine and thromboxane acting on vascular smooth muscle cell receptors (9). Systemic vasodilation and hypotension secondary to A_3 adenosine receptor activation are mediated in part by circulating histamine, which triggers nitric oxide release from endothelial cells.

Wester, W. R. Tracey, D. R. Knight, R. A. Buchholz, and S. P. Kennedy. Cloning, expression and pharmacological characterization of rabbit adenosine A(1) and A(3) receptors. *J. Pharmacol. Exp. Ther.* **280:**122–128 (1997).
6. Olah, M. E., C. Gallo-Rodriguez, K. A. Jacobson, and G. L. Stiles. ^{125}I-4-Aminobenzyl-5′-*N*-methylcarboxamidoadenosine, a high affinity radioligand for the rat A_3 adenosine receptor. *Mol. Pharmacol.* **45:**978–982 (1994).
7. Gilfillan, A. M., G. A. Wiggan, and A. F. Welton. Pertussis toxin pretreatment reveals differential effects of adenosine analogs on IgE-dependent histamine and peptidoleukotriene release from RBL-2H3 cells. *Biochim. Biophys. Acta* **1052:**467–474 (1990).
8. Ramkumar, V., G. L. Stiles, M. A. Beaven, and H. Ali. The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J. Biol. Chem.* **268:**16887–16890 (1993).
9. Shepherd, R. K., J. Linden, and B. R. Duling. Adenosine-induced vasoconstriction in vivo: role of the mast cell and A(3) adenosine receptor. *Circ. Res.* **78:**627–634 (1996).
10. Bjorck, T., L. E. Gustafsson, and S. E. Dahlen. Isolated bronchi from asthmatics are hyperresponsive to adenosine, which apparently acts indirectly by liberation of leukotrienes and histamine. *Am. Rev. Respir. Dis.* **145:**1087–1091 (1992).
11. Cushley, M. J., and S. T. Holgate. Adenosine-induced bronchoconstriction in asthma: role of mast cell-mediator release. *J. Aller. Clin. Immunol.* **75:**272–278 (1985).
12. Linden, J. Cloned adenosine A_3 receptors: pharmacological properties, species differences and receptor functions. *Trends Pharmacol. Sci.* **15:**298–306 (1994).
13. Caughey, G. H., S. C. Lazarus, N. F. Viro, W. M. Gold, and J. A. Nadel. Tryptase and chymase: comparison of extraction and release in two dog mastocytoma lines. *Immunology* **63:**339–344 (1988).
14. Chapman, K. R., K. Ljungholm, and A. Kallen. Long-term xanthine therapy of asthma: enprofylline and theophylline compared: International Enprofylline Study Group. *Chest* **106:**1407–1413 (1994).
15. Linden, J., A. Patel, and S. Sadek. [^{125}I]Aminobenzyladenosine, a new radioligand with improved specific binding to adenosine receptors in heart. *Circ. Res.* **56:**279–284 (1985).
16. Cullen, B. R. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* **152:**684–703 (1987).
17. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
18. Marquardt, D. M. An algorithm for least-squares estimation of nonlinear parameters. *J. Soc. Indust. Appl. Math.* **11:**431–441 (1963).
19. Linden, J. Calculating the dissociation constant of an unlabeled compound from the concentration required to displace radiolabel binding by 50%. *J. Cyclic Nucleotide Res.* **8:**163–172 (1982).
20. Schwartz, L. B., K. F. Austen, and S. I. Wasserman. Immunologic release of *beta*-hexosaminidase and *beta*-glucuronidase from purified rat serosal mast cells. *J. Immunol.* **123:**1445–1450 (1979).
21. Marquardt, D. L., L. L. Walker, and S. Heinemann. Cloning of two adenosine receptor subtypes from mouse bone marrow-derived mast cells. *J. Immunol.* **152:**4508–4515 (1994).
22. Motulsky, H. J., and L. A. Ransnas. Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.* **1:**365–374 (1987).
23. Peachell, P. T., M. Columbo, A. Kagey-Sobotka, L. M. Lichtenstein, and G. Marone. Adenosine potentiates mediator release from human lung mast cells. *Am. Rev. Respir. Dis.* **138:**1143–1151 (1988).
24. Ali, H., O. H. Choi, P. F. Fraundorfer, K. Yamada, H. M. S. Gonzaga, and M. A. Beaven. Sustained activation of phospholipase D *via* adenosine A_3 receptors is associated with enhancement of antigen- and Ca^{++}-ionophore-induced secretion in a rat mast cell line. *J. Pharmacol. Exp. Ther.* **276:**837–845 (1996).
25. Carruthers, A. M., and J. R. Fozard. Effect of pertussis toxin treatment on the putative adenosine A_3 receptor-mediated hypotensive response in the rat. *Eur. J. Pharmacol.* **250:**185–188 (1993).
26. Brackett, L. E., and J. W. Daly. Functional characterization of the A_{2b} adenosine receptor in NIH 3T3 fibroblasts. *Biochem. Pharmacol.* **47:**801–814 (1994).
27. Robeva, A. S., R. Woodard, X. Jin, Z. Gao, S. Bhattacharya, H. E. Taylor, D. L. Rosin, and J. Linden. Molecular characterization of recombinant human adenosine receptors. *Drug Dev. Res.* **39:**243–252 (1996).
28. Clarke, H., M. J. Cushley, C. G. Persson, and S. T. Holgate. The protective effects of intravenous theophylline and enprofylline against histamine- and adenosine 5′-monophosphate-provoked bronchoconstriction: implications for the mechanisms of action of xanthine derivatives in asthma. *Pul. Pharmacol.* **2:**147–154 (1989).
29. Figler, R. A., S. G. Graber, M. A. Lindorfer, H. Yasuda, J. Linden, and J.C. Garrison. Reconstitution of bovine A_1 adenosine receptors in Sf9 cell membranes with recombinant G proteins of defined composition. *Mol. Pharmacol.* **50:**1587–1595 (1996).
30. Cheng, Y., and W. H. Prusoff. Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22:**3099–3108 (1973).
31. Martin, P. L., R. J. Wysocki, R. J. Barrett, J. M. May, and J. Linden. Characterization of 8-(N-methylisopropyl)amino-N^6-(5′-endohydroxy-endonorbornyl)-9-methyladenine (WRC-0571), a highly potent and selective, nonxanthine antagonist of A_1 adenosine receptors. *J. Pharmacol. Exp. Ther.* **276:**490–499 (1996).
32. Yakel, J. L., R. A. Warren, S. M. Reppert, and R. A. North. Functional expression of adenosine A_{2b} receptors in *Xenopus* oocytes. *Mol. Pharmacol.* **43:**277–280 (1993).
33. Katsuyama, M., Y. Sugimoto, T. Namba, A. Irie, M. Negishi, S. Narumiya, and A. Ichikawa. Cloning and expression of a cDNA for the human prostacyclin receptor. *FEBS Lett.* **344:**74–78 (1994).
34. Hughes, P. J., S. T. Holgate, and M. K. Church. Adenosine inhibits and potentiates IgE-dependent histamine release from human lung mast cells by an A_2-purinoceptor mediated mechanism. *Biochem. Pharmacol.* **33:**3847–3852 (1984).
35. Lohse, M. J., K. Maurer, H. P. Gensheimer, and U. Schwabe. Dual actions of adenosine on rat peritoneal mast cells. *Naunyn-Schmiedebergs Arch. Pharmacol.* **335:**555–560 (1987).
36. Nyce, J. W., and W. J. Metzger. DNA antisense therapy for asthma in an animal model. *Nature (Lond.)* **385:**721–725 (1997).
37. Doyle, M. P., J. Linden, and B. R. Duling. Nucleoside-induced arteriolar constriction: a mast cell-dependent response. *Am. J. Physiol.* **266:**H2042–H2050 (1994).
38. Hoffman, H. M., L. L. Walker, and D. L. Marquardt. Mast cell adenosine induced calcium mobilization via G(i3) and G(q) proteins. *Inflammation* **21:**55–68 (1997).
39. Shin, Y., J. W. Daly, and K. A. Jacobson. Activation of phosphoinositide breakdown and elevation of intracellular calcium in a rat RBL-2H3 mast cell line by adenosine analogs: involvement of A(3)-adenosine receptors. *Drug Dev. Res.* **39:**36–46 (1996).
40. Marquardt, D. L., and L. L. Walker. Alteration of mast cell responsiveness to adenosine by pertussis toxin. *Biochem. Pharmacol.* **37:**4019–4025 (1988).
41. Feoktistov, I., and I. Biaggioni. Adenosine A_{2b} receptors evoke interleukin-8 secretion in human mast cells: an enprofylline-sensitive mechanism with implications for asthma. *J. Clin. Invest.* **96:**1979–1986 (1995).

Send reprint requests to: Joel Linden, Ph.D., Box MR4 6012, Health Sciences Center, University of Virginia, Charlottesville, VA 22908. E-mail: jlinden@virginia.edu