Characterization of Human A2B Adenosine Receptors: Radioligand Binding, Western Blotting, and Coupling to Gq in Human Embryonic Kidney 293 Cells and HMC-1 Mast Cells

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ABSTRACT

Recombinant human A2B adenosine receptors (A2BARs) and receptors extended on the amino terminus with hexahistidine and the FLAG epitope, DYKDDDDK (H/F-A2B), were stably overexpressed in human embryonic kidney 293 cells (HEK-A2B). By Western blotting, the H/F-A2B receptor runs as a 34.8-kDa glycoprotein. Pharmacological properties of A2BARs were characterized with 125I-3-aminobenzyl-8-phenyl-(4-oxoacetic acid)-1-propylxanthine (K0, 36 nM). In competition binding assays, the affinity of agonists is reduced by substitution on either the N6- or the C-2 position of the adenine ring, whereas 5'-substitutions increase affinity, resulting in the potency order: 5'-N-ethylcarboxamido-adenosine; CPA, N6-ethylcarboxamido-adenosine; NECA, N9-potency order: 5

Of the four subtypes of adenosine receptors (ARs), A1, A2A, A2B, and A3 (Linden et al., 94), radioligand binding assays have been extensively used for all except the A3AR. Consequently, the pharmacological characterization of A3ARs has been based primarily on functional assays of CAM accumulation in tissue slices or cultured cells. A2BARs have been functionally evaluated in NIH3T3 cells (Brackett and Daly, 1994) and demonstrated in aorta (Martin, 1992), chromaffin cells (Casado et al., 1992), astrocytes (Peakman and Hill, 1994), and mast cells (Frehdholm and Altijk, 1994), mast cells (Fredholm and Altiok, 1994), mast cells (Robeva et al., 1996a). Here we describe a binding assay improved by the generation of new HEK-A2B cell line that expresses a high density of A2BARs. A second improvement in radioligand binding to A2BARs derives from the use of a high-specific-radioactivity radioligand, 125I-3-(3-iodo-4-aminobenzyl)-8-(4-oxoacetate)phenyl-1-propyl-xanthine (125I-ABOPX).

AABBREVIATIONS: AR, adenosine receptor; DPX, 1,3-diethyl-8-phenylxanthine; CPX, 8-cyclopentyl-1,3-dipropylxanthine; XAL, 8-(4-(2-aminoethyl)aminomethyl)-1,3-dipropylxanthine; ABOXP, 3-(3,4-aminobenzyl)-8-(4-oxoacetate)phenyl-1-propyl-xanthine; H/F-A2B, recombinant human A2B adenosine receptor extended on the amino terminus with hexahistidine and the FLAG epitope (DYKDDDDK); N6-ethylcarboxamidoadenosine; CPA, N6-cyclopentyladenosine; CGS21680, 2-(4-carboxyethyl)-5N-ethylcarboxamidoadenosine; XAC, 8-(4-(2-aminoethyl)aminomethyl)-1,3-dipropylxanthine; ABA, N6-ethylcarboxamidoadenosine; BW-A1433, 8-(4-carboxyethenylphenyl)-1,3-dipropylxanthine; AB-NECA, aminobenzyl-5'-N-ethylcarboxamidoadenosine; DMEM, Dulbecco’s modified Eagle’s medium; APE, 2-(2-(4-amino-phenyl)ethylamino)adenosine; TBS/T, Tris-buffered saline/Tween 20; CHO, Chinese hamster ovary; IP3, inositol trisphosphate; IB-MECA, 6-(2-iodo)benzyl-5N-methylcarboxamido-adenosine; HK37942, 6-(2-iodo)benzyl-5N,9N,N-methylcarboxamidoadenosine.

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Antipeptide antibodies raised against the A2B adenosine receptor recognize a putative nonglycosylated receptor with a molecular mass of 57 kDa (Puffinbarger et al., 1995). This differs from the 33-kDa glycoprotein predicted by the A2BAR molecular mass of 57 kDa (Puffinbarger et al., 1995). Here we show that a purified, recombinant human A2B adenosine receptor extended on the amino terminus with hexahistidine and the FLAG epitope (DYKD-DDDK) (H/F-A2B) is a glycoprotein with an apparent molecular mass of 34.8 kDa.

Adenosine stimulates bronchoconstriction and the degranulation of mast cells in people with asthma, but not in normal subjects (Cushley and Holgate, 1985). The A2AR has been implicated as the receptor that is activated by adenosine to trigger degranulation of rodent perivascular mast cells (Jin et al., 1997) and rat RBL-2H3 mast-like cells (Ramkumar et al., 1993). However, A2ARs appear to play a predominant role in the regulation of canine BR mast cell degranulation (Auchampach et al., 1997) and the slow release of interleukin-8 from human HMC-1 mast cells (Feoktistov and Biagioni, 1995). It is curious, however, that A2ARs are not involved in the regulation of human mast cell degranulation (Lohse et al., 1987; Jin et al., 1997). Here we present evidence that in addition to coupling to Gs, A2AR receptors couple to G\(\alpha\), and calcium mobilization in HEK-A2B cells and HMC-1 mast cells. The antiasthma drugs theophylline and enprofylline effectively block human A2ARs and receptor-mediated mast cell degranulation at therapeutic concentrations.

**Experimental Procedures**

**Materials.** Wizard Megaprep columns and competent JM109 cells were obtained from Promega Corp. (Madison WI); R-N\(^6\)-(2-phenylisopropyl)adenosine, 5'-N-ethylcarboxamidoadenosine (NECA), CPA, peptatin A, leupeptin, aprotinin, phenylmethylsulfonyl fluoride, benzamidine, and forskolin were obtained from Sigma Chemical Corp. (St. Louis, MO). 2-(4-(2-Carboxyethyl)phenethylamino)-5N-N-ethylcarboxamidoadenosine (CGS21680), 2-chloroadenosine, DPX, and 8-(4-((2-aminoethyl)aminocarbonylmethyl)oxy)phenyl)-1,3-dipropylxanthine (XAC) were obtained from Research Biochemicals International (Natick, MA); adenosine deaminase was obtained from Boehringer Mannheim (Indianapolis, IN); lipofectin, G418, tissue culture media and serum, were obtained from Gibco BRL (Gaithersburg, MD). N\(^6\)-Iodoaminobenzyladenosine (I-ABA), 8-(4-carboxyethylphenyl)-1,3-dipropylxanthine (BW-A1433), 8-cyclopentyl-1,3-dipropylxanthine (CPX) and \(^{125}\)I-ABOPX (BW-A522) were gifts from Dr. Susan Daluge of Glaxo Wellcome (Research Triangle Park, NC). Aminobenzyl-5'-N-ethylcarboxamidoadenosine (AB-NECA) was a gift from Dr. Ray Olsson of the University of South Florida (Tampa, FL); anti-FLAG m2 antibodies were obtained from Kodak IBI (New Haven, CT).

**Stable Transfection of HEK 293 Cells.** cDNA for human A2B receptors was prepared by polymerase chain reaction of human brain cDNA (Clontech, Palo Alto, CA) and sequenced on both strands. cDNAs encoding human A1, A2A, and A3 ARs were gifts of Marlene Jacobson (Merck & Co, West Point, PA). DNA sequencing was carried out in the University of Virginia Biomolecular Research Facility with an ABI Prism 377 Automated DNA Sequencer. The four wild-type human adenosine receptor cDNAs were subcloned into the expression plasmid CLDN10B. To prepare H/F-A2B, the cDNA was subcloned into the pDoubleTrouble plasmid (Robeva et al., 1996b). The plasmids were amplified in competent JM109 cells and plasmid DNA isolated by using Wizard Megaprep columns (Promega Corporation, Madison, WI). Recombinant receptors were introduced into HEK 293 cells by lipofectin. Colonies were selected by growth of cells in 0.6 mg/ml G418. Stably transfected cells were maintained in Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/ F12 medium) with 10% fetal calf serum, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 0.3 mg/ml G418. G418 was omitted from the last passage before harvest.

**Radioligand Binding.** To prepare \(^{125}\)I-ABOPX, 10 \(\mu\)l of 1 mM ABOPX in methanol/1 M NaOH (20:1) was added to 50 \(\mu\)l of 100 mM phosphate buffer, pH 7.3. One or 2 \(\mu\)Ci of Na\(^{22}\)I were added, followed by 10 \(\mu\)l of 1 mg/ml chloramine T freshly prepared in water. After incubating for 20 min at room temperature, 50 \(\mu\)l of 10 mg/ml Na-metabisulfite in water was added to quench the reaction. The reaction products were applied to a C18 HPLC column and eluted for 5 min with 4 mM phosphate, pH 6.0/methanol (65:35). The methanol concentration was then ramped to 100% over 15 min. ABOPX elutes in 11 to 12 min and \(^{125}\)I-ABOPX elutes at 18 to 19 min in a yield of 50 to 60% of the initial \(^{125}\)I. For equilibrium binding assays, the specific activity of \(^{125}\)I-ABOPX was diluted 10- to 20-fold by the addition of \(^{125}\)I-ABOPX. Radioligand binding assays were conducted at 21°C for 2 to 3 h in 100 \(\mu\)l of buffer containing 10 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl\(_2\), 20 \(\mu\)g/ml protein, and 1 \(\mu\)M adenosine deaminase. Nonspecific binding was measured in the presence of 10 \(\mu\)M 8-(4-((2-aminoethyl)aminocarbonylmethyl)oxy)phenyl)-1,3-dipropylxanthine (XAC). Competition binding experiments were carried out with 0.5 to 0.6 nM \(^{125}\)I-ABOPX. To detect bound radioligand, membranes were filtered over Whatman GF/C.
filters by using a Brandel cell harvester (Gaithersburg, MD) and washed 3 times over 15 to 20 s with ice-cold buffer (10 mM Tris, 1 mM MgCl₂, pH 7.4). It is important to maintain the wash buffer in an ice-slurry and to prime the filtration apparatus with ice-cold wash buffer to prevent dissociation of specific binding during the wash of glass fiber filters. 

To characterize additional recombinant human adenosine receptor subtypes, radioligands and compounds used to detect nonspecific binding, respectively, were: A₁, [³H]CPX, 10 μM CPX; A₂A, 125I-2-[2-(4-amino-phenyl)ethylamino]adenosine (125I-APA) or 125I-ZM241385, 10 μM NECA, or 1 μM ZM241385; and A₃, 125I-ABA, 100 μM NECA. In competition binding assays, IC₅₀ values for competing compounds were derived by fitting the data to a four-parameter logistic equation: B = specific/(1+(IC₅₀)ⁿ) × nonspecific, where B = specific binding and n = the Hill slope. Kᵢ was derived from IC₅₀ as described previously (Linden, 1982).

Western Blots. Membranes expressing H/F-A₂BARs were solubilized in digitonin and purified by anti-FLAG affinity chromatography (Robeva et al., 1996b). Purified receptors were subjected to SDS polyacrylamide gel electrophoresis. In some instances, receptors were incubated with 0.5 U of N-glycosidase F for 18 h at 37°C. Electrophoresed receptors were transferred to Westtran polyvinylidene difluoride membranes. The membranes were incubated overnight at 4°C in 10% milk Blotto (50 mM Tris, 80 mM NaCl, 20% milk, 0.2% Tween 20, pH 8). Blots were rinsed with Tris-buffered saline/Tween 20 (TBS/T) buffer (20 mM Tris, 137 mM NaCl, 0.3% Tween 20, pH 7.6), incubated with anti-FLAG antibody (5 μg/ml) in TBS/T containing 1 mg/ml BSA for 1 h at room temperature, washed in TBS/T, and incubated for 1 h with horseradish peroxidase-conjugated sheep antimouse IgG Fab'₂ fragments diluted 1:10,000. After washing in TBS/T, blots were visualized by enhanced chemiluminescence. If untransfected HEK cells replaced HEK-A₂B cells, no detectable protein or immunoreactivity was eluted from anti-FLAG affinity columns.

cAMP Measurements. HEK-A₂B or Chinese hamster ovary (CHO)-K₁ cells were grown to near confluence on 150-mm diameter plates. The cells were removed by replacing the medium with PBS containing 5 mM EDTA for 5 min. Cells were pelleted by centrifugation at 250 g for 5 min, washed once in DMEM, and resuspended in DMEM supplemented with 1 U/ml adenosine deaminase and 10 mM HEPES, pH 7.2, resulting in a cell density of 250,000 cells/ml. The cells were lysed by the addition of 500 μl of 0.15 N HCl. After centrifugation at 2,000 g for 10 min, 500 μl of supernatant was removed, acetylated, and acetyl-cAMP measured by automated radioimmunoassay. The pA₂ of antagonists was determined by the method of Schild (Schild, 1957).

Inositol Trisphosphate (IP₃) Measurements. HEK-A₂B or HEK-A₁ cells were grown on 100-mm tissue culture plates. The medium was removed and the cells were washed once with inositol-free DMEM/F12 and incubated for 24 to 48 h with 2.5 μCi/ml [3H]-myo-Inositol in inositol-free DMEM/F12 plus 2% fetal calf serum. For treatment of cells with pertussis toxin, 200 ng/ml was added for 18 h. After tritium labeling, the cells were washed and resuspended in HEPES-buffered DMEM (20 mM HEPES, pH 7.2) plus 1 U/ml adenosine deaminase and 100 mM LiCl and pipetted into test tubes (10⁶ cells/0.2 ml). Cells were maintained at 37°C for 30 min in a shaking water bath. Assays were terminated by the addition of 400 μl of stop solution (0.5 M HClO₄, 5 mM EDTA, and 1 mM diethylenetriaminepentaacetic acid) and 1 mg/ml phytic acid. The tubes were placed on ice for 30 min and 5 M K₂CO₃ was added to raise the pH to 8 to 9. Samples were centrifuged to remove the KClO₄ precipitate and the supernatants were collected and filtered through 0.45-μm Gelman Acrodisc filters and then applied to columns containing 1 ml of anion exchange resin (AG 1-X̂₈, chloride form, 200 to 400 mesh; Bio-Rad Laboratories, Richmond, CA). Columns were washed with 5 ml of water, then 5 ml of 40 mM HCl. IP₃ fractions were eluted with 5 ml of 170 mM HCl and radioactivity counted by liquid scintillation counting.

Measurement of Intracellular Ca²⁺. Cells on tissue culture plates were loaded with 1 μM 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxyl]-2'-amino-5'-methylphenoxy)-ethane-N,N',N'-tetraacetic acid/acetoxymethyl ester in a buffer containing 100 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM KH₂PO₄, 25 mM NaHCO₃, 0.5 mM CaCl₂, 2.7 g/liter D-glucose, 20 mM Na-HEPES, pH 7.4, and 0.25% BSA for 45 min. Cells were washed, detached, and resuspended in the same buffer without BSA, plus 1 U/ml adenosine deaminase to a density of 10⁶ cells/ml. Fluorescence was monitored in an SLM 1100 spectrofluorometer in a thermostatted stirred cuvette at 37°C at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm.

Results

Western Blotting of H/F-A₂B Adenosine Receptors. To estimate the molecular mass of H/F-A₂B ARs, receptors were solubilized in digitonin, purified over anti-FLAG affinity column and detected by anti-FLAG western blotting. To determine whether the A₂B AR was a glycoprotein, some of the purified receptors were incubated with N-glycosidase F before electrophoresis. The results are illustrated in Fig. 2. The H/F-A₂B AR had an apparent molecular mass of 34.8 kDa reduced to 31.3 kDa after enzymatic deglycosylation. Some purified deglycosylated receptors seemed to form dimers with an apparent molecular mass of 65 kDa. The amount of dimerized receptors detected in purified receptor preparations increased with incubation time, receptor concentration, and temperature and may be an artifact of receptor purification, without physiological significance. The properties of the H/F-A₂B AR monomeric glycoprotein are consistent with its expected size and the existence of consensus sites for N-linked glycosylation, based on the receptor cDNA sequence.

Radioligand Binding Studies. Clonal lines of HEK-A₂B cells grown in G418 were screened for maximal 125I-ABOPX binding. Figure 3 shows equilibrium binding of 125I-ABOPX to membranes derived from the clone that was found to have...
the highest level of expression of the wild-type A2B receptors, with a
$B_{\text{max}}$ in excess of 20,000 fmol/mg protein. A single saturable
binding site was detected in transfected, but not in untransfected
cells. There is an endogenous A2B receptor that stimulates
cAMP production in untransfected HEK 293 cells, but the
density of this receptor is too low to be detected by radioli-
gand binding with $^{125}$I-ABOPX. The pharmacological prop-
erties of the $^{125}$I-ABOPX binding site were characterized in
competition binding studies with a series of 11 agonists and
eight antagonists, as summarized in Table 1. The binding
affinity of agonists was reduced by substitutions on either
the N$^6$- or the C2 position of adenine. NECA, which is un-
substituted on the N$^6$ or the C2 position, binds to A2B receptors
with more than 60-fold higher affinity than any of the other
agonists tested.

Among antagonists, the xanthines used therapeutically
to treat asthma, enprofylline and theophylline, were both found
to compete for $^{125}$I-ABOPX binding to A2B receptors with $K_i$
values <10 μM (Fig. 4A; Table 2). It is notable also that
ZM241385 and CPX (Fig. 4A), compounds reported to be
selective antagonists of A2AARs (Poucher et al., 1995) and
A1ARs (Lohse et al., 1993), respectively, bind with high affi-
cinity to A2B receptors. The adenosine receptor subtype selectively
of I-ABOPX, ZM241385, CPX, theophylline, and enprofylline
were determined in radioligand binding assays for the four
AR subtypes (Table 2). If A2B receptors are excluded, ZM241385
and CPX are highly selective for A2AARs and A1ARs, respec-
tively, as reported previously. However, ZM241385 has only
22.1-fold selectivity for human A2A over A2B receptors, and
CPX has only 22.4-fold selectivity for A1 over A2B receptors. The
high potency of CPX for A2B receptors was confirmed in
CHO-A2B cells (Fig. 4B). The stable expression of recombi-
nant human A2B receptors in CHO cells was lower than in
HEK cells and could not be accurately measured by radioli-
gand binding. Nevertheless, NECA stimulated cAMP accu-
culation in CHO-A2B cells, whereas it had only a slight effect
in untransfected cells. By Schild analysis, CPX blocked the
action of NECA in CHO-A2B cells with a $K_i$ of 22 nM (Fig.
4B). The high affinity of ZM241385 also has been confirmed.
In a recent study, $[^{3}H]ZM241385$ was found to bind to re-
combiant human A2B receptors with a $K_i$ of 34 nM (Ji and
Jacobson, 1999).

**Schild Analyses of Theophylline and Enprofylline**

Effects. We next set out to estimate $K_i$ values from pA2
values for enprofylline and theophylline as antagonists of
NECA-stimulated cAMP accumulation in HEK-A2B cells to
determine whether this estimate of binding affinity agrees
with $K_i$ values derived by radioligand binding assays. As
illustrated in Fig. 5, $K_i$ values for theophylline and enprofyl-
line are both 10 to 17 μM, in reasonably good agreement with
the binding constants reported in Table 1. Enprofylline and
theophylline caused an increase in the slope of NECA does
response curves. It is notable that basal cAMP in HEK-A2B
cells was dose-dependently decreased by either theophylline
or enprofylline from a level of about 20 pmol/ml to about 6
pmol/ml. This may be because of a small degree of constitu-
tive activity by the overexpressed receptor, or the effects of
endogenous adenosine released by the cells and not degraded
by added adenosine deaminase. Inhibition of phosphodi-
terase by these xanthines may also contribute to this effect on
the slope of the NECA dose–response curve.

**Potency Order of Agonists in cAMP Assays.** As a fur-
ther validation of the new radioligand binding assay, we
determined the potencies of three agonists in cAMP assays.
The absolute potencies of all three agonists is greater in
cAMP than in radioligand binding assays, but the ratio of

![Figure 3](https://example.com/image3.png)

**Fig. 3.** Equilibrium binding of $^{125}$I/$^{127}$I-ABOPX to membranes derived
from HEK-A2B cells. A. Specific (●) and non-specific equilibrium binding
□□ were determined with isotope dilution as described in the methods. B.
Scatchard transformation of specific binding. The $B_{\text{max}}$ value is 21,400
fmol/mg protein; the $K_i$ value is 37 nM. Each point is the mean ± S.E.M.
of triplicate determinations. The results are typical of quadruplicate
experiments.

**TABLE 1**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>$K_i$ (μM)</th>
<th>$N$</th>
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<tbody>
<tr>
<td>Agonists</td>
<td>$\mu M$</td>
<td></td>
</tr>
<tr>
<td>NECA</td>
<td>0.33 ± 0.06</td>
<td>3</td>
</tr>
<tr>
<td>$N^6$-Substituted Adenosines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AB-NECA</td>
<td>21.5 ± 0.58</td>
<td>3</td>
</tr>
<tr>
<td>R-NECA-(2-phenylisopropyl) adenosine</td>
<td>33.7 ± 6.8</td>
<td>4</td>
</tr>
<tr>
<td>CPA</td>
<td>34.4 ± 11.1</td>
<td>4</td>
</tr>
<tr>
<td>2-Cl-CPA</td>
<td>40.1 ± 16.4</td>
<td>3</td>
</tr>
<tr>
<td>IB-MECA</td>
<td>55.5 ± 5.6</td>
<td>3</td>
</tr>
<tr>
<td>S-NECA-(2-phenylisopropyl) adenosine</td>
<td>62.8 ± 21.6</td>
<td>4</td>
</tr>
<tr>
<td>2-Substituted Adenosines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Chloroadenosine</td>
<td>25.5 ± 6.2</td>
<td>3</td>
</tr>
<tr>
<td>2-Cl-CPA</td>
<td>40.1 ± 16.4</td>
<td>3</td>
</tr>
<tr>
<td>I-APE</td>
<td>115 ± 24</td>
<td>4</td>
</tr>
<tr>
<td>CGS21680</td>
<td>361 ± 21</td>
<td>4</td>
</tr>
<tr>
<td>Antagonists</td>
<td>$nM$</td>
<td></td>
</tr>
<tr>
<td>XAC</td>
<td>7.3 ± 2.8</td>
<td>7</td>
</tr>
<tr>
<td>DPX</td>
<td>12.3 ± 2.8</td>
<td>3</td>
</tr>
<tr>
<td>ZM241385</td>
<td>31.6 ± 6.0</td>
<td>6</td>
</tr>
<tr>
<td>I-ABOPX</td>
<td>40 ± 6.3</td>
<td>9</td>
</tr>
<tr>
<td>CPX</td>
<td>50.0 ± 3.7</td>
<td>7</td>
</tr>
<tr>
<td>BW-A1433</td>
<td>51.6 ± 6.7</td>
<td>7</td>
</tr>
<tr>
<td>Enprofylline</td>
<td>6,400 ± 71</td>
<td>3</td>
</tr>
<tr>
<td>Theophylline</td>
<td>7,850 ± 88</td>
<td>3</td>
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affinities derived from functional and binding assays is similar. Thus, IB-MECA is 169- and 162-fold less potent than NECA in functional and binding assays, respectively. CGS21680 is 530- and 1090-fold less potent than NECA.

Coupling of A2B ARs to Phospholipase C. The activation of A2B ARs in HEK-A2B cells was found to increase the cellular content of IP₃. This response was not affected by pretreating the cells with pertussis toxin (Fig. 6A). In contrast, pertussis intoxication to inactivate Gᵢ and Gₒ family G proteins abolished the ability of IB-MECA to stimulate phospholipase C in HEK-A3 cells (Fig. 6B). The data are consistent with the idea that A3 receptors are coupled to phospholipase C via Gₒ₁₁. A dose-response curve for NECA to stimulate IP₃ in HEK-A2B cells is shown in Fig. 6C. The ED₅₀ value for NECA, 19 nM, is close to its ED₅₀ to stimulate cAMP accumulation (29 nM; Fig. 7). We considered the possibility that there might be cross-talk between the cAMP and IP₃ pathways in HEK cells. However, as illustrated in Fig. 8, two activators of adenylyl cyclase, isoproterenol and forskolin, increase cAMP levels in HEK-A2B cell and minimally influence IP₃ levels, whereas UTP increases IP₃ but not cAMP. The effects of UTP to stimulate phospholipase C or NECA to stimulate cAMP accumulation are not affected by pretreatment of cells with pertussis toxin (data not shown). The data are consistent with the interpretation that A2B ARs are dually coupled to Gₒ₁₁ and to Gₒ₁₁.

The coupling of A2B ARs to PLC was further characterized by measuring calcium mobilization in response to A2B AR activation in HEK-A2B cells. Figure 9 shows that the response to NECA is not affected by intoxication of cells with pertussis toxin but is largely attenuated by 100 μM theophylline or enprofylline. The data are consistent with the interpretation that NECA stimulates calcium mobilization as a consequence of activating A2B ARs that are coupled to Gₒ₁₁.

Coupling of A2B ARs to Calcium Mobilization in Human Mast Cells. Adenosine stimulates the degranulation of rodent and canine mast cells by activating A2 and A2B receptors, respectively (Ramkumar et al., 1993; Auchampach et al., 1997; Jin et al., 1997). A2B ARs are thought to be responsible for triggering interleukin-8 release in the human mast cell tumor line, HMC-1 (Feoktistov and Biaggioni, 1995). Figure 10 shows that calcium mobilization and cAMP accumulation in HMC-1 cells are stimulated by NECA > IB-MECA. The response to NECA was not affected by pretreating HMC-1 cells with pertussis toxin. These data are consistent with the involvement of an A2B AR in human mast cell calcium mobilization. However, we could not detect specific ¹²⁵I-ABOPX binding to HMC-1 cell membranes, which suggests that A2B receptor density is too low to be detected with this radioligand.

**Discussion**

Physical Properties of A2B Adenosine Receptors. In this study, we characterized the physical, pharmacological, and coupling characteristics of recombinant human A2B ARs. Based on their cDNA sequences, the wild-type and H/F-A2B ARs are predicted to have molecular masses of 36 and 38.5 kDa, respectively. There are consensus sites for N-linked glycosylation on asparagines 153 and 163 in the second extracellular loop of the human receptor. The first site is conserved in the A2B sequences of all species that have been cloned to date (Linden and Jacobson, 1998). In a previ-

**TABLE 2**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Kᵢ (nM)</th>
<th>A₂A</th>
<th>A₂B</th>
<th>A₃</th>
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<tbody>
<tr>
<td>I-ABOPX</td>
<td>70 ± 3</td>
<td>95 ± 15</td>
<td>30 ± 2</td>
<td>15 ± 2</td>
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<tr>
<td>ZM214385</td>
<td>530 ± 63</td>
<td>1.4 ± 0.6</td>
<td>31 ± 1.0</td>
<td>269 ± 91</td>
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<tr>
<td>CPX</td>
<td>2.5 ± 0.1</td>
<td>156 ± 73</td>
<td>8 ± 16</td>
<td>509 ± 109</td>
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<tr>
<td>Theophylline</td>
<td>6,200 ± 1,500</td>
<td>4,200 ± 240</td>
<td>9,200 ± 750</td>
<td>52,300 ± 16,000</td>
</tr>
<tr>
<td>Enprofylline</td>
<td>44,000 ± 11,000</td>
<td>32,000 ± 7,800</td>
<td>6,300 ± 1,800</td>
<td>158,000 ± 70,000</td>
</tr>
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**Fig. 4.** A, competition by the enprofylline and theophylline for ¹²⁵I-ABOPX binding to HEK-A₂B membranes. Each tube contained 208,000 cpm (0.56 nM) ¹²⁵I-ABOPX and 20 μg of membrane protein. Each point is the mean ± S.E.M. of triplicate determinations; where omitted, the S.E.M. is smaller than the symbols. Kᵢ values derived from triplicate experiments are summarized in Table 1B. Competition by CPX to inhibit NECA-stimulated cyclic AMP accumulation in CHO-A₂B cells. The concentration of CPX (nM) is 0 (●), 30 (●), 150 (●), and 750 (●). The inset is a Schild plot, the pA₂ is 7.66.
ous study, Western blots conducted with antipeptide antibodies derived from the human A2BAR sequence detected immunoreactivity in 50- to 55-kDa nonglycoproteins found in various human tissues (Puffinbarger et al., 1995). Hence, we considered the possibility that the antipeptide immunoreactivity might detect a cross-reacting epitope on a nonreceptor protein. To explore this possibility, the physical properties of the purified H/F-A2BAR were investigated. Figure 1 indicates that the purified H/F-A2BAR has characteristics close to those expected from the cDNA sequence (i.e., by western blotting with anti-FLAG antibodies it appears as a 35-kDa glycoprotein). These data imply that the immunoreactivity previously detected in tissues may not represent the A2BAR.

**125I-ABOPX Binding to A2BARs.** 125I-ABOPX is described here as a new and improved radioiodinated ligand for the detection recombinant A2BAR. It is nonselective; hence, it cannot be used to detect A2BAR on cells or tissues that possess multiple subtypes of adenosine receptors. It has an affinity ($K_D = 37$ nM) that is low enough to require isotope dilution to saturate binding sites. The radioligand cannot be used to detect low levels of endogenous receptors on untransfected HEK cells or HMC-1 mast cells. Nevertheless, the results indicate that 125I-ABOPX binds with a high signal-to-noise ratio.

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**Fig. 5.** Schild analysis of theophylline and enprofylline to inhibit NECA-stimulated cAMP accumulation in HEK-A2B cells. A, competition by theophylline. B, competition by enprofylline. Each point is the mean ± S.E. of triplicate determinations. Basal levels for cAMP ranged from 3 to 20 pmol/ml; the maximum response to NECA was 240–270 pmol/ml. C, Schild analyses of the data derived from A and B; $pA_2$ values for theophylline and enprofylline ranged from 10 to 17 μM in triplicate experiments.

**Fig. 6.** Stimulation of phospholipase C by recombinant adenosine receptors. HEK-A2B (A) or HEK-A3 (B) cells were pretreated for 16 h without or with 100 ng/ml pertussis toxin (PTX) and then stimulated with NECA or IB-MECA. Control concentrations of [3H]IP3 were 500–600 cpm/tube. C, dose-response curve for NECA to stimulate [3H]IP3 accumulation in HEK-A2B cells. The ED50 value of NECA is 19 nM. Each bar or point is the mean ± S.E. of triplicate determinations. The results are typical of three to five experiments.

**Fig. 7.** Dose-response curves of agonists to stimulate cAMP accumulation in HEK-A2B cells. Each point is the mean ± S.E. of triplicate determinations. ED50 values are: NECA, 28.6 nM; IB-MECA, 4.8 μM; CGS21680, 15.2 μM. The results are typical of triplicate experiments.
to-noise ratio to recombinant A2B ARs on HEK-A2B cells. When used in conjunction with a new HEK-A2B cell line that has a high density of A2B ARs (in excess of 20,000 fmol/mg protein), specific binding of 125I-ABOPX is >80% of total binding (Fig. 4). Hence this compound can used efficiently to screen competing ligands in competition radioligand binding assays.

**Agonist Binding to A2B ARs.** The results of competition binding assays generally confirm the assessment, based on functional data, that A2B ARs have a relatively low affinity for agonists (Daly et al., 1983). The affinity of agonists for the A2B AR is reduced by the presence of bulky substituents on either the N6- or the C2-position of the adenine ring. Thus, N6-aminobenzyl-NECA binds with 65-fold lower affinity than NECA. The introduction of a relatively small chlorine atom at the C2-position has a minor effect on affinity, thus 2-Cl-CPA binds with only 1.17-fold lower affinity than CPA. However, CGS21680, which consists of NECA with a bulky 2-aryl substituent, binds with an affinity more than 1000 times lower than NECA. The data also are consistent with the previous observation that 2-phenylethoxy-9-methyladenine is a 2-substituted adenine-analog antagonist that discriminates well between A2A receptors on coronary arteries and A2B receptors on the aorta (Martin et al., 1993). The introduction of N-ethylcarboxamide on the 5’-position of the ribose ring of adenosine moderately enhances agonist affinity for A2B ARs inasmuch as the affinity of N6-aminobenzyl-5’-NECA (AB-NECA) is 5.8-fold higher than the affinity of the same compound lacing the 5’ substitution, ABA.

**Antagonist Binding to A2B ARs.** K1 values for antagonists noted in this study generally agree with literature K1 estimates for human receptors based on functional assays (Brackett and Daly, 1994; Alexander et al., 1996; Cooper et al., 1997). We found in this study that A2B ARs have a high affinity for CPX and ZM241385. It is significant in this regard that CPX or ZM241385 are often used in the concentration range of 0.1 to 1 μM to selectively block A1 or A2A receptors, respectively. At 1 μM, either of these antagonists would occupy more than 90% of human A2B ARs. The K1 value of enprofylline for binding to A2B receptors, 6 μM, agrees closely with functional estimates of K1 value based in inhibition of agonist-stimulated cAMP accumulation in NIH-3T3 cells (Brackett and Daly, 1994) and HEL cells (Feoktistov and Biaggioni, 1995). It was noted in 1982 that the K1 value of enprofylline needed to inhibit cAMP accumulation in rat hippocampal slices, approximate 7 μM, is 20 times more potent than the K1 for binding to rat A1 receptors. Hence, it seems likely that the receptor responsible for cAMP accumulation in hippocampal slices is A2B.

**Coupling Characteristics of A2B ARs.** We demonstrate in this study that the over-expressed recombinant human A2B ARs in HEK 293 cells couple not only to cAMP accumu-

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**Fig. 8.** Stimulation of IP3 (A) and cAMP (B) accumulation by various compounds in HEK-A2B cells. Cells were treated with 1 μM isoproterenol (ISO), 10 μM forskolin (Forsk), 10 μM UTP, or 10 μM NECA, and cAMP and IP3 accumulation were determined as described in Experimental Procedures. Each bar is the mean ± S.E. of duplicate (IP3) or triplicate (cAMP) determinations. The results are typical of three to four replicate experiments.

**Fig. 9.** NECA stimulation of calcium mobilization in HEK-A2B cells. Cells in suspension (106/ml) were stimulated with 1 μM NECA (arrows). A, control cells or cells pretreated with 200 ng/ml pertussis toxin (PTX) for 18 h. B, control cells or cells pretreated with enprofylline or theophylline for 5 min. The results are typical of triplicate experiments.
lution, but also to phospholipase C activation and calcium mobilization. It is possible that the coupling of recombinant receptors to one or both of these signaling pathways could be an artifact of receptor over-expression, resulting in indiscriminate coupling of recombinant receptors to various HEK cell G proteins. However, there is an endogenous A2BAR on HEK 293 cells (Townsend-Nicholson, 1997) that also couples to the accumulation of cAMP and calcium as well as to the activation of the mitogen-activated protein kinase isofrom extracellular-signal regulated kinase (Gao et al., 1999). We provide evidence here that calcium mobilization in response to NECA in HEK-A2B cells is not a result of cAMP accumulation (Fig. 8); rather, it is probably caused by the direct coupling of A2BARs to G_{q/11}.

The ED_{50} values of NECA required to stimulate cAMP accumulation in untransfected HEK and HEK-A2B cells are 2.7 μM (Gao et al., 1999) and 28.6 nM (Fig. 7), respectively, a difference of approximately 100-fold. Spare receptor theory dictates that under some circumstances the potency of an agonist in functional assays is proportional to receptor density (Nickerson, 1956; Nickerson, 1956). If this relationship holds for the endogenous A2BAR of HEK cells versus HEK-A2B cells, than the endogenous level of receptor expression in HEK cells can be approximated as about 200 fmol/mg protein. This should be considered a high estimate because lower levels of receptors are likely to have coupling efficiency greater than (or similar to) overexpressed receptors.

**A2BARs and Mast Cell Function.** The A2BAR has been implicated as the adenosine receptor that is responsible for stimulating the degranulation of canine BR mastocytoma cells (Auchampach et al., 1997) and for triggering the slow release of interleukin-8 from human HMC-1 mast cells (Feoktistov and Biaggioni, 1995). In this study, we show that A2BARs also trigger a rapid mobilization of calcium in HMC-1 cells. The agonist potency order (NECA > IB-MECA) is consistent with calcium mobilization in HMC-1 cells being mediated by an A2BAR. It is notable that a different AR subtype, the A3AR, mediates the degranulation of hamster perivascular mast cells (Shepherd et al., 1996; Jin et al., 1997) and rat RBL-2H3 mast-like cells (Ramkumar et al., 1993). It is calcium mobilization rather than cAMP accumulation that is likely to trigger mast cell degranulation in response to A2BAR activation, because cAMP seems to inhibit mast cell degranulation (Church and Hughes, 1985; Hughes and Church, 1986; Hughes et al., 1987; Jin et al., 1997).

**Xanthines in the Treatment of Asthma.** We found that enprofylline and theophylline block human A2BARs with K_{i} values in the range of 6 to 8 μM. Optimal plasma levels of theophylline have lower affinities for the A1AR, A2AR, and A3AR subtypes, whereas enprofylline is somewhat selective for the A2AR subtype (Table 2). Blockade of A2AARs by theophylline may be counterproductive in the treatment of asthma because activation of A2AARs has inhibitory effects on mast cells and several other types of inflammatory cells (Sullivan and Lindingen, 1998). Adverse side effects of theophylline that are not shared by enprofylline may be mediated principally by A1AR blockade. These include diuresis, free fatty acid release, gastric secretion, and central nervous system stimulation (Per-ssen, 1986).

The observation that enprofylline is effective in the treatment of human asthma (Chapman et al., 1990, 1994) suggests that activation of the A2AR may contribute to bronchoconstriction in human disease. The radioligand 125I-ABOPX cannot be used to detect low levels of endogenous receptors on untransfected HEK cells or HMC-1 mast cells. Nevertheless, the availability of improved radioligand binding assays described here should facilitate the search for new A2B-selective antagonists that may be useful for the treatment of asthma.

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