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# Characterization of Two Affinity States of Adenosine $A_{2a}$ Receptors with a New Radioligand, 2-[2-(4-Amino-3-[$^{125}$I]iodophenyl)ethylamino]adenosine

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## SUMMARY

Adenosine analogs substituted in the 2-position with arylamino groups have been found to have high affinity and selectivity for $A_{2a}$ adenosine receptors. Two such compounds, 2-[2-(4-aminophenyl)ethylamino]adenosine and 2-[2-(4-amino-3-iodophenyl)ethylamino]adenosine (I-APE), were synthesized and found to be potent coronary vasodilators ($ED_{50}$ < 3 nM). These compounds bind weakly to $A_1$ adenosine receptors of rat cortex ($K_i$ > 150 nM). $^{125}$I-APE was synthesized and the new radioligand was found to bind to two affinity states of rat striatal $A_{2a}$ adenosine receptors ($K_d$ = 1.3 ± 0.1 nM and 19 ± 4.5 nM). The high affinity site represents a previously unrecognized small (15–20%) fraction of $A_{2a}$ adenosine receptors coupled to G proteins. Guanosine 5′-*O*-(3-thio)triphosphate (GTPγS) reduces specific binding of $^{125}$I-APE half-maximally at a concentration of 45 ± 2 nM. [$^3$H]CGS21680 also binds to two affinity states of $A_{2a}$ receptors on striatal membranes ($K_d$ = 3.9 ± 0.9 and 51 ± 5.5 nM), although in previous studies single $K_d$ values ranging from 5 to 15 nM have been reported. This high affinity site is substantiated by the finding that the $IC_{50}$ of CGS21680 in competition with $^{125}$I-APE binding to striatal membranes is shifted leftward in membranes diluted for 4 min before filtration, to selectively dissociate radioligand from low affinity receptors. Assuming that agonist radioligands bind to both coupled and uncoupled forms of striatal $A_{2a}$ adenosine receptors, we could simulate with the computer the finding that the decrease in specific binding induced by GTPγS (100 μM) is variable and depends on radioligand concentration, ranging from 20 to 90%. Unlike $^{125}$I-APE, [$^3$H]CGS21680 is charged at physiological pH, and treatment of membranes with the pore-forming antibiotic alamethicin uncovers cryptic [$^3$H]-CGS21680 but not $^{125}$I-APE binding sites. We conclude that the GTPγS-sensitive high affinity form of the $A_{2a}$ adenosine receptor can be preferentially labeled by $^{125}$I-APE, due to both its high specific activity and its physicochemical properties. Possible functional manifestations of poor coupling of $A_{2a}$ adenosine receptors to G proteins are discussed.

Four subtypes of adenosine receptors, referred to as $A_1$, $A_{2a}$, $A_{2b}$, and $A_3$, have been cloned (1). In the central nervous system $A_{2a}$ adenosine receptors are found in high density in striatum (2), and in the periphery they are known to produce adenosine-mediated coronary dilation (3, 4). Radioligand binding to striatal receptors was characterized initially by using [$^3$H]NECA (5) or [$^3$H]PD 115,199 (6), compounds that bind with similar affinities to $A_1$ and $A_{2a}$ adenosine receptors, in combination with ligands that selectively block $A_1$ adenosine receptors (see Fig. 1 for structures). Subsequently, [$^3$H]CGS21680 was developed as an agonist radioligand that binds selectively to $A_{2a}$ receptors (7–9). A relatively hydrophobic radioiodinated derivative of this ligand, $^{125}$I-PAPA-APEC, was synthesized and utilized in a few studies (10, 11).

CGS21680 and PAPA-APEC are substituted in the 2-position of adenine (see Fig. 1). A number of other 2-substituted adenosines have been characterized as high affinity ligands with selectivity for $A_{2a}$ adenosine receptors (3, 4, 12). In this study we report on the synthesis and characterization of $^{125}$I-APE, a novel, 2-substituted, radioiodinated, $A_{2a}$-selective radioligand that is less hydrophobic than $^{125}$I-PAPA-APEC (Fig. 1).

The adenosine receptors belong to the large superfamily of receptors that are coupled to G proteins (13). Two populations of receptors make up the membrane pool; those coupled to G proteins have a high affinity for agonists that is blunted by

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**ABBREVIATIONS:** NECA, 5′-*N*-ethylcarboxamidoadenosine; APE, 2-[2-(4-aminophenyl)ethylamino]adenosine; I-APE, 2-[2-(4-amino-3-iodophenyl)ethylamino]adenosine; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; PAPA-APEC, 2-[4-[2-[2-[(4-aminophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine; PD 115,199, *N*-[(2-dimethylamino)ethyl]-*N*-methyl-4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)benzenesulfonamide; CGS21680, 2-[4-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high performance liquid chromatography; PMSF, phenylmethylsulfonyl fluoride; ABA, $N^6$-aminobenzyladenosine.

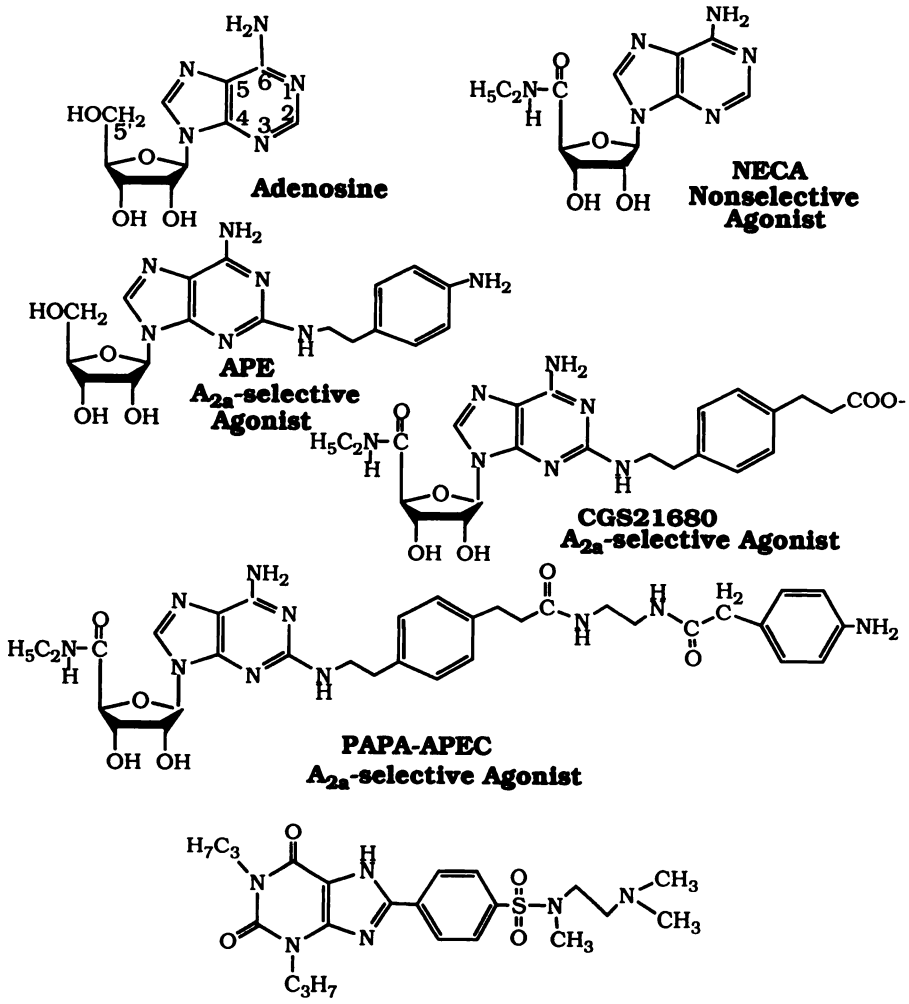


Fig. 1. Chemical structures of some adenosine $A_{2a}$ receptor ligands referred to in the text.

guanine nucleotides, and those uncoupled from G proteins are in a low affinity state. The binding of $^{125}$I-PAPA-APEC to striatal $A_{2a}$ receptors has been reported to be largely insensitive to guanine nucleotides and hence to be "tightly coupled" to $G_s$ (11). In contrast, [$^3$H]CGS21680 binding to striatal $A_{2a}$ receptors has been reported to be variably sensitive to inhibition by guanine nucleotides; usually specific binding is inhibited by about 50% (7–9). We now report that, under appropriate conditions, $^{125}$I-APE and [$^3$H]CGS21680 bind both to G protein-coupled $A_{2a}$ adenosine receptors and to a major fraction (85%) of uncoupled $A_{2a}$ adenosine receptors. Consequently, shifts in binding induced by GTP$\gamma$S depend upon the concentration of radioligand.

## Materials and Methods

### Chemicals

[$^3$H]CGS21680 (40.5 Ci/mmol) was purchased from DuPont-New England Nuclear, Na$^{125}$I from Amersham Corp. (Arlington Heights, IL), adenosine deaminase from Boehringer Mannheim Biochemicals (Indianapolis, IN), chloramine T from Kodak Chemicals, and CGS21680 and NECA from Research Biochemicals (Natick, MA). PMSF, leupeptin, pepstatin A, trypsin inhibitor, HEPES, EDTA, benzamidine, $MgCl_2$, alamethicin, and GTP$\gamma$S were from Sigma Chemical Co. $^{125}$I-ABA was synthesized as described previously (14). 2-Chloroadenosine was synthesized as described (15); other materials for the synthesis of APE and I-APE were from Aldrich and were used as supplied.

### Chemical Characterization of Ligands

All newly synthesized compounds were characterized by melting point, NMR, and elemental analyses. Melting point determinations employed a Thomas Hoover apparatus. $^1$H NMR spectra of solutions of nucleosides in dimethylsulfoxide-$d_6$ were recorded at 60 MHz and were consistent with the reported structures. Elemental analyses were performed by MHW Laboratories (Tucson, AZ). Preparative reverse phase HPLC employed a Rainin Autoprep system fitted with a column of $C_{18}$ silica. The eluant was a linear gradient of 50–70% methanol in water.

### Synthesis of Ligands

**APE.** A mixture of 2-chloroadenosine (2 g, 6.6 mmol), 2-(4-aminophenyl)ethylamine (10 g, 73.4 mmol), and *N,N'*-diisopropylethylamine (10 ml), in a stainless steel reaction vessel, was heated for 4 hr at 140°. After cooling and decantation of excess amine, the syrupy residue was taken up in 50% methanol/water for purification by preparative HPLC. Evaporation of appropriate fractions yielded 2.1 g (79%) of pure product as yellow needles (m.p. 90–93°; analysis for $C_{18}H_{23}N_7O_4$: formula weight, 401.33; calculated, C, 53.85; H, 5.76; N, 24.44; found, C, 53.86; H, 5.87; N, 24.21).

**I-APE.** A solution of iodine monochloride (224 mg, 1.38 mmol) in 2 ml of glacial acetic acid was added dropwise to a solution of APE (0.5

g, 1.25 mmol) in 10 ml of glacial acetic acid. The product, which separated out almost immediately, was filtered off, taken up in 50% methanol/water, and purified by preparative HPLC. Concentration of appropriate fractions yielded 150 mg (23%) of product as golden-yellow needles (m.p. 117–118°; analysis for $C_{15}H_{22}IN_7O_4$: formula weight, 545.24; calculated, C, 39.64; H, 4.44; I, 23.27; N, 17.98; found, C, 39.80; H, 4.68; I, 23.12; N, 17.85).

**$^{125}$I-APE.** A solution of Na$^{125}$I (1 mCi; Amersham), 5 μl of 1 mM APE, and 5 μl of aqueous chloramine T (1 mg/ml) in 50 μl of 100 mM $NaH_2PO_4$, pH 7.3, was incubated for 15 min at 21°. The reaction was terminated by the addition of 50 μl of 10.5 mM $NaHSO_3$. Preparative HPLC with a $C_{18}$ silica column eluted isocratically with methanol/5 mM $NaH_2PO_4$, pH 6 (45:55), separated the radioiodinated product, which was identified by monitoring the γ activity of the effluent (Fig. 2). APE and the major product were eluted at 4.8 and 9.5 min, respectively. $^{125}$I-APE accounted for 50–70% of the total $^{125}$I added.

### Coronary Perfusion Pressure

The aortae of rat hearts were cannulated and retrogradely perfused with Krebs-Henseleit buffer equilibrated with 95% $O_2$/5% $CO_2$. The hearts were paced at 4 Hz by ventricular stimulation and perfused at a constant flow rate of 9–10 ml/min/g. Coronary perfusion pressure was monitored through a side port in the aortic cannula.

### COS Cell Expression Studies

Rat $A_{2a}$ adenosine receptor cDNA subcloned into pcDNA1 (Invitrogen, San Diego, CA) was a generous gift from Dr. Stephen Reppert (Massachusetts General Hospital, Boston, MA). Rat $A_{2a}$ adenosine receptor DNA was introduced into COS-7 (COS/$A_{2a}$) cells using the DEAE-dextran method (16). COS-7 cells were grown in Dulbecco's modified Eagle's medium with 7.5% fetal calf serum for 60 hr after transfection.

### Membrane Preparation and Radioligand Binding

To prepare membranes for radioligand binding assays, cells were initially washed in phosphate-buffered saline, pH 7.4. A mixture of protease inhibitors (100 μM PMSF, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 1 μg/ml trypsin inhibitor) was added to all buffers used during and after homogenization. Cells or striata dissected from fresh or frozen rat brains (Pel-Freez)[1] were homogenized in 10 mM EDTA, 10 mM Na-HEPES, pH 7.4, 0.1 mM benzamidine, and centrifuged at 20,000 × $g$ for 20 min. Pellets were resuspended and washed twice in HE buffer (10 mM Na-HEPES, pH 7.4, 1 mM EDTA, 0.1 mM benzamidine) and were resuspended in the same buffer with 10% (w/v) sucrose at a membrane protein concentration of 5 mg/ml. Membranes were frozen in aliquots at −20°. For radioligand binding to $A_{2a}$ receptors, triplicate aliquots of cell membranes were diluted to 1 mg of protein/ml and incubated in 0.1 or 0.2 ml at 21° with 5 mM $MgCl_2$, 1 unit/ml adenosine deaminase, and 0.1–25 nM $^{125}$I-APE for 1–2 hr or 0.2–15 nM [$^3$H]CGS21680 (DuPont-NEN) for 4–5 hr. When used at concentrations above 5 nM, the specific activity of $^{125}$I-APE was diluted 10–20-fold with nonradioactive I-APE. To minimize errors, both the radioactive and nonradioactive forms of the ligand were simultaneously serially diluted. $^{125}$I-ABA was used as a radioligand for $A_1$ adenosine receptors (14). Nonspecific binding was measured in the presence of 100 μM NECA. In preliminary experiments, nonspecific binding was always found to be linearly related to the free concentration of radioligand. Therefore, to achieve greatest accuracy, in some experiments nonspecific binding was determined in sextuplicate at two radioligand concentrations and other values were calculated by linear extrapolation. The $IC_{50}$ values of ligands in competition for radioligand binding were fit to the equation $B/B_o = 1 - [(\Sigma i = 1 \text{ to } n)F_i(1 - NS)[I]/(IC_{50i} + [I])]$, where $B$ is specific binding, $B_o$ is specific binding in the absence of inhibitor, [I] is the concentration of inhibitor, $n$ is the number of receptor affinity states, $F_i$ is the fraction of receptors in each affinity state, and $NS$ is nonspecific binding. Assumptions of this model are that all ligands bind to equilibrium and that, for $n > 1$, there are independent binding sites.

### Data Analysis

Binding data were fit by the method of Marquardt (17) to equations describing one or two binding sites. Data are presented as means ± standard errors of at least three experiments, unless otherwise noted. For one- versus two-site comparisons, a method described by Motulsky and Ransnas (18) was used. Two-site fits were determined to be significantly better than one-site fits if $F$ tests evaluated at $p \leq 0.05$ indicated significant improvement in the goodness of fit.

## Results

The structures of APE and I-APE (Figs. 1 and 2) are consistent with elemental analyses and $^1$H NMR spectra. $^{125}$I-APE, purified by HPLC, co-chromatographs with nonradioactive I-APE. A minor peak of $^{125}$I elutes just after the major peak and probably represents APE radioiodinated in the *meta*-position, relative to the aromatic amino group. This minor product was found to bind poorly to striatal $A_{2a}$ adenosine receptors (data not shown).

APE, I-APE, and CGS21680 were evaluated on coronary arteries, because the dilator response to adenosine is thought to be mediated primarily by $A_{2a}$ receptors (19). All three compounds produce similar 45–50% maximal decreases in coronary perfusion pressure. The average $ED_{50}$ values from closely agreeing duplicate determinations are CGS21680 (1.6 nM) < APE (2.8 nM) = I-APE (2.7 nM).

Equilibrium binding of $^{125}$I-APE to rat striatal and COS/$A_{2a}$ membranes consistently results in curvilinear Scatchard plots, and the binding data are statistically better fit to a two-site than to a one-site model. Fig. 3 depicts representative equilibrium binding curves for $^{125}$I-APE binding to rat striatal membranes in the absence and presence of GTPγS (50 μM). The high and low affinity $K_d$ values of $^{125}$I-APE binding to striatal membranes are 1.3 ± 0.1 nM and 19 ± 4.5 nM, respectively (three experiments). The $B_{max}$ values for the high and low affinity sites are 129 ± 28 and 674 ± 246 fmol/mg, respectively.[1] In the presence of GTPγS, $^{125}$I-APE binds to a single site with a $K_d$ of 14.6 ± 0.25 nM and a $B_{max}$ of 261 ± 95 (three experiments). GTPγS also appears to reduce somewhat the number of low affinity $^{125}$I-APE binding sites in striatal membranes, perhaps due to some binding of high concentrations of $^{125}$I-APE to G protein-coupled striatal $A_1$ adenosine receptors. $^{125}$I-APE does not bind specifically to untransfected COS cells (data not shown). In COS/$A_{2a}$ membranes, the high affinity $K_d$ of $^{125}$I-APE binding to COS/$A_{2a}$ membranes is 1.2 nM (data not shown). Quantification of low affinity receptors in COS/$A_{2a}$ membranes with $^{125}$I-APE is difficult, due to high nonspecific binding at radioligand concentrations above 25 nM; hence, low affinity $K_d$ values range from approximately 40 to 110 nM in triplicate experiments. The $K_d$ values for high and low affinity $^{125}$I-APE binding sites are similar in striatum and COS/$A_{2a}$ membranes, but the percentage of coupled receptors is only about 2% in COS/$A_{2a}$ membranes ($B_{max1}$ = 174 fmol/mg of protein, $B_{max2}$ = 8260 fmol/mg of protein). This is consistent with the possibility that recombinant $A_{2a}$ receptors are very poorly coupled to $G_s$ in COS cell membranes.

The affinity of I-APE and a series of other 2-substituted adenosine analogs for the high affinity state of $A_1$ adenosine

[1] The number of $A_{2a}$ receptors ($B_{max}$) detected with either [$^3$H]CGS21680 or $^{125}$I-APE in the striata obtained from frozen rat brains from Pel-Freez was variable among batches, ranging from 400 to 900 fmol/mg of protein.

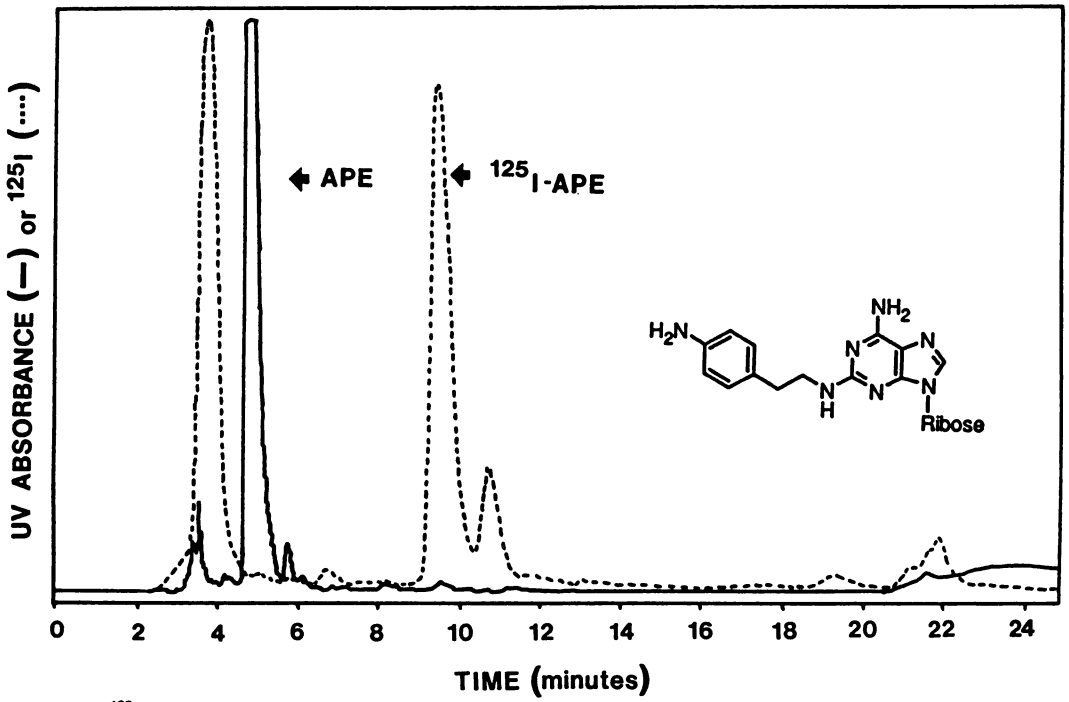


**Fig. 2.** HPLC purification of $^{125}$I-APE. APE was radioiodinated as described in Materials and Methods. UV absorbance at 260 nm and $^{125}$I (monitored with an in-line $\gamma$ detector) were continuously monitored. Fractions containing purified $^{125}$I-APE were pooled and stored in 50% methanol at −20° for use in radioligand binding assays.

receptors was evaluated on the basis of their ability to compete for $^{125}$I-ABA binding to receptors of rat cortex. The potency order for $A_1$ adenosine receptors is as follows (three experiments): I-APE (158 ± 16 nM) ≥ APE (308 ± 45 nM) > CGS21680 (1.3 ± 0.27 μM). Thus, APE and I-APE are not as selective for $A_{2a}$ receptors as is CGS21680. These data confirm that I-APE is only about 10-fold selective for low affinity $A_{2a}$ receptors, compared with high affinity $A_1$ adenosine receptors, but is >100-fold selective for high affinity $A_{2a}$ adenosine receptors versus high affinity $A_1$ adenosine receptors.

Examination of the high affinity component of $^{125}$I-APE binding ($K_d$ = 1.3 nM) in rat striatal membranes reveals that the number of binding sites is substantially reduced (~75–80%) by the addition of 50 μM GTPγS (Fig. 3), with a shift from two affinity states in the absence of GTPγS to a single lower affinity state in the presence of GTPγS. The $IC_{50}$ of GTPγS to reduce $^{125}$I-APE binding is 45 ± 2 nM (Fig. 4). These data suggest that high affinity binding sites of $^{125}$I-APE represent receptors that are coupled to G proteins.

Because $^{125}$I-APE and [$^3$H]CGS21680 bind to two affinity states of $A_{2a}$ receptors, we reasoned that CGS21680 should bind to two sites in competition for $^{125}$I-APE binding. As shown in Fig. 5, although a two-site competition model does not fit the binding data significantly better than a single-site model, a Hill coefficient of 0.78 does suggest that two binding sites might be present. To selectively detect the putative high affinity site in competition assays, striatal membranes were washed for 4 min at 21° in buffer containing 5 μM NECA. In preliminary kinetic experiments (two experiments) (data not shown), we estimated that the two dissociation constants for $^{125}$I-APE binding are 0.033–0.038/min and 1.3–1.8/min for high and low affinity sites, respectively, at 21°. Four minutes was chosen as a sufficient time to dissociate >99% of radioligand from low affinity binding sites but only about 15% from high affinity binding sites. The 4-min wash produces a reproducible 2.6–3-fold decrease (three experiments) in the $IC_{50}$ of CGS21680 (3.4 versus 1.2 nM with normal versus long wash, respectively) and an increase in the Hill coefficient to near unity ($n_H$ = 0.92). Applying the Cheng-Prusoff (20) equation to the high affinity $IC_{50}$, a $K_i$ for CGS21680 of 1.1 nM is calculated, somewhat lower that the high affinity $K_d$ of 2–4 nM calculated in equilibrium [$^3$H]-CGS21680 binding assays fit to a two-site equation (Fig. 6) and substantially lower than estimates of [$^3$H]CGS21680 binding to striatal membranes estimated from single site fits, 5–15 nM (7, 9, 21–23).

We reasoned that [$^3$H]CGS21680 should also bind with two affinity states to striatal $A_{2a}$ receptors. However, because the specific activity of [$^3$H]CGS21680 is much lower than that of $^{125}$I-APE, high affinity binding sites are difficult to detect in saturation binding assays. We have noted previously that the pore-forming antibiotic alamethicin increases specific binding of [$^3$H]CGS21680 to striatal membranes (31). We believe this is because CGS21680 is charged at physiological pH values and hence is poorly accessible to receptors on the inside of membrane vesicles. To increase the number of receptors specifically bound at low nanomolar concentrations of [$^3$H]CGS21680, several saturation binding assays were performed in the presence of 15.6 μg/ml alamethicin, a concentration determined to maximally increase specific binding. At this concentration of alamethicin, total specific binding of a single concentration of [$^3$H]CGS21680 (6–10 nM) in four separate experiments is increased to 176 ± 7.6% of control. Alamethicin increases the high affinity and to a lesser extent the low affinity $B_{max}$ values of [$^3$H]CGS21680 binding without affecting the $K_d$ values (Fig. 6). In both saturation isotherms (i.e., with and without alamethicin), two affinity sites are detected, with $K_d$ values of 3 and 45 nM for the high and low affinity sites, respectively. The selective $A_1$ adenosine receptor antagonist 1,3-dipropyl-8-cyclo-

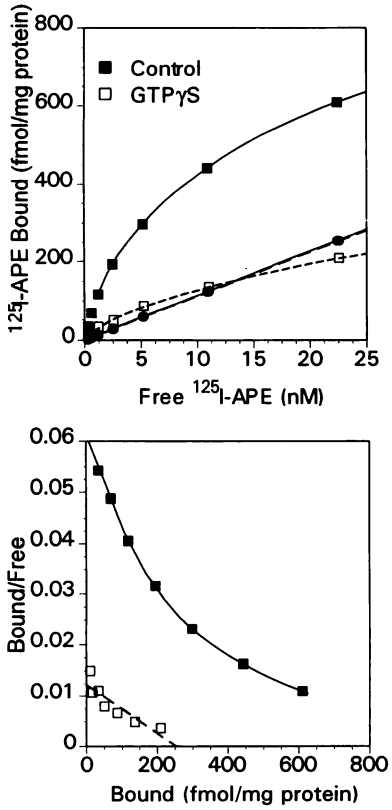


**Fig. 3.** $^{125}$I-APE binding to rat striatal membranes. A saturation isotherm (*top*) and Scatchard plot (*bottom*) of $^{125}$I-APE specifically bound (fmol of specifically bound $^{125}$I-APE/mg of total membrane protein) to rat striatal membranes, in the absence or presence of 50 μM GTPγS, are shown. Rat striatal membranes (80 μg/tube) were incubated with 0.25–23 nM $^{125}$I-APE. The specific activity of $^{125}$I-APE was diluted with 20 nM I-APE added to the highest concentration of radioligand and was serially diluted to allow for detection of low affinity binding sites. Nonspecific binding was measured in the presence of 100 μM NECA. Values shown are means from a representative experiment performed in triplicate. In three separate experiments, mean $K_d$ and $B_{max}$ values for control membranes were $K_{d1}$ = 1.30 ± 0.1 nM, $B_{max1}$ = 129 ± 28 fmol/mg of protein, $K_{d2}$ = 19 ± 4.5 nM, and $B_{max2}$ = 674 ± 246 fmol/mg of protein. In the presence of GTPγS, $^{125}$I-APE bound with a single affinity, with $K_d$ = 14.6 ± 0.25 nM and $B_{max}$ = 261 ± 95 fmol/mg of protein. □, Specific $^{125}$I-APE binding in the absence of GTPγS; ■, specific $^{125}$I-APE binding in the presence of GTPγS; ○, nonspecific binding in the absence of GTPγS; ●, nonspecific binding in the presence of GTPγS.

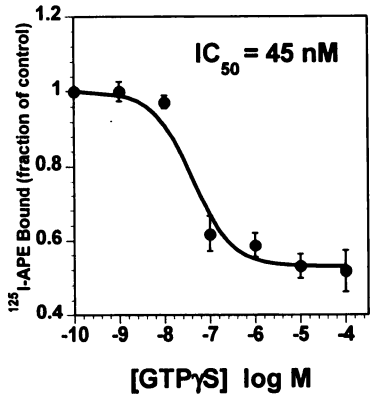


**Fig. 4.** Inhibition by GTPγS of $^{125}$I-APE binding to rat striatal membranes. Binding is plotted as a fraction of control specific binding. Striatal membranes (~100 μg/tube) and 0–10 μM GTPγS were incubated with 0.3 nM $^{125}$I-APE for 3 hr at room temperature. Values shown are means ± standard errors of a representative experiment performed in triplicate. The $IC_{50}$ of GTPγS is 45 ± 2 nM (three experiments).

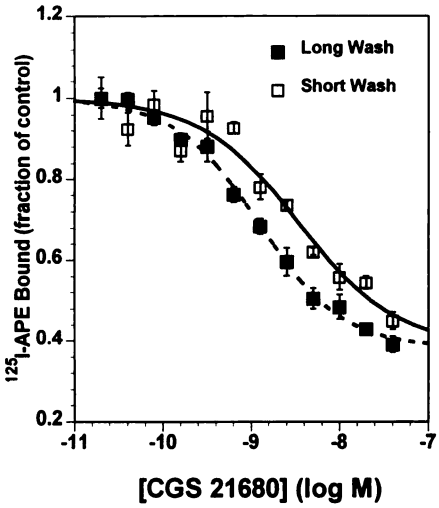


**Fig. 5.** Competition by CGS21680 with $^{125}$I-APE binding to rat striatal membranes. Binding is plotted as a fraction of control specific binding. Striatal membranes (~100 μg/tube) were incubated with 0–50 nM CGS21680 and were then incubated with 0.6 nM $^{125}$I-APE for 3 hr at room temperature. After the preincubation, membranes either were rapidly washed with ice-cold Tris/$Mg^{2+}$ buffer (short wash, 15 sec) (□) (see Materials and Methods) or were diluted in buffer containing 5 μM NECA and incubated for an additional 4 min at 21° (long wash) (■). $IC_{50}$ values are 3.4 and 1.2 nM with the short and long washes, respectively. Hill coefficients ($n_H$) are 0.78 and 0.92 with the short and long washes, respectively. Values are means ± standard errors of triplicate determinations.

pentylxanthine (100 nM) has no effect on [$^3$H]CGS21680 binding to striatal membranes (data not shown), indicating that the low affinity site is not a striatal $A_1$ receptor. Alamethicin increases the percentage of coupled receptors bound by [$^3$H]-CGS21680 from 22% to 34%. Alamethicin at this concentration has no effect on $^{125}$I-APE binding to rat striatal membranes (data not shown).

In some previous studies, guanine nucleotides have been reported to have only small effects on the binding of [$^3$H]-CGS21680 or $^{125}$I-PAPA-APEC (11). We reasoned that, because only a small fraction of $A_{2a}$ receptors are coupled to G proteins, the magnitude of the shift in binding produced by the addition of GTPγS would depend on the concentration of radioligand. High affinity receptors would be preferentially occupied by lower concentrations of radioligands. This hypothesis was verified in the experiment shown in Fig. 7. The effect of GTPγS to reduce radioligand binding to $A_{2a}$ receptors in a particular batch of rat striatal membranes varies from 13% at a high radioligand concentration (11 nM [$^3$H]CGS21680) to 65% at a low concentration of radioligand (36 pM $^{125}$I-APE). Furthermore, the degree of the shift is well predicted based upon a computer simulation using the binding parameters derived from equilibrium binding studies with the same membranes. Although not examined systematically, the reduction of binding produced by GTPγS is variable, depending on such factors as temperature, $Mg^{2+}$ concentration, and protein concentration.

## Discussion

We have synthesized and characterized a new agonist radioligand, $^{125}$I-APE, that binds with high affinity and selectivity

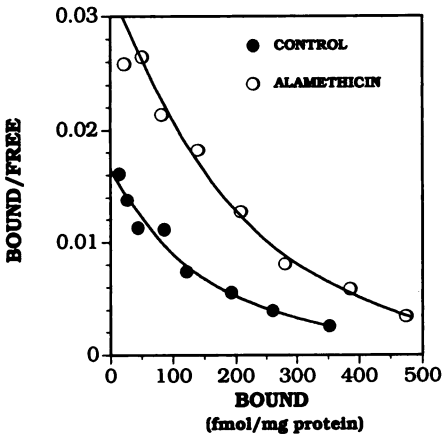


**Fig. 6.** Binding of [$^3$H]CGS21680 to rat striatal membranes. Scatchard plots of [$^3$H]CGS21680 binding in the absence (●) or presence (○) of alamethicin (15.6 μg/ml) are shown. Rat striatal membranes (88 μg/tube) were incubated in HE buffer containing 5 mM $MgCl_2$, 1 unit/ml adenosine deaminase, and 0.4–60 nM [$^3$H]CGS21680. Values shown are means of a single experiment that is representative of three experiments, each assayed in triplicate. For the experiment shown, $K_d$ values are 3 nM and 45 nM for the high and low affinity sites, respectively. $B_{max}$ values are $B_{max1}$ = 105 fmol/mg of protein (without alamethicin) and 170 fmol/mg of protein (same membranes with alamethicin) and $B_{max2}$ = 470 fmol/mg of protein (without alamethicin) and 502 fmol/mg of protein (with alamethicin). In three experiments $K_{d1}$ = 3.9 ± 0.9 nM and $K_{d2}$ = 51 ± 5.5 nM.

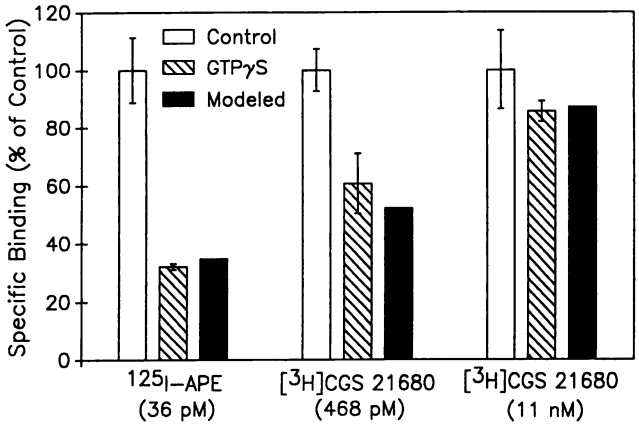


**Fig. 7.** Effect of radioligand concentration on the ability of GTPγS to modify binding to rat striatal membranes. Rat striatal membranes (~100 μg/tube) were incubated with 0.036 nM $^{125}$I-APE or 0.47–11 nM [$^3$H]-CGS21680 in the presence or absence of 50 μM GTPγS. Theoretical binding based on equilibrium binding parameters also is plotted (*Modeled*). The ratios of radioligand binding to high and low affinity sites were calculated from $K_d$ values, $B_{max}$ values, and radioligand concentrations ([L]) in a two-site binding model, $B = B_{max1}[L]/(K_{d1} + [L]) + B_{max2}[L]/(K_{d2} + [L])$. Values are means ± standard errors of triplicate determinations.

to a G protein-coupled form of $A_{2a}$ adenosine receptors. It is notable that the two affinity states of the $A_{2a}$ receptor that have not been clearly delineated in previous studies are detected with this new radioligand. In terms of the ratio of specific to nonspecific binding, $^{125}$I-APE does not have any advantage over [$^3$H]CGS21680, which displays excellent specific binding and superior selectivity for $A_{2a}$ versus $A_1$ adenosine receptors. The advantage of the new radioiodinated ligand lies in its much higher specific radioactivity (2200 versus 40.5 Ci/mmol), which is desirable for the purpose of using subnanomolar concentrations of radioligand to selectively measure high affinity binding sites or when tissue mass is limiting, e.g., to measure binding to small structures such as rat striatum. In addition, the charged compound CGS21680 may have limited access to receptors that are in cryptic pools (i.e., vesicles), as evidenced by the ability of alamethicin to increase particularly the high affinity and to a lesser extent the low affinity $B_{max}$ values of [$^3$H]CGS21680 binding to striatal membranes. In contrast, alamethicin has no effect on $^{125}$I-APE binding to striatal membranes.

Several lines of evidence suggest that the high affinity binding site corresponds to a G protein-coupled form of the $A_{2a}$ adenosine receptor. (i) Both $^{125}$I-APE and [$^3$H]CGS21680 label high and low affinity sites in striatum. (ii) $^{125}$I-APE also labels two populations of binding sites in membranes of COS cells transfected with rat $A_{2a}$ receptors. (iii) GTPγS produces a decrease in high affinity radioligand binding. (iv) We have noted recently that [$^3$H]CGS21680 binds to purified recombinant $A_{2a}$ receptors that are uncoupled from G proteins with a $K_d$ (50 nM) that is characteristic of the low affinity binding site in striatal membranes and COS/$A_{2a}$ membranes.[2]

Coupling of $A_{2a}$ receptors to G proteins appears to be particularly poor in transfected COS-7 cells. This has been observed previously in COS cells transfected with other receptors that couple to $G_s$ and apparently reflects low levels of $G_s$ in these cells and expression of a large number of receptors in a small percentage of cells. For example, Strader *et al.* (25) also found evidence that transiently overexpressed β-adrenergic receptors inefficiently couple to endogenous $G_{s\alpha}$ in COS-7 cells. More recently, Piersen *et al.* (26) reported that GTPγS was unable to reduce specific binding of [$^3$H]CGS21680 to COS cell membranes transfected with $A_{2a}$ receptors, suggesting that the low levels of endogenous $G_{s\alpha}$ are poorly coupled to the overexpressed receptors.

In previous studies, [$^3$H]CGS21680 has been reported to bind with high affinity to a single site in striatum, with $K_d$ values ranging between 5 and 15 nM (7, 9, 21–23). More recently, [$^3$H]CGS21680 was reported to bind to two sites in striatum, a high affinity site and a very low affinity site, with $K_d$ values of 5.6 and 343 nM, respectively (27). The latter, very low affinity, binding site may reflect, in part, binding of CGS21680 to $A_1$ receptors, which in the present study was found to occur with a $K_i$ of 1.3 μM. It may be that the previously reported $K_d$ values for single affinity sites represent a mixture of these two difficult to resolve $A_{2a}$ binding sites, with an average $K_d$ value of approximately 10 nM.

In addition to the high specific activity of $^{125}$I-APE, this radioligand may be capable of detecting more GTPγS-sensitive binding sites than is [$^3$H]CGS21680 for another reason. It is likely that receptors on the outside of membrane vesicles are most accessible to [$^3$H]CGS21680. If such receptors are coupled to G proteins, the G proteins would reside on the inside of vesicles, where they would be poorly accessible to GTP or GTPγS. This might contribute to the very small and quite variable shifts in [$^3$H]CGS21680 binding caused by the addition of guanine nucleotides that are noted in some instances. Alternatively, inhibition by guanine nucleotides of [$^3$H]CGS21680 binding to rat striatum has been shown to be markedly sensitive to $Mg^{2+}$ concentration (23), possibly accounting for the small and variable degrees of guanine nucleotide inhibition of [$^3$H]-CGS21680 binding reported in previous studies.

Studies of a number of $A_{2a}$ adenosine receptor agonists by means of bioassays and radioligand binding assays show that

[2] R. Woodard and J. Linden, unpublished observations.

the $EC_{50}$ for coronary vasodilation in rats or guinea pigs is 10–100 times lower than the $K_i$ for competition with [$^3$H]NECA for binding to the $A_{2a}$ adenosine receptors of either rat striatum or PC-12 cells (28, 29). Factors such as differences in receptor density, the degree of receptor-effector coupling, and other parameters have been invoked to explain the systematic difference between the two estimates of activity. By identifying the high affinity form of the $A_{2a}$ adenosine receptor and showing that the binding of [$^3$H]NECA reflects the affinity of the low affinity state of the $A_{2a}$ adenosine receptor, the present study appears to reconcile the two estimates of activity.

The finding that only a small fraction of $A_{2a}$ receptors in rat striatal membranes are coupled to G proteins suggests that the coupling between the $A_{2a}$ receptors and $G_s$ may be somehow limited. Limited coupling of $A_{2a}$ adenosine receptors in platelet membranes could provide an explanation for the unusual relationship between receptor number and activation of platelet adenylyl cyclase. A reduction of the number of $A_{2a}$ adenosine receptors in human platelet membranes as a result of photoaffinity labeling was reported to lead to a less than proportional decrease of both the extent and rate of adenylyl cyclase activation (30). Because there is generally a large excess of $G_s$ and effectors over receptors, the activation of adenylyl cyclase by agonists is usually found to be first order with respect to receptor number. Gross and Lohse (31) proposed a "restricted collision-coupling" model to account for the apparent receptor reserve in platelet membranes. Our data suggest an alternative possible mechanism for receptor reserve. If only a small fraction of receptors interact with $G_s$, then the G protein may be limiting in the activation of adenylyl cyclase. This does not necessarily imply that receptor number is in excess over $G_s$. Rather, only a subset of $G_s$ molecules may be capable of interacting with receptors, possibly because of the subunit structure of the G proteins, because of some covalent modification of the receptor or G protein, or because there is a limited compartment of G proteins that can interact with receptors. It will be of interest to determine why $A_{2a}$ receptors apparently are so poorly coupled to G proteins and whether this coupling is somehow regulated.

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