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# A Carboxyl-Terminally Truncated Mutant and Nonglycosylated $A_{2a}$ Adenosine Receptors Retain Ligand Binding

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## SUMMARY

The amino acids that comprise the ligand binding sites of adenosine receptors have not been identified. Adenosine and its agonist analogues differ from ligands for the well studied biogenic amine receptors and rhodopsin in that the adenosine receptor agonists are larger, contain a ribose moiety, and are uncharged at physiological pH. Thus, the locations of the ligand binding pockets of the adenosine receptors could differ significantly from those of the biogenic amine receptors. This report describes the characterization of a purification-amenable truncated mutant of the canine $A_{2a}$ adenosine receptor and demonstrates that neither the long carboxyl-terminal tail nor the glycosidic moiety appears to be required for ligand binding. The dog thyroid $A_{2a}$ adenosine receptor cDNA (RDC8) was subcloned into the mammalian expression vector pCMV4. A mutant $A_{2a}$ construct, in which six histidines replaced residues 310–412 as the carboxyl terminus of the protein, also was prepared. When overexpressed transiently in COS M6 cells, the wild-type and mutant $A_{2a}$ receptors exhibited similar 2-[*p*-(2- [$^3$H]carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine saturation binding and competition curve profiles. The following biochemical techniques confirmed that the COS M6 cells were transcribing and translating $A_{2a}$ receptors of the expected molecular masses: (a) immunoblotting with an antipeptide antibody directed against the putative carboxyl-terminal side of the second extracellular loop (Tyr$^{155}$–Val$^{172}$) of the canine $A_{2a}$ adenosine receptor, (b) photoaffinity labeling with the $A_{2a}$-selective agonist $^{125}$I-2-[4-[2-[2-[(4-azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine ($^{125}$I-azido-PAPA-APEC), and (c) partial purification of the hexahistidine-tagged receptor on $Ni^{2+}$·nitrilotriacetic acid resin. A presumed $A_{2a}$ receptor (44 kDa) from rabbit striatal membranes also was detected with the antisera against amino acids Tyr$^{155}$–Val$^{172}$ of the RDC8 receptor. Not only could the mutant $A_{2a}$ receptor be photolabeled specifically with $^{125}$I-azido-PAPA-APEC but so too could unglycosylated $A_{2a}$ receptors (i.e., from tunicamycin-treated COS M6 cells), either full length or truncated. In all of these cases, photolabeling was attenuated by both agonist and antagonist competitors.

Extracellular adenosine ultimately arises from a fall in the energy state within a cell (1). Adenosine then interacts with cell surface receptors to function as a neuromodulator in the central and peripheral nervous systems or as a local "retaliatory metabolite" throughout the body (2). $A_2$ adenosine receptors, in particular, stimulate adenylyl cyclase via coupling to $G_s$ and thereby restore energy and oxygen supplies to a tissue or a cell type (3, 4). $A_2$ receptors share the agonist rank order of potency of NECA > (*R*)-PIA > (*S*)-PIA but have been clearly divided into two subtypes. $A_{2a}$ receptors bind adenosine analogues with nanomolar affinities and are localized primarily in the striatum, whereas $A_{2b}$ receptors bind adenosine analogues with micromolar affinities and have a more widespread distribution in the brain and body (5). Two $A_{2a}$-selective radioligands, [$^3$H]CGS21680 and $^{125}$I-PAPA-APEC, have been developed by derivitizing NECA at the adenine C2-position (6, 7). Photolabeling with the light-sensitive $^{125}$I-PAPA-APEC azido analogue $^{125}$I-azido-PAPA-APEC first established the approximate molecular mass of the $A_{2a}$ receptor and demonstrated that the receptor is glycosylated (8, 9). The primary amino acid se-

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**ABBREVIATIONS:** NECA, 5′-*N*-ethylcarboxamidoadenosine; CGS21680, 2-[*p*-(2-carboxyethyl)phenylethylamino]-5′-*N*-ethylcarboxamidoadenosine; PAPA-APEC, 2-[4-[2-[2-[phenylmethylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine; azido-PAPA-APEC, 2-[4-[2-[2-[(4-azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine; PIA, $N^6$-(2-phenyl-1-methylethyl)adenosine; GPCR, G protein-coupled receptor; NTA, nitrilotriacetic acid; ADA, adenosine deaminase; 2-CADO, 2-chloroadenosine; CHA, $N^6$-cyclohexyladenosine; CV1808, 2-phenylaminoadenosine; DMEM, Dulbecco's modified Eagle's medium; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPBS, Dulbecco's phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DMSO, dimethylsulfoxide; TBS, Tris-buffered saline; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

quences (all close to 45 kDa) of $A_{2a}$ receptors from several species have been predicted from the corresponding cDNAs.

Canine (RDC8), rat, and human $A_{2a}$ adenosine receptor cDNAs have been isolated using degenerate, polymerase chain reaction primers based on other GPCRs (10–14). Secondary structural analyses of the primary sequences derived from these clones suggest that each receptor spans the plasma membrane seven times. The three species homologues are approximately 97% identical in their putative transmembrane spans and bind adenosine receptor agonists in similar manners (13–15). Minor sequence variations manifest themselves primarily in the second exofacial loops and carboxyl termini, regions that, based on precedents in the literature for the biogenic amine receptors, probably do not participate in ligand binding. Hydrophilic regions of the $\beta_2$-adrenergic receptor, for instance, can be deleted without significantly altering ligand binding (16, 17). Cloned $A_{2a}$ adenosine receptors resemble cloned $A_1$ and $A_{2b}$ adenosine receptors in their possession of short amino termini, potential *N*-linked glycosylation sites in interhelical region IV–V, and conserved histidines in transmembrane spans VI and VII (10, 18–23). Preliminary modeling of and a mutagenesis study on the $A_1$ adenosine receptor indicate that transmembrane residues, with the two histidines possibly included, constitute the $A_1$ binding cavity (21, 24–26). Although glycosylation is not essential for ligand binding to monoamine receptors such as the $\beta_2$-adrenergic receptor (27–31), the $A_{2a}$ ligand binding pocket could extend outside the transmembrane span boundaries traditionally observed for rhodopsin and the biogenic amine receptors; the adenosine ligand is distinctive in its larger size, incorporation of a ribose moiety, and uncharged character at physiological pH.

This report describes the characterization of a purification-amenable, truncated mutant of the canine $A_{2a}$ receptor (Fig. 1 highlights pertinent features of the canine wild-type $A_{2a}$ adenosine receptor and the truncated hexahistidine version of the $A_{2a}$ receptor created in our laboratory). Coding sequence for a hexahistidine affinity tag and a stop codon were inserted into the recombinant receptor cDNA 3′ of the codon for $Arg^{309}$, to facilitate purification of the protein on $Ni^{2+}$·NTA resin (32, 33). We examined whether the loss of 102 of the 121 carboxyl-terminal residues, nearly one quarter of the length of the wild-type receptor, had any effect on ligand binding through a series of [$^3$H]CGS21680 radioligand binding studies. Polyclonal antibodies were generated against RDC8 amino acids $Tyr^{155}$–$Val^{172}$, a segment of the putative second extracellular loop leading up to transmembrane span V. Finally, nonglycosylated $A_{2a}$ receptors from tunicamycin-treated COS M6 cells were tested to see whether they retained the ability to bind $^{125}$I-azido-PAPA-APEC and other agonist and antagonist ligands. For future peptide mapping studies, it will be necessary to know how, or whether, glycosylation affects $A_{2a}$ receptor mobility in our COS M6 expression system. Potentially, the mutant receptor hexahistidine tag and the $A_{2a}$ receptor antipeptide antibodies also will assist in the identification of radiolabeled peptides.

## Experimental Procedures

### Materials

The pBSK+/$A_{2a}$ plasmid, which originated in the laboratories of Drs. Jacques Dumont and Gilbert Vassart (Université Libre de Bruxelles), was provided by Dr. Richard Clark (University of Texas Health Science Center, Houston, TX). The pCMV family of plasmids was developed in the laboratory of Dr. David Russell (University of Texas Southwestern Medical Center, Dallas, TX). DNA enzymes were purchased from either New England Biolabs or Promega. RNase A (from bovine pancreas) was obtained from Sigma, but DNase-free RNase (also from bovine pancreas) was from Boehringer Mannheim. The Sequenase version 2.0 DNA sequencing kit and M13 reverse primer were obtained from United States Biochemical Corp. Plasmid DNA was occasionally purified on Qiagen Tip 2500 columns. COS M6 cells, developed by Edith Womack in the laboratories of Drs. Michael Brown and Joseph Goldstein (University of Texas Southwestern Medical Center), were obtained from Dr. Lee Limbird (Vanderbilt University, Nashville, TN), grown in medium containing heat-inactivated fetal bovine serum (Sigma), and transiently transfected using DEAE-dextran ($M_r$ 5 × $10^5$; Pharmacia). Hexahistidine $A_{2a}$ protein was partially purified on the $Ni^{2+}$·NTA agarose resin developed by Qiagen. Endoglycosidase F/N-glycosidase F (6 units/120 μl) was bought from Boehrin-

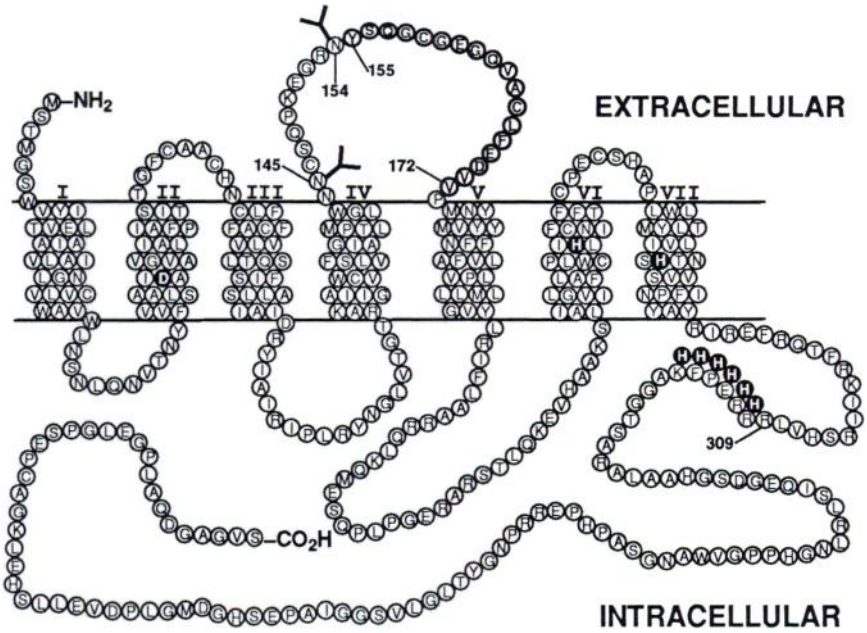


**Fig. 1.** Schematic representation of the membrane topology of the canine $A_{2a}$ (RDC8) adenosine receptor. Potential asparagine glycosylation sites, $Asn^{145}$ and $Asn^{154}$, are shown with carbohydrate symbols. Antipeptide antibodies were generated against amino acids $Tyr^{155}$–$Val^{172}$, and the hexahistidine-tagged mutant was truncated after $Arg^{309}$. Two histidines, $His^{250}$ and $His^{278}$, and $Asp^{52}$ are conserved throughout the adenosine receptor family.

ger Mannheim, and tunicamycin was purchased from Calbiochem. Leupeptin was purchased from Peptides International. ADA (calf intestinal type VIII), 2-CADO, NECA, (*S*)-PIA, and CHA were Sigma products. (*R*)-PIA was from Boehringer Mannheim, theophylline from Merck, and CV1808 and CGS21680 from Research Biochemicals Inc. Western blotting materials included Whatman 3-MM chromatography paper, the peroxidase Vectastain Elite ABC kit from Vector Laboratories, and 0.45 μm nitrocellulose paper and 4-chloro-1-napthol from Bio-Rad. Young rabbit brains (unstripped) were bought from Pel-Freez. [$^3$H]CGS21680 (39.6–48.6 mCi/μmol) and γ-$^{35}$S-dATP (1250 mCi/μmol) were purchased from DuPont-NEN. PAPA-APEC was a generous gift from Dr. Kenneth Jacobson (National Institutes of Health, Bethesda, MD). All other reagents were purchased from Sigma or Fisher Scientific Co.

## Methods

**Construction of eukaryotic pCMV4 expression vectors containing wild-type and hexahistidine $A_{2a}$ receptor cDNAs.** A short form of the dog thyroid $A_{2a}$ clone was subcloned from pBSK+ into pCMV4. The pBSK+/$A_{2a}$ plasmid included base pairs 258 through 1819 (*NciI-XbaI* restriction fragment) of the original RDC8 cDNA, with 35 5′ noncoding bases and 288 3′ noncoding bases surrounding the receptor-encoding DNA. The 1561 base pairs (plus 74 neighboring bases of pBSK+) were inserted between the *KpnI* and *XbaI* restriction enzyme sites of pCMV4 using a sticky/sticky strategy. The hexahistidine/pCMV4 construct was prepared by annealing the two overlapping oligonucleotides shown below:

```
                 His His His His His His    Stop
TG AGG CAT CAT CAC CAC CAC CAT TAA CCG CGG T
   CC GTA GTA GTG GTG GTG GTA ATT GGC GCC AGA TC
Bsu361                                SacII   XbaI
```

The resulting double-stranded DNA was ligated into the unique *Bsu*361 (base pair 1215 corresponds to $A_{2a}$ carboxyl-tail amino acid 308) and the polylinker *XbaI* restriction enzyme sites of the pCMV4/$A_{2a}$ construct. A *Sac*II site was incorpprated into the mutant sequence past the stop codon, for diagnostic purposes. Mutated $A_{2a}$ DNA was cloned back into pBSK+ for sequencing using the Sequenase Version 2.0 kit and M13 reverse primer.

**Maintenance of COS M6 cells and transient transfection with pCMV4/$A_{2a}$ constructs.** Cells were grown in monolayer cultures in DMEM supplemented with 10% fetal bovine serum, 20 mM HEPES, pH 7.4, 3 mM NaCl, 100 units/ml penicillin, and 100 μg/ml streptomycin. The incubator was maintained at 37° with a 95% air/5% $CO_2$ atmosphere. Wild-type and hexahistidine $A_{2a}$ plasmid DNA was transiently transfected into COS M6 cells by the DEAE-dextran method. Cells were plated at $4 \times 10^6$ cells/150-mm dish. The next day each dish was rinsed with 11 ml of DPBS (137 mM NaCl, 2.68 mM KCl, 1.47 mM $KH_2PO_4$, 8.20 mM $NaH_2PO_4$, pH 7.4), covered for 6 hr at 37° with 18 ml of DMEM containing 22 μg of plasmid DNA and 250 μg/ml DEAE-dextran, rinsed again with DPBS, incubated for 3 min in 7 ml of 10% DMSO/DPBS (v/v) at room temperature, incubated for an additional 2 hr in 18 ml of DMEM/0.1 mM chloroquine, and rinsed twice with DPBS. The cells were then grown in 18 ml of supplemented DMEM for 60–68 hr before harvesting.

**Tunicamycin treatment of $A_{2a}$ receptor-transfected COS M6 cells.** Two days before harvesting, DMEM was aspirated from transfected cells and replaced with 18 ml of DMEM containing 1 or 5 μg/ml tunicamycin. Control dishes received 15 μl of DMSO (vehicle) in 18 ml of fresh medium.

**Preparation of rabbit striatal membranes.** The following protocol was modified from the method of Nanoff *et al.* (34). Striata were dissected from young rabbit brains, placed in 20 volumes of ice-cold buffer (50 mM Tris·HCl, 5 mM EDTA, pH 7.4, containing 0.1 mM PMSF, 100 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1 μg/ml pepstatin A), and homogenized for 20 sec with a Brinkmann Polytron homogenizer at setting 6. The ground membranes were centrifuged at 49,460 × *g* for 10 min at 4°, and the pellet was resuspended in approximately 20 volumes of 50 mM Tris·HCl, 6 mM $MgCl_2$, pH 7.4, with a full complement of protease inhibitors. Aliquots of membrane homogenate were frozen in liquid $N_2$ and stored at −70°. Protein concentrations were determined using the Bio-Rad protein assay with ovalbumin standards.

**Preparation of $A_{2a}$ receptor-transfected COS M6 membranes.** Approximately 2.5 days after transfection, cells were confluent on the 150-mm culture dishes. Each dish was rinsed once with 10 ml of DPBS at room temperature, transferred to ice, covered with 10 ml of DPBS/1 mM EDTA, and scraped with a rubber policeman. After another EDTA/scraping cycle, cells were combined and pelleted by centrifugation at 49,460 × *g* for 10 min at 4°. The pellet was resuspended in 10 ml of 50/10/protease inhibitor buffer (50 mM Tris·HCl, 10 mM $MgCl_2$, pH 7.4, containing 0.1 mM PMSF, 100 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1 μg/ml pepstatin A) by repeatedly aspirating/expelling the mixture through a 20-gauge needle with a plastic syringe. The membranes were recentrifuged, brought up in 500 μl of 50/10/protease inhibitor buffer, frozen in liquid $N_2$, and stored at −70° until use. Protein concentrations were determined using the Bio-Rad protein assay with ovalbumin standards.

**[$^3$H]CGS21680 radioligand binding assays.** Membranes were treated for 20 min at 30° with 1.0 unit of ADA/40 μg of membrane protein to reduce the concentration of endogenous adenosine before the binding assays. Assays were carried out in a 250-μl reaction volume in 12- × 75-mm polystyrene tubes (Kew Scientific). Incubation ingredients included 2.5–20 μg of COS M6 membranes, Tris·HCl/$MgCl_2$ buffer to a final concentration of 50 mM/10 mM (designated 50/10), pH 7.4, varying amounts of competitor in competition binding assays, 20 μM 2-CADO in nonspecific binding tubes, and an appropriate concentration of [$^3$H]CGS21680. The concentration of [$^3$H]CGS21680 ranged from 0 to 120 nM in saturation binding assays. The radioligand concentration remained constant at 8 or 40 nM in competition binding assays and at 16 nM when the effect of $Na^+$ concentration on agonist binding was examined. 8-(4-Azidophenyl)-1,3-dipropylxanthine inhibition assays were performed under subdued lighting. The 90-min incubation at 24° was terminated by quenching with 3.5 ml of ice-cold 50/10 buffer. Bound radioligand and free radioligand were separated by pouring the tube contents over Whatman GF/C glass microfiber filters (presoaked in 50/10 buffer) situated on a vacuum filtration manifold. The filters were rinsed twice with 3.5 ml of cold buffer, air dried, transferred to scintillation vials, covered with 7 ml of Universol scintillant, shaken overnight, and counted in a liquid scintillation counter operating at 30–40% efficiency. Saturation and competition curves were analyzed using LIGAND, a nonlinear least squares model-fitting program.

**Synthesis of $^{125}$I-PAPA-APEC.** The synthesis of $^{125}$I-PAPA-APEC was performed as reported in the literature, with minor modifications (35). A 16-fold molar excess of PAPA-APEC to [$^{125}$I]NaI (NEZ-033H; DuPont NEN Products, Boston, MA) gave 65% incorporation of $^{125}$I. Separation from nonradioactive impurities and purification were carried out on a Waters (Milford, MA) high performance liquid chromatography system, using a Zorbax Rx $C_{18}$ column (Mac Mod, Chadds Ford, PA). A linear gradient of acetonitrile/0.1% trifluoroacetic acid in water yielded 99% radiochemically pure $^{125}$I-PAPA-APEC (2200 mCi/μmol). The product coeluted with a nonradioactive standard of PAPA-APEC.

**Synthesis of $^{125}$I-azido-PAPA-APEC.** This preparation was a slight modification of that reported previously (8). $^{125}$I-PAPA-APEC in methanol (200 μCi, 90 pmol) was dried under a stream of nitrogen gas, and the residue was dissolved in 300 μl of 6 N acetic acid. After the solution had been chilled on ice, 13.8 μl of 10 mg/ml aqueous $NaNO_2$ (0.138 mg, 2 μmol) were added. The reaction was allowed to proceed for 10 min on ice, and then the lighting was subdued. Ten microliters of 10 mg/ml $NaN_3$ (0.100 mg, 1.5 μmol) were pipetted into the mixture, the mixture was allowed to react for 5 min on ice, another 10 μl of 10 mg/ml $NaN_3$ were added, and the reaction vial was removed from the

ice. After 5 min at room temperature, the reaction mixture was alkalinized with 240 μl of concentrated $NH_4OH$. The Sandmeyer reaction completely converted $^{125}$I-PAPA-APEC to $^{125}$I-azido-PAPA-APEC, as verified by thin layer chromatography. In a solvent system of 85% chloroform/10% methanol/5% acetic acid, on silica gel 60, $^{125}$I-PAPA-APEC and $^{125}$I-azido-PAPA-APEC migrated with $R_f$ values of 0.28 and 0.37, respectively. Irradiated $^{125}$I-azido-PAPA-APEC migrated with an intermediate $R_f$ value of 0.31.

**Photoaffinity labeling of $A_{2a}$ receptors with $^{125}$I-azido-PAPA-APEC.** Our photolabeling procedure was adapted from that of Barrington *et al.* (8). Frozen COS M6 membranes were thawed and treated for 20 min at 30° with 1.0 unit of ADA/40 μg of membrane protein. Incubations were carried out in a 250-μl reaction volume in foil-covered polycarbonate tubes. Incubation ingredients included COS M6 membranes (50–300 μg of protein, depending on $A_{2a}$ expression levels; 1.5 pmol of receptor), 2.5 μl of DMSO or competitor (CGS21680 or theophylline), and 2 nM $^{125}$I-azido-PAPA-APEC (0.5 pmol), brought up to volume in HEPES buffer (50 mM HEPES, 10 mM $MgCl_2$, pH 6.8, containing 0.01%, w/v, CHAPS, 0.1 mM PMSF, 100 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, and 1 μg/ml pepstatin A). After a 1-hr incubation at 37°, the membrane suspension was diluted 5-fold with ice-cold HEPES buffer containing 0.03% CHAPS and was centrifuged at 49,460 × *g* for 10 min at 4°, and the pellet was then resuspended in 1 ml of HEPES buffer containing 0.01% CHAPS. The tube contents were transferred to quartz cuvettes and irradiated at 254 nm for 3 min in a Rayonet photochemical minireactor. After photoincorporation, membranes were washed a final time in HEPES buffer without CHAPS. Pellets were solubilized in 10% SDS sample buffer at room temperature and subjected to electrophoresis on 12% SDS-PAGE gels or on low molecular weight resolving gels (16.5% total/3% cross-linker SDS-PAGE gels with Tricine cathode buffer) (36). Coomassie blue-stained and dried gels were exposed overnight to XAR-5 Kodak film or DuPont-NEN Reflection film, typically with a single DuPont Quanta III intensifying screen enclosed in the cassette.

**Endoglycosidase F/N-glycosidase F treatment of labeled $A_{2a}$ receptor-containing membranes.** Labeled membranes were resuspended for the final time in 10 μl of 1% SDS. This suspension was heated in boiling water for 2 min before 90 μl of incubation buffer (20 mM $Na_2HPO_4 \cdot 7H_2O$, 10 mM $NaN_3$, 0.5%, v/v, Nonidet P-40, pH 7.2) were added. The tube was immersed again in boiling water for 2 min, and 0.25–0.50 unit of endoglycosidase was added when the tube and its contents had cooled to room temperature. After overnight incubation at 37°, 50 μl of 10% SDS sample buffer were pipetted into the 100-μl mixture, and the 150-μl samples were loaded onto gels.

**Generation of antipeptide antibodies to the $A_{2a}$ receptor.** The octadecapeptide YSQGCGEGQVACLFEDVV was synthesized and then purified by high performance liquid chromatography. The structure was verified by amino acid sequencing by Dr. Tom Lucas (Vanderbilt University, Nashville, TN). Antibody production was contracted to East Acres Biologicals (Southbridge, MA). Keyhole limpet hemocyanin-coupled peptide was prepared as the antigen, whereas bovine serum albumin-conjugated peptide was synthesized to test antisera reactivities. Two New Zealand white rabbits were injected subcutaneously with 1.0 mg of conjugated peptide suspended in Freund's complete adjuvant. Booster injections (subcutaneous injections with 0.5 mg of conjugated peptide suspended in Freund's incomplete adjuvant) were administered 3, 7, and 10 weeks later. As early as 8.5 weeks after the primary immunization, test bleed antisera recognized peptide and bovine serum albumin-peptide in dot blot and enzyme-linked immunosorbent assays.

**Immunoblotting of $A_{2a}$ receptors.** Rabbit striatal membranes (750 μg, ≈0.5 pmol of receptor) were pelleted (49,460 × *g* for 10 min at 4°) and solubilized in 75 μl of a buffer similar to that used by Palmer *et al.* (37) (100 mM $Na_2HPO_4$, 50 mM EDTA, pH 6.5, containing 0.8%, v/v, Triton X-100, 0.1 mM PMSF, 100 μg/ml soybean trypsin inhibitor, 2.5 μg/ml leupeptin, and 1 μg/ml pepstatin A). The mixture was kept on ice for 1 hr except for 15-sec bursts of sonication every 15 min. Particle-free supernatant was collected after centrifugation (14,000 × *g* for 15 min at 4°) and combined with an equal volume of 16% SDS sample buffer. Samples were loaded onto 0.75-mm, 15% SDS-PAGE minigels, and after electrophoresis the gel proteins were transferred to nitrocellulose paper for 45 min at 200 mA (constant voltage) on an American Bionetics Polyblot apparatus. The semidry transfer was accomplished using a discontinuous tribuffer system (anode buffer 1, 300 mM Tris, pH 10, 30% methanol; anode buffer 2, 25 mM Tris, pH 10.4, 20% methanol; cathode buffer, 25 mM Tris, 40 mM 6-aminohexanoic acid, 20% methanol). The membrane was soaked in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and then, in order, blocked with 3% gelatin in TBS for 1 hr, washed twice (5 min/wash) with water, exposed to 6 M urea denaturing solution for 45 min, again washed twice with water, reblocked for 30 min, washed twice (5 min/wash) with TBS/0.05% (v/v) Tween-20, and incubated with primary antibody overnight. Rabbit sera were diluted 500-fold in TBS/Tween-20 containing 1% gelatin. Detection was carried out according to the instructions supplied with the peroxidase Vectastain Elite ABC kit from Vector Laboratories.

$A_{2a}$ receptor-transfected COS M6 membranes (1 mg, ≈25 pmol of receptor) were pelleted (49,460 × *g* for 10 min at 4°) and solubilized in 75 μl of buffer (10 mM Tris, 1 mM EDTA, pH 7.4, containing 0.2%, w/v, deoxycholic acid, 1%, w/v, digitonin, 0.1 mM PMSF, 100 μg/ml soybean trypsin inhibitor, 2.5 μg/ml leupeptin, and 1 μg/ml pepstatin A). Untransfected or sham-transfected (pCMV4-transfected) COS M6 membranes (1 mg) were also solubilized as controls. From here, the immunoblotting protocol adhered to the steps outlined in the preceding paragraph. In later studies, we found it was more expedient to solubilize COS M6 membrane pellets (50–100 μg, ≈1.5 pmol of receptor) directly into 10% SDS sample buffer immediately before loading onto the gel.

**Partial purification of hexahistidine $A_{2a}$ receptor protein.** Hexahistidine $A_{2a}$ receptor-transfected COS M6 membranes (25 μg, 700 pmol of receptor), a fraction of which had been photolabeled, were collected by centrifugation (49,460 × *g* for 10 min at 4°) and resuspended in 5 ml of buffer A (6 M guanidine hydrochloride, 100 mM $Na_2HPO_4 \cdot 7H_2O$, 10 mM Tris·HCl 200 mM NaCl, 2 mM imidazole, pH 8.0). The slurry was sonicated on ice for 30 min and then rotated at 3° for 1 hr. Particle-free supernatant was obtained by centrifugation (10,000 × *g* for 10 min at 4°) and was circulated overnight, with a peristaltic pump, through a column holding a 300-μl bed of $Ni^{2+}$·NTA agarose resin (binding capacity, 1.5–3.0 mg of tagged protein). The column was washed sequentially with 20 column volumes of buffer A, buffer B (8 M urea, 100 mM $Na_2HPO_4 \cdot 7H_2O$, 10 mM Tris·HCl, 50 mM NaCl, 2 mM imidazole, pH 8.0), buffer C (buffer B prepared with $NaH_2PO_4 \cdot 2H_2O$ at pH 6.3), buffer D (buffer C with the concentration of imidazole increased to 20 mM), and buffer E (buffer C with the concentration of imidazole increased to 250 mM). Fractions (500 μl) were collected in Eppendorf tubes at a flow rate of approximately 65 μl/min, counted in a Beckman Gamma 4000 counter, concentrated if necessary in Microcon 10 concentrators, and subjected to electrophoresis on 12% SDS-PAGE gels. Total protein was visualized by silver staining. Receptor protein was visualized by autoradiography.

## Results

**Characterization of wild-type and hexahistidine $A_{2a}$ adenosine receptor ligand binding.** Because the pCMV4 mammalian expression vector functions very effectively in SV40-transformed cells (38), we transiently overexpressed $A_{2a}$ adenosine receptors in COS M6 cells, a cell line derived from simian kidney fibroblasts. We, and others, have not detected endogenous $A_1$ or $A_{2a}$ receptors in parental or vector-transfected COS cells via radioligand binding (data not shown) (13, 15). However, as might be expected for a fibroblast cell line, mRNA for the RFL9 $A_{2b}$ receptor has been found in COS M6 cells (23). LLC-$PK_1$ cells were permanently transfected with the

wild-type $A_{2a}$/pCMV4 construct (along with a neomycin resistance plasmid for selection), but none of the viable clones expressed the $A_{2a}$ adenosine receptor at a level higher than 1 pmol/mg of membrane protein, a density lower than that needed for eventual peptide mapping studies. Furthermore, even in the presence of the adenosine receptor antagonist 1,3-dipropyl-8-(4-sulfophenyl)xanthine the stable transfectants multiplied extremely slowly; prolonged stimulation of adenylyl cyclase can cause growth inhibition of LLC-$PK_1$ cells (39).

To determine whether removal of most of the carboxyl tail of the $A_{2a}$ adenosine receptor is detrimental to ligand binding, wild-type and hexahistidine $A_{2a}$ receptors were assayed for ligand binding using [$^3$H]CGS21680. Saturation isotherm analysis indicated that [$^3$H]CGS21680 bound to the wild-type receptor with a $K_d$ of 18 ± 4 nM and to the truncated receptor with a $K_d$ of 30 ± 7 nM (Fig. 2). This 2-fold difference in affinity was not disturbing; GPCRs mutated outside of the binding pocket often display slightly altered but nearly normal binding profiles (16, 17). In the set of transient transfections characterized in Fig. 2, the two $A_{2a}$ receptor types were expressed at statistically indistinguishable levels (16 ± 2 and 13 ± 6 pmol/mg of membrane protein). In other batches of COS M6 membranes, either wild-type or hexahistidine transfected, receptor densities routinely ranged from 10 to 50 pmol/mg of membrane protein. These $B_{max}$ values obscured the fact that the $A_{2a}$ adenosine receptors probably were being dramatically overexpressed in a small percentage of the COS M6 cells. Estimating that only 5–10% of cells typically express a gene introduced by transient transfection, select cells were probably producing 100–1000 pmol of receptor/mg of membrane protein. GTPγS experiments did appear to reflect the swamping of $G_s$ in this subset of cells; 100 μM GTPγS was unable to reduce specific binding of [$^3$H]CGS21680 to $A_{2a}$ receptor-transfected membranes (data not shown).

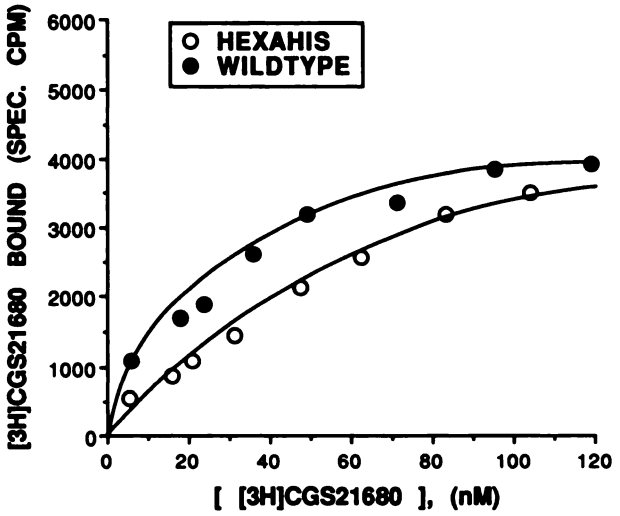


**Fig. 2.** Saturation binding of [$^3$H]CGS21680 to wild-type versus hexahistidine $A_{2a}$ receptor-transfected COS M6 membranes. [$^3$H]CGS21680 saturation binding assays were performed on 5 μg of either wild-type (●) or hexahistidine (○) $A_{2a}$ receptor-transfected COS M6 membrane protein. LIGAND analysis yielded the following one-site parameters: wild-type receptor, $K_d$ = 18 ± 4 nM and $B_{max}$ = 16 ± 2 pmol/mg; hexahistidine receptor, $K_d$ = 30 ± 7 nM and $B_{max}$ = 13 ± 6 pmol/mg. The saturation isotherms shown here were comparable to those seen for many sets of transient transfections; [$^3$H]CGS21680 always bound to the wild-type and hexahistidine $A_{2a}$ receptors with nearly equal avidity (≤2-fold difference in $K_d$ values).

$A_{2a}$ adenosine receptor binding is sensitive to sodium (9). Typically, $Na^+$ influences receptor-ligand interactions for GPCRs linked to inhibition, not stimulation, of adenylyl cyclase. This modulation is allosteric in nature and involves, at least for the $\alpha_2$-adrenergic receptor, an aspartate residue ($Asp^{79}$) in transmembrane span II (40). $Asp^{52}$ may serve the same role in the $A_{2a}$ adenosine receptor. In our hands, maximal inhibition of [$^3$H]CGS21680 binding to the $A_{2a}$ receptor occurred at a NaCl concentration of 150 mM. *N*-Methyl-D-glucamine chloride (150 mM) was included in control tubes to eliminate ionic strength as a variable. For both the wild-type and truncated $A_{2a}$ receptors, $Na^+$ approximately halved receptor affinity for the agonist [$^3$H]CGS21680, thereby reducing the apparent $B_{max}$ values by 40–60% (data not shown). The finding that wild-type and carboxyl-terminally truncated $A_{2a}$ receptors show comparable regulation by $Na^+$ indicates that residues in the carboxyl terminus of the $A_{2a}$ adenosine receptor are not required for the putative allosteric regulation by sodium.

[$^3$H]CGS21680 competition binding analysis yielded similar pharmacological profiles for the full length and truncated $A_{2a}$ adenosine receptors (Fig. 3; Table 1). Truncation of the $A_{2a}$ carboxyl tail or addition of the hexahistidine tag lowered the receptor affinities of most agonists tested [(*R*)-PIA excluded] by a factor of 1.2–2.1. However, the agonist rank order of potency remained unchanged, NECA > 2-CADO > [(*R*)-PIA/CHA] > (*S*)-PIA. Native canine $A_{2a}$ receptors have not been studied extensively, but the wild-type $K_i$ values listed in Table 1 agree with the values that are available, as well as with $A_{2a}$ receptor values obtained in other mammalian species (15, 41). CV1808, an $A_{2a}$ as opposed to an $A_{2b}$ receptor-selective agonist, displaced [$^3$H]CGS21680 specific counts with a $K_i$ of 41 ± 6 nM. 8-(4-Azidophenyl)-1,3-dipropylxanthine, the only antagonist included in the competition series, also experienced a 2-fold reduction in affinity for the $A_{2a}$ receptor upon deletion of the carboxyl terminus of the receptor. Finally, the hexahistidine $A_{2a}$ receptor demonstrated a diminished stereoselective preference for (*R*)-PIA over (*S*)-PIA. It can be inferred, therefore, that the mutated receptor existed in a subtly altered conformation that was less conducive to *R* (D)-enantiomer binding.

**Immunoblotting of $A_{2a}$ adenosine receptors.** The full length and truncated canine $A_{2a}$ adenosine receptor cDNAs encode proteins of 412 amino acids (45,031 Da) and 315 amino acids (35,427 Da), respectively. Immunoblotting first confirmed that COS M6 cells were expressing $A_{2a}$ adenosine receptors of the appropriate molecular masses. Rabbit antipeptide antibodies were directed against YSQGCGEGQVACLFEDVV, an octadecapeptide corresponding to the carboxyl-terminal side of the putative second extracellular loop of RDC8. This amino acid stretch is highly conserved among $A_{2a}$ species homologues (rat $A_{2a}$ receptor, 17 of 18 residues identical; human $A_{2a}$ receptor, 13 of 18 residues identical) but varies widely between adenosine receptor subtypes. Furthermore, it does not extend across the two RDC8 consensus sites for *N*-linked glycosylation, $Asn^{145}$ and $Asn^{154}$.

Fig. 4A shows a Western blot of solubilized membrane protein from untransfected, hexahistidine $A_{2a}$ receptor-transfected, or wild-type $A_{2a}$ receptor-transfected COS M6 cells. Diffuse bands of 29–33 kDa and 37.5–41 kDa predominated in the hexahistidine and wild-type $A_{2a}$ lanes, respectively. The unpurified rabbit antiserum also recognized a faint band at approximately 41 kDa in the untransfected lane. In several other

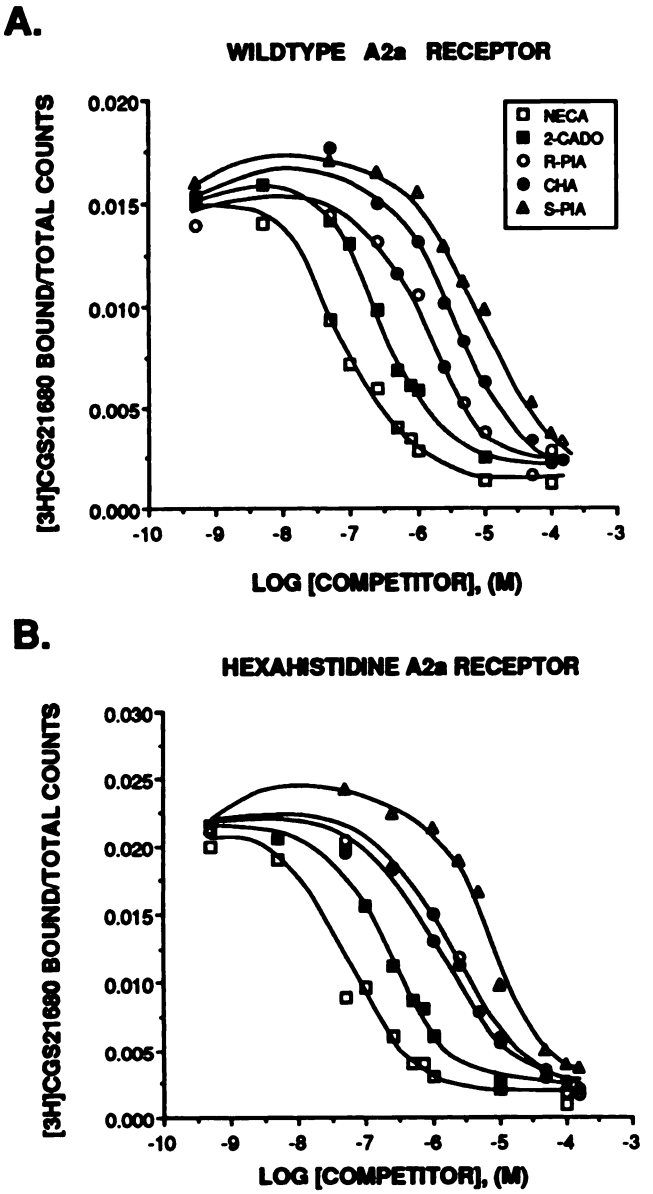


**Fig. 3.** [$^3$H]CGS21680 competition binding profiles of wild-type versus hexahistidine $A_{2a}$ adenosine receptors. Pictured are typical competition curves, showing the displacement of [$^3$H]CGS21680 binding to wild-type (A) and hexahistidine (B) $A_{2a}$ receptors by a series of agonist ligands. The same rank order of potency was observed for both full length and truncated $A_{2a}$ adenosine receptors, i.e., NECA > 2-CADO > [(*R*)-PIA/CHA] > (*S*)-PIA. Specific $K_i$ values, as the averaged means of three or more experiments, are given in Table 1.

TABLE 1

**Inhibition of [$^3$H]CGS21680 binding to wild-type $A_{2a}$ receptor-transfected versus hexahistidine $A_{2a}$ receptor-transfected COS M6 membranes**

$K_i$ values represent the mean ± standard error of at least three separate experiments.

| Inhibitor | $K_i$ | |
|---|---|---|
| | Wild-type | Hexahistidine |
| | *nM* | |
| NECA | 36 ± 6 | 66 ± 11 |
| 2-CADO | 116 ± 12 | 232 ± 63 |
| (*R*)-PIA | 474 ± 22 | 2256 ± 173 |
| CHA | 1528 ± 95 | 2071 ± 290 |
| (*S*)-PIA | 4880 ± 571 | 6071 ± 231 |
| CV1808 | 41 ± 6 | 86 ± 4 |
| $N_3$DPPX[a] | 47 ± 6 | 75 ± 13 |

[a] $N_3$DPPX, 8-(4-azidophenyl)-1,3-dipropylxanthine.

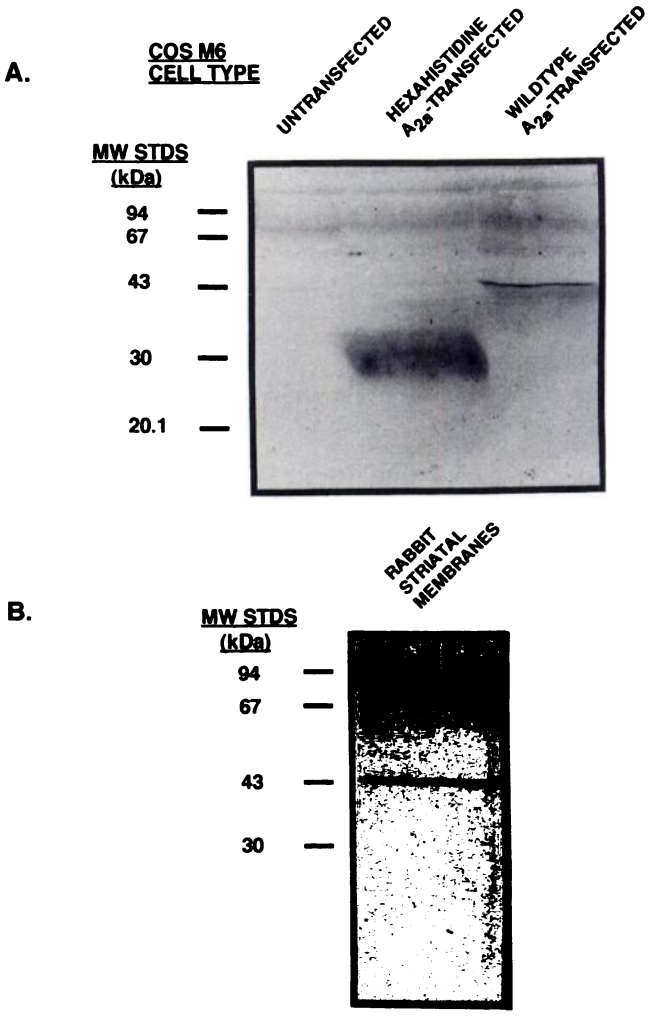


**Fig. 4.** Western blotting of $A_{2a}$ receptors expressed in transfected COS M6 cells and rabbit striatum. A, Immunoblot of solubilized membrane protein from untransfected, hexahistidine $A_{2a}$ receptor-transfected, and wild-type $A_{2a}$ receptor-transfected COS M6 cells. B, Immunoblot of solubilized membrane protein from rabbit striatum.

experiments, protein from untransfected or pCMV4-transfected COS M6 cells failed to produce strong signals anywhere in the 30–50-kDa region. A 70-kDa signal, as well as some other high molecular mass signals, consistently extended across treatment lanes. These bands probably represented nonspecific binding or multimers of endogenous $A_{2a}$ receptors, rather than novel $A_{2a}$ receptor-like proteins.

When rabbit striatal membranes were immunoblotted (Fig. 4B), antibodies recognized proteins at 44 and 90.5 kDa, sizes that may well correspond to the rabbit $A_{2a}$ receptor and its dimer. Given this strong interaction, the rabbit $A_{2a}$ receptor sequence must closely resemble the canine $A_{2a}$ receptor sequence, at least in the second extracellular loop. Although we did not study the physiology of the immunized rabbits, they would have been interesting models because they may have experienced *in vivo* effects due to the blockade of platelet, heart, or arterial smooth muscle $A_{2a}$ adenosine receptors by circulating antibodies.

**Photolabeling of glycosylated and unglycosylated wild-type and hexahistidine $A_{2a}$ adenosine receptors with $^{125}$I-azido-PAPA-APEC.** Photoaffinity labeling with the $A_{2a}$ receptor-selective probe $^{125}$I-azido-PAPA-APEC also verified that the wild-type and mutant pCMV4 constructs were being transcribed and translated correctly in COS M6 cells. Not only did Western blot receptor bands coincide with specifically labeled $^{125}$I-azido-PAPA-APEC bands, but photolabeling allowed us to explore the glycoprotein nature of the two receptor proteins. One day after they were transfected, COS M6 cells were cultured in the presence of vehicle or tunicamycin, a nucleoside antibiotic that inhibits *N*-glycosylation by preventing the synthesis of dolichol pyrophosphate *N*-acetylglucosamine (42). Vehicle-treated full length and truncated $A_{2a}$ adenosine receptors were visualized as >40 (≥42.5)- and 31.5-kDa proteins through $^{125}$I-azido-PAPA-APEC photolabeling (Fig. 5, *lanes 1*). The glycosylated form(s) of the full length receptor created a smear that extended upward from the 40-kDa position (Fig. 5A, *lane 1*). (Close inspection of the original autoradiograph revealed a doublet of bands at 40 and 42.5 kDa.) Tunicamycin treatment increased the mobility of both receptor types; unglycosylated full length receptor appeared as a distinct band at 40 kDa (Fig. 5A, *lanes 3*, *5*, and *7*) and unglycosylated truncated receptor assumed an apparent molecular mass of 28.5 kDa (Fig. 5B, *lanes 3*, *5*, and *7*). Forty-eight-hour exposure to 1 μg/ml tunicamycin (Fig. 5, *lanes 3*) was sufficient to inhibit virtually all *N*-linked glycosylation of either receptor type. In vehicle-treated cells, 10–20% of the receptors were unglycosylated, perhaps because the post-translational modification machinery of the COS M6 cells was overburdened. Minor bands were seen at 34 and 30 kDa in the full length receptor lanes, but analogous bands (≈9.6 kDa lower) were absent from the truncated receptor lanes. $^{125}$I-Azido-PAPA-APEC labeling also identified ≥67-kDa bands on both autoradiographs. The exact sizes of these bands could not be assessed using this gel system, however, because the bands were located on the spacer/resolving gel border of each Tricine-SDS-PAGE gel, an area where high molecular mass markers are poorly resolved. The hexahistidine $A_{2a}$ receptor-related high molecular mass species, but not the wild-type $A_{2a}$ receptor-related high molecular mass species, was sensitive to tunicamycin treatment, as evidenced by the radioactive band at 58 kDa in Fig. 5B, *lanes 3*, *5*, and *7*. (Similarly, tunicamycin treatment did not reproducibly reduce the appearance of high molecular mass bands on Western blots.) Photoincorporation of $^{125}$I-azido-PAPA-APEC into the various polypeptides was competed with to a large extent by both 1 mM theophylline (Fig. 5, *lanes 2*, *4*, and *6*) and 20 μM CGS21680 (Fig. 5, *lanes 8*). Untransfected or pCMV4-transfected COS M6 membranes displayed no labeling (specific or nonspecific) by $^{125}$I-azido-PAPA-APEC (blank lanes not shown in Fig. 5).

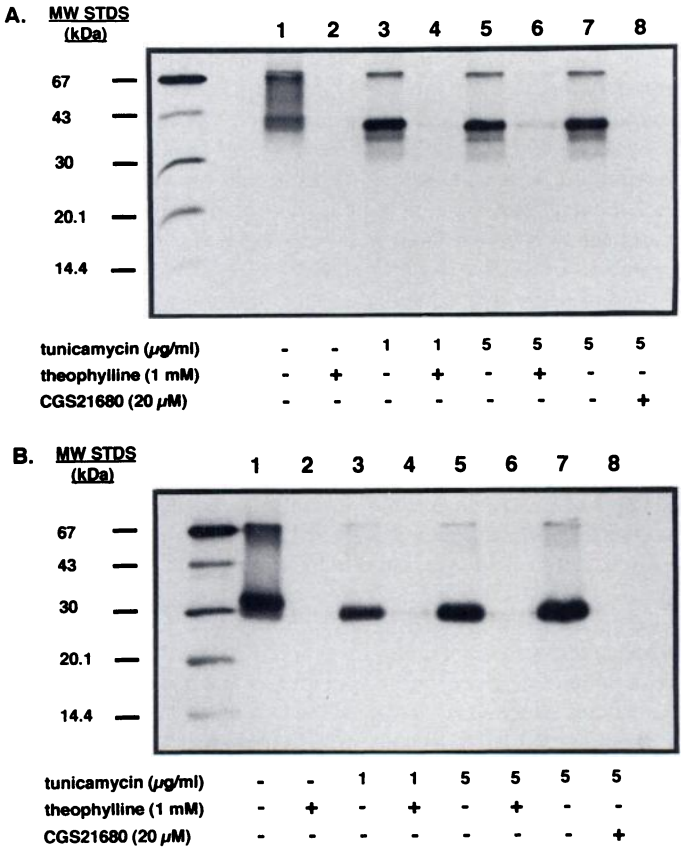


**Fig. 5.** Tunicamycin treatment of wild-type and hexahistidine $A_{2a}$ receptors. Twenty-four hours after they were transfected, wild-type $A_{2a}$ receptor-transfected COS M6 cells (A) and hexahistidine $A_{2a}$ receptor-transfected COS M6 cells (B) were exposed to vehicle (*lanes 1* and *2*) or tunicamycin at a concentration of 1 μg/ml (*lanes 3* and *4*) or 5 μg/ml (*lanes 5-8*). The cells were harvested, and the corresponding membranes were photolabeled with $^{125}$I-azido-PAPA-APEC in the absence of competitor (*lanes 1*, *3*, *5*, and *7*) or in the presence of 1 mM theophylline (*lanes 2*, *4*, and *6*) or 20 μM CGS21680 (*lanes 8*).

The tunicamycin experiment results were corroborated by the results of a second experimental strategy; photolabeled membranes were treated overnight under denaturing conditions with or without endoglycosidase F/N-glycosidase F, enzymes that remove *N*-linked carbohydrates. Glycosylated full length and truncated receptors migrated on 12% SDS-PAGE gels with apparent molecular masses of >39.5 and 30.5 kDa, respectively (autoradiograph not shown). Deglycosylated full length and truncated receptors appeared as 39.5- and 28-kDa proteins, respectively. Again, less prominent bands emerged at 33 and 30 kDa in the lanes for the wild-type receptor, but not the mutant receptor. In the tunicamycin experiments, unlike the endoglycosidase experiments, the glycoprotein properties of the $A_{2a}$ adenosine receptors were manipulated before photolabeling. This protocol reversal had no effect in the end; unglycosylated full length and truncated receptors clearly retained the ability to bind both agonist and antagonist ligands. To further test this finding, [$^3$H]CGS21680 saturation binding assays were performed on control and tunicamycin-treated full length $A_{2a}$ receptors. The agonist radioligand bound to wild-type receptors with a $K_d$ of 27 nM and to unglycosylated receptors with a $K_d$ of 33 nM (data not shown). Tunicamycin treatment did not change the $B_{max}$ range of $A_{2a}$ expression that was measured via [$^3$H]CGS21680 binding.

**Partial purification of the hexahistidine $A_{2a}$ adenosine receptor on $Ni^{2+}$·NTA resin.** One more line of biochemical evidence suggested that the COS M6 cells were expressing the genetically engineered $A_{2a}$ adenosine receptor, i.e., the hexahistidine-tagged protein could be partially purified on $Ni^{2+}$·NTA agarose resin. The high affinity interaction ($K_d \approx 10^{-13}$ M) between this resin and the hexahistidine affinity tag is unaffected by strong denaturants, so hexahistidine $A_{2a}$ receptor-transfected membranes, a fraction of which had been photolabeled with $^{125}$I-azido-PAPA-APEC, were solubilized in 6 M

guanidine hydrochloride. With several exceptions, purification followed the Qiagen protocol for *Escherichia coli*-expressed, membrane-associated proteins. To compensate for the fact that the hexahistidine $A_{2a}$ receptor was being expressed in mammalian cells (i.e., mammalian cells produce many proteins that can compete with tagged protein for resin sites), the column was loaded and washed under very stringent conditions. In the final wash, urea buffer containing 50 mM NaCl and 20 mM imidazole was run over the column until the fractions met two criteria, (a) protein could no longer be detected by silver staining and (b) $^{125}I$ counts reached a base-line level. Application of 250 mM imidazole to the resin resulted in prompt elution of all resin-associated proteins, including the desired $A_{2a}$ receptor. Electrophoresis of the concentrated fractions yielded silver-stained and autoradiographic bands that overlapped at 31 kDa, 74 kDa, and an undetermined high molecular mass. The most prominent silver-stained band, a 37-kDa protein that could easily have been mistaken for the receptor, did not pair with an autoradiographic band. Based on the tracer counts that were eluted and our knowledge that 1–2% of the $^{125}I$-azido-PAPA-APEC had been incorporated into the hexahistidine receptor during photolabeling, approximately 9% of the labeled receptor was recovered in the 250 mM imidazole fractions. This estimate was made under the assumption that the upper two radioactive bands represented aggregates of the receptor. If labeled and unlabeled receptors chromatographed identically, then only 2 $\mu$g of the 25 $\mu$g of hexahistidine protein that were loaded were recovered.

## Discussion

In conclusion, our carboxyl-terminally truncated $A_{2a}$ adenosine receptor remained capable of binding both agonist and antagonist ligands. Although the receptor affinities of most agonists tested were reduced about 2-fold [that of (*R*)-PIA by almost 5-fold], the rank order of potencies determined by inhibition of [$^3H$]CGS21680 binding was the same for both wild-type and mutant receptors, i.e., NECA > 2-CADO > [(*R*)-PIA/CHA] > (*S*)-PIA. Thus, amino acids critical for ligand binding to the $A_{2a}$ receptor cannot reside within the carboxyl terminus. Although the COS M6 cells glycosylated the mutant as well as the wild-type receptors, this *N*-linked glycosylation was not essential for ligand binding. Unglycosylated receptors could be photolabeled with $^{125}I$-azido-PAPA-APEC, and this incorporation was eliminated by the presence of either theophylline or CGS21680 in the incubation tubes. It seems unlikely, therefore, that any sort of sugar-sugar interaction that has an impact on receptor affinity occurs between $A_{2a}$ receptor *N*-glycosides and the ribose moiety of adenosine. Unfortunately, because the majority of the COS M6 recombinant receptors were uncoupled from $G_s$, the possibility cannot be eliminated that *N*-linked glycosylation and the carboxyl terminus of the $A_{2a}$ adenosine receptor play a role in high affinity agonist recognition.

For the most part, our immunoblotting, photolabeling, and purification results concurred with each other as well as with published observations. Glycosylated full length and truncated $A_{2a}$ adenosine receptors consistently migrated with apparent molecular masses of >40 (≥42.5) and about 31 kDa, respectively. Deglycosylated or unglycosylated full length and truncated receptors migrated with molecular mass values of approximately 40 and 28 kDa, respectively. Even though the $A_{2a}$ receptor proteins migrated further than was expected on SDS-PAGE gels, compared with molecular mass markers, they were separated by a distance of about 10 kDa; the theoretical molecular masses of the two receptors differ by 9.6 kDa. The hexahistidine receptor, which was tagged at its truncated carboxyl terminus, migrated at only 31 kDa but still bound to the $Ni^{2+}$·NTA resin, further suggesting that the COS M6 cells were producing full length translation products. The diffuse bands observed in the Western blotting experiments (i.e., 37.5–41 kDa for the wild-type receptor and 29–33 kDa for the mutant receptor) may have encompassed both the glycosylated and nonglycosylated forms of the $A_{2a}$ receptors.

With such a long carboxyl tail, the $A_{2a}$ adenosine receptor is particularly susceptible to proteolysis. In rabbit striatal membranes, for example, $^{125}I$-azido-PAPA-APEC labels a 38-kDa proteolytic product in addition to the 47-kDa glycosylated receptor (34). In human striatal membranes, $^{125}I$-azido-PAPA-APEC labels 43- and 37-kDa fragments in addition to the 45-kDa nonproteolyzed $A_{2a}$ receptor (9). Despite our efforts to inhibit protease activity in COS M6 membrane preparations, the minor 33–34- and 30-kDa bands accompanying the wild-type receptor bands were likely receptor fragments. Proteolysis of the "intracellular" carboxyl-terminal segment, however, cannot be the sole explanation for variations in $A_{2a}$ receptor mobility. Photolabeling of membranes from canine striatum, a tissue thought to express RDC8, reveals a single specific band at 34 kDa (37). Moreover, this protein can be detected by immunoblotting with an antibody against carboxyl-terminal amino acids $Ala^{347}$–$Tyr^{361}$ of the cloned canine $A_{2a}$ receptor.

Apparent receptor molecular masses must be interpreted with caution, but several conclusions could still be drawn from the tunicamycin and endoglycosidase F experiments. First, for either the wild-type or mutant canine $A_{2a}$ receptor, tunicamycin treatment or incubation with endoglycosidase F/N-glycosidase F produced one major band, evidence that was consistent with glycosylation at a single residue. Thus, *N*-linked glycosylation appeared to occur at one asparagine, probably $Asn^{154}$, given the proximity of $Asn^{145}$ to the membrane and to the disulfide bond candidate $Cys^{146}$ (25). Beyond the core sequence common to all *N*-linked oligosaccharides, the carbohydrate structures of the two receptor types may have differed. Whereas glycosylation of the hexahistidine receptor uniformly caused an apparent molecular mass increase of 2.5 kDa, it was difficult to discern a glycosylated wild-type band in the smear above 40 kDa. So far, no characteristic branch pattern has emerged for the $A_{2a}$ adenosine receptor *N*-glycosides. Canine liver plasma and striatal $A_{2a}$ receptors display mobility shifts of several kilodaltons (exact sizes not given) after endoglycosidase F treatment, but the bovine striatal $A_{2a}$ receptor decreases from an apparent molecular mass of 45 kDa to 38 kDa upon loss of its carbohydrate chain(s) (8, 37). Interestingly, glycosylation did not interfere with the ability of our antipeptide antibody to recognize the canine $A_{2a}$ receptor. Even more surprising, an antibody against the amino-terminal two thirds of RDC8 interhelical loop IV–V (amino acids $Trp^{143}$–$Gly^{162}$ encompass both $Asn^{145}$ and $Asn^{154}$) can immunoblot and immunoprecipitate canine liver plasma $A_{2a}$ receptors (37). Carbohydrate processing of the $A_{2a}$ adenosine receptor by COS M6 cells may or may not be physiologically relevant.

Dimerization, and sometimes higher order oligomerization, of the $A_{2a}$ adenosine receptor complicated the Western blot,

photolabeling, and purification results. On Western blots, suspected dimer bands usually, but not always, coincided with $^{125}$I-azido-PAPA-APEC-labeled bands. Furthermore, the high molecular mass bands were introduced by wild-type or hexahistidine $A_{2a}$ receptor transfection of the COS M6 cells; untransfected or pCMV4-transfected membranes acquired no labeling. Because "multimer" bands demonstrated the appropriate $A_{2a}$ receptor pharmacology, i.e., their photolabeling was attenuated by theophylline and CGS21680, they did not seem to be merely $A_{2a}$ receptor-associated proteins. Two-dimensional electrophoresis provided strong evidence for the dimerization phenomenon; excised, $^{125}$I-azido-PAPA-APEC-labeled, hexahistidine receptor bands reemerged in part as radioactive ≥67-kDa bands in the second electrophoretic dimension (43). Palmer *et al.* (37) also have noticed the tendency of $A_{2a}$ adenosine receptors to aggregate. When those authors subjected canine striatal membranes to endoglycosidase F treatment for 10 hr at 37°, nearly all of the $A_{2a}$ receptor migrated as a dimer (≈70 kDa). We found that hexahistidine $A_{2a}$ receptor oligomerization was most pronounced after partial purification, a process that took several days to complete. Receptor aggregation may not occur via intermolecular disulfide bond formation; 715 mM β-mercaptoethanol, 5 mM dithiothreitol, and successive 15 mM *N*-ethylmaleimide/5 mM dithiothreitol treatments failed to prevent the appearance of high molecular mass species. It remains unclear, then, how $A_{2a}$ receptors oligomerize or even whether aggregation is an intrinsic property of $A_{2a}$ adenosine receptors *in vivo*. $A_{2a}$ receptor multimers may form more readily in transfected COS M6 cells than in other cells types, perhaps as an artifact of overexpression.

In the future, we hope to apply the knowledge gained from these experiments to peptide mapping studies that will help to determine which amino acids comprise the ligand binding pocket of the $A_{2a}$ adenosine receptor. Because glycosylation is not necessary for ligand binding, the hexahistidine-tagged $A_{2a}$ receptor may now be mass-produced in a bacterial expression system, an advance that should facilitate affinity purification of the protein on nickel chelate resin. In addition, it may be possible to immunopurify or immunoprecipitate $A_{2a}$ adenosine receptors using our rabbit antisera raised against the second exofacial loop of the receptor.

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