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Immunological Identification of A_2 Adenosine Receptors by Two Antipeptide Antibody Preparations

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SUMMARY

Two antipeptide antibody preparations were raised against deduced amino acid sequences within the presumed second extracellular loop (antibody TP/1) and the carboxyl-terminal domain (antibody TP/2) of the canine-derived A_2 adenosine receptor (A_2AR) cDNA species termed RDC8. Immunoblotting of canine liver plasma membranes with both TP/1 and TP/2 identified a single band of 52 kDa, which co-migrated with ^{125}I-2-[4-[2-[2-[(4-azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine-labeled receptor. However, in membranes prepared from canine striatum, photoaffinity labeling and immunoblotting with TP/2, but not TP/1, revealed a single band of 34 kDa; the identity of the band observed on the immunoblot as an A_2AR was confirmed by the ability of TP/2 to specifically immunoprecipitate photoaffinity-labeled receptor from crude canine striatal membranes. The size difference between liver and striatal A_2ARs was not due to tissue-specific proteolysis, because membranes from striatum were prepared with a protease inhibitor cocktail previously shown to be effective in inhibiting endogenous A_2AR proteolysis during membrane preparation. Also, the protease-sensitive carboxyl-terminal region of the receptor had remained intact, because the peptide used to raise TP/2 antibodies resides in this domain of the molecule. The difference in size was also not due to a greater carbohydrate content of the liver receptor, because treatment of liver and striatal membranes with endoglycosidase F produced small mobility shifts for both receptors. Removal of *N*-linked carbohydrate chains also did not alter the inability of TP/1 to recognize the striatal A_2AR. Hence, we conclude that the A_2AR present in liver, which displays the predicted immunoreactivity of RDC8, is immunologically distinct from the A_2AR expressed in striatum and that the latter may represent an additional A_2AR subtype.

Adenosine is a ubiquitously distributed nucleoside that mediates several important physiological processes, including platelet aggregation, smooth muscle vasodilation, and neurotransmission (1). These effects are mediated by the binding of adenosine to either of two receptor subtypes, which have been classified pharmacologically as A_1 and A_2 (2). Whereas A_1ARs are linked to the inhibition of adenylyl cyclase activity via coupling to one, or more, inhibitory G_i proteins (3, 4), A_2ARs stimulate adenylyl cyclase activity in membranes from several sources, including human platelets (5), turkey erythrocytes (6), and rat liver (7), by coupling to G_s. Until recently, our understanding of the molecular structure of the A_2AR had been hampered by the lack of any selective ligand for this receptor. However, use of the high affinity A_2AR-selective agonist ^{125}I-PAPA-APEC, and its azide derivative, in photoaffinity cross-linking experiments has allowed us to determine the molecular sizes of A_2ARs from several sources (8, 9) and to define the glycoprotein nature of this receptor (10).

Recent advances in the molecular cloning of G protein-linked receptors have resulted in the isolation of canine, rat, and bovine A_1AR cDNAs (11–13), as well as the isolation from thyroid tissue of a canine cDNA species, termed RDC8, that exhibits significant overall homology with the A_1AR species (11). Secondary structural analyses of primary sequences obtained from both A_1AR and RDC8 cDNA species predict that these proteins are members of the superfamily of G protein-linked seven-transmembrane domain receptors, which includes rhodopsin and the adrenergic receptors, among many others (14). The predicted amino acid sequence of RDC8 also demonstrates that the cDNA codes for a protein of a predicted

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ABBREVIATIONS: A_1AR, A_1 adenosine receptor; A_2AR, A_2 adenosine receptor; G protein, guanine nucleotide-binding regulatory protein; G_s, stimulatory guanine nucleotide-binding protein of adenylyl cyclase; G_i, family of three guanine nucleotide-binding proteins originally ascribed the function of inhibition of adenylyl cyclase activity; PAPA-APEC, (−)-N^6-[(*R*)-1-methyl-2-phenylethyl]adenosine; azido-PAPA-APEC, 2-[4-[2-[2-[(4-azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine; SANPAH, *N*-succinimidyl 6-(4′-azido-2′-nitrophenylamine)hexanoate; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

molecular size of 45 kDa, which, when expressed transiently in COS cells, exhibits the appropriate A_2AR pharmacology (11, 15). The identification of this G protein-linked receptor as an A_2AR potentially allows a much greater understanding of this physiologically important receptor, because knowledge of the primary amino acid sequence facilitates the synthesis of specific probes to study aspects of receptor function and regulation. Here we describe the generation and characterization of two polyclonal antisera raised against different regions of RDC8 and demonstrate their potential usefulness in the immunoblotting and immunoprecipitation of A_2ARs.

Experimental Procedures

Materials

Soybean trypsin inhibitor, leupeptin, PMSF, benzamidine, chloramine T, CHAPS, cholic acid, Triton X-100, Protein A-agarose, rabbit IgG, and HEPES (sodium salt) were from Sigma. SANPAH was from Pierce Chemical Co. Cyanogen bromide-activated Sepharose 4B was from Pharmacia. All electrophoresis reagents were from Bio-Rad Laboratories. Endoglycosidase F was from Boehringer-Mannheim. ^{125}I-Protein A (120 μCi/ml) was from NEN, and [^{125}I]NaI (carrier-free; 100 mCi/ml) was from Amersham. All other chemicals were of the highest grade commercially obtainable.

Methods

Antibody generation and affinity purification. Antipeptide antisera were generated in New Zealand White rabbits by Immunodynamics Inc. (La Jolla, CA). The peptides used for immunization (WNNCSQPKEGRNYSQGCGEG and ANGSAPHPERRPNGY) were synthesized with an extra amino-terminal cysteine residue, to facilitate conjugation with keyhole limpet hemocyanin via *m*-maleimidobenzoyl-*N*-hydroxysuccinimide; the composition, but not the sequence, of each of the peptides was verified by high performance liquid chromatographic analysis. Keyhole limpet hemocyanin-coupled peptide was injected into two rabbits, and reactivities of the resulting antisera with the appropriate peptides were tested by enzyme-linked immunosorbent assay.

Purification of antibodies from whole rabbit serum was achieved by affinity chromatography, using the immunizing peptide coupled to Sepharose 4B, basically as described by Mumby and Gilman (16). Columns were made by using 3 mg of peptide/ml of swollen CNBr-activated Sepharose, in accordance with the manufacturer's instructions. For antibody purification, between 2 and 5 ml of antiserum were diluted 3-fold with 20 mM Tris·HCl, pH 7.5, 100 mM NaCl, and this material was passed twice through 1 ml of peptide-Sepharose (column dimensions, 0.7 cm × 4 cm; Bio-Rad Econo-column). The column was then washed with the Tris/NaCl buffer until the absorbance at 280 nm of the flow-through fractions reached zero. Antibodies were eluted by the addition of 5 ml of 0.2 M glycine, pH 2.2; 0.5-ml fractions were collected and immediately neutralized by the addition of 50 μl of 1 M Tris base. Fractions containing protein, as determined by monitoring of absorbance at 280 nm, were pooled and dialyzed against 1500 volumes of a 10 mM Tris·HCl, pH 7.5, 0.1 mM EDTA buffer. Dialyzed fractions were aliquoted for storage at −80°.

Preparation of dog liver plasma membranes. Fresh dog liver was obtained from the Department of Medicine (Cardiology Division), Duke University Medical Center; ~15 g wet weight of tissue was used for each membrane preparation. After fine chopping, the material was washed extensively with ice-cold 5 mM Tris·HCl, pH 7.5, 1 mM EDTA, in order to remove as much blood as possible. The pieces were then frozen and powdered in liquid nitrogen for storage at −80°. After rapid thawing, the wet tissue was homogenized with a Polytron by two 20-sec bursts at setting 7, before filtering through two layers of cheesecloth. The filtered homogenate was diluted to 300 ml with 5 mM Tris·HCl, pH 7.5, 1 mM EDTA, and centrifuged at 2000 × *g* for 10 min at 4°. The resulting loose pellets were mixed with sucrose in 5 mM Tris·HCl, pH 7.5, 1 mM EDTA, to form a 60% (w/v) solution. Portions (18 ml) of this mixture were applied to discontinuous sucrose gradients (11 ml of 48% sucrose and 5.9 ml of 42.5% sucrose) and centrifuged for 3 hr at 4°, in the AH629 swing-out rotor of a Sorvall Ultra 80 ultracentrifuge. Membranes were collected at the 48/42.5% sucrose interface and were diluted in an equal volume of 5 mM Tris·HCl, pH 7.5, 1 mM EDTA, before centrifugation at 43,000 × *g*, to sediment a membrane pellet. The pellet was washed several times until pale (to remove residual erythrocyte contamination) and was resuspended in 5 mM Tris·HCl, pH 7.5, 1 mM EDTA, to a final concentration of 3–6 mg/ml, for storage at −80°.

Preparation of striatal synaptosomal membranes. These membranes were prepared by the method of Booth and Clark (17). Briefly, fresh canine striatum was finely chopped and washed in 10 mM Tris·HCl, pH 7.4, 1 mM EDTA, 0.32 M sucrose (isolation medium), before Dounce homogenization (12 up and down strokes) and centrifugation at 1300 × *g* at 4°. The supernatants were recentrifuged at 17,000 × *g*, and the resulting crude synaptosomal pellets were mixed with Ficoll and isolation medium, to give a final Ficoll concentration of 12% (w/v). Portions (17.5 ml) of this mixture were applied to discontinuous gradients, each containing 8.8 ml of 7% Ficoll and 7.8 ml of isolation medium. After centrifugation at 100,000 × *g* for 1 hr at 4° in the AH629 swing-out rotor of a Sorvall Ultra 80 ultracentrifuge, membranes were removed from the 7%/12% interface, diluted 2-fold in 5 mM Tris·HCl, pH 8.0, 1 mM EDTA, sonicated for 30 sec, and left on ice for 45 min. Synaptosomal membranes were pelleted by centrifugation at 100,000 × *g* for 45 min at 4° and were resuspended in 5 mM Tris·HCl, pH 7.5, 1 mM EDTA, for storage at −80°. A protease inhibitor cocktail described by Nanoff *et al.* (9) was included in all buffers, to minimize endogenous proteolysis of the A_2AR; this cocktail consisted of 100 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 1 μg/ml pepstatin A, and 0.1 mM PMSF (final concentrations).

Other membrane preparations. Crude canine striatal membranes were prepared as previously described for rabbit striatum, with the inclusion of the protease inhibitor cocktail described above (9).

Radioiodination of PAPA-APEC and azido-PAPA-APEC. The synthesis and purification of ^{125}I-PAPA-APEC and ^{125}I-azido-PAPA-APEC have been previously described in several publications (8–10).

Photoaffinity labeling of A_2ARs. Photoaffinity labeling using ^{125}I-PAPA-APEC and the bifunctional cross-linking agent SANPAH, as well as ^{125}I-azido-PAPA-APEC, was carried out as previously described (8–10).

SDS-PAGE. Discontinuous electrophoresis was performed as described by Laemmli (19), using homogeneous 10% (w/v) or 12% (w/v) polyacrylamide resolving gels and 3% (w/v) polyacrylamide stacking gels. Samples were solubilized by the addition of electrophoresis sample buffer, consisting of 25 mM Tris base, 10% (w/v) SDS, 4.5 M urea, and 5% (v/v) β-mercaptoethanol, and incubation at room temperature for 1 hr, except for samples from dog synaptosomal membranes, which were incubated for 15 min. Gels were run overnight at 6 mA/gel or for 5 hr at 25 mA.

Immunoblotting of A_2ARs. Initial experiments involved solubilization of plasma membranes in CHAPS/cholate, as follows. Membrane protein (200–500 μg) was sedimented, and the pellet was solubilized in 500 μl of solubilization buffer [20 mM HEPES, pH 6.8, 10 mM $MgCl_2$, 5 mM EDTA, 100 mM NaCl, 1% (w/v) CHAPS, 0.1% (w/v) cholic acid, 5 μg/ml soybean trypsin inhibitor, 5 μg/ml leupeptin, 100 μM benzamidine, 0.1 mM PMSF] for 1 hr at 4°, with continuous rotation at 60 rpm. These samples were then centrifuged at 100,000 × *g* at 4°, to obtain a soluble fraction. After desalting of the supernatant on G-50 columns, to exchange the buffer to one consisting of 10 mM Tris·HCl, pH 7.4, and 0.2% (w/v) SDS, the samples were frozen rapidly in liquid nitrogen and lyophilized overnight. The resulting sample was solubilized in electrophoresis sample buffer before loading onto polyacrylamide gels.

Later studies used a membrane solubilization buffer containing 0.8% (v/v) Triton X-100 in 100 mM Na_2HPO_4, pH 6.5, 50 mM EDTA, with the protease inhibitor cocktail previously described. Sedimented membrane protein (1 mg) was resuspended in 100 μl of solubilization buffer, and the mixture was left on ice for 1 hr. After centrifugation at 14,000 × *g* for 30 min at 4°, an appropriate amount of supernatant was taken and added to an equal volume of SDS-PAGE sample buffer, containing 16% (w/v) SDS, before electrophoresis.

Proteins on the gel were transferred to nitrocellulose (0.2 μm, BA83; Schleicher & Schuell) over 3 hr at a current of 100 mA, using a Bio-Rad Transphor dry blotter. Nonspecific protein binding sites on the nitrocellulose were blocked by incubation for 2 hr at room temperature in 5% (w/v) skimmed milk powder, 1 mM EDTA, PBS (PBS contained 136 mM NaCl, 2.7 mM KCl, 8.05 mM Na_2HPO_4, and 14.7 mM KH_2PO_4, pH 7.5), containing 0.2% (v/v) Triton X-100. Affinity-purified A_2AR antibody was incubated with the nitrocellulose overnight at 4°, at a final concentration of either 4 or 8 μg of IgG/ml, in fresh blocking solution. The filter was then washed five times with blocking solution (10 min/wash) before incubation for 2 hr with 5×10^5 cpm/ml ^{125}I-labeled recombinant Protein A, in 0.5% (w/v) skimmed milk, 1 mM EDTA, PBS, 1% (v/v) Triton X-100, 0.1% (w/v) SDS. The filter was washed with blocking solution, as described above, followed by two 10-min washes with PBS alone. The nitrocellulose paper was then air-dried and exposed to Kodak XAR film, with dual intensifying screens, for 1–2 days.

Immunoprecipitation of A_2ARs. Immunoprecipitation of A_2ARs was assessed either by using photoaffinity-labeled receptors or by immunoblotting.

After photoaffinity labeling of A_2ARs, as described above, one tube of labeled dog striatal membranes (~700 μg of protein) was resuspended in 450 μl of HPEN (50 mM HEPES, pH 7.2, 10 mM $Na_4P_2O_7$, 100 mM NaCl, 4 mM EDTA, 5 mM *N*-ethylmaleimide, 0.1 mM PMSF, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 5 μg/ml soybean trypsin inhibitor). After resuspension by pipette trituration and passage through a 27-gauge syringe needle, 50 μl of 20% (v/v) Triton X-100 were added and the mixture was vortexed before being allowed to solubilize on ice for 1 hr. Lysates were then centrifuged in a Beckman benchtop ultracentrifuge for 60 min at 180,000 × *g*, to sediment insoluble material. Four hundred microliters of the supernatant were subsequently used for immunoprecipitation. Solubilized material was incubated with affinity-purified A_2AR antibodies (between 5 and 20 μg of IgG/ml) for at least 3 hr on ice, with nonspecific immunoprecipitation being assessed either by using nonimmune rabbit IgG at identical concentrations or by using A_2AR antibodies plus excess antigenic peptide. Protein A-agarose (20 μl; 10 μl of packed gel) was then added, and immune complexes were precipitated after a 2-hr incubation at 4°, with constant rotation. Immunoprecipitates were washed three times with 0.5 ml of HPEN, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, and once with 0.5 ml of HPEN. A_2ARs were eluted from the Protein A-agarose by the addition of 60 μl of 10% (w/v) SDS-PAGE sample buffer and incubation on ice for 1 hr. Samples were analyzed by SDS-PAGE and autoradiography.

Exactly the same protocol was followed for detection of immunoprecipitated A_2AR from canine liver membrane preparations by immunoblotting, except that, after electrophoresis, samples were transferred to nitrocellulose for immunoblotting with 4 μg/ml TP/2.

Endoglycosidase F treatment. Plasma membranes (500 μg) were resuspended in 40 μl of 0.8% (v/v) Triton X-100, 100 mM Na_2HPO_4, pH 6.5, 50 mM EDTA, with the protease inhibitor cocktail previously described for membrane solubilization. Ten microliters of stock endoglycosidase F (6 units/120 μl) were added to the lysate, and the mixture was incubated at 37° for the times indicated in the figure legends. Soluble fractions were collected by centrifugation for 30 min at 15,000 × *g* and were added to an equal volume of 16% (w/v) SDS-PAGE sample buffer before analysis by SDS-PAGE and immunoblotting. Preliminary experiments determined that the presence of such a high concentration of EDTA in the digestion buffer was essential to prevent proteolytic degradation of deglycosylated receptors (data not shown).

Results

Choice of immunizing peptides. Two peptides were chosen to raise polyclonal antisera against RDC8 (Fig. 1). Initially, an antiserum (TP/1) was raised against a 20-amino acid peptide that corresponded to a region of the presumed second extracellular loop of the receptor and encompassed the two consensus sites for *N*-linked glycosylation. A second antiserum (TP/2) was raised against a 15-amino acid peptide that corresponded to a region of the carboxyl-terminal tail of the receptor (Fig. 1). These sequences were chosen on the basis of their hydrophilicity, their predicted antigenicity, and their differences from any sequences present in the A_1AR proteins cloned to date (Fig. 1).

Immunoblotting of A_2ARs. Initially, optimal conditions were determined for immunoblotting experiments using the raised antisera as primary antibodies. To optimize the signal obtained, relative to background, it was found to be necessary to solubilize purified plasma membranes in either a 1% (w/v) CHAPS/0.1% (w/v) cholate detergent mix or a 0.8% (v/v) Triton X-100 detergent buffer before SDS samples of the supernatant were made. It was also necessary to use affinity-purified anti-A_2AR antibodies, to minimize the presence of nonspecific bands on immunoblots. Initially, we studied a canine liver preparation, because liver is known to possess adenosine receptors that stimulate adenylyl cyclase activity with the appropriate A_2AR pharmacology (7) and also because we wanted to avoid potential problems that could arise due to interspecies variation in the peptide sequences we chose as immunogens; this was a particularly important consideration because the only published A_2AR sequence available was that of the canine-derived RDC8 protein. When sucrose-purified dog liver plasma membranes were immunoblotted under these conditions with either TP/1 or TP/2, a major band of 52 kDa was recognized by both antibodies, and this band co-migrated with photoaffinity-labeled A_2AR from the same membrane preparation (Fig. 2A). The identification of the photoaffinity-labeled species as an A_2AR was confirmed by the ability of theophylline to specifically block the labeling of this band (Fig. 2A). Interestingly, after prolonged storage of some liver membrane preparations, two bands, of 52 kDa and 40 kDa, were observed on immunoblotting with TP/1, whereas only one band (52 kDa) was observed in parallel experiments using TP/2 (data not shown). Considering the fact that the sequence to which TP/2 was raised represents a region within the carboxyl-terminal domain of the receptor, whereas the sequence used for TP/1 is in the middle of the primary sequence (Fig. 1), it is possible that the 40-kDa species observed with TP/1 represents a population of A_2ARs in which the carboxyl-terminal region containing the TP/2 sequence has been cleaved during prolonged storage. A similar event may account for the two A_2AR species observed by photoaffinity labeling in rabbit striatal membranes prepared in the absence of protease inhibitors (9). Indeed, the carboxyl-terminal location of such a proteolytic event would account for the altered coupling between the A_2AR and G_s observed in rabbit striatal membranes prepared in the absence of protease inhibitors (9). In membranes prepared from dog striatum, photoaffinity labeling using the agonist photoaffinity probe ^{125}I-azido-PAPA-APEC identified a broad band of 34–36 kDa, whose labeling was completely blocked by the inclusion of theophylline in the reaction mixture (Fig. 2B). A single band of identical molecular mass was observed when

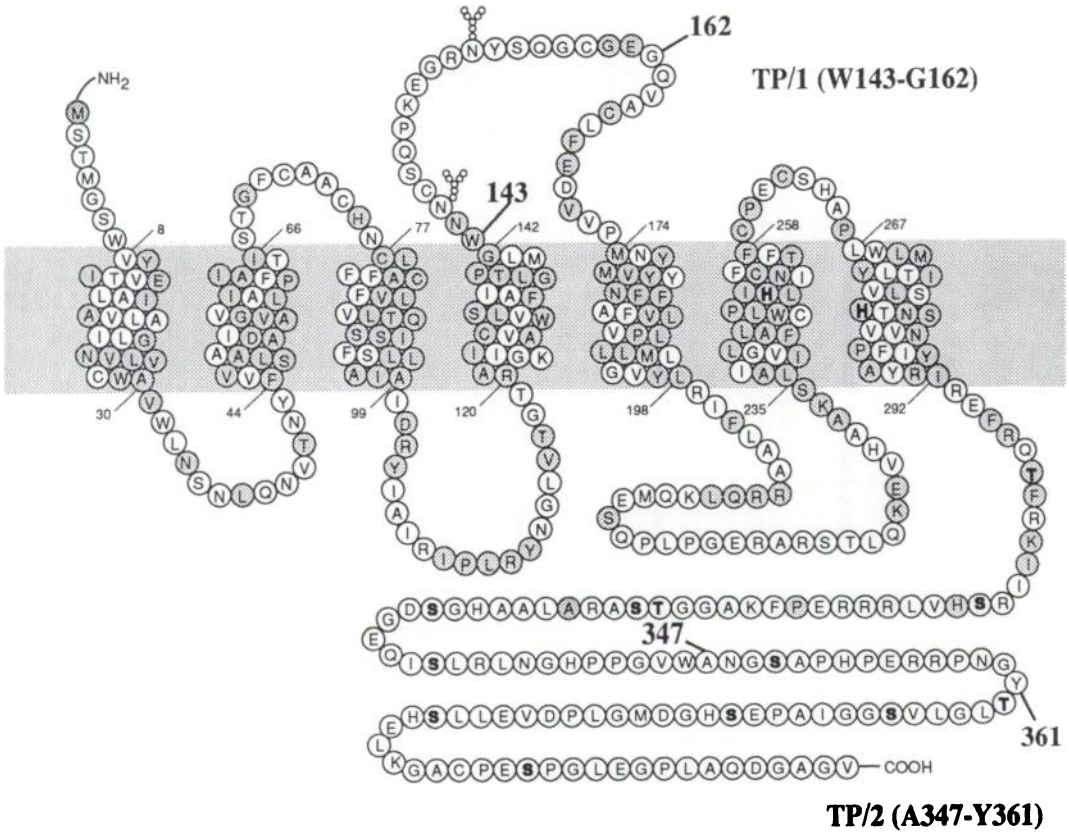

Fig. 1. Presumed membrane topography of the canine A_2AR. The predicted membrane-spanning topography of the canine A_2AR is shown, with the amino acid sequences used for antibody generation indicated. Branched amino acids indicate predicted sites for *N*-linked glycosylation. *Shaded amino acids* represent residues that are conserved between the canine A_1AR and A_2AR; serine and threonine residues in *bold type* represent potential phosphorylation sites. The figure is adapted from ref. 24.

purified synaptosomal membranes from dog striatum were immunoblotted with TP/2. However, in parallel experiments using the same membrane preparations, TP/1 was unable to identify this protein under conditions where it recognized the liver receptor (Fig. 2B).

Immunoprecipitation of A_2ARs. Unequivocal identification, as *bona fide* A_2ARs, of the species observed in immunoblots was achieved by immunoprecipitation of photoaffinity-labeled receptors. TP/1 was unable to immunoprecipitate A_2ARs from striatal membranes, using either nondenaturing or denaturing solubilization protocols (data not shown). As stated above, photoaffinity labeling of dog striatal membranes with ^{125}I-azido-PAPA-APEC identified a protein of 34–36 kDa, whose labeling was completely blocked by theophylline (Fig. 2B). The labeling of the lower prominent band, of 25 kDa, was not blocked by theophylline, and it cannot, therefore, be considered an A_2AR. Indeed, affinity-purified TP/2, but not an equivalent concentration of nonimmune IgG, was capable of specifically immunoprecipitating the 34–36-kDa species, confirming its identity as an A_2AR (Fig. 3A). Furthermore, immunoprecipitation of this protein was completely blocked when TP/2 antibodies to which immunizing peptide had been added were used, further confirming the specificity of the antibody-antigen interaction (Fig. 3B). The lower, nonspecifically labeled, band observed in Fig. 3 is present to a similar extent in comparable lanes, showing that equal amounts of solubilized labeled membranes were subjected to immunoprecipitation within each experiment.

Endoglycosidase F sensitivity of different A_2ARs. Previous photoaffinity labeling studies have shown that the A_2AR from bovine striatum is a glycoprotein consisting of a 38-kDa protein and a single *N*-linked carbohydrate chain (10). Photoaffinity labeling and immunoblotting experiments suggest that the molecular size of the A_2AR from canine liver is 52 kDa, whereas the canine striatal A_2AR migrates at 34 kDa. Considering the fact that the predicted molecular size of RDC8 is 45 kDa, it was important to assess how this figure relates to both of these endogenous canine A_2ARs, because the size of the receptor differs between liver and striatum.

Because the photoaffinity labeling signal was poor in dog liver, A_2ARs were subjected to immunoblotting with TP/2 after exhaustive treatment with 10 units/ml endoglycosidase F, to remove *N*-linked carbohydrate chains. After a 4-hr incubation, a band of 49 kDa could be recognized, along with the intact 52-kDa species. By 8 hr of treatment, only the lower band was present and no other smaller bands had been generated, suggesting the existence of a single *N*-linked carbohydrate chain attached to a 49-kDa receptor protein (Fig. 4A). Digestion of the canine striatal receptor under conditions that produced a shift in mobility of the liver receptor produced a small but detectable increase in mobility of this receptor, with a single band again being generated, as detected by immunoblotting with TP/2 (Fig. 4B). Under these conditions, TP/1 was still incapable of recognizing the canine striatal A_2AR, even though the carbohydrate that may have been masking the antigenic peptide sequence had been removed (Fig. 4B). However, during

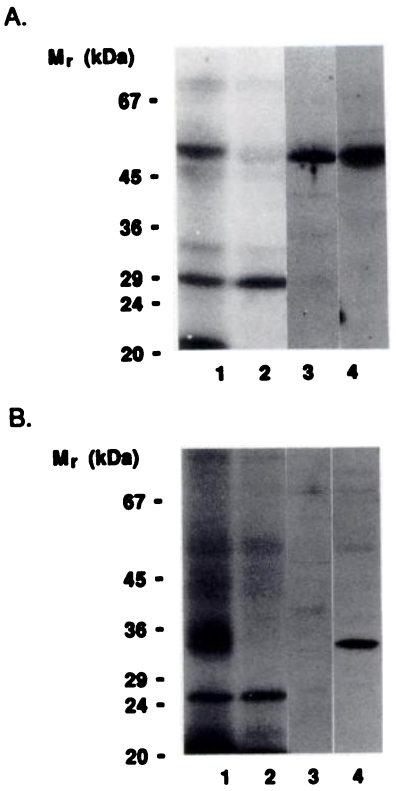

Fig. 2. Co-migration of A_2ARs identified by photoaffinity labeling and immunoblotting. Membranes from canine liver (A) or canine striatum (B) either were photoaffinity-labeled using ^{125}I-azido-PAPA-APEC, in the absence (*lane 1*) or in the presence (*lane 2*) of 5 mM theophylline, or were immunoblotted with 8 μg/ml affinity-purified TP/1 (*lane 3*) or 4 μg/ml affinity-purified TP/2 (*lane 4*). B, Crude membranes were used for the photoaffinity-labeling experiments and Ficoll gradient-purified synaptosomal membranes were used for the immunoblotting experiments. Each panel is representative of at least three experiments performed on different membrane preparations, using at least three preparations of affinity-purified antibodies.

Fig. 3. Immunoprecipitation of canine striatal A_2ARs with TP/2. Crude membranes prepared from canine striatum were subjected to photoaffinity labeling using the A_2AR-selective agonist ^{125}I-azido-PAPA-APEC. A, Each 700-μg sample of labeled membranes was solubilized in 500 μl of HPEN containing 2% Triton X-100 and, after centrifugation to sediment insoluble material, 400 μl of the supernatant were subjected to immunoprecipitation with either 20 μg/ml TP/2 (*lane 1*) or 20 μg/ml nonimmune IgG (*lane 2*), as described in Experimental Procedures. B, Samples were immunoprecipitated with 20 μg/ml TP/2 in the absence of added peptide (*lane 1*) or in the presence of carboxyl-terminal domain peptide at 20 μg/ml (*lane 2*). Samples were also immunoprecipitated with 20 μg/ml TP/2 in the presence of second extracellular loop peptide at 20 μg/ml (*lane 3*). The sequences of the peptides are given in Experimental Procedures and Fig. 1.

the prolonged incubation time necessary for removal of carbohydrate, virtually all of the glycosylated and deglycosylated immunoreactive striatal A_2AR aggregated to form a dimer. Such aggregation of transmembrane proteins after prolonged incubation above 4° has been previously noted for human erythrocyte glucose transporters, which are also multiple-transmembrane domain proteins (20, 21), and was also observed to a small but variable degree with other A_2ARs we have studied (data not shown). In the case of canine striatal membranes, the phenomenon is not a reflection of sample preparation for electrophoresis, because Triton-soluble canine striatal A_2ARs to which sample buffer has been added migrate at 34 kDa as long as the time between sample buffer addition and running of the gel is <30 min. Also, the aggregation does not reflect the presence of nonionic detergent, because incubation of native membranes for 10 hr at 37° in the buffer used for the deglycosylation reaction minus detergent also results in aggregation, as determined by immunoblotting with TP/2 (data not shown). Indeed, previous studies on the glucose transporter showed that aggregation was prevalent in a variety of nonionic detergents and occurred in the presence of 100 mM dithiothreitol, which would rule out aggregation via intermolecular disulfide bond formation (20); the sample buffer used in our studies contains 5% (v/v) β-mercaptoethanol (equivalent to ~700 mM). Therefore, a tendency to form aggregates may also be an intrinsic property of A_2AR proteins, in particular the A_2AR from canine striatum.

Discussion

In this study, we have demonstrated the ability of two antipeptide antibody preparations, raised against sequences encoded by the canine A_2AR protein cDNA RDC8, to recognize A_2ARs from two different tissues. Use of the agonist photoaffinity probes ^{125}I-PAPA-APEC and ^{125}I-azido-PAPA-APEC, as well as immunoblotting and immunoprecipitation of A_2ARs with TP/1 and TP/2, has demonstrated that the molecular size of A_2ARs is variable between different species and also between different tissues from the same species. In bovine striatum, the A_2AR migrates as a 44–45 kDa protein on SDS-PAGE; the intact A_2AR has been identified as a 45-kDa protein in several sources, including rabbit striatum, frog erythrocytes, and PC12 cells (9). However, the A_2AR from DDT$_1$ MF-2 cells migrates as a smaller protein on SDS-PAGE analysis (36–38 kDa),

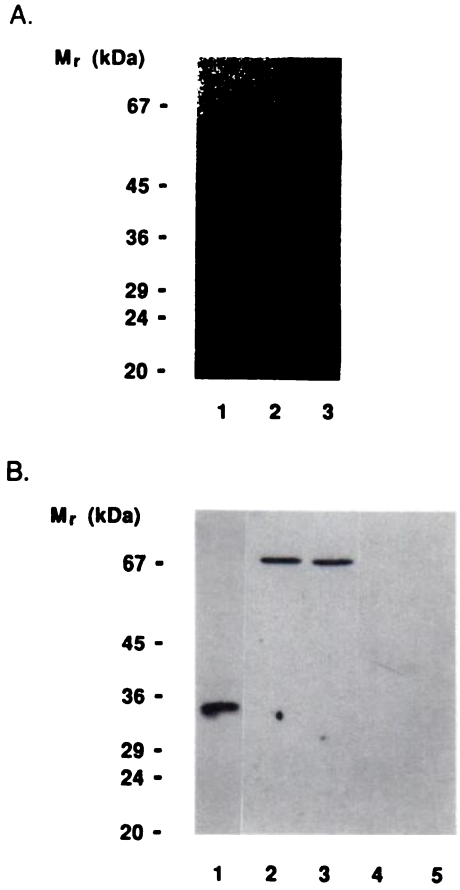

Fig. 4. Sensitivity of endogenous canine A_2ARs to endoglycosidase F. A, Purified plasma membranes (500 μg) from canine liver were treated in the absence (*lane 1*) or presence (*lanes 2* and *3*) of 10 units/ml endoglycosidase F, at 37°, for 4 hr (*lane 2*) or 8 hr (*lane 3*) before samples were prepared with SDS for electrophoresis and immunoblotting with 4 μg/ml TP/2. B, Purified synaptosomal membranes (500 μg) from canine striatum were treated in the absence (*lanes 2* and *4*) or presence (*lanes 3* and *5*) of 10 units/ml endoglycosidase F for 10 hr at 37° before analysis by SDS-PAGE and immunoblotting with either 4 μg/ml TP/2 (*lanes 1, 2,* and *3*) or 8 μg/ml TP/1 (*lanes 4* and *5*). B, *lane 1*, result of solubilizing 500 μg of canine striatal synaptosomal membranes in Triton X-100 on ice for 1 hr, followed by the addition of sample buffer to the supernatant and incubation at room temperature for 15 min before loading for electrophoresis.

whereas the A_2AR studied from canine sources can exist in one of two distinct sizes. The A_2AR from canine liver migrates as a 52-kDa polypeptide, as judged by both photoaffinity labeling and immunoblotting techniques. In contrast, the A_2AR from canine striatum migrates as a 34-kDa polypeptide, as judged by immunoblotting and photoaffinity labeling experiments. We do not believe that the 34-kDa band observed in striatum is a proteolytic product of the 52-kDa species, for two reasons. First, both crude and highly purified plasma membranes were prepared from canine striatum using a protease inhibitor cocktail that was previously shown to prevent the degradation of A_2ARs (9), as well as β- and α_1-adrenergic receptors (22, 23), during membrane preparation. Second, the finding that TP/2 recognizes the 34-kDa species may be a particularly important observation, because it has been shown that the A_2AR may be particularly sensitive to proteolysis in the domain responsible for coupling to G_s, which presumably is intracellular and which may involve some region of the carboxyl-terminal domain. Indeed, secondary structural analyses predict that RDC8 has a relatively long carboxyl-terminal domain (24), a feature it shares with other G_s-linked receptors (14, 25). Also, we have noted that the carboxyl-terminal domain of the canine liver A_2AR is prone to cleavage upon prolonged storage in the absence of protease inhibitors. Because the carboxyl terminus of the striatal A_2AR has remained intact, we consider it unlikely that proteolysis can account for the size difference observed. Therefore, if such size differences do not reflect proteolysis-derived artifacts, they may reflect tissue-specific post-translational processing of the same A_2AR protein or, alternatively, it is possible that liver and striatum express distinct A_2AR proteins; the latter possibility is suggested by the recognition of the liver receptor, but not the striatal receptor, by TP/1. Secondary structural analysis predicts the existence of two sites for *N*-linked glycosylation on the RDC8 protein (Fig. 1) (24), although the endoglycosidase F digestions performed in these studies suggest that only one of these is utilized, because both liver and striatal A_2ARs produce only one deglycosylated product (Fig. 4). However, the presence of carbohydrate on the second extracellular loop does not affect the ability of TP/1 to recognize the liver A_2AR, and the striatal A_2AR remains incapable of interacting with TP/1, even after digestion with sufficient endoglycosidase F to alter the mobility of the receptor, compared with untreated membranes, as assessed by immunoblotting with TP/2. It is theoretically possible that a form of extracellular post-translational modification other than glycosylation is masking the TP/1 sequence in the striatal A_2AR, but the nature of any such modification(s) is unknown. It is also possible that the observed aggregation of the canine striatal A_2AR is preferentially masking the epitope on the second extracellular loop once carbohydrate has been removed. However, we believe that the most likely explanation for the tissue-specific immunoreactivity observed is that the canine striatal A_2AR identified by photoaffinity labeling, immunoblotting, and immunoprecipitation with TP/2 is distinct from the A_2AR protein expressed in liver, which displays the predicted immunoreactivity of RDC8. Indeed, it was noted by Libert *et al.* (11) that the agonist-binding characteristics of the transiently expressed RDC8 protein were significantly different from those of the A_2AR in native dog striatal membranes; our experiments may provide one potential explanation for these differences. Also, further cloning of A_2AR subtypes may resolve this issue. Of course, subtype-specific tissue expression has been noted for several receptors, most notably the β_1-, β_2-, and β_3-adrenergic receptors and the α_2-adrenergic receptors (26, 27).

As discussed above, it seems unlikely that a post-translational modification can account for this lack of reactivity of the canine striatal A_2AR with TP/1. Nevertheless, it is possible that a post-transcriptional modification specific to the canine striatal A_2AR modifies the sequence at the second extracellular loop, either inserting or deleting certain amino acids by splicing of a single primary RNA transcript common to both liver and striatum. However, in the absence of any detail at present, regarding the regulation of A_2AR expression, these hypotheses are merely speculation.

In conclusion, we have demonstrated the potential usefulness of two antipeptide antibody preparations for the study of endogenous A_2ARs by both immunoblotting and immunoprecipitation. Preliminary studies using these antibodies suggest that, even within the same species, some heterogeneity may exist within the population of A_2ARs expressed, which may indicate

the presence of an immunologically distinct canine striatum-specific A_2AR subtype in addition to the RDC8 protein, which has already been cloned. Clearly, in the absence of any selective antagonists, immunoprecipitation of the A_2AR with antibody probes will be of great use in the study of unique aspects of A_2AR processing and functioning, in particular the relatively slow onset of desensitization (28) and the relation between its unusually 'tight' coupling to G_s (6, 7, 9) and the elevated constitutive activity of adenylyl cyclase observed when RDC8 is expressed either in *Xenopus* oocytes (15) or in thyroid tissue of transgenic mice (29).

References

1. Olsson, R. A., and D. Pearson. Cardiovascular purinoceptors. *Physiol. Rev.* **70**:761–845 (1990).
2. Londos, C., D. M. F. Cooper, and J. Wolff. Subclasses of external adenosine receptors. *Proc. Natl. Acad. Sci. USA* **77**:2551–2554 (1980).
3. Freissmuth, M., E. Selzer, and W. Schutz. Interactions of purified bovine brain A_1-adenosine receptors with G-proteins: reciprocal modulation of agonist and antagonist binding. *Biochem. J.* **275**:651–656 (1991).
4. Freissmuth, M., W. Schutz, and M. E. Linder. Interactions of the bovine brain A_1-adenosine receptor with recombinant G-protein α-subunits: selectivity for $rG_{i\alpha\text{-}3}$. *J. Biol. Chem.* **266**:17778–17783 (1991).
5. Haslam, R. J., and G. M. Rosson. Effects of adenosine on levels of cyclic 3′,5′-monophosphate in human blood platelets in relation to adenosine incorporation and platelet aggregation. *Mol. Pharmacol.* **11**:528–544 (1975).
6. Braun, S., and A. Levitzki. Adenosine receptor permanently coupled to turkey erythrocyte adenylate cyclase. *Biochemistry* **18**:2134–2138 (1979).
7. Johnson, R. A. Mn^{2+} does not uncouple adenosine "R_a" receptors from the liver adenylate cyclase. *Biochem. Biophys. Res. Commun.* **105**:347–353 (1982).
8. Barrington, W. W., K. A. Jacobson, A. J. Hutchison, M. Williams, and G. L. Stiles. Identification of the A_2 adenosine receptor binding subunit by photoaffinity cross-linking. *Proc. Natl. Acad. Sci. USA* **86**:6572–6576 (1989).
9. Nanoff, C., K. A. Jacobson, and G. L. Stiles. The A_2 adenosine receptor: guanine nucleotide modulation of agonist binding is enhanced by proteolysis. *Mol. Pharmacol.* **39**:130–135 (1990).
10. Barrington, W. W., K. A. Jacobson, and G. L. Stiles. Glycoprotein nature of the A_2 adenosine receptor binding subunit. *Mol. Pharmacol.* **38**:177–183 (1990).
11. Libert, F., S. N. Schiffman, A. Lefort, M. Parmentier, C. Gerard, J. E. Dumont, J.-J. Vanderhaegen, and G. Vassart. The orphan receptor cDNA RDC7 encodes an A_1 adenosine receptor. *EMBO J.* **10**:1677–1682 (1991).
12. Mahan, L. C., L. D. McVittie, E. M. Smyk-Randall, H. Nakata, F. J. Monsma, Jr., C. R. Gerfen, and D. R. Sibley. Cloning and expression of an A_1 adenosine receptor from rat brain. *Mol. Pharmacol.* **40**:1–7 (1991).
13. Olah, M. E., H. Ren, J. Ostrowski, K. A. Jacobson, and G. L. Stiles. Cloning, expression and characterization of the unique bovine A_1 adenosine receptor: studies on the ligand binding site by site-directed mutagenesis. *J. Biol. Chem.* **267**:10764–10770 (1992).
14. Strosberg, A. D. Structure/function relationships of proteins belonging to the family of receptors coupled to GTP-binding proteins. *Eur. J. Biochem.* **196**:1–10 (1991).
15. Maenhaut, C., J. Van Sande, F. Libert, M. Abramowicz, M. Parmentier, J.-J. Vanderhaegen, J. E. Dumont, G. Vassart, and S. Schiffmann. RDC8 encodes for an adenosine A_2 receptor with physiological constitutive activity. *Biochem. Biophys. Res. Commun.* **173**:1169–1178 (1991).
16. Mumby, S. M., and A. G. Gilman. Synthetic peptide antisera with determined specificity for G protein *alpha* or *beta* subunits. *Methods Enzymol.* **195**:215–233 (1991).
17. Booth, R. F., and J. B. Clark. A rapid method for the preparation of relatively pure metabolically competent synaptosomes from rat brain. *Biochem. J.* **176**:365–370 (1978).
18. Ramkumar, V., W. W. Barrington, K. A. Jacobson, and G. L. Stiles. Demonstration of both A_1 and A_2 adenosine receptors in DDT$_1$ MF-2 smooth muscle cells. *Mol. Pharmacol.* **37**:149–156 (1990).
19. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
20. Lienhard, G. E., J. H. Crabb, and K. J. Ransome. Endoglycosidase F cleaves the oligosaccharides from the glucose transporter of the human erythrocyte. *Biochim. Biophys. Acta* **769**:404–410 (1984).
21. Gould, G. W., and G. I. Bell. Facilitative glucose transporters: an expanding family. *Trends Biochem. Sci.* **15**:18–23 (1990).
22. Leeb-Lundberg, K. M., K. E. Dickenson, S. E. Heald, G. E. S. Wikberg, P. O. Hagen, G. T. DeBernadis, M. Winn, D. L. Arendson, R. J. Lefkowitz, and M. G. Caron. Photoaffinity labelling of α_1-adrenergic receptors. *J. Biol. Chem.* **259**:2579–2588 (1984).
23. Benovic, J. L., G. L. Stiles, R. J. Lefkowitz, and M. G. Caron. Photoaffinity labelling of mammalian *beta*-adrenergic receptors: metal-dependent proteolysis explains apparent heterogeneity. *Biochem. Biophys. Res. Commun.* **110**:504–511 (1983).
24. Van Galen, P. J. M., G. S. Michaels, G. L. Stiles, and K. A. Jacobson. Sequence analysis of the canine A_1 and A_2 adenosine receptors and comparison with other G-protein-linked receptors. *Med. Res. Rev.* **12**:423–471 (1992).
25. Dohlman, H. G., M. G. Caron, and R. J. Lefkowitz. A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry* **26**:2657–2664 (1987).
26. Muzzin, P., J.-P. Revelli, F. Kuhne, J. D. Gocayne, W. R. McCombie, J. C. Venter, J.-P. Giacobino, and C. M. Fraser. An adipose tissue-specific *beta*-adrenergic receptor: molecular cloning and down-regulation in obesity. *J. Biol. Chem.* **266**:24053–24058 (1992).
27. Lorenz, W., J. W. Lomasney, S. Collins, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Expression of three α_2-adrenergic receptor subtypes in rat tissues: implications for α_2 receptor classification. *Mol. Pharmacol.* **38**:599–603 (1990).
28. Ramkumar, V., M. E. Olah, K. A. Jacobson, and G. L. Stiles. Distinct pathways of desensitization of A_1- and A_2-adenosine receptors in DDT$_1$ MF-2 cells. *Mol. Pharmacol.* **40**:639–647 (1992).
29. Ledent, C., J. E. Dumont, G. Vassart, and M. Parmentier. Thyroid expression of an A_2 adenosine receptor induces thyroid hyperplasia and hyperthyroidism. *EMBO J.* **11**:537–542 (1992).

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