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^{125}I-2-[4-[2-[2-[(4-Azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine Labels Transmembrane Span V of the A_{2a} Adenosine Receptor

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SUMMARY

We have shown previously that ^{125}I-2-[4-[2-[2-[(4-azidophenyl)methylcarbonylamino]ethylaminocarbonyl]ethyl]phenyl]ethylamino-5′-*N*-ethylcarboxamidoadenosine (^{125}I-azido-PAPA-APEC) specifically and selectively photolabels RDC8 A_{2a} adenosine receptors that have been overexpressed in COS M6 cells. Glycosylated, ^{125}I-azido-PAPA-APEC-labeled, wild-type (412 residues; 45,031 Da) and carboxyl-terminally truncated (315 residues; 35,427 Da) receptors migrate with apparent molecular masses of >40 and 31.5 kDa, respectively, whereas unglycosylated or deglycosylated wild-type and truncated A_{2a} receptors migrate with apparent molecular masses of 40 and 28.5 kDa, respectively. Because nonspecific photoincorporation is not a complication, the present peptide mapping studies of the full length and truncated canine A_{2a} adenosine receptors were carried out on unpurified COS M6 membrane preparations. After partial proteolysis it became clear that glycosylation increased the apparent molecular mass of either the wild-type or mutant A_{2a} receptor by approximately 3 kDa. Although the A_{2a} receptor was readily cleaved by a variety of chemical reagents and proteases, trypsin and endoproteinase Glu-C generated the most reproducible and, in the case of trypsin, the most complete fragmentation patterns. Radiolabeled peptides were identified by their apparent molecular masses, (in)abilities to be recognized by an antipeptide antibody to amino acids Tyr^{155}–Val^{172} of the presumed second extracellular loop of the receptor, and (in)sensitivities to endoglycosidase F and tunicamycin treatments. A prominent, 7-kDa, radiolabeled peptide that was generated by trypsin digestion implicated putative α-helix V in the binding of ^{125}I-azido-PAPA-APEC.

The adenosine receptor family is becoming an increasingly well recognized member of the G protein-coupled receptor superfamily. The amino acid sequences of the 10 adenosine receptors cloned to date suggest a common receptor structure; all four adenosine receptor subtypes, like other G protein-coupled receptors, are believed to span the plasma membrane seven times (1–13). The A_{2a} and A_{2b} adenosine receptor subtypes stimulate adenylyl cyclase via coupling to G_s (14, 15) and exhibit the following agonist rank order of potency: 5′-*N*-ethylcarboxamidoadenosine > (*R*)-*N*^{6}-(2-phenyl-1-methylethyl)adenosine > (*S*)-*N*^{6}-(2-phenyl-1-methylethyl)adenosine (16). Two potent A_{2a}-selective radioligands, [^{3}H]CGS21680 and ^{125}I-PAPA-APEC, have been developed by adding hydrophobic extensions at the C2 adenine position of 5′-*N*-ethylcarboxamidoadenosine (17,18). Even before the cloning of the canine (RDC8), rat, and human A_{2a} adenosine receptor cDNAs (1, 8–10), ^{125}I-azido-PAPA-APEC was instrumental in establishing the apparent molecular masses of glycosylated A_{2a} receptors at about 45 kDa (19, 20). *N*-linked glycosylation seems to be a common feature of A_{2a} adenosine receptors (20, 21) and is believed to occur at a single asparagine, probably Asn^{154} for RDC8, given the proximity of Asn^{145} to the membrane and to the disulfide bond candidate Cys^{146} (22).

A_{2a} adenosine receptor species homologues are approximately 97% identical in their putative transmembrane spans. Based on precedents in the literature for rhodopsin and the biogenic

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ABBREVIATIONS: 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; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

amine receptors (23–26), A_{2a} receptor ligand binding pockets are probably composed of amino acids from the transmembrane spans. Most sequence variations among the A_{2a} receptors are concentrated in the putative second extracellular loops and carboxyl termini, regions that are not believed to participate directly in ligand binding. 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. Indeed, treatment with diethylpyrocarbonate, a histidyl-modifying reagent, inactivates both rat A_1 and rabbit A_{2a} adenosine receptors (27, 28). Mutagenesis studies on the bovine A_1 receptor further suggest that histidine residues may be involved in the binding of agonists and antagonists, but parallel experiments have not been carried out on the A_{2a} receptor (6). IJzerman and co-workers (22, 29) have prepared helical wheel models of the canine A_1 (RDC7) and A_{2a} (RDC8) adenosine receptors and constructed a preliminary three-dimensional model of the canine A_1 receptor. The A_1 binding cavity is predicted to partition into two domains, a hydrophilic region bordered by transmembrane spans I, VI, and VII and a hydrophobic region bordered by spans II–VI. This model may carry over to the A_{2a} adenosine receptor because the canine A_1 and A_{2a} receptors have 64% of their presumed transmembrane core amino acids in common.

In this report, we describe peptide mapping studies of the canine A_{2a} adenosine receptor (RDC8). It was possible to conduct these experiments on unpurified receptor preparations because ^{125}I-azido-PAPA-APEC photolabels COS M6 cell-expressed A_{2a} receptors in a highly specific manner; there was little or no photoinduced incorporation of this ligand in the presence of 1 mM theophylline. Proteolysis of the A_{2a} receptor with trypsin and endoproteinase Glu-C produced the most informative results in terms of identifying the stretches of the receptor involved in ligand binding. Fig. 1 illustrates the potential cleavage sites of these two proteinases on the canine A_{2a} receptor. Radioactive peptides were identified by their apparent molecular masses, (in)abilities to be recognized by immunoblotting, and (in)sensitivities to endoglycosidase F and tunicamycin treatments.

Experimental Procedures

Materials

COS M6 cells, developed by Edith Womack in the laboratories of Drs. Michael Brown and Joseph Goldstein (University of Texas Southwestern Medical Center, Dallas, TX), 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 × 10^6; Pharmacia). Endoglycosidase F/N-glycosidase F (6 units/120 μl) was purchased from Boehringer Mannheim, and tunicamycin was purchased from Calbiochem. Leupeptin was purchased from Peptides International. Trypsin (L-1-tosylamide-2-phenylethylchloromethyl ketone-treated type XII from bovine pancreas) and adenosine deaminase (calf intestinal type VIII) were Sigma products. Theophylline came from Merck and CGS21680 from Research Biochemicals Inc. Endoproteinase Glu-C (*Staphylococcus aureus* V8 protease) (excision grade) was purchased from Calbiochem. Sigma myoglobin fragments (peptide molecular weight marker kit MW-SDS-17S) were used to calibrate protein migration on Tricine-SDS-PAGE gels. Prestained, low range protein standards (Bio-Rad) were run on some gels to check transfer efficiencies of the Western blot procedures. 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. PAPA-APEC was donated generously by Dr. Kenneth Jacobson (NIH, Bethesda, MD), and ^{125}I-PAPA-APEC and ^{125}I-azido-PAPA-APEC were prepared as described in the preceding paper (30).

Methods

Procedures for the construction of pCMV4 expression vectors containing wild-type and hexahistidine A_{2a} receptor cDNAs, maintenance of and transient transfection of COS M6 cells, treatment of A_{2a} receptor-transfected COS M6 cells with tunicamycin, preparation of membranes, photoaffinity labeling, antibody production, and deglycosylation with endoglycosidase F/N-glycosidase F are detailed in the preceding paper (30).

Proteolysis of membrane-imbedded A_{2a} receptor protein. Membranes (50–300 μg of protein, depending on expression levels; 1.5 pmol of receptor) were photolabeled as described previously and resuspended for the final time in the appropriate digestion buffer. Digestions were conducted in Dulbecco's phosphate-buffered saline, pH 7.8, for trypsin (1–10 μg of enzyme/1 μg of total protein, ≥12-hr incubation at 37°), 10 mM NH_4HCO_3, pH 7.8, for endoproteinase Glu-C (1 μg of enzyme/8 μg of total protein, ≥12-hr incubation at room temperature), and Na_2HPO_4, pH 8.5, for kallikrein (1 μg of enzyme/2.5–25 μg of total protein, ≥12-hr incubation at 37°). Digested membranes were pelleted (49,460 × *g* for 30 min at 4°) and taken up in 10% SDS sample buffer for electrophoresis on peptide-resolving gels. In those instances where membrane protein had been deglycosylated after photolabeling, proteases and, finally, electrophoresis sample buffer were added directly to the denatured endoglycosidase F/N-glycosidase F mixture.

Chemical fragmentation of membrane-imbedded A_{2a} receptor protein. Membranes (50–300 μg of protein, depending on expression levels; 1.5 pmol of receptor) were photolabeled and resuspended for the final time in the appropriate treatment buffer. Hydroxylamine and 2-nitro-5-thiocyanobenzoic acid treatments were carried out according to published procedures (31, 32). Chemically treated membranes were pelleted (49,460 × *g* for 30 min at 4°) and taken up in 10% SDS sample buffer, in preparation for electrophoresis on peptide-resolving gels.

Proteolysis of A_{2a} receptor protein in SDS-PAGE gel slices: two-dimensional peptide mapping. Proteolyzed or nonproteolyzed A_{2a}-transfected COS M6 membranes were electrophoresed in the first dimension on 12% SDS-PAGE or 16.5% Tricine-SDS-PAGE gels. The desired undigested receptor or receptor fragment band was located via autoradiography and excised from the gel. The gel slice was frozen in liquid N_2 and pulverized using a Crescent Wig-L-Bug dental amalgamator, and the resultant powder was suspended in 750 μl of RIPA buffer (150 mM NaCl, 50 mM Tris·HCl, pH 8.0, containing 1%, v/v, Nonidet P-40, 0.05%, w/v, deoxycholic acid, and 0.1%, w/v, SDS) in an Eppendorf tube. Trypsin (1 mg) was added in great excess; an excised gel piece spanned approximately 3% of the gel length, but it was difficult to estimate how many micrograms of protein (of the original ~100 μg of total protein) had resolved to that area. After overnight incubation at 37°, the gel powder was sedimented by centrifugation in a Microfuge. Because the majority of ^{125}I-azido-PAPA-APEC counts remained embedded in the gel pellet, the RIPA supernatant was discarded. Powdered gel was mixed with 10% SDS sample buffer and subjected to electrophoresis in the second dimension on peptide-resolving gels. Runs were started at 30 V for 1.5 hr (until samples had completely left the wells) and then continued at 90 V for approximately 16 hr.

Immunoblotting of A_{2a} receptor fragments. Labeled digested samples were loaded on 0.75-mm, 16.5% Tricine-SDS-PAGE gels. A continuous unibuffer system was used in the semidry transfer; anode and cathode filter papers were both soaked in 48 mM Tris, 39 mM glycine, 20% methanol, 0.037%, w/v, SDS. The current was initially set at 2.5 mA/cm^3 of gel, and transfer took 45 min. The membrane was soaked in TBS (20 mM Tris, 500 mM NaCl, pH 7.5) for 10 min and

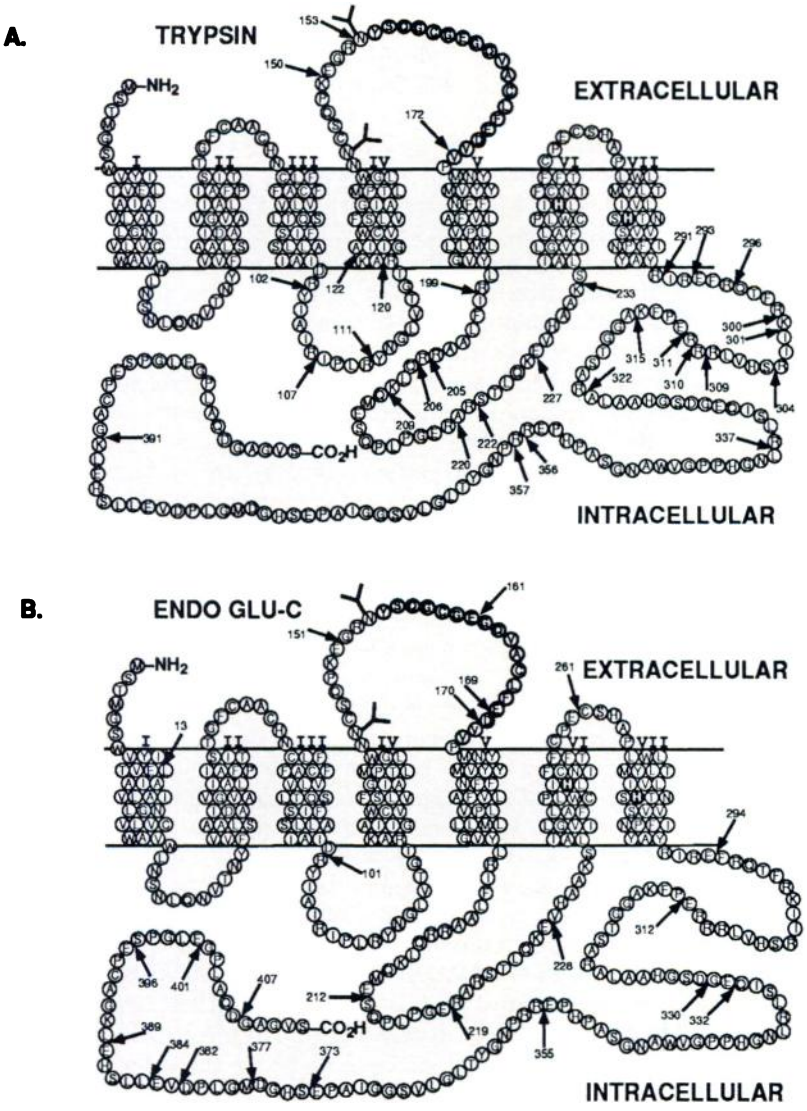

Fig. 1. Potential cleavage sites for trypsin and endoproteinase Glu-C on the canine A_{2a} adenosine receptor (RDC8). A, Canonical trypsin cut sites are indicated by *arrows* pointing to the carboxyl side of lysine (*K*) and arginine (*R*) residues. B, Canonical endoproteinase Glu-C sites are indicated by *arrows* pointing to the carboxyl side of glutamate (*E*) residues. Endoproteinase Glu-C can also cleave proteins after aspartate (*D*) residues under certain conditions. Potential asparagine (*N*) glycosylation sites, Asn^{145} and Asn^{154}, are shown with carbohydrate symbols. Two histidines, His^{250} and His^{278}, are conserved throughout the adenosine receptor family. *Bold letters*, amino acid sequence used to generate antipeptide antibodies (residues 155–172).

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% Tween-20, and incubated with primary antibody overnight. Rabbit sera were diluted 500-fold in TBS/0.05% Tween-20 containing 1% gelatin. Detection was carried out according to the instructions supplied with the peroxidase Vectastain Elite ABC kit from Vector Laboratories. After the horseradish peroxidase color detection was finished and the membrane had been rinsed and dried, it was subjected to autoradiography.

Results and Discussion

Peptide mapping with trypsin. Trypsinization of wild-type and hexahistidine A_{2a} adenosine receptors, treated with or without tunicamycin, produced distinctive fragmentation patterns (Fig. 2A). The presence of numerous trypsin-sensitive lysine and arginine residues within the carboxyl tail of the A_{2a} receptor resulted in apparently identical tryptic fragmentation patterns for the wild-type and hexahistidine A_{2a} receptors. Accordingly, we were unable to rely upon Ni^{2+}·nitrilotriacetic acid chromatography to resolve carboxyl-terminal fragments of the hexahistidine-tagged receptor and were unable to use the difference in mass between wild-type and mutant receptors to help identify fragments. The conformations of the wild-type and hexahistidine receptors must have been similar enough that the same residues were accessible to trypsin, and the same transmembrane units, if not the same amino acid or acids, were photolabeled. Prominent bands at 21.3 and 13.7 kDa in the lanes for vehicle-treated receptors (Fig. 2A, − *tunicamycin*), either full length or truncated, had counterparts at 18.8 and 7.4 kDa in the tunicamycin-treated receptor lanes (Fig. 2A, + *tunicamycin*). The 2.5-kDa shift between the 21.3- and 18.8-kDa peptides, but not the 6.3-kDa shift between the 13.7- and 7.4-kDa peptides, could be explained by the loss of a carbohydrate moiety.

Steric hindrance of proteolysis by an *N*-glycoside presented itself as a possible explanation for the 6.3-kDa shift; unglycosylated receptors could have been susceptible to additional proteolysis at Lys^{150} or Arg^{153}, sites that likely were blocked by glycosylation at Asn^{154} [see the preceding paper (30)]. Based on this hypothesis and the potential A_{2a} cleavage sites of trypsin, the 13.7-kDa fragment was proposed to span interhelical loop IV–V as well as transmembrane spans IV and V. One possible sequence assignment for the 13.7-kDa peptide was amino acids 108–209 (M_r 11,454; M_r 11,454 + 2,500 sugar =

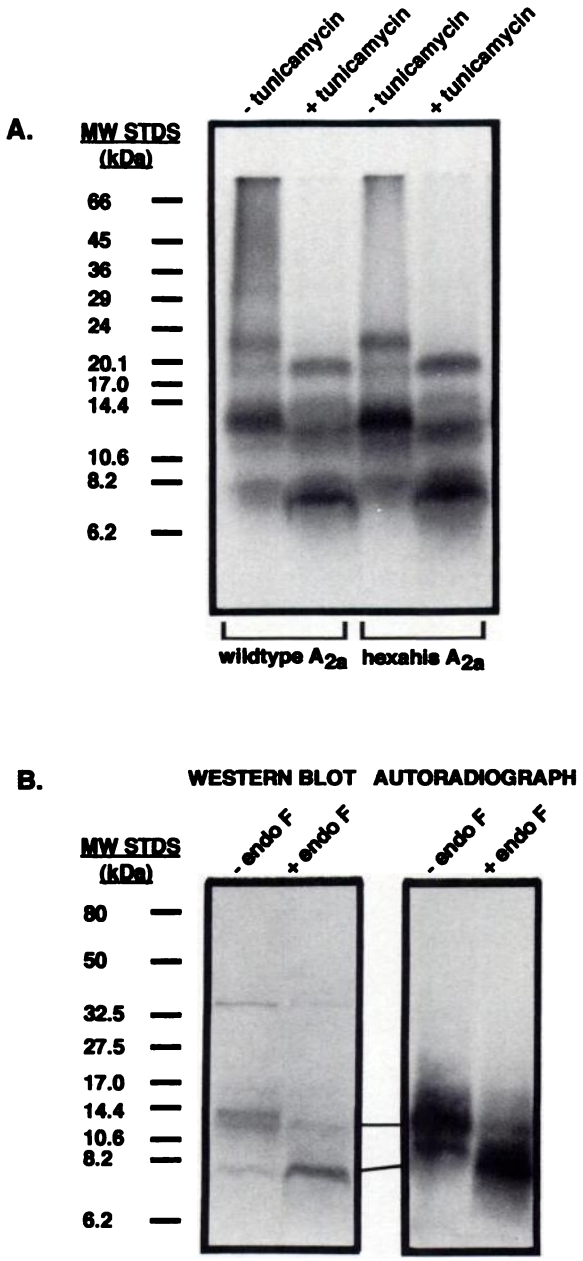

Fig. 2. Peptide mapping of the A_{2a} adenosine receptor (RDC8) with trypsin. A, Wild-type A_{2a} receptor-transfected or hexahistidine A_{2a} receptor-transfected COS M6 cells were treated with tunicamycin (5 μg/ml for 48 hr) or vehicle. The corresponding membranes (150–340 μg, 1.5 pmol of receptor) were photolabeled with ^{125}I-azido-PAPA-APEC and subsequently digested with trypsin (5-fold excess, w/w, of trypsin over membrane protein; overnight incubation at 37°). Protein fragments were subjected to electrophoresis on a 16.5% Tricine-SDS-PAGE gel. This autoradiograph was typical of four others from independent experiments. B, Hexahistidine A_{2a} receptor-containing COS M6 membranes (45 μg, 1.5 pmol of receptor) were photolabeled with ^{125}I-azido-PAPA-APEC treated with endoglycosidase F/N-glycosidase F (*endo F*) (0.25 unit of enzyme, overnight incubation at 37°), and then digested with trypsin (5-fold excess, w/w; 12-hr incubation at 37°). Protein fragments were subjected to electrophoresis on a 16.5% tricine-SDS-PAGE gel and immunoblotted as described in Experimental Procedures. The Western blot (*left*) and its corresponding autoradiograph (*right*) were representative of sets from three other experiments. The blot and the autoradiograph bands are offset because of shrinkage of the nitrocellulose paper between the time of photography and autoradiography.

13,954). The 7.4-kDa species probably represented transmembrane span V (amino acids 151–209 and 154–209 have molecular masses of 6811 and 6469 Da, respectively), rather than transmembrane span IV (amino acids 108–153; M_r 4985). Other researchers have suggested that serine proteases cannot hydrolyze bonds within about 10 residues of the lipid bilayer (33), but this was not an obvious conclusion from our work. Trypsin must have cleaved somewhere within interhelical loop III–IV, a region where no cut sites are located more than eight amino acids away from the putative transmembrane sequences. On the basis of molecular mass data alone, the nonglycosylated 18.8-kDa species corresponded to either the first five or the last three or four transmembrane spans of the A_{2a} receptor. In any of these scenarios, the photolabeled transmembrane unit included α-helix V. Minor bands may have represented or been derived from the small percentages of (a) vehicle-treated receptors that lacked glycosylation, (b) tunicamycin-treated receptors that were glycosylated, (c) glycosylated receptors that were nonetheless proteolyzed at Lys^{150} or Arg^{153}, or (d) unglycosylated receptors that escaped proteolysis at Lys^{150} or Arg^{153}.

Trypsinization of endoglycosidase F-treated or untreated hexahistidine A_{2a} adenosine receptors yielded radioactive peptides similar to those seen in Fig. 2A (see Fig. 2B). Again, removal of the A_{2a} carbohydrate moiety caused a distinct shift in the radioactive content of the various peptides (Fig. 2B, *autoradiograph lanes*). Whereas the majority of radioactivity resided in a 13.0-kDa species in the control lane (Fig. 2B, – *endo F*), a 7.0-kDa band predominated in the deglycosylated receptor lane (Fig. 2B, + *endo F*). Most importantly, our antibody to A_{2a} receptor amino acids Tyr^{155}–Val^{172} recognized both of these peptides (Fig. 2B, *Western blot lanes*), thereby confirming our theory that transmembrane span V was modified by ^{125}I-azido-PAPA-APEC. The Western blot also revealed an immunoreactive peptide at 11.5 kDa, in addition to 13.0- and 7.0-kDa species. It was difficult to discern whether the 11.5-kDa immunoblot band contributed to the radioactivity of the "13.0"-kDa autoradiograph band, but minor bands around 10 kDa displayed both immunoreactivity and radioactivity in other Western blot experiments. The minor, endoglycosidase-insensitive, radioactive fragment at 8.4 kDa was not recognized by our antipeptide antibody. Despite the confusion introduced by minor receptor fragments, one result was invariant: "limit" proteolysis of unglycosylated or deglycosylated A_{2a} receptors always produced an immunoreactive peptide of ≈7 kDa that accounted for ≥60% of ^{125}I-azido-PAPA-APEC photoincorporation (as determined by lane versus band counts). Assuming that trypsin cleaved only at arginine or lysine residues, the data are consistent with the interpretation that ^{125}I-azido-PAPA-APEC modified one or more amino acids in or near transmembrane span V.

Trypsinization of the hexahistidine A_{2a} receptor may have been more thorough in the mapping experiments with endoglycosidase F than in the mapping experiments with tunicamycin because enzymatic digestion was conducted under denaturing conditions. Further breakdown of the 21.3- and 18.8-kDa peptides in the tunicamycin experiments must have led to the increased formation of the 13.0- and 7.0-kDa "end products," respectively. Indeed, when the 21.3- and 18.8-kDa bands were excised from the gel, retrypsinized, and re-electrophoresed on a second Tricine-SDS-PAGE gel, the familiar 21/18/13–14/(10)/7-kDa fragmentation pattern was observed (data not

shown). In the mapping protocol with and without endoglycosidase F, photolabeled membranes were not centrifuged after proteolysis in the endoglycosidase F buffer. Even though any hydrophilic receptor fragments would have been present in the electrophoretic samples, no new radioactive bands appeared during gel autoradiography. Clearly, cleaved hydrophilic receptor segments could not have constituted a significant fraction of the total labeled protein.

Peptide mapping with endoproteinase Glu-C. Endoproteinase Glu-C generated A_{2a} adenosine receptor fragmentation patterns that were clear-cut but nevertheless difficult to interpret. From the start, peptide identification was complicated by the fact that endoproteinase Glu-C (*S. aureus* V8) may or may not have cut the A_{2a} receptor after aspartate as well as glutamate residues. Trial digests were carried out in buffers known either to favor cleavage after glutamate residues (10 mM NH_4HCO_3, pH 7.8) or to enhance cleavage after aspartate residues (phosphate-buffered saline, pH 7.8), but identical, and therefore inconclusive, results were obtained from the two experiments. Tunicamycin treatment, however, did have a pronounced effect on wild-type and hexahistidine A_{2a} receptor fragmentation patterns (Fig. 3A). For either receptor type, a 23.1-kDa fragment was converted to a 20.0-kDa fragment. On the basis of size alone, this presumed glycosylated/unglycosylated peptide pair represented either amino acids 1–212 (M_r 23,334) or 152–315 (M_r 19,081). A 14.6-kDa radiopeptide was insensitive to tunicamycin treatment, suggesting that it corresponded to amino acids 1–151 (M_r 16,264) or 162–294 (M_r 15,359). Typically, this particular peptide accounted for 30–35% of the total fragment counts. Some "starting material," i.e., undigested receptor, remained in the hexahistidine lanes and the wild-type tunicamycin-treated lane.

Endoglycosidase F/Western blotting (or tunicamycin/Western blotting) experiments on hexahistidine A_{2a} receptor protein assisted only marginally in the identification of the endoproteinase Glu-C radioactive fragments (Fig. 3B). As could have been predicted from our tentative peptide assignments, antibody recognized the 33.0/30.5-kDa set of undigested bands along with the unglycosylated member of the 23.1/20.0-kDa peptide pair. The 23.1-kDa species was not recognized on this particular gel. In this and other experiments, there was no indication that the 14.6-kDa peptide could be detected by antisera against the second extracellular loop of A_{2a}. Occasionally, a radioactive peptide was visible at approximately 16.1 kDa in lanes not treated with endoglycosidase F (or tunicamycin). This band possessed a curious combination of attributes: (a) it had no obvious partner in the endoglycosidase F (or tunicamycin)-treated lanes and, (b) although it appeared to be sensitive to deglycosylation, it was not immunoreactive. Because endoproteinase Glu-C could potentially cleave A_{2a} at three sites within the antibody recognition sequence (Glu^{162}, Glu^{169}, and Asp^{170}), peptides retaining only part of the YSQGCGEGQVACLFEDVV sequence may not have been detected by Western blotting. Thus, if the endoproteinase Glu-C results were to agree with the trypsinization results, most of the photolabeling of the 23.1/20.0-kDa peptide pair had to have occurred on transmembrane span V.

Two-dimensional peptide mapping with endoproteinase Glu-C and trypsin. To test the aforementioned hypothesis, the A_{2a} adenosine receptor was subjected to two-dimensional peptide mapping (Fig, 4). Specifically, 23.1- and 20.0-

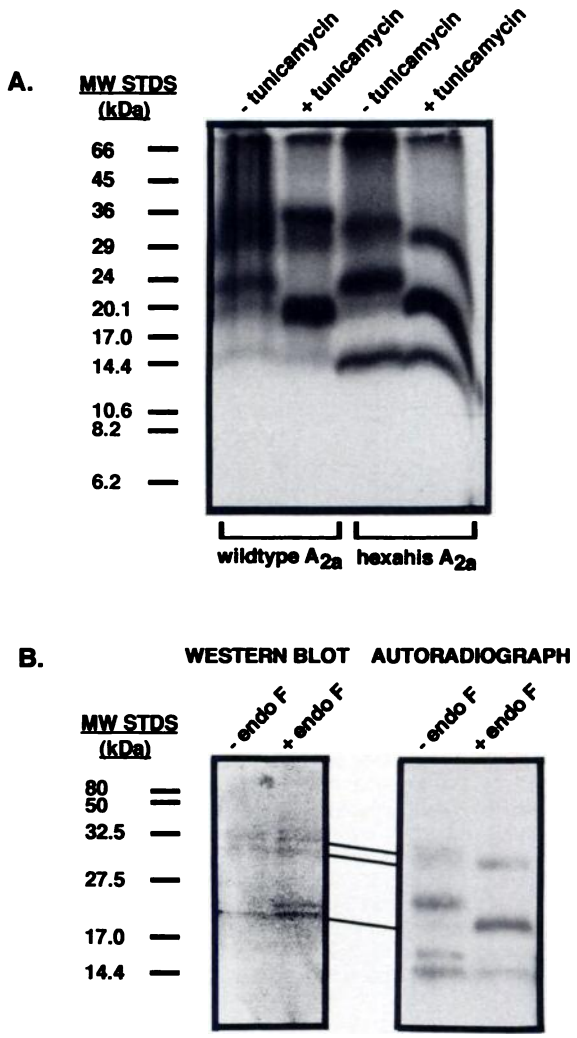

Fig. 3. Peptide mapping of the A_{2a} adenosine receptor (RDC8) with endoproteinase Glu-C. A, Wild-type A_{2a} receptor-transfected or hexahistidine A_{2a} receptor-transfected COS M6 cells were treated with tunicamycin (5 μg/ml for 48 hr) or vehicle. The corresponding membranes (150–340 μg, 1.5 pmol of receptor) were photolabeled with ^{125}I-azido-PAPA-APEC and digested with *S. aureus* V8 (1 μg of enzyme/8 μg of membrane protein, overnight incubation at room temperature). Protein fragments were subjected to electrophoresis on a 16.5% Tricine-SDS-PAGE gel. This autoradiograph was typical of two others from independent experiments. B, Hexahistidine A_{2a} receptor-transfected COS M6 membranes (45 μg, 1.5 pmol of receptor) were photolabeled with ^{125}I-azido-PAPA-APEC, treated with endoglycosidase F/N-glycosidase F (*endo F*) (0.25 unit of enzyme, overnight incubation at 37°), and digested with *S. aureus* V8 (1 μg of enzyme/8 μg of membrane protein, 12-hr incubation at room temperature). Protein fragments were subjected to electrophoresis on a 16.5% Tricine-SDS-PAGE gel and immunoblotted as described in Experimental Procedures. A representative Western blot (*left*) and its corresponding autoradiograph (*right*) are shown; these were typical of those obtained in two other independent experiments.

kDa hexahistidine receptor fragments were excised from a first gel, trypsinized in their powdered gel slices, and subjected to electrophoresis, next to each other, on a second Tricine-SDS-PAGE gel (Fig. 4, *lanes 3* and *4*). Control lanes contained trypsinized protein from radioactive bands at 32.5 and 29.7 kDa, the putative glycosylated and deglycosylated forms of the hexahistidine receptor retrieved from another gel (Fig. 4, *lanes 1* and *2*). Not only were the 32.5- and 29.7-kDa receptors hydrolyzed as they had been during membrane trypsinization

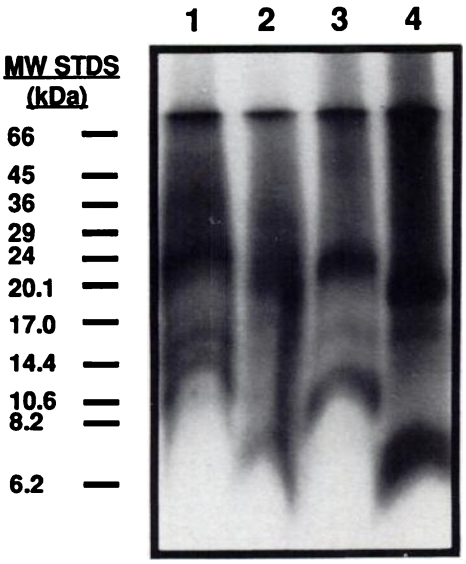

Fig. 4. Two-dimensional peptide mapping of the A_{2a} adenosine receptor (RDC8) with endoproteinase Glu-C and trypsin. Hexahistidine A_{2a} receptor-transfected COS M6 membranes (110 μg, 3.0 pmol of receptor) were photolabeled with ^{125}I-azido-PAPA-APEC, treated (*lanes 2* and *4*) or not treated (*lanes 1* and *3*) with endoglycosidase F/N-glycosidase F (0.50 unit of enzyme, overnight incubation at 37°), proteolyzed (*lanes 3* and *4*) or not proteolyzed (*lanes 1* and *2*) with *S. aureus* V8 protease (1 μg of enzyme/8 μg of membrane protein, 12-hr incubation at room temperature), and subjected to electrophoresis in the first dimension on a 16.5% Tricine-SDS-PAGE gel. Glycosylated and deglycosylated hexahistidine receptor bands (apparent molecular masses of 31 and 28 kDa, respectively) and endoproteinase Glu-C-derived fragment bands (apparent molecular masses of 23 and 20 kDa, respectively) were excised, and the incorporated proteins were digested with trypsin (1 mg, overnight incubation at 37°) as described in Experimental Procedures. Samples were then re-electrophoresed in the second dimension on another 16.5% Tricine-SDS-PAGE gel. These results were reproduced on another set of gels.

experiments, but also the 23.1- and 20.0-kDa proteins apparently were fragmented into the same ^{125}I-azido-PAPA-APEC-labeled peptide components as the glycosylated and deglycosylated receptors, respectively. Principally, counts observed at 13.8 kDa for the glycosylated species (Fig. 4, *lanes 1* and *3*) shifted to 7.5 kDa for the unglycosylated species (Fig. 4, *lanes 2* and *4*). If, as suggested by the earlier trypsin mapping experiments, the 13.8-kDa fragment encompassed both transmembrane spans IV and V, then the 23.1- and 20.0-kDa endoproteinase Glu-C fragments represented amino acids 1–212, as opposed to amino acids 152–315, of the A_{2a} receptor. The "bands" above 66 kDa highlighted the spacer/resolving gel border of the Tricine-SDS-PAGE gel, so it was unclear whether this radioactivity represented an artifact or fragment aggregates. Western blotting of the second resolving gel was not feasible because a considerable amount of protein was lost during our version of the Cleveland technique (i.e., radioactive peptides could be identified by size and sensitivity to endoglycosidase F but not by immunoblotting). Indirectly then, proteolysis of A_{2a} adenosine receptors with endoproteinase Glu-C implicated transmembrane span V in ^{125}I-azido-PAPA-APEC binding.

Minor labeling of transmembrane spans VI and VII. To lesser extents, transmembrane spans VI and VII of the wild-type and hexahistidine A_{2a} adenosine receptors also may have been labeled by ^{125}I-azido-PAPA-APEC. Digestion with kallikrein, a serine protease that cleaves after phenylalanine-arginine or leucine-arginine amino acid combinations, produced a minor 13.7-kDa fragment that, based on size, corresponded most logically to hexahistidine A_{2a} amino acids 200–315 (M_r 13,534). Moreover, this peptide was insensitive to either endoglycosidase F or tunicamycin treatments. After hexahistidine receptors were treated chemically with hydroxylamine, virtually all radioactivity was resolved in bands at 25.5 and 22.2 kDa. These two peptides represented amino acids 114–315 (M_r 22,431) with or without a carbohydrate moiety; theoretically, hydroxylamine could have severed the A_{2a} receptor only once, at the bond between Asn^{113} and Gly^{114} (32). Photolabeling that was not attributable to covalent modification of transmembrane span V must have occurred on α-helices IV, VI, or VII. Lastly, when 2-nitro-5-thiocyanobenzoic acid-treated wild-type and hexahistidine A_{2a} membranes were electrophoresed in adjacent lanes, a wild-type receptor fragment at 27.5 kDa appeared as an 18.2-kDa fragment of the mutant receptor. Given this apparent molecular mass difference of 9.3 kDa, both of these fragments probably spanned from amino acid 160 to the carboxyl terminus of their respective receptor types (amino acids 160–315, M_r 18,096; amino acids 160–412, M_r 27,700). 2-Nitro-5-thiocyanobenzoic acid has been reported to cleave proteins specifically at the carboxyl side of cysteine residues (31). Aryl azide photoaffinity probes often react with multiple residues, so ^{125}I-azido-PAPA-APEC may well have labeled several different residues in a region composed of amino acids from several transmembrane spans.

Conclusions. In summary, our peptide mapping studies tracked ^{125}I-azido-PAPA-APEC binding only to the transmembrane span level. They did not reveal which individual amino acid(s) in α-helix V of the canine A_{2a} adenosine receptor had been modified. Because the azido group of ^{125}I-azido-PAPA-APEC resides at the very end of a functionalized chain that contributes to A_{2a} selectivity, the photogenerated nitrene may not have modified the specific amino acids involved in binding of either the hydrophobic side chain or its terminal aromatic moiety. ^{125}I-Azido-PAPA-APEC-labeled amino acids are undoubtedly not the same residues that are involved in adenosine recognition. The identification of the modified residues, however, could indirectly help to establish the atomic coordinates of the adenosine binding site by establishing the position of those amino acids of putative transmembrane span V within a three-dimensional model of the receptor protein. Several generalizations can be made about what types of residues might form the adenosine binding pocket. First, most binding domain residues presumably would be conserved in all A_{2a} clones. Next, residues critical to ligand binding would be expected to face the transmembrane cavity. Assuming that the wheel model proposed by van Galen *et al.* (22) is correct, seven residues from α-helix V fulfill the aforementioned predictions regarding both oriintation and A_{2a}-wide conservation, i.e., Met^{174}, Asn^{175}, Val^{178}, Phe^{182}, Pro^{189}, Leu^{192}, and Met^{193}. Only three of these candidates (Met^{174}, Asn^{175}, and Leu^{192}) qualify as "A_{2a}-unique" amino acids, i.e., they might play a role in the binding of A_{2a}-selective ligands such as ^{125}I-azido-PAPA-APEC.

In the future, amino acid composition or mass spectroscopic studies on labeled peptide fragments may confirm that transmembrane span V forms part of the A_{2a} receptor ligand binding pocket. Because we have shown previously that glycosylation is not essential for ligand binding, we are now free to mass-

produce the A_{2a} receptor in a bacterial expression system (30). This technology should facilitate affinity purification of the receptor on Ni^{2+}·nitrilotriacetic acid resin. Given these goals for the future, it was mandatory that we demonstrate the similar photolabeling/fragmentation patterns of wild-type and hexahistidine A_{2a} receptors. Ultimately, microsequencing analyses will be necessary to determine exactly where ^{125}I-azido-PAPA-APEC incorporates into the A_{2a} adenosine receptor.

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