Cloning, Expression, and Characterization of the Unique Bovine $A_1$ Adenosine Receptor

STUDIES ON THE LIGAND BINDING SITE BY SITE-DIRECTED MUTAGENESIS*

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The bovine brain $A_1$ adenosine receptor (A1AR) is distinct from other $A_i$ARs in that it displays the unique agonist potency series of $N^{6}$-R-phenylisopropyladenosine (R-PIA) > $N^{6}$-S-phenylisopropyladenosine (S-PIA) > $5'$-N'-ethylcarboxamidoadenosine and has a 5-10-fold higher affinity for both agonists and antagonists. The cDNA for this receptor has been cloned from a size-selected (2-4-kb) bovine brain library and sequenced. The 2.0-kb cDNA encodes a protein of 326 amino acid residues with a molecular mass of 38,570 daltons. The amino acid sequence fits well into the seven-transmembrane domain motif typical of G protein-coupled receptors. Northern analysis in bovine tissue using the full length cDNA demonstrates mRNAs of 3.4 and 5.7 kb with a tissue distribution consistent with A1AR binding. Subcloning of the cDNA in a pCMV5 expression vector with subsequent transfection into both COS7 and Chinese hamster ovary cells revealed a fully functional A1AR which could inhibit adenylylcyclase and retained the unique pharmacologic properties of the bovine brain A1AR.

The A1AR was found to have a single histidine residue in each of transmembrane domains 6 and 7. Histidine residues have been postulated by biochemical studies to be important for ligand binding. Mutation of His-278 to Leu-278 (seventh transmembrane domain) dramatically decreased both agonist and antagonist binding by >90%. In contrast, mutation of His-251 to Leu-251 decreased antagonist affinity and the number of receptors recognized by an antagonist radioligand. In contrast, agonist affinity was not perturbed but the number of receptors detected by an agonist radioligand was also reduced. These data suggest that both histidines are important for both agonist and antagonist binding, but His-278 appears critical for ligand binding to occur.

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‡ A, adenosine receptors are integral membrane proteins which bind extracellular adenosine and then initiate a transmembrane signal via G proteins to modulate the activity of a number of effector systems including adenylcyclase (inhibition), K+ channels (opening), and Ca2+ channels (close) (1-3). These receptors are found in a diverse selection of cell types and tissues and are known to be dynamically regulated by a variety of pathophysiological conditions including agonist-induced desensitization (4, 5), antagonist-induced sensitization (6), and in altered thyroid states (7). Subpopulations of $A_1$ adenosine receptors have been postulated to exist based on distinct pharmacological profiles and on absolute agonist and antagonist affinities (8-11).

One such subtype is the bovine A1AR which differs from the classical A1AR found in most tissues and species in that it binds antagonists such as XAC with subnanomolar affinities and has a unique agonist potency series of R-PIA > S-PIA > NECA (8-11). This contrasts with the typical A1AR which has 5-10-fold lower affinities for antagonists and agonists and a potency series of R-PIA > NECA > S-PIA (1, 2).

We have previously documented that these unique properties of the bovine brain A1AR are displayed by the membrane-bound, solubilized, and purified receptor suggesting these characteristics are intrinsic to the receptor protein itself and not the environment in which it resides (10, 11). A1ARs of canine thyroid (12) and rat brain (13, 14) have recently been cloned. For both clones, the expressed receptor displays the pharmacological properties described above for the classical A1AR.

Biomedical studies have documented that the A1AR is a glycoprotein which contains complex-type carbohydrate chains (10, 15), likely contains disulfide bonds (16, 17), and contains histidine residues in the ligand binding site (17). Klotz et al. (17) have suggested that there are at least 2 histidines in the receptor binding pocket and that agonists and antagonists interact with distinct transmembrane domain recognition sites.

In this paper, we report the cloning, expression, and characterization of the unique bovine brain A1AR, and through site-directed mutagenesis studies begin to probe the important amino acids in the receptor binding pocket.

1 The abbreviations used are: G protein, guanine nucleotide regulatory protein; $A_i$AR, $A_i$ adenosine receptor; R-PIA, $N^{6}$-R-phenylisopropyladenosine; S-PIA, $N^{6}$-S-phenylisopropyladenosine; NECA, 5'-N'-ethylcarboxamidoadenosine; XAC, xanthine amine congener; APNEA, 5'-2-(4-aminophenyl)ethyladenosine; SDS, sodium dodecyl sulfate; kb, kilobase(s); CHO, Chinese hamster ovary; bp, base pair(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.
EXPERIMENTAL PROCEDURES

Materials—[α-32P]ATP, [α-35S]ATP, [α-32P]cDNA, and [α-H]XAC were from Du Pont-New England Nuclear. Restriction enzymes and T4 DNA ligase were from either Boehringer-Mannheim or Promega. The pCMV6 expression vector was obtained from Dr. Marc Caron (Duke University) and was originally described by Dr. David Russell (University of Texas). [α-32P]ATP was prepared as previously described (18). All cell culture supplies were from GIBCO.

Library Screening—A 60-mer antisense oligonucleotide based on a region (amino acids 201-221) of the putative third intracellular loop of the protein encoded by the canine RDC7 clone (19) was synthesized and labeled with [α-32P]ATP on the 5'- hydroxyl end by T4 polynucleotide kinase. This probe was used to screen approximately 1×10^9 recombinants in a size-selected (10% to 20% of size-selected) 10-bp minimum insert size, lambda gt11 cDNA library (ZAP) generously provided by Richard Dixon (Merck, Sharp & Dohme Research Laboratories) and Robert Lefkowitz (Duke University). Duplicate nylon filters (Biotrans, ICN) were used for phage lifts and were prehybridized in 6× SSPE (1× SSPE is 10 mM NaCl, 1 mM NaH2PO4, and 0.1% SDS) at 45°C. Hybridization with labeled probe (850,000 cpm/ml) was conducted in 6× SSPE, 6× Denhardt's solution, 0.1% SDS, 0.5 mg/ml salmon sperm DNA for 5 h at 45°C. Hybridization was performed via the DEAE-dextran method (20). COS7 cells were grown in 75-cm² flasks in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and gentamicin (50 mg/ml G418 (Geneticin, GIBCO), and, after 14 days of treatment, resistant cells were scored (18). All cell culture supplies were from GIBCO.

DNA Sequencing—The rescued insert contained in the phaged library vector was sequenced in a run sequence analysis. Both strands of the cDNA insert were sequenced using [32P]dATP and T7 DNA polymerase (Sequenase 2.0, U. S. Biochemical). The double-stranded DNA was denatured immediately prior to sequencing by the protocol supplied by U. S. Biochemical.

Receptor Expression in Mammalian Cells—A KpnI/PstI fragment containing nearly the entire cDNA insert was excised from pBluescript and subcloned into the pCMV5 expression vector using T4 DNA ligase with the reaction performed at 15°C for 15 h. For transient expression of the cDNA, ~1×10^7 COS7 cells were transfected with 15 μg of expression plasmid via the DEAE-dextran method (21). Cells were harvested 48-72 h posttransfection for radioligand binding assays. COS7 cells, grown in Ham's F-12 media supplemented with 10% fetal bovine serum and penicillin (100 units/ml) and streptomycin (100 μg/ml) were employed for stable expression of the cDNA. Approximately 300,000 cells in 25-cm² flasks were transfected with 30 μg of the pCMV5 expression vector containing the A1AR cDNA insert, and 5 μg of pSVneo by a calcium phosphate precipitation technique (22) with subsequent washes and modifications. Cells were then maintained in the presence of 250 μg/ml G418 (Geneticin, GIBCO), and, after 14 days of treatment, resistant colonies were selected and replated. Clonal cell lines expressing physiological levels of A1AR, as determined by radioligand binding assays, were used in adenylyl cyclase studies.

Site-directed Mutagenesis of the A1AR—The method for the creation of mutated cDNA was based on that described by Nelson and Long (22). Two oligonucleotides complementary to the sense strand of the cloned A1AR were synthesized in order to produce two mutant A1ARs. The first oligonucleotide (5' ATG AGT ACG AAG GGC AGC GAG GTC TGC GGG CTC G at 287) was substituted at histidine at position 251 (sixth transmembrane domain) with leucine. The second oligonucleotide (5' GCC GAG TTC CGG AGT AAG ATG ATG TTC GAG GT at 257) was used to construct a mutated A1AR in which the histidine at position 278 (seventh transmembrane domain) was replaced with leucine. Both His-251 and His-278 mutants, an oligonucleotide complementary to the cDNA located 60 bp upstream from a unique BsmI site and the oligonucleotide coding for the His-Leu substitution were used as primers in a polymerase chain reaction using 100 ng of A1AR/pCMV5 plasmid as the template. The fragment created in the first-step polymerase chain reaction was gel purified and used as a primer in a second polymerase chain reaction (one cycle only) for the extension of the mutated cDNA with expression vector DNA used as template. This fragment was then amplified by continuing the polymerase chain reaction with the BsmI site oligonucleotide and an oligonucleotide complementary to a region of the cDNA located down stream from a unique BstEII site used as primers. The final product (~750 bp was extracted with phenol and chloroform and ethanol-precipitated. Sequencing of the mutant cDNA was verified by double strand sequencing. COS7 cells were transfected with His-251 and His-278 expression constructs as described above.

Radioiodide Binding Assays—Membrane from COS7 cells were prepared as follows. Media was aspirated from the flask, and the cells were washed with 10 ml of saline buffer (10 mM Tris, 5 mM EDTA, pH 7.4 at 22°C). A fresh 5 ml of ice-cold lysis buffer was added to the flask, and cells were scraped and transferred to a dounce homogenizer on ice. The cells were homogenized with 20 strokes, spun at 38,500 × g for 5 min, and resuspended in 6 ml of 50/10/1 buffer (50 mM Tris, 10 mM MgCl2, 1 mM EDTA, pH 7.2 at 5°C). The cells were further diluted 3-4-fold with 50/10/1 buffer.

For saturation binding experiments, each assay tube contained 50 μl of H2O or 10 mM theophylline (to define nonspecific binding), 50 μl of radioligand, 50 μl of 50/10/1 buffer, and 100 μl (~5-10 μg of protein) of membrane suspension. In assays with mutagenized A1AR, 10 mM theophylline and 0.1 mM β-PIA gave similar results for nonspecific binding. For competition binding studies, each tube contained 50 μl of competing ligand, 50 μl of radioligand, 50 μl of 50/10/1 buffer, and 100 μl of the membrane suspension. All incubations were at 2°C for 90 min and terminated by the addition of ice-cold 50/10/1 saline buffer containing CHAPS and 0.03% polyethyleneimine-treated filters using a Brande cell harvester. Data analysis was performed via a nonlinear least-squares fitting technique as previously described (23). Protein determinations were performed via the Bradford method (24).

Adenylyl cyclase Assays—Membrane from stably transfected CHO cells were prepared exactly as described above for COS7 cells. Adenylyl cyclase assays were performed as previously described (25). CAMP accumulation experiments were performed using the cAMP radioimmunoassay kit as described by the manufacturer (Amersham). In both assays forskolin (50 μM) was used to activate the adenylyl cyclase system.

Northern Analysis—RNA was prepared from bovine tissues that had been frozen in liquid nitrogen immediately upon dissection. Tissues were homogenized in ice-cold buffer consisting of 0.1 M Tris HCl, pH 7.5, 4 M guanidinium thiocyanate, and 1% β-mercaptoethanol with a Polytron at full speed for 30 s bursts. Sodium lauryl sarcosinate was added to the homogenate to give a final concentration of 0.5%, and the suspension was layered onto CsCl (5.7 M CsCl, 0.01 M EDTA, pH 7.5) in an ultracentrifuge tube. Samples were centrifuged at 175,000 × g for 18 h. The resulting RNA pellet was resuspended in 10 mM Tris, 1 mM EDTA, pH 7.5, and 0.1% SDS; diluted with an equal volume of 1× SSC (1× SSC is 0.15 M NaCl, 0.01 M sodium citrate, pH 7.0) in 0.5% formamide, 6× SSPE (1× SSPE is 150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4), 5× Denhardt's solution, 0.5% SDS, and 0.25 mg/ml salmon sperm DNA at 42°C for 10 min. The full-length bovine brain A1AR cDNA was labeled with [32P]ATP using the Amersham multiprime DNA labeling system and diluted in hybridization buffer (identical procedures (21)). Poly(A)⁺ RNA was eluted with 0.5 ml of water and ethanol-precipitated and electrophoresed on a 1% agarose and 6.6% formaldehyde/2.2 M urea gel (3-5 μg of RNA per lane). RNA was transferred from the gel to a Zeta-probe membrane (Bio-Rad) with 10 × SSC (1× SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0) over 12 h. The RNA was fixed to the membrane by UV cross-linking and incubated in a prehybridization solution of 50% formamide, 6× SSPE, and 50% deionized mercaptoethanol at 50°C overnight. The gel was placed in a 50°C water bath overnight to allow for cross-linking. The brain A1AR cDNA was labeled with [32P]ATP using the Amersham multiprime DNA labeling system and diluted in hybridization buffer (identical to prehybridization except for the omission of 5× Denhardt's solution and increase of salmon sperm DNA to 0.5 mg/ml) to a final activity of 9.5 × 10⁶ cpm/ml. Hybridization was conducted at 42°C for 24 h, after which the membrane was washed twice in 2× SSC, 0.5% SDS at 22°C for 15 min. This was followed by three sequential washes in the 0.1× SSC, 0.1% SDS for 15 min at 55, 60, and 66°C.

RESULTS

Screening of a bovine brain cDNA library resulted in the cloning of an A1AR with a unique pharmacological profile compared to previously cloned A1ARs (12-14). The clone contained an insert of ~2.0 kb. The nucleotide sequence of this clone along with the deduced amino acid sequence of the
longest open reading frame is shown in Fig. 1. As shown in
Fig. 2, the encoded protein of 326 amino acids (36,570 daltons)
possesses many of the structural characteristics of the G
protein-coupled receptor family (26–28) and is highly homol-
ogous to the recently cloned A1ARs of canine thyroid gland
(12) and rat brain (13, 14). Putative transmembrane segments
1, 2, and 6 of the bovine brain, rat brain, and canine thyroid
A1ARs are 100% identical, while the bovine A1AR differs by
a total of 7 (rat) and 8 (canine) amino acids in transmembrane
domains 3, 4, 5, and 7. The majority of the differences in the
translated proteins exist in extracellular loops 2 and 3 and the
carboxyl terminus tail. Unlike the canine A1AR, the bovine
A1AR contains two potential sites for glycosylation, both of
which exist in the second extracellular loop. In com-
parison to many inhibitory G protein receptors, the third
intracellular loop of the bovine A1AR is extremely short,
composed of only 34 amino acids. This cytoplasmic loop
contains potential phosphorylation sites for protein kinase C

and protein kinase A. A single consensus sequence for protein
kinase A phosphorylation is also present in the second intra-
cellular loop. These sites may represent important regions for
A1AR regulation as a recent study has demonstrated the
in vivo phosphorylation of the A1AR of DDT1 MF2 smooth
muscle cells in association with agonist-induced desensitiza-
tion (29). The cytoplasmic carboxyl terminus of the bovine
brain A1AR is also relatively short which is a feature shared
by several other receptors that are linked to the inhibition of
adenylylcyclase via the Gi protein (28). This region of the
receptor also contains a cysteine at position 309 which rep-
resents a possible site for palmitoylation (30).

To validate that the isolated cDNA did indeed code for the
bovine brain A1AR, nearly the entire 2.0-kb insert was
subcloned into the pCMV5 expression vector and the plasmid
was transiently expressed in COS7 cells. As shown in Fig. 3A,
membranes prepared from transfected COS7 cells displayed
high affinity binding for the A1AR selective antagonist 3H
XAC (Kd = 0.37 ± 0.05 nM) with a Bmax of 2.56 ± 0.53 pmol/
mg (n = 6). This high affinity for 3H XAC is consistent with
that described for the bovine A1AR (10, 11). Likewise, binding
of the A1AR agonist, 32P]APNEA, was saturable and of high
affinity with a Kd of 0.57 ± 0.16 nM and a Bmax of 1.00 ± 0.28
pmol/mg (n = 5). A representative saturation curve is shown
in Fig. 3B. Neither ligand bound to membranes prepared from
nontransfected COS7 cells.

To document that the expressed receptor has the unique
pharmacological profile expected, the ability of A1AR agonists
to compete for 32P]APNEA binding sites was determined

![Fig. 2.](image-url) Comparison of the deduced amino acid sequences of bovine, dog, and rat A1AR. Dashes represent identical amino acids with differences noted in canine and rat sequences. Putative transmembrane domains (I-VII) are marked with a solid line. Italicized amino acids represent changes in the A1ARs in these transmembrane regions. Possible sites for glycosylation in extracellular loop 2 are marked by asterisks. His-251 and His-278 are marked by °.

![Fig. 3.](image-url) Representative curves depicting [3H]XAC (A) and [32P]APNEA (B) saturation binding assays with membranes prepared from COS7 cells transiently transfected with bovine A1AR cDNA. For both curves, nonspecific binding was defined with 10 mM theophylline. Assays were performed as described under "Experimental Procedures." Each curve was replicated at least five times.
In five experiments, the IC₅₀ values for R-PIA, S-PIA, and NECA were 0.37 ± 0.06, 5.05 ± 0.80, and 30.0 ± 7.3 nM, respectively. This potency order of R-PIA > S-PIA > NECA is identical to that observed for the membrane, soluble, and purified forms of bovine cortex A₁AR (10, 11). The A₁AR is known to inhibit the enzyme adenylylcyclase. To document that the expressed A₁AR was capable of functioning in this capacity, both cAMP accumulation and adenylylcyclase experiments in stably transfected CHO cells were performed. In these cells, the stably expressed receptor demonstrated high affinity [³H]XAC and [¹²⁵I]APNEA binding as described above. Fig. 5 demonstrates a dose-dependent inhibition of adenylylcyclase by R-PIA. The ~30% attenuation is similar to that observed in previous studies of A₁AR-mediated adenylylcyclase inhibition (4, 25). Similar results were obtained using cAMP accumulation as a determinant of A₁AR functional activity (data not shown).

The distribution of the A₁AR mRNA in bovine tissues by Northern analysis is presented in Fig. 6 and is in agreement with the findings from previous ligand binding and functional studies (31). Two transcripts of 3.4 and 5.7 kb are abundant in brain with a significant amount also in heart. In the kidney, a 3.4-kb transcript was detected. The thyroid contained only a 5.5-kb message. No transcript was detected in bovine spleen, lung, or liver. DDT and MF-2 cells, a cell line known to contain A₁AR, also contained two transcripts of 3.4 and 5.6 kb.

As mentioned in the Introduction, biochemical studies have suggested that histidine residues may be important for adenosine binding (17). Indicated in Fig. 2 are 2 histidines within the putative transmembrane domains (one each in transmembrane domains 6 and 7). In order to assess if these amino acids are important for agonist or antagonist binding, site-directed mutagenesis was utilized to individually change the histidines to leucines as described under “Experimental Procedures.” The mutagenized receptors were then expressed in COS7 cells, radioligand binding was performed, and the results were compared to the wild-type receptor. Antagonist ([³H]XAC) and agonist ([¹²⁵I]APNEA) binding to the His-278 → Leu-278 (seventh domain) mutant were both dramatically decreased compared to wild-type receptor (specific binding <10%). This mutant was, therefore, not evaluated further. Much different results were observed for the His-251 → Leu-251 mutant. In [³H]XAC saturation assays (Fig. 7), a 74% decrease in antigen binding (B₅₀) was observed and the Kᵣ changed increased from 0.17 ± 0.02 to 0.65 ± 0.15 nM in mutant as compared to wild-type A₁AR (n = 4). Agonist binding as assessed by [¹²⁵I]APNEA saturation assays (Fig. 8) decreased by 74%, while the Kᵣ was not affected (1.68 ± 0.4 and 1.23 ± 0.14 nM in wild-type and His-251 → Leu-251, respectively, n = 3). Full R-PIA competition curves versus [³H]XAC were also constructed to completely examine the effect of the histidine substitution on agonist binding in that [¹²⁵I]APNEA saturation assays may only provide information regarding high affinity binding. In these experiments, the IC₅₀ for R-PIA was 56.6 ± 17 nM (n = 4) and 50.7 ± 14.3 nM (n = 6) for wild-type and His-251 → Leu-251, respectively. It should be pointed out that R-PIA affinity for the A₁AR when

**Fig. 4.** Agonist competition versus [¹²⁵I]APNEA (~0.3 nM) in membranes prepared from COS7 cells transiently transfected with bovine brain A₁AR cDNA. Competing ligand was added at the concentration shown on the abscissa. The amount of [¹²⁵I]APNEA bound is on the ordinate. Assays are described under “Experimental Procedures.” This experiment is representative of four similar assays.

**Fig. 5.** Representative experiment demonstrating R-PIA-mediated inhibition of forskolin-stimulated adenylylcyclase activity in membranes prepared from CHO cells stably expressing bovine brain A₁AR cDNA. Assays were performed as described under “Experimental Procedures” using ~100 µg of protein per assay tube. Forskolin was present at 50 µM where indicated and the concentration of R-PIA was as shown. This experiment was performed four times with similar results.

**Fig. 6.** Northern blot analysis of tissue distribution of A₁AR mRNA. Poly(A)⁺ RNA (3–5 µg) was run on agarose/formaldehyde gels, transferred to nylon membranes, and probed with [³²P]-labeled full-length bovine A₁AR cDNA. Washing conditions were described under “Experimental Procedures.” Tissues analyzed were bovine brain (B), heart (H), thyroid (T), spleen (S), kidney (K), lung (Lg), and liver (Lv). A is a photograph of film exposed to the blots for 12 h. B is a selected panel from the same blot exposed to the film for 48 h to allow visualization of transcripts present in heart, thyroid, and kidney. For comparison, an analysis is shown of transcript levels in DDT and MF-2 smooth muscle cells (D). Position of the 18 and 28S ribosomal RNA is shown to the left of A.
cells, the cDNA codes for an adenosine receptor that displays a pharmacologic potency series which is identical to that reported for the bovine brain AR in both its membrane (11) and purified (10) forms. This potency series of R-PIA > S-PIA > NECA is unique for bovine cortex as it differs from that reported for ARs obtained from tissues of several other species in which NECA possesses a significantly greater affinity than S-PIA. The expressed receptor has all the pharmacological characteristics expected including high affinity agonist and antagonist binding and selective and stereospecific binding. In addition, the expressed receptor functionally couples to its G protein and can inhibit adenylcyclase activity.

The bovine brain AR has also been considered unique based on the binding of the radiolabeled antagonist, [H]XAC. The affinity of this compound for bovine brain AR is several-fold greater than that reported for several other ARs including those present in rat fat (8) and brain (9) and OGGT, MP-2 smooth muscle cells (25). COST7 cells transfected with the bovine brain AR cDNA bind [H]XAC with a Kd of ~0.4 nM in agreement with that displayed for the native receptor. This would indicate that the greater affinity of this receptor for the antagonist is due to distinct intrinsic properties of the bovine AR protein and not merely differences provided by the milieu of the membrane in its native tissue.

Recently, the cloning of ARs from canine thyroid (12) and rat brain (13, 14) cDNA libraries has been reported. Mahan et al. (13) observed the typical potency order of R-PIA > S-PIA > NECA for displacement of [H]PCPX from binding sites in A9-L cells transiently expressing the rat brain AR cDNA. Reppert and coworkers (14) reported this same potency order following the expression of a rat brain AR cDNA in COS-6 M cells. This agonist series was not used in the characterization of the canine AR clone, RDC7, expressed in COS7 cells (12). However, this study reported a Kd value of 4.55 nM for NECA in competition assays with [H]CHA. This value is similar to that reported for the high affinity agonist state of NECA (Kd = 4.3 nM) for the rat brain AR (13) and several fold lower than that found in the present study for bovine brain AR.

This tissue distribution of AR mRNA in bovine tissues as determined by Northern analysis corresponds with the results of radioligand binding and functional studies. The AR is abundant in brain while heart, thyroid and kidney also contain appreciable amounts of message. Northern analysis in rat (13, 14) and canine (12) tissues also demonstrates AR mRNA in brain and heart. However, unlike the bovine tissue, neither canine nor rat kidney contained detectable AR mRNA. Also, rat spleen appeared to possess AR transcripts while no message is present in bovine spleen.

Based on the unique ligand binding properties of the bovine AR and apparent differences in tissue distribution of the mRNA for this receptor, we propose that the bovine AR is a distinct subclass of this receptor. The existence of distinct subtypes of ARs is in keeping with what has been found in other G protein-coupled receptors. The presence of multiple-receptor subtypes has recently been appreciated in almost all receptor families. Cloning techniques have allowed for the recognition of multiple subtypes of ARs which is in keeping with what has been found in other G protein-coupled receptors. The presence of multiple-receptor subtypes is not unexpected. Such receptor subtypes are often demonstrated to be distinct either pharmacologically, functionally, or on the basis of tissue distribution.

The differences in pharmacology described above exist despite the remarkable amino acid homology between the bovine brain AR and those recently cloned. The encoded proteins are nearly identical in molecular mass with 326 amino acids constituting all of the cloned ARs. The three ARs now cloned are 92% identical overall and in the putative trans-membranes prepared from COS7 cells transiently expressing wild-type and mutagenized (His-251 → Leu-251) AR cDNA. Nonspecific binding was defined with 10 mM theophylline. The best-fit lines for nonspecific binding for both groups were superimposable. Approximately equivalent amounts (~3.5 pg) of protein were used per assay tube for both groups. Other experimental details are described under “Experimental Procedures.” This experiment was repeated three times.

**DISCUSSION**

The bovine brain AR has long been recognized as a distinct form of the AR in that it binds agonists with a different potency series and has an ~10-fold higher affinity for both agonists and antagonists compared to rat or other ARs (8-11). In this paper we describe the cloning and characterization of the cDNA for the bovine brain AR and through site-directed mutagenesis studies have begun to probe the binding pocket of the receptor.

Radioligand binding studies demonstrate that in COS7 cells, the cDNA codes for an adenosine receptor that displays a pharmacologic potency series which is identical to that...
membrane regions the bovine A1AR differs from the rat and canine receptors by 7 and 8 amino acid substitutions, respectively. In comparison, only 3 amino acid differences exist in the transmembrane regions between the rat and canine A1ARs. It is generally believed that the composition of the transmembrane domains of the G protein-coupled receptors determine their ligand binding characteristics and, therefore, confer the specificity of receptor subtype activation (26, 27). It is possible that very few or perhaps only a single amino acid difference in these regions is (are) responsible for the differences in bovine brain A1AR pharmacology relative to other A1ARs. Suryanarayana et al. (44) recently demonstrated that a single point mutation (Phe to Asn) in the seventh transmembrane domain of the α1-adrenergic receptor dramatically reduced the receptor’s affinity for selective agonists while increasing its affinity for a β-adrenergic receptor antagonist 3000-fold.

Site-directed mutagenesis provides a powerful technique for assessing the importance and role of individual or groups of amino acids in the functioning of proteins. Klotz et al. (17) have previously documented that diethylpyrocarbonate, a histidine-specific agent, was capable of specifically decreasing both agonist and antagonist binding to the A1AR of rat brain membranes. Diethylpyrocarbonate treatment decreased the number but did not alter the affinity of the receptors for antagonists. This suggests a modification of the ligand binding site itself. The fact that there was a selective protective effect of agonist binding by antagonists but not agonists and similarly a protective effect of agonist binding by agonists but not antagonists suggested that there were at least 2 histidine residues near the binding pocket.

In this study we have now been able to directly study the role of the 2 histidine residues in transmembrane domains 6 and 7. Our data demonstrate that the His-278 (seventh domain) appears to be extremely important for the binding of both agonists and antagonists. Substituting leucine for this histidine leads to the almost but not quite complete loss of agonist and antagonist binding. In contrast, His-251 is important for both agonist and antagonist binding in that agonist and antagonist bindings are both decreased by substitution of Leu for His, but not to the same extent as that of His-278 → Leu-278. At the present time, no antibodies to the A1AR are available for immunodetection of the receptor protein. Therefore, agonist and antagonist radioligand binding studies are the most suitable methods available to quantitate membrane A1ARs. It is possible, therefore, that the reduction in receptor number as assessed by [125I]APNEA and [3H]XAC binding may indicate a decline in the actual amount of A1AR protein rather than the loss of a specific ligand-receptor interaction. Such a decline could result if the process by which the mutant A1AR cDNA is transcribed, processed, and translated in the COS-7 cells is different from that for the wild-type cDNA. In addition, the mutated protein may not undergo the proper folding necessary for transport to and insertion in the membrane in the appropriate orientation. This latter possibility would be difficult to detect, for even immunologic techniques would not likely be able to distinguish between A1ARs differing only in their three-dimensional structure. To minimize the occurrence of the possibilities described above, leucine, which is an aliphatic and relatively nonbulky amino acid that is prevalent in protein transmembrane domains, was selected to substitute for the histidine. However, even with all these caveats we believe that the mutant studies provide new insights into A1AR structure and function.

In addition to the effects on A1AR Bmax, the His-251 → Leu-251 substitution has differential effects on agonist and antagonist binding affinity. As judged from Ki values for [3H]XAC, the mutated A1AR demonstrates approximately a 4-fold decline in antagonist affinity. However, both [125I]AP-NEA and R-P1A versus [3H]XAC competition curves indicate that the His-251 → Leu-251 substitution produces no effect on agonist affinity. The ability of His-251 → Leu-251 to bind agonists with high affinity demonstrates that the functional properties of the ligand binding pocket remain intact, and, furthermore, the mutated receptor does couple to a G protein, presumably Gi. The latter finding is reasonable based on previous analysis of structure and function of G protein-coupled receptors which indicates that transmembrane domain regions are involved in ligand binding, whereas intracellular regions are critical for G protein activation (25, 26).

The differential effects of the His-251 → Leu-251 substitution on agonist/antagonist binding are in agreement with previous studies on ligand-receptor interactions of the A1AR. These findings extend those of Klotz et al. (17) by specifically examining the histidine residues involved. Not only has sequence allowed the location of the histidine to be determined but site-directed mutagenesis permits their individual contributions to be studied. The histidine at position 251 appears to be directly involved in antagonist binding since antagonist affinity is significantly altered. Mutation at this site does not alter agonist affinity, only receptor number. Changes in Bmax are much more complicated in this system (A1AR) than changes in KH since it is known that the quantity of receptor detected by both agonist and antagonist radioligands is dependent on receptor-G protein coupling, ion concentration, and the presence or absence of guanine nucleotides (45). Interestingly, Barrington et al. (46), using proteolytic digestions of agonist and antagonist occupied A1AR in native bovine brain membranes, demonstrated that although these ligands may share the same binding pocket they induce unique conformational changes of the A1AR. The conformational changes induced will of necessity determine not only the apparent affinity for a given ligand but also its coupling to the G protein and hence the number of receptors detected.

In summary, this paper provides evidence for the existence of a subtype of the A1 adenosine receptor which is in agreement with previous data obtained with the native bovine receptor. Apparently, minor variations in amino acid sequence may account for the differences in pharmacologic properties of the bovine A1AR as compared to the canine and rat A1ARs previously described. Furthermore, this study significantly extends previous findings regarding A1AR-ligand binding interactions. His-278 in transmembrane domain 7 is apparently crucial for ligand recognition. His-251 in transmembrane domain 6, though involved in part in agonist binding, plays a much more important role in A1AR-antagonist interaction. Future studies probing other domains of the A1AR should provide a more detailed description of its ligand binding pocket.

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