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Cloning and expression of a bovine adenosine A_1 receptor cDNA

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A bovine brain adenosine A_1 receptor cDNA encoding a 326 amino acid protein has been identified. This cDNA, which encodes a protein >90% identical to analogous rat and dog receptors, was transiently expressed in COS-1 cells. Recombinant receptors exhibited the features of bovine A_1 receptors that distinguish it from rat and canine receptors, including subnanomolar K_i for 1,3-dipropyl-8-cyclopentylxanthine, *R*-phenylisopropyladenosine (*R*-PIA) and xanthine amino conjugate, and the distinct potency order: *R*-PIA > *S*-PIA >> 5'-*N*-ethylcarboxamidoadenosine > 2'-chloroadenosine. The results indicate that the pharmacological differences between A_1 adenosine receptors among species result from only minor differences in receptor structures.

Purinergic; G protein-coupled receptor; RDC7

1. INTRODUCTION

Adenosine modulates a number of physiologic functions and has important effects on the cardiovascular [1] and nervous systems [2]. The actions of adenosine appear to be mediated through specific G protein-coupled cell surface receptors [3]. Based on biochemical studies of adenylate cyclase activity and pharmacologic studies involving relative affinities for adenosine analogs, two major subtypes of adenosine receptors have been identified. The adenosine A_1-type receptors typically inhibit adenylate cyclase and show higher affinity for N^6-substituted adenosine analogs than for 5'-substituted analogs. Conversely, adenosine A_2- type receptors stimulate adenylate cyclase and show the reverse potency order [1,3,4]. That there are two subtypes of A_2 receptors (A_{2a} and A_{2b}) seems clear from pharmacological data [5]. The A_{2a} subtype has a distribution limited to the striatum, nucleus accumbens and olfactory tubercle and has been cloned [6]. Whether or not there are subtypes of A_1 receptors is uncertain. Binding to A_1 receptors varies qualitatively from tissue to tissue [7], and also varies markedly among species [3].

Abbreviations: G proteins, guanine nucleotide binding proteins; PIA, *N*-6-phenylisopropyladenosine; $NECA_a$, 5'-*N*-ethylcarboxamidoadenosine; ABA, N^6-aminobenzyladenosine; CPA, N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; CADO, 2-chloroadenosine; BW-A844, 3-aminophenethyl-1-propyl-8-cyclopentylxanthine; BW-A1433, 1,3-dipropyl-8-phenyl(*p*-acrylate)xanthine; CPT, cyclopentyltheophylline; IC_{50}, concentration required to inhibit binding by 50%.

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A_1 adenosine receptor cloning has been reported for two species, the dog and the rat [8–11]. Binding data from recombinant rat receptors expressed in COS cells correlated well with published data on binding to rat brain receptors [10–14]. Dog recombinant receptors expressed in COS cells bind ligands differently from rat receptors (little discrimination between *R*-PIA and NECA [9]) but similar to native receptors found on canine brain membranes. The bovine receptor pharmacology, which is very distinct from rat, canine and human [12] receptors, is maintained when purified bovine receptors are reconstituted into human platelet membranes [15]. These data suggest that structural differences in A_1 adenosine receptors among species are responsible for the differences seen in structure–activity profiles. We describe the cloning of an A_1 adenosine receptor from bovine brain. A detailed comparison of the pharmacology of recombinant receptors with that seen in native brain membranes reveals a strong similarity between native and recombinant receptors, indicating that although there is considerable similarity in receptor structure among species, the unusual bovine pharmacology can be attributed to the structure of the bovine receptor and not to the composition of membrane lipids or G proteins.

2. MATERIALS AND METHODS

Drugs were obtained from the Sigma Chemical Co. with the following exceptions: BW-A844, BW-A1433 and DPCPX were gifts of Dr. Susan Daluge of the Burroughs Wellcome Co. NECA, CPA, XAC and CPT were from Research Biochemicals Inc. CHA was from Calbiochem.

2.1. *cDNA library screening and DNA sequencing*

Two oligonucleotides (5'TGTCCTCATCCTCACCCAGAG-

CTCCAT and 5'GGTAGCGGTCCACGGCAATCGCCAG-CAGGGCCAGGATGGAGCTC) were designed. These encode a portion of the putative third transmembrane region of the dog adenosine A_1 receptor (RDC7) sequence [9]. The 3' terminal nonamers of the oligonucleotides are complementary; a radiolabeled probe was made by the action of the Klenow fragment of DNA polymerase I on the annealed oligonucleotide templates in the presence of [^{32}P]dCTP and three unlabeled deoxynucleoside triphosphates. The resulting residue DNA fragment, which had an average specific activity of 4×10^6 dpm/pmol, was denatured and used to screen duplicate nitrocellulose filters lifted from a bovine brain cDNA library (a gift from R.A.F. Dixon) in the vector λZAP. One million recombinants were screened. Filters were hybridized at 42°C in 5× SSPE [16], 5× Denhardt's solution [16], 0.2% sodium dodecyl sulfate, 0.05% sodium pyrophosphate, and 0.1 mg/ml sheared herring sperm for 36 hours. Filters were washed in a final wash buffer of 0.2% SSPE and 0.05% sodium pyrophosphate for 1 h at 42°C. Phage generating positive signals were plaque purified, followed by in vivo excision of the nested Bluescript plasmids using helper phage according to the Stratagene protocol. DNA sequence was determined along both strands using the Sanger dideoxynucleotide chain termination method [17]. Oligonucleotide primers used were either vector-specific or designed from conserved regions of known sequences from the canine and rat adenosine A_1 receptors. Sequencing primers were synthesized by Dr. John Zysk of the American Cyanamid Co. Sequence analysis was done using the GCG software.

2.2. *Expression studies*

The bovine adenosine A_1 receptor cDNA was subcloned into the expression plasmid CLDN10B (a gift from Dr. M. Reff, SK&F Laboratories). This plasmid, which contains the immediate early CMV promoter and an SV40 *ori* was used to transfect COS-1 cells using the DEAE-dextran method of Cullen [18]. 5 μg of CsCl-purified plasmid DNA were used for each 60 mm tissue culture plate containing approximately 2×10^6 cells. 72 h after transfection cell monolayers were scraped into 2.5 ml of Buffer A (1 mM EDTA, 0.1 mM benzamidine, 10 mM HEPES, pH 7.4) per plate. In parallel with frozen bovine brain (Pel-Freeze), the cells were homogenized using a Brinkmann polytron, setting 5, for 10 s. The homogenates were centrifuged at 20,000 × g for 30 min and resuspended in HE buffer (10 mM HEPES, 10 mM EDTA, 0.1 mM benzamidine and 0.1 mM phenylmethylsulfonylfluoride). The membranes were washed twice by centrifugation. The resulting pellets were resuspended in HE at a final protein concentration of 1 mg/ml. Membranes were aliquotted and frozen until use, at which time they were diluted to concentrations reported for each assay. Proteins were determined from fluorescamine fluorescence using bovine serum albumin standards.

Binding assays were performed using an antagonist, [^{125}I]BW-A844 [19], and an agonist, [^{125}I]ABA [20]. For binding assays membranes were thawed and 5 U/ml of adenosine deaminase (Sigma) was added. Membranes (50 μl) were added to 50 μl of HE buffer containing 4.9 mM $MgCl_2$, 0.01% CHAPS, and radioligand with or without the indicated concentrations of unlabeled competitors. Non-specific binding was measured in the presence of 10 μM *R*-PIA. Binding reactions were incubated at 25°C for 120 min and terminated by rapid filtration through Whatman glass fiber (GF/C) filters using a Brandel Cell Harvester. Filters were washed 3-times with 3 ml of ice-cold buffer consisting of 10 mM Tris-Cl, 1 mM $MgCl_2$, pH 7.4.

2.3. *Data analysis*

Saturation data were analyzed using a single-site binding model. IC_{50} values were derived from competition curves and K_i values calculated as described previously [21].

3. RESULTS AND DISCUSSION

An oligonucleotide probe encoding the putative third transmembrane region of the canine A_1 adenosine receptor was used to screen 10^6 recombinants from a bovine brain cDNA library. Ten positive signals were plaque-purified, and a portion of the DNA sequences was determined. This analysis showed that eight of the clones were derived from the same mRNA population; some of these cDNAs were replicates (i.e. sibs). One 2.8 kbp cDNA clone (pBOV13, Genbank accession no. X63592), which contained a complete extended translational reading frame encoding a protein of 326 amino acids, was chosen for further analysis. Fig. 1 shows the conceptualized amino acid sequence of pBOV13 compared with the analogous dog and rat sequences [9–11]. As is the case with numerous members of the seven transmembrane helix superfamily, the amino acid sequences of the different species are very similar (>90% identity). These receptors share features common to other G protein-linked receptors including 1 or 2 glycosylation consensus sites (although these occur in the 2nd exofacial loop rather than near the amino terminus) and several amino acids that are highly conserved in the seven transmembrane helix superfamily [22]. Notably absent from all three species are clusters of serines and threonines near the carboxyl terminus that are present in several G protein-linked receptors and are thought to be phosphorylated during receptor down-regulation. Interestingly, the divergence among the three sequences occurs in three small clusters: amino acids 151–156 (second exofacial loop), 261–270 (third exofacial loop), and at the carboxyl terminus.

To determine the binding characteristics of bovine

```
      1                                                  50
cow   MPPSISAFQA AYIGIEVLIA LVSVPGNVLV IWAVKVNQAL RDATFCFIVS
rat   ...Y...... .......... .......... .......... ..........
dog   ...A...... .......... .......... .......... ..........

      51                                                 100
cow   LAVADVAVGA LVIPLAILIN IGPRTYFHTC LKVACPVLIL TQSSILALLA
rat   .......... .......... ...Q...... .......... ..........
dog   .......... .......... .......... .M........ ..........

      101                                                150
cow   MAVDRYLRVK IPLRYKTVVT PRRAVVAITG CWILSFVVGL TPMFGWNNLS
rat   I......... .......... Q...A...A. .....L.... ..........
dog   I......... .......... ....A...A. .......... ..L....R.G

      151                                                200
cow   AVERDWLANG SVGEPVIECQ FEKVISMEYM VYFNFFVWVL PPLLLMVLIY
rat   V..Q..R... .......K.E .......... .......... ..........
dog   EAQ.A.A... .G.....K.E .......... .......... ..........

      201                                                250
cow   MEVFYLIRKQ LSKKVSASSG DPQKYYGKEL KIAKSLALIL FLFALSWLPL
rat   L......... .N........ .......... .......... ..........
dog   L.......R. .G........ .......... .......... ..........

      251                                                300
cow   HILNCITLFC PSCHMPRILI YIAIFLSHGN SAMNPIVYAF RIQKFRVTFL
rat   .......... .T.Q...... .......... .......... ...H......
dog   .......... ...RK.S..M ......T... .......... ..........

      301                  326
cow   KIWNDIIFRCQ PAPPIDEDAP AERPDD
rat   .......... .K......L. E.KAE.
dog   .......... .T..V...P. E.A.H.
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Fig. 1. Comparison of pBOV13 amino acid sequence to that of rat and dog adenosine A_1 receptors. The bovine sequence is shown on top with rat and dog below. Dots (.) indicate amino acid identity. Letters are used to indicate changes betweem the rat or dog from and the bovine sequence.

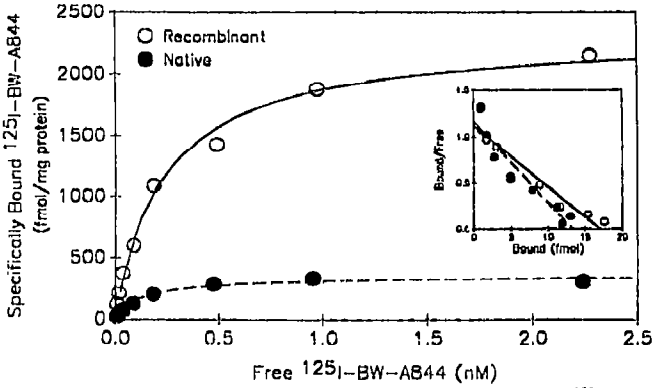

Fig. 2. Saturation isotherm and Scatchard plot (inset) of [^{125}I]BW-A844 binding to membranes prepared from transfected COS-1 cells (○, 0.008 mg protein/tube) and to membranes prepared from native bovine brain (●, 0.39 mg protein/tube). Only specific binding is shown. Non-specific binding was measured in the presence of 10 μM *R*-PIA and represented less than 15% of total binding at the radioligand K_D. Each point is the mean of triplicate determinations. Bound units for the Scatchard plots are expressed as fmol per assay tube.

adenosine receptors, the cDNA was expressed in COS cells and binding compared with native brain receptors assayed in parallel. Untransfected cells did not show specific adenosine analog binding (data not shown). Fig. 2 shows high affinity, specific, and saturable binding of [^{125}I]BWA844 [19]. The maximum binding capacity (B_{max}) of the transfected COS-1 cell membranes was 2.31 pmol/mg protein compared to 0.35 pmol/mg protein for bovine brain membranes. K_D values were 0.24 nM and 0.13 nM for transfected and native membrane systems, respectively. To estimate the relative fraction of coupling of the adenosine A_1 receptors to G proteins in the two systems, saturation binding was also performed using [^{125}I]ABA [20] over a concentration range chosen to selectively label only high affinity coupled receptors (Fig. 3). The B_{max} values for recombinant and native membranes was 0.91 pmol/mg protein and 0.18 pmol/mg protein, respectively; K_D values were 0.94 nM and 0.60 nM. Comparison of the antagonist to the agonist B_{max} suggested a coupling fraction of 39%

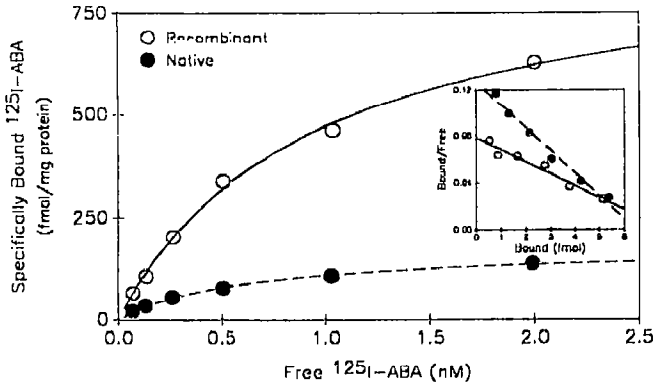

Fig. 3. Saturation isotherm and Scatchard plot (inset) of [^{125}I]ABA binding. The protocol is analogous to that described in Fig. 2. Non-specific binding is less than 5% of total binding at the radioligand K_D.

Table I

Competition by various drugs for binding of [^{125}I]ABA to recombinant receptors expressed in COS cells and to receptors in native bovine brain membranes*

	K_i (nM)	
	Recombinant	*Native*
Antagonists		
XAC	0.93	0.25
DPCPX	1.74	0.5
BW-A1433	3.02	1.35
8-CPT	4.57	3.39
BW-A844	6.46	2.14
Theophylline	26,000	6,460
Agonists		
R-PIA	0.26	0.50
CHA	0.69	2.04
CPA	1.17	1.86
S-PIA	1.58	3.39
NECA	6.46	14.5
CADO	56.2	89.1

*IC_{50} determinations were derived from competition curves consisting of eight concentrations of competing drugs assayed in triplicate and were fit to the equation: $B=B_{max}-(B_{max})-NS)\cdot D^n/(IC_{50}^n + D^n)$, where B = bound radioligand, NS = non specifically bound radioligand, D = the concentration of drug, and n = slope factor. K_i values were determined from IC_{50} values with correction for binding of radioligand and competing drugs to receptors. For each competing drug, recombinant and native receptors were assayed in the same experiment. Each assay tube (0.1 ml) contained 17.4 fmol of [^{125}I]ABA and 5 μg of COS membranes or 24.5 μg of bovine brain membranes.

in the transfected COS cell membranes vs. 50% in the brain membranes. In additional experiments (not shown) competition by the agonist *R*-PIA for [^{125}I]BW-A844 binding was biphasic and monophasic in the absence and presence of 30 μM GTPγS, respectively, which is also indicative of partial coupling of both native and recombinant receptors to G proteins.

To characterize further the pBOV13 encoded receptor, binding competition curves were performed using six adenosine A_1 agonist (Fig. 4) and six antagonist (Fig. 5) ligands. The rank order of potency of the drugs used was similar for native and recombinant receptors. For agonists, the affinity order of *R*-PIA ≥ *S*-PIA > NECA agrees with published data for A_1 adenosine receptors purified from bovine brain using an XAC affinity column [23]. In contrast, the 'classic' [14] A_1 subtype binding order, as seen in brain receptors from rat and several other species, is *R*-PIA ≥ NECA > *S*-PIA [12,13]. Of the antagonists tested, XAC exhibited the highest affinity, followed by DPCPX > BWA1433 = CPT > BWA844 >> theophylline (K_i values are listed in Table I). Characteristic of bovine receptors is the very high affinity (<1 nM K_D) of several of these ligands (*R*-PIA, XAC, [^{125}I]BW A844, [^{125}I]ABA and DPCPX).

The sequence of the pBOV13 clone suggests strongly

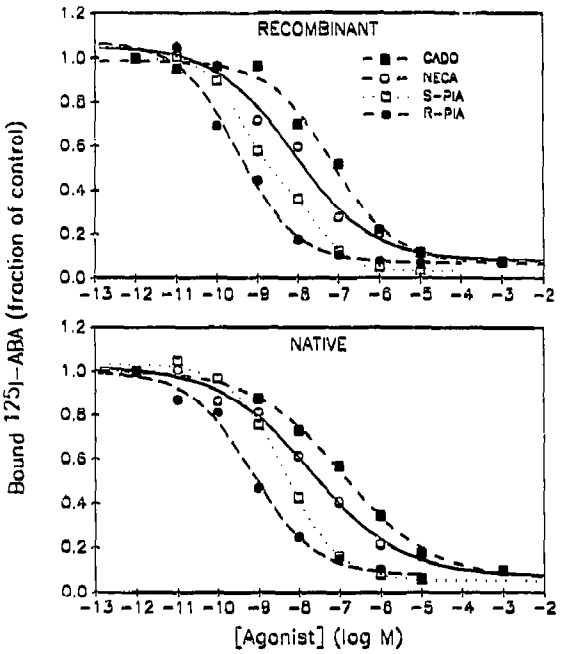

Fig. 4. Competition by agonists for binding to BOV13-transfected COS-1 cell membranes and bovine brain membranes. [^{125}I]ABA (17.4 fmol) and eight concentrations of unlabeled competing drugs were assayed in triplicate. Binding conditions, equations used for curve fitting and derived K_i values are found in Table 1.

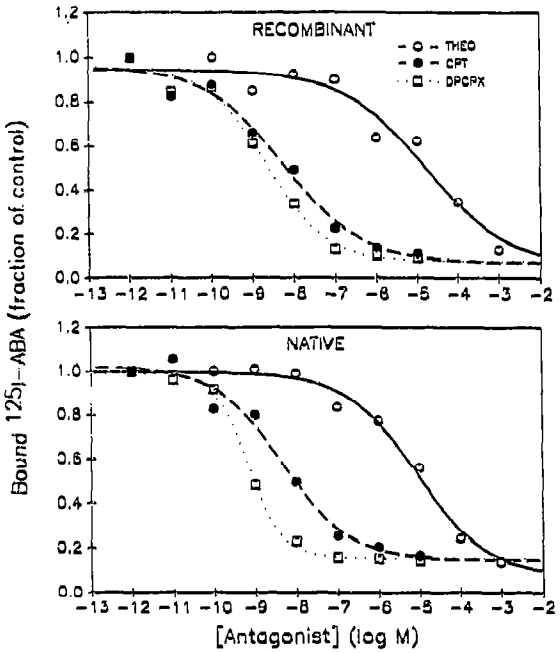

Fig. 5. Competition by antagonists for binding to BOV13-transfected COS-1 cell membranes. Conditions are analogous to Fig. 4.

that it represents the bovine homolog of the cloned rat and dog brain adenosine A_1 receptors. The pBOV13 protein binding profile, as determined with both agonists and antagonists, reflects that seen with the native receptor in bovine brain membranes. It has long been known that there are species-specific binding profiles for adenosine A_1 receptors. The differences seen in the native membranes are mimicked in the recombinant receptors, suggesting structural differences in the receptors are responsible.

A_1 receptors couple to several known effector systems and seem to be able to associate with different G proteins in vivo [24]. The affinities of ligands for A_1 receptors can vary not only from species to species, but also within a given species from tissue to tissue. For example, NECA affinity is approximately 100-fold less in rat adipose tissue than in heart or brain [3], raising the possibility of subtypes. Whether binding differences are secondary to structural differences in receptors or to environmental factors such as coupling can be addressed in part by molecular cloning and subsequent expression of the receptors. The cloning of the rat and dog, and now cow, A_1 receptors allows comparison of binding to recombinant receptors expressed in similar tissue environments. From Fig. 1 it can be seen that the amino acid sequences of the rat, dog and cow receptors are highly conserved over most of their length, with the exception of the second and third exofacial loops and the carboxy terminus. Perhaps these regions are responsible for unique binding profiles between species; alternatively, these regions may be hypervariable because they are not important in ligand recognition, and species differences may be due to the minor differences in amino acid sequences in the transmembrane reqions. The high affinity of the bovine brain clone for N^6-substituted adenosine analogs and for 8-substituted xanthine antagonists, as well as its distinct rank order potency, will make it a useful reagent in the study of structure–activity relationships and ligand binding domains of the A_1 receptor.

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