Biochemical Pharmacology, Vol. 58, pp. 1269–1277, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00214-2

A_1 Adenosine Receptor of Human and Mouse Adipose Tissues

CLONING, EXPRESSION, AND CHARACTERIZATION

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ABSTRACT. The aberrant functioning of the A_1 adenosine receptor of adipose tissue has been implicated as a factor in obesity. To begin to address questions concerning this relationship, the possibility of a unique A_1 adenosine receptor in adipose tissue must be investigated. Therefore, cDNAs encoding the A_1 adenosine receptors of adipose tissues of a mouse and an obese human were isolated, sequenced, and expressed in eukaryotic cells. We found their sequences to be 90% identical and each identical to published sequences of the receptors in brain preparations of the two species. The two cDNAs were transiently expressed in 293T cells, a human kidney cell line. Despite the 90% identity in their sequences, the ligand binding properties of the human and mouse cDNAs expressed in the 293T cell line differed markedly. With respect to amino acid differences in the extracellular loops, four occur in the second extracellular loop, which has been implicated in binding by other studies. The ligand binding characteristics of the recombinant receptors matched those of native receptors from human and mouse adipose tissue. The human A_1 receptor cDNA was also expressed in ob17 preadipocyte cells to investigate reported influences of cellular environment on binding characteristics. We compared ligand binding of the expressed receptor in the two cell lines (ob17 and 293T). We also compared ligand binding of native receptors from mouse brain and adipose tissue preparations. In both studies, cellular environment had no affect on binding characteristics. This conclusive evidence resolves earlier conflicting reports in the literature. BIOCHEM PHARMACOL **58**;8:1269–1277, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. cloning; RT–PCR; A_1 adenosine receptor; [^{3}H]DPCPX; adipose tissue; ligand-binding properties

A large segment of the population suffers from obesity and accompanying disorders such as hypertension and type 2 diabetes. The pathophysiological mechanisms that contribute to the development and maintenance of these disorders still are not defined clearly, although major strides have been made in studies on leptin and its receptor [1, 2]. Answers to these problems lie in the unique biochemistry of fat cells, and there is evidence that the activity of the A_1AR† may be involved [3–6].

Data suggest that in obesity there is abnormal signaling of the A_1 receptor to adenylate cyclase [5–8]. The receptor, its G protein, or both, are functioning abnormally. It has been suggested [6] that a mutation may exist in the sequence of the receptor of obese subjects. Or perhaps, at least in human obesity, there are simply excessive amounts of adenosine in adipose tissues. These are questions that need to be answered to further understand the possible connection of the adenosine receptor to obesity.

Libert and colleagues [9] and Maenhaut *et al.* [10] have reported the cloning and sequencing of an A_1 and an A_2 receptor from a dog thyroid cDNA library by screening with consensus sequences of known G protein seven-transmembrane receptors. Others have since cloned A_1 receptors from bovine [11] and rat brain [12, 13], which share greater than 90% sequence similarity. A_2 receptors of rat brain also have been cloned [14], as well as novel A_3 receptors in human, rat, and sheep brain (72–85% similar), which have about 58% sequence identity with the A_1 and A_2 subtypes [15, 16].

Missing is any report of cloning the A_1 receptor of adipose tissues. A genomic sequence for the human A_1AR was characterized from brain, along with a human brain cDNA sequence [17]. Evidence was presented for six exons in the genomic sequence with an alternative splicing mechanism determining the structure and level of expression of the receptor in various tissues. Their studies indicated that exons 4, 5, and 6 were expressed in adipose tissue, and they supported this with a partial sequence of

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† *Abbreviations*: A_1AR, A_1 adenosine receptor; BMI, body mass index; [^{3}H]DPCPX, tritiated 8-cyclopentyl-1,3-dipropylxanthine; KRB, Krebs–Ringer–bicarbonate buffer; *R*-PIA, N^6-phenylisopropyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; RT-PCR, reverse transcriptase-polymerase chain reaction; and CADO, 2-chloroadenosine.

Received 11 November 1998; accepted 18 February 1999.

the fat receptor, excluding more than half of the coding region in exon 6. Obtaining the complete cDNA of this receptor from fat would indicate if there is a unique fat-specific receptor sequence, as suggested by binding studies [18, 19], and if the receptor structure is different in obese individuals. Expression studies should provide insight into normal adenosine receptor functioning mechanisms in fat tissue and to the relationship between aberrant function and the etiology of obesity.

In this study, we report the cloning, sequencing, expression, and characterization of the A_1 receptors of mouse and human adipose tissues and examine the possible influences of membrane environment on binding characteristics.

MATERIALS AND METHODS

Materials

HUMAN TISSUE. Human omental fat was donated by Dr. Jeanine Albu of St. Luke's–Roosevelt Hospital. The sample was taken from a 40-year-old Hispanic woman of BMI 36.7. The BMI is obtained by dividing weight (kg) by the square of the height (m), and values of 20–25 kg/m^2 are considered normal. The subject had volunteered to have fat samples removed during gastric bypass surgery for a research study.

HUMAN OLIGONUCLEOTIDE PRIMERS. Custom oligonucleotides were made by Gibco BRL Life Technologies. Primers for cDNA synthesis and PCR were synthesized based on the human brain genomic A_1AR sequence [17]. The upstream primer corresponded to bases 366–389 (HA1AR-13) and the downstream primers corresponded to bases 1389–1412 (HA1AR-1036) and bases 1406–1431 (HA1AR-dp).

MOUSE TISSUES. Dr. Frank Costantini of the Department of Genetics and Development, Columbia University, donated abdominal fat tissue from five 5-month-old female B6CBAF1/J mice. This strain is derived from the following cross: C57B6/J × CBA. Mouse brains were obtained from eight 3-week-old female B6CBAF1/J mice, also from Dr. Costantini. All animal studies were conducted according to the Guidelines for Care and Use of Experimental Animals.

MOUSE OLIGONUCLEOTIDE PRIMERS. Custom oligonucleotides were made by Gibco BRL Life Technologies. Primers for PCR amplification were synthesized based on the mouse brain A_1AR cDNA sequence [20]. Two sets of primers were used. The first upstream primer, ATGCCGCCGTACATCTCG (m5), and the first downstream primer, TGAGGAAGATGGCAATGTAGATGA (m4), correspond to bases 1–18 and bases 806–829, respectively. The second pair of primers, ATGTACTGGTGATTTGGGCTGTGA (m3) and AGTCTAGTCCTCAGCTTTCTCCTC (m6), correspond to bases 80–103 and bases 961–984, respectively.

RT–PCR

Total RNA was isolated from 200 mg of human omental fat tissue and 50 mg of murine abdominal fat tissue using Qiagen's RNeasy kit, after first homogenizing tissue with a Virsonic 50 sonicator (Virtis Co.).

Total RNA (1 μg) was heated to 90° for 10 min, and then cooled on ice. Next oligonucleotide (40 pmol), dNTPs (10 mM), dithiothreitol (0.2 M), and 5x PCR reaction buffer (Promega) were added to RNA, mixed gently, and incubated at 42° for 2 min before adding 200 units of Superscript II enzyme (Gibco). The reverse transcription was carried out in a volume of 20 μL at 42° for 50 min, and the reaction was inactivated by heating at 70° for 15 min. Then 2 μL of this reaction mixture was mixed with water, 10x PCR reaction buffer, dNTPs (20 mM), Mg^{2+} (1.5 mM), appropriate oligonucleotides (50 pmol), and 2.5 units of Taq polymerase in a 100-μL final volume.

The polymerase chain reaction was carried out in a Perkin Elmer Cetus DNA thermal cycler. For the human cDNA, it was programmed as follows: 95° 1 min and 70° 2 min for 45 cycles.

The mouse A_1AR was cloned as two overlapping pieces using two pairs of primers. This required two separate PCRs. For each reaction, 1 μL of the RT reaction mixture was mixed with water, 10x PCR reaction buffer, dNTPs (20 mM), Mg^{2+} (2.0 mM), appropriate oligonucleotides (64 pmol for m5/m4 or 46 pmol for m3/m6), and 2.5 units of Taq polymerase in a 100 μL final volume. Fifty microliters of mineral oil was overlaid on the reaction mixture. The PCR was carried out in a Perkin Elmer Cetus DNA thermal cycler. Each primer pair required a different annealing temperature for amplification: 60° for the m5/m4 pair and 63° for the m3/m6 pair. The thermal cycler was programmed as follows: 95° 1 min, 60° or 63° 1 min, and 72° 2 min, for 40 cycles. Taq polymerase was added into the reactions after the temperature had reached 80° to avoid the possibility of non-specific priming. The PCR products were separated in a 1.0% agarose gel for visualization of the desired band.

Reagents

dNTPs, RT reaction buffer, dithiothreitol, and Superscript II enzyme for cDNA preparations were obtained from Gibco BRL. PCR reaction buffer and $MgCl_2$ were obtained from Promega. Taq polymerase was obtained from Fisher Scientific.

PCR Fragment Isolation and Subcloning

For the human clone, the PCR products were separated in a 2.0% agarose gel for visualization of the desired band. Then PCR products were separated in 0.75% Seaplaque low melt agarose (FMC). The identified band was excised and subcloned directly into the pCRII vector (Invitrogen) by TA cloning (Invitrogen).

For the mouse clone, the PCR products were subcloned directly from the PCR reaction mixture into the pCR2.1 vector (Invitrogen) by TA cloning (Invitrogen). Restriction mapping of the two subcloned bands was performed, indicating a similar pattern to the mouse brain sequence. The two gene fragments were connected to obtain a complete cDNA clone. Both plasmids were digested with *Acc*I and *Hin*dIII restriction enzymes (New England Biolabs) to generate 4390- and 502-bp bands. These two bands were electrophoresed on a 0.75% Seaplaque low melt agarose gel, and they were excised and ligated in low melt gel using T4 Ligase (New England Biolabs).

Sequencing

Plasmid DNA was purified, using Qiagen's plasmid midi preparation kit, and was sequenced at the Cancer Center core facility, using an ABI Perkin Elmer automated sequencer.

Transient Expression and Radioligand Binding of the Human and Mouse A_1ARs

The human and mouse fat A_1AR cDNAs were transferred into the eukaryotic expression vector pcDNA3 (Invitrogen). 293T Cells derived from human kidney were transiently transfected using the Calcium Phosphate Precipitate method. Cells were harvested after 42 hr, and crude plasma membrane preparations were made as described previously [21]. Protein concentration was measured by the BCA protein assay kit (Pierce). Radioligand binding assays with [^{3}H]DPCPX were carried out according to the procedure of Dong *et al*. [21].

Stable Expression and Radioligand Binding of the Human A_1AR

ob17 Cells were transfected with the pcDNA3 vector containing the human adipose A_1AR cDNA, using the Lipofectamine reagent (Gibco BRL). ob17 Cells were grown in 10-cm dishes to 50% confluence (3.5×10^6 cells) in normal growth medium. Eight micrograms of plasmid DNA was diluted into Opti-Mem I Reduced Serum Medium (Gibco BRL No. 31985) to a total volume of 0.8 mL. One hundred twenty-eight microliters of Lipofectamine also was diluted into Opti-Mem I Reduced Serum Medium to a total volume of 0.8 mL. The two solutions were combined together, mixed gently, and incubated at room temperature for 15 min to allow DNA–liposome complexes to form. While complexes formed, cells were rinsed with 8 mL of the reduced serum medium. For each transfection, 6.4 mL of reduced serum medium was added to the tube containing the complexes. This was mixed gently and overlaid onto the rinsed cells. The cells were incubated with the complexes for 7 hr, after which the medium was changed to 10 mL of normal growth medium with 10% fetal bovine serum. Forty-eight hours after transfection, the medium was changed to selection medium consisting of normal growth medium with 500, 750, or 1000 μg/mL of Geneticin (Gibco BRL No. 11811). Cells were treated with selection medium for 3 weeks to select for stably transfected cells displaying neomycin resistance. Between 2 and 3 weeks into the selection process, resistant cells began to appear. They were separated out by serial dilution and allowed to grow from single cells. Three such cell lines were established. Stable cells that were not separated were also grown and harvested for binding assays, as described for the 293T cells.

Fat Biopsy and Cell Isolation

Fat biopsy and cell isolation were based on the method of Carey and Sidmore [22] and Etherton and Chung [23] with modifications from Dong *et al*. [21]. Abdominal fat tissue was removed from the mice and washed immediately with 37° saline (0.9% NaCl) to remove excess blood. Then the fat tissue was placed into a plastic beaker with modified KRB: 125 mM NaCl, 5 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgCl_2$, 1 mM KH_2PO_4, 25 mM HEPES, 25 mM $NaHCO_3$, pH 7.4, containing 1 μM adenosine, 5 mM glucose, 0.5 mM ascorbic acid, and 4% fatty acid-free BSA (fraction V, Sigma A-7906).

Fat tissue was rinsed with warm saline, and obvious connective tissue was removed. The sample was minced quickly with scissors in a disposable plastic cup with warm saline. Fat pieces were rinsed over a 250-μm nylon mesh with abundant warm saline to remove blood connective tissue and free fatty acids. The underside of the mesh was blotted quickly on Whatman filter papers, and then 1.0 to 1.5 g of fat tissue was rinsed into a plastic flask with 3.0 to 4.5 mL of warm (37°) KRB and put into a 37° shaking water bath. Preweighed collagenase A (Boehringer-Mannheim Biochemicals) was dissolved in warm KRB and added to each flask to obtain a final concentration proportion of 12 mg collagenase A:4 mL KRB:1 g fat tissue. Each flask was sealed with paraffin, and the fat tissue was digested for 30 min at 37°, while shaking. After dissociation, flask contents were filtered through a 250-μm nylon mesh into a plastic beaker. Then each flask was rinsed twice with warm KRB and poured gently through the 250-μm nylon mesh, while stirring tissue chunks gently to release cells into the plastic beaker. Then fat cells were swirled gently into suspension in the plastic beaker and poured along the side into a 50-mL polypropylene centrifuge tube containing a 4-inch piece of polyethylene tubing. All flasks were handled in the same manner, and were centrifuged quickly in a table-top centrifuge. As soon as the speed reached 400 rpm, the brake was applied. The samples were removed from the centrifuge and placed into a 37° water bath. A 60-mL disposable syringe was attached to the polyethylene tubing of each tube, and cells that rose into the tubing were expelled gently. The supernatant was removed by aspiration and discarded. Cells were resuspended in 30 mL of fresh, warm KRB and transferred gently back and forth four

times between two centrifuge tubes to break apart cell clumps and wash cells. The tubes were centrifuged again. The cells were washed one more time. After the third centrifugation and removal of the supernatant, the fat cells were resuspended in 10 mL of warm KRB. The tube was centrifuged again, and the infranatant was removed as completely as possible. The cells obtained were used immediately for preparation of the crude plasma membrane.

Membrane Protein Preparation

Crude membrane protein was prepared from mouse fat cells as previously described [21]. For mouse brains, the procedure is exactly as described for the cell lines.

Experimental Animals

All animal studies were carried out according to the highest standards, as outlined in the Guidelines for Care and Use of Experimental Animals.

RESULTS

Cloning of the Human A_1AR cDNA

Adipose tissue-specific A_1AR cDNA was prepared from total RNA obtained from human omental adipose tissue of an obese subject and reverse transcribed into first strand cDNA using a gene-specific downstream primer, based on the human brain sequence (HA1AR.dp; see Materials and Methods). Then a cDNA fragment of 1047 bp was amplified using a set of primers corresponding to the human brain sequence (HA1AR-13 and HA1AR-1036), with the downstream primer internal to the one used for reverse transcription. The size of the PCR product was consistent with the predicted size, and the sequence was identical to the human brain receptor [17]. The sequence shared 80% sequence similarity with an A_1AR from chicken adipose [24], 88% sequence similarity with the mouse brain receptor [20], and greater than 90% sequence similarity with A_1 subtypes from dog, cow, and rat [9, 11–13]. Of importance is the fact that the A_1AR in human adipose tissue is not unique to that organ.

Cloning of the Mouse A_1AR cDNA

To isolate the murine version of the A_1AR cDNA from fat, total RNA was prepared from mouse abdominal adipose tissue and reverse transcribed into first strand cDNA using oligo$(dT)_{12–18}$ as a downstream primer. Then two overlapping cDNA fragments of 828 and 904 bp were amplified using two sets of primers (m5 and m4; m3 and m6) corresponding to sequences found in mouse brain. The two PCR products were joined together to form the complete coding region for the mouse fat receptor. The size of the final product was 983 bp, identical in size and sequence to mouse brain A_1 receptor [20]. The cDNA sequence shared 80% sequence similarity with A_1AR of chicken adipose tissue [24] and 88% sequence similarity with our human fat clone sequence at the nucleotide level, as well as greater than 90% sequence similarity with A_1 subtypes from dog, cow, and rat [9, 11–13] at the amino acid level. Of importance is the fact that the A_1AR in mouse fat, like the human receptor (above), is not tissue-specific; it is identical to the receptor in mouse brain [20].

Expression of the Human A_1AR in 293T Cells

The human receptor cDNA was transfected transiently into 293T cells, and ligand binding studies were carried out on membrane preparations. The binding assays were conducted with the A_1AR-selective antagonist [^{3}H]DPCPX. The results are shown in Fig. 1. K_D and B_{max} values for the [^{3}H]DPCPX binding saturation curve are shown in Table 1. The K_D was comparable to the binding of [^{3}H]DPCPX to membranes prepared from human adipose tissue (K_D = 3.3 nM) [8]. Untransfected 293T cells showed negligible binding (data not shown).

Competition studies were carried out using adenosine analogues (Fig. 2). The IC_{50} values for *R*-PIA, NECA, and CADO versus [^{3}H]DPCPX are shown in Table 2. Our results indicated a potency order of *R*-PIA = NECA > CADO, and agree with the order found in binding studies of the brain receptor expressed in COS-7 cells [17].

In previous studies by others on rat brain and adipose tissues, significant differences were seen in ligand potency order [18, 19]. For adipose tissue, *R*-PIA > CADO ≥ NECA (NECA was 100-fold less potent than *R*-PIA), and for brain it was reported that *R*-PIA ≥ NECA > CADO [18, 19]. These differences led to the suggestion that the two receptors might differ in structure and, perhaps, not be of the same subtype. No comparative data exist for human fat and brain receptors, but from our study, we can conclude that if there are differences in ligand binding between the two tissues they are not related to differences in amino acid sequence.

Perhaps the difference lies not in the receptor itself, but in its cellular environment, i.e. tissue-specific differences in plasma membrane components might influence receptor binding characteristics. There is evidence that membrane lipid fluidity can alter membrane protein lateral and rotational movements, thus affecting protein surface exposure [25, 26]. Membranes from brain and adipose tissue should differ in composition, and this may account for their apparent variability in how the A_1AR interacts with various ligands. To test this possibility, a fat cell line (ob17) was also transfected with the human A_1AR and treated in the same manner as the 293T cells to generate binding saturation and competition curves.

Expression of the Human A_1AR in ob17 Cells

K_D and B_{max} values for specific binding of [^{3}H]DPCPX to the human A_1AR stably expressed in ob17 cells are shown in Table 1, and were calculated based on a nonlinear

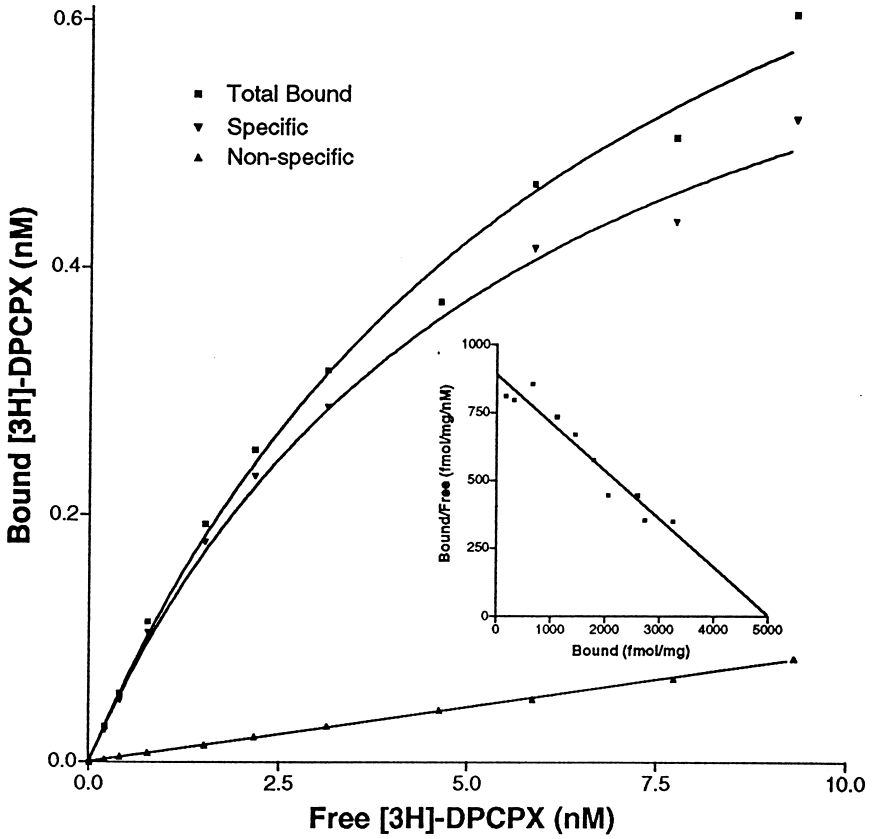

FIG. 1. Saturation curve and Scatchard plot for binding of [^{3}H]DPCPX to 293T cell membranes transfected with the human A_1AR. The data are representative of four experiments. Non-specific binding was carried out with 100 μM unlabeled *R*-PIA. Specific binding data were used for Scatchard analysis.

regression analysis of the saturation curve. The K_D was comparable to the binding of [^{3}H]DPCPX to membranes from human adipose tissue (K_D = 3.3 nM) [8] and was not markedly different from membranes of 293T cells expressing the human receptor (Table 1). As the B_{max} indicates, expression of the receptor was very low in this cell line. A problem encountered with the stably transfected cells was slow growth, resulting in fewer cells and a low yield of membrane protein. Untransfected ob17 cells demonstrated no binding (data not shown).

Competition studies were then carried out with *R*-PIA, NECA, and CADO. The IC_{50} values for *R*-PIA, NECA, and CADO are shown in Table 2. Our results with ob17 cells indicated a potency order of *R*-PIA = NECA > CADO, the same as we found with 293T cells.

TABLE 1. Binding characteristics of human and murine A_1Ars

	K_D (nM)	B_{max} (fmol/mg protein)
Human (293T)	4.60 ± 0.70	4700 ± 300
Human (ob17)	1.43 ± 0.27	67.29 ± 3.50
Mouse (293T)	0.57 ± 0.10	460 ± 20
Mouse brain	0.57 ± 0.10	380 ± 13.9
Mouse fat	1.09 ± 0.12	133 ± 4.2

K_D and B_{max} values were calculated based on nonlinear regression analysis of the specific binding data. Standard error, K_D, and B_{max} were calculated by Graphpad Prism Software. All experiments were conducted twice; human (293T) was conducted four times. All data points were run in duplicate for each experiment. (Graphpad Prism Software is a Product of Graphpad Software, San Diego, CA 92121.)

Expression of the Mouse A_1AR in 293T Cells

The mouse A_1AR cDNA was transfected transiently into 293T cells. Crude plasma membrane preparations from transfected and untransfected cells were assayed for binding to the A_1-selective antagonist [^{3}H]DPCPX.

Figure 3 shows the [^{3}H]DPCPX saturation curve for total, specific, and non-specific binding and the Scatchard analysis for specific binding. K_D and B_{max}, calculated based on a nonlinear regression analysis of the saturation curve, are shown in Table 1. The K_D was comparable to the binding of [^{3}H]DPCPX to membranes from rat adipose tissue (epididymal fat, K_D = 0.19 nM) [27]. Expression of the receptor was good, as indicated by the B_{max}. Untransfected cells demonstrated negligible binding (data not shown).

To assess the agonist potency series of standard adenosine analogues, binding assays were conducted in which *R*-PIA, NECA, or CADO was used to displace [^{3}H]DPCPX binding by expressed mouse fat A_1ARs (Fig. 4). The IC_{50} values for *R*-PIA, NECA, and CADO are shown in Table

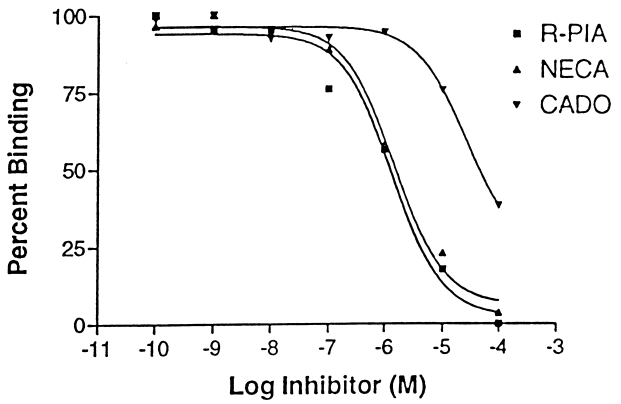

FIG. 2. Competitive inhibition by adenosine agonists of [^{3}H]D-PCPX binding to 293T cell membranes transfected with the human A_1AR. Reactions were carried out with 1 nM [^{3}H]D-PCPX. The data are representative of two experiments. Absolute values (dpm) for inhibitor concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} are as follows: R-PIA, 9102.90, 8609.95, 8593.81, 6944.38, 5253.42, 1953.79, and 461.49; NECA, 9102.90, 10141.22, 8763.09, 8324.00, 5576.09, 2485.35, and 776.81; CADO, 9102.90, 9102.90, 8445.90, 8458.20, 8599.40, 6992.13, and 3764.27.

2. Our results showed a potency order of *R*-PIA > NECA > CADO. This is the potency order found with rat [18] and human [17] brain A_1 receptors.

Binding Studies on Native A_1ARs from Mouse Brain and Fat Tissues

To round out these studies, membranes were prepared from mouse brain and adipose tissues; binding characteristics were compared with the cDNA from transfected cells. K_D and B_{max} values for specific binding of [^{3}H]DPCPX to brain and fat membranes are shown in Table 1. The K_D values for both tissues were comparable to those found in membranes from 293T cells transfected with the mouse fat A_1AR.

Competition assays were conducted in which *R*-PIA, NECA, or CADO was used to displace [^{3}H]DPCPX binding to mouse brain and fat membranes. The IC_{50} values for *R*-PIA, NECA, and CADO in each tissue are shown in Table 2. The potency order of these agonists was *R*-PIA > NECA > CADO for both brain and adipose tissues.

TABLE 2. IC_{50} (μM) values of adenosine agonists binding to human and murine A_1ARs

	IC_{50} (μM)		
	R-PIA	NECA	CADO
Human (293T)	1.30 ± 1.00	1.45 ± 1.00	25.40 ± 1.00
Human (ob17)	2.05 ± 1.65	1.29 ± 1.54	54.28 ± 3.47
Mouse (293T)	0.99 ± 1.31	5.29 ± 1.69	31.73 ± 3.36
Mouse brain	0.26 ± 1.40	1.46 ± 1.25	34.16 ± 1.21
Mouse fat	0.16 ± 1.61	0.97 ± 1.53	27.70 ± 2.18

IC_{50} values and standard error were calculated by Graphpad Prism Software. All experiments were conducted twice. All data points were run in duplicate for each experiment.

Therefore, unlike previous reports (reviewed in Ref. 18), we found no differences in binding patterns of the A_1 receptor in the two tissues.

DISCUSSION

cDNAs of human and mouse adipose tissue A_1ARs have been prepared and sequenced. Their sequences were identical to sequences reported earlier for the receptor in human [17] and mouse [20] brain and in accord with an alternative splicing mechanism, postulated in a paper that appeared while this work was in progress, that predicted expression of the same A_1AR coding region in brain and fat tissues [17]. Our human adipose tissue preparation was obtained from an obese individual, thus providing no evidence for obesity being related to a mutation in the A_1AR [6]. The cloning strategy described here was conducted after other methods failed to isolate any A_1AR cDNA of adipose tissue. These included fat library screenings and RT–PCR using non-degenerate and degenerate probes or primers, under conditions of low stringency. We are confident, therefore, that adipose tissue does not express a unique receptor.

The cDNAs were expressed in 293T cells and found to be functional in that membrane preparations could bind the A_1 receptor antagonist [^{3}H]DPCPX, and binding could be inhibited by the agonists *R*-PIA, CADO, and NECA. However, despite greater than 90% identity in amino acid sequence, their binding properties differed markedly (Tables 1 and 2). Their respective amino acid sequences and mismatches are shown in Fig. 5. The locations of the differences in this seven-transmembrane protein are shown in Fig. 6 (the structure of the figure is taken from Ref. 28). Four of the seven in the extracellular regions occur in the second loop, where the changes from human to mouse are as follows: alanine-151 to glutamate; arginine-154 to glycine; alanine-157 to valine; and methionine-162 to valine. The changes result in a charge difference of +1 for human to −1 for the mouse, by no means a conservative change.

A number of investigators have sought to identify regions in the various adenosine receptors that are important for binding specificity and the key amino acids that participate [29–33]. Most of these studies have involved the construction of chimeric receptors [29, 30]; others have used site-directed mutagenesis or have just compared a particular subtype from two different species [32, 33]. Generally, emphasis was on transmembrane sequences, regions known to be involved with binding specificity [31–33]. In our case, we have only one amino acid difference, in the fourth transmembrane region: Leu in the human is Phe in the mouse.

However, there is a study in which the second extracellular loop was implicated as important for ligand binding [30]. Chimeras composed of bovine A_1 and rat A_3 receptors were constructed. Substitution of the second extracellular loop of the A_1 into the A_3 receptor resulted in enhanced affinities for both agonists and antagonists relative to wild-type A_3. In their studies it was the distal amino acids

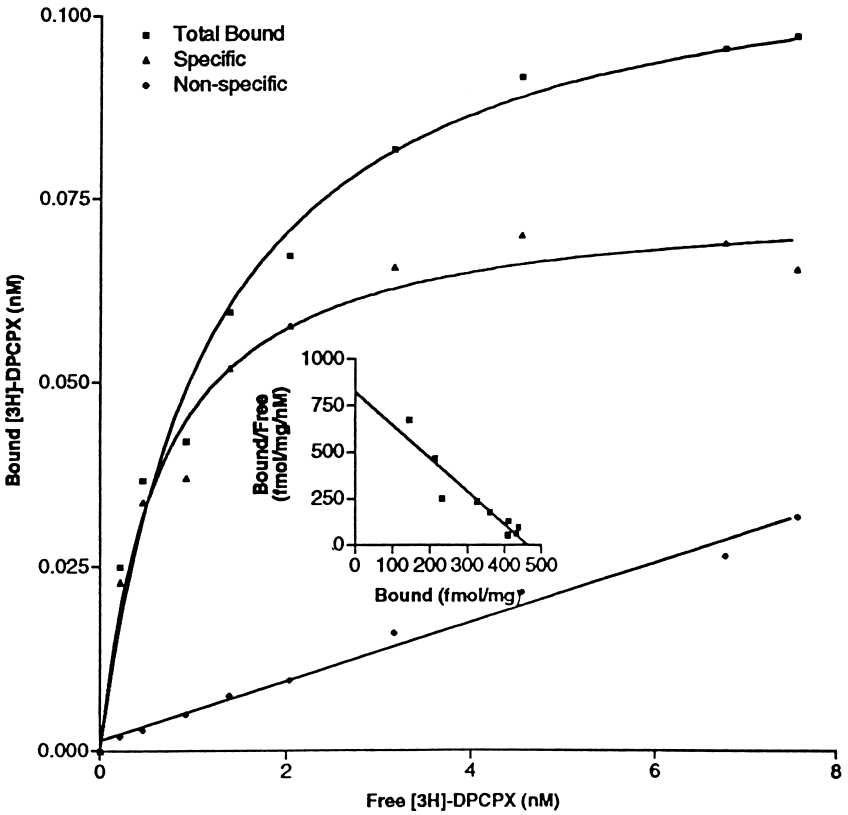

FIG. 3. Saturation curve and Scatchard plot for binding of [^{3}H]DPCPX to 293T cell membranes transfected with the mouse A_1AR. The data are representative of two experiments. Non-specific binding was carried out with 100 μM unlabeled R-PIA. Specific binding data were used for Scatchard analysis.

of the second extracellular loop that had the most influence on binding by the A_1, A_3 chimera.

The exact role of the second extracellular loop in the binding of agonists and antagonists has not been defined. Amino acid residues in this region may interact with the ligand directly or the loop may contribute to the overall architecture of the receptor protein, influencing the conformation of transmembrane-spanning regions of the receptor that provide the contact points for specific ligands. Additional mutagenesis studies targeting individual amino acids of the second extracellular loop may help to elucidate its role in ligand binding.

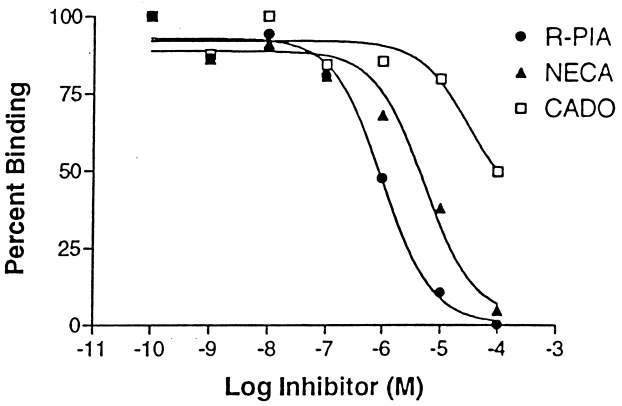

FIG. 4. Competitive inhibition by adenosine agonists of [^{3}H]DPCPX binding to 293T cell membranes transfected with the mouse A_1AR. Reactions were carried out with 1 nM [^{3}H]DPCPX. The data are representative of two experiments. Absolute values (dpm) for inhibitor concentrations of 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} are as follows: R-PIA, 3248.14, 2834.96, 3071.83, 2667.78, 1647.52, 527.21, and 213.43; NECA, 3248.14, 2829.05, 2976.33, 2654.79, 2269.49, 1360.50, and 353.82; CADO, 3248.14, 2875.57, 3248.14, 2771.16, 2800.83, 2632.89, and 1721.79.

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MPPSISAFQAAYIGIEVLIALVSVPGNVLVIWAVKVNQALRDATFCFIVSLAVA
...Y.....................................................

DVAVGALVIPLAILINIGPQTYFHTCLMVACPVLILTQSSILALLAIAVDRYLR
.....................................................

VKIPLRYKMVVTPRRAAVAIAGCWILSFVVGLTPMFGWNNLSAVERAWAANG
........T...Q.............L.............E..Q..I...

SMGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFYLIRKQLNKK
.V.....................................................

VSASSGDPQKYYGKELKIAKSLALILFLFALSWLPLHILNCITLFCPSCHKPSI
.....................................................T.Q....

LTYIAIFLTHGNSAMNPIVYAFRIQKFRVTFLKIWNDHFRCQPAPPIDEDLPEE
.I.....................H.................K...E..I...

RPDD
KAE.
```

FIG. 5. Comparison of human and mouse fat A_1AR amino acid sequences. The human sequence is shown on top and that of the mouse on the bottom. Dots depict identical residues; amino acids that differ in the mouse are indicated.

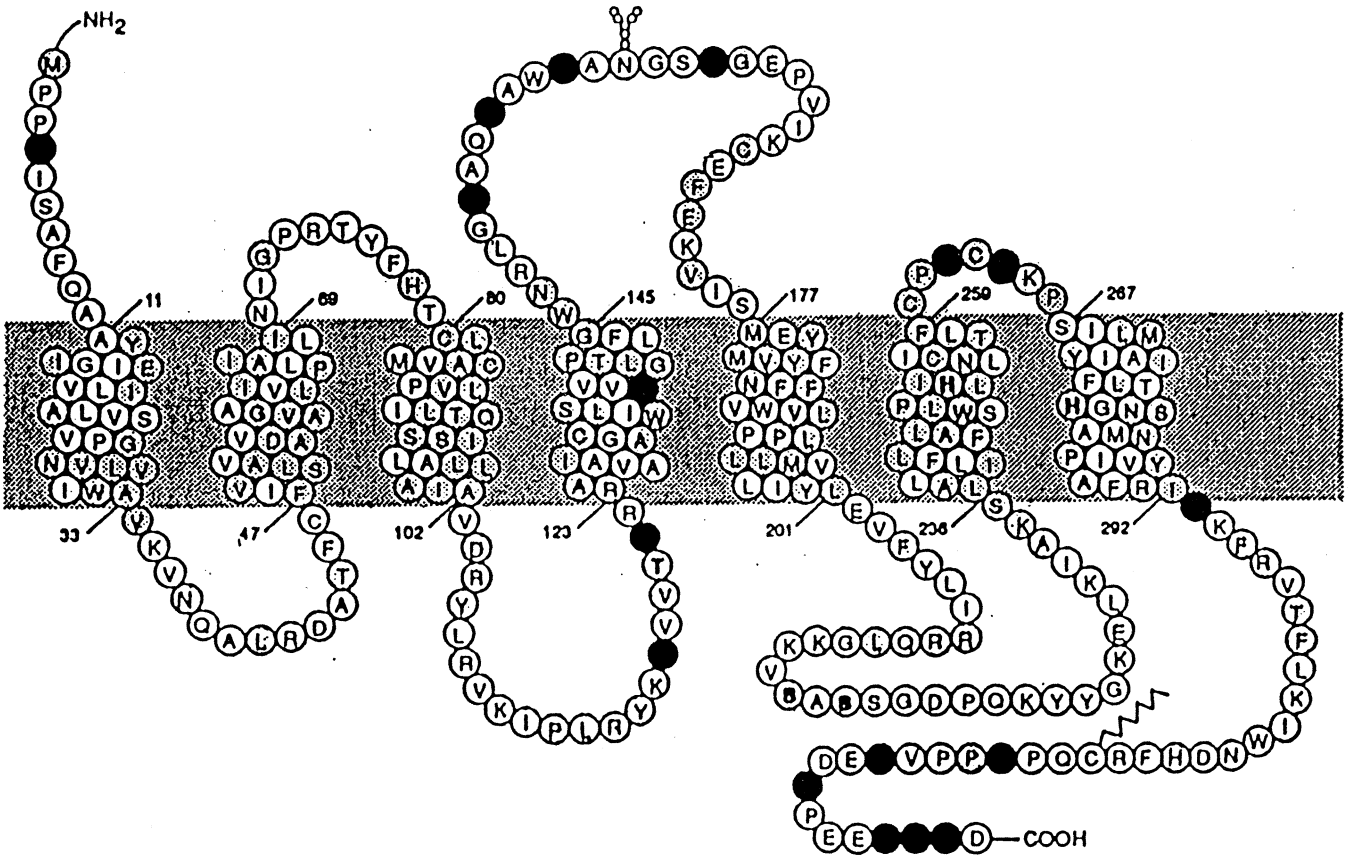

FIG. 6. Locations of amino acid residues that differ in the human and mouse fat A_1AR sequences. This diagram shows the protein structure of the A_1AR, generated by computer modeling. The sequence depicted is of the rat. Its purpose is to show the locations of residues that differ between the murine and human species (shown in black).

Early reports on rat tissues had claimed differences in ligand potency orders for A_1 receptors in fat and in brain. For example, the order for adipose tissue was *R*-PIA > CADO ≥ NECA (NECA is 100-fold less potent than *R*-PIA); for brain *R*-PIA ≥ NECA > CADO [18, 19, 34, 35]. We found that differences in ligand potencies between the two tissues cannot be related to differences in primary sequence since they are identical. To test the possibility that cellular environment could influence ligand potencies, we transfected two types of cells with the A_1AR from human adipose tissue: a human kidney-derived cell line (293T) and a murine fat cell line (ob17). The binding potency orders were the same in both lines.

To further validate our results, we conducted similar binding and competitive inhibition assays on membranes prepared from mouse adipose and brain tissues. The binding potency orders for *R*-PIA, NECA, and CADO did not differ significantly in the two tissues. This supports our findings with the human receptor and clearly shows that cellular environment does not influence significantly the binding characteristics of the human and mouse A_1ARs. Therefore, it is unlikely that the earlier binding studies on rat brain and fat membranes are correct [18, 19, 34, 35]. It should be noted that they were not all done in the same laboratory.

The availability of the mouse and human A_1AR cDNAs makes possible the breeding of transgenic mice overexpressing or underexpressing this receptor in adipose tissue. These experiments are now in progress and should lead to information concerning its functions *in vivo*.

I.T-K. was supported by an NIH Training Grant, T-3-DK07647, to the Institute of Human Nutrition, Columbia University. Support is also acknowledged from NIH Grant R01-HL47377. This report is part of a dissertation submitted by I.T-K. in partial fulfillment of the Ph.D. Degree in the Institute of Human Nutrition of Columbia University.

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