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Molecular cloning, expression and characterization of cDNA encoding a mouse α_{1a}-adrenoceptor

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1 In this study, we have cloned, expressed, and characterized an α_{1a}-adrenoceptor gene from the mouse. We designed oligonucleotide PCR primers complementary to regions of the rat α_{1a}-adrenoceptor sequence and amplified cDNA fragments from total RNA of mouse cerebral cortex, liver and kidney by reverse transcription-polymerase chain reaction (RT–PCR).

2 Both the nucleotide and deduced peptide sequences of the cDNA showed high sequence identity with those of cloned α_{1a}-adrenoceptors from other species. The cDNA clone had an open reading frame of 1398 nucleotides encoding a 466 amino acid peptide which had 97%, 92% and 90% identity with the deduced amino acid sequences of the rat, human and bovine α_{1a}-adrenoceptor, respectively.

3 The amplified mouse cDNA was inserted into a mammalian expression vector pcDNA3.1(+) and expressed in COS-1 cells. The pharmacological properties of the mouse cDNA clone were examined in radioligand binding studies and functional assays. The expressed mouse protein had a high affinity for [^{3}H]-prazosin ($K_d = 0.48$ nM) and pattern of affinity for antagonists in competition studies that is similar to that of the rat α_{1a}-adrenoceptor. Chloroethylclonidine (CEC) could slowly alkylate the expressed protein, with a rate similar to that of the rat α_{1a}-adrenoceptor.

4 The expressed receptors were able to mediate noradrenaline (NA) stimulation of the production of inositol phosphates in COS-1 cells, consistent with coupling to phospholipase C. This response to NA could be reversed by pretreatment of the transfected cells with prazosin.

5 Based on the above evidence, we concluded that the cloned cDNA is that of the mouse α_{1a}-adrenoceptor.

Keywords: Mouse α_{1a}-adrenoceptor gene; α_1-adrenoceptor subtypes; reverse transcription; polymerase chain reaction; [^{3}H]-prazosin binding; inositol phosphate; chloroethylclonidine

Introduction

α_1-Adrenoceptors are a family of G protein-coupled signalling proteins that mediate the actions of the sympathetic neurotransmitter noradrenaline, and the adrenal medullary hormone and central neurotransmitter adrenaline. They are widely distributed in many peripheral tissues and within the central nervous system. α_1-Adrenoceptor stimulation plays an important role in the regulation of the function of many tissues, especially in the cardiovascular system, such as blood pressure, cardiac function and circulatory homeostasis. Three distinct α_1-adrenoceptor subtype genes have been identified through molecular cloning from different species (Cotecchia *et al*., 1988; Schwinn *et al*., 1990; Lomasney *et al*., 1991; Perez *et al*., 1991), designated as α_{1a}-, α_{1b}- and α_{1d}-adrenoceptors. Emerging evidence suggests that these subtypes are discretely distributed among tissues and may serve discrete actions in the regulation of tissue functions (Bylund *et al*., 1994; Hieble *et al*., 1995). However, since many tissues contain a mixed population of α_1-adrenoceptor subtypes and the currently available α_1-adrenoceptor competitive antagonists are not highly selective, the function of each individual α_1-adrenoceptor subtype in tissue is still unknown.

At the present time, three α_1-adrenoceptor subtypes have been cloned from the rat and man (Vigot *et al*., 1990; Lomasney *et al*., 1991; Perez *et al*., 1991; 1994; Hirasawa *et al*., 1993; Laz *et al*., 1994; Weinberg *et al*., 1994), and most physiological and pharmacological studies on cardiovascular function have been performed in rat and dog. However, modern methods of genetic manipulation, such as transgenic models or antisense oligodeoxynucleotide techniques usually utilize the mouse. In order to use these modern molecular biological techniques to investigate the physiological function of each α_1-adrenoceptor subtype and their potential roles in disease states, the genetic sequences and pharmacological properties of murine α_1-adrenoceptor subtypes must first be investigated. Here, we describe the molecular cloning, sequencing, functional expression and pharmacological characterization of mouse α_{1a}-adrenoceptor subtype.

Methods

Tissue isolation and total RNA preparation

Male or female CD-1 mice (Sasco, Omaha, NE) weighing 15 to 18 g were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Three tissues including: brain, liver and kidney were rapidly removed and dissected in ice-cold saline (0.9% NaCl) containing 2 units ml^{-1} of recombinant ribonuclease inhibitor (RNasin, Promega Corp., Madison, WI). The tissues were frozen in liquid nitrogen and then stored at -70°C until extraction of total RNA. Total cellular RNA from each tissue was isolated by extraction with TRIzol Reagent (Life Technologies, Gaithersburg, MD).

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Cloning of partial cDNA fragment of mouse α_{1a}-adrenoceptor by RT–PCR

Oligonucleotide primers complementary to the cDNA sequence of the rat α_{1a}-adrenoceptor subtype (Scofield *et al.*, 1995), were used to amplify the majority of the third intracellular loop of the mouse α_{1a}-adrenoceptor cDNA. The cDNA sequence of the third intracellular loop and sixth transmembrane domain of the rat α_{1a}-adrenoceptor was used as the basis for the designing of the upstream or sense primer, UP1 (628 to 647) and the corresponding downstream or antisense primer, DN1 (820 to 839) (Figure 1). The numbers in parentheses following the primers refer to the position of the primers relative to the first nucleotide of the putative ATG translation start codon, and the corresponding cDNA sequences are indicated in Table 1. Reverse transcription (RT) reactions on total mouse cellular RNA were performed in a 20-μl reaction mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 500 μM of dNTP, 1.25 μM antisense primer, 20 μg total RNA, 20 units RNase inhibitor and 50 units M-MuLV reverse transcriptase (Perkin Elmer, Foster City, CA). The total cellular RNA used in the RT reaction was isolated from mouse cerebral cortex, kidney and liver. The cDNA samples produced were used immediately in PCR with the addition of 80 μl of the PCR mixture containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 125 μM of dNTP, 2.5 units AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA) and 312.5 nM sense primer. The RT reactions were performed under the following conditions: 40 min at 37°C, temperature increased 0.3°C min^{-1} over the next 16 min, 5 min at 42°C, 5 min at 95°C. The cDNA fragments were amplified by PCR under the following conditions: 8 min at 95°C, followed by 15 s at 95°C, 1 min at 60°C, 1 min at 72°C, for 39 cycles. A counterpart cDNA fragment of the expected size (212 bp) was amplified from mouse total RNA as shown in Figure 1. This result was confirmed in PCR experiments with rat α_{1a}-adrenoceptor primers in the mouse brain 5′-STRETCH PLUS cDNA library (Clontech, Palo Alto, CA).

Cloning of α_{1a}-adrenoceptor cDNA sequence from the mouse by RT–PCR

From the mouse α_{1a}-adrenoceptor sequence of the 212 bp PCR fragment, a 20 mer antisense primer DN3 (709 to 728) was synthesized (Figure 1 and Table 1). In addition, two other 20 mer oligonucleotide primers were synthesized based on the rat α_{1a}-adrenoceptor cDNA. One was complementary to the sense strand in a region 5′ of the translation start codon, UP2 (−17 to 3); and the other was complementary to the antisense strand in a region 3′ of the stop codon, DN2 (1402 to 1421) (Figure 1). The corresponding cDNA sequences of these primers are indicated in Table 1. RT–PCR was performed on total RNA isolated from mouse cerebral cortex, kidney and liver by use of primer sets either of the rat specific UP2 and mouse specific DN3, or the two rat specific primers UP1 and DN2 to isolate the 5′ half (745 bp) and the 3′ half (794 bp), respectively, of the mouse α_{1a}-adrenoceptor (Figure 1). The experimental conditions of RT–PCR were the same as described above, except that the cDNA fragments were amplified by PCR under the following conditions: 8 min at 95°C, followed by 30 s at 95°C, 1 min at 54°C, 2 min at 72°C, for 37 cycles. The cDNA amplification products were purified and sequenced. Two new oligonucleotide primers which are complementary to the sequence of the above cloned mouse α_{1a}-adrenoceptor cDNA fragments in the 5′ and 3′ regions were then synthesized (Figure 1). The corresponding cDNA sequences of the upstream primer (UP3, 1 to 20) and the downstream primer (DN4, 1382 to 1401) are indicated in Table 1. RT–PCR was performed on total RNA isolated from mouse cerebral cortex, kidney and liver by use of primers UP3 and DN4 and resulted in the amplification of a full length of mouse α_{1a}-adrenoceptor cDNA of the expected size (1401 bp) (Figure 1). The cDNA amplification products were purified and all products were determined to have identical sequences. The experimental conditions of RT–PCR were the same as described above, except the cDNA fragments were amplified by PCR under the following conditions: 8 min at 95°C, followed by 1 min at 95°C, 1 min at 48°C, 3 min at 72°C, for 37 cycles.

Sequencing

The cDNA PCR products were subcloned into TA cloning vector pCR2.1 (Invitrogen, San Diego, CA). Recombinant double stranded plasmids served as templates for cycle sequencing with M13 forward and reverse primers and fluorescence-based dideoxynucleotides (PRISM Ready Reac-

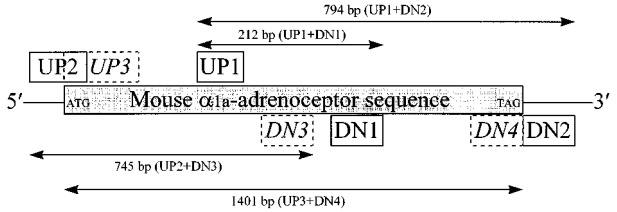

Figure 1 The synthetic oligonucleotide primers and the corresponding PCR products. The upstream primers (UP1, UP2 and UP3) are sense primers, and the downstream primer (DN1, DN2, DN3 and DN4) are antisense primers. Primers complementary to the cDNA sequence of either rat or mouse α_{1a}-adrenoceptor subtype are shown in either solid or dashed boxes, respectively. The PCR products generated from different sets of the above primers are shown in the diagram, and their sizes and corresponding locations with respect to the cDNA sequence of mouse α_{1a}-adrenoceptor are also indicated.

Table 1 Sequences and species specificity of oligonucleotide primers

Primer	*Sequence*	*Species*
UP1	5′-GTAGCCAAGAGAGAAAGCCG-3′	Rat/Mouse
DN1	5′-CAACCCACCACGATGCCCAG-3′	Rat
UP2	5′-GCCCCCTCCCTGAGACCATG-3′	Rat
DN2	5′-CCTGTGACCTTTCCCACTCT-3′	Rat
UP3	5′-ATGGTGCTTCTTTCTGAAAA-3′	Mouse
DN3	5′-GCAGGGACATTTTTACGGTG-3′	Mouse
DN4	5′-CTAGACTTCCTCCCCGTTTT-3′	Mouse
UP*Hin*d	5′-AAGCTTAAGCTTGCCACCATGGTGCTTCTTTCTGAAAA-3′	Mouse
DN*Bam*H	5′-GGATCCGGATCCCTAGACTTCCTCCCCGTTTTCACCGA-3′	Mouse

tion Dye Deoxy Terminator Cycle Sequencing Kit, Perkin Elmer, Inc.). Sequences were determined by use of a DNA Sequencer (ABI Model 373, Applied Biosystems, Foster City, CA). Sequences were validated by sequencing RT–PCR products from three separate RT–PCR reactions.

Cloning of mouse α_1-adrenoceptor cDNA into expression vectors

The full length of mouse α_{1a}-adrenoceptor cDNA was subcloned and inserted into a mammalian expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA) for expression studies. Two 38-bp chimeric oligonucleotide primers were synthesized to facilitate the subcloning. The chimeric upstream primer (UP*Hind*) includes two immediately adjacent recognition sequences of restriction endonuclease *Hind*III (12-bp sequence), a 6-bp Kozak sequence and a 20-bp sequence complementary to the mouse α_{1a}-adrenoceptor cDNA sequence 1 to 20. The chimeric downstream primer (DN*Bam*H) includes two immediately adjacent recognition sequences of restriction endonuclease *Bam*H I (12-bp sequence) and a 26-bp sequence complementary to the mouse α_{1a}-adrenoceptor cDNA sequence 1376 to 1401 (Table 1). PCR with the above two chimeric primers results in a 1431-bp product. The purified PCR product and expression vector pcDNA3.1(+) were digested with *Hind*III and *Bam*H I, ligated, and transformed into INVαF′ (Invitrogen, San Diego, CA). The transformed cells were then screened for ampicillin resistant recombinant plasmids.

cDNA plasmid purification and transfection

Ampicillin-resistant colonies were isolated and grown overnight in Luria-Bertani medium with 50 μg ml^{-1} ampicillin, and plasmid DNA was extracted according to QIAGEN Endofree Plasmid Maxi Protocol (QIAGEN, Chatsworth, CA). The cloned rat α_{1a}-adrenoceptor cDNA was subcloned into the modified eukaryotic expression plasmid pMT2′ (kindly provided by Dr Dianne Perez). COS-1 cells (American Type Culture Collection, Rockville, MD) were transiently transfected with 100 μg of plasmid per 150 mm^2 culture dish containing either cloned mouse or rat α_{1a}-adrenoceptor cDNA by the DEAE-dextran method (Cullen, 1987). Cells were harvested 48–72 h after transfection. All the cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum, 100 units ml^{-1} of penicillin and 100 μg ml^{-1} of streptomycin, at 37°C in a humidified incubator under an atmosphere containing 5% CO_2. Media were changed every other day.

Cell membrane preparation

Transfected cells were removed from culture flasks by washing twice with ice-cold PBS solution and scraping the cells twice from each flask in ice-cold PBS solution. The intact cells were pelleted by centrifugation at 1,500 × *g* for 10 min at 4°C. The cell pellet was resuspended in 50 mM Tris buffer (2 mM EDTA, pH = 7.4), and homogenized with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY). The mixture was then centrifuged at 1,260 × *g* for 5 min at 4°C and the pellet was discarded. The crude cell membranes were isolated by centrifugation of the supernatant fraction at 50,000 × *g* for 30 min at 4°C. The final membrane pellets were stored immediately at −70°C and were used within a week. Protein concentration was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Radioligand binding

The K_d and B_{max} of mouse or rat α_{1a}-adrenoceptor on COS-1 cell membranes were determined by use of [^{3}H]-prazosin saturation binding assays (0.02–2.0 nM), the non-specific binding was determined in the presence of 100 μM phentolamine as described previously (Feng *et al.*, 1993; Xiao *et al.*, 1997; Xiao & Jeffries, 1998). The K_i values of mouse and rat α_{1a}-adrenoceptors for other agonists and antagonists were determined by competition with [^{3}H]-prazosin. Membrane pellets were resuspended in 50 mM Tris buffer (2 mM EDTA, pH = 7.4). The final incubation volume was 1 ml and each incubation was allowed to proceed to equilibrium at 37°C in duplicate for 30 min. Reactions were stopped by addition of ice-cold 50 mM Tris buffer (2 mM EDTA, pH = 7.4) and were filtered onto S&S #32 glass fibre filter (Schleicher and Schuell, Keene, NH) with a Brandel MB-48R cell harvester (Brandel, Gaithersburg, MD). Filters were washed four times with 5 ml ice-cold Tris buffer, and bound radioactivity on the filter was determined by Beckman LS 5000 TD liquid scintillation counter (Beckman Instruments, Fullerton, CA). Binding data were analysed by the iterative curve-fitting programme LIGAND (Biosoft, Ferguson, MO) and Prism (GraphPAD, San Diego, CA). All the results are presented as mean ± s.e.mean from at least three individual experiments performed in duplicate.

CEC inactivation

A kinetic analysis of chlorethylclonidine (CEC) alkylation for mouse α_{1a}-adrenoceptor was conducted, which was similar to the method used for rat α_1-adrenoceptor described previously (Xiao & Jeffries, 1998). Cell membranes containing approxi-

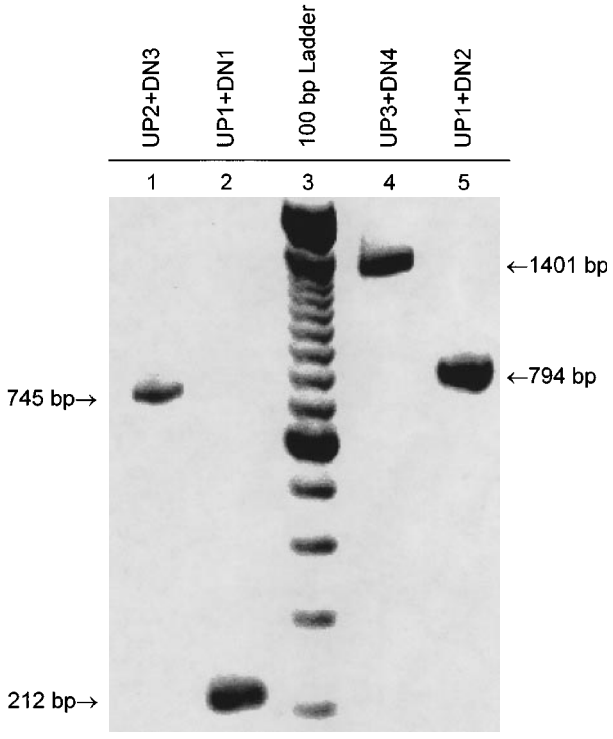

Figure 2 RT–PCR products of total RNA from mouse brain, liver and kidney. Lane 1, Product of primers UP2 and DN3 (745 bp). Lane 2, Product of primers UP1 and DN1(212 bp). Lane 3, 100 bp DNA ladder. Lane 4, Product of primers UP3 and DN4, which includes the entire mouse α_{1a}-adrenoceptor cDNA coding region (1401 bp). Lane 5, Product of primers UP1 and DN2 (794 bp).

mately 360 to 400 fmol of α_{1a}-adrenoceptor were incubated with 100 μM CEC in a final volume of 200 μl 50 mM Tris buffer (2 mM EDTA, pH = 7.4) at 37°C. After CEC treatment for various times from 0 to 60 min, the reaction was stopped by rapid washing with 1.4 ml ice cold buffer and rapid centrifugation in an Optima TL-100 ultracentrifuge (Beckman Instruments, Palo Alto, CA) at $86{,}000 \times g$ for 5 min at 4°C. This washing step was repeated 3 times. The fraction of receptors alkylated was determined by measuring specific [^{3}H]-prazosin binding (0.8 nM). Non-specific binding was determined with 100 μM phentolamine. The final incubation volume was 1 ml and each incubation was allowed to proceed at 37°C in duplicate for 30 min. The remainder of the radioligand binding assay procedure was similar to that described above. The experimental data were analysed with the curve fitting programme GraphPAD Prism which was used to determine the rate constants (K) for alkylation of mouse α_{1a}-adrenoceptor at 37°C. All results are presented as mean ± s.e.mean from three individual experiments performed in duplicate.

Formation of [^{3}H]-inositol phosphates

Assays were conducted essentially as described previously (Berridge *et al*., 1983; Jeffries *et al*., 1988; Gonzalez-Cabrera *et al*., 1996). Accumulation of total inositol phosphates was measured in 6-well plates (1×10^7 cells/plate). Five different experimental groups were used in this protocol: COS-1 cells, COS-1 cells with NA, COS-1 cells transfected with mouse α_{1a}-adrenoceptor (COS-α_{1a}), COS-α_{1a} with NA, and COS-α_{1a} with NA and a 1 μM prazosin pretreatment (10 min before NA). The cells were labelled with [^{3}H]-myo-inositol at 3 μCi ml^{-1} for 24 h in inositol-free DMEM. The monolayers were then washed twice with HEPES-buffered Krebs buffer (20 mM HEPES, 4 mM $NaHCO_3$, pH 7.4, 37°C) and preincubated at 37°C for 10 min in the same buffer containing 20 mM LiCl isoosmotically substituted for NaCl. After the preincubation, either vehicle or 10 μM (−)-NA was added to the appropriate groups for 10 min at 37°C. Incubations were stopped by the addition of 1 ml of ice-cold chloroform : methanol solution (1 : 2, vol/vol). Following a 2 h incubation at −20°C, the cell monolayers were collected and lysed by sonication. After the sample had been mixed with 0.5 ml of water, one ml of the aqueous phase was applied to columns containing 3 ml Dowex AGI-X8 anion exchange resin (200–400 mesh, formate form). The inositol phosphates were eluted as described previously (Jeffries *et al*., 1988). Radioactivity was quantified in a 0.5 ml aliquot of the final elution by scintillation spectrometry.

Materials

Chloroethylclonidine HCl, WB4101 (2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane), methoxamine, 5-methylurapidil, prazosin, phentolamine, (+)-niguldipine, spiperone, BMY 7378 (8-[2-[4-(2-methyoxyphenyl)-1-piperazinyl]-8-azaspiro[4,5]decane-7,9-dione di hydrochloride) and phentolamine mesylate were purchased from RBI (Natick, MA). (−)-Adrenaline, noradrenaline, phenylephrine, oxymetazoline, myo-inositol, HEPES, Krebs-Henseleit buffer and EDTA were purchased from Sigma Chemical Co. (St. Louis, MO). Tris hydroxymethyl aminomethane hydrochloride and Tris hydroxymethyl aminomethane were purchased from Fisher Scientific (Fair Lawn, NJ). DEAE-dextran was purchased from Pharmacia Biotech (Piscataway, NJ). [^{3}H]-prazosin and [^{3}H]-myo-inositol were purchased from DuPont NEN (Boston, MA). Dulbecco's modified Eagle's medium, antibiotic-antimycotic, trypsin EDTA, geneticin and foetal bovine serum were purchased from Gibco BRL Lifetechnologies (Grand Island, NY).

Results

Cloning of partial cDNA fragment of the mouse α_{1a}-adrenoceptor by RT–PCR

The rat-specific primers (UPI + DN1) used in this study amplify a PCR product that spans the majority of the nucleotide sequence of the rat α_{1a}-adrenoceptor third intracellular loop and includes a portion of the sixth transmembrane region of the α_{1a}-adrenoceptor (Scofield *et al*., 1995). In the present study, these same primers were used to amplify a RT–PCR product (212 bp) from total RNA isolated from mouse cerebral cortex as seen in Figure 2. An identical 212-bp product was obtained after amplification of the mouse brain 5′-STRETCH PLUS cDNA library (Clontech, Palo Alto, CA) with the same primers. The cDNA amplification product was sequenced and had a 95.35% nucleotide identity with the comparable sequence of rat α_{1a}-adrenoceptor.

Cloning of α_{1a}-adrenoceptor cDNA sequence from the mouse by RT–PCR

The 5′ half of the mouse α_{1a}-adrenoceptor was amplified by use of the rat specific primer UP2 and the mouse specific primer DN3 resulting in a PCR product of 745–bp as seen in Figure 2. The 3′ half of the mouse α_{1a}-adrenoceptor was amplified with the rat specific primer DN2 and the rat and mouse specific primer UP1 resulting in a PCR product of 794 bp as seen in Figure 2. Finally, the complete coding region of the mouse α_{1a}-adrenoceptor was amplified by RT–PCR from the total RNA isolated from mouse cerebral cortex, kidney and liver by use of mouse specific primers UP3 and DN4, and each reaction resulted in a 1401 bp PCR product as seen in Figure 2. Sequencing of the 1401 bp PCR product showed that it contained a 1398 bp open reading frame which had a 95.85% identity with the cDNA nucleotide sequence of rat α_{1a}-adrenoceptor (Table 2). This coding region encoded a 466-amino acid peptide and the deduced peptide sequence had 97.42%, 92.06% and 89.70% identity with rat, human and bovine α_{1a}-adrenoceptor, respectively (Table 3). Comparison of the amino acid sequence of the mouse to that of other species shows that the purported mouse α_{1a}-adrenoceptor protein also contains seven hydrophobic segments (Kyte & Doolittle, 1982), a feature shared by all of the members of the G-protein-coupled adrenoceptor family (Table 2).

Radioligand binding

The K_d values of prazosin for the expressed mouse clone and rat α_{1a}-adrenoceptor were similar, 0.47 ± 0.12 and 0.26 ± 0.11 nM, respectively. The B_{max} values for mouse and rat α_{1a}-adrenoceptor expressed in COS-1 cells were 1.72 ± 0.31 and 1.81 ± 0.40 pmol mg^{-1} protein, respectively. The K_i values of the mouse clone and rat α_{1a}-adrenoceptor for a series of agonists and antagonists obtained in competition studies are shown in Table 4.

CEC inactivation

Figure 3 demonstrates the alkylation of a cloned mouse α_{1a}-adrenoceptor with 100 μM CEC at 37°C. The log-linear nature

Table 2 Nucleotide and amino acid sequences of the mouse α-$_{1a}$-adrenoceptor subtype (GenBank accession number AF031431)

```
1    ATGGTGCTTCTTTCTGAAAATGCTTCTGAAGGCTCCAACTGCACCCACCCGCCAGCACAG   60
     M  V  L  L  S  E  N  A  S  E  G  S  N  C  T  H  P  P  A  Q

61   GTGAACATTTCTAAGGCCATTCTGCTTGGGGTGATCTTGGGGGGCCTCATCATTTTCGGG  120
     V  N  I  S  K  A  I  L  L  G  V  I  L  G  G  L  I  I  F  G
                         I

121  GTCTTGGGGAACATTTTAGTGATCCTCTCGGTGGCCTGTCATCGGCATCTGCACTCGGTG  180
     V  L  G  N  I  L  V  I  L  S  V  A  C  H  R  H  L  H  S  V

181  ACTCACTACTACATTGTCAACCTGGCTGTGGCAGACCTCCTCCTCACCTCCACCGTGCTG  240
     T  H  Y  Y  I  V  N  L  A  V  A  D  L  L  L  T  S  T  V  L
                   II

241  CCCTTCTCTGCCATCTTTGAGATCCTGGGATACTGGGCCTTTGGCAGGGTGTTCTGCAAC  300
     P  F  S  A  I  F  E  I  L  G  Y  W  A  F  G  R  V  F  C  N
                                                              III

301  ATCTGGGCGGCGGTGGACGTCTTATGCTGCACAGCGTCCATCATGGGCCTCTGCATCATC  360
     I  W  A  A  V  D  V  L  C  C  T  A  S  I  M  G  L  C  I  I

361  TCCATCGACCGATACATTGGTGTGAGCTACCCGCTGCGCTACCCCACCATTGTCACCCAG  420
     S  I  D  R  Y  I  G  V  S  Y  P  L  R  Y  P  T  I  V  T  Q

421  AGGAGGGGCGTCAGGGCTCTGCTCTGCGTCTGGGCGCTTTCCTTGGTCATCTCCATCGGA  480
     R  R  G  V  R  A  L  L  C  V  W  A  L  S  L  V  I  S  I  G
              IV

481  CCCCTGTTCGGCTGGAGGCAGCAGGCTCCGGAGGATGAGACCATCTGCCAAATCAATGAG  540
     P  L  F  G  W  R  Q  Q  A  P  E  D  E  T  I  C  Q  I  N  E

541  GAGCCAGGATACGTGCTGTTCTCAGCGCTGGGCTCTTTCTACGTGCCACTGACCATCATC  600
     E  P  G  Y  V  L  F  S  A  L  G  S  F  Y  V  P  L  T  I  I
           V

601  CTGGTTATGTACTGTCGAGTCTACGTGGTAGCCAAGAGAGAAAGCCGAGGCCTCAAGTCC  660
     L  V  M  Y  C  R  V  Y  V  V  A  K  R  E  S  R  G  L  K  S

661  GGCCTCAAGACCGACAAGTCAGACTCAGAGCAAGTGACGCTCCGTATCCACCGTAAAAAT  720
     G  L  K  T  D  K  S  D  S  E  Q  V  T  L  R  I  H  R  K  N

721  GTCCCTGCAGAAGGCAGCGGAGTAAGCAGTGCCAAGAATAAGACTCACTTCTCCGTGAGG  780
     V  P  A  E  G  S  G  V  S  S  A  K  N  K  T  H  F  S  V  R

781  CTGCTCAAGTTTTCCCGAGAGAAGAAAGCCGCCAAGACGCTGGGCATTGTGGTGGGATGC  840
     L  L  K  F  S  R  E  K  K  A  A  K  T  L  G  I  V  V  G  C
                                           VI

841  TTCGTCCTCTGCTGGCTGCCGTTCTTCCTCGTGATGCCCATTGGGTCCTTCTTCCCGAAT  900
     F  V  L  C  W  L  P  F  F  L  V  M  P  I  G  S  F  F  P  N

901  TTCAAGCCACCGGAAACAGTTTTCAAAATAGTATTTTGGCTTGGGTACCTAAATAGTTGC  960
     F  K  P  P  E  T  V  F  K  I  V  F  W  L  G  Y  L  N  S  C
                    VII

961  ATCAACCCTATCATATACCCATGCTCCAGCCAGGAGTTCAAGAAAGCCTTTCAGAATGTG 1020
     I  N  P  I  I  Y  P  C  S  S  Q  E  F  K  K  A  F  Q  N  V

1021 CTGCGAATCCAGTGTCTTCGCAGAAGGCAGTCTTCCAAGCATGCCCTGGGCTACACTCTG 1080
     L  R  I  Q  C  L  R  R  R  Q  S  S  K  H  A  L  G  Y  T  L

1081 CACCCACCCAGCCAGGCTGTAGAGGAACAGCACAGAGGCATGGTGCGTATCCCGGTGGGC 1140
     H  P  P  S  Q  A  V  E  E  Q  H  R  G  M  V  R  I  P  V  G

1141 TCAGGAGAGACTTTCTATAAGATCTCCAAGACAGATGGAGTCTGTGAATGGAAGTTTTTC 1200
     S  G  E  T  F  Y  K  I  S  K  T  D  G  V  C  E  W  K  F  F

1201 TCTTCCATGCCCCAGGGATCGGCCAGGATTACCATGCCGAAGGACCAATCCGCCTGTACC 1260
     S  S  M  P  Q  G  S  A  R  I  T  M  P  K  D  Q  S  A  C  T

1261 ACAGCCCGGGTGAGAAGTAAAAGCTTTTTGCAGGTCTGCTGCTGTGTGGGGTCGTCGACC 1320
     T  A  R  V  R  S  K  S  F  L  Q  V  C  C  C  V  G  S  S  T

1321 CCACGCCCTGAAGAAAATCACCAAGTTCCAACCATTAAGATCCACACCATCTCCCTCGGT 1380
     P  R  P  E  E  N  H  Q  V  P  T  I  K  I  H  T  I  S  L  G

1381 GAAAACGGGGAGGAAGTCTAG                                       1401
     E  N  G  E  E  V  *
```

Proposed membrane spanning segments TM1-TM7 are underlined.

Table 3 Comparison of amino acid sequences of mouse, rat (GenBank accession number U071126), human (L31774) and bovine (J05426) α_{1a}-adrenoceptors

Mouse	1	MVLLSENASEGSNCTHPPAQVNISKAILLGVILGGLIIFGVLGNILVILSVACHRHLHSV	60
Rat	1	.....P.....	60
Human	1	..F..G...DS....Q...P.....L.....	60
Bovine	1	..F..G...DS.....PP.....L.....	60
Mouse	61	THYYIVNLAVADLLLTSTVLPFSAIFEILGYWAFGRVFCNIWAAVDVLCCTASIMGLCII	120
Rat	61	.....	120
Human	61	.....V.....	120
Bovine	61	.....V.....	120
Mouse	121	SIDRYIGVSYPLRYPTIVTQRRGVRALLCVWALSLVISIGPLFGWRQQAPEDETICQINE	180
Rat	121	.....V.....P.....	180
Human	121	.....LM.....P.....	180
Bovine	121	.....K..LM.....P.....	180
Mouse	181	EPGYVLFSALGSFYVPLTIILVMYCRVYVVAKRESRGLKSGLKTDKSDSEQVTLRIHRKN	240
Rat	181	.....A.....	240
Human	181	.....L..A.....	240
Bovine	181	.....	240
Mouse	241	VPAEGSGVSSAKNKTHFSVRLLKFSREKKAAKTLGIVVGCFVLCWLPFFLVMPIGSFFPN	300
Rat	241	.....G.....D	300
Human	241	A..G...MA...T.....D	300
Bovine	241	AQVG....T.....D	300
Mouse	301	FKPPETVFKIVFWLGYLNSCINPIIYPCSSQEFKKAFQNVLRIQCLRRRQSSKHALGYTL	360
Rat	301	...S.....	360
Human	301	...S.....K.....	360
Bovine	301	.R.S.....A.....K.....T.....	360
Mouse	361	HPPSQAVEEQHRGMVRIPVGSGETFYKISKTDGVCEWKFFSSMPQGSARITMPKDQSACT	420
Rat	361	.....L.G...D.....V.....	420
Human	361	.....G..KD.....R....R.....R.....VS....S..	420
Bovine	361	.A..HVL.G..KDL.....A.....I...L.R....MAVAR.P....	420
Mouse	421	TARVRSKSFLQVCCCVGSSTPRPEENHQVPTIKIHTISLGENGEEV	466
Rat	421	.....A.....	466
Human	421	.....P...SLDK.....V.....S.....	466
Bovine	421	.....L.P...SHG....I.....S.....	466

Proposed membrane spanning segments are underlined and identical amino acids are substituted with dots in the sequences.

of the data indicated a first order reaction and the rate constant (K) for CEC alkylation at 37°C was determined by a non-linear regression analysis. We compared our alkylation data for the mouse α_{1a}-adrenoceptor with those previously obtained for the three cloned rat α_1-adrenoceptor subtypes (Xiao & Jeffries, 1998). The expressed mouse α_{1a}-adrenoceptor was slowly alkylated by to CEC alkylation with alkylation rate constant ($50.02 \pm 1.40\ 10^{-3}\ min^{-1}$) similar to that of the rat α_{1a}-adrenoceptor ($43.46 \pm 0.37\ 10^{-3}\ min^{-1}$), and much slower than the rat α_{1a}-adrenoceptor ($205.61 \pm 5.39\ 10^{-3}\ min^{-1}$) or the rat α_{1d}-adrenoceptor ($182.02 \pm 2.87\ 10^{-3}\ min^{-1}$) (Figure 3).

Formation of [^{3}H]-inositol phosphates

Figure 4 shows the accumulation of [^{3}H]-inositol phosphates in non-transfected and transfected COS-1 cells. NA produced no increase in accumulation of [^{3}H]-inositol phosphates in non-transfected cells. However, NA significantly increased accumulation of [^{3}H]-inositol phosphates in COS-1 cells transfected with the mouse α_{1a}-adrenoceptor. The NA-induced increase of total cellular inositol phosphates in the transfected cells was prevented by pretreatment of the cells with the α_1-adrenoceptor-selective antagonist prazosin.

Discussion

We have cloned a cDNA for mouse α_1-adrenoceptor from mouse total RNA by RT–PCR. The cloned cDNA fragment contains a 1398 bp open reading frame encoding a 466-amino acid peptide, which has the same nucleotide and peptide sizes as those of cloned rat, human and bovine α_{1a}-adrenoceptors. Both the nucleotide and deduced amino acid sequences of the mouse α_1-adrenoceptor cDNA showed high identity to those of cloned α_{1a}-adrenoceptors from other species. The deduced amino acid sequence of this mouse α_1-adrenoceptor cDNA has 97%, 92% and 90% identity with the rat, human and bovine α_{1a}-adrenoceptor, respectively, and appears to have seven hydrophobic segments, a feature shared by all of the members of the G-protein-coupled adrenoceptor family. When cloning with PCR, caution must be exercised to prevent the introduction of error in the sequence because of an occasional loss of the fidelity of the DNA polymerases used in DNA amplification (Keohavong & Thilly, 1989). There is a small possibility that the AmpliTaq Gold DNA polymerase used in our PCR reactions introduced sequence errors. To account for this possibility, we sequenced α_{1a}-adrenoceptor RT–PCR products from three different tissues of the mouse. Since the results of each of these experiments were identical, the possibility of errors existing in the cDNA sequence is unlikely.

Table 4 Pharmacological characteristics of mouse and rat α_{1a}-adrenoceptors

	Mouse α_{1a}-adrenoceptor		*Rat α_{1a}-adrenoceptor*	
	pK_i (M)			
Compound	pK_{iH}	pK_{iL}	pK_{iH}	pK_{iL}
Agonist				
Adrenaline	6.56±0.83	5.36±0.10	6.37±0.37	5.28±0.06
(−)-Noradrenaline	6.17±1.26	5.12±0.16	6.19±0.04	5.13±0.40
(−)-Phenylephrine	6.05±0.91	4.78±0.07	6.73±0.48	5.03±0.01
Methoxamine	5.79±0.77	4.83±0.17	5.45±0.85	4.31±0.10
Oxymetazoline	8.45±0.50	7.47±0.11	8.87±0.81	8.13±0.39
Antagonists				
Prazosin	9.32±0.09		9.58±0.08	
Phentolamine	8.48±0.02		8.71±0.05	
WB-4101	8.85±0.04		9.05±0.04	
5-Methylurapidil	8.56±0.03		8.81±0.01	
(+)-Niguldipine	10.41±0.04		10.50±0.03	
Spiperone	8.13±0.04		7.28±0.05	
BMY 7378	6.14±0.02		6.58±0.05	

COS-1 cell membranes transfected with either the mouse or rat α_{1a}-adrenoceptor cDNA were incubated with [^{3}H]-prazosin, in the absence or presence of increasing concentrations of various agonists or antagonists. Twelve concentrations of each ligand were used. K_i values were analysed by the iterative, nonlinear, curve-fitting programme GraphPAD Prism. K_i values are shown as K_{iH} (high affinity site) and K_{iL} (low affinity site) where two binding sites fit better than one site. Data for the K_d values of prazosin were generated from [^{3}H]–prazosin saturation study and analysed by the iterative, nonlinear, curve-fitting programme LIGAND. Data are presented as mean±s.e.mean from at least three individual experiments performed in duplicate.

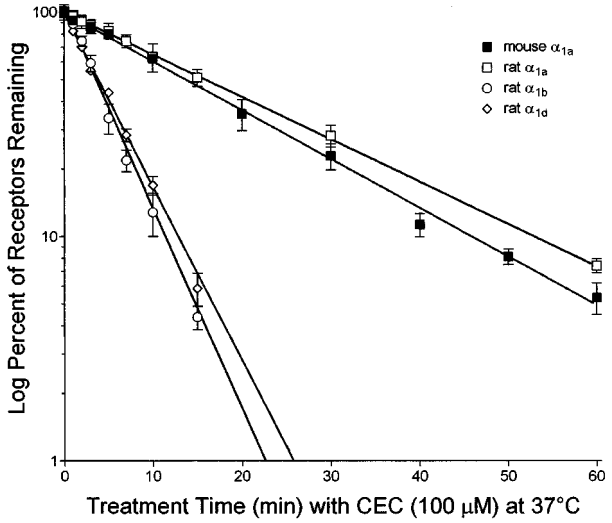

Figure 3 Alkylation of cloned mouse α_{1a}-adrenoceptor by 100 μM CEC at 37°C. Cell membranes were incubated with CEC at 37°C at time intervals from 0 to 60 min. After each incubation time, the fraction of receptors remaining was determined by measuring specific [^{3}H]-prazosin binding. Data were analysed by the iterative, nonlinear, curve-fitting programme GraphPAD Prism, and are presented as mean±s.e.mean from three individual experiments performed in duplicate. Data for mouse α_{1a}-adrenoceptor are superimposed on three previously obtained for cloned rat α_1-adrenoceptor subtypes, by use of a similar alkylation protocol (Xiao & Jeffries, 1998).

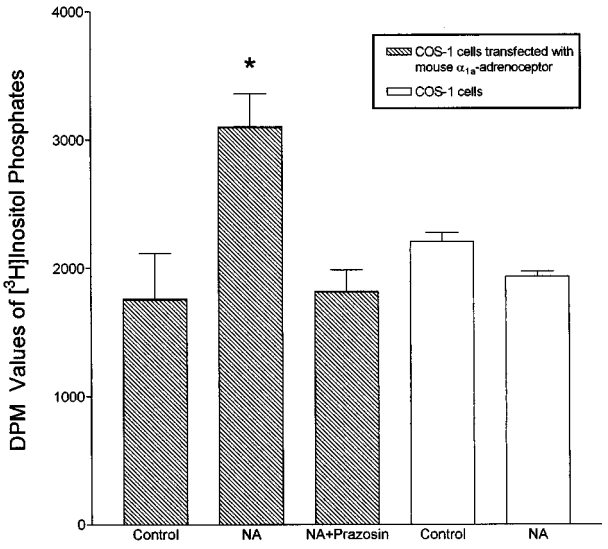

Figure 4 Accumulation of [^{3}H]-inositol phosphates in non-transfected and transfected COS-1 cells. Cells were treated with vehicle alone or 10 μM NA (10 min, 37°C) as noted. Prazosin (1 μM) was added in one group 10 min before the addition of NA. Columns represent mean±s.e.mean of d.p.m. values from three individual experiments, $^*P<0.05$. See text for experimental details.

The amino acid sequences of the seven putative transmembrane domains of α_{1a}-adrenoceptor are highly conserved between the four species (Table 3). The variant sites of the amino acid sequence of the four species commonly occur at the carboxyl, amino terminus and the third intracellular loop of α_{1a}-adrenoceptor. Both the alignment and percentage of amino acid identity of mouse α_{1a}-adrenoceptor relative to the cloned mouse α_1-adrenoceptor from other species strongly suggest that this mouse cDNA clone is the α_{1a}-adrenoceptor subtype. Other structural features of the α_{1a}-adrenoceptor of the mouse and other species are the presence of three potential sites for N-linked glycosylation in the amino terminus (asparagine residues 7, 13 and 22), and the presence of several serine and threonine residues in the carboxyl terminus and intracellular loops, which may serve as potential sites for phosphorylation by protein kinase C and protein kinase A.

The expressed mouse α_{1a}-adrenoceptor had a high affinity for the α_1-adrenoceptor selective antagonist [^{3}H]-prazosin, similar to that of the rat. Investigation of other pharmacological characteristics of mouse α_{1a}-adrenoceptor in competition studies indicated a pattern of affinity for other α_1-adrenoceptor-selective antagonists and agonists that was also similar to that of the cloned rat α_{1a}-adrenoceptor. As shown previously (Xiao & Jeffries, 1998), the cloned rat α_{1a}-adrenoceptor was sensitive to CEC alkylation, but at a markedly slower rate compared to rat α_{1b}- and α_{1d}-adrenoceptors. The expressed

mouse α_1-adrenoceptor was slowly alkylated by CEC with a rate constant similar to that of the rat α_{1a}-adrenoceptor. The expressed mouse α_{1a}-adrenoceptor responded to NA stimulation with significantly increased accumulation of [^{3}H]-inositol phosphates in COS-1 cells. This indicates that the expressed receptors could couple to G-proteins (G_q) and activate the downstream secondary messenger system phospholipase C, which is a common characteristic shared by the α_1-adrenoceptor family. Furthermore, the NA-induced increase of total cellular inositol phosphates observed with the expressed mouse receptors could be reversed by the α_1-adrenoceptor selective antagonist prazosin. Therefore, all the above pharmacological and functional properties of the expressed mouse α_{1a}-adrenoceptor confirm its α_{1a}-adrenoceptor identity.

Recently, several different groups have demonstrated the existence of splice variants of the human α_{1a}-adrenoceptor (Hirasawa *et al*., 1995; Chang *et al*., 1997), suggesting the existence of different isoforms of α_{1a}-adrenoceptors. These isoforms were generated by alternative mRNA splicing within the carboxyl terminus of the α_{1a}-adrenoceptor and differ from each other in their length and sequences of the C-terminal domain. In our studies, the downstream primers of the C-terminal region were specifically designed according to the sequence of the original cloned rat α_{1a}-adrenoceptor (Laz *et al*., 1994; Perez *et al*., 1994). Thus, when the cDNA sequence was compared with the α_{1a}-adrenoceptors in other species, the cloned mouse α_{1a}-adrenoceptor in our studies is analogous to the original cloned isoform of α_{1a}-adrenoceptors in man and rat (Hirasawa *et al*., 1993; Laz *et al*., 1994; Perez *et al*., 1994). Since there is a high degree of sequence identity of the cloned α_{1a}-adrenoceptors between the different species, it is highly possible that the mouse α_{1a}-adrenoceptor has splice variants and that our cloned mouse cDNA is only one of the different isoforms. Such possibilities await investigation.

α_1-Adrenoceptors play a critical role in the autonomic regulation of the function in many tissues, especially in the cardiovascular system. α_1-Adrenoceptors may also play an important role in the autonomic dysfunction seen in hypertension, heart failure, oedema and coronary artery disease (Bylund *et al*., 1994; Graham *et al*., 1996). Most of our knowledge of autonomic pharmacology and cardiovascular disease are from rats and dogs. However, since modern methods of genetic manipulation, including the use of antisense oligodeoxynucleotides, transgenic knock-out and gene therapy, usually utilize the mouse, it was an essential first step to clone the α_{1a}-adrenoceptor from this species. This work will allow us to study the physiological function of each individual subtype in mouse better and could also lead to the examination the function of α_1-adrenoceptors in newly emerging mouse models of human disease, which have been created by transgenic techniques.

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