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Cloning, functional expression and tissue distribution of human cDNA for the α_{1C}-adrenergic receptor

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We have cloned human α_{1C}-adrenergic receptor from human prostate cDNA library. The deduced amino acid sequence of the clone (P2C4) encodes a protein of 466 amino acids that showed strong sequence homology to the previously cloned bovine α_{1C}-adrenergic receptor. The radioligand binding properties of P2C4 expressed in COS-7 cells were very similar to those of bovine α_{1C}-adrenergic receptor. With reverse-transcription polymerase chain reaction assay, we observed α_{1C}-adrenergic receptor transcripts in heart, brain, liver and prostate, but not in kidney, lung, adrenal, aorta and pituitary. The data show that the clone P2C4 encodes a human α_{1C}-adrenergic receptor cDNA, and the receptor subtype is expressed not widely but localized in several human tissues.

α_1-adrenergic receptors play critical roles in the regulation of a variety of physiologic processes. Pharmacologic characteristics of α_1 receptors include an overall agonist potency series of epinephrine $\geq$ norepinephrine >> isoproterenol. The specific α_1 antagonist is prazosin. Recently, heterogeneity of α_1-adrenergic receptors has been suggested by several lines of pharmacologic evidence, based predominantly on different ligand binding properties (1-4). Specifically, two subtypes of α_1 receptors have been postulated, α_{1A} and α_{1B}. The α_{1A} subtype has high affinity for the agonist oxymetazoline, and antagonists WB 4101 and phentolamine, compared to the α_{1B} receptor. In addition, the alkylating agent chlorethylclonidine (CEC) binds covalently and irreversibly to inactivate the α_{1B} subtype completely; thus, it is considered

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Abbreviations:
CEC, chlorethylclonidine; [^{125}I]HEAT, ^{125}I-(2-β-(4-hydroxyphenyl)-ethylaminomethyl)-tetralone; WB 4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4 benzodioxane; RT-PCR, reverse-transcription polymerase chain reaction.

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sensitive to CEC. The α_{1A} receptor, however, is completely insensitive to this compound. This has been used as a criterion for differentiating between the α_{1A}- and α_{1B}-adrenergic receptors in various tissues. Furthermore, using molecular biological approach, the cDNA that encodes a novel α_1-adrenergic receptor subtype, the α_{1C} receptor, has been recently cloned from a bovine brain cDNA library (5); however, very few informations are still available regarding the pharmacological properties and the tissue distribution of this new receptor subtype in human in particular.

We now report the cloning of a human cDNA encoding an α_{1C}-adrenergic receptor. COS-7 cells transfected with the cloned cDNA expressed specific, high-affinity binding sites, which pharmacological character is similar to those of bovine α_{1C}-adrenergic receptor. Also, with RT-PCR assay, tissue distribution of α_{1C}-adrenergic receptor mRNA was examined in human.

Materials and Methods

cDNA library screening: Human prostate λgt 11 library ($1x10^6$ recombinants; Clontech, Palo Alto, CA) was screened with the 450 bp PstI-XhoI fragment of human α_{1A}-adrenergic receptor (6) labeled with digoxigenin DNA labeling kit (Boehringer Mannheim GmbH, Germany). A total of $1x10^6$ plaques were screened, using duplicate nylon membrane filters NY13N (Schleicher & Schuell). Hybridization and visualization of positive plaques are carried out using DIG luminescent detection kit (Boehringer Mannheim GmbH, Germany) as described by the manufacturer.

DNA sequencing: Cloned cDNA, enzyme digested fragments and PCR products were subcloned into pUC18, pUC119 (TaKaRa, Kyoto, JAPAN) and pBluescript KS II(+) (Stratagene, La Jolla, CA). Nucleotide sequence analysis was performed using overlapping templates by the ABI 373A DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) for both complete strands.

Transient expression in COS-7 cells: To facilitate construction of the expression vector for the human α_{1C}-adrenergic receptor, EcoRI-PstI 2100 bp fragment of P2C4 was ligated into the EcoRI-PstI-digested SRα promoter-based mammalian expression vector pME18S (7). The resulting construct pME18S-P2C4, contained a 436 bp of 5'-untranslated region, a 1398bp of coding region and a 250 bp of 3'-untranslated region. The constructs were transfected into COS-7 cells by the DEAE-dextran method (8), and cells were harvested 48-72 h after transfection.

Radioligand binding assay: COS-7 cell membranes were prepared as described previously by Lomasney et al.(9). Briefly, the cells were collected and disrupted by the Branson sonicator (model SONIFIER 250, setting 5 for 8 s). The mixture was then centrifuged at 3000 x g for 10 min. The supernatant fraction was centrifuged at 35,000 x g for 20 min. The resulting pellet was resuspended in binding buffer (50 mM Tris-HCl pH7.4, 12.5mM $MgCl_2$, 10mM EGTA). The protein concentration was measured using the BCA protein assay kit (PIERCE, Rockford, IL). Membrane aliquots (10μg of protein) were incubated for 60 min at 25°C with [^{125}I]HEAT, with or without a competing ligand, in a final volume of 250 μl of binding buffer. After dilution with ice-cold buffer, samples were immediately filtered through Whatmann GF/C glass fiber filters with a Brandel cell harvester (Model-30, Gaithersburg, MD). Competition experiments were carried out at about 70 pM [^{125}I]HEAT which is Kd of the ligand. At this concentration nonspecific binding, defined as that occurring in the presence of 10μM phentolamine, represented about less than 10% of total binding. Competition binding experiments were analyzed with the LIGAND programs (10). Treatment with CEC was performed as described by Lomasney et al.(9). 100μM CEC was incubated in 3 ml of hypotonic buffer (5 mM Tris, 5 mM EDTA. pH 7.5) with membranes made from COS-7 cells transtected with pME18S-P2C4. After treatment

for 30 min at 37°C, the reaction was stopped by centrifugation at 40,000 x for 20 min at 4 °C. The membranes were washed with 3 ml of ice-cold buffer, then they were resuspended in binding butfer and residual binding was assessed by incubation with 0.5nM [^{125}I]HEAT.

Tissue distribution of mRNA expression: For Northern analysis, human poly(A)$^+$ RNA blotted membrane were purchased from Clontech (Palo Alto, CA) and hybridized to ^{32}P-labeled P2C4 clone as a probe. For reverse-transcription polymerase chain reaction (RT-PCR), total cellular RNA from various human tissues was isolated by the cesium chloride gradient method (11). In preliminary experiments, the integrity of the purified RNA collected with this method was confirmed by visualization of the 28S and 18S ribosomal RNA bands after the electrophoresis of RNA through a 1% agarose-formaldehyde ethidium bromide gel.

RT-PCR analysis was performed as described previously (12). The primers were synthesized on a model 391A (Applied Biosystems, Inc., Foster City, CA) DNA synthesizer (using β-cyano-methylphosphoramidate derivatives). Oligonucleotide primers were constructed from the cDNA sequences of cloned human α_{1C}-adrenergic receptor and β-actin cDNA (13). β-actin served as a cell cycle-independent internal standard for the efficacy of RNA isolation and cDNA synthesis.

The sequences of the human α_{1C}-receptor primers were
5'-CATCGTGGTCGGCTGCTTCGTCCTCTGCTG-3' (coding sense) corresponding to bases 1261-1290 of the cloned full-length sequence,
5'-TCCCACGGGGATGCGCACCATGTCCTTGTG-3'(anticoding sense) which anneals to bases 1547-1576.

The sequences of the β-actin primers used were
5'-ATCATGTTTGAGACCTTCAACACCCCAGCC-3' (coding sense) corresponding to bases 2158-2187 of the cloned full-length sequence,
5'-AAGAGAGCCTCGGGGCATCGGAACCGCTCA-3' (anticoding sense) corresponding to bases 2550-2579. The predicted sizes of the amplified human α_{1C}-adrenergic receptor and human β-actin PCR products were 316 and 421 bp, respectively.

The PCR amplification profiles consisted of denaturation at 94 °C for 1 min, primer annealing at 55°C for 30 sec, and extension at 72 °C for 1 min. Negative control reactions without template were routinely included in PCR amplifications with both primer sets.

Each PCR product was purified after the agarose gel electrophoresis by the SUPREC-01 (TaKaRa, Kyoto, Japan), subcloned into the EcoRV site of Bluescript II KS(+) plasmid by TA-cloning method (14), and then sequenced.

Drugs: Sources of drugs were as follows: [^{125}I]HEAT (Du Pont - New England Nuclear); methoxamine, phenylephrine, norepinephrine bitartrate, (-)epinephrine, (+)epinephrine, oxymethazoline (Sigma); WB4101, chlorethylclonidine, 5-methylurapidil (Research Biochemicals Inc.); phentolamine HCl (Ciba-Geigy); prazosin (Pfizer); yohimbine (Aldrich).

Results and Discussion

Screening of human prostate cDNA library identified three clones (Fig.1A). The first clone, P2, contained a 1660 bp cDNA fragment which encoded for the N-term portion of a protein with seven hydrophobic clusters of amino acids. These seven putative transmembrane domains, when compared with the sequences of known G-protein coupled receptors, had the highest similarity with transmembrane regions I-VII of α_{1C}-adrenergic receptor. This P2 clone, however, did not contain either C-term hydrophilic region or the stop codon. The second and third clones, C4 and C7 contain VI-VII transmembrane domains, C-term region and stop codon. The 300 bp overlapping region of the nucleotide sequence for P2, C4 and C7 were identical,

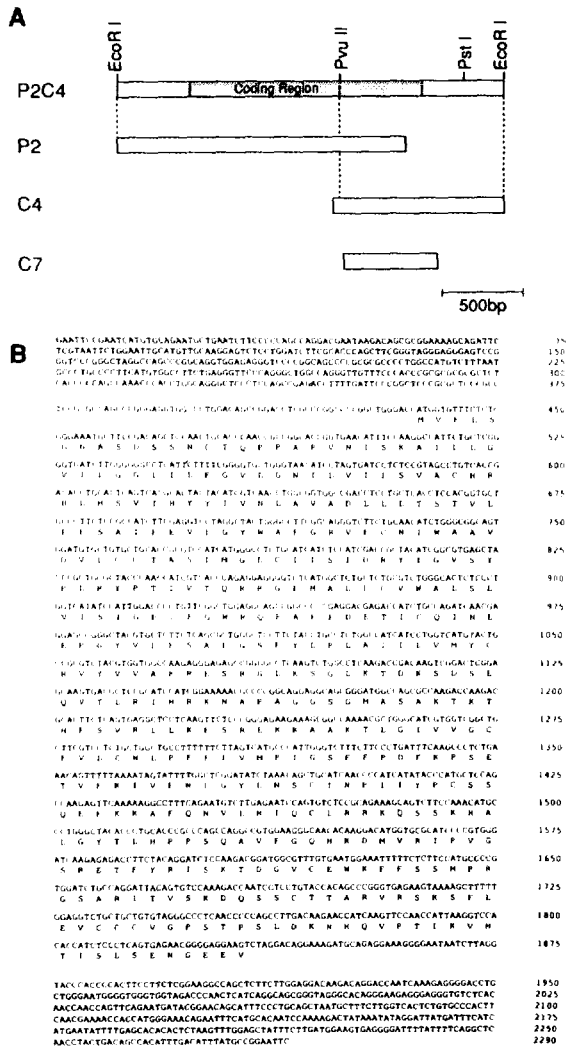

Fig. 1.

A: Restriction maps of human α_{1C}-adrenergic receptor clones.

B: Nucleotide sequence, and deduced amino acid sequence of the human α_{1C}-adrenergic receptor clone (P2C4). 5'-untranslated region = 436 bp, open reading frame = 1398 bp, and 3'-untranslated region = 480 bp. The single-letter amino acid code is used.

indicating that both sequences derived from the same gene. To facilitate constructing the full length clone, EcoRI-PvuII 1450 bp restriction fragment from the clone P2 was ligated with a PvuII-PstI 650 bp restriction fragment from the clone C4 , and the resulting construct was named P2C4.

The clone P2C4 contains a 1398 bp open reading frame encoding a protein of 466 amino acids, which number is the same as that of bovine α_{1C}-adrenergic receptor amino acids (Fig.1B). The percentage of the amino acid identity of P2C4 is 92% compared with the bovine α_{1C}-

adrenergic receptor, while it is 59% with hamster α_{1B}-adrenergic receptor (15) and 58% with human α_{1A}-adrenergic receptor (6). Both the alignment and percentage of amino acid identity relative to other members of the adrenergic receptor family strongly suggest that this receptor is belong to the α_{1C}-adrenergic receptor subtype (Fig.2). Other structural features of this clone are the presence of three potential sites for N-linked glycosylation in the amino terminus (asparagine residues 7, 13 and 21), 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.

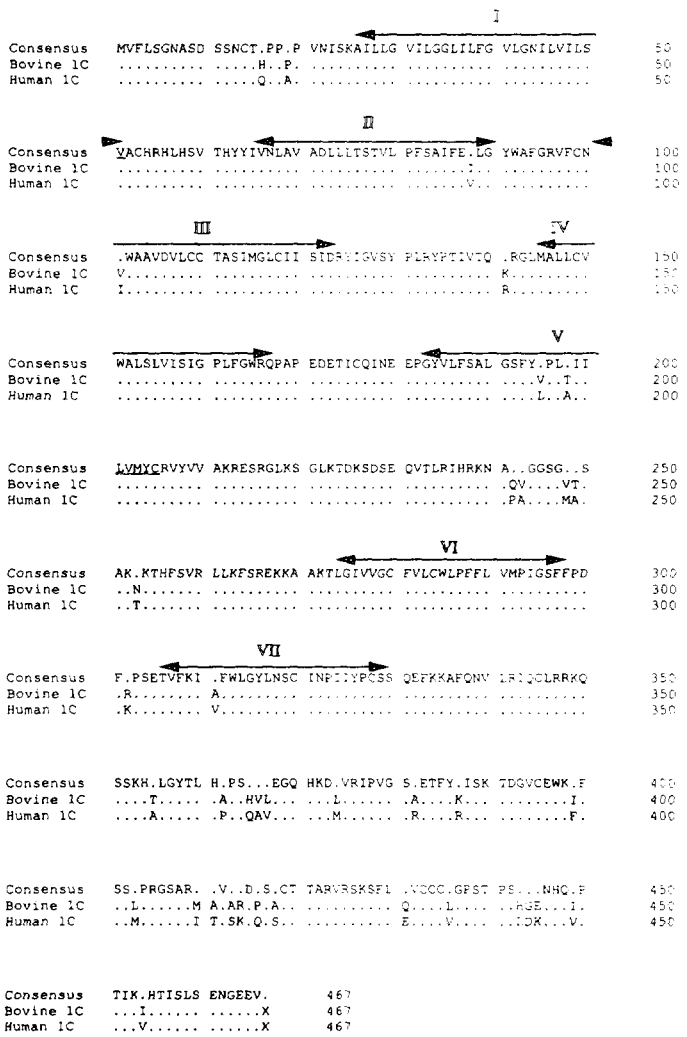

Fig. 2.

Alignment of the amino acid sequences of human and bovine α_{1C}-adrenergic receptors. Identical amino acids between the two sequences are not indicated. Putative membrane-spanning domains are indicated by I to VII and solid bars.

To examine the pharmacological characteristics of the clone P2C4, EcoRI-PstI 2100 bp fragment containing the entire 1398 bp coding region was inserted into the mammalian expression vector pME18S and the resulting construct pME-P2C4 was used to transfect COS-7 cells. COS-7 cells transfected with this vector was able to bind the antagonist radioligand [^{125}I]HEAT in a saturable manner with a high affinity (Kd, approximately 70 pM), and the level of expression for pME-P2C4 was approximately 600 fmol/mg of protein. Nontransfected COS-7 cells, or COS-7 cells transfected with a plasmid showed no specific [^{125}I]HEAT binding.

Table 1 summarizes ligand binding properties of COS-7 cells transfected with pME-P2C4. The agonist potency series confirm that P2C4 encodes α_1-adrenergic receptor. Also, the affinities of this clone for the agonists oxymetazoline and methoxamine and antagonists WB4101 and phentolamine were found to be approximately 10- to 100-fold more potent compared to those previously reported for the α_{1B} subtype (15). Furthermore, treatment with CEC inactivated 97% of the α_1 binding in COS-7 cells expressing the P2C4 clone. These data are consistent with the idea that this cDNA clone P2C4 encodes the human α_{1C}-adrenergic receptors.

To explore the tissue expression of the human α_{1C}-adrenergic receptor, Northern blot analysis was performed; however, the P2C4 probe detected very faint bands only in liver and

Table 1

Pharmacological characterization of expressed human α_{1C}-adrenergic receptors

	Ki (nM)
Agonists	
Oxymetazoline	28
(-)Epinephrine	2500
(+)Epinephrine	48000
Norepinephrine	7500
Methoxamine	19000
Isoproterenol	370000
Antagonists	
Prazosin	0.21
WB4101	0.23
Phentolamine	1.4
Yohimbine	770
5-Methylurapidil	4.2
Chlorethylclonidine	1200

COS-7 cell membranes transfected with the pME18s expression vector containing human α_{1C}-adrenergic receptors were incubated with the α_1-adrenergic receptor antagonist [^{125}I]HEAT, in the absence or presence of increasing concentrations of various agonists or antagonists. Each point represents the mean of at least two individual experiments, in duplicate. Ten concentrations of each ligand were tested, and the points were chosen to be on the linear portion of the displacement curve. Ki values were generated using the iterative curve-fitting program LIGAND.

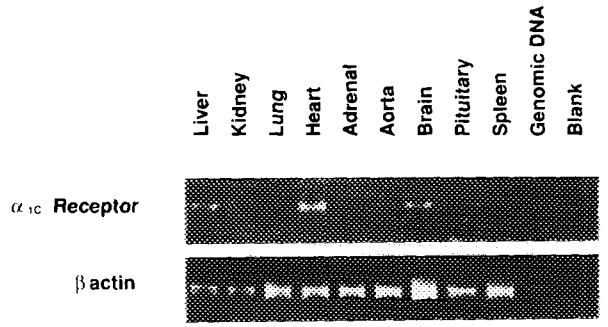

Fig. 3.
RT-PCR assay of α_{1C}-adrenergic receptor mRNA expression in human tissues. RT-PCR was performed with the primer set as described under ***Material and Methods***. cDNAs from various human tissues, genomic DNA and buffer (blank) were used as templates for PCR.

heart (data not shown). To detect α_{1C}-adrenergic receptor mRNA more sensitively, we assessed the tissue distribution with RT-PCR assay. Since it is reported that a large intron exists in the 6th. hydrophobic region for human α_{1B}-adrenergic receptor gene (16), and our preliminary experiment showed that human α_{1C}-adrenergic receptor gene has an intron in the same position, we designed a pair of primers which corresponds to the exon sequences on either side of the splice site. Using the primer sets, the PCR products were detected in brain, heart and liver; however, we could not find any PCR products in kidney, lung, adrenal, aorta, pituitary and spleen, and genomic DNA (Fig.3). Further, we confirmed that the PCR products obtained were originated from α_{1C}-adrenergic receptor transcripts by sequencing. We, therefore, concluded that this receptor expresses in human brain, heart, liver and prostate (data not shown). The tissue distribution of human α_{1C}-adrenergic receptor assessed with RT-PCR assay was partly confirmed by the previous *in situ* hybridization study of human brain (5) and a recent report of human prostate by RNA protection assay (17). Interestingly, the tissue distribution of human α_{1C}-adrenergic receptor is markedly different from those reported in rats and rabbits; thus, Northern blot analysis showed that no signal was observed in any rat tissue, but was present only in rabbit liver (5, 18).

In summary, we have isolated a cDNA for human α_{1C}-adrenergic receptor. With the information regarding pharmacological properties and tissue distribution of the receptor subtype, cloning of the human receptor cDNA would be instrumental in the developing selective agents for this not well-characterized receptor subtype.

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