

Cloning, functional expression and tissue distribution of human α_{1C} -adrenoceptor splice variants

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Abstract We report the cloning and characterization of two isoforms of human α_{1C} -adrenoceptor cDNA (α_{1C-2} , α_{1C-3}). These isoforms are generated by alternative splicing and differ from the clone we previously isolated (α_{1C-1}) in their length and sequences of the C-terminal domain. Tissue distribution of mRNAs showed that these variants co-express with α_{1C-1} in the human heart, liver, cerebellum and cerebrum. Despite the structural differences, functional experiments in transfected CHO cells showed that the three isoforms have similar ligand binding properties, and all couple with phospholipase C/Ca²⁺ signaling pathway.

Key words: α_1 -Adrenoceptor; Splice variant; Cloning; Tissue distribution

1. Introduction

α_1 -Adrenoceptors play critical roles in the regulation of a variety of physiologic processes. α_1 -Adrenoceptors comprise a heterogeneous family [1–3]. Two natively expressed subtypes (α_{1A} and α_{1B}) can be distinguished pharmacologically, while three subtypes (α_{1B} , α_{1C} , $\alpha_{1A/D}$) have been cloned [4–7]. The relationship between the cloned and native subtypes is currently uncertain, and it is postulated that α_{1C} -adrenoceptors codes the pharmacologically defined α_{1A} subtype. Alternatively, the pharmacologically defined α_{1A} subtype is suggested to be the result of splice variant(s) or some cell-specific posttranslational modification of one of the cDNAs already isolated [8,9]. However, so far any splice variant has never been reported for α_1 -adrenoceptors.

We recently isolated the human α_{1C} -adrenoceptor cDNA from human prostate cDNA library [10]. The receptor subtype is expressed abundantly in human tissues such as liver, heart, prostate and brain [10,11]. In the process of the cloning of α_{1C} -adrenoceptor, we obtained two isoforms (named α_{1C-2} and α_{1C-3}). Here we report that they can be produced through alternative splicing, and differ only in the C-terminal tail from the previously cloned α_{1C} -adrenoceptor (named α_{1C-1}). Also, we examined the distribution of mRNA of the three isoforms

in several human tissues in a quantitative manner using RT-PCR with mini-gene construct. Further, we constructed CHO cells stably expressing the three isoforms and compared their signal transduction and pharmacological properties.

2. Materials and methods

2.1. cDNA cloning

cDNA clones were isolated as described previously [10]. Briefly, human prostate λ gt11 library (1×10^6 recombinants; Clontech, Palo Alto, CA, USA) were screened with the 450 bp *Pst*I-*Xho*I fragment of human α_{1A} -adrenoceptor labeled with digoxigenin DNA labeling kit (Boehringer-Mannheim GmbH, Germany). A total of 1×10^6 plaques was 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. Cloned cDNAs, enzyme digested fragments and PCR products were subcloned into pUC18, pUC19 (TaKaRa, Kyoto, Japan) and pBluescript KS II(+) (Stratagene, La Jolla, CA, USA). Nucleotide sequence analysis was performed using overlapping templates by the ABI 373A DNA Sequencer (Applied Biosystems Inc., Foster City, CA, USA) for both complete strands.

2.2. RT-PCR assay

RT-PCR analysis was performed as described previously [10,12]. Total cellular RNA from human tissues (heart, liver, cerebrum, cerebellum and pituitary) were isolated by cesium chloride gradient method. 10 μ g of RNA was treated with RNase-free DNase I and reverse transcribed. Specific oligonucleotide primers were constructed from the cDNA sequences of cloned human α_{1C} -adrenoceptor isoforms α_{1C-1} , α_{1C-2} and α_{1C-3} . Mini-gene construct served as an internal standard for the efficacy of PCR amplification (Fig. 3A). The sequences of the human α_{1C} -adrenoceptor primers were: *primer-1* 5'-ATGCTCCAGC-CAAGAGTTCA-3' (coding sense) corresponding to bases 1417–1437 of the cloned full-length sequence; *1C-1* 5'-TCCAAGAAGAGCTG-GCCTTC-3' (anticoding sense) which anneals to bases 1917–1898 of the α_{1C-1} ; *1C-2* 5'-TGCCTTAGTCAGATGGATGC-3' (anticoding sense) which anneals to bases 1893–1874 of the α_{1C-2} ; *1C-3* 5'-GAG-ATCGAGATCATCTCTGGG-3' (anticoding sense) which anneals to bases 1924–1905 of the α_{1C-3} .

The predicted sizes of the amplified human α_{1C} -adrenoceptor isoforms, α_{1C-1} , α_{1C-2} and α_{1C-3} PCR products are 501, 477, and 508 bp, respectively. The sizes of mini-gene constructs amplified with primer sets of 1 and each primer specific for each isoform are 349, 329, and 309 bp, respectively. The PCR amplification profiles consisted of denaturation at 94°C for 1 min, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min, for 30 cycles. Negative control reactions without any template were routinely included in PCR amplifications for each primer set. For quantification of the amplified products, PCR was performed in the presence of [³²P]dCTP and 0.1 ng of minigene construct. PCR products were subjected to the PAGE and analyzed using BAS 2000 (Fuji, Tokyo, Japan). The amplified cDNA/minigene product ratio was used as the relative amount of mRNA of each isoform [13].

2.3. Southern blotting and PCR analysis

Human genomic DNA (10 μ g) prepared from placenta was digested

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Abbreviations: [¹²⁵I]HEAT, [¹²⁵I](2- β -(4-hydroxyphenyl)-ethylamino-methyl)-tetralon e; WB 4101, 2-(2,6-dimethoxyphenoxyethyl)-amino-methyl-1,4-benzodioxane; U-73122, 1-[6-[[17 β -3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrrole-2,5-dione; CEC, chlorethylclonidine; [Ca²⁺]_i, the intracellular free Ca²⁺ concentration; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse-transcription polymerase chain reaction; CHO cells, Chinese hamster ovary cells; NE, norepinephrine.

with *Bam*HI, *Eco*RI or *Hind*III. The resultant fragments were separated by electrophoresis on an 0.8% agarose gel, transferred onto a nylon membrane (Hybond-N⁺, Amersham Japan, Tokyo, Japan), then hybridized with a ³²P-labeled fragment (790–1311 bp) of α_{1C} -adrenoceptor cDNA clone. Hybridization was carried out for overnight at 65°C in buffer composed of 5 × saline-sodium citrate buffer (SSC), 5 × Denhardt's solution, 0.5% SDS, and heat-denatured salmon sperm DNA (100 µg/ml) and the filter was washed at 65°C in 0.1 × SSC/0.1% SDS for 30 min. PCR analysis was performed using the primers described above, LA-PCR kits (TaKaRa, Kyoto, Japan), and human genomic DNA as a template. Products were also analyzed by Southern blotting.

2.4. Cell culture and transfection

Cell culture of CHO cells and transfection were performed as described previously [14]. Briefly, CHO cells, grown in 5% CO₂ at 37°C, were plated at a density of 5 × 10⁶ cells/10-cm plate in 10 ml of medium F12 (Gibco/BRL, Life Technologies, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and penicillin/streptomycin, and allowed to attach overnight. The cloned α_{1C-1} , α_{1C-2} and α_{1C-3} were ligated into the pSVK-neo-R expression vector which contained neomycin resistance gene for selection of stable transformants. Cells were transfected with the plasmid by lipofection method. After overnight transfection, cells were fed with fresh medium, allowed to grow for 2 days, adjusted to a density of 2 × 10⁶ cells/plate, and incubated for an additional 24 h. Cells were continually selected for 3–4 weeks in medium containing 200 µg/ml levels of the antibiotic G418. Single colonies were then isolated, expanded, and harvested for radioligand binding assays to measure expression of receptors. Clones which express 1–2 pmol/mg membrane protein were selected for further experiments. Cells were maintained in the medium containing 200 µg/ml of G418. The medium was changed every 3 day, and the cells were subcultured after trypsinization.

2.5. Membrane preparation and [¹²⁵I]HEAT-binding assay

Membrane preparation of CHO cell and [¹²⁵I]HEAT-binding assay were performed as described previously [10,14]. Briefly, the cells were collected and disrupted by the sonicator (model Sonifier 250; setting 5 for 8 s) in ice-cold buffer containing 5 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 10 mM EGTA, 250 mM sucrose. The mixture was then centrifuged at 3,000 × g for 10 min. The supernatant fraction was centrifuged at 35,000 × g for 20 min. The resulting pellet was resuspended in binding buffer (50 mM Tris-HCl, pH 7.4, 12.5 mM MgCl₂, 10 mM EGTA). All buffers contain protein inhibitors of 1 mM PMSF, 100 µM benzamide, 1 µg/ml pepstatin A and 1 µg/ml leupeptin. The protein concentration was measured using the BCA protein assay kit (Pierce, Rockford, IL, USA).

Membrane aliquots (10 µg of protein) were incubated for 60 min at 25°C with [¹²⁵I]HEAT 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, USA). Specific [¹²⁵I]HEAT binding was experimentally determined from the difference between counts in the absence and presence of 10 µM phentolamine. From preliminary series of experiments, specific binding showed the kinetics, stereospecificity, and rank order of potency of agonists and antagonists characteristic of ligand-binding to α_1 -adrenoceptor [10,14]. *K_i* values were generated by the iterative curve-fitting program LIGAND [15].

2.6. [⁴⁵Ca²⁺]_i response

[Ca²⁺]_i response was measured as described previously [14]. Briefly, transfected CHO cells at 50% confluence in 100-mm dishes were trypsinized and washed with BSS buffer (140 mM NaCl, 4 mM KCl, 1 mM MgCl₂, 1.25 mM CaCl₂, 1 mM NaH₂PO₄, 5 mM HEPES, 11 mM glucose and 0.1% BSA, pH 7.4), and incubated in the buffer containing 4 µM fura-2/AM for 30 min at 25°C. The cells were then washed twice and incubated at room temperature for 10 min in BSS with or without 10 mM of U-73122, an inhibitor for agonist induced phospholipase C activation [16]. Mobilization of [Ca²⁺]_i evoked by 1 µM of norepinephrine were monitored by a Jasco CAF-110 fluorescence spectrophotometer (Nihon Bunkoh, Tokyo, Japan) with dual excitation at 340 nm/380 nm and emission at 500 nm. Calibration of the fluorescence levels was performed for every aliquot by addition of 5 µl of 10% Triton-X100 followed by addition of 5 µl of 300 mM EGTA/3 M Tris buffer (pH 9.0).

2.7. CEC treatment

For intact cells, cell suspension of two flasks in 20 ml of BSS buffer was incubated with or without 10 µM of CEC at 37°C for 30 min. After incubation, cells were washed three times with BSS and subjected to the binding assay. For the membrane, plasma membrane from CHO cells were incubated in 1 ml of hypotonic buffer (5 mM Tris, 5 mM EDTA, pH 7.6) with 10 µM of CEC at 37°C for 30 min, then the reaction were stopped by adding 16 ml of ice-cold buffer, and centrifuged at 35,000 × g for 20 min at 4°C. The membranes were washed and residual binding was assessed. Saturation analysis of specific [¹²⁵I]HEAT binding, or total and nonspecific binding of saturation concentration (400 pM) of [¹²⁵I]HEAT, was determined in duplicate.

2.8. Drugs

Sources of drugs were as follows: [¹²⁵I]HEAT (DuPont–New England Nuclear); methoxamine, phenylephrine, norepinephrine bitartrate, (–)-epinephrine, (+)-epinephrine, oxymetazoline, yohimbine (Sigma, St. Louis, MO, USA); WB4101, CEC, 5-methylurapidil and (+)-niguldipine (Research Biochemicals Inc., Natick, MA, USA); phentolamine-HCl (Ciba-Geigy, Summit, NJ, USA); prazosin (Pfizer, Sandwich, Kent, UK); U-73122 (Upjohn, Kalamazoo, MI, USA).

3. Results and discussion

Screening of human prostate cDNA library identified two other clones of C6 and C1A besides the previously reported three clones of P2, C4 and C7 (Fig. 1A) [10]. The clones, P2, C4 and C7 were used to construct the full length clone of α_{1C} -adrenoceptor (named α_{1C-1}) [10]. Sequencing of C6 and C1A clones showed that they encode the part of α_{1C} -adrenoceptor, while 3' portion is not identical to α_{1C-1} . To facilitate constructing the full-length clone, *Eco*RI–*Pvu*II 1450 bp restriction fragment from the clone P2 was ligated with the *Pvu*II–*Eco*RI restriction fragments from the clone C6 and C1A, and the resulting construct was named α_{1C-2} and α_{1C-3} , respectively (Fig. 1B). (The nucleotide sequences for α_{1C-2} and α_{1C-3} have been submitted to the GenBank Data Bank, with accession number D32202 and D32201, respectively.) Human α_{1C-1} , α_{1C-2} and α_{1C-3} are proteins of 466, 499 and 429 amino acids, respectively (Fig. 1C).

The three α_{1C} -adrenoceptor isoform proteins have the common transmembrane domain part and isoform-specific C-terminal (Fig. 1B), suggesting that mRNA of the three isoforms is derived from a single gene. To confirm this, Southern blot and PCR analysis of human genomic DNA were further con-

Table 1
Pharmacological characterization of expressed human α_{1C} -adrenoceptor isoforms

	α_{1C-1} <i>K_i</i> (nM)	α_{1C-2} <i>K_i</i> (nM)	α_{1C-3} <i>K_i</i> (nM)
Norepinephrine	1000	1300	1800
Methoxamine	6000	11000	7300
5-Methylurapidil	0.89	1.1	1.8
Phentolamine	2.7	2.3	2.2
WB-4101	0.21	0.21	0.20
Prazosin	0.17	0.13	0.16
(+)-Niguldipine	0.87	0.97	0.86

Membranes prepared from CHO cells stably expressing each α_{1C} -adrenoceptor isoform were incubated with the α_1 -adrenoceptor antagonist [¹²⁵I]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 ligands were tested, and the points were chosen to be the linear portion of the displacement curve. *K_i* values were generated by the iterative curve-fitting program LIGAND [15].

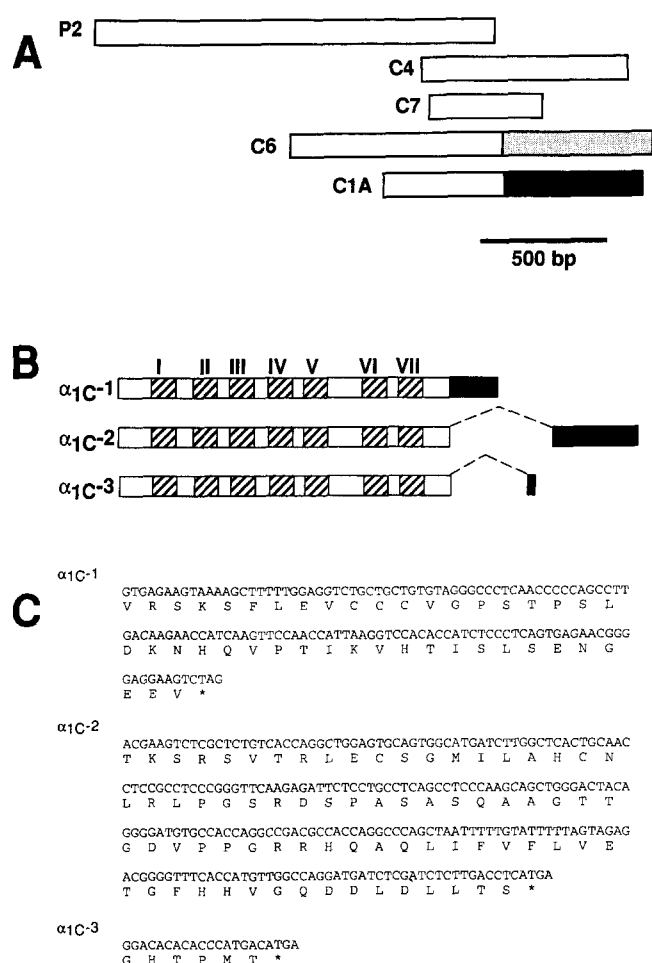


Fig. 1. Comparison of the cDNA structures of the three α_{1C} -adrenoceptor isoforms. (A) Schematic representation of the clones of human α_{1C} -adrenoceptor. The clones, C4, C6 and C1A are ligated with clone P2 and the full-length construct of α_{1C-1} , α_{1C-2} and α_{1C-3} were obtained, respectively. (B) Schematic representation of the sequence of human α_{1C-1} , α_{1C-2} and α_{1C-3} receptors. Boxes represent coding sequences; the open boxes are the common sequences among the three cDNAs, followed by sequences encoding different carboxyl-terminal-tail amino acid sequences (filled boxes). The putative transmembrane domains are indicated by Roman numerals (hatched boxes). (C) Nucleotide and amino acid sequences of the carboxyl-terminal regions of the α_{1C} -adrenoceptor isoforms. The deduced amino acid sequence is shown under the nucleotide sequence using the single letter code. The termination codons are indicated by asterisks.

ducted. As shown in Fig. 2A, all three lanes on treatment with the restriction enzymes tested, a single hybridization band was observed. While a standard PCR analysis showed that only the α_{1C-1} product was amplified from the human genomic DNA (Fig. 2B), a 13 kbp products was obtained by long-PCR analysis [17] using primer-1 and 1C-3 (Fig. 2C). Sequence and Southern hybridization analysis showed that the 13 kbp long-PCR product contains both C terminal portions of α_{1C-1} and α_{1C-3} , but does not contain that of α_{1C-2} (Fig. 2D). Also, no product was obtained by the same long-PCR condition when primer-1 and 1C-2 were used, and the C-terminal portion of α_{1C-2} did not hybridize the 13 kbp long-PCR product, suggesting that the C-terminal portion of α_{1C-2} does not locate within the 13 kbp region. These results revealed that mRNA of the three

α_{1C} -adrenoceptor isoforms originated from a single gene, and can be generated through alternative RNA splicing.

Next, expression of α_{1C-2} and α_{1C-3} in various human tissues was investigated by RT-PCR, using primers that were specific for each α_{1C} -adrenoceptor isoforms. The identity of these products as α_{1C} -adrenoceptor isoforms was confirmed by DNA sequencing (data not shown). To estimate the abundance of each transcript, we have synthesized mini-gene constructs (Fig. 3A) and added the constructs into the PCR as internal standards [13]. As shown in Fig. 3B, mRNA of the three α_{1C} -adrenoceptor isoforms is expressed in all the tissues examined. Also, the transcript of α_{1C-1} is the most abundant, while α_{1C-2} and α_{1C-3} are less (Fig. 3B).

To examine whether α_{1C} -adrenoceptor isoforms differ in the functional properties, CHO cells stably expressing α_{1C-1} , α_{1C-2} and α_{1C-3} receptors were compared in their ligand binding properties, the sensitivity to CEC alkylation, and also their coupling to $[Ca^{2+}]_i$ signaling. As summarized in Table 1, the ligands examined inhibited the $[^{125}I]$ HEAT binding at the three α_{1C} -adrenoceptor isoforms with similar potencies. Treatment of intact CHO cells with 10 μ M CEC decreased the $[^{125}I]$ HEAT binding sites, but with only 47%, 38% and 32% inhibition ($n = 3$ each) for the α_{1C-1} , α_{1C-2} and α_{1C-3} , respectively. The same CEC treatment of intact CHO cells stably expressing the human $\alpha_{1A/D}$ - and α_{1B} -adrenoceptors resulted in almost complete inactivation of the $[^{125}I]$ HEAT binding sites (94% and 93%, respectively; $n = 2$ each). Furthermore, hypotonic CEC (10 μ M) treatment of membrane preparations from CHO cells expressing the α_{1C-1} , α_{1C-2} and α_{1C-3} resulted in 74%, 73% and 83% inactivation ($n = 3$ each), respectively. The results showed that all human α_{1C} -AR isoforms are inactivated less by CEC compared to other α_1 -AR subtypes.

Next, norepinephrine (NE)-induced $[Ca^{2+}]_i$ responses were examined in CHO cells stably expressing α_{1C-1} , α_{1C-2} and α_{1C-3} receptors. As shown in Fig. 4, 1 μ M NE increased the $[Ca^{2+}]_i$ in all the three α_{1C} -adrenoceptor isoforms similarly. In all cells, NE-induced $[Ca^{2+}]_i$ responses were blocked by U-73122 (10 μ M) and prazosin (1 μ M, data not shown), and the wild-type CHO-K1 cells did not show any detectable response to NE (data not shown). Thus, all the three α_{1C} -adrenoceptor isoforms seem to couple to the phospholipase C/ $[Ca^{2+}]_i$ signaling pathway.

The α_{1C} -adrenoceptor isoforms shown in this study should be taken into account in considering the current confusing state of α_1 -adrenoceptor classification. As indicated in section 1, none of the three clones so far cloned ($\alpha_{1A/D}$, α_{1B} , α_{1C}) [4–7,10,18–20] can yet fully account for the pharmacologically defined α_{1A} subtype, and indeed, alternative splicing of multiple exons, resulting in multiple splice variants, could conceivably contribute to the current confusion. Our present study for the first time demonstrated the presence of splice variants of one of the cDNAs already isolated, α_{1C} -adrenoceptor, and clearly provided the supportive evidence for the above-mentioned possibility.

Since expression of α_{1C} -adrenoceptor cDNA had a high affinity for α_{1A} -selective drugs (the present study and [19,20]) and the tissue distribution of mRNA for the α_{1C} clone in rat is well correlated to that for the pharmacologically defined α_{1A} -adrenoceptor [21], most investigators are now considering that the cloned α_{1C} cDNA may encode the pharmacologically defined α_{1A} -adrenoceptor. The most critical issue that discourages

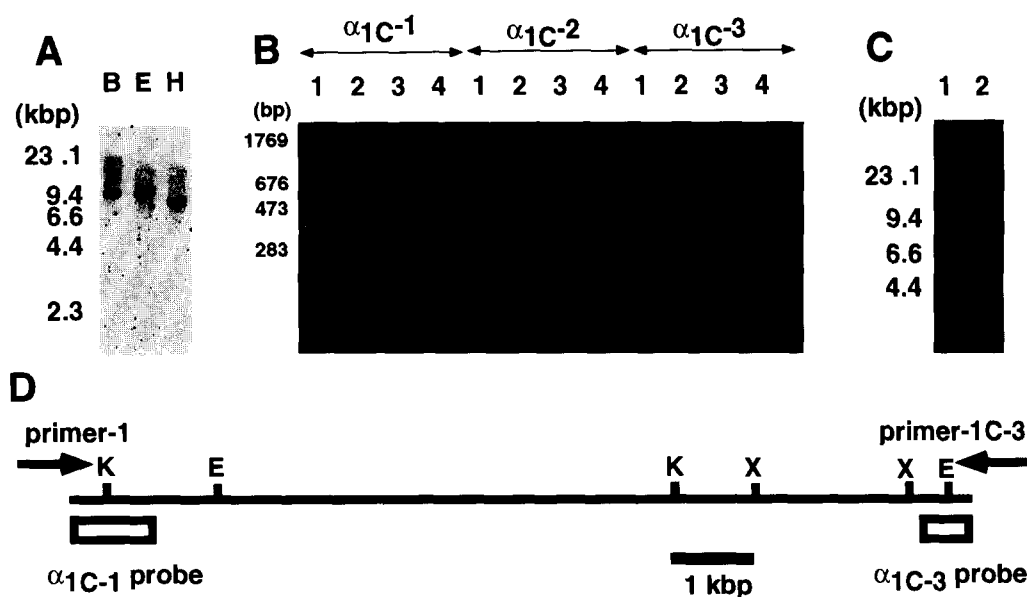


Fig. 2. Southern blot and PCR analysis of human genomic DNA. (A) Human genomic DNA (10 μ g) was digested with *Bam*HI (B), *Eco*RI (E) or *Hind*III (H). Hybridization analysis was carried out with the α_{1C} -adrenoceptor PCR fragments as a probe, as described in section 2. (B) Mini-gene construct (0.1 ng; lane 1), human genomic DNA (1 ng; lane 2), positive control cDNA (0.1 ng; lane 3) and negative control (without any template; lane 4) were amplified with specific sets of primers and analyzed by PAGE. (C) Long-PCR analysis of human genomic DNA with primer set of 1 and 1C-3 (lane 1); lane 2 shows a negative control. (D) Restriction enzyme map of the long-PCR product with primer set of 1 and 1C-3. E = *Eco*RI, X = *Xba*I, K = *Kpn*I.

to definitely conclude this idea is that the expressed α_{1C} -adrenoceptors are sensitive to inactivation by CEC, although the expressed α_{1C} is relatively insensitive to CEC inactivation compared to other two clones of α_{1B} and $\alpha_{1A/D}$ (the present study

and [18]). However, as recently reviewed by Ford et al. [9], we must interpret the present results of CEC inactivation with caution, since sensitivity to CEC is not an absolute measure [5,22], and also alkylation of cloned α_{1C} -adrenoceptor that are

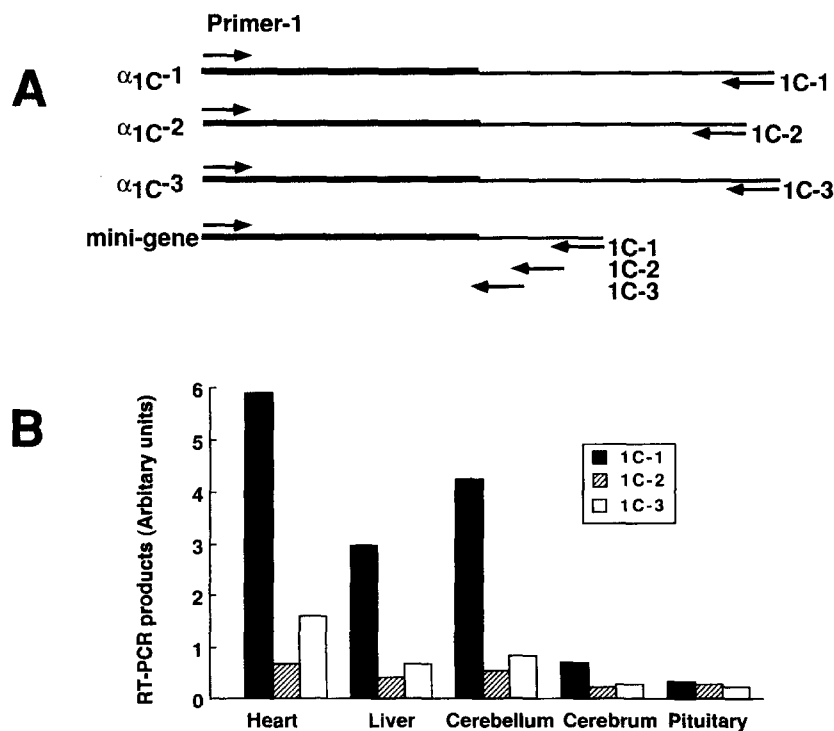


Fig. 3. Detection and quantitation of alternative spliced transcripts of the α_{1C} -adrenoceptor by RT-PCR. (A) Schematic presentation of the primer positions for splice variants. Annealing positions for the common primer (primer 1) and the primers specific for each isoform (1C-1, 1C-2, 1C-3) were indicated by arrow. (B) Relative amounts of the α_{1C} -adrenoceptor RT-PCR products determined as described in section 2. Each point is the mean value of at least 3 experiments.

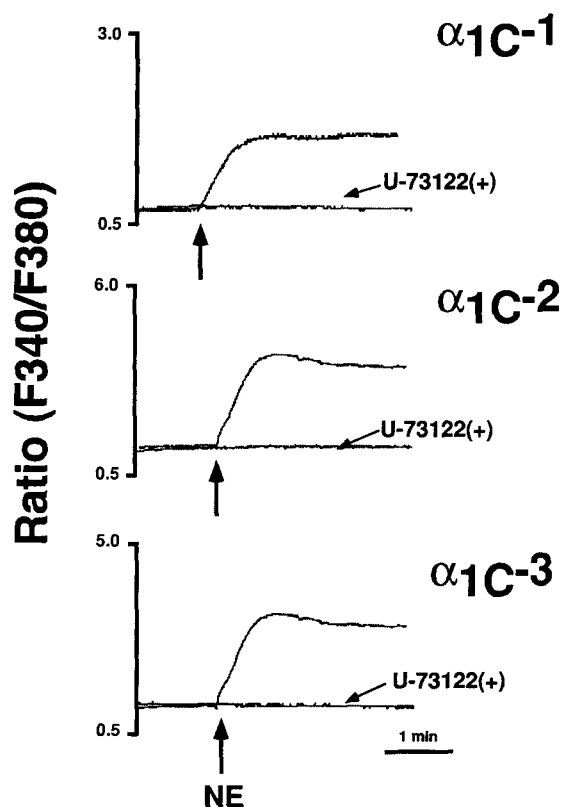


Fig. 4. Norepinephrine (NE)-induced $[Ca^{2+}]_i$ responses in CHO cells stably expressing α_{1C-1} , α_{1C-2} and α_{1C-3} receptors. Cells were loaded with fura-2/AM, pretreated with or without 10 μ M of U-73122 for 10 min, and NE (1 μ M)-induced $[Ca^{2+}]_i$ was determined by a fluorescence spectrophotometer with dual excitation at 340 nm/380 nm and emission at 500 nm, as described in section 2. The results presented are representative experiments of at least three similar experiments performed for each receptor.

overexpressed in CHO cells can hardly be compared directly with alkylation of the classical α_{1A} -adrenoceptor expressed in native tissues. As all the three α_{1C} -adrenoceptor isoforms appear to be generally localized in the same tissues, with different abundance though, and have similar pharmacological characters, all the isoforms may act as a group and can be pharmacologically defined as a single receptor site. This is important to note in assessing the relationship between the cloned cDNAs and the pharmacologically defined receptors. Furthermore, the three α_{1C} -adrenoceptor isoforms may differentially localize even within a tissue, which may confer different sensitivity to the very hydrophilic drug of CEC.

In conclusion, we have isolated, for the first time, splice variants of α_{1C} -adrenoceptors, and examined their pharmacological characters. The cloned variants are widely distributed in

native tissues with different abundance. These variants of α_{1C} -adrenoceptor give new insight into our understanding of the α_1 -adrenoceptor classification.

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