Molecular cloning, genomic characterization and expression of novel human α_{1A}-adrenoceptor isoforms

David J. Chang*, Thomas K. Chang, Susan S. Yamanishi, F.H. Rick Salazar, Alan H. Kosaka, Reena Khare, Sunil Bhakta, Jeffrey R. Jasper, Ing-Shih Shieh, John D. Lesnick, Anthony P.D.W. Ford, Donald V. Daniels, Richard M. Eglen, David E. Clarke, Chinh Bach, Hardy W. Chan

Center for Biological Research, Neurobiology Unit, Roche Bioscience, 3401 Hillview Avenue, Palo Alto, CA 94304, USA

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Abstract We have isolated and characterized from human prostate novel splice variants of the human α_{1A}-adrenoceptor, several of which generate truncated products and one isoform, α_{1A-4}, which has the identical splice site as the three previously described isoforms. Long-PCR on human genomic DNA showed that the α_{1A-4} exon is located between those encoding the α_{1A-1} and α_{1A-3} variants. CHO-K1 cells stably expressing α_{1A-4} showed ligand binding properties similar to those of the other functional isoforms as well as agonist-stimulated inositol phosphate accumulation. Quantitative PCR analyses revealed that α_{1A-4} is the most abundant isoform expressed in the prostate with high levels also detected in liver and heart.

Key words: α_1-Adrenoceptor; Splice variant; cDNA cloning; Genome organization; Expression

1. Introduction

Alpha$_1$-adrenoceptors are G-protein coupled transmembrane receptors which mediate various actions of the peripheral sympathetic nervous system through the binding of the catecholamines, epinephrine and norepinephrine [1,2]. Molecular cloning has identified three subtypes of the α_1-adrenoceptor class in several species including human [3–6], and these have been classified as α_{1A}- (previously α_{1C}-), α_{1B}- and α_{1D}-adrenoceptors [7]. A putative fourth subtype, the α_{1L}-adrenoceptor, which has low affinity for the antagonist, prazosin, has been defined in functional but not in molecular studies. The α_{1L}-adrenoceptor is postulated to mediate the contraction of several lower urinary tract tissues, including the smooth muscle in the human prostate, suggesting a role for this subtype in the bladder outlet obstruction observed in patients with benign prostatic hypertrophy [8,9].

Recent in-house data have shown that the cloned human α_{1A}-adrenoceptor exhibits the pharmacological profile of the α_{1L}-adrenoceptor in functional studies but not in ligand binding studies [10]. These observations suggest that the α_{1A}-adrenoceptor gene may encode gene products that exhibit both the α_{1A} and α_{1L} pharmacology. In addition, Tsujimoto and colleagues [11] have identified in human prostate two isoforms of the α_{1A}-adrenoceptor cDNA generated by alternative splicing, which differ from the 'original' α_{1A}-adrenoceptor cDNA in length and sequence at the carboxy-terminal region. Following the nomenclature for α_1-adrenoceptors recommended by the Nomenclature Committee of the International Union of Pharmacology (NC-IUPHAR) [7,12], these isoforms are named α_{1A-1}, α_{1A-2} and α_{1A-3}. In this paper we describe the identification of additional α_{1A}-adrenoceptor splice variants from human prostate, many of which give rise to truncated products, and also a fourth isoform containing a unique carboxy-terminus, α_{1A-4}. In addition, we have localized the 'isoform-specific' exons on human genomic DNA and examined functional expression and tissue distribution of these isoforms.

2. Materials and methods

2.1. cDNA library construction and screening

Two RNA sources were used to prepare the cDNA library, one from a freshly frozen non-tumor prostate tissue collected from a patient undergoing radical prostatectomy, and the other from commercially obtained human prostate mRNA (Clontech, Palo Alto, CA). The cDNA was ligated into lambda ZAP II vector and packaged with Gigapack Gold packaging extract (Stratagene, La Jolla, CA). 3.5×10^6 recombinant clones were screened with a 1.3 kbp *Hin*dIII fragment of the published human α_{1A}-adrenoceptor cDNA [3]. Hybridization was carried out according to standard procedures [13]. Positive phage clones were converted into phagemids by in vivo excision and coinfection of the R408 helper phage (Stratagene). Nucleotide sequence analysis was performed on the ABI 373A DNA Sequencer (Applied Biosystems, Foster City, CA) and determined on both DNA strands.

2.2. PCR isolation of α_{1A}-adrenoceptor isoforms

A sense primer derived from nucleotides 753–775 (5′-CAAGACCAAGACGCACTTCTCAG-3′) in the third cytoplasmic loop region between transmembrane domains V and VI and antisense primers consisting of random hexamer oligonucleotides were used to generate PCR products from human prostate Quickclone cDNA (Clontech). PCR conditions consisted of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min for 35 cycles. Fragments greater than 1 kbp in length were subcloned in PCR II vector (Invitrogen, San Diego, CA) and subjected to sequence analysis. The full-length coding sequence of the α_{1A-3}-adrenoceptor was constructed by ligating the 5′ portion of the α_{1A-1}-adrenoceptor cDNA with the PCR-generated 3′ fragment of the α_{1A-3}-adrenoceptor.

2.3. Quantitative PCR analysis

Expression levels of the α_{1A}-adrenoceptor isoforms were quantitated by competitive PCR using Quickclone cDNAs from human heart, kidney, liver and prostate as templates. PCR amplification of

*Corresponding author. Fax: (1) (650) 855-1238.
E-mail: david-j.chang@roche.com

Abbreviations: PCR, polymerase chain reaction; CHO, Chinese hamster ovary; EDTA, ethylene diamine tetraacetic acid; RS-17503, *N*-[2-(2-cyclopropylmethoxy-phenoxy)ethyl]-5-chloro-α,α-dimethyl-1*H*-indole-3-ethanamine hydrochloride; WB4101, (2,6-dimethoxyphenoxyethyl) aminomethyl-1,4-benzodioxane hydrochloride

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the targets was performed in the presence of truncated versions of the target (Δ standards) which contained excisions of 50–100 bp in the regions flanked by the PCR primers. The sequences of the primers are as follows: sense $\alpha_{1A-1/-2/-3}$: 5′-GCATCGTGGTCGGCTGCTTC-GTCCTCTGCTG-3′; sense α_{1A-4}: 5′-CTAGAATGCTGATCTCC-AGGT-3′; antisense α_{1A-1}: 5′-TCTTTCCTGTCCTAGACTTCCT-CC-3′; antisense α_{1A-2}: 5′-CACCAGTCACCATGGAGAGAATG-3′; antisense α_{1A-3}: 5′-AGGACAACAGTCGTGGACG-3′; antisense α_{1A-4}: 5′-CTCCTGAGCCACACGAAAAG-3′. PCR products were run on 1% agarose gel, stained with ethidium bromide and analyzed on a molecular imager (PDI). The ratio between each amplified cDNA/Δ standard was used as the amount of mRNA present for each isoform.

2.4. Determination of genomic organization

PCR on human genomic DNA was performed using a sense primer corresponding to the common transmembrane domain VII region of the α_{1A}-adrenoceptor from nucleotides 981–1000 (5′-ATGCTC-CAGCCAAGAGTTCA-3′), a sense primer in α_{1A-4} (5′-CCA-GAATGCTGATCTCCAGGT-3′), and antisense primers in α_{1A-4} (5′-CTCCAGCTACCTGGAGATCAG-3′) and α_{1A-3} (5′-TCATGT-CATGGGTGTGTGTC-3′). Products were run on agarose gels and the lengths were estimated by standard markers. The length of the intron between α_{1A-1} and α_{1A-4} exonic sequences was confirmed by DNA sequencing.

2.5. Stable expression cell lines

The cloned α_{1A}-adrenoceptor isoform cDNAs were ligated into pSW104 expression vector which contains the SR-α promoter [14] and Bluescript KS^+ plasmid multiple cloning site. The constructs were co-transfected with a pSV2neo plasmid into CHO-K1 cells using the lipofection method [15]. The cells were grown in Ham's F12 medium (Gibco-BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum and penicillin/streptomycin (30 U/ml, 30 μg/ml) at 37°C in 5% CO_2. Stable single clones were selected for resistance to G418 at 400 μg/ml concentration, expanded and harvested for radioligand binding and functional studies.

2.6. Radioligand binding assay

Cells were lysed by Polytron (Brinkman, New York, NY) homogenization in ice-cold 50 mM Tris-HCl, 5 mM EDTA pH 7.4 buffer. The lysate was centrifuged and the pelleted membrane was resuspended in 50 mM Tris-HCl, 0.5 mM EDTA pH 7.4 buffer. The membranes were incubated with [^{3}H]prazosin, specific activity 82 Ci/mmol (New England Nuclear, Boston, MA) for 60 min at 25°C in the presence or absence of competing drugs. Incubation was terminated by vacuum filtration through GF/B filters pretreated with 0.1% polyethyleneimine. Bound radioligand was counted using liquid scintillation spectroscopy.

2.7. Inositol phosphate assay

Inositol phosphate accumulation was determined using a modification of described procedures [16]. Cells were pre-loaded overnight with [^{3}H]*myo*-inositol (2 μCi/ml), washed and resuspended in inositol-free Ham's medium. Antagonist was then added for 20 min at 37°C followed by the addition of 2 μM norepinephrine with LiCl (10 mM) and incubation at 37°C for 10 min. Reactions were terminated by addition of ice-cold perchloric acid (20% w/v) and neutralized samples were decanted into a Dowex AG 1×8 (chloride form) column and [^{3}H]inositol phosphates were eluted for liquid scintillation counting.

3. Results and discussion

Screening of a mixed human prostate cDNA library with a 1.3 kbp fragment comprising the coding sequence of the human α_{1A}-adrenoceptor yielded a total of 28 positive clones. DNA sequencing of all the clones revealed that they fall into five different classes. Several clones contained either full-length coding or partial sequences identical to that of the original published human α_{1A}-adrenoceptor cDNA, the α_{1A-1} isoform [11]. Five clones were isolated and found to contain sequences virtually identical to the α_{1A-2} isoform except at one position. A deletion of a G at position 1393 results in a shift in the reading frame after amino acid residue 464 and introduces a stop codon following amino acid residue 475 (Fig. 1A). This nucleotide change was confirmed by sequencing both DNA strands from all the clones and the reason for this difference is unclear.

Two clones, #10 and #15, were identified to contain coding sequences which terminate in transmembrane domain VI (Fig. 1A). Close examination of the nucleotide sequences reveals a divergence occurring at position 883 which has been identified as an intron/exon junction by Tseng-Crank and colleagues [17] and suggests that these clones are generated through alternative RNA splicing at the acceptor sites. Furthermore, clone #15 contains one extra T residue between nucleotide positions 866 and 867 resulting in a shift in reading frame, which was also observed in human liver-derived α_{1A} clones in the aforementioned study [17].

Most interestingly, a 4.5 kbp clone, #13, was identified with a nucleotide sequence identical to that of the published isoforms up to position 1269 (including all the transmembrane domains) but followed by a novel sequence. Since this is also the location where the published isoforms diverge in their carboxy-terminal sequences, we believe clone #13 is a novel isoform and have named it α_{1A-4} (GenBank accession number AF013261). The deduced amino acid sequence of α_{1A-4} (Fig. 1B) reveals that it encodes a protein of 455 amino acids and contains almost 1.7 kbp of 3′ untranslated region. The unique portion of the carboxy-terminal tail contains two Ser/Thr res-

Table 1
Pharmacological profiles of human α_{1A}-adrenoceptor isoforms

	α_{1A-1}	α_{1A-2}	α_{1A-3}	α_{1A-4}
Prazosin[a]	9.9 ± 0.0	9.7 ± 0.4	9.2 ± 0.2	9.8 ± 0.1
Tamsulosin[a]	10.4 ± 0.2	10.5 ± 0.4	10.0 ± 0.1	10.4 ± 0.2
RS-17053[a]	9.2 ± 0.1	8.9 ± 0.4	8.8 ± 0.1	8.9 ± 0.0
WB 4101[a]	9.8 ± 0.1	9.6 ± 0.3	9.2 ± 0.2	9.4 ± 0.2
5-Me-urapidil[a]	9.2 ± 0.1	8.9 ± 0.4	8.4 ± 0.2	9.0 ± 0.1
Prazosin[b]	8.7 ± 0.0			8.8 ± 0.1
Tamsulosin[b]	10.5 ± 0.1			10.1 ± 0.1
RS-17053[b]	8.2 ± 0.1			8.0 ± 0.4
WB 4101[b]	8.9 ± 0.1			8.5 ± 0.2
(+)Niguldipine[b]	8.4 ± 0.3			8.2 ± 0.1

[a]Affinities (pK_i) of ligands for α_{1A}-adrenoceptor isoforms. Data are the means ± S.E.M. ($n = 3$) from radioligand displacement experiments using recombinant receptors expressed in CHO-K1 cells.
[b]Affinity estimates (pK_b) of ligands for α_{1A-1} and α_{1A-4} receptors. Data are the means ± S.E.M. ($n = 3$) from antagonist inhibition curves obtained against (−)norepinephrine-stimulated [^{3}H]inositol phosphate accumulation in CHO-K1 cells expressing the isoforms.

A

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        287  TM VI            TM VII                 336
α1A-1   PFFLVMPIGS FFPDFKPSET VFKIVFWLGY LNSCINPIIY PCSSQEFKKA
α1A-2   PFFLVMPIGS FFPDFKPSET VFKIVFWLGY LNSCINPIIY PCSSQEFKKA
α1A-3   PFFLVMPIGS FFPDFKPSET VFKIVFWLGY LNSCINPIIY PCSSQEFKKA
   10   PFFLVMPIDE QRGEGSGFST HH..... ..... ..... .....
   11   PFFLVMPIGK K..... ..... ..... ..... .....
   15   PFFLSHAHWN GSCILSS... ..... ..... ..... .....
   21   PFFLVMPIDE VSLCHQAGVQ WHDLGSLQPP PPGFKRFSCL SLPSSWDYRD

        337                                          386
α1A-1   FQNVLRIQCL CRKQSSKHAL GYTLHPPSQA VEGQHKDMVR IPVGSRETFY
α1A-2   FQNVLRIQCL CRKQSSKHAL GYTLHPPSQA VEGQHKDMVR IPVGSRETFY
α1A-3   FQNVLRIQCL CRKQSSKHAL GYTLHPPSQA VEGQHKDMVR IPVGSRETFY
   10   ..... ..... ..... ..... ..... ..... ..... .....
   11   ..... ..... ..... ..... ..... ..... ..... .....
   15   ..... ..... ..... ..... ..... ..... ..... .....
   21   VPPGRRHQAQ LIFVFLVETG FHHVGQDDLD LLTS..... .....

        387                                          436
α1A-1   RISKTDGVCE WKFFSSMPRG SARITVSKDQ SSCTTARVRS KSFLQVCCCV
α1A-2   RISKTDGVCE WKFFSSMPRG SARITVSKDQ SSCTTARTKS RSVTRLECSG
α1A-3   RISKTDGVCE WKFFSSMPRG SARITVSKDQ SSCTTARGHT PMT
   10   ..... ..... ..... ..... ..... ..... ..... .....
   11   ..... ..... ..... ..... ..... ..... ..... .....
   15   ..... ..... ..... ..... ..... ..... ..... .....
   21   ..... ..... ..... ..... ..... ..... ..... .....

        437                          466
α1A-1   GPSTPSLDKN HQVPTIKVHT ISLSENGEEV .....
α1A-2   MILGHCNLRL PGSRDSPASA SQAAGTTGMC HQADATRPS
α1A-3   ..... ..... ..... ..... ..... ..... ..... .....
   10   ..... ..... ..... ..... ..... ..... ..... .....
   11   ..... ..... ..... ..... ..... ..... ..... .....
   15   ..... ..... ..... ..... ..... ..... ..... .....
   21   ..... ..... ..... ..... ..... ..... ..... .....
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B

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1270  AGGGGAATGGATTGTAGATATTTCACCAAGAATTGCAGAGAGCATATCAA  1319
 424  R  G  M  D  C  R  Y  F  T  K  N  C  R  E  H  I  K   440

1320  GCATGTGAATTTTATGATGCCACCGTGGAGAAAGGGTCTAGAATGCTGA   1368
 441   H  V  N  F  M  M  P  P  W  R  K  G  L  E  C  *     455
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Fig. 1. A: Deduced carboxy-terminal amino acid sequences of truncated α_{1A}-adrenoceptor isoforms isolated from human prostate cDNA. The corresponding amino acid sequences of α_{1A-1}, α_{1A-2} (differences from published sequence are underlined) and α_{1A-3} receptors are also shown. B: Nucleotide (top) and amino acid (bottom) sequences of the carboxy-terminal region of α_{1A-4} which differs from the three other functional isoforms. The nucleotide and amino acid positions (from the translation start site) are shown at both ends.

idues as compared to nine in the unique portion of α_{1A-1}, 12 in α_{1A-2} and two in α_{1A-3}. Whether any of these residues are sites of phosphorylation which might dictate differences in down-regulation of the isoforms is not known.

Since we did not isolate the α_{1A-3} isoform from screening the prostate lambda phage cDNA library and because there may be additional α_{1A}-adrenoceptor isoforms, we performed PCR on prostate cDNA with random and isoform-specific primers. Using this method, we successfully isolated α_{1A-3} and also identified two more isoforms which encode truncated proteins. Both clones #11 and #21 (Fig. 1A) show sequence divergence following the intron/exon junction in transmembrane domain VI. Clone #11 contains a 180 bp insert with an in-frame stop codon after which the sequence matches that of α_{1A-1} (data not shown). A human hippocampus-derived α_{1A}-adrenoceptor clone (1088-7) with similar if not identical sequence has been previously described [17]. In contrast, clone #21 utilizes the same splice acceptor site as α_{1A-2} (data not shown) but with a shift in reading frame which results in a different amino acid sequence.

To determine the genomic organization of the exon cassettes which contain the unique 3′ sequences of the non-truncated isoforms ($\alpha_{1A-1/-2/-3/-4}$), human genomic DNA was subjected to long-range PCR analysis. PCR using a sense primer from the common transmembrane domain VII sequence and antisense primers in the unique sequences for α_{1A-4} and α_{1A-3} yielded products with sizes of approximately 4.5 kbp and 13 kbp, respectively (Fig. 2A). The distance between α_{1A-4} and α_{1A-3} was also deduced when a 9 kbp product was generated in the PCR reaction with sense primer in the α_{1A-4} sequence and antisense primer in the α_{1A-3} sequence. Direct sequencing of genomic DNA by primer walking confirmed the locations of the exon cassettes for α_{1A-1}, α_{1A-4} and α_{1A-3} as well as the lengths of the introns (Fig. 2C). The exact length of the α_{1A-3} exon cassette could not be determined because this clone was derived by PCR.

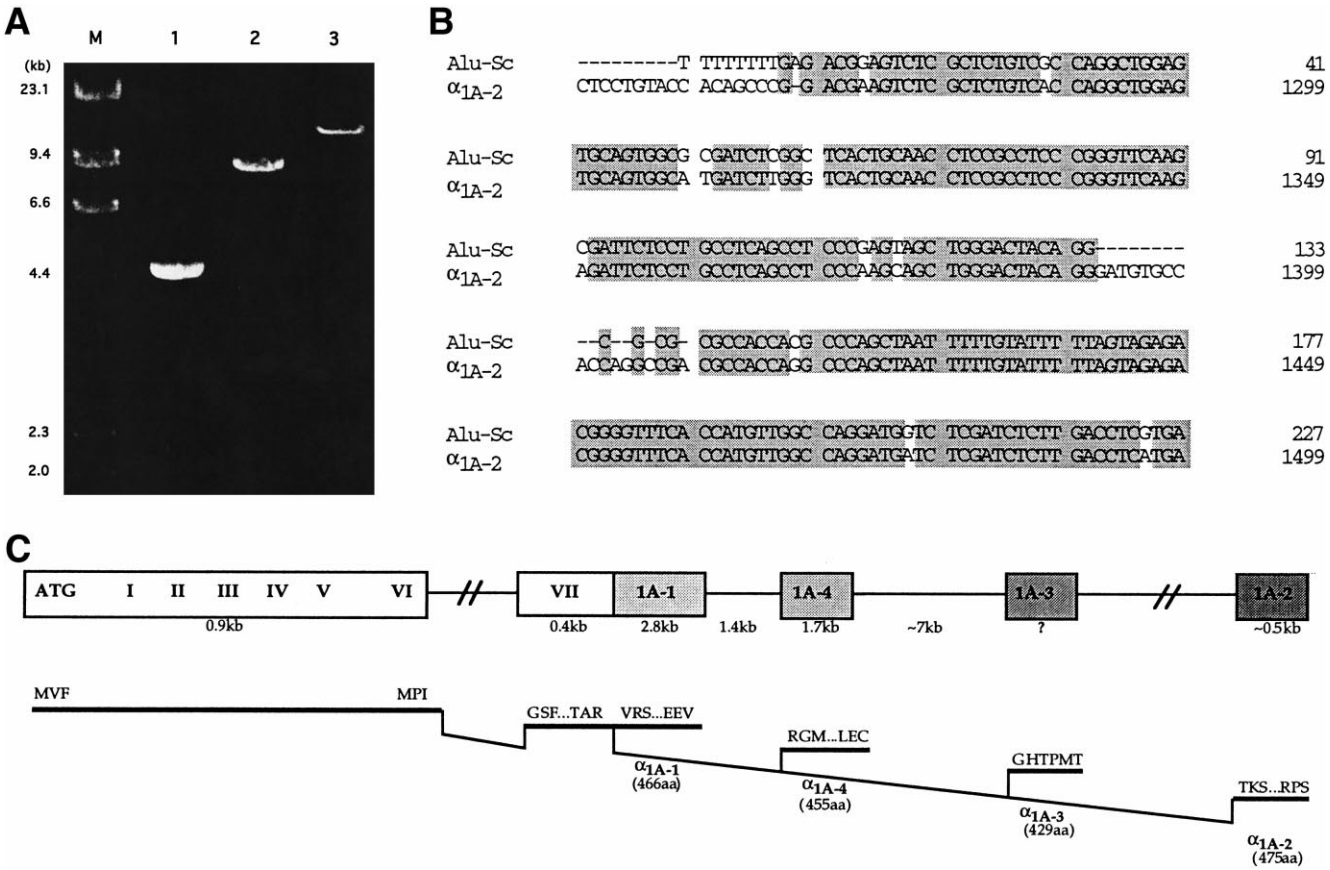

Fig. 2. A: PCR analysis of human genomic DNA using primers described in Section 2. Lanes are: (M) lambda *Hin*dIII marker, (1) common TM VII sense primer with α_{1A-4} antisense primer, (2) α_{1A-4} sense primer with α_{1A-3} antisense primer, (3) common TM VII sense primer with α_{1A-3} antisense primer. B: Comparison between the 3′ sequence of α_{1A-2} and the consensus Alu-Sc sequence (GenBank accession number U14571). The overall homology between the two sequences within this region is 87%. C: Genomic organization of the human α_{1A}-adrenoceptor isoforms. The top figure is a schematic representation of the human α_{1A}-adrenoceptor gene with boxes representing exons and lines representing introns. The numbers below the boxes and lines represent approximate lengths of exons and introns in nucleotide base pairs. The bottom figure shows the location of splice sites, and the amino acid sequences at the amino-terminus, donor and acceptor sites, and at the carboxy-termini. The full-size lengths of each splice variant product are shown in parentheses.

Attempts to localize the α_{1A-2} exon cassette by PCR using α_{1A-2}-specific primers were unsuccessful. Close examination of the unique 3′ sequence of this isoform reveals a very high homology to the Alu-Sc gene family (Fig. 2B) which are interspersed throughout the human genome [18]. Although the presence of an Alu element in an open reading frame could be the result of an artifact from cDNA cloning, we do not believe α_{1A-2} was generated this way for two reasons. First, the splice site is identical to that for α_{1A-3} and α_{1A-4}. Second, α_{1A-2} can be identified from both cDNA library screening and PCR amplification of tissue mRNA (described below). Since Alu elements can be actively transcribed by RNA polymerase III, the splicing mechanism in the generation of α_{1A-2} may not necessarily involve an intramolecular reaction (*cis*-splicing) but an intermolecular splicing of exons between two different RNAs (*trans*-splicing), which has been observed more commonly in genes in lower organisms and in mitochondria [19,20].

All the newly isolated α_{1A}-adrenoceptor isoforms were transiently expressed in Cos-7 cells and tested for ligand binding activities. As expected, α_{1A-4} displayed appropriate binding affinity to [^{3}H]prazosin but none of the truncated isoforms (#10, 11, 15, 21) exhibited any specific radioligand binding (data not shown). It is not known, however, whether these truncated isoforms are actually expressed on the plasma membrane following transient transfection and so we cannot state that the generated products are non-functional.

Next, the pharmacological profiles of the recombinant α_{1A-1}-, α_{1A-2}-, α_{1A-3}- and α_{1A-4}-adrenoceptors, stably expressed in CHO-K1 cells, were compared using radioligand binding. In addition, functional estimates of affinity were determined for several ligands at the α_{1A-1}- and α_{1A-4}-adrenoceptors by measuring antagonism of (−)norepinephrine-stimulated accumulation of inositol phosphates. As seen in Table 1, binding studies showed that all four isoforms display the classical α_{1A}-adrenoceptor profile (subnanomolar affinities for prazosin and WB4101) with similar affinities for all the tested ligands. In functional studies, both α_{1A-1}- and α_{1A-4}-adrenoceptors exhibited lower affinities for prazosin and RS-17053, indicative of α_{1L}-adrenoceptor pharmacology. These two receptors were virtually indistinguishable in their operational profiles (Table 1). Recent data have shown that α_{1A-2}- and α_{1A-3}-adrenoceptors also demonstrate α_{1L}-receptor pharmacology in functional studies [21]. Interestingly, norepinephrine also stimulates cyclic AMP accumulation in cells transfected with the four adrenoceptor isoforms (S. Yamanishi and D. Daniels, unpublished observations) suggesting that the α_{1A}-adrenoceptor in a recombinant system can couple

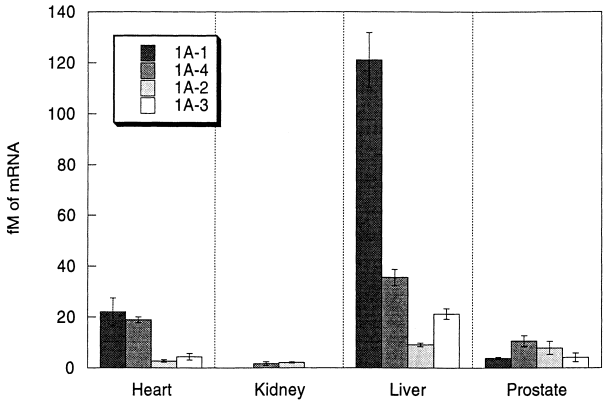

Fig. 3. mRNA levels of the four functional isoforms of the α_{1A}-adrenoceptor in the human heart, kidney, liver, and prostate. The expression levels were estimated using truncated standards in competitive PCR experiments and recalculated as femtomolar (fM) concentration of the target present in the cDNAs. Each value is an average of two separate experiments with standard errors shown.

to Gs in addition to Gq, an observation previously shown for the hamster α_{1B}-adrenoceptor [22].

The expression levels of the four functional isoforms in human heart, kidney, liver and prostate were determined by quantitative PCR with truncated constructs as internal standards. As seen in Fig. 3, total α_{1A}-adrenoceptor mRNA level was highest in the liver, followed by heart, prostate and kidney. The abundance of each isoform varied in the different tissues, with α_{1A-1} showing the highest levels in liver and heart. Interestingly, the newly identified α_{1A-4} was the most abundant isoform in the prostate and α_{1A-1} was the least abundant. This observation was rather surprising since the prostate has been used in several studies to clone the human α_{1A}-adrenoceptor [3,5,11] and the α_{1A-4} isoform has not been identified until now. We did not measure mRNA levels of the truncated isoforms except for clone #21 which showed extremely low (<1 fM) amounts in all four tissues examined (data not shown).

The physiological significance of α_{1A}-adrenoceptor splice variants is presently unknown. Since the four functional isoforms differ only in their carboxy-terminal regions, they may differ in their preference for G-protein coupling as observed in the isoforms of the prostaglandin EP3 receptor [23] or in rates of desensitization mediated by phosphorylation as seen in the α_{1B}- [24] and β_2- [25] adrenergic receptors. The role of the truncated isoforms is equally if not more perplexing. Assuming the truncated proteins reach the plasma membrane, the possibility that there may be inter-receptor interactions, as shown for the muscarinic receptors [26,27], which not only restore function but also introduce novel pharmacology is intriguing. Finally, the results of the mRNA expression study raise the possibility that relative levels of the isoforms in a tissue may change depending on its pathological state (for example, normal vs. hyperplastic prostate) and provide a molecular explanation for the initiation or development of a disease.

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