ELSEVIER

European Journal of Pharmacology 396 (2000) 9–17

ejp

www.elsevier.nl/locate/ejphar

Cloning of rabbit α_{1b}-adrenoceptor and pharmacological comparison of α_{1a}-, α_{1b}- and α_{1d}-adrenoceptors in rabbit

Hailan Piao, Takanobu Taniguchi, Seigo Nakamura, Jun Zhu, Fumiko Suzuki, Daisuke Mikami, Ikunobu Muramatsu *

Department of Pharmacology, School of Medicine, Fukui Medical University, Matsuoka, Fukui 910-1193, Japan

Received 26 August 1999; received in revised form 21 February 2000; accepted 25 February 2000

Abstract

We have isolated a cDNA clone of the rabbit α_{1b}-adrenoceptor which has an open reading frame of 1557 nucleotides encoding a protein of 518 amino acids. The sequence shows higher identity to those of hamster, human, and rat α_{1b}-adrenoceptors than to those of rabbit α_{1a}- and α_{1d}-adrenoceptors. The pharmacological binding properties of this clone expressed in Cos-7 cells showed a characteristic profile as α_{1b}-adrenoceptor; high affinity for prazosin ($pK_i = 10.3$), relatively high affinity for tamsulosin (9.5) and low affinity for (−)-(*R*)-1-(3-hydroxypropyl)-5-[2-[[2-[2-(2,2,2-trifluoroethoxy)phenoxy]ethyl]amino]propyl]indoline-7-carboxamide (KMD3213) (8.5), 2-(2,6-dimethoxy-phenoxyethyl)-aminomethyl-1,4-benzodioxane hydrochloride (WB4101) (8.7), and 8-[2-[4-(2-methoxy-phenyl)-L-piperazinyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride (BMY7378) (7.3). We have compared the levels of mRNA expression of three α_1-adrenoceptor subtypes in rabbit tissues using the competitive reverse transcription/polymerase chain reaction (RT/PCR) assay. In most rabbit tissues except heart, α_{1a}-adrenoceptor mRNA was expressed 10 folds more than the other two subtypes. However, binding experiments with [^{3}H]prazosin and [^{3}H]KMD3213 in rabbit tissues revealed a poor relationship between binding density and mRNA level. Especially, α_{1b} binding sites were exclusively predominant in spleen, whereas the α_{1b} subtype was minor at the mRNA level. These results indicate a high identity of structural and pharmacological profiles of three distinct α_1-adrenoceptor subtypes between rabbit and other species, but there are species differences in their distribution.

Keywords: α_{1a}-Adrenoceptor; α_{1b}-Adrenoceptor; α_{1d}-Adrenoceptor; Rabbit; Molecular cloning; Pharmacology; Tissue distribution

1. Introduction

α_1-Adrenoceptors are a heterogeneous family (Minneman and Esbenshade, 1994; Michel et al., 1995; Hieble et al., 1995; Graham et al., 1996). Presently, at least three distinct α_1-adrenoceptor subtypes (α_{1a}, α_{1b}, and α_{1d}, with lowercase letters) have been cloned that exhibit pharmacological equivalence to the native α_{1A}-, α_{1B}-, and α_{1D}-adrenoceptors (with uppercase letters). Each subtype shows wide tissue distribution and coexistence at the mRNA level in humans and rats, but the functional dominance in adrenergic responses varies among tissues; for example, a predominant involvement of α_{1D} in thoracic aorta (Kenny et al., 1995; Deng et al., 1996; Muramatsu et al., 1998), α_{1A} in kidney and caudal artery (Eltze et al., 1991; Blue et al., 1995; O'Malley et al., 1998), and α_{1B} in spleen (Burt et al., 1995) of rats. The contractile response to noradrenaline has been demonstrated to be mediated through α_{1B}-adrenoceptors in human mesenteric artery (Testa et al., 1996; Takahashi et al., 1999). In rabbit also, the functional predominance of the α_{1B} subtype has been demonstrated in the circulatory system such as thoracic aorta and carotid artery (Muramatsu 1991; Oshita et al., 1993). However, the rabbit α_{1b}-adrenoceptor has not yet been cloned although the other α_1 subtypes (α_{1a} and α_{1d}) have been done (Miyamoto et al., 1997; Suzuki et al., 1997).

In the present study, we cloned the rabbit α_{1b} subtype, and its pharmacological characterization and tissue distribution were compared with those of rabbit α_{1a} and α_{1d} subtypes.

* Corresponding author. Tel.: +81-776-61-3111; fax: +81-776-61-8130.

E-mail address: muramatu@fmssa.fukui-med.ac.jp (I. Muramatsu).

0014-2999/00/$ - see front matter
PII: S0014-2999(00)00171-0

2. Methods

2.1. cDNA library screening and sequencing

Rabbit brain cDNA library in λgt11 (Clontech) was screened with a 800 bp DNA fragment which was polymerase chain reaction (PCR) amplified from rabbit genomic DNA using a pair of primers designed after the hamster α_{1b}-adrenoceptor sequence; 5′-GGACACCGGCCACAACACATC-3′ and 5′-CCCTTGGCCTTGGTACTGCTGAG-3′. An isolated clone was subcloned into pCR3 mammalian expression vector (Invitrogen) and the nucleotide sequence was analyzed using overlapping templates by the dideoxy chain termination method in an ABI 373A DNA sequencer.

2.2. Cos-7 cell transfection

The plasmid clone constructed in pCR3 was transfected into Cos-7 cells with Lipofectamine (GIBCO) and the cells were harvested 72 h after transfection and stored at −80°C until the binding experiment.

2.3. Membrane preparation and binding experiment

The harvested cells were resuspended with ice-cold assay buffer (50 mM Tris–HCl, 1 mM EDTA, pH 7.4), homogenized by sonication and centrifuged at $3000 \times g$ for 10 min at 4°C. The supernatants were further centrifuged at $75{,}000 \times g$ for 30 min at 4°C and the resulting pellets were resuspended in the assay buffer for binding experiments. The saturation experiment was done in the range of 10–2000 pM of [^{3}H]prazosin. The displacement experiment was done in the presence of 200 pM of [^{3}H]prazosin with various concentrations of unlabelled drugs. Assays were done in duplicate and non-specific binding was defined in the presence of 0.3 μM tamsulosin. Membranes were incubated for 45 min at 30°C in a final 1 ml volume and then filtered onto Whatmann GF/C glass filters presoaked in 0.3% polyethyleneimine for 15 min. The filters were washed three times with ice-cold wash buffer (50 mM Tris–HCl, pH 7.4) and the bound radioactivity was determined in a liquid scintillation counter. Binding affinities of [^{3}H] ligands and unlabelled drugs were expressed as the negative logarithm of the equilibrium dissociation constant (pK_d and pK_i, respectively). Binding data were analyzed in a GraphPAD Prism. When the slope factor was close to unity, concentrations of competing agent producing 50% displacement of [^{3}H]prazosin (IC_{50}) were converted to K_i values with the Cheng–Prusoff approximation (Cheng and Prusoff, 1973). Protein was measured with bovine serum albumin as a standard (Bradford, 1976).

For preparation of native receptors, Japanese white rabbits were killed under pentobarbital anesthesia, and the tissues (thalamus, cortex, prostate, liver, spleen, kidney, aorta, and heart) were isolated immediately thereafter. The tissues were homogenized in 20 vol of ice-cold buffer (Tris–HCl 50 mM, NaCl 100 mM, EDTA 2 mM, pH 7.4) with a Polytron (setting 8, 15 s × 3) and filtered through four layers of cheesecloth. The supernatants were centrifuged at $80{,}000 \times g$ for 30 min, and the resulting pellets were suspended in the ice-cold assay buffer mentioned above, and then again centrifuged at $80{,}000 \times g$ for 30 min. All procedures were done at 4°C, and the final pellets were resuspended in assay buffer. Saturation experiments were done with various concentrations (30–2000 pM) of [^{3}H]prazosin or [^{3}H]KMD3213. Non-specific binding was determined with 0.3 μM tamsulosin for [^{3}H]prazosin and with 0.3 μM prazosin for [^{3}H]KMD3213, respectively. The other experimental conditions were the same as those described for recombinant receptor binding.

2.4. Total RNA preparation

Male Japanese white rabbits were anesthetized with sodium pentobarbital and were killed by exanguination. Tissues were rapidly removed, dissected, and frozen in liquid nitrogen and then stored at −80°C. Total cellular RNA was extracted according to the procedure of Chomczynski and Sacchi (1987).

2.5. Construction and RNA synthesis of competitor

Tissue distribution of the three α_1-adrenoceptor subtypes was examined with reverse transcription (RT)/PCR assays using a competitive internal standard. The competitor was constructed as follows. At first, a 60-bp Sma I/Rsa I fragment of pBluescript II was inserted into the Msc I site of rabbit α_{1d}-adrenoceptor clone. Next, it was connected with α_{1a} specific sequences and then α_{1b} specific sequences by PCR (Fig. 5A). The resulting competitor DNA fragment was subcloned into the Sma I site of pBluescript SK$^+$II and was transcribed with T7 RNA polymerase (GIBCO BRL). Competitor RNA was isolated from the transcription reaction by the method of Chomczynski and Sacchi (1987).

The following primers were used 5′-CATCGTGGTCGGCTGCTCGTC-3′ as forward primer and 5′-GGCTGTAGGCAGGCTGATT-3′ as reverse primer for α_{1a}-adrenoceptor; 5′-AGGAGCCGGCACCCAATGATGA-3′ as forward and 5′-GGCACTGGCACCCGAGGAT-3′ as reverse for α_{1b}-adrenoceptor; 5′-CTCCGTGCGCCTGCTCAAGT-3′ as forward and 5′-GGGTAGATGAGTGGGTTCAC-3′ as reverse for α_{1d}-adrenoceptor.

The size of the resulting PCR products (competitor/target) was 490:442, 604:549, and 270:210 bp for α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptor subtypes, respectively.

2.6. Competitive RT / PCR assay

To quantify the mRNA level of an adrenoceptor subtype in rabbit tissues, a trace of competitor RNA was co-transcribed and co-amplified in a RT/PCR assay. Briefly, the tissue RNA (250 ng) was premixed with the competitor RNA (1 pg) and was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (GIBCO BRL) using a random primer (dN6) at 37°C for 1 h. The resulting cDNA was amplified with Pwo polymerase (Boehringer Mannheim) using a subtype-specific pair of primers under the following conditions: one cycle at 98°C for 3 min; 32 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 10 s, and extension at 72°C for 15 s. PCR products were electrophoresed in 3.5% polyacrylamide gel which was stained with ethidium bromide. The

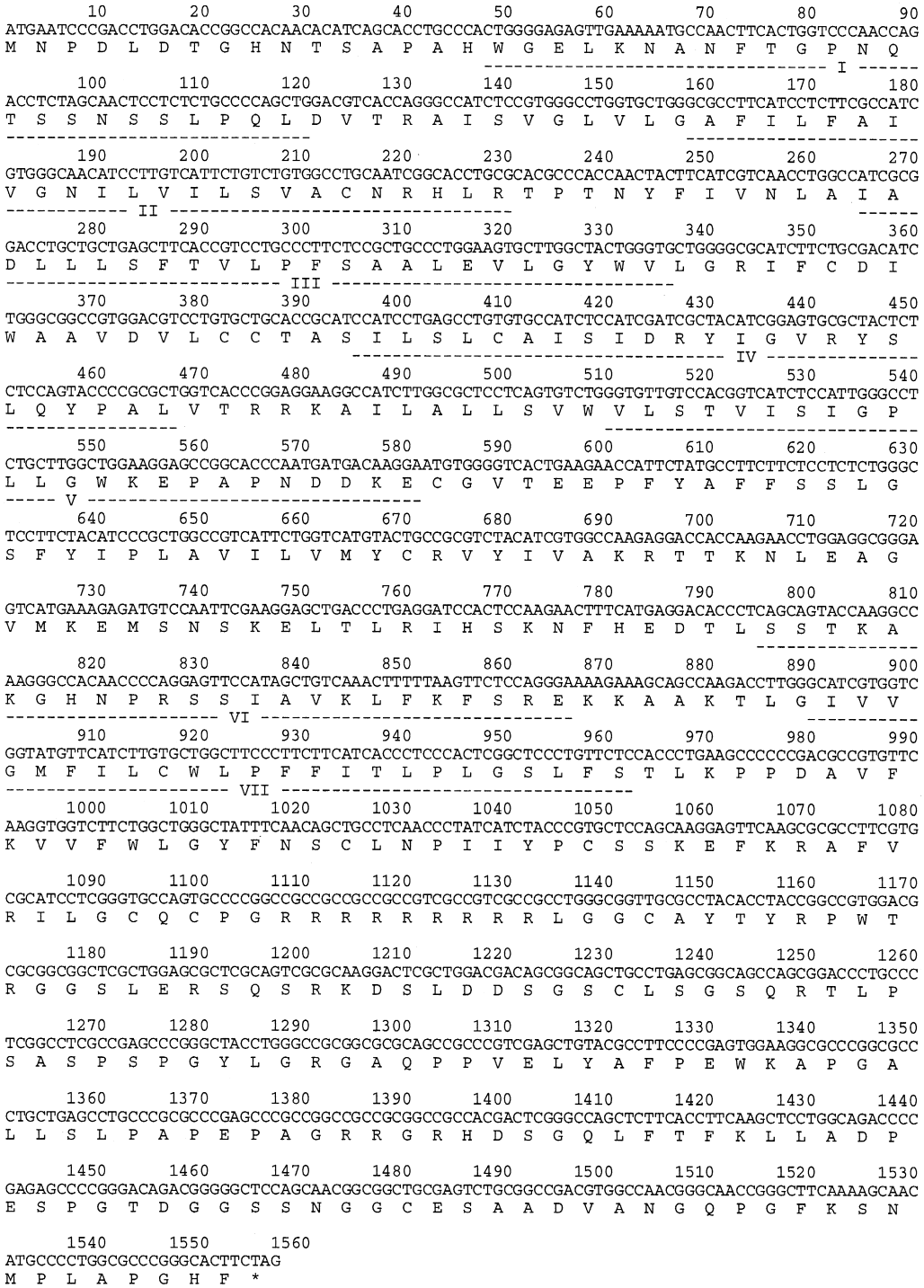

Fig. 1. Nucleotide and amino acid sequences of rabbit α_{1b}-adrenoceptor. Dashed lines indicate transmembrane domains I–VII.

intensity of bands was quantitated with the ATTO Densitograph System.

To compare the expression levels of mRNA of three α_1-adrenoceptor subtypes, competitive RT/PCR was done in cerebellum with a pair of primers specific for each subtype, using 500 ng of total RNAs premixed with serially diluted competitor RNA. The ratios of intensity of competitor band and target band were plotted against the dose of competitor RNA and the apparent equivalent point for each subtype was determined as the ratio was estimated to be one. The equivalence of subtypes mRNA to competitor RNA was finally calculated, taking band size factors into account, i.e. multiplying apparent equivalence by size factor. The size factor is a ratio of sizes of competitor band and target band and was 1.11, 1.10, and 1.29 for α_{1a}-, α_{1b}- and α_{1d}-adrenoceptor, respectively.

In preliminary experiments, RT/PCR products from the competitor and tissue RNA were digested with band-specific restriction endonucleases (EcoR V, Bgl II, BamH I, and Msc I for competitor and α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptors, respectively) to confirm their authenticity (data not shown).

2.7. Drugs

The drugs used and their sources were the following: (−)-(R)-1-(3-hydroxypropyl)-5-[2-[[2-(2,2,2-trifluoroethoxy) phenoxy]-ethyl]amino]propyl]indoline-7-carboxamide (KMD3213) and tamsulosin HCl (tamsulosin), from Kissei Pharmaceutical (Matsumoto, Japan); prazosin HCl, (−)-noradrenaline bitartrate, (−)-adrenaline hydrogen tartrate, oxymetazoline hydrochloride, and methoxamine hydrochloride, from Sigma (St. Louis, USA); 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4-benzodioxane hydrochloride (WB4101), 8-[2-[4-(2-methoxy-phenyl)-L-piperazinyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride (BMY7378), rauwolscine hydrochloride, and 5-methylurapidil, from Research Biochemicals (Natick, USA); (±)-propranolol hydrochloride, from Nacalai (Kyoto, Japan); [^{3}H]KMD3213 (49–52 Ci mmol^{-1}), from Amer-

```
                                                  ---------- I ----------          --------
rabbit    1:MNPDLDTGHNTSAPAHWGELKNANFTGPNQTSSNSSLPQLDVTRAISVGLVLGAFILFAIVGNILVILSVACNRHLRTPTNYFIVNLAIA 90
hamster   1:..............Q.....D............T.................................................. 90
human     1:................................T.....I.............................................M. 90
rat       1:.....................DD............T.................................................. 90

            -- II -----------          ---------- III ---------                    ---------- IV ----
rabbit   91:DLLLSFTVLPFSAALEVLGYWVLGRIFCDIWAAVDVLCCTASILSLCAISIDRYIGVRYSLQYPALVTRRKAILALLSVWVLSTVISIGP 180
hamster  91:.............T.....................................................T..................... 180
human    91:.....................................................................T..................... 180
rat      91:.............T.........LSF.....................................T..................... 180

            ------              ----------- V ----------
rabbit  181:LLGWKEPAPNDDKECGVTEEPFYAFFSSLGSFYIPLAVILVMYCRVYIVAKRTTKNLEAGVMKEMSNSKELTLRIHSKNFHEDTLSSTKA 270
hamster 181:.......................L.......................................................................... 270
human   181:.......................L.......................................................................... 270
rat     181:.......................L.......................................................................... 270

                                    ---------- VI ----------       ---------- VII ----------
rabbit  271:KGHNPRSSIAVKLFKFSREKKAAKTLGIVVGMFILCWLPFFITLPLGSLFSTLKPPDAVFKVVFWLGYFNSCLNPIIYPCSSKEFKRAFV 360
hamster 271:.........................................A.....................................................M 360
human   271:.........................................A..................................................... 360
rat     271:.........................................A.....................................................M 360


rabbit  361:RILGCQCPG-RRRRRRRRRLGGCAYTYRPWTRGGSLERSQSRKDSLDDSGSCLSGSQRTLPSASPSPGYLGRGAQPPVELYAFPEWKAPG 449
hamster 361:.......R-SG.........A.............................M.....................L..C.Y....-S. 448
human   361:.......R.R.....................................................................P.....C..... 450
rat     361:.......R-GG.........A.............................M..T.................T......C......-.. 448


rabbit  450:ALLSLPAPEPAGRRGRHDSGQLFTFKLLADPESPGTDGGSSNGGCESAADVANGQPGFKSNMPLAPGHF                    518
hamster 449:......E.-.-.....L...P.......GE......E.DA.....DATT.L.....................                 515
human   451:..........P.........P.......TE.........A......A...................Q.                      519
rat     449:......E.-.-.....L...P.......G.......E.DT.....DTTT.L.....................                 515
```

Fig. 2. Comparison of amino acid sequence of α_{1b}-adrenoceptors of rabbit, hamster, human, and rat. Dots are substituted for identical amino acids.

sham (England); and [^{3}H]prazosin (77.2 Ci mmol^{-1}), from NEN (Boston, USA).

3. Results

3.1. Cloning of rabbit α_{1b}-adrenoceptor

A positive clone was isolated from the screening of 5×10^5 recombinants. This had a 2.5 kbp insert containing an open reading frame of 1557 bp that encodes a 518-amino-acid peptide as shown in Fig. 1. Comparing the whole amino-acid sequence of this peptide with those of α_{1b}-adrenoceptors of hamster (Cotecchia et al., 1988), human (Ramarao et al., 1992) and rat (Voigt et al., 1990), the identity was more than 98% (Fig. 2). However the identity between this peptide sequence and those of α_{1a}- and α_{1d}-adrenoceptors of rabbit (Miyamoto et al., 1997; Suzuki et al., 1997) was low (less than 57%, Fig. 3) in whole length. We concluded that the clone obtained encodes the rabbit α_{1b}-adrenoceptor. This nucleotide sequence was submitted to GenBank with an accession number, AF156106.

3.2. Comparison of pharmacological profiles of rabbit α_1-adrenoceptor subtypes

Three rabbit α_1-adrenoceptor subtypes were expressed in Cos-7 cells and their binding characters were investigated by using [^{3}H]prazosin as a radioligand. Rabbit α_{1a}-adrenoceptor showed high affinity for prazosin, KMD3213, WB4101, tamsulosin, 5-methylurapidil, oxymetazoline and methoxamine and low affinity for BMY7378. Rabbit α_{1b}-adrenoceptor exhibited high affinity for prazosin and tamsulosin and low affinity for KMD3213, BMY7378, 5-methylurapidil, oxymetazoline, and methoxamine. Rabbit α_{1d}-adrenoceptor displayed high affinity for prazosin, tamsulosin, BMY7378, and noradrenaline and low affinity for KMD3213, 5-methylurapidil, and oxymetazoline. All three subtypes had low affinity for rauwolscine and propranolol (specific ligands for α_2- and β-adrenoceptors, respec-

Table 1
Pharmacological profile of recombinant α_1-adrenoceptor subtypes of rabbit
Data are means ± S.E. from three experiments. Negative log of the equilibrium dissociation constant (pK_i) for each drug was calculated.

	pK_i		
	α_{1a}	α_{1b}	α_{1d}
Prazosin	9.4 ± 0.05	10.3 ± 0.07	9.2 ± 0.03
KMD3213	9.8 ± 0.25	8.5 ± 0.09	8.1 ± 0.23
WB4101	9.4 ± 0.16	8.7 ± 0.15	8.7 ± 0.07
Tamsulosin	9.7 ± 0.27	9.5 ± 0.17	9.2 ± 0.09
BMY7378	6.6 ± 0.15	7.3 ± 0.12	8.5 ± 0.04
5-Methylurapidil	8.4 ± 0.02	6.7 ± 0.80	7.1 ± 0.29
Rauwolscine	< 6	< 6	< 6
Propranolol	< 5	< 5	< 5
Noradrenaline	5.8 ± 0.07	5.3 ± 0.18	7.6 ± 0.39
Adrenaline	6.1 ± 0.18	5.7 ± 0.12	6.6 ± 0.45
Oxymetazoline	8.4 ± 0.36	7.3 ± 0.15	6.6 ± 0.32
Methoxamine	5.2 ± 0.16	< 4	4.5 ± 0.03

Fig. 3. Comparison of amino acid sequence among three adrenoceptor subtypes in rabbit. Dots are substituted for identical amino acids.

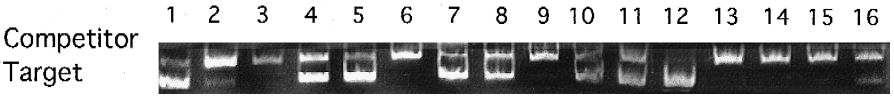

Fig. 4. Tissue distribution of α_{1b}-adrenoceptor mRNA in rabbit. Total RNA from each tissue was mixed with competitor RNA and was reverse transcribed, PCR amplified and separated by electrophoresis as described in Section 2. The products from competitor (604 bp) and from tissue RNA (549 bp) are indicated by arrows on the left. Rabbit tissues analyzed were heart, kidney, liver, lung, spleen, skeletal muscle, thoracic aorta, cerebellum, hippocampus, brainstem, cerebral cortex, thalamus, vas deferens, prostate, submaxillary gland, and parotid gland from lanes 1 to 16, respectively.

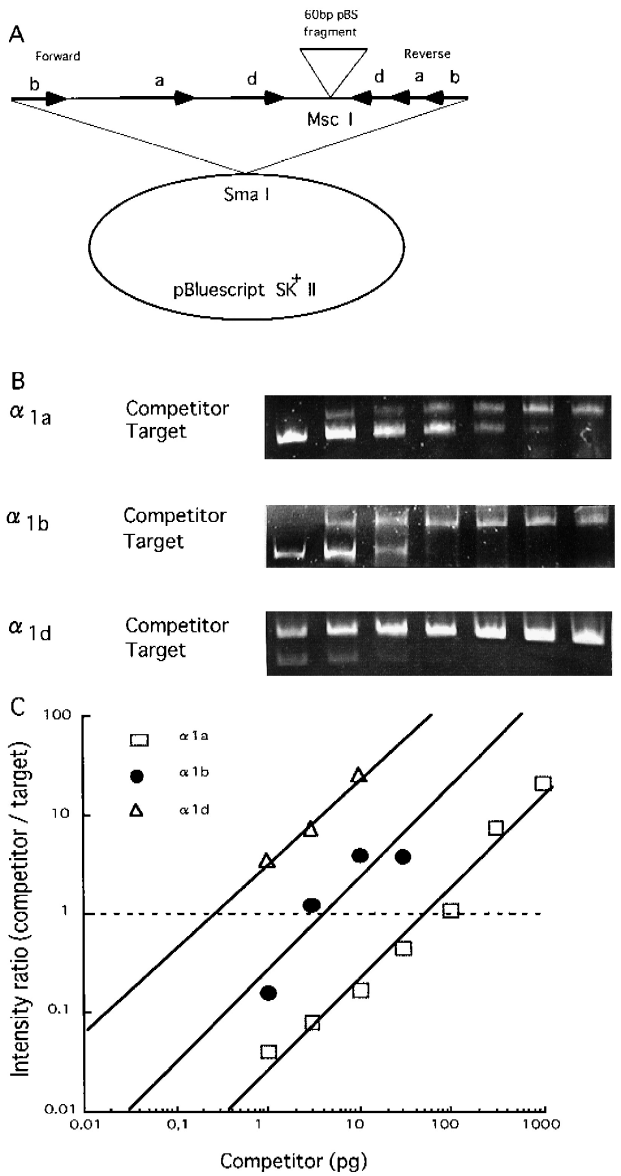

Fig. 5. Tissue distribution of mRNA of α_1-adrenoceptor subtype in rabbit. (A) Illustration of competitor in which three α_1-subtype-specific primer pairs are constructed. Arrows shown as forward and reverse of a, b and d represent PCR primers for α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptor sequences. (B) A representative result of competitive RT/PCR in rabbit cerebellum is shown. The total RNA was mixed with serially diluted competitor RNA (1, 3, 10, 30, 100, 300, and 1000 pg from the left), and was co-transcribed with random hexamer. Then, it was PCR-amplified with subtype-specific pair of primers. (C) amount of mRNA of three α_1-adrenoceptor subtypes in rabbit cerebellum. The ratios of intensity of competitor band and target band are plotted based on data from three independent experiments and the apparent equivalent point was defined as where the ratio is one.

tively). These binding affinities at rabbit α_1-adrenoceptor subtypes are summarized in Table 1. [^{3}H]KMD3213 at 0.6 and 1 nM bound to α_{1a}- but not to α_{1b}- and α_{1d}-adrenoceptors (data not shown).

3.3. Competitive RT / PCR analysis

A representative result of competitive RT/PCR for α_{1b}-adrenoceptor in rabbit tissues is shown in Fig. 4. Relatively high expression of α_{1b}-adrenoceptors was observed in thalamus, thoracic aorta, brain stem, spleen, and heart. However, while a significant amount of mRNA expression of the α_{1b}-adrenoceptor was reported in both human (Faure et al., 1995) and rat (Scofield et al., 1995) liver, it was not detected in rabbit liver.

Next, in order to compare the levels of mRNA expression of three subtypes of the α_1-adrenoceptor in various tissues, the equivalence of each subtype mRNA was evaluated against competitor RNA. As shown in Fig. 5, the apparent equivalence of mRNA of the subtypes as 52, 4.1, and 0.43 pg competitor RNA and the final equivalence was estimated as 57.7, 4.5, and 0.55 pg RNA in rabbit cerebellum, taking size factor into account, for α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptors, respectively. Previously, we reported relative mRNA levels of α_{1a}- (Miyamoto et al., 1997) and

Table 2
Relative level of α_1-adrenoceptor subtype mRNA in various rabbit tissues
Relative mRNA levels in each tissue were normalized, taking the level of α_{1b} mRNA in cerebellum as one. Data shown are means ± S.E. from three experiments.

Tissue	Relative mRNA Level		
	α_{1a}	α_{1b}	α_{1d}
Heart	< 0.1	1.7 ± 0.2	< 0.1
Kidney	2.5 ± 1.3	0.1 ± 0.1	< 0.1
Liver	96.1 ± 62.7	< 0.1	< 0.1
Lung	16.6 ± 5.1	0.8 ± 0.1	0.4 ± 0.0
Spleen	9.0 ± 2.5	1.7 ± 0.2	< 0.1
Skeletal muscle	< 0.1	< 0.1	< 0.1
Thoracic aorta	57.7 ± 14.1	2.6 ± 1.3	0.6 ± 0.1
Cerebellum	12.8	1	0.1 ± 0.0
Hippocampus	19.3 ± 1.3	< 0.1	< 0.1
Brain stem	192.0 ± 9.0	1.9 ± 0.6	0.1 ± 0.0
Cerebral cortex	20.5 ± 2.5	1.0 ± 0.1	0.4 ± 0.1
Thalamus	157.4 ± 21.8	13.5 ± 2.0	< 0.1
Vas deferens	19.3 ± 2.5	< 0.1	0.7 ± 0.2
Prostate	3.8 ± 1.3	< 0.1	0.5 ± 0.1
Submaxillary gland	< 0.1	< 0.1	< 0.1
Parotid gland	< 0.1	0.3 ± 0.1	< 0.1

α_{1d}-adrenoceptor (Suzuki et al., 1997) in various rabbit tissues. In the case of rabbit α_{1a}-adrenoceptor, we now repeated the competitive RT/PCR because the pair of primers was distinct from that used in the previous experiments. For the rabbit α_{1d}-adrenoceptor, we used the data from the previous report because the conditions for RT/PCR were identical. Based on the equivalence in rabbit cerebellum (Fig. 5), we normalized the relative mRNA level of each subtype, taking the level of α_{1b} mRNA in cerebellum as one (Table 2). Excepting heart, the α_{1a} subtype was dominant while the α_{1d} subtype was very minor in most tissues. This is quite unique to rabbit, since this kind of subtype distribution was reported for neither human (Hirasawa et al., 1993; Graham et al., 1996) nor rat (Rokosh et al., 1994; Graham et al., 1996). Notably, mRNA of α_{1a}-adrenoceptor was not detected in rabbit heart in contrast to human and rat hearts.

3.4. [^{3}H]prazosin and [^{3}H]KMD3213 binding to rabbit native tissue membranes

α_1-Adrenoceptor density at the protein level was evaluated in the binding study with [^{3}H]prazosin (1 and 2 nM) and [^{3}H]KMD3213 (0.6 and 1 nM) for total α_1- and α_{1A}-adrenoceptor, respectively. Since the binding affinities of these drugs are high (see Table 1 and Murata et al., 1999), it is possible to estimate the densities as approximations. As shown in Fig. 6A, the distribution of total α_1- and α_{1A}-adrenoceptors varied among rabbit tissues tested. Most α_1-adrenoceptors were α_{1A} subtype in thalamus, prostate, and liver, whereas α_{1B} subtype was detected in the heart and spleen. BMY7378 (0.1 μM) did not significantly inhibit the binding of [^{3}H]prazosin and [^{3}H]KMD3213 in any of the tissues tested (data not shown), indicating that the α_{1D} subtype is negligible at the protein level.

Some discrepancy was seen between mRNA level and binding density of α_1-adrenoceptor subtypes in several tissues including spleen, where the α_{1a}-adrenoceptor is a major subtype at the mRNA level but the α_{1B}-subtype is predominant at the protein level (Table 2 and Fig. 6A). We, thus ran saturation experiments in spleen (Fig. 6B). These showed that specific binding was detectable in [^{3}H]prazosin but not in [^{3}H]KMD3213, confirming that the α_{1B}-subtype is predominant at the protein level in spleen. B_{max} value of [^{3}H]prazosin was 100.6 ± 5.8 fmol/mg protein ($n = 3$) which was in good agreement with that obtained in the two-point assay (106.6 ± 12.8 fmol/mg protein, Fig. 6A).

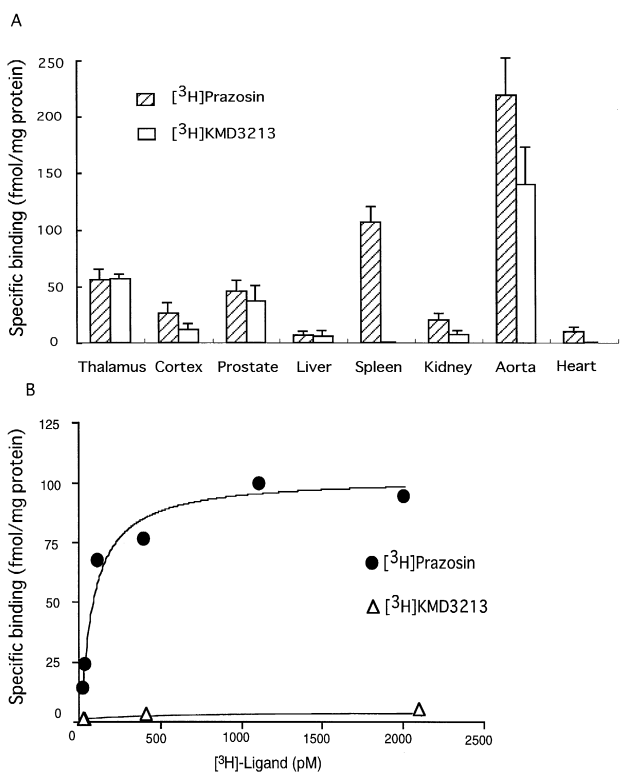

Fig. 6. Tissue distribution of binding sites for α_1-adrenoceptor subtypes. (A) shaded columns represent [^{3}H]prazosin binding (total α_1-adrenoceptor density) and open columns represent [^{3}H]KMD3213 binding (α_{1A}-adrenoceptor density). Data are means ± S.E. of three experiments. (B) saturation binding experiments with [^{3}H]prazosin and [^{3}H]KMD3213 in rabbit spleen membranes.

4. Discussion

4.1. Peptide sequence and binding character

The peptide sequence of the α_{1b}-adrenoceptor of rabbit had a high identity to those of hamster, human, and rat (Fig. 2), whereas it showed a lower identity to those of α_{1a}- and α_{1d}-adrenoceptors of rabbit. Pharmacological characterization also revealed the expected profile as α_{1b}-adrenoceptor, showing high affinity for prazosin and tamsulosin and low affinity for KMD3213, BMY7378, 5-methylurapidil, and methoxamine. The profiles of three α_1-adrenoceptor subtypes of rabbit were compatible with those of humans (Tseng-Crank et al., 1995; Weinberg et al., 1994; Esbenshade et al., 1995) and of rats (Perez et al., 1994). We concluded that rabbit has a similar α_1-adrenoceptor system to that of other mammalian species.

4.2. Distribution

Next, we investigated the mRNA distribution of three α_1-adrenoceptor subtypes in rabbit tissues. As shown in Table 2, the α_{1a}-subtype was a predominant subtype in most tissues tested. However, the α_{1b}-subtype was the main α_1-adrenoceptor in rabbit heart and was relatively abundant in thalamus, thoracic aorta, and spleen. In the case of the α_{1d}-subtype, the level of mRNA expression was very low compared to that of other subtypes in all rabbit tissues tested. This pattern of subtype distribution was distinct from that of rats or humans (Scofield et al.,

1995; Faure et al., 1995; Lomasney et al., 1991; Graham et al., 1996). Then, we examined α_1-adrenoceptor distribution at the protein level by using [^{3}H]prazosin and [^{3}H]KMD3213. Based on the binding affinity profiles for both ligands (Table 1 and Murata et al., 1999), specific binding of [^{3}H]prazosin and of [^{3}H]KMD3213 was speculated to correspond to total α_1- and α_{1a}-adrenoceptor densities, respectively. Comparison of mRNA level (Table 2) and binding density (Fig. 6A) shows that these parameters do not always correlate. For example, α_{1b} binding sites were not detected in thalamus in spite of significant expression of α_{1b} mRNA. On the other hand, α_{1b} binding sites alone were detected in the spleen, where greater amounts of α_{1a} mRNA than of α_{1b} mRNA were found. These results suggest that there is a tissue- and subtype-specific mechanism(s) to regulate expression of mRNA and protein of the α_1-adrenoceptor in rabbit. The lack of α_{1d}-binding sites we found may be related to the low level of its mRNA and is also consistent with the reports that the α_{1d}-adrenoceptor is not detectable in binding experiments (Yang et al., 1997; Muramatsu et al., 1998).

4.3. Conclusions

In conclusion, the present study showed a high identity of structural and pharmacological characters of three subtypes in the α_1-adrenoceptor system between rabbit and other mammalian species. However, there are species differences in their tissue distribution, implying that α_1-adrenoceptor subtypes are heterologously involved in physiological responses among mammals.

Acknowledgements

The authors would like to thank Ms. N. Aoki for secretarial assistance and Ms. N. Saito for technical assistance. This work was supported in part by a grant from the Smoking Research Foundation of Japan and by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References

Blue, D.R. Jr., Bonhaus, D.W., Ford, A.P., Pfister, J.R., Sharif, N.A., Shieh, I.A., Vimont, R.L., Willams, T.J., Clarke, D.E., 1995. Functional evidence equating the pharmacologically-defined alpha 1A- and cloned alpha 1C-adrenoceptor: studies in the isolated perfused kidney of rat. Br. J. Pharmacol. 115 (2), 283–294.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Burt, R.P., Chapple, C.R., Marshall, I., 1995. Evidence for functional alpha 1A-(alpha 1C-) adrenoceptor mediating contraction of the rat epididymal vas deferens and an alpha 1B-adrenoceptor mediating contraction of the rat spleen. Br. J. Pharmacol. 115 (3), 467–475.

Cheng, Y., Prusoff, W.H., 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I50) of an enzymatic reaction. Biochem. Pharmacol. 22 (23), 3099–3108.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162 (1), 156–159.

Cotecchia, S., Schwinn, D.A., Randall, R.R., Lefkowitz, R.J., Caron, M.G., Kobilka, B.K., 1988. Molecular cloning and expression of the cDNA for the hamster α1-adrenergic receptor. Biochemistry 85, 7159–7163.

Deng, X.F., Chemtob, S., Varma, D.R., 1996. Characterization of alpha 1D-adrenoceptor subtype in rat myocardium, aorta and other tissues. Br. J. Pharmacol. 119 (2), 269–276.

Eltze, M., Boer, R., Sanders, K.H., Kolassa, N., 1991. Vasodilatation elicited by 5-HT1A receptor agonists in constant-pressure-perfused rat kidney is mediated by blockade of alpha 1A-adrenoceptors. Eur. J. Pharmacol. 202 (1), 33–44.

Esbenshade, T.A., Hirasawa, A., Tsujimoto, G., Tanaka, T., Yano, J., Minneman, K.P., Murphy, T.J., 1995. Cloning of the human alpha 1d-adrenergic receptor and inducible expression of three human subtypes in SK–N–MC cells. Mol. Pharmacol. 47 (5), 977–985.

Faure, C., Gouhier, C., Langer, S.Z., Graham, D., 1995. Quantification of alpha 1-adrenoceptor subtypes in human tissues by competitive RT-PCR analysis. Biochem. Biophys. Res. Commun. 213 (3), 935–943.

Graham, R.M., Perez, D.M., Hwa, J., Piascik, M.T., 1996. Alpha 1-adrenergic receptor subtypes. Molecular structure, function, and signaling. Circ. Res. 78 (5), 737–749.

Hieble, J.P., Bylund, D.B., Clarke, D.E., Eikenburg, D.C., Langer, S.Z., Lefkowitz, R.J., Minneman, K.P., Ruffolo, R.R. Jr., 1995. International Union of Pharmacology: X. Recommendation for nomenclature of alpha 1-adrenoceptors: consensus update. Pharmacol. Rev. 47 (2), 267–270.

Hirasawa, A., Horie, K., Tanaka, T., Takagaki, K., Murai, M., Yano, J., Tsujimoto, G., 1993. Cloning, functional expression and tissue distribution of human cDNA for the α_{1c}-adrenergic receptor. Biochem. Biophys. Res. Commun. 195 (2), 902–909.

Kenny, B.A., Chalmers, D.H., Philpott, P.C., Naylor, A.M., 1995. Characterization of an alpha 1D-adrenoceptor mediating the contractile response of rat aorta to noradrenaline. Br. J. Pharmacol. 115 (6), 981–986.

Lomasney, J.W., Cotecchia, S., Lefkowitz, R.J., Caron, M.G., 1991. Molecular biology of alpha-adrenergic receptors: implications for receptor classification and for structure–function relationships. Biochim. Biophys. Acta 1095 (2), 127–139.

Michel, M.C., Kenny, B., Schwinn, D.A., 1995. Classification of alpha 1-adrenoceptor subtypes. Naunyn-Schmiedeberg's Arch. Pharmacol. 352 (1), 1–10.

Minneman, K.P., Esbenshade, T.A., 1994. Alpha 1-adrenergic receptor subtypes. Annu. Rev. Pharmacol. Toxicol. 34, 117–133.

Miyamoto, S., Taniguchi, T., Suzuki, F., Takita, M., Kosaka, N., Negoro, E., Okuda, T., Kosaka, H., Murata, S., Nakamura, S., Akagi, Y., Oshita, M., Watanabe, Y., Muramatsu, I., 1997. Cloning, functional expression and tissue distribution of rabbit α_{1a}-adrenoceptor. Life Sci. 60 (23), 2069–2074.

Muramatsu, I., 1991. Relation between adrenergic neurogenic contraction and alpha 1-adrenoceptor subtypes in dog mesenteric and carotid arteries and rabbit carotid arteries. Br. J. Pharmacol. 102 (1), 210–214.

Muramatsu, I., Murata, S., Isaka, M., Piao, H.L., Zhu, J., Suzuki, F., Miyamoto, S., Oshita, M., Watanabe, Y., Taniguchi, T., 1998. Alpha 1-adrenoceptor subtypes and two receptor systems in vascular tissues. Life Sci. 62 (17–18), 1461–1465.

Murata, S., Taniguchi, T., Muramatsu, I., 1999. Pharmacological analysis of the novel, selective α_{1d}-adrenoceptor antagonist, KMD-3213, and its suitability as a tritiated radioligand. Br. J. Pharmacol. 127 (1), 19–26.

O'Malley, S.S., Chen, T.B., Francis, B.E., Gibson, R.E., Burns, H.D., DiSalvo, J., Bayne, M.L., Wetzel, J.M., Nagarathnam, D., Marzabadi, M., Gluchowski, C., Chang, R.S., 1998. Characterization of specific

binding of [125I]L-762,459, a selective alpha 1A-adrenoceptor radioligand to rat and human tissue. Eur. J. Pharmacol. 348 (2–3), 287–295.

Oshita, M., Kigoshi, S., Muramatsu, I., 1993. Pharmacological characterization of two distinct alpha 1-adrenoceptor subtypes in rabbit thoracic aorta. Br. J. Pharmacol. 108 (4), 1071–1076.

Perez, D.M., Piascik, M.T., Malik, N., Gaivin, R., Graham, R.M., 1994. Cloning, expression, and tissue distribution of the rat homology of the bovine alpha 1C-adrenergic receptor provide evidence for its classification as the alpha 1A subtype. Mol. Pharmacol. 46 (5), 823–831.

Ramarao, C.S., Denker, J.M., Perez, D.M., Galvin, R.J., Riek, R.P., Graham, R.M., 1992. Genomic organization and expression of the human alpha 1B-adrenergic receptor. J. Biol. Chem. 267 (30), 21936–21945.

Rokosh, D.G., Bailey, B.A., Stewart, A.F., Karns, L.R., Long, C.S., Simpson, P.C., 1994. Distribution of alpha 1C-adrenergic receptor mRNA in adult rat tissues by RNase protection assay and comparison with alpha 1B and 1D. Biochem. Biophys. Res. Commun. 200 (3), 1177–1184.

Scofield, M.A., Liu, F., Abel, P.W., Jeffries, W.B., 1995. Quantification of steady state expression of mRNA for alpha-1 adrenergic receptor subtypes using reverse transcription and a competitive polymerase chain reaction. J. Pharmacol. Exp. Ther. 275 (2), 1035–1042.

Suzuki, F., Miyamoto, S., Takita, M., Oshita, M., Watanabe, Y., Kakizuka, A., Narumiya, S., Taniguchi, T., Muramatsu, I., 1997. Cloning, functional expression and tissue distribution of rabbit α_{1d}-adrenoceptor. Biochim. Biophys. Acta 1323 (1), 6–11.

Takahashi, M., Taniguchi, T., Murata, S., Okada, K., Moriyama, N., Yamazaki, S., Muramatsu, I., 1999. New alpha 1-adrenoceptor antagonist, JTH-601, shows more than 10 times higher affinity for human prostates than arteries. J. Urol. 16 (4), 1350–1354.

Testa, R., Guarneri, L., Taddei, C., Poggesi, E., Angelico, P., Sartani, A., Leonardi, A., Gofrit, O.N., Meretyk, S., Caine, M., 1996. Functional antagonistic activity of Rec 15/2739, a novel alpha-1 antagonist selective for the lower urinary tract, on noradrenaline-induced contraction of human prostate and mesenteric artery. J. Pharmacol. Exp. Ther. 277 (3), 1237–1246.

Tseng-Crank, J., Kost, T., Goetz, A., Hazum, S., Roberson, K.M., Haizlip, J., Godinot, N., Robertson, C.N., Saussy, D., 1995. The alpha 1C-adrenoceptor in human prostate: cloning, functional expression, and localization to specific prostatic cell types. Br. J. Pharmacol. 115 (8), 1475–1485.

Voigt, M.M., Kispert, J., Chin, H.M., 1990. Sequence of a rat brain cDNA encoding an alpha-1B adrenergic receptor. Nucleic Acids Res. 18 (4), 1053.

Weinberg, D.H., Trivedi, P., Tan, C.P., Mitra, S., Perkins-Barrow, A., Borkowski, D., Strader, C.D., Bayne, M., 1994. Cloning, expression and characterization of human alpha adrenergic receptors alpha 1a, alpha 1b and alpha 1c. Biochem. Biophys. Res. Commun. 201 (3), 1296–1304.

Yang, M., Verfurth, F., Buscher, J.R., Michel, M.C., 1997. Is alpha 1D-adrenoceptor protein detectable in rat tissues? Naunyn-Schmiedeberg's Arch. Pharmacol. 355 (4), 438–446.