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Distribution of alpha-1 adrenoceptor subtypes in RNA and protein in rabbit eyes

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1 We investigated subtypes of alpha-1 adrenoceptor (AR) in rabbit ocular tissues using reverse transcription-polymerase chain reaction (RT–PCR), *in situ* hybridization (ISH) and binding studies.

2 Competitive RT–PCR assays specific for the subtypes of alpha-1 AR revealed that the mRNA expression of alpha-1a AR was dominant, and that of each alpha-1b and alpha-1d was less than 10% and 0.5% of total alpha-1 ARs mRNA, respectively, in the iris, ciliary body, choroid and retina.

3 In alpha-1a AR splice isoform-specific RT–PCR assays, we found a distinct proportion of each isoform mRNA in the iris, ciliary body and choroid.

4 The results of the ISH assays for alpha-1a AR subtype showed that hybridization signals were clearly observed in the iris dilator muscle and in the epithelium of the ciliary processes.

5 In binding studies, alpha-1A AR was a dominant subtype in the iris, choroid and retina in contrast to the ciliary body that had more alpha-1B than alpha-1A AR subtype at protein level.
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Abbreviations: AR, adrenoceptor; IOP, intraocular pressure; ISH, *in situ* hybridization; RT–PCR, reverse transcription-polymerase chain reaction

Introduction

Alpha-1 ARs constitute a heterogeneous family of receptors and play critical roles in the regulation of the sympathetic system. Molecular cloning techniques revealed the existence of three alpha-1 AR subtypes (alpha-1a, alpha-1b and alpha-1d AR) in many species (Cotecchia *et al*., 1988; Schwinn *et al*., 1990; Lomansney *et al*., 1991; Perez *et al*., 1991) and pharmacological studies suggested that these recombinant subtypes correspond to native alpha-1A, alpha-1B, and alpha-1D AR subtypes, respectively (Bylund *et al*., 1994; Hieble *et al*., 1995). Furthermore, splice isoforms of the alpha-1a AR subtype were found in humans; alpha-1a HSA.1-, 2-, 3- and 4-AR (Hirasawa *et al*., 1995; Chang *et al*., 1998) and in rabbits; alpha-1a OCU.1-, 2- and 3-AR (Suzuki *et al*., 2000).

Alpha-1 ARs play important functions in the eyes. The contraction of the iris dilator muscle in rabbit eyes is mediated by alpha-1 ARs (Konno & Takayanagi, 1986; Nakamura *et al*., 1999). *In vivo* studies have revealed that alpha-1 ARs are involved in the regulation of intraocular pressure (IOP) in rabbits and monkeys (Kiuchi *et al*., 1992; Nishimura *et al*., 1993; Wang *et al*., 1997; Zhan *et al*., 1998; Okada & Gregory, 2001). Moreover, alpha-1 ARs have been found to regulate cellular functions in cultured endothelial cells from various ocular regions in several species (Walkenbach *et al*., 1992; Moroi-Fetters *et al*., 1995; Ryan *et al*., 1998). However, which subtype(s) of alpha-1 AR is involved in the ocular functions remains to be clarified.

The purpose of this study was to identify and characterize alpha-1 AR subtypes in rabbit ocular tissues by RT–PCR, ISH and binding studies.

Methods

Animals

Tissues were obtained from adult male albino rabbits that weighed 2–2.5 kg. Rabbits were anaesthetized with intravenous sodium pentobarbital (50 mg kg^{-1}) and sacrificed. The eyes were enucleated promptly and dissected under a surgical microscope to isolate the iris, ciliary body, choroid and retina and then frozen immediately on dried-ice or liquid nitrogen and stored at $-80^{\circ}C$ until used for the following experiment.

Competitive RT–PCR assays

To evaluate mRNA levels of each alpha-1 AR subtype or those of alpha-1a AR splice isoforms in rabbit ocular tissues, we performed competitive RT–PCR assays as previously described (Piao *et al*., 2000; Suzuki *et al*., 2000). Briefly, the total RNA from each tissue was co-

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transcribed and co-amplified with a competing RNA in RT–PCR assays. Figure 1 showed the schematic structures of each competing DNA.

We first constructed two kinds of control RNA that should be reverse transcribed and amplified with a pair of subtype- or isoform-specific primers as a competitive internal standard (Figure 1). The total RNA (500 ng) was extracted from each rabbit ocular tissue according to the procedure of Chomczynski & Sacchi (1987), premixed with the competitor RNA and reverse transcribed with moloney murine leukaemia virus reverse transcriptase (GIBCO BRL) with random hexamer (dN_6) at 37°C for 1 h. The resulting cDNA was amplified with a subtype- or isoform-specific pair of primers by PWO DNA polymerase (Boehringer Mannheim). For each experiment, negative controls were amplified, in which all of the components except reverse transcriptase were included. PCR products were electrophoresed in 3.5% polyacrylamide gel that was stained with ethidium bromide. The intensity of bands was quantitated with the ATTO Densitograph System and the ratios of competing products to target products were plotted against the amount of the competing template that was added to each sample on a logarithmic scale. Then the amount of target mRNA in each tissue was calculated by interpolation of the resultant linear regression to the equivalent point, where the ratio of the competitor and native product was 1.

Synthesis of cRNA Probes and in situ *hybridization*

A specific probe of rabbit alpha-1a AR which could detect all alpha-1a AR isoforms was designed for ISH (shown in Figure 1B), and the homology of the probe to both alpha-1b and alpha-1d AR was about 55%. The cDNA fragment was PCR amplified with a pair of primers; 5′-AGT GTT TTG GCT CGG ATA CCT AAA-3′ and 5′-GGC TGT AGT GCA GGC TGA TT-3′, and was subcloned into pBluescript II SK(+) vector in both directions. Antisense and sense cRNA probes were transcribed with T7 RNA polymerase (GIBCO BRL) in the presence of [^{35}S]-UTP (Daiichi kagaku).

Slides-mounted tissue sections (15 μm) were fixed in 4.6% formaldehyde in 0.1 M phosphate buffer (pH 7.2) for 20 min, soaked in 1% of triethanolamine for 10 min and treated in chloroform. Then they were hybridized at 55°C for 16 h with hybridization buffer (50% formamide, 20 mM Tris-HCl (pH 8.0), 10 mM phosphate buffer, 300 mM NaCl, 5 mM EDTA, 1 × Denhardt's solution, 0.2 mg ml^{-1} salmon sperm DNA, 10% dextran sulphate, 0.2% N-lauroylsarcosine sodium salt) containing each probe (5×10^6 c.p.m.). After that, they were washed to remove the non-specific signals with a high stringency wash buffer (50% formamide, 2 × SSC, 10% β-mercaptoethanol) for 30 min at 65°C, treated with RNase solution (RNaseA 0.2 mg ml^{-1} in 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.5 M NaCl buffer) for 30 min at 37°C and washed again. The slides were dipped in photographic

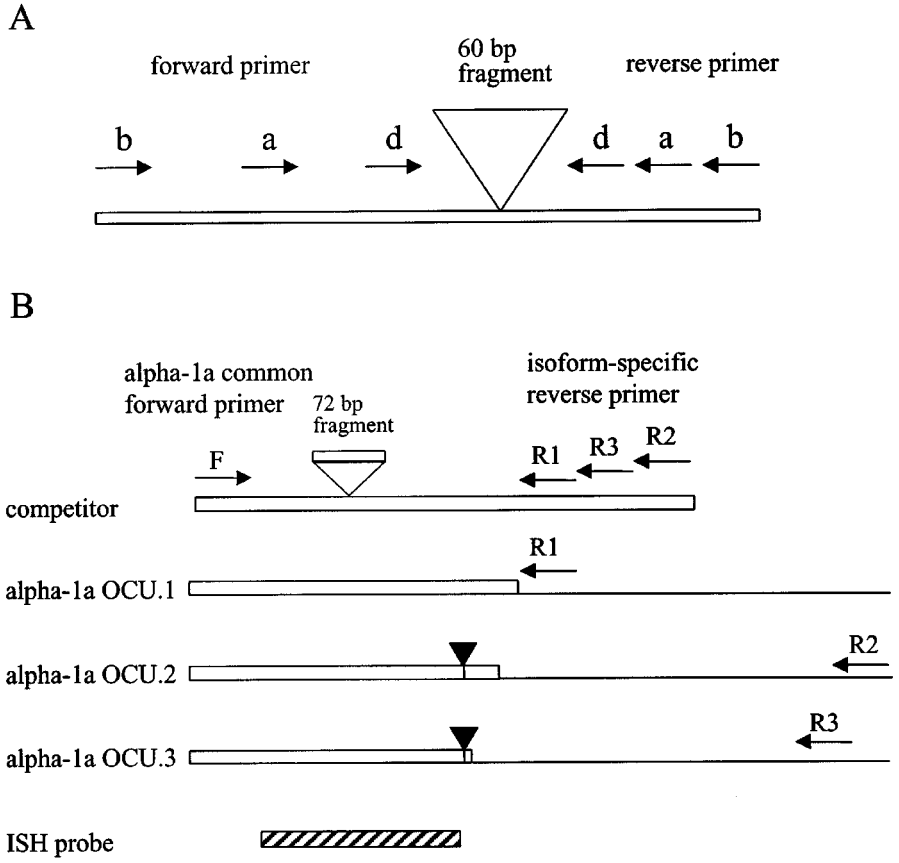

Figure 1 Schematic structure of the competitor for alpha-1 ARs (A) and for alpha-1a AR splice isoforms (B). The arrows indicate the primer's position and direction. The closed inverse triangles indicate alternative splice sites in alpha-1a AR and the ISH probe is indicated by a hatched box (B).

emulsion (50% in water, Eastman Kodak), exposed for 3 weeks at 4°C, and then developed in Kodak D19. All sections were examined under light- or dark-field illumination with a microscope.

Membrane preparation and binding experiment

For the binding experiment, each isolated ocular tissue was homogenized in 20 vol. (10 vol. only for the retina) of ice-cold homogenization buffer (mM: 50 Tris-HCl, 100 NaCl, 2 EDTA, pH 7.4) with a Polytron homogenizer (setting 8, 15 s × 3) and centrifuged at $3000 \times g$ for 15 min. The supernatant was filtered through four layers of surgical gauze and centrifuged at $80{,}000 \times g$ for 30 min at 4°C. The resulting pellet was suspended in ice-cold assay buffer (mM: 50 Tris-HCl, 2 EDTA, pH 7.4) and was immediately used for binding experiments.

In saturation binding experiments, the membranes were incubated with various concentrations of [^{3}H]-prazosin (100 pM – 2 nM) or [^{3}H]-KMD (500 pM – 1 nM). Nonspecific binding was defined as binding in the presence of a masking ligand (10μM phentolamine for [^{3}H]-prazosin and 3 μM prazosin for [^{3}H]-KMD). In competition binding experiments, the membranes were incubated with about 500 pM [^{3}H]-prazosin and unlabelled drugs (KMD-3213 or BMY7378). All assays were performed in duplicate and incubated for 45 min at 30°C with an incubation volume of 1 ml. Reactions were terminated by rapid filtration through Whatman GF/C filters presoaked in 0.3% polyethylenimine. The filters were then washed three times with 4 ml of ice-cold wash buffer (50 mM Tris-HCl, pH 7.4) and dried. The filter-bound radioactivity was determined by liquid scintillation counting. Binding affinities of [^{3}H]-prazosin and unlabelled drugs were expressed as a negative logarithm of the equilibrium dissociation constant (pK_D and pK_i, respectively). Protein determination was assayed according to the method of Bradford (1976) with bovine serum albumin as standard.

Data analysis

Analysis of radioligand binding data was performed with a curve-fitting program of GraphPAD PRISM (San Diego, CA, U.S.A.). Competition binding data were first fitted to a one- and then a two-site model, and the two-site model was accepted only if it resulted in a significant improvement of the fit as judged by an F-test with a $P < 0.05$. The pK_i values were calculated using the equation of Cheng & Prusoff (1973). Statistical difference in the B_{max} values was analysed using a one-factor ANOVA, and was decided significant with a $P < 0.01$.

Materials

The drugs used and their sources were following: (−)-1-(3-hydroxypropyl)-5-((2R)-2-{[2-(2,2,2-trifluoroethyl)oxy]phenyl}oxy)ethyl]amino}propyl)-2,3-dihydro-1H-indole-7-carboxamide (KMD-3213) from Kissei Pharmaceutical Co. Ltd. (Matsumoto, Japan); BMY7378 from research Biochemicals Inc. (Natick, U.S.A.); prazosin hydrochloride and phentolamine hydrochloride from Sigma (St. Louis, U.S.A.); [^{3}H]-prazosin (80 Ci mmol^{-1}) from NEN (Boston, U.S.A.); [^{3}H]-KMD-3213 (49 Ci mmol^{-1}) from Amersham (England) as a custom synthesis.

Results

Competitive RT – PCR assays

We first performed the subtype-specific competitive RT – PCR assays. Figure 2 illustrates the result of representative experiments using total RNA from rabbit iris and shows that alpha-1a AR was dominant and alpha-1d AR was very minor. The results of four regions (iris, ciliary body, choroid and retina) are summarized in Table 1, showing the main alpha-1 AR in the rabbit eye was the alpha-1a subtype at the mRNA level.

Next, we focused on the splice isoforms of the alpha-1a subtype in three regions (iris, ciliary body and choroid). The retina was not examined because of the low mRNA level of alpha-1 ARs. Figure 3 shows the experimental result in the rabbit iris, and the summarized results (Table 2) show distinct proportions of each alpha-1a isoform in three regions.

In situ *hybridization*

Since alpha-1a AR mRNA was dominant in RT – PCR assays, its tissue localization was examined by ISH methods. As shown in Figure 4, discernible hybridization signals for alpha-1a AR were observed in the iris dilator muscle (Figure 4A) and in the epithelium of the ciliary processes (Figure 4B) and moderate signals were observed in the choroid and retina (Figure 4C). There was a good correlation between the intensity of the hybridization signals and the results obtained in subtype-specific RT – PCR assays.

Binding study

We first performed saturation experiments with two ligands: [^{3}H]-prazosin (subtype non-selective drug for alpha-1 ARs) and [^{3}H]-KMD (selective drug for alpha-1A subtype). The receptor densities (fmol mg^{-1} protein) estimated with both ligands were very similar in the iris, choroid or retina; however, the Bmax value for [^{3}H]-KMD in the ciliary body was 38% of that for [^{3}H]-prazosin (Figure 5).

In the displacement experiments, [^{3}H]-prazosin binding sites were identified by BMY7378 (a selective drug for the alpha-1D AR subtype) and KMD-3213. BMY7378 inhibited [^{3}H]-prazosin binding monophasically with low affinities in the four regions of the rabbit eye (Figure 6 and Table 3). KMD-3213 also inhibited monophasically the binding, but the affinity was high in the iris, choroid and retina. In contrast, KMD-3213 showed a biphasic inhibition curve in the ciliary body, suggesting the presence of two distinct affinity sites. KMD-3213 has been used to separate alpha-1A from alpha-1B and alpha-1D subtypes with its high specificity to the alpha-1A subtype (Shibata *et al*., 1995; Murata *et al*., 1999). Taking these findings together with the saturation results, the pK_i values for KMD-3213 suggested that alpha-1A AR was a dominant subtype in the iris, choroid and retina; however, the ciliary body contained more alpha-1B (60 ~ 70% of total alpha-1 ARs) than the alpha-1A subtype at the protein level (Figure 5 and Table 3).

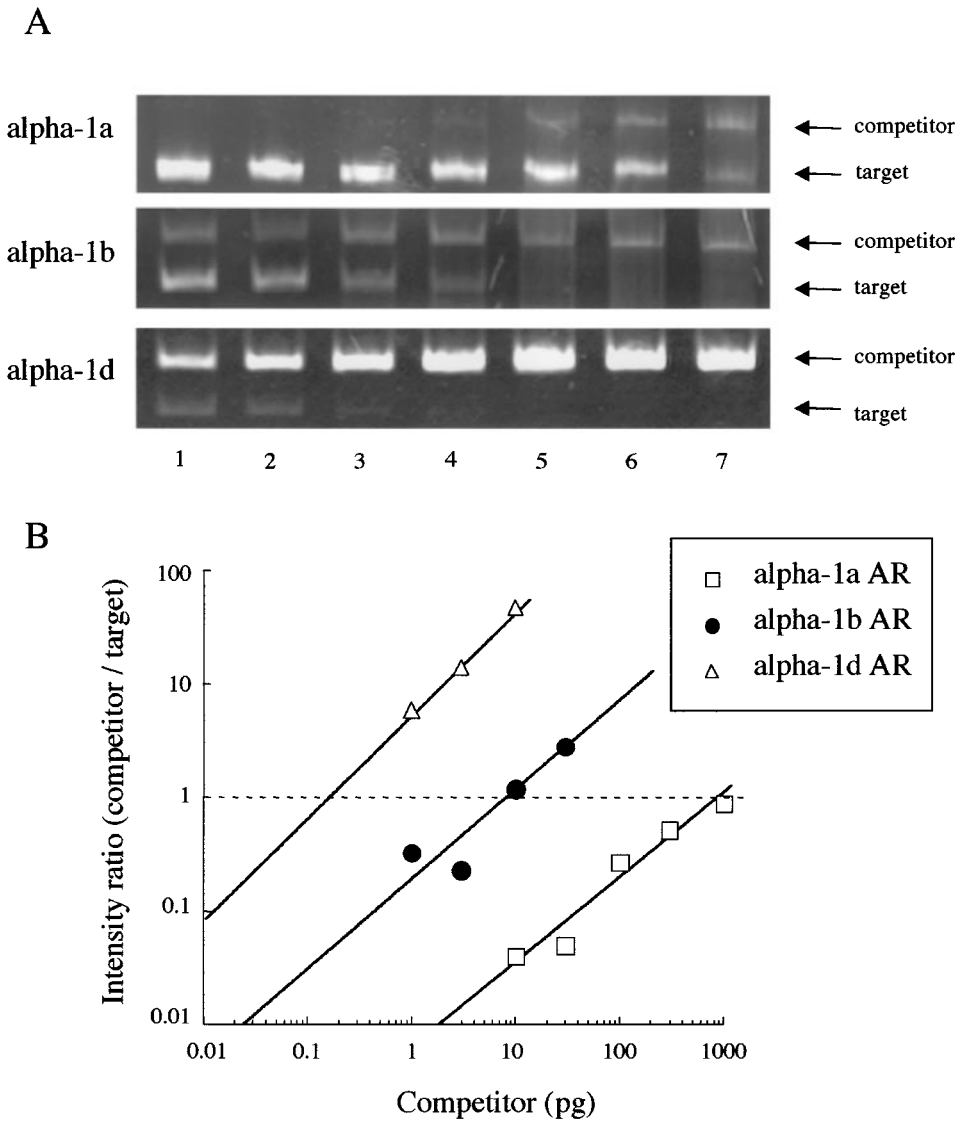

Figure 2 Subtype-specific competitive RT–PCR analysis of alpha-1 ARs mRNA. A representative result of the rabbit iris is shown. (A) Gel electrophoresis of RT–PCR products. Lanes 1–7 show the RT–PCR products of each alpha-1 AR subtype from 500 ng of the iris total RNA in the presence of 1, 3, 10, 30, 100, 300 and 1,000 pg of the competitor RNA. The sizes of the resulting PCR products (competitor/target) were 490/442, 604/549 and 270/210 bp for alpha-1a, alpha-1b and alpha-1d AR subtypes, respectively. (B) An estimation of the mRNA levels of each alpha-1 AR subtype in rabbit iris. The ordinate represents the ratio of the intensity of PCR products (competitor/target) and the abscissa represents the concentration of the competitor RNA in the reaction. Equivalent point where the ratio equals 1 means the same amount of each alpha-1 AR subtype mRNA as that of the added competitor.

Discussion

The findings of the current study clearly showed that alpha-1 ARs are widely expressed in rabbit eyes. The main subtype of alpha-1 ARs detected in this tissue appeared to be alpha-1A AR, which was confirmed at both mRNA and protein levels in the iris, choroid and retina. In addition, each alpha-1a isoform shows a distinct proportion at the mRNA level in the iris, ciliary body and choroid. Considering the possibility of their different functional profiles (Suzuki *et al*., 2000), it suggests that the different expression levels may reflect the distinct physiological significance of each alpha-1a AR isoform in the eye. On the other hand, in the ciliary body, alpha-1B AR was the dominant subtype at the protein level despite the negligible mRNA level of alpha-1b compared with alpha-1a AR. It was previously reported that alpha-1 AR mRNA levels are not necessarily correlated to the protein levels in several tissues (Piao *et al*., 2000; Michelotti *et al*., 2000).

Alpha-1 ARs play a role in the regulation of pupil diameter by contracting the iris dilator muscle. In addition to the predominant amount of the alpha-1A subtype in the whole iris tissue at the protein level, an intense ISH signal for alpha-1a mRNA was observed at the dilator muscle. These observations suggest that alpha-1 AR involved in mydriasis is the alpha-1A subtype. Nakamura *et al*. (1999) demonstrated that alpha-1A AR was a major subtype in the rabbit iris by binding and RT–PCR studies, but that the contractions of

Table 1 Relative mRNA levels of each alpha-1 AR subtype in rabbit eye tissues

	Iris	*Ciliary body*	*Choroid*	*Retina*
alpha-1a AR	99.1	99.2	98.6	92.6
alpha-1b AR	0.9	0.7	1.4	7.1
alpha-1d AR	0.0	0.1	0.0	0.3

The mRNA levels of alpha-1 AR subtypes in rabbit eye tissues were examined with subtype-specific competitive RT–PCR assays as shown in Figure 2. Values represent a percentage of each subtype of total alpha-1 ARs shown by mean of 2–3 independant experiments. The mRNA content of each alpha-1 AR subtype (competitor equivalent pg/500 ng total RNA) was as follows: 593, 5.2 and 0.1 in iris; 461, 3.2 and 0.3 in ciliary body; 283, 4.1 and 0.0 in choroid and 55, 4.2 and 0.2 in retina for alpha-1a, alpha-1b and alpha-1d AR, respectively. It was shown that the alpha-1a AR was a major subtype in rabbit eye tissues at the mRNA level.

Table 2 Relative mRNA levels of each alpha-1a AR splice isoform in rabbit eye tissues

	Iris	*Ciliary body*	*Choroid*
alpha-1a OCU.1-AR	44.4	55.2	36.1
alpha-1a OCU.2-AR	13.7	17.2	50.1
alpha-1a OCU.3-AR	41.9	27.7	13.8

The mRNA levels of alpha-1a AR splice isoforms in rabbit eye tissues were examined with isoform-specific competitive RT–PCR assays as shown in Figure 3. Vlaues represent a percentage of each isoform of total alpha-1a AR subtype shown by the mean of two independant experiments.

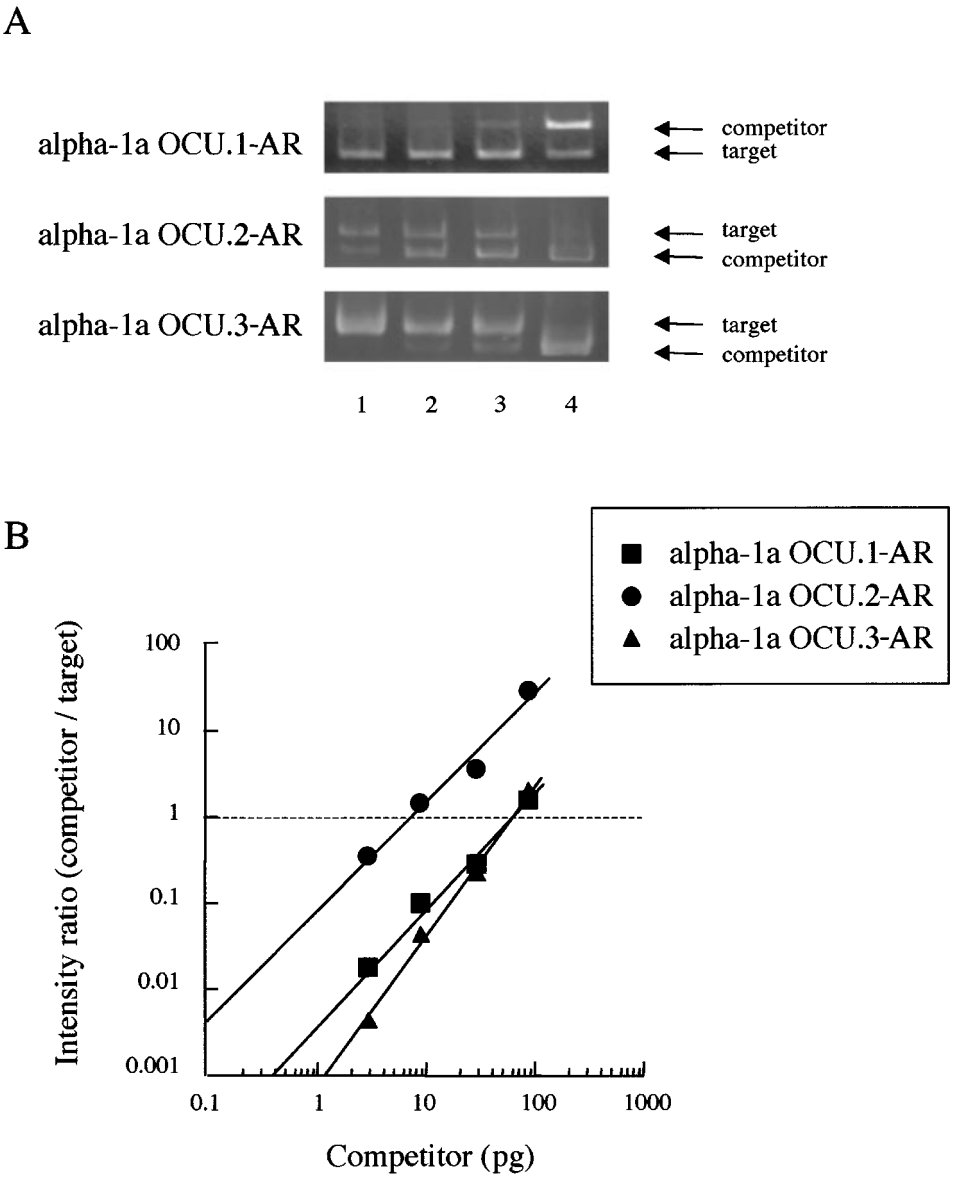

Figure 3 Isoform-specific competitive RT–PCR analysis of alpha-1a AR splice isoform mRNA. A representative result of the rabbit iris is shown. (A) Gel electrophoresis of RT–PCR products. Lanes 1–4 show the RT–PCR products from 500 ng of the iris total RNA in the presence of 3, 10, 30, and 100 pg of competitor RNA. The sizes of the resulting PCR products (competitor/target) were 676/604, 722/798 and 701/750 bp for alpha-1a OCU.1-, 2- and 3-AR, respectively. (B) An estimation of the mRNA levels of each alpha-1a AR splice isoform.

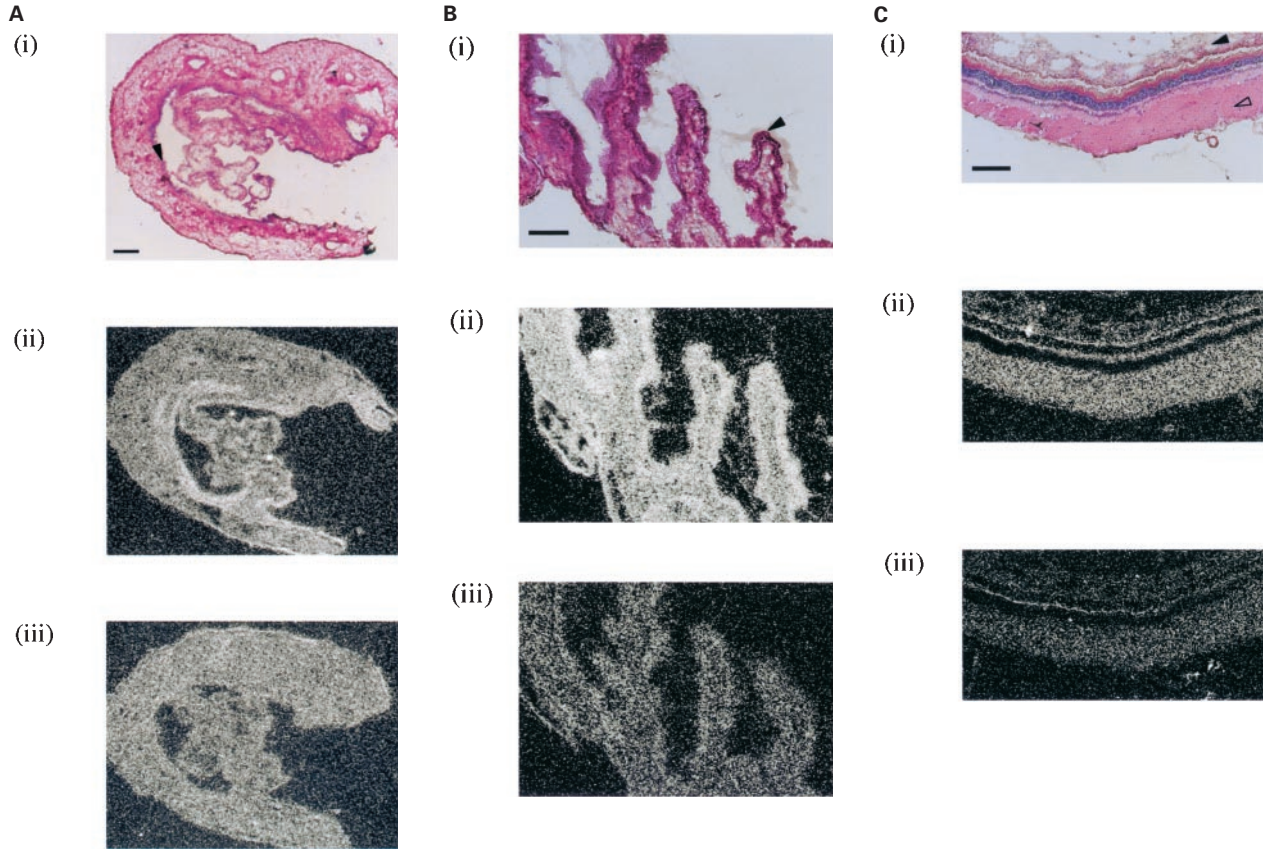

Figure 4 Alpha-1a AR mRNA distribution in rabbit eye tissues by ISH. High-magnification darkfield with both antisense (ii) and sense (iii) probes and corresponding hematoxylin and eosin (HE) stained images (i) of a rabbit iris (A), ciliary process (B), and choroid and retina (C). Closed arrowheads indicate iris dilator muscle (A), epithelium of ciliary process (B) and choroid (C), and an open arrowhead indicates the retina (C). The sclera was removed (C). Scale bars; 0.1 mm (A and C) and 0.05 mm (B).

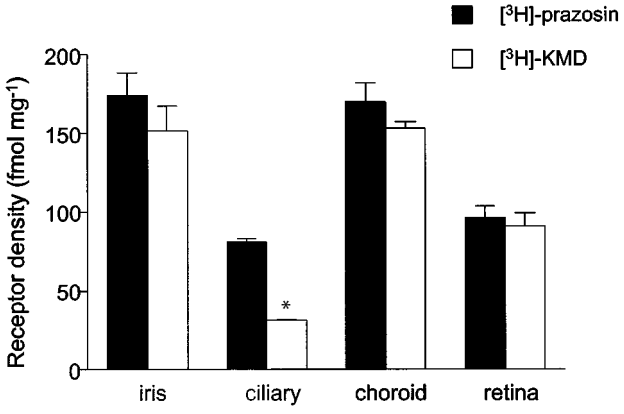

Figure 5 Receptor densities (B_{max}) of alpha-1 ARs in rabbit eye tissues determined by the saturation experiments using both [^{3}H]-prazosin and [^{3}H]-KMD as a radioligand. The black columns represent the results of [^{3}H]-prazosin binding (total alpha-1 AR densities) and the open columns represent that of [^{3}H]-KMD binding (alpha-1a AR densities). The B_{max} values are shown as mean-s$\pm$s.e.mean of 3–4 independent experiments and an asterisk indicates a significant difference ($P<0.01$) between the receptor density of [^{3}H]-prazosin binding and [^{3}H]-KMD binding.

the iris dilator muscle were mediated *via* the alpha-1 AR subtype which had low affinity for prazosin (putative alpha-1L AR subtype). Ishikawa *et al.* (1996) also reported that alpha-1 ARs in the iris dilator muscle of humans, and albino and pigmented rabbits showed low sensitivity to prazosin in contractile studies. *In vivo*, a topical application of bunazosin, a quinazoline analogue of prazosin, was relatively ineffective for pupillary size compared with the effect of decreasing IOP in rabbits (Nishimura *et al.*, 1993). These lines of evidence suggest the presence of putative alpha-1L AR as a functioning alpha-1 AR in the iris dilator muscle (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990). However, the corresponding gene of alpha-1L AR has not been identified. Recently, Daniels *et al.* (1999) proposed that recombinant alpha-1a AR exhibits a functional profile as alpha-1L AR. According to this theory, native alpha-1A AR in the rabbit iris exhibits two different characters; the binding profiles as alpha-1A AR and the functional profiles as alpha-1L AR. However, the mechanisms describing how a single receptor shows two different profiles are not clarified.

Alpha-1 ARs are also involved in aqueous humor dynamics. Topical application of specific agonists for alpha-1 ARs increases IOP, and that of specific antagonists for the receptors suppresses this phenomenon or decreases IOP in rabbits and monkeys (Kiuchi *et al.*, 1992; Nishimura *et al.*, 1993; Wang *et al.*, 1997; Zhan *et al.*, 1998; Okada & Gregory, 2001). IOP is regulated by two mechanisms; aqueous humor production and outflow. Krupin *et al.* (1980) demonstrated that prazosin lowered IOP in the rabbit eye by decreasing the rate of aqueous humor production. Ryan *et al.* (1998)

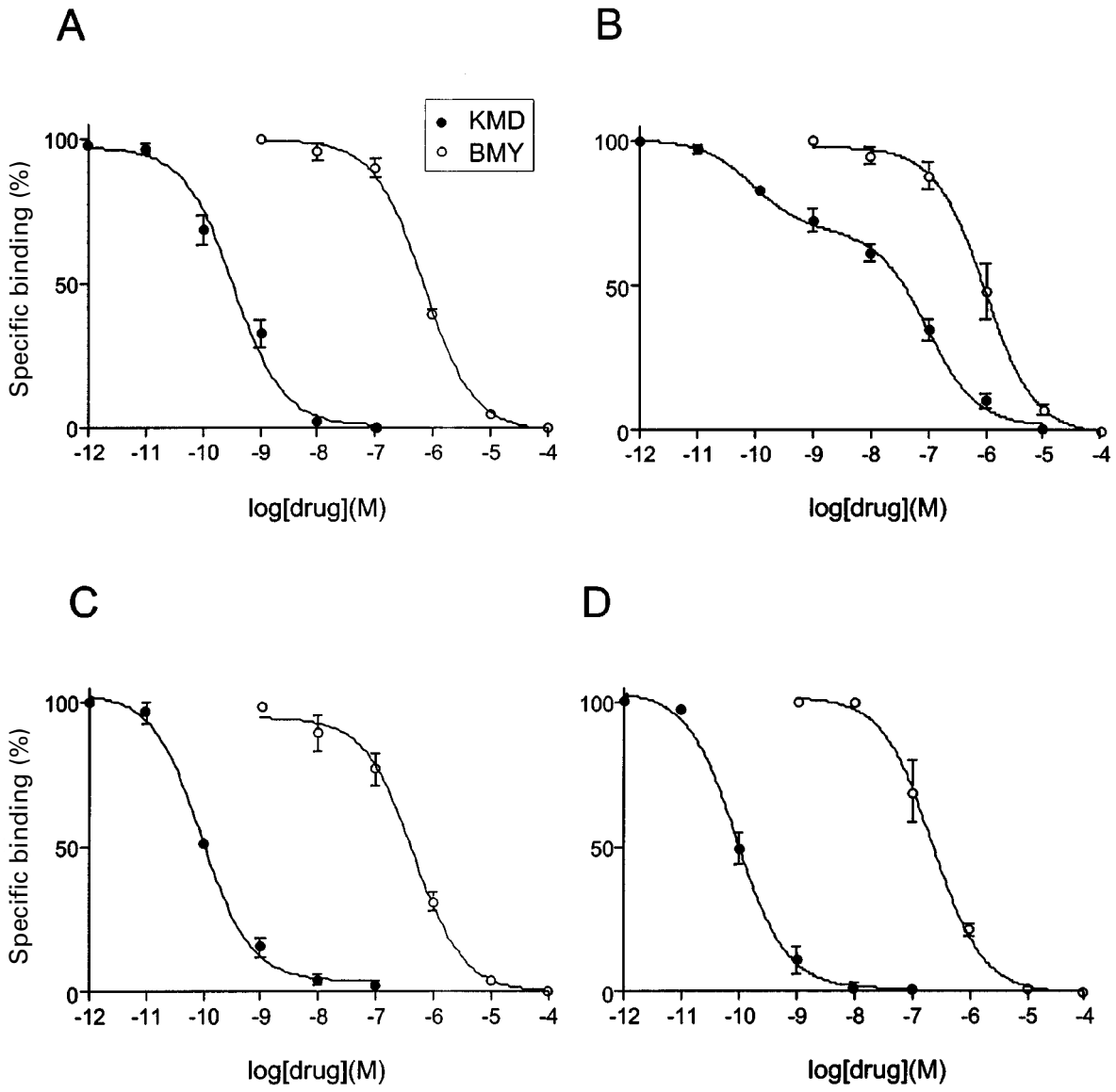

Figure 6 Displacement experiments of [^{3}H]-prazosin binding by KMD-3213 (closed circle) and BMY7378 (open circle) in rabbit eye tissues. The graphs show the results of a rabbit iris (A), ciliary body (B), choroid (C) and retina (D), respectively. Competition curve of KMD-3213 in the rabbit ciliary body was significantly fitted to the two-site model, and the other curves were fitted to the one-site model. Values are shown as means ± s.e.mean of 3–4 independent experiments.

Table 3 Characteristics of binding experiments to rabbit eye tissues

	Iris	*Ciliary body*	*Choroid*	*Retina*
Radioligand		pK_D		
[^{3}H]-prazosin	9.61 ± 0.03	9.50 ± 0.13	9.28 ± 0.05	9.34 ± 0.07
[^{3}H]-KMD	9.95 ± 0.36	9.78 ± 0.03	9.87 ± 0.12	9.81 ± 0.16
Antagonist		pK_i		
KMD-3213	10.06 ± 0.04	10.25 ± 0.17(32%) 7.16 ± 0.25	10.28 ± 0.06	10.28 ± 0.10
BMY7378	6.70 ± 0.07	6.51 ± 0.24	6.61 ± 0.10	6.95 ± 0.13

pK_D and pKi values were estimated from the results of the saturation and the displacement experiments. In the two-site models, the percentage of a high affinity site is shown in parenthesis. Values are shown as means ± s.e.mean of 3–4 experiments with different membrane preparations.

suggested that potassium channels coupled to alpha-1 ARs might modulate fluid secretion by the ciliary epithelium. In addition, Zhan *et al*. (1998) showed that bunazosin lowered IOP with increased outflow.

The current study clearly showed the ISH signal for alpha-1a mRNA in the epithelium of the ciliary processes, supporting the previous functional evidence. Collectively, it is likely that IOP regulation mediated *via* alpha-1A AR is caused by changing the rate of aqueous humor production and outflow. However, since cases have been reported that intra- and extrajunctional alpha-1 AR subtypes are heterogenous in some tissues (Hill *et al*., 1993; Honner & Docherty, 1999; Yang & Chiba, 2001), the physiological significance of minor expressed alpha-1 ARs may not be ignored.

In conclusion, the findings of the present study demonstrated that alpha-1A AR is a dominant subtype in rabbit iris, choroid and retina; however, alpha-1B AR function can not be neglected in the ciliary body. Moreover, alpha-1A AR selective drug may have a therapeutic potential in the eye.

GenBank accession numbers are as follows: U81982 for α_{1a}-OCU.1-AR, AF157505 for α_{1a}-OCU.2-AR and AF157506 for α_{1a}-OCU.3-AR, respectively.

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