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Evaluation of α_1-adrenoceptors in the rabbit iris: pharmacological characterization and expression of mRNA

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1 Subtypes of α_1-adrenoceptor in rabbit iris have been examined in functional, binding and molecular biological experiments.

2 In functional studies, exogenous and endogenous noradrenaline produced contractions of the iris dilator muscle. The contractile responses to noradrenaline were competitively antagonized by a range of α_1-adrenoceptor antagonists (pA_2 values): prazosin (8.1), WB4101 (8.2), BMY7378 (5.9), YM617 (9.5), JTH-601 (8.8), HV723 (7.8) and KMD-3213 (9.8). The same order of inhibitory potency was seen in the adrenergic responses to electrical stimulation. This affinity profile corresponds well to that of the putative α_{1L}-adrenoceptor, which has been proposed in lower urinary tract tissues.

3 In binding studies on rabbit iris membrane however, prazosin, KMD-3213 and WB4101 displayed high affinity (pK_d or pK_i: 9.6, 10.3, 9.6, respectively), and BMY7378 displayed low affinity (pK_i: 6.9). These results show that the binding sites typically correspond to α_{1A}-adrenoceptor subtype in character, and we could not detect the significant amount of α_{1L}-adrenoceptor subtype.

4 The expression of the three distinct mRNAs that encode proteins of α_{1a}-, α_{1b}- and α_{1d}-adrenoceptors was studied using reverse transcription-polymerase chain reaction (RT–PCR). RT–PCR demonstrated the strongest expression of the α_{1a}-adrenoceptor, weak expression of the α_{1b}-adrenoceptor and undetectable expression of the α_{1d}-adrenoceptor.

5 The present study suggests that α_{1A}-adrenoceptor is a major subtype detectable in binding and RT–PCR studies in rabbit iris, but that the adrenergic contractions of iris dilator muscle are mediated *via* activation of α_1-adrenoceptor subtype having low affinity for prazosin and WB4101.

Keywords: Rabbit; iris; dilator muscle; α_1-adrenoceptor; subtype; radioligand; mRNA

Abbreviations: CHO, Chinese hamster ovary; RT–PCR, reverse transcription-polymerase chain reaction; TES, transmural electrical stimulation

Introduction

Pharmacological and molecular cloning studies have established operational and structural heterogeneity among α_1-adrenoceptors (Minneman, 1988; Ford *et al*., 1994; Bylund *et al*., 1994). The current classification recognizes the existence of three α_1-adrenoceptors (α_{1A}, α_{1B} and α_{1D}) all of which meet well the criteria (i.e., sequence, second messenger and pharmacological profile: Hieble *et al*., 1995). A fourth α_1-adrenoceptor, α_{1L}-adrenoceptor, which is mainly based on the results of functional pharmacological studies, has been postulated (Holck *et al*., 1983; Flavahan & Vanhoutte, 1986; Muramatsu, 1992). However, the corresponding gene has not been identified yet. Being widely distributed in heart, liver, lower urinary tract tissues, vascular and ocular smooth muscle tissues, α_1-adrenoceptors play an important physiological role in peripheral organs (Lefkowitz & Caron, 1988; Minneman, 1988; Muramatsu, 1992; Ford *et al*., 1996; Miyamoto *et al*., 1997; Suzuki *et al*., 1997).

The iris, which controls the size of the pupil and thus regulates the amount of light admitted, is innervated by sympathetic and parasympathetic nerves; the former regulates the contraction of dilator muscle *via* α_1-adrenoceptors (Malmfors, 1965; Van Alphen *et al*., 1965; Narita & Watanabe, 1982; Konno & Takayanagi, 1986; McGregor, 1994) and the latter controls that of sphincter muscle *via* muscarinic receptors (Choppin *et al*., 1998) in many mammalian species. Thus, agonism of α_1-adrenoceptors in the iris dilator leads to mydriasis, conversely antagonism results in miosis. Ishikawa *et al*., (1996) reported that the α_1-adrenoceptor subtype in rabbit iris dilator has high affinity for prazosin, while that in the iris dilator of human or pigmented rabbit has low affinity for prazosin. Mittag & Tormay (1985) reported that the pK_d value of [^{3}H]-prazosin is 9.3 in rabbit iris-ciliary body. However, because of a small amount of this tissue, very few binding studies have been made in the iris so far, and it still remains unclarified which subtype(s) of α_1-adrenoceptors are involved in the contraction of the dilator muscle.

The purpose of this study is to demonstrate the α_1-adrenoceptor subtype responsible for the rabbit iris dilator contraction in the functional study, and to characterize the α_1-adrenoceptor subtype of whole rabbit iris in the binding study and RT–PCR assay.

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 killed. The eyes were enucleated promptly and dissected under a surgical microscope to isolate iris in ice-cold modified Krebs-Henseleit solution of the following composition (mM): NaCl 112, KCl

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5.9, $MgCl_2$ 1.2, $CaCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1.2, and glucose 11.5.

Functional experiments

A strip of iris dilator muscle was prepared in approximately 2 mm width and 4 mm length and was mounted vertically in an organ bath containing 20 ml of modified Krebs-Henseleit solution, continuously bubbled with 95% O_2 and 5% CO_2 at 37°C. All preparations were equilibrated for 90 min with a resting tension of 50 mg before the experiments were begun. Changes in isometric tension were recorded on a pen-writing oscillograph (FJG-4128, Nihon Koden, Tokyo, Japan) in the following experiments.

Concentration-response curves for agonists were obtained by adding the drugs directly to the bath in cumulative fashion. The concentration-response curves for noradrenaline were obtained four times in the same strip at 60 min washout intervals. The second concentration-response curves were used as the control and α_1-adrenoceptor antagonists were added for 30 min before and during the third and fourth responses. In all experiments desipramine (0.1 μM), deoxycorticosterone acetate (1 μM) and propranolol (1 μM) were added in the bath to block neural and extraneural uptake of noradrenaline and to block β-adrenoceptors, respectively.

For the transmural electrical stimulation (TES), the preparation was placed between a pair of platinum electrodes. Gaps between the electrodes were wide enough to accommodate the strip and yet sufficiently narrow to stimulate the intramural nerve terminals effectively. To block presynaptic α_2-adrenoceptor, DG-5128 (10 μM) was added to the bath (Muramatsu *et al*., 1983). Although Yamanaka *et al*. (1984) showed that IC_{50} value of this compound at α_1-adrenoceptors of rat brain homogenates is approximately 4 μM, we confirmed that noradrenaline-induced contractions of the rabbit dilator muscle are not affected by DG-5128 up to 10 μM in preliminary examination (data not shown). A train of rectangular pulses (7.5 mV intensity, 130 μs duration, 10 Hz frequency, for 10 s) was applied every 15 min. Antagonists to be tested were added to the organ chamber 30 min before the stimulation.

The affinities for α_1-adrenoceptor antagonists were estimated according to Arunlakshana & Schild (1959). Data were plotted as the $-\log$ [antagonist concentration] vs the log [concentration ratio (CR) -1], and pA_2 values with mean slopes and 95% confidence limits (95% CL) were calculated and first regression lines were drawn by least square method.

Binding experiments

For membrane preparation the irides were minced with scissors and homogenized in 20 volume of buffer (Tris HCl 50 mM, NaCl 100 mM, EDTA 2 mM, 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 subjected to centrifugation at $80{,}000 \times g$ for 30 min. The pellet was resuspended in ice-cold assay buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4) and the resuspension was centrifuged at $80{,}000 \times g$ for 30 min again. All centrifugation was done at 4°C. The resultant pellet was resuspended in assay buffer as a membrane fraction and was immediately used for binding assay.

In saturation binding experiments, the membranes were incubated with various concentrations of [^{3}H]-ligand ([^{3}H]-prazosin; 30 pM – 5 nM or [^{3}H]-KMD; 10 pM – 1 nM) for 45 min at 30°C with incubation volume 1 and 2 ml, respectively. Nonspecific binding was defined as binding in the presence of a masking ligand (0.3 μM YM617 for [^{3}H]-prazosin and 0.3 μM prazosin for [^{3}H]-KMD). In competition binding experiments, the membranes were incubated with about 100 pM [^{3}H]-KMD and unlabelled drugs for 45 min at 30°C. Reactions were terminated by rapid filtration through Whatman GF/C filters presoaked in 0.3% polyethylenimine for 45 min. The filters were then washed four times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and dried. The filter-bound radioactivity was determined by liquid scintillation counting. All assays were performed in duplicate. Binding affinities of [^{3}H]-ligands and unlabelled drugs were expressed as negative logarithm of the equilibrium dissociation constant (pK_d and pK_i, respectively). Displacement binding data were analysed by GraphPAD Prism. When the slope factor was close to unity, concentrations of competing agent producing 50% displacement of [^{3}H]-KMD (IC_{50}) were converted to K_i values with the Cheng & Prusoff approximation (1973). Protein determination was assayed according to the method of Bradford (1976) with bovine serum albumin as standard.

RT–PCR assays

The iris was frozen in liquid nitrogen immediately after isolation and then stored at −80°C until extraction of RNA. Total cellular RNA was extracted according to the procedure of Chomczynski & Sacchi (1987). All RNA transcripts for three adrenoceptor subtypes were synthesized from the appropriate plasmid DNA with the use of T7 RNA polymerase (Gibco BRL). To produce the cDNA, total RNA (500 ng) from iris and cRNA (300 pg) from clones were individually reverse transcribed by moloney murine leukaemia virus reverse transcriptase (Gibco BRL) with random hexamer. Three μl of cDNA was amplified in a 25 μl PCR mixture containing (mM) KCl 50, Tris-HCl 10, pH 8.3, $MgCl_2$ 2, 200 μM each of dNTP, 0.25 μM sense and antisense primers and 2.5 U Pwo DNA polymerase (Boehringer). The subtype specific primers were designed from published sequences of rabbit α_{1a}- and α_{1d}-adrenoceptors (Miyamoto *et al*., 1997; Suzuki *et al*., 1997) and unpublished sequence of rabbit α_{1b}-adrenoceptor, as follows: α_{1a}: 5′-CATCGTGGTCGGCTGCTTCGTC-3′, 5′-GGCTGTAGTGCAGGCTGATT-3′, α_{1b}: 5′-AGGAGCCGGCACCCAATGATGA-3′, 5′-GGCACTGGCACCCGAGGAT-3′, α_{1d}: 5′-CTCCGTGCGCCTGCTCAAGT-3′, 5′-GGGTAGATGAGTGGGTTCAC-3′. PCR was performed in a Perkin-Elmer DNA Thermal Cycler 2400 with the following cycle parameters: one denaturation cycle for 3 min at 98°C; 40 cycles of 30 s at 95°C, annealing for 10 s and extension for 15 s at 72°C. Annealing temperatures were 68°C for the α_{1a}-adrenoceptor, 64°C for the α_{1b}-and α_{1d}-adrenoceptor. For each experiment, negative controls were amplified, in which all of the components except reverse transcriptase were included. The PCR products were separated on 3.5% polyacrylamide gels and stained with ethidium bromide.

Drugs

The drugs used and their sources were following: (−)-(R)-1-(3-hydroxypropyl)-5-[2-[[2-[2-(2,2,2-trifluoroethoxy) phenoxy] ethyl]amino]propyl]indoline-7-carboxamide (KMD-3213) and tamsulosin hydrochloride (YM-617) from Kissei Pharmaceutical Co. Ltd. (Matsumoto, Japan); 3-[N-[2-[4-hydroxy-2-isopropyl-5-methylphenoxy] ethyl] -N-methylaminomethyl] - 4-methoxy-2,5,6-trimethylphenol hemifumarate (JTH-601) from Japan Tobacco Inc. (Osaka, Japan); α-ethyl-3,4,5-trimethoxy-α-[3-[[2-2-methoxyphenoxy]ethyl] -amino] -propyl] benzeneacetonitrile fumarate] (HV723) from Hokuriku Seiyaku (Katsuya-

ma, Japan); [2-[2-[4,5-dihydro-1H-imidazol-2-yl]1-phenylethyl] pyridine dihydrochloride sesquihydrate] (DG-5128) from Daiichi Seiyaku (Tokyo, Japan); prazosin hydrochloride, (−)-noradrenaline bitartrate, (−)-phenylephrine hydrochloride, oxymetazoline and desipramine hydrochloride, from Sigma (St. Louis, U.S.A.); WB4101 and BMY7378 from Research Biochemicals Inc. (Natick, U.S.A.); (−)-propranolol hydrochloride and deoxycorticosterone acetate from Nakalai (Kyoto, Japan); clonidine hydrochloride from Funakoshi (Tokyo, Japan); [^{3}H]-KMD-3213 (49 Ci mmol^{-1}), from Amersham (England); and [^{3}H]-prazosin (77.2 Ci mmol^{-1}) from NEN (Boston, U.S.A.).

Results

Functional study

Figure 1 shows the concentration-response curves for noradrenaline, phenylephrine, oxymetazoline and clonidine in the rabbit iris dilator muscle. All agonists produced concentration-dependent contraction in dilator muscle. The maximal amplitude of contraction induced by phenylephrine was almost equal to that induced by noradrenaline, whereas those by oxymetazoline or clonidine were smaller than that induced by noradrenaline. The pEC_{50} values and relative contractions of these agonists are summarized in Table 1, in which the maximal amplitude of contraction was compared with that induced by noradrenaline in the same strip. The following order of potency was observed: oxymetazoline > noradrenaline > phenylephrine > clonidine. Several α_1-adrenoceptor antagonists (prazosin, WB4101, BMY7378, YM617, JTH-601, HV723 and KMD-3213) were tested for their ability to inhibit noradrenaline-induced responses and their functional affinity estimates (pA_2 values) are summarized in Table 2. The concentration-response curves for noradrenaline were antagonized by these compounds in a concentration-dependent manner, with parallel rightward displacements. Figure 2 shows representative results with prazosin and KMD-3213. The Schild slopes were not significantly different from unity for all these antagonists, indicating that the antagonism was competitive in nature. KMD-3213, YM617 and JTH-601 displayed higher affinity than the other antagonists (Table 2). Rauwolscine (1 μM) had no effect on noradrenaline-induced contraction (data not shown).

TES at 10 Hz for 10 s produced a monophasic and transient contraction of dilator muscle. The responses to TES were abolished by 0.5 μM tetrodotoxin. The α_1-adrenoceptor antagonists mentioned above inhibited but not abolished the contraction induced by TES; thus 15% of contractile amplitude remained as the resistant component (Muramatsu *et al*., 1994). Their functional affinities (pIC_{50} which was estimated at the level of 50% reduction of α_1-adrenoceptor antagonist-sensitive contraction) are also summarized in Table 2. The rank order of pIC_{50} was the same as pA_2 values estimated in noradrenaline-response.

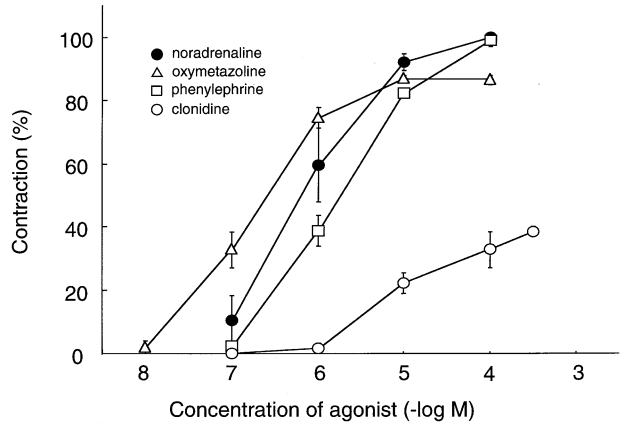

Figure 1 Concentration-response curves for four α-adrenoceptor agonists in isolated rabbit iris dilator muscle. The maximal contraction induced by 100 μM noradrenaline (267.2±19.4 mN; $n=8$) was taken as 100% in each preparation. The data shown are means±s.e.mean of 4–8 experiments. For pEC_{50} values and maximal contractions see Table 1.

Binding study

Figure 3 shows representative data obtained in saturation experiments with [^{3}H]-prazosin (a) and [^{3}H]-KMD (b), respectively. Both [^{3}H]-prazosin and [^{3}H]-KMD meet the criteria for binding to a single set of sites. The binding isotherms

Table 1 Contractile parameters for various α-adrenoceptor agonists in isolated rabbit iris dilator

Agonist	*pEC_{50}*	*Max. contraction* (%)*	n
Noradrenaline	6.16±0.11	100	8
Phenylephrine	5.78±0.05	99.0±1.00	7
Oxymetazoline	6.77±0.09	86.5±1.50	4
Clonidine	5.41±0.09	38.5±1.50	4

Values shown by means±s.e.mean; *n*, number of experiments. *Ratio of the maximal contraction induced by agonists to the maximal contraction induced by noradrenaline in the same preparation.

Table 2 Functional affinities for α_1-adrenoceptor antagonists in isolated rabbit iris dilator

Antagonist	*Noradrenaline-response* *pA_2* (Slope, 95% CL)*	n	*TES-response* *pIC_{50}†*	n
Prazosin	8.08±0.05 (0.88, 0.78–0.99)	6	7.66±0.07	6
WB4101	8.17±0.13 (1.00, 0.81–1.19)	4	8.08±0.07	7
BMY7378	5.90±0.09 (1.00, 0.65–1.34)	4	4.99±1.35	5
YM617	9.45±0.06 (1.14, 1.01–1.26)	4	9.49±0.03	4
JTH-601	8.82±0.21 (0.82, 0.56–1.06)	3	8.53±0.08	6
HV723	7.83±0.09 (1.10, 0.92–1.27)	4	7.61±0.09	5
KMD-3213	9.84±0.06 (0.92, 0.79–1.04)	4	9.68±0.02	4

Values shown are means±s.e.mean; *n*, number of experiments. *pA_2 value, slope factor and 95% confidence limit (CL) estimated from Schild analysis. All Schild slopes were not significantly different from unity ($P>0.05$). †Negative logarithm concentration of α_1-adrenoceptor antagonists which inhibited the TES-induced contractions to 50% in isolated rabbit iris dilator muscle.

for these ligands were hyperbolic, as shown in Figure 3. The pK_d values and maximum binding sites (B_{max}) of both ligands are shown in Table 3. Although we could not carry out many experiments because of limitation of tissue amount, there was no difference between the two independent experiments (Experiment 1 and Experiment 2). The pK_d values of [^{3}H]-prazosin and [^{3}H]-KMD are 9.6 and 10.3 (mean of two experiments), respectively, and B_{max} values of [^{3}H]-prazosin and [^{3}H]-KMD were almost same (Table 3). The specific bindings of [^{3}H]-KMD were displaced by simultaneous addition of nonradioactive ligands (Figure 4). The slope factors of the drugs were not significantly different from unity, suggesting the presence of a single type of binding site. The pK_i values for drugs in two independent experiments were almost same (Table 3).

RT–PCR study

Figure 5 illustrates the results of a representative experiment using total RNA from rabbit iris and cRNAs from cloned α_1-adrenoceptor subtypes. The RT–PCR products derived from clone cRNAs are observed for α_{1a}-adrenoceptor at 442 bp (A, lane 4), α_{1b}-adrenoceptor at 549 bp (B, lane 4) and α_{1d}-adrenoceptor at 210 bp (C, lane 4), respectively. The cDNA generated from total RNA of irides was amplified and expression of α_1-adrenoceptor subtypes mRNA in rabbit iris is shown in lanes 2. The α_{1a}-adrenoceptor specific oligonucleotide primers amplified a large amount of cDNA (A, lane 2). A faint band was generated with the α_{1b}-adrenoceptor specific primers (B, lane 2) while no detectable PCR product was amplified with the α_{1d}-adrenoceptor specific primers (C, lane 2). All PCR fragments corresponded to the predicted fragment size for each receptor subtype. No PCR product was seen in the control experiments which did not contain reverse transcriptase in the reverse transcriptase-reaction (A,B,C, lanes 3 and 5), suggesting no DNA contamination was present. Two other experiments conducted under same conditions showed the identical results.

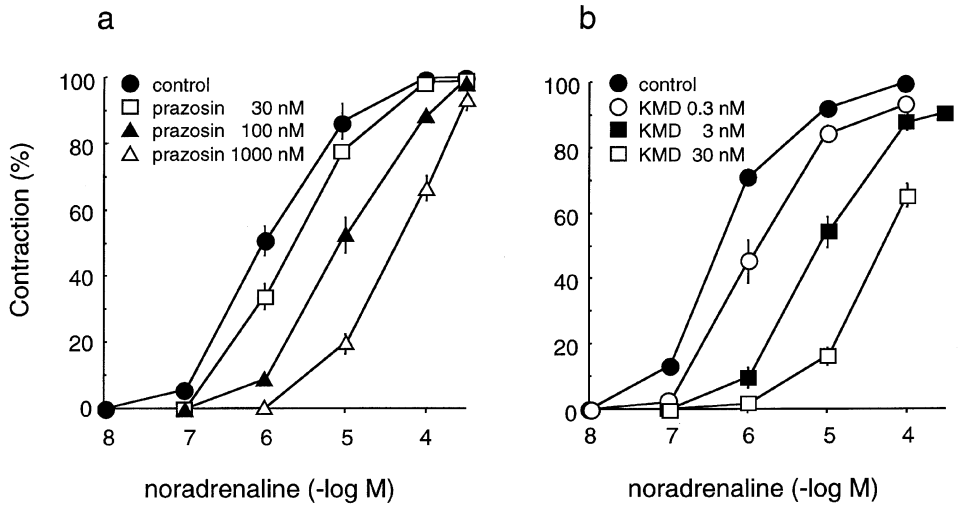

Figure 2 Concentration-response curves of isolated rabbit iris dilator muscle for noradrenaline and effects of prazosin (a) and KMD-3213 (b). Responses were plotted as percentages of maximal contractile response for noradrenaline in control. The data shown are means±s.e.mean of 3–6 experiments. For pA_2 values see Table 2.

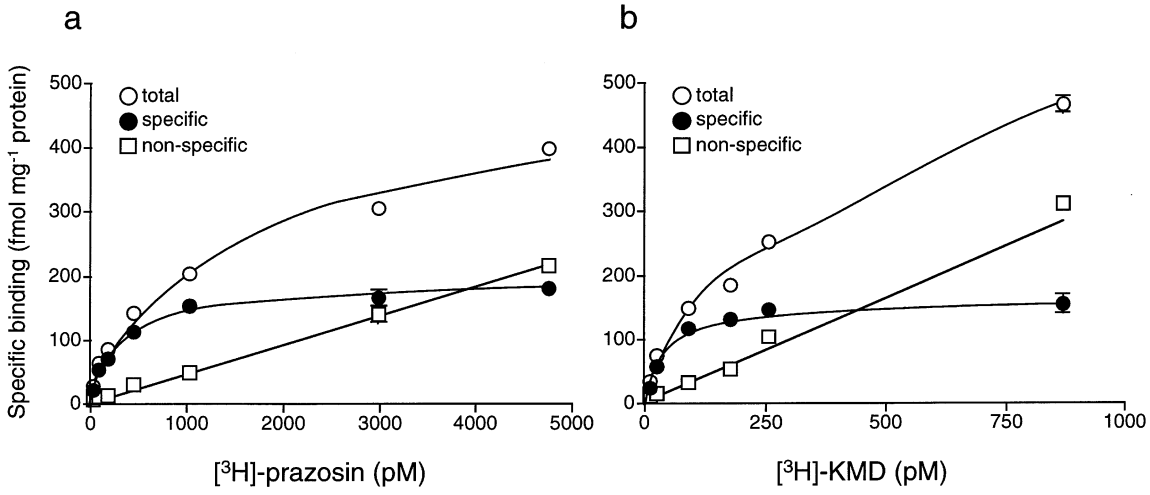

Figure 3 Saturation experiments of [^{3}H]-prazosin (a) and [^{3}H]-KMD (b) binding to rabbit iris membranes. The data are obtained from a single experiment (Experiment 1) where each point is the mean of duplicate determinations. Another experiment was also carried out with different membrane preparation and the data obtained from two independent experiments were shown in Table 3.

Table 3 Binding parameters estimated with [^{3}H]-prazosin and [^{3}H]-KMD in rabbit iris membranes

A. Saturation experiments

	pK_d		B_{max} (fmol mg^{-1} *protein*)	
[^{3}H]-ligand	*Experiment 1*	*Experiment 2*	*Experiment 1*	*Experiment 2*
[^{3}H]-prazosin	9.64	9.57	189.9	186.6
[^{3}H]-KMD	10.34	10.27	167.9	166.8

B. Displacement experiments

	pK_i	
Drug	*Experiment 1*	*Experiment 2*
Prazosin	9.28	9.22
WB4101	9.53	9.63
Oxymetazoline	8.44	7.94
BMY7378	6.91	6.79

Two independent experiments were conducted (Experiment 1 and Experiment 2), in which the irides isolated from 124 rabbits were pooled and used. A: Saturation experiments were carried out with [^{3}H]-prazosin and [^{3}H]-KMD in two different membrane preparations. B: Displacement experiments were carried out against 100 pM [^{3}H]-KMD binding.

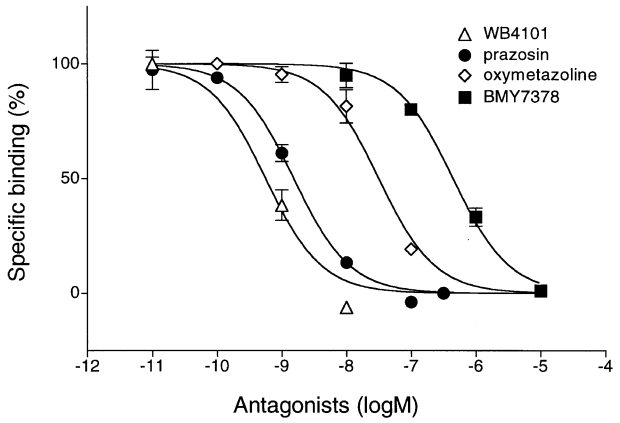

Figure 4 Displacement of [^{3}H]-KMD binding to rabbit iris membranes by α_1-adrenoceptor antagonists. The concentration of [^{3}H]-KMD used was 100 pM. The data are obtained from a single experiment (Experiment 2) where each point is the mean of duplicate determinations. The data obtained from two independent experiments were shown in Table 3.

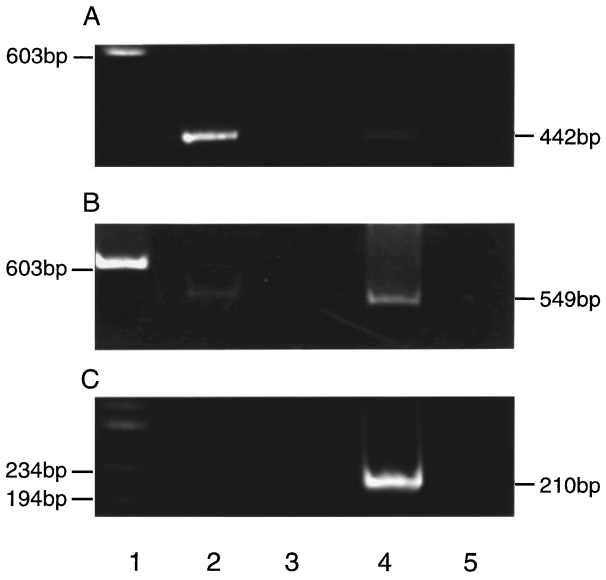

Figure 5 Polyacrylamide gel electrophoresis of 5 μl of RT–PCR product. Lane 1 shows DNA size markers ϕX174/*Hae*III. Lanes 2 and 3 represent adrenoceptor RT–PCR products from iris total RNA. Lanes 4 and 5 represent α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptor RT–PCR products from 300 pg of each adrenoceptor clone cRNA. Bands of lane 4 442 bp (A), 549 bp (B), 210 bp (C) is a product of the α_{1a}-, α_{1b}- and α_{1d}-adrenoceptor clone cRNA respectively. No PCR products were seen in the control experiments, which contained no reverse transcriptase in the reverse transcriptase-reaction (lanes 3 and 5).

Discussion

In the functional study, a series of α-adrenoceptor agonists and antagonists, which should be able to discriminate the subtype involved (Hieble *et al*., 1995; Muramatsu *et al*., 1998), were used to characterize the α-adrenoceptors in the dilator smooth muscle of rabbit iris. Noradrenaline and phenylephrine acted as full agonists, while oxymetazoline and clonidine behaved as partial agonists (Figure 1 and Table 1). The contractile responses to noradrenaline and TES were inhibited in a concentration-dependent and competitive fashion by several α_1-adrenoceptor selective antagonists (Figure 2). Whereas the responses to noradrenaline were not inhibited by 1 μM of rauwolscine (a selective antagonist for α_2-adrenoceptor). These results indicate that the adrenergic responses in rabbit iris dilator are mediated through α_1-adrenoceptors. However, the potencies or affinities varied among the agonists or antagonists used (Tables 1 and 2), suggesting an involvement of unique α_1-adrenoceptors in the adrenergic responses. Since Schild slopes for tested antagonists were close to unity and the range of effective concentrations of tested agonists were less than three log units, it appears that the adrenergic contractions are predominantly mediated by a single α_1-adrenoceptor subtype.

In the series of antagonists tested, KMD-3213 (pA_2: 9.8), YM617 (9.5) and JTH-601 (8.8) showed high affinity, while WB4101 (8.2), prazosin (8.1), HV723 (7.8) and BMY7378 (5.9) showed low affinity. These affinity estimates of antagonists correlated most closely with those in lower urinary tract tissues of rabbit or human (pA_2: prazosin 7.85–8.26; WB4101 8.4–8.9; BMY7378 6.4; YM617 9.31–9.49; JTH-601 8.8; HV723 7.63–8.05; KMD-3213 9.9–10.05) (Honda *et al*., 1985; Ford *et al*., 1996; Yamagishi *et al*., 1996; Leonardi *et al*., 1997; Takahashi *et al*., 1998; Murata *et al*., 1999), where putative α_{1L}-adrenoceptor has been proposed in the adrenergic responses. Furthermore, the affinity values for these antagonists against the responses to TES showed the same trend (Table 2). These results suggest that exogenous and endogenous noradrenaline acts on the same subtype (pre-

sumably α_{1L}-adrenoceptor) to cause contraction in rabbit iris dilator.

The α_{1L}-adrenoceptor subtype is characterized according to low affinity for prazosin and other antagonists (Flavahan & Vanhoutte, 1986; Muramatsu *et al*., 1990). Marshall *et al*. (1996) argued that one of the main problems in determining whether prazosin does distinguish between α_1-adrenoceptor subtypes is that the pA_2 values published cover a continuous range of values rather than falling into discrete groups. For example, pA_2 values of 8.3, 8.2 and 8.1 have been documented for α_1-adrenoceptors in the rabbit trigone, urethra and prostate, respectively (Honda *et al*., 1985), 8.5 in rabbit prostate (Williams & Clarke, 1993), 8.9 in rat anococcygeus (Ford *et al*., 1993) and rat portal vein (Schwietert *et al*., 1991), 9.2 in rat epididymal vas deferens, spleen and portal vein (Burt *et al*., 1995), 9.3, 9.5, and 9.6 in rat perfused kidney, perfused mesentery and aortic rings respectively (Ford *et al*., 1996) and 9.8 in rat aorta (Kenny *et al*., 1995). In this study, however, the pA_2 value of 8.1 obtained for prazosin on the rabbit iris dilator is low enough to show the involvement of α_{1L}-adrenoceptors in the contractile response of iris dilator muscle.

In the binding study, however, not only [^{3}H]-KMD but also [^{3}H]-prazosin bound to the iris membranes with subnanomolar high affinity (pK_d: 10.3 for [^{3}H]-KMD and 9.6 for [^{3}H]-prazosin, respectively) and with the same density (Figure 3 and Table 3). Furthermore, [^{3}H]-KMD binding sites were recognized as prazosin- and WB4101- high affinity sites (Figure 4 and Table 3). Originally, KMD-3213 was reported as an α_{1A}-adrenoceptor-selective antagonist (Shibata *et al*., 1995). In the experiments with human recombinant adrenoceptors, KMD-3213 showed a pK_i value of 10.4 for the α_{1a}-adrenoceptor, but has 580- and 56 fold lower affinity for α_{1b} and α_{1d}-adrenoceptors, respectively. Recently, it was also reported that KMD-3213 shows high affinity not only for α_{1a}- but also for α_{1L}-adrenoceptor (pA_2: 9.9) (Murata *et al*., 1999). This high selectivity of KMD-3213 to α_{1L}-adrenoceptor was confirmed in the present functional studies (Table 2). So it is likely that Bmax of [^{3}H]-KMD consist of major α_{1A} and minor α_{1L} site, whereas considering the result from RT–PCR studies, B_{max} of [^{3}H]-prazosin consist of major α_{1A} and minor α_{1B} site. Although we could not carry out further analysis because of limitation of tissue amount, the results obtained clearly show that the binding sites correspond mainly to α_{1A}-adrenoceptor subtype in character, and that the significant amount of α_{1L}-adrenoceptor subtype is undetectable.

The results on mRNA expression of α_1-adrenoceptor subtypes agrees with the result of binding experiments. RT–PCR studies demonstrated that expression of mRNA for the α_{1a}-adrenoceptor is major and that those of the α_{1b}- and α_{1d}-adrenoceptors are minor or negligible, respectively. Although mRNA levels do not always correlate with protein expression, the predominance of mRNA for α_{1a}-adrenoceptor suggests that this receptor may play an important role in irideal function.

Thus, there is a discrepancy for α_1-adrenoceptor subtypes between functional and binding or RT–PCR studies. How do we consider this matter? First of all, we must consider experimental conditions. Williams *et al*. (1996) demonstrated that in binding studies of the human cloned α_{1A}-adrenoceptor, temperature and cellular integrity appear to influence affinity estimates for several key antagonists, resulting in an apparent profile as α_{1L}-adrenoceptor. We tested various conditions including those employed in this study, however, rabbit cloned α_{1A}-adrenoceptor expressed in Chinese hamster ovary (CHO) cells displayed the binding affinity profile as α_{1A}-adrenoceptor (data not shown).

Second, characteristics of compounds such as high lipophilicity, chemical instability and extra binding sites, are likely to underestimate the affinity constant. For example, a low affinity estimate of S-niguldipine in the human lower urinary tract is probably influenced by its high lipophilicity (Ford *et al*., 1996). For another example, the human α_1-acid glycoprotein can inhibit prazosin and WB4101 binding to α_{1A}-adrenoceptors and therefore reduce their affinity for these receptors (Chiang *et al*., 1991; Qin & Øie, 1994). However, because the Schild regressions performed for all antagonists used in our experiments validated that the inhibition curve design and the slopes were close to unity, these influences may be negligible.

Third, in most binding studies, the concentrations of [^{3}H]-prazosin were up to 1 nM and then a single high affinity constant for prazosin (pK_d close to 10) has been estimated. However, it should be noted that affinity constant (K_B) of the α_{1L}-adrenoceptors found in the functional studies is close to 10 nM. Thus, it is likely that α_{1L}-adrenoceptors will be overlooked when low concentrations (<1 nM) of the [^{3}H]-prazosin are used. Although in this study we used up to 5 nM of [^{3}H]-prazosin, revealing the apparently saturated binding to α_1-adrenoceptors of rabbit iris (Figure 3a), this concentration might not be enough to detect α_{1L}-adrenoceptor subtype.

Fourth, it is likely that α_{1A}-adrenoceptor is a predominant subtype while α_{1L}-adrenoceptor is a minor in whole rabbit iris. The iris consists of four layers; anterior border layer, stroma and sphincter muscle layer, anterior epithelium and dilator muscle layer, posterior pigmented epithelium layer. In these, the dilator muscle is a very minor element (in human; 60 μm long and 7 μm wide) whereas the major structural element is the stroma which contains the vessels, nerves and cellular compartments (Bron *et al*., 1997). Previous reports showed that prazosin and bunazosin lower intraocular pressure in rabbits by a reduction in the rate of aqueous humor formation, which could occur by a vascular mechanism without affecting the pupillary diameter (Krupin *et al*., 1980; Green & Hatchett, 1987; Nishimura *et al*., 1993; Hong *et al*., 1998). These results suggest that there are at least two sets of α_1-adrenoceptors; one is high sensitive to prazosin in blood vessels and another shows low affinity to prazosin located in iris dilator muscle. Thus, it may be possible to speculate that α_{1A}-adrenoceptor distributes in the stroma, probably in the vessels, and shares the major part of α_1-adrenoceptor in whole iris while α_{1L}-adrenoceptor locates in the dilator muscle, as suggested in this study, and shares the small part.

Finally, a possible explanation for the discrepancy between functional and binding or molecular experiments is based on findings that functional estimates of antagonist affinity for the α_{1a}-adrenoceptor correlate closely with those established for the α_{1L}-adrenoceptor (Ford *et al*., 1997). Ford *et al*. compared binding affinities estimated by displacement of [^{3}H]-prazosin in membrane homogenates of CHO cells stably expressing the human α_{1a}-, α_{1b}-, and α_{1d}-adrenoceptors with affinity estimates obtained functionally in identical cells by measuring inhibition of noradrenaline-stimulated accumulation of [^{3}H]-inositol phosphates. And they suggested that upon functional pharmacological analysis, the cloned α_{1a}-adrenoceptor displays pharmacological recognition properties consistent with those of the putative α_{1L}-adrenoceptor. Recently, four isoforms of the α_{1a}-adrenoceptor generated by alternative splicing, which differ in length and sequence at the carboxy-terminal region, have been identified in human (Hirasawa *et al*., 1995; Chang *et al*., 1998). The α_{1a}-adrenoceptor of rabbit also have at least three splice variants (unpublished result), however, the physiological significance of α_{1a}-adrenoceptor splice variants

is presently unknown. Since the isoforms differ only in their carboxy-terminal regions, they may differ in their preference for G-protein coupling or in rates of desensitization mediated by phosphorylation. Recent data have implicated that some splice variants may demonstrate α_{1L}-adrenoceptor pharmacology in functional studies (Chang *et al*., 1998). However, why functional profile differs from that obtained in binding study requires further explanation.

In conclusion, we have found that α_{1A}-adrenoceptor is a major subtype in the α_1-adrenoceptors distributing in rabbit iris from binding study and that mRNA encoding this subtype is predominant in whole rabbit iris. While the dilator muscle, one of the constituents of the iris contracts *via* activation of α_1-adrenoceptor having low affinity for prazosin, WB4101 and BMY7378. This discrepancy is highly debatable, including the controversy whether or not the gene encoding α_{1L}-adrenoceptor exist. Also in human, α_1-adrenoceptor of iris dilator is characterized as α_{1L}-adrenoceptor (pA_2: prazosin 7.3) (Ishikawa *et al*., 1996). Therefore, rabbit may be the good strain for the drug research which is relevant to human ocular therapeutics. For example, α_1-adrenoceptor antagonists which have high affinity to the iris dilator may be available to reverse the effect of mydriasis rapidly and safely after diagnostic mydriasis (Mapstone, 1977; Relf *et al*., 1988; Allinson *et al*., 1990). Further study may reveal subtypes of α_1-adrenoceptor in ocular tissues with different locations and profiles for various agonists and antagonists, leading to development of a subtype selective drugs with therapeutic advantages.

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