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Alpha-1 adrenoceptor up-regulation induced by prazosin but not KMD-3213 or reserpine in rats

[1]Li Zhang, [1]Takanobu Taniguchi, [1]Takashi Tanaka, [2]Kazumasa Shinozuka, [2]Masaru Kunitomo, [3]Masahiko Nishiyama, [3]Koji Kamata & *,[1]Ikunobu Muramatsu

[1]Department of Pharmacology, School of Medicine, Fukui Medical University, Matsuoka, Fukui 910-1193, Japan; [2]Department of Pharmacology, Faculty of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Hyogo 663-8179, Japan and [3]Pharmacokinetics Research, Kissei Pharmaceutical Co., Ltd., 19–48 Matsumoto, Nagano 399-8710, Japan

1 We have investigated the effects of chronic administration of prazosin (a subtype-nonspecific alpha-1 AR antagonist), KMD-3213 (an alpha-1A AR subtype-specific antagonist) and reserpine (a catecholamine depletor) on the density of alpha-1 AR subtypes in various rat tissues (liver, kidney, submaxillary gland, heart and spleen).

2 Administration of prazosin (2 mg kg^{-1} day^{-1}, i.p.) for 2 weeks did not affect K_D values for [^{3}H]-prazosin or [^{3}H]-KMD-3213 of alpha-1 ARs in five rat tissues tested. However, it caused 52% up-regulation of alpha-1B AR in the spleen, and 84% and 107% up-regulation of alpha-1A- and alpha-1B ARs, respectively, in the heart. Although major subtypes of alpha-1 AR are alpha-1A AR in the submaxillary gland, alpha-1B AR in the liver, and alpha-1A and alpha-1B ARs in the kidney, these tissues showed no up-regulation. The mRNA levels of alpha-1 AR subtypes were not affected by prazosin administration in any tissue tested.

3 Neither administration of KMD-3213 (2 mg kg^{-1} day^{-1}, i.p.) nor reserpine (0.5–1 mg kg^{-1} day^{-1}, i.p.) for 2 weeks caused any change in either the binding affinity for [^{3}H]-prazosin or [^{3}H]-KMD-3213 or the density of the alpha-1 AR subtypes in the five rat tissues.

4 Neither prazosin nor KMD-3213 treatment reduced the noradrenaline content in the five rat tissues, in contrast to reserpine treatment, which markedly reduced it.

5 The findings of the present study demonstrated that up-regulation of alpha-1 AR is selectively caused by prazosin treatment in some tissues but neither by KMD-3213 treatment nor by chemical denervation with reserpine. These results suggest that up-regulation of alpha-1 ARs is not caused by a simple blockade of sympathetic tone.

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Abbreviations: AR, adrenoceptor; i.p., intraperitoneally; KMD-3213, (−)-1-(3-hydroxypropyl)- 5-((2*R*)-2- {[2-(2,2,2-trifluoroethyl) oxy]phenyl}oxy)ethyl] amino} propyl) -2,3-dihydro-1*H*-indole-7-carboxamide; RT–PCR, reverse transcriptase-polymerase chain reaction

Introduction

In general, activation of a receptor by agonists or neurotonic condition promotes a 'down-regulation' phenomenon while inhibition of a receptor by antagonists or denervation produces an 'up-regulation' phenomenon (Insel, 1989). These effects have been considered to be related to receptor states; e.g., the active state becomes more susceptible to phosphorylation resulting in desensitization and down-regulation, and the inactive state stabilizes the receptor resulting in up-regulation (Milligan & Bond, 1997; Leff, 1995; Barker *et al*., 1994; Lefkowitz *et al*., 1993; De Ligt *et al*., 2000). These phenomena are explained with a two-state receptor model, which gives a constitutive activity of receptor (Leff, 1995; Milligan *et al*., 1995; Milligan & Bond, 1997).

The alpha-1 adrenoceptors (alpha-1 ARs) are members of the G protein-coupled receptors and play critical roles in the regulation of a variety of physiological processes. At present, three distinct alpha-1 adrenoceptors are cloned and designated as alpha-1a (Lomasney *et al*., 1991; Schwinn *et al*., 1990; 1991), alpha-1b (Cotecchia *et al*., 1988) and alpha-1d subtypes (Perez *et al*., 1991; Schwinn & Lomasney, 1992), which are now recognized to correspond respectively to alpha-1A, alpha-1B and alpha-1D adrenoceptors detected pharmacologically in native tissues (Hieble *et al*., 1995; Muramatsu *et al*., 1995; 1998). Recently, all three subtypes have been shown to have constitutive activity when they are expressed in culture cells (Rossier *et al*., 1999; Garcia-Sainz & Torres-padilla, 1999; McCune *et al*., 2000). It has been reported that piperazinyl quinazolines derivatives, such as prazosin and terazosin, produce tolerance in their vasodilating effect, which is suggested to be due to up-regulation of alpha-1 ARs in vasculature (Cambridge & Greengrass, 1980; von Bahr *et al*., 1982; Vincent *et al*., 1992). Chemical denervation with reserpine or 6-OH dopamine also has been shown to induce up-regulation of alpha-1 ARs in the brain (Hanft & Gross, 1990; Grimm *et al*., 1992; Blendy *et al*., 1988).

In this study, we investigated the up-regulation of alpha-1 ARs in several rat tissues, comparing the effects of receptor

*Author for correspondence;
E-mail: muramatu@fmsrsa.fukui-med.ac.jp

blockade by alpha-1 AR antagonist treatment and of catecholamine deprivation by reserpine treatment *in vivo*. We administered prazosin (an alpha-1 AR subtype-nonselective antagonist) and KMD-3213 (an alpha-1A AR selective antagonist; Murata *et al*., 1999; Shibata *et al*., 1995) and the effects on alpha-1 ARs were compared with those seen in chemically denervated rats with reserpine.

Methods

Animals and chronic treatment with drugs

Male Wistar rats, 7 weeks old, about 210–240 g, were housed in groups of two or three animals with free access to the usual chow diet and tap-water. Prazosin and KMD-3213 were dissolved in saline (2 mg ml^{-1}) and was intraperitoneally administered at a dose of 2 mg kg^{-1} once a day for 2 weeks. Reserpine (1 mg ml^{-1}) was also intraperitoneally administered at a dose of 1 mg kg^{-1} once a day for the first week and 0.5 mg kg^{-1} for the following week. The control group was injected with saline. Twelve hours after the final injection, rats were exsanguinated and tissues (liver, kidney, submaxillary gland, heart and spleen) were rapidly isolated and used for the following experiments.

Measurement of endogenous noradrenaline content

The isolated rat tissues were weighed and homogenized in a homogenizer (setting three for 40 s) in 3.0 ml perchloric acid solution (0.4 M) containing 1.3 mM Na_2EDTA and 5.3 mM $Na_2S_2O_5$. The homogenates were then centrifuged ($12{,}000 \times g$; 4°C, 15 min), and the supernatant (0.1 ml) was collected into a sample tube containing 0.1 ml internal standard solution (10 ng ml^{-1} of 3,4- dihydroxybenzylamine). Noradrenaline in the sample tube was isolated using batch alumina chromatography and analysed using high-performance liquid chromatography - electrochemical detection (Shinozuka *et al*., 2001).

Determination of KMD-3213 concentration in rat plasma

Twelve or twenty-four hours after KMD-3213 injection, rats were exsanguinated and plasma samples were collected in 2 mg ml^{-1} 3-t-Butyl-4-hydroxyanisole solution. The plasma samples were mixed with an internal standard and were extracted by 10 mM ammonium acetate (pH 4.5)/acetonitrile. Concentrations of KMD-3213 were determined in a LC/MS/MS (Liquid Chromatography-tandem Mass Spectrometry) system.

Binding experiments

Rat tissues which had been kept at −80°C after isolation were homogenized in 10 ~ 20 vol of homogenization buffer (in mM) Tris-HCl 50, NaCl 100, EDTA 2; pH 7.4) with a polytron. The homogenate of tissues was subjected to centrifugation at $3\,000 \times g$ for 15 min. The supernatant was filtered through four layers of gauze and centrifuged at $80{,}000 \times g$ for 30 min. The pellet was resuspended in the same volume of assay buffer (in mM): Tris-HCl 50, EDTA 1; pH 7.4 and centrifuged at $80{,}000 \times g$ for 30 min again. The final pellet was resuspended in assay buffer and used for the binding study. All procedures to prepare membranes were conducted at 4°C and ice-cold buffers were used. The membranes were incubated with [^{3}H]-prazosin or [^{3}H]-KMD-3213 for 40 min at 30°C. The incubation volume was 1 ml for [^{3}H]-prazosin binding and 2 ml for [^{3}H]-KMD-3213 binding. Reactions were terminated by rapid filtration on to Whatman GF/C filters using a Brandel cell harvester. The filters were then washed three times with ice-cold washing buffer (Tris-HCl 50 mM; pH 7.4) and dried. The filter-bound radioactivity was then determined by means of a liquid scintillation counter (Aloka, Japan). Non-specific binding was defined as binding in the presence of 10 μM phentolamine during [^{3}H]-prazosin binding and as that in the presence of 0.3 μM prazosin during [^{3}H]-KMD-3213 binding. Assays were conducted in duplicate.

Binding data were analysed by the program LIGAND (Munson & Rodbard, 1980) and Prism 3.0. The data were first fitted to one- and then two-site models. If the residual sums of squares, as determined by an *F*-test comparison, were significantly less for a two-site fit of the data than for a one-site fit, the two-site model was accepted. Proteins were assayed according to the method of Bradford using bovine serum albumin as the standard (Bradford, 1976). Protein concentrations in each tube of the binding experiments were about 100 μg protein of rat liver and spleen, 80 μg protein of rat kidney, 50 μg protein of rat submaxillary and 200 μg protein of rat heart.

Total RNA preparation

Rat tissues were rapidly removed, frozen in liquid nitrogen and then stored at −80°C. Total cellular RNA was extracted according to the procedure of Chomczynski & Sacchi (1987). The concentration of total RNA was determined by spectrophotometry.

Construction and RNA synthesis of competitor

The levels of mRNA of the alpha-1 AR subtypes were examined in competitive RT–PCR assays using a competitive internal standard (competitor), as described previously (Piao *et al*., 2000). The competitor was constructed as follows. At first, a 50 bp *Sma*I/*Rsa*I fragment of pBluescript II SK (+) was inserted into the *Msc*I site of human alpha-1b AR clone. Next, it was connected with alpha-1d AR specific sequences and then alpha-1a AR specific sequences by the PCR technique. The resulting competitor DNA fragment was subcloned into the *Sma*I site of pBluescript II SK (+) and was transcribed with T7 RNA polymerase (GIBCO BRL).

The following primers were used in PCR experiments: 5′-AGCAAGTGACGCTCCGCATCCA-3′ as forward primer and 5′-TCTCACCCGGGCTGTGGTACAGG-3′ as reverse primer for alpha-1A AR; 5′-GTCATCTCCATCGGGCCTCTCCT-3′ as forward primer and 5′-GTAGCCCAGCCAGAACACCACCTT-3′ as reverse primer for alpha-1B AR. 5′-ATGTACTGCCG-CGTGTACGTGGTC-3′ as forward primer and 5′-CGCCAGTGGTGGCCGTAGAC-3′ as reverse primer for alpha-1D AR.

The size of the resulting PCR products (target/competitor) were 587/633 bp, 492/542 bp and 500/588 bp for alpha-1A, alpha-1B and alpha-1D AR subtypes, respectively.

Competitive RT–PCR assay

To quantify the mRNA level of alpha-1 AR subtypes, a trace of competitor RNA was co-transcribed and co-amplified with rat tissue total RNA in the RT–PCR assay. Briefly, the tissue RNA (500 ng) was premixed with serially diluted concentrations of competitor RNA and was reverse transcribed with Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (GIBCO BRL) using a random primer (dN6) at 37°C for 1 h.

The resulting cDNA was amplified with Pwo polymerase (Roche Molecular Biochemicals) using a subtype-specific pair of primers by PCR. The PCR was performed with a Perkin-Elmer GeneAmp PCR System 2400 under the following cycle parameters: For the alpha-1A AR subtype, 40 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 10 s, and extension at 72°C for 15 s. For the alpha-1B AR subtype, 40 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 10 s, and extension at 72°C for 15 s. For the alpha-1D AR subtype, 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 10 s, and extension at 72°C for 15 s.

PCR products were separated in a 4.0% agarose gel, which was stained with ethidium bromide. The intensity of the bands was quantitated using an ATTO Densitograph System. The ratios of intensity of the competitor band and the target band were plotted against the dose of competitor RNA and the apparent equivalent point was determined as the ratio estimated to be one. Equivalence of subtypes mRNA to competitor RNA was finally calculated (Suzuki *et al*., 2000).

Statistical analysis

All values were expressed as means ± s.e.m. Statistical analysis was carried out using Student's *t*-test or one-way analysis of variance (ANOVA) followed by a *post hoc* test of Fisher's PLSD (Protected Least Significant Difference), where appropriate. A P value less than 0.05 was accepted as significant.

Materials

The drugs used and their sources were as follows: (−)-1-(3-hydroxypropyl)-5-((2*R*)-2-{[2-(2,2,2-trifluoroethyl) oxy]phenyl}oxy)ethyl] amino} propyl) -2,3 -dihydro- 1*H*-indole-7-carboxamide (KMD-3213), from Kissei Pharmaceutical Co. Ltd. (Matsumoto, Japan); prazosin hydrochloride, (−)-noradrenaline bitartrate and phentolamine, from Sigma (St. Louis, U.S.A.); reserpine, from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan); 2-(2,6-dimethoxy-phenoxyethyl)-amino-methyl-1,4-benzodioxane hydrochloride (WB4101) and 8-[2-[4-(2-methoxy-phenyl)-L- piperazinyl]-8-azaspiro [4,5] decane-7,9-dione dihydrochloride (BMY7378), from Research Biochemicals Inc. (Natick, U.S.A.); [^{3}H]-prazosin (specific activity 77.2 Ci mmol^{-1}), from NEN(Boston, U.S.A.); [^{3}H]-KMD-3213 (specific activity 49 ~ 52 Ci mmol^{-1}), synthesized by Amersham Pharmacia Biotech (Buckinghamshrie, U.K.).

Results

Endogenous noradrenaline content in rat tissues

As shown in Table 1, the contents of noradrenaline varied significantly among the tissues tested (atria and ventricle of the heart, liver, kidney, submaxillary gland and spleen). Chronic treatment with prazosin and KMD-3213 did not change the noradrenaline content. However, reserpine treatment markedly reduced the noradrenaline levels.

Saturation binding studies with [^{3}H]-prazosin

[^{3}H]-prazosin (30–2,000 pM) bound single sites with high affinity in five rat tissues (Table 2). The B_{max} values varied among the tissues: the lowest in the heart and the highest in submaxillary gland. Chronic treatment with prazosin increased the density in the heart and spleen without changing the affinity (Figure 1 and Table 2). However, there was no significant change in the density and affinity in the other tissues tested. Administration of KMD-3213 or reserpine did not produce any changes in density or affinity of the five tissues tested.

Table 1 Noradrenaline contents in rat tissues

Tissue	*Control*	*Prazosin treatment*	*KMD-3213 treatment*	*Reserpine treatment*
Heart–atrium	24.6 ± 3.7	31.3 ± 2.3	23.4 ± 0.8	0.2 ± 0.0
Heart–ventricle	19.4 ± 1.7	21.6 ± 1.5	15.5 ± 2.7	0.1 ± 0.0
Kidney	6.1 ± 1.0	4.5 ± 0.7	5.8 ± 0.3	0.1 ± 0.0
Spleen	44.4 ± 5.1	52.9 ± 5.9	50.7 ± 8.4	0.3 ± 0.2
Submaxillary gland	16.3 ± 1.3	19.5 ± 0.9	19.6 ± 1.4	0.1 ± 0.1
Liver	0.7 ± 0.2	0.8 ± 0.2	0.9 ± 0.1	0.0 ± 0.0

Rats were treated with Prazosin (2 mg kg^{-1}), KMD-3213 (2 mg kg^{-1}) or reserpine (0.5–1 mg kg^{-1}) for 2 weeks. Results are means ± s.e.m. (n = 4) (pmol mg^{-1} wet weight). Noradrenaline content in each tissue of reserpine-treated rats was significantly lower than those in other groups (P < 0.05).

Table 2 Total α_1-adrenoceptor density and affinity in rat tissues ([^{3}H]-Prazosin experiments)

	Affinity (pK_D)				*Total α_1-AR density* (B_{max})			
Tissue	*Control*	*Prazosin*	*KMD-3213*	*Reserpine*	*Control*	*Prazosin*	*KMD-3213*	*Reserpine*
Liver	10.2 ± 0.1	10.0 ± 0.0	9.9 ± 0.1	10.3 ± 0.1	130 ± 9	133 ± 10	134 ± 5	135 ± 6
Kidney	10.1 ± 0.1	9.8 ± 0.1	10.0 ± 0.0	10.2 ± 0.0	228 ± 7	236 ± 10	215 ± 6	210 ± 10
Submaxillary gland	10.2 ± 0.1	10.0 ± 0.0	9.9 ± 0.1	10.2 ± 0.0	248 ± 10	264 ± 8	232 ± 7	221 ± 17
Heart	10.0 ± 0.0	9.6 ± 0.1	10.0 ± 0.1	10.1 ± 0.0	101 ± 5	199 ± 7*	103 ± 5	114 ± 9
Spleen	10.3 ± 0.1	9.8 ± 0.1	10.5 ± 0.1	10.4 ± 0.1	109 ± 4	166 ± 14*	121 ± 6	118 ± 16

Rats were treated with Prazosin (2 mg kg^{-1}), KMD-3213 (2 mg kg^{-1}) or reserpine (0.5–1 mg kg^{-1}) for 2 weeks. Results are means ± s.e.m. (n = 3 ~ 6). Total density (B_{max}) means the maximum number of [^{3}H]-prazosin binding sites (fmol mg^{-1} protein). *Significantly different from the control group (P < 0.05).

Saturation binding studies with [^{3}H]-KMD-3213

[^{3}H]-KMD-3213 (alpha-1A AR selective ligand, 10–2000 pM) bound single sites in the heart, submaxillary gland and kidney of rats (Table 3). However, specific binding of [^{3}H]-KMD-3213 was not detected in the liver and spleen. The B_{max} values of [^{3}H]-KMD-3213 in the submaxillary gland were similar to those of [^{3}H]-prazosin. In contrast, the binding density of [^{3}H]-KMD-3213 was approximately 40% of that of [^{3}H]-prazosin binding in the heart and 60% in the kidney (Tables 2 and 3). Prazosin treatment significantly increased the density of [^{3}H]-KMD-3213 binding sites in the heart but did not change the densities in the submaxillary gland and kidney (Figure 2). Chronic treatment with KMD-3213 or reserpine had no significant effect on the density and affinity of alpha-1 AR in any of the tissues tested.

Competition binding studies at [^{3}H]-prazosin binding sites

The pharmacological profiles of [^{3}H]-prazosin binding sites in five tissues of the control and prazosin treated rats (heart, liver, kidney, spleen and submaxillary gland) were examined in competitive experiments. Five drugs (BMY7378, WB4101, KMD-3213, prazosin and noradrenaline) were used to identify alpha-1 AR subtypes labeled with 200 pM [^{3}H]-prazosin (Table 4). Prazosin, BMY-7378 and noradrenaline produced a monophasic inhibition in all tissues. However, KMD-3213 and WB4101 showed a biphasic inhibition in the kidney and heart. The proportion of high affinity sites for either drug was approximately 60% in the kidney and 40% in the heart, which were estimated as alpha-1A AR, and another proportion with low affinity was estimated as alpha-1B AR (Table 4). Since alpha-1D selective ligands (BMY-7378, noradrenaline) showed low affinity, alpha-1D AR was not detectable in this binding study.

Competition binding studies at [^{3}H]-KMD-3213 binding sites

The pharmacological profiles of [^{3}H]-KMD-3213 binding sites in the heart, kidney and submaxillary gland were also examined in competitive experiments. The binding of 100 pM [^{3}H]-KMD-3213 was monophasically inhibited by all five drugs tested (Table 5). WB4101, prazosin and KMD-3213 showed high affinity ($pK_i > 9.0$).

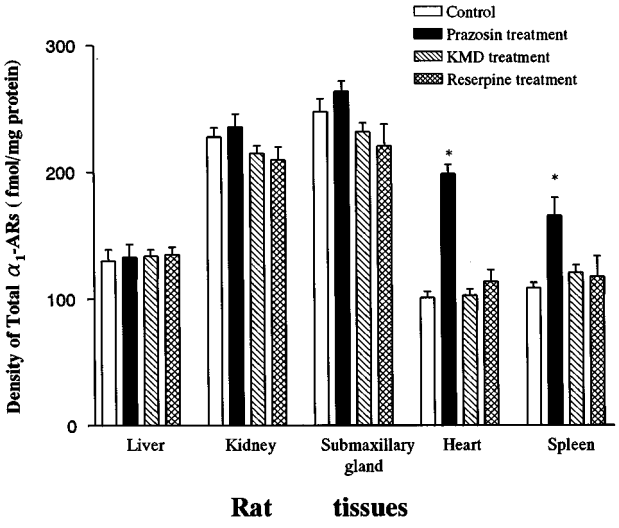

Figure 1 Total alpha-1 AR density in rat tissues. Total alpha-1 AR density was determined in saturation experiments with [^{3}H]-prazosin (a subtype non-selective antagonist) in five tissues isolated from rats, which had been treated for 2 weeks with prazosin, KMD-3213 or reserpine. Findings are summarized in Table 2. *Significantly different from the control group ($P<0.05$).

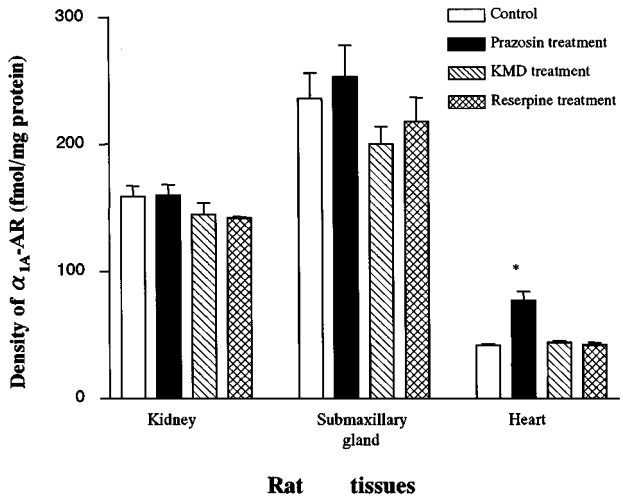

Figure 2 Alpha-1A AR density in rat tissues. Alpha-1A AR density was determined in saturation experiments with [^{3}H]-KMD-3213 (an Alpha-1A AR selective antagonist) in three tissues isolated from rats treated with prazosin, KMD-3213 or reserpine. Findings are summarized in Table 3. *Significantly different from the control group ($P<0.05$).

Table 3 α_{1A}-Adrenoreceptor density and affinity in rat tissues ([^{3}H]-KMD-3213 experiments)

	Affinity (pK_D)				*α_{1A}-AR density* (B_{max})			
Tissue	*Control*	*Prazosin*	*KMD-3213*	*Reserpine*	*Control*	*Prazosin*	*KMD-3213*	*Reserpine*
Kidney	10.4±0.2	10.3±0.1	10.1±0.0	9.9±0.0	159±8	160±8	145±9	142±1
Submaxillary gland	10.2±0.2	10.4±0.1	10.0±0.2	10.0±0.0	236±20	253±25	200±14	218±19
Heart	10.4±0.1	10.0±0.1	9.9±0.1	9.6±0.1	42±1	77±7*	44±1	42±2

Rats were treated with Prazosin (2 mg kg^{-1}), KMD-3213 (2 mg kg^{-1}) or reserpine (0.5–1 mg kg^{-1}) for 2 weeks. Results are means±s.e.m. ($n=3-6$). α_{1A}-AR density (B_{max}) means the maximum number of [^{3}H]-KMD-3213 binding sites (fmol mg^{-1} protein). *Significantly different from the control group ($P<0.05$).

Table 4 Pharmacological characterization of [^{3}H]-prazosin binding sites in rat tissues

Tissue (Subtypes)	*Drug*	*Control* pK$_i$	*Control* (%)	*Prazosin treatment* pK$_i$	*Prazosin treatment* (%)
Liver (α_{1B})	BMY7378	7.1±0.1		6.8±0.1	
	WB-4101	8.4±0.1		8.1±0.0	
	KMD-3213	7.7±0.1		7.5±0.0	
	Prazosin	10.1±0.1		9.8±0.0	
	Noradrenaline	5.5±0.1		5.2±0.1	
Spleen (α_{1B})	BMY7378	7.4±0.2		7.0±0.2	
	WB-4101	8.7±0.1		8.4±0.0	
	KMD-3213	8.0±0.1		7.6±0.1	
	Prazosin	10.3±0.1		9.8±0.1	
	Noradrenaline	5.7±0.1		5.5±0.1	
Kidney ($\alpha_{1A}+\alpha_{1B}$)	BMY7378	6.8±0.1		6.4±0.1	
	WB-4101	9.8±0.1	67±4 (α_{1A})	9.6±0.1	67±5 (α_{1A})
		8.3±0.2	33±4 (α_{1B})	7.8±0.1	33±5 (α_{1B})
	KMD-3213	10.4±0.1	58±6 (α_{1A})	10.0±0.1	59±4 (α_{1A})
		7.5±0.1	42±6 (α_{1B})	7.1±0.1	41±4 (α_{1B})
	Prazosin	9.9±0.1		9.4±0.1	
	Noradrenaline	5.9±0.1		5.5±0.0	
Submaxillary gland (α_{1A})	BMY7378	6.9±0.1		6.7±0.0	
	WB-4101	9.6±0.1		9.4±0.0	
	KMD-3213	10.6±0.2		10.3±0.3	
	Prazosin	9.9±0.1		9.8±0.0	
	Noradrenaline	6.1±0.1		5.9±0.1	
Heart ($\alpha_{1A}+\alpha_{1B}$)	BMY7378	6.6±0.1		6.2±0.0	
	WB-4101	9.5±0.2	35±7 (α_{1A})	9.4±0.2	34±7 (α_{1A})
		8.1±0.1	65±7 (α_{1B})	7.6±0.1	66±7 (α_{1B})
	KMD-3213	9.9±0.2	36±4 (α_{1A})	9.6±0.2	37±3 (α_{1A})
		7.2±0.1	64±4 (α_{1B})	6.9±0.0	63±3 (α_{1B})
	Prazosin	9.9±0.1		9.5±0.0	

Results are means±s.e.m. ($n=3-5$). %, Proportion of α_{1A}- and α_{1B}-ARs, respectively.

Table 5 Pharmacological characterization of [^{3}H]-KMD-3213 binding sites in rat tissues

Tissue (Subtype)	*Drug*	pK$_i$ *Control*	pK$_i$ *Prazosin treatment*
Kidney (α_{1A})	BMY7378	6.9±0.1	6.6±0.1
	WB-4101	9.8±0.0	9.7±0.0
	KMD-3213	10.3±0.1	10.1±0.1
	Prazosin	9.8±0.1	9.6±0.1
	Noradrenaline	5.7±0.1	5.4±0.0
Submaxillary gland (α_{1A})	BMY7378	6.7±0.0	6.7±0.0
	WB-4101	9.8±0.1	9.7±0.0
	KMD-3213	10.4±0.1	10.2±0.1
	Prazosin	10.0±0.1	9.9±0.1
	Noradrenaline	5.8±0.1	5.4±0.1
Heart (α_{1A})	BMY7378	6.7±0.1	6.2±0.1
	WB-4101	9.5±0.1	9.2±0.1
	Prazosin	9.6±0.1	9.3±0.1

Results are means±s.e.m. ($n=3\sim5$).

Table 6 mRNA level of α-AR subtypes in rat tissues

Tissue	*Chronic treatment*	*mRNA* α_{1a}	*mRNA* α_{1b}	*mRNA* α_{1d}
Kidney	Control	0.3±0.0	0.3±0.0	n.d.
	Prazosin	0.3±0.0	0.3±0.0	n.d.
	KMD-3213	0.3±0.0	0.3±0.0	n.d.
Liver	Control	n.d.	1.6±0.2	n.d.
	Prazosin	n.d.	1.6±0.1	n.d.
	KMD-3213	n.d.	1.7±0.1	n.d.
Spleen	Control	n.d.	0.5±0.0	n.d.
	Prazosin	n.d.	0.6±0.1	n.d.
	KMD-3213	n.d.	0.6±0.0	n.d.
Heart	Control	3.6±0.1	9.0±0.7	n.d.
	Prazosin	3.4±0.2	9.7±0.9	n.d.
	KMD-3213	3.2±0.3	8.9±0.4	n.d.

mRNA, pg of competitor cRNA per 500 ng of total tissue RNA. n.d., not detected (less than 0.1 pg of competition cRNA per 500 ng total RNA). Results are means±s.e.m. ($n=3\sim6$).

Competitive RT–PCR analysis at alpha-1 AR subtypes

The results of competitive RT–PCR for alpha-1 AR subtypes in rat tissues are summarized in Table 6. Four tissues (heart, spleen, kidney and liver) were chosen, because the former two tissues showed up-regulation by prazosin treatment while the latter two tissues did not. mRNA for alpha-1A AR was detectable only in the kidney and heart. mRNA of alpha-1D AR was undetectable in any tissues (less than 0.1 pg of competition cRNA per 500 ng total RNA). Chronic treatment with prazosin or KMD-3213 caused no significant change in mRNA level of alpha-1 AR subtypes (Table 6). The mRNA level in reserpine-treated tissues was not examined.

Determination of KMD-3213 concentration in rat plasma

Since KMD-3213 showed no detectable effects, we evaluated the concentration in plasma. The KMD-3213 concentration was 1.9 ± 0.3 and 0.3 ± 0.0 nM in rat plasma at 12 and 24 h after injection of 2 mg kg^{-1} KMD-3213, respectively ($n = 5-6$). Since KMD has a high affinity to alpha-1a AR (K_D: about 0.03 nM; Table 4 and Murata *et al*., 1999;), it was unlikely that the regimen of KMD-3213 administration was ineffective in blocking the alpha-1A AR.

Discussion

In the present study, we used [^{3}H]-prazosin, a subtype-nonselective alpha-1 AR antagonist, to evaluate total alpha-1 AR density (Hieble *et al*., 1995; Muramatsu *et al*., 1995; 1998) and [^{3}H]-KMD-3213, an alpha-1A selective antagonist, to estimate alpha-1A AR density, respectively (Shibata *et al*., 1995; Murata *et al*., 1999). The present findings showed good agreement with previous binding results in rats: alpha-1A AR in the submaxillary gland (Michel *et al*., 1989); alpha-1B AR in the liver and spleen (Minneman, 1988; Murata *et al*., 1999), and coexistence of alpha-1A AR and alpha-1B AR in the heart and kidney (Michel *et al*., 1994; Feng *et al*., 1991), and undetectable alpha-1D AR in any of the tissues tested (Yang *et al*., 1997). Furthermore, in the present study, there was no relation between the density or distribution pattern of alpha-1 ARs and the noradrenaline content in each tissue (Tables 1–3).

Chronic treatment with prazosin for 2 weeks increased the total density of alpha-1 AR in the heart and spleen without changing the noradrenaline content. The present study further revealed that the up-regulation was caused at both alpha-1A and alpha-1B subtypes in the heart and at alpha-1B subtype in the spleen. The extent of up-regulation varied among tissues: an approximately 100% increase in the heart but a 50% increase in the spleen. In contrast to the heart and spleen, no up-regulation was detected in the liver (alpha-1B subtype), kidney (alpha-1A and alpha-1B subtypes) or submaxillary gland (alpha-1A subtype). In the kidney, the proportions of alpha-1A and alpha-1B subtypes did not change after prazosin treatment, indicating no alteration in density of either subtype.

It is unclear why prazosin caused up-regulation in the heart and spleen but not in the other tissues. We measured mRNA of alpha-1 AR subtypes (Table 6). However, prazosin treatment did not produce any changes in the mRNA level of the heart and spleen as well as the liver and kidney. Therefore, it is unlikely that the up-regulation in the heart and spleen is produced by an enhancement of transcription, although the relation between mRNA and protein expression is not always obvious (Zhong & Minneman, 1999; Piao *et al*., 2000). Since sympathetic nerves innervate many tissues, tissue-specific up-regulation might in part reflect differences in the degree of adrenergic influence (Stassen *et al*., 1998). That is, alpha-1 ARs in the heart and spleen might be more dominantly and/or tonically stimulated by endogenous catecholamines among the tested tissues, resulting in accelerated down-regulation, which would be prevented by prazosin treatment. If this were true, other alpha-1 AR antagonists and chemical denervations would also produce up-regulation. However, neither KMD-3213 (an alpha-1A AR selective antagonist) (Murata *et al*., 1999; Zhu *et al*., 2000) nor reserpine (a catecholamine depletor) caused up-regulation of alpha-1 ARs in the heart, spleen or other tissues. It has been previously reported that reserpine treatment does not induce up-regulation of alpha-1 ARs in the heart (Chess-Williams *et al*., 1987; Latifpour & MacNeill, 1984). These previous and the present results thus suggest that something about prazosin rather than the simple blockade of sympathetic influence is important in the observed effects.

One possible explanation is a deduction from two-state receptor theory in which receptor density is affected by receptor state: the active state becomes more susceptible to phosphorylation resulting in sequesterization and degradation (Milligan & Bond, 1997; Leff, 1995; Barker *et al*., 1994; Lefkowitz *et al*., 1993). Recently, wild-types of alpha-1A, alpha-1B and alpha-1D ARs expressed in COS-7 cells and rat-1 fibroblasts were shown to be constitutively active without agonist (Rossier *et al*., 1999; Garcia-Sainz & Torres-Padilla, 1999; McCune *et al*., 2000). An *in vitro* study also showed that the rat thoracic aorta had a persistent contraction related to the constitutive activity of alpha-1 AR (probably alpha-1D subtype) (Noguera *et al*., 1996; Gisbert *et al*., 2000). Since prazosin is an inverse agonist for three alpha-1 AR subtypes (Rossier *et al*., 1999; Garcia-Sainz & Torres-padilla, 1999), tissue-specific up-regulation observed after prazosin treatment in the present study may reflect different degrees of constitutive activity of alpha-1 ARs in the heart, spleen and other tissues. This hypothesis is in accordance with the concept or finding that constitutive activity of receptors may not be necessarily maintained at the same level in different types of cells or tissues and may be modified even by their different environments (Costa *et al*., 1992; Kenakin, 1996). In fact, it was recently reported that constitutive activity of glutamate receptor is regulated by accessory protein (Ango *et al*., 2001). Since KMD-3213 is a neutral antagonist for alpha-1a AR (Zhu *et al*., 2000), i.e. it has no inverse activity, and no detectable effects of KMD-3213 on AR are not discordant with this hypothesis. However, many other possibilities that affect drug availability in a tissue-specific manner like distribution, metabolism and so on would cause the effects as observed here. Further investigation is required to clarify the issues regarding inverse activity *in vivo*.

In conclusion, the present study shows that prazosin caused up-regulation of alpha-1 ARs in a tissue-specific manner but that another antagonist KMD-3213 and a catecholamine depletor reserpine did not induce up-regulation. These results suggest that up-regulation of alpha-1 ARs by prazosin is not caused by a simple blockade of sympathetic tone.

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