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Two distinct α_1-adrenoceptor subtypes in rabbit liver: a binding study

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1 The characteristics of α_1-adrenoceptor subtypes present on rabbit liver membranes were determined by radioligand binding and compared with the characteristics of binding in rat liver.

2 In saturation experiments using rabbit liver, [^{3}H]-prazosin bound to two distinct affinity sites ($pK_D = 10.3 \pm 0.19$ and 8.13 ± 0.17, $B_{max} = 11.6 \pm 3.3$ and 657.8 ± 198.0 fmol mg^{-1} protein, respectively). In studies using rat liver, [^{3}H]-prazosin bound to a single affinity site ($pK_D = 9.98 \pm 0.27$, $B_{max} = 190.5 \pm 38.5$ fmol mg^{-1} protein).

3 In competition experiments, unlabelled prazosin displaced biphasically the binding of 200 pM [^{3}H]-prazosin to the rabbit liver; the resulting two pK_I values (9.85 ± 0.08 and 8.01 ± 0.09) were consistent with the affinity constants obtained in the saturation experiments. Two sites were also recognized by doxazosin (pK_I 9.73 ± 0.78 and 8.12 ± 0.34), 2-(2,6-dimethoxy phenoxyethyl)-aminomethyl-1,4-benzodioxane (WB4101) pK_I (9.74 ± 0.32 and 7.57 ± 0.34) and 5-methylurapidil (pK_I 8.69 ± 0.27 and 6.75 ± 0.35), and the population of low affinity sites for the three antagonists was approximately 70%. Two distinct affinity constants (pK_I 8.55 ± 0.09 and 7.90 ± 0.09) were also calculated for α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy) ethyl)-amino)-propyl)-benzeneacetonitrile fumarate (HV723).

4 By contrast, [^{3}H]-prazosin binding sites of rat liver membranes were detected as a single population with a high affinity for prazosin (pK_I 10.01 ± 0.08), and doxazosin (pK_I 9.67 ± 0.20) but with a low affinity for WB4101 (pK_I 8.25 ± 0.09), 5-methylurapidil (pK_I 7.22 ± 0.01) and HV723 (pK_I 8.88 ± 0.05).

5 These results indicate the presence of two distinct α_1-adrenoceptor subtypes in the rabbit liver, but only a single site in rat liver. The pharmacological characteristics of prazosin-high and -low sites in rabbit liver suggest identity with α_{1A} and putative α_{1L} subtypes, respectively. The site in rat liver is of the α_{1B} subtype.

Keywords: α_1-Adrenoceptor subtype; rabbit liver; α_{1A} subtype; α_{1L} subtype; [^{3}H]-prazosin binding

Introduction

α_1-Adrenoceptors are not homogeneous, and different subclassifications have been proposed. Two subtypes of α_1-adrenoceptors (α_{1A} and α_{1B}) were first defined on the basis of binding experiments (Morrow & Creese, 1986; Minneman, 1988; Hanft & Gross, 1989; Oshita *et al.*, 1991). The α_{1A}-adrenoceptor subtype showed high affinity for 2-(2,6-dimethoxy-phenoxyethyl)-aminomethyl-1, 4-benzodioxane (WB4101) and 5-methylurapidil, while the α_{1B} subtype displayed low sensitivity to these drugs. Both α_{1A} and α_{1B} subtypes exhibited a high affinity for prazosin. On the other hand, another subclassification of α_1-adrenoceptors suggested the existence of three subtypes (α_{1H}, α_{1L} and α_{1N}) (Drew, 1985; Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990; Oshita *et al.*, 1991). Prazosin had a higher affinity for the α_{1H}-adrenoceptor subtype than for the α_{1L} and α_{1N}-adrenoceptor subtypes. The α_{1N}-adrenoceptor subtype had a low affinity for prazosin but a higher affinity for α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy)ethyl)-amino)-propyl) benzeneacetonitrile fumarate (HV723), as compared with α_{1L}-adrenoceptor subtype.

Molecular cloning studies have now confirmed the existence of three α_1-adrenoceptor subtypes. The cloned α_{1B}-adrenoceptor isolated from DDT1 MF-2 cells is predominantly expressed in rat liver, heart and cerebral cortex and the pharmacological features are similar to those of the classical α_{1B} subtype mentioned above (Cotecchia *et al.*, 1988; Lomasney *et al.*, 1991). In addition, α_{1C}- and α_{1d}-adrenoceptor clones have also been described (Schwinn *et al.*, 1990; Perez *et al.*, 1991). Recently, Laz *et al.* (1994) and Perez *et al.* (1994) showed that the cloned α_{1C}-adrenoceptor corresponds to the classical α_{1A}-adrenoceptor (Morrow and Creese, 1986; Minneman, 1988). Thus, the new nomenclature of α_1- adrenoceptor with high affinity for prazosin (native and recombinant receptors: α_{1A} and α_{1a}, α_{1B} and α_{1b}, α_{1D} and α_{1d}, respectively) was recently acknowledged by IUPHAR (Hieble *et al.*, 1995).

Sympathetic innervation of the liver has been postulated as functionally important in regulating the hepatic metabolism and the activity of some specific enzymes (Jarhult *et al.*, 1979; 1980). For example, α_1-adrenoceptor activation increases acid transport, glycolysis and gluconeogenesis, and enhances inactivation of glycogen synthase (Jarhult *et al.*, 1979, 1980; Kunos, 1984). Although the existence of α_{1A}-adrenoceptor mRNA was demonstrated in rabbit liver by Northern blot analysis (Schwinn *et al.*, 1990; Lomasney *et al.*, 1991), detailed operational analysis of α_1-adrenoceptors in rabbit liver has not been carried out. In the present study, we have characterized the α_1-adrenoceptor subtypes of rabbit liver according to the recent α_1-adrenoceptor subclassification (Hieble *et al.*, 1995; Muramatsu *et al.*, 1995). Rat liver was used as a control because exclusive expression of α_{1B} subtype was demonstrated.

Methods

Binding experiments

Rabbits (2.0-2.5 kg) or rats (180-250 g) were anaesthetized with pentobarbitone and killed by exsanguination. The livers were isolated immediately and homogenized in 10 or 40 vol. of buffer (Tris HCl 50 nM, NaCl 100 mM, EDTA 2 mM, pH 7.4) with a polytron (setting 8, 15 s × 6). The homogenate of rabbit liver was subjected to centrifugation at 10,000 *g* for 10 min, and the supernatant further centrifuged at 80,000 *g* for 40 min

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at 4°C. Homogenates of rat liver were filtered through 4 layers of gauze and subjected to centrifugation at 80,000 *g* for 40 min at 4°C. The pellets were resuspended in the same volume of assay buffer (Tris HCl 50 mM, EDTA 1 mM, pH 7.4) and centrifuged 80,000 *g* for 40 min at 4°C. All procedures to prepare membranes were conducted at 4°C and ice cold buffers were used. The final pellet was resuspended in assay buffer and used for the binding assay. The membranes were incubated with [^{3}H]-prazosin for 45 min at 30°C. Incubation volume was 1 ml in all experiments. Reactions were terminated by rapid filtration on to Whatman GF/C filters using a Brandel cell harvester. The filters were then washed 4 times with 4 ml of ice-cold 50 mM Tris-HCl buffer (pH 7.4) and dried, and the filter-bound radioactivity determined. Non-specific binding was defined as binding in the presence of 0.3 μM prazosin unless mentioned elsewhere. Assays were conducted in duplicate. Proteins were assayed according to the method of Bradford with bovine serum albumin used as standard (Bradford, 1976).

Data analysis

Saturation binding data were analysed by the weighted least-squares iterative curve fitting programme LIGAND (Munson & Rodbard, 1980). The data were first fitted to a one- and then a two-site model, and if the residual sum of squares was statistically less for a two-site fit of the data than for a one-site fit, as determined by *F*-test, the two-site model was accepted. *P* values less than 0.05 were considered significant. Displacement binding data were first analysed by EBDA programme (Biosoft, Elsevior) (McPherson, 1985); then, when the slope factor was not different from unity, the calculated IC_{50} values were converted to K_I values with the Cheng-Prusoff approximation (Cheng & Prusoff, 1973). However, when the slope factor deviated from unity, two distinct K_I values were determined with the LIGAND programme from high and low affinity constants (K_D) for [^{3}H]-prazosin obtained in saturation experiments. Experimental values are given as a mean ± s.e.mean.

Drugs

The following drugs were used: [^{3}H]-prazosin (specific activity 76.6 Ci mmol^{-1}, NEN, Boston, U.S.A.), prazosin hydrochloride, doxazosin mesylate (Taito-Pfizer, Tokyo, Japan), 2-(2,6-dimethoxy-phenoxyethyl)-aminomethyl-1,4-benzodioxane hydrochloride (WB4101), 5-methylurapidil (Funakoshi, Tokyo, Japan) and α-ethyl-3,4,5-trimethoxy-α-(3-((2-(2-methoxyphenoxy) ethyl)-amino)-propyl) benzeneacetonitrile fumarate (HV723, Hokuriku Seiyaku, Katsuyama, Fukui, Japan), methoxamine hydrochloride, (−)-noradrenaline bitartrate (Nacalai tesque, Kyoto, Japan), phentolamine mesylate (Regitine, Ciba, Basal, Switzerland). As a stock solution, prazosin (0.5 mM) and 5-methylurapidil (1 mM) were dissolved in 50% ethanol or dimethylsulphoxide, respectively. The other drugs was dissolved in distilled water. Before use, the stock solution was diluted with the assay buffer.

Results

Saturation experiments with [^{3}H]-prazosin

Rabbit liver membranes: [^{3}H]-prazosin at concentrations ranging from 20 to 5,000 pM was used to label α_1-adrenoceptors of rabbit liver. The specific binding was approximately 60% of the total binding of 200 pM [^{3}H]-prazosin when the non-specific binding was defined as binding in the presence of 0.3 μM prazosin, and showed a gradual increase with an increase of the ligand concentrations (Figure 1a, ■). A Scatchard plot of the binding data was curvi-linear, suggesting more than a single class of binding site (Figure 2a, ■ and two straight lines). LIGAND analysis fitted the data to a two site model. The pK_D values of high and low affinity sites were 10.3 ± 0.19 and 8.13 ± 0.17, and the B_{max} value for each site was

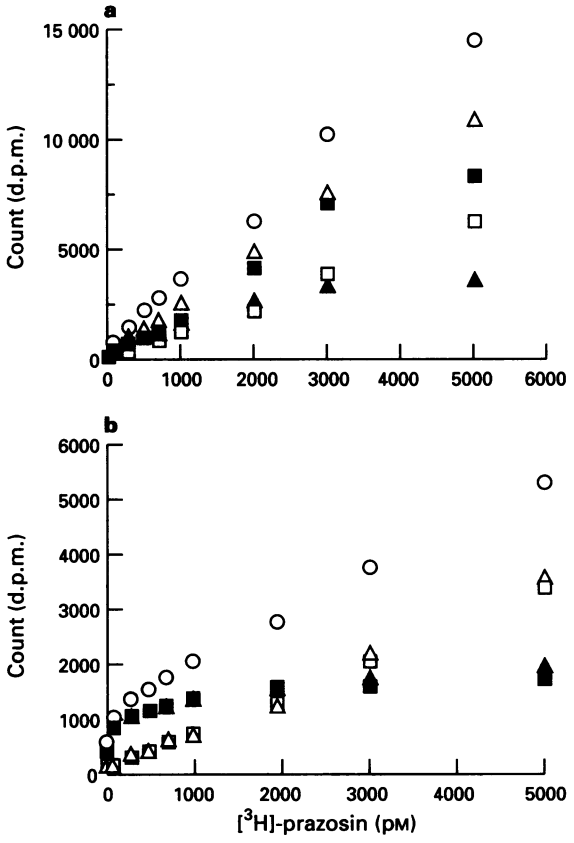

Figure 1 Saturation curves of the [^{3}H]-prazosin binding to rabbit (a) and rat (b) liver membranes. (○) total binding (□) and (△): non-specific binding in the presence of 0.3 μM prazosin and 10 μM phentolamine; (■) and (▲) specific binding determined by 0.3 μM prazosin and 10 μM phentolamine, respectively. The data were obtained from a single experiment with the same membrane preparations, where each point was the mean of duplicate determinations.

11.6 ± 3.3 and 657.8 ± 198.0 fmol mg^{-1} protein, respectively (n = 8) (Table 1). In contrast, using 10 μM phentolamine to determine non-specific binding, the amount of specific binding was less than that determined by 0.3 μM prazosin (Figure 1a, ▲). Scatchard analysis of the binding data was linear, suggesting the detection of a single class of binding site in this case (Figure 2a, ○ and dotted line). LIGAND analysis fitted the data to a one site model. The pK_D and B_{max} values were intermediate between the values of prazosin-high and low affinity sites detected in the presence of 0.3 μM prazosin (n = 8) (Table 1). These discrepant results in rabbit liver suggest either that phentolamine at 10 μM cannot sufficiently displace [^{3}H]-prazosin binding to α_1-adrenoceptors in this preparation or that binding in the presence of 0.3 μM prazosin enables [^{3}H]-prazosin binding sites in addition to the α_1-adrenoceptors to be detected. These possibilities were examined by comparing the inhibitory potencies of various α_1-adrenoceptor antagonists and agonists against the binding of a high concentration (1000 pM) of [^{3}H]-prazosin. As shown in Figure 3, phentolamine at 10 μM was less potent in inhibiting the [^{3}H]-prazosin binding than prazosin (0.3 μM), doxazosin (0.3 μM), WB4101 (10 μM), methoxamine (100 μM) and noradrenaline (1000 μM, in the presence of 0.5 mM ascorbic acid). The inhibitory potency of prazosin was the same as those of the tested drugs except for phentolamine. Thus, phentolamine seemed unsuitable for the determination of specific binding of [^{3}H]-prazosin to α_1-adrenoceptors of rabbit liver membranes and 0.3 μM prazosin was used to determine the non-specific binding in the following experiments.

Rat liver membranes: [^{3}H]-prazosin at concentrations ranging from 20 to 2,000 pM bound to the α_1-adrenoceptors of rat liver.

Table 1 Binding of [^{3}H]-prazosin to the α_1-adrenoceptors of rabbit and rat livers

Animal	*Specific binding*[a]	$pK_{D\ high}$	$pK_{D\ low}$	R_{high}	R_{low}	*%low*
Rabbit	Prazosin	10.3±0.19	8.13±017	11.6±3.3	657.8±198.0	98
	Phentolamine	9.07±0.20	–	134.2±37.8	–	–
Rat	Prazosin	9.98±0.27	–	190.5±38.5	–	–
	Phentolamine	10.2±0.07	–	264.5±38.5	–	–

Data shown are mean±s.e.mean of 3–9 experiments.
pK_{Dhigh} and pK_{Dlow}: negative log of equilibrium dissociation constants (-log M) at prazosin-high and -low affinity sites for [^{3}H]-prazosin.
R_{high} and R_{low}: maximum number of prazosin-binding at high and low affinity sites (fmol mg^{-1} protein).
%low: population binding at the low affinity site compared to the total specific binding sites.
[a]Specific binding was determined by 0.3 μM prazosin or 10 μM phentolamine.
–: not detected

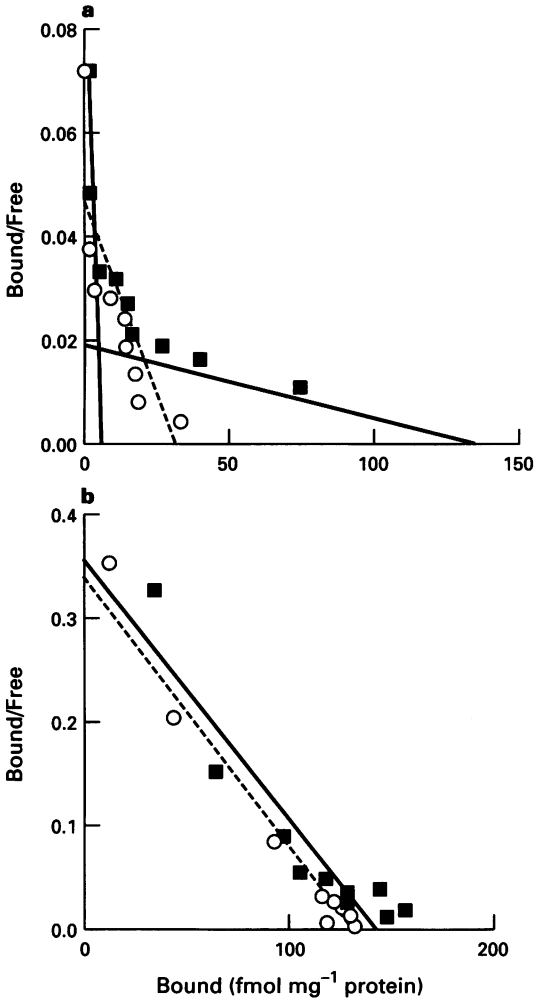

Figure 2 Scatchard plots for specific [^{3}H]-prazosin binding to rabbit (a) and rat (b) liver membranes in saturation experiments. The data of Figure 1 were analysed with LIGAND programme. Specific binding defined by 0.3 μM prazosin (■) was analysed with a two site model (two straight lines) in (a) but with a one site model (a straight line) in (b), while that defined by 10 μM phentolamine (○) was analysed with a one site model (dotted line) in (a) and (b).

The specific binding saturated at approximately 500 pM [^{3}H]-prazosin and the amounts were not significantly different even though the non-specific binding was defined by 0.3 μM prazosin or 10 μM phentolamine (Figure 1b, □ and △). Scatchard plots of specific binding were linear, suggesting a single class of binding site (Figure 2b). Similar pK_D and B_{max} values were estimated irrespective of different determinations of non-specific binding (Table 1).

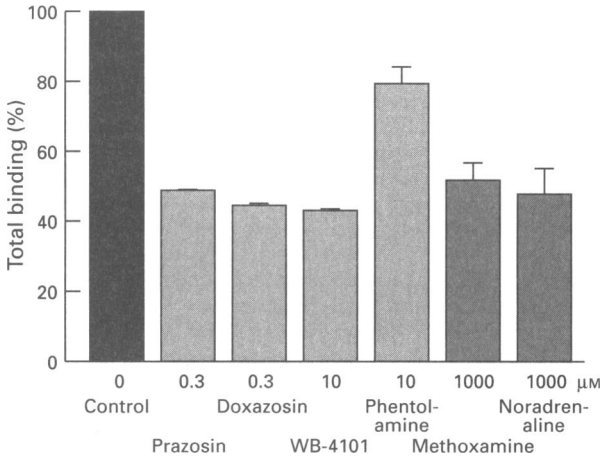

Figure 3 Inhibition by prazosin (0.3 μM, $n=22$), doxazosin (0.3 μM, $n=4$), WB-4101 (10 μM, $n=4$), phentolamine (10 μM, $n=3$), methoxamine (1000 μM, $n=4$), noradrenaline (1000 μM, $n=3$) of [^{3}H]-prazosin (1000 pM) binding to rabbit liver membranes. The total binding of the same membrane preparations in the absence of antagonist or agonist is represented as 100% (Control). Each column shows mean and s.e.mean.

Effects of competitive antagonists on [^{3}H]-prazosin binding

Rabbit liver membranes: Unlabelled prazosin, doxazosin, WB4101 and 5-methylurapidil produced a concentration-dependent inhibition of 200 pM [^{3}H]-prazosin binding. The maximum inhibition was approximately 60% of total binding, which was larger than that produced by phentolamine (Figure 4). Computerized analyses revealed that prazosin, doxazosin, WB4101 and 5-methylurapidil bound to two distinct affinity sites. The high and low pK_I values for prazosin were respectively the same as the pK_D values obtained in the saturation experiments with [^{3}H]-prazosin. The pK_I values at high and low affinity sites for WB4101 affinity were also not significantly different from the values at the corresponding sites for prazosin. The proportion of the low affinity sites for each antagonist amounted to approximately 70% of total specific binding (Table 2).

On the other hand, HV-723 displaced the binding of 200 pM [^{3}H]-prazosin in a monophasic manner. From a single IC_{50} value and two distinct K_D values, high and low pK_I values were estimated with the Cheng-Prusoff equation (Table 2).

Rat liver membranes: When 200 pM [^{3}H]-prazosin was used, unlabelled prazosin, doxazosin, WB4101, 5-methylurapidil and HV-723 displaced the binding in a monophasic manner. The pK_I values for prazosin or doxazosin were high; however, the affinities for the other antagonists were relatively low (Table 2).

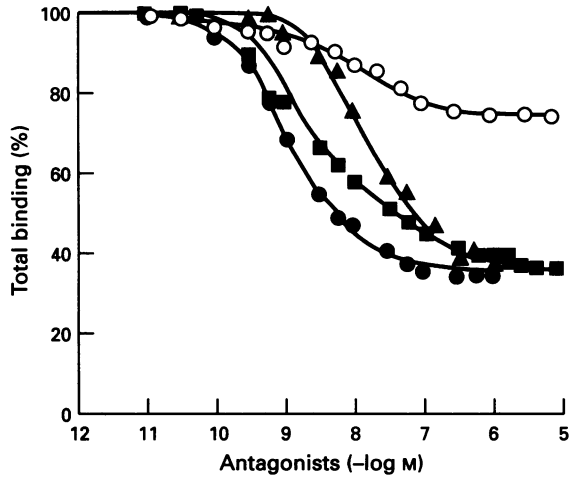

Figure 4 Inhibition of [^{3}H]-prazosin binding to rabbit liver membranes by prazosin (●), WB4101 (■), HV723 (▲) and phentolamine (○). The concentration of [^{3}H]-prazosin used was 200 pM. The figure represents a single experiment for each drug, where each point is the mean of duplicate determinations. Total bindings in the presence of vehicle are represented as 100%.

Discussion

In rat liver, [^{3}H]-prazosin apparently bound to a homogeneous population of sites with a high affinity for prazosin. The binding of [^{3}H]-prazosin was inhibited by WB-4101, 5-methylurapidil, HV723 monosphasically with relatively low affinities. These results clearly support the previous view that the α_1-adrenoceptor subtype in rat liver is α_{1B} (Hanft & Gross, 1989; Garcia-Sainz *et al.*, 1992; Taddei *et al.*, 1993; Hiramatsu *et al.*, 1994).

In the rabbit liver, only a single affinity site for [^{3}H]-prazosin was detected when the non-specific binding was determined in the presence of 10 μM phentolamine. The affinity constant obtained in this study accords with the values of a previous paper in which the non-specific binding was also defined with 10 μM phentolamine (Taddei *et al.*, 1993). In contrast, two distinct affinity sites were detected when the non-specific binding was determined in the presence of 0.3 μM prazosin and the total number of specific binding sites was much larger than that determined by phentolamine. These conflicting results may be due to a difference in the antagonists used to determine specific binding. This point was checked, and phentolamine at 10 μM was found to be less potent in inhibiting the [^{3}H]-prazosin binding than prazosin, doxazosin, WB4101, methoxamine, noradrenaline, suggesting that phentolamine was unable to inhibit completely the binding of [^{3}H]-prazosin to α_1-adrenoceptors in rabbit liver (Figures 3 and 4). Thus it seems that the specific binding of [^{3}H]-prazosin to the α_1-adrenoceptors of rabbit liver must be determined by other α_1-antagonists or -agonists instead of phentolamine (Muramatsu *et al.*, 1994; Noguchi *et al.*, 1995). In the present study, we used 0.3 μM prazosin because noradrenaline, methoxamine, WB4101 and doxazosin produced the same level of inhibition as that of prazosin.

The density of high affinity sites for prazosin was much lower than that of low affinity sites (less than 10% of total binding site). Accurate quantification of a subpopulation of less than 10% is impossible with the analysis used (DeLean *et al.*, 1982). However, both high and low affinity sites for prazosin were recognized not only in the saturation experiments but also in the displacement experiments with 200 pM [^{3}H]-prazosin, and the affinity constants of prazosin obtained were consistent between both the experiments. HV723 (an α_{1N} selective drug) displaced 200 pM [^{3}H]-prazosin binding in a monophasic manner, resulting in relatively low affinities. Rauwolscine (an α_2-antagonist) also showed a low affinity against the binding sites. Therefore, the prazosin-high and -low affinity sites of rabbit liver seem to correspond respectively to α_{1H} and α_{1L} subtypes in the α_{1H}, α_{1L} and α_{1N} subclassification (Flavahan & Vanhoutte, 1986; Muramatsu *et al.*, 1990; Oshita *et al.*, 1991). Recently, we confirmed the existence of the high and low affinity sites for prazosin in the rabbit hepatocyte isolated by collagenase digestion (Ohmura & Muramatsu, unpublished observation).

According to the new nomenclature of α_1-adrenoceptors (Hieble *et al.*, 1995), the α_1- adrenoceptors with high affinity for prazosin (α_{1H} sites) are subdivided into 3 subtypes (α_{1A}, α_{1B} and α_{1D}), where the α_{1A} and α_{1D} subtypes are more sensitive to WB4101 ($pK_B > 9$) as compared with the α_{1B} subtype. 5-Methylurapidil shows higher affinity toward the α_{1A} ($pK_B > 8.5$) (Morrow & Creese, 1986; Han *et al.*, 1987; Lomasney *et al.*, 1991; Cotecchia *et al.*, 1988; Schwinn *et al.*, 1990). The molecular biological study revealed the presence of α_{1a}-subtype in rabbit liver, and the α_{1d} and α_{1b} subtypes were not detected (Schwinn & Lomasney, 1992). The prazosin-high affinity sites ($pK_I = 9.85$) in the present study also showed high affinity for WB-4101 ($pK_I = 9.74$) and 5-methylurapidil ($pK_I = 8.69$); the pK_I values being consistent with the affinity constants obtained

Table 2 Inhibition of [^{3}H]-prazosin binding to α_1-adrenoceptor of rabbit or rat liver

Antagonists	*Slope factor*	*$pK_{I\ high}$*	*$pK_{I\ low}$*	*%low*
Rabbit liver				
Prazosin	0.60	9.85±0.08	8.01±0.09	79
Doxazosin	0.73	9.73±0.78	8.12±0.34	72
WB4101	0.48	9.74±0.32	7.57±0.34	64
5-Methylurapidil	0.48	8.69±0.27	6.75±0.35	65
HV723	0.87	8.55±0.09[a]	7.90±0.09[a]	–
Rauwolscine	–	<6	–	–
Rat liver				
Prazosin	0.91	10.01±0.08[a]	–	–
Doxazosin	0.92	9.67±0.20[a]	–	–
WB4101	0.99	8.25±0.09[a]	–	–
5-Methylurapidil	0.95	7.22±0.01[a]	–	–
HV723	0.98	8.88±0.05[a]	–	–

Data shown are mean±s.e.mean of 3–6 experiments.
Displacement experiments were done with 200 pM [^{3}H]-prazosin. pK_{Ihigh} and pK_{Ilow}: negative log of equilibrium dissociation constants (-log M) at prazosin-high and -low affinity sites for antagonists tested.
%low: population binding at the low affinity site compared to the total specific binding sites.
[a]Each pK_I value was calculated with the Cheng & Prusoff equation from a single IC_{50} value and the K_D value determined in saturation experiments.
–: not detected

for the α_{1a}-subtypes (Schwinn & Lomasney, 1992). Therefore, it is likely that the prazosin-high affinity sites of rabbit liver are of the α_{1A} subtype as recently proposed by Garcia-Sainz *et al.*, (1992) and Taddei *et al.* (1993), while the characteristics of additional prazosin-low affinity sites do not fit with those of α_{1A}, α_{1B} or α_{1D}-subtypes.

α_1-Adrenoceptors with low affinity for prazosin (α_{1L}) are evident from pharmacologial studies in many tissues (Muramatsu *et al.*, 1995). Therefore it is interesting to compare the characteristics of α_{1L}-subtypes between rabbit liver and other tissues. Affinity estimates for prazosin are similar in a wide range of tissues, whereas the affinities of WB4101 ($pK_I = 7.57$) and 5-methylurapidil (6.75) determined in rabbit liver are much lower than those determined in rabbit aorta or human prostate (8.2–8.5) (Oshita *et al.*, 1993; Muramatsu *et al.*, 1994). This may suggest heterogeneity of α_{1L}-adrenoceptor subtypes. We are now attempting to clone the α_{1A} and α_{1L}-subtypes of rabbit liver with a view to addressing this problem.

The activation of α_1-adrenoceptors causes a variety of effects such as increases in amino acid transport and glycolysis, inactivation of glycogen synthase and gluconeogenesis. These effects have been largely studied in rat liver (Kunos, 1984) which possessed only α_{1B} receptors (Gross *et al.*, 1988; Lomasney *et al.*, 1991; Torres-Marquez *et al.*, 1991). The present study shows the coexistence of two distinct α_1-adrenoceptor subtypes (presumably α_{1A} and α_{1L}) in the rabbit liver and the higher density of α_{1L} subtype compared with α_{1A} subtype. Therefore, in the rabbit liver, the α_{1L} subtype may play an important role in the physiological responses upon adrenergic stimulation.

In conclusion, the present study demonstrates, for the first time, the presence of two distinct α_1-adrenoceptor subtypes in the rabbit liver, which show, respectively, high and low affinities for prazosin, doxazosin, WB4101 and 5-methylurapidil, and presumably correspond to the α_{1A} and putative α_{1L} subtypes according to the recent α_1-adrenoceptor subclassification.

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