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RESEARCH PAPER

Pharmacologically distinct phenotypes of α_{1B}-adrenoceptors: variation in binding and functional affinities for antagonists

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BACKGROUND AND PURPOSE

The pharmacological properties of particular receptors have recently been suggested to vary under different conditions. We compared the pharmacological properties of the α_{1B}-adrenoceptor subtype in various tissue preparations and under various conditions.

EXPERIMENTAL APPROACH

[^{3}H]-prazosin binding to α_{1B}-adrenoceptors in rat liver (segments, dispersed hepatocytes and homogenates) was assessed and the pharmacological profiles were compared with the functional and binding profiles in rat carotid artery and recombinant α_{1B}-adrenoceptors.

KEY RESULTS

In association and saturation-binding experiments with rat liver, binding affinity for [^{3}H]-prazosin varied significantly between preparations (K_D value approximately ten times higher in segments than in homogenates). The binding profile for various drugs in liver segments also deviated from the representative α_{1B}-adrenoceptor profile observed in liver homogenates and recombinant receptors. L-765,314 and ALS-77, selective antagonists of α_{1B}-adrenoceptors, showed high binding and antagonist affinities in liver homogenates and recombinant α_{1B}-adrenoceptors. However, binding affinities for both ligands in the segments of rat liver and carotid artery were 10 times lower, and the antagonist potencies in α_{1B}-adrenoceptor-mediated contractions of carotid artery were more than 100 times lower than the representative α_{1B}-adrenoceptor profile.

CONCLUSIONS AND IMPLICATIONS

In contrast to the consistent profile of recombinant α_{1B}-adrenoceptors, the pharmacological profile of native α_{1B}-adrenoceptors of rat liver and carotid artery varied markedly under various receptor environments, showing significantly different binding properties between intact tissues and homogenates, and dissociation between functional and binding affinities. In addition to conventional 'subtype' characterization, 'phenotype' pharmacology must be considered in native receptor evaluations *in vivo* and in future pharmacotherapy.

Abbreviations

ALS-77, 3-(4-(1*H*-indol-3-yl)piperidin-1-yl)-*N*,*N*-dimethylpropan-1-amine dihydrochloride; BMY 7378, (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride; L-765,314, ((2S)-4-(4-amino-6,7-dimethoxy-2-quinazolinyl)2-[[(1,1-dimethylethyl)amino]carbonyl]-1-piperazinecarboxylic acid, phenylmethyl ester hydrate)

Table of Links

TARGETS	LIGANDS
α_{1B}-adrenoceptor	Noradrenaline
β-adrenoceptor	Phentolamine
M_3 receptor	Desipramine
	Propranolol
	Phenylephrine
	Probenecid
	Adrenaline
	Silodosin
	Tamsulosin
	5-methylurapidil
	Deoxycorticosterone
	[^{3}H]-prazosin

This Table lists key protein targets and ligands in this document, which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Antagonist affinity for a given receptor was previously considered to be relatively constant under all conditions, irrespective of the cell/tissue background in which the receptor is expressed. Therefore, antagonist affinity has traditionally been used as an important pharmacological index to characterize and identify each receptor subtype (Kenakin, 1995; Nelson and Challiss, 2007; Alexander *et al.*, 2013). Furthermore, as pharmacological profiles are relatively consistent between native and recombinant receptors, genome-based 'subtype' classification and the recombinant screening system have been widely applied for drug development. However, this central dogma has recently been challenged, as there is emerging evidence that a receptor can show multiple properties under different conditions or states (Kenakin, 1995; 2003; Muramatsu *et al.*, 2005; Baker and Hill, 2007; Nelson and Challiss, 2007; Kenakin and Miller, 2010; Mohr *et al.*, 2010; Anisuzzaman *et al.*, 2013; Lane *et al.*, 2013). In particular, pharmacological specificity for some receptors may differ markedly between intact cells and cell-free preparations. For example, more than 30 years ago, Tuong *et al.* (1980) reported that the drug-discriminatory properties of histamine H_2-receptors differed markedly between slices and homogenates of guinea pig hippocampus. Recently, we demonstrated that the M_3-muscarinic ACh receptors in rat cerebral cortex are insensitive to representative M_3-selective antagonists (darifenacin and solifenacin) in segments, while the antagonists recognized the M_3-muscarinic ACh receptors with high affinity after homogenization (Anisuzzaman *et al.*, 2011). From these lines of evidence, we considered that pharmacological properties of some receptors may be different at various tissue/cell backgrounds, and furthermore may be modified by different assay conditions.

The α_{1B}-adrenoceptor is one of three cloned α_1-adrenoceptor subtypes (α_{1A}, α_{1B} and α_{1D}) (Lomansney *et al.*, 1991; Hieble *et al.*, 1995; Michel *et al.*, 1995; Graham *et al.*, 1996; Alexander *et al.*, 2013), and is involved in numerous physiological functions such as BP regulation (Cavalli *et al.*, 1997; Michelotti *et al.*, 2000; Cotecchia, 2010). In pharmacological criteria of α_1-adrenoceptor subtypes, α_{1B}- as well as α_{1A}- and α_{1D}-subtypes, have high affinity for a specific α_1-adrenoceptor antagonist, prazosin. However, the α_{1B}-adrenoceptor shows low sensitivity to many drugs; for example, silodosin and 5-methylurapidil (α_{1A} antagonists), tamsulosin (α_{1A} and α_{1D} antagonist) and BMY 7378 (α_{1D} antagonist). To date, a few α_{1B}-selective antagonists, such as L3040 and ALS-77 (Figure 1), have been developed using recombinant α_{1B}-adrenoceptors and rat liver membranes; however, their hypotensive potency is extremely low *in vivo*, and clinical applications have not yet been successful (Testa *et al.*, 1997; Patane *et al.*, 1998; Hayashi *et al.*, 2000). We believe that the low potency *in vivo* may reflect pharmacologically distinct profiles of native α_{1B}-adrenoceptors. Therefore, we compared the pharmacological properties of α_{1B}-adrenoceptors under various assay conditions. Firstly, we conducted a binding study in intact segments and homogenates of rat liver and in hepatocytes. Rat liver is known to be a representative tissue predominantly expressing α_{1B}-adrenoceptors (Minneman, 1988; Michel *et al.*, 1989; Ford *et al.*, 1996; Testa *et al.*, 1997; Murata *et al.*, 1999). The pharmacological profiles of the α_{1B}-adrenoceptor were then compared with those in rat carotid artery and recombinant α_{1B}-adrenoceptors. Our results show the presence of pharmacologically distinct phenotypes in native α_{1B}-adrenoceptors and suggest the importance of phenotype-based characterization, in addition to genome-based subtype classification.

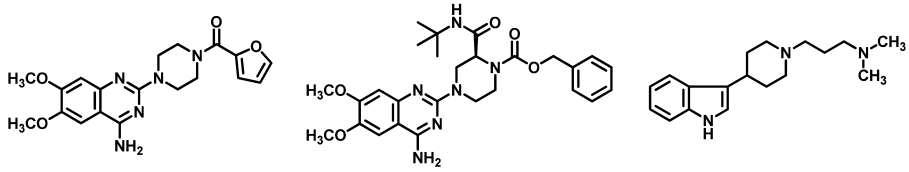

Figure 1
Chemical structures of the typical α_1-adrenoceptor antagonist prazosin and two α_{1B}–adrenoceptor-selective antagonists developed recently: L-765,314 and ALS-77.

Methods

Animals and tissue isolation

Male Wistar rats weighing 250–350 g (Japan SLC, Inc., Hamamatsu, Japan) were used in the present study, which was conducted in accordance with the Guidelines for Animal Experiments of the University of Fukui (accredited by the Ministry of Education, Culture, Sports, Science and Technology, Japan). The total number of rats used in this study was 120. Rats were anaesthetized with isoflurane and killed by cervical dislocation. The liver and carotid artery were rapidly isolated and immersed in modified Krebs–Henseleit solution containing (in mM): NaCl, 112.0; KCl, 5.4; $MgCl_2$, 1.2; $CaCl_2$, 2.0; NaH_2PO_4, 1.2; $NaHCO_3$, 25.5; and D-(+)-glucose, 11.5 (pH 7.4). The solution was oxygenated with a mixture of 95% O_2 and 5% CO_2 and was kept on ice. To obtain hepatocytes, rats were anaesthetized with pentobarbital sodium (total 50 mg kg^{-1}: i.p. injection of 30 mg kg^{-1} followed by s.c. injection of 20 mg kg^{-1}) and collagenase-containing Hanks solution was perfused through the portal vein at 37°C, and hepatocytes were dispersed (Berry *et al.*, 1992). The level of anaesthesia was confirmed by a lack of muscle response. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Binding experiments with rat liver segments

After removal of tunica serosa hepatic, liver parenchyma was cut into small pieces (approximately 3 × 3 × 3 mm). Each piece was incubated at 20 or 37°C with [^{3}H]-prazosin for various periods of time. The incubation medium used was the modified Krebs–Henseleit solution mentioned earlier, which was vigorously bubbled with a mixture of 95% O_2 and 5% CO_2 before use. In saturation-binding experiments, the concentrations of [^{3}H]-prazosin were 50–5000 pM. Non-specific binding was determined in the presence of 30 μM phentolamine. In the competition experiments, 1000 pM [^{3}H]-prazosin, which showed approximately 80% of maximum binding in saturation curves, was used. After incubation, tissue segments were quickly moved into a plastic tube containing 1.5 mL of ice-cold incubation buffer (4°C) and were carefully washed by vortex mixing for 45 s. This procedure resulted in the release of most of the unbound radioligand from the segments into the buffer medium and its rapid absorption to the plastic tube. After being washed, the segment was moved into a glass tube, and was then solubilized in 0.3 M NaOH solution. Bound radioactivity was measured using a liquid scintillation counter LSC-6100 (Aloka, Tokyo, Japan). Protein concentrations in each tissue segment were measured according to the method of Bradford using BSA as a standard. Experiments were performed in duplicate for each concentration of [^{3}H]-prazosin for binding-saturation experiments or for each concentration of competing ligand for competition binding experiments.

Binding experiments with rat liver homogenates and hepatocytes

Rat liver without tunica serosa was minced with scissors and homogenized in 50 volumes (v w^{-1}) of Krebs incubation buffer using a polytron homogenizer (setting 8, 3 × 20 s at 4°C). The Krebs incubation buffer was the same as that used in the tissue segment binding experiments. To avoid protein loss, whole homogenates without fractionation were used. Homogenates were incubated at 20 C in 1 mL of Krebs incubation buffer for various periods. [^{3}H]-prazosin (50–2000 pM) was used for binding-saturation experiments. In competition binding experiments, 200 pM [^{3}H]-prazosin, which showed approximately 80% of maximum binding in saturation curves was incubated in the presence or absence of unlabelled competing ligands. Reactions were terminated by rapid filtration, using a Brandel cell harvester (model V-30, Biomedical Research and Development Laboratories, Gaithersburg, MD, USA), onto Whatman GF/C filters presoaked in 0.3% polyethyleneimine for 30 min, and the filters were then washed three times with 2 mL of ice-cold Krebs incubation buffer. The resulting filters were dried and the trapped radioactivity was quantified by liquid scintillation counting. Non-specific binding of [^{3}H]-prazosin was determined with 30 μM phentolamine. Experiments were performed in duplicate at each radioligand concentration for a binding-saturation experiment or at each concentration of competing ligand for a competition binding experiment. The protein content of homogenates was measured as described earlier.

The experimental procedure for binding experiments with rat hepatocytes was essentially the same as that for liver homogenate experiments. Briefly, hepatocytes were incubated at 20°C in 1 mL of Krebs incubation buffer for 3 h. Incubation was then terminated by rapid filtration, using a Brandel cell harvester.

Binding experiments with rat carotid artery segments and homogenates

Isolated carotid artery was opened, and the endothelium was removed by rubbing with filter paper. Sixteen segments

(approximately 5 × 1 mm in each) were prepared from one rat and applied to one saturation or competition binding assay. In the homogenate binding assay, carotid arteries isolated from 20 rats were pooled and homogenates were applied to one saturation experiment. Experimental conditions were the same as for binding assays with liver segments or homogenates respectively. However, the concentrations of [^{3}H]-prazosin used were low (10–500 pM in saturation experiments, and 100 pM in competition experiments), because of the high affinity for [^{3}H]-prazosin of these carotid artery preparations.

[^{3}H]-prazosin binding to recombinant α_{1B}-adrenoceptors

CHO cell lines stably transfected with human α_{1B}-adrenoceptors subcloned in the mammalian expression vector pCR3 (Invitrogen™, Carlsbad, CA, USA) were cultured in α-minimum essential medium supplemented with 10% FBS and 200 μg·mL^{-1} G418. Cells were harvested by gentle scraping without trypsin. Whole-cell and homogenate-binding assays were essentially the same as described earlier and previously (Yoshiki *et al.*, 2013). Incubation was carried out at 20°C for 1 h.

Functional experiments with rat isolated carotid artery

Rat carotid artery was cut into ring preparations of 2 mm in length. Ring preparations were placed in organ baths containing a modified Krebs–Henseleit solution at 37°C. Desipramine (0.3 μM), deoxycorticosterone acetate (5 μM) and propranolol (1 μM) were added to inhibit neural and extraneural uptake and to block β-adrenoceptors. N$^{\omega}$-nitro-L-arginine methyl ester (100 μM) was also added to inhibit NO release from endothelial cells. After equilibration for 2 h, phenylephrine was applied cumulatively three times at 2 h intervals in the same ring and the isometric tension changes were recorded through a force displacement transducer. Antagonists were added 40 min before and preparations were treated during the third response to phenylephrine. The third response to phenylephrine in the absence of antagonists was used as a time control. All of the third concentration–response curves were normalized to the maximum amplitude of the second response in each preparation.

Measurement of intracellular Ca^{2+} concentration in CHO cells

Intracellular calcium concentration ($[Ca^{2+}]_i$) was measured as described previously (Yoshiki *et al.*, 2013). Briefly, CHO cells expressing human α_{1B}-adrenoceptors were loaded with 5 μM Fura-2 AM (Dojindo, Kumamoto, Japan) together with 0.02% pluronic F-127 and 1.4 mM probenecid for 45 min, and were then washed and resuspended in Ca^{2+} assay buffer with 1.4 mM probenecid and 3% FBS. Measurements were performed at 37°C with Fura-2 ratio fluorometry using a CAF-110 fluorescence spectrophotometer (JASCO, Tokyo, Japan). Cells were exposed to single concentrations of noradrenaline and changes in $[Ca^{2+}]$i were measured for 5 min. At the end of the experiment, maximum and minimum fluorescence ratios were determined by subsequent addition of Triton X-100 and EGTA, and $[Ca^{2+}]_i$ concentrations were then calculated. Antagonist was added 5 min before the application of noradrenaline.

Data analysis

Binding data were analysed using PRISM (version 5.01; Graph Pad Software, La Jolla, CA, USA), as described previously (Muramatsu *et al.*, 2005; Anisuzzaman *et al.*, 2011). Briefly, data from saturation-binding studies were fitted with a one-site saturation-binding isotherm (binding-saturation equation in PRISM), and K_D values and binding capacity were then calculated. The abundance of α_1-adrenoceptors is presented as the maximum binding capacity mg^{-1} of total tissue protein (Bmax). For competition studies, data were analysed using the binding-competitive equation of PRISM. As different K_D values for [^{3}H]prazosin were calculated between saturation-binding experiments using tissue segments and homogenates, the corresponding K_D value estimated for each preparation was used for analysis of the competition binding data to calculate K_i values for competitors and their proportions. A two-site model was adopted only when residual sums of squares were significantly less ($P < 0.05$) for a two-site fit to the data than for a one-site fit on *F*-test comparison. Kinetic experiments were also analysed using the association kinetics equation of PRISM.

In the $[Ca^{2+}]_i$ responses of CHO cells and the contractile responses of rat carotid artery, the antagonist affinity (pK_B) was determined for a single concentration of antagonist using the concentration–ratio method (Furchgott, 1972). Antagonist affinity estimates (pK_B values) and Schild slopes were also obtained by plotting the data according to Arunlakshana and Schild (1959).

Data are shown as means ± SEM of number of experiments (*n*) and were statistically analysed using Student's *t*-test.

Reagents

The following drugs were used in the present study: [7-methoxy-^{3}H]-prazosin ([^{3}H]-prazosin, specific activity, 3156 GBq·mmol^{-1}: PerkinElmer, Inc., Boston, MA, USA); prazosin hydrochloride, BMY 7378 and 5-methylurapidil, phentolamine hydrochloride, L-765,314 (Sigma-Aldrich Co., St. Louis, MO, USA); L-adrenaline hydrogen tartrate, L-noradrenaline bitartrate and phenylephrine hydrochloride (Nacalai Tesque, Kyoto, Japan); and silodosin and tamsulosin (donated by Kissei Pharmaceutical Co., Ltd., Matsumoto, Japan). ALS-77 was synthesized by Toray Industry Inc. (Kamakura, Japan).

Results

[^{3}H]-prazosin binding in rat liver segments, homogenates and hepatocytes

Association experiments were initially conducted in the segments and homogenates of rat liver at 20°C. Figure 2A and B shows the representative time courses of specific binding at three different concentrations of [^{3}H]-prazosin to the intact segments or homogenates of rat liver, which showed markedly slower binding kinetics in the segments than in homoge-

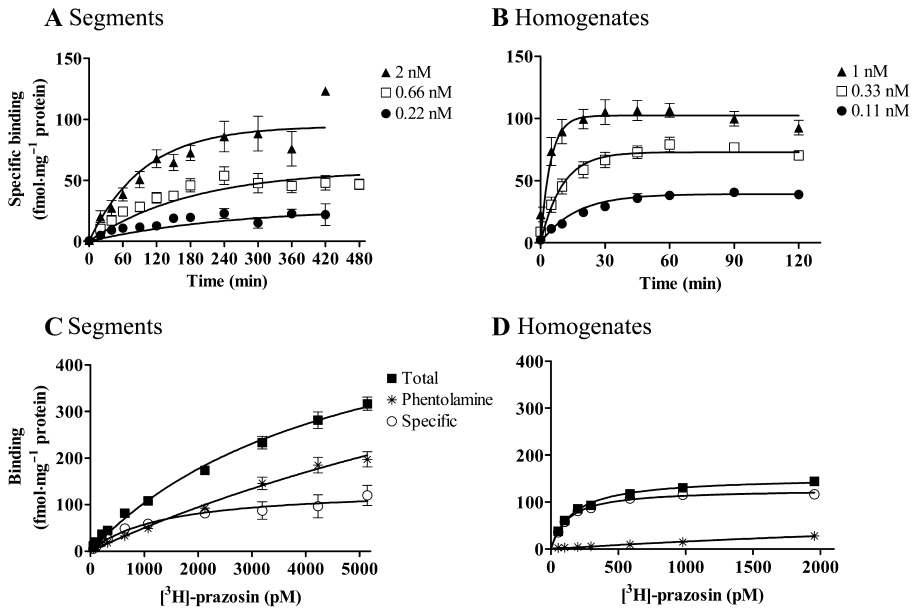

Figure 2

Binding of [^{3}H]-prazosin to intact segments and homogenates of rat liver at 20°C. (A and B) Association experiments using three different concentrations of [^{3}H]-prazosin in intact segments (A) and homogenates (B). (C and D) Saturation-binding curves for [^{3}H]-prazosin in intact segments (C) and homogenates (D). Each figure shows representative data from four to seven experiments and each point shows the mean±SEM of duplicate results.

nates. The calculated association and dissociation rate constants (K_{on} and K_{off}) were $6.6 \pm 1.2 \times 10^6$ M^{-1} min^{-1} and 6.0 ± 1.4 × 10^{-3} min^{-1} in the segments, and 1.9 ± 0.4 × 10^8 M^{-1}·min^{-1} and $4.4 \pm 0.8 \times 10^{-2}$ min^{-1} in the homogenates, respectively (n = 4). Thus, distinct dissociation constants (K_D) were estimated in the segments and homogenates (1093 ± 39 pM and 254 ± 41 pM, $P < 0.05$, n = 4). Association experiments were also conducted at 37°C in the segments, with K_{on} and K_{off} values of $3.7 \pm 1.3 \times 10^7$ M^{-1} min^{-1} and 5.8 ± 1.5 × 10^{-2} min^{-1} and K_D = 1827 ± 324 pM being estimated. Binding kinetics were faster at 37°C than at 20°C in the segments, becoming steady after 8 h and 1 h of incubation at 20°C or 37°C respectively.

Figure 2C and D shows the saturation curves for [^{3}H]-prazosin in the segments and homogenates of rat liver at 20°C. Non-specific binding was significantly higher in the segments (approximately 50% of total binding at 2000 pM [^{3}H]-prazosin) than in homogenates (less than 20%). Specific binding in segments increased gradually with [^{3}H]-prazosin concentration, while specific binding in the homogenates reached a plateau at low concentrations of [^{3}H]-prazosin. Thus, an approximately 10-fold higher K_D value was estimated in the segments as compared with the homogenates, despite the same density estimates being seen in both preparations (Table 1). Affinity for [^{3}H]-prazosin was also low in the liver segments at 37°C (Table 1).

As different binding kinetics and parameters were observed between the segments and homogenates of rat liver, whole-cell binding experiments were also conducted with the dispersed hepatocytes. As shown in Table 1, low affinity for [^{3}H]-prazosin was also estimated, but the binding density was approximately two times higher than in liver segments or homogenates (Table 1). This difference in density was considered to be due to a lack of contamination with connective tissues in hepatocyte preparations.

Pharmacological profiles of [^{3}H]-prazosin-binding sites in rat liver

As the binding affinity (K_D) for [^{3}H]-prazosin differed between the segments and homogenates of rat liver, the pharmacological profiles of [^{3}H]-prazosin-binding sites in both preparations were examined in competition experiments with several ligands. All tested ligands, except L-765,314, monotonically competed with [^{3}H]-prazosin binding, indicating the presence of single binding sites for each ligand in both liver preparations. However, as shown in Figure 3, slightly higher concentrations of prazosin, silodosin and ALS-77 were necessary for competition in the segments than in the homogenates. However, competition by L-765,314 was incomplete in the segments, in contrast to full competition in the homogenates; approximately 40% of [^{3}H]-prazosin-binding sites were insensitive to L-765,314 in the segments (Figure 3C).

Table 2 shows the dissociation constant (pK_i) of each competitor, which was calculated using different K_D values for [^{3}H]-prazosin obtained from saturation experiments with the segments and homogenates. The affinities for prazosin, silodosin, tamsulosin, adrenaline and ALS-77 were approximately 10-fold lower in the segments than in the homogenates. In contrast, 5-methylurapidil, BMY 7378 and L-765,314 (at its sensitive sites) showed the same affinity between both preparations.

Table 1

Binding parameters estimated from saturation experiments with [^{3}H]-prazosin in rat liver, carotid artery and recombinant α_{1B}-adrenoceptors

		K_D (pM)	Bmax (fmol·mg^{-1})
Liver			
Intact segments	20°C	1180 ± 128[a]	136 ± 10
	37°C	1338 ± 352[a]	101 ± 21
Homogenates	20°C	120 ± 13	135 ± 6
Hepatocyte (whole cells)	20°C	981 ± 97[a]	278 ± 28[a]
Carotid artery			
Intact segments	20°C	80 ± 10	146 ± 21[a]
Homogenates	20°C	85	18
Recombinant α_{1B}-adrenoceptor			
Whole cells	20°C	102 ± 8	456 ± 19
Homogenates	20°C	105 ± 14	426 ± 30

Data represent means ± SEM of four to seven experiments, but one saturation experiment with carotid artery homogenates was conducted in the arteries isolated from 20 rats. Saturation-binding experiments were carried out at 20 or 37°C. Bmax indicates maximum binding capacity mg^{-1} of total protein.
[a]Significantly different from homogenates ($P < 0.05$).

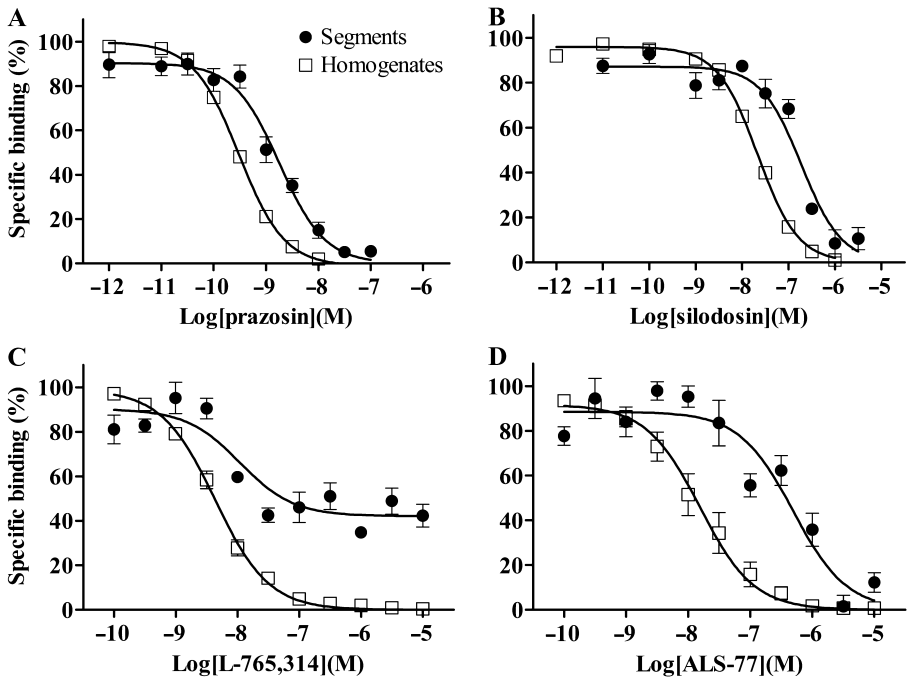

Figure 3

Competition curves for prazosin (A), silodosin (B), L-765,314 (C) and ALS-77 (D) at [^{3}H]-prazosin-binding sites of the segments and homogenates of rat liver. Each figure shows representative data from three to seven experiments and each point shows the mean ± SEM of duplicate results.

Binding and functional profiles of α_1-adrenoceptors in rat carotid artery

Binding experiments were then conducted in rat carotid artery. In contrast to rat liver, [^{3}H]-prazosin bound at high affinity not only in the segments, but also in the homogenates of carotid artery (Table 1). However, the binding amount was extremely low in the homogenates; therefore, we conducted one saturation experiment in homogenates prepared from arteries isolated from 20 rats. Binding sites in the segments of carotid artery competed biphasically with BMY 7378, but the other tested ligands displaced [^{3}H]-prazosin-binding monotonically (Figure 4, Table 3).

Table 2

Binding affinities of various ligands at [^{3}H]-prazosin-binding sites in segments and homogenates of rat liver and in recombinant α_{1B}-adrenoceptors expressed in CHO cells

	Segments	Homogenates	Recombinant α_{1B}-adrenoceptor
Drug (selective subtypes)	**pK_i**	**pK_i**	**pK_i**
Prazosin (α_{1A}, α_{1B}, α_{1D})	9.00 ± 0.06[a,b]	10.00 ± 0.04	10.17 ± 0.03
Silodosin (α_{1A})	7.00 ± 0.11[a,b]	8.29 ± 0.08	8.37 ± 0.03
5-methylurapidil (α_{1A})	6.70 ± 0.15[b]	7.19 ± 0.06	7.63 ± 0.03
Tamsulosin (α_{1A}, α_{1D})	8.27 ± 0.14[a,b]	9.49 ± 0.07	9.83 ± 0.03
BMY 7378 (α_{1D})	5.98 ± 0.13[b]	6.14 ± 0.06	6.70 ± 0.06
Adrenaline (α_{1D})	5.25 ± 0.10[a,b]	6.25 ± 0.03	6.97 ± 0.03
L-765,314 (α_{1B})	8.23 ± 0.12 (59%)[c]	8.65 ± 0.18	8.55 ± 0.03
ALS-77 (α_{1B})	6.90 ± 0.39[a,b]	8.10 ± 0.23	8.44 ± 0.11

Competition binding experiments with rat liver (segments and homogenates) and recombinant α_{1B}-adrenoceptor were carried out at 20°C. Whole-cell binding experiments were applied to recombinant α_{1B}-adrenoceptors. Data represent means ± SEM from three to nine experiments. pKi: negative logarithm concentration of dissociation constant for tested drug. (selective subtypes): The subtype selectivity was characterized in recombinant α_{1A}-, α_{1B}- and α_{1D}-adrenoceptors (see text).
[a]Significantly different from homogenates ($P < 0.05$).
[b]Significantly different from recombinant α_{1B}-adrenoceptor ($P < 0.05$).
[c]Proportion of L-765,314-sensitive sites (see Figure 3C).

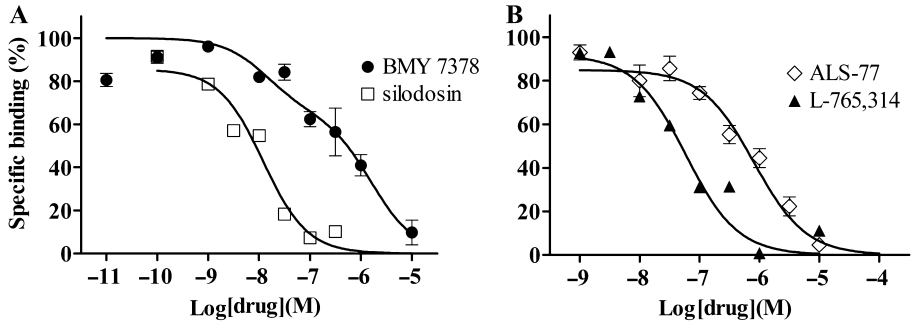

Figure 4

Competition curves for BMY 7378 and silodosin (A), and L-765,314 and ALS-77 (B) at [^{3}H]-prazosin-binding sites in rat carotid artery segments. Each figure shows representative data from four experiments and each point shows the mean ± SEM of duplicate results.

Next, a functional bioassay was carried out in ring preparations of carotid artery. Phenylephrine produced concentration-dependent contractions in rat carotid artery (pEC_{50} = 7.77 ± 0.07, n = 12). These contractions were inhibited by BMY 7378. However, as shown in Figure 5A, the rightward shift in concentration–response curves for phenylephrine by BMY 7378 ranging from 3 nM to 3 μM was biphasic. Thus, the Schild plots showed two-step competition, estimating two distinct pK_B values (approximately 8.6 and 7.0) (Figure 5B). In contrast, prazosin (1–100 nM) inhibited the response to phenylephrine in a simple concentration-dependent manner (Figure 5C), resulting in a Schild slope near unity and a high pK_B estimate (Figure 5B, Table 4). These results suggest that the concentration–response curves for phenylephrine in rat carotid artery are constructed through two distinct α_1-adrenoceptor subtypes, which are discriminated by BMY 7378, probably α_{1D}- and another α_1-adrenoceptor subtype. We then analysed the responses to phenylephrine in the absence or presence of 20 nM BMY 7378, in order to unmask or mask α_{1D}-adrenoceptors respectively. In the presence of BMY 7378, the contractions induced by phenylephrine were slightly less sensitive to silodosin, tamsulosin and 5-methylurapidil than the contractions in the absence of BMY 7378, whereas prazosin showed the same sensitivity irrespective of the presence or absence of BMY 7378 (Table 4, Figure 5C and D). From these results and based on the criteria of subtype classification (Table 2), it was more likely that the contractions induced by low concentrations of phenylephrine in the carotid artery were mediated through α_{1D}-adrenoceptors, but BMY 7378-resistant contractions were predominantly induced through α_{1B}-adrenoceptors. In the presence or absence of 20 nM BMY 7378, L-765,314 (1 and 10 μM) shifted the concentration–response curves for phenylephrine to the right; the pK_B value

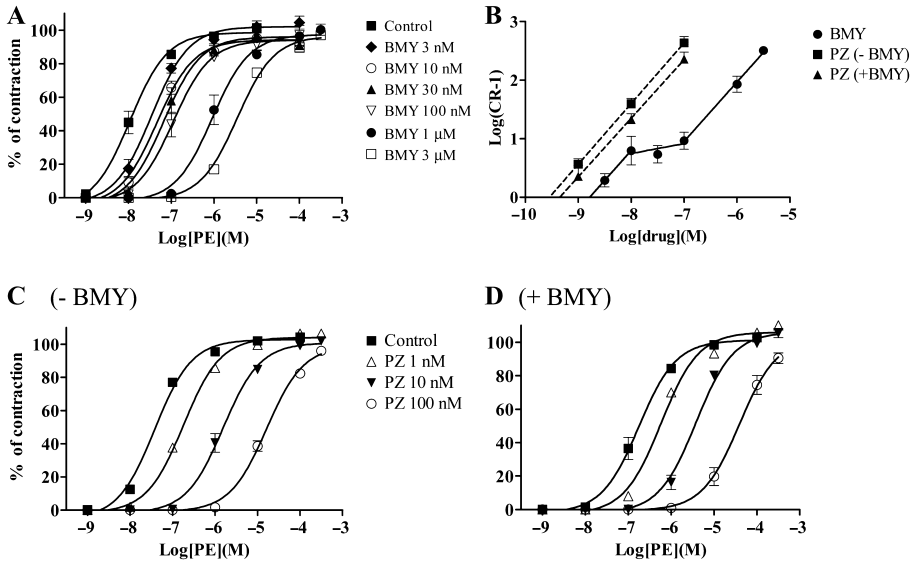

Figure 5

Effects of BMY 7378 and prazosin (PZ) on concentration–contraction curves for phenylephrine (PE) in rat carotid artery rings. (A) Antagonistic effects of BMY 7378. (C and D) Antagonistic effects of prazosin (in the absence or presence of 20 nM BMY 7378). (B) Schild plots of data in A, C and D. Each figure shows the data obtained from five to seven experiments (means ± SEM).

Table 3

Binding affinities of various ligands at [^{3}H]-prazosin-binding sites in segments of rat carotid artery

Drug	pK_i	
Prazosin	10.05 ± 0.30	
Silodosin	8.20 ± 0.16	
BMY 7378	8.20 ± 0.43 (35%)	6.24 ± 0.56
L-765,314	7.65 ± 0.16	
ALS-77	6.48 ± 0.06	

Competition binding experiments with rat carotid artery segments were carried out at 20°C. Data represent means ± SEM from four to five experiments. The α_1-adrenoceptors in carotid artery showed high and low affinities for BMY 7378, and the proportion of high-affinity sites was 35%.

Table 4

Functional affinities for four drugs in rat carotid artery

	pK_B (Schild slope)	
	–BMY 7378	+BMY 7378
Prazosin	9.66 ± 0.07 (1.036)	9.34 ± 0.22 (1.000)
Silodosin	8.45 ± 0.21 (0.974)	7.60 ± 0.08[a] (0.969)
5-methylurapidil	7.56 ± 0.15 (1.117)	6.83 ± 0.21[a] (0.907)
Tamsulosin	10.22 ± 0.04 (1.036)	8.92 ± 0.33[a] (1.069)

Concentration-contraction curves for phenylephrine were produced in the absence or presence of 20 nM BMY 7378 in rat carotid artery rings, and antagonist affinities were estimated from Schild plots. Data represent means and SEM from four to five experiments.
[a]Significantly different from the value in the absence of BMY 7378 ($P < 0.05$).

was approximately 6.6 (Figure 6A, Table 5). In contrast, ALS-77 (1 and 10 μM) showed little inhibitory effects on phenylephrine-induced contraction (Figure 6B, Table 5). Furthermore, additional treatment with 10 μM ALS-77 in the presence of 20 nM BMY 7378 had no effect on the antagonistic potency of prazosin or L-765,314 (Table 5).

Binding and functional profiles of recombinant α_{1B}-adrenoceptors

At the recombinant α_{1B}-adrenoceptors expressed in CHO cells, [^{3}H]-prazosin at low concentrations bound monotonically and there were no differences in K_D and Bmax values between whole cells and homogenates (Table 1). [^{3}H]-prazosin binding competed with all tested ligands, and the affinities for competitors were in good agreement with those in rat liver homogenates, but not those in segments (Table 2).

In response to noradrenaline, the recombinant α_{1B}-adrenoceptors increased $[Ca^{2+}]_i$ in a concentration-dependent manner (pEC_{50} for noradrenaline = 7.95 ± 0.18, n = 7). The concentration–response curve for noradrenaline was competitively inhibited by low concentrations (0.1 and 0.3 nM) of prazosin. In contrast, L-765,314 at 10 nM produced an insurmountable inhibition, pK_B = 8.42 being estimated (Figure 6C, Table 5). ALS-77 at 100 nM shifted the concentration–response curves for noradrenaline to the right and a pK_B value of approximately 8.2 was calculated (Figure 6D, Table 5).

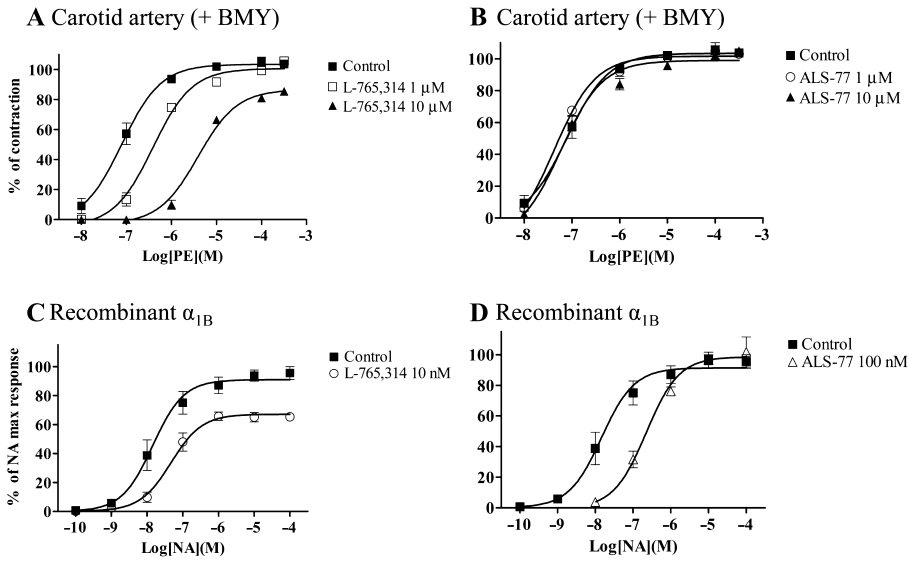

Figure 6

Effects of L-765,314 and ALS-77 on contractile responses to phenylephrine in rat carotid artery and on the $[Ca^{2+}]_i$ increases induced by noradrenaline in CHO cells expressing α_{1B}-adrenoceptors. (A and B) Concentration–response curves for phenylephrine (PE) in rat carotid artery were obtained in the presence of 20 nM BMY 7378. (C and D) The $[Ca^{2+}]_i$ responses were normalized against the maximum response to noradrenaline (NA) in the same batch cells in the absence of an antagonist. Data show means ± SEM of three to five experiments.

Table 5

Functional affinities for prazosin, L-765,314 and ALS-77 in rat carotid artery and recombinant α_{1B}-adrenoceptors

		Carotid artery pK_B		
		–BMY 7378	+BMY 7378 (+ALS-77)	Recombinant α_{1B}-adrenoceptor pK_B
Prazosin	0.1 nM	–	–	10.95 ± 0.21
	0.3 nM	–	–	10.78 ± 0.39
	1 nM	9.57 ± 0.06	9.36 ± 0.06 (9.10 ± 0.05)	–
	10 nM	9.85 ± 0.17	9.31 ± 0.05 (9.06 ± 0.06)	–
	100 nM	9.63 ± 0.07	9.34 ± 0.10 (9.25 ± 0.13)	–
L-765,314	10 nM	–	–	8.42 ± 0.15
	1 μM	6.65 ± 0.15	6.56 ± 0.09 (6.00 ± 0.17)	–
	10 μM	6.71 ± 0.23	6.68 ± 0.03 (6.42 ± 0.06)	–
ALS-77	0.1 μM	–	–	8.17 ± 0.09
	1 μM	N.I.	N.I.	8.24 ± 0.04
	10 μM	5.06 ± 0.10	N.I.	–

Experimental conditions are the same as those in Table 4. Antagonist affinity (pK_B) was calculated using a single concentration of antagonist by the concentration–ratio method (Furchgott, 1972).
(+ALS-77): Affinities for prazosin or L-765,314 were examined in the presence of 10 μM ALS-77 in addition to 20 nM BMY 7378. Data represent means and SEM from three to five experiments.
–, not tested. N.I., no inhibition.

Discussion

The present study suggests that the native α_{1B}-adrenoceptor is able to take pharmacologically distinct states/phenotypes, which may strongly depend on receptor environments. First, binding affinity of [^{3}H]-prazosin in rat liver α_{1B}-adrenoceptors was shown to be significantly different between intact segments and homogenates. The low affinity for [^{3}H]-prazosin in the intact segments was not due to poor accessibility of the ^{3}H-ligand to the binding sites, as low affinity was observed despite slow- or fast-binding association rates at 20 and 37°C, respectively, and also in the dispersed hepatocytes. Under

equilibrated binding conditions, tested drugs such as silodosin, tamsulosin and ALS-77 also showed lower affinity in the segments than in the homogenates, and L-765,314 caused incomplete competition at [^{3}H]-prazosin-binding sites in the segments. However, such binding properties in the segments disappeared after homogenization, converting to higher affinity properties for prazosin, silodosin, tamsulosin, adrenaline and ALS-77. L-765,314 also completely competed with [^{3}H]-prazosin binding with a high affinity in the homogenates. In contrast to the segments, the affinity estimates in the homogenates conformed well with those in recombinant human and rat α_{1B}-adrenoceptors (Lomansney *et al.*, 1991; Michel *et al.*, 1995; Ford *et al.*, 1996; Graham *et al.*, 1996; Testa *et al.*, 1997). Thus, it is likely that the pharmacological profile of α_{1B}-adrenoceptors observed in rat liver segments corresponds with the inherent properties under native tissue conditions, but that the drug-discriminatory properties are easily modified depending on the changes in receptor environments (here, intact tissue segments vs. homogenized membranes).

In contrast to rat liver, however, the α_{1B}-adrenoceptors in rat carotid artery showed a high affinity for [^{3}H]-prazosin in both segments and homogenates. In our preliminary experiments, a wide variation in binding affinity for [^{3}H]-prazosin was observed among the segments of various tissues, whereas a relatively high and uniform affinity was estimated in homogenates among various tissues (see Supporting Information Table S1). Thus, it is likely that variant affinities for [^{3}H]-prazosin binding reflects distinct tissue-conferred properties under intact tissue conditions.

Another interesting finding in the present study was the dissociation between binding affinity and functional affinity, which was clearly observed for L-765,314 and ALS-77. Both ligands were originally developed as α_{1B}-selective antagonists showing high affinity for recombinant α_{1B}-adrenoceptor, as well as rat liver membranes (Patane *et al.*, 1998; Hayashi *et al.*, 2000), and this profile was also confirmed in the present binding and functional experiments with rat liver homogenates and/or recombinant α_{1B}-adrenoceptors. However, both antagonists showed extremely low potency in inhibiting α_{1B}-adrenoceptor-mediated contractions in rat carotid artery; the functional affinity for L3040 in the artery was approximately 10 times lower than the binding affinity in the artery and 100 times lower than the functional and binding affinities in the recombinant α_{1B}-adrenoceptors. ALS-77 was essentially inactive on α_{1B}-adrenoceptor-mediated contractions, and furthermore did not affect the antagonistic potency of prazosin on the arterial contractions, even though ALS-77 did compete with [^{3}H]-prazosin binding. These paradoxical results suggest pharmacological dissociation between functional and binding activities at native α_{1B}-adrenoceptors. Such functional and binding dissociation at α_{1B}-adrenoceptor has also been found for spiperone, risperidone, (+)-cyclazosin and ketanserin (Burt *et al.*, 1995; Eltze, 1996; Stam *et al.*, 1998; Sathi *et al.*, 2008). Although the underlying mechanisms remain unknown, it is interesting to remember the 'mobile or floating' receptor concept put forward by Cuatrecasas and his group in the mid-1970s, in which the 'effectors' with which a receptor interacts in different cell settings can have a direct impact on the affinities of a ligand for a given receptor (Jacobs and Cuatrecasas, 1976). The present dissociation between functional and binding activities at native α_{1B}-adrenoceptors may also explain why L-765,314 and ALS-77 have extremely low potency in hypotensive action *in vivo* (Patane *et al.*, 1998; R. Hayashi, unpubl. obs.) and have not been successfully applied in clinical settings to date.

Recently, receptors have been demonstrated to possess multiple binding sites (e.g. orthosteric, allosteric and bitopic sites) in the same receptor molecule, where binding properties at each site may affect one another and be influenced by the agonists and antagonists used, or by coupling of distinct signalling pathways (Baker and Hill, 2007; Kenakin and Miller, 2010; Mohr *et al.*, 2010; Lane *et al.*, 2013). The pharmacological diversity of α_{1B}-adrenoceptors observed in the present study may be in part related to such distinct binding sites in the same receptor molecule. In addition to this possibility, the present study confirms that the multiple binding sites are also influenced by receptor environment (tissue integrity and tissue/cell background), resulting in distinct phenotypes/states (Baker and Hill, 2007; Nelson and Challiss, 2007; Nishimune *et al.*, 2012). Like the present α_{1B}-adrenoceptor, atypical properties have been found to date in several receptors and tissues under intact cell/tissue conditions: histamine H_2-receptor in guinea pig hippocampus (Tuong *et al.*, 1980), β_3-adrenoceptor in CHO cells (Arch, 2002), M_3-muscarinic ACh receptors in rat cerebral cortex (Anisuzzaman *et al.*, 2011) and α_{1L}-adrenoceptors in lower urinary tract of humans and rodents and in rat cerebral cortex (Morishima *et al.*, 2007; 2008; Muramatsu *et al.*, 2008), all of which converted into traditional/representative receptor phenotype showing high affinity for selective antagonists of each receptor in cell-free preparations.

A significant variation in receptor pharmacology between *in vivo* or intact tissues and cell-free preparations or recombinant system would provide important information on pharmacokinetics analysis, PET analysis and in drug development, because conventional binding affinity estimates obtained from homogenates or recombinant receptors have been routinely used in *in vivo* analyses and drug development. In parallel with this point, it might be interesting to report our preliminary results, in which distribution of i.v. administered [^{3}H]-prazosin into various rat tissue α_1-adrenoceptors varied in proportion with the individual affinities for prazosin estimated from various tissue segments, but not with the uniform affinity estimated from various tissue homogenates (Supporting Information Table S1 and Figure S1). However, the mechanisms leading to atypical states/phenotypes under native tissue conditions remain uncertain, and require further investigation.

In summary, the present study suggests that α_{1B}-adrenoceptors, and probably other receptors, exist as pharmacologically distinct phenotypes under native tissue conditions. In addition to conventional criteria on receptor 'subtype' classification, phenotypic variation must therefore be considered in native receptor evaluation *in vivo* and in future pharmacotherapy.

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Author contributions

H. Y., J. U. and I. M. designed the experiments; H. Y., A. S. M. A, H. U., T. M, M. N. and I.M. obtained and analysed the data; R. H. and M. K. designed and synthesized the compounds; and all authors contributed to the preparation of the paper.

Conflicts of interest

The authors have no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1 $[^3H]$-prazosin binding in various rat tissues *in vivo*. $[^3H]$-prazosin (400 pmol) was slowly injected through femoral vein for 10 min under pentobarbital anaesthesia, and after 1 h the radioactivity in each tissue was measured. As described in tissue segment binding assay (see text), isolated tissues were cut into small pieces and were then washed in modified Krebs–Henseleit solution at 4°C for 45 s in order to eliminate non-specific accumulation of ^3H ligand. Segments were then solubilized in 0.3 M NaOH, and the radioactivity and protein were measured. Plasma concentration of $[^3H]$-prazosin 1 h after injection was 0.35 ± 0.02 nM (n = 5), which was extrapolated from the plasma ^3H-radioactivity without consideration of $[^3H]$prazosin metabolism. To estimate the non-specific binding of $[^3H]$-prazosin, phentolamine was administered i.v. (10 mg·kg^{-1}) and s.c. (10 mg·kg^{-1}) at 30 min before injection of $[^3H]$-prazosin. A: Total (closed column) and non-specific (open column) binding in each tissue. ^3H-count in cerebral cortex was negligible, indicating lack of penetration of $[^3H]$-prazosin through the blood–brain barrier during 1 h *in vivo*. Data represent means ± SEM of five rats. B: Closed symbols: the *in vivo* specific binding in various tissues (shown in A) was plotted as a proportion per Bmax estimated from segment binding assay in each tissue (Supporting Information Table S1). Abscissa shows $[^3H]$-prazosin affinities (K_D) estimated from segment binding assay (Supporting Information Table S1). Open symbols: binding proportion was extrapolated from Bmax and affinity obtained from segment binding assay in each tissue and was shown in order to compare with actual specific binding proportion (closed symbols). Except thoracic aorta and carotid artery, higher binding proportions in higher affinity tissues and a good relationship between both proportions (closed and open symbols) in each tissue were observed. Lower proportions of specific binding in thoracic aorta (closed square, 34%) and carotid artery (closed circle, 29%) than the extrapolated proportions (approximately 80%, open square and open circle) might be resulted from competition by plasma catecholamines. On the other hand, if $[^3H]$-prazosin bound at a relatively high and uniform affinity estimated in tissue homogenates (approximately 100 pM, see Supporting Information Table S1), α_1-adrenoceptor-binding proportion at more than 80% should be extrapolated in various peripheral tissues at the present plasma concentration of $[^3H]$-prazosin (0.35 ± 0.02 nM).

Table S1 Binding parameters of $[^3H]$prazosin in segments and homogenates of various tissues of rats.