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Alpha-1 adrenoceptors: evaluation of receptor subtype-binding kinetics in intact arterial tissues and comparison with membrane binding

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1 The binding kinetics of [^{3}H]-prazosin were measured using intact segments of rat tail artery (RTA) and thoracic aorta (RAO), and the data were compared with those obtained using a conventional membrane ligand-binding method.

2 In intact RTA and RAO segments, [^{3}H]-prazosin bound reversibly in a time-dependent and receptor-specific manner at 4°C to alpha-1 adrenoceptors (ARs) of the plasma membrane, with affinities (pK_D: 9.5 in RTA; 9.9 in RAO) that were in agreement with values estimated by a conventional membrane ligand-binding method. However, nonspecific binding was considerably higher in RAO than RTA, failing to detect clearly the specific binding at high concentrations (>300 pM) of [^{3}H]-prazosin in binding experiments with RAO segments and membranes.

3 The abundance of receptor in the RTA and RAO (B_{max} mg^{-1} of total tissue protein), estimated using the tissue segment-binding approach (527 ± 14 fmol mg^{-1} for RTA; 138 ± 4 fmol mg^{-1} for RAO), was about 25-fold higher than values estimated using a conventional membrane-binding method (22 ± 5 fmol mg^{-1} for RTA; 5 ± 1 fmol mg^{-1} for RAO).

4 Binding competition experiments using intact tissue segments or membranes derived from RTA tissue yielded comparable data, indicating a coexistence of alpha-1A AR (high affinity for prazosin, KMD-3213 and WB4101 and low affinity for BMY 7378) and alpha-1B AR (high affinity for prazosin but low affinity for KMD-3213, WB4101 and BMY 7378).

5 In RAO tissue, careful evaluation of the tissue segment-binding assay revealed the coexpression of alpha-1B AR (high affinity for prazosin, but low affinity for KMD-3213 and BMY 7378) and alpha-1D AR (high affinity for prazosin and BMY 7378, but low affinity for KMD-3213), whereas the membrane-binding approach failed to detect these receptor subtypes with certainty.

6 The present study indicates that previous estimates of alpha-1 AR density and alpha-1 AR subtypes obtained by a conventional membrane-binding approach, as opposed to our improved tissue segment-binding assay, may have substantially underestimated the abundance of receptors present in arterial tissues, and may have failed to identify accurately the presence of receptor subtypes. Advantages and disadvantages of the tissue segment-binding approach are discussed.
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Keywords: Radioligand-binding experiment; intact tissue segment binding; membrane binding; [^{3}H]-prazosin; rat tail artery(RTA) and thoracic aorta(RAO)

Abbreviations: AR, adrenoceptor; BMY 7378, (8-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-8-azaspiro[4,5]decane-7,9-dione dihydrochloride; KMD-3213, (−)-(*R*)-1-(3-hydroxypropyl)-5-[2-[[2-[2-(2,2,2-trifluorpethoxy)phenoxy]ethyl]amino]propyl]indoline-7-carboxiamide; RAO, rat thoracic aorta; RTA, rat tail artery; WB4101, 2-([2,6-dimethoxyphenoxyethyl]aminomethyl)-1,4-benzodioxane

Introduction

The radioligand-binding method is one of the most important techniques in studying the biochemical identification and pharmacological characterization of many types of receptors (Fields *et al*., 1977; Yamamura *et al*., 1985; Bylund & Yamamura, 1990; Bylund & Toews, 1993). Most homogenate-binding studies have been done with microsomal membrane fractions prepared from tissue. However, there are some limitations in this method, such as limited amounts of tissue in small animals or small organs and a low yield of receptor-bearing membranes after fractionation (Colucci *et al*., 1981; Kwan *et al*., 1981; Faber *et al*., 2001). Originally, the radioligand-binding technique was applied to the intact strips of intestinal smooth muscle, in order to study the properties of acetylcholine receptors by Paton & Rang (1964). Morel & Godfraind (1989) thereafter developed this technique for intact rat brain microvessels to study the interaction of dihydropyridine drugs with calcium channels. Sayet *et al*. (1993) and Stassen *et al*. (1997; 1998) applied this method to quantitate alpha-1 adrenoceptors (ARs) of rat portal vein, aorta and mesenteric small arteries. These studies indicated that the tissue segment-binding technique could be applicable to study ligand binding not only for channels but also for receptors present in small tissues.

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Alpha-1 ARs are now classified into three subtypes, alpha-1A, -1B and -1D. These alpha-1 ARs are widely distributed in many tissues and many species (Hieble *et al*., 1995; Michel *et al*., 1995; Muramatsu *et al*., 1995; 1998; Graham *et al*., 1996). Among the three subtypes, the alpha-1D subtype, which is readily measured by a functional pharmacology approach, is difficult to detect using a radioligand-binding method with membrane fractions (Yang *et al*., 1997). In addition to the three subtypes, another alpha-1 AR (alpha-1L subtype) showing relatively low affinity for prazosin has been proposed in the functional studies with many tissues (Flavahan & Vanhoutte, 1986; Muramatsu *et al*., 1990; 1995; Oshita *et al*., 1993; Leonardi *et al*., 1997; Testa *et al*., 1997). However, the presence of alpha-1L AR is controversial because detection of this subtype is relatively difficult using the conventional membrane ligand-binding method (Hiraoka *et al*., 1999; Nakamura *et al*., 1999). Therefore, there is a discrepancy between the affinities obtained in membrane binding and functional approaches. We have speculated that intact tissue binding without homogenization may be a more useful method to detect the native states of receptors with less modification, as compared with the conventional membrane-binding method. The purpose of the present study was to establish the intact tissue segment-binding method and to compare it with a conventional membrane-binding method in the same tissues.

Several drugs specific for each alpha-1AR subtype have been developed. Prazosin is a classical alpha-1 AR antagonist which shows no selectivity for alpha-1A, -1B and -1D subtypes (Hancock, 1995; Hieble *et al*., 1995; Muramatsu *et al*., 1995; 1996). Therefore, [^{3}H]-labeled prazosin has been used for the detection of alpha-1ARs. KMD-3213 and BMY 7378 are well characterized as highly selective antagonists for alpha-1A and -1D subtypes, respectively (Goets *et al*., 1995; Saussy *et al*., 1996; Yamagishi *et al*., 1996; Murata *et al*., 1999). WB4101 shows high or intermediate affinity for alpha-1A and -1D subtypes (Michel *et al*., 1995; Muramatsu *et al*., 1998). In addition to these antagonists, catecholamines and phenylephrine have a relatively high affinity for the alpha-1D AR subtype (Lomasney *et al*., 1991; Piascik *et al*., 1994; Graham *et al*., 1996; Piascik & Perez, 2001).

In the present study, we examined alpha-1 ARs of the rat tail artery (RTA) and thoracic aorta (RAO) with an intact tissue segment-binding method. The results using this approach were compared directly with data obtained using a conventional ligand-binding method, employing microsomal membrane fractions.

Methods

Animals and tissue isolation

Male Wistar rats (Charles River Japan Inc.) weighing approximately 320 g (10–12 weeks old) were used. The rats were killed by exsanguination, and the thoracic aorta (RAO) and tail artery (RTA) were carefully isolated. Then, fat and connective tissues were removed from the arteries bathed in a modified Krebs–Henseleit solution (NaCl 120.7 mM, KCl 5.9 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 2 mM, $NaHPO_4$ 1.2 mM, $NaHCO_3$ 25.5 mM and glucose 11.5 mM, pH 7.4) gassed with 95% O_2 and 5% CO_2 at 4°C.

Tissue segment-binding experiments

Isolated RTA and RAO immersed in the gassed modified Krebs–Henseleit solution were cut along lengthwise and the endothelium was removed by gentle rubbing with filter paper in order to avoid the possible accumulation of [^{3}H]-prazosin. Then, the tissues were cut into small pieces under a stereoscopic microscope. Usually, 28 or more pieces of RTA (3–3.5 mm in length) and 24 pieces of RAO (3.0–3.5 mm square) were prepared from one rat (see Results). Each piece was incubated at 4°C in 1 ml of incubation buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl, pH 7.4) with [^{3}H]-prazosin for 10 h, unless otherwise mentioned elsewhere. After incubation, the pieces were gently blotted and rinsed during vortexing for 1 min with 2 ml incubation buffer at 4°C. The pieces were then blotted and solubilized in 0.3 N NaOH solution to estimate the radioactivity and protein content. The specific binding was determined by subtracting the radioactivity bound per mg protein in the presence of 30 μM phentolamine from the total radioactivity bound per mg protein, unless mentioned elsewhere. Experiments were done in duplicate at each concentration of radioligand for a binding-saturation experiment, or at each concentration of competing ligand for a binding-competition experiment. Radioactivity was measured by liquid scintillation counting using a water-miscible scintillation fluid (ULTIMA GOLD, Packard Bioscience, Groningen, Netherlands). The protein of tissue segment in each tube was measured using the method of Bradford (1976).

Membrane-binding experiments

RTA and RAO tissues were isolated from rats and stored at −80°C before use. These tissues were homogenized in 50 volumes by weight (V/W) of homogenization buffer (50 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, pH 7.4) containing proteinase inhibitors (complete EDTA-free proteinase inhibitor cocktail tablets, Roche, Mannheim, Germany), using a polytron homogenizer (specify setting 8, 10×60 s at 4°C). The tissue homogenate was subjected to centrifugation at $3000 \times g$ for 15 min at 4°C. The supernatant was filtered with four layers of gauze (type I) and then centrifuged at $80{,}000 \times g$ for 30 min at 4°C. The resulting pellet was resuspended in binding buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 100 mM NaCl, pH 7.4) and used for binding experiments. The membranes prepared from the RTA of 10 rats and RAO of 15 rats were used for one saturation or competition experiment. All these procedures were carried out at 4°C. The microsomal fractions obtained were incubated at 4°C in 1 ml of incubation buffer with [^{3}H]-prazosin for 4 h in the absence or presence of an excess of unlabelled competing ligand. Reactions were terminated by rapid filtration onto Whatman GF/C filters using a Brandel cell harvester, and filters were then washed rapidly with 3×5 ml aliquots of ice-cold washing buffer (50 mM Tris-HCl, pH 7.4). The resulting filters were dried and the trapped radioactivity was quantified by liquid scintillation counting. Nonspecific binding of [^{3}H]-prazosin was determined by measuring the radioactivity bound in the presence of 30 μM phentolamine. The protein contents of homogenates before centrifugation and of the microsomal fractions were determined by the method of Bradford (1976).

Table 1 pK_D B_{max} values estimated from [^{3}H]-prazosin-binding experiments with tissue segments or membranes of rat tail artery (RTA) and thoracic aorta of rats (RAO)

Tissue	*Affinity : pK_D*		*B_{max} fmol mg^{-1} total tissue protein*	
	Tissue segments	*Membranes*	*Tissue segments*	*Membranes*
RTA	9.5±0.1	9.3±0.3	527±14	22±6 (1100±248)
RAO	9.9±0.1	10.2±0.1	138±4	5±1 (245±2)

Mean±s.e.m. of five experiments except thoracic aorta membranes ($n=3$). ( ): B_{max} value per mg of microsomal membrane protein.

Data analysis

Binding data were analyzed using commercially available software (Graph Pad PRIZM®, Ver. 3.00, Graph Pad Software, San Diego, U.S.A.). Briefly, the data were first fitted to a one- and then two-site model, and if the residual sums of squares were statistically less for a two-site fit of the data than for a one-site, as determined by an F-test comparison, then the two-site model was accepted. *P*-values less than 0.05 were considered significant.

The abundance of the receptor was represented as the binding capacity mg^{-1} of total tissue protein (B_{max} mg^{-1} of total tissue protein), unless mentioned elsewhere. Thus, binding of fractionated membranes was corrected to the value per mg of the original homogenate protein. In this case, protein content in homogenates before fractionation was regarded as total tissue protein and the binding capacity of fractionated membranes was extrapolated by a yield of the membrane protein against the protein content of original homogenates (Table 1). Usually, 1 mg of membrane protein was obtained from approximately 50 mg of total tissue protein in both RTA and RAO.

Experimental values are given as the mean±s.e.m. Results were analyzed for statistical significance using the Student's *t*-test. A probability of less than 0.05 was considered significant.

Drugs

The following drugs were used in this study: (−)-phenylephrine HCl, methoxamine HCl, (−)-adrenaline bitartrate, prazosin HCl from Sigma (St Louis, U.S.A.), WB4101 and BMY 7378 from Research Biochemical Inc. (Natrick, U.S.A.). KMD-3213 was kindly provided from Kissei Pharmaceutical Co., Ltd (Matsumoto, Japan). [^{3}H]-Prazosin was purchased from Dupont-New England Nuclear Inc. (Boston, U.S.A.). The stock solutions of prazosin and KMD-3213 were prepared with ethanol and dimethylsulfoxide, respectively, and then diluted with distilled water in functional experiments and with binding buffer in binding experiment.

Results

[^{3}H]-prazosin binding to tissue segments of RTA and RAO

The potential segmental differences in binding capacity were first examined. Six small tissue pieces (3.0–3.5 mm in length) were prepared every 2 cm from a proximal portion of the RTA. The RAO, between aortic arch and diaphragm, was first divided into four parts, and then into six small pieces (approximately 3.0–3.5 mm square) were prepared from each part. Then, the small pieces of RTA and RAO were, respectively, incubated with 500 and 100 pM [^{3}H]-prazosin at 4°C for 10 h. Nonspecific binding was compared in the presence of lipophilic phentolamine (30 μM) and hydrophilic adrenaline (100 μM with 0.1 mg ml^{-1} ascorbic acid). As shown in Figure 1, the total binding capacity was not significantly different in proximal and intermediate portions (10 cm in length) of the RTA and in the entire RAO between the aortic arch and diaphragm. Nonspecific binding in the presence of phentolamine and adrenaline was the same in all of the areas of the RTA or RAO tissue tested. Subsequently, 28–32 pieces (3.0–3.5 mm in length) and 24 pieces (3.0–3.5 mm square) were, respectively, prepared from proximal and intermediate portions of the RTA, and the whole RAO was isolated from one rat and used in one saturation or binding competition experiment. Nonspecific binding was determined using 30 μM phentolamine.

Figure 2 shows the representative binding saturation curves obtained using RTA (Figure 2a) and RAO segments (Figure 2b). For the tail artery segments, [^{3}H]-prazosin at concentrations ranging from 30 to 2000 pM was used to label alpha-1ARs. The specific binding of [^{3}H]-prazosin was concentration-dependent, showing saturation at concentrations of about 1000 pM (Figure 2a). The specific binding was higher than the nonspecific binding at concentrations of [^{3}H]-

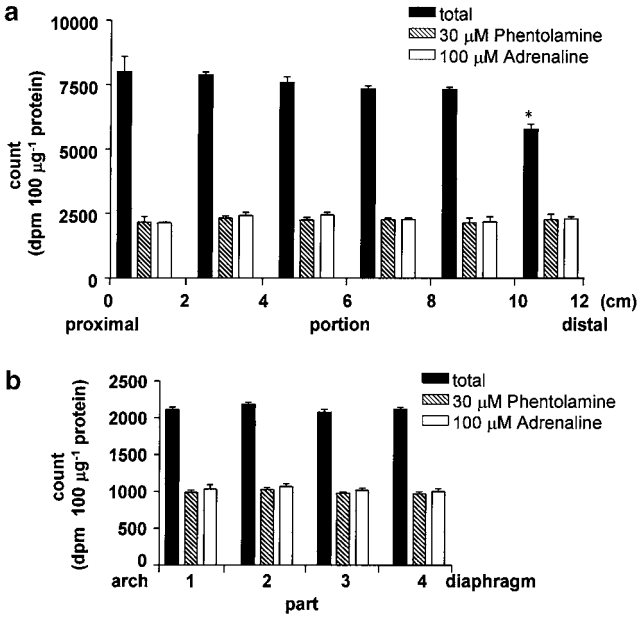

Figure 1 Binding of [^{3}H]-prazosin binding to sequential segments of RTA (a) and RAO tissues (b). The RTA was divided every 2 cm from the proximal portion and then six small pieces derived from each portion were incubated with 500 pM [^{3}H]-prazosin. The RAO between the aortic arch and diaphragm was cut into four parts and then six small segments prepared from each part were incubated with 100 nM [^{3}H]-prazosin. Total and nonspecific binding was measured in the absence (closed column) or presence of 30 μM phentolamine (shaded column) or 100 μM adrenaline (open column). Binding was determined in duplicate in each portion. Mean±s.e.m. of five independent experiments. *Significantly different from total binding (dpm 100 μg^{-1} protein) in other portions ($P<0.05$).

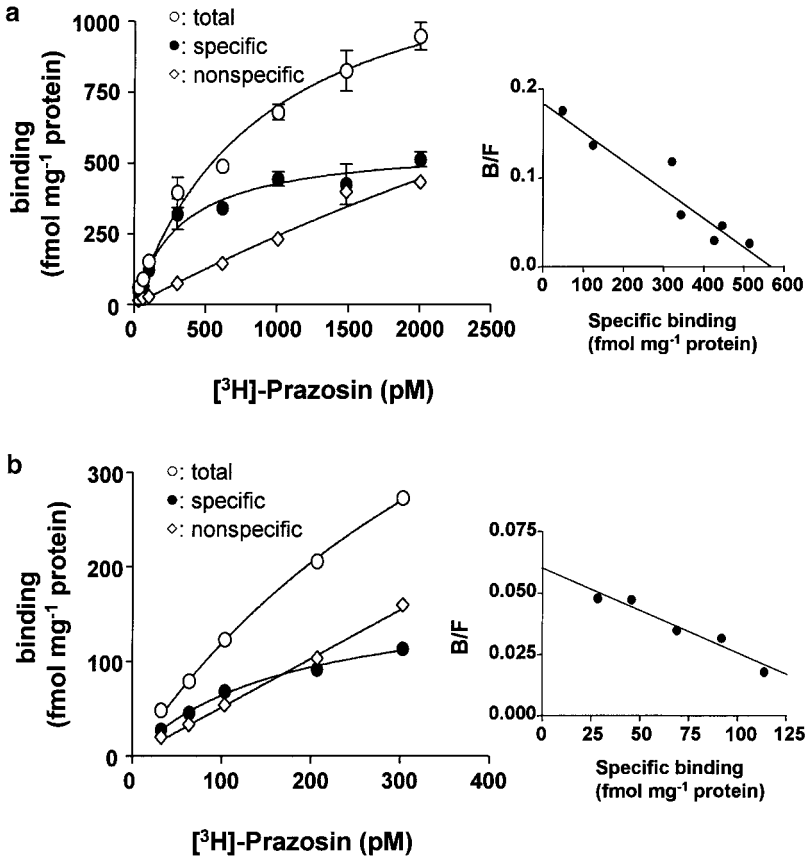

Figure 2 Representative results obtained from binding saturation experiments with [^{3}H]-prazosin in RTA segments (a) and RAO segments (b). Each point represents the mean of duplicate determinations. The right represents the Scatchard plot of the data. The experiments were replicated five times with similar results.

prazosin up to 1000 pM. Scatchard plot analysis (Scatchard, 1949) of the binding data for the RTA segments resulted in a straight line, suggesting a single class of binding sites for [^{3}H]-prazosin (Figure 2a right). The dissociation constant (pK_D) and maximal binding capacity (B_{max}) were: $pK_D = 9.5 \pm 0.1$ and $B_{max} = 527 \pm 14$ fmol mg^{-1} of total tissue protein ($n = 5$) (Table 1). In the RAO segments, [^{3}H]-prazosin also showed a concentration-dependent binding, but the nonspecific binding was significantly higher than the specific binding at concentrations higher than 300 pM (Figure 2b). Therefore, Scatchard analysis of the binding of [^{3}H]-prazosin to RAO segments was done for concentrations ranging from 30 to 300 pM: a pK_D of 9.9 ± 0.1 and a B_{max} of 138 ± 4 fmol mg^{-1} of total tissue protein were estimated ($n = 5$) (Table 1 and Figure 2b right).

The kinetics of binding of [^{3}H]-prazosin to RTA segments are shown for a representative experiment in Figure 3. Binding reached a plateau after 8 h (Figure 3a). A semilogarithmic plot of the time course of binding was linear, as expected for a pseudo-first-order reaction (Weiland & Molinoff, 1981), resulting in $K_{on} = 0.048 \pm 0.006$ nM^{-1} min^{-1} ($n = 4$). The binding of [^{3}H]-prazosin was reversible, as observed by the addition of 30 μM phentolamine to the binding reaction after [^{3}H]-prazosin binding was at equilibrium (Figure 3b). The dissociation kinetics were consistent with a first-order reaction ($K_{off} = 0.078 \pm 0.013$ min^{-1}, $n = 3$). Therefore, the dissociation constant (pK_D) calculated from the kinetic data was 9.3, which was in good agreement with the pK_D obtained from binding saturation experiments (Table 1). The same results were observed in the kinetic studies with RAO, with a pK_D of 9.2 being estimated from the kinetic studies.

The pharmacological profiles of [^{3}H]-prazosin-binding sites were examined in binding competition experiments with several drugs, as summarized in Table 2. All drugs tested inhibited the specific binding of [^{3}H]-prazosin in a concentration-dependent manner. Figure 4 shows the representative competition curves for KMD-3213 and BMY 7378. In the RTA segments, KMD-3213 biphasically inhibited 500 pM [^{3}H]-prazosin binding. The proportion of high-affinity sites ($pK_i = 9.7 \pm 0.1$) was approximately 60% (Table 2). WB4101 also competed for binding with a shallow inhibition curve. Prazosin showed a high-affinity binding site ($pK_i = 9.4 \pm 0.1$), but BMY 7378, phenylephrine and methoxamine showed low affinities in the RTA segments. These results suggest the coexistence of alpha-1A and -1B AR subtypes in the RTA, with high and low affinities for both KMD-3213 and WB4101.

On the other hand, in the RAO segments, KMD-3213 competed for [^{3}H]-prazosin binding monotonically with a low affinity ($pK_i = 7.1 \pm 0.4$), while the competition of BMY 7378 was biphasic ($pK_i = 8.9 \pm 0.2$ and 6.4 ± 0.2) (Figure 4b). WB4101 competed for binding with an intermediate affinity. The affinity for phenylephrine, but not methoxamine, was approximately 300 times higher in the RAO than in the RTA (Table 2). These results indicate the presence of alpha-1D and -1B AR subtypes in RAO.

[^{3}H]-prazosin binding to membranes prepared from RTA and RAO

The microsomal membrane fractions of RTA isolated from 10 rats or of RAO isolated from 15 rats were used in one binding saturation or binding competition experiment, respectively. The RTA membranes bound [^{3}H]-prazosin with a high affinity ($pK_D = 9.3 \pm 0.3$). The specific binding was approximately 90% of total binding at 200 pM [^{3}H]-prazosin (Figure 5a). The density of binding sites estimated by Scatchard analysis of the data was 1100 ± 248 fmol mg^{-1} protein for the microsomal fraction, which was calculated to be 22 ± 5 fmol mg^{-1} of total tissue protein (that is, the homogenate protein before fractionation; see Methods) (Table 1). KMD-3213 showed a shallow binding competition curve ($pK_i = 10.7 \pm 0.2$ and 8.2 ± 0.2) (Figure 5b). The proportion (56%) of high-affinity

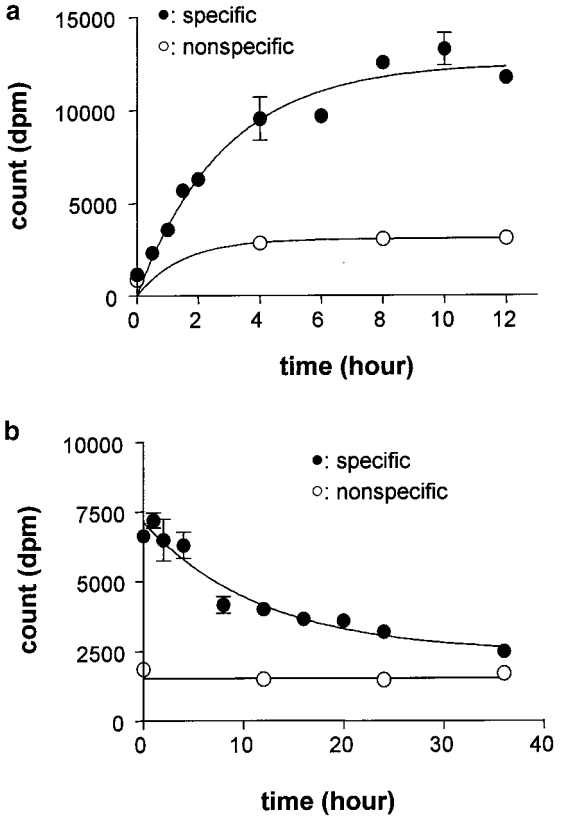

Figure 3 Association and dissociation kinetics of 500 pM [^{3}H]-prazosin binding to RTA segments. Association (a) was started by addition of 500 pM of [^{3}H]-prazosin and the reaction was stopped by washing the segments after different incubation periods. The dissociation rate (b) was determined by addition of 30 μM of phentolamine after a 12 h pre-equilibration with [^{3}H]-prazosin. Each point represents the mean of duplicate determinations. The experiments were replicated three times with similar results.

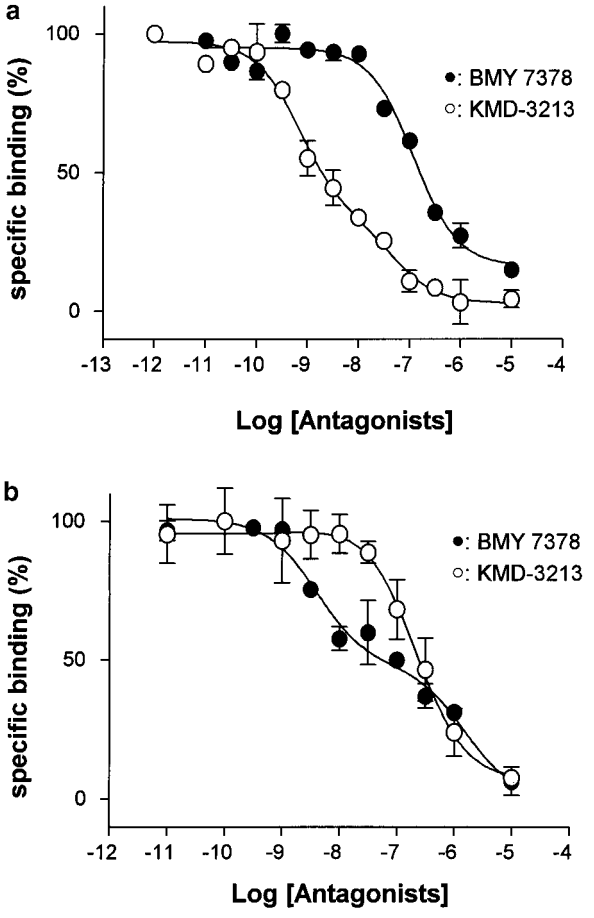

Figure 4 Competition curves for KMD-3213 and BMY 7378 in RTA segments (a) and RAO segments (b). [^{3}H]-prazosin at 500 or 100 pM was used in RTA and RAO segments, respectively. Each point represents mean $\pm$ s.e.m. of five independent experiments.

Table 2 Binding affinities for various drugs estimated in tissue segment- or membrane-binding experiments with rat tail artery (RTA) and thoracic aorta (RAO)

Drug	*RTA*				*RAO*			
	Tissue segments		*Membrane*		*Tissue segments*		*Membrane*	
	pK_i high	*pK_i low*	*pK_i high*	*pK_i low*	*pK_i high*	*pK_i low*	*pK_i high*	*pK_i low*
KMD-3213	9.7 ± 0.1 ($61 \pm 2\%$)	7.5 ± 0.1	10.7 ± 0.2 ($56 \pm 2\%$)	8.2 ± 0.2	7.1 ± 0.4			ND
BMY 7378	6.4 ± 0.1		6.7 ± 0.2		8.9 ± 0.2 ($48 \pm 2\%$)	6.4 ± 0.2	8.6 (33%)	6.1
Prazosin	9.4 ± 0.1			ND	9.9 ± 0.1			ND
WB4101	9.4 ± 0.3 ($63 \pm 5\%$)	7.4 ± 0.1		ND	8.7 ± 0.5			ND
Phenylephrine	4.3 ± 0.2			ND	6.8 ± 0.1			ND
Methoxamine	4.6 ± 0.2			ND	4.6 ± 0.4			ND

Mean $\pm$ s.e.m. of five experiments except thoracic aorta membrane ($n = 1$). ( ): Proportion of high-affinity sites. ND: not determined.

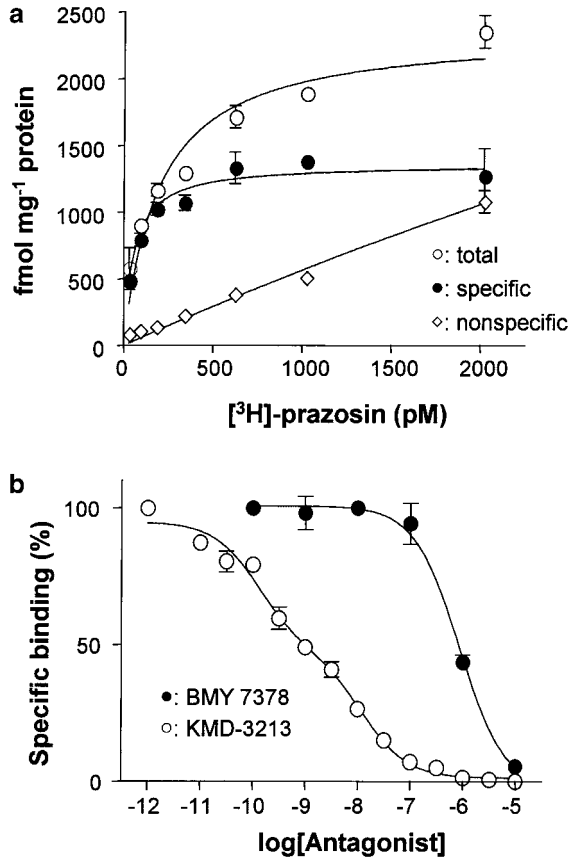

Figure 5 Representative results of a binding saturation experiment (a) and a binding competition experiment (b) with RTA membranes. Each point represents the mean of duplicate determinations. The experiments were replicated five times with similar results.

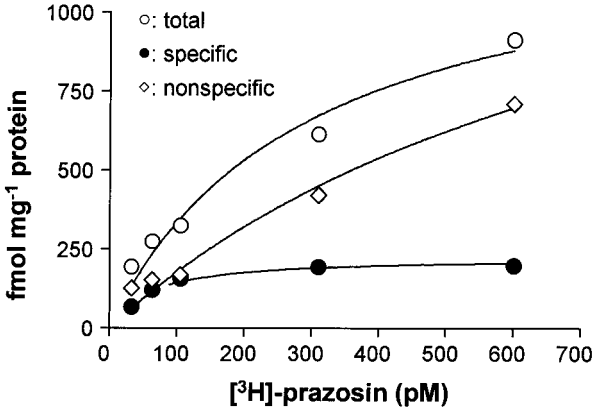

Figure 6 Representative results of a binding saturation experiment with RAO membranes. Each point represents the mean of duplicate determinations. The experiments were replicated with similar results in three experiments.

sites was in agreement with that (61%) estimated from binding data obtained using tail artery segments. BMY 7378 showed a low affinity. Competition experiments with prazosin, WB4101, phenylephrine and methoxamine were not done, because of the limited amount of membranes available.

In binding experiments using the RAO membranes, however, the proportion of nonspecific binding was extremely high, and thus the specific binding of [^{3}H]-prazosin was not clearly detected in two out of five experiments. Even in the remaining three experiments, the specific binding was approximately 20% of total binding at 100 pM [^{3}H]-prazosin (Figure 6). Careful analysis of the binding data yielded a pK_D of 10.2 ± 0.1 from three saturation experiments. The maximum density of binding sites was estimated to be 245 ± 2 fmol mg^{-1} membrane protein, which corresponded to 5 ± 1 fmol mg^{-1} of total tissue protein. The binding of [^{3}H]-prazosin was biphasically inhibited by BMY 7378 ($pK_i = 8.6$ and 6.1) in a binding competition experiment. In other competition experiments, concentration-dependent competition could not be clearly observed.

Discussion

The tissue segment-binding approach has been used in a limited way to date, and has been used successfully to characterize the alpha-1 ARs of rat portal vein (Sayet *et al*., 1993) and RAO and rat mesenteric artery (Stassen *et al*., 1997; 1998). However, a detailed comparison between the tissue segment method and a 'classical' ligand-binding method using 'microsomal' membranes derived from the same tissue has yet to be described in any depth. In the present study, we compared both binding methods directly using preparations of RTA and RAO.

At first, we validated the characteristics of [^{3}H]-prazosin binding to tissue segments from both RTA and RAO. The binding of [^{3}H]-prazosin to RTA and RAO segments was time-dependent, reversible and equivalent along the length of the artery. Appreciable amounts of receptor-specific ligand binding were estimated using phentolamine as an AR probe to determine 'nonspecific' binding. The dissociation constants calculated from the kinetic data (on and off rates) were in good agreement with those obtained from equilibrium binding measurements in both tissues studied. Furthermore, the binding affinities estimated by the tissue segment approach for a number of alpha-1 AR ligands were in close accord with the values obtained using the membrane-binding method. These results show that the [^{3}H]-prazosin-binding sites of RTA and RAO detected in tissue segment-binding method have pharmacological profiles of alpha-1 ARs, supporting the previous reports using a tissue segment-binding method (Sayet *et al*., 1993; Stassen *et al*., 1997).

However, some differences were observed between tissue segment- and membrane-binding methods. In the tissue segment-binding experiments, we used whole tissue without homogenization. Thus, the protein denominator used to calculate the binding density of the segments refers to their total protein, including not only plasma membrane but also all intracellular proteins and associated connective tissues. In contrast, the value for the protein denominator used in the membrane-binding method refers only to the microsomal fraction after homogenization and fractionation. Therefore, in order to compare the receptor-binding capacities (B_{max} mg^{-1} protein) between the two methods at the same level, the value taken as the protein 'denominator' must be adjusted to the level of intact tissue, that is, total tissue protein. We had measured the (total tissue) protein in homogenates before fractionation and adjusted the binding capacity of fractionated membranes to B_{max} mg^{-1} of the total tissue protein (see

Methods). As a result, even though the binding capacity of membranes after fractionation (for example, 1100 fmol mg^{-1} protein of RTA membranes) was two-fold higher than that per tissue segment protein (527 fmol mg^{-1} protein of RTA segment), the 'corrected' binding capacity of the microsomal membranes (22 fmol mg^{-1} protein in RTA homogenates) was reduced to less than 5% of the B_{max} value obtained from tissue segment-binding method (Table 1). This result is surprising, indicating that there is a large difference in the binding capacity estimated by the two binding methods. In agreement with this large difference, RAO and RTA segments obtained from a single rat (24 and 28 or more pieces, respectively) proved to be sufficient to perform an entire saturation- or competition-binding experiment using the tissue segment approach, while the conventional membrane-binding method required the tissues harvest from 10 or more rats to provide for a single binding saturation or competition experiment. Faber *et al*. (2001) also reported that approximately 70 rats were required to harvest sufficient membranes for binding studies with RAO.

Why might there be such a large difference in binding capacity caused between the two distinct binding methods? At least two possibilities can be considered. One possibility is that there may be an overestimate of 'specific' binding using the tissue segment-binding method. A second possibility is that there may be a substantial loss of receptor due to a low yield of receptor-bearing membranes from intact tissue during the microsomal membrane isolation procedure used for the 'classical' membrane ligand-binding method.

Recently, [^{3}H]-prazosin has been shown to penetrate the plasma membrane and bind to intracellular components (the binding sites which might also represent intracellular receptors: Cogé *et al*., 1999; McGrath *et al*., 1999; Mackenzie *et al*., 2000). Therefore, a large amount of binding capacity in tissue segments might possibly reflect the intracellular binding of [^{3}H]-prazosin. However, [^{3}H]-prazosin binding to tissue segments at 4°C was inhibited to the same level by the various drugs including not only a lipophilic ligand, phentolamine, but also a hydrophilic ligand, adrenaline (Cogé *et al*., 1999). Furthermore, if intracellular binding sites were significant, the sites would also be present in membrane fractions isolated by differential centrifugation. Therefore, it is likely that the large binding capacity estimated with the tissue segment-binding method under the present experimental conditions reflects specific binding to receptors present in the plasma membrane, rather than specific intracellular binding or nonspecific intra- and extracellular accumulation. Thus, the present results strongly suggest that homogenization and fractionation may cause a substantial loss in the yield of receptor-bearing membranes. Such a loss has been suggested to be especially important for small tissues (Colucci *et al*., 1981; Kwan *et al*., 1981; Faber *et al*., 2001).

In the present study, two different arterial preparations (RTA and RAO) were tested. The proportion of nonspecific binding sites was found to be significantly greater in the RAO tissue, compared with the RTA preparation, regardless of the binding method used. A comparable high proportion of nonspecific binding of [^{3}H]-prazosin has been observed in the tissue binding of RAO (Stassen *et al*., 1997), rabbit ear artery (Hiraizumi *et al*., 2003) and rabbit thoracic aorta (unpublished observations), but not in rat portal vein (Sayet *et al*., 1993) and rat mesenteric artery (Stassen *et al*., 1997). This difference could be due to different proportions of smooth muscle and connective tissue in the different vascular preparations. High amounts of nonspecific binding would interfere with the detection of ligand-specific binding sites. A loss of receptor yield due to homogenization and fractionation would further compromise the ability to detect specific binding, as shown in RAO tissue.

Competition experiments with the tissue segment-binding method clearly showed the coexistence of two distinct alpha-1 AR subtypes. In RTA, the pharmacological profiles of the alpha-1A subtype with high affinity for prazosin, KMD-3213, WB4101, and of the alpha-1B subtype with a low affinity for BMY 7378 and KMD-3213 were consistent with data obtained using the membrane-binding method. However, in the RAO, the alpha-1D subtype showing high affinity for BMY 7378 and alpha-1B subtype were clearly detected using the tissue segment binding, but not the membrane-binding method. This result suggests that the tissue segment-binding method used in the present study is useful to identify receptor subtypes that coexist in the plasma membrane of the same tissue. Although the contractile response to noradrenaline or phenylephrine is mainly mediated through the alpha-1A subtype in the RTA (Lachnit *et al*., 1997; Murata *et al*., 1999; Gisbert *et al*., 2003), involvement of an additional subtype (probably alpha-1B AR from the present study) has been suggested (Lachnit *et al*., 1997). In the RAO, alpha-1D AR is a major subtype producing noradrenaline-induced contraction (Burt *et al*., 1995; Muramatsu *et al*., 1995; 1998; Buckner *et al*., 1996; Saussy *et al*., 1996), but Muramatsu *et al*. (1998) reported alpha-1B AR mediation in oxymetazoline-induced contraction. In the mouse aorta and tail artery, Daly *et al*. (2002) showed by using the alpha-1B AR knockout that alpha-1B ARs make a small contribution to the contractile effect of phenylephrine, in addition to the major response *via* alpha-1D ARs and alpha-1A ARs, respectively. The present work provides direct evidence to support these pharmacological data. In addition to vascular contraction, alpha-1 ARs are now known to be involved in many physiological responses, such as cell growth and hypertrophy (Michelotti *et al*., 2000). Such physiological significance of coexisted alpha-1 AR subtypes would be unmasked by subtype-selective agonists and antagonists (Muramatsu *et al*., 1998).

Finally, the advantages and disadvantages of the tissue segment-binding method can be summarized, in comparison with the conventional membrane-binding method. The most important advantage is to avoid the low yield of receptor resulting from the homogenization and fractionation procedure, as mentioned above. Therefore, the tissue segment-binding method would be of particular value for dealing with limited amounts of tissue and/or small animals such as knockout mice. Another significant merit of tissue segment binding is the ability to detect receptors in a more physiological state, without changing the receptor environment. This approach may shed light on discrepancies observed between binding and functional studies. As mentioned in the introduction, it has been reported that adrenergic responses of several tissues are caused *via* a putative alpha-1L AR (Flavahan & Vanhoutte, 1986; Muramatsu *et al*., 1990; 1995; Leonardi *et al*., 1997; Testa *et al*., 1997). However, some binding data are not necessarily supportive of the bioassay data (Hiraoka *et al*., 1999; Nakamura *et al*., 1999). In preliminary work, we used the tissue segment-binding method to detect the putative

alpha-1L AR in several tissues, confirming a good relationship between binding and functional affinities (Hiraizumi *et al*., 2003; unpublished observations). On the other hand, the tissue segment-binding method may not be applicable for all vascular tissues. As mentioned above, a high amount of nonspecific binding of the radioligand to connective tissues would mask specific binding. In the RAO segments, nonspecific binding of [^{3}H]-prazosin was so high even at moderate concentrations (>300 pM) that specific binding could only be detected at lower concentrations of [^{3}H]-prazosin (Figure 2b in the present study; Stassen *et al*., 1997). Secondly, tissue uniformity is required to avoid segmental differences. Preliminary experiments must be done to rule out segmental tissue differences, as shown in Figure 1. Furthermore, binding to tissue segments reflects the binding to all types of cells and connective tissues included in the segment. Recently, Faber *et al*. (2001) reported that alpha-1 ARs distribute in not only the smooth muscle cells but also adventitial fibroblasts of RAO tissue. Thus, binding to tissue segments as well as fractionated membranes does not reflect the selective binding to any specific cell type in the tissue. In addition, the tissue-binding method requires the complexity of measuring the protein content of all tissue segments to normalize the binding data between incubation tubes.

In conclusion, the present study strongly suggests that the abundance of alpha-1 ARs has been considerably underestimated by the classical binding method using isolated membranes. Thus, in spite of some disadvantages, it appears that the tissue segment-binding method is of considerable utility to identify alpha-1 ARs in small tissue samples by minimizing the loss of receptor upon membrane isolation and by avoiding a modification of the receptor environment. Further studies are warranted to clarify whether this method is applicable to other types of receptors or to ARs present in other tissues.

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