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HETEROGENEOUS PROPERTIES OF ALPHA$_2$ ADRENOCEPTORS IN PARTICULATE AND SOLUBLE PREPARATIONS OF HUMAN PLATELET AND RAT AND RABBIT KIDNEY

Y.-D. CHEUNG,* D. B. BARNETT† and S. R. NAHORSKI
Department of Pharmacology and Therapeutics, University of Leicester, Leicester, U.K.

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Abstract—Alpha$_2$ adrenoceptors of the human platelet and rat and rabbit renal cortex were compared in binding studies using the selective antagonist ligand [^{3}H]rauwolscine. Significant differences in the pharmacological characteristics of the alpha$_2$ adrenoceptor were observed between the tissues with reference to both absolute drug affinities as well as rank order of drug potency. Two subsets of the alpha$_2$ adrenoceptor sites could be described: one exhibiting equal affinity for the alpha$_2$ selective diastereoisomers, yohimbine and rauwolscine, and low affinity for the alpha$_1$ antagonist prazosin (human platelet); the other displaying significantly higher affinity for rauwolscine than yohimbine but also relatively high affinity for prazosin (rat and rabbit kidney). Digitonin solubilised alpha$_2$ adrenoceptors from these tissues identified by [^{3}H]rauwolscine generally displayed reduced drug affinities. This was most apparent for agonists (10–50-fold lower) indicating separation of the soluble receptors from the guanine nucleotide binding proteins. However, the solubilised alpha$_2$ adrenoceptors retained the overall pharmacological properties of their respective membrane receptors, including rank order of drug potency, reflecting similar inter-tissue differences. These results suggest that the pharmacological differences in alpha$_2$ adrenoceptors observed are not species specific and are not related to variation in receptor effector coupling mechanisms or problems of ligand access due to membrane constraints. This may reflect true intrinsic differences in the molecular structure of these receptors.

In recent years, the selective alpha$_2$ antagonist ligands, [^{3}H]yohimbine and [^{3}H]rauwolscine, have been used as the major probes for alpha$_2$ adrenoceptor sites in a variety of mammalian tissues. In a previous study, we used these two ligands to characterise alpha$_2$ adrenoceptors in the human platelet and rat cerebral cortex [1]. In either tissue, both ligands labelled pharmacologically identical sites that could be classified as the alpha$_2$ sub-type. However, there were significant differences between the alpha$_2$ adrenoceptor sites of the human platelet and rat brain, not only with respect to absolute drug affinities, but also to the overall rank order of drug potency. For example, significantly lower affinities for yohimbine and rauwolscine and relatively high affinity for prazosin were observed in the rat cerebral cortex, compared to human platelet. The possibility was raised that heterogeneity of the mammalian alpha$_2$ adrenoceptors should be considered [1].

Species differences seemed apparent when, in a subsequent study, the rat renal cortical alpha$_2$ adrenoceptor sites were found to possess pharmacological properties similar to those in the rat cerebral cortex [2] whereas those in human cerebral cortex closely resembled those in human platelet [3]. Pharmacological differences in alpha$_2$ adrenoceptors have also been reported between rodent and porcine species [4] and more recently, Kawahara and Bylund [5] have examined the characteristics of alpha$_2$ adrenoceptors solubilised from human platelet and rat cerebral cortex and show that the pharmacological differences persist in the solubilised preparation.

In this study we have compared the pharmacological characteristics of alpha$_2$ adrenoceptors labelled with [^{3}H]rauwolscine in human platelet and rat and rabbit kidney both on membranes and in the soluble form free of membrane constraints. Our findings indicate that the solubilised receptors maintain the differential pharmacological behaviour of the respective particulate receptor sites. These results, therefore, provide additional evidence for a structural heterogeneity of mammalian alpha$_2$ adrenoceptors.

MATERIALS AND METHODS

Human platelet membranes were prepared as previously described [1]. Rat and rabbit kidneys were excised from freshly killed animals and the renal cortex was dissected. Tissue was chopped into small pieces before being homogenised in 20 vol ice-cold Tris–HCl 5 mM, EDTA 5 mM, pH 7.5, using an Ultra-Turrax homogeniser. The homogenate was fil-

* Present address: Faculty of Medicine, The Health Sciences Centre, Memorial University of Newfoundland, St John's, Newfoundland, Canada A1B 3V6.

† Address correspondence to: Professor D. B. Barnett, Department of Clinical Pharmacology, Clinical Sciences Building, Leicester Royal Infirmary, Leicester LE2 7LX, U.K.

tered through double-layer cheesecloth. The filtrate was centrifuged at 27,000 *g* for 15 min at 4° and the resultant pellet was washed once more in the same buffer by resuspension and centrifugation. The pellet was then washed in ice-cold Tris–HCl 50 mM, EDTA 0.5 mM, pH 7.5, by homogenisation, followed by centrifugation at 27,000 *g* for 10 min at 4°. The final pellet was resuspended in appropriate volume of assay buffer (Tris–HCl 50 mM, EDTA 0.5 mM, 0.1% ascorbic acid, pH 7.5) and used directly in binding assays or for solubilisation.

Solubilisation of alpha$_2$ receptors. Membranes were resuspended in assay buffer at a protein concentration of 6–25 mg/ml (approximately 6–8 mg/ml for human platelet, 16–20 mg/ml for rat kidney and 20–24 mg/ml for rabbit kidney). Aliquots of the membrane suspension were then added to an equal volume of buffer containing digitonin 10–26 mM and NaCl 0.2–0.4 M (final concentrations for digitonin and NaCl, respectively, were 5 mM and 0.2 M for platelet, 10 mM and 0.1 M for rat kidney and 13 mM and 0.1 M for rabbit kidney). The mixture was thoroughly shaken and left for 15 min on ice with occasional agitation. Subsequently, the mixture was homogenised for 1 min with an Ultra-Turrax homogeniser. The homogenate was then centrifuged at 4° for 1 hr at 50,000 *g*. The supernatant, representing the solubilised receptor preparation was carefully harvested and kept chilled on ice until used for assays. The concentrations of digitonin and NaCl used for each tissue were determined from preliminary studies in which optimal solubilising conditions were evaluated.

Radioligand binding assays. The binding assays with membrane preparations were performed as previously described [1, 2]. Assays of solubilised alpha$_2$ receptors were carried out at room temperature (22–25°) in a total volume of 250 μl assay buffer containing 50 μl [^{3}H]rauwolscine and 100 μl soluble receptor protein (2–9 mg/ml). Final assay concentration of NaCl was adjusted to 80 mM in all samples. Incubations were initiated upon the addition of solubilised receptor and continued for 40 min. Reaction was stopped by addition of 0.25 ml ice-cold bovine gamma globulin (0.5 mg/0.25 ml) followed by 1 ml of 15% ice-cold polyethylene glycol (PEG) (mol. wt. 6000–8000) in assay buffer. The contents were quickly and thoroughly mixed and left in the cold for 10–15 min before being filtered on GF/B filters under vacuum. Filters were rapidly washed with ice-cold 10% PEG (3 × 5 ml) and then allowed to dry. The precipitate collected on the filters was counted for radioactivity in a liquid scintillation counter at an efficiency of 45%. Specific binding was assessed in parallel incubations as binding of [^{3}H]rauwolscine in the presence of 5 μM phentolamine and represented approximately 70% of total binding at a ligand concentration of 4–6 nM. Protein was assayed by the method of Lowry *et al.* [6].

Data analysis. Binding curves were analysed using computer-assisted curve fitting [7, 8]. Data was analysed by unpaired Student's *t*-test to determine significance of difference between group means. Data is expressed as mean values ± SEM.

Materials. [^{3}H]Rauwolscine (specific activity 80–90 Ci/mmol) was purchased from New England Nuclear, Boston, MA.; adrenaline, noradrenaline, isoprenaline and dopamine were purchased from Sigma, St. Louis, MO.; corynanthine and rauwolscine were purchased from Carl Roth; and yohimbine was from Medochem. Phentolamine was a gift from Ciba Geigy and prazosin from Pfizer (Sandwich, U.K.).

RESULTS

Characteristics of alpha$_2$ adrenoceptors in particulate preparations

The binding of [^{3}H]rauwolscine in membranes of all three tissues was saturable with Scatchard analysis producing a single straight line, consistent with binding to a single class of non-interacting sites (Fig. 1). However, some differences in the affinity (K_d) of [^{3}H]rauwolscine for the binding sites in the different tissues were seen (Table 1) with human platelet exhibiting an approximately five-fold higher K_d than rabbit renal cortex. Binding was rapid (reaching equilibrium in 45 min at room temperature) and readily reversible. Analysis of the kinetics of the binding revealed a kinetically derived K_d of the same order as that indicated by Scatchard analysis (Table 2).

The binding of [^{3}H]rauwolscine in all three tissues showed the overall pharmacological characteristics of the alpha$_2$ adrenoceptor as indicated by the hierarchical potencies of adrenergic agents assessed in displacement experiments (Figs. 3–5 and Table 3). Thus the affinity of corynanthine (alpha$_1$ selective) for the binding sites was approximately two orders of

Table 1. Results of saturation analysis of [^{3}H]rauwolscine binding to alpha$_2$ adrenoceptors in particulate and solubilised preparations of human platelet and rat and rabbit renal cortex*

	Human platelet	Rat renal cortex	Rabbit renal cortex
Membrane preparation			
B_{max} (fmol/mg/protein)	144 ± 5	120 ± 10.2	76 ± 6.3
K_d (nM)	0.6 ± 0.1	2.7 ± 0.3	5.6 ± 0.4
Solubilised receptors			
B_{max} (fmol/mg/protein)	177 ± 20.6	63 ± 5.2	34 ± 5.3
K_d (nM)	4.6 ± 0.5	6.4 ± 1.1	7.1 ± 1.4

* Results presented are means ± SEM of three or four separate experiments performed in duplicate. Data analysed by Scatchard plots.

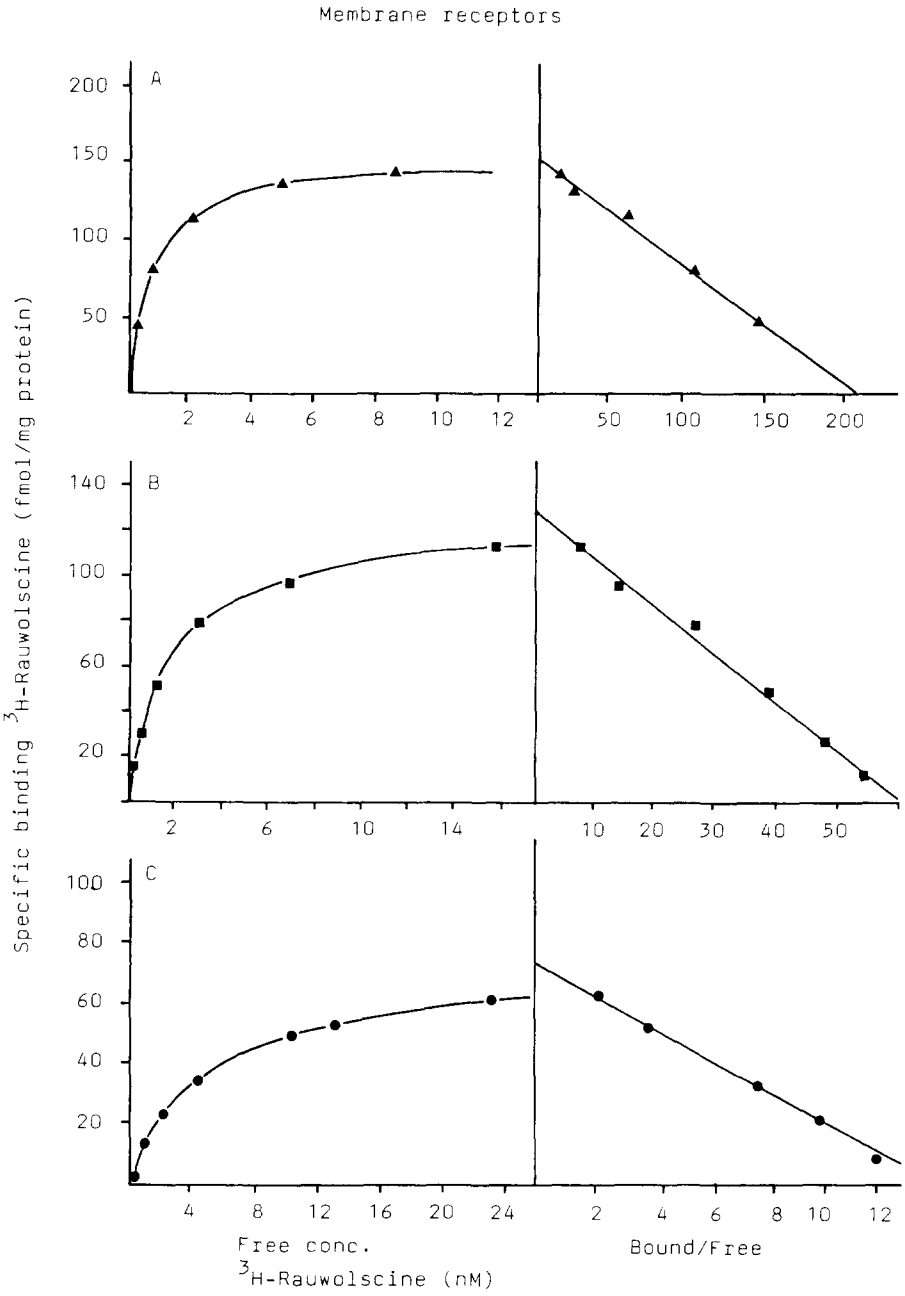

Fig. 1. Specific binding of [^{3}H]rauwolscine to membrane particulate preparations of (A) human platelet (B) rat renal cortex (C) rabbit renal cortex. Data presented is from representative experiments of saturation binding isotherms and Scatchard analysis. Each point is the mean of duplicate assay values. Mean B_{max} and K_d values (3–5 experiments) are shown in Table 1.

magnitude lower than its diastereoisomer yohimbine (alpha$_2$ selective) in all cases. However, as we have previously shown [1] considerable differences in the absolute affinities of some agents were apparent between the tissues studied. Thus the K_i values for rauwolscine and yohimbine are very similar in human platelet in the region of 0.5–1.0 nM, whereas in rat and rabbit kidney, yohimbine is 2–3-fold weaker than rauwolscine and the absolute affinities are in the region of 4–12 nM (Table 3). Further evidence of this inter-tissue difference was seen in the affinity of the alpha$_1$ selective agent prazosin. Thus in human platelet, prazosin is considerably weaker than in either of the other tissues (Table 3). These differences are particularly well seen if the hierarchy of potencies of the various agents are compared. Thus for human platelet (Fig. 3) rauwolscine = yohimbine > corynanthine > prazosin whereas in rat and rabbit

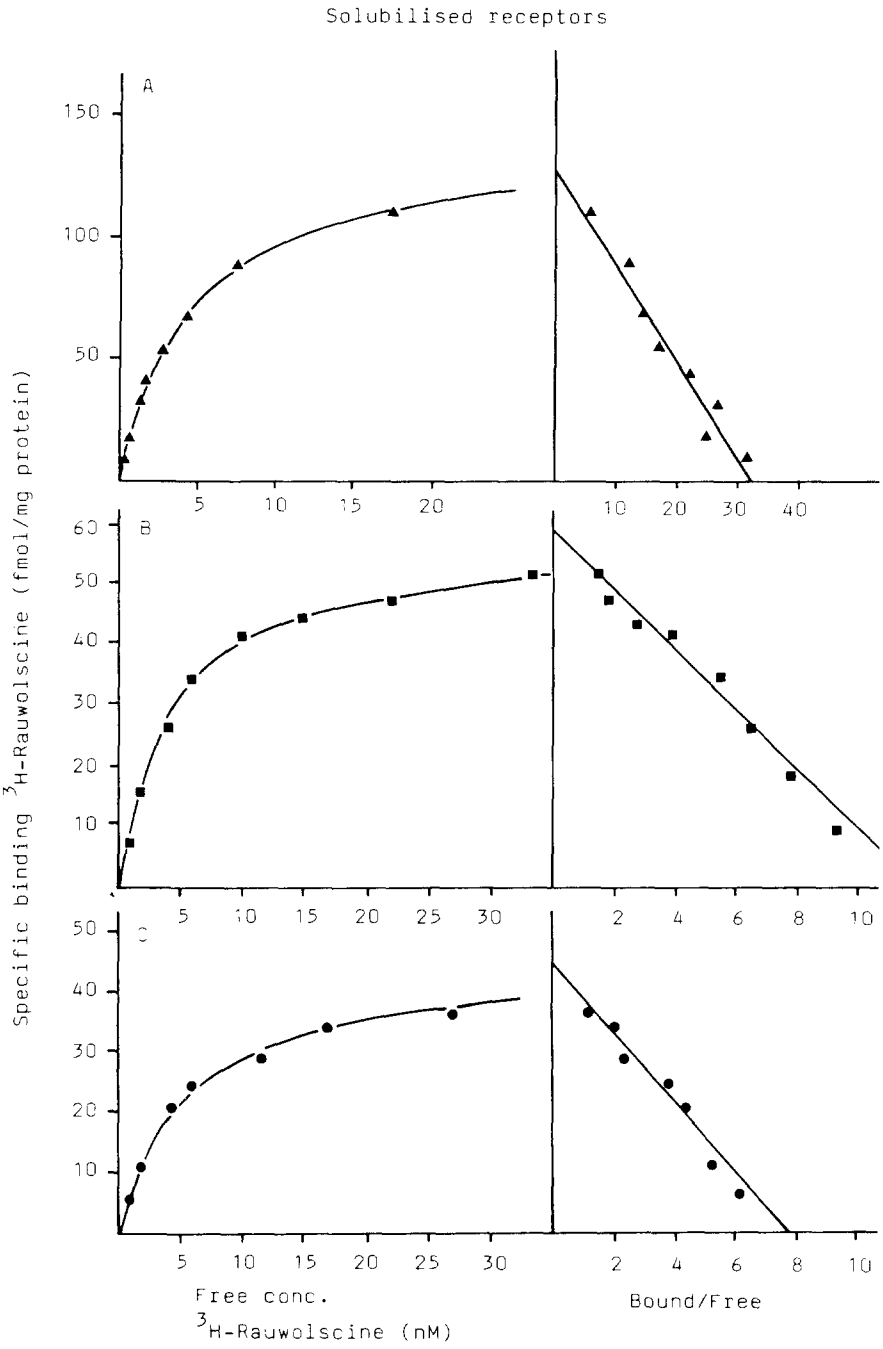

Fig. 2. Specific binding of [^{3}H]rauwolscine to soluble receptor preparations of (A) human platelet (B) rat renal cortex (C) rabbit renal cortex. Data presented is from representative experiments of saturation binding isotherms and Scatchard analysis. Each point is the mean of duplicate assay values. Mean B_{max} and K_d values (3–5 experiments) are shown in Table 1.

kidney (Figs. 4, 5) rauwolscine > yohimbine > prazosin > corynanthine. In all situations the slope factors of the antagonist displacement curves were close to unity indicating interaction at a single homogeneous class of binding sites.

Characteristics of soluble alpha$_2$ adrenoceptors

Alpha$_2$ adrenoceptors were effectively solubilised from the three tissues examined. Receptor yields determined by comparing specific binding of fixed ligand concentrations to total starting membranes and soluble products were in the region of 90% for human platelet and 40% for rat and rabbit kidney. The solubilised receptor preparation satisfied the criteria defining the soluble nature of the receptor. Thus without the addition of PEG specific

Table 2. Kinetic analysis* of [^{3}H]rauwolscine binding to alpha$_2$ adrenoceptors in particulate and solubilised preparations of human platelet and rat and rabbit renal cortex

	k_1 (1/nM/min)		k_2 (1/min)		$k_2/k_1 = K_d$ (nM)	
	Membrane	Soluble	Membrane	Soluble	Membrane	Soluble
Platelet	0.073	0.011	0.03	0.03	0.4	3.0
Rat kidney	0.04	0.018	0.082	0.04	2.0	2.2
Rabbit kidney	0.02	0.015	0.06	0.047	3.0	3.1

* Data derived from association/dissociation experiments. In these experiments groups of tubes were set up containing a fixed quantity of [^{3}H]rauwolscine (4 nM), incubation initiated by addition of membranes or soluble receptor preparation and finally terminated at various times by filtration. Other incubations were allowed to proceed well into equilibrium (40–50 min) and then after addition of an excess of phentolamine (5 μM) dissociation allowed to proceed for various times up to 1 hr before termination by filtration. Kinetic parameters were then calculated graphically from the rates of association and dissociation as described by Williams and Lefkowitz [14].

[^{3}H]rauwolscine binding to solubilised preparations was not retained on GF/B filters. Furthermore, prolonged ultracentrifugation at 100,000 g or filtration through 0.2 μM millipore filters of the 50,000 g supernatant did not result in any significant loss of specific binding.

Binding of [^{3}H]rauwolscine to soluble receptors was rapid (reaching equilibrium at 40–45 min) and reversible with dissociation following a monophasic pattern. Scatchard analysis of the equilibrium binding suggests interaction with a single class of binding sites (Fig. 2). B_{max} for soluble rat and rabbit renal cortical preparations were lower compared to respective particulate preparations probably related to the smaller specific receptor yields relative to total protein in these tissues (Table 1). The K_d in all three solubilised preparations was higher than respective particulate preparations and showed close similarity

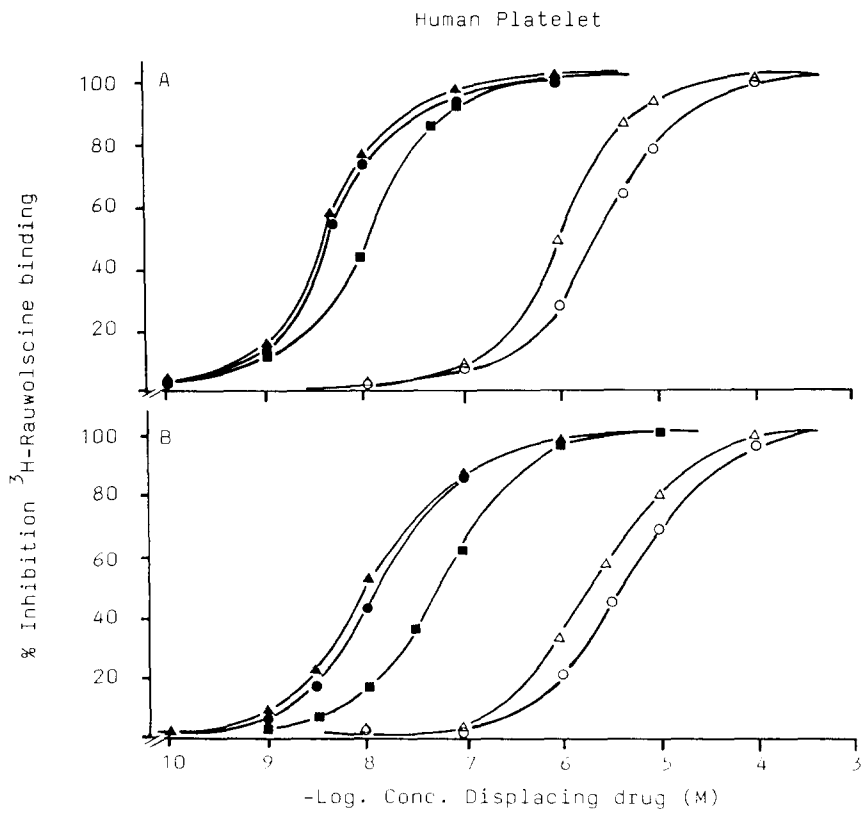

Fig. 3. Inhibition of specific [^{3}H]rauwolscine binding to particulate (A) and solubilised (B) preparations of the human platelet by various alpha adrenoceptor antagonists. Data for each curve represents the mean results of 3–5 separate experiments performed in duplicate. SEM for each point $< \pm 5\%$. ▲ yohimbine; ● rauwolscine; ■ phentolamine; △ corynanthine; ○ prazosin.

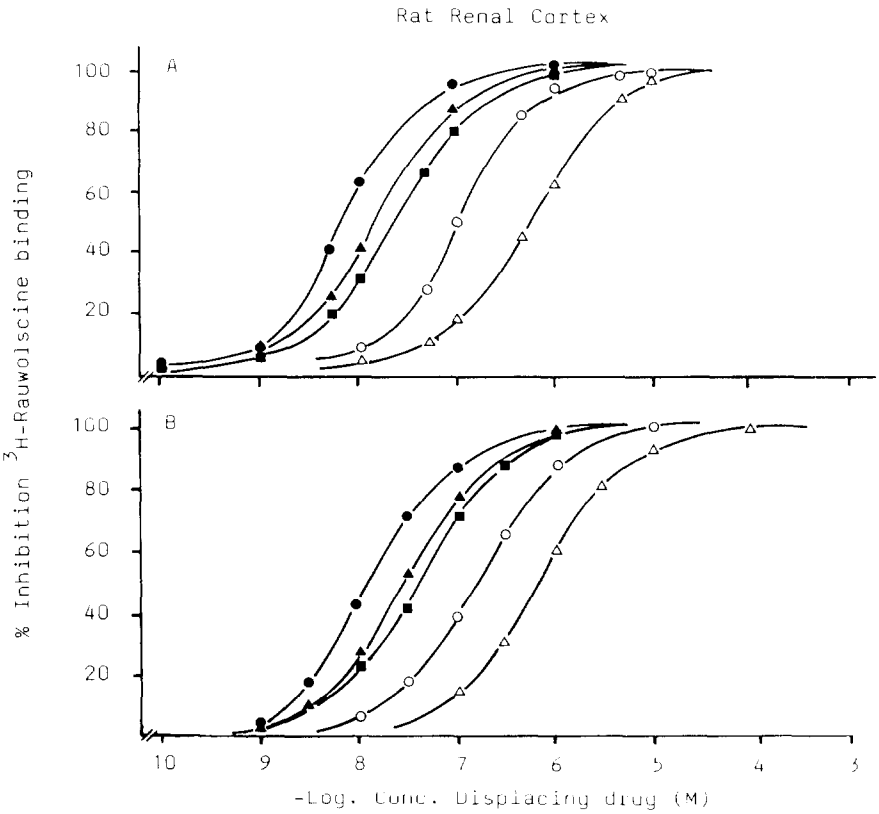

Fig. 4. Inhibition of specific [^{3}H]rauwolscine binding to particulate (A) and solubilised (B) preparations of the rat renal cortex by various alpha adrenoceptor antagonists. Data for each curve represents the mean results of 3–5 separate experiments performed in duplicate. SEM for each point $< \pm 5\%$. ▲ yohimbine; ● rauwolscine; ■ phentolamine; △ corynanthine; ○ prazosin.

whether estimated by equilibrium studies or by analysis of binding kinetics (Tables 1 and 2).

The soluble receptor sites from each tissue maintained the pharmacological properties of their respective particulate receptors, as indicated by the patterns of drug displacement of [^{3}H]rauwolscine binding (Table 3 and Figs. 3–5). All soluble [^{3}H]rauwolscine binding sites satisfied the requirement for the alpha$_2$ adrenoceptor classification, with respect to the relative potency of the selective alpha-adrenergic agents, although drug affinities were, in general, lower than those of the particulate preparations (Table 3). This was particularly marked in the case of agonists, with 10–50-fold lower apparent affinities observed at soluble sites, whereas antagonists were generally only up to two-fold less potent in these preparations (Table 3). Inter-tissue differences in pharmacological properties between the particulate receptors were similarly reflected in the soluble form. Thus yohimbine exhibited an affinity significantly lower than that of rauwolscine in both rat and rabbit soluble kidney preparations, whereas these two diastereoisomers were of almost identical affinity in the human platelet (Table 3). The alpha$_1$ antagonist prazosin was more potent at the alpha$_2$ adrenoceptor binding sites in solubilised rat and rabbit renal cortex than in the human platelet with the hierarchy of potency of alpha adrenoceptor subtype selective agents being of the same order as in the corresponding particulate preparations (Figs. 3–5). All antagonists displayed steep displacement curves indicating that these drugs were interacting with a single class of binding sites. Comparison of the ratio of affinities of yohimbine and prazosin for the [^{3}H]rauwolscine binding sites illustrates the marked differences between human platelet and the other two tissues studied. These differences are consistent between the tissues in both particulate and solubilised preparations (Table 4).

DISCUSSION

There is now substantial evidence from a number of laboratories indicating that the absolute affinity of certain antagonists at alpha$_2$ binding sites can differ between tissues and perhaps between species. Furthermore, in addition to these dramatic differences in affinity for antagonists, changes in the rank order of potency have also been reported [1–4, 9]. On the basis of these data we considered the possibility that this could indicate the existence of discrete subgroups of the alpha$_2$ adrenoceptor rather than, for example, possible antagonist affinity states of the same receptor resulting from an interaction between the receptor and putative effector (G) proteins. In the present study we have examined

Table 3. Affinities of adrenergic agents for [^{3}H]rauwolscine labelled alpha$_2$ adrenoceptors binding sites in membrane and solubilised preparations of human platelet and rat and rabbit renal cortex*

Drug	Human platelet		Rat renal cortex		Rabbit renal cortex	
	Membranes	Soluble	Membranes	Soluble	Membranes	Soluble
Rauwolscine	1.1 ± 0.1 (1.0)	5.2 ± 0.7 (0.99)	3.6 ± 0.2 (1.0)	7 ± 0.2 (1.0)	4.5 ± 0.7 (1.1)	5.3 ± 0.7 (1.0)
Yohimbine	0.85 ± 0.1 (0.98)	4.1 ± 0.5 (0.95)	7.9 ± 0.7 (0.92)	15 ± 1.7 (1.0)	14.5 ± 1.5 (1.0)	18 ± 2.8 (1.0)
Phentolamine	4.0 ± 1.2 (0.99)	21.4 ± 1.5 (0.97)	13 ± 0.2 (0.93)	28 ± 4.4 (0.95)	13 ± 0.6 (1.0)	42 ± 6 (0.96)
Corynanthine	192 ± 23 (1.0)	1274 ± 197 (0.96)	336 ± 2 (0.93)	421 ± 50 (1.0)	456 ± 46 (0.97)	878 ± 171 (0.97)
Prazosin	760 ± 190 (1.0)	1875 ± 169 (0.99)	53 ± 6 (1.0)	74 ± 6.2 (1.0)	303 ± 11 (1.1)	326 ± 25 (0.98)
Clonidine	8.8 ± 0.5 (0.83)	122 ± 11 (0.93)	38 ± 6.7 (0.93)	55 ± 6.6 (0.89)	70 ± 7 (0.76)	109 ± 10 (0.74)
Adrenaline	29 ± 4.9 (0.79)	2420 ± 147 (0.98)	94 ± 5 (0.75)	3898 ± 361 (0.63)	254 ± 16 (0.72)	6523 ± 323 (0.76)
Noradrenaline	88 ± 13 (0.90)	6887 ± 457 (0.97)	74 ± 4 (0.73)	6490 ± 560 (0.63)	193 ± 10 (0.68)	6700 ± 397 (0.85)

* Experiments were performed using increasing concentrations of cold drugs in competition with [^{3}H]rauwolscine (4–6 nM) in radioligand binding assays as described in the text. Results represent K_i (nM) calculated according to Cheng and Prussoff [15] from the IC_{50} value of each drug derived from the displacement curves. Values shown are means ± SEM of 3–6 experiments in each case performed in duplicate. Numbers in parentheses are slope factors of the mean displacement curves.

this in more detail comparing both different tissues and species as well as using both membrane and soluble preparations for alpha$_2$ adrenoceptor ligand binding.

In our hands, solubilised preparations of human platelet and rat and rabbit kidney displayed saturable, high-affinity [^{3}H]rauwolscine binding that possessed the appropriate pharmacological profile of an alpha$_2$ adrenoceptor. Similar results have been previously reported in human platelet [10] and calf [11] and rat [5] cerebral cortex. There was a generally small reduction in affinity (2–3-fold) of antagonists in soluble preparations compared to particulate perhaps reflecting a change in receptor conformation when freed from membrane constraint. However, the different pharmacological profiles (e.g. ratio of affinity of prazosin:yohimbine) observed in membranes were clearly maintained in solution. This suggests that the differences are not due to problems of ligand accessibility in the membrane. The data also suggests that the differences in pharamcological profile of antagonist drugs probably do not reflect different guanine nucleotide sensitive affinity states of the same receptor, since in the soluble preparation agonist affinities were dramatically (10–50-fold) reduced indicating that the receptor is divorced from the guanine nucelotide protein (Ni) after solubilisation with digitonin. In addition, in our soluble preparations divalent ions such as Mg^{2+} as well as guanine nucleotides do not influence agonist or antagonist binding affinities, nor do they alter the differential affinity displayed by prazosin at membrane or soluble alpha$_2$ adrenoceptors (data not shown).

Other possibilities for the differences should also be considered. In view of the lower affinity ratio of yohimbine and prazosin in rat and rabbit kidney it might be suggested that a portion of the [^{3}H]rauwolscine binding is to alpha$_1$ receptors. However, this can be effectively ruled out as the selectivity of this ligand for alpha$_1$ over alpha$_2$ sites is greater than 100-fold in all tissues indicating that few if any alpha$_1$ sites would be labelled at concentrations of radioligand used (4–6 nM). Furthermore, all antagonist competition curves possessed slopes close to unity. We have also considered the possibility that prazosin does not interact in a simple competitive way with alpha$_2$ sites in all the tissues. However, a detailed study in which complete [^{3}H]rauwolscine saturation curves were performed in the presence of increasing concentrations of prazosin indicated a simple competitive interaction in both rat kidney and human

Table 4. Ratio of affinities of prazosin and yohimbine* for [^{3}H]rauwolscine labelled alpha$_2$ adrenoceptor binding sites in membrane and solubilised preparations of human platelets and rat and rabbit renal cortex

	Membranes	Solubilised receptors
Human platelet	894	469
Rat renal cortex	7	5
Rabbit renal cortex	20	18

* Ratios calculated from mean K_i values shown in Table 3.

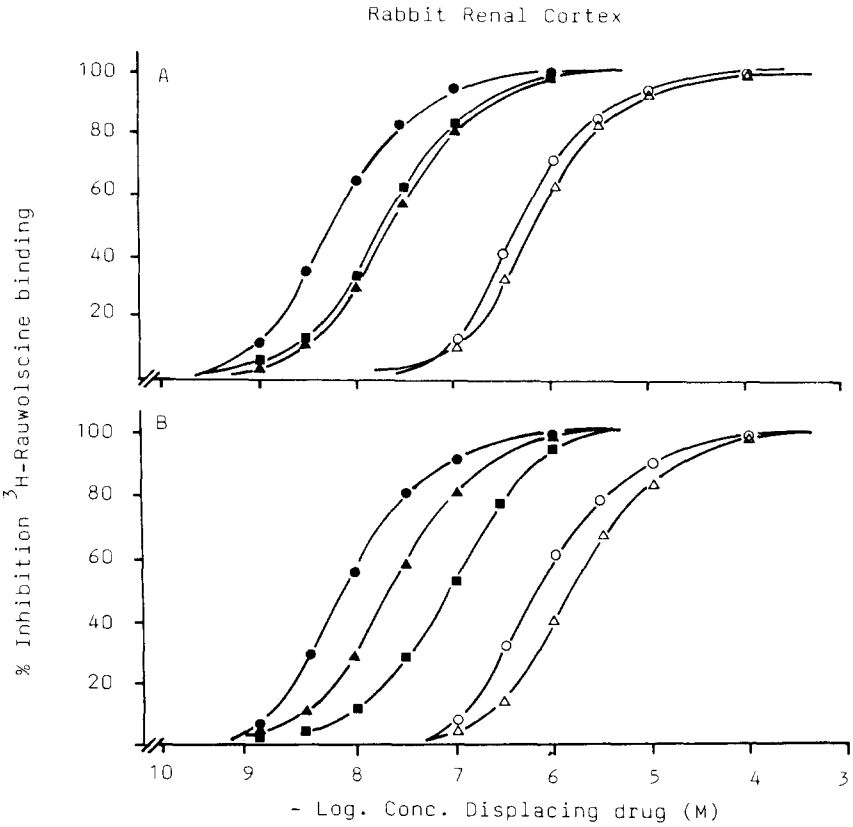

Fig. 5. Inhibition of specific [^{3}H]rauwolscine binding to particulate (A) and solubilised (B) preparations of the human platelet by various alpha adrenoceptor antagonists. Data for each curve represents the mean results of 3–5 separate experiments performed in duplicate. SEM for each point $< \pm 5\%$. ▲ yohimbine; ● rauwolscine; ■ phentolamine; △ corynanthine; ○ prazosin.

platelets but with apparent PA_2 values similar to those obtained from displacement curves and still differing by 20-fold (e.g. rat kidney PA_2 6.98, slope 1.17; human platelet PA_2 5.73, slope 1.18). Possible allosteric interactions of prazosin could also be eliminated since this antagonist did not alter the rate of dissociation of [^{3}H]rauwolscine following 'infinite' dilution of either human platelet or rat kidney membranes (data not shown). Kawahara and Bylund [5] have also been able to eliminate the possibility of differential proteolysis of the receptor and degradation of the radioligand.

Earlier studies from this laboratory [1–3] suggested that there may be a species difference in alpha$_2$ adrenoceptors with binding sites in rodent species possessing higher affinity for the alpha$_1$ antagonist prazosin and lower affinity for yohimbine and rauwolscine. Although further support for this rodent/non-rodent division of alpha$_2$ adrenoceptors was provided recently by Kawahara and Bylund [5], the present study and that of Neylon and Summers [9] suggest that this is probably an oversimplification. In this study, the prazosin/yohimbine affinity ratios with both soluble and particulate preparations of rat and rabbit kidney were substantially lower than that in human platelet (Fig. 4). Similar ratios calculated from the membrane data of Neylon and Summers [9] for kidneys of different species were human 228, rabbit 39, dog 17, rat 4, mouse 4.

Although the present data, together with those from several other studies suggest there are probable differences in alpha$_2$ adrenoceptors between tissues and species, it is somewhat discomforting to note that rather than simply falling into two or three discrete groups of sites, there appears to be a wide spectrum of affinities for drugs like prazosin. These vary from a K_i of 5 nM in neonatal lung membranes [12] through 53 nM in rat kidney to 760 nM in human platelet (this paper). Indeed, Neylon and Summers [9] also report a variety of K_i values for prazosin at [^{3}H]rauwolscine sites in kidneys from various species. The possibility that this spectrum of apparent affinities for prazosin reflects an admixture of two distinct sub-sites has been raised by Bylund [13] and Kawahara and Bylund [5]. These workers suggest that whereas human platelet possesses only one subtype (A) and neonatal rat lung only the other (B), several tissues display complex displacement curves for prazosin that are indicative of a mixture (A + B) of sites. However, the present studies and others [1, 3, 9] are not compatible with this model since prazosin displacement curves possess slopes of unity and show simple competition with [^{3}H]rauwolscine-labelled sites though with very different affinity.

In conclusion, the present data suggests that in both membrane and solubilised preparations, human platelet, rat and rabbit kidney show significant dif-

ferences in the properties of alpha$_2$ adrenoceptor sites labelled with [^{3}H]rauwolscine. These differences do not appear to be related to receptor–effector coupling, problems of ligand access or complex ligand–receptor interaction. Whether they reflect true intrinsic differences in the molecular structure of alpha$_2$ adrenoceptors will, however, require further purification and peptide sequencing.

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