Biochemical Pharmacology, Vol. 35, No. 20, pp. 3517–3523, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

HETEROGENEITY BETWEEN SOLUBLE HUMAN AND RABBIT SPLENIC α_2-ADRENOCEPTORS

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(*Received* 30 *July* 1985; *accepted* 22 *April* 1986)

Abstract—The pharmacological and biochemical characteristics of soluble α_2-adrenoceptors were investigated to determine whether differences observed in membranes were maintained in solution and to probe the nature of any such differences. α_2-Adrenoceptors were solubilized from purified plasma membrane preparations of human and rabbit spleen using digitonin. [^{3}H]yohimbine bound to one population of α_2-adrenoceptors in the preparations with dissociation constants of 2.4 nM and 7.8 nM respectively. The pharmacological profile of the α_2-adrenoceptors has been examined. Upon solubilization the affinity of the α_2-adrenoceptors for yohimbine was unchanged. In contrast, the potency of idazoxan and RX 811066 were increased, whereas the potency for prazosin (human only), phentolamine and WY 26392 was decreased 2–3-fold. The potency of the agonists oxymetazoline, UK 14304 and adrenaline were all reduced upon solubilization of α_2-adrenoceptors. The selectivity of yohimbine, idazoxan, RX 811066 and WY 26392 for human rather than rabbit α_2-adrenoceptors was maintained in solution.

Possible sources of heterogeneity between human and rabbit α_2-adrenoceptors were investigated. The protein structure was probed by comparing the susceptibility of the receptors to inactivation by sulphydryl modifying agents. No differences were observed in the potency of *N*-ethylmaleimide or *p*-chloromercuribenzoate to inactivate the receptor. The carbohydrate component of the receptors was investigated using agarose-linked lectins. Rabbit splenic α_2-adrenoceptors had a lower affinity for the lectins wheatgerm agglutinin (*Triticum vulgaris*) and soybean (*Glycine max*) which bind the sugars *N*-acetyl *d*-glucosamine and *N*-acetyl *d*-galactosamine respectively.

These findings suggest that heterogeneity of the α_2-adrenoceptor derives from its structural characteristics rather than its environment in the membrane.

Work from a number of laboratories has demonstrated heterogeneity of α_2-adrenoceptors between species [1–4] and between tissues [2, 5].

Differences have been revealed in the affinities and rank order of potencies of a variety of compounds to inhibit the binding of [^{3}H]yohimbine and [^{3}H]rauwolscine to membrane bound α_2-adrenoceptors. It is possible that the differential potencies of drugs for the receptors may relate to: (1) their lipid solubility, as has been shown for some β-adrenoceptor systems [6], (b) interaction of the compound with excessive non-specific sites in the membrane [4], (c) the membrane environment of the receptor which may exert modulatory effects, or (d) *bona fide* differences in the protein or carbohydrate nature of the receptors. To investigate these possibilities binding to soluble preparations of human and rabbit spleen α_2-adrenoceptors were compared. These two tissues were selected as models because they have been demonstrated to have markedly different characteristics in membranes [1]. The nature of the differences between the two species was further investigated by comparing the effects of sulphydryl modifying agents on adrenoceptor binding; and comparing the affinity of the receptor for agarose linked lectins in order to probe their protein and carbohydrate composition respectively.

MATERIALS AND METHODS

Tissue preparation. Rabbit spleen was obtained from Buxted Ltd. Human spleen was resected at surgery and both were frozen immediately in liquid N_2. Tissue was thawed into 10 vol. of 10 mM bicarbonate buffer, containing 5 mM EDTA, 0.1 mM PMSF,† pH 7.5. After cleaning extraneous fat and connective tissue, the spleen was homogenized by 3 × 10 sec bursts using an Ultraturrax homogenizer. The tissue was centrifuged at 10,000 *g* for 9 min at 4° in a Sorvall RC-5B centrifuge, the supernatant was removed, filtered through two layers of muslin and centrifuged at 40,000 *g* for 20 min at 4°. The pellet was resuspended in 20 mM imidazole buffer containing 300 mM KCl and 0.1 mM PMSF using a Potter Elvejhem homogenizer. This high salt wash was to extract the contractile proteins. Membranes were pelleted and the pellet washed by resuspension and centrifugation at 40,000 *g* for 20 min at 4° in 50 mM Tris, 0.5 mM EDTA, pH 7.5. This preparation was used for binding experiments in membranes and for α_2-adrenoceptor solubilization.

Receptor solubilization. Splenic membranes (20–40 mg of protein) were resuspended at a digitonin:protein ratio of 5:1 in buffer of composition

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† Abbreviations used: PMSF, phenylmethylsulphonylfluoride; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate.

50 mM Tris, 5 mM EDTA, 100 mM NaCl, 0.1 mM PMSF, 1% digitonin (pH 7.5). Membranes were homogenized with five strokes of a Potter Elvejhem homogenizer and then stirred on ice at 4° for 1 hr. Remaining membranes were centrifuged at 40,000 *g* for 1 hr. The supernatant, containing soluble α_2-adrenoceptors was filtered through 0.22 μM filters, and desalted by chromatography on Sephadex G-50 into buffer containing 50 mM Tris, 0.5 mM EDTA, 0.1% digitonin before assay. Digitonin at concentrations above 0.2% was found to inhibit [^{3}H]yohimbine binding (data not shown).

Binding assays. (a) Binding of [^{3}H]yohimbine ([^{3}H]YOH) to membrane bound α_2-adrenoceptors was carried out as previously described [1]. Briefly, incubations (200 μl) were carried out at room temperature for 40 min, then terminated by filtration through Whatman GF/C filters and washed with 3 × 4 ml aliquots of ice-cold buffer. Non-specific binding was determined in samples containing 10 μM phentolamine. Displacement studies were conducted using K_D concentration of ligand. K_i values were calculated according to Cheng and Prusoff [7].

(b) Binding of [^{3}H]YOH to soluble α_2-adrenoceptors was carried out in a total volume of 0.5 ml either at 4° for 3 hr or for 1 hr at room temperature, steady state being reached under both conditions. Incubations were terminated and bound and free ligand separated by gel filtration through Sephadex G-50 (fine) columns [8]. One ml void volumes were collected from the column which contained the soluble α_2-adrenoceptors and radioactivity was counted by liquid scintillation spectrometry.

Experiments to investigate the effect of sulphydryl reagents were conducted by incubating the soluble receptor with NEM, DTT or PCMB at 22° for 1 hr. The sulphydryl reagents were removed by gel filtration and the soluble α_2-adrenoceptors assayed as described above. Results were analysed by Students *t*-test.

Lectin affinity experiments. Aliquots of agarose bound lectins containing 0.15 mg of the lectin were washed twice in 50 mM Tris–HCl buffer pH 7.5. Soluble α_2-adrenoceptors were incubated with the lectin beads for 1 hr at room temperature after being desalted into buffer containing no EDTA since this removes the divalent cations essential for the binding of some lectins. After incubation, the lectin beads were removed by centrifugation and the supernatant assayed for soluble α_2-adrenoceptors as described above.

Source of chemicals. Drugs used in the study were from the following sources: Yohimbine (Sigma, London, U.K.), phentolamine mesylate (Ciba, Horsham, U.K.), prazosin HCl (Pfizer, Sandwich, U.K.), idazoxan, RX-811066 (2-[2-(2-allyloxy-1,4,benzodiozanyl)]-2-imidazoline) (Reckitt & Colman, Hull, U.K.), WY-26392 (Wyeth), adrenaline (Sigma), UK 14304 (Pfizer) and oxymetazoline (Schering, Bloomfield, N.J.). Agarose linked lectins and sulphydryl reagents were obtained from Sigma Chemicals Ltd. Digitonin (puriss) was from Fluka (Switzerland). Protein assays were conducted using Bio-Rad protein reagent, which was unaffected by the presence of digitonin at concentrations <0.2%.

RESULTS

Binding of [^{3}H]YOH to splenic membrane and soluble preparations

The saturation analysis of [^{3}H]YOH binding (shown in Fig. 1) demonstrates that in both human and rabbit spleen membrane preparations binding is to a single population of sites in a saturable and specific manner. The affinity of [^{3}H]YOH for human splenic α_2-adrenoceptors is approximately fourfold greater than for rabbit splenic α_2-adrenoceptors. Upon solubilization the K_D values for [^{3}H]YOH are largely unchanged (K_D 1.6 nM and 2.4 nM in human, 7.3 nM and 7.8 nM in rabbit membranes and soluble preparations respectively). Moreover, binding is to one set of sites, since Hill coefficients are not significantly different from unity. Solubilization with digitonin liberates approximately 50% of the membrane protein, but varying amounts of receptor (for summary see Table 1). A significantly higher percentage of α_2-adrenoceptors was solubilized from human (92%) compared with rabbit spleen (57%). Other studies of α_2-adrenoceptor solubilization from human platelet membranes using the same method of estimation recorded recoveries of 47% [9] and 85% [10].

Pharmacological profile of soluble α_2-adrenoceptors

The affinities of a number of α_2-adrenoceptor antagonists and agonists were determined in soluble splenic preparations from rabbit and human tissue (see Table 2). These were compared with K_i values

Table 1. Binding of [^{3}H]yohimbine in membrane and soluble α_2-adrenoceptor preparations.

	Human spleen	Rabbit spleen
Membranes		
B_{max} (fmoles/mg protein)	368 ± 55	854 ± 108
K_D (nM)	1.6 ± 0.2	7.3 ± 0.7
Soluble		
B_{max} (fmoles/mg protein)	671 ± 47	770 ± 107
K_D (nM)	2.4 ± 0.4	7.8 ± 0.9
% Solubilization of receptor	92 ± 12	52 ± 6
% Solubilization of protein	52 ± 4	48 ± 4

Data is the mean ± SEM of 6 separate experiments.

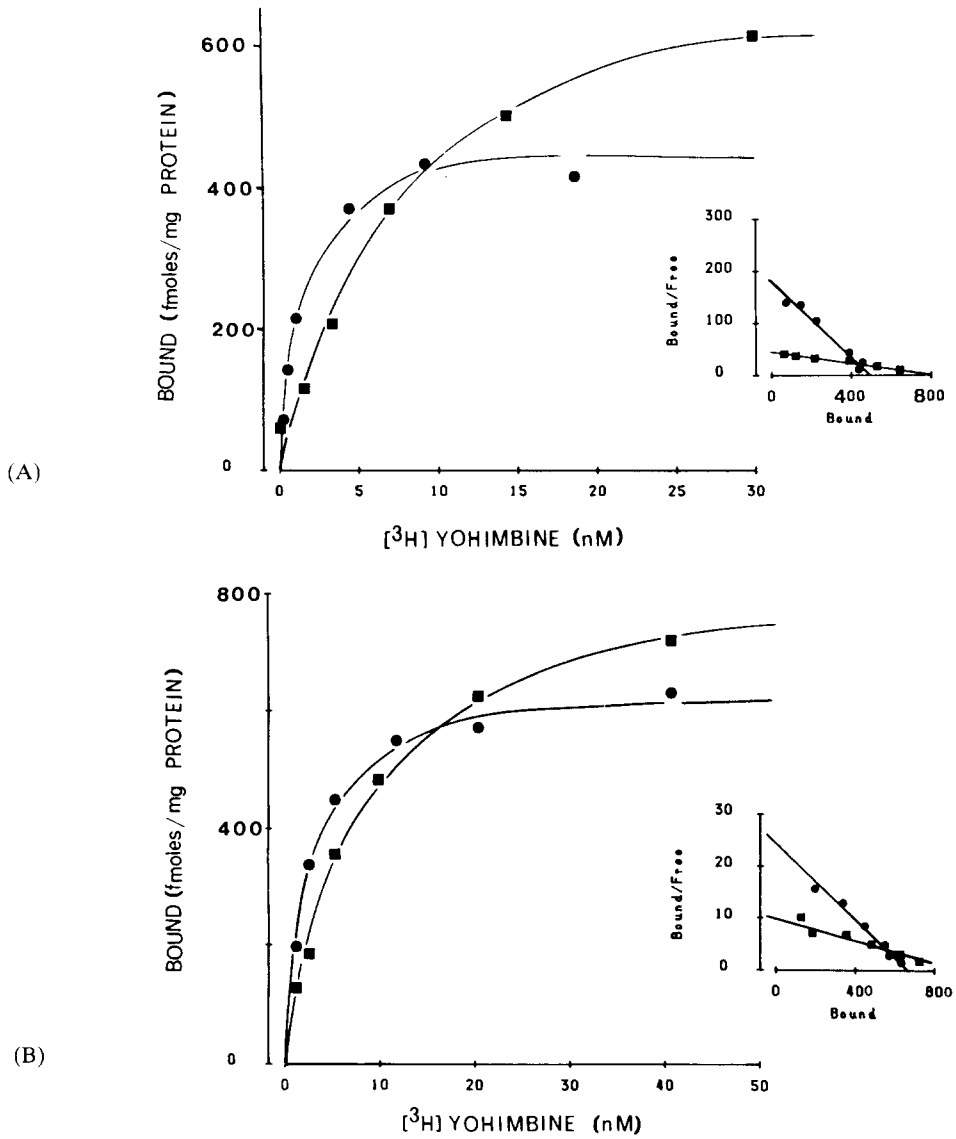

Fig. 1. Binding of [^{3}H]YOH membrane bound (A) and soluble (B) α_2-adrenoceptors from rabbit spleen (■) and human spleen (●). Specific binding represented 90–95% of total binding at the K_D in all preparations.

previously determined in membrane preparations [1]. Solubilization of the α_2-adrenoceptor resulted in variable effects on the affinity of certain drugs for the receptor. Thus, the K_i for yohimbine was unchanged, in agreement with unchanged K_D values of [^{3}H]yohimbine observed in the saturation analysis. For membrane preparations WY 26392 [11] exhibited a 10-fold selectivity for human splenic α_2-adrenoceptors when compared with rabbit α_2-adrenoceptors [1] and this selectivity was maintained in solution. The potency of idazoxan and its analogue, RX 811066 [12], was approximately three-fold greater for soluble α_2-adrenoceptors than for membrane bound receptors. These compounds exhibit a 3–5-fold selectivity for particulate human α_2-adrenoceptors and this property is maintained in soluble receptor preparations. The affinity of prazosin for soluble rabbit spleen α_2-adrenoceptors was unchanged, whereas it was decreased 2–3-fold at the soluble human splenic α_2-adrenoceptor. Phentolamine and WY 26392 were also 2–3-fold weaker at the soluble α_2-adrenoceptor compared with membrane bound receptors. The Hill coefficients (n_H) for all reversible antagonists were not significantly different from unity indicating one homogeneous population of sites.

All the agonists studied were equipotent at human and rabbit α_2-adrenoceptors, and characteristic low Hill slopes were observed when competition curves were performed in membranes. Upon solubilization, the K_i values were increased and the Hill slopes were not significantly different from unity.

The effect of sulphydryl modifying agents on soluble α_2-adrenoceptor binding

In order to probe the heterogeneities revealed by the displacement studies the effect of three sulphydryl modifying agents on soluble α_2-adreno-

Table 2. Inhibition constants of α_2-adrenoceptor antagonists for membrane bound and soluble α_2-adrenoceptors

	Inhibition constant K_i (nM) (n_H)					
	Membranes			Soluble		
	Human	Rabbit	H/R	Human	Rabbit	H/R
Yohimbine	2.5 ± 0.2	10.3 ± 1.4	0.24	2.4 ± 0.5 (0.96)	8.7 ± 0.5 (0.93)	0.27
Phentolamine	7.6 ± 1.4	14.7 ± 6.2	0.52	21.1 ± 2.0 (1.08)	40.1 ± 2.0 (0.98)	0.52
Prazosin	1112 ± 194	5251 ± 645	0.21	3196 ± 217 (1.0)	3098 ± 470 (0.86)	1.03
Idazoxan	9.7 ± 1.9	64 ± 1.7	0.15	3.5 ± 0.3 (0.92)	16.2 ± 3.5 (0.88)	0.21
RX 811066	1.1 ± 0.1	9.5 ± 2.1	0.11	0.34 ± 0.1 (1.05)	3.3 ± 1.2 (0.92)	0.10
WY 26392	4.8 ± 1.7	58 ± 12	0.08	9.7 ± 3.4 (1.09)	77 ± 9 (0.95)	0.12
Oxymetazoline	7.0 ± 2 (0.73)	12.7 ± 4 (0.69)	0.55	15 ± 4 (1.05)	42 ± 5 (0.86)	0.35
UK 14304	14.7 ± 4 (0.78)	22 ± 3 (0.64)	0.66	697 ± 216 (1.1)	666 ± 150 (0.89)	1.04
Adrenaline	117 ± 21 (0.71)	273 ± 90 (0.75)	0.42	2125 ± 204 (0.92)	1100 ± 240 (0.99)	1.93

IC_{50} values were derived from Hill analysis and K_i values calculated according to Cheung and Prussof [7]. Hill slope factors (n_H) are shown in parentheses. The ratio of K_i values for human vs rabbit α_2-adrenoceptors (H/R) demonstrates that selectivity is maintained on solubilization. These values represent the mean ± SEM of 3–11 separate determinations.

ceptors was investigated (see Fig. 2). Prior incubation with either NEM or PCMB results in alkylation of a thiol group preventing binding of [^{3}H]YOH to the receptor. This is in agreement with the work of Quennedy *et al.* [13] who demonstrated inhibition of α_2-adrenoceptor binding to membranes from rat cerebral cortex by the same agents. The IC_{50} values for inhibition of [^{3}H]YOH binding were similar in human and rabbit spleen (NEM IC_{50}, human spleen 6×10^{-4} M versus 8×10^{-4} M for rabbit spleen; PCMB IC_{50}, in human spleen 3×10^{-5} M versus 2.5×10^{-5} for rabbit spleen). DTT was ineffective on [^{3}H]yohimbine binding to both rabbit and human soluble receptor preparations. This indicates that for both the α_2-adrenoceptors investigated there is/are thiol group(s) but no disulphide bonds associated with the ligand binding site for [^{3}H]YOH. There is no evidence from this experiment to suggest

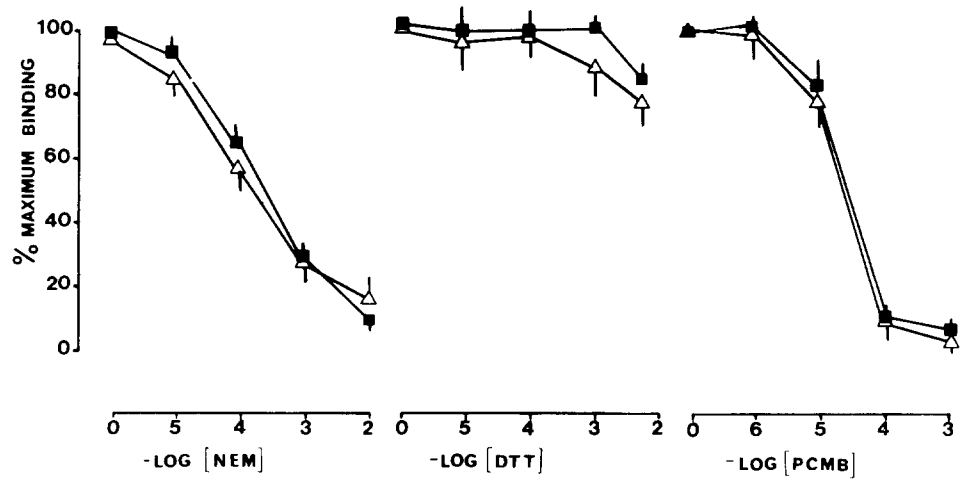

Fig. 2. The effects of preincubation of soluble α_2-adrenoceptors with the sulphydryl modifying agents, NEM, PCMB and DTT. Experiments were carried out as described in Methods. Data shown is the mean + SEM of 3–4 separate experiments performed on rabbit spleen (△) and human spleen (■).

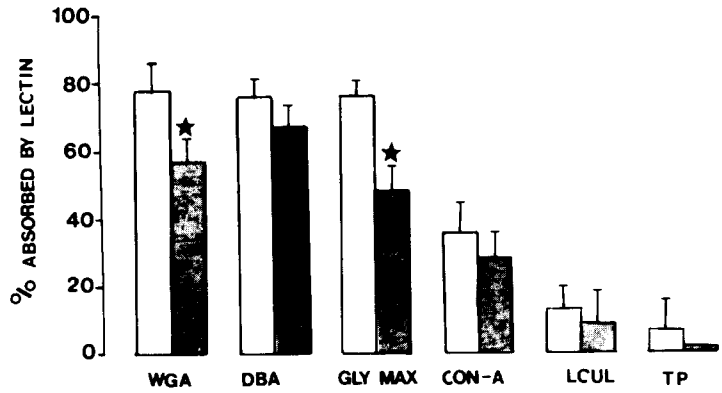

Fig. 3. Binding of human and rabbit α_2-adrenoceptors to a variety of agarose linked lectins. Experiments were carried out as described in Methods. Results are expressed as percentage of soluble receptor removed from solution by incubation with 0.15 mg of lectin for 1 hr at room temperature. Binding to human α_2-adrenoceptor is represented by the open blocks and to rabbit α_2-adrenoceptors by the shaded blocks. A significant difference between human and rabbit at $P < 0.05$ is denoted by a star (★). Data shown is the mean ± SEM of 3–5 independent experiments. The abbreviations are as follows: (Sugar specificity) WGA, Wheatgerm agglutinin (*N*-acetyl β-D-glucosamine); DBA, *Dolichos biflorus* (*N*-acetyl D-galactosamine); GLYMAX, glycine max or soybean (*N*-acetyl D-galactosamine); CON-A, concanavalin A (α-D mannose and α-D glucose); LCUL, *Lens culinarus* or lentil (α-D mannose and α-D glucose); TP, *Tetragonolobus purpureas* or Asparagus pea (α-L fucose).

that there is any variation in the position or accessibility of sulphydryl moieties between human and rabbit α_2-adrenoceptors.

The affinity of human and rabbit splenic α_2-adrenoceptors for a variety of lectins

The carbohydrate chains associated with the α_2-adrenoceptors were investigated by assessing the affinity of the soluble receptors for a variety of agarose-linked lectins (see Fig. 3). Both human and rabbit α_2-adrenoceptors bound readily to lectins with a high affinity for complex type carbohydrate chains, i.e. wheatgerm agglutinin, *Dolichos biflorus* and *Glycine max*. Their affinity for concanavalin A and *Lens culinaris* linked lectins was much less, indicating a lower availability of glucose or mannose residues, or alternatively the presence of fewer high mannose type carbohydrate chains. Neither human nor rabbit α_2-adrenoceptors had any significant affinity for *Tetragonolobus purpureas* indicating a lack of available α-L fucose residues. There was a small difference in the affinity of human and rabbit α_2-adrenoceptors for two of the lectins, wheatgerm agglutinin and *Glycine max*, both of which exhibited greater affinity for human α_2-adrenoceptors.

DISCUSSION

α_2-Adrenoceptors have been solubilized from human and rabbit splenic membranes. The location of α_2-adrenoceptors in the spleen is not clear. While a large proportion are present on splenic platelets, they must also be present in other areas, e.g. smooth muscle, to explain the greater density of receptors in spleen than on platelets. Interestingly, a high proportion of receptors were solubilized by this protocol. The yield of soluble receptors from human spleen membranes was occasionally greater than 100%. This unexpected finding may have several possible explanations: (i) The presence of Na^+ in the solubilizing buffer may increase the binding of [^{3}H]YOH [14]. (ii) Cryptic α_2-adrenoceptors exist in human splenic membranes which are freely expressed upon solubilization. (iii) Tightly bound endogenous catecholamines, present in the membranes which depress [^{3}H]YOH binding in a pseudo competitive manner [15, 16], are removed by solubilization of the receptor.

We favour the latter possibility, that solubilization with digitonin disrupts the α_2-adrenoceptor complex, removing tightly bound catecholamines. This explanation is supported by our observation that even if solubilization is carried out in the presence of Mg^{2+} there is negligible binding of the α_2-adrenoceptor agonist [^{3}H]UK 14304 (data not shown) and the report that α_2-adrenoceptors are solubilized only in the low affinity state when digitonin [10], but not CHAPS [17], is used as detergent. Moreover, it has been suggested that β-adrenoceptor binding in rat heart may be masked by tightly bound endogenous noradrenaline [18].

The data presented here are consistent with the hypothesis that heterogeneity of α_2-adrenoceptors exists and this is supported by other studies using both antagonists [1–5] and agonists [19]. α_2-Adrenoceptors obtained from human and rabbit spleen were selected for comparison because of their markedly different affinities for the compounds yohimbine, idazoxan, RX 811066 and WY 26392. Other workers [3, 5] have compared human and rat α_2-adrenoceptors because of their differential affinities for yohimbine, prazosin and oxymetazoline. Interestingly, while oxymetazoline and prazosin can differentiate between human and rat receptors, they are equipotent at human and rabbit receptors. Similarly, while idazoxan and WY 26392 can select between human and rabbit receptors they are equipotent at human and rat cerebral cortex receptors [1], but

differentially selective between human platelet and rat kidney α_2-receptors [20]. This supports the hypothesis that α_2-adrenoceptors exist as a spectrum [1] rather than as two distinct populations [3].

Selectivity of WY 26392, idazoxan and RX 811066 was maintained upon solubilization, although it was accompanied by alteration in the absolute potency of some agents. The compounds yohimbine, phentolamine and prazosin exhibited K_i values at soluble α_2-adrenoceptors in reasonable accord with those in the literature [9, 10, 29] given variation in incubation media and assay conditions between laboratories. Novel compounds such as idaxozan and its analogue RX 811066 were more potent at soluble receptors. This increased affinity may result from small conformational changes in the α_2-adrenoceptors such that it can more readily interact with a thermodynamically flavoured conformation of the idazoxan compounds. Chapleo and Stillings [12] have indicated the importance of a skewed conformation of the imidazole ring with the aromatic ring system of idazoxan for potency at α_2-adrenoceptors. Conformational alterations of the α_2-adrenoceptors may also be responsible for the decrease potencies of WY 26392 and phentolamine at soluble α_2-adrenoceptors. Interestingly, prazosin which exhibits a small selectivity for human α_2-adrenoceptors is not selective for the soluble receptor of either species. This may be related to putative accessory binding sites for prazosin in membranes which may not be coincident with [^{3}H]YOH binding sites [21, 4]. Since the selectivity of compounds such as idazoxan and WY 26392 for human α_2-adrenoceptors is maintained upon solution of the receptor, but the absolute affinity is altered this may be suggestive of more than one variation in the receptor conformation or structure. The α_2-adrenoceptor represents a novel and interesting system in that upon solubilization, when the constraints of the membrane are removed, the K_i values of antagonists for the receptor are increased in some cases and decreased in others. In other systems such as the β-adrenoceptor [22] and α_1-adrenoceptor [23, 24], antagonists are generally equipotent or less potent at soluble receptors. The potency of agonists are greatly reduced upon solubilization of the receptors. This is explained by the observation that solubilization with digitonin separates the α_2-adrenoceptor from its GTP-binding protein, Ni, preventing the formation of a high affinity state. The finding that agonists compete with a low Hill slope in membranes, but a hill slope close to unity in soluble preparations is consistent with this. Our findings are largely in agreement with those of Kawahara and Bylund [3] who demonstrate that heterogeneity between human platelet and rat cerebral cortex is maintained upon solubilization.

No gross differences were detected in the ability of sulphydryl modifying reagents to inhibit [^{3}H]YOH binding, indicating a lack of involvement of thiol groups in the structural differences between the two receptors. Some differences in the carbohydrate portion of the receptors were demonstrated. It is not clear to what extent the carbohydrate portion of the molecule is involved in ligand binding. In other systems, such as the β_2-adrenoceptor, the carbohydrate chains associated with receptor from two different species, the hamster lung and the rat erythrocyte, have been demonstrated to be heterogeneous [25]; however, there is no evidence for heterogeneity between the adrenoceptors in binding studies [26, 27]. Additionally, treatment of human cultured astrocytoma cells with tunicamycin produces aglycosylated receptors with similar binding characteristics to the native glycosylated receptors [28].

In summary, since the selectivity of some compounds for the α_2-adrenoceptor are maintained in soluble preparations, we favour the suggestion that the differences between human and rabbit α_2-adrenoceptors arise from heterogeneities in the molecular architecture of the receptor. Further understanding of the origin of these heterogeneities will only be possible after greater purification of the receptor and the development of further probes of receptor structure.

Acknowledgements—This work was supported by the British Heart Foundation. We thank the surgeons of St. Mary's Hospital for splenic tissue, Dr N. Shepperson for the kind gift of WY 26392 and Dr C. Chapleo for RX 811066 and Idazoxan.

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