Br. J. Pharmac. (1986), **88**, 607–614

Comparison of potency of α_2-adrenoceptor antagonists *in vitro*: evidence for heterogeneity of α_2-adrenoceptors

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1 A comparison has been made of affinity of α-adrenoceptor antagonists for α_2 binding sites in radioligand binding assays, and functional antagonist activity at pre- and postjunctional α_2-adrenoceptors in various *in vitro* preparations.

2 The antagonists displaced [^{3}H]-rauwolscine from rat brain and rabbit spleen membranes but there were substantial differences in rank order and absolute potency in the two tissues.

3 pA_2 values for yohimbine, phentolamine and Wy26703 against the selective α_2 agonist UK-14,304 were determined in the rat left atrium, rat and rabbit vas deferens and rabbit saphenous vein preparations. The pA_2 values varied substantially between the tissues, differing by two orders of magnitude in the case of Wy26703. Yohimbine was more potent in rabbit preparations while Wy26703 was markedly more potent in all the rat preparations.

4 Yohimbine and Wy26703 were compared in the dog saphenous vein preparation where pre- and postjunctional α_2 antagonist activity can be compared in the same tissue. As in the rabbit preparations, yohimbine was more potent than Wy26703 at both sites but the absolute potencies were different.

5 It is concluded that α_2-adrenoceptors are a heterogeneous population, different subgroups being more apparent between species rather than between tissue types or location.

Introduction

α-Adrenoceptors are classified on a pharmacological basis into α_1 and α_2 subgroups. Thus α_1-adrenoceptors are defined as those α-receptors at which prazosin is a more potent antagonist than yohimbine or its stereoisomer analogue rauwolscine, whereas at α_2-sites, yohimbine and rauwolscine are more potent than prazosin. α_1-Adrenoceptors are found at postjunctional sites whereas α_2-adrenoceptors can be located either prejunctionally where they can affect transmitter release, or postjunctionally for example in some vascular smooth muscle where they mediate contraction (Starke 1981).

Although the general characteristics of pre- and postjunctional α_2-adrenoceptors are the same, there are some observations indicating a possibility of discriminating between these receptors at different locations. Thus, for example, within a series of 2,5 disubstituted clonidine analogues, some derivatives appeared to distinguish between pre- and postjunctional α_2-adrenoceptors in the pithed rat. The steric bulk of the 5-substituent governed activity with respect to cardiac prejunctional α-adrenoceptors but no such relationship was apparent for activity on vascular postjunctional α_2-adrenoceptors (De Jonge *et al.*, 1981). Data from radioligand binding studies added weight to the hypothesis that α_2-adrenoceptors could be further differentiated. Preliminary studies in our laboratories on rat brain and rabbit spleen membranes showed that the ability of various α-antagonists to displace [^{3}H]-rauwolscine varied markedly between the tissue preparations (Alabaster & Brett 1983). The affinity of the antagonist prazosin for [^{3}H]-rauwolscine binding was also reported to depend on the tissue, being much higher in rat cerebral cortex than in human platelets (Cheung *et al.*, 1982) and in rat compared to pig lung membranes (Latifpour *et al.*, 1982).

There is much evidence, especially from studies in pithed rats, that α_2-adrenoceptor-mediated responses are dependent on the physical environment (for references see Alabaster & Davey, 1984). It has been suggested that the α_2-adrenoceptor exists in different conformational states depending on tissue or species, according to the presence of co-factors in the immediate vicinity of the receptor. The possibility existed therefore that α_2-adrenoceptors could differ sufficient-

ly to allow the identification of antagonists which could be targeted to different tissues or different α_2-sites. A study was undertaken to investigate further the characteristics of α_2-adrenoceptors in different tissues. In addition to radioligand binding studies, the functional activities of three α-antagonists were compared at both pre- and post-junctional α_2-sites. Phentolamine (a mixed α_1- and α_2-receptor antagonist), yohimbine (relatively selective for α_2 – as opposed to α_1-adrenoceptors) and Wy26703 (very selective for α_2 as opposed to α_1-adrenoceptors; Lattimer *et al.*, 1982) were the compounds studied. Preliminary accounts of some of these data have been presented to the British Pharmacological Society (Alabaster & Brett, 1983; Alabaster & Peters, 1984).

Methods

Radioligand binding studies

[^{3}H]-rauwolscine (82 Ci mmol^{-1}, New England Nuclear) binding to rat cerebral cortical membranes was determined according to the method of Perry & U'Prichard (1981). Rat brains, minus cerebellum, were homogenized in 50 mM Tris-HCl buffer (pH 7.7 at 4°C) with a Polytron PCV-2. After two centrifugations at 4°C (50,000 *g*, 10 min) with intermediate resuspension in fresh buffer, membranes were suspended in 50 mM Na-K phosphate buffer (36mM $Na_2HPO_4.12H_2O$, 14 mM KH_2PO_4; pH 7.4). Aliquots of tissue (standard assay volume 1.0 ml) were incubated in triplicate with [^{3}H]-rauwolscine for 60 min at 4°C. Incubation was terminated by filtration under reduced pressure over Whatman GF/B filters which were then rinsed with 2 × 5 ml ice-cold buffer. Filters were placed in plastic scintillation vials, 8 ml of Instagel (Packard) added and counted by liquid scintillation spectrometry with an efficiency of 45%.

Rabbit spleens were homogenized in 0.32 M sucrose solution and centrifuged at 1000 *g* for 15 min. The spleen membranes were then prepared as outlined for rat cerebral cortex membranes.

Specific binding (defined by phentolamine 10 μM) to cortex and spleen membranes was saturable (65–75% specific binding) with K_Ds of 3.7 and 3.2 nM respectively.

Displacement curves for antagonists were constructed by employing a wide range of concentrations, using triplicate incubations for each concentration, and K_i values calculated according to the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

pA_2 determinations

Functional α_2-adrenoceptor antagonist potency of yohimbine, phentolamine and Wy26703 was evaluated by comparing the ability of these compounds to antagonize the effects of the selective quinoxaline-imidazoline α_2-agonist UK-14,304 in various preparations (Cambridge 1981).

Rat atrium The method was based on that used by Vizi *et al.* (1973). The left atrium, under a resting tension of 0.5 g, was bathed in Tyrode solution (at 31°C) of the following composition (mM): NaCl 138, Na_2CO_3 1.5, KCl 5.7, $CaCl_2$ 1.8, NaH_2PO_4 0.4, $MgCl_2$ 1.1 and glucose 5.6 and containing atropine 1 μM and equilibrated with 95% O_2: 5% CO_2. The tissue was paced at 1 Hz, pulse width 0.1 ms and at a voltage just above threshold. After 30 min equilibration, the atrium was field stimulated at the same frequency as the pacing electrodes but with a pulse width 2 ms duration (5–6 V) by electrodes placed on either side of the preparation. The two stimuli were synchronous. The field stimulus was applied for 1 min at intervals of 10 min and produced an inotropic response equivalent to 0.5 g tension. In separate experiments, it was shown that propranolol 1 μM abolished responses to field stimulation but had no effect on pacing contractions. Following the establishment of consistent responses, cumulative doses of the α_2-adrenoceptor agonist UK-14,304 were added to produce dose-dependent inhibition of field stimulation responses. Separate atria were pretreated with a single concentration of antagonist for 30 min before adding UK-14,304.

Rat vas deferens The prostatic halves of rat vasa deferentia were mounted in magnesium-free Krebs-Henseleit solution of the following composition (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 11.1, gassed with 95% O_2:5% CO_2 at 37°C, under a basal tension of 0.5 g. The tissue was field stimulated via parallel platinum electrodes by single pulses at 0.1 Hz, 2 ms duration using a supramaximal voltage. One dose-response curve to UK-14,304 was obtained in vasa deferentia 30 min after treating with a single concentration of antagonist or solvent control.

Rabbit vas deferens The prostatic ends of the vasa deferentia (1.5–2 cm lengths) were used and responses to field stimulation obtained as described for the rat vas deferens preparation.

Rabbit and dog saphenous veins Rings of saphenous vein were prepared and studies carried out as previously described (Alabaster *et al.*, 1985). Each ring was mounted in a 15 ml organ bath containing Krebs-Henseleit solution of composition (mM) NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.9, KH_2PO_4 1.2, glucose 11.1, gassed with 95% O_2:5% CO_2 at 37°C. The solution also contained the catecholamine uptake inhibitors desipramine (0.1 μM) and

normetanephrine (10 μM) and the β-adrenoceptor blocker propranolol (0.04 μM). After 2 control dose-response curves to UK-14,304 had been obtained, a single concentration of antagonist was added and after 30 min a third dose-response curve obtained.

At least three concentrations of each antagonist were studied and pA_2 values were calculated from the dose-ratios according to the method of Arunlakshana & Schild (1959). The regression of the Schild slope was calculated with 95% confidence limits. Antagonism was considered to be competitive if the slope of the regression of the Schild plot was not significantly different from unity.

Overflow experiment in dog saphenous vein

The method was based on that of Su & Bevan (1970), as adapted by Starke *et al.* (1975). Spiral strips of dog saphenous vein were incubated in Krebs-Henseleit solution (composition as for saphenous veins) containing 5 μCi ml^{-1}, 147 nM [^{3}H]-(−)-noradrenaline together with ascorbic acid (0.1 mM) for 60 min at 37°C. The tissues were then washed, mounted between parallel electrodes and placed under 2 g tension and superfused at 6 ml min^{-1} with Krebs-Henseleit solution containing desipramine (0.6 μM), propranolol (0.04 μM) and normetanephrine (10 μM). After a 1 h stabilization time, the perfusate was collected in 3 min samples before and during field stimulation at 5 Hz, 1 ms, 25 V. Each strip was stimulated up to 6 times, the α-adrenoceptor antagonists being added to the perfusion fluid in increasing concentrations (10^{-9} to 10^{-6}M) 15 min before the next stimulation period. Aliquots (1 ml) were taken from the samples and tritium measured by liquid scintillation spectrometry. Stimulation-induced tritium overflow was calculated as a percentage of the total tritium in the tissue as described by Starke *et al.* (1975).

Drugs

The following drugs were used: corynanthine hydrochloride (Sigma), desipramine hydrochloride (Ciba-Geigy), [^{3}H]-(−)-noradrenaline sp.act 35 Ci mmol^{-1} (Amersham), normetanephrine hydrochloride (Sigma), phentolamine hydrochloride (Ciba-Geigy), prazosin (Pfizer), doxazosin (Pfizer), (±)-propranolol hydrochloride (ICI), rauwolscine hydrochloride (Roth), [^{3}H]-rauwolscine, sp.act. 82 Ci mmol^{-1} (New England Nuclear), Rx781094-idazoxan (Reckitt & Coleman), UK-14,304 (5-bromo-6- [2-imidazolin-2 ylamino]-quinoxaline; Pfizer), yohimbine hydrochloride (Sigma), Wy26703' (N-((2β,11bα)-1,3,4,6,7,11b-hexahydro-2H-benzo [a]-quinolizin-2-yl)-N-methylisobutanesulphonamide, Wyeth). All drugs were dissolved in distilled water except prazosin and doxazosin which were dissolved in lactic acid (40 mM) and diluted with distilled water.

Results

[^{3}H]-rauwolscine binding studies

All the antagonists displaced [^{3}H]-rauwolscine from rat brain and rabbit spleen membranes in a concentration-dependent way. The K_i values, calculated from the displacement curves, were however different in the two membrane preparations (Table 1). The difference in compound sensitivity between tissues was most marked with prazosin and Wy26703 which were 63 and 11 fold respectively less potent in the spleen compared to the brain preparation. Representative experiments are illustrated in Figure 1. Thus while rauwolscine and Wy26703 were approximately

Table 1 Displacement of [^{3}H]-rauwolscine by α-adrenoceptor antagonists

Antagonist	*Rat brain membranes* K_i (nM)	*n*	*Rabbit spleen membranes* K_i (nM)	*n*
Rauwolscine	2.9 ± 1.4	4	2.1 ± 0.4	5
Yohimbine	8.1 ± 1.8	4	3.4 ± 0.8	3
Phentolamine	21.4 ± 2.6	4	4.9 ± 1.4	4
Wy26703	3.6 ± 1.1	5	40 ± 4.4	5
Idazoxan	3.4 ± 0.5*	4	21 ± 8.7	3
Prazosin	45 ± 5.2†	5	2850 ± 100	4
Doxazosin	449 ± 61	4	2360 ± 500	3
Corynanthine	370 ± 98	3	405	2

K_i values are the mean ± s.e.mean. All slope factors were in the range 0.80 to 1.10 with the exception of * and † which were 0.6 and 0.7 respectively.

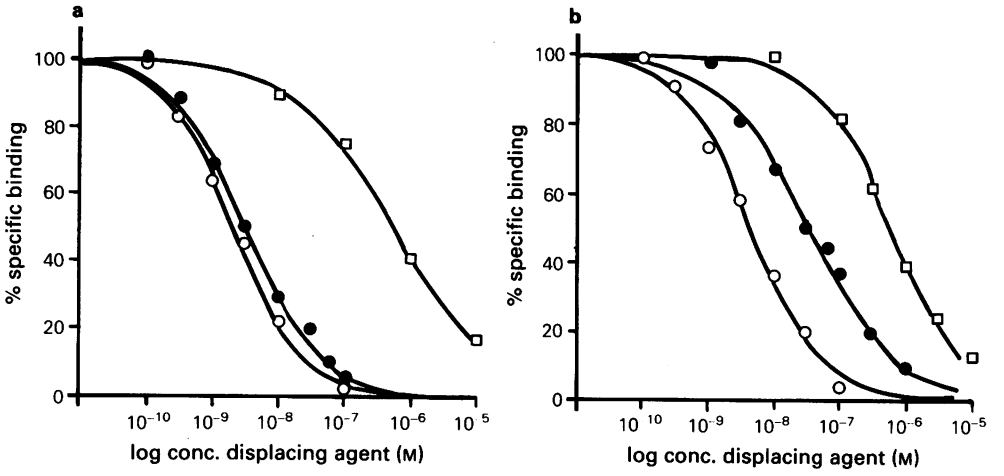

Figure 1 Inhibition of [^{3}H]-rauwolscine binding (2 nM) in rat brain (a) and rabbit spleen (b) membranes at 0°C (60 min) by increasing concentrations of rauwolscine (○), Wy26703 (●) and coryanthine (○). The data represent examples of single experiments with each antagonist, the points being a mean of triplicate incubations.

equipotent in rat membranes, Wy26703 was much less potent in displacing [^{3}H]-rauwolscine in rabbit spleen membranes. All compounds produced Hill-plot slope factors close to unity, with the exception of idazoxan and prazosin in the rat brain membranes.

Functional antagonist potency

The pA_2 values obtained for yohimbine, phentolamine and Wy26703 against UK-14,304 are given in Table 2. The antagonists gave parallel shifts of the dose-response curves and Schild plot slopes were not significantly different from unity.

The absolute potencies varied markedly between tissues especially with Wy26703. Thus for example, in the rat left atrium it had a pA_2 value of 8.52 while in the rabbit vas deferens it was 380 fold less potent with a pA_2 of 5.94. In all the rat preparations used, Wy26703 was consistently more potent in antagonizing UK-14,304 compared to rabbit preparations while the

Table 2 Comparison of pA_2 values obtained in various isolated preparations

Tissue	*Yohimbine*		*Phentolamine*		*Wy26703*	
	pA_2	Slope	pA_2	Slope	pA_2	Slope
Rat left atrium	7.77 (7.34–8.81)	1.05 (0.58–1.53)	7.50 (7.18–8.18)	0.92 (0.55–1.28)	8.52 (8.14–9.13)	0.99 (0.74–1.24)
Rat vas deferens	7.96 (7.67–8.40)	0.96 (0.72–1.13)	8.18 (7.83–8.73)	0.94 (0.75–1.17)	8.21 (7.93–8.44)	0.87 (0.79–1.06)
Rabbit vas deferens	6.34 (6.00–6.91)	1.02 (0.73–1.30)	NT	NT	5.94 (5.61–6.52)	0.85 (0.60–1.10)
Rabbit saphenous vein	7.67 (7.32–8.40)	0.92 (0.56–1.28)	7.42 (6.69–8.99)	0.87 (0.40–1.34)	6.25 (6.07–6.55)	1.16 (0.86–1.46)
Dog saphenous vein	8.33 (7.93–9.02)	0.92 (0.66–1.12)	NT	NT	7.47 (7.15–7.98)	0.91 (0.70–1.12)

pA_2 values with 95% confidence limits were derived from Schild plots and were taken from at least 9 concentrations points.
NT = not tested.

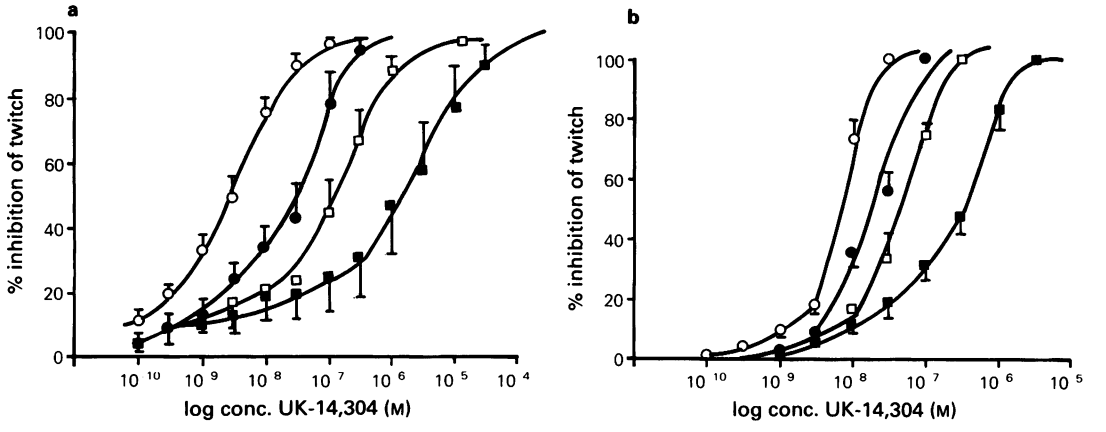

Figure 2 (a) Dose-response curves for UK-14,304 in the electrically stimulated rat vas deferens preparation in the absence (○) and presence of Wy26703 (●) 60 nM, (□) 300 nM and (■) 6 μM. (b) Dose-response curves for UK-14,304 in the electrically stimulated rabbit vas deferens preparation in the absence (○) and presence of Wy26703 (●) 3 μM, (□) 20 μM and (■) 100 μM.

potency in the dog saphenous vein was between the two. In rabbit and dog tissues, yohimbine and phentolamine were more potent than Wy26703 while in rat tissues Wy26703 was the most potent antagonist. The dose-response curves to UK-14,304 and inhibition by Wy26703 in rat and rabbit vas deferens is shown in Figure 2. Examples of Schild plots derived from dose-response curves, in this case from rat atrium and rabbit saphenous vein, are illustrated in Figure 3.

Stimulation-induced overflow in dog saphenous vein

Wy26703 and phentolamine increased tritium overflow induced by field stimulation over the concentration range 1–1000 nM. At the highest concentration used, Wy26703 and phentolamine increased overflow by 140% and 260% respectively. In contrast, the response curve for yohimbine was bell-shaped (as has previously been reported by Rhodes *et al.*, 1983), the peak increase in overflow occurring at a concentration of 10 nM. The absolute potencies of the antagonists is shown in Table 3. The rank order of antagonist potencies in this preparation was yohimbine>phentolamine> Wy26703.

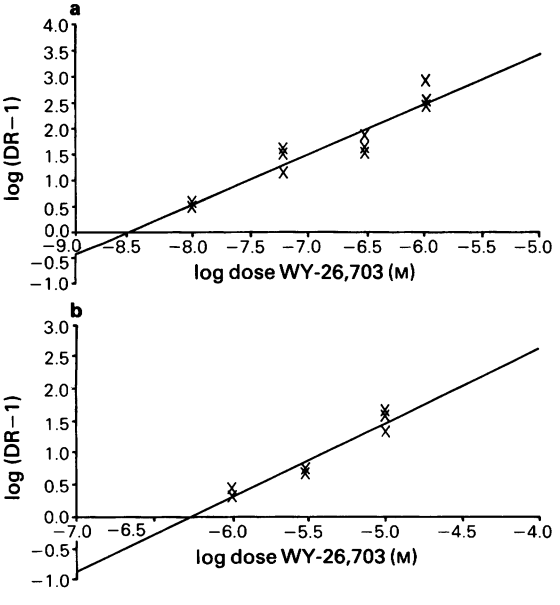

Figure 3 Schild plots derived from dose-response curves to UK-14,304 in the absence and presence of increasing doses of Wy26703 in rat atrium (a) and rabbit saphenous vein (b) preparations. In (a) $pA_2 = 8.52$, slope = 0.99 and correlation coefficient = 0.94; in (b) $pA_2 = 6.25$, slope = 1.16 and correlation coefficient = 0.97.

Discussion

Radioligand binding studies in rat brain and rabbit spleen membranes showed that K_i values for displacing [^{3}H]-rauwolscine differed markedly, depending on the tissue. Most slope factors for competition curves in

Table 3 Effect of antagonists on tritium overflow induced by nerve stimulation in the dog saphenous vein

Compound	*% change in overflow at 10^{-8} M*	*n*	*Conc. producing 50% increase in overflow* nM
Yohimbine	+75 ± 7	3	1.0
Phentolamine	+70 ± 14	4	4.2
Wy26703	+35 ± 14	3	16.0
Vehicle controls	−15 ± 5	6	–

% change in overflow is given as mean ± s.e.mean.

both brain and spleen tissue were close to unity indicating that the antagonists were interacting with a single population of binding sites. (The two exceptions being for idazoxan, which is known to have partial agonist activity, and prazosin in rat brain membranes). The different ranking of affinities of antagonists for rauwolscine binding sites indicated that α_2-sites are not homogeneous between tissues.

The classification of receptor types by radioligand binding can sometimes be misleading since not only can 'non-functional' sites be labelled in certain tissues but also many factors can alter the characteristics of binding in membrane preparations. Thus α_2-adrenoceptors are claimed to exist in multiple affinity states, the conformation being governed by the external conditions, e.g. ionic milieu and presence of guanine nucleotides (Michel *et al.*, 1980). Interpretation of data, therefore, can be complicated by the fact that membranes from different tissues or species may contain different proportions of high and low affinity sites and may vary in other factors which could affect binding characteristics, e.g. in amount of endogenous substances present. In an attempt to circumvent these problems, functional tests were therefore carried out to assess the potency of α_2-adrenoceptor antagonists at both pre- and postjunctional sites.

The prejunctional α_2-adrenoceptor on sympathetic nerve endings in rat left atria and the postjunctional α_2-adrenoceptor in rabbit saphenous vein were studied initially (Alabaster & Peters, 1984). Yohimbine, phentolamine and Wy26703 were all potent antagonists of the prejunctional α_2-receptor in rat atria. However, while yohimbine and phentolamine were equipotent in the atria and saphenous vein, Wy26703 showed an 186 fold selectivity for the receptor in the atria. This substantial difference in rank order and absolute potency was initially considered to indicate the existence of functionally distinct α_2-adrenoceptor subtypes in pre- and postjunctional loci.

However, further studies at the prejunctional α_2-adrenoceptor in the rabbit vas deferens preparation showed that Wy26703 was about 200 times less potent than in the rat vas deferens preparation, suggesting that the difference may in fact be one between species rather than between pre- and postjunctional location. This conclusion would appear to be substantiated by a more systematic comparison of a range of α_2-adrenoceptor antagonists (Lattimer & Rhodes, 1985). Thus, while yohimbine and rauwolscine were of equal potency, the benzoquinolizines and benzodioxans were very weak antagonists in the rabbit vas deferens compared with their potency in the rat vas deferens.

Interpretation of α_2-adrenoceptor antagonist activity has been complicated by the fact that prejunctional data are usually compared with postjunctional data in different species. Therefore the isolated saphenous vein of the dog was chosen for further studies since both pre- and postjunctional α_2-adrenoceptors can be studied in the same tissue. Although the data from the two assays were not strictly comparable due to differences in experimental design (agonists were not used so pA_2 values cannot be calculated from the overflow studies), the relative antagonist potency was the same at both sites, yohimbine being more potent than Wy26703. The absolute potencies of antagonists were dissimilar to those in both rat and rabbit tissues, but relative potency in the dog was the same as that found in the rabbit tissues. A similar ranking was also found in rabbit pulmonary artery overflow studies by Rhodes *et al.* (1983).

It has been proposed previously that α_2-adrenoceptors can be differentiated by radioligand binding studies on the basis of differing relative affinity of yohimbine and prazosin. (Nahorski *et al.*, 1985; Kawahara & Bylund, 1985). Thus for example the prazosin/yohimbine ratio in binding studies was 5–18 in the rat lung and brain but 800 in both the pig lung and human platelet (Latifpour *et al.*, 1982; Cheung *et al.*, 1982). Since the species differences are retained after solubilization of the α-receptor, differences in absolute conformation may exist between α_2-adrenoceptor subgroups (Kawahara & Bylund, 1985). Further evidence of differences in pharmacological characteristics between rodent and non-rodent α_2-adrenoceptors comes from functional studies measuring noradrenaline release from rat and rabbit brain slices (Ennis, 1985) and rat submandibular gland (Turner *et al.*, 1984). Further, it appears that this difference can be observed *in vivo* since Wy26703 was found to be much less potent than rauwolscine in pithed rabbits compared to pithed rats when α_2-induced pressor responses to catecholamines were studied (Bulloch *et al.*, 1985).

However, the subdivision of α_2-adrenoceptors into two groups, rodent and non-rodent is clearly an oversimplification. A recent study in kidney membranes from five different species reported marked

differences between species in the characteristics of α_2-sites labelled by [^{3}H]-rauwolscine (Neylon & Summers, 1985). The differences reported do not merely represent differences in species sensitivity to α antagonists since the rank order of potency of antagonists also varied with the tissue/site studied. Thus for example, yohimbine was more potent in kidney membranes of man followed by rat, mouse, rabbit and dog while idazoxan was more potent in membranes from rat followed by man, dog, rabbit and mouse. In addition, Nahorski *et al.* (1985) reported a difference in the affinity of prazosin for [^{3}H]-rauwolscine binding sites between tissues in the same species, in that prazosin was much less potent in rabbit platelets and cerebral cortex compared to rabbit kidney membranes.

In conclusion, data from our studies add to increasing evidence (see Waterfall *et al.*, 1985; Nahorski *et al.*, 1985; Kawahara & Bylund, 1985) that α_2-adrenoceptors are a heterogeneous population of receptors. Current knowledge would support the existence of α_2-isoceptors, i.e. heterogeneity between different species (Kawahara & Bylund, 1985) but further functional studies are required, using a wider range of tissues, to test the possibility that more than one sub-type of α_2-adrenoceptor can be present within the same species.

α-Adrenoceptor antagonists may have therapeutic potential in a number of disease areas, e.g. depression, diabetes and various cardiovascular indications. An important issue in the identification and evaluation of such drugs is to determine which animal tissues and models can predict α_2-adrenoceptor antagonist activity in man and indeed if α_2-adrenoceptors can be differentiated in man. Preliminary reports indicated that α_2-binding sites in human spleen, kidney, colon and platelets differed significantly from those in either rabbit spleen and kidney or in rat brain membranes (Dickinson *et al.*, 1985).

Further work is required to achieve a better understanding and clearer definition of α_2-adrenoceptor sub-groups. The identification of more selective antagonists should help to achieve this. In the meantime, care should be taken when interpreting and comparing data from different species and tissues on potency and receptor selectivity of α_2-adrenoceptor antagonists.

We are grateful to Mrs S. Boughton for her invaluable technical assistance.

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(*Received November 11, 1985.*
Revised February 18, 1986.
Accepted March 25, 1986.)