Br. J. Pharmac. (1984), **82**, 769–781

α-Adrenoceptor antagonism by apoyohimbine and some observations on the pharmacology of α-adrenoceptors in the rat anococcygeus and vas deferens

J.C. McGrath

Institute of Physiology, University of Glasgow, Glasgow G12 8QQ

1 α-Adrenoceptor antagonism of several test drugs was assessed against adrenergic contractile responses to field stimulation in rat vas deferens and anococcygeus, the prejunctional inhibitory effect of xylazine in vas deferens and the contractile effects of α-adrenoceptor agonists in anococcygeus.

2 Against the adrenergic nerve-induced contraction in vas deferens, the potency series was WB 4101 > prazosin > apoyohimbine > corynanthine > yohimbine > rauwolscine.

3 Against the inhibitory effect of xylazine in vas deferens the potency series was apoyohimbine > rauwolscine = yohimbine > WB 4101 ➢ prazosin and corynanthine.

4 In anococcygeus, against the contractile responses to adrenergic nerve stimulation or to the agonists amidephrine, noradrenaline and xylazine, the potency series was apoyohimbine > corynanthine > rauwolscine.

5 These results show that apoyohimbine is more potent than the yohimbine sterioisomers as an antagonist at α_1- and α_2-adrenoceptors but is no more selective.

6 The assay methods employed confirm the current classification of 'α'-receptors and drugs.

Introduction

Yohimbine and its stereoisomers, corynanthine and rauwolscine (α-yohimbine) have been recognized as adrenolytic for almost fifty years (see review by Bovet & Bovet-Nitti, 1948) and are now classified as α-adrenoceptor antagonists (Weitzell *et al.*, 1979). This may be the pharmacological mechanism behind their inclusion in mixtures of alkaloids, which are employed as anti-hypertensives, particularly on the continent of Europe.

In experimental pharmacology these compounds have played a key role in distinguishing sub-groups of α-adrenoceptors since yohimbine and rauwolscine are relatively selective for α_2- and corynanthine for α_1-adrenoceptors. Weitzell *et al.* (1979) examined these and several other compounds related chemically to yohimbine but found no others which were more selective or more potent than corynanthine and rauwolscine. The present study found that a derivative of yohimbine, apoyohimbine, was more potent, though no more selective, than the yohimbine isomers. The above compounds and some other α-antagonists were tested against pre- and post-junctional α-adrenoceptor agonism at sympathetic neuromuscular junctions in rat vas deferens and against α-adrenoceptors which mediate contraction of smooth muscle in rat anococcygeus.

An attempt is made to assess the suitability of the preparations for such purposes. The results also demonstrate that quantitative differences between the potencies of drugs, which, at first appear to indicate subdivisions among the receptor populations, can arise from the experimental protocol.

Preliminary accounts of these observations have been published (McGrath, 1981 a, b; 1982a).

Methods

Vasa deferentia or anococcygeus muscles were isolated from male Wistar rats (250–300g), killed by a blow on the head and exsanguination. All vasa were bisected transversely into two portions of equal length. The whole of each anococcygeus was taken excluding the ventral bar. Tissues were placed in Krebs-bicarbonate solution at 38°C and gassed with 95% O_2 plus 5% CO_2. Field stimulation was applied

via Ag: AgCl 'ring and hook' electrodes as described previously (Grass S88 or Square One Instruments stimulators, supramaximal voltage; pulse widths in text) (Gillespie, 1972; Anton *et al.*, 1977; McGrath, 1978).

Tissues were connected by thread to Grass FTO3 transducers and isometric tension was recorded on Grass Polygraph recorders.

Vas deferens

As shown previously (McGrath, 1978), the response of each portion of vas to a single supramaximal stimulus (0.5 ms) consists of two components, an early phase, Is, which is non-adrenergic and a later phase, IIs, which is α-adrenergic. In the prostatic portion, Is is dominant, reaching a peak at 200–250 ms after stimulation, and is followed by a minor second component (IIs) at 500–700 ms. In the epididymal portion, Is is the minor component and is followed by a dominant second phase (IIs).

It is thus convenient, in the epididymal portion, to assess the effects of α-adrenoceptor antagonists against the postjunctional receptors which are activated by endogenous noradrenaline (NA) Conversely, in the prostatic portion, the main response is non-adrenergic but is still susceptible to inhibition by prejunctional α-receptor agonism. The small postjunctional α-receptor component can be eliminated by reserpinization leaving a response against which prejunctional α-adrenoceptor antagonism can be assessed without interference of the test drug at the postjunctional site. In this case the agonist used is xylazine which has no detectable postjunctional effect within its prejunctional concentration-response range (MacDonald & McGrath, 1982).

Postjunctional α-adrenoceptors Contractile responses to single pulses were obtained every 5 min in epididymal portions. Antagonists were added cumulatively, each concentration just after a response had been obtained. Twenty minutes incubation was sufficient to produce equilibrium with the yohimbine analogues but prazosin and the WB compounds required up to 1 h at each concentration. The response to nerve stimulation was measured as the increment in tension above baseline at 650 ms after the stimulus. Antagonism was assessed as the percentage reduction in this response produced by the test drug.

Prejunctional α-adrenoceptors Contractile responses were obtained to single pulses every 5 min in prostatic portions from reserpinized rats. Increasing concentrations of xylazine were added cumulatively at 30 min intervals to produce graded inhibition. Paired preparations from the same rat were used to obtain dose-response curves for xylazine with and without a given concentration of antagonist. Fresh tissues were used for each concentration of antagonist since complete reversal of xylazine's effect could not be obtained consistently on wash-out. Responses were measured at 250 ms after the stimulus. For each pair of tissues an agonist dose-ratio was obtained at the concentrations producing 40% reduction in the response, which was within the steepest part of the concentration-response curve and took into account a residual response of approximately 15% remaining after 'maximal' inhibition.

Anococcygeus

Postjunctional α-adrenoceptors Contractile responses to single field stimuli were obtained and antagonists were added cumulatively as for the vas deferens. Responses were measured at the later time of 2 s since this corresponds to the peak in controls. Antagonists were also tested against trains of pulses as indicated in the text.

Smooth muscle α-adrenoceptors activated by exogenous agonists Non-cumulative concentration-response curves were constructed for the contractions to the agonists, xylazine, amidephrine and noradrenaline. Starting at the lowest concentration, the tissue was exposed to each concentration for 5 min. The bath was washed out several times until baseline had been re-established for at least 5 min before adding the next concentration. One concentration-response curve for one agonist was obtained from each tissue. Antagonism was assessed by comparison with the contralateral, antagonist-free control. Multiple concentration-response curves from single tissues were not employed since exposure to concentrations producing maximal contractions resulted in desensitization of the responses to low concentrations. For the same reason it was not possible to obtain an antagonist-free maximum response in tissues which were subsequently exposed to antagonists: curves of agonist concentration versus percentage of maximum response had, therefore, to be constructed from the maximum in the presence of antagonist. This was done only if such a maximum differed by less than 10% from that in the drug-free control. This precaution against possible depression of the maximum produced by non-competitive antagonism did not, in fact, prove necessary with the drugs employed. For each antagonist, at least twelve pairs of tissues were employed, normally as four pairs at each of three concentrations (in 10 times steps) and pA values were calculated by linear regression of log agonist dose-ratio minus one versus $-\log$ antagonist concentration.

Stock solutions of drugs were dissolved in Krebs solution and were added to the bath to give the required molar concentration. Exceptions were:

prazosin (distilled water); noradrenaline (distilled water with EDTA 20 μM); rauwolscine (mixed with w/w ascorbic acid in distilled water). The following drugs were used: (−)-amidephrine hydrochloride (Mead Johnson); apoyohimbine hydrochloride (Aldrich), BHT 933 hydrochloride 2 - amino - 6 - ethyl - 5,6,7,8 - tetrahydro - 4H - oxazolo - [4,5 - d] - azepine.2HCl (azepexole; Thomae), clonidine hydrochloride (Boehringer Ingelheim), corynanthine tartrate (Aldrich), guanabenz acetate (Wyeth), M7 2 - N,N - dimethylamino - 5,6 - dihydroxy - 1,2,3,4 - tetra - hydronaphthalene.HBr (gift, Dr R. Whiting), nifedipine (Bayer), noradrenaline bitartrate (Koch-Light), piperoxan (933F) and prosympal (883F) (gifts from Prof. A.G.H. Blakeley), prazosin hydrochloride (Pfizer), reserpine, crystalline (Koch-Light), raubasine base (Inverni Della beffa), rauwolscine base (α-yohimbine) (Inverni della Beffa), RS 21361 2 - (1 - ethyl) - 2 - imidazolyl methyl) - 1,4 - benzodioxan (imiloxan; Syntex), Sgd 101/75 4(2 - imidazoline - amino) - 2 - methylindazol-chlorhydrate (Siegfried), WB 4101 N - [- (2,6 dimethoxyphenoxy) ethyl] - 1,4 benzodioxane - 2 - methylamine (Ward Blenkinsop) and WB 4093 N - (phenoxyethyl) - 1,4 benzodioxane - 2 - methylamine (Ward Blenkinsop), xylazine hydrochloride (Bayer).

Reserpine pretreatment of rats Reserpine was dissolved in 2% (w/v) ascorbic acid and, when appropriate, was administered intraperitoneally (3 mg kg^{-1}) 18 h before killing the rat. This schedule reduces the noradrenaline content of the rat vas deferens by more than 98% (Gillespie & McGrath, 1974a).

Chemical sympathectomy 6-Hydroxydopamine was dissolved in de-oxygenated saline containing ascorbic acid (1 mg kg^{-1} to animal). Three intraperitoneal doses of 6-hydroxydopamine were given; day 1, 100 mg kg^{-1}; day 4, 2 × 100 mg kg^{-1}. Rats were killed on day 5.

The Krebs-bicarbonate solution had the following composition (mM): NaCl 119, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, glucose 11.1 and was gassed with 95% O_2 plus 5% CO_2.

Results

Vas deferens

Postjunctional α-adrenoceptors The effects of antagonists against the epididymal portions' responses to single pulses are shown in Figure 1. WB 4101 was the most potent of the compounds tested. Apoyohimbine was less potent than prazosin but more potent than any of the stereoisomers of yohimbine, e.g. it was 10 times as potent as yohimbine. The stereoisomers were of a similar potency: corynanthine was only 1.3 times as potent as yohimbine and 2.1 times as potent as rauwolscine. Yohimbine sulphuric acid, which is the intermediate in the synthesis of apoyohimbine from yohimbine had no detectable effect up to 10 μM. Raubasine was tested: this is analogous to apoyohimbine, having a planar but heterocyclic ring E. It inhibited responses with a threshold of between 300 nM and 1 μM and produced a 'maximum' effect at around 300 μM but quantification was difficult since it was insoluble in water, requiring either tartaric acid or alcohol, each of which, given as a solvent control reduced responses.

WB 4093, a close relation of WB 4101, was very potent; more than prazosin but less than WB 4101 (Figure 1c). It was noticeable that the three most potent compounds, WB 4101, WB 4093 and prazosin, required a long time to reach equilibrium when given at low concentrations. At the concentrations producing 50% inhibition of the responses, this time was between 40 and 60 min. Before this was appreciated an earlier series of experiments with only a 20 min incubation, had suggested a much lower potency for prazosin and WB 4101 (Figure 1a and c). Since these compounds are chemically dissimilar, the slow onset may be explained by a reduced rate of diffusion from the saline to the receptors due to the relatively small concentration gradient.

Two of the long-known benzodioxan-based antagonists were tested. 933F (piperoxan) and 883F (prosympal) were of a comparable potency to corynanthine, i.e. two to three orders of magnitude less potent than the WB series (Figure 1c).

A new 'selective' antagonist, RS 21361 (imiloxan), which is another benzodioxan derivative (Michel & Whiting, 1981), was less potent than any of the other antagonists tested (Figure 1c).

Prejunctional α-adrenoceptors

Antagonists versus xylazine In reserpinized vasa, xylazine-induced inhibition of Is in the prostatic portion was antagonized in a competitive manner by apoyohimbine as shown by the parallel displacement of the log concentration-response curve and a Schild plot with a slope near to unity (Figure 2).

The other antagonists did not provide such a simply analysed, classical picture. Rauwolscine and yohimbine were of similar potency at 100 nM but at higher concentrations yohimbine appeared to be more potent. Consequently, the Schild plot for yohimbine was steeper so that extrapolation might suggest a lower pA_2 value than for rauwolscine. Up to the highest concentrations which could be tested, corynanthine and prazosin were virtually devoid of antagonism against xylazine in this system. At very high concentrations, all of the α-adrenoceptor antagonists which we have tested produce an increase in the nerve-induced contraction, presumably due to an excitatory effect on the smooth muscle (McGrath, 1978). This effect had started to appear with prazosin

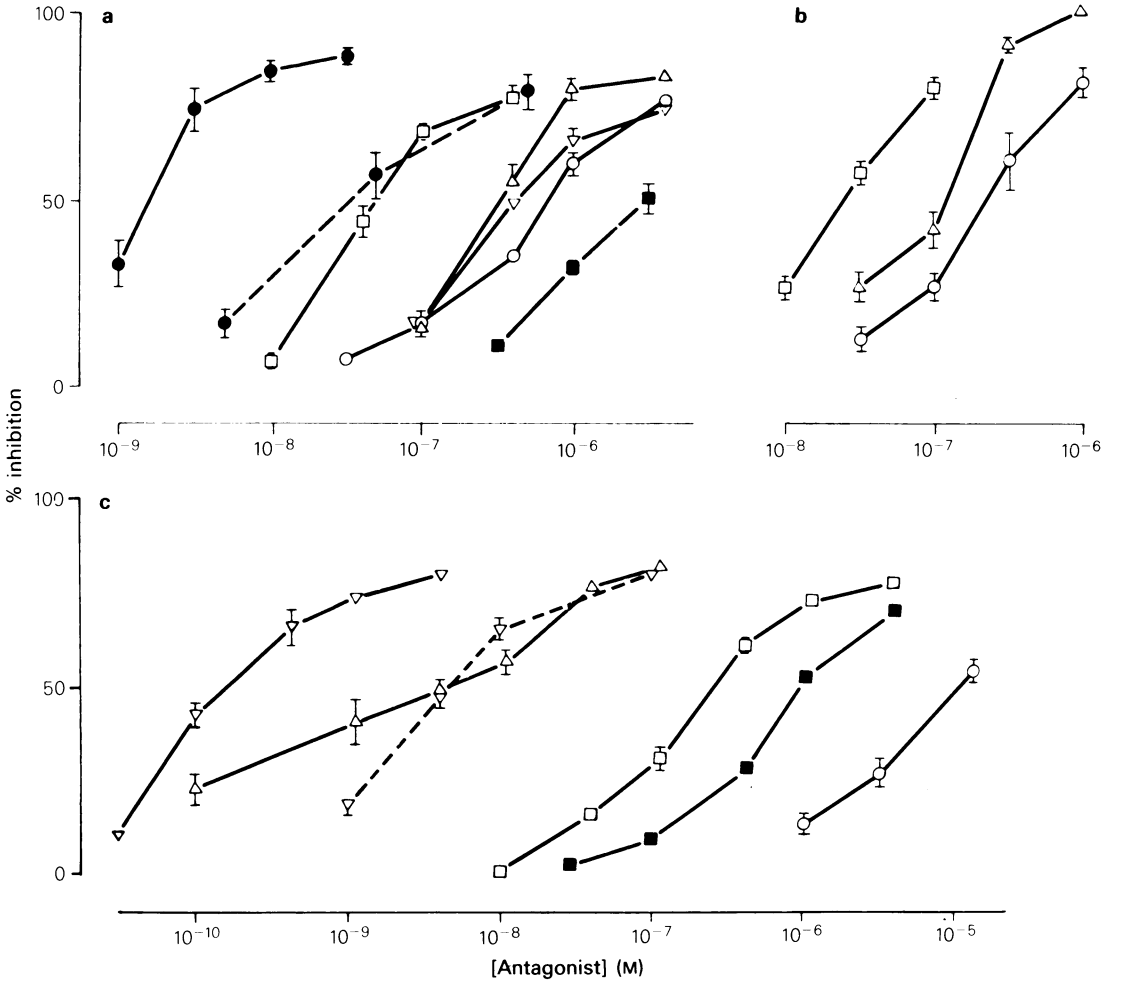

Figure 1 Effects of α-receptor blockers on contractile responses to single field stimuli (0.5 ms, supramaximal voltage, 5 min intervals) of epididymal portions of rat vas deferens (a and c) and rat anococcygeus muscles (b). Response expressed as percentage of the drug-free control 'peak', were obtained 20 min after addition of the antagonist and were measured at a fixed time after stimulation (vas, 650 ms; anococcygeus, 2 s). This represents 'equilibrium' for all antagonists except the WB compounds and prazosin. For WB 4101 and prazosin the responses are shown both after 20 min (dashed lines) and 60 min (solid lines). Points and vertical bars represent the means ± s.e.means for sets of at least 6 experiments (s.e.means are omitted when smaller than symbols). (a) and (b) Prazosin(●), apoyohimbine (□), corynanthine (△), yohimbine (▽), rauwolscine (○), and raubasine (■). (c) WB 4101 (▽), WB 4093, (△), piperoxan (□), prosympal (■) and RS 21361 (○).

1 μM and corynanthine 10 μM, rendering assessment of antagonism impracticable from these concentrations upwards. In this respect, yohimbine was the most potent of the stereoisomers, producing 20 ± 4% ($n = 6$) potentiation of the Is response at 1 μM compared with rauwolscine's 4 ± 2% ($n = 6$): this is sufficient to account for the apparent difference in inhibitory potency between yohimbine and rauwolscine. Apoyhimbine 6 μM produced an increase of 164 ± 28% ($n = 6$), which is the highest increase recorded with the exception of azapetine (McGrath, 1978). Raubasine (1 μM) did not significantly antagonize the effect of xylazine and at higher concentrations it was difficult to disentangle drug and solvent-induced effects. The WB compounds were comparable in potency to the yohimbine series (Figures 2 and 6).

Antagonists versus endogenous NA It has previously been shown that α-adrenoceptor antagonists can in-

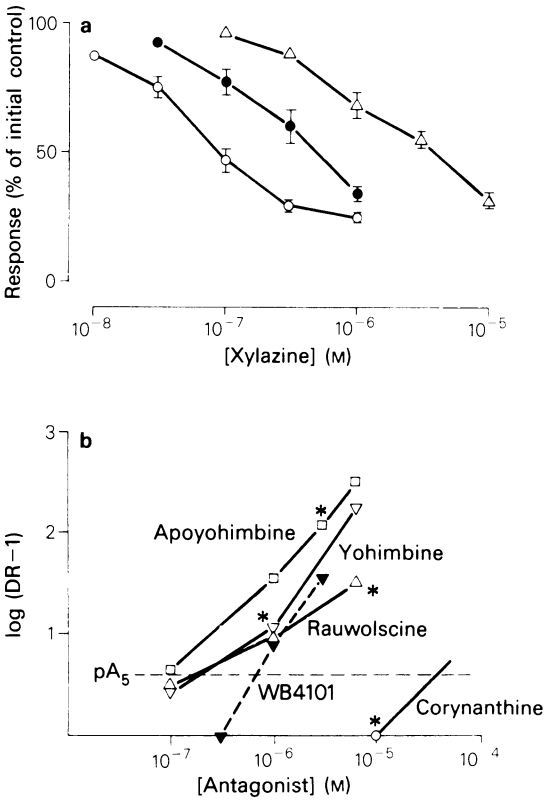

Figure 2 Antagonism of the inhibitory effect of xylazine on nerve-induced contractile responses of prostatic portions of vasa from reserpinized rats (single supramaximal stimuli, 0.5 ms). (a) Concentration-response relationship for inhibition by xylazine; with no antagonist present (○), apoyohimbine 0.1 μM (●), apoyohimbine 1 μM (△), ($n=6$). (b) An attempt at construction of Schild plots for various antagonists; apoyohimbine (□), yohimbine (▽), rauwolscine (△), WB 4101 (▼) and corynanthine (○). Symbols indicate mean values for log (DR − 1) obtained at fixed concentrations of antagonists in experiments as shown in (a) ($n=4$ to 10). Asterisks denote the lowest concentration of each antagonist which produced an increase in the contractile response of at least 10% when given in the absence of xylazine. The pA_5 level indicated by the horizontal dashed line was used to obtain interpolated estimates of antagonist potency, which were plotted in Figure 6. Standard errors are omitted for clarity but were never more than twice the width of the symbols.

crease the contractile response of the rat vas deferens to a train of pulses and that, for an antagonist which lacks selectivity, this can be demonstrated more easily if the postjunctional α_1-adrenoceptors are blocked (Brown *et al.*, 1979; 1983). Since apoyohimbine comes into this later category, its potentiating effect is demonstrated in the presence of WB 4101 (0.1 μM) Figure 3c. The external Ca^{2+} concentration is critical for the demonstration of the endogenous, α_2-receptor mediated, prejunctional modulation of NA release in guinea-pig vas deferens (Alberts *et al.*, 1981). Increasing the $[Ca^{2+}]$ decreases the effectiveness of an α_2-receptor mediated inhibition of transmitter release but also increases the output of transmitter, hence increasing the concentration of NA available to activate the α_2-receptors. The effectiveness of endogenous feedback is, thus, subject to opposing influences. In practice, in rat vas deferens, it was easier to demonstrate feedback at $[Ca^{2+}] = 2.5$ or 5.0 mM rather than the more physiological 1.25 mM (Figure 3e).

Anococcygeus

Nerve stimulation

Single pulses The potency series (apoyohimbine > corynanthine > rauwolscine) was similar to that found in vas deferens (Figure 1a, b). Rauwolscine was less potent than corynanthine with or without cocaine (3 μM). If the concentration of apoyohimbine was increased from 10 μM to 30 μM or more, the responses recovered towards control levels. This did not occur in the presence of cocaine (30 μM) and so might be due to blockade of the neuronal uptake of NA.

Trains of pulses At a concentration of 1 μM, at which each antagonist abolished the response to a single pulse, rauwolscine did not reduce the response to a train of 40 pulses at 8 Hz (control/drug-present = 108 ± 7%, $n=7$) whereas corynanthine did (22 ± 8%, $n=8$). At 100 μM, rauwolscine showed a potentiation of the response of variable size (5–30%), whereas corynanthine produced only blockade. Potentiation could not be demonstrated with apoyohimbine at 10 nM to 10 μM, graded blockade occurring. As would be expected from the above and from the concept of α_2-adrenoceptor-mediated negative feedback, rauwolscine blocked the response to the first few pulses in a train despite increasing the later peak (Figure 3).

Agonists

Noradrenaline In order to compare NA with other agonists it was necessary to block the neuronal uptake of NA with cocaine and to prevent the oxidation of NA by including ascorbic acid or EDTA. Since the presence of these compounds is neither necessary nor desirable when examining other agonists, the specificities of their effects were tested.

Without ascorbic acid or EDTA, the contractions to NA were short-lived. Contraction was maintained for longer as the concentration increased but even at

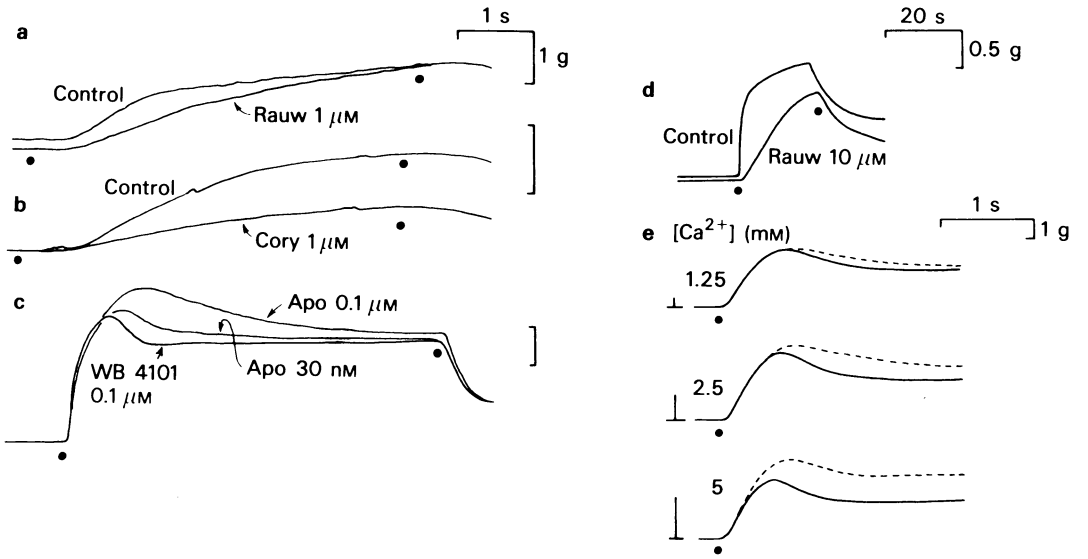

Figure 3 The effects of antagonists, rauwolscine (Rauw), corynanthine (Cory) and apoyohimbine (Apo), on the contractile responses to trains of pulses in the rat ancoccygeus and vas deferens (supramaximal pulses, 0.5 ms, trains between dots). Traces have been superimposed to allow close comparison of time courses.
(a, b and d) Anococcygeus, (c and e) vas deferens (epididymal portions). In (c) three sequential responses are shown: first in the presence of WB 4101 (0.1 μM) then with the further addition of apoyohimbine at 30 nM and finally 300 nM. In (e) three pairs of responses are shown at different levels of Ca^{2+} in the bathing solution. Solid line, control; dashed line, in the presence of rauwolscine 0.1 μM. Vertical lines preceding the main response represent the heights of the responses to single stimuli in this vas at the different $[Ca^{2+}]$. Stimulation was continued beyond the time shown: only in 5 mM Ca^{2+} did the response remain potentiated after 5 s.

NA 1 μM equilibrium was not established. This could be attributed entirely to breakdown of NA and was prevented by addition of ascorbic acid or EDTA or by continuously washing the bath with freshly diluted NA in Krebs solution. Consequently ascorbic acid (10 or 20 μM) was employed in all early experiments with NA until it was demonstrated that EDTA was more effective in preventing oxidation, viz. A sample of NA (5 ng ml^{-1}, 15 nM) was incubated in Krebs solution at 37°C; after 9 min none was detectable in the presence of ascorbic acid (20 μg ml^{-1}), while 52% remained if EDTA (10 μg ml^{-1}) was present (MacRae, 1983). Neither substance is ideal. Each attenuates adrenergic transmission in vas deferens and anococcygeus to some extent (A. MacDonald & J.C. McGrath, unpublished observations), ascorbic acid has sympathomimetic actions in higher concentrations (Gillespie & McGrath, 1975) and EDTA in higher concentrations will chelate a significant proportion of the Mg^{2+} and Ca^{2+}. There was also evidence for a potentiation by ascorbic acid of responses to low concentrations of NA, which could not be accounted for entirely by attenuation of NA breakdown; this produces a spurious increase in the pD_2 values for NA (see next paragraph). Alteration of the redox potential by these concentrations of ascorbic acid may have effects upon intracellular enzymes such as guanylate cyclase and this could increase smooth muscle sensitivity to contractile agents. Cocaine was used to block the neuronal uptake of NA since its side-effects seem no worse than those with other such compounds. Above 3 μM, cocaine contracts the anococcygeus (Gillespie & McGrath, 1974b; 1975) and, even at 3 μM, it often contracted the tissue on first addition to the bath. This concentration was, however, employed since it shifted the concentration-response curve for NA to the left to the same degree as sympathectomy with 6-hydroxydopamine, suggesting that uptake has been effectively removed as a factor modifying the concentration of NA at the smooth muscle α-adrenoceptors. In the presence of cocaine but the absence of an anti-oxidant, e.g. ascorbic acid or EDTA, the contraction to low concentrations of NA (<100 nM) was very transient, returning to baseline within one minute. Cocaine (3 μM) did not affect the EC_{50} to xylazine or amidephrine although the response to the threshold concentration of xylazine was increased indicating some excitatory action of cocaine.

Cocaine (3 μM) shifted the concentration-response curve for NA to the left; pD_2 5.9 ± 0.2 ($n = 10$) to

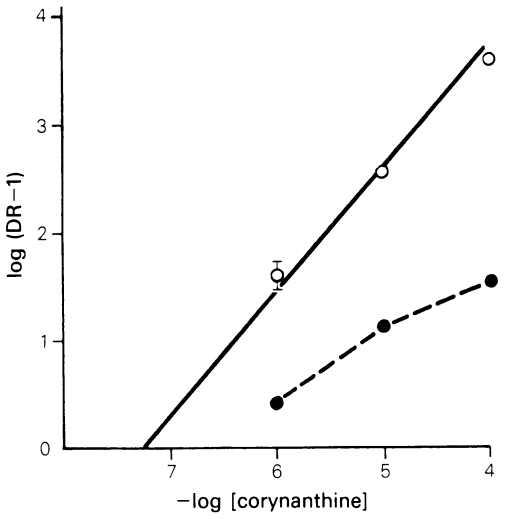

Figure 4 The effect of cocaine (3 μM) on antagonism by corynanthine of the contractile response to NA in anococcygeus. Points shown are the mean values of log (DR−1) at each concentration of antagonist (n= 4 to 10). With cocaine (open symbols) the regression line is shown. Without cocaine, the dashed line simply connects the means. The vertical bar represents the s.e.mean when this is greater than the height of the symbol.

7.2±0.1 (n= 23) (EDTA 20 μM present); or 5.9±0.2 (n=6) to 8.2±0.2 (n=6) (ascorbic acid 20 μM present). After pretreatment with 6-hydroxydopamine (EDTA 20 μM present), the pD_2 for NA was 7.1±0.1; after cocaine (3 μM) the pD_2 was 7.2±0.2, which was not significantly different from either its own control or from tissues from untreated rats in the presence of cocaine ($P>0.05$, n=6).

Apoyohimbine produced a parallel rightward displacement of the concentration-response curve to NA giving a pA_2 of 7.6 (EDTA present here and in all following experiments involving NA).

Corynanthine was tested against NA with and without cocaine. The rightward displacement of the concentration-response curve was non-parallel, the slope becoming steeper, and producing a curving Schild plot. With cocaine, displacement was parallel and a linear Schild plot had a slope near to unity and a pA_2 = 7.3 (Figure 4)

Prazosin was tested against NA and had a pA_2= 9.3.

Amidephrine Amidephrine was used without the addition of cocaine or anti-oxidants. It was slightly more potent than NA with a pD_2 of 7.7±0.1 (n= 15). Antagonism by the test compounds was straightforward, with parallel displacement and classical Schild plots, revealing pA_2 values of:

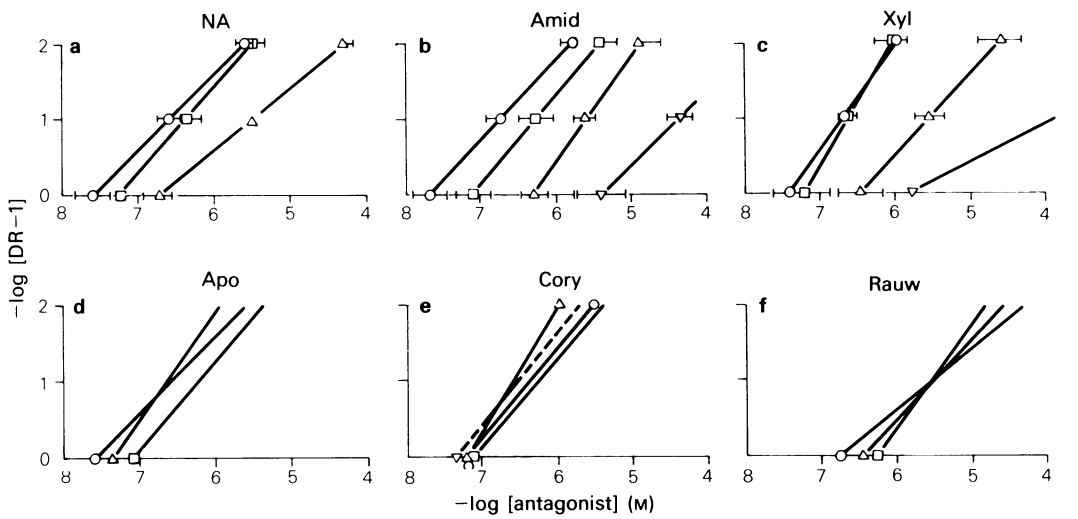

Figure 5 Schild plots for antagonism of α-adrenoceptor agonists by antagonists in the rat anococcygeus. (a–c) Each graph compares different antagonists , apoyohimbine (○), corynanthine (□), rauwolscine (△) and RS 21361 (▽) against a single agonist. In (a) the agonist is noradrenaline, (b) amidephrine and (c) xylazine. (d–f) Each graph compares the effects of a single antagonist, (d) apoyohimbine, (e) corynanthine and (f) rauwolscine, against different agonists; (○) noradrenaline, (□) amidephrine, (△) xylazine, (▽) Sgd 101/75. Regression lines are shown with 95% confidence limits indicated by horizontal bars in (a–c). The dashed line in (e) indicates the agonist Sgd 101/75.

apoyohimbine, 7.8; corynanthine 7.3; rauwolscine, 6.3; raubasine, 7.5. Corynanthine (1 μM) and rauwolscine (10 μM) were tested with and without cocaine (3 μM); this made no significant difference ($P > 0.05$; $n = 6$) to their antagonism against amidephrine.

Xylazine Xylazine was less potent ($pD_2 = 5.5 \pm 0.1$, $n = 24$) and produced a smaller, more variable maximum (5.5 ± 1.5g, $n = 24$) than either NA (7.42 ± 0.45g, $n = 23$) or amidephrine (7.52 ± 0.58g, $n = 15$). However no other evidence could be found that it was a partial agonist for the receptors activated by NA, in the sense of acting as an antagonist against it. Extensive investigation of the interaction between NA(1nM–0.1 mM) and xylazine (0.1 μM–0.1 mM) revealed only that contractions were additive or more than additive. The time course of the contraction to xylazine was characteristic. After addition to the bath it produced either a quiescent delay or a slow gradual rise according to concentration, followed after several minutes (up to eight minutes with threshold concentrations) by a sharp rise which then developed into rhythmic activity. This property was shared by other non-phenylethanolamine α-adrenoceptor agonists, e.g. M7 (> 0.3 μM), guanabenz (> 1 μM), BHT 933 (10 μM) and clonidine (> 0.1 μM). Nevertheless the concentration-response curve to xylazine was displaced by antagonists in the classical manner with results similar to those against amidephrine: pA_2 values; apoyohimbine 7.5, corynanthine 7.5, rauwolscine 6.4. Similarly, the responses to the other non-phenylethanolamine agonists were antagonized by corynanthine (1 μM), indicating activation of α_1-adrenoceptors.

In a further attempt to identify an α_2-mediated response, RS 21361 was tested against amidephrine and xylazine. It did not differentiate between the two agonists, producing only a small degree of non-competitive antagonism at a high concentration (Figure 5).

Sgd 101/75 Responses to this compound are antagonized by phenoxybenzamine relatively more powerfully than are those to NA (Coates *et al.*, 1982). Under the present experimental conditions, it had a pD_2 of 5.62 ± 0.05 and a maximum response of 4.7 ± 0.4 g ($n = 6$). Corynanthine competitively antagonized Sgd 101/75 ($pA_2 = 7.5$).

Discussion

In the vas deferens it is relatively simple to examine α-adrenoceptor antagonism at pre- and post-junctional sites. The results with known compounds are as expected and the previously untested apoyohimbine is revealed as more potent at both sites than the yohimbine stereoisomers but without any improvement in selectivity.

Despite highlighting some problems in comparing the effects of α-adrenoceptor antagonists, this study has indicated that apoyohimbine is more potent at both postjunctional α_1- and prejunctional α_2-adrenoceptors than yohimbine stereoisomers. Based on this observation, further requirements for α-adrenoceptor blockade have been proposed and published elsewhere (McGrath, 1982b). Although apoyohimbine has been recognized as an adrenolytic and hypotensive agent for many years (Bovet & Bovet-Nitti, 1984), it does not seem to have been employed as an anti-hypertensive agent, in contrast to rauwolscine and raubasine which are included in many anti-hypertensive mixtures of alkaloids. According to the present results, neither of the latter drugs is highly potent against either α_1-or α_2-adrenoceptors and might require high doses, particularly for peripheral, as opposed to CNS, effects. However, rauwolscine does have more selectivity for α_2-receptors relative to the other compounds tested in this and other systems (Weitzell *et al.*, 1979; McGrath, 1982b) and would, thus, be expected to produce a different range of effects *in vivo*. Raubasine presented solubility problems, had some selectivity for α_1-receptors and was less potent than the yohimbine stereoisomers, confirming, in general, another recent comparison with yohimbine on rat vas deferens (Demichel *et al.*, 1981).

If the pre- and post-junctional potencies are graphed against each other it is easy to see the characteristics of each compound (Figure 6). In this way, the potency at each receptor can be seen together with the ratio of post/pre-junctional activity. For example, among the yohimbine stereoisomers the only striking difference is the impotence of corynanthine at the prejunctional site. Among the compounds which are selective for the postjunctional site it is clear that corynanthine and prazosin achieve this by lacking any detectable effect at prejunctional receptors while WB 4101 has reasonable prejunctional potency gauged against other compounds but has such extreme potency against the postjunctional receptors that its ratio of selectivity remains high. Among those 'selective' for prejunctional receptors, rauwolscine has a slight advantage over yohimbine due to a marginal decrease in postjunctional and marginal increase in prejunctional actions. RS 21361, which has been tested using the same methods employed here (Michel & Whiting, 1981) is no more potent than rauwolscine but gains selectivity through its very weak postjunctional effect. At the time of testing, apoyohimbine was the most potent antagonist at α_2-adrenoceptors that had been described.

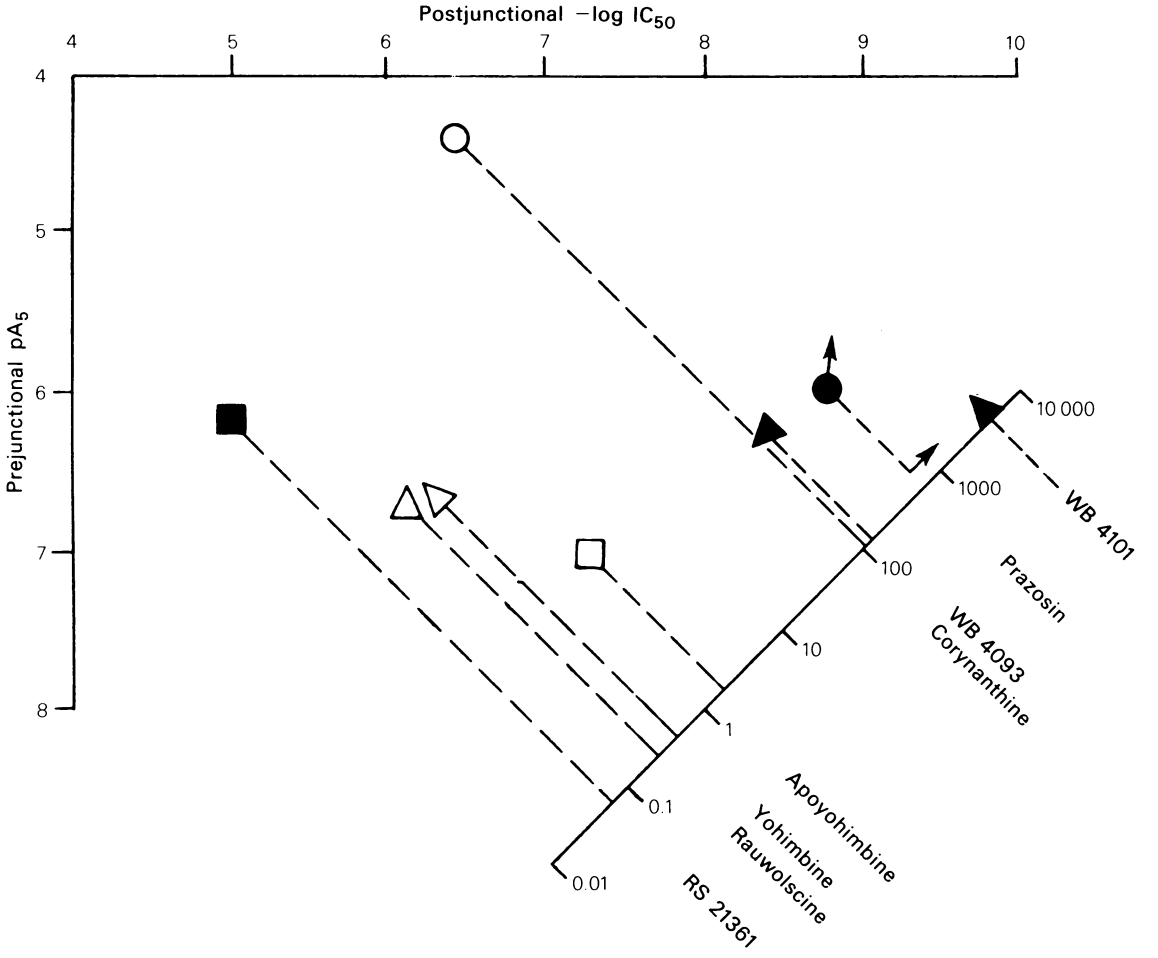

Figure 6 Comparison of the pre- and post-junctional α-adrenoceptor blocking potencies of antagonists in the rat vas deferens. Prejunctional pA_5 values were derived as in Figure 2. Postjunctional potencies (−log concentration producing 50% inhibition of the adrenergic contraction to a single pulse) were derived from Figure 1. The method did not allow estimation of a prejunctional value for prazosin so the arrow indicated that the appropriate point should lie somewhere above the symbol. Extrapolation via the dashed lines to the diagonal axis indicates the ratio of post: pre-junctional potency.

Thus in the rat vas deferens, the postjunctional receptors activated by nerves fit the α_1-adrenoceptor and the prejunctional receptors the α_2-adrenoceptor category.

Since this type of plot involves pre- and post-junctional receptors within the sympathetic neurotransmission system in the same organ it should have predictive value for whether an antagonist will potentiate or inhibit responses to a train of nerve stimuli. On its own, the response to a train can be used to confirm selectivity which is already known, but leads to complications as a predictor (Brown *et al*, 1979). In fact the situation is more complex in the vas deferens than in other tissues since part of the nerve-mediated response is resistant to postjunctional α-receptor antagonism. Nevertheless, rauwolscine, the compound with the greatest selectivity, was the only one which would increase the response to a train as its first effect at a low concentration. Yohimbine would do this in individual experiments but even its small increase in postjunctional potency compared to rauwolscine was sufficient to balance the prejunc-

tional effect. In a separate study, the non-adrenergic response was eliminated by the Ca^{2+} entry blocker nifedipine, and it was found that the prejunctional effect of endogenous NA was exerted against both adrenergic and non-adrenergic elements of transmission (Brown *et al.*, 1983). Thus, the present results lend support to the hypothesis that potentiation of responses to trains of stimuli by α-receptor antagonists is due to withdrawal of an endogenous restraint rather than any other effect of the drugs (see Kalsner, 1982; Rand *et al.*, 1982).

The increased size of the response to a single pulse, produced by high concentrations of antagonists, indicates that potentiation can come from an excitatory effect of such drugs, which is unrelated to antagonism at α-adrenoceptors. Thus an antagonist may appear to be more potent at reversing the inhibitory effect of a prejunctional agonist than it actually is. Conversely, a postjunctional excitatory agonist can increase the nerve-induced response by such a mechanism and could be mistaken for a prejunctional antagonist. This has already been demonstrated with the present method for the α_1-receptor agonist cirazoline (Docherty & McGrath, 1984). This places a limitation on the use of this type of preparation, particularly for the assessment of prejunctional antagonism. However, this problem can be detected if the effect of the test drug is tested against the nerve–induced response in the absence of the agonist. The method is unsuitable only when the excitatory action occurs at lower concentrations than the antagonism under scrutiny.

The effects of low concentrations of the antagonists against responses of the vas deferens to trains of pulses show that the prejunctional receptors activated by endogenous noradrenaline are similar to those activated by exogenous xylazine and are α_2-receptors. Similarly, in the anococcygeus, the concept of endogenous feedback is supported by the effect of rauwolscine. This drug blocked the response to the first few pulses while increasing the contraction to the later part of the pulse train. The physiological significance of this feedback system, under normal circumstances is, however, called into question since it could not be clearly demonstrated at physiological levels of ionized calcium.

The extreme potency of WB 4101 was interesting and accords with its effect against exogenous NA (Kapur & Mottram, 1978). WB 4093 has been found to be selective for α_2-adrenoceptors in some (Kapur & Mottram, 1978) but not all (Drew, 1979) circumstances. The present results suggest that one contributing factor may be the slow onset of action. The greatest difference between WB 4101 and WB 4093 on the one hand and the older benzodioxan derivatives piperoxan and prosympal is the much greater potency of the former for α_1-adrenoceptors, potency at α_2-adrenoceptors being of a similar order for each (see also Drew, 1977; Kapur & Mottram, 1978). The N-substituent seems to be of greater value for α_1-receptor rather than α_2-receptor antagonism. This remains true for the selective α_2-antagonist, RS 21361 (imiloxan), which loses α_1-receptor but retains α_2-receptor potency relative to piperoxan.

The contribution of postjunctional α-adrenoceptors to sympathetic, noradrenergic transmission is currently under debate since the potency series for antagonists and their absolute potencies is often different against agonists compared with nerve-induced contraction. This takes three main forms. (i) The γ-adrenoceptor hypothesis, in which NA is the neurotransmitter but the junctional receptors are not α-receptors (e.g. Hirst & Neild, 1980). (ii) Co-transmission, in which another transmitter supplements NA (Burnstock, 1976). (iii) 'Intra-junctional' and 'extra-junctional' receptors, each of which may be an α-receptor but which mediate responses to neurotransmitter and exogenous agonist (or hormone), respectively, and which thus belong to subgroups of a functional and possibly also of a pharmacological nature (Ariens & Simonis, 1976). These hypotheses prompted the present comparison of the α-adrenoceptors which mediate nerve- and agonist-induced responses. This comparison was made in the anococcygeus rather than on the vas deferens because it was clear at the outset that the activation processes involved with nerve- and agonist-induced contractions of the vas are radically different from each other and, furthermore, no equilibrium can be attained for agonist-induced contraction (MacDonald & McGrath, 1982; Brown *et al.*, 1983). In the anococcygeus, the effects of the antagonists against three agonists provided a potency series which closely resembled that against endogenous noradrenaline in both the anococcygeus itself and the vas deferens, i.e. apoyohimbine > corynanthine > rauwolscine, indicating α_1-adrenoceptors. This close similarity between the receptors activated by the neurotransmitter in the two tissues and the three agonists in the anococcygeus suggests that there is no need here to invoke anything other than a single population of α_1-adrenoceptors to explain the mediation of adrenergic neurotransmission or the contractions to exogenous sympathomimetic agonists. The possibility of some further transmitter in the vas, which mediates the α-receptor blocker-resistant phase Is of the response, is, of course still open. This does not, however, seem to influence the adrenergic response itself, which remains unaffected after the Is response has been eliminated by nifedipine, α, β-methylene ATP or $ANAPP_3$ (Brown *et al.*, 1983; Sneddon & Westfall, 1984; Burnstock & Sneddon, 1984; T. Cunnane & J.C.McGrath, unpublished observations). Thus, the α-receptor-mediated transmis-

sion is not facilitated by the second transmitter as has been suggested for other cases where two transmitters function alongside each other (Lundberg & Hokfelt, 1983).

Xylazine was included in an attempt to identify any postjunctional α_2-adrenoceptors which might have been present. The potency series for the antagonists, however, proved to be virtually identical against xylazine and amidephrine confirming that no postjunctional α_2-adrenoceptors could be demonstrated in this tissue (see also Docherty & Starke, 1981). Furthermore, RS 21361, an α_2-adrenoceptor antagonist did not differentiate between agonists and exhibited little potency. There is, thus, no evidence for anything other than α_1-adrenoceptors.

A subdivision of α_1-adrenoceptors in the rat anococcygeus has been suggested on the basis of the relative susceptibility of Sgd 101/75 to phenoxybenzamine at concentrations which have relatively little effect on NA (Coates *et al.*, 1982). The present study shows that corynanthine is more potent against Sgd 101/75 than against NA, if the neuronal uptake of NA is not blocked. However, this difference disappeared in the presence of cocaine: the antagonism of Sgd 101/75 by corynanthine was of the same order as that against amidephrine, noradrenaline or xylazine. On the basis of the present results, the actions of Sgd 101/75 and xylazine on the anococcygeus are, essentially, similar. Each produces a smaller maximum than NA and the response to each is relatively more susceptible to the Ca^{2+}-entry blocker nifedipine than is that to NA (McGrath, 1983; 1984). Although this inability to induce the maximum response suggests partial agonism, the other main criterion for partial agonism, antagonism of the response to a full agonist, could not be demonstrated with either compound (Coates *et al.*, 1982; this paper). This has been shown for oxymetazoline versus NA (Kenakin, 1984). However, oxymetazoline (pD_2, 8.87. Gibson & Pollock, 1973) is much more potent than Sgd 101/75 or xylazine, allowing assessment of its pK_d (6.5, Kenakin, 1984). It is not feasible to derive the pK_d for xylazine or Sgd 101/75 since their pD_2 values (5.5 and 5.62) are so high. The experimental protocol necessary to derive a pA_2 value (and hence an estimate of pK_d) for oxymetazoline, required concentrations of the drug 300 to 3000 times the ED_{50}. Thus, for xylazine or Sgd 101/75 this would be in the region of 1–10 mM, concentrations at which their effects are no longer selective. Thus the available data are consistent with the conclusions of Kenakin (1984), in his parallel study, that no subdivision of α-adrenoceptors in the anococcygeus is necessitated by agonist/antagonist interactions.

It was necessary to reappraise the use of some of the adjuncts which are commonly employed in adrenergic studies, since, by allowing the appearance of responses to lower concentrations of NA, they induced qualitative as well as quantitative changes in the contractile response. This enables a proper comparison of NA with other agonists. The response to NA becomes more akin to that of xylazine (and the other non-phenylethanolamine agonists). There is an increase in the degree of rhythmic activity and in the contribution from the nifedipine-sensitive Ca^{2+} channel (McGrath, 1983; 1984). For this reason, care is necessary with any additive which might affect Ca^{2+} disposition or the enzymes involved in excitation-contraction coupling. To preserve NA from oxidation it is necessary to include either ascorbic acid or EDTA. Since ascorbic acid was less effective than EDTA, had no additive effect with the latter (MacRae, 1983) and modified muscle responsiveness to nerve stimulation or agonists, it seems prudent to discontinue its use. Thus it is necessary to use EDTA, provided that a check is made on its effects against nerve-mediated responses and against agonists which are not susceptible to oxidation. The concentration of EDTA should be kept as low as possible, particularly when the object of the experiment is to assess responses involving movement of Ca^{2+} into the cell.

Prevention of oxidation and of the neuronal uptake of NA is an essential precaution if quantitative studies on NA responses are planned. Without such precautions, no equilibrium response to NA $< 1\ \mu M$ can be obtained. Thus the 'control' pD_2 value for NA is a function of the effectiveness of elimination of these factors. Both factors have less influence on the disposition of NA when it is present in the bath at high concentrations. In the presence of antagonists, when the concentration-response curve is displaced rightward, the pD_2 value will be influenced by the adjuncts to a lesser degree. Consequently, the dose-ratio will vary according to the 'control' pD_2 value. The pA_2value will be too low by approximately the size of the error in the 'control' pD_2 value. This type of technical detail may account for some of the quantitative differences found for NA in different tissues, between NA and other agonists and in the pA_2 values obtained for antagonists when they are tested against NA rather than other agonists.

The technical assistance of Mr Craig Daly is gratefully acknowledged. This work was supported by the Research Funds of The University of Glasgow and by a Wellcome Trust Research Leave Fellowship. Drugs were kindly donated by Bayer, Inverni della Beffa, Mead-Johnson, Syntex, Pfizer, Ward-Blenkinsop and Professor A.G.H. Blakeley. The author is grateful to the late Professor Z.M. Bacq for discussing the results: his early observations on the dissociation of adrenolytic and sympatholytic properties of yohimbine and 933F are echoed throughout this and many other recent papers (Bacq & Fredericq, 1934).

References

ALBERTS, P., BARTFAI, T. & STJÄRNE, L. (1981). Site(s) and ionic basis of α-autoinhibition and facilitation of [^{3}H] noradrenaline secretion in guinea-pig vas deferens. *J. Physiol.*, **312,** 297–334.

ANTON, P.G., DUNCAN, M.E. & McGRATH, J.C. (1977). An analysis of the anatomical basis for the mechanical response to motor nerve stimulation of the rat vas deferens. *J. Physiol.*, **273,** 23–43.

ARIENS, E.J. & SIMONIS, A.M. (1976). Receptors and Receptor Mechanisms. In *Beta-Adrenoceptor Blocking Agents.* ed. Saxena, P.R., &Forsyth, R.P. pp. 3–27 Amsterdam. North-Holland.

BACQ, Z.M. & FREDERICQ, H. (1934). Action adrenolytique d'un derivé du dïoxane (933F). *C.R. Soc. Biol.*, **117,** 806–808.

BOVET, D. & BOVET-NITTI, F. (1948). *Structure et activité pharmacodynamiques des Médicaments du Système Nerveux Vegetatif,* Editions Karger, S., Bale.

BROWN, C.R., McGRATH, J.C. & SUMMERS, R.J. (1979). The effects of α-adrenoceptor agonists and antagonists on responses of transmurally stimulated prostatic and epididymal portions of the isolated vas deferens of the rat. *Br. J. Pharmac.*, **66,** 553–564.

BROWN, D.A., DOCHERTY, J.R., FRENCH, A.M., MACDONALD, A., McGRATH, J.C. & SCOTT, N.C. (1983). Separation and analysis of the adrenergic and non-adrenergic contractile responses to nerve stimulation in the rat vas deferens. *Br. J. Pharmac.*, **79,** 379–393.

BURNSTOCK, G. (1976). Do some nerve cells release more than one transmitter? *Neuroscience,* **1,** 239–248.

BURNSTOCK, G. & SNEDDON, P. (1984). Evidence that excitatory junction potentials in some smooth muscles are mediated by ATP acting as a cotransmitter with noradrenaline. *J. Physiol. Proc. of the Physiological Society,* February, 1984.

COATES, J., JAHN, U. & WEETMAN, D.F. (1982). The existence of a new sub-type of α-adrenoceptor on the rat anococcygeus is revealed by Sgd 101/75 and phenoxybenzamine. *Br. J. Pharmac.*, **75,** 549–552.

DEMICHEL, P., GOMOND, P, & ROQUEBERT, J. (1981). Pre- and post-synaptic adrenoceptor blocking activity of Raubasine in the rat vas deferens. *Br. J. Pharmac.*, **74,** 739–745.

DOCHERTY, J.R. & McGRATH, J.C. (1984). The actions of cirazoline on the rat vas deferens. *Br. J. Pharmac.*, **82,** 9–14.

DOCHERTY, J.R. & STARKE, K. (1981). An examination of postsynaptic α-adrenoceptor subtypes in rabbit blood vessels and rat anococcygeus. *J. cardiovasc. Pharmac.*, **3,** 854–867.

DREW, G.M. (1977). Pharmacological characterisation of the presynaptic α-adrenoceptor in the rat vas deferens. *Eur. J. Pharmac.*, **42,** 123–130.

DREW, G.M. (1979). Is WB 4093 a selective presynaptic α_2-adrenoceptor antagonist? *Br. J. Pharmac.*, **67,** 487P.

GIBSON, A. & POLLOCK, D. (1973). The absence of cocaine- and 6-hydroxydopamine-induced supersensitivity to oxymetazoline in the rat anococcygeus muscle. *Br. J. Pharmac.*, **49,** 726–727.

GILLESPIE, J.S. (1972). The rat anococcygeus muscle and its response to nerve stimulation and to some drugs. *Br. J. Pharmac.*, **45,** 404–416.

GILLESPIE, J.S. & McGRATH, J.C. (1974a). The effect of pithing and of nerve stimulation on the depletion of noradrenaline by reserpine in the rat anococcygeus muscle and vas deferens. *Br. J. Pharmac.*, **52,** 585–590.

GILLESPIE, J.S. & McGRATH, J.C. (1974b). The response of the cat anococcygeus muscle to nerve or drug stimulation and a comparison with the rat anococcygeus. *Br. J. Pharmac.*, **50,** 109–118.

GILLESPIE, J.S. & McGRATH, J.C. (1975) The effects of lysergic acid diethylamide (LSD) on the response to field stimulation of the rat vas deferens and the rat and cat anococcygeus muscle. *Br. J. Pharmac.*, **54,** 481–488.

HIRST, G.D.S. & NEILD, T.O. (1980). Evidence for two populations of excitatory receptors for noradrenaline on arterial smooth muscle. *Nature,* **283,** 301–302.

KALSNER, S. (1982). The presynaptic receptor controversy. *Trends in pharmac. Sci.*, **3,** 11–16.

KAPUR, H. & MOTTRAM, D.R. (1978). A comparative study on the pre- and post-synaptic alpha blocking activity of a series of benzodioxanes. *Biochem. Pharmac.*, **27,** 1879–1880.

KENAKIN, T.P. (1984). The relative contribution of affinity and efficacy to agonist activity: organ selectivity of noradrenaline and oxymetazoline with reference to the classification of drug receptors. *Br. J. Pharmac.*, **81,** 131–141.

LUNDBERG, J.M. & HOKFELT, T. (1983). Coexistence of peptides and classical neurotransmitters. *Trends in Neurosciences,* **6,** 325–333.

MacDONALD, A. & McGRATH, J.C. (1982). Characterization and location of α-adrenoceptors in the rat vas deferens. *Br. J. Pharmac.*, **76,** 211P.

McGRATH, J.C. (1978). Adrenergic and 'non-adrenergic' components in the contractile response of the vas deferens to a single indirect stimulus. *J. Physiol.*, **283,** 23–39.

McGRATH, J.C. (1981a). Effects of yohimbine analogues on neuronal and smooth muscle α-adrenoceptors in rat vas deferens. *Br. J. Pharmac.*, **72,** 526P–527P.

McGRATH, J.C. (1981b). Apoyohimbine: a pattern for α-adrenoceptor antagonists or a template for the α-adrenoceptors? *Br. J. Pharmac.*, **73,** 283–284P.

McGRATH, J.C. (1982a). Is there more than one α_1-adrenoceptor or is this the wrong question? *Br. J. Pharmac.*, **76,** 210P.

McGRATH, J.C. (1982b). Commentary: Evidence for more than one type of postjunctional α-adrenoceptor. *Biochem. Pharmac.*, **31,** 467–484.

McGRATH, J.C. (1983). Ca^{2+}-free and Ca^{2+}-requiring responses in rat anococcygeus have differing agonist potency series. *Br. J. Pharmac.*, **79,** 227P.

McGRATH, J.C. (1984). Is the Ca^{2+}-free α_1-adrenoceptor-mediated response of smooth muscle limited to full agonists? *Br. J. Pharmac.*, Proc. of Pharm. Soc. Birmingham, April, 1984.

MACRAE, I.M. (1983). The release of catecholamines by nerve stimulation from the guinea-pig vas deferens. Ph.D. Thesis, University of Glasgow.

MICHEL, A.D. & WHITING, R.L. (1981). 2-(2-imidazolyl methyl)-1, 4-benzodioxans, a series of selective α_2-adrenoceptor antagonists. *Br. J. Pharmac.*, **74,** 255P–256P.

RAND, M.J., McCULLOCH, M.W. & STORY, D.F. (1982).

Feedback modulation of noradrenergic transmission. *Trends in pharmac. Sci.*, **3**, 8–11.

SNEDDON, P. WESTFALL, D.P., (1984). Pharmacological evidence that adenosine triphosphate and noradrenaline are co-transmitters in the guinea-pig vas deferens. *J. Physiol.*, **347**, 561–580

WEITZELL, R., TANAKA, T. & STARKE, K. (1979). Pre- and post-synaptic effects of yohimbine sterioisomers on noradrenergic transmission in the pulmonary artery of the rabbit. *Naunyn Schmiedebergs Arch. Pharmac.*, **308**, 127–136.

(*Received June 20, 1983.*
Revised February 29, 1984.)