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Separation of adrenergic and non-adrenergic contractions to field stimulation in the rat vas deferens

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1 Adrenergic and 'non-adrenergic' nerve-induced contractions in rat vas deferens were separated pharmacologically.

2 Responses to single stimuli comprised two components, an α-noradrenergic component (*II*s), dominant in the epididymal portion, and a 'non-adrenergic' component (*I*s), dominant in the prostatic portion. *I*s but not *II*s was blocked by nifedipine. A combination of adrenergic blockade and nifedipine virtually abolished all components. After cocaine, a third component (*III*s) emerged which was abolished by either adrenergic blockade or nifedipine.

3 The response to trains of stimuli consisted of 'twitch' and 'secondary' components. This biphasic time course was modified by adrenergic blockade or nifedipine to reveal the time course of the 'non-adrenergic' and adrenergic components, respectively: these did not correspond to the 'twitch' and 'secondary' components. A combination of adrenergic blockade and nifedipine virtually abolished the whole response.

4 Prejunctional α_2-adrenoceptor-mediated inhibition of the contractile responses could be blocked by selective α_2-adrenoceptor antagonists. The adrenergic contractile response demonstrated this 'feed-back' even on the second pulse at 0.5 Hz. Endogenous inhibition of the 'non-adrenergic' contraction required higher frequencies or enhancement of the extracellular concentration of noradrenaline by blockade of its neuronal uptake.

5 Contractile responses to exogenous noradrenaline were abolished by nifedipine, at a concentration that did not affect the adrenergic (*II*s) neurotransmission.

6 These results reinforce the view that part of the motor transmission in rat vas deferens is non-adrenergic and allow the disentanglement of the various postjunctional and prejunctional elements contributing to the complex response to a train of stimuli.

Introduction

The dense adrenergic innervation of their smooth muscle layers has led to the widespread use of rodent vasa deferentia in the study of 'adrenergic' mechanisms. In particular, over the last few years, nerve-induced contractions have proved useful in assaying compounds which act at prejunctional receptors to inhibit transmitter release, e.g. opioid receptors, α-adrenoceptors (Henderson, Hughes & Kosterlitz, 1972; Drew, 1977). This has been able to proceed in ignorance of the neurotransmitter. However, since the receptors present at nerve terminals may vary according to the transmitter, it seems worthwhile to establish how much of the response to nerve stimulation can be proved to be adrenergic.

Stimulation of the extrinsic nerves or field stimulation of the intramural nerves produces rapid and powerful contractions of the smooth muscle, which are accompanied by the release of noradrenaline. Although exogenous noradrenaline contracts the muscle via α-adrenoceptors, it is not easy to prove that the entire contraction to nerve stimulation is initiated by noradrenaline. Part of the contractile response persists after elimination of noradrenaline stores by reserpine or after α-adrenoceptor blockade (Ambache & Zar, 1971). To complicate matters, two distinct types of response can be demonstrated, each of which is dominant in one end of the organ. In the epididymal end, the pharmacological requirements

for adrenergic transmission are obeyed. In the prostatic end, none of these criteria is satisfied by the major part of the response, which appears, in every respect, to be 'non-adrenergic' (Anton, Duncan & McGrath, 1977; McGrath, 1978). However, a calcium entry blocker, nifedipine, can block the 'non-adrenergic' response but leave the adrenergic component intact (French & Scott, 1981a). This enables, for the first time, the pharmacological isolation of each component.

This paper describes the isolation of these two types of transmission in bisected vasa using nifedipine and drugs which block adrenergic transmission. First, each postjunctional element was isolated from within the responses to single stimuli or to trains of pulses. Secondly, the influence of prejunctional α-adrenoceptor activation on each component was sought, employing α-adrenoceptor antagonists. Thirdly the effects of the blocking drugs on the responses to exogenous noradrenaline were examined. The object was to clarify the extent to which nerve-induced contractions represent 'adrenergic' phenomena.

Preliminary communications of part of these results have been published (Booth, Connell, Docherty & McGrath, 1978; Docherty & McGrath, 1980; French & Scott, 1981b,c).

Methods

Vasa deferentia were isolated from male Wistar rats (250–300 g) killed by a blow on the head and exsanguination. All vasa were bisected transversely into two portions of equal length or in which the epididymal portion was two thirds of the total, as indicated in the text. Tissues were placed in Krebs bicarbonate solution at 38°C and gassed with 95% O_2:5% CO_2 as described previously (Anton *et al.*, 1977).

The Krebs bicarbonate solution had the following composition: (mM) NaCl 119, KCl 4.7, $MgSO_4$ 1.0, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25.0, and glucose 11.1; and was gassed with 95% O_2: 5% CO_2. Field stimulation was applied via parallel platinum electrodes or Ag: AgCl 'ring and hook' electrodes as described previously (Grass S88 or S44, or Square One Instruments stimulators, supramaximal voltage, see below; pulse widths in text) (Anton *et al.*, 1977; McGrath, 1978).

It has been pointed out by Stjarne (1977) that in guinea-pig vas deferens pre-loaded with [^{3}H]-noradrenaline it is difficult to achieve 'supramaximal' stimulation of the intramural nerves, gauged as the overflow of tritium. In the course of carrying out experiments on rat vas deferens in a number of different laboratories and with varying electrodes and electrical stimulators, we have found the same phenomenon in the contractile responses. 'Supramaximal' is thus used to indicate the largest response which could be obtained at short pulse durations ($< = 1$ ms) with the particular stimulator employed. For this reason the absolute sizes of control responses vary between figures. Absolute comparisons are made only between responses obtained at the same time using the same equipment. All such responses in rat vasa were abolished by tetrodotoxin (10^{-7} M to 10^{-6} M) confirming that they were initiated by action potentials induced by electrical stimulation of the intramural fibres. Furthermore, each component of the response could be obtained in pithed rats, stimulating the nerves as they left the vertebral column, several centimetres from the vas (McGrath, 1978).

Vasa were connected by thread to Grass FT03 or Ether UF1 transducers and isometric tension was recorded on Grass Polygraph recorders and storage oscilloscopes.

The following drugs were used and were added to the Krebs solution to give the appropriate molar concentration: corynanthine tartrate (Aldrich), 6-hydroxydopamine hydrochloride (Sigma), nifedipine (Bayer), noradrenaline bitartrate (Koch-Light), pargyline (Abbot), prazosin hydrochloride (Pfizer), reserpine, crystalline (Koch-Light), rauwolscine base (α-yohimbine) (Inverni della Beffa), WB 4101 (Ward-Blenkinsop).

For reserpine pretreatment of rats, the drug 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 over 98% (Gillespie & McGrath, 1974).

To produce chemical sympathectomy with 6-hydroxydopamine, it was dissolved in de-oxygenated 0.9% w/v NaCl solution (saline) containing ascorbic acid (1 mg/kg) and, when appropriate, was administered intraperitoneally (100 mg kg^{-1} on day 1; 2×100 mg kg^{-1} on day 4; rats killed on days 5 or 6). The effects of this treatment on the adrenergic innervation of the smooth muscle layers of the vasa were compared to reserpinization (above) by the histofluorescence technique of Hillarp and Falck. In each case the treatments led to the abolition of fluorescence. Following incubation with a high concentration of noradrenaline (10^{-4} M) in the presence of a monoamine oxidase inhibitor (pargyline, 10^{-5} M), faintly fluorescent nerve terminals became visible in the reserpinized tissue confirming that the adrenergic terminals were intact but depleted of catecholamines. In the vasa from 6-hydroxydopamine-treated rats, no terminals reappeared but fluorescence did appear in preterminal axons where they begin to invade the muscle layers. The destruction of the adrenergic

nerve terminals by 6-hydroxydopamine was confirmed by examining the vasa using the electron microscopic technique of Tranzer & Richards (1976). With this technique, control vasa contained a dense plexus of varicose nerve terminals, which contained dense-cored vesicles. Following reserpine the varicose terminals remained but contained few dense-cored vesicles. After 6-hydroxydopamine, however, it was very difficult to find any varicosities at all, although there were some damaged, 'ghost' profiles indicating the residue of destroyed varicosities. No consistent evidence was found for a population of nerve terminals that were resistant to 6-hydroxydopamine despite the persistence of a nerve-mediated contractile response *in vitro* (see Results) or in pithed rats (Docherty, 1979). While this is puzzling, a negative finding with the electron microscope does not mean that there are no nerves, but rather that we cannot find them.

Results

Responses to single stimuli

The response to a single supramaximal stimulus (0.5 ms) consists of two consecutive components, *I*s and *II*s, but their proportions differ in the two portions (Figure 1).

'Adrenergic' blockade In either portion, *I*s was resistant but *II*s was susceptible to corynanthine, a stereoisomer of yohimbine which is a preferential α_1-adrenoceptor antagonist (Weitzell, Tanaka & Starke, 1979; McGrath, 1981).

Following reserpine or 6-hydroxydopamine pretreatment, the response to a single stimulus consisted of a single contractile component with a magnitude and time course to peak which, in each portion, was similar to *I*s in controls. No component corresponding to *II*s was found (Figure 2). The relaxation phase was more prolonged than in controls: this was particularly noticeable in the prostatic portion in which the *I*s response was larger.

Blockade with nifedipine Nifedipine (10^{-8}–10^{-5} M) produced a concentration-dependent reduction in *I*s in each portion but at least up to 10^{-5} M it did not significantly affect *II*s (Figure 1).

Combined blockade The combination of an α-adrenoceptor antagonist and nifedipine produced virtually complete abolition of both contractile components, irrespective of order of addition of the drugs. All that remained was a small, slow, monophasic response which was slightly more evident in the epididymal portion (Figure 1).

The monophasic response, which remained after reserpine or 6-hydroxydopamine, was abolished by nifedipine.

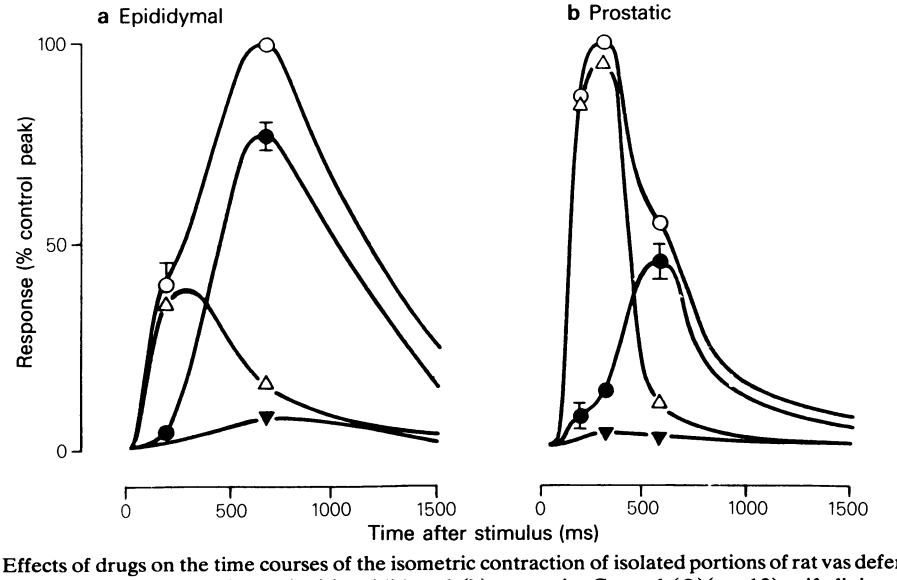

Figure 1 Effects of drugs on the time courses of the isometric contraction of isolated portions of rat vas deferens to single supramaximal field stimuli (0.5 ms): (a) epididymal (b) prostatic. Control (○)($n = 12$); nifedipine 10^{-5} M (●)($n = 6$); corynanthine 3×10^{-6} M (△)($n = 6$); nifedipine 10^{-5} M + corynanthine 3×10^{-6} M (▼) $n = 12$. Symbols with I-bars, indicating mean ± s.e.mean, are shown only at times corresponding to peaks of different phases. Graphs were plotted from measurements taken at 50 ms intervals. Square One stimulator, 0.5 ms pulses, maximal voltage for stimulator (800 mA), 'Ring and Hook' electrodes.

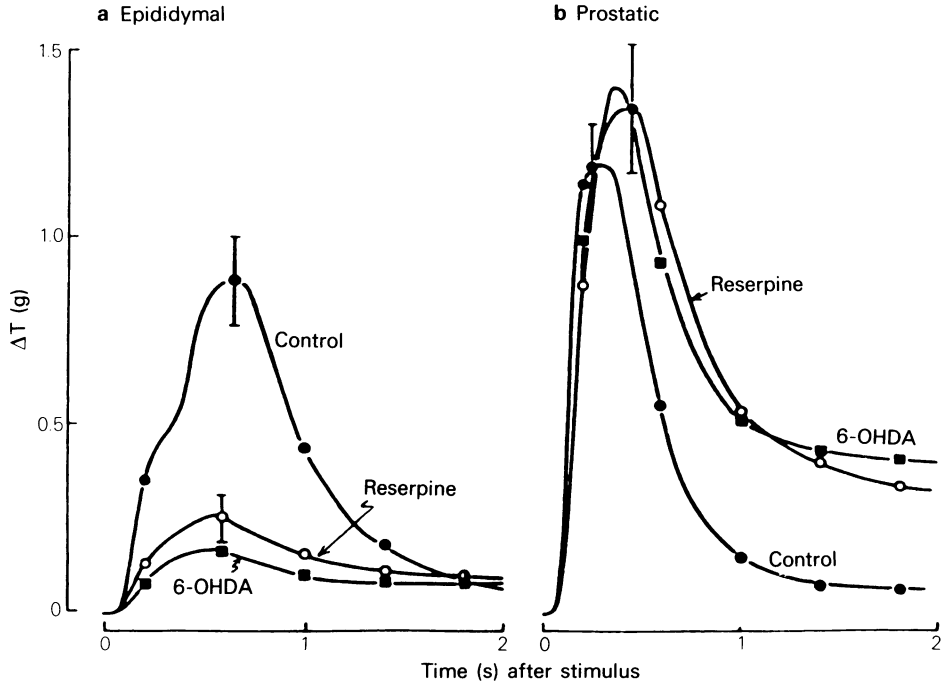

Figure 2 The isometric tension response of the bisected vas deferens to single pulse transmural stimulation *in vitro*: (a) epididymal half, (b) prostatic half. Response in control tissues (●); response in tissues taken from 6-hydroxydopamine (6-OHDA)-treated rats (■): responses in tissues taken from reserpinized rats (○). Stimulation was given at time zero. The graphs were constructed from the means of data taken every 0.1 s. Δ T indicates the increase in tension above the resting baseline. For the sake of clarity, symbols with error bars (s.e.mean) are shown only at certain points. $n=4-8$. Grass S88 stimulator, 0.5 ms pulses, maximal voltage for stimulator (150 mA). 'Ring and Hook' electrodes.

Effect of cocaine In the presence of cocaine (3×10^{-6} M) the response to a single stimulus, particularly in the epididymal portion, was increased in height and duration (Figure 3a). In the additional presence of nifedipine (10^{-5} M), the prolongation was abolished but the increase in the height of the peak remained (Figure 3b,c). This cocaine-induced, prolonged phase was nifedipine-sensitive, and is sensitive also to α-blockers (McGrath, 1978), the former property distinguishing it from the usual adrenergic response to a single stimulus (*IIs*): it is discussed below as a third component of the response to a single stimulus (*IIIs*).

Responses to trains of stimuli

Time course The response consists of two clearly discernible phases; an initial 'twitch' declines to be replaced by a better maintained 'secondary' contraction. The 'twitch' and 'secondary' responses are dominant in the prostatic and epididymal portions respectively (Anton *et al.*, 1977).

The measurement of responses to trains presents different problems according to the frequency of stimulation. At low frequencies (<0.1 Hz) the responses consist of discrete contractions to each pulse. These summate as frequency increases, but as Figure 4 indicates, two complications arise: (i) particularly in the epididymal portion, the time course of the biphasic response to each subsequent pulse changes after the first; (ii) after the first few pulses, the peak attained by each response may fall off; in the case of Figure 4a, after the second. At high frequencies the responses merge (threshold 5–10 Hz) so that the 'fall off' after a few pulses corresponds to the declining phase of the 'twitch', defined above. For this reason the responses to trains at low frequencies are expressed as the peak produced by each pulse and those to higher frequencies as the tension attained at times after the start of the train.

'Adrenergic' blockade At low frequencies of stimulation (<0.5 Hz), particularly in the epididymal portion, each of the two components of the response to each pulse visibly contributes (Figure 4c–f) to contraction. At such frequencies 'facilitation' of the *Is*

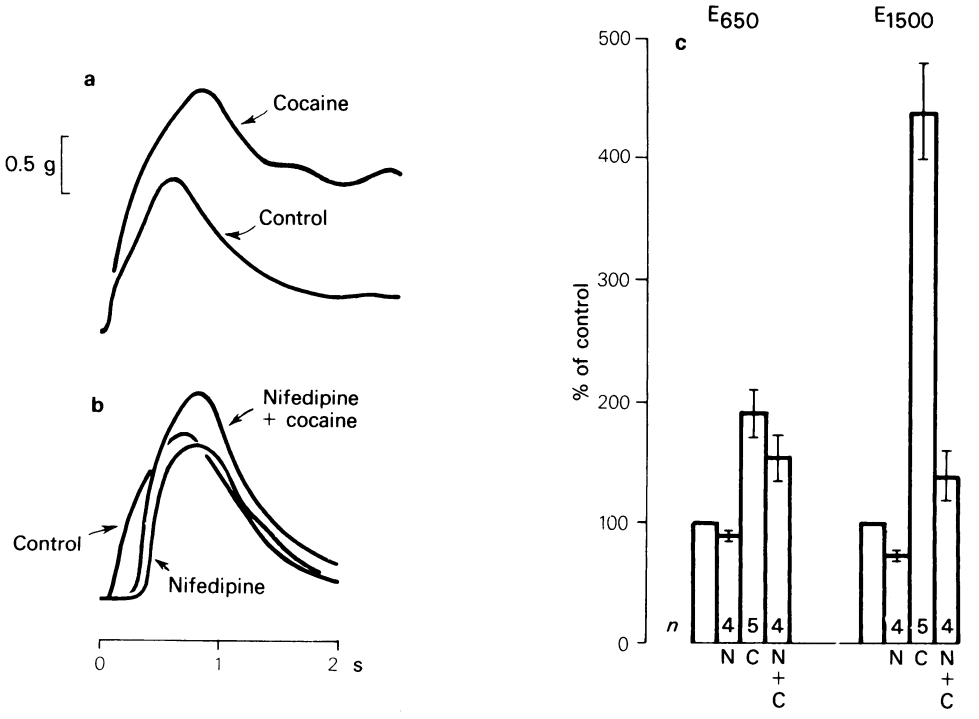

Figure 3 The effect of nifedipine (10^{-5} M) on the nerve-induced contraction of epididymal halves in the presence of cocaine (3×10^{-6} M). (a) and (b) Time course of isometric contractions to single stimuli (as in Figure 1) applied at time zero. (a) A control was followed by a response in the presence of cocaine; (b) control followed by nifedipine then nifedipine and cocaine. Note that in (b) cocaine still increases the height but not the duration of the response. (c) Experiments as in (a) and (b): these show the tension at 650 ms (E_{650}) and 1500 ms (E_{1500}) after the stimulus, expressed as a percentage of that in the drug-free controls. E_{650} illustrate the change in the peak; E_{1500} the extent of prolongation. First columns represent control; N, nifedipine; C, cocaine. I-bars represent s.e.mean. The numbers in each experimental group are shown in histogram.

component is more marked than that of *II*s. This is made clearer after *II*s is blocked by an α-adrenoceptor antagonist (Figure 4h & j). It now becomes obvious, even in the epididymal portion, that the 'peak' to each pulse in a train consists mainly of *I*s (Figure 4i).

At higher frequencies, following an α-blocker, the response should be 'non-adrenergic', formed by the summation and facilitation of the many *I*s components. An additional factor, however, is the inhibitory prejunctional effect produced by noradrenaline at α_2-adrenoceptors on the nerve terminals. If a selective α_1-antagonist is used which lacks the prejunctional action e.g. corynanthine or prazosin, then 'selective' removal of the postjunctional adrenergic influence (*II*s) can be achieved (Figure 7): under these conditions, addition of an α_2-antagonist, e.g. yohimbine, can reveal that the 'non-adrenergic' *I*s component is 'restrained' by a prejunctional α_2 influence (Figure 7).

After reserpine or 6-hydroxydopamine pretreatment, at low frequencies of stimulation ($< = 1$ Hz), the responses to each pulse summated to reach tensions greater than in controls (Figures 4b and 5). The increase in the height of response was relatively greater in the prostatic portion but was the more remarkable in the epididymal portion since, here, the response to the first pulse was reduced.

At higher frequencies of stimulation ($> = 5$ Hz) responses had time courses which were quite distinctly different from the 'twitch and secondary' components in untreated controls. Responses were (i) monophasic, (ii) slower to rise than in controls, (iii) reached a peak at 3–4 s after the start of stimulation and tended to decline thereafter (an exception to this was at high frequencies (10 or 20 Hz) after reserpine, in which the peak was earlier than 3 s but later than in controls), (iv) at the end of the train, relaxation was slower than in controls (Figure 5).

Qualitatively, the time course of the response was similar after reserpine, 6-hydroxydopamine or combined α_1- and α_2-blockade. After reserpine or 6-

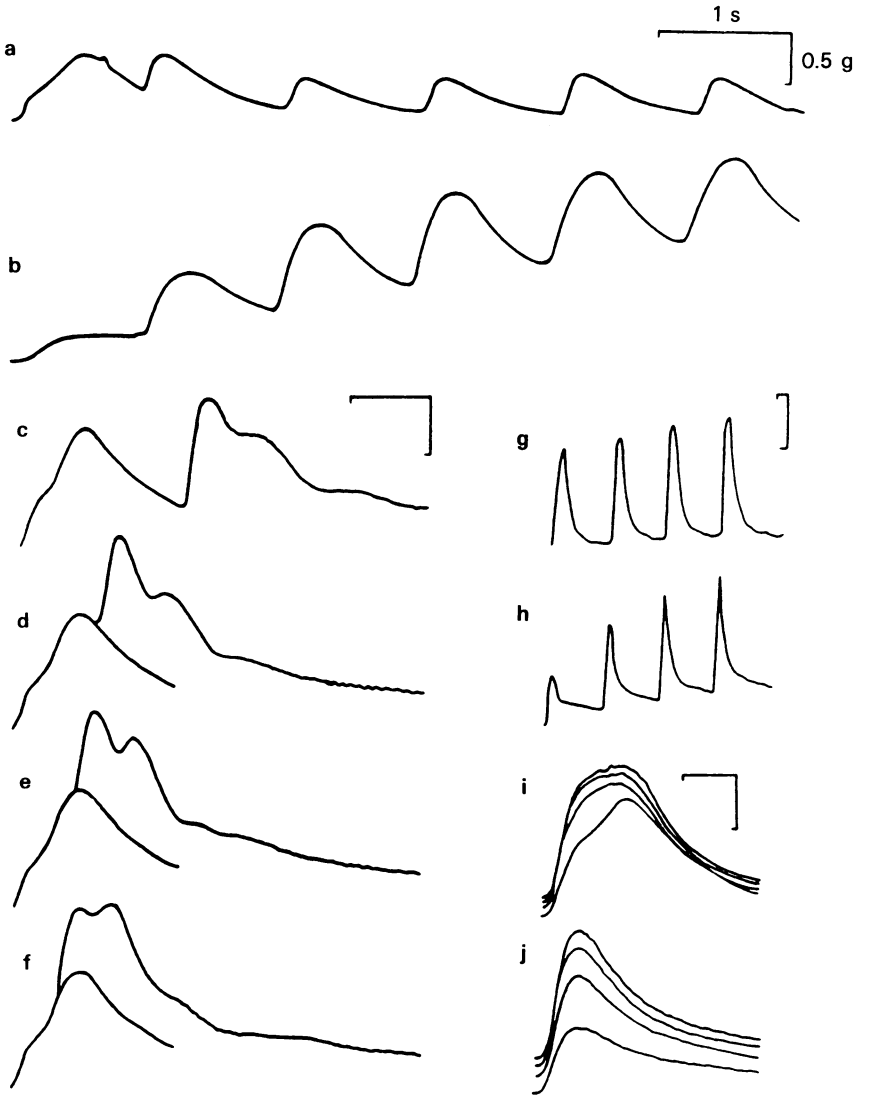

Figure 4 Illustration of the interaction between responses of epididymal halves to sequences of individual stimuli (a,b) 5 pulses at 1 Hz. (a) Control; (b) reserpinized; (c–f) a single stimulus was followed by a second, similar stimulus at an interval of (c) 2 s, (d) 0.7 s, (e) 0.6 s and (f) 0.5 s. In (d–f) the response to the first pulse from (c) is superimposed to allow comparison. (g–j) Four pulses at 0.2 Hz; (g) and (i) are controls, (h) and (j) are from the same portion of vas in the presence of phentolamine, 10^{-6} M. In (i) and (j) the trace was speeded up and the oscilloscope was triggered by each stimulus: this shows that only *Is* remains after phentolamine as it does after reserpinization, cf. (b). Grass S88 stimulator, 0.5 ms pulses, maximal voltage for stimulator (150 mA).

hydroxydopamine treatment neither α_1- nor α_2-adrenoceptor antagonists altered the response to trains of pulses: this confirms the elimination of pre- and postjunctional adrenergic influences (e.g. Figure 6e).

Blockade of the 'non-adrenergic' response In the presence of nifedipine (5×10^{-6} M), the responses to trains at low frequency (≤ 1 Hz) consisted only of adrenergic (*IIs*) components, which were susceptible to α_1-antagonists, e.g. prazosin (10^{-7} M), corynanthine (10^{-6} M), WB 4101 (10^{-7} M). In trains of several pulses, these responses showed a rapid fall-off, which was partly attributable to α_2-mediated pre-

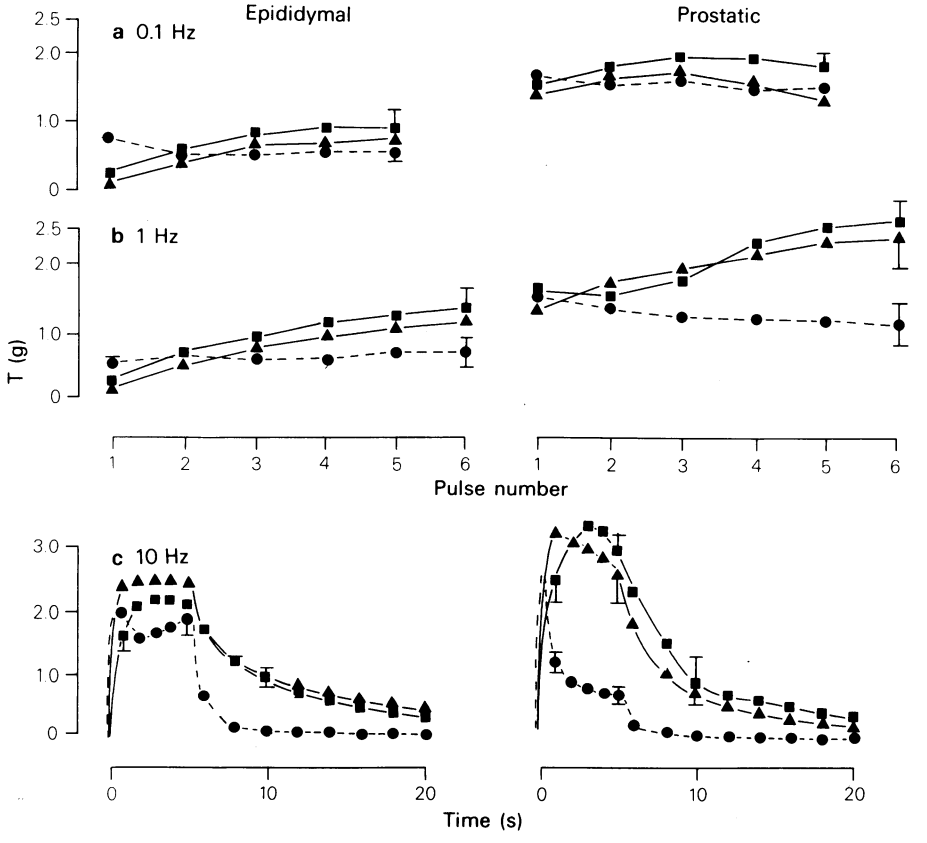

Figure 5 The effects of depletion of catecholamines on the isometric tension responses of the bisected vas deferens to stimulation at different frequencies. Left panel, epididymal half: right panel, prostatic half. In (a) and (b) the peak response to each stimulus pulse is shown: (a) 5 pulses at 0.1 Hz; (b) 6 pulses at 1 Hz. (c) Responses to stimulation for 5 s at 10 Hz. Stimulation started at time zero. Graphs were constructed from the means of data taken every 10 ms during rapid changes and otherwise at 1 s intervals. Controls (●); 6-hydroxydopamine-treated rats (■); reserpinized rats (▲). For the sake of clarity, symbols with I-bars (s.e.mean) are shown only at certain points. $n = 4-8$. Stimulus parameters as in Figure 2.

junctional inhibition (see Figure 8). This is seen most clearly in the epididymal portion in which the adrenergic, post-junctional response is greater.

At higher frequencies (1–10 Hz) the time courses of the responses to trains were markedly altered by nifedipine. The response consisted of a single component which started after a longer than usual delay, rose relatively slowly, attained a peak at 0.6–0.8 s (prostatic) or 1.0–1.2 s (epididymal) and declined thereafter, despite continued stimulation (Figure 6a,c). The fall-off could be off-set only in part by prejunctional α_2-antagonism (Figure 6c).

Combined blockade The combination of nifedipine with either an α_1-antagonist or reserpinization or 6-hydroxydopamine pretreatment produced virtually complete abolition of the response to a train of pulses (Figure 6); all that remained was a small, slow, monophasic contraction, that was greater in the epididymal portion.

Demonstration of pre-junctional α_2-adrenoceptor mediated effects

In trains of stimuli

In the absence of postjunctional blockade: rauwolscine, a 'selective' α_2-adrenoceptor antagonist (Weitzell, *et al.*., 1979), increased the height of responses to trains at 1–10 Hz. However, the increases were small and the effect was best characterized as a change in the time course; the peak was achieved more slowly, but was of longer duration, than the control 'twitch' (Figure 7b).

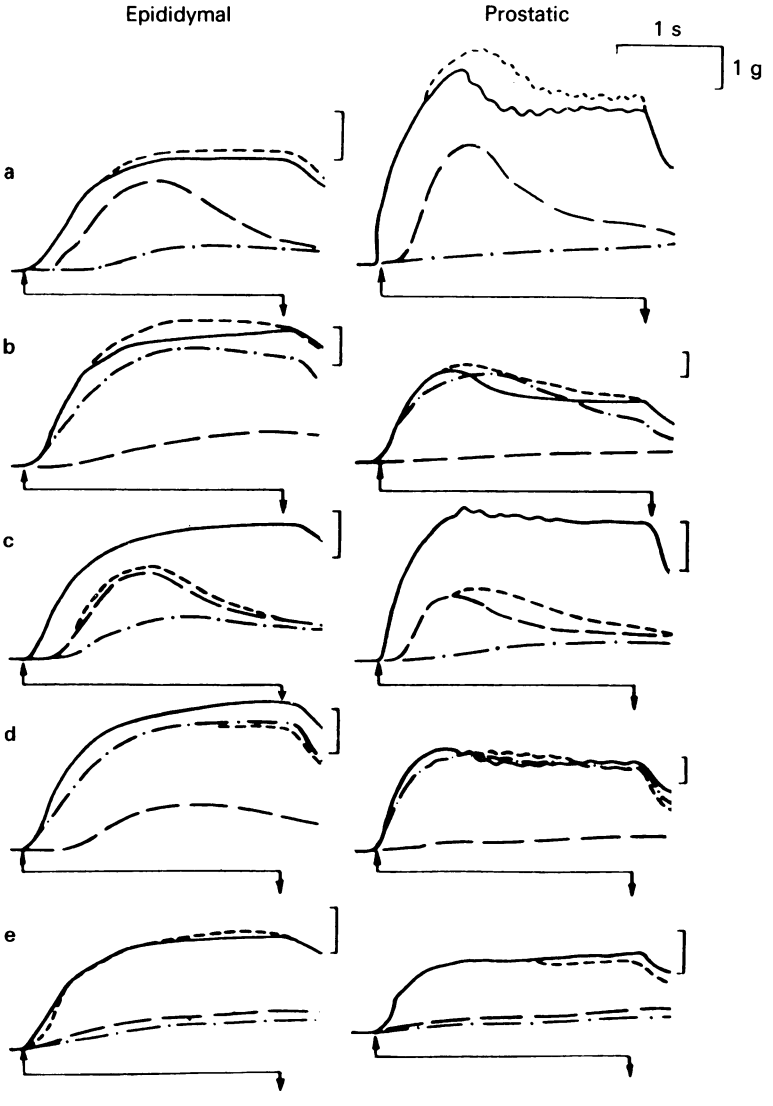

Figure 6 The effects of α-adrenoceptor antagonists and nifedipine on the responses of portions of vasa to trains of field stimuli (between arrows, 6 Hz, 15 pulses, 0.5 ms, Square One Stimulator, maximal voltage for stimulator = 800 mA). Left panel: epididymal halves. Right panel: prostatic halves from same vasa. In each experiment three drugs were administered in varying order. Different lines indicate the most recently added drug. Drug-free control, solid line: rauwolscine (R, 3×10^{-7} M), small dashes: corynanthine (C, 3×10^{-6} M), dots and dashes: nifedipine (N, 10^{-5} M), large dashes, (a–d) untreated animals; (e) reserpinized. Order of addition: (a) R, N, C; (b) R, C, N; (c) N, R, C; (d) C, R, N; (e) as (a).

Following post-junctional α_1-blockade: In the presence of an α_1-adrenoceptor antagonist, e.g. corynanthine, potentiation by an α_2-adrenoceptor antagonist could be more easily demonstrated (Figure 7c).

Figure 7b illustrates that α-antagonists reduce the rate of rise of the 'twitch', by removing the adrenergic, *I*Is component from the response to each pulse. Thus the 'twitch' does contain an α-adrenergic element. Furthermore, after blockade of pre- and post-junctional α-adrenoceptors, the peak occurs at a later time than in the original control 'twitch'. The response remaining after combined α_1- and α_2-blockade, is not, therefore a residual 'twitch' but is the sum of the *I*s components, freed from pre-

junctional, α_2-restraint (thus augmented) but without reinforcement from the post-junctional α_1-mediated contraction (thus curtailed).

Following nifedipine: Potentiation by rauwolscine could be demonstrated in the presence of nifedipine (Figure 6c). Following either α_1-blockade or nifedipine, the degree of potentiation by rauwolscine varied between vasa from different rats but was always more clearly seen in the prostatic portions (Figure 6a,c).

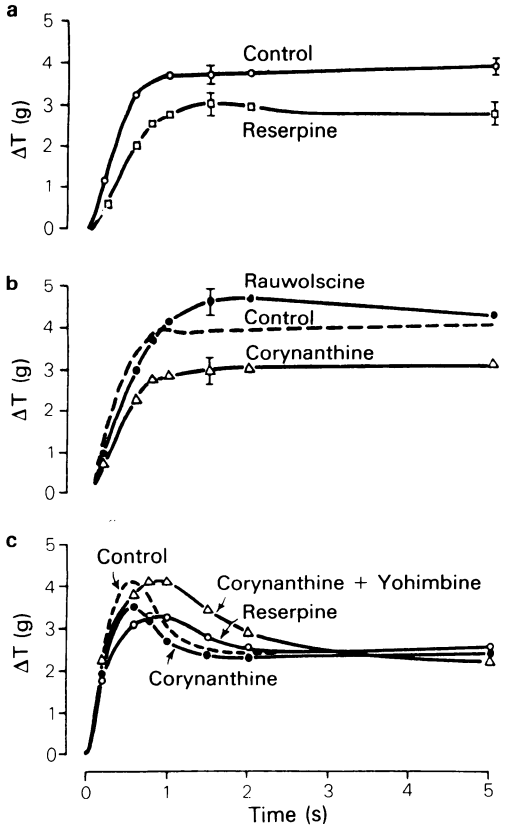

Figure 7 Comparison of the effects of α-adrenoceptor antagonists and reserpine on the time course of the isometric response to trains of stimuli at high frequency (10 Hz for 5 s). (a) Epididymal halves: untreated controls ($n = 6$), (○); reserpinized ($n = 8$), (□). (b) Epididymal halves: pooled controls ($n = 12$), dashed line; rauwolscine (10^{-6} M) ($n = 6$), (●); separate experiments, corynanthine (10^{-6} M) ($n = 6$), (△). (c) Prostatic halves: reserpinized ($n = 6$), (○): untreated rats ($n = 6$), control, dashed line; corynanthine (10^{-6} M), (●); subsequent addition of yohimbine (10^{-6} M), (△). Note that after reserpine pretreatment the time course of the response is similar to that after blockade of both groups of α-adrenoceptors. The high concentration of rauwolscine in (b) blocks both sets of receptors (McGrath, 1981). Stimulus parameters as in Figure 1.

Following reserpine or 6-hydroxydopamine: No potentiation by rauwolscine could be demonstrated (Figure 6e) at concentrations that were sufficient to potentiate under control conditions and which could antagonize the inhibitory effects of α_2-agonists (see MacDonald & McGrath, 1980). At higher concentrations all α-antagonists which have been tested produce a post-junctional excitatory effect which increases the responses to single stimuli and eventually leads to spontaneous contraction of the vasa (Brown, McGrath & Summers, 1979). This can give a false impression of prejunctional antagonism.

With pairs of stimuli

α_2-Mediated feedback could be demonstrated even between pairs of pulses. The extent of pre-junctional α_2-mediated restraint of the adrenergic and 'non-adrenergic' components was compared by isolating each response; the adrenergic in the epididymal portion, in the presence of nifedipine; the non-adrenergic in the prostatic 'third' of the organ (see Anton *et al.*, 1977). The adrenergic component could be shown to be under prejunctional restraint via α_2-adrenoceptors: the response to the second pulse was smaller than that to the first but could be restored by yohimbine (Figure 8a). In contrast, the non-adrenergic response to the second pulse was little smaller than that to the first (Figure 8b). It was, however, possible to enhance the pre-junctional α_2-restraint of the non-adrenergic response by blocking the neuronal uptake of noradrenaline with cocaine: in this case it was also necessary to block post-junctional α_1-adrenoceptors with prazosin since this response also is potentiated by cocaine (Figure 8c). After the adrenergic influence had been removed by reserpine pretreatment, no restoration by yohimbine could be demonstrated (Figure 8d).

Effect of an α_2 agonist on the adrenergic and 'non-adrenergic' responses

In order to compare the susceptibilities of the adrenergic and 'non-adrenergic' components to inhibition via α_2-adrenoceptors, the inhibitory effects of an α_2-adrenoceptor agonist, clonidine, were tested. The concentration of clonidine required to produce a 50% reduction in each response was calculated from dose-response curves.

In the absence of nifedipine: prostatic third, i.e. *Is*, $3.24 \pm 0.53 \times 10^{-9}$ M ($n = 6$); epididymal two thirds, i.e. mainly *IIs*, $2.03 \pm 0.26 \times 10^{-9}$ M ($n = 3$).

Nifedipine (5×10^{-6}M) present: epididymal two thirds, i.e. *IIs*, $1.79 \pm 0.22 \times 10^{-9}$M ($n = 8$).

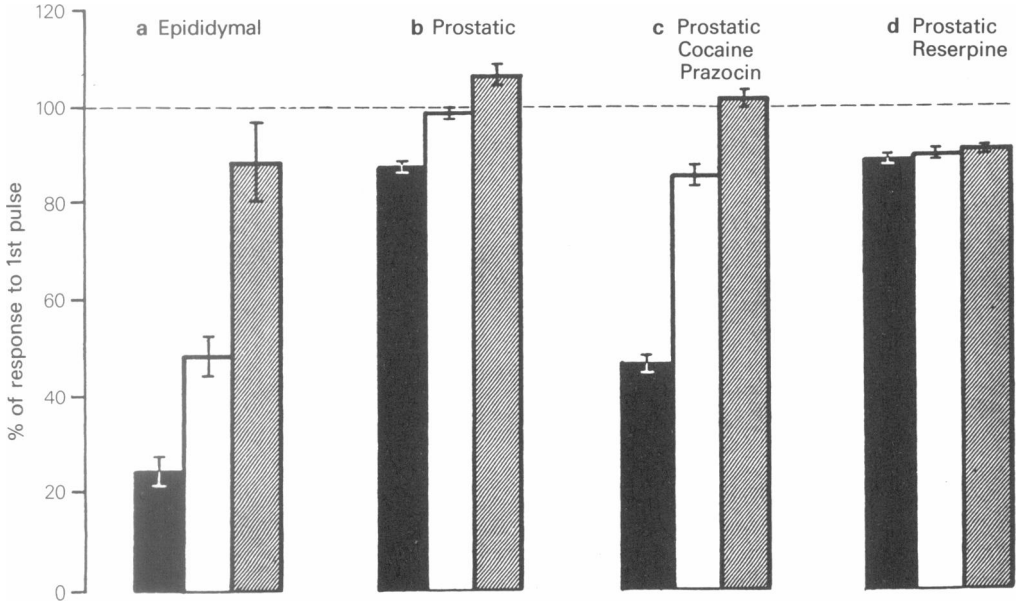

Figure 8 Demonstration of α_2-adrenoceptor-mediated inhibition of the response to the second of two pulses delivered 2 s apart. The peak of the response to the second stimulus is expressed as a percentage of that to the first. Solid column, drug-free: open column, yohimbine 5×10^{-8} M; hatched column, yohimbine 5×10^{-7} M. (a) Epididymal two thirds; (b) prostatic third; (c) prostatic third; cocaine 5×10^{-7} M, prazosin 5×10^{-9} M both present; (d) prostatic third, reserpinized. Columns represent the mean with s.e.mean shown by I-bar ($n = 6$ throughout). Parallel electrodes, 0.5 ms, Grass S44 stimulator, maximal voltage for stimulator (150 mA).

This similarity in sensitivity to clonidine concurs with an earlier study employing xylazine as the α_2-agonist, isolating *I*s with reserpine and separating *II*s by the time of its peak (MacDonald & McGrath, 1980).

Responses to noradrenaline

Nifedipine, in concentrations up to (10^{-5} M) did not inhibit the 'adrenergic' component of the response to a single electrical stimulus. However, nifedipine (10^{-5} M) produced complete abolition of the contractile response to exogenous noradrenaline ($< = 10^{-4}$ M): the normal threshold for contraction by noradrenaline is 3×10^{-6}M. It is possible that uptake of noradrenaline into the adrenergic nerve terminals might deny access of exogenous noradrenaline to receptors lying on the muscle within the narrow synaptic cleft and that these might be the receptors activated by the noradrenaline from nerves. However, when the neuronal uptake of noradrenaline was blocked with cocaine (3×10^{-6} M) the organ became more sensitive to noradrenaline (threshold 3×10^{-7} M) but nifedipine still abolished the response to noradrenaline (10^{-4} M).

With or without cocaine, α_1-antagonists blocked the responses to noradrenaline, e.g. WB 4101 (10^{-7} M) shifted the dose-contractile response curve, for noradrenaline in the epididymal portion, to the right by a factor of 18.

Discussion

Single stimuli

These results confirm that the contraction of rat vas deferens to a single stimulus consists of two separate components with different time courses (McGrath, 1978). The first of these (*I*s) survives, (i) blockade of α-adrenoceptors, (ii) depletion of tissue catecholamines, (iii) destruction of the entire population of adrenergic nerve varicosities which could be detected by fluorescence or electron microscopy, but (iv) is readily blocked by nifedipine. The second, (*II*s), is resistant to nifedipine but is susceptible to the other manoeuvres. When *II*s is blocked by anti-adrenergic drugs, the residual response is susceptible to nifedipine. On this evidence, *I*s is independent of either the whole, or at least of the major part, of the adrenergic innervation, does not act through a post-junctional α-adrenoceptor and has no properties that provide evidence in favour of an adrenergic nature. *II*s, in contrast, is α-noradrenergic by several tests

and is independent of *I*s since it is unaffected by nifedipine.

Trains of stimuli

The responses to trains of pulses are more complex and can, no longer, be interpreted solely in terms of a 'non-adrenergic twitch' and an 'adrenergic secondary contraction' (Swedin, 1971; Anton *et al.*, 1977). They can, however, be interpreted in terms of the two responses to single stimuli and a third element, which may correspond to the type of response produced by exogenous noradrenaline.

When each component (*I*s or *II*s), in the response to trains at low frequency, is isolated pharmacologically, each summates in a distinct way. The *I*s components readily summate and, even when the intervals between pulses are sufficient to allow recovery of basal tension before the next stimulus, the contractions increase in size indicating 'facilitation'. The *II*s component, however, tends to decrease as the train proceeds.

If the 'peaks' of the responses to each pulse are followed, in controls there is an initial rise, a fall as the *II*s component declines and a further rise as the *I*s components continue to summate. If the recorded tension signal is damped electronically or the response itself is damped by employing isotonic recording, this results in a 'twitch' and a 'secondary response' (Anton *et al.*, 1977). This is the main basis of the biphasic response to short trains of pulses. After reserpine or 6-hydroxydopamine pretreatment, the summation and facilitation of the *I*s component is particularly marked; this is not due entirely to the loss of a pre-junctional α-mediated inhibition but is partly due to prolongation of the relaxation phase of each contraction. Following reserpine, no 'twitch' is present and the response is monophasic. Previously the effect of reserpine could not be clearly interpreted since it was thought that the 'twitch' was 'non-adrenergic' (Ambache & Zar, 1971), yet it was reduced by reserpine (Gillespie & McGrath, 1974). This can now be explained by the loss of both the pre- and the postjunctional adrenergic effects, i.e. the residual response after reserpine is neither a 'twitch' nor a 'secondary' response but is the sum of *I*s components, which have been relieved of prejunctional α_2-mediated restraint.

At higher frequencies of stimulation, in the presence of an α_1-adrenoceptor antagonist, the postjunctional adrenergic effects are blocked but 'non-adrenergic' transmission continues. By observing the time course, it becomes clear that the inhibitory effect of α_1-blockade is greatest within the first 2 or 3 s. Hence, a substantial postjunctional adrenergic response occurs at this time. As a corollary, when nifedipine blocks the 'non-adrenergic' response, it leaves a monophasic contraction, slower to rise than the control 'twitch', reaching a peak at within 1.5 s and decaying rapidly, thereafter; this residual response is blocked by α_1-adrenoceptor antagonists and, thus, represents an 'adrenergic' component. It spans the period of time including the 'twitch' and the start of the 'secondary response' and thus contributes to each (Figure 6a,c). Thus it is possible to remove the adrenergic component pharmacologically, by blocking it with α_1-antagonists or to isolate it by removing the non-adrenergic response with nifedipine.

Prejunctional interactions

When each component within the response to a train is isolated (by nifedipine or an α_1-blocker) and then an α_2-adrenoceptor antagonist is given, the extent of α_2-mediated restraint of each component can be assessed. There is little difference in the sensitivity of the two types of response to the inhibitory effects of exogenous α_2-agonists (present study; MacDonald & McGrath, 1980). Interestingly, at relatively high frequencies, it was easier to demonstrate the prejunctional effect of nerve stimulation in the prostatic portion, where the contractile response is mainly non-adrenergic but which has the densest adrenergic innervation (Anton *et al.*, 1977). In contrast, at low frequencies, the adrenergic (*II*s) component is under greater α_2-restraint. Thus, α_2 'feedback' within the 'adrenergic' system could be shown at lower frequencies than were required for effective α_2-mediated inhibition of non-adrenergic transmission. Possibly, inhibition of non-adrenergic transmission requires diffusion of noradrenaline over a greater distance and thus requires the greater concentration of extraneuronal noradrenaline which will be produced with a high frequency train. In support of this, blockade of the neuronal re-uptake of noradrenaline, which also should allow greater diffusion, increased the endogenous inhibition of the non-adrenergic response.

After reserpine or 6-hydroxydopamine, no potentiation by α_2-adrenoceptor antagonists could be found. This is expected if no noradrenaline is there to be released and confirms the specific nature of the potentiation by α_2-adrenoceptor antagonists. After removal of noradrenaline, the response to a train at high frequency (>5Hz) consists of summating, facilitating and unrestrained *I*s components, each of which is, in addition, a prolonged response. Consequently the response is large, monophasic and relaxes slowly at the end of the stimulus train. It is interesting that this response reaches a peak after 3 to 4 s and then is not maintained, even after blockade of the prejunctional α_2-adrenoceptors. It seems that the 'non-adrenergic' component can be maintained for

longer than the adrenergic component but still wanes during prolonged stimulation. We have no explanation for the prolongation of responses after reserpine or 6-hydroxydopamine. This may be an early manifestation of 'denervation supersensitivity'.

The physiological role of prejunctional α_2-adrenoceptor activation cannot be deduced from these experiments. However, it has been shown that noradrenaline, released from the adrenergic nerves, has the ability to attenuate both the adrenergic and 'non-adrenergic' aspects of motor transmission in the rat vas deferens. There is thus support both for the 'α_2-feedback hypothesis' and for the concept that adrenergic nerves can exert an α_2-mediated inhibitory effect against transmission at neighbouring 'non-adrenergic' junctions.

Differential effects of nifedipine

Although the greater part of the adrenergic nerve-induced contraction occurs within the first few seconds of a train and is nifedipine-resistant, there is a small, maintained, α_1, adrenergic component throughout the duration of the train, which is responsible for the well-documented but small reduction of the 'secondary' response to trains by α_1-blockade (Swedin, 1971; Anton *et al.*, 1977; Brown *et al.*, 1979). This can be regarded as a third component (*III*) of nerve transmission and is the 'second' which is adrenergic. Since nifedipine extinguishes the later part of the response to a train, this 'phase *III* adrenergic' response must be nifedipine-sensitive. Like the response to exogenous noradrenaline, this component (*III*) is susceptible to α_1 blockade and to nifedipine. Thus prolonged stimulation of α_1-adrenoceptors by noradrenaline, whether exogenous or endogenous, results in one type of response which is susceptible to nifedipine, while there is another, nifedipine-resistant response (*II*s), which is activated rapidly by the noradrenaline released by the first few pulses in a train.

If the isolated vas deferens is used as an experimental tool, the effects of nifedipine suggest that exogenous noradrenaline, added to the bath, do not mimic the effects of discrete, adrenergic nerve stimuli. Perhaps it is more realistic to classify the effects of exogenous noradrenaline along with the relatively minor 'phase *III*' adrenergic nerve response. Phase *III* and exogenous noradrenaline may activate a different sub-group of the population of α_1-adrenoceptors from that involved in *II*s. Similar differentiation of nerve and agonist-mediated adrenergic responses has been shown on the pithed rat blood pressure and the rat anococcygeus where α_1-receptors are involved with both stimuli (Flavahan & McGrath, 1982; Bowman & McGrath, 1982). This might depend on the anatomical location of the different receptors and/or reflect different excitation-contraction coupling systems (McGrath, 1982; 1983).

The site of action of nifedipine has been investigated in the guinea-pig vas deferens: nifedipine does not affect the e.j.ps but prevents the initiation and

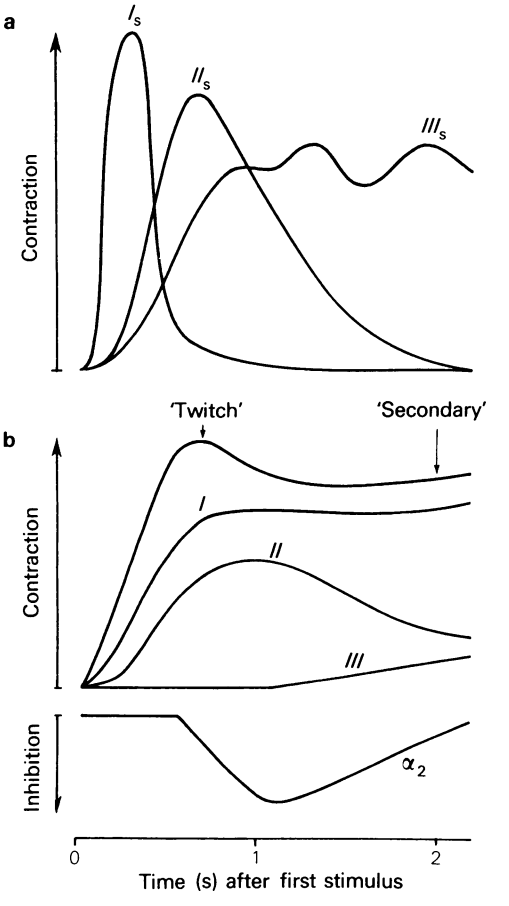

Figure 9 Schematic representation of the events following stimulation of the rat vas deferens based mainly on data from Figures 1, 3 and 7. (a) Single stimulus *I*s = the 'non-adrenergic' contraction; *II*s = the α_1-noradrenergic, nifedipine-resistant contraction; *III*s = the α_1-noradrenergic but nifedipine-sensitive contraction which is seen only after blockade of noradrenaline's uptake into nerves. (b) Continuous stimulation at 6 Hz. Upper curve control response showing 'twitch' and 'secondary' contractions. *I* = the 'non-adrenergic' contraction; *II* = the α_1-noradrenergic, nifedipine-resistant contraction; *III* = the α_1-noradrenergic, nifedipine-sensitive contraction which contributes significantly only with prolonged stimulation; α_2 = the prejunctional, α_2-adrenoceptor-mediated inhibition of the contractile responses. The 'contraction' scale in (a) is 3 times larger than in (b).

propagation of the muscle action potential, presumably calcium borne, and blocks the 'non-adrenergic' but not the 'adrenergic' contraction (Blakeley, Brown, Cunnane, French, McGrath & Scott, 1981). Thus the non-adrenergic contraction requires the action potentials initiated by e.j.ps. On the other hand, if these features occur also in the rat vas, the *II*s adrenergic component may not require the action potential and may utilize a different 'calcium mechanism' compared with the other stimuli: viz. exogenous noradrenaline, prolonged adrenergic nerve stimulation (i.e. *III*) and the 'non-adrenergic' *I*s. Differential effects on different Ca^{2+}-channels may explain why some other 'Ca^{2+} entry blockers', such as verapamil do block *II*s (French & Scott, 1981a).

Susceptibility to nifedipine may indicate those adrenergic responses which require an action potential and help to separate responses initiated by pharmacologically similar receptors but involving different activation systems.

Identification of the different components and their 'use' in pharmacology

It is possible, by considering the effects of all these drugs, to devise a scheme for the various effects induced by nerve stimulation (Figure 9).

The response to a single pulse provides a much simpler test preparation than that to a train: with intervals of 2 min or more, no interaction between pulses is detected (McGrath, 1978). The adrenergic response can be isolated in the epididymal portion without the use of drugs but the small, accompanying, non-adrenergic component can be removed by nifedipine. In the presence of nifedipine, there is the additional, fortuitous factor that postjunctional excitatory effects of test-drugs are eliminated. Thus, a variety of drugs can be tested for their effects against adrenergic transmission, despite their possessing undesirable excitatory actions, e.g. prejunctional α_2-agonists or postjunctional α_1-antagonists. On the other hand, prejunctional α_2-antagonism cannot be safely assayed on the epididymal portion's adrenergic *II*s component since any concurrent α_1-antagonism will block the contractile response: this can, however, be tested on the *I*s response by using the prostatic portion and, if it is required, blocking α_1-adrenoceptors with the highly selective prazosin or corynanthine. Thus, even when drugs have pre- and postjunctional actions, these can be safely estimated in the rat vas.

In the case of drugs that have relatively weak postjunctional effects such as opiates or purine nucleotides, the prejunctional effects can be tested against adrenergic or non-adrenergic transmission. This may seem an esoteric exercise but has advantages when attempting to correlate effects against contractile responses with other guides to neurotransmission. For example, in the vas deferens there is no solid evidence to connect e.j.ps, and the action potentials which they initiate, with the adrenergic contraction. However, these electrical events are implicated in the non-adrenergic contraction (Blakeley *et al.*, 1981). In contrast, sympathetic transmitter output, measured as the nerve-induced overflow of noradrenaline (tritiated or endogenous), is more likely to correlate with an 'α-adrenergic' contraction. Although the α_2-adrenoceptor agonists produce no significant distinction between the two responses (*I*s and *II*s), differences are produced by opiates, ATP and β-adrenoceptor agonists (MacDonald & McGrath, 1980).

Clearly, interpretation of the effects of drugs on the complex responses to trains of pulses provides a challenge. Figure 9 is an attempt to outline the elements involved. As a useful test preparation, however, the repetitively stimulated vas is severely limited. One interesting point which does emerge is that, within a train of pulses at 5–10 Hz, the timing of the most effective α_2 prejunctional effect corresponds to the most effective α_1, postjunctional effect. Does this suggest that noradrenaline release falls off rapidly after the first few pulses? Von Euler (1969) suggested that the acute effects of reserpine on guinea-pig vas deferens could be explained by its preventing the filling of a 'readily-releasable pool' of noradrenaline, perhaps membrane-bound from the main 'storage pool'. A similar concept might explain the rapid decline of adrenergic effects within a train of pulses. However, there could be other explanations. For example, a fall off at the postjunctional level could arise if the *II*s response used a limited store of Ca^{2+} to initiate contraction (the nifedipine-resistant α_1 system) which cannot sustain a prolonged response. A prolonged response might require the integrity of a nifedipine-sensitive Ca^{2+} channel, which is activated by the action potential. Similarly the absence of α_2-mediated prejunctional effects after the first few seconds could be due to complex interactions between the agonist (noradrenaline), the antagonist and the prejunctional release system. On balance, a fall off in noradrenaline output has the benefit of simplicity. Unfortunately studies on the output of transmitter to a single pulse or short trains, measured as overflow, have not so far been reported for rat vas deferens. In the mouse vas (T.O. strain) no such fall off occurs (Baker & Marshall, 1982) but in this strain, the *II*s response is very weak (McGrath, 1978), so this phenomenon of an initial 'extra' output may be lacking.

Interpretation of the response in the whole vas is also complex, whether this is to nerve stimulation or to agonists and its employment now seems inexpe-

dient. Comparison of the two ends of the organ in the presence of nifedipine confirms that, in the prostatic portion, the adrenergic contractile component is present but relatively small. A prejunctional adrenergic response could be demonstrated, i.e. inhibition by exogenous α_2-adrenoceptor agonists, presumably exerted at the elusive terminals which release the non-adrenergic transmitter. Even this effect is relatively weakly engaged by endogenous noradrenaline unless neuronal uptake of noradrenaline is blocked or high frequencies of stimulation are employed. Thus, in this portion, which has the densest adrenergic innervation, not only in the vas, but in any other smooth muscle tissue, the 'adrenergic' contractile responses are weak, of unknown physiological significance and possibly, are unique so that they do not serve as a useful model for other tissues. The major effect of noradrenergic nerves here may be restraint of the 'motor non-adrenergic nerves', as postulated by Ambache, Dunk, Verney & Zar (1972) or may be some, as yet, undetected effect.

The survival of the 'non-adrenergic' component after 6-hydroxydopamine treatment appears, at first, to conflict with the suggestion, in guinea-pig vas, that this response may be due to ATP, released from adrenergic nerves as a co-transmitter (Fedan, Hogaboom, O'Donnell, Colby & Westfall, 1981). These observations could be reconciled if the 'non-adrenergic' response can be initiated by only a small proportion of the nerves in the organ, which is possible since it involves the spread of the action potential (Blakeley *et al.*, 1981).

In conclusion, it is not surprising that conflicting results have emerged from experimentation with the rat vas deferens. An independent, non-adrenergic system seems to exist alongside the adrenergic one; the adrenergic stimuli can activate at least two types of muscle contraction and inhibit the two types of neurotransmission. However, these complexities are now avoidable.

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