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The contractile response to and the release of noradrenaline by transmural nerve stimulation in the guinea-pig vas deferens and a comparison with the response to noradrenaline

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1 The release of noradrenaline from the guinea-pig vas deferens by transmural stimulation was measured electrochemically after separation by h.p.l.c. Variations in release with different durations of stimulation were then compared with the mechanical response during stimulation.

2 During long periods of 270 s of stimulation at 10 Hz the average release of noradrenaline in the final 250 s remained at about 50% of that released during the first 20 s yet the mechanical response throughout the later time declined to near zero.

3 Superfusion with constant concentrations of noradrenaline produced mechanical responses similar to those from transmural nerve stimulation, an initial rapid contraction declining to a low level of maintained activity. During the later period, when the response to infused noradrenaline was low, the twitch response to transmural stimulation was potentiated.

4 Phentolamine 10^{-6} M reduced the potentiation of the twitch response to transmural stimulation by noradrenaline and propranolol 10^{-6} M enhanced the potentiation.

5 These results suggest that the decline in the adrenergic component of the response to prolonged transmural nerve stimulation is not the result of a decline in noradrenaline release but mainly due to desensitization to the contractile effect of noradrenaline. Since the similar desensitization produced by infused noradrenaline does not reduce the twitch response to transmural nerve stimulation it is unlikely that the transmitter responsible for that component is noradrenaline.

Introduction

The mechanical response to brief stimulation of the nerves to the vas deferens is a contraction made up of two components, an initial rapid contraction and partial relaxation (the twitch) followed by a slower more sustained contraction (Swedin, 1971). In spite of the high density of adrenergic nerves within the vas deferens there is considerable doubt as to whether noradrenaline is the sole motor transmitter (Ambache & Zar, 1971; von Euler & Hedqvist, 1975). The relative magnitudes of the twitch and secondary contraction vary along the length of the vas, the twitch dominant in the prostatic end and the slow component in the epididymal end, suggesting some regional differences in neurotransmission, and α-adrenoceptor antagonists reduce mainly the secondary contraction suggesting it, but not the twitch, is due to noradrenaline release (Ambache, Dunk, Verney & Zar, 1972; Duncan & McGrath, 1976; McGrath, 1978). By contrast, Doggrell (1981) showed that prazosin inhibits both components of the mechanical response in the rat vas deferens and in the epididymal half the two components were equally reduced suggesting both components are adrenergic.

The mechanical responses to nerve stimulation have mostly been studied with either single stimuli or short periods of stimulation not exceeding 30 s. If stimulation is continued for several minutes then the mechanical response disappears almost completely after about 30 s. If noradrenaline is the sole motor transmitter this decline could be due either to a decline in the release of noradrenaline or to a rapidly developing insensitivity to the amine. We report in this paper measurements of noradrenaline release during short, 20 s, and prolonged, 4.5 min, periods of stimulation and have compared the mechanical re-

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sponse to nerve stimulation with that to superfusion with a constant concentration of noradrenaline. In addition, we have tested whether desensitization with superfused noradrenaline would reduce the twitch response to nerve stimulation. The results suggest that the decline in the mechanical response is not due to a failure of noradrenaline release but mainly to a decline in sensitivity to noradrenaline. The desensitizing effect of noradrenaline superfusion did not affect the twitch response which was potentiated, suggesting that this component is not due to noradrenaline.

Methods

Adult male Dutch guinea-pigs were stunned and bled. The two vasa deferentia were removed from the epididymis to the prostate, their mesenteric coat stripped and a fine silver wire electrode inserted in the lumen of each along its entire length. Both tissues were then mounted vertically in a 2 ml perspex chamber through which warm Krebs solution could be perfused and then removed by suction at the upper surface of the chamber. The chamber and fluid were kept at 37°C by heat exchange channels in the perspex block through which warm water was circulated. The composition of the Krebs solution in mM was: NaCl 118, KCl 4.7, $CaCl_2$ 2.4, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11, the flow rate was 1.1 ml min^{-1} and the saline was gassed with a mixture of 95% O_2 and 5% CO_2. The intramural nerves were stimulated with 1 ms square wave pulses delivered from a Grass S44 stimulator and applied between the intraluminal silver electrode and an open coil of fine silver wire surrounding the entire length of the preparation. The frequency of stimulation was always 10 Hz and three train lengths were used, 20 s (200 pulses), 4.5 min (2,700 pulses) and brief trains of 1 s (10 pulses). The mechanical responses were recorded isometrically with Grass FT03 tension transducers and displayed on a Grass Polygraph. During nerve stimulation, perfusion of the bath was stopped to allow the noradrenaline to accumulate and the collection period was standardized at the duration of stimulation plus an additional 1.5 min, i.e 110 s for 200 pulses and 360 s for 2,700 pulses. The possibility that released noradrenaline was chemically oxidized was reduced by incorporating ethylenediaminetetraacetic acid (EDTA) 3×10^{-5} M and ascorbic acid 1.14×10^{-4} M into the Krebs solution and tissue inactivation by including desmethylimipramine (DMI), 17β-oestradiol and tranylcypromine all at a concentration of 10^{-5} M to block neuronal and extraneuronal uptake and monoamine oxidase.

Catecholamines were measured electrochemically after chromatographic separation by h.p.l.c. on an ion-exchange column. The catecholamines in the 2 ml samples from the bath were first adsorbed on 50 mg of acid-washed alumina by adjusting the pH to 8.6 with 0.5 ml of 0.5 M Tris buffer and shaking for 10 min (Anton & Sayre, 1962). The alumina was allowed to settle, the supernatant aspirated and discarded and the alumina washed once with a dilute 5 mM Tris buffer. The wash solution was discarded and the noradrenaline eluted from the alumina with 300 μl of 0.1 N perchloric acid containing 4×10^{-4} M sodium metabisulphite as antioxidant (Keller, Oke, Mefford & Adams, 1976). All of this eluate was injected into the h.p.l.c. system which consisted of a 50 cm long 0.25 cm i.d. stainless steel column packed with Waters CX/Corasil cation exchange resin (37–50 μm particle size). The mobile phase was citrate-acetate buffer pH 5.2 filtered and degassed and perfused at a flow rate 1.5 ml min^{-1}. This system will separate adrenaline, noradrenaline and dopamine and the quantities of each amine were measured electrochemically using a Bioanalytical System TL-5 glassy carbon cell. The threshold level for assay of noradrenaline in this system was about 100 pg.

Adrenergic nerves liberate noradrenaline in the absence of nerve impulses and in the presence of the antioxidants, chelators and uptake blockers this spontaneous release gives rise to measurable quantities of noradrenaline. It was therefore necessary to measure spontaneous release over the same period of time as was used to collect the stimulated samples and to subtract this from the stimulated value to get a measure of the additional release by nerve stimulation. Spontaneous release was found to vary with the interval since setting-up the preparation in the bath and as a result a minimal interval of 45 min was allowed before nerve stimulation.

Drugs

Drugs used were: L-ascorbic acid (BDH, desmethylimipramine hydrochloride (Geigy), dopamine hydrochloride (Koch-Light), ethylenediaminetetraacetic acid disodium salt—EDTA (Sigma), (−)-noradrenaline bitartrate (Koch-Light), 17β-oestradiol (Sigma), phentolamine (Ciba), propranolol (ICI) and tranylcypromine sulphate (SKF).

Results

Recovery of catecholamines

Catecholamines are chemically unstable and liable to oxidation especially in warm Krebs solution gassed with 95% oxygen. In addition, there are several

Table 1 Factors affecting the recovery of noradrenaline and dopamine

Sample	n	*% Recovery* NA	DA
Control (immediate extraction)	11	71.6±2.2	70.6±3.3
[*Chemical oxidation (Krebs at 37°C for 9min)*]			
Control	6	0	0
Ascorbic acid (1.14×10^{-4} M)	6	0	0
EDTA (3×10^{-5} M)	5	57.9±3.6	51.6±4.3
Ascorbic acid (1.14×10^{-4} M) + EDTA (3×10^{-5} M)	12	65.5±2.5	65.8±3.5
Ascorbic acid (2.3×10^{-4} M) + EDTA (6×10^{-5} M)	8	61.1±2.8	64.3±2.2
[*Biological inactivation (37°C for 6 min)*]			
Control (no tissue)	6	66 ±4.0	73 ±5.0
Tissue present	20	55 ±3.6	38 ±1.9
Tissue + DMI + 17β-oest + Tranyl	8	93.1±4.8	45.9±2.1

Catecholamines were either extracted immediately from Krebs solution, kept in the warm organ bath at 37°C and bubbled with 95% O_2 for nine minutes to test the effect of anti-oxidants ascorbic acid and ethylenediaminetetraacetic acid (EDTA) or incubated in the presence of the vas deferens to test the inactivation due to tissue. In this latter group ascorbic acid 1.14×10^{-4} M and EDTA 3×10^{-5} M were always present. Finally, the combined effect of the neuronal and extraneuronal uptake blockers desmethylimipramine (DMI 10^{-5} M) and 17β-oestradiol (17β-oest 10^{-5} M) together with the monoamine oxidase inhibitor tranylcypromine (Tranyl 10^{-5} M) on tissue inactivation is shown.

biological disposal mechanisms available to the tissues. All of these would reduce recovery of released noradrenaline. We, therefore, examined the effects of inhibiting these various routes of inactivation on the recovery of noradrenaline and dopamine by adding 10 ng of each amine to the bath and then extracting and assaying the catecholamines in the bath fluid after a period of time equal to the collection times used in later experiments. Dopamine as well as noradrenaline was added since the experiments were part of a separate investigation into the release of dopamine. The results are shown in Table 1. Catecholamines added to the Krebs solution and immediately extracted gave recoveries for both amines of just over 70%; added to Krebs solution at 37°C and left for 9 min resulted in a complete loss of both. Ascorbic acid 1.14×10^{-4} M alone was ineffective as a protectant but the addition of 3×10^{-5} M EDTA produced recoveries of over 50%. The combination of both ascorbic acid and EDTA did show additional protection and increased recoveries to about 65%. Doubling the concentration of both EDTA and ascorbic acid did not significantly increase these recoveries suggesting the lower concentrations provided adequate protection from chemical oxidation in the bath. This lower concentration of EDTA and ascorbic acid was used to examine the additional biological inactivation from the presence of the vasa deferentia in the bath. In these experiments the time of exposure was reduced to 6 min and this with the addition of EDTA and ascorbic acid gave control recoveries of about 70% and similar to immediate extraction. In the presence of tissue, recoveries fell and the noradrenaline (55%) and dopamine (38%) were now significantly different. This difference was exaggerated when the uptake and enzyme blocking drugs were added, noradrenaline rose to 93% and dopamine only to 46%. The explanation for these differences lies in the spontaneous release of noradrenaline. When this is protected from oxidation and particularly when this is combined with uptake block, noradrenaline but not dopamine from the tissues accumulates in significant quantities and gives an apparent recovery of noradrenaline which is fallaciously high. To demonstrate this we measured the extent of this spontaneous accumulation in the absence of added amines.

Spontaneous overflow of noradrenaline

Krebs solution containing antioxidants only and removed after 6 min contact with a pair of vasa deferentia always contained measurable amounts of noradrenaline but never dopamine. The amount depended on how soon after setting-up the preparation the six minute stopflow sample was taken. This is illustrated in Figure 1 for a single preparation in which four 6 min samples were collected at different times. In

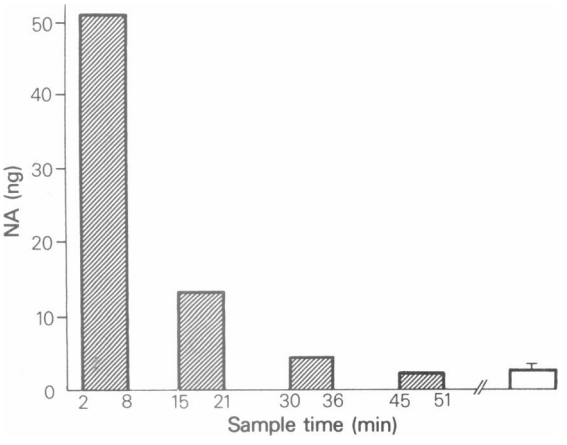

Figure 1 The accumulation of noradrenaline in six minute stopflow samples as a result of spontaneous overflow from a pair of vasa deferentia. The hatched columns are from a single experiment and show the progressive decline with time. The final open column shows the mean spontaneous overflow in 22 similar experiments but measured at least 45 min after setting-up the preparations. Ascorbic acid 1.14×10^{-4} M and EDTA 3×10^{-5} M present.

the first, beginning 2 min after the preparation was suspended in the bath, over 50 ng of noradrenaline accumulated in 6 min. In subsequent periods this accumulation declined, initially rapidly to 14 ng and, finally, to 2.4 ng after 45 min. This is apparently a base line since the average of 22 experiments in which spontaneous overflow was measured, 45 min or more after setting-up the preparation, averaged 2.8 ng as shown in the final column of Figure 1. This amount of noradrenaline is sufficient to explain the apparently greater recovery of noradrenaline in Table 1 if one allows for the fact that recovery was usually measured after less than 45 min when spontaneous accumulation would be greater.

Noradrenaline overflow and the mechanical response to nerve stimulation

The contractile response to short, 20 s, and prolonged, 4.5 min, field stimulation is illustrated in Figure 2c. The response to prolonged stimulation consists of three components. The first two are the well-known initial twitch and the secondary contraction. These largely disappear after about 20 s of stimulation and, for the remainder of the long period of stimulation, the response consists of a third component, a maintained small elevation of the base line on which small intermittent contractions are superimposed. When stimulation stops this residual activity disappears. Both the secondary slow contrac-

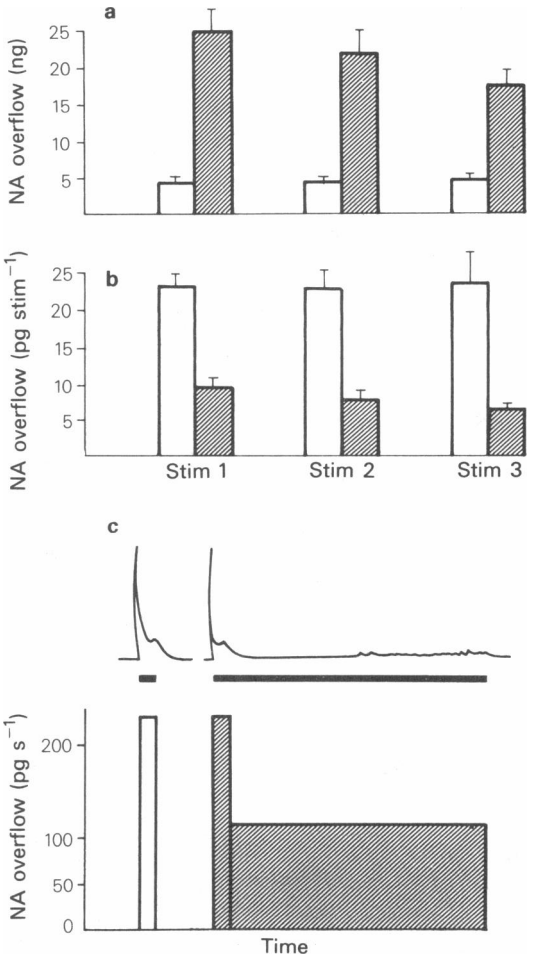

Figure 2 A comparison of the release of noradrenaline and the mechanical response to short and long trains of transmural stimulation of the guinea-pig vas deferens; values have been corrected for spontaneous release and for recovery. The histogram in panel (a) shows the mean total noradrenaline overflow in three successive periods of stimulation with either 200 stimuli (open columns) or 2,700 stimuli (hatched columns) both at 10 Hz. The histogram in panel (b) shows these same results expressed as noradrenaline overflow per stimulus (stim) which is now greater for the shorter trains. Panel (c) shows recordings of the mechanical response to short and long periods of stimulation and below this the average rate of noradrenaline release during the short period of stimulation and during the early and late phase of the long period of stimulation. During the long train the mechanical response after the first 30 s falls to near zero but the noradrenaline overflow falls only by 50% Release is based on eight experiments and bars show the s.e.mean. All experiments in the presence of ascorbic acid 1.14×10^{-4} M, EDTA 3×10^{-5} M, DMI 10^{-5} M, 17β-oestradiol 10^{-5} M and tranylcypromine 10^{-5} M.

tion and this residual mechanical activity are completely abolished by phenoxybenzamine and are presumably both due to noradrenaline acting on postsynaptic α_1-adrenoceptors. If noradrenaline is the transmitter and there is no change in sensitivity of the muscle, one would expect a considerable fall in transmitter release with continued stimulation and a corresponding fall in overflow between the first 20 s and the remainder of the stimulation period. We have measured the overflow of noradrenaline produced by three successive periods of stimulation at 10 Hz with train lengths of 200 (20 s) or 2,700 (4.5 min) pulses. The results are shown as histograms in Figure 2. The top panels show the total overflow of noradrenaline and the second the overflow expressed as pg stimulus^{-1}. As the upper panel shows, about six times more noradrenaline appeared in the bath fluid with 2,700 pulses than with 200 pulses. These total amounts conceal differences in the rate of release which is more likely to be related to the mechanical response. Expressed as output/stimulus, which compares the rate of release, the advantage is reversed and the average rate of release during the 200 pulse trains was more than twice that during the 2,700 pulse train. Figure 2 shows a second difference between the two train lengths. With 2,700 pulses there is a progressive decline in release in successive periods of stimulation but with 200 pulses the release is constant. The final panel in Figure 2c is derived from the data on the average release during a first period of stimulation with either 200 or with 2,700 pulses on the assumption that, during the first 200 pulses of a 2,700 pulse train, the rate of release of noradrenaline will be the same as that measured with a 200 pulse train. By subracting the value for the 200 pulse train from the total for the 2,700 pulse train, the average rate of release over the final 2,500 pulses can be calculated. As Figure 2c shows, during this later period when the tissue showed hardly any contractile activity the average rate of release remained at about 50% of that during the first 20 s when the contractile response was intense.

The response to noradrenaline

The decline in the release of noradrenaline as judged by its overflow from the tissue seemed insufficient to explain the almost complete loss of contraction during the third component of the response to a long stimulus train. We, therefore, examined the response to similarly prolonged exposure to constant concentrations of noradrenaline. To ensure a constant concentration, fresh noradrenaline in Krebs solution was continuously superfused over the tissue by a Watson-Marlow flow pump. The response to superfusion with increasing concentrations of noradrenaline is illustrated in Figure 3. The response, especially to higher

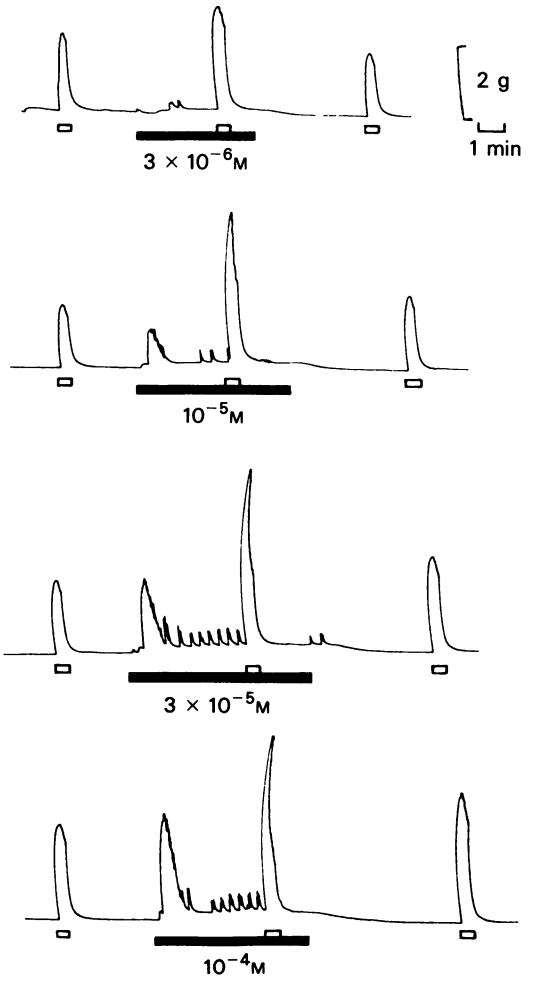

Figure 3 The effect of superfusing noradrenaline over the guinea-pig vas deferens on its response to transmural nerve stimulation. Transmural stimulation as indicated by the open rectangles was at 10 Hz for 30 s. Noradrenaline was superfused during the periods indicated by the black bar and at four different concentrations as shown under each bar. At all noradrenaline concentrations the nerve response is potentiated and with the higher concentrations this potentiation persists after the noradrenaline has been washed away. Noradrenaline also directly stimulates the preparation but this response declines after an initial rapid and powerful contraction during the first 30 s of superfusion. Ascorbic acid 1.14×10^{-4} M, EDTA 3×10^{-5} M, DMI 10^{-5} M, 17β-oestradiol 10^{-5} M and tranylcypromine were present throughout.

concentrations, resembles that to nerve stimulation, an initial rapid contraction decaying to a low level of tone with intermittent small contractions. At the higher concentrations the rate of contraction during

the initial response was almost as great as that during the twitch response to transmural stimulation. The decay was not due to tissue inactivation of infused noradrenaline since the addition of uptake and monoamine oxidase (MAO) blockers made no difference to the response.

If noradrenaline is the transmitter of both components of the response to nerve stimulation, then during a prolonged infusion of noradrenaline when the tissue loses the ability to respond to the amine, one would expect the twitch response to transmural stimulation similarly to decline. This was not so, as Figure 3 shows the response to short trains of stimuli at 10 Hz during the infusion of noradrenaline was potentiated at all concentrations from 3×10^{-6} M to 10^{-4} M and, with the higher concentrations of noradrenaline, this potentiation persisted, at a declining level, for at least ten minutes after removing the amine by washing.

While potentiation of the motor response to nerve stimulation by exogenous noradrenaline has been described, the more common effect, at least in recent papers, has been inhibition attributed to a presynaptic action on transmitter release. In our experiments with trains of 300 pulses, we never found any inhibitory effect. Ambache & Zar (1971) used short trains of a few pulses repeated at relatively short intervals to demonstrate the inhibitory effects of noradrenaline on the response to nerve stimulation. We, therefore, modified our experiments to this pattern with stimulation at one minute intervals with short trains of 10 pulses at 10 Hz. The results are shown in Figure 4. Once again the predominant effect of a noradrenaline superfusion was to potentiate the response to transmural stimulation. On rare occasions (Figure 4) a brief inhibition of the first response was seen followed by potentiation. This potentiating effect of noradrenaline was almost abolished by phentolamine 10^{-6} M (Figure 4a) and is presumably mediated by α-adrenoceptors. In the presence of phentolamine the initial inhibitory effect of noradrenaline is more often observed. The possibility of effects mediated by β-adrenoceptors was examined by studying the effects of isoprenaline and propranolol. As Figure 4c shows, isoprenaline inhibits the response to transmural stimulation, an effect abolished by prop-

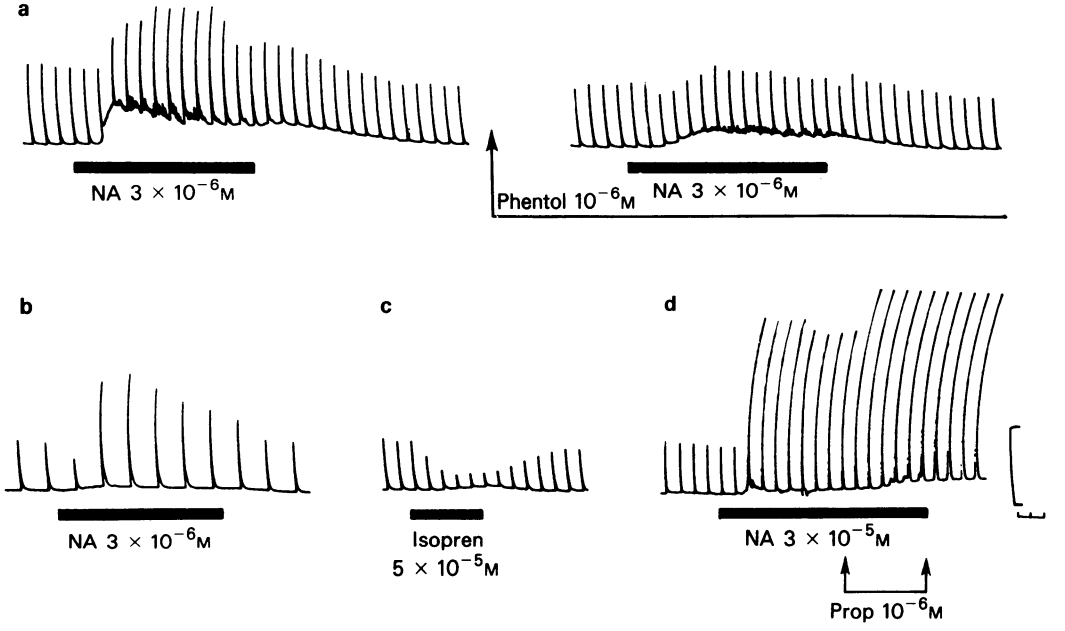

Figure 4 The effect of noradrenaline or isoprenaline superfusion on the twitch response to 10 stimuli at 10 Hz repeated at 1 min intervals. The top panel (a) shows the potentiation of the nerve response and the direct contractile action of noradrenaline 3×10^{-6} M perfused during the period indicated by the black bar; phentolamine 10^{-6} M reduced both the potentiation and the direct contractile effect. The initial effect of noradrenaline is now to inhibit the twitch. (b) Illustrates in another preparation the initial inhibitory effect of 3×10^{-6} M noradrenaline followed by potentiation. The inhibitory effect on the twitch of isoprenaline is shown in (c). In the last panel (d) the potentiating effect of noradrenaline 3×10^{-5} M is increased by propranolol 10^{-6} M indicating an underlying inhibitory effect mediated by β-adrenoceptors. Calibration 1 g and 2 min in (a), (c) and (d); 2.5 g and 1 min in (b). Ascorbic acid 1.14×10^{-4} M, EDTA 3×10^{-5} M, DMI 10^{-5} M, 17β-oestradiol 10^{-5} M and tranylcypromine 10^{-4} M present throughout.

ranolol. The possibility that noradrenaline also acted on β-adrenoceptors was examined by examining the effect of propranolol on the potentiating effect of noradrenaline (Figure 4d). Superfusion with noradrenaline 3×10^{-5} M produced the expected potentiation, the subsequent addition of propranolol 10^{-6} M augmented this potentiation still further, suggesting that the noradrenaline can stimulate both α-adrenoceptors, potentiating the nerve response, and β-adrenoceptors, inhibiting the response, but normally the effect of the former dominates.

Discussion

The neural mechanism underlying the contractile response to nerve stimulation in the vas deferens remains a puzzle. Most investigations have used short periods of stimulation up to about 30 s and have identified, in these circumstances, two components to the response, an initial rapid contraction or twitch which declines quickly, followed by a slower re-establishment of tension, the secondary component. Few studies have described the response to prolonged stimulation where, as the present results show, even the secondary contraction declines to a low level of maintained tone with superimposed brief contractions. A similar sequence of events has been recorded by Wadsworth (1974). Recent studies have shown the secondary contraction is abolished by α blocking drugs including prazosin, implying an adrenergic effect through postsynaptic α_1-adrenoceptors (Boyd, Chang & Rand, 1960; Brown, McGrath & Summers, 1979). The present results confirm this and extend it to the prolonged low level activity which replaces the secondary contraction during longer periods of stimulation. The decline in the secondary contraction might owe something to a reduction in transmitter release with prolonged stimulation but since the average noradrenaline concentration as judged by overflow does not fall below half the peak level this is probably not an important cause of the decline. In any case, since the response to exogenous noradrenaline superfused at a constant concentration suffers a similar decline, the major cause would seem to be a decline in the effectiveness of noradrenaline in causing a contraction.

The origin of the first, twitch component in the response to noradrenaline is less clear. At first sight exogenous noradrenaline seems to mimic this component reasonably well. However, this component fails to decline, as the tissue becomes desensitized to the contractile effect of noradrenaline but on the contrary shows a maintained potentiation, arguing against noradrenaline as transmitter. It has been argued that the junctional receptors close to the nerve varicosities differ from the extra-junctional receptors elsewhere on the muscle membrane (Hotta, 1969; Hirst & Neild, 1980). If so, one could postulate that exogenous noradrenaline acts only on the extra-junctional receptors and does not have access to the junctional receptors. The junctional receptors must be assumed to desensitize if continuously exposed to transmitter since both the twitch and the secondary adrenergic component of the response to nerve stimulation decline as rapidly and, if anything, to a lower level than the response to exogenous noradrenaline. Such a barrier to exogenous noradrenaline reaching junctional receptors seems unlikely since transmitter released spontaneously or by nerve stimulation is recovered in the bath fluid. While it is not possible to exclude completely the possibility that noradrenaline is the sole transmitter, the likeliest explanation still seems to be that, first suggested by Ambache & Zar (1971) and supported by Hedqvist & von Euler (1976), that a second transmitter is responsible for the twitch.

The interaction of noradrenaline and nerve stimulation is particularly complex in the vas deferens. Hedqvist & von Euler (1976) described three effects; noradrenaline could potentiate the twitch response by a postsynaptic action on α-adrenoceptors on the muscle membrane; inhibit the twitch by reducing transmitter release through a presynaptic action on αadrenoceptor and reduce the twitch by an action on both pre- and postsynaptic β-adrenoceptors. In the present experiments convincing evidence for the first and last of these effects only was obtained. The predominant effect was potentiation of the twitch by an α-adrenoceptor mediated effect. This potentiation was restrained by a simultaneous inhibitory effect mediated by β-adrenoceptors. Removal of this inhibition by propranolol enhanced the potentiation. Potentiation by noradrenaline has been attributed to postsynaptic depolarization of the smooth muscle membrane through activation of α-adrenoceptors (Sjöstrand, 1973; Hedqvist & von Euler, 1976). We have no direct measurement of membrane potential but the persistence of potentiation up to ten minutes after removing the noradrenaline from the bath and, at a time when there was no remnant of a direct motor response to noradrenaline, might suggest that depolarization was not the only mechanism.

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