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AN INHIBITORY ROLE FOR NORADRENALINE IN THE MOUSE VAS DEFERENS

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1 Noradrenaline (0.1–3.0 μM) inhibited the twitch responses to single pulse field stimulation of the isolated vas deferens of the mouse. The higher concentrations of noradrenaline (ca. 0.3–3.0 μM) were required to make the tissue contract.

2 Phentolamine (10 μM) abolished the contractor response to higher concentrations of noradrenaline and antagonized the inhibitory effect of lower concentrations on the twitch response.

3 Propranolol (10 μM) potentiated both the contractor and the inhibitory effect of noradrenaline on the twitch response.

4 Isoprenaline (0.1–3.0 μM) and salbutamol (1.0–3.0 μM) both inhibited the twitch response. Their effects were antagonized by propranolol (10 μM), but not by practolol (10 μM).

5 The effects of uptake$_1$ and uptake$_2$ blocking agents were determined. Cocaine (10 μM) reduced the size of the twitch response in 2 out of 4 experiments. Imipramine (0.18 μM) also reduced the size of the twitch, as did oestradiol (3.7 μM) and a combination of cocaine and oestradiol.

6 Contractor responses to exogenous noradrenaline showed tachyphylaxis, but when this was not very marked, the response could be shown to be potentiated by uptake blocking agents.

7 The inhibitory effect of noradrenaline on the twitch response was greatly potentiated by cocaine (10 μM) and much less so by oestradiol (3.7 μM).

8 It is concluded that the transmitter responsible for the twitch response is either an unknown substance released from the sympathetic neurone, or noradrenaline acting upon a receptor with none of the characteristics of known α- or β-adrenoceptors. In either case, noradrenaline can inhibit the output, probably by stimulation of presynaptic α-adrenoceptors.

Introduction

Ambache & Zar (1971) have provided evidence that noradrenaline is not the motor transmitter in the guinea-pig vas deferens. This view has also been taken by von Euler & Hedqvist (1975). Nevertheless, many species including the mouse have a high concentration of noradrenaline in the vas deferens (Sjöstrand, 1962, 1965; Blakeley, Dearnaley & Harrison, 1970) and it seems inconceivable that this catecholamine has no part to play in the transmission of nerve impulses. Ambache & Zar (1971) showed that exogenous noradrenaline inhibited the 'twitch' responses of the field stimulated guinea-pig vas deferens and proposed an inhibitory role for the amine. In 1972, Ambache, Dunk, Verney & Zar extended their observations to include vasa deferentia from rats, rabbits and hamsters. In none of these animals did phenoxybenzamine block motor transmission in the vas deferens, though it did block some or all of the effects of the indirectly acting amine, tyramine.

Showing that guanethidine, phentolamine and 6-hydroxydopamine pretreatment blocked the responses to field stimulation, Jones & Spriggs (1975) concluded that noradrenaline is the motor transmitter in the mouse vas deferens. The results described in this paper, some of which formed a preliminary communication (Jenkins, Marshall & Nasmyth, 1976) lead to the conclusion that noradrenaline is an inhibitory rather than a motor transmitter in the mouse vas deferens.

Methods

Preparation of isolated vas deferens

Male T.O. strain mice (20–30 g) were killed by a blow on the head and exsanguinated. The abdominal wall was opened and the testes exposed together with the

epididymis and the vas deferens. The vas was cut free from the epididymis and dissected out as close as possible to its junction with the urethra. At this point it was sectioned and dropped into a Petri dish containing Krebs-Henseleit solution of the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4 7H_2O$ 1.2, $NaH_2PO_4 2H_2O$ 1.2, $NaHCO_3$ 25 and glucose 0.2% w/v. In some cases magnesium was omitted because in agreement with Hughes, Kosterlitz & Leslie (1975), it was easier to obtain good responses in its absence. The vas was carefully desheathed whilst it was in the Petri dish, all mesenteric tags and connective tissue being cut off to ensure as far as possible, that any scattered ganglion cells lying close to the vas (Sjöstrand, 1965) were removed. The vas was then mounted in a 2 ml organ bath between gutter electrodes as described by Birmingham & Wilson (1963) except that the distance between the electrodes was approximately 6.0 mm. A tension of 500 mg was applied to the tissue which was bathed in the solution described above and was gassed in the organ bath and in the reservoir with 95% O_2 and 5% CO_2. A fine stream of bubbles in the organ bath was obtained from a 23 gauge hypodermic needle. Contractions were recorded isometrically on a Grass polygraph using a Grass FTO3 linear displacement transducer.

Stimulation. Intermittent field stimulation was provided by a Grass S48 stimulator. Unless otherwise stated, single pulses of 1 ms duration were applied at a current density of 150–250 mA every 10 seconds.

An experiment to ensure that only nerve fibres were being stimulated with these parameters was performed using the higher current density. Tetrodotoxin (0.63 μM), which paralyses nerve fibres, abolished all responses using pulse widths varying from 0.1–2.0 milliseconds. There was no evidence of a direct response from the muscle until the pulse width reached 50 milliseconds.

Drugs

The following drugs were used: cocaine hydrochloride, imipramine hydrochloride (Tofranil, Geigy), isoprenaline sulphate (BDH), practolol (Eraldin, ICI), salbutamol (Allen & Hanbury), noradrenaline bitartrate (Levophed, Winthrop Laboratories), oestradiol (1 mg/ml in propylene glycol as a stock solution), phentolamine mesylate (Rogitine, Ciba), and tetrodotoxin (Koch-Light).

The receptor blocking agents (phentolamine and propranolol) and the noradrenaline uptake blocking agents (cocaine, oestradiol and imipramine) were included in the bathing fluid as indicated in the results section and were allowed to remain in contact with the tissue for 20 min before making any observations. Other drugs were added to the bath with a micrometer syringe in volumes not exceeding 0.02 ml. The strength of all drug solutions is expressed as the molar concentration in the bath.

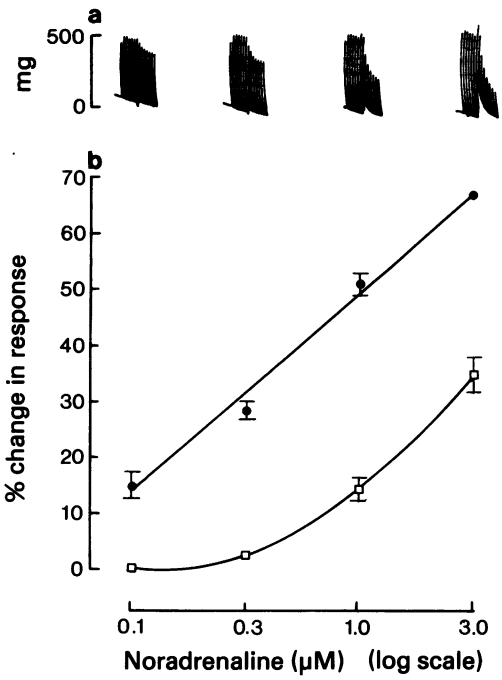

Figure 1 (a) Inhibition of twitch responses of the mouse isolated vas deferens to electrical stimulation (0.1 Hz, 1.0 ms, 250 mA) by noradrenaline (0.1–3.0 μM). Note the contraction induced by the higher concentrations. (b) Concentration-response curves for the inhibition (●) and the contraction (□). Bars represent s.e. mean (4 observations); where no bars are shown they are contained within the point.

Results

The effect of noradrenaline on the stimulated mouse vas deferens

When six single pulses were applied to the vas at 0.1 Hz the responses were constant except for the first response which was usually slightly weaker than the rest. After the sixth pulse, noradrenaline was added to the bath to give concentrations varying from 0.1–3.0 μM and was allowed to remain for 80 seconds. When the drug was washed out, the response to stimulation recovered within 8–10 min and the time cycle could be adjusted accordingly.

Figure 1 shows that noradrenaline (0.1 μM) produced only inhibition of the response to stimulation. As the concentration was increased, the

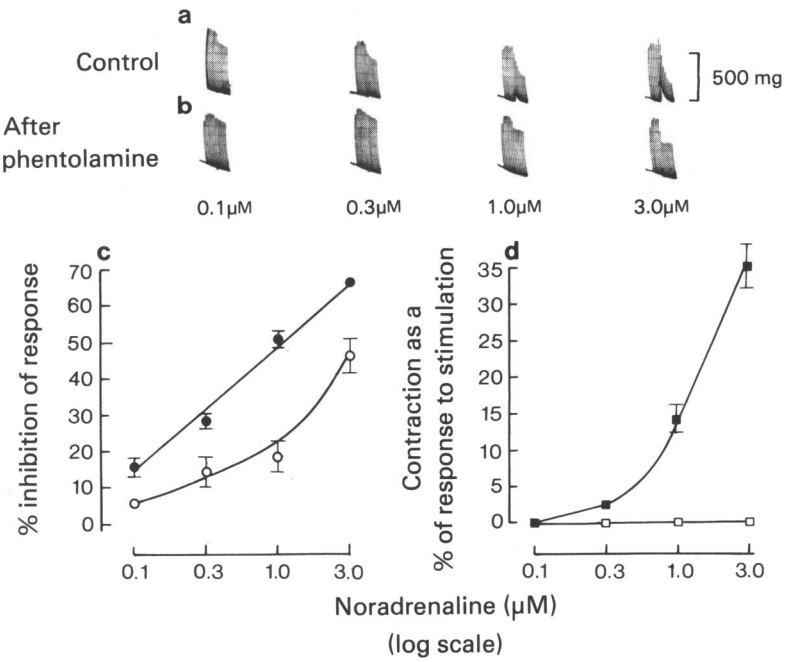

Figure 2 (a) Inhibition of twitch responses of the mouse isolated vas deferens to electrical stimulation (0.1 Hz, 1.0 ms, 250 mA) by noradrenaline (0.1–3.0 μM). In (b) the antagonism of both the inhibitory and contractor effects of noradrenaline, by phentolamine (10 μM) is shown. (c) Antagonism of the inhibitory effect of noradrenaline by phentolamine. Before phentolamine (●); after phentolamine (○). (d) Blockade of the contraction to noradrenaline by phentolamine. Before phentolamine (■); after phentolamine (□). The bars indicate the s.e. mean (4 observations). Where no bars are shown the error is contained within the point.

inhibition of the response to stimulation increased and the tissue began to contract in response to the drug. At these concentrations the duration of the contraction never exceeded the 80 s contact time, but the inhibition of the twitch always did. The same result was obtained in 5 separate experiments. Sometimes the contractor responses exhibited tachyphylaxis.

The influence of receptor blocking agents on the noradrenaline effects

Figure 2 shows that the inclusion of the α-adrenoceptor blocking agent phentolamine (10 μM) in the Krebs solution completely abolished the contractor response to noradrenaline and shifted to the right by 0.5 log units the concentration-response curve for the inhibitory effect of noradrenaline on the twitch response. Similar results were obtained in 5 separate experiments.

When the β-adrenoceptor blocking agent propranolol (10 μM) was included in the Krebs solution both the contractor response and the inhibitory effect of noradrenaline on the response to stimulation were increased. The concentration-response curve for the inhibitory effect shifted to the left by 0.5–1.0 log units in 5 separate experiments. In only 2 of these experiments could a contractor response be obtained which did not show tachyphylaxis and in these two experiments the concentration-response curve was moved to the left by about 0.25 log units.

The effect of β-adrenoceptor agonists and antagonists on the stimulated mouse vas deferens

Isoprenaline inhibited the twitch response of the mouse vas deferens to field stimulation to about the same extent as noradrenaline in the same molar concentrations. Results similar to those shown in Figure 3 were obtained in 4 other experiments. Salbutamol produced rather less inhibition of the twitch response than isoprenaline in 10 times higher molar concentrations. Similar results were obtained in 2 experiments.

The β-adrenoceptor blocking agent practolol (10 μM) was without effect on the inhibition produced by either isoprenaline or salbutamol. However, the less selective β-adrenoceptor blocking agent propranolol

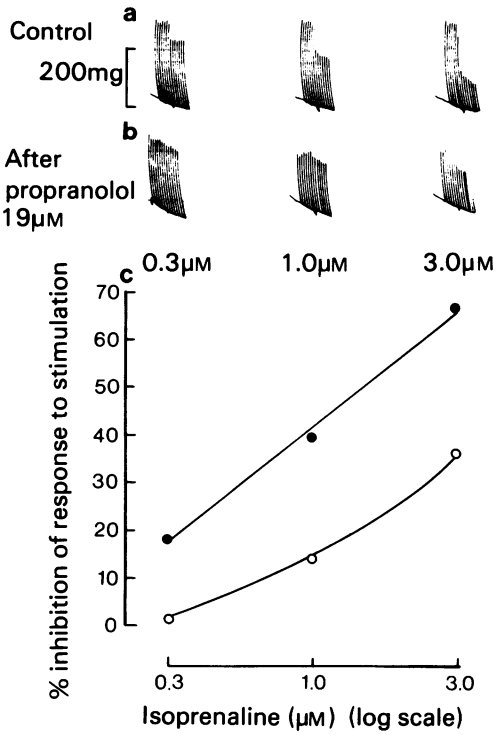

Figure 3 (a) Inhibition of the twitch response of the mouse isolated vas deferens to electrical stimulation (0.1 Hz, 1.0 ms, 250 mA) by isoprenaline (0–3–3.0 μM); (b) shows antagonism of the effect by propranolol (10 μM). (c) Antagonism of the inhibitory effect of isoprenaline by propranolol. Before propranolol (●); after propranolol (○). The s.e. mean (4 observations) is contained within the point in each case.

(10 μM) inhibited the effects of both isoprenaline (Figure 3) and salbutamol.

The effect of uptake blocking agents on twitch responses of the mouse vas deferens

The effect of cocaine (10 μM) on the twitch response to stimulation with pulse widths varying from 0.15–1.0 ms was observed in 4 experiments. In none of these experiments was the twitch response potentiated. In two, the cocaine was without effect and in the other two the responses were significantly reduced by amounts ranging from 13 to 94% ($P < 0.05$).

In two experiments with imipramine (0.18 μM) as the uptake blocking agent, the twitch responses were not potentiated but were reduced by amounts ranging from 16 to 38% ($P < 0.05$). In all the experiments the uptake blocking agents produced the largest percentage inhibition at the lower pulse widths.

When the uptake blocking agent oestradiol (3.7 μM) was included in the Krebs solution the responses to field stimulation were reduced by amounts ranging from 24.8 to 46.5%. The reduction of 46.5% at the pulse width of 0.3 ms was not significant but all the succeeding values were significant ($P < 0.01$). In two experiments the uptake blocking agent cocaine (10 μM) was combined with oestradiol (3.7 μM). In both of these experiments the responses to field stimulation were reduced by amounts ranging from 35 to 96% ($P < 0.001$).

The effect of uptake blocking agents on contractions of the vas deferens in response to noradrenaline

Concentrations of noradrenaline varying from 0.3 to 1.5 μM were used to contract the vas deferens in these experiments. The drug was allowed to remain in contact with the tissue for 30 s and doses were repeated once every 7 minutes. Tachyphylaxis to the motor effect of noradrenaline was a common phenomenon at the lower concentrations and was the cause of wide variations in the results. In two experiments contractions were produced to noradrenaline (0.37 μM, 0.74 μM, and 1.5 μM). The responses to each concentration were potentiated by 71%, 37% and 46% in one experiment and by 75%, 35% and 22% in the other by the addition of cocaine (10 μM) to the Krebs solution. The effect was only significant at the concentration of 1.5 μM where the tachyphylaxis had not induced wide variation.

In two experiments oestradiol (3.7 μM) was added to the Krebs solution. In the first of these, the contractions to noradrenaline (0.3 μM and 0.6 μM) were both potentiated but tachyphylaxis induced considerable variation at the lower concentration and the potentiation (221%) was not significant. Tachyphylaxis at the higher concentration was less obstructive and the potentiation (34%) was significant ($P < 0.05$). In the second experiment there was no tachyphylaxis and the same concentration of oestradiol was without effect on the response to noradrenaline.

The effect of uptake blocking agents on inhibition of the twitch response by noradrenaline

In two experiments noradrenaline (0.1, 0.3 and 1.0 μM) inhibited the twitch responses but in the presence of cocaine (10 μM) the potentiation of the effect was so large that smaller concentrations of noradrenaline had to be used to avoid extinguishing the responses altogether. In the first experiment the concentration-response curve was shifted by one log unit and in the second by one and a half log units.

Oestradiol (3.7 μM) also potentiated the inhibition of the twitch responses caused by noradrenaline (0.01,

0.03 and 0.1 μM). In this case, however, the shift in the response curve was only about one-fifth of a log unit and it was significant only at the two higher concentrations.

Discussion

Ambache & Zar (1971) and von Euler & Hedqvist (1975) have concluded that motor transmission in the vas deferens of a variety of species other than the mouse is non-adrenergic. Jones & Spriggs (1975), on the other hand, have concluded that motor transmission in the mouse vas deferens is noradrenergic.

In our experiments the following results support the view that the twitch response to single stimuli in the mouse vas deferens is unlikely to be mediated by noradrenaline: (a) The twitch response is inhibited by lower concentrations of noradrenaline than are required to make the tissue contract. (b) Twitch responses are not affected by concentrations of phentolamine which are quite adequate to block the motor responses to exogenous noradrenaline. (c) Concentrations of the uptake blocking agents cocaine and oestradiol which are adequate to potentiate the motor responses to exogenous noradrenaline, inhibit twitch responses to field stimulation.

The vas deferens is innervated by the hypogastric nerve and there is no doubt that the nerve fibres are conventionally noradrenergic. When treated by the method of Falck (1962) the fluorescence obtained has the typical characteristcs of noradrenaline. The mouse vas deferens, in common with other species, has been found to have the very high noradrenaline content of 5.4 μg/g (Sjöstrand, 1965). Using a modification of Falck's technique, described by Spriggs, Lever, Rees & Graham (1966), Jones & Spriggs (1975) have shown that the intravenous injection of 6-hydroxydopamine (6-OHDA, 250 mg/kg) destroyed the neurones, removed the fluorescence and virtually eliminated the response to field stimulation in the mouse vas deferens. We repeated this procedure with 6-OHDA in 3 animals and found no loss of response to field stimulation. Electron microscopic examination of one vas from each of these animals by Dr A.D. Hoyes revealed no damage to the neurones. The reason for the disagreement between these results and those of Jones & Spriggs (1975) is obscure. In addition to the certainty of a dense sympathetic innervation, it is also known that field stimulation of the mouse isolated vas deferens causes noradrenaline to appear in the bathing fluid (Henderson, Hughes & Kosterlitz, 1972; Hughes *et al.*, 1975; Jenkins, Marshall & Nasmyth, 1975).

The great difficulty in the way of acceptance of noradrenaline as the motor transmitter in the mouse vas deferens is the failure of phentolamine to block the twitch response both in these experiments and in those of others. Indeed, Hughes *et al.* (1975) reported that in concentrations of 30 μM, phentolamine increased the twitch height by 30–80%. However, Jones & Spriggs (1975) using trains of pulses (0.3 ms duration, supramaximal voltage at 20 Hz for 10 s) found that the responses of the mouse vas were blocked by a very high concentration of phentolamine (53 μM). Phenoxybenzamine and phentolamine also failed to block electrically induced contractions in isolated vasa differentia of rat and guinea-pig (Ambache *et al.*, 1972). Swedin (1971) reported that phentolamine potentiated the twitch response in the guinea-pig vas deferens. In these experiments, Swedin used trains of pulses and noted that the response consisted of two phases; namely a twitch followed by a slower and more prolonged contraction. This latter more prolonged contraction was blocked by phentolamine and in vas deferens from reserpine-treated animals this part of the response was absent. More recently Birmingham & Freeman (1976) have described the biphasic character of the response of the vasa deferentia of rats and guinea-pigs when stimulated with trains of pulses. They too showed that the second phase had the characteristics of a noradrenaline response.

The distances between the nerve varicosities and the smooth muscle cells in the vas deferens are estimated to be 10–30 nm compared with 50–80 nm in blood vessels (Burnstock & Costa, 1975). On this basis it has been suggested (Holman, 1970) that the α-adrenoceptor blocking agents do not gain access to the receptor sites. This is very unlikely as Ambache *et al.* (1972) found that contractions to tyramine, presumably mediated by release of endogenous noradrenaline, were completely blocked by low concentrations of phenoxybenzamine or phentolamine. Similarly, Furness, Campbell, Gillard, Malmfors, Cobb & Burnstock (1970) noted that isolated vasa from rats treated with 6-OHDA 250 mg/kg showed spontaneous contractions due, presumably, to noradrenaline released from the disintegrating neurones as they could be inhibited by phentolamine (1 μg/ml) and blocked by the same concentration of phenoxybenzamine. Despite the fact that α-adrenoceptor blocking agents do not antagonize the twitch response, guanethidine and bretylium, which block sympathetic neurones, completely eliminate the twitch response (Boyd, Chang & Rand, 1961; Birmingham & Wilson, 1963; Bhargava, Kar & Parmar, 1965; Bentley, 1965; Ambache & Zar, 1971; Jones & Spriggs, 1975; Jenkins *et al.*, 1976).

It is clear that noradrenaline is released on field stimulation of the mouse vas deferens, but it does not appear to be responsible for the twitch response to single pulses, at least by an action on any known adrenoceptors. The primary effect of exogenous noradrenaline in the mouse vas deferens is to inhibit twitch responses. The inhibition appears to be mediated by α-adrenoceptors since the effect is

inhibited by phentolamine and not blocked by propranolol. β-Adrenoceptors are present and are inhibitory because both salbutamol and isoprenaline inhibited the twitches and their effect was blocked by propranolol but not by practolol suggesting, in agreement with von Euler & Hedqvist (1975), that they are β_2-receptors. The enhancement of the inhibition due to noradrenaline by propranolol suggests that at the concentrations used, no part of its effect was due to stimulation of these β-adrenoceptors. The most likely explanation of the enhancement of the effect would seem to be that some blockade of uptake by the relatively high concentration of propranolol had increased the activity at the inhibitory α-adrenoceptors. The only known inhibitory α-adrenoceptors are pre-synaptic (Kirpekar & Puig, 1971; Farnebo & Hamberger, 1971; Enero, Langer, Rothlin & Stefano, 1972), where their effect is to inhibit the release of noradrenaline when the nerve fibre is stimulated. This is consistent with the observation that uptake$_1$ and uptake$_2$ blocking agents, individually or combined and in concentrations adequate to potentiate the contraction to exogenous noradrenaline, inhibited the twitch response or were without effect on it. It is less easy to accept the concept of an α-adrenoceptor responsible for motor transmission, but not susceptible to blockade with concentrations of α-adrenoceptor blocking agents which are demonstrably quite adequate to block motor responses to exogenous noradrenaline. If this were the case, then it is also necessary to suppose either that the twitch response is maximal and therefore not affected by uptake blockade, or that the increased concentration of noradrenaline caused by the blockade occurs only at the location of the inhibitory presynaptic receptors. Since there is no evidence to resolve this question, the possibility of the existence of an unconventional post-synaptic α-adrenoceptor responsible for the twitch remains. The alternative explanation is that an unknown motor transmitter is liberated from the same neurone as the noradrenaline. In either case its output seems to be controlled by presynaptic α-adrenoceptors.

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