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TRANSMISSION TO THE LONGITUDINAL MUSCLE OF THE GUINEA-PIG VAS DEFERENS: THE EFFECT OF PRETREATMENT WITH GUANETHIDINE

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1 Tissue was taken from guinea-pigs that had been injected with guanethidine (100 mg/kg, i.p.) 24 h before they were killed, and from paired control animals.

2 Pretreatment with guanethidine caused a significant, substantial, and sometimes complete reduction of the nerve-mediated contractions of the vas deferens. There were no significant changes in the responses of the ileum to stimulation of cholinergic nerves or of the distal colon to stimulation of intrinsic (non-adrenergic) inhibitory nerves. Responses of the vas deferens and ileum to acetylcholine were unchanged, but contractions of the vas deferens elicited by exogenous noradrenaline were enhanced.

3 The nerve-mediated contractions of the vas deferens were restored by exposing it to (+)-amphetamine followed by noradrenaline *in vitro*.

4 It is concluded that noradrenaline is the transmitter released from motor nerves to the longitudinal muscle of the guinea-pig vas deferens. Possible explanations for the ineffectiveness of receptor blocking agents in antagonizing transmission are discussed.

Introduction

The belief that noradrenaline is the excitatory transmitter to the smooth muscle of the vas deferens has now been challenged (Ambache & Zar, 1971; Ambache, Dunk, Verney & Zar, 1972); in fact, these authors state that their results exclude the possibility of noradrenergic transmission. Some supporting evidence has been advanced by Wakade & Krusz (1972). Two of the principal reasons for the proposal are that small concentrations of noradrenaline inhibit the nerve-mediated contractions of the vas deferens and that α-adrenoceptor antagonists produce a substantial reduction in the ability of noradrenaline to contract the muscle, without decreasing the contractions caused by nerve stimulation. The authors confirm that guanethidine abolishes the nerve-mediated responses, but they emphasize that this drug has pharmacological properties not related to that of blocking transmission from noradrenergic nerves. Non-specific effects (which might be presumed to explain the antagonism of transmission to the vas deferens) have been documented by Rand & Wilson (1967). These actions have the common property of being reversed as guanethidine is washed from the tissue, while blockade of transmission from noradrenergic neurones is maintained. Injected guanethidine quickly disappears from the circulation, although effects on noradrenergic nerves persist for more than 24 h (Cass, Kuntzman & Brodie, 1960; Chang, Costa & Brodie, 1965). Therefore, the present experiments were undertaken to see if the injection of guanethidine would cause a selective and maintained reduction in the response of the longitudinal muscle of the vas deferens to nerve stimulation. A substantial reduction was observed at 24 h and it was further discovered that the contraction could be restored by exposure of the tissue to noradrenaline. The discussion evaluates this and other evidence which suggests that the excitatory transmitter is indeed noradrenaline, and also explores some of the possible reasons for the results with α-adrenoceptor blocking agents.

Methods

Transmission from intramural nerves to the longitudinal muscle of the vas deferens was examined in tissue removed from normal guinea-pigs and from guinea-pigs that had been given intraperitoneal injections of guanethidine sulphate (100 mg/kg), 24 h beforehand. Body weights were 180-340 g. Guinea-pigs of similar weights (to within 10 g) were taken in pairs, one being injected with guanethidine and the other providing control preparations. Ileums and distal colons from the paired animals were also used.

The connective tissue sheaths were carefully dissected from the vasa deferentia. Pieces of intestine were washed out with cold Krebs solution and the mesentery was cut away. Tissue which could not be used immediately was kept in an organ bath containing oxygenated modified Krebs solution at 36° C for up to 4 h without being subjected to any tension. The solution was replaced at intervals of 1 h or less.

The pieces of tissue to be examined were anchored at one end in a 50 ml organ bath containing modified Krebs solution (Furness, 1970a) bubbled with 95% O_2: 5% CO_2 and maintained at 36° C. The other end of each preparation was connected to a transducer and changes in tension were recorded by a polygraph chart recorder. Paired specimens were anchored to a single point and were drawn through the same electrodes so that their stimulation and exposure to drugs was as close to identical as possible. The electrodes consisted of two silver wire rings, 0.75 cm in diameter and 1.8 cm apart.

Two regimens were used for the transmural stimulation of the paired preparations; these were five pulses of 0.5 ms duration given at 10 Hz with a strength just sufficient to elicit the maximum response from the control tissue, and 10 pulses of 0.5 ms duration given at 50 Hz, again with just maximal strength. The ileum was stimulated with single, just maximal, pulses of 0.2 ms duration and the distal colon was stimulated at 10 Hz with just maximal pulses of 0.2 ms duration, but in the presence of hyoscine (1 μg/ml).

The following drugs (concentrations expressed in terms of the salts) were used: acetylcholine chloride, (+)-amphetamine sulphate, atropine sulphate, bretylium tosylate, guanethidine sulphate, hyoscine hydrobromide, and tetrodotoxin. Concentrations of (−)-noradrenaline bitartrate are given in terms of the base.

The fluorescence histochemical technique (Falck, 1962) was used to examine the distribution of catecholamines in the tissue.

Results

Tissue was removed for examination 24 h after the intraperitoneal injection of 100 mg/kg guanethidine. At this stage there was no catecholamine fluorescence in the nerves of the longitudinal muscle of the vas deferens prepared by the histochemical method of Falck and Hillarp.

Figure 1 shows the comparison of the responses of paired (control and guanethidine-treated) vasa deferentia to the stimulation of intramural nerves, to exogenous noradrenaline and acetylcholine and of similarly paired segments of intestine to

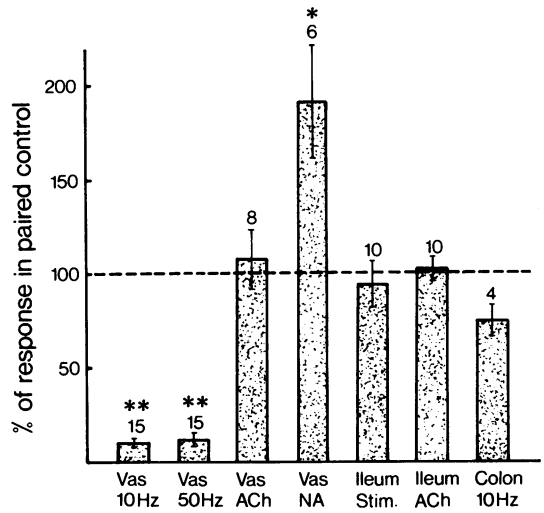

Fig. 1 The columns represent response amplitudes in tissue taken from guinea-pigs injected with guanethidine (100 mg/kg, 24 h before the experiment) expressed as percentages of responses in paired controls. Figures above each column indicate the number of experiments and the bars give the standard error of the mean. The contractions of the vas deferens to nerve stimulation at 10 and 50 Hz were significantly reduced by guanethidine ($P < 0.001$) (**) and the sensitivity of the vas deferens to exogenous noradrenaline (NA) was significantly increased ($P < 0.05$) (*). The responses of the vas deferens to acetylcholine (ACh), of the ileum to stimulation of cholinergic nerves and ACh, and of the distal colon to stimulation of intrinsic inhibitory nerves, were not significantly changed (taking the 5% level of probability to indicate significant difference). Statistical comparisons were made with the *t*-test for paired data (Croxton, 1959).

stimulation of cholinergic nerves, to stimulation of intrinsic, non-adrenergic inhibitory nerves and to exogenous acetylcholine. The nerve-mediated contractions of the vasa were reduced to an average of 10% of normal by the injection of guanethidine. In two cases out of 15 no response was obtained in the treated preparations; in the present experiments, in which there was always some baseline noise, 'no response' indicates a reduction to less than one-fivehundredth of control. In preparations in which injected guanethidine was not completely effective, the remaining response was entirely abolished by tetrodotoxin (0.1 μg/ml), by guanethidine (1-2 μg/ml) or by bretylium (5-10 μg/ml), but was not significantly affected by atropine (1 μg/ml) or hyoscine (1 μg/ml). The injection of guanethidine did not modify the response of the vas deferens to

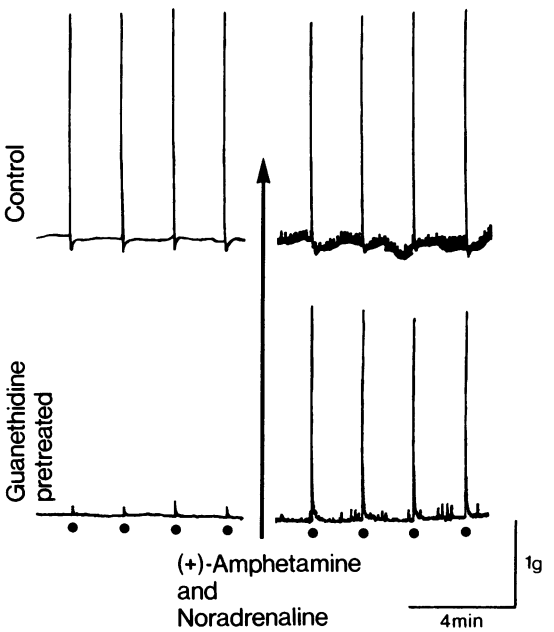

Fig. 2 Restoration, by noradrenaline (NA), of the responses to nerve stimulation of a guanethidine-treated vas deferens. Contractions were recorded simultaneously from a control vas deferens (top) and from a vas deferens taken from a guinea-pig which had been injected with guanethidine (100 mg/kg, 24 h before). The records on the left were taken soon after the tissue was set up and show responses to transmural stimulation at 10 Hz (dots). The records on the right show the contractions caused by equal stimuli after both preparations had been exposed to (+)-amphetamine (1 μg/ml for 15 min) followed by NA (0.5 μg/ml for 6 minutes).

acetylcholine (1 μg/ml) and it potentiated the contraction evoked by exogenous noradrenaline (1 μg/ml); for the intestine, transmission from cholinergic excitatory nerves, as well as contractions evoked by acetylcholine (50 ng/ml), were unaffected (Figure 1). There was a slight reduction (significant at the 10% but not at the 5% level) in the amplitude of the relaxation in response to transmural stimulation of the distal colon in the presence of hyoscine (1 μg/ml). This effect of guanethidine could have arisen because there are inhibitory noradrenergic, as well as intrinsic (non-adrenergic) inhibitory nerves in the colon (Furness, 1970b).

In four vasa taken from guinea-pigs treated with guanethidine, recovery of transmission was achieved by means of the following protocol: preparations were taken from injected guinea-pigs and from matched controls and changes in length were recorded while they were stimulated together by the same pair of electrodes; after the sizes of initial contractions had been recorded, (+)-amphetamine (1 μg/ml) was included in the organ bath for 15 min while the nerves to the vas deferens were stimulated at 2 min intervals with 500 ms trains of pulses at 10 Hz; the amphetamine was washed out and the preparations were exposed to (−)-noradrenaline (0.5 μg/ml) for 6 minutes. The purpose of stimulation in the presence of amphetamine was to displace guanethidine from the nerves (Flegin, Morgan, Oates & Shand, 1970). In these preparations, the response of the guanethidine-treated vasa averaged 8% of the control when first tested in the organ bath. After the amphetamine/noradrenaline treatment (in one of the four experiments the sequence was followed twice) the responses in the vasa taken from treated guinea-pigs increased and were no longer different from control. Records from one of the experiments are shown in Figure 2. Responses which had been restored in the manner described above were completely abolished by tetrodotoxin (0.1 μg/ml).

Discussion

Ultrastructural observations of the innervation of the longitudinal muscle of the guinea-pig vas deferens show that essentially all the axons in close contact with the muscle cells contain small granular vesicles after treatment with 5-hydroxydopamine (Furness & Iwayama, 1972). This characteristic would normally indicate that the axons are noradrenergic (Tranzer & Thoenen, 1967; Tranzer, Thoenen, Snipes & Richards, 1969). In addition, the axons show ultrastructural signs of degeneration (and transmission to the vas deferens is abolished) after treatment with 6-hydroxydopamine (Furness, 1971; Furness & Iwayama, 1971; Wadsworth, 1973). This drug is thought to act specifically on noradrenergic axons (Thoenen & Tranzer, 1968; Tranzer *et al.*, 1969; Malmfors & Thoenen, 1971). Histochemically, noradrenaline is found in a dense network of fibres innervating the longitudinal muscle (Falck, 1962; Jacobowitz & Koelle, 1965; Owman & Sjöstrand, 1965). Thus, the longitudinal muscle seems to be innervated only by axons containing noradrenaline. Furthermore, when these axons are stimulated, noradrenaline can be recovered from the solution bathing the vas deferens (Johnson, Thoa, Weinshilboum, Axelrod & Kopin, 1971; Weinshilboum, Thoa, Johnson, Kopin & Axelrod, 1971); and it is well known that noradrenaline contracts the muscle. Therefore, it would seem reasonable to suppose that the contractions elicited when nerves to the vas deferens are stimulated are mediated by

the release of noradrenaline. In the present study, it was found that, 24 h after the injection of guanethidine, the contractions of the vas deferens in response to nerve stimulation were substantially and specifically reduced. Noradrenaline is displaced from noradrenergic nerves by guanethidine (Cass *et al.*, 1960; Chang *et al.*, 1965) and, in the present study, histochemically demonstrable noradrenaline disappeared from fibres in the longitudinal muscle of the vas deferens after guanethidine treatment. However, after the vas deferens was exposed to noradrenaline *in vitro* the responses were restored. Therefore, if noradrenaline, which is normally present in and released from the nerves, is depleted, transmission is substantially or completely abolished, and if it is made available to restock the nerves, transmission recovers. There seems little alternative but to conclude that noradrenaline is the natural transmitter substance.

A combination of reasons can account for the ineffectiveness of α-adrenoceptor antagonists in reducing nerve-mediated contractions. Firstly, the probable inability of these agents to penetrate in sufficient concentrations to the neuromuscular junctions; secondly, their antagonism of the reuptake of noradrenaline into adrenergic axons; thirdly, their facilitation of transmitter release. The first reason, the proximity theory advanced by Dale & Gaddum (1930), implies that the tissue provides a barrier which inhibits the free diffusion of antagonists into regions of transmitter release and/or the transmitter is released from nerve endings in such intimacy with the muscle cells that it overcomes any receptor blockade. Hotta (1969) and Ambache *et al.* (1972) have considered this point, but conclude that there is no morphological basis for a diffusion barrier. However, it has recently been shown (Furness & Iwayama, 1971, 1972), that noradrenergic axons in the guinea-pig vas deferens often lie in grooves or deep invaginations of the muscle cells, forming junctions such as that shown in Figure 3. The axons, loaded with small granular vesicles, come within 10-20 nm of the muscle cell surface and in this junctional gap there is no basement membrane. A 'seal' is formed at the orifice of the groove or invagination by the confluence of basement membrane associated with the axon and muscle cell. The basement membrane is composed of a material which is sufficiently dense to appear as an electron-opaque band, 20-40 nm thick. It is reasonable to suppose that it provides a barrier to free diffusion of reactive substances such as noradrenaline and phenoxybenzamine. Even without this postulated barrier, the narrowness of the junctional gap is a likely impediment. The concentration of noradrenaline which might be

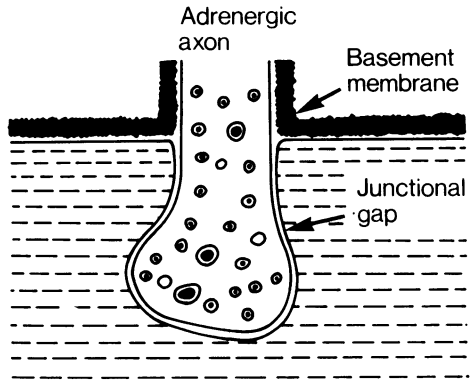

Fig. 3 Representation of the relation between noradrenergic axons and smooth muscle cells in the outer longitudinal layer of the guinea-pig vas deferens, based on electron-microscopical studies (Furness & Iwayama, 1971, 1972). The noradrenergic axon lies in an invagination of the muscle cell. Basement membrane covers the surface of the cell and the axon, but is excluded from the junctional gap. The width of the gap is 10-20 nm and of the basement membrane is 20-40 nm. The axon contains numerous small granular vesicles.

reached in the junctional gap during transmission is difficult to estimate. If the noradrenaline content of a vesicle is 5 fg (Dahlström, Häggendahl & Hökfelt, 1966) and the complete contents of a vesicle are released at a point into a gap 20 nm wide, it may be calculated (assuming equal diffusion along and across the gap) that the local concentration could reach 5 fg per $\pi \times (2 \times 10^{-6})^3$ cm^3, i.e. about 200 g/ml. It is necessary to regard this as an uppermost estimate of transmitter concentration, because noradrenaline may be released slowly from the vesicle, the complete contents might not be expelled, and some of the transmitter might be inactivated before it can have an effect. Nevertheless, this simple calculation does indicate that massive concentrations of transmitter can be contemplated at the junctional receptors. There is the additional possibility that the sensitivity to noradrenaline is greater at the junction than it is in extrajunctional regions. From these considerations, it seems possible that noradrenaline or α-receptor antagonists, injected into an organ bath, react with an extrajunctional set of receptors, which excludes most of those involved in neuromuscular transmission. The problem of penetration does not apply to drugs such as guanethidine and 6-hydroxydopamine, which block transmission by actions on the axons. Even if they need to be transported from their points of uptake to the remotest terminals, there

would seem to be no difficulty. Both drugs are taken up into axonal storage granules (Chang *et al.*, 1965; Lundborg & Stitzel, 1968; Malmfors & Thoenen, 1971). The storage granules are transported down the axon at about 1.4 μm/s (Smith, 1971); therefore, it would take a little more than 2 min for a compound incorporated into axonal vesicles at the surface to be transported 200 μm – the approximate thickness of the longitudinal muscle of the guinea-pig vas deferens. In the same manner, reloading depleted terminals with noradrenaline can be quickly accomplished.

α-Adrenoceptor blocking agents contribute to their own ineffectiveness by increasing the amount of noradrenaline released; phenoxybenzamine (3×10^{-5} M) increased the noradrenaline recovery from the bathing medium (when nerves to the vas deferens were stimulated) by more than 40 times (Johnson *et al.*, 1971). This is probably due to two effects – antagonism of noradrenaline uptake by the nerves and true potentiation of release. Phenoxybenzamine is a potent antagonist of noradrenaline uptake into noradrenergic nerves and phentolamine is rather less effective (Iversen, 1965). Both drugs increase the actual release of noradrenaline, but their relative potencies are reversed (de Potter, Chubb, Put & de Schaepdryver, 1971; de Potter, Chubb & Schaepdryver, 1972; Farnebo & Hamberger, 1971). In addition, phenoxybenzamine inhibits the enzymatic degradation of noradrenaline (Eisenfeld, Axelrod & Krakoff, 1967; Iversen & Langer, 1969).

The possibility that the absolute increase in transmitter release caused by α-receptor blockade might be due to antagonism of feed-back mechanism by which noradrenaline inhibits its own secretion, has been investigated by Starke (1972). He showed that even 10 ng/ml noradrenaline was effective in reducing stimulus-evoked release from noradrenergic nerves in the rabbit heart. In the guinea-pig vas deferens, antagonism of transmission by noradrenaline (Ambache & Zar, 1971) could result if the exogenous compound can reach neuronal receptors (which are not necessarily at the terminals) in sufficient concentration to inhibit release, but cannot effectively reach receptors to promote contraction.

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