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POSTGANGLIONIC PARALYSIS OF THE GUINEA-PIG HYPOGASTRIC NERVE-VAS DEFERENS PREPARATION BY *CLOSTRIDIUM BOTULINUM* TYPE D TOXIN

BY

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Clostridium botulinum type D toxin can, in certain preparations, cause a failure of the response to sympathetic nerve stimulation (Rand & Whaler, 1965). For the guinea-pig isolated hypogastric nerve-vas deferens preparation it might be argued that the block occurs in ganglion-cells within the hypogastric trunk, rather than at postganglionic nerve endings. Although the existence of ganglion-cells in the nerve trunk is accepted, their anatomical position in relation to the vas deferens itself is less clear. Ferry (1963, 1967) has shown that the hypogastric trunk has most of its ganglion-cells in the last 2 cm of the nerve trunk, mainly concentrated 0.4–0.9 cm from the muscle tissue. Thus, unless particular care is taken to have stimulating electrodes closer to the muscle than 0.4 cm, both preganglionic and postganglionic fibres will be stimulated.

For the correct interpretation of results from experiments on the action of botulinum toxin on adrenergic nerve endings, the possibility that ganglion-cells are interposed between the point of nerve stimulation and the release of noradrenaline must be ruled out because transmission through such a ganglion cell to the postganglionic fibre is cholinergic and therefore toxin-sensitive. This is especially important when the results are contrary to the accepted belief that adrenergic nerves are not sensitive to botulinum toxin (Ambache 1949, 1951 ; Vincenzi, 1967).

The experiments reported here were designed to exclude as completely as possible any preganglionic component: the results confirm that botulinum toxin blocks the response of the vas deferens preparation to postganglionic nerve stimulation.

METHODS

Hypogastric nerve-vas deferens preparation

Vasa deferentia, obtained from guinea-pigs weighing 300–650 g, were suspended in 50 ml. organ baths as described by Hukovič (1961) ; where possible, paired vasa deferentia from the same animal were used. The bath fluid (usually 40 ml.) was McEwen's (1956) solution, gassed with a mixture of 95% oxygen and 5% carbon dioxide and maintained at either 32° C or 36°–37° C. Isotonic contractions of the muscle were recorded on smoked paper by mean of a frontal writing lever.

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A muscle holder carrying an electrode assembly similar to that described by Birmingham & Wilson (1963) was used. The hypogastric nerve was pulled through the electrodes until the distance between the electrodes and the muscle was as short as possible; it varied a little between different preparations, the precise distance being determined by the amount of fatty and connective tissue present near the nerve-vas deferens junction, but in most cases it was between 2 and 6 mm. The free end of the nerve was then drawn through a second pair of electrodes of the type described by Burn & Rand (1960); here the stimulated region of the nerve trunk was some 15–25 mm from the muscle. Finally, for transmural stimulation of the vas deferens, a third pair of electrodes, similar to those described by Birmingham & Wilson (1963, Fig. 1d), was put into position.

With this arrangement the preparation could be stimulated at four sites. When the electrodes were 15–25 mm from the muscle the stimulation of the nerve trunk was considered to be largely preganglionic and with the electrodes 2–6 mm from the muscle, largely postganglionic. When the preparation was stimulated transmurally with low voltages (10–40 V), responses were obtained which were caused mainly by excitation of postganglionic nerve fibres in the muscle tissue. Higher voltages (70–100 V) were used to stimulate directly the muscle cells in those experiments in which nerve-induced responses had been blocked by procaine, bretylium and botulinum toxin.

Electronic square-wave stimulators (C. F. Palmer (London) Ltd.) were used in all experiments. For all four types of stimulation described, the pulse width was usually 1 msec and the frequency of stimulation was 20/sec or 50/sec. Trains of impulses lasting 3.8–6.0 sec were given every 45–100 sec, these times being kept as constant as possible within a particular experiment or group of experiments. The length of trains of impulses was controlled by a recording drum with contact arms on the spindle operating a series of micro-switches. This system was convenient but the switches were difficult to adjust, hence the range given above for duration of trains of impulses. The micro-switches carried the live side of the stimulator outputs and were connected to the electrodes. Stimulus strength was adjusted, up to 100 V, to give a maximum response.

Botulinum toxin

Pure type D toxin was used. The working solution was prepared by diluting the stock solution with cold phosphate buffer (0.05M, pH 6.5); it was stored in glass vessels (1–2 ml.) at −23° C until required. The specific toxicity of this solution was approximately 4×10^5 mouse (I.P.) LD50 doses/ml.; 1.2 ml. was added to the bath. Control preparations received either a similar volume of diluent buffer or, less frequently, an equivalent volume of toxin solution which had been heated in a boiling water bath for 5 min.

Drugs

Drugs used were bretylium tosylate and (+)-tubocurarine chloride (Burroughs Wellcome Ltd.), guanethidine monosulphate (Ismelin, Ciba Laboratories Ltd.), hexamethonium bromide and dexamphetamine sulphate (May and Baker Ltd.), atropine sulphate, hyoscine hydrobromide and procaine hydrochloride (Boots Pure Drug Co. Ltd.) and hemicholinium No. 3 (HC-3).

These were made up in 0.9% sodium chloride solution and the final bath concentration obtained by adding up to 1 ml. of the stock solution. Doses are given as weight of the salts.

RESULTS

Normal responses to stimulation

Under the conditions of stimulation described, good responses were normally obtained for both "preganglionic" and "postganglionic" stimulation as well as transmural stimulation with low voltages (Figs. 1 and 6). Responses to "preganglionic" stimulation were generally smaller than those to "postganglionic" stimulation and, as reported by Birmingham & Wilson (1963), transmural stimulation usually, but by no means invariably, gave the largest response.

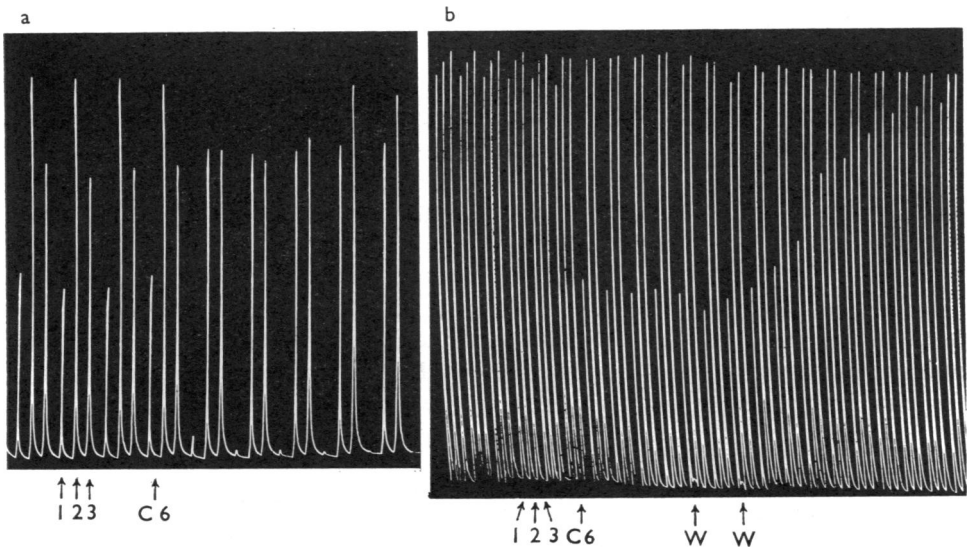

Fig. 1. Effects of hexamethonium on the responses of the vas deferens preparation to electrical stimulation at different sites, 20 pulses/sec, 1 msec pulse duration, for about 4 sec every 50–60 sec. Two experiments, a and b. 1, Preganglionic stimulation ; 2, postganglionic stimulation ; 3, low voltage transmural stimulation ; 20 V (a) and 12 V (b) ; C6, hexamethonium added to the bath (200 μg/ml.) ; W, hexamethonium washed out.

In control experiments, responses were maintained for some hours and any decline in response was reversed by small increases in stimulus strength. Stable responses to " preganglionic " stimulation were the most difficult to obtain and the contraction frequently declined fairly rapidly to only 10–50% of that given by " postganglionic " stimulation, in spite of increases in stimulus strength. On some occasions the response to " preganglionic " stimulation failed soon after setting up the preparation and could not be overcome by changes in the stimulation parameters, although the responses to " postganglionic " stimulation were unaffected. When this occurred, the addition of choline chloride (10–20 μg/ml. bath fluid) often brought about a rapid return of the contractions to their normal size, which was then maintained without considerable decrease even when the choline was washed out of the bath.

Effects of drugs

So far it has been assumed that the electrode arrangements used distinguished satisfactorily between " preganglionic " and " postganglionic " stimulation. The experimental basis for this assumption will now be presented, and the inverted commas discarded.

Hexamethonium. The addition to the bath fluid of hexamethonium 200 μg/ml. brought about an almost immediate failure of the response to preganglionic stimulation ; this effect was reversed readily after washing out the drug. Responses to postganglionic and transmural stimulation were not much affected by the drug (Figs. 1 and 3). On a few occasions only partial block was produced by hexamethonium (Fig. 1b). Because ganglion-cells are present along an appreciable length of the hypogastric nerve and because

it was not always possible to have the postganglionic electrodes very close to the vas deferens, reliable distinction between preganglionic and postganglionic stimulation could not always be expected.

Partial or complete block by hexamethonium of the response to stimulation with the preganglionic electrodes sometimes resulted in a small increase in the response to either postganglionic (Fig. 3) or transmural (Fig. 1a) stimulation. This increase is thought to be due to the less frequent stimulation of the postganglionic pathway because trains of impulses occur only twice instead of three times in each cycle. A similar increase can be obtained by lengthening the time interval between successive trains of impulses from, say, 40–60 sec and is probably the result of a change in the amount of transmitter released. While such an increase is not particularly important in these experiments, it should be appreciated that such an increase could either mask a drug-induced reduction in the remaining responses, or even give the impression of a potentiating effect.

Hemicholinium. At a concentration of 50–100 μg/ml. the response to preganglionic stimulation failed after 5–15 min (Fig. 2). In most cases the response to postganglionic stimulation did not change much and in only one out of twelve experiments was there a block of the response to postganglionic stimulation. Where a reduction occurred, the response eventually stabilized at the new level; on a few occasions hemicholinium caused a slight increase similar to that found after hexamethonium. When the response to preganglionic stimulation had been blocked by hemicholinium, an increase in the concentration to 200 μg/ml. for 60 min did not markedly alter the response to postganglionic stimulation.

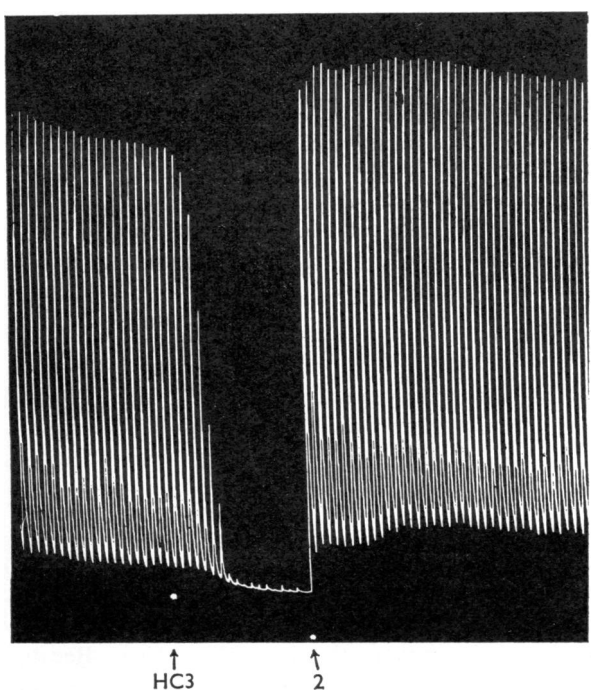

Fig. 2. Effect of hemicholinium on the response to preganglionic electrical stimulation, 20 pulses/sec, 1 msec pulse duration for about 4 sec every 46 sec. HC-3, Hemicholinium added to the bath (100 μg/ml.); at 2, following failure of the preganglionic stimulus to give a response, stimulation through the postganglionic electrode; this was as effective as it had been (not shown in the figure) before the addition of HC-3.

(+)-*Tubocurarine.* After the addition of 50–100 μg/ml., the response to preganglionic stimulation more or less disappeared within a few minutes; responses to postganglionic and transmural stimulation were not much altered (Fig. 3). Only when the preparation was exposed to concentrations of tubocurarine of 400 or 750 μg/ml. for 20 min was some reduction of the response to postganglionic stimulation observed.

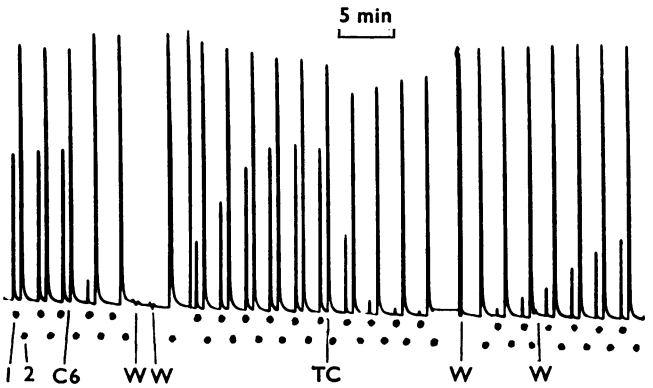

Fig. 3. Effects of hexamethonium and of (+)-tubocurarine on the responses of the vas deferens preparation to electrical stimulation (50 pulses/sec, 1 msec pulse duration for about 4 sec). 1, preganglionic stimulation; 2, postganglionic stimulation; C6, hexamethonium (200 μg/ml.) added to the bath; TC, (+)-tubocurarine (200 μg/ml.) added to the bath; W, drugs washed out. The addition of either drug led to a reversible block of the response to preganglionic stimulation.

Atropine and hyoscine. At bath concentrations of 1–10 μg/ml., the response to preganglionic stimulation was slowly abolished. The response to postganglionic stimulation was reduced but by not more than 20% and usually less. These effects appeared only slowly, so it was difficult to be certain that the change in the response to preganglionic stimulation was related to the addition of the drug, rather than being a non-specific failure of the type referred to earlier. Washing out the drug led to some recovery but this was very slow and incomplete.

Procaine. Procaine (300–400 μg/ml.) abolished the responses to low-voltage transmural as well as to preganglionic and postganglionic stimulation (Fig. 4). At lower concentrations the block was not always complete. The effects caused by the addition of procaine occurred rapidly and were readily reversed on washing out the drug. During the block, it was difficult to obtain any response to transmural stimulation below a strength of 40 V. Above this strength responses could be obtained, the size increasing with increasing voltage of stimulation.

In these experiments, as in others with bretylium and botulinum toxin, the responses to high-voltage transmural stimulation showed considerable variation in size. They were rarely larger than those obtained by postganglionic or low-voltage transmural stimulation, but could be much less and, on occasion, were reduced by as much as 90%. In some experiments, slight repositioning of the electrodes resulted in a much larger response, so we believe that much of this variation reflects the position of the electrodes rather than an effect upon different nervous elements in the tissue.

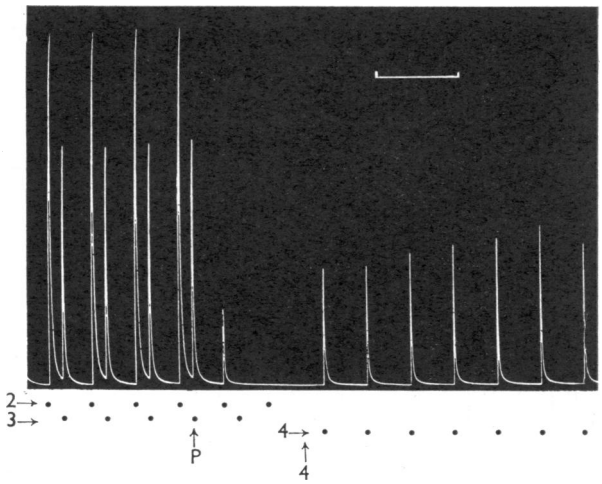

Fig. 4. Effect of procaine on the responses of the vas deferens to postganglionic and to transmural stimulation, 20 pulses/sec, 1 msec pulse duration for about 5.8 sec. 2, Postganglionic stimulation; 3, low-voltage (10 V) transmural stimulation. The addition of procaine to the bath (P, 400 μg/ml.) abolished these responses but increasing the transmural stimulus strength to 50 V (at 4) partially restored the response. A similar increase in the strength of the stimulus applied to the postganglionic electrode had no effect. Time scale, 5 min.

In all experiments, high-voltage transmural stimulation was attempted only after block of nerve-induced responses; thus no "controls" are shown (for example, in Figs. 4–6) and the responses obtained by this method of stimulation can be compared only with those to nerve or low-voltage transmural stimulation.

Bretylium. In the presence of bretylium 4–12 μg/ml., responses to both preganglionic and postganglionic stimulation declined and usually disappeared in 10–30 min (Fig. 5). After washing the drug out of the bath some recovery of the response took place but this was slow and incomplete. The addition of dexamphetamine (5–10 μg/ml.) after the removal of bretylium stimulated a rapid recovery of responses back to, and often exceeding, those obtained before treatment with bretylium.

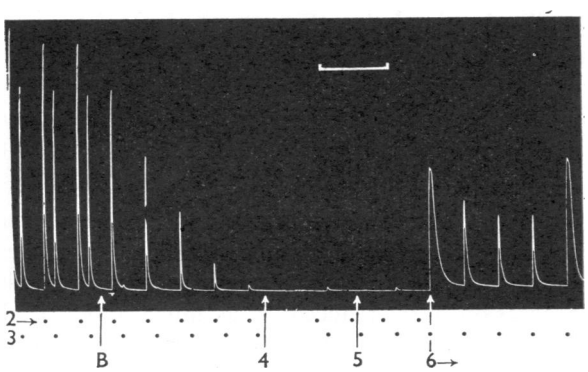

Fig. 5. Effect of bretylium on the responses of the vas deferens preparation to postganglionic and to transmural stimulation, 20 pulses/sec, 1 msec pulse duration for about 5 sec. 2, Postganglionic stimulation; 3, low-voltage (12 V) transmural stimulation. The addition of bretylium (B, 8 μg/ml.) virtually abolished these responses. Increase in strength of the transmural stimulus to 50 V (4) and 70 V (5) had no effect but with 90 V (6) a response was obtained. Increases in the strength of the stimulus applied to the postganglionic electrode (2) from 8 V through to 90 V were ineffective. Time scale, 5 min.

During blockade by bretylium, high-voltage transmural stimulation resulted in responses of 10–120% of the normal size. As with procaine, it was necessary to increase the voltage from the 10–25 V range to about 50 V before any response was obtained, and then to 80–100 V in order to obtain large contractions (Fig. 5). In contrast, increasing the voltage applied through the postganglionic electrodes did not bring about a contraction.

Guanethidine. On the few occasions when this drug was used, results similar to those described for bretylium were obtained. Block of postganglionic and low-voltage transmural stimulation occurred in the presence of 4–16 μg/ml., when high-voltage transmural stimulation was still effective.

Effects of botulinum toxin

Following the addition of toxin (1.2×10^4 LD50 doses/ml. bath concentration) there was progressive failure in response first to preganglionic and then to postganglionic

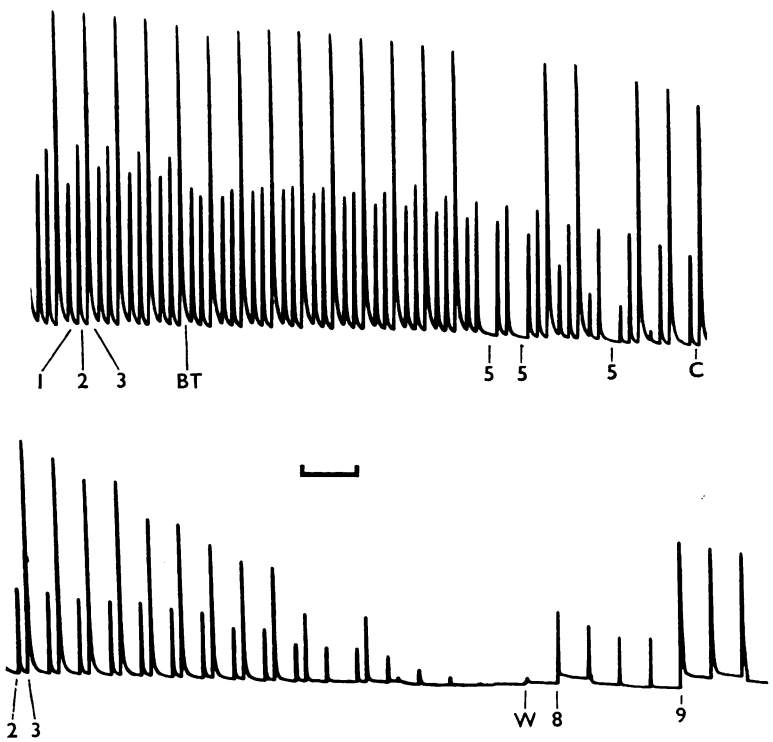

Fig. 6. Effect of botulinum toxin on the responses of the vas deferens to 1, preganglionic, 2, postganglionic and 3, low voltage transmural stimulation, 50/sec, pulse duration 200 μsec, for 5 sec. At BT, botulinum toxin (1.2×10^4 LD50 doses/ml.). When the response to preganglionic stimulation began to decline, transmural stimulation was occasionally omitted (at 5) but this did not affect the other responses. Choline (C, 10 μg/ml.) did not reverse block to preganglionic stimulation. Later, the responses to postganglionic and transmural stimulation ceased. After washing (W) the strength of the transmural stimulus was increased (90 V, 200 μsec at 8 ; 100 V, 400 μsec at 9). Continuous record. Time scale, 5 min.

stimulation. At a time when little or no response was obtained to preganglionic stimulation there was only a small reduction in the response to postganglionic stimulation. Subsequently the response to postganglionic stimulation declined rapidly and finally disappeared; low-voltage transmural stimulation at this stage sometimes produced a response but this was only transient. When preganglionic, postganglionic and low-voltage transmural stimulation were no longer effective, high-voltage transmural stimulation still caused a contraction (Fig. 6).

Procaine (300 μg/ml.) or bretylium (16 μg/ml.) was used to determine whether block of nervous transmission was complete at a time when only high-voltage transmural stimulation was effective. Usually these drugs produced no appreciable change in the height of the contraction. Occasionally there was a reduction in the response after procaine, which suggests that the block of the nerve-ending might have been incomplete in spite of the cessation of response to postganglionic stimulation of the hypogastric nerve.

Control experiments with either phosphate buffer diluent or heat-treated toxin were regularly carried out; the other vas deferens from the animal was normally used for this. In these experiments, while preganglionic responses often declined—as described earlier—the much more stable postganglionic pathway remained open, the contractions showing only a small decrement during the experiment.

In some experiments responses to added noradrenaline in a range of doses between 1 and 200 μg/ml. were tested immediately before the addition of toxin or control fluids, and again 4.5–7 hr later, when stimulation of the toxin-treated preparation was largely ineffective. Four of eight controls and three of seven preparations treated with toxin showed no appreciable change in sensitivity to noradrenaline. In the remainder the sensitivity decreased by 50–85%, except for two controls which showed a decrease of 90–95%. Thus the ultimate failure of postganglionic stimulation to evoke a response from preparations treated with toxin was not caused by any marked change in sensitivity of the muscle cells to noradrenaline.

DISCUSSION

In order to interpret correctly the results of experiments with botulinum toxin on the hypogastric nerve-vas deferens preparation, it is necessary to show that one can distinguish between preganglionic and postganglionic fibres in this preparation, and that the latter are toxin-sensitive. The use of two pairs of electrodes applied to the nerve at different sites giving a differential block by hexamethonium, hemicholinium, atropine or (+)-tubocurarine, and the effect of botulinum toxin, may justify the distinction between preganglionic and postganglionic stimulation. For example, under conditions where good responses were obtained from both pairs of electrodes, the addition of hexamethonium rapidly blocked the responses to stimulation by the distal or preganglionic electrodes but left the responses to stimulation by the other pair and to transmural stimulation almost unchanged. Observations with hexamethonium confirm the findings of Ohlin & Strömblad (1963).

These results are in general agreement with the findings of Birmingham & Wilson (1963). We stimulated with pulses of lower voltage but longer duration and obtained very stable responses. The possibility that muscle cells rather than nerve fibres were stimu-

lated is discounted by the fact that the drugs already mentioned, as well as procaine, bretylium and guanethidine, blocked the responses to both nerve and low-voltage transmural stimulation. Further, when such block had been obtained, an increase in the strength of the transmural stimulus had little or no effect until 50–60 V was reached, 70–100 V giving the maximum response. High-voltage stimulation of the nerve was ineffective in these conditions, so it was assumed that the muscle cells themselves were being stimulated.

There is considerable disagreement concerning the effect of hemicholinium on the hypogastric nerve-vas deferens preparation. Chang & Rand (1960), Bentley (1962) and Bentley & Sabine (1963) have shown that when the nerve was stimulated doses of 20–100 μg/ml. caused block of transmission. Bentley & Sabine (1963) using transmural stimulation did not obtain a reduction in the response greater than 50%, however, and Birmingham & Wilson (1963), also using transmural stimulation, found that the response fell by only 5–15% in the presence of hemicholinium. We found that hemicholinium blocks the response to preganglionic stimulation but that the responses to postganglionic nerve and transmural stimulation are largely unaffected.

Ferry (1967) has examined serial sections of the hypogastric nerve at intervals of 1 mm and shown that ganglion cells are present over the distal 25 mm and concentrated in the region 4–9 mm from the vas deferens. This being so, there is every likelihood that electrodes applied 10 mm or more from the muscle will stimulate mainly preganglionic fibres. This anatomical evidence, coupled with our findings and those of Birmingham & Wilson (1963) and Bentley & Sabine (1963), leads to the conclusion that the findings of Chang & Rand (1960) and Bentley (1962) with hemicholinium result from effects on the ganglion-cells in the hypogastric nerve and, further, that the postganglionic fibres are not cholinergic.

Our results with bretylium and guanethidine are similar to those of previous workers (Bentley, 1962 ; Bentley & Sabine, 1963 ; Birmingham & Wilson, 1963).

The results obtained with botulinum toxin show that this toxin abolishes the responses of the vas deferens to stimulation of the preganglionic and postganglionic fibres in the hypogastric nerve and of the intramural nerve fibres. These observations make it unlikely that the earlier findings of Rand & Whaler (1965) can be explained by an action of the toxin on the ganglion cells in the hypogastric nerve. The effect on the preganglionic cholinergic fibres was to be expected from the evidence of previous workers (Dickson & Shevky, 1923 ; Ambache, 1951 ; Wright, 1955). An important result of the present experiments is the observation that the block of postganglionic adrenergic transmission is not due to a reduced sensitivity of the muscle cells to the transmitter, noradrenaline.

Because the hypogastric nerve contains ganglion-cells very close to the muscle tissue of the vas deferens (Owman & Sjöstrand, 1965 ; Ferry, 1967), our postganglionic electrode may, in fact, stimulate some preganglionic fibres. Hexamethonium, hemicholinium and (+)-tubocurarine barely alter the responses to stimulation by the postganglionic electrodes, however, so these ganglion cells cannot contribute much to the innervation of the vas deferens ; moreover, they are by-passed when low-voltage transmural stimulation is used and cannot therefore be the cause of the blocking effect of botulinum toxin.

Bella, Benelli & Gandini (1964) have implied that some postganglionic cholinergic fibres enter the vas deferens and may be involved in the normal control of this organ, in addition to the postganglionic adrenergic innervation. This may be correct but we have shown that botulinum toxin completely suppresses contractions of the vas deferens caused by stimulation of postganglionic fibres, which must include adrenergic fibres.

The work of Ambache (1949, 1951) established for cat and rabbit pupillodilator fibres, and perhaps also for the cat nictitating membrane, as well as rabbit and guinea-pig ileum, that in these tissues transmission from adrenergic fibres to the smooth muscle cells is probably not affected by botulinum toxin. More recently, Vincenzi (1967) has shown that the responses of rabbit atria to stimulation of adrenergic nerves are unaffected by type D toxin. On the other hand, there are the findings of Rand & Whaler (1965) on the innervated rabbit ileum and cat pilomotor fibres, and the observations now obtained for the hypogastric nerve-vas deferens preparation. In the rabbit colon preparation (Garry & Gillespie, 1955), both the cholinergic and adrenergic nerve pathways are sensitive to the toxin (Whaler, 1967). With the *in vitro* preparations tested, the dose of toxin necessary to block adrenergic fibres is of the same order as that needed to block cholinergic postganglionic fibres, sympathetic ganglia and the rat phrenic nerve-diaphragm preparation. The time-course of the block is, however, somewhat different ; at cholinergic sites only 1–3 hr are required whereas the adrenergic endings which have been tested require 2–7 hr. It therefore seems necessary, at the present time, to assume that toxin-sensitive and toxin-insensitive adrenergic nerve fibres exist and that the basis for their difference is still unknown (Whaler, 1967).

The results presented in this paper are compatible with the concept of a cholinergic link in adrenergic transmission (Burn & Rand, 1965 ; Ferry, 1966) although not conclusive proof of such a mechanism (Rand & Whaler, 1965).

SUMMARY

1. The paralysing action of botulinum toxin on the vas deferens-hypogastric nerve preparation has been confirmed. The sites of action are at ganglion-cells present in the hypogastric nerve and at the postganglionic nerve-smooth muscle junction. The responses of the vas deferens to noradrenaline are not affected.

2. The effects of stimulation of preganglionic and postganglionic fibres in the hypogastric nerve have been distinguished by the use of hexamethonium, hemicholinium, (+)-tubocurarine, atropine, hyoscine and botulinum toxin.

3. The effects of transmural stimulation on intramural nerve fibres and on smooth muscle cells have been distinguished by the use of procaine, bretylium, guanethidine and botulinum toxin.

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