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THE POTENTIATION BY ANTICHOLINESTERASE DRUGS OF THE RESPONSES OF THE GUINEA-PIG ISOLATED VAS DEFERENS TO ALTERNATE PREGANGLIONIC AND POSTGANGLIONIC STIMULATION

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The potentiating effects of anticholinesterase drugs on the responses of the guinea-pig isolated vas deferens to electrical stimulation of the hypogastric nerve have been reported several times in recent years (Boyd, Chang & Rand, 1960; Burn & Weetman, 1963; Ohlin & Strömblad, 1963; Della Bella, Benelli & Gandini, 1964). It has now been established that most of the fibres in the hypogastric nerve are preganglionic (Sjöstrand, 1962; Bentley & Sabine, 1963; Kuriyama, 1963; Ohlin & Strömblad, 1963; Birmingham & Wilson, 1963) so that when considering the possible sites at which the potentiating effect is exerted, the effects of inhibiting cholinesterase in the ganglion must be taken into account. The feasibility of stimulating preganglionic or postganglionic nerve fibres in the guinea-pig isolated vas deferens was reported by Birmingham & Wilson (1963). It has therefore been possible to re-examine the effects of physostigmine and to describe the effects of diisopropylphosphorofluoridate (dyflos) on the isolated vas deferens stimulated alternately preganglionically and postganglionically. This paper describes the results of experiments which attempt to distinguish the sites of the potentiating effect of the anticholinesterase drug. Some of these results were reported to the July, 1964, meeting of the British Pharmacological Society at Bristol.

METHODS

The guinea-pig isolated vas deferens stimulated alternately through the hypogastric nerve and transmurally (Birmingham & Wilson, 1963). The vas deferens was dissected by the method of Huković (1961) and set up in a jacketed organ bath containing 75 ml. Krebs solution at 32° C bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Contractions were recorded on smoked paper with isotonic frontal writing levers loaded at 0.5 g with a magnification of four times. The vas was stimulated alternately preganglionically through the hypogastric nerve (0.1 msec pulse width, supramaximal voltage 40 v) or postganglionically by transmural stimulation between parallel platinum wires (0.1 msec pulse width, supramaximal voltage 120 v). Trains of 130 stimuli at frequencies of 3, 6, 12 or 25 shocks/sec were administered at 4 min intervals alternately through the hypogastric nerve and transmurally. Trains of 260 shocks at 50 shocks/sec were also included to establish the maximal response of the preparation. Changes from one frequency to another were not made until the responses remained uniform at that frequency; in most experiments this occurred within 30 min. The order in which the five frequencies were used was randomly varied

from one preparation to another but for any given preparation the sequence was the same for the three sections of the experiment. Both vasa deferentia were removed from each guinea-pig and set up under identical conditions; one served as the control and the other as the test preparation. To establish the baseline response of each preparation the five different frequencies were administered in the absence of any drugs. The anticholinesterase drug was then added to the Krebs solution bathing one of the preparations and the five frequencies were again tested against both vasa. For the third and final period of stimulation at the five frequencies, atropine was added to both preparations. Multitone "Ten Pulse" stimulators were used throughout this work.

The response of the isolated vas deferens to added drugs. The vas deferens was removed without the hypogastric nerve. The serous coat was stripped off to increase the sensitivity to drugs and the vas was suspended in a jacketed organ bath containing 20 ml. Krebs solution at 32° C bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Contractions were recorded on smoked paper with isotonic frontal writing levers loaded at 0.5 g with a magnification of four times. Agonist drugs were added for a one min contact time with a five min cycle. After the baseline responses to three doses of the agonist had been recorded, the doses were repeated in the presence of the anticholinesterase drug and then in the presence of the anticholinesterase drug and atropine. Both vasa deferentia were removed from each guinea-pig and set up under identical conditions; for one the agonist was acetylcholine, for the other it was noradrenaline.

Drugs. Those used were acetylcholine chloride, atropine sulphate, dyflos, (−)-noradrenaline bitartrate, physostigmine sulphate. All concentrations quoted are as final bath concentrations of the base in g/ml.

RESULTS

The isolated vas deferens stimulated alternately through the hypogastric nerve and transmurally

The responses of an alternately stimulated vas deferens to trains of 130 stimuli at frequencies of stimulation from 3 shocks/sec to 25 shocks/sec and then to 260 stimuli at 50 shocks/sec are shown in Fig. 1. At 3 shocks/sec the responses to hypogastric nerve stimulation were a few millimetres in height and the responses to transmural stimulation were two to three times larger. With increasing frequency of stimulation there was an increase in the height of the response up to the maximal response at 50 shocks/sec. Testing at 100 shocks/sec showed no further increase in height of contraction or usually showed a reduction in the response. The responses at any given frequency soon became uniform and would remain so for many hours. In the absence of drugs the response to transmural stimulation was always larger than the response to hypogastric nerve stimulation. The frequency changes were usually made in random order, instead of by the doubling sequence shown in Fig. 1: the same relation between frequency and response was seen with random frequency changes but it was necessary to allow more time for the responses to become uniform.

The relation between frequency of stimulation and height of response was expressed graphically as a log frequency-response curve (Figs. 3 and 4) in which the frequency of stimulation was plotted logarithmically as the abscissa and the response at any given frequency was plotted on the ordinate as a percentage of the height of the maximal contraction to stimulation at 50 shocks/sec during the baseline period.

Physostigmine. The tracing from a typical experiment is shown in Fig. 2. Firstly the baseline responses to the five different frequencies were established then, while the first frequency (6 shocks/sec) was used again, physostigmine was added to reach a final bath concentration of 10^{-6} g/ml. There was potentiation of the responses which was allowed to

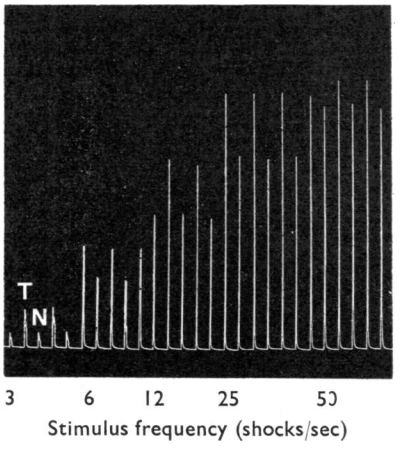

Fig. 1. The response of a guinea-pig isolated vas deferens-hypogastric nerve preparation to alternate transmural and hypogastric nerve stimulation at increasing frequency of stimulation. At T the vas deferens was stimulated transmurally (0.1 msec pulse width; supramaximal voltage) and at N the hypogastric nerve was stimulated (0.1 msec pulse width; supramaximal voltage). T and N were alternated at 4 min intervals. The total number of shocks was 130 for frequencies of 3, 6, 12 and 25 shocks/sec and 260 for the frequency of 50 shocks/sec.

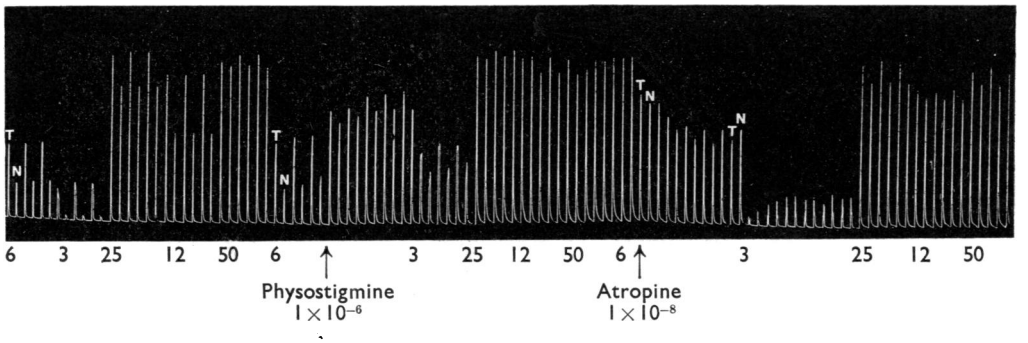

Fig. 2. The response of a guinea-pig isolated hypogastric nerve-vas deferens preparation to transmural stimulation at T alternated, at 4 min intervals, with hypogastric nerve stimulation at N. The pulse widths were 0.1 msec and the voltage was supramaximal. The responses to stimulation at the frequencies of 3, 6, 12, 25 and 50 shocks/sec administered in random order are shown first; then physostigmine was added in a final bath concentration of 1×10^{-6} g/ml. and the potentiation of the responses was allowed to develop to a maximum. The random frequencies were then repeated in the presence of physostigmine. Next, with the physostigmine still present, atropine (1×10^{-8} g/ml. final concentration) was added and the responses declined but, at 6 shocks/sec, the response to T declined more than the response to N so that N was then larger than T. The remaining frequencies of the random series were repeated in the presence of the physostigmine and atropine. As a control the other vas deferens from the same guinea-pig was treated in exactly the same way except that no physostigmine was added before the second series of random frequencies.

develop to a maximum at that frequency, then the responses to the other four frequencies were established in the order used for the baseline. Next, reverting to the first frequency (6 shocks/sec) with the physostigmine still present, atropine was added in a final concentration of 10^{-8} g/ml. and caused a reduction in the responses. When there appeared to be no further reduction in the responses the remaining four frequencies were tested. The

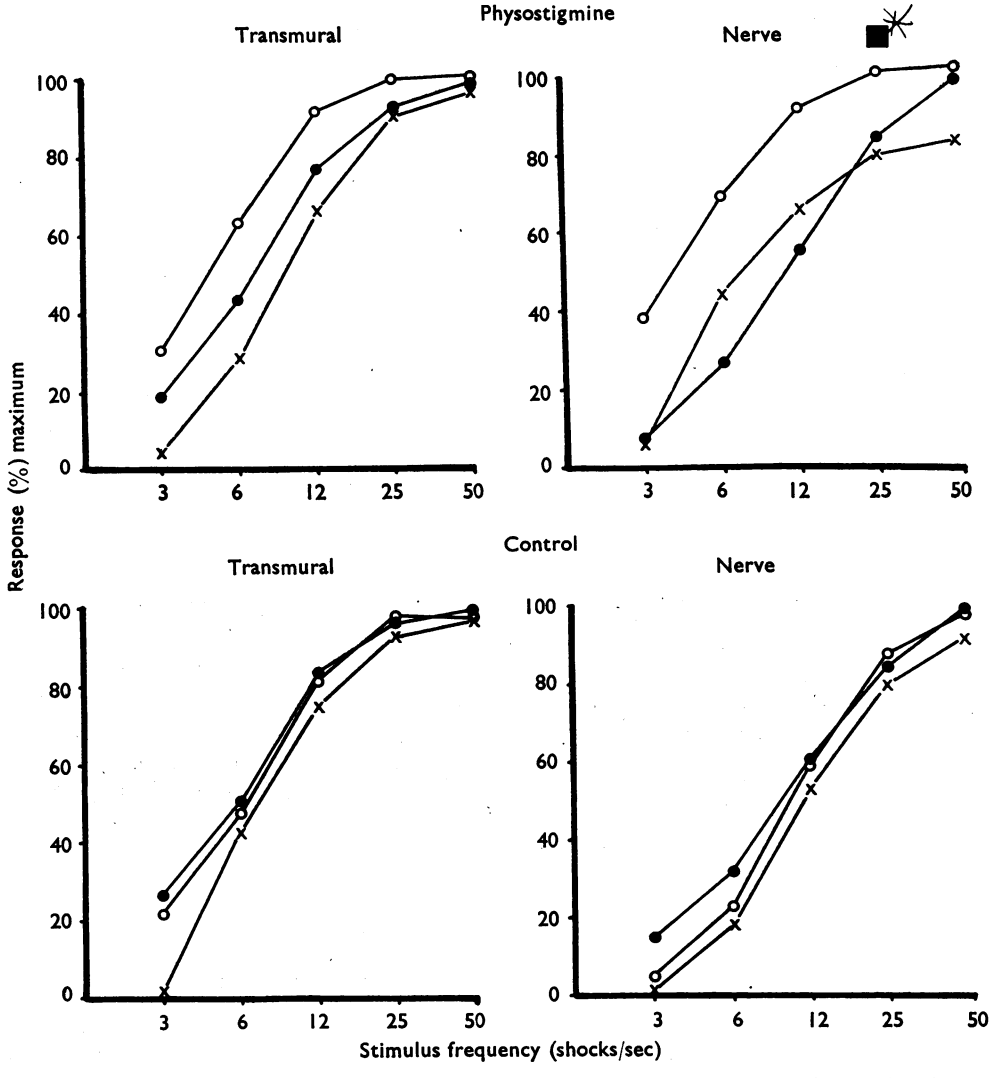

Fig. 3. Frequency-response graphs showing the effect of physostigmine on the alternately stimulated vas deferens. Each point is a mean derived from measurements of heights of contractions from experiments made on the vasa deferentia from four guinea-pigs. The measurements in mm were converted to percentages of the height of the baseline response to 50 shocks/sec. Upper graphs: ●——● before physostigmine; ○——○ in presence of 1×10^{-6} g/ml. physostigmine; ×——× in presence of physostigmine plus 1×10^{-8} g/ml. atropine. Lower graphs (Controls): ●——● no drug; ○——○ second period of stimulation, no drug: ×——× in presence of 1×10^{-8} g/ml. atropine.

other vas deferens from the guinea-pig was treated in exactly the same way at the same time except that physostigmine was not added after the baseline recordings, but atropine was added for the third series of frequencies.

Experiments were made on the vasa deferentia from four guinea-pigs with a different random sequence for each guinea-pig. The means of the results obtained are plotted in

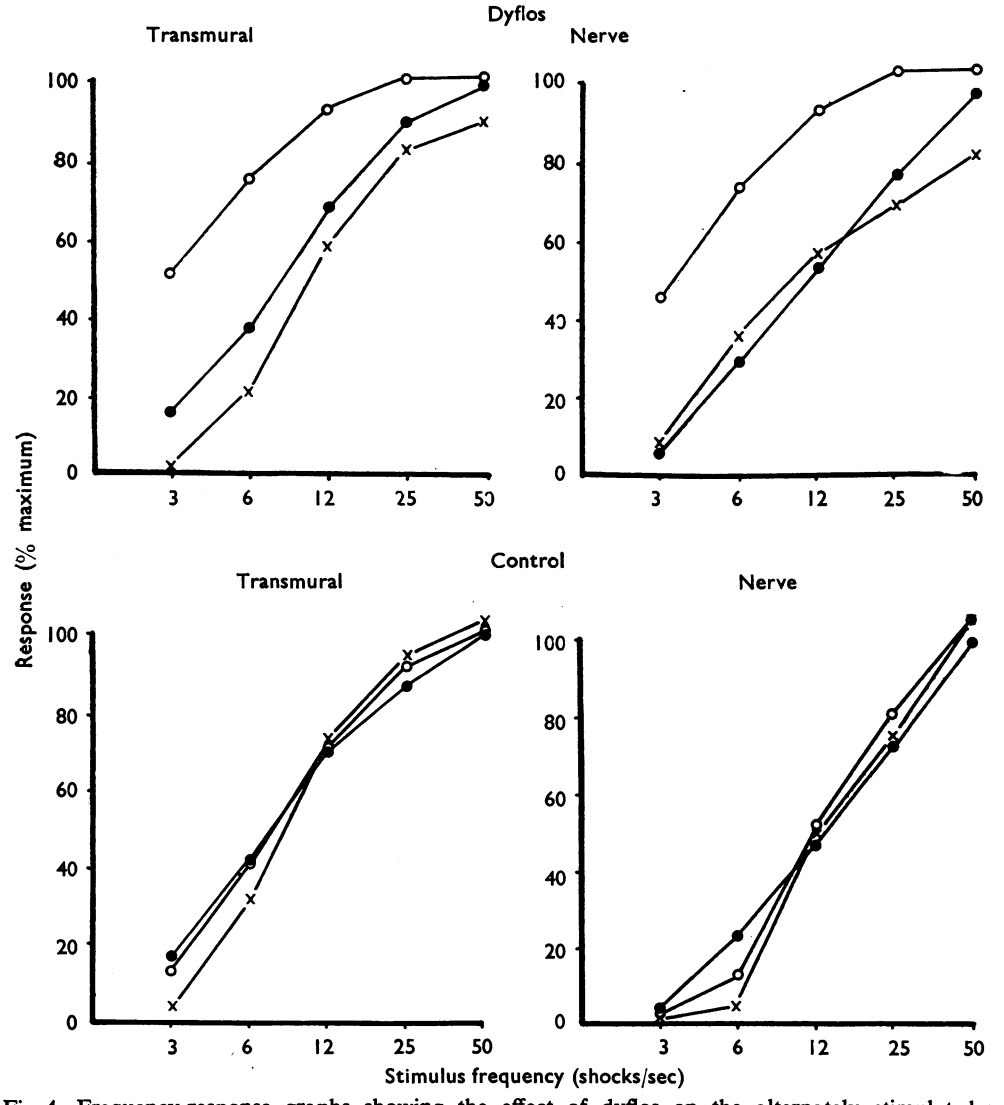

Fig. 4. Frequency-response graphs showing the effect of dyflos on the alternately stimulated vas deferens. Each point is a mean derived from measurements of heights of contractions from experiments performed on the vasa deferentia from four guinea-pigs. The measurements in mm were converted to percentages of the height of the baseline response to 50 shocks/sec. Upper graphs: ●——● before dyflos; ○——○ in presence of 1×10^{-5} g/ml. dyflos; ×——× in presence of dyflos and 1×10^{-8} g/ml. atropine. Lower graphs (Controls): ●——● no drug; ○——○ second period of stimulation, no drug; ×——× in presence of 1×10^{-8} g/ml. atropine.

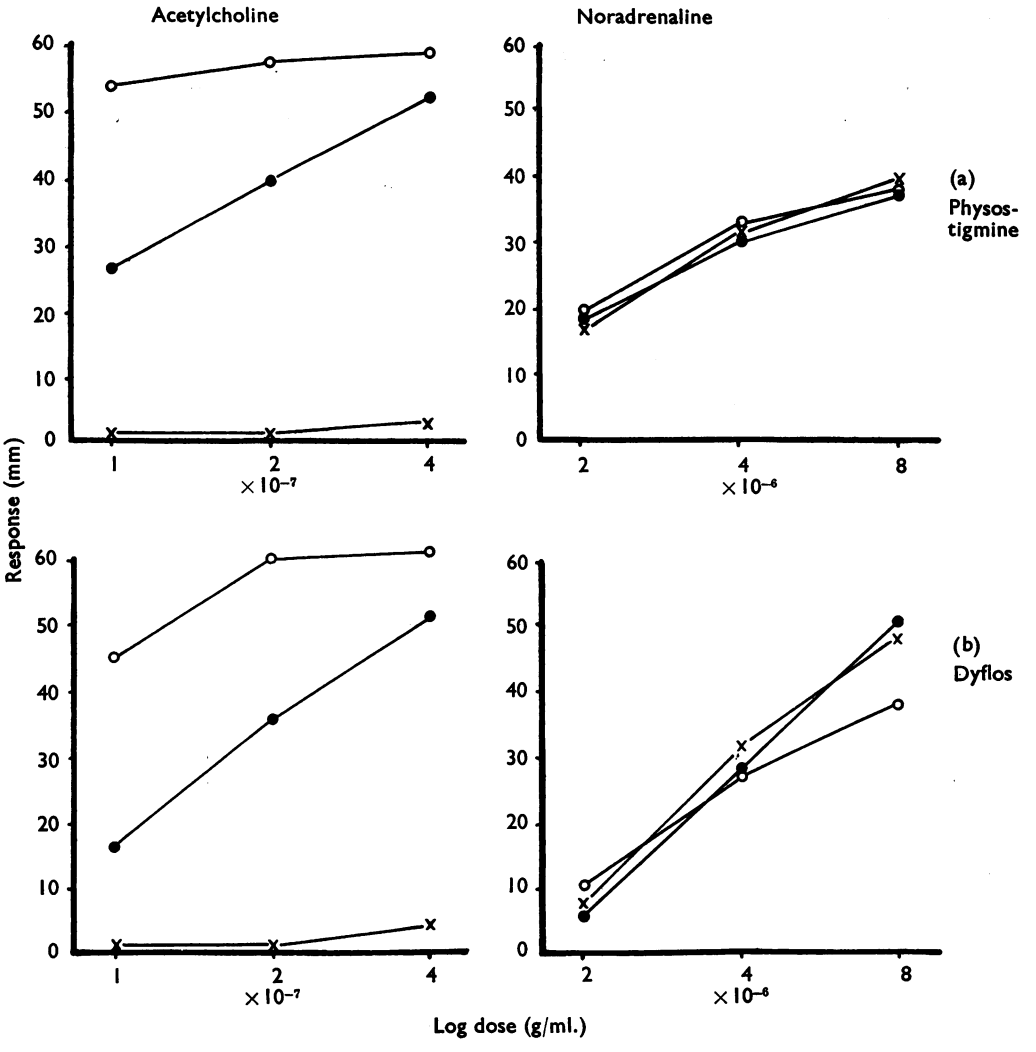

Fig. 5. Log dose–response graphs for acetylcholine and for noradrenaline on the guinea-pig isolated vas deferens, showing the effects of physostigmine or dyflos and the effects of atropine on the responses. Each point is a mean derived from measurements of heights of contractions to 1, 2 and 4×10^{-7} g/ml. acetylcholine or 2, 4 and 8×10^{-6} g/ml. noradrenaline on the vasa deferentia, stripped of serous coats, from four guinea-pigs. From each of the four guinea-pigs one vas was used for the acetylcholine responses and the other for the noradrenaline responses. Upper graphs (Physostigmine): ●——● agonists alone; ○——○ agonists repeated after 15 min exposure of the vas to, and in the presence of, 1×10^{-6} g/ml. physostigmine; ×——× agonists repeated with physostigmine still present after 20 min exposure to, and in the presence of, 1×10^{-8} g/ml. atropine. Lower graphs (Dyflos): ●——● agonists alone; ○——○ agonists repeated after 15 min exposure of the vas to, and in the presence of, 1×10^{-5} g/ml. dyflos; ×——× agonist repeated with the dyflos still present after 20 min exposure to, and in the presence of, 1×10^{-8} g/ml. atropine.

Fig. 3 as log. frequency-response curves. For the transmural method of stimulation the height of the response was increased at all frequencies in the presence of physostigmine but the control preparations showed little or no change in response during the corresponding periods. When atropine was added to the test preparations there was a reduction in the height of all the responses to a level below that of the baseline contractions. Atropine alone caused a reduction in the responses of the control preparations. The responses to hypogastric nerve stimulation were also increased by physostigmine and to an extent greater than the increase seen for transmural stimulation. However, when atropine was added the reduction in the height of the responses was, at the lower frequencies, much less, and at the higher frequencies, much greater, than the reduction seen with transmural stimulation. The responses to stimulation at 6 and at 12 shocks/sec, although reduced, were still above the baseline levels, whereas the corresponding control preparations showed a uniform reduction in the presence of atropine to below the baseline levels.

Dyflos. Experiments identical with those described for physostigmine were made on vasa deferentia from another four guinea-pigs with dyflos (D.F.P.) as the anticholinesterase present in a concentration of 10^{-5} g/ml. Dyflos caused a potentiation of the responses which had a more delayed onset and took longer to reach a maximum than that seen with physostigmine. The log. frequency-response graphs in Fig. 4 show that the mean results obtained with dyflos were similar to those obtained with physostigmine.

Physostigmine or dyflos after atropine. (Fig. 6.) When the two vasa deferentia from a guinea-pig were set up under similar conditions and stimulated at 12 shocks/sec, atropine, 1×10^{-7} g/ml., caused a small reduction in the height of the responses to transmural or hypogastric nerve stimulation. With the atropine still present, physostigmine (1×10^{-6}) or dyflos (1×10^{-5}) potentiated only the response to hypogastric nerve stimulation.

The effects of physostigmine or dyflos on the responses of the isolated vas deferens to acetylcholine or to noradrenaline

With the vas deferens stripped of its serous coat a linear relation was found between response and log. dose for the three doses of acetylcholine 1, 2 and 4 $\times 10^{-7}$ g/ml. or the three doses of noradrenaline 2, 4 and 8×10^{-6} g/ml. (Fig. 5).

Physostigmine. (Fig. 5a.) When the three doses of acetylcholine were repeated after 15 min exposure to and still in the presence of 1×10^{-6} g/ml. physostigmine the responses were greatly potentiated, whereas the other vas from the same guinea-pig showed no change in its responses to noradrenaline during similar treatment with physostigmine. With the physostigmine still present, 1×10^{-8} g/ml. atropine acting for 20 min before and during re-testing with the agonists, abolished or greatly reduced the responses to acetylcholine but did not reduce the responses to noradrenaline. Separate testing showed that the response to a maximal dose of noradrenaline was not reduced by physostigmine and atropine.

Dyflos. (Fig. 5b.) 1×10^{-5} g/ml. dyflos present for one hour before and during testing with the agonist drugs greatly potentiated the responses to acetylcholine but did not increase the responses to noradrenaline. Atropine (1×10^{-8}) abolished or greatly

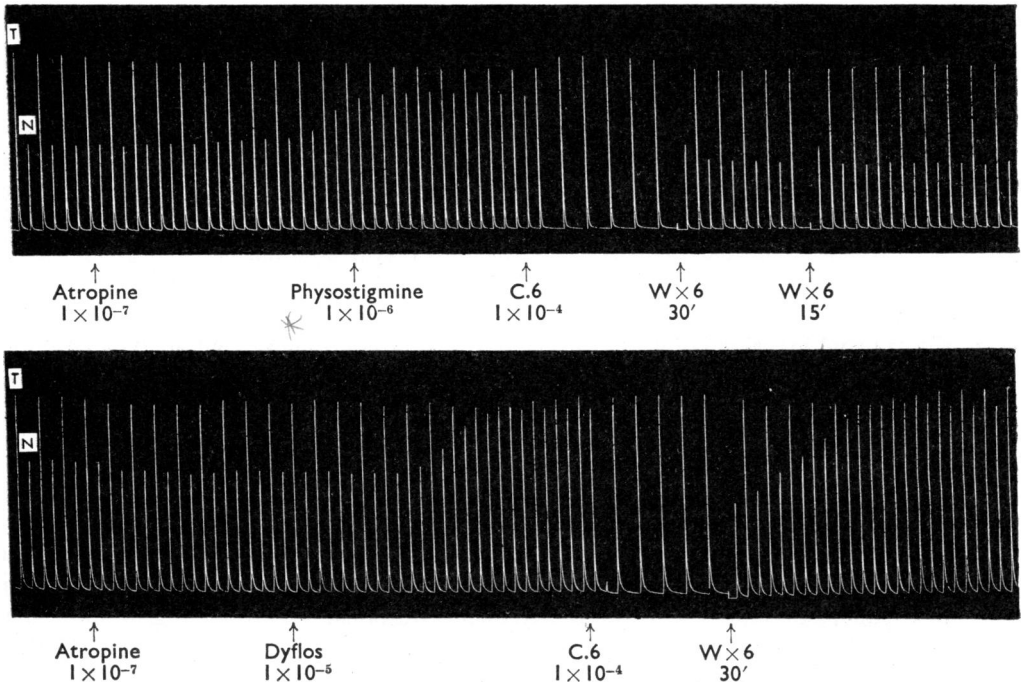

Fig. 6. The effect of physostigmine or dyflos on the alternately stimulated vas deferens in the presence of atropine. The Huković preparation of the vas deferens was stimulated between parallel wire electrodes at T (frequency 12 shocks/sec; pulse width 0.1 msec at supramaximal voltage) and the hypogastric nerve was stimulated with annular wire electrodes at N (frequency 12 shocks/sec; pulse width 0.1 msec at supramaximal voltage). T and N were alternated at 4 min intervals throughout the experiment. Baseline contractions were recorded, then atropine, 1×10^{-7} g/ml. final concentration, was added to the Krebs solution; it caused a small reduction in the height of the responses. When physostigmine 1×10^{-6} g/ml. was added (upper panel) it caused a gradual increase in the height of the response to hypogastric nerve stimulation but no increase in the height of the response to transmural stimulation. At the height of the potentiation hexamethonium (C.6., 1×10^{-4} g/ml.) was added and it blocked the response to hypogastric nerve stimulation without reducing the response to transmural stimulation showing that N was preganglionic stimulation and T postganglionic stimulation. The responses returned almost to baseline level on washing out the drugs. A similar experiment, on the other vas from the same guinea-pig, is shown with dyflos as the anticholinesterase in the lower panel. The result was the same as that seen with physostigmine except that the potentiation was slower to develop and remained after the ganglion blocking drug and anticholinesterase drug had been washed out, emphasizing the irreversible nature of the action of dyflos.

reduced the response to acetylcholine without reducing the response to noradrenaline. The response to a maximal dose of noradrenaline was not reduced by dyflos and atropine.

DISCUSSION

When the vas deferens was stimulated alternately through the hypogastric nerve and transmurally with frequencies of stimulation from 3 to 50 shocks/sec there was an increase in the height of the response up to the maximal response at 50 shocks/sec. The relation between frequency of stimulation and height of response was similar to that described by Day (1965) for either method of stimulation applied alone.

At all frequencies of stimulation the responses were increased in height by exposure to physostigmine or to dyflos but were reduced again when atropine was added. The transmurally induced responses were reduced to, or just below, the pre-anticholinesterase levels. But, for the contractions induced by stimulation of the hypogastric nerve, atropine effected a less uniform reduction of the potentiated responses. For the lower three frequencies the responses in the presence of atropine were the same as or greater than the pre-anticholinesterase responses whereas the responses to the upper frequencies were smaller than the baseline responses.

That the increased responses of the vas in the presence of anticholinesterase drugs were not due to an increased sensitivity of the effector cells to the adrenergic transmitter was shown by the lack of potentiating effect of physostigmine or dyflos on the response of isolated vasa deferentia to added noradrenaline. In the same way it was shown that the decreases produced by atropine were not due to a reduction in the sensitivity of the effector cells to the adrenergic transmitter, because atropine did not reduce the response of these vasa deferentia to noradrenaline, nor was the maximal contraction reduced in height. But it was shown that the concentrations of anticholinesterases used did increase the sensitivity of the vasa deferentia to added acetylcholine and that the low concentration of atropine used was sufficient to abolish or greatly reduce the response of the vas to acetylcholine even in the presence of anticholinesterase.

The effects of physostigmine or dyflos on the acetylcholine-destroying ability of the guinea-pig vas deferens were investigated by Birmingham & Underwood (1965). They found that after 20 min exposure to 10^{-6} g/ml. physostigmine or 10^{-5} g/ml. dyflos there was complete inhibition of the ability of the vas deferens to destroy acetylcholine. It seems, therefore, that the gradual potentiation of the responses of the electrically stimulated vas deferens by physostigmine or by dyflos may be coincident with the gradual inhibition of cholinesterase and that the maximal potentiation occurs at a time when it can be shown that the acetycholine-destroying ability of the vas has been abolished.

The involvement of acetylcholine in the potentiation of the transmural response is strongly suggested by the complete abolition of the potentiation by atropine in concentrations which block the response of the vas to added acetylcholine but not the response to added noradrenaline. For the transmural method of stimulation Birmingham & Wilson (1963) have shown that the contractions are produced by stimulation of postganglionic nerve fibres and from evidence obtained with hyoscine, atropine and hemicholinium they suggested that a small part of the transmural response was due to excitation of postganglionic cholinergic nerve fibres. Thus, if acetylcholine is involved, the part of the response increased by anticholinesterases and blocked by atropine could be due to acetylcholine liberated from postganglionic fibres to act on muscarinic receptors on the smooth muscle.

When the responses to hypogastric nerve stimulation are considered, it must be borne in mind that the responses depend on transmission through a ganglion. Most of the potentiation seen with anticholinesterase drugs still seems to involve cholinergic fibres and muscarinic receptors because it was greatly reduced by atropine in low concentrations. But there remains a component of the potentiated response which was resistant to atropine. This persisting potentiation on preganglionic stimulation, not seen on

postganglionic stimulation can be ascribed to an action of the anticholinesterase at the ganglion, so that by potentiating the effects of the pre-ganglionic nerve volleys more noradrenaline is liberated at the postganglionic nerve endings of the adrenergic nerves. Such an effect, unlike the potentiation involving postganglionic cholinergic nerves, would be resistant to atropine. This effect was seen, for physostigmine or for dyflos, only at 3, 6 or 12 shocks/sec. At higher frequencies atropine reduced the response to below the level seen before exposure to the anticholinesterase drug. This reduction could be a sign of impairment of ganglionic transmission associated with high frequency preganglionic stimulation under conditions of cholinesterase inhibition.

Although it was clear that atropine added after the potentiation had developed abolished postganglionic potentiation and reduced preganglionic potentiation, it was of interest to reverse the order of adding the drugs to the bath. When atropine was added before the anticholinesterase it prevented potentiation of the postganglionic response but still allowed potentiation of the preganglionic response.

Thus the phenomena of disproportionate depression of the response at high frequencies and of augmentation of the response to low frequencies were found not to be a feature of postganglionic nerve stimulation under conditions of cholinesterase inhibition and muscarinic blockade; they appeared to be associated with ganglionic transmission.

What is the relation of these findings to those of others who have used exclusively the guinea-pig vas deferens stimulated preganglionically? Potentiation by physostigmine of the responses of the Huković preparation was reported by Boyd, Chang & Rand (1960) and they considered this to be due to potentiation of acetylcholine acting as an intermediary in the release of noradrenaline from adrenergic nerves. Burn & Weetman (1963) extended these observations to include an analysis of frequency-response relations. With hyoscine present to exclude an action of acetylcholine at muscarinic receptors, they showed that physostigmine or neostigmine increased the height of the response to low frequencies and reduced the height of the response to high frequencies of stimulation. These results were interpreted to mean that the anticholinesterases were enhancing, through acetylcholine, the release of noradrenaline from the sympathetic nerve terminals. Della Bella, Benelli & Gandini (1964) confirmed the potentiating effect of physostigmine on the Huković preparation and noted that atropine reduced but did not abolish this potentiation. On the basis of some qualitative observations they inferred that physostigmine uncovered a parasympathetic cholinergic component in the autonomic innervation of the vas deferens.

The results now reported suggest that when the cholinesterase of the vas deferens is inhibited by physostigmine or by dyflos the responses to electrical stimulation are increased by at least two mechanisms. Firstly, stimulation of postganglionic nerves directly by transmural stimulation or indirectly by stimulation of the preganglionic hypogastric nerve invokes a response which, although mainly dependent on an adrenergic mechanism (Birmingham & Wilson, 1963), seems also to include a component involving an action of acetylcholine on muscarinic receptors on the smooth muscle. This response is increased by anticholinesterases and the increase is blocked by atropine. The site of action of the anticholinesterase drugs is presumably at or near the postganglionic

nerve terminals. Secondly, there is a component of the response to hypogastric nerve stimulation, seen at lower frequencies which is potentiated by anticholinesterases but not blocked by atropine. This potentiation could be due to an enhancement of the effectiveness of the preganglionic volleys in releasing noradrenaline from the postganglionic adrenergic neurones. For this component the site of action of the anticholinesterase drugs is presumably the cholinesterase enzyme in the ganglion. The potentiations observed by Boyd, Chang & Rand (1960) and by Burn & Weetman (1963) can be explained on the basis of the second mechanism outlined above. The results of Della Bella, Benelli & Gandini (1964) can be explained by one or both of these mechanisms. It no longer seems to be necessary to invoke a cholinergic link in the postganglionic adrenergic nerve endings to explain these results.

SUMMARY

1. The guinea-pig isolated hypogastric nerve-vas deferens preparation when stimulated alternately transmurally (postganglionic) or through the hypogastric nerve (preganglionic) at 4 min intervals responded with increased height of contraction to increased frequency of stimulation from 3 shock/sec to a maximum at 50 shock/sec. At any given frequency the response to transmural stimulation was always larger than the response to hypogastric nerve stimulation. The relation between frequency of stimulation and height of response was expressed as a log. frequency-response graph.

2. Physostigmine (1×10^{-6} g/ml.) or dyflos (1×10^{-5} g/ml.) increased the height of the responses to transmural and to hypogastric nerve stimulation but the increase was proportionately greater for hypogastric nerve stimulation than for transmural stimulation. When atropine (1×10^{-8} g/ml.) was added the potentiated responses were reduced; the transmural responses were reduced to or just below their pre-anticholinesterase level whereas the hypogastric nerve responses, at the lower frequencies, were reduced by a smaller amount so that they remained above the pre-anticholinesterase level.

3. Physostigmine or dyflos potentiated and atropine abolished the response of the isolated vas deferens to added acetylcholine. The responses to noradrenaline were not changed by physostigmine or dyflos nor by atropine.

4. It is concluded that inhibition of the cholinesterase of the vas deferens leads to an increase in height of contraction by at least two mechanisms. There is a cholinergic mechanism involving postganglionic nerves and muscarinic smooth muscle receptors. There is also a cholinergic mechanism involving preganglionic nerves, potentiation of which is partly resistant to atropine and could be due to an enhancement of the effectiveness of the preganglionic volleys in releasing noradrenaline from postganglionic adrenergic neurones.

5. The results and conclusions are discussed in relation to those of others obtained exclusively on the vas deferens stimulated preganglionically. It is concluded that it is no longer necessary to invoke a cholinergic link in the postganglionic adrenergic nerve endings to explain these results.

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