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THE EFFECTS OF PHYSOSTIGMINE ON THE MECHANICAL AND ELECTRICAL RESPONSES OF THE CAT NICTITATING MEMBRANE

BY

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The effects of physostigmine on the electrical and mechanical responses of the nictitating membrane elicited by single nerve shocks have been studied in cats anaesthetized with a mixture of chloralose and pentobarbitone. The results were variable but the most consistent effect, observed with large doses of physostigmine, was a depression and prolongation of the contractions which was sometimes associated with augmentation of the characteristic rhythmic electrical activity of the smooth muscle. Although the amplitude of single responses was depressed, incomplete tetanic contractions were sometimes increased in tension because of the greater fusion which occurred as a result of prolongation of the units of contraction. Possible mechanisms underlying the effect of physostigmine are discussed.

Burn, Rand & Wein (1963) found that, using repetitive stimulation and isotonic recording, physostigmine caused a pronounced increase in the amplitude of the contractions of the nictitating membrane elicited by postganglionic stimulation of the cervical sympathetic trunk. The effect was more marked the lower the frequency of stimulation. Gardiner, Hellmann & Thompson (1962) on the other hand, using similar experimental conditions, found little or no effect with physostigmine, but their graphs do suggest a slight potentiation when the number of stimuli delivered to the postganglionic nerve was low. Neither group of authors used single shock stimulation. In skeletal muscle, potentiation of contractions by anticholinesterases is seen only when single twitches are recorded ; tetanic responses are unaffected or depressed (Brown, 1937 ; Blaber & Bowman, 1963a). We decided, therefore, to study the effect of physostigmine on the mechanical and electrical responses of the nictitating membrane of the cat induced by single shocks applied to the pre- and postganglionic nerve trunks. Nystrom (1962) used the same preparation but he employed mainly preganglionic stimulation and, in his pharmacological studies, recorded only the electrical activity of the membrane.

METHODS

The experiments were performed on cats anaesthetized by intravenous or intraperitoneal injection of a mixture of chloralose (8 ml./kg of a 1% solution) and pentobarbitone sodium (6 mg/kg). The trachea was cannulated low in the neck, and the upper end, together with the larynx, the oesophagus and the overlying muscles, was removed. The superior cervical ganglion on the right side was exposed and the postganglionic fibres supplying the nictitating

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membrane were prepared for stimulation. The ganglion was either destroyed by crushing or was removed completely. All nerves in the vicinity of the ganglion other than that containing the supply to the nictitating membrane were then cut and stripped away. The external ocular muscles were cut and the eye-ball was removed after ligating its blood supply. Next, the eye-lids were sewn back and a thread tied through the free cartilaginous border of the nictitating membrane. The cat was laid on its back and the head fixed rigidly. Isometric contractions of the nictating membrane were recorded by attaching it to an RCA 5734 mechano-electric transducer valve in the mounting described by Bülbring (1955). Shielded bipolar platinum stimulating electrodes were placed on the postganglionic fibres with the cathode nearest the membrane. The skin flaps were raised and the neck region filled with warmed heavy liquid paraffin. Contractions of the membrane were elicited by rectangular shocks of 0.5 to 1 msec duration delivered, in most experiments, at a frequency of 1 shock/min from a modified Tektronix (type 160 series) stimulator. The strength of the shocks was about twice that necessary to produce maximal contractions and action potentials. Action potentials in the medial smooth muscle of the membrane were recorded between an electrode sewn under the external layer of the outer surface of the membrane and an earthed electrode inserted into the belly of the muscle near its origin. The recording electrodes consisted of 0.5 cm lengths of 22 gauge silver wire which had been turned in a lathe to a fine point and coated with silver chloride. The electrode in the medial muscle was attached to a light-weight screened lead mounted in a glass tube and clamped in position. The flexible screened lead from the electrode in the outer surface was so supported that its weight did not interfere with the contractions. The action potentials were fed to a Tektronix (type 122) battery-powered preamplifier (frequency response 0.2 cycles/sec to 40 kcycles/sec) and thence to a Tektronix double-beam oscilloscope (type 502) on which they were displayed simultaneously with the tension changes. The display was recorded photographically. An upward deflexion of the beam denoted a negativity of the earthed electrode in the smooth muscle with respect to that in the outer surface of the membrane.

Intra-arterial injections were made in a volume not exceeding 0.2 ml. into the carotid arterial blood stream through a fine polyethylene cannula tied into the cut stump of one of the branches of the common carotid artery. Control injections of 0.2 ml. of saline were without effect. Evans Blue injected by the same route at the end of some of the experiments rapidly stained the nictitating membrane and surrounding tissues. Some drugs were injected intravenously through a cannula in a femoral vein. When intra-arterial injection was used, no allowance was made for body weight.

In five experiments a similar procedure was carried out except that the ganglion was not destroyed and stimulation was applied to the preganglionic trunk of the cervical sympathetic nerve after separating it from the vagus and cutting it centrally. In these experiments intra-arterial injections were made through a cannula in one of the muscular branches of the common carotid artery lower in the neck. Injection of Evans Blue through the cannula stained the ganglion.

In five other animals, about a 1 cm length of the preganglionic cervical sympathetic trunk had been removed aseptically 10 to 12 days previously during intravenous pentobarbitone sodium anaesthesia (about 30 mg/kg). Stimulation was applied to the postganglionic fibres. These preparations are referred to as chronically decentralized preparations.

The drugs used were physostigmine salicylate (B.D.H.), atropine sulphate (B.D.H.), D-tubocurarine chloride (Burroughs-Wellcome), hexamethonium bromide (May & Baker), acetylcholine chloride (B.D.H.) and (−)-adrenaline base (B.D.H.). The drugs were diluted in saline (0.9% w/v). All doses are expressed in terms of the base or the cation.

RESULTS

Responses to single shocks

The responses recorded from the medial smooth muscle of the membrane elicited by single nerve shocks were essentially similar to those described by Eccles &

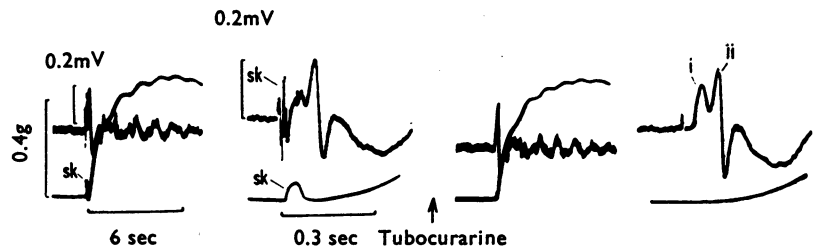

Fig. 1. Action potentials (upper trace) and isometric contractions (lower trace) in response to stimulation of the postganglionic nerve at 1 shock/min with a supramaximal shock. Responses were recorded alternately with a slow and fast sweep. The responses on the left are examples recorded before tubocurarine (0.5 mg/kg, intravenously). After tubocurarine the skeletal muscle components (labelled sk) disappeared. Calibrations: Action potentials, 0.2 mV; tension, 0.4 g; time, 6 sec for the slow sweep and 0.3 sec for the fast sweep.

Magladery (1937a). The initial main action potential complex usually consisted of two negative deflexions (labelled i and ii in Figs. 1, 3, 6 and 9) which preceded a positive deflexion. Contraction of the membrane followed the second negative deflexion. Such complex action potentials were recorded from normal preparations whether stimulated postganglionically or preganglionically and from chronically decentralized preparations stimulated postganglionically. In different experiments, the two negative deflexions were more-or-less fused, presumably on account of the relative positions of the two recording electrodes, but the form of the complex remained constant in any one experiment. The degree of fusion did not depend on whether the stimulation was pre- or postganglionic. With postganglionic stimulation, the onset of the electrical response occurred at 25 to 30 msec, while the onset of the tension response occurred at 150 to 200 msec after the stimulus artifact.

In most experiments, the initial main complex was followed by a rhythmic series of at least three gradually diminishing potentials, each of which could often be seen to precede a further small increment in tension so that the response to a single shock resembled an incomplete tetanus (Figs. 1, 2*a*, 6*a* and 8). The interval between each potential of the rhythmic series was 1 to 1.5 sec. When single shocks were delivered at intervals of 1 min, rhythmic activity usually became gradually less pronounced in the preparations which had not been chronically decentralized (Fig. 5); it occasionally disappeared completely after about 30 min of continuous stimulation at this frequency. It reappeared at full intensity when the preparation was allowed a rest of about 10 min from stimulation. In a few preparations, mostly from old male cats, very little or no rhythmic activity was evident at any stage in the experiment. The absence of rhythmic responses in these preparations may have been a consequence of the fact that, in life, the nictitating membrane of old males usually remains strongly contracted.

Rhythmic activity was evident in all five chronically decentralized preparations, one of which was from an old male cat, and it persisted unchanged throughout each experiment when the stimulus frequency was maintained at 1 shock/min. In all five of the chronically decentralized preparations, the tension developed by the membrane in response to a single shock was greater than that developed under

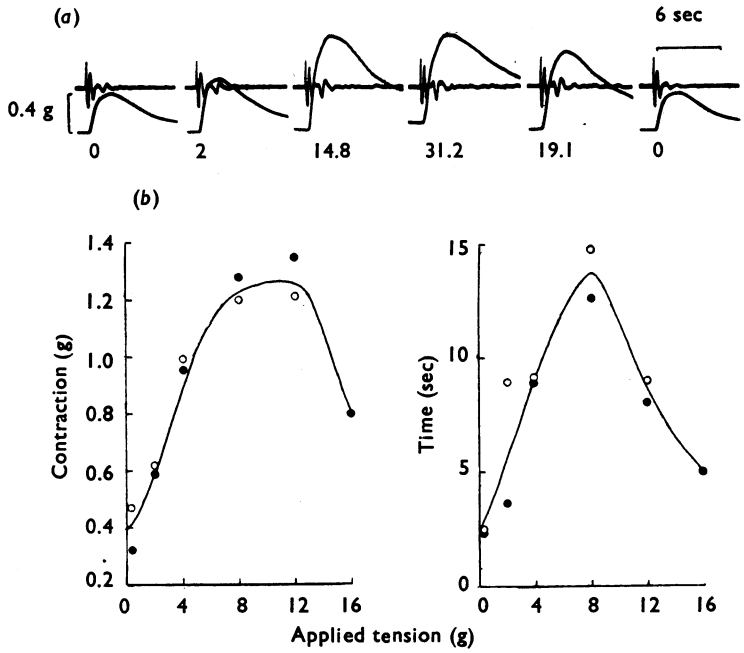

Fig. 2. Effects of varying the resting tension. (*a*) Recording as in Fig. 1 except that only a slow sweep speed was used. Each recording shown was taken when the responses had become constant after changing the resting tension. The numbers below the records denote the tension applied to the membrane in g. Calibrations: tension, 0.4 g; time, 6 sec. (*b*) The results of a similar experiment expressed graphically. Abscissae, tension applied to membrane in g. Ordinates, on the left, tension of contraction in g; on the right, time from start of contraction to half relaxation in sec. The closed circles represent responses taken while the resting tension was being increased and the open circles those during its return to zero.

identical recording conditions in any of the remaining experiments. The more powerful responses observed in the decentralized preparations may be explained by a greater release of transmitter as a result of its preservation in the nerve endings during the enforced rest, together with the increase in sensitivity of the membrane which results from chronic decentralization (Trendelenburg & Weiner, 1962).

With postganglionic stimulation, stimulus spread occasionally excited motor nerve fibres to some of the skeletal muscles in the orbit. An example of such a response is illustrated in Fig. 1 (labelled sk). The electrical and mechanical responses of the skeletal muscle fibres were much briefer and had a much shorter latency than those of the smooth muscle of the membrane. The skeletal muscle contractions were completely blocked by the intravenous administration of tubocurarine (0.3 to 0.5 mg/kg,) leaving the smooth muscle responses unaffected (Fig. 1).

In one experiment, the main smooth muscle action potential was preceded by a faster potential wave which did not appear to arise from skeletal muscle fibres. This potential was unaffected by the intravenous injection of tubocurarine (0.5 mg/kg) or phentolamine (5 mg/kg), or by the intra-arterial injection of guanethidine (1 mg), the last two of which abolished the smooth muscle response. It was depressed

by the intra-arterial injection of potassium chloride (1 mg in isotonic solution) and completely abolished by the intra-arterial injection of xylocaine (10 mg). The potential probably arose from one or more of the many fine nerve endings known to be distributed among the smooth muscle fibres of the membrane (Gardiner *et al.*, 1962).

Nystrom (1962) found it necessary in his experiments to ligate the ipselateral carotid artery in order to prevent pulsations in the nictitating membrane. Drugs were adminstered by topical application of high concentrations directly to the membrane. Minute oscillations in tension due to the arterial pulse were evident in most of our records but were not found troublesome since they were very small in comparison with the evoked tension responses, and cannot be discerned in the Figures. The circulation was therefore left intact and drugs were injected intra-arterially or intravenously. In many experiments in which the effects of drugs were studied, responses of the membrane to adrenaline as well as to nerve stimulation were recorded. Adrenaline, 0.5 to 1 μg/kg intravenously or 0.05 to 0.1 μg intra-arterially, produced an isometric tension response of the same order as that elicited by a single nerve shock although the adrenaline response was three- to five-times more prolonged (Figs. 3 and 9). The tension response to adrenaline was accompanied by irregular small oscillations in electrical potential occurring just before and during the rise in tension.

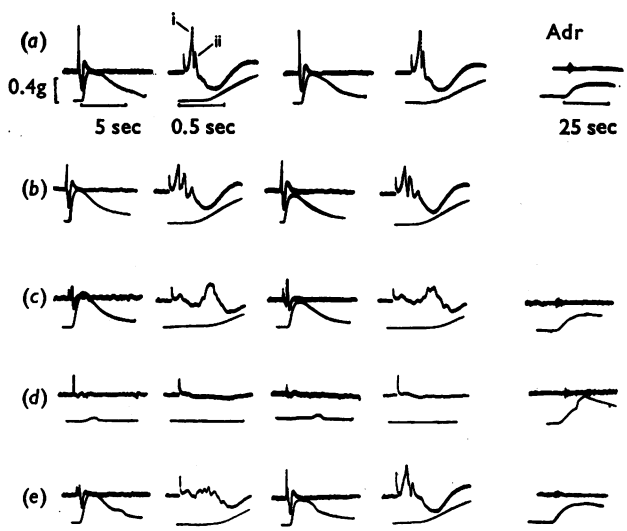

Fig. 3. Recordings as in Fig. 1. Effects of hexamethonium on the responses to preganglionic stimulation and to adrenaline. Responses of the membrane were elicited once every minute by single supramaximal shocks applied to the preganglionic nerve. Alternate slow and fast sweeps were used. Electrical stimulation was temporarily stopped about every 15 min and adrenaline (0.5 μg/kg) was injected intravenously. Responses to adrenaline (Adr) are shown on the extreme right. (*a*) Control responses; (*b*), (*c*) and (*d*) responses at the height of the blocking effect of intravenously injected hexamethonium (2.5, 2.5 and 5 mg/kg, respectively); (*e*) during recovery from hexamethonium, the first pair of responses 37 and 38 min and the second pair 49 and 50 min after the last injection. Calibrations: tension, 0.4 g; time, 5 sec for the slow sweep, 0.5 sec for the fast sweep and 25 sec for the adrenaline responses.

Effect of resting tension

In twelve cats, the effect of altering the resting tension on the mechanical and electrical responses was studied. Similar results were obtained whether or not the ganglion had been chronically denervated. Fig. 2*a* illustrates typical responses and Fig. 2*b* is a graphical representation of the results of a different experiment. Increase in resting tension caused an increase in the size of the contraction up to a maximum which varied in different experiments between 180 and 400% of the amplitude when the resting tension was zero. The times to peak tension and to half relaxation were correspondingly increased. Further increase in the resting tension decreased the responses. The amplitude of the main smooth muscle action potential was unaffected by changes in the resting tension, but rhythmic electrical activity was usually more pronounced the higher the resting tension (Fig. 2*a*). However, the increased contraction was not solely due to the increased rhythmic activity, since it also occurred in animals in which no rhythmic potentials were evident. The resting tension giving maximal contraction height varied in different experiments between 12 and 25 g. The smooth muscle of the nictitating membrane therefore resembles skeletal and cardiac muscle both of which give maximal responses at an optimal resting length.

Contractions of the membrane in response to adrenaline (0.5 to 1 μg/kg, intravenously) were altered by changes in the resting tension in a similar though less striking way to contractions produced by nerve stimulation, which showed that at least part of the effect was due to a change in the contractility of the smooth muscle rather than to improved neuro-effector transmission.

Effect of hexamethonium

In two experiments in which postganglionic stimulation was applied to preparations which had not been chronically decentralized, hexamethonium was injected intravenously in a total dose of 10 mg/kg. In one of these experiments, hexamethonium was without effect on the responses to postganglionic stimulation and in the other there was a barely perceptible reduction in the size of the main smooth muscle action potential complex. These results indicated that the number of aberrant ganglia scattered along the postganglionic trunk was too small to affect the responses significantly.

The same dose of hexamethonium, administered to a preparation in which preganglionic stimulation was applied, completely abolished the electrical and mechanical activity of the membrane (Fig. 3). The first effect of hexamethonium (2.5 mg/kg, intravenously) was to reduce and desynchronize the main smooth muscle potential (Fig. 3 row *b*). After a further similar injection, the configuration of the action potential was markedly changed (Fig. 3, row *c*), the initial negative deflexion being depressed and a second negative deflexion appearing 300 msec after the stimulus artifact. Contraction started after the delayed negative deflexion, that is about 400 msec after the stimulus artifact. Before hexamethonium, the latency of the contraction was 250 msec. A further 5 mg/kg of hexamethonium abolished almost completely the electrical and mechanical activity of the membrane in response to preganglionic stimulation (Fig. 3, row *d*). The responses then gradually recovered to normal over the next 30 to 40 min (Fig. 3, row *e*). The effect of small doses of

hexamethonium on the action potential was more striking than that on the contraction. Although its latency was prolonged, the tension of the contraction was only slightly depressed even when the action potential was markedly affected (Fig. 3, row *c*). Rosenblueth & Rioch (1933) showed that, if the stimulus frequency was increased, maximal contractions of the nictitating membrane to repetitive stimulation could still be obtained after partially sectioning the postganglionic nerve trunk. A similar mechanism might account for the effect of hexamethonium. A small dose will completely block transmission to some postganglionic fibres and delay transmission to others. The membrane will therefore receive a reduced and desynchronized volley which may resemble the conditions of the experiment of Rosenblueth & Rioch with the result that the contraction tension may not be much reduced.

The response of the membrane to adrenaline was not depressed by hexamethonium and in fact was slightly enhanced (Fig. 3), which confirms the observations of Mantegazza, Tyler & Zaimis (1958). The responses of the membrane to nerve stimulation were briefly restored towards normal after each injection of adrenaline. This effect, which is not shown in Fig. 3, may be explained by summation between injected adrenaline and released transmitter or by the ability of small doses of adrenaline to facilitate ganglionic transmission (Bülbring & Burn, 1942).

Effect of physostigmine and atropine

Preliminary experiments using physostigmine, atropine and intra-arterially injected acetylcholine were carried out to gain some idea of the range of effective doses of physostigmine and atropine. Acetylcholine, in an intra-arterial dose of 0.05 to 0.1 μg, produced a tension response of the same order as that produced by a single nerve shock or by a similar dose of adrenaline. The response to acetylcholine was completely abolished by atropine in doses of 10 μg intra-arterially or 100 μg/kg intravenously. After full atropinization (1 mg/kg, intravenously), intra-arterial doses of acetylcholine of the order of 5 to 10 μg produced a tension response similar to that produced by 0.05 to 0.1 μg given before atropine. The response to a large dose of acetylcholine in the presence of atropine was abolished by hexamethonium (2 mg/kg intravenously). Large doses of acetylcholine were effective in preparations in which the superior cervical ganglion had been removed at the start of the experiments. The size of the effect made it unlikely that it was due to stimulation of the few aberrant ganglia which may have been present. It was not analysed further, but was probably due to the sympathomimetic effect of acetylcholine, an action shared by nicotine (Thompson, 1958 ; Burn, Leach, Rand & Thompson, 1959) and which possibly arises from depolarization of sympathetic nerve terminals.

Physostigmine, in intravenous doses of 50 to 100 μg/kg or intra-arterial doses of 25 to 50 μg, increased and prolonged the responses of the membrane to acetylcholine both before and after atropine. The intra-arterial doses of physostigmine required were larger than might be expected, possibly because physostigmine combines relatively slowly with cholinesterase and, if the intra-arterial dose is small, insufficient combination will occur during the first passage through the tissue and the drug will then be diluted to sub-effective concentrations in the general circulation.

The intra-arterial route was chosen for most experiments in order to reduce the complications arising from an increased blood concentration of acetylcholine accumulating from all over the body.

The isometric recording conditions and the weak tension response of the membrane to a single shock (0.4 to 1 g) resulted in a number of difficulties when the action of physostigmine was studied. Firstly, any small skeletal muscle component, although unimportant in the absence of physostigmine, was potentiated after physostigmine to such a marked extent that it then began to interfere with the smooth muscle response. Results of experiments, in which skeletal muscle responses were present, became impossible to interpret and have been discarded. Secondly, the injection of physostigmine into the carotid artery frequently caused vigorous clonic convulsions. Occasionally, these were powerful enough to move the clamped head slightly, altering the resting tension on the muscle, and the results of these experiments too have been discarded. In most preparations, however, the resting tension on the membrane was not affected by the convulsions. The evoked contractions of the membrane could therefore be studied during the quiescent periods between the convulsions, and also after the convulsions had passed, for the effect of physostigmine on the smooth muscle usually outlasted the period of convulsions. The clonic convulsions produced by intra-arterial injection of physostigmine seemed to be central in origin and their intensity to be roughly inversely proportional to the depth of anaesthesia. They could be completely abolished by the injection of atropine (50 to 100 μg intra-arterially, or 0.5 mg/kg intravenously) and this difficulty was therefore not experienced with animals that had been previously atropinized.

In the first seventeen experiments on normal preparations using postganglionic stimulation, physostigmine was injected intra-arterially in doses ranging from 25 to 200 μg within 15 min of beginning stimulation at a frequency of 1 shock/min. In all experiments, the drug had either no effect on the amplitude of the contractions and action potentials or, in the larger doses, depressed them. These results therefore support those reported by Gardiner *et al.* (1962). However, in eight of these experiments, although the contraction was depressed in amplitude it was prolonged in duration (Fig. 4). In two of these eight experiments responses to adrenaline were

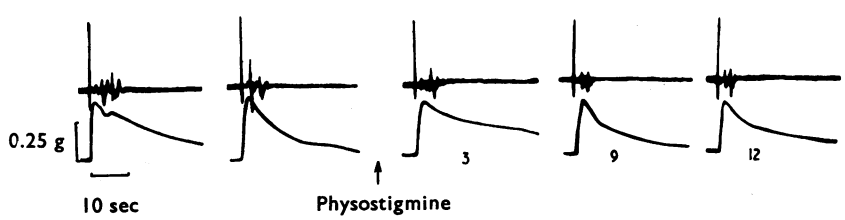

Fig. 4. Effect of physostigmine, recorded using a slow sweep speed, on the responses of the membrane to postganglionic stimulation with single supramaximal shocks once every minute. The first responses shown were the first of the experiment. The second were recorded 15 min later and immediately before injection of physostigmine (100 μg intra-arterially at the arrow). The numbers denote the times in minutes after physostigmine. Physostigmine depressed both responses and 3 min after injection the contraction was prolonged. Calibrations: tension, 0.25 g; time, 10 sec. Resting tension on membrane, 2 g.

also studied. They too were slightly depressed in height but were unaltered in duration.

Previous workers (Secker, 1937; Burn, Philpot & Trendelenburg, 1954) have reported that physostigmine may potentiate the response of the membrane to adrenaline. Out of a total of nine experiments in which responses to adrenaline were studied, slight potentiation (about 12%) was observed only twice and this was in the two experiments in which physostigmine (200 μg/kg) was injected intravenously. In one of these experiments, physostigmine (50 μg, previously injected intra-arterially) was without effect on the response to adrenaline. Fig. 9 illustrates the absence of potentiation of the response to adrenaline after intra-arterial injection of physostigmine.

In a subsequent experiment, physostigmine was not administered until 2.5 hr had elapsed since stimulation had commenced. The effect on the contractions of increasing and then decreasing the resting tension had been studied first in this experiment; a small degree of fatigue was evident and rhythmic activity was much reduced with supra-maximal stimulation. Physostigmine (100 μg, intra-arterially) then caused an increase in the contractions. In a further series of fifteen experiments physostigmine was not injected until a similar "fatiguing" process had been carried out. In all instances, maximal responses to nerve stimulation were still being produced. In nine of these fifteen experiments physostigmine, in doses of 100 to 150 μg intra-arterially, caused increases of 8 to 43% in the tension of the contractions. In the remaining six experiments, physostigmine was either without effect or, in large doses of 150 to 250 μg, it decreased the amplitude and prolonged the duration of the contractions. In all ten of the experiments in which physostigmine increased the amplitude of the contractions, the resting tension on the membrane was between 1 and 5 g, which was considerably below that which gave maximal responses to single stimuli, and rhythmical electrical activity had been present at the start of the experiment but was no longer so or was considerably reduced. However, these conditions were also fulfilled in three of the six fatigued preparations in which physostigmine did not increase the contractions.

In the ten experiments in which physostigmine increased the contractions in fatigued preparations, the onset of the effect occurred within 2 to 4 min, reached its peak in 4 to 8 min and lasted for 6 to 25 min. The potentiation was of two types and Figs. 5 and 6 illustrate the most pronounced effects obtained. In Fig. 5, an initial intra-arterial injection of physostigmine (40 μg, first arrow) restored the rhythmic electrical activity and slightly prolonged the contraction but did not increase the peak tension. A further dose of physostigmine (100 μg, second arrow) produced an additional slight increase in the rhythmical electrical activity and increased the height of the contractions. The amplitude of the initial main action potential complex remained unchanged. This effect of physostigmine occurred in six experiments and closely resembled the effect of increasing the resting tension, although there was no evidence that this had occurred. The second type of potentiation occurred in four experiments and one of these is illustrated in Fig. 6. Here the increase in tension was associated with an increase in the amplitude and a decrease in the width of the initial negative deflexion of the main smooth muscle action

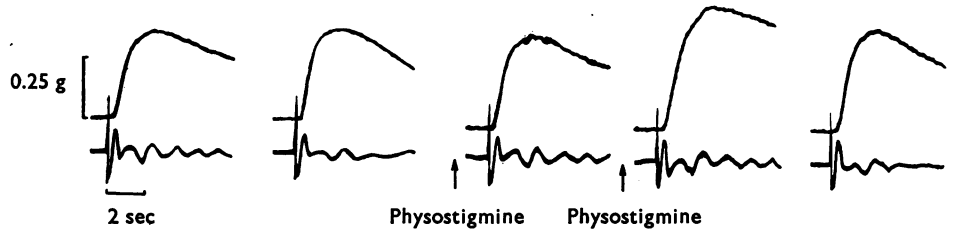

Fig. 5. Contractions (upper trace) and action potentials (lower trace) in response to stimulation of the postganglionic nerve with single supramaximal shocks once every minute. This preparation had been used previously to study the changes produced by altering the resting tension. The first response shown was that recorded after a rest from stimulation of 15 min. Rhythmic activity gradually diminished so that 12 min later (second response) it was much depressed. Rhythmic activity was restored by the first intra-arterial injection of physostigmine (40 μg, first arrow). A second injection of 100 μg (second arrow) increased the tension, the response shown being recorded 4 min after the second injection. The last record is that taken 20 min later. Calibrations: tension, 0.25 g; time, 2 sec. Resting tension on membrane, 4 g.

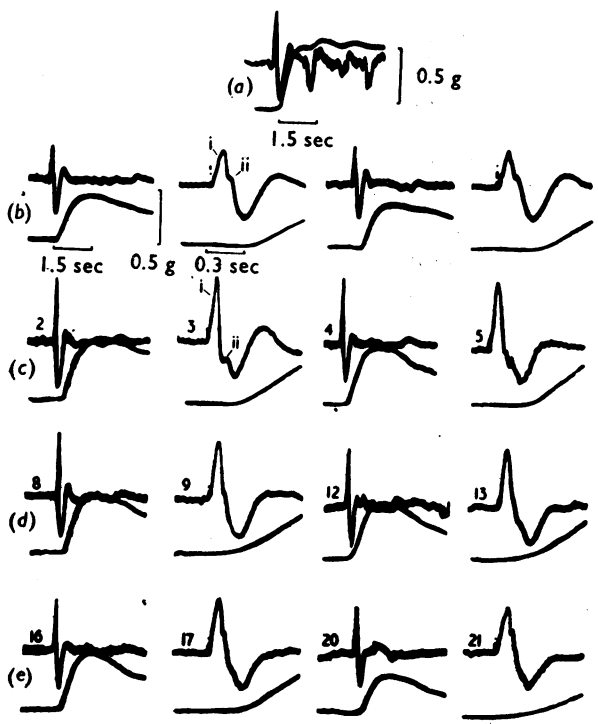

Fig. 6. Action potentials (upper trace) and contractions (lower trace) in response to supramaximal stimulation of the postganglionic nerve once every minute. (*a*) Response at the start of the experiment. Between (*a*) and (*b*) the preparation was used to study the effects of altering the resting tension. Between (*b*) and (*c*) physostigmine (100 μg) was injected intra-arterially. The numbers above the responses of rows (*c*), (*d*) and (*e*) denote the time in minutes after physostigmine. Note the waxing and waning of the increase in tension developed. The latency of the second negative component of the action potential (labelled ii) remained constant. The first component (labelled i) decreased in latency, increased in amplitude and decreased in duration after physostigmine. Calibrations: tension, 0.5 g; time, 1.5 sec for the slow sweep and 0.3 sec for the fast sweep. Resting tension applied to the membrane, 1.5 g.

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potential complex, suggesting that it was the result of improved synchronization of the smooth muscle fibre responses. The effect on the action potential was exerted entirely on the first negative component of the initial complex (labelled i in Fig. 6). The second negative component (labelled ii) was still evident but, because of the improved synchronization of the first component, it now appeared as a small interruption of the positive deflexion (Fig. 6, row *c*). This effect was similar to that recorded by Nystrom (1962) from the lateral smooth muscle stimulated preganglionically with submaximal single shocks.

In the chronically decentralized preparations, large doses of physostigmine usually caused a slowly developing increase in the background tone of the membrane upon which the evoked responses were superimposed (Figs. 8 and 11). This effect, which did not occur in the other experiments, lasted about 15 min after injection ; it was not an artifact due to movement of the head increasing the resting tension since it was gradual and smooth in its development and occurred in the absence of apparent head movements.

In three of the five animals in which the ganglion had been chronically denervated, a similar procedure to that already described for fatiguing the normal preparation was carried out before injecting physostigmine. However, in the decentralized preparations this procedure was inadequate to produce fatigue and rhythmic electrical activity remained evident. Physostigmine, in doses up to 250 μg intra-arterially, was without effect on the evoked responses in two experiments. In the third, a very large dose of physostigmine (0.5 mg, intra-arterially) was injected and this dose prolonged the contractions but did not increase their amplitude (Fig. 7). In another decentralized preparation fatigue of the responses was produced by stimulation at a frequency of 20 shocks/sec for 2 min. Subsequent single responses were depressed and rhythmic activity was abolished. Physostigmine, in a dose of 100 μg intra-arterially (P in Fig. 8), caused a very small and short lasting increase in the contractions superimposed on the increased tone of the membrane.

The absence of any definite increase in the amplitude of the contractions after physostigmine in the decentralized preparations suggested that the effect occasionally

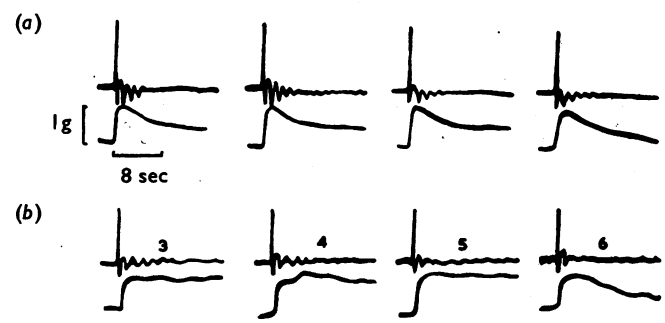

Fig. 7. Responses to postganglionic stimulation with single shocks delivered once every minute. The ganglion had been denervated 10 days earlier. Between (*a*) and (*b*), 0.5 mg of physostigmine was injected intra-arterially. The numbers in (*b*) denote the time in min after physostigmine. This preparation had been previously used to study the effect of alterations in the resting tension. Calibrations: tension, 1 g; time, 8 sec. Resting tension on membrane, 2 g.

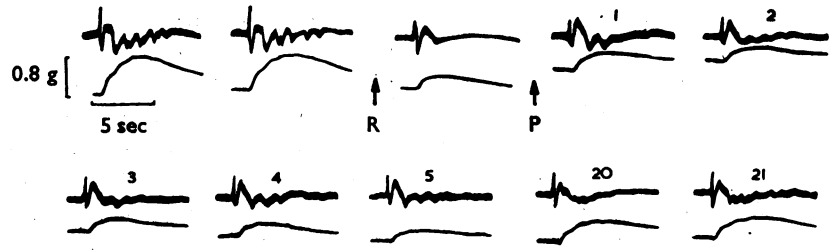

Fig. 8. Records as in Fig. 7, but fresh preparation. The ganglion had been denervated 11 days previously. The first two responses show controls at the start of the experiment. At R, repetitive stimulation (20 shocks/sec for 2 min) was applied to the postganglionic nerve and the subsequent responses to single supramaximal stimuli to the nerve were depressed and non-rhythmic. At P, physostigmine (150 μg) was injected intra-arterially and the numbers above the remaining records denote the time in minutes after injection of physostigmine. One minute after physostigmine, the tone of the membrane was increased and the amplitude of the contractions was slightly greater; 5 min later the effect of physostigmine had disappeared; 20 min later the responses were beginning to recover from the fatiguing effects of the repetitive stimulation. Calibrations: tension, 0.8 g; time, 5 sec. Resting tension, 3.5 g.

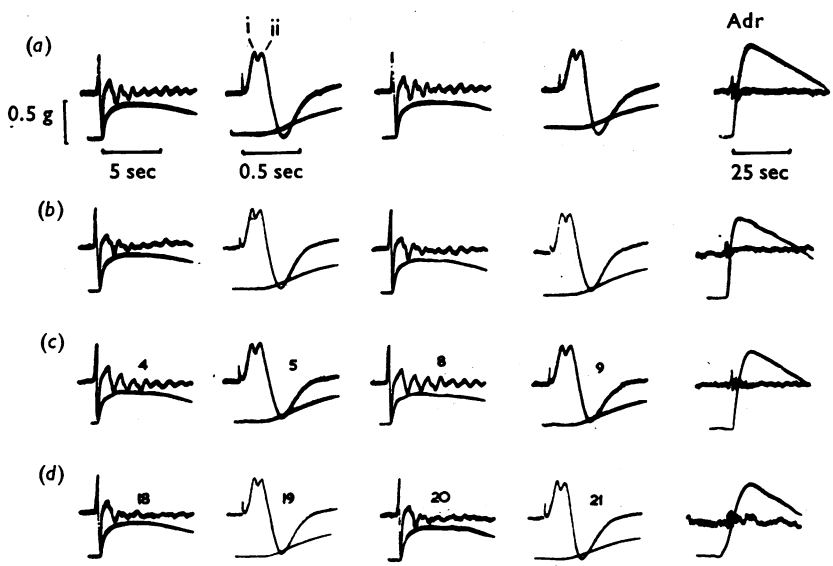

Fig. 9. Responses to preganglionic stimulation at a frequency of 1 shock/min recorded with alternate slow and fast sweeps, as in Fig. 1. Responses to adrenaline (1 μg/kg, intravenously, Adr) are shown on the extreme right hand side. (*a*) Responses at start of experiment; 1.5 hr elapsed between (*a*) and (*b*) and during this time the preparation was stimulated at 1 shock/min and the membrane was periodically stretched, rhythmic activity being depressed by this treatment; between (*b*) and (*c*) physostigmine (25 μg) was injected intra-arterially and rhythmic activity was temporarily restored (*c*). The numbers denote the time in minutes after physostigmine. The responses to adrenaline were not affected by physostigmine. Calibrations: tension, 0.5 g; time, 5 sec for the slow sweep, 0.5 sec for the fast sweep and 25 sec for the responses to adrenaline. Resting tension, 4 g.

observed in the normal preparations might have resulted from facilitation of transmission through aberrant ganglia, possibly scattered in greater numbers than usual along the postganglionic nerve trunk. If this were so, potentiation by physostigmine should be very pronounced when all the synapses are left intact, and for this reason five further experiments were carried out under similar conditions except that responses were evoked by preganglionic stimulation. In one of these experiments (Fig. 9) physostigmine (25 μg, intra-arterially) restored the rhythmic electrical activity of the membrane but the effect was not sufficiently marked to affect the contractions in any way. The initial main action potential complex of the membrane was completely unaffected by physostigmine. In the same experiment, a further dose of 100 μg of physostigmine slightly depressed and prolonged the contractions. In the second experiment, 100 μg of physostigmine increased the rhythmic activity and the amplitude of the contraction by 15%. In the third experiment, physostigmine (100 μg) depressed the contractions ; in the fourth the same dose was without effect ; in the fifth an increase in tension of 70% occurred 2 min after physostigmine (100 μg), but recording could not be continued as violent clonic convulsions displaced the electrodes. On the whole, the effects of physostigmine during preganglionic stimulation were little more striking than those recorded when postganglionic stimulation was used. These results confirm the findings of others (see Zaimis, 1963) that the effects of anticholinesterases on ganglionic transmission are difficult to demonstrate when contractions of the nictitating membrane are used as an index.

Atropine, in large doses of 100 to 200 μg intra-arterially or 1 to 2 mg/kg intravenously, depressed both the mechanical and the electrical responses of the membrane to single supramaximal stimuli applied to the nerve (Figs. 10 and 11).

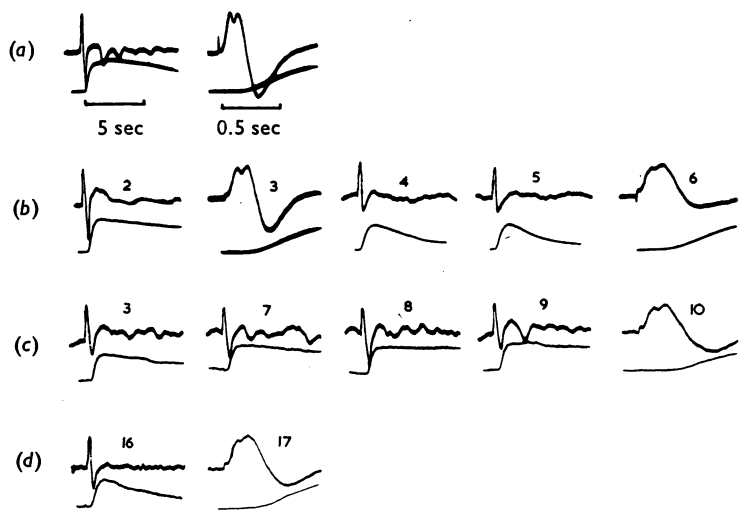

Fig. 10. Continuation of experiment illustrated in Fig. 9. (*a*) was recorded 30 min after the last record of Fig. 9. Between (*a*) and (*b*) atropine (1 mg/kg) was injected intravenously. The numbers in (*b*) denote the time in min after atropine. Between (*b*) and (*c*) physostigmine (100 μg) was injected intra-arterially. Rhythmic activity was temporarily restored by physostigmine and the duration of the contractions was increased. The numbers in (*c*) and (*d*) denote the time in minutes after physostigmine. Calibrations as in Fig. 9.

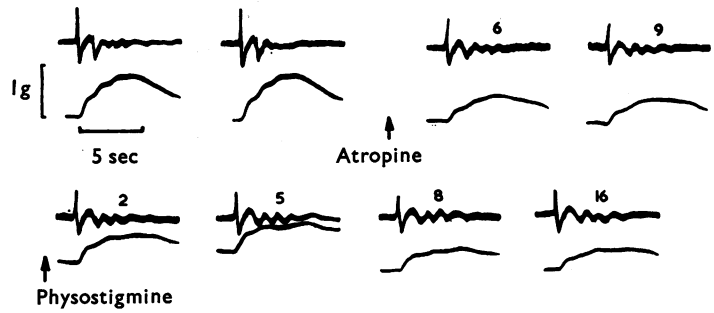

Fig. 11. Chronically decentralized preparation, ganglion denervated 10 days previously. Recordings as in Fig. 7. At the first arrow, atropine (150 μg) was injected intra-arterially and this depressed the responses. Responses 6 and 9 min after atropine are illustrated. At the second arrow physostigmine (100 μg) was injected intra-arterially and responses 2, 5, 8 and 16 min later are illustrated; 5 min after physostigmine the tone of the membrane was increased and the evoked contraction was slightly prolonged in duration. Calibrations: tension, 1 g; time, 5 sec.

Occasionally, even larger doses were required. The smallest effective doses often selectively depressed the rhythmic electrical activity and shortened the duration of the contraction without affecting its amplitude. These effects occurred during pre- or postganglionic stimulation, and in the chronically decentralized preparation. The responses of the membrane to adrenaline (1 μg/kg, intravenously) were also slightly depressed by these doses of atropine, confirming the results of Cervoni, West & Fink (1956), Thompson (1958) and Mirkin & Cervoni (1962).

The depressant effect of atropine on the initial main action potential complex was exerted on both of the negative deflexions (Fig. 10*b*). As well as being depressed, the initial complex was usually prolonged in duration and the latency of the contraction was increased. We obtained no convincing evidence for selective depression by atropine of the first of the two negative components of the initial main action potential, an effect previously described by Nystrom (1962) for the lateral smooth muscle. Since the doses of atropine required to affect the membrane were large and also depressed contractions produced by adrenaline, our results obtained with this drug cannot be taken as evidence for a cholinergic innervation of the membrane.

Atropine was injected in nine experiments and physostigmine (50 to 200 μg), subsequently administered intra-arterially, did not increase the height of the contractions in any of them. However, the duration of the contraction was temporarily prolonged in six of the experiments by the larger doses of physostigmine and this effect was associated with some restoration of rhythmic electrical activity. Fig. 10 illustrates this effect during preganglionic stimulation and Fig. 11 during postganglionic stimulation of the chronically decentralized preparation.

Intravenously injected physostigmine did not potentiate the responses to adrenaline in the two experiments in which adrenaline was administered to atropinized cats. This result confirms that reported previously by Burn *et al.* (1954).

The most consistent effect of physostigmine which we observed was an increase in the duration of the contraction rather than an increase in its height. This effect

was obtained in the normal preparation stimulated pre- or postganglionically, in the chronically decentralized preparation and in the preparation previously injected with atropine. It occurred in thirty-one out of a total of forty-five experiments in which the action of large doses of physostigmine was studied. That this effect can result in an increase in the tension of incomplete tetanic contractions is illustrated in Fig. 12. In this experiment nine shocks at a frequency of 1 every 3.2 sec were

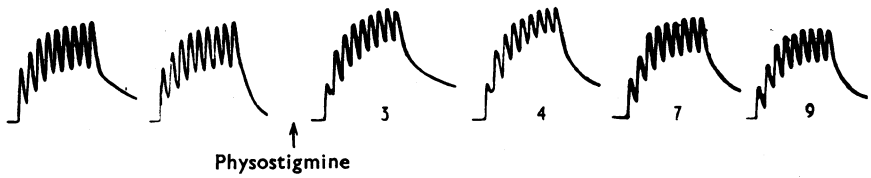

Fig. 12. Isometric contractions were elicited once per minute by stimulation of the postganglionic nerve with nine shocks delivered at a frequency of 1 every 3.2 sec. At the arrow, physostigmine (100 μg) was injected intra-arterially. Responses 3, 4, 7 and 9 min later are shown. Although the units of contraction were decreased, physostigmine temporarily increased the tension of the clonic contractions because of the increase in fusion which occurred.

delivered to the postganglionic trunk every 2 min. Physostigmine depressed the tension of the single response, as can be seen by comparing the heights of the first units of contraction before and after injection. However, the overall tension of the clonic contractions was slightly increased by physostigmine because of the increase in fusion which resulted from the prolongation of the individual responses.

DISCUSSION

When the responses of the preparation were impaired to a small extent by previous prolonged activity, physostigmine sometimes increased the contractions of the nictitating membrane elicited by single shocks applied to the postganglionic cervical sympathetic nerve. Since potentiation of the responses to preganglionic stimulation differed little from that recorded when postganglionic stimulation was used, it does not seem likely that the latter effect was due to facilitation of transmission through aberrant ganglia scattered along the postganglionic trunk. However, this possibility cannot be entirely excluded in view of the fact that no significant increase in contractions was produced when the ganglion had been chronically denervated so that any contribution by "ganglionic" acetylcholine was ruled out. This possibility also applies to the results of Nystrom (1962) who used mainly preganglionic stimulation and who recorded changes in the electrical response after physostigmine which were similar to those obtained in a few of the present experiments. He applied physostigmine directly to the smooth muscle of the membrane but this route does not exclude a ganglionic action, since the doses he used were large (1 mg) and Gardiner *et al.* (1962) have shown that much smaller amounts applied to the membrane are absorbed into the general circulation to exert effects elsewhere.

Another possibility which cannot be overlooked is that the increase in contractions was due to summation between subeffective amounts of acetylcholine, accumulating

from other sources in the presence of physostigmine, and noradrenaline released from the sympathetic nerve. Unpublished observations show that such summation between the action of adrenaline and the muscarinic action of acetylcholine can occur. When a dose of acetylcholine (0.025 μg), too small to be effective by itself, was mixed with adrenaline (0.1 μg), the resulting contraction on intra-arterial injection was greater than that produced by adrenaline itself. After a small dose of atropine (10 μg) the response to the mixture resembled that to adrenaline alone. It is possible that the potentiation of adrenaline contractions by physostigmine which we recorded was largely due to summation of this type, since it occurred only when large doses of physostigmine were injected intravenously and, as reported also by Burn *et al.* (1954), it did not occur in the presence of atropine. However, this may not be the entire explanation since, according to Cervoni *et al.* (1956), physostigmine also potentiates the response of the isolated membrane to adrenaline.

On no occasion was the effect of physostigmine on contractions elicited by nerve stimulation as great as that recorded by Burn *et al.* (1963) who used isotonic recording and repetitive stimulation. These authors regularly obtained striking potentiations after physostigmine even in the presence of atropine and when the ganglion had been chronically denervated. Under these conditions, but with isometric recording and single shock stimulation, we found no material increase. It is possible that the explanation of this discrepancy lies in the different methods for recording and stimulating, although Gardiner *et al.* (1962), who used methods similar to those of Burn *et al.* (1963), were unable to obtain potentiation by physostigmine. Burn *et al.* (1963), however, used large doses of physostigmine injected intravenously (0.5 mg/kg) or added to the fluid perfusing the head (10^{-6} g/ml.), while Gardiner *et al.* (1962) used much smaller doses injected intravenously (30 μg) and both lesser (5 μg) and larger doses (100 μg to 1 mg) applied directly to the membrane.

The most consistent effect of physostigmine, obtained with large doses under all conditions in our experiments, was a decrease in the contraction height associated with a prolongation of its time course, and this could often be seen to be accompanied by a restoration of rhythmical activity. It was also demonstrated that this effect on the single contraction could result in an increased tension of clonic contractions because of the increase in fusion which resulted. It would be expected that the lower the frequency of stimulation producing the clonic contraction, the greater would be the potentiation, and it is therefore possible than an increase in the duration of the unit contraction contributed to the potentiation observed by Burn *et al.* (1963). The higher frequencies of stimulation used by these authors, compared with those in our experiments, would have resulted in completely fused contractions under isometric conditions. However, as isotonic contractions are faster than isometric contractions, it is still possible that fusion may have been incomplete at least at the lower frequencies (0.5 to 2 shocks/sec) used by Burn *et al.* (1963).

Our results throw little light on the mechanism of the physostigmine effect especially as the origin of the rhythmic potentials is not known. In the absence of micro-electrode recordings from single cells, it cannot be stated with certainty whether the rhythmic responses reflect repetitive firing of the same smooth muscle fibres or activation of different fibres as conduction spreads through the tissue.

The following possible explanations of the effect of phygsostigmine may be considered.

1. The effect might be explained on the hypothesis of Burn & Rand (1959) that sympathetic nerve endings release acetylcholine which, acting by a "nicotinic" mechanism, subsequently causes the release of noradrenaline. Preservation of the acetylcholine by cholinesterase inhibition might then prolong the release of the transmitter and result in a lengthened tension response. The decreased amplitude of the contraction might be an independent effect, probably exerted on the smooth muscle fibres since the response of the membrane to adrenaline was also decreased.

2. It has been suggested by Cuthbert (1963) that, in some smooth muscles, excitation spreads from cell to cell by a cholinergic mechanism. It is possible that in the nictitating membrane only "trigger cells" are innervated. After their excitation by noradrenaline released from the nerve endings, the trigger cells would subsequently excite adjacent cells possibly by releasing acetylcholine upon them. The rhythmic potential waves may reflect the spread of excitation through the tissue by this mechanism which would explain the findings that relatively small doses of atropine sometimes selectively depressed rhythmicity and that physostigmine restored it. The response of the membrane to injected adrenaline was not prolonged by physostigmine, but here the tension response is already repetitive (Eccles & Magladery, 1937b).

3. When a twitch of a skeletal muscle is elicited by synchronous stimulation of all of its motor nerve fibres, the summed muscle action currents back excite the motor nerve. The back response in the nerve propagates in both directions from the site of its initiation and re-excites some of the muscle fibres which therefore fire repetitively in response to a single nerve shock (Brown & Matthews, 1960). The action potential of smooth muscle is much longer lasting than that of skeletal muscle and it is possible that the prolonged rhythmic potential waves in the nictitating membrane are the result of similar reverberating activity across the junction. Reverberating activity across the neuromuscular junction in skeletal muscle is enhanced in the presence of anticholinesterase drugs (Eccles, Katz & Kuffler, 1942). The mechanism of this enhancement is not fully understood (see Blaber & Bowman, 1963a, b) but it is possible that a similar effect of physostigmine at the nerve muscle junction in the nictitating membrane accounts for the accentuated rhythmic potential waves. The effect may not depend on preservation of acetylcholine.

4. The doses of physostigmine required to affect the contraction of the membrane were large, being several times greater than those necessary to potentiate responses to injected acetylcholine. This is the reverse of the situation at the neuromuscular junction in skeletal muscle which is known to be cholinergic. At this site, twitches elicited by nerve stimulation are potentiated by concentrations of anticholinesterase drugs smaller than those necessary to increase contractions produced by injected acetylcholine (Blaber, 1963). The large size of the effective doses may indicate that the effects of physostigmine recorded under the conditions of our experiments were independent of acetylcholine effects. Several workers have shown that anticholinesterase drugs may inhibit the active transport of ions across various cell

membranes (Rothenberg, 1950 ; Kirschner, 1953 ; Koch, 1954 ; Van der Kloot, 1956, 1958) and an action of this type might result in changes in excitability or contractility of the nictitating membrane.

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