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AN ELECTROPHYSIOLOGICAL STUDY OF THE EFFECTS OF ATROPINE AND PHYSOSTIGMINE ON TRANSMISSION TO THE GUINEA-PIG VAS DEFERENS

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SUMMARY

1. The effects of physostigmine and atropine on transmission to the longitudinal musculature of *in vitro* preparations of the guinea-pig vas deferens have been examined using intracellular micro-electrodes.

2. Atropine (5×10^{-7} to 10^{-6} g/ml.) increased the rate of decay of excitatory junction potentials (EJPs) in response to post-ganglionic stimulation.

3. Physostigmine (5×10^{-6} g/ml.) reduced the mean resting potential of the muscle cells from $-60{\cdot}5$ to $-51{\cdot}5$ mV and lowered the voltage of post-ganglionic stimulation necessary for initiation of an action potential in the muscle. In some but not all of the cells studied the time course of the EJP was markedly prolonged.

4. At concentrations which did not alter the response to post-ganglionic stimulation (5×10^{-7} to 10^{-6} g/ml.), physostigmine caused fully facilitated EJPs to appear with the first pulse of a preganglionic train of stimulation.

5. Atropine antagonized all the above effects of physostigmine.

6. Physostigmine (5×10^{-7} to 10^{-6} g/ml.) also lowered the voltage of preganglionic stimulation necessary for initiation of an action potential in the muscle. This effect was not antagonized by atropine.

7. The results are interpreted as being evidence for the existence of separate cholinergic and adrenergic motor fibres to the musculature of the guinea-pig vas deferens.

INTRODUCTION

The vas deferens appears to be innervated solely by fibres of sympathetic origin running in the hypogastric nerve (Gruber, 1933). The tissue of the vas deferens of the guinea-pig contains high levels of noradrenaline (Sjöstrand, 1962) which is localized in nerve fibres (Falck, 1962; Sjöstrand, 1965). The contractile response to hypogastric nerve stimulation is re-

duced by adrenergic blocking agents and reserpinization, and potentiated by inhibitors of mono-amine oxidase and catechol-*o*-methyl transferase (Huković, 1961; Bentley, 1962; Bentley & Sabine, 1963; Birmingham & Wilson, 1963; Bhargava, Kar & Parmar, 1965 and others). The excitatory junction potentials recorded in single muscle cells in response to hypogastric stimulation are also reduced by adrenergic blocking agents and by reserpinization (Burnstock & Holman, 1962; Kuriyama, 1963; Burnstock & Holman, 1964). These results support the view that the motor innervation of the musculature is largely by noradrenergic fibres.

The vas deferens also contains choline acetylase (Ohlin & Strömblad, 1963) and acetylcholinesterase which is associated with nerve fibres (Jacobowitz & Koelle, 1965). Furthermore, anticholinesterases and cholinergic blocking agents alter the mechanical responses to nerve stimulation (Boyd, Chang & Rand, 1960; Chang & Rand, 1960; Bentley, 1962; Burn & Weetman, 1963; Della Bella, Benelli & Gandini, 1964; and others). This has been interpreted variously as evidence for the participation of a cholinergic mechanism in adrenergic transmission (Chang & Rand, 1960; Burn & Weetman, 1963; Burn & Rand, 1965) and as evidence for the presence of independent cholinergic and adrenergic fibres (Birmingham & Wilson, 1963; Della Bella *et al.* 1964; Bhargava *et al.* 1965).

The majority of the fibres in the hypogastric nerve synapse at ganglia situated about 1 cm from the entry of the nerve into the vas deferens (Ferry, 1963; Sjöstrand, 1965; Kuriyama, 1963), and undoubtedly the potentiation of the mechanical response to hypogastric stimulation by physostigmine is partly due to an anticholinesterase action at the ganglionic synapse. However, anticholinesterases and cholinergic blocking agents also modify the response of the muscle to post-ganglionic stimulation (Birmingham & Wilson, 1963; Sjöstrand, 1965).

In the present paper, an examination has been made of the effects of atropine and physostigmine on transmission to single muscle cells in the longitudinal layer of the vas deferens.

METHODS

The vas deferens of the guinea-pig was isolated together with the hypogastric nerve and pinned down on a Perspex block in a 10 ml. bath. Modified Krebs solution (Huković, 1961), aerated with 95 % oxygen and 5 % carbon dioxide and maintained at 32° C was run through the bath at a rate of approximately 2 ml./min.

The drugs used were atropine and physostigmine (eserine) sulphates. These were added to the stock bottle of Krebs, and concentrations cited refer to the final concentration of salt in the bath.

Platinum ring electrodes embedded in Araldite were placed on the hypogastric nerve approximately either 2 cm or 2 mm from its junction with the vas deferens, for pre- and post-ganglionic stimulation. Square wave pulses of 200 μsec duration were applied with a Grass S4 stimulator at a frequency of 1 pulse/sec. The stimulation voltage was adjusted

so as to be just below the threshold strength for initiating a contraction of the muscle. Intracellular electrical events in the longitudinal musculature were recorded with capillary micro-electrodes filled with 2 M-KCl and having a tip resistance of 20-80 MΩ. The criterion used for successful impalement of a cell was an action potential with an overshoot of at least 10 mV.

The statistical significance of differences was tested using Student's *t*-test (Fisher, 1936) on the assumption that the difference was not significant.

RESULTS

Under the conditions of the present investigation resting membrane potentials of 50–80 mV (mean 60·5, S.E. ± 1·1, $n = 38$) were recorded from the smooth muscle cells of the vas deferens. Repetitive stimulation of the hypogastric nerve either pre- or post-ganglionically at 1 pulse/sec produced excitatory junction potentials (EJPs) (Burnstock & Holman, 1961) which showed facilitation over the first 4–10 pulses to reach a stable amplitude (Fig. 1*a*). Raising the voltage of nerve stimulation caused an increase in the amplitude of the EJPs elicited (Fig. 1*b*). At a critical voltage of stimulation which varied between 3 and 5 V in different preparations, stimulation at 1 pulse/sec produced EJPSs of sufficient amplitude to initiate an action potential (Fig. 1*c*). This caused contraction of the tissue and usually dislodged the micro-electrode from the recording cell. Under control conditions, the voltage of nerve stimulation necessary for initiation of a muscle action potential was stable in any one preparation. An action potential was only occasionally initiated unless the voltage of stimulation was sufficient to produce EJPs of at least 10 mV amplitude, and in these cases it appeared to be due to spontaneous contraction of the tissue. No summation of successive EJPs was produced by stimulation at 1 pulse/sec (Fig. 1*a*, *b*, *c*).

Effects of atropine on the response to post-ganglionic stimulation. In the presence of atropine (5×10^{-7} to 10^{-6} g/ml.) the duration of the falling phase of the EJP was reduced. The results obtained have been plotted in Fig. 2. The degree of shortening was found to be significant ($P < 0{\cdot}001$) for each point of the falling phase plotted. For practical reasons the measurement of the time course of the EJP in the presence of atropine was usually made in different cells from those in which control measurements were made. It was therefore not possible to determine whether the extent of the effect of atropine varied between cells. Atropine did not appear to have any effects on the mean resting membrane potential of the muscle cells or on the facilitatory process. No gross change in the amplitude of the fully facilitated EJP was observed. However, because measurements before and in the presence of atropine were usually recorded from different cells, any minor change of amplitude within an individual cell would not have been detected. Because of the variation of the maximal EJP amplitude

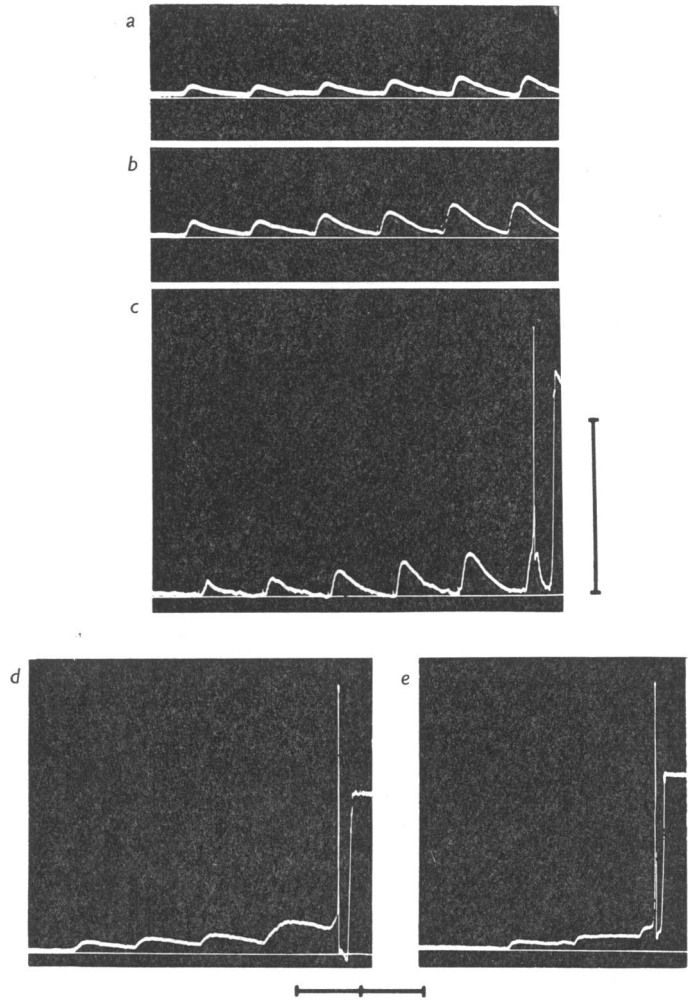

Fig. 1. (*a*, *b*, *c*) EJPs produced in a single cell by post-ganglionic stimulation of the hypogastric nerve at 1 pulse/sec, with increasing voltage (*a*) 2·5 V, (*b*) 4·0 V, (*c*) 4·5 V. Note increase in maximum amplitude of EJPs obtained with increasing voltage of stimulation, and lack of summation of successive EJPs. Membrane potential: 63 mV. (*d*, *e*) Records taken from the same preparation in the presence of physostigmine 5×10^{-6} g/ml. Post-ganglionic stimulation of the hypogastric nerve at 1 pulse/sec and 0·1 V. Note the prolongation of the falling phase of the EJPs, producing summation, and the small amplitude of the EJPs at a voltage of stimulation necessary for initiation of an action potential (cf. Fig. 1*c*). Membrane potentials: (*d*) 45 mV, (*e*) 48 mV. Calibration: 50 mV and 1 sec. The action potentials have been retouched.

obtainable in different cells, no estimate of change in amplitude after atropine could be made by averaging the amplitudes recorded from control and treated cells.

Effects of physostigmine and atropine on the response to post-ganglionic stimulation. Physostigmine at a concentration of 5×10^{-7} to 10^{-6} g/ml. had no apparent effect on the resting membrane potential of the muscle cells or on the response to post-ganglionic stimulation. In the presence of a

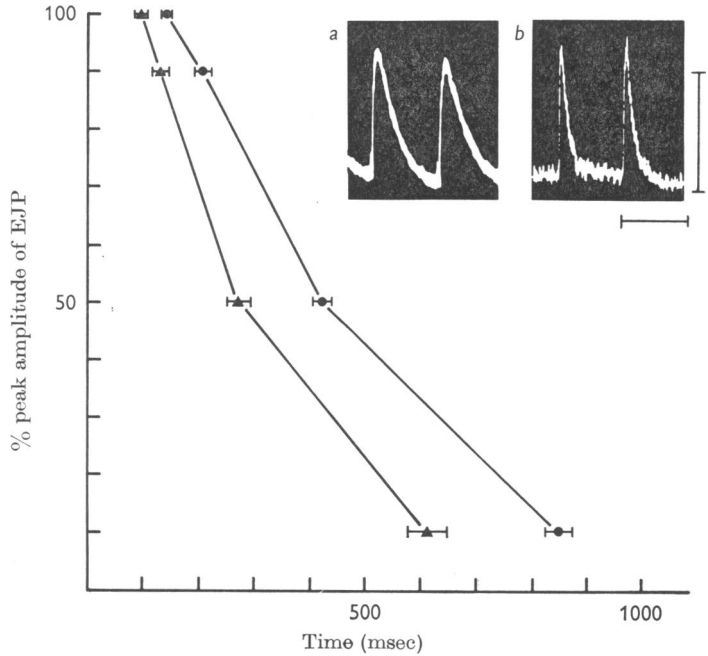

Fig. 2. Time course of decay of the fully facilitated EJP during post-ganglionic stimulation of the hypogastric nerve at 1 pulse/sec. Ordinate: % peak amplitude of EJP. Abscissa: msec after commencement of EJP. (●) control-average of twenty-two cells. (▲) atropine 10^{-6} g/ml.-average of seventeen cells. Limits = S.E. of the mean. Inset: (*a*) control EJP, (*b*) EJP in presence of atropine 10^{-6} g/ml. Calibrations: 10 mV and 1 sec.

higher concentration of physostigmine (5×10^{-6} g/ml.) the resting membrane potentials of the muscle cells were significantly ($P < 0{\cdot}001$) lower than under normal conditions, and were in the range 40–68 mV (mean 51·5, S.E. $\pm$ 1·1, $n = 30$). The voltage of nerve stimulation necessary for initiation of an action potential was lowered markedly, and the amplitude of the EJPs in response to stimulation at voltages just below this was considerably less than normal. On raising the voltage the amplitude of the EJPs was increased but usually an action potential was initiated before the pattern of successive EJPs could be observed.

In eight of the thirty cells impaled the duration of the falling phase of the EJP was lengthened, and stimulation at 1 pulse/sec caused marked summation of successive EJPs, as shown in Fig. 1 (*d*, *e*). In the remainder of the impaled cells no summation was apparent (Fig. 3*a*). However, the maximum amplitude of the EJPs observed in these cells was frequently less than 2 mV, and accurate determination of the duration of the falling phase was not usually possible. In these latter cells stimulation at 1 pulse/sec resulted in the firing of an action potential which frequently did not originate from the rising phase of the EJP and was probably propagated from an area in which summation occurred.

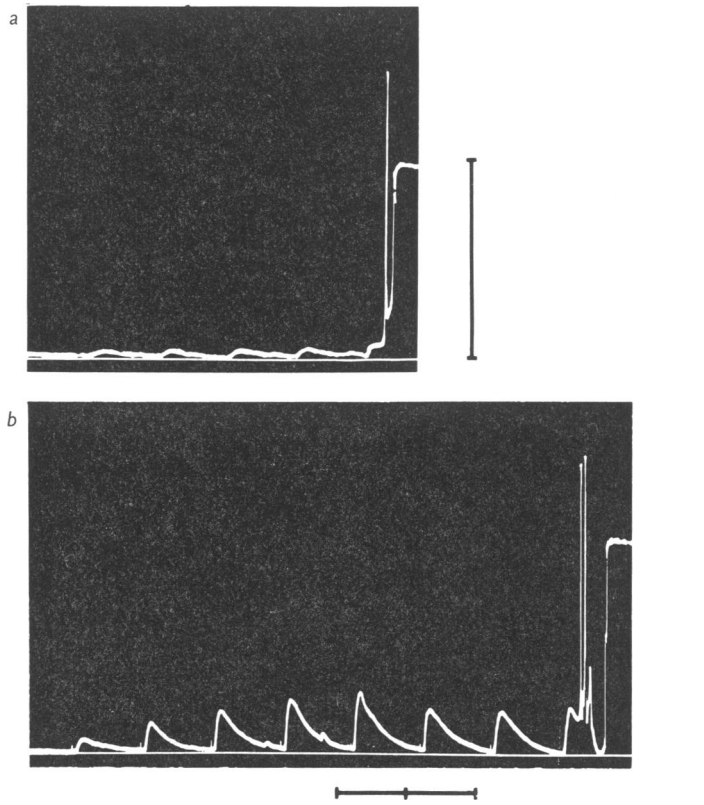

Fig. 3. Records taken from the same preparation as Fig. 1. Post-ganglionic stimulation of the hypogastric nerve at 1 pulse/sec. (*a*) In the presence of physostigmine 5×10^{-6} g/ml. Stimulation voltage 0·1 V. In comparison to Fig. 1 (*d*, *e*), the EJPs in this cell did not appear to summate. Membrane potential: 48 mV. (*b*) After addition of atropine 10^{-6} g/ml. in the presence of physostigmine 5×10^{-6} g/ml. Stimulation voltage 1·0 V. Note the increase in amplitude of EJPs obtained at a voltage of stimulation necessary for initiation of an action potential. Membrane potential: 53 mV. Calibrations: 50 mV and 1 sec. The action potentials have been retouched.

The action potentials initiated in the presence of physostigmine did not differ in amplitude from those recorded in control cells, although they arose from a depolarized resting potential. Figure 1 shows examples of 65 mV action potentials fired from (*c*) a control cell and (*d*) in the presence of physostigmine. The membrane potential of the control cell is 63 mV, that of the treated cell 45 mV.

Application of atropine (10^{-6} to 10^{-5} g/ml.) partially antagonized the effects of physostigmine (5×10^{-6} g/ml.) to an extent dependent on the concentration used. The voltage of nerve stimulation necessary for initiation of an action potential was increased, although never restored completely to the control value. In comparison to the situation in the presence of physostigmine alone, after addition of atropine it was possible to record a prolonged train of EJPs in response to nerve stimulation without firing of an action potential (Fig. 3*b*). A small degree of summation in response to stimulation at 1 pulse/sec was occasionally observed in the presence of 10^{-6} g atropine/ml., but never in the presence of 10^{-5} g atropine/ml. The physostigmine-induced reduction of the resting membrane potential was slightly raised in the presence of 10^{-6} g atropine/ml. (mean 54·5 mV, S.E. $\pm$ 1·1, $n = 30$), but this increase was not significant at the $P = 0{\cdot}05$ level. In the presence of 10^{-5} g atropine/ml. a greater increase in resting potential was produced (mean 56·8 mV, S.E. $\pm$ 0·76, $n = 26$). The maximum amplitude of the EJPs in response to nerve stimulation at voltages necessary for initiation of an action potential was consistently increased in the presence of either 10^{-6} or 10^{-5} g atropine/ml. (Fig. 3*b*).

Effects of physostigmine and atropine on the response to preganglionic stimulation. As reported above 5×10^{-7} to 10^{-6} g physostigmine/ml. produced no effects on the resting membrane potential of the muscle cells or on the response to post-ganglionic stimulation. In the presence of these concentrations of physostigmine, however, the voltage of preganglionic stimulation necessary for firing of an action potential was somewhat lowered. In eleven of sixteen cells impaled a marked change in the pattern of facilitation of EJPs in response to preganglionic stimulation at 1 pulse/sec was observed. Successive EJPs were irregular in size, and in general the amplitude of the EJP in response to the first pulse of a train was as large as those in response to later pulses (Fig. 4*b*, *c*). In the remainder of the cells impaled this phenomenon was not observed, and the facilitatory process was normal.

Atropine (5×10^{-7} to 10^{-6} g/ml.) had little effect in restoring the threshold voltage for firing an action potential towards the control value. On the other hand the normal pattern of facilitation was seen in all cells impaled following addition of atropine (Fig. 4*d*).

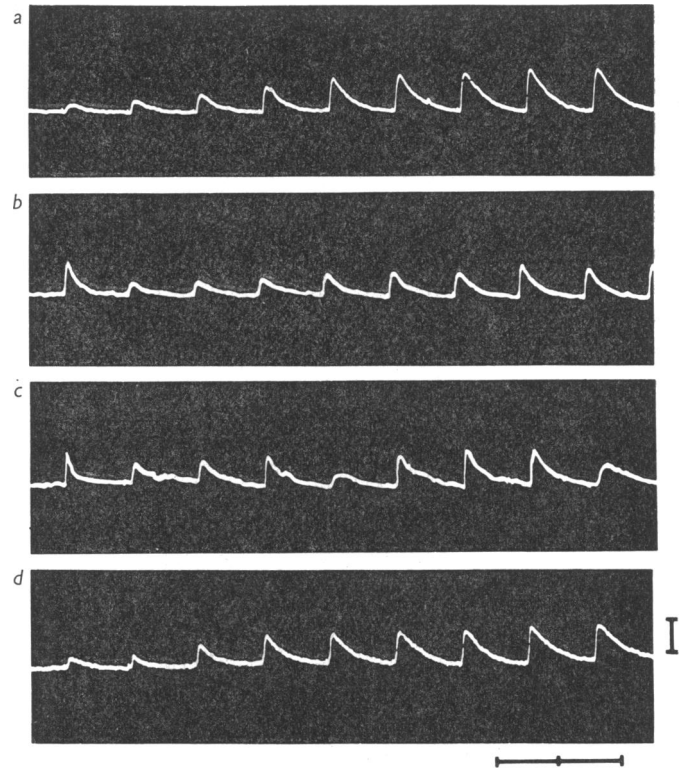

Fig. 4. EJPs in response to preganglionic stimulation of the hypogastric nerve at 1 pulse/sec: (*a*) control, stimulation voltage 4·0 V, membrane potential, 58 mV: (*b*), (*c*) in the presence of physostigmine 5×10^{-7} g/ml. stimulation voltage 1·0 V, membrane potential, (*b*) 57 mV, (*c*) 60 mV; (*d*) after addition of atropine 5×10^{-7} g/ml. in the presence of physostigmine 5×10^{-7} g/ml. stimulation voltage 1·2 V, membrane potential 59 mV. Note the irregularity and altered facilitatory pattern of the EJPs in the presence of physostigmine and the restoration of facilitation with atropine. Calibrations: 10 mV and 1 sec.

DISCUSSION

Physostigmine caused a marked increase in the duration of the falling phase of the EJP in some cells of the longitudinal musculature of the vas deferens, resulting in summation of successive depolarizations at a frequency of stimulation which normally did not produce summation. It has been suggested that two factors may be important in controlling the time course of the EJP (Bennett & Merrillees, 1966; Burnstock & Holman, 1966). These are the time course of transmitter release and the duration of post-junctional interaction between transmitter and receptor. The Burn–Rand theory (Burn & Rand, 1959, 1962) proposed that release of

noradrenaline from adrenergic sympathetic nerves is by an intermediate cholinergic mechanism. Thus it would be expected that application of a cholinesterase inhibitor such as physostigmine would increase the activity of the intermediate cholinergic mechanism, resulting in a greater liberation of noradrenaline in response to nerve stimulation (Burn & Rand, 1965). This might be expected to increase the time course of the EJP. If the EJP is partly due to a direct action of acetylcholine on the muscle membrane, prevention of its breakdown by inhibition of post-junctional cholinesterase would also increase the duration of the falling phase.

The evidence obtained to support the concept of an intermediary cholinergic mechanism in adrenergic fibres has indicated that this system is resistant to muscarinic blocking agents, and sensitive to nicotinic blocking agents (Burn & Rand, 1962, 1965). However, in the present study it has been demonstrated that the muscarinic blocking agent atropine, in low concentrations, reduces the time course of the EJP. Therefore it appears likely that the EJP is partly due to a direct action of acetylcholine on the muscle cell membrane. Burn & Weetman (1963) observed that the contractile responses of the vas deferens to stimulation of the hypogastric nerve were reduced by hyoscine when low frequency stimulation was employed, but that, as the frequency of stimulation was increased, the effect of hyoscine decreased. Burn, Dromey & Large (1963) suggested that at low frequencies of stimulation, acetylcholine can exert a direct action on muscle receptors, whereas at high stimulation frequencies it is completely utilized in the liberation of noradrenaline. In view of the low frequency of stimulation employed in the present study, it is possible that a mechanism such as suggested by Burn *et al.* (1963) may be involved. However, the fact that physostigmine did not increase the duration of the EJP in all the cells impaled is indicative of a mixed cholinergic and non-cholinergic, rather than a homogeneous innervation. This evidence is supported by recent histochemical studies using the electron microscope, which have shown that high levels of acetylcholinesterase are associated with some terminal axons running in the longitudinal musculature of the vas deferens, while other terminal axons in the same bundles are associated with only negligible levels of this enzyme. Both types of axon have been observed in close contact with muscle cells (P. M. Robinson & C. Bell, unpublished).

Bennett & Merrillees (1966) reported that depolarization of the muscle cells in the vas deferens reduced the amplitude of the fully facilitated EJP. In the present results, the amplitude of the EJP was usually extremely small in the presence of a concentration of physostigmine which depolarized the muscle membrane. This reduction in amplitude might therefore be partly explained by the low resting potential of the cells. However,

application of atropine after physostigmine increased the amplitude of the EJPs at a concentration which did not raise the mean resting potential of the muscle cells. The small amplitude of the EJP in the presence of physostigmine is therefore not due only to the low-resting membrane potential. It is likely that atropine antagonized the effect of physostigmine in producing summation of successive EJPs, so allowing EJPs of greater amplitude to be produced before contraction of the muscle occurred.

The effects of low concentrations of physostigmine (10^{-6} g/ml. or below) were seen with preganglionic but not post-ganglionic stimulation. Therefore these effects must have been at the ganglionic synapse. The voltage of preganglionic stimulation necessary for initiation of an action potential was lowered, and this effect was not antagonized by atropine. This is in accord with previous reports for the hypogastric and other ganglia that transmission is due to a nicotinic cholinergic mechanism (Eccles, 1964; Sjöstrand, 1965).

The second effect of physostigmine on ganglionic transmission was the production of a fully facilitated EJP in response to the first pulse of a preganglionic stimulating train. Facilitation of successive EJPs during low frequency stimulation has been attributed to increased liberation of transmitter from the post-ganglionic nerve with successive pulses (Burnstock, Holman & Kuriyama, 1964). Thus the modification of the facilitatory pattern observed in the presence of physostigmine may have been due to a greater effect of preganglionic impulses on the ganglion, resulting in maximal transmitter release post-ganglionically with the first stimulating pulse. This effect of physostigmine was fully antagonized by atropine, and was therefore due to an effect on ganglionic receptors other than those concerned with direct ganglionic transmission. Eccles & Libet (1961) reported that preganglionic stimulation of the superior cervical ganglion produced a complex wave in the ganglion cell. The last portion of this complex was a slow wave of depolarization which was selectively blocked by atropine. Takeshige & Volle (1962) showed that anticholinesterase agents produced a late discharge in the post-ganglionic fibre in response to either injected acetylcholine or a previous preganglionic tetanus. In view of the fact that the facilitation induced in the vas deferens by a conditioning train of impulses lasts for some seconds (Burnstock *et al.* 1964) it is tempting to postulate that the atropine-sensitive late response seen by Eccles & Libet (1961) and Takeshige & Volle (1962) may be concerned with the recruitment of transmitter during facilitation. In a few cells physostigmine did not affect the facilitatory process. This can be attributed to the fact that a certain percentage of fibres in the hypogastric nerve appear to be post-ganglionic (Kuriyama, 1963; Sjöstrand, 1965).

Although the most general interpretation of the Burn–Rand theory

involves the mediation of noradrenaline release by the presence of a cholinergic system in the same axon, the alternative was also proposed that acetylcholine may facilitate liberation of noradrenaline from a separate but adjacent fibre (Burn & Rand, 1962). The effects of physostigmine and atropine on the transmission process suggest that the first alternative is not applicable to the guinea-pig vas deferens. However, they are not incompatible with a possible interaction of adjacent cholinergic and adrenergic fibres which may also exert direct effects of themselves. Certainly there is a close correspondence between the distribution of nerve bundles containing high levels of noradrenaline and those staining intensely for acetylcholinesterase (Jacobowitz & Koelle, 1965).

Note added in proof. Birmingham (1966) has recently demonstrated that physostigmine and D.F.P. potentiate the contractile responses of the guinea-pig van deferens to both pre- and post-ganglionic nerve stimulation at both high and low frequencies. The post-ganglionic potentiation is abolished and prevented by atropine, whereas the preganglionic potentiation is largely atropine resistant. These results support the present conclusions that there are cholinergic fibres innervating the smooth muscle cells and that physostigmine enhances nicotinic transmission through the hypogastric ganglion.

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