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Neostigmine augments responses of the rat anococcygeus muscle to field stimulation

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1 The effects of neostigmine on noradrenergic transmission have been studied in the field stimulated, isolated anococcygeus muscle of the rat.

2 In those muscles where the excitatory response to field stimulation was not completely inhibited by guanethidine (5×10^{-6} to 10^{-5} M) or phentolamine (10^{-6} M), atropine (5×10^{-8} M) gave no further inhibition of the response.

3 The shape of the response to field stimulation was altered in a dose-dependent manner by neostigmine (5×10^{-7} to 5×10^{-6} M), such that a 'shoulder' appeared during the relaxation phase. The 'shoulder', present at all stimulation frequencies tested between 3 and 40 Hz, was abolished by atropine (5×10^{-8} M) and unaffected by tubocurarine (10^{-6} M).

4 Neostigmine (2.5×10^{-6} M), whether alone or in the presence of atropine (5×10^{-8} M), had no effect on the uptake or stimulation-induced release of [^{3}H]-noradrenaline.

5 Using electron microscopy, small Schwann/axon bundles close to smooth muscle cells rarely showed cholinesterase staining, whereas larger bundles at the outer serosal aspects of the muscle exhibited dense staining.

6 It is concluded that the observed effects of neostigmine are not due to a presynaptic effect on noradrenergic transmission.

Introduction

Anticholinesterases in the presence of atropine have been shown to potentiate the responses to low frequency stimulation of sympathetic nerves in rat mesenteric arteries (Malik, 1970), rabbit heart (Huković, 1966), dog retractor penis muscle (Armitage & Burn, 1967) and many other tissues (see Ferry, 1966; Burn, 1977). Burn (1977) interpreted such results as evidence for a cholinergic link in noradrenergic transmission, whereas other workers (Ferry, 1966; Kosterlitz & Lees, 1972) considered other explanations to be more plausible. The uncertainty as to the mechanism by which anticholinesterases potentiate responses to sympathetic stimulation arose because the end response to sympathetic stimulation (i.e. muscle contraction) was studied rather than the individual events (such as uptake, release, receptor activation) of the sympathetic transmission process itself.

The present experiments were designed to investigate the effects of anticholinesterases on sympathetic transmission processes. The rat anococcygeus muscle was chosen as the absence of a cholinergic innervation (Gillespie, 1972) would facilitate interpretation of the results.

Methods

The rat isolated anococcygeus muscle preparation

Anococcygeus muscles from male Wistar rats (220–300 g) were dissected out according to the method of Gillespie (1972). The muscles were mounted in 3.6 ml organ baths containing Krebs solution (see below) at 37°C, gassed with 5% CO_2 in O_2, and the resting tension maintained at 1 g. Tension was measured with Ether dynamometer UF1 4 oz isometric transducers, and displayed on a Devices M2 pen recorder. Field stimulation of intramural nerves was applied via parallel platinum wire elec-

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trodes mounted in the organ bath. Square wave pulses of 1 ms duration and supramaximal voltage at 30 Hz (except where stated in the text) were applied for 30 s every 6 min using an SRI 6053 stimulator.

[^{3}H]-noradrenaline uptake/release studies

The composition of the scintillation fluid is described below. Samples were counted in a Philips PW4540 liquid scintillation counter, and quench corrections made using an external standard channels-ratio technique.

Uptake of [^{3}H]-noradrenaline Groups of 4–6 muscles were freely suspended in 10 ml of Krebs solution at 37°C, and preincubated for 15 min before [^{3}H]-noradrenaline stock solution was added to give a final bath concentration of 1 μCi/ml, 1 μM. For those experiments investigating the effects of neostigmine and atropine on uptake, the drugs were present in the Krebs solution from the start of the 15 min preincubation period. After incubation with [^{3}H]-noradrenaline for the appropriate time, the tissues were removed, blotted dry and individually weighed and digested in 0.5 ml Protosol at 50°C overnight. The digested tissues were cooled, 10 ml of scintillation fluid without Triton X-100 was added, and the samples left to stand for at least 10 h before counting, to allow chemiluminescence due to the Protosol to fade. A 1 ml sample of each [^{3}H]-noradrenaline incubated medium was also counted, so that corrections could be made for variations in the bath concentration of [^{3}H]-noradrenaline between tissue groups.

Release of [^{3}H]-noradrenaline After a 30 min equilibration period, tissues were incubated with [^{3}H]-noradrenaline (1 μCi/ml, 1 μM) for 20 min and washed at 3 min intervals for 45 min. Desmethylimipramine (DMI, 10^{-7} M) and yohimbine (10^{-7} M), where used, were included in the Krebs solution immediately after the incubation with [^{3}H]-noradrenaline.

When the washing process was completed the bath fluid was changed every 3 min and a 1 ml aliquot added to 10 ml of scintillation fluid for counting. Field stimulation at 15 Hz, 1 ms pulse width and supramaximal voltage for 15 s was applied every seventh sample (21 min). One tissue from each animal was used as a control and the other tissue received neostigmine (2.5×10^{-6} M) after 3 or 4 control stimulation periods. The neostigmine was added in the seventh collection period after the last control stimulation, and the tissue was then stimulated six collection periods (18 min) later. The control tissue was treated in the same manner, but without receiving neostigmine.

Calculation of stimulation-induced tritium overflow. The mean basal overflow of tritium for the two collection periods immediately preceding each stimulation period were subtracted from the tritium overflow during the stimulation period and the two subsequent collection periods. This gave the stimulation-induced overflow during these three periods. The total stimulation-induced overflow was expressed as a percentage of the tritium present in the tissue at the start of the stimulation period (see Farnebo & Hamberger, 1971).

Electron microscopy

Rat anococcygeus muscles mounted under 1 g tension were immersed in cacodylate-buffered 2% glutaraldehyde fixative for 1 h, before being cut with sharp scissors into approximately 1 mm cubes which were exposed to the fixative for a further 3 h. Tissue pieces were processed for cholinesterase 'staining' using a method (Lewis & Shute, 1966) involving incubation with acetylthiocholine, and post-fixed in phosphate-buffered 1% osmium tetroxide for 2 h. Tissues were finally dehydrated through graded ethanols and embedded in araldite.

Thin sections (approximately 100 nm thick) were cut using an LKB ultratome III ultramicrotome, and stained with lead citrate before being examined using a Philips EM 300 electron microscope.

Choline acetyltransferase activity

Choline acetyltransferase activity in homogenates of anococcygeus muscles or vas deferens was measured by the method of Fonnum (1975), a radioenzymatic technique using ^{3}H-labelled acetyl-coenzyme-A (CoA).

Materials

Krebs solution had the following composition (mM):- NaCl 94.7, KCl 4.7, $MgSO_4.7H_2O$ 1.2, $CaCl_2$ 2.5, KH_2PO_4 1.8, $NaHCO_3$ 25 and glucose 11.7.

Scintillation fluid consisted of 5 g POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene, Sigma) and 0.3 g PPO (2,-diphenyloxazole, Sigma) dissolved in 1 l toluene (BDH), to which 500 ml Triton X-100 (Sigma) was added when counting aqueous samples.

Drugs used were (−)-[7-^{3}H]-noradrenaline (IRK342 Radiochemical Centre, Amersham), atropine sulphate (Sigma), desmethylimipramine hydrochloride (Ciba-Geigy), guanethidine sulphate (Ciba), neostigmine methyl sulphate (Sigma), (−)-noradrenaline bitartrate (Sigma), phenotolamine mesylate (Ciba), (+)-tubocurarine chloride (Sigma), yohimbine hydrochloride (Sigma).

Results

Motor responses of the rat anococcygeus muscle to field stimulation applied every 6 min remained constant over periods up to 6 h.

Antagonist drugs and the response to field stimulation

Guanethidine, added incrementally to reach concentrations of 5×10^{-6} M or 10^{-5} M over a period of 10 to 20 min, usually abolished motor responses to field stimulation without raising the resting tone of the muscle. In those experiments where the response to field stimulation was not completely abolished, the addition of atropine (10^{-7} M) failed to modify the residual response. If guanethidine was applied initially at a concentration of 10^{-5} M, the anococcygeus muscle showed a rise in tone and responses to field stimulation became biphasic initially, progressing to purely inhibitory responses as the tone continued to rise.

Phentolamine 10^{-6} M inhibited the responses to field stimulation by 65% and the addition of atropine (10^{-8} to 10^{-6} M) gave no further inhibition of the responses.

Neostigmine and the response to field stimulation

Neostigmine altered the shape of the response of the rat anococcygeus muscle to field stimulation, causing a 'shoulder' to appear during the relaxation phase (Figure 1). In 3 out of 3 experiments the neostigmine shoulder was present at all stimulation frequencies tested between 3 and 40 Hz; in addition neostigmine increased the tension developed at lower frequencies of stimulation (Figure 2). Pen recordings of the responses of the anococcygeus were photocopied and the copied responses cut out and weighed. Shoulder size was quantified by deducting the 'weight' of the control response from the 'weight' of the response in the presence of neostigmine and is expressed as a percentage of the maximum shoulder weight obtained in that tissue. Errors involved in this did not exceed 4% and were estimated by reproducing a single response in different positions on 5 separate sheets of paper, cutting out, weighing and comparing the weights of the responses. The size of the shoulder was dose-dependent in the range 5×10^{-7} M to 5×10^{-6} M neostigmine (Figure 3), was abolished by 5×10^{-8} M atropine (Figure 1), but was unaffected by 10^{-6} M (+)-tubocurarine. Atropine (5×10^{-8} M) alone did not affect the response to field stimulation, but prevented the development of a shoulder to the subsequent addition of 10^{-5} M neostigmine. When atropine was washed out, whilst retaining neostigmine, the response to field stimulation developed a shoulder which was abolished by readdition of 5×10^{-8} M atropine to the bath fluid.

With neostigmine concentrations of 5×10^{-6} M or more, the anococcygeus muscles showed a tendency to develop spontaneous activity.

[^{3}H]-noradrenaline-uptake studies

Figure 4 illustrates the effect of increasing incubation time on the uptake of [^{3}H]-noradrenaline by the rat anococcygeus muscle. The uptake increased rapidly for the first 20 min, but slowed without reaching a constant value during the next 40 min. Neostigmine (2.5×10^{-8} M) did not alter the amount of [^{3}H]-

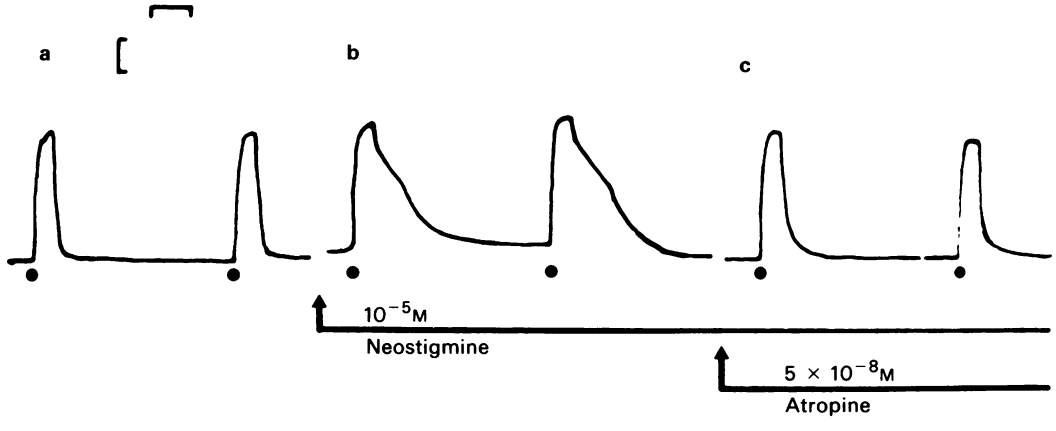

Figure 1 The effect of neostigmine on the response of the rat anococcygeus muscle to field stimulation. Field stimulation (supramaximal voltage, 30 Hz, 1 ms pulse width for 30 s) was applied at (●). (a) Shows control responses: (b) responses 6 min after addition of neostigmine 10^{-5} M to the bath, and (c) responses in the continued presence of neostigmine and 9 min after the addition of atropine 5×10^{-8} M to the bath. Calibrations: horizontal 1 min; vertical 1 g.

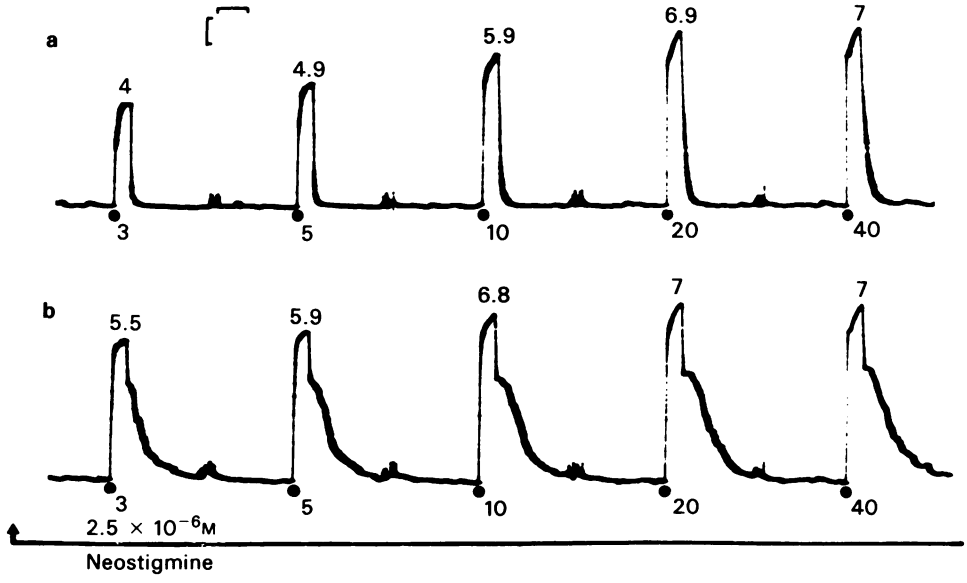

Figure 2 Responses of the rat anococcygeus muscle to field stimulation at various frequencies in the absence or presence of neostigmine. The numbers below each response refer to the stimulation frequency (Hz), and those above each response to the strength of the contraction (g). (a) Shows responses in the absence, and (b) responses in the presence of neostigmine 2.5×10^{-6} M. Calibrations; horizontal 1 min, vertical 1 g.

noradrenaline taken up by the anococcygeus muscle during a 15 min incubation period (Table 1).

[^{3}H]-noradrenaline release studies

The amounts of [^{3}H]-noradrenaline released in response to field stimulation in the presence of neostigmine (2.5×10^{-6} M) did not differ significantly from those released in control (untreated) tissues (Table 2). Neostigmine (2.5×10^{-6} M) failed also to modify significantly [^{3}H]-noradrenaline overflow in tissues equilibrated with 10^{-7} M desmethylimipramine plus 10^{-7} M yohimbine.

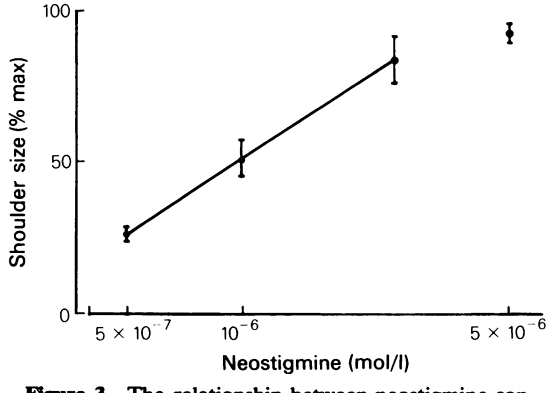

Figure 3 The relationship between neostigmine concentration and the size of the neostigmine 'shoulder' elicited in responses of the anococcygeus muscle to field stimulation at 30 Hz. The 'shoulder' size as a percentage of the maximum is plotted against the log molar concentration of neostigmine. Each point is the mean of 6 to 11 observations, and vertical bars represent s.e.mean.

Cholinesterase distribution

Using electron microscopy, axons profiles were visualiszd in anococcygeus muscles subjected to cholinesterase staining. In the smaller Schwann/axon bundles which were more closely adjacent to smooth muscle cells, axons which exhibited substantial cholinesterase reaction product were encountered only rarely although some axons showed sparse reaction deposits (Figure 5). In contrast, in larger Schwann/axon bundles located at the outer serosal aspects and distant to muscle cells, most of the axon profiles showed dense cholinesterase staining (Figure 6). Small agranular and both small and large granular vesicles were observed in both sparsely and densely stained axon profiles.

Occasionally cholinesterase staining was seen in the endoplasmic reticulum and nuclear envelope of smooth muscle cells.

Choline acetyltransferase

In the rat anococcygeus muscle the choline acetyltransferase activity ranged from 0.17 to

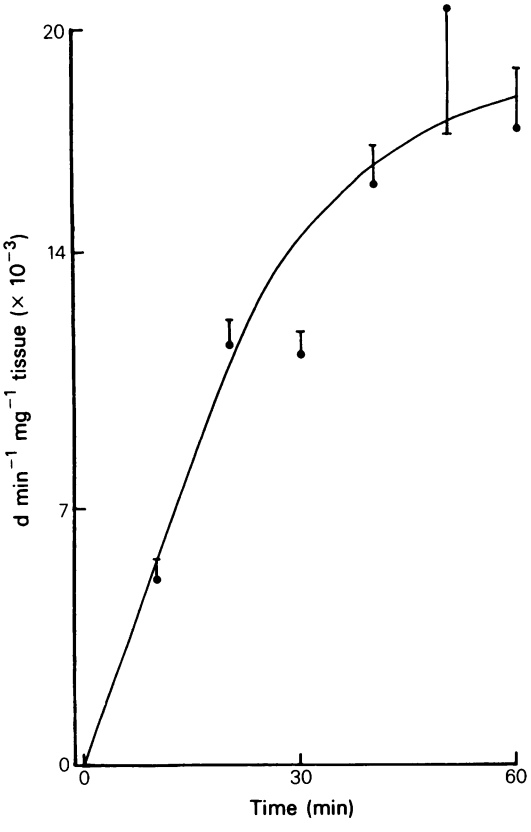

Figure 4 The effect of increasing incubation time on the uptake of [^{3}H]-noradrenaline by the rat anococcygeus muscle. The uptake of [^{3}H]-noradrenaline, expressed as $d\,min^{-1}\,mg^{-1}$ tissue, is plotted against incubation time in minutes. Each point is the mean of 4 or 5 observations, and vertical bars represent s.e.mean.

0.52 μmol h^{-1} g $tissue^{-1}$ (mean 0.27, s.e. mean 0.02, $n = 17$), and was similar to the activity measured in the rat vas deferens which ranged from 0.17 to 0.36 μmol h^{-1} g $tissue^{-1}$ (mean 0.26, s.e. mean 0.01, $n = 21$).

Discussion

A number of mechanisms were considered which might explain the effect of neostigmine in augmenting responses of the anococcygeus to field stimulation.

The most straightforward was a potentiation (by an anticholinesterase action) of a discrete cholinergic innervation of the anococcygeus muscle. However, the experiments with guanethidine, phentolamine and atropine, provided no evidence for this, in agreement with the observations of Gillespie (1972).

Alternatively, neostigmine may enhance noradrenergic transmission. Burn (1977) has interpreted the potentiation of tissue responses to low frequency sympathetic stimulation by anticholinesterases as evidence for a cholinergic link in noradrenergic transmission. The electron microscopy studies showed few axon profiles within the main body of the anococcygeus muscles to possess cholinesterase 'staining'. Despite this, homogenates of anococcygeus muscles contained choline acetyltransferase activities similar to those of rat vas deferens which has been shown by Mottram, Ivens, Lever & Presley (1973) to exhibit substantial cholinesterase staining in the axonal ground plexus. However, in the anococcygeus, the choline acetyl transferase activity may originate from cholinergic axons associated with ganglion cells present on the lateral surface of the anococcygeus (McKirdy & Muir, 1978). It is probably these axons, both myelinated and unmyelinated, which stain heavily for cholinesterase; the latter may be 'en passant' (i.e. have no functional relationship to the anococcygeus muscle).

Without invoking a cholinergic-link it is conceivable that neostigmine could enhance noradrenergic transmission by inhibiting the neuronal $Uptake_1$ process or by facilitating release. Doggrell (1981) has shown that neostigmine (10^{-6} M) did not alter contractile responses to (−)-noradrenaline itself.

In the [^{3}H]-noradrenaline incubation experiments the amount of tritium remaining in the tissue at the end of the experiment was used as a measure of the activity of the $Uptake_1$ process. A proportion of this tritium may have been in the form of noradrenaline metabolites, but as noradrenaline metabolism occurs intracellularly (Kopin, 1972), tritiated metabolites would have been in the form of [^{3}H]-noradrenaline at the time of uptake. Whilst it is widely appreciated that noradrenaline is susceptible to oxidation under

Table 1 The effect of neostigmine (Neo) and atropine (Atr) on [^{3}H]-noradrenaline uptake by rat anococcygeus muscle

Treatment	*Uptake* (mean $d\,min^{-1}\,mg^{-1}$ tissue)
Controls	13602 ± 1487 ($n = 6$)
Neo 2.5×10^{-6} M	12060 ± 1179 ($n = 6$) NS
Neo 2.5×10^{-6} M + Atr 5×10^{-8} M	13580 ± 669 ($n = 6$) NS

Values are given ± s.e.mean. NS = not significant.

Table 2 The lack of effect of neostigmine on stimulation-induced [^{3}H]-noradrenaline overflow in the the absence of inhibitors of Uptake$_1$ and presynaptic α-receptors

Stimulation period	1	2	3	4	5	6	7
Control tissues (mean ± s.e.mean)	12.1 ±1.1	5.9 ±1.0	4.2 ±1.9	3.1 ±0.4	2.8 ±0.5	3.0 ±0.5	2.7 ±0.2
Test tissues (mean ± s.e.mean)	9.2 ±2.3	5.5 ±1.4	3.2 ±1.2	3.8 ±1.3	3.1 ±0.8	3.1 ±0.6	2.7 ±0.5
t test	NS	NS	NS	NS	NS	NS	NS

Neostigmine (2.5×10^{-6} M) was added to the test tissues between stimulation periods 4 and 5. Stimulation-induced [^{3}H]-noradrenaline overflow is expressed as a percentage of the total tritium remaining in the tissue at the start of the stimulation period (see Farnebo & Hamberger, 1971). *P* values were calculated by Student's *t* test. NS = not significant.

the conditions prevailing in isolated organ baths (oxygenated physiological saline, pH 7.4, 37°C), Hughes & Smith (1978) showed that the presence of animal tissues in the bath was sufficient to protect noradrenaline against oxidation. Furthermore the inclusion of EDTA or ascorbic acid was not only superfluous, but had a detrimental effect on noradrenaline uptake. The experimental conditions in the present experiments were essentially similar to those used by Hughes & Smith (1978), and therefore EDTA and ascorbic acid were not included in the Krebs solution.

The accumulation of [^{3}H]-noradrenaline with time in the rat anococcygeus muscle (Figure 4) shows a slower time course than that reported by Nash, Gillespie & Robertson (1974). However, they stretched the muscle into a flat sheet exposing the maximum surface to the medium, whereas in the present experiments the muscles were free floating and were contracted by the noradrenaline (10^{-6} M). A 15 min incubation period was chosen for the uptake studies, as uptake was not maximal at this time enabling enhancement or inhibition to be observed. Neostigmine (2.5×10^{-6} M) had no effect on [^{3}H]-noradrenaline uptake, allowing the conclusion that the effects of this concentration of neostigmine on the response to field stimulation (Figure 3), cannot be ascribed to noradrenaline accumulating in the synaptic cleft subsequent to uptake-blockade.

Anticholinesterase drugs such as neostigmine, physostigmine and edrophonium, produce repetitive antidromic discharges in somatic motor nerves in response to single orthodromic shocks (Masland & Wigton, 1940; Riker, Roberts, Standaert & Fujimori, 1957). This action may be due to an effect on the after potentials in the motor nerve terminal (Blaber & Bowman, 1963; Blaber, 1972) and has been reviewed recently by Miyamoto (1978). However, in the present study, neostigmine (2.5×10^{-6} M) was without effect on the stimulation-induced overflow of tritium from the rat anococcygeus muscle, both in the presence and absence of 10^{-7} M DMI (to block re-uptake of [^{3}H]-NA) and 10^{-7} M yohimbine (to block presynaptic α-receptors). It was concluded therefore that the neostigmine 'shoulder' was not due to enhanced noradrenaline release consequential to repetitive firing of the sympathetic nerve terminals.

The samples collected in the release experiments were not analysed for tritiated metabolites of noradrenaline, so it is not known what proportion of tritium in the sample represents [^{3}H]-noradrenaline. Farnebo (1971) showed that 80–90% of the stimulation-induced tritium overflow from rat irides was unchanged [^{3}H]-noradrenaline, and Langer (1970) reported a figure of 50% at 4 Hz for rat vas deferens, which increased with increasing frequency of stimulation. It is reasonable, therefore, to assume for the present experiments that a high percentage of the stimulation-induced overflow elicited at 15 Hz from the rat anococcygeus muscle represents [^{3}H]-noradrenaline.

Our results provide strong evidence against a cholinergic link mechanism being involved in noradrenergic transmission in the rat anococcygeus muscle. In addition the present experiments have failed to reveal a presynaptic effect on noradrenergic transmission to account for neostigmine's action in enhancing responses to field stimulation.

Because of the paucity of cholinesterase staining revealed in the anococcygeus muscle in the electron microscopy studies it seems pertinent to ask whether the effects of neostigmine are a consequence of cholinesterase inhibition *per se* or whether another mechanism is involved.

This work was undertaken whilst J.A.S. was in receipt of an S.R.C. research studentship.

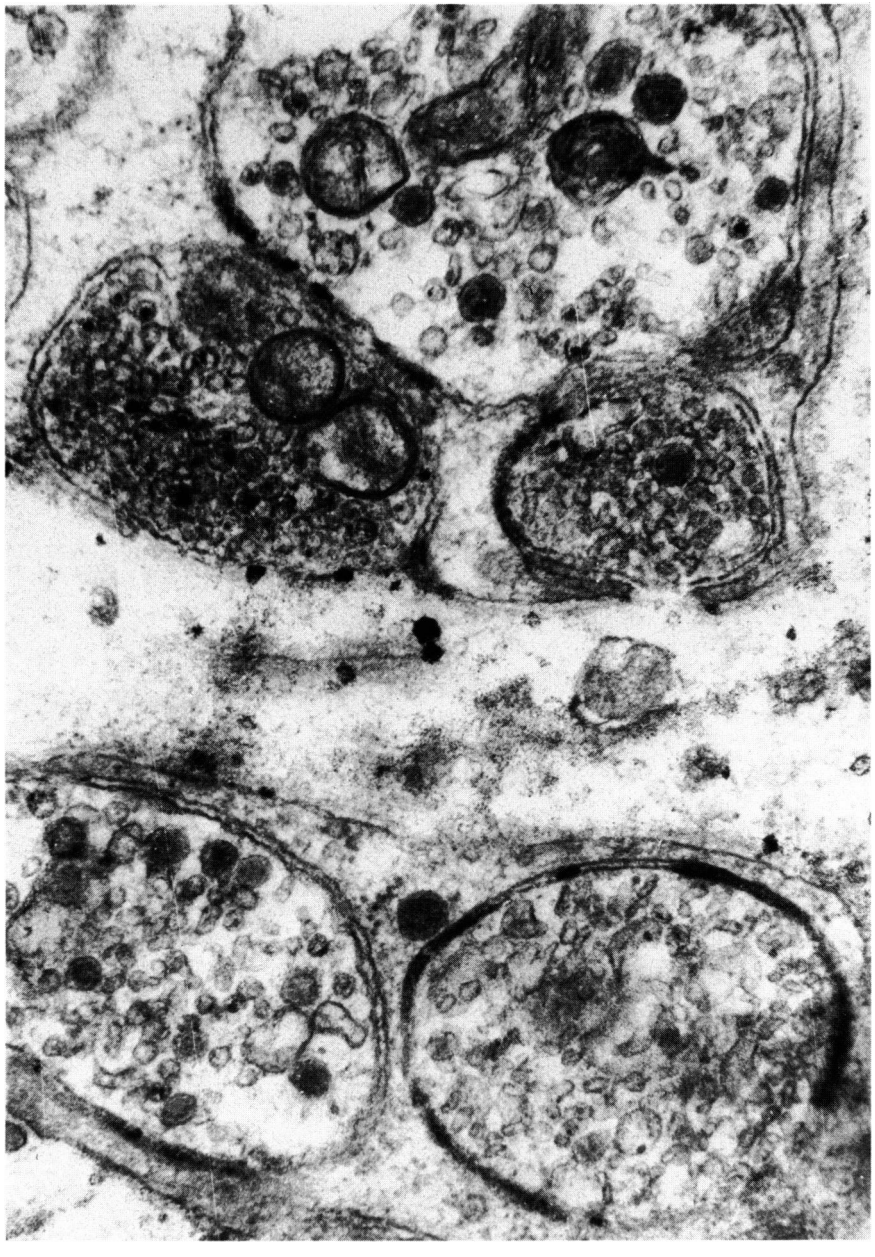

Figure 5 Electron micrograph of axon profiles in rat anococcygeus muscle stained for cholinesterase. Light deposits of cholinesterase reaction product are visible in relation to the axon profiles. Magnification × 58,725.

Figure 6 Electron micrograph of part of a nerve bundle in the outer serosal aspect of a rat anococcygeus muscle, stained for cholinesterase. Dense cholinesterase-reaction product is associated with most of the axon profiles. Magnification × 35,750.

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