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THE ISOLATED CIRCULAR MUSCLE LAYER OF THE VAS DEFERENS OF THE GUINEA-PIG

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1 A method is described for the removal of the outer longitudinal layer of muscle from the guinea-pig isolated vas deferens. The remaining tube of circular muscle was perfused at constant flow for recording changes in pressure in response to transmural electrical stimulation or to drugs.

2 The response to transmural stimulation was a rise in perfusion pressure at frequencies of stimulation of 5 to 50 Hz for trains of 16 to 256 pulses. At some frequencies and train lengths a second rise in pressure (the after-response) occurred after the cessation of the stimulus train.

3 Perfused vasa stripped of their longitudinal muscle did not develop longitudinal tension on electrical stimulation nor on intraluminal or extraluminal exposure to agonists.

4 Stripped perfused vasa gave concentration-dependent pressure rises to noradrenaline but not to acetylcholine.

5 Responses to transmural stimulation and to noradrenaline were antagonized by thymoxamine. Cocaine or desmethylimipramine increased the duration of the after-response to transmural stimulation. Reserpine pretreatment almost abolished the response and after-response. The after-response, but not the response, was increased by physostigmine and antagonized by atropine.

6 The results are discussed in relation to the known histochemical and electronmicroscopical demonstrations of dense noradrenergic and cholinergic populations of nerve terminals in the circular layer. It is suggested that mechanisms may exist for the separate control of the longitudinal and circular layers as a basis for propulsive activity.

Introduction

Two approaches to the study of the response of the circular muscle component of the vas deferens of the guinea-pig have been described by Anstey & Birmingham (1978). In one approach the vas was cut circumferentially to produce strips of tissue which could be mounted lengthwise for recording of circular muscle shortening; in the other the vas was perfused at constant flow and changes in pressure were taken to indicate changes in calibre. The continued presence of the longitudinal muscle layer in the preparations described posed some problems in interpreting the results. A solution to these problems would be physically to isolate the layer of circular muscle by removing the outer longitudinal muscle layer.

A technique was described by Evans & Schild (1953) for removing the longitudinal muscle coat of the cat jejunum. The longitudinal coat was peeled from tubular segments of jejunum; the mucous membrane and submucous plexus remained intact in this preparation.

A similar method has been devised for the removal of the longitudinal muscle from the vas. The outer longitudinal muscle layer was removed leaving a tube of circular muscle together with the inner submucosal and mucosal layers.

Methods

Removal of longitudinal muscle layer

Male albino guinea-pigs weighing over 600 g were killed by stunning and bleeding and the two vasa were removed without the hypogastric nerve. A stainless steel rod of suitable diameter (approx. 1.5 to 2.0 mm) was pushed through the lumen of a vas which was in a Petri dish of Krebs solution at room temperature, bubbled with 95% O_2 and 5% CO_2. Two diametrically opposed cuts running the length of the vas were made with a small scalpel blade. These cuts were made through the outer longitudinal layer only, the underlying circular muscle being left intact. With the scalpel blade the longitudinal muscle was then eased away from the underlying circular muscle in a sideways direction (Figure 1). The depth at which the cuts were to be made could roughly be estimated by

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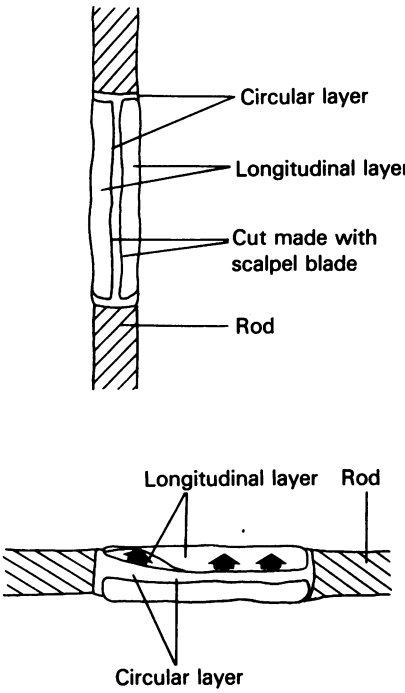

Figure 1 Diagram to show the technique for removing the outer longitudinal muscle layer from the vas deferens of the guinea-pig. The vas was slipped onto a stainless steel rod, two diametrically-opposed longitudinal cuts were made into the outer layer of muscle down to the inner layer and the outer layer was then peeled away from the inner layer.

observation of the circular muscle, which protruded slightly at each end of the vas tube from beneath the longitudinal layer of muscle. Other clues as to the exact position of the plane of cleavage between the longitudinal and circular layers became apparent after some experience. Slightly greater resistance was given to the scalpel blade by the circular muscle than by the longitudinal muscle. Also the two layers appeared to have slightly different colours. This became most apparent when some of the circular muscle had inadvertently been removed with the longitudinal muscle, the circular muscle appearing whiter in colour than the longitudinal muscle to which it adhered.

The vas, with the stainless steel rod, was kept under Krebs solution whenever possible, and the tissue was not allowed to dry. The dissection resulted in two strips of longitudinal muscle which had been removed, and a tube of circular muscle (with the submucosa and mucosa) which remained around the rod. This tube of circular muscle could be slipped off the rod and set up for perfusion in the manner described by Anstey & Birmingham (1978) for the perfused whole vas preparation. The tube of circular muscle was thin and had to be set up quickly to avoid its drying out.

Vasa in which the longitudinal muscle had been removed were examined histologically to see whether or not the longitudinal muscle had been removed completely. They were fixed in Souza's fixative, embedded in wax, sectioned at 6 μm and stained with haematoxylin and eosin.

Recording of pressure and longitudinal tension when perfused

Whole vasa with longitudinal and circular muscle layers intact and vasa from which the longitudinal muscle had been removed (referred to as stripped vasa) were used for the recording of the response, and any after-response, to transmural electrical stimulation or to drugs perfused through the lumen (intraluminal) or added to the organ bath (extraluminal). The technique was that described for whole vasa by Anstey & Birmingham (1978). The development of longitudinal tension was measured in addition to the measurement of changes in perfusion pressure. Whereas previously whole vasa had usually been fixed at their resting length to prevent shortening, the whole (unstripped) and stripped perfused vasa used in the experiments now described were fixed at the lower end by clamping the perfusion tube whilst the tube at the upper end was attached by a thread to an isometric force transducer.

Both stripped and whole vasa were mounted vertically between parallel wire electrodes in jacketed organ baths containing 100 ml Krebs solution at 32°C bubbled with 95% O_2 and 5% CO_2 and stimulated transmurally with pulses of supramaximal voltage, and 0.1 ms pulse width every 8 min. In some experiments a pulsegating unit was used which allowed the number of pulses to be varied. Tension and pressure responses to perfusion with Krebs solution at 32°C at a flow rate of 0.9 ml/min were measured and log frequency-response graphs were obtained.

Drugs

The following drugs (obtained from the usual commercial sources) were used: noradrenaline bitartrate, acetylcholine chloride, physostigmine sulphate, acetyl-β-methylcholine chloride, carbachol chloride, thymoxamine hydrochloride, cocaine hydrochloride, desmethylimipramine hydrochloride, reserpine and atropine sulphate. All drug concentrations are expressed as final concentrations in mol per litre.

The Krebs solution used had the following composition (mM): NaCl 119, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, NaH_2PO_4 0.9, $NaHCO_3$ 25 and glucose 11.1.

Results

Separation of longitudinal muscle and circular muscle

During the development of the technique for dissection of the longitudinal muscle layer from the tube of circular muscle, submucosa and mucosa, histological examination of sections of vasa deferentia subjected to the stripping procedure revealed, in a few instances, small sections of longitudinal muscle still adhering to the circular layer. As familiarity with the technique was gained, the incidence of unsuccessful stripping fell to zero. It also became apparent that on setting-up the vasa for perfusion and longitudinal tension recording, successfully stripped vasa did not develop tension when stimulated transmurally, those vasa that did develop a small degree of tension also revealed some remaining longitudinal muscle on histological examination. Thus an indication of successful stripping was the absence of tension response on transmural stimulation.

The response to transmural electrical stimulation

Log frequency-response curves for various train lengths of transmural stimulation are shown in Figure 2a. Increase in frequency from 5 to 50 or 100 Hz gave an increase in pressure response at train lengths from 16 pulses in a train to 256 pulses in a train. At some frequencies and train lengths a second rise in pressure (the after-response) occurred after the cessation of the train of stimuli during which the main rise in pressure had occurred. Log frequency-after response curves are shown in Figure 2b and Figure 4. For an illustration of an after-response see Anstey & Birmingham (1978) Figure 7.

The longitudinal tension response to transmural electrical stimulation

Stripped perfused vasa did not exhibit a tension response or tension after-response at any frequency or train length of transmural stimulation. Vasa from which the longitudinal muscle had not been removed (whole perfused vasa) developed tension at a threshold of 2 Hz, 8 pulses, increasing to a maximum at 50 Hz, 16 to 256 pulses, or 20 Hz, 8 pulses. No tension after-response was seen.

The response to noradrenaline or acetylcholine

Stripped perfused vasa gave a concentration-dependent pressure response to noradrenaline added to the bath (extraluminal) from a threshold at 8×10^{-6} M to a maximum at 1.25×10^{-4} M ($n = 3$). Concentration-related pressure rises were also obtained when noradrenaline was perfused intraluminally (1×10^{-7} to 1×10^{-4} M, $n = 3$). In a typical experiment the maximum pressure rises obtained were 25 and 15 mmHg for extraluminal and intraluminal noradrenaline respectively, to be compared with a pressure of 50 to 60 mmHg for electrical stimulation at 50 Hz.

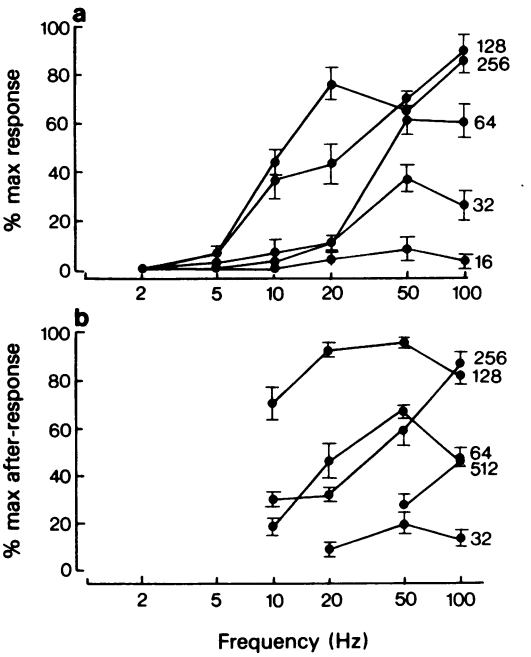

Figure 2 Log frequency-response graphs for transmurally stimulated stripped perfused guinea-pig vasa. Each point is the mean of measurements made on vasa from 6 guinea-pigs; vertical lines show s.e. means. Pulse width 0.1 ms, supramaximal voltage. Abscissa scale: frequency in Hz on a log scale. Ordinate scale: rise in pressure expressed as a % of the maximum response obtained. (a) The response obtained during the trains of stimuli (16 to 256 pulses in a train); (b) the after-response obtained from the same vasa after the cessation of the trains of stimuli (32 to 512 pulses in a train).

Stripped perfused vasa gave no pressure or tension responses to acetylcholine perfused intraluminally or added extraluminally (up to 1×10^{-4} M, $n = 6$). Pretreatment with physostigmine (2×10^{-5} M for 30 min) did not induce a response to acetylcholine. Neither acetyl-β-methylcholine nor carbachol produced pressure rises.

Whole perfused vasa gave graded tension responses to extraluminal acetylcholine from 1×10^{-7} M to a maximum at 3.2×10^{-6} M, but no pressure responses ($n = 4$). When perfused intraluminally neither tension nor pressure responses were seen up to 1×10^{-4} M acetylcholine concentration ($n = 4$).

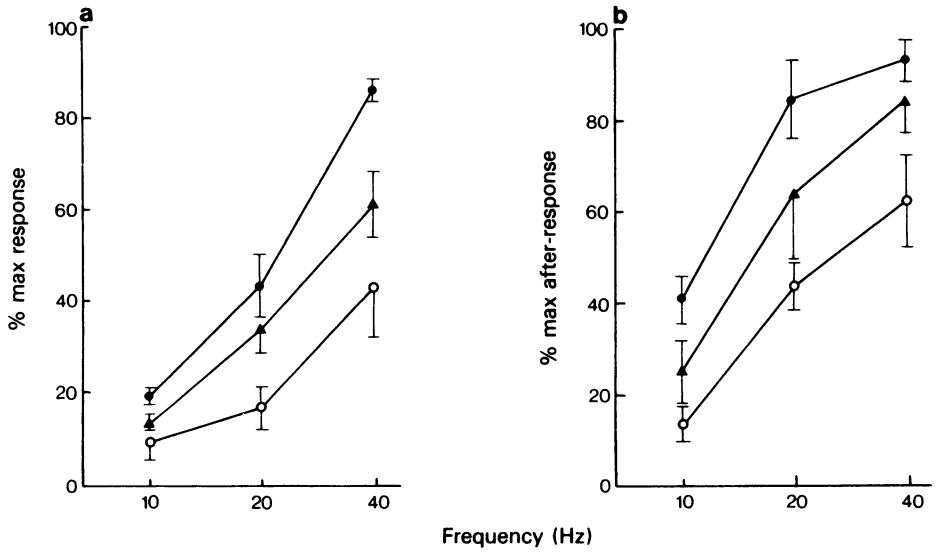

Figure 3 The effect of thymoxamine 10^{-7} (▲) and 10^{-6} M (○) on the log frequency-response (a) and log frequency-after-response (b) curves of transmurally-stimulated stripped perfused guinea-pig vasa; controls (●). Pulse width 0.1 ms, supramaximal voltage, 128 pulses. Each point is the mean of measurements made on vasa from 5 guinea-pigs; s.e. mean shown as vertical bars. Abscissa scale: frequency in Hz on a log scale. Ordinate scale: (a) rise in pressure during the stimulus train expressed as a % of the maximum obtained; (b) rise in pressure after the stimulus train expressed as a % of the maximum after-response.

Effects of drugs on response to transmural electrical stimulation

Thymoxamine The response and the after-response of stripped perfused vasa to transmural stimulation (Figure 3) and the response to extraluminal noradrenaline were all antagonized by thymoxamine (10^{-7} M and 10^{-6} M final bath concentration; at least 8 min equilibration).

Cocaine and desmethylimipramine The pressure response of the stripped vasa stimulated transmurally was not increased by exposure, for at least 8 min equilibration, to cocaine (1×10^{-5} M) or to desmethylimipramine (DMI) (3×10^{-7} M). The magnitude of the after-response was also not increased by these drugs but it lasted longer. Figure 4 shows the results of measurements made on the duration in seconds of the pressure rise above the baseline during the train of stimuli (upper graphs) and the duration of the second pressure rise occurring after the passage of the train of stimuli (lower graphs) in the absence or the presence of cocaine or DMI. It is apparent that the duration of the response was not changed at any of the 3 frequencies by either drug but the duration of the after-response was increased at each of the 3 frequencies by both drugs. The results shown in Figure 4 are single experiments which were typical of those obtained for vasa from 4 guinea-pigs for cocaine and 3 for DMI.

Reserpine When stripped perfused vasa ($n = 4$) from guinea-pigs pretreated with reserpine (2.5 mg/kg i.p., 48 and 24 h beforehand) were stimulated transmurally the pressure response was very small (maximum rise to 3 mmHg at 50 Hz) compared with that seen with vasa from control guinea-pigs (maximum rise usually over 60 mmHg at 50 Hz). Noradrenaline added extraluminally gave a pressure response from 1.25×10^{-5} M (threshold) to 1×10^{-4} M (maximum); the maximum pressure response of 13.0 mmHg was about half of the maximum usually obtained from control vasa.

Atropine The log frequency-response curve for stripped vasa ($n = 4$) was not shifted by 10^{-7} M or 10^{-6} M atropine; the log frequency after-response curve was moved to the right by 10^{-6} M atropine ($P < 0.01$).

Physostigmine The log frequency-response curve for stripped vasa ($n = 6$) was not shifted by 1×10^{-5} M physostigmine; the log frequency after-response curve was shifted to the left by 10^{-5} M physostigmine ($P < 0.05$) and this potentiation was reversed by 1×10^{-6} M atropine to below control levels.

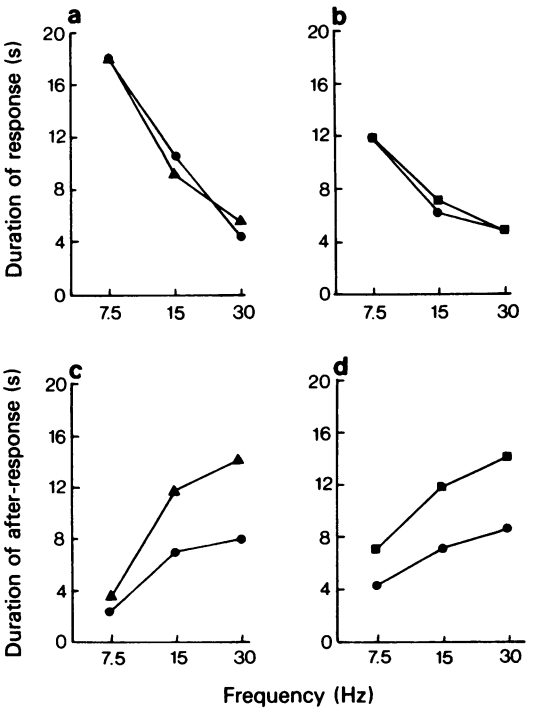

Figure 4 Effect of cocaine (10^{-5} M, ▲) and of desmethylimipramine (DMI, 3×10^{-7} M, ■) on the response (a and b) and the after-response (c and d) to transmural stimulation of stripped perfused vasa (0.1 ms pulse width, supramaximal voltage, 128 pulses); controls (●). Abscissa scale: (a and b) duration(s) of the pressure rise above the baseline occurring during the stimulus train; (c and d) duration(s) of the pressure rise above the baseline occurring after the stimulus train. Single vasa were used for the results shown in (a, c) and (b, d); almost identical results were obtained from 3 other experiments.

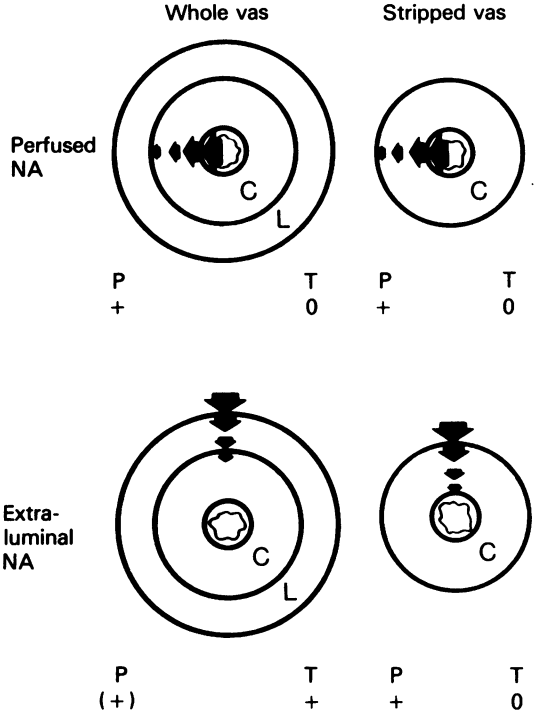

Figure 5 Diagrammatic representation of the effect of extraluminally (added to the bath) or intraluminally (perfused) administered noradrenaline (NA) on the pressure (P) and the tension (T) responses of whole and stripped perfused vasa: + indicates a response; (+) indicates a response only to very high concentrations; 0 indicates no response. C = circular muscle layer, L = longitudinal muscle layer. The arrows indicate direction of diffusion of NA.

Discussion

Several of the methods used by others to record the contractile response of the circular layer of smooth muscle of tubular organs were investigated by Anstey & Birmingham (1978) for the recording of the response of the circular muscle of the vas deferens of the guinea-pig. The most informative of these proved to be luminal perfusion (Anstey 1972) which has since been used by Illés, Rónai & Knoll (1976); Anton, Duncan & McGrath (1977); Anton & McGrath (1977); Jackson, Short & Tomlinson (1978) and Jackson & Tomlinson (1978). The experiments now described were carried out on guinea-pig vasa deferentia from which the outer longitudinal muscle layer had been removed thus confining the response to the circular muscle.

The simplest check on the completeness of removal of the longitudinal muscle was the absence, on transmural stimulation between parallel wire electrodes, of the development of longitudinal tension. Whenever a small degree of tension developed this proved, on histological examination, to be associated with the residual preserce of a small band of longitudinal muscle. Histological confirmation of completeness of removal was sought in all the early experiments and was found to correlate so completely with absence of longitudinal tension that it was found to be unnecessary in later work. There was never any evidence that the very thin layer, consisting of a mixture of circular, longitudinal and oblique fibres just beneath the muscosa, contributed to the pressure changes. Thus it was assumed that the changes in perfusion pressure recorded at a constant perfusion flow of about 1 ml/min were due to changes in calibre of the tube of circular muscle which reflected shortening of the circular muscle fibres.

The response of vasa stripped of their longitudinal muscle to transmural electrical stimulation over a range of frequencies at different train lengths were broadly similar to those reported by Anstey & Birmingham (1978) for luminally-perfused whole vasa with their longitudinal muscle intact. This confirms that the pressure changes described in the earlier paper were due to contraction of circular muscle. Whole vasa did, on the other hand, exhibit stimulus-related changes in longitudinal isometric tension which were never seen with stripped vasa. Like the perfused whole vas, the perfused stripped vas produced a pressure change (the response) during the passage of the stimulus train which declined on cessation of the train to be followed, at some frequencies and train lengths, by a second rise in pressure (the after-response) almost immediately after the end of the stimulus. The persistence of the after-response phenomenon in the absence of longitudinal muscle suggests the after-response to be a feature of circular muscle contraction. After-responses were never seen in longitudinal tension records from whole vasa.

The measurements on the stripped vas of the effects of noradrenaline, thymoxamine, reserpine, cocaine and desmethylimipramine, taken together with those on the whole vas reported by Anstey & Birmingham (1978), demonstrate that the response of the circular muscle to stimulation of intramural nerve terminals is noradrenergically-mediated. The apparent insensitivity of the pressure response of the whole vas to extraluminal noradrenaline seems to be due to difficulty of access to the inner circular layer since this insensitivity is not present in the stripped vas where the barrier of longitudinal muscle has been removed (Figure 5).

The pressure response of the stripped vas seems not to be due to a direct action of neuronally-released acetylcholine on the muscle. The circular muscle was insensitive to acetylcholine, by the intraluminal or extraluminal route, even after exposure to high concentrations of physostigmine. The same was true of the whole vas which, on the other hand, did develop longitudinal tension to acetylcholine. The response of the stripped vas to electrical stimulation was not significantly reduced by atropine nor was it increased by physostigmine.

The after-response is intriguing. It was seen only as a feature of circular muscle contraction in response to electrical stimulation, never in response to exogenously applied drugs. It was absent after treatment with reserpine, more resistant to guanethidine and to bethanidine than the response itself (Anstey & Birmingham 1978), increased in duration by cocaine or DMI (but like the response, not increased in magnitude) and unlike the response, increased by physostigmine (which latter increase was antagonized by atropine).

In looking for possible explanations for the differences in behaviour of the longitudinal and circular muscle layers, the previously reported differences in the morphology of the autonomic innervation of the two layers may be relevant. Gosling & Dixon (1972) and Furness & Iwayama (1972) found histochemical and electron microscopical evidence for the existence of a plexus of cholinergic nerve terminals in the circular layer but not in the longitudinal layer and confirmed the presence of dense plexi of noradrenergic terminals in both layers. Anstey, Birmingham & Tomlinson (1974) supplemented these findings with measurements of noradrenaline concentration and of cholinesterase activity in the separated layers of muscle. When the noradrenaline concentration of the longitudinal muscle stripped from vasa was measured it had a mean concentration ($n = 6$) of 13.54 ± 1.96 μg/g whereas the remaining circular muscle had a mean concentration of 16.46 ± 2.32 μg/g. Thus the circular muscle had some 1.2 times the concentration of noradrenaline compared with the longitudinal muscle. The difference was more striking for cholinesterase activity where the circular muscle ($n = 20$) had 2.6 times (18.57 ± 2.06 nmol acetylthiocholine hydrolysed min^{-1} mg^{-1} protein) the activity of the longitudinal muscle (8.13 ± 1.07).

If the after-response, characteristic of the circular muscle when it is stimulated transmurally, is a real reflection of a difference in functional innervation between the longitudinal and circular layers and not just an artefact of the *in vitro* methods of stimulation and pressure recording, it suggests that mechanisms exist for the separate control of the two muscle layers. If the vas acts as an organ of transport from epididymis to urethra, this separate control could allow a degree of peristaltic behaviour similar to that seen in the gut. Further investigation is needed to define the interactions between the noradenergic and cholinergic innervations which might bring about this separate control of the circular muscle. The identification of distinct populations of noradrenergic and cholinergic neurones in the hypogastric ganglion by Bell & McLean (1967) may provide a morphological basis for an explanation. Cholinergic modulation of noradrenergic mechanisms has been suggested for other tissues notably the heart (Lindmar, Löffelholz & Muscholl, 1968; Löffelholz & Muscholl, 1969) and peripheral blood vessels (Malik & Ling, 1969; Rand & Varma, 1970; Steinsland, Furchgott & Kirpekar, 1973).

We conclude from our results that it is possible to remove the outer longitudinal muscle from the guinea-pig isolated vas deferens to provide a tube consisting largely of circular muscle which develops no longitudinal tension on field stimulation, but does constrict. The results from measurements of response to agonists and antagonists (with or without simul-

taneous field stimulation) and the previously reported histochemical and chemical investigations, together indicate differences in the manner of innervation of the two layers of muscle which could allow for their separate control as a basis for propulsive activity in the vas deferens *in vivo*.

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