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THE INNERVATION OF SHEEP MESENTERIC VEINS

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SUMMARY

1. An isolated preparation of sheep mesenteric vein is described from which contractions of the longitudinal smooth muscle of the adventitia have been recorded in response to stimulation of intramural and peri-arterial nerves.

2. The preparation did not respond to single stimuli but the relation between amplitude of response and frequency of stimulation was characteristic of that described for other smooth muscles innervated by sympathetic nerves.

3. Responses were blocked by tetrodotoxin, guanethidine, and by a number of α-receptor blocking drugs. Responses to peri-arterial stimulation were unaffected by hexamethonium. It was concluded that the longitudinal smooth muscle is supplied with post-ganglionic noradrenergic motor nerves and the presence of such fibres in the adventitia was confirmed by fluorescence microscopy.

4. Acetylcholine also caused contractions. Although a direct excitatory action on the smooth muscle could not be excluded, results suggest that acetylcholine may release noradrenaline from the axons of the sympathetic ground plexus.

INTRODUCTION

Analysis of the effects of autonomic nerve stimulation on its various effector organs can be facilitated by the use of isolated nerve-muscle preparations maintained in a controlled environment. Most of the work on the neural control of vascular smooth muscle, however, has been carried out on the vessels *in situ*. The few recent studies on isolated neuromuscular preparations have dealt with arterial smooth muscle (e.g. Bevan, 1962; McGregor, 1965; Rogers, Atkinson & Long, 1965; de la Lande & Rand, 1965). The role of veins in the circulatory system as a whole, and the factors involved in the control of their capacity, have been studied by Folkow and co-workers (see Folkow, Lewis, Lundgren, Mel-

lander & Wallentin, 1964*a*, *b*). The actions of a number of drugs on isolated mammalian veins have been described by Franklin (see Franklin, 1937). However, there do not appear to have been any previous studies on isolated neuromuscular preparations of mammalian veins.

The walls of the larger veins of many mammalian species have a large component of longitudinal smooth muscle in the tunica adventitia (Franklin, 1937) and changes in the length or tension of this contractile component can be readily measured with simple mechanical transducers. This paper describes the contractile responses of isolated segments of the superior mesenteric vein of the sheep, in response to electrical stimulation of intramural nerve fibres according to the method of Paton (1955). The nerve supply to the vein was also stimulated by electrodes placed on the accompanying artery (peri-arterial stimulation).

The actions of a number of sympathetic nerve-blocking drugs on these contractions have been studied and compared with the effect of these drugs on contractions of the vein in response to noradrenaline and acetylcholine.

A preliminary account of this work was given to the Australian Physiological Society (February, 1966).

METHODS

Experiments were carried out on the superior mesenteric vein of yearling sheep (mainly dorset horns). Several centimetres of the vein with its accompanying artery were dissected from the animal immediately after slaughter at the local abattoir. Preparations were taken to the laboratory in cold physiological saline. Segments of vein, about 2·5 cm in length, were dissected free from the mesentery and the accompanying artery, and were mounted in an isolated organ-bath at 36° C containing physiological saline equilibrated with 95 % O_2 and 5 % CO_2 (composition: NaCl, 120 mM; KCl, 5 mM; $NaHCO_3$, 25 mM; NaH_2PO_4, 1 mM; $MgSO_4$, 1 mM, glucose, 10 mM, sucrose 10 mM, $CaCl_2$, 2·5 mM).

Tension was recorded isometrically from the majority of preparations using a Grass FTO3 force-displacement transducer. Semi-isotonic records were obtained from some preparations by attaching the vein to the long arm (30 cm) of a straw lever, the short arm (4 cm) being attached to the force transducer. Results were displayed on Grass or Offner polygraphs.

It seems likely that the records obtained in this way were due only to changes in tension (or length) of the longitudinal smooth muscle, and were not complicated by contractions of the circular muscle of the tunica media. Apart from the strip of vein running between the holder and the cotton thread by which the vein was attached to the transducer, the remainder of the wall of the vein was not under tension.

Unless otherwise stated all experiments were carried out on preparations which had been allowed to equilibrate in the isolated organ-bath for at least 1 hr.

Electrical stimulation. The two methods of electrical stimulation used in these experiments are illustrated diagrammatically in Fig. 1. The electrodes used for transmural stimulation (Fig. 1*a*) consisted of two Pt wires; one of these made connexion with the solution in the isolated organ-bath, while the other was insulated except for the final 1 cm which was placed within the lumen of the vein. Since there was a low electrical impedance between these

electrodes the voltage output of the stimulator (Grass Type S4) was reduced. When the stimulator was set for maximum output, the voltage measured between the electrodes did not exceed 4 V.

For peri-arterial stimulation, preparations were made in which the artery was left attached to the vein over a length of about 2 cm. A further length of artery, proximal to this segment, was cleared of vein and connective tissue and drawn through the electrodes as shown in Fig. 1*b*. These electrodes consisted of rings of Pt wire 4 mm in diameter, 6 mm apart.

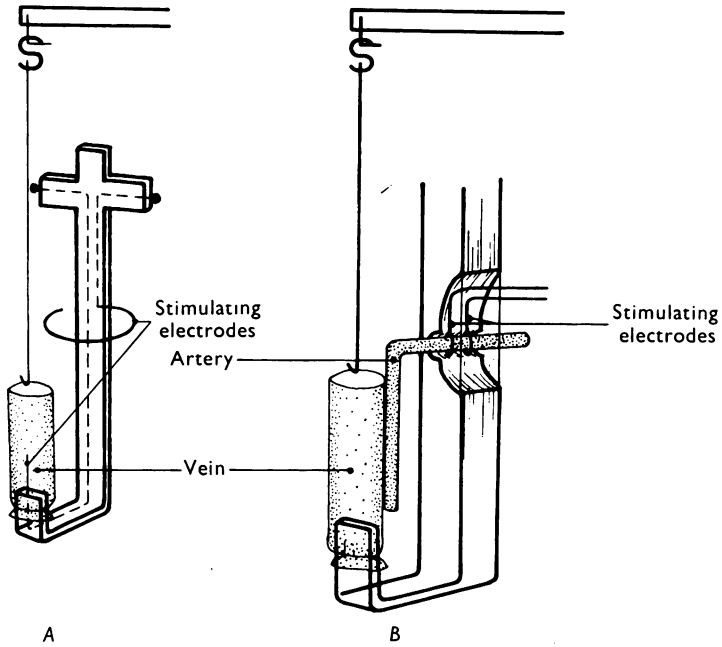

Fig. 1. Diagrams to illustrate the methods used for transmural stimulation (*A*) and peri-arterial stimulation (*B*). See text ('Methods' section) for explanation.

In all experiments trains of pulses of fixed duration (10 sec) and of varying frequency, pulse duration and intensity were used. The interval between stimuli was from 2 to 4 min.

Electrical recording. A modified sucrose-gap apparatus (S. Goldner, thesis for M.Sc., Monash University) was used to record the spontaneous electrical activity of strips of longitudinal muscle from the adventitia. Strips were mounted in a vertical chamber, 1 mm in diameter, which was divided into three compartments by thin rubber membranes. Each membrane was pierced with a hot needle to obtain a hole of suitable diameter to fit the muscle as snugly as possible without constricting it. The upper compartment was perfused with physiological saline at 34° C; the middle compartment was perfused with an isotonic solution of sucrose in de-ionized water and the lower compartment was perfused with isotonic K_2SO_4. The middle compartment, i.e. the sucrose-gap, was 5 mm long.

The potential difference between the upper and lower compartments was recorded with saline-agar bridges connected to calomel half-cells. The lower K_2SO_4 compartment was normally at earth potential and the upper compartment was connected, via a unity-gain pre-amplifier, to a Grass D.C. amplifier and polygraph.

Histology

Segments of vein, 1–2 cm long, were fixed in formal saline for about 2 weeks. These were washed, dehydrated and embedded in paraffin. Sections, 5–10 μ thick, were stained in two ways: haematoxylin and eosin and Mallory's aniline blue method, as described by McManus & Mowry (1960).

Blocks of the wall of the vein, not more than 5 mm square, were prepared for fluorescent microscopy from vessels which had been equilibrated in physiological saline for up to 4 hr. During this time they were stimulated at 2 min intervals in the usual way. The pieces of tissue were quenched in propane at the temperature of liquid N_2 and quickly transferred to the pre-cooled plates of a freeze-drying chamber. They were dried for 3 days at $-35°$ C at a pressure of 10^{-6} torr.

The dried tissue was exposed to standardized formaldehyde vapour at 80° C for 1 hr. The formaldehyde had been equilibrated with 70 % H_2SO_4 for 7–11 days at room temperature. The tissue was embedded in paraffin and 10 μ sections were cut which were cleared in a 50 % mixture of Entellen and xylol.

Sections were viewed with dark ground illumination with U.V. light (Hg vapour lamp HB 200 with a 2–7 mm Leitz BG 12 filter and a 3 mm Leitz BG 38 filter). Photo-micrographs were obtained using Agfa Isopan ISS film (ASA 200) (black and white negatives) and high speed Ectachrome (ASA 125) (colour transparencies).

The following drugs were used: acetylcholine chloride, atropine sulphate, cocaine hydrochloride, dimethyl phenyl piperazinium iodide (DMPP), guanethidine sulphate, hexamethonium chloride, isoprenaline hydrochloride, noradrenaline bitartrate, ouabain, phenoxybenzamine, phentolamine hydrochloride and reserpine.

RESULTS

Morphology. Conventional staining methods (haematoxylin and eosin, Mallory's triple stain) showed two layers of smooth muscle in this preparation: the inner circular layer of the tunica media and the adventitial layer of bundles of smooth muscle arranged longitudinally. The thickness of the longitudinal layer was 2–4 times that of the media. The Mallory stain indicated the presence of considerable amounts of nervous tissue in the wall, both along the medial-adventitial border and within the longitudinal layer.

The fluorescence method of Falck & Owman (1965) was used to study the localization of catecholamines within the vessel wall. Freeze-dried preparations were exposed to damp paraformaldehyde vapour at 80° C (see Methods) and, after embedding in paraffin, transverse and longitudinal sections were prepared for fluorescence microscopy. Dark-ground illumination was used to view the sections alternately with white and ultraviolet light. Numerous bright green fluorescent spots could be seen in transverse section of the adventitia. In longitudinal sections these appeared to be identical with the varicose fluorescent fibres described previously for other sympathetically innervated smooth muscles (see, for example, Falck, 1962). Varicose, fluorescent fibres were also seen running in a circular direction in the media.

These bright green profiles were absent from sections prepared from sheep which had been pre-treated with reserpine (0·1 mg/kg daily, for 3 days). The elastic lamina of the intima and media showed a pale yellow green fluorescence in both untreated and reserpinized preparations.

These results suggest that the adventitia and, to a lesser extent, the media of this blood vessel are supplied with noradrenergic nerves.

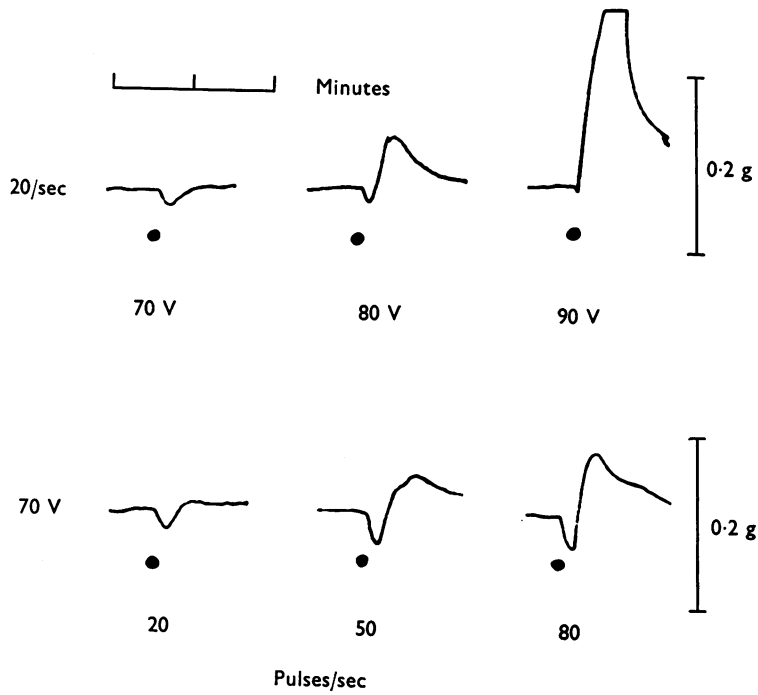

Fig. 2. Responses to transmural stimulation (10 sec trains of pulses of 0·8 msec duration) recorded from preparations immediately after they had been transferred to the isolated organ-bath. Upper row; the effect of increasing the stimulus voltage (70, 80 and 90 V refer to the settings of the stimulator and not to the voltage across the preparation). Lower row; the effect of varying frequencies of stimulation (20, 50 and 80/sec).

Responses of unequilibrated preparations to transmural stimulation. During the first hour of exposure to physiological saline at 35–36° C in the isolated organ-bath, both the length of the preparation and its responsiveness to transmural stimulation gradually increased. During this time the nature of the response to a standard stimulus (a 10 sec train of 0·8 msec pulses at 20/sec) changed from a diphasic response with relaxation preceding contraction to a pure contraction.

The nature of the response of unequilibrated preparations depended on the parameters of stimulation. Figure 2 shows the effect of increasing the

output voltage of the stimulator from a level which elicited a small relaxation (stimulator setting, 70 V) to that which gave a large contraction (90 V). Figure 2 also illustrates the effect of increasing the frequency of stimulation in unequilibrated preparations. In both these experiments the first response that was observed as the voltage or frequency was increased to a threshold level was relaxation. A further increase in voltage or frequency caused a diphasic response.

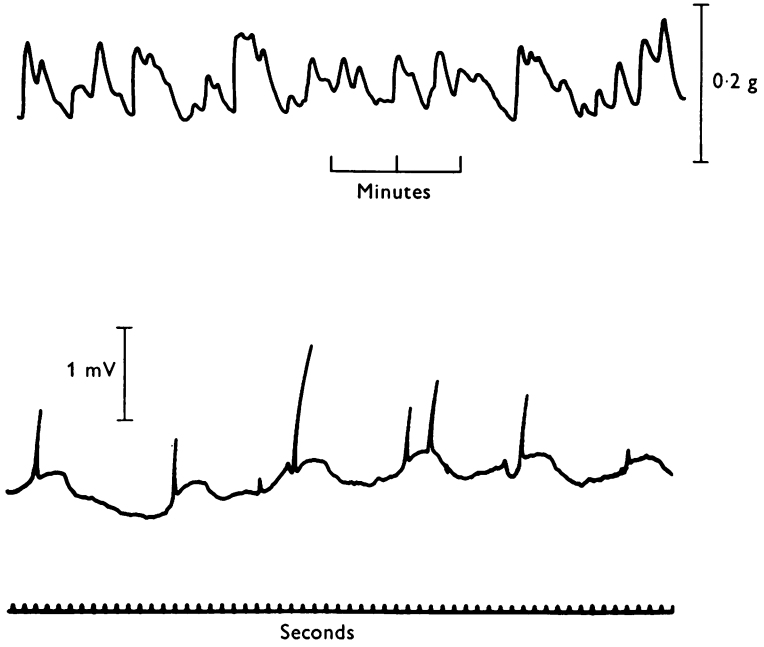

Fig. 3. Upper record: typical pattern of the fluctuations in tension recorded from a preparation showing spontaneous activity.
Lower record: sucrose-gap record showing changes in membrane potential during spontaneous activity (depolarization upwards). Note the difference in time scales.

Relaxation responses were never observed in preparations which had been set up for more than 1 hr. All the experiments described below were carried out on preparations which had been allowed to equilibrate for 1–2 hr.

Spontaneous activity. About 20% of the equilibrated preparations showed spontaneous contractions of variable magnitude of up to 0·5 g. In these veins, the amplitude of the spontaneous contractions increased if the muscle was stretched. Figure 3*a* illustrates a typical pattern of this activity. Contractions often occurred at a rate of about 2/min but were usually too irregular to permit frequencies to be measured for periods of more than a few minutes, in contrast to the findings of Franklin (1928).

The sucrose-gap apparatus was used to record the changes in membrane potential of preparations showing spontaneous mechanical activity (see 'Methods'). Figure 3 (lower record) shows a typical record of the slow waves and brief spikes which were characteristic of these veins. (Note that this sequence might correspond with one of the contractions shown in Figure 3 (upper record)). This and other records from sheep mesenteric veins were similar to those found for rabbit mesenteric veins (Cuthbert, Mathews & Sutter, 1961; Cuthbert & Sutter, 1965).

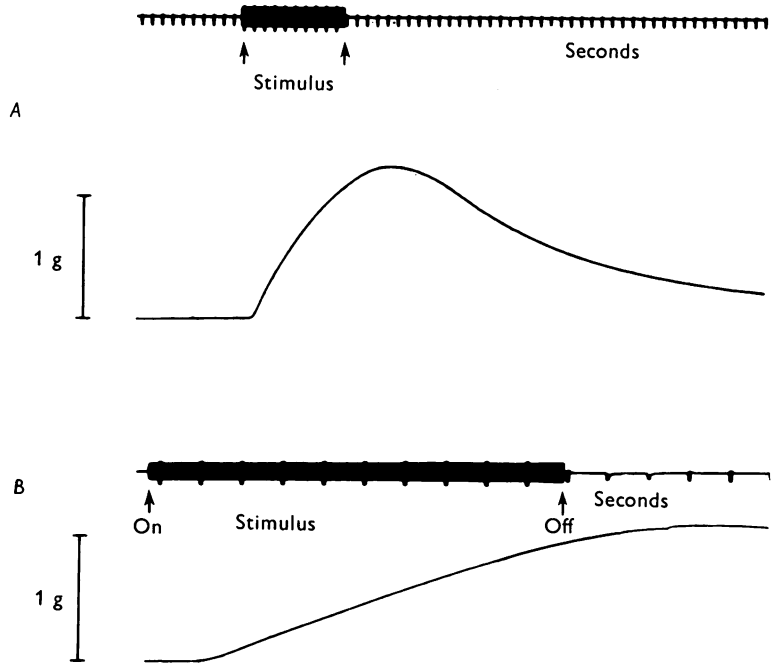

Fig. 4. Records illustrating the time course and latency of typical responses to stimulation with transmural electrodes (isometric recording). The upper tracing in each record shows 1 sec time marker and duration of stimulation.

Responses to transmural stimulation (equilibrated preparations). Figure 4 shows the time course of two typical isometric contractions in response to stimuli of submaximal voltage (frequency, 20/sec, pulse duration, 0·8 msec). Tension began to increase after a delay of $1\frac{1}{4}$ sec and continued to increase after the cessation of stimulation. Relaxation was slow, preparations taking from 60 to 100 sec to relax completely. The latency of these contractions was found to decrease as the frequency and voltage of stimulation were increased but, in the present experiments, the latency was always greater than 1 sec.

The amplitude of contraction was graded with stimulus voltage and pulse duration. For example, if the frequency was fixed at 20/sec and the voltage set at 50 V (i.e. about half the maximum output of the stimulator), the threshold pulse duration which was needed to elicit a contraction was found to be from 0·05 to 0·06 msec. If the pulse duration was increased, the amplitude of contraction increased up to a maximum value which was reached when the pulse duration was 0·5 msec. Pulses of much longer duration (around 20 msec) sometimes caused the amplitude of the contractile response to decrease.

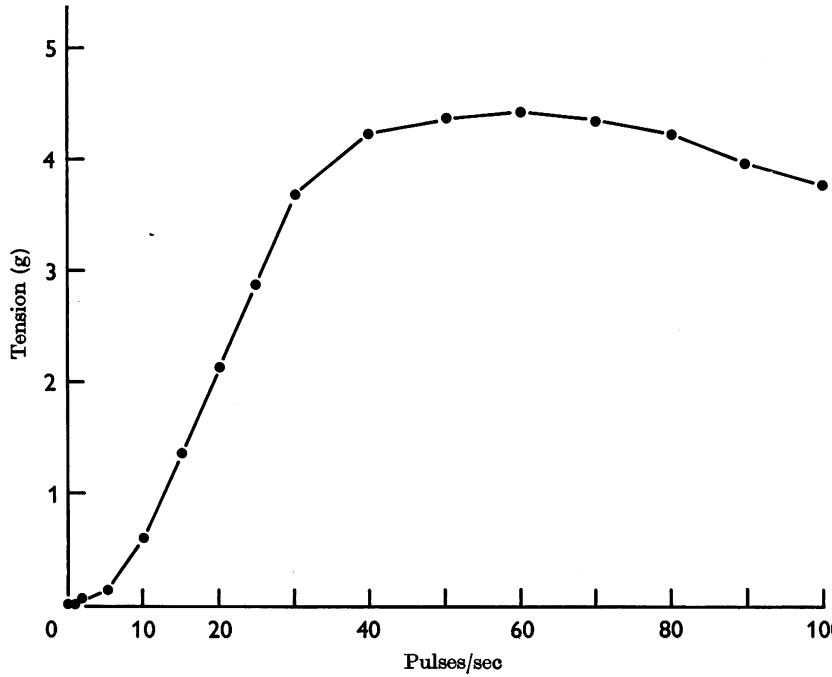

Fig. 5. Graph showing the relation between tension and frequency of stimulation. (10 sec trains of 0·8 msec pulses, voltage 50 V, and of variable frequency were given every 3 min.)

The amplitude of the response also varied with frequency of stimulation, as shown in Fig. 5. No responses were detectable for frequencies of stimulation lower than 1/sec. Contractions never occurred in response to single stimuli. In one series of experiments with single stimuli the duration of the pulse was increased up to 1 sec but again there was no detectable response. Figure 5 shows that the optimal frequency of stimulation at 36° C and 60/sec, the response declining if higher frequencies were used.

Responses to stimulation of peri-arterial nerves. Preparations were made

in which the artery which accompanies the mesenteric vein was left attached to the vein over a length of about 2 cm. The artery was placed in the stimulating electrode, as shown in Figure 1*b*. The same parameters of stimulation as those described for transmural stimulation were used. Responses to peri-arterial stimulation had the same time course as those in response to transmural stimulation (see Fig. 6). Threshold pulse duration and the relation between stimulus frequency and amplitude of response were also indistinguishable from those found for transmural stimulation. The only consistent difference was the maximum amplitude of the response. This was usually smaller when peri-arterial stimulation was used.

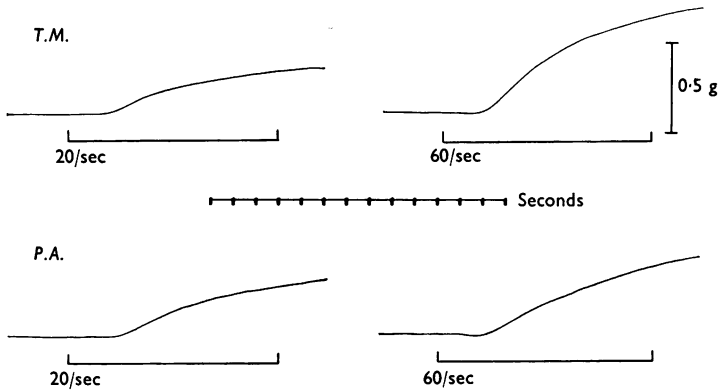

Fig. 6. Comparison of time course of the rising phase of isometric contractions in response to transmural (*T.M.*) stimulation (upper row) and peri-arterial (*P.A.*) stimulation (lower row) at 20/sec and 60/sec. Tension and time calibrations same for all records.

Drugs acting on α-adrenotrophic receptors. Noradrenaline had an excitatory effect on all preparations. Threshold doses (10^{-8} g/ml.) caused an increase in the amplitude of spontaneous contractions or the onset of mechanical activity in quiescent preparations. Larger doses caused prolonged contractions whose amplitude was often greater than the maximal response to nerve stimulation. The α-receptor blocking drugs, phentolamine and phenoxybenzamine, blocked the responses to both added noradrenaline and nerve stimulation (see Fig. 7, upper record). Concentrations of 10^{-6}–10^{-5} g/ml. were needed to block the response to nerve stimulation, phentolamine being somewhat more potent than phenoxybenzamine. Lower doses of these drugs (10^{-7} g/ml.) potentiated the response.

The contractions in response to added noradrenaline were blocked by lower concentrations of these α-receptor blocking drugs (e.g. noradrena-

line 10^{-7} g/ml. normally caused a strong contraction which was blocked by 10^{-8} g/ml. phentolamine).

Guanethidine. Responses to nerve stimulation were blocked within 10–20 min by guanethidine (5×10^{-5} g/ml.). In contrast to the α-receptor blocking drugs, guanethidine always caused potentiation of the effect of added noradrenaline, as shown in the lower record of Fig. 7.

Drugs which act on autonomic ganglia. Hexamethonium, in concentrations of up to 10^{-4} g/ml., did not depress the response to stimulation of intramural or peri-arterial nerves. In some preparations lower doses of hexamethonium caused a small potentiation of the nerve response.

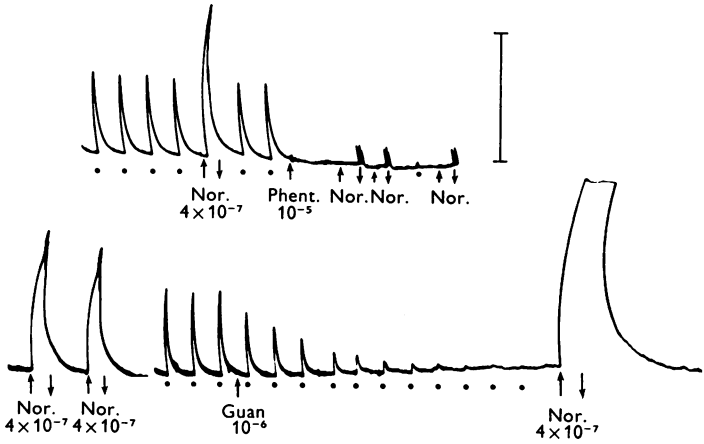

Fig. 7. Upper record: effect of phentolamine (10^{-5} g/ml.) on responses to transmural stimulation (•) and added noradrenaline (Nor.) Note 'wash-out' artifacts when noradrenaline was given after phentolamine.
Lower record: effect of guanethidine (10^{-6} g/ml.) on responses to nerve stimulation (•) and added noradrenaline (Nor.) Note the large potentiation of the response to noradrenaline in the presence of guanethidine.
Vertical calibration: 1·0 g for upper record, 0·5 g for lower record. Interval between nerve stimuli, 2 min.

DMPP (10^{-6} g/ml.) did not modify the response to nerve stimulation nor did it initiate or increase spontaneous activity.

These results suggest that there are few, if any, autonomic ganglion cells in the wall of the vein.

Acetylcholine. Acetylcholine had an excitatory effect on all preparations. The threshold concentration for contraction was 10^{-8} g/ml. Lower doses had no effect on the majority of preparations but caused some depression of spontaneous activity in a few cases. In general, responses to acetylcholine resembled those of noradrenaline in that contractions were often larger than those in response to maximal nerve stimulation.

The excitatory action of acetylcholine could be blocked by hexamethonium, as shown in Fig. 8. In this experiment, constant doses of acetylcholine (10^{-7} g/ml.) were alternated with stimulation of intramural nerves. Hexamethonium was added in increasing amounts (up to 5×10^{-5} g/ml.) which caused a progressive decline in the response to acetylcholine. This action of hexamethonium was rapidly reversed on washing out the bath.

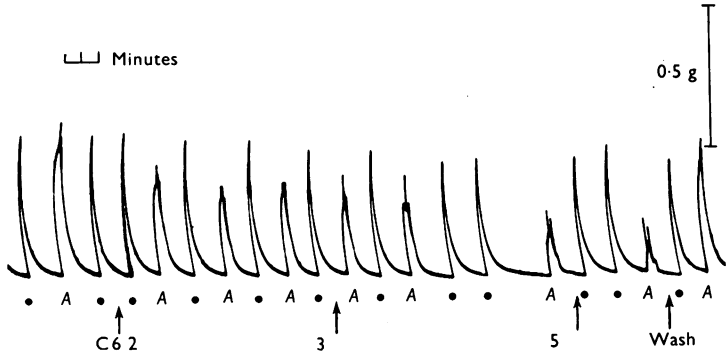

Fig. 8. Records from an experiment demonstrating the effects of varying doses of hexamethonium (C6, 2×10^{-5}, 3×10^{-5} and 5×10^{-5} g/ml.), on responses to nerve stimulation (•) and added acetylcholine (*A*).

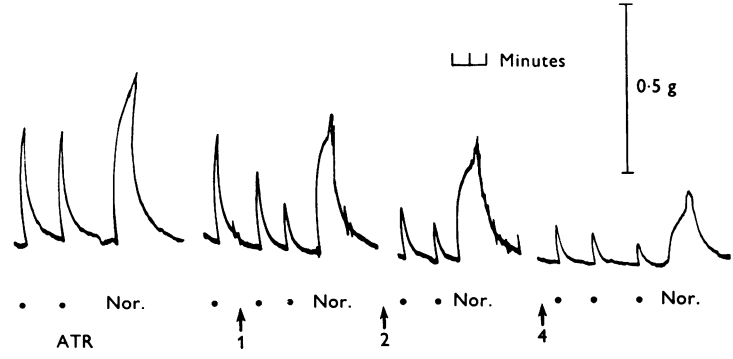

Fig. 9. Records from an experiment showing the effect of varying doses of atropine (ATR, 1×10^{-6}, 2×10^{-6} and 4×10^{-6} g/ml.) on responses to nerve stimulation (•) and added noradrenaline (Nor.)

In the presence of hexamethonium, acetylcholine caused depression of the response to nerve stimulation which was maintained for some minutes after washing out the acetylcholine. This is the reason for the decrease in amplitude of the contractions in response to nerve stimulation shown in Fig. 8.

Contractions in response to acetylcholine were blocked by phentolamine and phenoxybenzamine in concentrations similar to those needed to block

the response to nerve stimulation. Guanethidine, 10^{-5} g/ml., also blocked contractions due to added acetylcholine.

Atropine. The response to nerve stimulation was depressed by atropine (10^{-7} –10^{-5} g/ml). Nerve responses were never completely blocked by atropine but up to 90 % depression was recorded. Atropine also depressed the response to added noradrenaline, but again this response was never completely blocked. A typical experiment is shown in Fig. 9 in which the nerve response was depressed considerably more than the response to noradrenaline. Atropine (10^{-6} g/ml.), also reduced the contractions in response to added acetylcholine.

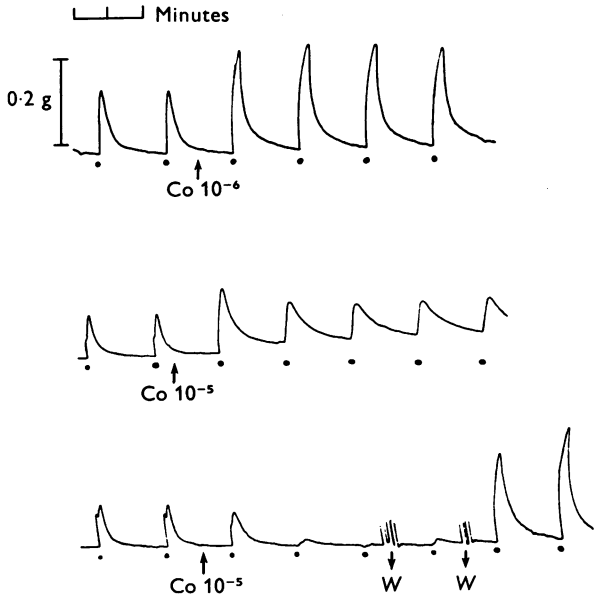

Fig. 10. The effect of cocaine (Co) on responses to nerve stimulation (•). Upper record shows potentiation by cocaine, 10^{-6} g/ml.; middle record shows potentiation followed by diminished relaxation caused by 10^{-5} g/ml.; lowest record shows the blocking action of 10^{-5} g/ml. in another preparation. Note the potentiation of the response after washing out the drug (*W*).

Cocaine and ouabain. Figure 10 shows the effect of cocaine on this preparation. In doses of up to 10^{-5} g/ml. the predominant effect was potentiation and prolongation of the response to nerve stimulation. Higher doses caused blockade. The initial effect of ouabain (10^{-7} g/ml.) was similar to that of low doses of covaine but prolonged exposure usually caused a marked increase in tone and decrease in responsiveness to nerve stimulation. This latter action of ouabain was not easily reversed.

Isoprenaline. The β-adrenotrophic drug, isoprenaline, caused a marked

reduction in the response to transmural stimulation. This finding is similar to that of Jowett & Holman (1964) for the vas deferens. They suggested that the stabilizing effect of the combination between catecholamines and β-receptors could depress the response to nerve stimulation.

Direct stimulation of the smooth muscle. In order to confirm the conclusion that these responses were due to nerve stimulation, tetrodotoxin 10^{-6} g/ml. was used. Responses to pulses of standard duration (0·8 msec) were completely blocked by the toxin. Prolonged stimulation (10 msec pulses at 20/sec for up to 30 sec) still produced a response. This differed in several ways from that described below. The preparation continued to shorten or develop tension for several minutes after the cessation of stimulation and took up to 30 min to relax back to the control level.

Similar responses have also been observed in the rabbit pulmonary artery (Bevan, 1962). They may be due to activation of the contractile components by a process which is not directly associated with any changes in the membrane potential of the smooth muscle cells.

DISCUSSION

Transmural stimulation of isolated segments of sheep mesenteric vein causes contractions of the longitudinal smooth muscle which appear to be mediated by post-ganglionic sympathetic nerves. The following evidence supports this view: (1) The characteristics of the stimuli which elicit these responses are typical of the adequate stimuli for autonomic C fibres innervating other preparations (Kuriyama, 1963; Paton, 1955). (2) Responses to transmural stimulation are similar to those in response to stimulation of peri-arterial nerves. (3) The action of drugs such as guanethidine, phentolamine, phenoxybenzamine and cocaine are typical of their effects on other preparations innervated by post-ganglionic sympathetic nerves.

The action of atropine in depressing the response to transmural stimulation is of interest. Although it caused a decrease in the response to noradrenaline, atropine was not as potent as the α-receptor blocking drugs in this respect. If the excitatory action of acetylcholine is considered together with the blocking action of atropine on responses to nerve stimulation and added acetylcholine it may be suggested that this smooth muscle is supplied with excitatory cholinergic nerves. Our present evidence does not rule out this possibility. However, the action of hexamethonium suggests that the predominant effect of acetylcholine is mediated through nicotinic receptors. The most likely site of these receptors would seem to be the sympathetic noradrenergic C fibres (Ferry, 1963). If acetylcholine caused the release of noradrenaline from these fibres, either directly or by their depolarization, the effect of acetyl-

choline should be blocked by phentolamine and phenoxybenzamine in concentrations which block the response to nerve stimulation, as shown in our results. However, it is not clear why the excitatory action of acetylcholine should also be blocked by atropine.

Guanethidine was effective in blocking the response to added acetylcholine. Since guanethidine potentiates the response to added noradrenaline, it must act by blocking the excitatory effects of acetycholine on the smooth muscle membrane, by blocking the action of acetylcholine on the prejunctional site from which it causes the release of noradrenaline, or by both these mechanisms.

Some evidence was found for an inhibitory action of acetylcholine. In the presence of hexamethonium, acetylcholine depressed both spontaneous activity and the response to nerve stimulation. The excitatory effects of acetylcholine were much more obvious in this preparation than its inhibitory effects, in contrast to the observations of de la Lande & Rand (1965) on the artery of the rabbit ear. This difference may be due to the different routes by which the drugs were given. In the rabbit ear artery preparation drugs were added to the solution which passed through the lumen of the vessel. In our experiments, drugs were added to the solution bathing the adventitia.

This preliminary survey of the pharmacology of the sheep mesenteric vein and its motor innervation suggests that the smooth muscle cells have both α- and β-adrenotrophic receptors, receptors for acetylcholine which are associated with inhibition and, possibly, receptors for acetylcholine which can bring about excitation. These results, together with morphological and physiological findings, suggest that this preparation may be a useful model for further studies on the innervation of venous smooth muscle.

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