J. Physiol. (1980), **309**, *pp.* 461–472
With 9 text-figures
Printed in Great Britain

NERVOUS MODULATION OF SPONTANEOUS CONTRACTIONS IN BOVINE MESENTERIC LYMPHATICS

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(*Received 12 March 1980*)

SUMMARY

1. Spontaneous isometric contractions were measured in 2 cm segments of bovine mesenteric lymphatics.

2. Field stimulation at 0·25, 1 and 4 Hz increased the frequency of contraction.

3. Tetrodotoxin 3×10^{-6} M blocked the response to electrical stimulation.

4. Phenoxybenzamine 3×10^{-7} M converted the excitatory effect of stimulation to an inhibitory one.

5. Propranolol 3×10^{-7} M potentiated the excitatory response to stimulation at 1 and 4 Hz.

6. Field stimulation was without effect in the presence of propranolol and phenoxybenzamine together.

7. Cocaine potentiated the response to stimulation at 0·25 and 1 Hz.

8. Atropine 3×10^{-7} M failed to block the excitatory effect.

9. Field stimulation at 0·5 and 1 Hz increased the frequency of spontaneous contractions and propulsion of fluid by 8 cm cannulated segments of lymphatic.

10. The results suggest that bovine lymphatic vessels have a noradrenergic innervation which is capable of controlling lymph flow.

INTRODUCTION

In 1927, Carleton & Florey demonstrated small non-myelinated nerve fibres in guinea-pig lacteals by vital staining with methylene blue while in the lacteals of squirrel they found a perilymphatic nerve network containing nerve cells. Florey (1927) also showed that the lacteals of cats contract in response to splanchnic sympathetic stimulation as well as to locally applied adrenaline. He found no response to vagal stimulation but quoted an observation by Bert & Laffont (1882) that vagal stimulation of dog lacteals produced a rapid dilatation followed by a contraction.

Most investigations into the nature of lymphatic innervation are based on the assumption that they constrict and dilate so as to alter either their resistance to flow (like arterial smooth muscle, e.g. Browse, 1968) or their capacity (like veins, e.g. Leandoer & Lewis, 1970). A third possibility which is less often considered is that lymphatics act as a series of hearts to propel lymph by their spontaneous contractions. The role of nerves in such a system would be to modulate their myogenic activity in a manner more like the autonomic innervation of the heart. Since there is now a wealth

0022-3751/80/2780-0849 $07.50

of evidence that lymphatics can propel lymph by their intrinsic contractions (Webb & Nicoll, 1944; Hall, Morris & Woolley, 1965; Campbell & Heath, 1973), the present study has approached the problem of lymphatic innervation from this third standpoint. Bovine mesenteric lymphatics were chosen as a suitable model since they are capable of pumping fluid in isolation (McHale & Roddie, 1976) and are known to be responsive to exogenous noradrenaline (Mawhinney & Roddie, 1973).

A preliminary account of this work has already been communicated to the Physiological Society (McHale, Roddie & Thornbury, 1979).

METHODS

Segments of lymphatic 2 cm in length and 2 mm in diameter were dissected from the mesenteries of freshly slaughtered cattle. These were mounted in an organ bath which was perfused at 35 °C with Krebs solution of composition (mM): NaCl, 120; $NaHCO_3$, 15·0; KCl, 5·9; NaH_2PO_4, 1·2; $CaCl_2$, 2·5; $MgCl_2$, 1·2; glucose, 11·0, gassed with 95% O_2, 5% CO_2. The resting tension was adjusted to 2–4 mN and the preparation was allowed to equilibrate for at least 30 min. Longitudinal tension changes were measured by means of an isometric tension transducer (Statham UC3) and recorded on a Devices M4 chart recorder. Regular spontaneous activity of the type described by Mawhinney & Roddie (1973) developed in approximately 60% of preparations. Electrical field stimulation was applied via platinum electrodes at top and bottom of the organ bath. For this a Square One modular stimulator was set to deliver pulses at a nominal voltage of 35 V and pulse width 0·3 msec. Trains of pulses of varying frequency were delivered for periods of either one or two minutes. The protocol used for most of the experiments consisted of a 1 min period of stimulation followed by 10 min without stimulation, and then another 1 min of stimulation and the cycle was repeated. Results were expressed as the average frequency for the 5 min period preceeding stimulation, the average frequency for the 1 min period of stimulation and the average frequency for the 5 min period immediately following stimulation.

In some experiments a different preparation was used. Longer 8 cm lengths of lymphatic were cannulated at both ends and set up in a horizontal organ bath so that fluid was pumped from a constant pressure reservoir at the inflow and through a drop-counter outflow as a result of the spontaneous contractions of the vessel (McHale & Roddie, 1976). Measurements were made of outflow pressure fluctuations using a pressure transducer (Statham P23H) and of flow by means of a drop counter. Electrical stimulation was as before except that the nominal voltage was at 95 V because of the larger organ bath volume and the stimulation period was 2 min to allow time to observe flow changes.

The drugs used were as follows: phenoxybenzamine hydrochloride (Dibenyline, S.K.F.); propranolol hydrochloride (Inderal, I.C.I.); atropine sulphate (Sigma); cocaine hydrochloride (Duncan Flockhart); tetrodotoxin (Sigma). All of these were made up to the final concentration in Krebs solution.

RESULTS

The effect of field stimulation

More than half of the preparations set up showed regular spontaneous activity similar to that observed by Mawhinney & Roddie (1973). This consisted of a rapid contraction, an almost equally rapid relaxation followed by a pause of 30 sec–1 min after which there was another similar contraction. When a 1 min period of field stimulation was applied (at a pulse width of 0·3 msec and 35 V nominal) at varying frequencies from 0·25 to 16 Hz the rate of spontaneous contractions increased (Fig. 1). At 0·25 Hz the response was barely perceptible but at 1 Hz increased by about 50% while during stimulation at 4 Hz rate of contraction almost doubled. At the higher

frequency of 16 Hz there was an initial transient increase in frequency of contraction but this led to a series of rapid fluctuations which fused to give a sustained increase in tension. At the end of the stimulation period base line tension decreased but discrete phasic contractions did not return for a further minute and then at reduced force. Contractions at prestimulation force returned five minutes later.

To establish whether the observed changes in frequency were mediated by nerves, tetrodotoxin in a dose of 3×10^{-6} M was perfused. Before drug addition a 1 min period of stimulation at 2 Hz increased contraction rate from 1·8 to 6 beats/min (lower record Fig. 1). Perfusion with tetrodotoxin had itself no effect on spontaneous activity

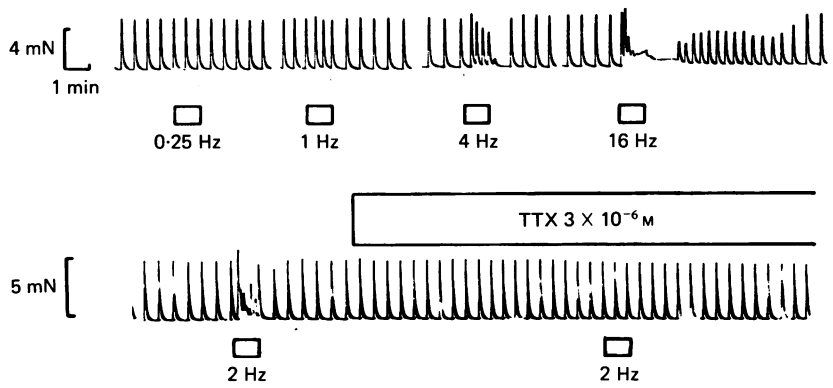

Fig. 1. Upper record, the effect on spontaneous contractions of 1 min periods of stimulation (0·3 msec pulses at 35 V nominal) at four different frequencies. Lower record, the effect of 1 min stimulation at 2 Hz, before and after the addition of tetrodotoxin.

but 10 min later when the vessel was again stimulated at 2 Hz for 1 min the effect was abolished. The response to all the stimulus frequencies used in the present study was similarly blocked by this dose of tetrodotoxin. In most subsequent experiments three stimulus frequencies were chosen, 0·25 Hz (which only just produced a response), 1 Hz and 4 Hz (this had an almost maximal effect on frequency of contraction).

The effect of 1 min periods of stimulation at the above three frequencies in sixteen experiments is summarized in Fig. 2. The diagram consists of three groups of bar charts. The first and third bar in each group represent the average frequency of spontaneous contraction for the 5 min period before and after stimulation respectively. The centre bar in each group represents the average contraction frequency during the 1 min stimulation period. During the six control periods spontaneous rate remained fairly constant at about 1·5–1·8 contractions per minute. When the vessels were stimulated, frequency increased slightly to 2/min at 0·25 Hz but the resting rate was doubled at 1 Hz and tripled at 4 Hz.

Effect of phenoxybenzamine

Phenoxybenzamine (3×10^{-7} M) converted the normal excitatory effect of field stimulation to an inhibitory one. A record from one such experiment is shown in Fig. 3. Before drug addition the lymphatic was beating spontaneously at between 2 and 3 beats/min. This was increased two and fourfold during stimulation at 1 and

4 Hz respectively. Following the addition of phenoxybenzamine, spontaneous activity was completely inhibited during the 1 Hz stimulation period and when it returned 30 sec later it was with reduced force and slightly increased frequency. Force then gradually returned to pre-stimulus levels. The 4 Hz stimulus had a more striking inhibitory effect. This time spontaneous activity did not return until 4 min after cessation of stimulation and again force was decreased. When six such experiments were summarized, the mean duration of inhibition of spontaneous activity after the beginning of stimulation was 1·4 min ± 0·22 (S.E.) at 1 Hz and 3 min ± 0·55 (S.E.) at 4 Hz. At 0·25 Hz contractions still occurred within the stimulus period but their rate was reduced.

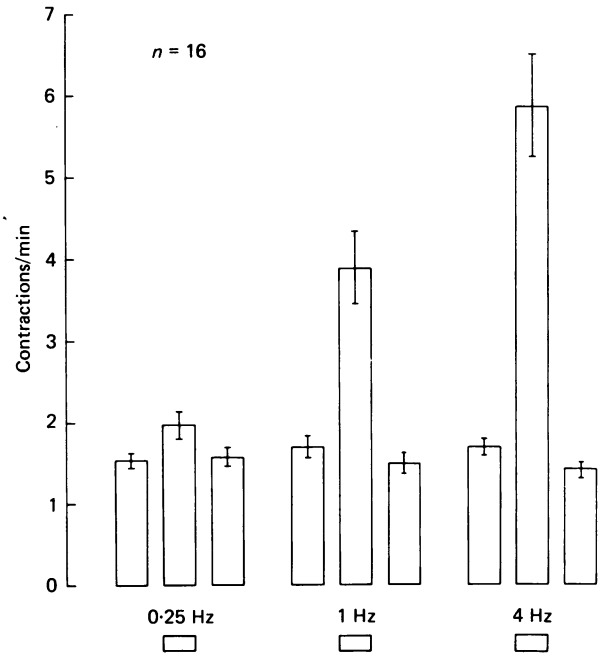

Fig. 2. Summary of the effects of field stimulation at the three frequencies shown. The first and third bar in each group of three represent the average rate of spontaneous contraction for the 5 min period before and after stimulation. The centre bar represents the average contraction rate during the 1 min period of stimulation. Vertical lines represent ± 1 S.E. of the means.

The effect of propranolol

In contrast to the above effect propranolol 3×10^{-7} M potentiated the excitatory effect of electrical stimulation. A typical experiment is shown in Fig. 4. The upper record shows that stimulation at 1 and 4 Hz increased the resting spontaneous rate from 2·7/min to 3 and 5/min respectively. In the presence of propranolol this increased to 5 and 9/min respectively, although the drug had itself no effect on the resting frequency of contraction. Six such experiments are summarized in Fig. 5. The layout of the diagram is similar to Fig. 2 except that on this occasion there are three groups of three paired bar charts. The first in each pair (plain) shows responses

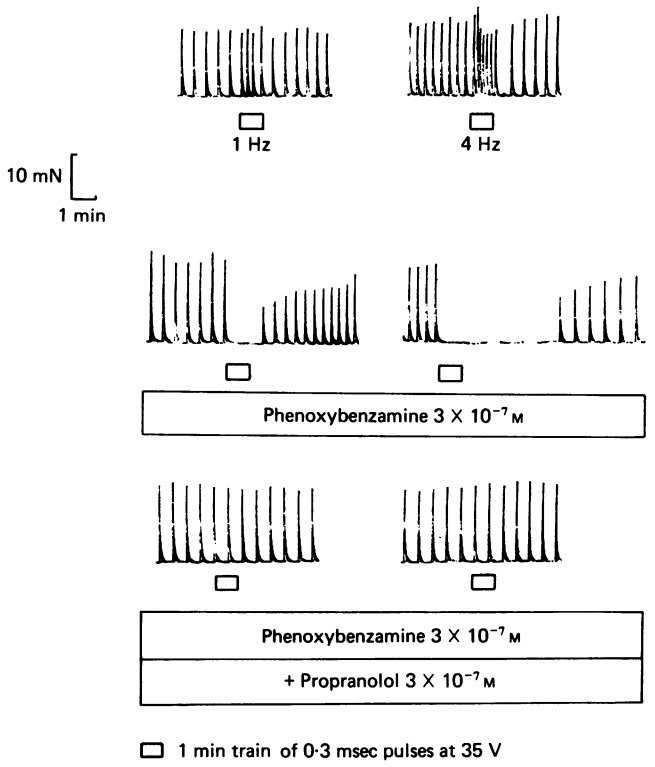

Fig. 3. The effect of stimulation at 1 and 4 Hz in Krebs solution (upper record) in the presence of phenoxybenzamine (middle record) and in the presence of both phenoxybenzamine and propranolol (bottom record).

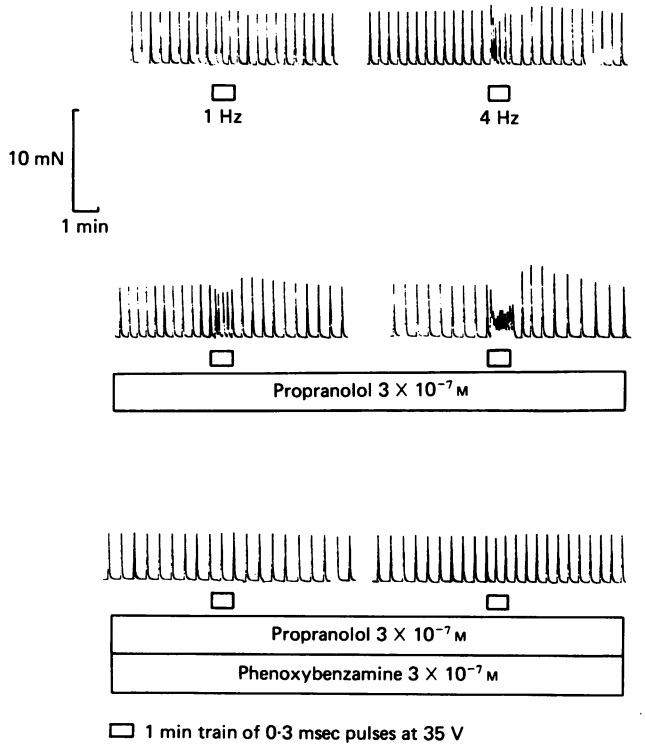

Fig. 4. The effect of stimulation at 1 and 4 Hz in Krebs solution (upper record) after addition of propranolol (middle record) and in the presence of both propranolol and phenoxybenzamine (bottom record).

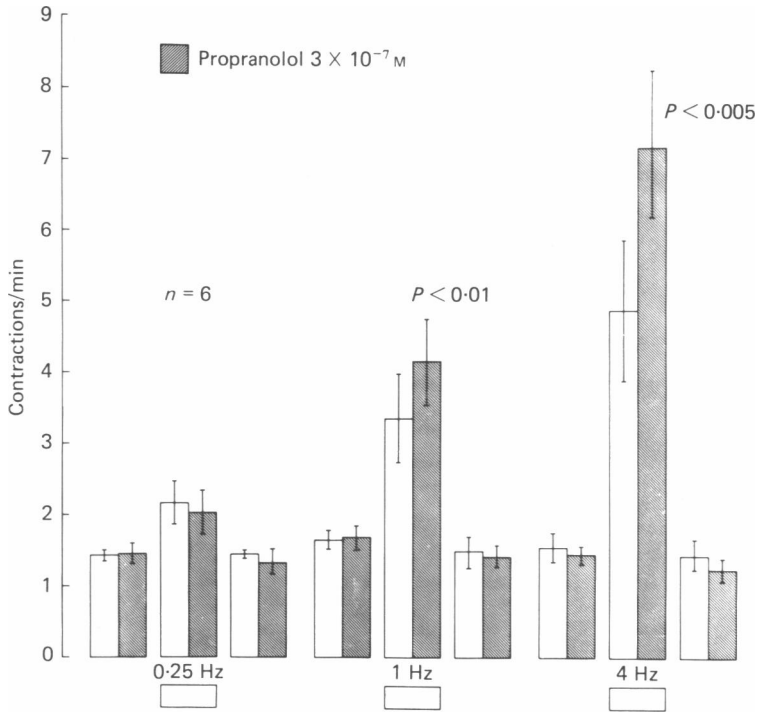

Fig. 5. Summary of the effect of propranolol at three stimulation frequencies. The layout is the same as that in Fig. 2. The plain bars summarize responses in Krebs solution. The hatched bars are those found after the addition of propranolol. The values during stimulation at 1 and 4 Hz were significantly different when compared using a paired t test. Vertical lines are ± 1 S.E. of the means.

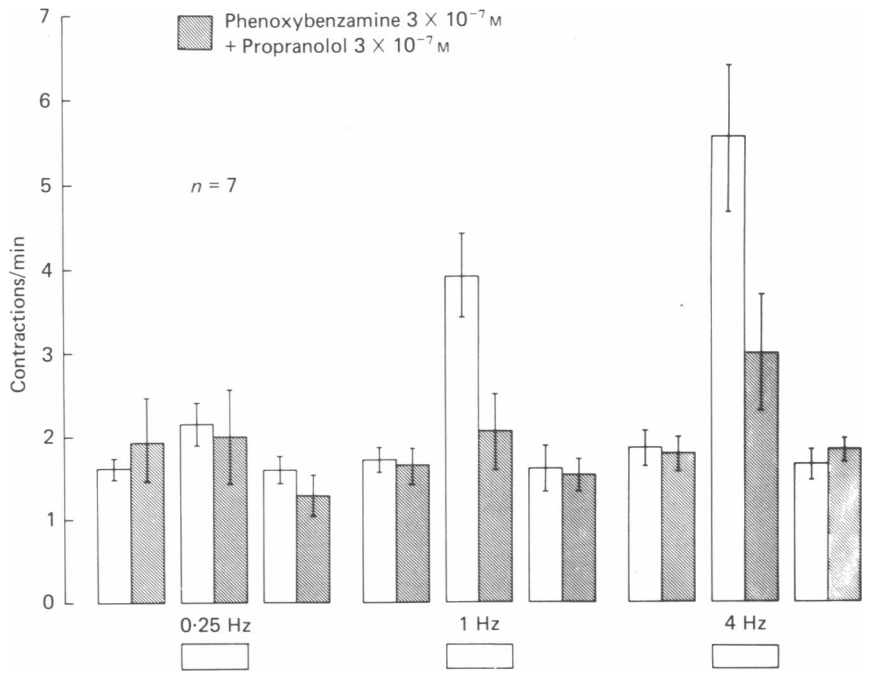

Fig. 6. Summary of the effect of field stimulation in the absence of drug (plain bars) and in the presence of both propranolol and phenoxybenzamine (hatched bars). Vertical lines are ± 1 S.E. of the means.

in the absence and the second (hatched) in the presence of 3×10^{-7} M-propranolol. The drug did not affect contraction frequency during the control periods before and after electrical stimulation but there was a significant potentiation of the effect of 1 min periods of field stimulation at 1 Hz ($P < 0{\cdot}01$) and 4 Hz ($P < 0{\cdot}005$) respectively. No such potentiation was observed during the 0·25 Hz stimulation period.

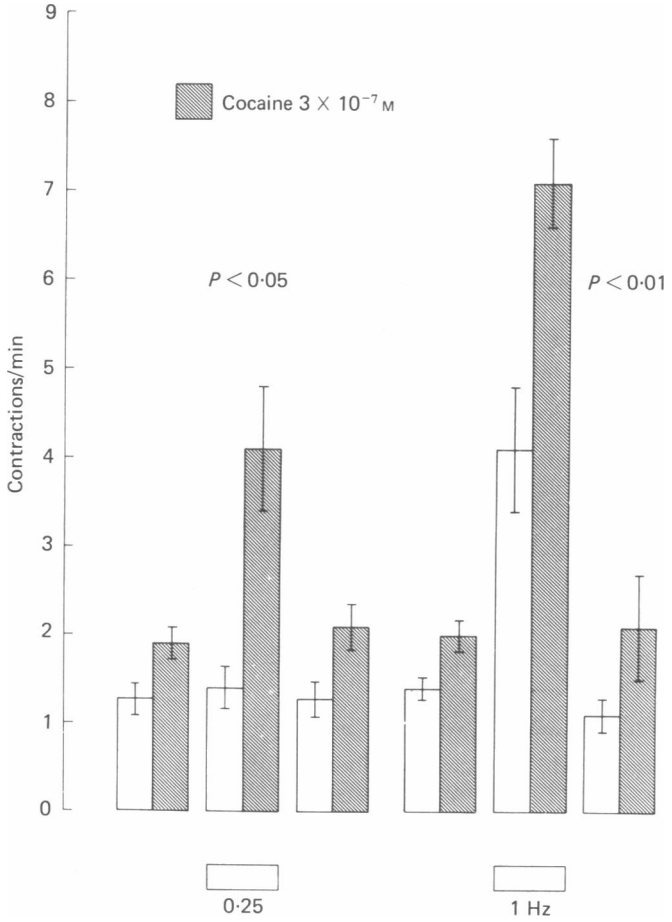

Fig. 7. Summary of the effect of cocaine. No significant differences were found when the control values were compared by a paired *t* test. The probabilities during the stimulation periods are shown.

Effect of both phenoxybenzamine and propranolol

This was examined either by adding propranolol 3×10^{-7} M to the phenoxybenzamine already present (bottom record, Fig. 3) or by adding phenoxybenzamine 3×10^{-7} M to the propranolol already present (bottom record, Fig. 4). It is evident in Fig. 3 that the inhibitory effect of electrical stimulation seen in the presence of phenoxybenzamine was abolished when propranolol was added. In fact, the effect of field stimulation was completely neutralized in the presence of both drugs. Similarly, the potentiation of excitation seen in the presence of propranolol (Fig. 4) was abolished

when phenoxybenzamine was added. Again field stimulation had no effect at 1 Hz in the presence of both drugs but there was a slight increase in frequency of contraction at 4 Hz. A summary of the effects of stimulation at 0·25, 1 and 4 Hz in seven experiments is shown in Fig. 6. The diagram layout is similar to Fig. 5. When both drugs were present, frequency of contraction during stimulation at 0·25 and 1 and 4 Hz did not differ significantly from that of the control periods.

The effect of cocaine

Cocaine is known to block re-uptake of noradrenaline into adrenergic nerve terminals (uptake 1, Iversen, 1967). One would expect, therefore, that if the innervation of these lymphatic vessels is noradrenergic, cocaine should potentiate the effect

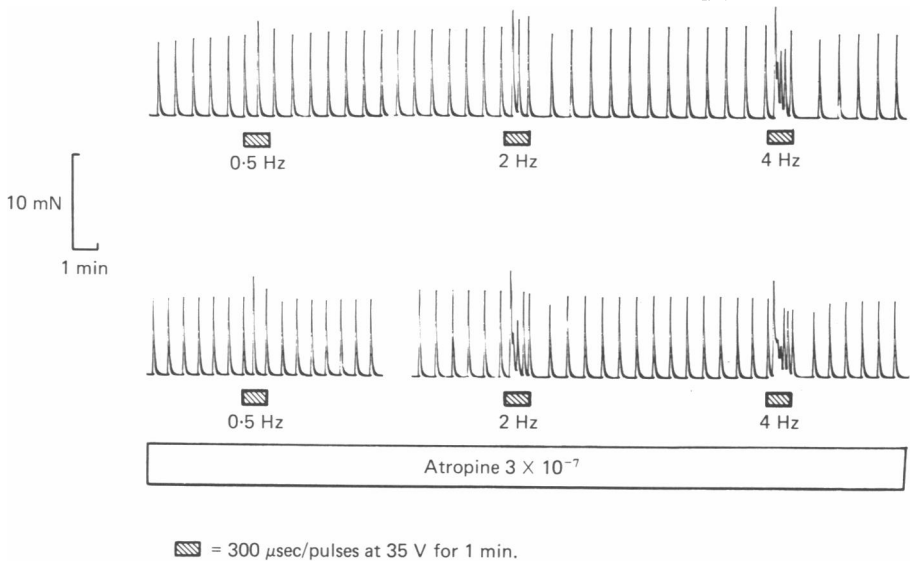

Fig. 8. The effect of field stimulation before and after the addition of atropine. The excitatory effect was not blocked.

of field stimulation. That this indeed is so can be seen in Fig. 7. In the six experiments summarized in the same manner as those in Fig. 5 by means of pairs of bars, it is evident that cocaine 3×10^{-6} M (hatched bars) increased the frequency of spontaneous activity both in control and stimulation periods. However, the response during stimulation at both 0·25 and 1 Hz was dramatically magnified increasing almost threefold at 0·25 Hz and almost twice at 1 Hz. The increase during control periods may well be due to an increase in the spontaneous release of transmitter as a result of uptake 1 blockade.

The effect of atropine

Fig. 8 shows three 1 min periods of field stimulation at 0·5, 2 and 4 Hz before and after the addition of 3×10^{-7} M-atropine. This dose failed to block the increase in contraction frequency induced by electrical stimulation and may indeed have potentiated it. This slight potentiation was not a constant response, occurring in only two out of five experiments. In none of the five experiments, however, did atropine block or reduce the excitatory effect of electrical stimulation.

Effect of field stimulation on pumping activity

A record typical of that obtained with the pumping preparation is shown in Fig. 9. The lower trace represents pressure fluctuations measured distal to the outflow cannula. The upper trace shows cumulative flow (i.e. the number of drops accumulated between resetting of the counter, expressed in ml.). The outflow pressure showed a rapid rise followed by a more gradual fall and then a slower rise to the resting transmural pressure of 4 cm H_2O where it remained till the next contraction. Flow

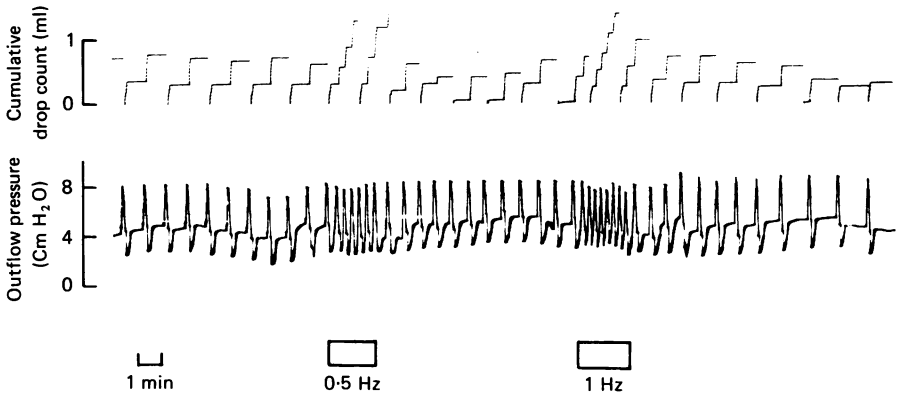

Fig. 9. The effect of 2 min periods of stimulation at 0·5 and 1 Hz. Flow was increased to a lesser extent at 1 Hz due to the decrease in stroke volume.

was intermittent; when the rise in outflow pressure overcame the resistance in the outflow tube a series of drops was expelled. This was followed by a pause until the next contraction produced a further series of drops. The average flow rate for the 5 min period before stimulation was 0·43 ml./min while the frequency was 1·3 beats/min. When stimulation was applied, frequency of contraction increased to 3 beats/min while average flow during the stimulation period increased to 1·37 ml./min. Stimulation at 1 Hz resulted in a greater increase in frequency of contraction (4 beats/min) than observed at 0·5 Hz but flow was not increased as much (1·15 ml./min) because of a decrease in stroke volume. Five such experiments consistently showed this decreased flow augmentation at increased stimulation frequencies. At frequencies of 16 Hz or higher, flow was quite markedly depressed during the stimulation period and often did not recover for 5–10 min afterwards.

DISCUSSION

The purpose of this investigation was to stimulate the intramural nerves believed to be present in bovine mesenteric lymphatics and to examine the effects of the transmitter or transmitters released, on spontaneous contractile activity. No attempt was made to identify substances released but the evidence presented is strongly suggestive of noradrenergic transmission. Field stimulation at short pulse widths has been used by many people (Paton, 1955; Patterson, 1965; Ljung, Bevan, Pegram, Purdy & Su, 1975) to selectively stimulate nerves in preparations where separation of smooth

muscle and nerve trunk is impossible. A pulse width of 0·3 msec at 35 V nominal was chosen in the present experiments because these values had been found to be supraliminal (Ohhashi, McHale, Roddie & Thornbury, 1980). Larger pulse widths and voltages were not used in case of direct muscle stimulation since Duckles (1979) found that increasing voltage at pulse widths as low as 0·3 msec could result in direct muscle stimulation. The fact that tetrodotoxin completely blocked field stimulation at all frequencies used in this study suggests that nerves were being selectively stimulated. The responses noted in the presence of phenoxybenzamine and propranolol were exactly those expected if noradrenaline were being released and they indicate the existence of both α and β adrenoceptors in lymphatic smooth muscle, the α excitatory effect normally predominating over a weaker β inhibitory effect. The blocking drugs in these doses showed no non-specific depressant effect on spontaneous contractility during the control periods. Cocaine did, however, increase spontaneous rate when it was added, perhaps by potentiating the effect of spontaneously released noradrenaline but the potentiation was not significant. No evidence was provided for the existence of an excitatory cholinergic innervation or for the existence of any other transmitter. The results demonstrate that spontaneously active isolated lymphatics respond to field stimulation by increasing their frequency of contraction and that, at low stimulus frequencies, this can result in an increase in fluid propulsion. Mawhinney & Roddie (1973) observed very similar increases in spontaneous rhythm of the same isolated vessels in response to noradrenaline. They found that a dose of noradrenaline 5 ng/ml. almost doubled frequency of contraction but slightly decreased force while 25 ng/ml. increased frequency about fourfold but at the expense of a dramatic reduction in force of contraction. They suggested that the increase in rate of contraction might more than compensate for the decrease in force and the net effect would be an increase in lymph flow. In a later study McHale & Roddie (1977) found that noradrenaline 50 ng/ml. consistently depressed flow in the isolated pumping preparation. This presented the interesting paradox that noradrenaline, if it were a transmitter released from lymphatic nerves (as seemed likely), was acting to inhibit lymph flow by decreasing the force of spontaneous contractions while at the same time exciting an increase in the frequency of those contractions.

McHale, Kirkpatrick & Roddie (1979) examined the effect of noradrenaline on spontaneous electrical and mechanical activity in the single sucrose gap apparatus. A dose of 100 ng/ml. produced a small depolarization, an increase in the slope of the prepotential and an increase in the rate of firing of the single action potentials which preceded each contraction but there was little change in spike amplitude and shape. Force of contraction was reduced out of proportion to the small change in electrical activity. This might indicate that noradrenaline impairs either excitation–contraction coupling or conduction between smooth muscle cells and thus the efficiency of contraction.

It appears from the evidence of this study that noradrenaline is released as a neurotransmitter in bovine mesenteric lymphatics, that its effect *is* an excitatory one and that when released in small amounts (such as during stimulation at 1 Hz) spontaneous propulsion of fluid is enhanced. Higher rates of stimulation and high doses of exogenous noradrenaline appear to decrease efficiency of spontaneous contractions and thus decrease flow despite an increase in rate. This may be because

of decreased synchrony of individual contractions (for reasons mentioned above) or because increase in rate above a critical value results in fusion of contraction and disappearance of the clear cut individual contractions necessary for effective pumping (a problem faced by a smooth muscle pump not shared by one consisting of cardiac muscle!).

This problem may not arise *in vivo*, however, since the impulse frequency in the nerves supplying lymphatics is probably quite low. It is known (Folklow, 1952) that sympathetic nerves in general fire at low rates *in vivo* and if lymphatic innervation is similar to that of veins one might expect firing rates even lower than in most sympathetic nerves. Browse, Lorenz & Shepherd (1966), for instance, found that stimulation of the sympathetic chain at 0·2 Hz was sufficient to produce an increased tension of the capacity vessels of the dog's hind limb while stimulation at 3 Hz produced a response that was 60 % of the maximum. The control of lymphatic pumping *in vivo* may consist of a balance of local regulatory forces (such as changes in transmural pressure and the effects of vasoactive hormones) and an innervation whose basal firing rate is low. Small changes in this impulse traffic if maintained for long periods could result in quite large changes in lymph flow.

We would like to thank Eric Welsh of the City of Belfast meat plant for the use of laboratory facilities, Smith, Kline & French for providing phenoxybenzamine and Miss Angela Kinnen for technical assistance.

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