Mediation of excitatory neurotransmission by the release of ATP and noradrenaline in sheep mesenteric lymphatic vessels

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1. Spontaneous isometric contractions were measured in rings of sheep mesenteric lymphatics. Field stimulation at short pulse widths increased the frequency of spontaneous contractions and this response was blocked by 3×10^{-7} M ω-conotoxin and by 10^{-6} M guanethidine.

2. Rings that had been incubated with [^{3}H]noradrenaline released ^{3}H in response to field stimulation in a frequency-dependent manner.

3. Exogenous ATP mimicked the response to field stimulation and this was blocked by 10^{-4} M suramin but not by prior desensitization with 10^{-6} M α,β-methylene ATP. Exogenous noradrenaline was not blocked by 10^{-4} M suramin.

4. The excitatory response to field stimulation was not blocked by 10^{-4} M suramin but a combination of 10^{-4} M suramin and 3×10^{-6} M phentolamine did block the response.

5. In rings taken from sheep that had been pretreated with reserpine, 10^{-4} M suramin alone blocked the response to field stimulation.

6. The results of this study suggest that the excitatory response to stimulation of intramural nerves in sheep mesenteric lymphatics is mediated by the release of both ATP and noradrenaline.

Stimulation of the splanchnic nerve in anaesthetized sheep increases the pumping activity of the main intestinal lymph duct (Harty, McGeown, McHale & Thornbury, 1988) but this response cannot be blocked by adrenergic antagonists (Harty, 1990). This is a surprising finding in view of the considerable body of evidence, both histochemical and functional, that lymphatics of other species have a noradrenergic innervation (Todd & Bernard, 1973; Alessandrini, Gerli, Sacchi, Pucci & Fruschelli, 1981; McHale, 1985, 1991, 1993; VanHelden, 1993). The studies of McHale, Roddie & Thornbury (1980) and Allen & McHale (1986) demonstrated that isolated bovine mesenteric lymphatics had a simple noradrenergic innervation which could be blocked completely by a combination of α- and β-antagonists. The possibility that the differences between responses to sympathetic stimulation in the sheep and *in vitro* responses in bovine vessels were due to differences in the behaviour of nerves in the living animal was excluded by the work of Harty, Thornbury & McHale (1993). These authors showed that the response to field stimulation in isolated rings of sheep mesenteric lymphatics could not be blocked with adrenergic antagonists but they did not establish the nature of the innervation.

The purpose of the present study was to take a closer look at the innervation of sheep lymphatic vessels and attempt to establish the identity of the transmitter or transmitters involved. A preliminary account of part of this study has been communicated to the Physiological Society (Hollywood & McHale, 1993*a*).

METHODS

Segments of main lymphatic duct 5 cm in length and 2 mm in diameter were dissected from the mesenteries of sheep approximately 10 min after slaughter. The vessels were transported in warmed oxygenated Krebs solution to the laboratory where the surrounding fat and connective tissue was removed from the lymphatic by sharp dissection. Rings of lymphatic 2 mm in diameter and 8 mm in length were then dissected from the main duct, suspended between stainless-steel hooks and placed into a water-jacketed organ bath (volume 5 ml) maintained at 37 °C. The rings were perfused with Krebs solution of composition (mM): NaCl, 120; $NaHCO_3$, 25·0; KCl, 5·9; Na_2HPO_4, 1·2; $CaCl_2$, 2·5; $MgCl_2$, 1·2; glucose, 11·0; gassed with 95% O_2, 5% CO_2. The rings were then adjusted to a tension of 2–4 mN and the vessels were allowed to equilibrate for at least 30 min. Isometric tension changes were measured with Statham UC3 or Dynamometer UF1 transducers and the output from these was written on Gould 2020 or 8000S chart recorders. When the vessels developed regular spontaneous activity, field stimulation was applied via platinum electrodes at the top and bottom of the organ bath. The pulse width was kept short to selectively stimulate nerves (0·3 ms pulse width, 35 V nominal) and 1 min trains of varying frequency were given at intervals of not less than 5 min. Results were expressed as the mean frequency of contraction for the 2 min period preceding stimulation and for the 1 min

during stimulation. Results were expressed as means ± 1 standard error of the mean (S.E.M.). Statistical comparisons were made using Student's paired t test, taking the $P < 0.05$ level as significant.

In a separate procedure reserpine was administered intraperitoneally for 5 days (2 mg kg^{-1} per day) to four sheep in the animal holding facility. The sheep were killed on the sixth day by intravenous injection of pentobarbitone. The main mesenteric lymphatic duct was dissected as above.

Drugs used were: phentolamine mesylate (Rogitine; Ciba, UK); α,β-methylene adenosine triphosphate, adenosine triphosphate (disodium salt), reserpine, cytochrome C (Sigma, UK); suramin (Germanin; Bayer, UK); noradrenaline bitartrate (Levophed; Winthrop Laboratories, UK); ω-conotoxin (GVIA conotoxin), NPY (porcine neuropeptide Y), PYX-2 (Peninsula Laboratories, UK). All of these drugs were made up to their final concentrations in Krebs solution with the exception of ω-conotoxin which was added to a Krebs solution containing 0·1 mg ml^{-1} bovine cytochrome C to prevent non-specific binding of ω-conotoxin to the containers and tubes (Feldman, Olivera & Yoshikami, 1987).

RESULTS

Approximately 50% of the preparations set up showed spontaneous contractions which were phasic in nature and consisted of a rapid contraction followed by a slower relaxation, similar in nature to those described by McHale *et al.* (1980) for bovine lymphatics. The typical effect of 1 min periods of field stimulation (pulse width 0·3 ms and 35 V nominal) at frequencies of 1 and 2 Hz is shown in the upper panel of Fig. 1. At 1 Hz contraction frequency increased by approximately 70%, and at 2 Hz it increased by 140% of control. To demonstrate that these responses were mediated by nerves, ω-conotoxin at a concentration of 3×10^{-7} M was added to the bathing solution. Application of ω-conotoxin did not affect spontaneous activity but when field stimulation was repeated in its presence (Fig. 1, lower panel) the response was greatly reduced, suggesting that the excitatory responses to field stimulation were nerve mediated.

The effects of putative neurotransmitters

The results of the study referred to above (Harty *et al.* 1993) made it clear that the excitatory response to field stimulation was not blocked by adrenergic or cholinergic blockers, or by α,β-methylene ATP, and this led to the tentative conclusion that the innervation was neither noradrenergic nor cholinergic nor purinergic. We therefore examined a range of possible transmitters including 5-HT and NPY. Although 5-HT has an excitatory effect similar to nerve stimulation in bovine vessels (Hutchinson, Hollywood, Burke, Allen & McHale, 1992), its effect in sheep lymphatics was to cause a concentration-dependent inhibition of spontaneous rhythm (Hollywood & McHale, 1993*b*), thus clearly ruling it out as a possible excitatory transmitter. NPY, on the other hand, did have an effect closely resembling field stimulation but the response to field stimulation could not be blocked by the NPY antagonist PYX-2 or by desensitizing with NPY.

The effect of guanethidine

Guanethidine is known to block sympathetic nerves by a complex mechanism that is as yet incompletely understood. The action is probably a combination of a local anaesthetic effect (blocking action potential propagation in nerve terminals; Brock & Cunnane, 1988) and depletion of neurotransmitter in nerve terminals. Figure 2 shows a summary of five experiments where the lymphatics were stimulated for 1 min at 0·5 Hz before and in the presence

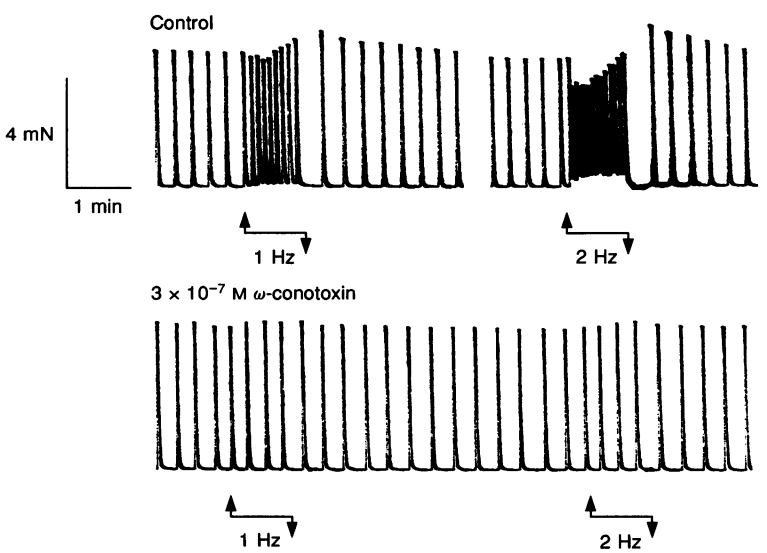

Figure 1. Blockade of nerve response by ω-conotoxin
The effects of 1 min periods of field stimulation at 1 and 2 Hz before and during perfusion with ω-conotoxin.

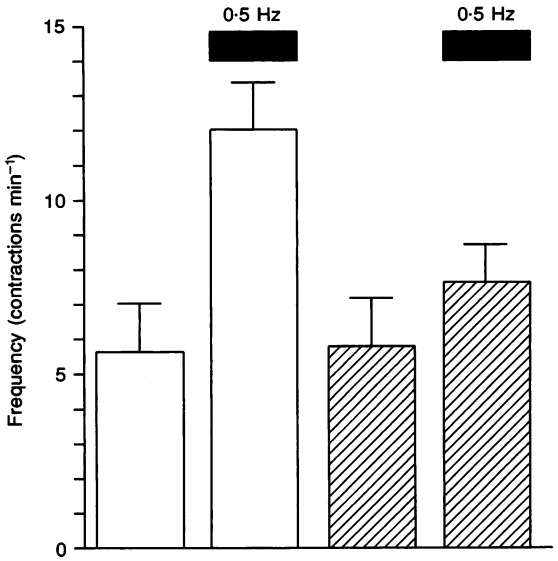

Figure 2. Effect of guanethidine on field stimulation
Summary of five experiments on the effect of field stimulation before and during perfusion with 10^{-6} M guanethidine. The paired columns represent mean contraction frequency (vertical bars are 1 S.E.M.) for the 2 min period before and the 1 min period during field stimulation in control conditions (□) and in the presence of guanethidine (▨).

of 10^{-6} M guanethidine. In control conditions spontaneous contraction frequency increased from $5{\cdot}6 \pm 1{\cdot}4$ to $12{\cdot}0 \pm 1{\cdot}4$ contractions min^{-1}. In the presence of guanethidine the response to field stimulation was significantly reduced (from $5{\cdot}8 \pm 1{\cdot}36$ to $7{\cdot}6 \pm 1{\cdot}12$ contractions min^{-1}, $P < 0{\cdot}05$).

The evidence presented so far suggests that these vessels have a sympathetic innervation but provides no indication of the nature of the transmitter or transmitters released by these nerves. Failure to implicate 5-HT or NPY led us to reappraise our previous conclusion that transmission was neither noradrenergic nor purinergic since noradrenaline and ATP would still appear to be the most likely transmitters. The next obvious step was to load the vessels with [^{3}H]noradrenaline to examine whether release of ^{3}H could be detected in response to field stimulation. Figure 3 shows such an experiment.

The upper panel shows the effect on frequency of isometric contraction of field stimulation at a range of frequencies from 0·5 to 8 Hz while the lower panel indicates the ^{3}H efflux from the tissue (in disintegrations min^{-1}). At the beginning of the experiment the vessel was contracting spontaneously at a rate of 5 contractions min^{-1}. At the points indicated below the record field stimulation was applied for 1 min periods at the frequencies shown. Frequency of spontaneous contractions and ^{3}H efflux increased in parallel with increasing

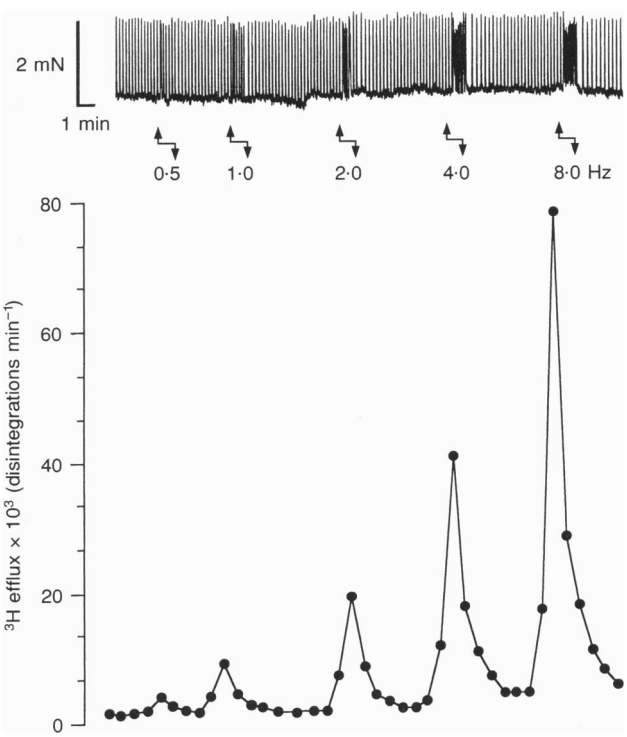

Figure 3. Relationship between increase in contraction frequency and ^{3}H efflux
Effect of five frequencies of field stimulation on contraction frequency (upper panel) and ^{3}H efflux (lower panel) in the same lymphatic vessel.

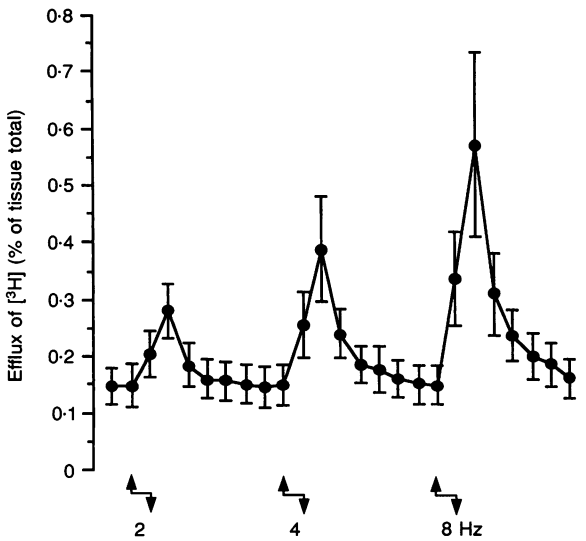

Figure 4. ^{3}H efflux in response to nerve stimulation
Summary of seven experiments where efflux of ^{3}H in response to three frequencies of field stimulation is expressed as a percentage of total ^{3}H in the tissue at the time of stimulation. Vertical bars represent ± 1 S.E.M.

stimulus frequency. Figure 4 shows a summary of seven such experiments where ^{3}H efflux is expressed as a percentage of the total ^{3}H in the tissue at the time of stimulation. Basal efflux was about 0·15 ± 0·03% and this increased to peaks of 0·28 ± 0·05, 0·39 ± 0·09 and 0·58 ± 0·16% in response to stimulation at frequencies of 2, 4 and 8 Hz respectively. These results are very similar to those found in bovine lymphatic vessels (Allen, McCarron, McHale & Thornbury, 1988) and suggest that noradrenaline was being released in response to field stimulation.

The effects of exogenous ATP

The demonstration by Chen, Fan, Hu & Brading (1992) that α,β-methylene ATP did not block the ATP-induced contractile response in rabbit bladder alerted us to the possibility that ATP-induced increases in spontaneous rhythm in sheep lymphatics might be similarly resistant to the desensitizing effect of α,β-methylene ATP. It was therefore essential to establish whether exogenously administered ATP could mimic the effects of field stimulation and, if it could, whether this response was blocked by 10^{-6} M α,β-methylene ATP. This latter drug has a potent excitatory effect on lymphatic vessels but the response desensitizes within 30 min in the continued presence of the drug (Harty *et al.* 1993).

A summary of nine experiments designed to answer these questions is shown in Fig. 5. ATP increased the frequency of spontaneous contractions from a mean of 6·1 ± 0·2 to 7·9 ± 0·46 contractions min^{-1} (open bars). This response was not blocked by 10^{-6} M α,β-methylene ATP (hatched bars) where the increase was from 6·2 ± 0·36 to 8·3 ± 0·4 contractions min^{-1}. The pattern of the ATP response was very similar to that of field stimulation in

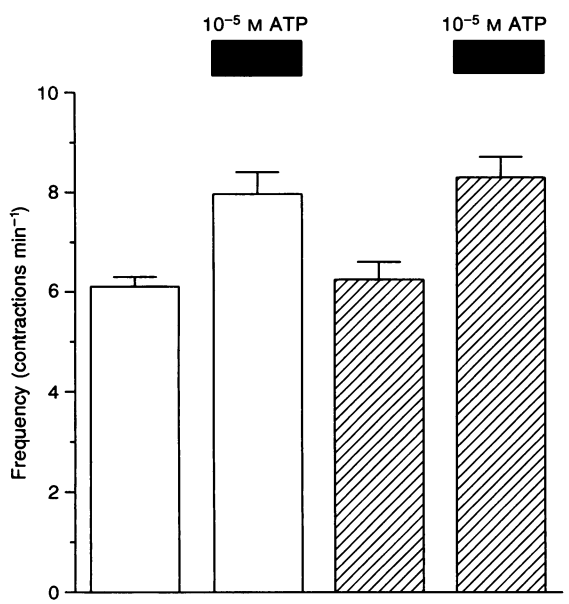

Figure 5. Failure of α,β-methylene ATP to block response to exogenous ATP
Summary of nine experiments showing the effect of exogenous ATP on spontaneous contraction frequency. The paired columns represent mean contraction frequency (vertical bars are 1 S.E.M.) for the 2 min period before and the 1 min period during ATP administration in control conditions (□) and in the presence of 10^{-6} M α,β-methylene ATP (▨).

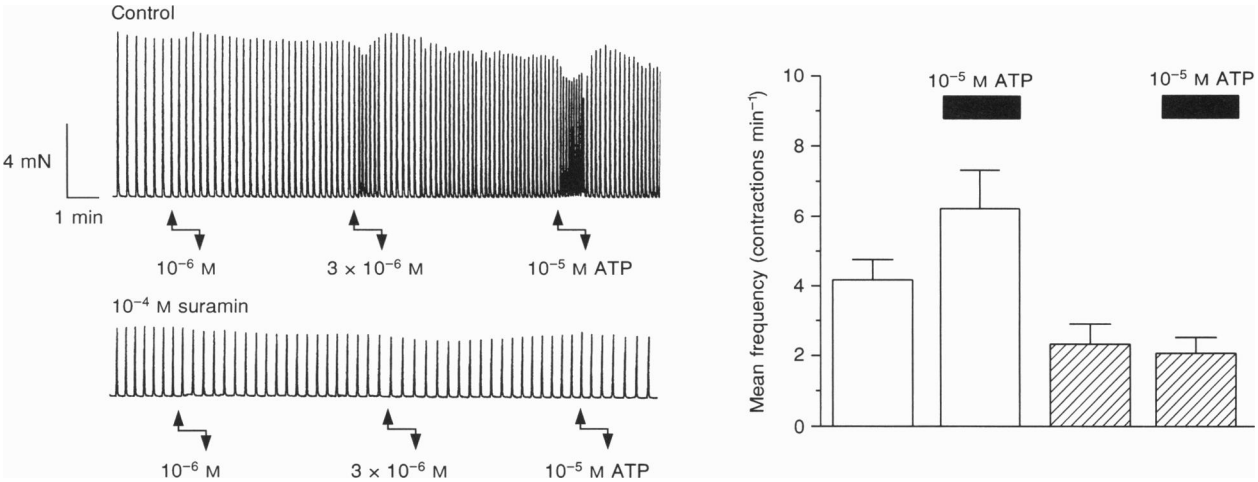

Figure 6. Suramin blocks the response to exogenous ATP
Effect of ATP on frequency of spontaneous contractions before (left panel, upper record) and in the presence of 10^{-4} M suramin (lower record). The right panel is a summary of four such experiments. The paired columns represent mean contraction frequency (vertical bars represent 1 S.E.M.) for the 2 min period before and the 1 min period during ATP administration in control conditions (□) and in the presence of 10^{-4} M suramin (▨).

that the increase in frequency of contraction was always accompanied by a depression of force of contraction (not shown).

Blockade of exogenous ATP

Since α,β-methylene ATP did not block the effect of exogenously applied ATP it might not be expected to modify a purinergic innervation, so it was clear that an antagonist that did block exogenous ATP would have to be found before we could assess the potential role of ATP as a neurotransmitter in these vessels. Suramin was chosen since it has been shown to block ATP and purinergic transmission in other tissues (Dunn & Blakeley, 1988). The left panel in Fig. 6 shows the effects of three concentrations of ATP on spontaneous contraction frequency before (Control) and in the presence of 10^{-4} M suramin. It is clear that the excitatory effect of ATP was blocked. The right panel is a summary of the effect in four experiments of 10^{-5} M ATP before and in the presence of 10^{-4} M suramin. In control conditions, frequency increased from $4{\cdot}2 \pm 0{\cdot}6$ to $6{\cdot}2 \pm 1{\cdot}1$ contractions min^{-1}. When suramin was introduced basal frequency decreased to $2{\cdot}4 \pm 0{\cdot}6$ contractions min^{-1} but ATP failed to increase this significantly ($2{\cdot}1 \pm 0{\cdot}4$ contractions min^{-1}).

Effect of suramin on exogenous noradrenaline

It was important to establish that the blocking effect of suramin was specific for ATP. This was done by examining its effects on the response to exogenous noradrenaline. Figure 7 shows the effects of a 1 min application of noradrenaline (10^{-6} M) before and in the presence of 10^{-4} M suramin. Suramin decreased basal frequency of contraction as before but noradrenaline still produced its typical excitatory effect. In five such experiments

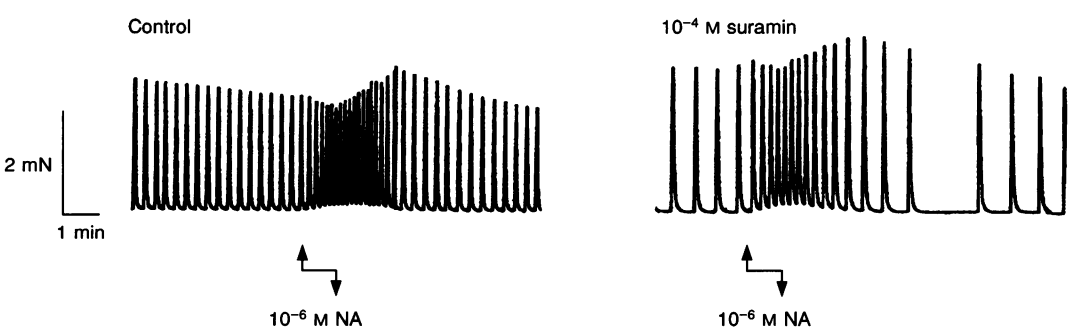

Figure 7. Suramin does not block response to noradrenaline
The effect of a 1 min application of 10^{-6} M noradrenaline (NA) before (left record) and in the presence of 10^{-6} M suramin (right record).

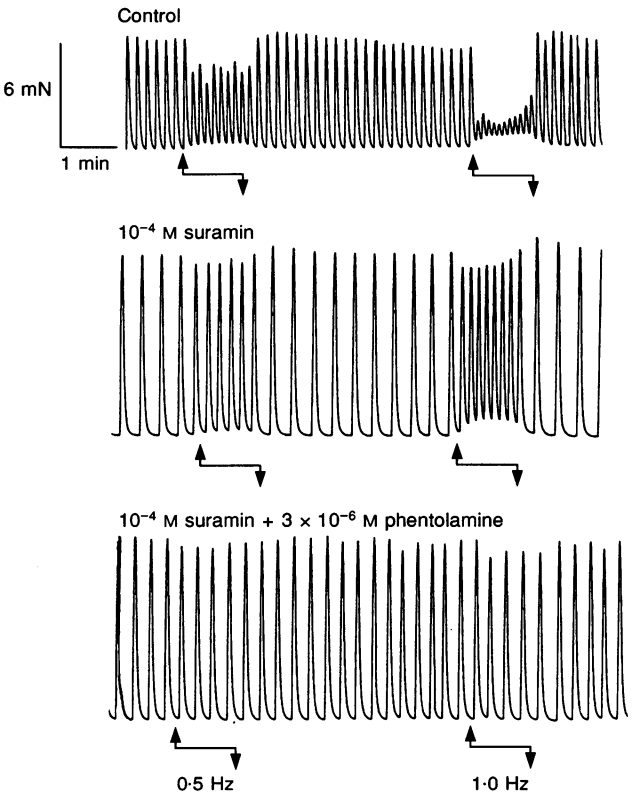

Figure 8. Suramin or phentolamine alone do not block the response to field stimulation
The effect of 1 min periods of field stimulation at 0·5 and 1 Hz under control conditions (top record), in the presence of suramin (middle record) and in the presence of both suramin and phentolamine (bottom record).

noradrenaline (10^{-6} M) significantly increased contraction frequency from $5{\cdot}4 \pm 1{\cdot}2$ to $11{\cdot}2 \pm 1{\cdot}3$ contractions min^{-1} ($P < 0{\cdot}005$) and from $2{\cdot}8 \pm 0{\cdot}58$ to $7{\cdot}7 \pm 0{\cdot}89$ contractions min^{-1} ($P < 0{\cdot}001$) in the absence and presence of suramin respectively.

The effect of suramin on field stimulation

Having established that suramin blocked the effects of exogenously applied ATP and did not block those of noradrenaline it was of interest to examine its effects on field stimulation.

Figure 8 shows an experiment where two 1 min periods of field stimulation were applied at 0·5 and 1 Hz in control conditions (top record) and in the presence of 10^{-4} M suramin. It is clear that suramin alone did not block the excitatory response at either frequency. However, when the α-blocker phentolamine (3×10^{-6} M) was also added, the excitatory effect of field stimulation was blocked. A summary of six such experiments is shown in Fig. 9. The mean frequency of contraction in the absence of either drug (open columns) was $5{\cdot}9 \pm 0{\cdot}9$ contractions min^{-1} and this was almost doubled to $10{\cdot}5 \pm 1{\cdot}1$ contractions min^{-1}

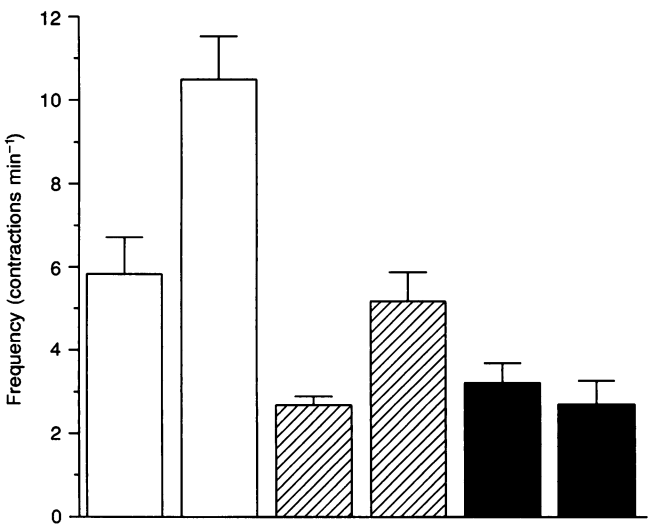

Figure 9. Suramin and phentolamine together block the effects of field stimulation
Summary of six experiments showing the effect of a 1 min period of field stimulation at 1 Hz on the frequency of spontaneous contractions The paired columns represent mean contraction frequency (vertical bars represent 1 S.E.M.) for the 2 min period before and the 1 min period during field stimulation in control conditions (□), in the presence of suramin (▨) and in the presence of both suramin and phentolamine (■).

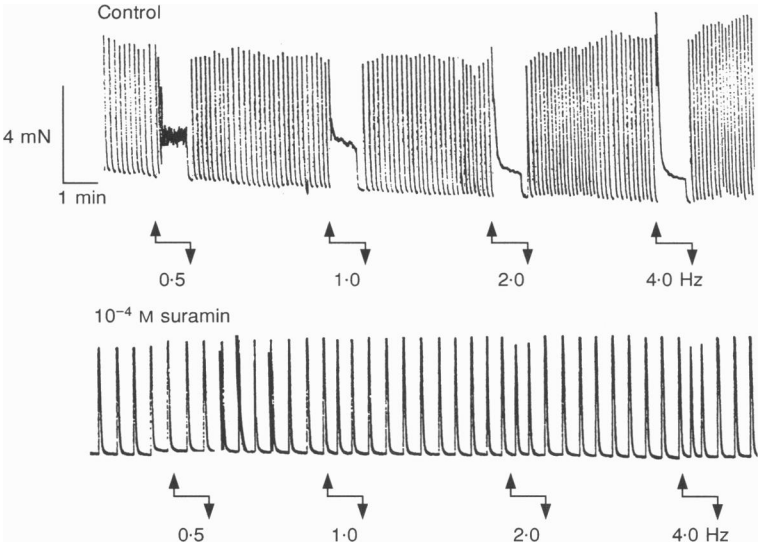

Figure 10. Response to field stimulation in reserpine-treated sheep
The effect of four frequencies of field stimulation on a spontaneously beating ring taken from a reserpine-treated sheep under control conditions (upper panel) and in the presence of 10^{-4} M suramin (lower panel).

when the vessel was stimulated at 1 Hz. When suramin was added (hatched columns) the basal frequency decreased to $2{\cdot}7 \pm 0{\cdot}2$ contractions min^{-1} but this was still almost doubled to $5{\cdot}3 \pm 0{\cdot}7$ contractions min^{-1} in response to 1 Hz field stimulation. Addition of phentolamine did not significantly change basal frequency (filled columns) but the excitatory response to field stimulation was now completely blocked ($3{\cdot}1 \pm 0{\cdot}3$ to $2{\cdot}7 \pm 0{\cdot}4$ contractions min^{-1}).

The effect of reserpine

Pretreatment of animals with reserpine is known to deplete adrenergic nerves of their noradrenaline stores. When rings were taken from such animals they still exhibited a vigorous excitatory response to field stimulation (top record, Fig. 10). When 10^{-4} M suramin was added to the perfusate this excitatory response was completely blocked in the absence of adrenergic blocker. In four such experiments (i.e. rings taken from four different animals) the mean frequency of contraction was $4{\cdot}5 \pm 0{\cdot}6$ contractions min^{-1} while during field stimulation at 0·5 Hz this increased to $7{\cdot}0 \pm 1{\cdot}2$ contractions min^{-1}. After addition of 10^{-4} M suramin to the bathing fluid, control frequency of contraction was $1{\cdot}78 \pm 0{\cdot}57$ contractions min^{-1} and this increased only to $2{\cdot}1 \pm 0{\cdot}17$ contractions min^{-1} during field stimulation at 0·5 Hz.

DISCUSSION

The results of this study confirm that the increase in frequency of spontaneous contractions in isolated sheep mesenteric lymphatics in response to field stimulation is nerve mediated (since it is blocked by ω-conotoxin and by the sympathetic blocker guanethidine). Our previous conclusion (Harty *et al.* 1993) that excitatory transmission was mediated by neither ATP nor noradrenaline was based on the failure of either α-adrenergic antagonists or α,β-methylene ATP, alone or in combination, to block the response. In the present study we have demonstrated that sheep lymphatics loaded with [^{3}H]noradrenaline released tritium in response to field stimulation in a manner very similar to that seen in bovine vessels (Allen *et al.* 1988) suggesting that noradrenaline is indeed a transmitter. This is further supported by the observation that, in the presence of the purinergic blocker suramin, phentolamine does block the excitatory response to field stimulation. Similarly lymphatics taken from a reserpine-treated sheep (where noradrenaline stores have, presumably, been depleted) still showed an excitatory response to field stimulation but this could be antagonized by suramin alone. Taken together these observations suggest that excitatory neurotransmission in sheep mesenteric lymphatics is mediated by both noradrenaline and ATP and that these act independently on specific postsynaptic receptors to mediate the excitatory response. Thus the effects of ATP could be blocked by suramin while those of noradrenaline could be blocked by phentolamine (Harty *et al.* 1993) but not by suramin.

Our failure to block the effects of field stimulation or exogenous ATP with α,β-methylene ATP was surprising but not without precedent. Thus Van Riper & Bevan (1991) were unable to desensitize ATP-mediated contractions of the rabbit middle cerebral artery with α,β-methylene ATP. Similarly Wiklund & Gustafsson (1988) showed that the contractile effect of ATP was not blocked by α,β-methylene ATP in the guinea-pig ileum and they speculated that ATP may activate a 'P_{2s}' receptor in

this tissue. Chen *et al.* (1992) showed that application of α,β-methylene ATP to strips of rabbit detrusor muscle caused an initial contraction followed by desensitization on continuous application of the drug. The response of the desensitized tissue to ATP was, if anything, greater than that prior to desensitization. It is known, however (Burnstock, Cocks, Crowe & Kasakov, 1978), that α,β-methylene ATP does block the effect of field stimulation in this tissue. This led Chen *et al.* (1992) to speculate that neurally released ATP may act on a different receptor from that activated by exogenous ATP.

It is not clear from the present results why both blockers were necessary to produce any reduction in the response to field stimulation. One might expect that phentolamine alone or suramin alone would cause a significant, though not complete, reduction in the nerve-induced acceleration in frequency of spontaneous contractions. This was not the case. There was no significant reduction in the frequency of contraction during field stimulation compared to the period before stimulation in the presence of phentolamine alone (Harty *et al.* 1993) or in the presence of suramin alone. In the case of suramin the interpretation is a little complicated by the fact that this drug caused a consistent slowing of spontaneous contraction frequency, but if one compares the relative change in frequency in response to field stimulation during suramin administration with the control it can be seen that this is almost exactly doubled in each case. Van Riper & Bevan (1991) showed that a similar phenomenon exists in the rabbit middle cerebral artery. The proposed transmitters in this case are noradrenaline and NPY but the pattern is similar to that observed in sheep lymphatics. Either transmitter alone was capable of producing the same contractile response as both together. The explanation given by these authors was that each transmitter was acting presynaptically to inhibit the release of the other. Normal transmission would produce a response reflecting the occupation of prejunctional and postjunctional sites by both substances. If one transmitter system were antagonized, the diminution of response from postjunctional blockade would be offset by enhanced release of the other transmitter because of blockade of inhibitory prejunctional sites. The resulting response would thus appear little changed. A similar explanation may account for our present results although we have no direct evidence to support it. We do know that activation of presynaptic α_2-receptors in bovine mesenteric lymphatics depresses the release of noradrenaline in response to field stimulation (Allen *et al.* 1988). It is also known that α_2-agonists can inhibit the field-evoked release of ATP (Sperlagh & Vizi, 1992) and that ATP can act presynaptically on P_3 receptors to inhibit the release of noradrenaline (Westfall, Kasumasa, Forsythe & Bjur, 1990). Thus such a mechanism might well exist but further study is needed to test the hypothesis.

It is not entirely clear what the consequences of this dual innervation are for the lymphatic system. The main function of these vessels is to propel lymph like a series of smooth muscle hearts. The normal effects of nerve stimulation are to modulate this pumping activity. This is normally achieved in bovine vessels by a depolarizing excitatory junction potential which in those vessels is due solely to the release of noradrenaline (Allen & McHale, 1986). What extra advantage there is in the co-release of ATP is not immediately apparent. The existence of this second transmitter has, however, advantages in the study of the nervous control of the lymph pump in the living sheep. Chief among these is the possibility of selectively antagonizing the effects of sympathetic nerve stimulation on blood vessels (with α-adrenergic antagonists) while leaving intact a functional innervation in lymphatic vessels (i.e. the purinergic component). This will make it possible to study the latter while blocking the effects of sympathetic nerve stimulation on blood vessel diameter and thus on lymph formation.

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