

5-HT Inhibits Spontaneous Contractility of Isolated Sheep Mesenteric Lymphatics via Activation of 5-HT₄ Receptors

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Spontaneous isometric contractions were measured in rings of sheep mesenteric lymphatic vessels *in vitro*. 5-Hydroxytryptamine (5-HT) produced a concentration-dependent decrease in spontaneous contraction frequency and force which was not antagonised by either the nonspecific 5-HT₁/5-HT₂ receptor antagonist methysergide (1 μ M) or the 5-HT₃ receptor antagonist ondansetron (1 μ M). The 5-HT₄ receptor agonist BIMU-8 mimicked the inhibitory effect of 5-HT and its effects were abolished by the 5-HT₄ receptor antagonist DAU 6285 (1 μ M). DAU-6285 also abolished the inhibitory effect of 5-HT and unmasked a weak excitatory response, which was mimicked by the 5-HT₂ receptor agonist α -methyl-5-hydroxytryptamine maleate. This excitatory response was, in turn, blocked by the 5-HT₂ receptor antagonist pirenperone (1 μ M). The results of this study suggest that sheep mesenteric lymphatics possess both 5-HT₄ receptors and 5-HT₂ receptors. The inhibitory 5-HT₄ receptor appeared to be the predominant subtype since the excitatory response to 5-HT could only be observed in the presence of the 5-HT₄ receptor antagonist DAU 6285. © 2000 Academic Press

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INTRODUCTION

5-Hydroxytryptamine (5-HT) has been demonstrated to have a variety of effects on smooth muscle which depend not only on the concentration of 5-HT applied, but also on the relative distribution of excitatory and inhibitory 5-HT receptor subtypes on the tissue in question. For example, Cocks and Arnold (1992) demonstrated that 5-HT (1 nM–1 μ M) relaxed isolated rings of sheep pulmonary veins whereas concentrations above 1 μ M contracted the same preparations. Similarly, in the gastrointestinal tract, 5-HT has both contractile (Prins *et al.*, 1997) and relaxant effects (McLean *et al.*, 1995).

In lymphatic smooth muscle the effects of 5-HT appear, at first, to be more uniform across the range of species studied. Reddy and Staub (1981) showed that pumping activity of an “isolated” segment of canine thoracic duct was enhanced by 5-HT while Hutchinson *et al.* (1992) showed that 5-HT increased contraction frequency of isolated bovine mesenteric lymphatic rings *in vitro*. In addition, Ferguson *et al.* (1993) and Sjoberg and Steen (1991) have demonstrated ex-

citatory effects of 5-HT in porcine and human lymphatics *in vitro*, respectively. These excitatory effects of 5-HT on lymphatic vessels would tend to increase pumping activity and presumably lead to an increase in lymph flow. However, preliminary experiments in the living sheep (M. A. Hollywood and N. G. McHale, unpublished observations) suggested that 5-HT actually reduced lymph flow.

The aim of the present study was to examine the effect of 5-HT on spontaneous contractility of isolated rings from sheep mesenteric lymphatics to determine whether the inhibitory effect on lymph flow in the living animal could be explained, at least in part, by inhibition of the spontaneous contractility of lymphatic vessels. When this was found to be the case, we then attempted to examine which 5-HT receptor subtype was responsible for mediating this inhibitory effect.

METHODS

Segments of main lymphatic duct 5–10 cm in length and 2–3 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 were removed from the lymphatic by sharp dissection. Rings of lymphatic 2–3 mm in diameter and 8 mm in length were suspended between stainless-steel hooks and placed into water-jacketed organ baths (volume 5 ml) maintained at 37°C. The rings were perfused at a rate of 5 ml min⁻¹ with Krebs solution of composition (mM): NaCl, 120; NaHCO₃, 25.0; KCl, 5.9; Na₂HPO₄, 1.2; CaCl₂, 2.5; MgCl₂, 1.2; glucose, 5.5 and gassed with 95% O₂, 5% CO₂. The rings were then adjusted to a tension of 2–4 mN and the vessels were allowed to equilibrate for at least 30 min. Resultant spontaneous contractions were measured with Statham UC3 and Dynamometer UF1 transducers as changes in isometric tension and the output from these were written on a Gould 2400S chart recorder.

The protocol used for most of the experiments consisted of perfusing agonists into the organ bath for 1

min. Increasing concentrations of agonist were usually applied at least 5 min apart. Antagonists were added to the fluid perfusing the tissue for at least 20 min before the agonists were reapplied. In the absence of receptor antagonists, the responses to repeated application of 5-HT and 5-HT receptor agonists were reproducible and did not significantly change with time. The results were calculated as the instantaneous contraction frequency (reciprocal of the time between successive contractions) for the 2-min period immediately preceding application of agonist and for the duration of inhibition after drug application. Results were expressed as the mean ± 1 standard error of the mean (SEM). Statistical comparisons were made using the Student paired *t* test, taking *P* < 0.05 as significant.

The drugs used were 5-hydroxytryptamine (5-hydroxytryptamine creatinine sulfate complex, Sigma), α -methyl-5-hydroxytryptamine maleate, methysergide, and pirenperone (Research Biochemicals Inc.). Ondansetron, DAU 6285 (*endo*-8-methyl-8-azabicyclo [3.2.1] oct-3-yl-2,3-dihydro-6-methoxy-2-oxo-1*H*-benzimidazole-1-carboxylate HCl), and BIMU-8 (*endo*-*N*-(8-methyl-8-azabicyclo[3.2.1]-oct-3-yl)-2,3-dihydro-(1-methyl)ethyl-2-oxo-1*H*-benzimidazole-1-carboxamide HCl) were obtained as gifts from Dr. C. Rizzi, Boehringer Ingelheim Italia. All of the drugs were made up to their final concentrations in Krebs solution.

RESULTS

Effect of 5-Hydroxytryptamine

Figure 1A shows a typical record of spontaneous activity from a ring of sheep mesenteric lymphatic in the absence and presence of 5-HT. As the trace in the top panel demonstrates, the frequency of spontaneous contractions was approximately 6 contractions min⁻¹ prior to drug application. Upon application of 30 nM 5-HT, the amplitude of contraction was decreased by approximately 20% and contraction frequency was reduced to 4 min⁻¹. Increasing the concentration of 5-HT to 100 nM resulted in cessation of spontaneous activity which returned approximately 3 min later.

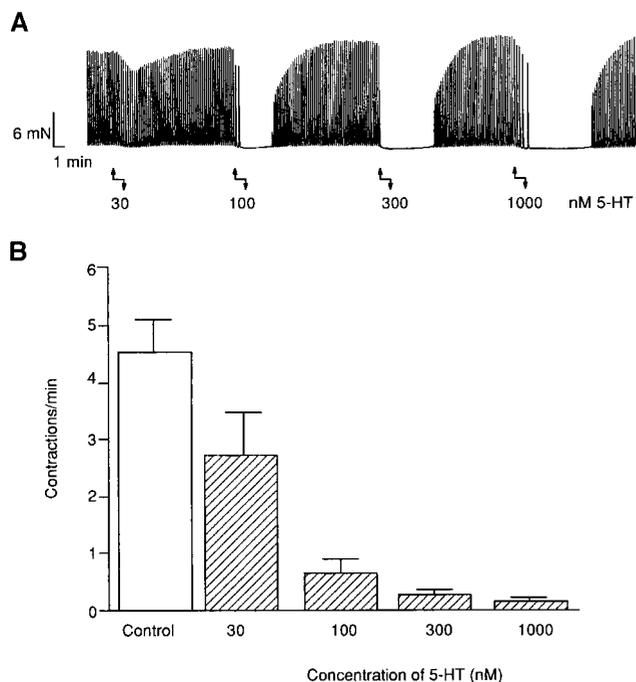


FIG. 1. The inhibitory effect of 5-HT on spontaneous contractility of sheep mesenteric lymphatics. (A) A typical trace from an experiment where the effects of 5-HT at concentrations 30, 100, and 300 nM and 1 μ M were examined. 5-HT caused a concentration-dependent decrease in contraction frequency. (B) A summary diagram for 13 similar experiments in which contraction frequency was plotted in the absence (plain bar) and presence of increasing concentrations of 5-HT (hatched bars). 5-HT significantly depressed contraction frequency at all concentrations shown ($P < 0.05$).

When the concentration of 5-HT was further increased to 300 nM and 1 μ M, spontaneous activity was abolished for 5 and 6 min, respectively. Figure 1B shows a summary of 13 similar experiments in which the mean instantaneous contraction frequency was plotted against increasing concentrations of 5-HT. Contraction frequency was significantly reduced by approximately 40% at 30 nM, 85% at 100 nM, 94% at 300 nM, and 97% at 1 μ M ($P < 0.05$).

Effect of Methysergide and Ondansetron on the Response to 5-HT

To test for the possibility that stimulation of either 5-HT₁ or 5-HT₃ receptors mediated the inhibitory response to 5-HT, the effects of both methysergide (1 μ M) and ondansetron (1 μ M) were examined. Figure

2A shows a summary of four experiments in which the effects of 5-HT were examined in the absence and presence of the nonspecific 5-HT₁/5-HT₂ receptor antagonist, methysergide. Neither the resting contraction frequency nor the response to 5-HT, at any concentration, was significantly decreased in the presence of methysergide as compared to control.

Figure 2B shows a summary of four experiments where the effects of 5-HT were examined in the absence and presence of the 5-HT₃ receptor antagonist ondansetron (1 μ M). The bar chart is of the same

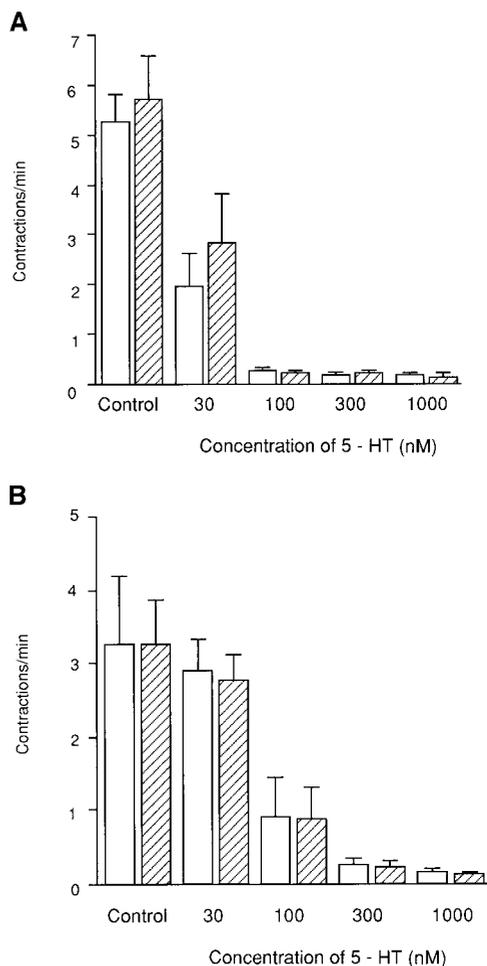


FIG. 2. (A) Effect of increasing concentrations of 5-HT on spontaneous contraction frequency in the absence (plain bars) and the presence (hatched bars) of 1 μ M methysergide ($n = 4$). (B) Effect of 5-HT in the absence (plain bars) and presence (hatched bars) of 1 μ M ondansetron ($n = 4$). Neither methysergide or ondansetron significantly affected the response to 5-HT. The bars in both bar charts represent the means \pm SEM (vertical lines).

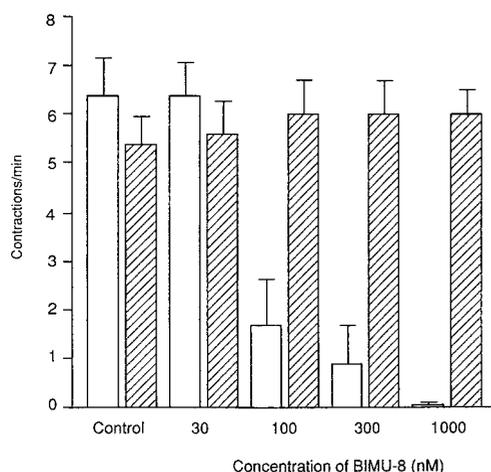


FIG. 3. Summary bar chart showing the effect of four concentrations of BIMU 8 on spontaneous contraction frequency, in the absence (plain bars) and presence (hatched bars) of 1 μ M DAU 6285 ($n = 5$). The bars represent mean \pm SEM (vertical lines). The inhibitory effect of BIMU 8 at all concentrations was antagonised by DAU-6285.

format as that described for Fig. 2A. Ondansetron did not significantly alter the resting contraction frequency or reduce the inhibitory effect of 5-HT at any concentration as compared to control.

Effect of the 5-HT₄ Receptor Agonist BIMU-8 on Contraction Frequency

A number of studies have demonstrated that the inhibitory effects of 5-HT on smooth muscle are mediated via activation of 5-HT₄ receptors (Cocks and Arnold, 1992; McLean *et al.*, 1995). To test if a similar mechanism was responsible for the inhibitory action of 5-HT observed in lymphatics, the effects of the 5-HT₄ receptor agonist BIMU-8 were examined. BIMU-8 produced a concentration-dependent decrease in contraction frequency and amplitude which resembled the inhibitory effects of 5-HT. Figure 3 shows a summary of five experiments in which contraction frequency was plotted against increasing concentrations of BIMU-8 in the absence (plain bars) and presence (hatched bars) of the 5-HT₄ antagonist DAU 6285 (1 μ M). In the absence of the antagonist, BIMU-8 reduced contraction frequency in a concentration-dependent manner.

In the presence of DAU 6285 alone, resting contraction frequency was unaltered but the inhibitory effects of BIMU-8 were abolished at all concentrations and were not significantly different from resting contraction frequency. These results suggest that BIMU-8 can mimic the effect of 5-HT in lymphatic smooth muscle and support the idea that this effect is mediated through activation of 5-HT₄ receptors.

Effect of the 5-HT₄ Receptor Antagonist DAU-6285 on the Response to 5-HT

To further test the possibility that the inhibitory effect of 5-HT was mediated via stimulation of 5-HT₄ receptors the response to 5-HT was examined in the presence of the 5-HT₄ receptor antagonist DAU 6285. The upper panel of Fig. 4 shows a representative trace from a series of five experiments in which the effect of 5-HT was examined before (upper panel) and during (lower panel) application of DAU 6285 (1 μ M). In the absence of the antagonist, 5-HT reduced contraction frequency in a concentration-dependent manner. In the presence of DAU 6285 not only was the inhibitory effect abolished at all concentrations, but a small excitatory response was unmasked at higher concentrations of 5-HT.

Figure 5 shows a summary of five similar experi-

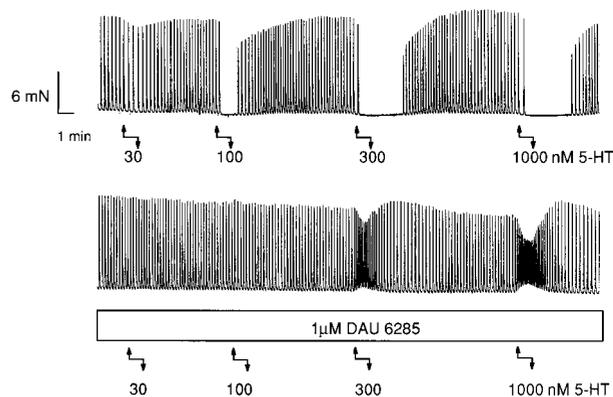


FIG. 4. Shows the typical effect of four 1-min applications of 5-HT at the concentrations shown in the absence (upper panel) and presence (lower panel) of the 5-HT₄ antagonist DAU 6285. In the absence of the antagonist 5-HT produced a concentration-dependent decrease in contraction frequency. In the presence of DAU 6285 the inhibitory effect of 5-HT was abolished and an excitatory response was unmasked at the higher concentrations of 5-HT.

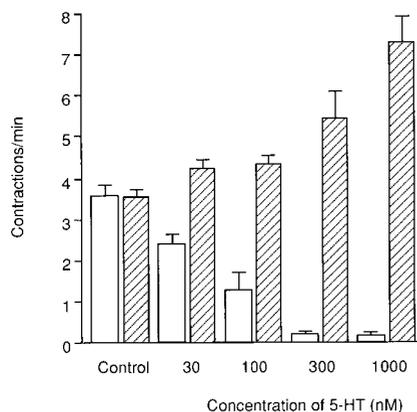


FIG. 5. Summary bar chart showing the effect of four concentrations of 5-HT on spontaneous contraction frequency, in the absence (plain bars) and presence (hatched bars) of 1 μ M DAU 6285 ($n = 5$). The bars represent mean \pm SEM (vertical bars). In the presence of DAU 6285 the inhibitory effect of 5-HT was clearly blocked and converted into an excitatory effect at concentrations of 5-HT above 0.1 μ M ($P < 0.05$).

ments. In the absence of DAU 6285, 5-HT produced a concentration-dependent decrease in contraction frequency. In the presence of DAU 6285, resting contraction frequency was unaltered and 5-HT at concentrations less than 300 nM had little effect on contraction frequency. However, in the presence of 300 nM and 1 μ M 5-HT, an excitatory response was unmasked and contraction frequency was significantly increased from 3.5 ± 0.2 to 5.4 ± 0.6 and 7.2 ± 0.7 min^{-1} , respectively ($P < 0.05$).

Effect of α Methyl 5-HT Maleate on Contraction Frequency

The weak excitatory effects of 5-HT observed in the presence of DAU 6285 were similar to those demonstrated in bovine mesenteric lymphatics (Hutchinson *et al.*, 1992) where 5-HT appears to act via 5-HT₂ receptors. We therefore examined the effect of the 5-HT₂ receptor agonist, α -methyl-5-hydroxytryptamine maleate on this weak excitatory response (Fig. 6A). In the absence of any drugs, the lymphatic ring contracted at a frequency of 6 min^{-1} and this was increased to 7, 11, and 13 min^{-1} in response to 1-min application of the agonist at concentrations of 300 nM and 1 and 3 μ M, respectively. Figure 6B shows a

summary of five similar experiments where contraction frequency was plotted against increasing concentrations of the 5-HT₂ receptor agonist. Application of 3 μ M α -methyl-5-hydroxytryptamine maleate significantly increased contraction frequency from a control value of 7.8 ± 0.4 min^{-1} to a maximum of 13 ± 0.8 min^{-1} ($P < 0.05$).

Effect of Pirenperone on the Excitatory Response to 5-HT

To confirm if the weak excitatory effect of 5-HT was mediated via activation of 5-HT₂ receptors, the response to 5-HT was examined in the presence of the 5-HT₂ receptor antagonist pirenperone (1 μ M). In the presence of DAU 6285 alone, 30 or 100 nM 5-HT had little effect on contraction frequency but 1 μ M 5-HT significantly increased contraction frequency ($P < 0.05$). Figure 7 shows a summary diagram for five experiments in which the effect of 5-HT was examined before (hatched bars) and during blockade of 5-HT₂ receptors with pirenperone (filled bars). Prior to application of pirenperone, 5-HT increased contraction frequency from 5.4 ± 0.7 to 7.4 ± 1.0 min^{-1} ($P < 0.05$).

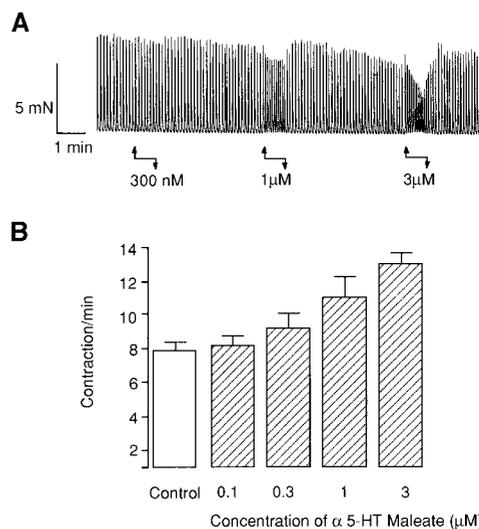


FIG. 6. (A) The excitatory effect of three concentrations of α -methyl-5-hydroxytryptamine maleate on contraction frequency. (B) A summary bar chart for five similar experiments in which the effect of four concentrations of α -methyl-5-hydroxytryptamine maleate (hatched bars) was compared to contraction frequency in the absence of any drugs (plain bar). The vertical lines represent $+1$ SEM.

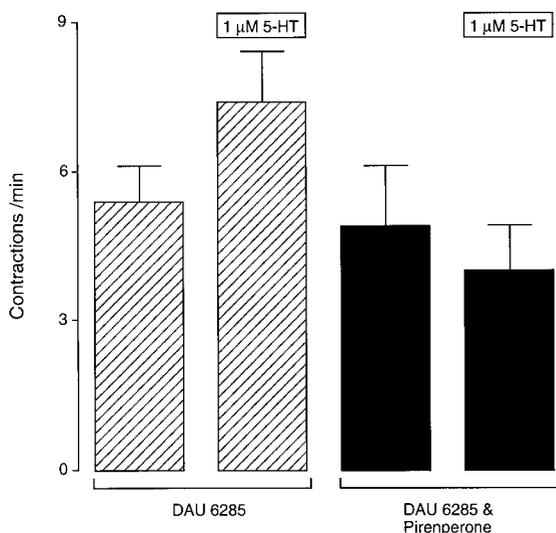


FIG. 7. Shows the effect of 5-HT in the presence of 1 μ M 5-HT in the presence of 1 μ M DAU6285 and in the presence of both DAU 6285 and 1 μ M pirenperone. The excitatory effect of 5-HT was blocked by pirenperone. The vertical lines represent +1 SEM.

In the presence of both DAU 6285 and pirenperone (filled bars), resting contraction frequency was not significantly different from control ($5.4 \pm 0.7 \text{ min}^{-1}$ in DAU 6285 compared with $4.9 \pm 1.2 \text{ min}^{-1}$ in DAU 6285 and pirenperone), but reapplication of 1 μ M 5-HT failed to significantly alter contraction frequency ($4.0 \pm 0.9 \text{ min}^{-1}$). These results support the idea that the excitatory response to 5-HT unmasked in the presence of DAU 6285 is mediated via activation of 5-HT₂ receptors.

DISCUSSION

The present study demonstrates that the predominant effect of 5-HT on isolated sheep lymphatic rings was to inhibit spontaneous contractility by depressing both the amplitude and the frequency of contractions. A number of pieces of evidence suggest that this inhibitory response was mediated via activation of 5-HT₄ receptors. Firstly, the inhibitory effect was mimicked by the 5-HT₄ receptor agonist BIMU-8 (Dumuis *et al.*, 1991). Secondly, the 5-HT₄ receptor antagonist DAU 6285 (Schivone *et al.*, 1991; Dumuis *et al.*, 1992)

abolished the inhibitory effect of 5-HT to unmask a weak excitatory response to 5-HT. In addition, the response was not blocked by the nonspecific 5-HT₁/5-HT₂ receptor antagonist methysergide or by the 5-HT₃ receptor antagonist ondansetron. These results suggest that the inhibition of spontaneous contractility is mediated via 5-HT₄ receptors and that this is the predominant 5-HT receptor subtype in sheep mesenteric lymphatics. These findings contrast with the reported effects of 5-HT on lymphatics from other species where a predominant excitatory response has been observed (Shim *et al.*, 1961; Reddy and Staub, 1981; Dabney *et al.*, 1984; Hutchinson *et al.*, 1992).

Although a predominant inhibitory response to 5-HT has not been previously demonstrated in lymphatic smooth muscle, relaxation in response to 5-HT has commonly been observed in blood vessels including the cat cranial artery and rabbit jugular vein (Edvinsson *et al.*, 1978; Feniuk *et al.*, 1984). In each of the above studies, 5-HT appeared to interact with 5-HT₁-like receptors to relax the tissue. These inhibitory effects of 5-HT were blocked by 5-HT₁ receptor antagonists such as methiothepin or the nonspecific 5-HT₁/5-HT₂ receptor antagonist methysergide. In other blood vessels such as rat mesenteric artery (Su and Urano, 1985), rat vena cava (Göthert *et al.*, 1986), and dog saphenous vein (McGrath, 1977), 5-HT appears to act indirectly via presynaptic 5-HT receptors to modulate the neuronal release of noradrenaline. The reduced release of noradrenaline from intramural nerves decreased sympathetic tone in these tissues, which in turn caused relaxation. If 5-HT mediated its effects via activation of presynaptic 5-HT₃ receptors in sheep lymphatics then ondansetron would be expected to abolish the response. No such effect of ondansetron was demonstrated thus arguing against an indirect 5-HT₃ receptor-mediated inhibition in this tissue.

More recent studies have demonstrated that 5-HT either inhibits spontaneous contractions or causes relaxation of gastrointestinal (Tuladhar *et al.*, 1996; Tam *et al.*, 1994) and some vascular smooth muscles (Cocks and Arnold, 1992) via direct activation of 5-HT₄ receptors on the smooth muscle. The results of the present study suggest that 5-HT may act through similar receptors in lymphatic smooth muscle to produce its

inhibitory effects. Although the mechanism underlying the inhibitory effect of 5-HT has not been examined in the present study, it is now well established that 5-HT₄ receptors are positively coupled to adenylyl cyclase and their activation leads to elevations in cyclic AMP (Bockeart *et al.*, 1992; McLean and Coupar, 1996). Some preliminary evidence suggests that the inhibition of sheep lymphatic contractility by 5-HT is also due to stimulation of cyclic AMP. Sergeant (1997) has demonstrated that 5-HT₄ receptor stimulation was mimicked by the adenylyl cyclase agonist, forskolin, and potentiated in the presence of the nonselective phosphodiesterase inhibitor IBMX but not by the cGMP phosphodiesterase inhibitor M&B 22948. These data provide tentative support for the idea that 5-HT may elevate cAMP levels in response to activation of 5-HT₄ receptors in sheep lymphatic smooth muscle.

The physiological significance of the inhibitory effect of 5-HT on sheep lymphatics is unclear. 5-HT plays an important role in inflammation (Sufka *et al.*, 1992) and can increase capillary permeability (Buzzi *et al.*, 1991) as well as constrict both veins and venules (Gilbert *et al.*, 1958). The combination of venous constriction and elevated capillary permeability could lead to an increased fluid load on the lymphatic system, which would normally respond by increasing its frequency and force of pumping. Inhibition of spontaneous activity by 5-HT would appear to neutralise the above response and limit the removal of excess tissue fluid. However, Elias and Johnston (1990) have suggested that inhibition of spontaneous activity may be a physiological response to an inflammatory stimulus which could serve to "reset" the lymph pump so that the lymphatic vessels may continue to pump at higher distending pressures. An increase in distending pressure causes an increase in frequency of spontaneous contraction and of flow up to a maximum after which flow declines (McHale and Roddie, 1976). Inhibitory substances could decrease the sensitivity of this response causing the maximum flow level to be achieved at a higher distending pressure. Such a rightward shift in the relationship between distension and flow was demonstrated by Elias and Johnston (1990) in doubly cannulated sheep mesenteric lymphatic vessels which were perfused with lymph taken from sheep that had been treated with endotoxin. It is in-

teresting to speculate that if 5-HT could produce a similar shift in the relationship between distension and flow in sheep lymphatics, then lymph flow may be maintained at higher distending pressures. Further research is required to examine if 5-HT can produce such an effect.

When the inhibitory effect of 5-HT was antagonised by blockade of 5-HT₄ receptors a weak excitatory response was unmasked. This excitatory response was similar to the excitatory response to 5-HT observed in the majority of lymphatic vessels from different species (Shim *et al.*, 1961; Reddy and Staub, 1981; Dabney *et al.*, 1984; Hutchinson *et al.*, 1992). Thus, the excitatory response was antagonised by pirenperone and mimicked by α -methyl-5-hydroxytryptamine maleate, supporting the idea that it was mediated via activation of 5-HT₂ receptors.

In conclusion, the present study demonstrates that 5-HT has both inhibitory and excitatory effects on sheep lymphatic smooth muscle. It is interesting to note that in contrast to its effects on lymphatics from other species, the prevalent effect of 5-HT in these vessels was inhibitory, presumably due to a predominance of 5-HT₄ receptors. These results may help to explain the observed reduction in lymph flow in response to 5-HT in the living sheep.

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