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5-HT decreases contractile and electrical activities in lymphatic vessels of the guinea-pig mesentery: role of 5-HT$_7$-receptors

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1 Constriction measurements and intracellular microelectrode recordings were performed *in vitro* on lymphatic vessels isolated from the guinea-pig mesentery to investigate whether 5-hydroxytryptamine (5-HT) affected lymphatic pumping and smooth muscle membrane potential.

2 5-HT decreased in a concentration-dependent manner the frequency of constrictions induced by intraluminal vessel perfusion. In nonperfused vessels, 5-HT hyperpolarized the lymphatic smooth muscle membrane potential and decreased the frequency and amplitude of spontaneous transient depolarizations (STDs).

3 The actions of 5-HT were significantly reversed by the 5-HT$_7$ receptor antagonist (2*R*)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine (SB269970, 0.5 μM) and by the 5-HT$_{1/2/5/7}$ receptor antagonists methysergide (0.5 μM), and were mimicked by the 5-HT$_{1/7}$-receptor agonist, 5-CT.

4 The 5-HT$_4$-receptor antagonists 1-methyl-1*H*-indole-3-carboxylic acid [1-2-[(methyl sulfonyl) amino] ethyl-4-piperidinyl] methyl ester (GR113808, 1 μM) and (1-piperidinyl) ethyl 1*H*-indole 3-carboxylate (SB203186, 1 μM) did not significantly affect the 5-HT-induced responses. The 5-HT$_4$-receptor agonist 1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-(2-methylsulfonylamino) ethyl-4-piperidinyl]-1-propanone hydrochloride (RS67506) decreased the constriction frequency, albeit only at 50 μM and without affecting the smooth muscle membrane potential.

5 Responses to 5-HT were attenuated by the nitric oxide synthase inhibitor N^G-nitro L-arginine (100 μM), whereas indomethacin (10 μM) and tetrodotoxin (1 μM) were without effects.

6 5-HT-induced responses were inhibited by the ATP-sensitive K^+ channel blocker, glibenclamide (10 μM) and the cAMP-dependent protein kinase inhibitor *N*-[2-(*p*-bromociannamylamino)-ethyl]-5-isoquinolinesulfonamide-dichloride (H89, 10 μM) blocked the hyperpolarization.

7 These results suggest that 5-HT modulates the rate of lymphatic vessel pumping by eliciting K_{ATP} channel-mediated smooth muscle hyperpolarization and decrease in STD activity, which appear to be mediated by activation of 5-HT$_7$ receptors coupled to cAMP production.
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Abbreviations: 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine; K_{ATP} channel, ATP-sensitive potassium channel; L-NOARG, N^G-nitro L-arginine; NO, nitric oxide; PKA, cAMP-dependent protein kinase; STD, spontaneous transient depolarization; TTX, Tetrodotoxin

Introduction

Efficient propulsion of lymph is essential for maintaining tissue fluid homeostasis. This function is mainly achieved by rhythmical transient constrictions of the valved, tubular chambers, which constitute the collecting lymphatic vessels. The constrictions are spontaneous and triggered by action potentials generated in the smooth muscle within the vessel wall (Kirkpatrick & McHale, 1977; Allen *et al*., 1983; Van Helden, 1993). Electrophysiological studies on small lymphatic vessels present in the mesentery of the guinea pig have revealed the existence of spontaneous transient depolarizations (STDs, Van Helden, 1993). Sufficient amplitude or summation of these events can trigger L-type Ca^{2+} channel-mediated action potentials and associated constrictions. STDs have thus been proposed as the pacemaker mechanism for spontaneous lymphatic pumping (Van Helden, 1993). STD activity is decreased in the presence of vasorelaxant agents such as nitric oxide (NO), released by the lymphatic endothelium, the β-adrenoceptor agonist isoprenaline, or the adenylate cyclase activator forskolin, which also reduce the frequency of constrictions. In addition, these agents hyperpolarize the smooth muscle resting membrane potential, which also contributes to attenuating lymphatic pumping (von der Weid & Van Helden, 1996; von der Weid *et al*., 1996; 2001). Hyperpolarizations to these chemicals occur via activation of K^+ conductances (Allen *et al*., 1986; von der Weid & Van Helden, 1996; von der Weid *et al*., 1996) through ATP-sensitive K^+ (K_{ATP}) channel opening (von der Weid, 1998).

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Lymphatic pumping activity adapts to changes in fluid load. This function is particularly important during inflammation when the lymphatic vessels need to increase their pumping activity to offset edema. Indeed, lymph flow is usually increased during inflammation as a consequence of edema induced by inflammatory mediators (Benoit *et al*., 1989). In addition, specific inflammatory mediators have been shown to affect lymphatic contractile mechanisms directly (Johnston, 1987; von der Weid, 2001). Among these mediators, 5-hydroxytryptamine (5-HT) is thought to play an important role as its concentration increased in the cat mesenteric lymph during brief mesenteric ischemia – reperfusion, suggesting that it was released and exported to the interstitial space (Fu *et al*., 1997). 5-HT is located in enterochromaffin cells in the gastrointestinal tract of most mammals (Sjolund *et al*., 1983) and stored in platelets (Meyers *et al*., 1982). It is also known to be present in rodent mast cells (Lehtosalo *et al*., 1984; Gershon & Tamir, 1985), making it a potent mediator of inflammation in these animals. 5-HT has been shown in several lymphatic vessel models to elicit tonic contraction (Williamson, 1969; Ohhashi *et al*., 1978; Takahashi *et al*., 1990; Ferguson *et al*., 1993; Hashimoto *et al*., 1994; Dobbins, 1998) or to increase spontaneous constrictions (Johnston *et al*., 1983; Sjoberg & Steen, 1991). Conversely, 5-HT decreased spontaneous contractile activity and relaxed preconstriction in lymphatic vessels of bovine and sheep mesentery (Miyahara *et al*., 1994; McHale *et al*., 2000). However, mechanisms underlying the effects of 5-HT and whether and how 5-HT affects the smooth muscle membrane potential and pacemaker activity are unknown.

Therefore, the aim of the present study was to examine the effects of 5-HT on spontaneous contractile activity of lymphatic vessel in the guinea-pig mesentery and to investigate the electrical and intracellular mechanisms underlying the mechanical responses. The data show that the 5-HT induced a slowdown in contractile activity and a decrease in electrical activity. These effects are mainly mediated by stimulation of a 5-HT receptor, which is proposed to be of the 5-HT_7 subtype. The results further support the proposal that STDs are involved in the initiation of lymphatic pumping.

Methods

Tissue preparation

Guinea pigs (7 – 15 days of age) of either sex were killed by decapitation during deep anesthesia induced by inhalation of halothane. This procedure has been approved by the University of Calgary Animal Care and Ethics Committee and conforms to the guidelines established by the Canadian Council on Animal Care. The small intestine with its attached mesentery was rapidly dissected and placed in a physiological saline solution (PSS) of the following composition (mM): $CaCl_2$, 2.5; KCl, 5; $MgCl_2$, 2; NaCl, 120; $NaHCO_3$, 25; NaH_2PO_4, 1; glucose, 11. The pH was maintained at 7.4 by constant bubbling with 95% O_2/5% CO_2.

Vessel constriction measurements

Lymphatic tissue was prepared as previously described (von der Weid *et al*., 1996; Fox & von der Weid, 2002). Briefly, small collecting lymphatic vessels (diameter $< 230\ \mu$m) from the jejunal and ileal regions were dissected together with their associated artery and vein and left intact within the surrounding mesentery. The mesentery was used to pin out the tissues upon the Sylgard-coated base of a 2-ml organ bath. The bath was mounted on the stage of an inverted microscope (CK40, Olympus) and continuously superfused at a flow rate of $3\ \text{ml min}^{-1}$ with PSS heated to 36°C. To induce a consistent rate of vessel constrictions, the vessel lumen was perfused through a fine micropipette glass cannula inserted into a cut opening of the vessel. The cannula was connected to an infusion pump via Teflon tubing allowing the vessel lumen to be perfused in the direction of the valves at a flow rate of $2.5\ \mu\text{l min}^{-1}$. This flow rate was selected from preliminary experiments as the most reliable in inducing a regular rhythmical contractile activity in lymphatic vessels in the range of diameter used and for the duration of the experiment (typically 3 – 4 h). As the Ca^{2+} concentration in normal PSS tended to block the cannula, a low-calcium solution, in which 0.3 mM $CaCl_2$ was substituted for 2.5 mM, was used. Perfusion with this solution did not alter vessel contractile activity nor endothelial responsiveness (see von der Weid *et al*., 1996). Lymphatic vessel chambers were observed by videomicroscopy, with diameter changes and constriction frequency continuously measured with a video-dimension analyzer (Model V94, Living Systems Instrumentation, Burlington, VT, U.S.A.). This device, designed to sense the optically denser wall of the vessel, at a chosen scan line seen on the monitor, followed any change in vessel diameter with a rapid (< 20 ms) time resolution. Data were then recorded on a computer via an analog-to-digital converter (PowerLab/4SP, ADInstruments, Mountain View, CA, U.S.A.). Preparations were allowed a 30-min equilibration period prior to the first agonist application. A 5-min control period of contractile activity was recorded prior to each test solution being administrated for 1 min and effects on vessel constriction frequency were assessed during the three consecutive minutes showing the greatest response. Data were averaged and expressed as a percentage of the mean of the 5-min control period immediately preceding the agonist application. Concentration – response relations to 5-HT and 5-CT were obtained by applying increasing concentrations of the agonist, with a washout period of at least 15 min between successive applications. The EC_{50} values were determined from individual concentration – response relations by manual graph interpolation. In experiments where the effects of inhibitors or antagonists were investigated, responses to agonists were evaluated after the inhibitor was present for at least 15 min in the superfusion solution and expressed as a percentage of 5-min control period preceding this agonist application in the presence of the antagonist. None of the inhibitors and antagonists used in this study had a noticeable effect on the control lymphatic vessel constriction rate.

Electrophysiology

The procedure has been previously described (von der Weid *et al*., 2001; Fox & von der Weid, 2002). Briefly, lymphatic vessels and attached mesentery were pinned onto a small organ bath (volume $100\ \mu$l), mounted on the stage of an inverted microscope and superfused (flow rate $3\ \text{ml min}^{-1}$) with PSS heated to 36°C. Impalements of smooth muscle cells were obtained from the adventitial side of a lymphatic vessel using

conventional glass intracellular microelectrodes (filled with 0.5 M KCl) with resistances of 150 – 250 MΩ. Electrodes were connected to an amplifier through an Ag – AgCl half-cell. Resting membrane potential was monitored on a digital oscilloscope and simultaneously recorded on a computer via an analog – digital converter. In order to ensure simplified electrical properties of the smooth muscle, vessels were cut into short segments (125 – 350 μm) with fine dissecting scissors. In this situation, electrical activity, even though generated at localized foci within the smooth muscle, produced a similar potential change in all the smooth muscle cells of the segment (Van Helden, 1993).

Lymphatic smooth muscle impalements were characterized by a sharp drop in potential that settled after 10 – 15 s to a value typically more negative than −45 mV. Impalements were maintained for more than 5 min in >90% of the cases and up to 1 – 3 h optimally. In experiments where the effects of agonists were studied in the presence of antagonists or inhibitors, agonists were applied first as a control and then, at least 20 min later, in the presence of the antagonist that had been superfused for at least 10 min. This protocol was usually performed during the same impalement. However, in some instances, successive impalements were obtained from neighboring cells in the same segment. In preliminary recordings, no significant difference in the responses was found during successive applications (20-min intervals) of the same agonist, at the same concentration. Depolarizing events greater than 1 mV were considered as STDs and their activity was assessed by measuring their frequency and amplitude. STD frequency and amplitude, occurring during an interval of 15 – 60 s (depending on the stability of the recording, but typically 30 s) before application of 5-HT (or other agonists), were compared with that occurring during a period of the same duration while the maximum response to the agonist was observed.

Destruction of the endothelium

The lymphatic endothelium was destroyed *in vitro* by repeatedly passing brief streams of air through the lumen of the vessel (five to six times for 5 – 10 s) as previously described (Fox & von der Weid, 2002). The success of the endothelial destruction was confirmed by applying acetylcholine (10 μM) followed by sodium nitroprusside (100 μM) in the superfusion solution, while the vessel lumen was perfused. A negative response to acetylcholine and a positive response to sodium nitroprusside were used as confirmation of the success of the procedure. Endothelial destruction based on this testing procedure proved successful in about 50% of treated vessels. The use of sodium nitroprusside was necessary, as it has been shown that 40% of guinea-pig mesenteric lymphatic vessels that had an intact endothelium did not respond in any way to either acetylcholine or sodium nitroprusside; the main reason shown to be due to a high basal production of nitric oxide (von der Weid *et al*., 1996).

Chemicals and drugs

The following drugs were used: 5-hydroxytryptamine (5-HT) from RBI; 5-carboxamidotryptamine (5-CT), L-NOARG, indomethacin, tetrodotoxin (TTX), glibenclamide from Sigma/Aldrich; H89 (*N*-[2-(*p*-bromociannamylamino)-ethyl]-5-isoquinolinesulfonamide-dichloride) and SB203186 ((1-piperidinyl)ethyl 1*H*-indole 3-carboxylate hydrochloride) from Alexis Corp. (San Diego, CA, U.S.A.); GR113808 (1-methyl-1*H*-indole-3-carboxylic acid [1-2-[(methyl sulfonyl) amino] ethyl]-4-piperidinyl methyl ester), methysergide, RS67506 (1-(4-amino-5-chloro-2-methoxy-phenyl)-3-[1-(2-methylsulfonyl-amino)ethyl-4-piperidinyl]-1-propanone hydrochloride) and SB269970 (2*R*)-1-[(3-hydroxyphenyl)sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine hydrochloride from Tocris Cookson (Ellisville, MO, U.S.A.), and α-methyl 5-HT from Biomol (Plymouth Meeting, PA, U.S.A.). Drugs were dissolved in distilled water, except H89 and glibenclamide (dimethylsulfoxide), L-NOARG (0.1 M HCl) and indomethacin (ethanol) to give 10 mM stock solutions, which were then diluted in PSS to achieve the appropriate concentration. The final concentration of each vehicle was always ⩽0.1%, a concentration that had no effects on lymphatic contractile and electrical functions.

Statistical analysis

Data are expressed as means ± one standard error of the mean (s.e. m.). Statistical significance was assessed using a two-tailed paired Student's *t*-test (unless specified in the text), with $P<0.05$ being considered significant.

Results

Effect of 5-HT on the constriction rate of perfused lymphatic vessels

During intraluminal perfusion, mesenteric lymphatic vessels spontaneously and rhythmically constricted at frequencies ranging from 3 to 20 min^{-1} (mean 8 ± 3 min^{-1}). Application of 5-HT for 1 min decreased the constriction frequency in a concentration-dependent manner (EC_{50} = 52.2 ± 0.8 nM, $n=5$) leading to a complete cessation of the constrictions for 3 – 4 min at concentrations higher than 0.5 – 1 μM (Figure 1).

Effect of 5-HT on the lymphatic smooth muscle membrane potential and activity of STDs

Intracellular microelectrode recordings were obtained in smooth muscle cells from short lymphatic vessel segments with the endothelium left intact. The mean resting membrane potential obtained from 31 recordings was −51.6 ± 1.1 mV. Application of 5-HT (1-min duration) hyperpolarized the lymphatic smooth muscle membrane potential in a concentration dependent manner (Figure 2). STDs, the electrical events underlying constrictions in these lymphatic smooth muscles (Van Helden, 1993), were observed in >90% of the recordings. Both frequency and amplitude of STDs were significantly decreased during 5-HT-induced hyperpolarizations (Figure 2d).

Role of the endothelium and innervation in the lymphatic vessel responses to 5-HT

Lymphatic vessel responses to acetylcholine, substance P, and ATP have been demonstrated to be mediated by endothelium-derived factors (von der Weid *et al*., 1996; Rayner & Van

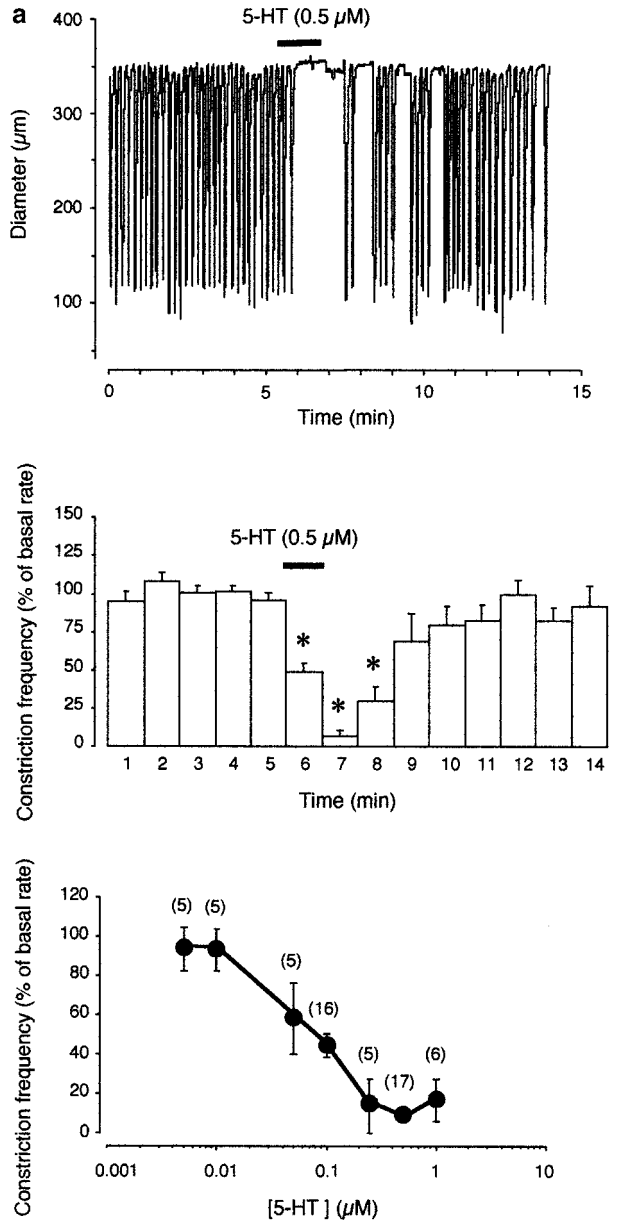

Figure 1 Effect of 5-HT on the contractile activity of lymphatic vessel in the guinea-pig mesentery. (a) Original traces of vessel diameter changes in an actively constricting lymphatic chamber, in response to 0.5 μM 5-HT applied for 1 min (horizontal bar). Downward deflections represent constrictions. (b) Time-course histogram showing the mean response ($\pm$s.e.m, $n=26$) to the same concentration of 5-HT. Columns represent constrictions per minute expressed as a percentage of the 5-min control period before application of 5-HT. *$P<0.05$ vs mean of 5 min of control (paired Student's t-test). (c) Concentration-dependent relation of the 5-HT-induced decrease in constriction frequency.

Helden, 1997; Gao *et al*., 1999). The endothelium thus plays an important role in modulating lymphatic vessel pumping. The role of the endothelium in the response to 5-HT was examined after the endothelium was made nonfunctional (see Methods). The results, illustrated in Figure 3a, showed that the response to 5-HT (0.1 and 0.5 μM) tended to be reduced by endothelium destruction. However, this effect did not attain statistical significance ($P=0.075$, unpaired Student's t-test). We then investigated the response to 5-HT in the presence and absence of the NO-synthase inhibitor L-NOARG. The decrease in constriction frequency caused by 5-HT (0.1 and 0.5 μM) was partially inhibited by L-NOARG (100 μM), with significance reached for the 0.5 μM concentration ($P=0.004$, $n=6$; Figure 3b). The membrane potential hyperpolarization to 0.1 μM 5-HT was significantly decreased in the presence of L-NOARG (100 μM; $P=0.004$). However, hyperpolarizations to 0.5 and 1 μM 5-HT and STD amplitude and frequency were not affected by this treatment (Figure 4).

The response to 5-HT was also assessed in the presence of indomethacin to test a possible involvement of cyclooxygenase products. No significant difference between the responses to 5-HT in the absence or presence of indomethacin could be demonstrated ($P>0.05$) at 0.1 μM (64.4$\pm$15.5% of control vs 82.6$\pm$19.6% of control in indomethacin, $n=4$) and 0.5 μM (32.7$\pm$9.1% of control vs 31.3$\pm$10.2% of control in indomethacin, $n=5$). The possibility of 5-HT mediating its effects by stimulating nerve terminals to release inhibitory neurotransmitters was examined by comparing the response to 5-HT prior and during treatment with tetrodotoxin (TTX, 1 μM). In the six vessels tested, 5-HT caused similar decreases in constriction frequency before and during application of TTX, at 0.1 μM (73.6$\pm$2.4% of control vs 76.1$\pm$4.4% of control in TTX, $n=4$) and 0.5 μM (50.8$\pm$5.2% of control vs 43.8$\pm$7.6% of control in TTX, $n=5$). This result indicates that actions of 5-HT on neural stimulation were not involved in this response.

Effect of 5-HT receptor agonists on lymphatic pumping

Lymphatic vessel constriction frequency was assessed during treatment with 5-CT, a very potent agonist for 5-HT_1 and 5-HT_7 receptors (Craig & Clarke, 1990; Ruat *et al*., 1993). 5-CT was observed to cause a concentration-dependent decrease of lymphatic constriction frequency (Figure 5a and b). The response to 5-CT was comparable to that of a 20-fold higher concentration of 5-HT (compare traces in Figure 1a and 5a, recorded from the same preparation). The 5-HT_4 receptor agonist RS67506 (Eglen *et al*., 1995) caused a decrease in the rate of lymphatic constrictions to 71.0$\pm$3.0% of control ($n=39$, $P<0.05$). This action was long-lasting, with the contractile activity depressed for more than 10 min, and was observed only at high concentrations (50 μM). The response to the 5-HT_2 receptor agonist, α-met-5-HT was also evaluated, but no change in lymphatic constriction frequency was observed up to concentrations as high as 50 μM. The constriction rate during application of 5 and 10 μM α-met-5-HT reached 91.1$\pm$6.4 and 88.7$\pm$6.4% of control, respectively ($P>0.05$, $n=11$ and 3).

Effects of 5-HT receptor agonists on the lymphatic smooth muscle membrane potential and activity of STDs

Consistent with its potent action in depressing vessel constriction rate, 5-CT also hyperpolarized the smooth muscle in a concentration-dependent manner (Figure 5c and e). Comparison of the left and right traces of Figure 6a illustrated that 5-CT induced a hyperpolarization similar to that of a 20-times higher concentration of 5-HT. 5-CT also significantly reduced the frequency and amplitude of STDs ($P<0.05$; Figure 5f). L-NOARG did not inhibit the 5-CT-induced hyperpolarization (7.7$\pm$1.8 *vs* 10.3$\pm$2.0 mV in control, Figure 5c and d) and the

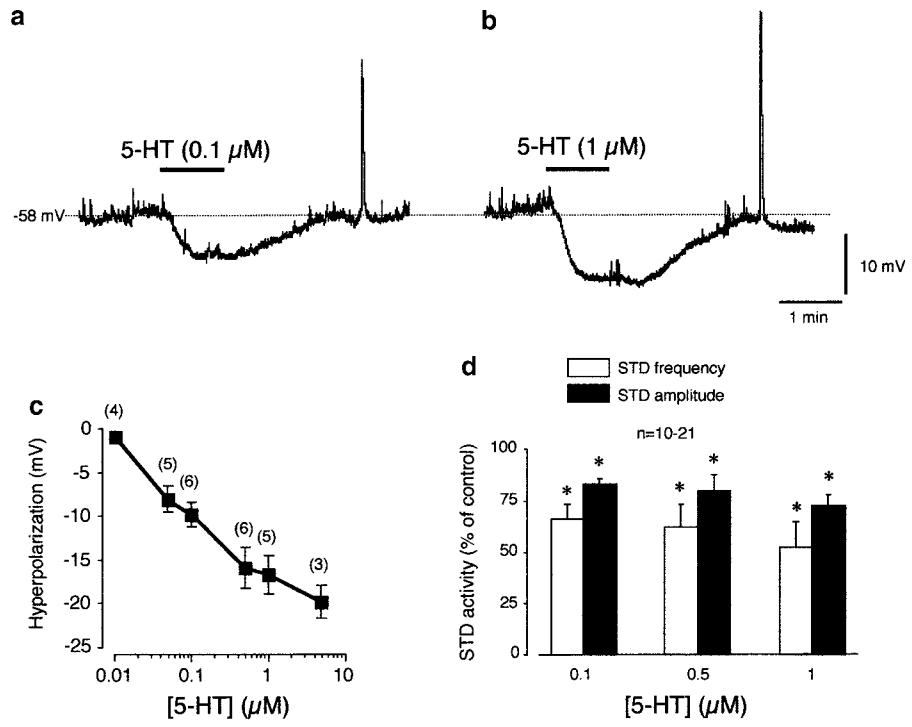

Figure 2 Effects of 5-HT on membrane potential and STD activity in guinea-pig mesenteric lymphatic smooth muscle. Original intracellular microelectrode recordings, displaying STDs and spontaneous action potentials (small and large upward deflections, respectively) in response to 0.1 (a) and 1 μM 5-HT (b) applied for 1 min (horizontal bars). Resting membrane potential value is indicated on the left-hand side. (c) Concentration-dependent relation of the 5-HT-induced hyperpolarization ($n=3-6$). (d) 5-HT-induced decrease in STD (small upward deflections in (a) & (b)) frequency and amplitude. STDs were measured during the maximum response to 5-HT (0.1, 0.5, and 1 μM) and expressed as a percentage of the values obtained during the same impalement, before 5-HT application. Columns are means$\pm$s.e.m, for n experiments. *$P<0.05$ vs control (paired Student's t-test).

5-CT-induced decrease in STD frequency (50$\pm$11 *vs* 45$\pm$10% in control) and STD amplitude (72$\pm$9 vs 70$\pm$6% in control; 5 – 500 nM, $n=6$). Figure 5c and d also shows that L-NOARG per se can cause an increase in STD amplitude and/or frequency, a finding already reported (von der Weid *et al.*, 1996), which suggests a constitutive release of NO in our experimental conditions. The 5-HT_4 agonist RS76065 (50 – 100 μM) did not hyperpolarize the smooth muscle or affect STD activity. However, in three out of four vessel segments, RS76065 caused a depolarization of 2 – 3 mV. In the presence of α-met-5-HT (5 μM), the membrane potential did not vary from its control value of -49.0 ± 3.1 mV ($n=6$).

Effect of 5-HT receptor antagonists on the 5-HT-induced decrease in lymphatic pumping

The effect of 5-HT in decreasing smooth muscle contractile activity has been shown to involve 5-HT_4 receptor activation in vascular (Cocks & Arnold, 1992), intestinal (Tam *et al.*, 1994; Tuladhar *et al.*, 1996; Prins *et al.*, 1999), and lymphatic smooth muscles (Miyahara *et al.*, 1994; McHale *et al.*, 2000). In order to investigate whether the 5-HT_4 receptor subtype was involved in the slowing of pumping in mesenteric lymphatic vessels of the guinea pig, we first tested the response to 5-HT in the presence of the 5-HT_4 receptor antagonist SB203186 (Parker *et al.*, 1995). Results, summarized in Table 1, show that SB203186 (1 μM) did not significantly inhibit the decrease in constriction rate induced by 5-HT (0.1, 0.5, and 1 μM; $P>0.05$). Consistently, the specific 5-HT_4 receptor antagonist GR113808 (1 μM, Gale *et al.*, 1994) did not significantly alter 5-HT-induced response ($P>0.05$). Similar results were obtained when lymphatic pumping was decreased by 5-CT (1, 5, and 10 nM; $P>0.05$; Table 1). Importantly, the RS67506-induced decrease in rate of constrictions was blocked neither by SB203186, nor by GR113808 ($n=4$ & 6, respectively, data not shown).

Recent investigations have reported 5-HT-induced relaxations mediated by 5-HT_7 receptors in smooth muscles (Prins *et al.*, 1999b; Terrón & Falcón-Neri, 1999; Ishine *et al.*, 2000). We addressed the possible involvement of 5-HT_7 in the action of 5-HT by using SB269970, a specific antagonist of 5-HT_7 (Lovell *et al.*, 2000), and methysergide, a 5-$HT_{1/2/5/7}$-receptor antagonist that has no effect on 5-HT_4-induced responses, but was shown to antagonize 5-HT_7-induced responses in smooth muscle preparations (Prins *et al.*, 1999a,b). As shown in Table 1, the decrease in constriction frequency caused by 5-HT ($n=4$), or by the 5-HT1/7 agonist 5-CT ($n=3$), was markedly decreased in the presence of SB269970 (0.5 μM). Similarly, in lymphatic vessels treated with methysergide (0.5 μM) the responses to 5-HT ($n=7$) and to 5-CT ($n=6$) were significantly inhibited (Table 1).

No difference between responses to 5-HT in control conditions and in the presence of the 5-HT_2 receptor antagonist, ketanserin (1 μM) were observed ($n=4$; Table 1).

Effect of 5-HT receptor antagonists on the 5-HT- and 5-CT-induced smooth muscle electrical responses

Electrical responses to 5-HT were not affected by the 5-HT_4 antagonist SB203186 (Figure 6). 5-HT (0.1 μM) hyperpolarized

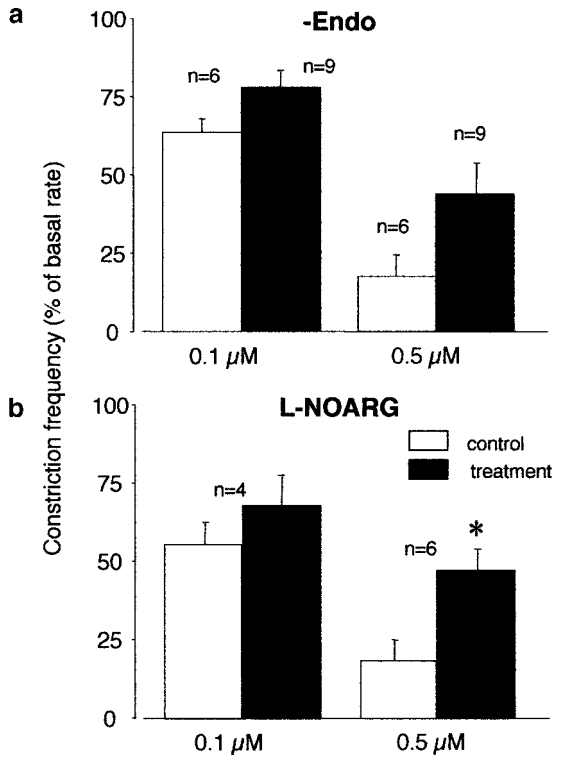

Figure 3 Effect of endothelium lysis (-Endo (a)) and NO-synthase inhibition by L-NOARG (100 μM (b) on the decrease in lymphatic constriction frequency induced by 5-HT (0.1 and 0.5 μM). Columns represent the mean ($\pm$s.e.m.) constrictions per minute in control conditions (open columns) and during treatment (closed columns). *$P<0.05$ vs control (unpaired (a) and paired Student's t-test (b).

the smooth muscle by 5.5 ± 1.7 mV in control, and by 6.0 ± 2.0 mV in the presence of SB203186 (1 μM, $n=4$). In addition, SB203186 did not inhibit the 5-HT-induced decrease in STD frequency (66 ± 10 vs 44 ± 7% in control) and STD amplitude (78 ± 4 *vs* 77 ± 12% in control). Similar results were obtained with 5-CT (Figure 6). Neither hyperpolarization to 5 nM 5-CT (7.3 ± 0.3 mV in control and 7.0 ± 1.5 mV in SB203136), nor the decrease in STD frequency (78 ± 9% in control and 54 ± 12% in SB203136) and STD amplitude (59 ± 12% in control and 66 ± 1% in SB203136) were inhibited in the presence of SB203136 ($n=3$). In the presence of methysergide, hyperpolarizations to 5-HT and 5-CT were strongly inhibited (Figure 6c). Values of 5.7 ± 2.2 and 4.5 ± 1.5 mV were reduced to 0.3 ± 1.5 and 0.5 ± 1.5 mV for 100 nM 5-HT and 5 nM 5-CT, respectively ($n=3$).

Effect of glibenclamide on the 5-HT-induced electrical and mechanical responses

Previous studies have demonstrated in the same preparation, a predominant role for K_{ATP} channels in lymphatic smooth muscle hyperpolarization caused by NO, isoprenaline, and forskolin (von der Weid, 1998). To determine the type of conductance(s) activated during the 5-HT-induced hyperpolarization, we thus examined the response to 5-HT in the presence of the K_{ATP} channel blocker, glibenclamide. In control conditions, lymphatic smooth muscle membrane potential was hyperpolarized by 5-HT (1 μM) from -49.8 ± 2.6 mV to a peak value of -59.5 ± 1.0 mV. The 5-HT-induced hyperpolarization was decreased to 21 ± 10% of control by 10 μM glibenclamide (hyperpolarization from

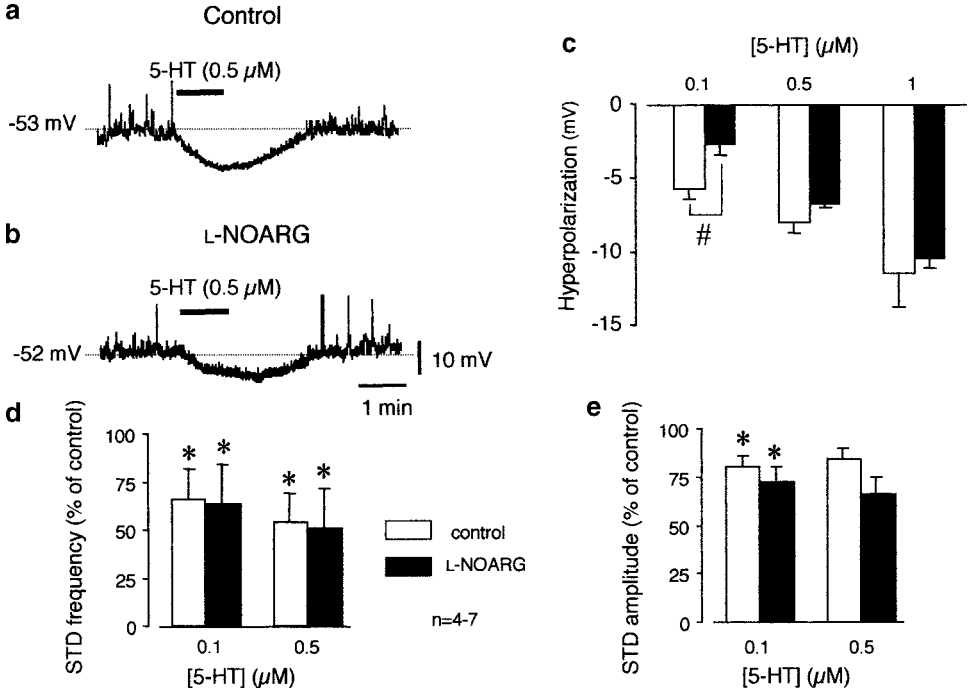

Figure 4 Effect of L-NOARG on the membrane potential responses to 5-HT. Original intracellular microelectrode recordings in response to 0.5 μM 5-HT applied for 1 min (horizontal bars) in control conditions (a) and in the presence of L-NOARG (100 μM (b)). Resting membrane potential values are indicated on the left-hand side of the traces. Summary histograms of the effect of L-NOARG on the hyperpolarization (c), changes in STD frequency (d) and STD amplitude (e) induced by 5-HT applied at the concentrations indicated. # and * indicate significant differences ($P<0.05$, paired Student's t-test) between 5-HT-induced hyperpolarization in the presence of L-NOARG vs control, and between STD activity during 5-HT-induced response vs control, respectively.

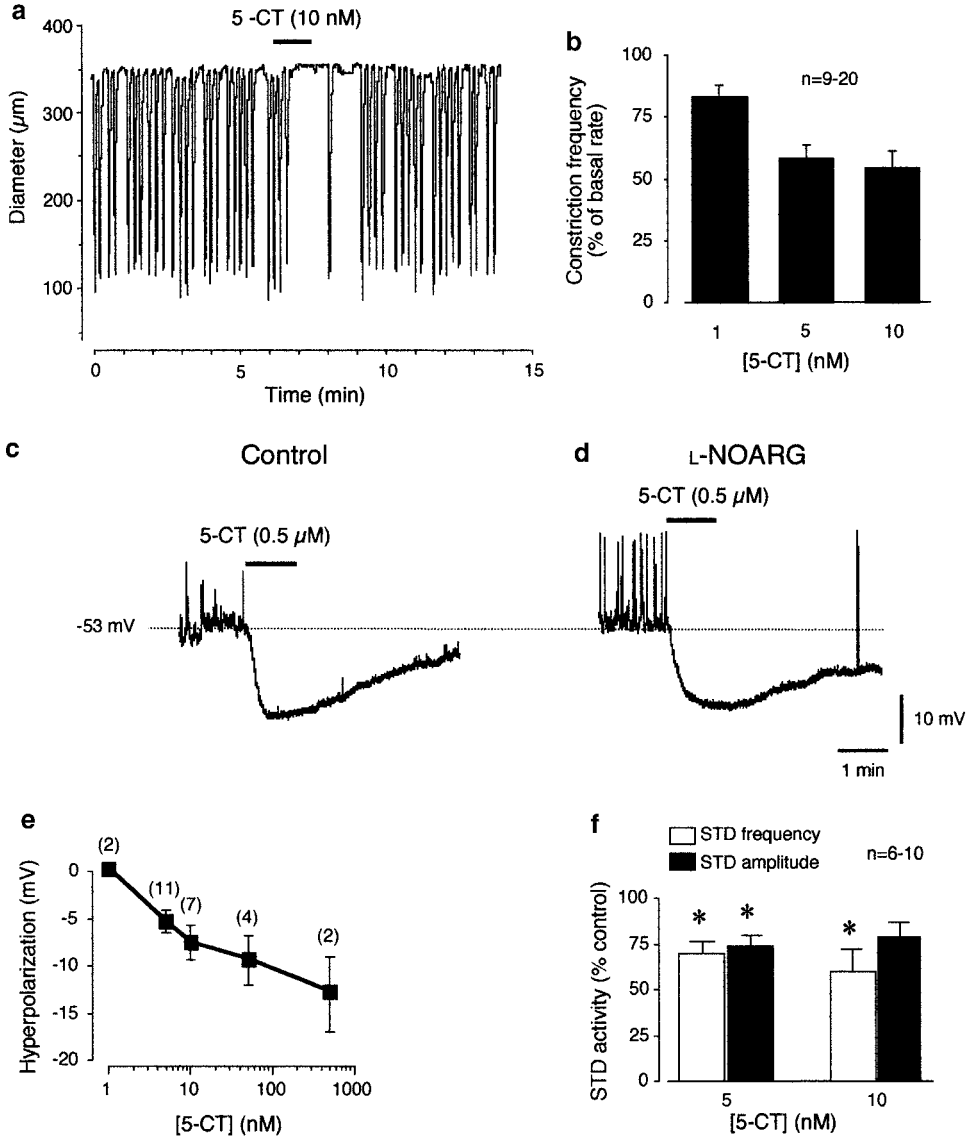

Figure 5 Effect of 5-CT on the contractile activity and membrane potential in guinea-pig mesenteric lymphatic vessel. (a) Original traces of vessel diameter changes in an actively constricting lymphatic chamber in response to 10 nM 5-CT. Downward deflections represent constrictions. (b) Concentration-dependent relation of the 5-CT-induced decrease in constriction frequency, with columns representing mean (±s.e.m.) constrictions per minute expressed as a percentage of the control period before application of 5-CT. (c, d) Original intracellular microelectrode recordings in response to 0.5 μM 5-CT applied for 1 min (horizontal bars), in control conditions (c) and in the presence of L-NOARG (100 μM, (d)). Resting membrane potential value is indicated on the left-hand side. (e) Concentration-dependent relation of the membrane potential response to 5-CT ($n=3-11$). (f) Summary histograms of the decrease in STD (upward deflections in (c, d) frequency and amplitude induced by 5-CT (5 and 10 nM). *$P<0.05$ vs control (paired Student's *t*-test).

-46.3 ± 1.9 to -48.8 ± 1.3 mV $n=4$ $P=0003$; Figure 7a and b). A similar result was obtained when glibenclamide was used at a concentration of 0.1 μM and the control hyperpolarization was blocked to $37\pm11\%$ of control ($P=0.03$, $n=3$). As already reported (von der Weid, 1998; von der Weid *et al.*, 2001), glibenclamide by itself caused a depolarization of about 4 mV. Although glibenclamide inhibited 5-HT-induced hyperpolarization, in the four preparations examined, it did not significantly affect the 5-HT-induced decrease in STD frequency ($P=0.304$) and amplitude ($P=0.297$, Figure 7c).

In perfused lymphatic vessels, glibenclamide (10 μM) significantly ($P<0.05$) altered the decrease in constriction frequency induced by 0.1 and 0.5 μM 5-HT ($n=5$, Figure 7d).

Effect of H89 on the 5-HT-induced hyperpolarization and STD activity

cAMP production and subsequent cAMP-dependent protein kinase (PKA) activation has been proposed to increase upon stimulation of 5-HT-receptors causing smooth muscle relaxation (Ford *et al.*, 1992; McLean & Coupar, 1996; Kitazawa *et al.*, 1998). In order to determine if PKA was involved in the 5-HT-induced hyperpolarization of the lymphatic smooth muscle, 5-HT was applied in the presence of the PKA inhibitor, H89. Hyperpolarization caused by 1 μM 5-HT in control conditions was reduced ($P=0.02$, $n=4$) in the presence of H89 (10 μM, Figure 8a and b). As illustrated in

Figure 8c, H89 also affected the 5-HT-induced decrease in STD frequency and amplitude ($n=3$). The lymphatic smooth muscle membrane potential was also significantly depolarized by H89 from a control value of -52.0 ± 1.2 to -44.3 ± 1.1 mV ($P<0.005$, $n=4$).

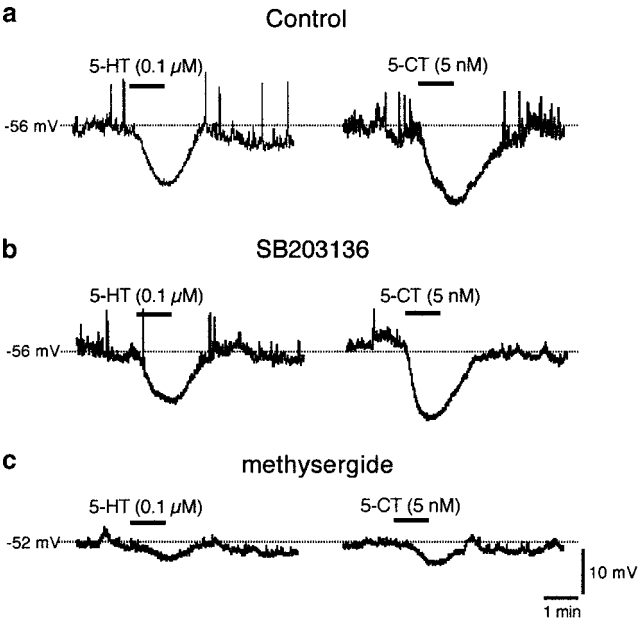

Figure 6 Hyperpolarizations induced by 5-HT (0.1 μM, left traces) and 5-CT (5 nM, right traces) in control conditions (a) and in the presence of the 5-HT receptor antagonists, SB203136 (1 μM, (b) and methysergide (0.5 μM, (c)). All recordings are from the same cell. Resting membrane potential values are indicated on the left-hand side of the traces and scale bars apply to all traces.

Discussion

Experiments have been conducted to investigate the effect of 5-HT on the contractile activity of guinea-pig mesenteric lymphatic vessels and to characterize the electrical and intracellular mechanisms underlying the mechanical responses. The main finding is that 5-HT decreased, in a concentration-dependent manner, the rate of constrictions induced by intraluminal perfusion of lymphatic vessels. This effect is associated with a hyperpolarization of the smooth muscle membrane potential and a decrease in the frequency and amplitude of STDs, the electrical events proposed to be involved the pacemaking of lymphatic contractile activity.

The 5-HT-induced decrease in lymphatic pumping shown in our study is in agreement with a recent report by McHale *et al.* (2000) showing that 5-HT primarily caused an inhibition of the spontaneous constrictions in sheep mesenteric lymphatic vessels. In contrast, 5-HT was shown to elicit tonic contraction in quiescent lymphatic vessels from isolated bovine mesenteric segments (Williamson, 1969; Ohhashi *et al.*, 1978), canine thoracic ducts (Takahashi *et al.*, 1990) and forelimbs (Dobbins, 1998), porcine tracheobronchial (Ferguson *et al.*, 1993), and hepatic lymphatic vessels (Hashimoto *et al.*, 1994). It was also observed to increase rhythmical constrictions in spontaneously contracting lymphatic vessels of the human lower leg and superficial groin (Sjoberg & Steen, 1991) and of bovine mesenteric vessels (Johnston *et al.*, 1983). Importantly, in the latter preparation, 5-HT caused a relaxation when the vessels were preconstricted (Johnston *et al.*, 1983; Miyahara *et al.*, 1994).

The 5-HT-induced relaxation and decrease in constriction frequency have been shown to be mediated by 5-HT_4 receptor stimulation located on the smooth muscle of sheep and bovine mesenteric lymphatic vessels (Miyahara *et al.*, 1994; McHale

Table 1 Effects of 5-HT-receptor antagonists on 5-HT- and 5-CT-induced decrease in rate of lymphatic vessel constriction

	Constriction rate (per cent of control before agonist application)		
(a) 5-HT	*0.1 μM*	*0.5 μM*	*1 μM*
Alone	80.6±5.5 (12)	54.6±7.2 (15)	46.8±6.2 (13)
+SB203186 (1 μM)	81.3±3.4 (12)	61.5±5.7 (15)	60.2±7.8 (13)
Alone	87.2±4.0 (4)	60.6±9.5 (5)	49.5±5.4 (3)
+GR113808 (1 μM)	80.1±8.3 (4)	52.1±8 (5)	27.4±6.5 (3)
Alone	70.5±11.4 (4)	54.7±5.4 (4)	n.d.
+SB269970 (0.5 μM)	89.6±6.2 (4)	95.2±3.8* (4)	n.d.
Alone	73.6±3.4 (7)	49.6±11.6 (7)	29.5±12.6 (3)
+Methysergide (0.5 μM)	88±4.8* (7)	82±3.2* (7)	73.6±12.3* (3)
Alone	69.5±8.6 (4)	47.5±14.4 (7)	49.3±18.9 (3)
+Ketanserin (1 μM)	97.8±7 (4)	80.2±8.5 (7)	82.8±9.2 (3)
(b) 5-CT	*1 nM*	*5 nM*	*10 nM*
Alone	73.3±4.4 (9)	72.3±5.4 (12)	53.7±8.8 (9)
+SB203186 (1 μM)	82.6±5.6 (9)	73.9±5.4 (12)	65.1±7.4 (9)
Alone	n.d.	70.5±12.4 (4)	55.1±23.4 (3)
+GR113808 (1 μM)	n.d.	52.1±8 (4)	27.4±6.5 (3)
Alone	n.d.	56.2±8.5 (3)	30.4±2.6 (3)
+SB269970 (0.5 μM)	n.d.	85.7±6.4 (3)	86.9±6.2* (3)
Alone	75.9±7.4 (6)	53.3±7.4 (6)	33.6±5.6 (4)
+Methysergide (0.5 μM)	83.6±6.5 (6)	76.6±6* (6)	64.7±10.1 (4)
Alone	n.d.	63.5±6.8 (4)	73.2±8.8 (4)
+Ketanserin (1 μM)	n.d.	86.1±2.7 (4)	78.2±4.5 (4)

Data are expressed as mean values±s.e.m. The number of experiments is indicated in brackets. *$P<0.05$ vs 5-HT or 5-CT alone (paired Student's *t*-test), n.d.; not determined.

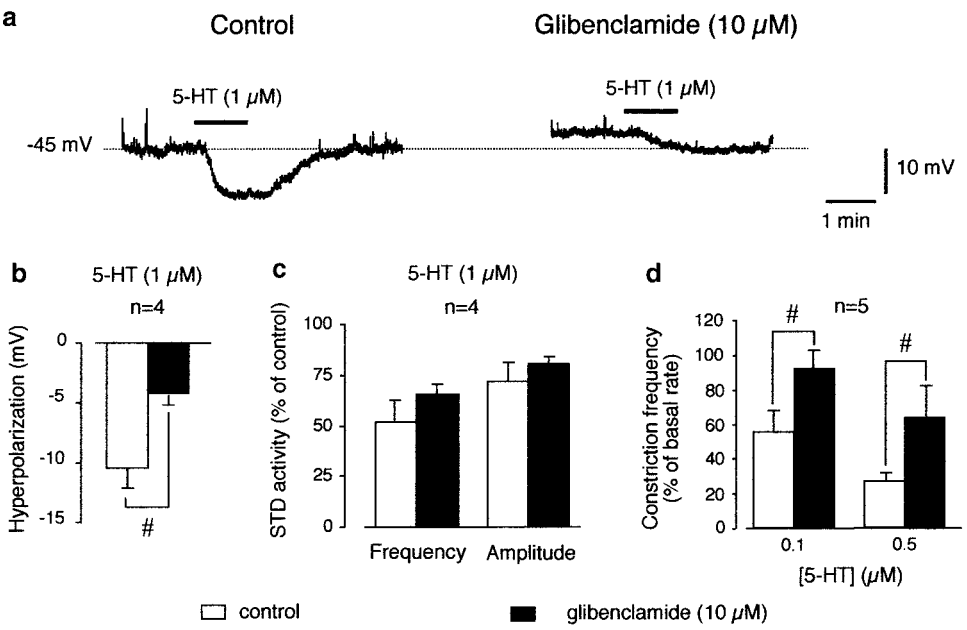

Figure 7 Effect of glibenclamide on the responses to 5-HT in guinea-pig mesenteric lymphatic vessels. (a) Original intracellular microelectrode recordings in response to 1 μM 5-HT in control conditions (left trace) and in the presence of glibenclamide (10 μM, right trace) applied for 1 min (horizontal bars). Resting membrane potential value is indicated on the left-hand side of the traces. Summary histograms of the effect of glibenclamide on the hyperpolarization (b), changes in STD frequency and amplitude (c) and on the decrease in constriction frequency of perfused vessel (d) induced by 5-HT applied at the concentrations indicated. $^{\#}P<0.05$ between control and treatment (paired Student's *t*-test).

et al., 2000). Although the insensitivity of the 5-HT-induced decrease in lymphatic pumping to TTX is indicative of a smooth muscle relaxant 5-HT receptor, the present results suggest that 5-HT_4 receptors, if involved, play a negligible role. This postulate is supported by the following observations: (1) The 5-HT_4 receptor antagonists SB203186 and GR113808 (at a concentration 100-fold higher than that effective in inhibiting 5-HT_4 receptors in canine isolated rectum circular smooth muscle, Prins *et al.*, 1999b) did not affect 5-HT-induced decrease in constriction rate. (2) Although RS67506 mimicked 5-HT-induced decrease in constriction rate, this effect was observed at high concentration (50 – 100 μM) only making it an agonist, 100 – 500 fold less potent than 5-HT. This observation is incompatible with the potent effect (pD_2 8.6) of RS67506 at stimulating 5-HT_4 receptors in the guinea-pig esophagus (Eglen *et al.*, 1995). Besides, RS67506 was not blocked by either SB203186 or GR113808 and did not mimic 5-HT-induced hyperpolarization.

In addition to the above, the following results further suggest that a different 5-HT receptor sub-type is implicated. (1) Responses to 5-HT were strongly inhibited by SB269970, a specific 5-HT_7 receptor antagonist (Lovell *et al.*, 2000) and by methysergide, a 5-$HT_{1/2/5/7}$ receptor antagonist, which is ineffective at blocking 5-HT_4 receptors (Prins *et al.*, 1999a). (2) 5-HT-induced responses were mimicked by the 5-$HT_{1/7}$ receptor agonist, 5-CT, which is not known to stimulate 5-HT_4 receptors (Craig & Clarke, 1990; Prins *et al.*, 1999a). Inhibition of lymphatic vessel contraction by 5-CT has been demonstrated in the bovine mesentery (Miyahara *et al.*, 1994). The authors used these data to support a role of 5-HT_4 receptors in their preparation as neither 5-HT- nor 5-CT-induced relaxant responses were blocked by methysergide. In our hands, 5-CT induced a decrease in constriction rate similar to that of a 100-fold higher concentration of 5-HT and this effect was abolished by SB269970 and methysergide. To our knowledge, 5-CT, SB269970, and methysergide are reliable tools to investigate the contribution of 5-HT_7 receptors in 5-HT-induced relaxation in smooth muscles (see Prins *et al.*, 1999a; Terrón & Falcón-Neri, 1999; Ishine *et al.*, 2000; Janssen *et al.*, 2002). However, the pharmacology of 5-HT_7 receptors is still difficult to assess. Despite these limitations, the pharmacological profile of the inhibitory 5-HT receptor in the lymphatic smooth muscle is consistent with that of the 5-HT_7 receptor.

Previous pharmacological investigations have revealed that lymphatic vessel contraction or increase in rhythmical constrictions are mediated by 5-HT_2 receptors (Miyahara *et al.*, 1994; McHale *et al.*, 2000). Such an excitatory action was never observed in response to 5-HT in the course of the present study. The involvement of excitatory 5-HT receptors in lymphatic vessels of the guinea-pig mesentery is thus unlikely. In addition, no response to α-met-5-HT and no significant effect of ketanserin could be revealed in our experimental conditions. Furthermore, depolarization or increase in STD activity that could account for 5-HT_2-receptor stimulation was never detected in response to 5-HT, even during inhibition of the hyperpolarization with glibenclamide. However, a transient increase in STD activity was sometimes detected during the washout period following 5-HT, when the membrane potential had returned to its resting value (see for example Figure 3). This effect was not investigated further, but may account for a possible 5-HT-mediated excitatory effect that was never translated into an increase in contractile activity in perfused vessels.

Hyperpolarization to 5-HT was inhibited by glibenclamide, suggesting the involvement of K_{ATP} channel. In contrast, 5-HT continued to induce a decrease in STD frequency and amplitude during glibenclamide treatment, in the quasi-absence of hyperpolarization. This action is reminiscent of the persistence of reduction in STD activity caused by NO and

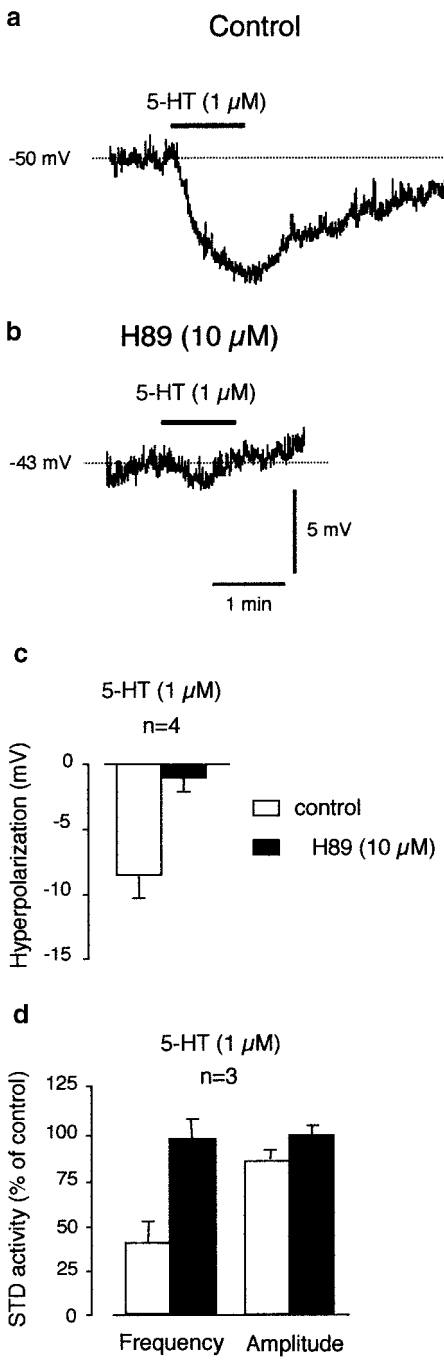

Figure 8 Effect of H89 on the responses to 5-HT in guinea-pig mesenteric lymphatic vessels. (a) Original intracellular microelectrode recordings in response to 1 μM 5-HT in control conditions (left trace) and in the presence of H89 (10 μM, right trace) applied for 1 min (horizontal bars). Resting membrane potential value is indicated on the left-hand side of the traces. Summary histograms of the effect of H89 on the hyperpolarization (b) and changes in STD frequency and amplitude (c).

forskolin in the presence of glibenclamide, and suggests that modulation of STD activity is independent of hyperpolarization (von der Weid *et al.*, 2001). However, both 5-HT-induced hyperpolarization and decrease in STD activity were blocked by the PKA inhibitor, H89, suggesting a role for the cAMP – PKA axis in the 5-HT-induced responses. This finding, again similar to that obtained with NO and forskolin (von der Weid *et al.*, 2001), is consistent with observations that other agents known to increase cytosolic cAMP concentration, like isoprenaline and isobutylmethylxantine, decreased lymphatic pumping (Mawhinney & Roddie, 1973; McHale & Roddie, 1983) and STD activity (von der Weid & Van Helden, 1996), and caused glibenclamide-sensitive hyperpolarizations (von der Weid, 1998). These results support the pivotal role of cAMP in the modulation of lymphatic contractile activity. Involvement of cAMP in 5-HT-induced responses also supports a role for 5-HT_7 receptors in these actions, as these receptors have been shown to mediate smooth muscle relaxation *via* cAMP production (Ford *et al.*, 1992; McLean & Coupar, 1996; Kitazawa *et al.*, 1998).

5-HT is located in enterochromaffin cells in the gastrointestinal tract of most mammals (Sjolund *et al.*, 1983) and stored in platelets (Meyers *et al.*, 1982). It was suggested to be released and exported to the interstitial space during brief mesenteric ischemia – reperfusion in the cat, as its concentration increased in the mesenteric lymph (Fu *et al.*, 1997). It is also known to be present in rodent mast cells (Lehtosalo *et al.*, 1984; Gershon & Tamir, 1985) making it a potent mediator of inflammation in these animals. In that context, the present finding that 5-HT directly alters lymphatic vessel pumping function, and thus lymph flow, is of considerable importance, as this action might counteract the edema-induced increase in lymph flow (Benoit *et al.*, 1989). Whether the action of 5-HT on lymphatic pumping plays a significant role in the resolution of edema during inflammation is difficult to assess, as many other inflammatory mediators have been shown to directly affect lymphatic contractile and electrical activities (von der Weid, 2001), but this aspect certainly needs to be considered.

In conclusion, 5-HT modulates the rate of lymphatic vessel pumping by eliciting a glibenclamide-sensitive hyperpolarization of the smooth muscle and a decrease in STD activity. These actions appear to be mediated by activation of 5-HT_7 receptors coupled to cAMP production. These results suggest that, although lymphatic vessel tone and contractile activity depend on the level of polarization of the smooth muscle membrane potential, decrease in STD activity is an important contributor to 5-HT-induced decrease in frequency of constrictions.

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