Endothelium-dependent modulation of pacemaking in lymphatic vessels of the guinea-pig mesentery

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1. Endothelial control of the rate of constrictions and the underlying pacemaker potentials has been studied *in vitro* in guinea-pig mesenteric lymphatic vessels.

2. ACh stimulated 60% of intraluminally perfused vessels to slow or abolish lymphatic constrictions. This action was inhibited by atropine and was likely to be due to the release of endothelium-derived nitric oxide (EDNO) as the effect was absent after endothelial lysis, mimicked by sodium nitroprusside (SNP), blocked by N^{ω}-nitro L-arginine (NOLA) and partially inhibited by Methylene Blue (MB).

3. The remaining 40% of perfused vessels did not mechanically respond to ACh or SNP. In four of seven such vessels this appeared to be due to excessive perfusion-associated release of EDNO, as incubation with NOLA restored the response to SNP.

4. Application of NOLA or MB in perfused vessels significantly increased constriction frequency, further indicating perfusion-associated release of EDNO.

5. ACh induced a marked increase in endothelial $[Ca^{2+}]_i$ of both mechanically responding and non-responding vessels. This ACh-induced increase could be repetitively induced when Ca^{2+} was present in the perfusate, but rapidly ran down when a Ca^{2+}-free EGTA perfusate was used.

6. Intracellular recordings from the smooth muscle of non-perfused vessel segments demonstrated an ACh-induced hyperpolarization and decrease in membrane resistance, changes which were prevented by atropine, NOLA, MB and endothelial lysis and mimicked by SNP.

7. ACh directly reduced the size of the underlying pacemaker potentials termed spontaneous transient depolarizations (STDs).

8. NOLA and MB enhanced STDs in non-perfused vessel segments indicating an endogenous release of EDNO.

9. It is concluded that the lymphatic endothelium produces and releases EDNO endogenously, during perfusion or after stimulation with ACh, to decrease the efficacy of STDs to generate action potentials and resultant constrictions.

The endothelium is an important regulator of smooth muscle tone in both blood and lymphatic vessels. In blood vessels the endothelium is known to release a variety of relaxing and contracting substances including endothelium-derived relaxing factor (EDRF), prostacyclin, endothelium-derived hyperpolarizing factor (EDHF) and endothelins (for review see Furchgott & Vanhoutte, 1989; Rubanyi, 1991). EDRF has been demonstrated to be nitric oxide (NO; Ignarro, Buga, Wood, Byrns & Chaudhuri, 1987; Palmer, Ferrige & Moncada, 1987) or an NO-containing moiety (Myers, Minor, Guerra, Bates & Harrison, 1990) and will henceforth be referred to as endothelium-derived nitric oxide (EDNO). EDNO has been shown to be released endogenously or in response to various stimuli (e.g. ACh, shear stress) and to cause smooth muscle relaxation by increasing production of muscular cyclic GMP (see Furchgott & Vanhoutte, 1989; Rubanyi, 1991). During stimulation with ACh, the relaxation caused by EDNO is often accompanied by a smooth muscle hyperpolarization generally attributed to EDHF (for review see Bény & von der Weid, 1993; Garland, Plane, Kemp & Cocks, 1994). However, in a few cases, ACh-induced hyperpolarizations

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have been shown to be due, in part, to ACh-induced release of EDNO (Tare, Parkington, Coleman, Neild & Dusting, 1990; Rand & Garland, 1992; Parkington, Tare, Tonta & Coleman, 1993; Vanheel, Van de Vorde & Leusen, 1994).

EDNO has also been shown to cause relaxation of pre-constricted lymphatic vessels (Ohhashi & Takahashi, 1991; Ferguson, 1992) similar to the effect on blood vessels. However, a major additional role of the lymphatic endothelium is to modulate the heart-like pumping action of the lymphatics which occurs through spontaneous constriction of vessels with smooth muscle in their wall. Endothelium-dependent slowing of these spontaneous constrictions has been reported in rings of bovine and porcine mesenteric lymphatic vessels (Yokoyama & Ohhashi, 1993; Reeder, Yang & Ferguson, 1994). Our preliminary studies indicate that this is also the case in mesenteric lymphatic vessels of the guinea-pig (Crowe, von der Weid & Van Helden, 1994), a preparation in which a mechanism underlying lymphatic pacemaking has been identified (Van Helden, 1993; Van Helden, von der Weid & Crowe, 1995). The present study considers the role and nature of endothelium-derived substances in lymphatic vessels and their action on the underlying pacemaker mechanism.

METHODS

Tissue preparation

Experiments were performed *in vitro* on mesenteric lymphatic vessels (< 300 μm diameter) from the small intestine of young guinea-pigs of either sex (age, 2–15 days). The wall of these vessels had an inner layer of endothelium with a single outer layer of smooth muscle cells. Animals were killed by an overdose of the inhalation anaesthetic halothane (5–10%), followed by decapitation. The small intestine and attached mesentery were rapidly removed and placed in a physiological saline solution of the following composition (mM): $CaCl_2$, 2·5; KCl, 5; $MgCl_2$, 2; NaCl, 120; $NaHCO_3$, 25; NaH_2PO_4, 1; and glucose, 11. The pH was maintained at 7·2 by constant bubbling with a 95% O_2–5% CO_2 gas mixture. A Ca^{2+}-free EGTA solution, which contained an equimolar substitution of $MgCl_2$ for $CaCl_2$ with 1 mM EGTA, was used in the Ca^{2+}-imaging experiments. A low-calcium solution, where 0·3 mM $CaCl_2$ was substituted for 2·5 mM $CaCl_2$, was used to perfuse the lumen of the vessels in the constriction and imaging studies. This solution reduced the possibility of Ca^{2+} precipitation and thus blockage of the cannula.

Constriction studies

The lymphatic vessel and attached mesentery were pinned onto the Sylgard-coated bottom of a perfusion bath (volume, 2 ml) and continuously superfused at a flow rate of 4 ml min^{-1} (90% bath change-over time, < 2 min) with physiological saline at 33–35 °C. The lumen of the vessel was perfused to enhance the frequency of vessel constrictions, as constriction frequency has been found to be increased by vessel filling (Florey, 1927; Smith, 1949). Perfusion was performed by cutting the vessel upstream and inserting a fine cannula (made from a glass micropipette) into the lumen so perfusion flow would be in the direction of the valves. The cannula was connected to an infusion pump and the lumen was perfused with 0·3 mM Ca^{2+} physiological saline at a rate of about 4 μl min^{-1} (range, 0·7–28·6 μl min^{-1}). Solutions containing higher Ca^{2+} concentrations tended to block the cannula. The endothelial responsiveness was not obviously inhibited with the use of the 0·3 mM Ca^{2+} perfusate as, for example, vessels loaded with a calcium-indicating dye showed consistent increases in $[Ca^{2+}]_i$ in response to ACh (see Results).

The vessels were observed using an inverted microscope with an attached video camera so that the contractile activity of the lymphatic vessels could be recorded and analysed at multiple locations in real time (or off-line) using an edge-detecting program (Beresford-Smith, Nesbitt & Van Helden, 1993). In these studies, single chambers were selected and either a single edge or both edges (for diameter measurements) were followed. A 10 min control period of contractile activity was recorded before test solutions (ACh or sodium nitroprusside (SNP)) were added to the bath. In those experiments where the effects of putative inhibitors were tested, two or three control applications of ACh or SNP were made at 10–15 min intervals and inhibitors applied for at least 10 min before, and during, application of ACh or SNP.

Confocal imaging

The lymphatic vessel and attached mesentery were pinned onto a small metal ring and placed in a glass-bottomed perfusion chamber (volume, 3 ml) and continuously superfused with physiological saline. The vessel lumen was perfused with the 0·3 mM Ca^{2+} solution containing the calcium-indicating dye Calcium Green (4 μM) for approximately 1 h at room temperature until the endothelium could be visualized. The vessel was observed using a laser-scanning confocal microscope (BioRad Laser-sharp MRC-600, Hemel Hempstead, UK) with a BHS filter set (excitation 488 nm, dichroic mirror 510 nm, emission 515 nm) attached to an inverted microscope (Zeiss Axiovert 10). The majority of images were taken using a ×40 oil-immersion objective (numerical aperture, 1·3) and gain and contrast levels set to ensure standardized images. Images of the loaded endothelium were taken at 0·5 s intervals. In all experiments nifedipine (1 μM) was present in the bathing solution to ensure that the vessel did not constrict and thus remained in the same focal plane. Images before, during and after bath application of ACh (10 μM) were continually saved. In the studies where the effect of ACh on the endothelium was observed in the presence of atropine or Ca^{2+}-free EGTA solution, a 10 min recovery period was left between the control ACh response and that obtained under test conditions.

Endothelial lysis

Removal of the endothelium was performed by a modification of the method described by Juncos, Ito, Carretero & Gavin (1993). Lymphatic vessels were intraluminally perfused for 25 min with bovine serum albumin (BSA; 5%, w/v), antibodies against human von Willebrand factor (factor VIII-related antigen, 1/1000 dilution) and rabbit complement (2%, v/v) added to the 0·3 mM Ca^{2+} saline solution. The preparation was then left at 4 °C for approximately 4 h before the lysing solution was washed out. For reasons unknown, this procedure was unreliable and often failed to lyse the endothelium. Endothelial lysis was confirmed by using the fluorescent calcium indicator Calcium Green (applied as described above). This indicator readily allowed visualizations of the endothelium of all intact vessels studied ($n = 19$). One vessel exposed to the antibody–complement solution was also fixed and prepared for examination by electron microscopy. In this tissue the endothelium appeared recognizable only as cell debris while smooth muscle appeared undamaged.

Electrophysiology

Lymphatic vessels were pinned by the mesenteric membrane surrounding the vessel to the Sylgard-coated bottom of a small perfusion chamber (volume, 100 μl). The tissue was continuously superfused at a flow rate of 3 ml min^{-1} with physiological saline causing 90% bath change-over time in < 7 s. Intracellular recordings were obtained by impaling smooth muscle cells with microelectrodes (filled with 0·5 M KCl and of resistance 150–200 MΩ) from the adventitial side of a lymphatic vessel. The vessel was previously cut into short segments (< 300 μm) with fine dissecting scissors to simplify the passive electrical properties to approximate those of a spherical cell (Van Helden, 1993). This was confirmed by establishing that the voltage response to injection of a constant current (typically −0·1 nA for 1 s) was exponential. To investigate the effect of inhibitors, ACh was superfused before, and 10–15 min after, addition of the inhibitor under study. Data were analysed for experiments in which the impalement was maintained throughout the entire experiment. When appropriate, SNP was used as control to test the responsiveness of the smooth muscle to exogenous NO.

Chemicals

The drugs used were: ACh, anti-human von Willebrand factor, atropine sulphate, BSA, complement, indomethacin, Methylene Blue (MB), nifedipine, N^{ω}-nitro-L-arginine (NOLA) and SNP, all from Sigma; Calcium Green (Molecular Probes); and halothane (Zeneca, Macclesfield, UK). Indomethacin was prepared as a 10 mM stock solution in ethanol. After dilution to the final concentration in physiological saline, the concentration of ethanol was $< 0{\cdot}1$% and had no measurable effect on the tested responses. NOLA was applied at a concentration of 1–100 μM from a stock solution of 10 mM containing 5 mM HCl, with the pH neutralized using NaOH. All other compounds were prepared as stock solutions in distilled water and appropriately diluted in physiological saline. When applied, drugs were always added to the superfusate only and hence, in the case of intraluminally perfused vessels, the effective drug concentration acting on the endothelium was less than that applied.

Data analysis

Experimental data are expressed as means ± S.E.M., with values compared using Student's paired t test. Drug-induced modulations of constriction frequency are expressed as a percentage, calculated as: (the constriction frequency during or just after application of the test drug/mean constriction frequency for the preceding 5 min control period before application of the test drug) × 100.

RESULTS

Constriction and confocal studies

Studies were performed on intact lymphatic vessels that displayed regular contractile activity during intraluminal perfusion.

Effects of ACh. Superfusion of active lymphatic vessels with ACh (10 μM) usually caused a complete inhibition or marked slowing of lymphatic constrictions. Not all perfused vessels responded to ACh, with thirty-six from ninety-two vessels investigated showing no observable response. These 'non-responding' vessels will be discussed in more detail later. Twelve vessels showed inconsistent responses to ACh, usually with an initial response that could not be replicated or variable responses from different chambers in the same vessel, with some chambers continuing to constrict while others were inhibited. These were not included in the data analysis. The remaining forty-four vessels responded to ACh in a consistent manner and observations on these shall now be considered.

Figure 1 shows examples of the two types of response to bath-applied ACh (10 μM) where constrictions were slowed (Fig. 1*A*) or abolished (Fig. 1*B*). The inhibition of contractile activity by ACh (10 μM) was due to stimulation of muscarinic receptors as it was blocked by atropine (0·1 μM, $n = 5$; see Fig. 1*C*). ACh at 10 μM appeared to affect constriction frequency and not force, because in those vessels which responded to ACh with a decrease in constriction frequency the amplitude of constrictions was not significantly altered (109 ± 11·5% of control, $n = 5$; $P = 0{\cdot}75$).

Endothelium dependence of the ACh response. To establish whether the ACh effect was endothelium dependent, experiments were carried out in vessels with lysed endothelium. These vessels exhibited typical contractile behaviour indicating that the lysis technique had not obviously damaged the smooth muscle. In the lysed vessels tested, application of ACh (10 μM) had no effect on vessel constriction with a frequency of 107 ± 9% of control during the 1 min application of ACh and 95 ± 10% of control during the first minute of wash ($n = 10$ applications to 3 vessels).

Effect of ACh on endothelial $[Ca^{2+}]_i$. ACh is known to increase the intracellular concentration of calcium ions ($[Ca^{2+}]_i$) in vascular endothelial cells in culture. Confocal microscopy and the calcium-indicating dye Calcium Green were used to investigate the direct action of ACh on $[Ca^{2+}]_i$ in the endothelium of intact lymphatic vessels. Bath application of ACh (10 μM) was observed to increase the endothelial $[Ca^{2+}]_i$ ($n = 18$). This was quantified in six vessels, with application of ACh increasing the mean intensity of fluorescence from the endothelium to 134 ± 12% of the control value ($P = 0{\cdot}011$). However, as Calcium Green is a non-ratiometric Ca^{2+} indicator, the absolute increase in $[Ca^{2+}]_i$ was not determined. A typical example of the change in endothelial $[Ca^{2+}]_i$ in response to ACh is presented in Fig. 2. The endothelial $[Ca^{2+}]_i$ increase was inhibited in the presence of atropine (0·1 μM, $n = 5$). When the tissue was perfused with a Ca^{2+}-free EGTA solution the ACh-induced increase in $[Ca^{2+}]_i$ was still observed, but the response faded, such that after the third application there was no detectable change in fluorescence ($n = 5$). This is in contrast to the observations made in control conditions using the 0·3 mM Ca^{2+} perfusing solution, where repetitive application of ACh (10 μM) continued to produce similar increases in $[Ca^{2+}]_i$, with all of the five vessels studied maintaining their response to ACh even after ten or more repeat applications.

Effects of NOLA, SNP, MB and indomethacin on constriction. The effect of the NO-synthase inhibitor, NOLA (1–100 μM) on the ACh response was investigated in nineteen vessels which had shown a clear initial response to ACh. At concentrations of 50 μM and greater, NOLA always prevented the ACh-induced inhibition of constrictions. The mean data from six vessels is shown in Fig. 3 where the response to ACh was blocked by 100 μM NOLA. NOLA competes with the substrate L-arginine for the catalytic site of NO-synthase, thus the addition of a high concentration of L-arginine following NOLA should overcome the inhibitory effects of this agent on the ACh response. However, in this preparation, it was only possible to reverse the NOLA inhibition of the ACh response with 1 mM L-arginine, in some cases, when lower concentrations of NOLA were used. The evidence for this derives from five vessels which had shown significant ACh responses (i.e. constriction frequency decreased, on average, to 50 $\pm$ 14% of control for a 3 min period, directly after a 1 min application of 10 μM ACh; $P = 0{\cdot}0008$) and no significant responses to ACh in the presence of either 10 ($n = 1$), 30 ($n = 3$) or 50 μM ($n = 1$) NOLA (constriction frequency, 81 $\pm$ 8% of control for the same measurement period). The addition of 1 mM L-arginine to the NOLA-containing solutions applied to these vessels significantly restored the ACh-induced responses (i.e. constriction frequency 64 $\pm$ 9% of control for the same measurement period, $P = 0{\cdot}009$). By comparison, L-arginine did not reverse the effects of NOLA in two other vessels exposed to 30 μM NOLA. Furthermore, in all cases tested, the ACh response was not restored by L-arginine in the presence of 100 μM NOLA ($n = 3$; see also Results, Electrophysiology). Taken together, these results indicate that the ACh response was mediated through EDNO as it was blocked by

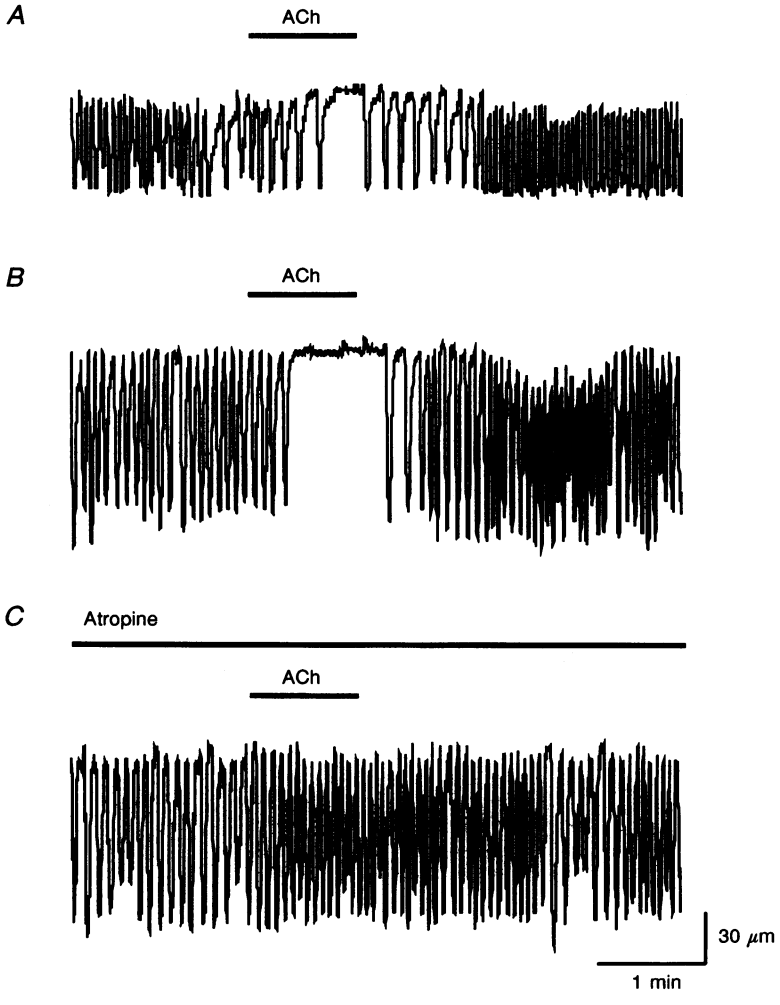

Figure 1. Effects of ACh on the contractile activity of two actively constricting lymphatic vessels, with downward deflection representing constriction

Bath application of ACh (10 μM) to the first vessel caused a marked slowing in the rate of constrictions (*A*). In the second vessel, ACh (10 μM) completely inhibited the contractile activity (*B*), an effect that was blocked in the presence of atropine (0·1 μM, *C*).

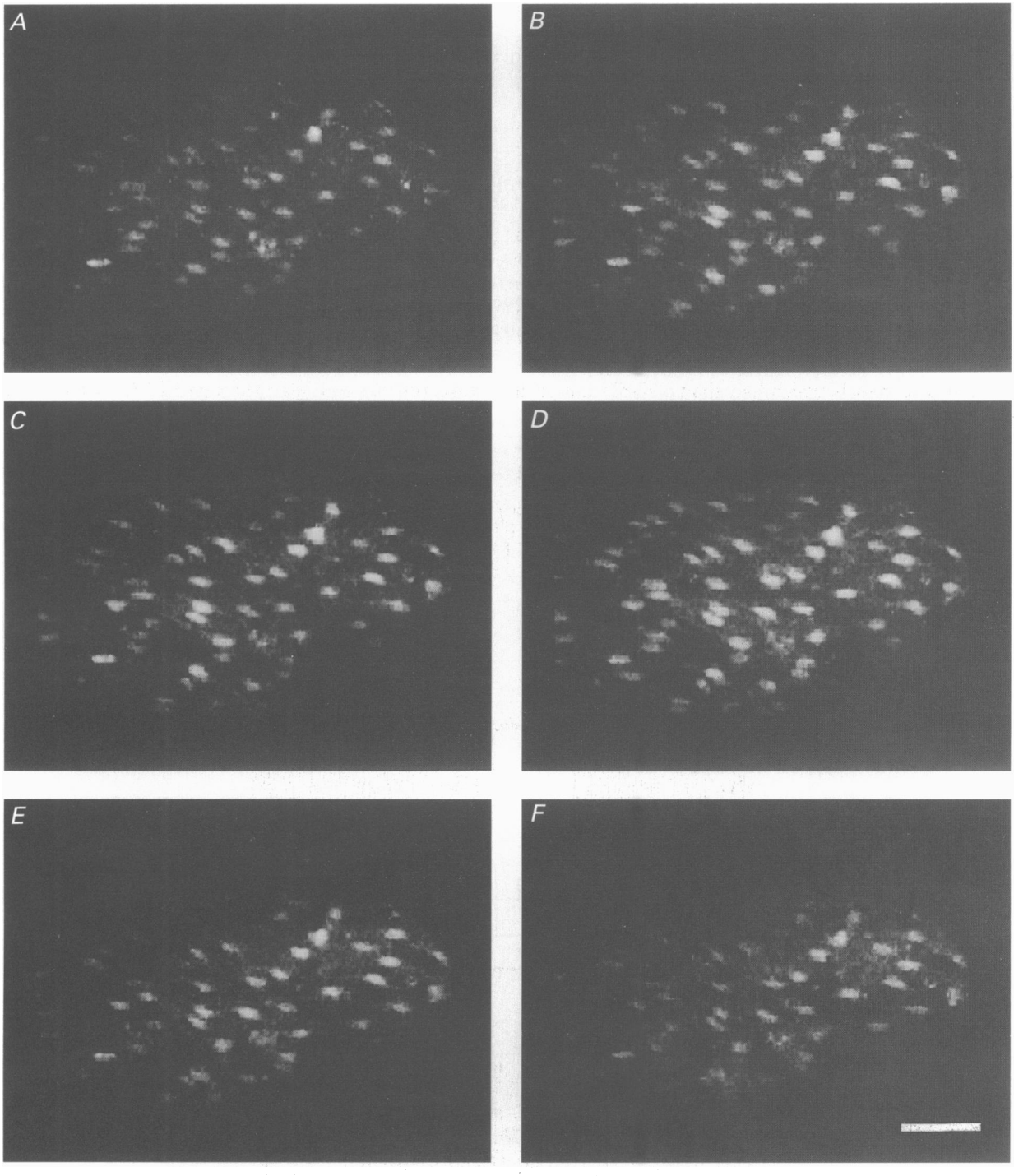

Figure 2. Confocal images showing the effect of ACh on endothelial $[Ca^{2+}]_i$ in an unperfused lymphatic chamber loaded with the calcium-indicating dye Calcium Green

ACh was applied in a large bath with a slow change-over rate (see Methods). The series of images shows the endothelial fluorescence under control conditions (*A*) and at times 1 min 30 s (*B*), 1 min 40 s (*C*), 3 min (*D*), 8 min (*E*) and 11 min (*F*) after an 80 s application of 10 μM ACh. The relative increases in fluorescence, expressed as percentages of control (*A*), were 113, 117, 130, 116 and 103% for *B*, *C*, *D*, *E* and *F*, respectively. Scale bar, 50 μm.

NOLA, a blockade which was reversed in the majority of cases (5 of 7) when lower concentrations (10–50 μM) of NOLA were used.

The possibility that the endothelial-derived relaxing factor was EDNO was further investigated by application of SNP. As for ACh, 1 min application of 100 μM SNP completely blocked, or markedly inhibited, lymphatic constrictions in those vessels which were responsive to ACh ($n = 6$). However, 10 μM SNP was less effective with only one of three vessels responding.

MB is known to inhibit the production of cGMP which mediates smooth muscle relaxation in response to NO. Thus, the effect of MB (25 and 50 μM) on the ACh response was tested and found to partially reverse the ACh-induced inhibition of lymphatic constrictions. The control application of ACh (10 μM) before the application of MB caused a reduction in constriction frequency to 52 ± 9% of control during a 1 min application of ACh and 19 ± 12% of control constriction frequency during the first minute of wash ($n = 7$). When these vessels were pretreated with MB (25 or 50 μM), the application of ACh had no immediate effect on constriction frequency with a mean frequency of 103 ± 14% of control, making this response significantly different from that of the ACh response in the absence of MB ($P = 0.032$). However, during the first minute of wash the constriction frequency was reduced to 53 ± 12% of control frequency. This change was markedly smaller than that observed before the application of MB. Thus, MB at these concentrations inhibited, but did not block, ACh action.

The possible role of prostaglandins in the ACh response was tested using the cyclo-oxygenase inhibitor, indomethacin. The addition of indomethacin (10 μM) had no effect on the ACh-induced inhibition of constrictions. The mean response to 1 min application of ACh (10 μM) during and in the first minute of wash of ACh was, respectively, 88 ± 16 and 43 ± 12% before, and 81 ± 7 and 42 ± 12% of control frequency during the addition of indomethacin ($n = 10$).

Endogenous release of endothelium-derived factors in perfused vessels. The application of NOLA or MB alone caused a significant increase in the frequency of constrictions in perfused vessels. Constriction frequency increased from 8·5 ± 1·7 to 11·5 ± 1·4 constrictions per minute in the presence of 50 μM MB ($n = 6$; Student's one-tailed paired t test, $P = 0.0319$) and similarly from 10·3 ± 1·2 to 12·2 ± 1·7 constrictions per minute in the presence of 100 μM NOLA ($n = 6$; Student's one-tailed paired t test, $P = 0.0189$). In addition, exposure of the tissue to indomethacin (10 μM) tended to decrease the rate of vessel constrictions from a control value of 8·8 ± 1·2 to 7·5 ± 1·2 constrictions per minute during application of indomethacin ($n = 10$), but these values were not significantly different (Student's two-tailed paired t test).

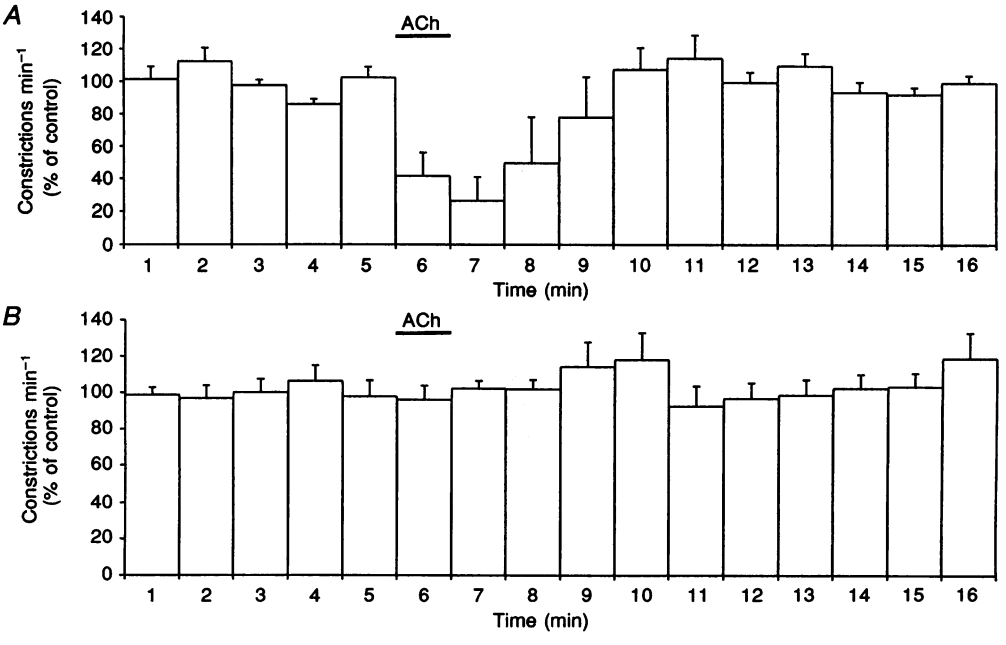

Figure 3. ACh-induced inhibition of constrictions is prevented by the NO-synthase inhibitor NOLA

The histograms present the mean response of 6 actively constricting lymphatic vessels to the addition of ACh (10 μM) under control conditions (*A*) and in the presence of NOLA (100 μM, *B*). Each column represents the constrictions per minute expressed as a percentage of the mean constriction frequency during the 5 min control period before application of ACh.

Non-responding vessels. Several experiments were performed in an effort to uncover the differences between the vessels that responded and those that showed no detectable response to ACh. Vessels that did not respond to 10 μM ACh also showed no response to 100 μM ACh ($n = 4$). This was not due to hydrolysis of ACh, as carbachol (10–100 μM) also failed to elicit a response in vessels which had not responded to ACh ($n = 4$). The action of ACh in these vessels was examined by monitoring the relative endothelial $[Ca^{2+}]_i$ using Calcium Green. Importantly, when five non-responding vessels were stimulated with 10 μM ACh there was a relative increase in the endothelial $[Ca^{2+}]_i$ to 122 ± 7% of the control value ($P = 0{\cdot}023$). Furthermore, EDNO was likely to be released because vessels that did not respond to ACh also did not respond to 100 μM SNP ($n = 17$), unlike ACh-responding vessels which always responded to 100 μM SNP (see above). The possibility of high endogenous release of EDNO was directly examined by inhibiting EDNO synthesis using NOLA (50 μM) applied continuously for at least 10 min before 100 μM SNP was added again. This protocol was applied to seven vessels that did not respond to either ACh or SNP. In four of them, the addition of SNP during application of NOLA resulted in a decrease in constriction frequency to 78 ± 8% of control, which further decreased to 68 ± 17% of control during the first 2–3 min of wash after SNP. The remaining three vessels did not respond to SNP in the presence of NOLA. The overall response from all seven vessels showed a decrease in mean constriction frequency that was marginally significant from control during SNP application ($P = 0{\cdot}053$) and significantly different during the first 2–3 min of wash ($P = 0{\cdot}017$). Taken together, these data support the postulate that some perfused vessels undergo high release of EDNO and hence fail to respond to either ACh or SNP. The reason for the insensitivity of the remaining three of seven vessels to ACh or SNP is not known.

Electrophysiology

Intracellular microelectrode recordings were made in smooth muscle cells from fifty-three short lymphatic segments with intact endothelium (41 tissues, 33 animals). The segments measured 205 ± 12 μm in length and 143 ± 5 μm in diameter ($n = 42$) and when measured, had an input resistance of 53 ± 4 MΩ ($n = 36$). The mean resting membrane potential (resting E_m) obtained from fifty-eight recordings in these segments was −57·5 ± 0·9 mV. Spontaneous transient depolarizations (STDs; see Van Helden, 1993) were recorded in fifty-five of the fifty-eight

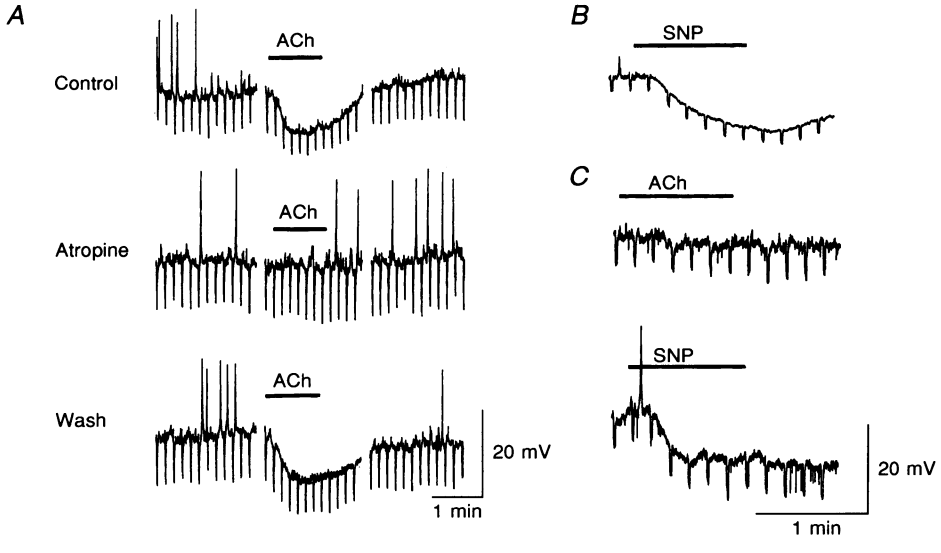

Figure 4. ACh hyperpolarizes lymphatic smooth muscle in an endothelium-dependent manner which requires activation of muscarinic receptors

A, hyperpolarization activated by 5 μM ACh was reversibly prevented by 0·1 μM atropine (resting E_m, −55 mV; same impalement throughout). The segment exhibited STDs and action potentials (small and large upward deflections, respectively). STDs were greatly decreased during the application of ACh and action potentials were not generated. *B*, SNP (50 μM) also hyperpolarized lymphatic smooth muscle (resting E_m, −60 mV). *C*, ACh (5 μM) caused no response (upper trace) while SNP (50 μM) hyperpolarized the smooth muscle (lower trace) of a segment in which the endothelium had been lysed (resting E_m, −47 mV; same impalement throughout). The downward deflections are the voltage responses to injection of current pulses (−0·1 nA). To clarify the traces, artefacts associated with application of current were blanked in this and subsequent figures.

recordings. The size and/or frequency of STDs were in the majority of cases too small to generate action potentials. This probably relates to the fact that vessel distension is normally necessary to activate constrictions. In all, action potentials were observed in seventeen of the fifty-eight recordings. The action potentials had an onset consistent with them being generated by one or more STDs, as would be expected if STDs were the pacemaker potentials (Van Helden, 1993).

Endothelium-dependent effects of ACh. Superfusion of the preparation with ACh induced a reversible hyperpolarization of the lymphatic smooth muscle in all recordings. A mean hyperpolarization of 11·4 ± 0·6 mV ($n = 41$) and 11·9 ± 0·7 mV ($n = 11$) occurred in response to 1 min applications of 5 and 10 μM ACh, respectively. An example is shown in Fig. 4*A*. The ACh-induced hyperpolarization was reproducible, as assessed by two successive applications of ACh (5 μM), each separated by a 10 min interval. The second response was 84 ± 7% of the first ($n = 7$). A decrease in input resistance was observed during the ACh-induced hyperpolarization (see Figs 4*A*, 5*A* and *C*). The mean input resistance measured at the peak of the hyperpolarization induced by 5 μM ACh was 75 ± 3% of control values ($n = 36$, $P < 0{\cdot}001$).

STD amplitude was decreased during application of ACh (Fig. 4*A*). STD amplitude recorded during a 20 s interval before, and during, the maximum hyperpolarization was compared in fourteen segments which exhibited high levels of STD activity. In these segments, the mean STD amplitude measured for events greater than 0·5 mV was decreased to 65 ± 7% of control amplitude. This decrease, while itself significant (Student's two-tailed t test,

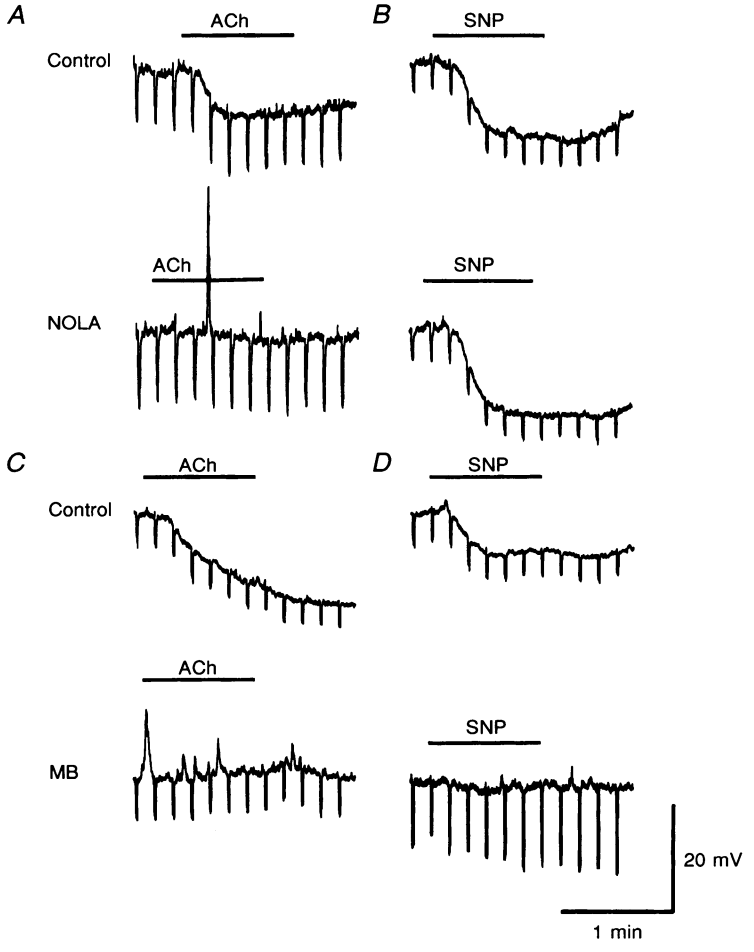

Figure 5. ACh-induced hyperpolarization of the smooth muscle is blocked by NOLA and MB
Hyperpolarizations induced by ACh (5 μM, *A*) and SNP (50 μM, *B*) in control conditions (upper traces) and in the presence of NOLA (100 μM, lower traces). Recordings in *A* and *B* were from 2 different segments with a resting E_m of −62 and −65 mV, respectively. Hyperpolarizations induced by ACh (5 μM, *C*) and SNP (50 μM, *D*) in control conditions (upper traces) and in the presence of MB (50 μM, lower traces; resting E_m, −51 mV, same impalement). The basis for the increase in input resistance appearing in *D* (lower trace) is unknown but was not observed in the other segments where this protocol was applied.

$P = 0{\cdot}0002$), was not different from the change in segment input resistance. However, it is likely that there is an additional suppressive factor as the hyperpolarization of approximately 10 mV should itself increase the size of STDs by about 40% (the reversal potential for STDs in lymphatic smooth muscle is ~ -35 mV; Van Helden, 1993). Hence, the real decrease in mean STD size is expected to be about twice that caused by the change in segment input resistance.

The ACh-induced hyperpolarization, increase in membrane conductance and decrease in STD size were abolished in the presence of 0·1 μM atropine. The mean hyperpolarization was $11{\cdot}0 \pm 1{\cdot}7$ mV before, and $1{\cdot}0 \pm 1{\cdot}0$ mV after superfusion with atropine for 10 min ($n = 3$). In two cells, the recording was maintained long enough to observe restoration of the ACh response after wash of atropine for more than 20 min (Fig. 4*A*). The NO donor SNP also induced hyperpolarization of the smooth muscle (Fig. 4*B*) with 50 μM SNP causing a mean hyperpolarization of $10{\cdot}2 \pm 1{\cdot}4$ mV ($n = 9$).

The responses to ACh and SNP were also studied in lymphatic segments in which the endothelium had been lysed. The smooth muscle studied in eleven such segments exhibited frequently occurring STDs, had an input resistance of 65 ± 6 MΩ and a resting E_m of $-48{\cdot}2 \pm 2{\cdot}7$ mV. ACh (5–10 μM) was applied to six of these segments with five of the six segments showing no obvious ACh-induced response (Fig. 4*C*) consistent with the assumption that ACh acted via the endothelium. In the remaining segment, ACh induced a very small hyperpolarization (1 mV) followed by a depolarization of 7 mV. The basis for this response was not investigated further. As for control tissues, hyperpolarization induced by SNP (50 μM) occurred in four lysed segments studied with a mean hyperpolarization of $8{\cdot}3 \pm 1{\cdot}1$ mV (Fig. 4*C*, lower trace). Taken together, these data confirm that the ACh-induced hyperpolarization, increase in membrane conductance and decrease in STDs in the smooth muscle occur through activation of factor(s) released from the endothelium consequent to stimulation of muscarinic receptors.

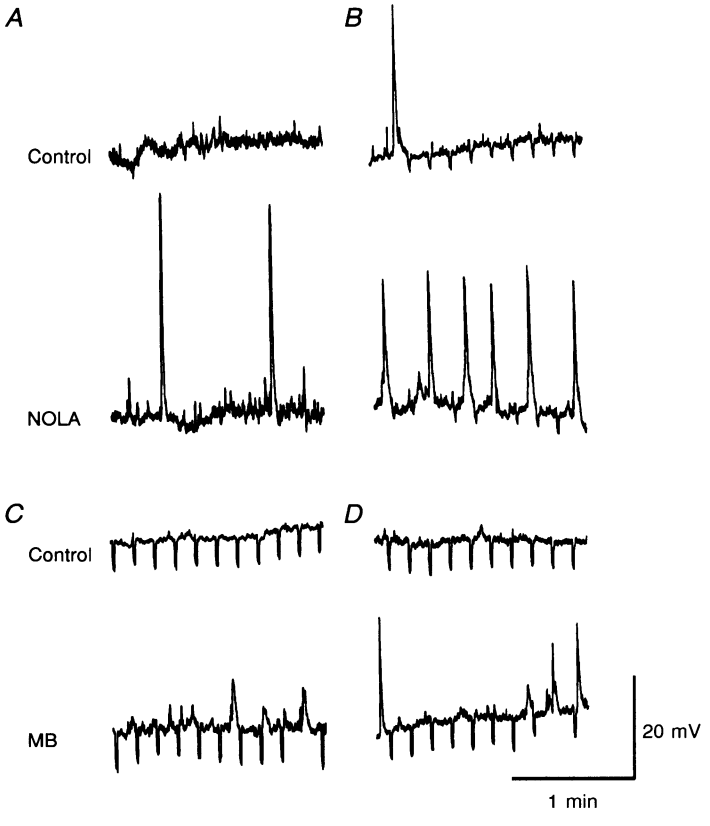

Figure 6. Spontaneous electrical activity is increased in the presence of NOLA and MB

A and *B*, membrane potential recordings in two segments, in control conditions (upper traces) and in the presence of NOLA (lower traces); resting E_m was, respectively, −60 and −58 mV in *A*, and −52 and −50 mV in *B*. *C* and *D*, membrane potential recordings in control conditions (upper traces) and in the presence of MB (lower traces) in two segments; resting E_m was, respectively, −55 and −50 mV in *C*, and −50 and −47 mV in *D*. Both NOLA and MB increased STD activity and caused generation of more action potentials (large depolarizations). Downward deflections appearing in *B*, *C* and *D* are voltage responses to injection of current pulses (−0·1 nA).

Effects of NOLA, MB and indomethacin on ACh and SNP-induced hyperpolarizations. The NO-synthase inhibitor NOLA applied before and during ACh, generally decreased the ACh-induced hyperpolarization (Fig. 5*A*). The effect of NOLA on the response to ACh was studied in nine segments with mean hyperpolarizations to 5 μM ACh of 12·0 ± 1·2 mV before, and 3·3 ± 1·7 mV in the presence of 100 μM NOLA. As reported in the constriction studies, the effects of NOLA at 100 μM were not reversed by L-arginine, with the ACh-induced hyperpolarization unable to be restored in the presence of L-arginine (1 mM) after it had been blocked by NOLA (100 μM, $n = 3$). The resting E_m of the smooth muscle was not significantly different in control, in 100 μM NOLA and in 100 μM NOLA plus 1 mM L-arginine with values of −63·7 ± 3·0, −63·7 ± 3·0 and −66·7 ± 4·4 mV ($n = 3$, continuous recordings), respectively.

In contrast to ACh, the response to SNP (50 μM) was not affected by NOLA (100 μM, Fig. 5*B*); the mean hyperpolarization induced by SNP before and in the presence of NOLA was 12·8 ± 0·9 and 12·3 ± 1·9 mV, respectively ($n = 4$). The ACh-induced hyperpolarization was significantly reduced after superfusion with a solution containing MB (Fig. 5*C*); the mean hyperpolarization to 5 μM ACh applied to segments bathed in MB (50 μM; exposure time, > 10 min) was decreased from 10·8 ± 2·1 mV to 1·8 ± 1·2 mV ($n = 6$). MB had a similar inhibitory action on the hyperpolarization to SNP (50 μM, Fig. 5*D*), which was decreased from 11·8 ± 1·7 to 2·3 ± 1·3 mV ($n = 4$). Indomethacin (10 μM), superfused for 10 min, did not markedly affect the hyperpolarization produced by 5 μM ACh, which was 14·2 ± 2·2 mV before and 12·0 ± 1·5 mV ($n = 3$, $P = 0·369$) during superfusion with indomethacin. These data support the postulate that ACh-induced endothelial release of EDNO caused the smooth muscle to hyperpolarize by increasing cGMP levels (see Discussion).

Endogenous release of endothelium-derived factors in non-perfused vessel segments. Application of NOLA (100 μM) to segments caused a significant increase in STD amplitude to 162 ± 14% of control ($n = 12$). This increase was observed after 1–8 min in the presence of NOLA in eleven of the twelve cells tested. STD activity recorded in two cells before and during application of NOLA is displayed in Fig. 6*A* and *B*. In some cases, the NOLA-induced increase in STD amplitude caused STDs to reach the threshold and generate action potentials (hence constrictions). STD enhancement was not due to an altered resting E_m or change in input resistance, which were little changed by NOLA, with values of −59·7 ± 1·8 and −58·8 ± 1·7 mV ($n = 12$; Student's one-tailed paired t test, $P = 0·107$), and 51 ± 9 and 57 ± 10 MΩ ($n = 11$; Student's two-tailed paired t test, $P = 0·069$) in control and 100 μM NOLA, respectively. STD activity was also increased during superfusion with 50 μM MB (159 ± 21% of control, $n = 9$; see Fig. 6*C* and *D*). In addition, MB depolarized the segments from a mean resting E_m of −55·4 ± 1·8 mV to −49·4 ± 2·5 mV ($n = 9$; Student's one-tailed paired t test, $P = 0·0002$), without obvious change in segment input resistance (51 ± 9 and 50 ± 8 MΩ in control and in 50 μM MB, respectively). These observations suggest there may be low-level release of EDNO under resting conditions sufficient to affect cGMP levels and to decrease STD activity.

Arachidonic acid metabolites produced in the presence of cyclo-oxygenase may also be endogenously released, as indomethacin (10 μM) caused a hyperpolarization from a control resting E_m of −54·6 ± 2·8 to −59·3 ± 2·1 mV during superfusion with indomethacin ($n = 7$; Student's two-tailed paired t test, $P = 0·007$). In four electrically active segments, STD activity was reversibly decreased. Consistent with this, the STD-induced action potentials, which occurred quite regularly in three of these segments, were reversibly inhibited during superfusion with indomethacin. This effect was not observed in tissues where the endothelium was lysed ($n = 2$).

DISCUSSION

The present study has shown that the endothelium of guinea-pig mesenteric lymphatic vessels can modulate contractile behaviour by ACh-induced release of EDNO. Unlike the situation in most blood vessels, EDNO causes hyperpolarization and a decrease in membrane resistance of lymphatic smooth muscle. These effects, plus a direct EDNO-induced inhibition of STDs, reduce the efficacy of these pacemaker potentials in generating action potentials and hence lymphatic phasic constrictions.

Agonists which cause endothelium-dependent relaxation are known to induce a rise in $[Ca^{2+}]_i$ in cultured endothelial cells. However, increases in $[Ca^{2+}]_i$ in response to ACh have not always been observed (for review see Himmel, Whorton & Strauss, 1993). Carter & Ogden (1994) recently showed, *in situ*, that endothelial cells from isolated rat aorta exhibited large, fast and repetitive $[Ca^{2+}]_i$ spikes in response to ACh. While such $[Ca^{2+}]_i$ spikes were not observed in the present study, it has shown that ACh induces a rise in $[Ca^{2+}]_i$ in the endothelium of intact lymphatic vessels, a response which could be repetitively activated. This increase was also observed with a Ca^{2+}-free EGTA perfusing solution but only for the initial two to three applications of ACh. Therefore, it is likely that both intracellular Ca^{2+} stores and exogenous Ca^{2+} entry pathways are involved in the endothelial response.

The ACh-induced effects must have been mediated via muscarinic receptors on the endothelium because, firstly,

the ACh-induced decrease in constriction frequency and hyperpolarization of the smooth muscle were inhibited by lysis of the endothelium and, secondly, both these effects and the ACh-induced increase in endothelial $[Ca^{2+}]_i$ were not observed in the presence of atropine. Endothelial release of EDNO was implicated, as the ACh effects were largely suppressed by NOLA, an effect which was competitively antagonized by L-arginine (1 mM), for the majority of cases, when lower concentrations of NOLA (10–50 μM) were used. The ACh effects were also mostly inhibited by MB, and were mimicked by the NO donor SNP. Endothelium-mediated relaxation in precontracted dog and porcine lymphatic vessels (Ohhashi & Takahashi, 1991; Ferguson, 1992) and decrease in spontaneous constrictions of bovine lymphatic vessels in response to ACh (Yokoyama & Ohhashi, 1993) have been suggested to be due to NO.

Our finding that the ACh-induced hyperpolarization is mostly mediated by the release of EDNO is of particular interest since endothelium-dependent hyperpolarizations induced by ACh in the majority of vascular smooth muscle preparations are not mediated by EDNO (see Bény & von der Weid, 1993; Garland *et al.* 1994). NO has been shown to contribute to the ACh-induced hyperpolarization in only a few blood vessels (Tare *et al.* 1990; Rand & Garland, 1992; Parkington *et al.* 1993; Vanheel *et al.* 1994).

Specific investigation of the channel(s) involved in the response to ACh and SNP was not undertaken in the present study. However, the observation that the input resistance was decreased during ACh-induced hyperpolarizations suggests that at least one K^+ conductance was involved. Studies on vascular smooth muscle have shown that NO activates K^+ channels either directly (Bolotina, Najibi, Palacino, Pagano & Cohen, 1994), or indirectly via cGMP or cGMP-dependent protein kinase (Williams, Katz, Roy-Contancin & Reuben, 1988; Robertson, Schubert, Hescheler & Nelson, 1993). The present result that ACh-induced hyperpolarizations were blocked by MB seems to exclude a direct channel activation by NO and suggests that the mediator is cGMP based.

A basal release of EDNO which affects the state of tension of vessels has been observed in many different vascular preparations (see Furchgott & Vanhoutte, 1989). In lymphatic vessels, superfusion with NO-synthase inhibitors has been shown to increase the force of spontaneous contractions in sheep mesenteric lymphatic vessels (Hollywood, Thornbury & McHale, 1992) and increase the amplitude and frequency of spontaneous constrictions in porcine mesenteric lymphatics (Reeder *et al.* 1994). However, basal release of EDNO was not demonstrated to play a significant role in regulating the active pumping mechanism in bovine mesenteric lymphatics (Yokoyama & Ohhashi, 1993). The present finding that superfusion with NOLA and MB increased the vessel constriction frequency provides evidence that EDNO is also released by the endothelium of perfused guinea-pig mesenteric lymphatic vessels. Indeed, the release of EDNO may be very high in perfused vessels because about 40% did not respond to ACh (10 μM). These vessels seemed to be exposed to saturating levels of NO since, unlike ACh-responding vessels, SNP (100 μM) had no action on these vessels. This was not a failure of the endothelium to respond to ACh because a comparable increase in $[Ca^{2+}]_i$ in response to ACh was observed in both ACh-responding and non-responding vessels. Where tested, four of seven non-responding vessels recovered responsiveness to SNP in the presence of the NO-synthase inhibitor NOLA (50 μM), proof that the perfusion-associated release of EDNO in these vessels was very high. However, the cause of the ACh insensitivity of the remaining three of seven vessels tested is not known.

Non-perfused vessels, as studied in vessel segments, also showed evidence of endogenous release of EDNO, however at a much reduced level. Demonstration of this came from microelectrode studies where NOLA caused an increase in the amplitude of STDs, but had little effect on the resting E_m. A differential effect on STDs compared with resting E_m may reflect different underlying mechanisms, with induction of hyperpolarization requiring higher levels of cGMP than the enhancement of STDs. STDs, or the underlying spontaneous transient inward currents (STICs), are highly sensitive to changes in $[Ca^{2+}]_i$ (Van Helden, 1991; Wang, Hogg & Large, 1992) probably due to $[Ca^{2+}]_i$ modulation of quantal release from intracellular stores (i.e. calcium-induced calcium release; see Berridge, 1993). Therefore, decreases in $[Ca^{2+}]_i$, such as are known to occur with an increase in cGMP production (see Lincoln, 1989), would decrease STD activity. Regulation of STDs by EDNO could also explain the direct ACh-associated inhibition of STDs.

Although indomethacin had no effect on the ACh-induced response, it caused a hyperpolarization of the smooth muscle and, in very active segments, a decrease in STD activity and action potential frequency. These effects were not observed after lysis of the endothelium. Thus, a prostaglandin and/or thromboxan is likely to be released by the endothelium in resting conditions. Indomethacin caused a decrease in the contractile activity of perfused vessels. This is consistent with the finding that spontaneous activity in lymphatic vessels is modulated by arachidonic acid metabolites (Johnston & Gordon, 1981) and is suppressed by the cyclo-oxygenase inhibitors indomethacin and aspirin (Johnston & Feuer, 1983; Allen, Burke, Johnston & McHale, 1984).

The results from the present study show that the endothelium-dependent modulation of constriction frequency induced by ACh stimulation is due to release of EDNO, which causes an increase in membrane conductance with associated hyperpolarization and a direct suppression of

STDs in the lymphatic smooth muscle, mediated by EDNO. Evidence has also been presented indicating that the lymphatic endothelium released low levels of EDNO as well as other factors under non-perfused conditions. The fact that some perfused vessels exhibited marked release of EDNO indicates that the lymphatic endothelium subserves an important functional role in the control of lymphatic pacemaking, with EDNO reducing the size and efficiency of the pacemaker potentials (STDs) in generating action potentials and hence constrictions.

ALLEN, J. M., BURKE, E. P., JOHNSTON, M. G. & MCHALE, N. G. (1984). The inhibitory effect of aspirin on lymphatic contractility. *British Journal of Pharmacology* **82**, 509–514.

BÉNY, J.-L. & VON DER WEID, P.-Y. (1993). Hyperpolarizing factors. In *Coronary Artery Disease. Reviews in Depth*, ed. BURTON, E. S., pp. 50–57. Bell & Bain, Glasgow.

BERESFORD-SMITH, B., NESBITT, K. V. & VAN HELDEN, D. F. (1993). Edge detection at multiple locations using a 'radar' tracking algorithm as exemplified in isolated guinea-pig lymphatic vessels. *Journal of Neuroscience Methods* **49**, 69–79.

BERRIDGE, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315–325.

BOLOTINA, V. M., NAJIBI, S., PALACINO, J. J., PAGANO, P. J. & COHEN, R. A. (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**, 850–853.

CARTER, T. D. & OGDEN, D. (1994). Acetylcholine-stimulated changes of membrane potential and intracellular calcium concentration recorded in endothelial cells in situ in isolated rat aorta. *Pflügers Archiv* **428**, 476–484.

CROWE, M. J., VON DER WEID, P.-Y. & VAN HELDEN, D. F. (1994). Endothelium-dependent modulation of lymphatic pumping in the guinea-pig mesentery. *Proceedings of the Australian Physiological and Pharmacological Society* **25**, 146P.

FERGUSON, M. K. (1992). Modulation of lymphatic smooth muscle contractile responses by the endothelium. *Journal of Surgical Research* **52**, 359–363.

FLOREY, H. (1927). Observations on the contractility of lacteals. Part II. *Journal of Physiology* **63**, 1–18.

FURCHGOTT, R. F. & VANHOUTTE, P. M. (1989). Endothelium-derived relaxing and contracting factors. *FASEB Journal* **3**, 2007–2018.

GARLAND, C. J. PLANE, F., KEMP, B. K. & COCKS, T. M. (1994). Endothelium-dependent hyperpolarization: a role in the control of vascular tone. *Trends in Pharmacological Sciences* **16**, 23–30.

HIMMEL, H. M., WHORTON, A. R. & STRAUSS, H. C. (1993). Intracellular calcium, currents, and stimulus–response coupling in endothelial cells. *Hypertension* **21**, 112–127.

HOLLYWOOD, M. A., THORNBURY, K. D. & MCHALE, N. G. (1992). Augmentation of sheep lymphatic contractility by L-*N*-nitro arginine methyl ester. *Journal of Physiology* **446**, 420*P*.

IGNARRO, L. J., BUGA, G. M., WOOD, K. S., BYRNS, R. E. & CHAUDHURI, G. (1987). Endothelium-derived relaxing factor produced and released from artery and vein is nitric oxide. *Proceedings of the National Academy of Sciences of the USA* **84**, 9265–9269.

JOHNSTON, M. G. & FEUER, C. (1983). Suppression of lymphatic vessel contractility with inhibitors of arachidonic acid metabolism. *Journal of Pharmacology and Experimental Therapeutics* **226**, 603–607.

JOHNSTON, M. G. & GORDON, J. L. (1981). Regulation of lymphatic contractility by arachidonate metabolites. *Nature* **293**, 294–297.

JUNCOS, L. A., ITO, S., CARRETERO, O. A. & GAVIN, J. (1993). A novel method for removal of the endothelium using anti-factor VIII-related antigen and complement. *Hypertension* **24**, 583.

LINCOLN, T. M. (1989). Cyclic GMP and mechanisms of vasodilation. *Pharmacology and Therapeutics* **41**, 479–502.

MYERS, P. R., MINOR, R. L. JR, GUERRA, R. JR, BATES, J. N. & HARRISON, D. G. (1990). Vasorelaxant properties of the endothelium-derived relaxing factor more closely resemble *S*-nitrosocysteine than nitric oxide. *Nature* **345**, 161–163.

OHHASHI, T. & TAKAHASHI, N. (1991). Acetylcholine-induced release of endothelium-derived relaxing factor from lymphatic endothelial cells. *American Journal of Physiology* **260**, H1172–1178.

PALMER, R. M. J., FERRIGE, A. G. & MONCADA, S. (1987). Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* **327**, 524–526.

PARKINGTON, H. C., TARE, M., TONTA, M. A. & COLEMAN, H. A. (1993). Stretch revealed three components in the hyperpolarization of guinea-pig coronary artery in response to acetylcholine. *Journal of Physiology* **465**, 459–476.

RAND, V. E. & GARLAND, C. J. (1992). Endothelium-dependent relaxation to acetylcholine in the rabbit basilar artery: importance of membrane hyperpolarization. *British Journal of Pharmacology* **106**, 143–150.

REEDER, L. B., YANG, L. H. & FERGUSON, M. K. (1994). Modulation of lymphatic spontaneous contractions by EDRF. *Journal of Surgical Research* **56**, 620–625.

ROBERTSON, B. E., SCHUBERT, R., HESCHELER, J. & NELSON, M. T. (1993). cGMP-dependent protein kinase activates Ca-activated K channels in cerebral artery smooth muscle cells. *American Journal of Physiology* **265**, C299–303.

RUBANYI, G. M. (1991). Endothelium-derived relaxing and contracting factors. *Journal of Cellular Biochemistry* **46**, 27–36.

SMITH, R. O. (1949). Lymphatic contractility: A possible mechanism of lymphatic vessels for the transport of lymph. *Journal of Experimental Medicine* **90**, 497–509.

TARE, M., PARKINGTON, H. C., COLEMAN, H. A., NEILD, T. O. & DUSTING, G. J. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from the endothelium. *Nature* **346**, 69–71.

UMANS, J. G. (1990). Inhibition of endothelium dependent relaxation by *N*-methyl arginine, *N*-nitroarginine and methylene blue. *European Journal of Pharmacology* **183**, 1613–1614.

VANHEEL, B., VAN DE VOORDE, J. & LEUSEN, I. (1994). Contribution of nitric oxide to the endothelium-dependent hyperpolarization in rat aorta. *Journal of Physiology* **475**, 277–284.

VAN HELDEN, D. F. (1991). Spontaneous and noradrenaline-induced transient depolarizations in the smooth muscle of guinea-pig mesenteric vein. *Journal of Physiology* **437**, 511–541.

VAN HELDEN, D. F. (1993). Pacemaker potentials in lymphatic smooth muscle of the guinea-pig mesentery. *Journal of Physiology* **471**, 465–479.

VAN HELDEN, D. F., VON DER WEID, P.-Y. & CROWE, M. J. (1995). Electrophysiology of lymphatic smooth muscle. In *Interstitium, Connective Tissue and Lymphatics*, ed. REED, R. K., MCHALE, N. G., BERT, J. L., WINLOVE, C. P. & LAINE, G. A., pp. 221–236. Portland Press, London.

WANG, Q., HOGG, R. C. & LARGE, W. A. (1992). Properties of spontaneous inward currents recorded in smooth muscle cells isolated from the rabbit portal vein. *Journal of Physiology* **451**, 525–537.

WILLIAMS, D. L. JR, KATZ, G. M., ROY CONTANCIN, L. & REUBEN, J. P. (1988). Guanosine 5′-monophosphate modulates gating of high-conductance Ca^{2+}-activated K^+ channels in vascular smooth muscle cells. *Proceedings of the National Academy of Sciences of the USA* **85**, 9360–9364.

YOKOYAMA, S. & OHHASHI, T. (1993). Effects of acetylcholine on spontaneous contractions in isolated bovine mesenteric lymphatics. *American Journal of Physiology* **264**, H1460–1464.

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