ET-1-associated vasomotion and vasospasm in lymphatic vessels of the guinea-pig mesentery

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1 *In vitro* experiments were performed to investigate the actions of endothelin-1 (ET-1) on vasomotion and vasospasm in guinea-pig mesenteric lymphatics.

2 ET-1 modulated lymphatic vasomotion independent of the endothelium, with lower concentrations ($\leqslant 10$ nM) increasing lymphatic vasomotion and higher concentrations ($\geqslant 100$ nM) causing vasospasm.

3 ET-1-induced increases in vasomotion were accompanied by an increase in tonic $[Ca^{2+}]_i$.

4 These actions were inhibited by the ET_A receptor antagonist BQ-123 (1 μM), the phospholipase C (PLC) inhibitor U73122 (5 μM), removal of extracellular Ca^{2+}, chelation of intracellular Ca^{2+} with BAPTA/AM (10 μM), the store Ca^{2+}-ATPase inhibitor thapsigargin (1 μM), caffeine (10 mM) and the inositol 1,4,5-trisphosphate (IP_3) receptor blocker heparin and 2-APB (30 μM). In contrast, the ET_B receptor antagonist BQ-788 (1 μM), ryanodine (1 & 20 μM), pertussis toxin (PTx) or Cs^+ had no significant actions on vasomotion or the magnitude of increase in tonic $[Ca^{2+}]_i$.

5 ET-1-induced vasospasm was accompanied by a transient increase in smooth muscle $[Ca^{2+}]_i$ followed by a sustained plateau, an action that was abolished by removal of extracellular Ca^{2+}, but only marginally inhibited by nifedipine (1 μM).

6 Caffeine (10 mM), SKF 96165 (30 μM) or U73122 (5 μM) together with nifedipine (1 μM) abolished ET-1-induced vasospasm and increase in $[Ca^{2+}]_i$.

7 These results indicate that ET-1 increases lymphatic vasomotion by acting on smooth muscle ET_A receptors and activation of G-protein-PLC-IP_3 cascade, which is known to cause pacemaker Ca^{2+} release and resultant pacemaker potentials. High concentrations of ET-1 cause a failure in Ca^{2+} homeostasis causing vasospasm, triggered by excessive Ca^{2+} influx primarily through store-operated channels (SOCs) with L-Ca^{2+} voltage-operated channels (VOCs) also contributing, but to a much lesser extent.

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Keywords: Endothelin-1; lymphatics; Ca^{2+} release; Ca^{2+} influx; vasomotion; vasospasm

Abbreviations: ACh, acetylcholine; 2-APB, 2-aminoethoxydiphenyl borate; ATP, adenosine 5′-trisphosphate; BAPTA, 1,2-bis(2-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid; BAPTA/AM, BAPTA acetoxymethylester; BQ-123, cyclic D-Trp-D-Asp-Pro-D-Val-Leu; BQ-788,N, *cis*-2,6-dimethylpiperidinocarbonyl-L-methylleucyl-D-l-methoxycarbonyltryptophanyl-D-norleucine; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; CPA, cyclopiazonic acid; DMSO, dimethylsulphoxide; EGTA, ethylene glycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid; ET-1, endothelin-1; F_x, fluorescence at wavelength x; fluo-3/AM, fluo-3 acetoxymethylester; fura-2/AM, fura-2 acetoxymethylester; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor; lymphangion, lymphatic chamber between adjacent valves; PLC, phospholipase C; PTx, pertussis toxin; RyR, ryanodine receptor; SOC, store-operated channel; U73122, 1-(6-((17-beta-methoxyestra-1,3,5(10)trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione; U73343, 1-[6-[[(17beta)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5 pyrrolidinedione; VOC, voltage-operated channel

Introduction

Many lymphatic and blood vessels exhibit vasomotion, a rhythmic constriction–dilation cycle of these vessels (Florey, 1927; Johnson, 1980). This mechanism is responsible for both the propulsion of lymph (each phasic constriction pumping lymph forward through unidirectional valves within lymphatic vessels; Mislin, 1983; Johnston & Elias, 1987; McHale, 1990), and for modulating the local vascular resistance and blood flow (see Haddock *et al.*, 2002). Ranges of neurotransmitters and paracrine or hormonal substances including ET-1 modulate vasomotion. In contrast, excessive stimulation of the smooth muscle of vessels can lead to vasospasm, a pathological condition of sustained vasoconstriction, observed both *in vivo* (Kurihara *et al.*, 1989; Wanebo *et al.*, 1998) and *in vitro* (Willette *et al.*, 1994; Kanashiro *et al.*, 2000).

ET-1 is a 21-amino-acid polypeptide that is produced primarily by the endothelium of blood and lymphatic vessels (Yanagisawa *et al.*, 1988; Reeder & Ferguson, 1996). It is a key smooth muscle activator known to either induce or enhance

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vasomotion, and at high concentrations cause vasospasm (Sakuma *et al.*, 1995; Toribatake *et al.*, 1997; Zhao & van Helden, 1997; Sakai *et al.*, 1999). ET-1, after being released from vascular endothelium, is known to act in a paracrine manner on ET_A and/or ET_{B2} receptors on smooth muscles and ET_{B1} receptors on the endothelium (Sokolovsky *et al.*, 1992; Reeder & Ferguson, 1996; Ortega Mateo & de Artinano, 1997; Sakai *et al.*, 1999). ET-1-induced constriction and/or vasomotion are primarily produced by stimulation of ET_A and/or ET_{B2} receptors, which tends to swamp a counter, vasodilatory response resulting from stimulation of endothelial ET_{B1} receptors. The second messenger pathway has been well established for ET_A receptors on vascular smooth muscle, where it is known that ET_A receptor activation results in PLC-mediated hydrolysis of phosphatidylinositol 4,5-bisphosphate, producing IP_3, which causes an increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} from intracellular stores (Marsden *et al.*, 1989; Berridge, 1993; Neylon, 1999).

Various mechanisms have been proposed to explain ET-1-induced vasospasm. Research using eicosanoids on single smooth muscle cells isolated from porcine coronary arteries (Sirous *et al.*, 2001), U46619 on pig skin flaps (Pang *et al.*, 2001) and 5-hydroxytryptamine on rings from rabbit abdominal aorta (Consigny, 1990) have shown that ET-1 may induce vasospasm by amplifying the effects of other vasoactive substances. Others have demonstrated a role of the endothelium in ET-1-induced vasospasm (Coessens, 1994; Volpe & Cosentino, 2000). Recent research on canine cerebral and porcine coronary arteries (Sirous *et al.*, 2001; Wickman *et al.*, 2001) have shown that ET-1 may cause vasospasm by activating PKC. Studies on smooth muscle from small mesenteric arteries of rabbit indicate that there is an increased sensitivity of the contractile apparatus to Ca^{2+} during vasospasm (Nishimura *et al.*, 1992). Other studies suggest that most of the effects of ET-1 on vascular tone can be accounted for by its potent ability to induce IP_3 formation, which mobilises Ca^{2+} from intracellular stores and stimulates Ca^{2+} entry (Neylon, 1999). There have also been reports that ET-1 may directly or indirectly activate VOCs (Van Renterghem *et al.*, 1988; Goto *et al.*, 1989; Chen & Wagoner, 1991; Ota & Oku, 1997). However, the failure of VOC antagonists to prevent ET-1-induced vasospasm suggests that other Ca^{2+} influx pathways result from the activation of ET-1 receptors. One other such pathway is through SOCs that, as indicated from studies of the basilar artery of a double subarachnoid haemorrhage rabbit model, play a pivotal role in the pathogenesis of ET-1-induced vasospasm (Zuccarello *et al.*, 1996b).

In the studies presented here, we examine the signal pathways underlying lymphatic vasomotion, demonstrating that ET-1 increases vasomotion by acting on ET_A receptors to enhance the production of IP_3, thus increasing pacemaker Ca^{2+} release and resultant vasomotion. Higher ET-1 concentrations herald an abrupt change from vasomotion to vasospasm, the latter arising by Ca^{2+} influx through both SOCs and VOCs. The most striking aspect of the data presented here is demonstration of a linkage between ET-1-induced vasomotion and vasospasm. We hypothesise that this transition represents a change from coordinated, synchronised store Ca^{2+} release to uncoordinated Ca^{2+} release arising through over stimulation. Some of these data have been presented previously in abstract form (Zhao & van Helden, 1997).

Methods

Lymphatic preparation

Young guinea-pigs (age <10 days) were euthanised by an overdose of the inhalation anaesthetic halothane (5–10% in air) followed by decapitation. Mesenteric lymphatic vessels were isolated from the ileal region of the intestine and pinned onto a sylgard-covered (Dow Corning) organ bath (volume 0.5 ml) mounted on an inverted microscope. Physiological saline solution of composition (mM): NaCl 120, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 2, $NaHCO_3$ 25, NaH_2PO_4 1, glucose 10, maintained at a pH of 7.2 by bubbling with a 95% : 5% $O_2 : CO_2$ gas mixture, was superfused over the tissue at a rapid rate (6 ml min^{-1}) at 34–36°C. Two bath types were used: one with a volume of 0.5–1 ml and the other with a volume of $\sim$0.2 ml. A reasonably rapid change in solutions could be effected with the latter, with $>95\%$ bath changeover in <10 s. In some cases, vessels were luminally perfused (Rayner & van Helden, 1997) at a rate of about 3 μl min^{-1} to destroy the endothelium, as effected by briefly (5–10 s) passing air through the vessels some 5–6 times. The relative success of the lysing procedure was then tested (see von der Weid *et al.*, 1996). Tissues were normally used within 1–4 h of isolation, and were stored at 4°C in physiological saline until used. Measurements were made by recording the constriction frequency and/or $[Ca^{2+}]_i$ of the smooth muscle of individual chambers formed by adjacent valves, and termed lymphangions (Mislin, 1983).

Ratiometric measurement of $[Ca^{2+}]_i$ with fura-2

$[Ca^{2+}]_i$ was measured photometrically using the ratiometric Ca^{2+} indicator fura-2. This dye was loaded into the smooth muscle by luminally perfusing endothelium-denuded vessels at 35°C with 2 μM fura-2/AM and pluronic acid (0.2%, w v^{-1}) for 30 min. Continuing vessel perfusion for a further 10 min washed out the extraneous dye. The tissue was then left for an equilibration time of at least 20 min, thus allowing the intracellular esterase to cleave fura-2/AM into active fura-2 (Goldman *et al.*, 1990). The tissue was viewed with an inverted microscope using an $\times 40$ oil-immersion objective (NA 1.3). A xenon lamp was used for illumination with the tissue alternately exposed to wavelengths of 340 and 380 nm for durations of 50 ms for each wavelength. The interval between wavelengths was 50 ms, with the cycle repeated at a frequency of 1–5 Hz (typically 5 Hz). Emission light was passed through a dichroic mirror (490 nm) and a bandpass filter (510 nm) and the light then recorded by a photomultiplier with the output responses, namely the fluorescence at 340 nm (F_{340}) and 380 nm (F_{380}), and the F_{340}/F_{380} ratio captured by an analogue-to-digital converter attached to a PC computer. Autofluorescence in the fura-2-loaded vessels was minor, representing only $2\pm0.6\%$ ($n=3$) of the total basal fura-2 fluorescence, and has not been further considered.

Measurement of vasomotion

Vasomotion of individual chambers was either monitored using a video camera attached to an inverted microscope or by monitoring the underlying Ca^{2+} transients during constriction. These experiments were made using lymphangions that exhibited spontaneous constrictions under control conditions.

The former measurement was effected either by a computer-based edge-detection system that determined the vessel edge positions from the video images (Diamtrack), or by recording the output on videotape and analysing the constrictions visually. System limitations during Ca^{2+} photometry experiments prevented videoscopic edge detection. However, fura-2-based measurements at the isosbestic point made either directly at F_{360} (Furuya *et al.*, 1994) or by calculating F_{360} from F_{340} and F_{380} data (Chiavaroli *et al.*, 1994) allows the measurement of movement. We used the latter procedure and found that constriction-associated movement correlated with the brief Ca^{2+} transients regularly recorded throughout our experiments. These increases in $[Ca^{2+}]_i$, measured relatively as F_{340}/F_{380} ratio, had a rapid rise phase of $<0.5\,s$ and duration at half amplitude between 0.5 and 1.0 s, values commensurate with independent measurements of vessel constriction (van Helden, 1993). Constriction was considered to have occurred when a Ca^{2+} transient of this wave shape presented with amplitude >1.5 standard deviations of the baseline noise. These transients were only used as a measure of vessel constriction and not of relative increases in $[Ca^{2+}]_i$. Should interpretation of the latter be of further interest to the reader, then inaccuracies in the relative $[Ca^{2+}]_i$ increases during the brief rise phases of the transients are to be noted. These inaccuracies arose because the kinetics of the constriction (as based on typical constrictions measured by edge tracking) were sufficiently fast during the onset phase to cause movement of $\sim 25\%$ of peak constriction between F_{340} and F_{380} measurements. However, such distortion was minimal during the remainder of the transient when movements between F_{340} and F_{380} measurements were less than $\sim 5\%$.

Heparin treatment of the tissue

Heparin ($8\,mg\,ml^{-1}$) was loaded into lymphatic smooth muscle using a reversible permeabilisation procedure (Kobayashi *et al.*, 1988). Fluo-3 ($1\,\mu M$) salt was simultaneously loaded with heparin to confirm that the tissue had been reversibly permeabilised, as confirmed using a confocal microscope laser-scanning system (Biorad MRC600) attached to an inverted microscope with an $\times 40$ oil-immersion objective (NA 1.3). Fluo-3-loaded tissues were excited using an argon ion laser at 488 nm with a 490 nm dichroic mirror and 515 nm band pass filter.

Pertussis toxin incubation

Tissues were superfused for 4 h at $35^{\circ}C$ with a physiological saline solution containing PTx ($100\,ng\,ml^{-1}$) (Burch *et al.*, 1988). Experiments to determine PTx sensitivities were performed subsequent to this incubation, but in the absence of PTx in the bathing solution.

Experimental protocols

Constriction and calcium photometric experiments were made (except where noted) using a 15 min control period, a 5 min test period during which ET-1 was applied, followed by a 15–30 min washout period. Measurements were made by recording the constriction frequency of individual lymphangions or $[Ca^{2+}]_i$ in the smooth muscle of these chambers for 4 min periods at the end of the initial control period, 1 min after application of the agonist and at the end of washout period. When pharmacological inhibitors were used, this protocol was repeated with the additional step of introducing the inhibitor, which was applied for a total of 20 min (15 min before and 5 min during the application of ET-1). In this case, constriction frequency or $[Ca^{2+}]_i$ was also monitored for 4 min in the inhibitor, just before application of ET-1. There followed a 30 min recovery period with the tissue exposed to control solution before ET-1 was reapplied. Throughout the experiments, an interval of at least 30 min was used between applications of ET-1. This minimised any tachyphylaxis observed by repeated use of ET-1 at shorter intervals (e.g. 10 min intervals). Except for the concentration–response studies, ET-1 was used at a concentration of 1 nM for the vasomotion studies. Analysis was based on comparisons of the test response of individual chambers to that of the relevant control immediately preceding the response, both averaged over a 4 min period.

The same protocol was followed for the vasospasm experiments, except that the tissue was normally exposed to 100 nM ET-1 now for a period of only 1 min to minimise tachyphylaxis (except where noted otherwise). The smaller bath (0.2 ml) was used throughout in these experiments. Analysis was now based on comparing control responses taken over 4 min to the response during the 1 min application of ET-1, allowing for the bath changeover time of $\sim 10\,s$.

Chemicals

The special chemicals used were 2-aminoethoxydiphenyl borate (2-APB) from Calbiochem, Sydney, Australia; acetylcholine (ACh), adenosine 5′-trisphosphate (ATP), BAPTA/AM, BQ-123, BQ-788, caffeine, ET-1, heparin, ionomycin and thapsigargin from Sigma, Sydney, Australia; cyclopiazonic acid (CPA), 1-(6-((17-beta-methoxyestra-1,3,5(10)trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5-dione (U73122), 1-[6-[[(17beta)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione (U73343) from Biomol Pty Ltd, PA, U.S.A.; PTx and ryanodine from RBI and fura-2 acetoxymethylester (fura-2/AM) and fluo-3/AM from Molecular probes, OR, U.S.A. 2-APB, CPA, fura-2/AM, fluo-3/AM, ionomycin, ryanodine, thapsigargin, U73122 and U73343 were dissolved into dimethyl sulphoxide (DMSO), whereas BQ-123, BQ-788, caffeine, ET-1, heparin and PTx were dissolved in water. Stock solutions were stored at concentrations of $1-20\,mM$ at $-20^{\circ}C$. For experiments where the vehicle DMSO was used, the DMSO concentration was always less than 0.1%, a concentration which had no significant effect on lymphatic vasomotion or smooth muscle $[Ca^{2+}]_i$.

Statistical analysis

Diameter-based measures of constriction frequencies and F_{340}/F_{380} fluorescence ratio-based amplitudes of Ca^{2+} transients, tonic $[Ca^{2+}]_i$ and frequency of $[Ca^{2+}]_i$ transients were normalised using the following equation:

$$f_N = (f/f_i) \times 100\%$$

where f_N is the normalised value, f is the actual value and f_i is the initial value measured just before application of the test solution. In some cases, the frequency of oscillatory Ca^{2+} transients was expressed as either numbers of transients per

minute or periods between two transients. The ET-1-induced changes in relative tone (ΔT) during vasomotion were measured as decreases in vessel diameter, normalised by the following relationship:

$$\Delta T \propto [(D_i - D)/D_i] \times 100\%$$

where D is the actual diastolic diameter and D_i is the initial diastolic diameter measured just before application of the test solution.

Dose–response relation curves were analysed by a computer-based curve-fit program (GraphPad prism 2.01), with the EC_{50} measured as the concentration giving half-maximal response (see Zhao & van Helden, 2002).

All values are presented as the mean$\pm$standard error of mean (s.e.m.) with n, the number of experiments. Statistical comparisons were made using a two-tailed paired Student's t-test, with $P<0.05$ (*) significant and $P<0.01$ (**) highly significant.

Results

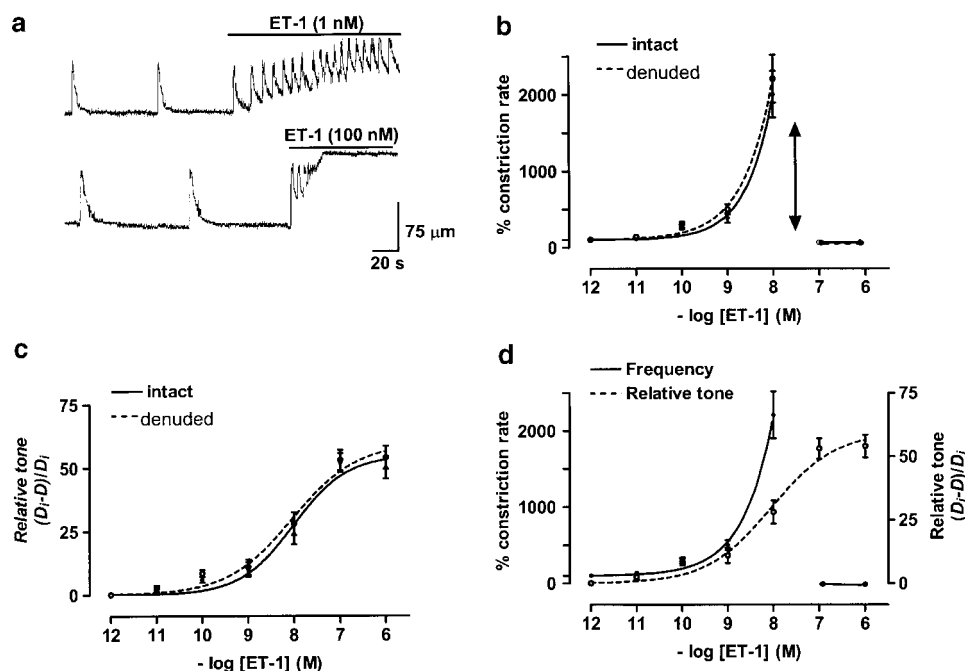### Effects of ET-1 on lymphatic vasomotion

Lymphatic vessels under resting, nonperfused conditions were either quiescent or exhibited spontaneous constrictions at low frequency (e.g. 1–4 constrictions min^{-1}). Analysis of 72 lymphangions (i.e. lymphatic chambers bounded by adjacent valves) provided a mean constriction rate of 1.5 ± 0.2 constrictions min^{-1}, corresponding to an average interval of 40 ± 5.4 s. The effects of ET-1 on lymphatic vasomotion were dose-dependent, with lower concentrations (e.g. 1 nM) increas-

ing vasomotion and higher concentrations (e.g. 100 nM), causing vasospasm (Figure 1a). The examples presented show that, at the two concentrations of ET-1 presented, there was a marked initial increase in the rate of vasomotion, which persisted in the 1 nM ET-1 solution but rapidly transformed into vasospasm in the 100 nM ET-1 solution. The concentration–response curve for the ET-1-induced increases in lymphatic vasomotion is presented in Figure 1b (solid curve, $n=9$). ET-1 concentrations as low as 0.1 nM tended to increase lymphatic vasomotion with maximal activation near 10 nM. Concentrations near 100 nM or higher abolished all vessel relaxation, resulting in sustained tonic contraction termed vasospasm.

ET-1 also acted to increase lymphatic tone in a dose-dependent manner, as estimated by measuring systolic lymphangion diameter with a maximum increase in estimated tone (i.e. $(D_i-D)/D_i$; Methods) of $52\pm3.7\%$ ($n=9$, $P<0.01$) at a concentration of 1 μM (Figure 1c, solid curve). Comparison of the concentration–response curves for vasomotion frequency and tone is made in Figure 1d. There was a parallelism between the curves for vasomotion (solid curve) and tone at lower concentrations (ET-1), though at higher concentrations vasomotion showed steeper ET-1 concentration dependence. The greatest difference was at the inflection where vasomotion transformed to vasospasm, the concentration-dependent increase in tone continuing in a predictable manner and showing no obvious inflection over this same ET-1 concentration range.

ET-1 action is independent of the endothelium

The role of the endothelium in ET-1-induced enhancement of vasomotion and induction of vasospasm was tested by

Figure 1 Effects of ET-1 on lymphatic vasomotion and vessel tone as measured from vessel diameter. (a) Sample traces indicating ET-1-induced lymphatic vasomotion (upper trace) and lymphatic vasospasm (lower trace; upward deflections indicate constrictions). (b, c) ET-1 concentration–response curves for lymphatic constriction rate and vessel tone (measured as $(D_i-D)/D_i$) in vessels with or without endothelium. The double-sided arrow in (b) is to highlight the abrupt change from vasomotion to vasospasm. (d) Comparison of concentration–response curves for ET-1 on the lymphatic constriction rate and tone. Data were normalised with respect to the corresponding control lymphangion constriction rate and diameter (D_i), respectively, with $n=9-12$ lymphangions for all points.

application of ET-1 to endothelium-denuded lymphatic vessels (Methods). The endothelium was not responsible for the ET-1 actions, as its absence caused no significant difference in the ET-1 concentration–response curves for vasomotion or vessel tone (Figures 1b, c, dashed curves, $n = 9$, $P > 0.05$ for each point between the $+$ and $-$ endothelium groups).

Effects of ET-1 on lymphatic smooth muscle $[Ca^{2+}]_i$

The mechanisms of ET-1 action were further investigated by measuring the effects of ET-1 on lymphatic smooth muscle $[Ca^{2+}]_i$, using the ratiometric Ca^{2+} indicator fura-2. These experiments were always performed on endothelium-denuded vessels to both facilitate loading of the calcium indicator fura-2/AM into the smooth muscle and to provide data specifically for the smooth muscle. System limitations during Ca^{2+} photometry experiments prevented videoscopic edge detection.

However, by estimating the fluorescence of fura-2 at its isosbestic point (F_{360}) using a linear combination of the F_{340} and F_{380} signal, it was possible to determine changes due to vessel movement (Methods). It was found that this movement correlated with the brief Ca^{2+} transients regularly recorded throughout the experiments, confirming that these transients reflected vasomotion (see also Zhao & van Helden, 2002). Examples of Ca^{2+} transients are presented in Figure 2a. The sample records presented here show the effects of application of 1 and 100 nM ET-1 on relative $[Ca^{2+}]_i$. The responses show similar characteristics to those of Figure 1a, obtained by measurement of lymphangion diameters, the Ca^{2+} transients reflecting vasomotion and the increase in tonic $[Ca^{2+}]_i$ paralleling diameter-based measures of the increase in lymphangion tone.

ET-1 increased the frequency of Ca^{2+} transients and hence vasomotion in a dose-dependent manner, with inhibition again
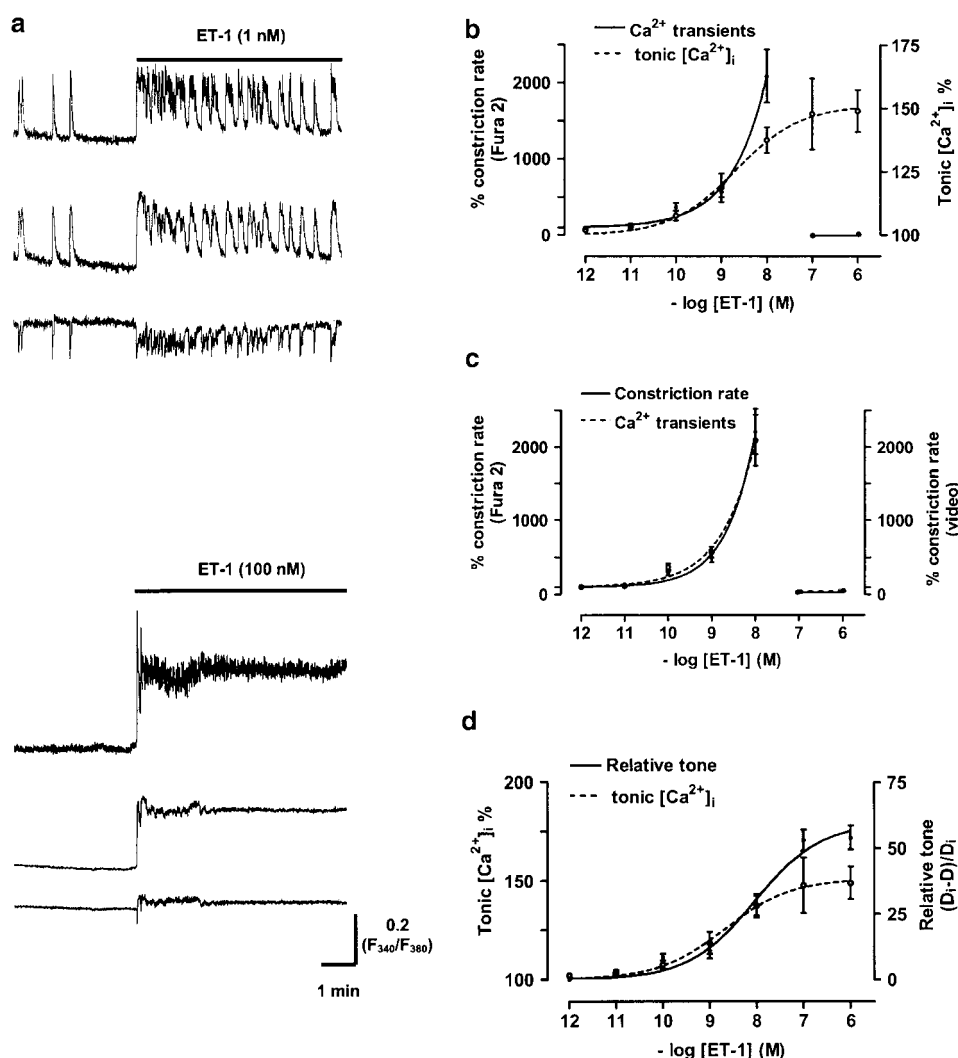

Figure 2 Effects of ET-1 on vasomotion (measured using Ca^{2+} transients) and relative tonic $[Ca^{2+}]_i$ in lymphatic smooth muscle. (a) Sample traces showing fura-2 F_{340} and F_{380} fluorescence measurements with the F_{340}/F_{380} ratio used to measure ET-1-induced increases in relative lymphatic smooth muscle $[Ca^{2+}]_i$ in response to low (1 nM) and higher ET-1 concentrations (100 nM). (b) ET-1 concentration–response curves for the % change in constriction rate and tonic $[Ca^{2+}]_i$. (c) Comparison of ET-1 concentration–response curves for the % change in constriction rate measured either using fura-2-based Ca^{2+} transients or by video-recorded movement (i.e. the endothelium-denuded record of Figure 1b). (d) Comparison of ET-1 concentration–response curves for the % change in tonic $[Ca^{2+}]_i$ and normalised change in vessel tone (i.e. $(D_i-D)/D_i$; endothelium-denuded record of Figure 1c). All data presented here are from endothelium-denuded vessels. Data were normalised with respect to the corresponding control tissues with $n = 6-9$. Vertical lines denote s.e.m. with $n = 9-12$ lymphangions for all points.

occurring at higher [ET-1], commensurate with the onset of vasospasm (Figure 2b, solid curve). The concentration–response curve for the relative increase in tonic $[Ca^{2+}]_i$ measured between Ca^{2+} transients is shown in Figure 2b (dashed curve). Comparison of the effects of ET-1 on the concentration-dependent action of ET-1 to increase Ca^{2+} transients with these then undergoing a vasospasm-associated inhibition parallels the findings made from measurements of vessel diameter (Figure 2c). The almost identical profiles of these curves both confirm the use of the Ca^{2+} transients as a measure of vasomotion, and indicates that loading vessels with the Ca^{2+} sensing fluorophore fura-2 or the acetoxymethylester carrier did not significantly alter the dependence of vasomotion and the transformation to vasospasm on [ET-1]. Comparisons of the ET-1 concentration–response curve for tonic $[Ca^{2+}]_i$ and for diameter-based lymphangion tone are presented in Figure 2d. There was a reasonable correlation between lymphatic tone (Figure 2d, solid line) and tonic $[Ca^{2+}]_i$ (Figure 2d, dashed line) up to an [ET-1] of $\sim 10\,nM$. However, higher [ET-1] produced steeper increases in lymphatic tone than in tonic $[Ca^{2+}]_i$. It is known that ET-1 can directly enhance smooth muscle contractility (Huang *et al.*, 1990), and this may provide some explanation as to the difference in these curves.

Pharmacological properties of ET-1-induced vasomotion

Pharmacological properties of the mechanisms underlying lymphatic vasomotion were examined using an [ET-1] of 1 nM. ET-1-activated receptor and intracellular pathways underlying vasomotion were investigated with either fura-2-based ratio-metric Ca^{2+} photometry of the smooth muscle of endothelium-denuded lymphatic vessels or videoscopic measurement of the diameters of endothelium-intact vessels.

ET-1 receptor subtypes   ET-1 actions were likely to be mediated by the ET_A receptor subtype, as BQ-123 (1 μM), a specific antagonist of the ET_A receptor (Eguchi *et al.*, 1992; Ihara *et al.*, 1992), abolished the effects of ET-1 (1 nM) both on the increase in frequency of the Ca^{2+} transients and on tonic $[Ca^{2+}]_i$ (Figures 3a, b). In contrast, BQ-788 (1 μM), a specific antagonist of the ET_B receptor (Ishikawa *et al.*, 1994), had no significant effect on the ET-1-induced responses (Figures 3c, d). Neither BQ-123 (1 μM) nor BQ-788 (1 μM) had a direct effect on either the frequency of oscillatory Ca^{2+} transients or on tonic $[Ca^{2+}]_i$ levels (Figure 3). Bath application of BQ-123 or BQ-788 also had no significant effects on the frequency and tone of lymphatic vasomotion, as measured by videoscopic vessel edge tracking (data not shown; $P > 0.05$, $n = 6$ for both).
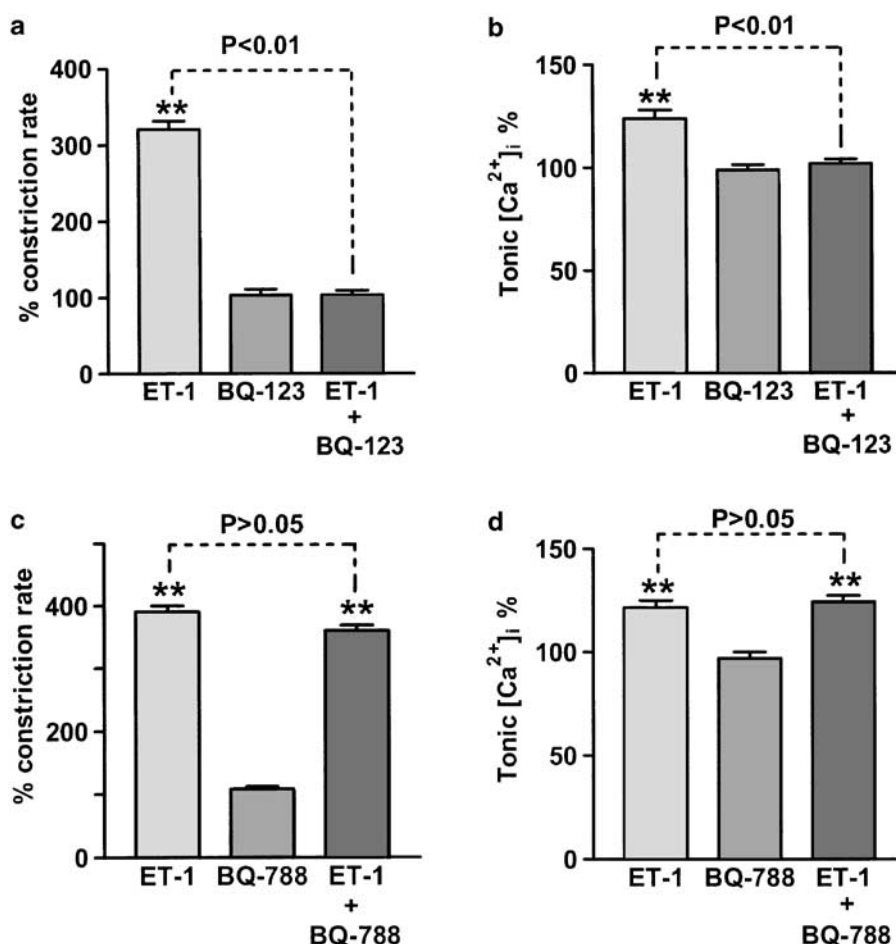
Figure 3 Effects of ET-1 receptor antagonists on ET-1-induced vasomotion and relative $[Ca^{2+}]_i$. BQ-123 (1 μM), an ET_A receptor blocker, inhibited vasomotion induced by 1 nM ET-1 (a) and prevented the associated increase in $[Ca^{2+}]_i$ (b). BQ-788 (1 μM), an ET_B receptor blocker, did not significantly alter vasomotion induced by 1 nM ET-1 (c) or associated increase in $[Ca^{2+}]_i$ (d). Direct application of the antagonists had no significant effects on either vasomotion or $[Ca^{2+}]_i$. Data were normalised with respect to the corresponding control lymphangions with $n = 6-9$. Vertical lines denote s.e.m. **$P < 0.01$.

Second messenger pathways   ET-1 receptors are known to be coupled to G-proteins. The possibility that this was of the G_i type was examined using PTx, an inhibitor of G_i-proteins (see Fields & Casey, 1997). Lymphatic vessels were superfused with physiological saline solution containing PTx ($100\,ng\,ml^{-1}$) for 4 h at $35^\circ C$, with the tissue then returned to control solution (Methods). PTx treatment of lymphatics had no significant effect on the 1 nM ET-1-induced increase in either vasomotion ($342\pm20\%$ of control; $n=5$, $P>0.05$) or tonic $[Ca^{2+}]_i$ ($125\pm2\%$ of control; $n=5$, $P>0.05$).

Application of the PLC inhibitor U73122 ($5\,\mu M$) (Bleasdale *et al.*, 1990; Thompson *et al.*, 1991) prevented the increase in both vasomotion and resting $[Ca^{2+}]_i$ induced by 1 nM ET-1 (Figures 4a, b). In contrast, U73343 ($5\,\mu M$), an inactive analogue of U73122 (Bleasdale *et al.*, 1990), had no measurable effect on these ET-1-induced increases (Figures 4a, b).
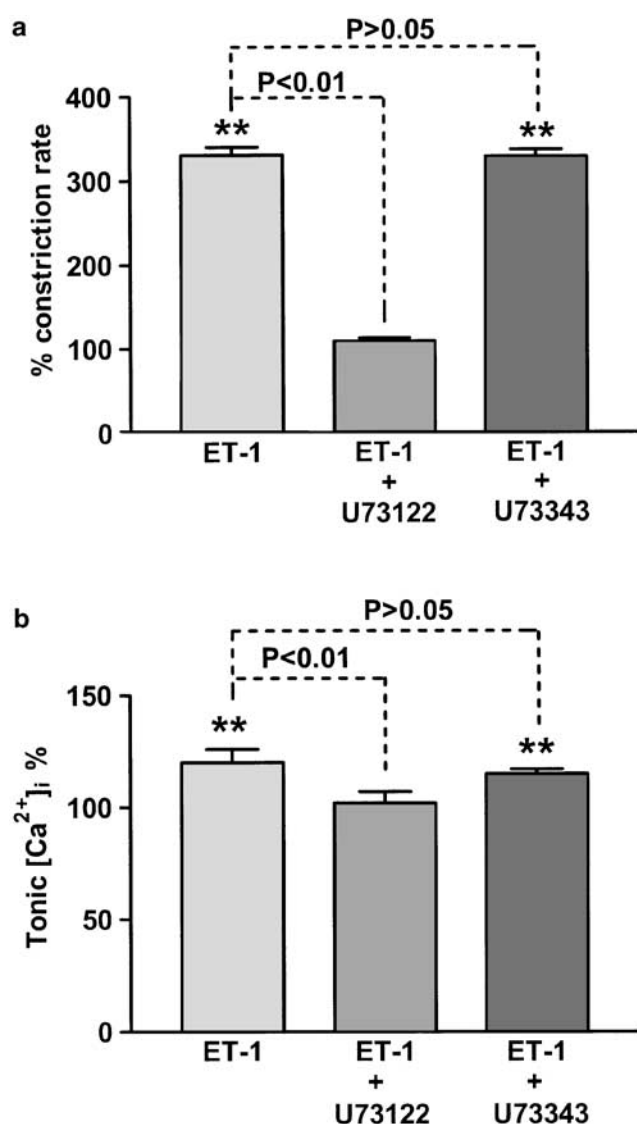
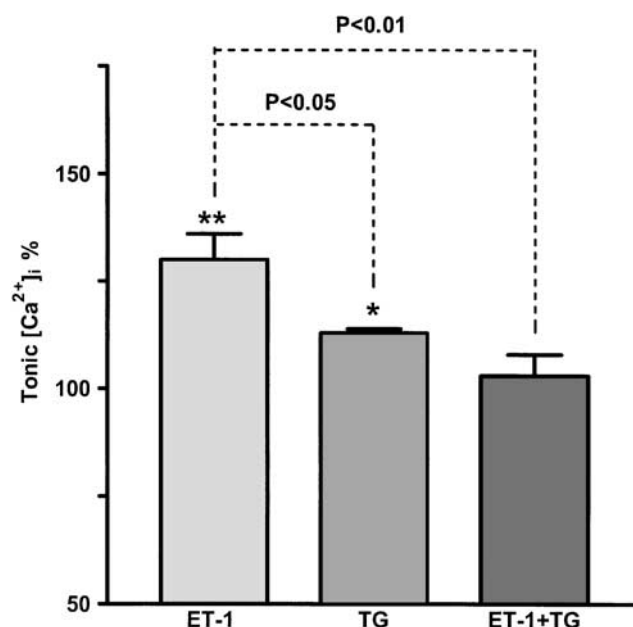U73122 itself had no significant effects on resting $[Ca^{2+}]_i$ ($100\pm2.6\%$ of control, $n=6$, $P>0.05$), but inhibited spontaneous vasomotion ($25\pm1.8\%$ of control, $n=6$, $P<0.01$). U73343 had no significant effects on both vasomotion and resting $[Ca^{2+}]_i$ ($n=6$, $P>0.05$, respectively).

$[Ca^{2+}]_i$ mobilisation and the role of the SR   Application of Ca^{2+}-free solution (i.e. no added $Ca^{2+}+1\,mM$ EGTA) abolished spontaneous and 1 nM ET-1-induced vasomotion, as determined by measurement of the frequency of Ca^{2+} transients ($n=8$). The Ca^{2+}-free solution did not alter resting $[Ca^{2+}]_i$ ($99\pm2.6\%$ of control, $n=8$, $P>0.05$), but inhibited the increase in tonic $[Ca^{2+}]_i$ ($103\pm3.2\%$ of control, $n=8$, $P>0.05$) induced by 1 nM ET-1. This indicates that extracellular Ca^{2+} is fundamental to ET-1 action.

Evidence that ET-1 action occurred through activation of SR Ca^{2+} release was provided by experiments using BAPTA/AM to chelate intracellular Ca^{2+} and thapsigargin, an irreversible inhibitor of the SR Ca^{2+} pump (Ghosh *et al.*, 1988; Takemura *et al.*, 1990; Darby *et al.*, 1993). BAPTA/AM ($10\,\mu M$) applied for 6 min before application of 1 nM ET-1, now without BAPTA/AM present, abolished both spontaneous and ET-1-induced lymphatic vasomotion ($n=3$) and the increase in tonic $[Ca^{2+}]_i$ ($n=3$). Thapsigargin ($1\,\mu M$) caused a small but significant increase ($P<0.05$) in the resting $[Ca^{2+}]_i$ of lymphatic smooth muscle (Figure 5), and inhibited spontaneous vasomotion in control conditions ($n=4$, $P<0.01$). Importantly, thapsigargin ($1\,\mu M$) abolished the increase in both vasomotion and tonic $[Ca^{2+}]_i$ induced by 1 nM ET-1 (Figure 5).

Evidence that ET-1 action occurred through activation of IP_3Rs was provided by studies with heparin, a known blocker of IP_3Rs (Ghosh *et al.*, 1988; Kobayashi *et al.*, 1988). Heparin, when introduced into the smooth muscle cells by reversible

Figure 4   Application of the PLC inhibitor U73122 ($5\,\mu M$) inhibited the increase in both vasomotion (a) and tonic $[Ca^{2+}]_i$ (b) induced by 1 nM ET-1. In contrast, the inactive analogue U73343 ($5\,\mu M$) had no significant effect. Data were normalised with respect to the corresponding control lymphangions with $n=9-12$. Vertical lines denote s.e.m. **$P<0.01$.

Figure 5   Application of the store Ca^{2+} pump inhibitor thapsigargin ($1\,\mu M$) inhibited the increase in both vasomotion (a) and tonic $[Ca^{2+}]_i$ (b) induced by 1 nM ET-1. Data were normalised with respect to the corresponding control lymphangions with $n=9-12$. Vertical lines denote s.e.m. **$P<0.01$ and *$P<0.05$.

a Control loaded

ET-1 (1 nM)

b Heparin loaded

ET-1 (1 nM)

0.2
(F_{340}/F_{380})

100 s
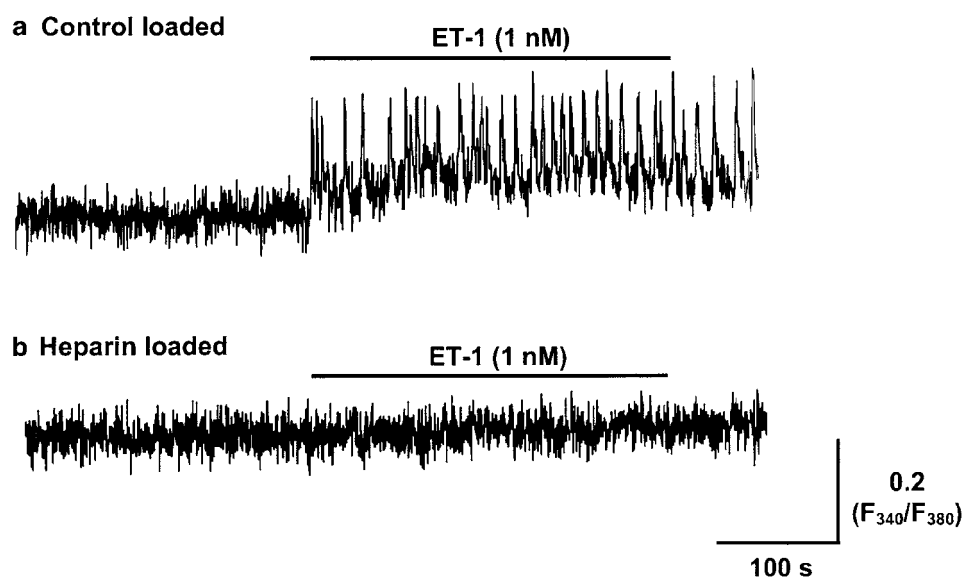
Figure 6 Intracellular heparin inhibits the increase in vasomotion and tonic $[Ca^{2+}]_i$ induced by 1 nM ET-1. Lymphatic smooth muscle was preloaded with heparin (8 mg ml^{-1}) by a reversible permeabilisation procedure. The records show the fura-2 F_{340}/F_{380} fluorescence ratio response to ET-1 in lymphatic smooth muscle without (a) and with heparin loading (b).

permeabilisation (Methods), inhibited both spontaneous and ET-1 (1 nM)-induced vasomotion ($n = 8$; Figure 6b). In contrast, 'control' tissues, exposed to the same loading procedure but in the absence of heparin, exhibited vasomotion of 2.0 ± 0.5 transients min^{-1} before and 7.0 ± 0.8 transients min^{-1} in the presence of 1 nM ET-1 ($n = 8$, $P < 0.01$; Figure 6a). Heparin-loaded tissues also showed no significant tonic increase in $[Ca^{2+}]_i$ in response to 1 nM ET-1 ($102 \pm 1.2\%$ of control; $n = 8$, $P > 0.05$; Figure 6b). By comparison, application of 1 nM ET-1 to 'control' tissues caused a significant increase in tonic $[Ca^{2+}]_i$ of $119 \pm 3\%$ ($n = 8$, $P < 0.05$; Figure 6a). Similar findings were made using 2-APB, which has been reported to block IP$_3$Rs and/or store-dependent Ca^{2+} entry (Maruyama et al., 1997; Ascher-Landsberg et al., 1999), with application of 30 μM 2-APB abolishing both spontaneous vasomotion and the ET-1-induced enhancement of vasomotion ($n = 3$).

The role of ryanodine receptors (RyRs) was investigated by application of ryanodine. Ryanodine (1 and 20 μM) itself had no measurable effect on vasomotion, with frequencies of 2.5 ± 0.6, 2.3 ± 0.3 and 2.2 ± 0.3 Ca^{2+} transients min^{-1} in control, 1 and 20 μM ryanodine, respectively (15 min exposure, $n = 5$ for each, $P > 0.05$), but caused a small but significant transient increase in $[Ca^{2+}]_i$ (113 ± 0.7 and $115 \pm 0.5\%$, $P < 0.05$, $n = 3$ and 5, respectively) (Figure 7Ab). Ryanodine (1 and 20 μM) had no significant effect on the enhancement of vasomotion and the magnitude of the increase in $[Ca^{2+}]_i$ induced by 1 nM ET-1 ($P > 0.05$, $n = 3$ and 5 for both), but may have caused a decrease in duration of the ET-1-induced increase in relative $[Ca^{2+}]_i$ (Figure 7Ac).

Caffeine (10 mM) abolished spontaneous vasomotion, despite causing a small but significant transient increase in $[Ca^{2+}]_i$ of $113 \pm 0.2\%$ ($P < 0.05$, $n = 5$, Figure 7Bb). This increase in $[Ca^{2+}]_i$ was likely to be due to the activation of RyRs, as it was abolished following treatment of the tissue with 20 μM ryanodine ($98 \pm 0.2\%$, $P > 0.05$, $n = 6$). Importantly, caffeine (10 mM) abolished spontaneous vasomotion and the enhancement of both vasomotion and tonic $[Ca^{2+}]_i$ induced by 1 nM ET-1 ($n = 6$; Figure 7Bc). This is a common action of caffeine, as observed for electrical rhythmicities in other smooth muscles (Hashitani et al., 1996; van Helden et al., 2000), and is likely to occur through its known actions to inhibit IP$_3$R-mediated Ca^{2+} release (Ehrlich & Watras, 1988) and/or to increase cAMP (Beavo & Reifsnyder, 1990).

ET-1-induced vasospasm

The data of Figures 1 and 2 demonstrate that ET-1 at concentrations higher than 10 nM produced a maintained maximal, tonic vessel constriction and increase in $[Ca^{2+}]_i$. Therefore, subsequent experiments were performed using ET-1 at a concentration of 100 nM to examine the mechanisms underlying this vasospasm.

Role of extracellular Ca^{2+}   Application of ET-1 for 1 min at a concentration of 100 nM markedly increased vasomotion upon application, which rapidly changed to a sustained contracture and underlying maintained increase in smooth muscle $[Ca^{2+}]_i$ (Figures 1a, 2a). Brief incubation of the tissue with Ca^{2+}-free solution (i.e. 0 Ca^{2+}, 1 mM EGTA solution) for

Figure 7 Effects of ryanodine and caffeine on the increase in vasomotion and tonic $[Ca^{2+}]_i$ induced by 1 nM ET-1. (A) The sample traces indicating the changes of the F_{340}/F_{380} fluorescence ratio in the smooth muscle of the same lymphatic vessel exposed to 1 nM ET-1 (a), ryanodine (20 μM) (b), and ET-1 (1 nM), in the presence of ryanodine (20 μM) (c) are shown. Ryanodine did not prevent the responses to 1 nM ET-1. (B) Sample traces indicating the changes of the F_{340}/F_{380} fluorescence ratio in the smooth muscle of a lymphatic vessel exposed to 1 nM ET-1 (a), 10 mM caffeine (b) and both 10 mM caffeine and 1 nM ET-1 (c) are shown. Caffeine inhibited the increase in vasomotion and tonic $[Ca^{2+}]_i$ induced by 1 nM ET-1.

1 min ($>95\%$ bath changeover in 20 s) abolished all vasomotion and the sustained but not transient increase in $[Ca^{2+}]_i$ induced by 1 min application of 100 nM ET-1 ($n = 5$, Figure 8).

These results suggest that the initial Ca^{2+} transient induced by ET-1 was primarily due to the release of Ca^{2+} from intracellular stores, while the sustained plateau was due to

ET-1 (100 nM)

OCa^{2+} + 1 mM EGTA
ET-1 (100 nM)

0.2
(F$_{340}$/F$_{380}$)

100 s
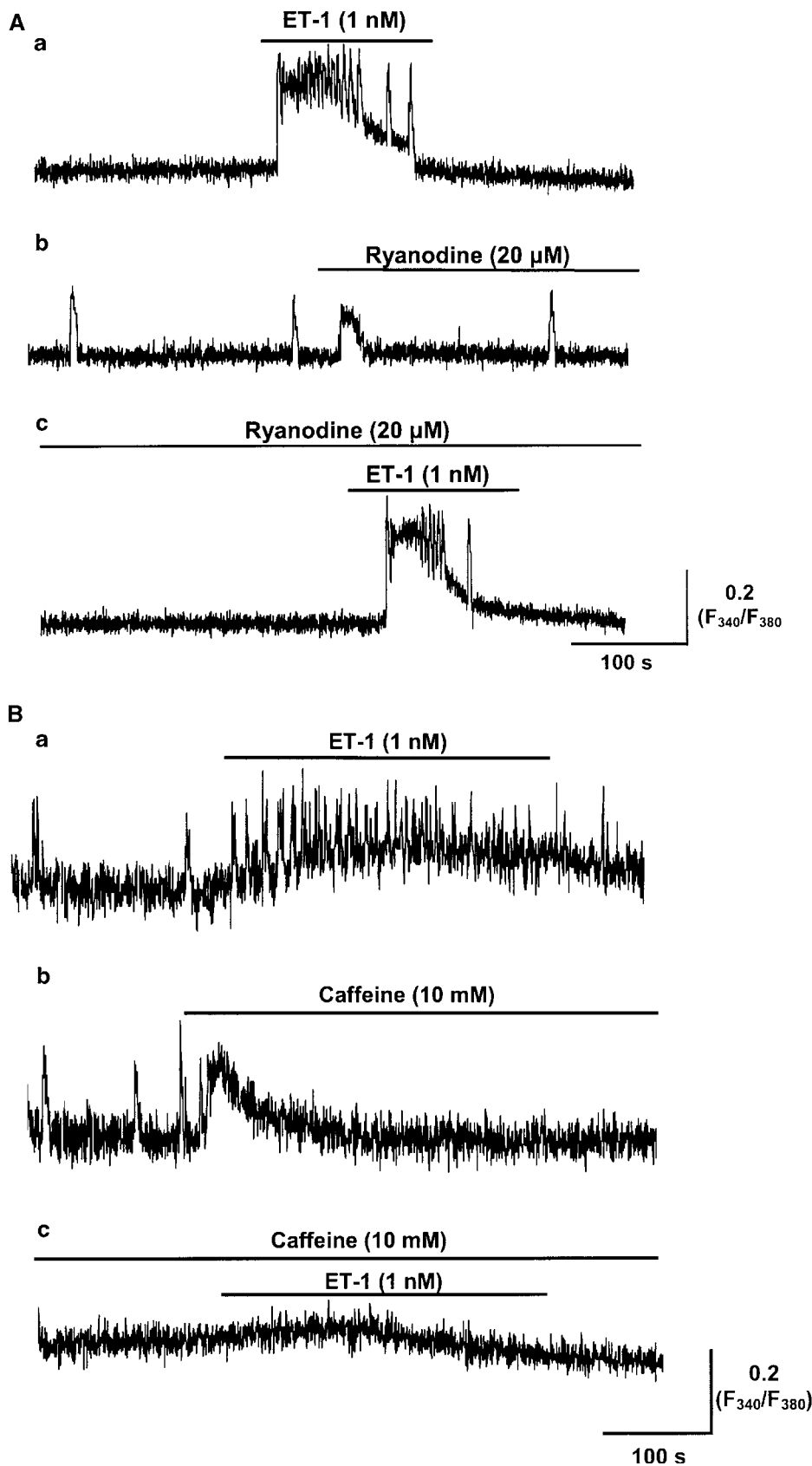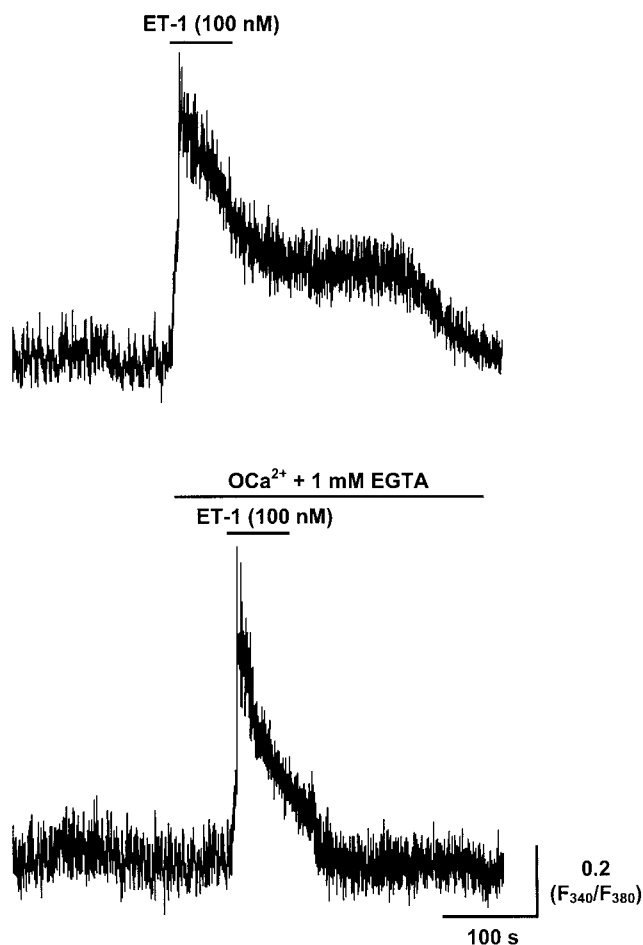
Figure 8 Effect of Ca^{2+}-free solution on ET-1-induced increase in [Ca^{2+}]$_i$. Application of 100 nM ET-1 caused a large transient followed by sustained increase in [Ca^{2+}]$_i$ measured as the fura-2 F$_{340}$/F$_{380}$ fluorescence ratio in lymphatic smooth muscle. The sustained increase in [Ca^{2+}]$_i$ became transient when 100 nM ET-1 was applied in a Ca^{2+}-free 1 mM EGTA-containing solution, with the tissue only briefly (i.e. ~1 min) preincubated in this Ca^{2+}-free solution.

the influx of external Ca^{2+}. The ET-1-induced transient increase in [Ca^{2+}]$_i$ was abolished when ET-1 (100 nM) was applied to tissues pretreated for 3 min (and then maintained) in Ca^{2+}-free solution ($n=6$). Commensurate with this, CPA (20 μM; $n=3$), while causing significant increases in [Ca^{2+}]$_i$ ($P<0.01$) when applied after 1 min exposure to Ca^{2+}-free solution, caused no response when applied after 3 min exposure. ATP (100 μM) also caused no response when applied after 3 min exposure, whereas we have previously reported a large response when ATP was applied upon changeover to Ca^{2+}-free solution (Zhao & van Helden, 2002). This suggests that maintained exposure to the Ca^{2+}-free solution rapidly depleted the SR stores. Significantly, application of the 100 nM ET-1 after 3 min exposure to the Ca^{2+}-free solution prevented the induction of vasospasm, as measured by edge detection ($n=4$), confirming that Ca^{2+} influx from the extracellular space is required for ET-1-induced vasospasm.

Role of Ca^{2+} entry channels Voltage-operated, receptor-operated and/or store-operated Ca^{2+} channels have each been suggested as candidates for the Ca^{2+} influx pathway(s)

underlying ET-1-induced vasospasm. The possibility of Ca^{2+} influx through VOCs, which in lymphatic smooth muscle are likely to be L-type Ca^{2+} channels (Ohhashi *et al.*, 1978), was examined by applying nifedipine. Application of nifedipine (1 μM) abolished spontaneous and ET-1-induced vasomotion (Figure 9a). However, this was likely to be through blockade of the L-Ca^{2+} channel-mediated action potentials, and not through blockade of ET-1-induced Ca^{2+} entry, as nifedipine (1 μM) reduced, but did not abolish, the sustained increase in [Ca^{2+}]$_i$ induced by 100 nM ET-1 (Figures 9a, b) and had no significant effect on vessel tone (Figure 9c). In contrast, nifedipine (1 μM) abolished the vasospasm induced by 100 mM KCl (Figure 9c). These results indicate that, while L-type Ca^{2+} channels are involved in the ET-1-induced vasospasm, their presence is not critical for vasospasm to occur.

The role of voltage-independent Ca^{2+} entry in vasospasm was examined using SKF 96365, a compound that has been shown to inhibit Ca^{2+} entry through either SOCs (Merritt *et al.*, 1990) or VOCs (Cuthbert *et al.*, 1994). Importantly, SKF 96365 (30 μM) had no obvious effect on vasospasm induced by 100 mM KCl ($n=6$), but caused vasospasm induced by 100 nM ET-1 to revert back to vasomotion (rate of 10 ± 2.5 constrictions min^{-1}, $n=6$, Figure 10). This indicates that the ET-1 was now less effective, as the outcome parallels lowering the [ET-1] back into the range of inducing vasomotion. The effect of SKF 96365 was reversible, as, consequent to 10 min washout, reapplication of 100 nM ET-1 produced vasospasm with lymphangion diameter decreased to $50\pm2\%$ of control ($n=6$).

These results suggest that ET-1-induced vasospasm primarily involves SOCs with residual Ca^{2+} influx through L-type Ca^{2+} channels. Further evidence for a primary role of SOCs is provided by the finding that 2-APB (90 μM), an inhibitor of IP$_3$ receptors and/or SOCs (Maruyama *et al.*, 1997; Gregory *et al.*, 2001), when applied in the presence of nifedipine (1 μM), abolished vasospasm induced by 100 nM ET-1 ($n=6$). However, it is possible that 2-APB at these high concentrations may have nonspecific effects. Therefore, we also investigated a role for SOCs using either caffeine or the PLC blocker U73122. Caffeine (10 mM) markedly blunted the action of 100 nM ET-1 to induce vasospasm, the relative tone now reduced from 51 ± 2.2 to $33\pm1.0\%$ of control diameter ($n=6-9$; Figure 11a). Further addition of nifedipine (1 μM) to the inhibitory 10 mM caffeine-containing solution abolished vasospasm ($n=6$; Figure 11a). Bath application of U73122 (5 μM) in the presence of 1 μM nifedipine abolished the vasospasm induced by 100 nM ET-1 (Figure 11b). In contrast, the same experiment made with nifedipine (1 μM) and U73343 (5 μM), an inactive analogue of U73122, had no significant effect on the ET-1-induced vasospasm (Figure 11b).

Discussion

This project has investigated lymphatic vasomotion and vasospasm induced by ET-1. The data provide a detailed account of the receptor and intracellular pathways involved.
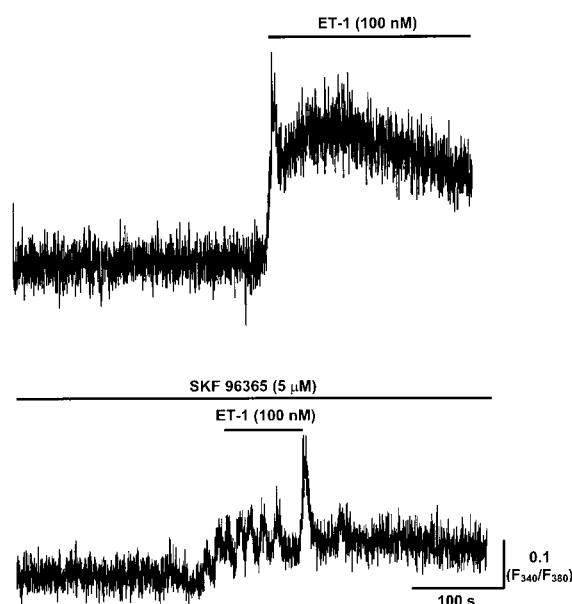
ET-1-induced vasomotion

The present study demonstrates that ET-1-induced enhancement of vasomotion occurs through activation of ET$_A$
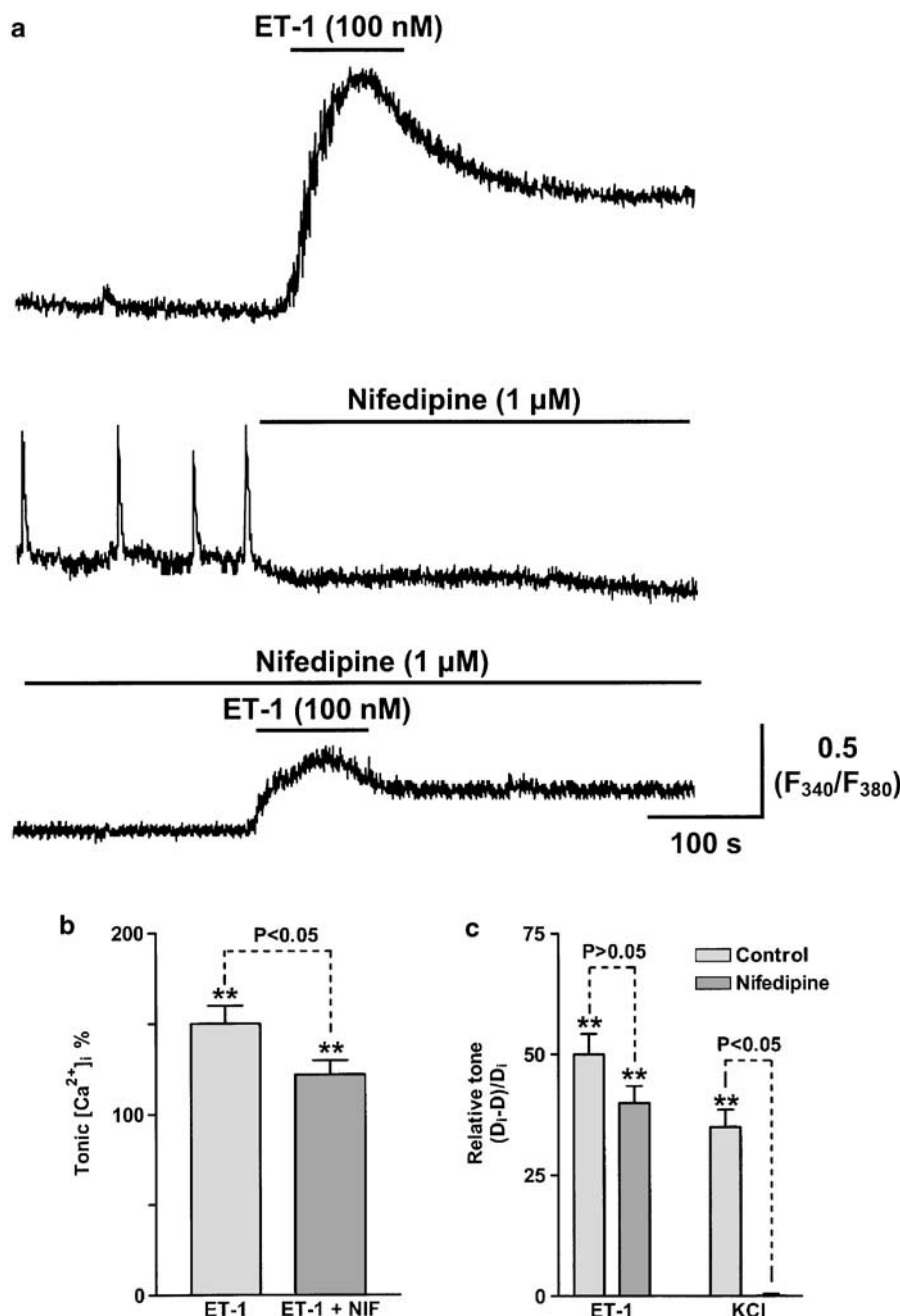
Figure 9 Effects of nifedipine on ET-1- and K^+-induced vasospasm. (a) Sample traces indicating the changes in lymphatic smooth muscle $[Ca^{2+}]_i$ measured as the F_{340}/F_{380} fura-2 fluorescence ratio in response to 100 nM ET-1, 1 μM nifedipine and 100 nM ET-1 applied in the presence of 1 μM nifedipine. (b) Bar graph indicating the mean effect of 100 nM ET-1 on tonic $[Ca^{2+}]_i$ before and in the presence of 1 μM nifedipine. (c) Bar graph showing the mean effects of both 100 nM ET-1 and 100 mM KCl solution on relative lymphangion tone (i.e. measured as $(D_i-D)/D_i)$) in the absence and presence of 1 μM nifedipine. Data bars in (b) and (c) were normalised with respect to the corresponding controls with $n = 5$ lymphangions for each value. Vertical lines denote s.e.m. **$P < 0.01$.

receptors on lymphatic smooth muscle, which stimulates production of PLC by a PTx-insensitive G-protein, leading to synthesis of IP_3 and release of Ca^{2+} from intracellular IP_3R-operated stores. Evidence for this comes from the findings that ET-1-induced increases in vasomotion were blocked by (1) the ET_AR antagonist BQ-123 but not the ET_BR antagonist BQ-788, (2) the PLC inhibitor U73122 but not the inactive analogue U73343, (3) the Ca^{2+} store ATPase inhibitor thapsigargin, (4) removal of extracellular Ca^{2+}, (5) the intracellular Ca^{2+} chelator BAPTA/AM and (6)

the IP_3R antagonists 2-APB and heparin. The ET-1-induced enhancement of vasomotion was also blocked by caffeine. This could result through direct inhibition of IP_3 channels (Bezprozvanny *et al.*, 1991), inhibition of PLC activity (Hamada *et al.*, 1997) and/or phosphodiesterase-induced formation of cAMP (Beavo & Reifsnyder, 1990; Tsugeno *et al.*, 1995). It did not occur through an action on RyRs, as RyRs while functionally present in the lymphatic smooth muscle did not abolish spontaneous or ET-1-induced vasomotion.

Figure 10 Effects of SKF 96365 on ET-1-induced vasospasm. Sample traces indicating the changes in lymphatic smooth muscle $[Ca^{2+}]_i$ measured as the F_{340}/F_{380} fura-2 fluorescence ratio in response to 100 nM ET-1 and both SKF 96365 (5 μM) and 100 nM ET-1.

The finding that heparin abolished spontaneous vasomotion is consistent with the view that IP_3-operated Ca^{2+} stores are required for vasomotion even under control conditions. Furthermore, 2-APB at its effective inhibitory concentrations (Maruyama *et al.*, 1997) abolished spontaneous vasomotion, consistent with a general involvement of IP_3R-operated stores.

There has been limited information about the role of endogenous ET-1 on lymphatic vasomotion both *in vivo* and *in vitro*. The fact that ET-1 antagonists BQ-123 and BQ-788 had no significant effects on spontaneous lymphatic vasomotion or smooth muscle $[Ca^{2+}]_i$, as studied here *in vitro*, suggests that, under these conditions, endogenous ET-1 does not modulate lymphatic vasomotion. Similar findings have been reported for blood vessels studied *in vivo*, where it was found that the concentration of circulating ET-1 was too low to produce any detectable vasoconstriction (Ortega Mateo & de Artinano, 1997). However, under some pathological conditions, it is likely that ET-1 concentrations become sufficiently elevated to either increase vasomotion or to cause vasospasm. Moderate enhancements of [ET-1] would increase lymphatic pumping and hence the propulsion of lymph. In contrast, high [ET-1] would cause vasospasm and interrupt the flow of lymph. The latter is unfortunately a common event for blood vessels where events such as haemorrhage can lead to vasospasm through marked enhancement of ET-1 production (Kwan *et al.*, 2002). While vasospasm would be a less catastrophic event in lymphatic vessels, it remains a phenomenon of considerable interest, as the underlying mechanisms are likely to share the same properties as for blood vessels.

ET-1-induced vasospasm

It has been reported in bovine mesenteric lymphatic vessels that ET-1 at higher concentrations induces vasospasm (Sakai
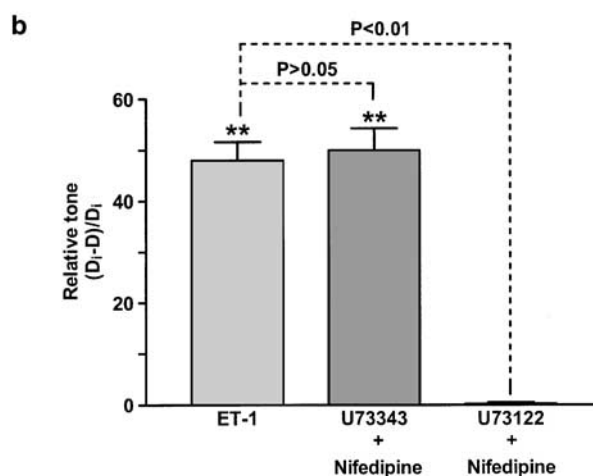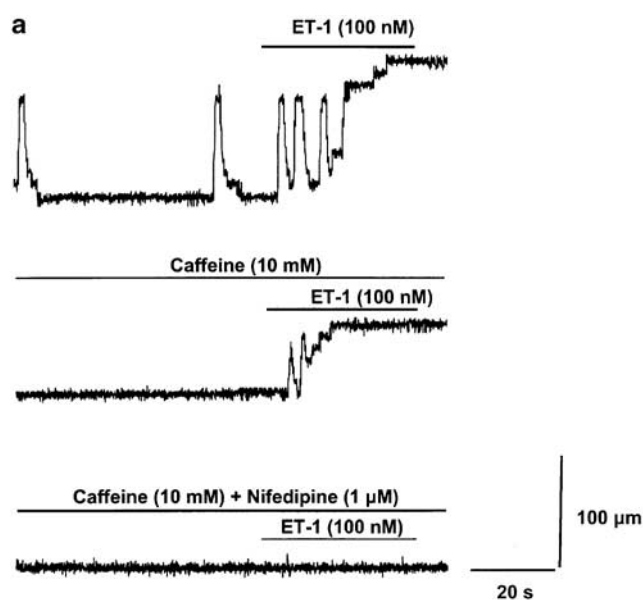
Figure 11 Effects of caffeine and the PLC antagonist U73122 on ET-1-induced vasospasm. (a) The effects of 100 nM ET-1 on constriction of a lymphangion in control, caffeine (10 mM) and caffeine (10 mM) together with nifedipine (1 μM). (b) Bar graphs showing the effects of U73343 (5 μM) and U73122 (5 μM) on the relative tonic increase in lymphangion constriction in response to 100 nM ET-1 in the presence of nifedipine (1 μM). Data in (b) were normalised with respect to the corresponding control tissues with $n = 6$ lymphangions for all points. Vertical lines denote s.e.m. **$P < 0.01$.

et al., 1999), but the underlying mechanisms were not elucidated. The present study provides the first evidence that ET-1 at concentrations greater than 10 nM causes vasospasm primarily by massive Ca^{2+} influx through SOCs, with a minor contribution by L-type Ca^{2+} channels.

ET-1 is known to induce depolarisation of vascular smooth muscle and associated biphasic elevation of $[Ca^{2+}]_i$ and cell constriction (see Haynes & Webb, 1993). This elevation of $[Ca^{2+}]_i$ consists of an initial rapid rise due to Ca^{2+} release from the SR, and is followed by a fall to plateau levels, which is maintained by the persistent influx of extracellular Ca^{2+} through membrane ion channels (Yanagisawa *et al.*, 1988;

Goto *et al.*, 1989). Results from the present experiments parallel these findings with high [ET-1] (e.g. $\geqslant 100$ nM), causing an initial Ca^{2+} transient due to release from intracellular Ca^{2+} stores, followed by a sustained plateau due to Ca^{2+} influx from the extracellular space.

The extracellular Ca^{2+}-dependent sustained component of the ET-1-induced $[Ca^{2+}]_i$ changes associated with vasospasm resulted from activation of several Ca^{2+} permeant channels. L-Ca^{2+} channels played a minor role in this, as blockade of these channels by nifedipine caused no obvious change in vasospasm and only partially reduced $[Ca^{2+}]_i$. Studies with the nonspecific SOC blocker SKF 96365 (Chernaya *et al.*, 1996) indicated that Ca^{2+} entry through either VOCs or SOCs was dominant, as application of SKF 96365 transformed ET-1-induced vasospasm to vasomotion. This action is equivalent to reducing the effective [ET-1] to less than 10 μM (Figure 1). Evidence that these channels were SOCs was provided by the finding that either the PLC inhibitor U73122 or the IP_3R and/or SOC blocker 2APB, when applied together with nifedipine, abolished vasospasm. Significantly, application of caffeine also markedly reduced ET-1-induced vasospasm and abolished all constriction with the further addition of nifedipine.

These findings are consistent with observations on enzymatically isolated lymphatic smooth muscle cells, where it was found that maximum concentrations of agonists caused a biphasic Ca^{2+} signal composed of an early transient phase due to Ca^{2+} release from intracellular Ca^{2+} stores, and a following sustained plateau phase due to Ca^{2+} influx primarily through SOCs (Shuttleworth, 1999). The findings are also consistent with findings that ET-1-induced vasospasm in arteries of a two-haemorrhage rabbit model was due to Ca^{2+} influx through VOCs and SOCs (Zuccarello *et al.*, 1996a).

Conclusions

Spontaneous lymphatic vasomotion occurs through a pacemaker mechanism dependent on Ca^{2+} release from intracellular IP_3R-operated stores and largely independent of RyRs. ET-1 enhances lymphatic vasomotion through activation of smooth muscle ET_A receptors, which stimulates the production of PLC by a PTx-insensitive G-protein, leading to synthesis of IP_3 and release of Ca^{2+} from intracellular IP_3R-operated stores.

A most striking observation of the present study was the abrupt transition from vasomotion to vasospasm. This occurred at [ET-1] in the range 10–100 nM, with supra maximal [ET-1] causing the frequency of vasomotion to increase to such an extent that reconstitution of $[Ca^{2+}]_i$ could no longer occur. The consequence of this now uncontrolled Ca^{2+} homeostasis was a failure of vasomotion and the onset of vasospasm. Importantly, pharmacological agents such as SKF 96365, which reduce ET-1-induced increases in $[Ca^{2+}]_i$, allowed recovery of cellular Ca^{2+} homeostasis and now vessels behaved physiologically, undergoing vasomotion at [ET-1] that would normally cause vasospasm. We propose that ET-1-induced vasospasm primarily results through overstimulation of Ca^{2+} stores such that they can no longer refill, this abolishing normal function (e.g. vasomotion) and inducing massive Ca^{2+} entry through store-operated Ca^{2+} channels with L-type Ca^{2+} channels exerting a lesser role.

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