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Comparison of the contractile and calcium-increasing properties of platelet-activating factor and endothelin-1 in the rat mesenteric artery and vein

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1 In the present study, the properties of endothelin-1 (ET-1) and platelet-activating factor (PAF) in inducing contraction and increased intracellular-free calcium level in rat mesenteric arteries and veins were studied. Furthermore, measurements of cytosolic ($[Ca]_c$) and nuclear ($[Ca]_n$) Ca^{2+} were performed by confocal microscopy.

2 PAF, at a concentration of 1 μM, and the selective ET_B agonists, IRL-1620 and sarafotoxin S6C (100 nM), induced a marked constriction and increase in $[Ca]_i$ in the mesenteric vein but not in the artery. On the other hand, endothelin-1 (1–100 nM) induced a significant concentration-dependent nifedipine-insensitive increase in tension and $[Ca]_i$ in both arteries and veins.

3 Those responses to endothelin-1 were significantly reduced by the ET_A receptor antagonist, BQ-123 (10^{-6} M), on both types of vessels, whereas the selective ET_B receptor antagonist, BQ-788, inhibited only the venous responses. The mixed ET_A/ET_B receptor antagonist, SB 209670, reduced the ET-1-induced venous responses to the same level of that found in presence of BQ-123 or BQ-788. However, concomitant applications of BQ-123 and BQ-788 reduced the vasoconstriction below to that induced by ET_A or ET_B blockade without further affecting $[Ca]_i$.

4 PAF and the selective ET_B agonists IRL-1620, induced a sustained increase of $[Ca]_c$ and $[Ca]_n$ solely in venous cells and ET-1 in both arterial and venous smooth muscle cells.

5 Thus, PAF increases total intracellular calcium concentration and tension on the smooth muscle cells from venous origin only. Furthermore, ET-1-induced vasoactive as well as $[Ca]_i$ and $[Ca]_n$ increasing effects are mediated by distinct receptors on venous and arterial smooth muscles.
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Keywords: Endothelin; platelet-activating factor; tension; calcium; mesenteric vessels; R-type Ca^{2+} channel

Abbreviations: ET, endothelin; PAF, platelet-activating factor; VSMC, vascular smooth muscle cell

Introduction

Impairment of hydrostatic pressure by vasoactive agents leading to capillary plasma extravasation is believed to be an initiating factor in pro-inflammatory conditions of the vasculature. Qualitatively different responses of pre and post-capillary vascularization to various autacoids and hormones may affect hydrostatic pressure in the capillary and modulate albumin extravasation (Filep *et al*., 1991; 1993; D'Orléans-Juste *et al*., 1996; Plante *et al*., 1996). Among the different mediators, the potent vasoconstrictor, endothelin-1, and the inflammatory mediator, PAF, were both shown, *via* their respective receptors, to cause an increase in vascular permeability (Eibl *et al*., 2000; Hirayama *et al*., 1998; Sirois *et al*., 1992). In contrast, ET-1 has been shown to directly decrease permeability in intact isolated microvessels (Victorino *et al*., 1999). Since both arterial and venous vasculatures contribute in modulating intra-capillary hydrostatic pressure, and due to the difficulty in studying these phenomena *in vivo* and to the conflicting reports in the literature, it is of importance to characterize the vasoactive properties of ET-1 and PAF in pre and post-capillary vasculatures. We have shown that endothelin-1 and PAF induced an endothelium-dependent arterial vasodilation and endothelium-dependent venoconstriction, respectively, in the mesenteric vasculature of the rat (Claing *et al*., 1994; D'Orléans-Juste *et al*., 1993). Interestingly, arterial vasodilation and venoconstriction to PAF were sensitive to the dual voltage-dependent R and L-type calcium channel blocker, isradipine (Bkaily *et al*., 1993), but not to the L-type calcium channel blocker, nifedipine (Claing *et al*., 1994).

It is now believed that cytosolic and nuclear-free Ca^{2+} levels play an essential role in excitation, contraction and secretion coupling of many cell types, including vascular smooth muscle and endothelial cells (Bkaily *et al*., 1997a; 2000b; Jacques *et al*., 2000). Earlier studies from our group have illustrated that PAF increases total intracellular-free calcium ($[Ca]_i$) in human and canine endothelial cells through the activation of the steady-state voltage-dependent sarcolemma R-type calcium channels (Bkaily *et al*., 1993). This increase of $[Ca]_i$ by PAF as well as by ET-1 was mainly nuclear (Bkaily *et al*., 1997a, b). A role for voltage-dependent calcium entry in endothelial cell-dependent

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secretion-coupling mechanisms is a controversial issue, as dihydropiridine-sensitive channels are generally absent in that particular cell type (Bkaily *et al*., 1993).

Given the importance of ET-1 and PAF in the pathogenesis characterized by capillary leakage, we have verified the qualitatively distinct vasoactive effects of both agonists and their capacity to increase $[Ca]_i$, $[Ca]_c$ and $[Ca]_n$ levels in tissues and cells of arterial and venous origins, the present study was performed in two steps. Firstly, we simultaneously monitored changes in tension and $[Ca]_i$ (Benchekroun *et al*., 1995; Claing *et al*., 1994) and, secondly, we visualized these changes using single cell confocal microscopy techniques, where Fluo-3 was used as an indicator of $[Ca]_c$ and $[Ca]_n$ levels (Bkaily *et al*., 1997a).

Our results in blood vessels and single cells allow us to conclude that ET-1 induces an increase in tension, $[Ca]_i$, $[Ca]_c$ and $[Ca]_n$ *via* ET_A receptors in the arterial vasculature, whereas ET_B receptors are solely responsible for the same responses in the mesenteric vein. In addition, PAF, albeit an activator of venous structures, is inactive on the smooth muscle of the arterial mesenteric circuit.

Methods

Tension and cytosolic-free calcium monitoring

Male Wistar rats (275–325 g) (Charles River) were sacrificed by stunning and exsanguination. The mesenteric artery and vein were carefully freed of connective tissues. The mesenteric vein was cut longitudinally, removed from the animal and placed in a physiological saline solution (PSS) gassed with a mixture of 95% O_2 and 5% CO_2 at room temperature. The mesenteric artery of the rat was cannulated with a polyethylene tube (PE-10), placed in the PSS and cut helicoidally. The endothelium of the vessels was gently removed with a humid cotton swab. The PSS solution contained (in mM): NaCl 123, KCl 4.67, $MgCl_2$, 1.2, $CaCl_2$, 2.5, KH_2PO_4 1.2, $NaHCO_3$ 15.5, glucose 11.5 and HEPES 10. The absence of vasodilatory responses to acetylcholine (10^{-7} M) was routinely controlled to ensure the complete removal of the endothelium. One artery and one vein per animal were tested. The loading of the vascular smooth muscle with Fura-2 AM was done using a standard technique (Benchekroun *et al*., 1995). In brief, the vessels were tied at one end with a metallic support and placed in a quartz cuvette containing the loading solution at room

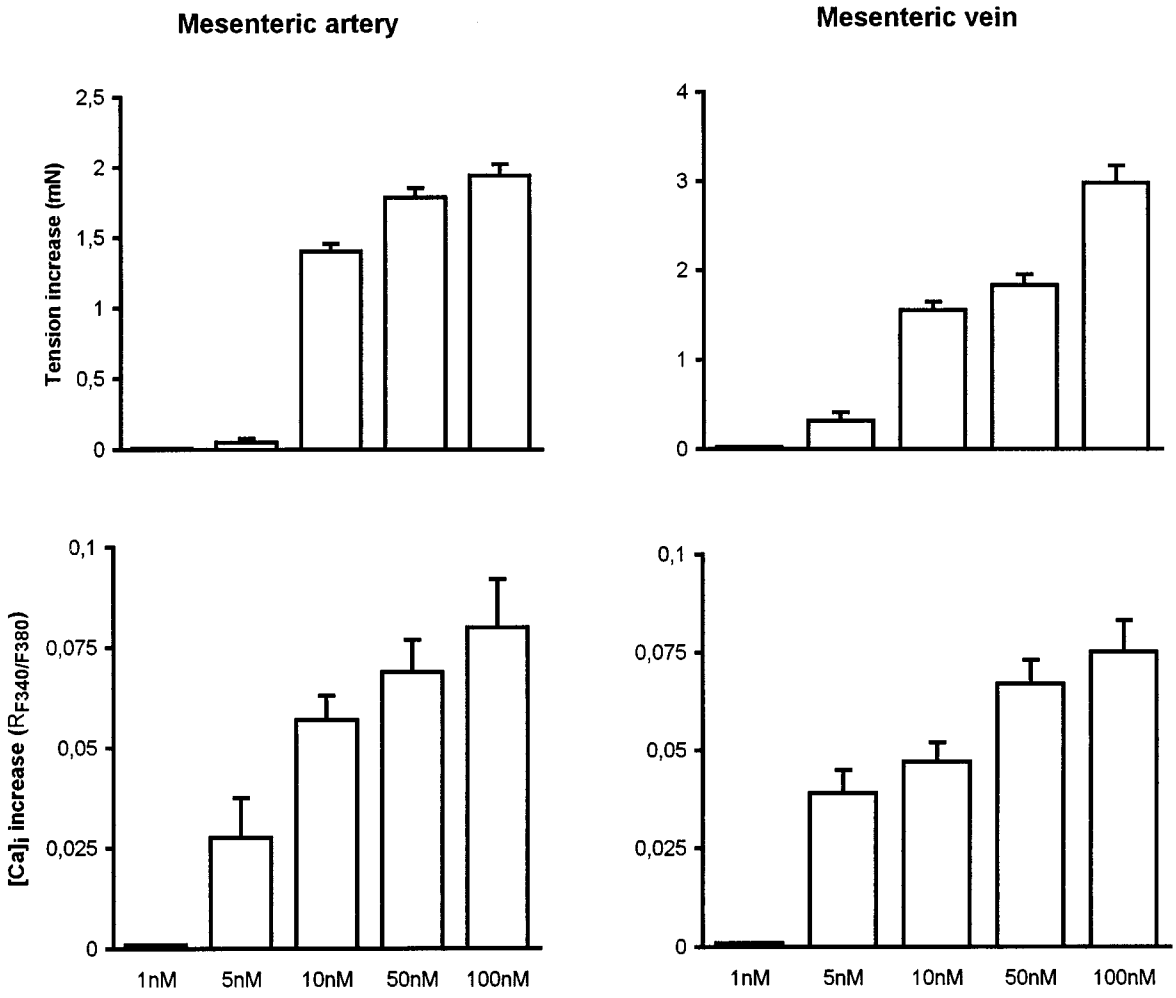

Figure 1 Concentration-dependent effect on tension and $[Ca]_i$ of ET-1 (1–100 nM) on the rat mesenteric artery and vein. Data are the mean ± s.e.mean from 6–15 different tissues.

temperature and oxygenated (12 μM Fura-2/AM in DMEM containing 5% FBS in the presence of 0.1% cremophor EL and 1 mM probenecid). After the loading period, the tissues were rinsed and allowed to equilibrate with normal PSS for 45 min. The initial tensions of the mesenteric artery and vein were 2 g and 1 g, respectively. Experiments were then performed with a double-wavelength excitation fluorometer (Photon Technology International Inc., PTI), where the Fura-2 loaded strips were fixed horizontally in a quartz cuvette and attached to an isometric transducer (Benchekroun *et al*., 1995; Claing *et al*., 1994). Simultaneously, 510 nm fluorescence emitted by 340 nm and 380 nm excitation (F340 and F380, respectively) was measured by successive alternating illumination and the ratio of F340 to F380 was automatically calculated. The responses were expressed as increase in vessel tension (nM) and the variation of ratio (F340/F380) reflected the total cytoplasmic-free calcium level (Benchekroun *et al*., 1995; Himpens & Somlyo, 1998; Sato *et al*., 1988), which is the sum of cytosolic and nuclear calcium (Bkaily *et al*., 1997a; 2000a). The tension of the vessels was measured by an isometric transducer and recorded on a grass physiograph. For the effect of BQ-123, BQ-788 and SB 209670 on the ET-1 response, the strips were preincubated for 20 min with the receptor blockers before the application of the agonist. The total intracellular-free Ca^{2+} ($[Ca]_i$) increase responses to PAF, ET-1, IRL-1620 and sarafotoxin S6C were analysed once both $[Ca]_i$ increase and contractions had reached a steady state. Furthermore, in the experiments where nifedipine was used, the calcium channel blocker was applied 30 min before the administration of ET-1. Finally, sodium nitroprusside was administered on the ET-1 precontracted vessels.

VSM culture

Cells were harvested from enzymatically dissociated rat mesenteric arteries and veins, according to the methods reported by Gunther *et al*. (1982) which were slightly modified in the present study. All procedures were carried out under aseptic conditions. Male Wistar rats were sacrificed by stunning and exsanguination. The superior mesenteric artery and vein were excised and placed in a petri dish containing ice-cold MEM with 0.2 mM added Ca^{2+}. The adherent fat and connective tissue was removed by blunt dissection and the cleaned vessels were transferred into a flask. The vessels were then rinsed and incubated at 37°C for

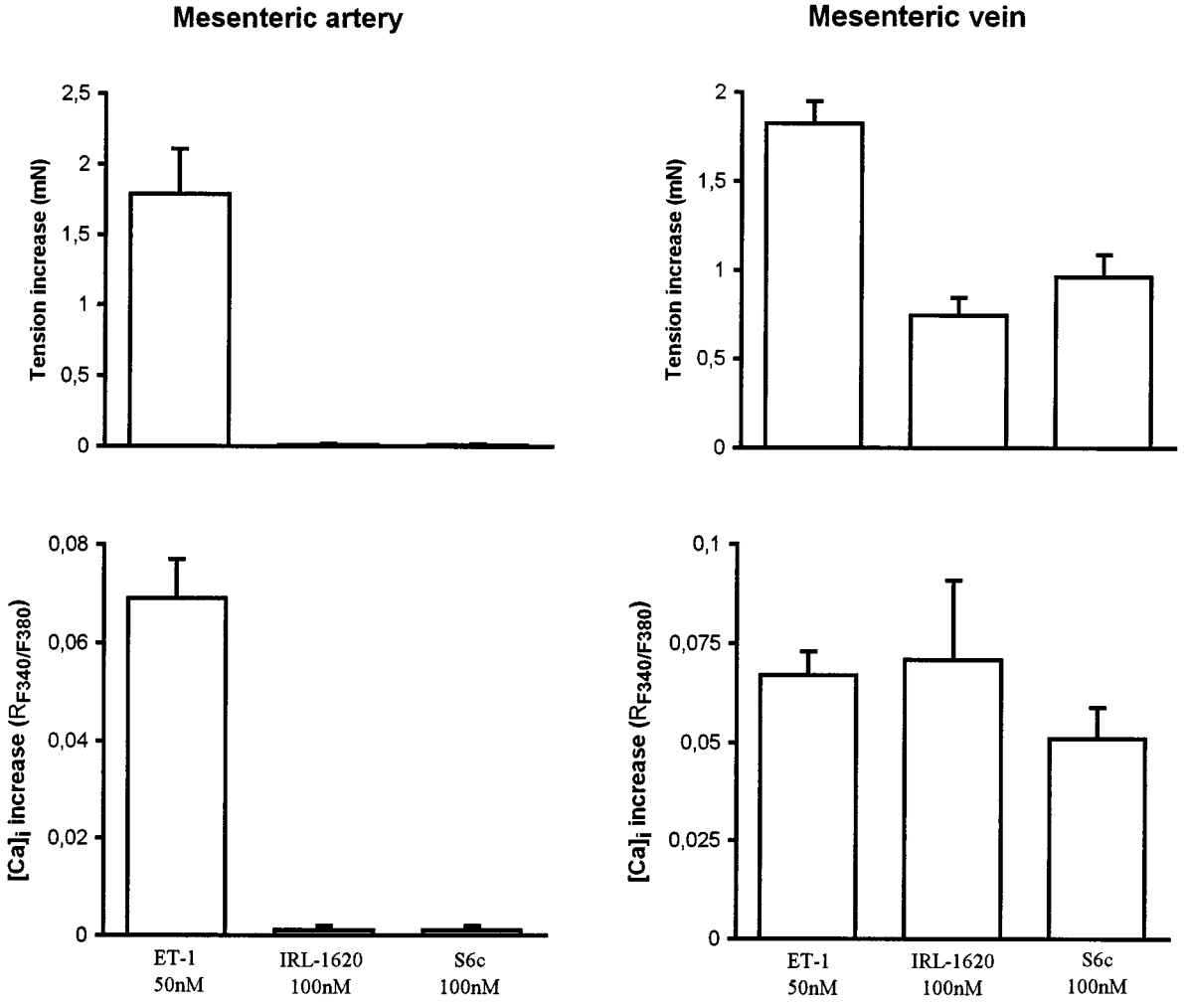

Figure 2 Two selective ET_B agonists, IRL-1620 and sarafotoxin S6C (S6C) (100 nM) induce a vasoconstriction and $[Ca]_i$ of the mesenteric vein but not artery. Data is mean ± s.e.mean of six different tissues.

1 h in a MEM enzyme dissociation mixture containing 0.2 mM added Ca^{2+}, 15 mM HEPES (pH 7.2–7.4), 0.125 mg/ml elastase, 0.375 mg/ml soybean trypsin inhibitor, 2 mg ml^{-1} collagenase and 2 mg ml^{-1} BSA. After incubation at 37°C for 60 min, the suspension was aspirated into a 10 ml plastic syringe and triturated 10 times. The suspension was centrifuged in a siliconized conical glass tube ($200 \times g$, 5 min) and the pellet resuspended in 6 ml of DMEM containing 10% FBS, 2 mM L-glutamine, 25 mM HEPES buffer, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. The dispersed cell suspension was aliquoted on 25 mm spheric lamelles used for confocal microscopy and then incubated at 37°C in a humidified 5% CO_2, 95% air solution. After 5 h, the cultures were washed once with sterile PBS to remove non-adherent cells and debris and fed with fresh medium. The medium was routinely changed at 48 h intervals thereafter. Cells were used after 3–4 days of culture. Isolated VSM cells of mesenteric arteries and veins were marked with an antibody raised against α-actin to confirm their muscular origin.

Cytosolic and nuclear Ca^{2+} imaging with confocal microscopy

For free cytosolic and nuclear Ca^{2+} imaging, VSMCs were loaded with Fluo-3 (13.6 μM) reconstituted in DMSO and diluted to a final concentration of 13.6 μM in Tyrode-BSA solution using a standard technique (Bkaily *et al*., 1997a; 1999). The cells were incubated for 30 min at room temperature, washed and further incubated for 15 min at room temperature to complete hydrolysis for acetoxymethyl ester groups.

Fluo-3 loaded cells were examined with a Molecular Dynamics (Sunnyvale, CA, U.S.A.) Multi Probe 2001 Confocal Argon Laser Scanning System (CSLM) equipped with a Nikon Diaphot epifluorescence inverted microscope and a 60× (1.4 NA) Nikon and Plan achromat objective. The Argon laser line (9.0 mV) was directed to the sample *via* a 510 nm primary dichroic filter and attenuated with a 1–3% neutral density filter to reduce photobleaching. The pinhole size was set at 100 μM. The image size was

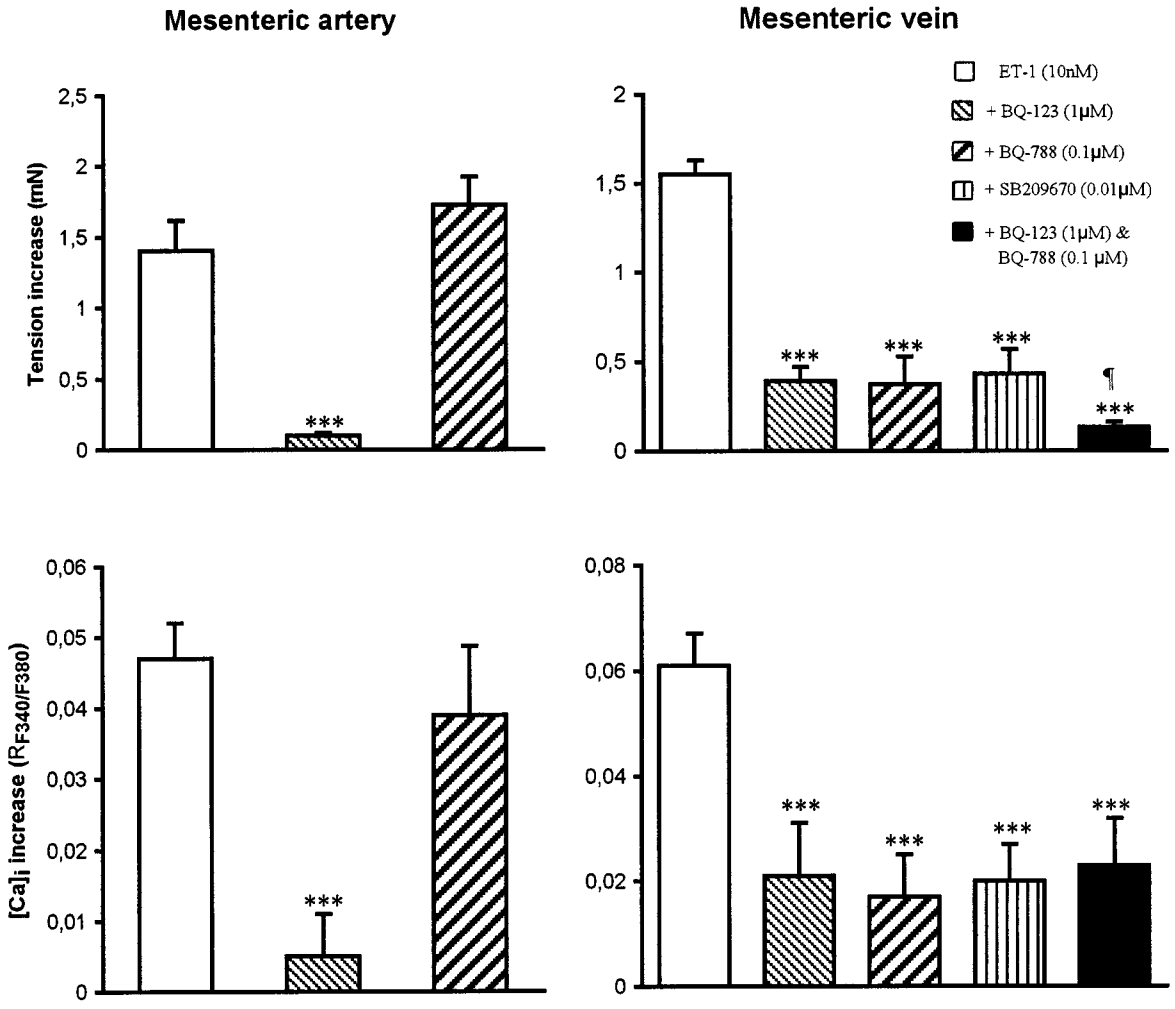

Figure 3 Effect of BQ-123 (1 μM), BQ-788 (0.1 μM), SB209670 (0.1 μM) and BQ-123 (1 μM) + BQ-788 (0.1 μM) on the response to ET-1 (10 nM) in the mesenteric artery and vein. $^{***}P<0.001$ responses to ET-1 in presence of BQ-123, BQ-788 or SB209670 are significantly different from control values (ET-1 without antagonists). The mixture of BQ-123 and BQ-788 induced an antagonism of ET-1 contraction, which is significantly greater than treatment with BQ-123, BQ-788 or SB209670 administered alone ($^{¶}P<0.05$). Data are the mean ± s.e.mean from 6–12 different tissues.

512×512 pixels with a pixel size of 0.11 μM. Laser line intensity, photometric gain, PMT setting and filter attenuation were kept constant throughout the experimental procedures. Serial optical scans were performed 2 min after addition of each agent. A total of 12–15 scans (512×512) were performed for each series with a step size of 0.8–1.0 μM. The number of sections and step size were rigorously maintained during the course of each experiment, in order to localize calcium variations within the boundaries of the nucleus (Bkaily *et al*., 1997a; 1999). Once agent additions completed, 30 mM KCl and 10 mM EGTA were added to the bath medium to ensure that cells were still responsive to external stimuli. Quantification of the intracellular ion concentration (cytosolic and nuclear) using the non-ratiometric dye, Fluo-3, was done using a procedure described previously (Bkaily *et al*., 2000a). In brief, sarcolemna and nuclear envelope membranes were perforated with 0.1% Triton $\times$ 100 solution. The perforated cells were then exposed to different concentrations of Ca^{2+} buffer containing 13.5 μM Fluo acid. The Ca^{2+}-Fluo-3 fluorescence intensity curve was then constructed (Bkaily *et al*., 2000a).

Nuclear staining

At the end of each experiment, the nucleus was stained with 100 nM of live cell nucleic acid stain Syto 11. Serial optical scans were taken immediately after development of the stain (approximately 8–10 min), while maintaining positioning, number of sections and step size identical to those used for calcium uptake. 3-D reconstructions of the nucleus were performed, as described by Bkaily *et al.* (1997a), and used as templates to delineate nuclear from cytosolic-free calcium.

Materials

The fluorescent probe, Fura-2/AM, was purchased from Calbiochem and Fluo-3 from Molecular Probes. Endothelin-1, sarafotoxin 6C, BQ-123 and BQ-788 were purchased from American Peptide Co. IRL-1620 and PAF were obtained from Bachem. All other materials, if not stated otherwise, were purchased from Sigma Chemical Co. Care of animals conform to the guiding principles for animal experimentation, as enunciated by the Canadian Council on Animal Care and

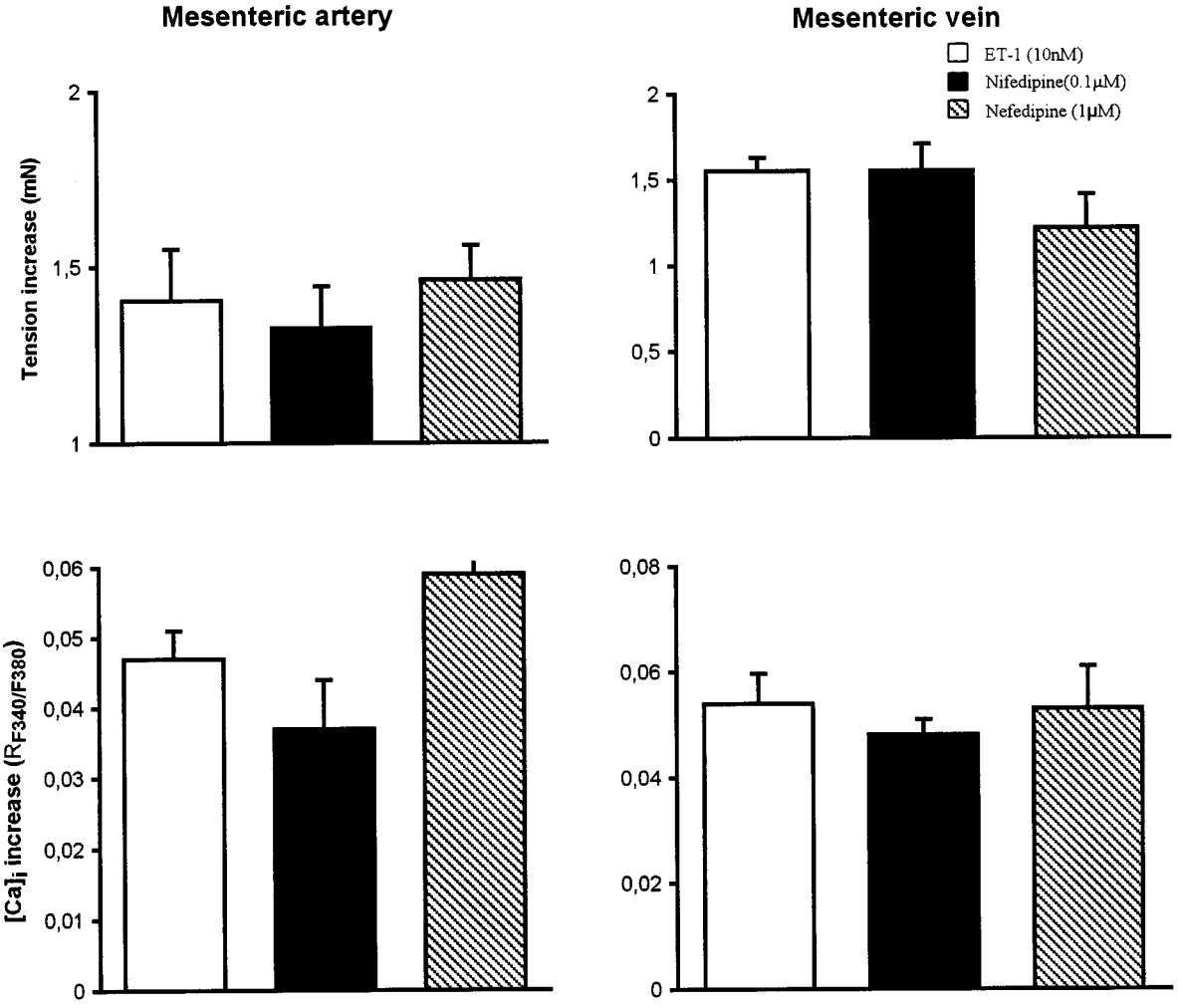

Figure 4 Effect of pre-treatment with nifedipine (0.1 μM and 1 μM) on the response to ET-1 (10 nM) on the mesenteric artery and vein. Data are the mean$\pm$s.e.mean from 6–10 different tissues.

approved by the Ethical Committee on Animal Research of the Medical School of the Université de Sherbrooke.

Statistics

All results are expressed as mean values ± s.e.mean. When applicable, statistical significance was determined using the variance analysis ANOVA for matched or unmatched values, followed by the proper multiple comparison test suggested by the program 'GraphPad Instat'™ (V2.04a) to assess statistical significance of the results. For Figure 5, a student *t*-test for matched values was performed. *P* values of less than 0.05 were considered significant.

Results

Effect of ET-1, selective ET_B agonists or PAF on the change of tension and total free calcium ($[Ca]_i$)

In the first series of experiments, the simultaneous measurement of tension and $[Ca]_i$ was performed in order to determine the effective concentrations of ET-1 and PAF on the mesenteric artery and vein of the rat.

Figure 1 illustrates the concentration-dependent increase in tension and $[Ca]_i$ level induced by ET-1 (1–100 nM) on the mesenteric artery and vein. Increasing the concentrations of ET-1 from 100 nM up to 1 μM did not further increase the tension and $[Ca]_i$ in both preparations (results not shown). Significant increase in both tension and $[Ca]_i$ in the mesenteric artery was seen at 10 nM of ET-1. However, in the mesenteric vein, significant increases in $[Ca]_i$ were detected at a concentration of 5 nM of ET-1. Thus, only concentrations of ET-1 that showed significant increase in both tension and $[Ca]_i$ were used in the series of experiments with ET receptor antagonists as well as those in single cell confocal microscopy. Figure 2 illustrates the contractile and $[Ca]_i$-increasing properties of two selective ET_B agonists, IRL-1620 and sarofotoxin S6C (0.1 μM each), in the mesenteric vein but not in the arterial counterpart. A higher concentration of IRL-1620 or sarafotoxin S6C (1 μM) did not further increase tension or $[Ca]_i$ levels in the mesenteric vein (results not shown).

Similarly, PAF at a concentration of 1 μM was able to induce a significant increase in tension (1.25 ± 0.29 mN) and $[Ca]_i$ (RF340/F380: 0.07 ± 0.013) in the mesenteric vein ($n = 6$

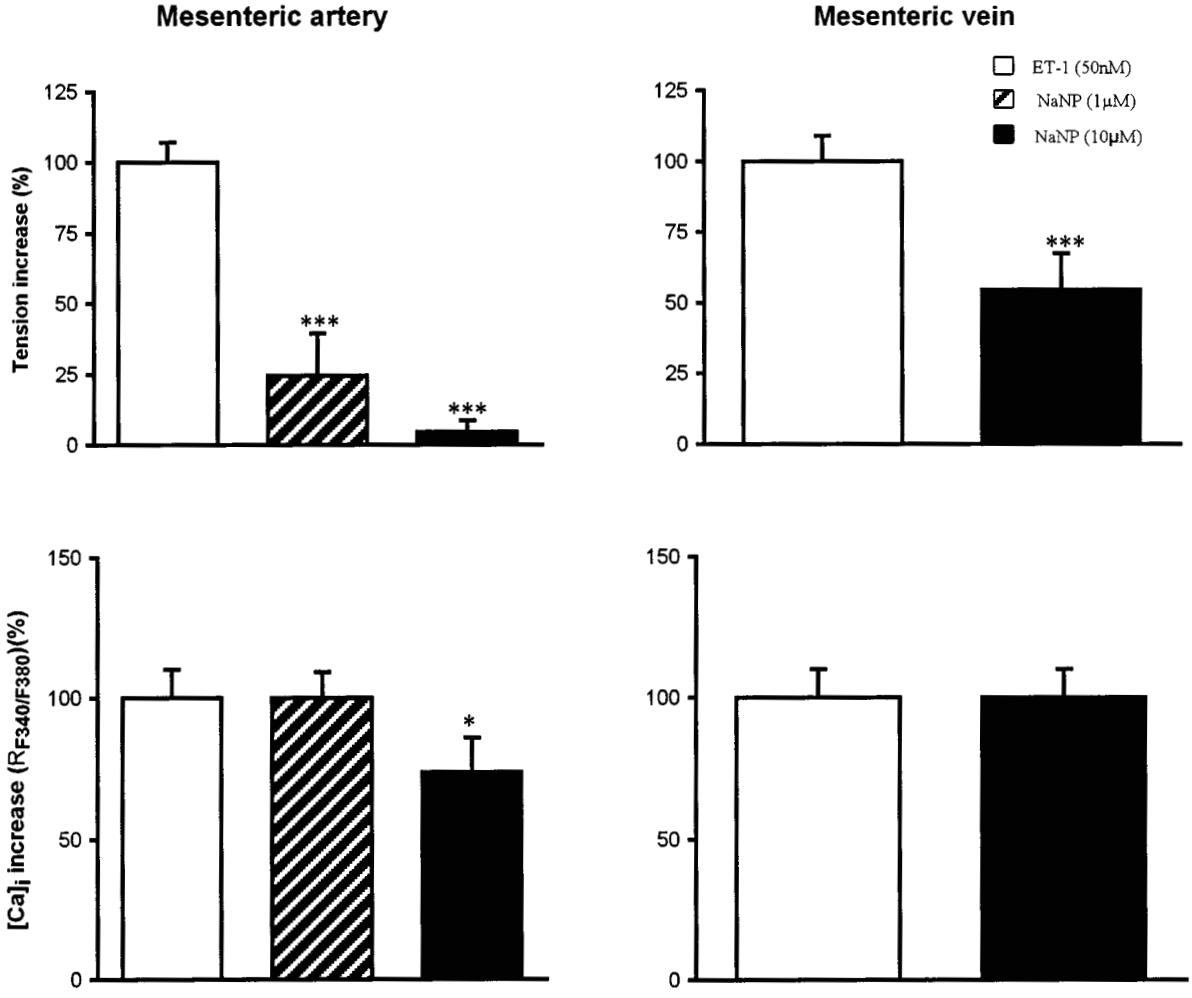

Figure 5 Effect of a curative treatment with sodium nitroprussiate (1 μM and 10 μM) against the response induced by ET-1 (50 nM). Data are the mean ± s.e.mean from 5–8 different tissues. ***$P < 0.001$ and *$P < 0.05$ when compared to control values with ET-1 alone.

different tissue preparations). In contrast, PAF (1 μM) was unable to change either tension or $[Ca]_i$ in the arterial vessels ($n=10$ different tissue preparations; results not shown). Thus, PAF at a concentration of 1 μM was used in the single cell confocal microscopy experiments.

Effect of selective agonists, antagonists and non-selective ET_A/ET_B antagonist on ET-1-induced increase of tension and $[Ca]_i$

In a third series of experiments, the contribution of ET_A and ET_B receptors in the endothelin-1-induced events was identified through the use of selective or non-selective antagonists.

The selective ET_A antagonist, BQ-123 (1 μM), markedly reduced the contraction induced by 10 nM of ET-1 by $93.0\pm5.3\%$ ($n=6$, $^{***}P<0.001$) and the increase of $[Ca]_i$ by $90.4\pm11.2\%$ ($n=6$, $^{***}P<0.001$) on the mesenteric artery. The blockade of the ET_A receptors also reduced the increase in tension induced by 10 nM of ET-1 on the mesenteric vein by $74.0\pm6.1\%$ ($n=8$, $^{***}P<0.001$) (Figure 3).

The selective ET_B receptor antagonist, BQ-788 (0.1 μM), did not alter the response induced by ET-1 (10 nM) on the mesenteric artery. In contrast, BQ-788 reduced by $73.4\pm10.0\%$ the increase of tension and by $72.2\pm7.6\%$ the total intracellular calcium level induced by ET-1 (Figure 3) in the mesenteric vein. The non-selective ET_A/ET_B antagonist, SB 209670 (0.01 μM), did not further reduce the contractile response nor the increase of $[Ca]_i$ induced by ET-1 (10 nM) on the vein (Figure 3), when compared to BQ-123 or BQ-788. However, the simultaneous treatment with BQ-123 (1 μM) and BQ-788 (0.1 μM) of the same preparation further reduced the contractile ($n=5$, $^{***}P<0.001$) but not the $[Ca]_i$ response triggered by ET-1 (Figure 3).

Effect of an L-type calcium channel blocker on ET-1-induced increase of tension and $[Ca]_i$

A 30-min pre-treatment with the specific L-type calcium channel blocker, nifedipine (0.1 – 1 μM), was unable to affect the contractile and $[Ca]_i$ response induced by ET-1 in both arteries and veins ($n=5$) (Figure 4), although it abolished (at 0.1 μM) the contractile response and the increase of $[Ca]_i$ induced by the selective L-type calcium channel opener, Bay K 8644, (5nM) in the artery (control tension: 0.72 ± 0.21 mN; $[Ca]_i$: 0.06 ± 0.01 R; + nifedipine : tension : 0; $[Ca]_i$: 0, $n=6$, $P<0.001$) as well as in the vein (control tension: 1.37 ± 0.09 mN; $[Ca]_i$: 0.1 ± 0.02 R; + nifedipine : tension :0; $[Ca]_i$: 0, $n=12$, $P<0.001$).

Effect of sodium nitroprusside on the vasoconstriction and increase of $[Ca]_i$ induced by ET-1 in the mesenteric artery and vein

In ET-1 precontracted arteries, sodium nitroprusside (1 and 10 μM) induced a concentration-dependent relaxation of the

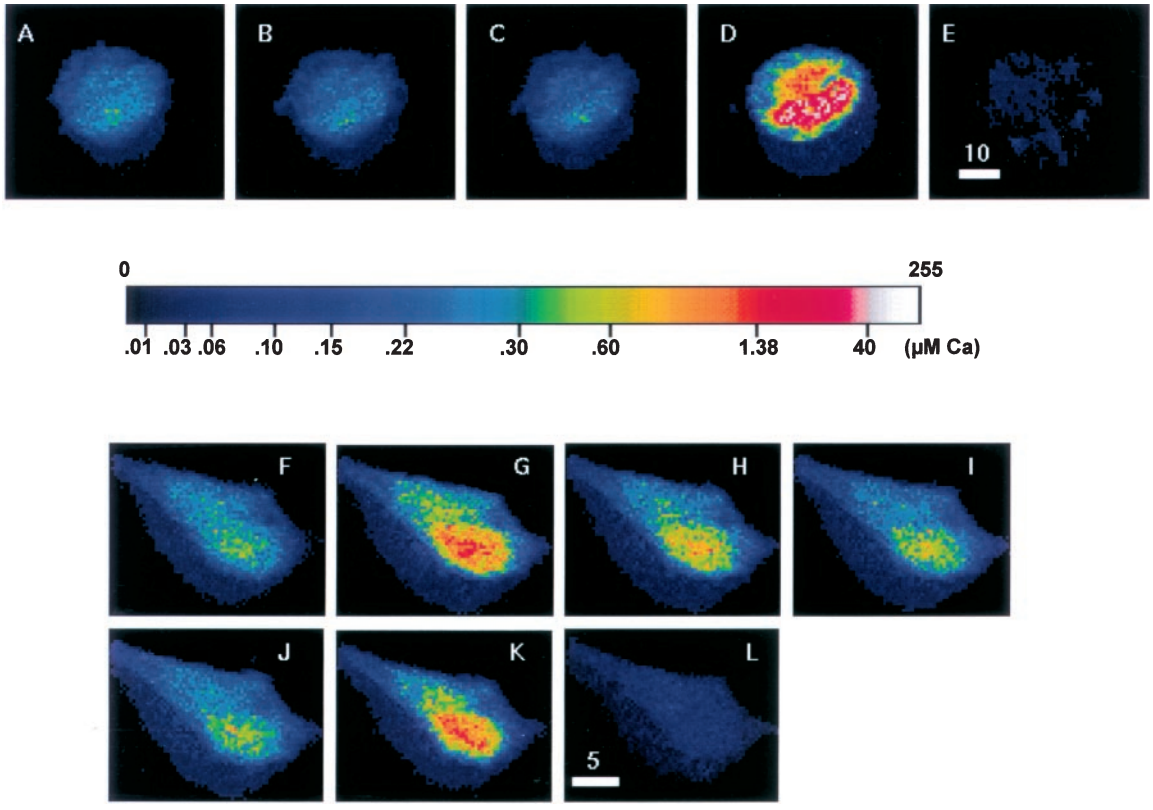

Figure 6 Cross-sectional view of a 3-D reconstructed isolated vascular smooth muscle cell from arterial (A – E) and venous (F – L) origin. This figure illustrates the intracellular calcium distribution of VSM cells isolated from arteries in response to PAF (1 μM; B,C,G,H,I and J) (A: control; B: 2 min; C: 10 min; D: KCl, 30 s and E: EGTA, 5 min) and VSM cells isolated from veins (F: control; G: 2 min; H: 5 min; I: 10 min; J: 15 min; K: KCl, 30 s and L: EGTA, 5 min). The white scale bar represents length in μm. The colour scale represents pseudocolor intensity levels of the complex Fluo-3 calcium fluorescence from 0 – 255 and [Ca] concentrations from 0.01 to 40 μM. This figure is representative of three other experiments.

vessel ($n=5-8$, $^{***}P<0.001$) and a reduction of $[Ca]_i$ at the highest concentration only. On the mesenteric vein, sodium nitroprusside reduced the contraction induced by ET-1 without affecting the increase of $[Ca]_i$ (Figure 5).

Effect of ET-1, IRL-1620 and PAF on $[Ca]_c$ and $[Ca]_n$

The following series of experiments were performed on single vascular smooth muscle cells in which changes in $[Ca]_c$ and $[Ca]_n$ induced by PAF, ET-1 or IRL-1620 were monitored through confocal microscopy. Figure 6 shows the cytosolic and nuclear-free calcium increase by PAF (1 μM) on VSM cells isolated from mesenteric arteries and veins. Similarly, ET-1 (0.1 μM) induced an increase in cytosolic and nuclear levels in both VSM cells from arteries and veins (Figure 7). The selective ET_B receptor agonist, IRL-1620 (0.1 μM), increased $[Ca]_c$ and $[Ca]_n$ in the VSM cells from the vein without any effect on the arterial cells (Figure 8). Furthermore, in all these cells, sustained depolarization of the cell membrane with continued superfusion with 30 mM $[K]_o$ induced sustained increase of $[Ca]_c$ and $[Ca]_n$; extracellular application of the Ca^{2+} chelator, EGTA (10 mM), decreased both levels to near the control values. Finally, staining with Syto 11 illustrates that a large portion of $[Ca]_i$ increases occur within the nucleus of venous smooth muscle cells, as previously demonstrated in arterial counterparts (Bkaily *et al*., 1999).

Discussion

The data presented here shows that endothelin-1 induces an increase in cytosolic calcium and a concomitant increase in tension. These results are in accordance with data reported in porcine coronary arteries (Yasutsune *et al*., 1999). Since the Ca^{2+} is the same for both cytosolic and nuclear-free Ca^{2+}, the increase of $[Ca]_i$ induced by 5 nM of ET-1 (in absence of changes in tension) in the mesenteric artery may substantially reflect the increase of nuclear Ca^{2+} due to the cytosolic Ca^{2+} buffering capacity of the nuclei (Bkaily *et al*., 1997a). On the other hand, our results suggest that the ET-1-induced constrictive effects are mediated through ET_A receptors on the arterial smooth muscle and through both ET_A and ET_B receptors on the venous smooth muscle. This observation supports previous studies from our laboratory (D'Orléans-Juste *et al*., 1993), illustrating the role of both receptor types in the constrictive properties of endothelins in both arterial and venous mesenteric beds of the rat. The ET_A-mediated vasoconstriction by ET-1 in the rat mesenteric arteries was also shown by Rizzoni *et al*. (1997). We have previously reported the predominant endothelium-dependant pre-capillary vasodilation and post-capillary constriction in the mesenteric circuit in response to endothelin-1 (D'Orléans-Juste *et al*., 1993). Nonetheless, other reports (Mickley *et al*., 1997; Touyz *et al*., 1995) suggested the presence of contractile ET_B receptors on rat mesenteric arteries.

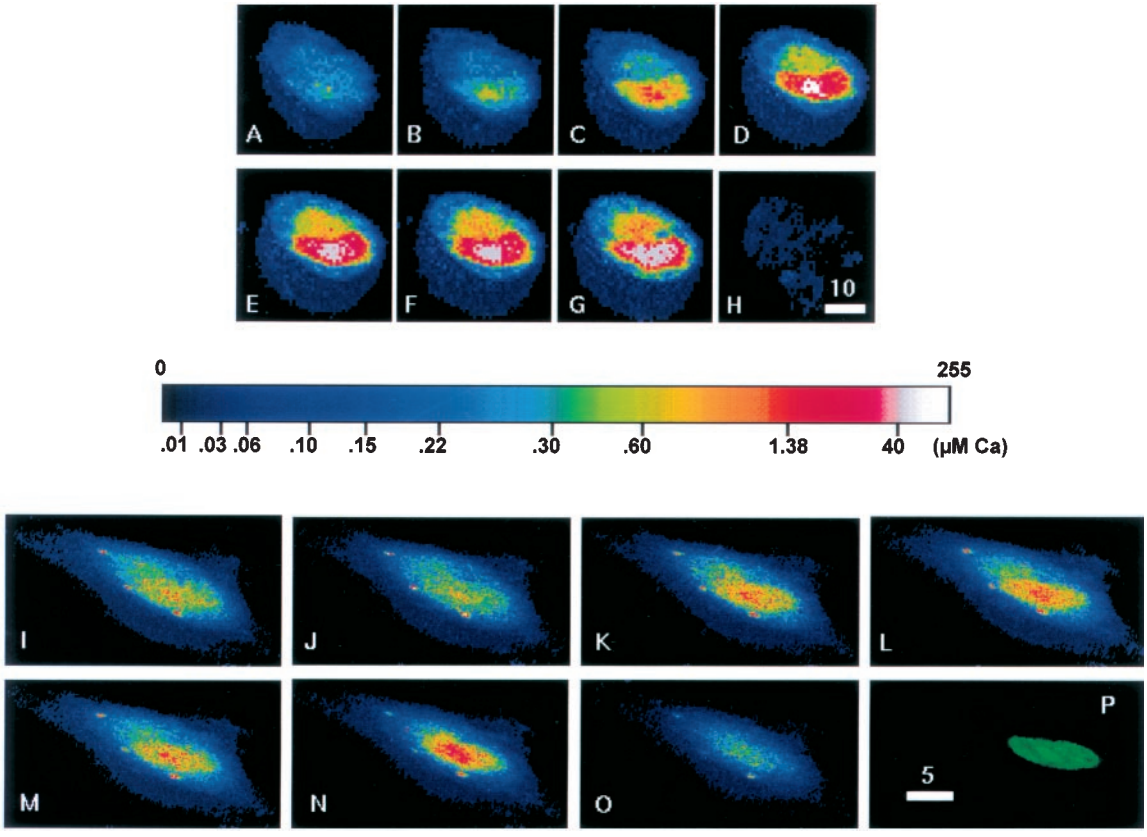

Figure 7 Cross-sectional view of a 3-D reconstructed isolated vascular smooth muscle cell from arterial (A to H) and venous (I to P) origin. This figure illustrates the intracellular calcium distribution of VSM cells isolated from arteries in response to ET-1 (0.1 μM) (B,C,D,E,F,J,K,L and M) (A: control; B: 2 min; C: 5 min; D: 10 min; E: 15 min; F: 18 min; G: KCl, 30 s and H: EGTA, 5 min) and VSM cells isolated from veins (I: control; J: 5 min; K: 15 min; L: 20 min; M: 25 min; N: KCl, 30 s; O: EGTA, 2 min and P: Syto-11). The white scale bar represents length in μm. The colour scale represents pseudocolor intensity levels of Fluo-3 dye from 0–255 and [Ca] concentrations from 0.01 to 40 μM. This picture is representative of three other experiments.

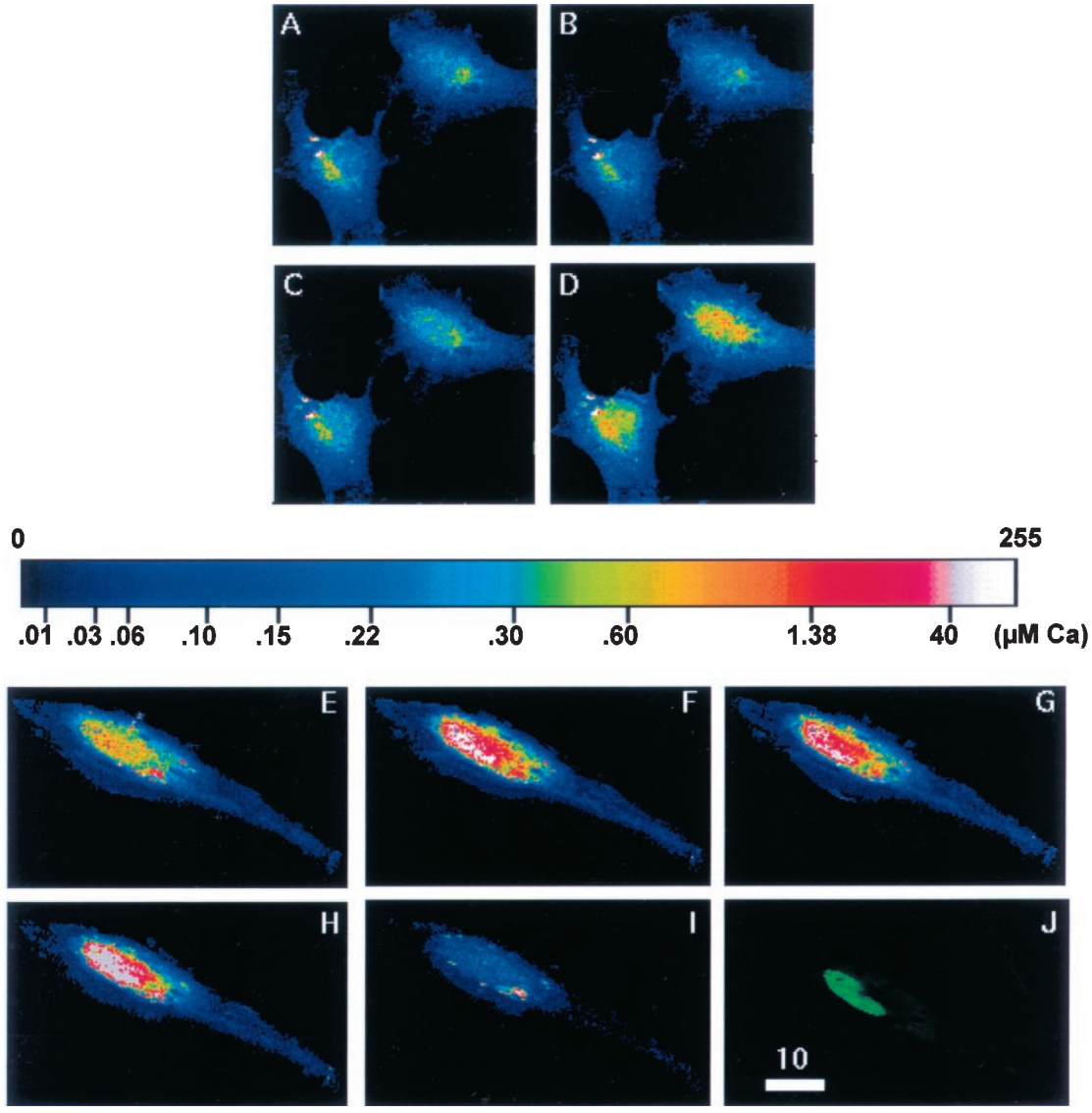

Figure 8 Cross-sectional view of a 3-D reconstructed isolated vascular smooth muscle cell from arterial (A to D) and venous origin (E to J). This figure illustrates the intracellular calcium distribution of VSM cells isolated from arteries in response to IRL-1620 (1 μM; B,C,F and G) (A: control; B: 5 min; C: 10 min) or ET-1 (0.1 μM) (D: 10 min) and VSM cells isolated from veins (E: control; F: 5 min; G: 10 min; H: KCl, 30 s, I: EGTA, 5 min; J: Syto: 11). The white scalebar represents length in μm. The colour scale represents pseudocolor, intensity levels of Fluo-3 from 0–255 and Ca concentrations from 0.01 to 40 μM. This figure is representative of three other experiments.

Recently, it was shown that in freshly isolated rat mesenterial segments, only ET_A receptors were characterized, while ET_B receptors only appeared following organ culture (Adner *et al*., 1998). These results would suggest that ET_B receptors, when present, only play a minor role, if any (Rizzoni *et al*., 1997). In the present study, we were unable to detect ET_B-dependent contractions in the rat mesenteric arterial strips or any IRL-1620-induced $[Ca]_i$ increase in muscle strips or cultured VSM cells of the same tissular origin. The conflicting observations reported among the different groups, including ours, could be attributed to differences in the size of the mesenteric arteries studied (1st *versus* 2nd or 3rd generation) and to the method of tissue preparation for contraction studies (rings *versus* strips).

The venous mesenteric vasculature of the rat predominantly responds to endothelin-1 *via* the activation of ET_A receptors. However, a small population of ET_B receptors was also illustrated, as demonstrated by the venoconstrictive effects of a selective ET_B agonist, IRL-1620 (D'Orléans-Juste *et al*., 1993). The same ET_B-dependent phenomenon seen in the intact mesenteric vasculature occurs at the level of the main venous, but not arterial, mesenteric vessels, as illustrated in the present study with IRL-1620 or sarafotoxin S6C. Both receptor types (ET_A on the arterial, ET_A and ET_B receptors on the venous side) contribute to the increased $[Ca]_i$ level and tension. The fact that the ET_A/ET_B receptor antagonist SB209670 decreased ET-1-induced increase of tension and $[Ca]_i$ to the same extent as BQ-123 or BQ-788, could be due to the fact that the former antagonist possesses a higher affinity for ET_A (pA_2 for 9.22) than ET_B receptors (pA_2 for 8.09) (Maurice *et al*., 1997). It is possible that the further decrease of tension and absence of effect on $[Ca]_i$ simultaneous blockade of ET_A and ET_B receptors by BQ-123

and BQ-788 could be due to the higher affinity of the mixture for ET_B receptors (pA_2 of BQ-788 9.01) than ET_A receptors (pA_2 of BQ-123 6.41) (Maurice *et al*., 1997). Finally, the superior effect of the mixture of BQ-123 and BQ-788 may not be due to a lack of selectivity of BQ-788, since that particular antagonist did not affect at all the contraction of ET-1 on the mesenteric artery in the present study.

In another series of experiments, we have shown that the mesenteric vein but not the artery responds to PAF by an increase in $[Ca]_i$ and a contraction. These results are in accordance with those reported by Claing *et al*. (1994), who have shown in the double-perfused arterial and venous vasculature of the rat that PAF induces an endothelium-dependent vasodilation solely in the pre-capillary and a constriction of the post-capillary circuits.

In order to avoid the contaminating contribution of elastin, collagen, endothelium and perivascular nerves, we have analysed single smooth muscle cells of arterial and venous origin challenged with endothelin-1 and the selective ET_B receptor agonist, IRL-1620. In absence of any drug, our results showed that in rat VSM cells, nuclear-free Ca^{2+} was higher than that of the cytosol. This finding is similar to those reported in the rabbit and human heart, VSM and endothelial cells (Bkaily *et al*., 1997a). In our experiments, endothelin-1 was found to markedly increase intracellular calcium, which was largely nuclear in both the arterial and venous single cells; IRL-1620 was found active only in smooth muscle cells of venous origin. These results are in accordance with those recently reported for ET-1 and PAF in human and rabbit aortic VSM cells (Bkaily *et al*., 1997a; 2000a).

Based on the present observations, we suggest that the potent vasoactive agents, PAF and endothelin-1, trigger tissue-specific cytosolic and nuclear calcium increase and myocontractions. The effect of these factors on single cells mimicks the response found in arterial and venous strips, as well as the intact mesenteric circuits (Claing *et al*., 1994; D'Orléans-Juste *et al*., 1993). It is also worthy of notice that both mesenteric arteries and veins possess functional L-type calcium channels, as illustrated by the nifedipine-sensitive contraction induced by Bay K-8644 in these tissues. However, this type of channel does not seem to be involved in the ET-1 and PAF-mediated actions.

Despite the observations that cytosolic calcium increase seems to be essential for the initiation of contraction, we report a reduction in vascular tone with sodium nitroprusside that was not correlated with a reduction in cytosolic calcium. These observations may be explained by the fact that although cytosolic calcium is important for the coupling of troponin and myosin elements *via* the activity of the myosin-light chain kinase (MLCK), vasodilation may occur at a step beyond the coupling of a cytosolic calcium-calmodulin entity to the MLCK, as recently shown in rabbit aortas (Bkaily *et al*., 2000b).

Finally, the contribution of vascular endothelium in the vasoactive effects of both PAF and endothelin was not studied. We have previously reported that the endothelium acts as a humoral barrier in the rat perfused mesenteric arterial and venous circuits (Claing *et al*., 1994) and can modulate vascular responses to vasoactive agents.

In summary, our study demonstrates that PAF, although inactive in the endothelium-denuded arterial blood vessels, is able to trigger an increase in tension and both cytosolic and nuclear calcium entry in the venous mesenteric vasculature. Our data suggests that this calcium entry is mediated by a nifedipine-insensitive calcium channel. The use of endothelin-1 and the selective ET_B receptor agonists, IRL-1620 and sarafotoxin S6C, demonstrates the functional presence of only ET_A receptors in the arterial vessels and of both ET_A and ET_B receptors in the venous smooth muscles which are responsible for increase in both tension and cytosolic calcium entry through a nifedipine-insensitive Ca^{2+} channel. Finally, it is of interest that PAF (venous VSM cells only) and ET-1 trigger a particularly marked increase in both $[Ca]_c$ and $[Ca]_n$. In fact, there is an increasing body of evidence supporting the contribution of nuclear and intracellular calcium in maintaining cellular calcium homeostasis. Furthermore, receptors such as *Ang*II and ET-1 have been shown to be present and functional within the nuclear envelope membranes (Bkaily *et al*., 1997a,b; 2000a; Jacques *et al*., 2000). Thus, it is possible that both PAF and ET-1 effects observed in our experiments at the tissular and cellular levels could also be due in part to ligand stimulation of nuclear membrane receptors (*via* receptor-ligand complex internalization) (Bkaily *et al*., 2000a). Further experiments are required in order to pursue that particular hypothesis.

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