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# Agonist-evoked calcium entry in vascular smooth muscle cells requires $IP_3$ receptor-mediated activation of TRPC1

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**Abstract**

Transient receptor potential canonical (TRPC) proteins have been proposed to function as plasma membrane $Ca^{2+}$ channels activated by store depletion and/or by receptor stimulation. However, their role in the increase in cytosolic $Ca^{2+}$ activated by contractile agonists in vascular smooth muscle is not yet elucidated. The present study was designed to investigate the functional and molecular properties of the $Ca^{2+}$ entry pathway activated by endothelin-1 in primary cultured aortic smooth muscle cells. Measurement of the $Ca^{2+}$ signal in fura-2-loaded cells allowed to characterize endothelin-1-evoked $Ca^{2+}$ entry, which was resistant to dihydropyridine, and was blocked by 2-aminoethoxydiphenylborate (2-APB) and micromolar concentration of $Gd^{3+}$. It was not activated by store depletion, but was inhibited by the endothelin $ET_A$ receptor antagonist BQ-123, and by heparin. On the opposite, thapsigargin-induced store depletion activated a $Ca^{2+}$ entry pathway that was not affected by 2-APB, BQ-123 or heparin, and was less sensitive to $Gd^{3+}$ than was endothelin-1-evoked $Ca^{2+}$ entry. Investigation of the gene expression of TRPC isoforms by real-time RT-PCR revealed that TRPC1 was the most abundant. In cells transfected with TRPC1 small interfering RNA sequence, TRPC1 mRNA and protein expression were decreased by 72±3% and 86±2%, respectively, while TRPC6 expression was unaffected. In TRPC1 knockdown cells, both endothelin-1-evoked $Ca^{2+}$ entry and store-operated $Ca^{2+}$ entry evoked by thapsigargin were blunted. These results indicate that in aortic smooth muscle cells, TRPC1 is not only involved in $Ca^{2+}$ entry activated by store depletion but also in receptor-operated $Ca^{2+}$ entry, which requires inositol (1,4,5) triphosphate receptor activation.


*Keywords:* TRPC [Transient receptor potential canonical]; Endothelin-1; Calcium; Vascular smooth muscle cell

## 1. Introduction

Intracellular calcium is considered as an important messenger in the control of vascular smooth muscle contractile tone. Activator $Ca^{2+}$ can be released from intracellular stores or can enter the cell from the extracellular space through several types of ion channels. In vascular smooth muscle cell, two main classes of plasmalemmal $Ca^{2+}$ channels have been identified based on the voltage sensitivity of the gating process. Voltage-operated $Ca^{2+}$ channels (VOC) are highly selective for $Ca^{2+}$. They have been widely studied, and their molecular structures have been identified (Catterall, 2000). The prominent role played by a second group of $Ca^{2+}$ channels that are not activated by membrane depolarisation, has been recognised more recently. Most of them have non-selective cation conductance. They are resistant to dihydropyridines, the well-known inhibitors of L-type VOC, and no reliable selective antagonist is available (Albert and Large, 2006; Beech et al., 2004; Inoue et al., 2003).

The mechanisms of activation and molecular composition of non-selective cation channels have not yet been conclusively identified. In most cells, depletion of intracellular $Ca^{2+}$ stores is pivotal for $Ca^{2+}$ entry commonly termed capacitive $Ca^{2+}$ entry (Berridge, 1995; Putney et al., 2001). Thapsigargin and cyclopiazonic acid are often used to study this pathway because they selectively inhibit the active uptake of $Ca^{2+}$ into the sarco-endoplasmic reticulum via $Ca^{2+}$-ATPase pump, allowing the stores to be passively depleted, without receptor activation. The

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capacitative pathway is not however, the only pathway through which $Ca^{2+}$ can enter cells in response to receptor activation. Non-selective cation channels are also activated in response to a signal generated by a range of intracellular messengers released after the binding of an agonist to its receptor including diacylglycerol (Albert and Large, 2006; Helliwell and Large, 1997), arachidonic acid (Munaron et al., 1997; Shuttleworth et al., 2004), inositol (1,4,5) triphosphate ($IP_3$) (Dong et al., 1995). The later channels have been called "receptor-operated channels or ROC", in contrast to "store-operated channels or SOC". However, whether this classification reflects distinct molecular entities is not yet determined.

The canonical transient receptor potential TRPC proteins have been proposed to form non-selective cation channels and considerable research has been carried out on the seven members of this family. RT-PCR analysis has revealed the expression of TRPC1, 4, 5, and 6 mRNA in vascular smooth muscle, including cerebral arteries (Bergdahl et al., 2005), aorta (Xu and Beech, 2001), mesenteric resistance artery (Hill et al., 2006), and pulmonary artery (McDaniel et al., 2001; Sweeney et al., 2002; Wang et al., 2004; Yu et al., 2003).

Several reports have proposed that TRPC1 forms SOC in vascular smooth muscle cell, as in several other cell types (Beech, 2005). TRPC1 specific antibody inhibits store-operated $Ca^{2+}$ influx in human, mouse and rabbit arterial smooth muscle cells (Xu and Beech, 2001). TRPC1 antisense oligonucleotides inhibit SOC in pulmonary artery cell line (Sweeney et al., 2002), while in rat cerebral artery in organ culture, increase in TRPC1 expression is associated with up-regulated SOC activity (Bergdahl et al., 2005). Similarly, overexpression of TRPC1 in pulmonary artery increases capacitative $Ca^{2+}$ entry (Kunichika et al., 2004). In pulmonary artery smooth muscle cell as in the stable A7r5 cell line, (small interfering) siRNA-mediated knockdown of TRPC1 selectively inhibits thapsigargin-activated $Ca^{2+}$ entry (Brueggemann et al., 2006; Lin et al., 2004).

On the other hand, the TRPC3/6/7 subfamily is generally proposed to form ROC. TRPC6 is activated by angiotensin II in rabbit mesenteric artery (Saleh et al., 2006), and by α1-adrenergic agonist in rabbit portal vein (Inoue et al., 2001). In A7r5 cell line, $Ca^{2+}$ current activated by Arg-vasopressin shares kinetic and pharmacological properties with heterologously expressed TRPC6 channel protein (Jung et al., 2002), and is suppressed by dominant-negative mutant TRPC6 (Maruyama et al., 2006). In rabbit ear artery, noradrenaline activates TRPC3 channels. TRPC3 is also activated by UTP in cerebral arteries (Reading et al., 2005) or, in association with TRPC7, by endothelin-1 in rabbit coronary artery (Peppiatt-Wildman et al., 2007).

The mechanism(s) of activation of $Ca^{2+}$ entry induced by store depletion or receptor activation in vascular smooth muscle cells and their relative contribution to $Ca^{2+}$ signalling are still not defined. One difficulty probably arises from the differences between vascular beds, that could be related to the expression of different TRPC isoforms. Moreover, many studies reported in literature were performed in overexpression systems, and their conclusions cannot be translated to native cells since the composition of the channels and by consequence their properties, depend on the relative expression of the TRPC proteins (Putney, 2004; Vazquez et al., 2003).

Discrepancy in the responses to vasopressin in A7r5 cells is a good example of the conflicting results that are found in literature: vasopressin has been reported to activate non-capacitative $Ca^{2+}$ entry (Broad et al., 1999; Jung et al., 2002), to activate capacitative $Ca^{2+}$ entry (Brueggemann et al., 2005; Brueggemann et al., 2006) or to reciprocally activate capacitative and non-capacitative $Ca^{2+}$ entry (Moneer et al., 2005; Moneer and Taylor, 2002). These puzzling results are suggested to be related to different expression in TRPC subtypes in variant cell lines: non-capacitative entry is observed in cells expressing TRPC6 (Jung et al., 2002), while TRPC1 is involved in capacitative $Ca^{2+}$ entry (Brueggemann et al., 2006; Moneer et al., 2005).

The present study was designed to characterize the $Ca^{2+}$ entry pathway activated by contractile agonist in rat aortic SMC and to investigate the role of TRPC1 in the response to agonist. The potent vasoconstrictor endothelin-1 was used to stimulate aortic cells. Endothelin-1 binds to endothelin $ET_A$ receptor on vascular smooth muscle cells (VSMC) (Oonuma et al., 2000) and induces an increase in intracellular $Ca^{2+}$ responsible for the development of contraction through the activation of non-selective cation channels (Miwa et al., 2005). Receptor-activated $Ca^{2+}$ entry evoked by endothelin-1 was compared to $Ca^{2+}$ entry activated by store depletion evoked by treatment of the cells with thapsigargin. Results indicated that, in aortic smooth muscle cells, endothelin-1 activates a $Ca^{2+}$ entry pathway that is distinct from SOC and requires $IP_3$ receptor activation. Inhibition of TRPC1 expression in cells transfected with TRPC1 small interfering RNA sequence abolished endothelin-1 activated $Ca^{2+}$ entry, suggesting that TRPC1 is an indispensable element of ROC in vascular smooth muscle.

## 2. Materials and methods

All procedures followed were in accordance with institutional guidelines.

### 2.1. Materials

Fura-2-AM, thapsigargin, endothelin-1, endothelin $ET_A$ receptor antagonist BQ-123, 2-aminoethoxydiphenylborate (2-APB), 1,6-bis(cyclohexyloximinocarbonylamino) hexane (RHC-80267), 3-[(4-octadecyl)benzoyl] acrylic acid (OBAA) were purchased from Calbiochem. Fura-2 acid was from Molecular Probes. All other reagents were purchased from Sigma. Commercially available antibodies were rabbit anti-TRPC1 antibody (Alomone, Jerusalem, Israel), mouse anti-α-smooth muscle actin monoclonal antibody, mouse anti-von Willebrand factor (vWF) and monoclonal antibody mouse anti-rabbit FITC (fluorescein isothiocyanate) secondary antibody were purchased from Dako A/S (Denmark).

### 2.2. Cell culture

Aorta was isolated from male Wistar rat weighing 150–200 g. Aorta was cleaned from adherent tissue and the endothelium was

mechanically removed by gently rubbing the lumen of the artery with forceps' tips. Pieces of aorta (2 $mm^2$) were cultured on 15-mm round glass coverslips coated with poly-L-lysine (10 μg/ml) in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM glutamine in an incubator at 37 °C with humidified 5% $CO_2$. Cells migrated from the pieces of aorta after 2–3 days. Aortic pieces were then removed and cells were left to proliferate for 5 days. They were used at 80% of confluence. Aortic smooth muscle cell primary culture will be further denominated VSMC.

### 2.3. siRNA

The siRNA sequence (www.idtdna.com) was as follows: silencing TRPC1-siRNA 5′-CTGGCCAGTCCAGCTC-TAATA-3′. Non-silencing control siRNA was used (Qiagen, Antwerpen, Belgium). The sense strand of each siRNA was labelled at the 3′ end with Alexa Fluor 647, to monitor transfection efficiency. VSMC, at 80% confluency, were transfected with siRNA at a final concentration of 5 μg per well (10 $cm^2$) using lipofectamine (Invitrogen). Control cells were treated with lipofectamine alone under the same condition.

After 48 h, transfection efficiency was assessed by comparison with non-transfected cells using fluorescence microscopy (Olympus IX70 microscope).

### 2.4. $Ca^{2+}$ imaging

VSMC were washed twice with physiological buffered solution (PBS) containing (in mM): NaCl, 137; KCl, 6; $CaCl_2$, 2; $MgCl_2$ 1.2; glucose, 10; HEPES, 10; pH 7.4 with Tris, and loaded for 1 h with fura-2-AM (2.5 μM). The coverslips were mounted in a chamber that was placed on the stage of an Olympus IX70 inverted microscope coupled to a CCD camera. Excitation light (340 and 380 nm) was obtained from a Xenon lamp coupled to a monochromator (TILL Photonics). The 510 nm emission was captured by the CCD camera. The images were transmitted to a computer and processed with the TILLvisION software.

10 to 30 cells were selected for analysis. When siRNA transfected cells were used, they were selected on the basis of siRNA conjugated Alexa Fluor 647 fluorescence. Cells were gently perfused at 37 °C with PBS at a slow speed (2.5 ml/min). Endothelin-1 (10 nM) was used to stimulate the cells. Cells were first superfused with $Ca^{2+}$-free solution (composition as above, without $Ca^{2+}$ and supplemented with 0.1 mM EGTA) for 5 min, and then challenged with the agonist in the $Ca^{2+}$-free solution. $Ca^{2+}$ was thereafter added into the perfusion solution in the continuous presence of endothelin-1.

The endoplasmic reticulum $Ca^{2+}$-ATPase inhibitor, thapsigargin, was used to deplete intracellular stores. Thapsigargin (1 μM) was applied for 5 min in the $Ca^{2+}$-free perfusion solution. Store-operated $Ca^{2+}$ entry was then assessed by adding $Ca^{2+}$ (2 mM) into the perfusion solution. Depletion of intracellular stores was confirmed by the absence of response to endothelin-1 in $Ca^{2+}$-free solution in thapsigargin-treated cells.

The background autofluorescence values at 340 and 380 nm were measured at the end of the experiment in the presence of 5 mM $MnCl_2$, and subtracted from all 340 and 380 fluorescence values. These corrected fluorescence values were used to calculate the F340/F380 ratio. All values were normalised to the F340/380 ratio measured at the beginning of the experiment.

For $Mn^{2+}$ quenching analysis, fluorescence was measured with excitation light at 360 nm (F360) in fura-2 loaded cells perfused with $Mn^{2+}$-quenching solution containing (in mM): $MnCl_2$ 0.1, NaCl 137, KCl 6, $MgCl_2$ 1.2, glucose 10, HEPES 10, EGTA 0.1, pH 7.4. After 30 s, endothelin-1 (10 nM) was added to the $Mn^{2+}$-quenching solution. 10 min later, $MnCl_2$ (5 mM) was added to measure background autofluorescence. This value was subtracted from all fluorescence data. $Mn^{2+}$ entry evoked by endothelin-1 was assessed from the rate of decrease of fura-2 fluorescence (normalised to the value recorded immediately before the addition of endothelin-1), measured by the slope of the linear regression line of F360 data recorded during the first 30 s after the addition of endothelin-1, expressed as fluorescence arbitrary unit per second ($s^{-1}$). $R^2$ values of the linear regression were between 0.95 and 0.97.

### 2.5. Microinjection of heparin

To test the effect of heparin on $Ca^{2+}$ signalling, a mixture of low-molecular weight heparin (200 mg/ml) and fura-2 acid (16.6 mM) was injected into VSMC cytoplasm using Femtotips and an Eppendorf Automated Microinjector 5442 system

Table 1
Oligonucleotide sequences of primers used for RT-PCR

| Target | Sequence (5′–3′) | | Amplicon |
|---|---|---|---|
| | Forward | Reverse | (bp) |
| rTRPC1 | ATGATTTTGCCGACCGGAAG | AATCTGCAGTGGGCCCAAAA | 143 |
| rTRPC2 | CGAGGACGATGCTGATGTAGA | GTTGTTAAAGTTCGGGATTGG | 217 |
| rTRPC3 | TGCTTGTGTTCAACGCCTCA | CCCGAGAACCCAGACCATAAT | 146 |
| rTRPC4 | CGTGGAAAAGGGGGACTATG | CGCATCGCCAACATAGACATTGAA | 181 |
| rTRPC5 | AACCTACTTCGGAGCCAATGTAC | CCGGATTTCACCTAACTCTACCTC | 158 |
| rTRPC6 | GTCGGTGGTCATCAACTACAATC | CCACATCCGCATCATCCTCAATT | 155 |
| rTRPC7 | ATCATCTCGGAAGGGCTGTAC | GCACCTCGGTAGTAGGAGTAC | 209 |
| RPL | AGGCACCAGTCGGACCGATA | GAAGCCGCTAGGCAGCATGT | 159 |

TRPC, transient receptor potential canonical.
RPL, ribosomal protein L32.

(Eppendorf; Brinkman Instruments Inc, Westbury, NY, USA). Injection time and intrapipette pressure were respectively: 0.2 s and 150 hPa. After injection, fura-2 injected cells for which the green signal was visible were examined for $Ca^{2+}$ measurement.

### 2.6. Real-time RT-PCR

Total RNA was isolated from VSMC and from freshly isolated intact or endothelium-denuded rat aorta using the Trizol Isolation Reagent (Invitrogen), and treated with DNase I (Invitrogen). The RNA sample was additionally purified by isopropanol precipitation and its concentration determined by measuring absorbance at 260 nm. DNase-treated RNA samples were reverse transcribed into cDNA by using Superscript III (Invitrogen).

PCR primers were designed based on published sequences in GenBank (Table 1).

Real-time RT-PCR (iCycler; BioRad, Hercules, CA, USA) analyses were performed in duplicate with 200 nM of both sense and antisense primers in a final volume of 25 µl using Supermix (BioRad). PCR conditions were settled as incubation at 94 °C for 3 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 61 °C, and 1 min at 72 °C. The melting temperature of PCR product was checked at the end of each PCR by recording SYBR Green fluorescence increase upon slowly renaturing DNA. A control PCR without template DNA was performed in each experiment. The melting curves of all PCR products showed single peaks, indicating the specificity of the primers. A control PCR without RT was performed in each experiment. We normalised mRNA expression of TRPC by ribosomal protein L32 (RPL32) as a housekeeping gene. Regression analyses of the Ct (threshold cycle) values of the standard dilution series were used to determine the amplification efficiency. Efficiencies and slopes were nearly similar and close to 100% and −3.33, respectively. The standardized ratio (TRPC mRNA/RPL mRNA) will be referred to as the relative amount of TRPC mRNA.

The expression of endothelial nitric oxide synthase (eNOS) and of vWF mRNA was assessed by semi-quantitative radioactive RT-PCR, as described (Krenek et al., 2006).

### 2.7. Immunofluorescence

Cells were fixed with paraformaldehyde 4% (v/v) for 15 min at room temperature and permeabilized thereafter with 1% Triton X-100 (v/v) in Tris-buffered saline (TBS; Tris–HCl 50 mM, NaCl 150 mM, pH 7,4) for 15 min. Non-specific immunostaining was prevented by a 1 h incubation in a TBS solution containing 3% non-fat dry milk at 37 °C. For TRPC1, fixed cells were then successively incubated in TBS/milk 3% for 1 h at room temperature with primary rabbit anti-TRPC1

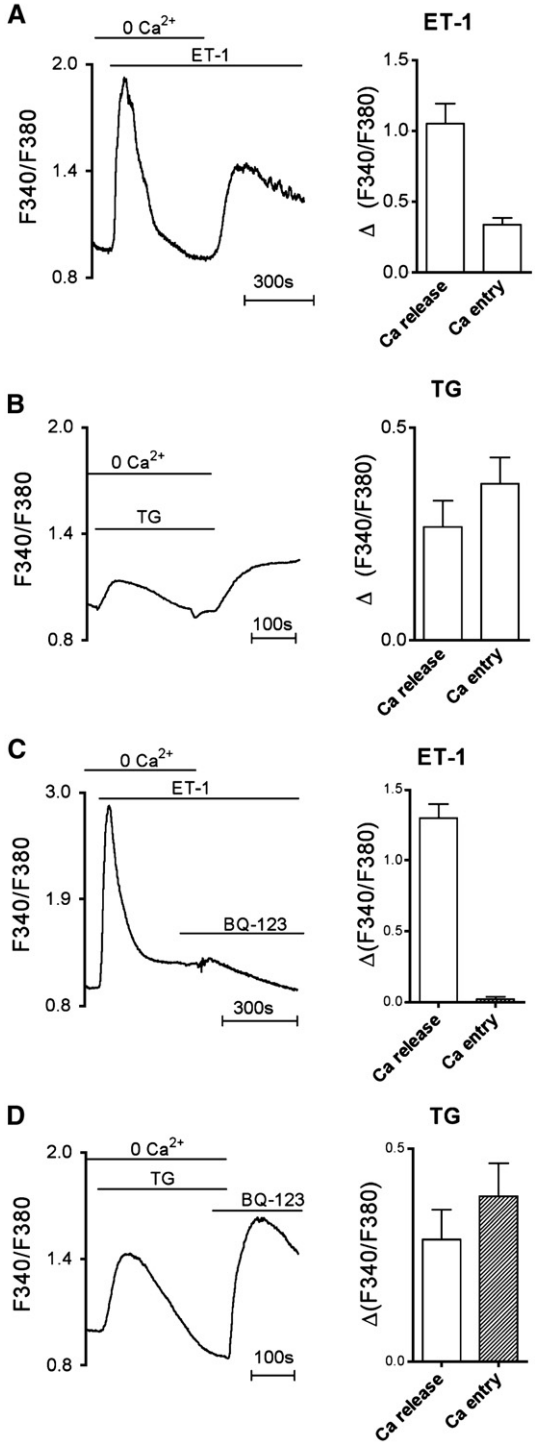


Fig. 1. Characterization of the $Ca^{2+}$ signal evoked by endothelin-1 and thapsigargin in aortic smooth muscle cells (VSMC). A. Effect of endothelin-1 in fura-2-loaded VSMC. *Left panel*: Typical experimental trace of the F340/F380 ratio. Cells were perfused with $Ca^{2+}$-free solution and endothelin-1 (ET-1, 10 nM) as indicated. *Right panel*: Mean values of the maximum increase in $Ca^{2+}$ signal induced by endothelin-1 in the absence and presence of $Ca^{2+}$ ($n=5$). B. Effect of thapsigargin in fura-2 loaded VSMC. *Left panel*: Typical experimental trace of the F340/F380 ratio. Cells were perfused with $Ca^{2+}$-free solution and thapsigargin (TG, 1 µM) as indicated. *Right panel*: Mean values of the maximum increase in $Ca^{2+}$ signal induced by TG in the absence and presence of $Ca^{2+}$ ($n=7$). C. Effect of BQ-123 on endothelin-1-evoked $Ca^{2+}$ signal in fura-2 loaded VSMC. *Left panel*: Typical experimental trace of the F340/F380 ratio. Cells were perfused with $Ca^{2+}$-free solution and endothelin-1 (ET-1, 10 nM) as indicated. BQ-123 (0.5 µM) was added in the perfusion solution after completion of $Ca^{2+}$ release. *Right panel*: Mean values of the maximum change in $Ca^{2+}$ signal induced by endothelin-1 in the absence and presence of $Ca^{2+}$ ($n=5$). D. Effect of BQ-123 on thapsigargin-evoked $Ca^{2+}$ signal in fura-2 loaded VSMC. *Left panel*: Typical experimental trace of F340/F380 ratio. Cells were perfused with $Ca^{2+}$-free solution and thapsigargin (TG, 1 µM) as indicated. BQ-123 (0.5 µM) was added in the perfusion solution after completion of $Ca^{2+}$ release. *Right panel*: Mean values of maximum increase in $Ca^{2+}$ signal induced by TG ($n=5$).

antibody (1/200), rinsed three times with TBS, and incubated for 1 h with a secondary anti-rabbit IgG FITC-conjugated antibody (1/500). Nuclei were stained for 3 min with the nuclear dye DAPI (4′-6-diamidino-2-phenylindole) (1/5000). After three rinses, preparations were mounted in Fluoprep medium (BioMerieux) and examined using an Olympus IX70 inverted microscope. Excitation lights were 488 and 400 nm for FITC and DAPI, respectively. Digital images were acquired using appropriate filters and combined using the TILLvisION software. To quantify the fluorescence intensity of TRPC1, the mean immunofluorescence intensity of cells was determined by measuring the fluorescence intensity using TILLvisION software.

### 2.8. Statistical analysis

Data are expressed as means±S.E.M. Number of experiments refers to the number of determinations performed with different cell cultures. Fura2 F340/F380 traces are mean values from 10 to 30 cells. Statistical analysis was performed by Student *t* test, or by analysis of variance, and Bonferroni's post test as indicated. Differences were considered to be significant when *P* was lower than 0.05. Log value of IC50 was calculated by non-linear fitting of experimental data, using the logistic equation with variable slope (Graph Pad Prism).

## 3. Results

### 3.1. Characterization of the cell culture

More than 95% of the cells stained positively for the muscular marker smooth muscle α-actin. None was positive for the endothelial marker, Von Willebrand factor (supplemental data). Absence of endothelial cells in the culture was confirmed by assessing the expression of eNOS mRNA by RT-PCR analysis. eNOS mRNA was not detected in primary culture of VSMC, while under the same condition marked expression was observed in intact aorta (supplemental data). Consequently, our cell culture was confirmed to be free of contamination with endothelial cells.

### 3.2. Characterization of receptor and store-operated $Ca^{2+}$ entry

Receptor-evoked $Ca^{2+}$ entry was estimated using endothelin-1. In the absence of extracellular $Ca^{2+}$, endothelin-1 (10 nM) evoked a rapid but transient increase in $Ca^{2+}$ signal (Fig. 1A). The $Ca^{2+}$ peak observed in $Ca^{2+}$-free solution was abolished after

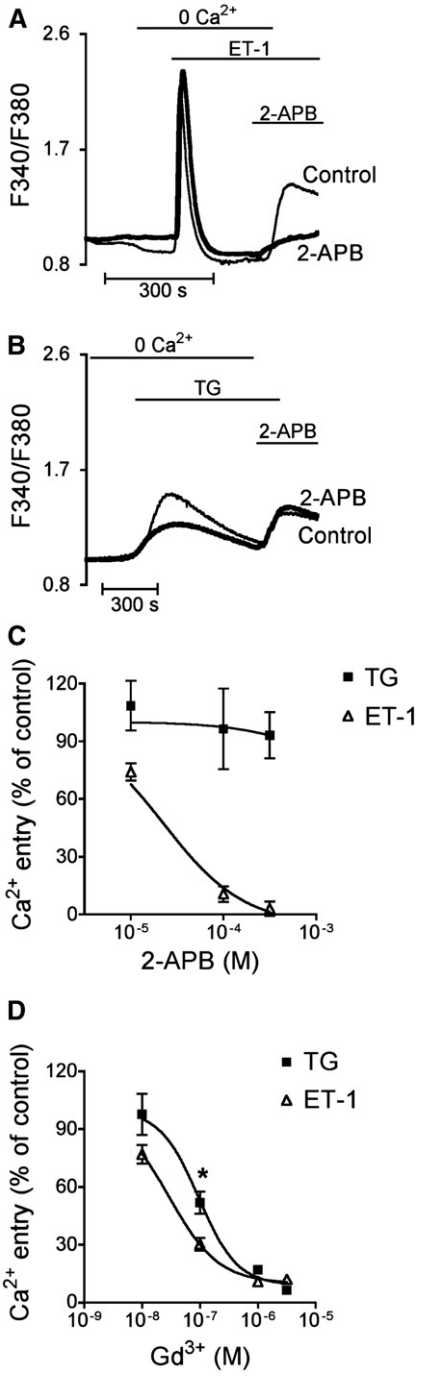


Fig. 2. Effect of 2-APB and $Gd^{3+}$ on endothelin-1 and thapsigargin-evoked $Ca^{2+}$ entry. A. Typical experimental traces showing the F340/F380 ratio in fura-2 loaded VSMC stimulated with endothelin-1. Cells were perfused with $Ca^{2+}$-free solution and stimulated with endothelin-1 (ET-1, 10 nM) as indicated. 2-APB (300 μM) or solvent (control) was added in perfusion solution after completion of $Ca^{2+}$ peak, as indicated by the horizontal bar. Traces are representative of at least 4 experiments. B. Typical experimental traces showing F340/F380 ratio in fura-2 loaded VSMC stimulated with thapsigargin. Cells were perfused with $Ca^{2+}$-free solution as indicated and $Ca^{2+}$ release was evoked with thapsigargin (TG, 1 μM). 2-APB (300 μM) or solvent (control) was added in perfusion solution after completion of $Ca^{2+}$ release, as indicated by the horizontal bar. Traces are representative of at least 4 experiments. C. Effect of 2-APB on $Ca^{2+}$ entry induced by endothelin-1 or thapsigargin. The increase in F340/F380 ratio measured after addition of $Ca^{2+}$ in the presence of 2-APB was expressed as a percentage of the value measured in the absence of 2-APB (control). Data are mean values±S.E.M. from 4 independent determinations. D. Effect of $Gd^{3+}$ on $Ca^{2+}$ entry induced by endothelin-1 or thapsigargin. Cells were stimulated with either endothelin-1 (ET-1, 10 nM) or thapsigargin (TG, 1 μM) in $Ca^{2+}$-free solution. $Ca^{2+}$ entry was evoked by the addition of $Ca^{2+}$ into the perfusion solution. $Gd^{3+}$ (3 nM–3 μM) was added to the perfusion solution after completion of $Ca^{2+}$ release. The increase in $Ca^{2+}$ signal measured after addition of $Ca^{2+}$ in the presence of $Gd^{3+}$ was expressed as a percentage of the value measured in the absence of $Gd^{3+}$ (control). Data are mean values±S.E.M. from 3–4 experiments. Curves were drawn by non-linear curve fitting of the experimental data.

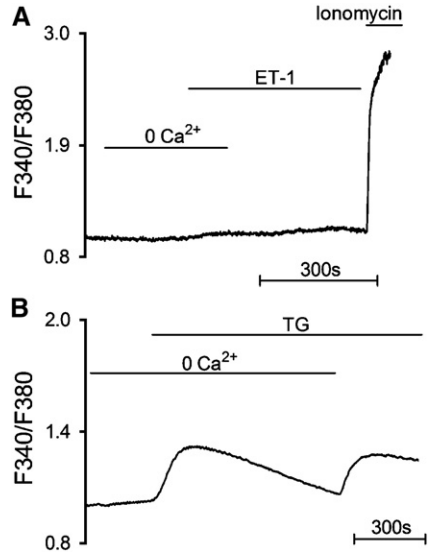


Fig. 3. Effect of heparin on endothelin-1 and thapsigargin-evoked $Ca^{2+}$ signal in fura-2-loaded VSMC. Typical experimental traces showing the fura-2 fluorescence F340/F380 ratio in cells injected with low-molecular weight heparin and fura-2 acid. A. Cells were perfused with $Ca^{2+}$-free solution and stimulated with endothelin-1 (ET-1, 10 nM) as indicated. Ionomycin (4 μM) was added at the end of the experiment. B. Cells were perfused with $Ca^{2+}$-free solution and stimulated with thapsigargin (TG, 1 μM) as indicated. Traces are representative of at least 4 experiments.

treatment of the cells with thapsigargin (data not shown), suggesting that it reflected the release of internal $Ca^{2+}$. Re-addition of $Ca^{2+}$ into the perfusion solution in the continuous presence of the agonist increased the $Ca^{2+}$ signal. The amplitude of the response evoked by the addition of $Ca^{2+}$ measured at the maximum of the $Ca^{2+}$ signal, was markedly smaller than the peak response obtained in $Ca^{2+}$-free solution ($\Delta R$ was 1.05±0.14 and 0.336±0.047 in $Ca^{2+}$-free medium and after addition of $Ca^{2+}$, respectively, $n=5$). Preincubation of VSMC with the endothelin $ET_A$ and $ET_B$ receptor antagonist bosentan (7 μM), or with the endothelin $ET_A$ receptor antagonist BQ-123 (0.5 μM) (Davenport and Battistini, 2002), completely abolished endothelin-1 evoked $Ca^{2+}$ signal (data not shown). When added immediately after the peak of $Ca^{2+}$ release induced by endothelin-1 in $Ca^{2+}$-free solution, the endothelin $ET_A$ receptor antagonist BQ-123 prevented the increase in $Ca^{2+}$ signal evoked by adding $Ca^{2+}$ into the external solution (Fig. 1C). The same effect was observed with bosentan (data not shown).

Store-operated $Ca^{2+}$ signal was measured by using thapsigargin to deplete SR $Ca^{2+}$ stores. Application of thapsigargin (1 μM) in $Ca^{2+}$-free solution produced a transient increase in $Ca^{2+}$ signal, which amplitude was variable from cell to cell, and in average smaller than the $Ca^{2+}$ peak evoked by endothelin-1 ($\Delta R$ was 0.267±0.061, $n=7$) (Fig. 1B). After 5 min in thapsigargin – $Ca^{2+}$-free solution, the re-addition of $Ca^{2+}$ into the perfusion solution increased the $Ca^{2+}$ signal above the basal value recorded before perfusion with $Ca^{2+}$-free solution, reflecting the activation of store-operated $Ca^{2+}$ entry (mean $\Delta R$ was 0.368±0.062, $n=7$). The increase in $Ca^{2+}$ signal resulting from thapsigargin-evoked store depletion was unaffected by the endothelin-1 receptor antagonists BQ-123 (0.5 μM) (Fig. 1D) or bosentan (7 μM, data not shown).

To further characterize the $Ca^{2+}$ entry activated by endothelin-1 and by thapsigargin-evoked store depletion, we tested their sensitivity to the inhibitor of $IP_3$ receptors 2-APB and the inhibitor of TRPC $Gd^{2+}$. Inhibitors were applied after completion of the $Ca^{2+}$ release process by endothelin-1 or thapsigargin, in order to prevent any effect of the inhibitors on the SR $Ca^{2+}$ content. Fig. 2A–C shows that 2-APB (300 μM) abolished endothelin-1 evoked $Ca^{2+}$ entry, but did not affect $Ca^{2+}$ entry activated after thapsigargin-evoked store depletion. The effect of 2-APB on endothelin-1 evoked $Ca^{2+}$ entry was concentration-dependent; log(IC50) value was −4.65±0.14. $Gd^{3+}$(10 nM–3 μM) depressed both endothelin-1 and thapsigargin-evoked $Ca^{2+}$ entry (Fig. 2D). However, endothelin-1 evoked $Ca^{2+}$ entry was significantly more sensitive to $Gd^{3+}$ than was thapsigargin-evoked $Ca^{2+}$ entry: $Gd^{3+}$ log(IC50) values were −7.54±0.05 and −7.04±0.06, respectively ($P<0.01$). Neither endothelin-1 nor thapsigargin-evoked $Ca^{2+}$ entry was affected by the L-type $Ca^{2+}$ channel blocker nimodipine (1 μM) (data not shown).

To investigate the involvement of $IP_3$ receptor in the activation of $Ca^{2+}$ entry, cells were injected with heparin, which blocks $IP_3$ receptor (Yamamoto et al., 1990), together with fura-2 acid. In heparin-injected cells, endothelin-1 did not produce any change in $Ca^{2+}$ signal in $Ca^{2+}$-free solution and after re-addition of $Ca^{2+}$ into the perfusion solution (Fig. 3A). The large increase in F340/F380 ratio evoked by ionomycin at the end of the experiment indicated that the absence of response to endothelin-1 was not caused by damage to the cells, or defect in fura-2 signalling. On the opposite, heparin did not affect either $Ca^{2+}$ release evoked by thapsigargin in $Ca^{2+}$ free solution, or $Ca^{2+}$ entry activated by addition of $Ca^{2+}$ after intracellular $Ca^{2+}$ store depletion (Fig. 3B).

Activation of phospholipase C following endothelin receptor stimulation also results in the production of DAG. Direct activation of $Ca^{2+}$ entry by DAG has been demonstrated (Helliwell and Large, 1997; Hofmann et al., 1999; Sydorenko et al., 2003). OAG was used to investigate the involvement of DAG in the $Ca^{2+}$ entry activated by endothelin-1 in VSMC. OAG (100 μM) was first applied in $Ca^{2+}$ free solution during 5 min, before $Ca^{2+}$ (2 mM) was added into the solution. Neither in $Ca^{2+}$ free solution, not after addition of $Ca^{2+}$, did OAG produce an increase in cytosolic $Ca^{2+}$.

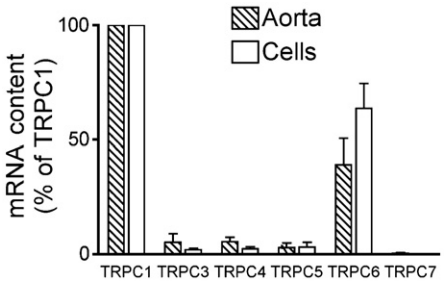


Fig. 4. Real-time RT-PCR analysis of TRPC1-7 in rat endothelium-denuded aorta and in primary culture of aortic smooth muscle cells. The mRNA levels were first adjusted to RPL, then normalised to the level of TRPC1 mRNA in the same sample, set at 100%. Data are means±S.E.M. from 4 independent experiments.

Involvement of arachidonic acid in the activation of $Ca^{2+}$ entry by endothelin-1 was assessed with a combination of the DAG lipase inhibitor RHC-80267 and of the phospholipase A2 inhibitor OBAA. At 10 μM, RHC-80267 and OBAA did not affect the $Ca^{2+}$ entry evoked by endothelin-1 ($\Delta R$ was 0.335±0.032 and 0.372±0.071, $n=3$ experiments, in the presence and in the absence of the inhibitors, respectively).

### 3.3. TRPC mRNA expression

The expression of TRPC1–7 isoforms was evaluated using real-time RT-PCR in VSMC and endothelium-denuded aorta. Preliminary experiments indicated that TRPC2, expressed in the vomeronasal organ used as positive control, was detected neither in VSMC nor in aorta (data not shown). All other TRPC isoforms were expressed in VSMC and in aorta (Fig. 4). TRPC1 was the most abundant in cultured aortic cells and in aorta. TRPC6 was expressed at intermediate levels, whereas TRPC3, TRPC4 and TRPC5 expressions were low. TRPC7 was hardly detectable.

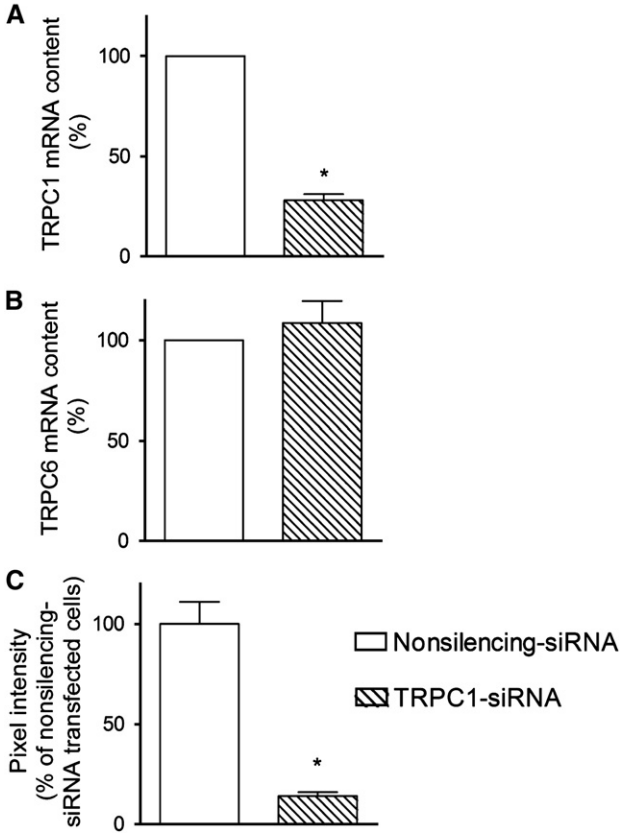


Fig. 5. Effect of TRPC1-siRNA on TRPC1 expression in VSMC. A–B. Real-time RT-PCR quantification of the mRNA expression of TRPC1 (A) and TRPC6 (B). The mRNA levels were first adjusted to housekeeping gene RPL mRNA, then normalised to the level of expression in cells transfected with non-silencing-siRNA set at 100%. mRNA expression was calculated from 4 independent experiments, *indicates significant difference ($n=4$). C. Mean value of anti-TRPC1 labelling intensity in VSMC transfected with non-silencing-siRNA ($n=26$ images from 3 independent experiments) and in VSMC transfected with TRPC1-siRNA ($n=39$ images from 4 independent experiments). Data were normalised to the pixel intensity measured in cells transfected with non-silencing-siRNA. *indicates significant difference ($P<0.01$, versus cells transfected with non-silencing-siRNA).

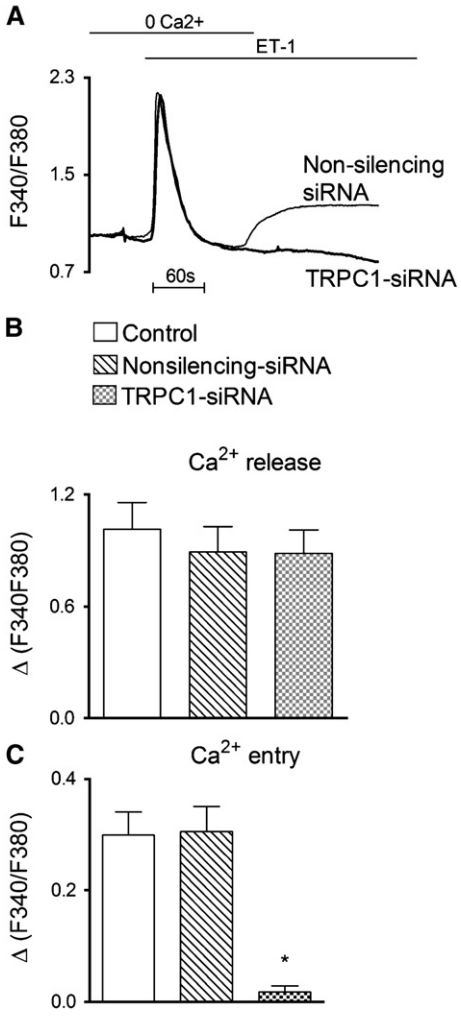


Fig. 6. Effect of the inhibition of TRPC1 expression on endothelin-1 induced $Ca^{2+}$ signal. A. Typical traces of F340/F380 ratio in fura-2-loaded VSMC stimulated with endothelin-1. Traces obtained in TRPC1-siRNA transfected cells and in non-silencing-siRNA transfected cells are superimposed. Cells were perfused with $Ca^{2+}$-free solution and endothelin-1 (ET-1, 10 nM) as indicated. Traces are means from 10 and 15 cells and are representative of at least 4 experiments. B. Mean values of the increase in F340/F380 ratio evoked by endothelin-1 in the absence of $Ca^{2+}$ C. Mean values of the increase in F340/F380 ratio evoked by the re-addition of $Ca^{2+}$ (2 mM) into $Ca^{2+}$-free solution in the presence of endothelin-1. Control non-transfected VSMC ($n=5$ experiments), VSMC transfected with non-silencing-siRNA ($n=4$ experiments), TRPC1-siRNA transfected VSMC ($n=4$ experiments).*indicates significant difference versus control ($P<0.01$).

### 3.4. Effect of inhibition of TRPC1 expression by siRNA on receptor and store-operated $Ca^{2+}$ entry

As shown in Fig. 5 (A–B), real-time RT-PCR analysis indicated that transfection of VSMC with TRPC1-siRNA inhibited mRNA expression of TRPC1 by 72±3% ($n=4$ different cell cultures), while the expression of TRPC6 was unaffected.

Protein expression of TRPC1 was estimated by immunocytochemistry (Fig. 5C and supplemental data). Quantification of the TRPC1 labelling indicated that TRPC1 protein expression

was decreased by 84±13% in TRPC1-siRNA transfected cells, compared with cells transfected with non-silencing-siRNA ($n$=4).

The function of TRPC1 in the activation of $Ca^{2+}$ signal was assessed in siRNA transfected cells loaded with fura-2. VSMC transfection with TRPC1-siRNA or with non-silencing-siRNA did not affect the intracellular $Ca^{2+}$ release evoked by endothelin-1 in $Ca^{2+}$-free solution (Fig. 6). The $Ca^{2+}$ signal evoked by re-addition of $Ca^{2+}$ into the $Ca^{2+}$-free solution was nearly abolished in TRPC1-siRNA transfected cells, while it was not different in cells transfected with non-silencing-siRNA and in control non-transfected cells (Fig. 6).

In order to provide a direct estimate of the activation of a $Ca^{2+}$ channel by endothelin-1, the effect of endothelin-1 on cation entry was monitored by measuring the quenching of fluorescence upon excitation at 360 nm (F360) evoked by $Mn^{2+}$. $Mn^{2+}$ is known to permeate through cation channels in the membrane, and when in the cell, to bind to fura-2 and to quench its fluorescence. The rate of fura-2 quenching by $Mn^{2+}$ upon excitation at the isosbestic point of fura-2 (360 nm) is a direct indication of the cation entry into the cell since $Mn^{2+}$ is not handled by pumps and transporters (Missiaen et al., 1990). Perfusion of the cells with the $Mn^{2+}$ quenching solution induced a slow decrease in F360 fluorescence, that could be analysed by linear regression to approximate the rate of F360 quenching. The slope of the decrease in F360 was $(-0.88\pm0.29)\cdot10^{-3}$ $s^{-1}$ ($n$=35 cells) and was not different in cells transfected with TRPC1-siRNA, non-silencing-siRNA, or in cells treated by the endothelin $ET_A$ receptor antagonist BQ-123. Addition of endothelin-1 in the perfusion markedly increased the quenching rate of F360. The rate of $Mn^{2+}$ entry was estimated from the slope of the fluorescence intensity decrease during the first 30 s after the addition of endothelin-1. As shown in Fig. 7, endothelin-1 caused an 8-fold increase in the quenching rate of F360, which was suppressed in the presence of the endothelin $ET_A$ receptor antagonist BQ123 ($P$<0.05 versus endothelin-1). Endothelin-1-evoked F360 quenching was significantly slowed down in cells transfected with TRPC1-siRNA compared to non-transfected cells, while in cells transfected with non-silencing-siRNA, the slope of F360 decrease after addition of endothelin-1 was not different from that observed in non-transfected cells (Fig. 7).

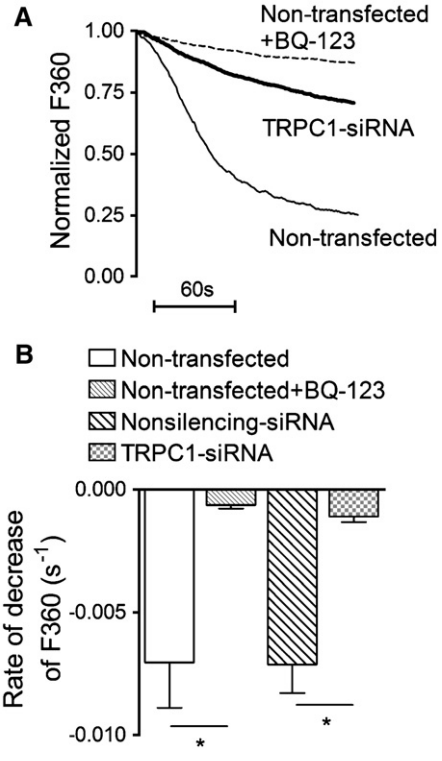


Fig. 7. Effect of TRPC1 inhibition on endothelin-evoked cation entry $Ca^{2+}$ entry was estimated by the quenching of fluorescence at 360 nm (F360) by $Mn^{2+}$ in fura-2-loaded cells. A. Decrease in F360 after addition of endothelin-1 (10 nM) in $Ca^{2+}$-free $Mn^{2+}$-containing solution, in control non-transfected cells, in TRPC1-siRNA transfected cells and in non-transfected cells preincubated with BQ-123 (0.5 μM). F360 values were normalised to the fluorescence measured at the time of endothelin-1 addition. Traces are means from 12–20 cells, measured in one experiment. B. Mean values of the rate of decrease of F360 measured during the first 30 s after the addition of endothelin-1. Data are means from 4 experiments, except for non-silencing-siRNA transfected cells, where data are means from 3 experiments. *indicates significant differences ($P$<0.01).

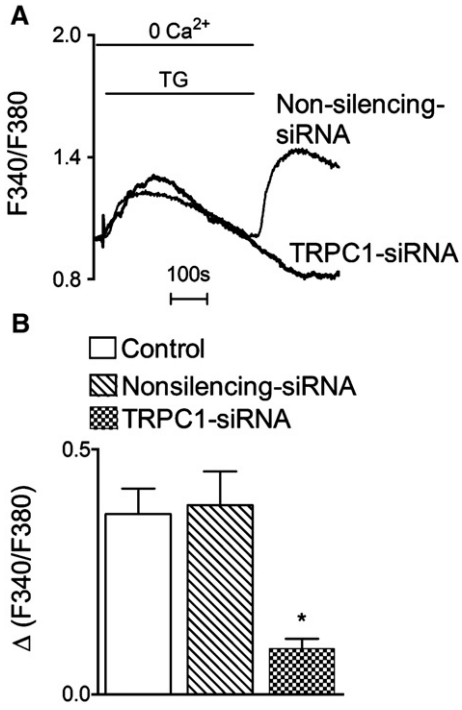


Fig. 8. Effect of inhibition of TRPC1 expression on thapsigargin-evoked $Ca^{2+}$ signal. A. Typical experimental traces showing the effect of thapsigargin on F340/F380 ratio in fura-2 loaded VSMC. Traces obtained in cells transfected with non-silencing-siRNA and in TRPC1-siRNA transfected cells are superimposed. Perfusion with $Ca^{2+}$-free solution and thapsigargin (TG, 1 μM) were applied as indicated. Traces are averages from 14–20 cells and are representative of at least 4 experiments. B. Mean value of the increase in F340/F380 ratio evoked by the re-addition of $Ca^{2+}$ (2 mM) into $Ca^{2+}$-free solution after thapsigargin-evoked intracellular $Ca^{2+}$ release. Data are means±S.E.M. from 4 experiments. *indicates significant difference versus control cells ($P$<0.01).

The functional role of TRPC1 in store-operated $Ca^{2+}$ entry in VSMC was assessed by measuring $Ca^{2+}$ entry after store depletion with thapsigargin in cells transfected with TRPC1-siRNA. Fig. 8 shows that store-operated $Ca^{2+}$ entry was depressed by 80±6% in cells transfected with TRPC1-siRNA, compared to non-

transfected cells but was not affected by cell transfection with non-silencing-siRNA ($n=4$).

## 4. Discussion

The present study shows that in VSMC, TRPC1 is not only involved in a store-operated entry of $Ca^{2+}$, but also in a receptor-activated $Ca^{2+}$ entry pathway that requires $IP_3$ receptor activation.

We used primary cultured aortic smooth muscle cells. Culture was principally composed of smooth muscle cells without contamination by endothelial cells, based on the absence of significant expression of markers of endothelial cells (eNOS, von Willebrand factor). This observation is important since endothelial cells in culture have been shown to express TRPC1–6 to varying degrees (Nilius et al., 2003; Tiruppathi et al., 2002). Culture has been reported to modify the expression of several ion channels (Bergdahl et al., 2005). Nevertheless, the comparison of the relative levels of TRPC transcripts in VSMC and in isolated, endothelium-denuded, aorta showed a similar pattern of expression of the different TRPC isoforms, indicating that VSMC is a good model for the study of $Ca^{2+}$ entry pathways. In agreement with Facemire et al. (2004), our results demonstrated that rat aorta and VSMC expressed mRNA for TRPC1, 3, 4, 5, 6 (Fig. 4). However, in contrast to this earlier report, we found that TRPC1 predominated, and that TRPC6, although it was also abundant, showed a significantly lower expression than TRPC1, in both VSMC and isolated aorta.

### *4.1. Endothelin-1 activates a calcium entry pathway distinct from capacitative calcium entry*

Endothelin-1 was used to investigate the properties of receptor-activated $Ca^{2+}$ entry in VSMC. In $Ca^{2+}$-free solution, endothelin-1 evoked a rapid and transient increase in $Ca^{2+}$ signal. Inhibition of this $Ca^{2+}$ peak after treatment with thapsigargin in $Ca^{2+}$ free solution indicated that it resulted from $Ca^{2+}$ release from the SR. This was confirmed by the abolition of the $Ca^{2+}$ peak after inhibiting the $IP_3$ receptor with heparin. The addition of $Ca^{2+}$ in the endothelin-1-containing perfusion solution produced a sustained increase in cytosolic $Ca^{2+}$, which was resistant to blockers of voltage-dependent $Ca^{2+}$ channels, in agreement with the activation by endothelin-1 of cation channels (Iwamuro et al., 1999; Kawanabe et al., 2002a; Peppiatt-Wildman et al., 2007). This increase in $Ca^{2+}$ signal reflected an entry of $Ca^{2+}$ into the cell, as confirmed by using extracellular $Mn^{2+}$ as a surrogate ion for $Ca^{2+}$. Quenching rate of fura-2 fluorescence by $Mn^{2+}$ was increased by endothelin-1, and this effect was inhibited by the endothelin $ET_A$ receptor antagonist BQ-123.

An important observation was that endothelin-1-evoked $Ca^{2+}$ entry was abolished when the endothelin $ET_A$ receptor antagonist BQ-123 was applied after the process of $Ca^{2+}$ release was completed, indicating that store depletion is not sufficient to activate $Ca^{2+}$ entry, which required the occupation of the endothelin $ET_A$ receptor by the agonist. Blockade of the channel by the endothelin $ET_A$ receptor antagonist BQ-123 might be ruled out because BQ-123 did not affect $Ca^{2+}$ entry activated after store depletion with thapsigargin, and because the same effect was obtained with the mixed endothelin $ET_A/ET_B$ receptor antagonist bosentan. This observation is in agreement with the report that in rabbit cerebral arteriolar smooth muscle, endothelin-1-induced $Ca^{2+}$ influx is separate from any capacitative $Ca^{2+}$ entry mechanism because its amplitude remains the same even when sarcoplasmic reticulum has previously been depleted of $Ca^{2+}$ (Guibert and Beech, 1999). Controversially, in rat aortic cells, endothelin-1 has been reported to activate both capacitative and non-capacitative $Ca^{2+}$ entry, on the basis of the sensitivity of the $Ca^{2+}$ response to SK&F 96365 and LOE 908 (Kawanabe et al., 2002a; Zhang et al., 1999).

A reciprocal effect of vasopressin on capacitative and non-capacitative $Ca^{2+}$ entry has been reported in A7r5 cells. In those cells, capacitative $Ca^{2+}$ entry is rapidly activated when vasopressin is removed from the bathing solution (Moneer and Taylor, 2002), an observation that has not been confirmed in other A7r5 cell lines variants (Brueggemann et al., 2005). Inhibition of capacitative $Ca^{2+}$ entry by endothelin-1 did not occur in VSMC, since the addition of the endothelin $ET_A$ receptor antagonist BQ-123 did not evoke any increase in $Ca^{2+}$ signal.

### *4.2. Endothelin-1-activated calcium entry requires $IP_3$ binding to the $IP_3$ receptor*

A second important property of endothelin-1-evoked $Ca^{2+}$ entry was its sensitivity to heparin, which is known to block $IP_3$ receptor (Peppiatt-Wildman et al., 2007). On the other hand, heparin did not affect thapsigargin-induced $Ca^{2+}$ entry. This observation indicates that, unlike store-operated $Ca^{2+}$ entry, $Ca^{2+}$ entry activated by endothelin-1 requires $IP_3$ binding to its receptor. This is in the line of the observation that endothelin-1-evoked $Ca^{2+}$ entry required the activation of the endothelin $ET_A$ receptor, which results in the G protein-mediated activation of phospholipase C (Kawanabe et al., 2002b).

In coronary artery myocytes, the signal transduction mechanism linking endothelin-1 to activation of cation channel current involves PLC coupled to endothelin $ET_A$ receptor (Peppiatt-Wildman et al., 2007). PLC is also involved in noradrenaline-induced cation channel activation in the rabbit portal vein (Helliwell and Large, 1997) and in angiotensin II in rabbit mesenteric smooth muscle cells (Saleh et al., 2006), but in those cells cation channel conductance was activated by DAG. In coronary artery cells, $IP_3$ potentiated the response to OAG, an effect that was blocked by heparin (Peppiatt et al., 2003).

The involvement of DAG in the activation of $Ca^{2+}$ entry by endothelin-1 in aortic myocytes is unlikely, because (i) heparin completely blocked endothelin-1 evoked $Ca^{2+}$ entry, (ii) inhibition of DAG degradation with RHC-80267 did not change endothelin-1 evoked $Ca^{2+}$ entry and (iii) OAG did not generate any $Ca^{2+}$ signal. In agreement with the last result, Brueggemann et al. (2006) reported that 100 μM OAG did not activate Isoc current in A7r5 cells.

In several cell types including smooth muscle cells, human platelets and jurkat T lymphocytes, store-operated $Ca^{2+}$ entry is blunted when the activity of phospholipase A2 is inhibited (Broad et al., 1999; Smani et al., 2004), and direct activation of cation channel by arachidonic acid has been reported (Shuttleworth

et al., 2004). In our study, endothelin-1 TRPC-1 induced $Ca^{2+}$ entry was not affected by the phospholipase A2 inhibitor OBAA, or by DAG lipase inhibition, which inhibits the release of arachidonic acid from DAG, suggesting that arachidonic acid was not involved in the activation of $Ca^{2+}$ entry by endothelin-1.

### *4.3. TRPC1 is involved in endothelin-1-activated calcium entry and in capacitative calcium entry*

In order to test the hypothesis that TRPC1 contributes to the $Ca^{2+}$ entry pathway activated by endothelin-1, the expression of the channel protein was inhibited by siRNA transfection. Using real-time RT-PCR and immunocytochemistry, we showed that TRPC1-siRNA significantly reduced the expression of endogenous TRPC1, while the level of expression of TRPC6 was not affected.

TRPC1-siRNA transfection of VSMC led to the inhibition of thapsigargin-evoked $Ca^{2+}$ entry. This observation is in agreement with several reports that demonstrate, by different experimental approaches, that TRPC1 functions as store-operated channel (see Introduction). Interestingly, in our study, inhibition of TRPC1 expression markedly blunted endothelin-1-induced $Ca^{2+}$ influx, while $Ca^{2+}$ release was not affected, providing strong evidence that TRPC1 is involved in the $Ca^{2+}$ entry generated by endothelin receptor activation. This was confirmed by measuring the rate of fura-2 fluorescence quenching at 360 nm in the presence of $Mn^{2+}$, showing that in TRPC1-siRNA transfected cells, quenching of F360 by endothelin-1 was significantly depressed, to a level similar to that observed in the presence of BQ-123 in non-transfected cells. The involvement of TRPC1 in endothelin-1-evoked $Ca^{2+}$ signal is in agreement with the report that in caudal artery, TRPC1 antibody inhibits the contraction evoked by endothelin-1 by 40% (Bergdahl et al., 2003). However, in rabbit coronary artery myocytes, anti-TRPC1 antibody did not inhibit endothelin-1 evoked cation channel activity, while anti-TRPC3 and anti-TRPC7 antibodies markedly inhibited endothelin-1 evoked current (Peppiatt-Wildman et al., 2007).

Taken together the present results indicate that in primary cultured VSMC, TRPC1 contributes to both SOC and ROC-mediated $Ca^{2+}$ entry. Pharmacological tools were used to further characterize those $Ca^{2+}$ entry pathways. $Gd^{3+}$ is widely used to block non-selective cation channels (McFadzean and Gibson, 2002), but has been reported to lack selectivity against VOC, ROC or SOC (Poburko et al., 2004). It blocks TRPC1 (Brueggemann et al., 2006; Zitt et al., 1996), TRPC3 (Albert et al., 2006), TRPC6 channels (Inoue et al., 2001) but activates and potentiates TRPC4/5 (Jung et al., 2003) and TRPV1 (Tousova et al., 2005). We have observed that at concentration as low as 1 μM, $Gd^{3+}$ abolished $Ca^{2+}$ entry activated by endothelin-1 as well as that activated by store depletion. However, $Gd^{3+}$ was two times more potent on endothelin-1-induced $Ca^{2+}$ entry ($IC_{50}$ 40 nM) compared to thapsigargin-activated $Ca^{2+}$ entry ($IC_{50}$ 110 nM). The latter value is in the range of the $IC_{50}$ on SOC (101 nM) reported by Flemming et al. (2003) in cerebral arteriolar smooth muscle cells. Higher concentrations of $Gd^{3+}$ are needed to inhibit cation current activated by α-adrenergic receptor in rabbit portal vein smooth muscle, which has been shown to be mediated through TRPC6 channel protein ($IC_{50}$ 2 μM) (Inoue et al., 2001), or to inhibit SOC in renal artery, which requires 100 μM $Gd^{3+}$ (Wilson et al., 2002). In A7r5 embryonic aortic cells, capacitative $Ca^{2+}$ entry is irreversibly blocked by low concentration of $Gd^{3+}$, which does not affect Arg-vasopressin-evoked $Ca^{2+}$ entry (Broad et al., 1999).

Difference in the sensitivity of store depletion and receptor-activated $Ca^{2+}$ entry to blockers was confirmed with 2-APB, which inhibited $Ca^{2+}$ entry activated by endothelin-1, but not store-operated $Ca^{2+}$ entry activated by thapsigargin. This selective effect of 2-APB against endothelin-1 evoked $Ca^{2+}$ entry might be related to the reported effect of the antagonist on $IP_3$ receptor (Ma et al., 2000; Maruyama et al., 1997; Peppiatt et al., 2003; Soulsby and Wojcikiewicz, 2002). This property has been challenged by the report that 2-APB inhibits TRPC3, 6 and 7 channels by a mechanism not involving $IP_3$ receptor in HEK293 cells (Lievremont et al., 2005). 2-APB also inhibits *I*CRAC and store-operated entry in a mutant DT40 cell line in which $IP_3$ receptors are not expressed (Ma et al., 2001), indicating that $IP_3$ receptors are not required for 2-APB block of $Ca^{2+}$ entry.

The lack of inhibition of thapsigargin-evoked $Ca^{2+}$ entry by 2-APB is not consistent with 2-APB being regarded as selectively inhibiting the capacitative entry of $Ca^{2+}$ in a variety of different cell types, including A7r5 cells (Brueggemann et al., 2006; Moneer and Taylor, 2002) and vascular smooth muscle (Kumar et al., 2006). Interestingly, in rabbit vena cava, 2-APB does not affect the elevation in intracellular $Ca^{2+}$ following SR store depletion but inhibits the generation of intracellular $Ca^{2+}$ concentration oscillations in response to α1-adrenoceptor activation (Lee et al., 2002). The molecular target of this compound remains to be further characterized.

Despite the lack of selectivity of the pharmacological inhibitors used, their action confirms that endothelin-1 and store depletion activate distinct $Ca^{2+}$ entry pathways, although both involve TRPC1. $Ca^{2+}$ entry evoked by endothelin-1 and store depletion in VSMC also differed by the mechanism of their activation. The present results indicate that in primary cultured aortic smooth muscle cells, activation of endogenous TRPC1 by endothelin-1 requires $IP_3$ receptor activation. Several experimental approaches have provided evidence indicating that $IP_3$ receptor and TRPC1 are physically associated, either directly or through binding partners like Homer or Rho (Lockwich et al., 2000; Mehta et al., 2003; Peppiatt et al., 2003; Rosado and Sage, 2000; Yuan et al., 2003). Such an interaction may be responsible for the activation of TRPC1 by endothelin-1 in VSMC.

The present data show that TRPC1 contributes to both SOCs and ROCs in VSMC, which have different pharmacological properties. TRPC channels have been reported to be heteromeric complexes (Maruyama et al., 2006; Strubing et al., 2003; Zagranichnaya et al., 2005). In particular, TRPC1 can associate with TRPC4 or 5 (Hofmann et al., 2002; Strubing et al., 2001) and with TRPC3 (Lintschinger et al., 2000; Liu et al., 2005). Association of different isoforms has been reported to confer distinct physiological and pharmacological properties to the channel (Liu et al., 2005; Trebak et al., 2002). Further studies will have to test whether formation of heteromeric channels

could explain the different functional and pharmacological properties of TRPC1 in VSMC.

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