Plasticity of TRPC expression in arterial smooth muscle: correlation with store-operated Ca$^{2+}$ entry

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The smooth muscle cells in the vascular wall are normally kept in a stable contractile phenotype under the influence of the tissue environment (23). Disturbance of the functional integrity of the tissue, such as intimal damage and/or inflammation, results in modulation toward a less differentiated synthetic phenotype, which is thought to be a starting point for atherosclerotic lesions or restenosis after angioplasty (24). The modulation from a contractile to a synthetic phenotype is marked by the loss of contractile and cytoskeletal proteins, but changes in membrane properties, receptor population, and Ca$^{2+}$ handling are also known to occur. These latter changes may in fact precede altered contractile properties and could potentially play a role in the modulation process itself. To test this hypothesis, a reference state of differentiated cells in the normal tissue environment is needed upon which environmental factors may be applied to influence cell differentiation.

Organ culture of intact vascular tissue has been shown to preserve contractile differentiation for several days, although subtle changes in cellular properties occur (11). Progression of cellular alterations is considerably slowed when vessels are cultured in the absence of added growth factors such as fetal calf serum (17), although endothelial integrity is disturbed and changes in membrane properties occur as observed with respect to receptor expression for PDGF and endothelin (ET) (20). Prolonged culture of human and porcine vessels has been shown to cause the formation of a neointima, which is a lesion characteristic of restenosis after angioplasty (15, 25). Phenotypic modulation of smooth muscle cells in organ culture may therefore offer insight into the early pathogenetic factors involved in vascular disease. The slow progression of membrane alterations in organ culture also allows the study of interventions, such as mechanical injury to the vessel wall, that may accelerate altered gene expression.

In rat caudal and cerebral arteries, the Ca$^{2+}$ release upon depletion of sarcoplasmic reticulum (SR) stores is increased after serum-free organ culture (4, 5). This is not associated with any increase in basal intracellular Ca$^{2+}$ stores ([Ca$^{2+}$]$_i$). Organ-cultured cerebral arteries showed a slightly decreased current via voltage-dependent Ca$^{2+}$ channels but a greatly augmented store-operated Ca$^{2+}$ entry (SOCE) that is insensitive to L-type channel blockade (4). Capacitative Ca$^{2+}$ entry upon the addition of extracellular Ca$^{2+}$ after store depletions did not result in contraction in freshly dissected arteries, whereas a prominent contractile response was seen after culture. Ca$^{2+}$ might possibly enter a discrete compartment not eliciting a contractile response (6), although increased store-operated channel (SOC) activity in cultured vessels also might raise Ca$^{2+}$ in the vicinity of contractile-regulatory proteins. These results suggest that alterations in membrane channel properties and intracellular Ca$^{2+}$ handling are characteristic features of the response to organ culture.

The molecular identity of SOCs is the subject of intense investigation. In mammals, genes homologous to the Drosophila trp gene have been suggested to code for SOC proteins (3).
Among the identified families of transient receptor potential canonical (TRPC) proteins (21), the TRPC family (TRPC1–TRPC7) has been suggested to be involved in SOCE as well as in receptor-mediated \( \text{Ca}^{2+} \) inflow. Evidence obtained using an antibody targeted to the outer vestibule of TRPC1 channels implicates this isoform as a subunit of SOCE in native vascular smooth muscle cells (31). However, TRPC1 itself may not be able to form functional channels and, in intact tissue, may associate with other isoforms, primarily TRPC4 or TRPC5 (1, 13). Other TRPC isoforms have been associated with SOC activity but may require cofactors such as diacylglycerol (12) or inositol trisphosphate (18). The cellular context may be important, because TRPC3 expressed in different cell lines is activated either by store depletion or by a phospholipase C-dependent mechanism (27).

TRPC isoforms may have specific physiological effects, both by virtue of a restricted tissue distribution and as components of ion channels with different properties. Evidence has been presented for TRPC6 as an essential component of \( \alpha_{1} \)-adrenoceptor-activated cation channels in vascular smooth muscle cells (14). With respect to the cell types in the vascular wall, TRPC4 may have a special role in endothelial cells because TRPC4\(^{-/-} \) mice are characterized primarily by endothelial dysfunction (8).

We have investigated the effects of organ culture on SOC currents and TRPC isoform expression in rat cerebral arteries. To investigate the basis of observed effects and assess their possible clinical significance, segments of human internal mammary artery were exposed to balloon dilatation in vitro and the ensuing TRPC expression was determined. The results indicate that vascular injury enhances plasticity in TRPC expression, that TRPC expression correlates with cellular \( \text{Ca}^{2+} \) handling, and that TRPC1 is a subunit of upregulated SOCs.

**METHODS**

**Preparation and tissue culture.** Sprague-Dawley rats weighing 200–300 g were killed by cervical dislocation using a procedure approved by the Animal Experimentation Ethics Committee of Lund University. The brain was removed and placed into ice-cold physiological salt solution (PSS). The cerebral (midcerebral, posterior, cerebellar, and basilar) arteries were removed and cleaned of connective tissue. To denude vessels of endothelium, an air bubble was allowed to remain in the lumen of the artery for 2 min, followed by a 1-min wash with PSS; alternatively, an insect needle was slid back and forth through the lumen. Both methods yielded the same results. With patients’ informed consent, we obtained internal mammary artery segments from nine patients undergoing bypass surgery as approved by the local ethical committee. All patients were males (mean age, 73 yr), and six of them had ischemic heart disease that was being treated with appropriate medication. Rat and human arterial (mean age, 73 yr), and six of them had ischemic heart disease that was approved by the local ethical committee. All patients were males and allowed to remain in the lumen of the artery for 2 min, followed by a

**Electrophysiology.** Using standard patch-clamp techniques, whole cell currents in single smooth muscle cells were recorded using an Axopatch-200 amplifier, a TL-1 direct memory access interface, and pClamp 6 software (Axon Instruments, Foster City, CA). Patch pipettes (\( R_{f} = 3–5 \, M \Omega \)) were filled with a solution containing (in mM) 140 CsCl, 1 MgCl\(_2\), 20 tetraethylammonium (TEA)-Cl\(^{-}\), 10 HEPES, 10 phosphocreatine, 5 ATP, and 5 BAPTA (pH 7.2 with CsOH). The experiments were conducted at room temperature (20–22°C). For 45–60 min before recording, cells were allowed to adhere to a glass coverslip placed at the bottom of a 30-mm culture dish. Test solutions were applied by gravity flow from two parallel pipettes positioned close to the cell being studied. These pipettes were attached to a manifold (Perfusion Fast-Step SF-77B; Warner Instruments, Hamden, CT), which allowed for rapid exchange between superfusion solutions by lateral displacement of the pipettes. Force experiments. Cultured basilar segments were incubated with a TRPC1 pore-specific antibody [T1E3 (Ref. 31), 1:500 dilution in PSS] at 4°C overnight. Controls were treated with preimmune serum from the rabbit used to generate T1E3 (1:500 dilution). After incubation, preparations were mounted in a myograph (610M; Danish Myo Technology, Aarhus, Denmark) on 40- \( \mu \)m stainless steel wires and stretched as previously described (2). After equilibration for at least 1 h, a reference contraction was elicited using high-K\(^{+} \) solution (140 mM). After relaxation from the contraction, intracellular stores were depleted of \( \text{Ca}^{2+} \) by the addition of caffeine (10 mM), followed by thapsigargin (10 \( \mu \)M) for 10 min. \( \text{Ca}^{2+} \) (2.5 mM) was then added in the presence of 1 \( \mu \)M verapamil.

**RT-PCR assay.** Arteries or collected single cells were transferred to nuclease-free microfuge tubes, frozen in liquid nitrogen, and stored at \(-80^\circ \text{C} \) before RNA isolation. Total RNA was prepared from cerebral vessels using TRIzol reagent (Life Technologies, Carlsbad, CA), according to the manufacturer’s protocol. RNA (0.5–0.9 \( \mu \)g) was treated with DNase and then reverse transcribed using oligo(dT) primers and the Sensiscript RT kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Reverse transcription negative controls were prepared without the addition of Sensiscript to exclude the presence of contaminating genomic DNA. PCR reactions were performed using the HotStarTaq Kit (Qiagen) with primer pairs based on known rat TRPC sequences specific for the different TRPC isoforms (Table 1). An Eppendorf Mastercycler personal thermal cycler (Brinkmann Instruments, Westbury, NY) was programmed to perform initial denaturation at 94°C for 12 min followed by 39 cycles of denaturation at 94°C for 1 min, annealing at 54.5°C for 30 s, and extension at 72°C for 2 min, followed by prolonged extension at 72°C for 10 min. Amplicons were separated on 2% agarose gels and visualized using ethidium bromide staining.

**Real-time PCR.** Real-time PCR reactions on RNA from human vessels were performed with primer pairs designed using Primer3 software (available online from the Whitehead Institute for Biomedical Research: http://frodo.wi.mit.edu/primer3/primer3_code.html) specific for the different human TRPC isoforms. Amplified regions were chosen to span one or more introns in the genomic sequence (Table 2). The specificity of the PCR products was verified by subjecting the amplicons to 2% agarose gel electrophoresis followed by sequencing. Transcribed...
Oligonucleotide sequences of human primers used for real time RT-PCR

Table 2. Oligonucleotide sequences of rat primers used for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward (5’-3’ )</th>
<th>Reverse (5’-3’ )</th>
<th>Amplicon, in bp</th>
</tr>
</thead>
<tbody>
<tr>
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<td>GTCGCCAGAGATGTTACAGAGTTTGGG</td>
<td>GCCGAACCTCCACTCTTATCTCTAGT</td>
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<td>CGAAGCAGATGCTGTTGACCC</td>
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<tr>
<td>rTRPC4</td>
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<tr>
<td>rTRPC5</td>
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<td>CTACAGGGGAGATGCTGTTATAGT</td>
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</tr>
<tr>
<td>rTRPC6</td>
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<tr>
<td>GAPDH</td>
<td>TGACCCCTTCTGAGGAGGA</td>
<td>GCAATGCGGAGAGCTGTTG</td>
<td>293</td>
</tr>
</tbody>
</table>

TRPC, transient receptor potential canonical.

cDNA was amplified using a LightCycler (Roche, Basel, Switzerland) in PCR mixtures (20 μl) consisting of 3 mM MgCl₂, 0.5 μM of each primer (Tables 1 and 2), and 1× LightCycler DNA Master SYBR Green I mix (Roche). The samples were incubated for initial denaturation at 95°C for 600 s, which was followed by 45 PCR cycles. For rat samples, each cycle consisted of 95°C for 1 s, 57°C for 6 s, and 74°C for 23 s; for human samples, each cycle comprised 95°C for 1 s, 51°C for 6 s, and 74°C for 23 s. LightCycler DNA Master SYBR Green I fluorescence emission was monitored after each cycle, and mRNA levels of the TRPC isofoms were quantified using the second-derivative maximum method of the LightCycler software. For each experiment, a baseline was set just above the background, and the quantitative results were obtained by determining crossing point values, which mark the cycle number at which sample fluorescence crosses a predetermined value. The amount of TRPC was expressed relative to the housekeeping gene GAPDH. To confirm amplification of specific transcripts, melting curve profiles (cooling the sample to 65°C and heating slowly to 95°C with continuous measurement of fluorescence) were produced at the end of each PCR reaction. The specificity of the PCR was further verified by subjecting the amplification products to agarose gel electrophoresis.

Western blotting. Cerebral arteries from two to four rats were pooled in each experiment and homogenized in lysis buffer. Proteins were separated by SDS-PAGE on 10% gels, transferred to nitrocellulose membrane, and blocked by rocking for 1.5 h at room temperature in blocking buffer [0.1% Tween 20 and 5% nonfat dry milk in Tris-buffered saline (TBS); see below]. Blots were exposed overnight to the primary rabbit antibodies T1E3 (1:500 in blocking buffer), anti-TRPC1 and anti-TRPC6 (1:200 and 1:500 dilutions, respectively; Alomone Labs, Jerusalem, Israel), or von Willebrand factor (DAKO, Carpinteria, CA); washed several times with 0.1% Tween 20 in Tris-buffered saline, 0.1% SDS, 1% nonfat dry milk, 5% BSA in PBS), anti-TRPC1 (1:100), and anti-TRPC6 (1:250) were applied overnight at 4°C. The secondary antibody, Cy5 anti-rabbit IgG (1:500 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA), was applied for 1 h at 25°C. The fluorescent nucleic acid dye YOYO-1 (1:40,000 dilution) was used for identification of nuclei. After being washed, preparations were mounted and examined with a ×40 oil-immersion lens objective in a Zeiss LSM 510 laser scanning confocal microscope. TRPC expression was detected by monitoring Cy5 fluorescence using an excitation wavelength of 650 nm and an emission wavelength of 670 nm. The pinhole setting yielded optical slices of 0.9 μm in thickness. For quantitation, isolated cells and multiple fields for each vessel were imaged and counted under blinded conditions. Three boxes of defined dimensions (10 μm² for single cells, 20 μm² for arterial whole mounts, and 200 μm² for arterial cross sections) were randomly positioned within the sample, and mean pixel intensity (range, 0–255 gray scale values) after background subtraction was calculated using Zeiss LSM 510 Pascal Analysis software. The number of cells, vessels, and sections analyzed, as well as the number of rats studied, is indicated in each figure.

Solutions and chemicals. All drugs and chemical agents were purchased from Sigma unless otherwise specified. Nominally Ca²⁺-, Mg²⁺-free modified Krebs solution (PSS) contained (in mM) 135.5 NaCl, 5.9 KCl, 1.2 MgCl₂, 11.6 glucose, and 11.6 HEPES, pH 7.35, at 37°C. DM solution used to dissociate cells contained (in mM) 0.16 CaCl₂, 110 NaCl, 5 KCl, 2 MgCl₂, 10 HEPES, 10 NaHCO₃, 0.5 KH₂PO₄, 0.5 NaH₂PO₄, 10 glucose, 0.04 phenol red, and 10 taurine. The pH was adjusted to 7.0 with NaOH. Lysis buffer used in Western blotting contained (in mM) 150 NaCl, 10 EDTA, 15 MgCl₂, 40 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 0.25% deoxycholate, 0.2% SDS, 1% NP-40, 10% glycerol, 1 Na⁺-orthovanadate, 1 NaF, 2.5 urea, 1:100 mammalian protease inhibitor cocktail, and 1 DTT. The TBS buffer used for Western blot analysis contained (in mM) 25 Trizma, 192 glycine, and 20% methanol.

Statistics. Results are expressed as means ± SE where applicable. Statistical significance was determined using a two-tailed Student’s t-test and was set at P < 0.05.
RESULTS

SOC currents in cerebral artery myocytes were studied using the whole cell patch-clamp technique. Cells were enzymatically dispersed from fresh vessels or from vessels that had been cultured for 3 days. After patch rupture and establishment of whole cell recording conditions, cells were maintained at −80 mV to deactivate voltage-gated Na+ and Ca2+ channels. Cell size, estimated on the basis of capacitance, was not significantly affected by the culture conditions (13.2 ± 0.9 pF, n = 8, and 13.0 ± 0.5 pF, n = 10, for fresh and cultured preparations, respectively). Figure 1A shows activation of an inward current in a cell from a cultured artery after depletion of intracellular Ca2+ stores using cyclopiazonic acid (CPA; 10 μM). The data shown in Fig. 1 were obtained using a low Ca2+ concentration (0.1 mM) in the extracellular medium, whereas pilot experiments using a higher concentration (10 mM) produced considerably smaller currents. CPA was added 8–10 min after establishment of whole cell recording, and current transients on addition of CPA were never observed. Consistently, a slowly developing, sustained but noisy current with a latency of 2–3 min was observed (Fig. 1). The mean value over 4 min was calculated, starting 3 min after the addition of CPA. As shown in Fig. 1B, current in cells from cultured vessels was twofold that in cells isolated from freshly dissected tissue. The CPA-induced current was essentially voltage independent and had a reversal potential close to 0 mV (Fig. 1C).

Depletion of intracellular Ca2+ stores by caffeine (10 mM) activated an inward current with a latency similar to that induced by CPA. Although the current usually developed more abruptly than it did with CPA, an overshooting transient possibly representing a Ca2+-activated Cl− current was never observed. The current was 70% increased by culture and insensitive to inhibition of L-type Ca2+ channels by nifedipine (1 μM), but it was moderately reduced (∼25%) by Ni2+ (5 mM) as shown in Fig. 1D.

To explore the molecular basis of the increased SOC current after organ culture, we studied the expression of TRPC isoforms. TRPC1–TRPC7 expression was evaluated using RT-PCR in single smooth muscle cells isolated from cultured vs. fresh arteries (Fig. 2A). With the use of the same dissociation protocol as that used for electrophysiological experiments and a differential interference contrast objective, ∼50 elongated, spindle-shaped smooth muscle cells were collected for each condition. Cells were chosen according to their shape, size, and brightness, which are the criteria that normally yield viable cells for patch-clamp experiments. Bands for all of the amplified TRPC products were of the expected sizes (Table 1). As shown in Fig. 2B, there was a dramatic upregulation of TRPC1 expression (57 vs. 7%) as well as increased TRPC6 expression (47 vs. 8%), whereas TRPC3 levels were found to decrease upon culture (6 vs. 18%).

TRPC expression in intact tissue is influenced by the presence of endothelial cells in addition to smooth muscle. Using real-time quantitative PCR, we compared TRPC isoform expression in cerebral arteries before and after 3 days of culture. In contrast to the results observed in isolated cells, TRPC4 was abundantly expressed in freshly dissected intact arteries (Fig. 3A) and also was detectable at low levels after endothelial
rubbing (Fig. 3B). No TRPC4 was detected in arteries rubbed after culture. The extent of removal of endothelium by rubbing was estimated on the basis of Western blot analysis demonstrating the persistence of 46% of von Willebrand factor after denudation in fresh arteries, decreasing to 37% in arteries denuded after culture (Fig. 4A). The decrease in TRPC4 after rubbing and culture is thus consistent with a predominantly endothelial localization of TRPC4 (8).

In contrast to results in isolated cells (Fig. 2A), changes in TRPC1 and TRPC6 mRNA expression in denuded arteries were not significant, whereas TRPC3 was significantly decreased (Fig. 3C). This discrepancy suggests that increases in TRPC1 and TRPC6 expression in smooth muscle cells during culture is masked in the whole vessel wall by a loss of endothelium and a consequent decrease of an endothelial pool of these isoforms. Indeed, confocal imaging of the endothelial cell layer in whole mounts of intact cerebral arteries confirmed abundant TRPC1 expression (Fig. 4B). Of the other isoforms studied, mRNA for TRPC5 was inconsistently detected in cerebral vessels, while TRPC2 and TRPC7 were not found.

Because only TRPC1 and TRPC6 mRNA levels were found to increase in cerebral arterial cells after culture, one or both of these isoforms might underlie the increased SOC currents induced upon store depletion. To confirm this hypothesis, we studied the effect of culture on these isoforms at the protein level. Western blotting of endothelium-denuded cultured arteries compared with fresh arteries showed increased TRPC1 protein contents, while TRPC6 levels did not differ between fresh and cultured preparations (Fig. 4C). Western blotting of nondenuded vessels showed unchanged TRPC1 and decreased TRPC6 after culture (data not shown), consistent with loss of endothelial TRPC expression during culture.

To further investigate the expression of TRPC1 and TRPC6, we stained arterial whole mounts and cross sections as well as isolated smooth muscle cells. Immunofluorescence experiments showed a highly significant TRPC1 upregulation upon culture, which is in agreement with our PCR data. Representative images of whole vessels and isolated cells stained with anti-TRPC1 are shown in Fig. 5A. Nuclei were stained with the nucleic acid binding fluorescent dye YOYO-1 to visualize the individual cells. Notice that in contrast to the endothelial staining shown in Fig. 4B, the direction of the smooth muscle cells stained in Fig. 5A is perpendicular to the vessel direction of flow. Fluorescence intensity was measured as described in METHODS. Briefly, boxes of defined size were randomly placed on the vessels/cells, and mean pixel intensity was calculated after background subtraction. Summarized data for each measurement condition are shown in Fig. 5B. In stained arterial whole mounts and isolated smooth muscle cells, we found a significant increase in both TRPC1 and TRPC6. In addition to the anti-TRPC1 antibody used in the images shown in Figs. 4B and 5A, we immunostained arterial cross sections using the T1E3 antibody raised against TRPC1 and shown to block SOCE into vascular cells (31). This again showed massive
upregulation of TRPC1 after culture (Fig. 5B). Western blotting demonstrated that both antibodies were specific for a protein of the mass predicted for TRPC1, which ranges between 87 and 92 kDa depending on the splicing variant (Fig. 5C).

To directly test the involvement of TRPC1 in upregulated SOC activity after culture, we investigated the effect of overnight pretreatment with the TRPC1-specific antibody T1E3 on contraction in cultured vascular segments mounted in a myograph. While freshly prepared vessels did not exhibit any contractile responses to store depletion, cultured cerebral vessels developed a verapamil-insensitive contraction (4). As shown in Fig. 6, contractions elicited by store depletion in cultured vessels were reduced by 50±12% after T1E3 pretreatment. This finding shows that there is a large SOC component that can contribute to force generation in the cultured arteries. This response appears to involve a TRPC1 protein.

The results obtained using rat cerebral arteries indicate that serum-free culture by itself is sufficient to cause increased TRPC expression. The mechanism behind this effect is not known but hypothetically might involve a reaction to the trauma associated with dissection and the change of environment. If so, altered TRPC expression might be found in vessel injury leading to clinical manifestations such as restenosis after angioplasty. We tested this hypothesis in human internal mammary artery, taking care to separate the effects of injury from those of the culture itself. Paired segments of human internal mammary artery were cultured for 24 h to equilibrate to the culture environment, and then one segment was exposed to balloon dilatation for 2 min (see METHODS) before further culture for up to 48 h. The control segment was cultured without dilatation. Figure 7 shows real-time RT-PCR determinations of TRPC expression at 6, 24, and 48 h after dilatation. While control segments showed no clear trend with time after the initial 24 h of culture, dilated segments showed a progressive increase in TRPC1 and TRPC6. These results suggest that increased TRPC expression is a feature of vascular injury and may be characteristic of the reaction to angioplasty.

**DISCUSSION**

The present study has shown plasticity in TRPC expression in smooth muscle of rat cerebral arteries during organ culture, correlating with increased SOC-induced currents and contraction. Serum-free organ culture of intact vessels preserves contractility and does not stimulate cell proliferation (17) but is associated with altered Ca²⁺ handling (4, 5), suggesting that a slow shift in phenotype occurs. Dissection of a blood vessel and placement in an artificial medium necessarily constitutes trauma, which is expected to cause a tissue reaction. In this respect, organ culture may reproduce early processes occurring in vivo, leading to neointima formation (15, 30). However, it provides a sufficiently stable background to reveal effects of a superimposed vessel injury as demonstrated in the present study by balloon dilatation of human
arteries in organ culture after equilibration to the culture conditions. Compared with uninjured control segments, the dilated arterial segments showed increased expression of TRPC1 and TRPC6 similar to the effects of culture alone. Comparison of TRPC expression in isolated cells and in the intact vascular wall revealed considerable differences. The endothelium expresses abundant TRPC and cannot be removed easily as demonstrated by the persistence of von Willebrand factor after mechanical denudation, even though this procedure has proved to be effective in abolishing endothelium-dependent relaxation in rat cerebral vessels (2). To study TRPC expression specifically in smooth muscle cells, we performed RT-PCR in pooled cells picked after dispersion of either fresh or cultured vessels. This experiment showed massive upregulation of TRPC1 and TRPC6 as well as downregulation of TRPC3.

Whole cell voltage-clamp experiments in cells isolated from freshly dissected and organ-cultured tissue confirmed upregulation of an inward current activated by store depletion. The reversal potential was close to 0 mV as expected of a nonselective cation current. The current-voltage relationship was essentially linear, consistent with observations of only weak rectification of store-operated currents in native vascular cells (10, 22, 26, 28). It is not likely to reflect a Ca\(^2+\)-activated Cl\(^-\) current, because Ca\(^2+\) was well buffered by the pipette solution, long (8–10 min) cell dialysis was allowed before store depletion, and a transient inward current was not observed upon application of CPA or caffeine. Similar results have been reported in rat pulmonary artery cells (22) with the use of essentially the same recording conditions as we used in the present study, while in the previous study CPA addition in perforated patch recordings caused a large inward transient (22).

Our laboratory (4) previously showed that voltage-dependent L-type currents are reduced after organ culture of cerebral arteries. The present findings instead indicate an increase in a nifedipine-insensitive voltage-independent current with properties consistent with those of a store-operated current. To date, no selective pharmacological inhibitors of store-operated currents have been reported, but the T1E3 antibody used in the present study to show inhibition of contraction due to store depletion in cultured arteries was previously demonstrated to inhibit store-operated current in vascular smooth muscle cells (31).

It should be noted that the protocol for store depletion in the contractility experiments in intact arteries was different from that used in single cells. A combination of caffeine and thapsigargin applied for a limited time (10 min) was used to deplete Ca\(^2+\) stores and irreversibly block reuptake during subsequent Ca\(^2+\) addition, while in the electrophysiological experiments with single cells, store depletion was achieved during continued recording. A latency of several minutes was observed both with CPA and with caffeine, but the more abrupt onset of the current after latency subsequent to caffeine application may reflect a more rapid and complete store depletion with the use of this compound than with the reversible Ca\(^2+\)-ATPase inhibitor CPA.
While there is evidence of a role for TRPC1 in SOCE in vascular smooth muscle cells (26, 31), TRPC6 is activated by diacylglycerol (12) and has been implicated in adrenergic (14) and PDGF-mediated (32) responses. A systematic study of possible multimers of TRPC isoforms expressed in human embryonic kidney HEK-293 cells suggested that whereas TRPC3, TRPC6, and TRPC7 could combine, TRPC1 forms complexes only with TRPC4 and TRPC5 (13). In our present study, mRNA for TRPC5 was detectable in human arteries, while TRPC5 mRNA was inconsistently detected in rat cerebral arteries. Also, low levels of a 110-kDa protein corresponding to the predicted molecular mass of TRPC5 (111.5 kDa) were detected in rat cerebral arteries by performing Western blot analysis (unpublished results). These results suggest that this potential partner for TRPC1 may be present in both preparations, although at a low level. Other possible partners of TRPC1 include the intracellular Ca^{2+} channel protein polycystin-2 (1, 29) and an inhibitor of the myogenin family, which has been shown to associate with TRPC1 and to negatively regulate SOCE in transfected cells (19). While possible binding partners to TRPC1 are not identified in the present study, it is significant that a blocking TRPC1 antibody (31) prevented the increase in SOCE after culture. This strongly indicates that TRPC1 is a component of the native channel accounting for Ca^{2+} inflow in response to store depletion.

The increase in TRPC6 expression during culture and after vessel injury suggests that this isoform contributes to altered vascular responsiveness under these conditions. TRPC6 has been shown to be associated with receptor-operated Ca^{2+} inflow in vascular smooth muscle (14, 32). It may thus contribute to plasticity of receptor responses associated with phenotype modulation. Increased TRPC6 expression after PDGF stimulation in pulmonary artery smooth muscle cells has been shown to be associated with increased SOC activity (32). Incubation with TRPC6 antisense DNA reduces pressure-dependent myogenic tone and osmotically induced currents in rat cerebral arterial cells (30), suggesting that TRPC6 is involved in stretch-sensing mechanisms in this tissue. Our present findings do not specifically support or rule out any of these possibilities, and it is therefore likely that organ culture, and by implication vascular injury, is associated with TRPC-mediated effects in addition to those shown in the present study to be associated with increased TRPC1 expression.

Upregulation of TRPC1 in association with increased SOC current during growth stimulation of cultured human pulmonary arterial myocytes was previously demonstrated in a study by Golovina et al. (10). In their study, basal [Ca^{2+}], was found to be increased and chelation of Ca^{2+} inhibited growth. In a subsequent study by the same laboratory (26), inhibition of TRPC1 protein synthesis using an antisense oligonucleotide was shown to decrease SOC currents and cell growth. Investigators at our laboratory (33) have recently shown that the stimulation of protein synthesis elicited by stretch of the vascular wall is dependent on intact caveolae and endogenous release of ET-1. Our laboratory (2) also has demonstrated that TRPC1 is functionally coupled to ET-1 type A receptors in

**Fig. 6.** Effect of a TRPC1-blocking antibody on store-operated channel (SOC)-dependent contraction in rat cerebral arteries. Cerebral arteries were cultured for 3 days and then incubated overnight at 4°C with T1E3 antibody or with preimmune serum (control). Force in response to high-K+ and in response to addition of Ca^{2+} (2.5 mM) in the presence of verapamil (1 mM) after depletion of intracellular stores by caffeine (10 mM) and thapsigargin (10 mM) is shown. Summary data show SOC-induced mean force integrated over a 5-min period and relative to high-K+ response (n = 4). *P < 0.05.

**Fig. 7.** Time course of trauma-induced changes in TRPC mRNA expression in human internal mammary artery. Summary data from real-time PCR data of TRPC isoforms normalized to GAPDH, showing increased TRPC1 (A) and TRPC6 mRNA (B) after balloon dilatation. Paired control and balloon-dilated denuded arterial segments (open and shaded bars, respectively) were studied at 6, 24, and 48 h after injury. For each time point, PCR experiments from three to four patients were analyzed. *P < 0.05. **P < 0.01.
intact arteries and is localized to cholesterol-rich membrane caveolae. In addition, organ culture of cerebral arteries was found to increase sensitivity of both SOCE and ET-1 responses to disruption of caveolae by cholesterol depletion (2). These results suggest an association of SOCE with trophic signals in native vascular smooth muscle. The present findings provide evidence for an association of TRPC1 with SOCE, and thus the plasticity of TRPC1 expression may have implications for growth stimulation and altered smooth muscle phenotype in response to vascular injury.

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