Diverse properties of store-operated TRPC channels activated by protein kinase C in vascular myocytes

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In vascular smooth muscle, store-operated channels (SOCs) contribute to many physiological functions including vasoconstriction and cell growth and proliferation. In the present work we compared the properties of SOCs in freshly dispersed myocytes from rabbit coronary and mesenteric arteries and portal vein. Cyclopiazonic acid (CPA)-induced whole-cell SOC currents were sixfold greater at negative membrane potentials and displayed markedly different rectification properties and reversal potentials in coronary compared to mesenteric artery myocytes. Single channel studies showed that endothelin-1, CPA and the cell-permeant Ca^{2+} chelator BAPTA-AM activated the same 2.6 pS SOC in coronary artery. In 1.5 mM $[Ca^{2+}]_o$ the unitary conductance of SOCs was significantly greater in coronary than in mesenteric artery. Moreover in 0 mM $[Ca^{2+}]_o$ the conductance of SOCs in coronary artery was unaltered whereas the conductance of SOCs in mesenteric artery was increased fourfold. In coronary artery SOCs were inhibited by the protein kinase C (PKC) inhibitor chelerythrine and activated by the phorbol ester phorbol 12,13-dibutyrate (PDBu), the diacylglycerol analogue 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) and a catalytic subunit of PKC. These data infer an important role for PKC in activation of SOCs in coronary artery similar to mesenteric artery and portal vein. Anti-TRPC1 and -TRPC5 antibodies inhibited SOCs in coronary and mesenteric arteries and portal vein but anti-TRPC6 blocked SOCs only in coronary artery and anti-TRPC7 blocked SOCs only in portal vein. Immunoprecipitation showed associations between TRPC1 and TRPC5 in all preparations but between TRPC5 and TRPC6 only in coronary artery and between TRPC5 and TRPC7 only in portal vein. Finally, flufenamic acid increased SOC activity in coronary artery but inhibited SOCs in mesenteric artery and portal vein myocytes. These data provide strong evidence that vascular myocytes express diverse SOC isoforms, which are likely to be composed of different TRPC proteins and have different physiological functions.

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Vascular smooth muscle cells possess non-selective cation channels, which are activated by several stimuli including vasoconstrictor agents (Large, 2002). When stimulated these channels allow the entry of cations, primarily Na^+ and Ca^{2+} ions, to produce membrane depolarization. These events have been implicated in several physiological functions such as vasoconstriction and cell growth and proliferation. (Large, 2002; Beech *et al.* 2004; Dietrich *et al.* 2007; Firth *et al.* 2007). We have been investigating the physiological properties of native conductances by studying ion channel activity with whole-cell and single channel recording in freshly dispersed vascular myocytes. Our experiments have revealed that agonists such as noradrenaline (NA), angiotensin-II (Ang II) and endothelin-1 (ET-1) evoke two distinct classes of membrane non-selective cation conductances. First, in rabbit portal vein and mesenteric, coronary and ear arteries these vasoconstrictors activate cation channels with unitary conductances between 13 and 70 pS that are mediated by diacylglycerol (DAG) in a protein kinase C (PKC)-*independent* manner (Helliwell & Large, 1997; Albert & Large, 2001; Albert *et al.* 2003; Saleh *et al.* 2006; Peppiatt-Wildman *et al.* 2007). These channels are not stimulated by depletion of intracellular Ca^{2+} stores with sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors such as cyclopiazonic acid (CPA) and hence these conductances are termed receptor-operated channels (ROCs). However, in the same preparations these vasoconstrictors also induce channel currents with much smaller unitary conductances (about 2 pS) that

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are mediated by DAG via a PKC-*dependent* mechanism. Since these conductances are also evoked by CPA and other procedures to deplete Ca^{2+} stores they are called store-operated channels (SOCs; Albert & Large, 2002*a*,*b*; Saleh *et al.* 2006; present work). Moreover, the fact that these channel currents can also be evoked by phorbol esters and calmodulin (CaM) suggests that they may be gated in a polymodal manner (Albert *et al.* 2007).

A notable feature is that ROCs display diverse properties that are likely to be associated with distinctive physiological functions. For example, the ROC evoked by noradrenaline in rabbit ear artery myocytes has high constitutive activity and therefore contributes to the resting membrane conductance as well as functioning as a ROC (Albert *et al.* 2003). In contrast, in rabbit mesenteric and coronary artery myocytes, Ang II and ET-1, respectively, evoke cation currents that demonstrate little spontaneous activity and are only active in the presence of the agonists (Saleh *et al.* 2006; Peppiatt-Wildman *et al.* 2007). Therefore these latter conductances are classical ROCs and are unlikely to contribute to the resting membrane potential. Also, inositol 1,4,5-trisphosphate (IP_3), which is produced by receptor stimulation, potentiates and accelerates agonist-evoked ROCs in rabbit portal vein (Albert & Large, 2003) and coronary artery (Peppiatt-Wildman *et al.* 2007) but has no effect on the ROC in mesenteric artery (Saleh *et al.* 2006). ROCs also demonstrate distinctive pharmacological profiles in that the agent flufenamic acid (FFA) potentiates ROCs in portal vein and mesenteric artery (Inoue *et al.* 2001; Saleh *et al.* 2006) but inhibits the conductance in rabbit ear and coronary arteries (Albert *et al.* 2006*a*; Peppiatt-Wildman *et al.* 2007). It is probable that these diverse properties result from different molecular compositions of the underlying ion channels. There is increasing evidence that canonical transient receptor potential (TRPC) proteins are components of non-selective cation channels in smooth muscle including TRPC1 as a component of SOCs (Xu & Beech, 2001; Large, 2002; Beech *et al.* 2004; Albert & Large, 2006; Brueggemann *et al.* 2006; Saleh *et al.* 2006; Albert *et al.* 2007). Moreover there is evidence that TRPC channels can exist in both homo- and heterotetrameric structures (e.g. Strubing *et al.* 2001; Goel *et al.* 2002; Hofmann *et al.* 2002; Mio *et al.* 2005; Zagranichnaya *et al.* 2005) and therefore the diverse characteristics of native channels may result from different TRPC compositions.

Considerably less is known about the physiological properties of SOCs in freshly dispersed myocytes but it is becoming evident that these conductances may also exhibit diverse characteristics. Recently during experiments on rabbit coronary artery myocytes we observed that ET-1 evoked a SOC that appeared to have significantly different characteristics from the SOC previously described in rabbit mesenteric artery. In the present work we show that SOCs in these two vascular preparations possess distinct biophysical properties in terms of unitary conductance and sensitivity to external Ca^{2+} ions. In addition the conductances exhibit distinguishing pharmacology and differential sensitivity to anti-TRPC antibodies. These results suggest that these SOC isoforms may consist of different TRPC subunits in different blood vessels. Some of these data were published in preliminary form (Albert *et al.* 2007).

Methods

Cell isolation

New Zealand White rabbits (2–3 kg) were killed using I.V. sodium pentobarbitone (120 mg kg^{-1}, in accordance with the UK Animals (Scientific Procedures) Act 1986). Right and left anterior descending coronary arteries, first to fifth order mesenteric arteries and portal vein tissue were dissected free from fat and connective tissue in physiological salt solution containing (mM): NaCl (126), KCl (6), glucose (10), Hepes (11), $MgCl_2$ (1.2), $CaCl_2$ (1.5), pH 7.2 adjusted with 10 M NaOH. An incision was made along the longitudinal axis of the blood vessels and the exposed endothelium was gently removed using a cotton bud. Enzymatic digestion and smooth muscle cell isolation were subsequently carried out using methods previously described (Saleh *et al.* 2006).

Electrophysiology

Whole-cell and single SOC currents were recorded in voltage-clamp mode using whole-cell recording, cell-attached, inside out and outside-out patch configurations (Hamill *et al.* 1981) with a HEKA EPC 8 patch-clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany) at room temperature (20–23°C). Whole-cell membrane potential recordings were made in current-clamp mode. Patch pipettes were manufactured from borosilicate glass to produce pipettes with resistances of about 5 MΩ for whole-cell recording and 6–10 MΩ for isolated patch recording when filled with patch pipette solution. To reduce 'line' noise the recording chamber (vol. *ca* 150–200 μl) was perfused using two 20 ml syringes, one filled with external solution and the other used to drain the chamber, in a 'push and pull' technique. The external solution could be exchanged twice within 30 s. When recording whole-cell and single SOC currents the holding potential was routinely set at 0 mV and −80 mV, respectively. To evaluate current–voltage (I–V) characteristics of whole-cell currents, membrane potential ramps were applied every 20–30 s between −100 mV and +100 mV (0.2 V s^{-1}) and in single channel recordings the membrane potential was manually altered between −120 mV and +100 mV.

Whole-cell SOC currents and single SOC currents were initially recorded onto digital audiotape (DAT) using a Biologic DRA-200 digital tape-recorder (BioLogic Science Instruments, Claix, France) at a bandwidth of 5 kHz (HEKA EPC 8 patch-clamp amplifier) and a sample rate of 48 kHz. For off-line analysis whole-cell SOC currents and membrane potentials were filtered at 1 kHz and single SOC currents at 100 Hz (see below, −3 db, low pass 8-pole Bessel filter, model LP02, Frequency Devices, Ottawa, IL, USA) and acquired using a Digidata 1322A and pCLAMP 9.0 (Molecular Devices/MDS Analytical Technologies) at sampling rates of 5 kHz and 1 kHz, respectively. Data were captured with a personal computer (Dell Dimension 5150).

Single SOC current amplitudes were calculated from idealized traces of at least 60 s in duration using the 50% threshold method and analysed using pCLAMP v.9.0 software with events lasting for < 6.664 ms ($2 \times$ rise time for a 100 Hz, −3 db, low pass filter) being excluded from analysis. Single channel current amplitude histograms were plotted and fitted with Gaussian curves with the peak of these curves determining the unitary amplitude of the single channel currents. Open probability (NP_o) was calculated using the equation:

$$NP_o = \frac{\sum (O_n n)}{T}$$

where $n =$ number of channels in the patch, $O_n =$ time spent at each open level and $T =$ total recording time. Figure preparation was carried out using Origin 6.0 software (OriginLab Corp., Northampton, MA, USA) where inward single SOC currents are shown as downward deflections.

Immunoprecipitation and Western blotting

Dissected tissues were either flash frozen and stored at −80°C for subsequent use or immediately placed into 10 mg ml^{-1} RIPA lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) supplemented with protease inhibitors and then homogenized on ice by sonication for at least 3 h. The total cell lysate (TCL) was collected by centrifugation at 1000*g* for 10 min at 4°C and then protein content was quantified using the Bio-Rad protein dye reagent (Bradford method). TCL was pre-cleared using A/G agarose beads (Santa Cruz biotechnology) and immunoprecipitated overnight at 4°C using the appropriate antibody and A/G agarose bead conjugate. Alternatively, the immunoprecipitation protocol was carried out using the Catch and Release kit (Upstate/Millipore), where spin columns were loaded with 500 μg of cell lysates, 4 μg of antibody and immunoprecipitated for 2 h at room temperature.

Protein samples were eluted with Laemmli sample buffer and incubated at 95°C for 2 min. One-dimensional protein gel electrophoresis was performed in 4–12% Bis-Tris gels in a Novex mini-gel system (Invitrogen) with 20 μg of total protein loaded in each lane. Separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes in a Bio-Rad trans-blot SD semi-dry transfer cell or using the iBlot apparatus (Invitrogen). Blots were incubated for 1–4 h with 5% (weight/volume) non-fat milk in phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) to block non-specific protein binding. Membranes were then incubated with appropriate primary antibody overnight at 4°C. (Where possible alternative antibodies raised against different epitopes were used for immunoprecipitation and Western blot analysis.) Following antibody removal blots were washed for 2 h with milk/PBST. Blots were subsequently incubated with horseradish peroxidase-conjugated goat anti-rabbit (Sigma) or sheep anti-goat (Santa Cruz) IgG secondary antibody diluted 1 : 1000–1 : 5000 in milk/PBST, washed 3 times in PBST, and treated with ECL chemiluminescence reagents (Pierce Biotechnology, Inc., Rockford, IL, USA) for 1 min and exposed to photographic films.

All antibodies used for immunoprecipitation and Western blotting were tested with positive control samples (rat brain extracts from Santa Cruz Biotechnology for Alomone TRPC1, C5, C6 and Santa Cruz TRPC5, C6; rat testis extract from Santa Cruz Biotechnology for Santa Cruz TRPC1) and negative control samples where the antigenic peptide was included with the precipitating antibody or TCL was omitted from the procedure. In all instances the procedure was repeated with both different antibodies alternately used for the precipitation or the Western blotting step and data shown represent *n*-values of at least three separate experiments.

Anti-TRPC antibodies

Polyclonal TRPC1, C3, C4, C5 and C6 antibodies generated in rabbits against intracellular epitopes were purchased from Alomone Laboratories (Jerusalem, Israel; n.b. Chemicon, Sigma and Biomol Laboratories retail anti-TRPC antibodies raised against the same epitopes as Alomone Laboratories). The selectivity and negligible cross-reactivity of these anti-TRPC antibodies for their target proteins have been previously confirmed (Liu *et al.* 2005; Zagranichnaya *et al.* 2005; Sours *et al.* 2006; Ju *et al.* 2007). Alternative polyclonal antibodies for TRPC1, TRPC3, TRPC4, TRPC5 and TRPC6 generated in goats against different intracellular epitopes were purchased from Santa Cruz Biotechnology. The specificity of these antibodies has also been previously confirmed (Liu *et al.* 2005; Zagranichnaya *et al.* 2005). Polyclonal

TRPC7 antibody was generated against the sequence Glu-Lys-Phe-Gly-Lys-Asn-Leu-Asn-Lys-Asp-His-Leu-Arg-Val-Asn corresponding to positions 843–857 of human and mouse TRPC7 (Accession nos NP_065122 and NP_036165, respectively) and previously characterized by Goel *et al.* (2002). The synthetic purified peptide was conjugated and used to immunize rabbits and all procedures were carried out by Sigma Genosys labs (Cambridge, UK). The antiserum was affinity purified using the AminoLink® Plus immobilization kit and the Melon Gel IgG purification kit (Pierce) and assessed using Western blotting with an enzyme-linked immunosorbent assay where pre-immune serum had no activity.

Solutions and drugs

The bathing solution used to measure whole-cell SOC currents and single SOC currents in outside-out patches was K^+ free and contained (mM): NaCl (126), $CaCl_2$ (1.5), Hepes (10), glucose (11), DIDS (0.1), niflumic acid (0.1) and nicardipine (0.005), pH adjusted to 7.2 with NaOH. In cell-attached patch experiments the membrane potential was set to approximately 0 mV by perfusing cells in a KCl external solution containing (mM): KCl (126), $CaCl_2$ (1.5), Hepes (10) and glucose (11), pH adjusted to 7.2 with 10 M KOH. Nicardipine (5 μM) was also included to prevent smooth muscle cell contraction by blocking Ca^{2+} entry through voltage-dependent Ca^{2+} channels.

The patch pipette solution used to measure whole-cell SOC currents and SOCs in outside-out patches (intracellular solution) was also K^+ free and contained (mM): CsCl (18), caesium aspartate (108), $MgCl_2$ (1.2), Hepes (10), glucose (11), BAPTA (10), $CaCl_2$ (4.8, free internal Ca^{2+} concentration approximately 100 nm as calculated using EQCAL software), Na_2ATP (1), NaGTP (0.2), pH 7.2 with Tris. The patch pipette solution used for both cell-attached and inside-out patch recording (extracellular solution) was K^+ free and contained (mM): NaCl (126), $CaCl_2$ (1.5), Hepes (10), glucose (11), TEA (10), 4-AP (5), iberiotoxin (0.0002), DIDS (0.1), niflumic acid (0.1) and nicardipine (0.005), pH adjusted to 7.2 with NaOH. In some experiments 1.5 mM $CaCl_2$ was removed and 1 mM BAPTA was added (< 10 nM free Ca^{2+}). The composition of the bathing solution used in inside-out experiments (intracellular solution) was the same as the patch pipette solution used for whole-cell and outside-out patches except that 1 mM BAPTA and 0.48 mM $CaCl_2$ were included (free internal Ca^{2+} concentration approximately 100 nM). Under these conditions VDCCs, K^+ currents, swell-activated Cl^- currents and Ca^{2+}-activated conductances are abolished and non-selective cation currents could be recorded in isolation.

All drugs were purchased from either Calbiochem (UK), Sigma (UK) or Tocris (UK) and agents were dissolved in distilled H_2O or DMSO (0.1%). DMSO alone had no effect on SOC activity. The values are the mean of n cells ± S.E.M. Statistical analysis was carried out using paired (comparing effects of agents on the same cell) or unpaired (comparing effects of agents between cells) Students' t test with the level of significance set at $P < 0.05$.

Results

Distinctive properties of CPA-evoked whole-cell cation conductances in coronary and mesenteric artery myocytes

In the first series of experiments we compared whole-cell currents evoked by CPA in coronary and mesenteric arteries. In these studies internal Ca^{2+} was clamped at 100 nM with a high concentration of BAPTA (10 mM), which is the conventional method of recording SOC activity with whole-cell recording.

It was evident that whole-cell SOC currents in coronary and mesenteric arteries exhibited markedly different properties that were highlighted by different current amplitudes at negative membrane potentials and current–voltage (I–V) relationships with distinct rectification properties and reversal potentials (E_r). Figure 1 shows that bath application of 10 μM CPA activated whole-cell SOC currents in both preparations at −80 mV and illustrates that the mean peak amplitude of -6.4 ± 1.4 pA pF^{-1} ($n = 10$) in coronary artery was sixfold greater than the mean peak amplitude of -1.1 ± 0.5 pA pF^{-1} ($n = 10$) in mesenteric artery. Moreover Fig. 1*B* and *C* illustrates that CPA-evoked SOC currents in coronary artery had a relatively linear mean current–voltage (I–V) relationship with an E_r of $+4 \pm 1$ mV ($n = 10$) whereas in mesenteric artery CPA-induced SOC currents displayed a pronounced outwardly rectifying I–V relationship and an E_r of $+22 \pm 4$ mV ($n = 10, P < 0.001$). Moreover, current ratios between coronary and mesenteric artery at −100 and +100 mV were about 10 and 1, respectively, which further illustrated differences between the I–V relationships in these two preparations (Fig. 1*C*).

These pronounced differences in the biophysical properties of CPA-evoked whole-cell SOC currents in coronary and mesenteric arteries strongly indicates that there are likely to be fundamental differences between the ion channels underlying the SOCs in these two arterial preparations.

Single channel properties of SOCs in coronary and mesenteric arteries

To gain more precise information on the SOCs in the two preparations we compared the properties of SOCs at the single channel level.

Figure 2*Aa–c* shows that bath application of the endogenous coronary vasoconstrictor ET-1 (100 nM, $n = 8$) and CPA (10 μM, $n = 8$) evoked cation channel currents in cell-attached patches from coronary artery myocytes, which had slope conductances of 2.6 and 2.7 pS, respectively, and E_r of about 0 mV. Moreover the cell-permeant Ca^{2+} chelator BAPTA-AM (50 μM), which passively depletes internal Ca^{2+} stores, also evoked channel currents with similar properties in cell-attached patches ($n = 8$, data not shown). These data show that ET-1, CPA and BAPTA-AM activate the same cation conductance in coronary artery myocytes which is termed a SOC.

A notable result was that single SOC currents had different biophysical properties in coronary and mesenteric arteries. In 1.5 mM $[Ca^{2+}]_o$ SOCs in mesenteric artery had a significantly smaller unitary conductance of 1.9 ± 0.2 pS ($n = 8$, Table 1, and previous data, Saleh *et al.* 2006) than SOCs in coronary artery, which had a conductance of 2.6 pS ($P < 0.05$, Fig. 2*Ac*, Table 1). Additionally, in 1.5 mM $[Ca^{2+}]_o$ SOCs in mesenteric artery also had a significantly different mean E_r of $+15 \pm 4$ mV ($n = 6$) compared to -2 ± 3 mV ($n = 7$, $P < 0.05$) in coronary artery (Table 1).

Previous work has shown that a distinctive feature of SOCs in mesenteric artery and in portal vein is that removing external Ca^{2+} ions increases unitary conductance (Albert & Large, 2002*a*; Saleh *et al.* 2006) and therefore we compared the effect of removing $[Ca^{2+}]_o$ on the unitary properties of SOCs in coronary and mesenteric arteries. Figure 2*Ba–c* shows that in 0 $[Ca^{2+}]_o$ the unitary conductance of SOCs in coronary artery was 2.7 ± 0.1 pS ($n = 7$) and similar to the value in 1.5 mM $[Ca^{2+}]_o$. In contrast in mesenteric artery in 0 $[Ca^{2+}]_o$ the conductance of SOCs was increased fourfold to 7.5 ± 1.4 pS ($n = 6$, $P < 0.05$, Table 1). Moreover the mean E_r of SOCs in mesenteric artery was significantly shifted from $+15 \pm 4$ mV ($n = 6$) in 1.5 mM $[Ca^{2+}]_o$ to -4 ± 1.3 mV in 0 mM ($n = 6$, $P < 0.05$, Fig. 2*Bc*, Table 1), which corresponds to a permeability ratio of Ca^{2+} to Na^+ ions (P_{Ca}/P_{Na}) of about 66 (using equation 3 of Jatzke *et al.* 2001). In contrast the mean E_r of SOCs in coronary artery was similar in 1.5 mM and 0 mM $[Ca^{2+}]_o$ suggesting P_{Ca}/P_{Na} of about 1 (Table 1). These different properties of single channels indicate that there are two SOC isoforms in coronary and mesenteric arteries.

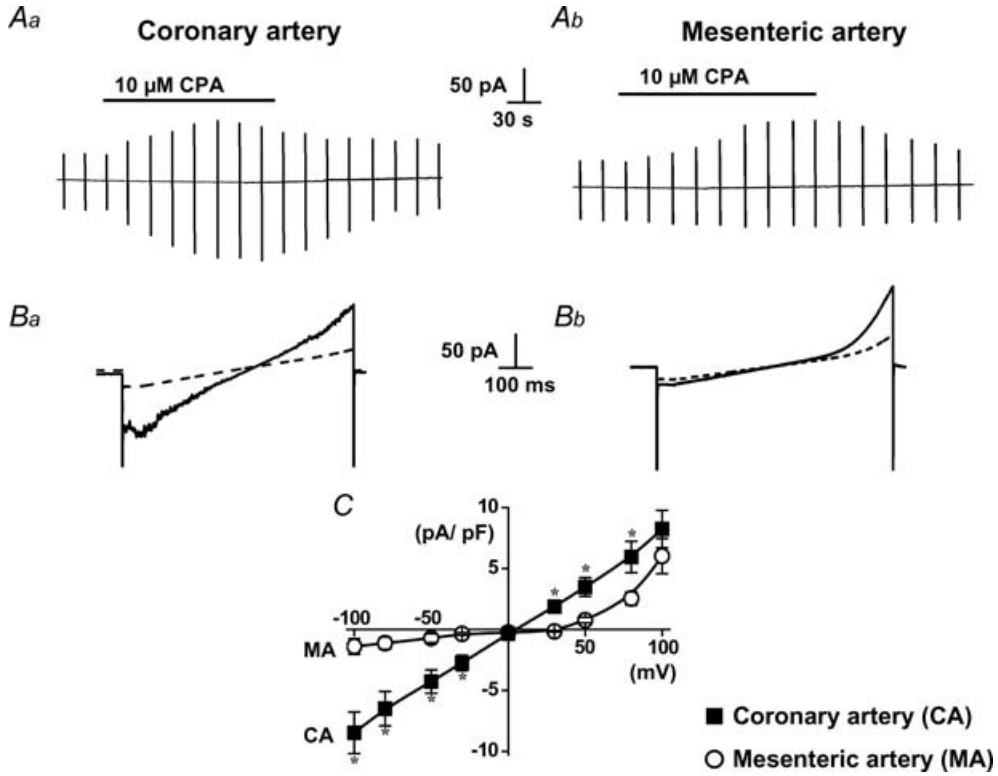

Figure 1. CPA-evoked whole-cell cation conductances in coronary and mesenteric artery myocytes
Aa and *b*, original traces showing that 10 μM CPA activated whole-cell SOC currents in coronary and mesenteric arteries. Vertical deflections represent current responses to voltage ramps from −100 mV to +100 mV. *Ba* and *b*, individual *I–V* relationships before (dashed line) and during (continuous line) CPA-induced whole-cell SOC currents in coronary and mesenteric arteries. *C*, mean *I–V* relationships of whole-cell SOC currents ($n = 10$ of each).

Table 1. Comparison of properties of PKC-activated SOCs in vascular smooth muscle

	Coronary artery	Mesenteric artery	Portal vein
Amplitude of CPA-induced −80 mV whole-cell SOC currents at −50 mV	6.5 pA pF^{-1}*	1.1 pA pF^{-1}	1.0 pA pF^{-1}[a]
I–V relationships	Linear	Outwardly rectifying	Outwardly rectifying
Whole-cell E_r in 1.5 $[Ca^{2+}]_o$	+4 mV*	+22 mV	+25 mV[a]
Unitary conductances			
1.5 mM $[Ca^{2+}]_o$	2.6 pS*	1.9 pS	2.1 pS[a]
0 mM $[Ca^{2+}]_o$	2.7 pS*	7.5 pS	7.4 pS[a]
E_r in 1.5 mM $[Ca^{2+}]_o$	−2 mV*	+15 mV	+23 mV[a]
E_r in 0 mM $[Ca^{2+}]_o$	−2 mV	−4 mV	−4 mV[a]
Response to IP_3	—	—	↑[b]
Response to FFA	↑	↓	↓
Sensitivity to anti-TRPC antibodies	TRPC1, TRPC5, TRPC6	TRPC1, TRPC5	TRPC1, TRPC5, TRPC7

*$P < 0.05$ (coronary *versus* mesenteric artery and portal vein). [a]Albert *et al.* 2002*a*; [b]Liu *et al.* (2005*a*).

At −80 mV SOC activity induced by 10 μM CPA in coronary artery had a mean open probability (NP_o) of 1.03 ± 0.27 ($n = 8$), which was approximately threefold greater than the mean NP_o of 0.38 ± 0.17 ($n = 8, P < 0.05$) of SOCs evoked by the same stimulus in mesenteric artery. This increase in SOC activity in coronary artery does not appear to be a difference in the number of channels per patch since in both preparations there were at least four to five channels per patch. Moreover kinetic analysis indicated that SOCs had two open times of about 5 ms and 30 ms in both preparations, which are similar to values in portal vein (data not shown, Albert & Large, 2002*a*).

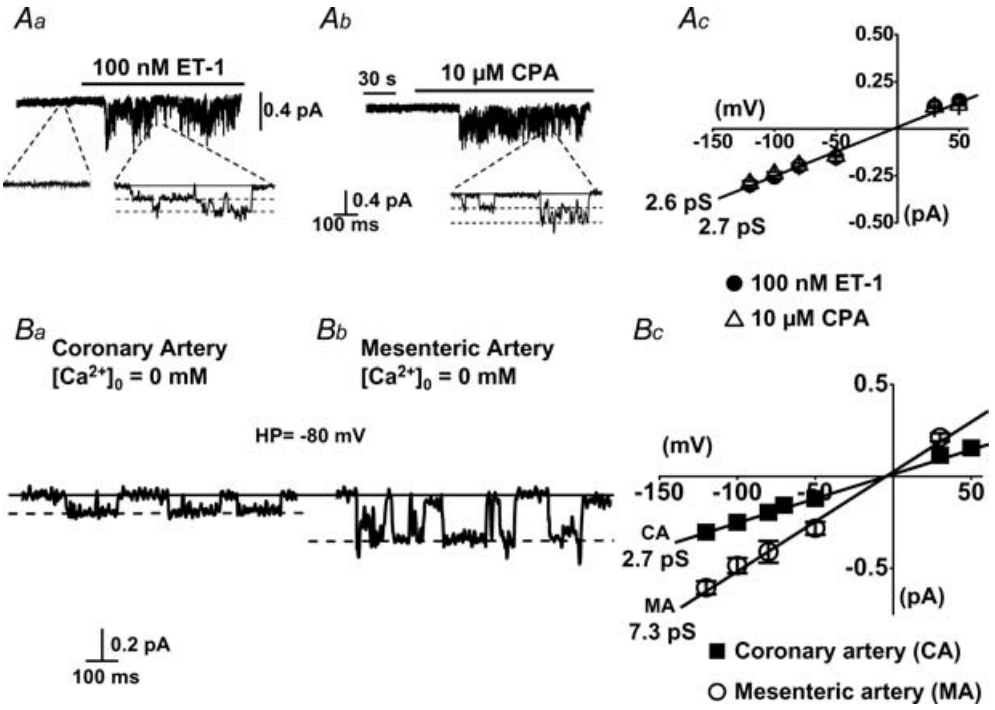

Figure 2. Activation of single channel currents by ET-1 and CPA in cell-attached patches from coronary artery

Aa and *b*, bath application of 100 nM ET-1, and 10 μM CPA activated channel currents at −80 mV. *Ac*, mean *I–V* relationships of ET-1- and CPA-evoked channel currents in coronary artery showing similar conductances of 2.6 pS and 2.7 pS and E_r of about 0 mV. *Ba* and *Bb*, comparison of CPA- induced single channel currents in coronary and mesenteric arteries recorded with 0 mM $[Ca^{2+}]_o$ illustrating that SOCs in mesenteric artery have larger unitary amplitudes at −80 mV. *Bc*, mean *I–V* relationships of CPA-evoked SOCs recorded in 0 mM $[Ca^{2+}]_o$ showing SOCs had conductances of 2.7 pS and 7.3 pS in, respectively, coronary and mesenteric arteries and E_r of about 0 mV in both preparations.

Therefore the greater probability of channel opening together with a larger unitary conductance may explain the much larger inward whole-cell SOC currents observed at negative potentials in coronary compared to mesenteric artery. However a thorough comparison between depletion of internal Ca^{2+} stores and channel activation, expression levels and activity of PKC and expression levels of TRPC proteins is needed between these two preparations to confirm these ideas.

SOCs in coronary artery are activated by PKC

We have previously shown that PKC is important in activating SOCs in both mesenteric artery and portal vein (Albert & Large, 2002*b*; Saleh *et al.* 2006). However, our present work indicates that SOCs in coronary artery have different biophysical properties from SOCs in mesenteric artery and therefore we investigated whether this kinase is also involved in activating SOCs in coronary artery, especially since Ca^{2+}-independent phospholipase A_2 has been implicated in SOC activation in rat coronary artery (Smani *et al.* 2007) and calmodulin also has been proposed to be involved in action of SOCs in portal vein (Albert *et al.* 2006*b*).

Bath application of 3 μM chelerythrine, a PKC inhibitor, significantly reduced BAPTA-AM-, CPA- and ET-1-evoked SOC activity in cell-attached patches held at -80 mV by, respectively, $91 \pm 19\%$ ($n = 4$, $P < 0.05$, Fig. 3*A*), $92 \pm 10\%$ ($n = 4$, $P < 0.05$), and $80 \pm 13\%$ ($n = 5$, $P < 0.05$). In addition in both 1.5 and 0 mM $[Ca^{2+}]_o$ bath application of 1 μM phorbol 12,13-dibutyrate (PDBu), a PKC activator, induced channel currents in cell-attached patches with unitary conductances of about 2.6 pS and E_r of about 0 mV ($n = 6$, Fig. 3*B*), which are similar to the properties of CPA-induced SOCs. Moreover PDBu-induced SOC activity ($n = 5$) and SOC activity evoked by the cell-permeant DAG analogue 1-oleoyl-2-acetyl-*sn*-glycerol (OAG, 10 μM, $n = 4$), an activator of endogenous PKC, were inhibited by chelerythrine by over 90% in cell-attached patches (data not shown). Figure 3*C* and *D* also shows that bath application of a PKC catalytic subunit (0.05 U ml^{-1}) to the cytosolic surface of inside-out patches from both coronary and mesenteric arteries activated channel currents with similar properties to SOCs induced by CPA.

We have previously shown that IP_3 potentiated the activity of SOCs in portal vein (Liu *et al.* 2005*b*) but had no effect on SOCs in mesenteric artery (Saleh *et al.* 2006). Therefore we also investigated the effect of IP_3 on SOC activity in coronary artery and showed that bath application of 10 μM IP_3 had no effect on CPA- ($n = 6$) or PDBu-evoked SOCs in inside-out patches (data not shown).

These results show that although SOCs in coronary artery have different biophysical properties from SOCs in

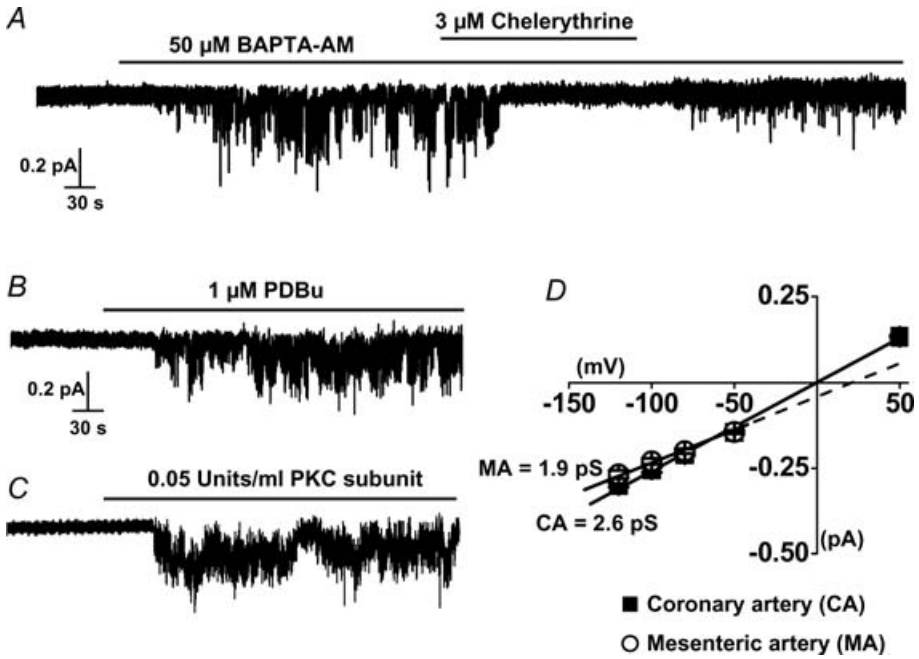

Figure 3. SOCs in coronary artery are activated by PKC
A, BAPTA-AM (50 μM) evoked channel activity in a cell-attached patch held at -80 mV, which was reversibly inhibited by chelerythrine (3 μM). *B* and *C*, show that PDBu (1 μM) and a PKC catalytic subunit (0.05 U ml^{-1}) activated single channel currents in cell-attached and inside-out patches, respectively. *D*, mean *I–V* relationships of channel currents induced by a PKC catalytic subunit in coronary and mesenteric arteries.

mesenteric artery there appears to be a common activation mechanism involving PKC stimulation.

Effect of anti-TRPC antibodies on SOC activity in coronary and mesenteric arteries and portal vein

There is evidence that TRPC proteins are components of SOCs in vascular smooth muscle (Large, 2002; Beech *et al.* 2004; Albert & Large, 2006; Albert *et al.* 2007) and therefore we investigated whether the diverse properties of native SOCs in coronary and mesenteric arteries were associated with different sensitivity to anti-TRPC antibodies. This approach has already demonstrated that ROCs in vascular smooth muscle have marked differences in sensitivity to these antibodies indicating diverse TRPC properties (Albert *et al.* 2006*b*; Saleh *et al.* 2006; Peppiatt-Wildman *et al.* 2007). We also investigated the effect of anti-TRPC antibodies on SOC activity in portal vein since IP_3 potentiates SOCs in this preparation (Liu *et al.* 2005*b*) but not in coronary and mesenteric artery (Saleh *et al.* 2006; present work) suggesting that the channel in portal vein is different from the SOCs in these arteries. In these experiments SOCs were initially evoked by CPA in the cell-attached mode and then patches were excised into the inside-out configuration where channel activity was maintained without any appreciable rundown for up to 30 min in control conditions. Anti-TRPC antibodies raised against different intracellular epitopes for each TRPC subtype were then bath applied to the cytosolic surface of the patch to measure directly the effect of antibodies on single channel activity.

Figure 4*A–C* shows that SOC activity in coronary and mesenteric arteries and portal vein was markedly inhibited by over 80% using anti-TRPC1 and anti-TRPC5 antibodies. However there was differential sensitivity to anti-TRPC6 and anti-TRPC7 antibodies. Anti- TRPC6 antibody inhibited SOC activity in coronary artery (Fig. 4*Aa* and *b*) but not in mesenteric artery or portal vein (Fig. 4*B* and *C*). In addition anti-TRPC7 antibody markedly decreased SOC activity in portal vein (Fig. 4*Ca* and *b*) but had no effect in coronary or mesenteric arteries (Fig. 4*A* and *B*). The inhibitory actions of all anti-TRPC

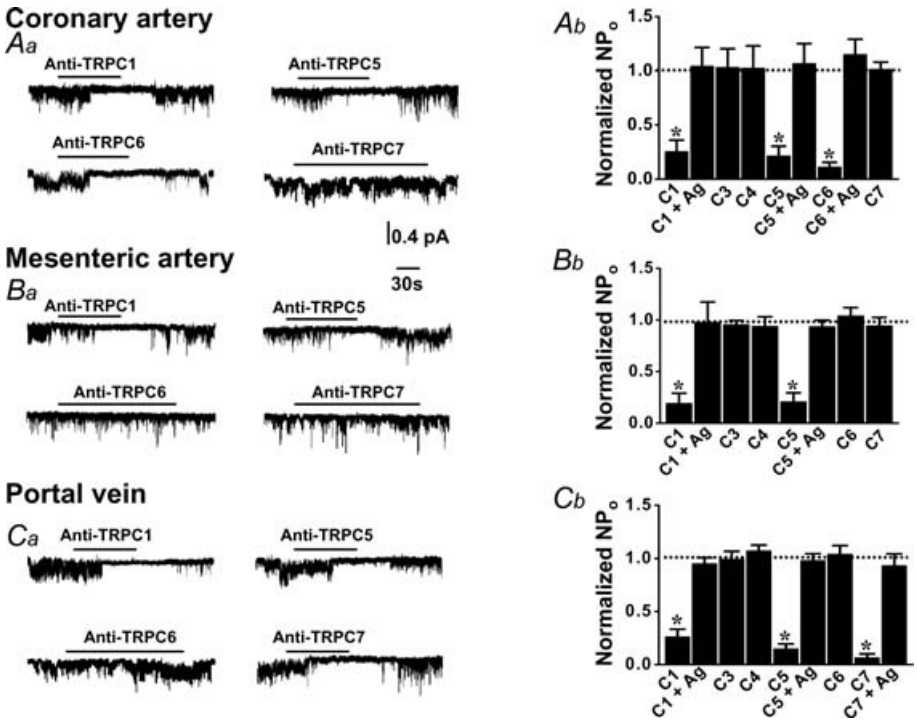

Figure 4. Differential sensitivity of SOCs in coronary and mesenteric arteries and portal vein to anti-TRPC antibodies

Aa, in coronary artery bath application of anti-TRPC1, -TRPC5 and -TRPC6 antibodies at 1: 200 dilution significantly inhibited SOC activity in inside-out patches in a reversible manner whereas anti-TRPC7 had no effect. *Ab*, mean data showing the effect of antibodies raised against all TRPC proteins on SOC activity in coronary artery. Note that in the presence of their antigenic peptides (Ag) the inhibitory action of anti-TRPC1, -TRPC5 and -TRPC6 were blocked. *Ba* and *b*, anti-TRPC1 and -TRPC5 antibodies inhibited SOCs in mesenteric artery whereas anti-TRPC6 and -TRPC7 had no effect. *Ca* and *b*, anti-TRPC1, -TRPC5 and -TRPC7 antibodies inhibited SOCs in portal vein whereas anti-TRPC6 had no effect. Note anti-TRPC3 and -TRPC4 antibodies had no effect in any preparation (*Ab*, *Bb* and *Cb*).

antibodies were completely blocked by preincubation with their respective antigenic peptides (Fig. 4*Ab*, *Bb* and *Cb*) and anti-TRPC3 and -TRPC4 antibodies had no effect on SOC activity in myocytes from any blood vessel.

These data indicate that SOCs in coronary and mesenteric arteries and portal vein have different sensitivities to anti-TRPC antibodies, which further demonstrates that SOCs in these vascular preparations have different properties and may also suggest that these SOC isoforms are composed of different TRPC subunits.

Coimmunoprecipitation of TRPC subunits from coronary and mesenteric arteries and portal vein vascular smooth muscle

Previous studies utilizing coimmunoprecipitation techniques have demonstrated that TRPC channels can combine to form heterotetrameric channel structures (Goel *et al.* 2002; Hofmann *et al.* 2002; Strubing *et al.* 2003). We therefore used this technique to see if TRPC proteins identified from the functional effects of anti-TRPC antibodies on channel activity shown above could associate with one another in native coronary and mesenteric arteries and portal vein.

Figure 5*Aa* shows that tissue lysates from coronary artery immunoprecipitated with either anti-TRPC1 or anti-TRPC5 antibodies and then blotted with a different anti-TRPC1 antibody exhibited similar protein bands of 90 kDa. TRPC1-mediated 90 kDa bands were present in positive control blots using rat testes (RT) cell lysates and were absent in negative control experiments when the anti-TRPC1 antibody was preincubated with its antigenic peptide (Fig. 5*Aa*). Furthermore Fig. 5*Ab* shows that in blots from coronary artery and rat brain (RB) tissue using anti-TRPC5, antibodies produced bands of 100 kDa following immunoprecipitation with either an alternative anti-TRPC5 antibody, or an anti-TRPC6 antibody. These experiments provide evidence that association between TRPC1 and TRPC5 and between TRPC5 and TRPC6 proteins can occur in coronary artery.

Figure 5*B* and *C* illustrates similar experiments using lysates from mesenteric artery and portal vein and shows that associations between TRPC1 and TRPC5 proteins can also occur in both these vascular tissues. However Fig. 5*Bc*

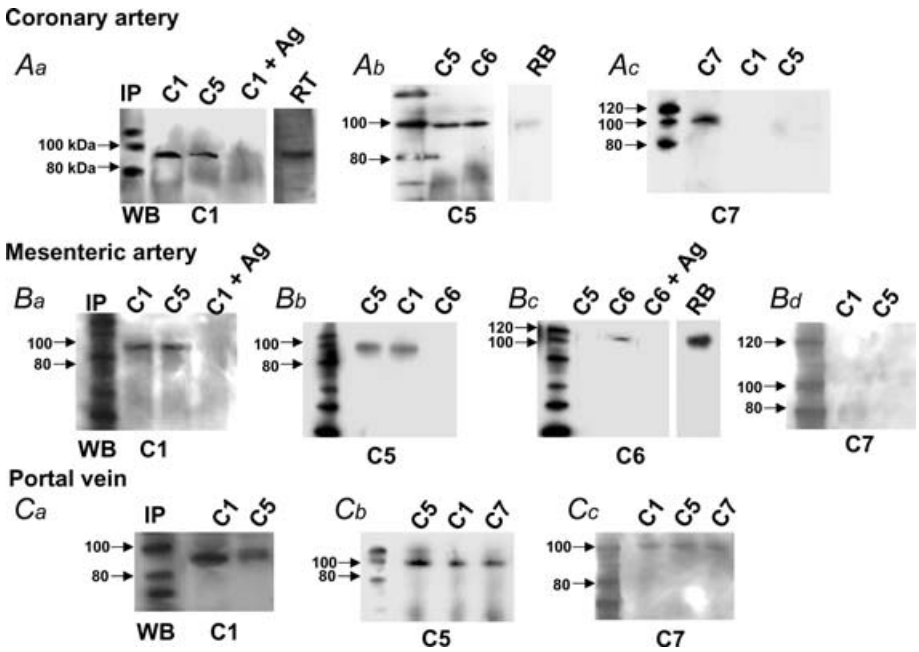

Figure 5. Coimmunoprecipitation of TRPC channel proteins in coronary and mesenteric arteries and portal vein

A, total tissue lysates from coronary artery were immunoprecipitated and then Western blotted (WB) using the corresponding anti-TRPC antibodies. Note RT (rat testes) relates to positive control WB using anti-TRPC1 giving bands of 90 kDa. Bands of 90 kDa were observed when Western blots were carried out with an anti-TRPC1 antibody in samples immunoprecipitated with TRPC1 and TRPC5 but not TRPC1 and antigenic peptide (Ag, *Aa*) showing TRPC1 and TRPC5 protein can associate together. Western blots conducted using an anti-TRPC5 antibody detected a band of 100 kDa when immunoprecipitation was carried out using anti-TRPC5 or anti-TRPC6 antibodies demonstrating TRPC5 and TRPC6 protein can also associate in coronary artery. In mesenteric artery TRPC1 and TRPC5 proteins are shown to coimmunoprecipitate with each other (*Ba* and *b*) whereas TRPC5 and TRPC6 protein do not (*Bc* and *d*). In portal vein TRPC1 and TRPC5 and TRPC5 and TRPC7 were shown to associate with each other (*Ca–c*).

and *d* shows that in mesenteric artery there is no resolvable combination of TRPC5 and TRPC6 or TRPC5 and TRPC7 proteins whereas Fig. 5*Cb* and *c* shows that in portal vein associations between TRPC5 and TRPC7 proteins do occur but combinations between TRPC5 and TRPC6 proteins do not.

These data provide evidence that association between TRPC1 and TRPC5 proteins occurs in coronary and mesenteric arteries and portal vein whereas association between TRPC5 and TRPC6 proteins only occurs in coronary artery and association between TRPC5 and TRPC7 proteins only occurs in portal vein. Interestingly these results correspond exactly to the differential sensitivities of single SOC currents to anti-TRPC antibodies in these preparations (see above), which further indicates that SOC isoforms described in the present work may be produced by different compositions of TRPC proteins.

Effect of flufenamic acid on SOCs in coronary and mesenteric arteries and portal vein

To further investigate the diverse properties of SOCs in these vascular preparations we studied the effects of flufenamic acid (FFA) on channel activity induced by PDBu in outside-out patches. In expression systems FFA has been shown to increase TRPC6 and inhibit the activity of all other TRPC channels (Inoue *et al.* 2001).

Figure 6*A* and *D* shows that the mean NP_o of PDBu-evoked SOC activity in coronary artery was significantly increased by about twofold from a control value of 0.09 ± 0.04 to 0.18 ± 0.07 in the presence of 100 μM FFA ($n = 5$, $P < 0.05$). In contrast Fig. 6*B*, *C* and *D* illustrates that FFA significantly reduced PDBu-evoked SOCs in mesenteric artery and portal vein by $92 \pm 10\%$ ($n = 4$, $P < 0.05$) and $91 \pm 9\%$ ($n = 5$, $P < 0.05$), respectively.

These data demonstrate pharmacological differences between the SOCs in coronary and mesenteric arteries and portal vein and also support the idea that TRPC6 proteins contribute to the molecular structure of SOCs in coronary artery but not in mesenteric artery or portal vein.

Discussion

In the present work we have investigated the physiological properties of native SOCs in myocytes from different blood vessels and the major finding is that SOCs in coronary and mesenteric arteries have markedly different properties. These characteristics are summarized in Table 1 and indicate the existence of various SOC isoforms, which may be explained by diverse structures involving TRPC subunits.

Biophysical properties of SOCs in coronary and mesenteric arteries

Comparison of whole-cell currents demonstrated that CPA evoked a conductance with a linear *I–V* relationship in coronary artery whereas a pronounced outwardly rectifying curve was observed in mesenteric artery (Table 1). The clear difference in the rectifying properties and reversal potential values of the whole-cell currents suggested that the CPA-induced conductance was not identical in the two preparations. A notable feature was

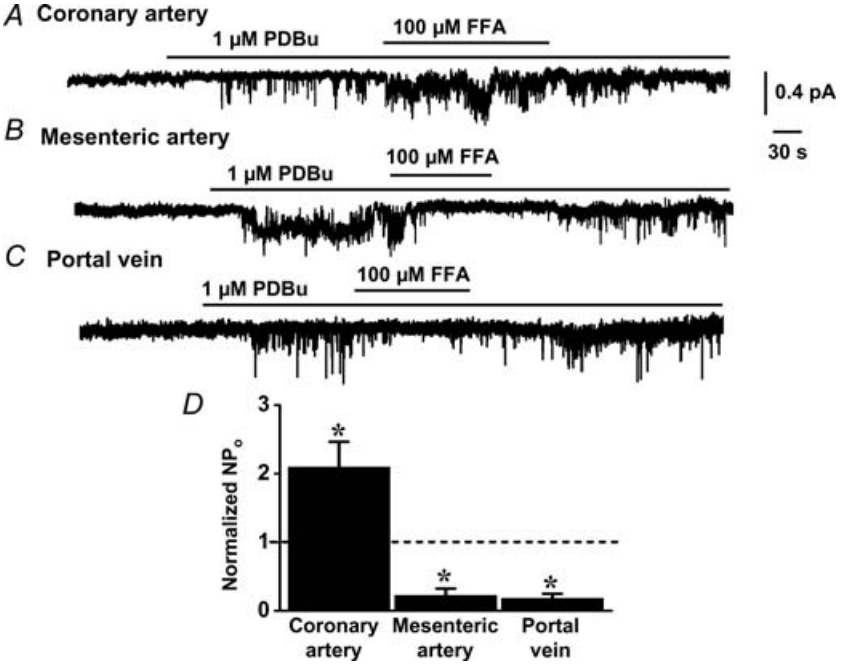

Figure 6. Differential effects of FFA on SOCs in outside-out patches from coronary and mesenteric arteries and portal vein
A, PDBu-evoked SOCs at −80 mV were significantly enhanced in a reversible manner by FFA (100 μM) in coronary artery. *B* and *C*, FFA decreased PDBu-induced SOC activity in mesenteric artery and portal vein. *D*, mean data of the effect of FFA on SOC activity in three vascular preparations ($n = 5$ of each).

that in the physiological range of membrane potentials (−50 to −60 mV) the CPA-evoked inward current was approximately sixfold greater in coronary compared to mesenteric artery. In the latter preparation the rectifying properties of the conductance permitted little inward current at physiological membrane potentials.

Single channel recording also illustrated different properties of the unitary conductance in coronary and mesenteric arteries. The single channel conductance was significantly larger in coronary compared to mesenteric artery in 1.5 mM $[Ca^{2+}]_o$ (Table 1) and this difference was even more marked in 0 mM $[Ca^{2+}]_o$ in which the unitary conductance was about 2.7 pS and 7.5 pS, respectively, in coronary and mesenteric arteries (Table 1). It appears that extracellular Ca^{2+} ions have a blocking effect on the SOC in mesenteric artery but not in coronary artery myocytes. However these differences in single channel properties strongly indicate a difference in the structure of the SOCs in coronary and mesenteric artery myocytes.

The NP_o value in coronary artery was threefold greater than in mesenteric artery myocytes. There were the same number of channels per patch in the two preparations and therefore there appears to be a greater probability of channel opening in coronary artery myocytes. The larger unitary conductance coupled with increased probability of SOC opening is likely to contribute to the larger inward current observed in coronary compared to mesenteric artery.

PKC-mediated activation of SOCs

Despite the differences in biophysical properties of SOCs in coronary and mesenteric arteries there appeared to be a common activation mechanism. Thus, PKC inhibitors markedly reduced CPA- and BAPTA-AM-evoked channel activity in coronary artery (present work) and in mesenteric artery (Saleh *et al.* 2006). Also phorbol esters, the DAG analogue OAG and a PKC catalytic subunit potently stimulated SOC activity in both preparations. These results implicate an important role for PKC in activation of SOCs. However, an alternative pathway involving calcium-influx factors, Ca^{2+}-independent phospholipase A_2 and production of lysophospholipids have been proposed to activate SOCs in other vascular myocytes (Smani *et al.* 2004, 2007; Park *et al.* 2008). The ability of PKC and lysophospholipids to activate these channels may be related to multimodal gating of SOCs, which has been recently reviewed (Albert *et al.* 2007).

Differential sensitivity of SOCs in coronary and mesenteric arteries and portal vein to anti-TRPC antibodies

There is much evidence that TRPC proteins form SOCs in several cell types although little is known about the role of TRPCs in mediating SOCs in vascular smooth muscle (Large, 2002; Beech *et al.* 2004; Albert *et al.* 2007). We explored TRPC properties of native SOCs in freshly dispersed myocytes by applying anti-TRPC antibodies to the cytoplasmic surface of inside-out patches in which SOCs had been stimulated. Using this approach we have observed a high degree of selectivity with several available anti-TRPC antibodies (Albert *et al.* 2006*a*; Saleh *et al.* 2006; Peppiatt-Wildman *et al.* 2007). In the present experiments we also investigated the sensitivity of a SOC to anti-TRPC antibodies in rabbit portal vein myocytes for comparison since we have previously characterized the whole-cell and single channel properties of this conductance (Albert & Large, 2002*a*,*b*; Liu *et al.* 2005*a*,*b*). Anti-TRPC1 and anti-TRPC5 antibodies (two different antibodies raised against different epitopes for each TRPC subunit) were found to produce marked inhibition of SOC activity in both arteries and portal vein (Table 1). This is not a non-specific action of these antibodies against non-selective cation channels since the same anti-TRPC1 and -TRPC5 antibodies have no effect against native TRPC3 channels in rabbit ear artery, TRPC6 channels in rabbit mesenteric artery or TRPC3/TRPC7 channels in rabbit coronary artery myocytes (Albert *et al.* 2006*a*; Saleh *et al.* 2006; Peppiatt-Wildman *et al.* 2007). A particularly novel finding was that anti-TRPC6 antibody (two different forms) reduced channel activity in coronary but not in mesenteric artery or portal vein. In addition anti-TRPC7 antibody reduced channel activity in portal vein but not in coronary or mesenteric arteries (Table 1). In all cases inhibitory effects of antibodies were blocked by preincubation with their respective antigenic peptides, which were designed from predicted intracellular regions of their respective TRPC channel proteins. Anti-TRPC3 and anti-TRPC4 antibodies had no effect on SOCs in any preparation although the anti-TRPC3 antibody has been shown to block the constitutively active ROC in ear artery (Albert *et al.* 2006*b*) and ET-1-induced ROC in coronary artery (Peppiatt-Wildman *et al.* 2007). These data showing that the native SOCs display differential sensitivities to anti-TRPC antibodies provide evidence that the three preparations express SOCs which exhibit distinct TRPC properties. It was clear in all three preparations that CPA evoked only a single resolvable conductance in our single channel recordings. Therefore although we are extremely cautious about assigning precise molecular identification, a simple explanation is that the SOC in each vascular preparation is composed of different heterotetrameric TRPC structures as detected by different antibodies. Accordingly one interpretation of our data is that SOCs contain TRPC1, TRPC5 and TRPC6 in coronary artery, TRPC1 and TRPC5 in mesenteric artery and TRPC1, TRPC5 and TRPC7 in portal vein and the diverse compositions confer different properties on the ion channels. Support for this was provided by

coimmunoprecipitation experiments in which evidence for association of TRPC1 and TRPC5 was found in all three preparations but interactions between TRPC5 and TRPC6 only occurred in coronary artery and TRPC5 and TRPC7 only associated in portal vein, even though TRPC6 and TRPC7 proteins are highly expressed in portal vein and coronary artery, respectively (Inoue *et al.* 2001; Peppiatt-Wildman *et al.* 2007). Interestingly the conductance value of co-expressed TRPC1 and TRPC5 proteins (about 5 pS, Strubing *et al.* 2001) is similar to the conductance values of SOCs in coronary and mesenteric arteries and portal vein (between 2 and 3 pS), which we show have TRPC1 and TRPC5 properties. Further evidence for TRPC6 as a component of SOCs in coronary artery was that FFA potentiated SOC activity in this preparation but inhibited SOC activity in mesenteric artery and portal vein. It has been shown that FFA selectivity increases expressed TRPC6 activity but inhibits all other TRPC channels in expression systems (Inoue *et al.* 2001). Also IP_3 has been shown to potentiate expressed TRPC7 channel activity but not TRPC6 (Estacion *et al.* 2004; Shi *et al.* 2004). IP_3 potentiated SOC activity in portal vein (Liu *et al.* 2005*b*), which was inhibited by anti-TRPC7 antibody, but not in coronary or mesenteric arteries where SOC activity was unaffected by anti-TRPC7 antibodies. Hence it seems that differences in important physiological characteristics of SOCs in the three vascular preparations can be explained by diverse TRPC composition of the native channels.

The above data suggesting combinations of TRPC5 and TRPC6 and of TRPC5 and TRPC7 proteins are involved in composing native SOCs are novel interactions that go against the original principles of TRPC heteromultimerization that suggested TRPC1, TRPC4 and TRPC5 or TRPC3, TRPC6 and TRPC7 may associate to form ion channels (Goel *et al.* 2002; Hofmann *et al.* 2002). However, more recently it has been proposed that functioning cation channels may be composed of subunits from both these groups (Strubing *et al.* 2003; Liu *et al.* 2005; Poteser *et al.* 2006). However, for more precise information on the molecular make-up of these native SOCs, comparison of the proposed heterotetrameric structures in expression systems with native SOCs would be required. Molecular techniques to elucidate the structure of these native TRPC channels will be problematic since these experimental approaches require cell culture during which vascular smooth muscle cells undergo fundamental changes (e.g. see Owens, 1995 for review). Also recently it has been shown that there is reorganization of intracellular Ca^{2+} stores during culture of vascular myocytes (Ng *et al.* 2008), which is likely to exacerbate difficulties in investigating store-operated events. Moreover it has been demonstrated that TRPC expression alters during cell culture of vascular myocytes (Bergdahl *et al.* 2005), which would amplify the difficulties of using siRNA approaches with heterotetrameric channel structures. Recently it was shown that CPA-evoked responses in wild-type and $TRPC1^{-/-}$ mice were similar, indicating that TRPC1 is not an essential component of SOCs in vascular smooth muscle (Dietrich *et al.* 2007). However the present work indicates that not only TRPC1 but also TRPC5, as also suggested by Xu *et al.* (2006), and TRPC6 and TRPC7 may be components of native SOCs. Consequently in $TRPC1^{-/-}$ mice it is possible that these other TRPC proteins can compensate for the loss of TRPC1 as was previously seen with $TRPC6^{-/-}$ mice (Dietrich *et al.* 2005).

Conclusion

It is concluded that there are several native SOC isoforms in vascular smooth muscle and we provide evidence that these differences may results from a diversity of TRPC composition. It is increasingly evident that vascular smooth muscle contains a complex array of TRPC ion channels with different physiological properties. For example, in rabbit coronary artery ET-1 activates two distinct conductances, one with TRPC3 and TRPC7 properties (Peppiatt-Wildman *et al.* 2007) and the second with TRPC1, TRPC5 and TRPC6 characteristics (present work). However, the marked pharmacological differences suggest that it might be possible to develop selective inhibitors of these TRPC conductances to therapeutic advantage.

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