British Journal of Pharmacology (2003) 139, 955–965
www.nature.com/bjp

Pharmacological profile of store-operated channels in cerebral arteriolar smooth muscle cells

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1 In this study, we determined a pharmacological profile of store-operated channels (SOCs) in smooth muscle cells of rabbit pial arterioles. Ca^{2+}-indicator dyes, fura-PE3 or fluo-4, were used to track $[Ca^{2+}]_i$ and 10 μM methoxyverapamil (D600) was present in all experiments on SOCs to prevent voltage-dependent Ca^{2+} entry. Store depletion was induced using thapsigargin or cyclopiazonic acid.

2 SOC-mediated Ca^{2+} entry was inhibited concentration dependently by Gd^{3+} (IC_{50} 101 nM). It was also inhibited by 10 μM La^{3+} (70% inhibition, $N=5$), 100 μM Ni^{2+} (57% inhibition, $N=5$), 75 μM 2-aminoethoxydiphenylborate (66% inhibition, $N=4$), 100 μM capsaicin (12% inhibition, $N=3$) or preincubation with 10 μM wortmannin (76% inhibition, $N=4$). It was completely resistant to 1 μM nifedipine ($N=5$), 10 μM SKF96365 ($N=6$), 10 μM LOE908 ($N=14$), 10–100 μM ruthenium red ($N=1+2$), 100 μM sulindac ($N=4$), 0.5 mM streptomycin ($N=3$) or 1 : 10,000 dilution *Grammostolla spatulata* venom ($N=4$).

3 RT–PCR experiments on isolated arteriolar fragments showed expression of mRNA species for TRPC1, 3, 4, 5 and 6.

4 The pharmacological profile of SOC-mediated Ca^{2+} entry in arterioles supports the hypothesis that these SOCs are distinct from tonically active background channels and several store-operated and other nonselective cation channels described in other cells. Similarities with the pharmacology of TRPC1 support the hypothesis that TRPC1 is a subunit of the arteriolar smooth muscle SOC.
British Journal of Pharmacology (2003) **139,** 955–965. doi:10.1038/sj.bjp.0705327

Keywords: Calcium stores; calcium channels; store-operated channels; cerebral arteriole; smooth muscle

Abbreviations: 2-APB, 2-aminoethoxydiphenylborate; CPA, cyclopiazonic acid; D600, methoxyverapamil; DMSO, dimethyl sulphoxide; Gd^{3+}, gadolinium; La^{3+}, lanthanum; Ni^{2+}, nickel; RBL cells, rat basophilic leukaemia cells; ROCs, receptor-operated channels; RT–PCR, reverse transcriptase polymerase chain reaction; SOCs, store-operated channels; TG, thapsigargin; WT, wortmannin

Introduction

Store-operated channels (SOCs) are Ca^{2+}-permeable plasma membrane channels that open in response to depletion of sarco/endoplasmic reticular Ca^{2+} stores ('store-depletion'). They are distinct from voltage-gated Ca^{2+} channels and open in the absence of receptor activation, and thus would appear not to be receptor-operated channels (ROCs). Although it remains plausible that store depletion and receptor activation are parallel signalling pathways to the same channel, in pial arterioles complete store depletion does not alter ROC activation, as if SOCs and ROCs are distinct (Guibert & Beech, 1999). Pharmacological distinctions between SOCs and ROCs have also been described in rat aorta smooth muscle and in the A7r5 smooth muscle cell line (Iwamuro *et al*., 1999; Zhang *et al*., 1999). Many cell types, including vascular smooth muscle cells, also express stretch-operated Ca^{2+}-permeable channels (Welsh *et al*., 2000; Wu & Davis, 2001) and it is unclear if or how these are related to SOCs.

In several types of smooth muscle, SOC-mediated Ca^{2+} entry elicits contraction (Gibson *et al*., 1998). In others, however, there is a lack of contractile function, as if there is a tight association with the refilling of Ca^{2+} stores (Casteels & Droogmans, 1981; Flemming *et al*., 2002). Ca^{2+} entry through SOCs could also have other specific functions. For example, a role in smooth muscle proliferation has been proposed (Magnier-Gaubil *et al*., 1996; Golovina, 1999; Golovina *et al*., 2001; Yu *et al*., 2003) and it can be hypothesised that SOCs have a discrete function in relation to gene expression, as certain other Ca^{2+} channels do in neurones (Crabtree, 1999; Hunton *et al*., 2002). SOCs may also have general, housekeeping functions, for example, to regulate sarco/endoplasmic reticulum Ca^{2+} concentration. Thus, in many respects, the function of SOCs in smooth muscle is poorly understood and several possibilities remain to be explored.

Even within the vascular smooth muscle cell type, evidence for SOC diversity is emerging. For example, SOCs are outwardly rectifying nonselective cation channels in aortic smooth muscle cells, whereas they are inwardly rectifying with a marked selectivity for Ca^{2+} in portal vein smooth muscle cells (Trepakova *et al*., 2001; Albert & Large, 2002). SOCs in pulmonary artery smooth muscle cells are nifedipine-resistant, whereas those in retinal arterioles are nifedipine-sensitive (Curtis & Scholfield, 2001; Ng & Gurney, 2001). SOCs also exist in other cell types, a striking example of which is the highly Ca^{2+}-selective calcium-release activated calcium (CRAC) channel of rat basophilic leukaemia (RBL) and Jurkat cells (Barritt, 1999; Lewis, 1999). SOC diversity may be

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explained by differential expression of channel subunits. The primary candidate subunits of mammalian SOCs are homologues of the *Drosophila* TRP protein. These mammalian TRP proteins are numerous (at least 19) and on the basis of amino-acid sequence can be divided into three subfamilies (Clapham *et al*., 2001; Montell *et al*., 2002). One of these (the TRPV subfamily) includes the well-characterised vanilloid or capsaicin receptor (or TRPV1; Caterina *et al*., 1997) and the CaT1 protein (TRPV6), which has been proposed as a subunit of the CRAC channel (Yue *et al*., 2001, but see Voets *et al*., 2001). Most studies, however, have focused on the hypothesis that the TRPC subfamily of TRP proteins is involved in SOC formation. For example, endothelial cells of TRPC4 knockout mice have reduced SOC-mediated signals (Freichel *et al*., 2001), and a TRPC1 blocking antibody and antisense DNA targeted to TRPC1 mRNAs inhibit native SOCs (Liu *et al*., 2000; Brough *et al*., 2001; Xu & Beech, 2001; Beech *et al*., 2003).

There is a paucity of data on the pharmacology of SOCs and related channels in smooth muscle (McFadzean & Gibson, 2002). In this study, we aimed to produce a pharmacological profile of SOCs in cerebral arteriolar smooth muscle cells based on the known pharmacology of a range of other Ca^{2+}-permeable and nonselective cation channels. The data enable comparisons with related channels and heterologously expressed TRPC proteins, testing of the hypothesis that SOCs are diverse, and provide a foundation both for future improvements in selective SOC pharmacology and studies aimed at revealing SOC functions.

Methods

Male Dutch dwarf rabbits (1–1.5 kg) were killed by an intravenous overdose of 70 mg kg^{-1} sodium pentobarbitone in accordance with the Code of Practice, U.K. Animals Scientific Procedures Act 1986. The brain was placed in ice-cold oxygenated Hanks solution and fragments of pial membrane dissected from across the cortical surface and incubated in Hanks solution containing 0.032 mg ml^{-1} protease and 0.2 mg ml^{-1} collagenase (type 1A) for 10 min at 37°C. The mixture was placed at 4°C for 15 min and mechanically agitated to isolate fragments of arterioles. After centrifugation (1000 r.p.m.) for 5 min, the supernatant was replaced with fresh Hanks solution. Arterioles were resuspended and dropped onto polylysine-coated coverslips and stored at 4°C. Experiments were performed within 10 h. Arteriole fragments used in recordings had an external diameter of $<45\,\mu$m, and lacked visible adventitia or endothelial cells (Cheong *et al*., 2001).

For Ca^{2+}-imaging experiments, isolated arterioles were pre-incubated with 1–5 μM fura-PE3 AM (Vorndran *et al*., 1995) or 1 μM fluo-4 AM (Gee *et al*., 2000) at 30–37°C for 1 h in standard bath solution. This was followed by a 0.5–1 h wash period in standard bath solution at room temperature. The fura-PE3 AM and fluo-4 AM incubation and wash periods and all experiments were in the presence of 10 μM methoxyverapamil (D600), unless $[Ca^{2+}]_i$-elevation in response to raised $[K^+]_o$ was studied. In elevated $[K^+]_o$ experiments, wortmannin (1 μM) was included in the fura-PE3 incubation solution to prevent contraction without effect on voltage-gated Ca^{2+} channels (Unno *et al*., 1998). Preincubation with 1 μM thapsigargin (TG) occurred for 0.5–1.5 h, and during the fura-PE3 and fluo-4 loading and washing periods when applicable.

Fura-PE3 and fluo-4 fluorescence was observed with an inverted epifluorescence microscope (Nikon, Japan; or Zeiss, Germany). A xenon arc lamp provided excitation light, the wavelength of which was selected by a monochromator (Till Photonics, Germany). Emission was collected via 510 and 530 nm filters for fura-PE3 and fluo-4, respectively. A cooled CCD camera (Hamamatsu, Japan) sampled the collected light, producing images at 12-bit (Orca-ER) or 14-bit (H4880-82) resolution. Every 10 or 20 s, images were sampled in pairs for two fura-PE3 excitation wavelengths (340, 345 or 355 and 380 nm) or singularly for the fluo-4 excitation wavelength (494 nm). Images were analysed off-line using regions of interest (ROIs), which selected parts of the image frame corresponding to individual smooth muscle cells within arterioles. Three ROIs were also selected that were distant from the arteriole and used for background subtraction. $[Ca^{2+}]_i$ is expressed as the ratio of the background-subtracted emission intensities for the two fura-PE3 excitation wavelengths ((340, 345 or 355 nm)/(380 nm)) or as the background-subtracted emission intensity for the single fluo-4 excitation wavelength (494 nm). With the Nikon imaging system, 345 or 355/380 nm excitation wavelengths were used while those used with the Zeiss imaging system were 340/380 nm. This was because of different light transmission and capture efficiencies. Thus, baseline R values varied between imaging systems. Imaging was controlled by Openlab 2 software (Image Processing & Vision Company Ltd, U.K.). Analysis and image presentation utilised Origin, Excel and Powerpoint software. Agents were applied to arterioles using a continuous bath perfusion system with a flow rate of 4 ml min^{-1}. All experiments were at room temperature.

Hanks solution contained (mM): NaCl 137, NaH_2PO_4 0.34, KCl 5.4, K_2HPO_4 0.44, D-glucose 8, *N*-[2-hydroxyethyl]piperazine-*N*′-2-ethanesulphonic acid (HEPES) 5, $CaCl_2$ 0.01. Standard bath solution contained (mM): NaCl 130, KCl 5, D-glucose 8, HEPES 10, $MgCl_2$ 1.2, $CaCl_2$ 1.5. Ca^{2+}-free solution was standard bath solution in which 0.4 mM EGTA replaced the $CaCl_2$. When the extracellular K^+ concentration was raised, the NaCl concentration in the bath solution was reduced by the equimolar amount. All solutions were titrated to pH 7.4 with NaOH.

Two protocols were used to deplete Ca^{2+} stores and activate SOCs. In the first protocol, arterioles were pretreated with 1 μM TG in standard (1.5 mM Ca^{2+}) bath solution. In the second protocol, 10 μM cyclopiazonic acid (CPA) was applied in Ca^{2+}-free solution and store-operated Ca^{2+} entry was observed on the subsequent readdition of 1.5 mM Ca^{2+}.

For arteriolar diameter measurements, a video edge-detection system (Living Systems Instrumentation, Inc., Burlington, U.S.A.) was used. Arterioles were placed on a bath on the stage of an inverted trinocular microscope (Nikon TMS, Japan) with a CCD camera (Sony, Japan). Signals were captured by an A-D converter (Picolog software, Pico Technology, Cambridge, U.K.) and stored on a computer.

Messenger RNA was isolated from arteriole fragments with Dynabeads® Oligo $(dT)_{25}$ according to the manufacturer's instructions (Dynal). Bead complexes were washed and transferred to a 20 μl SuperScript™ reverse transcriptase (Gibco-BRL) reaction at 42°C for 60 min. Forward and reverse PCR primer sequences (accession numbers are in

parentheses) were β-actin (V01217) TTGTAACCAACTGGGACGATATG and GATCTTGATCTTCATGGTGCTGG, TRPC1 (U31110) TGGTATGAAGGGTTGGAAGAC and GGTATCATTGCTTTGCTGTTC, TRPC3 (U47050) TGACTTCCGTTGTGCTCAAATATG and CCTTCTGAAGTCTTCTCCTTCTGC, TRPC4 (AF175406) TCTGCAGATATCTCTGGGAAGGATGC and AAGCTTTGTTCGAGCAAATTTCCATTC, TRPC5 (AF029983) TGAGAATGA-GAACTTGGAG and TACTCAGCCTTGAACTCATTC, and TRPC6 (AF057748) AGTTTTAAGACACTGTTCTGG and TTCTGATATTGTCTTGGAGG. Thermal cycling was 95°C (5 min), 40 cycles at 94°C (30 s), 53–60°C (1 min), 72°C (1 min), and 72°C (7 min). Products were detected on a 1.5% agarose gel. Product identities of TRPC1, TRPC4 and β-actin were also confirmed by direct sequencing using an ABI PRISM dye terminator.

Data sets are expressed as mean ± s.e.m. for the number of cells (ROIs) from which measurements were made. This involved up to five cells from each arteriole and so numbers of arterioles are also given: n/N, where n is the number of cells and N the number of arterioles. Statistical comparisons between two groups were made using unpaired Student's t-tests in which a significant difference was assumed if $P<0.05$. Some data sets were fit with the Hill equation, $\{(D_{max}-D_{min})x^s/(x^s+EC_{50}^s)\}+D_{min}$, where x is the concentration, s the slope, EC_{50} the concentration giving half-maximal response and D_{max} and D_{min} are the maximum and minimum values.

General salts were from BDH (U.K.) or Sigma (U.K.). Protease, collagenase, TG, D600, Gd^{3+}, La^{3+}, Ni^{2+}, capsaicin, nifedipine, streptomycin and sulindac were from Sigma. 2-Aminoethoxydiphenylborate (2-APB) was from Tocris Cookson Limited (U.K.) or Sigma. Fura-PE3 AM, endothelin-1 (ET-1) and SKF96365 were from Calbiochem (U.K.). Fluo-4 was from Molecular Probes (U.K.). CPA was from Affiniti Research Products Limited (U.K.) or Tocris Cookson Limited. Ruthenium red was from Calbiochem or Sigma. Wortmannin (WT) was from Tocris Cookson Limited. *Grammostolla spatulata* venom was from Spider Pharm Inc. (Yarnell, AZ, U.S.A.). LOE 908 was a kind gift from Boehringer Ingelheim (Germany). Chemical structures of some of these agents are shown in Figure 1. Ruthenium red is $[(NH_3)_5RuORu(NH_3)_4ORu(NH_3)_5]Cl_6$. LOE 908 is (*RS*)-(3,4-dihydro-6,7-dimethoxyisoquinoline-1-γl)-2-phenyl-*N*,*N*-di-[2-(2,3,4-trimethoxyphenyl)ethyl]acetamide. SKF96365 is 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1*H*-imidazole hydrochloride.

Fura-PE3 AM, fluo-4 AM, capsaicin, TG, 2-APB, LOE 908, sulindac and WT were made up as stock solutions in 100% dimethyl sulphoxide (DMSO) and, with the exception of experiments involving capsaicin, the final DMSO concentration did not exceed 0.2%. To avoid precipitation of capsaicin (100 μM), bath solutions contained a total of 1% DMSO. In these experiments, 1% DMSO was present throughout (i.e. including the period before application of capsaicin). Nifedipine was made up as a stock solution in 100% ethanol. Other chemicals were prepared in water/salt solution.

Results

Viability of the enzymatically isolated rabbit arteriolar fragments was confirmed by contraction studies. Arterioles contracted to 60 mM K^+ or endothelin-1 in the extracellular bath solution (Figure 2a–c). The concentration dependence of the response to endothelin-1 was similar to our previous findings and to that observed in human pial arteries (Guibert & Beech, 1999; Pierre & Davenport, 1999).

SOCs were investigated in the presence of 10 μM D600, which completely blocks 60 mM K^+ evoked Ca^{2+} entry in these arterioles (Guibert & Beech, 1999) and avoids complications due to Ca^{2+} entry through voltage-gated Ca^{2+} channels. SOCs activated by store depletion with TG were inhibited concentration dependently by Gd^{3+} with an IC_{50} of 101 nM (Figure 3a, b). Acute store depletion with CPA in standard (1.5 mM Ca^{2+}) bath solution similarly induced Gd^{3+} sensitivity ($n/N=32/8$) (data not shown). Gd^{3+} (100 μM) had no effect in the absence of store depletion (Figure 3c) ($n/N=43/10$).

In the absence of D600, activation of voltage-gated Ca^{2+} channels by 60 mM K^+ solution evoked a $[Ca^{2+}]_i$ rise that was inhibited by 1 μM nifedipine (Figure 3d). There was 91.5 ± 4.1% inhibition compared with the baseline prior to application of 60 mM K^+ ($n/N=19/4$). SOCs, however, were not sensitive to 1 μM nifedipine (Figure 3e) ($n/N=24/5$). In the same arterioles, SOC-mediated Ca^{2+} entry was inhibited by 97.2 ± 2.1% by 10 μM Gd^{3+} (Figure 3e). Store depletion conferred sensitivity to 0.1–1 mM Ni^{2+} or 10 μM La^{3+} (Figure 4a–f). Thus, store depletion induced Gd^{3+}, Ni^{2+} and La^{3+} sensitivity, but not nifedipine sensitivity.

2-APB inhibits SOCs or CRACs in other cell types (Dobrydneva & Blackmore, 2001; Gregory *et al*., 2001; Kukkonen *et al*., 2001; Prakriya & Lewis, 2001). In four experiments on store-depleted arterioles 75 μM 2-APB strongly reduced $[Ca^{2+}]_i$ (Figure 5a). There was 66.5 ± 2.7% inhibition compared with $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} ($n/N=20/4$). In two other store-depleted arterioles, there was no effect of 75 μM 2-APB even though Gd^{3+} was effective (Figure 5b). There are stimulatory effects of low micromolar concentrations of 2-APB on CRAC channels (Prakriya & Lewis, 2001). However, 7.5 μM 2-APB had no effect on four store-depleted arterioles (data not shown) and reduced $[Ca^{2+}]_i$ in two arterioles (Figure 5c). There were no effects of 75 μM 2-APB on $[Ca^{2+}]_i$ in fura-PE3-loaded arterioles without store depletion ($n/N=35/7$) or on the autoflourescence of arterioles without fura-PE3 loading ($n/N=15/3$) (data not shown). Preincubation with micromolar concentrations of Wortmanin (WT)–a kinase inhibitor (Ui *et al*., 1995)–inhibits store operated Ca^{2+} entry in various cell types (Jenner *et al*., 1996; Broad *et al*., 2001; Tran *et al*., 2001). Arterioles were pretreated with or without 10 μM WT for 1 h prior to incubation with TG in Ca^{2+}-free solution. The increase in $[Ca^{2+}]_i$ upon re-addition of 1.5 mM Ca^{2+} was significantly inhibited by WT (Figure 5d–f).

SKF96365 or LOE 908 inhibits SOCs and ROCs in several cell types and may distinguish between different subtypes of these channels (Merritt *et al*., 1990; Krautwurst *et al*., 1993; 1994; Iwamuro *et al*., 1999; Zhang *et al*., 1999). Using the CPA store-depletion protocol, 10 μM SKF96365 had no effect on SOC-mediated Ca^{2+} entry (Figure 6a; $n/N=16/6$). At 30 μM, SKF96365 had no inhibitory effect ($n=3/2$) or produced a 17.7 ± 0.8% transient inhibition ($n/N=3/1$) (data not shown). Using the TG depletion protocol, 30 μM SKF96365 caused a slow increase in $[Ca^{2+}]_i$ in all cells, which was preceded by a transient reduction in 21% of cells ($n/N=19/4$). There was no

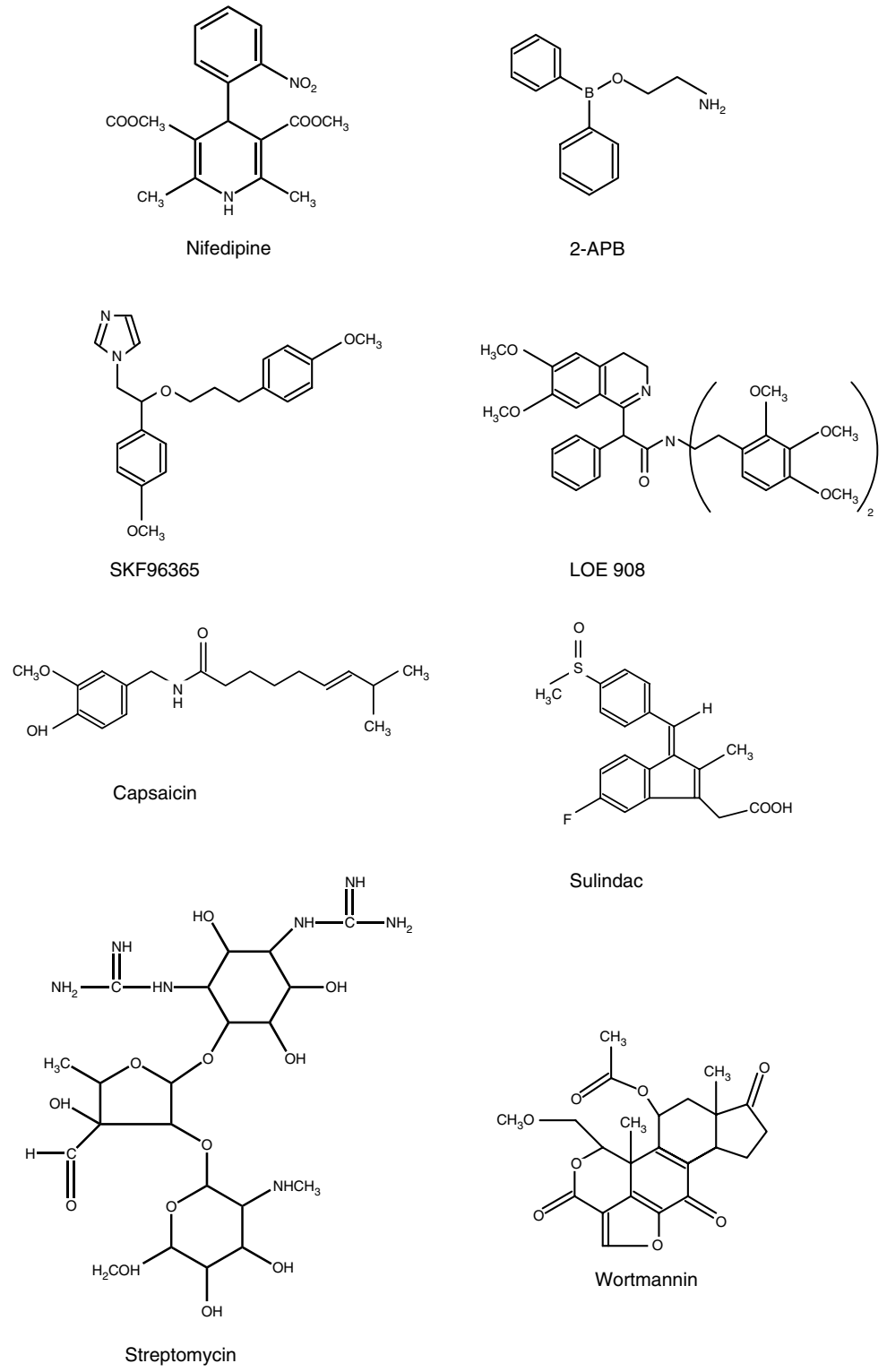

Figure 1 Chemical structures of agents tested for activity against cerebral arteriolar SOCs.

effect of 30 μM SKF96365 in arterioles without fura-PE3 loading ($n/N = 10/2$).

To study the effect of LOE 908, we used fluo-4 instead of fura-PE3 because there are LOE 908-mediated artefacts associated with fura-2 (Iwamuro *et al*., 1999) and in our fura-PE3 experiments LOE 908 apparently strongly reduced arteriolar $[Ca^{2+}]_i$ even in the absence of extracellular Ca^{2+} ($n/N = 6/2$) (data not shown). No such reductions in $[Ca^{2+}]_i$ occurred when using fluo-4. In store-depleted arterioles (TG protocol) in the presence of extracellular Ca^{2+} LOE 908 (10 μM) had no effect, even though Gd^{3+}-sensitivity was evident in all cases (Figure 6b; mean inhibition by 10 μM

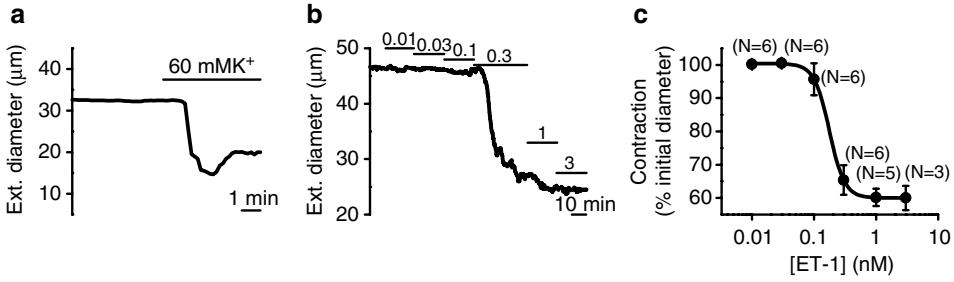

Figure 2 Contractile response of isolated rabbit cerebral arterioles to elevated external K^+ concentration and endothelin-1 (ET-1). (a, b) Plots of external diameter of arterioles against time. (a) An isolated arteriole contracted to 60 mM K^+ and then partially relaxed in the continued presence of 60 mM K^+. (b) ET-1 caused concentration-dependent contraction of arterioles. Concentrations are in nM. (c) Concentration–response curve for ET-1, N = number of arterioles. $EC_{50} = 0.18$ nM. Data shown are mean ± s.e.m. and the smooth curve is the fitted Hill equation.

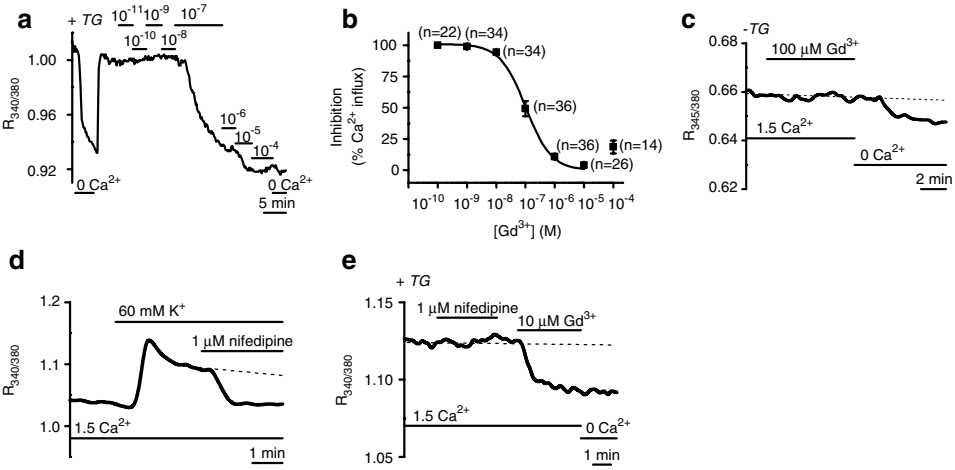

Figure 3 Store depletion activates Gd^{3+}-sensitive, nifedipine-resistant SOCs in smooth muscle cells of isolated cerebral arterioles. $[Ca^{2+}]_i$ is given as the ratio of fura-PE3 signals. Arterioles were in standard (1.5 mM Ca^{2+}) bath solution unless indicated. Ca^{2+}-free bath solution ('0 Ca^{2+}) contained 0.4 mM EGTA. (a–c) All solutions contained 10 μM D600. (a) Inhibition of SOC-mediated $[Ca^{2+}]_i$ rise by increasing Gd^{3+} concentrations in a TG-pretreated arteriole. (b) Concentration–inhibition curve for the effect of Gd^{3+} on arteriolar SOCs. n = number of individual smooth muscles. N (number of arterioles) was ⩾4 for each Gd^{3+} concentration. $IC_{50} = 101$ nM, slope = 1.00. Data shown are mean ± s.e.m. and the smooth curve is the fitted Hill equation. (c) No effect of 100 μM Gd^{3+} without TG pretreatment. Ca^{2+}-free solution reduced $[Ca^{2+}]_i$. (d) In the absence of D600, the $[Ca^{2+}]_i$ rise induced by 60 mM K^+ was inhibited by nifedipine. (e) All solutions contained 10 μM D600. There was no effect of nifedipine following TG pretreatment. Gd^{3+} lowered $[Ca^{2+}]_i$. (c–e; and in Figures 4–6). The broken line shows a prediction of the trend in $[Ca^{2+}]_i$ if the blocker had not been applied.

Gd^{3+} was 81.2 ± 6.6%, $n/N = 13/3$). Similarly, there was no effect of LOE 908 on $[Ca^{2+}]_i$ in arterioles treated with CPA (Figure 6c; $n/N = 43/11$).

Ruthenium red (0.03–10 μM) inhibits a number of Ca^{2+}-permeable channels including VR1 (TRPV1; Caterina *et al*., 1997), VRL-1 (TRPV2; Caterina *et al*., 1999), OTRPC4 (TRPV4; Strotmann *et al*., 2000), ECaC1 (TRPV5; Nilius *et al*., 2001) and CaT1 (TRPV6; Hoenderop *et al*., 2001). $[Ca^{2+}]_i$ in store-depleted arterioles (TG protocol) was, however, resistant to 10 μM ($n/N = 5/1$) (Figure 6d) or 100 μM ($n/N = 10/2$) ruthenium red. Subsequent application of Gd^{3+} inhibited SOC-mediated Ca^{2+} entry ($n/N = 15/3$). At submicromolar concentrations capsaicin is a vanilloid receptor (VR1) agonist. However, 10–100 μM capsaicin also inhibits SOC-mediated Ca^{2+} entry in PC12 and HL-60 cells (Choi & Kim, 1999; Choi *et al*., 2000) and CRAC-mediated Ca^{2+}-entry in Jurkat T cells (Fischer *et al*., 2001). SOC-mediated Ca^{2+}-entry in arterioles was nevertheless relatively resistant. Capsaicin (100 μM) reduced $[Ca^{2+}]_i$ by 12.3 ± 3.7% ($n/N = 10/3$) in store-depleted arterioles (Figure 6e). The nonsteroidal anti-inflammatory sulindac (60 μM) inhibits SOC-mediated Ca^{2+} entry in HRT-18 cells (Weiss *et al*., 2001). However, SOC-mediated Ca^{2+} entry was resistant to 100 μM sulindac (Figure 6f; $n/N = 19/4$). In the same arterioles, 10 μM Gd^{3+} reduced $[Ca^{2+}]_i$ by 84.6 ± 5.2% ($n/N = 19/4$).

Streptomycin at low micromolar concentrations inhibits stretch-activated cation channels in vestibular hair cells and ventricular myocytes (Ohmori, 1985; Gannier *et al*., 1994; Belus & White, 2001). SOC-mediated Ca^{2+} entry in arterioles was, in contrast, resistant to even 0.5 mM streptomycin (Figure 6g; $n/N = 15/3$). In the same arterioles, 10 μM Gd^{3+} caused 96.6 ± 3.4% reduction of the SOC-mediated Ca^{2+} signal. *Grammostolla spatulata* venom (1 : 100,000 dilution) inhibits stretch-activated cation channels in coronary arteriolar

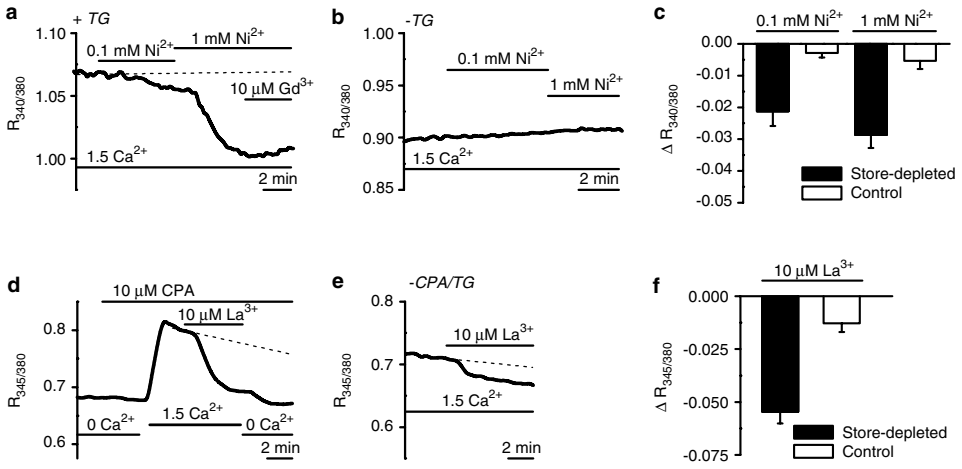

Figure 4 Cerebral arteriolar SOCs are Ni^{2+}- and La^{3+}-sensitive. Arterioles were in standard (1.5 mM Ca^{2+}) or Ca^{2+}-free (0.4 mM EGTA) bath solution. All solutions contained 10 μM D600. (a–c) Ni^{2+} reduced $[Ca^{2+}]_i$ following TG pretreatment (a), but had no effect in control conditions (b). (c) Mean $\pm$ s.e.m. Ni^{2+}-induced reduction of fura-PE3 ratio in the TG-pretreated 'store-depleted' (as in a; 0.1 and1 mM Ni^{2+}, $n/N = 19/5$) and control (as in b; 0.1 and 1 mM Ni^{2+}, $n/N = 27/6$) groups. (d–f) La^{3+} caused a large reduction in $[Ca^{2+}]_i$ after store depletion with CPA in Ca^{2+}-free solution (d), and had a smaller effect in control conditions (e). (f) Mean $\pm$ s.e.m. La^{3+}-induced reduction of fura-PE3 ratio in CPA/0 Ca^{2+} 'store-depleted' (as in D, $n/N = 15/3$) and control (as in e, $n/N = 58/12$) groups.

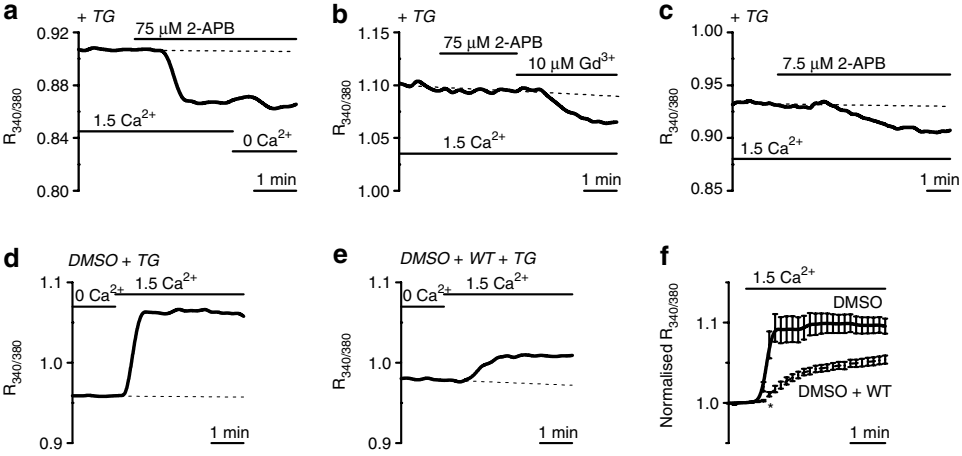

Figure 5 2-APB and WT inhibit cerebral arteriolar SOCs. Arterioles were in standard (1.5 mM Ca^{2+}) or Ca^{2+}-free (0.4 mM EGTA) bath solution. All solutions contained 10 μM D600. (a–c) Effects of 2-APB in TG-pretreated arterioles. (a) 2-APB (75 μM) rapidly reduced $[Ca^{2+}]_i$. (b) In another arteriole there was no effect of 75 μM 2-APB but Gd^{3+} reduced $[Ca^{2+}]_i$, confirming SOC activation. (c) 2-APB (7.5 μM) slowly reduced $[Ca^{2+}]_i$. (d–f) Preincubation with WT inhibited Ca^{2+} entry in TG-pretreated arterioles. Arterioles were first incubated with either 0.1% DMSO (d) or 0.1% DMSO and 10 μM WT (e) in standard (1.5 mM Ca^{2+}) bath solution. After this period, arterioles were incubated with TG in Ca^{2+}-free (0.4 mM EGTA) bath solution. The increase in $[Ca^{2+}]_i$ associated with the readdition of 1.5 mM Ca^{2+} was reduced in WT-pretreated arterioles. (f) Mean $\pm$ s.e.m. data for experiments as shown in (d) and (e), normalised to the value of $R_{340/380}$ at the point of addition of 1.5 mM Ca^{2+}. DMSO group, $n/N = 18/4$. WT group, $n/N = 20/4$. $P < 0.001$ for the data point marked * and all subsequent data points.

smooth muscle cells (Wu & Davies, 2001), but at 1 : 10,000 dilution it had no effect on SOC-mediated Ca^{2+} entry (Figure 6h; $n/N = 20/4$).

In summary, SOC-mediated Ca^{2+} entry in arterioles is inhibited by Gd^{3+}, La^{3+}, Ni^{2+}, 2-APB and WT, and is largely resistant to nifedipine, SKF96365, LOE 908, ruthenium red, capsaicin, sulindac, streptomycin or *G. spatulata* venom (Figure 7).

The determination of a pharmacological profile of SOCs in arterioles enables a comparison with the known pharmacology of heterologously expressed TRPC genes, which are suggested to encode SOC subunits. TRPC1 is already known to contribute to the SOCs of cerebral arterioles (Xu & Beech, 2001), but it may not account for all of the SOC signal and it may form heteromultimers with other TRPCs. Therefore, we used RT–PCR analysis of isolated arteriolar fragments to explore if additional TRPC genes are expressed. TRPC3, 4, 5 and 6 were detected (Figure 8). Genomic DNA was absent from the samples as indicated by the absence of a β-actin PCR product when the reverse transcriptase was excluded.

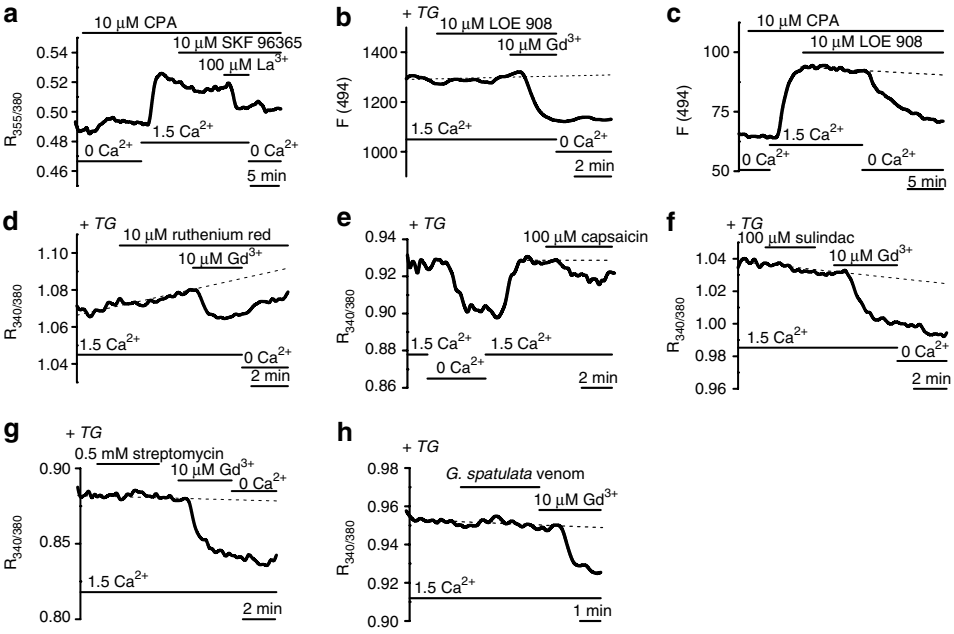

Figure 6 Effects of SOC and other nonselective cation-channel inhibitors on cerebral arteriolar SOCs. Arterioles were in standard (1.5 mM Ca^{2+}) or Ca^{2+}-free bath solution (0.4 mM EGTA). All solutions contained 10 μM D600. (a) No effect of 10 μM SKF 96365 in a zero Ca^{2+}/CPA-treated arteriole, but La^{3+} did reduce $[Ca^{2+}]_i$. (b) In a TG-pretreated arteriole, 10 μM LOE 908 had no effect although Gd^{3+} reduced $[Ca^{2+}]_i$. (c) In an arteriole treated with zero Ca^{2+}/CPA, LOE 908 had no effect on $[Ca^{2+}]_i$. (d–h) Effects of 10 μM ruthenium red (d), 100 μM capsaicin (e), 100 μM sulindac (f), 0.5 mM streptomycin (g) and 1:10,000 *G. spatulata* venom (h) on $[Ca^{2+}]_i$ in TG-pretreated arterioles. Only capsaicin (e) had any effect on $[Ca^{2+}]_i$. For agents having no effect, SOC activation was confirmed by Gd^{3+}-induced reduction of $[Ca^{2+}]_i$.

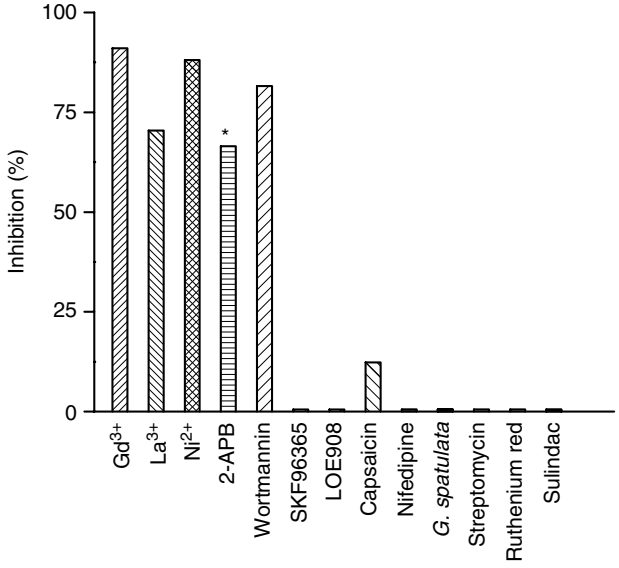

Figure 7 Pharmacological profile of SOCs in cerebral arteriolar smooth muscle cells. Data are the mean inhibition of Ca^{2+} entry in store-depleted arterioles. Concentrations (μM): Gd^{3+} (10), La^{3+} (10), Ni^{2+} (1000), 2-APB (75), WT (10), SKF 96365 (10), LOE 908 (10), capsaicin (100), nifedipine (1), streptomycin (500), ruthenium red (100) and sulindac (100). *G. spatuluta* venom (1 : 10,000 dilution). All agents were applied topically except WT, which was preincubated with arterioles before SOC activation. Store depletion was achieved using the TG protocol for all agents except La^{3+} and SKF 96365 for which the CPA store-depletion protocol was used. Both protocols were used for experiments with LOE 908. *The mean inhibition given for 2-APB is from experiments in which only clear inhibitory effects occurred.

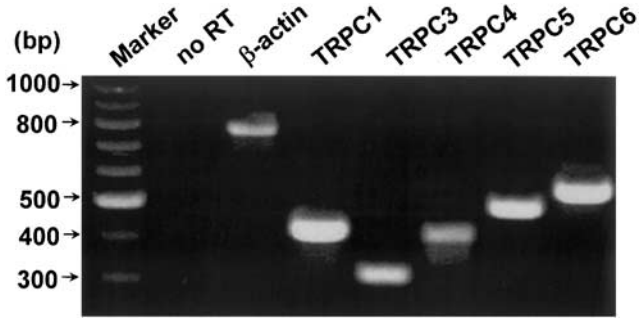

Figure 8 Detection of multiple TRPC mRNAs in isolated rabbit pial arterioles. Agarose gel electrophoresis of DNA amplicons produced from RT–PCR experiments. The predicted sizes of the amplicons are 763 bp (β-actin), 423 bp (TRPC1), 318 bp (TRPC3), 415 bp (TRPC4), 489 bp (TRPC5) and 551 bp (TRPC6). Positions of DNA ladder markers and a β-actin 'no RT' control are shown on the left.

Discussion

Through these experiments we have shown that SOC-mediated Ca^{2+}-entry in cerebral arteriolar smooth muscle is inhibited by Gd^{3+}, La^{3+}, Ni^{2+}, 2-APB and WT, and slightly by high concentrations of capsaicin. Importantly, sensitivity to Gd^{3+}, Ni^{2+} or 2-APB did not exist in control arterioles that were not store-depleted. This is compelling evidence for the activation of a distinct set of ion channels in response to store depletion that are pharmacologically distinct from tonically active background channels. The SOCs were resistant to a range of other agents (Figure 7), which have been shown to inhibit

SOCs and Ca^{2+}-permeable cation channels activated by other mechanisms in different cell types.

An important new observation has been that SOCs in smooth muscle cells of retinal arterioles are nifedipine-sensitive (Curtis & Scholfield, 2001). Although a defining characteristic of many SOCs is the opposite of this (i.e. resistance to classical Ca^{2+} antagonists) there are other examples of SOCs with sensitivity to Ca^{2+} antagonists – particularly some dihydropyridines (Hopf *et al*., 1996; Willmott *et al*., 1996; Auld *et al*., 2000; Young *et al*., 2001). Nevertheless, consistent with many other studies we found SOCs that were resistant to dihydropyridine and phenylalkylamine Ca^{2+} antagonists. One of several differences between our study and that of Curtis & Scholfield (2001) is that we included 10 μM D600 throughout the experiments. Although this ensured that we did not study voltage-dependent Ca^{2+} entry, we cannot exclude that D600 prevented us from observing nifedipine-sensitive SOCs (assuming they are also D600-sensitive). What is clear is that the SOC pharmacology we describe is for nifedipine-resistant SOCs, and we have no evidence for nifedipine-sensitive SOCs in pial arterioles.

A comparison of our SOC pharmacological profile with that of SOCs described in other types of smooth muscle suggests there is SOC diversity. Sensitivity to block by Ni^{2+} may be a common feature (McDaniel *et al*., 2001; Ng & Gurney, 2001; Trepakova *et al*., 2001; Wilson *et al*., 2002). However, in marked contrast to SOCs in arterioles, SOCs in anococcygeus smooth muscle cells are resistant to Gd^{3+} or La^{3+} at concentrations up to 400 μM (Wayman *et al*., 1996). SOCs in smooth muscle of renal arteries show some sensitivity to 100 μM Gd^{3+}, while those in pulmonary arterial smooth muscle do not (Wilson *et al*., 2002). In intrapulmonary arterial smooth muscle, SOCs are sensitive to 1 μM La^{3+} yet those in main pulmonary artery are blocked only by La^{3+} concentrations $\geqslant$100 μM (Robertson *et al*., 2000; Ng & Gurney, 2001).

Experiments with the highest concentration of SKF96365 (30 rather than 10 μM) were complicated by a stimulatory effect–a slowly developing but pronounced elevation of $[Ca^{2+}]_i$. A similar effect occurs in human left internal mammary artery smooth muscle cells in primary culture (T.J.P. Bachelor & D.J. Beech, unpublished obervations). We have not explored the mechanism, but it may be related to inhibition of Na^+/Ca^{2+} exchange and/or induction of Ca^{2+} release from endoplasmic reticulum (Leung *et al*., 1996; Jan *et al*., 1999; Arakawa *et al*., 2000). Activation of Ca^{2+}-permeable channels has also been observed in response to 15–200 μM SKF 96365 (Dietl & Volkl, 1994; Schwarz *et al*., 1994; Leung *et al*., 1996; Jan *et al*., 1999). A stimulatory effect of 30 μM SKF96365 could have masked an inhibitory action on arteriolar SOCs, but in this regard it is significant that 10 μM SKF96365 had neither inhibitory nor stimulatory effects. This contrasts with the marked inhibitory effect of 10 μM SKF96365 on SOCs in smooth muscle cells of the anococcygeus or pulmonary artery and on ROCs in ileal smooth muscle cells (Wayman *et al*., 1996; Zhang *et al*., 1999; Zholos *et al*., 2000; Ng & Gurney, 2001).

2-APB has emerged as a potential basis for small molecule inhibitors of SOCs, although as with many of the other SOC inhibitors it lacks specificity and has effects on the IP_3 receptor (Maruyama *et al*., 1997), SERCA (Bilmen *et al*., 2002) and voltage-gated K^+ channels (Wang *et al*., 2002). In common with SOCs in other cell types, including the CRAC channel, we found 2-APB had a blocking effect in the mid-to-high micromolar concentration range. However, the effects of this compound are not simple. In some instances (albeit with a low sample number), we found SOCs were resistant to 2-APB. This may be explained by the existence of multiple types of SOCs, if regulation of SOCs alters sensitivity to 2-APB, or by the existence of a counter-balancing stimulatory effect of 2-APB. SOCs with poor or no sensitivity to 2-APB have been observed, as have stimulatory effects of 2-APB (Kukkonen *et al*., 2001; Prakriya & Lewis, 2001). Our experiments were complicated by a slowly developing 2-APB (75 μM)-induced elevation of $[Ca^{2+}]_i$ in about 50% of cells (R. Flemming & D.J. Beech, unpublished).

Inhibition of store-operated Ca^{2+} entry with the fungal metabolite WT has variously indicated roles for tyrosine kinases, myosin light chain kinase and cellular polyphosphoinositides in the coupling mechanism between store depletion and activation of SOCs (Jenner *et al*., 1996; Broad *et al*., 2001; Tran *et al*., 2001). Pretreatment with 10 μM WT inhibited store-operated Ca^{2+} entry in arterioles. Various cellular kinases are inhibited by this concentration of WT and so a role for one or more kinase in the activation of arteriolar SOCs is indicated. Further investigation is required. In contrast, TG induced store-operated Ca^{2+} entry in porcine endothelial cells is resistant to preincubation with WT at concentrations up to 100 μM (Kuroiwa-Matsumoto *et al*., 2000).

Further evidence for SOC diversity comes from comparisons with SOCs in other cell types. Insensitivity of pial arteriolar SOCs to LOE 908 distinguishes these channels from SOCs in endothelial cells (Encabo *et al*., 1996). Sulindac-sensitive SOCs in HRT-18 (Weiss *et al*., 2001) contrast with pial arteriolar SOCs that are completely resistant to this drug. The poor sensitivity of pial arteriolar SOCs to capsaicin distinguishes them from SOCs in PC12, HL-60 and Jurkat T cells (Choi & Kim 1999; Choi *et al*., 2000; Fischer *et al*., 2001).

Stretch-activated cation channels mediate the depolarisation response to stretch in various cell types. These nonselective cation channels are inhibited by low micromolar concentrations of Gd^{3+} (Hamill & McBride, 1996) including the channel in cerebral artery smooth muscle cells (Welsh *et al*., 2000). The antibiotic streptomycin and venom of *G. spatula* inhibit stretch-activated cation channels (Ohmori, 1985; Gannier *et al*., 1994; Belus & White, 2001; Wu & Davis, 2001), but have no effect on pial arteriolar SOCs.

The suggestion that some TRPCs are subunits of SOCs (reviewed by Zitt *et al*., 2002) and the detection of mRNAs for several TRPCs in the isolated arterioles (Figure 8) helps justify a comparison of the pharmacology of arteriolar SOCs with that available for heterologously expressed TRPCs. TRPC1 has been found to be sensitive to block by 0.02–1 mM Gd^{3+} or 0.1–1 mM La^{3+} (Zitt *et al*., 1996; Sinkins *et al*., 1998; Liu *et al*., 2000). Thus, sensitivity of arteriolar SOCs to similar concentrations of Gd^{3+} and La^{3+} is consistent with our earlier suggestion that TRPC1 is a subunit of these SOCs (Xu & Beech, 2001). Intriguingly, there was a slight stimulation of Ca^{2+} entry with a higher (100 μM) Gd^{3+} concentration (Figure 3b). We have also noticed that following sustained application of 10–100 μM La^{3+} an increase in $[Ca^{2+}]_i$ is sometimes seen subsequent to its strong blocking effect (R. Flemming, S.-Z. Xu & D. J. Beech, unpublished observations). TRPC4 and TRPC5 are stimulated by La^{3+} and Gd^{3+}

(Schaefer *et al*., 2000; Strübing *et al*., 2001; Jung *et al*., 2003). Both were detected in arterioles and hence may be candidates for subunits of the pial arteriolar SOC. TRPC7, however, is insensitive to 0.1 mM Gd^{3+} (Okada *et al*., 1999) and thus would seem an unlikely candidate. Although TRPC6 is inhibited by La^{3+} or Gd^{3+}, it is also inhibited by 4 μM SKF96365 (Inoue *et al*., 2001), suggesting that TRPC6 is also not a subunit of the pial arteriolar SOC. Ruthenium red inhibits many types of channel: ryanodine receptors, some types of K^+ channel and members of the TRPV family of TRP proteins including VR1, VRL-1, OTRPC4, ECaC1 and CaT1 (Caterina *et al*., 1997; 1999; Tominaga *et al*., 1998; Strotmann *et al*., 2000; Hoenderop *et al*., 2001; Nilius *et al*., 2001). The resistance of pial arteriolar SOCs to a high concentration of ruthenium red indicates that TRPVs do not comprise subunits of the arteriolar SOC. A potential weakness in making these pharmacological comparisons is that TRPs are known to be able to form hetermultimers (Strübing *et al*., 2001; Hofmann *et al*., 2002). It will be important to determine not only the expression of TRP proteins in vascular smooth muscle, but also their tendency to heteromultimerise in native cells.

Thus, SOCs in arterioles have a distinct pharmacological profile. Knowledge of this profile provides support for the hypothesis that there are multiple types of SOC in smooth muscle and will facilitate comparisons with heterologously expressed genes that encode putative subunits of SOCs. The results presented in this study support the proposal that TRPC1, possibly along with TRPC4 and/or TRPC5, comprises the arteriolar SOC. The existence of distinct SOCs in smooth muscle may aid the targeting of novel therapeutic drugs to specific vascular beds.

We thank the British Heart Foundation and Wellcome Trust for support and Boehringer Ingelheim for kindly providing LOE 908.

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(Received March 4, 2003)
Accepted March 24, 2003)