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Pharmacological profile of store-operated Ca^{2+} entry in intrapulmonary artery smooth muscle cells

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Abstract

Store-operated Ca^{2+} entry (SOCE) plays an important role in the contraction and proliferation of pulmonary artery smooth muscle cells (PASMCs). The aim of this study was to characterise the pharmacological properties of the SOCE pathway in freshly isolated PASMCs from rat lung and to determine whether this Ca^{2+} entry pathway is sensitive to nitric oxide donor drugs. Following depletion of Ca^{2+} from the sarcoplasmic reticulum, by treating cells with thapsigargin, re-addition of Ca^{2+} produced an increase in cytosolic fluo-4 fluorescence that was sustained for the period that extracellular Ca^{2+} was present. Thapsigargin also increased the rate of quench of fura-2 fluorescence, confirming that SOCE was activated. The SOCE pathway was not affected by nifedipine or verapamil; however, it was inhibited by the divalent cations Ni^{2+} (10 μM) and Cd^{2+} (10 μM) by 47±5% and 49±5% respectively. SOCE was also inhibited 42±5% by 2-aminoethoxydiphenyl borate (2-APB; 75 μM) and 58±4% by Gd^{3+} (10 μM), although La^{3+} (100 μM) had little effect. None of the NO donors examined, including sodium nitroprusside, glyceryl trinitrate, and 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide had any effect on SOCE. Thus, the pulmonary vasorelaxation produced by NO does not involve direct inhibition of SOCE in PASMCs. Western blot and immunocytochemistry using antibodies directed against specific TRPC subunits detected the presence of TRPC1, 3, and 6 in pulmonary artery and the pharmacological profile of SOCE in PASMCs favours a role for TRPC1 in mediating the underlying channels that are activated by store depletion.

Keywords: Store-operated Ca^{2+} entry (SOCE); Nitric oxide; Pulmonary artery; Transient receptor potential (TRP); Smooth muscle cells

1. Introduction

Store-operated Ca^{2+} entry (SOCE) is activated in response to the depletion of Ca^{2+} from the sarcoplasmic/endoplasmic reticulum (Parekh and Putney, 2005; Parekh, 2006). The major function of this Ca^{2+} influx pathway is likely to be refilling of the intracellular stores, but it is becoming clear that it has additional roles (Parekh and Putney, 2005). For instance, in the pulmonary artery Ca^{2+} entering through store-operated channels can initiate contraction (Gonzalez De La Fuente et al., 1995; Ng and Gurney, 2001; Snetkov et al., 2003) and may play a role in hypoxic pulmonary vasoconstriction (Ward et al., 2005; Weigand et al., 2005). Furthermore, SOCE is enhanced during proliferation of pulmonary artery smooth muscle cells (PASMCs) and this is thought to be important in the pathophysiology of pulmonary hypertension (Landsberg and Yuan, 2004).

There is increasing evidence that SOCE is mediated by channels encoded by the canonical transient receptor potential (*TRPC*) gene family, of which there are seven members, denoted TRPC1-7 (Parekh and Putney, 2005). Pulmonary arteries express several of these genes (Dietrich et al., 2007), but there is incomplete agreement on which ones. For example, one study on rat intrapulmonary arteries found mRNA and protein expression for TRPC1, 3 and 6, but did not detect TRPC4 or 5 (Lin et al., 2004), while another reported finding TRPC1, 2, 4, 5 and 6, but not 3 (McDaniel et al., 2001) and a third study found only TRPC1 and 4, but not 3 (Wang et al.,

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2004). These discrepancies could reflect non-uniform expression of TRPC isoforms along the pulmonary arterial tree. Cell culture is also known to influence TRPC expression (Golovina et al., 2001; Bergdahl et al., 2005) and since most of these studies employed primary cultured PASMCs, the disparate results could reflect differences in culture conditions. In freshly isolated smooth muscle cells from the main pulmonary artery outside the lung, TRPC1, 3, 4, 5 and 6 were all detected, although TRPC4 and 5 were at low levels (Ng and Gurney, 2001); however, whether or not distinct TRPC genes are preferentially expressed in different parts of the pulmonary arterial tree remains to be determined.

Much of our understanding of the functional roles of SOCE and TRPC channels has relied upon interventions with pharmacological tools that block their activity. Unfortunately, although several agents are known to inhibit store-operated channels, they do not generally do this in a selective manner (Parekh and Putney, 2005). SKF96365 is a non-selective drug that consistently blocks around 50% of SOCE in PASMC at 7–10 μM (Ng and Gurney, 2001; Wang et al., 2004). Other inhibitors have proved to be less consistent. Although trivalent and divalent cations inhibit many Ca^{2+} influx pathways, they show some selectivity for store-operated channels at low micromolar concentrations, especially La^{3+} and Gd^{3+}. Variable effects of La^{3+} have been reported on store-operated channels or Ca^{2+} entry in the pulmonary artery, with the concentration producing 50% inhibition ranging from 0.1 μM (Snetkov et al., 2003) to 40 μM (Wang et al., 2004) in rat small intrapulmonary arteries, to 600 μM in rat main pulmonary artery (Ng and Gurney, 2001) and >100 μM in canine PASMCs (Ng et al., 2005). This variability could reflect different expression patterns of *TRPC* genes or different protocols for measuring channel activity. Similarly, while 1 μM Gd^{3+} blocked store-operated channels in the rat pulmonary artery almost completely (Snetkov et al., 2003), it was poorly effective in the dog even at 100 μM (Ng et al., 2005). The drug 2-aminoethoxydiphenylborate (2-APB) is a direct inhibitor of store-operated channels, although it has several additional effects, including IP_3 receptor antagonism. 2-APB was found to partially inhibit the store-operated current induced by thapsigargin in rat PASMCs and almost completely abolish the thapsigargin-induced contractile response (Snetkov et al., 2003).

The pharmacological properties of SOCE in PASMC are therefore far from clear, and this is especially so for freshly isolated smooth muscle cells. The lack of clearly selective inhibitors that can act as markers for the underlying channels highlights the importance of compiling a pharmacological profile of the Ca^{2+} entry pathway, from a wide range of agents that interact with it. The initial aim of this study was therefore to pharmacologically characterise the SOCE pathway in freshly isolated smooth muscle cells from small intrapulmonary arteries of the rat, and to determine which TRPC subunits are expressed. This information should help to identify the molecular make up of the underlying channels and provide a basis for studies aimed at understanding their physiological functions.

A pharmacological agent of particular interest is nitric oxide (NO), a potent endogenous vasodilator used in the treatment of pulmonary hypertension (Griffiths and Evans, 2005). NO relaxes smooth muscle in part by lowering the intracellular Ca^{2+} concentration (Carvajal et al., 2000). Several mechanisms may contribute to this action, including the inhibition of SOCE. Recent studies have shown that store-operated channels are regulated by multiple gating mechanisms, involving a number of protein kinases and intracellular messengers (Albert et al., 2007). Moneer et al. (2003) showed that NO can directly inhibit SOCE in A7r5 smooth muscle cells through a mechanism involving cGMP and G-kinase. A similar direct effect of NO was observed on recombinant TRPC3 channels, which may underlie store-operated channels in some cell types (Thyagarajan et al., 2001; Kwan et al., 2004). In contrast, the NO-induced relaxation of the aorta and reduction of cytosolic [Ca^{2+}] in aortic smooth muscle cells were prevented when the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) was inhibited by thapsigargin or cyclopiazonic acid (CPA), implying that they resulted from enhanced Ca^{2+} accumulation into the sarcoplasmic reticulum (Cohen et al., 1999). This would inhibit SOCE indirectly by preventing store depletion. In profiling the pharmacology of SOCE in pulmonary artery, a second aim of this study was therefore to determine whether or not NO donor drugs have a direct effect on SOCE, which could potentially underlie their pulmonary vasodilator action.

2. Materials and methods

2.1. Isolation of intrapulmonary artery smooth muscle cells

Male Sprague Dawley rats (~150–200 g) were sacrificed by cervical dislocation in accordance with the current UK Home Office guidelines on animal experimentation. The heart and lungs were rapidly removed *en bloc* and placed in ice cold dissecting solution of the following composition (in mM): NaCl 119, KCl 4.7, KH_2PO_4 1.18, $NaHCO_3$ 25, $MgSO_4$ 1.17, HEPES 10, glucose 5.5, pH adjusted to 7.4 with 1 M NaOH. Intrapulmonary arteries (300–800 μm outside diameter) were carefully dissected from the left lobe of the lung, cleaned of connective tissue and placed in fresh dissecting solution. Single smooth muscle cells were obtained using an enzymatic dissociation procedure previously described by Drummond and Tuft (1999). Briefly, the artery was cut into rings approximately 1 mm in length, which were then cut open and placed in enzyme solution, prepared by adding 1.5 mg/ml papain and 1.5 mg/ml dithiothreitol (DTT) to dissociation solution of the following composition (in mM): NaCl 128, KCl 5.4, KH_2PO_4 0.95, Na_2HPO_4 0.95, $NaHCO_3$ 4.16, $MgSO_4$ 1.17, HEPES 10, glucose 10, sucrose 5.5; pH adjusted to 7.3 with 1 M NaOH. The solution containing the tissue was then placed in a refrigerator at 4 °C for 1 h, after which it was transferred to a shaking water bath at 37 °C for 6 min. The tissue was then transferred to fresh dissociation solution containing collagenase (Sigma type VIII, 1.5 mg/ml) and incubated in the shaking water bath for a further 5 min at 37 °C. After washing the tissue several times in fresh dissociation solution, gentle trituration with a wide-bore, fire-polished Pasteur pipette yielded single smooth muscle cells, which remained viable for up to 6 h.

2.2. Ca^{2+} imaging

Freshly isolated PASMCs were loaded with the Ca^{2+} sensitive fluorescent indicator fluo-4 AM (5 μM) for 45 min at room temperature in the dark. A BioRad Radiance 2000MP laser scanning confocal microscope (BioRad®, Hemel Hempsted, UK), mounted on a Nikon eclipse E600FN upright microscope (Nikon, UK) equipped with a 20× NA 0.75 Plan Fluor water immersion lens (Nikon, UK), was used for imaging. Excitation of fluo-4 was achieved using the 488 nm line of an argon laser, and the emitted light was passed through a 510–530 nm band pass filter to a photomultiplier tube. For capturing rapid transient events, such as Ca^{2+} release induced by 20 mM caffeine, images were acquired at a rate of 100–200 frames/min. With slower events, such as the fluorescence increase during Ca^{2+} re-addition experiments, the acquisition rate was 5–10 frames/min.

2.3. Experimental protocol

In preliminary experiments, it was noticed that the increase in fluo-4 fluorescence produced by SOCE was significantly greater and more consistent when cells were exposed to thapsigargin (1 μM) for a longer period of time (~1 h), rather than the several minute period that is typically used in most studies (Takemura et al., 1989). This may be because intracellular stores in PASMCs are not particularly "leaky", so that short periods of exposure to thapsigargin do not fully deplete them. Thus for the majority of experiments reported here, the intracellular stores were depleted by treating cells with thapsigargin (1 μM) during the fluo-4 loading period, meaning cells were exposed to thapsigargin for at least 45 min. Thereafter, 30 μl of either the thapsigargin-treated or the untreated (control) cell suspension was added to a low profile, glass-bottom chamber of 0.5 ml volume (Warner Instruments, Harvard Apparatus, UK) and the cells allowed to settle. The chamber was then filled with a Ca^{2+}-free bath solution of the following composition (in mM): NaCl 150, KCl 5.4, $MgCl_2$ 3, HEPES 10, glucose 10, EGTA 1; pH 7.4.

The bath solution was changed during the course of experiments using a gravity-based perfusion system (flow rate ~8 ml/min). At the beginning of any experimental protocol, cells were imaged for 3–5 min in Ca^{2+}-free bath solution to provide a measure of basal fluo-4 fluorescence. To observe SOCE, the cells were perfused for 10 min with a Ca^{2+}-containing bath solution, comprising (in mM): NaCl 150, KCl 5.4, $MgCl_2$ 1.2, HEPES 10, glucose 10, $CaCl_2$ 1.8; pH 7.4. The bath solution was then returned to Ca^{2+}-free. The effects of various substances on SOCE were examined by adding them immediately prior to and during the perfusion with Ca^{2+}-containing bath solution. In all experiments (except those in Fig. 1A and B), nicardipine (10 μM) was included in the bath solutions to inhibit voltage-operated calcium channels. As all experiments employed a HEPES-buffered salt solution without bicarbonate or phosphate, chelation or precipitation of Gd^{3+} or La^{3+} was not a problem.

2.4. Mn^{2+} quench

Freshly isolated PASMCs were loaded with fura-2 AM (10 μM) for 45 min at room temperature in the dark. Thereafter, the cells were allowed to settle in a 1 ml glass-bottom chamber before being perfused with bath solution. Fura-2 fluorescence was visualised in a field of cells by excitation at 360 nm using a Cairn Optoscan monochromator (Cairn Research Ltd., Kent, UK) coupled to a Nikon Eclipse TE300 inverted microscope with a 40× NA 1.3 Plan Fluor oil immersion objective lens (Nikon, UK). Images were captured every 20 s at 500 nm using an intensified, cooled, frame-transfer CCD camera (iPentamax Gen IV, Roper Scientific, Marlow, Buckinghamshire, UK) and Winfluor image acquisition and analysis software (John Dempster, University of Strathclyde). $MnCl_2$ (200 μM) was initially added to untreated cells to determine the basal rate of fura-2 quenching, then thapsigargin (10 μM) was added to activate SOCE and determine it's effect on the rate of quenching.

2.5. Western blotting

Intrapulmonary arteries were dissected from the lungs of five adult male Sprague Dawley rats and the endothelium removed. The brains from three, 5-day old rat pups were also dissected out as a control for TRPC subunit protein expression. Tissues were cut into ~5 mm pieces and placed in cold solubilisation buffer comprising (in mM): Tris–HCl 20, NaCl 50, EDTA 1, EGTA 0.5, NaF 20, sodium orthovanadate 0.5, β-glycerophosphate 20, phenylmethanesulfonyl fluoride solution (PMFS) 1, glycerol 10% (v/v), triton-X100 1% (w/v), brij35 0.1% (w/v), leupeptin 10 mg/ml, aprotinin 10 mg/ml, pepstatin A 10 mg/ml; pH 7.6 with 1 M NaOH. The samples were then homogenised in a Retsch mixer mill (Haan, Germany) for 4 min prior to storage at −80 °C for 3–4 days before use. Aliquots (70 μl) of brain or pulmonary artery homogenate were added to 30 μl sample buffer, comprised of (in mM): Tris–HCl 252, $Na_4P_2O_7$ 8, EDTA 20, glycerol 40% (v/v), SDS 8% (w/v), bromophenol blue 0.028% (w/v); pH 6.8. The samples were then boiled for 5 min before being subjected to SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane and probed with TRPC1, 3, 4, 5 or 6 antibodies (~1:10,000 dilution). Protein bands were detected with a secondary, horseradish peroxidase-linked antibody.

2.6. Immunocytochemistry

Freshly dissociated PASMCs were allowed to adhere to 22 mm diameter glass coverslips coated with poly-L-lysine (1 mg/ml in dissociation solution) and fixed by incubating with 0.4% w/v paraformaldehyde in dissociation solution (lacking glucose and sucrose) for 20 min at room temperature. The cells were washed twice for 10 min then exposed for 1 h to dissociation solution containing 0.1% Triton-X100 and 1% BSA. After overnight incubation at 4 °C with anti-TRPC1, 3 or 6 antibody (1:200), cells were washed twice for 10 min then incubated at room temperature with Alexa fluor™ 488-conjugated secondary antibody (1:200, Molecular Probes) for 1 h, followed by thorough washing. The coverslips were then mounted on glass slides using a drop of Immu-mount (Thermo, Shandon, Pittsburgh, USA) and allowed to dry before staining was visualised using a BioRad confocal microscope, with a 60× 0.85 NA Plan Fluor objective lens (Nikon, Japan).

2.7. Materials

Papain, DTT, collagenase type VIII, nicardipine (prepared as 10 mM stock in dry DMSO) and verapamil hydrochloride (prepared as 10 mM stock in bath solution) were purchased from Sigma-Aldrich (Poole, UK). Thapsigargin and 2-APB were from Calbiochem (CN Biosciences, Nottingham, UK) and both were prepared as 10 mM stocks in dry DMSO. 2-(*N*,*N*-diethylamino)-diazenolate-2-oxide (DEANO) was from Alexis Biochemicals (Axxora, Nottingham, UK) and prepared as a 10 mM stock in 0.1 M NaOH. Sodium nitroprusside (SNP) from Sigma-Aldrich and glyceryl trinitrate (GTN) from Lipha Pharmaceuticals (West Drayton, UK) were prepared as 10 mM stocks in Ca^{2+}-free bath solution. Fluo-4/AM and Fura-2/AM were purchased from Molecular probes and prepared as 10 mM stocks in dry DMSO. TRPC1, 3, 4, 5 and 6 antibodies were from Alomone (Jerusalem, Israel) and Alexa fluor™ 488-conjugated goat, anti-rabbit secondary antibody from Molecular Probes All other reagents were of analytical grade from BDH (VWR, UK).

2.8. Data analysis

Fluo-4 and fura-2 fluorescence intensities were analysed using Metamorph image analysis software (Molecular Devices, UK). Individual PASMCs in the field of view were identified and a region of interest (ROI) drawn around an area in the middle of the cell. Within the ROI, mean pixel intensity was measured for each frame and expressed as $\Delta F/F_0$, where F_0 is the basal fluorescence and ΔF the change in fluorescence intensity ($F-F_0$) evoked by the experimental procedure.

Data are expressed as the mean sustained or peak increase in fluo-4 fluorescence ± S.E.M. with n denoting the number of cells from at least 3 different animals. The sustained increase in fluo-4 fluorescence was determined as the average $\Delta F/F_0$ between 2 and 10 min after the addition of extracellular Ca^{2+}. Statistical comparisons between means were made using Student's paired and unpaired *t*-tests as indicated. Differences between means were considered significant when $P<0.05$.

3. Results

3.1. SOCE in freshly isolated intrapulmonary artery SMCs

By using confocal microscopy and a low magnification objective lens, several freshly isolated PASMCs could be imaged simultaneously in the field of view during each experimental protocol. Individual PASMCs were selected on the basis of their classical spindle shape and only those cells that were in a relaxed state at the beginning of the experiment were subsequently analysed. Having depleted the sarcoplasmic reticulum by exposing the cells to thapsigargin (1 μM) for at least 45 min, subsequent re-addition of Ca^{2+} resulted in an increase in fluo-4 fluorescence (Fig. 1A), which was sustained as long as extracellular Ca^{2+} was present. Fig. 1B shows the average data from 20 cells, where the re-addition of Ca^{2+} produced an increase in fluo-4 fluorescence of $\Delta F/F_0=2.2\pm0.2$ ($n=20$). Cells that had not been treated with thapsigargin showed a small, gradual increase in fluo-4 fluorescence ($\Delta F/F_0=0.5\pm0.1$, $n=16$); however, this was significantly less than the increase observed in thapsigargin-treated cells (Fig. 1B).

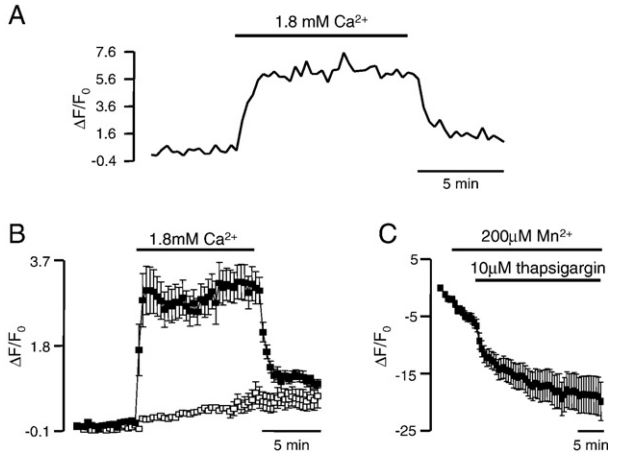

Fig. 1. Store-operated Ca^{2+} entry (SOCE) in pulmonary artery smooth muscle cells (PASMCs). A. Representative record from a single PASMC treated with thapsigargin (1 μM) for 45 min to deplete the intracellular stores of Ca^{2+} and activate SOCE. The cell was then exposed to 1.8 mM Ca^{2+} for 10 min, with fluo-4 being used to monitor intracellular Ca^{2+}. The increase in fluorescence during Ca^{2+} re-addition reflects influx of Ca^{2+} through the SOCE pathway. B. Average data from PASMCs that had been treated with thapsigargin and then exposed to external Ca^{2+} (■, $n=20$) and from control PASMCs that were not treated with thapsigargin (□, $n=16$), during a similar Ca^{2+} re-addition protocol. C. Thapsigargin increases the rate of Mn^{2+} influx into PASMCs. Pulmonary artery smooth muscle cells were loaded with fura-2 and quenching of fura-2 fluorescence by Mn^{2+} examined following addition of thapsigargin (10 μM), to activate SOCE ($n=90$).

A difficulty in interpreting the increase in fluo-4 fluorescence in response to Ca^{2+} re-addition in cells treated with thapsigargin is that it may simply reflect disrupted buffering of basal Ca^{2+} influx by the superficial sarcoplasmic reticulum when the Ca^{2+} ATPase of the sarcoplasmic reticulum is inhibited (Van Breemen et al., 1995). In order to determine if this was the case in PASMCs, the ability of Mn^{2+} to enter cells via Ca^{2+}-permeable channels and quench fura-2 fluorescence was examined. The fura-2 fluorescence signal decreased at a low, but steady rate in the absence of Mn^{2+} or thapsigargin (0.012 ± 0.005 $\Delta F/F_0$ s^{-1}, $n=90$) (Fig. 1C). Upon the addition of 200 μM Mn^{2+} the rate was not significantly altered (0.016 ± 0.003 $\Delta F/F_0$ s^{-1}). However, with the addition of 10 μM thapsigargin, the rate of decrease of fura-2 fluorescence due to Mn^{2+} quench significantly increased (0.033 ± 0.006 $\Delta F/F_0$ s^{-1}, $P<0.05$), indicating the opening of Mn^{2+}-permeable channels. After a period of 2–3 min, the rate of fluorescence decrease gradually reduced to a level significantly lower than that observed prior to cells being exposed to Mn^{2+} or thapsigargin (0.007 ± 0.002 $\Delta F/F_0$ s^{-1}, $P<0.05$).

To confirm that pre-incubation with thapsigargin resulted in store depletion, 20 mM caffeine was applied to thapsigargin-treated cells while they were maintained in a Ca^{2+}-free bath solution. None of the cells ($n=28$) that had been incubated with thapsigargin exhibited any change in fluo-4 fluorescence upon application of caffeine (Fig. 2A). Subsequent addition of Ca^{2+} to the extracellular solution resulted in a sustained increase in fluo-4 fluorescence ($\Delta F/F_0=1.7\pm0.2$), but a second application of

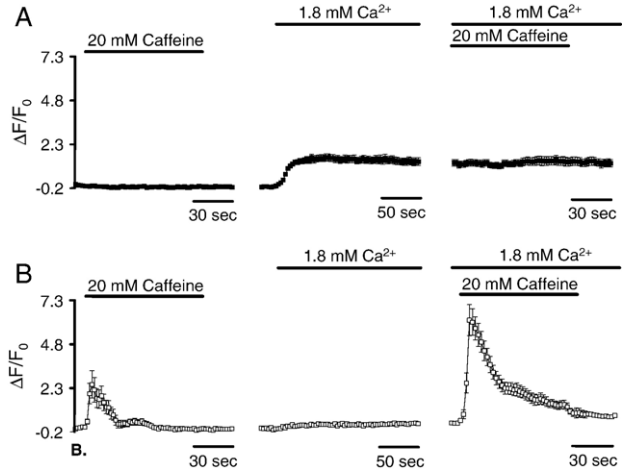

Fig. 2. Prolonged treatment with thapsigargin depletes the sarcoplasmic reticulum of Ca^{2+} in PASMCs. A. PASMCs were loaded with fluo-4 to monitor intracellular Ca^{2+}, while at the same time being treated with thapsigargin for at least 45 min to deplete the sarcoplasmic reticulum of Ca^{2+}. Application of caffeine (20 mM) had no effect on fluo-4 fluorescence (*left hand trace*). External Ca^{2+} was then re-added (*middle trace*) and following a 5 min equilibration period caffeine (20 mM) was re-applied (*right hand trace*) ($n=28$). Note that the experimental record is discontinuous because of the change in time scale for each of the traces. B. Control PASMCs that were not treated with thapsigargin show a small Ca^{2+} transient on application of caffeine (*left hand trace*). After re-addition of Ca^{2+} for 5 min (*middle trace*), a second application of caffeine produced a much larger increase in fluo-4 fluorescence (*right hand trace*) ($n=17$).

20 mM caffeine applied in the continuing presence of external Ca^{2+} had no effect on fluo-4 fluorescence. In control cells that had not been treated with thapsigargin, addition of 20 mM caffeine in the absence of extracellular Ca^{2+} resulted in a transient increase in fluo-4 fluorescence in 16 out of 17 cells studied, with peak $\Delta F/F_0=2.5\pm0.8$ (Fig. 2B). Subsequent addition of Ca^{2+} to the extracellular solution had a small effect upon basal fluo-4 fluorescence and a second application of 20 mM caffeine, applied in the continuing presence of external Ca^{2+} resulted in a significantly larger transient increase in fluo-4 fluorescence in all 17 cells, with peak $\Delta F/F_0=6.2\pm0.2$ ($P<0.05$).

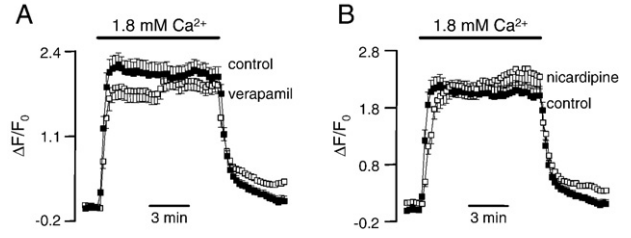

Fig. 3. The Ca^{2+} channel blockers, verapamil and nicardipine, have no effect on the increase in fluo-4 fluorescence during Ca^{2+} re-addition. A. SOCE in PASMCs following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} (■, $n=28$), and in matched PASMCs treated with verapamil (10 μM) prior to the re-addition of Ca^{2+} (□, $n=28$). B. SOCE in PASMCs following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} (■, $n=28$), and in matched PASMCs treated with nicardipine (10 μM) prior to the re-addition of Ca^{2+} (□, $n=30$). The control records (■) in A and B are the same and are repeated for comparative purposes.

3.2. Effect of Ca^{2+} channel blockers on SOCE

To determine whether voltage-operated Ca^{2+} channels (VOCCs) contribute to the increase in fluo-4 fluorescence following Ca^{2+} re-addition, the effect of the VOCC blockers verapamil and nicardipine was examined. In the presence of verapamil (10 μM) the increase in fluo-4 fluorescence following Ca^{2+} re-addition ($\Delta F/F_0=1.8\pm0.15$, $n=28$) was not significantly different from that observed in the absence of verapamil ($\Delta F/F_0=2.1\pm0.16$, $n=28$) (Fig. 3A). Nicardipine (10 μM) also had no effect on the increase in fluo-4 fluorescence following Ca^{2+} re-addition ($\Delta F/F_0=2.2\pm0.20$, $n=30$), when

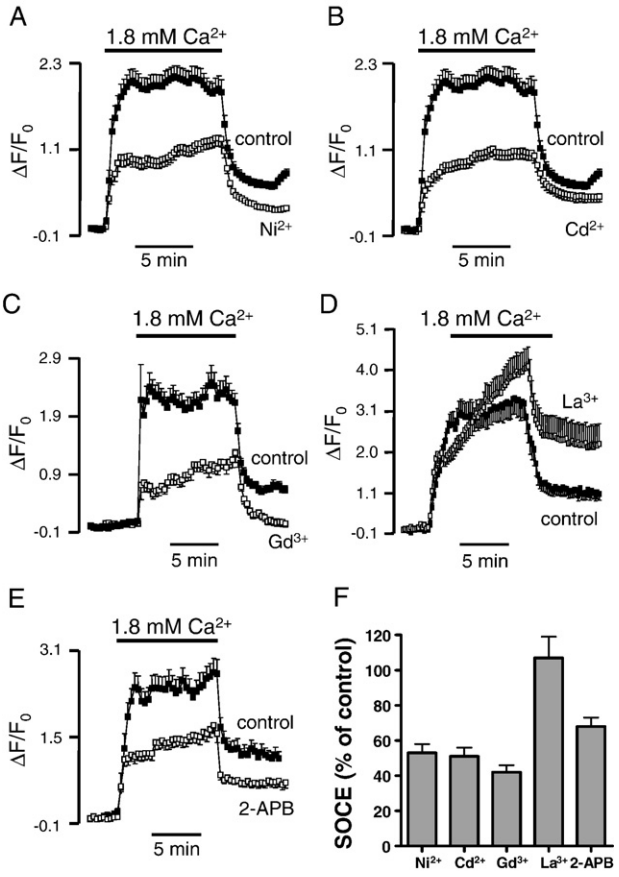

Fig. 4. Pharmacological properties of SOCE in PASMCs. Fluo-4 was used to monitor the intracellular Ca^{2+} and the increase in fluorescence during Ca^{2+} re-addition reflects SOCE. A. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=40$), and in matched PASMCs treated with Ni^{2+} (10 μM) prior to the re-addition of Ca^{2+} (□, $n=35$). B. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=40$), and in matched PASMCs treated with Cd^{2+} (10 μM) prior to the re-addition of Ca^{2+} (□, $n=35$). The control records (■) in A and B are the same and are repeated for comparative purposes. C. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=36$), and in matched PASMCs treated with Gd^{3+} (10 μM) prior to the re-addition of Ca^{2+} (□, $n=30$). D. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=54$), and in matched PASMCs treated with La^{3+} (100 μM) prior to the re-addition of Ca^{2+} (□, $n=47$). E. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=26$), and in matched PASMCs treated with 2-APB (75 μM) prior to the re-addition of Ca^{2+} (□, $n=28$). F. Summary showing the inhibitory effect of the various agents used on SOCE in PASMCs.

compared to the increase obtained under control conditions (Fig. 3B).

3.3. Pharmacological properties of SOCE in PASMCs

As the divalent cations Ni^{2+} and Cd^{2+} have been reported to inhibit CPA-induced contractions and SOCE in rat main pulmonary artery at low concentrations (Ng and Gurney, 2001), we examined their effects on PASMCs isolated from smaller blood vessels within the lung. Ni^{2+} (10 μM) caused a 47±5% ($P<0.05$, $n=35$) reduction in the increase in fluo-4 fluorescence following the re-addition of Ca^{2+} from $\Delta F/F_0=2.0\pm0.2$ ($n=40$) under control conditions to $\Delta F/F_0=1.1\pm0.1$ in the presence of Ni^{2+} ($n=35$) (Fig. 4A). Cadmium (10 μM) produced a similar inhibitory effect on SOCE, reducing the increase in fluo-4 fluorescence by 49±5% to $\Delta F/F_0=1.0\pm0.1$ ($P<0.05$, $n=35$) (Fig. 4B).

Since there are mixed reports of the potencies of Gd^{3+} and La^{3+} as blockers of SOCE in PASMC, we also investigated their effects on PASMC freshly isolated from small vessels. In the presence of Gd^{3+} (10 μM), the increase in fluo-4 fluorescence upon Ca^{2+} re-addition to PASMCs was inhibited by 58±4%, from $\Delta F/F_0=2.0\pm0.1$ ($n=36$) under control conditions to $\Delta F/F_0=0.8\pm0.1$ ($n=30$) in the presence of Gd^{3+} (Fig. 4C). Increasing the concentration of Gd^{3+} to 100 μM appeared to have a greater effect initially, with a 72±3% ($n=41$) reduction in the increase in fluorescence occurring during the first 30 s following Ca^{2+} re-addition. However, over the period that extracellular Ca^{2+} was present, there was a gradual increase in fluorescence such that SOCE was only inhibited by 18±6% at the end of the 10 min period of Ca^{2+} exposure. In contrast to the inhibitory effect of Gd^{3+}, when cells were treated with La^{3+} (100 μM) prior to Ca^{2+} re-addition no inhibition of SOCE was observed. The initial rise in fluo-4 fluorescence, during the first minute after addition of extracellular Ca^{2+}, was not different from that observed in control cells. Furthermore, the fluo-4 fluorescence gradually increased over the ensuing 10 min period to $\Delta F/F_0=4.2\pm0.5$ ($n=47$), which was significantly greater than the increase in fluo-4 fluorescence observed in control cells ($\Delta F/F_0=2.9\pm0.4$, $n=54$). This effect of La^{3+} was poorly reversible, so that upon removal of extracellular Ca^{2+}, the fluo-4 fluorescence decreased only slightly to $\Delta F/F_0=2.2\pm0.5$ in the La^{3+} treated cells (Fig. 4D), whereas it returned close to basal levels in control cells.

The IP_3 receptor antagonist 2-APB has been reported to inhibit store-depletion activated currents in PASMCs and we have now examined its effects on SOCE in PASMCs from the intrapulmonary artery. At 75 μM, 2-APB significantly inhibited the increase in fluo-4 fluorescence following Ca^{2+} re-addition by 42±5% ($P<0.05$; $\Delta F/F_0=2.3\pm0.2$ for control, $n=26$ and $\Delta F/F_0=1.3\pm0.1$ in the presence of 2-APB, $n=28$) (Fig. 4E).

3.4. Effect of nitric oxide donors on SOCE

To examine the effect of nitric oxide (NO) donors on SOCE, cells were exposed to the NO donor for 1–2 min prior to and during the re-addition of Ca^{2+}. SNP (10 μM) had no significant effect on SOCE with the increase in fluo-4 fluorescence during Ca^{2+} re-addition ($\Delta F/F_0=1.9\pm0.2$, $n=30$) being similar to the increase in fluorescence ($\Delta F/F_0=2.2\pm0.2$, $n=28$) observed under control conditions (Fig. 5A). To examine whether SNP may be a more effective inhibitor of SOCE during the sustained phase of the response, SNP was also applied to cells 10 min after the addition of extracellular Ca^{2+}. In cells in which SOCE had been pre-activated by the re-addition of Ca^{2+}, a sustained increase in fluo-4 fluorescence ($\Delta F/F=2.13\pm0.20$, $n=28$) was observed, which was unaffected when SNP (10 μM) was subsequently applied ($\Delta F/F=2.36\pm0.18$) (Fig. 5B). The effects of two other nitric oxide donors on SOCE in PASMCs were also investigated. In the presence of GTN (100 μM), re-addition of Ca^{2+} produced an increase in fluo-4 fluorescence ($\Delta F/F=1.45\pm0.11$, $n=28$) that was not different from that observed under control conditions ($\Delta F/F=1.64\pm0.15$, $n=29$) (Fig. 5C). Similarly, DEANO (10 μM) had no effect on the increase in fluo-4 fluorescence following Ca^{2+} re-addition to PASMCs. The SOCE dependent increase in fluorescence in the presence of DEANO ($\Delta F/F=2.85\pm0.19$, $n=70$) was similar to that obtained under control conditions ($\Delta F/F=3.05\pm0.22$, $n=56$) (Fig. 5D). The thapsigargin-treated PASMCs usually contracted when Ca^{2+} was re-applied and none of the NO donors examined in these studies prevented the contraction, although at the concentrations used the drugs all caused pronounced relaxation of intact pulmonary arteries.

3.5. TRPC expression in PASMCs

Western blot analysis of extracted proteins indicated the presence of TRPC1, 3, and 6 in PASMC (Fig. 6A). Strübing

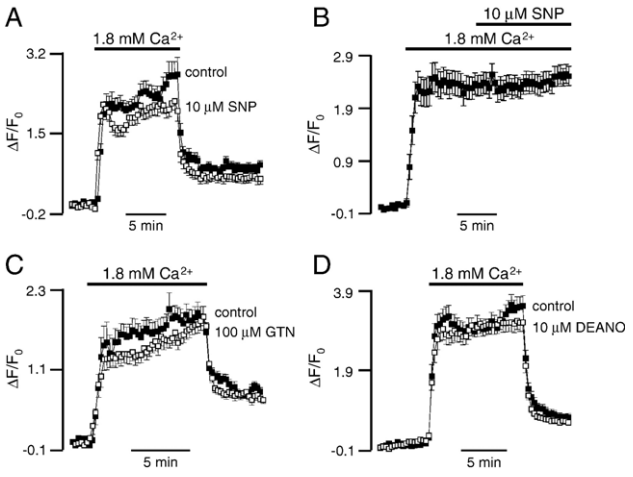

Fig. 5. Role of nitric oxide donors in regulating SOCE in PASMCs. Fluo-4 was used to monitor the intracellular Ca^{2+} concentration and the increase in fluorescence during Ca^{2+} re-addition reflects SOCE. A. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=28$), and in matched PASMCs treated with SNP (10 μM) prior to the re-addition of Ca^{2+} (□, $n=30$). B. Effect of SNP (10 μM) applied to PASMCs during the sustained phase of SOCE ($n=28$). C. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=29$), and in matched PASMCs treated with GTN (100 μM) prior to the re-addition of Ca^{2+} (□, $n=28$). D. SOCE following treatment with thapsigargin and subsequent addition of 1.8 mM Ca^{2+} in control cells (■, $n=56$), and in matched PASMCs treated with DEANO (10 μM) prior to the re-addition of Ca^{2+} (□, $n=70$).

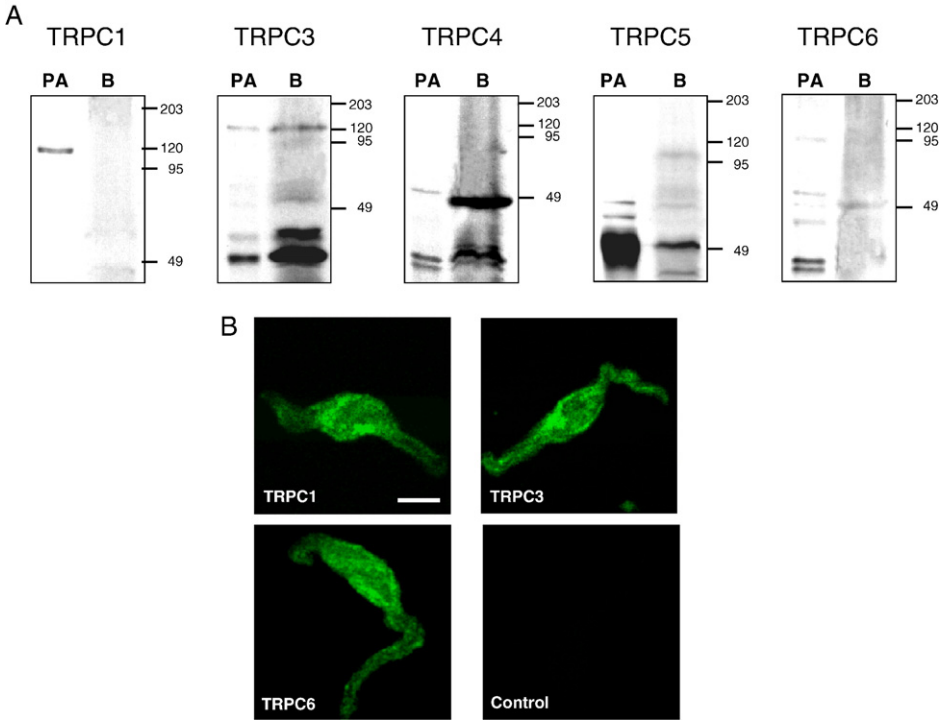

Fig. 6. TRPC channel expression in single PASMCs. A. Immunoblots showing the binding of TRPC1, 3, 4, 5, and 6 subunit antibodies to specific protein bands that have been separated from a sample of homogenised tissue. PA represents the sample from pulmonary artery and B represents the sample from rat pup brain. Data are representative of three independent experiments. B. Projected confocal images of PASMCs incubated with or without (control) rabbit primary antibody directed against the TRPC1, 3 or 6 subunit and visualised using Alexa fluor™ 488-conjugated goat, anti-rabbit secondary antibody. Scale bar is 10 μm.

et al. (2001) reported that the expression of TRP subunits is much higher in foetal than adult rat brain, thus, the brain from 5-day old rat pups was used as a control. In PASMCs the TRPC1 antibody yielded a single strong signal for a protein with a molecular weight of ~110 kDa. A protein of ~120 kDa was detected with the TRPC3 antibody in both pulmonary artery and brain. The TRPC3 antibody also labelled proteins of less than 50 kDa in both tissues. The TRPC4 antibody labelled three distinct proteins in pulmonary artery, one of ~55 kDa and two of less than 50 kDa, all corresponding with similar proteins in brain. A very strong signal was detected for a 50 kDa protein in pulmonary artery with TRPC5 antibodies, again corresponding with a similar protein in brain, but two larger proteins of ~55 and 60 kDa that labelled in pulmonary artery were not detected in brain. A protein of ~95 kDa, as well as a number of smaller proteins, was detected with the TRPC6 antibody in pulmonary artery, yet there were no corresponding bands in brain.

The TRPC1, 3 and 6 antibodies bound to protein bands in Western blots that were similar in molecular weight to previously published reports for the respective TRPC subunits (Zhang and Saffen, 2001; Babich et al., 2004; Goel et al., 2006). They were therefore used in immunocytochemistry experiments to probe the expression of TRPC1, 3 and 6 in freshly isolated PASMCs (Fig. 6B). With each antibody, the fluorescence signal appeared relatively punctate and was present in both the cytosol and plasmalemma. In control cells that had not been incubated with the primary anti-TRPC1 antibody, no fluorescence could be detected (Fig. 6B), indicating the absence of non-specific binding by secondary antibodies.

4. Discussion

4.1. Pharmacology of SOCE in rat PASMCs

The sustained increase in fluo-4 fluorescence following re-addition of Ca^{2+} to PASMCs treated with the SERCA inhibitor thapsigargin, is characteristic of a SOCE pathway being activated in these cells. The finding is consistent with previous studies of SOCE and store-operated channels in cultured PASMCs from human (Golovina, 1999; Golovina et al., 2001), rabbit (Kang et al., 2003), dog (Doi et al., 2000) and rat (Shimoda et al., 2000; McDaniel et al., 2001; Sweeney et al., 2002; Yu et al., 2003; Kunichika et al., 2004; Lin et al., 2004; Wang et al., 2004) as well as freshly isolated PASMCs from the dog (Wilson et al., 2002) and rat (Ng and Gurney, 2001; Snetkov et al., 2003). The small increase in fluo-4 fluorescence that was seen in cells not treated with thapsigargin probably reflects the increase in basal $[Ca^{2+}]_i$ that would normally be expected when restoring Ca^{2+} to the bath solution after a period in Ca^{2+}-free solution.

The rate of Mn^{2+} influx, as measured by the quenching of intracellular fura-2 fluorescence, is often used as a measure of Ca^{2+}-channel activity (Sage et al., 1989; Cho et al., 1997). Thus, the ability of Mn^{2+} to quench fura-2 fluorescence provides strong evidence that inhibition of SERCA and subsequent store depletion, resulted in the opening of cation channels in PASMCs.

The increased rate of Mn^{2+} quenching following the addition of thapsigargin is in agreement with previous studies of SOCE activation in PASMCs (Golovina, 1999; Ng and Gurney, 2001; Wilson et al., 2002).

All of the ionic currents found to underlie SOCE in PASMCs have been described as non-selective to cations (Golovina et al., 2001; Ng and Gurney, 2001; Snetkov et al., 2003). Therefore, it could be envisaged that following activation of store-operated channels, the influx of Ca^{2+} and Na^{+} may lead to depolarisation of the plasmalemma and subsequent activation of VOCCs. Neither verapamil nor nicardipine affected the increase in fluo-4 fluorescence following Ca^{2+} re-addition, indicating that the increase in intracellular Ca^{2+} almost entirely reflects Ca^{2+} entry through store-operated channels, consistent with published studies reporting no effect of nifedipine on SOCE in cultured PASMCs from human (Golovina, 1999) and rat (Wang et al., 2004), and no effect of nitrendipine on SOCE in freshly isolated PASMCs from the rat main pulmonary artery (Ng and Gurney, 2001). Thus, SOCE in PASMCs is insensitive to block by both dihydropyridine and phenylalkylamine calcium antagonists.

Both Ni^{2+} and Cd^{2+} were found to inhibit SOCE in PASMCs isolated from the intrapulmonary artery by approximately 50% at 10 μM. This is similar to the inhibitory effect of these ions on SOCE and store-depletion activated current in smooth muscle cells isolated from the main pulmonary artery (Ng and Gurney, 2001). In other studies, the sensitivity of SOCE in PASMCs to inhibition by divalent cations appears to be variable. Ni^{2+} has been reported to completely inhibit CPA-activated Ca^{2+} influx in rat and human cultured PASMCs at 0.5 mM (Golovina et al., 2001; McDaniel et al., 2001), although these studies did not investigate the effects of lower concentrations. In cultured PASMCs from intrapulmonary arteries of the rat, Ni^{2+} was reported to have an IC_{50} of 191 μM (Wang et al., 2004), demonstrating <20% inhibition of SOCE at 50 μM Ni^{2+}. Thus, the sensitivity of SOCE to divalent cations appears to be similar in large and small pulmonary arteries, but culturing cells appears to reduce their sensitivity, suggesting either a change in store-operated channel subunit or accessory protein composition, or alterations in their regulation (e.g. phosphorylation). The sensitivity to divalent cations may, however, be species dependent, because in freshly dissociated canine PASMCs Cd^{2+} (0.5 mM) only slightly inhibited Ca^{2+} entry, while Ni^{2+} caused strong inhibition, albeit at 0.1–10 mM (Wilson et al., 2002).

A number of studies describe sensitivity of SOCE in PASMCs to the lanthanides La^{3+} and Gd^{3+} (Robertson et al., 2000; Ng and Gurney, 2001; Kang et al., 2003; Snetkov et al., 2003; Wang et al., 2004). In the present study, Gd^{3+} inhibited SOCE by approximately 60% at 10 μM; however, at higher concentrations the degree of inhibition was reduced to approximately 20%. In canine PASMCs, 100 μM Gd^{3+} had no effect on either Ca^{2+} (Wilson et al., 2002) or Mn^{2+} influx activated in response to store depletion (Ng et al., 2005). While the explanation for this finding is unclear, 100 μM Gd^{3+} has previously been reported to slightly stimulate SOCE in cerebral arteriolar smooth muscle cells (Flemming et al., 2003), although lower concentrations (0.1–10 μM) did produce block (Flemming et al., 2002, 2003).

Complex effects were also observed with La^{3+}. At a concentration of 100 μM it had no inhibitory effect on SOCE. Rather, the increase in fluo-4 fluorescence upon Ca^{2+} re-addition seemed somewhat potentiated, although this did not reach statistical significance over the 10 min recording period. It was also apparent that following removal of extracellular Ca^{2+}, fluo-4 fluorescence remained elevated in approximately 40% of the cells, with the other 60% showing incomplete recovery, suggesting that Ca^{2+} removal was impaired. La^{3+} is known to inhibit the plasma membrane Ca^{2+}-ATPase at concentrations similar to those used in this study (Weiner and Lee, 1972; Schatzmann et al., 1986) and this may have contributed to the effects observed. At 100 μM, La^{3+} was similarly ineffective at inhibiting store depletion-induced Mn^{2+} influx in canine PASMCs (Ng et al., 2005), while in cultured PASMCs from intrapulmonary arteries of the rat, it increased the basal cytosolic Ca^{2+} concentration (Wang et al., 2005) and caused a concentration-dependent vasoconstriction in perfused rat lungs *in vitro* (Weigand et al., 2005). These effects of La^{3+} could also be due to inhibition of Ca^{2+} efflux mechanisms. In contrast, several studies clearly show an inhibitory effect of La^{3+} on SOCE in cultured PASMCs (Kang et al., 2003; Lin et al., 2004; Wang et al., 2004), albeit with varying sensitivity.

There is only one previous report indicating effects of 2-APB on SOCE in pulmonary artery. Snetkov et al. (2003) showed the complete inhibition of thapsigargin-induced contraction in rat intrapulmonary artery with 75 μM 2-APB, which also partially blocked thapsigargin activated inward currents in freshly isolated PASMCs. Similarly, 75 μM 2-APB was shown in this study to inhibit SOCE in PASMCs by 42%.

4.2. NO donors and SOCE

The lack of a direct effect of NO donors on SOCE in PASMCs observed here is consistent with a number of studies on different cell types (Gilon et al., 1995; Okamoto et al., 1995; Bischof et al., 1997; Trepakova et al., 1999), including cultured smooth muscle cells (Cohen et al., 1999). NO may well have an indirect effect in regulating SOCE, which contributes to its vasorelaxant activity. By stimulating the sarcoplasmic reticulum Ca^{2+} ATPase, NO donors could promote refilling of the sarcoplasmic reticulum, leading to inhibition of the SOCE pathway (Cohen et al., 1999). The lack of a direct effect contrasts, however, with other studies. NO, or NO donors, were reported to inhibit SOCE in A7r5 cells (Moneer et al., 2003), endothelial cells (Dedkova and Blatter, 2002), and the pulmonary artery (Jernigan et al., 2006). In the latter study the NO donor spermine NONOate was found to inhibit SOCE in control arteries, but not arteries from chronic hypoxic animals (Jernigan et al., 2006). However, these experiments were carried out on intact arteries where, without actually imaging the smooth muscle cells within the vessel, there is uncertainty as to the origin of the fluorescence signal reporting Ca^{2+} concentration. Furthermore, in multicellular preparations both thapsigargin and CPA require considerable time to produce complete inhibition of the sarcoplasmic reticulum Ca^{2+} ATPase (Baudet et al., 1993), possibly allowing NO to act indirectly by stimulating sarcoplasmic reticulum Ca^{2+} uptake.

4.3. TRPC expression in rat PASMCs

The TRPC antibodies used in this study were used previously to assess expression in pulmonary arteries (Wang et al., 2004; Lin et al., 2004). The single band of ~110 kDa identified by the TRPC1 antibody in Western blots of intrapulmonary arteries is slightly larger than the predicted size of the TRPC1 subunit (~90 kDa), but consistent with previous work on these vessels (Lin et al., 2004), although one report on the same vessels indicated interaction with a larger protein of 170 kDa (Wang et al., 2004). There may be variation in the size of TRPC1 protein expressed in pulmonary arteries, because in cultured human PASMCs it was reported to be as large as 220 kDa, possibly due to the detection of TRPC1 dimers (Sweeney et al., 2002). Immunostaining also indicated TRPC1 expression in the smooth muscle cells. Staining was apparent around the periphery of the cell, consistent with membrane expression, but was not restricted to the membrane. The punctate nature of the staining suggests possible clustering of subunits in certain areas or organelles. Interestingly, no TRPC1 antibody binding was detected in the 5-day old rat brain, although studies on adult rat brain found that TRPC1 antibody labelled a protein of comparable size to that detected here (Lin et al., 2004; Wang et al., 2004).

Immunocytochemistry also gave positive staining with the TRPC3 and 6 antibodies. Although both antibodies detected additional bands in Western blots, below 60 kDa, these may be breakdown products of TRPC subunits rather than non-specific binding of the antibody. The ~120 kDa protein detected by the TRPC3 antibody is larger than the predicted size of the TRPC3 subunit (97 kDa), but TRPC3 proteins of variable size have been reported. The same antibody detected a 160 kDa protein in rat cultured PASMCs (Lin et al., 2004), whereas in rat aorta and pre-glomerular afferent arterioles it bound to a protein of ~107 kDa (Facemire et al., 2004). While the TRPC6 antibody detected several protein bands, the band at ~100 kDa is likely to represent the intact TRPC6 protein. In previous studies TRPC6 antibodies labelled proteins of a similar size in rat intrapulmonary arteries, cultured PASMC, aorta and pre-glomerular arterioles (Facemire et al., 2004; Lin et al., 2004; Wang et al., 2004), although binding to smaller proteins (65 kDa) has also been reported (Wang et al., 2004).

Western blotting failed to detect TRPC4 expression in two previous studies of rat intrapulmonary arteries, although it did detect proteins of the appropriate molecular mass (~90 kDa) in brain, rat aorta, pre-glomerular arterioles and cultured PASMC (Facemire et al., 2004; Lin et al., 2004; Wang et al., 2004). The proteins detected in the present study were much smaller, although similar in intrapulmonary artery and brain, and a weak band at ~55-60 kDa is compatible with a 66 kDa protein reported in cultured human PASMCs (Zhang et al., 2004). The lack of a band at 112 kDa, the predicted size of the TRPC4 protein, along with the apparent absence of TRPC4 in earlier studies, suggests however that, like the main pulmonary artery (Ng and Gurney, 2001), TRPC4 is expressed at low levels, if at all, in intrapulmonary arteries. TRPC5 protein expression in pulmonary arteries has not previously been investigated. The TRPC5 antibody recognised a prominent band at ~50 kDa in both intrapulmonary artery and brain, although two bands of larger size were also apparent in the intrapulmonary artery. A protein of the size predicted for TRPC5 (110 kDa) is apparent in brain, but not intrapulmonary artery. Moreover, none of the protein bands labelled in the artery homogenate correspond with the 97 kDa protein detected in rat aorta (Facemire et al., 2004) or the 125 kDa protein expressed by recombinant TRPC5 channels in HEK293 cells (Shimizu et al., 2006; Xu et al., 2005). It is not clear what the smaller proteins represent, but they are unlikely to be the intact TRPC5 protein. Thus TRPC5 expression may also be low in intrapulmonary arteries, as found in the main pulmonary artery (Ng and Gurney, 2001). Overall, the results indicate that, like rat main pulmonary artery, TRPC1, 3 and 6 are the major TRPC isoforms expressed in intrapulmonary arteries.

5. Conclusions

This study has shown that the properties of SOCE are similar in rat PASMCs freshly isolated from large (Ng and Gurney, 2001) or small blood vessels, but that they change when cultured and may also vary in different species. This may reflect differential expression of different TRPC subunits. In freshly isolated rat PASMC, 2-APB, Ni^{2+} and Cd^{2+} are effective inhibitors of SOCE, while dihydropyridine and phenylalkylamine calcium antagonists had no effect. Although La^{3+} has been reported to potently inhibit SOCE in pulmonary arteries, we find it to be a poor inhibitor in freshly isolated rat PASMCs. Another lanthanide, Gd^{3+}, inhibited SOCE at 10 μM, but its effect was reduced at higher concentrations. The complex and inconsistent effects of the lanthanides, which may be due to additional actions on the cell, clearly limit their usefulness in functional studies aimed at identifying the physiological and pathophysiological roles of SOCE in the pulmonary circulation. We could find no evidence for direct modulation of SOCE by NO donors, although indirect inhibition could contribute to NO-induced pulmonary vasodilation as proposed previously for other smooth muscle cells. TRPC1 and 3 are both present in PASMCs and can act as store-depletion activated channels (Parekh and Putney, 2005). The pharmacological profile of SOCE in pulmonary artery does not allow distinction between these channels. However, TRPC3 contains phosphorylation sites for G-kinase, which mediate cGMP-dependent inhibition of the channel (Kwan et al., 2004), arguing against its role in pulmonary artery SOCE. Since TRPC6 is considered to act as a receptor-operated channel (Parekh and Putney, 2005), its involvement is also unlikely. That leaves TRPC1 as the most likely candidate to mediate the store-depletion activated channels that underlie SOCE in pulmonary artery, although we cannot rule out a role for heteromeric TRPC3/6.

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