Store-Operated Channels Mediate Ca\textsuperscript{2+} Influx and Contraction in Rat Pulmonary Artery

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Abstract—Cation channels activated by Ca\textsuperscript{2+} store depletion have been proposed to mediate Ca\textsuperscript{2+} influx in vascular smooth muscle cells. The aim of this study was to determine if store-operated channels have a functional role in pulmonary artery smooth muscle cells (PASMCs). In intact rat pulmonary artery rings, cyclopiazonic acid (CPA) produced a sustained contraction that was resistant to inhibition by nifedipine, but abolished in Ca\textsuperscript{2+}-free solution and 50% blocked in the presence of 6 \mu mol/L Cd\textsuperscript{2+}, 10 \mu mol/L Ni\textsuperscript{2+}, 600 \mu mol/L La\textsuperscript{3+}, and 7 \mu mol/L SKF96365. In freshly isolated PASMCs loaded with fura-2, CPA increased the intracellular Ca\textsuperscript{2+} concentration by stimulating dihydropyridine-resistant Ca\textsuperscript{2+} influx, which was ≈50% blocked by 10 \mu mol/L Ni\textsuperscript{2+} and 7 \mu mol/L SKF96365. In perforated-patch recordings, CPA activated a sustained inward current at negative membrane potentials, which persisted in cells dialyzed with BAPTA, showed a near linear dependence on membrane potential when Cs\textsuperscript{+} was the main intracellular cation, and was blocked by Ni\textsuperscript{2+}, Cd\textsuperscript{2+}, and SKF96365 at concentrations preventing contraction. The current showed a bimodal dependence on extracellular Ca\textsuperscript{2+}, being enhanced 2-fold in the absence of Ca\textsuperscript{2+} and around 10-fold on reducing Ca from 1.8 to 0.2 mmol/L. RT-PCR revealed the expression of Trp1, Trp3, Trp4, and Trp5 mRNA, whereas immunostaining identified Trp1, Trp3, Trp4, and Trp6 channel proteins in isolated PASMCs. At least one of these subunits may contribute to cation channels in PASMCs, which are activated by store depletion to bring about Ca\textsuperscript{2+} influx and contraction. (Circ Res. 2001;89:923-929.)

Key Words: store-operated channel ■ capacitative calcium entry ■ pulmonary artery smooth muscle ■ cation channel ■ Trp channel

In nonexcitable cells, the depletion of intracellular Ca\textsuperscript{2+} stores activates Ca\textsuperscript{2+} entry from the extracellular space via store depletion–activated channels.\textsuperscript{1,2} There is increasing evidence that such a pathway, distinct from voltage-operated Ca\textsuperscript{2+} channels or receptor-operated Ca\textsuperscript{2+}-permeable channels, contributes to Ca\textsuperscript{2+} entry in vascular smooth muscle. Numerous reports\textsuperscript{3} suggest that calcium store depletion, brought about by agents like thapsigargin or cyclopiazonic acid, which inhibit the Ca\textsuperscript{2+}-ATPase (SERCA) in the sarcoplasmic reticulum (SR), increases Ca\textsuperscript{2+} influx and induces tone in smooth muscle preparations. In mouse anococcygeus muscle, store depletion was shown to activate a nonselective cation current, which gave rise to sustained contraction.\textsuperscript{4} More recently, Trepakova et al\textsuperscript{5} described a 3-pS Ca\textsuperscript{2+}-conducting channel in aorta smooth muscle cells, which could be activated in cell-attached membrane patches by thapsigargin or by chelating intracellular Ca\textsuperscript{2+} with BAPTA, and is therefore likely to be a store-operated channel. Perhaps the best evidence that store-operated channels play a functional role in vascular smooth muscle was provided by an antibody directed against an extracellular domain of the Trp1 protein, which labeled the membrane of smooth muscle cells in resistance arterioles and inhibited Ca\textsuperscript{2+} influx activated by thapsigargin.\textsuperscript{6} The trp gene was originally associated with a transient receptor potential in Drosophila photoreceptors and shown to encode a Ca\textsuperscript{2+}-permeable channel. Since then, 7 mammalian homologues (trp1 through 7) have been identified that encode cation channels with varying selectivity for Ca\textsuperscript{2+}.\textsuperscript{2,7} Trp1, Trp2, Trp4, and Trp5 subunits have all been suggested as components of store depletion–activated Ca\textsuperscript{2+} influx.\textsuperscript{6,8–10}

Store depletion–activated channels could be important in pulmonary arteries, where SERCA inhibitors induce contractions that are resistant to nifedipine.\textsuperscript{11} Recent evidence suggests that store-operated channels are expressed in cultured PASMCs, with pharmacological properties consistent with a role in mediating agonist-induced contraction.\textsuperscript{12} In fact, it has been suggested that store-operated Ca\textsuperscript{2+} influx contributes to hypoxic pulmonary vasoconstriction, which is inhibited by agents that cause store depletion or inhibit store-operated channels.\textsuperscript{13–15} The aim of the present study was to determine if acutely dissociated rat PASMCs express...
Ca\(^{2+}\)-permeable channels that are activated by SR depletion and if these channels can support Ca\(^{2+}\) influx and smooth muscle contraction.

**Materials and Methods**

**Tissue and Cell Preparation**

Animal care and procedures conformed to the requirements of the UK Animals (Scientific Procedures) Act 1986. The main pulmonary artery was removed from male Sprague-Dawley rats (150 to 250 g, supplied by Bantin & Kingman Universal, UK) euthanized by cervical dislocation. Isometric tension was measured in endothelium-denuded rings, as previously described, using physiological salt solution (PSS) composed of the following (mmol/L): NaCl 122, KCl 5.0, MgCl\(_2\) 1.0, KH\(_2\)PO\(_4\) 0.5, NaH\(_2\)PO\(_4\) 0.5, HEPES 10, glucose 11, and CaCl\(_2\) 1.8 (pH 7.3). Ca\(^{2+}\)-free solutions were identical but with CaCl\(_2\) omitted and 0.1 mmol/L EGTA added. Smooth muscle cells were isolated as previously described, except that tissue was incubated at 37°C for 1 hour with 6.9 to 9.2 U/mL papain, 0.04% bovine serum albumin, and 0.4 mmol/L dithiothreitol, and for a further 10 to 15 minutes after adding collagenase (0.6 to 0.7 mg/mL, type IA, Sigma). Cells were dispersed in enzyme-free solution and used within 7 hours.

**Measurement of Intracellular Ca\(^{2+}\)**

PASMCs were incubated for \(\geq\)45 minutes with 1 \(\mu\)mol/L fura-2 acetoxymethyl ester then superfused with PSS at room temperature (\(\approx\)22°C). An epifluorescence microscope was used with a filter wheel rotating at 0.25 Hz to excite fluorescence alternately at 340 nm and 380 nm. Emitted light passed through a 510 nm filter to a photomultiplier and was recorded using Phocal software (version 5; Applied Imaging). Limiting excitation to 20- to 30-second periods in each experimental condition minimized photobleaching. Signals were corrected for background fluorescence and minimized by an image mask placed around the cell. The ratio of fluorescence (R) excited at the 2 wavelengths was used to calculate \([\text{Ca}^{2+}]_i\), as described, with in situ calibration providing minimum (R\(_{\text{min}}\)=0.219±0.002, n=37) and maximum (R\(_{\text{max}}\)=1.7±0.1, n=17) ratios, the ratio of fluorescence excited at 380 nm in Ca\(^{2+}\)-free and saturating conditions (β=4.9±0.4, n=17), and the dissociation constant for Ca\(^{2+}\) binding (K\(_D\)=361 nmol/mL). The \([\text{Ca}^{2+}]_i\) in each condition was determined as the average from 5 to 10 cells, and experiments were repeated in cell preparations from at least 3 animals. Results are expressed as mean±SEM of n animals. Mn\(^{2+}\)-induced quenching of fura-2 fluorescence was recorded during excitation at 360 nm in Ca\(^{2+}\)-free PSS containing 1 \(\mu\)mol/L nitrindipine. The slope of the linear decline in fluorescence was measured before and after adding 20 \(\mu\)mol/L MnCl\(_2\) and then after adding 30 \(\mu\)mol/L CPA.

**Electrophysiology**

Cells were superfused at 0.5 mL/min with PSS at room temperature and currents recorded using the perforated-patch or whole-cell recording technique. Pipettes for perforated-patch recording contained 0.3 mg/mL amphoterin B in 130 mmol/L KCl, 1 mmol/L MgCl\(_2\), and 15 mmol/L HEPES at pH 7.2. Pipettes for whole-cell recording usually contained the following (in mmol/L): KCl 112, HEPES 10, adenosine 5'-triphosphate (Mg salt) 3, guanosine triphosphate (Na salt) 0.3, BAPTA 5; pH 7.2 with KOH. The K\(^{+}\) was replaced with Cs\(^+\) to measure the current versus voltage relationship. Pipette resistance was 4 to 8 MΩ. Series resistance was routinely compensated (~80%) in perforated-patch experiments. Cells were voltage clamped at ~80 mV, and the current response to ~10 mV steps used to measure cell capacitance, against which current amplitudes were normalized to control for variations in cell size. Drugs were applied from a multiharrel pipette positioned close to the cell using a rapid perfusion system (Cell MicroControls).

**Expression of Trp Subunits**

RT-PCR was performed on 1.4 \(\mu\)g total RNA (260 to 280 nm optical density ratio 2.02) extracted from rat PASMCs, as previously described, using primers specific for rat Trp1, Trp3, Trp4, Trp5, and Trp6 subunits. Cycle parameters were 95°C for 10 minutes, 35 cycles at 95°C for 1 minute, 54°C for 30 seconds, and 68°C for 2 minutes. Samples were then heated at 68°C for 10 minutes. Reverse transcriptase was omitted from control cDNA reactions. Amplicons were resolved by agarose gel electrophoresis, purified, and verified by sequencing. To determine the relative expression of Trp mRNA, semiquantitative RT-PCR was performed through 5 to 50 cycles using primers for β-actin (sense, 5'-TACCCCATGACACCGGC-3'; antisense, 5'-TGGGCAAGTGTGGGTGAC-3') as an internal control, with serial dilutions of rat PASMC cDNA (1- to 10,000-fold). After gel electrophoresis, products were quantified using a BioRad GS-690 densitometer, and a series of standard curves obtained by plotting β-actin levels (arbitrary units) against cycle number. Steady-state Trp transcripts were then estimated relative to β-actin standards. Data are expressed as the ratio Trp/β-actin to indicate the relative expression of different Trp subunits.

Antibodies directed against unique domains in rat Trp1, Trp3, Trp4, and Trp6 (Alomone, Jerusalem, Israel) were used to investigate protein expression in fixed and permeabilized rat PASMCs as previously described. Fluorescence images were obtained with a BioRad MRC-1024 MP confocal microscope.

**Drug Solutions and Data Analyses**

CPA was prepared as a 20 mmol/L stock in dimethyl sulphoxide (DMSO). Nifedipine and nitrindipine were prepared as 10 mmol/L stocks in DMSO. Other drugs were dissolved in deionized water. Data are expressed as mean±SEM of n tissues, cells or animals as indicated. Statistical comparisons used one way analysis of variance (ANOVA), with probability value corrected by Tukey’s pairwise comparison or Student’s paired or unpaired t tests as appropriate. A value of P<0.05 was considered significant.

**Results**

**Contractile Response to Store Depletion**

Endothelium-denuded rat pulmonary arteries contracted in response to agents that cause SR store depletion. Ryanodine, which produces a small and maintained leak of Ca\(^{2+}\) from smooth muscle SR at low concentrations, produced concentration-dependent contraction amounting to 9±1% (n=4) of the response to 1 \(\mu\)mol/L phenylephrine (PE) at 10 mmol/L. The response took up to 30 minutes to develop and declined slowly on washing out the drug. Removing extracellular calcium reduced the response to 1.6±0.6% (n=4, P<0.01) of the PE contraction, implying that it involved Ca\(^{2+}\) influx. SERCA inhibitors also produced contraction. The response to thapsigargin was variable and poorly reversible on washout: it contracted only 50% of the arteries tested, but in responding vessels the contraction to 1 \(\mu\)mol/L thapsigargin was 10±2% (n=8) of the response to PE. CPA caused a more pronounced contraction that was readily reversed on washout (Figure 1A). In 40 pulmonary artery rings, the peak contraction evoked by 30 \(\mu\)mol/L CPA amounted to 31±3% of the response to PE. After the peak, tension declined to a lower level, equal to 12±1% (n=37) of the PE response, which was maintained for at least 1 hour. When applied consecutively to the same vessels, the maximum contractions induced by 5 \(\mu\)mol/L (14±2%, n=10), 10 \(\mu\)mol/L (14±2%), or 30 \(\mu\)mol/L (16±3%) CPA did not significantly differ. The rate at which contraction developed did, however, vary with the CPA concentration. Thus, the contraction evoked by 5
was mediated by Ca\(^{2+}\)/H11021

shown in Figure 1B, the contraction was largely abolished

less than 40 or 20 minutes at 10 and 30

Ca\(^{2+}\) extracellular calcium and then after readmission of 1.8 mmol/L

phenylephrine, in physiological solution (1.8 Ca), after removal of

subsequent experiments to induce store depletion.

channels in PASMCs. 21 In contrast, nifedipine did not inhibit

amplitude of the peak and sustained phases of contraction

blockers of these channels1 was tested. Figure 1D shows that

(CP) took 1 hour to reach maximum, compared with

5 mmol/L CPA caused a transient increase in [Ca\(^{2+}\)]\(_i\) (Figure 2A). The subsequent addition of 1.8 mmol/L

CaCl\(_2\) caused a gradual increase in [Ca\(^{2+}\)]\(_i\) (Figure 2A) from

free medium and restored on readmitting

Ni\(^{2+}\) and La\(^{3+}\), as well as the nonspecific

\(0.25 \text{ g} \quad 20 \text{ min}\)

A

B

C

D

Figure 1. CPA contracts rat pulmonary artery. A, Acetylcholine (1 \(\mu\)mol/L) had no effect on contraction activated by phenylephrine (1 \(\mu\)mol/L), indicating the absence of endothelium. In the same vessel, CPA (30 \(\mu\)mol/L) evoked a contraction, which peaked within 20 minutes then declined to a lower, sustained level. B, Histogram showing contraction amplitude evoked by CPA, measured as % of the maximum response to 1 \(\mu\)mol/L phenylephrine, in physiological solution (1.8 Ca), after removal of extracellular calcium and then after readmission of 1.8 mmol/L Ca\(^{2+}\). **P<0.01 (ANOVA, n=5). C, Histogram showing the mean amplitude of the peak and sustained phases of contraction induced by CPA under control conditions (C) and after exposure to 1 \(\mu\)mol/L nifedipine (N). ***P<0.001 (paired t test, n=21). D, Concentration dependence of inhibition of the nifedipine-resistant contraction to CPA by Cd\(^{2+}\) ( ), Ni\(^{2+}\) ( ), La\(^{3+}\) ( ), and SKF96365 ( ). Points show mean±SEM of at least 4 vessels.

\(\mu\)mol/L CPA took 1 hour to reach maximum, compared with

Because of the greater reversibility, reproducibility, and rapidity of its action, 30 \(\mu\)mol/L CPA was used in all subsequent experiments to induce store depletion.

The response to CPA required Ca\(^{2+}\) influx because, as shown in Figure 1B, the contraction was largely abolished (P<0.01) in Ca\(^{2+}\)-free solution and restored on readmitting Ca\(^{2+}\) in the continued presence of CPA. Part of the response was mediated by Ca\(^{2+}\) influx through voltage-gated L-type Ca\(^{2+}\) channels because nifedipine significantly (P<0.001) reduced the peak contraction (Figure 1C) when applied at a concentration (1 \(\mu\)mol/L) causing maximal inhibition of these channels in PASMCs. 21 In contrast, nifedipine did not inhibit the sustained contraction measured 30 minutes after the peak response (Figure 1C), implying that it required Ca\(^{2+}\) influx through a different pathway. To determine if this component involved store-operated channels, its sensitivity to known blockers of these channels1 was tested. Figure 1D shows that the cations Cd\(^{2+}\), Ni\(^{2+}\), and La\(^{3+}\), as well as the nonspecific

inhibitor SKF96365, all produced concentration-dependent inhibition of the contraction evoked by CPA in the presence of nifedipine. Half-maximal inhibition (IC\(_{50}\)) occurred at 6 \(\mu\)mol/L Cd\(^{2+}\), 10 \(\mu\)mol/L Ni\(^{2+}\), 600 \(\mu\)mol/L La\(^{3+}\), and 7 \(\mu\)mol/L SKF96365.

\([\text{Ca}^{2+}]_i\) Responses to CPA

To determine if store depletion–activated Ca\(^{2+}\) influx raises \([\text{Ca}^{2+}]_i\), the effects of CPA were tested on the fluorescence of PASMCs loaded with fura-2. When applied in Ca\(^{2+}\)-free solution, 30 \(\mu\)mol/L CPA caused a transient increase in \([\text{Ca}^{2+}]_i\) (Figure 2A). The subsequent addition of 1.8 mmol/L CaCl\(_2\) caused a gradual increase in \([\text{Ca}^{2+}]_i\) (Figure 2A) from 19±5 mmol/L to 563±168 mmol/L (n=5, P<0.05), which was well maintained. Figure 2B shows that this increase in \([\text{Ca}^{2+}]_i\) was little affected by 1 \(\mu\)mol/L nitrendipine, a photostable dihydropyridine calcium antagonist. When applying the same protocol to intact vessels, nifedipine also failed to inhibit the contractile response to readmitting Ca\(^{2+}\) in the continued presence of CPA, which amounted to 14±8\% (n=6) of the PE response before and 12±6\% after adding nifedipine; therefore, it involved a pathway distinct from L-type Ca\(^{2+}\) channels. The nitrendipine-resistant Ca\(^{2+}\) influx was 49±12\% (n=4) inhibited by 10 \(\mu\)mol/L Ni\(^{2+}\) and 69±11\% (n=3) by 7 \(\mu\)mol/L SKF96365 (Figure 2C), concentrations that halved the CPA-induced contraction. Cd\(^{2+}\) could not be tested because it interfered directly with fura-2 fluorescence. 22

To determine if CPA increased \([\text{Ca}^{2+}]_i\), by recruiting a new Ca\(^{2+}\) influx pathway or because the SR failed to buffer Ca\(^{2+}\)
entering the cell, the effect of CPA on Mn$^{2+}$ quenching of fura-2 fluorescence was tested under comparable conditions. During continuous excitation at 360 nm, fluorescence declined slowly over several minutes due to photobleaching. The addition of 20 μmol/L MnCl₂ to PASMCs bathed in nominally Ca$^{2+}$-free solution containing 1 μmol/L nitrendipine, caused the decline in fluorescence to increase only slightly. The subsequent addition of 30 μmol/L CPA caused a marked 308±65% (n=14, P<0.01) increase in the rate of decline (Figure 3), indicating enhanced Mn$^{2+}$ quenching and, by extrapolation, Ca$^{2+}$ influx.

**CPA-Induced Membrane Current**

When rat PASMCs were voltage clamped at −80 mV using the perforated-patch technique, 30 μmol/L CPA evoked an inward current composed of transient and sustained phases (Figure 4A). The peak amplitude of the transient current, normalized against cell capacitance, was 21±5 pA/pF (n=11), whereas the sustained current had an amplitude of 1.3±0.2 pA/pF (n=11). The transient component was lost when recording with the conventional whole-cell technique and pipettes containing 5 mmol/L BAPTA to buffer [Ca$^{2+}$], (Figure 4B). Of 76 cells, only 16 showed any trace of transient current, with an amplitude of only 1.3±0.2 pA/pF. CPA did, however, induce an inward current that usually took at least 2 minutes to reach maximum (Figure 5). The current was characteristically noisy and had a mean amplitude of only 0.41±0.04 pA/pF (n=76), but it was sustained while CPA was present. In the absence of CPA, a similar holding current was recorded at −80 mV using either the perforated-patch (1.9±0.4 pA/pF, n=11) or whole-cell method (0.82±0.06 pA/pF, n=76).

The pharmacology of the sustained current induced by CPA is illustrated in Figure 5. At concentrations maximally inhibiting the nifedipine-resistant contraction to CPA, NiCl₂ (200 μmol/L) reduced the current by 107±12% (Figures 5A and 5D; n=12), CdCl₂ (200 μmol/L) reduced it by 117±13% (Figures 5B and 5D; n=6) and SKF96365 (50 μmol/L) caused 96±9% inhibition (Figures 5C and 5D; n=7). Washout reversed the inhibition (eg, Figure 5A). The inhibition observed with each agent did not differ significantly from 100%.

The voltage dependence of the CPA-induced current was determined using the whole-cell technique, with BAPTA and Cs$^+$ in the internal solution. The current activated during 1.5-second voltage ramps from −80 to 80 mV was increased

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**Figure 3.** CPA accelerates Mn$^{2+}$ quenching of fura-2 fluorescence. Fluorescence excited at 380 nm was continuously recorded and 20 μmol/L MnCl₂, followed by 30 μmol/L CPA, added as indicated. Inset shows mean rate of decline of fluorescence in 14 cells exposed to MnCl₂ immediately before and after adding CPA. **P<0.01 (paired t test).**

**Figure 4.** CPA activates inward current at negative potentials. A, Current recorded before and during exposure to 30 μmol/L CPA in a freshly isolated PASMCs clamped at −80 mV using the perforated-patch technique. B, CPA-induced current recorded using the perforated-patch technique (n=11) or after intracellular dialysis with 5 mmol/L BAPTA using the conventional whole-cell technique (n=76). Mean amplitudes, normalized against cell capacitance, show the control current, the peak current measured within the first minute of CPA application, and the sustained current remaining 2 minutes after CPA application. **P<0.01 compared with control (paired t test).**

**Figure 5.** Sensitivity of CPA-induced current to blockers of store-operated channels. Currents were recorded from PASMCs clamped at −80 mV using the whole-cell patch-clamp technique and pipettes containing 5 mmol/L BAPTA. Current activated by 30 μmol/L CPA was reversibly reduced by 200 μmol/L NiCl₂ (A), 200 μmol/L CdCl₂ (B), or 50 μmol/L SKF96365 (C). D, Histogram showing the mean inhibition produced on adding NiCl₂ (n=12), CdCl₂ (n=6), or SKF96365 (n=7).
Properties of the CPA-induced current. A, Voltage dependence of the current recorded under control conditions during a 1.5-second ramp from −80 mV to +80 mV, and then after the current induced by 30 μmol/L CPA was maximal. Records were obtained using the whole-cell technique with Cs+ pipette solution. Ramps were applied at 5-second intervals; each record is the average of 5 consecutive ramps. B, Mean current–voltage relationship of the CPA-induced current determined by subtracting the control current at each voltage from that in the presence of CPA (n=4). C, Effect of replacing PSS (1.8 mmol/L Ca) with nominally Ca2+-free solution on the current recorded during continuous exposure to 30 μmol/L CPA. D, Effect of reducing the extracellular Ca to 0.2 mmol/L on the current activated by CPA. E, Histogram comparing the amplitudes of currents evoked by CPA when the extracellular calcium was reduced from 1.8 mmol/L to 0 (n=6), 0.2 mmol/L (n=4), or 0.2 mmol/L Ca2+ (paired t test). *P<0.05 compared with 1.8 mmol/L Ca2+.

Expression of Trp Channels

As shown in Figure 7A, RT-PCR amplified products from rat PASMCs with the predicted sizes for rat Trp1, Trp3, Trp4, Trp5, and Trp6, and each was confirmed to have the correct nucleotide sequence. Figure 7B shows the expression levels of each of the transcripts measured relative to β-actin (arbitrary units) using semiquantitative RT-PCR. Although Trp4 and Trp5 were consistently transcribed, the levels of their relative expression (Trp4 0.00002±0.00001; Trp5 0.00003±0.00001) were too low to register on the histogram of Figure 7B. The expression of Trp1 and Trp6 was significantly greater than Trp3, Trp4, or Trp5 (n=3, P<0.05).

Immunocytochemistry provided further evidence for the expression of Trp channel subunits in rat PASMCs. Strong fluorescent labeling was obtained with antibodies directed against Trp1 (Figure 7C), Trp3 (Figure 7D), Trp4 (Figure 7E), and Trp6 (Figure 7F). Staining was absent from control cells treated in the same way but without exposure to anti-Trp antibody. Staining was consistently observed in PASMCs from each of 3 preparations.

Discussion

This is the first direct demonstration that depletion of intracellular Ca2+ stores causes contraction of arterial muscle by activating Ca2+-permeable cation channels and promoting Ca2+ influx. That these events were linked is indicated by the inhibitory effects of low concentrations of known blockers of store-operated channels on the ionic current, increase in [Ca2+]i, and sustained contraction caused by CPA. At the IC50 for their...
effects on contraction, Ni²⁺ and SKF96365 also halved the rise in [Ca²⁺], and concentrations of Cd²⁺, Ni²⁺, and SKF96365 that abolished contraction also abolished the current. Although La³⁺ inhibited the CPA-induced contraction, it was much less potent. This pharmacological profile matches the store depletion–activated cation current identified in anocogyecous smooth muscle, but contrasts with receptor-coupled cation channels in vascular preparations, which appear less sensitive to Cd²⁺ and more sensitive to La³⁺. The high La³⁺ sensitivity of pulmonary artery contraction activated by Ca²⁺ after depleting stores with phenylephrine in Ca²⁺-free medium and inhibiting α-receptors with phenolamine, therefore suggests that receptor-operated channels may be involved, rather than store-operated channels as proposed. The current and [Ca²⁺], took several minutes to reach maximum following CPA application, which is compatible with the time course of contraction.

The inward current activated at negative potentials by CPA showed little voltage sensitivity at negative potentials, with slight inward rectification at positive potentials. The reversal potential, close to 0 mV, indicates that it was carried by cations, along with sensitivity to La³⁺ and sensitivity to external Ca²⁺.23,24 The high La³⁺ sensitivity of pulmonary artery contraction activated by Ca²⁺ after depleting stores with phenylephrine in Ca²⁺-free medium and inhibiting α-receptors with phenolamine, therefore suggests that receptor-operated currents may be involved, rather than store-operated channels as proposed.12 The current and [Ca²⁺], took several minutes to reach maximum following CPA application, which is compatible with the time course of contraction.

Rat PASMCs were found to express Trp1, Trp3, Trp4, Trp5, and Trp6. The presence of so many Trp subunits suggests that they play an important role in pulmonary arterial function. In heterologous expression systems, Trp 6 channels, which are activated by diacylglycerol rather than store depletion, appear to be essential components of α₁-adrenoceptor-mediated, but store depletion–independent, Ca²⁺ influx in rabbit portal vein,23 and they may serve this function in pulmonary arteries. Studies using antisense cDNA indicate that Trp1 and Trp3 are functional subunits of store-operated channels,26–28 Moreover, an antibody directed against the predicted outer vestibule of Trp1 channels blocked the rise in [Ca²⁺], induced by thapsigargin in isolated arterioles. Along with the high relative expression of Trp1 in PASMCs, this suggests that Trp1 could contribute to store depletion–activated Ca²⁺ entry in these cells. How channels incorporating Trp1 or Trp3 might participate in the response to store depletion is less clear because, when expressed in heterologous systems, these channels were sometimes,19 but not always,29,30 activated by thapsigargin: they lack the long COOH-terminal domain of Drosophila trp that is necessary for thapsigargin activation.31 Trp1 and/or Trp3 could form channels by coassembly with each other or with other Trp subunits because functional Trp channels most likely exist as tetramers.7

A feature of the cation current induced by CPA in rat PASMCs was its unusual sensitivity to extracellular Ca²⁺, a substantially larger current being recorded in the presence of 0.2 mmol/L Ca compared with either zero or physiological (1.8 mmol/L) levels. A similar sensitivity to external Ca²⁺ was reported for the noradrenaline-activated cation current in rabbit portal vein32 and was explained by the presence of 2 distinct Ca²⁺ binding sites that mediate dual effects of Ca²⁺ at different concentrations. Thus, a high affinity site mediating facilitation could explain current enhancement at intermediate levels of extracellular Ca²⁺, whereas another, low affinity site could mediate inhibition at higher levels, as found in other cation and Ca²⁺-selective channels.33 This bimodal effect of extracellular Ca²⁺ on the CPA-activated current is a novel finding. Although extracellular Ca²⁺ is known to inhibit mammalian Trp channels,24,35 this type of dual modulation has not previously been described in store-operated or Trp channels. It could be a useful property for future identification of the molecular makeup of the PASMC channel.

Not only CPA, but also thapsigargin and ryanodine could evoke pulmonary artery contraction. This suggests that store-operated channels in pulmonary artery may be activated by depletion of either inositol 1,4,5-trisphosphate-sensitive or ryanodine-sensitive calcium stores, as found in anocogyecous muscle.38 The variable effect of thapsigargin was surprising, given its similar action to CPA and previous reports that it contracts pulmonary arteries.11,12 It could not be explained by simultaneous release of endothelium-derived NO because responses were little affected by the NO synthase inhibitor, N⁶-nitro-L-arginine methyl ester (not shown). Because 5 μmol/L thapsigargin caused pronounced contraction in the same vessels,13 it is possible that poor penetration into the intact tissue37 necessitates higher concentrations to block SERCA than in isolated cells.38 Substantial store depletion may be needed before store-activated channels can open,39 so perhaps at 1 μmol/L thapsigargin, store depletion was insufficient in some vessels to reach the threshold for channel activation. Alternatively, the variable effect may reflect the inhibitory effect of thapsigargin on store depletion–activated Ca²⁺ entry and L-type Ca channels.30

As found previously in anocogyecous smooth muscle cells,4 BAPTA failed to activate cation current in the absence of CPA. This was unexpected, as passive store depletion with BAPTA is often used to activate store-operated channels. However, a recent study showed that SERCA pumps are very effective at preventing the activation of store-operated channels and that, as a result, BAPTA depletes stores slowly even at high concentrations.41 In addition, providing cells with ATP, as in this study, was found to slow channel activation even further. Store depletion depends on the relative rates of Ca²⁺ accumulation and release from the SR. Therefore, because the passive leak of Ca²⁺ from the SR in canine pulmonary artery is slow,14 a higher degree of SERCA inhibition than is achieved with BAPTA alone may be required to induce store depletion in PASMCs. CPA was usually applied shortly after the onset of recording; perhaps BAPTA would have been seen to cause channel activation if dialysis had continued for longer periods before applying CPA.
In conclusion, by causing store depletion, CPA activates a Ca\(^{2+}\)-permeable cation channel through which it stimulates Ca\(^{2+}\) influx leading to contraction in rat PASMCs. The main function of this channel may be to replenish Ca\(^{2+}\) stores. Store-operated channels in PASMCs could provide a novel means to regulate the tone of pulmonary arteries and may be useful targets for the development of new vasodilators to treat pulmonary hypertension.

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**References**

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