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Capacitative calcium entry as a pulmonary specific vasoconstrictor mechanism in small muscular arteries of the rat

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1 The effect of induction of capacitative Ca^{2+} entry (CCE) upon tone in small (i.d. 200–500 μm) intrapulmonary (IPA), mesenteric (MA), renal (RA), femoral (FA), and coronary arteries (CA) of the rat was examined.

2 Following incubation of IPA with 100 nM thapsigargin (Thg) in Ca^{2+}-free physiological salt solution (PSS), a sustained contraction was observed upon reintroduction of 1.8 mM Ca^{2+}, which was unaffected by either diltiazem (10 μM) or the reverse mode Na^+/Ca^{2+} antiport inhibitor KB-R7943 (10 μM). An identical protocol failed to elicit contraction in MA, RA, or CA, while a small transient contraction was sometimes observed in FA.

3 The effect of this protocol on the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was assessed using Fura PE3-loaded IPA, MA, and FA. Reintroduction of Ca^{2+} into the bath solution following Thg treatment in Ca^{2+}-free PSS caused a large, rapid, and sustained increase in $[Ca^{2+}]_i$ in all the three types of artery.

4 100 nM Thg induced a slowly developing noisy inward current in smooth muscle cells (SMC) isolated from IPA, which was due to an increase in the activity of single channels with a conductance of ~30 pS. The current had a reversal potential near 0 mV in normal PSS, and persisted when Ca^{2+}-dependent K^+ and Cl^- currents were blocked; it was greatly inhibited by 1 μM La^{3+}, 1 μM Gd^{3+}, and the IP_3 receptor antagonist 2-APB (75 μM), and by replacement of extracellular cations by $NMDG^+$.

5 In conclusion, depletion of intracellular Ca^{2+} stores with Thg caused capacitative Ca^{2+} entry in rat small muscular IPA, MA, and FA. However, a corresponding contraction was observed only in IPA. CCE in IPA was associated with the development of a small La^{3+}- and Gd^{3+}-sensitive current, and an increased Mn^{2+} quench of Fura PE-3 fluorescence. These results suggest that although CCE occurs in a number of types of small arteries, its coupling to contraction appears to be of particular importance in pulmonary arteries.

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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; CA, coronary artery; CCE, capacitative Ca^{2+} entry; FA, femoral artery; IPA, intrapulmonary artery; MA, mesenteric artery; PSS, physiological salt solution; RA, renal artery; SERCA, sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase; SMC, smooth muscle cell; SR/ER, sarco/endoplasmic reticulum; Thg, thapsigargin; TRP, transient receptor potential protein

Introduction

Capacitative Ca^{2+} entry (CCE) is activated upon depletion of intracellular Ca^{2+} stores and is demonstrable in a variety of nonexcitable cell types (e.g. Putney, 1990; Berridge, 1995). This pathway is thought to be the key regulatory mechanism in the maintenance of Ca^{2+} loading of intracellular stores within the sarcoplasmic/endoplasmic reticulum (SR/ER; for a review see Berridge, 1995). However, in recent years it has become apparent that CCE may also be of importance in the regulation of a number of diverse cellular functions such as apoptosis, secretion, and gene transcription (for review see Parekh & Penner, 1997). Although the precise molecular identity of the ion channels involved in CCE is not known, it has been suggested that CCE in vascular smooth muscle is mediated by one or more of a family of seven proteins homologous to the transient receptor potential (TRP) protein of *Drosophila* (Hardie & Minke, 1993; Birnbaumer *et al*., 1996; Inoue *et al*., 2001; Xu & Beech, 2001).

A number of reports have provided evidence for the involvement of CCE in the regulation of smooth muscle tone in a variety of preparations, including mouse anococcygeus (Wallace *et al*., 1999), guinea-pig and cat gastric fundus (Petkov & Boev, 1996a, b), and rat ileum (Ohta *et al*., 1995),

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urinary bladder (Munro & Wendt, 1994), and spleen (Burt *et al*., 1995; reviewed by McFadzean & Gibson, 2002). Activation of CCE, usually *via* the application of the selective SR/ER ATPase (SERCA) inhibitors thapsigargin (Thg) or cyclopiazonic acid, has also been shown to modulate tone in vascular smooth muscle preparations. The inhibition of SERCA, and subsequent induction of CCE, has been reported to result in constriction of several conduit arteries, including rat pulmonary (de la Fuente *et al*., 1995), femoral (Nomura *et al*., 1997), and carotid arteries (Sekiguchi *et al*., 1996) and also isolated rat aorta (Kwan *et al*., 1994; Tepel *et al*., 1994; Low *et al*., 1996).

We have previously presented preliminary evidence that activation of CCE in smooth muscle cells (SMC) isolated from the rat intrapulmonary artery (IPA) results in the activation of a Ca^{2+}-permeable cation channel with a conductance of $\sim$30 pS (Snetkov *et al*., 2001). Similar results have also been reported by Ng & Gurney (2001), who found that SMC isolated from rat main pulmonary artery possess a small inward current associated with Ca^{2+} influx that is activated upon inhibition of SERCA. McDaniel *et al*. (2001) demonstrated a much larger CCE-associated current in cells cultured from the same preparation. These currents appeared to differ not only in amplitude, but also in their sensitivity to La^{3+}, which blocked the current described in the former report with an IC_{50} of 600 μM, but virtually abolished the current in the cultured PA cells at a concentration of 50 μM.

Little information exists as to the functional effects of CCE in the resistance vasculature, although Flemming *et al*. (2002) have recently shown that store depletion causes Ca^{2+} influx, but no constriction in rabbit pial arterioles. Curtis & Scholfield (2001) have also demonstrated the presence of a nifedipine-sensitive CCE-associated current in rabbit choroidal arteriolar myocytes, but did not assess whether this current contributed directly to contraction. In the present study, we have examined the effects of induction of CCE upon tone in small intrapulmonary (IPA), mesenteric (MA), renal (RA), femoral (FA), and coronary (CA) arteries of the rat. We report that although induction of CCE is uniformly associated with a rise in $[Ca^{2+}]_i$, it elicits contraction only in the small pulmonary arteries. CCE in these arteries is associated with the activation of a Na^+- and Ca^{2+}-permeable channel with a conductance of $\sim$30 pS. In contrast to the work of Ng & Gurney (2001), we observe that both the contraction and channel activity associated with CCE are potently blocked by La^{3+}.

Methods

Tension measurement in small muscular arteries

Male Wistar rats (250–350 g) were anaesthetised with sodium pentobarbital (55 mg kg^{-1} IP) and killed by cervical dislocation as approved by the local Home Office Inspector. The heart, lungs, intestines, kidneys and hind limbs were excised and placed in cold physiological salt solution (PSS, see below). Small (150–600 μm internal diameter (i.d.), median $\sim$300 μm) IPA, CA, MA, RA, and FA were dissected free of adventitia, mounted in a temperature-controlled myograph at 37°C (Cambustion AM10, Cambustion Ltd, Cambridge, U.K.) or dual channel Mulvany–Halpern myograph, (Danish Myo Technology, Aarhus, Denmark), and gassed continuously with 95% air/5% CO_2 (pH 7.35). After 30 min, a length–tension curve was obtained, and the artery stretched to 90% of the diameter that is equivalent to a transmural pressure of 30 mmHg for pulmonary and 100 mmHg for systemic arteries as previously described (Leach *et al*., 1994). The viability of the preparation was assessed by measuring the response to 80 mM K^+ PSS (KPSS; isotonic replacement of NaCl by KCl), and was deemed suitable for use in experiments only when they produced at least 4 and 8 mN mm^{-1} length for pulmonary and systemic arteries, respectively. Prior to beginning the experiments, arteries were equilibrated with four 2 min exposures to KPSS. Tension recording from small arteries has previously been described in detail (Leach *et al*., 1994; Robertson *et al*., 1995).

Measurement of intracellular $[Ca^{2+}]_i$ in isolated arteries

In some experiments, $[Ca^{2+}]_i$ was measured simultaneously with tension. In this case, following mounting, stretching, and assessment of viability, an artery was incubated with PSS containing 2 μM Fura PE-3/AM for 90 min at room temperature, followed by a further 30 min at 37°C to facilitate intracellular hydrolytic cleavage of the dye. The preparation was then washed several times with fresh PSS and stimulated repeatedly with high KPSS till a stable tension response was achieved. The myograph was mounted on an inverted microscope equipped with spectrofluorimeter (Cairn Research Ltd, Faversham, U.K.) and illuminated alternately with a UV light source at 340 and 380 nm, while measuring the intensities of emitted light using a photomultiplier tube. The ratio of intensities of emitted light (at $>$510 nm) corresponding to excitation wavelengths of 340 and 380 nm ($R_{340/380}$) was taken as a measure of $[Ca^{2+}]_i$.

Protocol for induction of CCE with Thg

Following the equilibration procedure, the myograph bath solution was exchanged for nominally Ca^{2+}-free PSS for a period of 10 min. Thg (100 nM) was then added to the myograph bath for a period of 10 min, after which 1.8-mM Ca^{2+} was then reintroduced into the bath solution for 10 min. The myograph bath solution was then replaced again with Ca^{2+}-free PSS. As shown in Figure 1, CCE-related contraction stabilized at the third reintroduction of Ca^{2+}; therefore, in all experiments only the third response was included in the analysis.

To prevent precipitation, experiments involving La^{3+} were performed with HEPES-buffered PSS gassed with air, containing (mM): NaCl 130, $MgCl_2$ 1, glucose 5.56, $CaCl_2$ 1.8, KCl 4, and HEPES 10, with pH adjusted to 7.4 with NaOH.

Cell isolation for electrophysiology

Dissected small arteries were cut open and placed in 2 ml of Ca^{2+}-free HEPES-buffered PSS, containing (mM): NaCl 135, KCl 5, $MgCl_2$ 2, glucose 11, and HEPES 10, pH adjusted to 7.4 with NaOH at 37°C for 20 min. Tissues were then placed in the same solution containing in addition 2 mg ml^{-1} collagenase (Sigma type XI), 1 mg ml^{-1} papain, 1 mg ml^{-1} soyabean trypsin inhibitor, and 1 mM dithiothreitol for 40 min. The tissue was then washed with an enzyme-free dispersion solution four times and left at 37°C for another 20 min. The solution volume

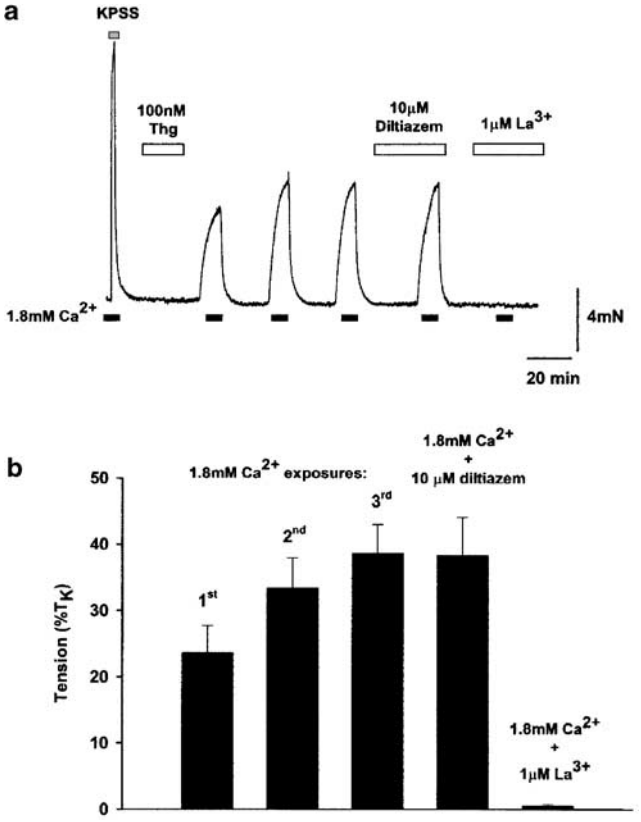

Figure 1 Thg induces sustained constriction in rat small isolated IPA. (a) The trace shows a typical response of rat 232 μm IPA to 100 nM Thg (applied in Ca^{2+}-free PSS). Upon the reintroduction of Ca^{2+} to the myograph bath, a sustained constriction was observed, which was reversed upon removal of Ca^{2+} from the myograph bath. The response increased with repeated Ca^{2+} exposures, yet was reproducible subsequent to the third exposure. This response was unaffected by the voltage-gated channel antagonist diltiazem (10 μM), but was completely abolished by 1 μM La^{3+}. (b) shows the mean response from 10 to 26 IPA.

was then reduced to $\sim$0.5 ml and the tissue was gently triturated with a fire-polished Pasteur pipette to release single cells. These were stored in a dispersion solution at 4°C, and then allowed to settle onto a coverslip, which formed the base of the recording chamber, prior to application of the HEPES-buffered solution used for current recording.

Current recording

Single SMC were studied with standard patch-clamp techniques using an Axopatch-1C amplifier (Axon Instruments Inc., Foster City, CA, U.S.A.). The experiments were performed at room temperature. The bath ($\sim$0.2 ml volume) was continuously superfused (1–2 ml min^{-1}) with HEPES-buffered PSS. It should be noted that EGTA was not added to external solutions containing La^{3+} and Gd^{3+} in order to avoid chelation. Agonists and drugs were applied *via* the perfusion system. Whole-cell currents were filtered with −3 dB, low-pass 80 dB dec^{-1}, Bessel-type built-in filter, and digitized using a CED 1401 interface and PATCH v.6 software (Cambridge Electronic Design, Cambridge, U.K.) as appropriate. Whole-cell current–voltage relationships were obtained using a voltage ramp protocol, where the holding potential was maintained at −60 mV, and a 0.5 s ramp from −100 to +100 mV was applied every 5 s. All traces shown are representative of at least four similar experiments.

Measurement of Fura PE-3 quench in SMC isolated from IPA

Single SMC isolated from IPA as above were loaded with Fura-PE3 *via* incubation of the cells with Fura PE-3/AM (1 μM) for 30 min at room temperature. A drop of cell suspension was placed on the bottom of the chamber mounted on an inverted microscope (Nikon Diaphot, Nikon U.K. Ltd, Kingston, U.K.) equipped with a spectrofluorimeter. SMC were allowed to attach to the bottom of the chamber, washed to remove excess Fura PE-3/AM, and left for another 20 min. Fluorescence recordings were made at room temperature in HEPES-buffered solution with excitation wavelengths of 340, 360, and 380 nm. Changes in $[Ca^{2+}]_i$ were assessed by calculating the ratio of the light intensities emitted (>510 nm) when the vessel was illuminated at 340 and 380 nm, respectively. Fluorescence quench was recorded at 360 nm using either 1 mM Mn^{2+} to study the basal influx or 0.1 mM Mn^{2+} for Thg-induced influx, where the quench rate was much higher.

Solutions

PSS used for tension recording contained (mM): NaCl 118, $NaHCO_3$ 24, KCl 4, $CaCl_2$ 1.8, $MgSO_4$ 1, NaH_2PO_4 0.434, and glucose 5.56. HEPES-buffered PSS used for tension recording in the presence of lanthanides contained (mM): NaCl 130, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, glucose 5.56, and HEPES 10, pH adjusted to 7.4 with NaOH. Low Na^+ PSS contained: NMDG 118, choline bicarbonate 24, $CaCl_2$ 1.8, $MgSO_4$ 1, NaH_2PO_4 0.434, and glucose 5.56, pH adjusted to 7.4 with HCl.

Electrophysiology The HEPES-buffered bath solution contained (mM): NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2, glucose 5, and HEPES 10, pH adjusted to 7.4 with NaOH. In experiments designed to study cation permeability, the latter solution was modified by replacing 140 mM Na^+ and 5 mM K^+ with 140 mM K^+, 140 mM Cs^+, or 80 mM Ca^{2+}, respectively. Ca^{2+}-free solution had Ca^{2+} omitted; no EGTA was used except when 0.1 mM EGTA was added to remove La^{3+} or Gd^{3+}. Ca^{2+}-, Mg^{2+}-free solution had no Ca^{2+} or Mg^{2+} and contained 1 mM EDTA. The pipette solution contained (mM): 140 mM KCl, 2 mM $MgCl_2$, 0.1 mM EGTA, and 10 mM HEPES, pH adjusted to 7.2 with KOH. In experiments designed to minimize K^+ and Cl^- currents, and with strong intracellular Ca^{2+} buffering, the pipette solution contained (mM): Cs methanesulphonate 120, BAPTA 10, and HEPES 10, pH adjusted to 7.2 with CsOH. $NMDG^+$, La^{3+}, Gd^{3+}, and Mn^{2+} were used as chloride salts.

Tension is presented as a percentage of the maximum tension (T_K) obtained to the final exposure to KPSS during the equilibration procedure. Mean changes in $[Ca^{2+}]_i$ are expressed as a percentage of the change in $R_{340/380}$ induced by KPSS; although not linearly related to $[Ca^{2+}]_i$, this provides a reasonable qualitative index. Results are expressed as mean ± s.e.m., and means were compared using paired or unpaired Student's *t*-test (SigmaStat, SPSS Inc., Chicago, U.S.A.). A difference was deemed significant if $P<0.05$.

Results

Comparison of the effects of Thg in rat small arteries

Following incubation of rat small IPA with 100 nM Thg in Ca^{2+}-free PSS, reintroduction of Ca^{2+} resulted in a significant sustained contraction ($23.7 \pm 4.0\%$ T_K, $n=26$). The size of this response increased with repeated 10 min exposures to Ca^{2+} (10 min apart), typically stabilizing after the third exposure to Ca^{2+} (Figure 1; second Ca^{2+} exposure $= 33.5 \pm 4.5\%$ T_K, $n=24$; third Ca^{2+} exposure $= 38.6 \pm 4.4\%$ T_K, $n=18$; fourth Ca^{2+} exposure $= 38.4 \pm 5.7\%$ T_K, $n=10$). In all experiments described subsequently, IPAs were given three 10 min exposures to 1.8 mM Ca^{2+}, 10 min apart, prior to experimental protocols, and time-matched controls were performed in every experiment. In accordance with our previous findings (Robertson *et al*., 2000), this constrictor response was completely blocked by preincubation with the trivalent cation La^{3+} at 1 μM (Figure 1b; Thg response to 1.8 mM Ca^{2+} in the presence of 1 μM $La^{3+} = 0.6 \pm 0.2\%$ T_K, $n=8$, $P<0.0001$). This response, however, was unaffected by the voltage-gated Ca^{2+} channel inhibitor diltiazem (10 μM, Figure 1a, $n=4$).

As illustrated in Figure 2, no sustained contractile response could be observed to Thg in any of the 'systemic' arteries examined in this study. However, in about 1/3 of the FA examined, a small transient contraction that decayed to baseline within 2–3 min was observed (peak response $\sim 9\%$ T_K, see Figures 4, 5). Removal of the endothelium had no effect on the response of either IPA or MA studied using this protocol (data not shown).

The above experiments do not rule out the possibility that the apparent absence of CCE-related contraction in the systemic arteries was due to a less effective Ca^{2+} store depletion by Thg in these arteries compared to IPA. In order to address this possibility, we used the procedure illustrated in Figure 3a. Arteries were preincubated for 90 s in Ca^{2+}-free solution containing 0.5 mM EGTA, to remove extracellular Ca^{2+}, and then challenged with 10 μM PE (maximally effective concentration). This caused a transient contraction, dependent on Ca^{2+} release, which provided an index of the amount of releasable Ca^{2+} in the store. Arteries were then placed in normal PSS for 10 min. This led to a large initial transient contraction, which was presumably caused by the brief simultaneous presence of PE and Ca^{2+} in the bath, since it was absent if PE was removed 2 min before Ca^{2+} was reapplied ($n=8$, data not shown). Thg (100 nM) was then applied for 10 min, after which the arteries were again incubated in Ca^{2+}-free PSS before being exposed to a second

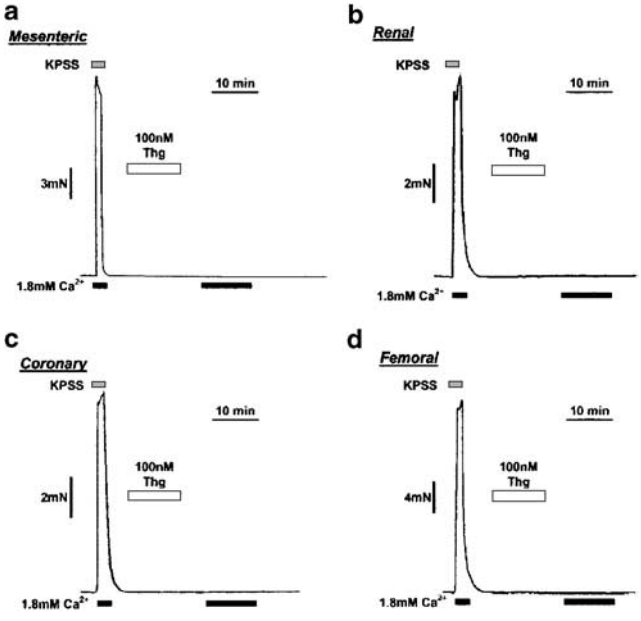

Figure 2 CCE-induced constriction is not observed in rat small MA, RA, CA, or FA. (a, b, c, d) traces show typical responses to 100- nM Thg and subsequent reintroduction of Ca^{2+} in rat MA (210 μm i.d.), RA (303 μm i.d.), CA (187 μm i.d.), and FA (324 μm i.d.), respectively, to 100 nM Thg. No constriction was ever observed in MA, RA, or CA upon reintroduction of Ca^{2+} to the myograph bath ($n=8$–12). A small transient contraction was observed in four of the 12 FA examined (see Figure 4).

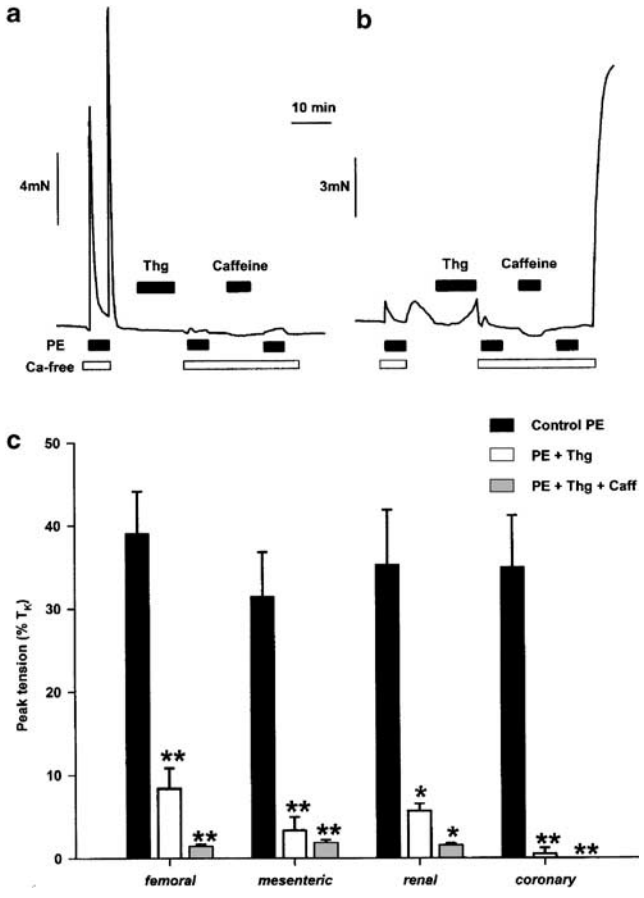

Figure 3 Contraction after depletion of cellular Ca^{2+} store with Thg (100 nM), caffeine (10 mM), and phenylephrine (PE, 10 μM). (a) The trace shows three responses to PE, administered 90 s after the removal of Ca^{2+} from the solution (with addition of 0.5 mM EGTA), in a rat mesenteric resistance artery. After a typical transient control PE contraction (left), stores were refilled by returning Ca^{2+} to the solution, and Thg was applied. This almost abolished the subsequent response to PE in Ca^{2+}-free solution. After the application and removal of caffeine and then PE in the absence of Ca^{2+} to ensure further Ca^{2+} store depletion, readdition of Ca^{2+} to the solution (at the end of the long open bar) did not cause contraction. (b) A protocol identical to that described above was carried out in a rat IPA. Here, Ca^{2+} reapplication after store depletion caused a large contraction (right). (c) The mean $\pm$ s.e.m. amplitude of the three PE contractions were recorded as described above in small FA, MA, RA, and CA. $n=4$; $^*P<0.05$; $^{**}P<0.01$.

PE challenge. The arteries, still in Ca^{2+}-free PSS, were then exposed to 10 mM caffeine for 2 min, before a third and last PE challenge, after which normal Ca^{2+}-containing PSS was reapplied (Figure 3a).

Thg itself had no effect on tension in MA, RA, FA, or CA, but the tension induced by the subsequent PE challenge was markedly reduced, reflecting a substantially reduced content of the PE-releasable Ca^{2+} store (Figure 3a, c, solid and open columns). In all types of arteries studied, subsequent treatment with caffeine induced no increase in tension, and the following PE challenge was also without significant effect (Figure 3c). These data strongly suggest that the releasable Ca^{2+} stores were essentially empty at this point. Nevertheless, in MA, RA, FA, and CA, reapplication of Ca^{2+}-containing PSS caused no increase in tension whatsoever (Figure 3a), whereas in IPA it caused a rapid and powerful contraction (Figure 3b). These experiments show firstly that Thg was effective in depleting intracellular stores of Ca^{2+} in all the five types of resistance artery, and secondly that the difference between IPA and the systemic arteries persisted even when store depletion was further assured by treating the arteries with PE and caffeine in addition to Thg.

It should be noted that consistent with previous reports (Priest *et al*., 1997), PE caused only a very small transient contraction in IPA in the absence of extracellular Ca^{2+}, although this was also reduced following application of Thg. Additionally, in IPA, Thg caused a gradually developing contraction in normal PSS (Figure 3b) in contrast to the systemic arteries where it had no effect on tension.

Effect of Thg on $[Ca^{2+}]_i$ in isolated arteries

Effects of Thg treatment on $[Ca^{2+}]_i$ were investigated in IPA, FA, and MA. Figure 4 illustrates the simultaneous records of tension and $[Ca^{2+}]_i$ obtained in an IPA (top), MA (middle), and FA (bottom). In each case, readmission of Ca^{2+}, following treatment of the artery with Thg in Ca^{2+}-free PSS, caused an increase in $R_{340/380}$, which was comparable to (and in the case of FA, much larger than) the increase observed during application of KPSS. In the FA, the rise in $[Ca^{2+}]_i$ in response to KPSS was consistently smaller than in the IPA or MA. Conversely, as described above, tension development upon Ca^{2+} readmission was negligible in the MA, substantial in the IPA, and small and transient, or absent, in the FA. Pooled measurements from a number of arteries of the CCE-mediated increases in $[Ca^{2+}]_i$ and tension are presented in Figure 5, with the bar for tension development in the FA representing the peak of the transient response. These results indicate that CCE occurs in each type of artery, but that it appears to be differentially coupled to tension development.

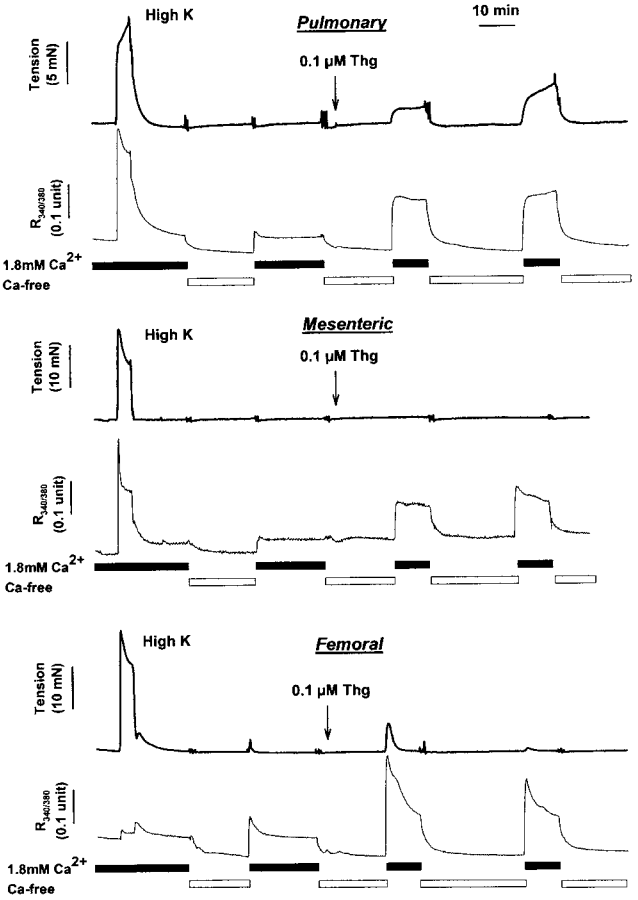

Figure 4 Simultaneous recording of tension development and $[Ca^{2+}]_i$ during application of KPSS and Thg in IPA (upper traces), MA (middle traces), and FA (lower traces). Following addition of KPSS, arteries were exposed to a nominally Ca^{2+}-free solution for 10 min. Subsequent reapplication of Ca^{2+} caused a small transient increase in $[Ca^{2+}]_i$. Arteries were then exposed to Ca^{2+}-free solution, and 100 nM Thg was applied. Reapplication of Ca^{2+} in the presence of Thg caused a large and sustained increase in $[Ca^{2+}]_i$, which was stable when the cycle of Ca^{2+} removal and replacement was repeated. This increases in $[Ca^{2+}]_i$ was associated with a sustained contraction in IPA, with no contraction in MA. A transient contraction was sometimes observed in FA, but tended to wane with further cycles of Ca^{2+} removal and replacement.

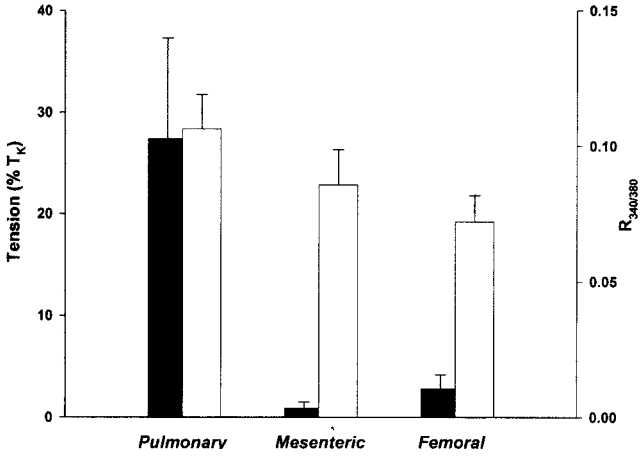

Figure 5 Summary of CCE-related tension development and $[Ca^{2+}]_i$ in IPA, MA, and FA. Effects of reapplication of extracellular Ca^{2+} following Thg treatment on tension development and $[Ca^{2+}]_i$ obtained in individual IPA ($n=5$), MA ($n=6$), and FA ($n=4$) during experiments similar to those shown in Figure 4. The mean ± s.e.m. of these responses are shown, with the solid bars representing tension (left axis) and the open bars representing the increase in $[Ca^{2+}]_i$, expressed in terms of the raw $R_{340/380}$ values (right axis).

Effect of Thg upon inward currents in SMC isolated from small IPA

The effect of Thg on the membrane current was studied in cells isolated from the IPA. The high input resistance of these cells meant that the holding current needed to maintain the membrane potential of the whole cell at −80 mV was very small, and occasionally spontaneous single-channel events could be detected in whole-cell mode as inward currents of ~3 pA (Figure 6). Thg (100 nM) in Ca^{2+}-free solution caused a progressive increase in the frequency of openings, the overlapping of which resulted in a strongly fluctuating inward current with amplitude of 20–30 pA within 3 min (Figure 6). The development of the inward current in the presence of Thg occurred both with standard KCl-based intracellular solution and with a Cs methanesulphonate pipette solution containing 10-mM BAPTA to minimize the contribution of Ca^{2+}-dependent K^+ and Cl^- currents (not shown).

The trivalent cations La^{3+} or Gd^{3+} (both 1 μM) were found to block the Thg-induced current rapidly (Figure 7a); for example, in four cells La^{3+} inhibited the current by 84±4%. Their effect could be reversed by washing with Ca^{2+}-free extracellular solution containing 0.1 mM EGTA, which strongly chelates lanthanides (Halaszovich *et al*., 2000). The replacement of all extracellular cations by $NMDG^+$ also inhibited the Thg-induced current by more than 90% (Figures 7b, c).

Thg-induced current was not significantly different in Ca^{2+}-free extracellular solution ($n=8$). However, the current increased considerably when both Ca^{2+} and Mg^{2+} were removed using a solution containing 1 mM EDTA to chelate any traces of divalent cations (Figure 8a). Both K^+ and Cs^+ supported the current to a certain extent and a current of ~40% of the normal amplitude persisted when 80 mM Ca^{2+} was the only extracellular cation (Figure 8b). This would be consistent with the channel having a somewhat smaller permeability to Ca^{2+} than to Na^+.

When cells were ramped from −100 to +100 mV in the absence of both intra- and extracellular K^+, Thg treatment caused the development of a current which reversed at ~0 mV (Figure 8c). This current was strongly inhibited at negative

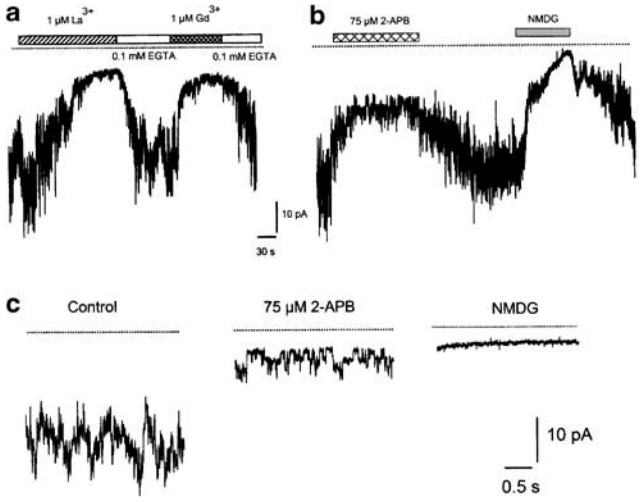

Figure 7 Block of Thg-induced inward current. (a) Trivalent cations La^{3+} and Gd^{3+} block Thg-induced inward current; this block could be reversed by chelating lanthanides with EGTA. (b) 2-APB caused partial and reversible block of the Thg-induced inward current, while replacement of all extracellular permeable cations by $NMDG^+$ abolished the current completely. (c) Single channels underlying Thg-induced whole-cell current were revealed in the presence of 2-APB, which reduced the opening frequency without affecting conductance. No activity could be observed in $NMDG^+$-based solution. Holding potential −80 mV, intracellular solution – Cs methanesulphonate, 10 mM BAPTA. Lines indicate zero current level. Traces shown are typical of six to experiments.

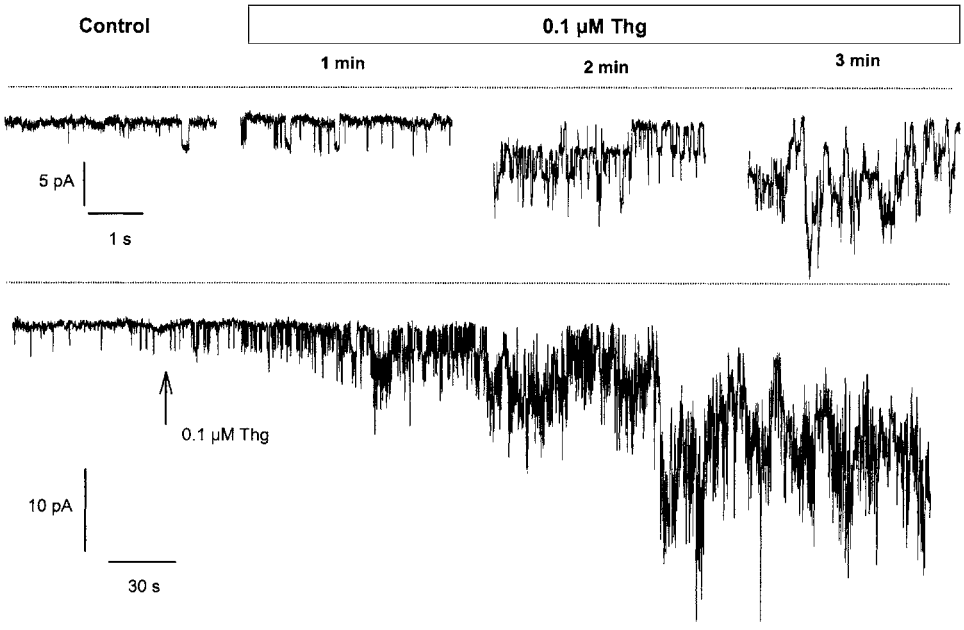

Figure 6 Thg causes an inward current in SMC isolated from rat small IPA. Below, the typical trace of the noisy inward current developing in SMC isolated from small IPA in response to 100 nM Thg. Above, the expanded sections of the same trace showing the dramatic increase of single-channel activity. Holding potential −80 mV, intracellular solution – Cs methanesulphonate, 10-mM BAPTA. Lines indicate zero current level. Traces shown are typical of five to seven experiments.

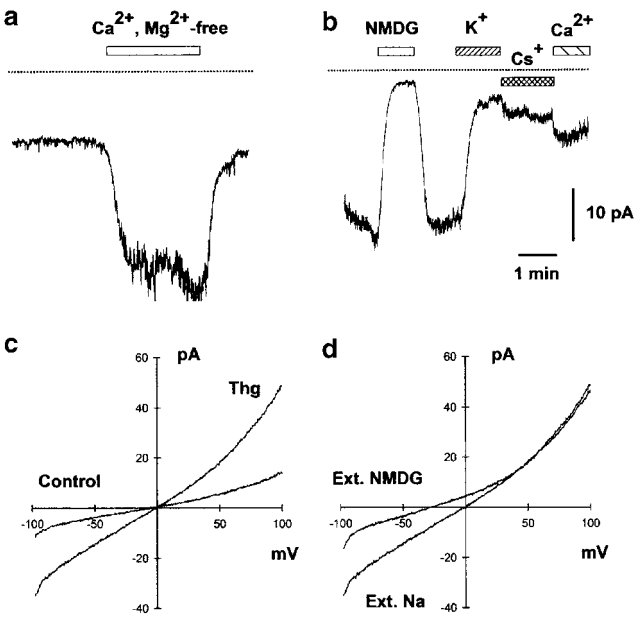

Figure 8 Ion permeability of Thg-induced inward current. (a) Removal of divalent cations with 1 mM EDTA considerably enhanced Thg-induced current. (b) 140 mM K^+, 140 mM Cs^+, and 80 mM Ca^{2+} supported the inward current to some extent, although it was smaller than with 140 mM Na^+ (control). The lines indicate zero current level. Traces shown are typical of six to eight experiments. (c) When the membrane potential was ramped from −100 to +100 mV in the absence of both intra- and extracellular K^+, Thg treatment (1 μM, 3 min) caused the development of nonrectifying current. (d) After treatment with Thg, the replacement of extracellular Na^+ for $NMDG^+$ suppressed the inward current. Holding potential −80 mV, intracellular solution – Cs methanesulphonate, 10 mM BAPTA. Typical traces are from four to eight experiments.

potentials by replacement of extracellular Na^+ with $NMDG^+$ (Figure 8d). In five cells, the inward current at −80 mV was reduced by 76.5±5.6%, whereas the outward current at +80 mV was reduced by only 19.2±7.1%, when $NMDG^+$ replaced Na^+.

Effect of 2-aminoethoxydiphenyl borate (2-APB) and KB-R7943

The putative membrane-permeable IP_3 receptor antagonist 2-APB has been reported to block both endogenous store-operated channels and heterologously expressed TRP3 channels in HEK293 cells (Ma *et al*., 2000). We examined the effect of 2-APB on both IPA constriction and Thg-induced inward currents. 2-APB (75 μM) was found to reduce the inward current (Figure 7b), and in doing so revealed individual channel openings (Figure 7c) similar in amplitude to those recorded spontaneously, and following Thg treatment. 2-APB (75 μM) also completely abolished the Thg-induced contraction in small IPA ($n=14$), and caused a small but significant inhibition of the KPSS contractile response (KPSS in the presence of 75- μM 2-APB = 78.4±11.8% T_K, $P<0.05$, $n=5$).

Since the current observed after Thg treatment was predominantly due to Na^+ influx, it is possible that a subsarcolemmal increase in Na^+ could cause an increase in intracellular $[Ca^{2+}]$ and therefore contraction, by activating reverse mode Na^+/Ca^{2+} exchange, as suggested by Lee *et al*. (2001) for rabbit inferior vena cava. However, the inhibitor of reverse mode Na^+/Ca^{2+} exchange, KB-R7943 (10 μM), did not significantly reduce the Thg-induced contraction in IPA (contraction in the presence of KB-R7943 = 85±5% of control response, $n=5$, $P>0.05$). Moreover, the rise in $[Ca^{2+}]_i$ induced by reintroduction of extracellular Ca^{2+} was unchanged in low Na^+ PSS (data not shown; $n=4$). However, KB-R7943 was found to inhibit the KPSS-induced contraction (contraction in the presence of KB-R7943 = 31.7±4.6% of control response, $n=5$, $P<0.05$), which is mediated by voltage-gated Ca^{2+} channels. This suggests that this compound is not selective for the reverse mode Na^+/Ca^{2+} exchanger in these arteries.

Basal Mn^{2+} permeability of SMC from IPA

In SMC freshly isolated from IPA and loaded with Fura-PE3, application of extracellular Mn^{2+} (1 mM) caused a relatively fast quenching of fluorescence under basal conditions. Application of 1 μM La^{3+} did not affect the rate of quenching (106±8% of control, $n=6$); however, 10 μM La^{3+} caused a significant reduction in the rate of quenching (25±4%, $n=6$), and 10 μM Gd^{3+} suppressed it entirely (2±1%, $n=6$). Diltiazem (10 μM) had no effect (98±8%, $n=4$), while 2-APB (75 μM) markedly blocked basal quenching (25±4%, $n=5$) (Figure 9a). These results indicate that IPA SMC possess a constitutively active Ca^{2+} influx pathway permeable to Mn^{2+}. Pretreatment of cells with Thg (100 nM) increased the rate of quenching to 625±96% ($n=8$, $P<0.001$) of control, and this increase was abolished by 75 μM 2-APB ($n=5$) and by 1 μM La^{3+} ($n=4$) (Figure 9b).

Discussion

CCE subsequent to inhibition of the SERCA has been proposed as a ubiquitous mechanism in smooth muscle controlling not only the Ca^{2+} load of stores within the ER/SR, but also modulating tone (for review see McFadzean & Gibson, 2002). We have recently reported that the transient portion of the vasoconstrictor response to hypoxia in rat-isolated IPA may be mediated in part *via* an activation of CCE (Robertson *et al*., 2000). In the present study, we applied Thg in the absence of Ca^{2+} to deplete Ca^{2+} stores, and then restored Ca^{2+} to the solution to examine CCE. Among the five types of small arteries which we studied, Ca^{2+} restoration caused a substantial sustained contraction in the IPA, sometimes caused a small transient contraction in the FA, and was not associated with contraction in the MA, RA, or CA. Ca^{2+} restoration was, however, associated with substantial increases in $[Ca^{2+}]_i$ in MA, FA, and IPA, each of the three arteries representing a different pattern of tension development.

CCE and arterial contraction

The majority of investigations into the possible role of CCE in regulating vascular tone have been performed in large conduit arteries. Induction of CCE, *via* inhibitors of the SERCA or Ca^{2+}-releasing agonists, has consistently been shown to induce a prompt and marked contraction in the rat main pulmonary artery (de la Fuente *et al*., 1995; McDaniel *et al*., 2001; Ng & Gurney, 2001), which is associated with a rise in $[Ca^{2+}]_i$

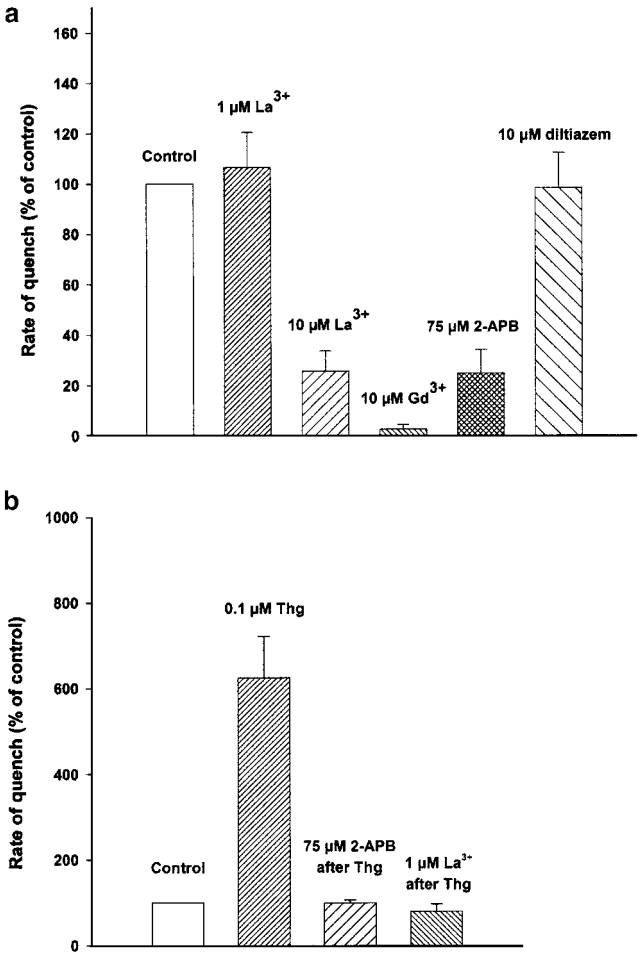

Figure 9 SMC isolated from IPA possess a Mn^{2+}-permeable, La^{3+}-, Gd^{3+}-, and 2-APB-sensitive basal and Thg-induced influx pathways. (a) Relative rates of basal Fura-PE3 fluorescence quenching with 1 mM Mn^{2+} in unstimulated SMC freshly isolated from IPA. La^{3+} (10 μM, but not 1 μM) caused a significant reduction in the rate of quenching and 10 μM Gd^{3+} suppressed it entirely. Diltiazem (10 μM) had no effect, while 2-APB (75 μM) attenuated the basal quenching. (b) Thg (100 nM) increased the rate of quenching (measured with 0.1 mM Mn^{2+}) more than six-fold, and this increase was abolished by 75 μM 2-APB or 1 μM La^{3+}. $n=10-16$, $^{***}P<0.001$.

(McDaniel *et al*., 2001; Ng & Gurney, 2001). However, agents that induce CCE tend to cause inconsistent, small, and/or more slowly developing contractions in systemic conduit arteries, including rat aorta (Noguera *et al*., 1998; Shima & Blaustein, 1992), tail artery (Kwan *et al*., 1994), carotid artery (Sekiguchi *et al*., 1996), and FA (Nomura *et al*., 1997), and mouse aorta (Cohen *et al*., 1999), in spite of the fact that, when measured, $[Ca^{2+}]_i$ has been shown to rise substantially in these arteries (Sekiguchi *et al*., 1996; Tosun *et al*., 1998; Cohen *et al*., 1999). The few analogous studies in smaller systemic arteries suggest that the rise in $[Ca^{2+}]_i$ is completely dissociated from contraction. Naganobu & Ito (1994) observed that cyclopiazonic acid caused a sustained rise in $[Ca^{2+}]_i$ with no contraction in rat mesenteric resistance arteries, and Flemming *et al*. (2002) reported a similar effect in rabbit pial arterioles. The present study confirms and extends these findings, showing firstly that CCE is associated with a marked and sustained contraction in smaller pulmonary arteries from rats, and secondly that CCE causes no or a minimal transient contraction in rat small MA, FA, RA, and CA.

The observations of Naganobu & Ito (1994) and Flemming *et al*. (2002), as well as the results shown in Figures 4 and 5, indicate, however, that Thg-induced store depletion causes a marked and sustained increase in $[Ca^{2+}]_i$ in small systemic as well as pulmonary arteries. The basis of the difference between IPA and the other arteries remains obscure. The Thg-induced current appeared to be mainly carried by Na^+ (see below), a finding that is consistent with the report of Arnon *et al*. (2000) that store release greatly elevated $[Na^+]_i$ in rat cultured mesenteric arterial myocytes. It therefore seemed possible that Na^+ influx through store-operated channels could cause an increase in intracellular $[Ca^{2+}]$ and contraction by activating reverse mode Na^+/Ca^{2+} exchange (Lee *et al*., 2001) in IPA but not the other arteries, especially because our preliminary observations suggest that Na^+/Ca^{2+} exchange is prominent in the IPA, but not in the MA (Moir & Aaronson, unpublished observations). However, we found no evidence for this mechanism, as KB-R7943, an inhibitor of reverse mode Na^+/Ca^{2+} exchange, did not significantly reduce the Thg-induced contraction, and removal of extracellular Na^+ did not affect the CCE-associated increase in $[Ca^{2+}]_i$. It should be noted that KB-R7943 inhibited KPSS-induced contractions in IPA, which we have previously found are completely abolished by voltage-gated Ca^{2+} channel blockers (Robertson *et al*., 2000). This suggests that KB-R7943 is not selective for reverse-mode Na^+/Ca^{2+} exchange in these arteries, and is consistent with its ability to block a variety of voltage-gated ion channels (Tanaka *et al*., 2002).

Although IPA responded to CCE with a contraction, it is apparent from Figure 4 that even in these arteries, the CCE-mediated contraction was much smaller than that evoked by high K^+, even though both stimuli were associated with comparable increases in $[Ca^{2+}]_i$. This disproportion between increases in $[Ca^{2+}]_i$ and tension, an extreme form of which apparently occurs in small systemic arteries and arterioles (Figure 4, see also Naganobu & Ito, 1994; Flemming *et al*., 2002), was initially highlighted in the rat aorta by Tosun *et al*. (1998), who proposed that Ca^{2+} entering during CCE is confined to a compartment that has limited access to the contractile apparatus. The case of the FA is particularly interesting, since here CCE caused an increase in $[Ca^{2+}]_i$ that was much larger than that caused by high K^+ depolarization, even though it caused a contraction that was negligible compared to the high K^+ response. It seems that even in the absence of Ca^{2+} uptake into the sarcoplasmic reticulum, SMC retain the ability to selectively direct Ca^{2+} entering the cell *via* different pathways either towards or away from the contractile apparatus.

Properties of CCE in rat IPAs

Treatment of isolated IPA SMC with Thg caused the development of a noisy inward current in cells held at −80 mV. The current amplitude was ~30 pA in cells held at, or ramped to, this potential. At negative potentials, the current was not affected when Ca^{2+} was removed in the presence of a normal $[Na^+]$, but was greatly reduced when Na^+ was replaced by $NMDG^+$, suggesting that it was mainly carried by Na^+ ions. A current of reduced amplitude was, however, observed when Na^+ was replaced by 80 mM Ca^{2+}, indicating

that the channel is also Ca^{2+}-permeable. The current was also supported by other monovalent cations, and reversed near 0 mV, suggesting that it is mediated by a nonselective cation channel.

During the onset of the current, and following its block by 2-APB, it was possible to observe individual channel openings of $\sim$3 pA at $-$80 mV, indicating a channel conductance of $\sim$30 pS. Although we did not further characterize the properties of these channels, they appear to differ from the store-operated channels in rabbit portal vein, which were recently described in greater detail by Albert & Large (2002), as the latter demonstrated a conductance of $\sim$2 pS and reversed at a more positive potential under similar conditions.

The current observed in IPA appears to be similar, however, to that previously reported in rat main pulmonary artery by Ng & Gurney (2001), with the difference that the current in IPA is much more potently inhibited by La^{3+}. It seems likely, however, that the relative La^{3+} insensitivity ($IC_{50} \sim 600\ \mu M$) reported by Ng & Gurney (2001) can be explained by their use of a phosphate-containing buffer system. Since lanthanum phosphate is known to be insoluble (Moeller, 1961), the use of phosphate-containing buffers in experiments where La^{3+} is used would result in the formation of lanthanum phosphate, thereby greatly decreasing the concentration of free La^{3+} in solution.

It has been reported that La^{3+} can directly prevent the inhibition of SERCA by Thg (Papp *et al*., 1991). It is therefore possible that the inhibitory effects of La^{3+} upon Thg-induced contraction, and channel activation, in pulmonary arteries may be due to stabilization of SERCA by La^{3+}. However, our results suggest that the blockade of the CCE-associated current by La^{3+} is extracellular since the pipette solution contained EGTA, which would strongly chelate any La^{3+} entering the cells. The inclusion of EGTA in the pipette solution would also appear to preclude the possibility that La^{3+} may block CCE in pulmonary arteries *via* an action on the cytosolic side of the plasma membrane, as reported for the blockade of TRP3 channels by La^{3+} in Chinese hamster ovary cells (Halaszovich *et al*., 2000).

In marked contrast to our results, McDaniel *et al*. (2001) reported a store release-activated current of over 300 pA in pulmonary artery SMC. The most likely explanation for this discrepancy is that McDaniel *et al*. (2001) utilized cells that had been maintained in primary culture, a procedure that is known to enhance CCE greatly (Golovina, 1999; Dreja *et al*., 2001).

It is interesting to note that infrequent openings of channels apparently similar to those underlying the Thg-induced current could be recorded prior to Thg exposure (see Figure 6a). It is possible that cell dialysis with high concentrations of Ca^{2+}-chelating agents, such as EGTA or BAPTA, could itself cause depletion of intracellular stores and subsequent activation of CCE (Albert & Large, 2002). However, in nondialysed cells a Mn^{2+}-permeable influx pathway was observed under basal conditions (Figure 9a), which was blocked by 10 μM La^{3+} but not by 1 μM La^{3+}. Thg preincubation elicited an approximately six-fold increase in Mn^{2+} quenching of the fluorescence signal, which was abolished by 1 μM La^{3+} (Figure 9b). There was, therefore, a difference in the La^{3+} sensitivity of the basal and Thg-associated influx pathways. This suggests the involvement of separate channels, although this requires additional confirmation.

2-APB was effective in abolishing the Thg-induced increases in both inward currents, and constrictor response in rat-isolated small IPA. This agent, which blocks the IP_3 receptor (Maruyama *et al*., 1997), was initially used to assess the possible role that this receptor could play in the regulation of CCE (e.g. Ma *et al*., 2000). Subsequent observations suggested, however, that 2-APB possesses effects upon CCE that are independent of any action upon the IP_3 receptor (Dobrydneva & Blackmore, 2001; Iwasaki *et al*., 2001; Prakriya & Lewis, 2001; Ma *et al*., 2002). The present results are consistent with these more recent findings. Irrespective of the mechanism by which 2-APB is acting to inhibit CCE, its effect on the Thg-induced current and contraction in IPA provides additional evidence linking these effects, although the minor inhibition of the contraction to KPSS by 2-APB illustrates its lack of complete selectivity for CCE.

In summary, CCE-induced vasoconstriction appears to be especially prominent in pulmonary arteries, at least at the level of small muscular arteries. The results suggest that the extent to which CCE contributes to agonist-induced vasoconstriction may vary widely between different vascular beds, and underline the importance of this pathway in the pulmonary vasculature. The CCE-associated current in rat IPA involves a nonselective cation channel with a conductance of $\sim$30 pS, and is sensitive to relatively low concentrations (1 μM) of the trivalent cations La^{3+} and Gd^{3+}. Further studies are required to determine the basis of the heterogeneity of the contractile response of various arteries to Ca^{2+} store release.

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