Ca²⁺ channel-sarcoplasmic reticulum coupling: a mechanism of arterial myocyte contraction without Ca²⁺ influx

Alberto del Valle-Rodríguez, José López-Barneo[1] and Juan Ureña

Laboratorio de Investigaciones Biomédicas, Departamento de Fisiología and Hospital Universitario Virgen del Rocío, Universidad de Sevilla, E-41013, Seville, Spain

[1]Corresponding author
e-mail: Jose.L.Barneo.sspa@juntadeandalucia.es

Contraction of vascular smooth muscle cells (VSMCs) depends on the rise of cytosolic [Ca²⁺] owing to either Ca²⁺ influx through voltage-gated Ca²⁺ channels of the plasmalemma or receptor-mediated Ca²⁺ release from the sarcoplasmic reticulum (SR). We show that voltage-gated Ca²⁺ channels in arterial myocytes mediate fast Ca²⁺ release from the SR and contraction without the need of Ca²⁺ influx. After sensing membrane depolarization, Ca²⁺ channels activate G proteins and the phospholipase C–inositol 1,4,5-trisphosphate (InsP₃) pathway. Ca²⁺ released through InsP₃-dependent channels of the SR activates ryanodine receptors to amplify the cytosolic Ca²⁺ signal. These observations demonstrate a new mechanism of signaling SR Ca²⁺-release channels and reveal an unexpected function of voltage-gated Ca²⁺ channels in arterial myocytes. Our findings may have therapeutic implications as the calcium-channel-induced Ca²⁺ release from the SR can be suppressed by Ca²⁺-channel antagonists.
Keywords: arterial myocytes/Ca²⁺ channels/calcium-channel-induced Ca²⁺ release/inositol trisphosphate/voltage sensor

Introduction

Vascular smooth muscle cells (VSMCs) control blood flow and pressure. Contraction of these cells depends on the rise of cytosolic [Ca²⁺], which can occur owing to either Ca²⁺ entry through membrane channels or to receptor-mediated Ca²⁺ release from the sarcoplasmic reticulum (SR) (Somlyo and Somlyo, 1994; Bolton *et al*., 1999). It has previously been reported that exposure to high extracellular K⁺ can induce elevation of cytosolic [Ca²⁺] in VSMCs without the need of significant transmembrane Ca²⁺ influx (Kobayashi *et al*., 1986). In addition, it is known that agonist-induced Ca²⁺ release from internal stores can be altered by modifications of the membrane potential in several cell types (Itoh *et al*., 1992; Ganitkevich and Isenberg, 1993; Kukuljan *et al*., 1994; Jaimovich *et al*., 2000; Mason and Mahaut-Smith, 2001). However, it is not known whether membrane electrical events in vascular smooth muscle can *per se* activate the Ca²⁺ release channels of the SR as well as the underlying mechanisms. Herein we show that in myocytes dispersed
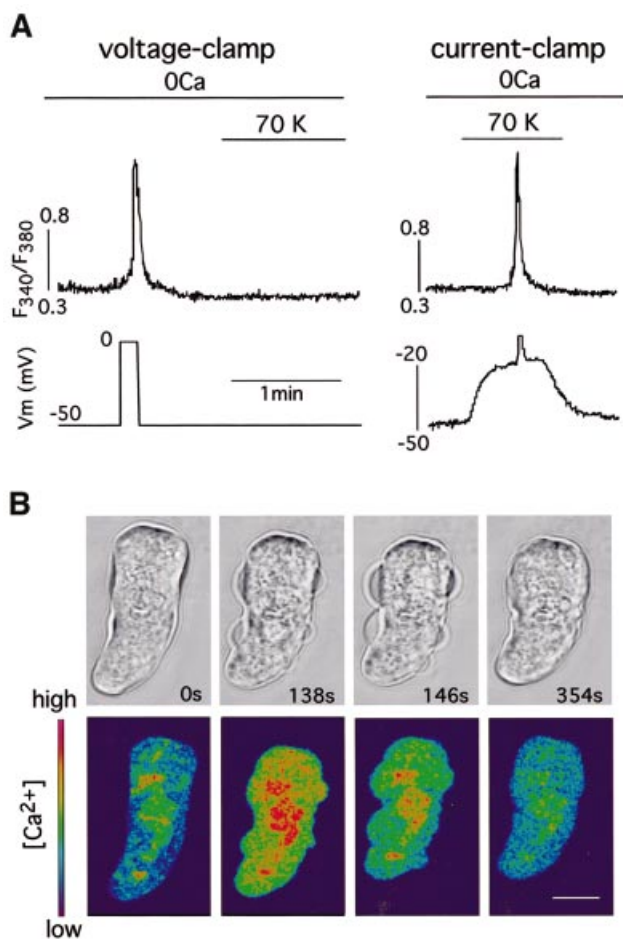
from basilar brain arteries, membrane depolarization can evoke Ca²⁺ release from stores that are sensitive to inositol 1,4,5-trisphosphate (InsP₃) and ryanodine (Ry) (Ehrlich and Watras, 1988; Blatter and Weir, 1992; Berridge, 1993; Pozzan *et al*., 1994) in a graded manner and can produce cell contraction in the absence of agonists or extracellular Ca²⁺ influx. Coupling of myocyte depolarization to Ca²⁺ release from the SR depends on voltage-gated Ca²⁺ channels that, after sensing the change of membrane potential, activate G proteins and subsequently the phospholipase C–InsP₃ pathway. Ca²⁺ released through InsP₃-dependent channels activates Ry receptors to amplify the cytosolic Ca²⁺ signal. These observations demonstrate a new mechanism of signaling SR Ca²⁺-release channels and reveal an unexpected functional feature of plasmalemmal voltage-gated Ca²⁺ channels in VSMCs. In addition to their well-known role as Ca²⁺-selective membrane pores, they act as voltage sensors capable of triggering fast G-protein-dependent Ca²⁺ release from the SR and myocyte contraction.

Results

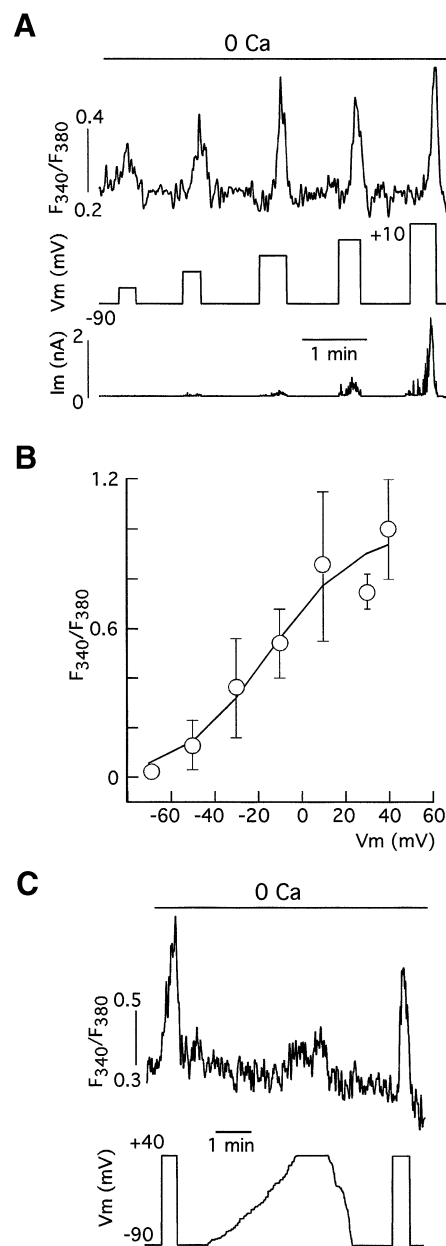### Calcium-channel-induced Ca²⁺ release and myocyte contraction in the absence of extracellular Ca²⁺ influx

In voltage-clamped basilar arterial myocytes, loaded with Fura-2 and bathed in solutions without external Ca²⁺, membrane depolarization induced a fast transient Ca²⁺ signal due to release of the cation from internal stores (Figure 1A, left). The signal normally had a slow foot lasting a few seconds preceding a sharp rise of [Ca²⁺]. This depolarization-induced Ca²⁺ release was also triggered in current-clamped cells (Figure 1A, right) or in intact undialyzed, myocytes (see below) by depolarization with 70 mM extracellular K⁺. However, high external K⁺ failed to elicit depolarization-induced Ca²⁺ release in voltage-clamped cells with the membrane potential held at –50 mV (Figure 1A), suggesting that it primarily requires a change in the cell's membrane potential. Depolarization-induced Ca²⁺ release has been observed in 81% of the basilar myocytes studied ($n = 232$) bathed in either Ca²⁺-free solutions (0 mM Ca²⁺ plus 2 mM EGTA) or in external solutions with cadmium (0.4 mM) and nickel (3 mM) added to block transmembrane Ca²⁺ influx. Depolarization-induced Ca²⁺ release was also present in Ca²⁺- and Na⁺-free solutions (Na⁺ substituted for choline; $n = 13$ cells tested) but totally abolished by previous exposure to thapsigargin (300 nM; $n = 7$ cells tested), a blocker of SR Ca²⁺-ATPase (Thastrup *et al*., 1990), thus indicating that the SR was the source of the Ca²⁺ released by membrane depolarization. In myocytes bathed in an external solution without Ca²⁺ and with 2 mM EGTA added, depolarization was able to produce contraction, as

demonstrated by confocal microscopy studies in which we simultaneously monitored changes in cell shape and cytosolic [Ca^{2+}] (Figure 1B). Depolarization-induced Ca^{2+} release was observed as a graded phenomenon whose magnitude increased in parallel with that of membrane depolarization. In voltage-clamped myocytes depolarizing pulses of increasing amplitude elicited progressively larger Ca^{2+}-release signals that triggered outward currents due to activation of Ca^{2+}-dependent maxi-K^+ channels known to be present in arterial myocyte membranes (Figure 2A) (Nelson *et al.*, 1995; Smani *et al.*, 2001). The amplitude of the Ca^{2+}-release signal (measured as change of fluorescence ratio) as a function of the membrane potential is plotted in Figure 2B using data from several voltage-clamped cells held at –90 mV. In these conditions, the threshold for the appearance of depolarization-induced

Ca^{2+} release was ~–70 mV and the midpoint of the fitted Boltzmann function was at ~–15 mV. The similarity of the curve in Figure 2B to the charge movement versus membrane potential relation for the L-type Ca^{2+} channels (Hadley and Lederer, 1991) suggested that these channels, highly expressed in vascular smooth muscle, might be the voltage sensors coupling the changes of myocyte

Fig. 1. Membrane depolarization induces Ca^{2+} release and contraction in myocytes bathed in Ca^{2+}-free solutions. (**A**) Left: membrane-depolarization-induced sharp Ca^{2+}-release signal in a voltage-clamped basilar myocyte bathed in Ca^{2+}-free external solution (0Ca plus 2 mM EGTA added). Addition of 70 mM external K^+ (70K) failed to produce membrane depolarization and Ca^{2+} release. Right: Ca^{2+} release induced by depolarization with external K^+ (70K) in the same myocyte subjected to current clamp. (**B**) Time course of contraction (top) and cytosolic [Ca^{2+}] (bottom) in a myocyte loaded with Fluo3-AM and bathed in a Ca^{2+}-free external solution for 5 min. At time zero, the cell was depolarized by application of 70 mM external K^+ (Ca^{2+}-free) for 140 s. The color scale (arbitrary units) is shown in the lower left corner. Calibration bar, 10 μm. Average reduction of cell length measured in the larger axis was 16 ± 6% (*n* = 4 cells).

**Fig. 2.** Graded voltage dependence of Ca^{2+} release induced by membrane depolarization. (**A**) Progressive increase in the Ca^{2+} release signal (expressed as change in the fluorescence ratio) in a voltage-clamped myocyte in parallel with the amplitude of depolarizing steps. (**B**) Average changes of fluorescence ratio versus membrane potential relationship in basilar myocytes. Data points represent the mean ± SE from *n* = 13 cells. The values were normalized to the amplitude at +40 mV. The curve is a least-squares fit to a Boltzmann function. $V_{1/2}$ = –15 mV and k = 20 mV. (**C**) Depolarization-induced Ca^{2+} release is abolished when the membrane depolarization is changed slowly. Representative example of qualitatively similar experiments performed on six cells.
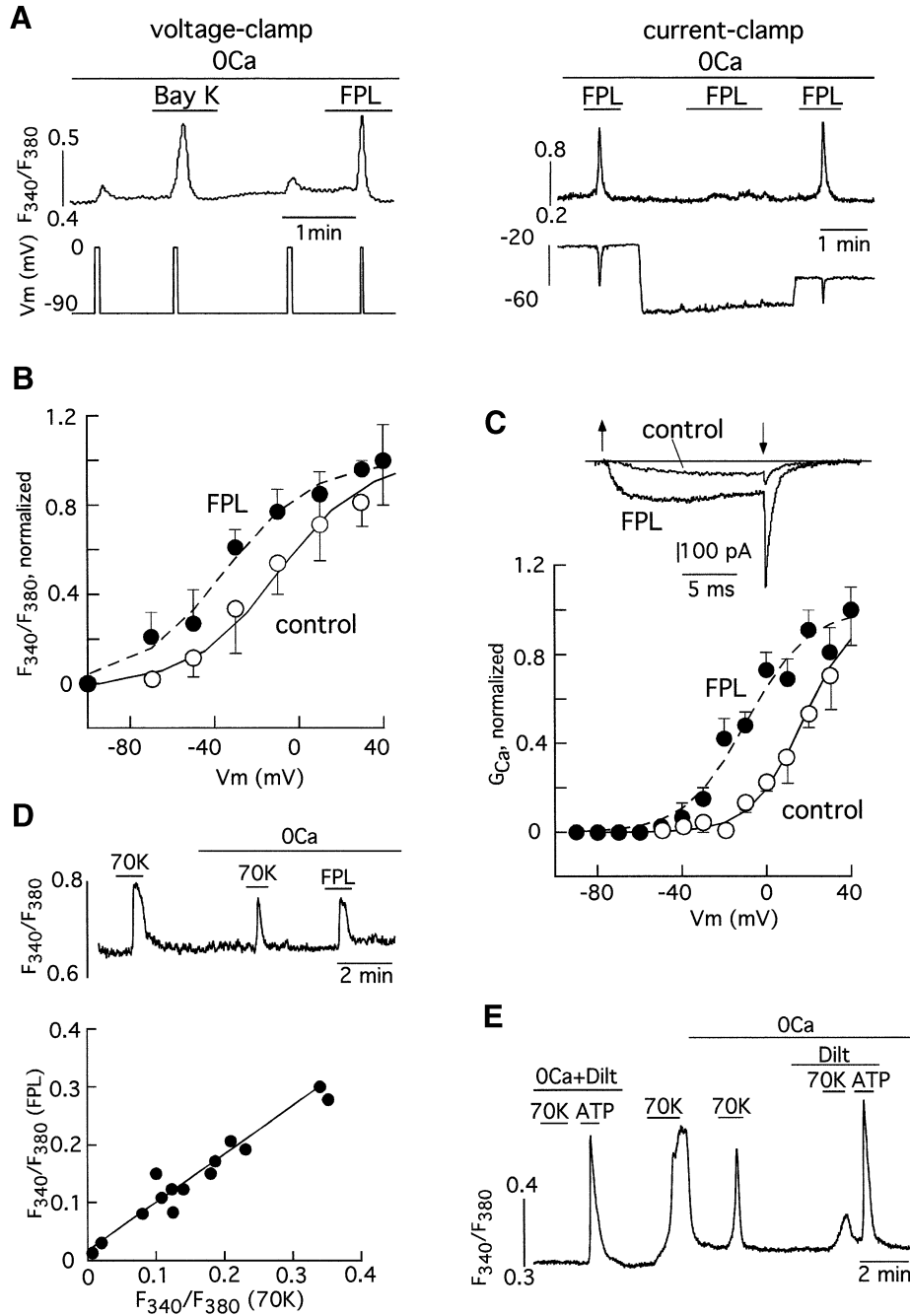
Fig. 3. Calcium-channel-induced Ca^{2+} release and effects of Ca^{2+}-channel agonists and antagonists. (**A**) Left: Ca^{2+} release evoked by the application of membrane-depolarizing pulses in a voltage-clamped myocyte bathed in the Ca^{2+}-free solution and potentiation of this effect by addition of the Ca^{2+}-channel agonists Bay K (200 nM) and FPL (200 nM). Right: Ca^{2+} release evoked by the application of FPL (1 µM) in a current-clamped myocyte is prevented by membrane hyperpolarization. Fast hyperpolarizations occurring in parallel with the Ca^{2+} transients are due to activation of Ca^{2+}-activated K^+ channels. In the first current injection (75 pA) the membrane potential was changed from –20 to –90 mV, thus yielding a value of input resistance of 0.93 GΩ. (**B**) Average Ca^{2+} release (indicated as fluorescence ratio) versus membrane potential relationship in basilar myocytes before (open symbols; see Figure 2B) and after (filled symbols; mean ± SE from $n = 11$ cells) exposure to FPL (30 nM). Curves are least-squares fits to Boltzmann functions (see Figure 2). For curve in FPL, $V_{1/2} = -35.2$ mV and $k = 20.9$ mV. Note that FPL shifted $V_{1/2}$ by –20.2 mV. Fluorescence values obtained at –30 and –10 mV in the two sets of data were statistically different ($p = 0.03$ and $p = 0.018$ respectively). (**C**) Normalized calcium conductance versus membrane potential relationship obtained in the barium solution before (open symbols) and after (filled symbols) addition of 200 nM FPL ($n = 5$ cells). Curves are least-squares fits to Boltzmann functions. For curve control, $V_{1/2} = 17.2$ mV and $k = 12.1$ mV, and for curve FPL $V_{1/2} = -9.1$ mV and $k = 14.6$ mV. Note that FPL shifted $V_{1/2}$ by –26.3 mV. The inset shows superimposed examples of calcium currents generated by depolarization (between the arrows) to +20 mV from –80 mV in the two experimental conditions. (**D**) Top: Ca^{2+} release induced by FPL (1 µM) and by depolarization with 70 mM K^+ in a representative cell. Bottom: relationship between the amplitude of the Ca^{2+} signals evoked by FPL (ordinate) and by 70 mM K^+ (abscissa) in 14 cells. Linear regression fit ($r = 0.95$). (**E**) Blockade of Ca^{2+}-release induced by membrane depolarization in the presence of diltiazem (10 µM). Note that Ca^{2+} release induced by 100 µM ATP (0.26 ± 0.09 increase in fluorescence ratio, $n = 12$) is not significantly affected by the presence of 10–50 µM diltiazem (0.22 ± 0.025 increase in fluorescence ratio, $n = 12$, $p > 0.05$).
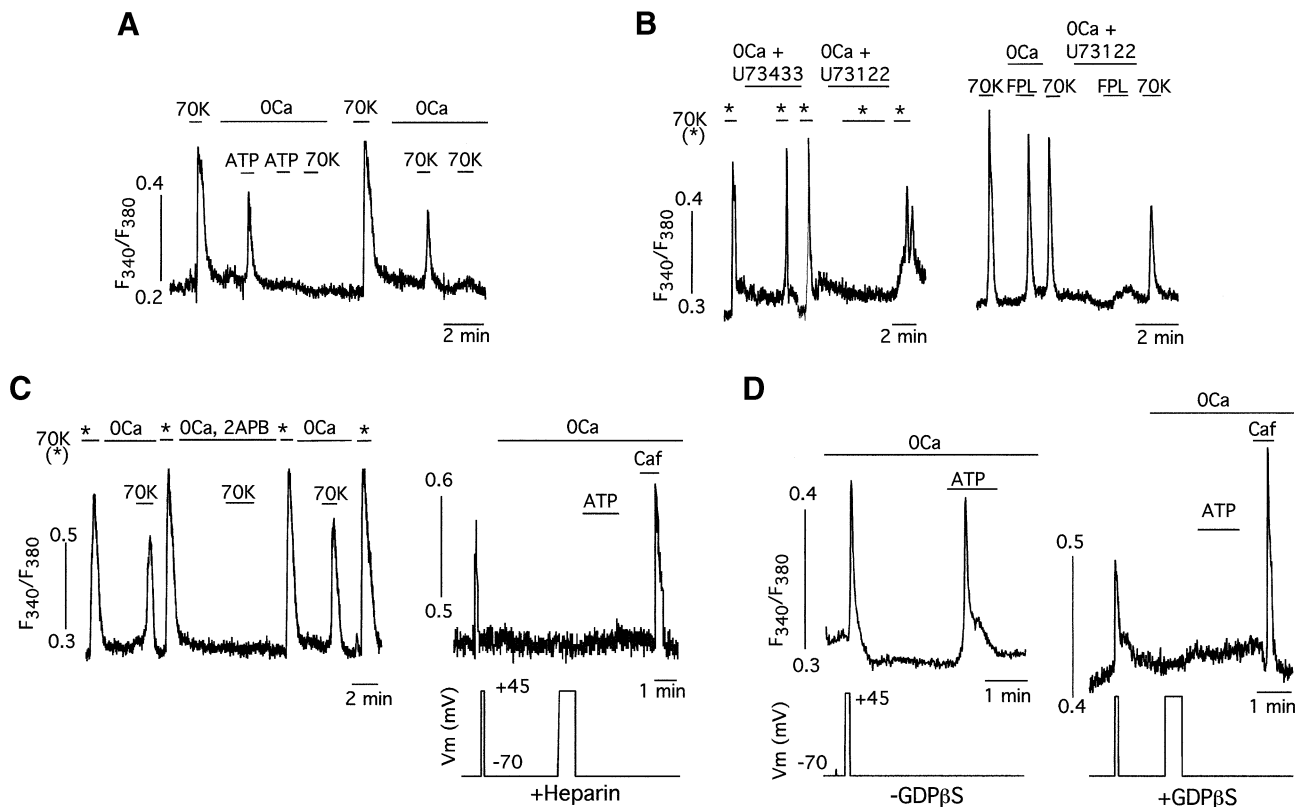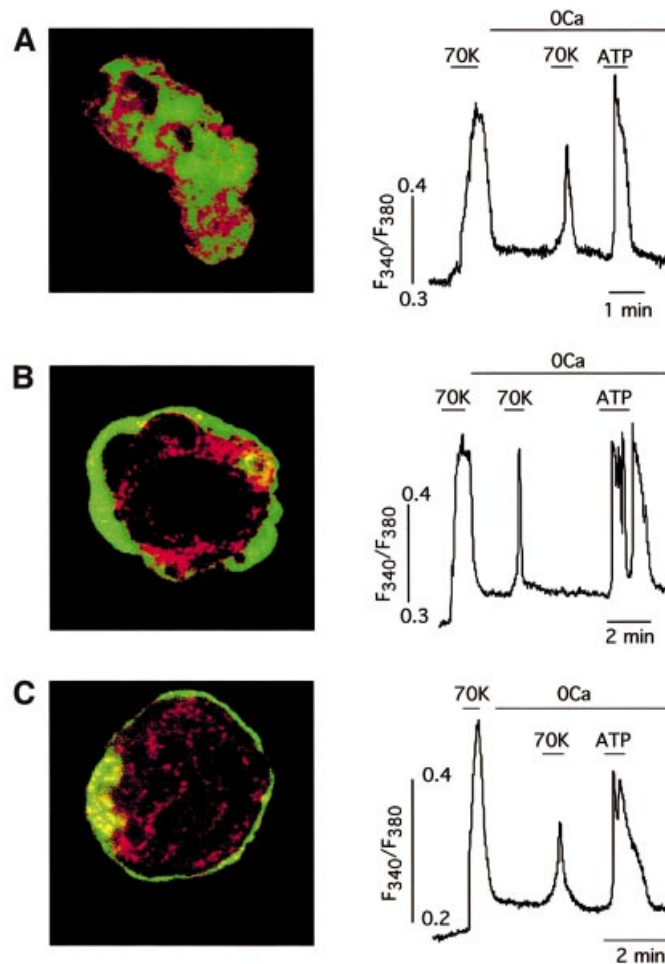
Fig. 4. Calcium-channel-induced Ca^{2+} release from sarcoplasmic reticulum is mediated by G-protein-dependent $InsP_3$ production. (**A**) Abolition of CCICR observed with 70 mM K^+ after repeated exposure of a myocyte to ATP (100 μM) in Ca^{2+}-free external solutions. (**B**) Suppression of CCICR evoked with either 70 mM K^+ (left) or FPL (1 μM, right) by application of an inhibitor of phospholipase C (U73122, 1 μM). Note that the analog U73433 (1 μM) has no effect. (**C**) Left: abolition of CCICR evoked with 70 mM K^+ by application of a blocker of $InsP_3$ receptors (2APB, 50 μM). Right: suppression of depolarization- and ATP (100 μM)-induced Ca^{2+} release in a representative patch–clamped myocyte loaded with heparin (200 μg/ml) to inhibit $InsP_3$ receptor activation. Note that the response to caffeine (Caf, 5 mM), a Ry receptor agonist, was unaltered. (**D**) Left: CCICR elicited in voltage-clamped cells dialyzed with the normal internal solution (–GDPβS). A similar Ca^{2+} release signal is induced by membrane depolarization or the application of ATP (100 μM). Right: abolition of depolarization- or ATP-induced Ca^{2+} release in cells dialyzed with GDPβS (1 mM, added to the patch pipette solution). Note that before switching to the Ca^{2+}-free solution (0Ca) a brief depolarizing pulse produced a Ca^{2+} signal due to influx through membrane channels and that G protein blockade did not prevent Ca^{2+} release induced by caffeine (Caf, 5 mM). GDPβS abolished CCICR in 100% of the cells tested ($n = 25$). In the same series of experiments, ~90% of the patch–clamped cells dialyzed without GDPβS ($n = 33$) responded with Ca^{2+} release to membrane depolarization.

membrane potential to Ca^{2+} release from the SR. In fair agreement with this hypothesis, depolarization-induced Ca^{2+} release elicited by fast depolarizing pulses was markedly reduced (by $82.2 \pm 8.1\%$, $n = 6$) when the same level of depolarization was obtained by slowly changing the membrane potential (Figure 2C). This observation suggested that, similar to what occurs in voltage-gated Ca^{2+} channels, the slow ramp-like depolarization resulted in inactivation of the voltage sensor.

The resemblance between the voltage sensor that mediates depolarization-induced Ca^{2+} release and the voltage-dependent Ca^{2+} channels was further evidenced by pharmacological experiments. Depolarization-induced Ca^{2+} release was potentiated by the selective Ca^{2+}-channel agonists Bay K-8644 and, with higher potency, FPL-64176, a benzoylpyrrole derivative that alters Ca^{2+}-channel gating by binding to a site other than the dihydropyridine receptor (Figure 3A, left) (Bean *et al.*, 1986; Zheng *et al.*, 1991). Figure 3A (right) illustrates that Ca^{2+} release induced by FPL was prevented by membrane hyperpolarization in a current-clamped myocyte. FPL-induced Ca^{2+} release was abolished in cells maintained at

membrane voltages more negative than –70 mV ($n = 6$ cells tested), thus indicating that the effect of the drug was to reduce the membrane potential threshold required for depolarization-induced Ca^{2+} release. In fact, addition of FPL produced a displacement of ~20 mV to negative voltages of the curve relating Ca^{2+} release amplitude and membrane potential (Figure 3B) and a qualitatively similar shift in the Ca^{2+} conductance versus voltage relation (Figure 3C). The differences observed in the half activation voltage for depolarization-induced Ca^{2+} release (open symbols in Figure 3B) and calcium currents (open symbols in Figure 3C) can be explained, at least in part, by the different concentration of external divalent cations used for these experiments (respectively, 0 mM Ca^{2+} versus 10 mM Ba^{2+}). In addition, it is expected that the intramembranous charge movement triggering depolarization-induced Ca^{2+} release could occur at voltages more negative than those required for the actual opening of the calcium channels. In addition to the similarity of the effects of FPL on depolarization-induced Ca^{2+} release and Ca^{2+}-channel gating, the magnitude of the Ca^{2+}-release signals evoked by high K^+ or FPL varied in parallel in

Fig. 5. Cytoskeletal reorganization does not alter calcium-channel-induced Ca^{2+} release in arterial myocytes. (**A**) Example of depolarization (70 mM K$^+$) and ATP (100 μM) induced Ca^{2+} release in a myocyte and confocal fluorescent staining of the cell with markers of the SR (antibodies against Ca ATPase, red) and cytoskeletal microfilaments (antibody against actin, green). (**B**), (**C**) Similar experiments as in (A) performed in cells subjected to prior incubation with calyculin A [(B) 100 nM, 30 min, $n = 8$ experiments] or jasplakinolide [(C) 1 μM, 120 min, $n = 7$ experiments]. Note in (B) and (C) the actin ring separating the plasma membrane and the cytosolic organella.

different cells (Figure 3D), thus suggesting that both stimuli (membrane depolarization and pharmacological activation of Ca^{2+} channels) act through a common mechanism. Finally, depolarization-induced Ca^{2+} release was almost completely abolished by diltiazem (10–50 μM; $87.8 \pm 1.7\%$ inhibition, $n = 13$ cells tested), a widely used L-type Ca^{2+}-channel antagonist in vascular smooth muscle (Kuga *et al*., 1990; Kraus *et al*., 1998), although this drug did not alter ATP-induced Ca^{2+} release (see Figure 3E). Other Ca^{2+}-channel antagonists as well as diltiazem reduced depolarization-induced Ca^{2+} release with variable potency. D600 (10–50 μM) was as effective as diltiazem and blocked depolarization-induced Ca^{2+} release by $96.5 \pm 1.5\%$ ($n = 10$). Interestingly, nifedipine (5 μM) significantly reduced depolarization-induced Ca^{2+} release amplitude in only seven of 17 cells tested and had a relatively low potency to block the macroscopic calcium current ($52.7 \pm 7\%$ reduction of peak current amplitude at +20 mV, $n = 15$). However, nifedipine, similarly to diltiazem and D600, completely abolished FPL-induced Ca^{2+} release in all the cells tested ($n = 4$). These findings demonstrate that in the complete absence of transmembrane Ca^{2+} influx, activation of L-type Ca^{2+} channels of

basilar VSMCs by either membrane depolarization or pharmacological agonists triggers a fast Ca^{2+}-release signal from intracellular stores of sufficient amplitude to produce contraction of the cells. Therefore we will refer to this phenomenon as calcium-channel-induced calcium release (CCICR) in the rest of this article.

Calcium channel-induced Ca^{2+} release requires G-protein-mediated activation of the phospholipase C–inositol trisphosphate pathway

To identify the transduction pathway and the internal stores involved in CCICR we performed the series of experiments summarized in Figure 4. Pretreatment of the cells with ATP, which is known to elicit InsP$_3$-dependent Ca^{2+} release in VSMCs of the basilar artery (Sima *et al*., 1997), depleted the intracellular stores and prevented any further CCICR upon exposure to high K$^+$ (Figure 4A). These observations suggested that both ATP-induced Ca^{2+} release and CCICR operate through the same Ca^{2+} stores. Ca^{2+} release induced by either high K$^+$ or FPL was fully prevented by incubation of the cells with U73122, an inhibitor of phospholipase C (Smith *et al*., 1990), whereas U73433, a related drug that does not affect the enzyme,

was ineffective (Figure 4B; $n = 9$ cells tested). CCICR was also reversibly abolished by 2APB, a blocker of the InsP$_3$-sensitive Ca^{2+}-release channels (Figure 4C left; $n = 10$ cells tested) (Maruyama *et al.*, 1997). In addition, CCICR or ATP-evoked Ca^{2+} release were totally abolished in patch–clamped cells dialyzed with heparin, an inhibitor of the InsP$_3$ receptors (Ehrlich and Watras, 1988) (Figure 4C, right; $n = 7$ cells tested). Together, these findings indicated that CCICR resulted from phospholipase C activation and InsP$_3$ production, which in turn elicited Ca^{2+} release from SR. Activation of phospholipase C by membrane depolarization involved G-protein function, as cell dialysis with 1 mM GDPβS prevented depolarization-induced or ATP-dependent Ca^{2+} release in all cells studied ($n = 25$). Both voltage-dependent extracellular Ca^{2+} influx and Ry receptor stimulation by caffeine were unaltered by dialysis with GDPβS (Figure 4D).

In full agreement with the data described above, CCICR elicited by step depolarizations to +20 mV or higher in voltage-clamped myocytes had a relatively long latency (6.8 ± 1.2 s measured to the onset of the foot preceding the fast rise of [Ca^{2+}], $n = 36$ cells), thus further suggesting that, after the change of membrane potential and calcium-channel activation, several biochemical events obligatorily precede Ca^{2+} release and therefore that Ca^{2+} channels are not directly coupled to InsP$_3$ or Ry receptors. This idea was strongly supported by experiments performed in cells treated with calyculin A and jasplakinolide, drugs that induce cytoskeletal reorganization and the formation of an actin ring separating the plasma membrane from the cytosolic organella (Hosoya *et al.*, 1993; Patterson *et al.*, 1999). Normally, VSMC actin filaments (Figure 5A, green) are homogeneously distributed and overlap the distribution of the SR (Figure 5A, red), which can appear near the plasma membrane. However, after incubation with calyculin A (Figure 5B, left) or jasplakinolide (Figure 5C, left), the SR seemed to be excluded from the vicinity of the membrane by a dense actin barrier. This cytoskeletal rearrangement maintained, however, either the CCICR induced by 70 mM external K$^+$ or the ATP-dependent Ca^{2+} release unaltered (Figure 5B and C, right; see also Patterson *et al.*, 1999). The amplitude of CCICR induced by 70 mM K$^+$ in cells treated with calyculin A (0.11 ± 0.02 increase in fluorescence ratio, $n = 11$) or jasplakinolide (0.11 ± 0.01 increase in fluorescence ratio, $n = 5$) was not significantly different from that measured in control cells recorded in the same set of experiments (0.13 ± 0.02 increase in fluorescence ratio, $n = 5$).

Ryanodine receptor activation is needed to evoke fast calcium-channel-induced Ca^{2+} release

Ca^{2+} release from the SR in VSMCs is mediated by two separate sets of channels: InsP$_3$ receptors, activated by ATP and other agonists, and Ry receptors, which can be pharmacologically activated by application of caffeine (Ehrlich and Watras, 1988; Blatter and Weir, 1992; Berridge, 1993; Pozzan *et al.*, 1994). Repeated exposure of basilar arterial myocytes to caffeine resulted in depletion of the ATP-sensitive store (Figure 6A), thus suggesting that, as described for other cell types (Blatter and Weir, 1992; Khodakhah and Armstrong, 1997; Boittin *et al.*, 1999), InsP$_3$ receptors in basilar myocytes use the
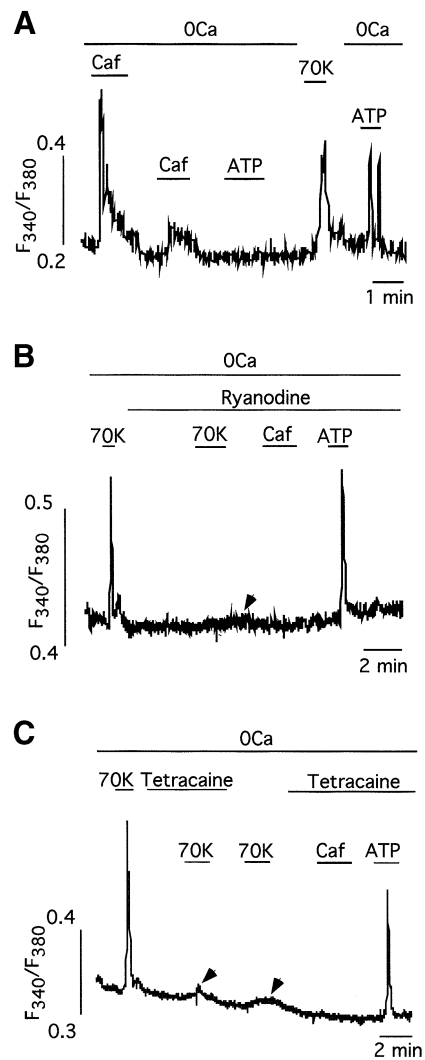
Fig. 6. Calcium-channel-induced Ca^{2+} release requires the activation of ryanodine receptors. (**A**) Depletion of the ATP-sensitive Ca^{2+} store by repeated application of caffeine (Caf, 5 mM). The response to ATP (100 μM) was restored after refilling the intracellular stores ($n = 7$ cells). (**B**) Application of ryanodine (20 μM) to the external solution blocks caffeine-induced Ca^{2+} release but maintains unaltered the Ca^{2+} release signal evoked by ATP (100 μM). Note that in cells treated with ryanodine, Ca^{2+} release induced by membrane depolarization (70K) is also strongly inhibited. The remaining slow signal (arrow) possibly represent the initiatory CCICR signal due to InsP$_3$-dependent CCICR. (**C**) Abolishment of depolarization (70K)- and caffeine (5 mM) induced Ca^{2+} release by application of tetracaine (100 μM), a blocker of ryanodine receptors, to the external solution. In these experiments the Ca^{2+} release signal evoked by ATP (100 μM) was maintained. As in the middle panel, the arrows indicate slow CCICR signals resistant to tetracaine block.

same functional Ca^{2+} pool as that utilized by Ry receptors. Using the appropriate concentration of externally applied Ry (20 μM in our dispersed myocyte preparation) to produce selective blockade of Ry receptors (Sutko *et al.*, 1997), we could abolish the caffeine-induced Ca^{2+} release although the InsP$_3$ receptor-dependent Ca^{2+}-release signal induced by ATP was left intact (Figure 6B). Interestingly, in these conditions CCICR evoked by depolarization with 70 mM K$^+$ was also almost completely blocked ($97.5 \pm 2.5\%$ reduction of CCICR amplitude, $n = 4$ cells tested; Figure 6B), thus indicating that, besides InsP$_3$-receptor

activation, the generation of the CCICR signal also requires Ca^{2+} release through Ry receptors. This fact was confirmed by experiments performed with tetracaine, a membrane-permeant blocker of Ry receptors in smooth muscle (McCarron et al., 2002). In all cells tested (n = 10) tetracaine selectively suppressed the caffeine-induced Ca^{2+} release and inhibited by $86 \pm 4.7\%$ the fast CCICR signal evoked by depolarization with 70 mM K^+, although the ATP-dependent Ca^{2+} signal was maintained (Figure 6C). In the presence of ryanodine or tetracaine a small slow CCICR signal was observed in numerous cells (arrows in Figure 6B and C). This signal, as well as the foot preceding CCICR in voltage-clamp cells, probably represents the initiatory $InsP_3$-evoked calcium release due to depolarization when the Ry receptors are inhibited. These results strongly suggest that, although CCICR primarily requires activation of $InsP_3$ receptors, stimulation of Ry receptors is necessary for the generation of the fast CCICR signal observed in arterial myocytes. It is most likely that, in basilar myocytes, Ca^{2+} released through $InsP_3$ receptor channels activates Ry receptors to induce further Ca^{2+} release, as described for other VSMCs (Blatter and Weir, 1992; Boittin et al., 1999). Therefore the two Ca^{2+}-release mechanisms interact to evoke full CCICR and myocyte contraction.

Discussion

The results reported here describe a new mechanism of signaling $InsP_3$ and Ry receptors in vascular smooth muscle, called CCICR, which can produce myocyte contraction. CCICR is initiated by the activation of voltage-gated Ca^{2+} channels which, after sensing membrane depolarization, trigger fast Ca^{2+}-release from the SR in the absence of extracellular Ca^{2+} influx. CCICR can be triggered by L-type Ca^{2+}-channel agonists and blocked by antagonists. CCICR requires stimulation of the G protein–phospholipase C pathway and activation of $InsP_3$ receptors; however, a full CCICR is only seen if Ca^{2+} is also released through Ry receptors. Sequential activation of $InsP_3$ and Ry receptors is probably achieved by spatial overlapping of both receptor types so that, as described before in VSMCs (Blatter and Weir, 1992; Boittin et al., 1999), Ca^{2+} released through $InsP_3$-receptor channels activates Ry receptors to induce further Ca^{2+} release. This form of coupling VSMC electrical excitation to contraction is reminiscent of the classical mechanism existing in skeletal muscle where, after sensing membrane depolarization, dihydropyridine receptors directly activate Ry receptors of the SR to induce fast Ca^{2+} release and contraction (for review see Rios and Pizarro, 1991). In vascular myocytes, however, the coupling between membrane-voltage-gated Ca^{2+} channels and Ry receptors of the SR described here is not direct but involves G-protein activation and $InsP_3$ production. It has recently been shown in a study performed on skeletal muscle cells that dihydropyridine receptors also mediate $InsP_3$-dependent slow Ca^{2+} waves, occurring mainly in the nucleus, that regulate early gene expression (Jaimovich et al., 2000; Araya et al., 2003).

As well as in rat basilar myocytes (the preparation used in all the experiments described in this article), we have also observed CCICR in myocytes dispersed from mouse basilar artery and aorta as well as from pig coronary arteries. Hence it appears that CCICR is a general phenomenon, broadly represented among the various types of VSMCs. It has previously been reported in visceral smooth muscle (Suzuki and Hirst, 1999; van Helden et al., 2000) and vascular smooth muscle (Itoh et al., 1992; Ganitkevich and Isenberg, 1993) as well as in other cell types (Vergara et al., 1985; Kukuljan et al., 1994; Jaimovich et al., 2000; Mason and Mahaut-Smith, 2001) that membrane depolarization can increase $InsP_3$ production and modulate agonist-induced Ca^{2+} release. In VSMCs of the rabbit mesentery artery it has been shown that hyperpolarization induced by pinacidil (an activator of K_{ATP} channels) reduces phosphatidylinositol hydrolysis and subsequent $InsP_3$ production induced by norephinephrine. This effect of hyperpolarization is inhibited by high extracellular K^+ (Itoh et al., 1992). Similarly, it has been reported in coronary myocytes that acetylcholine-induced $InsP_3$-production or activation of phospholipase C by intracellular dialysis with GTP-γS are both potentiated by membrane depolarization (Ganitkevich and Isenberg, 1993). Therefore, the existence of a voltage-sensitive step in the $InsP_3$ signaling pathway downstream from the surface membrane receptors (either at the level of G protein or phospholipase C) had been proposed (Itoh et al., 1992; Ganitkevich and Isenberg, 1993; Bolton et al., 1999; Suzuki and Hirst, 1999; Mason and Mahaut-Smith, 2001). Our results demonstrate that in arterial myocytes CCICR occurs without the presence of any stimulatory agonist or cell dialysis with GTP-γS, and they strongly suggest that CCICR depends on the sensing of membrane depolarization by Ca^{2+} channels and subsequent activation of G proteins. In the absence of membrane depolarization, CCICR can be triggered by Ca^{2+}-channel agonists, thus indicating that sensitivity to membrane potential of the phosphatidylinositol cascade is not the primary cause of CCICR. Therefore, in cells having CCICR, voltage- and receptor-mediated pathways can independently increase $InsP_3$ synthesis and Ca^{2+} release. It was already known that Ca^{2+} channels can be directly regulated by G proteins (Dolphin, 1998); however, the opposite phenomenon, regulation of G protein downstream of Ca^{2+}-channel activation was unexpected. The mechanism whereby Ca^{2+} channels interact with G proteins appears to depend on the change of conformation of the voltage sensor and adjacent regions induced by membrane depolarization. These Ca^{2+}-channel regions could possibly activate G protein by direct allosteric interactions, as it is known that membrane receptor, G proteins and L-type Ca^{2+} channels are in close proximity within membrane microdomains (Davare et al., 2001). Interestingly, suppression of Ca^{2+} influx by open-channel blockers like Cd^{2+} or Zn^{2+} does not prevent CCICR; however, this phenomenon is inhibited by Ca^{2+}-channel gating modifiers such as diltiazem, D600 or nifedipine. The weak stimulatory effect of Bay K as compared with FPL to induce CCICR, as well as the differential potency of the Ca^{2+}-channel antagonists to inhibit CCICR (diltiazem and D600 being more efficacious than nifedipine) is probably due to the fact that they interact with different regions of the L-type Ca^{2+}-channel molecule. Ca^{2+}-channel blockers of the benzothiazepine (diltiazem) and phenylalkylamine (D600) families share common molecular determinants

with Ca^{2+}-channel inactivation, thus explaining their potent inhibitory effect on CCICR (Hering *et al.*, 1997, 1998; Bournaud *et al.*, 1998; Kraus *et al.*, 1998).

Finally, we have shown that CCICR is functionally relevant as rat basilar myocytes can be contracted in the absence of extracellular Ca^{2+}. Therefore, in myocytes expressing CCICR, contraction due to membrane depolarization and extracellular Ca^{2+} influx is potentiated by Ca^{2+} release from the SR. These findings indicate that voltage-gated Ca^{2+} channels regulate vascular tone, not only permitting extracellular Ca^{2+} influx but also through modulation of Ca^{2+} release from the SR. They also explain previous reports (Somlyo and Somlyo, 1994; Kalsner, 1997; A.del Valle-Rodríguez, J.López-Barneo and J.Ureña, unpublished data) that some vascular territories contract without the need of external Ca^{2+}. Abnormal activation of CCICR might participate in the production of vasospasms or transient increases in vascular resistance observed in pathological conditions (Sima *et al.*, 1997). Since some Ca^{2+}-channel gating modifiers, such as diltiazem and D600, modulate CCICR, the search of Ca^{2+}-channel antagonists with a selective effect on CCICR should have therapeutic relevance.

Materials and methods

Cell preparation and treatments
Wistar rats (250–300 g) were deeply anesthetized and decapitated. Basilar arteries were dissected from the brain, cut into pieces and incubated at 4°C for 12–14 h in Hanks' solution containing 1.3 mg/ml papain, 1.2 mg/ml collagenase, 0.2 mg/ml elastase and 0.75 mg/ml bovine serum albumin (BSA). After this time period cells were mechanically dispersed using a fire-polished Pasteur pipette and plated on pieces of coverslip. Cells were ready to use for the experiments within the next 2–3 h. For some experiments cells were incubated with either calyculin A (100 nM for 30 min) or jasplakinolide (1 µM for 2 h) before recording. These drugs were added to Hanks' solution with 0.2 mg/ml BSA maintained at 37°C.

Cytosolic Ca^{2+} measurements and electrophysiological recordings
Cytosolic [Ca^{2+}] was estimated in intact and dialyzed cells. In the first case, myocytes were incubated in Hanks' solution with 1 µM Fura2-AM added for 15 min at room temperature. In dialyzed cells Fura2 potassium salt (50 µM) was added to the patch pipette solution. For the experiments a coverslip with cells was placed on a recording chamber (~0.2 ml) mounted on the stage of an inverted microscope (Axiovert 35, Zeiss) equipped with epifluorescence and photometry. For fluorescence excitation, we used a monochromator (Polychrome IV, T.I.L.L., Photonics) and the light was deflected by a dichroic mirror (BSP 430 nm) into the microscope objective (Plan-Neofluar 40×; NA 0.75, Zeiss). Fluorescence from the myocytes was filtered twice, first by a Zeiss LP470 filter and then by a short-pass filter (540 SP, Zeiss), and detected by a CCD camera (C4880–80, Hamamatsu). The software used was AquaCosmos 2.0 (Hamamatsu). Alternating excitation wavelengths of 340 and 380 nm were used and background fluorescence was subtracted before obtaining the F$_{340}$/F$_{380}$ ratio. In the text and figures, variations of cytosolic [Ca^{2+}] are given as changes of fluorescence ratio. Voltage- and current-clamp recordings were obtained using the whole-cell configuration of the patch–clamp technique. Details of our experimental setup have been given previously (Franco-Obregón *et al.*, 1995). We used an EPC-7 patch–clamp amplifier (List Electronics) and electrodes with resistance between 2 and 5 MΩ. When cytosolic [Ca^{2+}] and membrane-holding potential or current were recorded simultaneously, the signals were digitized at a sampling interval of 500 ms and filtered at 3 kHz. All the experiments were performed at room temperature (~22°C).

Confocal microscopy and immunocytochemistry
To study myocyte contraction in parallel with cytosolic [Ca^{2+}], we used a confocal microscope (Leica, TCS SP2). Cells were loaded with 1 µM Fluo3-AM following the same protocol as indicated above for Fura2-AM.

Excitation was done with an argon–krypton laser (488 nm) and the emitted fluorescence (500–600 nm) was recorded through an oil immersion objective (40 × 1.25). An image (512 × 512 pixels) was acquired every 2 s. Immunocytochemical studies were done on fixed cells following standard protocols. Briefly, isolated cells were placed on coverslips and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) (137 mM NaCl, 2.68 mM KCl, 4.02 mM Na$_2$HPO$_4$, 1.76 mM KH$_2$PO$_4$) for 20 min. Subsequently, cells were rinsed in PBS and incubated for 60 min in non-specific binding solution [PBS, 3% fetal bovine serum (FBS), 0.1% Triton X-100]. We used anti-SERCA-2 antibody (ABR-MA3-910, Alexis) and donkey antimouse Texas red (715-075-150, Jackson) secondary antibody to stain the SR. Muscle actin was stained with a monoclonal antibody fluorescein isothiocyanate (FITC) conjugate (F3777, Sigma Aldrich). Cells were incubated overnight at 4°C in mouse anti-SERCA-2 diluted 1:1000 in PBS, 3% FBS and 0.1% Triton X-100. Cells were rinsed in PBS and incubated for 60 min in donkey antimouse Texas red diluted 1:500. Coverslips were rinsed in PBS and incubated overnight at 4°C in mouse anti-α-actin FITC diluted 1:1000. Finally coverslips were rinsed in PBS and mounted on slides with DPX. Fluorescence was observed with the confocal microscope.

Solutions
The composition of Hanks' solution was 125 mM NaCl, 5.36 mM KCl, 5 mM NaHCO$_3$, 0.34 mM Na$_2$HPO$_4$, 0.44 mM KH$_2$PO$_4$, 10 mM glucose, 1.45 mM sucrose and 10 mM HEPES pH 7.4. During the experiments the recording chamber was continuously superfused with a control external solution containing 140 mM NaCl, 2.7 mM KCl, 2.5 mM CaCl$_2$ 1 mM MgCl$_2$, 10 mM HEPES pH 7.4. Unless otherwise noted, all the drugs used were added to this solution at the indicated concentration. The 70K solution was obtained by replacing 70 mM of NaCl with KCl. The 0Ca solution was obtained by substituting Mg^{2+} for Ca^{2+} and adding 2 mM EGTA. Patch–clamp experiments were done by dialyzing the cells with an internal solution containing 83 mM K glutamate, 47 mM KCl, 1 mM MgCl$_2$, 10 mM HEPES 4 mM ATP-Mg, 50 µM Fura2-potassium salt pH 7.2. To record whole-cell calcium currents the solutions used were as follows: external (140 mM NaCl, 2.7 mM KCl, 10 mM BaCl$_2$, 10 mM HEPES, pH 7.4); internal (100 mM CsCl, 30 mM CsF, 2 mM MgCl$_2$, 4 mM ATP-Mg, 10 mM EGTA, 10 mM HEPES pH 7.2).

Data analysis
Data are expressed as mean ± SE and the statistical significance was estimated using Student's *t* test. Values of $p < 0.05$ were considered significant. Normalized calcium conductance was estimated by measuring the amplitude of the repolarizing tail current pulses. The data points relating calcium conductance or fluorescence ratio to membrane potential were least squares fitted to a Boltzmann function of the form

$$Y = 1/[1 + \exp(V_{1/2} - V_m)/k]$$

where V_m is the membrane potential during each pulse, $V_{1/2}$ is the potential at which 50% of maximal amplitude is obtained and k is a slope factor that indicates the steepness of the curve.

Acknowledgements

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