The Foot Structure from the Type 1 Ryanodine Receptor Is Required for Functional Coupling to Store-operated Channels*

Received for publication, February 8, 2005, and in revised form, April 22, 2005
Published, JBC Papers in Press, May 4, 2005, DOI 10.1074/jbc.M501487200

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In the present study we have explored structural determinants of the functional interaction between skeletal muscle ryanodine receptor (RyR1) and transient receptor potential channel 1 (TRPC1) channels expressed in Chinese hamster ovary cells. We have illustrated a functional interaction between TRPC1 channels and RyR1 for the regulation of store-operated calcium entry (SOCE) initiated after releasing calcium from a caffeine-sensitive intracellular calcium pool. RNA interference experiments directed to reduce the amount of TRPC1 protein indicate that RyR1 associates to at least two different types of store-operated channels (SOCs), one dependent and one independent of TRPC1. In contrast, bradykinin-induced SOCE is completely dependent on the presence of TRPC1 protein, as we have previously illustrated. Removing the foot structure from RyR1 results in normal caffeine-induced release of calcium from internal stores but abolishes the activation of SOCE, indicating that this structure is require for functional coupling to SOCs. The footless RyR1 protein shows a different cellular localization when compared with wild type RyR1. The later protein shows a higher percentage of colocalization with FM-464, a marker of plasma membrane. The implications of the foot structure for the functional and physical coupling to TRPC and SOCs is discussed.

Depletion of the endoplasmic reticulum (ER) stimulates calcium influx from the extracellular space, a phenomenon known as store-operated entry (SOCE) (1, 2). Although SOCE appears to be ubiquitous in non-excitable cells, recent experimental evidence indicates that SOCE is also present in skeletal muscle (3), smooth muscle (4), and neurons (5, 6). Thus, it appears that store-operated calcium entry is a mechanism present in excitable and non-excitable cells, although it has been more extensively explored in the latter (5).

Because our original finding demonstrating that the trp gene from Drosophila melanogaster encoded a calcium-permeable cationic channel activated upon depletion of the ER (7), many members of the TRP superfamily have been identified in a wide variety of organisms, from yeast to humans (for review see Refs. 8–10). The plethora of mechanisms modulating TRP channel function has made the study of the physiological role of many of these channels difficult (8, 11). However, the activity of some of the members from the TRPC subfamily appears to be linked to the depletion of the ER via interactions with inositol triphosphate (IP3R) receptors (12–16). All members of the TRPC subfamily possess sequences at their carboxyl-terminal domain that bind selectively IP3R (17). Moreover, we have shown recently functional interactions between peptides corresponding to the amino-terminal domain of IP3R and TRPC1 channels (18).

The conformational coupling hypothesis was proposed to explain the communication between the TRPC channel at the plasma membrane and the IP3R at the ER, a system homologous to the coupling between the dihydropyridine receptor and the ryanodine receptor (RyR) in skeletal muscle (19). In skeletal muscle cells, the RyR is an essential player in the excitation-contraction (E-C) coupling mechanism. The RyR associates to the dihydropyridine receptor localized at the plasma membrane via protein-protein interactions (19). This association is responsible for coupling the depolarization at the plasma membrane with the activation of the type 1 ryanodine receptor (RyR1) and the concomitant release of calcium from the sarcoplasmic reticulum.

Interestingly, it has been recently shown that activation of RyR opens human TRPC3 channels (hTRPC3) in HEK293 cells (20), suggesting a functional coupling between RyR and hTRPC3 similar to what has been previously reported for TRPC3 and IP3R (21). Furthermore, depletion of the ER via activation of RyR in cells lacking IP3R initiates SOCE, illustrating functional coupling between RyR and store-operated channels (SOCs) in the absence of IP3R (22).

Depletion of the sarcoplasmic reticulum stimulates calcium influx in non-excitable cells (3), similar to the activation of calcium influx in non-excitable cells. The physiological role of SOCE in skeletal muscle remains to be uncovered. Nevertheless, recent experimental evidence suggests that altered regulation of TRPC channels may play a role in muscle disease (23).

In the present work functional association is demonstrated between the depletion of internal calcium stores via activation
of skeletal muscle RyR (RyR1) and activation of SOCE in Chinese hamster ovary (CHO) cells stably transfected with RyR1 constructs. Furthermore, it is shown that the foot structure from the RyR1 is required for functional coupling between RyR1 activity and SOCE channel activation. The footless RyR1 (RyR-COOH) protein shows a different cellular localization when compared with wild type RyR1. The later protein shows a higher percentage of co-localization with FM-4-64, a marker of plasma membrane.

In addition, we also show that part of the SOC requires TRPC1 channels, since elimination of TRPC1 with RNA interference significantly reduces SOCE induced by the depletion of the ER via activation of RyR1. The remaining SOC activity is inhibited by lanthanum, whereas the TRPC1-dependent SOC activity is insensitive to this trivalent cation. Based on this evidence, RyR1 appears to associate to at least two different types of SOCs; one requires TRPC1 protein, whereas the other does not. Eliminating the foot structure prevents functional associations between RyR-COOH and all SOCs.

MATERIALS AND METHODS

Reagents and Solutions—All salts used were analytical grade purchased from Sigma), with the exception of xestospongin C and BAPTA-AM, which were purchased from A. G. Scientific (San Diego, CA). FM-4-64 was purchased from Molecular Probes (Eugene, OR). The phosphate-buffered saline (extracellular) solution contained 140 mM NaCl, 10 mM tetraethylammonium chloride, 5 mM KCl, 2 mM MgCl2, 1.8 mM CaCl2, 10 HEPES mM adjusted to pH 7.2 with NaOH. For patch clamp experiments the intracellular (pipette) solution contained 140 mM potassium aspartate, 10 mM CsCl, 5 mM NaCl, 2 mM MgCl2, 10 mM HEPES, pH 7.2, adjusted with NaOH. The CsCl and tetraethylammonium chloride were used to eliminate endogenous outward potassium currents, which interfere with the measurements of currents via TRP1 and SOC channels.

Cell Culture—CHO cells were purchased from American Type Culture Collection (ATCC) and maintained in culture using Dulbecco's Modified Eagle's medium (Sigma) supplemented with 1% antibiotics, 2 mM glutamine, and 10% bovine fetal serum (Invitrogen). Cells were grown on plastic Petri dishes and maintained with 5% CO2 in a humidity-controlled incubator (Nuaire, Plymouth, MN), as previously described (18).

Stable Expression of RyR1 Constructs in CHO Cells—The full-length cDNA encoding the rabbit RyR1 and the amino-terminal domain containing the pore region (RyR1-COOH) lacking amino acids 280–3661 were cloned into the in-house-produced IRES-NEO expression vector. The expression of the RyR1 constructs is driven by the immediate early promoter of the housekeeping glyceraldehyde-3-phosphate dehydrogenase, which is followed by a neomycin resistance gene and a polyadenylation sequence. Individual colonies obtained from cells transfected with these constructs were collected after 3 weeks in culture in the continuous presence of 1 mg/ml G418. Colonies were expanded and tested by PCR for the RyR1 constructs and used for Western blotting and confocal microscopy analysis.

Double-stranded RNA Interference—Double-stranded RNA (dsRNA) from TRP1C1 was produced as previously described (18). Briefly, a fragment from isolated CHO cells and corresponding to TRP1C1 was cloned in phBlueScript (Stratagene, La Jolla, CA) and linearized with BamHI to produce a single-stranded RNA using the MEGAscript transcription kit (Ambion, Austin, TX) in the presence of dTTP, GTP, CTP and TTP. The transcription reaction was stopped by treating the reaction mixture with DNase I and the dsRNA was purified on a 1% agarose gel followed by ethidium bromide staining of the gel. The resulting dsRNA was linearized with EcoRV to produce the complementary RNA strand with the T7 promoter. Equal amounts of both single-stranded RNA products were mixed and heated at 60 °C and let cool slowly for 30 min until room temperature was reached. 5 μg of dsRNA was mixed with Lipofectamine plus (Invitrogen), and the mixture was placed on 60-mm Petri dishes containing 50% confluent CHO cells. After 48 and 72 h at room temperature was reached, 5 μg of dsRNA obtained from the enhanced green fluorescence protein (N1 vector, Clontech, Palo Alto, CA). CHO cells were transfected three times with the dsRNA using a similar procedure to the one described above.

Cellular Fractionation—Membrane fractionation of CHO cells was performed as reported previously (24). Briefly, cells were sonicated and homogenized in 10 volumes of 10 mM HEPES, pH 7.4, 20 mM KCl, 0.5% CHAPS, 1 mM EGTA, and one tablet of the complete peptidase-inhibitors (Roche Applied Science). After a centrifugation at 650 × g for 10 min, the supernatant was ultracentrifuged at 120,000 × g for 90 min to obtain the total membrane fraction. The total membrane fraction was resuspended in 0.3 m sucrose, 10 mM imidazole, pH 7.4, in the presence of the peptidase inhibitors.

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Received for publication July 27, 2018 and in revised form October 25, 2018, and accepted December 10, 2018

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Fig. 1. Functional expression of RyR type 1 constructs in CHO cells. A, representative Western blot illustrating the presence of human type 2 bradykinin receptor (BKR; lane 1), full-length type 1 RyR (lane 3), and the carboxyl-terminal (pore) domain of RyR1 (RyR-COOH, lane 4). Lane 2 shows untransfected CHO cells exposed to the anti-histidine antibody. The right side of panel A shows schematic drawings illustrating the two RyR1 constructs used in this study and the histidine tag at their amino-terminal domains. aa, amino acids. B, pharmacology of full-length RyR illustrating the characteristic increment in ryanodine binding by a non-hydrolyzable ATP analog (AMP-PCP), the reduction of ryanodine binding by ruthenium red (RuR), and the increment of ryanodine binding by caffeine (+CAFF). C, effect of two IP3R inhibitors on the IP3 binding to microsomal membranes from RyR-WT cells. Heparin final concentration was 1 mg/ml, and xestospongin C was 5 μM. Bars indicate the mean ± S.D. D, representative confocal microscopy images obtained with the endoplasmic reticulum marker brefeldin conjugated to fluorescein (green signal) and ryanodine conjugated to rhodamine (red signal). The panel on the left illustrates control CHO cells (RyR−), and that on the right illustrates RyR-WT cells (RyR+, cells expressing full-length RyR1). The yellow signal on the right indicates co-localization of brefeldin and ryanodine. The calibration bar shows 10 μm.

RESULTS

Functional Expression of RyR1 Constructs—Cells stably expressing the full-length type 1 ryanodine receptor from rabbit skeletal muscle (RyR-WT) or the construct missing the foot structure (Δ280–3661 amino acids) and containing the carboxyl-terminal (pore) domain (RyR-COOH) were analyzed by Western blot to determine the presence of both proteins.

Fig. 1A illustrates a representative Western blot illustrating the presence of the human type 2 bradykinin receptor (lane 1), RyR-WT (lane 3), or the RyR-COOH (lane 4). Lane 2 shows the Western blot obtained from cells not transfected with any of the constructs mentioned above (wild type CHO cells). The right side of panel A shows the two constructs used in this study. A polyclonal sequence (histidine tag) was inserted at their amino-terminal ends to facilitate the identification of the proteins by Western blotting using a commercial antibody (Materials and Methods).

The RyR-WT expressed in CHO cells show a typical RyR pharmacology (Fig. 1B). The binding of [3H]ryanodine increased with the non-hydrolyzable ATP analogue AMP-PCP by more than 2-fold. This binding was inhibited by 5 μM ruthenium red (RuR). As expected, [3H]ryanodine binding was stimulated with 5 mM caffeine (+CAFF) in the presence of low calcium (50 nM).

Fig. 1C shows [3H]IP₃ binding from the same cells expressing RyR-WT. The [3H]IP₃ binding was inhibited by heparin (1 mg/ml) and was not affected by the IP₃R inhibitor xestospongin C (5 μM). This result was expected since heparin interferes with the IP₃ binding site in the IP₃R, whereas Xestospongin C does not (26).

Simultaneous Measurements of Intracellular Calcium and Electrophysiology—Fig. 2 shows a typical experiment where simultaneous measurements of intracellular calcium (Fig. 2A) and whole-cell currents (B) were obtained in real time in response to 50 nM Bk.

All experiments described in this manuscript followed the same procedure; first the cell was exposed to Bk or caffeine in the absence of extracellular calcium with the intention of releasing calcium from intracellular storage compartments. In all cases this initial maneuver resulted in a rapid increment in fluorescence followed by a decline of fluorescence to a basal level similar to the one observed before the stimulation. During this part no changes in whole-cell currents were observed, consistent with the notion that this initial maneuver stimulates release of calcium from intracellular stores.

After calcium reached a new steady state level near baseline, extracellular calcium was rein corporated in the bath solution to initiate influx of calcium. This later maneuver resulted in a new increment in fluorescence and activation of inward currents, which reflected the influx of calcium from the extracellular space via SOCs. The second increment in fluorescence was also transient, slowly declining toward base-line...
levels. In all experiments, the inward current declined too, following a similar time course of that observed for the changes in fluo-4 fluorescence.

Depletion of Intracellular Calcium Stores via IP$_3$R or RyR1 Initiates Calcium Influx and Activates SOC—Using the experimental procedure described in the legend to Fig. 2, we proceeded to explore the role of RyR1 activation in the initiation of calcium influx via SOCs.

Activation of SOCs after depletion of intracellular calcium stores via stimulation of RyR1 has been previously reported. A previous study showed functional interactions between RyR1, the human TRPC3 channel (hTRPC3), and I$_{rev}$ channels (20).

Fig. 3A illustrates a typical experiment using Bk to release calcium from the ER and monitor calcium influx after the addition of calcium to the extracellular solution. Fig. 3B shows the effect of incubating the cell with the IP$_3$R selective inhibitor, xestospongin C (Xestos C). As illustrated in the figure, incubating the cell for 5 min with Xestos C (2 μM) inhibited completely the release of calcium from the ER induced by Bk. The addition of calcium to the extracellular solution failed to induce calcium influx. This behavior is expected since SOCs are only activated upon depletion of intracellular calcium stores, and it has been extensively shown that maneuvers that interfere with the release of calcium from the ER prevent activation of SOCs. To further demonstrate that the effect observed was the result of interfering with IP$_3$R activity, we used a structurally and mechanistically different IP$_3$R inhibitor, heparin. Because heparin does not cross the plasma membrane, this type of experiment was not conducted in the perforated patch mode (like all the experiments described in this report) but in the whole-cell configuration, and heparin (1 mg/ml) was included in the pipette solution. Heparin was also very effective in inhibiting IP$_3$R (in this regard Xestos C is a cleaner IP$_3$R inhibitor), and (b) using heparin as IP$_3$R inhibitor would imply working in the whole-cell mode, and we wanted to reduce the interference of the pipette solution on the intracellular milieu. Because Xestos C is membrane-permeable, we could conduct all experiments in the perforated patch mode.

We next explored the effect of caffeine in control CHO cells (RyR1−, cells not expressing the RyR constructs). In these cells, 5 mM caffeine (CAF) was ineffective in inducing release of calcium from internal stores and failed to activate SOCs, as illustrated in the representative experiment shown in Fig. 3D. In contrast, CHO cells expressing the full-length type 1 ryanodine receptor (RyR1+) showed a typical transient elevation of intracellular calcium in response to 5 mM CAF in the absence of extracellular calcium (reflecting release of calcium from internal stores). The addition of calcium resulted in a second increment in fluo-4 fluorescence, reflecting a transient elevation of intracellular calcium. Simultaneous activation of inward currents was observed that followed a similar time course to the fluorescence.

To eliminate any possible contribution of IP$_3$R to the increments in intracellular calcium and currents induced with caffeine, RyR1− cells were incubated with 2 μM Xestos C. Under these experimental conditions Bk stimulation failed to induce changes in intracellular calcium (Fig. 3F) as expected since the IP$_3$R was inhibited by 2 μM Xestos C. However, CAF stimulation induced a typical transient increment in intracellular calcium. The subsequent addition of calcium to the extracellular solution produced a transient calcium influx and concomitant activation of inward currents.

These results show that release of calcium from internal stores via activation of RyR1 is sufficient to induce SOC activity and calcium influx in cells where the IP$_3$R is inhibited and, therefore, does not contribute significantly to the release of calcium under these conditions. This result shows a functional interaction between RyR1 and SOC in CHO cells expressing the skeletal muscle type 1 ryanodine receptor. A similar interaction has been previously reported for hTRPC3 and for the calcium-release activated calcium current (I$_{rev}$) (20).

Depletion of Internal Calcium Stores via IP$_3$R or Caffeine—To evaluate if bradykinin (via the production of IP$_3$) and caffeine were equally effective in depleting internal calcium stores, we performed experiments utilizing the calcium ionophore, ionomycin.
The Foot Structure from RyR1 Is Required for Functional Coupling to SOCs—There is significant evidence in the literature showing functional and physical interactions between different TRPC channels and IP$_3$R (13, 14, 16–18, 27, 28). The requirement of IP$_3$R activation for the induction of SOC activity has also been shown. More recently, we have demonstrated that a sequence in the amino-terminal domain from the IP$_3$R is sufficient to induce SOC activity and activate calcium influx in CHO cells via TRPC1 channels (18).

Because IP$_3$Rs and RyRs fulfill similar functions as calcium release channels and in fact have similar structures, we wanted to explore if the foot structure (amino-terminal domain) from the RyR1 was required for the functional association to SOC illustrated in the previous results. To do this, we produced a RyR1 construct lacking the entire foot region by deleting the nucleotide sequence corresponding to amino acids 280–3661 from skeletal muscle RyR (identified in this study as RyR-COOH, Fig. 1).

Fig. 4 shows the results obtained with RyR-COOH and wild type RyR1 (RyR-WT). As illustrated in Fig. 5A, 5 mM CAF induced a typical transient calcium increment followed by a second increment induced after the addition of extracellular calcium in CHO cells expressing RyR-WT (as we have shown in previous figures). The second calcium increment was accompanied by activation of inward currents.

CHO cells stably expressing RyR-COOH also responded to 5 mM CAF with a transient increment in intracellular calcium (in the absence of extracellular calcium). However, the addition of calcium to the extracellular solution failed to induce the typical second transient increment in calcium (Fig. 5B). This maneuver failed also to activate inward currents in these cells.

Fig. 5C shows the integral of fluorescence (arbitrary fluorescence units (AFU) obtained from RyR-WT ($n = 19$) and RyR-COOH ($n = 25$) cells. Fig. 4D shows the inward current obtained at $-100 \, \text{mV}$ for RyR-WT ($4.4 \pm 1 \, \text{pA/\mu F}$) and RyR-COOH ($0.8 \pm 0.6 \, \text{pA/\mu F}$) cells.

The integral of fluorescence obtained in the absence of extracellular calcium (reflecting the release of calcium form the ER) was indistinguishable for wild type CHO cells (stimulated with Bk), RyR-WT, and RyR-COOH cells (Fig. 4D).

These results suggest that Bk and CAF were incapable or releasing all releasable calcium from internal stores or that a calcium pool lacking IP$_3$R and RyRs (but sensitive to ionomycin) is present in these cells. We did not further investigate these two possibilities in the present study.

The Foot Structure from RyR1 Is Required for Functional Coupling to SOCs—There is significant evidence in the literature showing functional and physical interactions between different TRPC channels and IP$_3$R (13, 14, 16–18, 27, 28). The requirement of IP$_3$R activation for the induction of SOC activity has also been shown. More recently, we have demonstrated that a sequence in the amino-terminal domain from the IP$_3$R is sufficient to induced SOC activity and activate calcium influx in CHO cells via TRPC1 channels (18).

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The integral of fluorescence obtained in the absence of extracellular calcium (reflecting the release of calcium form the ER) was indistinguishable for RyR-WT and RyR-COOH cells, 450 ± 41 and 492 ± 32 AFU s$^{-1}$, respectively. These results indicate that, although the RyR-COOH is capable of releasing calcium from internal stores in response to CAF, this is not sufficient to activate calcium influx and SOCs.

To determine that cells expressing RyR-COOH could respond normally to Bk, experiments were performed in the
absence of Xestos. As illustrated in Fig. 5E, these cells responded with a typical release of calcium and calcium influx in response to Bk. In fact, the integral of fluorescence (Fig. 5F) and inward current (Fig. 5G) obtained from wild type CHO cells and RyR-COOH-expressing cells in response to Bk were indistinguishable.

These results indicate that the foot structure from the RyR1 is required for functional coupling to SOCs, since only calcium influx is affected in RyR-COOH cells, which show a typical calcium release from internal stores in response to caffeine. These cells responded normally to Bk, showing that the coupling between SOCs and IP₃Rs was not affected by the expression of RyR constructs.

**The Increment in Intracellular Calcium Is Not Responsible for Activation of SOCs**—To explore if the increment in intracellular calcium resulting from the release of calcium from internal stores was responsible for the activation of calcium influx in RyR-WT cells, we conducted experiments in cells incubated with the calcium chelator BAPTA-AM. Fig. 6A shows a typical response to caffeine in a cell incubated with Xestos C. Fig. 6B shows the response obtained in cells incubated with BAPTA-AM (25 μM) for 10 min and Xestos C. As illustrated in the figure, no significant increment in intracellular calcium was observed in cells exposed to BAPTA-AM. However, the activation of inward current (reflecting SOC activity) was unaltered by BAPTA-AM.

Fig. 6C shows the integral of fluorescence after the addition of extracellular calcium to the bath solution obtained from control cells (not exposed to BAPTA-AM) and cells incubated with BAPTA-AM for 10 min. Under these conditions the integral of fluorescence was 830 ± 24 AFU s⁻¹ (n = 18) and 30 ± 9 AFU s⁻¹ (n = 23) for control and BAPTA-AM-treated cells, respectively.

Fig. 6D shows the peak inward current obtained from control and BAPTA-AM-treated cells. As illustrated in the figure, the increments in calcium were abolished by BAPTA-AM, but the activation of inward current was not affected. The mean peak inward current for the control cells was 4.8 ± 0.7 pA/pF (n = 18) and 5.4 ± 0.2 pA/pF (n = 23) for BAPTA-AM-treated cells.

These results strongly suggest that the activation of the current is the result of release of calcium from the ER and not the elevation of calcium in the cytosol. This type of result is expected for SOCs and has been extensively reported in the literature (5, 30, 31).

**Different Spatial Distribution of RyR-WT and RyR-COOH Constructs in the Cell**—While performing three-dimensional reconstructions from confocal images obtained from cells expressing RyR-WT and RyR-COOH, we observed that the spa-
tial distribution of the labeling of ryanodine coupled to fluorescein isothiocyanate was clearly different between both constructs (Fig. 7A and supplemental videos).

Colocalization studies with brefeldin (a marker of ER and Golgi) showed that the entire ryanodine label colocalized with brefeldin in cells expressing constructs (RyR-WT and RyR-COOH), Figs. 1D and 7A). However, when the FM-464 compound (a marker of plasma membrane) was utilized, a higher percentage of the ryanodine label colocalized with this compound in RyR-WT-expressing cells when compared with RyR-COOH (Fig. 7B). Using a quantitative method to determine percentages of colocalization as previously described (32), we obtained pixel colocalization for ryanodine-FM-464 labeling in RyR-WT cells of 11/110064% and only 1/110063% with RyR-COOH cells (Fig. 7B, n = 64).

These results indicate that removing the foot structure from the RyR1 alters the cellular distribution of the channels, resulting in less RyR-COOH near the plasma membrane. These results may explain the previous observation illustrating that the foot structure is required for functional coupling to SOCs. Because SOCs are located in the plasma membrane, close proximity to the channel might be a prerequisite for functional association to the RyR. This proximity was observed in cells expressing RyR-WT, where more than 10% of all ryanodine labeling showed colocalization with FM-464. Although this is purely speculative, the different cellular distribution of both constructs and the lack of functional association between RyR-COOH and SOCs are, nevertheless, worth mentioning.

Functional Coupling of RyR1 to Different SOCs—

We have previously shown that the majority of the calcium influx and the inward current induced by Bk stimulation in CHO cells depend on the presence of TRPC1 protein (18). Using RNAi, we have significantly reduced the amount of TRPC1 protein by nearly 98% in CHO cells, as previously described (18). Fig. 8, A–B, illustrates the effect of reducing TRPC1 protein (TRPC1-) on the Bk-induced calcium influx and the activation of inward currents (18).

Interestingly, the influx of calcium induced by CAF in CHO cells expressing the RyR-WT is only partially affected by the reduction in TRPC1 protein content, as illustrated in Fig. 8, C–D. This result was consistent in more than 30 cells explored, as shown in Fig. 8, E–F. The integral fluorescence in cells stimulated with Bk after the addition of extracellular calcium (reflecting calcium influx) in control cells (TRPC1+) was 798 ±
Fig. 6. Increments in intracellular calcium are not responsible for SOCs activation. A, representative experiment obtained from a RyR-WT cell showing increments in calcium and whole-cell currents in response to 5 mM CAF. B, a similar experiment in a cell previously incubated with the calcium chelator BAPTA-AM. Notice that BAPTA prevented the increments in calcium but not the activation of inward currents. C, calcium influx measured as the integral of fluorescence in response to the addition of extracellular calcium after caffeine stimulation. The control (empty bar) reflects RyR-WT cells not treated with BAPTA and BAPTA-AM-treated cells (filled bar). D, peak inward current measured at −100 mV from control and BAPTA-AM-treated cells. Data shows the mean ± S.D. obtained from 18 and 23 independent cells, respectively.

55 AFU s⁻¹ (n = 22); this value was reduced to 75 ± 19 AFU s⁻¹ (n = 32) for TRPC− cells (cells treated with dsRNA to reduce TRPC1 messenger RNA).

In contrast, the integral fluorescence from cells exposed to 5 mM CAF from RyR-WT cells under control conditions was 910 ± 108 AFU s⁻¹ (n = 14); this value was reduced to 470 ± 72 AFU s⁻¹ (n = 33) in TRPC− cells (Fig. 8E). These results indicate that ~60% of the calcium influx induced by CAF is independent of the presence of TRPC1 protein in the cells. These experiments were carried out in the presence of Xestos C to minimize the contribution of IP₃R in the activation of SOCs.

Similar results were obtained when measuring the inward current induced by Bk or CAF stimulation, where nearly 60% of the current induced by CAF in RyR-WT cells was unaltered by the reduction in TRPC1 protein content by RNAi. For example, as illustrated in Fig. 8D, the time course of the activation of inward current in fluorescence reflecting calcium influx were significantly slowed down in TRPC1− cells.

To determine whether RNAi did indeed alter TRPC1 protein content, Western blotting analysis were performed with control (TRPC1+) and TRPC1− cells expressing RyR-WT. Fig. 9A shows a representative Western blot illustrating the effect of RNAi on TRPC1 and RyR-WT (used as control) protein content. As illustrated in the figure, RNAi significantly reduced the amount of TRPC1 protein and showed no effect on RyR-WT protein content. This is more clearly illustrated in Fig. 9B, showing the relative TRPC1 protein content from six independent RNAi experiments.

Fig. 9C shows that the mRNA levels of TRPC1 were abolished by RNAi. RT-PCR experiments were ineffective in amplifying TRPC1 from RNAi-treated cells. These results demonstrate the efficiency of RNAi in reducing TRPC1 mRNA and protein contents. Because a significant amount of current and calcium influx remained in RyR-WT cells after the RNAi experiments strongly reduced TRPC1 protein content, we explored the characteristics of the TRPC1-independent SOC to determine whether this current was produced by a different channel.

As illustrated in Fig. 9D, the TRPC1-independent current activated by CAF in RyR-WT cells was very sensitive to the trivalent cation lanthanum. In contrast, the TRPC1-dependent current was insensitive to lanthanum; in fact, at higher concentrations lanthanum appears to increase the amount of TRPC1-dependent current (Fig. 9D). Although we cannot determine whether the TRPC1-independent current depends on other TRPC members, it is worth mentioning that lanthanides potentiates TRPC5 activity (33). Interestingly lanthanides are potent inhibitors of TRPC3 (34). Thus, although lanthanides are not selective blockers, they can be used to separate TRPC channel members.

A dose-response curve obtained from RyR-WT TRPC1− and TRPC1+ cells is illustrated in Fig. 9E. The half-inhibitory constant of 13 ± 4 μM (n = 17) for lanthanum in RyR-WT TRPC1− cells was obtained after fitting the curve to a Hill equation. The time course of the activation of inward current from TRPC1− cells was significantly slower when compared with TRPC1+ cells (Fig. 9D). After fitting to exponential functions, activation constants of 150 ± 27 and 12 ± 6 s were obtained for TRPC1− and TRPC1+ cells, respectively (n = 24).

Based on the sensitivity to lanthanum and the slow activation of inward current observed with TRPC1− cells, we conclude that it is possible that RyR1 may be associating to at least two types of SOCs, one that requires the presence of TRPC1 channel to function and one independent of the presence of TRPC1 and very sensitive to blockade by lanthanum. The lat-
ter represents about 60% of the SOC activity induced after RyR1 stimulation, as illustrated in Fig. 8.

**DISCUSSION**

Even though the best characterized SOC is the calcium release-activated calcium current (\(I_{\text{crac}}\)) (31), there is abundant experimental evidence based on electrophysiological properties, selectivity, and pharmacology indicating that there are more than one SOC in the wide variety of cells and tissues studied (for review, see Refs. 30 and 35).

Over the last few years major emphasis has been placed on the characterization of SOCs from non-excitable cells. Many if not all tissues and cells explored show at least one type of store-operated current (30). More recently, several reports have illustrated the existence of SOCs in excitable cells (for review, see Refs. 5 and 6).

In smooth muscle several SOCs have been identified over the last few years, many of which are activated after stimulation with agonists coupled to the IP\(_3\) cascade and responsible for muscle contraction (4). The role of SOCs in muscle contraction appears to be more important after prolonged exposure to agonists activating G-protein-coupled receptors linked to the IP\(_3\) cascade, although some studies suggest that SOCs may also play a role in smooth muscle proliferation (4). The major difficulty has been (as with other tissues) the separation between agonist-stimulated and store-operated channels, especially since the pathways in both scenarios share common modulators such as phospholipase C and IP\(_3\).

In mouse skeletal muscle depletion of the sarcoplasmic reticulum (SR) results in the activation of a calcium influx that is not sustained by voltage-gated calcium channels and favors the refilling of the SR (3). Furthermore, RyR1 activation induces release of calcium from SR and activation of SOC in primary human skeletal muscle cells (36).

This result was rather unexpected for two reasons; (a) there is a limited exchange of extracellular calcium in muscle fibers (in fact, muscle fibers can twitch for a long time in the absence of extracellular calcium), and (b) in general the SR is rather impermeable, and very little leakage of calcium occurs under...
physiological conditions. Nevertheless, these careful studies show activation of calcium influx even with partial depletion of the SR (3, 36).

Interestingly, recent work has shown that store-operated calcium influx is impaired in myotubes lacking RyRs (37). Furthermore, gating of store-operated and TRPC3 channels by conformational coupling to RyRs has recently been demonstrated in heterologous expression systems (20). In the same study the authors show biochemical evidence suggesting the presence of TRPC3-RyR complexes in plasma membrane microdomains (20).

In the present study we have shown for the first time the functional association of TRPC1 channels with RyR1. We further show that the foot structure from RyR1 is required for this functional association. Interestingly, the footless construct RyR-COOH shows reduced colocalization with plasma membrane markers when compared with wild type RyR1.

These results provide further evidence of TRPC-RyR plasma membrane microdomains previously suggested based on biochemical evidence (20) while providing clues about the structural determinants required for this association. We have found that RyR1 may associate to at least two types of SOCs,
one dependent and one independent on TRPC1 protein. The latter was inhibited by micromolar concentrations of lanthanum, with a half-inhibitory constant of 13 μM. The TRPC1-dependent SOC was potentiated by lanthanum at concentrations of 100 μM and higher. Removing the foot structure prevents functional associations to both SOCs.

Although lanthanides are not selective blockers, they have been used to differentiate TRPC channels. For instance, lanthanides potentiate TRPC5 currents (33), whereas they are potent inhibitors of TRPC3 (34). In fact, lanthanides have been used to separate TRPC channel subunit composition in a recent study (38).

Differential co-assembly of TRPC members that form different types of SOCs has been recently reported (39). Although we did not show that the lanthanide-sensitive, TRPC1-independent SOC, which functionally associates to RyR1, is sustained by a TRPC member, we cannot discard this possibility. We are currently exploring the possible involvement of other TRPC members in this SOC.

Yet another level of complexity in the regulation of the interactions between TRPCs and RyRs may involve calmodulin (CaM). CaM was one of the first proteins identified to bind RyRs (25). Also, CaM plays a crucial role in modulating SOC activity, as we have previously shown (29). In fact, all TRPC members possess a common CaM-IP3R binding domain. Activation of TRPC3 by IP3R occurs by displacement of inhibitory calmodulin from this common binding domain (28).

We have previously shown that the TRPC1-dependent SOC described here is modulated by CaM (18). Furthermore, peptides corresponding to sequences from the amino-terminal domain of IP3R gate TRPC1-dependent SOC. This information together with the results presented here showing the role of the foot structure (amino-terminal domain) from RyR suggest a common mechanism for the coupling between IP3R or RyR to TRPC and SOCs. Ongoing experiments may help to determine whether the TRPC1-independent SOC induced after RyR activation is modulated by CaM also.

Acknowledgments—The services from the Molecular Biology and Microscopy units and from the Library at the Institute of Cellular Physiology are greatly appreciated.

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