Adult rat cardiomyocytes exhibit capacitative calcium entry

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Hunton, Dacia L., LuYun Zou, Yi Pang, and Richard B. Marchase. Adult rat cardiomyocytes exhibit capacitative calcium entry. Am J Physiol Heart Circ Physiol 286: H1124–H1132, 2004. First published November 20, 2003; 10.1152/ajpheart.00162.2003.—Capacitative Ca2+ entry (CCE) refers to the influx of Ca2+ through plasma membrane channels activated on depletion of endoplasmic-sarcoplasmic reticulum Ca2+ stores. We utilized two Ca2+-sensitive dyes (one monitoring cytoplasmic free Ca2+ and the other free Ca2+ within the sarcoplasmic reticulum) to determine whether adult rat ventricular myocytes exhibit CCE. Treatments with inhibitors of the sarcoplasmic endoplasmic reticulum Ca2+-ATPases were not efficient in releasing Ca2+ from stores. However, when these inhibitors were coupled with either Ca2+-ionophores or angiotensin II (an agonist generating inositol 1,4,5-trisphosphate), depletion of stores was observed. This depletion was accompanied by a significant influx of extracellular Ca2+ characteristic of CCE. CCE was also observed when stores were depleted with caffeine. This influx of Ca2+ was sensitive to four inhibitors of CCE (glucosamine, lanthanum, gadolinium, and SKF-96365) but not to inhibitors of L-type channels or the Na+/Ca2+ exchanger. In the whole cell configuration, an inward current of ~0.7 pA/pF at ~90 mV was activated when a Ca2+-chelator or inositol (1,4,5)-trisphosphate was included in the pipette or when Ca2+ stores were depleted with a Ca2+-ATPase inhibitor and ionophore. The current was maximal at hyperpolarizing voltages and inwardly rectified. The channel was relatively permeant to Ca2+ and Ba2+ but only poorly to Mg2+ or Mn2+. Taken together, these data support the existence of CCE in adult cardiomyocytes, a finding with likely implications to physiological responses to phospholipase C-generating agonists.

IN CARDIOMYOCYTES the concentration of cytoplasmic free Ca2+ ([Ca2+]i) is the fundamental regulator of myofibrillar contractility and relaxation, primarily due to the cyclical release and sequestration of Ca2+ by the sarcoplasmic reticulum (SR) (2). The efflux from the SR that mediates excitation-contraction coupling occurs through Ca2+-activated Ca2+ channels termed ryanodine receptors (RyRs). Working in parallel with this inwardly rectified. The channel was relatively permeant to Ca2+ and angiotensin II (ANG II) (9). Despite the limitations noted above, these agonists result in increases in [Ca2+]i (25, 28) and contribute to positive inotropic responses (14, 33) arrhythmias (15, 35), cardiac damage following ischemia-reperfusion (36), and changes in gene expression (18).

The initial increase in [Ca2+]i in response to InsP3-generating agonists is due to the release of Ca2+ from the endoplasmic reticulum (ER) and/or the SR (termed ER-SR). In most cell types prolonged or repetitive stimulation and the subsequent depletion of ER-SR Ca2+ stores results in an influx of extracellular Ca2+ into the cytoplasm that allows for a sustained elevation in [Ca2+]i, a process termed store-operated or capacitative calcium entry (CCE) (21). This process is regulated by the depletion of ER-SR Ca2+ stores and can be activated by store depletion even in the absence of agonist. Such depletion can be achieved by inhibiting the sarco(endo)plasmic reticulum Ca2+-ATPases (SERCAs) responsible for the recovery of Ca2+ by the ER-SR, because in most cell types there is a substantive rate of leakage of sequestered ER Ca2+ (21). Recently, CCE was identified and shown to be dependent on SR Ca2+ stores in both smooth (6, 30) and skeletal (11, 31) muscle.

Hunton et al. (7) recently showed that CCE characterizes neonatal rat cardiomyocytes and linked CCE to myocyte hypertrophy through the calcineurin/nuclear factor of activated T-cells (NFAT) pathway. These authors asked here whether SR Ca2+ store depletion, achieved in adult rat ventricular myocytes (ARVMs) through treatment with SERCA inhibitors and an ionophore, an InsP3-generating agonist, or caffeine, activates CCE. We also utilized whole cell patch-clamp and inhibitors to partially characterize the channels responsible for CCE.

MATERIALS AND METHODS

Buffers and solutions. Tyrode solution contained (in mM) 137 NaCl, 5.0 KCl, 1.2 MgSO4, 1.8 CaCl2, 0.5 NaH2PO4, 5.0 glucose; pH was adjusted to 7.3 with NaOH. The potassium solution contained (in mM) 70 KOH, 40 KCl, 20 KH2PO4, 5.0 glutamic acid, 3 MgCl2, 20 taurine, 0.5 EGTA, 10 HEPES, and 10 glucose; pH was adjusted to 7.3 with KOH. The patch-clamp bath solution contained (in mM) 133 NaCl, 5 CsCl, 2 MgCl2, 10 HEPES, 10 tetrathylammonium chloride, 10 glucose, and 2 CaCl2; pH was adjusted to 7.4 with CsOH. The patch-clamp pipette solution contained (in mM) 140 CsCl, 2 MgCl2, 1 Mg-ATP, 10 EGTA, 10 HEPES, and 4.5 CaCl2; pH was adjusted to 7.3 with CsOH. All biochemicals were from Sigma unless otherwise specified.

Primary cardiomyocyte cultures. Animal procedures conformed to the Guide for Care and Use of Laboratory Animals, issued by The National Institutes of Health. Primary cultures of ARVMs were obtained from 250- to 300-g male Sprague-Dawley rats. Myocytes were isolated from ventricular tissue by enzymatic dissociation as previously described (38). Briefly, hearts were rapidly excised, and a

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Langendorff retrograde perfusion was performed at 37°C for 5–7 min with Ca²⁺-free Tyrode solution equilibrated with 95% O₂-5% CO₂. The perfusion buffer was then changed to Tyrode solution containing collagenase (0.3 mg/ml collagenase type I, Sigma) and 25 μM Ca²⁺ for 30–40 min. The dissociated cells were filtered and then centrifuged. Resuspended cells were incubated with 0.1 mg/ml protease at 37°C for 3–10 min. Rod-shaped healthy myocytes were used after 1 h. All experiments were carried out at room temperature.

**Patch-clamp recording.** The whole cell configuration of the patch-clamp technique was used to record ensemble membrane currents. Patch-clamp electrodes were pulled by using a two-step puller (Narishige PP-830), and after being filled with pipette solution, the electrodes had a tip resistance of 3–5 MΩ. The junction potential was set at zero before formation of the membrane-pipette seal. After the whole cell configuration was achieved, electrical signals were recorded with a patch amplifier (Axopatch 200B), digitized, and analyzed using PClamp (8.02) and PClampfit software (Axon Instruments).

**Calcium imaging.** ARVMs were isolated as described in Primary cardiomyocyte cultures and maintained in 10 mM HEPES-buffered Ca²⁺-free Hank’s balanced salt solution (HBSS) for 2 h. They were then made Ca²⁺ tolerant by increasing the HBSS Ca²⁺ concentration in four graded steps to a final concentration of 1.8 mM over 1 h at room temperature. Cells were then loaded for 20 min with the acetoxyethyl ester of fura-2 (2 μM) (dissociation constant (Kd) for Ca²⁺ = 0.14 μM; Molecular Probes) in 10 mM HBSS at room temperature. The solution was then exchanged to dye-free HBSS, and the cells were allowed to deesterify the indicator for an additional 30 min. To assess the intraluminal SR Ca²⁺ concentration simultaneously with [Ca²⁺], cells were incubated with 5 μM mag-fluo-4 AM (Kd = 22 μM; Molecular Probes) at 37°C for 40 min, washed with HBSS, and stored 6–8 h at 4°C to preferentially localize mag-fluo-4 to the SR (26). The cells were then loaded with 2 μM fura-2 AM at room temperature for 20 min and maintained in HBSS for an additional 40 min. Cells were allowed to settle on laminin-coated coverslips in a perfusion chamber and imaged on an Olympus IX70 inverted microscope through a ×20 Uplan APO objective. Cells were illuminated at 340 and 380 nm for fura-2 excitation and at 465 nm for mag-fluo-4 excitation. Emissions were monitored at 510 nm. Excitation duration for each wavelength was 50 ms with a 1-s delay between frame sets. For analysis, the perimeter of each cell was traced and de-convolved to identify the component of the [Ca²⁺] response in the absence of extracellular Ca²⁺ to identify the component of the [Ca²⁺], response dependent on Ca²⁺ influx.

In the absence of extracellular Ca²⁺, cells responding to 1 μM ANG II displayed a peak increase in fluorescence 30–60 s after treatment (Fig. 1). The average peak response of 21 cells from three experiments was an increase in the fura-2 fluorescence ratio of 13 ± 2%. Without exception, all the cells returned to within 2% of the original baseline within 2 min after the addition of ANG II. In the presence of extracellular Ca²⁺, the average peak response to 1 μM ANG II of 26 cells was 17 ± 3% and was not significantly different from the response in the absence of extracellular Ca²⁺. However, there was a surprising degree of variability with respect to the capacity of the cells to maintain sustained elevations in [Ca²⁺]. In 6 of 26 cells, a sustained plateau 10% or more greater than the original baseline persisted for minutes. In another 7 cells the plateau was >2% but <10% of the initial fluorescence ratio, whereas in 13 cells no sustained plateau was seen. An overall plateau average of 6% was calculated, which is consistent with the previous report (25) and reinforcing the conclusion that despite substantial cell-to-cell variability, treatment with ANG II in the presence of 1.8 mM extracellular Ca²⁺ results in an average sustained [Ca²⁺], increase in ARVMs not seen in the absence of extracellular Ca²⁺. This averaged response is thus similar to that seen in other cell types in which InsP₃-generating agonists initiate a release of Ca²⁺ from the ER, which in turn triggers a sustained increase in [Ca²⁺], due to CCE (21). Cardiomyocytes are not the only cell type in which experiments with fura-2 that produce smooth results when performed in suspension and with dilutions of thousands of cells yield significant variability when individual cells are followed, both with respect to a single cell’s temporal response and among different cells (24).

**ANG II depletes sequestered Ca²⁺ stores and activates calcium influx in selected ARVMs.** To further investigate the mechanism responsible for the persistent elevation in [Ca²⁺], in response to ANG II and perhaps to elucidate the unexplained cell-to-cell variability, we employed a differential dye-loading protocol similar to that used by Shmilgel et al. (26) in primary uterine smooth muscle cells to determine how [Ca²⁺], changes were also carried out in the presence and absence of extracellular Ca²⁺ to identify the component of the [Ca²⁺], response dependent on Ca²⁺ influx.

![Fig. 1. Sustained elevation of cytoplasmic free Ca²⁺ concentration ([Ca²⁺]) responses to angiotensin II (ANG II) depends on extracellular Ca²⁺. The [Ca²⁺], signal in response to 1 μM ANG II in fura-2-loaded adult rat ventricular myocytes (ARVMs) is shown in the presence and absence of 1.8 mM extracellular Ca²⁺. Data shown are the averaged responses and means ± SE of 21 (no extracellular Ca²⁺) and 26 (1.8 mM Ca²⁺) cells from three independent experiments.](http://ajpheart.physiology.org/)
correlated with \( \text{Ca}^{2+} \) store depletion. This permitted the simultaneous assessment of \([\text{Ca}^{2+}]_i\), responses using the high-affinity dye fura-2 and of SR \( \text{Ca}^{2+} \) stores with low-affinity mag-fluo-4. A conventional \( \text{Ca}^{2+} \) add-back protocol, in which the experiment is initiated in the absence of extracellular \( \text{Ca}^{2+} \), was used to identify the extracellular component of the \([\text{Ca}^{2+}]_i\) increases (21). Exposure to 1 \( \mu \text{M} \) ANG II in the absence of \( \text{Ca}^{2+} \) resulted in a transient increase in \([\text{Ca}^{2+}]_i\) in all cells that were analyzed and a decrease in sequestered \( \text{Ca}^{2+} \) that was partially reversed (Fig. 2A), likely due to reuptake by SERCA following \( \text{ANG II} \) release (27). This level of \( \text{Ca}^{2+} \) store depletion was sufficient upon the readaddition of extracellular \( \text{Ca}^{2+} \) to activate a \( \text{Ca}^{2+} \) influx leading to a change in the fluorescence ratio of \( 5 \% \) in only 7 of 36 cells (Fig. 2A and data not shown), consistent with the experiments reported in Fig. 1 in which \( \text{Ca}^{2+} \) was always present. There were no significant differences in the extent of store depletion observed between the cells exhibiting such a plateau and those not. Thus an explanation for the observed cell-to-cell variability was not forthcoming. In control experiments in which extracellular \( \text{Ca}^{2+} \) was removed, no agonist was added, and then extracellular \( \text{Ca}^{2+} \) was replaced, none of the tested cells showed an increase in ratio of \( 2 \% \) upon the readaddition of \( \text{Ca}^{2+} \) (data not shown).

To achieve more complete SR store depletion, \( \text{Ca}^{2+} \) store refilling was prevented by including the SERCA inhibitor thapsigargin. Interestingly, this addition to the protocol greatly reduced the cell-to-cell variability described above. In the presence of 5 \( \mu \text{M} \) thapsigargin the average decrease in the mag-fluo-4 signal 5 min after the addition of 1 \( \mu \text{M} \) ANG II was \( 9 \% \), and the influx of \( \text{Ca}^{2+} \) on add back caused an average \([\text{Ca}^{2+}]_i\) increase in the fura-2 ratio of 16 \% (\( n = 17 \)). When CPA was washed out before \( \text{Ca}^{2+} \) repletion, there was evidence of \( \text{Ca}^{2+} \) store refilling, based on an increase in the mag-fluo-4 signal, upon readaddition of extracellular \( \text{Ca}^{2+} \) (Fig. 2C). This demonstrates that the presence of SERCA inhibitors facilitates ANG II-mediated \( \text{Ca}^{2+} \) store depletion and leads to a significantly larger influx of extracellular \( \text{Ca}^{2+} \).

**Store depletion with thapsigargin and ionomycin also leads to calcium influx.** We next asked whether \( \text{Ca}^{2+} \) store depletion in the absence of \( \text{InsP}_3 \)-generating agonists could activate a calcium influx. Initial experiments indicated that, similar to skeletal muscle (11), treatment with 5 \( \mu \text{M} \) thapsigargin or 5 \( \mu \text{M} \) CPA alone was insufficient to cause rapid \( \text{Ca}^{2+} \) store depletion in ARVMs. To facilitate more rapid store depletion in conjunction with 5 \( \mu \text{M} \) thapsigargin, 100 \( \text{nM} \) ionomycin was used, a concentration that selectively permeabilizes ER-SR \( \text{Ca}^{2+} \) stores (21). In the absence of extracellular \( \text{Ca}^{2+} \), sequestered \( \text{Ca}^{2+} \) levels rapidly decreased by an average of 15 \% at 5 min, whereas the concurrent increase in \([\text{Ca}^{2+}]_i\) was transient (Fig. 3A). The modest and short-lived response in \([\text{Ca}^{2+}]_i\), relative to the magnitude of the release of \( \text{Ca}^{2+} \) from stores likely reflects both the high level of \( \text{Ca}^{2+} \) buffering that characterizes the cytoplasm as well as the removal of cytoplasmic \( \text{Ca}^{2+} \) by \( \text{Na}^+ / \text{Ca}^{2+} \) exchangers and plasma membrane \( \text{Ca}^{2+} \) ATPases (2). The subsequent addition of extracellular \( \text{Ca}^{2+} \) resulted in a sustained increase in \([\text{Ca}^{2+}]_i\), averaging 32 \% in 61 cells, consistent with activation of CCE. Because of the irreversible effects of thapsigargin, there was no significant increase in sequestered \( \text{Ca}^{2+} \) after \( \text{Ca}^{2+} \) repletion (Fig. 3A).
Depletion of caffeine-sensitive SR Ca$^{2+}$ stores activates calcium influx. We next asked whether triggering Ca$^{2+}$ store depletion through RyRs would activate a calcium influx. ARVMs were treated with both 5 μM CPA and 20 mM caffeine in Ca$^{2+}$-free HBSS. Simultaneous assessments of sequestered Ca$^{2+}$ and [Ca$^{2+}$], showed a drop in mag-fluo-4 fluorescence that averaged 21 ± 2%, significantly larger than that seen with ANG II in the presence of SERCA inhibition.

The transient increase in the fura-2 ratio was also larger. When extracellular Ca$^{2+}$ was restored, an increase in the fura-2 ratio of 21 ± 2% (n = 27) was observed (Fig. 3B). These data indicate that depletion of caffeine-sensitive Ca$^{2+}$ stores activates CCE, although this level of Ca$^{2+}$ depletion is clearly not physiological.

Previous studies in ARVMs (13) and other cell types (3) suggest that there may be spatially and functionally distinct stores of sequestered Ca$^{2+}$. To address whether caffeine and ANG II were accessing the same or different SR Ca$^{2+}$ pools, we used 5 μM thapsigargin to prevent refilling and measured the extent of SR Ca$^{2+}$ store depletion by each agonist. Treatment with 1 μM ANG II in the absence of extracellular Ca$^{2+}$ and in the presence of thapsigargin caused a rapid drop in stored Ca$^{2+}$ that was followed by a continuing gradual decline (Fig. 3C). At 5 min after the addition of ANG II, this amounted to a 7% decrease in the mag-fluo-4 signal (n = 19). Concurrently, there was a transient increase in [Ca$^{2+}$]. Subsequent addition of 20 mM caffeine generated a much larger drop in stored Ca$^{2+}$ (14%), indicating that ongoing (Fig. 3C) or repetitive (data not shown) treatment with ANG II does not efficiently empty all caffeine-sensitive stores. Interestingly, the partial Ca$^{2+}$ store depletion with ANG II was sufficient to activate a calcium influx, although an equivalent degree of Ca$^{2+}$ store depletion achieved with caffeine in the absence of CPA was ineffective (data not shown).

Next, the order of treatments was reversed to determine whether depletion of caffeine-sensitive Ca$^{2+}$ stores eliminated ANG II-sensitive stores. In none of the 52 cells examined was more than a slight additional drop in stored Ca$^{2+}$ apparent when treatment with 1 μM ANG II followed 20 mM caffeine in the presence of 5 μM thapsigargin (data not shown). These data are consistent with the findings of Lipp et al. (13) using immunofluorescent antibodies demonstrating that InsP$_3$ receptors are present only on a portion of the SR, but that RyRs are present on all stores, both positive and negative for InsP$_3$ receptors.

L-type channels or the Na$^+/Ca^{2+}$ exchanger do not mediate store depletion-induced Ca$^{2+}$ influx. ARVMs loaded with fura-2 were treated with 20 mM caffeine in the presence of the reversible SERCA inhibitor CPA (10 μM) in the absence of extracellular Ca$^{2+}$. As noted above, this protocol was determined to maximally deplete Ca$^{2+}$ stores and to activate CCE. At the same time that 1.8 mM Ca$^{2+}$ was restored to the medium, CPA was removed so as to allow the Ca$^{2+}$ stores to partially refill following Ca$^{2+}$ entry. After 90 s, cells were subjected to an additional 20 mM caffeine treatment to assess the extent to which SR stores had been refilled and then to 30 mM KCl to assess influx due to L-type voltage-gated channels. Control cells showed a robust increase in [Ca$^{2+}$], after Ca$^{2+}$ was restored to the media and an additional increase in [Ca$^{2+}$], in response to caffeine, indicating that the SR Ca$^{2+}$ stores had been at least partly refilled. Additionally, a robust [Ca$^{2+}$] increase following depolarization with KCl showed that L-type channels were functional (Fig. 4A).

In other cell types (32) as well as in neonatal cardiomyocytes (7), glucosamine (GlcNH$_2$) has been shown to inhibit CCE. To determine its effects on calcium influx in ARVMs, we repeated this protocol with 5 mM GlcNH$_2$ being added 2 min before caffeine. The response to caffeine was normal, showing that GlcNH$_2$ had little effect on SR Ca$^{2+}$ stores. However, [Ca$^{2+}$].
did not increase when Ca\(^{2+}\) was returned to the media, and caffeine failed to induce a response. This suggests that GlcNH\(_2\) was able to efficiently block Ca\(^{2+}\) entry and SR store refilling. A subsequent exposure to 30 mM KCl produced a robust [Ca\(^{2+}\)] increase, indicating, as seen previously in neonatal cells (7), that L-type channels were not impayed by the presence of GlcNH\(_2\). (Fig. 4B).

Treatment with another inhibitor of CCE, 20 \(\mu M\) gadolinium (21), also effectively blocked Ca\(^{2+}\) influx and SR Ca\(^{2+}\) store refilling without affecting L-type channels (Fig. 4C). SKF-96365, still another CCE inhibitor (21), did the same (Fig. 4D). Lanthanum at 100 \(\mu M\) (21) was also effective, although a gradual increase in fluorescence ratio, probably due to the entry of lanthanum via the Na\(^+/Ca\(^{2+}\) exchanger and its interaction with fura-2 (22), confounded the analysis (data not shown). In contrast, neither nifedipine (10 \(\mu M\)) nor verapamil (10 \(\mu M\)) prevented the influx of Ca\(^{2+}\) or refilling of SR stores, although L-type channels were clearly blocked as depolarization failed to elicit a response (Fig. 4, E and F).

Previous evidence has suggested a role for the Na\(^+/Ca\(^{2+}\) exchanger working in reverse mode in controlling [Ca\(^{2+}\)], responses to InsP\(_3\)-generating agonists (12). However, treatment with unspcific inhibitors of the Na\(^+/Ca\(^{2+}\) exchanger amiloride (10 \(\mu M\)) and benzamil (10 \(\mu M\)) caused a delay in the removal of Ca\(^{2+}\) following treatment with caffeine but failed to prevent [Ca\(^{2+}\)], increases on Ca\(^{2+}\) repletion, suggesting that the reverse mode of the Na/Ca\(^{2+}\) exchanger is unlikely to be contributing substantially to the calcium influx observed with store depletion (Fig. 4, G and H).

A compound that has received much attention in studies of calcium overload recently is KB-R7943 (8), which preferentially inhibits the reverse mode of the Na\(^+/Ca\(^{2+}\) exchanger responsible for Ca\(^{2+}\) influx. As with the unspcific inhibitors of the exchanger, KB-R7943 at 5 \(\mu M\) was ineffective in blunting the increase in [Ca\(^{2+}\)], resulting from the Ca\(^{2+}\) influx that followed caffeine-induced store depletion (Fig. 5A).

To complement this point, we asked whether the agents we used to inhibit the putative CCE influx pathway had effects on Ca\(^{2+}\) influx attributable to the reverse mode of the Na\(^+/Ca\(^{2+}\) exchanger in ARVMs. We utilized the protocol of Eigel and Hadley (4), in which the concentration of intracellular Na\(^{+}\) is elevated by removing K\(^+\) from the bath. K\(^+\) (5 \(\mu M\)) is then returned to the medium, and Na\(^{+}\) is removed. The resulting Ca\(^{2+}\) influx (Fig. 5B) is attributable to the reverse mode of the Na\(^+/Ca\(^{2+}\) exchanger (4). This increase in [Ca\(^{2+}\)], was sensitive to 5 \(\mu M\) KB-R7943. However, neither 5 mM GlcNH\(_2\) nor 1 \(\mu M\) SKF-96365 resulted in a significant decrease in this mode of entry. These results provide further evidence that the Ca\(^{2+}\) influx observed with store depletion is independent of the Na\(^+/Ca\(^{2+}\) exchanger.

Whole cell patch-clamp experiments detect an inward Ca\(^{2+}\) current activated by SR store depletion. The whole cell patch-clamp configuration was achieved by utilizing a pipette solution buffered to between 0 and 80 nM Ca\(^{2+}\) and that also contained 2 mM MgCl\(_2\) and 1 mM Mg\(^{2+}\)-ATP. A holding potential of +20 mV that had previously been shown to be optimal for store-operated calcium channels (SOCs) (1) and that leads to the rapid inactivation of L-type channels (23) was utilized. Extracellular Ca\(^{2+}\) was 2 mM. Voltage ramps from −100 to +60 mV were applied every 2 s. When the pipette solution was buffered to 80 nM Ca\(^{2+}\) (Fig. 6A), a current of −0.2 pA/pF at −90 mV developed spontaneously over ~4 min and remained stable for at least 30 min thereafter (32 of 34 cells) (Fig. 6A). When SR Ca\(^{2+}\) stores from this population of cells were depleted with 5 \(\mu M\) thapsigargin and 100 nM ionomycin, the inward current at −90 mV rapidly increased (Fig. 6A). Little change was seen at +60 mV. The magnitude of the current at −90 mV, usually 0.5–1.5 pA/pF, is small compared with the per beat influx through L-type channels (14) but not dissimilar to currents due to capacitative entry in other cell types in the presence of 2 mM extracellular Ca\(^{2+}\) (21). An average of the additional current induced by store depletion for five cardiomyocytes is shown as a function of time after the addition of thapsigargin and ionomycin in Fig. 6B.

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levels of current were seen when store depletion was achieved with 5 μM thapsigargin and 20 mM caffeine (data not shown). Figure 6 also shows that the additional current induced by store depletion is blocked by 100 μM lanthanum, as are many SOCs (21).

We further examined the changes in current observed within the first few minutes of achieving the whole cell configuration. When the cytoplasmic solution was buffered to near-zero [Ca$^{2+}$], with no Ca$^{2+}$ and the chelator EGTA at 10 mM, conditions shown in other cell types to lead to so-called passive store depletion (21), activation of a current comparable to that conditions shown in other cell types to lead to so-called passive store depletion (21), activation of a current comparable to that seen with thapsigargin and ionomycin or caffeine was observed (6 of 6 cells) (Fig. 6). We also facilitated store depletion at an intracellular Ca$^{2+}$ of 80 nM by including 20 μM InsP$_3$ in the pipette (Fig. 6C). Within 5 min, a current averaging 0.7 ± 0.1 pA/pF at -90 mV was observed (5 cells) when compared with the ~0.2 ± 0.04 pA/pF with the 80 nM Ca$^{2+}$ buffer alone. The initial decrease to 0.2 pA/pF could be lessened by increasing the intracellular Ca$^{2+}$ above 80 nM (data not shown), but further elucidation of the mechanism underlying the activation of this current was not achieved.

The current-voltage relationship of the store depletion-induced current was further defined. Utilizing voltage ramps, the results were confounded by the presence of both voltage-gated Ca$^{2+}$ channels and voltage-gated Na$^+$ channels. The former rapidly inactivated at the +20 mV holding potential and could be effectively inhibited by 50 μM nifedipine and/or 50 μM verapamil. The Na$^+$ channel was also inhibited by nifedipine-verapamil or by 50 μM tetrodotoxin. The current that developed in the presence of these inhibitors in response to thapsigargin and caffeine or ionomycin minus the current remaining after inhibition by 100 μM lanthanum was computed (Fig. 6D) and averaged ~0.6 pA/pF at -90 mV (n = 5). The computed current displayed an inward rectification characteristic of many SOCs (21). In the absence of inhibitors, the current at -90 mV averaged 0.7 pA/pF.

A series of experiments were then performed in which the SOCs were activated with 5 μM thapsigargin and 100 mM ionomycin in the presence of 2 mM Ca$^{2+}$. The extracellular buffer was then exchanged for one with a higher concentration of Ca$^{2+}$, an alternate divalent cation, or the complete absence of divalent cations (Fig. 6E). When Ca$^{2+}$ was increased to 5 mM, the current increased to 162% of that seen with 2 mM Ca$^{2+}$. When Ba$^{2+}$ was exchanged for Ca$^{2+}$, there was an increase of 80%. However, when either Mg$^{2+}$ or Mn$^{2+}$ was utilized instead of Ca$^{2+}$, the current was decreased to 40 and 10% of the original value, respectively. When all divalents were removed, the current increased to ~300% of the original value. This increase in the absence of divalent cations is seen in a variety of SOCs (21).

**DISCUSSION**

CCE is a ubiquitous mechanism in nonexcitable cell types that facilitates both repletion of depleted ER Ca$^{2+}$ stores and the sustained elevation of [Ca$^{2+}$]$_i$ in response to the ongoing presence of agonist (21). However, CCE has now been shown to coexist with voltage-dependent Ca$^{2+}$ entry pathways in smooth muscle cells (6, 30), skeletal muscle cells (11, 31), neurons (3), and cardiomyocytes (7). This demonstrates a more universal distribution and suggests broader function.

A previous study utilizing thapsigargin to deplete stores in cardiomyocytes did not detect the prolonged increases in [Ca$^{2+}$]$_i$ that characterize CCE (10), possibly because the concentration of the inhibitor was only 100 nM and/or because the rate of leak from stores in these cells is less than that in others. This may not have resulted in sufficient store depletion to activate CCE. In contrast, several studies have shown a prolonged elevation of [Ca$^{2+}$]$_i$ in response to InsP$_3$-generating agonists such as endothelin-1, phenylephrine, and ANG II (25, 29). These agents only partially deplete Ca$^{2+}$ stores, but the subset of Ca$^{2+}$ stores containing InsP$_3$ receptors (13) appears to be more closely linked to CCE activation.

The level of expression of InsP$_3$ receptors in cardiomyocytes is about 50-fold less than that of RyRs (19), and previous reports (13) have described a limited distribution of InsP$_3$ receptors within cardiomyocytes. Blaustein and Golovina (3) have discussed the concept that a subset of the ER-SR might be preferentially linked to the role of Ca$^{2+}$ in regulating events at the cell surface. In cardiomyocytes it is critical, of course, to allow Ca$^{2+}$ cycling of troponin C to proceed so as to permit excitation-contraction coupling. It appears, however, that in distinct areas of the cytoplasm, [Ca$^{2+}$]$_i$ remains sufficiently elevated to allow for the sustained activation of calmodulin and calcineurin, allowing, for instance, for the nuclear translocation of NFAT and the hypertrophic response (18). Interestingly, in smooth muscle cells, flux through SOCs has been shown to increase dye-detected [Ca$^{2+}$], without causing constriction, even though a similar overall increase of [Ca$^{2+}$], induced by depolarization resulted in constriction (6). The authors suggest Ca$^{2+}$ influx into a “noncontractile” compartment. Such compartmentalization of Ca$^{2+}$ may allow ARVMs to discriminate between different Ca$^{2+}$ signals as well. The putative signaling compartment likely communicates with the “contractile” com-

**Fig. 5.** Capacitative Ca$^{2+}$ entry (CCE) inhibitors do not suppress the reverse mode of the Na$^+$/Ca$^{2+}$ exchanger. A: in the presence of 5 μM KB-R7943 (KBR), 20 mM caffeine and 5 μM CPA were applied to fura-2 AM-loaded ARVMs to deplete Ca$^{2+}$ stores. CPA was then washed out, and 2 mM Ca$^{2+}$ was restored. Trace shows caffeine response and a robust increase in fluorescence after Ca$^{2+}$ restoration (n = 7). B: reverse mode of the Na$^+$/Ca$^{2+}$ exchanger was activated by exposing cells to K$^+$-free Tyrode solution, followed by exposure to Na$^+$-free Tyrode solution (26). SKF, SKF-96365. Indicated inhibitors were present throughout the experiment. Data shown are averaged normalized fluorescent changes across multiple experiments (n ≥ 8) from 3 independent isolations with error bars representing means ± SE.
partment during the signal transduction leading from InsP$_3$-generating agonists to positive inotropic responses. Recent publications have determined that the increases in L-type currents that characterize the response to such agonists are detected in perforated patch recordings but not in the whole cell configuration (14, 33). This suggests that the internal integrity of the cardiomyocyte may be critical. Recent results (Pang et al., unpublished observations) have determined that...
CCE inhibitors partially inhibit this response, perhaps by disrupting the link between InsP$_3$ generation and activation of L-type channels.

The previous publication with neonatal cardiomyocytes (7) was the first to suggest that CCE might be active in this cell type, but the notion was not without precedent. Others have previously observed a Ca$^{2+}$-permeant channel that was activated by InsP$_3$-generating agonists, not voltage-gated, and distinct from L-type channels. Merle et al. (17) found that such channels were activated with basic fibroblast growth factor, whereas Felzen et al. (5) found similar responses to the Fas ligand or a Fas-specific antibody. In the latter report, the channel could be opened by intracellular delivery of InsP$_3$ as was also shown here. Whereas neither of the previous contributions linked channel activation to depletion of SR Ca$^{2+}$ stores, such a mechanism could easily apply.

We thus suggest that CCE is likely to mediate InsP$_3$-induced and calcineurin-mediated changes in gene expression, such as those responsible for the hypertrophic response (18), and, indirectly, positive inotropic responses to these agonists (14, 33). In addition, transient receptor potential family members, those responsible for the hypertrophic response (18), and, in planar lipid bilayers, L-type channels and calcium-permeable channels open at negative membrane potentials. Voltage-dependent conductance changes in rat ventricular myocytes; perforated patch-clamp recordings. Am J Physiol Heart Circ Physiol 281: H2207, 1998.


