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STORE-OPERATED CALCIUM ENTRY IS PRESENT IN HL-1 CARDIOMYOCYTES AND CONTRIBUTES TO RESTING CALCIUM

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Abstract

Store-operated Ca^{2+} entry (SOCE) has recently been shown to be of physiological and pathological importance in the heart, particularly during cardiac hypertrophy. However, measuring changes in intracellular Ca^{2+} during SOCE is very difficult to study in adult primary cardiomyocytes. As a result there is a need for a stable and reliable in vitro model of SOCE which can be used to test cardiac drugs and investigate the role of SOCE in cardiac pathology. HL-1 cells are the only immortal cardiomyocyte cell line available that continuously divides and spontaneously contracts while maintaining phenotypic characteristics of the adult cardiomyocyte. To date the role of SOCE has not yet been investigated in the HL-1 cardiac cell line. We report for the first time that these cells express stromal interaction molecule 1 (STIM1) and the Ca^{2+} release-activated Ca^{2+} (CRAC) channel Orai1, which are essential components of the SOCE machinery. In addition, SOCE is tightly coupled to sarcoplasmic reticulum (SR)- Ca^{2+} release in HL-1 cells, and such response was not impaired in the presence of voltage dependent Ca^{2+} channels (L-type and T-type channels) or reverse mode $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX) inhibitors. We were able to abolish the SOCE response with known SOCE inhibitors (BTP-2 and SKF-96365) and by targeted knockdown of Orai1 with RNAi. In addition, knockdown of Orai1 resulted in lower baseline Ca^{2+} and an attenuated response to thapsigargin (TG) and caffeine, indicating that SOCE may play a role in Ca^{2+} homeostasis during unstressed conditions in cardiomyocytes. Currently, there is little knowledge about SOCE in cardiomyocytes, and the present results suggest that HL-1 cells will be of great utility in investigating the role of SOCE in the heart.

Keywords

Store-operated calcium entry; cardiomyocytes; HL-1 cells; Stim; Orai

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Introduction

Store-operated calcium entry (SOCE) represents a pathway for calcium (Ca^{2+}) entry that is activated by a release of endoplasmic/sarcoplasmic reticulum (ER/SR) Ca^{2+} . Reduced ER/SR Ca^{2+} triggers the formation of subplasmalemmal clusters of the SR protein, stromal interacting molecule 1 (STIM1) [1; 2]. Thereby, STIM1 couples to the plasma membrane channel pore-forming subunit of the Ca^{2+} release-activated Ca^{2+} (CRAC) channel protein, Orai1 [1]. The interaction between STIM1 and Orai1 allows Ca^{2+} entry upon release of SR- Ca^{2+} [3; 4; 5].

While the role of SOCE has been shown to be important in non-excitable cell types, in striated muscle, particularly cardiac muscle, SOCE has not been extensively studied. Uehara et al. [6] were the first to show that SOCE was present in embryonic and neonatal myocytes. Later, Hunton et al. [7] clearly demonstrated that SOCE was also present in adult cardiomyocytes via Ca^{2+} imaging. More recently, the molecular machinery that mediates SOCE has been shown to contribute to both physiological and pathological cardiac hypertrophy [7; 8; 9; 10]. However, since the work of Hunton et al. [7], SOCE has not been demonstrated using Ca^{2+} imaging in adult cardiomyocytes.

While the use of adult primary myocytes is ideal, there are several problems with using them to measure SOCE. Primary myocytes can become overgrown by non-myocytes after a few days in culture, they do not divide after the neonatal period, genetic manipulation is difficult [11; 12], and they lose their phenotype in long-term culture. In all cases, testing adult primary myocytes in a typical fashion for SOCE (i.e. removing and replacing extracellular Ca^{2+}) is complicated by the fact that myocytes tend to contract and die. In addition to these factors, it is necessary to use pharmacological inhibitors of contraction, 2,3-butanedione monoxime (BDM) or N-benzyl-p-toluenesulfonamide (BTS), which alter Ca^{2+} regulation [13; 14; 15]. The cumulative effect of these factors makes the study of SOCE in primary cardiomyocytes technically challenging and more difficult to interpret. To avoid these problems, groups studying SOCE have avoided Ca^{2+} imaging in adult primary cardiomyocytes. Although embryonic and neonatal primary cardiomyocytes are relatively easy to isolate, their utility is limited because they lack many of the characteristics of adult cardiomyocytes. Thus, HL-1 cells are a useful surrogate for studying SOCE because they display spontaneous electrophysiological and mechanical activity, cyclic Ca^{2+} movements [16; 17], maintain a differentiated cardiac phenotype, and are resilient to death from the Ca^{2+} paradox.

Therefore, because of the increasing importance of SOCE in cardiac tissue and the difficulty in studying these Ca^{2+} currents in primary adult cardiomyocytes, we sought to test the hypothesis that the HL-1 cell line would exhibit SOCE. Furthermore, it is currently unknown what role SOCE plays in maintaining Ca^{2+} homeostasis at rest and thus we sought to determine if Orai1 was important in maintaining intracellular Ca^{2+} levels in unstressed cardiomyocytes.

Materials and Methods

Cell Culture

HL-1 cardiomyocytes were cultured in Claycomb media (Sigma-Aldrich) as previously described [18].

RT-PCR

Total RNA from HL-1 cells was extracted by use of the RNeasy Fibrous Tissue Kit (QIAGEN). Reverse transcriptase polymerase chain reaction (RT-PCR) was performed on

mRNA isolated from cells following the protocol of the TaqMan RNA-to CT 1-step kit (ABI). STIM1 (ABI; Mm00486423_m1) and Orai1 (ABI; Mm00774349_m1) primer sets span an exon-exon junction and are expected to yield a product size of 140 and 150 bp, respectively. PCR products were run on a 1.8% ethidium bromide agarose gel and visualized by use of a commercially available UV system (GE).

Western Blot

Protein extraction, quantification, and subsequent Western blotting using STIM1 (BD Laboratories) and Orai1 (Millipore) antibodies were performed as previously described [18].

Calcium Imaging

Prior to experiments (48–72 h), HL-1 cells were washed twice with PBS and plated on 35 mm optical dishes (5.0×10^3). Calcium imaging using the Ca^{2+} indicator dye, fura-2-AM (Invitrogen) (2 μM) in 0.025% Pluronic F-127 was performed on attached populations of HL-1 cells as previously described [18; 19]. To test for SOCE, plates were perfused with 10 μM thapsigargin (TG) (AG Scientific) in HBSS (0 mM Ca^{2+}) to trigger SR Ca^{2+} release. At the peak of the TG response, plates were perfused with caffeine (Caff; 10 mM) in HBSS (0 mM Ca^{2+}) to test if the RyR-sensitive Ca^{2+} pool was depleted. SOCE was triggered upon reperfusion with HBSS (1.8 mM Ca^{2+}) and myocytes were tested at the conclusion of the experiment with KCl (80 mM). Data was only included if the KCl response was greater than a 100% increase above the baseline fluorescence. In the next series, plates were pre-treated with 10 μM of SOCE inhibitors (BTP-2 or SKF-96365; EMD Chemicals) 10 min before application of TG and Caff. In the last series of experiments, cells were pre-treated for 10 min with 10 μM Verapamil (Sigma-Aldrich) (Verap) to block L-type Ca^{2+} channels, 10 μM Mibefradil (Sigma-Aldrich) (Mib) to block T-type Ca^{2+} channels, or 5 μM SN-6 (Tocris Biosciences) to block the reverse mode of the NCX prior to testing for SOCE. Mean data are presented as the peak increase in ratiometric fluorescence after treatment. All diluted drugs were carefully perfused to the plates at a rate of 0.3 ml/min. Six plates were tested per experimental treatment. Each plate represents the average ratiometric changes from 10–15 cells. The averages from these 6 plates were used for statistical analysis.

Plasmid Construction

A plasmid expressing a small hairpin (sh)RNA probe targeting the mouse Orai1 mRNA (NM_175423) was produced by subcloning two complimentary oligonucleotide sequences into a custom-made pU6r-RFP vector containing a monomeric red fluorescence protein (RFP) expression cassette with its own constitutive promoter used to identify transfected cells [20]. The sequences of oligonucleotides used were:

Sense: 5'-

GATCGTCCTGGCGCAAGCTCTACTTAATTCAAGAGATTAAGTAGAGCTTGCG
CCAGGACTTTTTT-3

Antisense: 5'-

AATTAAAAAAGTCCTGGCGCAAGCTCTACTTAATCTCTTGAATTAAGTAGAG
CTTGCGCCAGGAC-3'

Transfection

HL-1 cells were plated on 35mm dishes (4×10^5) in 1 ml of Claycomb Medium (without norepinephrine and penicillin/streptomycin). Cells were transfected with lipofectamine according to the manufacturer's protocol (Invitrogen).

Electrophysiology

HL-1 cells were lightly trypsinized, suspended in HBSS, and aliquots placed in the well of a laminar perfusion chamber. Cells were superfused at 1 ml/min with bath buffer (in mM: 140 N-methyl-D-glucamine, 10 BaCl₂, 2 MgCl₂, 10 glucose, 10 HEPES with pH adjusted to 7.4 using HCl). Micropipettes (3–5 MΩ resistance) were filled with pipette buffer (in mM: 140 CsCl, 1.0 MgCl₂, 10 EGTA: $\sim 6 \times 10^{-15}$ M free Ca²⁺, and 10 HEPES) with pH adjusted to 7.2 using CsOH). Whole-cell patch clamping was performed as described previously [21].

Statistical Analysis

Statistical procedures were performed using Graphpad Prism 5.0. Data are presented as means $\pm$ SEM. Data were compared using either a paired t-test or one-way ANOVA where appropriate. Significance was set at the $P = 0.05$ level. When necessary the one-way ANOVA was followed up with post hoc testing using Dunnetts multiple comparison test.

Results

We first investigated the expression of two key components of SOCE in HL-1 cardiomyocytes. Fig. 1a demonstrates positive detection of both STIM1 and Orai1 mRNA (n=3) and Fig. 1b demonstrates positive detection of both STIM1 and Orai1 proteins from HL-1 cardiomyocyte protein extracts as well as from a positive control, adult mouse skeletal muscle (n=3).

To test for the activation of SOCE, calcium imaging was conducted in the HL-1 cardiomyocytes (Fig. 2a). Fig. 2b shows a raw tracing of the ratiometric Ca²⁺ response of a HL-1 cell. Treatment of HL-1 cells with TG slowly elevated the peak cytosolic Ca²⁺ which then returned to baseline, indicating Ca²⁺-depletion. Reperfusion with Ca²⁺, elevated ($p < 0.05$) the peak cytosolic Ca²⁺ above baseline suggesting that HL-1 cells may exhibit SOCE. Fig. 2c shows the average ratiometric Ca²⁺ level at baseline, and the peak SOCE response following Ca²⁺-depletion for all experiments (n=6).

To determine if the Ca²⁺ entry seen following Ca²⁺-depletion was regulated via SOCE channels, we pre-treated HL-1 cardiomyocytes with known inhibitors of SOCE. Fig. 3a shows a raw tracing of the ratiometric Ca²⁺ response of a HL-1 cell that was pre-treated with BTP-2. BTP-2 and SKF-96365 prevented a SOCE response ($p < 0.01$) following Ca²⁺-depletion when compared to control. Fig. 3b shows the average ratiometric Ca²⁺ level during SOCE in untreated HL-1 cells, as well as HL-1 cells pre-treated with BTP-2 and SKF-96365 (n=6/treatment). The dotted line represents the average baseline ratiometric Ca²⁺ level for these cells. Treatment of HL-1 cardiomyocytes with BTP-2 and SKF-96365 had no effect on the TG response, eliminating the possibility of reduced Ca²⁺ entry due to reduced Ca²⁺-release. Further, to ensure that this Ca²⁺ entry did not arise from voltage sensitive Ca²⁺ channels or NCX, we conducted concurrent experiments pre-treating cells with Verap (L-type inhibitor), Mib (T-type inhibitor), and SN-6 (NCX inhibitor). None of these drugs reduced Ca²⁺ entry following Ca²⁺-depletion, as compared to control (Fig. 3c). Fig. 3d shows a raw trace of a HL-1 cell during a whole cell patch clamp experiment. HL-1 cells were pretreated with TG 10 min prior to patching. Cells were voltage-clamped at -100 mV to inactivate L-type and T-type Ca²⁺ channels in a Na⁺ and Ca²⁺ free bath buffer. Perfusion with extracellular Ba²⁺ (Ca²⁺ surrogate) caused an inward current (8.70 ± 2.8 pA/pF, n=7 cells) carried by Ba²⁺, which was undetectable following pre-incubation with BTP-2.

To confirm our pharmacological and electrophysiological data showing the inhibition of SOCE in HL-1 cells, we used RNA interference (RNAi) to suppress the expression of Orai1. RT-PCR experiments using RNA from all cells treated for targeted knockdown of Orai1

demonstrated cells collectively reduced ($p < 0.05$) Orai1 mRNA by 45% (0.55 ± 0.01) 48 hr following transfection when compared to the parental plasmid (1.00 ± 0.1) ($n = 2$ plates/treatment). We also investigated the effect of Orai1 knockdown on STIM1 expression 48 hr following transfection and found that neither the parental plasmid nor the targeted Orai1 plasmid had any effect on the expression of STIM1 ($p = 0.56$). Only transfected cells (identified by the expression of red fluorescent protein) were used for Ca^{2+} imaging 96 hr following transfection. Fig 4A presents a raw tracing of a HL-1 cell treated with the parental plasmid. The transfection procedure itself did not impair normal Ca^{2+} handling of HL-1 cardiomyocytes as the baseline ratiometric Ca^{2+} levels (0.64 ± 0.04), as well as the SOCE responses (1.62 ± 0.17) of the parental plasmid were not statistically different from HL-1 cells that did not undergo transfection (baseline: 0.52 ± 0.03 and SOCE: 1.40 ± 0.36). Fig 4B presents a raw tracing of the same SOCE experiment performed in a HL-1 cell targeted for Orai1 knockdown. We investigated the effect of Orai1 knockdown on resting Ca^{2+} . Baseline Ca^{2+} levels in the Orai1 knockdown cells were lower ($p < 0.01$) than parental plasmid controls (Fig 4C). The average TG/Caff response from HL-1 cells treated with Orai1 plasmid were also lower ($P < 0.01$) than parental plasmid controls (Fig 4D). Furthermore, the average SOCE response of HL-1 cardiomyocytes targeted for Orai1 knockdown was inhibited ($p < 0.01$) when compared to cells transfected with the parental plasmid (Fig 4E).

Discussion

This is the first study to examine the role of SOCE in the HL-1 cardiomyocyte cell line and to investigate the effects of Orai1 expression on baseline Ca^{2+} levels. In the past few years, there has been a focus on the role of SOCE in excitable tissues, particularly skeletal [22; 23] and cardiac muscle [7; 9; 24; 25]. In the cardiomyocyte, intracellular Ca^{2+} functions as an important signal to regulate muscle contraction, apoptosis, proteolysis and hypertrophy. Recently, the molecular machinery of SOCE and the CRAC current has been elucidated with the discovery of STIM1 [26] and Orai1 [10]. Using RT-PCR and western blotting we found that both of these essential components of the molecular machinery are present in HL-1 cells. Our results are in agreement with previous research which has shown that STIM1 and Orai1 channels are present in primary cardiomyocytes [8; 9; 10; 27].

HL-1 cells showed significant intracellular Ca^{2+} accumulation following TG treatment and subsequent extracellular Ca^{2+} entry following perfusion with Ca^{2+} . We verified that the Ca^{2+} refilling was truly an SOCE current through pharmacological blockade and genetic inhibition of Orai1. Furthermore, SOCE was specifically responsible for the Ca^{2+} current as blockade of L-Type channels, T-type channels and the NCX inhibitors did not attenuate the SOCE current. These results are in agreement with previous research in testing SOCE in adult cardiomyocytes [7]. To extend our findings beyond Ca^{2+} imaging, a similar experiment was performed with whole cell patch clamping. After treatment with TG, perfusion with extracellular Ba^{2+} (Ca^{2+} surrogate) caused an inward current carried by Ba^{2+} , which was eliminated by pre-incubation with BTP-2. This provides further support that HL-1 cells exhibit SOCE. These results agree with the limited reports of SOCE in patch-clamped primary neonatal cardiomyocytes [24; 28].

While SOCE has been shown to play a role in cardiac hypertrophy [8; 9; 10; 28], no studies have determined if SOCE chronically regulates resting levels of intracellular Ca^{2+} . Interestingly, cells identified with Orai1 knockdown had substantially reduced baseline Ca^{2+} levels, as well as a reduced response to TG/Caff. These effects on basal SR- Ca^{2+} stores were not noted in the parental plasmid (control) or cells acutely treated (10min) with SKF-96365 and BTP-2, suggesting that they are directly linked with chronically impaired SOCE. These data suggest that SOCE channel Orai1 helps maintain the resting levels of SR- Ca^{2+} in

unstressed cardiomyocytes. In cardiomyocytes the fluctuations in free cytosolic Ca^{2+} serve as a potent regulator of signal transduction, physiological growth and cardiac contractility. Currently it is thought that SOCE may not play an important role in cardiac function under normal physiological conditions [24]. A similar dogma downplaying the importance of SOCE was previously held in skeletal muscle physiology. Now SOCE is considered as an important regulator of muscle contractility and fatigability of skeletal muscle [20]. Our data begin to challenge this dogma and we suggest that SOCE, particularly with regard to Orai1, plays an important role in maintaining intracellular Ca^{2+} in cardiac cells on a beat to beat basis.

In summary, we believe that HL-1 cells are an ideal model to circumvent most of the difficulties encountered while studying SOCE in primary cardiomyocytes. HL-1 cells have been used as a model system for cellular signaling, electrophysiology, Ca^{2+} handling [17], and hypertrophy [29; 30]. Using this model, we show for the first time that HL-1 cardiomyocytes exhibit SOCE, and that SOCE (via Orai1) is involved in maintaining baseline Ca^{2+} . Given the emerging role of SOCE in mediating cardiac function during health and disease, we feel HL-1 cells may serve as an important model to help examine the role of SOCE in cardiomyocytes.

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Highlights

- SOCE is of physiological and pathological importance in the heart.
- Investigators need a model to investigate $[Ca^{2+}]_i$ during SOCE in the heart.
- HL-1 cardiomyocytes express Orai1/Stim1 and exhibit SOCE.
- SOCE plays a role in maintaining resting Ca^{2+} homeostasis via Orai1.

Figure 1. STIM1 and Orai1 are expressed in HL-1 cardiomyocytes

Panel A, RT-PCR was conducted using RNA isolated from HL-1 cardiomyocytes with the use of primers designed for mouse STIM1 and Orai1 (expected sizes, 140 and 150 bp respectively). There was no significant amplification in the no-template control (NTC) or the no-reverse transcriptase (NRT; Taq polymerase only) samples (HW Lad= 100 bp ladder; LW Lad= 50 bp ladder). **Panel B**, Western blot using STIM1, Orai1, and β -tubulin antibodies and total protein isolated from HL-1 cardiomyocytes as well as a positive control (skeletal muscle).

Figure 2. SOCE is present and activated following SR- Ca^{2+} depletion in HL-1 cardiomyocytes
Panel A, ratiometric fluorescent images of HL-1 cardiomyocytes starting at baseline (perfusion with 0 mM Ca^{2+}), at the peak ratiometric response to TG (10 μM) and Caff (10 mM), at the peak of SOCE following reperfusion with Ca^{2+} (1.8 mM), and at the peak KCl (80 mM) response. **Panel B**, a raw tracing of the ratiometric fluorescent measurements of a HL-1 cell during testing for SOCE. Treatment of cells with TG (10 μM , in 0mM Ca^{2+}) increased intracellular Ca^{2+} . Following a return to baseline, the cells were perfused with Ca^{2+} (1.8 mM), resulting in SOCE. **Panel C**, shows the average change in ratiometric fluorescence following SR-depletion and subsequent reperfusion with Ca^{2+} to initiate SOCE (n=6 experiments). * indicates a significant increase.

Figure 3. SOCE is effectively blocked by selective SOCE inhibitors

Panel A, shows a raw tracing of the ratiometric fluorescent measurements of a HL-1 cell pretreated and continually perfused with BTP-2 (10 μ M). Treatment of cells with TG (10 μ M, in 0 mM Ca²⁺) created a large accumulation of Ca²⁺ in the cells. Following a return to baseline, the cells were perfused with Ca²⁺ (1.8 mM), and showed minimal Ca²⁺ entry, until stimulated with KCl. **Panel B**, shows the average change in ratiometric Ca²⁺ entry following SR-stimulation and reperfusion with Ca²⁺ (n=6 experiments) in HL-1 cells pre-treated with BTP-2 and SKF-96365. **Panel C**, shows the average ratiometric fluorescence following SR-stimulation and subsequent reperfusion with Ca²⁺ (n=6 experiments) in HL-1 cells pre-treated with Verap, Mib, and SN-6. **Panel D**, shows a raw tracing from a whole cell patch clamp experiment with HL-1 cardiomyocytes. Cells were voltage clamped at -100 mv and then perfused with Ba²⁺ (arrow; 1.8 mM). We were able to detect a small inward Ba²⁺ current (n=5 experiments), which was completely eliminated by pre-incubation of BTP-2 (n=5 experiments). Dashed lines represent the average baseline Ca²⁺ levels of these cells. * indicates significant reduction from control.

Figure 4. SOCE is abolished following Orai1 knockdown (KD)

Panel A, shows a raw tracing of the ratiometric fluorescent measurements of a HL-1 cell transfected with the parental plasmid (48 hr) during testing for SOCE. Treatment of cells with TG (10 μ M, in 0mM Ca^{2+}) created a large accumulation of Ca^{2+} in the cells. Following a return to baseline, the cells were perfused with Ca^{2+} (1.8 mM), resulting in SOCE. **Panel B**, a raw tracing of the ratiometric fluorescent measurements of a HL-1 cell targeted for Orai1 KD (48 hr) during testing for SOCE. Note that SOCE is inhibited. **Panel C**, shows the average ratiometric fluorescence at baseline. HL-1 cells targeted for Orai1 knockdown had lower baseline levels of Ca^{2+} (n=6 experiments) when compared to control (parental plasmid). **Panel D**, shows the average ratiometric fluorescence following treatment with TG and Caff. The basal Ca^{2+} levels and SR-stimulated release of Ca^{2+} were significantly inhibited in HL-1 cardiomyocytes targeted for Orai1 KD. **Panel E**, shows the average ratiometric fluorescence following treatment with TG and reperfusion with Ca^{2+} in HL-1 cells targeted for Orai1 knockdown. n=6 experiments; * indicates significant reduction from control (parental plasmid).