

TRPC1, STIM1, and ORAI Influence Signal-Regulated Intracellular and Endoplasmic Reticulum Calcium Dynamics in Human Myometrial Cells¹

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ABSTRACT

To explore the relationship between signal-stimulated increases in intracellular calcium ($[Ca^{2+}]_i$) and depletion and refilling of the endoplasmic reticulum (ER) Ca^{2+} stores ($[Ca^{2+}]_L$) in human myometrial cells, we measured simultaneous changes in $[Ca^{2+}]_i$ and $[Ca^{2+}]_L$ using Fura-2 and Mag-fluo-4, respectively, in PHM1-41 immortalized and primary cells derived from pregnant myometrium and in primary cells derived from nonpregnant tissue. Signal- and extracellular Ca^{2+} -dependent increases in $[Ca^{2+}]_i$ (SRCE) and ER refilling stimulated by oxytocin and cyclopiazonic acid were not inhibited by voltage-operated channel blocker nifedipine or mibefradil, inhibition of Na^+/Ca^{2+} exchange with KB-R7943, or zero extracellular Na^+ in PHM1-41 cells. Gadolinium-inhibited oxytocin- and cyclopiazonic acid-induced SRCE and slowed ER store refilling. *TRPC1* mRNA knockdown specifically inhibited oxytocin-stimulated SRCE but had no statistically significant effect on ER store refilling and no effect on either parameter following cyclopiazonic acid treatment. Dominant negative *STIM1ERM* expression attenuated oxytocin- and thapsigargin-stimulated SRCE. Both *STIM1* and *ORAI1–ORAI3* mRNA knockdowns significantly attenuated oxytocin- and cyclopiazonic acid-stimulated SRCE. The data also suggest that reduction in *STIM1* or *ORAI1–ORAI3* mRNA can impede the rate of ER store refilling following removal of SERCA inhibition. These data provide evidence for both distinct and overlapping influences of TRPC1, STIM1, and ORAI1–ORAI3 on SRCE and ER store refilling in human myometrial cells that may contribute to the regulation of myometrial Ca^{2+} dynamics. These findings have important implications for understanding the control of myometrial Ca^{2+} dynamics in relation to myometrial contractile function.

calcium, endoplasmic reticulum calcium stores, intracellular calcium, myometrial cells, myometrium, ORAI, STIM1, TRPC1

INTRODUCTION

Intracellular Ca^{2+} signals play important roles in myometrium in the regulation of cellular function and contraction [1,

2]. The myometrium is an excitable tissue in which spontaneous depolarization and associated action potentials give rise to spontaneous contractions [3]. Increases in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) are correlated with increases in contractile activity. Increases in $[Ca^{2+}]_i$ in myometrium occur primarily as a result of the entry of extracellular Ca^{2+} through plasma membrane ion channels and release of Ca^{2+} from the endoplasmic reticulum (ER) via inositol 1,4,5-trisphosphate (IP_3) receptors following G protein-coupled receptor (GPCR)-stimulated phospholipase C activation, or by inhibition of the ER Ca^{2+} ATPase (SERCA), or by passive leakage [2], but there is little contribution of Ca^{2+} -induced Ca^{2+} release and no evidence of associated sparks in myometrial cells [1, 4, 5]. $[Ca^{2+}]_i$ is lowered through the combined activities of SERCA, the plasma membrane Ca^{2+} ATPase, and Na^+/Ca^{2+} exchangers [6, 7].

Influx of extracellular Ca^{2+} into cells occurs through voltage-dependent and signal-regulated (variously termed capacitative, store-operated, or receptor-operated) ion channels in the plasma membrane [8, 9]. The signal for store-operated Ca^{2+} entry has been attributed to ER Ca^{2+} depletion following SERCA inhibition and variously also to Ca^{2+} entry resulting from GPCR stimulation and IP_3 production. The term signal-regulated Ca^{2+} entry (SRCE) is operationally defined here as an increase in $[Ca^{2+}]_i$ that is dependent on extracellular Ca^{2+} and a prior stimulus, such as GPCR stimulation or SERCA inhibition, regardless of mechanism.

The myometrial ER functions as an important intracellular Ca^{2+} store that contributes to both increases and decreases in $[Ca^{2+}]_i$. The concentration of ER luminal Ca^{2+} ($[Ca^{2+}]_L$) has been estimated to be submicromolar, in contrast to that of resting cytoplasmic $[Ca^{2+}]_i$, which is in the nanomolar range [7]. Simultaneous measurements of Ca^{2+} dynamics in myometrial cells by using the high- and low-affinity calcium indicators Fura-2 and Mag-fluo-4, respectively, revealed that there were no detectable changes in $[Ca^{2+}]_L$ during spontaneous $[Ca^{2+}]_i$ oscillations [10]. Moderate decreases in $[Ca^{2+}]_L$ abolished agonist-induced $[Ca^{2+}]_i$ transients, whereas increasing $[Ca^{2+}]_L$ did not increase the size of agonist-induced $[Ca^{2+}]_i$ transients [11].

Human myometrial cells express canonical transient receptor potential (TRPC) channels, with *TRPC1*, *TRPC4*, and *TRPC6* mRNAs in highest relative abundance [12]. TRPC proteins form homo- or heterotetrameric ion channels with various properties and have been implicated in SRCE [8, 13, 14]. We have previously reported that knockdown of endogenous TRPC4 in myometrial cells specifically attenuates GPCR-stimulated but not thapsigargin- or diacylglycerol (OAG)-stimulated SRCE [15]. In contrast, TRPC6 knockdown specifically reduces the OAG-mediated increase in $[Ca^{2+}]_i$ in a manner consistent with both an enhanced Na^+ entry coupled to activation of voltage-dependent Ca^{2+} entry channels and a nifedipine-independent Ca^{2+} entry mechanism [16].

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TABLE 1. Short-hairpin RNA (shRNA) sequences for targeting *ORAI1*, *ORAI2*, *ORAI3*, *STIM1*, and *TRPC1* mRNAs.^a

Target	Orientation	Oligonucleotide sequence (5'-3')	Location
O1sh ^b	Forward	5'TGCTGTTGACAGTGAGCGCACTGCTCTAAGAGATAAGCTGTGAAGCCAC AGATGGGCTTATCTCTTAGAGGACAGTTTGCCTACTGCCTCGGA3'	1264–1282 NM_032790.3
O2sh ^b	Forward	5'TGCTGTTGACAGTGAGCGCAGGGCATGGATTACCGGGACTCTGTGAAGCCAC AGATGGGAGTCCCAGTAATCCATGCCCTTTCCTACTGCCTCGGA3'	301–318 NM_001126340.1
O3sh ^b	Forward	5'TGCTGTTGACAGTGAGCGCTGGGTCAAGTTTGCCCATCTGTGAAGCCAC AGATGGGAATGGGCACAACTGACCCAATGCCTACTGCCTCGGA3'	718–736 NM_152288.2
Rsh	Forward	5'TGCTGTTGACAGTGAGCGCACTTCTTCGTCGAGACCATGCTGTGAAGCCAC AGATGGGCATGGTCTCGACGAAGAAGTTATGCCTACTGCCTCGGA3'	1227–1248 psiCHECK-2 vector
S1sh ^c	Forward	5'TGCTGTTGACAGTGAGCGCACAGTGAACACAGCACCCTCTGTGAAGCCAC AGATGGGGAAGGTGCTGTGTTTCACTGTTGCCTACTGCCTCGGA3'	873–891 NM_003156.3
TC1sh	Forward	5'TGCTGTTGACAGTGAGCGCTTCTCGTGAATTGGAAGTTATCTGTGAAGCCAC AGATGGGATAACTTCAATTACGAGAATTGCCTACTGCCTCGG3'	868–889 NM_003304
<i>ORAI1</i> PCR	Forward Reverse	5'TACTTGAGCCGCGCCAAGCTTAAA3' 5'ACCGAGTTGAGATTGTGCACGTTG3'	431–454 649–672 NM_032790.3
<i>ORAI2</i> PCR	Forward Reverse	5'ATGGTGGCCATGGTGGAGGT3' 5'TGCAGGCGCTGAAGGCAA T3'	349–368 409–427 NM_032831.2
<i>ORAI3</i> PCR	Forward Reverse	5'AAGCTCAAAGCTTCCAGCCGC3' 5'GGTGGGTACTCGTGGTCACTC T3'	384–404 481–460 NM_152288.2
<i>STIM1</i> PCR	Forward Reverse	5'ATCTCACAGCTCATGGTATGCTCC3' 5'GGAAGGTGCCAAAGAGTGTGTTTC3'	1576–1588 1728–1751 NM_003156.3
<i>STIM2</i> PCR	Forward Reverse	5'CCTCACACCCGCAACA3' 5'CAGAGAAGTAAATGGCCTCTTCCCTCCTC3'	2107–2123 2227–2200 NM_020860.2
pAdT-MCS ^d		5'CCGGTAGTACTCGATCGAAGTCATTCACGTGGCGCGCCTGCAGGCCGCGCCAG GCCTACGCGTATCGATA3'	

^a Short-hairpin RNA target sequences (in bold) were incorporated into the human miR-30 pre-micro RNA backbone.

^{b,c} Sequences adapted from reported siRNAs: ^bMotiani et al. [51]; ^cJones et al. [52].

^d The sequence of the pAdT-CMR multiple cloning site.

To assess the roles of TRPC1 alone and in relation to TRPC4 in myometrial SRCE, knockdown of *TRPC1* mRNA as well as the combined knockdown of these two mRNAs was achieved by expressing tandem Short-hairpin RNA (shRNA) in a new adenoviral vector targeting *TRPC1* alone or *TRPC1* plus *TRPC4* within a single adenovirus. This vector was modeled after the lentiviral vector created by Sun et al. [17] for expression of multi-microRNA hairpin constructs, effectively targeting knockdowns of either single or multiple mRNAs. A new multiple cloning site (MCS) inserted into the pAdTrack-CMV vector enables the potential targeting of single or multiple proteins through tandem shRNA expression and infection with a single adenoviral vector.

It has recently been recognized that the stromal interaction molecule (STIM) and calcium release-activated calcium modulator (ORAI) proteins constitute store-operated channels and are responsible for the highly selective Ca²⁺ release-activated Ca²⁺ (CRAC) channel in a number of cell types (see [18–21] for recent reviews). STIM proteins span the endoplasmic reticulum membrane and sense changes in ER Ca²⁺. In response to decreases in ER Ca²⁺, STIM1 protein oligomerizes and clusters into ER regions in close apposition to the plasma membrane. There STIM1 interacts with ORAI1 dimers and induces the formation of ORAI1 tetramers to produce the pore-forming unit of the CRAC channel. STIM1 and ORAI proteins have been shown to interact with TRPC proteins and have been implicated in GPCR-stimulated SRCE in some studies but not others [22]. Less work has been done on the functions of other STIM and ORAI isoforms.

To date, there have been no direct knockdown studies in myometrium that identify roles for TRPC1, STIM, and ORAI

proteins in cytoplasmic or ER Ca²⁺store dynamics. In this study, we used channel inhibitors and viral shRNA delivery systems to examine the effects of *TRPC1*, *STIM1*, and *ORAI1–ORAI3* mRNA knockdown on simultaneous [Ca²⁺]_i and ER [Ca²⁺]_L dynamics in response to GPCR activation and SERCA inhibition in human myometrial cells.

MATERIALS AND METHODS

Materials

Fura-2/acetoxymethyl ester (Fura-2/AM), Mag-fluo-4/AM and pluronic acid F127 were obtained from Invitrogen (Carlsbad, CA). KB-R7943 was obtained from Tocris Bioscience (Ellisville, MO). Thapsigargin, cyclopiazonic acid (CPA), nifedipine, mibefradil, gadolinium, oxytocin, and all other chemicals were obtained from Sigma (St. Louis, MO). Restriction enzymes were obtained from New England Biolabs Inc. (Beverly, MA) or Promega (Madison, WI). Cell culture medium and other tissue culture reagents were obtained from Invitrogen/GIBCO BRL (Carlsbad, CA). Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA).

Cell Culture

PHM1-41 immortalized myometrial cells derived from tissue collected from a nonlaboring pregnant woman at the time of cesarean section [23] were cultured in Dulbecco modified Eagle medium-high glucose with 10% fetal calf serum (FCS), 50 units/ml penicillin, 50 µg/ml streptomycin, and 2 mM L-glutamine and were used between passages 14 and 23. These cells retain many morphological and phenotypic responses in common with primary cells. Primary uterine smooth muscle cells (UtSMC) from nonpregnant human myometrium were purchased from Lonza (CC-2562, lot # 17590; Walkersville, MD).

Primary human myometrial cells (HMC) were isolated from myometrial tissue obtained at the time of cesarean section in uncomplicated pregnancies from 37–39-week pregnant women not in labor, with informed consent under

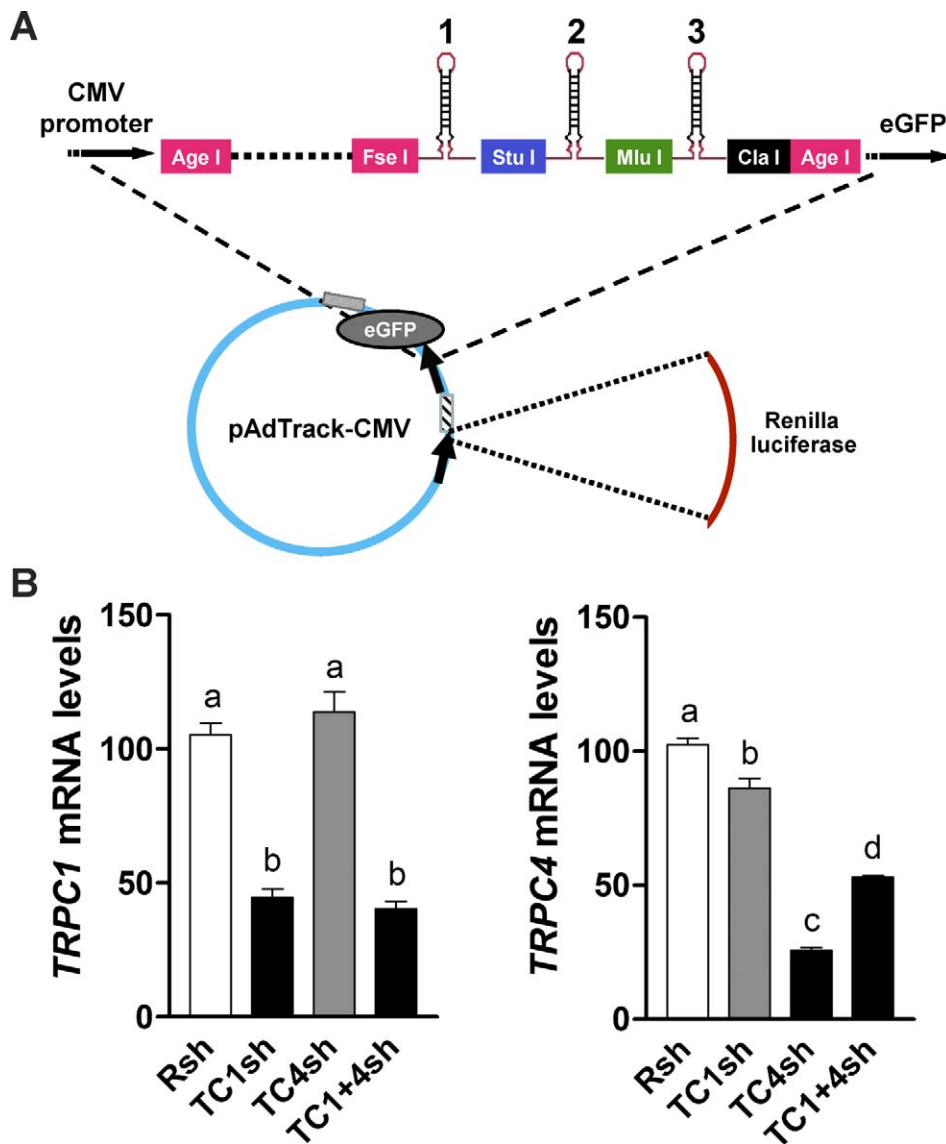


FIG. 1. **A**) Schematic representation of the pAdT-CMR vector construct shows the sites for tandem insertion of shRNAs at positions 1, 2, and 3. **B**) Specific *TRPC* mRNA knockdowns produced by adenovirus vector expressing tandem *TRPC1* (TC1sh), *TRPC4* (TC4sh), and *TRPC1+TRPC4* (TC1+4sh) shRNAs in UtsMC cells are shown. Left: *TRPC1* mRNA knockdown was achieved by tandem expression of TC1sh and TC1+4sh but not TC4sh, compared to effects of control shRNA (Rsh). Right: Tandem expression of TC4sh and TC1+4sh, but not TC1sh, induced a *TRPC4* mRNA knockdown when compared to Rsh. Data represent means \pm SEM (n = 4).

approved protocols at both institutions, and cultured as described previously [16]. Cells were used for experiments at passages 3–9.

Adenoviral Construct Synthesis and Adenoviral Infection

STIMAERM cDNA in pRK5/myc vector was obtained from Dr. P.F. Worley (The Johns Hopkins University School of Medicine, Baltimore, MD) and was cloned into pAdTrack-CMV vector using *EcoRI* and *NotI* restriction sites.

To generate a vector capable of accepting multiple shRNAs, the adenoviral vector pAdT-MCS was constructed by introduction of a new MCS (Table 1) between the CMV promoter in the pAdTrack-CMV vector (ATCC, Manassas, VA) and the sequence for green fluorescent protein (GFP) by using the *AgeI* restriction site (Fig. 1A). The MCS allows insertion of up to three shRNA hairpin sequences into the *FseI*, *StuI*, *MluI*, and *ClaI* sites. The *Renilla* luciferase cDNA sequence was excised from the psiCHECK-2 vector and cloned into this vector, using *NheI* and *XhoI* restriction sites, giving rise to pAdTrack-CMV/MCS/*Renilla*-luc (pAdT-CMR). The GFP reporter in this vector is expressed in cells, indicating infection, but fades with increasing numbers of shRNA constructs cloned upstream of the GFP transcription start site, presumably an indication of shRNA processing.

TRPC1, *STIM1*, *ORAI1*, *ORAI2*, *ORAI3*, and *Renilla Luciferase* shRNA sequences and the human pre-microRNA stem sequence (miR-30) used are shown in Table 1. Restriction sites used for insertion into the *FseI*, *StuI*, *MluI*, and *ClaI* restriction sites in the MCS were added to the shRNA

oligonucleotides by PCR using adapter primers (sequences are available on request), and the resulting products were cloned into the pAdT-CMR vector in various combinations. *TRPC1*, *STIM1*, and *Renilla* control shRNA (Rsh) constructs in pAdT-CMR express three copies of the respective sequences. The *TRPC1* plus *TRPC4* (*TRPC1+TRPC4*) construct expresses one copy each of *TRPC1* and *TRPC4* shRNA, along with one *Renilla* shRNA sequence. The triple shRNA construct targeting *ORAI1*, *ORAI2*, and *ORAI3* contains a single copy of each shRNA inserted into the *FseI*, *StuI*, and *ClaI* restrictions sites, respectively. Integrity was checked by DNA sequencing. The resulting pAdT-CMR clones were used to prepare adenovirus by recombination with the adenoviral backbone plasmid pAdEasy-1 (Stratagene, La Jolla, CA) as described previously [15]. Adenoviruses were crudely purified by passing the adenovirus-containing cell lysates through 0.45- μ m polyvinylidene fluoride filters (Millipore, Billerica, MA) after three freeze-thaw cycles in a methanol-dry ice bath and titered by end-point dilution.

The day before infection, myometrial cells (0.4×10^5 to 0.7×10^5 cells) were plated on 35-mm dishes with glass inserts (MatTek, Ashland, MA) in 1 ml of culture medium. The next day, cells were infected in 1 ml of medium containing 2% FCS with adenovirus at a multiplicity of infection of 500 for UtsMC and HMC cells and 1000 for PHM1-41 cells. After 4–6 h, 1 ml of medium containing 18% FCS was added. Cells were used 72 h postinfection and exhibited morphology similar to that of uninfected cells, as determined by visual inspection. The effectiveness of infection under these conditions was 90%–95%.

Gene Expression Analysis by Quantitative Real-Time RT-PCR

Myometrial cell mRNA was prepared using the RNeasy mini-kit including the RNase-Free DNase step (QIAGEN, Valencia, CA). cDNA was synthesized using the qScript cDNA SuperMix synthesis kit (Quanta Biosciences, Gaithersburg, MD) with 1 μ g of total RNA and the primers listed in Table 1. Each PCR reaction was carried out in duplicate and contained 2 μ l of cDNA template, 500 nM of forward and reverse primers for *TRPC1* [12], *STIM1*, *STIM2*, *ORAI1*, *ORAI2*, *ORAI3* (Table 1), and beta-glucuronidase (*GUS*) [15] and 10 μ l of PerfeCta SYBR Green FastMix for the iQ mixture (Quanta Biosciences, Gaithersburg, MD). In some cases, 100 ng of mRNA was used with the iScript one-step reverse transcriptase (RT)-PCR kit with SYBR Green (Bio-Rad, Hercules, CA). Amplification was conducted using the iCycler iQ real-time PCR detection system (Bio-Rad, Hercules, CA). The PCR protocol was as follows: 15 sec denaturation at 95°C, 30 sec annealing at 60°C, and 30 sec elongation at 72°C for 40 cycles. Sequence integrity of RT-PCR products was verified by direct sequencing. Melting curves for all products showed single peaks. The relative target gene copy number was quantified by the $\Delta\Delta C_t$ method, where a given RNA was first normalized to *GUS* in each sample and then expressed relative to the corresponding value in cells infected with control (Rsh) adenovirus for knockdown experiments or relative to *TRPC1* activity in expression experiments.

Measurement of Ca^{2+} in Cytoplasm and ER

Cells were prepared for imaging experiments, and studies using only Fura-2 were carried out as previously described [15, 16]. To monitor changes in ER $[Ca^{2+}]_L$ and cytoplasmic $[Ca^{2+}]_i$, respectively, cells were loaded with low-affinity Mag-fluo-4 ($K_d = 22 \mu$ M) and high-affinity Fura-2 ($K_d = 0.14 \mu$ M) indicators as described by Shmymol et al. [10, 11] with the following modifications. Cells were incubated in fluorescence buffer (FB; consisting of 145 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4 , 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, 5 mM glucose, pH 7.4) containing 4–5 μ M Mag-fluo-4/AM and 0.05% Pluronic F127 at 37°C for 40 min, washed with FB, and then incubated in FB with 5 μ M Fura-2 AM plus 0.05% Pluronic F127 at room temperature for 30 min. The cells were washed and incubated in FB at room temperature for an additional 30 min to allow de-esterification of the indicators and used immediately for measurements. Cells were placed in Ca^{2+} -free buffer (Ca^{2+} -free FB containing 100 μ M EGTA) and alternately illuminated at 340 and 380 nm (Fura-2) and 465 nm (Mag-fluo-4) excitation and 510 nm emission, using a model 101 Photon Technology International fluorescent imaging system. Cells exposed to CPA were washed by perfusion with Ca^{2+} -free FB for 10 min at 0.7–1 ml/min and incubated for another 5 min before SRCE was initiated by addition of 1 mM $CaCl_2$. Choline chloride was substituted for Na^+ in Ca^{2+} -free FB where indicated. Changes in $[Ca^{2+}]_i$ are reported as changes in the Fura-2 340:380 nm ratio. Relative changes in $[Ca^{2+}]_L$ are reported as the Mag-fluo-4 signal normalized to its value at the beginning of each experiment (F/F_0).

Data Collection and Analysis

In most of the studies, we present data obtained in both PHM1-41 immortalized and primary myometrial cells in order to minimize preparation artifacts. In each dish, 10–40 individual myometrial cells were examined. Because the results after viral infection were somewhat variable, in the studies using infected cells, we determined the mean of the responses from all the cells measured in a given dish and expressed the data as means \pm SEM of these values for n dishes in order not to introduce selection bias into interpretation of the data. In single-labeling experiments, changes in $[Ca^{2+}]_i$ were analyzed using numerical analyses software (CalciumComp; K. J. Bois, Fort Collins, CO) [15]. In dual-labeling experiments, the area of the $[Ca^{2+}]_i$ response was determined using features in Kaleidagraph software (Synergy Software, Reading, PA). The initial rate of ER Ca^{2+} store refilling was determined by linear regression analysis with Excel software (Microsoft, Seattle, WA), and the ER store refilling:ER store depletion ratio was determined from mean responses by using the equation, fraction of ER refilling = $[(F/F_0)_t - (F/F_0)_{min}]/[1 - (F/F_0)_{min}]$, where F/F_0 is the 465-nm fluorescence relative to time, t , zero, $(F/F_0)_t$ is relative fluorescence at time t , and $(F/F_0)_{min}$ is relative fluorescence at the point of maximal store depletion.

Data were analyzed by one-way ANOVA, and post hoc comparison of means was performed using Tukey multiple comparison tests with Prism (GraphPad Software Inc., San Diego, CA) or Kaleidagraph software or by Student t -test for unpaired samples using Kaleidagraph software. P values of ≤ 0.05 were considered significant and are indicated with different lowercase letters or an asterisk, as appropriate.

RESULTS

TRPC1 Is Specifically Involved in GPCR-Stimulated $[Ca^{2+}]_i$ Increases in Myometrial Cells

Infection of myometrial UtSMC with an adenoviral vector expressing three copies of *TRPC1* shRNA under the control of the cytomegalovirus (CMV) promoter produced a 57% *TRPC1* mRNA knockdown compared to cells infected with control vector (Rsh) without affecting *TRPC4* mRNA levels, whereas infection with a virus expressing three copies of *TRPC4* shRNA produced a 75% *TRPC4* mRNA knockdown without affecting *TRPC1* mRNA (Fig. 1B). *TRPC6* expression was not changed in either case (data not shown). The *TRPC1+TRPC4* shRNA tandem construct induced a knockdown of both *TRPC1* and *TRPC4* mRNA (61% and 48%, respectively). Similar results were obtained in PHM1-41 cells (data not shown). Hence, the tandem approach allows the knockdown of several mRNAs by using a single adenovirus, thus eliminating the ambiguity of multiple infections of the same cells, and is especially useful when working with myometrial cells that are difficult to transfect.

Expression of *TRPC1* shRNA attenuated oxytocin (OT)-stimulated SRCE in UtSMC (Fig. 2A, left panel) and PHM1-41 cells (Fig. 2A, middle panel), with an average of 56% and 50% inhibition of the $[Ca^{2+}]_i$ transient peak height and integrated area, respectively (Fig. 2A, right panel). Similar to our previous results using the U6-promoter virus [15], expression of *TRPC4* shRNA in the pAdT-CMR vector inhibited OT-stimulated SRCE (Fig. 2A). Simultaneous knockdown of both *TRPC1* and *TRPC4* mRNAs by using the tandem shRNA construct induced a decrease in OT-stimulated SRCE that was not significantly greater than the decrease obtained after knockdown of either *TRPC1* or *TRPC4* alone (Fig. 2A). Thapsigargin-stimulated SRCE was not significantly affected by *TRPC1*, *TRPC4*, or *TRPC1* plus *TRPC4* mRNA knockdown in either UtSMC or PHM1-41 cells (Fig. 2B). Similarly, none of these shRNA combinations had any effect on OAG-stimulated SRCE (data not shown). Therefore, *TRPC1* mRNA knockdown, like *TRPC4* knockdown [15], resulted in specific attenuation of GPCR-mediated SRCE.

Calcium Responses to GPCR Stimulation and SERCA Inhibition Are Consistent with Fura-2 and Mag-fluo-4 Measuring Changes in Myometrial Cell $[Ca^{2+}]_i$ and $[Ca^{2+}]_L$, Respectively

Differential loading of Mag-fluo-4 and Fura-2 has previously been reported to produce changes in $[Ca^{2+}]_L$ and $[Ca^{2+}]_i$, respectively, in pregnant rat uterine myocytes [10, 11]. Our results, obtained with human myometrial cells, are consistent with those observations and further validate the use of this approach. OT elicited a rapid but transient increase in $[Ca^{2+}]_i$ in PHM1-41 cells and a decrease in $[Ca^{2+}]_L$ in the absence of extracellular Ca^{2+} (Fig. 3A). Subsequent addition of 1 mM Ca^{2+} resulted in an increase in $[Ca^{2+}]_i$ (SRCE), as previously reported [24, 25]. This was accompanied by a return of $[Ca^{2+}]_L$ to basal levels, reflecting refilling of the ER store. Neither event occurred if Ca^{2+} -free buffer (0 Ca) was added instead, indicating that the changes were completely dependent on extracellular Ca^{2+} . Thapsigargin, which irreversibly inhibits SERCA pumps and elicits ER Ca^{2+} store depletion, increased $[Ca^{2+}]_i$ and produced a greater decline in $[Ca^{2+}]_L$ than OT (Fig. 3B). The addition of 1 mM extracellular Ca^{2+} after thapsigargin resulted in an increase in $[Ca^{2+}]_i$ (SRCE), but, consistent with the inhibition of SERCA, there was only a small increase in $[Ca^{2+}]_L$. This small increase in $[Ca^{2+}]_L$ was

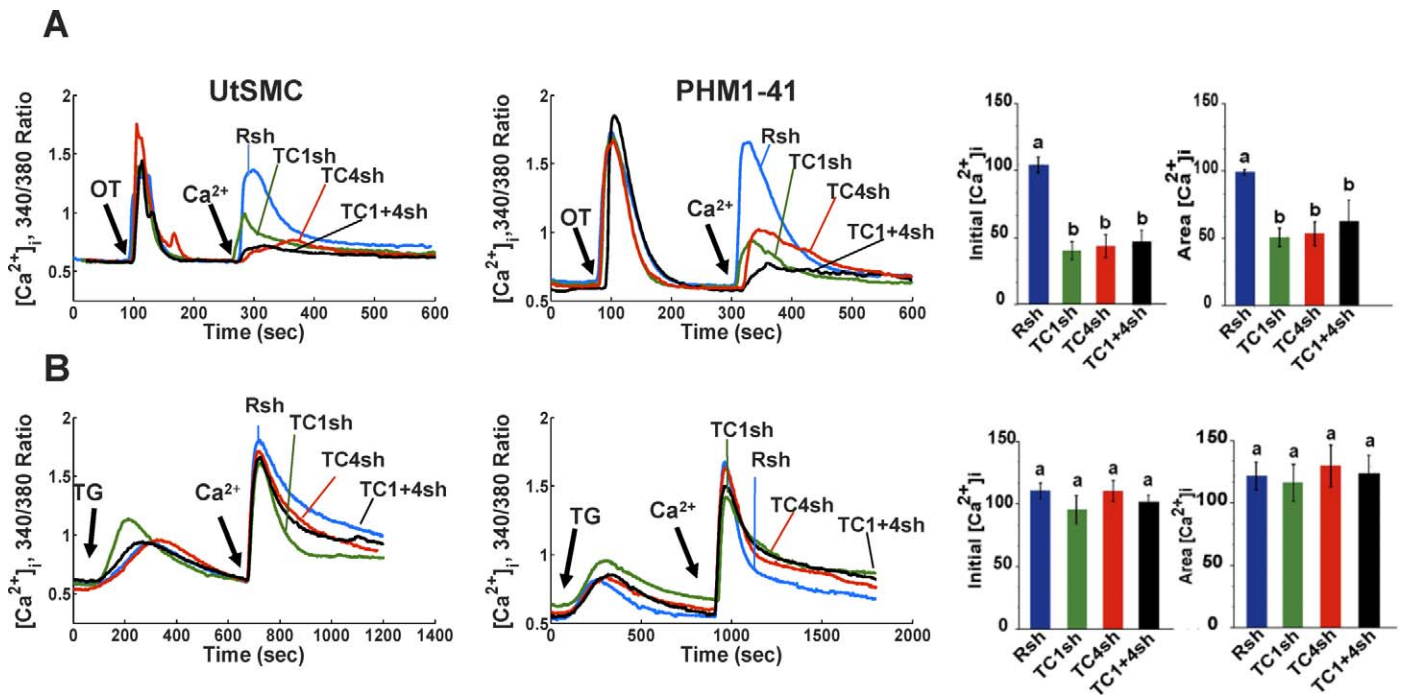


FIG. 2. *TRPC1*, *TRPC4*, and *TRPC1* plus *TRPC4* mRNA knockdown induces specific inhibition of OT-stimulated SRCE in UtsMC (left panels) and PHM1-41 (middle panels) cells. **A** Attenuation of SRCE induced by 100 nM OT in cells infected with a control shRNA Rsh (Rsh, blue lines) or adenovirus expressing *TRPC1* (TC1sh, green lines), *TRPC4* (TC4sh, red lines), or *TRPC1* plus *TRPC4* shRNAs (TC1+4sh, black lines) is shown. The addition of 1 mM Ca^{2+} that initiates SRCE is indicated. Traces represent the mean responses of 10–35 cells. **B** No effect of these shRNAs was observed on thapsigargin (TG, 100 nM)-stimulated SRCE. Right panels: Mean changes in $[\text{Ca}^{2+}]_i$ (**A** and **B**), calculated as peak height (initial $[\text{Ca}^{2+}]_i$) and integrated area under the curve ($[\text{Ca}^{2+}]_i$ area), are shown. As no significant differences were observed in responses from UtsMC and PHM1 cells, data from these sources were pooled for this analysis. Data are presented as means \pm SEM ($n = 6$ –8).

not eliminated by the use of a higher concentration of thapsigargin (1 μM) and was observed in cells exposed to an equivalent amount of vehicle (0.1% DMSO) (data not shown).

Similar to the effects of thapsigargin, the addition of 1 mM extracellular Ca^{2+} after exposure to CPA, a reversible SERCA inhibitor, produced an increase in $[\text{Ca}^{2+}]_i$ but only a small increase in $[\text{Ca}^{2+}]_L$ (Fig. 3C). However, when CPA was washed out before the addition of 1 mM extracellular Ca^{2+} , in addition to the increase in $[\text{Ca}^{2+}]_i$, significant ER store refilling also occurred. These data are consistent with prior reports [10, 11] that Fura-2 and Mag-fluo-4 are simultaneously measuring changes in $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_L$, respectively, and show that increases in both compartments occur following introduction of Ca^{2+} into the extracellular medium subsequent to stimulation of human myometrial cells as described.

SRCE and ER Ca^{2+} Store Refilling Are Not Inhibited by Inhibitors of L- or T-Type Channels or Reverse Mode $\text{Na}^+/\text{Ca}^{2+}$ Exchanger Activity But Are Attenuated by Gadolinium

Inhibitors were used to assess the contribution of different types of Ca^{2+} entry mechanisms to myometrial cell ER store refilling after decreases in $[\text{Ca}^{2+}]_L$. Gadolinium (10^{-4} M) inhibited OT-induced SRCE and slowed ER store refilling (Fig. 4A). The effect of gadolinium was concentration-dependent and was statistically different from that of control at 5×10^{-5} M and 10^{-5} M for OT-stimulated SRCE and ER store refilling, respectively (Fig. 4C). In contrast, OT-induced SRCE and ER refilling were not inhibited by 10^{-7} M nifedipine, a concentration of the L-type channel blocker that effectively inhibits OAG-

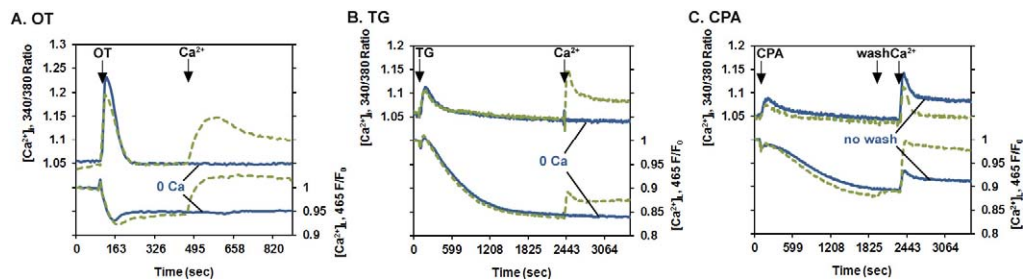


FIG. 3. Changes in Fura-2 and Mag-fluo-4 fluorescence are consistent with the expected changes in PHM1-41 myometrial cell ($[\text{Ca}^{2+}]_i$) and ($[\text{Ca}^{2+}]_L$), respectively. Cells loaded with the dyes were exposed to (A) OT (100 nM), (B) thapsigargin (TG, 100 nM), or (C) CPA (10 μM) in the absence of extracellular Ca^{2+} . After Ca^{2+} changes stabilized, cells were exposed to Ca^{2+} -free buffer (0 Ca, blue line) or 1 mM extracellular Ca^{2+} (green line) in **A** and **B**. **C** CPA was added as described for thapsigargin and was then removed from one group of cells by washing with calcium-free buffer (wash) at the time indicated. Both the washed (green line) and unwashed (blue line) cells were then exposed to 1 mM extracellular Ca^{2+} . Additions of OT, CPA, wash, and Ca^{2+} are indicated by arrows. Each line represents an average of the responses of 35–40 cells in one of three similar experiments.

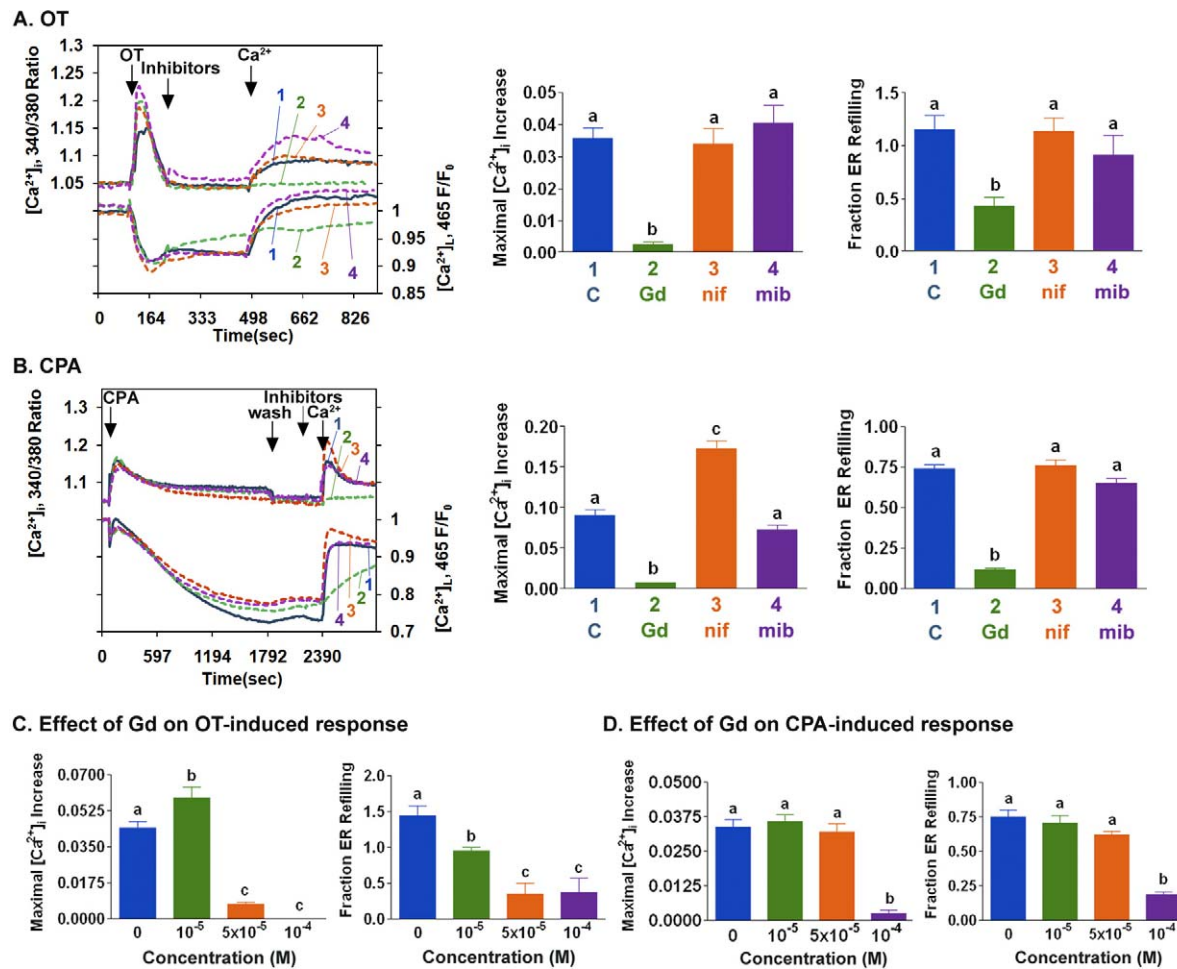


FIG. 4. **A)** After stimulation with OT (100 nM) in the absence of extracellular Ca²⁺, PHM1-41 cells were exposed to buffer (blue line, 1), 10⁻⁴ M gadolinium (green line, 2), 10⁻⁷ M nifedipine (orange line, 3), or 10⁻⁶ M mibefradil (purple line, 4) for 4 min before the addition of 1 mM Ca²⁺. The traces represent the mean of responses in 35–40 cells in one of three experiments. Bar graphs summarize the effect of SRCE inhibitors on OT-stimulated responses, expressed as maximal [Ca²⁺]_i increase and fraction of ER store refilling at 1 min. Data represent the means ± SEM of responses from 90–120 cells. **B)** After stimulation with CPA (10 μM) in the absence of extracellular Ca²⁺, PHM1-41 cells were washed with Ca²⁺-free buffer for 10 min (wash) and then exposed for 4 min to the same compounds as shown in **A** before addition of 1 mM Ca²⁺. Each line represents an average of the responses of 35–40 cells in one of three similar experiments. Bar graphs represent the means ± SEM of responses from 90–120 cells. **A** and **B**) Additions of OT, CPA, wash, and Ca²⁺ are indicated by arrows. Concentration dependence of the effect of gadolinium (Gd) on OT-induced (**C**) and CPA-induced (**D**) responses (means ± SEM, n = 35–40 cells) is shown.

stimulated increases in [Ca²⁺]_i in myometrial cells [16], or by 10⁻⁶ M mibefradil, a T-type calcium channel blocker that inhibits T-type electrical activity and contractions in human myometrium (Fig. 4A) [26]. CPA-induced SRCE was significantly inhibited, and ER store refilling was slowed by 10⁻⁴ M gadolinium (Fig. 4, B and D) but not by 10⁻⁷ M nifedipine or 10⁻⁶ M mibefradil (Fig. 4B). No effects on OT or CPA responses were observed at nifedipine concentrations of up to 10⁻⁶ M (data not shown). Similar effects of these compounds on CPA-stimulated SRCE and ER store refilling were also seen in HMC cells; modest inhibition of OT-stimulated SRCE by nifedipine and mibefradil was observed (Supplemental Fig. S1, available online at www.biolreprod.org).

Some TRPC channels appear to activate L-type Ca²⁺ channels as a consequence of facilitating Na⁺ entry and associated membrane depolarization [27, 28]. Neither OT- nor CPA-stimulated SRCE or ER store refilling following addition of 1 mM Ca²⁺ were significantly affected by the absence of extracellular Na⁺ or the presence of the Na⁺/Ca²⁺ exchanger reverse mode inhibitor KB-R7943 in PHM1-41 cells (Fig. 5), suggesting that these mechanisms do not pertain.

TRPC1 Knockdown Specifically Attenuates OT-Stimulated SRCE But Does Not Significantly Affect Myometrial ER Store Refilling

In PHM1-41 cells loaded with both Fura-2 and Mag Fluo-4, adenoviral-mediated reduction in *TRPC1* shRNA attenuated OT-stimulated SRCE (Fig. 6A, left panel). SRCE was reduced by 41% ($P < 0.001$) in PHM1-41 cells and by 52% in HMC cells ($P < 0.01$) (Fig. 6A, right panel). Because the amount of ER store depletion was relatively small and there was some store refilling in the absence of extracellular Ca²⁺, the sensitivity of our system did not permit accurate assessment of initial rates of ER store refilling following OT stimulation. Nonetheless, as shown in Figure 6B, there appeared to be a trend toward slower store refilling in PHM1-41 (Fig. 6B, upper graph) and HMC (Fig. 6B, lower graph) cells expressing *TRPC1* shRNA than in cells infected with control virus.

In contrast to the inhibitory effects on OT-stimulated SRCE, *TRPC1* knockdown did not significantly affect CPA-stimulated SRCE in PHM1-41 or HMC cells (Fig. 6C) and did not inhibit ER store refilling (data not shown). No effects of expression of

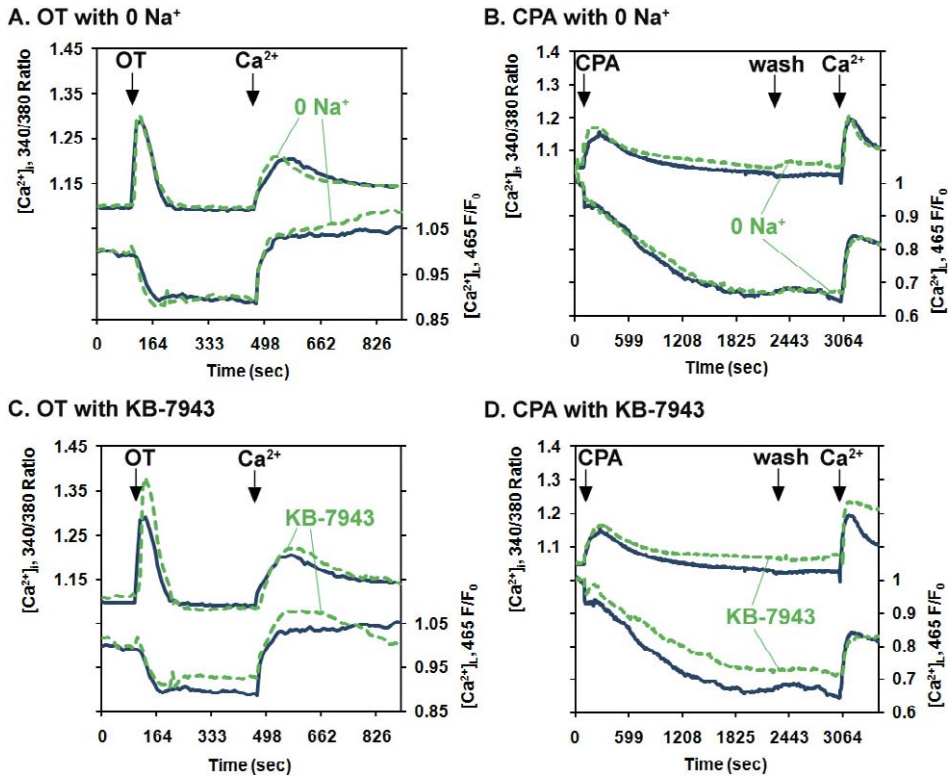


FIG. 5. Removing extracellular Na^+ or exposing PHM1-41 myometrial cells to the $\text{Na}^+/\text{Ca}^{2+}$ exchanger inhibitor KB-R7943 had no effect on SRCE and ER store depletion stimulated by oxytocin or CPA or the refilling of the ER stores following addition of 1 mM extracellular Ca^{2+} . Cells in medium in which choline chloride was substituted for NaCl (green line) were exposed to 100 nM OT (A) or 10 μM CPA (B) as described in the legend to Figure 4. Cells in normal FB were exposed to 10 μM KB-R7943 (green line) and then treated with OT (C) or with CPA (D). Each line represents an average of the responses of 35–40 cells in one of three similar experiments.

TRPC1 shRNA on the ability of OT or CPA to produce the initial increase in $[\text{Ca}^{2+}]_i$ in the absence of extracellular $[\text{Ca}^{2+}]$ were apparent in either cell type.

STIM1 and *Orai1–Orai3* Influence Myometrial SRCE and ER Store Refilling

In a number of other systems, *STIM1* and *Orai1* proteins have been implicated in store depletion-mediated Ca^{2+} entry

mechanisms. In order to design shRNAs to target the most abundant forms, we determined the relative expression of *STIM1* and *Orai1* mRNA isoforms in myometrial cells. Figure 7A shows that *STIM2* mRNA is significantly less abundant than *STIM1* mRNA in myometrial cells. Although *Orai2* and *Orai3* mRNAs were less abundant than *Orai1* mRNA in PHM1-41 cells, the differences were less apparent in HMC and UtSMC cells. Based on these data, we created *STIM1* and *Orai1–Orai3* shRNA tandem viruses expressing three copies

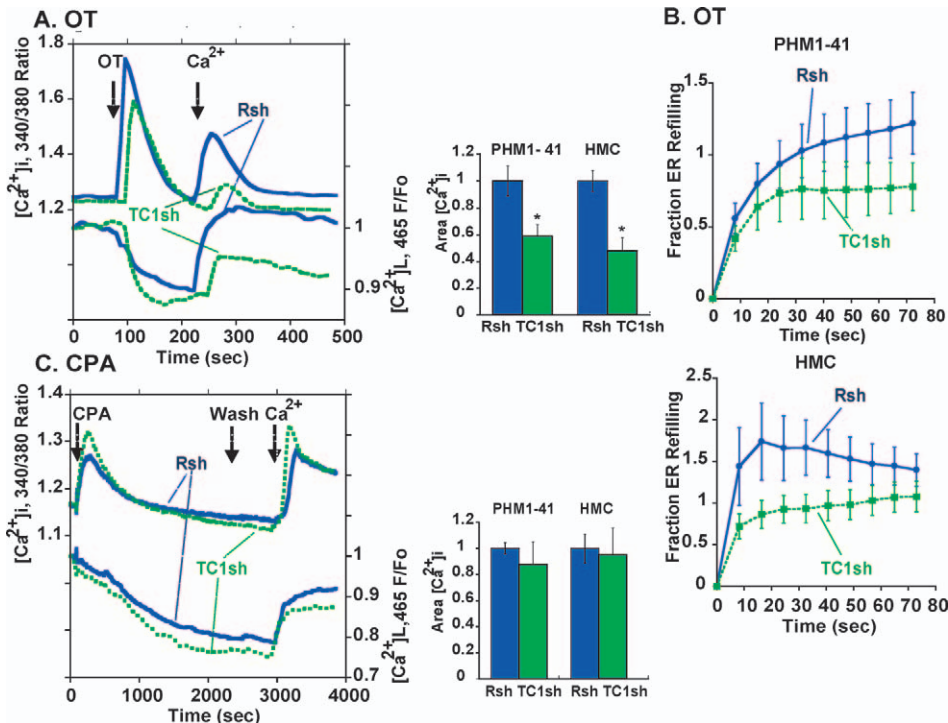


FIG. 6. Effects of *TRPC1* knockdown on SRCE and ER store depletion and refilling following treatment of myometrial cells with OT and CPA, as described in the legend to Figure 4, are shown. A) Tracings in the left panel represent the mean responses of 10–15 PHM1-41 cells infected with control virus (Rsh, blue lines) or adenovirus expressing *TRPC1* shRNA (TC1sh, green lines). The middle panel presents the mean changes in integrated SRCE area in PHM1-41 and HMC cells ($n = 10–11$). B) The fraction of ER refilling after OT stimulation and Ca^{2+} addition in cells infected with control (Rsh, blue line) or *TRPC1* (TC1sh, green line) shRNAs in PHM1 cells (upper graph) and HMC cells (lower graph) ($n = 9–11$). C) Effects of *TRPC1* mRNA knockdown on CPA-stimulated responses. Data are presented as described in the legend to A ($n = 4–6$).

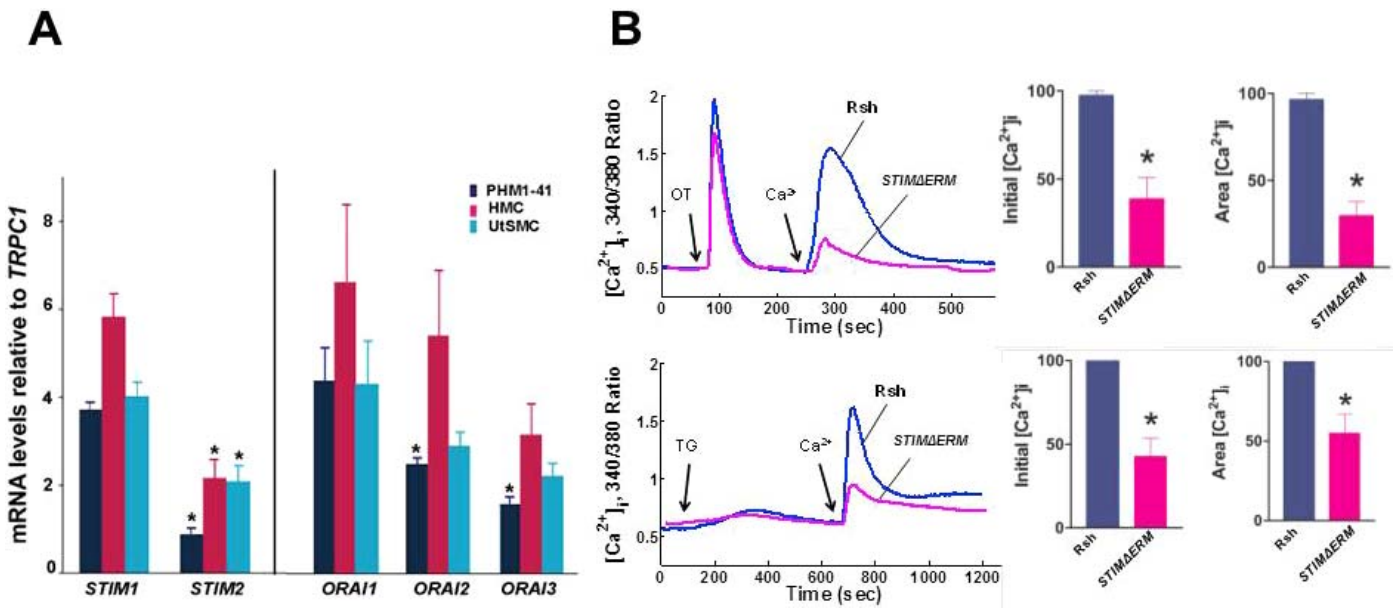


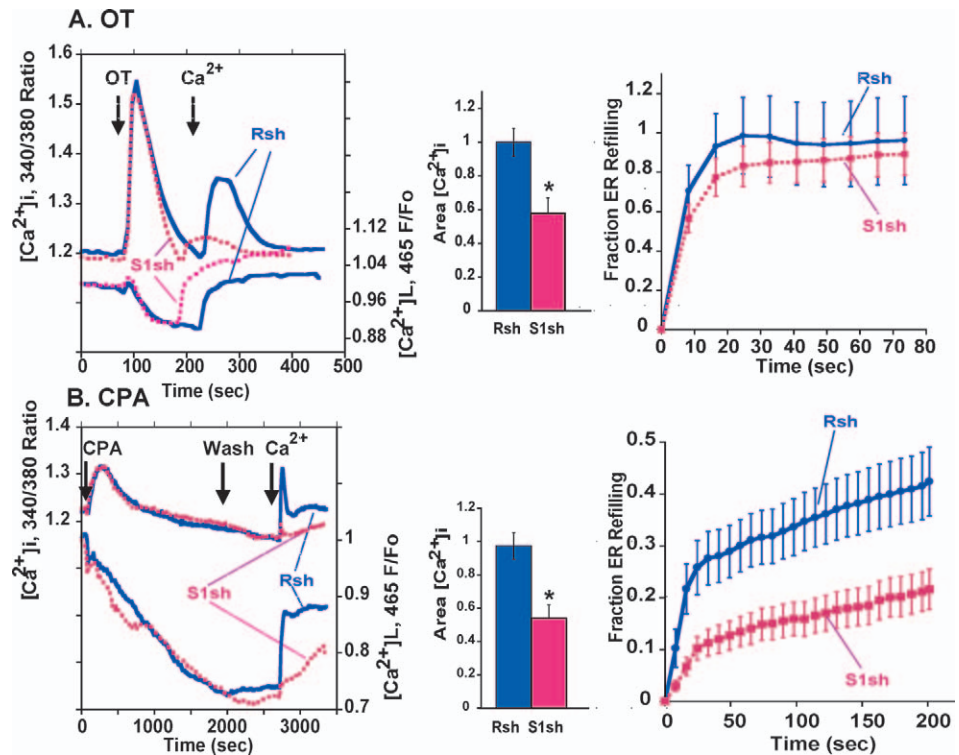
FIG. 7. A) Relative expression of *STIM* and *ORA1* mRNA isoforms are shown in PHM1-41, HMC, and UtsMC myometrial cells, each compared to *STIM1* and *ORA11* mRNA for that cell type ($n = 3-4$). B) *STIM1ΔERM* significantly inhibits OT and thapsigargin SRCE in UtsMC cells. Representative tracing (left) of mean SRCE induced by 100 nM OT or 100 nM thapsigargin (TG) in 10–15 cells infected with either control (Rsh, solid line) or adenovirus expressing *STIM1ΔERM* (dotted line) is shown. Mean changes in initial $[Ca^{2+}]_i$ peak height (middle) and integrated SRCE area (right) compared to control ($n = 5-7$).

of *STIM1* shRNA or one single copy each of *ORA11*, *ORA12*, and *ORA13* shRNAs. The *STIM1* shRNA vector achieved an average of 61% and 64% knockdown of *STIM1* mRNA in PHM1-41 and HMC cells, respectively. The tandem *ORA11-ORA13* shRNA vector produced knockdowns in *ORA11*, *ORA12*, and *ORA13* mRNAs of 94%, 55%, and 31%, respectively, in PHM1-41 cells and 93%, 37%, and 45%, respectively, in HMC cells. *STIM1* and *ORA11-ORA13* mRNA knockdowns did not affect the concentrations of *TRPC1*, *TRPC4*, or *TRPC6* mRNA (data not shown). In addition to

these constructs, we generated a recombinant adenovirus expressing *STIM1ΔERM*, a dominant negative *STIM1* form that interferes with the interaction between *STIM1* and *ORA11* proteins [29].

Infection with virus expressing *STIM1ΔERM* attenuated both OT- and thapsigargin-stimulated SRCE (Fig. 7B). Expression of *STIM1* shRNA attenuated CPA-stimulated SRCE and the rate of ER store refilling compared to control in PHM1-41 cells (Fig. 8B). Mean initial rates were 2.1 ± 0.6 versus 0.7 ± 0.2 arbitrary units/sec for control and *STIM1* shRNA, respectively

FIG. 8. Expression of *STIM1* shRNA attenuated OT- and CPA-stimulated SRCE in PHM1-41 cells is shown. A) Tracings (left panel) represent the mean responses to OT stimulation and Ca^{2+} addition of 10–15 cells infected with control virus (Rsh, blue lines) or adenovirus expressing *STIM1* shRNA (S1sh, pink lines). The middle panel presents the mean changes in integrated SRCE area ($n = 16-17$). The fraction of ER refilling in cells infected with control (Rsh, blue line) or *STIM1* (S1sh, pink line) shRNA is shown in the right panel ($n = 16-17$). B) Effects of *STIM1* mRNA knockdown on CPA-stimulated responses are shown. Data are presented as described in the legend to A ($n = 24-29$ dishes).



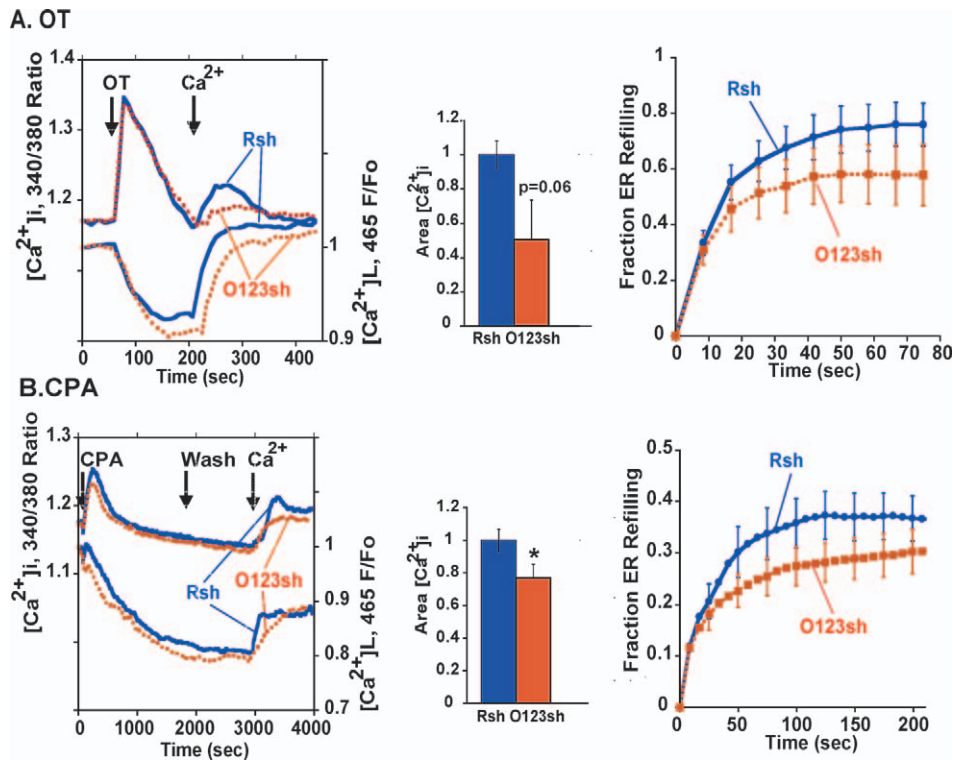


FIG. 9. Effects of *ORAI1*, *ORAI2*, and *ORAI3* tandem shRNA expression on OT- and CPA-stimulated SRCE and ER refilling in PHM1-41 cells are shown. **A)** Effects of *ORAI1–ORAI3* mRNA knockdown on OT-stimulated responses. Data are presented as described in the legend to Figure 8 (control adenovirus (Rsh, blue lines); *ORAI1–ORAI3* shRNA (O123sh, orange lines); $n = 10–11$). **B)** Effects of *ORAI1*, *ORAI2*, and *ORAI3* mRNA knockdown on CPA-stimulated responses are shown. Data are presented as described in the legend to **A** ($n = 16–17$).

($P < 0.05$, $n = 25$ and 29). *STIM1* mRNA knockdown also inhibited OT-stimulated SRCE but had no significant effect on ER store refilling in PHM1-41 cells (Fig. 8A). In HMC cells, *STIM1* shRNA knockdown also significantly attenuated CPA-stimulated SRCE (Supplemental Fig. S2B). While there was a trend toward decline in the rate of ER store refilling, neither the initial rate nor the values at selected time points were significantly different from those of control. *STIM1* knockdown attenuated OT-stimulated SRCE in HMC cells, and there was a trend toward a slowing of ER store refilling (Supplemental Fig. S2A).

Knockdown of *ORAI1*, *ORAI2*, and *ORAI3* mRNAs suppressed CPA-stimulated SRCE, and, whereas there was no significant effect on initial rate or at selected time points, there was a trend toward a slowing of ER store refilling in PHM1-41 cells (Fig. 9B). *ORAI1–ORAI3* suppression attenuated OT-stimulated SRCE but had no significant effect on ER store refilling (Fig. 9A). In HMC cells, knockdown of *ORAI1–ORAI3* mRNAs attenuated CPA-stimulated SRCE and significantly slowed store refilling (initial rates of 2.7 ± 0.5 versus 0.9 ± 0.2 arbitrary units/sec for control *ORAI1–ORAI3* shRNA, respectively; $n = 13$) (Supplemental Fig. S3B) and attenuated OT-stimulated SRCE but had no significant effect on ER store refilling. No consistent effects of *STIM1* or *ORAI1–ORAI3* mRNA knockdowns on OT- or CPA-stimulated increases in $[\text{Ca}^{2+}]_i$ in the absence of extracellular $[\text{Ca}^{2+}]$ were observed in either cell type.

DISCUSSION

Data presented here provide strong evidence for the involvement of TRPC1, STIM1, and ORAI1–ORAI3 proteins in OT-stimulated SRCE and of STIM1 and ORAI1–ORAI3 in CPA-stimulated SRCE, thus reinforcing a distinction in human myometrium between receptor-operated and classical store-operated SRCE mechanisms [15] while identifying some

commonalities in the regulation of cytoplasmic intracellular Ca^{2+} . Furthermore, the kinetic measurements presented here suggest that *STIM1* or *ORAI1–ORAI3* mRNA knockdowns slow the rate of ER store replenishment following removal of SERCA inhibition.

TRPC channels have been implicated in both GPCR-stimulated and store depletion-stimulated increases in $[\text{Ca}^{2+}]_i$ in response to addition of extracellular Ca^{2+} [8, 13, 14]. TRPC1 expression plays an important role in the formation of heterotetramers with other TRPCs and may contribute to the unique characteristics of these channels in a given cellular setting. The effect of *TRPC1* knockdown in human myometrial cells specifically on OT-stimulated SRCE is similar to the effect of *TRPC4* knockdown [15]. The combined knockdown of *TRPC1* plus *TRPC4* was no more effective in inhibiting OT-stimulated SRCE than responses obtained from single *TRPC1* or *TRPC4* knockdowns, suggesting that both proteins may be contributing to the same GPCR-mediated SRCE response, either together or separately. In agreement with these results, knockdown of either *TRPC1* or *TRPC4* had no effect on thapsigargin-stimulated $[\text{Ca}^{2+}]_i$ increases or on CRAC currents in endothelial cells [30], and single and combined *TRPC1*, *TRPC4*, or *TRPC6* knockdowns had no effect on thapsigargin-stimulated $[\text{Ca}^{2+}]_i$ increases in vascular SMCs [31]. In contrast, in a number of other cell types, shRNAs or antisense nucleotides targeted against *TRPC1* and/or *TRPC1* plus *TRPC4* decreased thapsigargin-induced membrane currents and $[\text{Ca}^{2+}]_i$ increases [32–36]. These apparently contradictory results in different cell types may be due to differences in the relative abundance of TRPC isoforms expressed and hence the nature of the TRPC channels formed, as well as to differences in regulatory coupling and modulation of activity.

The ER functions as an intracellular Ca^{2+} store that plays complex roles in the regulation of myometrial Ca^{2+} dynamics. In response to an increase in $[\text{Ca}^{2+}]_i$, SERCA contributes to the sequestration of a portion of this Ca^{2+} and, along with the

plasma membrane pump and $\text{Na}^+/\text{Ca}^{2+}$ exchanger, is responsible for the decline in $[\text{Ca}^{2+}]_i$ [1, 6, 7, 10]. Depending on the circumstances, the ER can refill its Ca^{2+} store and/or deliver Ca^{2+} to the plasma membrane pumps and exchangers for efflux, thus protecting the cell from the dangers of elevated $[\text{Ca}^{2+}]_i$ and dampening contractile activity.

Fura-2 and Mag-fluo-4 are effective for measuring relative changes in cytoplasmic and ER Ca^{2+} , respectively, both because of their differential affinities for Ca^{2+} and the buffering capacities that exist in both compartments [11, 37]. In the present study, the responses to agents known to elicit a decrease in $[\text{Ca}^{2+}]_L$ followed the expected dependence on extracellular Ca^{2+} for both rises in $[\text{Ca}^{2+}]_i$ and ER Ca^{2+} store refilling, demonstrating the usefulness of this approach for use in human myometrial cells. Thus, OT elicited a transient increase in $[\text{Ca}^{2+}]_i$ and a decrease in $[\text{Ca}^{2+}]_L$ in the absence of extracellular Ca^{2+} , as expected from its ability to stimulate phospholipase C activity and generate IP_3 [2]. The refilling of ER Ca^{2+} stores was partially dependent on addition of extracellular Ca^{2+} , similar to what has been reported for ATP-stimulated store depletion in rat myometrial cells [11]. The irreversible SERCA inhibitor thapsigargin produced a sustained decrease in $[\text{Ca}^{2+}]_L$ that was not reversed by addition of extracellular Ca^{2+} , whereas the reversible SERCA inhibitor CPA elicited a similar decrease in $[\text{Ca}^{2+}]_L$, but the store was rapidly refilled following addition of extracellular Ca^{2+} after CPA washout.

L-type Ca^{2+} channel blockers inhibit Ca^{2+} entry following myometrial cell membrane depolarization and have marked inhibitory effects on spontaneous and agonist-induced uterine contractile activity [1, 26, 38–40]. T-type Ca^{2+} channels have been implicated in the initiation of action potentials and in spontaneous contractile activity in myometrium [26, 40]. With time in culture, myometrial cells such as those used in this study tend to lose robust responses to iso-osmotic KCl-dependent depolarization, indicative of dampened voltage-dependent responses. Nonetheless, we have observed that these cells express mRNA for the α subunit of *Ca_v1.2* (our unpublished observations). Our previous finding of a TRPC6-mediated mechanism that is inhibited by removing extracellular Na^+ and by nifedipine [16], consistent with a previous report linking TRPC6 to Na^+ entry and L-type channel activation [27], is the only evidence to date that L-type channels are still functional to some extent in PHM1-41 and primary myometrial cells in culture. In the present study, CPA-stimulated increases in $[\text{Ca}^{2+}]_i$ and ER store refilling in PHM1-41 or primary myometrial cells were not inhibited by nifedipine or mibefradil, suggesting that voltage-activated channels do not play a direct role in either GPCR- or store depletion-stimulated SRCE or the subsequent ER store refilling following addition of extracellular Ca^{2+} in these cells. Similar effects were found in the primary cells, apart from a modest inhibition of OT-stimulated SRCE by nifedipine and mibefradil, which may be consistent with effects of these compounds on SRCE mechanisms under some conditions [41]. The responses in zero Na^+ and with KB-R7943 also rule out reverse-mode operation of $\text{Na}^+/\text{Ca}^{2+}$ exchangers in these mechanisms. It remains to be determined, however, whether these conclusions also pertain in acutely isolated human cells displaying robust L-type and/or T-type currents.

Lanthanides in μM concentrations are considered relatively specific inhibitors of store-operated channels, but TRPC4 and TRPC5 proteins are potentiated by μM and inhibited by mM concentrations of gadolinium [42]. Cells with TRPC3, TRPC5, or TRPC6 but not TRPC1 overexpression exhibited carbachol-stimulated SRCE in the presence of 5 μM gadolinium, which

was used to suppress endogenous store-operated channels [22]. In the present study, both OT- and CPA-stimulated SRCE and ER store refilling were attenuated by gadolinium, but it is not possible to infer with certainty which specific channels are affected, from these observations.

Thapsigargin- and CPA-stimulated SRCE in human myometrial cells is sensitive to reduction of *STIM1* and *ORAI1–ORAI3* mRNAs but is not attenuated by *TRPC1*, *TRPC4*, or *TRPC6* [16] mRNA knockdown. This finding is consistent with the identification of STIM and ORAI proteins as comprising store-operated channels that give rise to the CRAC current and are activated by SERCA inhibitors in other systems [18–21]. The attenuation of OT-stimulated SRCE by *STIM1* and by *ORAI1–ORAI3*, as well as by *TRPC1* and *TRPC4*, mRNA knockdowns is consistent with emerging evidence suggestive of potential interactions among STIM1, ORAI1, and TRPC [18, 19, 21, 33, 36, 43]. Interestingly, STIM1 uses different interaction domains to activate ORAI1 and TRPCs, and both STIM-dependent and STIM1-independent modes of TRPC function have been described [18, 19, 44, 45]. TRPC channels are organized into microdomains, and this can affect their assembly with STIM1 and ORAI1 [33, 36, 43]. These assemblies may depend on cell-specific properties and signals and remain to be defined in myometrium.

To our knowledge, there is only one study of the effects of STIM1 knockdown on the rate of ER store refilling in any cell type and no study of the effects of ORAI on this parameter. Jousset et al. [46] reported an inhibitory effect of STIM1 knockdown on both GPCR- and thapsigargin-mediated SRCE in HeLa cells. Using transfected reporters to measure $[\text{Ca}^{2+}]_i$ and $[\text{Ca}^{2+}]_L$ simultaneously, they found that STIM1 knockdown slowed the rate of ER refilling following histamine stimulation but that the ER store eventually refilled even though there was no detectable increase in $[\text{Ca}^{2+}]_i$. Overall, our data also support the concept that the ER stores in myometrial cells can refill, albeit at a slower rate, when *STIM1* or *ORAI* mRNA concentrations are reduced. Our findings and those of Jousset et al. [46] are consistent with the observation that in response to decreases in $[\text{Ca}^{2+}]_L$, STIM1 and ORAI1 form punctae indicative of close apposition of plasma membrane and ER membranes, making it possible to refill ER Ca^{2+} stores via channel-mediated Ca^{2+} influx through these microdomains, without significant increases $[\text{Ca}^{2+}]_i$ detectable by Fura-2.

Because of the marked dependence of prolonged myometrial spontaneous and hormone-stimulated activity on extracellular Ca^{2+} and L-type channel activity, a physiological role for capacitative Ca^{2+} entry in the myometrium has been questioned [1]. Nonetheless, a preliminary report of CPA-stimulated SRCE and increase in basal force that is nifedipine-insensitive but inhibited by SKF96365 in pregnant rat myometrium, slightly different responses in nonpregnant rat myometrium, and reference to unpublished effects of OT on voltage-independent calcium transients inhibited by CPA depletion of ER stores [5] suggest functionality of CPA- and GPCR-mediated capacitative mechanisms in rat myometrium. Furthermore, Shimamura et al. [47] reported that OT elicited a long-lasting nonselective cation current in late pregnant rat myometrium. Therefore, the evidence in favor of a physiological role for SRCE in myometrium is growing.

Our studies defining components of the SRCE mechanism in myometrium were carried out in primary and immortalized human myometrial cells to facilitate evaluation of individual cell responses. Assessing the effect of these same knockdowns on human myometrial tissue function is logistically more difficult and will take additional time to accomplish. Nonetheless, it is interesting to speculate on the potential significance of

these findings. Uterine contractants such as OT increase [Ca²⁺]_i by releasing ER Ca²⁺ and stimulating Ca²⁺ entry through SRCE mechanisms involving TRPC1, TRPC4, STIM1, and ORAI1–ORAI3. While these mechanisms are independent of L-type channel involvement, they also generate local OAG that could potentially stimulate TRPC6 and L-type channels through protein kinase C activation. STIM1 has also recently been shown to inhibit Ca_v1.2 L-type Ca²⁺ channels [48, 49], suggesting that GPCRs might stimulate the formation of complexes containing some combination of TRPC, STIM, and ORAI in microdomains where subtle temporal regulation of other proteins such as Ca_v1.2 could occur. In the myometrium such TRPC complexes in specialized subcellular environments might locally influence the pattern of [Ca²⁺]_i and, in turn, the pattern of contractions. Interestingly, the study by Shimamura et al. [47] reported an OT-stimulated nonselective cation current and also found that OT partially inhibited L-type currents. There are few clues in the literature as to what might be the physiological equivalent of chemical inhibition of SERCA. In this regard, Gehrig-Burger et al. [50] reported that high progesterone concentrations inhibit OT-stimulated uterine contractions and deplete intracellular ER Ca²⁺ stores in HEK293 cells, and they speculate that this action of progesterone may contribute to uterine quiescence during pregnancy. Clearly, there is still much to be learned about the interactions among and influence of the many components that regulate [Ca²⁺]_i and ER Ca²⁺ in the myometrium. Because of their ubiquitous nature, we consider it unlikely that targeting ORAI or STIM1 would produce myometrial-specific effects on Ca²⁺ dynamics. On the other hand, the species- and tissue-specific patterns of TRPC protein expression and the distinctive effects of TRPC1, TRPC4, and TRPC6 knockdowns on human myometrial cells suggest that they might be potential targets for tocolytic intervention if specific inhibitors can be developed.

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REFERENCES

- Wray S. Insights into the uterus. *Exp Physiol* 2007; 92:621–631.
- Sanborn B. Hormonal signaling and signal pathway crosstalk in the control of myometrial calcium dynamics. *Semin Cell Dev Biol* 2007; 18:305–314.
- Garfield RE, Maner WL. Physiology and electrical activity of uterine contractions. *Semin Cell Dev Biol* 2007; 18:289–295.
- Wray S, Shmygol A. Role of the calcium store in uterine contractility. *Semin Cell Dev Biol* 2007; 18:315–320.
- Noble K, Matthew A, Burdya T, Wray S. A review of recent insights into the role of the sarcoplasmic reticulum and Ca entry in uterine smooth muscle. *Eur J Obstet Gynecol Reprod Biol* 2009; 144(suppl 1):S11–S19.
- Shmygol A, Eisner D, Wray S. The role of the sarcoplasmic reticulum as a Ca²⁺ sink in rat uterine smooth muscle cells. *J Physiol* 1999; 520:153–163.
- Shmygol A, Wray S. Functional architecture of the SR calcium store in uterine smooth muscle. *Cell Calcium* 2004; 35:501–508.
- Ramsey S, Delling M, Clapham DE. An introduction to TRP channels. *Annu Rev Physiol* 2006; 68:619–647.
- Putney JW. New molecular players in capacitative Ca²⁺ entry. *J Cell Sci* 2007; 120:1959–1965.
- Shmygol AV, Eisner DA, Wray S. Simultaneous measurements of changes in sarcoplasmic reticulum and cytosolic. *J Physiol* 2001; 531:707–713.
- Shmygol A, Wray S. Modulation of agonist-induced Ca²⁺ release by SR Ca²⁺ load: direct SR and cytosolic Ca²⁺ measurements in rat uterine myocytes. *Cell Calcium* 2005; 37:215–223.
- Ku C, Babich L, Word R, Zhong M, Ulloa A, Monga M, Sanborn BM. Expression of transient receptor channel proteins in human fundal myometrium in pregnancy. *J Soc Gynecol Invest* 2006; 13:217–225.
- Gees M, Colsoul B, Nilius B. The role of transient receptor potential cation channels in Ca²⁺ signaling. *Cold Spring Harb Perspect Biol* 2010; 2:a003962.
- Wu LJ, Sweet TB, Clapham DE. International union of basic and clinical pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. *Pharmacol Rev* 2010; 62:381–404.
- Ulloa A, Gonzales AL, Zhong M, Kim YS, Cantlon J, Clay C, Ku CY, Earley S, Sanborn BM. Reduction in TRPC4 expression specifically attenuates G-protein coupled receptor-stimulated increases in intracellular calcium in human myometrial cells. *Cell Calcium* 2009; 46:73–84.
- Chung D, Kim YS, Phillips JN, Ulloa A, Ku CY, Galan HL, Sanborn BM. Attenuation of canonical transient receptor potential-like channel 6 expression specifically reduces the diacylglycerol-mediated increase in intracellular calcium in human myometrial cells. *Endocrinology* 2010; 151:406–416.
- Sun D, Melegari M, Sridhar S, Rogler CE, Zhu L. Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotechniques* 2006; 41:59–63.
- Cahalan MD. STIMulating store-operated Ca(2+) entry. *Nat Cell Biol* 2009; 11:669–677.
- Deng X, Wang Y, Zhou Y, Soboloff J, Gill DL. STIM and Orai: dynamic intermembrane coupling to control cellular calcium signals. *J Biol Chem* 2009; 284:22501–22505.
- Smyth JT, Hwang SY, Tomita T, DeHaven WI, Mercer JC, Putney JW. Activation and regulation of store-operated calcium entry. *J Cell Mol Med* 2010; 14:2337–2349.
- Lee KP, Yuan JP, Hong JH, So I, Worley PF, Muallem S. An endoplasmic reticulum/plasma membrane junction: STIM1/Orai1/TRPCs. *FEBS Lett* 2010; 584:2022–2027.
- DeHaven WI, Jones BF, Petranka JG, Smyth JT, Tomita T, Bird GS, Putney JW Jr. TRPC channels function independently of STIM1 and Orai1. *J Physiol* 2009; 587:2275–2298.
- Monga M, Campbell DF, Sanborn BM. Oxytocin-stimulated capacitative calcium entry in human myometrial cells. *Am J Obstet Gynecol* 1999; 181:424–429.
- Yang M, Gupta A, Shlykov SG, Corrigan R, Tsujimoto S, Sanborn BM. Multiple Trp isoforms implicated in capacitative calcium entry are expressed in human pregnant myometrium and myometrial cells. *Biol Reprod* 2002; 67:988–994.
- Shlykov SG, Yang M, Alcorn JL, Sanborn BM. Capacitative cation entry in human myometrial cells and augmentation by hTrpC3 overexpression. *Biol Reprod* 2003; 69:647–655.
- Young R, Zhang P. Inhibition of in vitro contractions of human myometrium by mibefradil, a T-type calcium channel blocker: support for a model using excitation-contraction coupling, and autocrine and paracrine signaling mechanisms. *J Soc Gynecol Invest* 2005; 12:e7–e12.
- Soboloff J, Spassova M, Xu W, He L, Cuesta N, Gill D. Role of endogenous TRPC6 channels in Ca²⁺ signal generation in A7r5 smooth muscle cells. *J Biol Chem* 2005; 280:39786–39794.
- Lemos VS, Poburko D, Liao CH, Cole WC, van Breemen C. Na⁺ entry via TRPC6 causes Ca²⁺ entry via NCX reversal in ATP stimulated smooth muscle cells. *Biochem Biophys Res Commun* 2007; 352:130–134.
- Huang G, Zeng W, Kim J, Yuan J, Han L, Muallem S, Worley P. STIM1 carboxyl-terminus activates native SOC, I(crac) and TRPC1 channels. *Nat Cell Biol* 2006; 8:1003–1010.
- Abdullaev IF, Bisaillon JM, Potier M, Gonzalez JC, Motiani RK, Trebak M. Stim1 and Orai1 mediate CRAC currents and store-operated calcium entry important for endothelial cell proliferation. *Circ Res* 2008; 103:1289–1299.
- Potier M, Gonzalez JC, Motiani RK, Abdullaev IF, Bisaillon JM, Singer HA, Trebak M. Evidence for STIM1- and Orai1-dependent store-operated calcium influx through ICRAC in vascular smooth muscle cells: role in proliferation and migration. *FASEB J* 2009; 23:2425–2437.
- Zagranichnaya T, Wu X, Villereal M. Endogenous TRPC1, TRPC3, and TRPC7 proteins combine to form native store-operated channels in HEK-293 cells. *J Biol Chem* 2005; 280:29559–29569.
- Ambudkar I, Ong H, Liu X, Bandyopadhyay B, Cheng K. TRPC1: the link between functionally distinct store-operated calcium channels. *Cell Calcium* 2007; 42:213–223.
- Ong H, Cheng K, Liu X, Bandyopadhyay B, Paria B, Soboloff J, Pani B, Gwack Y, Srikanth S, Singh B, Gill D, Ambudkar I. Dynamic assembly of TRPC1-STIM1-Orai1 ternary complex is involved in store-operated calcium influx. Evidence for similarities in store-operated and calcium release-activated calcium channel components. *J Biol Chem* 2007; 282:9105–9116.
- Sours-Brothers S, Ding M, Graham S, Ma R. Interaction between TRPC1/

- TRPC4 assembly and STIM1 contributes to store-operated Ca²⁺ entry in mesangial cells. *Exp Biol Med* 2009; 234:673–682.
36. Pani B, Ong HL, Brazer SC, Liu X, Rauser K, Singh BB, Ambudkar IS. Activation of TRPC1 by STIM1 in ER-PM microdomains involves release of the channel from its scaffold caveolin-1. *Proc Natl Acad Sci U S A* 2009; 106:20087–20092.
 37. Wray S, Burdyga T. Sarcoplasmic reticulum function in smooth muscle. *Physiol Rev* 2010; 90:113–178.
 38. Shmigol A, Eisner D, Wray S. Properties of voltage-activated [Ca²⁺]_i transients in single smooth muscle cells isolated from pregnant rat uterus. *J Physiol* 1998; 511:803–811.
 39. Longo M, Jain V, Vedernikov Y, Hankins G, Garfield R, Saade G. Effects of L-type Ca(2+)-channel blockade, K(+)(ATP)-channel opening and nitric oxide on human uterine contractility in relation to gestational age and labour. *Hum Reprod* 2003; 9:159–164.
 40. Lee SE, Ahn DS, Lee YH. Role of T-type Ca channels in the spontaneous phasic contraction of pregnant rat uterine smooth muscle. *Korean J Physiol Pharmacol* 2009; 13:241–249.
 41. Young RC, Schumann R, Zhang P. Nifedipine block of capacitative calcium entry in cultured human uterine smooth-muscle cells. *J Soc Gynecol Invest* 2001; 8:210–215.
 42. Jung S, Muhle A, Schaefer M, Strotmann R, Schultz G, Plant TD. Lanthanides potentiate TRPC5 currents by an action at extracellular sites close to the pore mouth. *J Biol Chem* 2003; 278:3562–3571.
 43. Liao Y, Erxleben C, Yildirim E, Abramowitz J, Armstrong D, Birnbaumer L. Orai proteins interact with TRPC channels and confer responsiveness to store depletion. *Proc Natl Acad Sci U S A* 2007; 104:4682–4687.
 44. Liao Y, Plummer NW, George MD, Abramowitz J, Zhu MX, Birnbaumer L. A role for Orai in TRPC-mediated Ca²⁺ entry suggests that a TRPC:Orai complex may mediate store and receptor operated Ca²⁺ entry. *Proc Natl Acad Sci U S A* 2009; 106:3202–3206.
 45. Lee KP, Yuan JP, So I, Worley PF, Muallem S. STIM1-dependent and STIM1-independent function of transient receptor potential canonical (TRPC) channels tunes their store-operated mode. *J Biol Chem* 2010; 285:38666–38673.
 46. Jousset H, Frieden M, Demaurex N. STIM1 knockdown reveals that store-operated Ca²⁺ channels located close to sarco/endoplasmic Ca²⁺ ATPases (SERCA) pumps silently refill the endoplasmic reticulum. *J Biol Chem* 2007; 282:11456–11464.
 47. Shimamura K, Kusaka M, Sperelakis N. Oxytocin induces an inward current in pregnant rat myometrial cells. *Can J Physiol Pharmacol* 1994; 72:759–763.
 48. Wang Y, Deng X, Mancarella S, Hendron E, Eguchi S, Soboloff J, Tang XD, Gill DL. The calcium store sensor, STIM1, reciprocally controls Orai and CaV1.2 channels. *Science* 2010; 330:105–109.
 49. Park CY, Shcheglovitov A, Dolmetsch R. The CRAC channel activator STIM1 binds and inhibits L-type voltage-gated calcium channels. *Science* 2010; 330:101–105.
 50. Gehrig-Burger K, Slaninova J, Gimpl G. Depletion of calcium stores contributes to progesterone-induced attenuation of calcium signaling of G protein-coupled receptors. *Cell Mol Life Sci* 2010; 67:2815–2824.
 51. Motiani RK, Abdullaev IF, Trebak M. A novel native store-operated calcium channel encoded by Orai3: selective requirement of Orai3 versus Orai1 in estrogen receptor-positive versus estrogen receptor-negative breast cancer cells. *J Biol Chem* 2010; 285:19173–19183.
 52. Jones BF, Boyles RR, Hwang SY, Bird GS, Putney JW. Calcium influx mechanisms underlying calcium oscillations in rat hepatocytes. *Hepatology* 2008; 48:1273–1281.