Video Article

Measurement of Calcium Fluctuations Within the Sarcoplasmic Reticulum of Cultured Smooth Muscle Cells Using FRET-based Confocal Imaging

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Abstract

Maintenance of steady-state calcium (Ca^{2+}) levels in the sarcoplasmic reticulum (SR) of vascular smooth muscle cells (VSMCs) is vital to their overall health. A significant portion of intracellular Ca^{2+} content is found within the SR stores in VSMCs. As the only intracellular organelle with a close association to the surrounding extracellular space through plasma membrane-SR junctions, the SR can be considered to constitute the first line of response to any irregularity in Ca^{2+} transients, or stress experienced by the cell. Among its many functions, one of the most important is its role in the transmission of Ca^{2+}-regulated signals throughout the cell to induce further cell-wide reactions downstream. The more common use of cytoplasmic Ca^{2+} indicators in this regard is overall insufficient for research into the highly dynamic changes to the intraluminal SR Ca^{2+} store that have yet to be fully characterized. Here, we provide a detailed protocol for the direct and clear measurement of luminal SR Ca^{2+}. This tool is useful for investigation into the nuanced changes in SR Ca^{2+} that have significant subsequent effects on the normal function and health of the cell. Fluctuations in SR Ca^{2+} content specifically can provide us with a significant amount of information pertaining to cellular responses to disease or stress conditions experienced by the cell. In this method, a modified version of a SR-targeted Ca^{2+} indicator, D1SR, is used to detect Ca^{2+} fluctuations in response to the introduction of agents to cultured rat aortic smooth muscle cells (SMCs). Following incubation with the D1SR indicator, confocal fluorescence microscopy and fluorescence resonance energy transfer (FRET)-based imaging are used to directly observe changes to intraluminal SR Ca^{2+} levels under control conditions and with the addition of agonist agents that function to induce intracellular Ca^{2+} movement.

Video Link

The video component of this article can be found at https://www.jove.com/video/53912/

Introduction

Ca^{2+} is a very important ion found in abundance within every single cell in the body. It has significant roles in many different cellular functions, including growth, proliferation, migration, and apoptosis[1-4]. It is well established that Ca^{2+} can affect these processes through both direct and indirect actions, and therefore, changes to the normal intracellular concentrations of this ion can easily result in negative outcomes for affected cells. The SR is considered as the largest intracellular Ca^{2+} store within the cell[5]. Steady state levels of Ca^{2+} in both the SR and cytoplasm are normally maintained through constant flux into and out of the Ca^{2+}-transporting channels of this organelle. The stressful conditions imposed upon cells due to any form of injury or disease are commonly associated with significant changes in SR Ca^{2+} that go on to have long-lasting effects on the health of the cell. In the most severe of cases, the inability of a stressed cell to recover and maintain steady state SR Ca^{2+} levels may even culminate in death by apoptosis[6-8].

Current research into cellular Ca^{2+} dynamics is limited by the fact that few studies test organelle Ca^{2+} store content directly[9-11]. The most common practice instead involves measurement of cytoplasmic Ca^{2+} levels as indirect measurements of changes in SR Ca^{2+} content [12-14]. In these experiments, Ca^{2+} is commonly induced to be released from the SR through the use of pharmacological agents causing the organelle's depletion (*e.g.* thapsigargin). Conclusions are then drawn with regard to changes to SR Ca^{2+} based on fluctuations in the cytoplasmic Ca^{2+} concentrations. Despite the ability remaining for investigators to draw such conclusions in a roundabout manner, this method of SR Ca^{2+} measurement is clearly an indirect way to glean such information, with many limitations concerning the interpretation of collected data. In order to bypass this clear restriction, it is necessary to measure the amount of Ca^{2+} found directly within the SR luminal network.

Vital to the final outcome of being able to directly record intraluminal SR Ca^{2+} levels are the cell culture tools and the Ca^{2+} indicator used. For the data referenced in the current manuscript, it is important to note that VSMCs used came from a frozen cell line. Cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) + 10% newborn calf serum (NCS) over passages 22-26 for the outlined experiments, incubated at a constant 37 °C with 5% CO_2 supply. Using the current method, for example, cells have been very successfully grown on a protein mixture that simulates the extracellular environment of many different types of tissue[15]. Important for success along the vein of SR Ca^{2+} research is the type of protein mixture used; in this case, a low growth factor variety was necessary to avoid components of this tool from affecting the regular Ca^{2+}

signaling and movements constantly occurring within the tested cells. Following successful growth of test cells, the Ca^{2+} indicator must also be effectively introduced to these cultured cells. This has become possible by using an adenoviral vector that carries the SR-residing Ca^{2+} indicator D1SR. To achieve a high transfection efficiency, cells must be incubated with viral vectors for at least 36-48 hr prior to imaging. This preparation provides a reliable tool to measure Ca^{2+} transients within the SR lumen with high accuracy and reproducibility.

D1SR indicator used in this protocol is a modified variant of the D1ER Ca^{2+} indicator that was originally created by Dr. Roger Y. Tsien's laboratory at the University of California, San Diego, USA[9,16]. The original D1ER belongs to a second generation of genetically encoded Ca^{2+} indicators called cameleons that display Ca^{2+} sensitivities over a much broader range (0.5-160 µM) as compared to previous Ca^{2+} indicators[9,16]. The new variant D1SR, a kind gift from Dr. Wayne Chen (University of Alberta, Canada), however, carries a mutant calsequestrin sequence (instead of a calreticulin sequence as in the original D1ER). The mutant calsequestrin has a reduced binding to Ca^{2+} that eliminates the issue of competing with endogenous calsequestrin in binding to Ca^{2+} within the SR lumen[11]. The D1SR indicator carries a truncated enhanced cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) that bind by a linker protein containing modified calmodulin (CaM) and M13 (the 26-residue peptide of myosin light-chain kinase that binds to CaM) sequences. The CaM-M13 sequence has been modified to prevent M13 from binding to endogenous calmodulin. Also, to ensure SR retention, a calsequestrin sequence has been added on the 5´ end of CFP[11]. When bound to Ca^{2+}, the CaM-M13 domain goes through conformational changes that result in an increase in the energy transfer between the flanking CFP and YFP, which is recorded as an increase in the FRET signal intensity. On the other hand, when the concentration of SR luminal Ca^{2+} drops, the CaM-M13 domain goes through reverse conformational changes, resulting in a decrease in the energy transfer between the flanking CFP and YFP, and a significant drop in FRET signal intensity.

Protocol

Ethics statement: All experiments and procedures were conducted in agreement with the Laboratory Biosafety guidelines of the University of British Columbia, Vancouver, Canada.

1. Preparation of 35 mm Glass-bottom Culture Dishes for FRET-based Confocal Microscopy

1. Prepare plates using the following procedure 36-48 hr prior to experiments.
2. Retrieve chosen gelatinous protein mixture and 0.25% trypsin-EDTA from -20 °C storage and keep them in mobile ice bath until use.
   NOTE: The coating protein mixture should only be exposed to RT for a brief period of time to prevent the mixture from solidifying before use. Trypsin solution should also be keep chilled until needed; alternatively, trypsin can be retrieved from -20 °C storage following completion of step 1.5.
3. Prepare a 1:25 dilution of protein mixture: DMEM (no added serum, chilled). Transfer appropriate volume of protein mixture/DMEM to each plate being prepared. For 35 mm glass-bottomed plates, add approximately 200 µl to fully cover inner glass circle on bottom of plate.
4. Leave plates to sit for at least 30 min inside the safety cabinet and at RT. Warm DMEM with 10% NCS to 37 °C in water bath during this time period.
5. Warm trypsin in water bath to 37 °C immediately prior to next steps.
6. Remove DMEM that is currently in culture flask using a glass pipette and suction. Wash floor of the flask with warm sterile PBS to remove remaining DMEM residue.
7. Dislodge previously cultured smooth muscle cells from flask bottom:
   NOTE: Flask was established days to this step to allow cells to reach desired confluency (approximately 80%) before plate preparation. Cells were originally suspended in the flask in 20 ml of DMEM with 10% NCS and incubated at 37 °C with 5% CO_2 supply for 24 hr, after which DMEM supply was refreshed and cells were returned to incubator. DMEM was refreshed every 48 hr following these initial steps until cells reached confluency.
   1. Wash flask quickly with 1 ml of trypsin and then remove 1 ml trypsin using suction.
   2. Add in another 1 ml of trypsin and leave for longer period (approximately 30-60 sec), swishing flask to ensure the enzyme makes contact with the entire bottom of the flask.
   3. Observe how much cells have detached under a microscope until satisfied with separation from flask. Alternate swishing trypsin solution and checking cell detachment until satisfied with proportion of cells separated from flask.
      NOTE: The flask can be returned to the incubator for approximately 30-60 sec to speed up this process at 37 °C.
   4. Once cells have been dislodged using trypsin, add fresh DMEM with 10% NCS to the flask to stop the trypsin reaction (sufficient volume so that trypsin forms $1/8^{th}$ of total volume in flask).
      NOTE: For example, when using a 250 ml culture flask, this would be 7 ml DMEM added to the 1 ml of trypsin to result in 8 ml of cell solution.
8. Transfer resulting cell solution from the flask to a clean (labeled) 50 ml conical tube.
9. Add 1 ml fresh DMEM plus 10% NCS to each plate being prepared (sufficient medium to last 24 hr).
10. Gently invert tube containing cell solution multiple times to break up any pellet that may have formed.
11. Pipette desired volume of gently mixed cell solution into each plate.
    NOTE: The recommended SMCs number plated for this protocol is 0.5-0.8 x 10^6 per 35 mm culture dishes. These numbers may vary depending on the cell type and should be tested and modified by each laboratory.
12. Swirl each plate thoroughly clockwise/counterclockwise to ensure equal distribution of cells across glass bottom.

2. Transient Transfection with Adenoviral Vector Carrying D1SR Ca^{2+} Indicator

1. Following addition of medium and cell solution to each plate, leave plates to incubate at 37 °C with 5% CO_2 supply for at least 1 hr to allow cells to attach properly to glass on bottom of dishes. Check plates under a microscope at 15 min intervals until cells are clearly attached.
2. Retrieve adenovirus-D1SR aliquot from -80 °C storage and keep in mobile ice bath to transport to biological safety cabinet.
3. Pipette desired dose (multiplicity of infection of 100) of chilled D1SR to each plate.

NOTE: The calculation for the volume of adenoviral solution needed per plate depends on the virus titer in the original stock solution, which is presented as the plaque forming unit (PFU). Based on our experience, we recommend a multiplicity of infection (MOI) of 100, which is interpreted as the number or virus particles needed to infect one smooth muscle cell (SMC) in the culture plate.
4. Incubate the infected cells at 37 °C with 5% CO_2 supply O/N.
5. Next day, replenish cells with 1 ml of fresh DMEM plus 10% NCS.
6. Incubate plates in a humidified incubator at 37 °C in 5% CO_2 O/N.

3. Measurement of SR Luminal Ca^{2+} Using FRET-based Confocal Imaging

1. Following O/N incubation, remove the culture medium from the plate.
2. Wash the culture plate with 1 ml warm physiological HEPES-PSS buffer containing (in $mmol \cdot L^{-1}$) NaCl 140, glucose 10, KCl 5, HEPES 5, $CaCl_2$ 1.5 and $MgCl_2$ 1 (pH 7.4) three times.
3. Add 1 ml fresh warm HEPES-PSS buffer immediately prior to initiation of recording.
4. Perform initial imaging using the FRET SE (Sensitized Emission) application (or similar feature) on the confocal microscope's associated software.
    1. Once application is open, click into the "Setup" tab to adjust settings to achieve desired experimental conditions.
    2. Manually change channel settings so that cells are excited in sequence at 440 nm (for the donor and FRET channels) and 513 nm (for the acceptor channel). Collect the emission wavelengths at 488 nm (for donor) and 535 nm (for FRET and acceptor).
    3. Set the intensity of light at 15% transmission, with 150 ms excitation exposure time of cells (recording time for three sequential CFP donor, FRET, and YFP acceptor channels) and 10 sec intervals between exposures.
5. Stabilize plate on the microscope stage.
6. Use the "Live" button to check image resolution. Tweak settings further until desired resolution is reached.
7. Still under the "Setup" tab, take an image of the sample using the "Capture image" button.
8. Moving on to the "Evaluation" tab, choose the appropriate method to calculate the FRET efficiencies for the experiments being done. Method 3, using the Ratiometric Calculation [$E_A(i) = B/A$], is recommended for experiments involving cameleons such as the D1SR indicator.
9. Switch over to the "Graph" tab to be able to observe the intensity values being recorded in graph form during the experiment.
10. Draw an ROI in the image viewer to observe the corresponding average intensity of this area of interest on the graph as the experiment proceeds.
    NOTE: Investigator may also choose to draw multiple ROIs and have corresponding intensities be recorded and displayed simultaneously on the graph. Alternatively, investigators may outline a single ROI for initial recording, and proceed to outline multiple ROIs at a later time by opening the experiment file on a data analysis-oriented version of the software.
    NOTE: Further detail on the steps required to set up FRET-based experiments can be found in FRET application wizard manuals. If the microscope is not equipped with the FRET Wizard, the following protocol can be used to set up the parameters for recording manually.
11. Start recording and allow data collection for at least 3 min to ensure a steady basal recording of the signal prior to introducing the agent of interest.
12. Introduce Ca^{2+}-moving agent (*e.g.* 2 µM thapsigargin; 100 nm endothelin-1) as a tool for depleting SR Ca^{2+}, by manually pipetting the predetermined dose directly into the plate at a spot as near to the region of interest being recorded as possible. Continue recording for a desired time post treatment.
13. Capture ratiometric FRET serial images (512 x 512 pixels) with a 63X oil-immersion objective of the confocal inverted microscope using the FRET SE application.
14. Collect data resulting from imaging from a cluster of 5-10 SMCs in each region of interest (ROI) and subsequently format and analyze using statistics-based software.
    1. To save data for later use, right-click over the recorded trace and choose the "Export" option to save the spreadsheet with recorded mean signal intensities on the investigator's chosen drive.
    2. Normalize data from each individual experiment by dividing each data point by the starting intensity value observed at time point 0 sec.
    3. Import normalized data from spreadsheets into a statistical program project file by manually copying and pasting relevant data rows from their original spreadsheet/sec.
    4. Automatically generate graphs corresponding to the data sets pasted into the program.
       NOTE: The program's default settings generate line graphs from imported data. Type of graph displayed can be modified by clicking the "Choose a different type of graph" button under the "Change" heading found within the main toolbar.
    5. Pool data representing multiple independent trials within a larger group of identical experiments into a single data sheet to produce an average trace for the specific test performed.

4. Preparation of the Cytoplasmic Ca^{2+} Indicator

1. Dissolve 0.0125 g Pluronic Acid (PA) in 1 ml of dimethyl sulfoxide (DMSO) in a black microcentrifuge tube (may have to warm the tube in mobile water bath to help dissolve PA).
2. Retrieve a tube (50 µg) of the cytoplasmic Ca^{2+} indicator dye (Fluo-4 AM) from -20 °C storage.
3. Wrap the tube of dye in foil to protect it as much as possible from contact with light.
4. Pipette 45 µl of the DMSO+PA solution into the tube containing the dye. Wrap DMSO+PA solution in foil and store at RT.
5. Mix contents of the tube thoroughly by pipetting solution up and down multiple times. Alternatively, sonicate the dye tube for 10 min.
   NOTE: Either method of thoroughly distributing the dye throughout the DMSO+PA solution will be sufficient; it is simply a matter of the investigator's preference. If investigator chooses to sonicate solution, it should be done in an isolated area/room where others will be unaffected by the noise. Investigator should also use heavy mufflers to protect ears.
    1. If sonicating solution, insert foil-wrapped tube containing the dye inside a foam tube holder and place inside the 2/3 full (with dH_2O) sonicator bath.

2. Place sonicator bath in isolated area/room and power machine on (ensure protective headwear is in place prior to initiating sonication process). Leave room and leave machine running for 10 min before shutting it off and retrieving the tube (keep wrapped in foil to protect from light for as long as possible).

6. Aliquot the now thoroughly mixed solution into dark microcentrifuge tubes (5 µl per tube, should be able to make 10 tubes total).
7. Wrap each aliquot tube in foil. Label tubes and store in desiccant at -20 °C conditions until use.

5. Measurement of Cytoplasmic Ca^{2+}

1. Follow steps in 1.1-1.12 to prepare culture plates of rat aortic SMCs.
2. Remove the culture medium from the plate at 48 hr post culture.
3. Remove the previously prepared aliquot of cytoplasmic Ca^{2+} indicator (5 µl) from -20 °C storage and maintain in mobile ice bath until use.
4. Wash the culture plate with 1 ml warm physiological HEPES-PSS buffer containing (in $mmol \cdot L^{-1}$) NaCl 140, glucose 10, KCl 5, HEPES 5, $CaCl_2$ 1.5 and $MgCl_2$ 1 (pH 7.4) three times.
5. Mix the indicator with 1 ml of warm HEPES-PSS and load it onto the culture plate.
6. Incubate SMCs with the indicator for 1 hr at RT (24 °C).
7. Remove indicator from culture plate and wash with 1 ml warm physiological HEPES-PSS buffer three times.
8. Add 1 ml fresh warm HEPES-PSS buffer immediately prior to initiation of recording.
9. Capture serial images (512 x 512 pixels) with a 63X oil-immersion objective of the confocal inverted microscope.
    1. Manually change settings so that cells are excited at 488 nm, and the emission wavelengths are collected at 555 nm with a scanning speed of 700 Hz.
    2. Set the intensity of light at 15% transmission, with 150 msec excitation exposure time of cells (the amount of time that cells are exposed to light at designated time intervals during recording) and 5 sec intervals between exposures.
10. Stabilize plate on the microscope stage and start recording.
11. Record for at least 3 min to ensure a steady basal recording of the signal prior to introducing the agent of interest.
12. Introduce Ca^{2+}-moving agent (*e.g.* 2µM thapsigargin) as a tool for depleting SR Ca^{2+} (as described in step 3.16) and continue recording for desired amount of time post treatment.
13. Record signal intensity using microscope-specific software by averaging fluorescence signals (F/F_0) from a cluster of 5-10 SMCs in each region of interest (ROI). Collect, format, and analyze data resulting from imaging using data analysis software. See Steps 3.18.1-3.18.5 for description of data collection and analysis using this software.

Representative Results

This section provides examples of the results that can be obtained using the described method to capture SR luminal Ca^{2+} concentration data, compared to the commonly employed manner of indirectly measuring changes to organelle Ca^{2+} store concentrations by observing fluctuations in cytoplasmic Ca^{2+}.

Figure 1A shows a snapshot of a region of interest (ROI) of the SMC culture transfected with D1SR adenovirus in the YFP channel (514 nm excitation/535 nm emission). As shown, although all SMCs are positive for D1SR expression, it is evident that the expression levels for D1SR indicator vary among different cells within the same ROI. **Figure 1B** shows a 2-D projection of a fluorescence image of a SMC transfected with the D1SR adenovirus (YFP channel), which presents a clear picture of Ca^{2+} distribution within the expansive SR luminal network. **Figure 1C** presents D1SR indicator distribution in the SMC using CFP (440/488 nm), FRET (440/535 nm), and YFP (514/535 nm) band-pass filters.

We also show examples of the Ca^{2+} signal traces obtained from monitoring the movement of this targeted ion in the SR, using the clear test of acute exposure of cells to an SR emptying agent thapsigargin (2 µM) (**Figure 2**). As shown in **Figure 2A** and **2B**, thapsigargin induces a drop in SR Ca^{2+} content ($[Ca^{2+}]_{SR}$).

In **Figure 3A**, we have shown representative images of cultured SMCs loaded with the cytoplasmic Ca^{2+} indicator treated with the SR emptying agent thapsigargin (2 µM). The observed transient peak in recorded Ca^{2+} signal (**Figure 3B**) is an indication of Ca^{2+} release into the cytoplasm of the SMC, even though the source of the Ca^{2+} transients cannot be explicitly identified.

Figure 4A shows representative snapshots of cultured SMCs expressing the SR calcium indicator D1SR and treated with 100 nm of endothelin-1. As shown in both **Figures 4A** and **4B**, endothelin-1 induces a slow, but steady drop in SR calcium content as measured by a drop in D1SR FRET signal. As evident, the ratiometric FRET protocol importantly allows study of Ca^{2+} movements directly related to the mode of action of the agent used. Directly measuring this ion's movement into/from the largest Ca^{2+} store in the cell in this manner is helpful in providing results representative of Ca^{2+} dynamics directly pertaining to the SR's actions or due to changes in SR lumen environment.

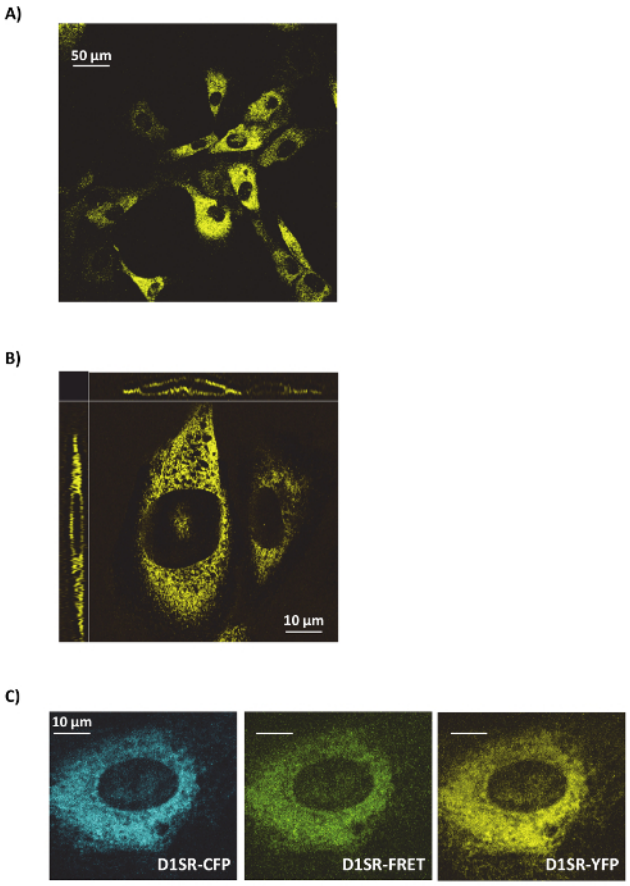

Figure 1. Distribution of the SR Ca^{2+} indicator D1SR in rat aortic SMCs. (**A**) Representative image depicting SMC culture transfected with D1SR andenoviral vector at 48 hr post infection. (**B**) 2-D projection of a fluorescence image of D1SR distribution in a cultured SMC using YFP channel (514 nm excitation/535 nm emission). (**C**) Representative snapshot of a SMC transfected with D1SR indicator using CFP (440/488 nm), FRET (440/535 nm), and YFP (514/535 nm) band-pass filters. Scale bars = 10 µm. Please click here to view a larger version of this figure.

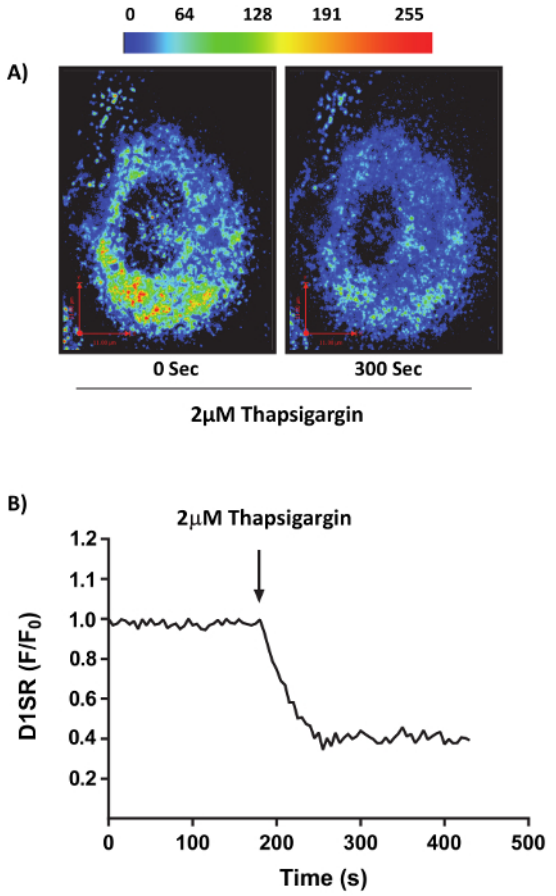

Figure 2. Thapsigargin causes a significant drop in $[Ca^{2+}]_{SR}$. (**A**) Real-time pseudocolor snapshots (FRET channel) of cultured rat aortic SMCs transfected with D1SR and treated with 2 μM of thapsigargin showing a decrease in signal intensity indicating a significant drop in SR Ca^{2+} content due to thapsigargin-induced Ca^{2+} release. (**B**) Representative trace for F/F_0 value in cultured SMCs treated with 2 μM thapsigargin confirming a significant drop in $[Ca^{2+}]_{SR}$. Please click here to view a larger version of this figure.

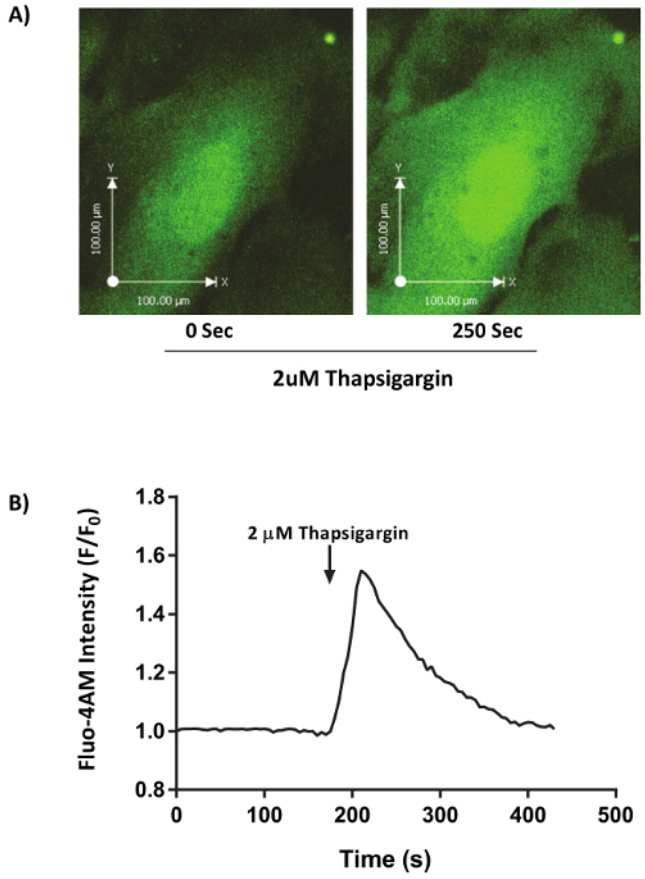

Figure 3. Thapsigargin causes a significant increase in cytoplasmic Ca^{2+} ($[Ca^{2+}]_i$). (**A**) Representative snapshots of cultured rat aortic SMCs loaded with cytoplasmic Ca^{2+} indicator, and treated with 2 µM of thapsigargin. As shown, thapsigargin causes a significant increase in signal intensity within the cytoplasm due to the release of Ca^{2+} from the SR. (**B**) Representative trace for F/F_0 value in cultured SMCs treated with 2 µM of thapsigargin. The depicted increase in Ca^{2+} signal is considered to be proportional to the amount of Ca^{2+} released from the SR. Please click here to view a larger version of this figure.

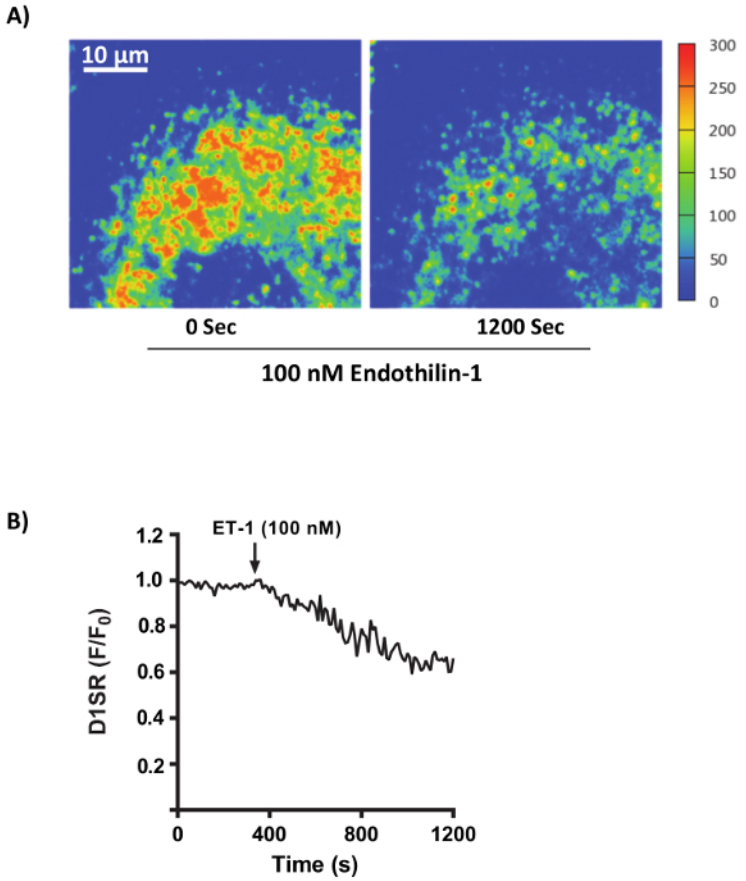

Figure 4. Endothelin-1 causes a significant drop in $[Ca^{2+}]_{SR}$. (**A**) Real-time pseudocolor snapshots (FRET channel) of cultured rat aortic SMCs transfected with D1SR and treated with 100 nm endothelin-1 showing a slow but steady decrease in signal intensity indicating a significant drop in SR Ca^{2+} content due to endothelin-induced Ca^{2+} release for the SR. (**B**) Representative trace for F/F_0 value in cultured SMCs treated with 100 nm of endothelin-1. Please click here to view a larger version of this figure.

Discussion

This protocol describes the transfection of VSMCs with the D1SR adenovirus to study Ca^{2+} concentrations within the lumen of the SR. This method allows for the direct measurement of Ca^{2+} within this organelle as well as its movement into/out of the SR as a result of the application of different calcium-moving agents. This method has many advantages for investigators working towards a comprehensive understanding of how changes to SR and cytoplasmic Ca^{2+} levels affect overall cellular health and how these changes are inter-related. Traditional observation of intracellular Ca^{2+} movement involves the tracking of changes to only cytoplasmic Ca^{2+} levels. An important advantage derived from using the D1SR or similar constructs, therefore, is the ability to monitor this ion's movement involving a specific organelle such as the SR, rather than having to draw vague conclusions on the effect of agonists on the SR by indirectly observing changes to cytoplasmic Ca^{2+} levels induced by such manipulation. Along the same vein, agents that serve to affect SR Ca^{2+} specifically can be more efficiently used to assess their effects on both organelle and cell-wide cellular Ca^{2+} concentrations and overall health. It is due to these advantages that transfection of cultured cells with D1SR provides a more direct, as well as complimentary, method of measuring changes to intraluminal SR Ca^{2+} levels. Also of importance is the fact that this model provides a tool for measuring changes in Ca^{2+} concentration within the SR network in response to pharmacological or physiological agents that are known to induce cellular stress or specific functional responses. This will be possible by generating a calibration curve for D1SR indicator in any cell of interest. Calcium concentration within the ER/SR can then be calculated by calibrating normalized D1SR ratio (F/F_0) against the standard calibration curve as described previously [10, 11].

A limitation of the protocol outlined here is the lack of information available on use of the D1SR construct in other cell types. This method has so far proved successful for rat SMCs in our studies multiple times [13-15], though its use with other cell types has to date been limited. For the success of this method, it is vital that all precautions are taken to ensure the healthy progression of the cell culture steps. It is therefore important to use an appropriate material allowing cell growth on plates, such as the many protein-based gelatinous substances available for purchase that have proven to promote sufficient growth of rat aortic SMCs on glass-bottomed microscopy plates. Variability in the success of transfection with D1SR and subsequent imaging can be observed if cells are not prepared during their optimal passages and at high confluency. Changes to phenotype inherent to prolonged use of the same cell line may result in suboptimal transfection and weak signal being obtained during the imaging stage. The limitations implicated in the use of this method are therefore mainly those made inevitable by the required cell culture work, as experiments using the D1SR construct must be prepared for at least 36-48 hr prior to their execution to ensure confluency of cells for imaging and sufficient time for incubation of growing cells with the adenovirus.

Disclosures

The authors declare that they have no competing financial interests.

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