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Corresponding Author:	Susan Treves SWITZERLAND
Corresponding Author's Institution:	
Corresponding Author E-Mail:	susan.treves@unibas.ch
Order of Authors:	Susan Treves
	Thierry Girard
	Francesco Zorzato
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TITLE:

Functional characterization of endogenously expressed human RYR1 variants

AUTHORS AND AFFILIATIONS:

Susan Treves[1,2,*], Thierry Girard[3], Francesco Zorzato[1,2]

[1]Department of Biomedicine, Basel University Hospital, Basel, Switzerland
[2]Department of Life Sciences, University of Ferrara, Ferrara, Italy
[3]Department of Anesthesia, Basel University Hospital, Basel, Switzerland

Thierry.girard@unibas.ch
fzorzato@usb.ch

*Corresponding author:
susan.treves@unibas.ch

KEYWORDS:

RYR1, mutations, functional characterization, endogenous expression, myotubes, EBV-lymphoblasts, calcium, dose response

SUMMARY:

Here methods used to study the functional effect of RYR1 mutations endogenously expressed in Epstein Barr Virus immortalized human B-lymphocytes and muscle biopsy derived satellite cells differentiated into myotubes are described.

ABSTRACT:

More than 700 variants in the RYR1 gene have been identified in patients with different neuromuscular disorders including malignant hyperthermia susceptibility, core myopathies and centronuclear myopathy. Because of the diverse phenotypes linked to RYR1 mutations it is fundamental to characterize their functional effects to classify variants carried by patients for future therapeutic interventions and identify non-pathogenic variants. Many laboratories have been interested in developing methods to functionally characterize RYR1 mutations expressed in patients' cells. This approach has numerous advantages, including: mutations are endogenously expressed, RyR1 is not over-expressed, use of heterologous RyR1 expressing cells is avoided. However, since patients may present mutations in different genes aside RYR1, it is important to compare results from biological material from individuals harboring the same mutation, with different genetic backgrounds. The present manuscript describes methods developed to study the functional effects of endogenously expressed RYR1 variants in: (a) Epstein Barr virus immortalized human B-lymphocytes and (b) satellite cells derived from muscle biopsies and differentiated into myotubes. Changes in the intracellular calcium concentration triggered by the addition of a pharmacological RyR1 activators are then monitored. The selected cell type is loaded with a ratiometric fluorescent calcium indicator and intracellular [Ca^{2+}] changes are monitored either at the single cell level by fluorescence microscopy or in cell populations using a spectrofluorometer. The resting [Ca^{2+}], agonist dose response curves are then compared

between cells from healthy controls and patients harboring RYR1 variants leading to insight into the functional effect of a given variant.

INTRODUCTION:

To date more than 700 RYR1 variants have been identified in the human population and linked to various neuromuscular disorders including malignant hyperthermia susceptibility (MHS), exercise induced rhabdomyolysis, central core disease (CCD), multi-minicore disease (MmD), centronuclear myopathy (CNM)[1-3]; nevertheless, studies to characterize their functional effects are lagging and only approximately 10% of mutations have been tested functionally. Different experimental approaches can be used to assess the impact of a given RyR1 variant, including transfection of heterologous cells such as HEK293 and COS-7 cells with plasmid encoding for the WT and mutant RYR1 cDNA[4,5], transduction of dyspedic mouse fibroblasts with plasmids and vectors encoding for the WT and mutant RYR1 cDNA, followed by transduction with myo-D and differentiation into myotubes[6], generation of transgenic animal models carrying mutant RyR1s[7-9], characterization of cells from patients expressing the RYR1 variant endogenously[10-12]. Such methods have helped established how different mutations functionally impact the RyR1 Ca^{2+} channel.

Here, methods developed to assess the functional effects of RYR1 mutations are described. Various parameters of intracellular calcium homeostasis are investigated in human cells endogenously expressing the RyR1 calcium channel, including myotubes and Epstein Barr Virus (EBV) immortalized B-lymphocytes. Cells are obtained from patients, expanded in culture and loaded with ratiometric fluorescent calcium indictors such as fura-2 or indo-1. Parameters which have been reported to be altered because of pathogenic RYR1 mutations including the resting [Ca^{2+}], the sensitivity to different pharmacological agonists and the size of the intracellular Ca^{2+} stores are measured either at the single cell level, using fluorescence microscopy, or in cell populations using a fluorimeter. Results obtained in cells from mutation carriers are then compared to those obtained from healthy control family members. This approach has demonstrated that: (i) many mutations linked to MHS lead to an increase in the resting [Ca^{2+}] and a shift to the left in the dose response curve to either KCl-induced depolarization or pharmacological RyR1 activation with 4-chloro-m-cresol[10-13]; (ii) mutations linked to CCD lead to a decrease in the peak [Ca^{2+}] released by pharmacological activation of the RyR1 and decreased size if the intracellular Ca^{2+} stores[12-15]; (iii) some variants do not impact Ca^{2+} homeostasis[13]. Advantages of this experimental approach are: the RyR1 protein is not over-expressed and physiological levels are present, cells can be immortalized (both muscle cells and B-lymphocytes) providing cell lines containing mutations. Some disadvantages relate to the fact that patients may carry mutations in more than one gene encoding proteins involved in calcium homeostasis and/or excitation contraction coupling (ECC) and this may complicate experimental conclusions. For example, two JP-45 variants were identified in the MHS and control population and their presence were shown to impact the sensitivity of the dihydropyridine receptor (DHPR) to activation[16]. Patients need to be available, biological material needs to be freshly collected and ethical permits need to be obtained from the local ethical boards.

PROTOCOL:

The protocols described below comply with the ethics guidelines of the Ethikkommission Nordwest- und Zentralschweiz EKNZ.

1. Preparation of Epstein Barr immortalized B-lymphocyte cell lines[11]

1.1. After informed consent, collect 30 mL of whole blood in EDTA-treated sterile tubes from the proband carrying a RYR1 mutation and from healthy family members with no mutation.

NOTE: Keep all solutions sterile and work in a tissue culture hood.

1.2. Isolate mononuclear cells from whole blood by density gradient centrifugation media (e.g., Ficoll-Hypaque, .077 g/L).

1.2.1. Place 30 mL of sterile blood in a 50 mL conical sterile tube.

1.2.2. Place the tip of a Pasteur pipette containing the density gradient centrifugation media at the bottom of tube and layer 20 mL sterile of density gradient centrifugation media solution slowly underneath the blood.

1.2.3. Centrifuge for 30 min at 900 x *g* at 18°-20°C, with no break.

NOTE: The mononuclear cell layer appears as a cloudy ring at the interphase between the density gradient centrifugation media layer and the top layer containing platelet enriched plasma.

1.3. With a sterile pipette gently remove the interphase layer containing mononuclear cells (approximately 3-5 mL) and transfer the solution to a clean 50 mL sterile conical tube.

1.4. Add 20 mL of phosphate buffer saline (PBS) to rinse cells, centrifuge for 10 min at 600 x *g* at room temperature and resuspend the pellet in PBS. Repeat for a total of three times; this ensures that all the media is removed.

1.5. After the last wash, resuspend cells in 1-2 mL of tissue culture medium (RPMI medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 units of penicillin and streptomycin). Place mononuclear cells (approximately 1 x 10^6 cells) in a T125 tissue culture flask containing 20 mL of tissue culture medium.

1.6. Infect mononuclear cells with Epstein-Barr virus.

1.6.1. Use supernatants from B95.8 cell line cultures (containing 10^2-10^3 transforming units/mL stocked at -80 °C) as a source of EBV.

1.6.2. Resuspend 1 x 10^6 mononuclear cells from step 1.5 in 20 mL of tissue culture medium and expose them to 2 mL of supernatant from the B95.8 cell line in the presence of cyclosporin A (0.2 µg/mL final concentration) for infection.

1.7. Place the flask in a 37 °C cell culture incubator and allow the cells to grow. After one week change the culture medium.

NOTE: Once B-cells start proliferating they form recognizable clumps and grow rapidly so that the cultures can be expanded and frozen.

1.8. Extract the genomic DNA from the EBV immortalized B-lymphocyte cell lines[11] to confirm the presence or absence of the given mutation.

2. Intracellular Ca^{2+} measurements

NOTE: Changes in the intracellular calcium concentration of the EBV-transformed B-lymphocyte cell lines can be monitored in cell populations, with a spectrofluorometer equipped with a magnetic stirrer and cuvette holder set to 37 °C. Alternatively Ca^{2+} changes can be monitored in single cells by fluorescence microscopy. In both cases cells are removed from the tissue culture flask, washed twice with Krebs Ringer's solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 1 mM $NaHPO_4$, 5.5 mM glucose, pH 7.4 containing 1 mM $CaCl_2$) and counted**.**

2.1. For experiments in cell populations using a spectrofluorometer[11,13,14]

2.1.1. Resuspend cells at a final concentration of 1 x 10^7 cells/ mL in Krebs Ringer's solution and incubate at 37°C for 30 min with a final concentration of 5 µM Fura-2/AM.

2.1.2. Centrifuge cells at 900 x *g* for 10 min and resuspend them in Krebs Ringer's solution at a concentration of 2 x 10^6 cells/mL.

2.1.3. Measure fluorescence changes (ratio 340/380 nm) using a spectrofluorometer equipped with a magnetic stirrer set at maximal velocity and set to 37 °C.

2.1.4. Just before the experiment spin cells at 900 x *g* for 5 min in a microcentrifuge and quickly resuspend the pellet in 1.5 mL of Krebs Ringer's solution with 0.5 mM EGTA but no added Ca^{2+}.

2.1.5. Place cells in a 3 mL glass spectrofluorometer cuvette and record the fluorescence ratio (340 nm/380 nm excitation, 510 nm emission).

2.1.6. Achieve a stable base line (approximately 30 seconds), add the selected concentration of RyR1 agonist (4-chloro-m-cresol, or 4-cmc) and record the calcium transient.

NOTE: A 300 mM stock solution of 4-cmc made in DMSO is use used as a starting reagent. This solution can be made in advance, aliquoted and stored at -20 °C for several months.

2.1.7. Perform experiments for different 4-cmc concentrations.

NOTE: Include 75 µM, 150 µM, 300 µM, 450 µM, 600 µM, 750 µM to 1 mM to generate a dose response curve of agonist versus change in [Ca^{2+}]. The different 4-cmc concentrations are obtained by adding the appropriate volume of 4-cmc from the stock solution, directly into the cuvette containing the fura-2 loaded cells. For example, for a final concentration of 300 µM 4-cmc, 1.5 µL of the stock solution are added to the cuvette containing 1.5 mL of cells in Krebs Ringer's solution. For lower agonist concentrations, the 300 mM stock solution should be diluted to 75 mM with DMSO and the appropriate volume added to the cuvette containing 1.5 mL of cells in Krebs Ringer's solution.

2.1.8. Add 400 nM thapsigargin to cells to calculate the total amount of Ca^{2+} present in intracellular stores. Record the peak Ca^{2+}.

2.1.9. Plot the peak calcium induced by a given 4-cmc concentration versus the peak calcium induced by thapsigargin, which is considered 100% and construct a 4-cmc dose response curve comparing cells from a proband and healthy relative

2.2. For experiments on single cells[13]

2.2.1. Dilute poly-L-lysine 1:10 in sterile H_2O and pre-treat the glass coverslips for 30 min. Allow to air-dry under a sterile tissue culture hood.

2.2.2. Re-suspend EBV-transformed B-lymphocytes to a final concentration of 1 x 10^6 cells/mL in Krebs Ringer's solution containing 1 mM $CaCl_2$ and add a final concentration of 5 µM Fura-2/AM.

2.2.3. Place 1 mL of cells on the poly-L-lysine treated coverslips and incubate at 37 °C in a humidified cell culture incubator for 30 min to allow the EBV cells to stick to the glass coverslip during loading.

2.2.4. Place the coverslip in the perfusion chamber and start perfusion (at a rate of 2 mL/min) with Krebs Ringer's solution containing 1 mM Ca^{2+}.

2.2.5. Use an inverted fluorescent microscope (equipped with a 40x oil-immersion objective (0.17 numerical aperture), filters (BP 340/380, FT 425, BP 500/530) to record on-line measurements, with a software-controlled charge coupled device (CCD) camera attachment.

2.2.6. Acquire Images at 1 s intervals at a fixed exposure time (100 ms for both 340- and 380-nm excitation wavelengths. Use imaging software to analyse changes in fluorescence. Measure the average pixel value for each cell at excitation wavelengths of 340 and 380 nm[13].

2.2.7. To achieve cell stimulation, use a cell perfusion stimulator with 12 valves and add different concentrations of 4-cmc. The flush valve contains Krebs Ringer's solution with no added Ca^{2+} plus 100 µM La^{3+} to monitor calcium release from intracellular stores only.

2.2.8. Construct a dose response curve of 4-cmc versus change in [Ca^{2+}], as described above.

3. Preparation of human myotubes from muscle biopsies[10,12,15]

NOTE: Different methods have been used by different laboratories to obtain satellite cell-derived myoblasts and myotubes. Below is the description of the method used in Basel.

3.1. Rinse muscle biopsy with sterile PBS to remove excess blood and cut into small fragments of about 0.5-1 mm.

3.2. Prepare 6 well tissue culture dishes with insert. Add 1.5 mL of human muscle growth medium to each well and 0.5 mL of human muscle growth medium to each insert.

NOTE: The growth medium is made up as follows: 500 mL of Dulbecco's modified Eagle's medium with high glucose, or DMEM (4.5 mg/mL), containing 10% horse serum, 5 ng/mL insulin, 3 mM glutamine, 600 ng/mL penicillin G and streptomycin, and 7 mM HEPES, pH 7.4. Commercially available skeletal muscle growth medium can also be used.

3.3. Place 2-3 small muscle fragments into each insert (**Figure 1A**) and place culture dishes into the cell culture incubator (5% CO_2, 37 °C). After approximately 8-10 days satellite cells can be seen growing out of and surrounding the muscle biopsy, attached to the insert (**Figure 1, B** and **C**, arrows).

3.4. Release a sufficient number of cells from the biopsy (after approximately 10-14 days), trypsinize as follows:

3.4.1. Remove all culture medium, rinse the cells once with 1 mL of PBS, add 0.5 mL of trypsin/EDTA solution (0.025% trypsin and 0.01% EDTA) and incubate at 37 °C for 5 min.

3.4.2. Add 1 mL of growth medium to the cells to neutralize the effect of trypsin and transfer the satellite cells into a new T25 cell culture flask; add 3 mL of growth medium and place the cells in a cell culture incubator (5% CO_2, 37 °C).

3.4.3. Change the growth medium the next day to remove the EDTA and subsequently change the medium once per week.

3.5. When myoblasts are approximately 75% confluent, trypsinize and transfer them onto laminin-treated glass coverslip.

NOTE: As a proportion, cells growing in one T25 flask should be transferred onto one 43 mm diameter laminin-treated glass coverslip. The glass coverslip should be placed within a 60 mm diameter tissue culture plate containing 3 mL of growth medium.

3.6. Grow cells on the glass coverslip in growth medium in a cell culture incubator (5% CO_2, 37 °C) changing the medium once per week. At 90% confluency, switch to differentiation medium made up as follows: high glucose DMEM (4.5 mg/mL), 0.5% bovine serum albumin, 10 ng/mL epidermal growth factor, 0.15 mg/mL creatine, 5 ng/mL insulin, 200 mM glutamine, 600 ng/mL penicillin G and streptomycin, and 7 mM HEPES, pH 7.4). Commercially available differentiation medium may also be used. Change the differentiation medium once per week.

3.7. After 7-10 days in differentiation medium, multinucleated myotubes are visible. Assess for changes in $[Ca^{2+}]$ within one week, as described below.

4. $[Ca^{2+}]_i$ ratio measurements determined with Fura-2

4.1. Load glass coverslip grown myotubes with Fura-2/AM (final concentration of 5 µM) diluted in DMEM for 30 min at 37 °C. Briefly, remove the differentiation medium from the glass coverslip grown cells and add 2 mL fresh differentiation medium. Add 10 µL of Fura-2 AM from a stock solution of 1 mM and incubate 30 min in a cell culture incubator (5% CO_2, 37 °C).

4.2. Transfer glass coverslip to the perfusion chamber and rinse cells with Krebs Ringer's solution containing 2 mM $CaCl_2$.

4.3. Perform on-line $[Ca^{2+}]$ measurements as described above in the EBV single cell section with the use a 20x water immersion FLUAR objective (0.17 numerical aperture).

REPRESENTATIVE RESULTS:

$[Ca^{2+}]_i$ measurements in populations of EBV-immortalized B lymphocytes

Primary B-lymphocytes express the RyR1 isoform that functions as a Ca^{2+} release channel during B cell antigen receptor stimulated signaling processes[17]. Immortalization of B-cells with EBV, a procedure routinely used by geneticists to obtain cell lines containing genomic information of patients, provides the advantage of generating cell lines that express mutant RyR1 Ca^{2+} channels in patients harboring RYR1 mutations[11,13]. $[Ca^{2+}]_i$ changes brought about by the addition of specific RyR1 agonists such as 4-chloro-m-cresol[18] and caffeine can be easily monitored in order to establish whether a given RYR1 mutation alters the sensitivity to an agonist, the amount of calcium released, the resting $[Ca^{2+}]$, or other parameters that have shown to be impacted by mutations. $[Ca^{2+}]_i$ changes can be monitored either in populations of fura-2 loaded cells in suspension with a spectrofluorometer or on groups of cells attached to glass coverslips and examined by epifluorescence. **Figure 2** shows a representative experiment carried out on cell suspensions. Immediately before being placed in the cuvette, cells were spun to remove fura-2 that may have leaked out; cells were then resuspended to a final concentration of 1 x 10^6 cells/mL in warm (37°C) Krebs Ringer's solution containing no additional Ca^{2+} plus 0.5 mM EGTA and placed in the spectrofluorometer. The magnetic stirrer was switched on to position 4 (the highest

position) to keep cells in suspension and fluorescence was recorded. After a stable trace was obtained, the selected agonist was added (in **Figure 2A** this was 300 µM 4-chloro-m-cresol) leading to a rapid Ca^{2+} increase which then slowly declined back to resting levels. The transient nature of the change in fluorescence is important as it indicates (i) that it is not an artefact caused for by the addition of a fluorescent or quenching compound, (ii) that it is not due to calcium binding to extracellular fura-2 and (iii) that the cells are healthy and can actively remove calcium from their cytoplasm.

The same experiment needs to be repeated several times to be analyzed statistically. For each cell line and each day, the experiments are carried out and the total amount of rapidly releasable calcium in the stores needs to be determined by adding the SERCA inhibitor thapsigargin[11,13,14]. As shown in **Figure 2B**, the addition of 400 nM thapsigargin causes a large calcium transient reaching a peak fluorescence value of 2.4 arbitrary units (a.u.); thus the total amount of calcium that can be released from the intracellular stores of the EBV-immortalized B- cells shown in **Figure 2B** equals 2.4 a.u. (thapsigargin peak) - 1.45 a.u. (resting ratio) or 0.95 This fluorescence value was considered 100% when constructing the dose response curve shown in **Figure 2C.**

Single cell $[Ca^{2+}]_i$ measurements in EBV-immortalized B lymphocytes

This second approach relies on the availability of a fluorescence microscope and microperfusion set up allowing the stimulation of a single cell or small groups of cells with a given concentration of agonist and the simultaneous recording of the fluorescence changes. The syringes of the microperfusion system are loaded with different concentrations of the selected RyR1 agonist (either caffeine or 4-chloro-m-cresol) which will be used to generate dose response curves. In the example shown in **Figure 3**, cells were stimulated with 0.5-10 mM caffeine dissolved in Krebs Ringer's solution containing no added calcium plus 100 µM La^{3+} in order to monitor Ca^{2+} release from intracellular stores. Glass coverslips on which fura-2 loaded EBV-immortalized B lymphocytes were allowed to attach are placed in the perfusion chamber and perfused with Krebs Ringer's solution containing 1 mM Ca^{2+}. Most of the cells will have adhered to the poly-L-lysine treated coverslip and small groups of cells should be identified and checked for fura-2 loading. The tip of the perfusion system is placed close to the cells to bathe them (and not all the cells on the coverslip) with caffeine. Normally cells are stimulated starting from the lowest to the highest concentration of caffeine; for each concentration, a new cell or group of cells are selected. Ratiometric fluorescence measurements (excitation at 340 nm and 380 nm, emission at 510 nm) are recorded every second for up to 2 min. A few images are obtained before perfusion in order to obtain a steady baseline, subsequently cell perfusion is initiated, first by flushing cells with a solution of Krebs Ringer's solution containing 100 µM La^{3+} for 5 seconds. This should not result in a change in florescence and is a control to assure that the cell(s) stick to the coverslip throughout the experiment; subsequently a solution containing the selected agonist concentration is flushed over the cell(s). In the example shown in **Figure 3A**, the cells were stimulated with 5 mM caffeine for 20 seconds. The arrow shown indicates when the caffeine valve was opened and this results in an immediate increase in the 340/380 nm fluorescent ratio. After 20 seconds, the caffeine valve closes, and cells are flushed with Krebs Ringer's solution containing 100 µM La^{3+}; fluorescence is recorded until the baseline is reached. For each caffeine concentration the ΔF, that is the caffeine induced peak 340/380 nm fluorescence – the initial

resting 340/380 nm fluorescence is calculated and used to construct a dose response curve as shown in **Figure 3B**. Mean ΔF from 5-10 cells is averaged for each caffeine concentration. The amount of calcium in intracellular stores can also be monitored. In this case, cells are rinsed with Krebs Ringer's solution containing 0.5 mM EGTA and a solution of 1 µM thapsigargin, 1 µM ionomycin and 0.5 mM EGTA is added by hand to the cells (not through the microperfusion system as ionomycin sticks to the tubing and cannot be washed off) and the fluorescence is recorded for approximately 4-5 min. To calculate the ΔF, a region of interest (ROI) outlining a cell is obtained and the changes in fluorescence within the ROI are calculated using an imaging software.

In summary, when using EBV immortalized B-cells to measure the sensitivity of the RyR1 to a specific agonist, repeated experiments should be performed on cells from one individual, on different days. For measurements on cell populations, the status of the intracellular calcium stores needs to be assessed and the EC_{50} to agonist induced calcium release is plotted relative to the total amount of calcium that can be released from the stores. One advantage of using this approach is that the $[Ca^{2+}]$ response of millions of cells is averaged; additionally, no microperfusion system and fluorescent microscopes are necessary. The single cell method allows the use of a smaller number of cells and the on-line visualization of changes in $[Ca^{2+}]$ of selected cells.

Single cell $[Ca^{2+}]_i$ measurements in human satellite cell derived myotubes

Glass coverslip grown and differentiated myotubes are loaded with fura-2, transferred to the perfusion chamber and bathed in Krebs Ringer's solution containing 2 mM Ca^{2+} as described above for EBV cells. The syringes of the perfusion system are filled with the selected agonist and myotubes are stimulated as described above. Since not all cells on the coverslip are multinucleated myotubes, it is important to select the appropriate cell(s) that will be measured. Small groups of myotubes can be stimulated simultaneously. In the example shown in **Figure 4**, a single myotube was flushed with a solution containing KCl, different concentrations of 4-chloro-m-cresol and finally caffeine, however, normally dose response curves to agonists are constructed, as indicated in the previous section, in order to compare the agonist sensitivity of cells from different individuals[12,15,16]. KCl is used as a way to depolarize the plasma membrane. In skeletal, muscle plasma membrane depolarization is sensed by the voltage sensing DHPR which thereby undergoes a conformational change leading to activation and opening of the RyR1. On the other hand, 4-chloro-m-cresol and caffeine are direct pharmacological activators of the RyR1.

FIGURE AND TABLE LEGENDS:

Figure 1: Generation of primary human muscle biopsy-derived myoblast cultures. (**A**) Small fragments of muscle (arrows) are placed in inserts containing 0.5 mL growth medium. After 7-14 days satellite cells can be seen (small arrows) adjacent to the muscle tissue and growing on the bottom of the insert. Image taken through a 10x objective (**B**) and 20x objective (**C**). The grey boxes in panel A were used to cover the identity of the patient.

Figure 2: Calcium release experiments in EBV immortalized B lymphocytes. Ratiometric $[Ca^{2+}]_i$ measurements in a population of fura-2 loaded cells in suspension. **A)** The addition of 300 µM 4-

chloro-m-cresol (arrow) causes an immediate increase in the cytoplasmic [Ca^{2+}], which subsequently decays back to resting levels within 500 seconds. In this example the ΔF induced by the addition of 300 µM 4-chloro-m-cresol is 1.7 fluorescence units- 1.4 fluorescence units = 0.3 fluorescence units. **B)** The addition of the SERCA inhibitor thapsigargin (400 nM, arrow) causes a larger increase in the fura-2 fluorescence ratio which peaks at 2.4 fluorescence units, and subsequently decays to resting levels at 1.45 fluorescence units. The thapsigargin induced [Ca^{2+}] transient represents the total amount of rapidly releasable calcium in the intracellular stores present in the cell population. The peak transient obtained (2.4-1.45= 0.95 units) is used to calculate the percentage of calcium released by a given concentration of 4-chloro-m-cresol. **C)** Representative dose response curves correlating the ΔF as a percentage of the total amount of rapidly releasable calcium in intracellular stores. For 300 µM 4-chloro-m-cresol this value is 0.3/0.95x100=31.6%. Each symbol represents the mean± SEM% of 5-10 values from EBV immortalized cells from a control (closed circles, dotted line) and an MHS individual (closed squares, continuous line) carrying a RYR1 mutation. The curves were generated using a sigmoidal dose–response curve function. Panels A and B are adapted from Girard et al.[11].

Figure 3: Caffeine-induced Ca^{2+} release in individual EBV-immortalized B lymphocytes from a control individual. A) Top panels, time lapse images (**A**) Phase-contrast; (**B–E**), single-cell $[Ca^{2+}]_I$ measurements of fura-2-loaded EBV-immortalized lymphocytes: (B) t=0, (C) t=36 s, (D) t=50 s and (E) t=77 s after the application of 5 mM caffeine. Cells were individually stimulated by the addition of caffeine diluted in Krebs–Ringer solution. Scale bar=10 µm. Bottom panel, representative trace obtained after stimulation of a single cell with 5 mM caffeine (arrow). **B)** Dose response curves showing the caffeine-dependent change in $[Ca^{2+}]_i$, expressed as change in fluorescence ratio (peak ratio 340/380 nm−resting ratio 340/380 nm). Each point represents the mean ± SEM of the change in fluorescence of 4-15 cells. The curves were generated using a sigmoidal dose–response curve function. Closed squares, dotted line, control cells; closed triangles, continuous line, MHS individual carrying a RYR1 mutation. This figure is an adaptation from Ducreux et al.[13].

Figure 4: Calcium release stimulated by KCl, 4-chloro-m-cresol and caffeine in human myotubes from a control individual. **Left panels**: **A)** phase contrast (B–H), single cell intracellular Ca^{2+} measurements of Fura-2-loaded human myotubes. **B)** resting $[Ca^{2+}]_i$; **C)** t=2 s after the application of 150 mM KCl. **D)** t= 2 s after the application of 150 µM 4-chloro-m-cresol; **E)** t=2 s after the application of 300 µM 4-chloro-m-cresol; **F)** t=2 s after the application of 600 µM 4-chloro-m-cresol; **G)** t=2 s after the application of 10 mM caffeine; **H)** t=20 s after the application of caffeine. **Right panel**: Plot of time (s) versus fluorescence ratio (340/380 nm) in the stimulated cell. Myotubes were individually stimulated by addition of the agonist in Krebs-Ringer buffer containing 100 µM La^{3+}, thus the increase in $[Ca^{2+}]_i$ represents only release of calcium from intracellular stores.

DISCUSSION:

The protocols described in this paper have been successfully utilized by several laboratories to study the impact of RYR1 mutations on calcium homeostasis. The critical steps of the approaches outlined in this paper deal with sterility, cell culturing skills and techniques and availability of

biological material. In principle, the use of EBV-immortalized B lymphocytes is simpler and allows one to generate cell lines containing mutant RyR1 channels. The cells can be frozen and stored in liquid nitrogen for many years and cultures can be re-started at any time. Additionally, one can chose whether to monitor calcium homeostasis in cell populations or at the single cell level. The former method is simpler, does not require a fluorescence microscope and allows the investigator to test cell lines generated from different individuals within a short period of time. The limitation being the velocity of cell growth and the availability of a fully equipped (heated and with magnetic stirrer) spectrofluorometer. As an alternative approach flow cytometry in combination with fluorescent calcium indicators can be used to measure calcium fluxes in EBV-immortalized B lymphocytes; in such a way changes in the intracellular calcium concentration can be determined[19]. If a fluorescent microscope, perfusion chamber and microperfusion system are available, then single cell imaging has the advantage of being more sensitive and giving more detailed information including cell to cell variability, kinetic analysis and identification of subcellular domains involved in calcium release. The latter approach is technically more challenging and requires more equipment.

There are multiple advantages to using EBV-immortalized B lymphocytes, including that other parameters aside $[Ca^{2+}]_i$ homeostasis can be measured. For example 4-chloro-m-cresol induced acidification of B cells has been used to differentiate cells from control individuals from MHS patients[20,21]. Nevertheless one must keep in mind (i) that B cells do not express many of the proteins involved in skeletal muscle excitation contraction coupling that may indirectly influence calcium release, (ii) they are non-excitable cells thus cannot be activated physiologically by plasma membrane depolarization, and finally a report by Monnier et al. found that a mutation identified in a patient was not expressed in EBV-immortalized B cells because of a cryptic splice site[22].

Patient derived primary muscle cell cultures have been used by several groups interested in studying the effect of mutations in different genes encoding proteins involved in calcium homeostasis[10,23,24]. These cells can be differentiated into multinucleated myotubes, respond to plasma membrane depolarization and can be assessed by electrophysiological means. In addition, they can be immortalized in order to obtain cell lines carrying RYR1 mutations[25], though the procedure is far more complex than for B-lymphocytes. While it is also true that the muscle cells are slow growing and the time necessary from taking a biopsy to having a sufficient number of cells can be more than one month, it is also possible to store the small pieces of muscle biopsies in freezing medium in liquid nitrogen in order to obtain myoblasts years later. The long culturing times have the added risk of contamination by bacteria, yeasts or molds and as soon as a sufficiently large number of myoblasts have been obtained, it is important to freeze and store them in liquid nitrogen. Our laboratory has successfully applied the same technique outlined above for myotubes, to study calcium changes in human skin-derived fibroblasts transduced with myoD and differentiated into myotubes[26]. This was done to study the functional effect of RYR1 mutations when muscle biopsy-derived myoblasts were not available. As for B-lymphocytes, 4-chloro-m-cresol induced acidification of myotubes from patients with RYR1 mutations linked to MHS has been tested successfully[27].

In conclusion, the use of biological material from patients to study the genotype-phenotype correlation has many advantages the drawback and can be used successfully to study the effect of mutations in RYR1. When using this approach however, it is important to keep in mind that mutations present in other genes may influence calcium homeostasis; therefore, the use of cells from different families harboring the same mutation as well as from family members not harboring the RYR1 mutation as controls, should be performed.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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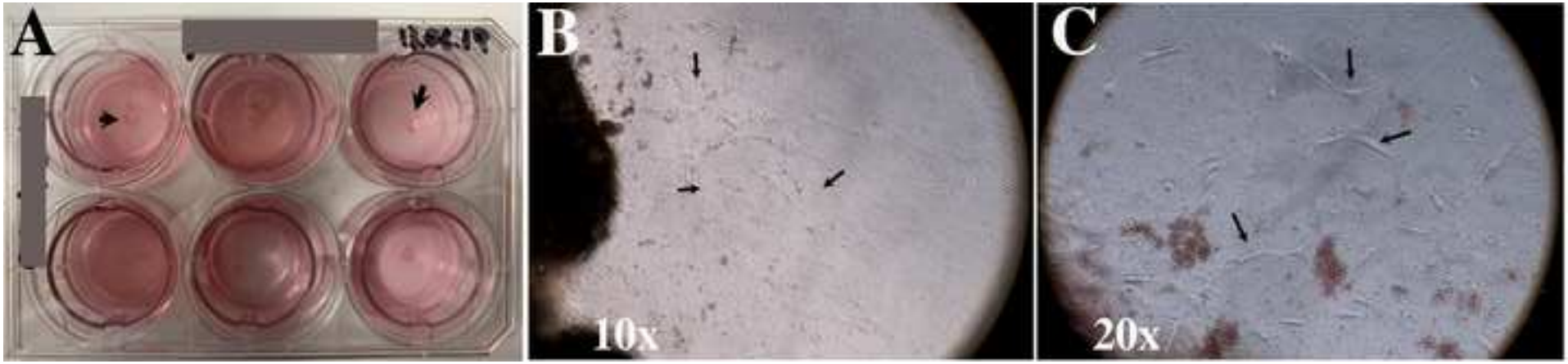

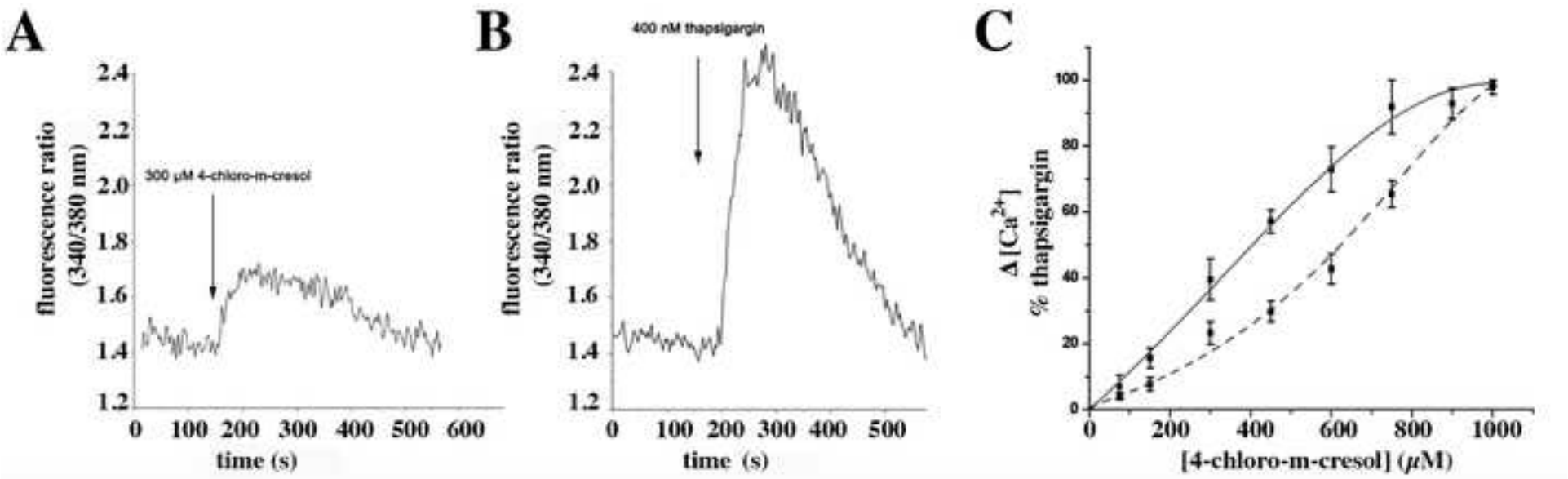

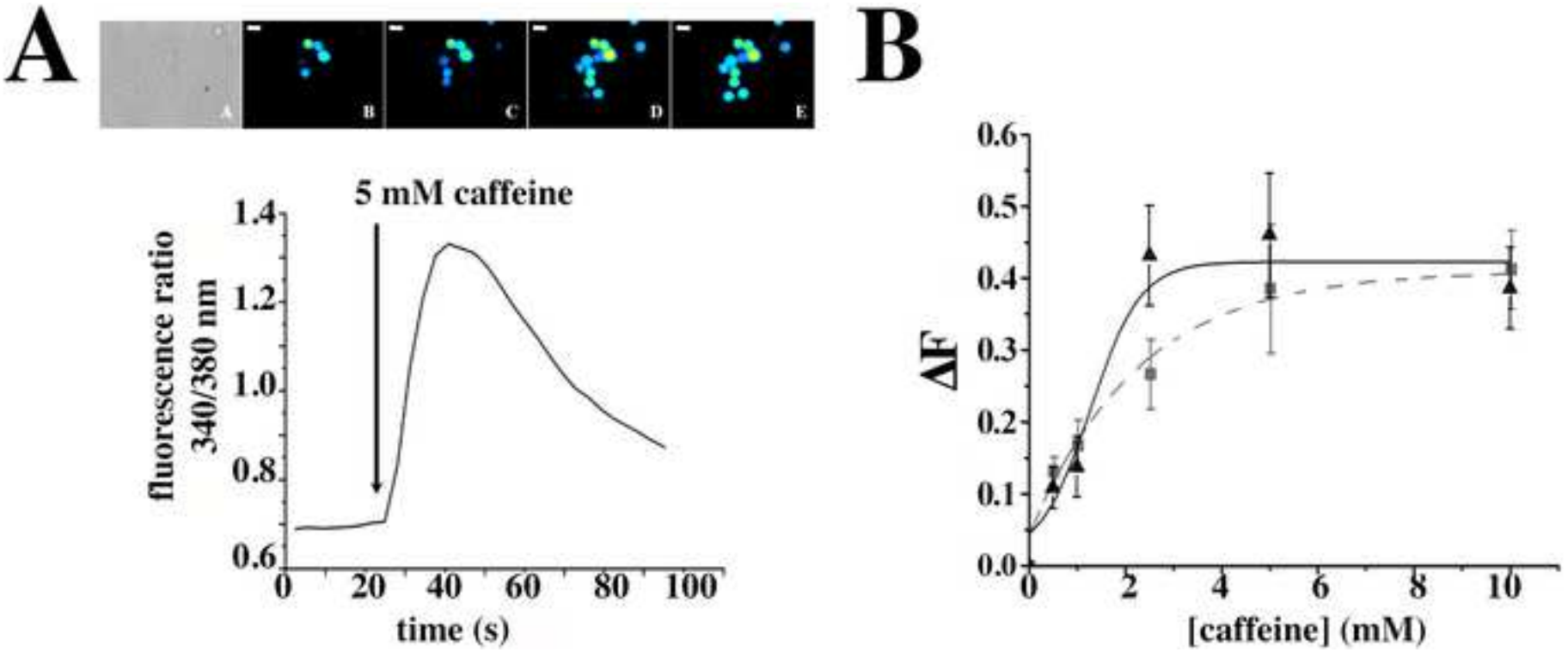

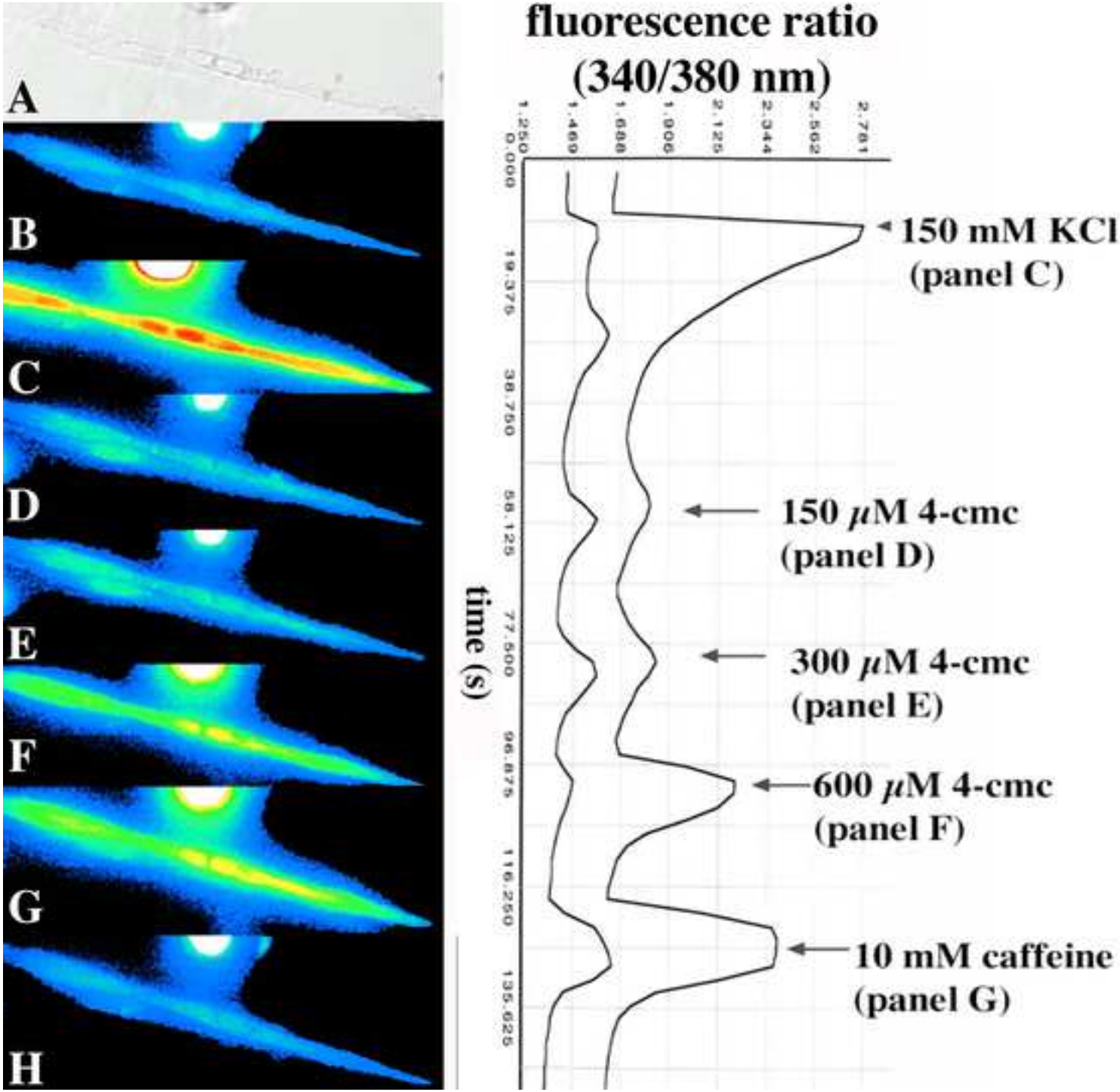

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
4-chloro-m-cresol	Fluka	24940	
Blood collection tubes	Sarstedt	172202	
Bovine serum albumin (BSA)	Sigma-Aldrich	A7906	
caffeine	Merk	102584	
Cascade 125+ CCD camera	Photometrics		
Cascade 128+ CCD	Photometrics		
Creatine	Sigma-Aldrich	C-3630	
DMEM	ThermoFisher Scientific	11965092	
DMSO	Sigma	41639	
EGTA	Fluka	3778	
Epidermal Growth Factor (EGF)	Sigma-Aldrich	E9644	
Ficoll Paque	Cytiva	17144002	
Foetal calf serum	ThermoFisher Scientific	26140079	
Fura-2/AM	Invitrogen Life Sciences	F1201	
Glutamax	Thermo Fisher Scientific	35050061	
HEPES	ThermoFisher Scientific	15630049	
Horse serum	Thermo Fisher Scientific	16050122	
Insulin	ThermoFisher Scientific	A11382II	
Ionomycin	Sigma	I0634	
KCl	Sigma-Aldrich	P9333	
Laminin	ThermoFisher Scientific	23017015	
Lanthanum	Fluka	61490	
Microperfusion system	ALA-Scientific	DAD VM 12 valve manifold	
Origin Software	OriginLab Corp	Software	
Pennicillin/Streptomycin	Gibco Life Sciences	15140-122	
Perfusion chamber POC-R	Pecon	000000-1116-079	
poly-L-lysine	Sigma-Aldrich	P8920	
RPMI	ThermoFisher Scientific	21875091	
Spectrofluorimeter	Perkin Elmer	LS50	
Thapsigargin	Calbiochem	586005	
Tissue culture dishes	Falcon	353046	
Tissue culture flask	Falcon	353107	

Tissue culture inserts	Falcon	353090
Trypsin/EDTA solution	ThermoFisher Scientific	25300054
Visiview	Visitron Systems GmbH	Software
Zeiss Axiovert S100 TV microscope	Carl Zeiss AG	
Zeiss glass coverslips	Carl Zeiss AG	0727-016

Basel 09.12.2020

Dear Dr Vineeta Bajaj,
enclosed you will find our revised manuscript N° JoVE62196. We have revised the manuscript as requested below in the Editorial comments and by the Reviewers. All changes made to the text are highlighted in yellow in the revised version of the manuscript. We will answer all queries on a point by point basis:

Editorial comments:

Q1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
R1. We have checked the text and have corrected all the typos and grammar mistakes.

Q2. Y axis label: Please be consistent in the ratio description. Figure 3A calls it fluorescent ratio but figure 2A/B refers to it as a fluorescence ratio. Figure 4 refers to the fluor ratio.
R2. We have changed the Y axis labels of figures 3 and 4. They now read fluorescence ratio (340/380 nm).

Q3. Figure 1: Please discuss the gray boxes or remove them. Please include scale bars for panel B and C. Additionally, please make clear in the figure that B is 10x and C is 20x magnification of Panel A.
R3. We have a sentence to the legend of figure 1 (line 331 revised manuscript) explaining that the grey boxes were added to cover the identity of the patient. Additionally, 10x and 20x (magnification) have been added onto panels B and C of figure 1.

Q4. Figure 2: Please use SI abbreviations for time: s instead of sec. Please include a space between the number and the unit: 380 nm instead of 380nm.
R4. The abbreviation of “sec” has been changed to “s” and a space has been added between 380 and nm.

Q5. Figure 3: Please use SI abbreviations for time: s instead of sec.
R5. This has been done on the revised figure 3.

Q6. Figure 3A: Please include scale bars for the subpanels.
R6. A scale bar of 10 µm has been added to panel A sub-panels B to E of figure 3; this is also indicated in line 354 of the revised Figure Legend section.

Q7. Figure 3B: 0.0 on the y axis is cut off. Please revise.
R7. The text has been corrected in panel B.

Q8. Figure 4: Please use SI abbreviations for time: s instead of sec. Can the panel labels be moved to the left side of the panel instead of the right? This would maintain consistency with the other figures.
R8. We have complied with this comment and Figure 4 has been modified as suggested.

Q9. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "ourThe protocls " etc.).

R9. The personal pronouns have been removed from the text. All changes are highlighted in yellow.

Q10. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.
R10. The ethical statement has been added to the revised manuscript (line 88-89).

Q11. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: Axiovert, Falcon, etc.
R11. Commercial language does not appear in the manuscript.

Q12. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?
R12. More details to the protocols have been added (see sections highlighted in yellow)

Q13. Line 88: How much blood is collected?
R13. 30 ml, this has been added to line 91, revised manuscript.

Q14. Line 91: How is the density centrifugation done? What is the concentration of Ficoll used and how long is the centrifugation and at what speed in x g?
R14. This information has been added to points 2 and 3 of the revised manuscript, lines 95-103.

Q15. Line 93: Rinse the cell pellet? How much PBS is used for the rinse? Are the cells counted and how many cells per flask are there? How much culture medium is in the flask?
R15. This information has been added to point 4 of the revised manuscript, lines 104-110.

Q16. Line 96: What is the ratio of cell to virus used?
R16. We do not know the ratio since we used the supernatant from B95.8 cells as a source of virus. We used 2 ml supernatant per million mononuclear cells in 20 mls. This information appears in point 5, lines 111-112, revised manuscript.

Q17. Line 101: How is the extraction performed? A citation would suffice here.
R17. We have added a reference on line 120, revised manuscript.

Q18. Line 113: What are the centrifugation parameters used: speed and time?
R18. Centrifugation parameters have been added to point 2, line 133, revised manuscript.

Q19. Line 114: What is the stir rate and temperature for the measurement?
R19. The stir rate was set to maximal velocity and the temperature was set to 37°C. This information has been added to lines 136-137, revised manuscript.

Q20. Please specify all volumes used throughout the experiment.

R20. The information has been added in the revised text. Please note that volumes can be easily calculated by the experimenter running the test, if the stock solution concentration is known. See point 6 lines 141-145, revised manuscript.

Q21. Line 120: What volume and concentration of the agonist is used here?
R21. The volume and concentration depend, as a dose response curve is being generated. All concentrations between 75 µM and 1 mM need to be tested. This information has been added to lines 146-154, revised manuscript.

Q22. Line 123: Please provide the different concentrations used for the response curve.
R22. See reply to point Q21 above.

Q23. Line 135: How many cells are placed?
R23. A total of $1.0x10^6$ cells in 1.0 ml, see line 164, revised manuscript.

Q24. Line 138: What is the perfusion rate?
R24. The perfusion rate is 2 ml/min (line 169, revised manuscript.

Q25. Please use SI abbreviations: s instead of sec.
R25. The abbreviation has been corrected.

Q26. Line 166: Did you mean Figure 1B/C?
R26. Yes, this was a mistake and has been corrected in the revised manuscript, line 197.

Q27. Line 169: How is trypsinization done? How much of what is used for how long? How many cells are placed in a what size flask and incubate at what conditions?
R27. The requested information has been added to points 4, lines 199-205, revised manuscript.

Q28. Line 170: How many myoblasts per glass coverslip?
R28. We do not count the cells, but transfer all cells from a T25 onto a 43 mm coverslip. This is detailed in point 5, lines 206-210, revised manuscript.

Q29. Line 171: Grow at what conditions?
R29. Grow cells on the glass coverslip in growth medium in a cell culture incubator (5% CO_2, 37°C) changing the medium once per week, lines 211-212, revised manuscript.

Q30. Line 179: Please revise for clarity. What is being done here?
R30. Myotubes are being loaded with fura-2. The paragraph has been expanded to explain in more detail. See lines 223-226 revised manuscript.

Q31. Please include a one line space between each protocol step.
R31. This has been done in the revised manuscript.

Q32. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted

Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Q33. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

R32-33. I am not sure that a video can be made. Our lab is off limits because of Coronavirus and people are allowed to work within 2 meters. I am afraid we will have to skip the video altogether???

Q34. Please spell out journal titles.
R34. This has been done in the revised manuscript.

Reviewers' comments:

Reviewer #1:

Major Concerns:

The major concern is the focus on EBV immortalized B cells, where the process of immortalization is not well described, and spending as much of the manuscript to describe a method which uses a cell type that does not express many ECC related proteins which modify RyR function seems excessive. In addition based on this reviewers experience with the single cell method as described I found that the technique is not as easily done as described in the manuscript, despite the fact that the experimenters were relatively experienced in performing fluorescence Ca2+ imaging in single cells.

Reply:

We have added more details concerning the immortalization process (see lines 95-115 revised manuscript).
While it is true that EBV immortalized cells do not express many ECC related proteins, they do express the RyR1 and thus can be used to directly assess the effect of *RYR1* mutations on calcium homeostasis. We would like to point out that EBV cells have been successfully used by many investigators to test alterations of RyR1 function brought about by mutations identified in patients.
We were sorry to hear that the reviewer found the technique not as easily done as described in the manuscript. Like all techniques, it may require a bit of time before it is optimized, however it is straightforward and simple to perform.

Minor Concerns:

There are a few type editing issues

Reply

We have corrected the editing issues in the revised manuscript.

Reviewer #2:

Manuscript Summary:

A manuscript by Treves et al. describes methods to evaluate ryanodine receptor (RyR) ion channel function. Particularly, the group uses these strategies to characterize skeletal RyR1 channels derived from patient biopsies harboring skeletal disease-associated RyR1 mutations. The skeletal diseases include malignant hyperthermia, centronuclear myopathy, and different types of core myopathies. These techniques have been greatly contributing during the past ~20 years for identifying skeletal calcium signaling pathology and characterizing genotype/phenotype correlations of RyR1 mutants; therefore, the manuscript is well suitable for JoVE. I have a few minor questions/suggestions.

Reply: We would like to thank the reviewer for his/her kind words.

Minor Concerns:
In Fig. 4, chemical depolarization-induced Ca2+ release is much larger (>1.5 times) than caffeine-induced Ca2+ release in the same cell. Considering 10 mM caffeine is believed to deplete SR Ca2+ store, how could this happen?

Reply: The Reviewer is correct in his/her reasoning. However, in the experiment depicted in figure 4 we show the same cells responding ***to consecutive stimulations,*** starting from KCl, followed by three concentrations of 4-cmc and ending with 10 mM caffeine. Since the experiments were performed in the presence of 100 µM La^{3+}, the stores of the myotube cannot replenished after the addition of each agonist since La3+ blocks calcium influx, and therefore the peak calcium transients are smaller after the addition of each agonist.
Under experimental conditions used to construct dose response curves such as those referred to (References 12,15 and 16) , a single cell receives ***only one concentration*** of an agonist, and the increase in fluorescence (ΔF) from 5-10 cells is averaged to make the dose response curve.

Also, please describe the method for Fig. 4 in detail. How did you give 150 mM KCl stimulus? All salt in Krebs Ringer solution (total 145 mM for KCl + NaCl) was replaced with 150 mM KCl? What is the expected ΔmV (~+85 mV?) caused by this procedure?

Reply: That is correct. We prepare a 10 x stock solution of Krebs Ringer ***without added NaCl or KCl***. We then dilute this solution by 80%, bring adjust the pH to 7.4 with 1 N NaOH, add KCl in order to reach the required final KCl concentration. We then add enough NaCl to bring the ***combined*** concentration of NaCl and of KCl, to 150 mM, then add the remaining volume of H_2O in order to have a 1 x solution. For example, when stimulating cells with 100 mM KCl, the solution is made up with Krebs Ringer base, to which 100 mM KCl and 50 mM NaCl are added.
The expected ΔmV caused by the addition of 150 mM KCl should be roughly +80 mM as calculated using the Nernst equation.

In introduction, ECC and DHPR are not common abbreviations. Please spell out.
Reply: Thank you for pointing this out. This has been amended in the revised manuscript (lines 81 and 83).

In the section of "Preparation myotubes from muscle biopsies", 200 mM glutamine seems to be too high.

Reply: Thank you for pointing this out, the final concentration is 3 mM glutamine. This has been corrected on line 192, revised manuscript.

Table is difficult to read. A section for "Comments/Description" is probably not needed. Please proofread. Figure 1 legend: (A) 300 µM, not mM; (B) 400 nM, not nm. Figure 4 legend: (D) 150 µM, not mM.

Reply. Thank you for pointing this out. Corrections have been made to the text

Reviewer #3:
Manuscript Summary:
The manuscript reports three methods to analyze the functional effect of RYR1 mutations endogenously expressed in patient cells, i.e., calcium release measurements in EBV immortalized B lymphocyte populations or at single cell level, and in human satellite cell derived myotubes at single cell level.
This topic is of current interest because mutations in the Ryr1 calcium channel are linked to several distinct skeletal muscle disorders, and RYR1-related myopathies are probably the most frequent form of congenital myopathies in several populations.

Reply: We would like to thank the reviewer for his comments.

Minor Concerns:
Page 3 line 166. Fig 1 and not Fig 2.
Reply: This has been corrected in the revised manuscript (line 198, revised manuscript).

Page 5 lines 239-240. "In the example shown in Fig.3 A, the cells were stimulated with 5 mM caffeine for 20 seconds." In the legend to fig 3 A it is reported that the top panels refer to EBV-immortalized lymphocytes stimulated with 10 mM caffeine and the bottom panel to cells stimulated with 5 mM caffeine. Please, clarify this point in the text.
Reply: Thank you for spotting this. The figure legend has been corrected (line 353, revised manuscript).

Page 7 line 332. Please, cite papers where the flow cytometry was used to functionally characterize Ryr1 mutant channels.
Reply: The reference has been added (new reference 19) and has been cited on line 388.

Page 6, line 270. Briefly describe the reason for using KCl and the significance of the response to this stimulation.
Reply: A sentence has been added to the revised manuscript (lines 320-324)

In addition to some grammar errors, I have noticed some inaccuracies, such as unexplained acronyms/abbreviations (e.g. page 1 line 80, ECC; page 2 line 93, PBS; page 2 line 94, RPMI; page 3 line 160, DMEM) or the lack of use in the text of introduced abbreviations (e.g. 4-cmc), which I believe will be checked and corrected by the copy-editing services.
Reply: Thank you, some of these issues were also pointed out to by the Editor and other reviewers.

We would like to thank the Editor and Reviewers and hope that in its present format our manuscript is now acceptable for publication in JoVE.

Kind regards,

Susan Treves.