Tracking the sarcoplasmic reticulum membrane voltage in muscle with a FRET biosensor

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Ion channel activity in the plasma membrane of living cells generates voltage changes that are critical for numerous biological functions. The membrane of the endoplasmic/sarcoplasmic reticulum (ER/SR) is also endowed with ion channels, but whether changes in its voltage occur during cellular activity has remained ambiguous. This issue is critical for cell functions that depend on a Ca2+ flux across the reticulum membrane. This is the case for contraction of striated muscle, which is triggered by opening of ryanodine receptor Ca2+ release channels in the SR membrane in response to depolarization of the transverse invaginations of the plasma membrane (the t-tubules). Here, we use targeted expression of voltage-sensitive fluorescence resonance energy transfer (FRET) probes of the Mermaid family in differentiated muscle fibers to determine whether changes in SR membrane voltage occur during depolarization–contraction coupling. In the absence of an SR targeting sequence, FRET signals from probes present in the t-tubule membrane allow calibration of fibers to determine whether changes in SR membrane voltage occur during depolarization–contraction coupling. In the absence of an SR targeting sequence, FRET signals from probes present in the t-tubule membrane allow calibration of the voltage sensitivity and amplitude of the response to voltage-clamp pulses. Successful SR targeting of the probes was achieved using an N-terminal domain of triadin, which completely eliminates voltage-clamp–activated FRET signals from the t-tubule membrane of transfected fibers. In fibers expressing SR-targeted Mermaid probes, activation of SR Ca2+ release in the presence of intracellular ethyleneglycol-bis(β-amino-ethyl ether)-N,N,N′,N′-tetra acetic acid (EGTA) results in an accompanying FRET signal. We find that this signal results from pH sensitivity of the probe, which detects cytosolic acidification because of the release of protons upon Ca2+ binding to EGTA. When EGTA is substituted with either 1,2-bis(o-aminophenoxy)ethane-N,N,N′,N′-tetracetic acid or the contraction blocker N-benzyl-p-toluene sulfonamide, we find no indication of a substantial change in the FRET response caused by a voltage change. These results suggest that the ryanodine receptor–mediated SR Ca2+ efflux is well balanced by concomitant counterion currents across the SR membrane.

Introduction

Ion fluxes across the cell plasma membrane generate electrical activity that plays a key role in the function of excitable and non-excitable tissues (Hodgkin and Huxley, 1952; Bertrán et al., 1995; Armstrong and Hille, 1998). Ion channels, pumps, and transporters are also present in the membrane of the ER/SR, where they either play a clearly identified role (Berridge, 1993; Meissner, 1994; Martonosi, 1996) or are presumed to do so (Kourie et al., 1996; Picollo and Pusch, 2005; Yazawa et al., 2007; Kuum et al., 2012). One of the most classic examples is the role of Ca2+ release channels and SR/ER Ca2+-ATPase in Ca2+ cycling across the reticulum membrane to control contraction of smooth and striated muscles. In skeletal muscle, contraction is initiated when action potentials activate the voltage-sensing Ca1.1 proteins in the t-tubules, within a specific membrane region called the triad, where the membrane of one t-tubule is in close apposition with the membrane of two terminal cisternae of SR. In turn, activated Ca1.1s open up type 1 ryanodine receptor (RYR1) Ca2+ release channels in the junctional SR membrane, through a protein–protein interaction coupling process (Schneider, 1994; Samsó, 2015). The resulting SR Ca2+ efflux increases cytosolic [Ca2+], which triggers contraction. So far, it is believed that during Ca2+ release, the voltage across the SR membrane is maintained near a resting value close to 0 mV because of a countercurrent of other ions (Somlyo et al., 1981; Smith et al., 1988; Oetliker, 1989; Fink and Veigel, 1996; Gillespie and Fill, 2008). However, the ER/SR membrane is not accessible to standard electrophysiology in intact cells, which has so far prevented determining whether changes in its electrical potential occur during cellular activity. In skeletal muscle, this question was experimentally challenged in the 1980s (Vergara et al., 1978; Baylor et al., 1984). However, results...
were inconclusive, and because the approaches used at the time lacked target specificity, it could not be established whether the detected signals corresponded to a SR voltage change or resulted from other steps of excitation–contraction (EC) coupling. Here, we circumvented this problem by targeting voltage-sensing fluorescence resonance energy transfer (FRET) biosensors of the Mermaid family (Tsutsui et al., 2008) to the SR membrane of muscle fibers. Results suggest that the SR membrane voltage changes negligibly during muscle fiber activation, if at all.

Materials and methods
All experiments and procedures were performed according to the ethics principles of the French Department of Veterinary Services and the French Ministry for Higher Education and Research, in accordance with the guidelines of the local animal ethics committee of the University Claude Bernard Lyon 1, the French Ministry of Agriculture (decreet 87/848), and the revised European Directive 2010/63/EU. All experiments were performed at room temperature (20–22°C).

Plasmid constructs
Bio-sensor cDNAs were derived from the original Mermaid cDNA (Tsutsui et al., 2008) and were all inserted into the modified pCS2+ vector for mammalian expression. Rv-Mermaid cDNA was designed by inserting Mermaid voltage-sensing domain (VSD) coding sequence in 3′ of mUKG and mK0κ coding sequences. Rv-MermaidD129E/Y235R was designed by inserting mutations into codons 129 and 235 of the VSD S4-segment sequence to shift its voltage dependence toward more positive values (Tsutsui et al., 2013a,b). The VSDD129E/Y235R sequence was PCR amplified from pCS2+-MermaidD129E/Y235R and was used to replace WT VSD sequence by in-frame insertion using EcoRI and XbaI sites. To trigger retention of the biosensor at the triadic SR, we fused in a 201-residue-long N-terminal chain comprising two di-arginine motifs (residues 29–35). Di-arginine motif located in the N-terminal cytosolic tail has been shown to be responsible for membrane-associated transport (Sharma et al., 2010). After PCR amplification, the cDNA fragment corresponding to this so-called ER-N-terminal sequence was inserted in-frame and in place of the original 5′ end of Mermaid2 sequence using appropriate restriction enzymes.

In vivo transfection
The experimental protocol for in vivo transfection was approved by the Lyon University Animal Experimentation Committee. Expression was achieved by plasmid injection followed by electroporation according to previously described procedures (Lefebvre et al., 2011). In brief, Swiss OF1 male mice 8–16 wk old were used. Transfection was performed so as to target both the flexor digitorum brevis (fdb) and interosseus muscles of the animals. Mice were anesthetized by isoflurane inhalation using a commercial delivery system (Univentor 400 Anesthesia Unit; Univentor). Then, 20 µl of a solution containing 2 mg/ml hyaluronidase dissolved in sterile saline was injected into the footpads of each hind paw. 1 h later, the mouse was reanesthetized by isoflurane inhalation. A total volume of 20 µl of a solution containing 30–50 µg plasmid DNA diluted in NaCl 0.9% was injected into the footpads of the animal. After the injection, two gold-plated stainless steel acupuncture needles connected to the electroporation apparatus were inserted under the skin, near the proximal and distal portion of the foot, respectively. The standard protocol that we used consisted of 20 pulses of 130 V/cm amplitude and 20-ms duration delivered at a frequency of 2 Hz by a BTX ECM 830 square wave pulse generator (Harvard Apparatus). Muscle fiber isolation and experimental observations and measurements were performed 7–14 d later.

Preparation of muscle fibers and electrophysiology
Single fibers were isolated from the fdb and interosseus muscles using a previously described procedure (Jacquemond, 1997). Mice were anesthetized by isoflurane inhalation and killed by cervical dislocation before removal of the muscles. Muscles were treated with collagenase (type I; Sigma) for 60 min at 37°C in the presence of Tyrode’s solution (see Solutions). Single fibers were then obtained by gentle trituration of the muscles within a 50-mm-wide culture μ-dish (Ibidi) filled with culture medium containing 10% FBS (MI199; Eurobio). The bottom of which had been first covered with a thin layer of silicone grease. Single fibers were then partially insulated with silicone grease so that only a 50–100-µm-long portion of the fiber extremity was left out of the silicone, as described previously (Jacquemond, 1997). In case of local expression of the constructs (Fig. 4), care was taken so that the expressing fiber region was outside of the silicone. Voltage clamp was performed with a micropipette filled with an intracellular-like solution (see Solutions). The tip of the micropipette was inserted through the silicone within the insulated part of the fiber and was crushed against the bottom of the chamber to reduce series resistance and ease intracellular equilibration of the micropipette solution. The micropipette was connected to an RK-400 patch-clamp amplifier (Bio-Logic) used in whole-cell voltage-clamp configuration. Command voltage pulse generation was achieved with an analogue-to-digital converter (Digidata 1440A; Axon Instruments) controlled by pClamp 9 software (Axon Instruments). Fibers were bathed in the TEA-containing extracellular solution (see Solutions). Analogue compensation was adjusted to further decrease the effective series resistance. Voltage-clamp steps were applied from a holding command potential of either −80 or 0 mV. For intracellular Ca2+ measurements shown in Fig. 5, the micropipette was filled with the intracellular-like solution also containing 0.2 mM of the Ca2+-sensitive fluorescent indicator fluo-4 FF. In most experiments, muscle fiber contraction was prevented by the presence of a large concentration of EGTA in the intracellular-like solution (see Solutions). Alternatively, contraction...
was prevented by using either 1,2-bis(o-aminophenoxy)ethane-
N,N,N′,N′-tetraacetic acid (BAPTA) in the intracellular-like solu-
tion or N-benzyl-p-toluene sulphonamide (BTS) in the extracel-
lar solution, as specified in the text.

Fluorescence measurements in voltage-clamped muscle fibers
All experiments were conducted with a Zeiss LSM 5 Exci ter
confocal microscope equipped with a 63× oil-immersion ob-
tive (numerical aperture, 1.4). For x,y confocal imaging of fibers
expressing the Mermaid constructs, either excitation was pro-
vided by the 488-nm line of an argon laser and a 505-nm long-
pass filter was used on the detection channel, or excitation was at
543 nm from a HeNe laser and fluorescence was collected at >560
nm. Both configurations provided essentially identical images.
For detection of FRET changes in response to voltage-clamp
pulses, the line-scan mode (x,t) of the microscope was used with
the 458-nm line of the argon laser for excitation and simultane-
ous detection at 500 ± 25 nm (F500, green traces) and >560 nm
(F>560, red traces). The line was always oriented along the main
axis of the fibers. To improve detection and limit photobleaching,
records were taken with the pinhole fully open (1-mm diameter).
Even under these conditions, the signal-to-noise ratio of the volt-
age-induced FRET changes was in most cases rather poor; so that,
in a given fiber for each tested pulse protocol, routinely three
to five records (each at a different line position) were taken and
averaged. FRET signals were expressed as the ratio R = F556/F500
and normalized to the ratio R0 at either −80 or 0 mV, as speci-
fied in the text. Fluorescence records at 500 and >560 nm were
rectified to some extent by time-dependent changes including
photobleaching, the relative rate of which differed on the two
channels. In addition, with all Mermaid and Rv-Mermaid con-
structs, there was a transient rise in fluorescence at the begin-
ing of the records that was most obvious on the red channel
(Fig. 1, B and E). These changes were observed in the absence of
voltage-clamp pulses and were not systematically cancelled in
the F556/F500 ratio. To correct for them, we used two strategies
that gave essentially the same results. The first method consisted
of using bracketing records from the same fiber region, taken
with no voltage-clamp pulses applied. Corresponding ratios
were fitted with either a single exponential plus linear function
or with the sum of two exponentials, and the fits were then used
to normalize the test records. Alternatively, fits were achieved
directly on the test records, using only the portions of traces
where no voltage-induced response was present; the result was
then extrapolated to the full record duration and used for nor-
malization. Examples of such fits performed directly on the fluo-
rescence traces are shown as insets in Fig. 1 (B and E) and Fig. 2 B.

For detection of fluo-4 FF fluorescence, excitation was at 488
nm, whereas detection was >505 nm. Image processing and anal-
ysis was performed using ImageJ (National Institutes of Health)
and Microcal Origin (Microcal Software).

Fluorescence measurements in nigericin-treated muscle fibers
To test the effect of changes in pH on the FRET signal from SR-tar-
geted Mermaid probes, transfected muscle fibers were treated
with nigericin. For this, single isolated muscle fibers expressing
the construct of interest were partially embedded into silicone
grease so that they remained well maintained on the bottom of
the chamber during the experiment. A thin polyethylene capil-
lar perfusion system operating by gravity was used to change
the composition of the extracellular solution in the immediate
vicinity of the tested fiber. Fluorescence detection was per-
formed using the x,y scanning mode of the microscope with the
standard FRET configuration (458-nm excitation and simulta-
nous detection at 500 and >560 nm). Fibers were bathed in a
solution containing (in mM) 140 potassium glutamate, 2 MgCl2,
5 glucose, 5 Na2-ATP, 5 HEPES, and 0.01 nigericin adjusted to
pH 7.0 or 7.2, and the gravity perfusion system was used to test
the effect of solutions of identical composition adjusted to a dif-
ferent pH value.

Cell culture and immunolabeling
COS-7 and HeLa cells were grown in 4.5 g/l glucose-containing
Dulbecco’s Modified Eagle Media (Eurobio) supplemented with
10%–15% FCS, 100 mM sodium pyruvate, 100 U/ml penicillin,
and 100 µg/ml streptomycin at 37°C in a 5% CO2 environment.
Cells were seeded on glass coverslips 1 d before transfection and
transfected with corresponding plasmids using FuGENE HD
(Promega) or Polyfect (Qiagen). Immunolabeling was performed
48–72 h after transfection. Cells were first fixed with 2% para-
formaldehyde diluted in PBS supplemented with 300 mM MgCl2
and 30 mM CaCl2 (PBS-CaMg) and permeabilized with 0.1% Tri-
ton X-100/50 mM glycine in PBS-CaMg. Nonspecific sites were
blocked with 1% BSA in PBS-CaMg. Cells were then incubated
overnight at 4°C with the following primary monoclonal antibi-
dodies diluted in 1% BSA in PBS-CaMg: monoclonal anti-GM130
(clone 35; 1:1,000; BD Transduction), anti-KDEL (10C3; 1:250;
Abcam), or polyclonal anti-GRP78 BIP antibody (1:200; Abcam).
A second blocking step was performed using normal goat serum
1% in PBS-CaMg. Cells were then incubated with either Hilyte
Fluor647–conjugated anti-mouse secondary antibody (Anaspec)
or Alexa Fluor 594–conjugated anti–rabbit IgG (Molecular
Probes) diluted in 1% normal goat serum in PBS-CaMg (1:500 or
1:1,000, respectively) for 1 h at ambient temperature.
Incubations all took place in a humidified chamber, and cells
were extensively washed in PBS-CaMg after each incubation.
Immunolabeled samples were mounted in antifading medium
(Vectashield; Vector Laboratories; or Dako mounting medium)
and examined using a Zeiss LSM 5 Exci ter confocal microscope
equipped with a 63× oil-immersion objective.

Solutions
The extracellular solution used for voltage clamp contained (in
mM) 140 TEA-methanesulfonate, 2.5 CaCl2, 2 MgCl2, 1 4-amii-
nopyridine, 10 HEPES, and 0.002 tetrodotoxin. The standard
intracellular–like solution contained (in mM) 150 potassium glu-
tamate, 5 Na2-ATP, 5 Na2-phosphocreatine, 20 mM EGTA, 8 mM
CaCl2, 5.5 MgCl2, 5 glucose, and 5 HEPES. In some experiments,
BAPTA was used in place of EGTA at the same concentration
(specified in text). Current-clamp experiments were performed
in the presence of extracellular Tyrode’s solution containing (in
mM) 140 NaCl, 5 KCl, 2.5 CaCl2, 2 MgCl2, and 10 HEPES. In some
experiments, the extracellular solution contained 50 µM BTS to
block contraction. For all experiments with BTS, the intracellular
solution contained neither EGTA nor BAPTA. All solutions were adjusted to pH 7.20.

Statistics

Least-squares fits were performed using a Marquardt-Levenberg algorithm routine included in Microcal Origin (Originlab). Data values are presented as means ± SEM for n fibers.

Online supplemental material

Fig. S1 shows the sequence structure of plasma membrane- and SR membrane–targeted Mermaid-derived voltage sensors used in this project. Fig. S2 and Fig. S3 show the subcellular localization of Rv-MermaidD129E/Y235R and of T306-Rv-MermaidD129E/Y235R in COS-7 cells, respectively. Fig. S4 illustrates the disappearance and recovery of the FRET signal from T306-Rv-MermaidD129E/Y235R upon voltage-dependent inactivation and recovery from inactivation of SR Ca2+ release, respectively. Fig. S5 shows the response of ER-Mermaid2-D129E to t-tubule membrane depolarization. Fig. S6 shows the ER localization of ER-Mermaid2 in COS-7 and HeLa cells.

Results

FRET response of Mermaid biosensor to t-tubule membrane voltage changes

To measure changes in membrane voltage, we used fluorescent sensors of the Mermaid family composed of a VSD associated with two fluorophores mUKG and mKOκ as FRET donor and acceptor, respectively (Tsutsui et al., 2008; Fig. S1). Expression of Mermaid in mouse muscle fibers yielded a transverse-banded fluorescence pattern under the form of successive double peaks separated by 2 μm (Fig. 1A), consistent with the protein being present in the triadic region of the fibers. In response to voltage-clamp depolarizing steps from −80 mV, the detected changes in fluorescence (at 500 nm and >560 nm, upon 458-nm excitation) were consistent with voltage-dependent energy transfer between the two fluorophores mUKG and mKOκ (Fig. 1B), providing evidence for localization of Mermaid in the t-tubule membrane of the muscle fibers. Main traces in Fig. 1B have been corrected from time-dependent changes in fluorescence also observed when no voltage pulse was given. Similar changes were previously reported for the fluorescent proteins mOrange2 (Shaner et al., 2008) and mNeptune (Chu et al., 2014) and interpreted as resulting from photoactivation before photobleaching. It is thus possible that similar mechanisms operated in our conditions. Raw fluorescence traces and superimposed fits used for the correction are shown in the insets. Details of how fits were generated and corrections performed are described in Materials and methods. In each tested fiber, the resulting depolarization-dependent increase in the FRET ratio R/R0 (R0 being the value of the ratio at −80 mV) was adjusted with a Boltzmann function. On average, Mermaid yielded a voltage dependence centered at a mid-value (V0.5) of −38 mV (Table 1). Importantly, depolarizing and hyperpolarizing pulses of identical amplitude from −80 and 0 mV, respectively, generated symmetrical changes in the FRET ratio, with no sign of shift in the voltage sensitivity (Fig. 1C). These results establish the efficiency and reliability of membrane voltage detection in isolated muscle fibers using the FRET signal from Mermaid.

FRET response of Rv-Mermaid: a Mermaid sensor adapted to accommodate the T306 SR targeting sequence

The next step was to express Mermaid fused to an SR-specific sequence. To target the SR membrane, we used a protein domain encompassing the 306 residues (T306) of the N-terminal end of triadin, an archetypal protein of the junctional SR (Rossi et al., 2014). To avoid having the T306 domain fused to mKOκ, we constructed reverse-Mermaid (Rv-Mermaid), in which the FRET couple is fused to the S1 segment of the VSD. Fusion of T306 on the S4 segment was then expected to ensure proper topology of the biosensor in the SR membrane (with the FRET couple facing the cytosol) in concurrence with the native topology of the T306 domain (Fig. S1). Rv-Mermaid also yielded an expression pattern consistent with t-tubule localization (Fig. 1D), and this was confirmed by a FRET response qualitatively similar to that of Mermaid upon changes in membrane voltage (Fig. 1E). However, Rv-Mermaid yielded a very negative midvoltage (V0.5 ∼ −70 mV; Fig. 1F and Table 1), whereas the SR membrane voltage is presumed to be maintained at ~0 mV (Somlyo et al., 1981). The variant MermaidD129E/Y235R tested in Xenopus oocytes exhibits a midvoltage near +10 mV (H. Tsuttsui, personal communication). Thus, to match Rv-Mermaid’s

Figure 1. Detection of t-tubule membrane changes with Mermaid and Rv-Mermaid in muscle fibers. (A and D) Confocal pattern of the green fluorescence in muscle fibers expressing Mermaid and Rv-Mermaid, respectively. In each panel, the frame and graph on the right show a higher-magnification view together with the longitudinal profile of fluorescence within the white box. Expression of Mermaid and Rv-Mermaid yielded a transverse-banded pattern consistent with the proteins being present in the triadic region of the fibers. A schematic illustration of the structure of the proteins is shown at top. (B and E) Changes in mUKG (Fλ360 G) and mKOκ (Fλ560 R) fluorescence and corresponding FRET ratio in response to the voltage-clamp protocol shown at top, from muscle fibers expressing Mermaid and Rv-Mermaid, respectively. In response to the voltage-clamp depolarizing steps from −80 mV, the detected changes in fluorescence (at 500 nm and >560 nm, upon 458-nm excitation) were consistent with voltage-dependent energy transfer between the two fluorophores mUKG and mKOκ, providing evidence for localization of Mermaid in the t-tubule membrane of the muscle fibers. Main traces in B and E have been corrected from time-dependent changes in fluorescence also observed when no voltage pulse was given. Raw fluorescence traces and superimposed fits used for the correction are shown in the insets. Details of how fits were generated and correction performed are described in Materials and methods. (C) Changes in Mermaid FRET ratio in response to depolarizing (light, black trace) and hyperpolarizing (right, blue trace) steps from −80 and 0 mV, respectively, in the same fiber. The graph at bottom shows the mean voltage dependence of the change in FRET ratio established from three fibers stimulated with a series of successive voltage-clamp depolarizing steps from −80 mV (black circles) and then with a series of successive hyperpolarizing steps from 0 mV (blue circles). FRET ratio values were normalized to the value R0 at −80 mV. Step hyperpolarizing pulses from 0 mV generated symmetrical changes in the FRET ratio, with no detectable sign of shift in the voltage sensitivity. (F) Mean voltage dependence of the FRET response of Mermaid (n = 6) and Rv-Mermaid (n = 6) established using depolarizing steps from −80 mV. Error bars represent ± SEM.
V_{0.5} with the resting SR voltage, we designed and expressed Rv-Mermaid^{D129E/Y235R} and its confocal triadic pattern in muscle fibers. This construct also exhibited a triadic localization (Fig. 2 A) and had a voltage sensitivity centered at +5 mV, as established from FRET responses to voltage-clamp pulses applied from 0 mV (Fig. 2, B–D; and Table 1). The rate of change in FRET ratio at the onset and offset of the pulses was approximated by a single exponential function: the “on” time constant yielded a bell-shaped voltage dependence with a maximum value of ~150 ms at +10 mV and a minimum value near ~25 ms, whereas the “off” time constant was rather independent from the pulse voltage (Fig. 2 E).

**Evidence for mixed subcellular localization of Rv-Mermaid^{D129E/Y235R}**

Interestingly, the FRET response from fibers expressing Rv-Mermaid^{D129E/Y235R} to depolarizing pulses applied from ~80 mV qualitatively differed from that of Mermaid and Rv-Mermaid (Fig. 3). Indeed, depolarizing pulses to values ranging between ~40 and

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**Figure 2.** FRET response of Rv-Mermaid^{D129E/Y235R} to changes in t-tubule membrane voltage applied from 0 mV. (A) Schematic structure of Rv-Mermaid^{D129E/Y235R} and its confocal triadic pattern in muscle fibers. (B) Changes in mUKG (F_{500}, green) and mKOκ (F_{560}, red) fluorescence in response to the indicated voltage protocol. Insets show the corresponding raw records before correction for voltage-independent changes in fluorescence; the fit used for correction is shown in white. (C) Changes in the FRET ratio (F_{560}/F_{500}) elicited in response to the pulse protocols shown at top. The black trace was from the fluorescent responses shown in B. (D) Mean voltage dependence of the FRET response measured with voltage pulses from 0 mV (n = 6). FRET ratio values were normalized to the value at 0 mV (R_{0}). (E) Mean voltage dependence of the time constant of change in FRET ratio at the onset (τ_{on}) and offset (τ_{off}) of the pulses. Error bars represent ± SEM.
Rv-MermaidD129E/Y235R appeared to localize both in the plasma membrane and, to some extent, in the ER. This is illustrated in Fig. S2, which shows the results of immunolabeling experiments using antibodies directed against either a Golgi or an ER marker protein. The depolarization-induced negative FRET signal in the muscle fibers may thus originate from the SR membrane.

### Table 1. Mean values for the parameters obtained from fitting a Boltzmann function to the FRET ratio ($R/R_0$) versus voltage data obtained with the different Mermaid constructs

| Main subcellular distribution | Mermaid (and SR?) | Rv-Mermaid (and SR?) | Rv-Mermaid D129E/Y235R and SR | T306-Rv-Mermaid (and SR?) | T306-Rv-Mermaid D129E/Y235R (and SR?) |
|------------------------------|------------------|----------------------|-------------------------------|---------------------------|----------------------------------------|
| $n$                          | 6                | 6                    | 6                            | 6                         | 13                                     |
| Max                          | 0.17 ± 0.02      | 0.16 ± 0.02          | 0.17 ± 0.02                   | −0.075 ± 0.01             | −0.06 ± 0.01                           |
| Min                          | 0.99 ± 0.01      | 0.95 ± 0.01          | 0.93 ± 0.01                   | 1                         | 1                                      |
| $V_{0.5}$ (mV)               | −37.7 ± 7.1      | −69.0 ± 4.1          | 5.45 ± 3.4                    | −26.4 ± 1.2               | −26.2 ± 1.9                            |
| $k$ (mV)                     | 14.1 ± 0.46      | 14.9 ± 1.1           | 11.4 ± 0.5                    | 5.5 ± 0.9                 | 2.8 ± 0.9                              |

Individual sets of data points from each fiber were fitted with the following expression: $\text{Max}/(1 + \exp((V_{0.5} - V)/k)) + \text{Min}$. Data are mean ± SEM.

The voltage dependence was established from signals elicited by pulses from 0 mV.

~20 mV generated negative changes in FRET ratio (Fig. 3, A and B), whereas for more depolarized step levels, an additional component, similar to what was observed with Mermaid and Rv-Mermaid (Fig. 1), was present. Fig. 3C shows the voltage dependence of the FRET ratio measured in six fibers, at the time of the end of each pulse during the protocol. There was variability, with some fibers exhibiting a very prominent negative change in FRET ratio at intermediate voltages (e.g., Fig. 3C, triangles), whereas other ones had much less. We hypothesized that these complex responses resulted from two populations of Rv-MermaidD129E/Y235R, one in the plasma/t-tubule membrane and the other in a distinct membrane compartment experiencing a distinct voltage change. Assuming that only the t-tubule population would respond to a voltage-clamp hyperpolarization, adding the FRET signal elicited by a pulse from −80 to 0 mV to the one elicited by a pulse from 0 to −80 mV unmasks the pure contribution of the non-t-tubule population (Fig. 3D). Interestingly, when expressed in COS-7 cells, Rv-MermaidD129E/Y235R appeared to localize both in the plasma membrane and, to some extent, in the ER. This is illustrated in Fig. S2, which shows the results of immunolabeling experiments using antibodies directed against either a Golgi or an ER maker protein. The depolarization-induced negative FRET signal in the muscle fibers may thus originate from the SR membrane.

### FRET response of SR-targeted Mermaid biosensors to t-tubule membrane voltage changes

Mermaid biosensors fused to the T306 sequence were anticipated to specifically reveal electrical activity of the SR membrane during muscle fiber activation. In contrast with the other constructs tested in the present study that yielded a general distribution throughout the muscle fibers, the ones carrying the T306-targeting sequence provided a very different expression pattern (see Fig. S5, A and D), we believe it is reasonable to assume that most of the T306-fused probes did reside in the junctional SR membrane. In muscle fibers, the FRET response of T306-Rv-MermaidD129E/Y235R to voltage-clamp pulses was unique, as it had no sign, whatsoever, of a t-tubule contribution. In response to depolarizing pulses from ~80 mV, the FRET ratio experienced only a decrease during the pulses. The amplitude of this drop increased with the t-tubule depolarization, with a voltage-dependence centered near ~25 mV (Fig. 4, B and C; and Table 1). The time constant of onset of the signal decreased with the amplitude of depolarization within a 230–60-ms range, whereas the “off” time constant was voltage independent (Fig. 4D). Because the construct was designed to have a membrane topology with the FRET couple facing the cytosol, the simplest interpretation is that this FRET signal corresponds to a negative polarization of the cytosolic face of the SR membrane with respect to the lumen. Assuming that the resting SR membrane voltage is 0 mV and that the sensor response is identical in the t-tubule membrane (Fig. 2D) and in the SR membrane, then the maximum change in FRET ratio (6%; Fig. 4C) would be consistent with an SR voltage change of at least 20 mV. Further evidence that the FRET response from T306-Rv-MermaidD129E/Y235R to voltage-clamp depolarizing pulses is intimately linked to EC coupling is provided in Fig. S4, which shows that the FRET signal progressively collapses during a protocol of successive depolarizing pulses (Fig. S4A), in concurrence with Ca$^{2+}$ release. This occurs under these conditions because of voltage-dependent inactivation of EC coupling (Hodgkin and Horowicz, 1960). In response to hyperpolarizing pulses from 0 mV, T306-Rv-MermaidD129E/Y235R also produces a small FRET response (Fig. S4B), the onset of which coincides with t-tubule membrane repolarization to 0 mV and the amplitude of which increases as the hyperpolarization is made longer (Fig. S4C). Consistent with the FRET response being triggered upon recovery of EC coupling from inactivation.

To confirm that the change in fluorescence detected by T306-Rv-MermaidD129E/Y235R upon EC coupling activation did result...
from a change in FRET, we performed acceptor photobleaching experiments. The protocol was first tested on the resting fluorescence from fibers expressing a non-SR-targeted Mermaid construct. Fig. 4 E shows x,y fluorescence frames from a muscle fiber expressing Rv-Mermaid\textsuperscript{D129E/Y235R}. A square region in the middle of the fiber was scanned during several minutes at 543 nm with high laser power until substantial bleaching occurred (Fig. 4 E, left). As expected, upon subsequent excitation at 458 nm in FRET detection configuration, extinction of the acceptor fluorescence was accompanied by dequenching of the donor in the photobleached region (Fig. 4 E, right). Fig. 4 F illustrates the effect of acceptor photobleaching on the changes in fluorescence detected in response to a 0.5-s-long depolarizing pulse from −80 to 0 mV in FRET configuration (458-nm excitation) from a fiber expressing T306-Rv-Mermaid\textsuperscript{D129E/Y235R}; traces on the left and right were recorded before and after photobleaching with 543-nm light, respectively. Again, photobleaching induced an increase and decrease in the resting donor and acceptor fluorescence, respectively, and this was accompanied by a loss of the transient changes in fluorescence triggered by the pulse and of the corresponding change in FRET ratio. Fig. 4 G shows mean values for the resting fluorescence ratio (left) and the relative changes in F500 and F>560 fluorescence triggered by a strong depolarizing pulse, from five fibers expressing T306-Rv-Mermaid\textsuperscript{D129E/Y235R} and tested under the same conditions as described in Fig. 4 F. The photobleaching-induced loss of pulse-triggered changes in fluorescence establishes that these changes (and the corresponding change in ratio), result from FRET.

In an attempt to confirm the occurrence of an SR membrane-specific voltage-dependent FRET signal, we tested an alternative SR-targeted probe, ER-Mermaid\textsuperscript{D129E}, which makes use of a CFP–YFP FRET pair and of an arginine-based targeting sequence (ER;
expected to allow expression throughout the entire SR membrane. ER-Mermaid2D129E indeed exhibited a fluorescence pattern consistent with widespread expression in the SR membrane (Fig. S5, A and D; and Fig. S6). More specifically, ER-Mermaid2D129E exhibited a transverse striated pattern, but in contrast to the other constructs, the longitudinal fluorescence profile was composed of successive “single” bands separated by 2 µm (Fig. S5 D, green trace on the right corresponding to the fluorescence profile along the white box). Imaging of the t-tubule network with di-8-anepps (Fig. S5 D, red image and corresponding longitudinal profile along the same white box) indicated that the protein was concentrated around the Z line, consistent with the standard expression pattern of nonjunctional SR proteins (Sorrentino, 2011). However, ER-Mermaid2D129E responded to voltage-clamp steps from 0 mV (Fig. S5, B and C), demonstrating presence in the t-tubule membrane, and thus indicating that the ER signal was not as strong as T306 to ensure exclusive SR localization. Although, in response to depolarizing pulses from −80 mV, ER-Mermaid2D129E responded by a mixed signal (Fig. S5, E and F) similar to that observed with Rv-Mermaid2D129E/Y235R (Fig. 3), thus consistent with a response from probe molecules in the SR membrane, the nonstrict SR membrane targeting of ER-Mermaid2D129E was very limiting and we thus did not pursue investigations with this probe.

**T306-Rv-Mermaid provides a FRET response similar to that of T306-Rv-Mermaid2D129E/Y235R despite its distinct range of voltage sensitivity**

To further characterize the SR membrane–confined FRET change detected by Rv-Mermaid2D129E/Y235R upon Ca2+ release activation, we also tested the response of T306-Rv-Mermaid. Because the voltage sensitivity of Rv-Mermaid is centered near a much more negative value than that of Rv-Mermaid2D129E/Y235R (Figs. 1 and 2), very distant from the presumed resting SR membrane voltage, we expected a much reduced FRET response, if any. Fig. 5 A shows that T306-Rv-Mermaid exhibited a patchy expression pattern similar to the one of T306-Rv-Mermaid2D129E/Y235R. However, in response to depolarizing pulses from −80 mV, the FRET ratio also experienced a voltage-dependent decrease during the pulses, the amplitude and voltage dependence of which were very similar to those of T306-Rv-Mermaid2D129E/Y235R (Fig. 5, B and C; and Table 1). Considering the distinct range of sensitivity to voltage of the two probes, this result was unexpected.

**Figure 4. FRET response of T306-Rv-Mermaid2D129E/Y235R during Ca2+ release.** (A) Patchy expression (regions pointed by green arrows) and triadic confocal pattern (bottom image and associated graph showing the longitudinal fluorescence profile along the white box) of T306-Rv-Mermaid2D129E/Y235R in muscle fibers. Schematic structure of the protein is shown at top. (B) Changes in T306-Rv-Mermaid2D129E/Y235R FRET ratio in response to the indicated voltage-clamp depolarizing pulses. Inset shows the fluorescence traces in response to the pulse from −80 to −10 mV. (C) Mean voltage dependence of the peak amplitude of the drop in FRET ratio measured in response to single 0.5-s-long pulses from −80 mV. For this, successive single depolarizing pulses of increasing amplitude were applied, separated by a time interval of 30 s. Data points are mean values from several fibers ranging from a minimum of seven and a maximum of 13. (D) Mean voltage dependence of the time constant of change in FRET ratio at the onset (t_on) of the depolarizing pulses and upon return (t_off) to the holding value of −80 mV. (E) Acceptor photobleaching of the resting fluorescence from a fiber expressing Rv-Mermaid2D129E/Y235R. X/Y fluorescence frames after photobleaching of a square region in the middle of the fiber with excitation light at 543 nm. Bar, 20 µm. (F) Effect of acceptor photobleaching (postbleach) on the changes in fluorescence detected in response to a depolarizing pulse from −80 to 0 mV in FRET configuration (458-nm excitation) from a fiber expressing T306-Rv-Mermaid2D129E/Y235R. (G) Mean values for the resting fluorescence ratio (left) and the relative changes in F_p,0 and F_p,565 fluorescence (right) triggered by a strong depolarizing pulse, from five fibers expressing T306-Rv-Mermaid2D129E/Y235R. Error bars represent ± SEM.

**pH sensitivity of the FRET signal from Mermaid biosensors**

The similarity between the FRET response of T306-Rv-Mermaid and T306-Rv-Mermaid2D129E/Y235R makes it questionable whether this signal actually witnesses a change in SR membrane voltage. Because our standard experimental conditions make use of a large concentration of EGTA inside the muscle fibers to block contraction, a slight change in cytosolic pH is expected during Ca2+ release, because Ca2+ binding to EGTA releases protons. Although the Mermaid fluorescent proteins were initially designed to be pH resistant (Tsutsui et al., 2008), we tested whether a change in pH could affect the FRET signal using muscle fibers treated with the H+ ionophore nigericin. Under these conditions, we found that the FRET signal from T306-Rv-Mermaid2D129E/Y235R was very sensitive to pH. This is illustrated in Fig. 6 A, which shows fluorescence levels and the corresponding FRET ratio from an intact fiber challenged by successive changes in pH in the 6.7–7.5 range. Fig. 6 B shows values for the change in FRET ratio experienced by five nigericin-treated muscle fibers expressing T306-Rv-Mermaid2D129E/Y235R, upon various changes in pH. The FRET ratio was expressed either as R_R الا or R_R الا الا. Results clearly establish the sensitivity to pH of the FRET signal.

**FRET response of SR-targeted Mermaid biosensors to t-tubule membrane voltage changes under conditions limiting cytosolic changes in pH**

To circumvent the pH change caused by Ca2+ binding to EGTA, we performed experiments in the presence of BAPTA instead, which exhibits much less pH sensitivity (Tsien, 1980). Under these conditions, hardly any change in the T306-Rv-Mermaid2D129E/Y235R FRET signal could be detected after depolarizing pulses. This is illustrated in Fig. 7 A, which shows a family of FRET ratio traces collected while applying 0.5-s-long depolarizing pulses between −30 and +10 mV. Loss of the negative FRET signal was not caused by suppression of Ca2+ release, as fibers equilibrated with BAPTA still yielded robust voltage-activated Ca2+ transients, though of smaller amplitude than in the presence of EGTA. For instance, a 500-ms-long voltage-clamp depolarization applied to seven fibers equilibrated with EGTA and four fibers equilibrated with BAPTA triggered a rhod-2 Ca2+ transient of mean initial peak F/F₀ amplitude of 3.5 ± 0.4 and 1.7 ± 0.2, respectively (Fig. 7 B). In Fig. 7 A, the trace shown in the inset corresponds to the mean response to 6–10 0.5-s-long pulses to voltages ranging between +10 and +40 mV from four fibers expressing T306-Rv-Mermaid2D129E/Y235R; traces from each fiber were averaged, and the
mean was calculated from the four mean traces. This revealed a slight positive change in the ratio that did not exceed 0.015 \( R_0 \). According to the calibrated FRET response in Fig. 2 D, this could correspond to a <10 mV depolarization. Alternatively, according to the fit shown in Fig. 6 B, it could correspond to an alkalization of \( \sim 0.06 \) pH units. We also attempted to measure the FRET response in the absence of exogenous intracellular \( \text{Ca}^{2+} \) buffer, using the contraction blocker BTS in the extracellular medium. However, under our standard conditions of stimulation with hundreds of milliseconds–long voltage-clamp pulses, BTS never completely eliminated contraction, precluding measurements in response to large pulses that maximally activate \( \text{Ca}^{2+} \) release. Still, measurements in response to 0.5-s-long pulses up to \( -25 \) or \( -20 \) mV never elicited any negative FRET response. Instead, a slight positive change in the ratio was observed, as illustrated in Fig. 7 C, which shows FRET ratio traces from a fiber expressing T306-Rv-Mermaid\(^{D129E/Y235R}\) challenged by pulses up to \( -20 \) mV in the presence of BTS. Under these conditions, data from three fibers gave a mean change in FRET ratio of 0.03 ± 0.002 \( R_0 \). In our hands, BTS proved much more effective to block contraction triggered by trains of action potentials. We thus took measurements of FRET signals from T306-Rv-Mermaid constructs in such conditions. The response of two distinct fibers expressing T306-Rv-Mermaid\(^{D129E/Y235R}\) to a train of action potentials is shown in Fig. 7 (D and E); in Fig. 7 D, the fiber was equilibrated with our standard EGTA-containing solution in the voltage-clamp pipette, whereas in Fig. 7 E, no EGTA was used inside the pipette, but BTS was present in the extracellular solution. Results further confirm the EGTA dependence of the negative FRET signal upon EC coupling activation and the presence of a small FRET signal of opposite direction in the absence of EGTA. Fig. 7 F illustrates the fact that, in the absence of EGTA (and presence of BTS), T306-Rv-Mermaid also responded by a positive change in FRET of amplitude similar to the one observed with T306-Rv-Mermaid\(^{D129E/Y235R}\).
which, considering the very different midvoltage sensitivities of the two probes, tends to exclude that this signal corresponds to an SR membrane voltage change.

**Discussion**

The question of whether the membrane of the reticulum experiences voltage changes during cellular activity was experimentally addressed more than 30 yr ago in isolated frog muscle fibers, through detection of optical retardation signals and through the use of potentiometric dyes (Baylor and Oetliker, 1975, 1977; Vergara et al., 1978; Kovács et al., 1983; Baylor et al., 1984). However, these studies did not lead to coherent results, and no further attempt was made. In the same period, results from electron probe x-ray microanalysis of ion distribution and movements across the SR membrane established the currently admitted view, according to which there is no large voltage gradient across the SR membrane at rest or during Ca\(^{2+}\) release (Somlyo et al., 1977, 1981, 1985). This view was corroborated by the identification and characterization of K\(^+\) and Cl\(^-\) channels in the SR membrane that could participate to the counter-Ca\(^{2+}\) release current necessary to maintain the SR membrane voltage clamped near its resting value during Ca\(^{2+}\) release (Labarca and Miller, 1981; Rousseau et al., 1988; Abramcheck and Best, 1989; Wang and Best, 1994; Kourie et al., 1996; Yazawa et al., 2007). Conversely, it was also proposed that need for these channels may be limited, as the open RYR could itself carry most of the necessary counter-current (Gillespie and Fill, 2008; Guo et al., 2013). In this context, the now-available option of targeting a genetically engineered voltage-sensitive fluorescent tool to the SR membrane offered a unique opportunity to experimentally settle this point.

Mermaid biosensors in the SR membrane are sensitive to cytosolic acidification induced upon Ca\(^{2+}\) binding to EGTA

In the present conditions, Mermaid constructs devoid of SR targeting sequence were present in the plasma/t-tubule membrane of the transfected muscle fibers, allowing quantitative characterization of the properties of the voltage-induced changes in FRET. Use of the T306 sequence then proved successful to target the constructs to the junctional SR membrane, as no more plasma/t-tubule membrane–related FRET change was detected in fibers expressing the T306 constructs. Under these conditions, use of Rv-Mermaid and Rv-Mermaid\(^{D129E/Y235R}\), yielding distinct voltage sensitivity (centered near −70 and 0 mV, respectively), was anticipated to allow differentiation of any EC-coupling activated FRET signal caused by an actual change in SR membrane voltage from a change in any other parameter that could affect FRET. Indeed, the two constructs should provide a response of very different amplitude to a given voltage change, if voltage were the only parameter affecting FRET. This was obviously not the case: both constructs provided very similar responses in the different conditions tested. In the presence of intracellular EGTA,
they responded by a change in FRET consistent with sensitivity of the probes to acidification of the cytosolic face of the SR membrane. Because the response was very similar for the two probes, pH sensitivity was not a consequence of the mutations. A cytosolic pH change has been well documented in frog muscle fibers loaded with EGTA, where it was used to measure and quantify the EC coupling-activated cytosolic Ca2+ transients using the EGTA-phenol red method (Pape et al., 1995, 1998, 2002; Pizarro and Rios, 2004). Under conditions of voltage-clamp stimulation similar to the ones used here, Pizarro and Rios (2004) reported maximum cytosolic levels of increase in [H+] of 0.2–0.3 µM, which would translate into a change of 0.6–0.8 pH units. According to our calibration in nigericin-treated fibers (Fig. 6), the maximum decrease in FRET ratio (6%, Fig. 4) evoked by a large depolarizing pulse in fibers expressing Rv-MermaidD129E/Y235R would correspond to a change in pH from 7.2 to 6.97. One interesting point is that no sign of cytosolic acidification was detected with the non-SR-targeted Mermaid constructs (assuming that the mixed response of Rv-MermaidD129E/Y235R was caused by its mixed plasma/SR membrane localization). Yet, in both the plasma membrane and the SR membrane, the topology of the probe proteins yields the FRET pair facing the cytosol, this concurring with the native topology of the T306 domain for the SR-targeted forms. Then, absence of “pH response” of the probes residing in the plasma/t-tubule membrane has to indicate that the pH change detected in the vicinity of the SR membrane spared the cytosolic face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane. This is somewhat inconsistent with calculations by Pape et al. (1995), which suggest that, in the face of the t-tubule membrane.

**No evidence for an SR voltage change during muscle fiber activation under conditions limiting cytosolic changes in pH**

In the absence of intracellular EGTA (either replaced by BAPTA or in the presence of the contraction blocker BTS), there was no sign of negative change in FRET ratio from T306-Rv-Mermaid constructs during activation of Ca2+ release. Upon Ca2+ binding, BAPTA releases much fewer protons than EGTA (Fénelon and Pape, 2002), which thus further supports the pH nature of the negative FRET signal observed in the presence of EGTA. However, BAPTA yields substantially higher affinity and faster kinetics than EGTA (Pape et al., 2002; Sztretye et al., 2011). Thus, corresponding disappearance of the negative FRET signal (Fig. 7 A) may be speculated to result from circumvention of a positive feedback of the released Ca2+ ions on either Ca2+ release activity itself or on other Ca2+-dependent channels implicated in SR membrane voltage control (e.g., Pitt et al., 2010). Alternatively, large concentrations of BAPTA may also be suspected to have a deleterious pharmacological effect on Ca2+ release (Pape et al., 2002), which could also contribute to attenuate the negative FRET signal. The fact that robust Ca2+ transients were still elicited in the presence of BAPTA indicates that disappearance of the negative FRET signal did not result from failing SR Ca2+ release. Furthermore, the fact that experiments in the presence of BTS also showed no sign of negative FRET signal from the SR membrane–targeted probes tends to definitely eliminate the option that this signal witnesses an SR membrane voltage change.

Instead, in the absence of EGTA, both the native and the D129E/Y235R form of T306-Rv-Mermaid responded by a small increase in the FRET ratio that, again, can hardly be thought to result from an SR membrane voltage change, considering the differing midvoltage sensitivity of the two probes. The mean change in FRET ratio recorded in response to a voltage-clamp pulse to −20 mV was ~0.03 R0, and a response of similar amplitude was recorded in response to a train of action potentials (Fig. 7 E). According to the pH dependence of Rv-MermaidD129E/Y235R (Fig. 6), this change would correspond to a cytosolic alkalinization from 7.2 to 7.31. A small alkalinization of the cytosol was previously reported to occur in conjunction with Ca2+ release in frog muscle fibers, consistent with a proton flux from the cytosol into the SR (Baylor et al., 1982; Hollingworth and Baylor, 1990; Pape et al., 1990). It is thus possible that in our conditions, in the absence of intracellular EGTA, the FRET response of T306-Rv-Mermaid constructs reveals the same process.

In conclusion, voltage-sensitive Mermaid probes targeted to the SR membrane of intact muscle fibers exhibit changes in

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FRET upon activation of Ca\textsuperscript{2+} release that most likely report local changes in pH occurring in the vicinity of the SR membrane during the process. Results provide no evidence for additional FRET changes that would be caused by a substantial variation of SR membrane voltage during muscle EC coupling.

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Author contributions: C. Sanchez, C. Berthier, J. Perrot, C. Bouvard, H. Tsutsui, and Y. Okamura designed and/or prepared the plasmid constructs. C. Sanchez, C. Bouvard, and V. Jacquemond conducted the electrophysiological and fluorescence measurements on muscle fibers and performed the related analysis. C. Berthier, J. Perrot, and H. Tsutsui performed the cell culture and immunostaining experiments. All authors contributed to data analysis and interpretation and to manuscript preparation. Y. Okamura, C. Berthier, and V. Jacquemond conceived and coordinated the study, designed the experiments and wrote the manuscript.

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