Role of the Sequence Surrounding Predicted Transmembrane Helix M4 in Membrane Association and Function of the Ca\(^{2+}\) Release Channel of Skeletal Muscle Sarcoplasmic Reticulum (Ryanodine Receptor Isoform 1)*

Guo Guang Du‡, Guillermo Avila§, Parveen Sharma‡, Vijay K. Khanna‡, Robert T. Dirksen‡, and David H. MacLennan‡

From the ‡Banting and Best Department of Medical Research, University of Toronto, Toronto, Ontario M5G 1L6, Canada, and the §Department of Biochemistry, Cinvestav-IPN, AP 14-740, Mexico City DF 07000, Mexico, and the ¶Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, New York 14642

The role of the sequence surrounding M4 in ryanodine receptors (RyR) in membrane association and function was investigated. This sequence contains a basic, 19-amino acid M3/M4 loop, a hydrophobic 44–49 amino acid sequence designated M4 (or M4a/M4b), and a hydrophilic M4/M5 loop. Enhanced green fluorescent protein (EGFP) was inserted into RyR1 and truncated just after the basic sequence, just after M4, within the M4/M5 loop, just before M5 and just after M5. The A52 epitope was inserted into RyR2 and truncated just after M4a. Analysis of these constructs ruled out a M3/M4 transmembrane hairpin and narrowed the region of the membrane association to M4a/M4b. EGFP inserted between M4a and M4b in full-length RyR2 was altered conformationally, losing fluorescence and gaining trypsin sensitivity. Although it was accessible to an antibody from the cytosolic side, tryptic fragments were membrane-bound. The expressed protein containing EGFP retained caffeine-induced Ca\(^{2+}\) release channel function. These results suggest that M4a/M4b either forms a transmembrane hairpin or associates in an unorthodox fashion with the cytosolic leaflet of the membrane, possibly involving the basic M3/M4 loop. The expression of a mutant RyR1, Δ4274–4535, deleted in the sequence surrounding both M3 and M4, restored robust, voltage-gated L-type Ca\(^{2+}\) currents and Ca\(^{2+}\) transients in dyed muscle fibers, demonstrating that this sequence is not required for either orthorhode (DHPR activation of sarcoplasmic reticulum Ca\(^{2+}\) release) or retrograde (RyR1 increase in DHPR Ca\(^{2+}\) channel activity) signals of excitation-contraction coupling. Maximal amplitudes of L-currents and Ca\(^{2+}\) transients at 4274–4535 were larger than with wild-type RyR1, and voltage-gated sarcoplasmic reticulum Ca\(^{2+}\) release was more sensitive to activation by sarcolemmal voltage sensors. Thus, this region may act as a negative regulatory module that increases the energy barrier for Ca\(^{2+}\) release channel opening.

In our first analysis of the transmembrane (TM) sequences in the ryanodine receptor isoform 1 (RyR1), we predicted that five TM hairpin loops were present in the C terminus of RyR1, among which M3 was formed by amino acids 4277–4300 and M4 was formed by amino acids 4342–4362 (1). Later sequencing of other RyR isoforms (2, 3) showed that the sequence surrounding M3 and M4 is among the most diverse, leading to designation of the sequence between amino acids 4254 and 4631 as divergent region 1 or D1 (4). M3 is not hydrophobic in RyR3, although it is in RyR1 and RyR2. However, the hydrophobicity of a 41-amino acid sequence (5, 6), which includes predicted M4, is conserved in all three isoforms. A hydrophobic sequence of this length is considered to be sufficient to form a TM hairpin loop by itself. Indeed, the TMHMM2.0 program (7) predicts that this sequence of up to 49 amino acids will form a TM hairpin loop in each of RyR1, RyR2, and RyR3 (Fig. 1). The sequence separating the C terminus of M3 and the beginning of the hydrophobic M4 sequence is hydrophilic and very basic (1, 3).

In a previous study (8), we analyzed the topology of rabbit skeletal muscle RyR1 by determining the orientation of EGFP fused to the C terminus of truncated RyR1 sequences. Compelling evidence was obtained for the existence of three hairpin loops, M5/M6, M7a/M7b, and M8/M10. A fourth region of membrane association was detected upstream of the three hairpin loops, but its topological characteristics were not investigated fully. It was shown that an EGFP tag fused to amino acid 4300, at the C terminus of predicted helix M3 in RyR1 was located in the cytosol, and the truncated protein to which it was attached was soluble in the cytosol. The results of this experiment virtually ruled out the possibility that predicted TM sequences M4, M6, M1, M2, and M3 might exist as TM helices. However, when EGFP was fused to points in the sequence between predicted TM sequence M4 and demonstrated TM sequence M5, the truncated protein was membrane-bound, and EGFP was located in the cytosol. These observations raise the question of whether the long hydrophobic M4 sequence might form a TM hairpin loop or, alternatively, whether M3, which did not appear to form a signal anchor sequence by itself, might act as a signal anchor sequence in conjunction with a stop transfer

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†To whom correspondence should be addressed: Banting and Best Department of Medical Research, University of Toronto, Charles H. Best Institute, 112 College St., Toronto, Ontario M5G 1L6, Canada. Tel.: 416-978-5008; Fax: 416-978-8528; E-mail: david.maclennan@utoronto.ca.

‡ The abbreviations used are: TM, transmembrane; RyR, ryanodine receptor; RyR1, RyR2, and RyR3, ryanodine receptor isoform 1, 2, and 3, respectively; IP, inositol 1,4,5-trisphosphate receptor; HEK-293 cells, human embryonic kidney cell line 293; MEF, mouse embryonic fibroblast; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; PBS, phosphate-buffered saline; SLO, streptolysin-O; EC, excitation-contraction.
sequence formed by M4 to form a TM hairpin loop (9, 10).

In this study, we investigated the possibility that a TM hairpin loop might be formed in the M4 region. We inserted EGFP or an A52 epitope into sequences on either side of M4 in RyR1 and in the middle of the M4 sequence in RyR2, where a luminal loop might separate TM sequences M4a and M4b, as they do for M7. The results show that it is the M4 sequence that associates with the membrane, but it is not clear whether association occurs through the formation of a TM hairpin or through an unorthodox association with the membrane. An RyR2 construct that contained an EGFP insertion between M4a and M4b formed an active channel, and an RyR1 construct in which the M3/M4 region was deleted retained both ortho- and retrograde Ca\(^{2+}\) release channel function.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA restriction enzymes and modifying enzymes were purchased from New England Biolabs, MBI Fermentas, Amersham Biosciences, and Roche Applied Science. The pEGFP vectors were purchased from New England Biolabs, MBI Fermentas, Amersham Biosciences, and Roche Applied Science. The pEGFP vectors were from Clontech. All other reagents were of reagent grade and retrograde Ca\(^{2+}\) release channel function.

**Vector Construction**—To examine the contribution of the M4 sequence to the topology of RyR, the cDNA sequence encoding the extended M4 sequence was removed from the pEGFP-C2 cassette by AgeI and SmaI and inserted into a blunt-ended NruI fragment, located between the end of M4 and the beginning of M5, at Arg 4557. Since it was not possible to find a site for insertion of a tag in the very GC-rich M4 region in RyR1, we resorted to the insertion of tags into the M4 region of RyR2. The 44-amino acid M4 sequence in RyR4 was inserted into the pTMHM2.0 (7) programs is MVTAFSFSYSWFMTLHLHVAVSF- RGGFRRIVCSLLLGGGLVEAG (13). The nucleotide sequence encoding VAS4301, located in the middle of the M4 sequence, is predicted by the transmembrane hairpin loop, although a transmembrane hairpin loop has been demonstrated experimentally (9, 10).

For construct EGFP4329, the EGFP fragment was removed from the pEGFP-C2 vector by Eco47III and SmaI and inserted into a blunt-end NruI fragment, located just before M5 and, in EGFP4628, just after M5, a well defined, cytosol-to-lumen TM sequence (Fig. 1). The pEGFP-C3 vector by Eco47III and SmaI was digested with BstXIII site in the C10 cassette isolated from pEGFP-C1 by PCR using primers, some of which contained desired restriction endonuclease sites.

For **RyR1** fusion constructs EGFP4556 and EGFP4628, the EGFP sequence was removed from the EGFP-C2 cassette by AgeI and SmaI and blunt-ended. The fragment was inserted into an NruI (4556) site, previously introduced (11) into a full-length RyR1 cDNA clone in pEGFP-C1 by PCR using primers some of which contained desired restriction endonuclease sites.

**For construct EGFP4329, the EGFP fragment was removed from the pEGFP-C2 vector by Eco47III and SmaI and inserted into a blunt-end NruI fragment, located between M4a and M4b (Fig. 1). The cDNA sequence encoding the extended M4 sequence is predicted by the TMHMM2.0 program (7) clearly predicts that M4a/M4b/M4c is predicted to have a long transmembrane sequence, containing EGFP codons, were cloned back into the original C10 cassette position in full-length RyR1 cDNA. For construct EGFP4329, EGFP was fused at the end of a 22-amino acid sequence containing 10 positively charged residues (RGLSYRLRRR- VRRLLRRLRFAE) (10), which links the C terminus of M3 to the N terminus of the hydrophobic sequence constituting the extended M4 sequence (Ala\(^{4325}\)–Ala\(^{4370}\). EGFP in EGF43481 and EGF4452 was located between the end of M4 and the beginning of M5, at Arg\(^{4557}\).

Since it was not possible to find a site for insertion of a tag in the very GC-rich M4 region in RyR1, we resorted to the insertion of tags into the M4 region of RyR2. The 44-amino acid M4 sequence in RyR4 was inserted into the pTMHM2.0 (7) programs is MVTAFSFSYSWFMTLHLHVAVSF-RGGFRRIVCSLLLGGGLVEAG (13). The nucleotide sequence encoding VAS4301, located in the middle of the M4 sequence, is predicted by the TMHMM2.0 program (7) clearly predicts that M4a/M4b/M4c is predicted to have a long transmembrane sequence, containing EGFP codons, were cloned back into the original C10 cassette position in full-length RyR1 cDNA. For construct EGFP4329, EGFP was fused at the end of a 22-amino acid sequence containing 10 positively charged residues (RGLSYRLRRR-VRRLLRRLRFAE) (10), which links the C terminus of M3 to the N terminus of the hydrophobic sequence constituting the extended M4 sequence (Ala\(^{4325}\)–Ala\(^{4370}\). EGFP in EGF43481 and EGF4452 was located between the end of M4 and the beginning of M5, at Arg\(^{4557}\).
Caffeine-induced Ca\(^{2+}\) Release—Ca\(^{2+}\) fluorescence measurements of caffeine-induced Ca\(^{2+}\) release were carried out in a microfluorimetry system (Photon Technology Inc.). The Fura-2/AM fluorescence changes were monitored in transiently transfected HEK-293 cells, as described previously (17).

Confocai Microscopy—MEF cells (a kind gift from Dr. Tak Mak, University of Toronto) or HEK-293 cells were cultured, transfected, treated with saponin before or after fixation, and examined to determine the pattern of EGFP fluorescence by confocal microscopy, as described previously (18).

Immunoocytochemical Analysis of EGFP Orientation—Transfected MEF cells seeded on coverslips were fixed with 4% paraformaldehyde in PBS and, subsequently, permeabilized selectively or nonselectively with streptolysin-O or Triton X-100, according to methods described previously (18, 19). In brief, fixed cells were washed and treated for 15 min with either 1% Triton X-100 at room temperature or 200 units/ml streptolysin-O, previously activated by a 5-min incubation on ice with 10 mM dithiothreitol in PBS. Unbound streptolysin-O was washed away, and cells were incubated at 37 °C for 30 min in PBS containing 10 mM dithiothreitol. Triton X-100- and streptolysin-O-treated cells were then incubated sequentially with a primary antibody (monoclonal antibody JL-8 against EGFP) (1:1000) and a secondary antibody (Alexa Fluor 588, goat anti-mouse IgG, Molecular Probes) (1:1000) for 1 h each, with three 10-min washings in between. After washing with PBS, the coverslips were mounted on microscope slides and examined by confocal microscopy.

Alkaline Extraction—Sodium carbonate, which extracts secretory proteins and peripheral membrane proteins without solubilizing membrane proteins (20), was used to determine whether the expressed fusion proteins were associated peripherally or integrally with the membrane.

Protease Digestion—To determine whether EGFP, at the C terminus of truncated fusion proteins or within full-length RyR was located on the luminal or cytosolic surface, microsomal vesicles were digested by trypsin at protein/trypsin ratios between 6 and 20. The samples were incubated at 37 °C or 24 °C for 1 h before the addition of aprotinin and trypsin inhibitor at a concentration 10-fold (w/w) over trypsin. Insoluble membrane fractions were separated from the digest by centrifugation at 14,000 × g for 5 min at room temperature. Supernatants were collected, and pellets were washed once with PBS and suspended in 1 × loading buffer with 10 μg/ml trypsin inhibitor. The samples were subjected to SDS-PAGE and immunoblotting.

SDS-PAGE and Immunoblotting—Samples were separated by 6% SDS-PAGE or 5–15% discontinuous gradient SDS-PAGE, according to standard protocols (21). Proteins were detected by a standard Western blotting assay.

Preparation and Microinjection of Dyspedic Myotubes—Primary cultures of myotubes were prepared from newborn dyspedic mice with a gene, as described previously (22, 23). Expression of wild-type RyR1 and an RyR1 deletion mutant lacking residues 4274–4535 (Δ4274–4535) was achieved by nuclear microinjection of dyspedic myotubes with cDNAs encoding CD8 (0.2 μg/μl) and the appropriate RyR1 expression plasmid (0.5 μg/μl) 4–6 days after initial plating of myotoblasts (24). Myotubes expressing RyR1 were identified 2–4 days later through incubation with CD8 antibody beads (Dynabeads Myonek A52 immunofluorescence for 1 h). Subsequently, 30-ms test pulses were applied from a holding potential of ~80 mV. A conditioning prepulse designed to inactivate RyR1 sequences. An RyR2 clone was also truncated at the middle of the M4 sequence through the attachment of the A52 epitope to its new C terminus (Fig. 2, A and B). This RyR2 fusion protein, 1–4300-A52 (R2), could be detected by monoclonal antibody C3-33 against RyR2 and by monoclonal antibody A52. We also inserted EGFP at various positions in the full-length clone in an attempt to determine its topology within the longer clone. Analysis by confocal microscopy showed that soluble proteins such as EGFP and RyR1 truncated at amino acid 4300 (designated post-M3 in Ref. 8) could be washed out of HEK-293 or MEF cells if the cells were permeabilized with saponin before fixation. However, integral membrane proteins remained inside the cells and formed a pattern characteristic of the membrane in which they were situated (8).

EGFP fluorescence for truncation protein 1–4329-EGFP and A52 immunofluorescence for 1–4300-A52 (R2), were confined to regions outside of the nucleus, forming a uniform pattern in the cytoplasm (Fig. 3, A and c). This uniform fluorescence disappeared following saponin treatment, and only dense aggregates remained, which did not show a network pattern characteristic of the endoplasmic reticulum (Fig. 3, A and d). These results show that the sequence ending just before M4 and containing the basic M3/M4 loop sequence was insufficient to anchor the cytosolic portion of RyR1 to the membrane and that the sequence containing the first half of M4 (M4a) is insufficient to anchor the cytosolic portion of RyR2 to the membrane.

EGFP fluorescence for truncation protein 1–4381-EGFP and 1–4452-EGFP showed a network pattern (Fig. 3A, e and g), which was less well defined than that for wt RyR1 (not shown). However, fluorescence remained in the intracellular membranes after saponin treatment (Fig. 3A, f and h). These results demonstrate that RyR fusion proteins, truncated just before M4b, were soluble and not membrane-associated, whereas RyR fusion proteins, truncated just after M4b, were insoluble and associated with organelar membranes that included the ER.

Further analysis was carried out to determine whether these fusion proteins were associated with isolated microsomal membranes. Homogenates from HEK-293 cells were centrifuged at 8,000 × g for 30 min and then at 186,000 × g for 1 h. The proportion of the fusion protein in the high speed pellet, representing the microsomal membrane fraction, and in the supernatant, representing the soluble fraction, was determined by SDS-PAGE and Western blotting with either EGFP antibody JL-8 or the A52 antibody. The fusion proteins 1–4329-
EGFP and 1–4300-A52 (R2) were located largely in the supernatant fraction (not shown), whereas 1–4381-EGFP and 1–4452-EGFP were associated with the microsomal membrane fractions (not shown).

The ability of sodium carbonate to extract proteins from isolated microsomal membranes was also examined (Fig. 3B). Following treatment with 0.1 M Na2CO3, pH 11.5, more than 50% of the total protein from 1–4381-EGFP and 1–4300-A52 (R2) was extracted into solution, but less than 20% of total 1–4381-EGFP protein and less than 5% of total 1–4452-EGFP protein (Fig. 3B) were extracted into solution.

Fusion proteins 1–4329-EGFP, 1–4381-EGFP, and 1–4452-EGFP were tested for topological location of EGFP by measurement of its susceptibility to proteolytic digestion in microsomal fractions. Native EGFP is intrinsically resistant to digestion by proteases (25), and expressed EGFP migrates as a trypsin-resistant 28-kDa protein (Fig. 3C). Earlier, we demonstrated that 28-kDa EGFP could be detected in the supernatant when EGFP in the fusion protein lies on the cytoplasmic side of the membrane and that an increased mass is obtained in the membrane pellets when EGFP is translocated to the lumen, where it and its congruent transmembrane sequences are protected from trypptic digestion (8).

EGFP with a mass of 28 kDa was found in the supernatant for 1–4329-EGFP and 1–4381-EGFP (Fig. 3C), but EGFP from 1–4452-EGFP, which was also found in the supernatant, had a mass of ~32 kDa, suggesting that the adjacent sequence was insensitive to trypsin, either because it contained no trypsin-sensitive sites or because it was folded into the EGFP domain, where it was protected against trypsin. No EGFP signal was detected in the membrane pellets for any of these fusion proteins (Fig. 3C). These results show thatEGFP from 1–4329-EGFP, 1–4381-EGFP, and 1–4452-EGFP is released to the supernatant by trypsin digestion and is, therefore, located in the cytosol.

The data presented in Fig. 3 for 1–4329-EGFP and 1–4300-A52 (R2) indicate that neither M3, the sequence of basic amino acids immediately following M3, nor M4a can anchor the cytoplasmic domain to the membrane. In further testing, we inserted EGFP, in frame, into full-length RyR1 between M3 and M4 (EGFP4329). This construct was used to exclude the possibility that M3/M4 might form a hairpin loop, although M3 alone does not behave like a signal anchor sequence. We also inserted EGFP, in frame, into full-length RyR1 between M4 and M5 (EGFP4381, EGFP4452, and EGFP4556) to confirm that the entire M4/M5 loop is cytoplasmic and between M5 and M6 (EGFP4628) to confirm that the M5/M6 loop is located in the lumen (Fig. 2, A and B).

The question of whether M4a is a signal anchor could not be confirmed in RyR1 by our standard technique of fusing EGFP at the C terminus of M4a, since we could not find a site in the GC-rich sequence of M4 that could be used to insert EGFP, followed by a stop codon. A site in RyR2 in the middle of M4 at Ala4306 was suitable for EGFP insertion. However, we could not isolate any RyR2 construct in which EGFP was followed by a stop codon. Nevertheless, we were able to isolate a clone in which EGFP was inserted, in frame, into full-length RyR2 at a site between M4a and M4b to create EGFP4300(R2), with which we hoped to discriminate between the possibility that the C terminus of M4a lies in the lumen, consistent with the formation of a TM hairpin loop by the full-length M4, or lies in the cytosol.

Expression was confirmed by Western blotting of the microsomes isolated from transiently transfected HEK-293 cells or MEF cells. All five fusion proteins were expressed at readily detectable levels (Fig. 4A) by probing with the EGFP antibody JL-8, but no similar mass was detected in expression vector-only transfected cells (not shown) or in cells expressing wild type RyR1 and RyR2 (not shown). The masses of these fusion proteins were shown to be similar to wild type by probing with antibody C3-33 for RyR1 and antibody 34C for RyR2 (not shown).
EGFP fluorescence in MEF cells expressing insertion-truncation proteins 1–4329-EGFP (a and b), 1–4381-EGFP (e and f), 1–4452-EGFP (g and h), or 1–4300-A52(R2) (c and d). a, c, e, and g show transfected MEF cells fixed with formaldehyde prior to treatment with saponin treatment; b, d, f, and h show cells after treatment with saponin, which permeabilizes the cell membrane so that soluble components can leak from the cell (b and d), leaving aggregated (b and d) and membrane proteins in situ (f and h). c and d record immunofluorescence from monoclonal antibody A52 and Alexa-568. The left panel shows a schematic designation for each construct in the proposed topology for M4a/M4b in RyR1. B, alkaline extraction of microsomes isolated from HEK-293 cells expressing 1–4329-EGFP, 1–4300-A52(R2), 1–4381-EGFP, and 1–4452-EGFP. Microsomes were extracted with 0.1 M Na2CO3, pH 11.0. Lanes 1, 3, 5, and 7 proteins extracted into solution by alkali; lanes 2, 4, 6, and 8, proteins remaining in the microsomes of the corresponding samples. C, tryptic digestion of microsomes isolated from HEK-293 cells expressing 1–4329-EGFP, 1–4300-A52(R2), 1–4381-EGFP, and 1–4452-EGFP. Digested samples were centrifuged to separate membrane-associated or membrane-protected (luminal) proteins (left) from soluble peptides (right). Undigested wild type EGFP served as a control in both panels.

Resequencing of this construct did not reveal any coding error, and the fact that the JL-8 antibody recognized EGFP in the protein expressed from this construct indicated that EGFP was expressed in this construct. Moreover, the construct retained Ca2+ release channel function (see below), indicating that the EGFP sequence remained in frame. Therefore, it is most likely that the conformation of the inserted EGFP was strained to the point where fluorescence was not emitted (25). Immunofluorescence from cells expressing EGFP4300(R2), following staining with EGFP antibody JL-8 and the Alexa-568 secondary antibody, was located in intracellular membranes (Fig. 4D) and was not affected by saponin treatment (not shown).

We analyzed the orientation of EGFP in EGFP4300(R2) through the detection of EGFP immunofluorescence in transfected cells treated with streptolysin-O (SLO). SLO permeabilizes plasma membranes, but not intracellular membranes, and has been used successfully in several studies to identify the orientation of membrane sequences in intracellular membrane proteins (18, 19). Fixed cells were treated with 1% Triton-X-100 to permeabilize both cellular and intracellular membranes or with SLO to permeabilize the plasma membrane selectively and then labeled sequentially with JL-8 and Alexa-568. Cells expressing EGFP4300(R2) did not fluoresce at 488 nm (Fig. 4D, a and d). Cells treated with either Triton X-100 or SLO emitted Alexa-568 fluorescence at 568 nm from intracellular membrane networks (Fig. 4D, b, c, e, and f), indicating that EGFP was located on the cytoplasmic side. Similar results were obtained for EGFP4329, EGFP4381, EGFP4452, and EGFP4456, as expected (not shown).

Control experiments were performed using EGFP4628 in which EGFP is located after M5 in a known cytoplasm-to-lumen membrane sequence (8). Alexa-568 fluorescence was observed only for Triton X-100-treated cells and not for SLO-treated cells (Fig. 4D, b and e). Merged images at 488 and 568 nm showed an identical distribution of the expressed proteins (Fig. 4D, a–c). These results suggest that the EGFP of EGFP4628 is, indeed, located in the lumen and that the orientation of M5 is from cytoplasm to lumen.

These studies involving insertion of tag sequences and truncation show that M4 associates with the membrane, that EGFP inserted between M4a/M4b is altered conformationally, and that EGFP inserted at this site faces toward the cytoplasm. As a further test of the subcellular location of EGFP in EGFP4300(R2) and other insertion sites, trypsin digestion of micromsomal vesicles isolated from transfected HEK-293 cells was carried out.

Cleavage of EGFP4300(R2) at a trypsin/protein ratio of 1:20
at 37 °C for 1 h produced two major low molecular mass peptides of ~28 and ~16 kDa, which were stained by antibody JL-8. They were located only in the membrane fraction, and no JL-8-reactive fragments were found in the supernatant (Fig. 5, A and B). This study shows that EGFP was not folded properly and that it was membrane-associated. When we titrated the concentration of trypsin and incubated the reaction at 24 °C, several bands ranging from ~16 to ~32 kDa appeared in the membrane pellet at low concentrations of trypsin (Fig. 5 C). As the trypsin concentration was increased, the concentration of the 16-kDa band increased, but all bands disappeared eventually (Fig. 5 C). These results confirm that EGFP in EGFP4300(R2) was attached to the membrane but was exposed to the cytosol. In this membrane-associated, nonfluorescent conformation, it was susceptible to proteolytic digestion. Since the epitope for JL-8 is not known, it was not possible to determine which part of the EGFP sequence was exposed to the cytosol.

Tryptsin released EGFP to the supernatant in EGFP4329, EGFP4381, EGFP4452, and EGFP4556 (Fig. 5, A and B). These observations, together with the results obtained from the EGFP immunofluorescence study with SLO permeabilization, indicate that the sequences before M4a and after M4b are, indeed, located in the cytoplasm and rule out the existence of a TM sequence pre-M4a or of an M3/M4 loop. Similar to the results obtained with 1–4452-EGFP, the major product from the trypsin digestion of EGFP4452 had a mass of ~32 kDa. By contrast, EGFP from EGFP4628 was protected in the membrane fraction and had a mass of ~40 kDa, similar to the mass calculated for the predicted peptide length of the M5/M56 loop and M6, confirming that the M5/M6 loop is in the lumen and suggesting that EGFP inserted at amino acids 4628 does not disrupt the membrane integration of M5 and M6, although it caused loss of physiological channel function (see below).

Measurement of Channel Function in Mutant-expressed RyR1 and RyR2—Channel function for the various fusion proteins was examined by measurement of caffeine-induced Ca²⁺ release in transfected cells. Caffeine does not release Ca²⁺ from ER stores in nontransfected HEK-293 cells (17). Following the application of 10 μM caffeine, Ca²⁺ release was observed in HEK-293 cells expressing wild type RyR1 and RyR2 (not
shown), EGFP4329, EGFP4300(R2), EGFP4381, or EGFP4452 (Fig. 4B, a–d). No Ca²⁺ release was observed for EGFP4556 or EGFP4628 (Fig. 4B, e and f). It is thus clear that EGFP insertion at amino acids 4329 and 4300 (RyR2) or at amino acids 4381 and 4452 in divergent region 1 of RyR1 and RyR2 do not alter channel function. Nevertheless, insertions close to either side of the M5 membrane sequence (EGFP4556 and EGFP4628) disrupted channel function.

In earlier studies (12), we showed that the membrane-associated M3/M4 region of RyR1 is not essential to the formation of Ca²⁺ release channels but does exert a profound influence on certain functions of the release channel. Specifically, RyR1 sensitivity to activation by caffeine and Ca²⁺ was increased following expression in HEK-293 cells of an RyR1 deletion (Δ4274–4535) lacking the positively charged M3 linker, the entire M4 sequence, and much of the M4/M5 loop. Moreover, this deletion mutant exhibited markedly reduced Ca²⁺-dependent inactivation (12). However, the impact of this region of RyR1 on excitation-contraction (EC) coupling in skeletal muscle was not investigated.

In order to determine the ability of Δ4274–4535 to restore both orthograde and retrograde signals of skeletal muscle EC coupling, we expressed this RyR1 mutant in dyspedic myotubes in which the endogenous RyR1 gene was disrupted. Dyspedic myotubes lack voltage-gated SR Ca²⁺ release and appreciable L-type Ca²⁺ currents. However, expression of wild-type RyR1 restores both functions (Fig. 3 in Ref. 22). In our experiments, expression of the Δ4274–4535 deletion mutant also restored robust, voltage-gated L-type Ca²⁺ currents and Ca²⁺ transients (Fig. 6, A–D), demonstrating that the 4274–4535 region of RyR1 is not required for either the orthograde (DHPR activation of SR Ca²⁺ release) or retrograde (RyR1 increase in DHPR Ca²⁺ channel activity) signals of EC coupling. In fact, the maximal amplitude of L-type currents and Ca²⁺ transients in Δ4274–4535-expressing myotubes were actually larger than those observed in myotubes expressing wild-type RyR1. In addition, voltage-gated SR Ca²⁺ release was shifted to more negative potentials (−16 mV) in myotubes expressing Δ4274–4535 (Fig. 6E), indicating increased sensitivity to activation by sarcolemmal voltage sensors.

Fitting the experimental data in Fig. 6, C and D, to Boltzmann equations (Equations 1 and 2, respectively) yielded the following kinetic parameters (mean ± S.E.) for voltage-dependent activation of L-currents and Ca²⁺ transients in RyR1- (n = 11) and Δ4274–4535-expressing (n = 13) myotubes, respectively: Gₚ₅₀ (nanosiemens/nanofarads), 105 ± 15 and 188 ± 25; Vₐ₅₀ (mV), 22.8 ± 1.2 and 18.7 ± 1.2; kₐ (mV), 5.0 ± 0.6 and 6.3 ± 0.3; V₅₀ (mV), 72 ± 4 and 75 ± 2; (ΔF/F)ₚ₅₀ (mV), 2.0 ± 0.4 and 3.8 ± 0.6; Vₐ₅₀ (mV), 15.7 ± 1.4 and −0.2 ± 1.4; kₐ (mV), 6.2 ± 0.6 and 6.7 ± 0.7. Significant differences (p < 0.05; t test) were found for Gₚ₅₀ and (ΔF/F)ₚ₅₀ and Vₐ₅₀.

Discussion

In a previous study (8), we proposed that RyR1 contains six TM helices, formed into three TM helical hairpin loops, M5/M6, M7a/M7b, and M8/M10, together with a region of membrane association located around M4. This region of membrane association was localized between the C-terminal end of M3 (post-M3) and the N-terminal end of M5 (pre-M5). It contains a 41-amino acid hydrophobic region designated M4 (or M4a/M4b), but the possibility that M3 might act as a signal anchor sequence only in the presence of a stop transfer sequence formed by M4 was not excluded.

The M3/M4 loop is a 19–22-amino acid, well conserved, soluble, very basic sequence (SYRSLRRVRVRLLRTARE₄₃₂₆ in RyR1 and SLKSLKKQMKKKMKTVD₄₄₇₉ in RyR2). This sequence continues into the relatively hydrophobic sequence of 44–49 amino acids that surrounds M4 (₄₃₂₉–M₄b) and the possibility that M3 might act as a signal anchor sequence only in the presence of a stop transfer sequence formed by M4 was not excluded.

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immediately pre-M4. They also fail to support the formation of a hairpin loop by M3/M4.

Gly4381 in RyR1 lies just after M4, at the C terminus of the 44–49-amino acid hydrophobic sequence. Since 1–4381-EGFP was associated with the membrane, we could conclude that the first clear membrane association sequence is present between amino acids 4329 and 4381. To rule out the possibility that other sites of membrane association may lie between 4381 and 4556, we showed that EGFP was located in the cytoplasm in the deletion mutants 1–4381-EGFP, 1–4452-EGFP, and 1–4556-EGFP and in the insertion mutants EGFP4381, EGFP4452 and EGFP4556, which span the region from immediately post-M4 to immediately pre-M5. These data suggest that the entire M4/M5 loop sequence lies in the cytoplasm. This narrows the region of membrane association to the M4 region and raises the question of whether M4a/M4b can form a TM hairpin loop.

According to the TMHMM algorithms (6, 7, 26), amino acids 4322–4371 in RyR1 and 4280–4323 in RyR2 form a hydrophobic span of about 44–49 amino acids, long enough to form a TM hairpin loop (Fig. 1). In the TMHMM prediction, the VAS 4300 sequence in RyR2 forms a loop between two potential TM helices. Since we know that the N terminus of M5 is cytosolic and that M5 is a cytoplasm-to-lumen sequence (8), any TM sequences existing in the M4 region before M5 would have to be paired to form a hairpin loop. In this respect, the long hydrophobic sequence in M4 would have to resemble the 50-amino acid hydrophobic M7 sequence, which traverses the membrane twice with a short luminal loop (8).

In attempts to probe for the existence of an M4a/M4b TM hairpin loop, data obtained from the fusion protein 1–4300-A52 (R2), deleted after M4a, indicated that M4a is unable to anchor the protein to the membrane and is, therefore, unlikely to form a TM sequence. This result must be viewed cautiously, however, since some membrane sequences with weak topogenic function require support from a partner stop transfer TM sequence to complete the signal anchor stop transfer hairpin loop and achieve both integration into the membrane and proper orientation (9, 10). The first TM sequence of the inositol 1,4,5-trisphosphate receptor (IP3R) (19) is a case in point; IP3R, truncated after its first TM sequence, was soluble, but truncated IP3R containing both the first and second TM sequences was targeted to the ER (19). The first and second TM sequences in IP3R are likely to correspond to the M5/M6 TM sequences in RyR and not to the M4 region in RyR. Indeed, the whole sequence corresponding to divergent region D1 in RyR is absent from IP3R. Thus, we cannot gain insight into the TM nature of M4 by comparison with IP3R.

If M4a/M4b should form a hairpin loop, then EGFP inserted between M4a and M4b in EGFP4300(R2) would be expected to be translocated into the lumen. Unfortunately, EGFP was not fluorescent in this construct, probably because of altered con-
formation due to misfolding, and could only be detected by an EGFP antibody. Use of this antibody in SLO-permeabilized cells showed that the epitope in the misfolded EGFP was located in the cytosol. Nevertheless, proteolysis of EGFP4300(R2) in micromolar preparations did not release antibody reactive fragments into the supernatant. Instead, a ladder of EGFP antibody-reactive peptides with masses between 16 and 30 kDa was associated with the membrane pellet. These reactive bands disappeared upon further digestion, suggesting that the membrane-associated EGFP was fully accessible to trypsin. Trypsin accessibility was almost certainly due to the same misfolding of EGFP that is proposed to account for its loss of fluorescence.

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