Deletion of Amino Acids 1641–2437 from the Foot Region of Skeletal Muscle Ryanodine Receptor Alters the Conduction Properties of the Ca Release Channel

Manjunatha B. Bhat,* Jiying Zhao,* Salim Hayek,* Eric C. Freeman,* Hiroshi Takeshima,# and Jianjie Ma*
*Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106 USA, and #Department of Pharmacology, University of Tokyo Faculty of Medicine, Hongo, Bunkyo-ku, Tokyo 113, Japan

ABSTRACT The ryanodine receptor (RyR) of skeletal muscle contains two functional domains: a carboxyl-terminal hydrophobic domain that forms the putative conduction pore of the calcium release channel, and a large cytoplasmic domain that corresponds to the "foot structure." To understand the contribution of the foot structure to the function of the calcium release channel, we studied a RyR deletion mutant, $\Delta_{1641-2437}$-RyR, in which a region that is rich in glutamate and aspartate residues (a.a. 1641–2437) was removed. The wild-type and $\Delta_{1641-2437}$-RyR proteins were expressed in a Chinese hamster ovary (CHO) cell line, and functions of single calcium release channels were measured in the lipid bilayer membrane. The wild-type RyR forms functional calcium release channels with a linear current-voltage relationship similar to that of the native channel identified in the sarcoplasmic reticulum membrane of skeletal muscle, whereas the channels formed by $\Delta_{1641-2437}$-RyR exhibit significant inward rectification, i.e., currents moving from cytoplasm into SR lumen were ~20% less than that in the opposite direction. As in to the wt-RyR channel, opening of the $\Delta_{1641-2437}$-RyR channel has a bell-shaped dependence on the cytoplasmic calcium, but the calcium-dependent activation and inactivation processes of the $\Delta_{1641-2437}$-RyR channel are shifted to higher calcium concentrations. Our data show that deletion of a.a. 1641–2437 from the foot region of the skeletal muscle RyR results in changes in both ion conduction and calcium-dependent regulation of the calcium release channel.

INTRODUCTION

The ryanodine receptor (RyR) is an essential component of excitation-contraction coupling in striated muscle cells, whereby a depolarization across the transverse tubule (TT) membrane leads to rapid release of Ca from the sarcoplasmic reticulum (SR) membrane (Fleischer and Inui, 1989; Rios et al., 1991; McPherson and Campbell, 1993). RyR consists of a single polypeptide of ~560 kDa normally arranged in a homotetrameric structure (Takeshima et al., 1989; Zorzato et al., 1990), which contains two structural domains: a carboxyl-terminal hydrophobic domain that forms the putative conduction pore of the Ca release channel, and a large cytoplasmic domain that spans the junctional gap between the TT and SR membranes, referred to as the "foot structure" (Franzini-Armstrong and Jorgenson, 1994; Wagenknecht et al., 1989). The Ca release channel is a large conductance pore, with a linear conductance of ~100 pS for Ca and ~400 pS for monovalent cations (K, Cs) (Smith et al., 1988; Lai et al., 1988; Ma et al., 1988). The activity of the skeletal muscle Ca release channel reconstituted into lipid bilayers is controlled by cytoplasmic Ca through activation and inactivation mechanisms. Ca in the nanomolar to micromolar concentration range activates the channel, whereas in the micromolar to millimolar concentration range it inhibits the channel (Smith et al., 1985; Ma and Zhao, 1994).

The RyRs of skeletal and cardiac muscle are coded by different genes, *ryr1* (skeletal subtype) (Takeshima et al., 1989; Zorzato et al., 1990; Marks et al., 1989) and *ryr2* (cardiac subtype) (Otsu et al., 1990; Nakai et al., 1990), which share high homology with each other, with ~66% identity in amino acid sequence. Based on the primary sequence alignment, three divergent regions between RyR1 and RyR2 have been identified. These regions were referred to as D1, D2, and D3 (Takeshima, 1993; Sorrentino and Volpe, 1993). The D1 region comprises residues 4254–4631 in RyR1 and residues 4210–4562 in RyR2; thus it involves a domain just proximal to the putative four transmembrane segments at the carboxyl-terminal end (Takeshima et al., 1989). The D2 region occurs between residues 1342–1403 of RyR1 and residues 1353–1396 of RyR2, and D3 is between residues 1872–1923 in RyR1 and 1852–1890 in RyR2. The D3 region corresponds to a glutamate-rich region in RyR1, not in RyR2, which may be a potential low-affinity binding site for Ca (Zorzato et al., 1990) and thus may play a role in Ca-dependent inactivation of the skeletal muscle Ca release channel.

To test the role of a negatively charged cytoplasmic domain of skeletal RyR on the function of the Ca release channel, we constructed a RyR deletion mutant ($\Delta_{1641-2437}$-RyR) in which a 2.4-kb nucleotide sequence (amino acid residues 1641–2437) was removed from the wild-type RyR cDNA. The wild-type and $\Delta_{1641-2437}$-RyR were expressed in a Chinese hamster ovary (CHO) cell line, from which microsomal membrane vesicles were isolated for single-channel studies by the bilayer reconstitution technique. The

Received for publication 30 September 1996 and in final form 23 June 1997.

Address reprint requests to Dr. Jianjie Ma, Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 10900 Euclid Ave., Cleveland, OH 44106. Tel.: 216-368-2684; Fax: 216-368-5586; E-mail: jxm63@po.cwru.edu.

0006-3495/97/09/1320/09 $2.00

wild-type RyR expressed in CHO cells formed a linear conductance Ca release channel that is similar to the native Ca release channel from rabbit skeletal muscle. The $\Delta_{1641-2437}$-RyR also formed functional Ca release channels in the bilayer membrane, which displayed gating properties different from those of the wild-type RyR channel. Unlike the wild-type RyR channel, currents through the $\Delta_{1641-2437}$-RyR channel exhibited significant inward rectification, and furthermore, both Ca-dependent activation and inactivation of the $\Delta_{1641-2437}$-RyR channel were shifted to higher Ca concentrations. These results provide evidence that a.a. 1641–2437 of skeletal RyR, which is highly negatively charged, may fold close to the conduction pore of the channel, and thus may contribute to the ion conduction and Ca-dependent regulation of the Ca release channel.

MATERIALS AND METHODS

Subcloning of ryanodine receptor cDNA

The entire sequence of the rabbit skeletal muscle ryanodine receptor cDNA (15.3 kb, wt-RyR) was cloned into the pRRS11 expression vector, and the transcription occurs under the control of the SV40 promotor (Takeshima et al., 1989; Penner et al., 1989). Within the RyR cDNA there are four unique restriction sites for *Bsp*EI enzyme at nucleotide positions 2739, 4923, 6188, and 7311. A deletion mutant of pRRS11 was generated through digestion with *Bsp*EI and religation with T4 ligase. This mutant lacked the nucleotide sequence from 4923 to 7311, which corresponds to a deletion of amino acids from 1641 to 2437. The correct identity of this deletion mutant was verified through digestion with restriction enzymes (*Bsp*EI and *Bam*HI) and immunoblotting analysis of RyR expression in CHO cells (see Fig. 1).

Expression of ryanodine receptor in Chinese hamster ovary cells

CHO cells were grown at 37°C and 5% CO_2 in Ham's F12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. pRRS11(wt-RyR) or pRRS11($\Delta_{1641-2437}$-RyR) was introduced into the cells by the electroporation technique (Gene Pulser, Bio-Rad) (Imagawa et al., 1992). Twenty-four hours after transfection, the cell culture medium was changed, and neomycin (0.5 mg/ml) was added to the medium 24 h later for selection of cells stably transfected with the pRRS11 plasmids. The cells usually reached 90–100% confluency 4–6 days after treatment with the neomycin-containing medium. At that time, the cells were harvested and the microsomal membrane vesicles were isolated. The expression of wild-type and mutant RyR proteins was detected by immunoprecipitation and Western blot.

Immunoprecipitation and Western blot of ryanodine receptor

CHO cells (one flask of 90–95% confluency, 140 cm^2) transfected with RyR cDNAs were harvested and washed twice with ice-cold phosphate-buffered saline and lysed with 1 ml of ice-cold modified RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8.0, 1 mM EGTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% sodium deoxycholate) in the presence of protease inhibitors (0.5 mM diisopropyl fluorophosphate, 1 μM pepstatin

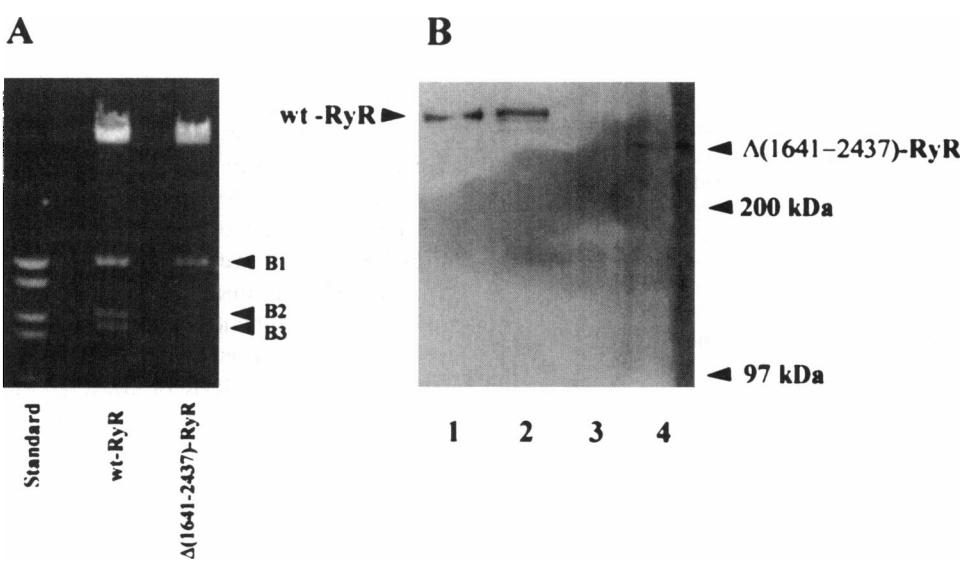

FIGURE 1 Expression of skeletal muscle ryanodine receptor in Chinese hamster ovary cells. The restriction enzyme *Bsp*EI cuts the ryanodine receptor cDNA four times, at nucleotide positions 2739, 4923, 6188, and 7311, thus releasing three cDNA fragments of 2.184 kb (B1), 1.265 kb (B2), and 1.123 kb (B3) from pRRS11 (*A*, *wt-RyR*). The B2 and B3 fragments correspond to amino acid residues 1641–2437, which include a highly negatively charged cytoplasmic domain of the ryanodine receptor protein (D3). $\Delta_{1641-2437}$-RyR is a subclone of pRRS11(wt-RyR), which contained a deletion of the B2 and B3 fragments, as digestion with *Bsp*EI released only the 2.184-kb fragment (*A*, $\Delta_{1641-2437}$-*RyR*). The correct orientation (5′→3′) of the B1 fragment in $\Delta_{1641-2437}$-RyR was verified by digestion with *Bam*HI enzyme (not shown). The standards are 2.176, 1.766, 1.230, 1.033, and 0.653 kb (*Lane 1*). pRRS11(wt-RyR) and pRRS11($\Delta_{1641-2437}$-RyR) were introduced into CHO cells by the electroporation method. Forty-eight to seventy-two hours after transfection, the CHO cells were harvested and microsomal membrane vesicles were isolated for detection of ryanodine receptor protein by the immunoprecipitation/Western blot assay (see Materials and Methods). CHO cells transfected with pRRS11 expressed a 560-kDa RyR protein (*B*, *Lane 2*), which is identical to the native ryanodine receptor from the rabbit skeletal muscle (*B*, *Lane 1*). CHO cells transfected with $\Delta_{1641-2437}$-RyR express a lower-molecular-mass protein of ~480 kDa, which can be recognized by monoclonal antibody against the skeletal muscle ryanodine receptor (*B*, *Lane 4*). The untransfected CHO cells did not contain any detectable amount of ryanodine receptor proteins (*B*, *Lane 3*).

A, 10 μM leupeptin, 1 μg/ml aprotinin). The cell lysate was incubated with 4 μg of anti-rabbit skeletal muscle RyR antibody (Upstate Biotechnology, Lake Placid, NY) at 4°C for ~2 h. Antibody complexes were then precipitated with 25 μl protein A-agarose beads (Boehringer-Mannheim Corp.). The beads were washed twice with ice-cold RIPA buffer, and the proteins were solubilized with 25 μl gel sample buffer (200 mM Tris-Cl, pH 6.7, 9% SDS, 6% β-mercaptoethanol, 15% glycerol, 0.01% bromophenol blue) and separated on a 3–12% linear sodium dodecyl sulfate-polyacrylamide electrophoesis gel after the sample was heated at 85°C for 5 min. The proteins were then transferred to a PVDF membrane and blotted with anti-rabbit skeletal muscle RyR antibody and horseradish peroxidase-linked antibody (sheep anti-mouse) by the enhanced chemoluminescence detection system (Amersham Corp.).

Isolation of microsomal membrane vesicles from CHO cells

CHO cells transfected with pRRS11(wt-RyR) or pRRS11($\Delta_{1641-2437}$-RyR) were harvested with versene solution (137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4 and 0.5 mM EDTA), followed by two washes with ice-cold phosphate-buffered saline. The cell pellet (600 × *g*, 5 min) was resuspended in ice-cold hypotonic lysis buffer (1 mM EDTA, 5 μM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 mg/ml benzamidine, 10 mM HEPES, pH 7.4) before lysis by 10 strokes in a tight-fitting Dounce homogenizer, followed by 15 strokes after the addition of an equal volume of sucrose buffer (500 mM sucrose, 10 mM HEPES, pH 7.2). Microsomes were collected by centrifugation of post-nuclear supernatant (10,000 × *g*, 15 min) at 100,000 × *g* for 45 min. The pellet was resuspended in a buffer containing 250 mM sucrose, 10 mM HEPES-Tris, pH 7.2. The membrane vesicles were stored at a protein concentration of 2–6 mg/ml at −75°C until use.

Reconstitution of calcium release channel in lipid bilayer membrane

Lipid bilayer membranes were formed across an aperture of ~200-μm diameter by the Muller-Rudin method, with a mixture of phosphatidylethanolamine:phosphatidylserine:cholesterol (6: 6: 1); the lipids were dissolved in decane at a concentration of 40 mg lipid/ml decane (Ma and Zhao, 1994). Incorporation of the Ca release channel in thebilayer was achieved by the addition of membrane vesicles containing either the wild-type or the $\Delta_{1641-2437}$-RyR proteins to the *cis* solution, under a concentration gradient of 200 mM (*cis*)/50 mM (*trans*) Cs-gluconate. Unless otherwise indicated, symmetrical 200 mM Cs-gluconate was used as a current carrier. The pH in both *cis* and *trans* solutions was buffered at 7.4 with 10 mM HEPES-Tris. The free Ca concentrations in both solutions were buffered with 1 mM EGTA. For each single-channel measurement, the starting free [Ca] in the *trans* solution was always 6–20 μM, and that in the *cis* solution was 220 μM.

To study the Ca-dependent activation of the wt-RyR and $\Delta_{1641-2437}$-RyR channels, a different amount of EGTA was added to the *cis* solution, together with a calibrated amount of Tris base to maintain the pH at 7.4. The free [Ca] in the *cis* solution ranges from 220 μM to 6 nM. To define the Ca-dependent inactivation properties of the wt-RyR and $\Delta_{1641-2437}$-RyR channels, a calibrated amount of $CaCl_2$ (and Trisbase) was added to the *cis* solution, which changes the free [Ca] from 220 μM to 30 mM. For the measurement of free [Ca] from 1 μM to 100 mM, a standard Ca-sensitive electrode was used (model 93-20; Orion, Boston, MA). To calibrate the free [Ca] in the range of 1 μM to 1 nM, a fluorescent Ca indicator, fura-2, was used (Grynkiewicz et al., 1985). This was performed with the help of Dr. George Dubyak at Case Western Reserve University.

Orientation of the Ca release channel in the lipid bilayer, usually in the *cis*-cytoplasmic translumenal SR manner, was determined by sensitivity of the channel to cytoplasmic Ca. Channels oriented in the opposite direction, which account for less than 10% of the experiments, were not used in the present study. Data presented in this paper were obtained from six different preparations of membrane vesicles isolated from CHO cells transfected with $\Delta_{1641-2437}$-RyR, and five different preparations of wt-RyR.

Analysis of single-channel data

To maintain the stability of the bilayer membrane and the Ca release channel activities, designed pulse protocols were used to measure currents through the single Ca release channels. The bilayer membrane was kept at a holding potential of 0 mV and pulsed to different test potentials of 0.5 to 1 s duration. Single-channel currents were recorded with an Axopatch 200A patch-clamp unit. Data acquisition and pulse generation are performed with a 486 computer and 1200 Digidata A/D-D/A convertor. The currents were sampled at 0.05 ms/point and filtered at 2 kHz, through an 8-pole Bessel filter. Open probabilities of the single wt-RyR and $\Delta_{1641-2437}$-RyR channels during consecutive test episodes were calculated with the TRANSIT program (purchased from Baylor College of Medicine); these are represented as "diary plots" in Fig. 4. All other single-channel data analyses were performed with pClamp and custom softwares.

RESULTS

Ryanodine receptor (RyR) proteins were expressed by transfecting pRRS11(wt-RyR) or pRRS11($\Delta_{1641-2437}$-RyR) into CHO cells by the electroporation method (Imagawa et al., 1992). To confirm RyR expression, immunoprecipitation/Western blot assay was performed on the transfected cells. Cells transfected with pRRS11(wt-RyR) expressed a readily detectable amount of high-molecular-mass protein of ~560 kDa (Fig. 1 *B*, *Lane 2*), which was identical to that of the native RyR from rabbit skeletal muscle (Fig. 1 *B*, *Lane 1*). Cells transfected with pRRS11($\Delta_{1641-2437}$-RyR) expressed a protein with lower molecular mass (~470 kDa; Fig. 1 *B*, *Lane 4*), reflecting the deletion of 798 amino acids from the foot region of RyR (residues 1641–2437). The appearance of RyR proteins in CHO cells could be detected as early as 5 h after gene transfection, and CHO cells growing under a selection medium containing neomycin (0.5 mg/ml) maintained a high level of expression of RyRs for as long as 7–8 weeks, which made possible the generation of sufficient amounts of wt-RyR and $\Delta_{1641-2437}$-RyR proteins for the functional studies.

Upon incorporation of microsomal membrane vesicles containing either wt-RyR or $\Delta_{1641-2437}$-RyR into the lipid bilayer membrane, functional channel activities could be routinely measured (Fig. 2). These single-channel currents resembled the Ca release channel based on the following criteria. First, opening of the channel absolutely required the presence of a micromolar concentration of Ca in the *cis*-cytoplasmic solution (Fig. 2 *A*), as buffering of free [Ca] from 220 μM to <40 nM resulted in complete closure of the channel (Fig. 2 *B*). Second, the channels were sensitive to ryanodine. In the presence of 5 μM ryanodine, the current amplitudes were reduced by ~50%, and the channel open lifetimes were increased by more than 20-fold (Fig. 2 *C*). These changes were characteristic of the native Ca release channel obtained from the rabbit skeletal muscle (Rousseau et al., 1987). Third, the channels displayed fast kinetics of transitions between open and closed states. Based on the open-time histogram analysis, the wt-RyR channels had

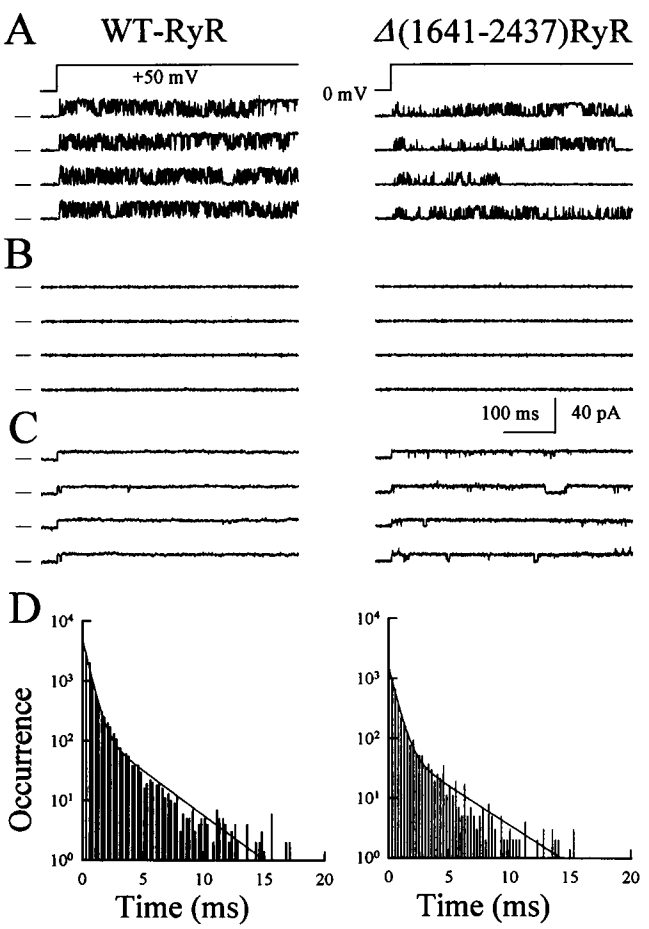

FIGURE 2 Lipid bilayer reconstitution of wild-type and $\Delta_{1641-2437}$-RyR Ca release channels. Currents through a single Ca release channel of the wt-RyR and $\Delta_{1641-2437}$-RyR are shown in the left and right panels, respectively, with 200 mM Cs-gluconate as the current carrier and a test potential of +50 mV. The control condition contained 220 μM free [Ca] in the *cis*-cytoplasmic solution (*A*). The addition of 10 mM EGTA to the *cis* solution, which reduced the free [Ca] to <40 nM, resulted in the complete closure of both wild-type and $\Delta_{1641-2437}$-RyR Ca release channels (*B*). The addition of 5 μM ryanodine to both *cis* and *trans* solutions led to a reduction in current amplitudes by ~50% and prolongation of channel open lifetimes by >20 fold, for both wt-RyR and $\Delta_{1641-2437}$-RyR channels (*C*). Traces shown in *A* and *B* were obtained from the same experiment, and those shown in *C* were taken from a separate experiment. The open time histograms were calculated at +50 mV, which contained data from 13 separate experiments for wt-RyR (*D*, *left*; total open events = 8860), and nine separate experiments for $\Delta_{1641-2437}$-RyR (*D*, *right*; total open events = 3246). Solid lines represent the best fits according to $y = W1/\tau o1\ \exp(-t/\tau o1) + W2/\tau o2\ \exp(-t/\tau o2)$, with the following parameters: W1 = 2348, $\tau o1$ = 0.493 ms, W2 = 511, $\tau o2$ = 2.90 ms for wt-RyR; W1 = 794, $\tau o1$ = 0.530 ms, W2 = 234, $\tau o2$ = 3.36 ms for $\Delta_{1641-2437}$-RyR. The relative occurrence of the $\tau o2$ state was W2/(W1 + W2) = 0.178 and 0.228, for wt-RyR and $\Delta_{1641-2437}$-RyR, respectively.

mean open lifetimes of $\tau o1$ = 0.49 ms and $\tau o2$ = 2.90 ms, and the $\Delta_{1641-2437}$-RyR channels had mean open lifetimes of $\tau o1$ = 0.53 ms and $\tau o2$ = 3.36 ms (Fig. 2 *D*). These time constants were similar to those of the native Ca release channels (Ma and Zhao, 1994). Thus the expressed wt-RyR and $\Delta_{1641-2437}$-RyR maintained functional Ca release channel activities in the bilayer membrane.

One of the notable differences between wt-RyR and $\Delta_{1641-2437}$-RyR is that the outward currents (movements of Cs ions from cytoplasm into SR lumen) through the $\Delta_{1641-2437}$-RyR channel were significantly smaller than that through the wt-RyR channel (Fig. 2 *A*). At +50 mV, the mean current amplitude was 20.24 ± 0.89 pA for the wt-RyR (n = 13) and 15.22 ± 0.69 pA for the $\Delta_{1641-2437}$-RyR (n = 9), which was significantly smaller ($p < 0.001$). This difference could be due to the different distribution of subconductance states between the wt-RyR and $\Delta_{1641-2437}$-RyR channels, or the altered ion conduction properties of the $\Delta_{1641-2437}$-RyR channel. Subconductance states are characteristic properties of the native Ca release channel recorded in the lipid bilayer membranes (Ma et al., 1988; Liu et al., 1989; Ma and Zhao, 1994), which were frequently observed with the wt-RyR and $\Delta_{1641-2437}$-RyR expressed in CHO cells (data not shown). An earlier study by Brillantes et al. (1994) showed the cytosolic receptors for immunosuppressant drugs, FK506 binding protein (FKBP12), are involved in stabilization of the full conductance states of the wt-RyR channel expressed in Sf9 cells. It is unlikely, however, that the 15-pA open state represents one of the subconductance states of the $\Delta_{1641-2437}$-RyR channel, because in over 56 successful experiments with the $\Delta_{1641-2437}$-RyR channel, we observed subconductance levels of ~8 pA and ~4 pA at +50 mV, but never observed any single-channel current larger than +15 pA.

To compare the size of the inward and outward currents and to construct the current-voltage relationship of the wt-RyR and $\Delta_{1641-2437}$-RyR channels, step pulses of negative and positive voltages of equal amplitude were applied to a single Ca release channel under a symmetrical ionic condition of 200 mM Cs-gluconate (Fig. 3 *A*). Clearly, the wt-RyR channel allowed the movement of Cs ions equally well in both the inward and outward directions (Fig. 3 *A*, *left*), such that the *I-V* curve was linear over the voltage range from −80 to +80 mV, with a slope conductance of 406.4 ± 4.0 pS (Fig. 3 *C*, *left*). The $\Delta_{1641-2437}$-RyR channel, however, had a lower conductance for the *cis* → *trans* movement of Cs ions, as the outward currents were consistently smaller than the inward currents (Fig. 3 *A*, *right*). As a result, the *I-V* curve of the $\Delta_{1641-2437}$-RyR channel displayed significant inward rectification, i.e., the channel had an inward conductance (417.2 ± 12.6 pS) that is larger than the outward conductance (338.8 ± 12.9 pS) (Fig. 3 C, *right*). The inward conductance of the $\Delta_{1641-2437}$-RyR channel was approximately the same as that of the wt-RyR channel. The fact that the same $\Delta_{1641-2437}$-RyR channel, under instantaneous transitions from negative to positive voltages, opened with unequal inward and outward currents (see Fig. 3 *A*, *trace a*) is inconsistent with the assumption that the reduced outward current was due to a transition to a subconductance state.

If the observed rectification with the $\Delta_{1641-2437}$-RyR channel was due to changes in local concentration of permeant ions (Cs), by measuring the single-channel current in high salt solutions that saturate the conductance-activity

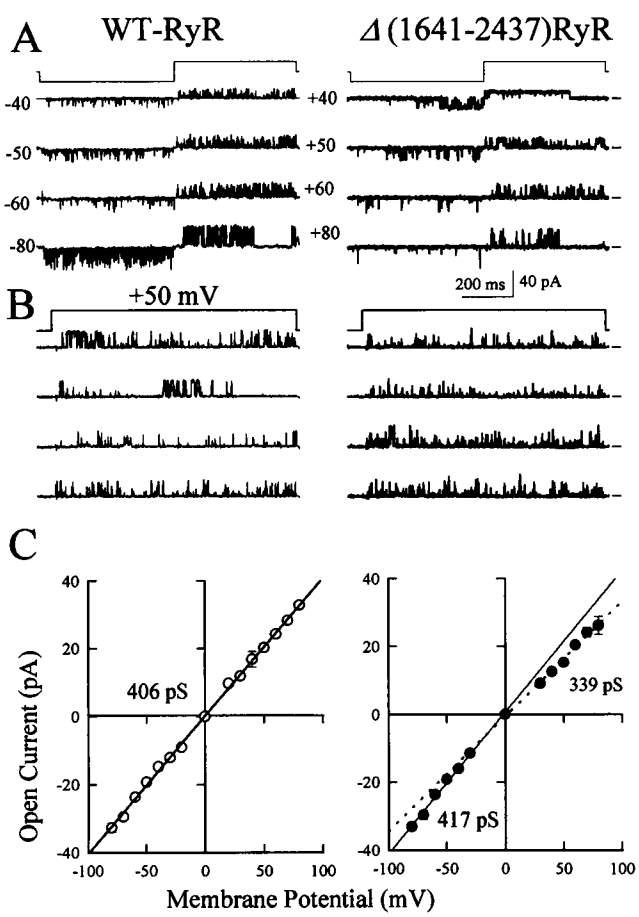

FIGURE 3 Inward rectification of the $\Delta_{1641-2437}$-RyR calcium release channel. Single-channel currents through the wt-RyR (*left panels*) and $\Delta_{1641-2437}$-RyR (*right panels*) Ca release channels were measured at a symmetrical solution of 200 mM Cs-gluconate (*A*) and 500 mM Cs-gluconate (*B*). The pulse protocols were −40 mV → +40 mV for trace labeled *a*, −50 → +50 mV for *b*, −60 → +60 mV for *c*, and −80 → +80 mV for *d*, respectively. Traces shown in *B* were of consecutive episodes at a test potential of +50 mV. Single-channel currents carried by 200 mM Cs were plotted as a function of test potential (*C*). ○, wt-RyR; ●, $\Delta_{1641-2437}$-RyR. The data points (mean ± SD; most standard deviations are smaller than the size of the symbol) represent the average of at least three experiments. The wt-RyR channel had a linear *I-V* curve with a slope conductance of 406.4 ± 4.0 pS (*C*, *left*). The $\Delta_{1641-2437}$-RyR channel exhibited inward rectification (*C*, *right*), with a slope conductance of 417.2 ± 12.6 pS for the inward currents (0 to −80 mV, ——), and 338.8 ± 12.9 pS for the outward currents (0 to +80 mV, ·····).

curve of the calcium release channel (Smith et al., 1988), we would expect to see no difference in the outward currents between the wt-RyR and $\Delta_{1641-2437}$-RyR channels. The single-channel currents shown in Fig. 3 *B* were obtained with 500 mM Cs-gluconate as the current carrier at a test potential of +50 mV. Unlike at 200 mM Cs-gluconate (Fig. 3 *A*), the full open state of the $\Delta_{1641-2437}$-RyR channel (i = 27.4 ± 2.3 pA at V = +50 mV, n = 8) was approximately equal to that of the wt-RyR channel (i = 26.8 ± 1.5 pA at V = +50 mV, n = 9) at 500 mM Cs-gluconate (Fig. 3 *B*). This result suggests that the reduced outward current through the $\Delta_{1641-2437}$-RyR channel is likely due to the altered conduction property of the channel.

Within the deleted 798 amino acids, there is a sequence of 18 consecutive glutamates (a.a. 1873–1890, rabbit RyR) that may form a potential Ca-binding site for the Ca release channel, as initially suggested by Zorzato et al. (1990). Using peptide overlay assays, Chen and MacLennan (1994) have located a putative Ca-binding domain between amino acid residues 1861 and 2094. Because $\Delta_{1641-2437}$-RyR lacks this potential Ca-binding site, we asked whether the Ca-dependent regulation of the Ca release channel was also altered in the $\Delta_{1641-2437}$-RyR channel.

First we tested the effect of increasing the concentration of cytoplasmic Ca on the open probabilities of both wt-RyR and $\Delta_{1641-2437}$-RyR channels (Fig. 4). Earlier studies have shown that the native Ca release channel from skeletal muscle exhibited Ca-dependent inactivation, in a Ca concentration range between 0.1 and 10 mM (Ma et al., 1988; Chu et al., 1993; Laver et al., 1995; Ma and Zhao, 1994). As shown in Fig. 4 *A*, the open probability of the wt-RyR channel expressed in CHO cells decreased progressively as the cytoplasmic [Ca] was increased from 220 μM to 12.5 mM (n = 6). Interestingly, a concentration of Ca (2.31 mM), which did not affect the activity of the wt-RyR channel, instead enhanced the activity of the $\Delta_{1641-2437}$-RyR channel (Fig. 4 *B*). The relative changes of channel activity after the increase in [Ca] from 0.22 to 2.31 mM were 0.86 ± 0.30 (n = 6) for wt-RyR and 2.22 ± 0.63 (n = 5) for $\Delta_{1641-2437}$-RyR.

The complete dose-response relationships of single-channel open probability as a function of cytoplasmic [Ca] was shown in Fig. 5. Clearly, the $\Delta_{1641-2437}$-RyR channels were less susceptible to inhibition by cytoplasmic Ca, as ~10-fold more Ca was required to inhibit the channel activity (Fig. 5 *A*). In addition, the sharp increase in open probability of the $\Delta_{1641-2437}$-RyR channel after the change in free [Ca] from 0.22 to 2.31 mM (Fig. 4 *B*) suggests possible changes in the Ca-dependent activation process of the $\Delta_{1641-2437}$-RyR channel.

To further characterize the Ca-dependent activation of the wt-RyR and $\Delta_{1641-2437}$-RyR channels, separate sets of experiments were performed (Fig. 5 *B*). Starting with a free [Ca] of 220 μM in the *cis*-myoplasmic solution, different amounts of EGTA were added to the *cis* solution (together with a calibrated amount of Tris base to maintain the pH at 7.4), which resulted in the reduction of free [Ca] to nanomolar concentrations. As expected, activities of both the wt-RyR and the $\Delta_{1641-2437}$-RyR channels decreased with the addition of EGTA to the *cis* solution. Complete closure of the wt-RyR channel occurred at ~10 nM [Ca], whereas that of the $\Delta_{1641-2437}$-RyR channel occurred at ~250 nM (Fig. 5 *B*). The dose-response relationship of P_o versus [Ca] indicated that activation of the $\Delta_{1641-2437}$-RyR channel was shifted to higher Ca concentrations, as the half-dissociation constant for Ca activation of the wt-RyR was ~0.25 μM and that of the $\Delta_{1641-2437}$-RyR channel was ~2 μM (Fig. 5 *B*).

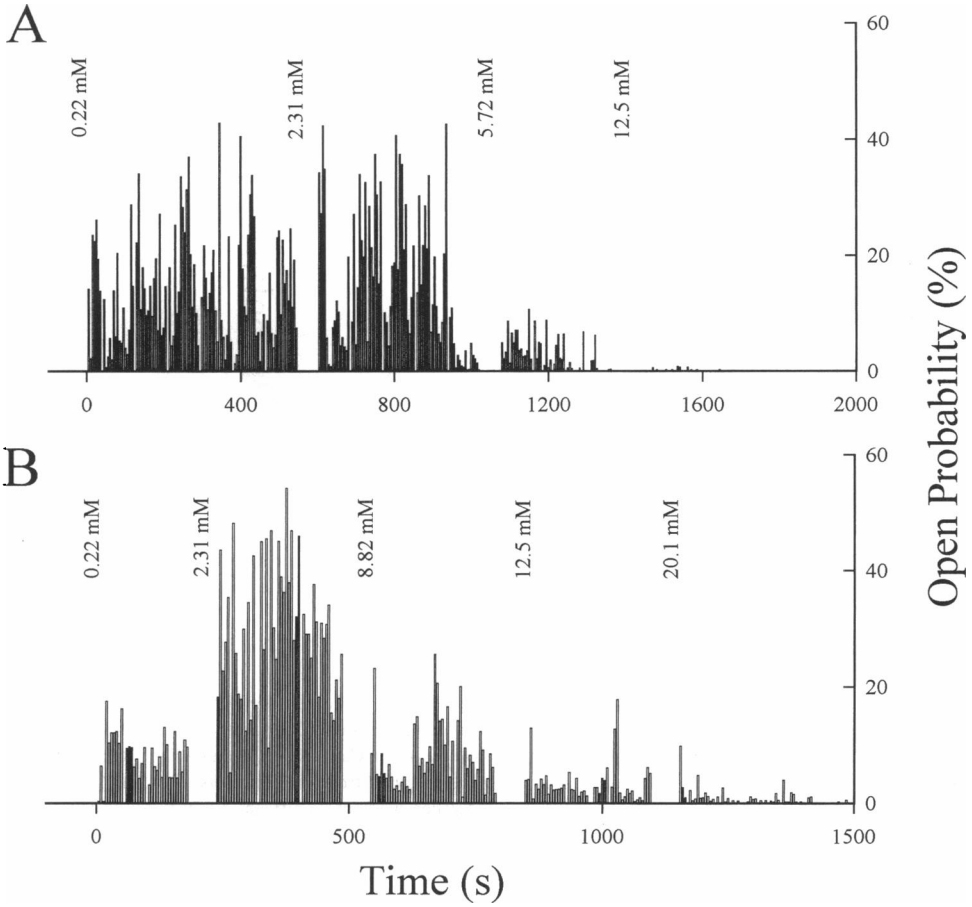

FIGURE 4 Ca-dependent inactivation of wild-type and $\Delta_{1641-2437}$-RyR calcium release channel. Channel open probabilities (P_o) were calculated at a test potential of +50 mV (0.5 s duration) from a continuous experiment for the wt-RyR channel (*A*) and the $\Delta_{1641-2437}$-RyR channel (*B*). The numbers indicated by the arrows represent the amount of free [Ca] (determined with a Ca-sensitive electrode) present in the *cis* solution. *A* is representative of five other complete experiments with the wt-RyR channel, and *B* is representative of four other experiments with the $\Delta_{1641-2437}$-RyR channel.

DISCUSSION

In this paper we characterized the single-channel properties of the skeletal muscle RyR heterologously expressed in a CHO cell line. To test the role of a negatively charged cytoplasmic domain of RyR on the function of the Ca release channel, the single-channel functions of a RyR deletion mutant, $\Delta_{1641-2437}$-RyR, were compared with those of the full-length RyR channel. Several differences between the wt-RyR and $\Delta_{1641-2437}$-RyR channels were identified in the present study. First, unlike the wt-RyR channel, which had a linear current-voltage relationship, the $\Delta_{1641-2437}$-RyR channel displayed a significant inward rectification, i.e., the movement of Cs ions from the SR lumen into the cytoplasm was similar to that in the wt-RyR channel, but the movement of Cs ions into the SR lumen was significantly smaller (Fig. 3 *C*). Second, although both wt-RyR and $\Delta_{1641-2437}$-RyR channels maintained Ca-dependent activation and inactivation properties, the dose-response relationship of P_o versus [Ca] of the $\Delta_{1641-2437}$-RyR channel was shifted to the right by approximately an order of magnitude (Fig. 5). In addition, although the wt-RyR and $\Delta_{1641-2437}$-RyR channels contained similar time constants of openings ($\tau o1$ and $\tau o2$), the relative occurrence of the longer open events ($y = W_2/W_1 + W_2$) was higher in the $\Delta_{1641-2437}$-RyR channel than in the wt-RyR channel (y = 0.178 and 0.228 for wt-RyR and $\Delta_{1641-2437}$-RyR, respectively) (Fig. 2 *D*). The fact that the apparent affinities for Ca-dependent activation and inactivation were reduced in the $\Delta_{1641-2437}$-RyR channel suggests that certain negatively charged residues within the deleted region (a.a. 1641–2437) may constitute a putative Ca-binding site(s) and thus may participate in the activation and inactivation process of the Ca release channel.

Examination of the primary sequence revealed that the amino acids deleted in $\Delta_{1641-2437}$-RyR (residues 1641 to 2437) contain 111 glutamates, 36 aspartates, 55 arginines, and 23 lysines. Thus a net of 69 negative charges were removed in $\Delta_{1641-2437}$-RyR. Assuming that a functional Ca release channel is formed by a tetrameric structure of RyR, the $\Delta_{1641-2437}$-RyR channel would have 64 × 4 fewer negative charges than the wt-RyR on the cytoplasmic side of the channel. It is possible that the negatively charged domain folds close to the conduction pore of the Ca release channel, which may create a negative surface potential near the cytoplasmic side of the channel. The presence of this negative potential may increase the local concentration of permeating ions (Cs), thus enhancing the outward currents through the wt-RyR channel. Alternatively, removal of the 798 amino acids may alter the foot structure allosterically, thus altering the distribution of charged amino acids near the conduction pore of the Ca release channel, and thus

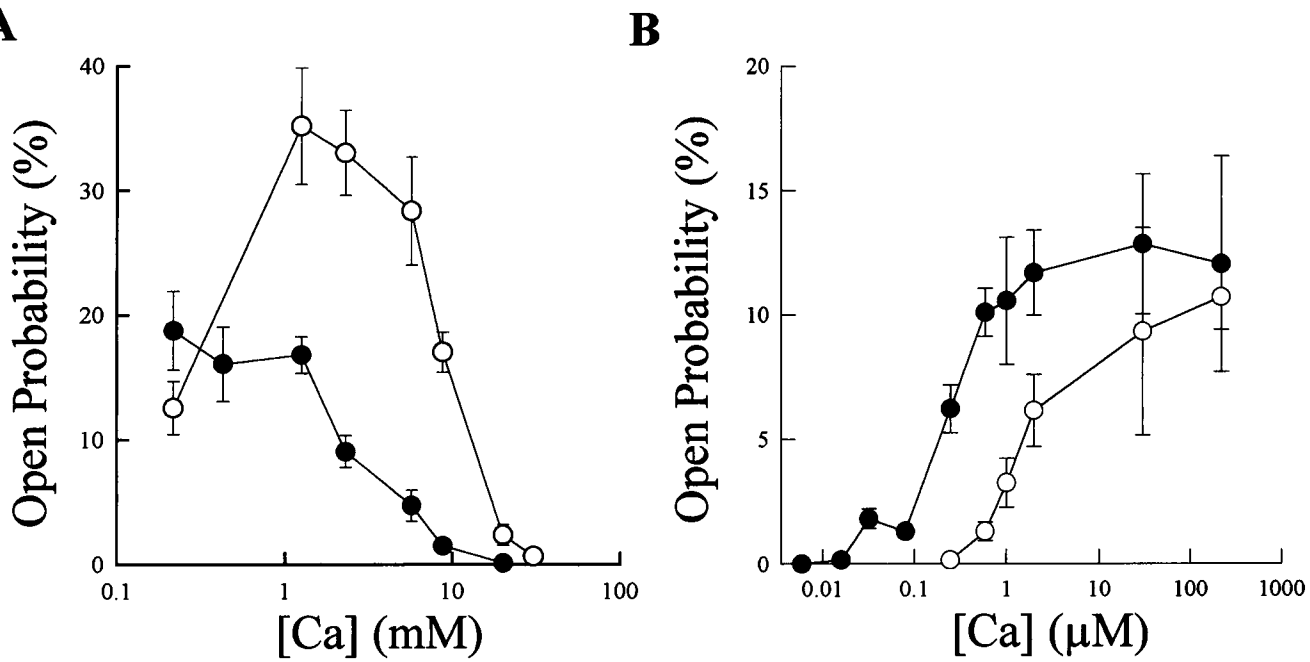

FIGURE 5 P_o of wt-RyR and $\Delta_{1641-2437}$-RyR channels as a function of cytoplasmic [Ca]. Single-channel open probabilities (P_o) were measured at a test potential of +50 mV, in the presence of different cytoplasmic calcium concentrations, for the wt-RyR channel (●) and the $\Delta_{1641-2437}$-RyR channel (○). (*A*) Ca-dependent inactivation and (*B*) Ca-dependent activation of the Ca release channels. Each data point represents the P_o value averaged over multiple experiments (n = 4–12). Data represented in *A* and *B* were obtained from different sets of experiments. The averaged P_o of the wt-RyR channel at 220 μM was 18.1 ± 3.2 for *A* and 12.1 ± 4.4 for *B*; the difference was not significant. Only bilayers containing single channels were included in the data analyses. The vertical bars represent standard errors of the mean.

leading to asymmetrical ion conduction behavior of the channel.

Another possibility or a potential problem with the heterologous expression of RyRs in CHO cells is the lack of muscle-specific proteins in the function of the expressed RyR channels. Early studies from other investigators have shown that triadin (Caswell et al., 1991; Knudson et al., 1993), calsequestrin (Ikemoto et al., 1989), and FKBP12 (Jayaraman et al., 1992; Chen et al., 1994; Brillantes et al., 1994; Ma et al., 1995) all bind to the native RyR and influence the function of the Ca release channel. A recent study by Nakai et al. (1996) showed that the dihydropyridine receptor does not function well as an L-type Ca channel in the absence of RyR, suggesting a direct interaction between the dihydropyridine receptor and ryanodine receptor in skeletal muscle. These interactions, which are absent in the CHO cells, could potentially alter the function of the wt-RyR and $\Delta_{1641-2437}$-RyR channels. Our present study does not rule out the possibility that deletion of a.a. 1641–2437 could lead to changes in the interaction of the mutant RyR protein with the accessory proteins, and thus could account, in part, for the observed difference in the conduction property of the $\Delta_{1641-2437}$-RyR channel.

In our previous studies, we showed that the native Ca release channel obtained from the rabbit skeletal muscle had affinities (K_d) for Ca activation and inactivation of ~20 μM and 300 μM, respectively (Ma and Zhao, 1994). However, in the present study with the wt-RyR channel expressed in CHO cells, openings of the Ca release channel were observed in a much wider range of cytoplasmic Ca, with an apparent affinity of ~0.25 μM for Ca-dependent activation and ~2 mM for Ca-dependent inactivation (Fig. 5). This observation is consistent with the role of accessary proteins, such as FKBP12, in the function of the Ca release channel (Timerman et al., 1993; Brillantes et al., 1994). A recent study by Ahern et al. (1997) showed that removal of FKBP12 from RyR of rabbit skeletal muscle altered the Ca sensitivity of the Ca release channel. Compared with the FKBP12-bound RyR channel, the FKBP12-depleted RyR channel required lower cytoplasmic Ca to open and higher cytoplasmic Ca to inactivate. The Ca-dependent regulation of the FKBP12-depleted RyR channel from skeletal muscle was similar to the wt-RyR channel expressed in CHO cells. At present, we do not know the level of FKBP12 in CHO cells, but it will be interesting to see whether over expression of FKBP12 could lead to changes in Ca-dependent gating properties of the Ca release channels in CHO cells.

A question remains here: What is responsible for the asymmetrical current-voltage relationship of the $\Delta_{1641-2437}$-RyR channel? An open ion channel is a pore that often behaves like an Ohmic conductor, i.e., the amount of ions moved through the channel, independent of the direction the ions move, is proportional to the amount of driving force applied across the membrane (Hille, 1992). A channel that exhibits a nonlinear current-voltage relationship (under symmetrical ionic conditions) can be the result of either

perturbation of the local concentration of permeant ions due to asymmetrical distribution of surface charges on the channel protein, or voltage-dependent block of the open channel through the competition of nonpermeant ions with the permeant ions, or of inherent asymmetry in the conduction pore of the channel. Voltage-dependent block of the Ca release channel does not seem to be responsible for the rectification of the $\Delta_{1641-2437}$-RyR channel, because under identical experimental conditions a linear *I-V* relationship was observed with the wt-RyR channel. To account for the asymmetrical ion-conduction properties, the $\Delta_{1641-2437}$-RyR channel would have to bare asymmetrical distribution of surface charges across the bilayer membrane. Interestingly, the RyRs of both skeletal and cardiac subtypes contain a stretch of negatively charged amino acids at the carboxyl-terminal end, which is predicted to extend into the lumenal side of the SR membrane—the two lumenal loops join transmembrane segments M1 and M2, and M3 and M4 (Takeshima et al., 1989). These negatively charged residues, in principle, could concentrate the permeating cations in the pore region of the Ca release channel and facilitate the movement of cations from the SR lumen to the cytoplasm, thus leading to the asymmetrical feature of the current-voltage relationship. Indeed, an earlier study by Tu et al. (1994) demonstrated that the cardiac Ca release channel did conduct more current from SR lumen to cytoplasm than from cytoplasm to SR lumen, particularly at low ionic conditions, as observed here with the $\Delta_{1641-2437}$-RyR channel. Consistent with this observation is our recent study that the carboxyl-terminal portion of skeletal muscle RyR forms a regulated Ca release channel that exhibits inward rectification in its current-voltage relationship. Furthermore, the skeletal carboxyl-terminal Ca release channel does not seem to be inactivatable by cytoplasmic [Ca] (Bhat et al., 1997). It is worth noting that the D3 region of the cardiac subtype of RyR contains fewer negative charges, and the cardiac Ca release channels are less susceptible to inactivation by cytoplasmic Ca (similar to $\Delta_{1641-2437}$-RyR) (Chu et al., 1993; Laver et al., 1995). It remains to be studied which particular negatively charged residues in the deleted region (a.a. 1641–2437) and which specific glutamate or aspartate residues in the lumenal side of the RyR are involved in the Ca-dependent regulation and ion conduction of the Ca release channel. Furthermore, additional studies are required to test whether specific residues within the deleted region (798 amino acids) or more global changes in protein conformation are responsible for the altered ion conduction property and Ca-dependent regulation of the $\Delta_{1641-2437}$-RyR channel.

We thank Drs. Junxia Xie and Mitchell L. Drumm for initial help with the expression and mutagenesis of RyR, and Dr. George Dubyak for help with the fura-2 measurement of Ca.

This work was supported by grants from the National Institutes of Health (AR42057), the Muscular Dystrophy Association, the Pilot Project of Howard Hughes Medical Institute, and an Established Investigatorship from the American Heart Association to JM.

REFERENCES

Ahern, G. P., P. R. Junankar, and A. F. Dulhunty. 1997. Subconductance states in single channel activity of skeletal muscle ryanodine receptors after removal of FKBP12. *Biophys. J.* 72:146–162.

Bhat, M. B., J. Zhao, H. Takeshima, and J. Ma. 1997. Functional Ca release channel formed by the carboxyl-terminal portion of ryanodine receptor. *Biophys. J.* 73:1329–1336.

Brillantes, A. B., K. Ondrias, A. Scott, E. Kobrinsky, E. Ondriasova, M. C. Moschella, T. Jayaraman, M. Landers, B. E. Ehrlich, and A. R. Marks. 1994. Stabilization of calcium release channel/ryanodine receptor function by FK506-binding protein. *Cell.* 77:513–523.

Caswell, A. H., N. R. Brandt, J-P. Brunschwig, and S. Purkerson. 1991. Localization and partial characterization of the oligomeric disulfide-linked molecular weight 95,000 protein (triadin) which binds the ryanodine and dihydropyridine receptors in skeletal muscle triadic vesicles. *Biochemistry.* 30:7507–7513.

Chen, S. R. W., and D. H. MacLennan. 1994. Identification of calmodulin, Ca, and ruthenium red-binding domains in the Ca release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 269:22698–22704

Chen, S. R. W., D. M. Vaughan, J. A. Airey, R. Coronado, and D. H. MacLennan. 1993. Functional expression of cDNA encoding the Ca release channel (ryanodine receptor) of rabbit skeletal muscle sarcoplasmic reticulum in COS-1 cells. *Biochemistry.* 32:3743–3753.

Chen, S. R. W., L. Zhang, and D. H. MacLennan. 1994. Asymmetrical blockade of the Ca release channel (ryanodine receptor) by 12-kDa FK506 binding protein. *Proc. Natl. Acad. Sci. USA.* 91:11953–11957.

Chu, A., M. Fill, E. Stefani, and M. L. Entman. 1993. Cytoplasmic Ca does not inhibit the cardiac muscle sarcoplasmic reticulum ryanodine receptor Ca channel, although Ca-induced Ca release is observed in native vesicles. *J. Membr. Biol.* 135:49–59.

Fleischer, S., and M. Inui. 1989. Biochemistry and biophysics of excitation-contraction coupling. *Annu. Rev. Biophys. Biophys. Chem.* 18: 333–364.

Franzini-Armstrong, C., and A. O. Jorgensen. 1994. Structure and development of E-C coupling units in skeletal muscle. *Annu. Rev. Physiol.* 56:509–534.

Grynkiewicz, G., M. Poenie, and R. Y. Tsien. 1985. A new generation of Ca indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260:3440–3450.

Hille, B. 1992. Ionic Channels of Excitable Membranes, 2nd ed. Sinauer Associates, Sunderland, MA.

Ikemoto, N., M. Ronjat, L. G. Meszaros, and M. Koshita. 1989. Postulated role of calsequestrin in the regulation of calcium release from sarcoplasmic reticulum. *Biochemistry.* 28:6764–6771.

Imagawa, T., J. Nakai, H. Takeshima, Y., Nakasaki, and M. Shigekawa. 1992. Expression of Ca-induced Ca release channel activity from cardiac ryanodine receptor cDNA in Chinese hamster ovary cells. *J. Biochem.* 112:508–513.

Jayaraman, T., A-M. Brillantes, A. P. Timerman, S. Fleischer, H. Erdjument-Bromage, P. Tempst, and A. R. Marks. 1992. FK506 binding protein associated with the calcium release channel (ryanodine receptor). *J. Biol. Chem.* 267:9474–9477.

Knudson, C. M., K. K. Stang, C. R. Moomaw, C. A. Slaughter, and K. P. Campbell. 1993. Primary structure and topological analysis of a skeletal muscle-specific junctional sarcoplasmic reticulum glycoprotein (triadin). *J. Biol. Chem.* 268:12646–12654.

Lai, F. A., H. Erickson, E. Rousseau, Q. Y. Liu, and G. Meissner. 1988. Purification and reconstitution of calcium release channel from skeletal muscle. *Nature.* 331:315–319.

Laver, D. R., L. D. Roden, G. P. Ahern, K. R. Eager, P. R. Junankar, and A. F. Dulhunty. 1995. Cytoplasmic Ca inhibits the ryanodine receptor from cardiac muscle. *J. Membr. Biol.* 147:7–22.

Liu, Q. Y., A. Lai, E. Rousseau, R. V. Jones, and G. Meissner. 1989. Multiple conductance states of the purified calcium release channel complex from skeletal sarcoplasmic reticulum. *Biophys. J.* 55:415–424.

Ma, J., M. Fill, C. M. Knudson, K. P. Campbell, and R. Coronado. 1988. Ryanodine receptor of skeletal muscle is a gap junction-type channel. *Science.* 242:99–102.

Ma, J., and J. Y. Zhao. 1994. Highly cooperative and hysteretic response of the skeletal muscle ryanodine receptor to changes in proton concentrations. *Biophys. J.* 67:626–633.

Ma, J., M. B. Bhat, and J. Zhao. 1995. Rectification of skeletal muscle ryanodine receptor mediated by FK506 binding protein. *Biophys. J.* 2398–2404.

Marks, A. R., P. Tempst, K. S. Hwang, M. B. Taubman, M. Inui, C. Chadwick, S. Fleischer, and B. Nadal-Ginard. 1989. Molecular cloning and characterization of the ryanodine receptor/junctional channel complex cDNA from skeletal muscle sarcoplasmic reticulum. *Proc. Natl. Acad. Sci. USA.* 86:8683–8687.

McPherson, P. S., and K. P. Campbell. 1993. The ryanodine receptor/Ca release channel. *J. Biol. Chem.* 268:13765–13768.

Nakai, J., R. T. Dirksen, H. T. Nguyen, I. N. Pessah, K. G. Beam, and P. D. Allen. 1996. Enhanced dihydropyridine receptor channel activity in the presence of ryanodine receptor. *Nature.* 380:72–75.

Nakai, J., T. Imagawa, Y. Hakamata, M. Shigekawa, H. Takeshima, and S. Numa. 1990. Primary structure and functional expression from cDNA of the cardiac ryanodine receptor/calcium release channel. *FEBS Lett.* 271:169–177.

Otsu, K., H. F. Willard, V. K. Khanna, F. Zorzato, N. M. Green, and D. H. MacLennan. 1990. Molecular cloning of cDNA encoding the Ca release channel (ryanodine receptor) of rabbit cardiac muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265:13472–13483.

Penner, R., E. Neher, H. Takeshima, S. Nishimura, and S. Numa. 1989. Functional expression of the calcium release channel from skeletal muscle ryanodine receptor cDNA. *FEBS Lett.* 259:217–221.

Rios, E., J. Ma, and A. Gonzalez. 1991. The mechanical hypothesis of excitation-contraction (EC) coupling in skeletal muscle. *J. Muscle Res. Cell. Motil.* 12:127–135.

Rousseau, E., J. S. Smith, and G. Meissner. 1987. Ryanodine modifies conductance and gating behavior of single Ca release channel. *Am. J. Physiol.* 253:364–368.

Smith, J. S., R. Coronado, and G. Meissner. 1985. Sarcoplasmic reticulum contains adenine nucleotide-activated calcium channels. *Nature.* 316: 446–449.

Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. 1988. Purified ryanodine receptor from rabbit skeletal muscle is the calcium-release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* 92:1–26.

Sorrentino, V., and P. Volpe. 1993. Ryanodine receptors: how many, where and why? *Trends Pharmacol. Sci.* 14:98–103.

Takeshima, H. 1993. Primary structure and expression from cDNAs of the ryanodine receptor. *Ann. N.Y. Acad. Sci.* 707:165–177.

Takeshima, H., S. Nishimura, T. Matsumoto, H. Ishida, K. Kangawa, N. Minamino, H. Matsuo, M. Ueda, M. Hanaoka, T. Hirose, and S. Numa. 1989. Primary structure and expression from complementary DNA of skeletal muscle ryanodine receptor. *Nature.* 339:439–445.

Timerman, A. P., E. Ogunbumni, E. Freund, G. Wiederrecht, A. R. Marks, and S. Fleischer. 1993. The calcium release channel of sarcoplasmic reticulum is modulated by FK-506-binding protein. Dissociation and reconstitution of the FKBP-12 to the calcium release channel of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 268:22992–22999.

Tu, Q., P. Velez, M. Cortes-Gutierrez, and M. Fill. 1994. Surface charges potentiates conduction through the cardiac ryanodine receptor channel. *J. Gen. Physiol.* 103:853–867.

Wagenknecht, T., R. Grassucci, J. Frank, A. Saito, M. Inui, and S. Fleischer. 1989. Three dimensional architecture of the calcium channel/foot structure of sarcoplasmic reticulum. *Nature.* 338:167–170.

Zorzato, F., J. Fujii, K. Otsu, N. M. Green, F. A. Lai, G. Meissner, and D. H. MacLennan. 1990. Molecular cloning of cDNA encoding human and rabbit forms of the Ca release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. *J. Biol. Chem.* 265: 2244–2256.