Functional Consequences of Mutations of Conserved, Polar Amino Acids in Transmembrane Sequences of the Ca\(^{2+}\) Release Channel (Ryanodine Receptor) of Rabbit Skeletal Muscle Sarcoplasmic Reticulum*

The potential role in Ca\(^{2+}\) release channel function of highly conserved, polar, and small amino acids in predicted transmembrane sequences in the rabbit skeletal muscle ryanodine receptor (RyR1) was investigated through mutagenesis. Acidic amino acids Asp\(^{4806}\), Asp\(^{4917}\), and Asp\(^{4938}\) and amidated residues Asn\(^{4805}\), Asn\(^{4806}\), Asn\(^{4807}\), Asn\(^{4896}\), and Gln\(^{4932}\), and Gly\(^{4033}\) were mutated to Ala, and Ala\(^{4888}\) was mutated to Val. When expressed in HEK-293 cells and challenged with either caffeine or 4-chloro-m-cresol, mutants E4032A, N4806A, D4815A, and D4917A did not respond, indicating that Ca\(^{2+}\) release channel function was impaired. None of these mutants exhibited specific binding of [\(^{3}\)H]ryanodine. Mutants N4805A and Q4933A showed a diminished response to both caffeine and 4-chloro-m-cresol, but [\(^{3}\)H]ryanodine binding was not altered. Other mutant responses and the responses of mutants E4032D, N4806Q or D, D4815N or E, and D4938N or E were unaltered when compared with RyR1. However, mutants E4032Q, D4917N or E, and Q4933N or E displayed neither caffeine nor 4-chloro-m-cresol response nor [\(^{3}\)H]ryanodine binding. Sedimentation assays indicated that the nonfunctional mutants did contain tetrameric complexes, implying that defects in the assembly of a functional channel did not occur with specific mutations in transmembrane sequences. These results support the view that amino acids Glu\(^{4806}\) (M7), Asp\(^{4815}\) (M7), Asp\(^{4917}\) (M10), and Gln\(^{4932}\) (M10) are involved in channel function and regulation.

In skeletal muscle, excitation-contraction coupling involves the interaction between two important proteins, the \(\alpha\)1-subunit of the L-type Ca\(^{2+}\) channel (dihydropyridine receptor or DHPR),\(^{1}\) located in the transverse tubular membrane, and the Ca\(^{2+}\) release channel (ryanodine receptor or RyR), located in the junctional terminal cisternae of the sarcoplasmic reticulum. The DHPR acts as a voltage sensor in response to depolarization of the transverse tube, whereas RyR responds as a release channel for Ca\(^{2+}\) stored in the sarcoplasmic reticulum. Ca\(^{2+}\) release channel function of the DHPR is probably irrelevant for excitation-contraction coupling in skeletal muscle (1–3). Analysis of the deduced amino acid sequence of rabbit skeletal muscle RyR has led to the prediction that transmembrane sequences near the COOH terminus form the Ca\(^{2+}\)-conducting pore and the binding sites for ryanodine and Ca\(^{2+}\) (4–6), whereas the remainder of the molecule forms a series of cytoplasmic domains.

The number of transmembrane sequences in the COOH-terminal region is still undefined. We proposed that there are 12 transmembrane sequences, but M’ and M” were tentative (5). Although we no longer consider M3 and M4 to be transmembrane sequences, since they are not conserved in the RyR/IP\(_3\)R family, we retain the Zorzato numbering system in this study. Studies using site-directed antibodies have confirmed that the NH\(_2\) and COOH termini of RyR1 are located in the cytosol (7, 8). Recent investigations of an expressed, truncated RyR1 protein have demonstrated that the COOH-terminal portion of RyR1, containing M1 to M10, is sufficient to form a functional Ca\(^{2+}\) release channel (9).

Studies of structure/function relationships in transmembrane sequences in the Ca\(^{2+}\)-ATPase (SERCA1) have revealed that acidic or amidated amino acids provide ligand-binding sites for Ca\(^{2+}\), whereas the juxtaposition of a small residue with an acidic residue (EG in M6, GE in M7, and DG in M8) is a critical motif in SERCA molecules (10–14). Analysis of the transmembrane sequences M1 to M10 of RyR1 and alignment of the sequences of 6 RyR and 5 IP\(_3\)R members of the Ca\(^{2+}\) release channel family (4, 15–23) revealed that several negatively charged and amidated residues are highly conserved. Among these, Glu\(^{4803}\), Asp\(^{4815}\), and Asp\(^{4917}\) are absolutely conserved (Fig. 1), suggesting that these amino acids might play an important role in structure/function relationships in Ca\(^{2+}\) release channels.

In this study, we mutated 14 negatively charged and amidated amino acids (plus vicinal Gly and Ala residues), which were conserved among 11 members of the RyR and IP\(_3\)R families. These residues are located in M1, M2, M5, M7, and M10 of RyR1. We used Ca\(^{2+}\) microfluorimetry to measure Ca\(^{2+}\) release in response to caffeine and 4-chloro-m-cresol for wild type and mutant RyR1 proteins transiently expressed in HEK-293 cells. We also measured [\(^{3}\)H]ryanodine binding by wild type and mutant RyR1 proteins, and sedimentionation was used to assess the oligomeric status of the expressed receptors. Our results indicate that residues in M2, M7, and M10 are involved in channel function.

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\footnote{The abbreviations used are: DHPR, dihydropyridine receptor; RyR, ryanodine receptor; SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; CHAPS, 3-[\(\{\)3-cholamidopropyl\]dimethylammonio]-1-propanesulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid; IP\(_3\)R, inositol 1,4,5-trisphosphate receptor.}
 EXPERIMENTAL PROCEDURES

Materials—Pfu polymerase, restriction endonucleases, and other DNA-modifying enzymes were purchased from Stratagene, Boehringer Mannheim, New England Biolabs, Promega, and Amersham Pharmacia Biotech. Fura-2 acetoxymethyl ester was from Molecular Probes. Caffeine was from Sigma and 4-chloro-m-cresol from Fluka. Unlabeled ryanodine was obtained from Calbiochem, and [3H]ryanodine was purchased from New England Nuclear Life Science Products. CHAPS was from Bio-Rad. All other reagents were from Sigma Chemical Company, Inc., and were of reagent grade or highest grade available.

Oligonucleotide-directed Mutagenesis—Site-directed mutagenesis was carried out in calf testes using the Quickchange kit (Stratagene) as described previously (24). Expression vector pcDNA 3.1(–) was from Invitrogen. Monoclonal antibody 34C was a kind gift from Dr. Judith Airey (24). Horseradish peroxidase- and alkaline phosphatase-conjugated goat anti-mouse secondary antibody (IgG) were obtained from Promega, and SuperSignal Ultra chemiluminescent substrate was obtained from Pierce. Nitrocellulose membranes were from Schleicher & Schuell. All other reagents were from Sigma Chemical Company, Inc., and were of reagent grade or highest grade available.

Mutagenesis and Expression of Mutant RyR1 cDNAs—Sequence of alignments in 6 RyR and 5 IP3R members of the Ca2+ release channel family (4, 15–23) that we have predicted to be transmembrane sequences (5, 15) showed that conserved acidic and amidated amino acids are present in predicted transmembrane sequences M1, M2, M5, M6, M7, and M10 in RyR1 and that Gly or Ala residues vicinal to an acidic amino acid are present in M1 (DA) and M2 (EG) (Fig. 1). Gly1632, Asp1633, and Asp1637 are 100% conserved in the RyR/IP3R family, but Asn4662 is poorly conserved and was not analyzed. Conserved, negatively charged amino acids, Asp1633, Gly1635, Asp1637, and Asp4615, amidated residues Asn4614, Asn4575, Asn4575, Asn4605, Asn4606, Gin4393, and Ala13888, and small residue Gly1633 were selected for mutagenesis. Two absolutely conserved residues Asp4593 in the COOH terminus and Asp4938 and Asp4969, located on the predicted cytoplasmic junction of M10, were also included. In initial studies, most residues were mutated to the small, non-polar residue Ala, whereas Ala13888 was mutated to Val.

Wild type and mutant RyR1 proteins were expressed in HEK-293 cells. Western blotting of whole cell lysates with monoclonal antibody 34C showed no expression of RyR1 in non-transfected or vector-transfected HEK-293 cells, but wild type and all of the mutants were expressed at readily detectable levels (Fig. 2). Although the extent of staining of Western blots of whole cell lysates varied from lane to lane, the differences averaged out over 5 independent experiments, so that we did not observe any significant differences in the expression level of any of the mutants described in this study. There was also no significant difference in apparent molecular mass between wild type and mutant RyR1 proteins (Fig. 2).

Fluorescence Measurement of Ca2+ Release in Response to Caffeine and 4-Chloro-m-cresol—Caffeine releases Ca2+ from internal stores through RyR, by sensitizing the Ca2+-release channel through an unknown mechanism (34). The peak amplitude of fura-2 fluorescence following each application of caffeine or 4-chloro-m-cresol was measured to estimate the function of each expressed wild type or mutant RyR1.

Fig. 3A shows an example of the fluorescence changes elicited by wild type RyR1-transfected cells in response to caffeine and 4-chloro-m-cresol. Fura-2-loaded cells were first challenged with 20 mM caffeine. This caused a significant upward shift in the fluorescence ratio, indicating that Ca2+ stored internally was released into the cytosol. After peak fluorescence was reached, caffeine was washed away by perfusion, allowing Ca2+ to return to the internal store before 4-chloro-m-cresol was added to obtain a second peak of Ca2+ release. We did not observe any changes in the 340/380 nm ratio after application of either 20 mM caffeine or 0.3 mM 4-chloro-m-cresol to either

L-phosphatidylcholine, and the protease inhibitor mix described above. Solubilized proteins were obtained by removing the debris through centrifugation at 4,000 rpm at 4 °C for 10 min. Microsome preparations were solubilized as described previously (31). The resulting supernatant from cells or microsomes was placed on the top of a 7.5–25% (w/v) linear sucrose gradient solution containing 50 mM Tris-Hepes, pH 7.4, 0.3 mM NaCl, 0.1 mM CaCl2, 0.3% CHAPS, 0.15% l-phosphatidylcholine, and protease inhibitor mix and was centrifuged at 28,000 rpm in a Beckman SW 40 rotor for 18–20 h at 4 °C. Fractions of 1.0 ml each were collected from bottom to top and measured for immunoreactivity.

Enzyme-Linked Immunoabsorbent Assay—Immunoreactivity in the sucrose density gradient fractions was determined by direct enzyme-linked immunoabsorbent assay using monoclonal antibody 34C (24), as described previously (31). Protein Assay—Protein concentration was determined by dye binding using bovine serum albumin as a standard (32).

RESULTS

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non-transfected or vector-transfected HEK 293 cells. We did, however, observe that concentrations of 4-chloro-m-cresol higher than 0.5 mM could induce a slow Ca\textsuperscript{2+} release phase in non-transfected cells.

Fig. 3B shows the results of fluorescence measurements for control, wild type, and mutant RyR1 proteins. Mutation to Ala of three absolutely conserved residues, Glu\textsubscript{4032}, Asp\textsubscript{4815} and Asp\textsubscript{4917}, and of the less well conserved Asn\textsubscript{4806}, led to the loss of response to caffeine and 4-chloro-m-cresol. Two mutants, N4805A and Q4933A, showed decreased response to both agents. The response of other mutants, to caffeine and 4-chloro-m-cresol, including control mutants Asp\textsubscript{4938} and Asp\textsubscript{4969}, was not different from wild type RyR1. Mutants A3988V and G4033A were also normally responsive to caffeine and 4-chloro-m-cresol. The location of Gly vicinal to an acidic amino acid, a critical feature in the formation of Ca\textsuperscript{2+}-binding sites in Ca\textsuperscript{2+}-ATPase molecules (12, 14), is obviously not critical to RyR1 function.

Since mutation to Ala changed size and charge for acidic residues, and size and polarity for amidated residues, it was of interest to determine the consequences of less drastic mutations. Asp was mutated to Asn and Glu, Glu to Gln and Asp, Asn to Gln and Asp, and Gln to Asn and Glu. These included Glu\textsubscript{4032} in M2; Asp\textsubscript{4815} and Asp\textsubscript{4969} was not different from wild type RyR1. Mutants A3988V and G4033A were also normally responsive to caffeine and 4-chloro-m-cresol. The location of Gly vicinal to an acidic amino acid, a critical feature in the formation of Ca\textsuperscript{2+}-binding sites in Ca\textsuperscript{2+}-ATPase molecules (12, 14), is obviously not critical to RyR1 function.

When Glu\textsubscript{4032} in M2 was mutated to Asp, wild type function was retained, but this was not the case for mutation to Gln, indicating that charge is important for RyR1 function. In M5, mutation of Asp\textsubscript{4815} to Gln and Asp and mutation of Asp\textsubscript{4917} to Asn and Glu did not result in any significant defect in response to caffeine or 4-chloro-m-cresol, implying that the size of these residues is more important than charge. Mutation of Asp\textsubscript{4917} in M10 to Ala, Asn, and Glu led to loss of caffeine and 4-chloro-m-cresol responses, implying that retention of Asp at position 4917 is essential. When Gln\textsubscript{4933} was mutated to Ala, a decrease in the peak amplitude of the fluorescence ratio was observed, but mutation to Asn and Glu, of similar size and polarity, abolished responses to both caffeine and 4-chloro-m-cresol. When Asp\textsubscript{4938} was mutated to Asn, the mutant response to caffeine and 4-chloro-m-cresol was not different from wild type, and when mutated to Gln, the response was diminished.

\textsuperscript{[3H]}Ryanodine Binding Assay—Binding of ryanodine to the single high affinity binding site on the ryanodine receptor requires channel opening (35–37). Microsomes from cells expressing each of the mutant RyR1 proteins were incubated with 60 nM \textsuperscript{[3H]}ryanodine under conditions promoting Ca\textsuperscript{2+} channel opening. As shown in Fig. 4, those mutants lacking response to caffeine and 4-chloro-m-cresol also had no significant \textsuperscript{[3H]}ryanodine binding, indicating that the channels were either closed, that they did not form a tetrameric complex, or that they did not fold and assemble properly and were vulnerable to proteolytic digestion.

Western blotting of wild type and nonfunctional mutant proteins in cell lysates (Fig. 2) or in microsomal membranes (not shown) revealed bands of comparable content and molecular
mass. Therefore, the defective [3H]ryanodine binding in microsome preparations from those mutants that were not responsive to caffeine was not due to their poor expression in HEK-293 cells. The fact that the proteins were located in the microsomal fraction indicated that the mutations did not affect membrane insertion. Mutants Q4933A and D4938E, which were weakly responsive to caffeine and 4-chloro-m-cresol, retained normal [3H]ryanodine binding capacity, confirming that an active tetrameric channel was formed.

Sedimentation—In order to determine whether ryanodine binding deficient channels were tetrameric, sedimentation assays in linear sucrose gradients were carried out. The location of wild type RyR1 proteins in gradient fractions was determined using monoclonal antibody 34C. Fig. 5 shows the distribution of the expressed RyR proteins in a linear sucrose density gradient. A single peak of immunoreactivity in the position corresponding to an oligomer in the gradient fractions was found in solubilized preparations from wild type RyR1, mutants G4033A and Q4933A, and all the non-functional mutants in M2, M7, and M10 (Fig. 5 and Table I). These expressed proteins clearly formed an oligomeric complex of similar size to that formed by the native receptor (31), indicating that they have normal structure but abnormal function.

DISCUSSION

Structure/Function Relationships in RyR1—In this study, alterations in function were observed following mutation of individual amino acid residues in transmembrane sequences M2, M7, and M10 of RyR1, as summarized in Figs. 3 and 4 and in Table I. In M2, mutation of Glu4032 to Ala or Gln, but not to Asp, led to loss of channel response to caffeine and 4-chloro-m-cresol and to loss of [3H]ryanodine binding, but tetramer formation was retained. In M7, mutation of Asn4806 to Ala, but not to Gln or Asp, and mutation of Asp4815 to Ala, but not to Asn or Glu, had similar deleterious effects. It is of special interest that neither M2 nor M7 are among the 4 most hydrophobic sequences first identified by Takeshima et al. (4). Clearly these sequences do play a role in functions associated with transmembrane sequences (38).

In M10, mutation of Asp4917 to Ala, Asn or Glu, and of Gln4933 to Asn or Glu led to loss of Ca2+ release channel function, whereas tetramer formation was retained. Asp4917 seemed to be irreplaceable, since change to Ala, Asn, or Glu abolished channel function. These observations highlight M10 as an interesting sequence for further detailed analysis of channel pore formation.
Ryanodine Receptor Transmembrane Mutations

The reasons for the defects in caffeine response and \(^3\text{H}\)ryanodine binding in our mutant channels are not known. The physical structure of the ion-conduction pathway may be altered in these mutants. Since transmembrane sequences of the Ca\(^{2+}\) release channel are likely to form the pore of the channel, substitutions of critical amino acids in the pore region may cause narrowing or distortion of the interior of the pore and thus not allow Ca\(^{2+}\) ions to pass through. It is well known that ryanodine binding to Ca\(^{2+}\) release channels requires opening of the channel. Thus our \(^3\text{H}\)ryanodine binding data indicate that those mutant channels that are inactive and do not respond to caffeine are closed. Channel opening is associated with a 4\(^\circ\) rotation of the transmembrane region with respect to the cytosolic region (39). Disruption of the channel pore might prevent the rotation of the transmembrane region that leads to channel opening. A second possibility is that transmembrane sequences M2, M7, and M10 form part of the ion selectivity filter (40–42) in the Ca\(^{2+}\) release channel and that alterations in ion selectivity occur in \(^3\text{H}\)ryanodine binding deficient mutants.

A third possibility is that the Ca\(^{2+}\) sensitivity of the mutant channels is decreased. Activation of the Ca\(^{2+}\) release channel by Ca\(^{2+}\) is mediated through high affinity Ca\(^{2+}\)-binding sites that are likely to be located within the channel. Although several Ca\(^{2+}\)-binding sites have been proposed and experimentally demonstrated (3), none of them has been confirmed as the critical site of Ca\(^{2+}\) activation. Strong evidence has been presented to show that negatively charged amino acids in transmembrane sequences are major contributors to the high affinity Ca\(^{2+}\)-binding sites in SERCA molecules (10, 14) and in the L-type Ca\(^{2+}\) channel (41). In fact, the high affinity binding site determines the selectivity of L-type Ca\(^{2+}\) channels for Ca\(^{2+}\) over other ions (41).

Support for the view that negatively charged amino acids in transmembrane sequences might be major contributors to the high affinity Ca\(^{2+}\)-binding sites in RyR1 comes from the work of Chen et al. (43) who reported that the mutation E3885A in M2 in RyR3 (equivalent to E4032A in RyR1) resulted in the loss of caffeine response in transfected HEK-293 cells, in agreement with our observations. They used single channel recordings in planar bilayers to show that the mutant RyR3 forms a functional channel with normal conductance in which Ca\(^{2+}\) sensitivity for activation is reduced by 10,000-fold. The mutant channel retained normal modulation by ryanodine, caffeine, and ATP. Formation of heterotetramers of wild type and mutant channels by coexpression led to channels with intermediate Ca\(^{2+}\) sensitivity. These observations led Chen et al. (43) to propose that Glu\(^{3885}\) is a key residue in formation of the Ca\(^{2+}\) sensor of RyR3. Our data for mutant E4032A in RyR1 are consistent with the observations of Chen et al. (43) and extend them to mutants N4806A, D4815A, and D4917A, which also did not respond to either caffeine or 4-chloro-m-cresol when expressed in HEK-293 cells.

A potential inconsistency lies in the fact that Chen et al. (43) demonstrated a response of the E3885A mutant to ryanodine, suggesting that the ryanodine-binding site is intact in activated channels, whereas we could not detect \(^3\text{H}\)ryanodine binding to our E4032A or E4032Q mutants in the presence of 10 mM ATP and 100 \(\mu\)M free Ca\(^{2+}\), recreating conditions that previously allowed Ca\(^{2+}\) release channel sensitivity. These observations led Chen et al. (43) to propose that Glu\(^{3885}\) is a key residue in formation of the Ca\(^{2+}\) sensor of RyR3. Our data for mutant E4032A in RyR1 are consistent with the observations of Chen et al. (43) and extend them to mutants N4806A, D4815A, and D4917A, which also did not respond to either caffeine or 4-chloro-m-cresol when expressed in HEK-293 cells.

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\(^2\) G. G. Du and D. H. MacLennan, unpublished observations.

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**TABLE I**

| Location | Amino acid change | Caffeine/cresol response | Ryanodine binding | Tetramer formation |
|----------|------------------|--------------------------|------------------|------------------|
| M1       | D3987A           | +                        | +                | ND\(^a\)          |
|          | A3989V           | +                        | +                | ND               |
| M2       | E4032A           | –                        | –                | +\(^b\)           |
|          | E4032Q           | –                        | +                | +\(^b\)           |
| M5       | N4574A           | +                        | +                | ND               |
| M7       | N4805A           | +                        | +                | ND               |
| M10      | D4917A           | –                        | –                | +                |
|          | D4917N           | –                        | –                | +                |
|          | D4917E           | –                        | –                | +                |
| COOH     | D4938A           | –                        | –                | ND               |
| terminus | D4938N           | +                        | +                | ND               |
|          | D4938E           | +                        | +                | ND               |
|          | D4969A           | +                        | +                | ND               |

\(^a\) ND, not determined.

\(^b\) Determined with solubilized microsomes.
Single channel recordings across a planar lipid bilayer provide an excellent assay for mutant RyR1 function. However, some mutants may not form functional channels in the bilayer system (44) and, since only a tiny fraction of molecules is sampled, errors can arise from inadvertent assay of endogenous channels. In unpublished studies using Ca$^{2+}$ imaging, we have noted that caffeine or halothane-stimulated Ca$^{2+}$ release occurs in less than 3% of untransfected cells, in line with the observation that RyR1 is not detectable through Western blotting of whole cell lysates (45). Ca$^{2+}$ photometry and ryanodine binding, in which fluorescence or binding data are gathered from a large number of intact cells, are very useful assays for the screening and partial characterization of RyR1 proteins carrying mutations. In earlier studies, we and others (25, 46–47) used Ca$^{2+}$ photometry to show enhanced caffeine, halothane, and 4-chloro-m-cresol sensitivity in RyR1 molecules carrying mutations associated with malignant hyperthermia. Thus Ca$^{2+}$ photometry and [3H]ryanodine binding provide a relatively rapid and efficient first screen for the detection of interesting mutants that can then be subjected to more time-consuming, detailed analyses.

**RyR1 Transmembrane Sequences**—Although there is no consensus regarding the number of transmembrane sequences in the COOH-terminal region of RyR1, hydrophobicity of four predicted transmembrane sequences is obvious. They are referred to as M1–M4 in the sequence of Takeshima et al. (4), and as M5, M6, M8, and M10 in Zorzato et al. (5). Transmembrane sequences are believed to be key determinants of both tetramer formation and channel pore formation (3, 38).

Attempts have been made to analyze the function of the COOH terminus of RyR1, containing transmembrane sequences. A truncated RyR1 transcript is expressed in brain (48). It is probably initiated at Met4382, resulting in the expression of the COOH-terminal 656 amino acid residues of RyR1, and would be missing M1 and M2, as defined in Fig. 1. This protein has been expressed in Chinese hamster ovary cells, where it was detectable in the endoplasmic reticulum membrane. However, neither caffeine response nor [3H]ryanodine binding activity could be detected in the expressed, truncated protein. A longer truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences. A truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences. A truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences. A truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences. A truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences. A truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences. A truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences. A truncated COOH-terminal fragment of RyR1, containing M1 and M2 (see Fig. 1), has also been expressed in Chinese hamster ovary cells (9). This expressed protein formed a functional channel that was shown in single channel recordings to be responsive to ryanodine, but to be otherwise unregulated. This fragment must form a functional tetramer and a channel pore, but regulation may involve other upstream sequence. The NH2-terminal portion of RyR1 clearly contributes to channel pore, but regulation may involve other upstream sequences.