A highly conserved amino acid sequence, GVRAGGGID4831, which may form part of the Ca\(^{2+}\) release channel pore in RyR2, was subjected to Ala scanning or Ala to Val mutagenesis; function was then measured by expression in HEK-293 cells, followed by Ca\(^{2+}\) photometry, high affinity \([\text{H}]\)ryanodine binding, and single-channel recording. All mutants except I4829A and I4829T (corresponding to the I4897T central core disease mutant in RyR1) displayed caffeine-induced Ca\(^{2+}\) release in HEK-293 cells; only mutants G4826A, I4829V, and G4830A retained high affinity \([\text{H}]\)ryanodine binding; and single-channel function was found for all mutants tested, except for G4822A and A4825V. EC\(_{50}\) values for caffeine-induced Ca\(^{2+}\) release were increased for G4822A, R4824A, G4826A, G4828A, and D4831A; decreased for V4823A; and unchanged for A4825V, G4827A, I4829V, and G4830A. Ryanodine (10 \(\mu\)M), which did not stimulate Ca\(^{2+}\) release in wild type (wt), did so in Ala mutants in amino acids 4823–4827. It inhibited the caffeine response in wt and most mutants, but enhanced the amplitude of caffeine-induced Ca\(^{2+}\) release in mutant G4828A. It also restored caffeine-induced Ca\(^{2+}\) release in mutants I4829A and I4829T. In single-channel recordings, mutants I4829V and G4830A retained normal conductance, whereas all others had decreased unitary channel conductances ranging from 27 to 540 picosiemens. Single-channel modulation was retained in G4826A, I4829V, and G4830A, but was lost in other mutants. In contrast to wt and G4826A, I4829V, and G4830A, in which divalent metals were preferentially conducted, mutants with loss of modulation had no selectivity of divalent cations over a monovalent cation. Analysis of Gly4822 to Asp4831 mutants in RyR2 supports the view that this highly conserved sequence constitutes part of the ion-conducting pore of the Ca\(^{2+}\) release channel and plays a key role in ryanodine and caffeine binding and activation.

Ryanodine receptors (RyRs)\(^1\) and inositol 1,4,5-trisphosphate (IP\(_3\)) receptors form a family of Ca\(^{2+}\) release channels that play an essential role in the regulation of intracellular Ca\(^{2+}\) levels, thus impacting on a variety of physiological functions (1–3). The RyR channels are homotetramers of ~565-kDa subunits located in the sarc(endo)plasmic reticulum of muscle and nonmuscle cells. Analysis of the deduced amino acid sequence, which consists of about 5000 amino acids in skeletal and cardiac muscle RyRs, has led to the prediction that transmembrane sequences near the COOH terminus form the Ca\(^{2+}\) conducting pore, while the remainder of the molecule forms a series of cytoplasmic domains (4–6).

Several important regions have been mapped with low resolution through structure/function analysis of the linear amino acid sequence of RyRs. Malignant hyperthermia (MH) and central core disease (CCD) mutations, found in the sequences lying between amino acids 35 and 614 (MH/CCD domain I), 2162 and 2458 (MH/CCD domain II), and 4793 and 4897 (MH/CCD domain III), alter sensitivity of the channel to caffeine and halothane (7–10). Several mutations, recently found in two regions equivalent to MH/CCD domains I and II in RyR1, and also a COOH-terminal region (amino acids 4104–4497) in human cardiac RyR2, are associated with catecholaminergic polymorphic ventricular tachycardia and arrhythmogenic right ventricular cardiomyopathy type 2 (11, 12).

Locations of some ligand binding sites in the molecule have also been mapped. Ca\(^{2+}\) activation sites (13, 14), Ca\(^{2+}\) inactivation sites (15–17), and high affinity \([\text{H}]\)ryanodine binding sites (18, 19) are localized to the COOH-terminal quarter of RyR1. Mutation of Glu3885 in predicted transmembrane sequence 2 (TM2) of RyR3 (equivalent to Glu1032 in RyR1) caused a huge decrease in Ca\(^{2+}\) sensitivity (14). Other mutations of acidic amino acids in TM2, TM7, and TM10 have also been shown to block caffeine and 4-chloro-m-cresol activation and high affinity ryanodine binding, but single-channel function was not analyzed (20). The COOH-terminal one-fifth of the molecule retains sufficient structure to form a functional Ca\(^{2+}\) release channel, but lack of regulation of this channel suggests that upstream sequences contain regulatory elements (13). Although several potential calmodulin binding sites have been identified (21, 22), Cys3635 in RyR1 appears to be critical for calmodulin binding and redox modulation (23).

One CCD mutation, I4897T, associated with severe clinical symptoms, abolished caffeine-induced Ca\(^{2+}\) release and high affinity \([\text{H}]\)ryanodine binding when RyR1 bearing this mutation was expressed in HEK-293 cells (10). It was proposed that the I4897T mutation was leaky, depleting Ca\(^{2+}\) stores. However, a later study of the I4897T mutant, expressed in dyspedic myotubes, demonstrated a functional uncoupling of sarcocellular excitation from Ca\(^{2+}\) release from the sarcoplasmic reticulum (24). In that study, Ca\(^{2+}\) stores were reported to be similar when wt RyR1 or I4897T mutant RyR1 were expressed...
in the dyspedic myotubes. The I4897T mutation is located in a highly conserved sequence in predicted TM9, between two of the most likely transmembrane helices (TM8 and TM10) (5). This sequence is GVRAGGGGD in the RyR family and GLR/S/NGGGG/IV/GD in the IP$_3$ receptor family. The GIG motif is similar to the K$^+$/channel pore region motif, GYG (25), leading to the proposal that this sequence might form part of the pore of the RyR Ca$^{2+}$/release channel (26, 27).

In support of this proposal, the mutation G4824A in mouse RyR2 reduced single-channel conductance from 798 pS for the wild type channel to 22 pS (27). Agents such as Ca$^{2+}$, Mg$^{2+}$/, ATP, caffeine, ruthenium red, and ryanodine modulated the mutant channel. Co-expression of wild type and G4824A mutant proteins yielded single channels with intermediate unitary conductances. Several mutations in this region also abolished high affinity [H]ryanodine binding. Selected mutations in this region or in adjacent regions in RyR1 altered channel conductance in a separate study, but some of these mutations were otherwise unregulated in single-channel recordings (28).

In this study, we carried out systematic Ala to Ala or Val scanning mutagenesis of all amino acids in this highly conserved and disease-inducing region. Mutant channel function was analyzed by measurement of ligand-induced Ca$^{2+}$/release in transfected HEK-293 cells using Ca$^{2+}$/ photometry. [H]Ryanodine binding in whole cell extracts, and single-channel recordings with partially purified proteins. We found alterations in regulation and in caffeine and ryanodine modulation of the channels, alterations in conductivity and ion selectivity, and abnormal interactions between ryanodine and caffeine that imply a close relationship between the effects of these two modulators.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction endonucleases and other DNA modifying enzymes were from Stratagene, Roche Molecular Biochemicals, New England Biolabs, Promega, and Amersham Pharmacia Biotech; Fura-2 acetoxyethyl ester (AM) was from Molecular Probes; caffeine and protease inhibitors were from Sigma; [H]ryanodine was from PerkinElmer Life Sciences; unlabeled ryanodine was from Calbiochem; CHAPS was from Bio-Rad; soybean phosphatidylcholine (PC) was from Northern Lipids. The expression vector pcDNA 3.1 (+) was from Invitrogen. Monoclonal antibody C3-33 was from Affinity Bioagents. All other reagents were of reagent grade or highest grade available.

**Oligonucleotide-directed Mutagenesis**—Site-directed mutagenesis of the pore region residues was carried out in a small fragment (SacI-BglII, 13692-14526) of RyR2 cDNA by $Phi_{29}$ polymerase-based polymerase chain reaction using the Quickchange kit (Stratagene). The complete sequence of the mutated fragment was confirmed by DNA sequencing. The fragment with the desired mutation was subcloned back into its original position in RyR2 by three steps of subcloning: to a vector containing SacII-BglII (13602-14880), then to a vector containing fragment SacII-Xhol (11206-15380), and, finally, to the full-length RyR2 in the pcDNA expression vector.

**Cell Culture and DNA Transfection**—The culture of HEK-293 cells and cDNA transfection by the calcium phosphate precipitation method were carried out as described previously (29).

**Fluorescence Measurements**—A microfluorimetry system (Photon Technologies Inc.) was used to monitor the Fura-2 AM fluorescence and mutant RyR2 cDNAs were transfected transiently into HEK-293 cells and cDNA transfection by the calcium phosphate precipitation method were carried out as described previously (29).

**Extraction of Recombinant RyR2 and Mutant Proteins**—Transfected HEK-293 cells grown in 100-mm Petri dishes were solubilized with 1% CHAPS, 5 mg/ml phosphatidylcholine, and a protease inhibitor mix (0.1 mM AEBSF, 1 mM benzamidine, 1 $mu$/ml each of leupeptin, pepstatin, aprotonin, and E64) at 4°C for 1 h, as described previously (30). After precipitation at 8000 × g (Sorvall model 34 rotor) for 20 min, the supernatant was used for [H]ryanodine binding assay or was further centrifuged at 45,000 × g for 1 h in a Beckman rotor T107.1. The resulting pellet was dissolved in 250 mM sucrose, 150 mM KCl, 25 mM HEPES, pH 7.1, for measurement of [H]ryanodine binding, as described below, or used for purification of RyR2 and mutant proteins by sucrose gradient centrifugation, as described below.

**[H]Ryanodine Binding**—Channel opening was analyzed using the [H]ryanodine binding assay described previously (30). In brief, about 50 $mu$/g of protein were added to a binding buffer composed of 0.5 $mu$/KCl, 1 mM ATP, 100 $mu$/mL free Ca$^{2+}$/, 0.2 mM EGTA, 50 mM HEPES, pH 7.1, and various concentrations of [H]ryanodine in a total volume of 0.25 mL. Non-specific binding was determined using a 1000-fold excess of unlabeled ryanodine. After 2 h at 37°C, the samples were diluted with 1 mL of ice-cold washing buffer composed of 25 mM HEPES, pH 7.1, and 0.25 $mu$/KCl and placed on Whatman GF/B membrane filters pre-soaked with 1% polyethyleneimine in washing buffer. Filters were washed three times with 6 mL of washing buffer. [H]Ryanodine bound to the filter was quantified by liquid scintillation counting. All binding assays were carried out in duplicate.

**Purification of Expressed RyR2 and Mutant Proteins for Single-channel Recording**—The pellet obtained after centrifugation at 45,000 × g (see above) was solubilized for 1 h in a buffer composed of 1 mM NaCl, 1% CHAPS, 5 mg/ml PC, a mix of protease inhibitors (0.1 mM AEBSF, 1 mM benzamidine, 1 $mu$/ml each of leupeptin, pepstatin, aprotonin, and E64) and 50 mM Heps, pH 8.0. After centrifugation at 30,000 × g for 30 min, the supernatant was placed on the top of a 7–25% (w/v) linear sucrose gradient solution containing 50 mM Tris-HEPES, pH 7.4, 0.3 mM NaCl, 0.1 mM CaCl$_2$, 0.3% CHAPS, 0.15% PC, and the protease inhibitor mix, and was centrifuged at 28,000 rpm in a Beckman SW-40 rotor for 16–18 h at 4°C. Fractions of about 0.75 mL each were collected from bottom to top and subjected to direct enzyme-linked immunosorbent assay using monoclonal antibody C3-33 to determine the amount that contained RyR protein. The protein fractions with peak immunoreactivity were collected and stored at −70°C.

**Single-channel Recording**—Single-channel activities were recorded after incorporation of sucrose density gradient-purified wild type and mutant RyR2 proteins into a planar lipid bilayer. The bilayer was formed by painting a lipid mixture (5:3 phosphatidylethanolamine: phosphatidylcholine, 35 mg/mL) across a 200-$mu$/m hole in a Delrin paraffin separating two chambers (Warner Instrument Corp.). The bilayer chamber was connected to the head stage input of an amplifier (model EPC-7, List Electronics). The cis chamber was virtually grounded. Unless stated otherwise, single-channel recordings were obtained with a symmetrical solution containing 250 mM KCl and 25 mM Heps, pH 7.4. After formation of the bilayer, a 3-$mu$/aliquot of the sample was added to the cis chamber with continuous stirring of both chambers. Voltage commands to the amplifier were given through a Digidata 1200 (Axon Instruments Inc.). Recordings were filtered at 1 kHz before being acquired at 5 kHz by the Digidata 1200.

**Data Analysis**—Ca$^{2+}$/ photometric data were analyzed with Felix software (Photon Technologies Inc.). Single-channel recording data were analyzed using pClamp 7.0 software (Axon Instruments Inc.). Single-channel analysis was used to determine the dissociation constant ($K_{D}$) for Ca$^{2+}$/ and maximal binding capacity ($B_{max}$) from equilibrium binding data. $EC_{50}$/ values were obtained by fitting the curves with an equation for logistic dose response using Microc Origin software (Microc Software Ltd., Northampton, MA). Data are expressed as mean ± S.E. A paired or unpaired Student t test was used for evaluation of the mean values. A value of p ≤ 0.05 was considered to be statistically significant.

**RESULTS**

**Site-directed Mutagenesis and Expression of Mutant RyR2**—In this study, most residues in the highly conserved sequence GVRAGGGGD in RyR2 were mutated to the smart, non-polar residue Ala, Ala$^{4897}$/T was mutated to Val, and Ile$^{4829}$/ was mutated to Leu$^{4829}$/, the residue equivalent to Ile$^{4897}$/ in RyR1, was mutated to Ala and Val and to Thr, a mutation that causes a clinically severe form of central core disease in humans (10). Wild type and mutant RyR2 cDNAs were transfected transiently into HEK-293 cells. All proteins were expressed at readily detectable levels, as judged by functional assays such as caffeine-induced Ca$^{2+}$/ release and [H]ryanodine binding and by Coo massie Blue staining and Western blotting of whole cell lysates with monoclonal antibody C3-33 (data not shown).

**Fluorescence Measurement of Caffeine-induced Ca$^{2+}$/ Release**—Fura-2 fluorescence was measured at excitation wavelengths of 340 and 380 nm. Changes in the 340/380 ratio provided a measure of ligand-induced Ca$^{2+}$/ release in HEK-293 cells transfected with wt or mutant RyR2 (30). Since the measurement was carried out by photometry, resting intracellular Ca$^{2+}$/ levels could not be measured accurately.
No significant \( \text{Ca}^{2+} \) release was observed with caffeine up to 30 mM in pcDNA-transfected cells (30), but caffeine-induced \( \text{Ca}^{2+} \) release was readily observed in cells transfected transiently with wt RyR2. Figs. 1 (A–C) show representative traces of fluorescence changes for wt, V4823A, and G4828A that represent changes in intracellular \( \text{Ca}^{2+} \) levels in response to incremental application of 0.03–30 mM caffeine. \( \text{Ca}^{2+} \) release occurred with 0.03–0.1 mM caffeine for wt and mutant V4823A, but was seen only with concentrations of caffeine above 3 mM for G4828A. Peak fluorescence amplitudes were measured following the incremental application of 0.03–30 mM caffeine and normalized to the peak amplitude of the peak response in fluorescence ratio (340/380 nm) caused by 30 mM caffeine. The resulting data for all the mutants in this study were averaged in D and expressed as mean ± S.E. \( \text{EC}_{50} \) values and Hill coefficients, presented in the inset to D, were obtained by fitting the dose-response curves with an equation for logistic dose response. *, \( p < 0.05 \) when compared with the \( \text{EC}_{50} \) value for wild type RyR2.

**Pore Region Mutants**

**Fig. 1.** Fluorescence measurements of \( \text{Ca}^{2+} \) release by incremental concentrations of caffeine in HEK-293 cells transfected with wild type and mutant RyR2 cDNAs. Representative fluorescence tracings are shown for HEK-293 cells expressing RyR2 (A), V4823A (B), or G4828A (C) following incremental additions of caffeine. Caffeine dose-response curves are shown in D. Cells cultured on a coverslip were loaded with 2 \( \mu \text{M} \) Fura-2 AM and mounted on the stage of an inverted microscope, where selected fields containing about 30 cells were challenged with caffeine (30). Caffeine was washed out to restore resting \( \text{Ca}^{2+} \) levels after measurement of each peak amplitude (peak of change in the ratio of fluorescence at 340/380 nm) indicated that peak changes in \([\text{Ca}^{2+}]_i\) had been obtained. Individual peak amplitudes of 340/380 nm ratio (fluorescence ratio at the highest response to caffeine minus the ratio at rest) were collected and normalized to the maximal amplitude of the peak response in fluorescence ratio (340/380 nm) caused by 30 mM caffeine. The resulting data for all the mutants in this study were averaged in D and expressed as mean ± S.E. \( \text{EC}_{50} \) values and Hill coefficients, presented in the inset to D, were obtained by fitting the dose-response curves with an equation for logistic dose response. *, \( p < 0.05 \) when compared with the \( \text{EC}_{50} \) value for wild type RyR2.

**Fluorescence Measurement of Ryanodine-induced \( \text{Ca}^{2+} \) Release**—As shown previously (30), 10 or even 100 \( \mu \text{M} \) ryanodine did not elicit \( \text{Ca}^{2+} \) release in wt RyR2-transfected cells (Figs. 2A and 3A) or pcDNA-transfected cells (data not shown) over a period of 3–4 min. Under the same conditions, no \( \text{Ca}^{2+} \) release was induced by 10 \( \mu \text{M} \) ryanodine in cells transfected with mutants G4822A, G4828A, I4829A, I4829T, and D4831A (Figs. 2B, H, and I–K) and 3 (B and C). By contrast, ryanodine-induced \( \text{Ca}^{2+} \) release in cells transfected...
with mutants V4823A, R4824A, A4825V, G4826A, and G4827A (Fig. 2, C–G). The most significant Ca$^{2+}$ release occurred in mutants R4824A and G4827A (Fig. 2, D and G). When mutants I4829A and I4829T were co-expressed with wt RyR2 in a ratio of 1:1 or 2:1, Ca$^{2+}$ release was induced by ryanodine in the heterozygote-transfected cells (data not shown).
Fluorescence Measurement of Interactions between Ryanodine and Caffeine to Induce Ca\textsuperscript{2+} Release—The effect of ryanodine on caffeine-induced Ca\textsuperscript{2+} release was then examined in wt and mutant RyR2. Caffeine-induced Ca\textsuperscript{2+} release could be obtained repeatedly in wt-transfected cells, provided that caffeine was washed out between applications. Table I shows that caffeine-induced release in transfected HEK-293 cells expressed wt and mutant RyR2. Caffeine-induced Ca\textsuperscript{2+} release was determined for each mutant (Fig. 4B). EC\textsubscript{50} values (pCa) for caffeine-induced Ca\textsuperscript{2+} release in transfected HEK-293 cell and \textsuperscript{3}H]ryanodine binding to recombinant expressed wt and mutant RyR2

| Caffeine-induced Ca\textsuperscript{2+} release | Caffeine sensitivity | Ryanodine binding \((K_b)\) | Ryanodine-induced Ca\textsuperscript{2+} release | Effect of ryanodine on caffeine activation |
|-----------------------------------------------|---------------------|------------------------|-----------------------------------------------|----------------------------------------|
| Wt RyR2                                       | +\textsuperscript{a} | N\textsuperscript{b}    | 2.2                                           | ND                                     |
| G4822A                                        | +                   | +\textsuperscript{c}    | 4.2                                           | ND                                     |
| V4823A                                        | +                   | +\textsuperscript{c}    | 2.4                                           | +\textsuperscript{d}                  |
| R4824A                                        | +                   | +\textsuperscript{c}    | 2.8                                           | +\textsuperscript{d}                  |
| A4825V                                        | +                   | N                     | 2.0                                           | +\textsuperscript{d}                  |
| G4826A                                        | +                   | N                     | 3.3                                           | +\textsuperscript{d}                  |
| G4827A                                        | +                   | N                     | 3.5                                           | +\textsuperscript{d}                  |
| G4828A                                        | +                   | N                     | ND                                            | ND                                    |
| I4829A                                        | ND                  | +\textsuperscript{c}    | ND                                            | ND                                    |
| I4829T                                        | ND                  | +\textsuperscript{c}    | ND                                            | ND                                    |
| I4829V                                        | +                   | N                     | ND                                            | ND                                    |
| G4830A                                        | +                   | N                     | ND                                            | ND                                    |
| D4831A                                        | +                   | N                     | ND                                            | ND                                    |

\textsuperscript{a} +, positive response.

\textsuperscript{b} N, normal.

\textsuperscript{c} ND, not detected.

\textsuperscript{d} \downarrow, decreased.

\textsuperscript{e} \uparrow, increased.

High Affinity Equilibrium Binding of \textsuperscript{3}H]Ryanodine to Mutant RyR2—The equilibrium properties of \textsuperscript{3}H]ryanodine binding to mutant RyRs were measured to determine whether the high affinity ryanodine binding site was preserved. Surprisingly, high affinity \textsuperscript{3}H]ryanodine binding could not be detected in most of the mutants. Scatchard analysis showed a single binding site only in mutants G4826A, I4829V, and G4830A (Fig. 4A). \(K_b\) values for these mutants were similar to wild type RyR2 (Ref. 30; Fig. 4A, inset). \(B_{max}\) values for mutants G4826A, I4829V, and G4830A were 1.6, 1.0, and 1.5 pmol/mg of lysate protein. These data indicate that the high affinity ryanodine binding site is normal only in mutants G4826A, I4829V, and G4830A.

The Ca\textsuperscript{2+} dependence of \textsuperscript{3}H]ryanodine binding was also determined for each mutant (Fig. 4B). EC\textsubscript{50} values (pCa) for Ca\textsuperscript{2+} activation for mutants G4826A, I4829V, and G4830A were 6.06, 6.01, and 6.01, respectively, compared with 5.98 for wt. No significant \textsuperscript{3}H]ryanodine binding was observed for the other mutants in the presence of Ca\textsuperscript{2+} at concentrations up to 1.5 pCa units, even when supplemented by 10 mM caffeine and 3 mM ATP, which enhance channel opening (data not shown).

Single-channel Unitary Conductance and Gating—Single-channel conductance, gating, modulation, and permeability were determined for wt RyR2 and most of the mutants using the planar lipid bilayer method with K\textsuperscript{+} as the current carrier. Typical single-channel current traces are shown in Fig. 5 for wt RyR, and mutants G4823A, R4824A, G4826A, G4827A, G4828A, I4829A, I4829T, I4829V, G4830A, and D4831A. Channel open probability was 0.09, 0.01, 0.08, and 0.02 for wt RyR2, G4826A, G4827, and G4830A in the presence of trace amounts (about 300 nM) of Ca\textsuperscript{2+}, and each of these channels displayed gating behavior that was similar to wt in terms of mean open and mean closed time (data not shown). Channel open probability, however, was much higher for all other mutant channels in the presence of trace (500 nM) amounts of Ca\textsuperscript{2+} (Fig. 5). These channels were not closed, even in the presence of 1–3 mM EGTA, indicating that they required almost no Ca\textsuperscript{2+} for opening under the conditions of the in vitro assay. Among these mutants, single-channel activity for G4828A was observed only after overnight treatment with 50–200 \mu M ryanodine.

Unitary single-channel conductances, calculated from the current/voltage curves shown in Fig. 6A for wt and mutants, are presented in Fig. 6B (see also Fig. 5). Mutants I4829V and G4830A had single-channel conductances similar to that of wt RyR2. All other mutants had significantly lower conductances than wt. Among them, G4826A had a conductance of about 27 pS, a 26-fold reduction when compared with the wt channel. This finding is consistent with observations for the same mutants in mouse RyR2 and rabbit RyR1 (27, 28).

Single-channel Modulation—Fig. 7 shows representative single-channel recordings of wt RyR2 and mutants G4826A, G4828A, and I4829V following the addition of a number of physiological and pharmacological agents that modulate native RyR2 channels. The response of CHAPS-solubilized and sucrose gradient-purified recombinant wt RyR2 to modulators such as Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, ATP, caffeine, ryanodine, and ruthenium red was unchanged from the responses of the native channel (Fig. 7A). In the presence of 100 \mu M Ca\textsuperscript{2+} in the cis chamber,
the Ca\(^{2+}\) release channel was activated with a \(P_o\) of 0.56. The addition of 1.0 mM EGTA to the cis chamber, lowering free Ca\(^{2+}\) to 0.05 \(\mu\)M, completely inhibited channel activity. Elevation of free Ca\(^{2+}\) to 100 \(\mu\)M by the addition of 1 mM CaCl\(_2\) increased the \(P_o\) to 0.33. The subsequent addition of 3 mM MgCl\(_2\) inhibited channel activity and reduced the \(P_o\) to 0.001. The addition of ATP to 3 mM, then caffeine to 3 mM, increased the \(P_o\) to 0.05 and 0.45, respectively. Ryanodine (10 \(\mu\)M) reduced channel conductance to 50% of the unmodified state and shifted it to a long-lived open state. Ruthenium red (10 \(\mu\)M) blocked the ryanodine-modified channel.

Among the mutants tested, G4830A, with normal conductance and gating behavior, responded to these modulators with a pattern similar to that displayed by wt RyR2 (Table II). Mutant G4826A, despite its greatly reduced conductance, and mutant I4829V also displayed wt modulation by Ca\(^{2+}\), Mg\(^{2+}\), ATP, caffeine, ryanodine, and ruthenium red (Fig. 7, panels B and D). However, mutant G4828A, which displayed caffeine-induced Ca\(^{2+}\) release in transfected HEK-293 cells, did not respond to any of the ligands at concentrations shown routinely to modulate wt RyR2 channels (Fig. 7C). In addition, mutants V4823A, R4824A, G4827A, I4829A, I4829T, and D4831A were not modulated by any of these ligands (Table II).

The effect of higher concentrations of both ryanodine and ruthenium red was tested on mutant channels that did not respond to ligands. Fig. 8 shows the effects of these two modulators on the single-channel activity of mutant G4828A when applied at higher concentrations. This experiment was carried out at very low Ca\(^{2+}\) concentrations, with 1 mM EGTA in both the cis and trans chambers; ryanodine and ruthenium red were added subsequently to both chambers. Under these conditions, the G4828A channel remained in the open state, even in the presence of 10 \(\mu\)M ryanodine or 10 \(\mu\)M ruthenium red (data not shown). Higher concentrations of ryanodine (60 and 100 \(\mu\)M) gradually induced a long-lived closed time but did not affect channel conductance. Ruthenium red at 100 and 400 \(\mu\)M blocked the channel. Thus, the purified G4828A, I4829A, or I4829T channels were all affected in atypical fashion by ruthenium red and ryanodine, the most prominent change being a substantial decrease in affinity (Table II). None of the other non-modulated mutants responded to ryanodine as high as 300 \(\mu\)M or to ruthenium red as high as 1.2 mM (Table II).

Heparin, an IP\(_3\) receptor antagonist, was tested on wt RyR2 and some mutants. Neither stimulatory nor inhibitory effects were found for wt or mutants V4823A, R4824A, G4828A, I4829A, or I4829T (Table II) after the addition of 100–1000 \(\mu\)g/ml heparin to both the cis and trans chambers.

In an attempt to determine whether cellular accessory factors might regulate the properties of the mutant channels, we extracted about 200 \(\mu\)l of HEK-293 cell fluid from five 100-mm plates of confluent cells by homogenization and centrifugation at 10,000 \(\times\)g for 30 min. Wild type RyR2, mutant V4823A, and mutants retaining modulatory function did not respond when up to 3 \(\mu\)l of the fluid were added to the cis and/or trans chambers (data not shown). However, the addition of 1–3 \(\mu\)l of cell fluid to the cis chamber in the presence of 0.5 mM EGTA or 100 \(\mu\)M Ca\(^{2+}\) blocked channel opening for mutants R4824A, G4827A, G4828A, I4829A, I4829T, and D4831A. Blockage was not reversed by the addition of Ca\(^{2+}\), ATP, or caffeine. Although the effective component in the cell fluid is not known and modulation is clearly not typical, the results provide a hint for why the mutants lost modulation in vitro.

**Single-channel Permeability—Ca\(^{2+}\) release channels are known to conduct divalent cations such as Ca\(^{2+}\), Mg\(^{2+}\), and Ba\(^{2+}\) with equal efficiency.** Fig. 9 (A–C) shows single-channel recordings for RyR2, I4829V, and G4828A obtained with 250 mM KCl in the cis chamber and 50 mM BaCl\(_2\) in the trans chamber. At 0 mV, RyR2 and I4829V, a mutant with normal conductance and modulation, showed a Ba\(^{2+}\) current of 3.5 ± 0.05 pA (n = 3) and 3.7 ± 0.3 pA (n = 5), respectively (second trace in Fig. 9, A and B). Under the same conditions, no measurable Ba\(^{2+}\) current was detected for G4828A (n = 4) (second trace in Fig. 9C). The current/voltage curves for RyR2, I4829V, and G4828A under these conditions are presented in Fig. 9 (D–F) and compared with the IV curves (from Fig. 6A) obtained with 250 mM symmetrical KCl in both chambers. The K\(^+\) conductance was 490 ± 23 and 487 ± 45 pS and the Ba\(^{2+}\) conductance was 186 ± 13 and 187 ± 11 pS for RyR2 and I4829V.
respectively. The K⁺ and Ba²⁺ conductances were similar for G4828A, with a value of 280 ± 27 pS. The reversal potential for these three channels, and hence the calculated permeability ratio (p(Ba²⁺)/p(K⁺)) (31), are presented in Fig. 9 (inset). They indicate that RyR2 and mutant I4829V have a limited preference for Ba²⁺ over K⁺, but that mutant G4828A has a similar selectivity for Ba²⁺ and K⁺.

The conductance for Mg²⁺ and/or Ca²⁺ was also determined for wt and selected RyR2 mutants. Current/voltage curves for wt, R4824A, and G4828A were obtained under two conditions with 250 mM symmetrical KCl in both chambers: (a) with contaminating Ca²⁺ and (b) with 10 mM MgCl₂ in the trans chamber. The I/V curves for K⁺ current in contaminating Ca²⁺ was linear for these three channels, showing ohmic voltage dependence (Figs. 6A and 10 (A–C)). The addition of 10 mM MgCl₂ (Fig. 10A) or 10 mM CaCl₂ (data not shown) to the trans chamber reduced the current at both positive and negative holding potentials and shifted the reversal potential leftward to about 5 mV. Such phenomena were not seen, however, for mutants R4824A and G4828A after the addition of 10 mM MgCl₂ to the trans side (Fig. 10, B and C). In wt RyR2 and the mutants with normal modulation (G4826A, I4829V, and G4830A), the addition of 3 mM MgCl₂ to the cis chamber resulted in a reduced current of about 30% at −30 mV holding potential (Fig. 10, inset). No change of current was observed in other mutants under the same conditions (Fig. 10, inset).

**DISCUSSION**

Various mutations in amino acids 4822–4831 of RyR2 Ca²⁺ release channels alter the sensitivity of channel opening to caffeine, the ability of the channel to bind [³H]ryanodine with high affinity, the ability of ryanodine to activate and inhibit channel function, the unitary conductance of single channels, the modulation of single channels by Ca²⁺ and other endogenous and exogenous agents, and ion permeability. These results demonstrate the critical role of this sequence in caffeine activation, ryanodine binding, and ryanodine activation of Ca²⁺ release and support the hypothesis that this region is the pore region of the RyR Ca²⁺ release channel, comparable to the P-loop of many voltage-gated ion channels in the plasma membrane.

This sequence, which connects highly hydrophobic transmembrane sequences, M8 and M10, was originally predicted to be a transmembrane sequence (M9) (5), but this sequence must now be considered to fold into the pore structure from the luminal side (27, 28). Since both the NH₂ and COOH termini of RyR molecules are located in the cytoplasm (32), there should be an even number of transmembrane sequences. Of the 10 transmembrane sequences originally proposed (5), M3 and M4 are not conserved in the family and can be eliminated. Recognition that former M9 probably folds into the pore region eliminates M9 as a transmembrane sequence. Thus, it is likely that at least one more proposed helix must also be eliminated to reduce the transmembrane sequences to an even number.

It is complicated to sort out and interpret the possible links among the large number of functional consequences of mutation of the 12 amino acids forming part of the pore region. Association was observed between loss or retention of high affinity [³H]ryanodine binding and single-channel modulation. Mutants G4826A, I4829V, and G4830A retained normal Kᵣ for high affinity [³H]ryanodine binding and normal single-channel modulation. All other mutants lost both [³H]ryanodine binding and normal single-channel modulation. We did not observe a correlation between retention of high affinity [³H]ryanodine.
binding and ryanodine-induced Ca\(^{2+}\) release in transfected HEK-293 cells. Mutants I4829V and G4830A, like wt RyR2, retained high affinity \(^{3}H\)ryanodine binding and did not display ryanodine-induced Ca\(^{2+}\) release, whereas mutant G4826A retained high affinity \(^{3}H\)ryanodine binding and gained the function of ryanodine-induced Ca\(^{2+}\) release in transfected HEK-293 cells.

Surprisingly, mutants that did not display modulation by agents such as caffeine, Ca\(^{2+}\), Mg\(^{2+}\), and ATP in single-channel recordings retained at least low levels of caffeine-induced Ca\(^{2+}\) release in transfected HEK-293 cells. Thus, there is a dilemma that channels that are active in whole cells appear inactive when measured as single-channels. This feature is further complicated by the fact that mutants that did not retain normal modulation in single-channel recordings did not retain detectable \(^{3}H\)ryanodine binding, even in the presence of caffeine, which favors channel activation. Loss of regulatory factors during the process of isolation of the protein in the presence of CHAPS is a possible reason (28), and preliminary results reported here support this idea.

**Single-channel Properties**—Analysis of the single-channel properties of all mutants in the predicted pore region sequence, except for G4822A and A4825V, showed that only mutants G4830A and I4829V retained both normal conductance and normal modulation. With the further exception of mutants G4830A and I4829V, all had decreased K\(^{+}\) conductance. Mutant G4826A, with a unitary K\(^{+}\) conductance of only 27 pS, retained normal modulation by endogenous and exogenous agents. Similar results have been reported previously for the same RyR2 mutant (27) and for the corresponding mutant in RyR1 (28). The unitary K\(^{+}\) conductance of mutants V4823A, R4824A, G4827A, G4828A, I4829A, I4829T, and D4831A varied between 340 and 540 pS, compared with 710 pS for the normal channel. Although this group of mutants retained a higher conductance, they lost regulatory properties. Mutants G4828A and I4829A or I4829T had reduced sensitivity to ryanodine and ruthenium red. Many of the mutated channels, similar to those mutated in the same region in RyR1 (28), remained open and retained a high P\(_{o}\), even in the presence of millimolar EGTA, indicating that loss of Ca\(^{2+}\) regulation was a common feature among these mutants. It must be stressed, however, that a high probability of channel opening in these mutant channels under the conditions of single-channel recording might not reflect the *in vivo* situation. These channels retained caffeine activation in cellular assays, but were not responsive in single-channel recordings. Unknown cellular factors could exist that fine-tune channel opening and channel modulation. These factors could be lost during protein isolation with CHAPS. Single-channel recording with isolated microsomes or direct patch clamping on the nuclear membrane of transfected HEK-293 cells (33) could be useful in elucidating the mechanisms underlying these events.

RyR Ca\(^{2+}\) release channels have a limited selectivity for divalent cations as compared with a permeability ratio of more than 1000 for divalent cations over monovalent cations observed for L, N, or T type Ca\(^{2+}\) channels (31). In L type Ca\(^{2+}\) channels, the molecular determinant of Ca\(^{2+}\) selectivity is a four-glutamate sequence, the EEEE locus, located in the pore region of each of the four internal repeats in the Ca\(^{2+}\) channel \(\alpha_{1}\) subunit (34). The results obtained in this study suggest that many amino acids in the pore region of the RyR Ca\(^{2+}\) release channel, not only those that are negatively charged, might contribute to the weak selectivity for divalent cations. K\(^{+}\) conductance by the mutants V4823A, R4824A, G4827A, G4828A, I4829A, I4829T, and D4831A was unimpaired by the presence of the divalent cations Ca\(^{2+}\), Ba\(^{2+}\), or Mg\(^{2+}\) in the *cis* chamber or in the *trans* chamber, suggesting that divalent and monovalent cations were conducted with similar efficiency. Residues adjacent to this region in RyR1 are also involved in channel selectivity (28).

**Single-channel Properties of Mutant I4829T**—Mutation I4897T in RyR1, equivalent to I4829T in RyR2, is a causal mutation for central core disease (10). The RyR1 mutation I4897T differed from other CCD and MH mutations found in MH/CCD domains I and II in the NH\(_{2}\)-terminal portion of RyR1, since it abolished caffeine-induced Ca\(^{2+}\) release in transfected HEK-293 cells in the homozygous state, although not in the heterozygous state, and it lost ryanodine binding. Thus it was proposed that it exists as a highly leaky channel, which continually depletes Ca\(^{2+}\) stores. The single-channel properties of the corresponding RyR2 mutant, I4829T, strongly support this view. The I4829T channel, in the absence of Ca\(^{2+}\) or any other modulator had an open probability of over 90%. It is, however, possible that channel opening is suppressed substantially in cells. Thus, even though its conductance was reduced to about 60% of wt, it would still function as a highly leaky channel, consistent with the hypothesis that CCD is caused by chronic elevation of intracellular Ca\(^{2+}\) (7, 35). This hypothesis has been disputed by Avila *et al.* (24), who believe that Ca\(^{2+}\) stores are unaffected by the mutation on the basis of experiments showing that cyclopiazonic acid-induced Ca\(^{2+}\) transients are similar in dyspedic myoblasts expressing wt or I4897T RyR1.
Both mutants I4829A and I4829T had abnormal single-channel properties. When I4829 was mutated to Val, the corresponding conserved residue in IP3 receptors, channel regulation and channel conductance were restored to normal. These results indicate the critical requirement for a hydrophobic residue in this position. The corresponding mutation, I4897V, has been reported to decrease channel conductance in RyR1 (28), whereas V2458I in IP3 receptor increased channel conductance (33). Our result for I4829V differs from these two reports. Isoform or species differences may contribute to phenotypic differences in the expressed proteins.

### Caffeine Activation

Caffeine, an exogenous activator of Ca$^{2+}$/H$^{1001}$ release, has been used as a tool for studying excitation-contraction coupling and the function of Ca$^{2+}$/H$^{1001}$ release channels (36). It is also used in the clinical diagnosis for malignant hyperthermia (7). Whereas caffeine sensitivity is useful for the diagnosis of CCD caused by mutations in MH/CCD domains I and II, recent evidence that CCD mutations in MH/CCD domain III retain normal caffeine sensitivity in heterozygous form limits the usefulness of caffeine sensitivity for the diagnosis of CCD (10).

The binding site for caffeine in RyR has not been defined, largely because mutations throughout the molecule cause alterations in caffeine sensitivity. All MH and CCD mutations located in the sequences lying between amino acids 35 and 614 and between 2162 and 2458 (7) had increased sensitivity to caffeine and halothane (8, 9), implying that these domains might be involved in caffeine binding. In support of this view, expression of the COOH-terminal one-fifth of the RyR1 sequence was sufficient to form a functional Ca$^{2+}$/H$^{1001}$ release channel, but the truncated channel was not caffeine-activated (37). Nevertheless, the COOH-terminal sequence has been linked to caffeine sensitivity, since deletion of amino acids 4272–4535 increased the sensitivity of the mutant RyR1 channel to caffeine and Ca$^{2+}$/H$^{1001}$ (17). The CCD mutation, I4897T in RyR1, found in MH region 3 and equivalent to I4829T in this study, abolished caffeine-induced Ca$^{2+}$ release in transfected HEK-293 cells when expressed as a homozygote, but displayed normal...
caffeine and halothane sensitivity when expressed as a heterozygote (10). These observations, together with the results obtained in this study, indicate that caffeine activation of RyR channels involves amino acids throughout the sequence and suggest that caffeine activation is a global and complicated process. Indeed, caffeine-induced Ca2+/H11001 release must include a number of processes such as ligand binding, consequent conformation changes, and, finally, channel opening. Mutations in the predicted pore region could alter structures related to any one of these processes, altering caffeine sensitivity.

In this study, more evidence is presented for a role of the pore region in caffeine activation. Mutations I4829A and I4829T abolished caffeine-induced Ca2+/H11001 release in transfected HEK-293 cells; mutation V4823A increased caffeine sensitivity; mutations G4822A, R4824A, G4826A, and G4828A decreased caffeine sensitivity; the remaining mutants retained normal caffeine sensitivity. It is of interest that loss of caffeine occurred with every other amino acid starting from G4822, mutants G4828 and I4829 being exceptions.

Ryanodine Binding and Activation—High affinity [3H]ryanodine binding occurs in the tetrameric form of RyR1 in CHAPS, but not in the monomeric form in Zwittergent 3-14 (38). The binding site has been localized to the COOH-terminal region in the linear sequence in biochemical studies (18, 19). Studies with ryanoids have predicted that pyrrole and isoproply groups are embedded deep inside a cleft in RyR molecules (39).

Ryanodine activation of Ca2+/H11001 release channels exhibits use dependence. For example, contracture was induced in rat muscles infused with ryanodine and receiving electrical stimuli, but not in unstimulated muscles (40), indicating that ryanodine did not bind to RyR in resting muscles. Consistent with this finding, high affinity [3H]ryanodine binding to RyR is observed only when the channel is activated, indicating that ryanodine...
binds to an open state conformation of the channel. Ryanodine is known to increase \( \text{Ca}^{2+} \) permeability of the sarcoplasmic reticulum at submicromolar concentrations and to decrease \( \text{Ca}^{2+} \) permeability at higher concentrations. In single-channel recordings, ryanodine either locks the channel in an open subconductance state with about 50% of normal conductance or closes the channel (see reviews in Refs. 1–3).

In this study, 10 \( \mu \text{M} \) ryanodine did not stimulate \( \text{Ca}^{2+} \) release in HEK-293 cells expressing wt RyR2 channels. By contrast, 10 \( \mu \text{M} \) ryanodine did stimulate \( \text{Ca}^{2+} \) release in HEK-293 cells expressing mutants in amino acids 4823–4827. \( \text{Ca}^{2+} \) release occurred under resting conditions that would not favor channel opening, normally considered to be a prerequisite to ryanodine binding. This was especially surprising, since most mutations in the pore region caused loss of high affinity \( ^{3} \text{H} \)ryanodine binding to CHAPS-solubilized proteins. These results imply that the high affinity binding site in RyR2 is retained in mutants of amino acids 4823–4837, but with possible alterations in affinity or dissociation rate that were not measurable in our binding assay. One exception is mutant G4826A, which possessed a normal \( K_d \) for \( ^{3} \text{H} \)ryanodine binding but gained the ryanodine activation function.

In order to exclude a possible effect of CHAPS, we tested \( ^{3} \text{H} \)ryanodine binding to isolated microsomes and to whole cells with plasma membranes either intact or permeabilized with saponin, in the presence and absence of 10 mM caffeine. No \( ^{3} \text{H} \)ryanodine binding to whole cells or to microsomes was observed for any of the mutants previously found to lack \( ^{3} \text{H} \)ryanodine binding, but binding was consistently positive for wild type (data not shown).

These observations raise the possibility that the pore region is involved in high affinity ryanodine binding in RyR. However, this region is also highly conserved among IP_3 receptors, which do not bind ryanodine. Thus, these amino acids are probably essential, but not the sole components of the high affinity ryanodine binding site. The location of these residues is consistent with the finding that the high affinity ryanodine and perhaps the low affinity binding sites are located in the COOH-terminal 76 kDa of the RyR molecule (18, 19).

**Ryanodine Restoration of Caffeine-induced \( \text{Ca}^{2+} \) Release**—The addition of 10 \( \mu \text{M} \) ryanodine to wt RyR2 did not inhibit caffeine-induced \( \text{Ca}^{2+} \) release in transfected HEK-293 cells. However, the addition of ryanodine did inhibit the amplitude of \( \text{Ca}^{2+} \) release induced by subsequent additions of caffeine, eventually leading to abolition of \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release. This pattern is believed to result from the fact that ryanodine binds to the open state of the channel, induced by caffeine, and blocks the channel in an open subconductance state, preventing the accumulation of a \( \text{Ca}^{2+} \) store. This behavior pattern was shared with mutants I4829V and G4830A. Mutants I4829A and G4829T, however, exhibited a different pattern. Neither 10 \( \mu \text{M} \) caffeine nor 10 \( \mu \text{M} \) ryanodine induced \( \text{Ca}^{2+} \) release when these mutants were transfected into HEK-293 cells. However, if these cells were incubated in the presence of 10 \( \mu \text{M} \) ryanodine, the subsequent addition of 10 mM caffeine restored and enhanced caffeine-induced \( \text{Ca}^{2+} \) release. When the two compounds were added simultaneously, a much slower rate of \( \text{Ca}^{2+} \) release was observed (data not shown). These results indicate that these mutant channels retain the ability to bind ryanodine (perhaps with lower affinity) and that binding of ryanodine to the molecule stabilizes a channel conformation that favors caffeine activation.

Mutation E4032A in RyR1 was found to be caffeine- and 4-chloro-\( m \)-cresol-insensitive when expressed in HEK-293 cells (20). The corresponding mutation in RyR3, E3885, was found to be more than 1000-fold less sensitive to \( \text{Ca}^{2+} \) activation than wt when subjected to single-channel analysis and was proposed to form part of the \( \text{Ca}^{2+} \) sensor site (14). Recently, prior addition of 200–500 \( \mu \text{M} \) ryanodine to cells expressing the E4032A mutant has been shown to restore caffeine and 4-chloro-\( m \)-cresol sensitivity to the mutant channel (41). These observations are similar to our observations with mutants G4828A and I4829A or I4829T. It was proposed that ryanodine binds to the E4032A mutant with low affinity and induces allosteric changes in the structure of the protein that restore caffeine activation (41). It is of interest that residues Gly4828 and Ile4829 are located in the pore region, whereas Glu4032 is located in predicted transmembrane sequence 2. If these residues interact to form a ryanodine binding site, TM2 might lie close to the pore sequence.

**Hypothesis for Ryanodine Restoration of Caffeine Activation in I4829T (CCD) Mutant**—If we consider wt and mutants I4829V and G4830A to form one functional class (class 1) and mutants G4828A, I4829A, and G4829T to form a second functional class (class 2) of RyR2 channels, we can compare several of their respective properties. Class 1 molecules have the following properties described in this paper: 1) low probability of opening in the presence of trace \( \text{Ca}^{2+} \); 2) high conductance; 3) caffeine-induced \( \text{Ca}^{2+} \) release induced by caffeine, \( \text{Ca}^{2+} \), and ATP and inhibition by Mg\(^{2+}\) and inhibition of the channel by EGTA; 5) high affinity \( ^{3} \text{H} \)ryanodine binding; 6) modulation of channel opening by low concentrations of ryanodine; 7) lack of ryanodine activation of caffeine-induced \( \text{Ca}^{2+} \) release. By contrast, class 2 mutants have the following, largely opposite characteristics: 1) high probability of opening in the presence of trace \( \text{Ca}^{2+} \); 2) ~25% reduced conductance; 3) lack of caffeine induction of \( \text{Ca}^{2+} \) release in HEK-293 cells; 4) lack of activation of channel opening by caffeine, \( \text{Ca}^{2+} \), and ATP and lack of inhibition by Mg\(^{2+}\) or by EGTA; 5) lack of high affinity \( ^{3} \text{H} \)ryanodine binding; 6) lack of modulation of channel opening by low concentrations of ryanodine; 7) ryanodine activation of caffeine-induced \( \text{Ca}^{2+} \) release.

The different behavior of class 1 and class 2 mutants might be explained by a series of simple hypotheses. In single-channel analysis, which does not mimic the ionic or protein composition of whole cells and organelles, class 2 mutants have a high, largely unregulated probability of opening. If these channels were also open in HEK-293 cells, then they would not respond to caffeine activation, not necessarily because the caffeine activation site had been altered, but simply because they would have emptied \( \text{Ca}^{2+} \) stores, creating a futile cycle of \( \text{Ca}^{2+} \) uptake and release. Class 2 mutations have very low affinity for ryanodine, and we propose that class 2 mutations affect the structure of the pore in a way that leads to a low affinity interaction with ryanodine. Low affinity interactions with ryanodine may be able to block \( \text{Ca}^{2+} \) conductance, but not hold the channel in a subconductance state. We propose that, in the cellular environment, the low affinity binding of ryanodine to open mutant channels leads to their full or partial blockage. Blockage of these channels by ryanodine would then allow a \( \text{Ca}^{2+} \) store to build up, which can be released by subsequent additions of caffeine. Ryanodine might be completely displaced from its altered, lower affinity binding site by allosteric effects of caffeine activation. Alternatively, low affinity ryanodine binding might induce closure, rather than blockage of the mutant channel, and it might, through allosteric mechanisms, induce a caffeine-sensitive state. It is unfortunate that we cannot directly equate results obtained in whole cells with results obtained in single-channel analysis, probably because cellular conditions cannot be recreated in isolation. Thus, we cannot reproduce ryanodine restoration of caffeine activation.
in single channels even though the phenomenon can be observed in whole cells. These hypotheses will be pursued in future studies.

Although these hypotheses might explain class 1 and class 2 channel behavior, at least two other classes of mutants can be identified that do not fall into these categories. Mutant G4826A is similar to class 1 mutants, but has a very low conductance and displays ryanodine-induced Ca\textsuperscript{2+} release. Mutants V4823A and R4824A have very high probability of unregulated opening, like class 2 mutants, but have ryanodine-induced Ca\textsuperscript{2+} release and do not have ryanodine restoration of caffeine activation. Thus different behaviors arise from mutations in the pore region. A better understanding of the structure of the pore and mutant-induced modification of this structure would be very useful in understanding how mutations modify structure and function.

In summary, our data demonstrate that different mutations in the highly conserved sequence, GVRAGGGID\textsuperscript{4831}, in the RyR2 Ca\textsuperscript{2+} release channel can alter single-channel conductance, ion selectivity, caffeine sensitivity, high affinity [\textsuperscript{3}H]ryanodine binding, and the ability of ryanodine to modulate Ca\textsuperscript{2+} release. These results support the proposal that this region contributes to the formation of the channel pore. The fact that other mutations in the predicted transmembrane sequences in our previous study (20) and mutations in the predicted pore region in the present study abolished caffeine activation and [\textsuperscript{3}H]ryanodine binding suggests that all of these amino acids are critical to channel function and its regulation.

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