Molecular Identification of the Ryanodine Receptor Pore-forming Segment*

Communication

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A sequence motif, GXRGGXXGXD, located in the putative channel-forming domain, is conserved in all known ryanodine receptors and inositol 1,4,5-trisphosphate receptors. The functional significance of this conserved region was investigated by using site-directed mutagenesis together with functional assays consisting of Ca2+ release measurements, [3H]ryanodine binding, and single channel recordings in planar lipid bilayers. We report here that single point mutations introduced into this region of the mouse cardiac ryanodine receptor reduce or abolish high affinity [3H]ryanodine binding. Single channel analysis revealed that a single substitution of alanine for glycine 4824 within this region reduced the single channel conductance by 97%, from 798 pS to 74 pS. Single substitu-
ces for the remaining residues of this motif similarly reduced the conductance. Single point mutants of the G4824A mutant channel were modulated by Ca2+, Mg2+, ATP, caffeine, ryanodine, and ryanodine red, and ryanodine red. Co-expression of the wild type and G4824A mutant proteins produced single channels that have intermediate unitary conductances of 516, 256, 176, and 60 pS. These data suggest that this conserved region constitutes an essential part of the ryanodine binding site and the channel conductance pathway of the ryanodine receptor.

Ryanodine receptors ( RyRs) are members of a superfamily of intracellular Ca2+ channels that include the inositol 1,4,5-trisphosphate receptors (IP3Rs). These channels play an essential role in intracellular Ca2+ signaling by virtue of releasing Ca2+ from the lumen of sarco(endo)plasmic reticulum to the cytosol of muscle and non-muscle cells (1, 2).

RyR is a homotetrameric structure composed of four identical subunits, each having ~5000 amino acids. Sequence analysis reveals that one-fifth of the COOH terminus of the mole-
cule is likely to form the channel conducting pore. The remaining ~4000 amino acid residues apparently constitute the cytoplasmic “foot” domain (3–7). A truncated RyR in which the foot domain has been deleted has not shown to function as a Ca2+ release channel. The truncated RyR channel was still regulated by Ca2+, was modified by ryanodine, and exhibited a single channel conductance similar to that of the full-length RyR (8). These studies indicate that the sites for Ca2+ activation and ryanodine binding, and the ion conduction pathway are located within the COOH-terminal ~1000 amino acid residues. A glutamate residue located in the putative transmembrane sequence M2 has recently been identified as the Ca2+ sensor of RyR (9). The locations of the ryanodine binding site and the pore-forming segment of RyR, however, have yet to be defined.

RyRs and IP3Rs share some sequence homology, in particu-
lar in the COOH-terminal channel-forming domain (10). Con-
sidering their sequence homology and similar conduction properties (11–13), RyRs and IP3Rs are likely to share similar structural features in the channel pore. A hydrophobic region between the M5 and M6 transmembrane sequences of the mouse type 1 IP3R has been proposed to be the pore-forming region (14). The equivalent region in RyR, corresponding to the M9 transmembrane sequence proposed by Zorzato et al. (15), is also hydrophobic. Sequence alignment of these regions reveals a GXRGGXXGXD motif that can be found in all known RyRs and IP3Rs (Fig. 1). To investigate its role in RyR function, we have introduced point mutations into this highly conserved region and examined the functional consequences of these point mutations. Our data indicate that this region is critical for ryanodine binding and ion conduction and probably constitutes the pore-forming segment of RyRs.

Experimental Procedures

Materials—Ryanodine was obtained from Calbiochem. [3H]Ryanodine was from NEN Life Science Products. Monoclonal antibody 34C was a generous gift from Dr. John L. Sutko (16).

Cloning of the Mouse Cardiac RyR cDNA—Total RNA from mouse heart tissue, isolated by the method of Chomczynski and Sacchi (17), was used to generate first strand cDNA using the SuperScript Preamplification System (Life Technologies, Inc.) with random primers. Using degenerate primers designed on the basis of the reported cDNA sequence of the rabbit (18, 19) and human (20) cardiac RyR, we obtained six short PCR fragments corresponding to nucleotides 1171–1661, 2386–3821, 5689–6256, 11029–11646, 13366–13599, and 13473–14919 of the mouse cardiac RyR (mRyR2) cDNA. These cDNA frag-

ments were then used as probes to screen a mouse cardiac cDNA library. Four overlapping clones, corresponding to nucleotides 5’–UTR–6486, 1891–8268, 7602–13,359, and 12,991–3’–UTR, covering the entire coding region and part of the 5’- and 3’-untranslated regions of the mRyR2, were obtained. These overlapping clones were used to construct the full-length mRyR2 cDNA in the expression vector pCDNA3 (Invitrogen) for functional expression studies.

Site-directed Mutagenesis—Point mutations were carried out by the overlap extension method (21) using the PCR. The NruI (14,237)–NotI (vector) cDNA fragment that contains the 3’ end of the mRyR2 cDNA was subcloned into the pBluescript vector and was used as a template for site-directed mutagenesis. PCR products were subcloned into pBlue-

script and the sequence of each PCR clone was confirmed by DNA sequencing. The NruI (14,237)–NotI (vector) fragment was removed from the PCR clone and subcloned into the full-length mRyR2 cDNA. Transfection of HEK293 cells were carried out using Ca2+ phosphate precipitation.

Co2+ Release Measurements—Free cytosolic Ca2+ concentration in transfected HEK293 cells was measured with the fluorescence Ca2+...
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RESULTS AND DISCUSSION

We first investigated the functional consequences of single point mutations in the putative pore-forming segment (Fig. 1) by measuring caffeine-induced Ca2+ release in HEK293 cells transfected with the wild type and various mutant cDNAs. Addition of 2 mM caffeine resulted in an increase in the fluo-3 fluorescence in cells transfected with the wild type and all mutant cDNAs, but not in cells transfected with the vector DNA (Fig. 2a). These data demonstrate that all mutants can function as Ca2+ release channels. To further characterize these mutants, we carried out [3H]ryanodine binding. Fig. 2b shows that mutations at R4822, G4825, G4828, and D4829 reduced or abolished high affinity [3H]ryanodine binding, while caffeine-induced Ca2+ release from these mutants was comparable with that from the wild type. On the other hand, mutation at G4824 retained [3H]ryanodine binding, but decreased caffeine-induced Ca2+ release. Mutations at G4820 and G4826 reduced caffeine-induced Ca2+ release and abolished [3H]ryanodine binding. It should be noted that the levels of expression of the wild type and mutant RyR proteins were similar as revealed by immuno blotting (Fig. 2b). These data indicate that this conserved region is critical for ryanodine binding and channel function.

Ryanodine binds to RyRs in an open state with high affinity. The binding of ryanodine modifies channel gating and ion conduction and reduces single channel conductance of RyRs (3–7, 25). The significance of this region in ion conduction was examined by determining the effect of these mutations on single channel conductance. Fig. 3a shows single channel recordings of the wild type and mutant G4824A channels after incorporating into planar lipid bilayers at different holding potentials. The unitary conductance of the mutant G4824A channel, determined in symmetrical 250 mM KCl, was 22 ± 1.1 pS (n = 4), compared with 798 ± 17 pS (n = 3) for the wild type channels. Thus, the G4824A mutation reduced the single channel conductance of mRyR2 by 97%. To examine whether the G4824A mutant channel is still sensitive to modulators of RyR,
wild type and mutant subunits and the structural characteristics of the channel pore. We detected a total of 29 single channels, which can be separated into six groups according to their single channel conductances. Of the 29 single channels detected, one exhibited a unitary conductance of 778 pS, three 516 pS, four 256 pS, nine 176 pS, eleven 60 pS, and one 21 pS. All these single channels were sensitive to EGTA and Ca²⁺ and were modified by ryanodine (not shown). The 778- and 21-pS single channels probably represent the homotetrameric wild type and mutant G4824A channels, respectively, whereas single channels with intermediate unitary conductances are most likely represent the wild type-mutant hybrid channels. For example, the 516-pS conductance may be produced by a hybrid channel with three wild type and one mutant subunits, while the 60-pS conductance may be generated by a hybrid channel with three mutant and one wild type subunits. The 256- and 176-pS channels may be formed by two wild type and two mutant subunits with different subunit arrangements (Fig. 4a). It is of interest that these intermediate conductances can not be derived from a simple additive of the

FIG. 3. Effects of G4824A mutation on single channel function and [³H]ryanodine binding. a, single channel activities of the wild type (WT) (A) and G4824A mutant (B) mRyR2 channels were recorded in a symmetrical recording solution containing 250 mM KCl and 25 mM Heps (pH 7.4). The holding potentials are shown on the left, and base lines are indicated by a short line to the right of each current trace. Current-voltage (I-V) relationships of the wild type (C) and G4824A mutant (D) are shown. b, a single mutant G4824A channel was inhibited by the addition of 100 μM EGTA (cis) (not shown), indicating that the channel was incorporated into the bilayer with its cytoplasmic side facing the cis chamber. All subsequent additions were then made to the cis (cytoplasmic) chamber. Single channel current recordings in the presence of 127 mM free Ca²⁺, and after sequential additions (cis) of 2 mM ATP and 2 mM caffeine were made from the same channel. Current recordings in the presence of 10 μM ryanodine were obtained from a different channel. Single channel activities shown in E (control) and F (after addition of 2 mM MgCl₂) were recorded in the presence of 120 nM free Ca²⁺. The open probability (Pₒ) at each condition is indicated on the top of each panel. The holding potential was +80 mV. C, [³H]ryanodine binding to the wild type and G4824A mutant mRyR2 was carried out in the presence of 120 mM free Ca²⁺ (Cont) or plus 2.5 mM ATP, 2.5 mM caffeine, 5 mM MgCl₂, 30 μM ruthenium red, 2.5 mM EGTA, or 150 mM CaCl₂. Data shown are mean ± average error from three separate experiments.
we have mutated G4729 in RyR3, corresponding to G4824 in mRyR2, to alanine. The G4729A mutant RyR3 showed decreased caffeine-induced Ca\(^{2+}\) release and a drastic reduction in single channel conductance, but retained \[^{3}H\]ryanodine binding, similar to those observed with the G4824A mutant mRyR2 (unpublished data). Recently, a mutation, I4889T, in RyR1, corresponding to I4827 in mRyR2, has been reported to be associated with severe central core disease (28). Introduction of this mutation into the rabbit RyR1 abolished \[^{3}H\]ryanodine binding and caffeine or halothane-induced Ca\(^{2+}\) release in HEK293 cells transfected with the mutant cDNA. Co-expression of the wild type and mutant RyR1 cDNA in a 1:1 ratio produced RyR channels with normal caffeine and halothane sensitivities, but reduced levels of Ca\(^{2+}\) release and \[^{3}H\]ryanodine binding (28). One explanation for these observations is that the I4849T mutation may disrupt the channel conducton pathway (29). Mutations near this isoleucine in RyR1 have also been reported to alter both \[^{3}H\]ryanodine binding and single channel conductance (30). Taken together, we propose that this conserved region (GVRAGGGIGD) is likely to be the pore-forming segment of RyR, and that the pore-forming segments of each subunit constitute the RyR channel conducton pathway (Fig. 4b), analogous to the K\(^{+}\) channel pore (26).

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"FIG. 4. Co-expression of mutant G4824A produced single channels with intermediate conductances (a) and proposed model for the transmembrane topology and ion conduction pathway of RyR. a, HEK293 cells were transfected with 6 \( \mu \)g of wild type cDNA plus 6 \( \mu \)g of mutant G4824A cDNA. Single channel current fluctuations were recorded in symmetrical 250 mM KCl. Four distinct types of single channels with unitary conductances of 516 \( \mu \)S (B), 176 \( \mu \)S (C), and 60 \( \mu \)S (D) are shown. The holding potential was +40 mV. Base lines are indicated by a short line to the right of each current trace. Proposed subunit compositions and arrangements of hybrid channels for each observed conductance are indicated schematically on the right. Each circle represents a subunit of wild type or mutant G4824A, \( \beta \), putative transmembrane segments (four or six) of RyR are depicted as open right cylinders. The remainder of the molecule is shown as the foot structure. The amino acid sequence of the proposed pore-forming segment (P segment), depicted as a loop inserted into the membrane, is shown in single amino acid codes. M5 and M10 (15) are likely to be the transmembrane sequences that flank the P segment. The sequences of the remaining two or four transmembrane segments have yet to be defined. A model for the ion conduction pathway of the tetrameric RyR, in which the P segments of each subunit form the channel pore similar to that of the potassium channels (26), is schematically shown at the bottom."

quarter conductance of the wild type (798/4 \( \mu \)S) and mutant (224/4 \( \mu \)S) in any possible ratios for a tetramer. Therefore, it is unlikely that the RyR channel conduction pathway is formed by four individual pores from each RyR subunit. Conversely, the glycines at position 4824 and the flanking residues of each monomer may act cooperatively to form the conduction pathway or the ion selectivity filter of the tetrameric RyR.

The equivalent region of other RyR isoforms (RyR1 and RyR3) and of IP\(_{3}\)Rs is most likely to be important in determining the single channel conductance. To examine this possibility,