Cryoelectron micrograph studies recently have identified a Ca\textsuperscript{2+}-binding site in the 2,200-kDa ryanodine receptor ion channel (RyR1) in skeletal muscle. To clarify the role of this site in regulating RyR1 activity, here we applied mutational, electrophysiological, and computational methods. Three amino acid residues that interact directly with Ca\textsuperscript{2+} were replaced, and these RyR1 variants were expressed in HEK293 cells. Single-site RyR1-E3893Q, -E3893V, -E3967Q, and -E3967V variants displayed cellular Ca\textsuperscript{2+} release in response to caffeine, which indicated that they retained functionality as caffeine-sensitive, Ca\textsuperscript{2+}-conducting channels in the HEK293 cell system. Using [\textsuperscript{3}H]ryanodine binding and single-channel measurements of membrane isolates, we found that single- and double-site RyR1-E3893 and -E3967 variants are not activated by Ca\textsuperscript{2+}. We also noted that RyR1-E3893Q/E3967Q and -E3893V/E3967V variants maintain caffeine- and ATP-induced activation and that RyR1-E3893Q/E3967Q is inhibited by Mg\textsuperscript{2+} and elevated Ca\textsuperscript{2+}. RyR1-T5001A exhibited decreased Ca\textsuperscript{2+} sensitivity compared with WT-RyR1 in single-channel measurements. Computational methods suggested that electrostatic interactions between Ca\textsuperscript{2+} and negatively charged glutamate residues have a critical role in transducing the functional effects of Ca\textsuperscript{2+} on RyR1. We conclude that the removal of negative charges in the recently identified RyR1 Ca\textsuperscript{2+}-binding site impairs RyR1 activation by physiological Ca\textsuperscript{2+} concentrations and results in loss of binding to Ca\textsuperscript{2+} or reduced Ca\textsuperscript{2+} affinity of the binding site.

Ryanodine receptor ion channels (RyRs)\textsuperscript{2} release Ca\textsuperscript{2+} from intracellular Ca\textsuperscript{2+}-storing compartments to regulate multiple cellular functions (1–4). There are three mammalian RyR isoforms. RyR1 is present in skeletal muscle, RyR2 is present in heart muscle, and RyR3 is present at low levels in many tissues, including brain and slow-twitch skeletal muscle. Calcium ions play a predominant role in the regulation of RyRs. Activation by micromolar Ca\textsuperscript{2+} and inhibition by millimolar Ca\textsuperscript{2+} suggest the presence of high-affinity Ca\textsuperscript{2+} activation and low-affinity Ca\textsuperscript{2+} inactivation sites. Additional regulation is mediated by ATP and caffeine, which increase Ca\textsuperscript{2+}-gated RyR activities. Mg\textsuperscript{2+} inhibits Ca\textsuperscript{2+}-activated RyRs by competing with Ca\textsuperscript{2+} for high-affinity Ca\textsuperscript{2+} activation sites and by binding to inhibitory low-affinity divalent cation sites (3).

Cryoelectron microscopy studies have provided detailed information about closed and open structures of the 2,200-kDa RyRs (5–12). Des Georges et al. (9) determined the location of RyR1-binding sites for endogenous channel activators Ca\textsuperscript{2+} and ATP and the exogenous activator caffeine (Fig. 1). Ca\textsuperscript{2+} or ATP/caffeine alone induced structural changes in the large cytosolic domain and primed RyR1 to nearly full open conformation in the presence of the three channel activators (9).

Three conserved amino acid residues contribute to the primary coordination sphere of bound Ca\textsuperscript{2+} in the large cytoplasmic domain of RyRs. Thr-5001 is conserved in RyRs, whereas RyR1 residues Glu-3893 and Glu-3967 are conserved in both RyRs and the related inositol trisphosphate receptor family members. The RyRs and IP\textsubscript{3} receptors are expressed in intracellular membrane compartments containing up to millimolar concentrations of bound and free Ca\textsuperscript{2+}. The release of Ca\textsuperscript{2+} is triggered by a Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release mechanism for the cardiac and brain RyR isoforms and by the action of IP\textsubscript{3} and Ca\textsuperscript{2+} for the IP\textsubscript{3} receptors (3, 13). A distinguishing feature of skeletal muscle is that voltage-sensing L-type Ca\textsuperscript{2+} channels (DHPRs, Cav1.1s) open juxtaposed RyR1s through direct protein–protein interactions (14). Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release is regulated on a slow time scale in mammalian skeletal muscle compared with physiological rates of Ca\textsuperscript{2+} release (15, 16). This suggests that while having a predominant role in the regulation of cardiac and brain RyR isoforms, Ca\textsuperscript{2+} may have a more confined role in the activation of mammalian skeletal-muscle RyRs.

The present study tested the hypothesis that the Ca\textsuperscript{2+}-binding site identified by cryo-EM (9) has a major role in transducing the functional effects of Ca\textsuperscript{2+} in RyR1. Five single-site RyR1 variants (E3893Q, E3893V, E3967Q, E3967V and T5001A) and two double-site RyR1 variants (E3893Q/E3967Q and E3893V/E3967V)
E3967V) were expressed as caffeine-sensitive, Ca$^{2+}$-conducting channels in HEK293 cells. Studies using membrane isolates suggested that Ca$^{2+}$ did not significantly activate single- and double-site RyR1-E3893Q and -E3967 variants, whereas RyR1-T5001A exhibited altered Ca$^{2+}$-dependent regulation compared with WT. Computational methods using cryo-EM micrograph densities provided structural information on the interaction between Ca$^{2+}$ and amino acid residues of the Ca$^{2+}$-binding sites of RyR1-WT and variant channels.

Results

Three conserved amino acid residues (Glu-3893, Glu-3967, and Thr-5001) were shown previously to directly interact with Ca$^{2+}$ in a Ca$^{2+}$-binding site in RyR1 (Fig. 1) (9). In the present study, we focused on the role of the two negatively charged glutamates in regulating RyR1 activity. Negative charges were removed by replacing one or both Glu residues with Gln or Val (RyR1-E3893Q, -E3893V, -E3967Q, -E3967V, and -E3893Q/E3967V) while maintaining residue volumes (17). RyR1 Thr-5001 was mutated to Ala, reducing the size of the side chain at this position. SDS-PAGE and immunoblot analysis indicated that single- and double-site RyR1-E3893 and -E3967 variants were expressed at variable, elevated levels compared with WT and RyR1-T5001A at a level comparable with WT (Fig. 2, top and Table 1).

The expression of functional RyR1 variant channels was monitored in HEK293 cells using the RyR1 agonist caffeine and fluorescence Ca$^{2+}$ indicator Fluo-4. Millimolar caffeine activates RyR1 (18) and has little effect on Fluo-4 fluorescence in HEK293 cells transfected with the pCMV5 plasmid (Fig. 2, bottom). A variable caffeine-induced Ca$^{2+}$ release was observed in 30–60% of HEK293 cells transfected with WT-RyR1. The variable response may have resulted from uneven exposure to caffeine and/or removal of released Ca$^{2+}$ by HEK293 cellular transport systems. The number of variant HEK293 cells showing caffeine-induced Ca$^{2+}$ release ranged from 46.5% for RyR1-E3967V to 119% for RyR1-E3967Q compared with WT (Table 1). These observations suggest that all variants expressed caffeine-sensitive, Ca$^{2+}$-conducting channels in HEK293 cells.

Two methods were used to determine WT and variant activities. Regulation by Ca$^{2+}$ was directly measured using the lipid bilayer method (19) (see below). In a second widely used but less direct method, Ca$^{2+}$-dependent RyR activity was determined using the RyR-specific plant alkaloid ryanodine (20).

Specific $[^{3}H]$ryanodine binding to WT showed a bimodal Ca$^{2+}$ activation/Ca$^{2+}$ inhibition profile with peak activity at $\sim$70 $\mu$M Ca$^{2+}$ (Fig. 3). [$^{3}H$]ryanodine binding to RyR1-T5001A was lower than WT. In contrast to RyR1-WT and -T5001A, RyR1-E3893Q and -E3967Q displayed the highest [$^{3}H$]ryanodine binding levels at submicromolar Ca$^{2+}$ (Fig. 3). RyR1-E3893Q/E3967Q bound the highest amount of [$^{3}H$]ryanodine at $<1$ $\mu$M Ca$^{2+}$ ($\sim$275 fmol/mg protein), followed by E3967Q and E3893Q ($\sim$55 and $\sim$40 fmol/mg protein, respectively), E3893V ($\sim$20 fmol/mg protein), and E3967V and E3893V/E3967V variants ($\sim$11 and 15 fmol/mg protein, respectively). Elevated Ca$^{2+}$ concentrations inhibited [$^{3}H$]ryanodine binding for all variants, with $IC_{50}$ values ranging from 50 to 200 $\mu$M Ca$^{2+}$.

We considered the possibility that the different [$^{3}H$]ryanodine binding levels of the variants in Fig. 3 could be accounted for by varying levels of expression in HEK293 cells and/or recovery of functional variants in the membrane isolates. We assessed this in recording single channels using the lipid bilayer method, which directly compares WT and variant activities. RyR1s were recorded as K$^{+}$-conducting channels using 0.25 mM KCl on both sides of the bilayer at cytosolic Ca$^{2+}$ ranging from 0.01 $\mu$M Ca$^{2+}$ to 10 mM Ca$^{2+}$.

RyR1-WT showed a biphasic Ca$^{2+}$ activation/inhibition profile with a maximum averaged channel open probability ($P_{o}$) of 0.16 at $\sim$20 $\mu$M cytosolic Ca$^{2+}$ (Fig. 4). RyR1-T5001A resulted in a rightward shift of the $P_{o}$–Ca$^{2+}$ activation response curve and reduced peak $P_{o}$ at $\sim$100 $\mu$M Ca$^{2+}$ compared with WT at 20 $\mu$M Ca$^{2+}$.

In agreement with the [$^{3}H$]ryanodine-binding data of Fig. 3, RyR1-E3893Q/E3967Q was not significantly activated by Ca$^{2+}$. Averaged $P_{o}$ was 0.02 at 0.01 $\mu$M Ca$^{2+}$ (Table 1) and half-maximal levels at $\sim$6 $\mu$M Ca$^{2+}$ (Fig. 4B) suggested that binding of elevated cytosolic Ca$^{2+}$ had an inhibitory effect ($p < 0.05$). One possibility we could not rule out is that the presence of Ca$^{2+}$-inhibitory sites interfered with the activation of RyR1-E3893Q/E3967Q by the binding of Ca$^{2+}$ to low-affinity sites. RyR1-E3893Q, -E3967Q, -E3967V, and E3967V/E3967V had very low averaged $P_{o}$, values of 0.002 and less at Ca$^{2+}$ ranging from 0.01 to 2 $\mu$M (Table 1). The results suggest that the two negatively charged Glu-3893 and Glu-3967 residues have a critical role in transducing the functional effects of Ca$^{2+}$ in RyR1.

The amino acid mutations could have potentially modified the RyR1 ion permeation properties, resulting in reduced K$^{+}$ conductances and loss of Ca$^{2+}$ conductances (3). However, measurement of the voltage dependence of variant channel currents indicated that K$^{+}$ conductances were similar to WT (Table 1). In the presence of 10 mM luminal Ca$^{2+}$, RyR1-E3893Q/E3967Q and T5001A variants conducted Ca$^{2+}$ and maintained a Ca$^{2+}$/K$^{+}$ permeability ratio similar to WT. The permeability ratio of the remaining variants could not be determined due to low open channel probabilities.

Des Georges et al. (9) determined single-channel activities of the purified RyR1–FK506-binding protein 12.6 (FKBP12.6, Calstabin2) complex reconstituted in lipid bilayer vesicles. FKBP12.6 and the related FKBP12 bind with nanomolar affinity to RyRs and are considered constitutive members of RyR ion channel complexes. Dissociation of FKBP12 from the homotrameric RyR1 complex increased channel open probability and

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Figure 1. Location of Ca$^{2+}$-, ATP-, and caffeine-binding sites of open RyR1 (PDB code 5TAL). The protein structure is shown as a transparent surface. Inset, structure of Ca$^{2+}$-binding site of open RyR1 (PDB code 5TAL).
induced substates in single-channel measurements (21). In other studies, full conductances were recorded (22, 23).

We addressed the presence of RyR1–FKBP12 complex by incubating membrane isolates for 30 min with 5 μM FKBP12 before addition to the cis bilayer chamber (23). We found that treatment with FKBP12 did not significantly alter channel open probability at 2 μM cytosolic of RyR1-WT ($P_\text{o} = 0.07 \pm 0.02$ and $0.11 \pm 0.02$ with and without FKBP12, respectively, $n = 8 – 9$) and RyR1-E3893Q/E3967Q ($P_\text{o} = 0.022 \pm 0.011$ and $0.030 \pm 0.019$ with and without FKBP12, respectively, $n = 6 – 7$).

RyR1 is activated by ATP and caffeine and inhibited by Mg$^{2+}$ (3). Single-channel traces (Fig. 5A) and averaged -fold changes of $P_\text{o}$ values (Fig. 5B) show that at 0.01 μM cytosolic Ca$^{2+}$, the initial addition of 5 mM caffeine increased RyR1-WT, RyR1-E3893Q/E3967Q, and E3893V/E3967V channel activities from their low $P_\text{o}$ values (Table 1). The addition of caffeine signifi-
Activation of RyR1 by Ca\textsuperscript{2+}

Table 1
Properties of WT and variant channels

| Immunoblot intensity | Caffeine-induced Ca\textsuperscript{2+} release | Single-channel properties |
|---------------------|-----------------------------------------------|--------------------------|
|                      | % WT\textsuperscript{a} | % WT\textsuperscript{b} | \(P_e\) at 0.01 \mu M Ca\textsuperscript{2+} | \(P_e\) at 2 \mu M Ca\textsuperscript{2+} | \(p_S\) | \(P_e^{-1}/P_K\) |
| WT                  | 100 | 100 | 0.0003 ± 0.0001 (7) | 0.10 ± 0.03 (10) | 766 ± 11 (12)\textsuperscript{f} | 6.6 ± 0.2 (8)\textsuperscript{f} |
| E3893Q              | 540 ± 218 (9) | 119.0 ± 7.2 (4) | 0.0019 ± 0.0008 (4) | 0.0015 ± 0.0003 (4) | 810 ± 22 (4) | ND |
| E3893V              | 772 ± 265 (3) | 101.2 ± 11.2 (3) | 0.0009 ± 0.0007 (4) | 0.0007 ± 0.0005 (4) | 809 ± 13 (4) | ND |
| E3967Q              | 298 ± 76 (8) | 77.6 ± 9.9 (4) | 0.0005 ± 0.0003 (3) | 0.0017 ± 0.0011 (4) | 780 ± 10 (4) | ND |
| E3967V              | 494 ± 212 (9) | 46.5 ± 5.9 (5)\textsuperscript{e} | 0.0005 ± 0.0003 (4) | 0.0011 ± 0.0004 (4) | 746 ± 18 (4) | ND |
| E3893Q/E3967Q       | 207 ± 70 (10) | 105.6 ± 11.0 (4) | 0.020 ± 0.008 (10) | 0.016 ± 0.006 (10) | 790 ± 7 (5) | 6.2 ± 0.3 (4) |
| E3893V/E3967V       | 728 ± 372 (9) | 48.9 ± 11.2 (4) | 0.0004 ± 0.0002 (7) | 0.0004 ± 0.0001 (8)\textsuperscript{f} | 774 ± 24 (3) | ND |
| T5001A              | 103 ± 40 (5) | 73.5 ± 11.5 (8) | 0.0004 ± 0.0003 (3) | 0.0009 ± 0.0007 (9) | 788 ± 14 (4) | 6.1 ± 0.3 (4) |

\(\textsuperscript{a}\) Intensities of RyR1 variant bands on immunoblots were normalized to RyR1-WT intensities.

\(\textsuperscript{b}\) Numbers of variant cells responding to 8 mM caffeine were normalized to WT cells showing a caffeine response; for each determination, 30–50 cells were examined.

\(\textsuperscript{c}\) From Xu et al. (30).

\(\textsuperscript{d}\) \(p < 0.05\) compared with WT by Kruskal–Wallis one-way ANOVA followed by Dunn’s method.

\(\textsuperscript{e}\) \(p < 0.05\) compared with E3893Q by Kruskal–Wallis one-way ANOVA followed by Dunn’s method.

\(\textsuperscript{f}\) \(p < 0.05\) compared with E3893Q/E3967Q by Kruskal–Wallis one-way ANOVA followed by Dunn’s method.

\(\textsuperscript{g}\) Numbers of variant cells responding to 8 mM caffeine were normalized to WT cells showing a caffeine response; for each determination, 30–50 cells were examined.

\(\textsuperscript{h}\) From Xu et al. (30).

\(\textsuperscript{i}\) \(p < 0.05\) compared with E3893Q by Kruskal–Wallis one-way ANOVA followed by Dunn’s method.

| \(\text{Bound [\textbf{3}H]ryanodine (fmol/mg protein)}\) | \([\text{Ca}^{2+}]\) (\(\mu M\)) |
|-----------------|------------------------|
| 0               | 0.01 \(\times 10^6\)   |
| 100             | 0.01 \(\times 10^6\)   |
| 1000            | 1000 \(\times 10^6\)   |

Figure 3. Effects of Ca\textsuperscript{2+} on \([\textbf{3}H]\)ryanodine binding to RyR1-WT and variants. Specific \([\textbf{3}H]\)ryanodine binding to RyR1-WT and RyR1-E3893Q, -E3893V, -E3967Q, -E3967V, -E3893Q/E3967Q, -E3893V/E3967V, and -T5001A variants was determined as described under "Experimental procedures" in the absence or presence of 10 \(\mu M\) unlabeled ryanodine in 0.25 \(M\) KCl, 20 \(mM\) imidazole, pH 7.0, containing 3 \(nM\) [\textbf{3}H]ryanodine, protease inhibitors, and the indicated concentrations of free Ca\textsuperscript{2+}. Data are the mean \(\pm\) S.E. (error bars) of 4–7 experiments.

\(\text{Activation of RyR1 by Ca}^{2+}\) in the high-affinity Ca\textsuperscript{2+}-binding site were mutated and characterized. The results suggest that the high-affinity Ca\textsuperscript{2+}-binding site plays a critical role in Ca\textsuperscript{2+}-dependent activation of RyR1. Neutralization of negatively charged Glu-3893 and Glu-3967 resulted in loss of Ca\textsuperscript{2+}-dependent activation of RyR1. Loss of activation appeared to be specific because other regulatory mechanisms, such as RyR1 ion permeation, inhibition by Mg\textsuperscript{2+} and elevated levels of Ca\textsuperscript{2+}, and activation by ATP and caffeine, were maintained in single-channel measurements. RyR1-T5001A differentially affected RyR1 Ca\textsuperscript{2+} activation by shifting the Ca\textsuperscript{2+} activation/inactivation curve rightward relative to WT in single-channel measurements. Computational analysis using cryo-EM densities determined the structure of the Ca\textsuperscript{2+}-binding sites of nominally Ca\textsuperscript{2+}-free and Ca\textsuperscript{2+}/ATP/caffeine-activated variant channels.

The Ca\textsuperscript{2+}-binding site is located in the large cytoplasmic side of RyR1 \(\sim 70\) nm from the transmembrane effector channel sites. This suggests that transition from the nominally Ca\textsuperscript{2+}-free closed to the Ca\textsuperscript{2+}-activated open channel involves additional sites. RyR1-E4032A mutation outside the Ca\textsuperscript{2+}-binding site (9) exhibited a reduced Ca\textsuperscript{2+}-dependent channel activity in lipid bilayers (24) and depolarization-induced Ca\textsuperscript{2+} transients in myotubes (25). Activation of the RyR1-\(\Delta\)183–4006 deletion variant by micromolar Ca\textsuperscript{2+} suggested a Ca\textsuperscript{2+}-activation site different from the one identified by cryo-EM (26). Other regions shown to be involved in activation and inactivation of RyR1 by Ca\textsuperscript{2+} include residues in the transmembrane helix S2 (27), S4–S5 linker (28, 29), and pore-lining S6 helix of skeletal-muscle and cardiac-muscle RyR isoforms (30, 31).

\(\text{Ca}^{2+}\)-induced Ca\textsuperscript{2+} release has been suggested to contribute little to the depolarization-induced Ca\textsuperscript{2+} release in adult mammalian skeletal muscle (15, 16). This raises the question of the physiological significance of Ca\textsuperscript{2+} activation of RyR1 seen with isolated RyR1s. We reported that the RyR1-G4941K mutation...
Activation of RyR1 by Ca$^{2+}$

Figure 5. Effects of caffeine, ATP, and Mg$^{2+}$ on RyR1-WT and E3893Q/E3967Q and E3893V/E3967V variant channel open probabilities in the absence of cytosolic Ca$^{2+}$. A and C, representative single-channel currents were shown at 20 mV as downward deflections from the closed state (c−) in symmetrical 0.25 mM KCl with 2 μM SR luminal Ca$^{2+}$, 0.01 μM cytosolic Ca$^{2+}$2− and the indicated cytosolic Ca$^{2+}$2− concentrations. B, Ca$^{2+}$−dependence of RyR1-WT and RyR1-T5001A and E3893Q/E3967Q variant channel open probabilities in the presence of ATP/caffeine, and the presence of Ca$^{2+}$−in−

The pore-lining S6 helix increased RyR1 sensitivity to luminal Ca$^{2+}$ (30). This suggested that luminal Ca$^{2+}$ activates RyR1 by accessing the cytosolic Ca$^{2+}$−binding site in the open channel. Thus an intriguing possibility is that in skeletal muscle, voltage−dependent activation of Cav1.1 renders the Ca$^{2+}$−binding site accessible to SR luminal Ca$^{2+}$−passing through the channel and amplifies depolarization−induced Ca$^{2+}$ release via a Ca$^{2+}$−induced Ca$^{2+}$ release mechanism.

Murayama et al. (32) reported results using RyR2 variants that correspond to RyR1-E3893 and E3967 while this paper was under revision. Alanine substitution of glutamates caused loss of [3H]ryanodine binding to RyR2-E3847D and E3921D. Decreasing size but not charge resulted in minimal [3H]ryanodine binding to RyR2-E3847I, whereas RyR2-E3921D had a Ca$^{2+}$−binding profile similar to RyR2-WT. The results suggest that, as in RyR1, the corresponding Ca$^{2+}$−binding site of RyR2 has a critical role in the regulation by Ca$^{2+}$.

Replacement of negatively charged glutamate residues Glu-3893 and Glu-3967 with glutamine and valine resulted in loss of Ca$^{2+}$−dependent activation of RyR1. Glu, Gln, and Val residues have a similar residue volume of 140, 147, and 139 Å$^{3}$, respectively (17), suggesting that electrostatic interactions between Ca$^{2+}$−and Glu residues contributed to transducing the functional effects of Ca$^{2+}$.

We determined single-channel and structural properties of the Ca$^{2+}$−binding site of RyR1-T5001A, E3893Q/E3967Q, and E3893V/E3967V variants under conditions similar to those reported by des Georges et al. (9). Single-channel activities and structure of the purified RyR1−FKBP12.6 (Calstabin2) complex were determined in the absence of Ca$^{2+}$, the presence of Ca$^{2+}$, the presence of ATP/caffeine, and the presence of Ca$^{2+}$/ATP/caffeine (9). Cryo−EM data sets were divided into four classes in an attempt to account for conformational heterogeneity. Ca$^{2+}$ or ATP/caffeine alone resulted in constricted closed−pore conformations comparable with the Ca$^{2+}$−free closed states, even though a partial opening of channels was observed in the presence of 30 μM cytosolic Ca$^{2+}$ or 2 mM ATP/5 mM caffeine in lipid bilayer studies. In contrast, in the combined presence of the three channel activators Ca$^{2+}$, ATP, and caffeine, channels were nearly fully activated in single−channel recordings. Analysis of cryo−EM micrographs of Ca$^{2+}$/ATP/caffeine−activated
Activation of RyR1 by Ca^{2+}

channels yielded two conformations with a dilated pore (class 1 and 2, PDB code 5TAL) and two with a constricted pore (class 3 and 4, PDB code 5TAQ), which suggested an equal number of open and closed channels. The data suggested that Ca^{2+} or ATP/caffeine alone primed RyR1 to open in the presence of the three channel activators (9). An alternative explanation was that in the presence of Ca^{2+} or ATP/caffeine, the number of open channels was too low to be scored as a separate class. In the combined presence of the three channel activators, the number of open channels was proposed to increase, as the equilibrium of channel openings/closings shifted toward the open state(s). In the RyR1-E3893Q/E3967Q variant channel open probabilities indicated that the T5001A mutation caused secondary structural changes in the presence of the three activating ligands Ca^{2+}, ATP, and caffeine. Together, the results suggest that electrostatic interactions between Ca^{2+} and glutamate residues of the RyR1 Ca^{2+}-binding site have a major role in transducing the functional effects of Ca^{2+} in RyR1.

In conclusion, our studies show that the removal of negative charges in a RyR1 Ca^{2+}-binding site impairs activation of RyR1 by physiological concentrations of Ca^{2+} and suggests loss of binding to or reduced Ca^{2+} affinity of the site. Ca^{2+} binding to inhibitory sites is expected to interfere with any low-affinity Ca^{2+} activation of RyR1. Hence, a more detailed understanding of the mechanism(s) resulting in dysfunctional Ca^{2+} activation may depend on identifying and eliminating low-affinity RyR1 Ca^{2+}-inactivation sites.

Experimental procedures

**Materials**

[^3]Ryandine was obtained from PerkinElmer Life Sciences, protease and phosphatase inhibitors from Sigma-Aldrich, and phospholipids from Avanti Polar Lipids.
Preparation of variant channels

RyR1-E3893 and -E3967 single- and double-site variants were prepared using Pfu polymerase-based chain reaction, mutagenic oligonucleotides, and the QuikChange II site-directed mutagenesis kit (Agilent, Santa Clara, CA). RyR1-T5001A was prepared using a gene synthesis method (Genewiz, Inc., South Plainfield, NJ). WT and variant RyR1s were transiently expressed in HEK293 cells using jetPRIME (Polyplus, New York) according to the manufacturer’s instructions. Transfected cells were harvested, and crude membrane isolates were prepared as described (33) in the presence of 1 mM GSSG.

SDS-PAGE and immunoblot analyses

Proteins in crude membrane isolates (20 μg of protein/lane) were separated using 3–12% acrylamide gradient SDS-PAGE, transferred overnight to nitrocellulose membranes, and probed using primary rabbit anti-RyR1 polyclonal antibody 6425 (30). Immunoblots were developed using peroxidase-conjugated anti-rabbit IgG, enhanced chemiluminescence, and quantified using the Bio-Rad ChemiDoc MP Imaging System and ImageQuantTL analysis software. Intensity of RyR1 variant bands on immunoblots was normalized to RyR1-WT intensities.

Cellular Ca^{2+} release

Release of stored Ca^{2+} was determined as described (34). Briefly, Ca^{2+} transients in HEK293 cells grown on coverslips were monitored with the fluorescence Ca^{2+} indicator Fluo-4. Cellular Ca^{2+} release was induced by the addition of ~8 mM caffeine and monitored in individual cells using the EasyRatioPro algorithm (Photon Technology International, Lawrenceville, NJ).

[^H]Ryanodine binding

Ryanodine binds with high specificity to RyR1 and is widely used to probe RyR activity and content (20). [^H]Ryanodine binding was measured by incubating crude membrane isolates for 20–24 h at 24 °C in 20 mM imidazole, pH 7.0, 0.25 M KCl, 3 mM [^H]ryanodine, and protease inhibitors at the indicated free Ca^{2+} concentrations. Non-specific binding was determined in the presence of 100 μM unlabeled ryanodine.

Figure 7. Interactions of Ca^{2+} with RyR1 residues in multiple functional states. Shown are the predicted interactions of Ca^{2+} with RyR1-WT and RyR1-E3893Q/E3967Q, E3893V/E3967V, and T5001A variants under nominally Ca^{2+}-free (PDB code 5TB0) and Ca^{2+}/ATP/caffeine (PDB code 5TAQ and 5TAL) conditions. Residues displaying electrostatic interactions with Ca^{2+} in the Ca^{2+}/ATP/caffeine state are depicted in a stick representation, and the backbone of RyR1 is shown in a ribbon representation. Ca^{2+} is shown as a green sphere in Ca^{2+}-bound states and as a hollow sphere in Ca^{2+}-free states. Strong electrostatic interactions (d < 3.5 Å) are shown as blue dashed lines, and weak electrostatic interactions (3.5 < d < 4 Å) are shown as red dashed lines between Ca^{2+} and RyR1 residues. Distances less than 3.5 Å formed interresidue contacts and are shown as blue dashed lines.
Activation of RyR1 by Ca\(^{2+}\)

Table 2

Interactions between Ca\(^{2+}\) and amino acids

| System                  | Variant | Ca\(^{2+}\) interactions | Distance (Å) | as interactions |
|-------------------------|---------|--------------------------|--------------|-----------------|
| EGTA (closed)           | WT      | Nil                      | Nil          | Nil             |
|                         | E3893Q/E3867Q | Nil                         | Q3893–T5001 |                 |
|                         | E3895V/E3867Y | Nil                      | Nil          |                 |
|                         | T5001A   | Nil                      | Nil          |                 |
| Ca/ATP/caffeine (closed)| WT      | E3893 (OE1)              | 2.4          | Nil             |
|                         |         | E3893 (OE2)              | 2.1          |                 |
|                         |         | E3893 (O)                | 2.8          |                 |
|                         |         | E3897 (OE1)              | 2.5          |                 |
|                         |         | E3897 (OE2)              | 2.2          |                 |
|                         |         | T5001 (O)                | 2.6          |                 |
|                         | E3893Q/E3867Q | Q3893 (O)                | 3.8          | Q3893–T5001     |
|                         |         | T5001 (O)                | 2.6          | Q3897–T3966     |
|                         | E3893V/E3867V | V3893 (O)                | 3.8          | Q3897–N3963     |
| Ca/ATP/caffeine (open)  | WT      | E3893 (OE1)              | 2.3          | Nil             |
|                         |         | E3893 (OE2)              | 2.2          |                 |
|                         |         | E3897 (OE1)              | 2.4          |                 |
|                         |         | E3897 (OE2)              | 2.4          |                 |
|                         |         | T5001 (O)                | 2.5          |                 |
|                         | E3893Q/E3867Q | Q3893 (O)                | 3.3          | Q3893–N3889     |
|                         |         | Q3897 (OE1)              | 2.3          |                 |
|                         |         | T5001 (O)                | 2.5          |                 |
|                         | E3893V/E3867V | V3893 (O)                | 3.9          | Nil             |
|                         |         | T5001 (O)                | 2.5          |                 |
|                         | T5001A   | E3893 (OE1)              | 2.3          | Nil             |
|                         |         | E3893 (OE2)              | 2.2          |                 |
|                         |         | E3897 (OE1)              | 2.4          |                 |
|                         |         | E3897 (OE2)              | 2.4          |                 |
|                         |         | A509 (O)                 | 2.5          |                 |

The presence of 10 μM unlabeled ryanodine. Amounts of bound [\(^{3}\text{H}\)] ryanodine were determined using a filtration assay (30). Single-channel recordings

Membrane isolates were added to the cis (cytosolic) chamber of the bilayer apparatus (35). Single-channel recordings took advantage of the impermeability of RyRs to Cl\(^{-}\) and high conductance of K\(^{+}\) relative to Ca\(^{2+}\). Channel activities were recorded using 0.25 M KCl, 20 mM KHEPES, pH 7.4, on both sides of the bilayer, 2 μM trans (SR luminal), and the indicated cis (cytosolic) Ca\(^{2+}\) concentrations. The trans side of the bilayer was defined as ground. Electrical signals were filtered at 2 kHz, digitized at 10 kHz, and analyzed at 50% threshold setting (35). Data acquisition and analysis of 2-min recordings were performed using commercially available software (pClamp, Axon Instruments). Channel activities were also recorded in symmetrical 0.25 M KCl solution with 10 mM Ca\(^{2+}\) in the trans bilayer chamber. The reversal potential was measured to determine Ca\(^{2+}\)/K\(^{+}\) permeability ratios using a modified form of the Goldman–Hodgkin–Katz equation (35).

Computational methods

The effects of RyR1 mutations on Ca\(^{2+}\) binding were modeled by performing in silico amino acid substitutions at chosen positions in three RyR1 cryo-EM structures solved under different physiological conditions (PDB entries 5TB0, 5TAQ, and 5TAL) (9). The position of Ca\(^{2+}\) in the Ca\(^{2+}\)-free RyR1 structure (PDB code 5TB0) was simulated by superposing the closed RyR1 structure (PDB code 5TAQ). In silico mutations at chosen residue positions in RyR1 structures were performed using the Mutagenesis tool in the PyMOL molecular visualization suite (36). We chose side-chain rotamers for substituted amino acids at selected positions based on backbone dependence and minimum clash score. For further refinement, side-chain optimizations were executed on mutated RyR1 structures using GROMACS version 4 (37). Optimized structures were visualized and analyzed for loss or gain of Ca\(^{2+}\) interactions with surrounding residues using PyMOL (36).

Biochemical assays and data analysis

Free Ca\(^{2+}\) concentrations were obtained by including in the solutions the appropriate amounts of Ca\(^{2+}\) and EGTA using a Ca\(^{2+}\)-selective electrode. Free Ca\(^{2+}\) concentrations following the addition of 2 mM ATP (Fig. 6) were calculated using MaxChelator and constants from Theo Schoenmakers’ Chelator. Differences between samples were analyzed using SigmaPlot 11 Statistics. Comparison of two groups was determined by Student’s t test or Mann–Whitney rank sum test (when data failed the normality test). Three sample groups or more were determined by one-way ANOVA with Tukey’s test, Kruskal–Wallis one-way ANOVA on ranks followed by Dunn’s method (when the normality test failed in one-way ANOVA), or two-way ANOVA using the Holm–Sidak method, where \(p < 0.05\) was considered significant.

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