Molecular Identification of the Ryanodine Receptor Ca\(^{2+}\) Sensor*

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We have investigated the molecular basis for ryanodine receptor (RyR) activation by Ca\(^{2+}\) by using site-directed mutagenesis together with functional assays consisting of Ca\(^{2+}\) release measurements and single channel recordings in planar lipid bilayers. We report here that a single substitution of alanine for glutamate at position 3885 (located in the putative transmembrane sequence M2 of the type 3 RyR) reduces the Ca\(^{2+}\) sensitivity, as measured by single channel activation, by more than 10,000-fold, without apparent changes in channel conductance and in modulation by other ligands (e.g. ATP and ryanodine). Co-expression of the wild type and mutant RyR proteins results in the synthesis of single channels that have intermediate Ca\(^{2+}\) sensitivities. These results suggest that the glutamates at position 3885 of each monomer may act in a coordinated way to form the Ca\(^{2+}\) sensor in the tetrameric structure corresponding to RyR.

Ryanodine receptors (RyRs) are a family of Ca\(^{2+}\) channels which mediate intracellular Ca\(^{2+}\) release that is essential for a variety of cellular functions including muscle contraction, egg fertilization, and synaptic transmission (1, 2). Three RyR isoforms (RyR1, RyR2, and RyR3) have been identified in mammalian tissues; all three are activated by Ca\(^{2+}\) (3–8). Activation of RyR by Ca\(^{2+}\) is the mechanism underlying Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the sarcoplasmic reticulum (9–11).

Of the many ligands known to modulate the activity of RyR, Ca\(^{2+}\) is the essential regulator. Most other ligands exert their effect on RyR activity by influencing the Ca\(^{2+}\) sensitivity of RyR (3–8, 12). Alterations in the Ca\(^{2+}\) sensitivity of RyR have been implicated in at least one disease, malignant hyperthermia (13). Thus, understanding the molecular mechanism that controls the Ca\(^{2+}\) sensitivity is fundamental to the understanding of RyR regulation and intracellular Ca\(^{2+}\) signaling.

RyR activation by Ca\(^{2+}\) is thought to be mediated by high affinity Ca\(^{2+}\) binding sites in the protein (14), but the molecular identity of these Ca\(^{2+}\) activation sites, the Ca\(^{2+}\) sensor, has yet to be defined. It has been shown that negatively charged residues within a transmembrane sequence are often involved in binding and translocation of cations across the membrane (15–17). Analysis of the amino acid sequences of RyRs reveals that of the 12 predicted transmembrane sequences of RyR (18), four (M1, M2, M7, and M10) contain negatively charged amino acid residues that are conserved in all known RyR isoforms (Fig. 1A) (19–26). To investigate their roles in RyR function, we have mutated these negatively charged residues in the rabbit type 3 RyR. The functional consequence of one of these point mutations, a glutamate-to-alanine mutation at position 3885 (E3885A) located in the M2 transmembrane sequence (Fig. 1B), was assessed. Our results demonstrate that glutamate 3885 plays an essential role in determining the Ca\(^{2+}\) sensitivity and provide important new insights into the Ca\(^{2+}\)-sensing mechanism of RyR.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis—** Cloning and sequencing of the rabbit uterus RyR3 cDNA have been described previously (27). Substitution of alanine for glutamate 3885 (E3885A) was carried out by the overlap extension method (28) using polymerase chain reaction (PCR). The “outer” two oligonucleotides used were: forward, 5’-TACTCCAGATGATGAG-3’; and reverse, 5’-TCCATGGCCCTTCTGGAAATTC-3’. The oligonucleotides for the E3885A mutation were: forward, 5’-CCTCCTGGCAAGGAGGT-3’; and reverse, 5’-ACATCCCCGTGGAGG-3’. The sequences of the PCR products were confirmed by DNA sequencing. The ApoI (11,469)–RcoI (11,833) fragment was removed from the PCR product and subcloned into the SpeI (10,590)–SpeI (12,795) subfragment and subsequently into the full-length RyR3 cDNA. Transfection of HEK293 cells were carried out using Ca\(^{2+}\)-phosphate precipitation.

**Ca\(^{2+}\) Release Measurements—** Free cytosolic Ca\(^{2+}\) concentration in HEK293 cells was measured with the fluorescence Ca\(^{2+}\) indicator dye fluo-3 (97). Cells grown for 16 h after transfection were three times with KRH buffer without MgCl\(_2\) and CaCl\(_2\) (KRH buffer: 125 mM NaCl, 5 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 6 mM glucose, 1.2 mM MgCl\(_2\), 2 mM CaCl\(_2\), and 25 mM Hepes, pH 7.4) and incubated in the same buffer at room temperature for 30 min and at 37 °C for 30 min. After being detached from culture dishes by pipetting, cells from two 100-mm tissue culture dishes were collected by centrifugation at 2,500 rpm for 2 min in a Beckman Ti70 and loaded. Cell pellets were suspended at 5 μM fluo-3 AM in Dulbecco’s modified Eagle’s medium at room temperature for 30 min followed by washing with KRH buffer three times and resuspended in 150 μl of KRH buffer plus 0.1 mg/ml bovine serum albumin and 250 μM sulpfynylpyrazone. The fluo-3-loaded cells (150 μl) were added to 2 ml (final volume) of KRH buffer in a cuvette. Fluorescence intensity of fluo-3 at 530 nm was measured in an SLM-Aminco series 2 luminescence spectrometer with 480 nm excitation at 25 °C (SLM Instruments, Urbana, IL).

**RyR Purification and Single Channel Recordings—** Preparation of microsomal membranes from transfected HEK293 cells and purification of the expressed RyR were carried out as described previously (27). Single channel recordings were carried out using CHAPS-solubilized and sucrose density gradient-purified wild type and mutant recombinant RyRs as reported previously (27). Free Ca\(^{2+}\) concentrations were calculated using the computer program of Fabiato and Fabiato (29).

**RESULTS AND DISCUSSION**

Fig. 1C shows that addition of 2 mM caffeine to HEK293 cells transfected with the wild type cDNA caused an increase in the fluo-3 fluorescence (n = 5), similar to that observed previously (27). In contrast, the fluo-3 fluorescence in HEK293 cells transfected with mutant E3885A cDNA failed to increase in response to 2 mM caffeine but did respond to 100 nm Ca\(^{2+}\) iono-

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‡ The abbreviations used are: RyR, ryanodine receptor; PCR, polymerase chain reaction; CHAPS, 3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonic acid.
Caffeine is known to sensitize RyR to Ca$^{2+}$ (12). The lack of caffeine response suggests that the E3885A mutation may interfere with the Ca$^{2+}$ activation pathway. To examine directly whether the E3885A mutation alters Ca$^{2+}$ activation, we incorporated the recombinant E3885A mutant proteins into planar lipid bilayers and determined the response of single mutant channels to a wide range of Ca$^{2+}$ concentrations. As shown in Fig. 2A, the E3885A mutant channel was essentially closed at micromolar Ca$^{2+}$ and remained closed until the Ca$^{2+}$ concentration was raised to about 500 μM. The mutant channel was further activated by increasing the Ca$^{2+}$ concentration, but the maximal open probability was low. On the other hand, the wild type channel was activated at about 100 nM Ca$^{2+}$ and reached full activation at micromolar Ca$^{2+}$ (Fig. 2, B and C) (27). Open times of the mutant channels were short and exponentially distributed with a time constant of 0.296 ± 0.095 ms (mean ± S.D.) (n = 10) (Fig. 2D), as compared with the open time constant of 1.16 ms of the wild type channels (27). Thus the E3885A mutant channel, although still regulated by Ca$^{2+}$, differs markedly from the wild type channel in the sensitivity to Ca$^{2+}$ activation, the extent of maximal Ca$^{2+}$ activation, and the gating kinetics.

To examine whether the mutant channel is still sensitive to other modulators of RyR, we assessed the effect of ATP, caffeine, and ryanodine on the single channel activity of the mutant channel (Fig. 2, E and F). In the presence of 1 mM cytoplasmic Ca$^{2+}$, the mutant channel was activated by the addition of 2 mM ATP (n = 3). The ATP-activated channel was inhibited by subsequent addition of EGTA. The mutant channel was also activated by caffeine and was sensitive to ryanodine (n = 3). The mutant channel exhibited a linear I-V relationship similar to that of the wild type (Fig. 2G). The unitary conductance of the mutant channel, determined in the presence of both ATP and caffeine, was 806 ± 16 pS (n = 4), compared with 777 pS of the wild type (27). These data suggest that the E3885A mutation does not cause gross alterations in channel function.

The E3885A mutation thus appears to alter specifically the Ca$^{2+}$ activation of the channel. Quantification of the extent of alteration in Ca$^{2+}$ activation of the mutant channel was difficult because of its low open probability. To overcome this problem, we assessed the relative sensitivity to activation by Ca$^{2+}$ of the mutant channel in the presence of 2 mM ATP and 4 mM caffeine. Under these conditions, the mutant channel was activated at micromolar Ca$^{2+}$, and it was fully activated at about 150–200 μM Ca$^{2+}$ (Fig. 3A). In comparison, under the same conditions, the wild type channel was activated at less than 1 nM Ca$^{2+}$, and it was fully activated at about 15–20 nM Ca$^{2+}$ (Fig. 3B). The Hill equation was used to fit the data obtained from two wild type and nine mutant single channels (Fig. 3C). This analysis revealed that half-maximal activation was achieved at a Ca$^{2+}$ concentration of 61.4 μM for the mutant channels and 4.7 nM for the wild type channels. Thus the sensitivity to Ca$^{2+}$ activation of the mutant channel is more than 10,000-fold lower than that of the wild type.

Based on the 10,000-fold difference in Ca$^{2+}$ sensitivity and the threshold of the wild type channel (about 100 nM) (27), the threshold for Ca$^{2+}$ activation of the E3885A mutant in the absence of ATP and caffeine would be about 1 μM. The high threshold for Ca$^{2+}$ activation of the mutant channel is close to the threshold for Ca$^{2+}$ inactivation (about 1 mM) (27). The very low activation by Ca$^{2+}$ of the mutant channel (Fig. 2, A and C) is, therefore, most likely because of simultaneous inactivation by Ca$^{2+}$. This high threshold may also explain why we could not detect caffeine-induced Ca$^{2+}$ release in E3885A-transfected HEK293 cells where the resting cytoplasmic Ca$^{2+}$ concentration is about 100 nM, although mutant channels in the lipid bilayers proved to be sensitive to caffeine. Taken together, these results indicate that glutamate 3885 is essential in determining the sensitivity of RyR to activation by Ca$^{2+}$.

The mechanism by which substitution of a single amino acid residue results in a more than 10,000-fold change in the Ca$^{2+}$ sensitivity of RyR is not clear. One possibility is that the four glutamates in M2 form a sensor in the homotetrameric RyR. To examine this possibility, we co-expressed the wild type and mutant cDNA in HEK293 cells and determined caffeine-induced Ca$^{2+}$ release. We reasoned that co-expression of the wild type and mutant

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**Fig. 1. A schematic diagram of the proposed transmembrane topology of RyR (A), amino acid sequences of the M2 segment of different RyR isoforms (B), and effect of caffeine on intracellular Ca$^{2+}$ release in transfected HEK293 cells (C).**

(A) A, up to 12 transmembrane sequences have been predicted (M', M', M1–M10) (18). The four negatively charged amino acid residues located in the transmembrane sequences M1, M2, M7, and M10 that are conserved in all known RyRs are depicted by single-letter amino acid codes. The remainder of the molecule has been predicted to form the cytoplasmic domain known as the “foot structure.” B, sequences shown are from Caenorhabditis elegans (CelRyR), Drosophila melanogaster (DroRyR), bullfrog (BfRyR2α and BfRyRβ), chicken (ChRyR3), and rabbit (RaRyR1, RaRyR2, and RaRyR3). Amino acid numbers for each RyR sequence are given at right. The conserved glutamates (E) in M2 are boxed. C, HEK293 cells were transfected with 10 μg of wild type (WT) and 10 μg of mutant E3885A cDNA, respectively. Fluorescence intensity of fluo-3-loaded cells was measured before and after sequential addition of 2 mM caffeine (solid arrows) and 100 nM Ca$^{2+}$ ionophore A23187 (open arrows).
E3885A would result in the formation of a Ca$^{2+}$ sensor with a mixture of glutamates and alanines. This hybrid Ca$^{2+}$ sensor would be expected to have reduced caffeine response and Ca$^{2+}$ sensitivity. Fig. 4A shows that the caffeine-induced Ca$^{2+}$ release in these co-transfected HEK293 cells was reduced as a result of the decreasing ratio of wild type over mutant cDNA ($n = 4$). Single-channel analysis revealed that co-transfection of HEK293 cells with a 1:1 ratio of wild type and mutant cDNA produced channels that have intermediate Ca$^{2+}$ sensitivities (Fig. 4B). Half-maximal activation of these hybrid single channels was achieved at Ca$^{2+}$ concentrations ranging from 36.6 nM to 7.5 μM. Most of the hybrid channels displayed half-maximal activation at about 288 nM Ca$^{2+}$. These different Ca$^{2+}$ responses most likely resulted from hybrid channels with different ratios of wild type and mutant subunits. Thus, these findings are consistent with the possibility that the glutamates at position 3885 of the monomers act coordinately to form the Ca$^{2+}$ sensor.

The putative transmembrane sequence M2 is highly conserved in all RyR isoforms sequenced to date (Fig. 1B). It is most likely that the corresponding glutamate in other RyR isoforms also plays a fundamental role in determining the Ca$^{2+}$ sensitivity. Further studies of the Ca$^{2+}$ sensor will provide clues to how the Ca$^{2+}$ sensitivity is modulated by various modulators and by disease states and how binding of Ca$^{2+}$ to the Ca$^{2+}$ sensor couples to channel opening. The oligomeric coordination of the Ca$^{2+}$ sensor may provide the basis for controlling and manipulating the Ca$^{2+}$ sensitivity of RyR.

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REFERENCES

1. Berridge, M. J. (1993) Nature 361, 315–325
2. Clapham, D. E. (1995) Cell 80, 259–268
3. Coronado, R., Morrissette, J., Sukhareva, M., and Vaughan, D. M. (1994) Am. J. Physiol. 266, C1655–C1564
4. Meissner, G. (1994) Annu. Rev. Physiol. 56, 485–508
5. Ogawa, Y. (1994) Crit. Rev. Biochem. Mol. Biol. 29, 229–274
6. Sorrentino, V. (1995) Adv. Pharmacol. 33, 67–90
7. Sutko, J. L., and Airey, J. A. (1996) Physiol. Rev. 76, 1027–1071
Fig. 3. Assessment of the relative Ca²⁺ sensitivity of the mutant channel. A, Ca²⁺ response of the mutant channel in the presence of ATP and caffeine. Single channel activities were first inhibited by cis addition of EGTA and reactivated by cis addition of 2 mM ATP and 4 mM caffeine. An aliquot of 100 mM EGTA or CaCl₂ solution was added to the cis chamber (cytoplasmic) to obtain various free Ca²⁺ concentrations. Single-channel current fluctuations of the ATP and caffeine-activated wild type channel were made in the presence of 1, 6, and 13 nM free caffeine. Single-channel recordings of the ATP and caffeine-activated wild type channel in the presence of 2 mM ATP and 4 mM caffeine. Data shown are obtained from two wild type channels (open circles) and nine mutant channels (solid circles). Curve fit was done using the Hill equation. The Pₒ values of the mutant channels at Ca²⁺ concentrations greater than 1 mM where Ca²⁺ inactivation was apparent were not included in the fitting.

13. Mickelson, J. R., and Louis, C. H. (1996) Physiol. Rev. 76, 537–592
14. Meissner, G., Darling, E., and Evelent, J. (1985) Biochemistry 24, 236–244
15. Clarke, D. M., Loo, T. W., Inesi, G., and MacLennan, D. H. (1989) Nature 343, 476–478
16. Heinemann, S. H., Terlau, H., Stuhmer, W., Imoto, K., and Numa, S. (1992) Nature 356, 441–443
17. Yang, J., Ellinor, P. T., Sather, W. A., Zhang, J-F., and Tsien, R. W. (1993) Nature 366, 158–161
18. Zorzato, F., Fujii, J., Otsu, K., Phillips, M., Green, N. M., Lai, F. A., Meissner, G., and MacLennan, D. H. (1990) J. Biol. Chem. 265, 2244–2256
19. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsu, H., Ueda, M., Hanoaka, M., Hirose, T., and Numa, S. (1990) Nature 349, 439–445
20. Otsu, K., Willard, H. F., Khanza, V. K., Zorzato, F., Green, N. M., and MacLennan, D. H. (1990) J. Biol. Chem. 265, 13472–13483
21. Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima, H., and Numa, S. (1990) FEBS Lett. 271, 169–177
22. Hakamata, Y., Nakai, J., Takeshima, H., and Imoto, K. (1992) FEBS Lett. 312, 229–235
23. Takeshima, H., Nishii, M., Iwabe, N., Miyata, T., Hosoya, T., Masai, I., and Hotta, Y. (1994) FEBS Lett. 337, 81–87
24. Oyama, H., Murayama, T., Takagi, T., Iino, M., Iwabe, N., Miyata, T., Ogawa, Y., and Endo, M. (1994) J. Biol. Chem. 269, 17206–17214
25. Ottini, L., Marziali, G., Conti, A., Charlesworth, A., and Sorrentino, V. (1996) Biochem. J. 315, 207–216
26. Sakube, Y., Ando, H., and Kagawa, H. (1997) J. Mol. Biol. 267, 849–864
27. Chen, S. R. W., Li, X., Ebisawa, K., and Zhang, L. (1997) J. Biol. Chem. 272, 24244–24246
28. Ho, S. N., Hunt, H. D., Morton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Amst.) 71, 51–59
29. Fabiato, A., and Fabiato, F. (1979) J. Physiol. (Paris) 75, 463–505

FIG. 4. Co-expression of mutant E3885A results in decreases in both the caffeine response and the sensitivity to activation by Ca²⁺. A, caffeine-induced Ca²⁺ release in HEK293 cells transfected with 2 μg of wild type (WT), 2 μg of wild type plus 2 μg of mutant E3885A (WT:E3885A (1:1)), or 2 μg of wild type plus 6 μg of mutant E3885A (WT:E3885A (1:3)) DNA. B, HEK293 cells were transfected with 5 μg of wild type plus 5 μg of mutant E3885A DNA (WT:Mutant). The Ca²⁺ responses of single hybrid channels were determined in the presence of 2 mM ATP and 4 mM caffeine. Solid circles indicate data obtained from a channel with relatively high Ca²⁺ sensitivity. Open squares display data from a channel with relatively low Ca²⁺ sensitivity. Open circles show data obtained from four channels with intermediate Ca²⁺ sensitivity. The curve fit of each group of data was performed using the Hill equation. The dashed curves depict the Pₒ-pCa relationship of the wild type and mutant channel in the presence of 2 mM ATP and 4 mM caffeine as shown in Fig. 3C.