Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 13, Issue of March 28, pp. 8161–8164, 1997


# A Region of the Ryanodine Receptor Critical for Excitation-Contraction Coupling in Skeletal Muscle*

(Received for publication, January 21, 1997)

**Toshiko Yamazawa, Hiroshi Takeshima, Misa Shimuta, and Masamitsu Iino‡**

*From the Department of Pharmacology, Faculty of Medicine, The University of Tokyo and CREST, Japan Science and Technology Corporation, Hongo 7-3-1, Bunkyo-ku, Tokyo 113, Japan*

**$Ca^{2+}$ release mediated by the ryanodine receptor (RyR) regulates many important cell functions including excitation-contraction (E-C) coupling in skeletal muscle, by which membrane depolarization controls the opening of RyR via the dihydropyridine receptor. Among the three RyR subtypes, RyR-1 mediates skeletal muscle E-C coupling, whereas RyR-2 and RyR-3 cannot substitute for RyR-1. We carried out expression experiments using cultured mutant skeletal myocytes not having intrinsic intracellular $Ca^{2+}$ release channels to study the structure-function relationship of amino acid residues 1303–1406 in RyR-1 (D2 region). In this region the amino acid sequences are highly divergent between RyR-1 and RyR-2, and the corresponding sequence is lacking in RyR-3. Expression of RyR-1 but not of RyR-2 rescued E-C coupling in the mutant cells. Deletion of either the entire D2 region or its N-terminal half from RyR-1 preserved the function of RyR-1 as a $Ca^{2+}$ release channel but resulted in the loss of E-C coupling. Substitution of the D2 region for the corresponding sequence of RyR-2 had no effect on the function of RyR-1. These results indicate that the presence of the D2 region is critical for E-C coupling in skeletal muscle, although the D2 region alone cannot determine the functional difference between RyR-1 and RyR-2.**

The ryanodine receptors (RyRs)[1] are a family of channels that mediate the release of intracellular $Ca^{2+}$ stores and comprise three subtypes derived from distinct genes (1–3). The function of RyRs was first recognized and most extensively studied in skeletal muscle, although the study of RyRs has been expanded to cardiac and smooth muscle cells, nerve cells, and certain nonexcitable cells. In skeletal muscle excitation-contraction (E-C) coupling, membrane depolarization reaches the interior of the cell through the transverse tubules (T-tubules), where the arrival of membrane depolarization is sensed by the dihydropyridine receptor (DHPR). A still unknown signal is then transmitted from the DHPR located on the T-tubule membrane to the RyR, which constitutes the foot structure forming a bridge between the T-tubules and the SR membrane (4, 5). Upon receipt of the signal from the DHPR, the RyR induces release of $Ca^{2+}$ from the SR (6–8). One of the characteristic features of this coupling mechanism is that it does not require the presence of extracellular $Ca^{2+}$ (9).

Two RyR subtypes, type 1 (RyR-1) and type 3 (RyR-3), are expressed in skeletal muscle cells. RyR-1 is the dominant subtype and mediates E-C coupling, whereas RyR-3 is the minor component the functions of which are still poorly understood. Indeed, targeted disruption of the gene encoding RyR-1 resulted in the loss of E-C coupling (10), whereas skeletal muscle cells in RyR-3-deficient mice show normal E-C coupling (11). Although all subtypes of RyR mediate $Ca^{2+}$-induced $Ca^{2+}$ release (CICR), neither RyR-2 nor RyR-3 is capable of substituting for RyR-1 in skeletal muscle E-C coupling (12, 13). Thus, the three subtypes of RyR have clear functional differences. Although the overall amino acid sequence identity among the RyR subtypes is 67–70% (14), there are several regions where the amino acid sequences significantly diverge. These regions are referred to as D1, D2, and D3 (15). It is an interesting possibility, therefore, that these divergence regions may correspond to the different functions of the RyR subtypes.

Among the divergence regions of the RyRs, the D2 region of RyR-1 (amino acid residues 1342–1403) is contained within the stretch of amino acid residues 1303–1406, the corresponding sequence of which is entirely absent in RyR-3 (14). In this study, we refer to the residues 1303–1406 of RyR-1 as the D2 region and examine its functional significance. First we cultured skeletal myocytes from mutant mice lacking both RyR-1 and RyR-3. The cells were then transfected with expression plasmids carrying mutated RyR-1 cDNAs with deletions or substituted with the region of RyR-2 corresponding to the D2 region to examine if the mutated RyR-1 can mediate E-C coupling. The results obtained indicate that the presence of the D2 region in RyR-1 is essential for E-C coupling in skeletal muscle but is not for the CICR *per se*.

## EXPERIMENTAL PROCEDURES

*Mutant Mice, Cell Culture, and Transfection Experiments*—The method of generation of mice carrying the disrupted RyR-1 or RyR-3 gene is described elsewhere (10, 11). Neonates simultaneously homozygous for both mutations were obtained by mating double heterozygotes. The genotypes of all the neonates used in the present experiments were determined by polymerase chain reaction analysis (10, 11). Primary cultured myocytes were prepared from newborn mice, and expression plasmids were co-transfected with the green fluorescent protein (GFP) expression plasmid (Life Technologies, Inc.) using LipofectAMINE (Life Technologies, Inc.) as described previously (13). After 3–5 days of culture in differentiation medium, myocytes were used in the experiments. Successfully transfected myocytes were identified by the fluorescence of GFP.

*Intracellular $Ca^{2+}$ Measurements*—Cultured cells were incubated in a physiological salt solution (13) containing 20 $\mu$M Fura-2 for 30–35 min. The fluorescence intensity of $Ca^{2+}$ indicator-loaded cultured cells was imaged using a cooled CCD camera (Photometrics) attached to an inverted Olympus IX70 microscope with a 40× (UPlanApo) objective. The excitation wavelength was 470 nm for GFP and 340 nm for Fura-2. Two-dimensional images composed of 128 × 128 pixels were acquired every 0.2 or 0.5 s (13).

*Construction of Expression Plasmids*—The plasmid pCAGGS (16)

* This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Mitsubishi Foundation, the Uehara Memorial Foundation, the Japan Heart Foundation, and the Naito Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 81-3-5802-8687; Fax: 81-3-3815-9360; E-mail: iino@m.u-tokyo.ac.jp.

[1] The abbreviations used are: RyR, ryanodine receptor; CICR, $Ca^{2+}$-induced $Ca^{2+}$ release; SR, sarcoplasmic reticulum; E-C coupling, excitation-contraction coupling; T-tubule, transverse tubule; DHPR, dihydropyridine receptor; GFP, green fluorescent protein.

was digested with *Hin*dIII, blunted using Klenow fragment, and self-ligated to delete the *Hin*dIII site. The resulting plasmid was cleaved with *Eco*RI and ligated to a synthetic polylinker carrying *Eco*RI, *Xho*I, *Hin*dIII, *Not*I, and *Nhe*I sites to generate the expression plasmid pCAGPL. The expression plasmid pYT-1 was constructed by inserting the 15-kilobase pair *Hin*dIII fragment from pRRS11 (17), containing the entire coding sequence of the rabbit RyR-1, into the *Hin*dIII site of pCAGPL in the same orientation with respect to the β-actin promoter. The plasmid pYT-2 was generated by deleting nucleotide residues 3907–4218 of the RyR-1 cDNA (see Ref. 18 for the residue numbers) from pYT-1; to generate the *Apa*LI site in pYT-2, a base conversion, T to A at position 4221, was introduced that causes no amino acid substitution. The plasmids pYT-3 and pYT-4 were generated by deleting residues 3907–4101 and 4069–4218 from pYT-1, respectively; base substitutions T to A at position 4104 in pYT-3, G to T at position 4068, and T to A at position 4221 in pYT-4 cause no amino acid changes. The plasmid pYT-5 was generated by inserting the fragment 3946–4200 of RyR-2 cDNA (see Ref. 19 for the residue numbers) into the *Apa*LI site of pYT-2.

## RESULTS AND DISCUSSION

*$Ca^{2+}$ Transients in Cultured Myocytes Lacking RyR-1 or RyR-3 or Both*—Myocytes isolated from the skeletal muscle of wild-type, RyR-1-deficient, and RyR-3-deficient mice as well as from double mutant mice lacking both RyR-1 and RyR-3 were cultured. The cultured cells from the wild-type and RyR-3-deficient mice responded with an intracellular $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) increase to electrical stimulation even in the absence of extracellular $Ca^{2+}$ (Fig. 1, *A* and *B*) in accordance with the results obtained in intact muscle bundles (11). However, electrical stimuli were unable to induce an increase in $[Ca^{2+}]_i$ in RyR-1-deficient and double mutant myocytes in the absence of extracellular $Ca^{2+}$ (not shown). These results confirm that RyR-1 is required for skeletal muscle E-C coupling.

Upon application of 25 mM caffeine, a $[Ca^{2+}]_i$ increase was observed in control, RyR-1-deficient, and RyR-3-deficient myocytes (Fig. 1, *A*, *B*, and *C*). However, no caffeine-induced $[Ca^{2+}]_i$ increase was observed in double mutant myocytes (Fig. 1*D*). When cyclopiazonic acid, an inhibitor of SR $Ca^{2+}$-ATPase (20), was applied to the double mutant myocytes, a $[Ca^{2+}]_i$ increase was observed in the absence of extracellular $Ca^{2+}$ (not shown). Therefore, even though $Ca^{2+}$ was stored in the SR, caffeine failed to induce $Ca^{2+}$ release in double mutant myocytes. Caffeine has been shown to induce $Ca^{2+}$ release through activation of one of the RyR subtypes (12, 21). Hence, the results obtained indicate that no RyR is expressed in the double mutant myocytes.

In our previous studies we showed that RyR-1-deficient myocytes responded with a slow $[Ca^{2+}]_i$ increase of long duration to electrical stimulation in the presence of 10 mM $Ca^{2+}$ and 10 μM Bay K-8644, a $Ca^{2+}$ channel agonist, in the extracellular solution (10, 12). However, it was not determined whether this $[Ca^{2+}]_i$ increase was due solely to an influx of $Ca^{2+}$ or to CICR from the SR secondary to the $Ca^{2+}$ influx. Here we show that upon electrical stimulation, a $[Ca^{2+}]_i$ increase that was similar in size and kinetics to that of RyR-1-deficient myocytes was induced in double mutant myocytes in the presence of the high concentration of extracellular $Ca^{2+}$ and Bay K-8644 (Fig. 1, *C* and *D*). The peak height of the Fura-2 fluorescence intensity increase relative to the resting fluorescence and the time to reach 50% of the peak height were 1.13 ± 0.02 and 0.32 ± 0.12 s for RyR-1-deficient myocytes and 1.13 ± 0.02 and 0.39 ± 0.14 s for double mutant myocytes (mean ± S.E., $n = 8$ for both cell types). There was no statistically significant difference in the $[Ca^{2+}]_i$ increase between the two types of mutant myocytes ($p > 0.3$, *t* test). These observations suggest that the electrically evoked $[Ca^{2+}]_i$ increase observed in RyR-1-deficient myocytes was due to an influx of $Ca^{2+}$ and was independent of CICR through RyR-3, the activation of which requires a relatively high $Ca^{2+}$ concentration (12).

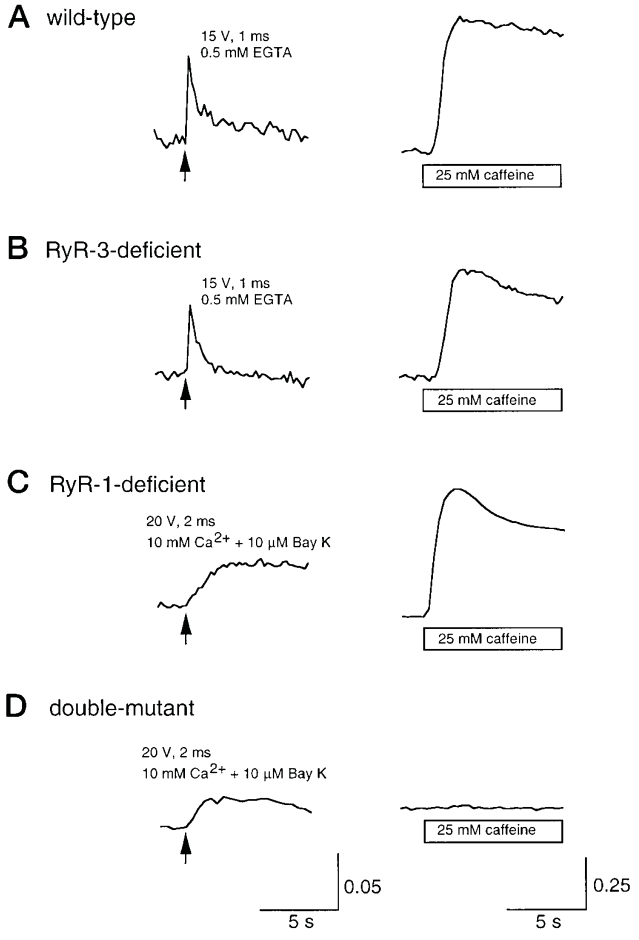


FIG. 1. **$[Ca^{2+}]_i$ responses in cultured skeletal myocytes isolated from wild-type and RyR-deficient mice.** Electrical stimulation in the absence of extracellular $Ca^{2+}$ induced a $[Ca^{2+}]_i$ increase in wild-type myocytes (*A*) and in RyR-3-deficient myocytes (*B*). The presence of extracellular $Ca^{2+}$ and Bay K-8644 was required for induction of an electrically evoked $[Ca^{2+}]_i$ increase in the RyR-1-deficient myocytes (*C*) and in the double mutant myocytes lacking both RyR-1 and RyR-3 (*D*). Note the difference in the time course of $[Ca^{2+}]_i$ changes between *panels A* and *B* and *panels C* and *D*. Caffeine (25 mM) induced a $[Ca^{2+}]_i$ increase in the wild-type and single-mutant myocytes (*A*, *B*, and *C*) but not in the double mutant myocytes (*D*). The results shown in each *panel* are representative of those obtained from more than 40 cells.

*$Ca^{2+}$ Response in Myocytes Transfected with Mutated RyR-1 cDNA*—In double mutant myocytes transfected with RyR-1 cDNA, a $[Ca^{2+}]_i$ increase was observed in response to electrical stimulation in the absence of extracellular $Ca^{2+}$ (see Fig. 3*A*). When RyR-2 cDNA was transfected in place of RyR-1, the myocytes failed to respond to electrical stimulation, although they showed spontaneous $Ca^{2+}$ oscillations (not shown). These results are in accordance with previous results obtained using RyR-1-deficient myocytes (13, 22).

To determine whether the D2 region is responsible for the functional difference among the RyR subtypes, we constructed four different expression plasmids (pYT-2, -3, -4, and -5) carrying mutated RyR-1 cDNAs. The entire D2 region (amino acid residues 1303–1406) was deleted in pYT-2, whereas either the N-terminal half (1303–1367) or the C-terminal half (1357–1406) of D2 was deleted in pYT-3 and pYT-4, respectively (Fig. 2). In pYT-5 the entire D2 region was replaced by the corresponding sequence of RyR-2 (1316–1400).

In all double mutant myocytes transfected with any of the mutated RyR-1 cDNAs, the $[Ca^{2+}]_i$ response to caffeine was recovered (Fig. 3, *B–E*). Therefore, the mutations in the D2 region had no obvious effect on the $Ca^{2+}$ release function and


FIG. 2. **Comparison of amino acid sequences of the D2 region of RyR subtypes.** The numbers at both ends of the one-letter-coded sequences indicate the amino acid residue numbers (14, 18). Amino acid residues identical to the corresponding residues in RyR-1 are indicated by *reversed letters*. Gaps (*dashes*) have been inserted to achieve maximum identity. *Overlines* indicate the sites deleted in expression plasmids pYT-3 and pYT-4. Both sites were contiguously deleted in pYT-2 or replaced by the corresponding sequence of RyR-2 in pYT-5.

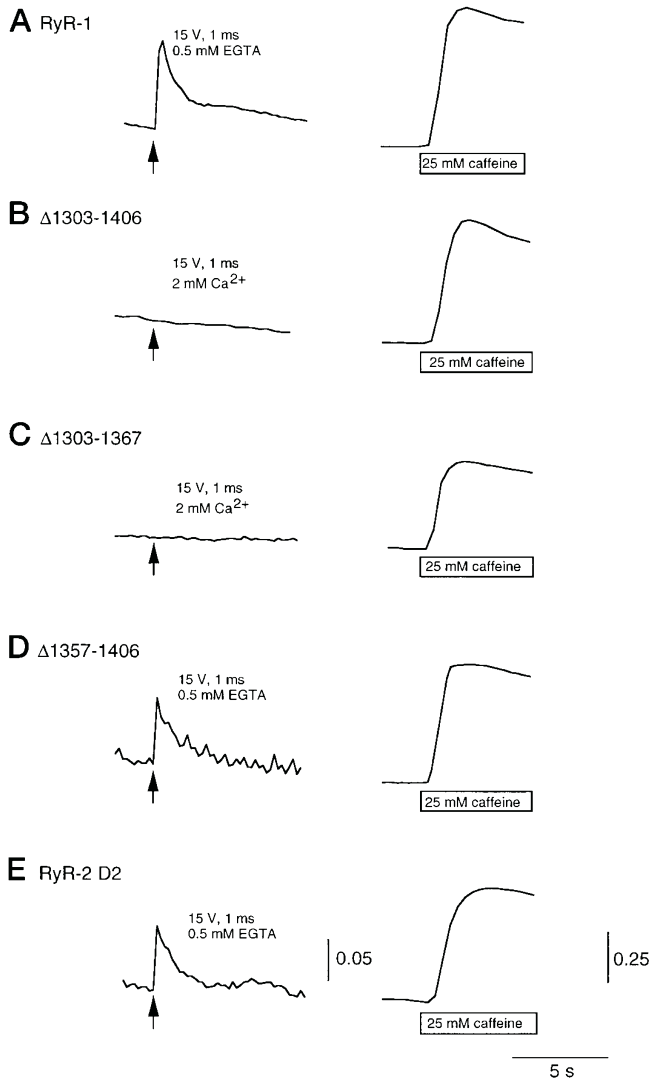


FIG. 3. **$[Ca^{2+}]_i$ responses in the double mutant myocytes transfected with mutated RyR-1 cDNAs.** Electrically evoked and caffeine-induced $[Ca^{2+}]_i$ increases were recovered in the RyR-1 cDNA transfected double mutant myocyte (*A*). Transfection of RyR-1 cDNA with the entire sequence encoding the D2 region deleted (Δ*1303–1406*, *B*) or the N-terminal half of the D2 region deleted (Δ*1303–1367*, *C*) resulted in the recovery of the caffeine-induced $[Ca^{2+}]_i$ increase but not of the electrically evoked response. When deletion was limited to the sequence encoding the C-terminal half (Δ*1357–1406*, *D*) or the entire D2 region coding sequence was replaced by the corresponding sequence of RyR-2 (*RyR-2 D2*, *E*), both caffeine-induced and electrically evoked responses were recovered after transfection with the mutated cDNA. Shown are the representative results from 3, 10, 11, 5, and 5 cells in *A*, *B*, *C*, *D*, and *E*, respectively.

responsiveness to caffeine. Double mutant myocytes transfected with either pYT-4 or pYT-5 responded with a $[Ca^{2+}]_i$ increase to electrical stimulation even in the absence of extracellular $Ca^{2+}$ (Fig. 3, *D* and *E*). However, electrical stimulation could not induce a $[Ca^{2+}]_i$ increase in myocytes transfected with pYT-2 and pYT-3 (Fig. 3, *B* and *C*). These results suggest that the presence of the D2 region in RyR-1, especially the C-terminal half of this region (amino acid residues 1303–1356), is indispensable for skeletal muscle E-C coupling. The lack of this region in RyR-3 could be one of the reasons for the inability of RyR-3 to mediate E-C coupling in skeletal muscle. The double mutant myocytes transfected with RyR-2 cDNA did not exhibit E-C coupling and showed $Ca^{2+}$ oscillations as in the equivalent experiment in RyR-1-deficient myocytes (13). However, E-C coupling in skeletal muscle was recovered when the double mutant myocytes were transfected with mutated RyR-1 cDNA carrying the sequence of the D2 region of RyR-2 (pYT-5). Therefore, the D2 region alone does not determine the functional difference between RyR-1 and RyR-2 in terms of E-C coupling.

In skeletal muscle E-C coupling, direct intermolecular coupling between the DHPR and RyR-1 is assumed to mediate the transmission of signal from the T-tubule to the SR, although the presence of an intervening molecule cannot be excluded at present. The D2 region is relatively hydrophilic (18) and overlaps with one of the protease-sensitive regions (amino acid residues 1220–1360) in RyR-1 (23). Furthermore, it has been shown that cleavage of RyR-1 within the D2 region by calpain results in enhancement of CICR activity (24–26). Therefore, the D2 region seems to be located on the cytoplasmic surface and may have important roles in the control of channel activity. Four negatively charged amino acid residues appear at every other position within the critical site of the D2 region (amino acid residues 1327–1333 in RyR-1) and are conserved between RyR-1 and RyR-2. It is an interesting possibility that the acidic region may participate in the protein-protein interaction in E-C coupling.

## REFERENCES

1. McPherson, P., and Campbell, K. (1993) *J. Biol. Chem.* **268,** 13765–13768
2. Meissner, G. (1994) *Annu. Rev. Physiol.* **56,** 485–508
3. Sutko, J. L., and Airey, J. A. (1996) *Physiol. Rev.* **76,** 1027–1071
4. Block, B., Imagawa, T., Campbell, K., and Franzini-Armstrong, C. (1988) *J. Cell Biol.* **107,** 2587–2600
5. Franzini-Armstrong, C., and Jorgensen, A. (1994) *Annu. Rev. Physiol.* **56,** 509–534
6. Adams, B. A., and Beam, K. G. (1990) *FASEB J.* **4,** 2809–2816
7. Ríos, E., and Pizarro, G. (1991) *Physiol. Rev.* **71,** 849–908
8. Schneider, M. (1994) *Annu. Rev. Physiol.* **56,** 463–483
9. Armstrong, C., Bezanilla, F., and Horowicz, P. (1972) *Biochim. Biophys. Acta* **468,** 31–50
10. Takeshima, H., Iino, M., Takekura, H., Nishi, M., Kuno, J., Minowa, O., Takano, H., and Noda, T. (1994) *Nature* **369,** 556–559
11. Takeshima, H., Ikemoto, T., Nishi, M., Nishiyama, N., Shimuta, M., Sugitani, Y., Kuno, J., Saito, I., Saito, H., Endo, M., Iino, M., and Noda, T. (1996) *J. Biol. Chem.* **271,** 19649–19652
12. Takeshima, H., Yamazawa, T., Ikemoto, T., Takekura, H., Nishi, M., Noda, T., and Iino, M. (1995) *EMBO J.* **14,** 2999–3006
13. Yamazawa, T., Takeshima, H., Sakurai, T., Endo, M., and Iino, M. (1996) *EMBO J.* **15,** 6172–6177
14. Hakamata, Y., Nakai, J., Takeshima, H., and Imoto, K. (1992) *FEBS Lett.* **312,** 229–235
15. Sorrentino, V., and Volpe, P. (1993) *Trends Pharmacol. Sci.* **14,** 98–103
16. Ishii, M., Tashiro, F., Hagiwara, S., Toyonaga, T., Hashimoto, C., Takei, I., Yamamura, K., and Miyazaki, J. (1994) *Endocr. J.* **41,** (suppl.) S9–S16

17. Penner, R., Neher, E., Takeshima, H., Nishimura, S., and Numa, S. (1989) *FEBS Lett.* **259,** 217–221
18. Takeshima, H., Nishimura, S., Matsumoto, T., Ishida, H., Kangawa, K., Minamino, N., Matsuo, H., Ueda, M., Hanaoka, M., Hirose, T., and Numa, S. (1989) *Nature* **339,** 439–445
19. Nakai, J., Imagawa, T., Hakamata, Y., Shigekawa, M., Takeshima, H., and Numa, S. (1990) *FEBS Lett.* **271,** 169–177
20. Goeger, D. E., Riley, R. T., Dorner, J. W., and Cole, R. J. (1988) *Biochem. Pharmacol.* **37,** 978–981
21. Endo, M. (1977) *Physiol. Rev.* **57,** 71–108
22. Nakai, J., Dirksen, R. T., Nguyen, H. T., Pessah, I. N., Beam, K. G., and Allen, P. D. (1996) *Nature* **380,** 72–75
23. Marks, A. R., Fleischer, S., and Tempst, P. (1990) *J. Biol. Chem.* **265,** 13143–13149
24. Rardon, D. P., Cefali, D. C., Mitchell, R. D., Seiler, S. M., Hathaway, D. R., and Jones, L. R. (1990) *Circ. Res.* **67,** 84–96
25. Iino, M., Takano-Ohmuro, H., Kawana, Y., and Endo, M. (1992) *Biochem. Biophys. Res. Commun.* **185,** 713–718
26. Shoshan-Barmatz, V., Weil, S., Meyer, H., Varsanyi, M., and Heilmeyer, L. M. (1994) *J. Membr. Biol.* **142,** 281–288