Identification of Calcium Release-triggering and Blocking Regions of the II-III Loop of the Skeletal Muscle Dihydropyridine Receptor*

(Received for publication, July 13, 1995, and in revised form, July 27, 1995)

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In an attempt to identify and characterize functional domains of the rabbit skeletal muscle dihydropyridine receptor α subunit II-III loop, we synthesized several peptides corresponding to different regions of the loop: peptides A, B, C, C1, C2, D (cf. Fig. 1). Peptide A (Thr671-Leu690) activated [H]ryanodine binding to, and induced Ca2+ release from, rabbit skeletal muscle triads, but none of the other peptides had such effects. Peptide A-induced Ca2+ release and activation of ryanodine binding were partially suppressed by an equimolar concentration of peptide C (Glu724-Pro760) but were not affected by the other peptides. These results suggest that the short stretch in the II-III loop, Thr671-Leu690, is responsible for triggering SR Ca2+ release, while the other region, Glu724-Pro760, functions as a blocker of the release trigger. A hypothesis is proposed to account for how these subdomains interact with the sarcoplasmic reticulum Ca2+ release channel protein during excitation-contraction coupling.

The electrical signal elicited at the T-tubule membrane is transmitted to the sarcoplasmic reticulum (SR) to induce Ca2+ release, which in turn leads to muscle contraction (1–8). According to the current widely accepted view, upon T-tubule depolarization a portion of the dihydropyridine receptor (DHPR), the voltage-sensing protein in the T-tubule, undergoes a conformational change to make contact with the ryanodine receptor (RyR) to open its Ca2+ release channel (9–13). The idea that the cytoplasmic loop linking Repeats II and III of the α subunit of the DHPR, the so-called II-III loop, may play an essential role in this process has emerged from an earlier finding that this portion of the DHPR is the critical determinant of the skeletal muscle-type Ca2+ current (14). This view has been further supported by recent findings that the expressed II-III loop (both skeletal and cardiac isoforms) enhanced the ryanodine binding to the skeletal muscle RyR (15). The site important for activation of ryanodine binding was localized in the region encompassing residues Glu696-Glu706 (16), which contains the phosphorylatable serine 687 (17). Furthermore, a recent study with lysogenic myotubes expressing the chimeric (skeletal/cardiac) DHPR has shown that the critical determinant of the skeletal muscle-type Ca2+ transient is localized in the stretch of residues Glu724–Pro742 (18). In this study, using synthetic peptides corresponding to different regions of the II-III loop of rabbit skeletal muscle DHPR α subunit, we identified the region responsible for triggering Ca2+ release and another region for blocking the release. The implication of these findings on the E-C coupling mechanism is discussed.

EXPERIMENTAL PROCEDURES

Preparation—Triad-enriched microsomal fractions (triads) were prepared from rabbit back paraspinal and hind leg skeletal muscles by differential centrifugation as described previously (19). Microsomes from the final centrifugation were resuspended in a solution containing 0.3 M sucrose, 0.15 M potassium gluconate, proteolytic enzyme inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 0.8 μg/ml antipain, 2.0 μg/ml soybean trypsin inhibitor), 20 mM MES, pH 6.8, to a final concentration of 20–30 mg/ml, frozen immediately in liquid N2, and stored at −70 °C.

Synthesis of II-III Loop Peptides—Peptides were synthesized on an Applied Biosystems model 431 A synthesizer employing Fmoc (fluorenyl)methoxycarbonyl as the α-amino protecting group. Peptides were cleaved and deprotected with 95% trifluoroacetic acid. Purification was carried out by reversed-phase high pressure liquid chromatography using a Rainin Instruments preparative C8 column. Ca2+ Release Assays—Triads (1 mg/ml) were incubated in a solution containing 0.15 M KCl, 1 mM Mg-ATP, an ATP-regenerating system, 20 mM MES, pH 6.8 (Solution A) for 6–7 min to load the SR moiety with Ca2+. Then, 1 volume of Solution A was mixed with 1 volume of Solution B containing 0.15 mM KCl, 20 mM MES, pH 6.8, and various concentrations of peptides. The Ca2+ concentration in both solutions was buffered at 3 μM using an EGTA-calcium buffer (4.22 mM CaCl2, 5 mM EGTA). The time course of SR Ca2+ release was monitored in a stopped flow apparatus (Bio-Logic SFM-3) using 10 μM arsenazo III as a Ca2+ indicator (20). Twenty to twenty-five traces (each representing 1000 data points) of the arsenazo III signal were averaged for each experiment. The arsenazo III signal was converted to nanomoles of Ca2+ released per mg of protein by determining the Δ arsenazo III signal/ΔCa2+ coefficient from a Ca2+ calibration curve. Time courses of Ca2+ release were determined at different peptide concentrations. Curves were fitted by a single exponential function, y = A(1 – e kt), where y is the amount of Ca2+ released at time t, A is the final amount of Ca2+ released at an infinite time, and k is the rate constant of release.

Ryanodine Binding Assay—Rabbit skeletal muscle triads (0.5 mg/ml) or porcine cardiac microsomes (1.0 mg/ml) were incubated in 0.1 ml of a reaction solution containing 8 mM [H]ryanodine (68.4 Ci/mmol, DuPont NEN), 0.3 mM KCl, 10 μM CaCl2, 20 mM Na-PIPES (pH 7.2), and various concentrations of the II-III Loop peptides for 120 min at 36 °C. The incubated reaction mixture was filtered through Whatman GF/A glass fiber filters and washed twice with 5 ml of 0.3 M KCl, 10 μM CaCl2, 20 mM Na-PIPES (pH 7.2). The specific binding was calculated as the difference between the binding in the absence (total binding) and in the presence (nonspecific binding) of 10 μM unlabeled ryanodine (21). Experiments were carried out in duplicate; each datum point is obtained by averaging the duplicates. Nonspecific binding was ~10% of total binding.
In an attempt to identify the subdomains of the II-III loop of the α₁ subunit of the DHPR that play important roles in excitation-contraction coupling, we synthesized several peptides corresponding to different regions of the loop as shown in Fig. 1 and investigated the effect of each of these peptides on [³H]ryanodine binding to, and Ca²⁺ release from, rabbit skeletal muscle triads. Fig. 2A depicts the extent of ryanodine binding activation/inhibition (expressed as percent of control) induced by various concentrations of these peptides. Of all the peptides investigated up to a concentration of 50 μM, only peptide A produced significant activation of ryanodine binding. Increasing concentrations of peptide A progressively increased ryanodine binding to a maximal level (about 230% of control). However, peptides B, C, C₁, C₂, and D produced virtually no effect on ryanodine binding. Mirroring the ryanodine binding experiments (Fig. 2A), only peptide A induced a significant amount of Ca²⁺ release from SR. However, equimolar concentrations of all the other peptides induced virtually no Ca²⁺ release.

Peptide A produced no appreciable effects on ryanodine binding to microsomes isolated from porcine cardiac muscle (percent of control: at 20 μM peptide A, 102 ± 7 (n = 3); at 50 μM,
104 ± 11 (n = 4)). This is in agreement with the recent report that the expressed II-III loop activates the skeletal muscle RyR but not the cardiac RyR isoform (15).

Under the same conditions as above, in which peptide A produced significant activation, the whole II-III loop expressed in Escherichia coli (22) had virtually no effects on ryanodine binding nor induced Ca\(^{2+}\) release, unless 5 mM AMP was added as done in the original study by Meissner and co-workers (15). This suggests that there might be an inhibitory domain countering the peptide A region within the II-III loop. Indeed, as shown by the experiments in Fig. 3, A and B, the presence of 50 \(\mu\)M peptide C, but not the other peptides (B, C1, C2, or D), produced significant suppression of the activation of ryanodine binding induced by 50 \(\mu\)M peptide A (Fig. 3A). Again mirroring the ryanodine binding experiments, an equimolar concentration (20 \(\mu\)M in this case) of peptide C produced significant inhibition of SR Ca\(^{2+}\) release induced by peptide A. However, peptides B, C1, C2, and D had no effect. It is particularly interesting that neither peptide C1 nor C2, which represent the two subdomains of peptide C, had any Ca\(^{2+}\) release blocking effect by themselves. This indicates that both C1 and C2 subdomains must be linked to exert the blocking function.

Several important new properties of the II-III loop of the DHPR are revealed in this study. Most importantly, we could localize the critical site for activating the RyR/Ca\(^{2+}\) channel to peptide A (Thr\(^{671}\)-Leu\(^{690}\)), which represents approximately one-third of the recently reported 61-residue ryanodine binding activating peptide of the II-III loop (16). Another important aspect of this study is the finding of peptide C, which antagonized the effect of peptide A on Ca\(^{2+}\) release or ryanodine binding. These results suggest that there are at least two functionally important subdomains in the II-III loop: an activator that is responsible for the stimulation of the RyR/Ca\(^{2+}\) release channel in E-C coupling and a blocker that antagonizes the activator. These results suggest an intriguing hypothesis as follows. In the resting state, the putative signal receptor site in the RyR is occupied by the blocker domain of the loop. Upon depolarization, the blocker domain (corresponding to peptide C) is removed from the site; then the activator domain (corresponding to peptide A) is allowed to interact with the site to trigger SR Ca\(^{2+}\) release. In the present study, the activation of SR Ca\(^{2+}\) release by peptide A was produced presumably by competitive binding with the blocker domain to the signal receptor (in the case of coupled RyR) or by direct binding (in the case of uncoupled RyR). Peptide C1 (Phe\(^{725}\)-Gly\(^{743}\)) used in the present study covers the 17-residue (Glu\(^{726}\)-Pro\(^{742}\)) region reported to be a critical determinant for the skeletal muscle-type regulation (18), which requires a physical contact of the II-III loop to the RyR (11). On this basis, we tentatively propose that the C1 subdomain may behave like a hinge for this blocker/activator exchange operation.

Acknowledgments—We would like to thank Drs. Renne C. Lu and Paul C. Leavis for their help in the synthesis and purification of the peptides, Dr. Timothy J. Connelly for his kind supply of the porcine cardiac muscle, and Dr. John Gergely for his valuable comments on the manuscript.

REFERENCES

1. Coronado, R., Morrissette, J., Sukhareva, M., and Vaughan, D. M. (1994) Am. J. Physiol. 266, C1485–C1504
2. Ogawa, Y. (1994) Crit. Rev. Biochem. Mol. Biol. 29, 229–274
3. Meissner, G. (1994) Annu. Rev. Physiol. 56, 485–508
4. Franzini-Armstrong, C. (1994) Annu. Rev. Physiol. 56, 509–534
5. McPherson, P. S., and Campbell, K. P. (1993) J. Biol. Chem. 268, 13765–13768
6. Fleischer, S., and Inui, M. (1989) Annu. Rev. Biophys. Biophys. Chem. 18, 333–364
7. Fill, M., and Coronado, R. (1988) Trends Neurosci. 11, 453–457
8. Catterall, W. A. (1991) Cell 64, 871–874
9. Chandler, W. K., Rakowsky, R. F., and Schneider, M. F. (1976) J. Physiol. (Lond.) 254, 285–316
10. Rios, E., and Pizarro, G. (1992) J. Physiol. Rev. 71, 849–908
11. Rios, E., Pizarro, G., and Stefani, E. (1992) Annu. Rev. Physiol. 54, 109–133
12. García, J., Tanabe, T., and Beam, K. G. (1994) J. Gen. Physiol. 103, 125–147
13. Catterall, W. A. (1995) Annu. Rev. Biochem. 64, 493–531
14. Tanabe, T., Beam, K. G., Adams, B. A., Niidome, T., and Numa, S. (1990) Nature 346, 567–569
15. Lu, X., Xu, L., and Meissner, G. (1994) J. Biol. Chem. 269, 6511–6516
16. Lu, X., Xu, L., and Meissner, G. (1995) Biophys. J. 68, A372 (abstr.)
17. Rohrkasten, A., Meyer, H. E., Nazáínczyk, W., Sieber, M., and Hofmann, F. (1988) J. Biol. Chem. 263, 13325–13329
18. Nakai, J., Tanabe, T., and Beam, K. G. (1995) Biophys. J. 68, A14 (abstr.)
19. Ikenoto, N., Kim, D. H., and Antoniu, B. (1988) Methods Enzymol. 157, 469–480
20. Ikenoto, N., Antoniu, B., and Kim, D. H. (1984) J. Biol. Chem. 259, 13151–13158
21. El-Hayek, R., Valdivia, C., Valdivia, H. H., Hogan, K., and Coronado, R. (1993) Biophys. J. 65, 779–789
22. Smith, D. B. (1993) Methods Mol. Cell Biol. 4, 220–229