Involvement of the Glu$_{724}^{760}$-Pro$_{760}$ Region of the Dihydropyridine Receptor II-III Loop in Skeletal Muscle-type Excitation-Contraction Coupling

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Our previous study (El-Hayek, R., Antoniu, B., Wang, J. P., Hamilton, S. L., and Ikemoto, N. (1995) J. Biol. Chem. 270, 22116–22118) suggested the hypothesis that skeletal muscle-type excitation-contraction coupling is regulated by two domains (activating and blocking) of the II-III loop of the dihydropyridine receptor α1 subunit. We investigated this hypothesis by examining conformational changes in the ryanodine receptor induced by synthetic peptides and by transverse tubular system (T-tubule) depolarization. Peptide A, corresponding to the Thr$_{671}^{690}$ region, rapidly changed the ryanodine receptor conformation from a blocked state (low fluorescence of the conformational probe, methyl coumarin acetamide, attached specifically to the ryanodine receptor) to an activated state (high methyl coumarin acetamide fluorescence) as T-tubule depolarization did. Peptide C, corresponding to the Glu$_{724}^{760}$ region, blocked both conformational changes induced by peptide A and T-tubule depolarization. Its ability to block peptide A-induced and depolarization-induced activation was considerably impaired by replacing the portion of peptide C corresponding to the Phe$_{725}^{742}$ region of the loop with cardiac muscle-type sequence. These results are consistent with the model that depolarization-induced activation of excitation-contraction coupling and blocking/repriming are mediated by the peptide A region and the peptide C region (containing the critical Phe$_{725}^{742}$ sequence) of the II-III loop, respectively.

According to a widely accepted hypothesis (1–7), skeletal muscle-type E-C coupling is triggered by the voltage-dependent binding of one of the cytoplasmic loops (II-III loop) of the dihydropyridine (DHP) receptor α1 subunit to the SR Ca$^{2+}$ release channel protein, also referred as ryanodine receptor (RyR). The critical role of the II-III loop was recognized first by an earlier finding of Tanabe et al. (8, 9) that replacement of the II-III loop of the cardiac DHP receptor with the skeletal muscle-type E-C coupling activity in dysgenic myotubes expressing chimeric DHP receptors. This concept was further supported by the finding that a recombinant peptide corresponding to the II-III loop activated ryanodine binding and Ca$^{2+}$ channel activity.

Further studies with shorter peptides corresponding to various regions of the II-III loop have permitted new insight into functionally important subdomains of the loop. A 61-residue recombinant peptide corresponding to the Glu$_{666}^{726}$ region activated the RyR, suggesting that the ability of activating E-C coupling is localized in this region (11). According to our studies with a series of synthetic peptides corresponding to different regions of the loop (12), only one peptide corresponding to the Thr$_{671}^{690}$ region (designated peptide A) activated ryanodine binding and induced Ca$^{2+}$ release from SR (12, 13). Activating functions of peptide A are retained in a truncated form of peptide A, corresponding to Arg$_{681}^{690}$ but further truncation abolished its activity (13). These findings indicate that an essential domain for the activation of E-C coupling (designated the “activator” of E-C coupling) is localized in the Arg$_{681}^{690}$ region of the II-III loop. The concept that there is another important domain of the II-III loop that may be involved in the regulation of E-C coupling has emerged from the findings as follows. As reported in our recent paper (12), a synthetic peptide corresponding to the Glu$_{724}^{760}$ region of the II-III loop (designated peptide C), but not other peptides, inhibited peptide A-induced activation of the RyR. This suggests that the Glu$_{724}^{760}$ region of the loop may serve as an antagonist of the activator described above. The idea that this portion is critical for E-C coupling has also emerged from the recent report by Nakai et al. (14). According to the report, chimeric replacement of the Phe$_{725}^{742}$ region of the II-III loop (the region corresponding to the N-terminal half of peptide C) from cardiac type to skeletal muscle type conferred the E-C coupling properties of skeletal muscle-type to dysgenic myotubes expressing cardiac-type DHP receptor. Thus, there appear to be at least two important domains of the II-III loop that are required for skeletal muscle-type E-C coupling.

The main purpose of the present study is to gain new insight into the mechanism by which E-C coupling is regulated by these two domains of the II-III loop. As shown in the present study, peptide A produces a rapid change of the RyR conformation from a resting state to an activated state, and peptide C reverses this process. Interestingly, these changes induced by peptide A and peptide C correspond to the conformational changes induced by T-tubule depolarization and polarization, respectively. This predicted that peptide C would counteract not only peptide A-dependent activation of the RyR but also depolarization-induced activation of E-C coupling. To test this, we investigated the effects of peptide C on depolarization-induced conformational change in the RyR and Ca$^{2+}$ release.

* This work was supported by grants from the National Institutes of Health (AR16922) and the Muscular Dystrophy Association. 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.

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‡ The abbreviations used are: DHP, dihydropyridine; E-C coupling, excitation-contraction coupling; MCA, methyl coumarin acetamide; MES, 2-N-morpholinooctanesulfonic acid; SAED, sulfosuccinimidyl 3-((2-(7-azido-4-methyl-coumarin-3-acetamido)ethyl)dithio)propionate; SR, sarcoplasmic reticulum; T-tubule, transverse tubular system; RyR, ryanodine receptor.
from the SR. As shown here, depolarization-induced changes in the RyR to an active conformational state and Ca\(^{2+}\) release from SR were blocked or reversed by peptide C. These results suggest a new concept, that depolarization-dependent activation of E-C coupling and polarization-dependent repriming of the system are modulated by the voltage-dependent alternative binding of the activator domain (the \textit{in situ} counterpart of peptide A) and the blocker/primer domain (the \textit{in situ} counterpart of peptide C), respectively, to the E-C coupling site(s) of the RyR.

**EXPERIMENTAL PROCEDURES**

**Preparation**—The triad-enriched microsomal fraction was prepared from rabbit leg and back muscles by differential centrifugation as described previously (15). After the final centrifugation, the sedimented fraction was homogenized in a solution containing 0.3 M sucrose, 0.15 M gluconate, proteolytic enzyme inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 10 \(\mu\)g/ml aprotinin, 0.8 \(\mu\)g/ml antipain, 2 \(\mu\)g/ml trypsin inhibitor) and 20 mM MES, pH 6.8 (PI buffer), to a final protein concentration of 20–30 \(\mu\)g/ml. The preparation was quickly frozen in liquid nitrogen and stored at \(-7^\circ\)C.

**Synthesis of Peptides**—Peptides were synthesized on an Applied Biosystems model 431 A synthesizer employing Fmoc (N-(9-fluorenylethoxycarbonyl) as the \(\alpha\)-amino protecting group. Amidated 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. Purified peptides were then dialyzed against water using a dialysis membrane with a cutoff of M, of 500. Five millimolar solutions of peptides were prepared in 10 mM HEPES, pH 7.2.

\[^{3}H\]Ryanodine Binding Assay—Trip vesicles (0.5 mg/ml) were incubated in 0.1 ml of a reaction solution containing 8–10 \(\times\) 10\(^{-6}\) \(^{3}\)H]ryanodine (55.4 Ci/ml, NEN Life Science Products), 0.3 M KCl, 100 \(\mu\)M EGTA, 64.4 \(\mu\)M CaCl\(_2\), 20 mM MOPS, pH 7.2 for 2 h at 36 °C in the absence or in the presence of various concentrations of peptide. Specific binding was calculated as the difference between the binding in the absence (total binding) and in the presence (non-specific binding) of 10 \(\mu\)M non-radioactive ryanodine (16). Experiments were carried out in duplicate, and each data point was obtained by averaging the duplicates.

**Site-specific Fluorescent Labeling of the RyR Moiety of the Triad**—Site-specific fluorescent labeling of the RyR moiety of the triad was performed using the cleavable hetero-bifunctional cross-linking reagent sulfoBocOsucloramidinyl 3-[2-(7-azido-4-methylcoumarin-3-acetamido)ethyl]dithio)propionate (SAED) (17, 18) with the aid of neomycin as a carrier conjugate (final neomycin concentration, 20 \(\mu\)M) containing the MCA-labeled triads were first polarized by incubating the triads (2.0 mg/ml) within a 150 mM sodium gluconate, 15 mM NaCl, 2.5 mM EGTA, 1.61 mM CaCl\(_2\), 20 mM imidazole, pH 6.8; \([\text{Ca}^{2+}] = 1.0 \mu\text{M}) using a stopped-flow apparatus (BioLogic SFM4).

To induce conformational changes in the RyR induced by T-tubule depolarization, we used the K\(^{+}\) to Na\(^{+}\) replacement protocol, which was originally devised in the skinned fiber system by Lamb and Stephenson (20, 21) and was adopted to our triad system (19, 22). The MCA-labeled triads were first polarized by incubating the triads (2.0 mg/ml) within the base solution (see above) containing 5.0 mM Mg-ATP and an ATP-regenerating system (2.5 mM phosphoenolpyruvate and 10 units/ml pyruvate kinase) for 10–15 min. Then, the T-tubule moiety was depolarized by mixing 15 \(\mu\)l of the solution containing the polarized triads with 5 \(\mu\)l of depolarization solution (150 mM sodium gluconate, 15 mM NaCl, 2.5 mM EGTA, 1.61 mM CaCl\(_2\), 20 mM imidazole, pH 6.8; \([\text{Ca}^{2+}] = 1.0 \mu\text{M}) using a stopped-flow apparatus (BioLogic SFM4).

The time courses of fluorescence change of the protein-bound MCA (excitation at 368 nm, emission at 440 nm using an interference filter with a 70-nm bandwidth) induced by peptide A or T-tubule depolarization were monitored with the stopped-flow fluorometer (BioLogic SFM4) using fluo-3 as the Ca\(^{2+}\) indicator (excitation at 437 nm, emission at 530 nm with a 510-nm cut-off filter).

**RESULTS AND DISCUSSION**

**Countercution between Peptide A and Peptide C in the Regulation of Conformational States of the RyR**—Peptide C, corresponding to the Thr\(^{721}\)-Leu\(^{729}\) region of the II-III loop, mimics some features of a physiologic activation of E-C coupling in skeletal muscle, whereas peptide C, corresponding to the Glu\(^{724}\)-Pro\(^{730}\) region of the loop, blocks the activation by peptide A (12). This has led us to the hypothesis that the RyR is regulated by these two portions of the II-III loop in a reciprocal fashion. Fig. 1A shows the effects of various concentrations of peptide C on the ryanodine binding activity in the absence or in the presence of peptide A. In agreement with our recent report (12), peptide C alone has virtually no effect on ryanodine binding in the range of the concentrations tested. In the presence of 30 \(\mu\)M peptide A, which produced a near maximal enhancement of ryanodine binding (see Fig. 1A, inset), peptide C blocked peptide A-dependent enhancement with an IC\(_{50}\) (the concentration for half maximal inhibition) of about 50 \(\mu\)M. At 200 \(\mu\)M, peptide C produced an almost complete blocking of the peptide A-dependent activation. Thus, peptide C reverses the activation of the RyR by peptide A, although peptide C alone has no direct effect on the RyR. In other words, peptide C seems to regulate the RyR by serving as an antagonist of the activating peptide A.

As described above, the N-terminal half of peptide C (see the underlined portion of peptide C in the sequence shown below) corresponds to the so-called “determinant of skeletal muscle-type E-C coupling” (the Glu\(^{726}\)-Pro\(^{742}\) region of the II-III loop) (23), the skeletal muscle-type sequence of which seemed to be essential for the skeletal muscle-type E-C coupling.

**Peptide C:**

\[
\text{SDFEHENKVDKVDFPSADPEGPDEEPEKPITPSPP}^{726}\text{EFNEKDSFGPVNPTTPDDEEPEKPITPSPP}^{730}\text{ }
\]

**Chimeric peptide C:**

\[
\text{BEFNEKDSFGPVNPTTPDDEEPEKPITPSPP}^{726}\text{BEFNEKDSFGPVNPTTPDDEEPEKPITPSPP}^{730}\text{ }
\]

**SEQUENCE 1**

In order to test the importance of the type of sequence in this segment, we synthesized a chimeric peptide C by changing this segment to cardiac type sequence (see the segment of chimeric peptide C with italic letters). As shown in Fig. 1A and Table I, the extent of blocking of peptide A-dependent activation by chimeric peptide C (chimeric C) was significantly less than that by peptide C, indicating that the skeletal muscle-type with this region had a significantly long function of peptide C. In light of the suggested importance of the skeletal muscle-type sequence of this segment for E-C coupling (I.c.), it is suggested that blocking function residing in the peptide C region of the II-III loop is in fact required for E-C coupling besides the activating function located in the peptide A region.
**E-C Coupling Peptide Probe**

**Peptide C**: 724\(^{E}FE\)SNVNE\(^{VK}D\)PYPSAD\(^{FP}GD\(^{D}\)E\(^{D}\)E\(^{D}\)PE\(^{IPVSPR\}PR\}5760\)

**Peptide C\(_{1}\)**: 724\(^{E}FE\)SNVNE\(^{VK}D\)PYPSAD\(^{FP}GD\)

**Peptide C\(_{2}\)**: 724\(^{D}\)FPGD\(^{D}\)E\(^{D}\)E\(^{D}\)PE\(^{IPVSPR\}PR\}P\)

**SEQUENCE 2**

**TABLE I**

| Peptide        | IC\(_{50}\) (\(\mu M\)) | S.D. |
|----------------|--------------------------|------|
| Peptide C      | 57.0 ± 10.8              |      |
| Chimeric C     | 245.7 ± 75.0             |      |
| C\(_{1}\)       | 3089.8 ± 1607.6          |      |
| C\(_{2}\)       | 289.3 ± 75.9             |      |
| C\(_{1}\) + C\(_{2}\) | 221.7 ± 20.0             |      |

We previously found that shorter peptides of peptide C, viz. peptide C\(_{1}\) and peptide C\(_{2}\) (corresponding to the Phe\(^{725}\_\)Gly\(^{743}\) and Asp\(^{740}\_\)Pro\(^{760}\) regions of the II-III loop, respectively; see the above sequence), had no appreciable effect on peptide A-dependent activation up to 50 \(\mu M\) (12). Further studies with higher concentrations of these shorter peptides revealed new features, shown in Fig. 1B. As seen, peptide C\(_{1}\) had again virtually no blocking effect even at higher concentrations, indicating that the N-terminal half of peptide C alone is not capable of competing with peptide A. Interestingly, however, peptide C\(_{2}\) produced a small but significant competition with peptide A at high concentrations. The addition of equimolar concentrations of peptide C\(_{1}\) to peptide C\(_{2}\) produced little or no additional change in the extent of blocking by peptide C\(_{2}\). From these findings, we propose that a relatively large region (presumably the whole length of peptide C) is required for an effective blocking of peptide A-dependent activation. As seen in the above scheme, peptide C\(_{2}\) contains a cluster of negatively charged residues (DDEEDE). Because the cluster of positively charged residues located in peptide A seems to play a key role for its activating function (13), the cluster of acidic residues located in the peptide C\(_{2}\) region likely plays a key role in the blocking function of peptide C. We tentatively propose that the blocking function is localized in the C\(_{2}\) region, but the C\(_{1}\) region with the skeletal muscle-type sequence is required for some other functions, such as the binding of peptide C to the RyR.

As seen in Fig. 1 and Table I, the extent of blocking by chimeric peptide C (Fig. 1A) is about the same as that by peptide C\(_{1}\), or an equimolar mixture of C\(_{1}\) and C\(_{2}\) (Fig. 1B). This indicates that the partial blocking of peptide A-dependent activation by chimeric peptide C described above is solely due to the inhibitory function localized in the C-terminal half of chimeric peptide C.

In the experiments shown in Fig. 1C, we investigated the effects of increasing concentrations of peptide C on enhancement of ryanodine binding by the generally used Ca\(^{2+}\) release-inducing reagents caffeine (24, 25) and polylysine (26, 27). As seen, peptide C had no effect on both caffeine- and polylysine-dependent enhancement, even at the high concentrations investigated. Thus, peptide C is a specific blocker of peptide A. This is consistent with the view that peptide C is competing with peptide A at the specific E-C coupling site(s) of the RyR.

Although ryanodine binding assays provide a convenient method for the assessment of conformational states of the RyR, the assay is not suitable for monitoring rapid conformational changes occurring in the RyR. Stopped-flow fluorometry of the fluorescent conformational probe, MCA, specifically attached to the RyR moiety (17, 19, 27) permits us to study rapid conformational changes in the RyR with a high temporal resolution. In the experiment shown in Fig. 2A, we investigated the effects...
Peptide A induces a rapid increase of the RyR-bound MCA fluorescence (i.e., formation of an active conformational state of the RyR). A, time courses of MCA fluorescence increase induced by various concentrations of peptide A. Each trace was obtained by signal averaging a total of 75 traces from three experiments. B, initial rates of peptide A-induced conformational change as a function of the concentration of peptide A. Traced line was obtained by fitting a single exponential function ($y = y_0 + A(1 - e^{-kt})$). The initial rate ($A\cdot k$) was calculated from the $A$ and $k$ values obtained from the fitting. Data represent the mean ± S.D. of three experiments.

Fig. 2. Peptide A-dependent formation of the active conformational state of the RyR is suppressed by peptide C in a concentration-dependent manner. Each trace was obtained by signal averaging a total of 75–90 traces from three experiments. B, the initial rates of the peptide A-induced MCA fluorescence increase as a function of the concentration of peptide C added. The initial rate was calculated as described in the legend to Fig. 2. Data represent the mean ± S.D. of three experiments.
manner. An increase of the peptide concentration up to 50 μM resulted in an increase in both the magnitude (A) and the rate constant (k) of ΔF. Upon further increase of peptide A concentration, however, the activation by peptide A was somewhat suppressed. Fig. 2B shows the [peptide A] dependence of the initial rate of ΔF (i.e., A-k value). Importantly, the general pattern of the dose-dependent activation seen here (Fig. 2B) shows a striking resemblance to that obtained from the ryanodine binding assay (cf. Fig. 1A, inset). This indicates that ryanodine binding and MCA fluorescence assays can provide us with the essentially identical information about the functional/conformational state of the RyR, although only the MCA fluorometry permits a sufficient temporal resolution for the studies of the activator-induced rapid conformational changes.

Fig. 3A depicts time courses of conformational changes of the RyR when the vesicles were incubated first with various concentrations of peptide C and then 30 μM peptide A was added to activate the RyR. As seen, peptide A-induced ΔF was blocked significantly by peptide C in a concentration-dependent manner. The [peptide C] dependence of the reduction of the initial rate of peptide A-induced fluorescence change is shown in Fig. 3B. The [peptide C] dependence of the blockage is similar to that shown in the ryanodine binding assay (Fig. 1), although the IC50 in the stopped-flow assay (approximately 30 μM, Fig. 3B) is somewhat lower than that in the ryanodine binding assay (approximately 50 μM, Fig. 1). These results indicate that peptide C blocks the peptide A activation by interfering with the binding of peptide A to the RyR.

The isolated triad preparation contains two classes of RyRs: coupled RyRs, which are linked with the T-tubule DHP receptors, and free RyRs, which are not linked with the DHP receptors. In this preparation, the population of free RyRs must be significantly larger than that of the coupled RyRs due to the fact that a significant portion of the previously coupled RyRs (which had been about 50% of the total RyR population [28-30]) must have been dissociated from T-tubules during fragmentation and isolation of the vesicles. Furthermore, the rapid accessibility of the added peptide A to the activating site(s), which seems to be critical for rapid activation of the RyR in those stopped-flow experiments, would be much higher in the free RyRs than the coupled RyRs. Therefore, these data in Fig. 3 seem to represent almost primarily the conformational response of the free RyRs to the added peptides, although peptide A may also be accessible to the coupled RyRs. Diagram 1A summarizes our interpretation of the above data. Binding of peptide A to the putative E-C coupling site(s) of the RyR produces a rapid conformational change from the state with low MCA fluorescence to the new state with high MCA fluorescence, which leads to the enhancement of ryanodine binding and Ca2+ release from the SR (cf. Refs. 19 and 27). Peptide C by itself produces virtually no change in the RyR conformation. However, activation of the RyR by peptide A, viz. the increase in the MCA fluorescence level, was blocked by peptide C owing to the competition between peptide A and peptide C to the specific E-C coupling site(s).

Effects of Peptide C on E-C Coupling Phenomena in the Coupled Triads—As described above, peptide A increases MCA fluorescence, and peptide C reverses the peptide A-induced increase of MCA fluorescence. This is reminiscent of similar fluorescence changes occurring in the coupled triads. Namely, the fluorescence intensity of the RyR-bound MCA increases upon T-tubule depolarization, whereas it decreases upon T-tubule polarization (31). Such a tight correlation between the two experiments, one in the free RyRs and the other in the coupled RyRs, suggests the following hypothesis (cf. Diagram 1B). Upon T-tubule depolarization, the region of the II-III loop corresponding to peptide A (activator of E-C coupling) will bind to the E-C coupling site(s) of the RyR, leading it to an active conformational state with a high MCA fluorescence (cf. Refs. 19 and 31). Upon T-tubule polarization, the RyR-bound activator will be replaced by the region of the II-III loop corresponding to peptide C, leading to a blocked (or primed) state of the RyR with a low MCA fluorescence.

In order to test this model, we monitored MCA fluorescence increase induced by T-tubule depolarization (at a level of G10, cf. Ref. 22) in the presence of various concentrations of peptide C. The important feature of this experiment is that only the RyRs linked with the T-tubule DHP receptor show voltage-dependent conformational (MCA fluorescence) changes. This is in sharp contrast to the activation by general activators of the
RyR (Ca\textsuperscript{2+}, caffeine, polylysine, etc.), which will react with both free and coupled RyRs. This provides us with a straightforward method for testing the effect of peptide C on the voltage-dependent operation of the II-III loop. In the experiment shown in Fig. 4A, the vesicles were first incubated with various concentrations of peptide C, and after priming the system (viz. after polarizing the T-tubule and loading the SR with Ca\textsuperscript{2+} (19, 22)), the T-tubule moiety was depolarized (see “Experimental Procedures”). As seen, depolarization-induced MCA fluorescence increase (i.e., the voltage-dependent formation of an active conformational state of the RyR) was blocked by peptide C in a concentration-dependent manner. The IC\textsubscript{50} was 20 \mu M as determined from the d\[F]/dt versus [peptide C] plot (Fig. 4B), which is essentially identical with the IC\textsubscript{50} for the peptide C inhibition of the peptide A-dependent activation (Fig. 3B). We also carried out the same type of experiments as in Fig. 4A by adding equivalent concentrations of peptide C simultaneously with T-tubule depolarization (rather than depolarizing after preincubation with peptide C). The degree of inhibition by peptide C without preincubation was almost identical with that after preincubation, although the former was somewhat smaller than the latter. These results suggest that the occupancy of peptide C at the E-C coupling site(s) of the RyR interfered with the voltage-dependent binding of the activator domain of the II-III loop to the site(s), although the voltage-dependent changes in the position of the II-III loop domains may be occurring in a normal way.

The magnitude of the relative MCA fluorescence increase (\Delta F/F\textsubscript{0}) induced by T-tubule depolarization (about 4\%, Fig. 4A) was significantly larger than that induced by a maximally activating concentration of peptide A (about 1\%, Fig. 3A). This is rather surprising, because the population of the coupled RyRs responsible for the former event is significantly smaller than that of the free RyRs responsible for the latter, as described above. The most reasonable explanation for this would be that the changes in the fluorescence intensity are greatly enhanced in the coupled RyRs because of their location in the space between the T-tubule and SR membranes, which is presumably a more hydrophobic environment than that of the free RyRs.

In the experiment shown in Fig. 4C, we compared the extent of inhibition of depolarization-induced MCA fluorescence by peptide C and that by chimeric peptide C at 200 \mu M. In good agreement with the results of ryanodine binding assay (cf. Fig. 1), the potency of blocking depolarization-induced conformational change is significantly less in chimeric peptide C compared with peptide C. This indicates again that the skeletal muscle-type sequence of the region corresponding to the Glu\textsuperscript{726}–Pro\textsuperscript{742} region of the II-III loop is important for peptide C to exert an efficient reversal of the in situ mechanism of activation.

Because the formation of active conformational state of the RyR (i.e., high MCA fluorescence state) is a prerequisite for the channel opening and Ca\textsuperscript{2+} release (cf. Refs. 19, 27), inhibition of this process by peptide C should also inhibit Ca\textsuperscript{2+} release from the SR. In the experiment shown in Fig. 5A, we investi-
II-III loop in the neighborhood of the peptide C region) had no effect on peptide A-induced Ca\(^{2+}\) release. As seen in Table II, neither peptide B nor peptide D had any appreciable effect on depolarization-induced Ca\(^{2+}\) release, even at 200 \(\mu\)M.

**Table II**

| Addition   | Initial Ca\(^{2+}\) release rate (nmol/mg/s) |
|------------|---------------------------------------------|
| 0          | 435.8 ± 73.9                                |
| Peptide B  | 440.5 ± 101.7                               |
| Peptide C  | 38.7 ± 29.0                                 |
| Peptide D  | 389.5 ± 65.3                                |

**Relationship of the Present Findings to Those by Others**—In a recent paper, Nakai et al. (14) described that there is a considerable amount of inconsistency in the literature concerning the proposed locations of critical domain(s) of the II-III loop. However, many (if not all) of those apparently inconsistent findings, together with the present findings, may be explained by a general hypothesis as elaborated below. First, the finding by Nakai et al. (14) that replacement of a short segment of the cardiac loop with the corresponding skeletal residues Phe\(^{725}\)-Pro\(^{742}\) was sufficient to produce skeletal type E-C coupling has led to their suggestion that this region may serve as an “agonist,” rather than the peptide A region being the activator. This suggestion was based upon the assumption that the cardiac sequence of the peptide A region of the loop would have no capability of activating the RyR1 (14). As a matter of fact, as shown in our recent report, both skeletal and cardiac sequences of the critical 10-residue portion of peptide A (peptide A-10) can activate the RyR1, although cardiac peptide A-10 is somewhat weaker than its skeletal counterpart (cf. Figs. 3 and 6 of Ref.13). We propose that both skeletal and cardiac loops have a common activating domain in the peptide A-10 region, which has a similar amino acid sequence (skeletal, RKRRKMSGRL; cardiac, KERKKLARTA, cf. Ref. 13). This concept can well explain an earlier finding that both skeletal and cardiac constructs of the II-III loop activated the RyR1 (10). This concept is also in accord with the recent report that the II-III loop construct subjected to mutations within the peptide A-10 region became incapable of interacting with the 37-residue construct of the RyR (32).

Then what makes the region of skeletal residues Phe\(^{725}\)-Pro\(^{742}\) essential for E-C coupling without having an agonist function? The present study provides some clues to this question. As shown (cf. Fig. 3A), peptide C blocked MCA fluorescence increase induced by both peptide A and T-tubule depolarization. Furthermore, according to our preliminary data, the addition of peptide C to the depolarized triads resulted in a decrease of MCA fluorescence (data not shown). These results would indicate that upon T-tubule polarization, the peptide C region of the II-III loop binds to the RyR in replacing the activator domain, which results in a blocked or resting conformational state of the RyR (cf. Diagram 1B). The binding of the blocker to the RyR to form its resting state is essential for E-C coupling, presumably for the purpose of priming the system. This is because without it, a new cycle of E-C coupling cannot take place (cf. Diagram 1B). The Phe\(^{725}\)-Pro\(^{742}\) region corresponds to the N-terminal half of peptide C (i.e., peptide C\(_1\)), and replacement of this portion to the cardiac sequence produced a significant reduction in the blocking (or priming) ability of peptide C, as shown in the present study. Furthermore, as
suggested from the present data (see Fig. 1B), the peptide C1 region may serve as a mechanism to link the inhibitory peptide C2 region to the RyR. Thus, it is not unreasonable to speculate that the Phe725–Pro742 segment may serve as a link of the whole loop to the RyR as well.

Some of the discrepancies in the literature may be at least partly ascribable to the difference in the method of approach. Synthetic or constructed peptides would be accessible to their designated sites in an unrestricted manner. On the other hand, in chimeras, all of those domains under discussion are parts of the II-III loop; hence, their accessibility to the RyR must be restricted and controlled in a voltage-dependent manner. Therefore, it is likely that the activator located in the Arg681–Leu690 region of the II-III loop becomes accessible to the RyR only after the two events have occurred: first, binding of the Phe725–Pro742 segment to the RyR, and second, depolarization of the T-tubule membrane.

Thus, the accumulated pieces of information in the literature, although apparently inconsistent, can be explained by a unified scheme. Namely, the regulation of skeletal muscle-type E-C coupling by the II-III loop is mediated not by a single particular domain, but by multiple domains with different roles. We tentatively propose three such domains: activator, linker, and blocker/primer, located in the A, C1, and C2 regions of the II-III loop, respectively.

Conclusion—The hypothesis that E-C coupling in skeletal muscle may be regulated by at least two domains of the II-III loop (activator and blocker, located in the Thr671–Leu690 and Glu724–Pro760 regions, respectively) was tested. Several pieces of new evidence shown here support this concept. First, peptide C (synthetic peptide corresponding to the blocker region) blocked T-tubule depolarization-induced conformational change in the RyR as well as Ca2+ release from the SR. Second, peptide C also blocked conformational changes in the RyR induced by peptide A (synthetic peptide corresponding to the activator region). The [peptide C] dependence of inhibition of depolarization-induced conformational change was similar to that of peptide A-induced conformational change, supporting the notion that voltage-dependent activation of the RyR is mediated by the region of the II-III loop corresponding to peptide A. Third, replacement of the portion of peptide C, which corresponds to the determinant of skeletal muscle-type E-C coupling (14), from the skeletal muscle-type sequence to the cardiac type produced a significant reduction of the blocking ability of peptide C. This suggests that skeletal-type E-C coupling requires not only the activating function localized in the peptide A region of the II-III loop but also the blocking function residing in the peptide C region. Based upon these findings, we propose the following mechanism (cf. Diagram 1B). Depolarization-induced activation of E-C coupling is mediated by the binding of the activator (located in the region of the II-III loop corresponding to peptide A) to the specific E-C coupling site(s) of the RyR. The binding of the blocker (located in the other region of the II-III loop corresponding to peptide C) to the E-C coupling site(s) removes the activator from the site(s) in a competitive manner. The binding of the blocker/primer is mediated by polarization of the T-tubule, and this step is a prerequisite to the next cycle of depolarization-induced activation. Therefore, this segment is one of the domains that are essential for E-C coupling. For the sake of simplicity of the model, we tentatively assume that the activator and the blocker bind to the same regulatory site of the RyR in a competitive manner. However, it is also possible that they bind to different sites of the RyR.

Acknowledgments—We thank Dr. Graham D. Lamb for comments on the manuscript and Dr. Renne C. Lu, Dr. Paul Leavis, Anna Wong, and Elizabeth Gowell for help in the synthesis and purification of the peptides.

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