Identification of the Minimum Essential Region in the II-III Loop of the Dihydropyridine Receptor α_1 Subunit Required for Activation of Skeletal Muscle-Type Excitation−Contraction Coupling†

Roque El-Hayek‡ and Noriaki Ikemoto‡,§

Boston Biomedical Research Institute, Boston, Massachusetts 02114, and Department of Neurology, Harvard Medical School, Boston, Massachusetts 02115

Received November 26, 1997; Revised Manuscript Received March 18, 1998

ABSTRACT: We have previously shown that among several peptides encompassing various regions of the II−III loop of the dihydropyridine receptor α_1 subunit, only one peptide corresponding to the Thr[671]-Leu[690] region (designated as peptide A) activated ryanodine binding to and induced calcium release from the sarcoplasmic reticulum [El-Hayek et al. (1995) *J. Biol. Chem. 270,* 22116−22118]. To further localize within peptide A the minimum unit essential for activating the sarcoplasmic reticulum calcium release channel, we synthesized variously truncated forms of peptide A and examined their ability to activate ryanodine binding. We found that the carboxy-terminal 10-residue region of peptide A encompassing Arg[681]-Leu[690] (peptide As-10; s, skeletal muscle-type sequence) activated ryanodine binding in a RyR1-specific manner and induced calcium release even more efficiently than the 20-residue peptide A. Further truncation of one or more residue(s) of peptide As-10 virtually abolished both functions of activating ryanodine binding and inducing Ca^{2+} release. The activating ability of As-10 seems to be determined by at least two factors: (1) the distribution of the positively charged residues, and (2) the skeletal muscle-type amino acid sequence, as deduced from the comparison of various peptides with modified structures. These results provide evidence that the minimum essential unit for the *in situ* trigger of skeletal muscle excitation−contraction coupling is localized in the Arg[681]-Leu[690] region of the II−III loop of the α_1 subunit of the dihydropyridine receptor.

The depolarization signal elicited in the plasma membrane of the muscle cell is transmitted *via* the T-tubule to the SR to induce calcium release (*1*−*8*). The molecular mechanism involved in this process has not yet been fully understood. According to the current hypothesis, T-tubule depolarization induces protein conformational changes first in the voltage-sensing dihydropyridine (DHP) receptor and then in the ryanodine receptor, leading to the opening of the SR calcium release channel (*9*−*11*). The concept that the cytoplasmic loop linking repeats II and III of the α_1 subunit of the DHP receptor plays an important role in skeletal muscle E−C coupling was originally derived from the work of Tanabe et al. with dysgenic myotubes expressing chimeric skeletal/cardiac DHP receptors (*12*, *13*). 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 (*14*−*16*). Further attempts have been made to localize the domain of the II−III loop critical for E−C coupling in a shorter region, but the results reported in the literature are rather controversial. The study with the DHP receptor system with various II−III loop chimeras suggested that the determinant of the skeletal muscle-type E−C coupling is localized in the Glu[726]-Pro[742] region (*17*), while the study with shorter recombinant peptides suggested that the activating function is localized in the region encompassing Glu[666]-Glu[726] (*15*). In agreement with the latter, our recent study (*18*) showed that among several synthetic peptides corresponding to different regions of the II−III loop, only one peptide corresponding to the Thr[671]-Leu[690] region (designated as peptide A) activated ryanodine binding and induced Ca^{2+} release from the SR. Thus, it appears that the putative activator of the skeletal muscle-type E−C coupling is localized in the Thr[671]-Leu[690] region, while some other yet unidentified function required for E−C coupling is in the Glu[726]-Pro[742] region.

The aims of the present study are first to settle the shortest region required for the activation of skeletal muscle-type E−C coupling, and second to characterize the structural requirements for this activating function. We report here that the ability of activating the RyR1 Ca^{2+} channel is localized in the carboxy-terminal 10-residue region of peptide A (As-10). Deletion of one or more amino acid residue(s) from the C-terminus of peptide As-10 virtually abolished

† This work was supported by a grant from the NIH (AR16922).

* Address to whom correspondence should be addressed at Boston Biomedical Research Institute, 20 Staniford Street, Boston, MA 02114. Tel: (617) 912-0384. Fax: (617) 912-0308. E-mail: Ikemoto@bbri.harvard.edu.

‡ Boston Biomedical Research Institute.

§ Harvard Medical School.

[1] DHP, dihydropyridine; E−C coupling, excitation−contraction coupling; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonylfluoride; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; T-tubule, transverse tubule.

S0006-2960(97)02907-3 CCC: $15.00
Published on Web 04/23/1998

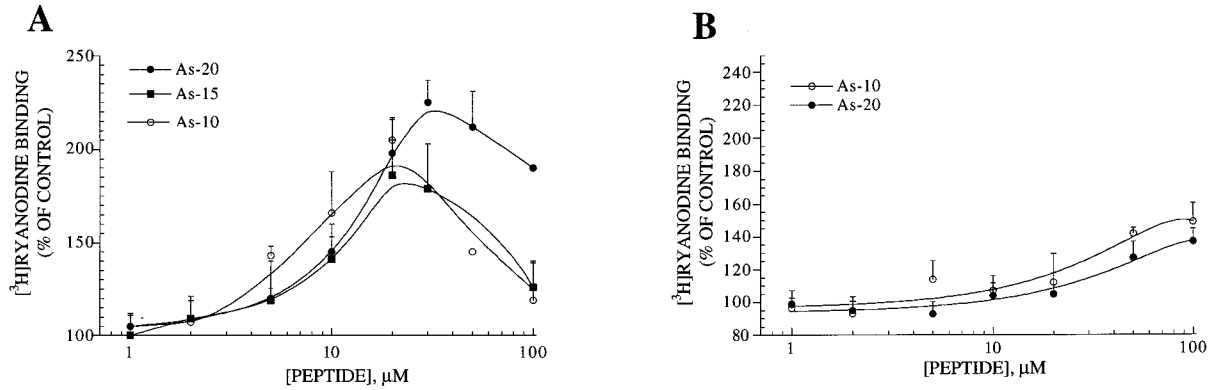

FIGURE 1: Effects of truncated forms of peptide As-20 on ryanodine binding to triads from rabbit skeletal muscle (A) or bovine cardiac muscle (B). Triads from rabbit skeletal muscle (0.5 mg/mL) or bovine heart (1.0 mg/mL) were incubated with 8 nM or 10 nM [^{3}H]-ryanodine, respectively, in a solution containing 0.3 M KCl, 10 μM $CaCl_2$, 20 mM MOPS, pH 7.2, and various concentrations of peptides as indicated. Ryanodine binding in the absence of peptides (control) was 1.31 ± 0.18 pmol/mg and 0.27 ± 0.05 pmol/mg for rabbit skeletal muscle and bovine cardiac triads, respectively. Each datum point represents the mean ±SD of at least four experiments carried out in duplicate.

its activating function, indicating that the decapeptide represents a minimum unit critical for the activation of skeletal muscle-type E−C coupling. We also modified the charge distribution as well as the amino acid sequence in the essential 10-residue peptide. These studies suggest that the charge distribution is one of the important determinants for the activation of skeletal muscle-type E−C coupling.

EXPERIMENTAL PROCEDURES

Preparation. Triad-enriched microsomal fractions were prepared from back paraspinous and hind leg rabbit skeletal or bovine cardiac muscle by a method of differential centrifugation as described previously (*19*). Microsomes from the final centrifugation were resuspended in a solution containing 0.3 M sucrose, 0.15 M Kgluconate, proteolytic enzyme inhibitors (0.1 mM PMSF, 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 N_2, and stored at −70 °C.

Synthesis of Peptides. Peptides were synthesized on an Applied Biosystems model 431 A synthesizer employing Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) as the α-amino protecting group. Peptides were cleaved and deprotected with 95% trifluoroacetic acid and purified by reversed-phase high-pressure liquid chromatography.

[^{3}H]Ryanodine Binding Assay. Triad vesicles (0.5 mg/mL rabbit skeletal, or 1.0 mg/mL bovine cardiac) were incubated in 0.1 mL of a reaction solution containing 8−10 nM [^{3}H]ryanodine (68.4 Ci/ml, DuPont NEN), 0.3 M KCl, 10 μM $CaCl_2$, 20 mM MOPS, pH 7.2 for 2 h at 36 °C in the presence or absence of various concentrations of peptides. Specific binding was calculated as the difference between the binding in the absence (total binding) and in the presence (nonspecific binding) of 10 μM nonradioactive ryanodine (*20*). Experiments were carried out in duplicate and each datum point was obtained by averaging the duplicates.

Assays of Peptide-Induced Ca^{2+} Release. Triads (1 mg/mL) were incubated in solution A (see below) to allow for active Ca^{2+} loading. After completion of Ca^{2+} loading (6−7 min), one volume of this solution was mixed with one volume of solution B (see below) containing various concentrations of peptides. The Ca^{2+} in both solutions was buffered at 3 μM using an EGTA-calcium buffer. The time course of SR Ca^{2+} release was monitored in a stopped flow apparatus (Bio-Logic SFM-3) using 20 μM arsenazo III as a Ca^{2+} indicator (*21*). Twenty to twenty-five traces (each representing 1000 data points of the time course of Ca^{2+} release) of the arsenazo III signal were averaged for each experiment. The arsenazo III signal was converted to nmoles of Ca^{2+} released/mg of protein by determining the Δ arsenazo III signal/Δ [Ca^{2+}] coefficient from a Ca^{2+} calibration curve. The data were plotted as time courses of Ca^{2+} release at respective peptide concentrations. The data were analyzed by computer fitting of a single-exponential function, $y = A(1 - e^{-kt})$ (where y is the amount of Ca^{2+} released at time t, A is the amplitude of release, and k is the rate constant of release).

Solution A consisted of 0.15 M KCl, 20 mM MES, 1 mM Mg-ATP, 10 units/mL pyruvate kinase, 5 mM phosphoenol pyruvate, pH 6.8. Solution B consisted of 0.15 M KCl, 20 mM MES, pH 6.8.

Statistical Analysis. Statistical analysis for the comparison of mean values was performed using an unpaired Student's *t*-test; $p < 0.05$ was accepted as statistically significant.

RESULTS

The Critical Activator Function of Peptide A Is Localized in Its C-terminal 10-Residue Region. A synthetic peptide corresponding to the Thr^{671}-Leu^{690} region of the DHP receptor α1 subunit II−III loop (peptide A) activates ryanodine binding and induces SR Ca^{2+} release, and represents the shortest activating peptide reported in the literature (see Introduction). To further localize the subdomain of peptide A essential for its activating function, we synthesized a series of peptides listed in Scheme 1, and examined their effects on ryanodine binding. As reported previously (*18*), peptide A (now called As-20 to distinguish it from shorter peptides we are using in the present study: s, skeletal muscle-type sequence of the II−III loop) activated ryanodine binding to the skeletal muscle isoform of RyR (RyR1) in a concentration-dependent manner (Figure 1A). Deletion of the N-terminal 5 residues (As-15) and 10 residues (As-10) did not impair the ability of the peptide to activate ryanodine binding to the RyR1, but even enhanced the activating

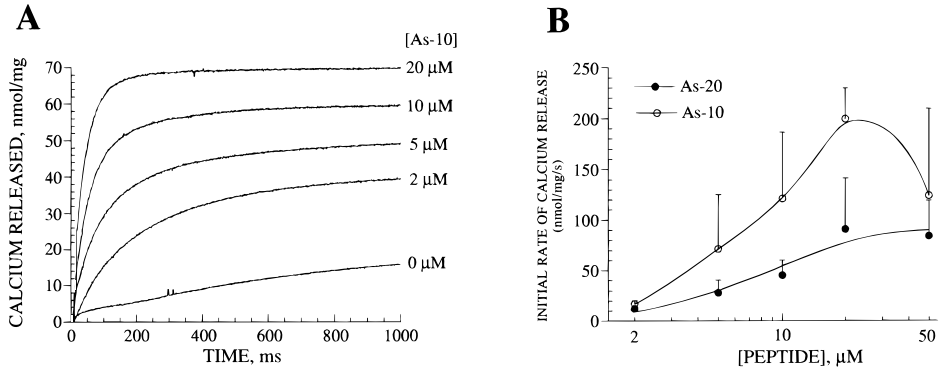

FIGURE 2: (A) Time courses of SR calcium release from rabbit skeletal muscle triad vesicles induced by various concentrations of peptide As-10. The SR moiety was first loaded with Ca^{2+} by ATP-dependent uptake and then mixed with a solution containing various concentrations of peptide As-10 to induce Ca^{2+} release (for details see Experimental Procedures). Each trace was obtained by signal averaging a total of 120−150 traces originating from four different experiments. (B) The initial rate of SR Ca^{2+} release induced by various concentrations of peptide As-20 or peptide As-10 as indicated. Each datum point represents the mean ±SD obtained from four different experiments.

function as evidenced by the fact that the dose-dependence of activation by peptide As-10 is slightly shifted to the left ($AC_{50} \approx 5$ μM; maximally activating concentration, 20 μM) compared with that by peptide As-20 (Figure 1A). Neither As-20 nor As-10 produced appreciable activation of ryanodine binding to the RyR2 (Figure 1B). These results indicate that not only the activating function but also the RyR isoform-specificity of activation are localized within the C-terminal 10-residue region (As-10) of peptide As-20.

The [peptide]-dependence of activation by these peptides is biphasic; namely, at concentrations higher than an optimum concentration (20−30 μM) the extent of activation decreased with an increase in the peptide concentration. We tentatively interpret this phenomenon as a reflection of the existence of two classes of peptide binding sites (for preliminary data, see ref. *22*): (1) specific activator site(s) that has a high affinity and is responsible for the activation at lower peptide concentrations, and (2) nonspecific sites which have a lower affinity and are responsible for the inhibition at higher peptide concentrations.

Peptide A (or peptide As-20) induces Ca^{2+} release from skeletal muscle SR, as described previously (*18*). If the subdomain critical for the activator function is localized within the As-10 region as deduced from the ryanodine binding experiments, peptide As-10 should be capable of inducing SR Ca^{2+} release. In fact this is the case, as seen in the time courses of Ca^{2+} release induced by various concentrations of peptide As-10 (Figure 2A). The initial rate of Ca^{2+} release induced by the maximally activating concentration was much higher with peptide As-10 than peptide As-20 (Figure 2B), and the AC_{50} of As-10 was significantly lower than that of As-20, indicating that the activating function was even enhanced in the 10-residue C-terminal portion of peptide As-20 after removing the N-terminal 10-residue portion.

Figure 3 depicts the dose-dependent activation of ryanodine binding to the RyR1 by a series of truncated peptides in which increasing numbers of C-terminal residues of As-10 were systematically deleted (As-9−As-5, see Scheme 1). As seen in Figure 3, a considerable loss of the ability to activate ryanodine binding to the RyR1 took place when only one C-terminal residue (Leu) was deleted from As-10. Further deletions produced small additional losses of the activating function of the peptide.

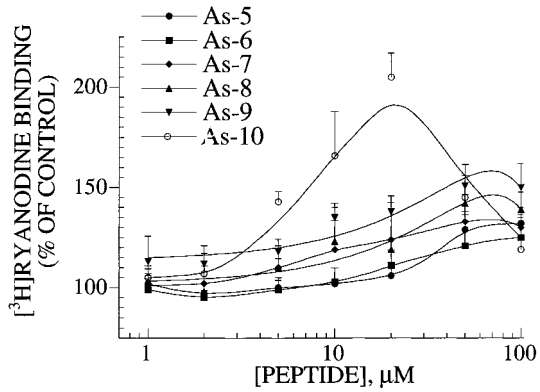

FIGURE 3: Effects of truncated forms of peptide As-10 on ryanodine binding to triads from rabbit skeletal muscle. Ryanodine binding assay conditions were as described in legend to Figure 1 (for details see Experimental Procedures). Each datum point represents the mean ±SD of at least three experiments carried out in duplicate.

Scheme 1: Amino Acid Sequences of Synthetic Peptide As-20 and Gradually Truncated Forms of I[a]

As-20	TSAQKAKAEERKRRKMSRGL
As-15	AKAEERKRRKMSRGL
As-10	RKRRKMSRGL
As-9	RKRRKMSRG
As-8	RKRRKMSR
As-7	RKRRKMS
As-6	RKRRKM
As-5	RKRRK

[a] As-20 is a peptide corresponding to the region encompassing the Thr^{671}-Leu^{690} sequence of the II-III loop of the skeletal muscle α_1 subunit of the dihydropyridine receptor.

We also investigated the Ca^{2+} release-inducing ability of these truncated peptides. As shown in Table 1, the ability of triggering Ca^{2+} release by 20 μM peptide was high in peptide As-10 as described above. Upon deletion of even one C-terminal residue, the ability of the peptide as a Ca^{2+} release trigger decreased sharply. These results altogether indicate that the C-terminal 10-residue segment of peptide As-20 (namely, As-10) represents the minimum essential region for the activation of skeletal muscle-type E−C coupling.

Clustered Basic Residues within the Activator Subdomain Play a Critical Role for Its Function. Peptide As-10 contains six positively charged residues (bold letters in Scheme 2)

Table 1: Initial Rates of SR Ca^{2+} Release Induced by 20 μM Peptide As-10 and Gradual C-Terminus Truncated Forms of the Peptide (As-9−As-5)[a]

peptide (20 μM)	initial rate of Ca^{2+} release (nmol/mg/s ± SD)
As-10	191 ± 32
As-9	34 ± 21*
As-8	18 ± 11*
As-7	8 ± 3*
As-6	13 ± 9*
As-5	9 ± 5*

[a] Ca^{2+} release was induced and monitored as described in Experimental Procedures. Data represent the mean ± SD of three experiments. Asterisk indicates $p < 0.001$.

Scheme 2: Amino Acid Sequences of Synthetic Decapeptides Used as Controls for Peptide As-10[a]

As-10	**RKRRK**MS**R**GL
As-10-M1	**RKR**L**R**G**K**SM**R**
As-10-M2	**RK**M**R**S**R**G**K**L**R**

[a] As-10-M1 and As-10-M2 are decapeptides having the same amino acid composition as As-10 but different degrees of scattering of the clustered positively charged groups (bold letters).

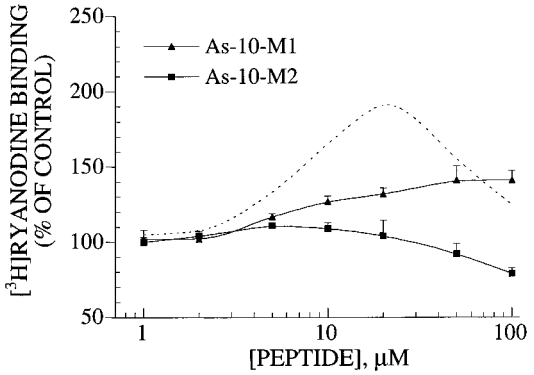

FIGURE 4: Effects of two control 10-residue peptides (As-10-M1 and As-10 M2, see Scheme 2) on ryanodine binding to rabbit skeletal muscle triads (solid lines) with a reference to that of peptide As-10 (dotted line). Ryanodine binding assay conditions were as described above. Each datum point represents the mean ±SD obtained from three different experiments carried in duplicate.

and no negatively charged group. Five out of the six positively charged residues are clustered (**RKRRK**, see Scheme 2). In an attempt to characterize structural determinants for the activator function, we synthesized several modified decapeptides as listed in Scheme 2 and examined the effects of these modifications on ryanodine binding activity. In these modifications, As-10-M1 and As-10-M2, we scrambled the sequence of As-10 to scatter the clustered positively charged residues to various extents. We investigated the dose-dependent activation of ryanodine binding to the RyR1 by these modified peptides. As shown in Figure 4, replacement of one of the five clustered basic residues with a neutral residue (As-10-M1) produced a significant reduction of the activating function. Upon replacing two of the five clustered positive residues with neutral residues (As-10-M2), the activating function was completely abolished; at higher concentrations As-10-M2 even inhibited ryanodine binding (Figure 4).

Control Experiments with a Poly-Basic Residue Peptide, Polylysine. Figure 5 shows the control experiment in which we examined the dose-dependent effects of a poly-basic

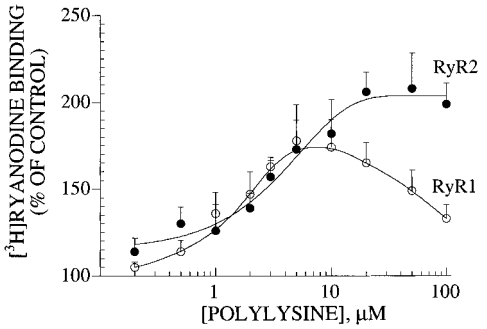

FIGURE 5: Effects of polylysine on ryanodine binding to triads from rabbit skeletal muscle (RyR1) or bovine cardiac muscle (RyR2). Ryanodine binding assay conditions were identical as in the experiments performed with the other various peptide A forms (for details see Experimental Procedures). Polylysine (4 kDa) was obtained from Sigma. Each datum point represents the mean ±SD of at least three experiments carried out in duplicate.

residue peptide polylysine (a potent Ca^{2+} release trigger by its specific binding to the RyR moiety of the triad, ref. *23*) on both RyR1 and RyR2. In agreement with our previous reports (*23*, *24*), polylysine produced a biphasic dose-dependent activation of ryanodine binding to the RyR1. An important new finding in this study is that polylysine produced the essentially identical dose-dependent activation of the RyR2 as it did to the RyR1, except that the dose-dependence of activation of the RyR2 was monophasic rather than biphasic (Figure 5). As deduced from the experiments shown in Figure 1A,B, the function of the activating loop peptides, As-20 and As-10, is clearly distinguishable from that of polylysine in that the activating function of the former peptides is specific for RyR1, but that of the latter is common to both RyR1 and RyR2. Thus, clustering of basic residues alone cannot explain the RyR1-specific activating property localized in As-10. It appears that some other factors such as specific amino acid sequence are required for the activating function of this subdomain within the II−III loop peptide.

Functions of Cardiac Counterparts of Peptide As-20 and Peptide As-10. The region of the cardiac DHP receptor α1 subunit II−III loop corresponding to peptide As-20 (designated as peptide Ac-20; c, cardiac muscle-type sequence of the II−III loop) shows a high degree of sequence homology with peptide As-20, but there are some differences (see Scheme 3). We examined the effect of the synthetic peptide Ac-20 on ryanodine binding to RyR1 (Figure 6). In contrast to peptide As-20, peptide Ac-20 had virtually no effect on ryanodine binding. Interestingly, however, the cardiac counterpart of peptide As-10 (designated as Ac-10) produced an appreciable extent of activation (Figure 6), although the AC_{50} for activation (30 μM) was significantly higher than that of peptide As-10 (5 μM). In peptide Ac-10, one of the five clustered basic residues seen in As-10 is replaced with Asp (see Scheme 3). To mimic the cluster of five basic residues seen in As-10, this Asp was changed to Lys (peptide Ac-10-M1, see Scheme 3). Upon forming the five-basic residue cluster in Ac-10-M1, the activating function was partially restored (AC_{50} = 13 μM), although the activating function of Ac-10-M1 was still less than that of As-10. These results further support the notion that the five-basic residue cluster present in the 10-residue subdomain of the II−III loop plays an important role in its activating function.

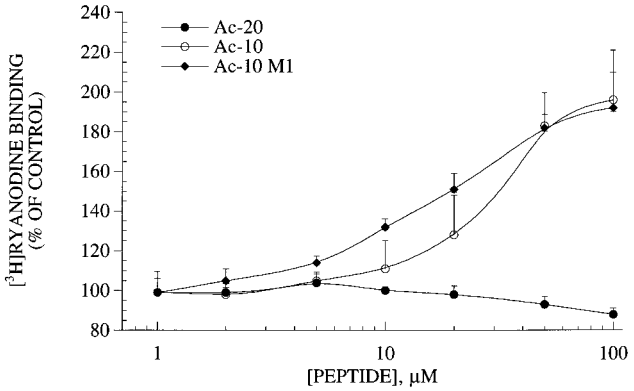

FIGURE 6: Effects of peptides Ac-20, Ac-10, and Ac-10-M1 on ryanodine binding to triads from rabbit skeletal muscle. Each datum point represents the mean ±SD of at least three experiments carried out in duplicate.

Scheme 3: Amino Acid Sequences of Synthetic Peptide Ac-20, Truncated and Chemically Mutated Forms of It[a]

Ac-20	TSAQKEEEEKERKKLARTA
Ac-N10	TSAQKEEEE
Ac-10	KERKKLARTA
Ac-10-M1	KKRKKLARTA

[a] Ac-20, the cardiac isoform of peptide As-20, is a peptide corresponding to the region encompassing the Thr^{671}-Ala^{690} sequence of the II-III loop of the cardiac muscle α_1 subunit of the dihydropyridine receptor. Ac-10 and Ac-N10 are decapeptides corresponding to the C- and N-terminal halves of Ac-20, respectively. Ac-10-M1 is a chemical mutant of Ac-10 in which Glu^{682} is replaced by Lys.

Table 2: Effect of 20 μM Peptides Ac-10 or As-10 on Ryanodine Binding to Triads from Rabbit Skeletal Muscle in the Absence or Presence of an Equimolar Concentration of Peptide Ac-N10[a]

	[³H]ryanodine binding (% of control)	
Ac-N10	Ac-10 (20 μM)	As-10 (20 μM)
0 μM	124 ± 19	212 ± 17
20 μM	101 ± 2*	161 ± 9*

[a] Data represent the mean ± SD of three experiments performed in duplicate. Asterisk indicates $p < 0.05$.

As described above, peptide Ac-20 totally lacks activating function, although its C-terminal 10-residue portion retains an appreciable level of activity. This is probably due to the fact that its N-terminal 10-residue portion which is enriched in acidic residues may counteract with the activating function of the C-terminal portion. To test this hypothesis, we synthesized a 10-residue peptide corresponding to the N-terminal half of peptide Ac-20, designated as Ac-N10 (see Scheme 3). Then, the effects of addition of an equimolar amount of peptide Ac-N10 to the activating decapeptides (Ac-10 and As-10) were investigated. As shown in Table 2, the addition of peptide Ac-N10 resulted in a considerable suppression of the activating function of peptide Ac-10 and peptide As-10 as well, indicating that the N-terminal half of peptide Ac-20 in fact works as a suppressor.

DISCUSSION

The molecular mechanism by which transient changes in the T-tubule membrane potential lead to rapid Ca^{2+} release from the SR is one of the most important unsolved questions in muscle physiology (*1*−*5*). Recent studies have resolved two important components in the E−C coupling process, namely the DHP receptor and the ryanodine receptor (*25*−*33*), and have firmly established their roles. For skeletal muscle E−C coupling, changes in the T-tubule membrane potential are sensed by the DHP receptor α_1 subunit (*12*, *13*, *34*), generating an electro-mechanical signal in the DHP receptor (*30*, *35*, *36*). The signal is then transmitted to the ryanodine receptor presumably by mediation of physical interactions between the two proteins (*11*), leading to the opening of the SR Ca^{2+} channel located within the ryanodine receptor (*27*). As described in Introduction, recent studies suggest that the II−III loop of the DHP receptor α_1 subunit is at least a part of the major sites involved in the interaction with the ryanodine receptor (*14*−*18*).

As shown in our previous study with several synthetic peptides corresponding to various regions of the II−III loop (*18*), of all the peptides we tested only the 20-residue peptide corresponding to the Thr^{671}-Leu^{690} region (named peptide A; in this study called As-20 to distinguish it from many other peptides used) produced significant activation of ryanodine binding and SR Ca^{2+} release. This suggested that the signal transmission site of the II−III loop is located within this region. One of the important findings in the present study is that peptide A activates RyR1 but produces virtually no effect on the RyR2. This is particularly significant since the mechanisms of physiological activation of the RyR1 and the RyR2 are different. In skeletal muscle, in contradistinction to cardiac muscle, a physical interaction between the DHP receptor and the RyR is believed to be responsible for stimulation of SR calcium release. Thus, this provides a strong evidence for the view that the region of the II−III loop corresponding to peptide A represents the putative trigger of the skeletal muscle-type E−C coupling.

The important questions we addressed in the present study were (a) whether an even shorter region of peptide A (or peptide As-20) retains the physiologic trigger function, and if so (b) what is the minimum essential unit for the function. It was found that the C-terminal 10-residue portion of peptide As-20 is sufficient to activate ryanodine binding and induce SR Ca^{2+} release. The activating function was even more enhanced in the 10-residue peptide than in the 20-residue peptide. Furthermore, the RyR1-specificity of peptide activation is retained in the 10-residue peptide. However, truncation of one or more amino acid residue(s) from the C-terminus of As-10 produced a significant reduction in the activating function of the peptide. Similarly, any truncation from the N-terminus of the peptide would produce a significant reduction in the peptide's activity, because as discussed below, the five consecutive positively charged residues located at the N-terminal portion of peptide As-10 seem to be essential for its activating function. From these results altogether, we propose that the As-10 region represents the minimum essential unit for triggering skeletal muscle-type E−C coupling.

The present study has permitted several new insights into the structural basis for the activating function of peptide A as outlined below. A cluster of the five basic residues located in the N-terminal side of As-10 (RKRRK) seems to be one of the determinants of the activating function. Scattering of the clustered basic residues to various extents produced corresponding decreases in the activator function. Conversely, reforming of the cluster of five basic residues by mutating the cardiac-10-residue II−III loop peptide (Ac-10)

restored a significant part of the activator function of Ac-10. Moreover, as revealed in this study the N-terminal half of peptide Ac-20 that is enriched in the negatively charged residues suppresses the activating function of its C-terminal half (that is, peptide Ac-10) as well as As-10. This is also consistent with the postulated role of the clustered positive charges in the activating function.

Although it is clear that the charge distribution is an important determinant for the activating function, more factors appear to be involved in the physiologic trigger mechanism, since the poly-basic residue peptide, polylysine, does not have the RyR1-specificity of activation, which is one of the important criteria for the physiologic trigger as described above. In view of the present finding that the potency of activation of the skeletal decapeptide (As-10) is much higher than that of the cardiac counterpart even after equalizing the charge distribution (namely, Ac-10-M1), we propose that the skeletal muscle-type amino acid sequence is another important determinant of the activating function. Thus, there are at least two requirements for the activating function of the 10-residue activator subdomain of the II−III loop: (a) the number and distribution of positive charges, and (b) skeletal muscle-type sequence.

In conclusion, the present study suggests that the 10-residue region of the DHP receptor α_1 subunit II−III loop encompassing Arg^{681}-Leu^{690} represents the minimum unit essential for the physiologic trigger of skeletal muscle-type E−C coupling. The number and the distribution of positive charges present as well as the skeletal muscle-type amino acid sequence of this region are important determinants for the ability of activating ryanodine binding and triggering SR Ca^{2+} release in a RyR1-specific manner.

ACKNOWLEDGMENT

The authors thank Dr. Graham D. Lamb for his comments on the manuscript, and Dr. Renne C. Lu, Dr. Paul Leavis, Ms. Anna Wong, and Ms. Elizabeth Gowell for their help in the synthesis and purification of the peptides.

REFERENCES

1. Fleischer, S., and Inui, M. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 333−364.
2. Ebashi, S. (1991) *Annu. Rev. Physiol. 53*, 1−16.
3. Rios, E., and Pizarro, G. (1991) *Physiol. Rev. 71*, 849−908.
4. Ashley, C. C., Mulligan, P., and Lea, T. J. (1991) *Q. Rev. Biophys. 24*, 1−73.
5. Rios, E., Pizzaro, G., and Stefani, E. (1992) *Annu. Rev. Physiol. 54*, 109−133.
6. Schneider, M. F. (1994) *Annu. Rev. Physiol. 56*, 463−484.
7. Meissner, G. (1994) *Annu. Rev. Physiol. 56*, 485−508.
8. Melzer, W., Herrmann-Frank, A., and Luttgau, H. Ch. (1995) *Biochim. Biophys. Acta 1241*, 59−116.
9. Chandler, W. K., Rakowsky, R. F., and Schneider, M. F. (1976) *J. Physiol. 254*, 285−316.
10. Garcia, J., Tanabe, T., and Beam, K. G. (1994) *J. Gen. Physiol. 103*, 125−147.
11. Rios, E., Ma, J., and Gonzalez, A. (1991) *J. Muscle Res. Cell Motil. 12*, 127−135.
12. Tanabe, T., Beam, K. G., Powell, J. A., and Numa, S. (1988) *Nature 336*, 134−139.
13. Tanabe, T., Beam, K. G., Adams, B. A., Niidome, T., and Numa, S. (1990) *Nature 346*, 567−569.
14. Lu, X., Xu, L., and Meissner, G. (1994) *J. Biol. Chem. 269*, 6511−6516.
15. Lu, X., Xu, L., and Meissner, G. (1995) *Biophys. J. 68*, A372 (abstract).
16. Lu, X., Xu, L., and Meissner, G. (1995) *J. Biol. Chem. 270*, 18459−18464.
17. Nakai, J., Tanabe, T., and Beam, K. G. (1995) *Biophys. J. 68*, A14 (abstract).
18. El-Hayek, R., Antoniu, B., Wang, J. P., Hamilton, S. L., and Ikemoto, N. (1995) *J. Biol. Chem. 270*, 22116−22118.
19. Ikemoto, N., Kim, D. H., and Antoniu, B. (1988) *Methods Enzymol. 157*, 469−480.
20. El-Hayek, R., Valdivia, C., Valdivia, H. H., Hogan, K., and Coronado, R. (1993) *Biophys. J. 65*, 779−789.
21. Ikemoto, N., Ronjat, M., Meszaros, L. G., and Koshita, M. (1989) *Biochemistry 28*, 6764−6771.
22. El-Hayek, R., and Ikemoto, N. (1997) *Biophys. J. 72*, A46 (abstract).
23. Cifuentes, M. E., Ronjat, M., and Ikemoto, N. (1989) *Arch. Biochem. Biophys. 273*, 554−561.
24. El-Hayek, R., Yano, M., and Ikemoto, N. (1995) *J. Biol. Chem. 270*, 15634−15638.
25. Caterall, W. A. (1991) *Cell 64*, 871−874.
26. Fleischer, S., Ogunbunmi, M., Dixon, M. C., and Fleer, E. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 7256−7259.
27. Imagawa, T., Smith, J., Coronado, R., and Campbell, K. (1987) *J. Biol. Chem. 262*, 16636−16643.
28. Campbell, K. P., Leung, A. T., and Sharp, A. H. (1988) *Trends Neurosci. 11*, 425−430.
29. Coronado, R., Morrissette, J., Sukhareva, M., and Vaughan, D. M. (1994) *Am. J. Physiol. 266*, C1485−C1504.
30. Rios, E., and Brum, G. (1987) *Nature 325*, 717−720.
31. Schwartz, L. M., McCleskey, E. W., and Almers, W. (1985) *Nature 314*, 747−751.
32. Caterall, W. A. (1988) *Science 242*, 50−61.
33. Lamb, G. D. (1992) *J. Muscle Res. Cell Motil. 13*, 394−405.
34. Knudson, M. C., Chaudhari, N., Sharp, A. H., Powell, J. A., Beam. K. G., and Campbell, K. P. (1989) *J. Biol. Chem. 264*, 1345−1348.
35. Beam, K. G., Adams, B. A., Niidome, T., Numa, S., and Tanabe, T. (1992) *Nature 360*, 169−171.
36. Adams, B. A., Tanabe, T., Mikami, A., Numa, S., and Beam, K. G. (1990) *Nature 346*, 569−572.

BI972907O