A Structural Requirement for Activation of Skeletal Ryanodine Receptors by Peptides of the Dihydropyridine Receptor II-III Loop*

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The solution structures of three related peptides (A1, A2, and A9) corresponding to the Thr<sup>671</sup>-Leu<sup>690</sup> region of the skeletal muscle dihydropyridine receptor II-III loop have been investigated using nuclear magnetic resonance spectroscopy. Peptide A1, the native sequence, is less effective in activating ryanodine receptor calcium release channels than A2 (Ser<sup>687</sup> to Ala substitution). Peptide A9, Arg<sup>681</sup>-Ser<sup>687</sup>, does not activate ryanodine receptors. A1 and A2 are helical from their N terminus to Lys<sup>685</sup> but are generally unstructured from Lys<sup>685</sup> to the C terminus. The basic residues Arg<sup>681</sup>-Lys<sup>685</sup>, essential for A1 activation of ryanodine receptors, are located at the C-terminal end of the α-helix. Peptide A9 was found to be unstructured. Differences between A1 and A2 were observed in the C-terminal end of the helix (residues 681–685), which was less ordered in A1, and in the C-terminal region of the peptide, which exhibited greater flexibility in A1. Predicted low energy models suggest that an electrostatic interaction between the hydroxyl of Ser<sup>687</sup> and the guanidino moiety of Arg<sup>685</sup> is lost with the Ser<sup>687</sup>Ala substitution. The results show that the more structured peptides are more effective in activating ryanodine receptors.

The ryanodine receptor (RyR)<sup>1</sup> calcium release channel in striated muscle sarcoplasmic reticulum (SR) is an essential component of excitation-contraction coupling (ECC). RyRs are activated during ECC by a signal originating in t-type Ca<sup>2+</sup> channels (dihydropyridine receptors (DHPRs)). The α<sub>1</sub> subunit of the DHPR contains charged residues in its S4 segment that sense plasmalemma or transverse tubule depolarization and either open the DHPR Ca<sup>2+</sup> channel to allow the influx of Ca<sup>2+</sup> ions, which activate RyRs in cardiac muscle, or initiate a protein-protein interaction with the RyR in skeletal muscle. The loop between the second and third repeats (II-III loop) of the skeletal DHPR is required for skeletal ECC, which is independent of cytoplasmic Ca<sup>2+</sup> (<sup>3, 4</sup>). Sites of interaction between the II-III loop and the RyR have been identified. A region of the DHPR II-III loop between Glu<sup>671</sup>-Leu<sup>690</sup> binds to the RyR in surface plasmon resonance studies (<sup>2</sup>). RyRs are activated by the full skeletal DHPR II-III loop and by the 20-amino acid peptide A1 corresponding to Glu<sup>671</sup>-Leu<sup>690</sup> (<sup>3–7</sup>). A peptide A1/II-III loop interaction site on the skeletal RyR between Arg<sup>679</sup> and Asp<sup>1112</sup> has also been reported (<sup>8</sup>). The peptide A1 region may be necessary for the DHPR II-III loop to bind to the RyR, whereas other regions of the loop are necessary for the transmission of the ECC signal from the S4 part of the DHPR to the II-III loop/RyR binding region (<sup>7</sup>). Evidence for this hypothesis is that the cardiac DHPR can support skeletal type ECC when the peptide A region contains the cardiac sequence, provided that residues 725–742 contain the skeletal sequence (<sup>9</sup>, suggesting that (a) both cardiac and skeletal peptide A1 regions bind to the RyR, and (b) residues 725–742 are essential for transmission of the ECC (<sup>7</sup>). If this hypothesis is correct, activation of RyR channels by peptide A1 or by the II-III loop may reflect binding to the RyR, rather than activation equivalent to ECC. The hypothesis is supported by the fact that the full cardiac II-III loop activated skeletal RyRs (<sup>3, 4</sup>).

Two regions of peptide A1 are important in its interaction with RyRs. The highly charged Glu<sup>685</sup>RKKRR<sup>690</sup> sequence is essential for RyR activation by peptide A1 (<sup>6, 10</sup>), and Ser<sup>687</sup> is important, although its precise role is not clear. The II-III loop does not activate RyRs when Ser<sup>687</sup> is either phosphorylated or replaced by alanine (<sup>4</sup>). In contrast, replacement of Ser<sup>687</sup> with Ala increases the ability of peptide A1 to activate RyRs (<sup>7</sup>), whereas a similar replacement in a 25-amino acid peptide (Glu<sup>666</sup>-Pro<sup>690</sup>) reduces [H]ryanodine binding (<sup>11</sup>). The different effects of Ser<sup>687</sup> to Ala substitution are likely to reflect structural differences between peptides of different lengths. The structure of the protein-protein interaction sites on the II-III loop and on the RyR must be understood in order to comprehend these functional observations and the structural changes that could occur during ECC. Neither structure has been determined.

Here, we address the structural basis for Ca<sup>2+</sup>+ release from the SR by the native DHPR sequence from Thr<sup>671</sup>-Leu<sup>690</sup> (peptide A1) and the enhanced ability of peptide A2 (S687A substitution) to release Ca<sup>2+</sup> from skeletal SR and to activate skeletal RyRs. We determined the solution structures for three peptides using NMR spectroscopy. These peptides are a small, seven-residue peptide encompassing the basic region essential for RyR activation (A9), peptide A1 (containing Ser<sup>687</sup>), and peptide A2 (containing a S687A substitution). The results show that the smaller A9 peptide is unstructured, whereas both peptides A1 and A2 have a propensity to form helical structures at their N-terminal end. The C-terminal part of A1 is highly mobile, whereas the C-terminal part of A2 is more constrained. Because we find that A9 is not effective in releasing Ca<sup>2+</sup> from...

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† The abbreviations used are: RyR, ryanodine receptor; SR, sarcoplasmic reticulum; DHPR, dihydropyridine receptor; ECC, excitation-contraction coupling; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; DQF-COSY, double quantum filtered correlated spectroscopy; CSI, chemical shift index; rmsd, root mean square deviation; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

* The hypothesis is supported by the fact that the full cardiac II-III loop activated skeletal RyRs (<sup>3, 4</sup>). The hypothesis is supported by the fact that the full cardiac II-III loop activated skeletal RyRs (<sup>3, 4</sup>).
SR or in activating RyR channels, the relative activity of the three peptides is closely correlated with their helical content.

**EXPERIMENTAL PROCEDURES**

**Peptides**—DHPR II-III loop peptides were synthesized as described by Dulhunty et al. (7). Test peptides were as follows: A1, ThrArgLys-Ala-Lys-Ala-Glu-Glu-Arg-Lys-Arg-Lys-Met-Glu-Glu-Lys-Arg-Arg-Glu-Arg-Asp-Met-Ala-Ara-Asp-Glu-Leu; A2, ThrArgLys-Ala-Lys-Ala-Glu-Glu-Arg-Lys-Arg-Lys-Met-Glu-Glu-Lys-Arg-Arg-Glu-Arg-Asp-Met-Ala-Ara-Asp-Glu-Leu; and A9, ArgArgArg-Lys-Arg-Lys-Met-See-See-See.

Optimum NMR conditions were established after multiple trials with various salt and pH conditions. For final NMR studies, all peptides were dissolved in water containing 10% D2O/90% H2O to a final concentration of 4 mM and adjusted to pH 5.0.

**NMR Spectroscopy**—All spectra were acquired on a Varian-Inova 600 spectrometer using a spectral width of 6000 Hz, a pulse delay of 7 ms (90°), and acquisition time of 0.130 s, collecting 2048 data points and 512 increments of 32 transients. NOESY and rotating frame NOE spectroscopy (12, 13) spectra (mixing time of 200–500 ms), total correlation spectroscopy (14) spectra (mixing time of 70 ms), and DQF-COSY (15) spectra were acquired at 278 K and used for the assignment of the 1H NMR resonances. Suppression of the H2O resonance for the NOESY and NOE spectroscopy (mixing time of 200–500 ms), total correlation spectroscopy (14) spectra (mixing time of 70 ms), and DQF-COSY (15) spectra was achieved using pulse field gradients (16, 17), whereas a presaturation pulse was employed for the rotating frame NOE spectroscopy and total correlation spectroscopy experiments. Temperature coefficients (18) and hence the patterns of backbone hydrogen bonding were assessed by monitoring the chemical shift values of the amide backbone protons with changes in temperature (277, 285, and 298 K) in the DQF-COSY experiments. The two-dimensional data were processed on an O3 Silicon Graphics workstation using the Felix 95 software package. Data sets were zero-filled to 4096 by 2048 K and multiplied by a phase-shifted sine-bell-squared function in both dimensions prior to transformation. An internal standard, 2,2-dimethyl-2-silapentane-5-sulfonate was used as a chemical shift reference (0.00 ppm). 3JHH coupling constants were derived from one-dimensional 1H spectra where possible and/or fitted from one-dimensional slices extracted from DQF-COSY spectra.

**Structure Calculations**—Distance constraints were derived manually from a two-dimensional NOESY spectrum acquired with a mixing time of 200 ms. Upper bounds were derived from NOE cross-peak intensities by counting the number of contour levels of nonoverlapped cross-peaks and using distances calibrated from Glu γ-methylene protons. NOE signals were classified into four categories of upper distance limits of 2.7, 3.5, 4.5, and 5.8 Å and lower distance limits of 1.9 Å or lower in all cases. Dihedral angle restraints based on the 3JNN coupling constant (2.7, 3.5, 4.5, and 5.8 Å) and lower distance limits of 1.9 Å in all cases. Results included in the final calculations, with a tolerance of 20°.

Structure calculations were performed with the X-PLOR program (19, 20) using the simulated annealing protocol on a Silicon Graphics O2 workstation. The “topallhdg.pro” topology file and “parallhdg.pro” parameters within the program were used. The sum averaging option parameter file within the program were used. The sum averaging option was used to treat equivalent and nonstereo specifically assigned protons. Calculations were conducted iteratively by checking violations after each round and adding or refining constraints accordingly. Hydrogen bonds were identified and introduced after initial calculations. These were simulated by two distance constraints: a 3.3-Å restraint between the amide nitrogen and carbonyl oxygen and a 2.3-Å restraint between the amide nitrogen and the carbonyl oxygen. Initially, 100 fully extended structures with random coordinates were generated. Following the calculations, structures were analyzed, and 20 were selected on the basis of distance and dihedral constraint violations and low energies. None of the 20 structures had NOE violations greater than 0.5 Å or dihedral violations greater than 5°. For structural comparisons, individual structures from the ensemble were superimposed onto the average structure.

Peptide modeling was performed using the MSI software Insight 97 and the α-helix predicted by the Chou-Fasman algorithm (21) modeled on an extended structure. The extended structure was then minimized by the program Discover, firstly by steepest descent and finally by the conjugate gradient method until the maximum derivative was less than 0.001 kcal K–1 A–1.

**Ca2+ Release from SR Vesicles and Single Channel Recording Techniques**—Rabbit skeletal SR vesicles were prepared, Ca2+ release from SR measured, and single channel experiments performed as described by Laver et al. (22) and Dulhunty et al. (7). Ca2+ release from SR vesicles partially loaded with Ca2+ was measured using antipyrilazo III as a Ca2+ indicator. In bilayer experiments, SR vesicles and peptides were added to the cis chamber. Bilayer potential is expressed as Vcis – Vtrans (i.e. Vcispriamus – Vlumam). RyR channel activity was recorded using a cissolution containing 230 mM cesium methanesulfonate, 20 mM CsCl, 100 mM CaCl2, and 10 mM N-tris(hyroxymethyl)methyl-2-aminooethanesulfonic acid (TES, pH 7.4, with CsOH) and a trans solution containing 230 mM cesium methanesulfonate, 20 mM CsCl, 1 mM CaCl2, and 10 mM TES (pH 7.4). RyR channel activity was recorded over a range of peptide concentrations at +40 mV and –40 mV. Potential was changed every 30 s, and activity was recorded (and currents were measured) for 2 min under control conditions and after each addition of peptide to the cis chamber.

**RESULTS**

**Functional Characteristics of Peptides A1 and A2**—Peptides A1 and A2 activate Ca2+ release from skeletal SR and skeletal RyR channels (7). Peptide A2 releases Ca2+ from SR vesicles at rates that are substantially greater than rates of release induced by A1 (Fig. 1A). Peptide A2 produces 2-fold greater activation than A1 at +40 mV (Fig. 1C). In contrast to A1 and A2, the shorter 7-amino acid peptide containing the essential basic residues had little effect on Ca2+ release from SR (Fig. 1A) and failed to activate RyR channels (Fig. 1B). Indeed, RyR...
NMR Studies of Peptides A1 and A2—The complete proton assignments for A1 and A2 are presented in Table I and were determined using standard two-dimensional methods (23), which involved two-dimensional total correlation spectroscopy and DQF-COSY experiments to identify spin types and NOESY experiments to make sequence specific assignments. An illustration of the quality of the spectra is shown for the amide proton Hα of Peptide A2 at 2.3 ppm (Fig. 3). The relative abilities of NH, Hα, and Hβ to release Ca2⁺ from the SR and to activate RyRs was A2 < A1. A2 > A1 > A9.

The different concentration dependence of activation of Ca2⁺ release from SR vesicles (i.e. activation of RyRs in vesicles) and activation of single RyR channels in bilayers has also been reported for the effects of the full II-III loop on RyR activity (3, 4).

### Table I

| Residue  | NH Hά Hβ Other 3J NH-Hα | Temperature coefficient Hz ppb/K |
|----------|--------------------------|-------------------------------|
| Peptide A1  |                          |                               |
| Thr^771   | 8.39 4.35 4.42 Hγ 1.27  | -8.1                          |
| Ser^772   | 8.83 4.31 3.93/3.96  | 5.1 -13.8                     |
| Ala^773   | 8.54 4.27 1.42  | 5.2 +5.8                     |
| Glu^774   | 8.17 4.11 2.08/2.15 Hγ 2.44/2.44, NH, 7.63/6.98 | 5.7 -8.5 |
| Lys^775   | 8.40 4.14 1.83/1.88 Hγ 1.40/1.50, Hδ 1.66, Hε 2.96, HeNH^+ 7.61 |
| Ala^776   | 8.23 4.22 1.47  | 4.3 -0.5                     |
| Lys^777   | 8.11 4.17 1.87 Hγ 1.48/1.55, Hδ 1.68, Hε 2.97, HeNH^+ 7.61 |
| Ala^778   | 8.15 4.16 1.49  | -a                          |
| Glu^779   | 8.34 4.13 2.15  | 5.3 -1.4                     |
| Glu^800   | 8.18 4.11 2.12  | 5.3 -0.7                     |
| Arg^801   | 8.13 4.10 1.78/1.89 Hγ 1.61, Hδ 3.21, NH 7.33 | 5.7 -a |
| Lys^802   | 7.95 4.13 1.85/1.92 Hγ 1.40/1.53, Hδ 1.68, Hε 2.95, HeNH^+ 7.61 |
| Arg^803   | 8.19 4.19 1.76/1.89 Hγ 1.62, Hδ 3.22, NH 7.50 | -a e |
| Arg^804   | 8.20 4.20 1.74/1.86  | 7.2 -3.3                      |
| Lys^805   | 8.21 4.22 1.82/1.87 Hγ 1.44/1.50, Hδ 1.66, Hε 2.98, HeNH^+ 7.63  |
| Met^806   | 8.33 4.48 2.05/2.14 Hγ 2.58/2.67, HCH, 2.14  | 7.2 -6.7                      |
| Ser^807   | 8.31 4.43 3.90  | 7.2 -2.9                     |
| Arg^808   | 8.44 4.34 1.80/1.91  | 7.2 -3.3                      |
| Gly^809   | 8.46 3.96/3.96  | 7.2 -2.8                     |
| Leu^900   | 8.30 4.30 1.66/1.66 Hγ 0.58, Hδ 0.86/0.92  | 7.2 -6.7                      |
| Peptide A2  |                          |                               |
| Thr^771   | 8.39 4.35 4.43  | 8.0 -7.6                     |
| Ser^772   | 8.83 4.32 3.94/3.98  | 4.4 -13.3                    |
| Ala^773   | 8.53 4.27 1.43  | 4.2 -7.6                     |
| Glu^774   | 8.16 4.10 2.09/2.17 Hγ 2.46/2.46, NH, 7.63/6.97 | 0 e |
| Lys^775   | 8.40 4.14 1.84/1.88 Hγ 1.42/1.53, Hδ 1.66, Hε 2.95, HeNH^+ 7.61  |
| Ala^776   | 8.22 4.23 1.48  | 5.3 -1.9                     |
| Lys^777   | 8.10 4.17 1.88 Hγ 1.50/1.57, Hδ 1.67, Hε 2.96, HeNH^+ 7.62  | 7.2 -1.4                      |
| Ala^778   | 8.13 4.17 1.50  | 5.3 -1.4                     |
| Glu^779   | 8.34 4.13 2.10  | 5.6 -1.4                     |
| Glu^800   | 8.17 4.12 2.12  | 5.4 -0.7                     |
| Arg^801   | 8.12 4.08 1.80/1.90 Hγ 1.63, Hδ 3.24, NH 7.33 | 5.4 -0.3 |
| Lys^802   | 7.93 4.13 1.89/1.94 Hγ 1.41/1.54, Hδ 1.70, Hε 2.95, HeNH^+ 7.61 |
| Arg^803   | 8.16 4.18 1.79/1.90 Hγ 1.65, Hδ 3.23, NH 7.53 | 4.2 -1.9                     |
| Arg^804   | 8.19 4.20 1.77/1.88 Hγ 1.65, Hδ 3.22, NH 7.47 | 7.2 -2.4                     |
| Lys^805   | 8.22 4.19 1.85/1.88 Hγ 1.44/1.51, Hδ 1.67, Hε 2.98, HeNH^+ 7.60  |
| Met^806   | 8.25 4.40 2.07/2.12 Hγ 2.59/2.68, HCH, 2.14  | 7.2 -1.0                     |
| Ala^807   | 8.21 4.30 1.43  | 7.2 -0.5                     |
| Arg^808   | 8.31 4.30 1.82/1.90  | 7.2 -3.9                      |
| Gly^809   | 8.42 3.97/3.97  | 7.2 -2.9                     |
| Leu^900   | 8.26 4.31 1.69/1.67 Hγ 1.62, Hδ 0.87/0.93  | 7.2 -6.2                      |

*a Temperature coefficients could not be quantified but are greater than -2.0.

*b Temperature coefficients could not be quantified but are less than -2.0.

activity was less than control in the presence of the peptides. The decrease in activity reflects the inhibitory action of the peptide, which is also seen as a decline in activation of single channels by A1 and A2 at [peptide] greater than 10 nM. This inhibitory action of the peptide is not seen in Ca²⁺ release from SR vesicles. The average mean current flowing through RyRs at +40 mV and −40 mV did not increase after addition of A9 at any peptide concentration up to 50 μM. The relative abilities of the peptides to release Ca²⁺ from the SR and to activate RyRs was A2 < A1 > A9.

Table I shows the different concentration dependence of activation of Ca²⁺ release from SR vesicles (i.e. activation of RyRs in vesicles) and activation of single RyR channels in bilayers has also been reported for the effects of the full II-III loop on RyR activity (3, 4).
Substitution of Ser^{687} for Ala gives rise to some NOE differences in the NOESY spectrum. This is most clearly seen in Fig. 3, where additional medium range NOEs (NH-NH, aH-NH, aH-NH, and aH-NH, aH-NH) are observed for A2 compared with the A1 peptide, particularly for the basic region stretching from Arg^{681} to Lys^{685}. More modest changes are observed for the NOEs at the C-terminal end of the peptide; this is accompanied by slight differences in the temperature coefficients, as well as chemical shift index data (data not shown), it is apparent that this peptide possesses little if any structure.

NMR analysis was also carried out on the A9 peptide. Complete assignment of the side chains was not possible due to the high degree of peak overlap. However, based on NOESY/rotating frame NOE spectroscopy cross-peak patterns, temperature coefficients (less than –2.0), 3J_{NH-H_\alpha} coupling constants (less than 6 Hz), and chemical shift index data (data not shown), it is apparent that this peptide possesses little if any structure.

Analysis of the NOESY spectra gave rise to a total of 216 and 236 distance constraints for peptides A1 and A2, respectively, the details of which are summarized in Table II. Apart from the 236 distance constraints for peptides A1 and A2, respectively, the resulting structures had distance violations greater than 0.5 Å or dihedral violations greater than 5°. In both structures, helical constraints were also included in the structure calculations, in which it was shown that the 3J_{NH-H_\alpha} coupling constant, temperature coefficient, and CSI data supported this constraint.

The structural statistics including average energies and rmsd values for peptides A1 and A2 are summarized in Table II. This shows that for the 20 calculated lowest energy structures, peptide A2 has a slightly smaller overall backbone rmsd value, whereas the converse is true for residues 671–680. However, when an additional 5 residues were examined, the rmsd values for residues 671–685 were marginally smaller for A2, suggesting that the helical structure of the basic region (681–685) was more ordered in A2. Fig. 4 shows the superposition of the 20 lowest energy structures for peptides A1 and A2. None of the structures had dihedral violations greater than 0.5 Å or dihedral violations greater than 5°. In both structures, helical segments are observed extending from the N-terminal ends to residue 685, with the remaining residues being disordered. It is clear that the A2 peptide has a more defined helical structure, particularly toward the N-terminal helix end.

**DISCUSSION**

General Structure of Peptides A1 and A2 Determined by NMR—Structural analysis by NMR of the A1 and A2 peptides is depicted in Fig. 5 and, in general, both models show good correlation with the experimentally derived NMR structures. The key features for both peptides is the \( \alpha \)-helical region extending from the N terminus to Lys^{685}. A key difference between the A1 and A2 peptides is seen toward the C terminus, where the hydroxyl group of Ser^{687} (A1) is capable of interacting with the helix via the guanidino group of Arg^{683}. Substitution of Ser^{687} for Ala in A2 precludes the possibility of such an interaction, and the C terminus extends away from the helix (see Fig. 5).
could directly correlate the structure with functional effects of these peptides on RyRs. The structures of A1 and A2 correlate well with the predicted model structures presented here but were substantially different from the previously predicted structures (10). The NMR data indicated that A1 and A2 consist of a helical segment extending from the N terminus to residue Lys685, which is then followed by an unstructured region through to the C terminus. In both A1 and A2, the basic residues Arg681–Lys685, which are essential for peptide A1 activation of RyRs (6, 10), are located at the C-terminal end of the helix and thus in a region of the peptide that is structured. The α-helix is stabilized by amide-carbonyl backbone interactions normally associated with helical-type structures, as well as various favorable side chain interactions. One such interaction involves the Thr671 side chain with the Gln674 backbone amide. It has been noted that an N-terminal threonine side chain is capable of interacting with an i + 3 backbone amide proton (25), thus aiding in helix stabilization. Several NOEs were seen between the Thr671 side chain and side chains of the residues Ala673 and Gln674, providing supporting evidence for such an interaction. Other key NOEs revealed side chain-side chain interactions between residues separated by one helix turn (i.e. three or four residues apart), so that their side chains are capable of participating in electrostatic interactions. Several of these interactions were between the two glutamate residues at positions Glu679 and Glu680 and between residues Lys682 and Arg684. It is worth noting that these side chain-side chain NOEs were absent when the NMR solution conditions were altered to pH 3.0; this was thought to be because the protonated glutamate side chains could no longer electrostatically interact with the arginine and lysine residues.2

### Table II

Summary of restraints, structural statistics, and atomic root mean square deviations for the family of 20 calculated lowest energy structures of peptide A1 and A2

| Restraints          | Peptide A1 | Peptide A2 |
|---------------------|------------|------------|
| Total NOEs          | 212        | 237        |
| Intra               | 106        | 118        |
| $i - i + 1$         | 55         | 53         |
| $i - i + 1$         | 51         | 66         |
| $^{3}N_{H-H}$       | 7          | 6          |
| Hydrogen bonds      | 4          | 3          |
| Atomic rmsd (Å)     |            |            |
| Ensemble average    |            |            |
| All                 | 3.06 (0.74) | 2.67 (0.68) |
| Residues 671–685    | 1.51 (0.48) | 1.43 (0.47) |
| Residues 671–680    | 0.60 (0.14) | 0.82 (0.36) |
| rmsds from experimental restraints | | |
| NOE (Å)             | 0.0056 (0.0006) | 0.019 (0.002) |
| Dihedral restraints (+) | 0.70 (0.10) | 0.14 (0.03) |
| rmsd from idealized covalent geometry | | |
| Bonds (Å)           | 0.0041 (0.0035) | 0.00196 (0.003) |
| Angles (+)          | 0.55 (0.034) | 0.50 (0.05) |
| Improper dihedral angles (+) | 0.41 (0.024) | 0.37 (0.02) |
| Xplor energies (kcal mol⁻¹ Å⁻¹) | | |
| $E_{NOE}$           | 75.54 (11.35) | 38.1 (7.3) |
| $E_{NOE}$           | 33.55 (7.38) | 4.54 (1.8) |
| $E_{CDIH}$          | 0.33 (0.07) | 0.0056 (0.002) |

* Standard deviation is given in parentheses.

a $N$, $Cα$, and $CO$ atoms.

b Force constants used to calculate $E_{NOE}$ and $E_{CDIH}$ were 50 kcal mol⁻¹ Å⁻² and 200 kcal mol⁻¹ rad⁻², respectively. $E_{NOE}$ is the NOE energy term, and $E_{CDIH}$ is the dihedral energy term.
Structural Differences between the A1 and A2 Peptides—

There are two main structural differences between peptides A1 and A2. The first involves the uniformity of the α-helix toward the C-terminal end at residues Arg681–Lys685, which are essential for peptide activation of RyRs (6, 11). It is evident from the ensemble of the 20 structures in Fig. 4 that this stretch of basic amino acids is less structurally ordered in peptide A1 compared with A2. In fact, the rmsds for the backbone atoms of the helical segments are supportive of this, implying that the helix in this region is more uniform for peptide A2. The second area of difference is the C-terminal region of the peptides. Although both peptides seem disordered in this region (Fig. 4), it is apparent that there is a modest degree of order for the C-terminal end of peptide A2. Subtle differences are observed in the NOEs, coupling constant, amide exchange, and CSI for this region (see Fig. 3). These latter differences are not surprising given that this is the region where the amino acid substitution is located and considering that alanine is noted as a helix promoting residue (28). However, what is more intriguing is why an amino acid substitution toward the C terminus of the peptide, and outside the helix, affect the structure of the helix. Although no NOEs were detected between the serine side chain and side chains of residues contained in the α-helix, the predicted low energy A1 peptide model (Fig. 5) does suggest that an electrostatic interaction is possible between the hydroxyl oxygen of Ser687 and the guanidino moiety of Arg683. It is likely that in solution, this conformation may indeed be present, but the high conformational flexibility of the terminal five C-terminal residues means that only a minor population of this conformation is present, and thus, no NOEs can be detected.

Comparison with Previous Models of Peptide A1—No previous structural analysis has been performed for the peptide A1 region of the skeletal DHPR. A structural model of a 26-amino acid segment of the skeletal DHPR II-III loop (Glu666–Pro691), encompassing peptide A1, has been proposed by Zhu et al. (10). Their model predicted a strong helical component, and our NMR data and predicted structures agree in that (a) the helix commenced at Thr677 and terminated at approximately Lys685, and (b) the C-terminal end was essentially random coil. One major difference is that the central amino acids Glu679 and Glu680 are predicted to assume a random coil conformation, whereas we observed that this is definitely not the case. Our NMR analysis shows that Glu679 and Glu680 are indeed helical, participating in typical backbone interactions found in helices as well as in electrostatic side chain–side chain interactions with basic amino acid residues one helix turn away. Also, no mention of the possibility of an interaction between Ser687 and Arg683 was made (10) for the 26-amino acid peptide, whereas in our study, this interaction offers an explanation for the stronger activation for the Ser687 to Ala substitution in the 20-amino acid A1 peptide (7). Interactions of Ser687 with other residues in the longer II-III loop segments may account for the inhibition seen with Ser687 to Ala substitution in other studies (5, 10).

Correlation of Structure and Function—Previous work in this laboratory has shown that both peptides A1 and A2 can activate the skeletal RyR with peptide A2 exhibiting an increase in activity compared with A1 (7) (Fig. 1). An additional observation presented here is that peptide A9 is a very poor activator of skeletal RyR, despite the fact that it contains the essential amino acids for activation. This result suggests that activation of RyRs requires not only the correct DHPR amino acid sequence but also the correct conformation. Preliminary experiments with fragments of the A1 peptide also indicate that the basic amino region must adopt a helical structure for effective activation of the RyRs. Consistent with this hypothesis, we find, in contrast to others (6), that the 10 residue peptide Arg681–Leu690, i.e. A9 plus 3 additional C-terminal amino acids, is relatively ineffective in activating either Ca2+ release from SR or RyR channels (30) and has no helical structure.2

The experimental observation that the presence or absence of Ser687 alters the ability of the II-III loop, peptide A1, and the Glu666–Pro691 peptide to enhance RyR activity (4, 11) suggests that this serine residue does play some role in the activation of the skeletal RyR. The nature of this role has up to now been unclear, but it has been assumed that this residue is an essential factor in activation. Our results have shown that Ser687 is not essential for activation by peptide A (7) and suggest that the effect of substituting Ala for Ser687 depends on the length of the II-III loop segment being examined and presumably on the conformational properties, which may well differ in segments of different lengths and amino acid composition. In the present study, the structural outcome of substituting Ser687 for an alanine (peptide A2) was to stabilize the C-terminal end of the helix; this substitution also had some structural effect on the C-terminal end of the peptide. Because the Ser687 side chain is not directly involved with the formation of the helix, this suggests that the hydroxyl group must somehow interact with the C-terminal end of the helix and that the flexibility of the C-terminal end of the peptide allows the interaction. The manner in which this may happen is presented in Fig. 5, which shows an interaction between the guanidino moiety of Arg683 and the hydroxyl group of the serine. The stretch of basic amino acid residues from 681–685 is critical for activation of the RyR (6, 10), and we propose that the interaction between Ser687 and Arg683 diminishes the capacity of this basic region of the peptide to activate the RyR. There are two possible ways that this may occur. First, an interaction between Ser687 and Arg683 within peptide A could lead to the disruption of the structural uniformity of the C-terminal end of the helix, which is required for activation. The second possibility is that this interaction would hinder the area of the peptide responsible for binding (681–685) from interacting with the RyR. Under these circumstances, a greater negative charge density at residue 687 through phosphorylation of the serine should enhance the interaction with Arg683 and thus reduce binding and RyR activation. Indeed, it has been demonstrated that phosphorylation of Ser687 in the II-III loop prevented activation of the RyR by the II-III loop (4). Preliminary investigations of the phosphorylation of Ser687 in peptide A1 also indicate that this modified peptide does not activate the RyR. It is not clear why the substitution of Ser687 to Ala totally abolished [3H]ryanodine binding in the case of the II-III loop (4) or why replacement of Ser687 with Ala or glutamate in the 25-amino acid Glu666–Pro690 peptide reduced binding (12). It is, however, likely that
the different lengths, different interactions between amino acid residues, and hence different conformational structures of these peptides may explain the conflicting results obtained with the $[^3H]$ryanodine binding studies.

In conclusion, the unstructured nature of peptide A9 and the structures of the A1 and A2 peptides obtained in this study suggest that a strong helical structure of the five consecutive basic amino acids extending from Arg$^{681}$ to Lys$^{685}$ is important in these peptides binding to skeletal RyRs. It may well be that activation of the RyR during ECC is achieved by conformational changes in the II-III loop that force the peptide A region to assume a more helical structure and interact more strongly with the RyR. A structural investigation of other II-II loop fragments are currently underway in this laboratory and should further elucidate the physical nature of interactions between the DHPR loop and its binding site on the RyR.

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