The three-dimensional structural surface of two β -sheet scorpion toxins mimics that of an α -helical dihydropyridine receptor segment

Daniel GREEN*, Suzi PACE*, Suzanne M. CURTIS*, Magdalena SAKOWSKA*, Graham D. LAMB†, Angela F. DULHUNTY* and Marco G. CASAROTTO*‡¹

*Division of Molecular Bioscience, John Curtin School of Medical Research, Australian National University, P.O. Box 334, Canberra, ACT, 2601, Australia, †Department of Zoology, La Trobe University, Kingsbury Drive, Victoria, 3086, Australia, and ‡Research School of Chemistry, Australian National University, P.O. Box 334, Canberra, ACT, 2601, Australia

An α -helical II–III loop segment of the dihydropyridine receptor activates the ryanodine receptor calcium-release channel. We describe a novel manipulation in which this agonist's activity is increased by modifying its surface structure to resemble that of a toxin molecule. In a unique system, native β -sheet scorpion toxins have been reported to activate skeletal muscle ryanodine receptor calcium channels with high affinity by binding to the same site as the lower-affinity α -helical dihydropyridine receptor segment. We increased the alignment of basic residues in the α -helical peptide to mimic the spatial orientation of active residues in the scorpion toxin, with a consequent 2–20-fold increase in the activity of the α -helical peptide. We hypothesized that, like the

native peptide, the modified peptide and the scorpion toxin may bind to a common site. This was supported by (i) similar changes in ryanodine receptor channel gating induced by the native or modified α -helical peptide and the β -sheet toxin, a 10–100-fold reduction in channel closed time, with a ≤ 2 -fold increase in open dwell time and (ii) a failure of the toxin to further activate channels activated by the peptides. These results suggest that diverse structural scaffolds can present similar conformational surface properties to target common receptor sites.

Key words: ion channel, NMR, peptide A, ryanodine receptor.

INTRODUCTION

Protein–protein interactions between the dihydropyridine receptor (DHPR) and ryanodine receptor (RyR) underlie excitation–contraction (EC) coupling in skeletal muscle. The DHPR L-type Ca^{2+} channel in the transverse tubule membrane detects surface depolarization and transmits a signal to the RyR channel in the sarcoplasmic reticulum (SR) via the cytoplasmic domains of the two proteins. The interacting region of the DHPR is located between the second and third transmembrane repeats in the $\alpha 1$ subunit [1]. Peptide fragments of this DHPR II–III loop can activate isolated RyRs [2–5]. One fragment (peptide A1, Thr⁶⁷¹–Leu⁶⁹⁰) induces Ca^{2+} release from the SR and enhances current flow through RyR channels [2–8]. The ability of peptide A to activate RyRs is strongly correlated with its capacity to adopt α -helical structures [3,4].

Among the scorpion toxins, imperatoxin A (IPTx) and maurocalcine mimic effects of peptide A on the RyR [8,9]. IPTx has been shown to bind to the same site on the RyR as a peptide corresponding to a segment of the II–III loop (Glu⁶⁶⁶–Leu⁶⁹⁰) containing the peptide A sequence [8]. Intriguing and unusual is the low sequence similarity ($\approx 18\%$) between the toxins and peptide A (Figure 1a) and the very different structures which engage the RyR (Figure 1b). The toxins have an inhibitor cysteine knot ('ICK') fold, a compact disulphide core and a β -sheet structure [10] (H. Valdivia, personal communication), whereas the native DHPR peptide (A1) and the Ser⁶⁸⁷–Ala mutant (A2), contain a single N-terminal α -helical structure [3].

A common stretch of basic amino acids (Figure 1a) is critical for RyR activation by IPTx and peptide A [3,7,8,11]. The sequence alone is not sufficient for RyR recognition; appropriate secondary structures must also be adopted [4].

IPTx and maurocalcine activate RyRs with nanomolar affinity, while peptide A activates at micromolar concentrations [8]. Our hypothesis is that these binding affinities reflect secondary structural rigidity and the alignment of positively charged residues along a surface of these molecules. Three disulphide linkages stabilize the β -sheet surface of the toxin that is responsible for RyR activation, while peptide A's helix is stabilized only by favourable side-chain interactions (Glu–Arg). Therefore, peptide A remains structurally less ordered than the toxins. The basic residues are tightly aligned along one surface of the toxin but are poorly aligned in peptide A (Figure 1b).

Our aim was to modify the basic residues on peptide A1 to resemble the electrostatic surface properties of the basic residues on the toxin, to create a more potent RyR activator. We previously manipulated the native peptide (A1) by converting Ser⁶⁸⁷ to an Ala residue to give peptide A2 [3]. This manipulation stabilized the helical structure, increased the alignment of the positively charged residues along one surface of the helix and modestly enhanced the peptide's ability to activate RyRs. In the present study, Arg⁶⁸⁸ was targeted because its positively charged side chain is located in the unstructured portion of peptide A1 and because it is adjacent to Ser⁶⁸⁷. We altered the chirality of Arg⁶⁸⁸ with the specific aim of producing a peptide [A1(D-R18)] with a positively charged surface that would mimic the positively

Abbreviations used: CSI, chemical shift index; CsMS, caesium methanesulphonate; DHPR, dihydropyridine receptor; DQF-COSY, double-quantum filtered-COSY; EC, excitation–contraction; IPTx, imperatoxin A; NOE, nuclear Overhauser effect; RyR, ryanodine receptor; SR, sarcoplasmic reticulum.

¹ To whom correspondence should be addressed, at the Division of Molecular Bioscience (e-mail Marco.Casarotto@anu.edu.au).

The co-ordinates for peptide A1(D-R18) have been deposited in the Protein Data Bank under accession code 1JZP.

Figure 1 Structural and sequence similarities of DHPR and toxin peptides

(a) Sequence alignment of IPTx, maurocalcine and the peptide A region of the DHPR. (b) Average solution structures of peptides A1, A2 [3] and maurocalcine [10]. The stretch of basic amino acids responsible for RyR activation is depicted in blue and the disulphide linkages are in yellow. Peptide A1 contains the native DHPR sequence and peptide A2 has a Ser⁶⁶⁷-to-Ala substitution.

charged surface of the toxin molecules. We show that A1(D-R18) is a higher-affinity activator of RyR channels than peptides A1 or A2 and provide additional evidence to support the hypothesis that peptide A1, peptide A1(D-R18) and IPTx may act at a common site on the RyR complex.

MATERIALS AND METHODS

Peptide A1(D-R18) was synthesized as in Dulhunty et al. [2]. IPTx was synthesized by Auspep Australia and folded using procedures outlined by Fajloun et al. [9].

NMR

Peptide A1(D-R18) was dissolved at $\approx 2\text{--}3\text{ mM}$ in 10% $^2\text{H}_2\text{O}/90\%$ H_2O and pH adjusted to a value of 5. ^1H Chemical shift values are relative to 2,2-dimethyl-2-silapentane-5-sulphonate. Spectra were acquired on a Varian Inova 600 spectrometer using a spectral width of 6000 Hz, a pulse width of 7 μs (90°) and an acquisition time of 0.130 s, collecting 4096 data points and 512 increments of 32 transients. NOESY [12] spectra (mixing time of 200–500 ms), TOCSY [13] spectra (mixing time of 70 ms) and double-quantum filtered-COSY (DQF-COSY) [14] spectra were acquired at 278 K and used for the assignment of the ^1H -NMR resonances. Suppression of the H_2O resonance for the NOESY and DQF-COSY spectra was achieved using pulse field gradients [15,16], while a presaturation pulse was employed for the TOCSY experiments. Temperature coefficients [17] 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, 298 K) in the DQF-COSY experiments. Two-dimensional data were

processed on a Silicon Graphics O2 workstation using Felix 98 software. Data sets were zero-filled to 4096×2048 data points and multiplied by a phase-shifted sine-bell-squared function in both dimensions prior to transformation. $^3J_{\text{NH}-\alpha\text{H}}$ coupling constants were derived from one- and two-dimensional DQF-COSY ^1H 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 nuclear Overhauser effect (NOE) crosspeak intensities by counting the number of contour levels of non-overlapped crosspeaks and using distances calibrated from glutamine 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, each with a 1.9 Å lower distance limit (1 Å = 0.1 nm). Dihedral angle restraints based on the $^3J_{\text{NH}-\text{H}\alpha}$ measurements were included in the final calculations with a tolerance of 20° .

Structure calculations were performed with the X-PLOR program [18,19], using the topallhdg.pro topology file and parallhdg.pro parameter file, with the simulated annealing protocol on a Silicon Graphics O2 workstation. The sum averaging option was used to treat equivalent and non-stereospecifically assigned protons. Calculations were iterative, checking violations after each round and adding or refining constraints. Hydrogen bonds were identified and introduced after initial calculations. These were simulated by a 3.3 Å restraint between the amide nitrogen and carbonyl oxygen and a 2.3 Å restraint between the amide hydrogen and the carbonyl oxygen. Initially, 100 fully extended structures with random co-ordinates were generated. Structures were analysed and 17 selected for distance and dihedral constraint violations and low energies. No structures had NOE violations $> 0.5\text{ Å}$ or dihedral violations $> 5^\circ$. For structural comparisons, individual structures were superimposed on to the average structure.

Ca^{2+} release and single-channel techniques

Preparation of SR vesicles, Ca^{2+} release from SR and single-channel techniques have been described [2]. Rabbit skeletal SR vesicles (100 μg of protein) were added to a cuvette, to a final volume of 2 ml of a solution containing 100 mM KH_2PO_4 (pH 7), 4 mM MgCl_2 , 1 mM Na_2ATP and 0.5 mM antipyrilazo III. Extravesicular $[\text{Ca}^{2+}]$ was monitored at 710 nm. Identical experiments at 790 nm (to test for non- Ca^{2+} -dependent changes in absorbance that may be induced by peptide or toxin addition) showed no changes in absorbance which would alter the rate of Ca^{2+} release measured at 710 nm.

Bilayers were formed from phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine (5:3:2, by weight; Avanti Polar Lipids, Alabaster, AL, U.S.A.) across an aperture with a diameter of 200–250 μm in the wall of a 1.0 ml Delrin cup (Cadillac Plastics, Sydney, Australia). Terminal cisternae vesicles (10 $\mu\text{g}/\text{ml}$) were added to the *cis* chamber. The cytoplasmic side of channels incorporated into the bilayer faced the *cis* solution. Bilayer potential was controlled and single-channel currents recorded using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, U.S.A.). Bilayer potential was expressed as $V_{\text{cis}} - V_{\text{trans}}$ (equivalent to $V_{\text{cytoplasm}} - V_{\text{lumen}}$).

Bilayers were formed and vesicles incorporated using *cis* solutions containing 230 mM caesium methanesulphonate (CsMS), 20 mM CsCl, 1.0 mM CaCl_2 and 10 mM Tes, pH 7.4, with CsOH; and a similar *trans* solution with 30 mM CsMS.

Following incorporation, (i) the *cis* solution was replaced with an identical solution, except that $[\text{Ca}^{2+}]$ was 300 nM, buffered with 2 mM BAPTA [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid], and (ii) 200 mM CsMS was added to the *trans* chamber to establish symmetrical conditions. Drugs were added to the *cis* chamber and removed by perfusion with 10 vol. of *cis* solution. Analysis of single-channel records (using Channel 2, courtesy of P. W. Gage and M. Smith, John Curtin School of Medical Research, Australian National University, Canberra, Australia) yielded mean current (I), probability (P_o), mean open time (T_o) and mean closed time (T_c). Channel activity was analysed over two 30 s periods of continuous activity at +40 mV and two 30 s periods at -40 mV. Measurements on mean current were performed on records from experiments containing either one or two channels. Use of this parameter allows inclusion of data from most experiments and increases the numbers of observations. The measurement of mean current includes all channel activity from the smallest subconductance openings to maximum openings. The other single-channel parameters, P_o , T_o and T_c were measured only in records in which when the opening of a single channel could be detected. Threshold levels for channel opening and closing were set to exclude baseline noise and were usually set at $\approx 20\%$ of the maximum single-channel conductance.

Skinned-fibre experiments

Segments of single muscle fibres from the extensor digitorum longus muscle of the rat were mechanically skinned, connected to a force transducer (AME801; SensoNor, Horten, Norway) and bathed in a high- $[\text{K}^+]$ solution mimicking the normal environment, as described previously [20]. All experiments were performed at room temperature ($23 \pm 2^\circ\text{C}$). All chemicals were obtained from Sigma. The high- $[\text{K}^+]$ solution contained 126 mM K^+ , 37 mM Na^+ , 50 mM 1,6-diaminohexane-*N,N,N',N'*-tetraacetic acid (HDTA; Fluka, Buchs, Switzerland), 8 mM total ATP, 10 mM total creatine phosphate, 8.5 mM total magnesium, 90 mM Hepes, 1 mM NaN_3 and 0.05 mM total EGTA, with a $p\text{Ca}$ (i.e. $-\log [\text{Ca}^{2+}]$) of 7.1 and $\text{pH } 7.10 \pm 0.01$. This solution had a free $[\text{Mg}^{2+}]$ of 1 mM and an osmolality of $295 \pm 5 \text{ mosmol} \cdot \text{kg}^{-1}$. The SR was depleted of Ca^{2+} by a 2 min exposure to a similar solution with 30 mM caffeine, 0.05 mM free Mg^{2+} and 0.5 mM EGTA ($p\text{Ca} > 8$). The SR was reloaded with Ca^{2+} by exposure for a set period (usually ≈ 15 s) to the standard solution with 0.5 mM total EGTA at $p\text{Ca } 6.7$. Fibres were then equilibrated in the standard solution (1 mM Mg^{2+} /0.05 mM EGTA, $p\text{Ca } 7.1$) for 20 s, with or without peptide, and exposed to a similar solution with 8 mM caffeine for 15 s, before fully depleting the SR again, as described in Lamb et al. [20]. Maximum Ca^{2+} -activated force in each fibre was determined using a solution with 50 mM Ca-EGTA ($p\text{Ca } 4.5$).

Statistics

Averaged data are given as means $\pm$ S.E.M. The significance of differences between control and test values were tested using the Student's *t* test.

RESULTS

NMR structure of A1(D-R18)

The stereochemical properties of peptide A1 were altered by replacing the L-Arg⁶⁸⁸ with its D-isomer [peptide A1(D-R18)]. The complete proton assignments for A1(D-R18) are presented in Table 1 and determined using standard two-dimensional methods [21] which involved two-dimensional TOCSY and DQF-COSY

experiments to identify spin types and NOESY experiments to make sequence-specific assignments. Short- and medium-range NOE patterns, $^3J_{\text{NH-H}\alpha}$ coupling constants, α -proton chemical shift index (CSI) [22] and hydrogen-bonding patterns (Figure 2a) all indicate the presence of an α -helix formation. $J_{\text{NH-H}\alpha}$ coupling constants < 6 Hz and temperature coefficients > -2.0 p.p.b./K were noted for several residues between 671 and 685. The upfield shift of α -protons is also indicative of helix formation within this region, particularly for residues 680–685 encompassing the basic residues required for activation [3,7,8]. Overall, the NMR profile for A1(D-R18) was similar to A1 and A2 [3], with notable differences in the C-terminal end where coupling constants and NOE data indicate an extended helical conformation.

Analysis of NOESY spectra from A1(D-R18) gave 216 distance constraints (Table 2). Apart from medium-range NOEs synonymous with helical structure, several side-chain–side-chain NOEs were detected for residue pairs Gln⁶⁷⁴–Lys⁶⁷⁷, Glu⁶⁷⁹–Arg⁶⁸³ and Glu⁶⁸⁰–Arg⁶⁸⁴ which add to the stability of the helix. Hydrogen-bond constraints were included in structure calculations when the $^3J_{\text{NH-H}\alpha}$ coupling constant, temperature coefficient and CSI data supported this constraint. Structural statistics for A1(D-R18) (Table 2) show the 17 lowest energy structures which have an overall backbone root-mean-square-deviation value that is similar to A2 and slightly less than A1 [2]. A1(D-R18) has the lowest root-mean-square-deviation values for residues in the C-terminal half of the peptide, again indicating greater structural order than in A1 or A2. The 17 lowest energy structures of A1(D-R18) are superimposed in Figure 2(b) and the average structure shown. Thus A1(D-R18) contains a high degree of helical content and, although the N- and C-terminal ends show modest disorder, the peptide has greater helical content than either A1 or A2. Of particular interest was the orientation of the basic amino acid residues which is best represented in the side-on and head-on views of the basic side chains (Lys⁶⁷⁷–Arg⁶⁸⁸) for peptides A1, A2 and A1(D-R18) in Figure 3. Three major features are evident. (i) The helical arrangement of the positive residues increases from A1 to A2 to A1(D-R18) (head-on views) with the central cavity more prominent for A1(D-R18). (ii) The distribution of Arg⁶⁸⁸ (red balls) is more scattered in A1 and A2 than A1(D-R18) (side-on views), indicating structural ordering and helix extension by converting Arg⁶⁸⁸ to a D-isomer. (iii) Most basic residues are aligned on one surface of the helix in A1(D-R18) (as highlighted by blue shading in Figure 3), compared with a more widespread distribution in A1 and A2 (side-on view).

Enhanced A1(D-R18)-induced Ca^{2+} release from SR

A1(D-R18) evoked stronger Ca^{2+} release from SR vesicles than either A1 or A2 (Figure 4). The Ca^{2+} release induced by peptide A1(D-R18) was markedly greater than that induced by peptide A1. Ruthenium Red added at the end of the records in Figure 4(A) terminated Ca^{2+} release by peptide A1 indicating that Ca^{2+} release was through RyR channels. Addition of Ruthenium Red during Ca^{2+} release induced by low concentrations of A1(D-R18) terminated release in all cases where Ca^{2+} release was submaximal (results not shown), indicating that this peptide also released Ca^{2+} via RyR activation.

Average rates of peptide-enhanced Ca^{2+} release are shown Figure 4(B). The broken lines show average data for A1 and A2 [2], which are included for comparison. There is significant Ca^{2+} release with $2 \mu\text{M}$ A1(D-R18) and $> 15 \mu\text{M}$ peptide with maximum values between 200 and 300 nmol/mg/min. This compares with maximum rates of 95 nmol/mg/min with $38 \mu\text{M}$ A1 and of 191 nmol/mg/min for $38 \mu\text{M}$ A2.

Table 1 Proton assignment for the A1(D-R18) peptide in 10% $^2\text{H}_2\text{O}$ /90% H_2O , pH 5.0, at 5 °C

Residue	NH	H α	H β	Other	$^3J_{\text{NH}-\alpha\text{H}}$ (Hz)	Temperature coefficient (p.p.b./K)
Thr ⁶⁷¹	8.40	4.43	4.36	H γ , 1.28		−3.5
Ser ⁶⁷²	8.83	4.33	3.94/3.98		4.5	−14.0
Ala ⁶⁷³	8.53	4.28	1.43		4.9	−8.0
Gln ⁶⁷⁴	8.17	4.11	2.12/2.18	H γ , 2.45; δNH_2 , 7.63/6.97	5.0	0.5
Lys ⁶⁷⁵	8.41	4.15	1.89/1.86	H γ , 1.41/1.53; H δ , 1.67	4.0	−5.0
Ala ⁶⁷⁶	8.22	4.24	1.48			0.5
Lys ⁶⁷⁷	8.11	4.18	1.89	H γ , 1.48/1.57; H δ , 1.70; H ϵ , 3.00	5.4	0.0
Ala ⁶⁷⁸	8.15	4.20	1.50			0.0
Glu ⁶⁷⁹	8.33	4.14	2.10/2.06	H γ , 2.37/2.37	4.4	−1.0
Glu ⁶⁸⁰	8.18	4.12	2.12/2.17	H γ , 2.30/2.37		−1.5
Arg ⁶⁸¹	8.14	4.10	1.90/1.78	H γ , 1.63/1.63; H δ , 3.23/3.23; NH, 7.33	4.6	−0.5
Lys ⁶⁸²	7.95	4.14	1.93/1.88	H γ , 1.41/1.53; H δ , 1.71; H ϵ , 3.01	5.3	0.0
Arg ⁶⁸³	8.14	4.20	1.90/1.77	H γ , 1.64/1.64; H δ , 3.23/3.23; NH, 7.50		−3.0
Arg ⁶⁸⁴	8.21	4.22	1.85/1.75	H γ , 1.65/1.65; H δ , 3.20/3.20; NH, 7.42		−2.5
Lys ⁶⁸⁵	8.23	4.25	1.91/1.70	H γ , 1.58/1.58; H δ , 1.67; H ϵ , 3.00		0.0
Met ⁶⁸⁶	8.34	4.47	2.15/2.07	H γ , 2.60/2.68; ϵCH_3 , 2.09	5.2	−0.5
Ser ⁶⁸⁷	8.27	4.47	3.92/3.92		6.8	−2.0
Arg ⁶⁸⁸	8.36	4.35	1.95/1.83	H γ , 1.64/1.70; H δ , 3.23/3.23; NH, 7.24	5.8	−6.0
Gly ⁶⁸⁹	8.58	3.94			6.2	−5.5
Leu ⁶⁹⁰	8.23	4.31	1.59/1.68	H γ , 1.68; H δ , 0.88/0.94		−6.5

Enhanced A1(D-R18)-induced activation of single RyR channels

The activity of single RyR channels, with 300 nM *cis* Ca^{2+} and 2 mM *cis* MgATP, at +40 and −40 mV, increases when peptide A1 (300 nM and 50 μM) is added to the cytoplasmic (*cis*) solution [2]. Single RyR channel activity in the present study was recorded under the same conditions, with 300 nM *cis* Ca^{2+} , in order to avoid the inhibitory effects of the peptides [2]. The single-channel records in Figure 5(A) show a substantial increase in RyR activity at +40 and −40 mV after addition of only 10 nM peptide A1(D-R18) to the *cis* chamber. Mean current is used as a measure of RyR channel activity in bilayers containing either one or two channels and thus allows examination of a larger population of channels than those available for single-channel analysis. Average relative mean current from six experiments plotted in Figure 5(B) is compared with previously reported activation by peptides A1 and A2: data for A2 were obtained with 10 μM *cis* Ca^{2+} and have been appropriately scaled for comparison with the data obtained with 300 nM *cis* Ca^{2+} for A1 and A1(D-R18) [2]. Activation of 50% by peptide A1(D-R18) was achieved with $\approx$ 10 nM peptide, compared with > 100 nM peptide A1 and the average activation by A1(D-R18) was double that achieved by A1 at −40 mV and 20-fold greater at +40 mV.

Examination of single-channel parameters at +40 and −40 mV, in five of the six experiments in which one channel only was open in the bilayer, showed that the average $\approx$ 10–50-fold increase in channel open probability at both potentials (Figure 6A) could be principally attributed to a dramatic decrease in the closed times of the channel (Figure 6C) coupled with an equally large increase in the number of openings (Figure 6D) and a modest 2-fold increase in channel open time (Figure 6B). Similar parameter values in the presence of A1(D-R18) were measured at +40 and −40 mV. The greater increase in relative mean current at +40 mV (Figure 5B) was due to the fact that the closed times were longer, and the frequency of events lower, in control activity at that potential than control activity at −40 mV. The effects of peptide A1(D-R18) on single-channel parameters (a strong decrease in closed times and an increase in event frequency, with only a small increase in mean open time) were the same as the effects seen with peptide A1 [2,23]. The major difference

between the peptides was in the magnitude of the effects, which was 2–10-fold greater with A1(D-R18) than with A1.

The activation of RyRs by peptide A1(D-R18) was not reversed for up to 6 min after peptide A1(D-R18) was removed from the *cis* chamber by perfusion with 10 ml of peptide-free solution. The slow reversal of activation is in contrast to the rapid reversal of pore block which occurs within the perfusion time. The slow reversal of activation is indicative of slow dissociation of the peptide from its interaction site and has also been seen with peptides A1 and A2 [2,23]. Slow dissociation may seem inconsistent with the relatively high concentrations of peptides A1 and A1(D-R18) required for activation. However, this result may mean that binding is not simply limited by free diffusion, because the binding to the activation site is restricted because either (i) the binding site is partially occluded by the complex three-dimensional folding in the RyR structure, and/or (ii) there are dynamic changes in the conformation of the protein in the vicinity of the binding site. Indeed, the toxin-binding site has been localized to a deep fold in the protein structure using relatively low-resolution cryo-electron microscopy [24]. An alternative possibility, or an additional factor, could be that the peptide may need to assume an appropriate conformation for binding, which might occur only infrequently (or for a short period of time). However, once bound to the RyR, the peptide remains tightly associated with the protein. Similar behaviour has been seen with other II–III loop peptides (C. S. Haarmann, A. F. Dulhunty and D. R. Laver, unpublished work).

The similar actions of A1(D-R18) at positive and negative potentials is also in contrast to data obtained with peptide A1 with 300 nM *cis* Ca^{2+} , where a weaker activation was seen at +40 mV and attributed to a secondary effect of peptide A1 in blocking the channel pore at that potential [2]. The pore block has been attributed to the positively charged residues in the peptide interacting with negatively charged residues within the pore [2]. One explanation for the failure of A1(D-R18) and IPTx to block the pore is that these more structurally constrained molecules lack the flexibility to interact with the negatively charged residues within the pore, in contrast to the more flexible A1 peptide. In other words, the negatively charged residues within the pore are distributed in a manner that favour interaction

Figure 2 NMR restraints and structure for the A1(D-R18) peptide

(a) Summary of the sequential and medium-range NOE connectivities, α H CSI, $^3J_{\text{NH-H}\alpha}$ coupling constants and temperature coefficients in 10% $^2\text{H}_2\text{O}/90\%$ H_2O . The NOE connectivities are indicated by black lines with thicknesses proportional to NOE intensity. Values of $^3J_{\text{NH-H}\alpha}$ less than 6 Hz are indicated by $\downarrow$. Temperature coefficients greater than -2.0 p.p.b./K are represented by $\bullet$ and those less than -2.0 p.p.b./K by $\circ$. For the CSI, a value of $+1$ or -1 is assigned for residues whose α H chemical shift deviates from tabulated random-coil values by more than 0.1 p.p.m., downfield and upfield, respectively [22]. (b) Best-fit superposition of backbone heavy atoms of residues Gln⁶⁷⁴-D-Arg⁶⁸⁸ of the 17 lowest-energy structures for A1(D-R18) calculated from the NMR data (left-hand image). Average structure of A1(D-R18) (right-hand image) with the side chains showing residues Lys⁶⁷⁷, Arg⁶⁸¹, Arg⁶⁸⁴ and D-Arg⁶⁸⁸.

with a less structured set of positively charged peptides over interaction with the more structured A1(D-R18) and IPTx.

Peptides A1, A1(D-R18) and IPTx modify RyR gating in the same way

The effects of IPTx on RyR activity were examined for comparison with effects of A1 and A1(D-R18). Ca^{2+} release from SR vesicles was dramatically increased by $1 \mu\text{M}$ IPTx (Figure 7A) to an average value of 889 ± 87 nmol/mg/min, compared with 332 ± 87 nmol/mg/min with $33 \mu\text{M}$ A1(D-R18) (Figure 4B) or

Table 2 Summary of restraints, structural statistics and atomic root mean square deviations (rmsds) for the family of lowest-energy structures

Where applicable, means $\pm$ S.D. are shown.

Restraints	Peptide A1(D-R18)
Total NOEs	222
Intra	108
$i \rightarrow (i+1)$	58
$i \rightarrow (i < 1)$	56
$^3J_{\text{NH-H}\alpha}$	12
Hydrogen bonds	5
Atomic rmsd backbone (ensemble versus average)	
All	2.66 ± 0.56
Residues 671–686	2.35 ± 0.61
Residues 674–690	4.58 ± 0.86
rmsd from experimental restraints	
NOE	0.022 ± 0.013
Dihedral restraints	0.33 ± 0.32
rmsd from idealized covalent geometry	
Bonds	0.0020 ± 0.001
Angles	0.51 ± 0.058
Improper dihedral angles	0.41 ± 0.080
Xplor energies ($\text{kcal} \cdot \text{mol}^{-1}$)	
E_{tot}	37.56 ± 7.45
E_{NOE}	3.22 ± 1.73
E_{CDIH}	0.24 ± 0.03
Ramachandran analysis	
Most favoured regions (%)	76.5
Additionally allowed regions (%)	17.6
Generously allowed regions (%)	5.9
Disallowed regions (%)	0

95 ± 8 nmol/mg/min for peptide A1 [2]. Examination of single-channel activity showed that, under our conditions (300 nM *cis* Ca^{2+} , 2 mM *cis* ATP, 1 mM *trans* Ca^{2+}), the actions of IPTx at 1–10 nM (Figure 7) added to the *cis* chamber on RyR channel activity were very similar to those of A1(D-R18) at 100 nM–10 μM (Figures 5 and 6). Mean current increased to a maximum at 10–100 nM IPTx (Figure 7B). The activating effects of IPTx and A1(D-R18) could not be distinguished in that (i) similar activation was seen at positive and negative potentials (results not shown; values from $+40$ and -40 mV are combined in the averaged data in Figure 7B) and (ii) there was large 10–100-fold reduction in the mean closed time (and increased frequency of events), with only a small ≈ 2 -fold increase in mean open time with each compound (Figures 7C–7E). Similar actions of peptide A1 have been reported [2,23]. Maintained sub-conductance openings, lasting several seconds [25] were not seen, possibly due to the relatively low activity with 300 nM Ca^{2+} and 2 mM MgATP. Brief submaximal conductance activity can be seen in the records in both Figure 5 and Figure 7 and is typical of current records from native RyR channels [26,27]. In our experience, subconductance openings might occupy anything from 2 to 80% of control channel openings with dwell times longer than 1 ms (sample frequency, 5 kHz), varying from channel to channel. This brief subconductance activity did not appear to vary significantly before and after exposure to either the peptides or the toxin at low concentrations (100 nM toxin or 1 μM peptides).

IPTx at higher concentrations (1–10 μM) inhibited RyR channel activity (Figure 7B) at $+40$ and -40 mV. This lower-affinity inhibition was rapidly reversible after perfusion of the *cis* chamber with a toxin-free solution. In all experiments, channel activity

Figure 3 Ball and stick representation of the 17 lowest energy NMR structures of peptides A1, A2 and A1(D-R18) (residues 671–690 superimposed)

The Figure shows side (left-hand side) and top (right-hand side) views of basic amino acids 677–688. Lys⁶⁷⁷, brown; Arg⁶⁸¹, blue; Lys⁶⁸², green; Arg⁶⁸³, yellow; Arg⁶⁸⁴, black; Lys⁶⁸⁵, purple; D-Arg⁶⁸⁸, red.

Figure 4 Ca^{2+} release from SR vesicles

(A) The effect of peptides on Ca^{2+} release from SR. Vesicles were partially loaded with Ca^{2+} and thapsigargin (200 nM) then added to block Ca^{2+} uptake by the SR $\text{Ca}^{2+}/\text{Mg}^{2+}$ -ATPase, leading to a background increase in extravesicular Ca^{2+} (due to Ca^{2+} leak through RyRs) at the start of each record. Peptide A1 (left-hand record) or peptide A1(D-R18) (right-hand record) was then added as indicated by the vertical bars. Finally Ruthenium Red (rr) was added before the Ca^{2+} ionophore A23187 (3 $\mu\text{g}/\text{ml}$) to assess remaining levels of Ca^{2+} within the vesicles (results not shown). The vertical calibration is 0.1 absorbance unit [addition of 10 μM Ca^{2+} (or 20 nmol) increased absorbance by 0.1 unit]. (B) Average initial rates of Ca^{2+} release (nmol/mg of terminal cisternae vesicles per min) induced by peptides (rate of Ca^{2+} release with peptide minus that with thapsigargin). ●, Means $\pm$ S.E.M. for peptide A1(D-R18) ($n = 3$, three different SR preparations). Short-dashed line and long-dashed line show average data for the native A1 and the mutant A2 [2], both included for comparison.

Figure 5 Peptide A1(D-R18) increases single RyR channel activity

(A) Single-channel activity at -40 mV (left-hand panel) and $+40$ mV (right-hand panel), before (control) and after [10 nM A1(D-R18)] addition of 10 nM peptide to the *cis* (cytoplasmic) solution bathing bilayers containing RyR channels in the presence of 300 nM *cis* Ca^{2+} . The continuous line indicates the zero (closed) current level (labelled C) and the broken line shows the maximum open conductance (labelled O). (B) Average relative mean current (I' ; i.e. mean current with peptide normalized to control mean current) at -40 (left-hand panel) and $+40$ mV (right-hand panel) as a function of peptide concentration (in nM) for six experiments. Relative mean current is used as an initial measure of changes in channel activity since data from records containing one or several channels can be included. The ● on continuous lines show means $\pm$ S.E.M. for channels exposed to A1(D-R18); ● and ○ on dotted lines show means for the native A1 and mutant A2 [2] respectively, both included for comparison. The data for A2 was obtained with 10 μM *cis* Ca^{2+} and has been appropriately scaled for comparison with data for A1 and A1(D-R18), which was obtained with 300 nM *cis* Ca^{2+} where the increase in relative mean current is ≈ 1.5 times that recorded with 10 μM *cis* Ca^{2+} .

Figure 6 Effects of peptide A1(p-R18) on single channel parameters measured in five experiments in which the opening of only one channel could be detected

Peptide A1(p-R18) increases channel open probability by dramatically reducing the mean closed time of the channel and increasing the frequency of events, and also by a modest increase in the mean open dwell time. The graphs show average parameter values for data obtained at -40 (left-hand panels) and $+40$ mV (right-hand panels) as a function of A1(p-R18) concentration in nM. (A) Channel open probability (P_o); (B) mean open time (T_o); (C) mean closed time (T_c); (D) frequency of open events (F_o). The symbols show means $\pm$ S.E.M.

recovered during the 20 s perfusion time and then remained high for 6–8 min before declining towards control levels. This slow reversal indicates a slow dissociation rate of the toxin from its binding site and is similar to the slow dissociation seen with peptides A1, A2 and A1(D-R18) (see above). Since reversal of inhibition is rapid, the activity following perfusion is indicative of the degree of activation achieved with the concentration of peptide that the channel had been exposed to immediately before perfusion. Channel activity following perfusion of $10 \mu\text{M}$ toxin from the *cis* chamber (Figure 7B, $\circ$) was similar to the activity recorded with 10–100 nM toxin. The similar activity indicates that maximum activation was achieved at 10–100 nM of the toxin as indicated by the curve fitted to the data in Figure 7(B).

Peptides A1, A1(D-R18) and IPTx also had similar actions on caffeine-induced Ca^{2+} release in skinned muscle fibres (Figure 8). Caffeine (8 mM) produced a small, highly reproducible, force response (see Figure 8A, first and second records). A1(D-R18)

Figure 7 IPTx acts with higher affinity, but has the same effects on Ca^{2+} release and single-channel parameters as peptides A1 and A1(p-R18)

(A) The rate of Ca^{2+} release from the SR, induced by $1 \mu\text{M}$ IPTx, is greater than that released by either A1 or A1(p-R18) at $30 \mu\text{M}$ (see Figure 4). (B) Average relative mean current through RyR channels ($n = 6$) is increased at 1 and 10 nM IPTx and then falls at higher toxin concentrations. The first point at position (C) shows control data, while the final point labelled (W) was obtained after washout of $10 \mu\text{M}$ toxin. The continuous line shows a sigmoid curve fitted to data at 1 nM, 10 nM and W (equivalent to activation with $10 \mu\text{M}$ toxin), assuming that the washout value is the true value for $10 \mu\text{M}$ toxin. Data points at 100 nM, $1 \mu\text{M}$ and $10 \mu\text{M}$ toxin were not included because they were clearly influenced by the inhibitory process (see text). (C) Single-channel records show that 10 nM IPTx causes a substantial increase in the number of channel openings without substantially increasing the duration of the channel opening [records at $+40$ mV, channel opening is upward from the closed level (C) to the maximum conductance (O)]. (D) Mean open time for individual channels. (E) Mean closed time for individual channels. In D and E, channels were exposed to 100 nM A1(p-R18) ($n = 3$, $\bullet$), $1 \mu\text{M}$ peptide A1 ($n = 4$, $\blacksquare$) or 10 nM IPTx ($n = 6$, $\blacklozenge$). Measurements at $+40$ and -40 mV were combined for each channel since there was no consistent effect of bilayer potential on the open or closed times. The increase in open times for all compounds is substantially less than the decrease in closed times.

($2 \mu\text{M}$) increased the size of the force response to 8 mM caffeine from 8% of maximum Ca^{2+} -activated force to 65% ($n = 3$ fibres, Table 3; and Figure 8A, third record). The response to caffeine returned to close to the control level after extensive washout of the peptide (results not shown) and the effect of re-application of $2 \mu\text{M}$ A1(D-R18) was similar to the first exposure (Figure 8A, fourth record). A1(D-R18) ($10 \mu\text{M}$) increased the caffeine response to near maximal force in every fibre (Figure 8A, fifth record; Table 3). Peptide A1 ($2 \mu\text{M}$) caused less potentiation of the caffeine response (from 6 to 20% of maximum force) than the same concentration of peptide A1(D-R18); on average

Figure 8 Effect of peptides and IPTx on caffeine-induced responses in skinned muscle fibres

Mechanically skinned segments of single extensor digitorum longus muscle fibres were subjected to repeated cycles in which the SR was fully depleted of Ca^{2+} , reloaded to a set level, briefly equilibrated in the standard solution with or without peptide and then exposed to the same solution with 8 mM caffeine, before fully depleting the SR again. The level of SR loading was similar to that present endogenously [20]. **(A)** The force response to 8 mM caffeine of a rat extensor digitorum longus fibre was greatly potentiated in the presence of 2 μM peptide A1(D-R18). Washout and re-application gave a similar response, and more potentiation occurred in 10 μM peptide A1(D-R18). The SR was fully depleted of Ca^{2+} and reloaded to a set level between each response. **(B)** 2 μM peptide A1(D-R18) caused greater potentiation than 2 μM peptide A. **(C)** IPTx caused comparable potentiation at 25 nM. Maximum or 50% maximum Ca^{2+} -activated force indicated for each skinned fibre.

Table 3 Force response of skinned muscle fibres to 8 mM caffeine

	Force response (% of maximum Ca^{2+} -activated force)	
	Control	Peptide
Peptide A1 (2 μM ; $n = 3$)	6 ± 4	20 ± 9
Peptide A1(D-R18) (2 μM ; $n = 3$)	8 ± 5	65 ± 7
Peptide A1(D-R18) (10 μM ; $n = 5$)	7 ± 3	91 ± 5
IPTx (25 nM; $n = 2$)	3, 5	46, 30

(Table 3) and in the same fibre (e.g. Figure 8B, second and third records). IPTx also potentiated the caffeine response, although with higher affinity (Figure 8C and Table 3), and the effect could not be readily reversed by washout.

Together these results show that the actions of peptide A1, A1(D-R18) and IPTx on RyR channel open and closed times and on caffeine-activated Ca^{2+} release from skeletal muscle SR are

Figure 9 IPTx can prevent RyR channel activation by peptide A1, while peptides A1 and A1(D-R18) can prevent the actions of IPTx

(A) 1 and 10 nM IPTx cause a significant increase in average mean current (I') and subsequent addition of 1 μM peptide A1 did not increase activity further. Washout of the peptide and toxin by perfusion (four experiments) of the *cis* chamber was followed by an increase in relative mean current in each experiment to a level similar to that with 10 nM toxin. **(B)** 1 μM peptide A1 alone caused a significant increase in average mean current, while subsequent addition of 1 or 10 nM IPTx did not increase activity further (five experiments). An increase in activity after washout of the peptide and toxin (perfusion) in one experiment restored channel activity to the level seen with 1 μM peptide A alone. **(C)** 100 nM peptide A1(D-R18) caused a significant increase in mean current, but subsequent addition of 1 or 10 nM IPTx did not increase activity further (six experiments). Washout of peptide and toxin (perfusion) in four experiments restored the average activity to levels seen with 100 nM peptide A1(D-R18) alone. Dashed horizontal lines show the relative mean current expected if the currents were achieved with 10 nM IPTx (shown in **A**, before addition of peptide A) was added to the relative mean current achieved with 1 μM peptide A1 (shown in **B**) or 100 nM A1(D-R18) (shown in **C**) before addition of toxin. *, indicate values significantly different from control.

similar, as might be expected if they acted at the same site on the RyR complex. The affinities of the compounds differed with the sequence being $\text{IPTx} > \text{A1(D-R18)} > \text{A1}$, which is the same as the sequence of decreasing structural stability of the peptides.

Competition between peptides A1, A1(D-R18) and IPTx

To test the hypothesis that the II–III loop peptides and the scorpion toxin might be acting at the same site, we examined

(i) the ability of peptide A1 to activate channels that were already activated by saturating concentrations (10 nM) of IPTx (Figure 9A) or (ii) the ability of IPTx to activate channels that were already activated by close to saturating concentrations of peptide A1 (1 μ M) or A1(D-R18) (100 nM) (Figures 9B and 9C). It is important to note that channel activity in all experiments remained low (P_o was well below 1.0 and mean current was well below the 12–15 pA expected if P_o were close to 1.0). Therefore the channel could potentially have been activated by a further 3–10-fold. The broken line in each of the graphs in Figure 9 shows the level of activity expected with summation of the activation achieved by each of the peptides or the toxin added alone in this series of experiments. In no case were additive effects of the peptide and the toxin seen. Indeed, there was a tendency for channel activity to fall when 10 nM IPTx was added after either peptide A1 or A1(D-R18), as it did with higher concentrations of IPTx alone (Figure 7B). This suggested that the inhibitory action of IPTx may have been enhanced in the presence of peptides A1 or A1(D-R18).

It was possible that an inhibitory effect of IPTx prevented additional channel activation by peptides A1 and A1(D-R18). Therefore we took advantage of the fact that the inhibitory action of the toxin and the blocking actions of the A peptides are rapidly reversed when the peptides are removed by perfusion from the *cis* solution, while activation by all three compounds remains for several minutes after perfusion (see above), to look at the actual degree of activation by each combination of peptides and toxin. The final bar in Figure 9 (labelled 'perfuse') shows the relative mean current measured between 2 and 3 min after perfusion of the *cis* chamber with peptide/toxin-free solution. Channel activity after perfusion is in each case equivalent to the activity seen with the combination of peptides and toxin, and no greater than the highest activity seen with the compound that was added initially. Thus there appears to be no additive effect of the peptide and toxin, which might have been masked by inhibition.

It has been shown that, even at saturating concentrations, peptide A and toxin exhibit additive effects with other RyR agonists (e.g. Ca^{2+} and ATP) and antagonists (e.g. Mg^{2+} ; M. G. Casarotto, E. M. Gallant and A. F. Dulhunty, unpublished work and [2,8,23,25]). A similar addition might be expected if the peptides and the toxin bound to independent sites on the RyR. In no case were additive effects seen (Figure 9). The fact that additive effects were not detected suggest that the peptides and the toxin are unlikely to bind to independent sites. Possible explanations for the results is that the peptides bind to the same site as the toxin or that they bind to separate sites in a dependent manner, i.e. sites that either physically or functionally interact with each other.

DISCUSSION

Signalling between macromolecular protein complexes often involves protein–protein interactions between discrete functional units. Structural and functional characterization of such interactions has enabled microdomains of biological significance to be identified and shown that they function as isolated entities in various biological situations [28,29]. However, these isolated segments of protein can often lose their structural integrity and hence their desired biological efficacy. In the case of the 170 kDa DHPR, a segment of protein (peptide A) located in the II–III loop region is capable of activating calcium release from the RyR as long as it maintains its helical structure [4]. Other activating peptides reported to target the same receptor site on the RyR have been derived from scorpion venom. Two scorpion toxins,

IPTx and maurocalcine, elicit very strong responses when applied *in vitro* to the RyR. Both the toxins and peptide A contain a common stretch of basic amino acid residues, leading to our speculation that these motifs may share a common conformational surface. On first glance this seems highly unlikely given that the overall structural topology of peptide A and the toxins are radically different (α -helix in contrast to β -sheet). However as seen in Figure 1, both secondary structure scaffolds have the potential to present a common positively charged surface to the RyR.

Modification of the Arg⁶⁸⁸ side chain from an L- to a D-isomer has the effect of increasing the helical character of the α -helix, particularly in the region where the positive residues are located (Arg⁶⁸¹–Arg⁶⁸⁸). The resultant structural effect of this isomeric change is to line one surface of the helix with positive charges, as seen in Figures 2 and 3. D-Arg¹⁸ now is an active participant of the α -helix where in peptides A1 and A2 this is not the case. D-Amino acid replacement has been a popular strategy used to alter the physical and functional properties of peptides [30] with particular emphasis on protecting these molecules from *in vivo* biodegradation [31]. In structural terms, it has been observed that the greatest destabilizing effect on α -helical peptides is introduced by substituting D-amino acids towards the centre of the helix compared with substitution towards the termini [31,32]. In our study, substitution of a residue that is located three away from the C-terminus has the unusual effect of enhancing the helical nature for this portion of the peptide. It is known that the presence of basic amino acids at the C-terminus of a helix results in the stabilization of that helix due to the interaction of the positive amino acid charge and negative dipole inherent in the helix [33]. It is quite conceivable that altering the stereochemistry of a basic residue so close to the C-terminus may promote the extension of the helix.

Although it is possible that the stabilization of the C-terminus of the helix alone may contribute to an increase in activity, it appears most likely that a change in the alignment of the surface charge is the dominant feature responsible for this result. We have previously shown that function can be abolished by removing key positively charged amino acids while maintaining the helical structure [4]. Conversely, the presence of key positively charged residues but loss of helical structure also results in a decrease in activity [4]. Therefore increasing helical structure alone, without simultaneous alignment of positively charged residues, is unlikely to be sufficient to increase activity. However, the extension of the helix increases the alignment of positively charged residues and includes residue D-Arg¹⁸ in the charged surface. Our hypothesis is that the 2-fold increase in activity of A1(D-R18), and 10-fold increase in affinity, compared with A1 is principally a result of extending the helical structure so as to include all five sequential residues (Arg⁷⁸¹–Arg⁷⁸⁵) and allowing Lys⁶⁷⁷, Arg⁶⁸¹, Arg⁶⁸⁴ and D-Arg⁶⁸⁸ to form a positively charged surface. The similarity between the positively charged surfaces on the peptide and toxins is consistent with this hypothesis.

The alignment of the positively charged residues along one surface of the peptide A helix allows the DHPR II–III loop peptide to activate Ca^{2+} release from the SR at rates that were at least ≈ 7 times greater than rates of Ca^{2+} release evoked by the native peptide A1 at peptide concentrations of $\approx 15 \mu\text{M}$. Thus both the alignment of certain amino acids (Lys⁶⁷⁷, Arg⁶⁸¹, Arg⁶⁸⁴ and D-Arg⁶⁸⁸) in peptide A1(D-R18) and its activity became closer to the surface structure and to the activity of the toxins. The fact that the β -sheet toxins are more active than the α -helical DHPR peptides is indicative of the importance of the stability of the secondary structures. Although the helical A1(D-R18) peptide has a higher helical content than the other helical peptides (A1

and A2), its structure remains significantly less stable than the β -sheet structure of the toxins because of the internal, disulphide-bridged linkages present in the toxin.

Previous studies suggested that a II–III loop peptide Glu⁶⁷⁷–Leu⁶⁹⁰, which contains the peptide A sequence, might bind to the same site as IPTx, since it could reduce the increase in [³H]ryanodine binding produced by IPTx and could displace the binding of [¹²⁵I]IPTx to the SR [8]. Here we provide supporting evidence that peptide A1, A1(D-R18) and IPTx might bind to the same site because they produce almost identical changes in RyR gating characteristics. Furthermore, their non-additive effects suggest that their binding sites are not independent. Nevertheless, the question of whether peptide A and related peptides bind to the same site as the toxin remains unresolved. Although published competition and binding studies and sequence alignment data [8], coupled with our functional profiling experiments and structural analysis, suggest that a common site, the definitive answer must await high-resolution (> 5 Å) structural studies of these compounds bound to the RyR. Such studies are not feasible at present. Current NMR experiments focusing on specific fragments of the RyR that bind to the peptide A region of the DHPR II–III loop [34] may help resolve the question.

The regions of the DHPR II–III loop responsible for structural and functional interactions between the DHPR and RyR during EC coupling have not been clearly defined. The physiological relevance of the A segment of the DHPR in EC coupling is unclear, and other regions of the II–III loop are also functionally important [32]. Clearly, a structural analysis of the full II–III loop is required as well as identification of all protein–protein interaction sites between the DHPR and RyR. The present study has elucidated the structural requirements for just one of these interactions.

Conclusions

This study raises the possibility that naturally occurring toxins can mimic the surface topology of parts of essential mammalian proteins. Based on published competition experiments between the toxin and DHPR peptides [8] as well as functional/structural studies outlined in this paper evidence is mounting that the toxins and mammalian peptides can activate a common receptor site of RyR because of their common surface topology. We have used this information to modify the stereochemistry of peptide A to significantly enhance its ability to activate the RyR. This raises the prospect of employing non-peptidic molecular scaffolds in an alternative therapeutic route for peptide-based drugs.

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