Activation of Ryanodine Receptors by Imperatoxin A and a Peptide Segment of the II-III Loop of the Dihydropyridine Receptor*

(Received for publication, October 7, 1998, and in revised form, December 2, 1998)

Georgina B. Gurrola, Carolina Arévalo, Raghava Sreekumar, Andrew J. Lokuta, Jeffery W. Walker, and Hector H. Valdivia†

From the Department of Physiology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Excitation-contraction coupling in skeletal muscle is believed to be triggered by direct protein-protein interactions between the sarcosomal dihydropyridine-sensitive Ca2+ channel and the Ca2+ release channel/ryanodine receptor (RyR) of sarcoplasmic reticulum. A 138-amino acid cytoplasmic loop between repeats II and III of the α1 subunit of the skeletal dihydropyridine receptor (the II-III loop) interacts with a region of the RyR to elicit Ca2+ release. In addition, small segments (10–20 amino acid residues) of the II-III loop retain the capacity to activate Ca2+ release. Imperatoxin A, a 33-amino acid peptide from the scorpion Pandinus imperator, binds directly to the RyR and displays structural and functional homology with an activating segment of the II-III loop (Glu666-Leu690). Mutations in a structural motif composed of a cluster of basic amino acids followed by Ser or Thr dramatically reduce or completely abolish the capacity of the peptides to activate RyRs. Thus, the Imperatoxin A-RyR interaction mimics critical molecular characteristics of the II-III loop-RyR interaction and may be a useful tool to elucidate the molecular mechanism that couples membrane depolarization to sarcoplasmic reticulum Ca2+ release in vivo.

In cardiac and skeletal muscle, the dihydropyridine receptor (DHPR) of the external membrane and the Ca2+ release channel/ryanodine receptor (RyR) of sarcoplasmic reticulum (SR) are key components of excitation-contraction (E-C) coupling, the series of events that link an electrical stimulus (depolarization) to a mechanical contraction (1). Skeletal and cardiac muscle express different subtypes of DHPR and RyR, which account for different E-C coupling mechanisms. In the heart, a small influx of Ca2+ through DHPRs triggers the opening of RyRs (2). In skeletal muscle, however, external Ca2+ is not required for Ca2+ release (3). Contractions are instead triggered by membrane depolarizations, and because Ca2+ release may be arrested immediately upon repolarization, a mechanical coupling between the DHPR and the RyR is thought to mediate E-C coupling (4, 5).

Compelling evidence indicates that the skeletal DHPR subtype is indispensable to elicit a Ca2+-independent (skeletal-type) contraction (6, 7) and that the 138-amino acid cytoplasmic loop between repeats II and III of the α1 subunit of the skeletal dihydropyridine receptor (the II-III loop) interacts with a region of the RyR to elicit Ca2+ release. In experiments with isolated peptides, the II-III loop activates purified RyRs (9), and a small fragment of the II-III loop (Thr771-Leu806) induces Ca2+ release from SR vesicles (10). In dysgenic myotubes, skeletal-type E-C coupling is partially restored by a chimeric DHPR that is entirely cardiac except for a short segment of skeletal II-III loop (Phe725-Pro726) (8). Although apparently contradictory in the identity of the activating region, these results suggest that specific domains of the II-III loop directly interact with the RyR to change its conformational state and produce Ca2+ release. Therefore, in skeletal muscle, the II-III loop stands as the strongest candidate among regions of the DHPR to bind to RyRs. However, the precise amino acid residues of the II-III loop that trigger Ca2+ release remain unknown. Furthermore, other DHPR segments (11) or subunits (12) have not been discarded as points of contact.

We have previously shown that Imperatoxin A (IpTxα), a 33-amino acid peptide from the scorpion Pandinus imperator, is a high-affinity activator of RyRs (13, 14). The biological significance of IpTxα is unknown, because the apparent target for this membrane-impermeable peptide is located intracellularly. Because some peptide toxins activate intracellular signaling pathways by mimicking surface receptors (15, 16), we tested the hypothesis that IpTxα activates RyRs by mimicking a domain of the DHPR that is critical to trigger Ca2+ release. We found that IpTxα, and a synthetic peptide with an amino acid sequence corresponding to a segment of the II-III loop (Glu666-Leu690) (10) activate RyRs in a similar manner and appear to compete for a common binding site on the channel protein. Both peptides bind to RyRs via a structural domain consisting of a cluster of basic amino acids (Arg681-Lys685) of the II-III loop and Lys19–Arg24 of IpTxα followed by a hydroxylated amino acid (Ser687 of the II-III loop and Thr27 of IpTxα). Thus, IpTxα presents an interesting case of toxin mimicry of effector proteins that may be used to identify regions of the RyR that trigger Ca2+ release. If the peptide segment emulated by IpTxα is an actual participant in the DHPR/RyR interaction, IpTxα may also be exploited to identify regions of the RyR involved in E-C coupling.

EXPERIMENTAL PROCEDURES

Materials—[3H]Ryanodine (60–80 Ci/mmol) was from NEN Life Science Products, agelenin and Tx2-9 were from The Peptide Institute, Inc. (Osaka, Japan), bovine brain phosphatidylethanolamine and phosphatidylserine were from Avanti Polar Lipids (Birmingham, AL), and Fmoc-amino acids were from Applied Biosystems. Polyclonal skeletal RyR antibody was from Upstate Biotechnology. Peroxidase-conjugated secondary antibody and α-conotoxin were from Calbiochem. The chemiluminescence detection kit was from Boehringer Mannheim. Pre-cast linear gradient polyacrylamide gels were from Bio-Rad. All other re-
agents were of high-purity reagent grade. [3H]Ryanodine and 125I-IpTxa, Binding Assay—[3H]Ryanodine (7 nM) was incubated for 90 min at 36 °C with 40–50 μg of rabbit skeletal SR vesicles in medium containing 0.2 mM KCl, 10 mM CaCl2, and 10 mM Na-Hepes (pH 7.2) in the absence and presence of peptides. Free ligand and bound ligand were separated by filtration on glass filters, as described previously (13, 14). Native IpTxa, (10-μg batches) was purified according to established procedures (13, 14) and iodinated to a specific activity of 60–80 Ci/mmol with the Bolton-Hunter radiolabeling method following the specifications of the manufacturer (New England Nuclear). The binding of 125I-IpTxa to skeletal SR and Chinese hamster ovary (CHO) cell homogenates was performed under conditions identical to those described for [3H]ryanodine, except that the protein concentration was 0.1–0.2 mg/ml in the case of CHO cells. Bmax and Kd of the 125I-IpTxa receptor complex were obtained by fitting data points with the following equation: B = Bmax × 125I-IpTxa/Kd + 125I-IpTxa, where B is the specific binding of 125I-IpTxa.

Transfection of CHO Cells with RyR—CHO cells were transfected by lipofection with plasmid pRS81, the rabbit skeletal muscle RyR (RyR1), as described previously (17). Expression of the RyR was confirmed by immunoblot analysis using monoclonal antibodies against the skeletal RyR and by [3H]ryanodine binding. Control and transfected cells were homogenized in 500 mM sucrose, 1 mM EGTA, and 10 mM Hepes-Tris (pH 7.4) and spun at 44,000 g for 30 min (17). The pellet was washed twice with the same buffer for [3H]ryanodine binding experiments.

Synthesis of Peptides—Linear analogs of IpTxa and the II-III loop of the 125I-IpTxa receptor complex were obtained by fitting data points with the following equation:

\[ B = B_{\text{max}} \times 125\text{I-IpTxa} / K_d + 125\text{I-IpTxa}, \]

where B is the specific binding of 125I-IpTxa.

Planar Bilayer Recording of RyRs—Recording of single RyR in lipid bilayers was performed as described previously (13, 19). Single channel data were collected at steady voltages (+30 mV) for 2–5 min in symmetrical 300 mM cesium methanesulfonate, 10 mM CaCl2, and 10 mM Na-Hepes (pH 7.2). IpTxa and the II-III loop peptide were added to the cis chamber, which corresponded to the cytosolic side of the channel (13, 19). The addition of the peptide to the trans (luminal) side of the channel was without effect. In some experiments, we added 10 mM CaCl2 to the trans solution. At 0 mM Ca2+, Ca2+ was the only charge carrier in the cis chamber, and both peptides were effective in inducing a subconductance state of about one-fourth of the full conductance level. However, the low signal:noise ratio obtained under these conditions made the analysis of the kinetic effect difficult. For the experiments presented here, we omitted Ca2+ in the trans solution. Signals were filtered with an 8-pole low pass Bessel filter at 2 kHz and digitized at 5 kHz. Data acquisition and analysis were done with Axon Instruments software (pClamp v6.0.2, Digidata 200 AD/DA interface), kHz. Data acquisition and analysis were done with Axon Instruments software (pClamp v6.0.2, Digidata 200 AD/DA interface).

RESULTS

Selective Activation of [3H]Ryanodine Binding by IpTxa among Ca2+ Channel Toxins—The amino acid sequence of IpTxa (14) exhibits no significant homology with the well-characterized Na+ and K+ channel scorpion toxins (data not shown). However, IpTxa does share 45% and 42% sequence identity with agelenin (21) and Tx2-9 (22), respectively, two spider toxins that block presynaptic (P-type) Ca2+ channels (Fig. 1A). The Cys residues, which stabilize the three-dimensional structure by forming disulfide bridges (16), are similarly arranged in these three peptides (gray boxes). Indeed, they may be used as a frame to align the amino acid sequence of α-conotoxin MVIIIC, a snail peptide that blocks P-type Ca2+ channels (23), and to reveal regions of homology (open boxes). Fig. 1B shows that, despite the demonstrated structural kinship among these peptides, only IpTxa is capable of enhancing [3H]ryanodine binding. ED50, the concentration of IpTxa required to produce a half-maximal effect (6 ± 3.1 mM, mean ± S.D.; n = 18), is only slightly higher than that exhibited by [3H]ryanodine among ligands of RyRs (24). This selective and high-affinity effect suggests that IpTxa possesses a unique structural motif that activates RyRs, which is not present even in structurally related peptides.

Physical Interaction of IpTxa with RyRs—To test whether IpTxa may be used independently as a high-affinity, specific ligand for RyRs, we radiolabeled IpTxa and conducted binding experiments in the absence of ryanodine. Fig. 2A shows that the radiolabeled derivative of IpTxa retained high affinity (Kd = 11 ± 3 mM) and bound to skeletal SR with a maximal receptor site density (Bmax) of 16.1 ± 1.9 pmol/mg protein (n = 3). In the same tissue, the Bmax for [3H]ryanodine was 3.7 ± 0.6 pmol/mg protein. Thus, assuming all 125I-IpTxa binding occurs to the RyR, the 125I-IpTxa/[3H]ryanodine binding site stoichiometry is 4.3:1. Because one [3H]ryanodine molecule binds with high affinity to the tetrameric RyR (24), this ratio suggests that about four IpTxa molecules bind to every RyR tetramer. In CHO cells transfected with the skeletal RyR (Fig. 2B, + RyR1), the 125I-IpTxa/[3H]ryanodine binding site stoichiometry is 4:6:1 (n = 2). In naive CHO cells, there is neither 125I-IpTxa (Fig. 2B, Untransfected) nor [3H]ryanodine binding (data not shown).

To confirm that IpTxa physically interacts with the RyR monomer in skeletal SR, we prepared photoactivatable IpTxa, a synthetic derivative of IpTxa in which Leu9 was replaced by the light-sensitive cross-linker p-benzoyl-phenylalanine (18). The photoactivative derivative retained high affinity for the RyR (Kd = 12 ± 4 mM; n = 3; data not shown). Fig. 3A shows a SDS-polyacrylamide gel electrophoresis profile of SR proteins that were radiated with ultraviolet light after incubation with 30 nM photoactivatable 125I-IpTxa in the absence (lane 1) and the presence (lane 2) of 50 mM unlabeled IpTxa. An immunoblot analysis using a skeletal RyR polyclonal antibody recognized only the high molecular weight band of SR proteins (Fig. 3B). The autoradiogram of the SDS-gel (Fig. 3C) shows clear labeling of the band corresponding to the RyR (lane 1). Other bands are also labeled, most likely from a nonspecific interaction with the toxin, because labeling persists in the presence of excess
IpTxα (lane 2). Together with data from Fig. 2, these results indicate that IpTxα makes direct protein-protein interactions with the RyR with a stoichiometry of four IpTxα molecules per single RyR channel.

**Functional Effect of IpTxα and a Short Segment of the II-III Loop**—The functional effect of the IpTxα-RyR interaction was tested in planar lipid bilayer and Ca_{2+} release experiments. Fig. 4A shows that 50 nM IpTXa added to the cytoplasmic (cis) side of the skeletal RyR induced the appearance of a subconductance state corresponding to ~25% of the full conductance as previously shown (25). Although of small amplitude, the subconductance state displayed a mean open time that was ~100-fold longer than that of unmodified channels. Ion flow would therefore be expected to be greater for an IpTxα-modified channel, despite its lower conductance. Fig. 4B shows that IpTXa elicited Ca_{2+} release from actively loaded SR vesicles in a dose-dependent manner. The effect of IpTXa was blocked by ruthenium red, consistent with Ca_{2+} release occurring through RyRs. Strikingly similar results were observed with a 25-amino acid synthetic peptide with primary sequence (Glu666-Leu690; Fig. 5A) overlapping that of peptide A (Thr671-Leu690), a segment of the II-III loop that activates RyRs (10). The 25-amino acid segment of the II-III loop (henceforth termed “the II-III loop peptide”), like IpTXa, induced the appearance of a small-
amplitude and long-lifetime subconductance state (Fig. 4C) and elicited Ca\(^{2+}\) release from SR (Fig. 4D). Thus, albeit with different affinity, the two apparently unrelated peptides exhibit similar functional effects on RyRs.

**Structural Analogy between IpTx\(_a\) and the II-III Loop Peptide**—A one-to-one residue alignment between IpTx\(_a\) and the II-III loop peptide does not reveal significant homology in their amino acid sequence (Fig. 5A). However, both IpTx\(_a\) and the II-III loop peptide display a structural motif consisting of a cluster of basic amino acids (boxes, Lys\(^{19}\)-Arg\(^{24}\) and Arg\(^{681}\), Lys\(^{8}\) and Glu\(^{685}\), respectively) followed by Thr or Ser, two hydroxylated amino acids (ovals, Thr\(^{26}\) and Ser\(^{687}\), respectively). In IpTx\(_a\), the cluster of basic amino acids is interrupted by Cys\(^{21}\) and encompasses the sequence KCK, which is also found in agelinin and Tx2-9 (Fig. 1A). Therefore, it is likely that the KCK sequence alone does not suffice to activate RyRs and that Cys\(^{21}\) stabilizes the peptide structure without intervening in protein-protein interactions with the RyR. Hydroxylated amino acids in a position close to Thr\(^{26}\) of IpTx\(_a\) are also found in agelinin (Ser\(^{28}\)) and in Tx2-9 (Thr\(^{26}\) and Thr\(^{24}\)), but none is preceded by a cluster of basic amino acids. Likewise, the motif RRG, which appears in IpTx\(_a\) and \(\alpha\)-conotoxin MVIIC, is only followed by a hydroxylated amino acid in the former peptide. Indeed, similar to Ser\(^{687}\) of the II-III loop (27), the distinctive arrangement of Thr\(^{26}\) of IpTx\(_a\) with the preceding residues produces a phosphorylation consensus for several protein kinases (28). As presented in Fig. 5B, the structural motif consisting of a cluster of positively charged amino acids followed by a hydroxylated residue is not found in other peptide toxins or in other regions of the DHPR, including the \(\beta\)-subunit. Thus, IpTx\(_a\) and the II-III loop peptide share a specific arrangement of amino acid residues that may be responsible for their similar functional effect on RyR channels.

**Competition Experiments between IpTx\(_a\) and the II-III Loop Peptide**—To test whether the analogous functional effects produced by IpTx\(_a\) and the II-III loop peptide (Fig. 4) result from activation of the same modulatory site on the RyR, we carried out competition experiments between the two peptides. Fig. 6A shows that the II-III loop peptide incrementally decreases the capacity of IpTx\(_a\) to activate RyRs. The ED\(_{50}\) for II-III loop inhibition of the IpTx\(_a\) effect was 1.3 ± 0.7 \(\mu\)M (n = 3), in agreement with the value calculated from direct activation of [\(^{3}H\)]ryanodine binding by the II-III loop peptide (Fig. 7B). In contrast, a scrambled II-III loop peptide (a synthetic peptide with amino acid composition identical to the II-III loop peptide but in random sequence) was incapable of stimulating [\(^{3}H\)]ryanodine binding (data not shown) or of abolishing the IpTx\(_a\) effect (Fig. 6B). Thus, the effects of the II-III loop peptide require a defined amino acid sequence and are unrelated to peptide mass or electrical charge. In other competition studies, the II-III loop peptide displaced the binding of 125I-IpTx\(_a\) to SR vesicles with an ED\(_{50}\) of 36 ± 4 \(\mu\)M (Fig. 5C). This reduced affinity may be due to displacement of 125I-IpTx\(_a\) from sites of nonequivalent affinity, or it may result from positive allosteric interaction between the II-III loop peptide and 125I-IpTx\(_a\). Nevertheless, the II-III loop-125I-IpTx\(_a\) competition appears to be specific, because the scrambled II-III loop had no significant effect at concentrations up to 300 \(\mu\)M (Fig. 6C).

**Effect of Mutations in IpTx\(_a\) and the II-III Loop Peptide**—If, by analogy with other peptide toxin-in channel associations (29, 30), the high-affinity IpTx\(_a\)-RyR interaction is mediated by electrostatic forces, then mutations in the binding domain of IpTx\(_a\) should alter its electrostatic potential and the measured affinity constant of the IpTx\(_a\)-RyR complex. Likewise, if IpTx\(_a\) and the II-III loop peptide bind to the skeletal RyR via a common structural motif, then corresponding mutations should evoke parallel changes in affinity for both peptides. Fig. 7A shows that synthetic IpTx\(_a\), a synthetic peptide with an amino acid sequence identical to that of native IpTx\(_a\); Ref. 14) activates [\(^{3}H\)]ryanodine binding to skeletal SR with potency and affinity identical to native IpTx\(_a\) (5 ± 3 \(nM\); n = 6). Other synthetic derivatives of IpTx\(_a\), in which Thr\(^{26}\) was replaced with Ala (T26A) or with the negatively charged residue Glu (T26E), increased [\(^{3}H\)]ryanodine binding with an affinity 12- and 160-fold lower (ED\(_{50}\) = 60 ± 5 and 800 ± 78 \(nM\), respectively). Mutations to the II-III loop peptide elicited qualitatively similar results (Fig. 7B). The replacement of Ser\(^{687}\) with Ala (S687A) or with Glu (S687E) decreased the affinity of the II-III loop peptide from 0.81 ± 0.2 \(\mu\)M (control) to 4.2 ± 1.1 and 22 ± 4 \(\mu\)M, respectively. Therefore, substituting Thr or Ser of IpTx\(_a\) and the II-III loop peptide with nonpolar or charged amino acids has a substantial impact on the ability of both peptides to activate RyRs. However, neither mutation results in a complete loss of peptide activity.

Mutations within the cluster of basic amino acids elicit more dramatic effects. In IpTx\(_a\), replacing Arg\(^{23}\) with Glu (R23E) abolished IpTx\(_a\) stimulation of [\(^{3}H\)]ryanodine binding (Fig. 8A). The corresponding substitution in the II-III loop peptide, Arg\(^{684}\) to Glu (R684E), also abolished its effect on [\(^{3}H\)]ryanodine binding (Fig. 8B). In contrast, replacing either Lys\(^{8}\) of IpTx\(_a\) or the comparable amino acid Lys\(^{675}\) of the II-III loop peptide with Glu (K6E and K675E, respectively) yielded ED\(_{50}\) values of 19 ± 4 \(nM\) and 1.5 ± 0.3 \(\mu\)M, respectively. Thus, the dramatic effect of mutations within the cluster of basic amino acids cannot be solely attributed to a change of electrical charge because mutations distant to the cluster but producing the same electrical change have relatively minor effects.

**DISCUSSION**

The molecular mechanism by which depolarization of the skeletal T-tubule membrane induces Ca\(^{2+}\) release from the SR remains an outstanding problem in E-C coupling, with a physical DHPR/RyR interaction being the most plausible hypothesis. Whereas the identification of the RyR region(s) involved in this interaction is in progress (31, 32), there is already substantial evidence to invoke the participation of the II-III loop of the \(\alpha_1\) subunit of the DHPR (6, 8–10). As discussed below, there...
is still controversy regarding the precise structural domain(s) of the II-III loop involved in this interaction. In this study, we identified a unique structural motif involved in activation of RyRs in IpTx₉ and in a short segment of the II-III loop. Although the participation of this structural motif in E-C coupling awaits further testing, our results provide a structural framework for a mechanical model in which specific amino acids of the II-III loop are capable of interacting with RyRs and triggering Ca²⁺ release.

Analysis of the amino acid sequence of IpTx₉ and the effect of
mutations strongly suggest that the structural motif encompassing Lys19-Arg24, the cluster of basic amino acids, followed by Thr 26, the hydroxylated amino acid, is involved in IpTxa binding to the RyR. Our results, however, do not allow us to ascertain the weight of individual amino acids within this structural domain for participation in binding. For instance, agelenin, Tx2-9, and $\gamma$-conotoxin, three structurally related peptides that are incapable of increasing $[^{3}H]$ryanodine binding (Fig. 1), bear resemblance to IpTxa in several regions, except in the proposed structural domain. However, agelenin and Tx2-9 do maintain the KCK motif, and thus the actual involvement of these residues in the binding of IpTxa, remains undetermined. Either the KCK motif is independent of the binding site or, in analogy to other peptide toxins (29, 30), it may be involved in IpTxa docking without participating in activation. The pair of Arg residues following the KCK motif is clearly unique to IpTxa. As shown in Fig. 8, replacing Arg 23 with Glu (R23E) totally abolished the capacity of IpTxa to activate RyRs. Because a similar substitution in a region distant to this cluster (K8E; Fig. 8) was without major functional consequences, the lack of effect of R23E was probably the result of local changes in the electrostatic potential of the toxin’s binding site, rather than global conformational changes. Lastly, the involvement of Thr26 was demonstrated by hydrophobic (T26A) and charged (T26E) substitutions (Fig. 5). Although the mutated peptides retained their ability to activate RyRs, there was a significant loss of affinity for both peptides.

Replacing equivalent amino acids in the II-III loop peptide
produced results that were qualitatively similar to those obtained with IpTXₐ mutants (Figs. 7 and 8). These data, plus the competition experiments in which the II-III loop peptide inhibited the effect of IpTXₐ (Fig. 6A) and displaced the binding of ¹²⁵I-IpTXₐ (Fig. 6C), strongly suggest that both peptides activate RyRs by a physical interaction with the channel protein via the aforementioned structural domain. If this structural domain of the II-III loop is important for the activation of RyR in vivo, then IpTXₐ is a peptide mimetic of an effector protein, and its structure-activity information may have direct implications for the mechanism of E-C coupling in skeletal muscle. In skeletal muscle cells, the DHPR-RyR interaction involves orthograde (DHPR→RyR) as well as retrograde (RyR→DHPR) signals (32). Thus, providing that the II-III loop peptide studied here is more similar to the activation produced by FK506 and ryanodine. FK506 and other immunosuppressants that strip RyRs of the accessory protein FKBP12 produced results that were qualitatively similar to those obtained with isolated peptides, it is still possible that the structural domain of peptide A postulated here as being important for activation of RyRs participates in the transmission signal from DHPR to RyR. A potential scenario would be that Phe₇₂₅-Pro₇₄₂ was the most important region of the skeletal DHPR to elicit a skeletal-type contraction. This region is located almost at the middle of the II-III loop, more than 30 residues downstream from peptide A. Although the results with DHPR chimeras are apparently contradictory to those obtained with isolated peptides, it is still possible that the structural domain of peptide A postulated here as being important for activation of RyRs participates in the transmission signal from DHPR to RyR. A potential scenario would be that Phe₇₂₅-Pro₇₄₂, which determines skeletal-type E-C coupling (8), binds to the skeletal RyR at rest to inhibit Ca²⁺ release and that depolarization of the T-membrane rearranges the II-III loop such that peptide A interacts with and activates the RyR. In agreement with this hypothesis, El-Hayek et al. (10) found that the activating effect of peptide A was antagonized by Glu₇₂₄-Pro₇₆₀, a fragment of the skeletal II-III loop encompassing the amino acid sequence identified by Nakai et al. (8). However, even in this simplified scenario, outstanding issues remain unsolved. For example, skeletal-type contractions do not require entry of external Ca²⁺, whereas activation of RyRs by peptide A (10), IpTXₐ (15), or the whole II-III loop (9) requires Ca²⁺, at least at suboptimal levels. Again, this may reflect the fact that these peptides are only partial effectors of the transmission signal, with other segments of the DHPR conferring Ca²⁺ independence to the DHPR-RyR interaction.

Toxin mimicry of surface receptors is not unprecedented. Mastoparan, a peptide from wasp venom, is a potent secretagogue that mimics an intracellular loop of G protein-coupled receptors (15). As previously shown (16), the capacity of foreign peptides to activate intracellular signals may yield insights into the molecular mechanisms of signal transduction in the target cells. In the case of IpTXₐ, the high affinity of ¹²⁵I-IpTXₐ
demonstrates a direct protein-protein interaction with the RyR and reveals a ~4:1 stoichiometry of IpTxa/RyR binding sites (Fig. 2). Assuming that IpTxa is a surrogate high-affinity ligand for the II-III domain that triggers Ca\(^{2+}\) release, these results suggest that in the intact muscle, up to four II-III loops directly gate one RyR. This would be consistent with the current structural model of E-C coupling in skeletal muscle (26), where every other foot protein (RyR) faces a tetrad of T-tubule particulates representing four DHPR molecules. Another inference based on the above assumption is that the II-III loop but not the carboxyl terminus of the \(a_1\) subunit (11) or the \(\beta\)-subunit (12) is structurally endowed to trigger Ca\(^{2+}\) release. This is because the structural domain identified as being responsible for activating RyRs (Fig. 4) is contained within the II-III loop sequence only. However, as mentioned above, our results do not rule out the possibility of multiple sites of interaction between the DHPR and the RyR because inhibitory sites of contact have been detected in other segments of the \(a_1\) subunit (11) and even within the II-III loop itself (10).

Regardless of its potential use as a peptide probe to study the molecular determinants of E-C coupling, it is important to keep in mind that IpTxa triggers conformational changes in the channel protein that ultimately lead to Ca\(^{2+}\) release (Fig. 4). The specificity, high affinity, and reversibility of the IpTxa-RyR interaction may thus be exploited to identify amino acids of the RyR directly responsible for channel opening.

Acknowledgments—We thank Hiroshi Takeshima and Jianjie Ma for providing CHO cells transfected with the RyR, Fernando Zamudio for help with bilayer experiments, and Joel Armstrong for iodination of IpTxa.

REFERENCES

1. Bers, D. (1991) in Excitation Contraction Coupling and Cardiac Contractile Force, Kluwer Academic Publishers, The Netherlands
2. Fabiato, A. (1986) J. Gen. Physiol. 88, 247–290
3. Armstrong, C. M., Bezanilla, F., and Horowicz, P. (1972) Biochim. Biophys. Acta 267, 605–612
4. Chandler, W. K., Rakowski, R. F., and Schneider, M. F. (1976) J. Physiol. (Lond.) 254, 283–316
5. Bioz, E., and Pizzarro, G. (1992) Physiol. Rev. 71, 849–908
6. Tanabe, T., Beam, K. G., Adams, B. A., Nidome, T., and Numa, S. (1990) Nature 346, 567–569
7. Tanabe, T., Mikami, A., Numa, S., and Beam, K. G. (1990) Nature 344, 451–453
8. Nakai, J., Tanabe, T., Konno, T., Adams, B., and Beam, K. G. (1998) J. Biol. Chem. 273, 24983–24986
9. Lu, X., Xu, L., and Meissner, G. (1994) J. Biol. Chem. 269, 6511–6516
10. El-Hayek, R., Antoniu, B., Wang, J., Hamilton, S. L., and Ikemoto, N. (1995) J. Biol. Chem. 270, 22116–22118
11. Slavik, K. J., Wang, J. P., Aghdasi, B., Zhang, J. Z., Malouf, N., and Hamilton, S. L. (1997) Am. J. Physiol. 274, C1475–C1481
12. Beurg, M., Ahern, C. A., Sukhareva, M., Perez-Reyes, E., Puwars, P. A., Gregg, R. G., and Coronado, R. (1998) Biophys. J. 74, A235
13. El-Hayek, R., Lokuta, A. J., Arévalo, C., and Valdivia, H. H. (1995) J. Biol. Chem. 270, 28696–28704
14. Zamudio, F. Z., Gurrola, G. B., Arévalo, C., Sreekumar, R., Walker, J. W., Valdivia, H. H., and Possani, L. D. (1997) FEBS Lett. 405, 385–389
15. Higashijima, T., Uzu, S., Nakajima, T., and Ross, E. M. (1988) J. Biol. Chem. 263, 6491–6494
16. Menez, A., Bontems, F., Roumestand, C., Gilquin, B., and Toma, F. (1992) Proc. R. Soc. Edinb. Sect. B (Biol.) 99B, 83–103
17. Bhat, M. B., Zhao, J., Zang, W., Balke, C. W., Takeshima, H., Wier, W. G., and Ma, J. (1997) J. Gen. Physiol. 110, 749–760
18. Wilson, C. J., Husain, S. S., Stimson, E. R., Dangett, L. J., Miller, K. W., and Maggio, J. E. (1997) Biochemistry 36, 4542–4551
19. Xiao, R.-P., Valdivia, H. H., Bogdanov, K., Valdivia, C., Lakatta, E. G., and Cheng, H. (1997) J. Physiol. 500, 343–354
20. Palade, P. (1987) J. Biol. Chem. 262, 6135–6141
21. Hagiwara, R., Sakai, T., Miwa, A., Kawai, N., and Nakajima, T. (1999) Biomed. Res. 1, 181–186
22. Cordeiro, M. do N., Diniz, C. R., Valentim, A. do C., von Eickstedt, V. R., Gilroy, J., and Richardson, M. (1992) FEBS Lett. 310, 153–156
23. Hilliard, D. R., Monje, V. R., Mintz, I. M., Bean, B. P., Nadasdi, L., Ramachandran, J., Miljanich, G., Azimiz-Zoonooz, A., McIntosh, J. M., and Cruz, J. L. (1992) Neuron 9, 69–77
24. Zucchi, R., and Ronca-Testoni, S. (1997) Pharmacol. Rev. 49, 1–51
25. Trypathy, A., Rees, W., Xu, L., Valdivia, H. H., and Meissner, G. (1998) J. Gen. Physiol. 111, 679–690
26. Block, B., Imagawa, T., Campbell, K. P., and Franzini-Armstrong, C. (1988) J. Cell Biol. 107, 2587–2600
27. Lu, X., Xu, L., and Meissner, G. (1995) J. Biol. Chem. 270, 18459–18464
28. Kemp, B. E., and Pearson, R. B. (1990) Trends Biol. Sci. 15, 342–346
29. Hidalgo, P., and MacKinnon, R. (1995) Science 268, 357–370
30. Dudley, S. C., Todt, H., Lipkind, G., and Fozzard, H. A. (1995) Biophys. J. 69, 1657–1665
31. Yamazawa, T., Takeshima, H., Shimuta, M., and Ino, M. (1997) J. Biol. Chem. 272, 3161–3164
32. Nakai, J., Sekiguchi, N., Rando, T. A., Allen, P. D., and Beam, K. G. (1998) J. Biol. Chem. 273, 13403–13406
33. Brilliante, A. M., Ordmar, R., Scott, A., Kabinsky, E., Ordmar, E., Moschella, M., Jayaraman, T., Landers, M., Ehrlich, B. E., and Marks, A. R. (1994) Cell 77, 513–523
34. Lindsay, A. R. G., Tinker, A., and Williams, A. J. (1994) J. Gen. Physiol. 104, 1–51
35. El-Hayek, R., and Ikemoto, N. (1998) Biochemistry 37, 7015–7020
36. Leong, P., and MacLennan, D. H. (1998) J. Biol. Chem. 273, 7791–7794