Three-dimensional Location of the Imperatoxin A Binding Site on the Ryanodine Receptor

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Abstract. Cryo-electron microscopy and three-dimensional, single-particle image analysis have been used to reveal the specific binding site of imperatoxin A (IpTxα) on the architecture of the calcium release channel/ryanodine receptor from skeletal muscle (RyR1). IpTxα is a peptide toxin that binds with high affinity to RyR1 and affects its functioning. The toxin was derivatized with biotin to enhance its detection with streptavidin. IpTxα binds to the cytoplasmic moiety of RyR1 between the clamp and handle domains, 11 nm away from the transmembrane pore. The proposed mimicry by IpTxα of the dihydropyridine receptor (DHPR) II-III loop, thought to be a main physiological excitation-contraction trigger, suggests that the IpTxα binding location is a potential excitation-contraction signal transduction site.

Key words: ryanodine receptor • imperatoxin A • cryo-electron microscopy • three-dimensional reconstruction • excitation-contraction coupling

The ryanodine receptor isoform 1 (RyR1) is a large multi-subunit transmembrane protein (four identical subunits of 565-kD and four 12-kD subunits) that functions as the calcium release channel in the sarcoplasmic reticulum (SR) of mammalian skeletal muscle (recently reviewed by Franzini-Armstrong and Protasi, 1997). Dihydropyridine receptors (DHPRs) are located in the tubular invaginations of the plasma membrane known as transverse (T) tubules. RyR1s and DHPRs meet at the triad junctions, specialized myofiber regions where the T tubules meet the terminal cisternae regions of the SR. At these junctions the RyR1s and DHPRs form two extended rows in their respective cisternae regions of the SR. A t these junctions the RyR1s and DHPRs form two extended rows in their respective membranes, appearing to form complementary lattices from which the relative geometry of the two receptors has been inferred (B lock et al., 1988). According to the currently favored mechanism of excitation-contraction (E-C) coupling in skeletal muscle, a neuronal voltage-induced conformational change in the DHPR, by means of direct interaction with RyR1, switches RyR1 from a closed to an open configuration, thereby releasing Ca2+ from the SR and provoking muscular contraction (Schneider and Chandler, 1973; Catterall, 1991; Rios and Pizzaro, 1991).

The three-dimensional (3D) structure of RyR1 as determined by cryo-EM and image processing (Radermacher et al., 1994; Serysheva et al., 1995; for review see Samsó and Wagenknecht, 1998) reveals a large symmetric square-prism shaped structure forming the cytoplasmic moiety with 10 well-defined domains. The cytoplasmic peripheral features are also known as “clamp” (domains 5-10) and “handle” (domain 3). A smaller transmembrane region bears the ion channel. The large cytoplasmic region, which occupies the gap between the T tubule and SR membranes at the triad junction, likely contains the site(s) of interaction with the DHPR, but the precise location of this putative interaction on the surface of the RyR1 is unknown.

A peptide toxin, imperatoxin A (IpTxα; 33 amino acid residues), isolated from the venom of scorpion Pandinus imperator, interacts specifically with the skeletal (RyR1) and cardiac (RyR2) isoforms of the RyR. It reversibly enhances binding of ryanodine to the receptors (El-Hayek et al., 1995b), and when added to the cytosolic side of single receptors reconstituted in lipid bilayers, induces long-lived subconductance states (Tripathy et al., 1998; Gurrola et al., 1999). The binding location of IpTxα on the RyR is unknown, and since the peptide toxin presumably mimics a DHPR domain that triggers RyR openings (Gurrola et al., 1999).
Materials and Methods

Biotinylated RyR1 was isolated from skeletal muscle by affinity chromatography on immobilized streptavidin agarose and gel electrophoresis. The gel was incubated with a molar excess of IpTx-B to facilitate its detection by cryo-EM using 3D reconstruction of the protein-ligand complexes. The isolated RyR1 was then incubated with a molar excess of IpTx-B and SA to localize the toxin binding site on the RyR1 3D structure, which should reveal the location of the switch for channel gating and thus provide insight into the fundamental principles of action of this macromolecule.

Affinity Chromatography and Gel Electrophoresis

Purified RyR1 (10 µg) was incubated with biotinylated or non-biotinylated IpTx-B in 100 µl binding buffer to yield the following final concentrations: 20 mM MOPS-NaOH (pH 7.4), 200 mM NaCl, 0.3% (vol/vol) CHAPS, 0.1 mM CaCl₂, 0.24 mM DTT, 1 mM NEM, 5 µg/ml leupeptin, and 2.6 µM IpTx-B. Freeequilibrated streptavidin (SA) agarose agarose complexes were prepared for cryo-EM in parallel with a control sample. The elution buffer was added to yield the following final concentration: 20 mM MOPS-NaOH (pH 7.4), 200 mM NaCl, 0.3% (vol/vol) CHAPS, 0.1 mM CaCl₂, and 5 µg/ml leupeptin. The mixture was shaken for 15 min at room temperature. The supernatant was recovered by centrifugation for 2 min at 1,000 g. The sedimented SA-agarose was resuspended in 100 µl washing buffer of the following composition: 20 mM MOPS-NaOH (pH 7.4), 200 mM NaCl, 0.3% (vol/vol) CHAPS, 0.1 mM CaCl₂, and 5 µg/ml leupeptin. The mixture was centrifuged and the supernatant saved. The step was repeated nine times. Subsequently, the RyR1 was eluted batchwise in three steps with 125 µl elution buffer that contained 0.1 M sucrose, 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 2 mM EDTA, 50 mM DTT, and 0.01% (wt/vol) bromophenol blue. All the supernatants recovered (100 µl each) were also mixed with 25 µl fivelfold concentrated elution buffer. A liquid (40 µl) was applied onto discontinuous SDS-polyacrylamide gels (3.5% stacking and 5% resolving gel). The resolved proteins were visualized by Coomassie staining.

Cryo-EM and Image Processing

RyR1-pTx-B-SA complexes were prepared in 20 mM Tris-HCl, pH 7.4, 0.15 M KCl, 0.1 mM CaCl₂, and incubated between 10 and 30 min at room temperature before cryo-grid preparation. A 2D-mold excess of IpTx-B and SA over RyR1 was used. Vitrified specimens were prepared on 300-mesh carbon-coated gold grids as described in Wagenknecht et al. (1997). Samples were examined on a Philips 420 electron microscope operated at 100 kV under low-dose conditions at a magnification of 52,000. Underfocus was 1.8 µm. Micrographs were scanned on a Hi-Scan microdensitometer (Hitachi) using a pixel size corresponding to 3.85 Å on the specimen. Images were processed using the software SPIDER/WEB (Frank et al., 1996). 3D reconstructions were obtained following the projection-matching method (Penczek et al., 1994), taking care that all eulerian angles in both control and experimental volumes were well represented (Buisson et al., 1998). The total number of particles used for the final volumes was 3,900 and 2,347 particles for the IpTx-B-containing and the control sample, respectively, after removing oversampled views and noisy images. 3D volumes were filtered to their limiting resolution, its value obtained using the Fourier shell correlation with a cutoff value at 0.5 (see appendix in Malhotra et al., 1998). The density threshold chosen for isosurface representation was the midpoint of the 3D boundary density profile.

Results

IpTx-B Activity and Stability of IpTx-B-SA Complex

IpTx-B was synthesized with a biotin group added to the NH₂ terminus to facilitate its detection by cryo-EM using SA. Biotin-derivatized IpTx-B (IpTx-B-SA) retained the ryanodine-binding enhancement property of the native toxin, although higher concentrations were needed to obtain the same half-maximal effect, and the plateau of maximal effect was 80% of that for native IpTx-B (Fig. 1a). The affinity of IpTx-B for RyR1 is within the range suitable for cryo-EM.

Precipitation of RyR1 by SA-agarose in the presence of IpTx-B confirms that a stable complex forms between IpTx-B and RyR1 (Fig. 1b). The SA-agarose resin does not precipitate RyR1 when the mixture contains IpTx-B instead of IpTx-B-SA (Fig. 1b).

Cryo-EM and IpTx-B-SA Difference Map

RyR1 was incubated with a molar excess of IpTx-B and SA, and prepared for cryo-EM in parallel with a control reaction consisting of RyR1 and SA. In the presence of IpTx-B and SA the receptors distributed homogeneously on the grid, but some dimers and a few higher oligomers formed, probably due to the tetravalent binding potential of both RyR1 and IpTx-B and SA for both protein. The control specimen (Fig. 2b) showed mostly individual channels and some small particles on the background, presumably corresponding to free SA (in the experimental sample, less free SA was observed because a significant fraction of the SA was presumably bound by the RyR1:IpTx-B-SA complex). Although these observations are indicative of the formation of RyR1:IpTx-B-SA complexes, it is impossible to assert by direct visual examination of the raw micrographs whether particular RyR1s contain ligand, and if so, where it is located.

Two-dimensional (2D) Analysis

2D image processing was performed on the frequently occurring square-shaped views of RyR1 as described in Wagenknecht et al. (1997). Both experimental and control 2D averages display the characteristic morphology with four protruding corners (clamps) and a central low-density cross (Fig. 3a and b). Careful inspection reveals an extra mass density in the case of RyR1 incubated with SA and IpTx-B versus the RyR1 incubated with SA only (Fig. 3a, arrowheads). The difference map (Fig. 3c) takes the form of a line of sharp, discrete, and elongated positive densities.
that are attributed to bound IpTxa-B:SA, and weaker differences that possibly correspond to minor conformational readjustments. The t test shows the regions of the difference map significant at the 98% confidence level (Fig. 3 d). These regions correspond to the main differences shown in Fig. 3 c.

3D Analysis

3D reconstructions were computed for both control and experimental samples. Surface renderings of the reconstructed volumes filtered to their limiting resolution of 29 Å (Fig. 4) show the characteristic square-prism cytoplas-
mic moiety containing 10 well-defined domains and the smaller transmembrane assembly that have been documented previously (Radermacher et al., 1994; Serysheva et al., 1995). A major difference between the two reconstructions occurs in the crevice delimited by domains 3 and 7/8 of the control reconstruction, which appears to be filled in by density in the reconstruction done in the presence of IpTx-a-B and SA (compare Fig. 4, a and b). When the two volumes were subtracted, and the difference volume is displayed at the same threshold as the control and experimental volumes, the only differences remaining localize in the above-mentioned crevice (Fig. 4 c, violet). We attribute these differences to bound IpTx-a-B:SA, and hereafter we refer to them as such. The fact that these differences appeared at the same threshold as the control and experimental volumes is indicative that most of the receptors had all four sites occupied. This finding is supported by the results of Gurrola et al. (1999) who have reported that 4 mol of IpTx-a bind per mole of tetrameric RyR1.

The attachment of IpTx-a-B:SA (Fig. 4 c, violet regions) to the RyR1 appears to occur near the base of the crevice (Fig. 5 a), indicating that IpTx-a-B, the link between RyR1 and SA, is probably located at this region of the difference map. The apparent size of the surface-rendered difference between the experimental and control reconstructions appears to be smaller than SA's dimensions. This discrepancy likely results from a loss of signal at the distal regions of the SA due to its mobility, and thus dilution of the signal through averaging. Similar effects have been seen in reconstructions using antibodies as ligands (e.g., Yu et al., 1998). Furthermore, analysis of the variance associated with the 2D average of RyR1:IpTx-a-B:SA shows locally high variance of the mass attributed to SA in the distal region, which would be consistent with mobility of this protein (not shown).

Although IpTx-a has been shown to induce subconductance states in the RyR1 (Tripathy et al., 1998), our 3D reconstruction of the RyR1 containing bound IpTx-a-B and SA does not reveal any major conformational changes such as were reported for ryanodine-modified RyR1 (Orlova et al., 1996). Perhaps minor differences exist, but they are unappreciable at our current resolution.

**Discussion**

### Allosteric Activation by IpTx-a

Binding of IpTx-a to the RyR1 induces the appearance of
Our finding that the IpTx binding locations are far (<11 nm) from the center of the cytoplasmic side of the transmembrane region of the channel supports an allosteric mechanism of action of IpTx, as opposed to a mechanism involving direct positioning of the toxin within the ion conducting channel (both models have been discussed by Tripathy et al. [1998]). IpTx is the third modulator ligand of RyR1 that has been localized by cryo-microscopy and 3D reconstruction, and intriguingly, all three bind to sites on the cytoplasmic region of the receptor that are far from the transmembrane portion of RyR1. The other mapped ligands are calmodulin, which binds at a site near that found here for IpTx, and FK 506-binding protein, which binds at the periphery of the cytoplasmic region on the opposite side of domain 3 from that of IpTx (Wagenknecht et al., 1997). It seems possible that domain 3 plays a key role in the allosteric mechanism of channel modulation by these ligands, perhaps by moving so as to affect the conformation of the transmembrane assembly to which it appears to be

Figure 4. (a) Solid body representations of the 3D reconstruction of RyR1 incubated in the presence of IpTx-B and SA. Resolution, 29 Å. (b) 3D reconstruction of RyR1 control incubated in the presence of SA. Resolution, 29 Å. (c) Control reconstruction (green) with differences (violet) obtained by subtracting reconstruction in a and b superimposed. This map shows the locations of bound IpTx-B:SA (violet). The volumes are seen from the T tubule-facing side (left), SR-facing side (middle), and from the side (right) with the cytoplasmic moiety of the receptor on top, and the transmembrane assembly at the bottom. The numerals point to distinguishable domains as described in Radermacher et al. (1994).
connected by a bridge of density (Fig. 5 b, short dashed line). IpTxA could also affect the transmembrane domain through the other bridge of density indicated by the long dashed line in Fig. 5 b, or through a concerted movement of both of them.

**Mimicry by the II-III Loop of the α1 Subunit of the DHPR**

Recently, convincing evidence has been reported in support of the hypothesis that IpTxA mimics the effects of RyR1-activating peptides derived from the DHPR (Gurrrola et al., 1999). Specifically, residues 681–687 (Arg-Lys-Arg-Arg-Lys-Met-Ser), which lie in the cytoplasmic II-III loop (residues 666–791; Tanabe et al., 1990) of the α1 subunit of the DHPR, are crucial for the activating effects that the isolated II-III loop and various derived subfragments have on the RyR1 (Lu et al., 1994, 1995; EI-H ayek et al., 1995a; EI-H ayek and Ikemoto, 1998). A similar cluster of basic amino acids followed by a hydroxyl-containing amino acid occurs at residues 19–26 of IpTxA (Lys-Lys-Cys-Lys-Arg-Arg-Gly-Thr) and is essential for the effects of IpTxA on RyR1 (Gurrrola et al., 1999). Although the precise role of the II-III loop in E-C coupling seems to be complex, including its relationship to other regions of the DHPR or other components of the triad junction (El-H ayek and Ikemoto, 1998; Leong and M aclennan, 1998b; Nakai et al., 1998), the mimicry shown by IpTxA suggests that the site of IpTxA binding on the 3D architecture of RyR1 can potentially correspond to a DHPR interaction site crucial for E-C coupling.

In this context, the 37-amino acid sequence Arg^{1076}-Asp^{1112} from RyR1 that interacts with the DHPR II-III loop identified by Leong and M aclennan (1998a,b) would locate at the base of the crevice between domains 3 and 7/8.

To correlate further our results with the work implicating IpTxA as a DHPR mimic, we examined how the density attributed to IpTxA-B:SA in our reconstruction (Fig. 4 c, violet) fits into the known quaternary arrangement of DHPRs at the triad junction (Block et al., 1988). The distance between the centers of mass of neighboring IpTxA-B:SA is 15 nm, which would lie within the boundaries defined by the morphologic units (DHPRs) comprising the tetrads seen by freeze-fracture EM (for this comparison we project the four IpTxA-B:SA per RyR1 and the four subunits of a tetrad into a plane normal to the axis of fourfold symmetry). In the orthogonal direction (i.e., parallel to the fourfold axis of the RyR1s), the differences attributed to IpTxA-B:SA are <5 nm from the T tubule-facing side of domain 4 of RyR1 (Fig. 4 c, side view). For the basic sequence of the II-III loop to extend to this region would apparently require a fully extended conformation for the first 15 residues of the II-III loop that precede it (El-H ayek et al., 1995a). Alternatively, a less extended configuration would suffice if the interaction between RyR1 and DHPR involves more interdigitation than supposed.

Regardless of the potential use of IpTxA as a tool to help elucidate E-C coupling, it is important to emphasize that IpTxA produces discrete functional effects on RyR1 that ultimately lead to calcium release in isolated SR vesicles (Gurrrola et al., 1999) and in skinned muscle cells (Shiftman et al., 1999). Thus, activation of the IpTxA binding domain identified here must lead to conformational changes in RyR1 that shift channel conductance.

In conclusion, we have found by cryo-E M and 3D reconstruction that IpTxA binds to RyR1 along the edges of the cytoplasmic assembly, in a crevice between the clamp and handle domains. We suggest that a subtle conformational change mediates pore gating and toxin binding, and we discuss the possibility that the toxin binding location represents one of the physiological activating sites of RyR1 during E-C coupling.

Figure 5. 3D reconstructions of the control RyR1 displayed in different orientations to illustrate specific features. (a) Likely site of attachment of IpTxA to RyR1. The difference map was displayed at a higher threshold (in violet) to determine the region of higher rigidity. This region remains connected to the RyR1 structure at one location, suggesting that this is the attachment site. (b) The dashed lines indicate bridges of mass connecting domain 3 and the transmembrane assembly. The asterisk indicates the likely site of attachment of IpTxA.
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