Activation of Skeletal Ryanodine Receptors by Two Novel Scorpion Toxins from *Buthotus judaicus*

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*Buthotus judaicus* toxin 1 (BjTx-1) and toxin 2 (BjTx-2), two novel peptide activators of ryanodine receptors (RyR), were purified from the venom of the scorpion *B. judaicus*. Their amino acid sequences differ only in 1 residue out of 28 (residue 16 corresponds to Lys in BjTx-1 and Ile in BjTx-2). Despite a slight difference in EC_{50}, both toxins increased binding of [3H]ryanodine to skeletal sarcoplasmic reticulum at micromolar concentrations but had no effect on cardiac or liver microsomes. Their activating effect was Ca^{2+}-dependent and was synergized by caffeine. *B. judaicus* toxins also increased binding of [3H]ryanodine to the purified RyR1, suggesting that a direct protein-protein interaction mediates the effect of the peptides. BjTx-1 and BjTx-2 induced Ca^{2+} release from Ca^{2+}-loaded sarcoplasmic reticulum vesicles in a dose-dependent manner and induced the appearance of long lived subconductance states in skeletal RyRs reconstituted into lipid bilayers. Three-dimensional structural modeling reveals that a cluster of positively charged residues (Lys^{1} to Lys^{16}) is a prominent structural motif of both toxins. A similar structural motif is believed to be important for activation of RyRs by imperatoxin A (IpTxA), another RyR-activating peptide (Gurrola, G. B., Arevalo, C., Sreekumar, R., Lokuta, A. J., Walker, J. W., and Valdivia, H. H. (1999) *J. Biol. Chem.* 274, 7879–7886). Thus, it is likely that *B. judaicus* toxins and imperatoxin A bind to RyRs by means of electrostatic interactions that lead to massive conformational changes in the channel protein. The different affinity and structural diversity of this family of scorpion peptides makes them excellent peptide probes to identify RyR domains that trigger the channel to open.

In cardiac and skeletal muscle, ryanodine receptor (RyR) of sarcoplasmic reticulum (SR) is a key component of excitation-contraction coupling, the process that links an electrical stimulus (depolarization) to Ca^{2+} release and cell contraction (1–3). The amino acid sequence of the three RyR isoforms has been completely elucidated, but due in part to their tremendous molecular size (~5,000 amino acids/monomer), the exact structural motif(s) of RyR involved in excitation-contraction coupling, the ATP and Mg^{2+} regulation sites, as well as the Ca^{2+} activation and inactivation domains still remain uncertain and controversial (4–6). The limited availability of antibodies and other ligands against specific functional sites has also hampered the elucidation of the channel’s most important structural domains.

To date, only a few molecular probes of RyRs have been well characterized. Ryanodine, a plant alkaloid, has been used for identification and characterization of RyRs because it preferentially binds to the open state of RyRs, thus serving as an index of channel activity (7). Because venoms from spiders, snakes, and scorpions are rich in peptide toxins, they have been extensively exploited as a source of peptide probes specifically targeted to various voltage- and ligand-gated channels (8–10).

From the African scorpion *Pandinus imperator*, two components (IpTx_{a} and IpTx_{b}) have been shown to activate and inhibit RyRs, respectively (11). IpTx_{a}, a 3.7-kDa basic peptide (12), increases [3H]ryanodine binding to skeletal RyR, but not cardiac RyR, although it induces the appearance of long lived subconductance states in both isoforms (13). Interestingly, IpTx_{a} shares strikingly similar structural and functional properties with peptide A (14), a peptide fragment from the II-III loop of the skeletal type dihydropyridine receptor α_{1}, subunit, which also triggers Ca^{2+} release from the SR (15). By mutating some amino acids in the cardiac and skeletal II-III loop peptide, we found that a cluster of positively charged amino acid residues on the surface of the peptide A molecule is important for activation of skeletal RyRs (16). Thus, identification of the common structural components for activation of RyR, as well as their binding sites in RyRs, will help us to better understand the mechanisms that control gating of the RyR.

Other peptide probes for the RyR have been isolated, but they are not as well characterized as the above mentioned peptides. A peptide fraction of ~4000–7000 Da molecular mass from the venom of the African scorpion *Buthotus hottentota* increases [3H]ryanodine binding and induces the appearance of subconductance states in skeletal RyRs (17). Likewise, Morrissette et al. (10) isolated ryanotoxin, an ~11.4-kDa peptide fraction, from *Buthotus judaicus* scorpion venom, which similarly induces the appearance of subconductance states in skel-
Peptide Activators of RyR from B. judaicus

EXPERIMENTAL PROCEDURES

MATERIALS—[3H]Ryanodine was purchased from PerkinElmer Life Sciences. Bovine brain phosphatidylethanolamine and phosphatidylserine were obtained from Avanti Polar Lipids (Birmingham, AL). Purification of B. judaicus Toxins—100 mg of lophylized B. judaicus venom (Alomone Laboratories Ltd., Jerusalem, Israel) was suspended in 2 ml of water and centrifuged at 14,000 rpm for 10 min to separate the soluble fraction and the mucus. The mucus was re-extracted two more times. All the supernatants were pooled and adjusted to 5 mg of protein/ml. 150 μl of sample was injected into a C18 reverse-phase HPLC column (System Gold, Beckman Instruments, San Ramon, CA). The peptide fractions were eluted with 0–67.5% acetonitrile at a flow rate of 1 ml/min. The eluted fractions were monitored at 214 nm. Each fractionated peak was collected and lyophilized. With the same amount of peptides (w/v) in each sample as determined by A214, the activity of HPLC peak fractions was determined by [3H]ryanodine binding.

Amino Acid Analysis and Sequencing of B. judaicus Toxins—Amino acid analysis of B. judaicus toxins was performed on samples hydrolyzed in 6 M HCl with 0.5% phenol at 110 °C in evacuated, sealed tubes as described (18). Reduction of B. judaicus toxins with dithiothreitol, and alkylation with iodoacetic acid was performed as described (18). The sequences of the intact native and reduced/carboxymethylated B. judaicus toxins were determined using a model 6400/6800 automatic liquid-phase protein sequencer (Milligen/BioSearch Sequencer) using standard Edman degradation programs and CD Immobilon membrane. To confirm the carboxyl-terminal sequence, 20 μg of B. judaicus toxin was hydrolyzed with Staphylococcus aureus V8 in 100 mM ammonium bicarbonate (pH 7.8). The peptide fragments were purified and sequenced as described for native B. judaicus toxins.

Computer Modeling—To generate the three-dimensional structure, we used Insight II Discover software from Biosym Technologies (San Diego, CA) running on a Silicon Graphics Octane RS10000 Workstation. Peptide P01 (19), the three-dimensional structure of which has been determined by NMR, is a natural peptide from the North African scorpion Androctonus mauretanicus mauretanicus. The primary structure of peptide P01 is quite similar to the B. judaicus toxins we purified, therefore it was chosen as the structural model for building. The three-dimensional structure of peptide P01 was obtained from the Entrez of NCBI (www.ncbi.nlm.nih.gov/Entrez). The electrostatic potential surface was analyzed using the Discover force field. Five hundred steps of minimization were performed using the steepest descent algorithm method until a root mean square deviation of 0.001 was obtained.

SR Microsome Preparation and Purification of RyR1—Porcine skeletal, cardiac, and liver SR-enriched microsomes were isolated as described (20). Microsomes from the last centrifugation were suspended in 0.3 M sucrose, 0.1 M KCl and 5 mM Na-PiPES (pH 7.2). RyR1 was purified from microsomes using continuous 5–20% sucrose density gradient centrifugation as described (21).

[3H]Ryanodine Binding Assay—[3H]Ryanodine binding to pig skeletal SR and other tissue homogenates was carried out as described previously (22). The standard incubation medium contained 0.2 M KCl, 10 μM CaCl2, 40 mM Na-HEPES (pH 7.2), 7 nM [3H]ryanodine, 40–50 μg of pig skeletal or cardiac SR vesicles, and different concentrations of modulators as stated in each specific experiment. Samples (0.1 ml) were run in duplicate at 37 °C for 90 min. For binding assays with different calcium concentrations, 10 μM CaCl2 in the standard assay was replaced by 1 mM EGTA and CaCl2 necessary to set free calcium from 1 nM to 10 mM. Unless otherwise indicated, data represent the mean ± S.E. with n = 2. Mathematical fitting of data was accomplished with the computer program Origin (version 7.0, Microcal Inc., Northampton, MA).

Planar Lipid Bilayer Technique—Pig skeletal RyRs were reconstituted into Muller-Rudin planar lipid bilayers as described previously (22). Single channel data were collected at steady voltages (+35 mV, cis chamber grounded) for 2 min in symmetrical 200 mM cesium methanesulfonate, 10 mM Na-HEPES (pH 7.2). The recording solution contained ~5 μM free Ca2+, as assessed by a calibration curve, which was sufficient to activate RyRs. Different concentrations of B. judaicus toxins were added to the cis chamber, which corresponded to the cytosolic side of the channel (22). Signals were digitized at 4 kHz and analyzed after filtering with a low-pass 8-pole Bessel filter at a sampling frequency of 1.5 kHz. Data acquisition and analysis were performed with Axon Instruments software and hardware (pClamp version 8.03, Digidata 2000/DA interface). The half and subconducting current values were obtained from Gaussian fits to the amplitude histograms, as described previously (23).

Spectrophotometric Ca2+ Release Assay—Skeletal SR vesicles (~50 μg of protein) were mixed in a 1 ml solution containing 95 mM KCl, 7.5 mM sodium pyrophosphate, 250 μM antipyrylazo III, 1.5 mM MgATP, 25 μg/ml creatine phosphokinase, 5 mM phosphocreatine and 20 mM K-MOPS, (pH 7.0). The solution was placed in a cuvette, incubated at 37 °C, and allowed to equilibrate for 2 min. Changes in free Ca2+ were monitored by changes in the absorbance of antipyrylazo at 710 nm and subtraction of the absorbance at 790 nm, at 1-s intervals, using a diode array spectrophotometer (Hewlett Packard Model 8452A). Initially, vesicles were actively loaded with CaCl2 until levels were close to the filling capacity (~2 μmol of total Ca2+/mg of protein, usually 10 μl of 1 mM CaCl2 added 4 times). Thapsigargin (1 μM) was then added to block Ca2+ uptake. The absorbance signals were calibrated by addition of a known amount of Ca2+ to the complete transport mixture in the presence of the Ca2+ ionophore A23187 to prevent Ca2+ accumulation.

RESULTS

Purification of B. judaicus Toxin Activators—[3H]Ryanodine binds with high affinity (Kd ~7 nM) to the open conformational state of the Ry R. Thus, we used the standard [3H]Ryanodine binding assay to characterize whole B. judaicus venom and to screen the peaks separated by HPLC fractionation (as described below). Whole B. judaicus venom stimulated [3H]Ryanodine binding in a dose-dependent manner, and the effective concentration (EC50) for the whole venom was 400 μg/ml (Fig. 1C). To purify the active component, soluble proteins from whole venom were loaded onto a C18 reverse-phase column. Two peaks, eluted at 32.8 min and 36.4 min (Fig. 1A, arrows), effectively increased [3H]Ryanodine binding, although most other peaks had no effect. (Two other peaks, one eluted at 6 min (not shown) and another eluted at 44 min, increased [3H]Ryanodine binding marginally.) Thus, the two peaks at 32.8 and 36.4 min were collected and subjected to another analytical HPLC separation to verify their purity. Both fractions appeared as single, homogenous, and symmetrical peaks (Fig. 1B), and their purity was greater than 95%. The molecular weights, determined by mass spectrometry analysis, were 2888.95 ± 0.12 Da for peptide 1 and 2874.46 ± 0.37 Da for peptide 2. Interestingly, the molecular mass difference between peptide 1 and peptide 2 is only 15 Da, suggesting that these two peptides may have similar primary structures. Both peptides stimulated [3H]Ryanodine binding in a dose-dependent manner (Fig. 1C). The EC50 for peptide 1 and peptide 2 was 8 and 20 μM, respectively. Because both peptides activating RyRs were purified from B. judaicus, we named them BjTx-1 and BjTx-2, respectively.

Primary Structures—The complete amino acid sequences of BjTx-1 and BjTx-2 were determined by direct automated sequencing (Fig. 2). Both peptides are composed of 28 amino acid residues with a difference of only 1 amino acid at position 16 (i.e. a Lys in BjTx-1 but an Ile in BjTx-2). The molecular difference between Lys and Ile is 15 Da, and this precisely matches the difference in molecular mass between the two toxins as determined by mass spectrometry. This difference also explains the observed HPLC elution profile. Because Ile is a hydrophobic amino acid but Lys is electrically charged, BjTx-1 is more hydrophilic than BjTx-2 and thus elutes earlier in the reverse-phase column. The calculated molecular weights for the linear sequences are 2895 and 2880, respectively, which
are 6 greater than the molecular masses determined by mass spectrometry. This suggests that both toxins may contain three pairs of disulfide bridges, which is a characteristic of most short scorpion toxins (forming a disulfide bridge removes two hydrogen atoms). However, unlike most small scorpion toxins, which are usually basic in nature, both \textit{B. judaicus} toxins contain four negatively charged amino acid residues (Asp or Glu), but only two or three positively charged amino acids (Lys), making them acidic in general (calculated pI values for BjTx-1, 5.48; BjTx-2, 4.75).

To compare the similarity of the two isolated toxins with other peptides, we searched GenBank™ of the National Center for Biotechnology Information (NCBI), using the program Blast (www.ncbi.nlm.nih.gov/BLAST/). \textit{B. judaicus} toxins are highly homologous with scorpion peptide Lqhpep 2, from \textit{Leiurus quinquestriatus hebraeus} (24), and PepII, from \textit{Buthus sindicus} (25). They also share some similarity with peptide P01 from the scorpion \textit{A. mauretanicus mauretanicus}, the three-dimensional structure of which has been determined by NMR (19). However, although the complete amino acid sequences of those three peptides have been determined, their biological function remains unknown. No significant similarity in primary structure is observed between both \textit{B. judaicus} toxins and IpTx a, except that all three peptides contain 6 cysteines, enabling them to form three pairs of disulfide bridges (Fig. 2).

Molecular Modeling of \textit{B. judaicus} Toxins—\textit{B. judaicus} toxins share some sequence similarities with scorpion peptide P01 and leiuropeptide II, the solution structures of which have been determined by NMR (19). However, although the complete amino acid sequences of those three peptides have been determined, their biological function remains unknown. No significant similarity in primary structure is observed between both \textit{B. judaicus} toxins and IpTx a, except that all three peptides contain 6 cysteines, enabling them to form three pairs of disulfide bridges (Fig. 2).

As shown in Fig. 3, the backbone model of BjTx-1 includes an α-helix connected by a tight turn to a β-sheet. The α-helix extends from residues Cys³ to Gln¹⁴, with a Pro residue located...
at position 7, which may act as an α-helix breaker (19). The antiparallel β-sheet is composed of two strands. The first strand extends from Ala15 to Asp20 and the second from Gly23 to Val230. The primary structures of both toxins are ordered as -Cys1-Cys2-Xaa-Xaa-Xaa-Cys5-Cys6-Cys7-, a well known cysteine motif common to most scorpion toxins, including all the peptides we previously mentioned, and also charybdotoxin (20), iberiotoxin (27), margatoxin (28), and kaliotoxin (28). The α-helix and β-sheet are connected by three disulfide bridges, with the pattern Cys1-S-Cys5, Cys3-S-Cys6, and Cys4-S-Cys7 (19).

The Corey-Pauling-Koltun model of BjTx-1 (Fig. 3, lower model) indicates the spatial orientation of all atoms of the amino acid residues forming the carbon backbone. Three pairs of disulfide bridges formed by 6 cysteine residues (yellow) remain buried inside the molecule and function to pull it together, whereas other amino acid residues are surface-exposed. Based on calculated electrostatic potentials, there appear to be two potential electrostatic zones. One of them is positive, localized in the region around Lys11 and Lys13 in the α-helix, whereas the two thick ribbons on the left side correspond to the two strands of anti-parallel β-sheets. The α-helix is connected to the β-sheet by a tight turn. In this orientation, the end of the string on the left and right sides correspond to the carboxyl- and amino-terminal segments, respectively. In the bottom models, the amino acid residues giving rise to the carbon backbone are shown as Corey-Pauling-Koltun structures. Color code: Cys, yellow; Glu, dark red; Asp, magenta; Lys, blue; Arg, dark blue; His, light blue; Ala, green; and Gly, white. Arrows show Lys11, Lys13, and Lys16, which encompass a positively charged zone and form the putative structural domain that binds to RyRs.

Selective Activation of Skeletal RyRs by B. judaicus Toxins—To ascertain whether the B. judaicus toxins had any effects on the various RyR isoforms, we carried out [3H]ryanodine binding assays with tissues expressing different isoforms. Fig. 4 shows that both BjTx-1 and BjTx-2 increased binding of [3H]ryanodine to skeletal SR (RyR1). Bindings increased to ~300% of control at a concentration of 8 and 20 μM (the Kd values of BjTx-1-RyR1 and BjTx-2-RyR1 interactions, respectively). Both toxins also slightly increased [3H]ryanodine binding to cardiac SR (RyR2), but to a much lesser extent than skeletal SR and had negligible effects on the binding of [3H]ryanodine to liver microsomes (mostly RyR3). These results suggest that B. judaicus toxins preferentially increase [3H]ryanodine binding to skeletal type RyR1 isoforms. Similarly, it has been shown that IpTv preferentially increases [3H]ryanodine binding to RyR1 and has a limited effect on RyR2 or RyR3 (22) (Fig. 4).

B. judaicus Toxins Interact with RyR1 Directly—To determine whether BjTx-1 and BjTx-2 had a direct interaction with
RyR1 or mediated their effects through a regulatory protein, we purified RyR1 from skeletal microsomes and tested the effects of BjTx-1 and BjTx-2 on [3H]ryanodine binding. Fig. 5A shows RyR1 identified as a single, high molecular weight band in a silver-stained SDS gel, with no other proteins present. To exclude the interference of high salt (1 M NaCl) and [Ca2+] (300 μM) present in the purification buffer, we adjusted the salt and calcium to a final concentration of 0.2 μM and 10 μM, respectively, by adding salt-free buffer and EGTA. BjTx-1 increased [3H]ryanodine binding to the purified RyR1 at concentrations of 2 μM (low dose) and 5 μM (high dose). BjTx-2, although having less affinity compared with BjTx-1, also increased [3H]ryanodine binding to the purified RyR1 at concentrations of 6 μM (low dose) and 20 μM (high dose) (Fig. 5B), suggesting that both toxins act on RyR1 based on a direct protein-protein interaction mechanism. This property is analogous to that of IpTxa, which also activates RyR1 by a direct interaction (14, 22) (Fig. 5B).

Effects of BjTx-1 and BjTx-2 on the Ca2+ Dependence of [3H]Ryanodine Binding—The Ca2+ dependence of [3H]ryanodine binding to RyR1 has been well established. In the range of pCa 9 to pCa 5, Ca2+ has an activating effect and increases [3H]ryanodine binding to the RyR. Whereas in the range of pCa 4 to pCa 2, Ca2+ has an inactivating effect. Fig. 6A (black line) shows the dual effect of Ca2+ on RyR activity, which gives rise to the characteristic bell-shaped curve (30, 31). In the presence of BjTx-1 (filled circles) or BjTx-2 (filled triangles), the binding curves maintained the same bell shape; however, the absolute [3H]ryanodine binding curves were increased above pCa 7. Both B. judaicus toxin curves maintained the same basal threshold as control, peaked between pCa 5 and pCa 4, and almost fully inhibited RyR activity at pCa 3. For further comparison, we normalized the data points with maximal binding at pCa 5 (Fig. 6B) and found no significant difference in Ca2+ sensitivity between the curves with or without the presence of B. judaicus toxins.

Combined Effects of Caffeine with B. judaicus Toxins—We tested the effect of caffeine, a classical agonist of RyRs, in the presence or absence of BjTx-1 and BjTx-2 using [3H]ryanodine binding assays. As shown in Fig. 7, millimolar caffeine increased [3H]ryanodine binding to RyRs, with the greatest effect observed above 10 mM. The presence of 8 μM BjTx-1 or 20 μM BjTx-2 (EC50 values for the toxins, respectively) increased [3H]ryanodine binding to caffeine-activated RyRs in a synergistic manner. The curves displayed a steeper slope when [caffeine] exceeded 1 mM. These results suggest that BjTx-1 and BjTx-2 do not share the same binding site with caffeine; instead, the toxin-binding site appears to display cooperative interaction with the caffeine-binding site so that occupation of one site promotes binding of ligand to the other.

Functional Activation of B. judaicus Toxins on Calcium Flux Assay—To determine whether the increase of [3H]ryanodine binding to skeletal SR vesicles by B. judaicus toxins had any functional correlation, we performed calcium flux assays. Antipyrilazo III was used to detect the changes of [Ca2+] outside of the SR vesicles. Fig. 8A shows that BjTx-1 induced Ca2+ release in a dose-dependent manner. In the absence of BjTx-1, the curve was flat, indicating that most of the releasable Ca2+ was stored in the SR until A23187, a Ca2+ ionophore, was added (Fig. 8A, asterisk). Similar effects were also observed for BjTx-2 (Fig. 8B).

Functional Activation of B. judaicus Toxins on Single Channels—To further investigate the functional properties of the B. judaicus toxins, we reconstituted skeletal RyRs into planar lipid bilayers to determine their effects on single channel activity. Before addition of peptide, the RyR1 channel remained mostly in a closed state, with only a few intermittent open events (Fig. 9A). The unitary conductance of the control channel corresponded to 640 pS. Current amplitude histograms representing 2 min of recording showed a symmetric peak centered at 5 pA (closed), and a small peak at 25 pA (open) (Fig. 9E). Addition of 1 μM BjTx-1 induced a small but significant increase in open events (Fig. 9B, C and F). However, when 5 μM (Fig. 9C) or 10 μM (Fig. 9D) BjTx-1 was added to the cis side, it induced subconductance states measuring 160 pS, which corresponded to ~25% of the full conductance openings. As reflected in the amplitude histograms (Fig. 9, G and H), the time the channel spent in a subconducting state was dose-dependent.

A similar effect was observed with BjTx-2 (Fig. 10). Addition of BjTx-2 into the cis side of the channel also induced subconductance states in a dose-dependent manner, as de-
the toxin-modified channels. Open time that is toxin-induced subconductance states displayed a long mean subconductance states. Despite their small amplitude, these that both B. judaicus F_{am} stocks (kept in 37 °C to prevent precipitation). The Ca2+ dependence of [3H]ryanodine binding. Data points are the mean (± S.E.) of three experiments. B, normalized data of A. Each data point was normalized to the binding at pH 5, which is the maximal binding for each group of data.

**DISCUSSION**

In this study we reported identification of two novel activators of RyR1 from the scorpion B. judaicus. These two toxins show no similarities in primary structure with any known peptide probes of RyR1. Both toxins increased binding of [3H]ryanodine to skeletal SR vesicles and induced subconductance states measuring 163 ± 12 pS in RyRs reconstituted into lipid bilayers. However, this does not mean that the two toxins we isolated here are ryanotoxin; in fact, we have evidence that shows they are separate toxins. First, using the same C18 reverse-phase column, ryanotoxin was eluted by 67.5% ACN (10), whereas BjTx-1 and BjTx-2 were eluted by 31 and 38% ACN, respectively. The shorter retention times reflect that the toxins we isolated have different hydrophobicity values than that of ryanotoxin. Another obvious difference between B. judaicus toxins and ryanotoxin is their molecular weight. The molecular masses of BjTx-1 and BjTx-2 are 2889 and 2874 Da, respectively, much less than that of ryanotoxin, which is 11.4 kDa as presented by Morissette et al. (10). Actually, based on our protein elution profile, it is possible that the ryanotoxin corresponds to the peak eluted at 44 min, which also showed an activation effect in [3H]ryanodine bindings. However, further studies must be done to confirm these data. Interestingly, Morissette et al. (10) did not detect the B. judaicus toxins that we isolated probably because they used a wavelength of 254 nm to detect peptides, which can resolve only 6 peaks from B. judaicus venom. Conversely, we used 214 nm to monitor the peptides, which separates the venom into more than 30 peaks.

When the functional effect of B. judaicus toxins is compared via the Ca2+ release assay versus the single channel recordings, we notice an obvious difference in dose dependence for RyR activation. In the Ca2+ release assay, low nanomolar toxin induced Ca2+ release from the SR, whereas in single channel recordings, micromolar toxin was necessary to induce the occurrence of subconductance states. Likewise, micromolar toxin was necessary to increase [3H]ryanodine binding to SR vesicles. This discrepancy could be explained by differences in Ca2+ loading on the luminal side of SR. In the Ca2+ release assay, SR vesicles
were fully loaded with Ca$^{2+}$, which increased the activity of RyRs and also increased their sensitivity to the challenge of toxin. One study that supports this explanation was done by Koizumi et al. (32), who investigated the properties of quantal Ca$^{2+}$ release evoked by activation of RyR in PC12 cells. They found that the sensitivity of RyR to caffeine was altered by luminal Ca$^{2+}$. Specifically, the threshold for RyR activation by caffeine was sensitized 10-fold as the Ca$^{2+}$ load increased from minimal to maximal loading. In addition, the fraction of Ca$^{2+}$ released by low caffeine concentrations was increased (32).

Surprisingly, both *B. judaicus* toxins and IpTx$_x$ share functional similarities, although there is no significant similarity in their primary sequences. *B. judaicus* toxins and IpTx$_x$ selectively activate [$^3$H]ryanodine binding to RyR1, have a moderate effect on RyR2, and have no effect on RyR3. Their interaction with RyR1 is direct and does not appear to be mediated by any accessory protein(s). In combination with caffeine, both the *B. judaicus* toxins and IpTx$_x$ increase [$^3$H]ryanodine binding in a synergistic manner, suggesting that the *B. judaicus* toxin binding sites may interact with the caffeine binding site and create a cooperative binding effect. All three toxins stimulate Ca$^{2+}$ release from SR vesicles and induce subconductance states in

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**Fig. 8.** *B. judaicus* toxins induce Ca$^{2+}$ release from skeletal RyRs. Ca$^{2+}$ release from actively loaded SR vesicles by BjTx-1 (A) or BjTx-2 (B) was measured with the spectrophotometric Ca$^{2+}$ indicator antipyrlyazo III, as described (22). Downward arrows indicate the addition of 10 nmol of Ca$^{2+}$ to the 1-ml reaction medium. Thapsigargin (1 μM) was added simultaneously with the peptides (upward arrows) to block Ca$^{2+}$ uptake by the SR. When equilibrium was reached, the Ca$^{2+}$ ionophore A23187 (5 μM) was added to assess SR Ca$^{2+}$ content (asterisks).
single RyR1 channels reconstituted into planar lipid bilayers. Nevertheless, there are also functional differences between the toxins. As seen in the [3H]ryanodine binding curves in Fig. 6, for example, B. judaicus toxins do not alter RyR Ca$^{2+}$/H$^{1+}$ sensitiv-
ity. However, IpTxa shifts the threshold of activation from pCa 7 to pCa 8 and the threshold of inactivation from pCa 3 to pCa 2. Consequently, IpTxs$_{a}$ exerts its stimulatory effect by sensitizing RyRs to Ca$^{2+}$/H$^{1+}$ activation and decreasing the inhibitory effect at low Ca$^{2+}$/H$^{1+}$ (22).

Despite significant differences in their primary sequences, structural modeling (Fig. 3) suggests that the position of the charge distribution at the level of the three-dimensional structures of B. judaicus toxins and IpTxs$_{a}$ are comparable, which may explain why they share functional similarity. In BjTx-1, there is a positively charged domain which consists of Lys$^{13}$, Lys$^{16}$, and Lys$^{16}$. A similar positively charged domain also exists in the three-dimensional structure of IpTxs$_{a}$ (Fig. 3). Indeed, structure-function relationship studies have demonstrated that the electrostatic effect plays a very important role in toxin-receptor interactions. For example, with IpTxs$_{a}$, replacement of Arg$^{684}$, localized in a cluster of five basic amino acids, with negatively charged Glu abolished its activation effect on [3H]ryanodine binding. However, replacement of Lys$^{6}$, which is far away from the positively charged domain, with Glu has a negligible effect on IpTxs$_{a}$ activity (14). Comparable results are obtained from point mu-
tations of peptide A, a peptide fragment within the II-III loop of the a1 subunit of dihydropyridine receptors, which is critical for Ca$^{2+}$/H$^{1+}$ release via activation of RyRs. Mutation of Arg$^{684}$, located in a cluster of five basic amino acids, with negatively charged Glu also abolished its function as a RyR activator (14), whereas mutation of Lys$^{675}$/Glu, has no effect (14). Taken together, it is plausible that the positive potential zones within IpTxs$_{a}$ and BjTx-1 are also involved in their interactions with the RyR. Sound support to this idea is the fact that BjTx-2, a natural mutant of BjTx-1 with Lys$^{16}$ replaced by Ile, displays lower affinity for interaction with RyR compared with BjTx-1, as shown in Fig. 1C.

In summary, peptide toxins are emerging as useful probes of RyR structure and function. The B. judaicus toxins that we isolated bind directly to the RyR and act rapidly and reversibly. These intrinsic properties confer upon them a unique set of attributes that make them useful tools to identify regulatory domains critical for channel gating.

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