

Primary structure and synthesis of Imperatoxin A (IpTx_a), a peptide activator of Ca²⁺ release channels/ryanodine receptors

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Abstract We present the complete amino acid sequence of Imperatoxin A (IpTx_a), a 33-amino-acid peptide from the venom of the scorpion *P. imperator* which activates Ca²⁺ release channels/ryanodine receptors (RyR) of sarcoplasmic reticulum (SR). The amino acid sequence of IpTx_a shows no homology to any scorpion toxin so far described, but shares some homology to the amino acid sequence of Tx2-9 and agelenin, two spider toxins that target neuronal P-type Ca²⁺ channels. We also describe the total synthesis of IpTx_a and demonstrate that it efficiently activates RyRs with potency and affinity identical to those of native IpTx_a. The use of synthetic IpTx_a should help in the identification of the structural motifs of RyR critical for channel gating.

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Key words: Ca²⁺ release channel; Sarcoplasmic reticulum; *Pandinus imperator* scorpion venom; Caffeine; Synthetic peptide

1. Introduction

Ryanodine receptors (RyR) are essential for maintaining the intracellular Ca²⁺ homeostasis in striated muscle and in a variety of non-excitabile cells [1,2]. Their role in excitation–contraction coupling of cardiac and skeletal muscle is well established [3], and their participation in stimulus–secretion coupling of secretory cells [4], and elevation of [Ca²⁺]_i in neurons [5], is increasingly evident. In all of these cells, RyR releases massive amounts of Ca²⁺ from intracellular Ca²⁺ pools in response to a variety of triggering signals.

To this point, ryanodine has been the major probe used for structural and functional studies of RyRs. The alkaloid binds to RyRs with high affinity and specificity, and there is a consensus that [³H]ryanodine binding is an indicator of the number of receptors that are in the open conformational state [6]. However, ryanodine also displays some undesirable features that limit its use in experiments with intact cells, such as its slow association and dissociation rates, and its behavior as both an agonist and a blocker depending on the concentration used [6,7].

Scorpion venoms have traditionally represented excellent sources of ionic channel-blocking peptides. In the venom of the scorpion *P. imperator* we found Imperatoxin A (IpTx_a), a short peptide that specifically and with high affinity increased

[³H]ryanodine binding and enhanced the activity of RyRs reconstituted in planar lipid bilayers [8,9]. At concentrations well above the half-maximal effective concentration (ED₅₀) exhibited for RyRs, IpTx_a did not affect other Ca²⁺ channels or ion transporters of muscle and brain [8]. Moreover, all of these effects could be seen only on skeletal-type RyR, suggesting that IpTx_a preferentially affects this particular RyR isoform [9]. However, the use of this promising agent has been hampered by the extremely small amount of peptide obtained from the whole venom.

In this paper we communicate the entire amino acid sequence of IpTx_a and describe the synthesis of an IpTx_a analog that displays functional properties identical to those of the authentic native IpTx_a. The design of a fully functional synthetic IpTx_a analog should alleviate the problem associated with the scarcity of IpTx_a and accelerate its use as a peptide probe of RyR function.

2. Materials and methods

2.1. Purification of IpTx_a

IpTx_a was purified from *P. imperator* scorpion venom in three chromatographic steps. Crude venom was extracted from CO₂-anesthetized scorpions kept alive in the laboratory, recovered with deionized water, and lyophilized. Batches (100 mg) of crude venom were dissolved in 2–3 ml of deionized water and applied onto a column (1.5 × 125 cm) of Sephadex G-50 fine. Fractions were eluted with 20 mM NH₄AcOH (pH 4.7) at a flow rate of 10 ml/h. Fraction 3 containing IpTx_a was applied to a column (1 × 25 cm) of carboxymethyl cellulose 32 (Pharmacia) equilibrated with 20 mM NH₄AcOH (pH 4.7). Peptides were eluted at a flow rate of 12 ml/h with a linear gradient of 250 ml of 20 mM NH₄AcOH (pH 4.7) and 250 ml of the same buffer containing 0.5 M NaCl. The peak containing IpTx_a eluted when the NaCl concentration at the top of the column reached 340 mM. This fraction was concentrated by vacuum centrifugation and injected into an Aquapore C₈ reverse-phase HPLC column (Pierce). IpTx_a was eluted with a linear gradient of 0–100% acetonitrile in 0.075% trifluoroacetic acid (TFA) run at 1 ml/min for 60 min. IpTx_a was quantified by absorbance at 280 nm (A_{280nm}) using an extinction coefficient (ε) = 1852 M⁻¹ cm⁻¹.

2.2. Preparation of sarcoplasmic reticulum vesicles and [³H]ryanodine binding assay

Heavy sarcoplasmic reticulum (SR) was prepared from rabbit white back and leg muscle using the procedure of Meissner [10]. [³H]ryanodine binding to rabbit skeletal SR was carried out as previously described [8,9]. Briefly, the standard incubation medium contained 0.2 M KCl, 1 mM Na₂EGTA, 10 mM Na-Pipes, pH 7.2 and CaCl₂ necessary to set [free Ca²⁺] in the range of 1 nM to 100 μM. Ca²⁺/EGTA ratios were calculated using the stability constants of Fabiato [11]. [³H]ryanodine (68.4 Ci/mmol, Dupont NEN) was diluted directly in the incubation medium to a final concentration of 7 nM. Protein concentration was in the range of 0.2–0.4 mg/ml and was determined by the Bradford method. Incubations lasted 90 min at 36°C. Samples (0.1 ml) were always run in duplicate, filtered on What-

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man GF/B glass fiber filters and washed twice with 5 ml of distilled water using a Brandel M24-R cell harvester (Gaithersburg, MD). Nonspecific binding was determined in the presence of 10 μ M unlabeled ryanodine and has been subtracted from each sample. Equilibrium binding data were fitted by nonlinear regression analysis with the functions specified in the text using the computer program Origin 4.0 (Microcal Inc., Northampton, MA).

2.3. Amino acid analysis and microsequencing of IpTx_a

Amino acid analysis of IpTx_a was performed on samples hydrolyzed in 6 N HCl with 0.5% phenol at 110°C in evacuated, sealed tubes as described [12]. Reduction of IpTx_a with dithiothreitol, and alkylation with iodoacetic acid was performed as described [12]. The sequence of the intact native and reduced/carboxymethylated IpTx_a was determined using a model 6400/6600 automatic liquid-phase protein sequencer (Milligen/BioSearch Prosequencer) employing standard Edman degradation programs and CD immobilon membranes. To confirm the correctness of the carboxy-terminal sequence, 20 μ g of IpTx_a was hydrolyzed with *S. aureus* V8 in 100 mM ammonium bicarbonate (pH 7.8). The peptide fragments were purified as described and directly sequenced as described for native IpTx_a.

2.4. Synthesis of IpTx_a

A linear analog of IpTx_a was synthesized by the solid-phase methodology with Fmoc-amino acids in an Applied Biosystems peptide synthesizer (model 432A). After cleavage with 90% trifluoroacetic acid

for 4 h at room temperature, the crude linear peptide was extracted with 5% acetic acid and dried by vacuum centrifugation. The cyclization reaction to make disulfide bridges in the molecule was carried out in 0.1 M NaCl, 5 mM reduced glutathione, 0.5 mM oxidized glutathione, 20 mM NaHPO₄ (pH 8.0) and 30 μ M of synthetic IpTx_a. The crude cyclized product was purified in a C₈ reverse-phase HPLC column using the conditions described in the text. The structure and the purity of the synthetic toxin were confirmed by analytical HPLC, amino acid analysis and mass spectrometry. For amino acid analysis, synthetic toxin was hydrolyzed in 350 μ l of 6 N HCl, 15 μ l of phenol at 165°C for 50 min, then analyzed in an Applied Biosystems analyzer (model 421). Mass spectrometry was carried out in a Bruker Reflex II MALDI-TOF spectrometer.

2.5. Sequence comparisons

The amino acid sequence of IpTx_a was compared with those of other proteins deposited in the protein database of GenBank (Los Alamos National Laboratory, Los Alamos, NM) by computer analysis using the program Blitz version 1.5 (Biocomputing Research Unit, University of Edinburgh, UK).

3. Results and discussion

Whole *P. imperator* scorpion venom completely inhibited

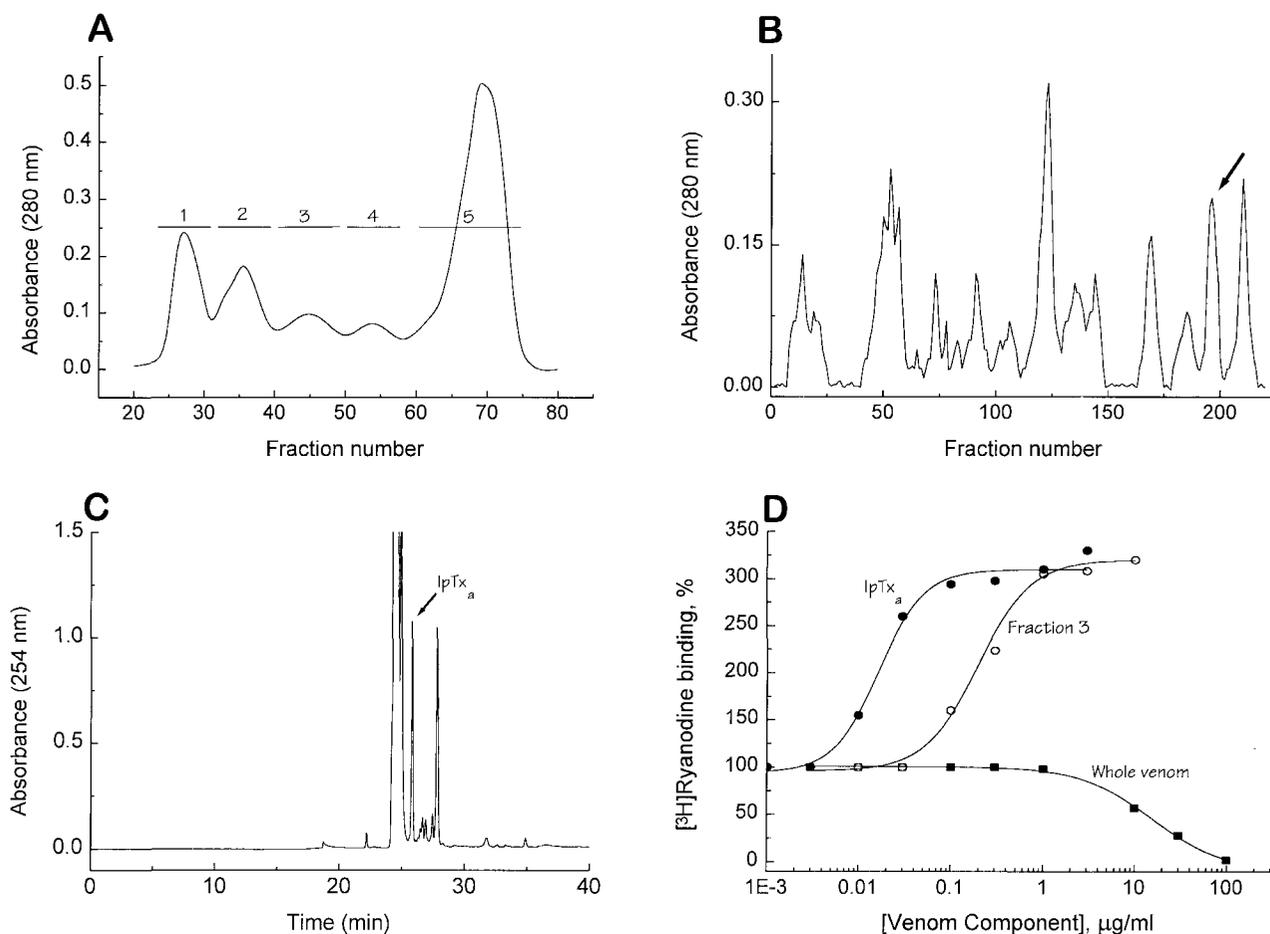


Fig. 1. Purification of IpTx_a. A: *P. imperator* soluble venom (120 mg protein) was applied to a Sephadex G-50 column (0.9×190 cm) equilibrated and run with 20 mM NH₄AcOH (pH 4.7). Samples (5 ml) were collected and tested for their capacity to modify [³H]ryanodine binding. B: Fraction 3 was further separated through a CM-Cellulose column (0.9×30 cm), equilibrated and run with 20 mM NH₄AcOH (pH 4.7). A linear gradient of sodium chloride resolved multiple sub-fractions, one of which (labeled with arrow) contained IpTx_a. C: The sub-fraction from the CM-cellulose column containing IpTx_a was lyophilized and injected into a C₈ reverse-phase HPLC column and eluted with a 0–100% linear gradient of acetonitrile containing 0.075% trifluoroacetic acid. D: Dose-response curve for whole venom and purified components. [³H]Ryanodine (7 nM) was incubated with skeletal SR protein in 0.2 M KCl, 10 μ M CaCl₂, 10 mM Na-HEPES pH 7.2, in the absence (control, 100%) and the presence of indicated concentrations of venom components. Nonspecific binding was determined in the presence of 20 μ M ryanodine and has been subtracted from this and subsequent results.

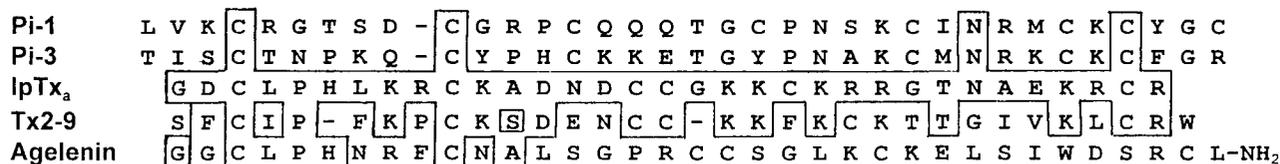


Fig. 2. Amino acid sequence of IpTx_a and comparison with other scorpion and spider toxin sequences. The amino acid sequence of IpTx_a was obtained by direct Edman degradation of the reduced and alkylated IpTx_a. Gaps (-) in the amino acid sequence of Pi-1 and Pi-3, two K⁺ channel blockers from the scorpion *P. imperator* [13,14], as well as in Tx2-9, a P-type Ca²⁺ channel blocker from the spider *P. nigriventer* [16], have been introduced to maximize homology. Agelenin, also a spider toxin that blocks P-type Ca²⁺ channel [17], is presented without gaps.

[³H]ryanodine binding to skeletal SR with a concentration of 15 µg/ml yielding the half-maximal effect (ED₅₀) (Fig. 1D, see also [8]). The presence of components that stimulate RyRs is only apparent after separation of the stimulatory and inhibitory components. The purification of IpTx_a, an activator of RyRs, was performed in three chromatographic steps as described in the Section 2 and shown in Fig. 1. After fractionation of whole venom in Sephadex G-50 (Fig. 1A), five fractions were collected and assayed for effects on [³H]ryanodine binding. Fraction 2 contained peptides in the range of 10–20 kDa that were responsible for the inhibitory effect exhibited by the whole venom. Fraction 3 increased [³H]ryanodine binding with an ED₅₀ = 0.19 µg/ml (Fig. 1D), but since it accounts for ≤5% of the total venom, its effect is probably masked in the whole venom by that of Fraction 2. Fraction 3 was subsequently applied to a carboxymethyl-cellulose and eluted with a gradient of NaCl (Fig. 1B). The peak containing IpTx_a (marked by an arrow) eluted late in the run, suggesting that IpTx_a was a strongly basic peptide. Fig. 1C shows the chromatographic profile of IpTx_a (marked with an arrow) after elution from a reverse-phase C₈ HPLC column. IpTx_a increased [³H]ryanodine binding with an ED₅₀ = 0.016 µg/ml. Given this value and the molecular weight of IpTx_a (3.7 kDa, see below), the apparent dissociation constant (K_d) was ~5 nM. The proportion of IpTx_a in the whole venom is very small, about 0.03%, but since it displays a high potency to stimulate RyRs, the [³H]ryanodine binding assay offers a sensitive method to track its functional activity.

Fig. 2 shows the complete amino acid sequence of IpTx_a as determined by direct automated microsequencing. IpTx_a is composed of 33 amino acids with a calculated M_r = 3765. As most short scorpion toxins, IpTx_a is a basic peptide containing three pairs of cysteine residues that stabilize the three-dimensional conformation by forming disulfide bridges. Fig. 2 also shows a comparison of the amino acid sequence of IpTx_a with those of Pi-1 and Pi-3, two peptide blockers of K⁺ channels from the same *P. imperator* venom [13,14], reportedly the smallest ionic channel-targeted scorpion peptides. Pi-3 is representative of a group of homologous toxins with three disulfide bridges such as noxiustoxin, charybdotoxin, iberiotoxin, etc. (for a review, see [15]); Pi-1 forms a K⁺ toxin subgroup on its own by having the remarkable characteristic of possessing four disulfide bridges [13]. No significant similarity is observed between IpTx_a and any of these toxins, even when gaps (-) are introduced at the level of Cys¹⁰ to maximize homology. Even less homology is found when the comparison involves the larger Na⁺ channel-selective peptides from scorpion venoms (not shown). Thus, IpTx_a constitutes a novel class, the smallest yet, of scorpion toxins targeted against ionic channels.

Albeit not dramatic, a higher sequence homology was

found when the comparison was made with Tx2-9 of the spider *P. nigriventer* [16], and agelenin of the spider *A. asperta* [17], two peptide blockers of neuronal P-type calcium channels (Fig. 2). If gaps are introduced to maximize homology, Tx2-9 is the most similar with 45% sequence identity. Agelenin has only limited resemblance (21% sequence identity), although higher homology may be ascribed if two-residue gaps are introduced. Regardless of the extent of similarity, it is clear that IpTx_a is more related to these two spider toxins than to other toxins present in scorpion venoms. Thus, IpTx_a adds to the emerging notion that peptide toxins found in

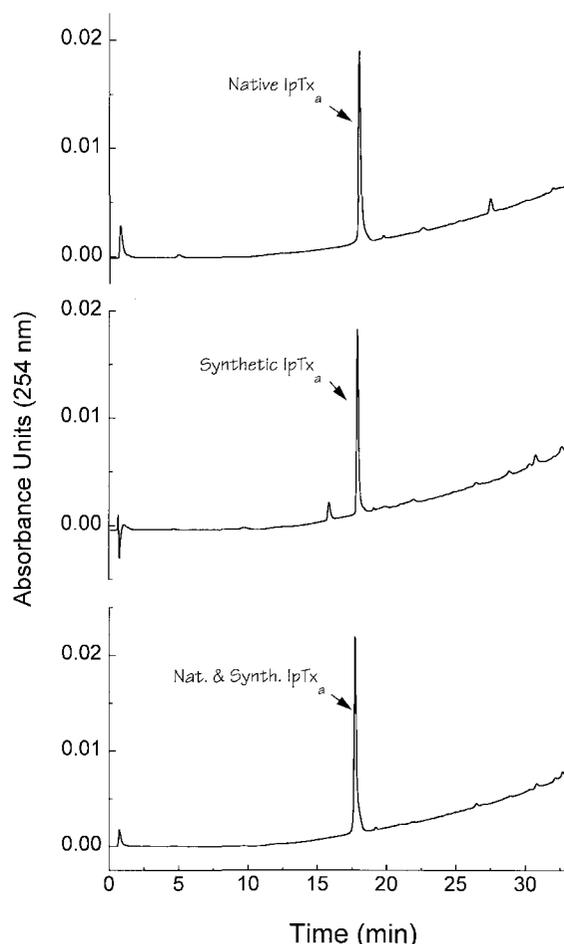


Fig. 3. HPLC comparison of native and synthetic IpTx_a. Native and Synthetic IpTx_a (~1.5 nmol each), as well as an equimolar mixture (~1 nmol each) were chromatographed on a Pierce C₈ analytical column. The peptides were eluted using 0.075% trifluoroacetic acid as solvent, applying a linear gradient from 0 to 100% (v/v) of acetonitrile at a flow rate of 1 ml/min.

venomous animals of different phyla contain similar structural motifs [18].

We used the IpTx_a amino acid sequence to synthesize a linear analog of IpTx_a. After purification and cyclization of the synthetic analog as described in Section 2, synthetic IpTx_a

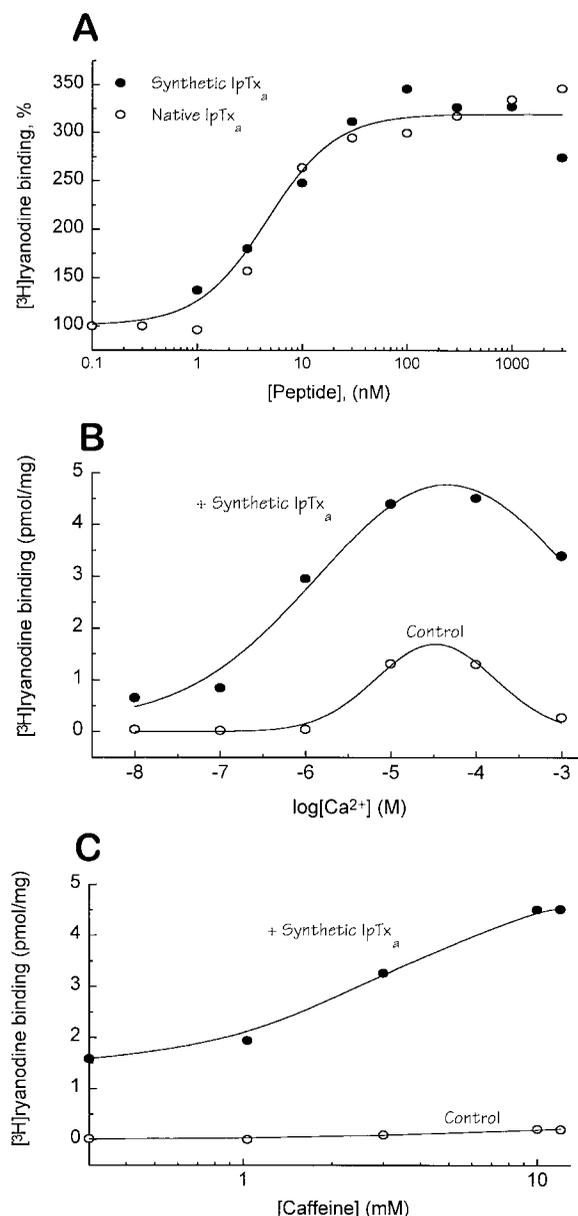


Fig. 4. Functional properties of synthetic IpTx_a. A: Dose-response relation for native (○) and synthetic IpTx_a (●). [³H]Ryanodine (7 nM) was incubated with skeletal SR in the standard incubation medium described in Section 2 in the presence of the indicated concentrations of IpTx_a. Both sets of data points were fitted with the same line. K_d was 5 nM and B_{max} was 325%, which corresponded to 2.36 pmol/mg protein. B: Effect of synthetic IpTx_a on the Ca²⁺-dependence of [³H]ryanodine binding. The standard binding medium contained 1 mM EGTA and several CaCl₂ concentrations to yield the desired [free Ca²⁺]. Synthetic IpTx_a (1 μM) was present throughout the incubation period. Smooth lines linking data points have no theoretical meaning. C: Potentiation of synthetic IpTx_a effect by caffeine. The binding of [³H]ryanodine was determined in incubation medium containing 100 nM free Ca²⁺ (1 mM EGTA and 385 μM CaCl₂). Caffeine was added at the beginning of the incubation as 10-μl aliquots from 100-fold stocks for concentrations up to 10 mM, and as powder form to reach 20 mM.

was analyzed by reverse-phase HPLC for comparison with native IpTx_a (Fig. 3). The elution time of synthetic IpTx_a was identical to that of native IpTx_a, and a single sharp peak was observed upon mixing synthetic and native IpTx_a.

We also tested the functional activity of synthetic IpTx_a. Fig. 4A shows that synthetic IpTx_a increased [³H]ryanodine binding to skeletal SR with the same potency and affinity as native IpTx_a. Both sets of data points were overlapping and could be fitted with the same line. We used the function: $B = B_{max}/1+(K_d+[IpTx_a])^n$ to fit the data, where B is the specific binding of [³H]ryanodine, B_{max} is the maximal activation of [³H]ryanodine binding evoked by IpTx_a (325%), K_d is the apparent dissociation constant of IpTx_a (5 nM), and n is the Hill number (1.58). Unlike this dramatic effect on skeletal RyR, no significant activation of [³H]ryanodine binding to cardiac SR was observed with synthetic IpTx_a (results not shown). Hence, synthetic IpTx_a also displays selectivity for skeletal-type RyRs, just as native IpTx_a does.

In a previous study [9], we reported that native IpTx_a increases [³H]ryanodine binding by sensitizing RyRs to Ca²⁺. Fig. 4B shows the Ca²⁺-dependence of [³H]ryanodine binding to skeletal SR and the effect of synthetic IpTx_a. Specific binding in the absence of the peptide (Fig. 4B, Control, ○) had a threshold for detection at pCa 7-6 and was maximal at -50 μM [Ca²⁺]. In the presence of 1 μM synthetic IpTx_a (Fig. 4B, +Synthetic IpTx_a, ●), the threshold for detection of [³H]ryanodine binding decreased to pCa 8 and the binding curve was dramatically augmented in absolute values. The EC₅₀ for the activation of [³H]ryanodine binding by Ca²⁺ (ascending limb of the curve) was -5 μM and 0.8 μM for control and synthetic IpTx_a, respectively. Thus, synthetic IpTx_a also activates [³H]ryanodine binding by sensitizing RyRs to Ca²⁺.

Another functional attribute of native IpTx_a was its ability to potentiate its effect with caffeine [9]. Fig. 4C shows the interaction of caffeine and synthetic IpTx_a. At pCa 7, binding was 0.02 pmol/mg in the absence of synthetic IpTx_a and caffeine and increased to 0.39 pmol/mg in the presence of 20 mM caffeine (Fig. 4C, ○). In the presence of 1 μM synthetic IpTx_a (Fig. 4C, ●), binding was 1.52 pmol/mg in the absence of caffeine and increased to 4.51 in the presence of 20 mM caffeine, i.e., a net gain of -3 pmol/mg. Since the binding increment evoked by the combined addition of synthetic IpTx_a and caffeine was larger than that evoked by caffeine or synthetic IpTx_a alone, this suggested a cooperative interaction between the synthetic IpTx_a- and caffeine-binding sites.

A mass spectrometry analysis of native and synthetic IpTx_a yielded the expected molecular mass (3765.8 Da) based on amino acid composition and sequence. Thus, by all structural and functional criteria applied, synthetic IpTx_a is identical to native IpTx_a. The design of a synthetic analog of IpTx_a with functional attributes identical to the native IpTx_a verifies the correctness of the amino acid sequence, since even single amino acid substitutions in analogous peptides result in large changes of affinity towards their acceptor site [13]. The availability of relatively large amounts of synthetic IpTx_a should accelerate its use as a peptide probe of RyR function.

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