Primary structure and synthesis of Imperatoxin A ($IpTx_a$), a peptide activator of Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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.**

Key words: Ca^{2+} release channel; Sarcoplasmic reticulum; *Pandinus imperator* scorpion venom; Caffeine; Synthetic peptide

1. Introduction

Ryanodine receptors (RyR) are essential for maintaining the intracellular Ca^{2+} homeostasis in striated muscle and in a variety of non-excitable 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 $[Ca^{2+}]_i$ in neurons [5], is increasingly evident. In all of these cells, RyR releases massive amounts of Ca^{2+} from intracellular Ca^{2+} 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 [^{3}H]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 [^{3}H]ryanodine binding and enhanced the activity of RyRs reconstituted in planar lipid bilayers [8,9]. At concentrations well above the half-maximal effective concentration (ED_{50}) exhibited for RyRs, $IpTx_a$ did not affect other Ca^{2+} 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 CO_2-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 NH_4AcOH (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 NH_4AcOH (pH 4.7). Peptides were eluted at a flow rate of 12 ml/h with a linear gradient of 250 ml of 20 mM NH_4AcOH (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 C_8 reverse-phase HPLC column (Pierce). $IpTx_a$ was eluted with a linear gradient of 0-100% acetonitrile in 0.075% trifluroacetic 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 [^{3}H]ryanodine binding assay

Heavy sarcoplasmic reticulum (SR) was prepared from rabbit white back and leg muscle using the procedure of Meissner [10]. [^{3}H]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 Na_2EGTA, 10 mM Na-Pipes, pH 7.2 and $CaCl_2$ necessary to set [free Ca^{2-}] in the range of 1 nM to 100 μM. Ca^{2+}/EGTA ratios were calculated using the stability constants of Fabiato [11]. [^{3}H]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 μM 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 μg 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 Byosystems 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 $NaHPO_4$ (pH 8.0) and 30 μM of synthetic $IpTx_a$. The crude cyclized product was purified in a C_8 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 μl of 6 N HCl, 15 μl 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

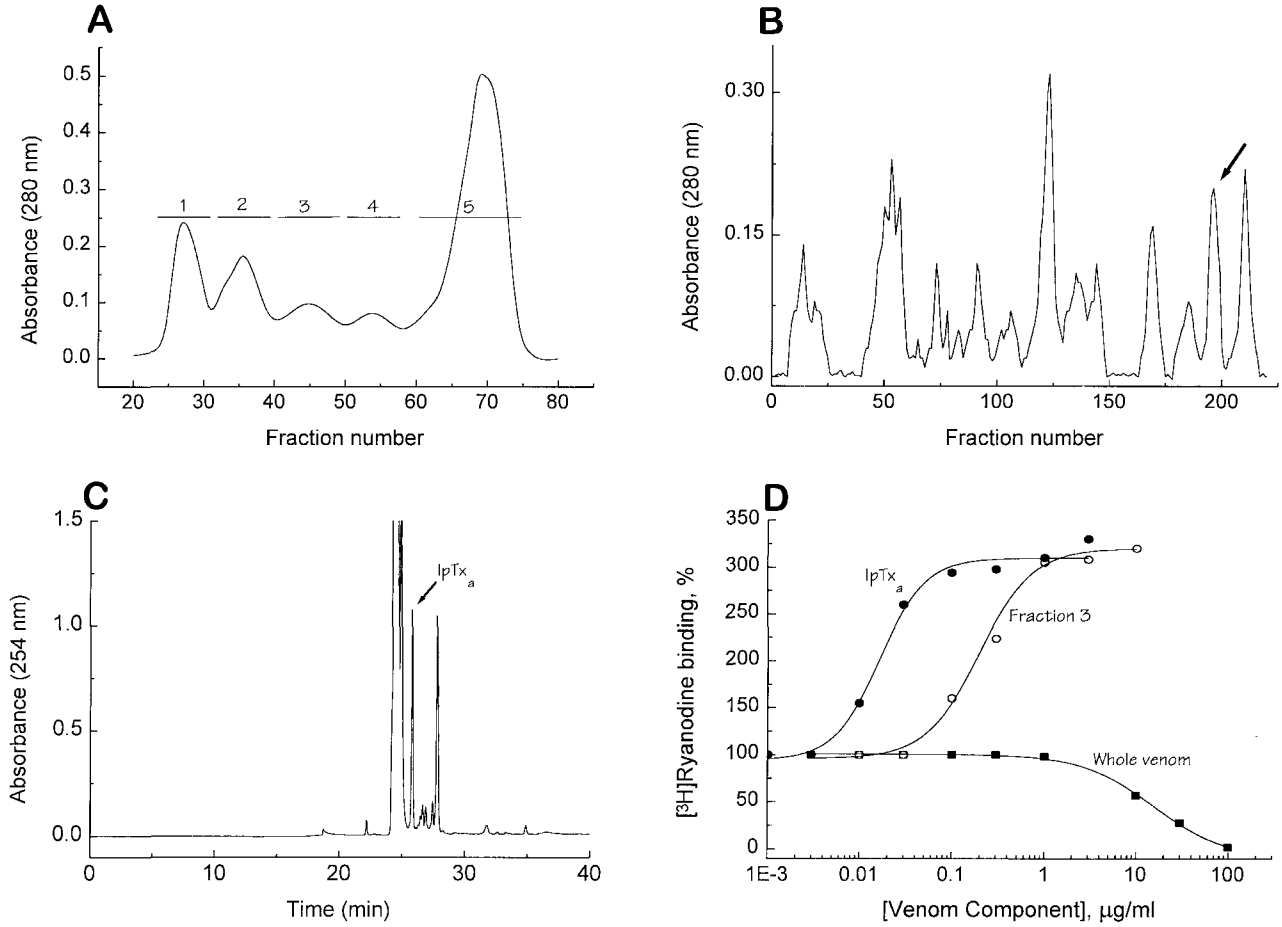

Fig. 1. Purification of $IpTx_a$. A: *P. imperator* soluble venom (120 mg protein) was applied to a Sephadex G-50 column (0.9×190 cm) equilibrated and run with 20 mM NH_4AcOH (pH 4.7). Samples (5 ml) were collected and tested for their capacity to modify [^{3}H]ryanodine binding. B: Fraction 3 was further separated through a CM-Cellulose column (0.9×30 cm), equilibrated and run with 20 mM NH_4AcOH (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 C_8 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. [^{3}H]Ryanodine (7 nM) was incubated with skeletal SR protein in 0.2 M KCl, 10 μM $CaCl_2$, 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 μM ryanodine and has been subtracted from this and subsequent results.

Pi-1	L V K C R G T S D - C G R P C Q Q Q T G C P N S K C I N R M C K C Y G C
Pi-3	T I S C T N P K Q - C Y P H C K K E T G Y P N A K C M N R K C K C F G R
$IpTx_a$	G D C L P H L K R C K A D N D C C G K K C K R R G T N A E K R C R
Tx2-9	S F C I P - F K P C K S D E N C C - K K F K C K T T G I V K L C R W
Agelenin	G G C L P H N R F C N A L S G P R C C S G L K C K E L S I W D S R C L-NH_2

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 Ca^{2+} channel blocker from the spider *P. nigriventer* [16], have been introduced to maximize homology. Agelenin, also a spider toxin that blocks P-type Ca^{2+} channel [17], is presented without gaps.

[^{3}H]ryanodine binding to skeletal SR with a concentration of 15 μg/ml yielding the half-maximal effect (ED_{50}) (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 [^{3}H]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* [^{3}H]ryanodine binding with an $ED_{50} = 0.19$ 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 C_8 HPLC column. $IpTx_a$ increased [^{3}H]ryanodine binding with an $ED_{50} = 0.016$ 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 [^{3}H]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 Cys^{10} 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

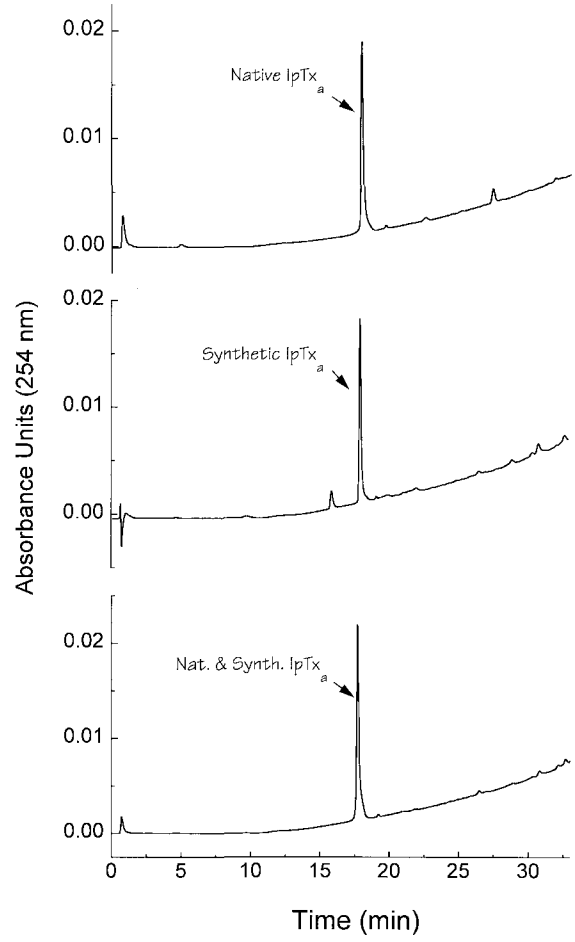

Fig. 3. HPLC comparison of native and synthetic $IpTx_a$. Native and Synthetic $IpTx_a$ (~1.5 nmol each), as well as an equimolar mixture (~1 nmol each) were chromatographed on a Pierce C_8 analytical column. The peptides were eluted using 0.075% trifluoroacetic acid as solvent, applying a linear gradient 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 $IpTx_a$ 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 $IpTx_a$.

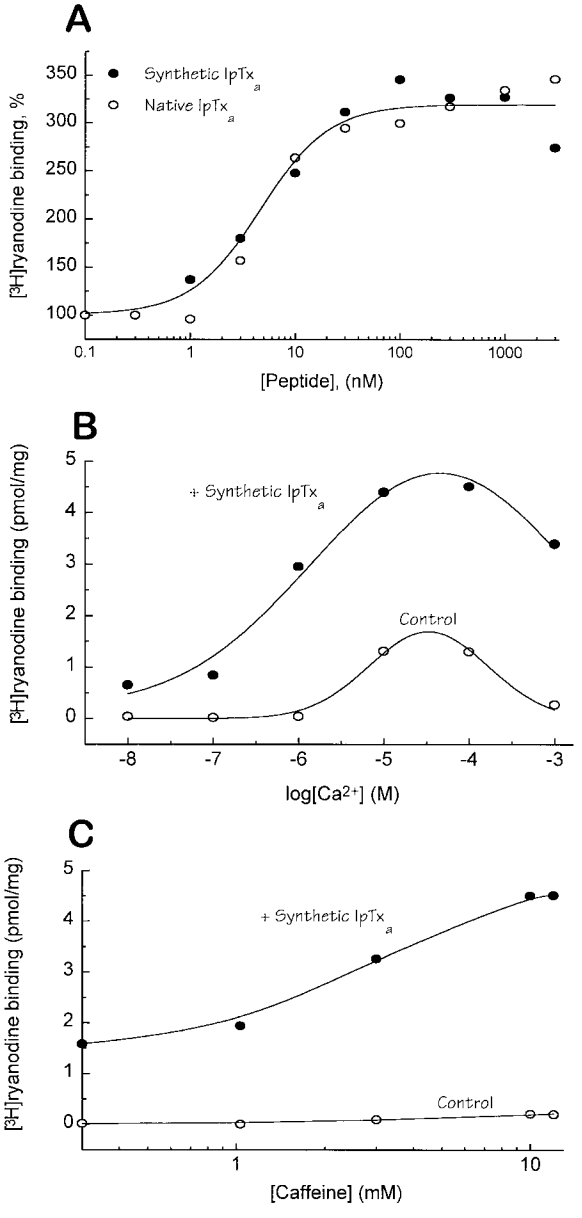

Fig. 4. Functional properties of synthetic $IpTx_a$. A: Dose-response relation for native (○) and synthetic $IpTx_a$ (●). [^{3}H]Ryanodine (7 nM) was incubated with skeletal SR in the standard incubation medium described in Section 2 in the presence of the indicated concentrations of $IpTx_a$. Both sets 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 Ca^{2+}-dependence of [^{3}H]ryanodine binding. The standard binding medium contained 1 mM EGTA and several $CaCl_2$ concentrations to yield the desired [free Ca^{2+}]. 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 [^{3}H]ryanodine was determined in incubation medium containing 100 nM free Ca^{2+} (1 mM EGTA and 385 μM $CaCl_2$). 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.

We also tested the functional activity of synthetic $IpTx_a$. Fig. 4A shows that synthetic $IpTx_a$ increased [^{3}H]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 [^{3}H]ryanodine, B_{max} is the maximal activation of [^{3}H]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 [^{3}H]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 [^{3}H]ryanodine binding by sensitizing RyRs to Ca^{2+}. Fig. 4B shows the Ca^{2+}-dependence of [^{3}H]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 *p*Ca 7-6 and was maximal at ~50 μM [Ca^{2+}]. In the presence of 1 μM synthetic $IpTx_a$ (Fig. 4B, +Synthetic $IpTx_a$, ●), the threshold for detection of [^{3}H]ryanodine binding decreased to *p*Ca 8 and the binding curve was dramatically augmented in absolute values. The EC_{50} for the activation of [^{3}H]ryanodine binding by Ca^{2+} (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 [^{3}H]ryanodine binding by sensitizing RyRs to Ca^{2+}.

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 *p*Ca 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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