A Spider Toxin That Induces a Typical Effect of Scorpion α-Toxins but Competes with β-Toxins on Binding to Insect Sodium Channels†

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Received July 22, 2004; Revised Manuscript Received September 30, 2004

ABSTRACT: δ-Palutoxins from the spider *Paracoelotes luctuosus* (Araneae: Amaurobiidae) are 36−37 residue long peptides that show preference for insect sodium channels (NaChs) and modulate their function. Although they slow NaCh inactivation in a fashion similar to that of receptor site 3 modifiers, such as scorpion α-toxins, they actually bind with high affinity to the topologically distinct receptor site 4 of scorpion β-toxins. To resolve this riddle, we scanned by Ala mutagenesis the surface of δ-PaluIT2, a δ-palutoxin variant with the highest affinity for insect NaChs, and compared it to the bioactive surface of a scorpion β-toxin. We found three regions on the surface of δ-PaluIT2 important for activity: the first consists of Tyr-22 and Tyr-30 (aromatic), Ser-24 and Met-28 (polar), and Arg-8, Arg-26, Arg-32, and Arg-34 (basic) residues; the second is made of Trp-12; and the third is made of Asp-19, whose substitution by Ala uncoupled the binding from toxicity to lepidopteran larvae. Although spider δ-palutoxins and scorpion β-toxins have developed from different ancestors, they show some commonality in their bioactive surfaces, which may explain their ability to compete for an identical receptor (site 4) on voltage-gated NaChs. Yet, their different mode of channel modulation provides a novel perspective about the structural relatedness of receptor sites 3 and 4, which until now have been considered topologically distinct.

Voltage-gated sodium channels (NaChs)[1] are responsible for the generation and propagation of electrical signals in the nervous system. There are at least nine different genes encoding distinct NaChs in mammals, but only one gene (*para*), which undergoes alternative splicing, was identified in various insect species (*1*). The structural diversity of NaChs coincides with variations in physiological and pharmacological properties (*2*). At least seven distinct toxin binding sites were characterized by radioligand binding studies on vertebrate and insect NaChs (*3*, *4*). Among them, binding sites for polypeptide toxins from scorpions, sea anemones, spiders, and cone snails are distributed on the extracellular surface of the NaCh and are instrumental in the study of NaCh topology, function, and pharmacology (*5*). Scorpion α-toxins bind to receptor site 3, which involves extracellular loops of domains I and IV of the NaCh (*6*, *7*). This site is also recognized by the sea anemone toxin, ATXII, and spider δ-atracotoxins (*7*−*9*), which differ structurally but induce a similar inhibitory effect on NaCh inactivation. Scorpion β-toxins bind to receptor site 4, which involves domain II in insect and mammalian NaChs (*10*−*12*). Two groups of scorpion β-toxins that show selectivity to insects were named according to the behavioral response they induced: the excitatory toxins (e.g., Bj-xtrIT and AahIT from *Butothus judaicus* and *Androctonus australis hector*, respectively) and the depressant toxins (e.g., LqhIT2 from *Leiurus quinquestriatus hebraeus*) (*13*, *14*). These toxins compete with other scorpion β-toxins that are active on both mammalian and insect NaChs (e.g., Ts1 from *Tityus serrulatus* and Lqhβ1) on binding to insect NaChs (*15*−*18*). Excitatory toxins cause a fast contraction paralysis of fly larvae, attributed to the induction of repetitive firing of the axon, which results from a shift in the voltage dependence of activation to more negative membrane potentials, a small increase in the peak sodium conductance, and slowing of its inactivation. In contrast, depressant toxins induce a slow,

† This work was supported by the Research for the Future Program from the Japanese Society for the Promotion of Science (JSPS) and by the Suntory Institute for Bioorganic Research. D.G. and M.G. are in part supported by United States−Israel Binational Agricultural Research and Development Grant IS-3259-01 and by the Israeli Science Foundation, Grants 508/00 and 733/01, respectively.

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[1] Abbreviations: NaCh, sodium channel; CD, circular dichroism; CZE, capillary zone electrophoresis; MALDI-TOF-MS, matrix-assisted laser desorption−ionization time-of-flight mass spectrometry; δ-PaluIT2 and δ-sPaluIT2, native and synthetic δ-palutoxins, respectively; RP-HPLC, reversed-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; TFE, trifluoroethanol; LD_{50}, lethal dose that kills 50% of the animals.

10.1021/bi048434k CCC: $30.25 © 2005 American Chemical Society
Published on Web 01/13/2005

flaccid paralysis of fly larvae, attributed to a block of evoked action potentials, which results from strong depolarization of the axonal membrane and induction of noninactivating sodium currents at negative membrane potentials (*5*, *14*, *19*).

We have previously described four anti-insect selective δ-palutoxins (variants 1−4) from the venom of the spider *Paracoelotes luctuosus* (*20*). Although weaker in activity (*20*), δ-palutoxins alter the inactivation properties of inward Na^+ currents in cockroach axons in a manner resembling that of insecticidal site 3 toxins, such as scorpion α-toxins [e.g., LqhαIT and BomIII (*21*−*23*)] and spider δ-atracotoxins (*8*). Here we show that δ-palutoxins compete with the excitatory scorpion β-toxin, Bj-xtrIT, on binding to receptor site 4 of cockroach NaChs. Ala scanning of the δ-PaluIT2 variant highlighted structural elements important for activity that also appear on the bioactive surface of Bj-xtrIT (*17*, *24*). Although this structural resemblance may explain the ability of both toxin types to compete for receptor site 4, the molecular basis of their different mode of action remains to be clarified.

MATERIALS AND METHODS

Materials. All chemicals used in the study were of analytical grade. δ-TxVIA was kindly provided by Dr. Legall, l'Oreal, France.

Neuronal Membrane Preparations. Insect synaptosomes were prepared from whole heads of adult *Periplaneta americana* cockroaches according to a previously described method (*25*). All buffers contained a cocktail of protease inhibitors composed of phenylmethanesulfonyl fluoride (50 μg/mL), pepstatin A (1 μM), iodoacetamide (1 mM), and 1,10-phenanthroline (1 mM). No loss in binding activity was observed after 6 months of storage at −80 °C. Membrane protein concentration was determined using the Bio-Rad protein assay, with bovine serum albumin (BSA) as standard (Bio-Rad, Hercules, CA).

Iodination of LqhαIT, Bj-xtrIT, and δ-PaluIT2. The three toxins were radioiodinated with bovine milk lactoperoxidase (EC 1.11.1.7; Sigma-Aldrich, Taufkirchen, Germany) using 0.7 lactoperoxidase unit, 1 nmol of toxin, and 0.5 mCi of carrier-free $Na^{125}I$ (Amersham, Surrey, U.K.). The monoiodinated forms of the toxins were purified using an analytical RP-HPLC C_{18} column (250 × 4.6 mm, 300 Å, 5 μm; Vydac, Hesperia, CA) with a gradient of buffer A (H_2O, 0.1% TFA) to buffer B (acetonitrile, 0.1% TFA) (0−15% buffer B in 5 min, 15−40% buffer B in 120 min). Iodinated toxins were eluted at acetonitrile percentages of 23%, 26%, and 30% for δ-PaluIT2, LqhαIT and Bj-xtrIT, respectively. The concentrations of the monoiodotoxins were determined according to the specific radioactivity of ^{125}I (2500−3000 dpm/fmol).

Binding Assays. Standard binding buffer composition was (in mM) choline chloride 130, $CaCl_2$ 1.8, KCl 5, $MgSO_4$ 0.8, Hepes 50, glucose 10, and 2 mg/mL BSA. Washing buffer composition was (in mM) choline chloride 140, $CaCl_2$ 1.8, KCl 5.4, $MgSO_4$ 0.8, Hepes 50, and 5 mg/mL BSA, pH 7.5. Cockroach neuronal membrane preparations were suspended in 0.2 mL of binding buffer containing iodinated toxins. After 60 min incubation, the reactions were terminated by dilution with 2 mL of ice-cold washing buffer. Separation of free from bound toxins was achieved by rapid vacuum filtration through GF/C filters (Whatman, Maidstone, U.K.) preincubated in 0.3% poly(ethylenimine) (Sigma-Aldrich, Taufkirchen, Germany). The filters were then rapidly washed twice with 2 mL of buffer. Nonspecific toxin binding was determined in the presence of a high concentration of the unlabeled toxin and was typically 10−30% of total binding. Equilibrium competition and cold saturation assays were performed with increasing concentrations of unlabeled toxins in the presence of a constant, low concentration of ^{125}I-toxins. The results of cold saturation experiments were analyzed using the iterative program Ligand (Elsevier Biosoft, Cambridge, U.K.) and "cold saturation" analysis. For IC_{50} determination, competitive binding experiments were analyzed by the KaleidaGraph software (Synergy Software, Reading, PA), with a nonlinear Hill equation. The K_i values were calculated using the equation $K_i = IC_{50}/[1 + (L^*/K_d)]$, in which L^* is the concentration of hot toxin and K_d is its dissociation constant (*26*).

Peptide Synthesis. The C-terminally amidated forms (identical to native) of δ-PaluIT2 and all of the δ-PaluIT2 analogues were chemically synthesized by a solid-phase method using the 0.1 mmol Fmoc methodology on an Applied Biosystems 433A peptide synthesizer. A Rink amide resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, was used to provide the amino group at the C-terminus. Chemical synthesis, cleavage, and deprotection of peptide resins were performed as previously described (*27*). The crude linear synthetic peptides were dissolved in 20% aqueous acetonitrile and purified by RP-HPLC on a semipreparative C_8 column (5C8MS, 10 × 250 mm; Nacalai Tesque, Osaka, Japan) to 90−95% homogeneity to eliminate synthetic byproducts and incorrectly assembled peptides. The eight free cysteine residues were allowed to oxidize in air for 24 h at 5 °C in a 2 M aqueous ammonium acetate solution containing 1 mM reduced glutathione/0.1 mM oxidized glutathione. The folded synthetic toxins were purified on the same semipreparative C_8 RP-HPLC column using a 30 min linear gradient from 20% to 35% aqueous acetonitrile/0.1% TFA (2 mL/min), followed by cation-exchange chromatography as was previously described for the native toxins (*20*). The structural identity between synthetic and native peptides was verified by capillary zone electrophoresis and cation-exchange HPLC coelution experiments and by MALDI-TOF mass spectrometry. Peptide synthesis yields of linear and oxidized peptides are reported in the Results section.

MALDI-TOF Mass Spectrometry. Peptides mixed with a α-cyano-4-hydroxycinnamic acid matrix (α-CHCA; Aldrich, Milwaukee, WI) were analyzed on an Applied Biosystems Voyager DE-PRO system in reflector mode. Mass spectra were calibrated with internal peptide standards (LaserBio Labs, Valbonne, France) and analyzed in the Data Explorer software.

Circular Dichroism (CD) Measurements. CD spectra were obtained on a Jasco J-725 spectropolarimeter (Jasco, Tokyo, Japan). The spectra were measured from 260 to 180 nm in 60% trifluoroethanol (pH 7.1) at room temperature with a 1 mm path-length cell. Data were collected at 0.1 nm with a scan rate of 100 nm/min and a time constant of 1 s. The concentration of the toxins determined by amino acid analysis was 30 μM.

Microinjection Assay. Insect paralytic activity was evaluated by microinjection of early third-instar larvae (2−3 mg) *Spodoptera litura* (tobacco cutworm) (*28*). Larvae were

Table 1: Binding Parameters of Spider and Scorpion Toxins to Cockroach Neuronal Membranes[a]

toxin	K_d (nM)	B_{max} (pmol/mg)	competition against (K_i values in nM)		
			^{125}I-δ-PaluIT2	^{125}I-Bj-xtrIT	^{125}I-LqhαIT
δ-PaluIT1			13.8 ± 0.3	161 ± 64	≫1000
δ-sPaluIT2	1.6 ± 0.3	1.9 ± 0.2	1.3 ± 0.4	12.7 ± 3.2	≫1000
δ-PaluIT2	1.8 ± 0.2	2.1 ± 0.3	1.9 ± 0.3		
LqhαIT			≫10000		0.076 ± 0.005
δ-TxVIA			≫10000		
Bj-xtrIT			0.85 ± 0.2	0.36 ± 0.1	≫10000
LqhIT2			1.6 ± 0.3		≫10000

[a] Data are the mean ± SD of two to six experiments.

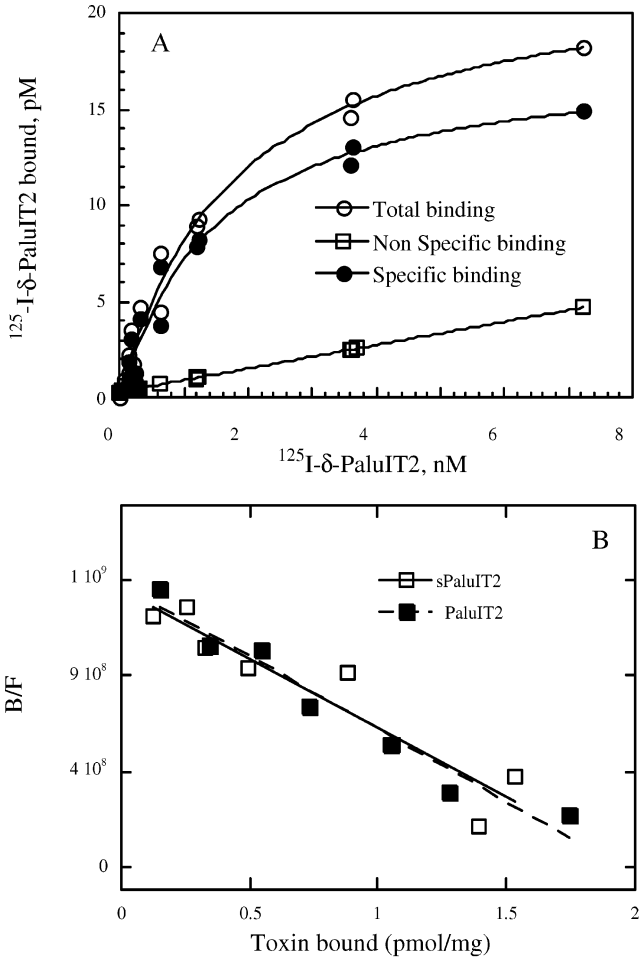

FIGURE 1: Binding of δ-palutoxins to *P. americana* neuronal membranes. (A) Hot saturation performed with ^{125}I-δ-PaluIT2. Cockroach neuronal membranes (14.7 μg/mL) were incubated for 60 min with increasing concentrations of ^{125}I-δ-PaluIT2 (0.1−7.2 nM). Identical curves were obtained with the synthetic toxin. The curves were analyzed using the program Kaleidagraph. (B) Scatchard representation of hot saturations performed by natural and synthetic ^{125}I-δ-PaluIT2. The program Ligand was used to analyze the data. The equilibrium binding parameters are $K_d = 1.8 \pm 0.2$ nM, $B_{max} = 2.1 \pm 0.25$ pmol/mg, and $n = 2$ and $K_d = 1.6 \pm 0.3$ nM and $B_{max} = 1.9 \pm 0.2$ pmol/mg for the natural and synthetic forms, respectively.

injected in the pronotum with up to 300 nL of peptide suspended in water using glass capillary pipets. They were then placed in 55 mm diameter plastic Petri dishes with artificial diet. Paralytic and lethal effects were observed at different time intervals up to 24 h. The LD_{50} was calculated using probit analysis with the PROBAN program.

Statistical Analysis. The Student *t*-test was used to determine whether statistically significant differences occurred between the mean values obtained in binding experiments or toxicity assays for the δ-palutoxin Ala analogues.

RESULTS

δ-Palutoxins Recognize Receptor Site 4 on Insect Sodium Channels. Due to the inhibitory effect of δ-palutoxins on the fast inactivation of cockroach NaChs (*20*) we further characterized δ-PaluIT2, the toxin variant with the highest affinity for neuronal membranes. Scatchard analysis of "hot saturation" binding experiments using native or synthetic ^{125}I-δ-PaluIT2 on cockroach neuronal membranes (Figure 1A) suggested a single binding site (Figure 1A,B) with site capacity similar to that reported for the binding sites of scorpion α- (e.g., LqhαIT) and β-toxins (e.g., Bj-xtrIT) in an identical neuronal preparation (*17*, *23*, *29*). The K_d of the synthetic ^{125}I-δ-sPaluIT2 was comparable to that of ^{125}I-δ-PaluIT2, and their K_i values, determined in competition with ^{125}I-δ-PaluIT2 on binding to cockroach synaptosomes (Table 1), indicated that iodination did not affect the biological activity of the toxin and that the synthetic δ-sPaluIT2 had binding properties similar to those of the native toxin.

The capability of δ-PaluIT2 to compete with toxins that induce a similar effect on channel inactivation upon binding to receptor site 3 or 6 was examined. ^{125}I-δ-PaluIT2 was subjected to competition against the site 3 scorpion toxins, LqhαIT (*21*) and Lqh3 (*30*), and against the site 6 conotoxin, δ-TxVIA, which is a toxin from the cone snail *Conus textil* that blocks the inactivation of the voltage-gated NaCh and could not be displaced by any of the known site 1−5 binders (*31*). Unexpectedly, none of the toxins that inhibit sodium current inactivation (LqhαIT, Lqh3, and δ-TxVIA) displaced ^{125}I-δ-PaluIT2 from its binding site (Figure 2A). On the other hand, two anti-insect selective β-toxins, Bj-xtrIT and LqhIT2, which bind to site 4 (*14*, *17*), inhibited the binding of ^{125}I-δ-PaluIT2 with K_i values of 0.85 and 1.6 nM, respectively (Figure 2A, Table 1). In reciprocal experiments, 10 μM δ-PaluIT2 did not displace ^{125}I-LqhαIT (Figure 2B, Table 1), whereas the binding of ^{125}I-Bj-xtrIT was inhibited by δ-PaluIT2 with a K_i value of 12.7 ± 3.2 nM (Figure 2C, Table 1). These results have clearly indicated that, despite their mode of action that resembles site 3 or 6 toxins, δ-palutoxins do in fact recognize receptor site 4 on insect NaChs.

Dissection of δ-PaluIT2 by Ala Scanning. To elucidate the molecular determinants involved in δ-PaluIT2 binding to receptor site 4 of insect NaChs, 25 Ala analogues were chemically synthesized. All amino acid residues in δ-PaluIT2 except Gly, Pro, Cys, and Ala were substituted. Although Gly and Pro could have a functional role, they have a high propensity to form reverse turns in high molecular weight

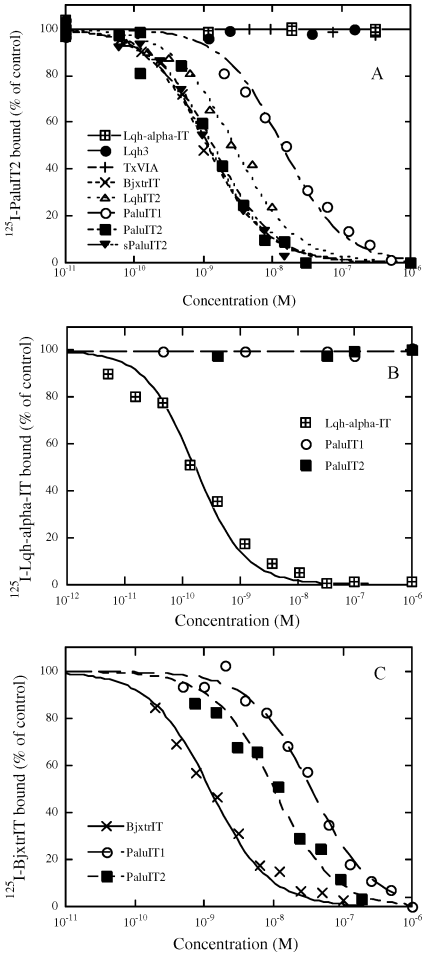

FIGURE 2: Identification of the receptor binding site of δ-palutoxins on the insect sodium channel. (A) Displacement of bound ^{125}I-δ-PaluIT2 by the site 3 toxins, Lqh3 and LqhαIT, the site 4 toxins, Bj-xtrIT and LqhIT2, the site 6 toxin, δ-TxVIA, and δ-palutoxins 1 and 2. Cockroach neuronal membranes (35 μg/mL) were incubated for 60 min at 22 °C with 112 pM ^{125}I-δ-PaluIT2 in the presence of increasing concentrations of toxins. The calculated K_i are ≫10 μM, $n = 2$; ≫10 μM, $n = 2$; 1.6 ± 0.3 nM, $n = 2$; 0.85 ± 0.2 nM, $n = 2$; 1.3 ± 0.4 nM, $n = 2$; 1.9 ± 0.3 nM, $n = 3$; and 13.8 ± 0.3 nM, $n = 3$ for LqhαIT, δ-TxVIA, LqhIT2, Bj-xtrIT, δ-sPaluIT2, δ-PaluIT2, and δ-PaluIT1, respectively. (B) Inhibition of binding of the site 3 scorpion toxin, ^{125}I-LqhαIT, by δ-palutoxins. Cockroach neuronal membranes (28 μg/mL) were incubated for 60 min at 22 °C with 85 pM ^{125}I-LqhαIT and increasing concentrations of toxins. The calculated K_i are 76 ± 5 pM for LqhαIT and more than 1 μM for δ-PaluIT1 and δ-PaluIT2. (C) Inhibition of binding of the site 4 scorpion toxin, ^{125}I-Bj-xtrIT, by δ-palutoxins. Cockroach neuronal membranes (32 μg/mL) were incubated for 60 min at 22 °C with 55 pM ^{125}I-Bj-xtrIT and increasing concentration of toxins. The calculated K_i are 0.36 ± 0.1 nM, $n = 5$; 12.7 ± 3.2 nM, $n = 6$; and 161.3 ± 64 nM, $n = 3$ for Bj-xtrIT, δ-PaluIT2, and δ-PaluIT1, respectively. Nonspecific binding in panels A, B, and C was determined in the presence of 1 μM δ-PaluIT2, 100 nM LqhαI, and 300 nM Bj-xtrIT and subtracted from all data points, respectively. The data points are fitted with the nonlinear Hill equation (Hill number of 1).

proteins (*32*) and are essential components of reverse turns in low molecular weight proteins folded according to the ICK motif (*33*). Thus, glycines and prolines, which were expected to have a structural role, were excluded from the Ala scanning. An additional δ-PaluIT2 analogue, named INV, in which the charge of positive and negative amino acid residues was inverted, was used to examine the effect of the entire charge of the toxin.

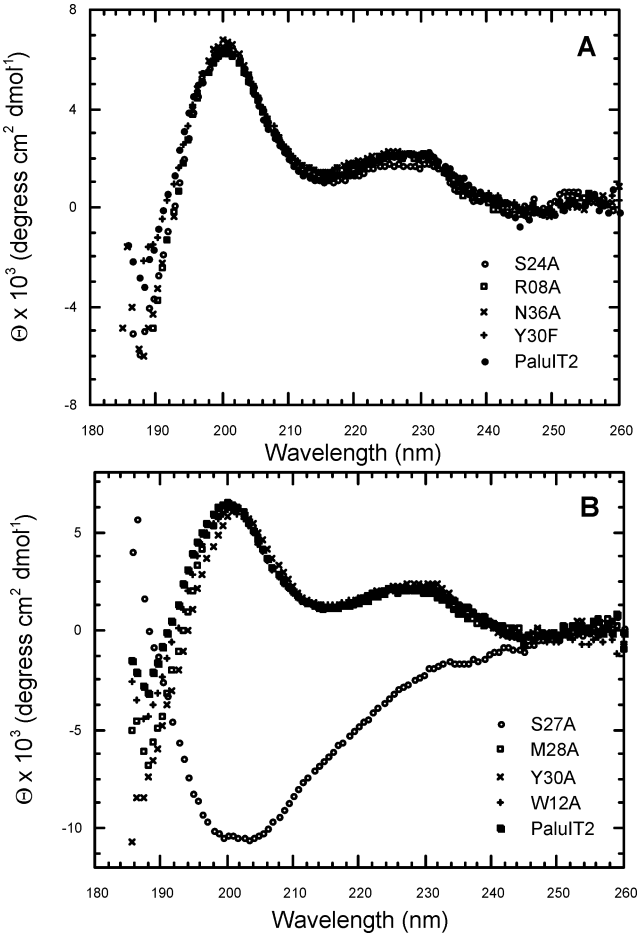

FIGURE 3: Circular dichroism comparison of the native δ-palutoxin IT2 with analogues S24A, R8A, N36A, and Y30F in (A) and with analogues S27A, M28A, Y30A, and W12A in (B).

The overall assembly and cleavage yield of all 25 synthetic peptides was 76% according to theoretical values of the peptidyl resin and the calculated mass increase for 0.1 mmol of peptide to give 315 mg of crude synthetic peptide. The final yield after chromatographic purification of the linear peptide and refolding was ca. 0.05−2.5%. Analogues V3A and W12A had the highest and lowest peptide yields, 2.5% and 0.05%, respectively.

The CD spectra of 21 out of 25 analogue toxins superimposed nicely with the CD spectrum of the native δ-PaluIT2 (Figure 3A), suggesting that their secondary structure was similar to that of the native δ-PaluIT2. Three substitutions (W12A, M28A, Y30A) caused a minor shift of the maximal peak in the range 200−205 nm (Figure 3B). Mutation Y30A revealed the largest shift in the maximal peak together with a small reduction in intensity. Substitutions M28A and W12A had a minor effect on the shift of the maximal peak with no effect on its intensity. The only substitution that caused a major change in the CD spectrum was S27A. Although the mass spectrometric analysis of S27A, before and after folding, indicated that all eight cysteine residues were oxidized into disulfide bridges, its CD spectrum differed markedly from that of the native δ-PaluIT2 (Figure 3B), suggesting improper folding or some structural alteration. Therefore, mutation S27A was excluded from the structure−function analysis. To examine the effect of Ala substitutions on toxin activity, each analogue was assayed for toxicity to

Table 2: Biological Activity of δ-PaluIT2 and Its Analogues

toxin variant	K_i(mut)/ K_i(native)[a]	LD_{50}(mut)/ LD_{50}(native)[b]	toxin variant	K_i(mut)/ K_i(native)[a]	LD_{50}(mut)/ LD_{50}(native)[b]	toxin variant	K_i(mut)/ K_i(native)[a]	LD_{50}(mut)/ LD_{50}(native)[b]
wild type	1	1	S13A	0.65	1.1	Y30A	58	5.1
V3A	1.1	0.9	Y16A	1.0	1.3	Y30F	16	1.4
D5A	0.76	0.9	D19A	0.87	2.9	R32A	24	2.3
Q7A	1.5	1	Y21A	4.2	1.4	R34A	18	1.7
R8A	51	1.3	Y22A	54	2.1	N35A	1.9	1.2
R8D	>80	5.0	S24A	40	1.8	N36A	2.0	1.2
S11A	1.8	1	R26A	16	1.7	S37A	1.0	1.1
W12A	>160	>8	M28A	14	1.6	INV[c]	>160	>8
W12F	13.5	1.2						

[a] K_i was determined on cockroach neuronal membrane preparations against ^{125}I-Bj-xtrIT. The K_i value of the wild type is 12.7 ± 3.2 nM (n = 6). [b] LD_{50} values were based on 50% mortality of injected insects (third-instar larvae of *S. litura*) 24 h postinjection, and they represent the mean of two independent experiments. The LD_{50} of the wild type is 25.2 ± 5.2 ng/100 mg body weight. [c] Analogue with inversion of all anionic and cationic amino acid residues (DRDRRR5,8,19,26,32,34RDRDDD).

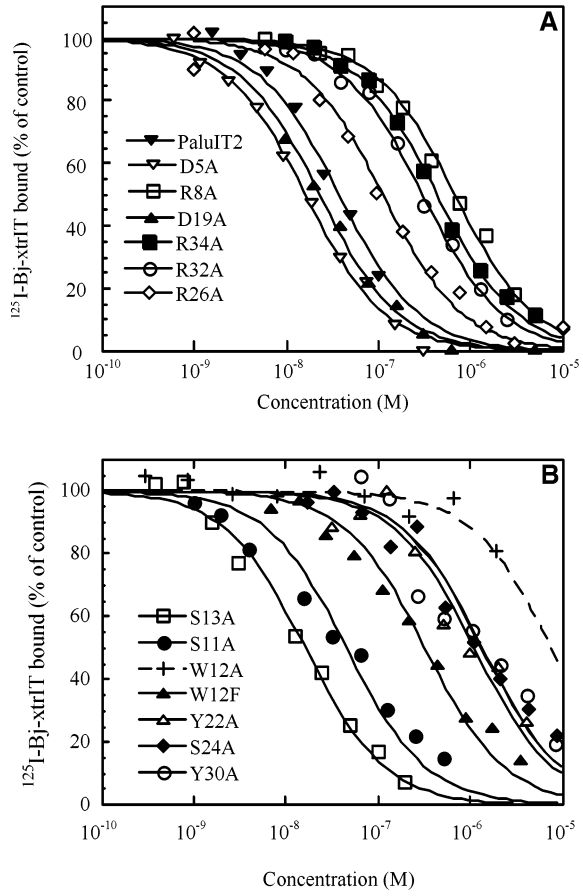

FIGURE 4: Competition assays for ^{125}I-Bj-xtrIT binding with various synthetic variants of δ-PaluIT2 (A and B). The same conditions were used as described in Figure 2, panel C.

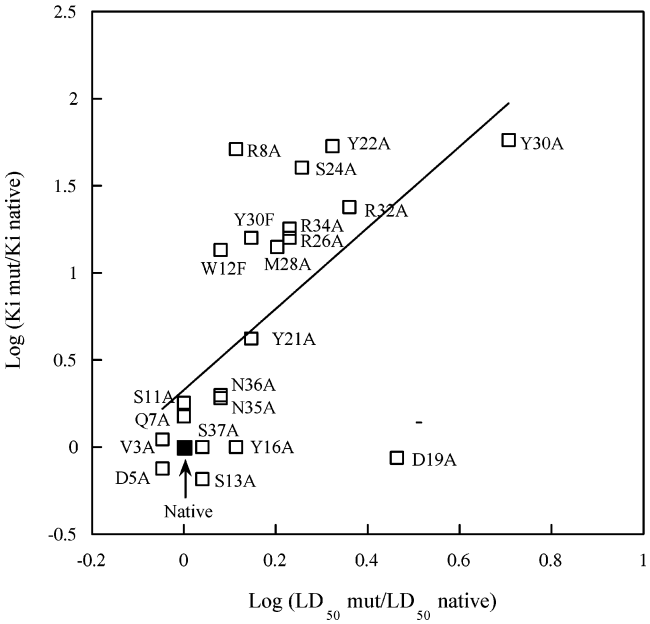

FIGURE 5: Correlation between the logarithmic values of K_i(mut)/ K_i(native) and LD_{50}(mut)/LD_{50}(native). Since the K_i(mut)/K_i(native) values of W12A, R8D, and INV are unknown, they are not represented.

lepidoptera larvae and binding properties on cockroach neuronal membranes.

Biological Activity of the δ-PaluIT2 Analogues. Since Bj-xtrIT is a suitable probe for site 4 of insect NaChs (*17*), the inhibition constant (K_i) values of δ-PaluIT2 analogues were calculated from competition binding experiments against ^{125}I-Bj-xtrIT using cockroach neuronal membranes (Figure 4A,B, Table 2). A level of significance of 0.0005 was used to narrow the number of amino acid residues critical for binding to the insect NaCh. Analogues with nonsignificant differences ($p > 0.0005$) in inhibition constants were V3A, D5A, Q7A, S11A, S13A, Y16A, D19A, Y21, N35A, and N36A. These analogues had K_i values within a range of 1−4-fold of that of the native toxin. Significant differences ($p < 0.0005$) were observed for R8A, W12A, Y22A, S24A, R26A, M28A, Y30A, R32A, and R34A (Table 2). The importance of positive charges was verified by their inversion: the binding affinity of R8D dramatically decreased and the INV analogue, in which all positive charges were inverted, lost completely the activity. In addition, the importance of the aromatic side chains at positions 12 and 30 was confirmed by conservative substitutions. W12F regained activity ca. 10-fold compared to W12A, and analogue Y30F was 4-fold more active than Y30A.

The 25 analogues were also injected into *S. litura* larvae to assess their biological activity in vivo. The LD_{50} values of δ-PaluIT2 analogues ranged from a minimum of 22 ng/mg body weight to more than 200 ng/mg of lepidoptera larvae (Table 2). On the basis of the insecticidal activity of native δ-PaluIT2 (25.2 ng/mg body weight), substitutions V3A, D5A, Q7A, R8A, S11A, W12F, S13A, Y16A, Y21A, Y22A, S24A, R26A, M28A, Y30F, R34A, N35A, and N36A had an insignificant effect ($p > 0.02$). Therefore, these residues appear to have low relevance to the insecticidal activity on lepidoptera larvae. The substitutions that signifi-

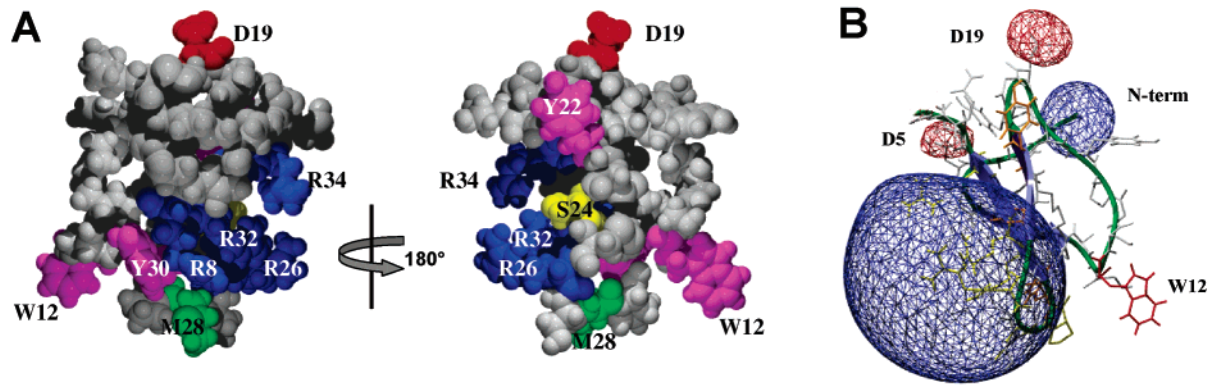

FIGURE 6: Structural model of δ-PaluIT2 (PDB code 1V91). Ball and stick representation (A). Amino acid residues are colored according to their chemical nature: aliphatic, green; aromatic, magenta; negative, red; polar, yellow; positive, blue. Electrostatic surface potential (B). The blue net shows the positive surface, and the red net shows the negative surface. Calculation of electrostatic potentials was carried out using the program DeepView (*40*). Estimation of model reliability, calculation of electrostatic potentials, and model exploration were carried out using DeepView.

cantly affected the insecticidal activity of δ-PaluIT2 ($p < 0.02$) were R8D, W12A, D19A, Y30A, R32A, and INV.

A correlation between the logarithmic values of K_i(mut)/K_i(native) and LD_{50}(mut)/LD_{50}(native) was created to observe graphically the substitutions that affected δ-PaluIT2 (Figure 5). Interestingly, analogue D19A decreased the toxicity with no effect on the binding affinity. Such unusual uncoupling between toxicity and binding was recently demonstrated in the excitatory toxin Bj-xtrIT when the negatively charged Glu-15 was substituted by Ala (*34*).

DISCUSSION

The ability of spider δ-palutoxins to compete with scorpion β-toxins on binding to an identical receptor site on NaChs is intriguing in light of the significant difference in their structure. Not only do these toxins provide another example for convergent evolution, clarification of the molecular basis of this ability may shed new light on the common receptor binding site on the NaCh. We approached this question by molecular dissection of the spider δ-PaluIT2 and comparison of its bioactive surface to that of the scorpion β-toxin Bj-xtrIT.

δ-Palutoxins differ structurally from scorpion β-toxins, but both compete on binding to receptor site 4. Upon injection of δ-PaluIT2 to lepidopteran larvae, paralysis develops slowly and is accompanied by muscle spasm and loss of water through the body wall. The insects then become flaccid and dry out. Although the toxic symptoms resemble those induced by scorpion depressant toxins, which bind to site 4 and affect the activation of insect NaChs (*35*), the effect of δ-PaluIT2 on a cockroach axon (*20*) is typical of that induced by scorpion α-toxins, which bind to receptor site 3 (*21*). This unexpected phenomenon motivated us to analyze the ability of δ-PaluIT2 to compete with various α- and β-toxins on binding to the insect NaChs. This analysis clearly indicated that the effect on cockroach axon, i.e., inhibition of sodium current inactivation (*20*), did not result from binding to receptor site 3 (see Figure 2B). The competition with Bj-xtrIT indicated that δ-PaluIT2 bound to receptor site 4 of the insect NaCh. Thus far, only scorpion β-toxins have been shown to bind receptor site 4 on insect NaChs. These toxins are divided into several groups, three of which are represented by the excitatory toxins, Bj-xtrIT and AahIT, the depressant toxin, LqhIT2, and the β-toxins active on both insect and mammalian NaChs, such as Lqhβ-1. All of these scorpion toxins have a similar α/β-scaffold cross-linked by four disulfide bridges (*13*, *24*, *36*, *37*) and compete with Bj-xtrIT for binding to receptor site 4 on cockroach neuronal membranes (*17*, *38*).

The Bioactive Surface of δ-PaluIT2 and Similarities to That of Scorpion β-Toxins. The bioactive surface of δ-PaluIT2 is discontinuous and consists of a main cluster of amino acid residues (Arg-8, Tyr-22, Ser-24, Arg-26, Met-28, Tyr-30, Arg-32, and Arg-34) and Trp-12 14.9 Å apart (Figure 6A). Together, the total surface area of these residues is 1168.5 $Å^2$, which is in the range of "small" interface areas proposed for protein−protein interactions (*39*). Most bioactive residues appear on the surface constituted by the antiparallel strands of the β-sheet. The four Arg residues at the bioactive surface seem to contribute significantly to the electrostatic potential of the toxin (Figure 6B), which may be important for approaching the receptor site. Most interesting is the putative role of Asp-19 located in the first β-turn. Its substitution (D19A) had a prominent effect on the activity but strikingly no effect on the binding affinity to cockroach synaptosomes (Table 2). A similar uncoupling of activity from binding has been recently shown upon substitution of Glu-15 in the scorpion excitatory toxin, Bj-xtrIT, and was explained in that Glu-15 is not involved in binding but rather in voltage sensor trapping upon activation of the NaCh (*34*). Therefore, we assume that Asp-19 in δ-PaluIT2 may function similarly and, hence, be part of the bioactive surface.

The bioactive surface of the excitatory toxin, Bj-xtrIT, was recently described (*29*). Its comparison with that of δ-PaluIT2 highlights a hydrophobic region containing a number of amino acid residues that seem to orient similarly on the molecule surface (Figure 7). Taking Asp-19 in δ-PaluIT2, and Glu-15 in Bj-xtrIT as a point of reference, their distance from the other functional residues on the toxin surface is similar. Asp-19 is 9.6 Å in distance from Tyr-22 or 27.2 Å from Trp-12; similarly, Glu-15 is 8.8 Å in distance from Tyr-26 or 25.8 Å from Ile-73 (Figure 7). The hydrophobic amino acid cluster at the C-tail of Bj-xtrIT (Val-71, Ile-73, and Ile74), which was suggested to confer the specificity for insects (*24*), superimposes with the hydrophobic region of δ-PaluIT2 (Met-28, Tyr-30, and Trp-12). Thus, it is

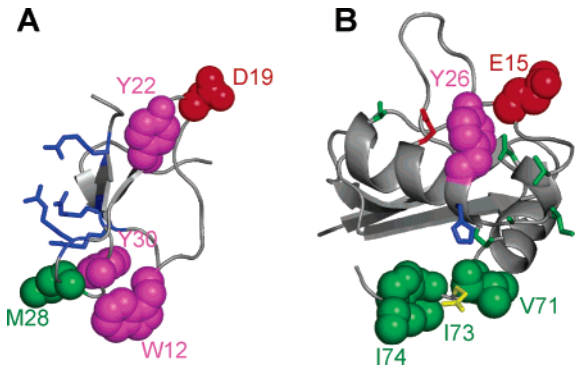

FIGURE 7: Commonality in the bioactive surfaces of δ-PaluIT2 (A) and Bj-xtrIT (B). Amino acid residues with similar spatial orientation are shown. Bj-xtrIT is PDB code 1bcg, and δ-PaluIT2 is PDB code 1V91. The three-dimensional model of δ-PaluIT2 and Bj-xrtIT was generated with the DeepView program (Swiss-PDB Viewer, http://www.expasy.ch/spdv/) (*40*). Amino acid residues are colored according to their chemical nature: aliphatic, green; aromatic, magenta; negative, red; polar, yellow; positive, blue.

conceivable that these bioactive residues in δ-PaluIT2 may be part of the hydrophobic/aromatic region equivalent to the C-tail region in Bj-xtrIT, and hence, it may be involved in the insecticidial activity of both toxins. The spatial resemblance of key residues in the bioactive surface of both toxins may explain their ability to displace one another from insect receptor site 4. Yet, other components of the bioactive surface, which differ mainly in charge between δ-PaluIT2 and Bj-xtrIT, expand our perspective of this receptor site, which until now could be envisioned as a mirror face to the bioactive surface of scorpion β-toxins. Not only do the differences between the bioactive surfaces of the spider and scorpion toxins suggest that receptor site 4 is an extended macrosite, they also show that the inhibitory effect of channel inactivation is obtainable upon binding to a site considered to be related to effects on channel activation. Therefore, the definition of toxins according to their mode of action should be reconsidered and perhaps rely on binding competition assays.

All in all, the unique features and small size of δ-palutoxins may be useful in further studies aiming to unravel receptor site 4 on insect NaChs and understand how this site is linked to the mechanism of NaCh inactivation.

ACKNOWLEDGMENT

We are very grateful to Ms. Maeda for insect rearing, to Dr. H. Minakata for advice during peptide synthesis, and to Drs. H. Darbon and G. Ferrat for providing the coordinates of δ-PaluIT2.

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BI048434K