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Genetic Polymorphism and Expression of a Highly Potent Scorpion Depressant Toxin Enable Refinement of the Effects on Insect Na Channels and Illuminate the Key Role of Asn-58†

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ABSTRACT: We isolated from the venom of the scorpion *Leiurus quinquestriatus hebraeus* an extremely active anti-insect selective depressant toxin, Lqh-dprIT$_3$. Cloning of Lqh-dprIT$_3$ revealed a gene family encoding eight putative polypeptide variants (a−h) differing at three positions (37A/G, 50D/E, and 58N/D). All eight toxin variants were expressed in a functional form, and their toxicity to blowfly larvae, binding affinity for cockroach neuronal membranes, and CD spectra were compared. This analysis links Asn-58, which appears in variants a−d, to a toxin conformation associated with high binding affinity for insect sodium channels. Variants e−h, bearing Asp-58, exhibit a different conformation and are less potent. The importance of Asn-58, which is conserved in other depressant toxins, was further validated by construction and analysis of an N58D mutant of the well-characterized depressant toxin, LqhIT$_2$. Current and voltage clamp assays using the cockroach giant axon have shown that despite the vast difference in potency, the two types of Lqh-dprIT$_3$ variants (represented by Lqh-dprIT$_3$-a and Lqh-dprIT$_3$-e) are capable of blocking the action potentials (manifested as flaccid paralysis in blowfly larvae) and shift the voltage dependence of activation to more negative values, which typify the action of β-toxins. Moreover, the stronger and faster shift in voltage dependence of activation and lack of tail currents observed in the presence of Lqh-dprIT$_3$-a suggest an extremely efficient trapping of the voltage sensor compared to that of Lqh-dprIT$_3$-e. The current clamp assays revealed that repetitive firing of the axon, which is reflected in contraction paralysis of blowfly larvae, can be obtained with either the less potent Lqh-dprIT$_3$-e or the highly potent Lqh-dprIT$_3$-a at more negative membrane potentials. Thus, the contraction symptoms in flies are likely to be dominated by the resting potential of neuronal membranes. This study clarifies the electrophysiological basis of the complex symptoms induced by scorpion depressant toxins in insects, and highlights for the first time molecular features involved in their activity.

Scorpion toxins that modulate the gating properties of voltage-gated sodium channels (NaChs) are 61−76-residue polypeptides that have greatly diversified in sequence, yet they share a spatially conserved scaffold cross-linked by four disulfide bonds (*1*−*4*). They are traditionally divided into two major classes, α and β, according to their mode of action and binding properties (*3*, *5*). α-Toxins inhibit the inactivation of the sodium current by binding to receptor site 3 assigned mainly to domain 4 of the channel pore-forming protein (*6*, *7*). β-Toxins bind to receptor site 4 assigned to domain 2 in the channel and shift the activation of sodium channels to more negative membrane potentials (*8*). Most α- and β-toxins affect mammalian and insect sodium channels with various affinities. However, two additional distinct toxin groups of the β-class, excitatory and depressant, affect selectively sodium channels of insects (*3*, *9*−*11*). Both groups induce apparent opposite symptoms on blowfly larvae (*9*, *12*) and bind to distinct yet closely related receptor sites (*13*).

Scorpion depressant toxins, typified by the symptoms they produce in blowfly larvae, comprise a large group of polypeptides, some of which have been characterized [e.g., BjIT$_2$ (*12*), LqhIT$_2$ (*14*, *15*), BaIT$_2$ (*16*), Bs-dprIT$_{1-4}$ (*17*), BotIT$_4$ and BotIT$_5$ (*18*), LqqIT$_2$ (*10*, *19*), and AaIT$_5$ (*20*)] and a few of which have been cloned (*4*). Immediately upon injection, they induce a transient phase of contraction of body musculature followed by an increased progressive flaccid paralysis, a dual effect that also has been described on a

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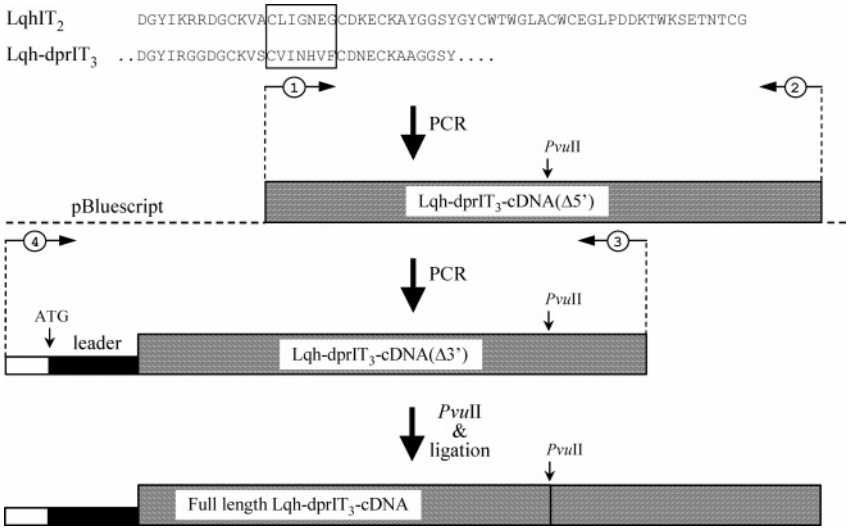

FIGURE 1: Cloning strategy of Lqh-dprIT$_3$. A degenerate oligonucleotide (primer 1), designed according to a toxin region that differed mostly from that of LqhIT$_2$ (boxed), was reacted via PCR with oligo-dT (primer 2) using *L. quinquestriatus hebraeus* cDNA library [in pBluescript (*25*, *26*)] as the template DNA to yield the 3′ half of Lqh-dprIT$_3$. On the basis of the new sequence, primer 3, designed according to a region that differed from its LqhIT$_2$ equivalent, was reacted via PCR with the KS primer of pBluescript using once more the *L. quinquestriatus hebraeus* cDNA library. This PCR product consisted of the 5′ half of Lqh-dprIT$_3$. Both PCR products were ligated at a unique *Pvu*II site to yield the full-length cDNA of Lqh-dprIT$_3$, which included the 5′ noncoding region, the region encoding the leader sequence, and the rest of the gene.

prepupal housefly neuromuscular preparation (*14*) and an isolated cockroach axon (*15*). In the axon, a progressive depolarization of the membrane, which initially produces a short burst of repetitive firing, proceeds into a block of the action potentials (*15*). Although the dual, opposing effects could be easily explained by assuming toxin recognition of two receptor sites (*14*), binding studies using the depressant toxins LqhIT$_2$ (*13*) and BaIT$_2$ (*16*) have revealed only one high-affinity receptor site on insect sodium channels, whereas another high-capacity site, to which LqhIT$_2$ binds with low affinity, has been shown to be unrelated to sodium channels (*13*, *21*). Although the insecticidal potency of depressant toxins is promising (*22*, *23*), and may be useful for studying structural features that are important for toxin specificity for insects, no information about their active site is available. Here we describe the isolation of a highly insecticidal depressant toxin, cloning of which revealed a gene family. Functional expression of all members of this gene family highlighted the importance of Asn-58 for toxin structure and activity, and shed light on the mechanism of action of depressant toxins.

EXPERIMENTAL PROCEDURES

Biological Material. *Leiurus quinquestriatus hebraeus* scorpions were collected at the Judaean desert of Israel, and their venom was collected by allowing them to sting a Parafilm membrane. *Sarcophaga falculata* blowfly larvae and *Periplaneta americana* cockroaches were bred in the laboratory. Albino laboratory ICR mice were purchased from the Levenstein farm in Yokneam. *Escherichia coli* DH5α and BL21 strains were used for plasmid construction and expression, respectively. The translational vector used, pET-11cK, is a derivative of pET-11c (*24*) bearing a kanamycin resistance gene.

Purification of Lqh-dprIT$_3$. Crude venom (7.7 mg) from 35 scorpions was lyophilized, dissolved in 3 mL of 10 mM ammonium acetate (pH 6.7), and subjected to three successive chromatographic steps. Anion-exchange chromatography on a 0.8 mL Sephadex-DEAE column (Sigma) equilibrated in 10 mM ammonium acetate (pH 8.0) was carried out. A linear gradient of 0.01 to 1.0 M ammonium acetate (pH 8.0) at a flow rate of 0.5 mL/min at room temperature was applied, and 1.5 mL aliquots were collected and lyophilized. Several fractions eluted by 10 mM ammonium acetate were found to be toxic to blowfly larvae, and one of them revealed an extremely high flaccid activity. This fraction was further purified by HPLC using a 3 mL RPC-Resource column (Pharmacia). The sample was loaded in 0.1% acetonitrile (buffer A), and a stepwise elution was conducted at 3 mL/min using a gradient of acetonitrile in 0.1 M TFA (buffer B): from 0.1 to 18% for 3 min, from 18 to 35% for 16 min, and from 35 to 100% for 5 min. A fraction with depressant type activity was eluted after 17 min and was further purified on an analytical Vydac C$_{18}$-RP-HPLC column (250 mm × 10 mm). Loading was carried out in 0.1% TFA, and elution at 1 mL/min was carried out with the same gradient described above: from 0.1 to 25% for 5 min, from 25 to 32% for 25 min, and from 32 to 100% for 5 min. The peak obtained after 30 min was highly active and contained a toxin, Lqh-dprIT$_3$, whose N-terminal sequence was determined by automated Edman degradation using an Applied Biosystem gas-phase sequencer connected to its corresponding PTH analyzer and data system.

Cloning of the Lqh-dprIT$_3$ Gene Family. Fifteen venom gland segments (telsons) were ground for RNA purification. Isolation of poly(A)$^+$ RNA and cDNA synthesis were performed as previously described (*25*, *26*). Fifty nanograms of cDNA was cloned into the *Sma*I polylinker site of pBluescript phagemid (Stratagene) to create a library in *E. coli* strain DH5α. Isolation of Lqh-dprIT$_3$ cDNA was achieved in several steps (Figure 1). A degenerate oligonucleotide (5′-TGC/T GTA/C/T/G ATA/C/T AAC/T CAC/T GTA/C/T/G TT-3′; primer 1 in Figure 1) was designed according to the determined N-terminal amino acid sequence

at a region of least similarity to the sequence of the most abundant depressant toxin in this scorpion, $LqhIT_2$ (*14*, *27*; Figure 1). This primer was reacted with primer 2 (5′-ATTCCTGCAGCCCTTTTTTT-3′) in a thermocycler (MJ Research) using the cDNA library as template DNA. Reaction conditions were as follows: 30 cycles of 1 min at 94 °C, 1 min at 50 °C, and 1 min at 72 °C. The PCR product, a 5′-truncated Lqh-dprIT3 cDNA, was blunt-ended, phosphorylated, cloned into the *Sma*I site of pBluescript, and subjected to sequence analysis using Sequenase II (United States Biochemicals). Primer 3 (5′-CGGGATCCCTATGAGGGTATTATTTGG-3′) was designed according to the sequence of the PCR product at a region encoding a *Pvu*II restriction site in reverse orientation and according to the noncoding strand. This primer was reacted with primer 4 (KS, Stratagene) using the entire cDNA library as a template to yield a 3′-truncated Lqh-dprIT$_3$ cDNA (Figure 1). Both truncated clones were combined in the overlapping *Pvu*II site, thus providing a full-length Lqh-dprIT$_3$ cDNA. Ten various cDNA clones encoding Lqh-dprIT$_3$ were pulled out of the cDNA library by colony hybridization (*28*) using Lqh-dprIT$_3$ cDNA as a probe under stringent hybridization conditions.

Expression and Functional Reconstitution. Oligonucleotide primers were used to reconstruct via PCR the termini of all eight cDNA clones of Lqh-dprIT$_3$. Primer 5 (5′-GGGAATTCCATATGGACGGATATATAAGAGGAGG-3′) was designed to (i) remove sequences encoding the putative leader, (ii) create an additional codon for methionine at position −1 of the mature polypeptide, and (iii) provide an *Nde*I restriction site (underlined) juxtaposed to the ATG start codon. Primer 6 (5′-GCGGATCCTTAACCGCATGTATCGGTTTC-3′), designed beforehand to contain a *Bam*HI restriction site, was used for reconstruction of the 3′ end. The PCR products were cloned into the corresponding *Nde*I and *Bam*HI sites in vector pET-11cK (*29*). Expression and in vitro folding were performed as described for depressant toxin $LqhIT_2$ (*27*).

Toxicity Assays. Four-day-old blowfly larvae (∼100 mg body weight) were injected intersegmentally at the rear side. A positive result was scored when a characteristic paralysis (transient immobilization and contraction replaced by gradually increasing flaccidity) was obtained and lasted for at least 15 min. Ten larvae were injected with five concentrations of each toxin in three independent experiments. ED_{50} (50% effective dose**)** values were calculated according to the sampling and estimation method of Reed and Muench (*30*). Female mice (20 g) were injected subcutaneously with toxin amounts of up to 0.2 mg.

Competition Binding Experiments. (1) Neuronal Membrane Preparations. Insect synaptosomes were prepared from heads of adult cockroaches (*P. americana*) according to a procedure described previously (*11*, *31*). The membrane protein concentration was determined using a Bio-Rad protein assay, with BSA as a standard. (2) Radioiodination. Bj-xtrIT and $LqhIT_2$ were radioiodinated by Iodogen (Pierce Chemical Co., Rockford, IL) using 5 μg of toxin and 0.5 mCi of carrier-free $Na^{125}I$, and the monoiodotoxins were purified using a Vydac RP-C_{18} column as described previously (*13*, *31*). The concentration of radiolabeled toxins was determined according to the specific activity of ^{125}I, ranging between 4200 and 3450 dpm/fmol of monoiodotoxin. (3) Binding Assays. Equilibrium competition assays were performed using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the radioactive toxin. The standard binding medium consisted of 135 mM choline chloride, 1.8 mM $CaCl_2$, 5.5 mM KCl, 0.8 mM $MgSO_4$, 50 mM HEPES (pH 7.3), 10 mM glucose, and 2 mg/mL BSA. The washing buffer was similar except for 5 mg/mL BSA and no glucose. Insect synaptosomes (10−14 mg/mL) were suspended in 0.2 mL of binding buffer containing [^{125}I]Bj-xtrIT. After incubation for 1 h at 22 °C, the reaction was terminated by rapidly filtering the solution through a GF/C filter (Whatman) under vacuum (*31*).

Electrophysiological Measurements. Recordings in current clamp and voltage clamp conditions were carried out on cockroach isolated giant axons using the double oil-gap single-fiber technique (*32*). Axon isolation and the recording technique were previously described in detail (*33*). Experiments were performed at room temperature (18−20 °C). The isolated axon was superfused with physiological saline (buffered with 1 mM HEPES to pH 7.2) containing 210 mM NaCl, 3.1 mM KCl, 5.2 mM $MgCl_2$, and 5.4 mM $CaCl_2$. Potassium currents were blocked under voltage clamp conditions by superfusion with 1.0 mM 3,4-diaminopyridine (Sigma), and when necessary, sodium currents were suppressed by 0.1 μM tetrodotoxin (Sigma). Lyophilized toxins were dissolved, prior to the assay, in normal saline in the presence of 0.25 mg/mL BSA. Fibers, providing action potentials of at least 95 mV, were used under current clamp conditions. Action potentials were evoked every 5 s by passing short (0.5 ms) current pulses (approximately 10 nA).

Circular Dichroism Spectroscopy. CD spectra were recorded at 25 °C using a model 202 circular dichroism spectrophotometer (Aviv Instruments, Lakewood, NJ). Toxins (140 μM) were dissolved in 5 mM sodium phosphate buffer (pH 7.0), and their spectra (190−260 nm) were measured three times using a 0.1 mm path length quartz cuvette. The blank spectrum of the buffer was determined under identical conditions and subtracted from each toxin spectrum.

RESULTS

Isolation and Characterization of Lqh-dprIT$_3$. Judicious fractionation of *L. quinquestriatus hebraeus* crude venom by traditional methods (see Experimental Procedures) revealed a minute amount of a toxin (molecular mass of 6677 Da) that was highly active on insects. Toxicity assays on blowfly larvae provided typical poisoning symptoms normally obtained with depressant toxins, i.e., an immediate transient contraction paralysis that briefly turns into a gradually increasing flaccidity and immobilization (*14*). Strikingly, the activity of the new toxin on insects was ∼10-fold higher (ED_{50} = 3−5 ng/100 mg body weight) compared to those of other known depressant toxins (*10*, *12*, *14*, *16*−*19*, *34*). On the basis of its effect and the similarity to the sequence of 30 amino acid residues of the N-terminal region of the depressant toxin $LqhIT_2$ (Figure 1; *14*), the new toxin was named Lqh-dprIT$_3$. Since Lqh-dprIT$_3$ was found in a relatively small amount in the venom of *L. quinquestriatus hebraeus* compared to the amounts of other depressant toxins, further analysis for understanding the basis of its high potency required cloning and functional expression.

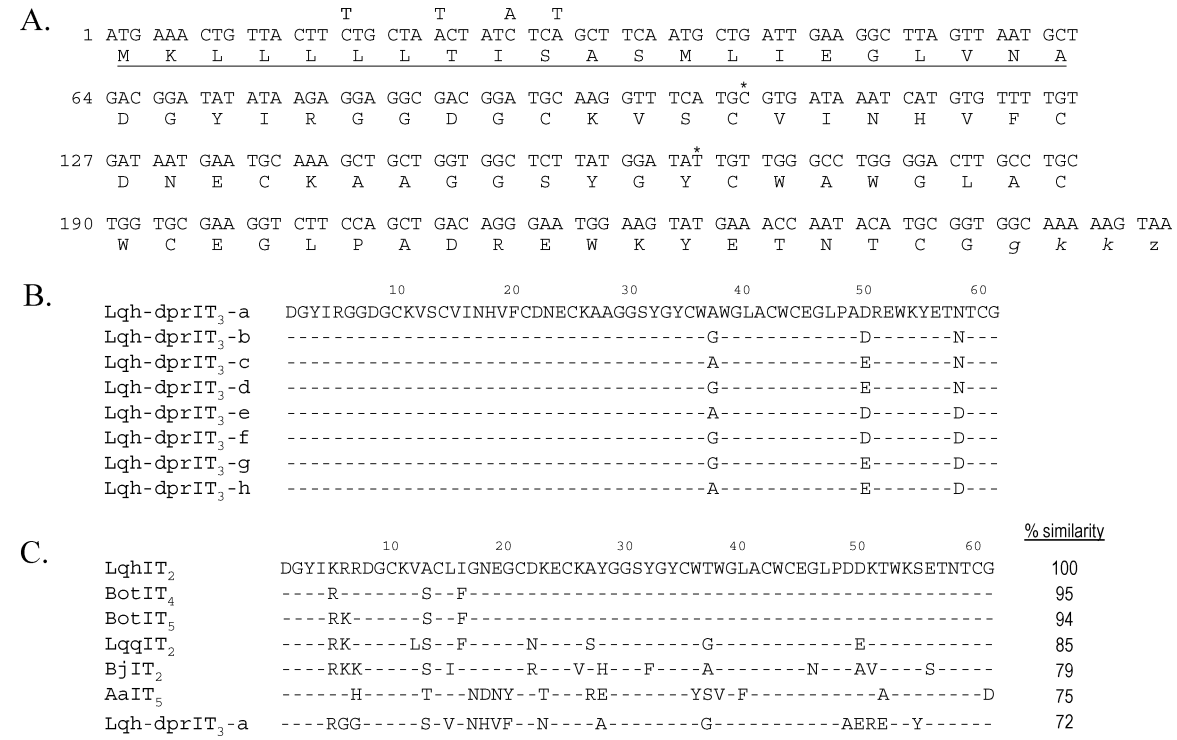

FIGURE 2: Genetic polymorphism of Lqh-dprIT$_3$. (A) Nucleotide and protein sequences of Lqh-dprIT$_3$. The putative leader is underlined. C-Tail residues that most likely are processed off post-translationally are designated by lowercase letters in italics. (B) Sequences of eight Lqh-dprIT$_3$ variants deduced from the nucleotide sequences of the isolated cDNA clones, which were pulled out from the cDNA library by colony hybridization (*28*) using Lqh-dprIT$_3$-cDNA(Δ5′) as a probe. (C) Sequence comparison among a variety of scorpion depressant toxins: LqhIT$_2$ from *L. quinquestriatus hebraeus* (*14*), BotIT$_4$ and BotIT$_5$ from *Buthus occitanus tunetanus* (*18*), LqqIT$_2$ from *Leiurus quinquestriatus quinquestriatus* (*19*), Bj from *Buthotus judaicus* (*26*), and AaIT$_5$ from *Androctonus australis hector* (*20*).

Lqh-dprIT$_3$ Is Encoded by a Gene Family Whose Products Vary in Activity. Ten distinct cDNA clones encoding Lqh-dprIT$_3$ were isolated from a cDNA library using PCR and colony hybridization techniques (Figure 2A). Interestingly, the deduced amino acid sequences varied at only three positions (37A/G, 50D/E, and 58N/D), providing a total of eight different toxin variants (Figure 2B). Each of these toxin variants was overexpressed in *E. coli*, and the resulting nonsoluble protein was denatured and then folded in vitro using an established protocol (*27*). Bioactive isoforms were purified using a C_{18}-RP-HPLC column, and their toxicity and K_i values were determined on blowfly larvae and cockroach neuronal membranes, respectively (Table 1). The native Lqh-dprIT$_3$ and recombinant toxin variants a−d, bearing Asn at position 58, were highly toxic to insects and generated in small doses (3 ng/100 mg larva) contraction paralysis, which subsequently developed into complete flaccidity. This high potency differs from that obtained with LqhIT$_2$, where 20 and 40 ng per 100 mg larva induce contraction and flaccid paralysis, respectively. Lqh-dprIT$_3$ variants a−d competed well with the formerly characterized excitatory and depressant toxins, [^{125}I]Bj-xtrIT (Figure 4 and Table 1) and [^{125}I]LqhIT$_2$ (not shown), respectively, on binding to the receptor site in cockroach NaChs, as was shown for other depressant toxins (*11*, *13*, *35*). The four toxin variants with Asp at position 58, Lqh-dprIT$_3$-e−h, were less active, being 21-fold weaker in their ability to induce flaccidity in blowfly larvae but only 5-fold weaker in their ability to provoke contraction paralysis (Table 1). This reduced potency correlated with a 2−3 order of magnitude decrease in their apparent binding affinity for the cockroach NaCh receptor site (Figure 4 and Table 1). The differences in toxicity and K_i values between the two groups of toxin variants may be attributed to the different systems used for assays. Analysis of all eight toxin variants by circular dichroism (CD) spectroscopy indicated a clear difference between the group of variants a−d, bearing Asn-58, and the group of variants e−h, bearing Asp-58 (Figure 3), which seemed to correlate with the striking difference in toxin potency.

Table 1: Activity of Lqh-dprIT$_3$ Variants[a]

toxin	ED$_{50}$ (ng/100 mg) contraction paralysis	ED$_{50}$ (ng/100 mg) flaccid paralysis	K_i (nM)
Lqh-dprIT$_3$-a	3−5	3−5	0.14 ± 0.04[b]
Lqh-dprIT$_3$-b	3−5	3−5	0.14 ± 0.05
Lqh-dprIT$_3$-c	3−5	3−5	0.13 ± 0.02
Lqh-dprIT$_3$-d	3−5	3−5	0.2 ± 0.05
Lqh-dprIT$_3$-e	19	85	116 ± 10
Lqh-dprIT$_3$-f	19	85	287 ± 50
Lqh-dprIT$_3$-g	19	85	488 ± 80
Lqh-dprIT$_3$-h	19	85	60 ± 10
LqhIT$_2$	20	40	0.7 ± 0.1
Bj-xtrIT	13	NE[c]	0.17 ± 0.03
LqhIT$_2$-N58D	750	>5500	696 ± 38

[a] The 50% effective dose (ED$_{50}$) was determined by injection of *Sarcophaga* larvae. The K_i values were determined in binding assays using *P. americana* neuronal membranes (see Experimental Procedures). LqhIT$_2$ is a well-characterized depressant toxin (*12*, *15*), and Bj-xtrIT is a well-characterized excitatory toxin (*11*, *55*) used here as the radiolabeled ligand (see Experimental Procedures). [b] Mean ± standard error of at least three independent experiments. [c] Not effective.

To assess whether Asn-58 has a similar role in other depressant toxins, we substituted it in the classical depressant toxin, LqhIT$_2$. Mutant N58D of LqhIT$_2$ was able to induce contraction paralysis of blowfly larvae in doses >37-fold

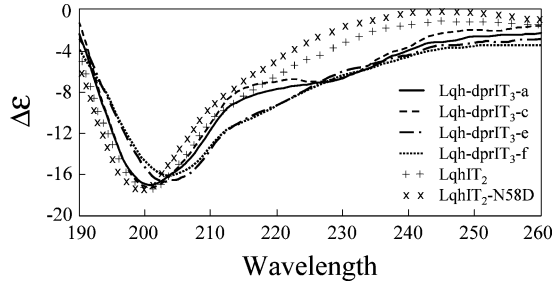

FIGURE 3: Circular dichroism spectra of Lqh-dprIT$_3$ variants and LqhIT$_2$. The spectra presented are of Lqh-dprIT$_3$-a and Lqh-dprIT$_3$-c, representing the a−d group, and Lqh-dprIT$_3$-e and Lqh-dprIT$_3$-f, representing the e−h group. The spectrum of LqhIT$_2$ is shown for comparison.

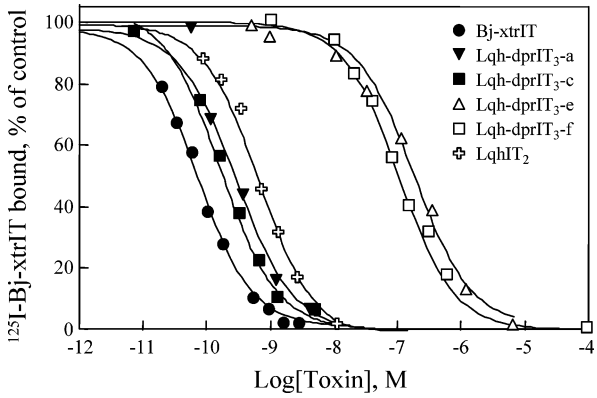

FIGURE 4: Competitive displacement of [^{125}I]Bj-xtrIT by Lqh-dprIT$_3$ variant representatives and by LqhIT$_2$ from cockroach sodium channels. Cockroach neuronal membranes (16 μg/mL) were incubated for 60 min at 22 °C with 180 pM [^{125}I]Bj-xtrIT and increasing concentrations of the indicated toxins. Nonspecific binding, determined in the presence of 1 μM Bj-xtrIT, was subtracted. The amount of bound ^{125}I-labeled toxin is provided as a percentage of the maximal specific binding without a competitor. The competition curves were analyzed using the nonlinear fit of the Hill equation (with a Hill coefficient of 1) for determining the IC_{50} values (see Experimental Procedures). The data points are means of two to three measurements from representative experiments. The K_i values are listed in Table 1.

larger than that of the unmodified toxin, and could hardly induce flaccidity even at ∼5500 ng/100 mg larva. In parallel, the binding affinity of this mutant for cockroach sodium channels decreased almost 1000-fold (Table 1). The drop in activity of this mutant correlated with a change in the CD spectrum, which, however, was different from those of Lqh-dprIT$_3$ variants e−h or variants a−d (Figure 3).

Electrophysiological Properties of Lqh-dprIT$_3$ Variants and LqhIT$_2$-N58D. Two recombinant Lqh-dprIT$_3$ toxins representative of the a−d and e−h groups were analyzed on the cockroach giant axon. Under current clamp conditions and in the absence of toxin, the resting potential (−60 mV) and evoked action potential (AP) were stable for at least 40 min (Figure 5Aa). Lqh-dprIT$_3$-a (of the a−d toxin group bearing Asn at position 58) at 1 μM induced a gradually growing depolarization of the membrane resting potential [Figure 5Ab,c; 10.2 ± 3.6 mV (n = 3) after 4 min], which was TTX-sensitive (Figure 5Ae). The amplitude of the evoked AP declined gradually until the axonal firing was blocked after 5 min (Figure 5Aa−c). Artificial repolarization to −60 mV, in the presence of the toxin, restored 75% of the AP amplitude (Figure 5Ad); however, the membrane depolarized again, and the AP disappeared once more within 3 min (not shown). The effects of Lqh-dprIT$_3$-a on the axon were different when the membrane potential was hyperpolarized to −70 mV. A short stimulation (0.5 ms) by a single current pulse induced repetitive APs and only a minor increase in the extent of resting depolarization (Figure 5B). Further exposure to the toxin (more than 8 min) at −70 mV generated spontaneous oscillations of membrane potential (Figure 5C).

Lqh-dprIT$_3$-e (of the e−h toxin group bearing Asp at position 58) at 1 μM did not affect the axonal bioelectrical activity, and therefore, we analyzed it at a higher concentration. Lqh-dprIT$_3$-e at 6 μM applied at a resting potential of −60 mV had two time-resolved effects. The first, recorded following a single stimulus and observed within 6−10 min of toxin application, was a short "train" of repetitive APs accompanied by growing membrane depolarization. Then, the membrane potential slowly returned to its resting level (long postdepolarization) (Figure 6B). The second effect, observed within 10−20 min in the presence of Lqh-dprIT$_3$-e, was apparently similar to that induced by Lqh-dprIT$_3$-a. The resting membrane potential depolarized progressively [by 8.4 ± 3.2 mV (n = 3) after 15−17 min] in parallel to a decrease in AP amplitude that finally disappeared (Figure 6Ca,b). Artificial repolarization to −60 mV in the presence of Lqh-dprIT$_3$-e restored 90% of the AP amplitude, which again was accompanied by postdepolarization (Figure 6D). Hyperpolarization to −70 mV increased the level of postdepolarization, which appeared as a long high plateau (Figure 6E). Such a long postdepolarization was never observed in the presence of Lqh-dprIT$_3$-a.

Under voltage clamp conditions, the effects of Lqh-dprIT$_3$-a were generally similar to those previously described for LqhIT$_2$ (*15*). The sodium peak current measured after a depolarizing pulse of −10 mV decreased progressively in the presence of 1 μM toxin and after 8−10 min was 70 ± 19% (n = 3) of that of the control. The toxin also generated a constant current at a holding potential of −60 mV, which after 8−10 min was 103 ± 26 nA (n = 3) (Figure 7A). Addition of 10^{-7} M TTX not only abolished the sodium peak current but also eliminated the constant current at the holding potential, indicating that this current was mediated by sodium channels (Figure 7B). Measurement of sodium currents under a broad range of membrane potentials revealed 10 min after toxin application a shift in the voltage dependence of activation to the negative direction (Figure 7C,D), which increased over time.

In comparison to Lqh-dprIT$_3$-a, higher concentrations of Lqh-dprIT$_3$-e were required to obtain an effect. In the presence of 6 μM Lqh-dprIT$_3$-e, the first observed effect was a development of maintained current, which after 10 min at −20 mV was 16.4 ± 4.3% (n = 4) of that of the peak current (Figure 8A). This maintained current was much more prominent upon depolarization steps to −40 and −30 mV (Figure 8B) and could still be observed after a depolarization step to −10 mV (Figure 8C). In parallel, an increasing tail current appeared upon repolarization to −60 mV (Figure 8A−C), while returning to a holding potential of −70 mV minimized markedly the tail current induced by Lqh-dprIT$_3$-e (Figure 8C). It is important to note that under such a voltage protocol, Lqh-dprIT$_3$-a never generated tail currents. Both the maintained and tail currents increased over time (Figure 8A). No shift in voltage dependence of activation was

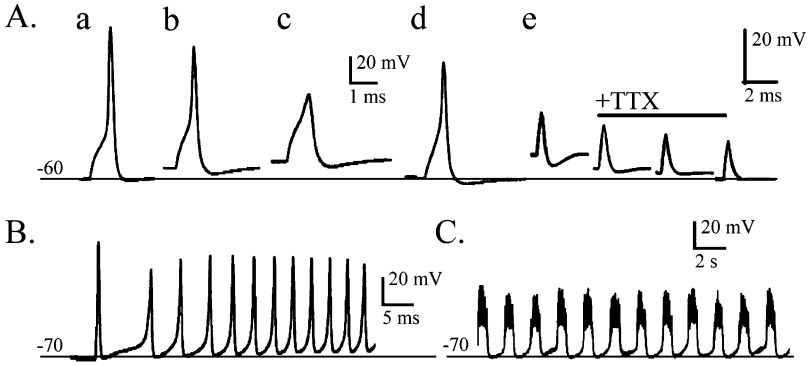

FIGURE 5: Effects of 1 μM Lqh-dprIT$_3$-a on the cockroach axon under current clamp conditions. (A) Action potentials evoked by a 0.5 ms, 10 nA current stimulation of an axon held at resting potential of −60 mV: (a) control, (b and c) decreases in the AP amplitude and increases in the resting depolarization in the presence of the toxin after 5 and 10 min, respectively, (d) artificial repolarization to −60 mV 12 min after toxin application restores part of the AP amplitude, and (e) repolarization of the resting potential by 0.1 μM TTX. (B) Salvos of action potentials obtained from an axon held at resting potential of −70 mV in the presence of toxin for more than 10 min. (C) Spontaneous oscillations of axonal membrane potential with a discharge of small amplitude action potentials recorded at a resting potential of −70 mV after 15−30 min in the presence of the toxin.

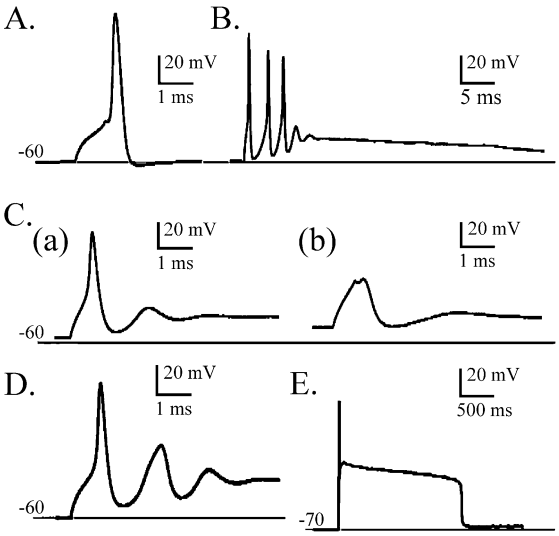

FIGURE 6: Effects of 6 μM Lqh-dprIT$_3$-e on the cockroach axon under current clamp conditions. (A) Control AP. (B) Short repetitive activity with long postdepolarization after less than 10 min in the presence of the toxin. (C) Effect of the toxin on the axon after a longer period: (a) 15 or (b) 20 min. (D) Artificial repolarization to −60 mV after more than 20 min from toxin application restores part of the AP amplitude and the postdepolarization. (E) Prominent postdepolarization obtained after further artificial repolarization of the axon to −70 mV.

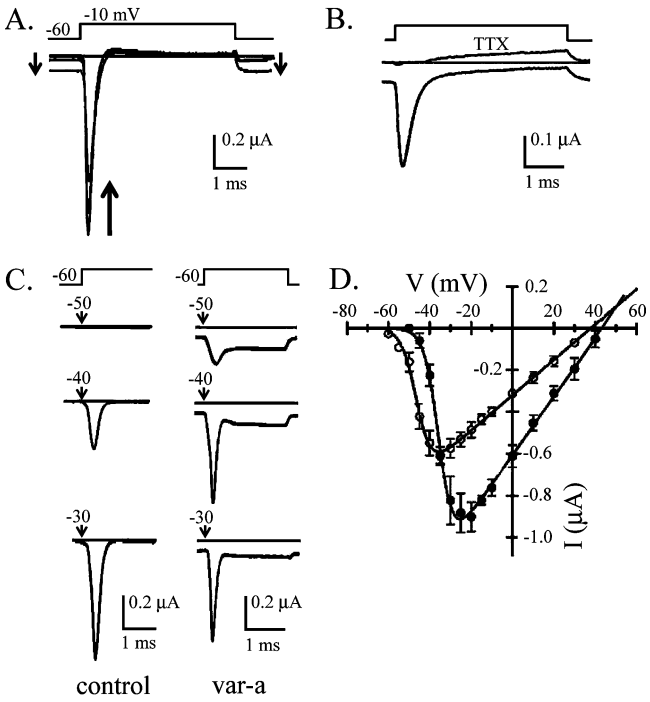

FIGURE 7: Effects of 1 μM Lqh-dprIT$_3$-a on the cockroach axon under voltage clamp conditions. (A) Under a depolarizing pulse of −10 mV, the sodium current amplitude decreases (up arrow) and a constant holding current develops within 10 min of toxin application (down arrows). (B) Elimination of the holding and peak currents by 0.1 μM TTX. (C) Sodium currents evoked by depolarization to −50, −40, and −30 mV after 10 min in the presence of the toxin compared to currents obtained in the absence of toxin. (D) Current−voltage relationship under control conditions (●; ±standard error of the mean, $n = 8$) and 10 min after toxin application (○; ±standard error of the mean, $n = 4$).

induced by Lqh-dprIT$_3$-e within 10 min, which is the opposite of Lqh-dprIT$_3$-a (Figure 8Da). However, after a longer period in the presence of the toxin, the peak current decreased markedly, and a constant current developed at a holding potential of −60 mV (Figure 8A). Indeed, a clear shift in the voltage dependence of activation is obtained after 20 min in the presence of Lqh-dprIT$_3$-e (Figure 8Db).

Analysis of the LqhIT2-N58D mutant revealed that under current clamp conditions 6 μM toxin induced during the first 10 min a short repetitive activity followed by long postdepolarization with no significant effect on the resting membrane potential (Figure 9Ab,c). Later, a progressive resting depolarization was observed together with a decrease in action potential amplitude (Figure 9Ad). Under voltage clamp conditions, a maintained current appeared during the depolarizing pulse (to −10 mV), and upon repolarization to a holding potential, a slowly decaying tail current was observed (Figure 9Bb). Over time, a constant current at a holding potential of −60 mV developed and the peak current decreased (Figure 9Bc). Hence, the effects of this mutant on the axon were very similar to those induced by Lqh-dprIT$_3$-e under both current clamp and voltage clamp conditions.

DISCUSSION

The toxin Lqh-dprIT$_3$ appears in minute amounts in the venom of the scorpion *L. quinquestriatus hebraeus* and is

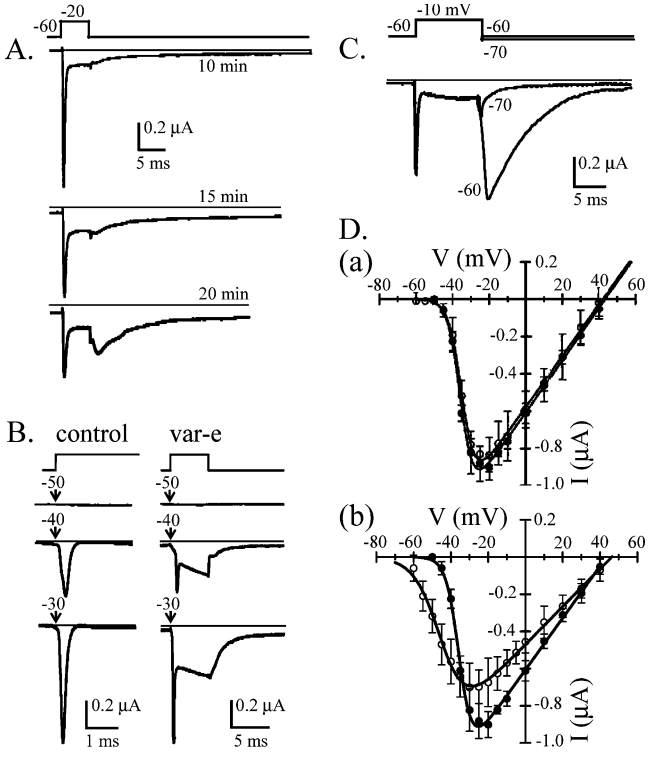

FIGURE 8: Effects of 6 μM Lqh-dprIT$_3$-e on the cockroach axon under voltage clamp conditions. (A) Development over time of maintained, tail, and holding currents. (B) Sodium currents evoked by depolarization to −50, −40, and −30 mV after 10 min in the presence of the toxin compared to currents in the absence of the toxin. (C) Decrease in tail current observed at −60 mV by hyperpolarization to −70 mV. (D) Current−voltage relationship under control conditions (●; ±standard error of the mean, $n = 8$) and 10 (a) and 20 min (b) after toxin application (○; ±standard error of the mean, $n = 3$).

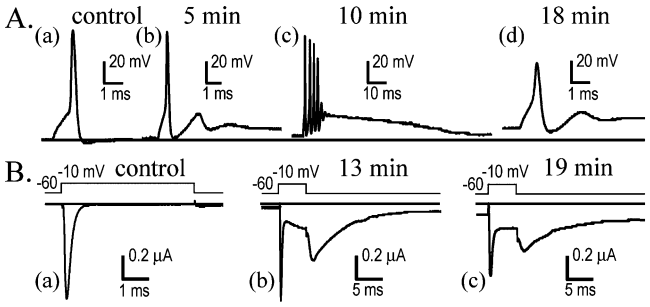

FIGURE 9: Effects of 6 μM LqhIT$_2$-N58D on the cockroach axon under current and voltage clamp conditions. (A) Response of the axon to a single 0.5 ms current stimulus in the presence of the mutant toxin: (a) control action potential, (b) postdepolarization obtained after 5 min, (c) short repetitive activity that changes into a long postdepolarization after 10 min in the presence of toxin, and (d) resting depolarization and decrease in the action potential amplitude after more than 15 min. (B) Modification of the axonal sodium current by the N58D mutant: (a) current under control conditions, (b) current recorded 13 min after toxin application, and (c) constant current and decreased peak current after 19 min. Note the resemblance in the effects of LqhIT$_2$-N58D and Lqh-dprIT$_3$-e.

extremely toxic to insects [ED$_{50}$ on blowfly larvae is 3−5 ng/100 mg body weight compared to known depressant toxins with a 1 order of magnitude higher ED$_{50}$ value (*12*, *14*, *17*, *34*)]. The fact that all scorpion toxins are encoded by gene families has been previously shown (*36*) and was explained by the extensive evolutionary process of the scorpion "pharmaceutical factory", enabling survival in an ever-changing environment. The various Lqh-dprIT$_3$ clones provided a "reverse genetics" tool for studying the effect of the natural mutations on toxin activity. Lqh-dprIT$_3$ purified from the venom and recombinant variants a−d (bearing Asn at position 58) were found to induce upon injection the typical effects of depressant toxins, i.e., transient contraction followed by flaccid paralysis of fly larvae, in unprecedented small doses (3−5 ng/100 mg larva). Lqh-dprIT$_3$ variants e−h (bearing Asp at position 58), which have not been identified in the venom, were substantially less potent in terms of their toxicity to blowfly larvae, their binding affinity for cockroach neuronal membranes (Table 1), and their effects on an isolated cockroach axon (Figures 5 and 6). The different CD spectra of Lqh-dprIT$_3$ variants a−d compared to those of Lqh-dprIT$_3$ variants e−h (Figure 4) suggest that residue 58 is important for the spatial arrangement of the toxin and hence for its function. Interestingly, in all scorpion depressant toxins, this residue, Asn-58, is conserved (*4*, *17*). In a previous report, a toxin very similar to Lqh-dprIT$_3$ was shown to induce contraction paralysis of insects (*37*). Sequence comparison has indicated that this toxin, LqhIT5, differs from Lqh-dprIT$_3$-b at a single position, Asp-17 rather than Asn. To verify whether this substitution accounts for the vast difference in activity, we substituted Asp-17 with Asn in Lqh-dprIT$_3$-b. The resulting mutant toxin was as active as the entire group of Lqh-dprIT$_3$ variants a−d and exhibited clear depressant characteristics (not shown). The lack of typical features of depressant toxins that were reported for LqhIT5 remains therefore unclear.

Despite the putative difference in structure and the different affinities, the two types of Lqh-dprIT$_3$ toxin variants induce flaccidity in blowfly larvae, are able to block the action potential (Figures 5Ac and 6C), and most likely bind to the same receptor site as LqhIT$_2$ (Figure 3). The decrease in action potential amplitude observed in the presence of both types of toxin variants may result from a reduced number of sodium channels being available for opening, because a fraction of the channels have been modified by the toxin and remain open at the resting membrane potential. This fraction depolarizes the membrane, which further leads to inactivation of more channels that were not modified by the toxin.

Still, the two groups of Lqh-dprIT$_3$ variants differ markedly in potency, as is evident from the binding and toxicity assays (Table 1), and the effects on the cockroach axon (Figures 5−8). Whereas depolarization of the axonal membrane held at −60 mV occurred instantaneously, and the level increased rapidly in the presence of Lqh-dprIT$_3$-a (Figure 5), higher concentrations and longer periods were required to achieve such an effect with Lqh-dprIT$_3$-e (Figure 8A,D). In the meantime, before depolarization reached a level where the action potential was blocked in the presence of Lqh-dprIT$_3$-e, salvos of action potentials were observed (Figure 6). This repetitive firing is most likely reflected in the initial contraction paralysis of blowfly larvae before flaccidity prevails. However, salvos of action potentials could also be obtained in the presence of Lqh-dprIT$_3$-a when the axon was stimulated from a potential of −70 mV (Figure 5B). This suggests that small differences in membrane potential dominate the mode of toxin effect, and since all Lqh-dprIT$_3$ variants produce upon injection a transient contraction of

fly larvae that proceeds into flaccid paralysis, it is likely that the resting membrane potential of neurons in flies is slightly below −60 mV.

Since the shift in current−voltage relations to the negative direction is attributed to the ability of β-toxins to trap the DIIS4 voltage sensor in its activated outward position (*38*, *39*), variations observed between both types of Lqh-dprIT$_3$ variants in the voltage clamp experiments may correlate with differences in their trapping capabilities and, hence, kinetics of deactivation. The tail current obtained in the presence of Lqh-dprIT$_3$-e (Figure 8), which corresponds to the long postdepolarization observed under current clamp conditions (Figure 6), indicates a delay in deactivation as a result of slow reversal movement of the voltage sensor that interacts with the toxin. Such an interaction between DIIS4 and the β-toxin Css4 (voltage sensor trapping) has been previously suggested (*38*, *40*). The marked decrease in the tail current upon repolarization to −70 mV (Figure 8C), despite the increase in sodium ion driving force, indicates that the deactivation is faster at a slightly more negative membrane potential, and implies that the inward movement of DIIS4 is voltage-dependent to a large extent. In comparison, deactivation of sodium channels in the presence of the highly potent Lqh-dprIT$_3$-a is extremely slow due to efficient trapping of the voltage sensor in its activated position, so apparently a fraction of the channels remains permanently open at the resting membrane potential (see resting depolarization in Figure 5Ac and the constant current at −60 mV in Figure 7).

Another salient difference between both Lqh-dprIT$_3$ variants is the extent of maintained current obtained during the depolarizing pulses. Whereas in the presence of Lqh-dprIT$_3$-a, inactivation of channels, which opened by the depolarization pulse, was almost complete (Figure 7), in the presence of Lqh-dprIT$_3$-e a large fraction of the channels were not inactivated (Figure 8). It is noteworthy that the incomplete inactivation is not a specific phenomenon for Lqh-dprIT$_3$ toxins, as it was observed previously with other β-toxins such as LqhIT$_2$ (*15*), BotIT$_2$ (*41*), CsE VII (*42*), CsE IV (*43*), TiTx-γ (Ts 1) (*44*), and BmKITb (*45*).

Since sodium channel inactivation has been shown to be coupled to activation (*46*−*52*), a putative explanation for the incomplete inactivation in the presence of Lqh-dprIT$_3$-e is that the inward movement of DIIS4 and the outward movement of DIVS4 are coupled (*53*). It is possible that this phenomenon does not occur in the presence of Lqh-dprIT$_3$-a because DIIS4 is trapped in its outward position, which enables almost complete inactivation when the channel is open.

The important role of Asn-58 in depressant toxins demonstrated with the Lqh-dprIT$_3$ variants was substantiated by the effects on the affinity, potency, and electrophysiological properties of the LqhIT$_2$-N58D mutant. Although LqhIT$_2$ is 1 order of magnitude less potent than the native Lqh-dprIT$_3$ and the a−d recombinant toxin variants, it practically induces similar effects in blowfly larvae and on the cockroach axon (*15*). In contrast, LqhIT$_2$-N58D hardly affected blowfly larvae (Table 1), its binding affinity for cockroach neuronal membranes decreased markedly (Table 1), and its effects on the cockroach axon were similar to those induced by Lqh-dprIT$_3$-e (Figure 9). Interestingly, the CD spectra of Lqh-dprIT$_3$-a, Lqh-dprIT$_3$-e, LqhIT$_2$, and LqhIT$_2$-N58D vary from one another, suggesting that their structures differ slightly. This possibility gains support from a recent NMR analysis of LqhIT$_2$, which revealed two major conformers in solution (*54*) that may vary in potency. Thus, depressant toxins exhibit structural flexibility that may alter their potency. Although any conclusion about their putative interaction with the receptor binding site requires comparison of toxin structures and/or toxin−receptor complexes, the results obtained in this study demonstrate the mandatory role of Asn-58 in depressant toxins, and correlate the effects observed on the axon with the intoxication symptoms of blowfly larvae.

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