A Scorpion Venom Neurotoxin Paralytic to Insects That Affects Sodium Current Inactivation: Purification, Primary Structure, and Mode of Action†

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ABSTRACT: A new toxin, LqhαIT, which caused a unique mode of paralysis of blowfly larvae, was purified from the venom of the scorpion *Leiurus quinquestriatus hebraeus*, and its structural and pharmacological properties were compared to those of three other groups of neurotoxins found in Buthinae scorpion venoms. Like the excitatory and depressant insect-selective neurotoxins, LqhαIT was highly toxic to insects, but it differed from these toxins in two important characteristics: (a) LqhαIT lacked strict selectivity for insects; it was highly toxic to crustaceans and had a measurable but low toxicity to mice. (b) It did not displace an excitatory insect toxin, ^{125}I-AaIT, from its binding sites in the insect neuronal membrane; this indicates that the binding sites for LqhαIT are different from those shared by the excitatory and depressant toxins. However, in its primary structure and its effect on excitable tissues, LqhαIT strongly resembled the well-characterized α scorpion toxins, which affect mammals. The amino acid sequence was identical with α toxin sequences in 55%–75% of positions. This degree of similarity is comparable to that seen among the α toxins themselves. Voltage- and current-clamp studies showed that LqhαIT caused an extreme prolongation of the action potential in both cockroach giant axon and rat skeletal muscle preparations as a result of the slowing and incomplete inactivation of the sodium currents. These observations indicate that LqhαIT is an α toxin which acts on insect sodium channels. It should be useful as a pharmacological tool for the study of sodium channels related to insect neuronal excitability and as a model for the clarification of the structural basis for animal group specificity of neurotoxins.

The venoms derived from scorpions belonging to the Buthidae family serve as a source for four main groups of polypeptide neurotoxins that modify axonal sodium conductance. The first group are the α toxins, which specifically affect mammals through an extreme prolongation of the action potentials due to a slowing or blockage of the sodium channel inactivation (Catterall, 1984; Rochat et al., 1979). α toxins bind in a voltage-dependent manner and show positive cooperativity with the lipid-soluble alkaloids such as veratridine (Catterall, 1984). The second group, the β toxins, affect sodium activation, bind in a potential-independent manner to sites distinct from those of the α toxins, and do not interact synergistically with veratridine (Couraud et al., 1982; Couraud & Jover, 1984). The third group of toxins are the depressant insect-selective toxins, which induce a progressive flaccid paralysis of insects by blocking action potentials, primarily by depolarizing the axonal membrane and suppressing the sodium current (Lester et al., 1982; Zlotkin et al., 1985). The fourth group are the excitatory insect-selective toxins, which cause an immediate spastic paralysis of insects. Excitatory insect toxins induce repetitive firing in the motor nerves (Walther et al., 1976) by causing an increase of the peak sodium current and a voltage-dependent slowing of sodium current inactivation (Pelhate & Zlotkin, 1981, 1982). Binding of insect excitatory toxins (as represented by AaIT[1]) to insect neuronal membranes is voltage-independent (Gordon et al., 1984) and shows no cooperativity with veratridine (Gordon et al., 1984). The interaction of the insect excitatory toxins with insect nervous tissue therefore strongly resembles the effect of β toxins on mammalian neuronal systems.

The insect toxins in scorpion venoms were first detected and their isolation has been monitored by assaying on blowfly larvae (Zlotkin et al., 1971a,b). In these larvae the excitatory toxins induce an immediate and transient contraction paralysis, while the depressant toxins cause a progressively developing flaccidity (Zlotkin, 1971a; Lester et al., 1982). Although depressant and excitatory insect toxins produce opposite effects, both bind to the same site on insect neuronal membranes and affect sodium conductance (Zlotkin et al., 1985; Gordon et al., 1984). The excitatory and depressant insect toxins are inactive on mice, while α toxins isolated by assaying for lethality to mice (Rochat et al., 1967) are nontoxic to blowfly larvae (Zlotkin et al., 1971c).

Sarcophaga falculata blowfly larvae display simple behavioral responses to different neurotoxins. Such behavioral responses have previously made it possible to distinguish and isolate the excitatory and depressant insect toxins present in Buthinae scorpion venoms (Zlotkin & Gordon, 1985). The

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[1] Abbreviations: AaIT, the excitatory insect toxin from the venom of the scorpion *Androctonus australis* Hector; AaH2, α toxin 2 isolated from the venom of *Androctonus australis* Hector, which affects mammals; ED_{50}, dose at which 50% of animals show symptoms characteristic of the assay; HPLC, high-performance liquid chromatography; LD_{50}, dose at which 50% of animals die; LqqIT2, the depressant insect-selective toxin isolated from the venom of *Leiurus quinquestriatus quinquestriatus*; PU_{50}, dose at which 50% of animals are paralyzed within 5 min; SDS, sodium dodecyl sulfate; STX, saxitoxin; TTX, tetrodotoxin.

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present study was initiated by the observation that a certain chromatographic fraction of scorpion venom induced in blowfly larvae a third set of symptoms, namely, a delayed and sustained contraction. We report here the isolation and characterization of the factor responsible for this effect and present evidence suggesting that it is an α toxin which affects insects.

Materials and Methods

Toxic Substances. Crude venom of the scorpion *Leiurus quinquestriatus hebraeus* was obtained by electrical milking (Zlotkin & Gordon, 1985) of field-collected scorpions, followed by lyophilization of the fluid. The venom was fractionated as described below.

The excitatory insect toxin AaIT was purified from the venom of the scorpion *Androctonus australis* as described by Zlotkin et al. (1971a). The mammalian α toxin AaH_2 was a generous gift from Professor H. Rochat (Faculté de Médecine, Biochimie, Marseilles, France). Following purification, scorpion toxins to be used for activity assays were lyophilized in the presence of a 10-fold excess of bovine serum albumin (BSA, fraction V, Armour Co.).

Test Animals. Larvae of the blowfly *S. falculata* (100–130-mg body weight) were bred in the laboratory as previously described (Zlotkin et al., 1971a). Isopods (terrestrial crustacean) *Hemilepistus* sp. (300–400-mg body weight) were field-collected, and albino laboratory mice (variant Sabra) were purchased from the laboratory animal farm of the Hadassah Medical School, Jerusalem, Israel.

Locust Synaptosomal Membrane Vesicles. Locust synaptosomes and membrane vesicles derived from them were prepared from homogenates of the dissected central nervous systems of *Locusta migratoria* locusts, according to a previously described method (Zlotkin & Gordon, 1985).

Bioassays. The lethal and paralytic effects of toxic substances were determined by subcuticular injections. In the assays on blowfly larvae, volumes of 2–10 μL/100 mg of body weight were injected into an abdominal intersegmental membrane; the desired activity induced a new symptomatology characterized by delayed and sustained spastic paralysis (Figure 4). Larvae were judged paralyzed if they were immobile and contracted. A paralytic unit (PU_{50}) was defined as the dose that paralyzes 50% of the animals within 5 min of injection. Isopods, dorsally injected between the thorax and the abdomen with volumes of 1–5 μL/300 mg of body weight, were used for the determination of toxicity to crustaceans; the PU_{50} was based on complete immobility 5 min after injection. The 50% lethal dose (LD_{50}) in mice was determined 24 h after subcutaneous injection. The sampling and calculation of 50% end points (PU_{50} and LD_{50} doses) were performed by the method of Reed and Muench (1938).

Column Chromatography. In the present study three different methods of column chromatography were employed: (a) molecular exclusion chromatography on Sephadex G50 fine (Pharmacia, Sweden) equilibrated in and eluted with 0.1 M ammonium acetate, pH 8.5 (Figure 1A); (b) cation-exchange chromatography on CM52 cellulose (Whatman, England) equilibrated in 0.01 M ammonium acetate, pH 6.4, and eluted with a linear gradient of 0.01–0.4 M ammonium acetate (Figure 1B); and (c) HPLC reverse-phase chromatography on a TSK-ODS-120T RP-C18 (4.6 × 250 mm) column (LKB, Sweden; Figure 1C). Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.1% trifluoroacetic acid and acetonitrile/2-propanol, 1:1. The column was equilibrated in 5% buffer B and eluted with a linear gradient of 5%–20% buffer B over the first 15 min followed by a 60-min linear gradient to 50% buffer B.

Electrophoretic Techniques. SDS–polyacrylamide gel electrophoresis in a 12.5% polyacrylamide gel was carried out in the presence of 8 M urea as described by Swank and Munkres (1971). Analytical isoelectric focusing in 7.5% polyacrylamide gels used pH 3–10 ampholines (LKB, Technical Bulletin 1217-2001ME). Appropriate markers (Sigma) were included on each gel. Gels were stained with Coomassie blue as described by Righetti and Chillemi (1978).

Protein Determination. Protein was determined by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard.

Primary Structure Determination. LqhαIT toxin was reduced and alkylated with 4-vinylpyridine: protein samples were incubated in 6 M guanidine hydrochloride, 1 M Tris-HCl, pH 8.6, 10 mM EDTA, and 20 mM dithiothreitol for 1 h at 37 °C. 4-Vinylpyridine (Sigma) was added to a final concentration of 50 mM, and incubation was continued for 1 h at room temperature. The modified protein was desalted by HPLC on a Vydac C-8 or Hypersil ODS column in 0.1% trifluoroacetic acid with a linear gradient of 0–80% 2-propanol/acetonitrile, 1:1.

Peptides were prepared by digestion of the reduced and alkylated protein with Asp-N, Lys-C, trypsin, and chymotrypsin (Boehringer-Mannheim) according to the manufacturer's directions. Additional peptides were produced by partial acid hydrolysis by the method of Inglis (1980). Peptides were separated by HPLC on a Hypersil ODS column in 0.1% TFA with a linear gradient of 0–60% 2-propanol/acetonitrile, 1:1.

Amino acid sequence analysis was performed by automated Edman degradation with an Applied Biosystems 470A gas-phase sequencer connected to an Applied Biosystems 120A PTH analyzer and M900 data system for on-line analysis of the PTH-amino acids. The chromatographic system was calibrated prior to each sequence analysis with PTH-amino acid standards. Each sequence was confirmed in at least two separate determinations.

The C-terminal arginine was identified by mass spectrometry carried out by M-Scan, Inc. (Westchester, PA). A chymotryptic digest was analyzed by fast atom bombardment on a VG Analytical ZAB-2SE high-field mass spectrometer operating at Vacc = 8 kV. A cesium ion gun was used to generate ions for the mass spectra, which were recorded on a PDP 11-250J data system. Mass calibration was performed with cesium iodide or cesium iodide/glycerol. The enzyme digest was analyzed by using glycerol/thioglycerol and *m*-nitrobenzyl alcohol as FAB matrices.

For sequence comparisons, amino acid sequences were aligned for maximum similarity with the aid of the University of Wisconsin Genetics Computing Group (UWGCG) Profile Analysis (Devereux et al., 1984). The percentage of the total positions containing identical residues was calculated by using the UWGCG "distance" algorithm. The isoelectric point was calculated by using the UWGCG "isoelectric" algorithm.

Binding Assays. ^{125}I-Labeled AaIT was prepared and competitive displacement binding assays were performed according to previously described procedures (Zlotkin & Gordon, 1985). The reaction mixture (210 μL) included 1.5 nM ^{125}I-AaIT, locust synaptosomal membrane vesicles (40 μg of protein), and increasing concentrations of the competing substance in the standard binding medium (0.15 M choline chloride, 1 mM $MgSO_4$, 2 mM $CaCl_2$, and 0.1% BSA). The membranes were incubated for 40 min at 22 °C. Free and membrane-bound ^{125}I-AaIT were separated by rapid filtration. The binding of the labeled toxin measured in the presence of

Table I: Toxicity to Blowfly Larvae and Mice of CM52 Column Fractions

assay	units (μg/g)	CM52 fraction c	d	e
blowfly larvae	PU_{50}[a]	0.54	0.28	7.0
mice	LD_{50}[b]	0.60	6.0	1.25

[a] An immobile and contracted larva 5 min after injection was considered as a positive response. PU_{50} was defined as the dose required to evoke this response in 50% of the larvae tested. [b] Lethality was determined after 24 h. The LD_{50} of Sephadex G50 fraction IV (Figure 1A) to mice corresponded to 2 μg/g of body weight.

a large excess (1 μM) of unlabeled toxin was defined as the nonspecific binding.

Electrophysiological Techniques. (A) Insect Axonal Preparation. Voltage- and current-clamp experiments were performed on giant axons dissected from abdominal nerve cords of the cockroach *Periplaneta americana* by using the double oil gap, single-fiber technique (Pichon & Boistel, 1967). Experiments were performed at 18–20 °C. Insect physiological saline had the following composition: 200 mM NaCl, 3.1 mM KCl, 5.4 mM $CaCl_2$, 5.0 mM $MgCl_2$, 2 mM $NaHCO_3$, and 0.1 mM NaH_2PO_4, pH 7.2. 3,4-Diaminopyridine was employed to selectively block the potassium current (Pelhate & Pichon, (1974), and synthetic saxitoxin (STX, 2×10^{-7} M) was used for selective, reversible blockage of sodium currents (Sattelle et al., 1979).

(B) Mammalian Skeletal Muscle Single-Fiber Preparation. Voltage- and current-clamp experiments were performed at room temperature (18–22 °C) on single muscle fibers isolated from the slow-twitch soleus muscle of the rat, *Rattus norvegicus*, using the double mannitol gap, single-fiber technique (Duval & Leoty, 1978). Rat physiological saline had the following composition: 140 mM NaCl, 6 mM KCl, 3 mM $CaCl_2$, 5 mM glucose, and 6.5 mM Tris-HCl, pH 7.3. Tetraethylammonium chloride (10 mM) and 3,4-diaminopyridine (2 mM) were added to the bath in order to block potassium current, and tetrodotoxin (1 μM) was used to block the sodium current (Duval & Leoty, 1980).

Results

Isolation of a Factor Inducing a Delayed and Sustained Contraction of Blowfly Larvae. The lyophilized crude venom of the scorpion *L. q. hebraeus* was successively subjected to the following treatments: (1) extraction with water; (2) chromatography of the lyophilized water extract on Sephadex G50 eluted with 0.1 M acetic acid, in order to remove high molecular weight mucoproteins (data not shown); and (3) chromatography on Sephadex G50 eluted with 0.1 M ammonium acetate buffer, pH 8.5, in order to remove nonprotein low molecular weight pigments (data not shown).

The lyophilized toxic fractions obtained in step 3 were separated by recycling chromatography on a series of four Sephadex G50 columns as described in the legend to Figure 1A. As shown in Figure 1A, four major fractions (I–IV) were obtained from recycling chromatography. Fraction IV contained about 12% of the protein loaded on the column and induced an unusual mixture of symptons when injected into blowfly larvae. These symptoms included an initial flaccidity (typical of the depressant insect toxins—see above) followed by a contraction that, in contrast to the contraction elicited by excitatory toxins, occurred after a delay and exhibited a prolonged duration. Fraction IV also proved moderately lethal to mice (LD_{50} = 40 μg/20 g of body weight) and induced in mice the excitatory symptoms of envenomation typical of Buthinae scorpion venoms and the mammalian toxins purified from them (Rochat et al., 1979).

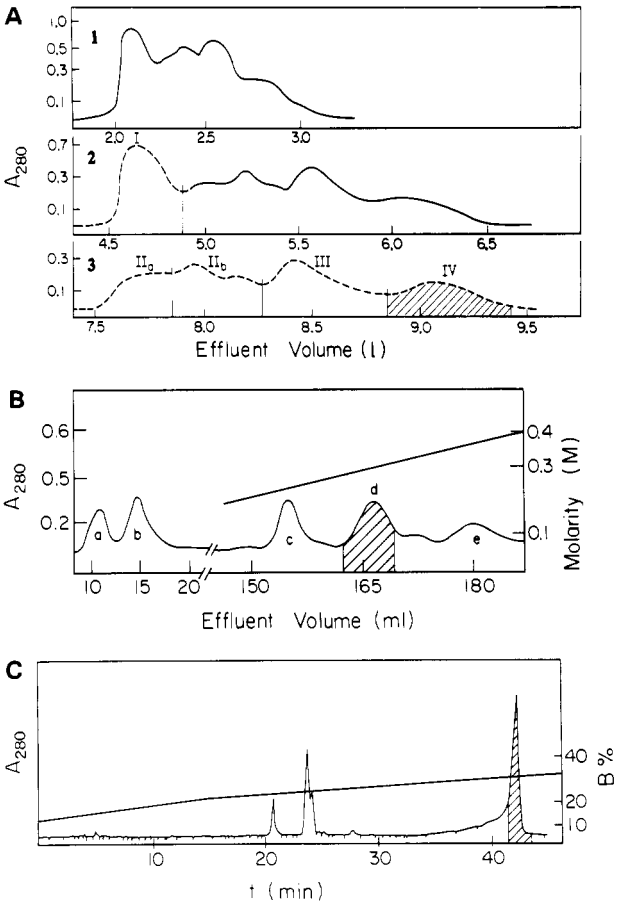

FIGURE 1: Purification of LqhαIT. (A) Separation of the *L. q. hebraeus* venom by recycling size-exclusion column chromatography. Two grams of crude *L. q. hebraeus* venom was extracted with water and separated by the initial two steps of gel filtration as described in the text. The active material (1650 A_{280} units) was applied to Sephadex G50 columns (four 3.2 × 100 cm columns in series) equilibrated in and eluted with 0.1 M ammonium acetate, pH 8.5, at a flow rate of 45 mL/h (Zlotkin et al., 1971b). The solid line represents elution, and the dotted line represents collection. The numbers 1, 2, and 3 indicate the successive cycles. The various fractions were collected according to the elution profile. Fraction IV (A_{280} = 200 units) had paralytic and lethal effects on blowflies, isopods, and mice. (B) Separation by CM52 chromatography of the factor inducing a delayed and sustained contraction of blowfly larvae. A 23-mg quantity (31.5 A_{280} units) of fracton IV (panel A) was applied to a 10-mL column of the cation exchanger CM52 cellulose (Whatman, England) equilibrated and eluted at 10 mL/h with 0.01 M ammonium acetate, pH 6.4. The first stage of elution was performed at initial conditions and resulted in fractions a and b, which induced flaccidity in *Sarcophaga* larvae. The second stage of elution was performed with a linear gradient of increasing molarity to 0.4 M ammonium acetate and resulted in fractions c–e. The toxicity of these fractions to fly larvae and mice is presented in Table I. (C) HPLC separation of 200 μg of CM52 fraction d (panel B) on a column of TSK-LDS-120T RP-C18 (4.6 × 250 mm, LKB, Sweden). Buffers: A, 0.1% trifluoroacetic acid (TFA); B, 0.1% TFA and acetonitrile/2-propanol, 1:1. Gradient: 0 min, 5% buffer B; 15 min, 20% buffer B; 75 min, 50% buffer B. The flow rate was 1 mL/min. Crosshatching indicates the toxic fraction.

Figure 1B shows the pattern resulting from the separation of Sephadex G50 fraction IV (Figure 1A) by cation-exchange (CM52) chromatography. Fractions a and b induced the flaccid paralysis of blowfly larvae typical of the depressant insect toxins (Lester et al., 1982; Zlotkin et al., 1985). Fractions c–e demonstrated toxicity to mice (Table I) and in blowfly larvae evoked a new symptomatology, namely, a delayed and sustained contraction paralysis (Figure 4). As shown in Table I, the ratio of insect to mammalian toxicity differed

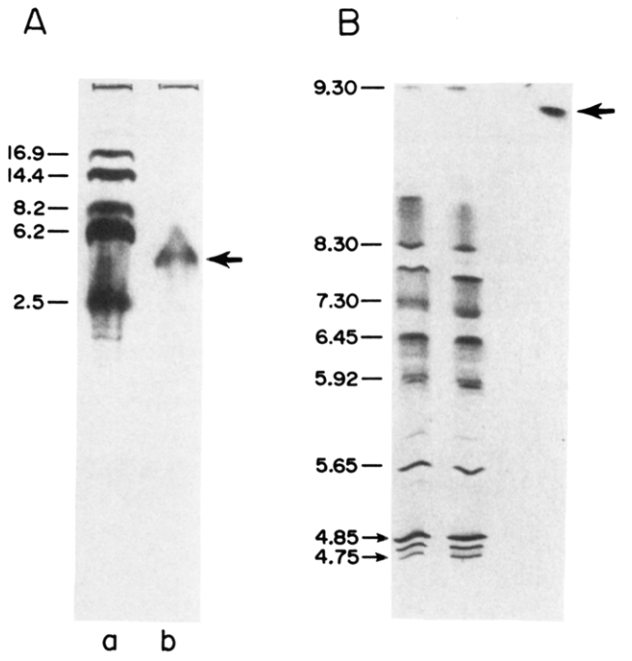

FIGURE 2: Estimation of molecular weight and isoelectric point of LqhαIT. (A) SDS–polyacrylamide gel electrophoresis of purified toxin was performed on a 12.5% polyacrylamide gel (60 × 80 × 1.5 mm) in the presence of 8 M urea. Lane a, molecular weight markers: myoglobin and its CNBr fragments; the numbers correspond to the molecular weight ($\times 10^3$). Lane b, 10 μg of LqhαIT. The arrow points to the LqhαIT, which migrates with a molecular weight of about 5000. (B) Isoelectric focusing analysis of LqhαIT. Bands represent p*I* markers in the range of pH 4.7–9.3. The two lanes of the markers result from loading the same mixture at two different locations on the gel. The arrow points to the toxin LqhαIT, the p*I* of which is in the range pH 9.0–9.2.

from each fraction. Fraction d, which possessed the highest toxicity to blowfly larvae and the lowest toxicity to mice (Table I), was chosen for further purification.

The final purification of the new factor was achieved by reverse-phase HPLC chromatography (Figure 1C). The final product, which eluted at about 45 min, is designated as LqhαIT toxin. Purified LqhαIT accounted for about 30% of the protein in CM52 fraction d and 60% of the activity to blowfly larvae. LqhαIT ran in an SDS–polyacrylamide gel as a single band with a molecular weight of about 5000; in analytical isoelectric focusing, the protein also migrated as a single band with an isoelectric point of about pH 9.0 (Figure 2).

Table II: Toxic Activity of LqhαIT Toxin

test animal	effect	ED_{50} value (ng/g of body wt)
blowfly larvae	delayed sustained contraction paralysis (PU_{50})	140
isopods[a]	paralysis within 5 min (PU_{50})	200
mice	lethality observed after 24 h (LD_{50})	5000[b]

[a] Terrestrial crustaceans. [b] At least 2 orders of magnitude less toxic than the common mammalian toxins derived from scorpion venoms (Rochat et al., 1979).

Primary Structure Determination. The amino acid sequence of LqhαIT toxin was determined by gas-phase sequencing of the reduced and pyridylethylated toxin and peptides derived by partial acid hydrolysis and enzymatic digestion as indicated in Figure 3. Only a single sequence was observed when the intact toxin was subjected to Edman degradation. Mass spectrometry of a chymotryptic digest confirmed the sequence, except that a peak was observed at mass 1737. The peak at mass 1737 was much larger than the peak of mass 1582 expected for the C-terminal peptide. MS/MS analysis showed that the 1737 species dissociated to a species of mass 1582. These data indicate that arginine is the C-terminus of the protein. While we cannot rule out microheterogeneity at the C-terminus, only one Asp-N peptide spanning this region was recovered from the HPLC separation of the digest. The inability to detect the C-terminal arginine during sequencing of this peptide is not surprising since the yields of the two preceding amino acids were low and arginine is consistently obtained in lower yields than most other amino acids. These data plus the fact that the peak height of the mass 1737 peak was more than three times the height of the 1582 peak support the conclusion of a single sequence ending in arginine. The molcular weight calculated from this amino acid sequence is 7257.

Biological Activity of the LqhαIT Toxin. (*A*) *Symptomatology.* In contrast to the excitatory insect toxins, which induce an immediate and transient contraction paralysis of blowfly larvae, the LqhαIT toxin induced a delayed and sustained contraction paralysis. This new symptomatology is shown in Figure 4, which presents *Sarcophaga* larvae at various times following injection of a paralytic dose (20 ng) of CM52 fraction d.

(*B*) *Toxicity.* As shown in Table II, LqhαIT displayed a similar potency to blowfly larvae and isopods. The toxicity of LqhαIT to mice was at least 2 orders of magnitude lower

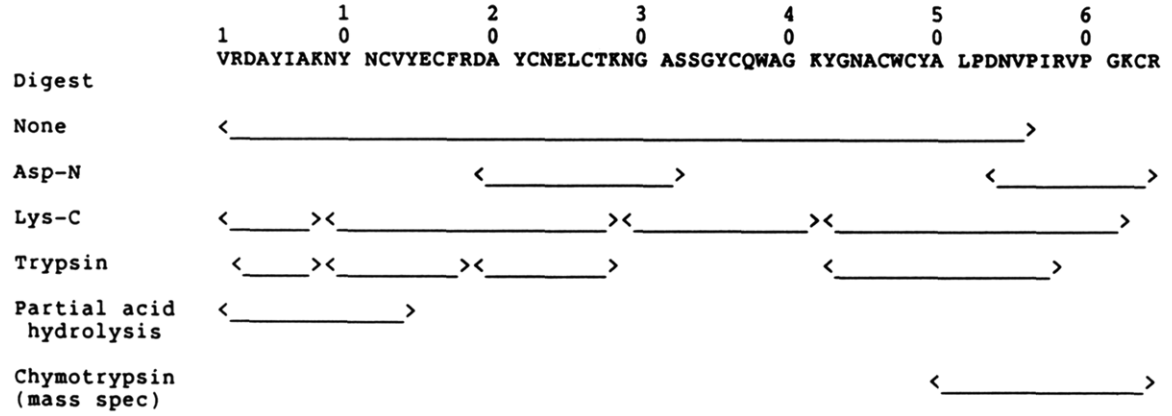

FIGURE 3: Summary proof of the sequence of LqhαIT toxin. The one-letter code designates the amino acids identified by Edman degradation of reduced and alkylated intact protein and the peptides obtained by Asp-N, Lys-C, and tryptic digestion. The length of the line indicates the portion determined in a single run. The C-terminal chymotryptic peptide was analyzed by mass spectrometry and the arginine assigned on the basis of the observed molecular weight.

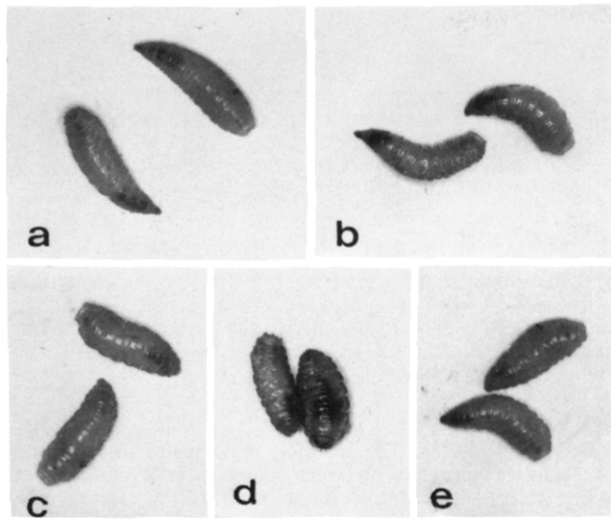

FIGURE 4: Response at various time intervals of *Sarcophaga* blowfly larvae to injection of a paralytic dose (PU_{50}; see Table I) of CM52 fraction d (Figure 1B). (a) Before injection; (b) 1 min after injection—mobile and without detectable change in body form; (c) 5 min—obvious contraction and immobility; (d) 8 min—fully contracted and paralyzed; (e) 20 min—still contracted but with partial recovery of mobility.

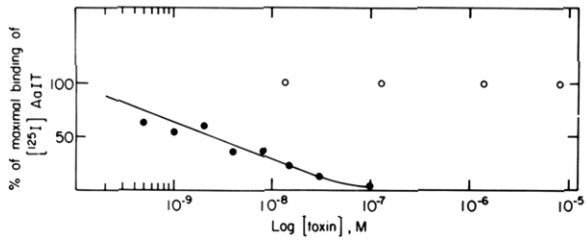

FIGURE 5: Competitive displacement of the specific binding of ^{125}I-AaIT by (●) the depressant toxin $LqqIT_2$ (Zlotkin et al., 1985) and (○) the LqhαIT toxin. Details are given under Materials and Methods.

than that of the common α toxins affecting mammals.

Binding Assays. The data presented in Figure 5 clearly indicate that the LqhαIT toxin was unable to displace ^{125}I-AaIT toxin (an excitatory toxin) in a binding assay using a locust synaptosomal preparation. The positive control, the depressant toxin LqqIT2, displaced ^{125}I-AaIT with 50% displacement at about 3×10^{-9} M. The $K_{0.5}$ for the depressant toxin was similar to the previously reported value of 2.2×10^{-9} M for displacement of ^{125}I-AaIT by unlabeled AaIT (Zlotkin et al., 1985). These results may indicate that the binding sites for LqhαIT toxin differ from those shared by the excitatory and depressant insect toxins.

Electrophysiological Studies. The LqhαIT toxin was assayed in current- and voltage-clamp conditions in two different preparations of excitable membranes: the giant isolated axon of *P. americana* (Figure 6A,B) and the isolated skeletal muscle fiber of the rat (Figure 6C,D). In both preparations the toxin induced essentially the same effect, namely, the prolongation of the evoked action potential (Figure 6A,C) due to an evident inhibition of sodium inactivation (Figure 6B,D). The toxin did not affect or modify the amplitude of the action potentials (Figure 6A,C) or the level of the membrane resting potential (Figure 6A,C). Likewise, potassium conductance was not affected in either rat muscle fiber treated with 1 μM LqhαIT (Figure 6D, graph c) or the insect axon treated with 2.8 μM LqhαIT (data not shown).

The concentration of LqhαIT toxin required to alter the action potential of the insect axon under current-clamp conditions was about 2 orders of magnitude less than the con-

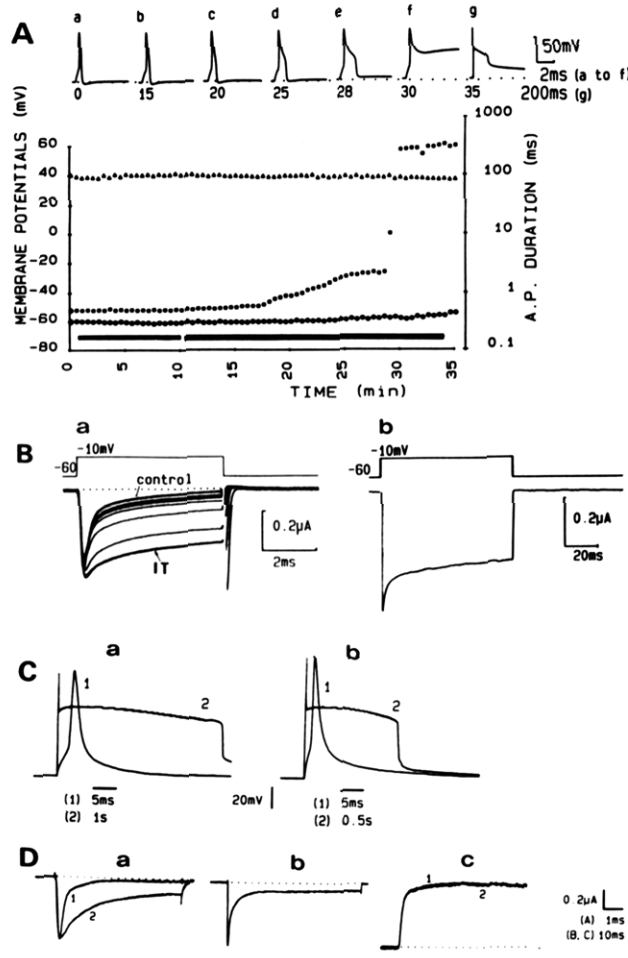

FIGURE 6: Effect of LqhαIT toxin on action potentials and sodium currents of isolated insect axon and mammalian muscle fiber. (A) Effect on action potentials of the isolated cockroach axon. (a–g) Records of action potentials evoked by a short (0.5 ms) depolarizing current pulse of 10 nA. The number under the records indicates the time in minutes. Note the different time scale in (g). The lower graph indicates the evolution of the resting potential (●), the value of the action potential peak (▲), and the duration of the action potential (■) in the presence of LqhαIT at 10^{-8} M (open bar) and 5×10^{-8} M (solid bar). (B) Effect of LqhαIT toxin (10^{-6} M) on the sodium current of the isolatd cockroach axon—a voltage-clamp experiment in the presence of 2×10^{-4} M 3,4-diaminopyridine. (a) Superimposed records of the sodium currents. In the control the sodium current completely inactivates in less than 6 ms. In the presence of LqhαIT toxin, the sodium inactivation is progressively slowed. The records were made every 20 s. (b) Record of the maintained sodium current. This record, which shows a very slow decrease in the sodium inward current during the voltage pulse, was taken 3 min after LqhαIT application. The slowest time constant was 250 ms instead of about 2.0 ms as observed in the absence of toxin. (C) Effect of AaH2 toxin (a) and of LqhαIT toxin (b) on action potentials in isolated rat soleus skeletal muscle fibers. For each fiber, superimposed traces were obtained for two time bases, in normal ringer (1) and 3 min after external application of the toxin (2). These records show the maximum effect obtained with saturating toxin concentrations of 10^{-7} M AaH2 (a) and 10^{-6} M LqhαIT (b). (D) Effect of LqhαIT on ionic currents of the voltage-clamped rat soleus muscle fiber. (a) Sodium currents associated with a step depolarization from a holding potential of −90 mV to −40 mV before (1) and 3 min after (2) adding 10^{-6} M LqhαIT toxin. In these records the TTX-insensitive current has been subtracted. (b) Sodium current in the same fiber as in (a) but associated with a long-duration step depolarization to −40 mV in the presence of LqhαIT. Under these conditions the current is maintained for 70 ms. (c) Effects of LqhαIT on potassium currents in rat soleus muscle fiber. The potassium current is detected as the delayed net outward current, which corresponds to the membrane current recorded in the presence of TTX (10^{-6} M) minus leakage and capacitive current. The figure shows superimposed traces of delayed net outward currents obtained during depolarization to +10 mV (holding potential −90 mV) in normal ringer (1) and 3 min after external application of 10^{-6} M of the LqhαIT toxin (2).

AMINO ACID SEQUENCES

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                1          2          3          4          5          6          7
          1     0          0          0          0          0          0          0
LqhαIT    .VRDAYIAKN YNCVYECFRD AYCNELCTKN G.ASSGYCQW AGKYGNACWC YALPDNVPIR VP.....GKC R.....
AaIT      .KKNGYAVDS SGKAPECLLS NYCNNQCTKV HYADKGYCCL L.....SCYC FGLNDDKKVL EISDTRKSYC DTTIIN
AaH2      .VKDGYIVDD VNCTYFCGRN AYCNEECTKL K.GESGYCQW ASPYGNACYC YKLPDHVRTK GP.....GRC H.....
Lqq4      GVRDAYIADD KNCVYTCGSN SYCNTECTKN G.AESGYCQW LGKYGNACWC IKLPDKVPIR IP.....GKC R.....
Lqq5      .LKDGYIVDD KNCTFFCGRN AYCNDECKKK G.GESGYCQW ASPYGNACWC YKLPDRVSIK EK.....GRC N.....
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PERCENT IDENTICAL RESIDUES

	LqαIT	AaIT	AaH2	Lqq4	Lqq5
LqhαIT	100	28	59	75	56
AaIT	28	100	33	29	31
AaH2	59	33	100	63	78
Lqq4	75	29	63	100	63
Lqq5	56	31	78	63	100

FIGURE 7: Comparison of amino acid sequences of several toxins derived from Buthinae scorpions. The excitatory insect toxins are represented by AaIT from *A. australis* Hector (Darbon et al., 1982). The α toxins affecting mammals are represented by *Leiurus quinquestriatus quinquestriatus* toxin 4 (Lqq4) (Kopeyan et al., 1982), *L. q. quinquestriatus* toxin 5 (Lqq5) (Kopeyan et al., 1978), and *A. australis* Hector toxin 2 (AaH2) (Rochat et al., 1972). Amino acid sequences were aligned for maximum similarity with the aid of the University of Wisconsin Genetics Computing Group Profile Analysis (Devereux et al., 1984). The percentage of aligned positions with identical residues is shown in the matrix below the sequences.

centration of the mammalian toxin AaH2 required to affect the same insect preparation [Figure 6A and Pelhate and Zlotkin (1981)]. The maximal effect was obtained at 10^{-7} M LqhαIT (n = 7 preparations) but 10^{-5} M AaH2 (n = 6). As shown in Figure 6C, with the rat skeletal muscle preparation, the concentration of LqhαIT required to elicit the maximum effect was at least 1 order of magnitude higher than the concentration required for AaH2. Furthermore, at saturating concentrations AaH2 caused a much greater prolongation (duration = 4700 ± 420 ms, n = 8) of the action potential than did LqhαIT (duration = 2234 ± 584 ms, n = 7).

Discussion

LqhαIT is a single polypeptide composed of 64 amino acids with a molecular weight of about 7000 (MW = 7257). The molecular size is typical of Buthinae scorpion toxins (Possani, 1984). The eight cysteines are presumed to form disulfide bonds as they do in related toxins. This hypothesis is supported by the observed high pI value (pI = 9), which is consistent with the presence of three more basic residues than acidic residues. If the sulfhydryls were free, they would be expected to lower the pI value; the calculated pI assuming free sulfhydryls is 8.02.

The amino acid sequence shows a high degree of similarity to sequences of other scorpion toxins; in particular, the positions of the eight cysteines fit the highly conserved pattern typical of such proteins (Possani, 1984). In Figure 7, the amino acid sequence of the LqhαIT toxin is compared to those of the excitatory insect toxin AaIT and three α toxins active on mammals. When aligned for maximum similarity, the LqhαIT sequence is identical with the mammalian α toxin sequences at 56%–75% of the positions. This degree of similarity equals that which occurs among the various α toxins themselves. The degree of similarity with the excitatory insect toxin AaIT is much lower; only 28% of the positions are identical. The primary sequence data give no clear indication of domains that correlate with insect or mammalian specificity. This is in agreement with a recent computer modeling study, which suggests that the specificity of the toxins is determined by rather subtle changes in the three-dimensional structure (Fontecilla-Camps, 1989).

In addition to the sequence similarity, the LqhαIT toxin resembles other α toxins in its effect on sodium conductance. LqhαIT shows a preference for interacting with insect nervious tissue while the previously described α toxins show a preference for mammalian sodium channels. Since these preferences are quantitative rather than qualitative differences, it is not surprising that LqhαIT has a low but observable toxicity to mammals.

The LqhαIT toxin was isolated by assaying with *S. falculata* (blowfly) larvae for a specific set of behavioral responses that distinguished it from the excitatory and depressant insect toxins. In the current-clamp assays on the cockroach isolated axon, LqhαIT induced an extreme prolongation of the action potential (Figure 6); this effect differed markedly from the repetitive firing induced by the excitatory toxins (Pelhate & Zlotkin, 1981) and the blocking of the action potential caused by the depressant toxins (Lester et al., 1982; Zlotkin et al., 1985). The inability of LqhαIT to displace ^{125}I-AaIT from its binding sites on insect neuronal membranes indicates that LqhαIT binds to sites different from those shared by the excitatory and depressant insect toxins. Furthermore, unlike the other types of insect toxins, LqhαIT has a low but measurable toxicity to mammals and affects the mammalian muscle sodium channel.

Scorpion toxins that affect mammals play an essential role in the pharmacological and chemical characterization of sodium channels in mammalian excitable tissues (Catterall, 1984) and are commonly subdivided into two categories, α toxins and β toxins. The α toxins, including AaH2, Lqq4, and Lqq5, affect sodium inactivation, bind in a voltage-dependent manner, and show positive cooperativity with the lipid-soluble alkaloids such as veratridine (Catterall, 1980, 1984; Catterall et al., 1976). These properties make the α toxins useful probes of the voltage-sensing mechanism of the sodium channel. The data presented above indicate that LqhαIT induces in two different excitable tissue preparations the "classical" effect on sodium inactivation previously observed in only the Buthinae scorpion venoms and the α toxins derived from them (Catterall, 1980). Thus LqhαIT is an insect α toxin. Insect α toxins are apparently also present in venoms of other scorpions. For example, the venom of *Buthus judaicus* is nontoxic to mammals but has a potent effect on sodium inactivation when assayed with an insect axonal preparation (Lester et al., 1982).

The potent toxicity of LqhαIT to insects coupled with its strong effect on sodium inactivation in insect axons may make LqhαIT useful as a tool to study sodium conductance related to insect neuronal excitability, just as the mammalian α toxins have proved so useful in studying the properties and functions of the vertebrate sodium channels (Catterall, 1980, 1984). In

addition, the activity of LqhαIT on arthropods suggests it will be an important reference point in the clarification of the structural features responsible for animal group specificity of scorpion venom toxins.

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