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Functional Duality and Structural Uniqueness of Depressant Insect-Selective Neurotoxins†

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ABSTRACT: Depressant insect-selective neurotoxins derived from scorpion venoms (a) induce in blowfly larvae a short, transient phase of contraction similar to that induced by excitatory neurotoxins followed by a prolonged flaccid paralysis and (b) displace excitatory toxins from their binding sites on insect neuronal membranes. The present study was undertaken in order to examine the basis of these similarities by comparing the primary structures and neuromuscular effects of depressant and excitatory toxins. A new depressant toxin (LqhIT2) was purified from the venom of the Israeli yellow scorpion. The effects of this toxin on a prepupal housefly neuromuscular preparation mimic the effects on the intact animal; i.e., a brief period of repetitive bursts of junction potentials is followed by suppression of their amplitude and finally by a block of neuromuscular transmission. Loose patch clamp recordings indicate that the repetitive activity has a presynaptic origin in the motor nerve and closely resembles the effect of the excitatory toxin AaIT. The final synaptic block is attributed to neuronal membrane depolarization, which results in an increase in spontaneous transmitter release; this effect is not induced by excitatory toxin. The amino acid sequences of three depressant toxins were determined by automatic Edman degradation. The depressant toxins comprise a well-defined family of polypeptides with a high degree of sequence conservation. This group differs considerably in primary structure from the excitatory toxin, with which it shares identical or related binding sites, and from the two groups of scorpion toxins that affect sodium conductance in mammals. The two opposing pharmacological effects of depressant toxins are discussed in light of the above data.

Buthinae scorpion venoms have been shown to contain two types of insect-selective neurotoxins that differ in the effects they produce in blowfly larvae. The excitatory toxins cause an immediate, fast, and reversible spastic-contractive paralysis attributed to the induction of repetitive firing in the toxin-accessible terminal branches of the motor nerves (Walther et al., 1976; Zlotkin, 1986; Zlotkin et al., 1988). These toxins, which include the AaITs[1] (Darbon et al., 1982; Loret et al., 1990) and LqqIT1 (Kopeyan et al., 1990), are single polypeptides of 70 amino acids cross-linked by four disulfide bridges, three of which are in positions identical with those found in scorpion toxins that affect vertebrates. The second group of insect-selective toxins are the depressant toxins, including BjIT2 (Lester et al., 1982) and LqqIT2 (Zlotkin et al., 1985). In contrast to the excitatory toxins, depressant toxins induce in blowfly larvae a slow, progressive, flaccid paralysis and complete relaxation of the body musculature.

The possibility of similarities between the two groups of toxins was suggested by two observations: (1) The depressant toxins competitively displace the excitatory toxin AaIT from its binding sites on insect neuronal membranes (Zlotkin et al., 1985; Gordon et al., 1984). (2) As shown below (see Results), depressant toxins induce in blowfly larvae a short transient phase of contraction that precedes the onset of flaccidity.

The present study was undertaken to examine these similarities more closely. Therefore, the primary structures of the two groups of toxins have been compared, and the effects on a fly larval neuromuscular preparation have been studied by use of a newly isolated depressant toxin.

Experimental Procedures

Bioassays. Toxicity to insects was assayed by use of laboratory-raised larvae of the blowfly *Sarcophaga falculata* (Zlotkin et al., 1971) and fifth instar *Spodoptera littoralis* larvae. Larvae of 100–130-mg body weight were injected through an abdominal intersegmental membrane. In our standard bioassay with *Sarcophaga* larvae, responses were scored 5 min after injection. Complete immobility of the larvae in an extended and flaccid position was considered a positive response. The amount of toxin inducing this response in 50% of the test animals was defined as a flaccid paralysis unit (FPU) (Lester et al., 1982; Zlotkin et al., 1985) and determined according to Reed and Muench (1938). Paralysis of *Spodoptera* larvae was scored 24 h after injection.

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[1] Abbreviations: AaH2, α mammal toxin 2 from the venom of the scorpion *Androctonus australis*; AaIT, excitatory insect toxin from the venom of *A. australis*; BjIT2, depressant insect toxin from the scorpion *Buthotus judaicus*; EJC, excitatory junction current; EJP, excitatory junction potential; LqhIT2 and LqqIT2, depressant insect toxins from the venom of the scorpions *Leiurus quinquestriatus hebraeus* and *L. q. quinquestriatus*, respectively; LqqIT1, excitatory toxin from the venom of *L. q. quinquestriatus*; MEJC, miniature excitatory junction current; Lqq4 and Lqq5, α mammal toxins 4 and 5 from the venom of *L. q. quinquestriatus*; SDS, sodium dodecyl sulfate; Ts7, β mammal toxin 7 from the venom of the scorpion *Tityus serrulatus*.

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Toxicity to mice was assayed by determining the 50% lethal dose (LD_{50}) (Reed & Muench, 1938) 24 h after subcutaneous injection into albino (variant sabra) laboratory mice. Toxicity to crustaceans was assayed by use of field-collected isopods (*Hemilepistus sp.*) of 200–400-mg body weight. Qualitative assessment of toxicity was made by injecting 5 μL of 1:10 dilutions of test fractions.

Venom and Toxins. The depressant insect toxins BjIT2 and LqqIT2 and the excitatory insect toxin AaIT were purified as previously described (Lester et al., 1982; Zlotkin et al., 1985, 1971).

Leiurus quinquestriatus hebraeus venom was obtained by electrical milking of field-collected scorpions and lyophilized. The venom was extracted with water and the water extract subjected to chromatography on Sephadex G-50 eluted with 0.1 M acetic acid in order to remove high molecular weight mucoproteins. The toxic fraction was applied to a second Sephadex G-50 column equilibrated in 0.1 M ammonium acetate, pH 8.5, in order to remove nonprotein low molecular weight pigments. The toxic fraction from this step constituted the starting material for purification of individual toxins.

Electrophoretic Techniques. SDS-polyacrylamide slab gel electrophoresis was performed in the presence of urea as described by Swank and Munkres (1971). Analytical isoelectric focusing was performed in 7.5% polyacrylamide prepared with LKB ampholines within the pH range 3.5–10 (LKB Technical Bulletin 1217-2001 ME). Proteins were visualized by staining with Coomassie Brilliant Blue G-250 as described by Righetti and Chillemi (1978).

Protein Determination. Protein concentrations were determined as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Primary Structure Determination. Each toxin was reduced, alkylated with 4-vinylpyridine, and desalted by RP-HPLC as described previously (Eitan et al., 1990). Peptides were prepared by digestion of the reduced and alkylated protein with the enzymes Asp-N, Lys-C, and trypsin (Boehringer-Mannheim, Indianapolis, IN) according to the manufacturer's directions. Partial acid hydrolysis was performed as described by Inglis et al. (1980). Peptides were separated by HPLC on a Hypersil-ODS column in 0.1% TFA using a gradient of 0–60% isopropyl alcohol/acetonitrile (1:1). Automated Edman degradations were performed with an Applied Biosystems 470A gas-phase sequencer connected to an Applied Biosystems Model 120A PTH analyzer and an M900 data system for on-line analysis of PTH-amino acids. Prior to each sequence analysis, the chromatographic system was calibrated with PTH-amino acid standards. Each sequence was confirmed in at least two separate determinations. The tryptophan at position 38 was obtained in very low yield; however, the assignment was confirmed by fast atom bombardment mass specrtrometry (performed by Research Triangle Institute, Research Triangle Park, NC) of the tryptic peptide 27–51. For sequence comparisons, amino acid sequences were aligned for maximum homology by use of the University of Wisconsin Genetics Computing Group (UWGCG) Profile Analysis software (Devereux et al., 1984). The percent of total positions containing identical residues was calculated for each pair of proteins by use of the UWGCG Distances program.

Binding Assays. ^{125}I-Labeled AaIT was prepared, and the competitive displacement binding assays were performed according to previously described procedures (Zlotkin & Gordon, 1985). Briefly, the reaction mixture (210 μL) included 1.5 nM ^{125}I-AaIT, 40 μg of locust synaptosomal membrane vesicles, and increasing concentrations of LqhIT2 in standard binding medium [0.15 M choline chloride; 1 mM $MgSO_4$; 2 mM $CaCl_2$; 0.1% BSA]. The membranes were incubated for 40 min an 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 unlabeled toxin (1 μM) was defined as the nonspecific binding.

Electrophysiological Techniques. The neuromuscular actions of LqhIT2 and AaIT were investigated with use of the longitudinal ventrolateral muscles 6A and 7A of prepupal *Musca domestica* (Irving & Miller, 1980; Adams et al., 1989). Excitatory junction potentials (EJPs) were neurally evoked by applying 0.5-ms suprathreshold current pulses to segmental nerves via a polyethylene suction electrode. Intracellular muscle recordings were made with glass micropipets filled with a mixture of 2 M potassium chloride and 2 M potassium acetate; tip resistances measured 15–20 MΩ. An Axoclamp 2A (Axon Instruments, Foster City, CA) preamplifier was used in bridge mode for synaptic potential recordings. Synaptic currents at neuromuscular terminals were recorded through a loose patch clamp as described by Dudel (1981) and modified by Bindokas and Adams (1989). Saline-filled pipets were positioned over synaptic areas so as to produce maximum amplitude records and were allowed to equilibrate 20–30 min to minimize effects of mechanical disturbances on release events. The loose seal permitted relatively rapid access of bath-applied substances to synaptic areas. Currents were amplified with an Axopatch 1B (Axon Instruments) amplifier, and output was filtered at 1 kHz through a 4-pole low-pass Bessel filter. Data were digitized and processed by use of Data 6000 waveform analyzer (Data Precision, Danvers, MA). Toxins were dissolved in physiological saline of the following composition: 140 mM NaCl, 5 mM KCl, 0.75 mM $CaCl_2$, 4 mM $NaHCO_3$, and 5 mM HEPES, pH = 7.2 (Adams et al., 1989).

Results

Separation of Toxic Activities Affecting Various Animals. Lyophilized venom of the scorpion *Leiurus quinquestriatus hebraeus* was treated as described in Experimental Procedures. The toxic fraction was separated by recycling chromatography on a series of four Sephadex G-50 columns. This produced five main fractions (I, IIa, IIb, III, and IV; Figure 1A), which differed in their toxicity to insects, crustaceans, and mammals (Table I).

Fraction I was the most potent in causing paralysis of blowfly and lepidopterous larvae but lacked toxicity to mice and crustaceans. In blowfly larvae, this fraction induced the flaccid paralysis characteristic of other depressant insect toxins and had a potency in the range of those of pure BjIT2 and LqqIT2 (Lester et al., 1982; Zlotkin et al., 1985; Table I).

Purification of a New Depressant Insect Toxin LqhIT2. Fraction I, which isoelectric focusing (Figure 2B) showed contained many proteins, was subjected to anion-exchange chromatography on DEAE-Sephadex A-25. This resulted in fraction F2 (Figure 1B), which contained about 14% of the protein content of fraction I and only about 11% of the depressant toxicity (FPU = 70 ng/100 mg of body weight). The remaining depressant toxicity can be eluted by a gradient of increasing ionic strength (data not shown).

The fraction F2 (Figure 1B) was applied to a cation-exchange column and eluted with 0.01 M ammonium acetate, pH 6.5 (Figure 1C). The entire toxicity of the F2 fraction eluted in a single peak that accounted for about 80% of the starting protein. This CM-52 fraction was designated LqhIT2. As shown in Figure 2, LqhIT2 migrated in SDS–polyacrylamide gel electrophoresis as a single species with a molecular

Table I: Toxicity of the Various Fractions Obtained by Recycling Sephadex G-50 Chromatography[a]

effect	toxicity unit (μg/100 mg or 20 g [mice] of body wt)	recycling fraction I	IIa	IIb	III	IV	crude venom
immediate contraction paralysis of blowfly larvae	CPU[b]	0.017	0.112	0.100	0.040	0.055	0.055
flaccid paralysis of blowfly larvae	FPU[c]	0.070	–	–	–	+	ND[g]
paralysis of lepidopterous larvae	PU_{50}[d]	2.60	50.0	50.0	40.0	50.0	8.5
lethality of mice	LD_{50}[e]	–	–	–	5.0	40.0	40.0
paralysis of isopods	NQD[f]	–	–	–	+	+	ND

[a] See Figure 1A. [b] One contraction paralysis unit (CPU) is defined as the amount of toxin that causes an immediate paralysis (within several seconds) in 50% of the tested larvae (Zlotkin et al., 1971a). [c] One flaccid paralysis unit (FPU) is defined as the amount of toxin that causes a flaccid paralysis in 50% of the larvae tested within 5 min of injection. [d] One paralytic unit (PU_{50}) is defined as the amount of the material that causes a loss of mobility in 50% of the larvae observed 24 h after injection. [e] One LD_{50} (lethal dose) is the amount of toxin that kills 50% of the test mice within 24 h of injection. [f] Not quantitatively determined. [g] Not determined.

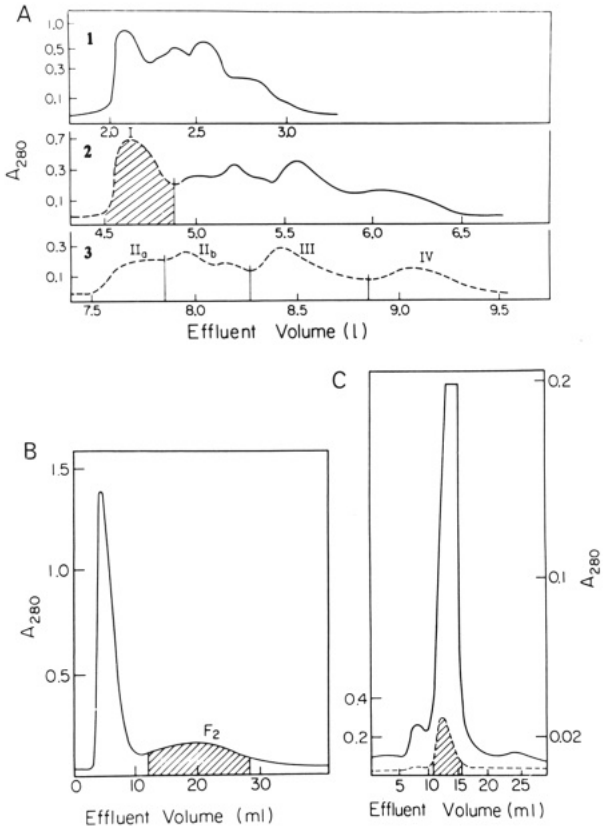

FIGURE 1: Purification of LqhIT2. (A) Separation of *L. q. hebraeus* venom by recycling gel filtration chromatography is shown. A total of 1650 A_{280} units of the toxic fraction resulting from pretreatment of 2 g of *L. q. hebraeus* venom (see Experimental Procedures) were recycled through a series of four columns (each 3.2 × 100 cm) packed with Sephadex G-50 (Zlotkin et al., 1985). The columns were equilibrated and eluted at a flow rate of 45 mL/h with 0.1 M ammonium acetate, pH 8.5. 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 I (600 OD_{280}) was collected at the beginning of the second cycle; fractions IIa (95 OD_{280}), IIb (375 OD_{280}), III (330 OD_{280}), and IV (200 OD_{280}) were continuously collected at the third cycle. (B) Separation by anion-exchange chromatography is shown. Twenty milligrams (33 OD_{280} units) of the recycling Sephadex G-50 fraction I was applied to a column (10 mL) filled with DEAE-Sephadex A-25 (Pharmacia, Sweden) and equlibrated and eluted at a flow rate of 10 mL/h with 0.01 M ammonium acetate, pH 8.5, buffer. The indicated toxic fraction (F2) contains 14% of the protein content and 11% of the toxicity of the applied material. (C) Separation by cation-exchange chromatography is shown. DEAE-Sephadex fraction F2 (5 OD_{280} units) was applied to a 10-mL column filled with carboxymethyl cellulose (CM-52; Whatman, England) equilibrated and eluted with 0.01 M ammonium acetate, pH 6.4, at 10 mL/h. The marked section (4.0 OD_{280} units) was collected and lyophilized. The specific activity in the fly larval paralysis assay of the final product was 50 ng/100 mg of body weight.

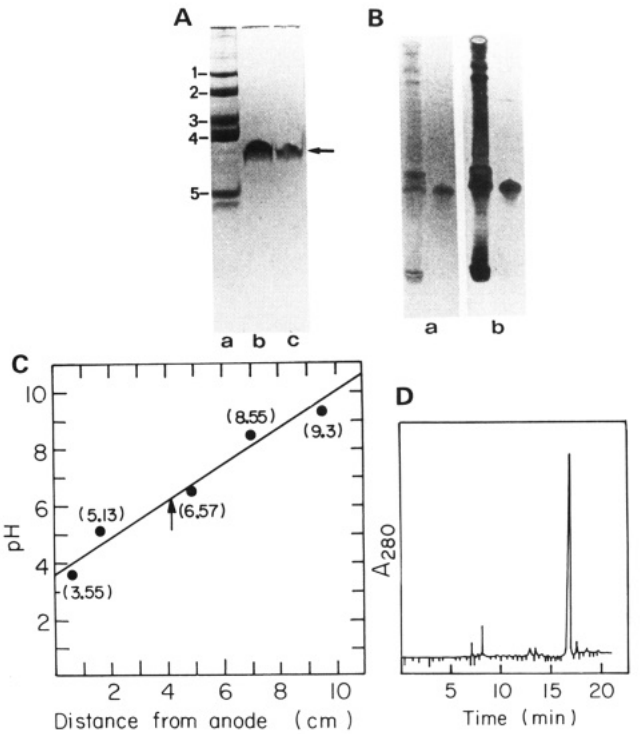

FIGURE 2: Characterization and assessment of the purity of LqhIT2. (A) The separation of LqhIT2 on SDS–polyacrylamide gel electrophoresis is shown. Polyacrylamide gel electrophoresis was performed by use of a 12.5% polyacrylamide minigel (60 × 80 × 1.5 mm) in the presence of 8 M urea and 0.1% SDS. (a) Fragments of myoglobin (Sigma, St. Louis, MO) served as molecular weight markers: (1) 16 950, (2) 14 400, (3) 8160, (4) 6210, (5) 2500. Lanes b and c represent 20 μg of LqhIT2 and 10 μg of LqhIT2, respectively. (B) Isoelectric focusing of LqhIT2 is shown. Proteins were focused in a 7.5% polyacrylamide gel by use of pH 3.5–10 ampholines. In (a), the gel was stained with Coomassie Brilliant Blue G-250: left lane, recycling Sephadex G-50 fraction I (40 μg); right lane, LqhIT2 (20 g). In (b), the gel was silver stained and the lanes are as in (a). (C) Calibration curve of p*I* markers is shown. The arrow indicates the position of the LqhIT2 toxin (p*I* = 6.4). The p*I* markers (Sigma, USA) are 3.55, *Aspergillus oryzae* amyloglucosidase; 5.13, milk β-lactoglobulin A; 6.57, human erythrocyte carbonic anhydrase B; 8.55, rabbit muscle lactic dehydrogenase; and 9.3, bovine pancreatic trypsinogen. (D) Analysis of LqhIT2 by reversed phase HPLC is shown. A total of 100 μg of the CM-52 fraction (Figure 1C) was separated on a TSK-ODS-120T RP-C18 column (4.6 × 250 mm; LKB, Sweden) and eluted with a two-part linear gradient. The solvents are (A) 0.1% trifluoroacetic acid and (B) 0.1% trifluoroacetic acid and acetonitrile/isopropyl alcohol (1:1). The gradient is (0 min) 5% B, (15 min) 20% B, and (75 min) 50% B. The single peak indicates homogeneity.

mass or approximately 5 kDa (Figure 2A), had an isoelectric point of 6.4 (Figure 2B), and eluted as a single peak during reversed phase HPLC (Figure 2C). This protein had a specific

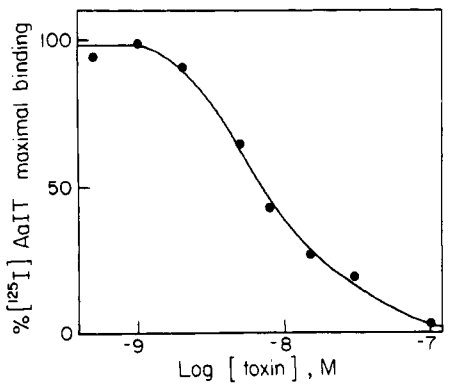

FIGURE 3: Competitive displacement of the specific binding of ^{125}I-AaIT by the depressant toxin LqhIT2. From the competitive displacement curve, the dissociation constant for LqhIT2 may be estimated from the following equation (Gordon et al., 1984; Chang & Prusoff, 1973) $K_D = K_{0.5}/1 + ([L^*]/K_D^*)$ where K_D is the dissociation constant of the competitor (LqhIT2), $K_{0.5}$ is the graphically determined concentration of the competitor inhibiting 50% of the binding of the radiolabeled ligand (7 nM), [L*] is the concentration of the radiolabeled ligand (^{125}I-AaIT = 1.5 nM), and K_D^* = the equilibrium dissociation constant of the radiolabeled ligand [1.2 nM (Gordon et al., 1984)]. From the application of this equation to the present data, the K_D for binding of LqhIT2 to insect neuronal membranes is calculated to be 3.1 nM.

toxicity to blowfly larvae of 50 ng/100 of mg body weight.

As shown in Figure 3, the LqhIT2 depressant insect toxin competitively displaced the excitatory insect toxin ^{125}I-AaIT from its binding sites in an insect neuronal membrane preparation. The K_D calculated for binding of LqhIT2 was 3.1 nM, which indicates that the depressant toxin has a high affinity for the sites that bind AaIT. This binding constant is very similar to that for AaIT itself (Gordon et al., 1984).

Primary Structure Determination of the Depressant Insect Toxins. The amino acid sequences of LqhIT2, LqqIT2, and BjIT2 are presented in Figure 4. The predicted amino acid compositions of these sequences are consistent with that determined experimentally (data not shown). A polymorphism was observed at position 15 in BjIT2. When the intact protein was sequenced, 262 pmol of isoleucine and 217 pmol of valine were recovered at this position. Both amino acids were also detected in nearly equimolar amounts when the fragment 9–55, generated by dilute acid hydrolysis, was sequenced. There was no evidence for two residues at any other position. The carboxy-terminal peptide of LqhIT2 was isolated from Lys-C, trypsin, and Asp-N digests; in all cases no residues were detected following the terminal glycine. Similarly, the carboxy-terminal peptides isolated from tryptic and dilute acid cleavages of LqqIT2 ended in cysteine–glycine as did the peptide obtained from dilute acid cleavage of BjIT2. The sequence of LqqIT2 is identical with that recently reported by Kopeyan et al. (1990), who confirmed the carboxy terminus by carboxypeptidase Y digestion. Thus, all three proteins are single polypeptides of 61 residues and contain eight cysteines that are in the conserved positions characteristic of scorpion toxins affecting mammals (Possani, 1984). The cysteines are presumed to be linked in intrachain disulfide bonds as are the

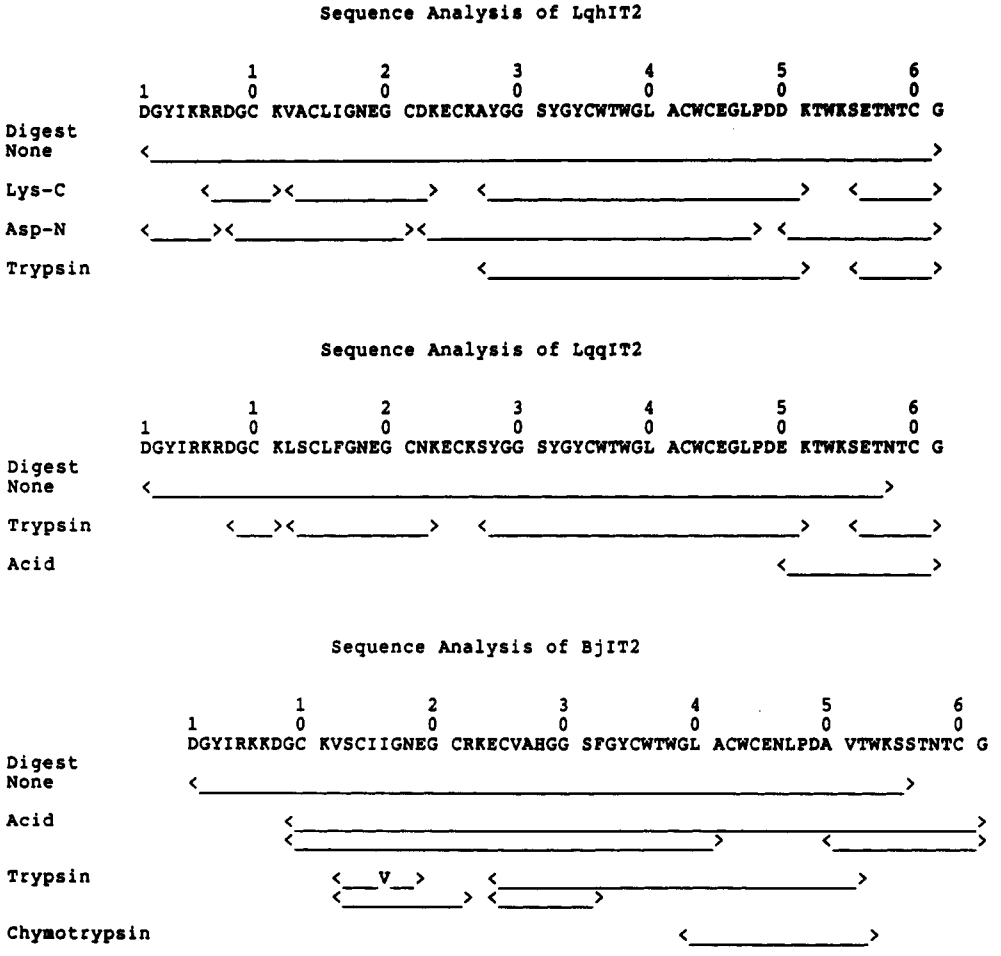

FIGURE 4: Summary proof of structures of the three depressant insect toxins LqhIT2, BjIT2, and LqqIT2. The sequences are presented in the one-letter code. In each case, the sequence was determined by automatic Edman degradation of the reduced and alkylated intact proteins (no digest) and peptides were obtained by reversed phase HPLC of digests with various enzymes and dilute acid hydrolysis. The solid line indicates the portion determined in a single sequencer run; < denotes the ends of the isolated fragments. Mass spectrometry of the tryptic fragment 27–51 confirmed its sequence.

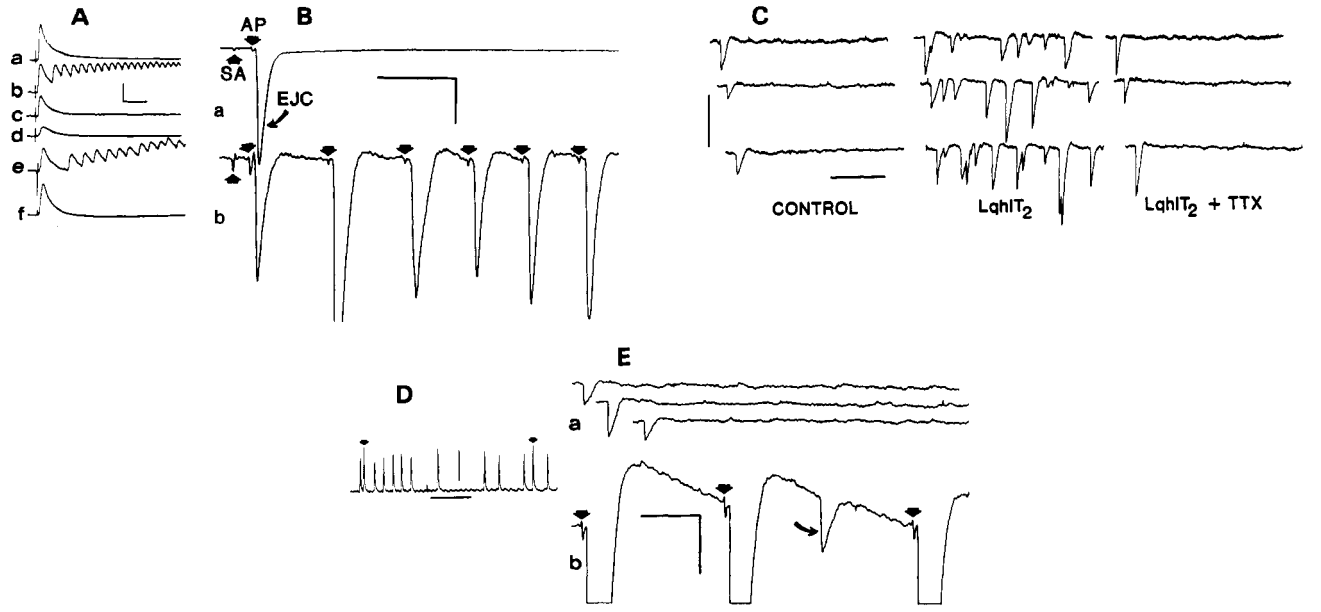

FIGURE 5: Neuromuscular effects of the depressant (LqhIT2) and excitatory (AaIT) insect toxins. (A) The depressant insect toxin LqhIT2 causes a brief phase of repetitive motor neuron activity followed by gradual suppression of excitatory junction potentials (EJPs) in prepupal *M. domestica*. Trace a shows the neuronally evoked EJP recorded in a longitudinal ventrolateral muscle prior to the addition of toxin. Trace b, recorded 2 min after the addition of 40 nM LqhIT2, shows a burst of repetitive activity coinciding with visible muscle spasms. Such repetitive activity disappears within several minutes, after which a progressive reduction in EJP amplitude is observed (traces c and d). The reduction in EJP amplitude is frequently, but not always, correlated with a 5–8-mV depolarization of the muscle resting potential. Upon removal of toxin with a saline wash, repetitive activity reappears again only briefly (trace e) and the amplitude of the EJP partially recovers over a period of 30–60 min (trace f). Vertical calibration is 20 mV; horizontal calibration is 30 ms. (B) LqhIT2 induces repetitive presynaptic action potentials and excitatory junction currents (EJCs) in housefly muscle. (a) Nerve stimulation (shown as a stimulus artifact, SA) generates a nerve terminal action potential (AP) resulting in an EJC of 2.7-nA amplitude. (b) Following treatment with 30 nM LqhIT2, a single stimulus leads to repetitive action potentials (arrows) and EJCs. Note that the control EJC amplitude is reduced from 2.7 nA to values as low as 0.6 nA. Vertical calibrations are (a) 1.0 nA and (b) 0.2 nA; horizontal calibration is 0.1 s. (C) LqhIT2 increases the spontaneous rate of neurotransmitter release, as measured by the frequency of miniature excitatory junction currents (MEJCs) recorded with a loose patch electrode. (Left) Three oscilloscope sweeps, each triggered by a single MEJC (1.2–0.5-nA amplitude), show the normally low frequency of spontaneous events (<1 Hz). (Middle) Bath application of 5 nM LqhIT2 results in a dramatic increase in MEJC frequency to 50–100 Hz within minutes, and giant events reaching several nA are occasionally observed. Due to summation and the occurrence of simultaneous MEJC events, it was not possible to quantify accurately the MEJC frequency after treatment with toxin and therefore our estimate of a 100-fold frequency increase is likely to be a conservative one. (Right) The effect of LqhIT2 is reversed upon addition of 60 nM tetrodotoxin (TTX) to the bath indicating the involvement of sodium channels. Vertical calibration is 0.5 nA and horizontal calibration is 30 ms. (D) AaIT induces repetitive activity in housefly motor neurons without suppressing EJPs or depolarizing the resting membrane potential of the muscle. Bath application of 125 nM toxin induces sporadic EJP activity. Arrows indicate neuronally evoked EJPs, showing that spontaneous EJP amplitudes are not greatly reduced by AaIT. Vertical calibration is 20 mV and horizontal calibration is 0.5 s. (E) Spontaneous junction current activity is shown before and after treatment with AaIT. (a) Each of the three oscilloscope sweeps is triggered by the infrequent appearance of a spontaneous MEJC under normal conditions. (b) Following treatment with 50 nM AaIT, spontaneous nerve terminal action potentials (arrows) evoke large amplitude (8–10 nA) EJCs (current traces are truncated to save space); note the appearance of a single spontaneous MEJC (curved arrow) between the EJCs. No increase in spontaneous MEJC frequency was observed after treatment with AaIT. Vertical calibration is 1 nA and horizontal calibration is 100 ms.

cysteines in other scorpion toxins. The numbers of negatively and positively charged residues in LqhIT2 are compatible with the p*I* estimated by analytical isoelectric focusing (Figure 2B).

Neuromuscular Action. Paralysis of Fly Larvae. Blowfly larvae were carefully observed immediately after injection while still mounted on the needle. A dose of 1 FPU of LqhIT2 caused a short (2–3 s) transient contraction of the body musculature that occurred several (3–6) seconds after injection. This contraction preceded the progressive development of the flaccid paralysis that began 30–50 s after injection; larvae were completely flaccid and immobile by 5 min after injection. Identical effects were evoked by the other depressant toxins BjIT2 and LqqIT2.

Effects on Fly Neuromuscular Junction. Bath perfusion of LqhIT2 in the concentration range 5–50 nM caused motor neuron excitation followed by suppression of neuromuscular transmission in housefly body wall muscle (Figure 5). The neuronally evoked EJPs presented in Figure 5A show that toxin application caused an initial state of brief and transient repetitive activity (Figure 5A, trace b) followed by a gradual reduction of EJP amplitude (Figure 5A, traces c and d). Both effects were reversed upon washing with toxin-free saline (Figure 5A, traces e and f). In several experiments, a 3–8-mV depolarization of the muscle resting potential was observed, although this was not always seen. In any case, muscle depolarization appeared not to be responsible for the reduction of EJP amplitude.

Loose patch recordings enabled measurement of presynaptic action potentials and the resulting synaptic currents (Figure 5B, trace a). After toxin treatment (30 nM), a single nerve stimulation led to repetitive nerve terminal action potentials and excitatory junction currents (EJCs). This indicates that the toxin-induced repetitive activity originates in the motor axons. Toxin treatment attenuated the amplitude of the EJCs (Figure 5B) just as toxin decreased the amplitude of the EJPs (Figure 5A). Thus, the depressant toxin decreased the amplitudes of synaptic events.

Low concentrations (5 nM) of LqhIT2 toxin increased, in a tetrodotoxin-sensitive manner (Figure 5C, right), the rate of spontaneous neurotransmitter release; the rate increased up to 100-fold over base-line levels (Figure 5C, left and middle). This result suggests that the mechanism of toxin action involves voltage-sensitive sodium channels. The toxin-induced increase in the spontaneous transmitter release may

AMINO ACID SEQUENCE

```
                1         2         3         4         5         6         7
        1       0         0         0         0         0         0         0
LqhIT2  ...DGYIKRR DGCKVACLIG NEG.CDKECK AYGG.SYGYC ...WTWGLAC WCEGLPDDKT WKS.ETNTCG ........
LqqIT2  ...DGYIRKR DGCKLSCLFG NEG.CNKECK SYGG.SYGYC ...WTWGLAC WCEGLPDEKT WKS.ETNTCG ........
BjIT2   ...DGYIRKK DGCKVSCIIG NEG.CRKECV AHGG.SFGYC ...WTWGLAC WCENLPDAVT WKS.STNTCG ........
AaIT    .KKNGYAVDS SGKAPECLLS N..YCNNQCT KVHYADKGYC CLL.....SC YCFGLNDDKK VLEISDTRKS YCDTTIIN
LqqIT1  .KKNGYAVDS SGKAPECLLS N..YCYNECT KVHYADKGYC CLL.....SC YCVGLSDDKK VLEISDARKK YCDFVTIN
Lqq4    GVRDAYIADD KNCVYTC.GS NS.YCNTECT KNGAE.SGYC QWLGKYGNAC WCIKLPDKVP IRI..PGKCR ........
Lqq5    .LKDGYIVDD KNCTFFC.GR NA.YCNDECK KKGGE.SGYC QWASPYGNAC WCYKLPDRVS IKE..KGRCN ........
AaH2    .VKDGYIVDD VNCTYFC.GR NA.YCNEECT KLKGE.SGYC QWASPYGNAC YCYKLPDHVR TKG..PGRCH ........
Ts7     ..KEGYLMDH EGCKLSCFIR PSGYCGRECG IKKGS.SGYC AWP.....AC YCYGLPNWVK VWDRATNKC. ........
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PERCENT IDENTICAL RESIDUES

	LqhIT2	LqqIT2	BjIT2	AaIT	LqqIT1	Lqq4	Lqq5	AaH2	Ts7
LqhIT2	100	87	79	30	31	36	43	38	39
LqqIT2		100	79	30	30	38	44	39	41
BjIT2			100	25	26	38	43	39	41
AaIT				100	87	29	33	34	31
LqqIT1					100	29	33	34	33
Lqq4						100	63	63	36
Lqq5							100	78	43
AaH2								100	44
Ts7									100

FIGURE 6: Comparison of scorpion toxin amino acid sequences. The depressant insect toxin sequences are compared with those of the excitatory toxins, AaIT (Darbon et al., 1982) and LqqIT1 (Kopeyan et al., 1990); Lqq4 (Kopeyan et al., 1978) and Lqq5 (Kopeyan et al., 1982), two mammalian α toxins isolated from *L. quinquestriatus*; AaH2, a mammalian α toxin isolated from *A. australis* (Rochat et al., 1972); and Ts7, a mammalian β toxin isolated from *T. serrulatus* (Possani et al., 1984). The sequences were aligned for maximum similarity with the aid of the University of Wisconsin Genetics Computing Group Profile Analysis (Devereux et al., 1984). The table below the sequences presents for each pair of proteins the percentage of aligned positions with identical residues.

explain the depolarization of the muscle membrane.

As shown in Figure 5D,E, the excitatory insect toxin AaIT (50–500 nM) induced sustained repetitive motor neuron activity that lasted for minutes to hours and correlated with visible, spasmodic contractions in the musculature. In contrast to the depressant LqhIT2 toxin, AaIT did not produce an appreciable reduction in the amplitude of the EJP (Figure 5D) nor did it cause a depolarization of the muscle membrane or an increase in the frequency of miniature excitatory junction currents (MEJCs) (Figure 5E, part a). The above data are in good accordance with previous information concerning the action of AaIT on a locust nerve muscle preparation (Walther et al., 1976).

Discussion

The present study was aimed at clarifying the neurophysiological and structural basis of the similarities and differences in the symptoms induced by the depressant and excitatory insect toxins. The two opposing types of symptoms induced by depressant toxins in intact blowfly larvae parallel the effects of the toxins on the housefly larval neuromuscular junction, which is a natural target for the scorpion neurotoxins (Walther et al., 1976; Zlotkin et al., 1988). The initial repetitive activity (Figure 5A, trace b) evoked in the motor nerve correlates with the brief contractive phase in intact larvae, while the reduction of synaptic potentials (Figure 5A, traces c and d) may account for the onset of flaccid paralysis.

AaIT causes repetitive firing of the motor nerve and contraction of the larvae; these effects are similar to the initial effects of depresssant toxin. The excitatory effects of both toxins are presynaptic in origin. Thus, the depressant toxins mimic the effects of the excitatory toxin both on the intact animal and in the neuromuscular preparation.

The transient excitatory effects of the depressant toxins are not likely to be the result of contaminating excitatory toxin for several reasons. First, analysis of the LqhIT2 depressant toxin preparation used for the neurophysiological studies by isoelectric focusing showed only a single component (Figure 2B). Reversed phase HPLC of either intact (Figure 2C) or reduced and alkylated material (data not shown) likewise revealed only one component. In addition, no evidence of heterogeneity was detected during amino acid sequence analysis. Most importantly, the concentration of depressant toxin required to induce the transient excitatory effects was equal to or lower than the concentration at which the excitatory toxin caused its effects.

The dominant effect of the depressant toxin was a complete block of neuromuscular transmission resulting in the final flaccid paralysis. In previous studies using the cockroach central nervous system giant axon, the insect-specific depressant toxins LqqIT2 and BjIT2 were shown to block evoked action potentials due to a tetrodotoxin-sensitive depolarization of the insect axonal membrane and suppression of the sodium current (Lester et al., 1982; Zlotkin et al., 1985). Is the dominant blocking action of LqhIT2 in the present preparation a consequence of membrane depolarization? While we were unable to measure directly toxin-induced changes in motor nerve terminal resting potential or action potential amplitude,

a clear increase in the frequency of spontaneous MEJCs was recorded following LqhIT2 exposure. Previous studies have indicated that a similar increase in MEJC frequency accompanies nerve terminal depolarization caused by elevated potassium ion concentrations or exposure to sodium channel toxins such as aconitine or pyrethroid insecticides (Salgado et al., 1983). Increased MEJC frequency following treatment with LqhIT2 thus provides indirect evidence for nerve terminal depolarization that, in turn, could sharply limit invasion of action potentials into nerve terminals and thereby suppress EJPs by reducing *evoked* transmitter release. The ability of very low concentrations (5 nM) of LqhIT2 to increase MEJC frequency suggests that depolarization may be a primary effect of the toxin on the motor system.

The absence of increased MEJC activity following AaIT treatment (Figure 5E) suggests that the excitatory toxin causes minimal, if any, depolarization of motor axon terminals. Its predominant effect is to produce sustained repetitive activity in the motor nerve (Figure 5, parts D and E) as previously shown in a locust neuromuscular preparation (Walther et al., 1976). Thus, the excitatory toxin has a single effect on the motor nerve, inducing sustained repetitive activity, while the depressant toxin induces a transient phase of repetitive activity followed by motor nerve terminal depolarization that produces the ultimate block of neuromuscular transmission. The distinctive characteristics differentiating excitatory (AaIT) from depressant (LqhIT2) insect toxins recall those previously observed with type I and type II pyrethroid insecticides. Like the excitatory toxins, the type I pyrethroids produce a slowing of sodium channel inactivation and repetitive action potential activity. In contrast, the type II pyrethroids, like the depressant insect toxins, primarily depolarize the resting membrane potential (Narahashi, 1986).

An additional similarity between the two types of insect toxins is suggested by the observation that LqhIT2 competitively displaces ^{125}I-AaIT from its binding sites on the insect neuronal membrane (Figure 3). The simplest explanation of this result is that the two types of toxins share identical binding sites. Alternatively, the two toxins could bind to separate sites which interact allosterically. A third possibility is that depressant toxins occupy two classes of binding sites, the first related to their excitatory effect and the second through which they exert their blocking–depressant effect. The two-site hypothesis is consistent with the dual nature of the depressant toxin activity. Future experimentation will be directed toward testing this hypothesis.

The pharmacologic similarities between the excitatory and depressant toxins are not evident in their primary structures. In Figure 6, the amino acid sequences of the three depressant toxins are compared with those of the excitatory toxins AaIT and LqqIT1 and those of representatives of two classes of scorpion toxins that affect vertebrates. Lqq4, Lqq5, and AaH2 are three α toxins that affect the sodium inactivation process; Ts7 is a β toxin (Couraud & Jover, 1984) that is highly toxic to mice but also induces a contraction paralysis in fly larvae and binds to the same sites in housefly head synaptosomes as does the excitatory insect toxin AaIT (DeLima et al., 1986). The data in Figure 6 show that from a structural point of view the depressant toxins comprise a well-defined family of polypeptides that differs considerably in sequence from the excitatory toxin family. The degree of sequence conservation within each of these families is strikingly high and exceeds that of the α toxin group. Although the depressant and excitatory toxins have similar effects on insect nervous tissue, no primary sequence regions are similar enough to suggest that they might account for the insect selectivity of these toxins.

It is difficult at the present stage of our knowledge to speculate on the structure–activity relationships among these toxins. Fontecilla-Camps (1989) has developed models of scorpion toxins based on crystallographic data, computer graphics, and energy minimization. These models indicate similar spatial arrangements for α toxins, β toxins, and the excitatory insect toxin. All toxins have a conserved "hydrophobic surface" that previous modification studies (Habersetzer et al., 1976; Sampieri et al., 1978; Fontecilla-Camps et al., 1982) suggested is involved in interaction with the membrane binding site. The specificity of such interactions seems likely to be modulated by small regions adjacent to the surface (Fontecilla-Camps, 1989). A more complete understanding of the structural basis for the differences and similarities of the depressant and excitatory insect toxins should be gained from future studies in which these toxins are modified, either chemically or genetically, at specific sites.

In summary, the primary sequences and neuromuscular effects of the depressant insect toxins are clearly distinguishable from other groups of scorpion toxins that affect sodium conductance. As such, these toxins may serve as tools for the study of insect excitability and models for future design of selective insecticides.

Registry No. LqhIT2, 130300-64-0; LqqIT2, 130300-67-3; BjIT2, 133164-84-8.

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Fluorescence Study of the Topology of Messenger RNA Bound to the 30S Ribosomal Subunit of *Escherichia coli*†

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ABSTRACT: Short RNAs (25–36 nucleotides in length) with sequences of the translational initiation region of bacteriophage R17 protein A mRNA were produced by chemical and in vitro transcription techniques and labeled at their 5′ or 3′ ends with fluorescent probes. The interaction of these labeled RNAs with the 30S subunit of *Escherichia coli* was studied by using fluorescence spectroscopic techniques. All the RNAs bound tightly to 30S subunits ($K_d \leq 200$ nM). Resonance energy transfer experiments demonstrated the proximity of the ends of the RNAs to each other and to two fluorescently labeled sites on the 30S subunit: the 3′ end of 16S rRNA and the cysteine residue of ribosomal protein S21. By using the distances calculated from energy transfer between the 3′ end of 16S rRNA and the ends of RNAs of varying lengths, a topological map of this region of mRNA on the 30S subunit was constructed.

The accumulation of structural information about the ribosome has provided a foundation for inquiries concerning its interaction with the other macromolecules required for protein synthesis. This paper addresses the interaction of mRNA with the 30S subunit. Specifically, fluorescence spectroscopy has been used to study the interaction of short model mRNAs with the subunit and to develop a map of the topology of the mRNA on the 30S subunit by using resonance energy transfer. The model mRNAs possess sequences of the translational initiation region of the A protein of RNA bacteriophage R17 (Steitz, 1969) and are of lengths comparable to the regions of mRNA protected from ribonuclease when bound to the ribosome (Kang & Cantor, 1985; Steitz, 1980).

The site on the ribosome most often implicated by electron microscopy in mRNA binding has been the platform/cleft structure of the 30S subunit, both by direct localization of bound mRNA (Evstafieva et al., 1983) and by localization of important interaction sites for the mRNA such as the 3′ end of the 16S rRNA (Lührmann et al., 1981; Olson & Glitz, 1979; Shatsky et al., 1979), the anti-Shine–Dalgarno region of the 16S rRNA (Olson et al., 1988) and the codon–anticodon interaction site (Gornicki et al., 1984; Oakes et al., 1986). [For a review of ribosome morphology, see Wittmann (1986) and Stöffler and Stöffler-Meilicke (1986).] Evstafieva et al. (1983) have proposed a loop or "U-turn" in the mRNA on the ribosome on the basis of their immunoelectron microscopy studies

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