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SHORT COMMUNICATIONS

POSITIVE COOPERATIVITY AMONG INSECTICIDAL SCORPION NEUROTOXINS

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R. Herrmann, H. Moskowitz, E. Zlotkin and B. D. Hammock. Positive cooperativity among insecticidal scorpion neurotoxins. *Toxicon* **33,** 1099–1102, 1995.—The insecticidal activity of scorpion neurotoxic polypeptides increased 5–10-fold with no apparent increase in mammalian toxicity when a combination of two toxins was injected. Synergistic combinations could be predicted from binding studies and competitive displacement assays. Our results indicate that simultaneous expression in baculovirus or other transgenic organisms of the synergistic combinations of insecticidal toxins may result in more potent insect-selective biopesticides.

Scorpions are known to use a cocktail of different peptide neurotoxins to immobilize their prey. These toxins can be broadly divided into insect and mammal toxins. The insect toxins are represented mainly by the excitatory neurotoxins (such as AaIT derived from the venom of the North African scorpion *Androctonus australis* (Zlotkin *et al.*, 1971), $LqhIT_3$ derived from the venom of the yellow Israeli scorpion *Leiurus quinquestriatus hebraeus*) and the depressant neurotoxins (such as $LqhIT_2$ also from *L. quinquestriatus hebraeus*; Zlotkin *et al.*, 1991). Mammalian toxins are represented by the α neurotoxins (such as AaHII from *A. australis*; Rochat *et al.*, 1979) and the β neurotoxins (such as CssII derived from the venom of the Mexican scorpion *Centruroides suffusus suffusus*; Couraud and Jover, 1984). The α and β toxins are the main factors in scorpion venom responsible for envenomation of vertebrates, but they also affect insects (Rathmayer *et al.*, 1978; DeLima *et al.*, 1986, 1989). Recently, a new toxin designated as LqhαIT, which strongly resembles α mammal toxins in its primary structure and electrophysiological effects, was isolated from the venom *L. quinquestriatus hebraeus* and was shown to affect mainly insects (Eitan *et al.*, 1990).

Binding studies of scorpion toxins have shown that α toxins bind in a voltage-dependent manner to mammalian sodium channels (Catterall, 1984). Unlike the α scorpion toxins,

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the β scorpion toxins bind in a voltage-independent manner to a distinct site other than that of the α scorpion toxins site on the mammalian sodium channels (Couraud *et al.*, 1982; Couraud and Jover, 1984). The insect toxins studied revealed no binding to rat brain neuronal membranes (Gordon *et al.*, 1984; Gordon and Zlotkin, 1993). The depressant and the excitatory insect selective toxins do not compete with the α insect toxin on its binding site (Gordon and Zlotkin, 1993). In contrast to locust or cockroach neuronal membranes there is no competitive displacement between the excitatory and the depressant insect toxins in neuronal membranes of lepidopterous larvae (Gordon *et al.*, 1992; Moskowitz *et al.*, 1994).

In the present study the various toxins AaIT, LqhIT$_3$, LqhIT$_2$ and LqhαIT were purified by established methods from the respective crude venoms (the purification and characterization of LqhIT$_3$ will be published elsewhere). The various toxins affecting insects were injected either separately or in combination and their toxicity towards mice, larvae of blow fly (*Sarcophaga falculata*) and caterpillars of a moth (*Heliothis virescens*) was determined according to the method of Reed and Muench (1938). Table 1 shows the activity of the toxins towards insects and mice in terms of the 50% end points (paralytic or lethal doses PU_{50}, LD_{50}, respectively). The PU_{50} values of the toxins to blow fly larvae were in accordance with previously published results (Zlotkin *et al.*, 1971, 1991; Eitan *et al.*, 1990; Herrmann *et al.*, unpublished results). The toxicity of those toxins towards the lepidopterous larvae of *Heliothis virescens* is comparable to their toxicity to larvae of *Spodoptera littoralis* (Herrmann *et al.*, 1990). LqhαIT showed higher toxicity towards mice (Swiss Webster) compared with the previous study using Sabra mice (Eitan *et al.*, 1990). All other toxins showed no toxicity to mammals (60 μg/20 g b.w. injected s.c. had no effect, in contrast to the LD_{50} of a mammalian toxin AaHII 0.018 μg/20 g b.w.; DeLima *et al.*, 1986). When a combination of toxins was injected simultaneously, the insecticidal activity of the toxins increased 5–10-fold (the amount of protein toxin needed to cause a fast paralytic response in insect was reduced 5–10-fold when two toxins were injected simultaneously compared to the amount of protein needed to cause paralysis by each toxin alone), and no increase in mammalian toxicity was observed (Table 2). In this study the toxin combinations included amounts corresponding to 1 PU_{50} unit of each toxin and their dilutions. Pairs of

Table 1. The activity of insect toxins derived from scorpion venom towards insects and mice

Toxins	PU_{50} to *Sarcophaga falculata* larvae (μg/100 mg b.w.)*	PU_{50} to *Heliothis virescens* larvae (μg/100 mg b.w.)†	LD_{50} to Swiss Webster mice (μg/20 g b.w.)‡
AaIT	0.0025	2.5	>60
LqhIT$_3$	0.050	2.5	>60
LqhIT$_2$	0.025	2.5	>60
LqhαIT	0.0025	2.5	8.0

* Three replicates of 25–40 blow fly larvae each were injected with each one of the toxins and the PU_{50} determined (using the method developed by Reed and Muench, (1938). The PU_{50} of the excitatory toxins AaIT and LqhIT$_3$ was determined as a concentration paralysis immediately after injection. The PU_{50} of the depressant toxin LqhIT$_2$ and of the α insect toxin LqhαIT were determined as a flaccid paralysis and a delayed and sustained contraction paralysis, respectively, 5 min after injection.

† Three replicates of 25–40 lepidopterous larvae each were injected with each one of the toxins and the PU_{50} was determined (Reed and Muench, 1938) as inability to move or turn when inverted on its back 24 hr after injection.

‡ Two replicates of eight mice were injected and the LD_{50} to mice was determined (Reed and Muench, 1938) 24 hr after injection.

Table 2. Interactions of insect toxins against insects and mice

Toxins	PU_{50} to *Sarcophaga falculata* larvae (μg/100 mg b.w.)* Dose	Change in potency§	PU_{50} to *Heliothis virescens* larvae (μg/100 mg b.w.)† Dose	Change in potency§	LD_{50} to Swiss Webster mice (μg/20 g b.w.)‡ Dose	Change in potency§
AaIT + $LqhIT_3$	0.0025 (AaIT) + 0.050 ($LqhIT_3$)	0.5 ×	2.5 (AaIT) + 2.5 ($LqhIT_3$)	0.5 ×	60 (AaIT) > + 60 ($LqhIT_3$)	No effect
AaIT + $LqhIT_2$	0.0025 (AaIT) + 0.025 ($LqhIT_2$)	0.5 ×	0.25 (AaIT) + 0.25 ($LqhIT_2$)	5 ×	60 (AaIT) > + 60 ($LqhIT_2$)	No effect
AaIT + LqhαIT	0.000125 (AaIT) + 0.000125 (LqhαIT)	10 ×	0.25 (AaIT) + 0.25 (LqhαIT)	5 ×	60 (AaIT) + 8.0 (LqhαIT)	Like lqhαIT alone (Table 1)
$LqhIT_3$ + $LqhIT_2$	Not determined		0.25 ($LqhIT_3$) + 0.25 ($LqhIT_2$)	5 ×	60 ($LqhIT_3$) > + 60 ($LqhIT_2$)	No effect
$LqhIT_3$ + LqhαIT	0.005 ($LqhIT_3$) + 0.00025 (LqhαIT)	5 ×	0.25 ($LqhIT_3$) + 0.25 (LqhαIT)	5 ×	60 ($LqhIT_3$) + 8.0 (LqhαIT)	Like LqhαIT alone (Table 1)

* Three replicates of 25–40 blow fly larvae each were injected with a combination of toxins (a 1:1 ratio of toxins PU_{50} in various dilutions was used) and the PU_{50} was determined (Reed and Muench, 1938) as fast contraction of the larvae within 1 min after injection.

† Three replicates of 25–40 lepidopterous larvae each were injected with a combination of toxins (a 1:1 ratio of toxins PU_{50} in various dilutions was used) and the PU_{50} was determined as inability to move or turn when inverted on its back. The PU_{50} was determined (Reed and Muench, 1938) 24 hr after injection.

‡ Two replicates of eight mice were injected s.c. and the LD_{50} to mice was determined (Reed and Muench, 1938) 24 hr after injection.

§ The potency was estimated as the ratio between a single toxin PU_{50} (given the value 1) and the sum of the PU_{50} fractions causing an effect when two toxins are injected simultaneously.

toxins that do not compete with each other on the same binding site and differ in their pharmacology were synergistic. In blow fly larvae it corresponded to the combinations AaIT + LqhαIT, $LqhIT_3$ + LqhαIT and in lepidopterous larvae this corresponded to the combinations AaIT + LqhαIT, AaIT + $LqhIT_2$, $LqhIT_2$ + $LqhIT_3$, LqhαIT + $LqhIT_3$ (Table 2). Thus, as shown in Table 2, the degree of cooperativity is dependent not only on the toxin combinations but also on the test animal. These data support previous information (Moskowitz *et al.*, 1994) suggesting a pharmacological diversity of sodium channels among various insects. However, the most significant implication of the present data concerns pest control. Classical insecticides are increasingly being scrutinized owing to problems associated with environmental contamination, toxicity to nontarget organisms and an increasing number of pest species becoming resistant to the available insecticides. Insect pathogens are tested as alternative control agents. However, many of these pathogens lack the fast action of chemical insecticides. Recently, the insect pathogenic virus *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV) was genetically modified to increase speed of kill, by the introduction of genes encoding insect-selective toxins into the viral genome (Maeda *et al.*, 1991; Stewart *et al.*, 1991; McCutchen *et al.*, 1991; Tomalski and Miller, 1991). Our results demonstrating synergism suggest that the speed of kill of recombinant microbial insecticides could be further increased by constructing transgenic organisms that simultaneously express two or more toxins. These

studies show that synergistic combinations of toxins can be predicted from binding data and toxin physiology. Combinations of known insect selective toxins with classical insecticides and or natural products found in crop plants or newly isolated peptide toxins will lead to the development of new, more potent biopesticides.

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