Modification of Synaptic Transmission and Sodium Channel Inactivation by the Insect-Selective Scorpion Toxin LqhαIT

DAEWOO LEE, MICHAEL GUREVITZ, AND MICHAEL E. ADAMS

1Departments of Entomology and Neuroscience, University of California at Riverside, Riverside, California 92521; and 2Department of Botany, Faculty of Life Sciences, Tel-Aviv University, Ramat-Aviv 69978, Tel-Aviv, Israel

Lee, Daewoo, Michael Gurevitz, and Michael E. Adams. Modification of synaptic transmission and sodium channel inactivation by the insect-selective scorpion toxin LqhαIT. J. Neurophysiol. 83: 1181–1187, 2000. The peptide LqhαIT is an α-scorpion toxin that shows significant selectivity for insect sodium channels over mammalian channels. We examined the symptoms of LqhαIT-induced paralysis and its neurophysiological correlates in the house fly (Musca domestica). Injection of LqhαIT into fly larvae produced hyperactivity characterized by continuous, irregular muscle twitching throughout the body. These symptoms were correlated with elevated excitability in motor units caused by two physiological effects of the toxin: 1) increased transmitter release and 2) repetitive action potentials in motor nerves. Increased transmitter release was evident as augmentation of neurally evoked synaptic current, and this was correlated with an increased duration of action potential–associated current (APAC) in loose patch recordings from nerve terminals. Repetitive APACs were observed to invade nerve endings. The toxin produced marked inhibition of sodium current inactivation in fly central neurons, which can account for increased duration of the APAC and elevated neurotransmitter release at the neuromuscular junction. Steady-state inactivation was shifted significantly to more positive potentials, whereas voltage-dependent activation of the channels was not affected. The shift in steady-state inactivation provides a mechanism for inducing repetitive activity in motorneurons. The effects of LqhαIT on sodium channel inactivation in motor nerve endings can account both for increased transmitter release and repetitive activity leading to hyperactivity in affected insects.

INTRODUCTION

Scorpions have evolved a diversity of peptide toxins comprising an effective biochemical strategy for prey capture. These toxins modify ion channels in nerve membranes, producing physiological changes leading to various types of excitatory or flaccid paralysis. A primary target of scorpion venom toxins is the voltage-sensitive sodium channel, and an extensive literature is available on their modification of mammalian (Couraud and Jover 1984; Martin-Eauclaire and Couraud 1995; Strichartz et al. 1987) and insect channels (Zlotkin et al. 1994). α-Scorpion toxins were some of the first to be described in detail, both with respect to biochemical characteristics and neurophysiological actions. To a rather surprising degree, the α-scorpion toxins are characterized by their specific modification of vertebrate sodium channels, despite the fact that scorpions prey primarily on insects. Indeed, the binding site for α-scorpion toxins (site 3) (Catterall et al. 1992) is defined exclusively by characteristic epitopes on the mammalian sodium channel (Rogers et al. 1996).

A variety of scorpion toxins specific for insect sodium channels have been identified (Zlotkin et al. 1971, 1994), but only recently was an α-scorpion toxin–like peptide with potency against insects discovered. This toxin, LqhαIT has high sequence-similarity to α-scorpion toxins and prolongs action potentials in a manner characteristic of this class of peptides (Adam et al. 1966; Eitan et al. 1990; Wang and Strichartz 1985). This effect results from inhibition of sodium channel inactivation (Catterall et al. 1992; Strichartz et al. 1987). Although also active against mammalian sodium channels, LqhαIT differs from previously described α-scorpion toxins in that it shows preference for insect channels (Eitan et al. 1990; Gordon et al. 1996; Gordon and Zlotkin 1993). Structural correlates of the toxin responsible for insect activity recently have been defined (Zilberberg et al. 1996, 1997).

In some respects, the α-scorpion toxins are among the most well-characterized elements of the scorpion venom cocktail. Many electrophysiological studies have documented their inhibition of mammalian sodium channel inactivation (Adam et al. 1966; Martin-Eauclaire and Couraud 1995; Wang and Strichartz 1985), and the characteristic α-scorpion toxin binding site (site 3) has been pinpointed through site-directed mutagenesis and photoaffinity labeling (Rogers et al. 1996). However, few studies have documented the symptoms associated with α-scorpion toxin action in the intact animals, and little has been done to relate these to their effects on synapses. It is interesting to note that α-scorpion toxins do not alter the sodium channel activation mechanism, nor are they associated with persistent depolarization of nerve membranes (Catterall 1980). Nevertheless, LqhαIT is reported to cause hyperexcitation in treated insects, the natural prey animals of scorpions (Eitan et al. 1990).

Here we provide a detailed examination of α-scorpion toxin action on an insect model at three levels: behavioral, synaptic, and sodium channel modification. We find that hyperexcitability produced in treated fly larvae is likely caused by two consequences of toxin on nerve terminals: increased transmitter release and repetitive firing of action potentials. These effects in turn can be attributed to prolongation of inward sodium current in nerve terminals and a shift of steady-state sodium channel inactivation to more positive potentials.
METHODS

Scorpion toxin

In most experiments, native LqhIT (MW = 7,258) purified from the venom of Leiurus quinquestriatus hebraeus was used (Eitian et al. 1990). In some experiments (Figs. 4 and 5), the recombinant toxin produced in Escherichia coli was employed (Zilberberg et al. 1996).

Bioassay

House flies (Musca domestica; NAIDM strain) were obtained from laboratory colonies maintained in the Department of Entomology, University of California, Riverside. For toxicity assays, third instar house fly larvae were injected with various concentrations of LqhIT dissolved in insect saline through an abdominal intersegmental membrane. All larvae used in this bioassay weighed ~20 mg.

Intracellular recording

House fly prepupae were dissected, pinned in silicone elastomer (Sylgard) dishes, and flooded with physiological saline. The saline formulation is (in mM) 135 NaCl, 5 KCl, 0.75 CaCl2, 1 MgCl2, 5 NaHCO3, and 5 HEPES, adjusted to pH 7.2. Muscles for recordings were obtained from the longitudinal ventrolateral muscles 6A and 7A of house fly (Bindokas and Adams 1989; Irving and Miller 1980). Excitatory junctional potentials (EJPs) were evoked by a suction electrode attached to the cut ends of motor nerves. Isolated voltage pulses at a rate of 0.4 Hz were generated by a Grass S88 stimulator. EJPs were measured intracellularly with glass microelectrodes (5–10 MΩ) filled with 3 M KCl. Voltages were amplified with an Axoclamp 2A (Axon Instruments) amplifier, and signals were digitized and processed with Data 6000A waveform analyzer (Analog Instruments, Woburn, MA).

Two electrode voltage clamp

Synaptic currents from house fly muscles were evoked by stimulation of a motor nerve via the suction electrode and recorded with an Axoclamp 2A in two-electrode voltage-clamp mode. Tip resistance of current passing and recording electrodes were around 2 and 5–10 MΩ, respectively. Output signals were filtered with a Warner Instruments LPF-100 low-pass filter at 0.5 kHz.

Loose patch-clamp recordings

Miniature excitatory junctional currents (MEJCs), excitatory junctional currents (EJC's), and action potential-associated currents (APACs) were measured extracellularly from house fly neuromuscular junctions by patch pipettes that were pulled and fire-polished to achieve an internal tip diameter of 10–15 μm as previously described (Bindokas and Adams 1989). The saline formulation for loose patch-clamp recordings was the same as that for intracellular recordings except for the addition of 4 mM MgCl2 to minimize minute muscle movements. Saline-filled pipettes were placed over neuromuscular junctions, located by manipulation of pipette tips to sites producing maximum amplitude records after nerve stimulation. Once a large amplitude synaptic current was obtained, slight downward force was applied to produce approximate seal resistance of ~0.5 MΩ. The loose seal permitted relatively rapid access to bath-applied toxins. Preparations were allowed to equilibrate for at least 20 min after positioning the pipette to minimize the effects of mechanical disturbance. To monitor APACs at high gain, saline containing 2–5 mM CoCl2 or CdCl2 (see figure captions) was perfused to block EJCs. MEJCs, EJC's, and/or APACs were amplified with an Axopatch 200A (Axon Instruments), and output was filtered at 1 kHz through a four-pole low-pass Bessel filter and processed with a Data 6000A.

RESULTS

LqhIT induces an excitatory paralysis

Injection of house fly larvae with microgram doses of LqhIT caused hyperactivity leading to paralysis and death. Larvae (~20 mg body wt) injected with 1.5 μg LqhIT exhibited moderate sporadic contractions; local twitching and rolling of the body occurred within 1–2 min of injection. Within 10 min intense hyperactivity, manifested as uncoordinated and continuous muscle twitches throughout the insect body, was observed. In the dose range 0.15–1.5 μg/larva, hyperactivity persisted for several days until death occurred. In contrast, larvae injected with an equivalent volume (1 μl) of distilled water (n = 3) or physiological saline (n = 7) showed none of the symptoms mentioned above.

Augmentation of synaptic responses and repetitive activity caused by LqhIT

We observed the effects of LqhIT on neuromuscular responses after bath application of toxin to the larval musculature in vitro. Body wall muscles of house fly prepupae typically respond to nerve stimulation with a single EJP, but do not contract (Fig. 1, dotted lines). At the lowest concentrations of LqhIT (300 pM), evoked synaptic responses increased in size...
LqhαIT prolongs the duration of the presynaptic action potential

We investigated further the physiological basis of increased excitability by measuring synaptic currents. Using two-electrode voltage clamp, we measured evoked synaptic currents in muscles 6A and 7A (Fig. 2A, trace a). As shown in Fig. 2A, trace b, application of 5 nM LqhαIT caused a significant increase in the amplitude of the evoked EJC after 5 min of exposure. After 10 min, the EJC was increased by threefold, and repetitive responses were registered (Fig. 2A, trace c).

Because α-scorpion toxins are known to affect neuronal sodium channels, we hypothesized that the increase in EJC amplitude resulted from a presynaptic action of LqhαIT, probably through elevated transmitter release. However, augmented EJC amplitudes also could result from increased sensitivity of the postsynaptic cell to released neurotransmitter, a postsynaptic effect. The latter possibility would be reflected as larger amplitudes of spontaneous MEJCs after toxin exposure. In loose patch-clamp recordings from junctional areas under control conditions, we observed MEJCs, the amplitudes of which remained unchanged after exposure to the toxin (Fig. 2B). We averaged a total population of 224 MEJCs accumulated from 4 independent experiments before and after application of 2.5 nM LqhαIT and found that the amplitudes remained unchanged. These data indicate that postsynaptic membrane sensitivity to released transmitter was unaltered by the toxin. We also observed no significant difference in the frequency of spontaneous MEJCs before and after toxin exposure (Fig. 2C).

We next analyzed the effect of LqhαIT on nerve terminal action potentials. Simultaneous measurements of presynaptic currents and postsynaptic potentials are shown in Fig. 3. Under normal conditions, nerve stimulation led to single EJCs and EJPs. Preceding each EJC was a small current that appeared in all-or-none fashion as stimulus intensity was increased. This current, which we refer to as APAC, appeared abruptly at the same stimulus intensity as the EJC and EJP. After application of LqhαIT (10 nM), two obvious changes in synaptic responses were observed: increased EJC and EJP amplitude, and repetitive activity. Notably, each EJC was preceded by an APAC, suggesting that repetitive postsynaptic events originated presynaptically.

To confirm that the APAC indeed represented the presynaptic action potential, we first blocked the EJC by application of 2 mM cadmium (Fig. 4A). No effect on the APAC was observed (Fig. 4B). Application of 5 nM LqhαIT to the nerve terminal caused a significant increase in the amplitude of the evoked EJC (Fig. 4C). This resulted in increased repetitive activity (Fig. 4D). The increase in EJC amplitude of 5 min after application of 5 nM LqhαIT (trace b). Trace c: after 10 min exposure, a further increase of the evoked EJC was evident as well as repetitive responses. B: averaged spontaneous miniature excitatory junctional currents (MEJCs) recorded using a loose patch-clamp technique before and after application of 2.5 nM toxin. Averaged MEJCs before and after toxin exposure are shown (224 events averaged over 4 experiments). C: the frequency of spontaneous MEJCs before (Control) and after (LqhαIT) application of toxin (2.5 nM). Error bars depict standard deviation for 4 experiments.
evident. Subsequent addition of tetrodotoxin (2 μM) to the bath abolished the APAC, confirming its dependence on voltage-activated sodium channels. We conclude from this experiment that the APAC represents the presynaptic action potential as it invades the nerve terminal.

Exposure of the preparation to LqhαIT (20 nM) led within 1 min to repetitive EJCs, each preceded by an APAC (Fig. 5, A–C). After addition of cadmium to the bath, repetitive APACs could be observed in the absence of EJCs. Again, APACs were abolished after TTX application (Fig. 5D). Under these conditions, we noted a significant increase in duration of the APAC. Further analysis of the APAC at high gain showed that LqhαIT increased APAC duration by more than twofold (n = 5; Fig. 6).

In some experiments, variability in the amplitude of the APAC was observed, probably due to slight changes in the position of the loose patch pipette caused by minute muscle movements. For this reason, APACs often were normalized for comparison. These results indicate that action potentials invading nerve terminals are prolonged after exposure to LqhαIT. To verify that these changes originate at the level of the sodium channel, we recorded whole cell currents from dissociated central neurons.

**Slowing of sodium channel inactivation by LqhαIT**

We recorded voltage-dependent sodium currents from dissociated central neurons (20–30 μm diam) of M. domestica using whole cell patch-clamp recordings. Because recordings from freshly dissociated fly neurons produced only minute sodium currents, a protocol was developed for short-term cultured central neurons dissociated from thoracic and abdominal ganglia (Fig. 7A). Robust, voltage-dependent sodium currents were measured in cultured neurons within 1–2 days (Fig. 7B). Currents activated in the range of −30 and −40 mV and inactivated rapidly. Peak currents occurred at around −5 mV (Fig. 8, A and D). All currents were uniformly sensitive to
TTX, and we observed no evidence of biophysical or pharmacological heterogeneity in any of the neurons sampled. LqhαIT (20 nM) produced a large increase in steady-state sodium current without changing the rising phase of the current (Figs. 7C and 8B). Higher concentrations (40 nM) of LqhαIT produced no further change..., indicating that the effect of the toxin at 20 nM was complete within 1 min of application (Fig. 7D). All neurons sampled had TTX-sensitive sodium currents, confirmed by application of this toxin at the conclusion of the experiment. Voltage-dependent activation was unaffected, but peak sodium currents were slightly augmented (Figs. 8D and 9A). Test potentials to activate 50% of sodium channels, were −19.6 ± 0.2 mV before and 20.8 ± 0.6 mV after application of the toxin, respectively. No changes in the reversal potential were evident.

Modification of sodium channel inactivation by LqhαIT was particularly obvious in comparisons of steady-state inactivation (Fig. 9B). For these curves, a double-pulse protocol was employed, consisting of 100-ms prepulses to various voltages followed by the test potential (−10 mV; Fig. 9B). Peak sodium currents evoked by the test potential were normalized to the maximum peak current and plotted as a function of prepulse potentials. The h∞ curve was fitted by the Boltzman equation: $h_\infty = (1-C)/(1+\exp[(V_p - V_{1/2})/k]) + C$, where $V_{1/2}$ is the voltage at half-inactivation, $V_p$ and $k$ are the prepulse potential and the slope factor, respectively, and $C$ is the non-inactivated fraction. LqhαIT (20 nM) shifted the $h_\infty$ curve to more positive potentials and produced a small percentage of steady-state current (~10%) that remained activated (Fig. 9B). We note in particular that, under normal conditions, a −20-mV prepulse inactivated virtually all available sodium channels in the absence of the toxin. After LqhαIT treatment, ~40% of the current remained at this prepulse potential.

**DISCUSSION**

We have shown that the behavioral effects of LqhαIT in the house fly are associated with modification of neuromuscular transmission. The early effects of LqhαIT are characterized by local twitching of the body wall and rolling of the whole insect. Subsequently, the body wall musculature throughout the insect becomes progressively more hyperactive, culminating in lethality. Our observations indicate that these symptoms can be explained by increased excitability of efferent motor units.
through two main mechanisms: 1) elevation of evoked neurotransmitter release and 2) repetitive firing of motor axons.

Elevated levels of transmitter release, evidenced here as increases in EJP and EJC amplitudes, are likely the result of a prolonged action potential duration in motor neurons. Evidence for this comes from the increased durations of action potential–associated current invading motor nerve terminals. Sustained depolarization of the nerve terminal allows for increased Ca$^{2+}$ influx and hence increased neurotransmitter release (Katz and Miledi 1967). The progressive increase in transmitter release caused by the toxin accounts for the gradual increases in twitch contractions observed in affected animals.

Repetitive activity caused by LqhαIT was evident both presynaptically in nerve terminals (APAC, EJCs) and postsynaptically in body wall muscles (EJPs). This repetitive activity originates in the nerve terminal because it persists in the presence of extracellular cobalt, which abolishes postsynaptic responses through blockade of transmitter release. Furthermore, each postsynaptic event was found to be preceded by an action potential–associated current. Repetitive motor-unit ac-

FIG. 8. Sodium current (I-V) relationships before (A) and after (B) application of LqhαIT (20 nM) evoked by test potentials from −45 to −10 mV in 5-mV increments. The holding potential was −105 mV. C: steady-state sodium currents were sampled at points indicated in A and B by arrows $I_{Na(s-s)}$, and plotted as a function of test potentials. D: typical I-V curves for peak currents before and after application of LqhαIT.
tivity combined with the appearance of muscle action potentials lead to intense and prolonged muscle contraction observed at the behavioral level.

The neuromuscular effects of Lqh\textit{aIT} can be understood at the level of the sodium channel as a modification of the sodium channel inactivation process. We found that steady-state inactivation in house fly neurons is increased after exposure to 20 nM Lqh\textit{aIT}. Furthermore, the steady-state inactivation curve is shifted markedly to more positive potentials and is incomplete even at test potentials greater than or equal to $+20$ mV. Similar concentrations of the toxin also inhibit inactivation of sodium channels in cockroach giant axons, mammalian muscle fibers (Eitan et al. 1990), and mammalian peripheral nerves (Martin-Eauclaire and Couraud 1995; Strichartz et al. 1987; Wang and Strichartz 1982, 1985). Such effects of the toxin are not observed in insect muscle, due to the absence of voltage-activated sodium current. Instead, calcium channels are responsible for excitatory electrogenesis in insect muscle.

We have shown that, under normal conditions, a significant portion of the h$\infty$ curve for housefly central neurons lies to the left of the m$\infty$ curve. However, after toxin exposure, the steady-state inactivation curve shifted to the right of the m$\infty$ curve, providing a plausible explanation for the appearance of repetitive firing in house fly motoneurons. The consequences of decreased steady-state sodium channel inactivation include changes in threshold and tendency to fire repetitively (Valbo 1964). We have shown that the specific effects of Lqh\textit{aIT} on house fly motor units include inhibition of sodium channel inactivation, prolonged action potential duration, and increased transmitter release. These observations provide a physiological basis for the in vivo effects of Lqh\textit{aIT}.

Although Lqh\textit{aIT} cannot be characterized as an “insect-specific” neurotoxin, it is reported to be more selective for insects (Eitan et al. 1990). These workers cited a 20-fold higher toxicity to insects over mammals. Data presented here show 20 nM Lqh\textit{aIT} produces maximal inhibition of sodium channel inactivation in house fly neurons. Much higher concentrations ($\geq 200$ nM) are needed to elicit similar effects on sodium channels in rat dorsal root ganglion neurons (Norris et al. 1995; Zilberberg et al. 1996). In combination, these data provide a molecular basis for the selective toxicity of Lqh\textit{aIT} in insects versus mammals.

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Present address of D. Lee: Dept. of Anatomy and Neurobiology, University of California, Irvine, CA 92697.

Address for reprint requests: M. E. Adams, Dept. of Neuroscience, 5149 Boyce Hall, University of California, Riverside, CA 92521.

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