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ELECTROPHYSIOLOGICAL ANALYSIS OF THE NEUROTOXIC ACTION OF A FUNNEL-WEB SPIDER TOXIN, δ-ATRACOTOXIN-HV1a, ON INSECT VOLTAGE-GATED Na^+ CHANNELS

FRANÇOISE GROLLEAU[1,*], MARIA STANKIEWICZ[2], LIESL BIRINYI-STRACHAN[3], XIU-HONG WANG[4], GRAHAM M. NICHOLSON[2], MARCEL PELHATE[1] AND BRUNO LAPIED[1]

[1]*Laboratoire de Neurophysiologie UPRES EA 2647 (RCIM), Université d'Angers, UFR Sciences, 2 Boulevard Lavoisier, F-49045 Angers Cedex, France,* [2]*Institute of Biology, Laboratory of Biophysics, N. Copernicus University, ul. Gagarina 9, 87-100 Torun, Poland,* [3]*Department of Health Sciences, University of Technology, Sydney, City Campus, Broadway, NSW 2007, Australia* and [4]*Department of Biochemistry, University of Connecticut Health Center, Farmington, CT 06032, USA*

*e-mail: francoise.grolleau@univ-angers.fr

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Summary

The effects of δ-ACTX-Hv1a, purified from the venom of the funnel-web spider *Hadronyche versuta*, were studied on the isolated giant axon and dorsal unpaired median (DUM) neurones of the cockroach *Periplaneta americana* under current- and voltage-clamp conditions using the double oil-gap technique for single axons and the patch-clamp technique for neurones. In parallel, the effects of the toxin were investigated on the excitability of rat dorsal root ganglion (DRG) neurones. In both DRG and DUM neurones, δ-ACTX-Hv1a induced spontaneous repetitive firing accompanied by plateau potentials. However, in the case of DUM neurones, plateau action potentials were facilitated when the membrane was artificially hyperpolarized. In cockroach giant axons, δ-ACTX-Hv1a also produced plateau action potentials, but only when the membrane was pre-treated with 3-4 diaminopyridine. Under voltage-clamp conditions, δ-ACTX-Hv1a specifically affected voltage-gated Na^+ channels in both axons and DUM neurones. Both the current/voltage and conductance/voltage curves of the δ-ACTX-Hv1a-modified inward current were shifted 10 mV to the left of control curves. In the presence of δ-ACTX-Hv1a, steady-state Na^+ channel inactivation became incomplete, causing the appearance of a non-inactivating component at potentials more positive than −40 mV. The amplitude of this non-inactivating component was dependent on the holding potential. From this study, it is concluded that, in insect neurones, δ-ACTX-Hv1a mainly affects Na^+ channel inactivation by a mechanism that differs slightly from that of scorpion α-toxins.

Key words: insect neurone, vertebrate neurone, Na^+ channel, spider neurotoxin, atracotoxin, funnel-web spider, *Hadronyche versuta*.

Introduction

Many venoms from different animal phyla (e.g. sea anemones, spiders, scorpions and snails) contain small neurotoxic peptides. Many of these venoms have been studied intensively and were found to target mammalian and insect neuronal ionic channels. The receptor site of a large variety of these neurotoxins is the voltage-gated Na^+ channel that is essential for the initiation and conduction of action potentials in most excitable tissues. For example, a number of polypeptide toxins have been extracted from various species of scorpion; those designated scorpion α-toxins mainly interfere with the inactivation of the Na^+ channel, and those designated β-toxins mainly interfere with the activation mechanisms of the Na^+ channel (Gordon, 1997; Pelhate et al., 1998). Some of these neurotoxins have been used as tools for functional mapping and characterization of the voltage-dependent Na^+ channel (for reviews, see Catterall, 1980; Catterall, 1992; Catterall, 1996; Fainzilber et al., 1994; Rogers et al., 1996; Marban et al., 1998).

Spider venoms also possess diverse types of neurotoxin (Jackson and Parks, 1989). Arguably, the most lethal spider toxins are those isolated from Australian funnel-web spiders (Araneae, Hexathelidae, Atracinae). These neurotoxins have been shown to target the voltage-gated Na^+ channel by slowing the inactivation process (Nicholson et al., 1994; Nicholson et al., 1998) and to share a similarity of action with polypeptide scorpion and sea anemone α-toxins by interacting with neurotoxin receptor site 3 of the voltage-gated Na^+ channel (Fletcher et al., 1997; Little et al., 1998a). Interestingly, they have also been shown to be moderately insecticidal and to compete for binding of the insect-selective scorpion α-toxin,

LqhαIT, to cockroach neuronal membranes (Little et al., 1998b). The spider toxins, however, were structurally unrelated to those of other invertebrates since their three-dimensional folding differed completely from the previously determined structure of the scorpion α-toxins Aah II (Fontecilla-Camps et al., 1982) and LqhαIT (Tugarinov et al., 1997) and the sea anemone toxin anthopleurin-B (Monks et al., 1995). Thus, these spider toxins define a new family of polypeptides that modulate the function of voltage-gated Na^+ channels.

Two species have been extensively studied. These are the Sydney funnel-web spider *Atrax robustus* and the Blue Mountain funnel-web spider *Hadronyche versuta*. Interest in venom toxicology has been concerned primarily with these two Australian species of funnel-web spider because they are neurotoxic to humans and, consequently, have direct medical relevance (Hodgson, 1997; Browne, 1997; Miller et al., 2000). Two peptide neurotoxins, δ-ACTX-Hv1a (formerly versutoxin) from *H. versuta* (Brown et al., 1988) and its homologue δ-ACTX-Ar1 (formerly robustoxin) from *A. robustus* (Sheumack et al., 1985), have been isolated. Both toxins cause neurotoxic symptoms in primates (Mylecharane et al., 1989), and δ-ACTX-Hv1a has also been shown to affect insects (Little et al., 1998a). This toxin causes a delayed contractile paralysis of blowfly larvae and crickets. With high concentrations, contractile paralysis is immediate and eventually leads to death 2–4 days later. The LD_{50} of δ-ACTX-Hv1a in crickets after 72 h is approximately 770 pmol g^{-1}. These symptoms are similar to those reported for LqhαIT (Eitan et al., 1990). Patch-clamp electrophysiological studies on mammalian dorsal root ganglion (DRG) neurones have shown that these toxins acted by slowing tetrodotoxin-sensitive Na^+ current inactivation (Nicholson et al., 1994; Nicholson et al., 1998). However, the effect on action potential excitability was not assessed.

Although many studies of arachnid venoms (e.g. scorpions) have focused on insect-specific neurotoxins, little is known about the effects of peptide spider toxins on insects, a natural prey of spiders. In the present study, we have therefore investigated the effects of δ-ACTX-Hv1a on the inward Na^+ currents of isolated giant axons and of neurosecretory cells identified as DUM neurones (Grolleau and Lapied, 2000) from the cockroach *Periplaneta americana*. For comparative purposes, the most active insect-specific α-toxin from the venom of the scorpion *Leiurus quinquestriatus hebraeus*, LqhαIT (Eitan et al., 1990; Pelhate et al., 1998), has also been investigated.

Materials and methods

Preparations

Experiments were carried out on isolated giant axons dissected from abdominal nerve cords (Pelhate and Sattelle, 1982) and on short-term cultured adult DUM neurones isolated from the terminal abdominal ganglion (Lapied et al., 1989) of the adult male cockroach *Periplaneta americana*. Rat dorsal root ganglion (DRG) neurones were acutely dissociated and maintained in short-term primary culture using the method described by Nicholson et al. (Nicholson et al., 1994; Nicholson et al., 1998).

Electrophysiology

Isolated giant axon

Electrophysiological recordings under current-clamp and voltage-clamp conditions were carried out using the double oil-gap single-fibre technique (Pichon and Boistel, 1967). The methods of axon isolation and the recording techniques have been described previously in detail (Pelhate and Sattelle, 1982). Isolated axons were superfused with a physiological saline solution containing (in mmol l^{-1}): 210 NaCl, 3.1 KCl, 5.4 $CaCl_2$ and 5.2 $MgCl_2$. The saline was buffered with 1 mmol l^{-1} Hepes, pH 7.2. When necessary, K^+ currents were blocked by 3,4-diaminopyridine (3,4DAP; Sigma Chemical, France). The current and voltage traces were visualised on a digital oscilloscope (Tektronix) and digitized. The digitized traces were subsequently transferred to a desktop computer (Hewlett Packard) and stored on floppy disks for further analysis. The current traces were usually corrected for non-specific capacitative and leakage currents using an analogue compensation circuit, as described by Hille and Campbell (Hille and Campbell, 1976).

Isolated neurones

We used the patch-clamp technique in whole-cell recording configuration (Hamill et al., 1981) to record electrical activity and inward Na^+ currents with an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA, USA). Micropipettes were pulled from borosilicate glass capillary tubes. The liquid junction potential between the internal and external solutions was always corrected before the formation of the gigaohm seal (>3 GΩ). For current-clamp recordings, rat DRG neurone action potentials were evoked by applying 1–2 ms supramaximal current pulses at 0.1 Hz. Evoked action potentials were recorded using micropipettes containing (in mmol l^{-1}): 110 KCl, 10 NaCl, 5 Hepes, with the pH adjusted to 7.0 with 1 mol l^{-1} KOH. The external solution contained (in mmol l^{-1}): 120 NaCl, 3 KCl, 1.8 $CaCl_2$, 1.8 $MgCl_2$, 10 D-glucose, 10 Hepes; with the pH adjusted to 7.4 with 1 mmol l^{-1} NaOH. Large round light-coloured DRG cells with diameters of 20–40 μm were selected for experiments. Cells were rejected if resting membrane potentials were less than −50 mV.

To record action potentials from isolated DUM neurones, patch pipettes were filled with a solution of the following ionic composition (in mmol l^{-1}): 160 potassium aspartate, 10 KF, 1 ATP-Mg, 0.5 $CaCl_2$, 15 NaCl, 1 $MgCl_2$, 10 EGTA, 10 Hepes, with the pH adjusted to 7.4 with KOH. The solution superfusing the cells contained (in mmol l^{-1}): 200 NaCl, 3.1 KCl, 5 $CaCl_2$, 4 $MgCl_2$, 10 Hepes, pH was adjusted to 7.4 with NaOH. For voltage-clamp experiments, cells were clamped at a holding potential of −90 mV, and 30 ms test pulses were applied at 0.3 Hz. Cells that exhibited an 'all-or-none' current/voltage relationship or a stepwise activation of currents

were presumed to be inadequately clamped and were discarded. Command potentials were generated by a programmable stimulator (SMP310, Biologic) or an IBM Pentium 100 computer (with software control pClamp, version 6.0.3, Axon Instruments) connected to a 125 kHz labmaster DMA data-acquisition system (TL-1-125 interface, Axon Instruments). Subtraction of capacitative and leak currents was performed using the P/6 procedure at the beginning of each experiment. Na^+ currents were recorded on-line on the hard disk of the computer (sampling frequency 30.3 kHz) for later off-line analysis using pClamp software. All experiments were performed at room temperature (21–25 °C). Data, when quantified, are expressed as means ± S.E.M.

Toxins

Preparation of δ-ACTX-Hv1a

Crude venom from *Hadronyche versuta* was 'milked' by direct aspiration from the chelicerae of live adult male or female spiders, maintained in a colony, using silanised (Coatasil, Ajax Chemicals, Australia) glass pipettes. Crude venom was washed from the pipettes with 0.1 % (v/v) trifluoroacetic acid (TFA), and δ-ACTX-Hv1a was isolated and purified by reverse-phase high-performance liquid chromatography (rpHPLC). Purification was achieved using a Pharmacia HPLC system employing a Vydac analytical rpHPLC column (C_{18}, 4.6 mm×250 mm, 5 μm). Pooled venom was applied to the column, and venom components were eluted at a flow rate of 1 ml min^{-1} using a linear gradient of 5 % to 25 % acetonitrile/0.1 % TFA over 22 min, followed by a gradient of 25 % to 50 % acetonitrile/0.1 % TFA over 48 min. Fractions containing δ-ACTX-Hv1a (which eluted at 30 % acetonitrile) were then further purified using a linear gradient of 23 % to 32 % acetonitrile/0.1 % TFA over 20 min at a flow rate of 1 ml min^{-1}. Toxin quantification was performed using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin as a standard. Absorbances were read at 570 nm on a Bio-Rad (model 450) microplate reader. The molecular mass was determined by electrospray ionisation mass spectrometry. The fraction containing δ-ACTX-Hv1a (M_r=4852) was stored lyophilised at –20 °C in 5–10 nmol samples.

When required, δ-ACTX-Hv1a was dissolved in 10 mmol l^{-1} Hepes-Tris buffer (pH 6.0), and a sample of this stock solution was diluted in the external solution. For comparative studies between the two insect neuronal preparations, the concentration of δ-ACTX-Hv1a was always 5–10 times higher for giant axon preparations than for isolated DUM neurones (Stankiewicz et al., 1996). This was explained by the method used for isolating the giant axon (Pelhate and Sattelle, 1982). The mechanical micro-dissection used in this case did not allow complete elimination of the glial environment surrounding the isolated axon, and this restricted the penetration of pharmacological agents. In contrast, isolated DUM neurone cell bodies were obtained after enzymatic treatment and mechanical dissociation of the median parts of the terminal abdominal ganglion (see above). Any unused δ-ACTX-Hv1a stock solution was kept at 4 °C and used within 2 weeks.

Preparation of LqhαIT

LqhαIT was produced by recombinant techniques in *Escherichia coli*, renatured and purified as described previously (Zilberberg et al., 1997), and was a generous gift of Professor M. Gurevitz, Tel-Aviv University, Ramat Aviv, Israël.

Results

Effects of δ-ACTX-Hv1a on DRG neurone action potentials

Under current-clamp conditions, δ-ACTX-Hv1a (30 nmol l^{-1}) initially produced a prolongation of the repolarizing phase of the action potential (Fig. 1Ai). The falling phase of the action potential developed a broad shoulder, resulting in a 'plateau' action potential lasting up to 100 ms (Fig. 1Ai,ii). Although a small depolarization of 3.2±0.8 mV (N=3) was observed, neither resting membrane potential nor action potential amplitude was significantly altered in the presence of the toxin. During the development of the plateau potential, δ-ACTX-Hv1a also caused the development of spontaneous repetitive firing (Fig. 1Aii,iii), which tended to increase in frequency throughout toxin application. Even prolonged washing of the preparation with toxin-free solution could not reverse these effects.

Effects of δ-ACTX-Hv1a on cockroach giant axon and DUM neurone action potentials

The effects of δ-ACTX-Hv1a on the action potential of a cockroach giant axon are illustrated in Fig. 1Bi and the effects on isolated DUM neurones in Fig. 1Ci. In the presence of the toxin, a shoulder progressively developed on the falling phase of the action potentials. As in DRG neurones, neither action potential amplitude nor resting membrane potential was modified by the toxin. In axonal preparations, the effect of 0.5 μmol l^{-1} δ-ACTX-Hv1a on action potential duration occurred only in the last third of the repolarization, resulting in a suppression of the afterhyperpolarization and a slight increase in spike duration (Fig. 1Bi). Interestingly, when the axonal preparation was pre-treated with 3-4 diaminopyridine (3-4DAP), which is known to slow down the falling phase of the action potential by selectively inhibiting the voltage-dependent K^+ current (Fig. 1Bii), 0.5 μmol l^{-1} δ-ACTX-Hv1a co-applied with 3-4DAP markedly prolonged the action potential duration (compare Fig. 1Bi with Fig. 1Biii). In this case, the action potential duration (approximately 50 ms) was more than 10 times longer than with 3-4DAP applied alone.

In the case of DUM neurones, action potentials could be evoked by injecting a depolarizing current pulse, but these cells were also capable of generating spontaneous action potentials whose characteristics have been described elsewhere (Grolleau and Lapied, 2000). As illustrated in Fig. 1Ci, 0.1 μmol l^{-1} δ-ACTX-Hv1a affected evoked action potentials by prolonging the repolarization. A plateau action potential progressively

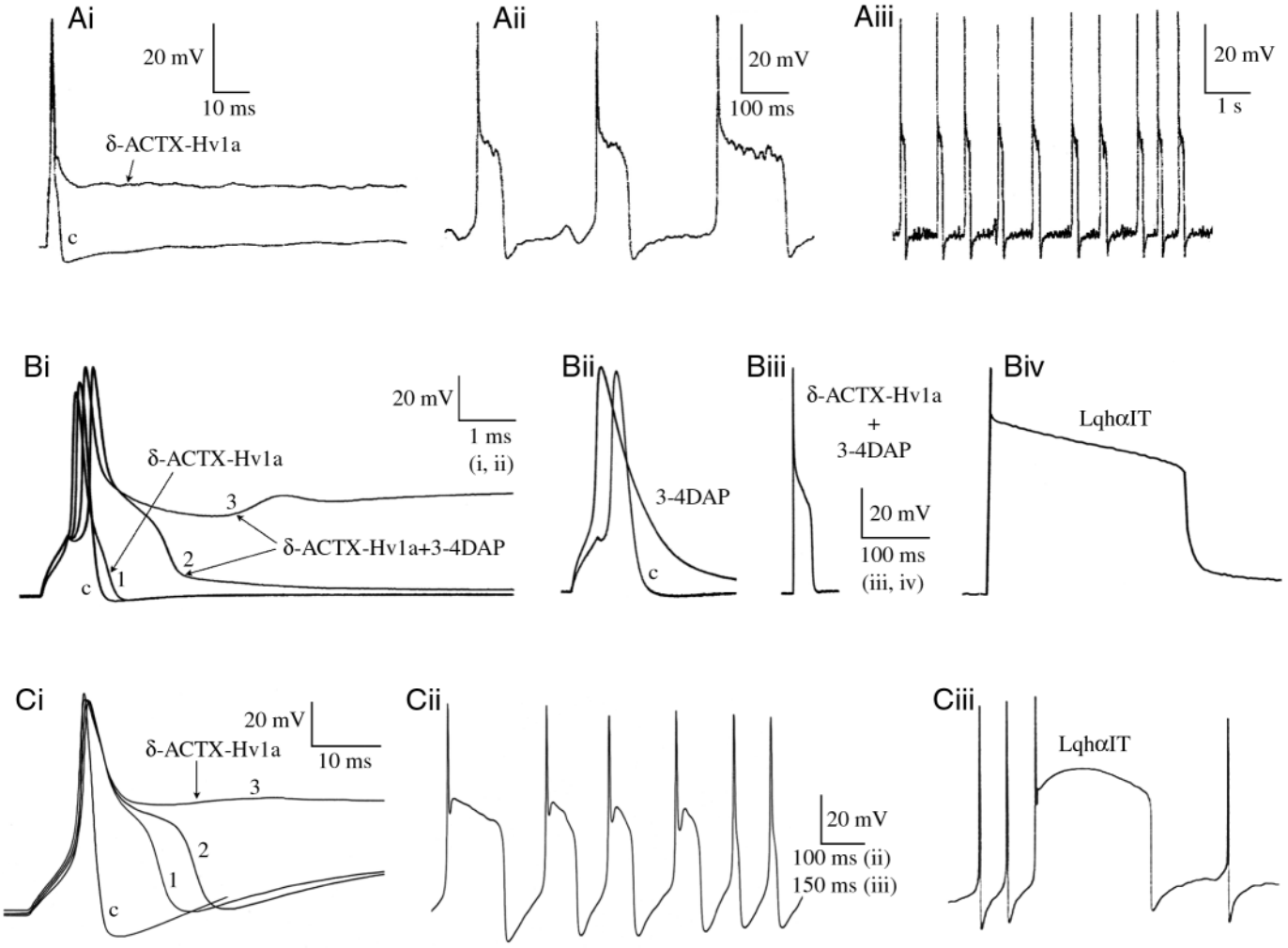

Fig. 1. Effects of δ-ACTX-Hv1a isolated from the venom of the funnel-web spider *Hadronyche versuta*, on action potentials recorded from isolated rat dorsal root ganglion neurones (A), cockroach giant axons (B) and cockroach dorsal unpaired median (DUM) neurones (C) in current-clamp mode. (Ai) Typical superimposed action potentials evoked by a short depolarizing current pulse (2 nA, 2 ms in duration) recorded before (c) and during superfusion with 30 nmol l^{-1} δ-ACTX-Hv1a from a cell held at −50 mV. Note the slowing of repolarization and the development of a 'plateau' action potential. (Aii,iii) Spontaneous repetitive action potentials showing a plateau. (Bi) In the presence of 0.5 μmol l^{-1} δ-ACTX-Hv1a, a slight prolongation of the evoked cockroach giant axon action potential (current pulse 10 nA, 0.5 ms in duration) was observed with no change in the resting axonal membrane potential (−60 mV). c, action potential before treatment. Treatment of the axonal membrane with δ-ACTX-Hv1a alone never induced a plateau action potential (1). In contrast, the development of a plateau action potential was observed after 2 min (2) and 3 min (3) when toxin was co-applied with 500 μmol l^{-1} 3,4-diaminopyridine (3-4DAP). For comparison, only an increase in the duration of the action potential, which exhibited a faster depolarizing phase, was observed when the selective K^+ channel blocker 3-4DAP was applied alone (Bii). (Biii,iv) A comparison of plateau action potentials induced by δ-ACTX-Hv1a plus 3-4DAP (Biii) and by 1 μmol l^{-1} LqhαIT (Biv). (Ci) Action potentials in an isolated cockroach DUM neurone were evoked by a current pulse (1 nA, 50 ms in duration) before (c; membrane potential −60 mV) and 10 s (1), 30 s (2) and 1 min (3) after 0.1 μmol l^{-1} δ-ACTX-Hv1a had been applied to a DUM neurone that had previously been hyperpolarized by applying a hyperpolarizing pulse from −60 mV to −100 mV for 1 min. (Cii,iii) Comparative effects of 0.1 μmol l^{-1} δ-ACTX-Hv1a (Cii) and 0.2 μmol l^{-1} LqhαIT (Ciii) on spontaneous electrical activity in a DUM neurone. Note that plateau action potentials induced by δ-ACTX-Hv1a in DUM neurones were preferentially observed after a short artificial hyperpolarization.

developed from a shoulder arising in the last two-thirds of the repolarizing phase. In all neurones studied, applying an artificial hyperpolarization to a level 20–40 mV more negative than the resting potential facilitated the appearance of plateau action potentials. Under these conditions, repetitive plateau action potentials, up to 100–150 ms in duration, could also occur spontaneously (Fig. 1Cii). However, when the membrane potential was stepped back to the resting level, spontaneous plateau action potentials were progressively replaced by modified action potentials exhibiting only a slight shoulder (Fig. 1Cii). It should be noted that, in contrast to the axonal preparation, the effect of δ-ACTX-Hv1a was the same on DUM neurones pre-treated with a K^+ channel blocker such as tetraethylammonium (not illustrated).

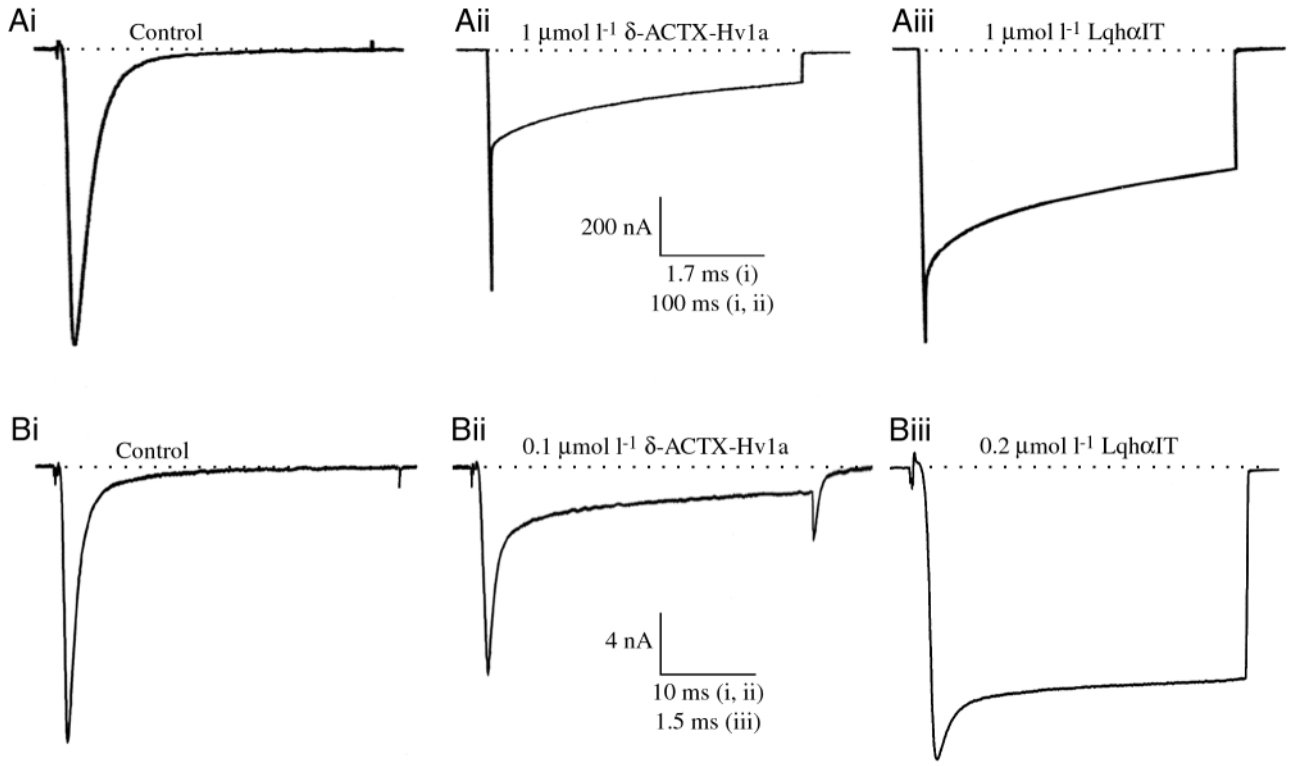

Fig. 2. Typical inward Na^+ currents recorded in voltage-clamp mode in a giant axon (A) and an isolated dorsal unpaired median (DUM) neurone cell body (B). (Ai,Bi) Control inward Na^+ currents elicited by a 5 ms depolarizing pulse to −10 mV from a holding potential of −60 mV in an isolated giant axon (Ai) and by a 30 ms depolarizing pulse to −10 mV from a holding potential of −90 mV in a DUM neurone (Bi). Comparative effects of δ-ACTX-Hv1a, the venom of the funnel-web spider *Hadronyche versuta*, and an insect-selective scorpion α-toxin, LqhαIT on inward Na^+ currents in a giant axon (Aii,iii) and an isolated DUM neurone (Bii,iii).

These observations indicate that δ-ACTX-Hv1a is an excitatory toxin, which alters insect axonal and cellular neuronal excitability in a manner similar to that of scorpion α-toxins (Pelhate et al., 1998). We were therefore interested in carrying out parallel tests, and under the same experimental conditions, with the most active insect-specific α-toxin, LqhαIT. When 1 μmol l^{-1} LqhαIT was bath-applied to axonal membranes, the falling phase of the action potential also developed a prolonged shoulder. Within 3 min, LqhαIT produced a very long plateau action potential lasting 5–10 times longer than those observed with the spider toxin (Fig. 1Biv). In the presence of LqhαIT, slowing of the repolarization commenced much earlier than that caused by δ-ACTX-Hv1a (Fig. 1Biii,iv). In DUM neurones, LqhαIT (0.2 μmol l^{-1}) induced a similar effect and transformed the short-duration action potentials (2.3±0.1 ms, N=30 under normal conditions; Lapied et al., 1989) into plateau action potentials lasting several hundred milliseconds (458±47 ms, N=6; Fig. 1Ciii). Like δ-ACTX-Hv1a, LqhαIT had no effect on the amplitude of the action potential of either the axon or DUM neurones.

General effects of δ-ACTX-Hv1a on cockroach giant axon and DUM neurone cell body inward Na^+ currents

The findings provided by the current-clamp experiments suggested that the mode of action of δ-ACTX-Hv1a resembles that of LqhαIT, even though LqhαIT induced longer plateau action potentials. Consequently, we performed additional experiments under voltage-clamp conditions to determine whether δ-ACTX-Hv1a affects insect voltage-gated Na^+ channels. This is the most likely site of action given (i) that LqhαIT-induced plateau action potentials are known to be the consequence of an alteration in the gating and kinetics of voltage-gated Na^+ channels (Pelhate et al., 1998), and (ii) that δ-ACTX-Hv1a had previously been shown to slow Na^+ channel inactivation in mammalian sensory neurones (Nicholson et al., 1994). Fig. 2 shows typical examples of the effect of δ-ACTX-Hv1a on voltage-gated Na^+ currents recorded in an isolated cockroach axon and in DUM neurones. In both cases, the inward current inactivated fully under control conditions. However, after treatment with the toxin, current inactivation remained incomplete, resulting in a sustained Na^+ current observed at the end of the test pulses (Fig. 2Aii,2Bii). In parallel, the peak current amplitude recorded at −10 mV was reduced by 20.3 % in the axon and by 15.3 % in the DUM neurone. The effects of LqhαIT on inward Na^+ currents from axon and DUM neurones are also illustrated in Fig. 2Aiii,Biii. In both preparations, LqhαIT (at 0.2 μmol l^{-1} for DUM neurones and at 1 μmol l^{-1} for axons) slowed Na^+ current inactivation, as demonstrated by a marked prolongation of the

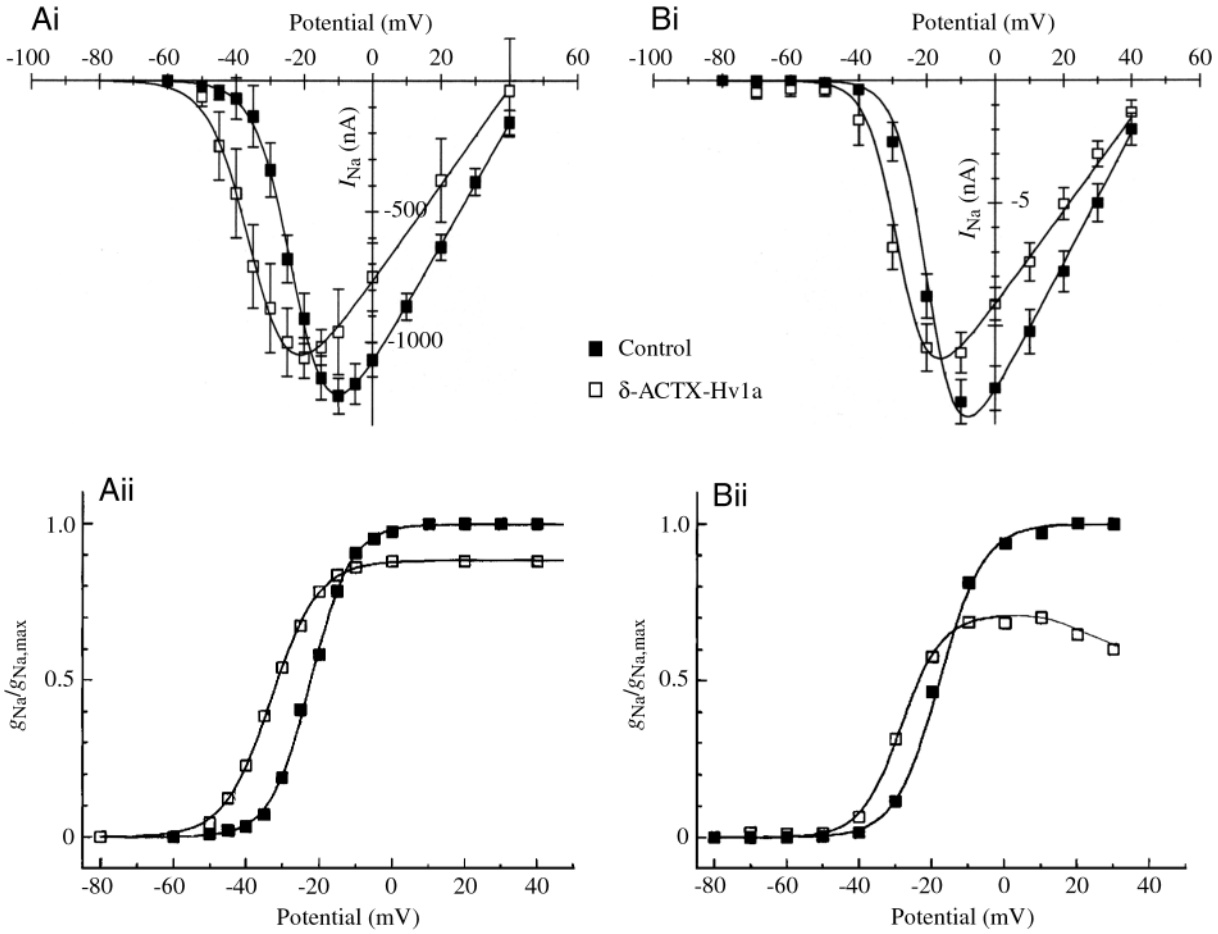

Fig. 3. Voltage-dependence of Na^+ currents (I_{Na}) and conductances in a giant axon (A) and in a dorsal unpaired median (DUM) neurone cell body (B). (Ai,Bi) Current/voltage relationship for the peak inward Na^+ current before (■) and after (□) bath application of δ-ACTX-Hv1a of the funnel-web spider *Hadronyche versuta*, on an axon (Ai) (1 μmol l^{-1} δ-ACTX-Hv1a, N=6–11) and on a DUM neurone (Bi) (0.1 μmol l^{-1} δ-ACTX-Hv1a, N=4). In the giant axon, inward Na^+ currents were elicited by a series of 5 ms depolarizations (between −50 and +40 mV) from the holding potential of −60 mV. In DUM neurones, inward currents were evoked by a series of 30 ms depolarizing pulses from a holding potential of −90 mV. Test voltages ranged from −80 mV to +40 mV in 10 mV steps. Values are means ± S.E.M. (Bi,ii) Voltage-dependence of the normalized Na^+ conductance ($g_{Na}/g_{Na,max}$) under control conditions (■) and following exposure to δ-ACTX-Hv1a (□) in an axon (Bi) and a DUM neurone (Bii). The Na^+ conductances were calculated from the data shown in Ai and Bi.

decay phase of the current. In contrast to δ-ACTX-Hv1a, the main effects of LqhαIT were (i) to increase the peak Na^+ current amplitude rather than to decrease it, as observed with δ-ACTX-Hv1a, and (ii) to produce a more pronounced maintained inward component during the test pulses.

The peak current/voltage (I/V) relationships of the Na^+ currents in axon and DUM neurones are shown in Fig. 3A,B. Mean values were fitted by an exponential function according to the following equation (Stühmer, 1988):

$$I_{Na} = g_{Na}(1 - 1/\{1+\exp[(E_m - E_{0.5})/k]\})(E_m - E_{Rev}), \quad (1)$$

where I_{Na} is the amplitude of the peak Na^+ current at a given test potential (E_m), $E_{0.5}$ is the voltage at half-maximal activation, k is the slope factor, g_{Na} is the maximum Na^+ conductance and E_{Rev} is the reversal potential. Under control conditions, the Na^+ currents recorded from axons activated at approximately −50 mV and reached a maximum amplitude at approximately −10 mV (Fig. 3Ai). In the presence of δ-ACTX-Hv1a, the I/V curve was shifted by 12 mV towards more negative potentials, with $E_{0.5}$ decreasing from −22.3 mV in controls to −34.5 mV in the presence of 1 μmol l^{-1} δ-ACTX-Hv1a. Consequently, the maximum peak inward current was shifted from −10.5 to −20.8 mV. In addition, g_{Na} decreased from 22.2 μS in controls to 18.5 μS in the presence of toxin. The reversal potential was reduced from +46.9 mV to +41.5 mV after δ-ACTX-Hv1a treatment. One possible explanation for this reduction could be the accumulation of Na^+ near the internal side of the clamped axonal membrane as a result of the slowing down and incomplete inactivation of the Na^+ current.

In isolated DUM neurones (Fig. 3Bi), the control inward Na^+ current activated at approximately −40 mV, reached maximum amplitude at approximately −10 mV, and then decreased to a reversal potential of +49.2 mV, a value close to the calculated Nernstian equilibrium potential for Na^+ (+48 mV). In the presence of toxin, the curve was also shifted

in the hyperpolarizing direction, with $E_{0.5}$ decreasing from −20.1 mV in controls to −28.8 mV in the presence of 0.1 μmol l^{-1} δ-ACTX-Hv1a. The toxin-modified inward current reached a maximum at approximately −17 mV and reversed at +47 mV, a value similar to that of control inward Na^+ currents. The maximum conductance (g_{Na}, see equation 1) was also reduced from 265 nS (control condition) to 195 nS. The hyperpolarizing shift in the voltage-dependence of the inward Na^+ currents and the decrease in the current amplitude, observed in both axonal and DUM neurone preparations, suggested that δ-ACTX-Hv1a affected the voltage-dependence of whole-cell Na^+ conductance (g_{Na}). To examine this possibility, g_{Na} was calculated before and after toxin treatment for each membrane potential according to the equation:

$$g_{Na} = I_{Na}/(E_m - E_{Na}), \quad (2)$$

where I_{Na} is the maximum current amplitude, E_m is the membrane potential at which the membrane was clamped and E_{Na} is the equilibrium potential for Na^+. Values of Na^+ conductance were obtained from the mean values shown in Fig. 3Ai,Bi and were normalized to the maximum Na^+ conductance ($g_{Na,max}$) (Fig. 3Aii,Bii). The smooth curves shown in Fig. 3Aii represent the best fits through the mean values using a single Boltzmann distribution:

$$g_{Na}/g_{Na,max} = 1/\{1 + \exp[(E_{0.5} - E_m)/k]\}, \quad (3)$$

where $E_{0.5}$ is the potential at which 50 % of the maximal conductance is activated and k is the slope factor. In axons, the values of $E_{0.5}$ for g_{Na} of currents recorded before and after δ-ACTX-Hv1a treatment were −22.2 mV and −33.1 mV, respectively. The slope factor was unchanged (5.5 mV for the control and 6.4 mV after δ-ACTX-Hv1a treatment), but maximum g_{Na} was decreased by 12 %.

In DUM neurones (Fig. 3Bii), only the normalized conductances obtained under control condition could be adequately fitted by a single Boltzmann equation using equation 3 with $E_{0.5}$=−18 mV and k=5.8 mV. In the presence of δ-ACTX-Hv1a, the peak Na^+ conductance reached its half-maximal value at approximately −28 mV and reached a maximum at approximately 0 mV. The 10 mV difference in the voltage-dependence of the Na^+ conductance was similar to that observed in axons and was consistent with the corresponding changes in the *I*/*V* curves.

Since we found that δ-ACTX-Hv1a-induced plateau action potentials were more evident in DUM neurones when the membrane had previously been hyperpolarized (Fig. 1C), we wished to determine whether the effect of the toxin under voltage-clamp conditions was also dependent on the holding potential. As illustrated in Fig. 4A,B, the amplitude of the δ-ACTX-Hv1a-modified Na^+ current measured at the end of the test pulse varied with the holding potential. The peak current amplitude increased slightly when the holding potential was varied between −90 and −110 mV (because of the increase in the driving force) and then stabilized between −110 and −160 mV. In contrast, the fraction of the sustained inward Na^+ current, measured at the end of the 30 ms depolarizing test pulse, compared with the peak current amplitude,increased markedly from 20 % at a holding potential of −90 mV to 34 % at −110 mV and reached a maximum of 53 % at −160 mV (Fig. 4B).

The effects of δ-ACTX-Hv1a on the voltage-dependence of steady-state Na^+ channel inactivation were also examined. A conventional two-pulse voltage-clamp protocol was used. Inactivation properties were examined by applying a 110 ms conditioning pulse between −100 mV and +20 mV, in 10 mV increments. The membrane potential was then stepped back to the holding potential of −90 mV for 2 ms, and a 30 ms test pulse was applied to −10 mV, a potential that evoked the inward Na^+ current. Steady-state inactivation curves (Fig. 4C) were obtained by plotting the amplitude of the peak inward current measured during the 30 ms test pulse against the conditioning potential in normal saline and following application of 0.1 μmol l^{-1} δ-ACTX-Hv1a. In controls, the mean values (N=6) were fitted to a single Boltzmann distribution according to equation 4:

$$h_\infty = 1/\{1 + \exp[(E_m - E_{0.5})/k]\}, \quad (4)$$

where h_∞ is steady-state Na^+ channel inactivation, $E_{0.5}$ is the potential at which half the Na^+ channels are inactivated, E_m is the membrane potential at which the membrane was clamped and k is the slope factor. In the presence of δ-ACTX-Hv1a, the voltage-dependence of steady-state inactivation was not modified for potentials more negative than −40 mV. However, the mean values could not be adequately fitted by the same Boltzmann distribution as in equation 4 because δ-ACTX-Hv1a caused a non-inactivating component at conditioning potentials more depolarized than −40 mV. This revealed an incomplete inactivation of the Na^+ current.

Discussion

The present study showed that in insect preparations, as in the mammalian preparation, δ-ACTX-Hv1a induced, under different experimental conditions, plateau action potentials that could be accompanied by spontaneous repetitive firing. This effect is a result of (i) a slowing of Na^+ current inactivation, (ii) a slight reduction in peak current amplitude, (iii) a negative shift in the voltage-dependence of activation, and (iv) a non-inactivating component in the steady-state Na^+ channel inactivation curve (h_∞). This study supports the conclusions of previous electrophysiological experiments performed on rat DRG neurones (Nicholson et al., 1996; Nicholson et al., 1998) and extends them to the insect tetrodotoxin-sensitive voltage-dependent Na^+ channels. It is concluded that the effects of δ-ACTX-Hv1a are similar to those of the scorpion α-toxin, including the representative LqhαIT used here for comparison.

δ-*ACTX-Hv1a differentially alters axonal and somatic electrical activity*

In DUM neurones, both δ-ACTX-Hv1a and LqhαIT were able to induce plateau action potentials without additional

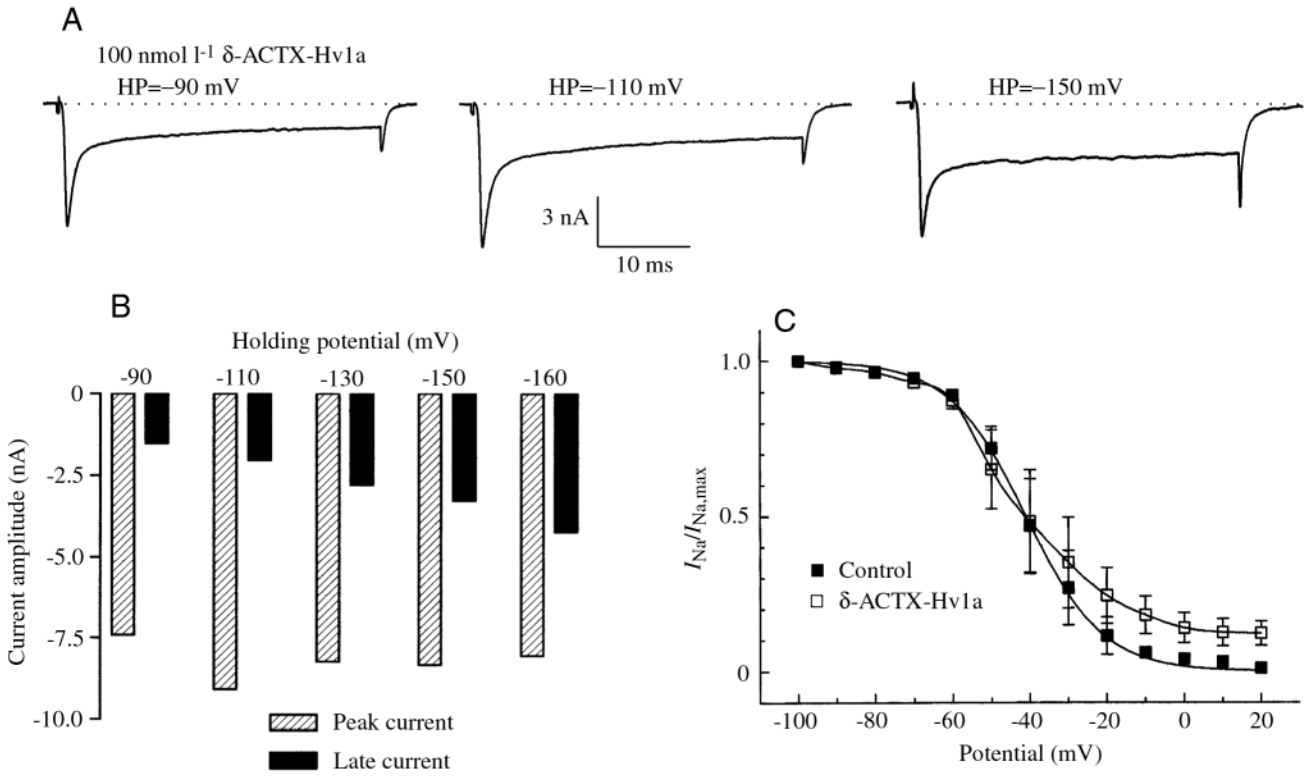

Fig. 4. Voltage-dependent effect of δ-ACTX-Hv1a isolated from the venom of the funnel-web spider *Hadronyche versuta*, on the inward Na^+ current in a dorsal unpaired median (DUM) neurone cell body. (A) Typical effect of δ-ACTX-Hv1a (0.1 μmol l^{-1}) on the amplitude of the sustained component of the inward Na^+ current elicited by a 30 ms depolarizing test pulse to −10 mV. The different holding potentials (HP) are indicated above each current trace. (B) Comparative histogram of the effects of δ-ACTX-Hv1a on both the sustained (late) component (measured at the end of the depolarizing pulse) and the peak inward Na^+ current recorded at different holding potentials. Unlike the peak current amplitude, the amplitude of the sustained component increased for holding potentials more negative than –90 mV. (C) Voltage-dependence of inward Na^+ current inactivation before (■) and after (□) application of δ-ACTX-Hv1a. A conventional two-pulse voltage-clamp protocol was used. Following 110 ms conditioning pulses applied between −100 mV and +20 mV, the membrane potential was stepped back to a holding potential of −90 mV for 2 ms, and 30 ms test pulses to −10 mV were then applied. Values are means ± S.E.M. of three experiments. $I_{Na}/I_{Na,max}$, normalized Na^+ current.

experimental manipulation. In contrast, in giant axons, the spider toxin on its own did not induce plateau action potentials, and the repolarizing phase of the action potentials was only slightly prolonged. However, plateau action potentials occurred in axons when the K^+ channel blocker 3-4DAP was added in the presence of toxin. This difference between the susceptibility of neuronal somata and axons of the same species could reflect quantitative differences in the magnitude of the Na^+ and K^+ conductances and/or the existence of ionic currents exhibiting distinct biophysical properties in the two types of membrane. In DUM neurones, Na^+- and Ca^{2+}-dependent K^+ conductances underlie action potential repolarization in addition to a delayed outwardly rectifying K^+ conductance (Lapied et al., 1989; Grolleau and Lapied, 1995). In axons, only a delayed outward K^+ current involved in the repolarizing phase of the action potential has been identified (Pelhate and Sattelle, 1982). In addition, it is well known that part of the action potential repolarization is due to the inactivation of voltage-gated Na^+ channels. However, participation of the Na^+ current inactivation process in action potential repolarization is probably not at the same level in axons and DUM neurones since the electrophysiological properties of Na^+ currents in these two preparations are not identical (Lapied et al., 1990). The kinetics of activation and inactivation of DUM neurone Na^+ currents are approximately four times slower and the activation threshold is 15 mV more positive than in axons (Lapied et al., 1990; this study). Furthermore, the inward Na^+ current in DUM neurones displays a bi-exponential decay, whereas the axonal Na^+ current decays mono-exponentially. Consequently, action potential duration is four times shorter in axons than in DUM neurones or rat DRG neurones. Such a disparity in ion channel subtypes or in the biophysical properties of ionic currents could explain why the neuronal excitability of the two distinct insect preparations could be differentially altered in the presence of the same toxin.

Distinct differences in the effects of δ-ACTX-Hv1a on the cockroach giant axon and DUM neurones may also result from a selective interaction of site 3 toxins with one or several Na^+ channel subtypes, even within a discrete subcellular region, as

has recently been proposed (Cestèle et al., 1995; Gilles et al., 1999). For instance, the scorpion α-like toxin Lqh III has been found to slow voltage-gated Na^+ channel inactivation in rat hippocampal neurones, but has no effect on certain expressed rat brain Na^+ channel subtypes (Gilles et al., 1999). Consideration of channel subtype specificity may help to explain the pathophysiological actions of the spider toxins since the different effects observed during spider envenomation could be related (i) to the contribution of differently acting neurotoxic components, but also (ii) to the species- or channel-subtype-specific properties of these compounds, as suggested above.

Voltage-dependence of δ-ACTX-Hv1a effects on DUM neurone Na^+ channel inactivation

Voltage-clamp experiments confirmed that the major effect of δ-ACTX-Hv1a was a slowing of Na^+ current inactivation. Analysis of the voltage-dependence of steady-state Na^+ channel inactivation (h_∞) revealed that δ-ACTX-Hv1a produced a non-inactivating component at pre-pulse potentials more depolarized than −40 mV. Interestingly, in DUM neurones, δ-ACTX-Hv1a was more potent in inhibiting Na^+ current inactivation when the membrane potential was held at the more hyperpolarized potentials that were usually required to reactivate unmodified Na^+ channels. The simplest explanation to account for this behaviour is that the toxin dissociated from the channel at more depolarised potentials. Dissociation of bound toxin during depolarisation has already been demonstrated in vertebrate Na^+ channels treated with toxins from the scorpion *Centruroides sculpturatus* (Strichartz and Wang, 1986) and the sea anemone *Anemonia sulcata* (Schreibmayer et al., 1987). The binding affinity of classical α-toxins such as Aah II to receptor site 3 in rat brain synaptosomes has also been shown to be highly voltage-dependent (Ray and Catterall, 1978; Jover et al., 1980; Cestèle and Gordon, 1998). Under these conditions, the voltage-dependent dissociation of the toxin could help to restore normal current inactivation following depolarisation of the membrane.

δ-ACTX-Hv1a exhibits similar actions to those of other receptor site 3 neurotoxins

Neurotoxins that modify voltage-gated Na^+ channels are known to interact with at least seven identified neurotoxin receptor sites determined by direct radiolabelled toxin studies (Catterall, 1992; Fainzilber et al., 1994; Trainer et al., 1997; for a review, see Gordon, 1997). Previous studies reported that δ-ACTX-Hv1a had comparable actions to scorpion α-toxins, especially with respect to its ability to alter the inactivation properties of the voltage-gated Na^+ channels (Nicholson et al., 1994; Nicholson et al., 1998; Little et al., 1998b) or to compete for the binding of the classical α-scorpion toxins Aah II and Lqh II to neurotoxin receptor site 3 on rat brain Na^+ channels (Little et al., 1998a; Little et al., 1998b). Furthermore, δ-ACTXs compete for binding to cockroach neuronal membranes with the insect-selective scorpion α-toxin LqhαIT (Little et al., 1998a). Like scorpion α-toxins, δ-ACTX-Hv1a also displayed a positive allosteric interaction with lipid-soluble alkaloids such as batrachotoxin and veratridine. However, unlike scorpion α-toxins, δ-ACTX-Hv1a decreased maximal batrachotoxin-activated $^{22}Na^+$ uptake (Little et al., 1998b). Despite this latter effect, the available evidence suggests that δ-ACTXs bind to receptor sites that overlap, at least partially, with those of scorpion α-toxins.

We have compared the electrophysiological effects of funnel-web spider toxin with those induced by LqhαIT. Although both δ-ACTX-Hv1 and LqhαIT markedly altered the inactivation properties of the inward Na^+ current in axons and DUM neurones, some differences in their action were revealed under voltage-clamp conditions. First, the sustained inward current induced by LqhαIT was greater in amplitude than that induced by the spider toxin. Second, δ-ACTX-Hv1a reduced peak Na^+ currents in both DRG neurones (Nicholson et al., 1994) and insect neuronal preparations (this study), whereas scorpion toxins from *Leiurus* spp. either failed to alter or increased peak Na^+ current amplitude (Wang and Strichartz, 1985; Eitan et al., 1990; Lee and Adams, 2000; this study). The LqhαIT-induced increase in peak Na^+ current can be partially explained by the delayed onset of inactivation. In contrast, δ-ACTX-Hv1a did not slow Na^+ current inactivation kinetics as markedly as LqhαIT and therefore failed to increase current amplitude. Another possible reason why δ-ACTX-Hv1a did not increase Na^+ current amplitude is that δ-ACTXs, like alkaloid toxins, may decrease a single-channel conductance.

In the present study, δ-ACTX-Hv1a was found to reduce the maximal whole-cell conductance and to produce a 10 mV shift towards hyperpolarized potentials in the voltage-dependence of both axonal and DUM neurone Na^+ channel conductance. Such effects were also produced by alkaloid toxins known to bind to receptor site 2 of the voltage-gated Na^+ channel (Gordon, 1997). These neurotoxins, for example batrachotoxin and veratridine, are characterized by a complex mode of action including (i) an inhibition of channel inactivation, (ii) a shift in the voltage-dependence of activation to more negative membrane potentials, (iii) a reduction in the single-channel conductance, and (iv) an alteration of the channel ion selectivity (Hille, 1992; Hille et al., 1987). Single-channel analysis of δ-ACTX-Hv1a-modified Na^+ currents are required to show any similarity in action between δ-ACTX-Hv1a and receptor site 2 neurotoxins. Such a study could demonstrate, for instance, whether, like alkaloid toxins, the decrease in peak current amplitude induced by δ-ACTX-Hv1a might result from a reduction in single-channel conductance in addition to a slowing of channel inactivation. In support of this possibility, δ-ACTX-Hv1a has been shown to decrease the maximal batrachotoxin-activated uptake of $^{22}Na^+$ (Little et al., 1998b), while this effect has never been observed with the scorpion α-toxins. However, unlike batrachotoxin or aconitine, δ-ACTX-Hv1a did not affect the reversal potential of the current, suggesting that the ion selectivity for Na^+ was not altered. In addition, α-ACTX-Hv1a enhanced [^{3}H]batrachotoxin binding in a positive allosteric fashion rather than inhibiting binding

(Little et al., 1998b). These effects prove that δ-ACTX-Hv1a does not interact directly with receptor site 2.

The shift in the voltage-dependence of Na^+ conductance induced by δ-ACTX-Hv1a was also observed with scorpion β-toxins (Wang and Strichartz, 1983; Gordon, 1997), which are known to bind to receptor site 4. However, in contrast with scorpion β-toxins, δ-ACTX-Hv1a failed to induce the large prolonged inward tail currents that are responsible for the induction of repetitive activity.

In summary, the present study indicates that δ-ACTX-Hv1a selectively modulates insect voltage-gated Na^+ channels both in the isolated cockroach giant axon and in isolated DUM neurones by a mechanism resembling that of scorpion α-insect toxins. It should be noted that this toxin seems to be more effective on Na^+ channels in vertebrates than in insects. This difference in efficiency may be related to the molecular structure of the toxin. It has recently been reported that δ-ACTX-Hv1b, a novel δ-ACTX isolated from the same venom, is also active in vertebrates. However, this toxin, which differs from δ-ACTX-Hv1a in its amino acid sequence, completely lacks insecticidal activity (Szeto et al., 2000). This confirms that such spider toxins could be of importance as tools for investigating the structural requirements for specific anti-insect *versus* anti-mammalian effects.

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