

Domain 2 of *Drosophila Para* Voltage-Gated Sodium Channel Confers Insect Properties to a Rat Brain Channel

Iris Shichor,^{1,3} Eliahu Zlotkin,³ Nitza Ilan,^{1,2} Dodo Chikashvili,¹ Walter Stuhmer,⁴ Dalia Gordon,² and Ilana Lotan¹

Departments of ¹Physiology and Pharmacology, Sackler School of Medicine and ²Plant Sciences, George S. Wise Faculty of Life Sciences, Tel-Aviv University, 69978 Ramat-Aviv, Israel, ³Department of Cell and Animal Biology, Institute of Life Sciences, The Hebrew University, 91904 Jerusalem, Israel, and ⁴Max-Planck-Institut für Experimentelle Medizin, D-37075 Göttingen, Germany

The ability of the excitatory anti-insect-selective scorpion toxin AahIT (*Androctonus australis hector*) to exclusively bind to and modify the insect voltage-gated sodium channel (NaCh) makes it a unique tool to unravel the structural differences between mammalian and insect channels, a prerequisite in the design of selective pesticides. To localize the insect NaCh domain that binds AahIT, we constructed a chimeric channel composed of rat brain NaCh α -subunit (rBIIA) in which domain-2 (D2) was replaced by that of *Drosophila Para* (paralytic temperature-sensitive). The choice of D2 was dictated by the similarity between AahIT and scorpion β -toxins pertaining to both their binding and action and the essential role of D2 in the β -toxins binding site on mammalian channels. Expression of the chimera rBIIA-ParaD2 in *Xenopus* oocytes gave rise to voltage-gated and TTX-sensitive NaChs that, like rBIIA, were sensitive to

scorpion α -toxins and regulated by the auxiliary subunit β_1 but not by the insect TipE. Notably, like *Drosophila Para*/TipE, but unlike rBIIA/ β_1 , the chimera gained sensitivity to AahIT, indicating that the phyletic selectivity of AahIT is conferred by the insect NaCh D2. Furthermore, the chimera acquired additional insect channel properties; its activation was shifted to more positive potentials, and the effect of α -toxins was potentiated. Our results highlight the key role of D2 in the selective recognition of anti-insect excitatory toxins and in the modulation of NaCh gating. We also provide a methodological approach to the study of ion channels that are difficult to express in model expression systems.

Key words: Na channel; insect selectivity; *Xenopus* oocytes; scorpion toxin; gating; *Drosophila Para*

Voltage gated sodium channels (NaChs) play a pivotal role in excitability. They are plasma membrane proteins composed of the large pore-forming α -subunit generally accompanied by one or two auxiliary subunits (Catterall, 1992; Feng et al., 1995). The α -subunit is composed of four repeated domains (D1–D4) consisting of six transmembrane α -helices (S1–S6) and a pore loop (between S5 and S6) (Catterall, 1995). Many modifiers of NaChs are used as drugs and insecticides (Kallen et al., 1993; Gordon, 1997a,b). However, being unable to distinguish between NaCh subtypes in various tissues and species, their use often renders adverse side effects or toxicity to nontargeted animals. However, some scorpion neurotoxins are able to exclusively identify insect NaChs (Zlotkin et al., 1978; Zlotkin, 1999) or distinguish among distinct mammalian neuronal subtypes (Gilles et al., 1999, 2000). Such selectivity indicates structural differences at toxin binding sites among NaChs that, when identified, can be used in the design of selective drugs and insecticides.

Scorpion toxins affecting NaChs bind to two receptor sites on the α -subunit and are divided into α - and β -classes according to their mode of action and binding properties (Gordon et al., 1998). α -Toxins inhibit sodium current inactivation during binding to receptor site-3, which involves extracellular loops S5–S6 of D1 and D4 and S3–S4 of D4 (Thomsen and Catterall, 1989; Rogers et al., 1996; Ma et al., 2000). β -Toxins shift the voltage dependence of activation to more negative potentials (Wang and Strichartz, 1983) (for review, see Gordon, 1997b) by binding to receptor site-4 (Jover et al., 1980), shown to be in domain-2 (D2) (Marcotte et al., 1997; Tsushima et al., 1999) and to include the external loop S3–S4 (Cestele et al., 1998).

Two additional groups of scorpion toxins, the excitatory and depressant, are highly selective to insects (Zlotkin et al., 1978; Zlotkin, 1997). Like the β -toxins acting on mammals, the excitatory and depressant toxins modify the activation of insect NaChs and compete for binding with other β -toxins (for review, see Gordon et al., 1998), properties that suggest their affiliation to the β -toxin class. The exclusive recognition of insect NaChs was best demonstrated using the excitatory toxin from *Androctonus australis hector* (AahIT). AahIT is composed of 70 amino acids cross-linked by four disulfide bridges (Zlotkin et al., 1978) and modifies the gating mechanism by binding to an external receptor site of insect NaChs (Pelhate and Zlotkin, 1981, 1982; Gordon et al., 1984, 1992). Its strict selectivity for insects, which has been documented by toxicity and electrophysiological and binding experiments (Gordon, 1997a; Zlotkin, 1997), makes AahIT a

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Correspondence should be addressed to either Prof. Ilana Lotan, Department of Physiology and Pharmacology, Sackler School of Medicine, Tel-Aviv University, 69978 Ramat-Aviv, Israel, E-mail: ilotan@post.tau.ac.il; or Dr. Dalia Gordon, Department of Plant Sciences, George S. Wise Faculty of Life Sciences, Tel-Aviv University, 69978 Ramat-Aviv, Israel, E-mail: dgordon@post.tau.ac.il.

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unique tool to study the basis of selective recognition of insect channels.

To clarify the basis of the selective interaction of AahIT with insect NaChs, aiming to localize the region that is targeted by AahIT, we constructed a chimeric mammalian–insect channel of the rat brain NaCh IIA (rBIIA) in which D2 was replaced with that of the *Para* (paralytic temperature-sensitive) *Drosophila* channel. The chimeric channel was functional, despite the large phylogenetic distance between insects and mammals, and acquired the sensitivity to the anti-insect selective toxin AahIT.

MATERIALS AND METHODS

Materials

Para NaCh DNA was a kind gift from Dr. J. Warmke (Merck, Rahway, NJ). The clone of *TipE* was a kind gift from Dr. M. Williamson (IACR-Rothamsted, Harpenden, UK). AahIT was purified by column chromatography from the crude venom of the North African scorpion *Androctonus australis hector* as described previously (Zlotkin et al., 1971). LqhαIT, Lqh-II (Ltx001) (from *Leiurus quinquestratus hebraeus*) was purchased from Latoxan (Valence, France). TTX was purchased from Sigma (Jerusalem, Israel). All reagents used were of molecular biology grade.

Construction of a chimeric cDNA, rBIIA-ParaD2

The rat brain NaCh α -subunit cDNA (rBIIA, pVA2580) (Gershon et al., 1992) was restricted by *Xba*I and *Bgl*II to excise a 1233 bp fragment corresponding to D2 and was isolated by gel electrophoresis. The 1380 bp parallel fragment of *Drosophila Para* cDNA corresponding to D2 was amplified by PCR using *Para Drosophila* cDNA as a template and two primers containing either *Xba*I or *Bgl*II restriction sites corresponding to those of the rat brain cDNA: primer 1 (sense), 5'-GCCTCCC-GGGGTCGTATACCTCACATGGCGATCTACTCGGC-3'; and primer 2 (antisense), 5'-GGACAGATCTTCCAGTTGCGTCTGCTC-CTTGATCCC-3'.

The PCR conditions were as follows: 27 cycles of 96°C for 1 min, 62°C for 45 sec, and 72°C for 2 min, using the PFU DNA polymerase (Stratagene, La Jolla, CA). The 1380 bp PCR product cDNA was restricted with *Xba*I and *Bgl*II, isolated by agarose gel electrophoresis, extracted by gel purification kit (QIAEX; Qiagen, Hilden, Germany), and ligated (T4 DNA ligase; New England Biolabs, Beverly, MA) to the excised rat brain NaCh cDNA lacking D2. The ligation products were transformed into competent bacteria cells (*Escherichia coli*; JM-109; Promega, Madison, WI). Colonies were examined by restriction analysis, and relevant ones were sequenced (automated sequencer; core facilities at Tel-Aviv University).

Generation of cRNA, injection into *Xenopus* oocytes, and electrophysiology

rBIIA cRNA was generated from the pVA2580 construct, linearized by *Cl*aI, and transcribed *in vitro* with T7 RNA polymerase as described previously (Dascal and Lotan, 1992; Gershon et al., 1992). Chimeric cRNA α -subunit was generated from the rBIIA-ParaD2 cDNA, linearized by *Pac*I, and transcribed with T7 RNA polymerase. β_1 -Subunit cRNA was linearized by *Nor*I and transcribed *in vitro* with T7 RNA polymerase (Wallner et al., 1993).

Para and *TipE* cRNAs were generated from the pGH19-13-5 *Para* (Warmke et al., 1997) and pGHTipE (Vais et al., 2000) constructs, respectively, linearized by *Nor*I, and transcribed with T7 RNA polymerase. Oocytes were injected with 0.3–0.8 ng of rBIIA cRNA or with 4–10 ng of rBIIA-ParaD2 cRNA with or without β_1 cRNA (1:1 ratio) (Catterall, 1995; Makita et al., 1996) or with 10 ng of *Para* cRNA with *TipE* (1:1 ratio) (Warmke et al., 1997; Vais et al., 2000). Injected oocytes were incubated at 22°C for 3–4 d in ND96 solution (in mM: 96 NaCl, 2 KCl, 1 MgCl₂, and 5 HEPES, pH 7.5) supplemented with 1.8 mM CaCl₂, 2.5 mM sodium pyruvate, and 100 μ g/ml gentamicin, as described previously (Gilles et al., 1999).

Sodium currents were recorded using a Warner Instruments (Hamden, CT) OC-725B two-electrode voltage-clamp amplifier with a series resistance compensation circuit and low-resistance agarose-plugged electrodes filled with 3 M KCl (0.2–0.5 M Ω). Experiments were done in a 150 μ l bath in ND96 solution supplemented with 1 mM CaCl₂, pH 7.6, at 20–22°C. Sodium currents were measured in single oocytes before and

after application of the relevant toxin. Toxins were kept in concentrated stock solutions and were diluted before the experiment in ND96 solution that contained 1 mg/ml BSA. The application of the toxin–BSA solution was done directly into the bath, in a volume up to 15 μ l (10% of the bath volume) to get the desired total concentration. To discard any application artifacts, in few oocytes, 1 mg/ml BSA solution was applied before the application of the toxin. Before impaling the oocyte with electrodes, the voltage base line was set to 0 mV. At the end of each experiment, the base line voltage was checked not to exceed \pm 2 mV, otherwise the experiment was discarded. The currents were filtered at 4 kHz and sampled at 11 kHz. Stimulation and data acquisition were done with an IBM computer using the pClamp software (Axon Instruments, Foster City, CA). Net current was estimated by subtraction of scaled leak current.

Data analysis

Voltage dependence of activation and inactivation. Current–voltage relationship (I – V) data and its transforming to activation curve, i.e., G/G_{\max} versus V_{test} , were fitted using a nonlinear least-square algorithm, to the modified Boltzmann equation (as by Dascal and Lotan, 1991) as follows: $G/G_{\max} = 1/\{1 + \exp[-(V_{\text{test}} - V_{1/2})/k]\}^3$ (Eq. 1), where at each membrane voltage (V_{test}) [$G = I/(V_{\text{test}} - V_{\text{rev}})$], I is the peak sodium current, V_{test} is the test potential, and V_{rev} is the reversal potential. The three free parameters were $V_{1/2}$ (the voltage at which the probability of a single gate opening is one-half), k (the slope factor, which corresponds to a change in voltage that produces an e -fold change in conductance), and G_{\max} (the maximal Na⁺ conductance attained at very positive voltages).

Steady-state inactivation was studied by holding the oocyte at –80 mV and stepping the voltage for 200 msec to various values from –90 mV up to –10 mV in 10 mV increments (V_{prepulse}), before measuring I_{Na^+} by a 50 msec voltage step that elicits maximal current (–10 mV for the wild-type rBIIA or 0 mV for the rBIIA-ParaD2). Fractional current (I/I_{\max}) was plotted as a function of V_{prepulse} and fitted (as by Dascal and Lotan, 1991) to the following equation: $I/I_{\max} = 1/\{1 + \exp[(V_{\text{prepulse}} - V_{1/2})/k]\}$ (Eq. 2). I_{\max} was the current obtained by a prepulse to –90 mV, and all other parameters was as above.

Analysis of the effect of α -toxins. Two parameters were checked to analyze the effect of the α -toxins. The first was the time constant of inactivation of the current described by the sum of two exponents (a fast component τ_1 and a slow component τ_2) and calculated in the same oocyte before and after application of the toxin. The second parameter was the estimated affinity (k_D) of the toxin to the channel, determined by the fraction of the maximal peak current (elicited at –10 or 0 mV for rBIIA or rBIIA-ParaD2, respectively) remaining 5 msec after the peak, before, and 13 min after application of the toxin. Because the fraction of conductance remaining 5 msec after the peak is proportional to the number of channels modified by α -toxins (Rogers et al., 1996), it can be used to estimate receptor occupancy and toxin affinity according to the following formula: $K_D = [\alpha \text{ toxin}] (F_G'/F_G - 1)$ (Eq. 3), where F_G is the fraction of Na⁺ current remaining 5 msec after beginning of the pulse, and F_G' is the maximum fraction of current 5 msec after the beginning of the pulse in the presence of a saturating concentration of α -toxin.

RESULTS

rBIIA is not sensitive to the excitatory insect-selective toxin AahIT in contrast to *Drosophila Para*

The rat brain rBIIA (Noda et al., 1986; Auld et al., 1988, 1990) and the insect *Drosophila Para* NaChs (Loughney et al., 1989; Warmke et al., 1997) have been functionally expressed in *Xenopus* oocytes, and their biophysical properties were well characterized. We examined the sensitivity of these two channels, expressed in *Xenopus* oocytes, to the scorpion anti-insect excitatory toxin AahIT, which is toxic to *Drosophila* flies (PD₅₀ of 2.3 ng/mg) (Zlotkin et al., 1999). AahIT (3–10 μ M) had no effect on Na⁺ current mediated by rBIIA, whereas 1–2 μ M AahIT increased significantly the Na⁺ current mediated by the *Drosophila Para* (Fig. 1) (for detailed characterization, see Fig. 4C). We aimed to confer AahIT sensitivity to the mammalian rBIIA by insertion of an insect channel domain from the *Drosophila Para*. AahIT shares biophysical and binding properties with other scorpion toxins of the β -class (Gordon et al., 1984, 1998; Zlotkin et

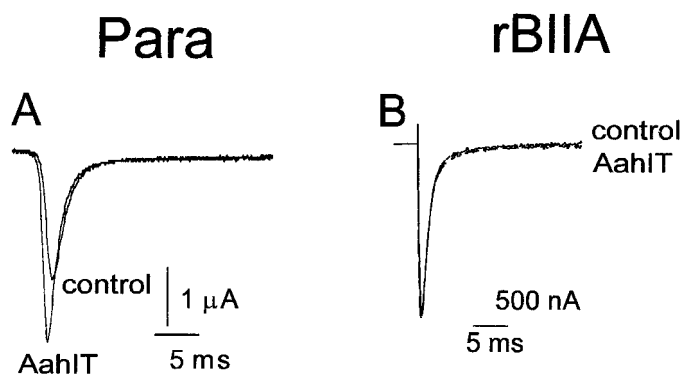


Figure 1. rBIIA is not sensitive to the excitatory insect selective toxin AahIT, in contrast to *Drosophila* Para. Oocytes expressing the *Drosophila* Para channel (together with the TipE subunit; *A*) or the rBIIA channel (together with the β_1 -subunit; *B*) were voltage clamped at a holding potential of -80 mV, and currents were elicited by a depolarizing pulse to -10 mV. Na^+ currents in single oocytes were recorded before (*control*) and 13 min after application of $1 \mu\text{M}$ (*A*) or $3 \mu\text{M}$ (*B*) AahIT.

al., 1985; De Lima et al., 1986; Lazdunski et al., 1986; Lee and Adams, 2000) (see Discussion). Because the receptor binding site for β -toxins (site-4) was localized to D2 on mammalian NaChs (Marcotte et al., 1997; Cestele et al., 1998), we constructed a chimeric channel rBIIA-ParaD2 formed from rBIIA (as a mammalian acceptor channel) and D2 of Para (as a donor of insect channel; see Materials and Methods).

Biophysical characterization of the chimeric channel rBIIA-ParaD2

Expression of the chimeric α -subunit rBIIA-ParaD2 in *Xenopus* oocytes gave rise to voltage-gated and TTX-sensitive Na^+ currents (Fig. 2*A,B*), indicating that NaCh activity was not disrupted by the insertion of the insect D2. Like rBIIA (Kontis and Goldin, 1993), the chimeric currents were modulated by coexpression of the mammalian auxiliary β_1 -subunit; namely, the inactivation was faster and the amplitudes increased (Isom et al., 1994; Makita et al., 1996) (Fig. 2*A,B*). This result indicates that the interaction between the mammalian α - and β_1 -subunits was not interrupted by the insect D2, in concert with the documented involvement of D4 in this interaction (Qu et al., 1999). Notably, coexpression of the insect auxiliary subunit TipE, required for the functional expression of the *Drosophila* Para NaCh α -subunit in *Xenopus* oocytes (Feng et al., 1995; Warmke et al., 1997), had no effect on the chimeric channel activity (data not shown). Thus, all subsequent electrophysiological experiments were performed with co-expressed α - and β_1 -subunits. To obtain the same amplitudes of Na^+ currents, 10-fold higher cRNA concentration of rBIIA-ParaD2 was required compared with that of rBIIA (see Materials and Methods). Expression of both proteins in the reticulocyte lysate system (Jing et al., 1999) revealed that rBIIA protein level of expression was ~ 10 -fold higher than that of rBIIA-ParaD2 (data not shown), suggesting that, at least partly, impaired expression of rBIIA-ParaD2 is responsible for the low current amplitudes.

Comparison between rBIIA and rBIIA-ParaD2 biophysical characteristics revealed a positive shift of $+11$ mV in the half-activation voltage ($V_{1/2}$) of the chimera, accompanied by an increase of 2.8 mV in the slope factor (k), compared with rBIIA (Fig. 2*C,D*), and both effects were statistically highly significant ($p < 0.001$). The activation characteristics of the rBIIA-ParaD2 ($V_{1/2} = -13$ mV; $k = 5.1$ mV) (Fig. 2*D*) were similar to those of

the Para *Drosophila* Na^+ channel, which was previously fully characterized in oocytes (Warmke et al., 1997). The α -subunit of the Para *Drosophila*, expressed with TipE, was shown to have $V_{1/2} = -16.9$ mV and $k = 5.43$ mV. Thus, it seems that the rBIIA-ParaD2 chimera acquired insect channel properties related to voltage-dependent activation, which is in concert with the importance of D2 in the voltage sensitivity of the NaChs (Auld et al., 1990; Marcotte et al., 1997; Qu et al., 1999). The steady-state inactivation characteristics of the rBIIA-ParaD2 were slightly different, but statistically significant, from those of rBIIA (Fig. 2*E*) and were within the range of values determined for the *Drosophila* Para channel expressed in *Xenopus* oocytes (Warmke et al., 1997).

rBIIA-ParaD2 is sensitive to AahIT

Comparison of the effects of AahIT on rBIIA and rBIIA-ParaD2 channels (Fig. 3) revealed that $3 \mu\text{M}$ AahIT had no effect on rBIIA currents at all test potentials, as shown in the I - V relationship (Fig. 3*A*, bottom). In contrast, $1.4 \mu\text{M}$ AahIT increased markedly the rBIIA-ParaD2 current, as shown for currents elicited at -30 and -20 mV (Fig. 3*B*, top). Analysis of the voltage dependence of activation of the rBIIA-ParaD2 revealed a statistically significant ($p \leq 0.003$) negative shift of 6 mV in $V_{1/2}$ in the presence of AahIT (Fig. 3*B*, middle and bottom). This effect resembles the negative shift in the voltage dependence of activation of Na^+ currents in insect neurons induced by AahIT (Lee and Adams, 2000). As described previously (Marcotte et al., 1997; Cestele et al., 1998; Tsushima et al., 1999), such an effect is typical to β -toxins acting on mammalian Na^+ channels.

Conditioning pulse potentiates the effect of AahIT on the rBIIA-ParaD2 channel

Depolarizing conditioning pulse was demonstrated to be crucial for the activity of the β -toxin Csx-IV (from *Centruroides suffusus suffusus*) on the rat brain NaCh rBIIA (Cestele et al., 1998) or to potentiate the effect of other β -toxins on mammalian NaChs (Tsushima et al., 1999). Based on the results showing that AahIT belongs to the β -toxin class (Fig. 3), we wanted to examine the effect of depolarizing conditioning pulse on the activity of AahIT. To this end, we compared in single oocytes the effect of AahIT with and without a 2 msec depolarizing prepulse to $+50$ mV (Cestele et al., 1998). The results obtained in a representative oocyte clearly show that the prepulse potentiated the effect of AahIT on rBIIA-ParaD2 currents (Fig. 4*A*, compare $-$ prepulse with $+$ prepulse). With the prepulse, AahIT not only shifted the voltage dependence of activation but also increased the maximal current (I_{max}). Steady-state activation analysis in several oocytes (Fig. 4*B*) not only revealed a prepulse-independent shift in the equilibrium activation potential (~ 5.5 mV) but also an increase in the maximal conductance (G_{max}) with the prepulse by $23 \pm 6\%$ ($n = 4$; $p \leq 0.033$). Thus, using the prepulse, we further revealed the similarity between the effects of AahIT on rBIIA-ParaD2 and that of β -toxins on mammalian NaChs.

Analysis of the effect of 1 – $2 \mu\text{M}$ AahIT on the *Drosophila* Para (the donor of D2) coexpressed with TipE revealed an increase in the maximal conductance by $35 \pm 10\%$ ($n = 4$; $p \leq 0.019$) and a shift in the equilibrium activation potential of ~ 3 mV, the former effect being larger and the latter effect being smaller than those observed in the chimera (Fig. 4*C*). Notably, the rBIIA channel remained insensitive to AahIT also with the prepulse (Fig. 4*D*).

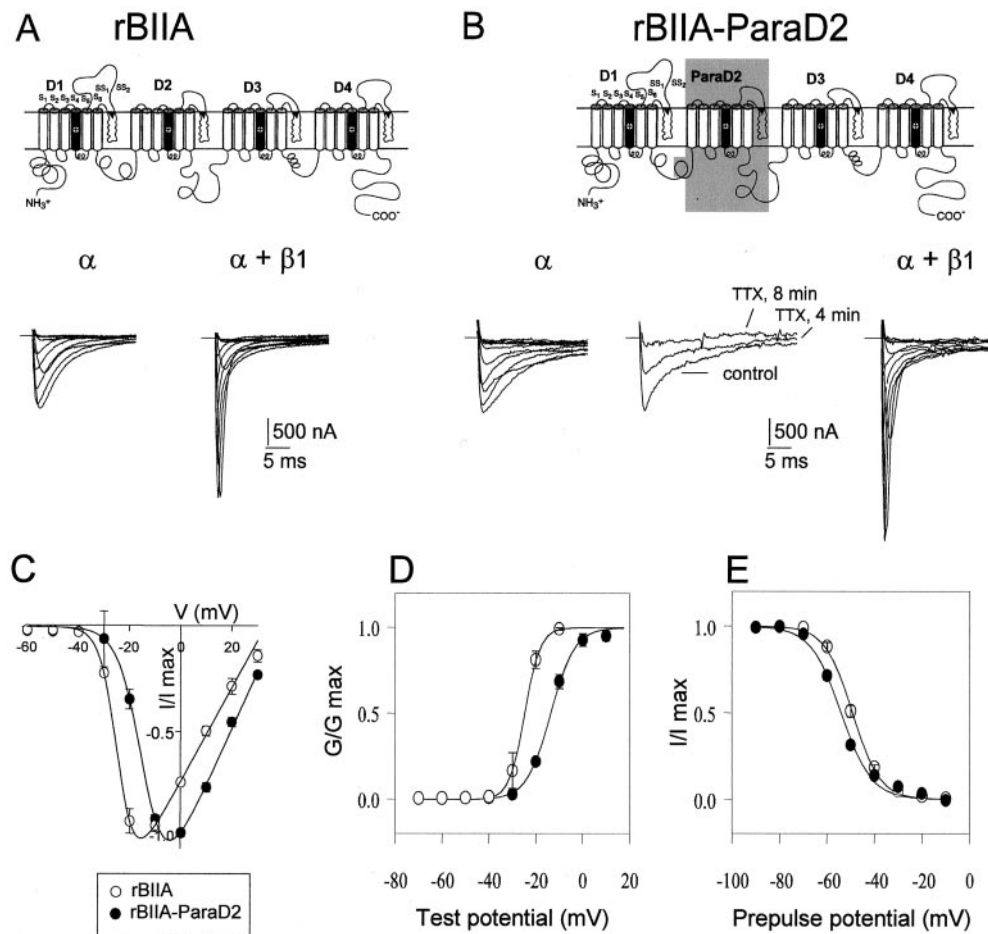


Figure 2. Comparison between the chimera, rBIIA-ParaD2, and the rBIIA currents. *A, B, Top*, A schematic presentation of rBIIA (*A*) and rBIIA-ParaD2 (*B*) channel domains (D1–D4), the latter having rBIIA D1, D3, and D4 and the Para *Drosophila* D2 (ParaD2; gray box). *Bottom*, Na⁺ currents elicited in oocytes expressing α -subunits of rBIIA (*A*) or rBIIA-ParaD2 (*B*) alone (α) or together with β_1 -subunit ($\alpha + \beta_1$; 1:1) as denoted. Oocytes were held at -100 mV, followed by 40 msec depolarizations to voltages varying from -80 to $+30$ mV in 10 mV increments. *Middle trace in B* shows rBIIA-ParaD2 currents elicited by stepping to 0 mV from -80 mV holding potential, before and 4 or 8 min after $1 \mu\text{M}$ TTX application. *C*, Normalized peak Na⁺ currents (I/I_{max}) of rBIIA (open circles) and rBIIA-ParaD2 (filled circles) plotted as a function of membrane voltage (V). Each value is mean \pm SEM from seven oocytes. The experimental results were fitted using a modified Boltzmann equation (Eq. 1 in Materials and Methods). *D*, Normalized conductance (G/G_{max})–voltage relationships derived from the data in *C*. Data were fitted using Equation 1 (see Materials and Methods). *E*, Steady-state inactivation of the rBIIA (open circles) and rBIIA-ParaD2 (filled circles). Oocytes were held at -80 mV, and 200 msec steps to prepulse potentials (V_{prepulse}) from -90 to -10 mV in 10 mV increments were given before eliciting currents by 50 msec steps to -10 or 0 mV (as detailed in Materials and Methods). Fractional current (I/I_{max}) was plotted as a function of V_{prepulse} and fitted to Equation 2 (see Materials and Methods). Each point represents mean \pm SEM values from four oocytes. Values for half-inactivation potential ($V_{1/2}$) and for the slope factor (k) were as follows: rBIIA, $V_{1/2} = -49 \pm 0.75$ mV, $k = 5.7 \pm 0.12$ mV; rBIIA-ParaD2, $V_{1/2} = -54 \pm 0.7$ mV, $k = 6.4 \pm 0.13$ mV; showing a significant difference between rBIIA and rBIIA-ParaD2 (paired two-tailed t test; $p < 0.005$ for $V_{1/2}$; $p < 0.01$ for k).

estimation of the K_D values for both channels (12.78 ± 4.2 nM, $n = 5$; 6.26 ± 1.76 nM, $n = 5$; for rBIIA and rBIIA-ParaD2, respectively) were found not to differ statistically ($p > 0.05$), suggesting that the change in D2 had no major effect on the binding site of the α -toxin Lqh-II. Analysis of the effect of Lqh-II on the time constants of inactivation showed a significant increase in the fast (τ_1) and slow (τ_2) constants of both rBIIA and rBIIA-ParaD2 channels (Table 1). Notably, the slowing of channel inactivation by Lqh-II was more profound in the rBIIA-ParaD2 channel chimera compared with rBIIA (Table 1, Fig. 5). Moreover, the potentiated effect of Lqh-II on rBIIA-ParaD2 was also manifested by the larger increase in current amplitude [by 3.6 ± 0.6 (mean \pm SEM) and 1.76 ± 0.09 , for rBIIA-ParaD2 and rBIIA, respectively] (Fig. 5*B*). These results resemble those obtained with another site-3 toxin from sea anemone (ATXII) on *Drosophila* Para NaCh expressed in oocytes (Warmke et al., 1997). Despite having comparable affinities to rBIIA and to *Drosophila* Para, ATXII had a larger effect on the insect channel, manifested in both larger increase in maximal conductance and larger slowing of inactivation. We also verified that 20 nM Lqh-II practically abolished inactivation and caused a marked increase in the maximal conductance (data not shown), similar to the effect of ATXII on this insect channel. Together, the high sensitivity of

Effects of scorpion α -toxins on the rBIIA-ParaD2 channel

Because rBIIA-ParaD2 was found to be sensitive to the insect-selective toxin AahIT, we wanted to examine whether this insect-mammalian chimera also acquired sensitivity to the α -toxin Lqh-II, which is highly active on insects (Eitan et al., 1990). rBIIA-ParaD2 was poorly sensitive to Lqh-II; the small decrease in τ_2 (Fig. 5*A*, Table 1), however, may be attributed to modifications of intrinsic gating properties rather than to a change in toxin sensitivity caused by the insertion of the insect D2 (Fig. 2) (see below). The rat brain channel rBIIA was practically insensitive to Lqh-II, even at concentrations as high as $5 \mu\text{M}$ (Fig. 5*A*, Table 1), as expected (Gilles et al., 1999). To rule out the possibility that the chimera became resistant to α -toxins in general, we tested the effect of the anti-mammalian α -toxin Lqh-II (Sautiere et al., 1998; Gilles et al., 1999, 2000). Lqh-II caused a typical inhibitory effect on the inactivation of both rBIIA and rBIIA-ParaD2 currents (Fig. 5*B*) (Rogers et al., 1996; Gilles et al., 1999, 2001; Chen et al., 2000). The K_D values (a measure of the apparent affinity of the toxin for its receptor) of Lqh-II to the two channels were estimated by measuring the fraction of the current remaining 5 msec after the beginning of the pulse in the absence and presence of the toxin (see Materials and Methods). The

estimated K_D values for both channels (12.78 ± 4.2 nM, $n = 5$; 6.26 ± 1.76 nM, $n = 5$; for rBIIA and rBIIA-ParaD2, respectively) were found not to differ statistically ($p > 0.05$), suggesting that the change in D2 had no major effect on the binding site of the α -toxin Lqh-II. Analysis of the effect of Lqh-II on the time constants of inactivation showed a significant increase in the fast (τ_1) and slow (τ_2) constants of both rBIIA and rBIIA-ParaD2 channels (Table 1). Notably, the slowing of channel inactivation by Lqh-II was more profound in the rBIIA-ParaD2 channel chimera compared with rBIIA (Table 1, Fig. 5). Moreover, the potentiated effect of Lqh-II on rBIIA-ParaD2 was also manifested by the larger increase in current amplitude [by 3.6 ± 0.6 (mean \pm SEM) and 1.76 ± 0.09 , for rBIIA-ParaD2 and rBIIA, respectively] (Fig. 5*B*). These results resemble those obtained with another site-3 toxin from sea anemone (ATXII) on *Drosophila* Para NaCh expressed in oocytes (Warmke et al., 1997). Despite having comparable affinities to rBIIA and to *Drosophila* Para, ATXII had a larger effect on the insect channel, manifested in both larger increase in maximal conductance and larger slowing of inactivation. We also verified that 20 nM Lqh-II practically abolished inactivation and caused a marked increase in the maximal conductance (data not shown), similar to the effect of ATXII on this insect channel. Together, the high sensitivity of

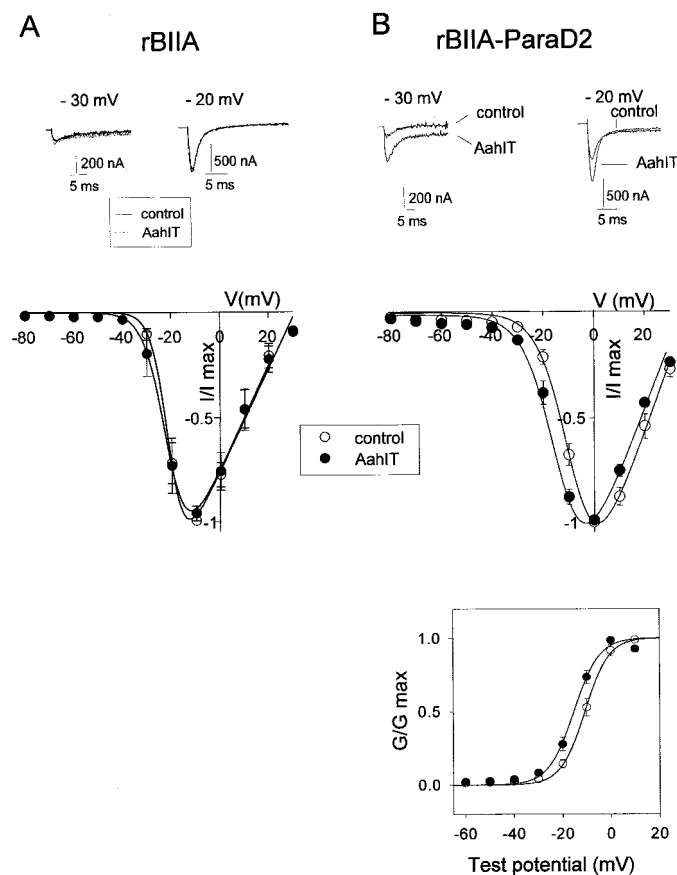


Figure 3. AahIT affects the rBIIA-ParaD2 but not the rBIIA channels. *A, B, Top*, rBIIA (*A*) and rBIIA-ParaD2 (*B*) currents elicited after step depolarizations to -30 or -20 mV (as denoted above traces) before and 10 min after application of $3 \mu\text{M}$ (*A*) or $1.4 \mu\text{M}$ (*B*) AahIT. Concentrations up to $10 \mu\text{M}$ were checked to give the same results. *Middle*, Normalized peak currents (I/I_{max}) of each oocyte plotted as a function of membrane voltage (V), before (*open circles*, control) and 10 min after (*filled circles*) application of AahIT. Each point represents the mean \pm SEM values from three oocytes treated with $3 \mu\text{M}$ (*A*) or of six oocytes treated with 1.4 – $2.8 \mu\text{M}$ (*B*) AahIT. Data were fitted using Equation 1 (see Materials and Methods). No significant difference between maximal currents of control and AahIT-treated oocytes was observed. *Bottom*, Normalized conductance (G/G_{max})–voltage relationships derived from the data in the *middle panel*, fitted to Equation 1. Values for $V_{1/2}$ and k were as follows: control, $V_{1/2} = -10 \pm 1.2$ mV, $k = 4.7 \pm 0.15$ mV; AahIT-treated oocytes, $V_{1/2} = -15.5 \pm 1$ mV, $k = 4.99 \pm 0.07$ mV; showing a significant difference ($p \leq 0.003$ using two-tailed paired t test) in $V_{1/2}$ between the control and AahIT.

the Para channel, as opposed to the resistance of the rBIIA-ParaD2, to Lqh α IT and the strong effect of Lqh-II on both rBIIA and the chimera indicate that D2 neither contribute to the binding of receptor site-3 or to the selectivity of α -toxins. On the other hand, the larger effects of Lqh-II on the chimeric channel indicate that D2 conferred insect properties related to both activation and inactivation gating.

DISCUSSION

In this study, using the insect-selective neurotoxin AahIT, we provide a first step toward deciphering the structural elements that are responsible for the selective recognition of insect versus mammalian NaChs by drugs and toxins. With the background of the close similarity in the primary structure, topological organization, and basic biochemical and pharmacological properties

between the mammalian and the insect NaChs (Gordon, 1997a), we used a mammalian–insect chimeric channel to identify the region that is recognized by AahIT and is responsible for its selective insect toxicity.

The choice of the chimera

The *Drosophila* Para NaCh, which is sensitive to AahIT (Figs. 1*A*, 4*C*), was chosen as a donor of an insect channel region that will confer AahIT sensitivity to the chimeric channel. The rat brain NaCh rBIIA, shown to be insensitive to high doses of AahIT (Fig. 1*B*), was chosen as the mammalian background. Both NaChs have been well characterized in *Xenopus* oocytes. To choose the appropriate region of the insect channel, which would confer the sensitivity to AahIT, we considered the following line of evidence that led us to assign AahIT to the β -toxin class, the receptor site of which is comprised mainly of a single channel domain, D2 (see introductory remarks): (1) like scorpion β -toxins active on mammals (Cahalan, 1975; Jaimovich et al., 1982; Wang and Strichartz, 1983; Vijverberg et al., 1984; Jonas et al., 1986), AahIT modified the activation process of sodium currents, as shown in cockroach axon (Zlotkin et al., 1985) and in lepidopterous (*Heliothis virescens*) neurons (Lee and Adams, 2000); (2) AahIT competed with the binding of the β -toxin Ts-VII (or γ -toxin, from *Tityus serrulatus*) (Lazdunski et al., 1986) on cockroach neuronal membranes (De Lima et al., 1986); and (3) like β -toxins binding to mammalian NaChs (Jover et al., 1980; Lazdunski et al., 1986), AahIT binding to insect (*Locusta migratoria*) neuronal membranes was not modified by membrane potential or by the alkaloid toxin veratridine (Gordon et al., 1984). Having assigned AahIT to the β -class of neurotoxins, we assumed that, like on mammalian channels (Marcotte et al., 1997; Cestele et al., 1998; Tsushima et al., 1999), the β -toxin site on insect channels also resides in D2 and thereby is targeted by AahIT. This assumed that homology between toxin receptor sites on insect and mammalian NaChs was supported by the corresponding homology demonstrated for the α -toxin receptor sites (Gordon and Zlotkin, 1993; Gordon et al., 1996, 2002; Gilles et al., 1999). Thus, we constructed the chimeric channel rBIIA-ParaD2 consisting of rBIIA and D2 of the insect Para *Drosophila*.

Functional NaCh integrity of the rBIIA-ParaD2 chimera

rBIIA-ParaD2 formed a functional, voltage-dependent and TTX-sensitive NaCh (Fig. 2*B*), despite the large phylogenetic distance between flies and rodents. rBIIA-ParaD2 was also regulated by the coexpressed β_1 -subunit (Fig. 2*A, B*), consistent with the finding that the binding site for β_1 was localized to D4 in rBIIA (Qu et al., 1999). The chimeric channel was, however, not sensitive to the coexpressed insect auxiliary subunit TipE, excluding the involvement of D2 in the interaction of the insect channel α -subunit with TipE. Scorpion α -toxin Lqh-II was used to further assess the functional NaCh integrity of the chimeric channel. The binding of α -toxins to an extracellular receptor site (see introductory remarks) affects fast inactivation, a process that involves internal channel regions (Stuhmer et al., 1989; Patton et al., 1992); thus, the toxin effect may reflect a chain of intermolecular changes (Catterall, 1992; Gordon, 1997b; Gilles et al., 2001). The fact that Lqh-II affected the inactivation of the chimeric channel (Fig. 5*B*) ensured that the overall assembly and activity of the channel were not impaired. This functional NaCh integrity provided a suitable setting for the study of insect characteristics, including AahIT sensitivity, that were gained by the chimera.

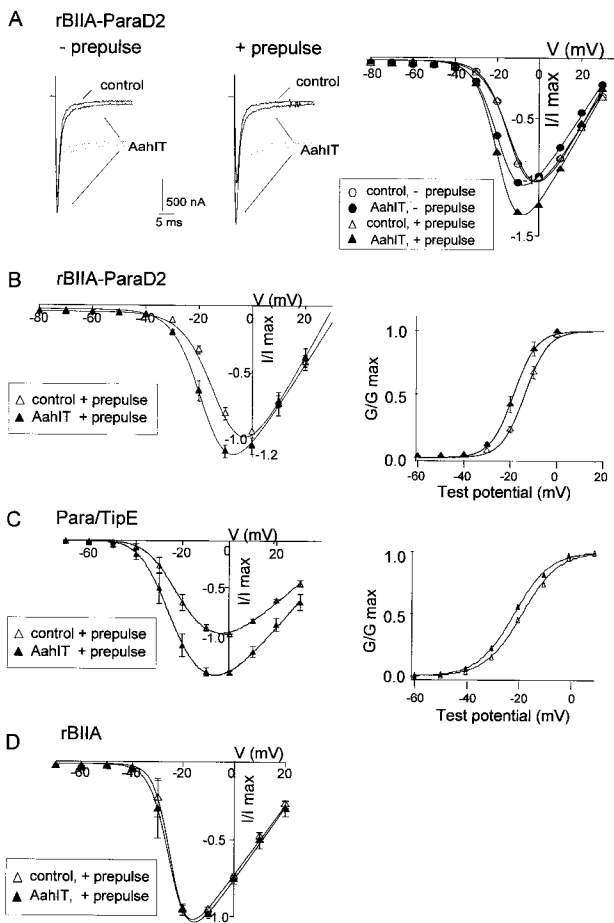


Figure 4. The effect of AahIT is potentiated by a conditioning depolarizing pulse. *A*, rBIIA-ParaD2 currents elicited by step depolarization to -10 mV without ($-$ prepulse, left) or after ($+$ prepulse, middle) a 2 msec prepulse to $+50$ mV, before (control) and after (AahIT) application of $1.4 \mu\text{M}$ AahIT. Right, Normalized rBIIA-ParaD2 current–voltage relationships measured in a single oocyte, before (open triangles, control) and after (filled triangles) application of $1.4 \mu\text{M}$ AahIT using the prepulse protocol. Data showing the effect of the toxin in the same oocyte without using the prepulse protocol (as in Fig. 3B) was superimposed (open and filled circles for control and AahIT, respectively). Currents were normalized to maximal current of control. Data were fitted using Equation 1 (see Materials and Methods). G_{max} values without prepulse were as follows: 53.2 and $52.4 \mu\text{S}$ for control and AahIT-treated oocytes, respectively. G_{max} values with prepulse were as follows: 43.5 and $50.6 \mu\text{S}$ for control and AahIT-treated oocytes, respectively. *B*, *C*, Left, Normalized peak currents (I/I_{max} of control) of rBIIA-ParaD2 (*B*) or Para/TipE (*C*) plotted as a function of membrane voltage (V), before (open triangles) and 10 min after (filled triangles) application of $1\text{--}2 \mu\text{M}$ AahIT, using a prepulse protocol. Each point represents the mean \pm SEM values from four oocytes. Right, Voltage dependence of activation derived from the current–voltage relationships (left) presented as an activation curve of control (open triangles) and AahIT-treated (filled triangles) oocytes. Normalized $G\text{--}V$ relationships were fitted using Equation 1 (see Materials and Methods). Values for rBIIA-ParaD2 (*B*) were as follows: control, $V_{1/2} = -14 \pm 0.67$ mV, $k = 4.7 \pm 0.09$ mV, $G_{\text{max}} = 31.48 \pm 3$; AahIT-treated oocytes, $V_{1/2} = -19.5 \pm 1$ mV, $k = 4.6 \pm 0.1$ mV, $G_{\text{max}} = 38.6 \pm 2.6$; showing significant differences (paired two-tailed t test) in $V_{1/2}$ ($p < 0.005$) and G_{max} ($p < 0.033$) between the control and AahIT-treated oocytes. Values for Para/TipE (*C*) were as follows: $V_{1/2} = -19 \pm 2.23$, $k = 6.5 \pm 0.3$, $G_{\text{max}} = 19.42 \pm 1.37$; AahIT-treated oocytes, $V_{1/2} = -22.2 \pm 2.7$, $k = 6.2 \pm 0.2$, $G_{\text{max}} = 26.31 \pm 2.84$; showing significant differences (paired two-tailed t test) in $V_{1/2}$ ($p < 0.007$) and G_{max} ($p < 0.019$) between the control and AahIT-treated oocytes. *D*, Normalized currents (I/I_{max}), elicited using the prepulse protocol, plotted as a function of membrane voltage (V) in either control (open triangles; $n = 7$) or $10 \mu\text{M}$ AahIT treated (filled triangles; $n = 4$) in rBIIA-expressing oocytes.

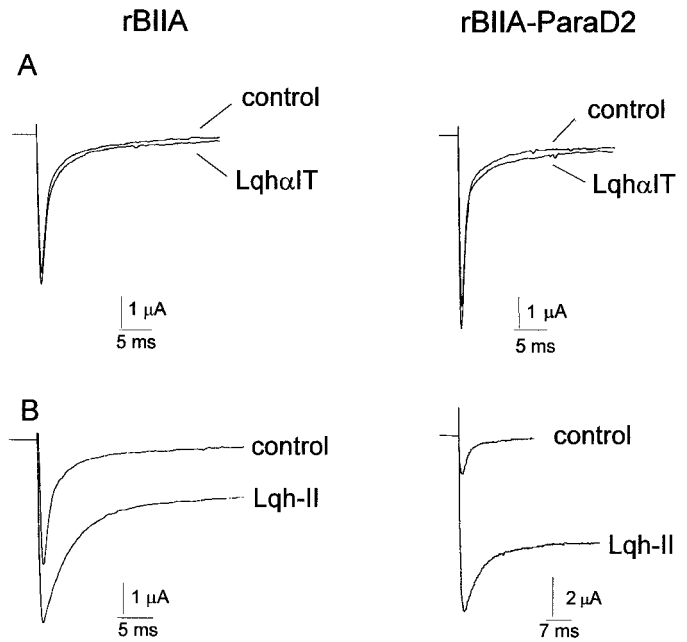


Figure 5. The effect of scorpion α -toxins on rBIIA-ParaD2 and rBIIA. Oocytes were held at -80 mV, and maximal rBIIA or rBIIA-ParaD2 currents, recorded every 1 min, were elicited by a depolarizing pulse to -10 or 0 mV, respectively. *A*, rBIIA-ParaD2 (right) and rBIIA (left) currents, before (control) and 13 min after application of $1 \mu\text{M}$ Lqh α IT. Same results were obtained with the toxin at $5 \mu\text{M}$. *B*, rBIIA-ParaD2 (right) and rBIIA (left) currents, before (control) and 13 min after application of 200 nM Lqh-II.

Table 1. The effect of scorpion α -toxins on the inactivation of sodium current

	Lqh-II ^a		Lqh α IT ^b	
	τ_1 (msec)	τ_2 (msec)	τ_1 (msec)	τ_2 (msec)
rBIIA				
Control	1.08 ± 0.1	10.42 ± 1.3	1.15 ± 0.1	11.6 ± 2
+ Toxin	$3.27 \pm 0.1^{**}$	$18.56 \pm 1.4^*$	1.2 ± 0.1	11.57 ± 1.6
<i>n</i>		5		6
rBIIA-ParaD2				
Control	1.2 ± 0.2	9.9 ± 1.4	1.06 ± 0.1	9.28 ± 1.1
+ Toxin	$3.6 \pm 0.2^{**}$	$30 \pm 3.1^*$	1.01 ± 0.1	$7.3 \pm 0.7^*$
<i>n</i>		4		7

Values were measured before and 15 min after application of the indicated toxin. *n* denotes number of oocytes. Values represent mean \pm SEM, and asterisks represent a significant difference between control and toxin-treated oocytes. * $p \leq 0.05$; ** $p \leq 0.01$.

^aAt least 100 nM was used to obtain a maximal effect of Lqh-II.

^bOne to $5 \mu\text{M}$ Lqh α IT were used.

Domain 2 of the insect channel confers NaCh sensitivity to AahIT

Two apparent effects on Na^+ current activation were documented for the anti-insect toxin AahIT in neuronal preparations of various insects: an increase of maximal current and a negative shift of the voltage dependence of activation (Zlotkin, 1997). Thus, AahIT increased the Na^+ peak current in cockroach axons and shifted the activation curve (Zlotkin, 1997), whereas, in intact insect neurons (*Heliothis virescens*, Lepidoptera), only the shift in the activation was observed (Lee and Adams, 2000). In the *Drosophila* (fruit fly) Para NaCh coexpressed with TipE in oo-

cytes, the main effect of AahIT was an increase of the peak current (by 35%) accompanied by a small shift (~ 3 mV) in the activation curve (Fig. 4C). It appears that the sensitivity to AahIT varies among the different insect species and may depend also on channel preparation (native channels in neurons vs expressed channels in oocytes). Notably, AahIT, at concentrations similar to those used for *Drosophila* Para NaCh, exerted both effects on the rBIIA-ParaD2: an increase in the peak current (by 20%) and a shift in the activation curve (by ~ 6 mV) (Fig. 3B). In view of the similar toxin sensitivity of the chimeric channel to the insect Para channel, together with the absolute toxin resistance of the mammalian channel rBIIA (Fig. 1), these results indicate that the receptor site for the insect-selective toxin AahIT resides mainly in ParaD2. Also, TipE is apparently not involved in the toxin selectivity to insect channels, because the chimera was sensitive to AahIT in its absence.

Interestingly, the concentrations of AahIT required to elicit the effects in neurons (50–100 nM) (Lee and Adams, 2000) were 10-fold lower than those required for the chimeric and *Drosophila* Para channels (1–2 μ M). These differences may be attributed to variations in susceptibility of various insects to the toxin (Fishman et al., 1997), which most probably vary in their NaCh Para gene homologs. Such variations may be exemplified by *Sarcophaga* blowfly larvae, which are at least 130-fold more sensitive to AahIT than *Drosophila melanogaster* wild-type flies. Moreover, in various *Drosophila* strains, which differ by a few substitutions in their gene encoding the Para channel, the sensitivity to AahIT is reduced by up to 2000-fold compared with blowflies (Zlotkin et al., 1999).

The negative shift in the voltage sensitivity of activation induced by AahIT on insect and the rBIIA-ParaD2 channels resembles the effect of scorpion β -toxins on mammalian channels (Cahalan, 1975; Meves et al., 1982; Wang and Strichartz, 1983; Vijverberg et al., 1984; Jonas et al., 1986; Marcotte et al., 1997; Cestele et al., 1998; Tsushima et al., 1999). Furthermore, the effects of AahIT on rBIIA-ParaD2 was potentiated by a depolarizing prepulse (Fig. 4), as was shown previously for some β -toxins (Cestele et al., 1998; Tsushima et al., 1999), thus establishing the similarity between AahIT and β -toxins modes of action.

The rBIIA-ParaD2 acquired additional insect NaCh properties

In addition to the sensitivity to AahIT, the rBIIA-ParaD2 acquired inherent activation parameters [a positive shift of the half activation voltage ($V_{1/2}$)] (Fig. 2D) that were similar to those of the intact *Drosophila* Para channel expressed in oocytes (Warmke et al., 1997) (Fig. 4C). The critical impact of the origin of D2 on the voltage-dependent activation of NaChs was demonstrated previously (Marcotte et al., 1997). In view of the 65% identity between the entire D2 of rBIIA and *Drosophila* Para, sharing, however, a 100% identity in their voltage sensors (D2–S4), our results highlight the importance of structural elements other than D2–S4 in the activation process of NaChs. Indeed, in addition to the previously demonstrated critical impact of mutations in D2–S4 (Stuhmer et al., 1989; Auld et al., 1990; Moran et al., 1994), changes in the external loops of D2 in mammalian NaChs were also shown to affect the voltage dependence of activation (Cestele et al., 1998; Qu et al., 1999).

Other insect properties conferred by the insect D2 were revealed by analyzing the effect of the site-3 α -toxin Lqh-II on the chimeric channel. Lqh-II affected the inactivation of rBIIA-ParaD2 more potently than that of rBIIA (Fig. 5B, Table 1). This

effect was not accompanied by a change in the toxin binding affinity (Fig. 5), supporting the notion that D2 does not contribute to receptor site-3 (Gordon et al., 2002). Because D2 has not yet been implicated in any phenomenon pertaining to NaCh inactivation, our results indicate involvement of D2 in inactivation, in addition to its expected involvement in activation (as detailed above).

Additional considerations

Two additional inferences regarding NaChs can be made on the basis of the results obtained in this study. (1) The findings that the binding of both α -toxins Lqh-II and Lqh α IT were not affected by the change in D2 (Fig. 5) indicate that the selectivity of α -toxins toward rat brain and insect NaChs is not conferred by D2. (2) The insect Para *Drosophila* NaCh is hardly expressed in oocytes without the coexpression of the auxiliary subunit TipE (Feng et al., 1995; Warmke et al., 1997). We show, however, that the mammalian channel rBIIA can acquire insect channel properties by virtue of insertion of a single domain of Para *Drosophila* channel without the need of TipE. Thus, the chimera approach presents a technological advantage for the study of NaChs in heterologous systems, which circumvents possible difficulties resulting from missing additional subunits.

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