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Voltage Sensor–Trapping: Enhanced Activation of Sodium Channels by β-Scorpion Toxin Bound to the S3–S4 Loop in Domain II

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Summary

Polypeptide neurotoxins alter ion channel gating by binding to extracellular receptor sites, even though the voltage sensors are in their S4 transmembrane segments. By analysis of sodium channel chimeras, a β-scorpion toxin is shown here to negatively shift voltage dependence of activation and enhance closed state inactivation by binding to a receptor site that requires glycine 845 (Gly-845) in the S3–S4 loop at the extracellular end of the S4 segment in domain II of the α subunit. Toxin action requires prior depolarization to drive the S4 voltage sensors outward, but these effects are lost in the mutant G845N. The results reveal a voltage sensor–trapping model of toxin action in which the IIS4 voltage sensor is trapped in its outward, activated position by toxin binding.

Introduction

Voltage-gated sodium channels mediate the voltage-dependent increase in sodium ion permeability that initiates action potentials (Hodgkin and Huxley, 1952). Sodium channels are composed of a pore-forming α subunit of 260 kDa associated with one or two auxiliary subunits, β1 of 36 kDa and β2 of 33 kDa (reviewed by Catterall, 1992). The α subunit consists of four repeat domains (I–IV), each containing six transmembrane segments (S1–S6) and one membrane reentrant segment (SS1/SS2) connected by internal and external polypeptide loops (Noda et al., 1986; Catterall, 1992). The S4 segments contain 4–8 positively charged residues at 3 residue intervals. They serve as voltage sensors and initiate the voltage-dependent activation of sodium channels by moving outward under the influence of the electric field (Armstrong, 1981; Stühmer et al., 1989; Yang et al., 1996). Inactivation is mediated by the short intracellular loop connecting domains III and IV (Vassilev et al., 1988, 1989; Stühmer et al., 1989; West et al., 1992). Transmembrane segments S5 and S6 and the SS1 and SS2 segments in the membrane reentrant loop between them form the walls of the pore and the narrow ion selectivity filter at its extracellular end (Noda et al., 1989; Terlau et al., 1991; Heinemann et al., 1992). The pore blocker tetrodotoxin binds to a receptor site within the ion selectivity filter of the channel (Terlau et al., 1991).

Although the voltage sensors of the sodium channel are located within the membrane, polypeptide toxins from scorpions, sea anemones, and other species strongly modify voltage-dependent activation and inactivation by binding to specific receptor sites on the extracellular surface of the channel (reviewed by Catterall, 1980, 1992). How does their binding to extracellular amino acid residues alter gating? Previous studies have shown that α-scorpion toxins and sea anemone toxins, which slow inactivation by interaction with neurotoxin receptor site 3, bind to amino acid residues in the S3–S4 loop at the extracellular end of the S4 voltage sensor in domain IV of the sodium channel α subunit (Rogers et al., 1996). In contrast to α-scorpion toxins, β-scorpion toxins bind to neurotoxin receptor site 4 (Jover et al., 1980; Barhanin et al., 1982) and shift the voltage dependence of activation to more negative potentials (Cahalan, 1975; Jaimovich et al., 1982; Meves et al., 1982; Wang and Strichartz, 1983; Vijverberg et al., 1984; Jonas et al., 1986). In these experiments, we show that a potent β-scorpion toxin binds to a receptor site including the S3–S4 extracellular loop in domain II of the sodium channel α subunit (IIS3–S4) and causes a prepulse-dependent negative shift in the voltage dependence of gating by a voltage sensor–trapping mechanism. A single point mutation (G845N) in IIS3–S4 reduces the affinity for toxin binding and prevents voltage sensor trapping. It is proposed that voltage sensor trapping is the fundamental mechanism of action of polypeptide toxins that alter the voltage-dependent gating of sodium, calcium, and potassium channels.

Results

The β-scorpion toxins are single chain polypeptides of 60–65 amino acid residues cross-linked by four disulfide bridges (Rochat et al., 1979; Dufton and Rochat, 1984). Their binding to neurotoxin receptor site 4 of sodium channels causes both a shift in the voltage dependence of activation in the hyperpolarizing direction and a reduction of peak current amplitude (Cahalan, 1975; Jover et al., 1980; Jaimovich et al., 1982; Meves et al., 1982; Wang and Strichartz, 1983; Vijverberg et al., 1984; Jonas et al., 1986). Cardiac and brain sodium channels are similar in structure but differ significantly in gating, ion permeation and block, and pharmacology (Fozzard and Hanck, 1996). Previous results show that β-scorpion toxins bind with higher affinity and have stronger functional effects on skeletal muscle sodium channels than on cardiac sodium channels (Marcotte et al., 1997). In this study, we have used chimeras constructed from cardiac and type IIA brain sodium channels to identify the molecular determinants responsible for high affinity binding and functional modulation by one of the most

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potent β-scorpion toxins, Css IV isolated from the venom of the Mexican scorpion *Centruroides suffusus suffusus*.

State-Dependent Modulation of Brain-Type IIA Sodium Channels by Css IV

Previous studies have shown that the negative shift in the voltage dependence of activation caused by β-scorpion toxins is only observed after the channel is "primed" with a preceding depolarizing prepulse in the presence of toxin (Cahalan, 1975; Jover et al., 1980; Jaimovich et al., 1982; Meves et al., 1982; Wang and Strichartz, 1983; Vijverberg et al., 1984; Jonas et al., 1986). To begin to analyze the effects of Css IV on brain-type IIA sodium channels expressed transiently in tsA-201 cells, we measured peak sodium currents elicited by strong depolarization and threshold sodium currents elicited by a test pulse to a negative membrane potential (−65 mV; see pulse protocol in Figure 1A) as a function of time after exposure to 40 nM Css IV in the whole-cell voltage-clamp configuration. In the absence of Css IV, the channels are closed at potentials between −80 and −60 mV. No Na^+ current is activated by depolarizations to −65 mV (Figure 1B), and no change in peak sodium current is observed with or without preceding depolarizations to +50 mV. Similarly, in the presence of Css IV without priming depolarizations, no sodium current is observed in test pulses to −65 mV (data not shown). In contrast, after a priming depolarization to +50 mV in the presence of the toxin, a test pulse to −65 mV resulted in a substantial sodium current (Figure 1B, trace 2). In addition, after several repetitions of this pulse protocol, the peak sodium current recorded in test pulses to 0 mV was decreased (Figure 1C). Thus, Css IV caused two different effects on brain-type IIA sodium channels, and both required prior depolarization.

The time course of the changes in sodium current at −65 mV and in peak sodium current at 0 mV are illustrated in Figure 1D as a function of time of toxin exposure. The time courses of onset and reversal of these effects are strikingly different. The sodium current at −65 mV appears after a single depolarization to +50 mV and is reversed upon washout within one or two depolarizations (Figure 1D, open circles). In contrast, the reduction in peak sodium current accumulates over many seconds and is slowly reversed upon washout (Figure 1D, closed squares). The results indicate that the toxin-modified sodium channel is trapped in a partially activated state after a single activation of the channel by a depolarizing prepulse in the presence of the Css IV toxin.

Analysis of current–voltage relationships for control and Css IV–modified sodium currents revealed a negative shift in the voltage dependence of activation after priming depolarizations in the presence of Css IV (Figure 1E). A low toxin concentration (40 nM) caused a biphasic current–voltage relationship with ~10% of the sodium current activating at negative membrane potentials relative to control (Figure 1E). A high toxin concentration (1 μM) caused an almost complete shift of the current–voltage relationship to more negative membrane potentials (Figure 1E). Replotting these results as conductance–voltage relationships showed that the rapid, prepulse-dependent appearance of sodium current in test pulses to −65 mV results from a toxin-dependent shift in the voltage dependence of activation by -31.7 ± 3.0 mV in the presence of 1 μM Css IV ($n = 3$; Figure 1F).

The voltage dependence of inactivation was measured by changing the holding potential in the range of −120 to −60 mV under control conditions or following a series of depolarizing prepulses in the presence of 40 nM Css IV (Figure 1G). The toxin caused a negative shift of 12 mV in the voltage dependence of steady state inactivation. Because 40 nM Css IV had no effect on sodium channels at a holding potential of −120 mV but reduced peak sodium currents at more positive holding potentials, it is likely that the toxin binds with higher affinity to the inactivated state than to the resting state. The shift in voltage dependence of inactivation is most readily explained by an allosteric mechanism involving preferential toxin binding to inactivated channels, analogous to the modulated receptor model for local anesthetic block of inactivated sodium channels (Hille, 1977; Bean et al., 1983). As predicted by this model, the shift in steady state inactivation was reversible and was dependent on the concentration of β-scorpion toxin (data not shown). Based on this model, we calculated a K_D value for Css IV binding to the inactivated state of 3.4 nM. The K_D for binding to the resting state is ~1–5 μM. Preferential binding to inactivated sodium channels would stabilize the inactivated state and increase inactivation at negative membrane potentials. This effect is responsible for the reduction of peak sodium current observed at holding potentials of −100 mV in Figures 1C and 1D. Thus, these experiments show that the voltage dependence of both activation and inactivation gating of brain-type IIA sodium channels is shifted to more negative membrane potentials by Css IV in response to priming depolarizations. The shifts of activation gating require priming depolarizations and occur more rapidly during trains of depolarizing stimuli.

The effects of Css IV described above were observed for sodium channels expressed from cDNA encoding only the α subunit. In contrast, previous photoaffinity labeling experiments with photoreactive derivatives of β-scorpion toxins have shown specific incorporation into the α or α plus β1 subunits of sodium channels (Barhanin et al., 1983; Darbon et al., 1983; De Lima et al., 1988). Radiation inactivation indicated a functional unit size of 34–45 kDa for the receptor site of β-scorpion toxin, suggesting that the β1 subunit of the sodium channel is required for the binding of this group of toxins (Angelides et al., 1985; Seagar et al., 1986). To assess the binding affinity of sodium channels containing α alone or α plus β1 subunits for Css IV under conditions more similar to these previous biochemical studies, we developed a radioligand binding assay for identification of the toxin binding site and measured high affinity binding of ^{125}I-labeled Css IV to cloned and expressed sodium channels. Scatchard transformation of competition binding curves indicates that there is no difference in K_D values ($K_D = 0.2 \pm 0.03$ nM) for Css IV binding to the α subunit alone or to the αβ1 complex (Figure 2A). This affinity is comparable to that observed on rat brain synaptosomes (Martin-Eauclaire et al., 1987). These data indicate that the α subunit of the type IIA brain

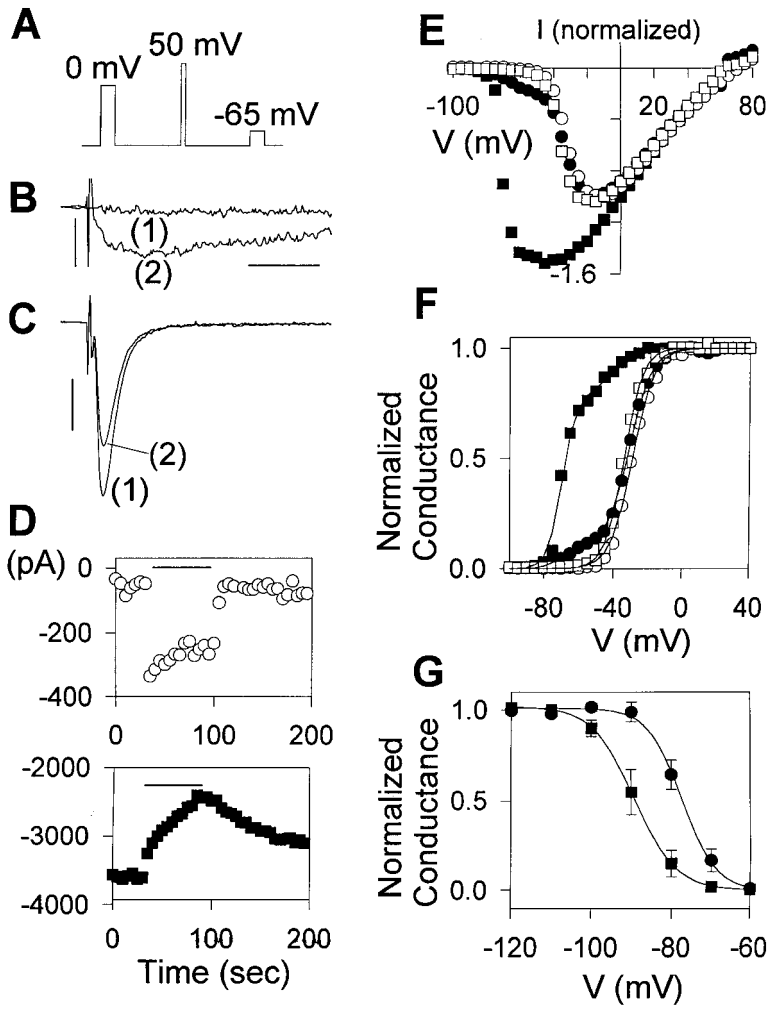

Figure 1. Effects of Css IV on Brain-Type IIA Na Current

(A) The pulse protocol for obtaining the data shown in panels (B), (C), and (D). A 15 ms test pulse to 0 mV was applied from a holding potential of −100 mV. This was followed 61.2 ms later by a 1 ms conditioning pulse to +50 mV, a second period of 61.2 ms at the holding potential, and a 15 ms test pulse to −65 mV. This protocol was applied once every 5 s.

(B) Currents recorded during the −65 mV test pulse in control (1) and 40 nM Css IV (2). Calibration bars, 1 ms; 300 pA.

(C) Currents recorded in response to the test pulse to 0 mV in control (1) and in the presence of 40 nM Css IV (2) after nine cycles of the depolarizing protocol in panel (A). Calibration bars, 1000 pA; time scale is the same as in panel (B).

(D) Time course of the Css IV effect on the amplitude of peak currents elicited by the test pulse to −65 mV (upper panel, open circles) and by the test pulse to 0 mV (lower panel, closed squares) from a holding potential of −100 mV. Css IV was applied during the period indicated by the solid lines.

(E) Normalized current–voltage relationships in the presence of 40 nM (circles) and 1 μM Css IV (squares) without a prepulse (open symbols) or with a +50 mV, 1 ms prepulse that preceded the test pulse by 61.2 ms (solid symbols). The holding potential was −100 mV. All data were normalized to the peak current amplitude in the presence of 40 nM Css IV in the absence of prepulses.

(F) The voltage dependence of activation derived from the data in (E) presented using the corresponding symbols. Data obtained without prepulses were fit with a single Boltzmann relationship, and data obtained with prepulses were fit with the sum of two Boltzmann relationships (solid lines). In the presence of 40 nM Css IV, $V_a = -33.7$ mV and $k = -5.4$ mV without prepulses, and with prepulses, $V_a = -70.5$ mV, $k = -4.8$ mV for the first component, which accounted for 9% of total activation, and $V_a = -30.8$ mV, $k = -6.4$ mV for the second component. In the presence of 1 μM Css IV, $V_a = -38.8$ mV, $V_a = -6.2$ mV without prepulses, and with prepulses, $V_a = -69.5$ mV, $k = -4.0$ mV for the first component, which accounted for 79% of the total, with $V_a = -40.4$ mV, $k = -6.0$ mV for the second component.

(G) Voltage dependence of inactivation derived with 30 s holding potential changes in the absence (closed circles) and presence (closed squares) of 40 nM Css IV. Data were normalized to the current amplitude elicited with a holding potential of 120 mV in control and shown as mean ± standard error (n = 5). Css IV caused no reduction in current at the most hyperpolarized potentials. The data were fit with single Boltzmann relationships (solid lines), with $V_h = -77.3$ mV, $k = 4.2$ in control and $V_h = -89.2$ mV, $k = 5.2$ in the presence of Css IV.

sodium channel contains the specific receptor site for β-scorpion toxin binding and that its affinity is not altered in the presence of the β1 subunit under our binding assay conditions.

Effects of Css IV on Cardiac Sodium Channels

We carried out an analogous series of experiments on cloned and expressed cardiac sodium channels. In contrast to the complex effects of Css IV on activation of brain sodium channels, this toxin did not cause a negative shift of the voltage dependence of activation of cardiac sodium channels, with or without priming depolarizations, at concentrations up to 1 μM (Figures 3A and 3B). In contrast, a small (3–5 mV) positive shift was observed in the presence of the toxin with or without prepulses. Peak sodium current was reduced by Css IV (Figure 3A), but priming depolarizations were not required for this effect, and both the onset and reversal of toxin action were rapid (data not shown). The reduction in peak sodium current of cardiac sodium channels was much less voltage-dependent than for brain sodium channels and had much lower affinity. Measurements of steady state inactivation showed little negative shift in the voltage for half-maximal inactivation (Figure 3C), and the reduction in peak sodium current persisted at the most negative holding potentials tested (−160 mV). The apparent K_D measured at a holding potential of −140 mV was 227 nM, and the K_D calculated for the inactivated state was 65.8 nM. Thus, Css IV has nearly 20-fold lower affinity for the inactivated state of cardiac sodium channels compared to brain sodium channels and has only small effects on the voltage dependence of activation or inactivation of cardiac sodium channels, but it does

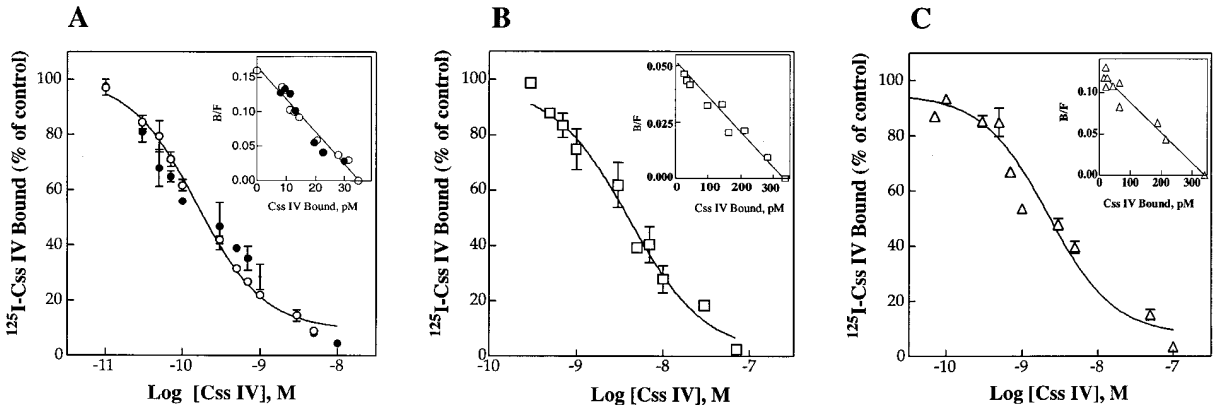

Figure 2. Competitive Binding Curves for Inhibition of ^{125}I–Css IV Binding to Transiently Expressed Sodium Channels by Unlabeled Css IV

(A) Binding of Css IV to transiently expressed rIIA sodium channel α subunits (open circles) or the $\alpha\beta1$ complex (closed circles).
(Inset) Scatchard transformation of the competitive binding curves, indicating that Css IV binds with the same affinity (K_D = 0.2 ± 0.03 nM) to cells expressing only the α subunit of the sodium channel or to those expressing the $\alpha\beta1$ complex.
(B) Binding of Css IV to transiently expressed rH1 sodium channel α subunits.
(Inset) Scatchard transformation of the competitive binding curve, indicating Css IV affinity of 3.8 ± 0.8 nM.
(C) Binding of Css IV to transiently expressed G845N sodium channel α subunits.
(Inset) Scatchard transformation of the competitive binding curve, indicating Css IV affinity (K_D = 2.6 ± 0.004 nM).

reduce peak sodium current substantially at all holding potentials.

We also analysed the binding affinity of Css IV to membranes from tsA-201 cells transfected with the cardiac (rH1) sodium channel α subunit. Scatchard transformation of the competition binding curves of Css IV on membranes prepared from these sets of transfected cells gave a K_D value of 3.8 ± 0.8 nM (Figure 2B), indicating that the α subunit of cardiac sodium channels binds β-scorpion toxins with 19-fold lower affinity than brain sodium channels. Experiments in which the $\beta1$ subunit was cotransfected gave similar results, indicating that the $\beta1$ subunit has no effect on Css IV binding to cardiac sodium channels (data not shown).

Amino Acid Residues Responsible for the Differences in β-Scorpion Toxin Binding to Brain and Cardiac Sodium Channels

We analyzed Css IV binding to chimeric sodium channels in which the amino acid residues within each of the 16 extracellular loops of the brain-type IIA α subunit had been converted to those in the cardiac rH1 isoform in order to identify regions involved in the formation of β-scorpion toxin receptor site. For some chimeras, the expression level was too low to perform binding experiments. For these, partial chimeras were constructed converting only one or two amino acids at a time, the sum of which contained the full amino acid differences between the two isoforms (Figure 4A). The binding affinity of each chimera for Css IV was tested using membranes prepared from tsA-201 cells expressing the chimeric sodium channel α subunits. The K_D values for the chimera or partial chimera in each extracellular loop that had the largest difference from wild-type rIIA are illustrated as a bar graph in Figure 4B. Results from these experiments indicate that substitutions in 4 of the 16 extracellular loops caused reductions in binding affinity for Css IV from 3-fold to 13-fold (Figure 4B): IS5–SS1 in domain I, IIS1–S2 and IIS3–S4 in domain II, and IIISS2–S6 in domain III. These extracellular loops are spread broadly across the primary structure of the α subunit (Figure 4A, arrows). The lack of effect of chimeric mutations in 12 of the 16 extracellular loops demonstrates that the effects of these mutations are specific.

The amino acid sequences and binding data for chimeras in each of these four extracellular loops are presented in Figure 5. In domain III, the conversion of the 5 amino acid residues in the SS2–S6 loop of the brain sodium channels to those in the cardiac isoform (Figure 5A; Chim IIISS2–S6) reduces Css IV affinity 3.5-fold (K_D= 0.7 ± 0.07 nM). One or more of the altered amino acid residues in the IIISS2–S6 loop of the brain sodium channels may play a significant role in the formation of the β-scorpion toxin receptor site and may contribute to the difference in binding affinity between brain and cardiac sodium channels.

In domain I, the chimera of the S5–SS1 loop (Figure 5A; Chim IS5–SS1) has reduced affinity for Css IV. This loop is the largest extracellular loop of the sodium channel and has the lowest sequence similarity between the two isoforms. The chimera corresponding to the conversion of all of the different amino acid residues between brain and cardiac sodium channels (Chim IS5–SS1) binds Css IV with an affinity 6-fold lower than brain sodium channels (Figure 4B). To identify which part of the loop is involved in β-scorpion toxin binding, the binding affinity of a chimera converting only the amino acid differences in the N-terminal part of the loop (Chim IS5–SS1a) was tested (Figure 5A). No significant difference in Css IV binding affinity between this chimera and the sodium channel rIIa was observed (Figure 5B), indicating that the amino acid residues responsible for the reduction in affinity of Css IV for Chim IS5–SS1 are localized in the C-terminal part of the IS5–SS1 loop. This part of IS5–SS1 loop differs by 6 amino acid residues between brain and cardiac sodium channels. As for domain III, we did not attempt to identify the single residues

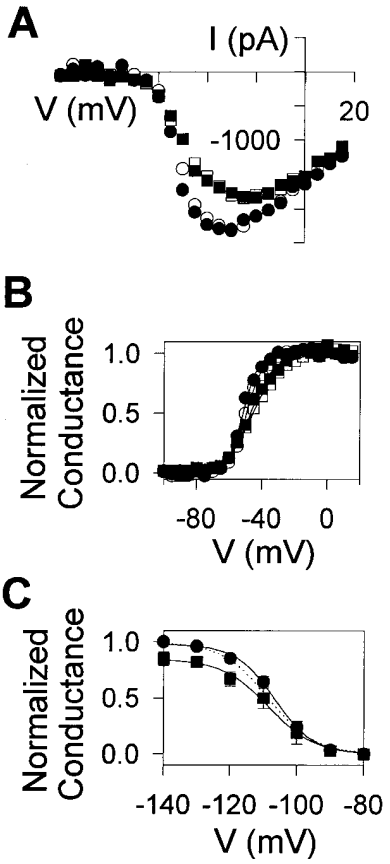

Figure 3. Effects of Css IV on Cardiac rH1 Na Current

(A) Current–voltage relationships in control (circles) and 40 nM Css IV (squares) in the absence (open symbols) and presence (solid symbols) of a 1 ms depolarizing prepulse to +50 mV. The protocol was identical to that of Figure 1E except that the holding potential was −120 mV.

(B) Voltage dependence of activation derived from the data in (A) plotted using the corresponding symbols. Data were fit with a single Boltzmann relationship (solid lines). In control, $V_a = -51.6$ mV, k = −4.7 mV with a prepulse, and $V_a = -49.4$ mV, k = −5.2 mV without a prepulse. In the presence of 40 nM Css IV, $V_a = -46.9$ mV, k = −7.8 mV with a prepulse, and $V_a = -45.2$ mV, k = −8.4 mV without a prepulse.

(C) Voltage dependence of inactivation obtained using 30 s prepulses to a variable voltage followed by a test pulse to 0 mV in control (closed circles) and 40 nM Css IV (closed squares). Data were normalized to the current amplitude elicited by the test pulse following the −140 mV prepulse in control and are shown as mean ± standard error (n = 3). A single Boltzmann relationship (solid lines) was fit to the data, with $V_h = -106.6$ mV, k = 5.9 mV in control and $V_h = -108.6$ mV, k = 7.0 mV in the presence of Css IV. The dotted line is the scaled version of the fit to the data in Css IV.

involved, because the reduction in binding affinity was relatively small.

In domain II, two chimeras had altered β-scorpion toxin binding. In the IIS1–S2 loop, three partial chimeras were constructed (Figure 5A). Chim P782N, which converts pro in position 782 to asparagine (Asn), binds Css IV with a 4-fold lower affinity ($K_D = 0.8 \pm 0.2$ nM), while the adjacent chimeric mutations E785A/Q786E and S788E/S789E/V790M had no detectable effect (Figure 5B). The chimera having a substitution of the entire IIS3–S4 loop did not express detectable binding activity in tsA-201 cells. Therefore, each amino acid that differed between the two isoforms was individually mutated (Figure 5A). Of the five mutants analyzed, only G845N strongly decreased the affinity of Css IV for sodium channels ($K_D = 2.6 \pm 0.004$ nM), whereas the others had no effect. Competition binding experiments and Scatchard transformation of the binding data for mutant G845N are shown in Figure 2C. The decrease in affinity (13-fold) was the largest detected in our study and indicates that glycine 845 (Gly-845) is an important determinant for β-scorpion toxin binding to sodium channels. If their effects are additive, the 2 single residue chimeric mutations in domain II (P782N and G845N) would be sufficient to account fully for the difference in affinity between brain and cardiac sodium channels, suggesting that these extracellular loops are directly involved in β-scorpion toxin binding.

Effects of Css IV on Mutant G845N

We analyzed the functional effects of Css IV on mutant G845N to determine whether any of the differences in toxin action on brain and cardiac sodium channels were caused by this single amino acid difference. The overall functional properties of the G845N chimera in the absence of Css IV were similar to those of rIIA. The time course of the current during the pulse was similar to rIIA and strikingly faster than that of the rH1 channel at the same potential (τ_h at 0 mV: rIIA, 0.36 ± 0.04 ms, n = 6; rH1, 0.75 ± 0.05 ms; G845N, 0.29 ± 0.01 ms, n = 7). The voltage dependence of current activation was similar to rIIA and differed by almost 27 mV from rH1 (Figures 6A and 6B; V_a (rIIA) = −30.4 ± 3.7 mV, n = 5; V_a (rH1) = −56.2 ± 4.3 mV, n = 4; V_a (G845N) = −29.6 ± 1.4 mV, n = 6). The voltage dependence of steady state inactivation was also similar for rIIA and G845N (Figure 6C; V_h (rIIA) = −52.7 ± 2.0 mV, n = 6; V_h (G845N) = −53.4 ± 1.2 mV, n = 7) and much more positive than for rH1. Thus, mutation G845N had little effect on the kinetics or voltage-dependent properties of the rIIA Na^+ channel.

In contrast, all effects of Css IV on the channel were strongly altered by mutation G845N. Application of Css IV (40 nM) to G845N channels had no effect on channel voltage dependence in the absence or presence of a prepulse (Figures 6A and 6B). Cells expressing G845N were challenged with prepulses in the presence of Css IV concentrations up to 1 μM, with no effect on the voltage dependence of activation. The toxin did cause a reduction in peak Na^+ current in G845N (Figure 6A). However, the concentration dependence of the reduction and its kinetic characteristics differed from the potential-dependent reduction of peak current observed for rIIA and were similar to the potential-independent reduction of current observed for rH1. In steady state inactivation experiments (Figure 6C), Css IV reduced peak sodium current at all holding potentials tested. Even hyperpolarization to −140 mV did not reverse the reduction in peak sodium current (data not shown). The apparent K_D for current block was 77.6 nM at −120 mV, and the K_D calculated for the inactivated state of the

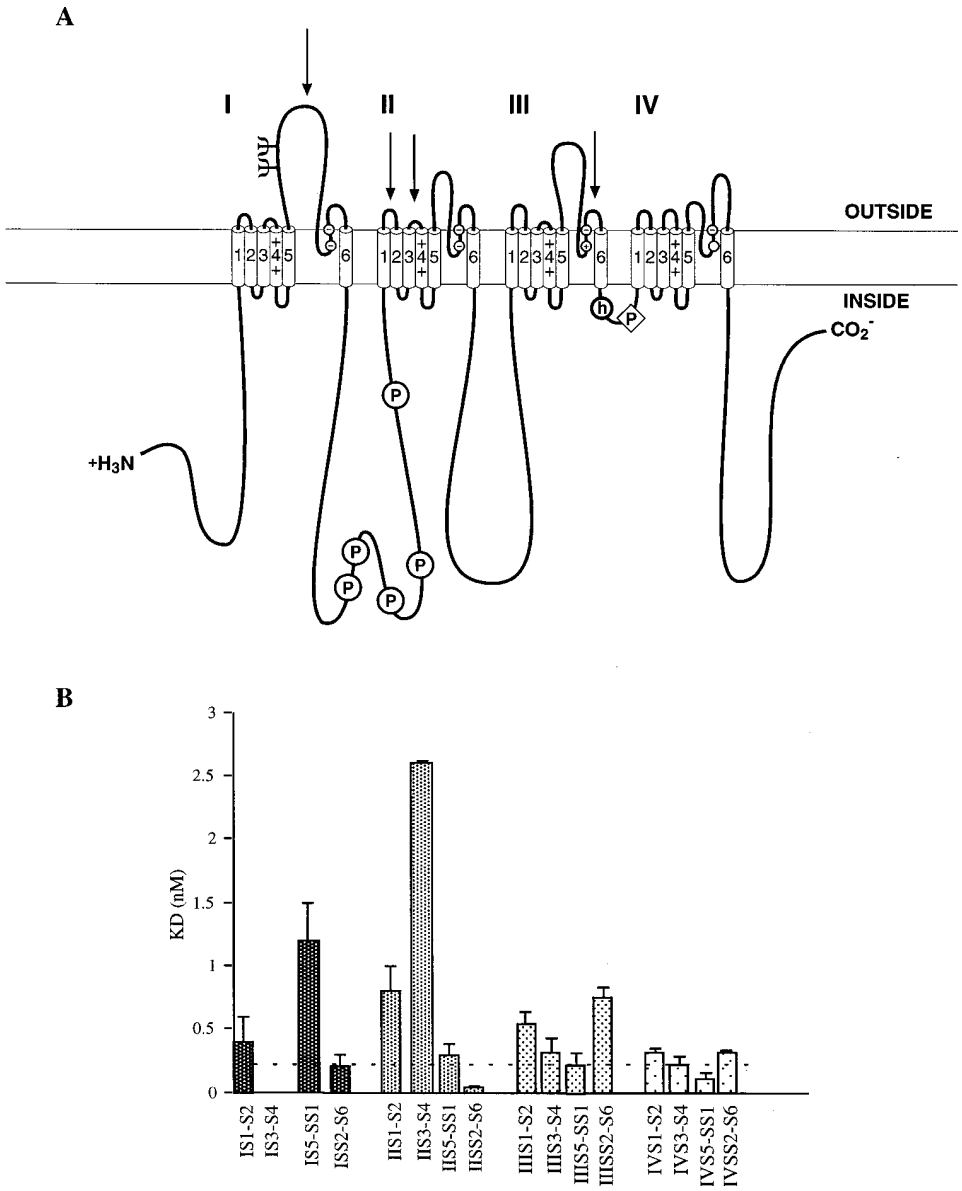

Figure 4. Css IV Binding to Chimeric Brain/Cardiac Na^+ Channels

(A) Proposed transmembrane arrangement of the α-subunit of Na^+ channels. Arrows indicate extracellular loops in which the substitution of amino acid residues of the rat brain rIIA by those in the cardiac rH1 isoform decreased Css IV affinity for its receptor site.

(B) Binding affinity of Css IV for rIIA Na^+ channels ($K_D = 0.2 \pm 0.03$ nM, dotted line) and chimera of the indicated extracellular loops. In cases where the indicated complete chimera could not be expressed as a functional sodium channel, the result for the mini-chimera having the largest change in Css IV binding is presented (e.g., L392Q for ISS2–S6, P782N for IIS1–S2, and G845N for IIS3–S4).

channel was 43.4 nM, approximately 13-fold greater than for wild type. These reductions in blocking affinity were accompanied by a change in the kinetics of inhibition. Css IV inhibition of peak current was complete within 20 s of beginning toxin exposure without priming prepulses and recovered upon washing toxin out of the bath with similarly rapid kinetics (data not shown). Thus, G845 not only controls the kinetics and affinity for binding of Css IV but also determines its physiological effect and its requirement for priming prepulses.

Discussion

The α Subunit of the Sodium Channel Contains the β-Scorpion Toxin Receptor Site

Previous photoaffinity labeling experiments with photoreactive derivatives of β-scorpion toxins have shown specific incorporation into both the α and $\beta 1$ subunits of sodium channels (Darbon et al., 1983) or into only the α subunit (Barhanin et al., 1983; De Lima et al., 1988). Radiation inactivation indicated functional unit sizes of

A

IS5-SS1	
rIIa	FNWDEYIEDKSHFYFLEGQNDALLCGNSSDAGQCPEGYICVKAGRNPNYGYTSFDTFSWAFLSLF
rH	NSL-V-LN-PANYLLKN-TT-V----------T-----R-L---E--DH------S-A----A--
Chim IS5-SS1	NSL-V-LN-PANYLLKN-TT-V----------T-----R-L---E--DH----------
Chim IS5-SS1a	NSL-V-LN-PANYLLKN-TT-V----------
IIS1-S2	
rIIa	FMAMEHYPMTEQFSSVLSVGNL
rH	---L---N--AE-EEM-Q----
Chim P782N	-------N----------
Chim E785A/Q786E	----------AE----------
Chim S788E/S789E/V790M	----------EEM------
IIS3-S4	
rIIa	IVSLSLMEGLGLANVEGLSVLRSF
rH	----------SRMGN------
Chim A841S	----------S----------
Chim N842R	----------R----------
Chim V843M	----------M---------
Chim E844G	----------G--------
Chim G845N	----------N-------
IIISS2-S6	
rIIa	GWMDIMYAAVDSRNVELQPKYEDNLYMY
rH	----------GY-E--QW-------
Chim IIISS2-S6	----------GY-E--QW-------

B

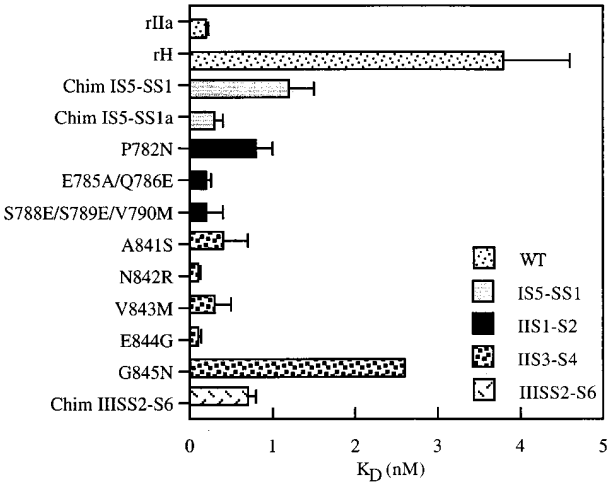

Figure 5. Analysis of Brain–Cardiac Chimera that Affect Css IV Binding Affinity to rIIA Sodium Channels

(A) Amino acid sequence alignment of the IS5–SS1, IIS1–S2, IIS3–S4, and IIISS2–S6 segments of brain (rIIA), cardiac (rH1), and chimeric sodium channels containing the indicated site-directed changes to substitute cardiac sequence.

(B) Effect of the different mutations on Css IV binding affinity to rIIA sodium channels.

34–45 kDa for the receptor site of β-scorpion toxins (Angelides et al., 1985; Seagar et al., 1986), consistent with an essential role of the β1 subunit. In contrast, our results show that β-scorpion toxins bind to receptor site 4 of voltage-gated sodium channels consisting of only α subunits expressed in tsA-201 cells with an affinity comparable to that observed on rat brain synaptosomes (Martin-Eauclaire et al., 1987) and modify channel function as previously observed in neuronal preparations. Evidently, neurotoxin receptor site 4, at which β-scorpion toxins bind, is located exclusively on the α subunit. This result agrees with findings for another β-scorpion toxin, TiTxγ, isolated from the venom of *Tityus serrulatus*, for which the coexpression of the β1 subunit with the α subunit of rSkM1 sodium channels in *Xenopus laevis* oocytes had no effect (Marcotte et al., 1997). Labeling of both the α and β1 subunits of sodium channels has also been observed with photoreactive derivatives of α-scorpion toxins (Beneski and Catterall, 1980; Darbon et al., 1983; Sharkey et al., 1984), but these toxins also bind with normal affinity to the sodium channel α subunit expressed alone (Rogers et al., 1996). Labeling of the β1 subunit by photoreactive derivatives of α- and β-scorpion toxins may indicate that the β1 subunit is

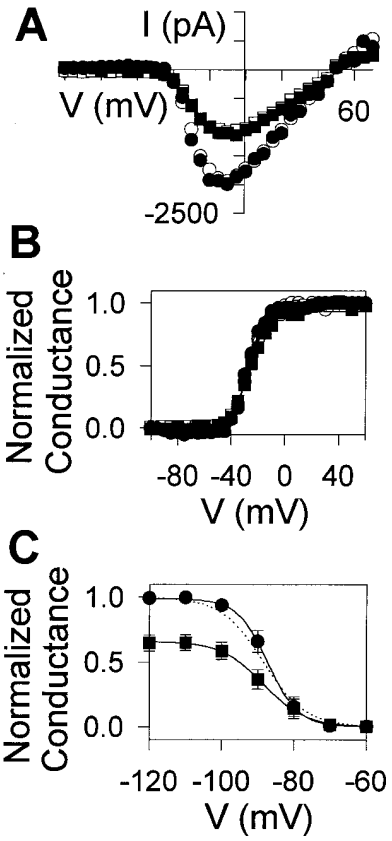

Figure 6. Effects of Css IV on Mutant G845N Na Current

(A) Current–voltage relationships in control (circles) and 40 nM Css IV (squares) in the absence (open symbols) and presence (solid symbols) of depolarizing prepulses to +50 mV (same protocol as Figure 1E).

(B) Voltage dependence of activation derived from the data in (A) plotted using the corresponding symbols. The data were fit with single Boltzmann relationships (solid lines). In control, $V_a = -27.1$ mV, $k = -5.7$ mV with a prepulse, and $V_a = -28.4$ mV, $k = -5.3$ mV without a prepulse. In the presence of 40 nM Css IV, $V_a = -27.3$ mV, $k = -6.5$ mV with a prepulse, and $V_a = -26.6$ mV, $k = -7.0$ mV without a prepulse.

(C) Voltage dependence of inactivation obtained using 30 s prepulses to a variable voltage followed by a test pulse to 0 mV in control (closed circles) and 40 nM Css IV (closed squares). Data were normalized to the current amplitude elicited by the test pulse following the −120 mV prepulse in control and shown as mean ± standard error ($n = 5$). A single Boltzmann relationship (solid lines) was fit to the data with $V_h = -87.2$ mV, $k = 4.2$ mV in control and $V_h = -88.4$ mV, $k = 5.8$ mV in the presence of Css IV. The dotted line is the scaled version of the fit to the data in Css IV.

localized in close proximity to both neurotoxin receptor sites 3 and 4 on the α subunit of sodium channels but that the β1 subunit does not contain amino acids required for high affinity toxin binding or action.

Multiple Extracellular Loops Contribute to the High Css IV Affinity for Brain Sodium Channels, but Domain II Is Dominant

The β-scorpion toxin Css IV binds with approximately 20-fold higher affinity to brain sodium channels than to cardiac sodium channels in isolated membrane preparations. The chimeric approach used here identified four extracellular loops (IS5–SS1, IIS1–S2, IIS3–S4, and IIISS2–S6) that contribute to this difference. Evidently, amino acid residues in loops IS5–SS1, IIS1–S2, IIS3–S4, and IIISS2–S6, which are from distant parts of the primary structure of the sodium channel, all influence binding of β-scorpion toxins. Similarly, the tetrodotoxin receptor site is formed by amino acid residues in four different domains (Terlau et al., 1991), and different extracellular loops in domains I, III, and IV are involved in the binding of α-scorpion toxins and insect-specific scorpion toxins (Tejedor and Catterall, 1988; Thomsen and Catterall, 1989; Gordon et al., 1992). The extracellular loops we have identified do not contribute equally to Css IV binding. Conversion of 5 or 6 amino acid residues in the IS5–SS1 and IIISS2–S6 loops has a much weaker effect than conversion of single amino acid residues in loops IIS1–S2 and IIS3–S4. Thus, our results point to a critical role for the IIS1–S2 and IIS3–S4 extracellular loops in the formation of neurotoxin receptor site 4 and suggest that domain II may have a primary role in β-scorpion toxin binding and action. Domain II was also implicated in the binding of a different β-scorpion toxin in studies of chimeras in which entire domains of skeletal muscle and cardiac sodium channels were exchanged (Marcotte et al., 1997).

Pro-782 and Gly-845 in Domain II Are Required for High Affinity β-Scorpion Toxin Binding

We have identified 2 amino acid residues in domain II that are responsible for the reduction of Css IV affinity for its receptor site. Conversion of proline 782 (Pro-782) and Gly-845 to Asn in loops IIS1–S2 and IIS3-S4, respectively, leads to a strong reduction of toxin affinity. Substitution of Asn for Pro-782 is expected to cause a change in the conformation of the IIS1–S2 loop. The side chain of Gly-845 cannot make a noncovalent interaction (e.g., hydrophobic interactions, hydrogen bonds, salt bridges, and or/polar interactions) with amino acid residues of the toxin. Therefore, the conversion of this residue to the larger, more hydrophilic Asn is likely to cause unfavorable steric or polar/nonpolar interactions when it is present in position 845. The replacement of Gly by Asn may also produce a conformation change in loop IIS3–S4 that affects β-scorpion toxin binding.

Gly-845 Is Required for the Voltage-Dependent Negative Shift in the Voltage Dependence of Activation Caused by Css IV

Mutation of Gly-845 in the rIIA α subunit to Asn markedly altered the electrophysiological effects of β-scorpion toxin Css IV. Peak sodium current is reduced, but no shift in the voltage dependence of activation to more negative potentials and no voltage-dependent reduction of the current amplitude are observed. Thus, even at toxin concentrations that bind to the sodium channel, the toxin effects on voltage-dependent activation and steady state inactivation and the slow kinetics of toxin action on rIIA sodium channels are lost. Since the voltage dependence of activation of the rIIA sodium channel is modified by β-scorpion toxin only after activation by a strong depolarizing prepulse, the interaction of the toxin with its receptor site must be dependent on the

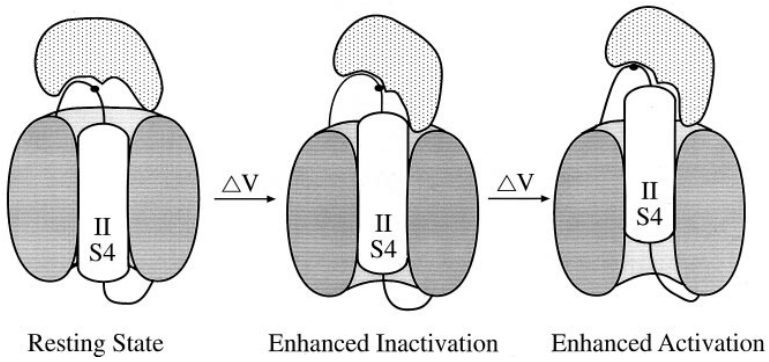

Figure 7. The Voltage Sensor–Trapping Model for Css IV Action

Domain II of the sodium channel and its S4 voltage sensor are represented. The three drawings show toxin binding to resting sodium channels (left) and to the partially (center) and fully (right) activated channels. Partial activation of the IIS4 voltage sensor at intermediate voltages allows tighter Css IV binding as the voltage sensor moves outward. This is depicted as an increased contact area between the voltage sensor and the bound toxin. Full activation of the IIS4 voltage sensor at positive voltages exposes more of the voltage sensor for binding, and the tight binding traps the voltage sensor in the outward position, preventing its inward movement.

conformational state of the toxin receptor site. Evidently, binding of Css IV to this high affinity conformation is disturbed by the mutation G845N.

It has been previously shown that domain II of sodium channels is important for β-scorpion toxin binding by using another β-toxin: TiTxγ isolated from the venom of *Tityus serrulatus* (Marcotte et al., 1997). Moreover, replacement of domain II of the rSkM1 sodium channel by that of the cardiac isoform also removes the shift of activation induced by TiTxγ. Our results demonstrate that Gly-845 in domain II is responsible for this effect of Css IV, and it seems likely that the same residue is responsible for the differences in sensivity to TiTxγ.

A Voltage Sensor-Trapping Model for Css IV Action

The steep voltage dependence of the activation of sodium channels implies that the transition from a resting, closed conformation to an open conformation is accompanied by the translocation of several positive charges outward across the membrane (Hodgkin and Huxley, 1952; Armstrong, 1981). The S4 transmembrane segments contain 4–8 basic residues that serve as gating charges (Stühmer et al., 1989; Seoh et al., 1996; Kontis et al., 1997a, 1997b) and are translocated across the membrane upon depolarization (Larsson et al., 1996; Yang et al., 1996; Starace et al., 1997; Mitrovic et al., 1998). One likely mechanism of action of β-scorpion toxin is interaction with the extracellular end of the IIS4 segment through its binding site on the IIS3–S4 loop. This idea is supported by the requirement for a depolarizing prepulse to observe the voltage-dependent reduction in peak sodium current and the negative shift in the voltage dependence of activation induced by Css IV. Since the IIS4 segment is thought to move outward during the prepulse, the toxin may bind to newly accessible amino acid residues in the IIS3–S4 loop and the extracellular end of the IIS4 segment as it moves outward during the conditioning prepulse. By binding to the extracellular end of the IIS4 segment, the toxin can stabilize and trap it in the outward (activated) position and thereby enhance channel activation in response to subsequent depolarizing pulses. The mutation G845N in the IIS3–S4 loop may abolish the effect of β-scorpion toxin on the voltage dependence of activation by preventing binding of the toxin to the extracellular end of the activated conformation of the IIS4 segment, because it introduces unfavorable steric or polar/nonpolar interactions.

The voltage sensor–trapping model for β-scorpion toxin action on brain-type IIA sodium channels is depicted in Figure 7. In the absence of depolarizing prepulses, the toxin binds to its receptor site, as observed in both electrophysiological and ligand-binding studies. Upon depolarization, the S4 segment is proposed to move outward in two steps (Baker et al., 1998). Moderate depolarization shifts the S4 voltage sensor partially outward, and the toxin enhances closed-state inactivation by binding to and stabilizing a partially activated closed state from which inactivation is preferred. This effect is responsible for the slow reduction of peak sodium current that is observed during toxin treatment and is reversed by strong hyperpolarization. Consistent with this interpretation, we find that block of fast sodium channel inactivation by reaction of a cysteine substituted for the key residue T1491 in the inactivation gate with a positively charged methanethiosulfonate reagent (Boeckman et al., 1998, Biophys. Soc., abstract) reduces the inhibition of peak sodium current by Css IV but does not alter its effect on the voltage dependence of activation (Y. Q., S. C., H. R., T. S., and W. C., unpublished data). We propose that toxin binding is enhanced at more depolarized holding potentials in the range of −120 to −80 mV, because the IIS4 voltage sensor is already shifted to a preferred position for toxin binding at these potentials. This effect develops slowly in our repetitive pulsing experiments (e.g., Figure 1), because the IIS4 segment resides in the partially activated position only very briefly during our strong depolarizing prepulses. Strong depolarizations that open sodium channels drive the IIS4 voltage sensor fully outward. In this position, the extracellular end of the IIS4 segment associates tightly with the bound Css IV and is held in its most outward position. Trapping the IIS4 voltage sensor in the outward, activated position enhances activation of the channel in subsequent depolarizations and therefore causes the negative shift in the voltage dependence of activation. This model predicts a prepulse-dependent immobilization of the activation gating charge movement associated with the IIS4 segment. In fact, β-scorpion toxins prevent a component of gating charge movement recorded at positive test potentials following a

conditioning prepulse (Meves et al., 1987). This model also predicts that hyperpolarization would induce reversal of the toxin effect on activation without toxin dissociation. We have found in kinetic experiments that hyperpolarization does indeed reverse the toxin effect on the voltage dependence of activation ($t_{1/2}$ = 200 ms at −140 mV). Moreover, longer hyperpolarization is required for recovery from inhibition of peak sodium current ($t_{1/2}$ = 15 s at −140 mV), and reestablishment of the toxin effect after that prolonged hyperpolarization is concentration-dependent, consistent with the idea that short hyperpolarization reverses voltage sensor trapping, while prolonged hyperpolarization causes toxin dissociation, and then reestablishment of toxin action requires rebinding (Y. Q., S. C., H. R., T. S., and W. C., unpublished data). Thus, both gating current results and the kinetics of the reversal of toxin action, toxin dissociation, and rebinding are consistent with the idea that IIS4 is trapped in the outward position following the conditioning prepulse in the presence of the toxin, as predicted by the voltage sensor–trapping model.

The S3–S4 Loop Is Required for Binding of Multiple Polypeptide Toxins that May Act by Voltage Sensor Trapping

Molecular determinants for high affinity binding of α-scorpion toxin and sea anenome toxins have been identified in the IVS3–S4 linker of sodium channels, indicating that this linker is important for the coupling of the channel activation to fast inactivation (Rogers et al., 1996). Similarly, the S3–S4 linker of the *drk1* voltage-gated potassium channel contains important residues for the binding of Hanatoxin, which inhibits the channel by altering the energetics of channel gating (Swartz and MacKinnon, 1997). High affinity binding of agatoxins to P/Q-type calcium channels shifts their voltage-dependence of activation to more positive membrane potentials (McDonough et al., 1997) and also involves binding to an S3–S4 segment in their α1 subunit (Bourinet et al., 1998, European Winter Conference on Brain Research, abstract).

All of these polypeptide toxins appear to act by a voltage sensor–trapping mechanism, but their actions are most consistent with trapping the S4 voltage sensor in its inward, not-activated position in contrast to β-scorpion toxin, which traps IIS4 in its outward, activated position. α-scorpion and sea anemone toxins are proposed to slow sodium channel inactivation by preventing outward movement of the IVS4 segment (Rogers et al., 1996) and by trapping it in a position that allows activation but not fast inactivation. Consistent with this idea, α-scorpion toxin immobilizes a slowly activated component of gating charge movement (Nonner, 1979), and recent mutagenesis analysis shows that neutralization of the outermost gating charge in segment IVS4 prevents that effect (Sheets et al., 1998, Biophys. Soc., abstract) Strong depolarization drives α-scorpion toxins off their binding site (Catterall, 1977; Rogers et al., 1996), presumably by steric interference of the outwardly moving IVS4 segment with toxin binding. Hanatoxin binds to the S3–S4 segment of *drk* potassium channels (Swartz and MacKinnon, 1997) and may inhibit those channels by trapping the S4 segment in the inward, not-activated position, similar to the actions of the α-scorpion toxins. Agatoxins, which inhibit P/Q-type calcium channels and are driven off their binding site by strong depolarization (McDonough et al., 1997), bind to S3–S4 segments of the calcium channel α1 subunit (Bourinet et al., 1998, European Winter Conference on Brain Research, abstract) and may inhibit channel activation by trapping an S4 voltage sensor in the inward position. Voltage-dependent relief of agatoxin block (McDonough et al., 1997) is likely to result from steric interference with binding by the outward movement of this S4 voltage sensor in the calcium channel as previously proposed for voltage-dependent reversal of α-scorpion toxin binding to sodium channels (Catterall, 1977; Rogers et al., 1996). Thus, voltage sensor trapping may be a widespread strategy used by natural polypeptide toxins to modify the gating function of voltage-gated ion channels. β-scorpion toxins are unique among this family of ion channel modulators, because they trap the IIS4 segment sodium channels in the outward, activated position and enhance channel activation.

Together, our results emphasize the importance of the S3–S4 linkers for the activation and inactivation of different classes of voltage-gated ion channels and for the function of the S4 voltage sensors in these two gating mechanisms. The specificity of β-scorpion toxins for activation and α-scorpion toxins for inactivation implicates the IIS3–S4 loop of sodium channels in activation gating and the IVS3–S4 loop in the coupling of activation to inactivation of sodium channels. These results correlate well with previous studies of the role of the gating charges of the S4 segments in channel gating. Neutralization of the positive charges in the IIS4 segment of the sodium channel reduces apparent gating charge (Stühmer et al., 1989; Kontis and Goldin, 1997b), and the reaction of a substituted cysteine residue in the extracellular end of IIS4 with methanethiosulfonate reagents alters the voltage dependence of activation, similar to the binding of β-scorpion toxins (Mitrovic et al., 1998). In contrast, neutralization of the charged residues in the IVS4 segment has a specific effect on channel inactivation (Chen et al., 1996). Thus, both the sites of action of these polypeptide neurotoxins on the S3–S4 loops and the effects of the mutations of the S4 gating charges themselves indicate that the IIS4 segment plays an essential role in the activation of sodium channels, while the IVS4 segment couples activation to inactivation. The voltage sensor trapping mechanism allows polypeptide toxins to alter either activation or inactivation by a common molecular interaction with the S3–S4 loops in appropriate domains of voltage-gated ion channels.

Experimental Procedures

Materials

β-scorpion toxin Css IV was purified from the venom of *Centruroides suffusus suffusus* (Martin-Eauclaire et al., 1987) and radiolabeled by the lactoperoxidase method of iodine-125 oxidation (Thomsen et al., 1995). The monoiodotoxin was purified according to the method described by Cestèle et al. (1997a). $Na^{125}I$ was from ICN. Antibiotics and lactoperoxidase were from Sigma. Dulbecco's modified Eagle's medium/F-12 cell culture medium was from Life Technologies/BRL.

Restriction endonucleases and other molecular biology reagents were from New England Biolabs and Boehringer Mannheim. pCDM8 vector and MC1016 *Escherichia coli* bacterial strain were from Invitrogen. Human embryonic kidney tsA-201 cells, a simian virus (SV40) large T-antigen–expressing derivative of HEK-293 cells, were kindly provided by Dr. Robert Dubridge (Cell Genesis, Foster City, CA).

Transient Expression in tsA-201 Cells

The tsA-201 cells were maintained in Dulbecco's modified Eagle's medium/F-12 media supplemented with 10% fetal bovine serum, 20 μg/ml penicillin, and 10 μg/ml streptomycin at 37°C in a 10% CO_2 incubator. The rIIA α subunit cDNA (Auld et al., 1990) was subcloned into the transient expression vector pCDM8, and cDNA encoding chimeras of cardiac and brain sodium channel were prepared as described by Rogers et al. (1996). Expression of sodium channels for ^{125}I-Css IV binding was begun by plating 20,000 tsA-201 cells/cm^2 in a 150 mm tissue culture plate on the day before transfection. Cells were transfected with 50 μg of pCDM8 vector containing the rIIA sodium channel cDNA by using calcium phosphate/DNA coprecipitation (Margolskee et al., 1993). Where indicated, 2 μg of cDNA encoding the β1 subunit in the pCDM8 vector was added to the transfection mixture. Cells were collected for membrane preparation 40–48 hr after transfection.

Membrane Preparation from Transfected tsA-201 Cells

Transfected cells on 150 mm dishes were rinsed twice with a phosphate-buffered saline solution, scraped into 10 ml of 0.32 M sucrose, 1 mM EDTA, and 20 mM HEPES (pH = 7.1), and placed in a 50 ml polystyrene tube. Cells were sedimented at 500 × g for 10 min at 4°C. The pellet was homogenized with 4 × 15 sec bursts of a Polytron homogenizer and with 10 strokes of a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 2,000 × g for 10 min; the supernatant was poured off and centrifuged at 45,000 × g for 30 min. The pellet was resuspended in 0.32 M sucrose, 1 mM EDTA, and 20 mM HEPES (pH = 7.1).

Binding Assays

Equilibrium saturation assays were performed using a series of concentrations of the unlabeled toxin in the presence of a fixed concentration of the radioactive toxin. To obtain saturation curves, the specific radioactivity and amount of bound toxin were calculated for each toxin concentration. The binding buffer was (in mM): choline Cl (130), KCl (5.4), $MgSO_4$ (0.8), HEPES (5) (pH = 6.5), glucose (5.5), and bovine serum albumin (BSA) (2 mg/ml). The membranes (35–65 μg for rIIA and 120–170 μg for rH1) were suspended in 0.3 ml of binding buffer containing 0.05–0.15 nM ^{125}I-Css IV. After incubation for 45 min at 21°C, the reaction mixture was diluted with 2.5 ml ice-cold washing medium (in mM): choline Cl (163), $MgSO_4$ (0.8), $CaCl_2$ (1.8), HEPES (5) (pH = 7.4, adjusted with Tris Base), and BSA (4 mg/ml)) and filtered over GF/C filters (Whatman, UK) under vacuum. The filters were washed twice in washing medium. Nonspecific binding was determined in the presence of 0.2–1 μM unlabeled toxin and was 10%–35% of total binding. Equilibrium saturation or competition experiments were analysed using the iterative computer program LIGAND (Elsevier Biosoft, UK).

Electrophysiological Recording

Whole-cell voltage clamp experiments were performed as described previously (Qu et al., 1994) using an extracellular solution containing (in mM) NaCl (140), CsCl (5), $CaCl_2$ (1.8), $MgCl_2$ (1), HEPES (10) (pH 7.4), and an intracellular (pipette) solution containing CsF (90), CsCl (50), CsEGTA (10), NaF (10), $MgCl_2$ (2), HEPES (10) (pH 7.4). Css IV was dissolved in the extracellular solution containing 1% fetal BSA at the final concentration and applied with an extracellular "sewer pipe" perfusion system (Cantrell et al., 1996). For some experiments at the 1 μM toxin concentration, appropriate volumes of 5× concentrated stock solution were added directly to the bath in order to conserve toxin. Data collection was initiated 10 min after achieving the whole-cell recording configuration. Electrodes were formed from glass micropipettes (VWR), and currents were recorded with an Axopatch 1C Amplifier (Axon Instruments). Cell and electrode capacitance and series resistance were compensated with internal voltage-clamp circuitry. Residual linear leak and capacitance were subtracted by using a P/4 protocol. Pulses were generated and data were recorded and analyzed with a Basic Fastlab interface and software (Indec Systems, Capitola, CA). Half activation and inactivation voltages, V_a or V_h, were derived from fits of a Boltzmann relationship, $1/(1+\exp((V-V_{a,h})/k)$ to normalized conductance–voltage relationships or inactivation curves, respectively, where k was a slope factor. Conductance–voltage relationships quantitating the voltage dependence of activation were obtained from current–voltage relationships by transforming according to conductance = $I/(V-V_{rev})$, where I was the peak test pulse current and V_{rev} was the measured reversal potential. The affinity of Css IV for the resting state of the sodium channel (K_R) was calculated from the extent of inhibition at the most negative holding potential, assuming 1:1 of the toxin to its receptor site. The affinity of the toxin for the inactivated state of the channel (K_i) was calculated from K_R and the measured shift in the inactivation curves as described by Bean et al. (1983). Results are reported as ± standard error of the mean.

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