**Partial Agonist and Antagonist Activities of a Mutant Scorpion β-Toxin on Sodium Channels**

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Scorpion β-toxin 4 from *Centruroides suurus suurus* (Css4) enhances the activation of voltage-gated sodium channels through a voltage sensor trapping mechanism by binding the activated state of the voltage sensor in domain II and stabilizing it in its activated conformation. Here we describe the antagonist and partial agonist properties of a mutant derivative of this toxin. Substitution of seven different amino acid residues for Glu15 in Css4 yielded toxin derivatives with both increased and decreased affinities for binding to neurotoxin receptor site 4 on sodium channels. Css4E15R is unique among this set of mutants in that it retained nearly normal binding affinity but lost its functional activity for modification of sodium channel gating in our standard electrophysiological assay for voltage sensor trapping. More detailed analysis of the functional effects ofCss4E15R revealed weak voltage sensor trapping activity, which was very rapidly reversed upon repolarization and therefore was not observed in our standard assay of toxin effects. This partial agonist activity ofCss4E15R is observed clearly in voltage sensor trapping assays with brief (5 ms) repolarization between the conditioning prepulse and the test pulse. The effects ofCss4E15R are fit well by a three-step model of toxin action involving concentration-dependent toxin binding to its receptor site followed by depolarization-dependent activation of the voltage sensor and subsequent voltage sensor trapping. Because it is a partial agonist with much reduced efficacy for voltage sensor trapping, Css4E15R can antagonize the effects of wild-type Css4 on sodium channel activation and can prevent paralysis by Css4 when injected into mice. Our results define the first partial agonist and antagonist activities for scorpion toxins and open new avenues of research toward better understanding of the structure-function relationships for toxin action on sodium channel voltage sensors and toward potential toxin-based therapeutics to prevent lethality from scorpion envenomation.

Voltage-gated sodium channels are the molecular targets for many paralytic neurotoxins, which have highly selective effects on sodium channel function (1–4). Scorpion α- and β-toxins inhibit fast inactivation of sodium channels and enhance their activation by interacting with neurotoxin receptor sites 3 and 4, respectively (1, 2, 4). Together, these effects cause persistent depolarization of nerve and muscle fibers and block action potential conduction, resulting in lethal paralysis. Understanding the molecular mechanisms of scorpion toxin action would give important insights into the mechanisms of voltage-dependent activation and inactivation of sodium channels and could potentially lead to development of antagonists of toxin action with therapeutic benefit.

Voltage-gated sodium channels are complexes of a pore-forming α subunit with one or two auxiliary β subunits (5). The α subunits consist of four homologous domains, each containing six transmembrane segments. The S1–S4 segments form the voltage-sensing module, whereas the S5 and S6 segments and the P-loop between them serve as the pore-forming module. The S4 segments bear the gating charges of the channel, which are responsible for voltage sensing, and these charges are arrayed in three-residue repeats of a positively charged residue (usually arginine), followed by two hydrophobic residues (6–9). Scorpion α- and β-toxins bind to sites that include the extracellular S3–S4 linkers in domains IV and II, respectively, and modify voltage sensor function (10–14). The receptor site for scorpion β-toxins includes amino acid residues in the adjacent S1-S2 linker in domain II in addition to the S3-S4 linker (10, 12). Scorpion β-toxins act by a voltage sensor-trapping mechanism, in which they bind with high affinity to the activated voltage sensor in domain II and trap it in an activated state (10, 12, 15). Strong depolarizations that activate the sodium channel greatly enhance toxin action by driving the voltage sensor into its activated state and allowing rapid voltage sensor trapping. This three-step process involves initial binding of the toxin, followed by depolarization-dependent activation of the voltage sensor and then rapid trapping of the activated voltage sensor (10, 12). This mechanism predicts that toxin derivatives that bind to neurotoxin receptor site 4, but do not preferentially bind to the activated state of the voltage sensor, would have reduced efficacy in voltage sensor trapping and would be partial agonists or antagonists of the actions of wild-type scorpion β-toxins. However, to our knowledge, no scorpion toxin derivatives having partial agonist and antagonist actions at mammalian sodium channels have been described. Here we show that the toxin

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mutant Css4E15R retains high affinity binding to sodium channels but has greatly reduced efficacy in voltage sensor trapping. As predicted from the voltage sensor-trapping model, this toxin derivative acts as a weak partial agonist in enhancement of sodium channel activation and can antagonize the functional effects of wild-type Css4 toxin on sodium channels in vitro as well as its lethal effects in vivo. Our results provide new support for the voltage sensor-trapping model of toxin action and proof of principle for potential development of toxin antagonists as therapeutic agents.

MATERIALS AND METHODS

Toxin Production and Mutagenesis—Production of Css4, PCR-driven mutagenesis, expression in Escherichia coli, in vitro folding, and purification of toxin derivatives were performed as described previously (16).

Binding Experiments—Rat brain synaptosomes were prepared from adult albino Wistar strain (300 g, laboratory-bred) as described previously (17). Membrane protein concentration was determined by a Bio-Rad protein assay, using bovine serum albumin (BSA) as a standard. Css4 was radioiodinated by lactoperoxidase (Sigma, catalog no. L8257; 7 units per 60 μl of reaction mix) using 10 μg of toxin and 0.5 mCi of carrier-free Na125I (Amersham Biosciences), and the moniodotoxin was purified as described previously (16). The compositions of the media used in the binding assays and terminations of the reactions were described elsewhere (17, 18). Nonspecific toxin binding was determined in the presence of 1–10 μM unlabeled toxin and was typically 10–30% of total binding. Equilibrium competition binding assays were performed and analyzed as described previously (16). Each experiment was performed in duplicate and repeated at least three times as indicated (n). Data are presented as mean ± S.D. of the number of independent experiments.

Expression and Whole-cell Patch Clamp Recording—Chinese hamster ovary (CHO) cells were maintained in F-12 medium, supplemented with 10% fetal calf serum, in a 5% CO2 incubator. Hamster ovary (CHO) cells were maintained in F-12 medium, supplemented with 10% fetal calf serum, in a 5% CO2 incubator. CHO cells were transfected using the calcium phosphate method, and the cell surface CD8 was identified by incubation with anti-CD8 Dynabeads. The expressed CD8 protein was used to identify cells that express wild type rNav1.2 channels. Transfected cells were subcloned 12–18 h after transfection. Electrophysiological recordings were performed 18–72 h after transfection. The whole-cell patch clamp configuration was utilized for sodium current recording with extracellular recording solution containing 150 mM NaCl, 10 mM Cs-HEPES, 1 mM MgCl2, 2 mM KCl, 1.5 mM CaCl2, and 0.1% BSA, pH 7.4, and intracellular solution containing 190 mM N-methyl-d-glucamine, 10 mM HEPES, 10 mM MgCl2, 10 mM NaCl, and 5 mM EGTA, pH 7.4. Linear leak and capacitance currents were subtracted using an online P/4 subtraction paradigm. Toxins were dissolved in extracellular solution at the desired concentration. When toxin Css4E15A or Css4E15R was used at concentrations below 1 μM, toxin-containing solution was added directly to the recording dish, and cells were analyzed by a whole-cell voltage clamp. When Css4E15A or Css4E15R was used at a concentration of 2 μM or higher, we found that the presence of the toxin impaired formation of stable high resistance seals. Therefore, we dissolved the toxin in 100 μl of extracellular solution at twice the desired concentration. Cells were initially incubated in 100 μl of toxin-free solution. Immediately after obtaining a high resistance seal and breaking the membrane to achieve the whole-cell voltage clamp configuration, we added the 100 μl of extracellular solution containing the toxin into the recording dish. Thus, the total volume of the extracellular solution was 200 μl with toxin at the desired final test concentration.

Conductance-Voltage Curves—The curves were derived from peak sodium current versus voltage measurements according to the equation, \( G = I/(V − V_R) \), where \( I \) is the peak current, \( V \) is the test voltage, and \( V_R \) is the apparent reversal potential estimated from the I/V curve. Normalized conductance-voltage curves for unmodified channels were fit with a one-component Boltzmann distribution of the following form,

\[
G(v) = \frac{1}{1 + e^{(v - V_o)/\kappa}}
\]

(Eq. 1)

where \( V_o \) is the voltage for half-maximal activation, and \( \kappa \) is the slope factor in mV. For toxin-modified channels, the activation data were fit with either a one- or a two-component Boltzmann distribution of the following form,

\[
G(v) = \frac{f_{mod} + 1}{1 + e^{(V - V_o)/\kappa}} + \frac{1 - f_{mod}}{1 + e^{(V - V_o)/\kappa}}
\]

(Eq. 2)

where \( f_{mod} \) corresponds to the fraction of channels modified by the toxin.

Onset and Reversal of Voltage Sensor Trapping—To measure the onset of voltage sensor trapping, cells were held at −100 mV, and priming depolarizations of various durations (0.2–50
ms) to 0 mV were applied, followed by a 50-ms pulse at holding potential and a test pulse to −60 mV. In the absence of toxin or without a priming depolarization, no current was observed during the test pulse. The test pulse amplitude at each time point is normalized to the maximal amplitude at 50 ms and is plotted as a function of priming depolarization duration. To measure reversal of voltage sensor trapping, channels were fully activated by a 50-ms prepulse to 0 mV in the presence of 5 μM toxin and then held at hyperpolarized membrane potential for variable durations with 5–10-ms intervals. Test pulses to −60 mV were applied to monitor the toxin-induced shift in channel activation at each time point. The current amplitudes were normalized to the maximal amplitude obtained after the prepulse. The decaying phase was fit with a monoexponential function.

**Steady-state Inactivation**—Steady-state inactivation was studied by applying from a holding potential of −100 mV a series of 50-ms conditioning pulses in the range of −100 to +20 mV, followed by a test pulse to −10 mV. Fractional current (I/I_{max}) was plotted against the conditioning pulse potential and fitted with a Boltzmann function of the following form,

\[
\frac{I}{I_{max}} = \frac{1}{1 + e^{V_{0.5} - V/k}}
\]

(Eq. 3)

where \( V_{0} \) and \( k \) are the voltage for half-maximal inactivation and the slope factor in mV, respectively.

**Toxicity Assays**—To determine toxicity to mammals, groups of 3–5 female mice (ICR Leven Farm, Israel) received subcutaneous injection with each toxin concentration in volumes up to 50 μL. Symptoms were monitored until paralysis was evident, and animals were euthanized with CO₂. When protection by toxin mutants was examined, the mutant Css4^E15R was co-injected with Css4, and the animals were monitored for 24 h to evaluate lack of paralysis and survival.

**RESULTS**

**Binding Affinity and Functional Activity of Css4 Mutants at Position 15**—Previous studies (16) have shown that amino acid substitutions for Glu^{15} alter the binding affinity and functional activity of Css4 toxin. Substitution of seven different amino acid residues at that position in recombinant Css4 yielded toxins with \( K_{d} \) values for displacement of {125}I-labeled Css4 from its receptor sites on synaptosomal sodium channels ranging from 0.1 to 5 nM, in comparison with the wild-type value of 1 nM (Fig. 1). When Glu^{15} of Css4 was substituted by Gln, Arg, or Lys, the mutant toxins exhibited an affinity for rat brain synaptosomes similar to that of the unmodified Css4 (\( K_{d} \approx 2 \pm 0.4, 2.8 \pm 1.6 \), and \( 1.9 \pm 0.3 \) nM, \( n \approx 3 \), respectively; wild-type Css4, \( K_{d} = 0.98 \pm 0.1 \) nM, \( n = 8 \); Fig. 1). In contrast, substitution of Glu^{15} by Ala, Gly, Ser, or Trp resulted in toxin mutants with higher apparent affinity (\( K_{d} = 0.08 \pm 0.01, 0.15 \pm 0.05, 0.08 \pm 0.01 \), and \( 0.3 \pm 0.1 \) nM, \( n \approx 3 \); respectively; Fig. 1). These results indicate that a range of amino acid residues is tolerated at position 15 and that substitution of both smaller ( Ala, Gly, and Ser) and more hydrophobic (Ala and Trp) residues can increase toxin affinity.

The functional effects of these mutant toxins were tested by measurement of enhanced activation of NaN_{a1.2a} channels expressed in CHO cells caused by voltage sensor trapping by the toxins. Higher toxin concentrations are required in this functional assay because the toxin must bind to the resting state at −100 mV rather than to sodium channels in depolarized membrane preparations. In the presence of 5 μM recombinant Css4 or mutants, the cells were depolarized from a holding potential of −100 to 0 mV to induce channel activation and voltage sensor trapping, repolarized to −100 mV for 60 ms to allow recovery from fast inactivation, and depolarized again to −60 mV, a membrane potential at which only toxin-modified sodium channels are activated (Fig. 2A). Under control conditions, no sodium current was observed at −60 mV using this protocol, but the addition of wild-type Css4 induced substantial sodium current (Fig. 2A). The effects of wild-type Css4 and Css4^E15R on the voltage dependence of activation of Na_{a1.2a} channels were tested using the protocol described in the legend to Fig. 2A with test pulses ranging from −100 to +40 mV (Fig. 2B). The recombinant wild-type Css4 exhibited the dual effect that typifies scorpion \( \beta \)-toxins in that it induced a hyperpolarizing shift in the voltage dependence of channel activation and a reduction of the peak current amplitude at more depolarized potentials (Fig. 2B). As reported for native Css4, a depolarizing prepulse was required to observe the effects of the recombinant Css4 (Fig. 2, 12, 16). Mutants Css4^E15K, Css4^E15Q, Css4^E15G, and Css4^E15W are all active in voltage sensor trapping (supplemental Fig. 1). However, in contrast to the wild type and these mutants, mutant Css4^E15R had no effect in our voltage sensor trapping assay (Fig. 2, C and D). These results show that Css4^E15R has lost its functional activity, as defined in this standard assay for voltage sensor trapping, although it retains high affinity binding to sodium channels.

**Reversal of Voltage Sensor Trapping by Repolarization**—To search for any remaining functional effects of Css4^E15R, we analyzed the kinetics of toxin action more completely. The decay of the Css4 effect upon repolarization of NaN_{a1.2a} was monitored using a protocol with a 50-ms depolarizing prepulse to 0 mV, in order to move the voltage sensors outward and induce voltage

**FIGURE 1. Effects of Glu^{15} substitution on binding of Css4 to sodium channels in rat brain synaptosomes.** Rat brain synaptosomes were incubated for 60 min at 22 °C with 0.1 nM {125}I-labeled Css4 and increasing concentrations of the various mutant toxins. Nonspecific binding, determined in the presence of 1 μM unmodified Css4, was subtracted. Representative experiments are shown (see "Materials and Methods" for details). The \( K_{d} \) values are as follows: Css4, 0.98 ± 0.1 nM (n = 8); E15A, 0.08 ± 0.01 nM (n = 7); E15S, 0.08 ± 0.01 nM (n = 6); E15Q, 2.0 ± 0.4 nM (n = 4); E15G, 0.15 ± 0.05 nM (n = 4); E15K, 1.9 ± 0.3 nM (n = 3); E15R, 2.8 ± 1.6 nM (n = 3); E15W, 0.3 ± 0.1 nM (n = 4).
sensor trapping; a hyperpolarizing pulse to −100 mV for increasing intervals to reverse voltage sensor trapping and allow toxin dissociation; and a test pulse to −60 mV to measure the sodium current through those channels that are still affected by the toxin (Fig. 3A). With increasing intervals at −100 mV (10–250 ms), the sodium currents elicited by the test pulse to −60 mV decreased progressively (Fig. 3B) with a time constant of 113 ms (Fig. 3C). The decay rate was voltage-dependent and increased 3.5-fold at −180 mV (Fig. 3C). These results define the time course of reversal of voltage sensor trapping for wild-type Css4.

To our surprise, tests of the activity of Css4E15R using the same protocol revealed substantial activation of sodium current when the test pulse to −60 mV was applied after only a short interval at −100 mV (Fig. 4A, squares). This activating effect of Css4E15R decayed with a time constant of 4 ms, which was 28-fold faster than that obtained in the presence of the unmodified toxin (Fig. 4A, circles). The fast decay in the presence of Css4E15R explains why the typical shift of voltage-dependent activation to more hyperpolarizing potentials was not detected under our standard protocol, which includes a 60-ms hyperpolarizing pulse between the conditioning prepulse and the test pulse (Fig. 2). When the duration of the interval at −100 mV was set to the minimum that allowed recovery from fast inactivation (5 ms), a detectable negative shift in voltage dependence of activation was observed (Fig. 4C). Thus, Css4E15R is not inactive, as was previously suggested (16), but acts as a partial agonist capable of weak voltage sensor trapping with a very rapid decay rate.

Kinetcs of Voltage Sensor Trapping by Css4E15R—On the basis of these results, the rate of the onset of voltage sensor trapping by Css4E15R could be measured using a shorter (5 ms) repolarization interval to −100 mV. The rate of onset of the wild-type Css4 effect was initially measured using priming depolarizations of variable duration (0.2–50 ms) to 0 mV, followed by repolarization to −100 mV for 60 ms and depolarization to a test potential (Fig. 5A). The Css4 effect increased with longer prepulse durations (Fig. 5B) and developed in a double exponential time course, with a minor fast component (τ = 0.77 ms, A = 0.34) and a major slow component (τ = 18.9 ms, A = 0.66) (Fig. 5C). The amplitude of the fast component increased with more depolarized prepulses to +60 mV (Fig. 5C). This suggests that the fast component of toxin action reflects binding to a channel population in which the voltage sensor is trapped in the presence of toxin immediately upon activation. The slow component of toxin effect developed at a constant rate, independent of the prepulse voltage (Fig. 5C), suggesting that this component reflects a voltage-independent rate-limiting step.
sodium channel gating processes are all highly voltage-dependent in this voltage range, the slower voltage-independent component of toxin action must represent binding of the toxin to the activated state of the sodium channel, which is followed by further voltage sensor trapping.

The biphasic time course of onset of voltage sensor trapping at 0 mV can be precisely fit by a kinetic model based on the three-step voltage sensor trapping mechanism (Fig. 6). In this kinetic model, Css4 (Tx)\(^2\) can bind to either the resting voltage sensor (R) or the activated voltage sensor (A), but only binding to the activated voltage sensor leads to voltage sensor trapping at a rate defined by \(k_6 \text{ Trap} \). In this model, the biphasic binding kinetics arise because Css4 first binds rapidly to an activated Tx complex to the activated voltage sensor leads to voltage sensor trapping. The close fit of the kinetic data for wild-type Css4 obtained with a 5-ms repolarization interval illustrated in Fig. 6A is based on a value for the rate constant for voltage

\[ 2 \] The abbreviations used are: Tx, Css4 toxin; R, voltage sensor in the resting state; A, voltage sensor in the activated state.
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FIGURE 6. A kinetic model for onset of voltage sensor trapping by Css4 and Css4E15R. A, the rates of onset of voltage sensor trapping by Css4 (filled circles) and Css4E15R (open squares) during a priming depolarization to 0 mV were measured using a test pulse to −60 mV following a 5-ms hyperpolarization to −100 mV as described in the legend to Fig. 5B. kinetic model used to generate the solid lines through the data in A. The rate constant k6 for the onset of voltage sensor trapping was reduced from 2 ms−1 for wild-type Css4 to 0.42 ms−1 for Css4E15R. The rate constant k5 for reversal of trapping was increased from 0.6 ms−1 for Css4 to 0.88 ms−1 for Css4E15R. Therefore, the ratio of rate constants controlling voltage sensor trapping (k6/k5) was decreased from 3.3 to 0.52 by the E15R mutation, a 6.4-fold reduction.

Sensor trapping (k6) of 2 ms−1 and the rate constant for reversal of voltage sensor trapping (k5) of 0.6 ms−1.

In comparison with wild-type Css4, the amplitude of the sodium current elicited by test pulses to −60 mV for channels incubated with Css4E15R was much smaller (Fig. 6A). This difference from wild-type Css4 reflects the poor efficacy of Css4E15R in voltage sensor trapping and the rapid reversal of the trapping process as illustrated in Fig. 4A. As for wild-type Css4, the rate of appearance of channels with negatively shifted voltage dependence of activation was fit by two exponentials. The population of trapped channels developed with a dominant fast component (Fig. 6A), consistent with the model that Css4E15R traps channels rapidly upon activation but to a lesser extent than the wild-type toxin (Fig. 6B). After this initial rapid phase of toxin binding and voltage sensor trapping, the rapid reversal of voltage sensor trapping by the mutant toxin reduces the second phase of accumulation of trapped channels during the prepulse. The results of Fig. 6A were fit with a value of k6 = 0.42 ms−1 for the onset of voltage sensor trapping and k5 = 0.9 ms−1 for reversal of voltage sensor trapping. The ratio of k6/k5 for wild-type toxin is 3.3, compared with 0.52 for Css4E15R, a difference of 6.4-fold. Thus, the effects of the E15R mutation can be quantitatively fit by our three-step kinetic model with only this specific change in the rate constants for onset and reversal of voltage sensor trapping, providing strong support for the conclusion that the E15R mutation specifically affects voltage sensor trapping.

Our measurements of reversal of voltage sensor trapping at −100 mV (Fig. 4A) indicate a much larger increase in k5 for the Css4E15R mutant (28-fold) than the 1.9-fold change observed for our measurements of the onset of voltage sensor trapping at 0 mV (Fig. 6A). The comparison of these results implies that the effects of the E15R mutation on voltage sensor trapping are strongly voltage-dependent, as expected if this amino acid residue interacts differentially with different functional states of NaV1.2a channels. At negative membrane potentials, the reversal of voltage sensor trapping may be much more dramatically affected by Css4E15R than at 0 mV because the channel is pulled along that pathway toward toxin dissociation.

Competitive Antagonism of Css4 Toxin Action by Css4E15R—
Partial agonists are often effective antagonists of the pharmacological actions of full agonists at their common receptor site. To test whether Css4E15R can function as an antagonist of Css4 action, we measured the voltage dependence of activation of NaV1.2a channels in the presence of the high affinity activator Css4E15A and increasing concentrations of Css4E15R (Fig. 7). The negative shift of the voltage dependence of activation caused by 500 nM Css4E15A (Fig. 7A) was not observed for

FIGURE 7. Antagonist activity of Css4E15R on voltage sensor trapping. Current-voltage relations in the presence of toxin obtained using pulses to the indicated potentials alone (open symbols) or preceded 60 ms earlier by a 1-ms prepulse to +50 mV (filled symbols) from rNaV1.2a channels expressed transiently in tsA-201 cells. A, 500 nM Css4E15A. B, 5 μM Css4E15R. C, 500 nM Css4E15A + 5 μM Css4E15R. Error bars, S.E.M.
Figure 8. Antagonist activity of Css4E15R on paralysis by wild-type Css4 toxin. All toxins were injected subcutaneously into adult mice. Injection of 32–33 μg (n = 4) and 35 μg (n = 5) Css4 per 20-g mouse paralyzed 50% (black bar, left) or 100% (black bar, right) of the injected animals, respectively. Coinjection with 160 μg (n = 6, left) or 350 μg of Css4E15R (n = 3, right) protected 100% of the mice from the paralytic effect of Css4 (open bars).

CSS4E15R, even at 5 μM (Fig. 7B). Moreover, CSS4E15R was able to effectively antagonize the effect of CSS4E15A, substantially reducing the negative shift of the voltage dependence of activation when both toxins were present (Fig. 7C). The results of these experiments show that CSS4E15R can indeed prevent the negative shift of the voltage dependence of activation of sodium channels by CSS4 and therefore demonstrate competitive antagonism of scorpion β-toxin action by CSS4E15R.

Antagonism of the Lethality of CSS4 by CSS4E15R—Scorpion β-toxins are lethal when administered to mice by intraperitoneal or subcutaneous injection (LD₅₀ of 1.6 mg/kg for CSS4) (16). Lethality is preceded by characteristic paralysis of the rear legs. Injection of 1.6 mg/kg CSS4 paralyzed 50% of injected mice (Fig. 8). In contrast, paralysis was completely prevented by concomitant injection of 16 mg/kg CSS4E15R (Fig. 8). A higher dose of 1.75 mg/kg CSS4 paralyzed 100% of the mice tested, and this higher level of paralysis was also completely prevented by 17.5 mg/kg CSS4E15R. Therefore, CSS4E15R is a weak partial agonist for activation of sodium channels and at the same time acts as an antagonist of the paralytic effects of wild-type CSS4 toxin.

Discussion

Gain or Loss of Affinity for Mutations of Glu¹⁵ in CSS4—Substitutions of amino acid residues at many positions in CSS4 cause partial or complete loss of binding affinity (16). In contrast to these previous results, we found that substitutions for Glu¹⁵ in CSS4 can cause either reduced affinity or substantial increases in binding affinity. The majority of substitutions resulted in increased binding affinities, suggesting a unique role for Glu¹⁵ in toxin binding and action. It is surprising that most substitutions for Glu¹⁵ cause increased binding affinity, because it would be expected that selective pressure during evolution of the toxin would assure that the most effective amino acid residue would be present in each position in its structure. Our results suggest that this residue has been conserved as a glutamate during evolution of the toxin, although it reduces binding affinity relative to other amino acid residues at this position. In light of the unexpected increases in toxin affinity upon substitution of Glu¹⁵, it is possible that this residue has been conserved in evolution because it is crucial for toxin action despite its negative effect on toxin affinity. Our finding that CSS4E15R has lost most of its agonist activity but retains high affinity is consistent with this hypothesis.

Partial Agonist Activity of CSS4E15R—The mutant CSS4E15R is of special interest among the many mutant toxins that have been described because it retains nearly normal binding affinity but has lost efficacy of toxin action. In our standard assay for voltage sensor trapping, CSS4E15R is completely inactive. Further analysis using a different pulse protocol showed that this low efficacy is caused by very rapid dissociation upon repolarization. These results indicate that bound CSS4E15R is unable to trap the activated state of the voltage sensor effectively and prevent return of the voltage sensor to its resting state. Most likely, this reflects loss of selective high affinity interaction of CSS4E15R with the activated/trapped state of the voltage sensor, as required for voltage sensor trapping (10, 12).

Partial Agonist Activity of CSS4E15R—Partial agonists serve as effective antagonists of the actions of full agonists at many drug receptor sites, but this type of competitive antagonism has not previously been described for scorpion toxins. Our results show that CSS4E15R antagonizes the functional effect of wild-type CSS4 by preventing the negative shift of the voltage dependence of activation in voltage sensor trapping assays. In addition, CSS4E15R prevents the lethal effects of wild-type CSS4 toxin in mice, indicating effective antagonism of the functional effects of wild-type CSS4 toxin in vivo. Toxin derivatives that effectively antagonize the paralytic effects of wild-type scorpion toxin without side effects would be potentially useful therapeutically in preventing deaths from envenomation, as was shown for another scorpion toxin in insects (20). Our results provide proof of principle that such agents can be developed by substitutions of specific amino acid residues in CSS4 toxin. Unfortunately, doses of CSS4E15R higher than those described here induce myotonia and other side effects, apparently caused by actions on Na⁺,1.4 channels in skeletal muscle. Therefore, further studies of other toxin derivatives and determination of their effects on a range of sodium channel subtypes are required to develop an antagonist of sufficient efficacy and specificity to be of therapeutic value.

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