A μ-Conotoxin-Insensitive Na^+ Channel Mutant: Possible Localization of a Binding Site at the Outer Vestibule

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ABSTRACT We describe a mutation in the outer vestibule region of the adult rat skeletal muscle voltage-gated Na^+ channel (μI) that dramatically alters binding of μ-conotoxin GIIIA (μ-CTX). Mutating the glutamate at position 758 to glutamine (E758Q) decreased μ-CTX binding affinity by 48-fold. Because the mutant channel showed both low tetrodotoxin (TTX) and μ-CTX affinities, these results suggested that μ-CTX bound to the outer vestibule and implied that the TTX- and μ-CTX-binding sites partially overlapped in this region. The mutation decreased the association rate of the toxin with little effect on the dissociation rate, suggesting that Glu-758 could be involved in electrostatic guidance of μ-CTX to its binding site. We propose a mechanism for μ-CTX block of the Na^+ channel based on the analogy with saxitoxin (STX) and TTX, on the requirement of μ-CTX to have an arginine in position 13 to occlude the channel, and on this experimental result suggesting that μ-CTX binds in the outer vestibule. In this model, the guanidinium group of Arg-13 of the toxin interacts with two carboxyls known to be important for selectivity (Asp-400 and Glu-755), with the association rate of the toxin increased by interaction with Glu-758 of the channel.

INTRODUCTION

The μ-conotoxins are a class of toxins consisting of three related 22 amino acid peptides produced by the piscivorous cone snail *Conus geographus* (Cruz et al., 1985; Gray et al., 1988; Olivera et al., 1990). The best studied of the three, μ-CTX GIIIA and GIIIB, have shown strong selectivity between voltage-gated Na^+ channel isoforms. Both toxins exhibit high-affinity (nanomolar) binding to the adult rat skeletal muscle and eel electroplax Na^+ channels but exhibit little affinity for highly homologous brain and heart channel isoforms (Cruz et al., 1985; Moczydlowski et al., 1986a,b; Ohizumi et al., 1986; Yanagawa et al., 1986; Chen et al., 1992). These toxins have a high degree of hydrophilicity, a net positive charge of 6 or 7 at neutral pH, and backbone structural rigidity conferred by three disulfide bonds. Table 1 shows an alignment of the primary sequences of the two μ-CTXs.

There are several similarities between the μ-CTXs and the guanidinium toxins saxitoxin (STX) and tetrodotoxin (TTX). In each case, binding of toxin produces complete blockade of the Na^+ channel current. A guanidinium group is required for binding (Kao and Walker, 1982; Strichartz, 1984; Kao, 1986; Sato et al., 1991; Becker et al., 1992; Yang and Kao, 1992). Finally, binding studies have shown competitive inhibition between STX and μ-CTX GIIIA or GIIIB, as if the binding sites overlap (Moczydlowski et al., 1986a; Ohizumi et al., 1986; Yanagawa et al., 1986, 1987; Becker et al., 1989).

In spite of these similarities, mutagenesis of several Na^+ channel sites known to be crucial for STX and TTX binding have shown little effect on μ-CTX affinity, and no mutations of the channel have been described yet that result in a substantial reduction in μ-CTX binding. For example, in domain I of the rat brain II Na^+ channel, mutation of Glu-387 to glutamine completely eliminates TTX and STX block (Noda et al., 1989; Terlau et al., 1991), but the analogous mutation (E403Q) in the adult rat skeletal muscle Na^+ channel (μI) results in less than a fourfold change in affinity for μ-CTX GIIIA (Stephan et al., 1994). Mutation of a tyrosine to cysteine (Y401C) in the outer vestibule region of the rat skeletal muscle clone Skm1 increases the half-maximal inhibitory concentration (IC_{50}) of TTX by 45-fold (35 nM to 1.5 μM; Chen et al., 1992). Similar reductions in TTX binding have been seen with the analogous mutation in a Na^+ channel brain isoform (Terlau et al., 1991; Heinemann et al., 1992) and in μI (Backx et al., 1992). Nevertheless, this mutation causes only a 3.6-fold increase in the IC_{50} of μ-CTX (Chen et al., 1992). These results pose a serious question for the overlap of the STX/TTX binding site with that of the μ-CTXs (Stephan et al., 1994).

We present evidence that a glutamate to glutamine mutation (E758Q) in the outer vestibule region of domain II of μI dramatically reduced the affinities for both TTX and μ-CTX GIIIA. The reduction in μ-CTX affinity resulted from a ~100-fold decrease in the association rate of the toxin after mutation of the channel. Because the mutation eliminated a negative charge at the mouth of the pore, the change in association rate was consistent with the possibility that Glu-758 contributes to electrostatic guidance of the toxin to its binding site on the native channel (Russell and Fersht, 1987; Russell et al., 1987; Sharp et al., 1987; Getzoff et al., 1992; Escobar et al., 1993).

Received for publication 11 May 1995 and in final form 15 August 1995.
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0006-3495/95/11/1657/00 $2.00

TABLE 1 Comparison of the two best studied μ-CTXs known to bind Na^+ channels

Toxin	Sequence	Charge*
μ-CTX GIIIA	RDCCTPPKKCKDRQCKPQRCCA	+6
μ-CTX GIIIB	RDCCTPPRKCKDRRCKPMKCCA	+7

Standard one letter codes are used for the amino acids. All prolines are *trans*-γ-hydroxlated, and the carboxy termini are amidated.
*Indicates the net charge at neutral pH.
Adapted from Gray et al. (1988).

The effect of E758Q suggests that the μ-CTX binding site indeed does overlap that of the other guanidinium toxins, STX and TTX, in the outer vestibule of the channel. The localization of the μ-CTX binding site to the same area as that of STX and TTX and the requirement for an arginine in position 13 of the toxin for blockade imply that μ-CTX may occlude the pore by binding to the selectivity filter in a manner similar to that proposed for TTX and STX. Using our previously described model of the outer vestibule of the Na^+ channel (Lipkind and Fozzard, 1994), we speculate that the three positively charged nitrogens of Arg-13 interact with a triad of carboxyl groups in the outer vestibule of μI, Asp-400, Glu-755, and Glu-758. This arrangement satisfies the currently available data concerning μ-CTX binding.

These results represent the first molecular evidence suggestive of a possible binding site for μ-CTX at the mouth of the pore. Localization of the binding site may allow for more efficient mutagenic mapping to define the molecular interactions of the Na^+ channel with μ-CTX. Such mapping might lead to refinements in the current understanding of the outer vestibule and selectivity filter.

MATERIALS AND METHODS

Mutagenesis of μI

A construct of the cDNA encoding the μI Na^+ channel cloned into the phagemid vector pBluescript SK- (Stratagene, La Jolla, CA) was provided as a gift from Dr. Gail Mandel and served as the template for mutagenesis. Oligonucleotide-directed mutagenesis was carried out by using a four primer polymerase chain reaction (PCR) technique (Higuchi, 1990). This technique involved the production of two PCR fragments that ranged from the site of the desired mutation to two separate outside primer sites. These initial products incorporated the desired mutation and together formed the template for a second PCR amplification reaction that yielded the full-length insert. Forward and reverse primers to the pore-forming region of domain II of μI were designed to introduce a glutamate (E) to glutamine (Q) substitution at amino acid 758 by substituting a guanine for cytosine at base pair (bp) 2272. This substitution also resulted in the addition of a silent *Bam*HI restriction site. The forward primer consisted of 26 nucleotides and corresponded to bp 2258–2283 (5′-gcggggaatggatccagaccatgtgg-3′). The reverse primer consisted of 25 nucleotides and corresponded to bp 2284–2260 (5′-cccacatggtctggatccattcccc-3′). The outside primers spanned unique restriction sites, *SphI* and *Aat2*, and were 5′-cgtggtgggcatgcagctgttc-3′ and 5′-gatgatggagacgtccacg-3′, respectively.

The first PCR reactions paired the forward primer with the *AatII* primer and, in a separate reaction, the reverse primer with the *SphI* primer. The reactions contained the following: 0.5 μg of each primer, 50 ng of μI template, 1 U of *Taq* DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each deoxynucleotide triphosphate and 2 mM $MgCl_2$ in a final volume of 50 μl. All PCR reagents were obtained from Perkin-Elmer (Norwalk, CT). Thirty-five cycles of amplification were undertaken under the following conditions: 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s. The last cycle was followed by a 7-min extension interval at 72°C. The products of each reaction were fractionated by low-melting-point agarose (Gibco-BRL, Gaithersburg, MD) gel electrophoresis in TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) at 4°C, isolated from the agarose by centrifugation through 0.22-μm cellulose acetate filters (Costar, Cambridge, MA), purified by chloroform:phenol extraction, concentrated by ethanol precipitation, and reconstituted in 20 μl of distilled water. These intermediate products were used as templates in a second PCR reaction, which contained the following: 1 μg of the *AatII* and *SphI* primers, 2 μl of each intermediate product, 2 U of *Taq* DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.2 mM of each deoxynucleotide triphosphate, and 1 mM $MgCl_2$ in a final volume of 100 μl. Thirty-five cycles of amplification were undertaken under the following conditions: 94°C for 30 s, 58°C for 1 min, and 72°C for 45 s. The last cycle was followed by a 7-min extension interval at 72°C. This reaction yielded a final product of approximately 1180 bp. The final construct, referred to as E758Q, was ligated directly into μI from the *SphI* to *AatII* restriction sites. Incorporation of the mutation was indicated by the presence of an additional *Bam*HI site and was confirmed by DNA sequencing. All restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA).

Electrophysiological recordings

Messenger cRNA was prepared in vitro using reagents from the mCAP RNA Capping Kit (Stratagene, La Jolla, CA). For transcription of the native rat skeletal muscle, a cDNA construct of μI inserted between flanking sequences from the *Xenopus laevis* globin gene 5′ and 3′ untranslated regions was used. This construct was provided as a gift from Dr. Randall Moorman. The phagemid containing μI was linearized with SalI and transcribed with SP6 DNA-dependent RNA polymerase. The phagemid containing E758Q was linearized with NotI and transcribed with T7 DNA-dependent RNA polymerase.

Stage V and VI *Xenopus* oocytes were isolated from female frogs (NASCO, Ft. Atkinson, WI), washed with Ca^{2+}-free solution (90 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, and 5 mM HEPES titrated to pH 7.6 with 1 N NaOH), treated with 2 mg/ml collagenase (Sigma, St. Louis, MO) for 1½ h, and had their follicular cell layers manually removed. Approximately 50–100 ng of cRNA was injected into each oocyte with a Drummond microinjector (Broomall, PA). Oocytes were incubated at 16°C for 12 h to 3 days before examination in a solution containing 90 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM NaH_2PO_4, 2.5 mM Na^+ pyruvate, 100 μg/ml gentamicin (Gibco-BRL, Grand Island, NY), 50 U/ml nystatin (Sigma), and 5 mM HEPES titrated to pH 7.6 with 1 N NaOH.

Recordings were made in the two-electrode voltage clamp configuration using either a Dagan CA-1 voltage clamp or a Dagan TEV-200 voltage clamp with a series resistance compensation circuit (Dagan, Minneapolis, MN). All recordings were obtained at room temperature (20–22°C). Oocytes were placed in recording chambers in which the bath flow rate was 100 ml/h, and the bath level was adjusted so that the total bath volume was less than 500 μl. Electrodes were filled with 3 M KCl and had resistances of less than 1 MΩ. Using pCLAMP6 (Axon Instruments, Foster City, CA) software, data were acquired at 71.4 kHz after low-pass filtration at 2 kHz (−3 dB). The holding potential in all experiments was −100 mV. Analysis of recordings, including curve fitting, was performed using standard equations in pCLAMP6. Curve fitting involved the minimization of the sum of squared errors by the simplex method.

With or without the addition of toxin, recordings were made in a standard bathing solution that consisted of (in mM): 90 NaCl, 2.5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 5 mM HEPES titrated to pH 7.2 with 1 N NaOH. μ-CTX GIIIA was obtained from either Research Biochemicals International (Natick, MA) or Sigma and stored at −20°C as a 1 mM stock solution in 1 mM HEPES (pH 7.0). The blocking efficacy of μ-CTX GIIIA

stored in this way and diluted before experiments did not change appreciably over a several-month period, suggesting that the peptide was stable under these conditions. Consistent with a previous report (Becker et al., 1992), addition of 50 μg/ml acetylated bovine serum albumin did not alter the observed values for μ-CTX block. TTX was obtained from Calbiochem (La Jolla, Ca) and stored frozen as a 3 mM stock solution in distilled water.

All data are presented as the mean ± SEM. Statistical comparisons were made using two-tailed Student's *t*-tests.

Molecular modeling

Molecular modeling was performed using Biosym software (Biosym Technologies, Inc., San Diego, CA) operating on a Silicon Graphics Elan 4000 workstation (Mountain View, CA). The coordinates for the model of the STX/TTX binding site were taken unaltered from our previous report (Lipkind and Fozzard, 1994). All energy calculations were based upon the Biosym consistent valence force field (cvff), wherein electrostatic interactions and hydrogen bonds are calculated as coulombic energies between charges or partial charges. Structural optimization was performed by minimizing the potential energy surface predicted by the cvff and involved the iterative methods of the steepest descents and conjugate gradients. The initial coordinates for the structure of μ-CTX in solution were obtained from the Protein Data Base (entry 1TCK; Brookhaven National Laboratory, Upton, NY; Lancelin et al., 1991.)

RESULTS

Blockade of native μI by μ-CTX

To obtain a preliminary estimate of the affinity of μ-CTX for the Na^+ channel in our experimental conditions, a dose-response relationship for inhibition of the native μI channel by μ-CTX was measured. The peak Na currents at 0 mV were recorded from oocytes expressing native μI and exposed to the standard bathing solution with the addition of 1, 10, 50, or 200 nM μ-CTX. These peak currents were normalized to the peak current observed in the absence of toxin. Because it has been previously shown that μ-CTX binding to rat skeletal muscle Na^+ channels results in complete inhibition of the ion permeation (Becker et al., 1992), the normalized peak current was taken to represent the fraction of unbound channels.

The relationship between the fraction of available channels and the applied μ-CTX concentration is shown in Fig. 1. This relationship was fitted to a Langmuir absorption isotherm of the form

$$\frac{I}{I_{max}} = \frac{1}{1 + ([\mu CTX]/IC_{50})^n}$$

where I is the observed peak current in the presence of μ-CTX, I_{max} is the peak current in the absence of μ-CTX, the IC_{50} is the concentration of μ-CTX resulting a half-maximal inhibition of current, and n is the Hill coefficient. The estimated IC_{50} was 5.1 ± 0.6 nM with a Hill coefficient of 0.9 ± 0.1. This IC_{50} for μ-CTX binding was consistent with previous reports for μI expressed in *Xenopus* oocytes (Chen et al., 1992; Stephan et al., 1994). A Hill coefficient close to 1 agrees with the previous binding (Moczydlowski et al., 1986a; Yanagawa et al., 1987) and electrophysiological studies (Cruz et al., 1985; Stephan et al., 1994) suggesting that μ-CTX has a single binding site on the Na^+ channel.

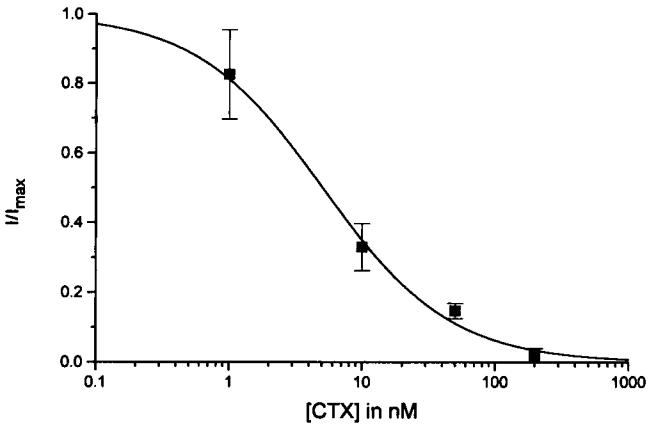

FIGURE 1 The dose-response relationship for μ-CTX block of μI Na^+ channels expressed in *Xenopus* oocytes. The peak Na^+ currents at 0 mV in the presence of varying concentrations of μ-CTX were normalized to the peak current observed in the absence of toxin. Data were fitted to a Langmuir absorption isotherm. The estimated IC_{50} was 5.1 ± 0.6 nM with a Hill coefficient of 0.9 ± 0.1. The data points at 1, 10, 50, and 200 nM represent the values from 8, 7, 7, and 4 separate determinations, respectively, from a total of 11 oocytes. Fitting was performed by minimizing the χ^2 for the unweighted means of the normalized currents. Repeating the fitting after weighting the means by the SEM^{-1} did not substantially change the result.

Subsequent determinations of the IC_{50} were made using a single concentration of μ-CTX (Chen et al., 1992; Chahine et al., 1994; Stephan et al., 1994). Estimates of the IC_{50} were made by recording currents before and after the addition of either 10 or 50 nM μ-CTX to the bathing solution. The average peak current was obtained from three separate voltage steps to 0 mV. Voltage steps were separated by 20 s to ensure that any reduction in current did not represent a partial recovery from inactivation. After a solution change, μ-CTX was allowed to equilibrate with the channel for at least 5 min before recording, and a steady state of block was confirmed by comparison of the peak currents from the separate trials before averaging. The IC_{50} was calculated from the ratio of the average current in the presence of μ-CTX (I_{CTX}) to the average current in control conditions (I_{max}) in the following manner:

$$IC_{50} = [\mu CTX]\frac{I_{CTX}/I_{max}}{1 - (I_{CTX}/I_{max})}.$$

This formula yielded an IC_{50} for μ-CTX binding of 17 ± 5 nM (n = 8). This result was in close agreement with the IC_{50} obtained by fitting the dose-response relationship.

Decreased μ-CTX blockade of a TTX-insensitive mutant channel

Competitive inhibition of μ-CTX binding by STX suggests that the binding sites for the toxins overlap (Moczydlowski

et al., 1986a; Ohizumi et al., 1986; Yanagawa et al., 1986, 1987; Becker et al., 1989). Nevertheless, mutations in the region of the outer vestibule that are known to affect TTX and STX binding result in minimal changes in the affinity to μ-CTX (Chen et al., 1992; Stephan et al., 1994). μ-CTX GIIIA has a net charge of +6 in solution and only one hydrophobic residue (Gray et al., 1988), so μ-CTX probably interacts with at least some negative charges on the channel. Substitution of a neutral glutamine for a glutamate in the domain II outer vestibule region significantly reduces STX binding to rat brain channels (Noda et al., 1989; Terlau et al., 1991; Kontis and Goldin, 1993). Therefore, we decided to test the analogous mutation in μI (E758Q) for its effect on μ-CTX blocking efficacy.

Na^+ channels in which the glutamate at position 758 was mutated to glutamine showed the predicted decrease in affinity for TTX and a dramatic reduction in μ-CTX binding. Fig. 2 shows current traces from oocytes expressing the mutant channel E758Q. The records were obtained in a single oocyte exposed to control conditions, 500 nM μ-CTX, or 3 μM TTX. Under all three conditions, current was elicited by stepping to 0 mV from a holding potential of −100 mV. As a preliminary test of the mutant channel's resistance to toxin block, concentrations of the toxins were chosen that greatly exceeded the known IC_{50}s, yet neither 3 μM TTX nor 500 nM μ-CTX resulted in more than a ~50% reduction in current. This dose of TTX was chosen because several previous reports have established that the native μI Na^+ channel has a nanomolar affinity for TTX under a variety of conditions (Moczydlowski et al., 1986a; Trimmer et al., 1989; Chen et al., 1992; Ukomadu et al., 1992; Stephan et al., 1994). At 145 μM μ-CTX, there was 97% suppression of the Na^+ current, implying that, although E758Q reduced μ-CTX affinity, μ-CTX binding still resulted in a complete blockade of the channel.

The equilibrium IC_{50}s for block of E758Q were determined for each toxin by the protocol described above. The IC_{50} at 0 mV was 13.4 ± 2.7 μM (n = 4) and 822 ± 105 nM (n = 11) for TTX and μ-CTX, respectively. As had been reported for the analogous mutation in the brain channels (Noda et al., 1989; Terlau et al., 1991; Kontis and Goldin, 1993), there were no significant changes in the macroscopic kinetics or Na^+ reversal potential as a result of E758Q (data not shown). These results suggest that Glu-758 interacts with some portion of μ-CTX and that the negative charge at this site contributes significantly to the binding energy. Furthermore, Glu-758 is likely to represent an area of overlap in the outer vestibule of the Na^+ channel between the TTX- and μ-CTX-binding sites.

Kinetics of μ-CTX blockade

To further define the effect of E758Q, the kinetics of μ-CTX block of the native channel were compared to the kinetics observed with E758Q. Peak currents were recorded every 20 s after voltage steps to 0 mV. Fig. 3 shows the typical reduction of peak currents after the addition of 500 nM μ-CTX to the bathing solution of oocytes expressing E758Q. μ-CTX block was always reversible, as demonstrated by the return of peak currents to near the starting level after removal of the toxin. Recovery of current during washout was typically >90%. Similar kinetics were observed when μ-CTX was exposed to oocytes expressing native μI Na^+ channels.

The kinetics of μ-CTX block and its relief upon washout in both native and mutant channels were fairly well de-

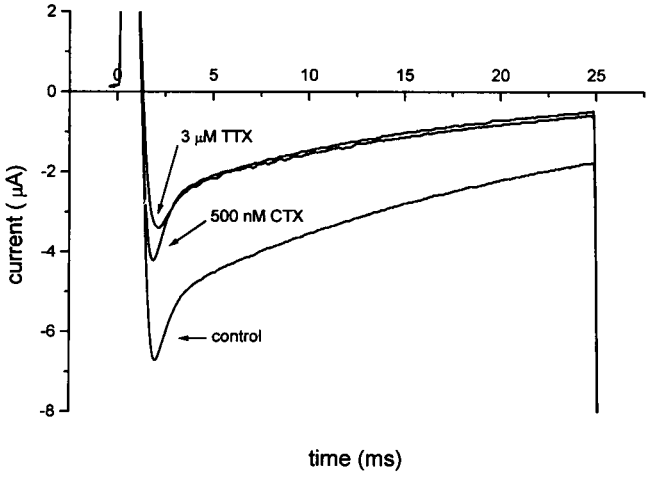

FIGURE 2 Macroscopic currents showing the resistance of the mutant channel, E758Q, to block by μ-CTX or TTX. Current traces during voltage steps to 0 mV from a holding potential of −100 mV in a single oocyte expressing E758Q were obtained after exposure to either control conditions, 500 nM μ-CTX, or 3 μM TTX. In concentrations greatly exceeding the IC_{50}s in native channels, neither μ-CTX nor TTX completely inhibited the channel.

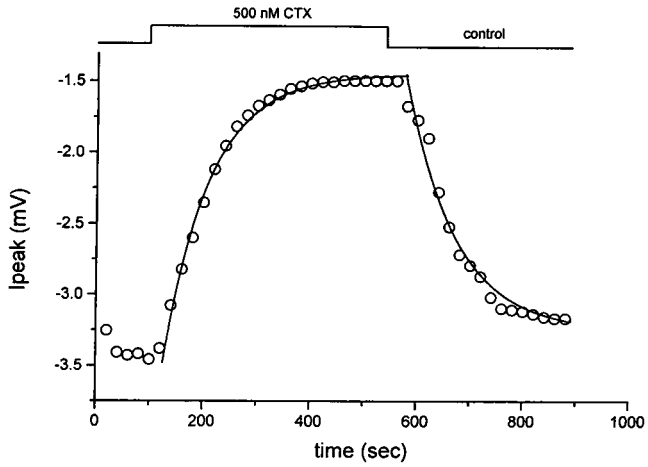

FIGURE 3 Application and washout of μ-CTX in an oocyte expressing E758Q. Peak currents at 0 mV were recorded every 20 s. Both binding and unbinding of μ-CTX were first-order processes fairly well fitted by single exponential functions. The time constants of these functions were used to calculate the on and off rates for μ-CTX. Records typically showed >90% recovery upon washout of the toxin for both the native and mutant channels.

scribed by single exponential functions. These simple exponential binding kinetics have been observed previously for native μI expressed in oocytes (Chahine et al., 1994) or for rat skeletal muscle channels reconstituted in lipid bilayers (Cruz et al., 1985). The time constant of inhibition of the channel was related to the pseudo-first-order rate constant of binding (k_{on}) by the formula

$$k_{on} = [\mu CTX]^{-1}\left(\frac{1}{\tau_{on}} - \frac{1}{\tau_{off}}\right)$$

where τ_{on} and τ_{off} are the time constants of the exponential functions describing the kinetics of μ-CTX binding and unbinding to the channel, respectively. The first-order rate constant for unbinding (k_{off}) was taken as the inverse of τ_{off}.

The results of such kinetic analysis of μ-CTX binding are shown in Table 2. The major effect of E758Q was to reduce k_{on} for toxin binding. Compared with the native μI channel, the k_{on} for μ-CTX binding to E758Q was decreased by 107-fold. Neutralizing the negative charge in the mouth of the vestibule modified the k_{off} by a factor of ~2, but this difference was not statistically significant ($p = 0.15$). Using both the k_{on} and k_{off} recorded from a single oocyte, a K_m for μ-CTX binding was calculated. The average kinetically defined values of K_m for native (seven oocytes) and E758Q (six oocytes) channels were statistically indistinguishable from the equilibrium values of IC_{50} ($p = 0.17$ and $p = 0.40$, respectively; see Table 2).

DISCUSSION

The large reduction in affinity for μ-CTX observed by mutating E758Q in the absence of obvious changes in macroscopic channel kinetics suggests that this residue is an important part of the μ-CTX-binding site. Because the mutation resulted in a channel with low affinity for both TTX and μ-CTX, it seems likely that μ-CTX blocks by binding to part of the TTX-binding site in the outer vestibule of the Na^+ channel. It is not possible to rule out an allosteric effect of the mutation, but its selective effect on both toxin affinities without additional kinetic effects supports the interpretation that Glu-758 participates directly in the μ-CTX-binding site.

Previous attempts to locate the μ-CTX-binding site on μI have led to equivocal results, and site-directed mutations have resulted in only limited changes in μ-CTX affinity (Chen et al., 1992; Stephan et al., 1994; see Table 3). Chen et al. (1992) tested chimeras constructed by switching domain I between the μ-CTX-sensitive adult rat skeletal muscle Na^+ channel (rSkM1=μI) and the μ-CTX-insensitive rat heart channel (rSkM2) and tested other block mutations, concluding that μ-CTX binding involved multiple domains of the channel. In addition, they observed a small effect when mutating Tyr-401 to cysteine, but the correlate mutation Cys-374 to tyrosine in rSkM2 did not result in improved μ-CTX binding. Other mutations of rSkM1 in the pore-forming regions of domains I and IV have failed to affect μ-CTX block (Chahine et al., 1994). Recently, a mutation of a glutamate at position 403 in the outer vestibule region of the μI Na^+ channel that is critical for TTX and STX block caused only a small change in μ-CTX affinity (Stephan et al., 1994), demonstrating that the μ-CTX-binding site was not identical to that of STX and TTX. In the experiments presented here, mutation of another negatively charged residue important for STX and TTX binding, E758Q, resulted in a mutant μI channel that showed low affinity for μ-CTX and TTX simultaneously. Consequently, it seems likely that the binding sites for these two toxins do overlap in this region.

The effect of neutralizing this carboxyl group mainly was to slow the association rate of the positively charged μ-CTX with the channel. This result is similar to the effect of charge-altering mutations at the outer vestibule region of the Shaker H4 K^+ channel on Lq2 scorpion toxin binding kinetics (Escobar et al., 1993). Escobar et al. (1993) interpreted the effect of these mutations to mean that diffusion of the toxin was rapid in comparison to the rate of formation of other short-range factors necessary for high-affinity binding. In this case, one plausible explanation for the observed experimental result would be if Glu-758 exerted an electrostatic force directly upon μ-CTX, facilitating a relationship with a large energy of interaction.

The contribution of Glu-758 to the free energy of μ-CTX binding can be estimated by using the equilibrium IC_{50}

TABLE 2 Comparison of μ-CTX binding to native μI and to E758Q

	Native μI	E758Q
Equilibrium IC_{50} (nM)	17 ± 5 (n = 8)	822 ± 105 (n = 11)
k_{on} (M^{-1} s^{-1})	1.4 ± 0.8 × 10^6 (n = 7)	1.3 ± 0.4 × 10^4 (n = 6)
k_{off} (s^{-1})	3.9 ± 1.9 × 10^{-3} (n = 9)	8.7 ± 1.1 × 10^{-3} (n = 6)
Kinetic K_m (nM)	9 ± 4 (n = 7)	1150 ± 340 (n = 6)

n, number of oocytes tested.

TABLE 3 The effect of selective mutations of μI on μ-CTX affinity

Channel mutation	Ratio of mutant/native IC_{50} for CTX
Y401C*	3.8
E403Q‡	3.8
N404R*	1.1
E758Q§	48.6

The ratio of mutant to native IC_{50}s is used to represent the effect of a mutation as a means to correct partially for variations in experimental conditions. Mutations of the channel are indicated by the single letter code for the amino acid, the number of that amino acid in the primary sequence, and the single letter code for the amino acid substituted.
*Chen et al. (1992).
‡Stephan et al. (1994).
§This work.

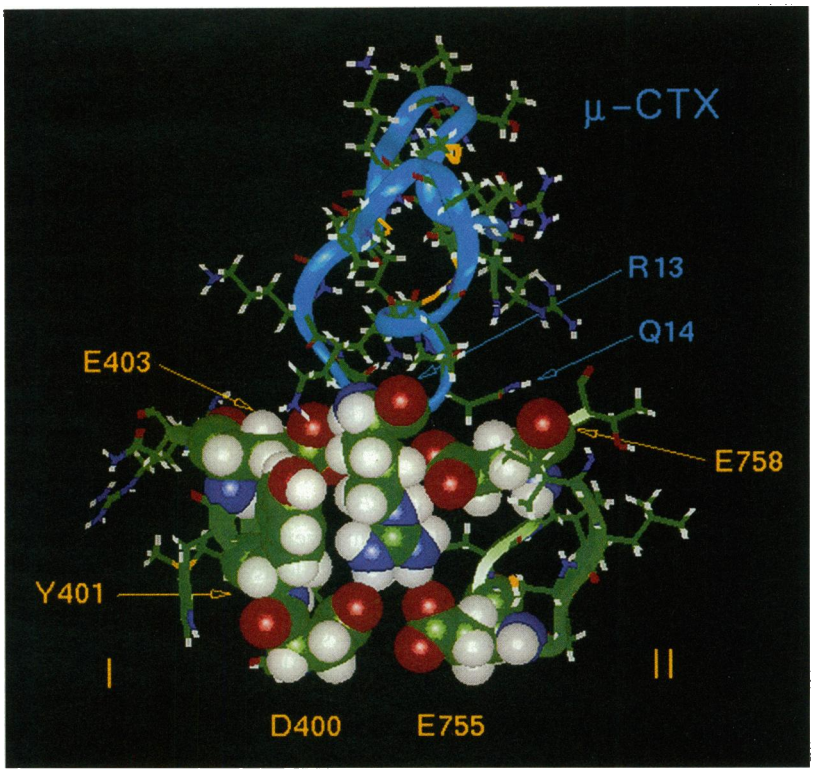

FIGURE 4 A possible mechanism for μ-CTX block of the Na^+ channel. In this figure, the guanidinium of Arg-13 of μ-CTX forms salt bridges with carboxyls of Asp-400, Glu-755, and Glu-758 from domains I and II (shown by space-filling models). Because Asp-400 and Glu-755 have been shown experimentally to be involved with selectivity, the interaction of these groups with Arg-13 could serve to occlude the pore. The backbone of μ-CTX is indicated in light blue and is shown with respect to the proposed structure for the TTX/STX binding site (Lipkind and Fozzard, 1994). The green ribbon structures represent the β-hairpins of domains I and II, which comprise part of the outer vestibule TTX/STX binding site. Each hairpin consists of a 10 amino acid portion of the S5–6 linker forming two β-strands linked by a type III β-turn. The hairpins of domains III and IV have been omitted from the picture for clarity. Amino acids of the channel and of μ-CTX are labeled in yellow and light blue, respectively. Oxygen, hydrogen, nitrogen, carbon, and sulfur are red, white, blue, green, and yellow, respectively.

values before and after the mutation E758Q. The relative free energy contributed by Glu-758 (ΔG) is related to the equilibrium IC_{50} values by

$$\Delta G = RT \ln \frac{IC_{50_1}}{IC_{50_2}}$$

where R is the gas constant and T is the temperature. The potential change resulting from E758Q was calculated to be 2.3 kcal/mol or, equivalently, 98 mV. If this free energy change was primarily the result of a loss of coulombic attraction and if the distance between Glu-758 and that part of μ-CTX interacting with the outer vestibule was known, it would be possible to estimate a local, effective dielectric constant (D_{eff}) for this region of the outer vestibule.

Several lines of evidence suggest that the Arg-13 of μ-CTX may be the positive charge interacting with the outer vestibule of the channel. μ-CTX contains four lysines (Lys-8, 9, 11, and 16), three arginines (Arg-1, 13, and 19), and an amino terminus that are positively charged, so it is reasonable to suppose that one of these positively charged residues interacts with the outer vestibule. Mutations altering Arg-13 repeatedly have been shown to have the largest effect on binding affinity (Sato et al., 1991; Becker et al., 1992; Chahine et al., 1994). μ-CTX with a mutation of Arg-13 to glutamine (R13Q) binds the channel with a substantially reduced affinity (μM), and binding no longer results in total occlusion of the pore (Becker et al., 1992). Furthermore, as is the case with TTX and STX (Kao and Walker, 1982; Strichartz, 1984; Kao, 1986; Yang and Kao, 1992), the inability of either lysine or ornithine to substitute effectively for Arg-13 implies that the special properties of a guanidinium group are necessary for block of the channel.

To further investigate this possibility, we evaluated the interaction of μ-CTX with our model of the outer vestibule of the Na^+ channel (Lipkind and Fozzard, 1994). This model is based upon the mapping of interactions of the channel with TTX and STX and consists of four antiparallel β-hairpins from each of the four pore-forming regions. By virtue of the shape of TTX and STX, the model is conical with a constriction at its base. This constriction is formed by Asp-400, Glu-755, Lys-1237, and Ala-1529 in domains I, II, III, and IV, respectively. Because these amino acids are known to be involved in Na^+ selectivity (Terlau et al., 1991;

TABLE 4 The effect of selected mutations of μ-CTX on the toxin binding affinity

Mutation of CTX	Ratio of mutant/native IC_{50}s
R13A*	234
R13Q‡	228
R13K‡	88
R13Orn‡	25

The ratio of mutant to native IC_{50}s is used to represent the effect of a mutation as a means to correct partially for variations in experimental conditions. Mutations of CTX are indicated by the single letter code for the amino acid, the number of that amino acid in the primary sequence, and the single letter code for the amino acid substituted. Orn stands for ornithine.
*Data from Sato et al. (1991) using a bioassay of the effect on rat diaphragm.
‡Data from Chahine et al. (1994) using rat skeletal muscle channels expressed in *Xenopus* oocytes.

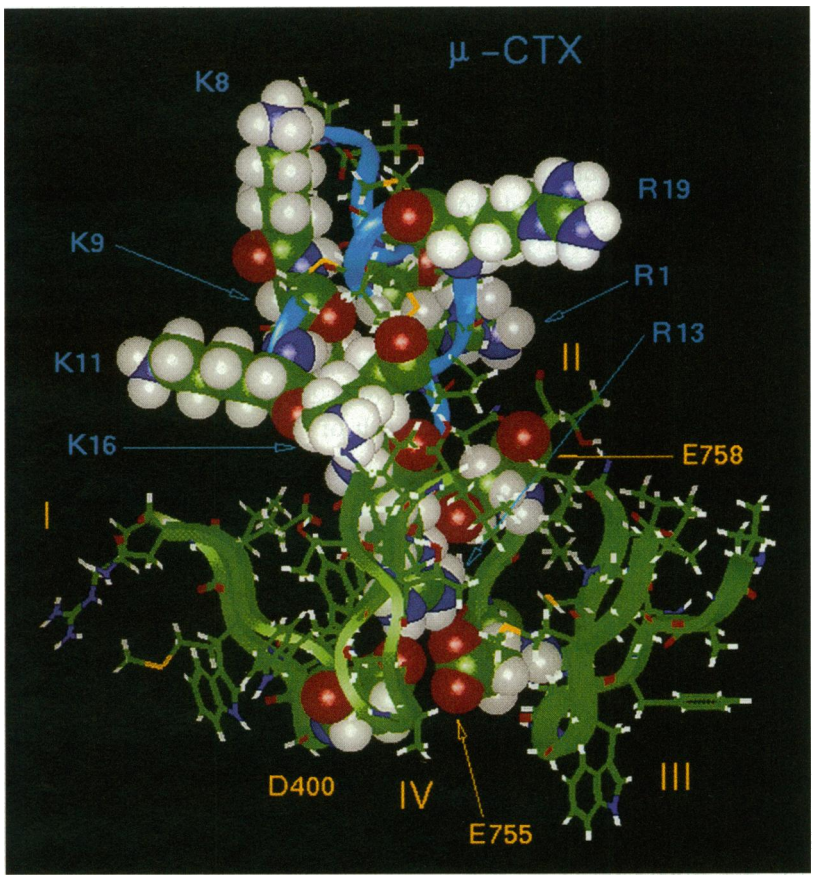

FIGURE 5 A view of the energetically preferred orientation of μ-CTX with respect to the putative outer vestibule of the Na^+ channel. The β-hairpins from domains I–IV, which form the outer vestibule, are indicated by the green ribbons and are labeled with roman numerals. Selective carboxyl groups of the channel are shown by space-filling models and are labeled in yellow. The backbone of μ-CTX is indicated in light blue, and the basic amino acid side chains of the toxin are shown by space-filling models and are labeled in light blue. To assess the most favorable orientation for μ-CTX, we rotated the rigid core of the toxin about the dihedral C^α–C^β bond (angle χ_1) of the side chain of Arg-13 and evaluated the energy of interaction between toxin and the channel. During this process, the remaining atoms of the side chain of Arg-13 were fixed in the arrangement shown in Fig. 4. The preferred orientation occurs when χ_1 of Arg-13 equals 72°, close to 70° found by Lancelin et al. (1991). The values of χ_1 for the other amino acid side chains of μ-CTX are the angles determined by Lancelin et al. (1991). Other χ angles correspond to the extended conformations. This view emphasizes the relative scale of μ-CTX with respect to the outer vestibule and the distances between various positively charged residues of the toxin and Glu-758 of the channel. The interaction of Arg-13 of μ-CTX with Asp-400, Glu-755, and Glu-758 is well visualized. Oxygen, hydrogen, nitrogen, carbon, and sulfur are red, white, blue, green, and yellow, respectively.

Heinemann et al., 1992), this constriction seems to be a good candidate for the selectivity filter.

The following constraints were used in construction of the model: 1) Arg-13 must have a low free energy of interaction with the outer vestibule, 2) binding of Arg-13 to the outer vestibule must result in occlusion of the pore, 3) mutating E758Q must result in lower binding affinities for both TTX and μ-CTX, and 4) the model must be consistent with the experimental results in Tables 3 and 4. There was only one orientation of Arg-13 that fulfilled all of these constraints (Fig. 4). In this orientation, N-H moieties of the guanidinium group of Arg-13 interacted with two carboxyl groups in the outer vestibule of μI (i.e., Asp-400 and Glu-755) in a manner similar to that of the association that we proposed between the channel and the guanidinium groups of STX and TTX (Lipkind and Fozzard, 1994). In place of the Glu-403 that we hypothesized was a third carboxyl group interacting with the guanidinium of STX and TTX, Glu-758 interacted with the guanidinium of Arg-13. The association of Arg-13 with the two carboxyls Asp-400 and Glu-755 resulted in the occlusion of the ion permeation pathway. The analogous amino acids in rat brain Na^+ channels are known to be important in TTX and STX binding and in Na^+ permeation (Noda et al., 1989; Terlau et al., 1991; Kontis and Goldin, 1993).

This orientation of Arg-13 is consistent with the results of the μI mutations on μ-CTX affinity, as noted in Table 3. Because the aliphatic portion of Arg-13 faces Glu-403, there is little interaction between Arg-13 and Glu-403. This could explain why the Glu-403 to glutamine mutation has only a small effect on μ-CTX binding (Stephan et al., 1994) compared with the effect of E758Q. Tyr-401 is oriented such that its partially negative π orbital is directed toward the positive guanidinium. In the model, replacement of Tyr-401 by cysteine reduces the interaction energy by less than 1.5 kcal/mol, which is significantly less than the effect of this mutation on TTX binding (~5 kcal/mol; Lipkind and Fozzard, 1994).

Assuming that Arg-13 interacted with Glu-758 in the manner suggested by the model, the predicted distance between Arg-13 and Glu-758 was used to estimate the effective dielectric constant, D_{eff}, by the following relation (Russell et al., 1987):

$$D_{\text{eff}} = 332\frac{\Delta q}{r\Delta G}$$

where ΔG is the change in potential in kcal/mol, Δq is the change in valence produced by the mutation, and r is 4.6 Å, the separation between the centers of the guanidinium group of Arg-13 and carboxyl oxygens of Glu-758 in the model. Estimated in this way, the D_{eff} was 31, a reasonable value intermediate between the aqueous and lipid phases. Using kinetically defined values of K_m, the cal-

culated D_{eff} was similar at 25. By varying the radius between 3 and 6 Å, the calculated values of D_{eff} ranged from 23 to 47. Radii on this order have been observed experimentally between carboxyl groups and a guanidinium (Bray et al., 1984), and these values of D_{eff} are similar to those of other enzyme-reactive sites (Sternberg et al., 1987; Warshel and Åqvist, 1991). As a second check of the consistency of the model, the difference in the energies of interaction between Arg-13 of the toxin before and after neutralization of Glu-758 were determined assuming a dielectric constant of 31. The model gave a calculated potential energy difference for μ-CTX binding after introduction of E758Q of ~2 kcal/mol, in agreement with our experimental result of a 48-fold reduction in block.

To this point, the calculations have assumed that Glu-758 of the channel interacts exclusively with Arg-13 of μ-CTX. It is possible that the decrease in binding affinity seen with the E758Q mutation results from the loss of coulombic interactions with more than one of the toxin's positively charged residues. In this regard, neutralization of five other positively charged residues of the toxin has an effect on the association rate similar to that of the mutation R13Q (i.e., Lys-8, 9, 11, 16, and Arg-19; Becker et al., 1992). In the proposed model, these positive charges are distant from Glu-758, however (Fig. 5). The distance estimates from the model suggest a separation from Glu-758 to Lys-8, 9, 11, 16, and Arg-19 of 22 Å, 9 Å, 17 Å, 14 Å, and 20 Å, respectively. Because these residues are on the surface of the toxin and surrounded by a high dielectric, aqueous medium, it seems reasonable to assume that the contribution of these charges to the toxin interaction with Glu-758 is small. This assumption is consistent with the findings of Stocker and Miller (1994) concerning the contribution of charges on the surface of charybdotoxin distant to the electrostatic interaction between Lys-11 of charybdotoxin and the Shaker K^+ channel residue 427. Presumably, these other charges on μ-CTX influence the association rate by interactions with other residues on the channel.

In summary, elimination of a negative charge at the outer vestibule of μI, E758Q, resulted in a decrease in μ-CTX binding. The predominant effect of this mutation was to slow the association rate between the toxin and the channel, suggesting that Glu-758 might be involved in electrostatically guiding the toxin to its binding site. We propose a model of the interaction of μ-CTX with the outer vestibule of the Na^+ channel that predicts that the toxin occludes the pore by the guanidinium group of Arg-13 interacting with two carboxyls at the putative selectivity filter. At this point, our current model of the Na^+ channel outer vestibule does not explain the strong preference of μ-CTX for skeletal muscle and eel electric organ Na^+ channels.

This work was supported in part by P01-HL20592, by a National Research Service Award (F32-HL08104) to S.C.D., and by the University of Chicago Cardiology Molecular Modeling Core.

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