Research Paper

Docking of μ-Conotoxin GIIIA in the Sodium Channel Outer Vestibule

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NOTE

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ABSTRACT

μ-Conotoxin GIIIA (μ-CTX) is a high-affinity ligand for the outer vestibule of selected isoforms of the voltage-gated Na⁺ channel. The detailed bases for the toxin’s high affinity binding and isoform selectivity are unclear. The outer vestibule is lined by four pore-forming (P) loops, each with an acidic residue near the mouth of the vestibule. μ-CTX has seven positively charged residues that may interact with these acidic P-loop residues. Using pair-wise alanine replacement of charged toxin and channel residues, in conjunction with double mutant cycle analysis, we determined coupling energies for specific interactions between each P-loop acidic residue and selected toxin residues to systematically establish quantitative restraints on the toxin orientation in the outer vestibule. Xenopus oocytes were injected with the mutant or native Na⁺ channel mRNA, and currents measured by two-electrode voltage clamp. Mutant cycle analysis revealed novel, strong, toxin-channel interactions between K9/E403, K11/D1241, K11/D1532, and R19/D1532. Experimentally determined coupling energies for interacting residue pairs provided restraints for molecular dynamics simulations of μ-CTX docking. Our simulations suggest a refined orientation of the toxin in the pore, with toxin basic side-chains playing key roles in high-affinity binding. This modeling also provides a set of testable predictions for toxin-channel interactions, hitherto not described, that may contribute to high-affinity binding and channel isoform selectivity.

INTRODUCTION

Voltage-gated sodium (Na⁺) channels are present in excitable cells from the nervous system to the heart, where they provide the main current underlying rapid signal propagation. They consist of a ~230 kD α-subunit that incorporates the ion-permeation pathway, the selectivity filter and binding sites for multiple drugs and toxins. The α-subunit is arranged in four homologous domains each containing six predicted α-helical transmembranous segments (S1–S6).1 The domains are thought to be organized circumferentially around the ion-conducting pore. The amino-acid chains that link the fifth and sixth helices appear to loop down into the pore from its extracellular side. These loops line the ion permeation pathway and are known as the P-loops.2 A water-filled region encompassed by the four P-loops forms the outer vestibule of the channel. The outer vestibule is the region of the α-subunit that includes the receptors for site 1 sodium channel blockers such as tetrodotoxin (TTX), saxitoxin (STX), and the μ-conotoxins. At its inner end, the vestibule narrows to form the selectivity filter, probably about 30% of the way along the ion-conducting pathway. TTX and STX are naturally occurring guanidinium toxins responsible for fugu and paralytic shellfish poisoning, respectively.3-6 Both toxins block Na⁺ channels with high affinities and, aside from representing an ongoing health hazard present in contaminated seafood, these toxins have been instrumental in isolation, purification, and description of these channels.

μ-Conotoxin GIIIA (μ-CTX) is a rigid 22-amino acid peptide toxin isolated from piscivorous cone snails, which binds to the outer vestibule of skeletal muscle Na⁺ channels at nanomolar concentrations.7-11 Compared with STX and TTX (each ~300 Da), μ-CTX (~2,500 Da) is larger and is more selective among different channel isoforms, suggesting that a fuller understanding of this toxin’s interactions may lead to useful information about the channel’s complementary binding surface that could be exploited to develop future isoform-selective pharmacology. Probing the high affinity interaction of μ-CTX with the channel’s outer vestibule already has led to insights into its structure. For example,
identification of several toxin-channel interactions by use of double mutant cycle analyses revealed that the four homologous domains are arranged in a clockwise manner, as viewed from the extracellular end.\textsuperscript{12,13} Despite evidence of the rotational positions of certain toxin residues around the pore axis, the full, three dimensional docking orientation of the toxin in the outer vestibule remains controversial and is a subject of debate.\textsuperscript{14}

Each P-loop is thought to contribute a negatively charged residue to an “outer ring” of charge lining the extracellular mouth of the vestibule. In the rat skeletal muscle Na\textsuperscript{+} channel (rNa\textsubscript{A},1.4), these are E403, E758, D1240 and D1532. These residues have an important role in \(\mu\)-CTX binding and have recently been shown to affect slow inactivation.\textsuperscript{15} \(\mu\)-CTX has seven positively charged residues that may participate in electrostatic interactions with the outer vestibule residues (R1, K8, K9, K11, R13, K16 and R19). Using double mutant cycle analysis, we determined specific toxin-channel interactions between each P-loop outer ring acidic residue and selected toxin residues to systematically fix the toxin orientation with respect to all four P-loops. All mutations were alanine substitutions of native charged residues, in an effort to remove interactions of a selected residue, without introducing new ones. This provided a systematic experimental dataset of energetic restraints for molecular dynamics (MD) simulations of \(\mu\)-CTX docking in the outer vestibule. The docking simulations suggest a refined orientation of the toxin in the pore, in which key basic side chains play a significant role in high affinity binding, and which differs significantly from previous working models. Also, the simulations predict a novel set of toxin-channel interactions that may contribute to high affinity, isoform-selective toxin binding.

**EXPERIMENTAL METHODS**

**Preparation and expression of Na\textsubscript{A},1.4 channel.** Most methods have been described previously in detail (see refs. 16 and 17). A brief description is provided. The rNa\textsubscript{A},1.4 cDNA flanked by the *Xenopus* globulin 5’ and 3’ untranslated regions (provided by J. R. Moorman, University of Virginia, Charlottesville, VA) was subcloned into either the Bluescript SK vector or pAlter vector (Promega, Madison, WI). These vectors have been used extensively for oocyte expression of Na\textsuperscript{+} channels. Oligonucleotide-directed point mutations were introduced into the adult rat skeletal muscle Na\textsuperscript{+} channel (rNa\textsubscript{A},1.4 or SCN\textsubscript{A}4a) by either the Unique Site Elimination Mutagenesis Kit (Pharmacia Biotech, Piscataway, NJ), or by four primer PCR.\textsuperscript{18} Oligonucleotides were designed with silent restriction site changes for rapid identification of mutants. DNA sequencing of the entire polymerized regions insured that only the intended mutations were present. The vectors were linearized and transfected with a DNA-dependent RNA polymerase. Stage V and VI *Xenopus* oocytes from female frogs (NASCO, Ft. Atkinson, WI or Xenopus 1, Ann Arbor, MI) were injected with approximately 50–100 ng of cRNA. Oocytes were incubated at 16°C for 12 to 72 hours prior to examination.

**Peptide synthesis.** Peptide synthesis has been described previously (see refs. 19 and 20). Briefly, linear peptides were produced by solid phase synthesis using 9-fluorenlymethoxycarbonyl (Fmoc) chemistry. Coupling of Fmoc amino acids was performed using the HBTU/HOBt/DIPEA method on an Applied Biosystems 431A synthesizer. The linear peptides were air oxidized and purified as described by Chang and collaborators.\textsuperscript{9} During oxidation, cyclization was monitored by analytical HPLC and usually complete after 2–3 days at 4°C. Following folding of the peptide by air oxidation, toxin derivatives were purified to near homogeneity by HPLC (\(-95\%\), based on analytical HPLC). Active toxin derivatives were isolated as a single major peak. The identity of purified peptides was confirmed by quantitative amino acid analysis and, for some derivatives, by electrospray mass spectroscopy molecular weight determination.

**Electrophysiology.** Recordings were made in the two-electrode voltage clamp configuration using Dagan CA-1B oocyte clamp (Dagan Corp., Minneapolis, MN). Data were collected using Axograph 4.4 software (Axon Instruments, Foster City, CA). All recordings were obtained at room temperature (20–22°C). The oocytes were placed in the center of a bath chamber designed to promote laminar flow, and the bath flow was typically 500 \(\mu\)L/min. Affinity measurements for wild-type channels were reproducible over the experimental period.

The standard bath solution consisted of (in mM): 90 NaCl, 2.5 KCl, 1 CaCl\textsubscript{2}, 2 MgCl\textsubscript{2} and 5 HEPES titrated to pH 7.2 with 1 N NaOH. The effect of toxin addition was monitored by recording the peak current elicited every 20 s by step pulses of 70 ms duration to 0 mV from a holding potential of -100 mV. This protocol allowed the observation of toxin blocking and unblocking, ensured equilibrium was reached at the holding potential, and avoided the development of use-dependent toxin block. There was no accumulation of inactivated channels with this stimulus rate for the wild-type or mutant channels studied. The IC\textsubscript{50} for toxin binding was calculated from the ratio of peak currents in the absence and presence of toxin based on a single-site Langmuir adsorption isotherm.

**Mutant cycle analysis.** Mutant cycle analysis is a method for determining interactions between a specific pair of residues by mutating each in turn and observing mutually dependent effects on binding when each residue in the pair is mutated. We defined the energy of interaction between a pair of residues, one on the toxin and one on the channel, as \(\Delta\Delta G\) where:

\[
\Delta\Delta G = (\Delta G_{WTChannel,CTX} - \Delta G_{WTChannel,MutCTX}) - (\Delta G_{MutChannel,CTX} - \Delta G_{MutChannel,MutCTX})
\]

The standard error of \(\Delta\Delta G\) was calculated as the square root of the sum of the variances of the mean values of RT \(\ln(\text{IC}_{50})\), divided by the square root of the sum of the total number of observations minus four.\textsuperscript{21} A value of \(\Delta\Delta G=0\) implies no coupling interaction. Note that \(\Delta\Delta G\) may be positive or negative, each representing a coupling interaction.\textsuperscript{22} In principle, mutations could lead to either an increase or a decrease in binding affinity, and either case might reflect a pair-wise coupling. An increase may occur as a result of relief of repulsion, or by addition of attraction.\textsuperscript{23} The mutations that we introduced were charge-neutralizing, deliberately chosen to avoid introduction of new electrostatic interactions between the mutant pair. In the data of Table 2, there are only two instances for which an interaction involving one mutant, either channel or toxin, results in binding of higher affinity than for the WT pair; no mutant pair shows a higher affinity than the WT pair. The \(\Delta\Delta G\) reported is the free energy of coupling, or interdependence, between a particular residue pair.

Data are presented as means \(\pm\) SEM. The number of observations \((n)\) was \(\geq 3\) for all reported data. Interaction energies greater than 0.5 kcal/mol were considered significant.\textsuperscript{13,24,25}
Molecular modeling and dynamics simulations. The structure of the μ-CTX has been resolved using NMR experiments. The atomic coordinates of μ-CTX were obtained from the Protein Data Bank (PDB) entry, 1TCG (minimized mean structure). A molecular dynamics (MD) simulation (10 ns) was carried out, for the NMR-determined μ-CTX mean structure in an aqueous environment, to perform a conformational analysis of the toxin. A cluster analysis, based on RMSD from the starting structure, was used, taking into account the backbone plus C\(_\alpha\) atoms with a cut off of 1.0 Å. This analysis showed two main conformations that represent 93% of the total population. These two conformations were used as initial structures for the docking experiments; one of these conformations included the minimized mean NMR structure (see Suppl. Material).

The starting model of the sodium channel outer vestibule used for MD docking simulations was based on the work of Lipkind and Fozzard, who developed it using the backbone positions of the KcsA structure, 29 together with considerations of functional biophysical data. The P-loop region sequence is summarized in Table 1. The P-loops used in our model are 23–26 amino acids long. The length of the modeled S5 and S6 segments are 15 and 16 amino acids, respectively (see Supplementary Material for the S5 and S6 sequences).

MD simulations of toxin-pore interactions were carried out as follows. The pore models were placed in a pre-equilibrated (2 ns) water-octane biphasic cell. The octane phase mimics the bilayer interior. Although this is a significant simplification of a more realistic lipid environment, for the purpose of docking the toxin to the channel model, this should be sufficient. The pore was placed in the center of the octane phase of the system. The system consists of a rectangular box of dimensions 80 x 80 x 80 Å\(^3\). The final biphasic cell contained approximately 400 octane and 7500 water molecules. The charge of the system was neutralized by adding Na\(^+\) ions. The amount of Na\(^+\) depended on the total charge of the pore model, including the P-loops and the S5 and S6 segments. A short equilibration MD simulation (0.5 ns) was carried out to obtain an initial structure (pore, octane, water and Na\(^+\)) for toxin docking. During the equilibration MD, the backbone structure of the pore was held fixed using a harmonic potential with a force constant of 2.5 kcal/molÅ\(^2\).

The toxin was given a random orientation and was directly placed above the pore, at a distance of ~ 20–30 Å from the upper level of the octane layer (see Suppl. Material, supporting Fig. 1). During the docking simulations (0.05–0.7 ns), the backbone atoms of the P-loops and the C\(_\alpha\) atoms for the side chains of the DEKA selective filter were restrained using a harmonic potential with a force constant of 2.5 kcal/molÅ\(^2\). Then, μ-CTX was docked onto the channel pore in the presence of a selected number of distance restraints derived from the experimental data. Distance restraint potentials were defined as a harmonic potential \(V_{DR}\) between the heavy atoms of side chains from the toxin (i) and the channel pore (j), as follows:

\[
V_{DR} = \frac{1}{2} k_{DR} (r_i - r_0)^2 \text{ for } r_i \leq r_0
\]

where \(V_{DR}\) is the distance restraint potential energy, \(k_{DR}\) is the distance restraint force constant, with values from 20–35.0 kcal/molÅ\(^2\), depending on the interaction type; \(r_0\) was assigned values from 2 to 6 Å, and is the distance above which a non-zero restraint potential is applied to the system; and \(r_i\) is the distance between the center of mass of individual heavy atoms, \(i\), of the toxin and \(j\), of the channel. Eriksson and Roux applied a similar docking protocol to study the interaction between agitoxin and the Shaker K\(^+\) channel.

The MD simulations were carried out using the GROMACS set of programs. We used the GROMOS96 43a2 force field for μ-CTX and the channel pore, and the octane parameters were taken from the CH\(_2\)/CH\(_4\) groups of the lipid force field of Berger et al. The Simple Point Charge model was used to represent water.

Bond lengths were constrained using the LINCS algorithm. Lennard-Jones interactions were calculated with a 0.9/1.4 nm twin-range cutoff. The electrostatic interactions were calculated using the Particle Mesh Ewald algorithm with a cutoff of 0.9 nm. The neighbor list was updated every 10 steps. Each component of the systems was coupled separately to a temperature bath at 300 K, using a Berendsen thermostat, with a coupling constant \(\tau_T = 0.1\) ps. In the water/octane simulation, the x and y dimensions (the area of the interface) of the system were held fixed, while the z dimension was coupled to a pressure of 1.0 bar with time constant \(\tau_p = 0.1\) ps. Simulations were run with a 2 fs time step. The data was collected every 1 ps. Molecular graphics were made using VMD.

RESULTS

The importance of the negatively charged residues in the outer ring of the Na\(^+\) channel outer vestibule (E403, E758, D1241 and D1532) and the positively charged residues on the toxin (R1, K11, R13, K16 and R19) for μ-CTX binding has been shown previously (see refs. 12 and 13). Residue R13 has been the most intensely studied toxin residue. It binds deeply in the outer vestibule, interacting both with outer ring charges and residues of the selectivity filter. It interacts strongly with Domain II, and is critical for high affinity toxin block. With this residue-channel interaction serving...
as a fixed point around which the toxin could be rotated, we reasoned that knowledge of other toxin interactions with respect to the outer vestibule negative charges of the four domains would allow us to establish a likely orientation of the body of the toxin with respect to the channel.

Previously, we have found that estimates of coupling energies between a pair of residues can vary substantially depending on what mutations are used in a double mutant cycle analysis. Therefore, in this study, we undertook a systematic evaluation of the interactions of selected basic toxin residues with the four channel outer ring residues, using alanine replacement of charged residues to evaluate the pair-wise couplings. This strategy yields a set of coupling energies that should be systematically related to distances between members of the interacting residue pair, and thus provide an optimal set of restraints for MD simulations of docking.

Representative current tracings obtained by two electrode voltage clamp experiments are shown in Figure 1. The binding affinities for the toxin-channel combinations are listed in Table 2, and a summary of interaction energies is presented in Figure 2. Of the outer vestibule mutations, the elimination of a negative charge in domain II had the largest effect on toxin affinity. In the toxin, the neutralizing substitution R19A led to the largest change in affinity, by 580-fold. Lys-8 interacts with domains I and IV. Neutralizing replacement of Lys-8 decreased toxin binding more than 20-fold. Nevertheless, K8 did not have strong interactions with the outer ring residues. Double mutant cycle analysis showed that K8 had weak interactions with E403 and D1532 in the channel.

Table 2: The effect of channel and toxin mutations on µ-CTX IC₅₀

| Residue | CTX (nM) | K8A (nM) | K9A (nM) | K11A (nM) | K16A (nM) | R19A (nM) |
|---------|----------|----------|----------|-----------|-----------|-----------|
| Native channel | 11.2 ± 2.7 | 247.1 ± 37.5 | 4.0 ± 0.4 | 325.0 ± 28.6 | 267.5 ± 24.1 | 6464.8 ± 635.6 |
| E403Q | 98.0 ± 9.1 | 9702.5 ± 561.7 | 1061.8 ± 135.3 | 2151.1 ± 108.7 | 576.2 ± 21.9 | 99497.8 ± 5510.7 |
| E758Q | 864.7 ± 74.6 | ND | 370.2 ± 58.2 | 4142.0 ± 243.0 | 6112.3 ± 728.8 | 71134.8 ± 7353.7 |
| D1241A | 110.6 ± 9.7 | ND | 65.7 ± 3.2 | 168.0 ± 23.6 | 118.9 ± 5.9 | 8809.2 ± 2179.3 |
| D1532N | 164.4 ± 22.7 | 1377.7 ± 50.5 | 43 ± 3.5 | 205.6 ± 30.7 | 438.1 ± 44.4 | 4077.2 ± 316.5 |

*Reported previously by Dudley et al.*

Lysine-9 interacts with domain I. The mutation K9A was used to study the interaction of this residue with E403, E758, D1241, and D1532 (IC₅₀ values in Table 2). Interestingly, mutation of this lysine did not impair toxin binding to the native channel, for reasons that are unclear but may involve compensatory alterations in binding energy. Nevertheless, it disproportionately worsened binding to the channel mutant, E403Q. Mutant cycle analysis showed K9 interacted significantly with E403 in domain I (|ΔΔG| = 1.8 ± 0.1 kcal/mol), but had no significant interaction with the other residues.

Lysine-11 interacts with domains III and IV. Mutation of K11 caused a substantial (28-fold) reduction in the binding affinity to the wild-type channel. This resulted from interactions with three residues in the outer ring. Mutant cycle analysis revealed that K11 had strong interactions with D1241 (|ΔΔG|: 1.9 ± 0.1 kcal/mol) and D1532 (|ΔΔG|: 1.9 ± 0.1 kcal/mol), medium interaction with E758 in domain II (|ΔΔG|: 1.1 ± 0.1 kcal/mol), and no significant interaction with E403 in domain I.

Lysine-16 interacts strongly with domains III and IV. Alanine substitution at the toxin K16 site resulted in a 24-fold reduction in binding with the wild-type channel. Mutant cycle analysis revealed that the 16 site interacted with all four domains, most strongly with D1241 (|ΔΔG|: 1.9 ± 0.1 kcal/mol). Its interaction energy with D1532 was previously reported to be 1.4 ± 0.1 kcal/mol. Weaker interactions were noted with E403 in domain I (|ΔΔG|: 1.0 ± 0.1 kcal/mol) and E758 in domain II (|ΔΔG|: 0.8 ± 0.1 kcal/mol).

Arginine-19 interacts strongly with domain IV. The mutation R19A had the most substantial impact on toxin binding of those.
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Figure 2. (A) A schematic of sodium channel outer vestibule with the four P-loops and outer ring of acidic residues and μ-CTX with the basic residues that were tested. (B) The interaction energies (ΔΔAG) calculated by mutant cycle analysis between five basic μ-CTX residues and the four acidic residues in the outer ring of the pore of the voltage-gated sodium channel. Interactions >0.5 kcal/mol are considered to be significant.

Figure 3. Alterations in the P-loops of the Lipkind and Fozzard model made to accommodate μ-CTX binding. Superimpositions of P-loop regions before (orange ribbons) and after modifications (mauve ribbons) as discussed in the text. The DEKA selective filter positions were held constant. The amino-acid side chains are displayed as sticks only in the P-loops of the extended model.

tested, closely followed by the R13 mutations. It interacted strongly with D1532 in Domain IV (ΔΔAG: 1.9 ± 0.1 kcal/mol), followed by E758 and D1241 (ΔΔAG: 1.3 ± 0.1 kcal/mol), and had no significant interaction with domain I E403.

MD simulations. In order to understand the structural implications of these couplings, we performed MD simulations using distance restraints based on experimental determinations of coupling energies (see Methods and Table 3).

A stable, single toxin/channel binding orientation was obtained with some minor alterations in the Lipkind and Fozzard model. These modifications consisted of: (1) Adding 2–5 residues to the C-terminus of the P-loops (see Table 1; added residues are underlined); and (2) Rotating each P-loop between 5–15° around the pore axis, in the membrane plane, to provide increased volume of the outer vestibule. A comparison of the model before and after the modifications is presented in Figure 3. The extensions were kept to the minimum necessary to resolve the toxin-channel orientation. This newly refined model successfully accommodated the smaller toxins, 11-SO₃-STX and TTX²⁴,²⁵ (Fig. 4). In each model, the diameter of the DEKA selectivity filter was held constant at 14 Å based on the β carbons of the side chains during the rotations. With this extended and modified model, a robust, consistent orientation of the toxin in the pore was obtained using 12 distance restraints (r₀), which are summarized in Table 3. They varied inversely with coupling energies in the range of 2.7 to 6 Å. The distance restraint force constant (kDR was in the range of 20–35 kcal/molÅ²), and
DISCUSSION

The high affinity, specific binding of \( \mu \)-CTX to certain isoforms of the voltage-gated sodium channel provides insight into the structure of the outer vestibule of the channel, which may pave the way to rational design of isotype-specific sodium channel blockers to treat neuronal, cardiac, or skeletal muscle hyperexcitability disorders. Initial studies used single mutation analyses of the toxin and/or the channel to determine the role of key residues involved in toxin binding.\(^9,10,43-46\) Subsequently, the outer vestibule was modeled based on either homology to \( \text{K} \text{v} \text{1.2} \) and/or its high affinity for toxins such as saxitoxin (STX) and tetrodotoxin (TTX).\(^28,47-49\)

Recent double mutant-cycle analysis studies have provided insight into specific toxin-channel interactions, revealed the clockwise orientation of the domains, and indicated a preliminary docking arrangement,\(^10,12,13,17,24,25,50-52\) but also they have revealed some apparent inconsistencies in interpretation. To help resolve these issues, we have exclusively used pair-wise alanine mutations, coupled with double mutant cycle analysis, to identify additional...
μ-CTX-Sodium Channel Interactions

High affinity interactions between the ring of negative charges at the mouth of the channel outer vestibule and several positively charged residues on the toxin.

Interactions of TTX and STX with the residues on the P-loops have enabled identification of plausible docking configurations and limits on the shape of the outer vestibule. μ-CTX is a substantially larger molecule than STX and TTX. A correct structural model of the outer vestibule must also accommodate the larger μ-CTX. To test this idea and to refine our knowledge of the outer vestibule, we mutated all negatively charged residues in the outer ring of charge in the outer vestibule, each of which is postulated to be in roughly the same plane parallel with the membrane surface. Since negatively charged residues in the channel are known to be involved in μ-CTX binding and positively charged residues in the toxin are also important for binding, we probed for toxin-channel pairs among these residues, hoping to identify the orientation of the toxin with respect to the channel outer ring residues. We found that R19 had the most substantial impact on toxin binding with R19A/WT channel affinities, similar to those reported by Li et al.12 In addition to R19, R13 has been shown to be very important for toxin binding,9 and for complete single-channel block.20,44 The effects of mutations K16A and K11A on toxin-binding with the wild-type channel were similar to previously reported data with these mutations.12,13

**Novel interactions.** Mutant cycle coupling results indicated novel, strong toxin-channel residue interactions between K9/E403, K11/D1241, K11/D1532, K16/D1241 and R19/D1532. In contrast, we found no substantial interaction between K8 and any of the tested channel residues. Since mutation of K8 to alanine had a modest effect on toxin binding to the native channel, it seems likely that K8 interacts with other residues in the pore not mutated in this study.

**Docking orientation of the toxin.** Using experimentally determined interaction constraints, a stable docking orientation between the toxin and the channel was obtaining with a modified Lipkind and Fozzard outer vestibule model. In developing their original model, Lipkind and Fozzard used the spatial orientation of the M1 and M2 helical segments of the KCAS channel to predict the backbone locations of the S5 and S6 helices of the sodium channel, and the P-loop region was modeled by an α-helix-turn-β-strand motif developed around the shapes of STX and TTX.28

In order to accommodate the larger μ-CTX, the outer vestibule needed to have a somewhat larger volume, which we achieved by twisting the P-loops slightly around an axis perpendicular to the plane of the membrane, and by flaring them outward at a wider angle to this axis. This kept the selectivity filter ring fixed, consistent with the biophysical requirements of this region, but provided a larger volume in the outer vestibule. Secondly, the larger μ-CTX molecule required a larger docking surface than the smaller toxins, achieved by inclusion, in the model, of additional residues from the C-terminal ends of the P-loops. We confirmed the ability of this newly refined model to successfully accommodate the smaller toxins, 11-SO₃-STX and TTX.24,25 (Fig. 4).

In the MD simulation of μ-CTX docking, we identified a general orientation of the docked toxin in the pore consistent with experimentally determined interactions. The docking orientation shows residue Q14 facing the domain II in proximity to D762 and E765. This is consistent with prior observations indicating that this is an important interaction that distinguishes the binding of GIIIA and GIIIB with the sodium channel.52

**Table 4** Summary of the predicted interactions between the toxin and the pore

| Toxin Residue | Interaction Residue | Interaction Strength |
|---------------|---------------------|---------------------|
| R1            | D1/D2              | Medium              |
| D2            | D2                 | Medium              |
| K8            | N1536              | Medium              |
| Q14           | D762               | Medium              |
| Q18           | I1242              | Medium              |
| A22           | N1536              | Medium              |

aD1: Domain 1, D2: Domain 2. bMedium interactions correspond to distances between 4 Å and 7 Å.

Figure 5. μ-CTX docking results with the refined model of the Na⁺ channel outer vestibule. Each view shows interactions between the toxin and two of the four domains viewed in a plane perpendicular to the membrane. The backbone positions for 6 independent results from molecular dynamics simulations are shown and were nearly identical. The channel selectivity filter amino acid side chains are shown in CPK format. Residue labels indicate selectivity filter residues for the two front domains in each of the four frames. In the lower frames, the short A1529 side chains are circled in red for clarity. Toxin residue side chains R13 (yellow) and R19 (white) are displayed in stick format. The axis of the pore is shown.
domain II was also observed by Dudley et al. This is reassuring, since Q14 was not included in the set of distance restraints used for the modeling. Another interaction that is consistent with our model is between R1 and domain II as reported by Dudley et al. In our docking model, the R1 residue faces up and away from the selectivity filter but is in proximity to domain II. The docking does not explain the observation by Li et al that A22 interacts with residues D762 and D765 of domain II. The spheres represent the Cα atom positions of K11 (blue) and K16 (red) that face the selectivity filter. The bottom panel shows the proximity of the K9 residue on the toxin to the channel domain I.

**Isoform selectivity.** In general, μ-CTX GIIIA has higher binding affinity for skeletal muscle Na+ channels than for neuronal Na+ channels, though there are species differences overlaid on this generalization. Recently, it has been reported that μ-CTX KIIIA, and some related toxins have higher affinity for certain neuronal isoforms than for rNaV1.4. The basis of isoform selectivity of the toxins remains to be fully explored. The most detailed study to date identifies residues, which account for differences in toxin sensitivity between rNaV1.4 and either rNaV1.1 or hNaV1.5. The residues identified as most important all lie in the DII S5-P linker, outside the model developed in our study (see Table 1, and Suppl. Table S.1). Important future tasks include: (1) confirming our docking simulations by testing predictions for a toxin, such as PIIIA, which is close to GIIIA in structure, and is only slightly more promiscuous in its interactions with neuronal channels; (2) extending the pore model to include more of the S5-P linker “turret” region; and (3) examining both experimentally, and in silico, the action of toxins, such as the KIIIA family, which show a strong preference for certain neuronal channel isoforms. In this context, the extended pore model proposed in this study, and the novel docking orientation of GIIIA, should provide an important framework on which to base a deeper analysis of specific interactions of μ-conotoxins with different channel isoforms.

**Limitations.** Even the alanine substitution mutations used for the channel and toxin residues could change the docking orientation of the toxin, leading to erroneous conclusions. We tried to avoid this by performing mutations of residues on all four domains of the channel to obtain multiple constraints on the channel-toxin interactions. Additionally, we used neutralizing, rather than charge reversal, mutations, so as to not induce new interactions, which might complicate energetic calculations, and confuse the interpretation. Furthermore, both the channel and toxin are flexible, so the docking arrangement may represent a unique configuration of both. Even if the channel outer vestibule remained fixed after toxin unbinding, the accuracy of our analysis would be unlikely to approach that of crystallographic or NMR techniques. Finally, some features of the docked structure might represent an induced fit specific to μ-CTX, since MD simulations were not performed with STX and TTX.

**Conclusions.** Using mutant cycle analysis, we identified 11 significant energetic interactions between the Na+ channel and μ-CTX. We obtained a consensus structure for the bound complex between an outer vestibule model and the toxin, using molecular dynamics together with restraints derived from these interactions. With refinements, an outer vestibule model based on STX and TTX binding was able to accommodate the larger μ-CTX. These experiments and the minimal refinements necessary to accommodate binding of all three toxins suggest that the broad structural conclusions implied about the docking orientation of the channel are likely to be correct. Moreover, this work could inform future experiments involving this important region of the channel.

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