A Specific Interaction between the Cardiac Sodium Channel and Site-3 Toxin Anthopleurin B*

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The polypeptide neurotoxin anthopleurin B (ApB) isolated from the venom of the sea anemone Anthopleura xanthogrammica is one of a family of toxins that bind to the extracellular face of voltage-dependent sodium channels and retard channel inactivation. Because most regions of the sodium channel known to contribute to inactivation are located intracellularly or within the membrane bilayer, identification of the toxin/channel binding site is of obvious interest. Recently, mutation of a glutamic acid residue on the extracellular face of the fourth domain of the rat neuronal sodium channel (rBr2a) was shown to disrupt toxin/channel binding (Rogers, J. C., Qu, Y. S., Tanada, T. N., Scheuer, T., and Catterall, W. A. (1996) J. Biol. Chem. 271, 15950–15962). A negative charge at this position is highly conserved between mammalian sodium channel isoforms. We have constructed mutations of the corresponding residue (Asp-1612) in the rat cardiac channel isoform (rH1) and shown that the lowered affinity occurs primarily through an increase in the toxin/channel dissociation rate constant. Further, we have used thermodynamic mutant cycle analysis to demonstrate a specific interaction between this anionic amino acid and Lys-37 of ApB (ΔAG = 1.5 kcal/mol), a residue that is conserved among many sea anemone toxins. Reversal of the charge at Asp-1612, as in the mutant D1612R, also affects channel inactivation independent of toxin (~14 mV shift in channel availability). Binding of the toxin to Asp-1612 may therefore contribute both to toxin/channel affinity and to transduction of the effects of the toxin on channel kinetics.

Members of a structurally diverse family of toxins isolated from several species of sea anemone and scorpion have proven useful tools in the functional characterization of mammalian voltage-gated sodium (Na) channels (1, 2). These toxins bind to the extracellular face of the channel at a locus termed site-3 (3) and selectively delay current inactivation (4–6), principally by inhibiting transitions of the channel from the open to the inactivated state (7–9). Several of the sea anemone toxins discriminate between different mammalian isoforms of the Na channel, generally binding to the cardiac isoform with highest affinity (10). Because these toxins are only active when applied extracellularly (5, 6) and because most regions of the channel known to affect inactivation are located intracellularly or within the plasma membrane (11–14), it is of particular interest to characterize the binding interface between toxin and channel.

Several regions of the channel have been implicated in toxin binding. Studies involving covalent attachment of scorpion toxin derivatives (15) and protection against such labeling by site-specific antibodies (16) have implicated regions in the first channel domain in toxin binding, while other studies involving antibodies (16) and chimeric channels (17) have pointed to regions in domain 4. Most recently, mutation of a negative amino acid at the outer end of the third helix in the fourth domain of rat brain sodium channels (Glu-1613) was shown to inhibit the binding of the sea anemone toxin ATX-II (from Anemonia sulcata) by 80-fold (18).

The sea anemone toxin anthopleurin B (ApB) was originally isolated from the Pacific coast sea anemone Anthopleura xanthogrammica (19, 20) and subsequently cloned and expressed heterologously (21). It has seven positive amino acid residues (22), and the ability of a subset of those to affect the binding affinity of the toxin (23–25) suggested the possibility of an electrostatic interaction between toxin and channel. We therefore attempted to determine which of these cationic toxin residues mediate the decrease in affinity observed with the channel mutation at the end of domain IV, helix 3 (18). Specifically, we constructed a series of mutations of residues on ApB and used the formalism of thermodynamic mutant cycle analysis (26, 27) to assess the degree to which these toxin mutations interacted with the mutation on the channel. Taking advantage of the higher affinity of ApB for cardiac sodium channels than for neuronal channels, we performed our experiments in cells transfected with the rat cardiac channel isoform rH1.

MATERIALS AND METHODS

Molecular Biology—The rat heart sodium channel cDNA (rH1) (28, 29) subcloned into the pSP64t vector was generously provided by R. Kallen (University of Pennsylvania). The aspartic acid at amino acid 1612 (corresponding to position 1613 in rBr2a) was mutated to asparagine (D1612N) or arginine (D1612R) using polymerase chain reaction (PCR) in a 4-primer strategy (30). Sequences in this region were as follows,

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The abbreviations used are: ApB, anthopleurin B; ApA, anthopleurin A; rBr2a, rat neuronal sodium channel; rH1, rat cardiac channel isoform; PCR, polymerase chain reaction; Ω, Ohm; ATX, sea anemone toxin.
where the capitalized residues denote the position of Glu-1613 (rBrIIa) and Asp-1612 (rH1) and asterisks denote the third and fourth helices of channel repeat IV (see Ref. 31). PCR primers included the desired mutations and an additional mutation to introduce a silent KpnI restriction site, which did not alter the amino acid sequence but facilitated conjugation of mutated PCR products. The final PCR product spanned unique BstEII (base pair 4632) and BspDI (base pair 5069) endonuclease restriction sites in rH1 and were subsequently used in the reconstruction of the full-length rH1 cDNA in pSP64t. The entire BstEII to BspDI PCR-generated region found in the full-length construct was completely sequenced by dideoxy methods (32) to confirm the presence of the desired mutations and the absence of spurious PCR-generated errors. Expression was first tested in Xenopus oocytes, and the entire coding region of rH1 was subsequently shuttled into the HindIII site in the polylinker of pREP9 (Invitrogen, Carlsbad, CA) using HindIII (in pSP64t) to HindIII (base pair 6371 in rH1) endonuclease cleavage sites.

Cell Culture—Channels from the rH1-pREP9 constructs were expressed in tsA201 cells (33) and maintained as described previously (17). Two to three days prior to recording, cells were seeded onto 60-mm culture dishes and transfected with 10 μg of plasmid pREP9-rH1 using a calcium phosphate coprecipitate. Cells were cotransfected with pHook1, and transfectants were visualized with the Capture-Tec bead system (Invitrogen) using supplied protocols. Approximately 30–50% of cells bound beads; virtually all cells binding two or more beads expressed Na+ current (I\(_{\text{Na}}\)).

Electrophysiological Solutions—Pipette solution contained (in mM) 140 Cs+ (0.5 CsOH), 10 Na+, 100 F-, 50 Cl-, 10 HEPES, with pH adjusted to 7.3 with CsOH. Bath solution contained (in mM) 140 Na+, 2 Ca2+, 144 Cl-, 10 HEPES, with pH adjusted to 7.3 with NaOH. Because toxin in solution tends to adhere to glassware and tubing, bath solutions containing toxin were supplemented with 5 mg/ml bovine serum albumin (Sigma) (17). Toxins ApB, ApA, and ApB mutants were produced in Escherichia coli strain BL21(DE3) transformed with the appropriate version of the plasmid pKB-13 as described previously (21, 25, 34) and characterized by amino acid analysis and matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Circular dichroism spectra of the toxin derivatives used in the present study to confirm proper folding of toxins were determined in a Jasco 710 spectropolarimeter (Easton, MD) equilibrated with camphor d\(_{10}\) sulfonic acid, and the spectra deconvoluted using the standard error of the estimate program supplied by the manufacturer.

Recording Techniques—Recording protocols were generated using Clampex 6.0.2 (Axon Instruments, Foster City, CA) running on a Pentium-based microcomputer and were imposed through a 12-bit DAC controlling an Axopatch 200B amplifier (Axon). The recording chamber was maintained at 16 °C with a Sorensen TS-4 controller (Clifton, NJ). Currents were 4-pole Bessel filtered at 5 kHz and digitized at 12-bit resolution at 100 kHz.

Recordings were made in the whole-cell patch clamp configuration with typical pipette resistances of 0.8–1.5 MΩ. Because the binding of these toxins is state-dependent, with significantly lower affinity for inactive states than for resting states (35–37), we held the cells at a sufficiently negative holding potential (−150 mV) to maintain complete channel availability during the duration of the experiment. Our experiments therefore probed the interactions of these toxins primarily with the closed conformation of the channel.

Data Analysis—Analysis and fitting were performed with locally written programs (GRB, DAH) running under Matlab 5.0 (The MathWorks, Natick MA). Traces were leak corrected based on the resistance calculated from the current at holding potential. The capacity transient was removed through subtraction of summed subthreshold pulses, and traces were digitally refiltered at 5 kHz using a zero-phase fitting algorithm.

When the rates of current modification or unmodification by toxin were too fast to resolve with pulse protocols, toxin/channel affinities were calculated using the currents produced by modifying channels with subsaturating concentrations of toxin. We assumed that partially modified \(I_{\text{Na}}\) consisted of the sum of a quickly decaying component arising from unmodified channels and a slowly decaying phase arising from toxin-modified channels. The current at late times after the start of depolarization was, therefore, assumed to be proportional to the total number of channels modified. Accordingly, we averaged the current over a 7.6–8.0 ms window from depolarization onset (\(I_{\text{Na, peak}}\)). Because peak currents increase during toxin modification, we normalized this late current by the initial value of peak current before toxin modification (\(I_{\text{Na, peak, low}}\)). This ratio was then divided by a similarly calculated ratio for a saturating dose of a higher affinity toxin from another cell (\(I_{\text{Na, peak, high}}\)), yielding the fraction of current modified (\(f_{\text{mod}}\)). That is as follows.

\[
\frac{I_{\text{Na}}}{I_{\text{Na, peak}} - \frac{I_{\text{Na, peak, low}}}{I_{\text{Na, peak, high}}}} = \frac{f_{\text{mod}}}{f_{\text{mod}}}.
\]

(\(\text{Eq. 1}\) )

Affinity was then calculated from the Langmuir adsorption isotherm.

\[
K_D = \frac{[T]}{[\text{mut}]} \left( \frac{1}{f_{\text{mod}}} - 1 \right)
\]

(\(\text{Eq. 2}\) )

Interactions between toxin and channel residues were calculated using the formalism of thermodynamic mutant cycle analysis (26, 27). In brief, wild-type and mutant forms of toxin and channel were used to calculate a coupling coefficient \(\Omega\).

\[
\Delta G = R T \ln \Omega
\]

(\(\text{Eq. 4}\) )

Because affinities were related to coupling energies logarithmically, and because affinities and rate constants both varied over a wide range of values, we chose to calculate errors and significance statistics based on the natural logarithms of the data.

RESULTS

Kinetics of Mutant Channels—We introduced mutations into rH1 at the position identified by Rogers and colleagues (18) in rat brain channels (rBrIIa). Channels rH1 D1612N and D1612R displayed currents similar to those of the wild-type channel (Fig. 1A). The mutations produced little change in voltage-dependence of conductance (Fig. 1B, left); the charge neutralization mutant D1612N was very slightly but significantly shifted in a depolarizing direction (\(p = 0.02\), Student's \(t\)-test), while the halfpoint of the charge reversal mutation was unchanged. Halfpoints of the conductance curve were as follows: wild-type rH1, −41 ± 1 (n = 14); D1612N, −38 ± 2 (n = 13); D1612R, −43 ± 2 (n = 9). Cells with conductance slope factors <6.0 mV were deemed to be inadequately voltage controlled and were excluded from any further study (38). On the other hand, the charge reversal mutation D1612R displayed a steady-state availability curve 14 mV negative to that of wild-type channels (\(p < 0.0001\)), whereas that of D1612N was similar to the wild-type channel (Fig. 1B, right). Halfpoints of availability were: wild-type rH1, −100 ± 2 (n = 22); D1612N, −101 ± 2 (n = 14); D1612R, −114 ± 2 (n = 9). Paralleling the shift in availability, the kinetics of recovery from inactivation of D1612R were shifted in a hyperpolarizing direction by −16 ± 3 mA across a range of recovery potentials with respect to rH1 (data not shown).

Neutralization (D1612N) or reversal (D1612R) of Asp-1612 decreased the affinity for ApB, consistent with results in the neuronal channel isoform (18). For example, 100 nM ApB modified wild-type rH1 channels to completion but caused only fractional modification of current from the charge reversal mutation D1612R (Fig. 1C).

Calculation of Toxin/Channel Interaction—The energies of interaction of the positive residues of ApB with channel residue Asp-1612 were calculated through thermodynamic mutant cycle analysis (26, 27). In short, the effects of point mutations of ApB on toxin/channel affinity were tested both in the context of wild-type rH1 channels and in the context of channels mutated at Asp-1612. Differences in the effects of the toxin mutation were quantified as coupling coefficients \(\Omega\), which are related logarithmically to the energy of interaction between the particular toxin and channel residues (see “Materials and Methods”).
Because association rates of these toxins are 3 orders of magnitude below those predicted by diffusion, we were usually able to calculate affinities using the rate constants of toxin/channel association and dissociation. Once stable whole-cell access was achieved, cells were exposed to and removed from a toxin-containing chamber. During wash-in and wash-out, modification was monitored by 11-ms depolarizations to −10 mV at ≤1 Hz, of which a current window of 7.6–8.0 ms after the onset of each pulse was averaged and plotted (Fig. 2A). For most combinations of toxin and channel, these relations were well resolved by single exponentials, yielding first order rate constants of association, \( k_{on} \), and dissociation, \( k_{off} \), respectively (Fig. 2A). From these values, the true second-order association rates, \( k_{nt} \), and the affinities, \( K_{y5} \), were calculated as follows,

\[
k_{nt} = k_{off} + k_{on} T
\]  

(Eq. 5)

where \( T \) denotes toxin concentration, and

\[
K_{y5} = \frac{k_{off}}{k_{on}}
\]  

(Eq. 6)

In most cases, toxin-channel affinities were sufficiently high that modification of \( I_{Na} \) was produced at measurable values of \( k_{mod} \). However, in the cases where toxin/channel affinity was quite low (specifically, the binding of all toxins to rH1 D1612R and the binding of ApA to D1612N), a concentration of toxin sufficient to cause appreciable modification produced values of \( k_{mod} \) too high to accurately resolve the modification process. In those cases, affinity was calculated from the currents of cells modified to steady-state with subsaturating toxin concentrations, as described under “Materials and Methods.” We verified this technique by calculating the binding affinity of toxin ApB H39A to rH1 D1612N channels both kinetically and by steady-state measurements. The kinetically calculated \( K_{y5} \), 26 nm (25–27 nm, \( n = 4 \)), agreed well with the equilibrium measurements of 20 nm (19–31 nm, \( n = 5 \)).

**Toxin Interactions with D1612N**—Including its α-amino group, ApB possesses six fully positive charges and two histidines (22), any of which might be a candidate for binding to the negative channel residue Asp-1612. Residues Arg-12, His-34, His-39, and Lys-48 have produced only small effects on toxin affinity when neutralized (25, 39) and so were relatively poor candidates for interacting with Asp-1612; similarly, short N-terminal extensions of the toxin have no measurable effect on its affinity (21, 40). We tested toxin ApB H39A as a representative of this group. Residues Arg-12 and Lys-49 are known to have a significant and cooperative effect on toxin affinity (23) but are not conserved among all anemone toxins; in particular, they are neutral in ATX-II, the toxin tested by Rogers and colleagues (18). We tested these residues by using toxin ApA, which has neutral residues at both positions (in addition to several more conservative differences which have been shown to have only minor effects on toxin affinity) (22, 40). The remaining residue, Lys-37, significantly decreased the affinity of ApB when neutralized (25) and is also conserved among a number of anemone toxins. It was, therefore, a strong candidate for the interaction and was tested with toxin ApB K37A. As a positive control, we also tested a toxin mutated at a hydrophobic position, toxin ApB W45F. This toxin has lower affinity for cardiac channels than does wild-type ApB (34) but would not be expected to interact with an anionic channel residue.

Values of \( k_{off} \) and \( k_{on} \) for wild-type channels and for rH1 D1612N are shown in Table I. The decrease in affinity with channel mutation D1612N arose primarily from an increase in \( k_{off} \), as did the difference in affinity between ApB and ApA, which was consistent with previous results (23). The effects of toxin mutation K37A, however, arose from both a decrease in \( k_{on} \) and an increase in \( k_{off} \). These kinetic values were used to calculate toxin/channel affinities (Fig. 2B), which were then translated into coupling coefficients (\( \Omega \)) and energies of interaction (Fig. 2C). Of the tested toxin mutations, only K37A interacted with channel mutation D1612N with an energy significantly different from zero (\( \Delta\Delta\Omega = 1.5 \text{ kcal/mol} \)). We conclude that toxin residue Lys-37 interacts positively with channel residue Asp-1612.

**Toxin Interactions with D1612R**—Having demonstrated an attraction between cationic toxin residue Lys-37 and anionic channel residue Asp-1612, we attempted to preserve this interaction by transposing the charges of the two residues. To this end, we constructed the charge-reversed toxin ApB K37D and the charge-reversed channel rH1 D1612R. Unfortunately, circular dichroism revealed that ApB K37D possessed a significantly less ordered secondary structure than that of wild-type ApB or the neutralization mutant ApB K37A (25) (Fig. 3).

Accordingly, ApB K37D bound to both wild-type rH1 channels and to rH1 D1612R with strikingly low affinity (see Table...
Although the calculated interaction was large ($\Delta G = 2.2$ kcal/mol), we interpret this “interaction” to arise from aberrant folding of the toxin rather than from the targeted disruption of an ionic interaction between Lys-37 on the toxin and Asp-1612 on the channel. Somewhat surprisingly, the energy of interaction of the neutralized toxin, ApB K37A, with the charge-reversed channel, rH1 D1612R, was only 0.9 kcal/mol, a lower value than that observed when both the channel and toxin residues were neutralized.

**DISCUSSION**

We have isolated a specific interaction involved in the binding of the site-3 toxin anthopleurin B to the rat cardiac sodium channel. Channel residue Asp-1612 and toxin residue Lys-37 display an energy of interaction of $\Delta G = 1.5$ kcal/mol. This
coupling energy is less than that expected for a hydrogen bond to a charged residue (41) but is well within the range predicted for electrostatic interactions (41). For example, Schreiber and Fersht (42) reported values ranging from a noise level of 0.4 kcal/mol up to values typical of H-bonds. Indeed, while neutralization of Lys-37 in the context of a wild-type channel decreased affinity by 15-fold, the mutant channel D1612N was almost insensitive to a charge at toxin position 37. Thus all of the effects of neutralizing toxin residue Lys-37 on the affinity of the toxin are accountable through the actions of channel residue Asp-1612. A comparison of the sequences of 15 homologous toxins isolated from sea anemone revealed all toxins but one, ATX-I from *A. sulcata* (43), to have a positive residue at or adjacent to position 37. Interestingly, toxin ATX-I is ineffective when applied to vertebrate sodium channels at 100 μM (5). Further, an anionic residue at channel position 1612 is highly conserved between mammalian sodium channel isoforms (31). These data strongly support the hypothesis that the interaction between Lys-37 and Asp-1612 comprises a conserved component of binding site-3 in mammalian channels.

Site-3 toxins act to delay sodium channel inactivation (4–6). Because the extracellular face of the channel is not typically thought to be associated with the inactivation process, the manner by which these toxins produce their kinetic effect remains unclear. In light of this ambiguity, it is interesting that the toxin are accountable through the actions of channel residue Asp-1612 produced a shift in the voltage dependence of steady-state availability. Since perturbation of this residue affects inactivation gating, it is possible that Asp-1612 participates in the control of both the affinity and the kinetic effects of toxin binding.

At present, little is known of the structure of the extracellular face of the sodium channel outside of the immediate region of the pore vestibule. The interaction between Asp-1612 and toxin residue Lys-37 provides a defined interaction between the channel and a molecule with a known three-dimensional structure (44). The determination of more such interactions should therefore increase our insight into the structure and function of the exterior of the channel.

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