Dependence of $\mu$-Conotoxin Block of Sodium Channels on Ionic Strength but Not on the Permeating [Na$^+$]

IMPLICATIONS FOR THE DISTINCTIVE MECHANISTIC INTERACTIONS BETWEEN Na$^+$ AND K$^+$ CHANNEL PORE-BLOCKING TOXINS AND THEIR MOLECULAR TARGETS

Ronald A. Liî‡, Kwokying Huiî, Robert J. French‡, Kazuki Sato**, Charles A. Henriksonî, Gordon F. Tomaselliî, and Eduardo Marbán‡‡‡

From the Institute of Molecular Cardiobiology, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205, the Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta T2N 4N1, Canada, and the Fukuoka Women's University, Fukuoka 813-8529, Japan

$\mu$-Conotoxins ($\mu$-CTXs) are Na$^+$ channel-blocking, 22-amino acid peptides produced by the sea snail Conus geographus. Although K$^+$ channel pore-blocking toxins show specific interactions with permeant ions and strong dependence on the ionic strength ($\mu$), no such dependence has been reported for $\mu$-CTX and Na$^+$ channels. Such differences would offer insight into the binding and blocking mechanism of $\mu$-CTX as well as functional and structural properties of the Na$^+$ channel pore. Here we studied the effects of $\mu$ and permeant ion concentration ([Na$^+$]) on $\mu$-CTX block of rat skeletal muscle ($\mu_1$, Na$_{1.4}$) Na$^+$ channels. $\mu$-CTX sensitivity of wild-type and E758Q channels increased significantly (by ~20-fold) when $\mu$ was lowered by substituting external Na$^+$ with equimolar sucrose (from 140 to 35 mM Na$^+$); however, toxin block was unaltered ($\mu > 0.05$) when Na$^+$ was maintained by replacement of [Na$^+$] with N-methyl-D-glucamine (NMG$^+$), suggesting that the enhanced sensitivity at low $\mu$ was not due to reduction in [Na$^+$]. Single-channel recordings identified the association rate constant, $k_{on}$, as the primary determinant of the changes in affinity ($k_{on}$ increased 40- and 333-fold for $\mu$-CTX D2N/R13Q and D12N/R13Q, respectively, when symmetric 200 mM Na$^+$ was reduced to 50 mM). In contrast, dissociation rates changed <2-fold for the same derivatives under the same conditions. Experiments with additional $\mu$-CTX derivatives identified toxin residues Arg-1, Arg-13, and Lys-16 as important contributors to the sensitivity to external $\mu$. Taken together, our findings indicate that $\mu$-CTX block of Na$^+$ channels depends critically on $\mu$ but not specifically on [Na$^+$], contrasting with the known behavior of pore-blocking K$^+$ channel toxins. These findings suggest that different degrees of ion interaction, underlying the fundamental conduction mechanisms of Na$^+$ and K$^+$ channels, are mirrored in ion interactions with pore-blocking toxins.

$\mu$-Conotoxins ($\mu$-CTXs)$^1$ are 22-amino acid peptides produced by the sea snail Conus geographus with well defined three-dimensional structures (1–4); these toxins block the Na$^+$ channel pore with high affinity and specificity (5–7). The selective binding of $\mu$-CTX to the channel pore depends not only on the intimate physical fit of the toxin molecule to its receptor surface but also on electrostatic interactions among numerous charged toxin and channel residues (2, 3, 8–17). Although the steric components of interactions are closely related to the pore and toxin geometries and thus are relatively short range, electrostatic interactions are longer range and should depend not only on the immediate ionic environment but also the proximity of complementary, interacting charges. Indeed, K$^+$ channel pore-blocking toxins such as agitoxin and charybdotoxin (ChTX) are known to block their target pores with strong dependence on both the ionic strength and the permeant ion concentration (18–23). However, the importance of these factors for Na$^+$ channel block by $\mu$-CTX has not been systematically explored. Does $\mu$-CTX block the Na$^+$ channel pore with comparable ion dependence as do its K$^+$ channel pore-blocking counterparts? What are the molecular bases underlying such similarities (or differences) in the biological actions of these Na$^+$ and K$^+$ channel pore-blocking toxins? Answers to these questions will not only provide insights into the mechanisms underlying the activities of these specific toxin blockers but also, by inference, into the functional and structural properties of ion channel pores.

We studied the effects of Na$^+$ concentration ([Na$^+$]) and ion $\mu$-CTX block of toxin-sensitive rat skeletal muscle ($\mu_1$ or Na$_{1.4}$) Na$^+$ channels. Unlike the action of K$^+$ channel toxins, we found that $\mu$-CTX block of Na$^+$ channels depends on ionic strength but not specifically on the Na$^+$ concentration. Using synthetic $\mu$-CTX derivatives, we further

---

$^*$ This work was supported in part by National Institutes of Health Grants R01 HL-52768 (to E. M. and R. A. L.) and R01 HL-90411 (to G. F. T.) and by Canadian Institutes of Health Research (CIHR) Grant MOP-10053, the Heart and Stroke Foundation of Alberta, NWT & Nunavut, and a fellowship from the Max Planck Institute of Experimental Medicine, Göttingen, Germany (to R. J. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of a Young Investigator Award from the North American Society of Pacing and Electrophysiology and a Research Career Development Award from the Cardiac Arrhythmias Research & Education Foundation. To whom correspondence may be addressed: Institute of Molecular Cardiobiology, The Johns Hopkins University School of Medicine, 720 Rutland Ave., Ross 844, Baltimore, MD 21205, Tel.: 410-614-0035; Fax: 410-955-7963; E-mail: ronald@jhu.edu.

$^1$ The abbreviations used are: $\mu$-CTX, $\mu$-conotoxin; ChTX, charybdotoxin; GFP, green fluorescent protein; NMG, N-methyl-D-glucamine; MOPS, 4-morpholinepropanesulfonic acid; WT, wild-type; D, domain; $\mu$, ionic strength.
identified toxin residues Arg-1, Arg-13 and Lys-18 as particularly important effectors of the dependence on ionic strength. These results offer novel insights into the mechanism by which µ-CTX interacts with the Na\(^+\)/H\(^+\) channel pore. A preliminary report of this work has appeared previously (24).

**MATERIALS AND METHODS**

**Site-directed Mutagenesis and Heterologous Expression**—The gene encoding the Nav1.4 sodium channel\(^+\) subunit (25) was cloned into the pGFP-IRES vector with an internal ribosomal entry site interposed between it and the GFP reporter gene, enabling translation of two independent proteins (GFP and Nav1.4) from a single plasmid. Mutagenesis was performed in pGFP-IRES using PCR with overlapping mutagenic primers. All mutations were made in duplicate and confirmed by sequencing. Na\(^+\)/H\(^+\) channel constructs were transfected into tsA-201 cells (26), which constitutively express t-antigen to boost the level of channel expression, using LipofectAMINE Plus (Invitrogen, Gaithersburg, MD) according to the manufacturer’s protocol. Briefly, plasmid DNA encoding the WT or mutant \(\alpha\) subunit (1 \(\mu\)g/60-mm dish) was added to the cells with LipofectAMINE, followed by incubation at 37°C in a humidified atmosphere of 95% O\(_2\)-5% CO\(_2\) for 48-72 h before electrical recordings.

**Synthesis of Point-mutated µ-CTX GIIIA—µ-CTX derivatives** were created as previously described (2, 8, 10). Briefly, peptides were synthesized using N-(9-fluorenyl)methoxycarbonyl chemistry and were high-performance liquid chromatography-purified. Peptide composition was verified by quantitative amino acid analysis and/or mass spectroscopy. One-dimensional proton NMR spectra of a number of synthesized toxins were compared with those of the native toxin to test for proper folding.

**Electrophysiology**—Macroscopic currents were recorded using the whole-cell patch clamp technique (27) at room temperature. Transfected cells were identified by their green epifluorescence. Pipette electrodes had final tip resistances of 1-3 MΩ. The bath solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 10 HEPES, 10 glucose, with pH adjusted to 7.4 with NaOH. Designated concentrations of toxin were added to the bath as indicated. External NaCl was substituted by either sucrose or N-methyl-D-glucamine (NMG) as noted. Osmolalities (in mosmol-kg\(^{-1}\)H\(_2\)O) of the solutions were: 140 Na, 313; 70 Na/105 sucrose, 269; 35 Na/105 sucrose, 259; 140 NMG, 295; the internal (pipette) solution contained (in mM): 35 NaCl, 105 CsF, 1 MgCl\(_2\), 10 HEPES, 1 EGTA, with pH adjusted to 7.2 with CsOH (286 mosmol-kg\(^{-1}\)H\(_2\)O).

**Single-channel recordings** were performed with bilayer-incorporated Na\(^+\)/H\(^+\) channels incubated with 50 \(\mu\)M batrachotoxin as previously described (8). Briefly, bilayers were formed from a 4:1 mixture of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (Avanti Polar-Lipids) solution dissolved in decane (Fisher Scientific). The decane-lipid mixture was painted on a 100- to 200-\(\mu\)m hole in a plastic partition between the two compartments of the experimental chamber. Sodium channels were incorporated into the bilayers from surface membrane vesicles from rat skeletal muscle. Sodium channel-containing plasmaemmal vesicles were isolated as described before (Becker et al. (8)), sonicated, and incubated with 50 \(\mu\)M batrachotoxin in a 0.3-\(\mu\)s sucrose, 20 mM HEPES solution
half-blocking concentrations (IC$_{50}$) for concentration was increased by addition of an identically buffered 2.3M IC$_{50}$ is the half-blocking concentration, reported values of the bath solution. Unlike Fig. 1, external /H9262
experiments to preserve both osmotic and /H9262
elicited by a brief test pulse (\( \pm 10 \) mV) before and after application of the /H9262
were assayed for their dependence on ionic strength. Comments on the choice of these derivatives are given under “Results.”

Data Analysis and Statistics—Ionic strength (\( \mu \)) was calculated using the following equation,

\[
\mu = \frac{1}{2} \Sigma x_i^2
\]

where \( x_i \) is the concentration of ion species i and \( z_i \) is its valence.

Toxin was superfused continuously during the whole-cell experiments. Equilibrium half-blocking concentrations (IC$_{50}$) for toxins were determined from the following binding isotherm,

\[
\frac{I_{I_0}}{I} = \frac{1 - R}{1 + ([\text{toxin}] / \text{IC}_{50})} + R
\]

where IC$_{50}$ is the half-blocking concentration, \( R \) is the residual current or sub-conductance when channels are fully blocked, \( I_0 \) and \( I \) are the peak currents measured from a step depolarization to \(-10\) mV from a holding potential of \(-100\) mV before and after application of the toxin, respectively.

For single-channel experiments, the unitary current amplitude was determined visually from the current records and was similar to Gaussian fits of the all-points amplitude histograms. Single-channel events were detected by the half-amplitude threshold technique using a minimum cutoff of 400 ms for blocked events. The kinetic parameters, \( k_{on} \) and \( k_{off} \), were determined from mean unblocked and blocked times, \( (t_{on}) \) and \( (t_{off}) \), respectively, as follows: \( k_{on} = 1 / (t_{on}) \) (toxin), and \( k_{off} = 1 / t_{off} \). The reported values of \( K_a \), \( k_{on} \), and \( k_{off} \) were determined from estimates at multiple voltages interpolated to 0 mV. All data reported are mean \( \pm \) S.E. Statistical significance was determined using a paired Student’s t test at the 5% level.

**RESULTS**

Effects of External Na$^+$ Concentrations and Ionic Strength on $\mu$-CTX Block of Na$^+$ Channels—We first studied the effects of ionic strength (\( \mu \)) on $\mu$-CTX block of WT Na$_{\alpha.1.4}$ Na$^+$ channels. Given the slow kinetics of toxin binding and dissociation (macrosopic time constants generally of many seconds), the current elicited by a brief test pulse (\( \pm 10\) ms) from a holding potential of \(-100\) mV reflects the fraction of channels that are not blocked by the toxin at the holding potential. Toxin-bound channels show subtle shifts of activation gating and perhaps of inactivation, which have previously been analyzed in detail, but these do not influence the assay of toxin binding by the protocol used here (28, 29).

External Na$^+$ was substituted by sucrose to reduce changes in the osmolarity while varying \( \mu \). Fig. 1 shows the effects of 140 mm Na$^+$, 70 mm Na$^+$/70 mm sucrose, and 35 mm Na$^+$/105 mm sucrose on the half-blocking concentration (IC$_{50}$) of $\mu$-CTX on WT Na$_{\alpha.1.4}$ channels. The toxin sensitivity increased \(-18\)-fold as \( \mu \) was lowered over the range examined. The channel mutation E758Q is known to significantly reduce $\mu$-CTX sensitivity (11); despite the lower toxin sensitivity of E758Q channels, $\mu$-CTX block also increased substantially (\( p < 0.05 \)) when \( \mu \) was lowered (Fig. 1). Indeed, the affinity of E758Q channels for $\mu$-CTX increased by \(-28\)-fold when the external solution was changed from 140 mm Na$^+$ to 35 mm Na$^+$/105 mm sucrose.

We next investigated whether the changes in toxin affinity were directly due to the lowering of \( \mu \), changes in the permeant concentration, or both. To distinguish among these possibilities, we studied $\mu$-CTX block of E758Q channels by substituting external Na$^+$ with NMG$^+$ so as to maintain both the osmotic and ionic strengths while varying [Na$^+$]. Fig. 2 summarizes these results. In contrast to the sucrose experiments, $\mu$-CTX sensitivity was not altered when external [Na$^+$] was lowered from 140, to 70, and to 35 mm (\( p > 0.05 \)). These results indicate that the changes observed in Fig. 1 were entirely due to the effects of ionic strength. Therefore, unlike K$^+$ channel toxins, $\mu$-CTX block depends on ionic strength but not, in an obligatory manner, on permeant ion concentration.

**Effects of Ionic Strength on $\mu$-CTX Block of Single Na$^+$ Channels**—To measure directly the kinetic changes, we performed single-channel recordings in neutral lipid bilayers to study the effects of ionic strength on the blockade by the $\mu$-CTX-based derivatives D2N/R13Q, D12N/R13Q, and R13W. This approach is advantageous for two reasons. First, it is much easier to symmetrically change solutions over a wide range of ionic strength than it is using single-channel patch recording. Second, the long steady-state recordings from batrachotoxin-mod-
ified channels enable toxin association and dissociation rate constants to be determined directly from the unblocked and blocked times in a single record, for toxin kinetics varying over a wide range. This avoids the need for continuous perfusion and step changes in concentration that are required to estimate the rate constants from macroscopic recordings and allows more detailed and precise kinetic measurements.

The three toxin derivatives studied in these experiments were chosen in part because they have shorter blocked times, than the native toxin, thus facilitating kinetic analysis. Wild-type µ-CTX shows mean blocked times on the order of minutes, whereas R13W (Fig. 3B) also shows rapidly rising and decaying kinetics. All of these derivatives cause incomplete block of single-channel currents and thus enabled us to monitor the efficacy of single-channel block by the bound toxin as a function of ionic strength, while simultaneously recording binding and unbinding kinetics.

Fig. 3A shows representative traces of single Na⁺ channel activities recorded in the absence and presence of D2N/R13Q at a test voltage of +60 mV. It is evident from these records that at low ionic strength the mean blocked and unblocked times increased and decreased, respectively. As shown in Fig. 3C, the association rate (k_{on}) decreased dramatically (~40-fold for D2N/R13Q), accompanied by a modest, ~2-fold increase in the dissociation rate constant (k_{off}), as [Na⁺] was increased from 50 to 200 mM. These changes in blocking kinetics conferred significant increases in the kinetically derived K_D with increasing ionic strength (Fig. 3D), consistent with the changes in steady-state IC_{50} of µ-CTX block of Na,1.4 channels observed at the whole-cell level when µ was changed (cf. Fig. 1).

In addition to the kinetics, the blocked and unblocked unitary conductances were also affected. Notably, the fractional residual conductance (R) increased with the increase in µ (Fig. 3B). Similar trends were observed for single-channel block and binding kinetics shown by the two other derivatives, D12N/R13Q and R13W (Fig. 3B-D). The different ranges over which the fractional residual current changed depend on both size and charge of the particular amino acids substituted into the toxin (see “Discussion” and Ref. 30).

The key points to note here are the consistent increases, with decreasing ionic strength, in both the toxin binding affinity, and completeness of single channel block. We suggest that both changes are directly related to the decreased screening of various charges on the toxin and/or the channel.

Arg-13 and Glu-758 Are Not Essential for Ionic Strength Dependence of Binding—Arg-13 is critical for high affinity, complete blockade of Na⁺ channels by µ-CTX (2, 3, 8, 10). We
therefore created the $\mu$-CTX derivative R13A by neutralizing the native basic toxin residue at position 13 by alanine substitution and studied the effects of ionic strength on its interactions with the pore. Under normal conditions (140 mM Na⁺), R13A, applied to WT Na⁺,1.4 channels, displayed significantly reduced affinity and showed incomplete block of the whole-cell current even at high toxin concentrations (Fig. 4, A and B) consistent with previous single-channel observations (8, 30). However, the leftward shift of its dose-response curve in Fig. 4B upon lowering of $\mu$ indicates that the affinity of R13A also increased significantly ($p < 0.05$) in a manner qualitatively similar to WT $\mu$-CTX block (cf. Fig. 1, and see Fig. 5 for quantification of this dependence on ionic strength). Consistent with our observations with D2N/R13Q, D12N/R13Q, and R13W block at the single-channel level, the asymptotic residual whole-cell current at high [R13A] block was also reduced at low ionic strength. Because Arg-13 is known to interact with the domain II (DII) pore residue Glu-758 (10), we studied also the ionic strength dependence of R13A block of E758Q channels (Fig. 4, C and D). Indeed, the magnitude of changes in affinity and residual currents were similar to those seen with WT Na⁺,1.4 channels.

**Interactions of Individual Toxin Charges Are Not Equally Dependent on Ionic Strength**—To quantify the dependence of $\mu$-CTX block on ionic strength, we examined the half-blocking concentrations ($IC_{50}$) of WT $\mu$-CTX and six alanine substitution derivatives on E758Q Na⁺ channels as a function of ionic strength (Fig. 5, A and B). Alanine was used to replace, separately, the charged residues Arg-1, Lys-9, Lys-11, Asp-12, Arg-13, and Lys-18. The data were fit by linear regressions, whose slopes (i.e. $\delta IC_{50}/\delta \mu$) reflect the dependence of toxin block on ionic strength (Fig. 5C). Interestingly, R13A displayed a somewhat weaker dependence than WT $\mu$-CTX, although its affinity did increase as $\mu$ was lowered. We next studied the ionic strength dependence of toxin block when the charged toxin residues Arg-1, Lys-9, Lys-11, Asp-12, and Lys-18 were individually neutralized by alanine substitution (Fig. 5B). The values of $\delta IC_{50}/\delta \mu$ for WT $\mu$-CTX and toxin derivatives are summarized in Fig. 5C. Similar to R13A, R1A also showed a weaker $\mu$ dependence than WT toxin. K16A exhibited the largest reduction in its dependence on $\mu$. Other derivatives studied (i.e. K9A, K11A, and D12A) were not different from WT $\mu$-CTX despite their vastly different affinities for the same channel. These observations suggest that Arg-1, Arg-13, and Lys-18 underlie major toxin-channel interactions that are sensitive to ionic strength.

**DISCUSSION**

**Peptide Blockers of K⁺ and Na⁺ Channels**—Hydrophilic pore-blocking toxins have been useful molecular probes for...
studying the structural and functional properties of ion channels. For instance, the study of K⁺ channel toxins such as agitoxin and ChTX, and Na⁺ channel toxins such as tetrodotoxin, saxitoxin, and \( \mu \)-CTX have provided significant insights into the three-dimensional configurations of their target channel pores (e.g. see Ref. 31). We demonstrate here that \( \mu \)-CTX block is not specifically dependent on \([Na⁺]\) but is strongly dependent on ionic strength. In contrast, K⁺ channel toxins are...
known to block with strong dependence on either the permeant ion concentration (i.e. $[K^+]$) or ionic strength (18–23). These findings are consonant with known, substantial differences between the structures and fundamental working mechanisms of Na$^+$ and K$^+$ channel pores. K$^+$ channel pores contain multiple high affinity K$^+$ binding sites whose occupancy depends critically on K$^+$ concentration. When the channel is multiply occupied, electrostatic repulsion among the occupying ions propels the conduction of ions at high throughput rates, thereby facilitating K$^+$ permeation (32). In an analogous manner, bound K$^+$ tends to “knock off” any bound toxin from the pore receptor and repels free toxin molecules (Fig. 6A). Kinetically, this destabilizes toxin block by accelerating dissociation rates, giving rise to the K$^+$ and voltage dependence of toxin block (for review, see Ref. 33). Interestingly, this K$^+$ dependence is highly localized on the toxin surface. In the case of ChTX block of large conductance K$^+$ channels, it arises entirely from the repulsion between a bound K$^+$ in the pore and the single toxin residue Lys-27 (21). Mutations elsewhere on the toxin molecule affect binding affinity but not the permeant ion dependence.

The interactions between Na$^+$ channels and µ-CTX are drastically different from the K$^+$ channel precedents (Fig. 6B). Here we show that µ-CTX block does not depend on [Na$^+$], per se, but rather on ionic strength. This supports the notion that Na$^+$ binds weakly to Na$^+$ channels with low occupancy (34–37). To a first approximation, the Na$^+$ channel operates as a single occupancy channel. Indeed, this interpretation is consistent with the faster association and dissociation rates of K$^+$ channel toxins relative to µ-CTX despite their similar nanomolar equilibrium dissociation constants (31). Differing toxin-ion interactions in Na$^+$ versus K$^+$ channels are also indicated by the facts that the primary influence of ionic strength on µ-CTX is to change the association rate constant, whereas K$^+$ selectively affects the dissociation rate of charybdotoxin from K$^+$ channels. Overall, it is apparent that Na$^+$ and K$^+$ channels have substantial differences in their pore structures and fundamental ionic interactions despite strong evolutionary conservation of functionally important pore domains (e.g. pore helices) (32, 38).

**Ionic Strength Dependence of Binding and Block**—As mentioned, µ-CTX binding has significant steric and electrostatic determinants. Because the latter are ionic in nature, it is not surprising that µ-CTX block is dependent on ionic strength, a feature shared with K$^+$ channel toxin counterparts. In fact, the membrane surface potential becomes more negative in low extracellular ionic strength because of diminished charge screening (39). Therefore, multivalent µ-CTX and other pore-blocking toxins are particularly sensitive to this change and become more potent at low ionic strength, partly as a result of their increased effective local concentrations at the external pore mouth. Additionally, the positively charged toxin and the negatively charged pore receptor would be “shielded” from each other in high ionic strength solutions, thereby increasing the activation energy required for forming the activated intermediate toxin-channel complex. Consistent with this premise, the toxin association rates are much more affected than the dissociation rates by ionic strength: $k_{\text{ass}}$ was orders of magnitude faster at low than high ionic strength, whereas $k_{\text{diss}}$ was only modestly changed.

**Different Contributions of Specific Toxin Residues**—Previous studies have demonstrated that individual charges and their particular positions rather than the overall charge of the toxin molecule itself are critical for modulating high affinity toxin block. For instance, neutral substitution of Arg-13, which protrudes into the pore and interacts with multiple anionic DII pore residues,2 induces the largest effect on toxin block of all cationic toxin residues (10). Our present findings are consistent with this notion. In addition to the vastly different effects on binding affinities of the toxin residues studied (all derivatives carry a net charge of +5 except for D12A (+7), D2N/R13Q (+6), and D12N/R13Q (+6), with dissociation constants ranging from $-10$ nM of D12A to $>10$ µM of R13A), they also do not contribute equally to the sensing of ionic strength. Whereas K9A, R11A, and D12A displayed dependence on ionic strength similar to that observed with WT toxin, the binding of R1A, R13A, and K16A (in particular) to Na$^+$ channels was less sensitive to changes in ionic strength. These results suggest that R13A and K16A have a role in guiding the toxin to its receptor and/or stabilizing the toxin-bound channel complex (8) as well as working in concert to prevent Na$^+$ from passing through the pore by creating an electrostatic exclusion volume (30).

It is of interest that the single-channel data reveal that the completeness of single-channel block, as well as the binding kinetics, depends systematically on ionic strength (Fig. 3). Fractional block increases with net charge on the toxin, with the most striking sensitivity to changes at residues 13 and 16, but measurable contributions from changes in other locations, e.g. neutralization of D2 or D12 (30). Although the data are limited, the lack of change in fractional residual current, $R$, for D2N/R13Q between 100 and 200 mM salt (Fig. 3B) may reflect that D2 is sufficiently distant from the “exclusion volume” critical for block that charge changes have no influence in this range of ionic strength. In contrast, where incomplete block is produced only by charge changes at positions 12 and 13, presumably nearer the narrow selectivity filter in the toxin-channel complex, changes in the residual current with ionic strength continue up to higher ranges (50–200 mM, D12N/R13Q; 200–400 mM, R13W). This suggests that the two latter charges are closer to the critical exclusion volume required for block. Important to this interpretation is that the previous study (30) showed empirically that the space constant, within the pore vestibule, for electrostatic contributions to block was close to the Debye length in the bulk solution. The Debye length varies inversely with the square root of ionic strength, covering a range of $\sim 4$–10 Å in our experiments. This range would allow discrimination between ionic strength dependence of charge changes at positions 2 and 12 or 13 on the toxin and gives a rough idea of the physical distances over which electrostatic interactions are likely to affect toxin association rates in different ionic strengths.

Interpretation of the kinetic changes is relatively straightforward. For all three toxin derivatives tested, the dissociation rate constant showed only minimal dependence on ionic strength. Association rates, on the other hand, increased dramatically, dominating changes in the equilibrium dissociation constant, as salt concentration was lowered. Together, this gives an increase in affinity of close to two orders of magnitude over the range of 200 to 50 mM (D2N/R13Q and D12N/R13Q), close to the relative changes seen in our whole-cell studies, in which ionic strength was changed only in the external solution. Charge changes produced by titrating an individual toxin residue also affect primarily the toxin association rate.3 Thus, relatively long range, electrostatic focusing seems to be a critical determinant of the toxin association rate, whereas the dissociation rate appears likely to be dominated by short range, non-electrostatic interactions.

Overall, our findings are the first to demonstrate that µ-CTX

---

2. Xue, T., Ennis, I. L., Sato, K., French, R. J., Li, R. A. (2003) Biophys. J., in press.

3. Hui, K., McIntyre, D., and French, R. J. (2003) J. Gen. Physiol., in press.
block of Na\textsuperscript{+} channels is ionic strength-dependent. This property rationalizes several quantitative discrepancies in \(\mu\)-CTX affinities reported in the literature, which might have arisen from the different ionic conditions used in studies on different preparations.

**CONCLUSION**

In summary, \(\mu\)-CTX block of Na\textsuperscript{+} channels depends on ionic strength but not specifically on the permeant ion concentration. These findings contrast those for K\textsuperscript{+} channel toxins and underscore the different roles of ion-ion interactions in the fundamental conduction mechanisms of Na\textsuperscript{+} and K\textsuperscript{+} channels and their interactions with pore-blocking toxins. Presumably because of their specific electrostatic contributions to binding interactions, toxin residues Arg-1, Arg-13, and Lys-18 make especially large contributions to the sensitivity of binding to ionic strength.

**REFERENCES**

1. Lancelin, J. M., Kohda, D., Tate, S., Yanagawa, Y., Abe, T., Satake, M., and Inagaki, F. (1991) *Biochemistry* **30**, 6908–6916
2. Sato, K., Ishida, Y., Wakamatsu, K., Kato, R., Honda, H., Ohizumi, Y., Nakamura, H., Ohy, M., Lancelin, J. M., Kohda, D., and Inagaki, F. (1991) *J. Biol. Chem.* **266**, 16889–16991
3. Wakamatsu, K., Kohda, D., Hatanaka, H., Lancelin, J. M., Ishida, Y., Oya, M., Nakamura, H., Inagaki, F., and Sato, K. (1992) *Biochemistry* **31**, 12577–12584
4. Hill, J. M., Aplewood, P. F., and Craik, D. J. (1996) *Biochemistry* **35**, 8824–8835
5. Cruz, L. J., Gray, W. R., Olivera, B. M., Zeikus, R. D., Kerr, L., Yoshikami, D., and Moczydlowski, E. (1985) *J. Biol. Chem.* **260**, 9280–9288
6. Olivera, B. M., Rivier, J., Clark, C., Ramilo, C. A., Corpuz, G. P., Abogadie, F. C., Mena, E. E., Woodward, S. R., Hillyard, D. R., and Cruz, L. J. (1990) *Science* **249**, 257–263
7. Nakamura, H., Kobayashi, J., Ohizumi, Y., and Hirata, Y. (1983) *Experientia* **39**, 590–591
8. Becker, S., Prusak-Sochaczewski, E., Zamponi, G., Beck-Sieking, A. G., Gordon, R. D., and French, R. J. (1992) *Biochemistry* **31**, 8229–8238
9. Chahine, M., Chen, L. Q., Fotuhi, N., Walsky, R., Fry, D., Santarelli, V., Horn, R., and Kallen, R. G. (1985) *Receptors Channels* **3**, 161–174
10. Chang, N. S., French, R. J., Lipkind, G. M., Fozzard, H. A., and Dudley, S., Jr. (1998) *Biochemistry* **37**, 4407–4419
11. Dudley, S. C., Jr., Toft, H., Lipkind, G., and Fozzard, H. A. (1995) *Biophys. J.* **69**, 1657–1665
Dependence of \( \mu \)-Conotoxin Block of Sodium Channels on Ionic Strength but Not on the Permeating [\( Na^+ \): IMPLICATIONS FOR THE DISTINCTIVE MECHANISTIC INTERACTIONS BETWEEN Na+ AND K+ CHANNEL PORE-BLOCKING TOXINS AND THEIR MOLECULAR TARGETS

Ronald A. Li, Kwokying Hui, Robert J. French, Kazuki Sato, Charles A. Henrikson, Gordon F. Tomaselli and Eduardo Marbán

J. Biol. Chem. 2003, 278:30912-30919.
doi: 10.1074/jbc.M301039200 originally published online May 21, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M301039200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 37 references, 13 of which can be accessed free at http://www.jbc.org/content/278/33/30912.full.html#ref-list-1