Pore Residues Critical for μ-CTX Binding to Rat Skeletal Muscle Na^+ Channels Revealed by Cysteine Mutagenesis

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ABSTRACT We have studied μ-conotoxin (μ-CTX) block of rat skeletal muscle sodium channel (rSkM1) currents in which single amino acids within the pore (P-loop) were substituted with cysteine. Among 17 cysteine mutants expressed in *Xenopus* oocytes, 7 showed significant alterations in sensitivity to μ-CTX compared to wild-type rSkM1 channel (IC_{50} = 17.5 ± 2.8 nM). E758C and D1241C were less sensitive to μ-CTX block (IC_{50} = 220 ± 39 nM and 112 ± 24 nM, respectively), whereas the tryptophan mutants W402C, W1239C, and W1531C showed enhanced μ-CTX sensitivity (IC_{50} = 1.9 ± 0.1, 4.9 ± 0.9, and 5.5 ± 0.4 nM, respectively). D400C and Y401C also showed statistically significant yet modest (approximately twofold) changes in sensitivity to μ-CTX block compared to WT ($p < 0.05$). Application of the negatively charged, sulfhydryl-reactive compound methanethiosulfonate-ethylsulfonate (MTSES) enhanced the toxin sensitivity of D1241C (IC_{50} = 46.3 ± 12 nM) while having little effect on E758C mutant channels (IC_{50} = 199.8 ± 21.8 nM). On the other hand, the positively charged methanethiosulfonate-ethylammonium (MTSEA) completely abolished the μ-CTX sensitivity of E758C (IC_{50} > 1 μM) and increased the IC_{50} of D1241C by about threefold. Applications of MTSEA, MTSES, and the neutral MTSBN (benzyl methanethiosulfonate) to the tryptophan-to-cysteine mutants partially or fully restored the wild-type μ-CTX sensitivity, suggesting that the bulkiness of the tryptophan's indole group is a determinant of toxin binding. In support of this suggestion, the blocking IC_{50} of W1531A (7.5 ± 1.3 nM) was similar to W1531C, whereas W1531Y showed reduced toxin sensitivity (14.6 ± 3.5 nM) similar to that of the wild-type channel. Our results demonstrate that charge at positions 758 and 1241 are important for μ-CTX toxin binding and further suggest that the tryptophan residues within the pore in domains I, III, and IV negatively influence toxin-channel interaction.

INTRODUCTION

Conotoxins (CTXs) are a group of toxins isolated from the venom of the piscivorous sea snail *Conus geographus* (Cruz et al., 1985; Gray et al., 1988; Olivera et al., 1990). Many of these toxins have specific actions on ion channels and neurotransmitter receptors. The μ-conotoxins (μ-CTXs) are a class of conotoxins that selectively blocks ion flux through voltage-gated skeletal muscle and electric eel sodium channels with high affinity compared to the brain, heart, or peripheral nerve subtypes (Cruz et al., 1985). μ-CTXs are peptides with 22 amino acids, including six cysteine residues (Nakamura et al., 1983; Gray et al., 1988; Olivera et al., 1990) that form three disulfide linkages, making the toxins very rigid (Cruz et al., 1985; Moczydlowski et al., 1986; Ohizumi et al., 1986; Yanagawa et al., 1986; Chen et al., 1992). At neutral pH, μ-CTXs are very hydrophilic and carry a net positive charge of either 6 or 7, depending on the subtype. Rigid peptide inhibitors have previously been used to study the molecular structures of receptors and ion channels (Park and Miller, 1992; Goldstein et al., 1994; Stampe et al., 1994; Hidalgo and MacKinnon, 1992). Consequently, μ-conotoxins should be useful molecular probes for testing various molecular models of Na^+ channel pore structure (Hille, 1992; Dudley et al., 1995; Stampe et al., 1994; Gross and MacKinnon, 1996), because the molecular structure of μ-CTXs has been determined at very high resolution (Lancelin et al., 1991; Sato et al., 1991; Wakamatsu et al., 1992; Hill et al., 1996). Because μ-CTX binds to skeletal muscle Na^+ channels with a 10^3-fold higher affinity compared to cardiac and nerve Na^+ channels (Cruz et al., 1985), this toxin is also useful for identifying structural differences between various Na^+ channel subtypes. In addition, shifts in steady-state activation after CTX application have recently been used to probe charge movements in the S4 voltage sensor (French et al., 1996).

Based on mutagenesis studies of the toxin, it has been suggested that the positively charged guanidinium group of Arg^{13} of μ-CTX is directly involved in the binding to sodium channels (Sato et al., 1991; Becker et al., 1992; Chahine et al., 1995). To further identify residues of the channel pore that might contribute to high-affinity μ-CTX binding, we constructed 18 cysteine mutants of the rat skeletal muscle Na^+ channel (rSkM1). All mutations (except E1524C) were constructed in the ascending portion (SS2) of the P-loop in the four homologous domains (DI–DIV). Cysteine mutants were initially tested for Cd^{2+} sensitivity to assess their side-chain accessibility and then tested for μ-CTX sensitivity. These studies therefore allowed us to identify pore-lining residues that influence the toxin binding.

In this report we located seven residues (D400, Y401, W402, E758, W1239, D1241, W1531) in the pore that

Received for publication 26 February 1997 and in final form 21 July 1997.
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0006-3495/97/10/1874/11 $2.00

significantly affect the binding of μ-CTX to Na^+ channels. These results are discussed in relationship to previous models of Na^+ channel pore structure.

MATERIALS AND METHODS

Mutagenesis

A 1.9-kb *Bam*HI-*Sph*I or a 2.5-kb *Sph*I-*Kpn*I fragment of the rSkM1 rat skeletal Na^+ channel cDNA (Trimmer et al., 1989) was subcloned into pGEM-11f$^+$ or pGEM-7f$^+$ (Promega, Madison, WI), respectively. Site-directed mutagenesis was performed, using uracil-enriched single-stranded DNA according to the method of Kunkel (1985). The mutation was confirmed by dideoxynucleotide sequencing (Sanger et al., 1977) before subcloning into the expression vector pGW1H (British Biolabs, Oxford, UK) containing the full-length Na^+ channel clone. Mutants were also resequenced after subcloning into the expression vector (i.e., pGW^{1}H) to ensure that the desired point mutation was present.

Heterologous expression

Xenopus laevis oocytes were removed from anesthetized frogs (3-aminobenzoic acid ethyl ester, 0.2%; Sigma) and digested with 2 mg/ml collagenase (Type 1A; Sigma) in OR-2 solution containing (in mM): 88 NaCl, 2 KCl, 1 $MgCl_2$, and 5 mM HEPES (pH 7.6). Oocytes were stored at room temperature in ND96 solution containing (in mM): 96 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES (pH 7.6) supplemented with 50 μg/ml gentamicin, 5 mM pyruvate, and 0.5 mM theophylline. Fetal bovine serum (2.5%) was added to promote the removal of the follicular layer. Stage V–VI oocytes were injected with a total of 50 nl of cDNA (10–100 ng/μl) consisting of the wild-type (rSkM1) α-subunit alone or a mixture of the rat brain β_1-subunit (Isom et al., 1992), along with either the rSkM1 or cysteine mutant α-subunits. In experiments involving the coexpression of β_1- and α-subunits, the concentration ratio of β_1-subunits to α-subunits was 5. Injected oocytes were incubated at room temperature for 48–72 h to allow for channel expression.

Electrophysiology

Whole-cell current recordings were performed at room temperature (20–22°C) using a two-microelectrode voltage-clamp amplifier (OC-725A; Warner Instruments, Hamden, CT) 2–3 days after injection. Agarose-plugged microelectrodes (TW120F-6; World Precision Instruments, Sarasota, FL) were filled with 3 M KCl and had a final resistance of 1–3 MΩ. The currents were digitized at 10 kHz and low-pass filtered at 1–2 kHz (−3 dB). A P/4 protocol was utilized for leak and capacitance subtraction. Analysis of current records was performed using custom-written software. Only oocytes expressing 2–5-μA currents were used in this study to ensure adequate voltage clamping.

Solutions

The recording solution contained (in mM): 96 NaCl, 2 KCl, 1 $BaCl_2$, 1 $MgCl_2$, and 5 HEPES (pH 7.6). For examination of the Cd^{2+} and μ-CTX sensitivities of rSkM1 and mutant channels, appropriate amounts of $CdCl_2$ (Sigma, Mississauga, ON) and GIIIB homolog of μ-CTX (Biomol, Plymouth, PA) were added to the bath recording solution from 1 M and 100 μM stock solutions, respectively. The purity of the toxin as stated by the supplier was 75% peptide content, >92% (HPLC). Sulfhydryl modification by the methanethiosulfonate (MTS) derivative (Toronto Research Chemical Co., Toronto, ON) MTSEA (MTS-ethylammonium), MTSES (MTS-ethylsulfonate), or MTSBN (benzyl methanethiosulfonate) was performed by exposing the cysteine mutants to 1 mM MTS-X for 3–10 min followed by a 5-min washout. Modification of the cysteinyl sulfhydryl side chain was reversed by adding 1 mM DTT (Fischer, Madison, WI) for 10–15 min. The MTS-X derivatives and DTT were prepared daily and dissolved in the recording solution.

Statistical analysis and curve fitting

Individual IC_{50} values were determined by fitting dose-response data, using the Marquardt-Levenberg algorithm in a nonlinear least-squares procedure, to the binding equation

$$I_B/I_O = 1/(1 + [\text{blocker}]/IC_{50})$$

where I_B and I_O represent measured Na^+ currents in the presence and absence, respectively, of μ-CTX or Cd^{2+}, and IC_{50} is the [blocker] at which $I_B = 0.5\ I_O$. In cases where incomplete substate block occurs, a modified form of the binding equation was used to accommodate the finite current recorded at high blocker concentrations. Such an equation is as follows:

$$I_B/I_O = (1 - (I_B/I_O)^\infty)/(1 + [\text{blocker}]/IC_{50}) + (I_B/I_O)^\infty$$

where $(I_B/I_O)^\infty$ represents the fraction of current remaining at very high [blocker]. All mean values presented were calculated by taking the average of at least three individual IC_{50} values, one for each mutant. Data presented are the means ± SEM. Statistical significance was determined using an unpaired Student's *t*-test, with $p < 0.05$ representing significance.

RESULTS

μ-Conotoxin sensitivity of rSkM1 and single cysteine mutants

We initially examined the μ-CTX sensitivity of wild-type rSkM1 channels expressed in *Xenopus* oocytes to block by μ-CTX. Fig. 1 *A* shows a typical whole-cell current recording from an oocyte coexpressing the rSkM1 α-subunit, along with a fivefold excess of the rat brain β_1 subunit. The currents were recorded using a two-electrode voltage clamp in the absence and presence of 30 nM μ-CTX after depolarization to −10 mV from a holding potential of −120 mV. The IC_{50} estimated by fitting the binding curve to the data (*filled circles*), shown in Fig. 1 *B*, was 17.4 ± 1.5 nM (n = 5), which falls within the range of 5–150 nM reported previously for expressed rSkM1 channels (Chen et al., 1992; Stephan et al., 1994; Dudley et al., 1995). Although the wide range of IC_{50} values measured previously could originate from differences in toxin purity, it is also conceivable that the level of coexpression with the rat brain β_1 Na^+ channel subunit could contribute to or be responsible for these differences. To address this question, we examined the effects of μ-CTX block on rSkM1 channels without coexpressing β_1. The representative current trace in Fig. 1 *A* (*right*) shows the expected slowing of inactivation kinetics when the rSkM1 α-subunit is expressed alone (Krafte et al., 1988; Zhou et al., 1991; Cannon et al., 1993; Nuss et al., 1995) (compare with Fig. 1 *A*, *left*). Despite these changes

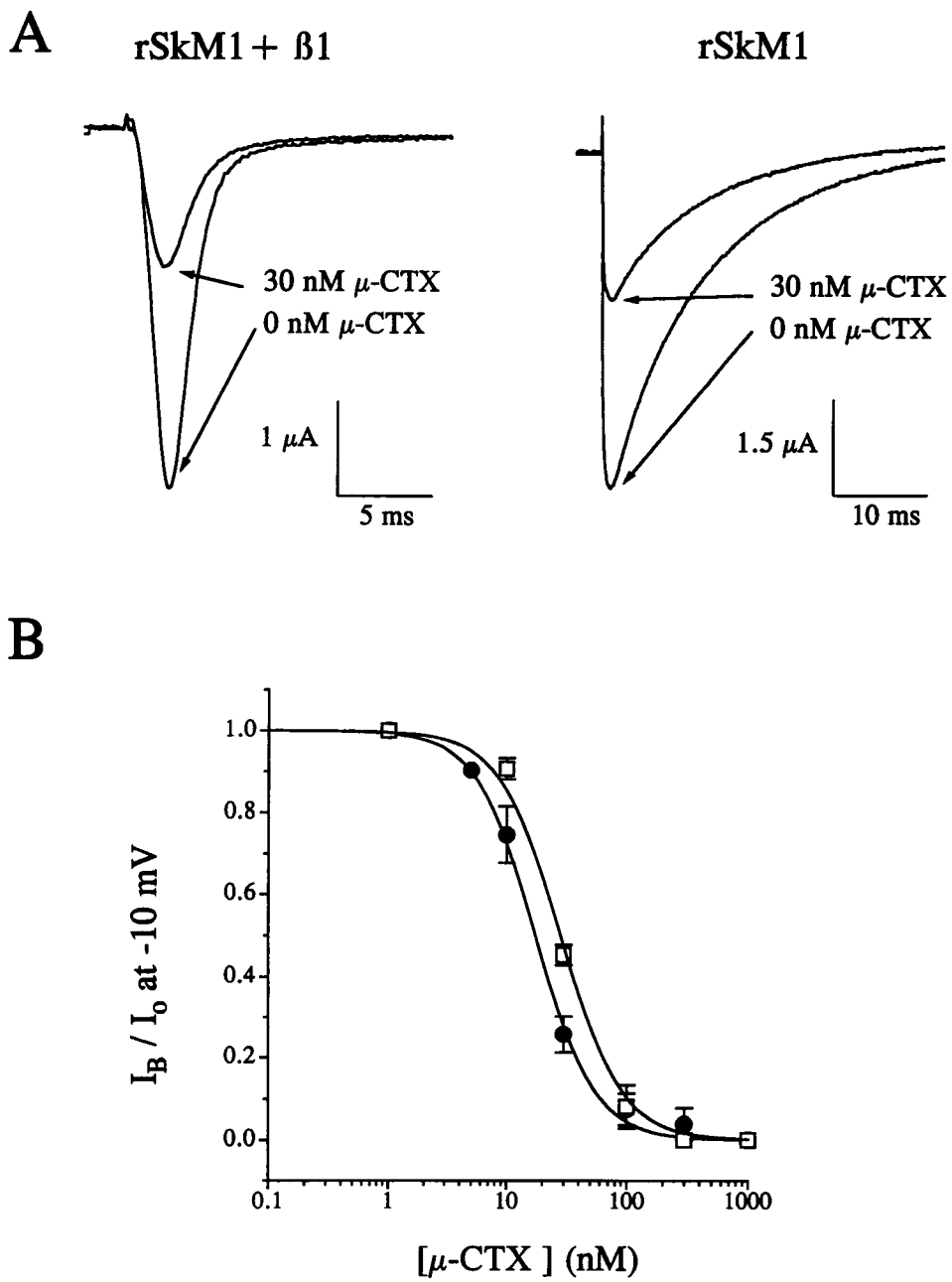

FIGURE 1 (*A*) Representative records of raw current traces of rSkM1 with (*left*) and without (*right*) coexpression with the rat brain β_1 subunit recorded in the absence and presence of 30 nM μ-CTX, as indicated by arrows. Lack of β_1 coexpression resulted in slower inactivation kinetics but did not significantly affect the toxin binding. (*B*) The dose-response relationship for μ-CTX binding to rSkM1 Na^+ channels with (●) and without (□) coexpression with β_1. Normalized peak Na^+ currents at −10 mV were plotted as a function of extracellular μ-CTX concentrations. The curve fitted with the binding equation allows estimation of the IC_{50} for μ-CTX block (see Materials and Methods). Data are plotted as mean ± SEM. IC_{50} values estimated were 17.4 ± 1.5 nM (n = 5) with β_1 and 27.9 ± 1.8 nM (n = 4) without β_1.

in channel gating, Fig. 1 *B* shows that β_1-subunits did not significantly ($p > 0.05$) affect the sensitivity for μ-CTX block (IC_{50} = 27.9 ± 1.8 nM, n = 4 without β_1).

All cysteine substitutions in the P-loops of the four internal repeats of rSkM1 expressed functional channels except G1238C. Furthermore, all mutant channels except W756C were sensitive to externally applied Cd^{2+} and became Cd^{2+}-insensitive after MTSEA modification, suggesting that these residues lie the extracellular face of the pore (Table 1) and thereby might influence toxin interaction with the channel.

Fig. 2 shows the ratios of the estimated IC_{50} values for μ-CTX block of our single cysteine mutants to the IC_{50} of WT. Mutants that were significantly different from wild type (i.e., ratios different from 1) are indicated by asterisks. All mutant channels were coexpressed with a fivefold excess of β_1. Most cysteine substitutions did not significantly alter sensitivity to block by μ-CTX compared to rSkM1. However, the negative charge substitution mutants E758C (IC_{50} = 220 ± 39 nM, n = 5) and D1241C (IC_{50} = 112 ± 24 nM, n = 6) were ~15- and 6-fold less sensitive to μ-CTX block than rSkM1. In contrast, the tryptophan substitution mutants W402C (IC_{50} = 1.9 ± 0.1 nM, n = 4), W1239C (IC_{50} = 4.9 ± 0.9 nM, n = 5), and W1531C (IC_{50} = 5.5 ± 0.4 nM, n = 5) were ~10-, 4-, and 3-fold more sensitive to μ-CTX block compared to rSkM1 channels. The estimated IC_{50} values for μ-CTX binding to D400C (38.8 ± 4.5 nM, n = 4) and Y401C (32.9 ± 2.1 nM,

TABLE 1 Half-blocking concentrations (IC_{50}) for Cd^{2+} of WT and mutant Na^+ channels before and after MTSEA modification

Mutants	IC_{50} (μM)	IC_{50} (μM) after MTSEA modification
WT (rSkM1)	1250 ± 224	1340 ± 250
D400C	880 ± 102	NA
Y401C	13.0 ± 2.5	1581 ± 86
W402C	199 ± 9	1235 ± 146
E403C	283 ± 20	1176 ± 227
E755C	55 ± 12	720 ± 120
W756C	1081 ± 62	NA
I757C	200 ± 23	858 ± 74
E758C	493 ± 34	1054 ± 112
K1237C	159 ± 23	1150 ± 152
G1238C	NE	NE
W1239C	37 ± 10	323 ± 25
M1240C	569 ± 30	1209 ± 125
D1241C	454 ± 46	1910 ± 262
A1529C	297 ± 58	800 ± 58
G1530C	68 ± 9	1722 ± 168
W1531C	46 ± 5	1625 ± 250
D1532C	602 ± 70	2325 ± 287
G1533C	33 ± 5	NA

NA, Not available; NE, no expression. Table adapted from Tsushima et al. (1997b).

$n = 3$) were only modestly yet significantly ($p < 0.05$) different from rSkM1 channels.

Effects of charge replacements on μ-CTX sensitivity

One simple explanation of the reduced potency of μ-CTX block of E758C and D1241C mutant channels is that the loss of negative charge disrupts the electrostatic interactions with the positively charged toxin. If electrostatic interactions are indeed important, introduction of a positive charge to the cysteine sulfhydryl might further destabilize toxin binding, whereas replacing a negative charge should strengthen toxin binding. Methane thiosulfonate derivatives MTSEA (positively charged) and MTSES (negatively charged), which have been used for many functional studies of ion channels (Akabas et al., 1992, 1994a,b; Kürz et al., 1995; Pascual et al., 1995; Li et al., 1996; Pérez-García et al., 1996), were chosen to introduce positive and negative charges to the pore of the cysteine mutants. In these experiments 1 mM MTSEA and MTSES were applied for 3–10 min and subsequently washed out for 5 min before the application of μ-CTX. This duration for MTS application was chosen to allow sufficient time to ensure complete modification of all channels. Complete charge insertion was established by two independent methods. First, after application and washout of MTSEA and MTSES, the peak current recorded in response to depolarizing pulses changed irreversibly (Pérez-García et al., 1996). As an example, Fig. 3 *A* depicts the peak current-voltage relationship recorded in E758C channels before (*solid square*) and after modification with 1 mM MTSEA (*left*) and MTSES (*right*) applied for 5 min. Reduction in peak current by MTSEA and the increase in peak current by MTSES recorded in E758C channels were reversed after treatment with 10 mM DTT (*open triangle*) for 10 min. Sulfhydryl modification was further verified by examining the sensitivity to Cd^{2+} block before and after treatment with MTSEA and MTSES. Because the MTS agents react with free sulfhydryls by forming disulfide bonds (Akabas et al., 1992) and because Cd^{2+} binds with much higher affinity to free sulfhydryls compared to disulfide-linked sulfhydryls (Torchinsky, 1981), successful modification of our cysteine mutant channels by MTSEA and MTSES should result in reductions in Cd^{2+} binding affinity. As an example, Fig. 3 *B* shows whole-cell current traces recorded in oocytes expressing E758C channels after depolarization to −10 mV from a holding potential of −120 mV before (*solid lines*) and after (*broken lines*) the application of 300 μM Cd^{2+} in the absence (*left*) and presence of sulfhydryl agents (MTSEA in the *middle panel* and MTSES in the *right panel*). Clearly, after the introduction of MTSEA and MTSES, only a small fraction of the current was blocked by 300 μM Cd^{2+}. This observation is quantified in Fig. 3 *C*, showing the fraction of peak current recorded with E758C channels expressed in oocytes after depolarization to −10 mV as a function of extracellular [Cd^{2+}] current; the average estimated IC_{50} (see Materials and Methods) for Cd^{2+} from Fig. 3 *C* was increased from 493.3 ± 34.2 μM ($n = 4$) to 1054 ± 112 μM ($n = 3$) by MTSEA and to 1120 ± 224 μM ($n = 3$) by MTSES application. Notice that the IC_{50} values estimated after the application of MTSEA and MTSES are not statistically different ($p > 0.05$) from those for WT channels (i.e., 1250 ± 224 μM). Similar analyses were used for all of the cysteine mutants studied to ensure successful sulfhydryl modification by the methanethiosulfonate derivatives (Table 1).

Inspection of Fig. 3 *C* demonstrates that E758C channels are not fully blocked by high [Cd^{2+}]. Similar results were also observed in E403C channels. Single-channel recordings reveal that the incomplete blockade at high [Cd^{2+}] results from the incomplete block of unitary current to a subconductance level, rather than a full conductance closure (Tsushima et al., 1997b). Nevertheless, the presence of incomplete block did not interfere with our ability to accurately determine the IC_{50} for Cd^{2+} block and thereby assess complete modification of the inserted cysteine by MTSEA and MTSES. See Materials and Methods.

Having confirmed that the cysteine mutant channels could be successfully modified by methanethiosulfonate derivatives, we next examined the effects of charge insertions using MTSEA and MTSES on μ-CTX binding (Fig. 4). MTSEA and MTSES application did not significantly affect our estimates of IC_{50} for μ-CTX block in wild type, E403C, or D1532C. These observations are anticipated for rSkM1 channels because of the absence of free sulfhydryls in the pores of these channels (Backx et al., 1992) and for E403C and D1532C channels, because toxin block of these channels was not different from that of rSkM1 channels.

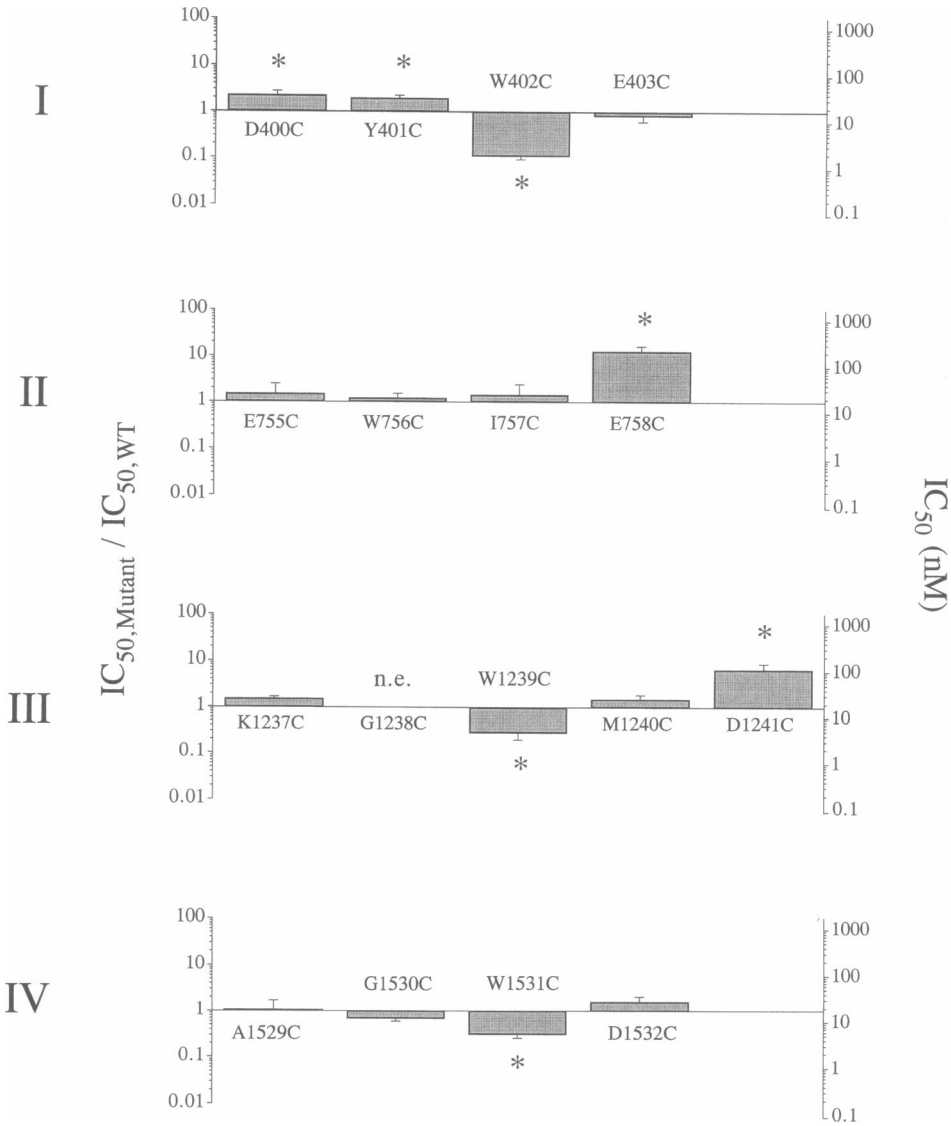

FIGURE 2 Summary of the half-blocking concentration (IC_{50}) for μ-CTX block of each of the single cysteine mutants. Roman numerals denote the corresponding domain numbers. Mutants not expressing currents are indentified as n.e. Data represent at least three individual IC_{50} determinations for each mutant. The left ordinate axis measures the ratio of the IC_{50} for mutant channels to the IC_{50} for rSkM1 channels on a logarithmic scale. The right ordinate represents the absolute IC_{50} (nM) plotted on a logarithmic scale. Mutants with IC_{50} values that are statistically different ($p < 0.05$) from rSkM1 are marked with asterisks. D400C, Y401C, E758C, and D1241C were less sensitive to μ-CTX block than WT, whereas W402C, W1239C, and W1531C were more sensitive.

By comparison, Fig. 4 shows that MTSEA application to D1241C channels further destabilized toxin binding, thereby increasing the IC_{50} for μ-CTX block to 334 ± 103 nM ($n = 3$, $p < 0.05$), whereas MTSES increased the μ-CTX sensitivity to 46 ± 12 nM ($n = 3$, $p < 0.05$), suggesting that charge at position 1241 is a determinant of toxin binding. More dramatic changes were observed with E758C; MTSEA modification completely abolished μ-CTX block, even at μ-CTX concentrations as high as 1 μM (Figs. 4 and 5), whereas MTSES treatment did not significantly affect μ-CTX sensitivity (IC_{50} = 199.8 ± 21.8 nM, $n = 4$, for MTSES compared to IC_{50} = 220 ± 39 nM, $n = 5$, for a nonreacted control). These results, combined with the results in Fig. 2, suggest that the charge at position 758 plays an important role in μ-CTX binding. However, the effects of MTSEA and MTSES are clearly disproportionate. That is, insertion of a positive charge has an enormous effect on toxin binding, whereas negative charge replacement causes no significant change, despite the similarity in molecular size and shape of MTSEA and MTSES. This observation demonstrates that factors other than just charge magnitude critically influence toxin binding.

Competitive binding between Cd^{2+} and μ-CTX in E758C and D1241C channels

To further test the role of charge and charge localization at positions E758 and D1241 in μ-CTX binding, we examined the ability of 1.0 mM Cd^{2+} to modify μ-CTX binding affinities. Because specific Cd^{2+} binding to cysteine sulfhydryls (Li et al., 1996; Pérez-García et al., 1996) will introduce a local positive charge, we anticipate that μ-CTX binding to E758C and D1241C channels might be destabilized in the presence of Cd^{2+}. Furthermore, the location of the added charge, because of the lack of an ethyl alkyl chain, should differ from the site of the positive charge introduced by MTSEA. However, rSkM1 and E758C chan-

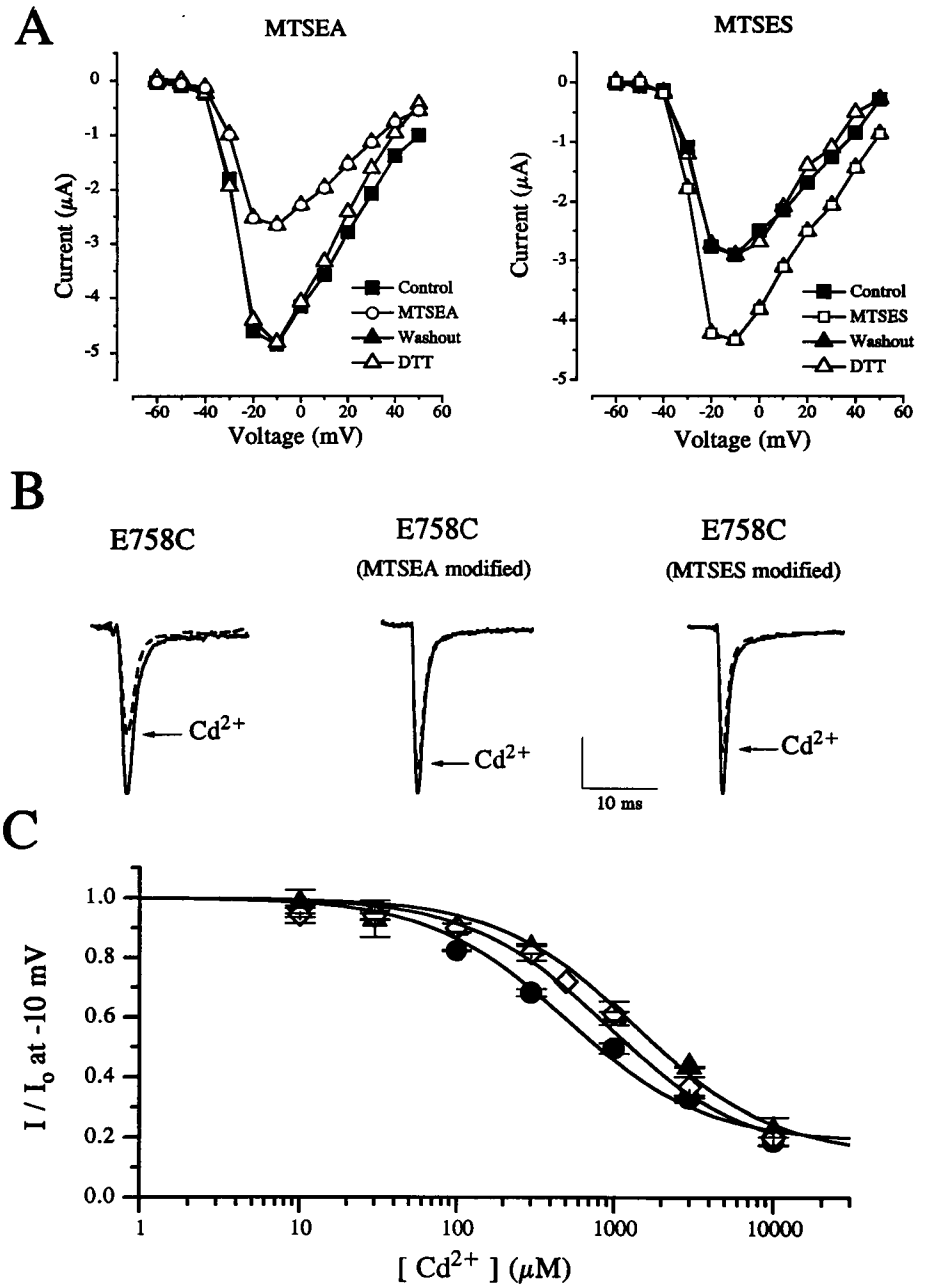

FIGURE 3 The effects of MTS-X modifications on E758C. (*A*) The current-voltage relationships of E758C before and after MTSEA (*left*) and MTSES (*right*) modifications. MTS-X modifications were irreversible in the absence of reducing agents and persisted even after prolonged washout. Both MTSEA and MTSES modifications could be reversed, however, by adding 1 mM DTT to the recording solution and incubating for 10 min. Other single mutants showed similar results. (*B*) Current traces of E758C in the absence (——) and presence (– – –) of 300 μM Cd^{2+} before (*left*) and after MTSEA (*middle*) and MTSES (*right*) modifications. Current records have been scaled such that the peak currents at the baseline are equal. The vertical scale bar represents 1.2, 0.8, and 1.3 μA for control, and after MTSEA and MTSES respectively. (*C*) Dose-response curves of the normalized peak Na^+ currents as a function of extracellular Cd^{2+} concentrations. MTSES (▲, n = 3) and MTSEA (◇, n = 3) shifted the Cd^{2+} binding curve of E758C channels (●, n = 4) to the right. Note the presence of a Cd^{2+}-resistant plateau at high Cd^{2+} concentrations, suggesting that these channels were blocked by Cd^{2+} to a subconductance state. A modified version of the binding equation was used to accommodate these steady-state components (see Materials and Methods).

nels became about twofold less sensitive to μ-CTX in the presence of 1.0 mM Cd^{2+} (31.8 ± 2.6 nM, n = 4, and 458 ± 150 nM, n = 4, respectively), whereas the IC_{50} for μ-CTX binding to D1241C was only modestly increased to 158 ± 29 nM (n = 6). Clearly, MTSEA and Cd^{2+} had differential effects on the sensitivities of E758C and D1241C channels to μ-CTX block. Nevertheless, these results are not inconsistent with the importance of charges at these positions to toxin binding, because the measured differences might reflect the dynamic nature of Cd^{2+} binding and unbinding. Unlike MTSEA, Cd^{2+} dynamically binds and unbinds to the pore. Hence, the kinetics of Cd^{2+} binding in relationship to μ-CTX binding could influence their interaction. Assuming no dramatic alterations in channel structure after Cd^{2+} binding, these observations are consistent with μ-CTX binding to the Na^+ channel pore.

Modification of μ-CTX binding to W402C, W1239C, and W1531C by sulfhydryl modification

To investigate the nature of toxin-channel interaction at positions 402, 1239, and 1531, we added MTSEA, MTSES, and MTSBN to W402C, W1239C, and D1531C channels. Despite their opposite charges, both MTSEA and MTSES increased the IC_{50} for μ-CTX block for the three tryptophan mutants (Fig. 6). After MTSEA modification, the IC_{50} values of W402C, W1239C, and W1531C increased from

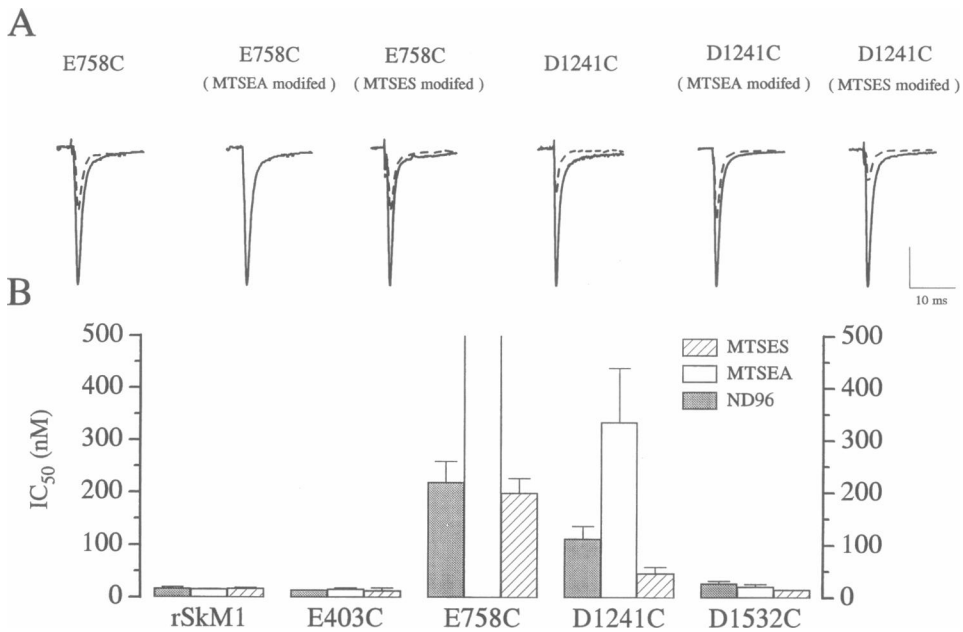

FIGURE 4 (*A*) Na^+ current tracings of E758C and D1241C mutant channels in response to depolarization to −10 mV from a holding potential of −120 mV in the absence (——) and presence (– – –) of 300 nM μ-CTX before and after MTSEA and MTSES modifications. Current amplitudes were normalized to the peak current for each mutant in the absence of μ-CTX. The vertical scale bar represents 1.5, 0.9, and 1.3 μA for E758C, E758C after MTSEA, E758C after MTSES, and 0.8, 1.1, and 1.3 μA for D1241C, D1241C after MTSEA and D1241C after MTSES, respectively. (*B*) Bar graphs summarizing the half-blocking concentrations (IC_{50}) for μ-CTX of WT (rSkM1), E403C, E758C, D1241C, and D1532C before and after MTSES and MTSEA modifications. μ-CTX sensitivities of WT, E403C, and D1532C were not significantly altered by applications of MTS-X ($p > 0.05$). E758C and D1241C became less susceptible to μ-CTX block after MTSEA modification. MTSES increased the μ-CTX sensitivity of D1241C, but had no effect on toxin binding to E758C. The data presented are the mean ± SEM from three to six oocytes. The bar labeled ND96 represents the bathing solution without sulfhydryl reagent.

1.9 ± 0.1 nM ($n = 4$), 4.9 ± 0.9 nM ($n = 5$), and 5.5 ± 0.4 nM ($n = 5$) to 12.5 ± 1.2 nM ($n = 4$), 15.1 ± 1.9 nM ($n = 5$), and 12.2 ± 3.4 nM ($n = 4$), respectively, whereas with MTSES the IC_{50} values changed to 7.0 ± 1.3 nM ($n = 4$), 8.2 ± 0.5 nM ($n = 4$), and 11.8 ± 3.1 nM ($n = 4$). Because both MTSEA and MTSES decreased μ-CTX sensitivity, it appears that electrostatic interactions at these positions are not important components of μ-CTX binding. Therefore, we hypothesized that the presence of any additional side-chain bulk at these positions will decrease toxin binding. To test this hypothesis, we applied the agent MTSBN (benzyl methanethiosulfonate), which tethers a neutral benzyl group, resembling the tryptophan side chain, to the inserted cysteine sulfhydryl group. In accordance with our postulate, MTSBN modification fully restored the lower wild-type μ-CTX sensitivity in W402C (12.7 ± 2.0 nM, $n = 4$) and W1239C (17.75 ± 0.6 nM, $n = 5$) while largely restoring the sensitivity of W1531C (8.5 ± 1.2 nM, $n = 4$). The incomplete restoration for W1531C may be attributed to the distant attachment of the benzyl group by an ethyl alkyl chain. Nevertheless, these observations suggest that side-chain bulkiness at these positions is vital to μ-CTX binding.

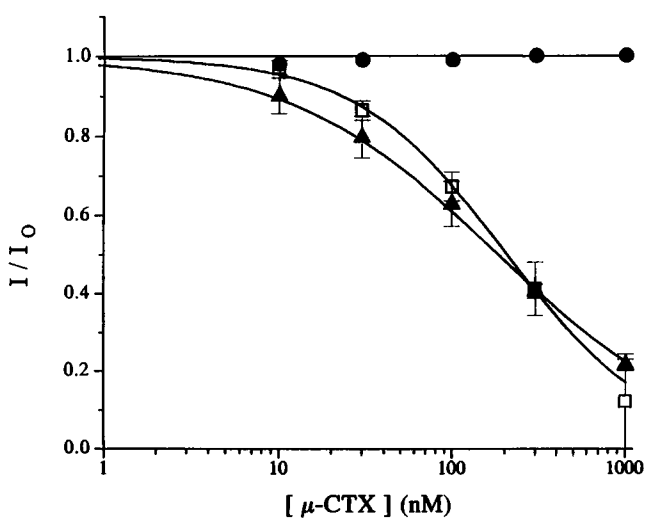

FIGURE 5 Dose-response curves of the normalized peak Na^+ currents at −10 mV as a function of extracellular μ-CTX concentrations before (□) and after MTSEA (●) and MTSES (▲) modification. MTSEA completely abolished μ-CTX sensitivity of E758C, whereas MTSES did not significantly affect μ-CTX binding to these channels. Data were fit to the binding equation as described in Materials and Methods.

E1524C shows wild-type sensitivities to Cd^{2+} and μ-CTX block

All P-loop residues studied above, except Y401C, are conserved between cardiac, nerve, and skeletal muscle Na^+ channels, despite much stronger binding of μ-CTX to skeletal muscle Na^+ channels. Previous results using chimeric exchanges of domains of cardiac and skeletal muscle Na^+

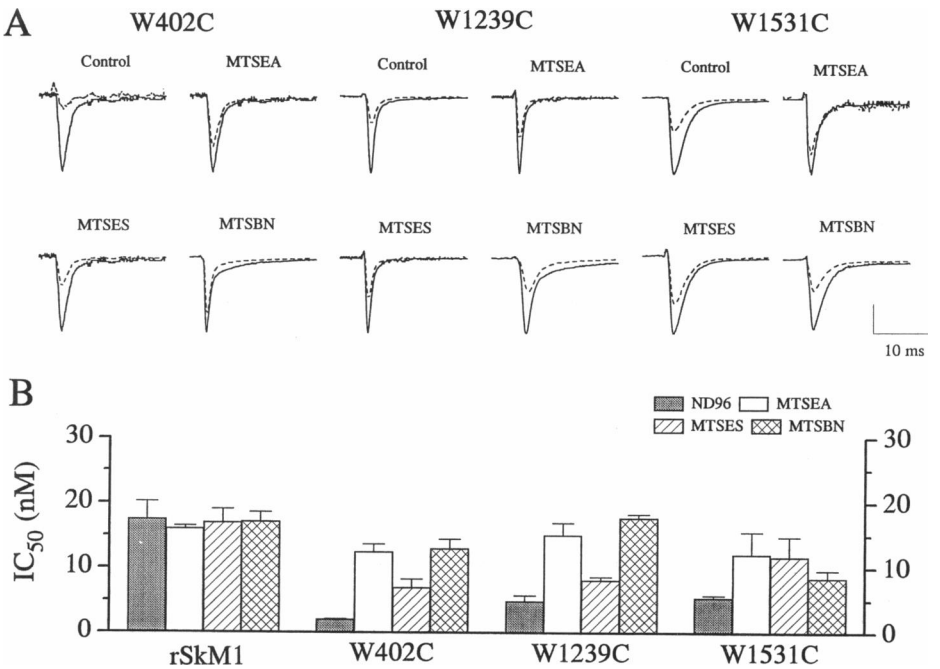

FIGURE 6 (*A*) Na^+ current tracings of W402C, W1239C, and W1531C mutant channels in response to depolarization to −10 mV from a holding potential of −120 mV in the absence (——) and presence (– – –) of 10 nM μ-CTX before and after MTSES, MTSEA, and MTSBN modifications. Peak control currents have been normalized to the same magnitude. Vertical scale bar represents currents ranging from 0.6 to 2.2 μA. (*B*) Bar graphs summarizing the half-blocking concentrations (IC_{50}) for μ-CTX of WT (rSkM1), W402C, W1239C, and W1531C before and after MTSES, MTSEA, and MTSBN modifications. All three agents were capable of partially or fully restoring WT μ-CTX sensitivity to W402C, W1239C, and W1531C. WT channel sensitivities to μ-CTX block were not modified by MTSEA, MTSES, or MTSBN. The bar labeled ND96 represents the bathing solution without sulfhydryl reagent.

channels established that domains I and II are primarily responsible for the differences observed in μ-CTX sensitivity in these Na^+ channel subtypes (Chen et al., 1992; Chahine et al., 1995). In the descending portion of the P-loop in domain IV, skeletal muscle Na^+ channels have a glutamate at position 1524 that is conserved at the equivalent position in electric eel Na^+ channels while being a neutral glutamine in heart and brain Na^+ channel subtypes. Because E1524 is negatively charged and is only found in the μ-CTX-sensitive Na^+ channels, we speculated that this residue might be crucial for the high-affinity toxin binding of the skeletal muscle and electric eel subtypes to all other cysteine mutants. E1524C channels were first probed with Cd^{2+} and then tested for μ-CTX sensitivity. This mutant channel showed the wild-type sensitivity to Cd^{2+} block (1120 ± 129 μM, n = 5), suggesting that the side chain of this glutamate residue does not lie within the permeation pathway. Despite the lack of Cd^{2+} sensitivity for this mutant, it is still conceivable that it may play a role in isoform-specific binding of μ-CTX. However, E1524C showed wild-type sensitivity to μ-CTX block (22.4 ± 5.8 nM, n = 4), demonstrating that this residue does not play a significant role in the specific high-affinity block of μ-CTX to the skeletal muscle Na^+ channels.

DISCUSSION

In the present study, we used cysteine mutagenesis to identify P-loop residues in the four internal repeat domains of rat skeletal muscle Na^+ channels that are important for binding μ-CTX, because this strategy enables identification of pore-lining residues and allows sulfhydryl-specific chemical modifications in charge and size at the residues under examination. Most cysteine replacements of P-loop made in our studies (except G1238C) produced functional channels with relatively normal macroscopic channel kinetics (Li et al., 1996; Pérez-García et al., 1996), suggesting preserved pore structure. Furthermore, all P-loop residues replaced by cysteine appeared to line the pore, as assessed by Cd^{2+} block and sulfhydryl modification. Our results identify seven pore-lining residues that significantly influenced μ-CTX binding to rSkM1 channels: D400, Y401, W402, E758, W1239, D1241, and W1531 (Fig. 2).

Expected and unexpected results were obtained with negative charge replacement mutants like E403C, E758C, D1241C, and D1532C channels. Modification of E403C and D1532C with MTSEA and MTSES had no affect on toxin binding, as expected from the identical μ-CTX binding of E403C, D1532C, and rSkM1 channels; apparently these residues do not participate in μ-CTX binding. Similarly, MTSEA modification of E758C and D1241C channels, whose IC_{50} values for μ-CTX binding were 15-fold and 6-fold higher than those for rSkM1, further increased the IC_{50} for μ-CTX binding, with E758C channels becoming completely resistant to block at concentrations up to 1 μM (Fig. 5). On the other hand, MTSES treatment of D1241C enhanced the toxin sensitivity, approaching the IC_{50} value of wild-type channels, whereas MTSES modification had little effect on toxin binding to E758C channels. Taken together, these results are consistent with electrostatic interactions between the charges at positions 758 and 1241 and μ-CTX. However, simple charge substitution at position 758 did not produce the expected effects; restoring the negative charge by using MTSES in E758C had modest effects on toxin binding, whereas insertion of a positive charge by MTSEA completely eliminated toxin binding.

One potential explanation for these unexpected findings is that the inserted charged groups are not localized to equivalent molecular positions with MTSES and MTSEA, because of their attachment via an ethyl alkyl chain (Akabas et al., 1992). Alternatively, modification by methanethiosulfonate compounds could induce local structural changes to the P-loops, thereby altering toxin binding.

To further test the role of charge and its localization within the channel pore on μ-CTX binding, we examined the effects of Cd^{2+} on toxin binding to E758C and D1241C channels. Similar competitive binding studies between Zn^{2+} and STX have previously been performed in cardiac Na^+ channels (Schild and Moczydlowski, 1991; Doyle et al., 1993). μ-CTX block was only slightly reduced in E758C and D1241C channels in the presence of Cd^{2+} compared to MTSEA treatment, possibly reflecting either the dynamic nature of the interaction of the channel with Cd^{2+} compared to irreversible channel modification by cysteine modifying agents or, alternatively, the differences in charge localization. Because μ-CTX block of rSkM1 was also impaired in the presence of Cd^{2+}, our results are consistent with μ-CTX binding to the channel pore.

Our data generally support previous hypotheses that electrostatic interactions between negatively charged residues within the channel pore and the positively charged μ-CTX are important factors for high-affinity μ-CTX binding (Becker et al., 1992; Stephan et al., 1994; Dudley et al., 1995). However, negatively charged residues at equivalent locations in the four internal repeat domains do not contribute equally to this interaction. Indeed, E403C and D1532C mutant channels showed wild-type behavior with respect to μ-CTX block, whereas E758C and D1241C channels were far less sensitive to μ-CTX block than rSkM1 channels. Therefore our results establish that these negatively charged residues do not contribute equally to toxin binding. The nonequivalence of the negatively charged residues located at equivalent alignment positions could reflect a nonsymmetrical arrangement of the residues within the pore (Chiamvimonvat et al., 1996; Tsushima et al., 1997b). Alternatively, the toxin might interact with a symmetrical pore in an off-axis fashion similar to that observed for AgTx binding to the *Shaker* K^+ channels (Gross and MacKinnon, 1996).

The large reductions in affinity for μ-CTX observed in E758C and D1241C are consistent with the suggestion that Arg^{13} of μCTX interacts with the negatively charged groups of glutamate and aspartate side chains within the pore (Becker et al., 1992; Dudley et al., 1995). Becker and co-workers (1992) suggested that glutamate residues within the pore interact with Arg^{13} of μ-CTX, which has been shown to be an essential residue for the toxin binding to the channel, because mutations of this toxin residue dramatically reduced the toxin binding affinity (Sato et al., 1991; Becker et al., 1992; Chahine et al., 1995). The potency of μ-CTX as a blocker is related to the positive charge of the guanidinium group of Arg^{13}, because replacement with lysine or ornithine had only moderate effects on affinity compared to less conservative substitutions using alanine and glutamine (Sato et al., 1991; Chahine et al., 1995). These observations support the hypothesis that interactions between positive charges on the toxin with negative charges within the channel pore are essential for high-affinity toxin binding. This electrostatic hypothesis is consistent with our observation that E758C had the largest effect on μ-CTX binding. This finding is also consistent with the model proposed previously by Dudley and colleagues (1995) in which E758 interacts with the N-H moiety of the guanidinium group of Arg^{13} (Dudley et al., 1995). However, this model predicts that channel occlusion is the direct result of the association of Arg^{13} with the carboxyl side chains of D400 and E755, whereas we observed a modest 2.2-fold decrease in toxin binding to D400C channels and no change in toxin binding to E755C channels ($p > 0.05$) compared to rSkM1 (Fig. 2). Mutation of E403 also showed little effect on μ-CTX binding, consistent with the suggestion that the aliphatic portion of Arg^{13} may face E403 (Stephan et al., 1994).

Interestingly, the tryptophan-to-cysteine mutants (W402C, W1239C, and W1531C) were more sensitive to μ-CTX block. Because both MTSEA and MTSES decreased μ-CTX sensitivity to about the same extent despite their opposite charges, we speculate that reintroduction of large side groups at those positions might negatively influence toxin binding. Consistent with this suggestion, modification of channels with MTSBN (which attaches an ethylbenzene side group to the inserted cysteine sulfhydryl) largely restored wild-type μ-CTX binding affinities to these mutants. Furthermore, we found that the sensitivity of W1531A (7.5 ± 1.3 nM, $n = 4$) to μ-CTX block was similar to that of W1531C, whereas that of W1531Y (14.6 ± 3.5 nM, $n = 5$) was indistinguishable from that of wild type.

Sequence alignment analysis of the voltage-gated Na^+ channels reveals a high homology within the four pore-forming regions, with very few nonconservative differences in amino acid composition. Two such notable differences are Y401 and E1524. The tyrosine at position 401 of rSkM1 is critical for TTX/STX sensitivity (Backx et al., 1992), whereas phenylalanine at the equivalent position in brain Na^+ channels also confers high-affinity TTX/STX binding on the channel (Heinemann et al., 1992b). Substitution of the Tyr or Phe residues for cysteine, which is the residue found in the TTX/STX resistant cardiac isoform at the equivalent position (Satin et al., 1992; Kallen et al., 1990), abolishes TTX sensitivity of both skeletal muscle and brain Na^+ channels while enhancing their sensitivity to Cd^{2+} block by 200-fold (Backx et al., 1992; Chen et al., 1992; Heinemann et al., 1992). This residue, however, cannot account for the 10^3-fold differences in sensitivity to μ-CTX between cardiac and skeletal muscle Na^+ channels, because Y401C reduced μ-CTX binding by only twofold (Chen et al., 1992; Chahine et al., 1995). Similarly, mutation of E1524 to cysteine had no effect on toxin sensitivity, suggesting that this residue is also not responsible for the differences in toxin affinity. These results were also con-

sistent with those reported recently by Chahine and colleagues (1995).

In summary, our results support models wherein μ-CTX interacts with E758 in the skeletal muscle Na^+ channel pore. Our results further suggest that the D-III residue D1241 is also an important determinant of toxin binding. The fact that the tryptophan mutants (W402C, W1239C, and W1531C) are more sensitive to μ-CTX block suggests that these large uncharged residue side chains appear to inhibit or weaken the toxin-channel interaction in WT channels. Finally, the differences in μ-CTX binding between cardiac and rat skeletal muscle Na^+ channels do not originate from absolutely conserved differences at positions 401 (i.e., Y401C) or 1524 (i.e., E1524Q).

This work was supported by the Medical Research Council of Canada. P.H.B. is a Medical Research Council of Canada Scholar. R.A.L. holds a research studentship from the Centre of Cardiovascular Research, The Toronto Hospital. R.G.T. was supported by a fellowship from the Department of Medicine, University of Toronto. Equipment support from the Tiffin Trust (Toronto, Canada) is gratefully acknowledged.

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