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The μI Skeletal Muscle Sodium Channel: Mutation E403Q Eliminates Sensitivity to Tetrodotoxin but not to μ-Conotoxins GIIIA and GIIIB

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Abstract. Voltage-sensitive Na channels from nerve and muscle are blocked by the guanidinium toxins tetrodotoxin (TTX) and saxitoxin (STX). Mutagenesis studies of brain RII channels have shown that glutamate 387 (E387) is essential for current block by these toxins. We demonstrate here that mutation of glutamate 403 (E403) of the adult skeletal muscle μI channel (corresponding to E387 of RII) also prevents current blockade by TTX and STX, and by neo-saxitoxin. However, the mutation fails to prevent blockade by the peptide neurotoxins, μ-conotoxin GIIIA and GIIIB; these toxins are thought to bind to the same or overlapping sites with TTX and STX. The E403Q mutation may have utility as a marker for exogenous Na channels in transgenic expression studies, since there are no known native channels with the same pharmacological profile.

Key words: Rat skeletal muscle Na channel — Tetrodotoxin — μ-Conotoxin — μI

Introduction

Voltage-sensitive Na channels are responsible for the inward currents of action potentials in nerve and muscle cells. When activated by membrane depolarization, these channels form a highly selective conductance pathway for Na entry. A variety of specific neurotoxins, including the small guanidinium toxins tetrodotoxin (TTX) and saxitoxin (STX), bind to receptor sites on the external channel surface and block Na conductance (*reviewed in* [1], [3] and [5]). The mechanisms of toxin blockade and the amino acid residues forming these receptor sites are beginning to be resolved through site-directed mutagenesis and expression of mutant Na channel cDNA's. These experiments offer the possibility of producing Na channels with unique pharmacological properties not encountered in native tissues. Such constructs may have utility in transgenic studies aimed at expressing wild-type or mutant exogenous Na channels in targeted tissues or at specific stages of tissue differentiation. The present report describes a mutant rat μI adult skeletal muscle Na channel which has lost sensitivity to the general Na channel neurotoxins TTX and STX but retains a high sensitivity to the muscle isoform-specific class of toxins, the μ-conotoxins GIIIA and GIIIB.

Na channels consist of an essential large α-glycopeptide subunit together with 0–2 smaller β-glycopeptide subunits (*reviewed in* [29]). Cloning studies confirm that the α-subunits are members of an extensive multigene family (*reviewed in* [32]). Beta subunits cloned from mammalian skeletal muscle and heart, however, appear to arise from the same gene, suggesting that they may not arise from a multigene family (J. Tong, J. Potts, J. Rochelle, M. Seldin and W. Agnew, *submitted*). Expression studies indicate that the α-subunits form the receptor sites for TTX, STX and μ-conotoxins [32]. It is well recognized that different isoforms exhibit marked differences in sensitivity to these toxins [32]. The μI channel is blocked by nanomolar concentrations of the guanidinium toxins and the peptide μ-conotoxins. Neuronal channels (RI, RII, RIIA and RIII) are blocked by TTX and STX, but not μ-conotoxin. Channels from heart and immature skeletal muscle (H1, also known as

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μII or SkMII) are strongly resistant to TTX and STX, and to μ-conotoxins as well. The biological significance of these neurotoxin receptor sites is at present unknown. Clearly, preservation of neurotoxin binding is not essential for preservation of the fundamental biophysical properties of the channels; despite this, developmental and tissue-specific differences have been evolutionarily preserved.

Inspection of the primary structure of the highly homologous α-subunits reveals four internally homologous subunit-like domains; these domains (*I–IV,* or *A–D*) each possess six hydrophobic segments predicted to form membrane-spanning α-helices (Fig. 1). Between helices 5 and 6 of each domain lie two shorter, conserved segments, SS1 and SS2; recent evidence suggests that the SS1–SS2 region penetrates the membrane and contributes to the lining of the ion conductance pathway not only in Na channels, but in other, homologous voltage-regulated channels. Mutations of K channels in the region corresponding to SS1–SS2 alter ion conductance, selectivity and the binding of external and internal conductance blockers ([9, 10]; *reviewed in* [15]). Mutations in this region in each domain of the rat brain RII Na channel affect TTX and STX blockade [19, 31], change permeation selectivity [11], and reduce conductance [19, 21, 31]. Naturally occurring differences in this region between brain, muscle and heart Na channels can also explain differences in sensitivity to conductance block by TTX, STX and metal ions [2, 24, 26].

In the rat skeletal muscle μI channel, glutamate 403 of the SS1–SS2 region in domain I is homologous to glutamate 387 of the rat brain RII channel. Mutations of RII have shown that this residue is essential for TTX blockade [19, 31]. Direct studies to show whether the toxin still binds to the mutant channel have not been reported; thus, although current models favor the SS1–SS2 region(s) forming the conductance pathway *per se,* TTX, STX and μ-conotoxin effects could be due to induced conformational changes. Binding studies demonstrate that these toxins displace one another, suggesting that they bind to the same or overlapping sites on the channel surface [8, 16]. We show here that mutation of glutamate 403 to glutamine (E403Q) results in loss of TTX, STX and neo-STX blockade of μI conductance. The mutant channel, however, retains a high level of sensitivity to μ-conotoxins GIIIA and GIIIB. The ability of TTX to displace the μ-conotoxins is also lost, strongly suggesting that actual binding of TTX is lost. These results are discussed in terms of current models of residues forming the conductance pathway. Also, because the E403Q construct of μI has a unique pharmacological profile (μ-conotoxin sensitive, TTX and STX insensitive), it should prove useful in transgenic experiments aimed at exploring normal and abnormal Na channel physiology.

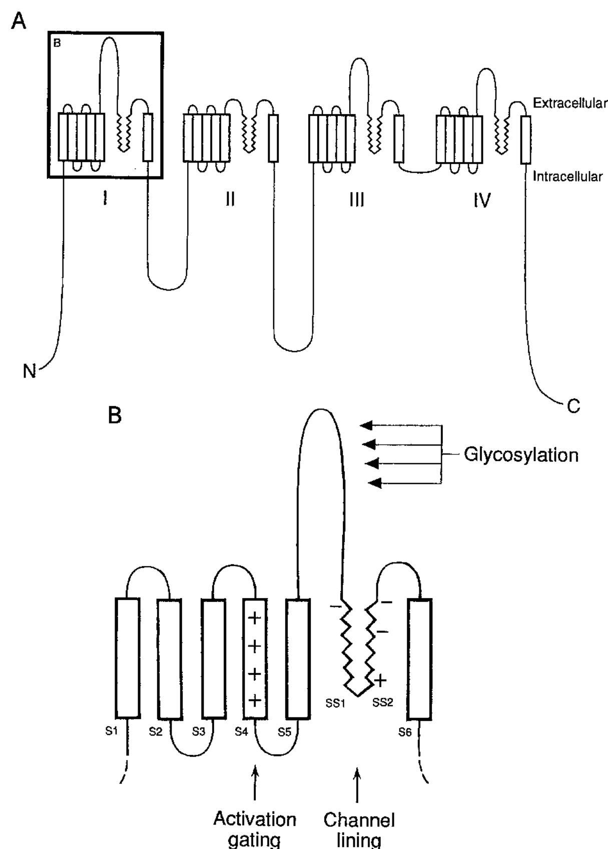

Fig. 1. (*A*) Schematic diagram of the μI Na channel, showing the four internally homologous domains (labeled *I–IV*). Rectangular boxes denote predicted α-helices and zig-zag lines represent the unknown conformation of the four SS1–SS2 regions. (*B*) Enlarged drawing of a single domain (the boxed region in *A*) showing the six predicted membrane-spanning α-helices and the SS1–SS2 region. Functions thought to be associated with specific sites on the channel are labeled.

Materials and Methods

Mutagenesis of μI cDNA

Oligonucleotide-directed, site-specific mutagenesis was performed on plasmid pMM12BR, a subclone of plasmid pμ1–2 that encodes the entire 5,520 bp μI coding region together with 95 bp of 5′ and 307 bp of 3′ untranslated regions [33]. pMM12BR contains an 860 bp ApaI fragment of pμ1–2, corresponding to the second half of domain I and a portion of the I–II interdomain region (bp 675–1,534), ligated into the ApaI site of the pBluescript SK$^+$ (Stratagene, La Jolla, CA) polylinker. Mutagenesis was performed by the polymerase chain reaction (PCR)-based "megaprimer" method [23], using the mutagenic oligonucleotide 5′-GGACTACTGGCAGAACCTTTTC-3′. The codon GAG encoding glutamate (E) 403 was changed to CAG (glutamine (Q)). The mutant PCR product was digested with ApaI and re-ligated into pBluescript. Recombinant plasmids were isolated from single colonies and sequenced to detect the presence of the desired mutation. Positive clones were fully sequenced between the downstream ApaI site and a unique upstream StuI site (bp 999) to detect any extraneous mutations introduced by Taq polymerase (Se-

quenase kit; United States Biochemical, Cleveland, OH). The 534 bp ApaI-StuI fragment was recovered and re-ligated into pμ1–2 to produce a μI expression vector identical to the original except for the presence of the point mutation.

Expression of Mutant μI cDNA in Oocytes

Messenger RNA was transcribed and capped with m^{7}G(5′)ppp(5′)G in vitro as previously described [33]. After transcription, the RNA was purified on glass beads with the RNAid kit according to the manufacturer's directions (Bio101, La Jolla, CA). Stage V and VI *Xenopus laevis* oocytes were isolated [6, 30] and microinjected with 50 nl of 0.5 μg/ml mRNA, and allowed to incubate in OR3 medium (50% Leibovitz's L15 cell culture medium [GIBCO BRL, Gaithersburg, MD], 15 mM HEPES [pH 7.5], 10 U/ml penicillin, 10 μg/ml streptomycin) for 3–4 days at 19°C. Na currents were recorded in frog Ringer's solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES [pH 7.2]) with a two-electrode voltage clamp (Warner Instrument, Hamden, CT) and software developed by InstruTech (Elmont, NY) for data acquisition and analysis.

Measurement of Toxin Blockade

The concentration dependence of peak Na current block by each neurotoxin was measured in a small volume (400 μl) recording chamber. A separate oocyte was used to measure the effect of each toxin concentration (n = 2–4), according to the following protocol: Each oocyte, in 200 μl of frog Ringer's solution, was first maintained at a holding potential of −120 mV and given a series of ten 50 msec test pulses to −10 mV to measure the amplitude of peak Na current before toxin addition. (Voltage-clamp measurements confirmed our observations [33] that peak currents were obtained at −10 mV.) The oocyte was then allowed to rest unclamped while toxin was added at two times the desired concentration in a second 200 μl aliquot of frog Ringer's solution. The toxin was allowed to equilibrate for 3 min, after which ten more test pulses were applied to measure the toxin-induced decrease in peak amplitude. (Control experiments demonstrated that equilibration of toxin block was complete within 2 min (*not shown*).) Extent of toxin blockade was then expressed for each oocyte as peak current after toxin/peak current before toxin (I/I_{max}). By the end of the test protocol, each oocyte had been impaled on the recording electrodes for 5 min. We observed that control oocytes, which underwent the same procedures without toxin, showed an intrinsic decline of peak current within the same time period (average decline = 8%, n = 11). I/I_{max} was normalized upward to correct for this rundown for all data points (*see* Results).

TTX (citrate-free) was purchased from Sankyo and made to 200 μM in 100 ml of distilled water acidified to pH 4 with a few drops of glacial acetic acid. μ-Conotoxin GIIIB was purchased from Research Biochemicals (Boston, MA) and GIIIA was the kind gift of Dr. Baldomero Olivera (University of Utah, Salt Lake City). Neo-saxitoxin was the kind gift of Dr. Edward Moczydlowski (Yale University School of Medicine, New Haven, CT). All other reagents were from Sigma Chemical (St. Louis, MO).

Results

Macroscopic Currents

The electrophysiological properties of the wild-type μI channel and the E403Q mutant were compared at the level of macroscopic current behavior. *Xenopus* oocytes were injected with cRNA from both constructs and the resulting Na currents were measured with the two-electrode voltage clamp. Results of these experiments are illustrated in Fig. 2. Figure 2*A* and *B* show a family of voltage-clamp traces and the peak current-voltage relationship for the wild-type channel. Figure 2*C* and *D* show comparable data for the mutant channel. The slight differences in current-voltage relationship between the wild-type and mutant channels are within the range of variation seen with wild-type channels alone [36]. In addition, there were no apparent differences in the kinetics of activation or inactivation, nor were there systematic differences in peak current amplitude. From the standpoint of macroscopic channel behavior, E403Q appeared to exert essentially no effect.

Effects of the E403Q Mutation on Binding and Blockade by TTX and μ-Conotoxin GIIIB

Initial screening experiments were performed to determine whether the E403Q mutation resulted in the predicted loss of sensitivity to nanomolar TTX. In the experiment illustrated in Fig. 3*A*, it is evident that exposure to 500 nM TTX resulted in essentially no reduction in peak Na current. The small decrement represents uncorrected rundown during the time of toxin equilibration in the chamber, as illustrated by Fig. 3*B*. In this experiment, the time course of peak current decline was followed from the time of initial impalement, with or without the addition of 500 nM TTX at 2 min. The time course of toxin blockade was so rapid that it was essentially complete within 30 sec of addition, corresponding to an approximately 80% decline in current amplitude. In contrast, the time course of intrinsic rundown shows only a 1–2% decline over the same time period. For all oocytes, data were collected within the first five minutes after impalement and normalized to correct for this rundown (*see* Materials and Methods).

The experiment in Fig. 3*A* also shows the result of adding 500 nM μ-conotoxin GIIIB to the chamber immediately following exposure to 500 nM TTX. As the traces illustrate, this toxin strongly suppressed the E403Q Na current. These initial experiments suggested that the mutation resulted in loss of the ability of TTX to block current; that μ-conotoxin was capable of blocking current in the presence of TTX suggested, first, that binding of the peptide toxin was largely unaffected by the mutation and, second, that the ability of TTX to prevent μ-conotoxin binding was eliminated.

Effects on Sensitivity to STX, neo-STX and μ-Conotoxin GIIIA

Similar experiments were performed to test whether the E403Q channel was sensitive to STX, neo-STX

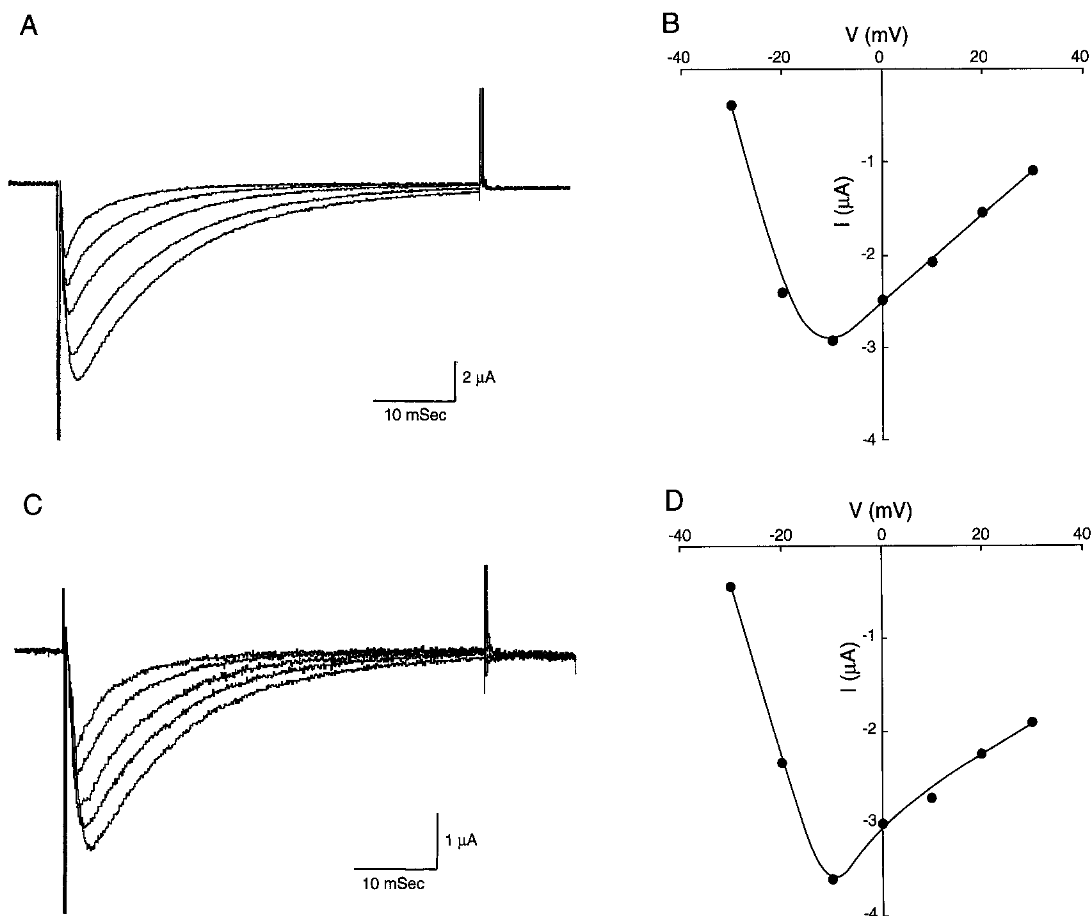

Fig. 2. Macroscopic currents recorded by a two-electrode voltage clamp of oocytes injected with wild type (*A*) or E403Q (*C*) cRNA (*see* Materials and Methods) and current-voltage relationships of wild-type (*B*) or E403Q (*D*) channels. Oocytes were clamped at a holding potential of −120 mV and depolarized for 50 msec to (starting from largest current in both *A* and *C*): −10, 0, +10, +20, and +30 mV. Each trace represents the average of 5–10 traces recorded at 3 sec intervals.

(neo-saxitoxin) and μ-conotoxin GIIIA. STX, which differs greatly from TTX in structure, contains two guanidinium groups. One of these, the 7,8,9 group, appears to correspond to the single guanidinium group of TTX as the active group in Na channel blockade [13]. Neo-saxitoxin differs from saxitoxin by the presence of a hydroxyl group at the N1 position of the 1,2,3 guanidinium group, which also plays a role in toxin binding [13, 18]. As illustrated in the traces shown in Fig. 4, the E403Q mutant was strongly resistant to both STX (*A*) and neo-STX (*B*), at least at concentrations of 500 nM. In contrast, Fig. 4*C* shows that the E403Q mutant remained sensitive to μ-conotoxin GIIIA. The GIIIA peptide differs from GIIIB at three positions: Arg8 in GIIIB corresponds to Lys in GIIIA, Arg14 to Gln, and Met18 to Gln; the net result being the presence of one fewer positive charges in the GIIIA form [7]. These differences did not apparently affect the interaction of the toxin with the E403Q mutant channel.

Dose-Response Sensitivity to Toxins

Further experiments were performed to quantitate the difference between wild-type and mutant channel sensitivities to TTX and μ-conotoxin GIIIB. The affinity for each neurotoxin ($K_{1/2}$) was estimated from a dose-response curve (Fig. 5) as the toxin concentration required for 50% reduction of peak current (*see* legend to Fig. 5). As illustrated in Fig. 5*A*, the $K_{1/2}$ for TTX was 24 nM for the wild-type channel, consistent with our previous findings of 5 and 35 nM for μI channels expressed in oocytes [33] and mammalian cells [34], and the report of 27 nM by Moczydlowski et al. (1986) for rat skeletal muscle channels studied in planar lipid bilayers [17, 18]. The E403Q mutant channel was completely insensitive to TTX. In contrast, high concentrations of μ-conotoxin GIIIB resulted in complete suppression of both wild type and mutant currents (Fig. 5*B*). The $K_{1/2}$ of GIIIB for the wild-type channel of 143 nM

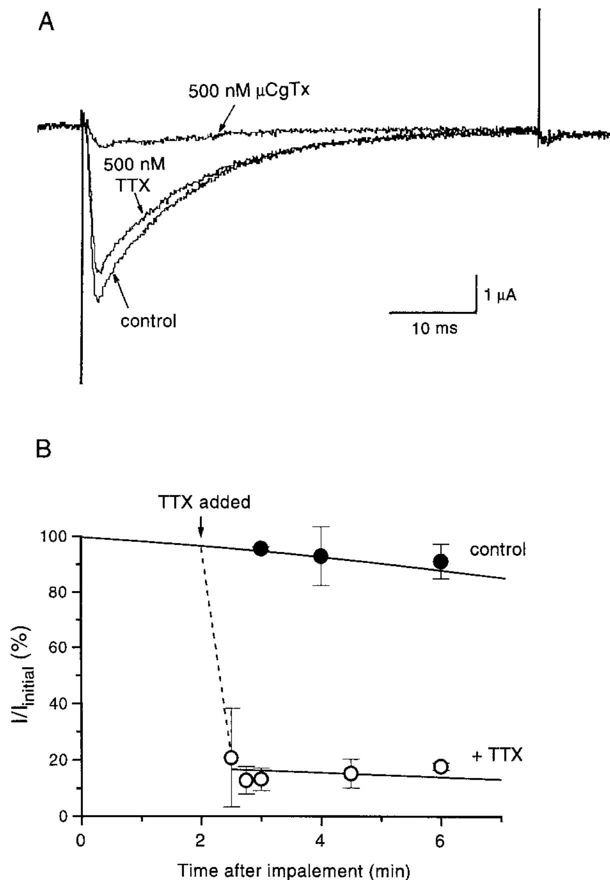

Fig. 3. (*A*) Effects of 500 nM TTX and 500 nM μ-conotoxin GIIIB on E403Q current. The oocyte was held at −120 mV and depolarized to −10 mV to record 10 control traces (*control*). TTX (500 nM) was then added and allowed to equilibrate for 3 min, after which 10 more traces were recorded (*500 nM TTX*). μ-Conotoxin GIIIB (500 nM) was subsequently added in the presence of TTX and allowed to equilibrate for another 3 min, after which 10 more traces were recorded (*500 nM μCgTx*). All other recording parameters were the same as for Fig. 2. (*B*) Time course of decay in peak current amplitude with (*+TTX,* open circles) and without (*control,* filled circles) 500 nM TTX. Each oocyte was impaled at 0 time, and the initial peak current measured. After two min, 500 nM TTX was added to one set of oocytes (*see* Materials and Methods) and the peak current was measured again at the soonest interval, 30 sec, and then again for several more time points. Another set of control oocytes was subjected to the same procedures without TTX, and the intrinsic rundown measured at several time points. All subsequent measurements of toxin effect (Figs. 4 and 5) were made within 5 min to minimize the effect of rundown on the data. Mean currents are shown as a percentage of the initial current for each oocyte (I/I_{initial}).

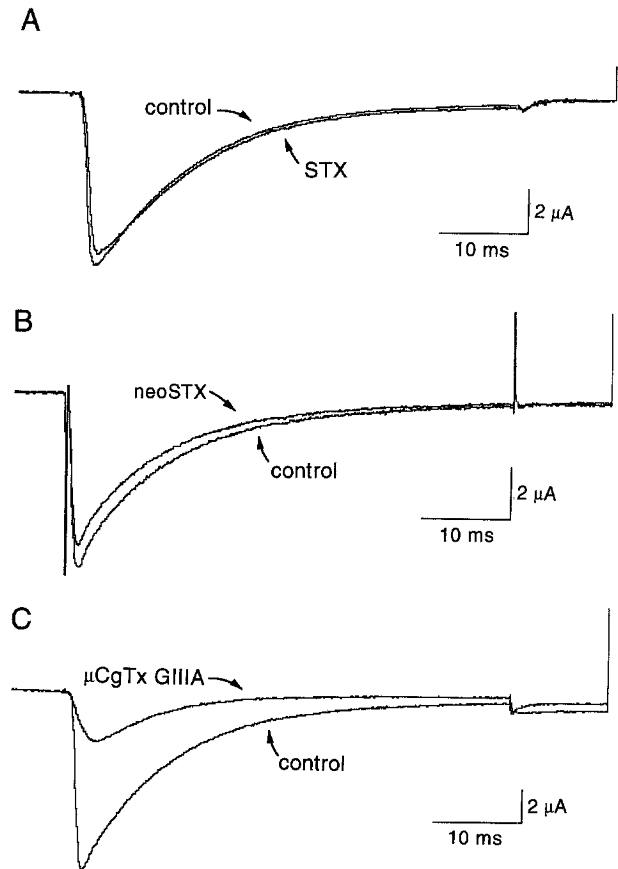

Fig. 4. Effects of 500 nM STX (*A*), neo-STX (*B*) and μ-conotoxin GIIIA (*C*) on E403Q peak current. Toxin effects on peak current were measured at equilibrium as described in Materials and Methods. The *control* trace in each panel shows the current before toxin addition, superimposed on another trace recorded after 3 min incubation with the indicated toxin. Recording parameters were the same as for Fig. 2.

was consistent with published reports [16, 18]; the $K_{1/2}$ for the mutant was shifted to 331 nM, slightly more than a twofold decrease in affinity.

Estimates were also made for the sensitivities of the E403Q construct to STX, neo-STX and μ-conotoxin GIIIA. These are illustrated in the Table. The control, wild-type values correspond well to published values estimated from both electrophysiological [18] and binding competition [16] studies of these toxins. The $K_{1/2}$ for μ-conotoxin GIIIA of the mutant was shifted about fourfold from the wild type $K_{1/2}$, similar in magnitude to the shift observed for GIIIB.

Discussion

The results described here show that, while glutamate 403 of the μI Na channel is essential for TTX and STX blockade [19, 31], this residue is not required for blockade by the muscle-specific μ-conotoxins. This result is unexpected in view of the close similarity in properties between the two classes of toxins. First, they have marked similarities of action. Both classes of neurotoxins block single channel conductance of skeletal muscle channels in lipid bilayers, with probabilities corresponding to single site, reversible binding [7, 18]. Second, they appear to compete for the same binding site. Binding competition studies have shown that μ-

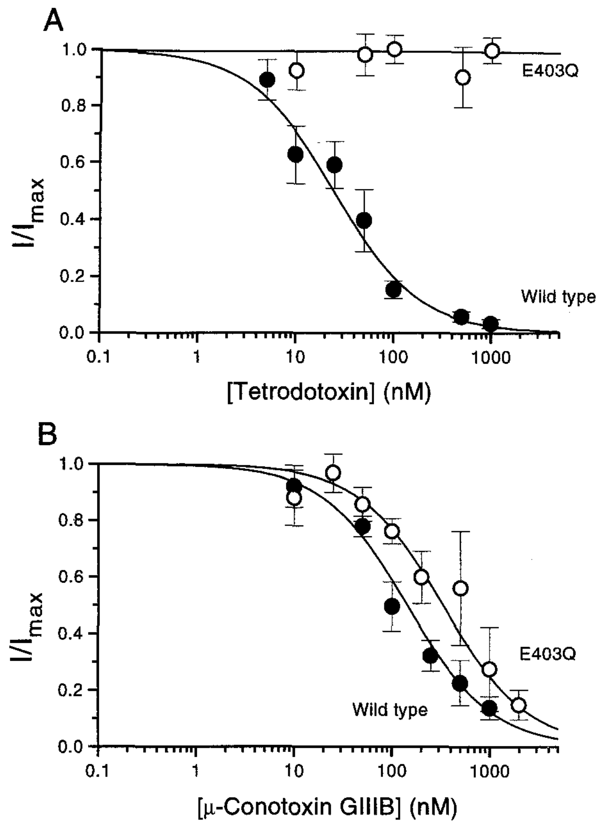

Fig. 5. Dose-response curves for the effects of TTX (*A*) and μ-conotoxin GIIIB (*B*) on wild type (filled circles) and E403Q (open circles) peak current amplitudes. Toxin concentrations ranging from 5 nM to 2 μM were applied and their effects measured as described in Materials and Methods. Peak current after toxin equilibration is shown as a fraction of peak current before toxin addition (I/I_{max}). Each curve was fitted to its entire data set using Origin (MicroCal Software, Northampton, MA) and the following equation:

$$y = 1 - [T]/(k_{1/2} + [T]),$$

where $y = I/I_{max}$, $[T]$ = toxin concentration, and $k_{1/2}$ = the toxin concentration which results in 50% reduction of peak current amplitude.

conotoxins directly inhibit binding of radiolabeled STX to Na channels in rat skeletal muscle and eel electroplax [16]. Preincubation with a high concentration of TTX also prevents binding of synthetic radiolabeled μ-conotoxin GIIIA to eel Na channels [8]. Third, each class of toxin carries a positively charged functional group which is required for Na channel blockade. Studies of TTX analogues have shown that the positively charged guanidinium group of this toxin is essential for block [13]. μ-Conotoxins GIIIA and GIIIB are 22 amino acid peptide toxins; site-directed mutagenesis has identified arginine 13 as the critical residue for blockade [25]. However, despite these similarities, our results show that the two classes of toxins do not have identical binding sites.

Table. Estimated toxin sensitivities of the E403Q mutant

Toxin	$K_{1/2}$ (nM)	
	Wild type	Mutant
Tetrodotoxin	24[a]	>2,000[c]
Saxitoxin	4[b]	>2,000[c]
Neo-saxitoxin	2[b]	>2,000[c]
μ-Conotoxin GIIIA	44[b]	165[b]
μ-Conotoxin GIIIB	143[a]	331[a]

[a] Estimated as the concentration required to reduce peak current amplitude by 50% (*see* legend to Fig. 5).
[b] Estimated from several data points giving 60–70% reduction in peak current amplitude, using the equation in the legend to Fig. 5.
[c] Half-maximal inhibition had not been reached at the highest concentration tested, 2,000 nM.

TTX blockade exhibits pH dependence [13, 35] and can be eliminated by treatment of the channel with carboxyl-modifying reagents [27, 28], suggesting that coulombic or hydrogen bonds could be formed between its guanidinium group and the carboxyl side chains of aspartate or glutamate residues near the mouth of the channel. Five such residues have been identified in the RII channel that, when mutated, give rise to TTX and STX resistance (E384, E387, E942, E945, and D1717) [31]. The E403 residue of μI, which corresponds to E387, is thus not the only candidate for interaction with arg13 of the μ-conotoxins. It is likely that a subset of these residues will be involved in the binding of each toxin, defining closely overlapping, but not identical, binding sites.

The above-mentioned mutagenesis studies of the RII channel characterized mutations which resulted in loss of TTX blockade of Na current. However, direct binding studies were not done to examine whether the mutation eliminated toxin binding, or simply altered an allosteric coupling between toxin binding and conductance block. The experiment illustrated in Fig. 3 demonstrates that not only was TTX blockade of conductance eliminated in the E403Q mutation, but the ability of excess (500 nM) TTX to competitively inhibit μ-conotoxin GIIIB blockade was also lost. This result indicates that both TTX binding and current block were disrupted by the mutation. Together with the evidence that residues involved in TTX binding are located in the region forming the ion conductance pathway [11, 21], these findings support the notion that the toxins act to physically occlude the ion pathway, rather than working through a conformational mechanism that alters gating.

Figure 6*A* shows a schematic diagram of some of the residues thought to make up the lining of the pore (the SS1–SS2 segment of domain I) of μI; they are drawn as they might appear in relation to the membrane (*see* figure legend for an explanation of the cri-

teria used). Figure 6*B* shows an alignment of the SS1–SS2 regions from domain I of muscle (μI), heart (H1 = μII = SkMII) and brain (RII) Na channels. At least one of the residues previously identified as critical for TTX block, D384 of RII, must lie well within the pore, suggesting that these neurotoxins may penetrate partway into the channel's interior. Mutagenesis studies of the heart (H1) Na channel also support this conclusion. H1 is blocked by the divalent metal ions Cd^{2+} and Zn^{2+}, but is relatively insensitive to TTX and STX (*reviewed in* [32]). These ions appear to displace TTX and STX competitively [26]. Studies with sulfhydryl reagents [26] indicated that Zn^{2+} and Cd^{2+} effects might involve a cysteine residue near the TTX/STX binding site. In H1, a cysteine, C374, lies between the residues corresponding to D384 and E387 of RII (Fig. 6*B*). Mutation of this residue to tyrosine, which is the corresponding residue in the μI channel (Y401), results in loss of metal ion sensitivity and a gain of TTX and STX sensitivity [24]. Complementary mutations were made in the μI skeletal muscle channel; mutation of Y401 to C conferred both TTX and STX resistance and Cd^{2+} and Zn^{2+} sensitivity to this channel [2]. It has been estimated that C374 lies 22–24% of the way through the transmembrane electric field, as measured by the weak voltage dependence of Cd^{2+} and Zn^{2+} blockade [2]. This again suggests that the guanidinium groups of TTX and STX also penetrate well into the pore. While it seems possible that the larger μ-conotoxin may bind more superficially to the mouth of the ion channel, it is interesting that the crucial arginine lies on a flexible loop of the peptide which might also be able to penetrate into the pore [14]. It seems possible that another negatively charged residue in this region, D401, is more directly involved in μ-conotoxin binding; alternatively, residues of the SS1–SS2 regions from the other domains might be involved. The larger size of the peptide toxin suggests that residues on the channel surface may also be important, perhaps not contributing significantly to the free energy of binding, but permitting close interaction of the essential arginine with the channel.

The pharmacological profile of the E403Q mutant of μI appears to be unique; the channel lacks sensitivity to the common toxin blockers TTX and STX, but retains the specialized sensitivity to μ-conotoxin peptides. From a practical standpoint, these unique properties may assist in experiments exploring the consequences of functional expression of otherwise wild-type or mutant channels in transgenic animals. For example, animals engineered to express in their muscle tissues the autosomal dominant mutations responsible for hyperkalemic periodic paralysis [20, 22] may exhibit abnormalities reminiscent of the corresponding clinical syndromes in human beings. The contributions of the exogenous channel to currents in the muscle fibers can

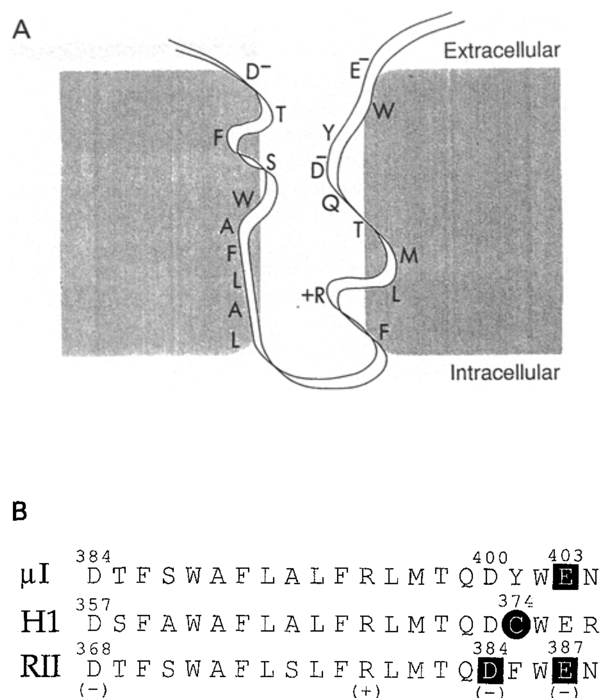

Fig. 6. (*A*) Proposed topographical disposition of the domain I SS1–SS2 region of the μI Na channel. The positions of the amino acid residues with respect to depth within the membrane are hypothesized according to the following considerations: recent evidence suggests that ion channel pores often contain rings of charged residues [4, 11, 12]. Thus, D384 and E403 are placed opposite one another at the mouth of the pore, with the residues in between them placed so as to reach the internal surface of the membrane and back again. This placement fits well with three pieces of experimental evidence: (i) C374 of the heart Na channel (which corresponds to Y401 shown here) is thought to lie 22–24% of the way across the membrane, as measured by the weak voltage dependence of its interaction with the pore-blocking metal ions Cd^{2+} and Zn^{2+} [2]. (ii) W402 of μI corresponds to T449 of the Shaker K channel, a residue which has been shown to be involved in the external binding of the pore-blockers tetraethylammonium (TEA) and charybdotoxin [9]. Since charybdotoxin is a very large, bulky molecule, this residue must likely lie on or near the external surface of the channel. (iii) F394 corresponds to T441 of the Shaker channel, a residue which must lie near the internal surface of the membrane since it is required for internal black by TEA [10]. (*B*) Alignment of the domain I SS1–SS2 regions from the μI, H1 and RII channels. Residues involved in TTX and STX sensitivity are boxed. The cysteine residue in the heart channel which is important for Cd^{2+} and Zn^{2+} blockade is circled.

be isolated by performing experiments with or without TTX or μ-conotoxin. Perhaps more interesting, we speculate that the neurotoxin receptor sites may play a yet undiscovered role in the physiology of nerve and muscle tissues, possibly interacting with endogenous equivalents to the neurotoxin blockers. Expression of channels containing apparently benign mutations in the receptor sites in otherwise normal tissues may result in unexpected developmental or functional disorders.

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