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Novel Site on Sodium Channel α-Subunit Responsible for the Differential Sensitivity of Grayanotoxin in Skeletal and Cardiac Muscle

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ABSTRACT

We searched for sites on the α-subunit of the fast Na^+ channel responsible for the difference in GTX (grayanotoxin) sensitivity of the skeletal- and cardiac-muscle Na^+ current. cDNA clones, encoding the skeletal or cardiac isoforms of the α-subunit, were inserted into a mammalian expression vector and transiently transfected into human embryonic kidney cells. The expressed channels were measured using whole-cell patch-clamp techniques and examined for GTX sensitivity. As a measure of GTX sensitivity, we used relative chord conductance (ratio of maximum chord conductance of noninactivating GTX-modified Na^+ currents to that of unmodified peak currents). Wild-type channels from skeletal muscle (μ1) were more sensitive to GTX modification than wild-type cardiac channels (rH1) by a factor of 1.6. To facilitate exploration of α-subunit sites determining GTX sensitivity, we used SHHH, a chimera of skeletal muscle (S) domain D1 and heart muscle (H) domains D2D3D4 with supernormal sensitivity to GTX I (1.5-fold of wild-type μ1). Successive replacement of Ser-251 (D1S4–S5 intracellular loop) and Ile-433 (D1S6 transmembrane segment), with corresponding rH1 residues Ala and Val, reduced, in a stepwise manner, the GTX sensitivity of the chimera and related mutants to that of wild-type rHI. We concluded that, in addition to Ile-433, known as the GTX-binding site, Ser-251 represents a novel site for GTX modification.

Voltage-dependent Na^+ channels modified by GTX open at membrane potentials considerably more negative than normal and lack the fast-inactivation process of unmodified channels (Seyama and Narahashi, 1981). It is possible to use the distinct pharmacological characteristics of the modified sodium channel, in conjunction with genetic techniques, to determine the site of action of GTX on the sodium channel protein. Knowledge of the site of action of GTX has the potential to yield new information about the molecular locus of the activation process and the region of the channel protein in which coupling between activation and inactivation occurs.

Previously, we reported on a sequence of six amino acid residues, found in the transmembrane segments of D1S6 (Ishii et al., 1999) and D4S6 (Kimura et al., 2000), which are required for GTX-binding to the sodium channel and partially overlap the binding domain for batrachotoxin (Linford et al., 1998; Wang and Wang, 1998, 1999). We also showed that the potency of GTX I differs significantly between the Na^+ channel isoforms μ1 and rH1 (Ishii et al., 1999; Yakehiro et al., 2000). In the present study, we set out to identify the regions in the α-subunit of the Na^+ channel critical to this difference in GTX sensitivity. We show herein that both Ser-251 in the intracellular loop of D1S4–S5 and Ile-433 in the transmembrane segment of D1S6 in μ1 are responsible for the differential action of GTX I.

Materials and Methods

Construction of Chimeras and Point Mutation of Na^+ Channels. Na^+ channel chimeras and point mutations were constructed using two cDNA clones coding the skeletal and cardiac α-subunits (μ1 and rH1). To construct the chimeras by substitution of D1 and D4, respectively, *Bsi*WI and *Cla*I sites were created in the cDNA clones as described previously (Ishii et al., 1999; Kimura et al., 2000). For introduction of point mutations in the transmembrane segment D1S6 and the extracellular D1S4–S5 loop, we used polymerase chain reaction-based and site-directed mutagenesis (Promega, Madison, WI). All of the resulting chimeras and point mutants were confirmed

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ABBREVIATIONS: GTX, grayanotoxin; D, domain; S, segment; HEK, human embryonic kidney; P_o, channel open probability; I-V, current-voltage; I_{Na}, Na^+ current; μ1, α-subunit of rat skeletal muscle Na^+ channel; rH1, α-subunit of rat heart Na^+ channel.

with restriction mapping and sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Each mutant channel will be referenced by the original amino acid followed by its number and introduced amino acid

Transient Transfection and Cell Culture. The constructed chimeras and point-mutated cDNA clones were inserted into mammalian expression vector pCI-neo (Promega) or pcDNA3.1 (Invitrogen, Carlsbad, CA) and were then transiently cotransfected with CD8 cDNA into HEK cells using the SuperFect transfection reagent (QIAGEN, Hilden, Germany). The cells were grown to 50% confluence in Dulbecco's modified Eagle's medium (Invitrogen), containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 30 units/ml penicillin G (Invitrogen) and 30 μg/ml streptomycin (Invitrogen), in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The transfected cells were used for electrophysiological experiments as late as 3 to 4 days after being replated in 35-mm tissue culture dishes. Transfection-positive cells were identified using CD8-Dynabeads (Dynal, Oslo, Norway) before I_{Na} recording.

Electrophysiological Recording. Macroscopic I_{Na} from the transfected cells was measured using the whole-cell variation of the patch clamp method. The bath solution contained 70 mM NaCl, 67 mM *N*-methyl-D-glucamine, 1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES, pH 7.4. The pipette solution contained 70 mM CsF, 60 mM CsCl, 12 mM NaF, 5 mM ethylene-bis-(oxonitrilo)-tetraacetic acid and 5 mM HEPES, pH 7.4. To assess the effects of GTX on whole-cell I_{Na}, different concentrations of GTX I were added to the pipette solution, because GTX is known to act intracellularly (Seyama et al., 1988). Single Na^+ channel currents were measured using the cell-attached variation of the patch-clamp technique (Hamill et al., 1981). Single-channel currents were filtered at 10 kHz and digitized at 50 kHz and 10 to 20 kHz for GTX-modified and unmodified Na^+ channels. Pipette solutions for cell-attached single-channel recordings contained 250 mM NaCl, 0.2 mM $CaCl_2$, 2.5 mM $MgCl_2$, 5 mM KCl, and 5 mM HEPES. The pH was adjusted to 7.4 with NaOH. Bath solution contained 150 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM glucose, and 5 mM HEPES. The pH was adjusted to 7.4 with KOH. Data are presented as "mean ± S.D. (number of observations)" unless otherwise indicated.

We obtained channel open probability (P_o), using essentially the same method as Yakehiro et al. (2000). In brief, we first averaged 200 to 500 traces at 0 mV to get an ensemble recording. Next, we estimated the number of channels in the patch (N) by dividing the individual current recording with the maximal number of simultaneous openings by the unitary channel current. We also divided the peak ensemble current by the unitary channel current to obtain NP_o (the number of channels × open probability in the patch). We obtained $P_{o,control}$ (i.e., open probability of unmodified sodium channels) by dividing NP_o by N. Therefore, $P_{o,control}$ represents the open probability of a single channel at the peak of the ensemble recording. $P_{o,control}$ was estimated to be 0.44 ± 0.03 (n = 4) for μ1, 0.44 ± 0.02 (n = 4) for rH1, and 0.48 ± 0.04 (n = 9) for chimeric SHHH Na^+ channels.

Results

Significant Difference between μ1 and rH1 in Sensitivity to GTX I. GTX I induced Na^+ channel modification after conventional application of repetitive depolarizing prepulses (Yakehiro et al., 2000). Figure 1 shows the effects of GTX I on the two Na^+ channel isoforms expressed in HEK cells (μ1 and rH1). After 100 repetitive depolarizing pulses, modified Na^+ channels of either type opened at a potential of around −100 mV and did not inactivate. Without repetitive prepulses, the vast majority of channels opened and inactivated normally, although a slight increase in noninactivating Na^+ current (I_{Na}) at the end of test pulses indicated that a small fraction of the channels were modified during the pulse itself. Because the number of Na^+ channels expressed on each HEK cell was variable, we used the maximum chord conductance as a measure of number of Na^+ channels expressed per cell. The I-V relationships for unmodified sodium currents through μ1 or rH1 isoforms are given in Fig. 1, A and B, ●. A straight line was fitted to peak I_{Na} at membrane potentials from 0 to +60 mV and the chord conductance was estimated from the slope (continuous line). GTX-modified I_{Na} at the end of a 160-ms test pulse, in which unmodified Na^+ channels should have completely inactivated, was plotted against the membrane potential (○). From the slope (dotted line) of the obtained I-V relationship between −50 and +50 mV for GTX-modified I_{Na}, the chord conductance of GTX-modified I_{Na} was estimated as described previously (Yakehiro et al., 2000). To provide a relative measure of GTX I-induced channel modification, we determined the ratio of chord conductances of GTX-modified/unmodified channels. We plotted in Fig. 1C the relative chord conductance for μ1 and rH1 against concentration of GTX I. The dose-response curves for μ1 and rH1 showed marked differences in the extent of GTX I-evoked modification. The values of the relative chord conductance for μ1 and rH1 (with 300 μM GTX I) are listed in Table 1.

Comparison of Kinetic Parameters of Channel Gating among Wild-Type and Mutant Channels. To determine whether the mutations introduced into Na^+ channels affected the gating properties of Na^+ channels, we measured the time constant of I_{Na} decay and the time-to-peak I_{Na} as indices of channel inactivation and activation, respectively. The kinetic properties of the chimeric or point-mutated channels (Fig. 2, Tables 1–3) did not differ significantly from those of wild-type channels and so did not impact measurements of relative chord conductance (Fig. 2).

The Meaning of Relative Chord Conductance. Yakehiro et al. (2000) reported that differences in GTX responsiveness of tetrodotoxin-insensitive (dorsal root ganglion neurons) and tetrodotoxin-sensitive (ventriculomyocytes) whole-cell Na^+ currents are related not to single channel conductance and open-channel probability but to number of Na^+ channels modified. We therefore tested whether the same rationale could apply to differences in GTX sensitivity of μ1, rH1, and SHHH Na^+ currents. Because the whole-cell sodium conductance is the product of three factors, $N \times P_o \times g$, (where N is the number of functioning Na^+ channels in a cell, g is the single-channel conductance), the relative chord conductance is expressed as $(N_{GTX} \times P_{o,GTX} \times g_{GTX})/(N_{control} \times P_{o,control} \times g_{control})$, where subscripts indicate unmodified (control) or GTX-modified channels. Treatment with 100 μM GTX I induced a characteristic, long-lasting opening of single Na channels (Fig. 3A) when rectangular pulses were applied. By applying linear regression analysis (Fig. 3, B and C1) to the records in Fig. 3A, single channel conductance (g_{GTX}) was estimated to be 7.7 ± 1.9 pS (n = 5) for μ1, 9.2 ± 1.1 pS (n = 5) for rH1, and 8.9 ± 2.2 pS (n = 4) for SHHH. Because the channel openings did not overlap one another, open probability ($P_{o,GTX}$) was determined by dividing the total time spent in the open state by the pulse length. Thus, open probability in GTX was estimated to be 0.74 ± 0.09 (n = 5) for μ1, 0.72 ± 0.09 (n = 5) for rH1, and 0.69 ± 0.05 (n = 4) for SHHH (Fig. 3C2). Values for $P_{o,control}$ were also determined, as described previously (Fig. 3C4). There were no statistically significant differences among channel isoforms (includ-

ing chimera) in any of the parameters required to calculate $(P_{o,GTX} \times g_{GTX})/(P_{o,control} \times g_{control})$. Thus, the only tenable explanation for the differences in relative chord conductance (recorded in the whole-cell configuration) lies in the ratio $N_{GTX}/N_{control}$. Therefore, we have concluded that differences in responsiveness of Na^+ channel isoforms to GTX are attributable to the number of channels modified. On this basis, we have justified the use of relative slope conductance as an index of GTX action.

The Tissue Origin of D1 Is a Determinant of the Potency Difference. To determine the structural basis for the marked difference in the sensitivity of μ1 and rH1 to GTX I, we constructed chimeric Na^+ channels and investigated the change in their sensitivity to GTX I. Because we showed previously that the receptor site for GTX is located in domains D1 and D4 (Ishii et al., 1999; Kimura et al., 2000), we thought that these domains should be critical to the difference in potency of GTX I. We therefore constructed two chimeric sodium channels by exchanging D1 domains between μ1 and rH1 and assayed the channels for their relative chord conductance. Each domain of the chimera is referred to by the name of the isoform of origin (H, heart; S, skeletal muscle) beginning at the N terminus of the chimera and proceeding in sequence to the C terminus. Of several chimeric channels made by domain-exchanging, the chimera SHHH (Fig. 1C, ●) was the most sensitive to effects of GTX I (300 μM), giving a relative chord conductance ~1.5 times that of the wild-type μ1. The reverse chimera HSSS had a lower sensitivity than that of the wild-type rH1 isoform (Fig. 1C, ▲), even though all but one

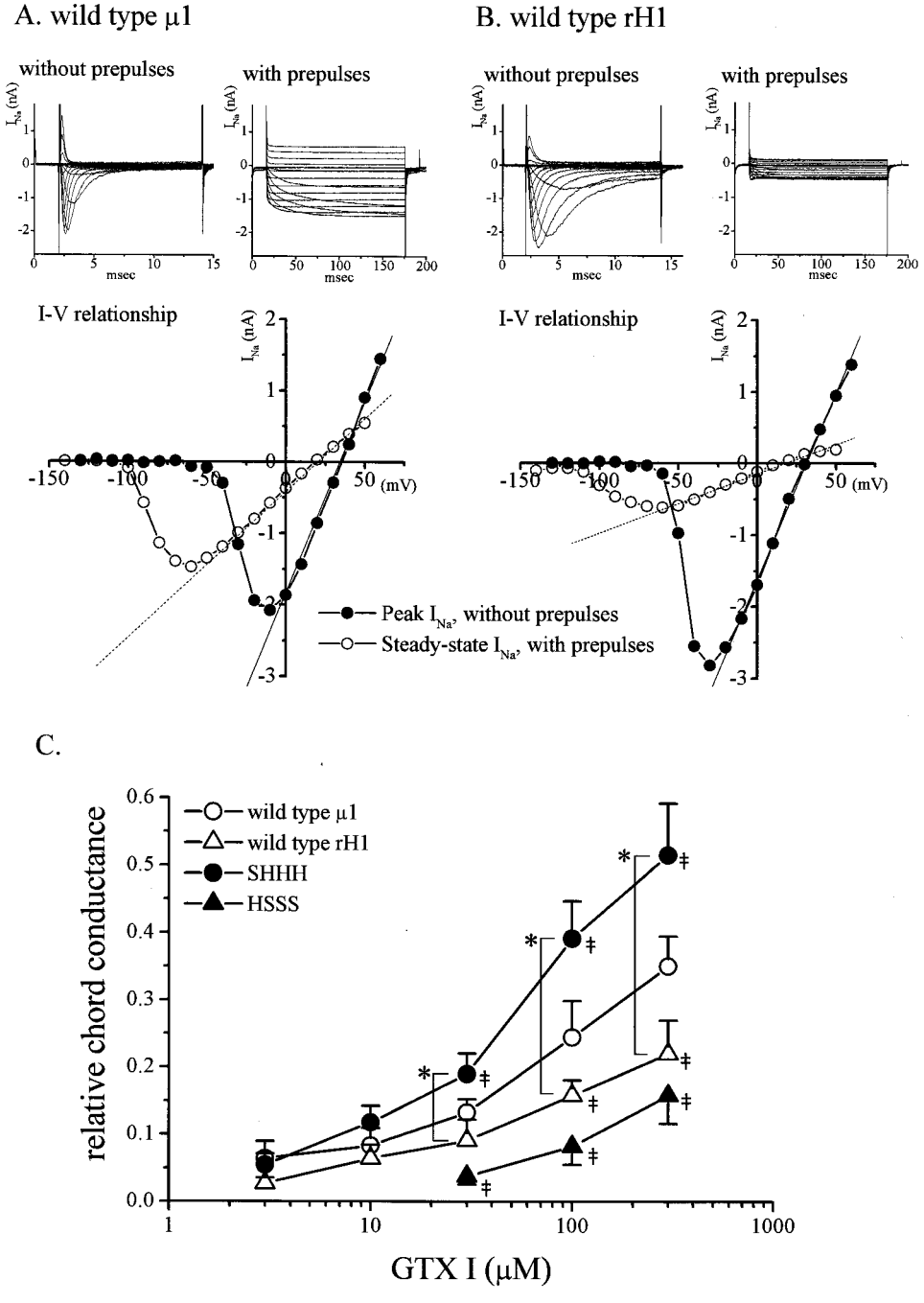
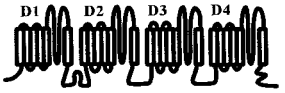
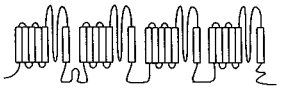
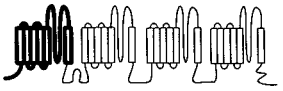
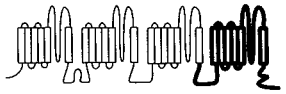
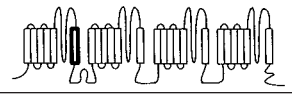


Fig. 1. Differences between μ1 and rH1 isoforms in potency of GTX I. I_{Na} families and I-V relations for unmodified peak I_{Na} and for GTX I-modified steady-state I_{Na} in wild-type μ1 (A) and rH1 (B). Pipette solutions contained 300 μM GTX I. Modified current (open symbols in I-V plots) was induced by a train of 100 conditioning prepulses (pulse potential, −20 mV; pulse duration, 6 ms; holding potential, −120 mV), and assayed with a 160-ms test pulse to variable potential between −140 mV and +60 mV, incremented in 10-mV steps. Predominantly unmodified current (filled symbols in I-V plots) was obtained without application of conditioning prepulses and measured as a peak I_{Na}. C, dose-response curves for GTX I-evoked Na^+-channel modification in the two wild-type isoforms studied and in two chimeric Na^+ channels. The degree of channel modification was expressed as relative chord conductance, as defined in the text. $^*p < 0.01$; statistically significant difference between data pairs indicated in brackets ; $‡p < 0.01$; statistically significant difference compared with wild-type μ1.

TABLE 1
Effect of GTX I on wild-type and chimeric sodium channels

Wild-Type/Chimera	Amino Acid Sequence	Relative Chord Conductance [mean ± S.D. (number of observations)]
Wild-type μ1		0.35 ± 0.04 (n = 9)
Wild-type rH1		0.22 ± 0.05 (n = 8)*
SHHH		0.52 ± 0.08 (n = 6)*
HSSS		0.16 ± 0.04 (n = 6)*
HHHS		0.13 ± 0.04 (n = 3)*
SSSH		Not Expressed
μ1 chim 1		0.23 ± 0.02 (n = 9)*
rH1 chim 1		0.34 ± 0.06 (n = 7)

* $p < 0.01$ vs. wild-type μ1 (Student's t test).

of the domains in this chimera came from the μ1 isoform. We also investigated the chimeras made by exchanging the D4 domain. The chimera HHHS exhibited the lowest GTX sensitivity of all chimeric Na^+ channels tested in this study. The chimera SSSH was not expressed in HEK cells. In Table 1, the values of relative chord conductance (300 μM GTX I) for the three chimeras studied are compared with those of the two wild-type isoforms. The results indicate that the source of D1 is an important determinant of the GTX-I sensitivity of chimeric channels. Hence, we decided to focus our subsequent investigation on sites within domain D1.

Ile-433 in the D1S6 Segment of μ1 Is a Key Molecular Determinant of GTX Sensitivity. Because some residues

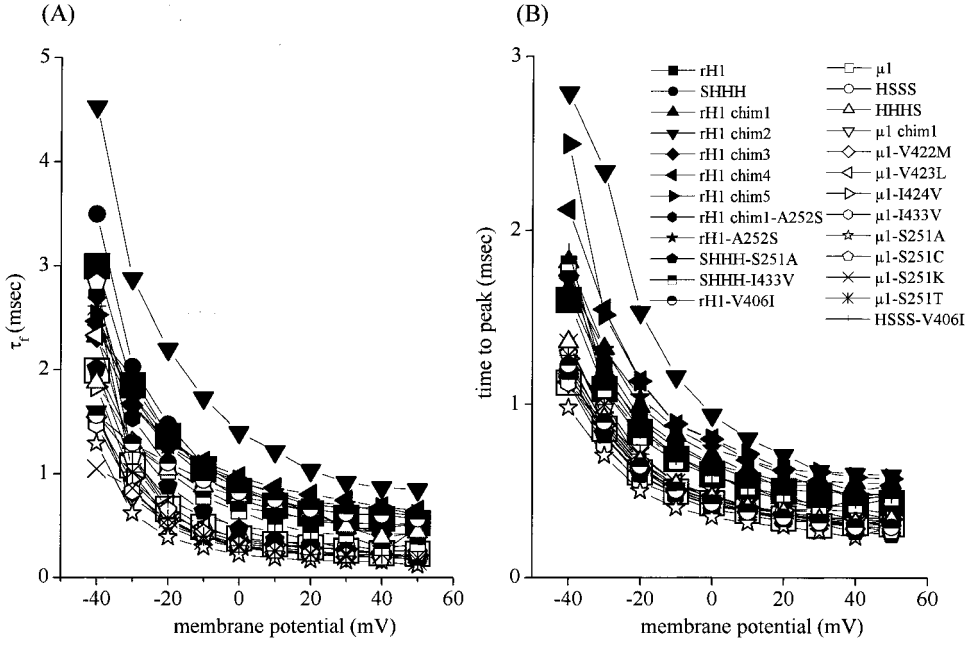


Fig. 2. Similar inactivation and activation kinetics of wild-type and mutant channels. A, relationship between the time constant (τ_f) for the falling phase of I_{Na} and membrane potential. B, relationship between time-to-peak I_{Na} and membrane potential. Symbols used specify names of wild-type and mutant sodium channels listed in Tables 1 to 3. For the sake of clarity, mean values without error bars are given. Number of observations was six for wild-type μ1 or rH1 and four for mutant channels.

critical for GTX-binding are localized to the D1S6 transmembrane segment (Ishii et al., 1999), we next constructed chimeric mutants by exchanging this segment of D1 between μ1 and rH1. The chimera of μ1 (μ1 chim1), constructed by replacement of D1S6 in μ1 with the corresponding segment from rH1, had the same GTX sensitivity as wild-type rH1. In contrast, the relative chord conductance of the reverse chimera (rH1 chim1) was increased to that of wild-type μ1. The shape of the dose-response curve for μ1 chim1 and rH1 chim1 resembled that of the corresponding wild-type isoform (μ1 and rH1, respectively; data not shown). The values of relative chord conductance for both chimeras, at 300 μM GTX I, are shown in Table 1. The findings suggest that at least one critical site for induction of GTX I sensitivity is located somewhere within segment D1S6. Four residues in the amino acid sequence for this region differ between μ1 and rH1. Therefore, we made four chimeras in which amino acid residues in rH1 were substituted for the corresponding residues in μ1. Replacement of Val-422, Val-423, or Ile-424 in μ1 with the corresponding Met, Leu, or Val from rH1 did not alter the GTX sensitivity of the resulting chimeras (Table 2). However, μ1-I433V had a reduced GTX (300 μM) sensitivity, yielding a relative chord conductance value of 0.24 $\pm$ 0.05 (n = 4). Because the introduction of Val into position 433 in μ1 had a deleterious effect on GTX I sensitivity, the reverse mutant, in which Val in the less sensitive rH1

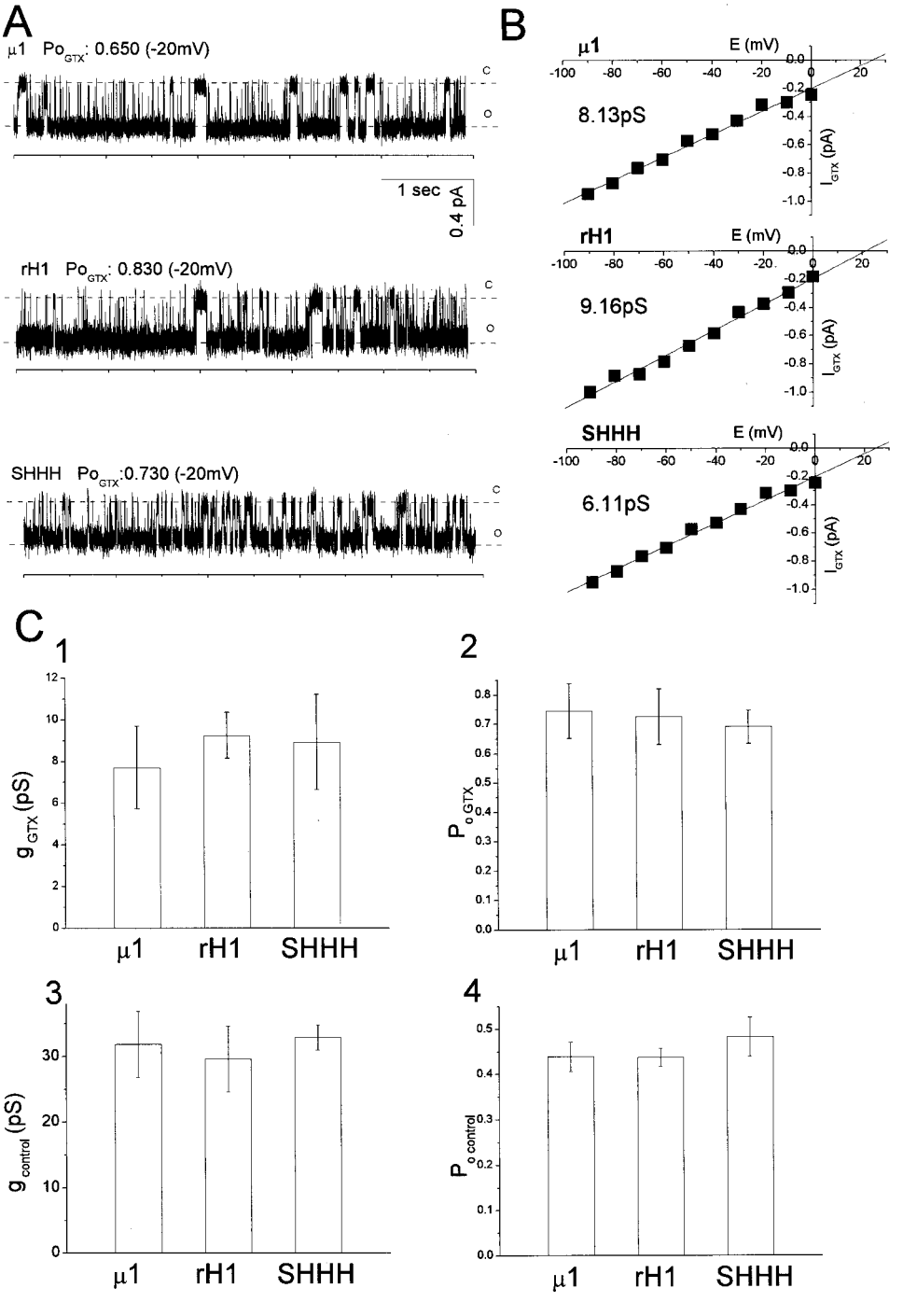


Fig. 3. Analysis of single-channel currents modified by GTX I in rH1, μ1, and mutant SHHH. A, representative recordings of single-channel currents from GTX-modified Na^+ channels, recorded at a membrane potential of -20 mV (holding potential of -120 mV). The closed (c) and open (o) states are indicated. Vertical and horizontal bars in inset indicate 0.4 pA and 1 s, respectively. These recordings were used in computations of single channel conductance and open-state probability. B, the single-channel conductance (in pS) was determined to be 9.16 (rH1), 8.13 (μ1), and 6.11 (SHHH) from best-fitting regression line. C, summary of all data in the three isoforms pertaining to single channel conductance and open probability both of unmodified ("control") and GTX-modified Na^+ channels.

isoform was replaced by Ile from more sensitive μ1 isoform, would have been expected to increase the sensitivity to the same level as that of wild-type μ1. However, that was not the case (Table 2). By contrast, the replacement of Val by Ile at position 406 of the chimera HSSS increased the relative effect of GTX I from 0.16 to 0.37 (Tables 1 and 2). Consistent with this observation, the introduction of I433V into SHHH reduced the relative effect of GTX I from 0.52 to 0.36. These results suggest that the site in the D1S6 segment is one of the major molecular determinants for the difference in potency between the two isoforms.

Ser-251 in the D1S4-S5 Loop of μ1 Is a New Site of Action for GTX I. During this study, we found that chimeric Na^+ channel SHHH is more sensitive to GTX I than wild-type μ1, giving a relative chord conductance of 0.52 ± 0.08 (n = 6) at 300 μM GTX-I (Table 1). This unique feature of SHHH afforded us the opportunity to detect a novel binding-site for GTX on D1 by determining the residue responsible for the marked increase in sensitivity of this chimera. Two chimeric Na^+ channels, rH1 chim2 and rH1 chim3, that each contained a μ1 intracellular-loop (between D1S4 and D1S5) and μ1 P-loop (between D1S5 and D1S6) showed exaggerated GTX-sensitivity similar to that of SHHH (Table 3). Because these findings suggest that sites in μ1 critical to GTX sensitivity should be located within the region extending from the intracellular loop of S4–S5 through S6, we constructed a series of chimeras with systematic substitutions in the D1 segments, as shown schematically in Table 3. rH1 chim4 and rH1 chim5 became less responsive to GTX I and gave a relative chord conductance of 0.38, similar to the value for wild-type μ1. These results suggest that the site determining the potency of GTX I may localize in the intracellular loop of D1S4–S5.

Comparison of the amino acid sequence of D1S4–S5 loop in rH1 with that in μ1 revealed that there is a difference of only one amino acid residue: Ala-252 in rH1 and Ser-251 in μ1. The introduction of Ser into position 252 of rH1 chim1 (rH1 chim1-A252S) markedly increased the relative chord conductance from 0.34 (rH1 chim1) to 0.50 (rH1 chim1-A252S) (Tables 1 and 3). Another line of evidence supportive of the involvement of Ser came from an experiment with the point-mutant rH1-A252S. This mutation increased the relative effect of GTX I from 0.22 to 0.35, which is near that of wild-type μ1 (Table 3). In the reverse mutant, μ1-S251A, the effect of GTX I was diminished to the level of wild-type rH1 (Fig. 4). Consistent with this result, introduction of S251A into SHHH also reduced the effect of GTX I to the level found in wild-type μ1. These results indicate that a site in the D1S4-S5 loop is another major molecular determinant of the difference in potency between the two Na^+ channel isoforms.

Discussion

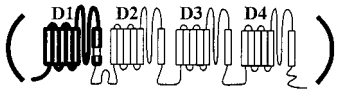
We showed that successive substitution of the Ser-251 and Ile-433 residues of μ1 channels and related mutants for the corresponding Ala and Val residues in rH1 channels produced stepwise reductions in relative chord conductance from 0.52 to 0.35 and finally to 0.22. These data arguably limit the site of action for GTX I to two loci within the D1S6 transmembrane segment and D1S4-S5 loop.

From the structure-activity relationship of GTX, we have deduced that the hydrophobicity of the α-surface of the GTX molecule is essential for GTX activity, because covalent modification of the α-surface, through addition of hydrophilic NH_2 groups, drastically reduced the potency of GTX (Masutani et al., 1981; Tsuji et al., 1991; Yakehiro et al., 1993). We also have shown that the binding sites for GTX on the μ1 Na^+-channel isoform include hydrophobic residues Ile-433, Asn-434, and Leu-437 in D1S6, and Ile-1575, Phe-1579, and Tyr-1586 in D4S6 (Ishii et al., 1999; Kimura et al., 2000). Thus, it is probable that hydrophobic interaction between the α-surface of GTX and the specified sites in D1 and D4 plays an important role in GTX binding. Because replacement of the amino acid at position 433 (μ1) or 406 (rH1) with a different lipophilic residue should not alter the hydrophobicity of the local chemical environment, reduction in sensitivity

TABLE 2

Effect of GTX I on wild-type and mutant sodium channels

Amino acids located in the transmembrane segment S6 of D1 are represented. The numbers of amino acids for each wild-type isoform are given on the right-hand side of the column.

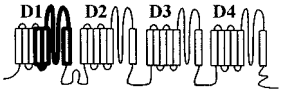
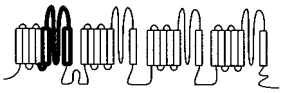
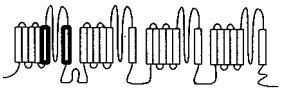
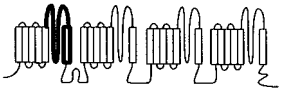
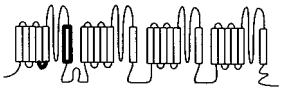
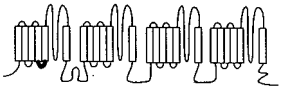
Wild-type/point mutant	Amino Acid Sequence	Relative Chord Conductance [mean ± S.D. (number of observations)]
Wild-type μ1	MIFFVVIIFLGSFYLINLILAVVA418–441	0.35 ± 0.04 (n = 9)
μ1-V422M	———M———————————	0.32 ± 0.04 (n = 8)
μ1-V423L	————L———————————	0.37 ± 0.09 (n = 6)
μ1-I424V	—————V———————————	0.31 ± 0.07 (n = 4)
μ1-I433V	————————————V———	0.24 ± 0.05 (n = 4)*
Wild-type rH1	MIFFMLVIFLGSFYLVNLILAVVA391–414	0.22 ± 0.05 (n = 8)*
rH1-V406I	————————————I———	0.21 ± 0.06 (n = 6)*
SHHH-I433V	MIFFVVIIFLGSFYLVNLILAVVA	0.36 ± 0.06 (n = 3)
		
HSSS-V406I	MIFFMLVIFLGSFYLIVNLILAVVA	0.37 ± 0.06 (n = 4)
		

* $p < 0.01$ vs. wild-type μ1 (Student's t test).

TABLE 3

Effect of GTX I on wild-type and chimeric sodium channels

Amino acids located in D1S4–S5 intracellular loop of both isoforms are as follows (the numbers represent amino acid sequence numbering from N terminus): rH1, KTIVGALIQSVKKLAD (238–253); μ1, KTIVGALIQSVKKLSD (237–252). Ala-252 in rH1 corresponds to Ser-251 in μ1.

Wild-Type/Chimera	Amino Acid Sequence	Relative Chord Conductance [mean ± S.D. (number of observations)]
rH1 chim2		0.54 ± 0.07 (n = 5)*
rH1 chim3		0.56 ± 0.08 (n = 4)*
rH1 chim4		0.38 ± 0.01 (n = 3)
rH1 chim5		0.38 ± 0.02 (n = 3)
rH1 chim1-A252S		0.50 ± 0.06 (n = 3)*
rH1-A252S		0.35 ± 0.03 (n = 3)

* $p < 0.01$ vs. wild-type μ1 (Student's t test).

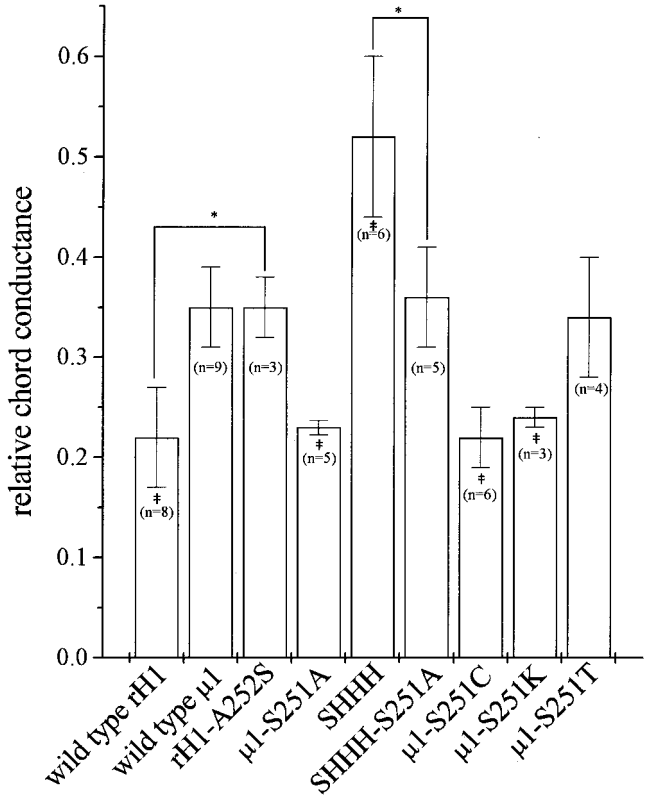


Fig. 4. Effect of GTX I on wild-type and mutant Na^+ channels. Degree of Na^+-channel modification at 300 μM GTX-I is expressed as relative chord conductance. *$p < 0.01$; statistically significant difference between data pairs indicated in brackets ; ‡$p < 0.01$; statistically significant difference compared with wild-type μ1.

to GTX I caused by replacement of Ile by Val in D1S6 can perhaps be attributed to spatial distortion of residues in the GTX binding pocket. The difference in GTX sensitivity (see Fig. 4) caused by replacement of Ser-251 (μ1) or Ala-252 (rH1) can be accounted for as follows. The hydroxyl group on the substituent residue could stabilize GTX binding through hydrogen-bonding with hydroxyl groups on the β-surface of the GTX molecule, leading to more efficient chemical coupling between the GTX-binding residues and the gating region of Na^+ channel. Hence, in the μ1 isoform, hydroxyl-containing residues (Ser or Thr) at position 251 effected higher GTX sensitivity than either Cys or Lys substituents (Fig. 4).

Recent evidence increasingly points to active involvement of segments D1S4 and D1S6 in regulation of both channel activation and inactivation. First, Stühmer et al. (1989) showed that neutralization of positive charge on S4 segments D1S4 and D2S4 induced a shift in the voltage dependence of activation. Second, Kontis et al. (1997) reported that charge-neutralizing and -conserving mutations of the S4 segment resulted in a large positive shift of half-maximal activation voltage, a significant reduction in gating valence, and substantial depolarizing shifts in the voltage dependence of the activation or deactivation rate. Third, in a painful form of congenital myotonia, substitution of Met for Val at position 445 in the D1S6 segment of the human skeletal-muscle Na^+ channel has recently been reported to induce a small noninactivating current during a brief test depolarization, a hyperpolarizing shift in the voltage-dependence of channel activation, and a slowing of the time course of recovery from inactivation (Takahashi and Cannon, 1999; Wang et al., 1999). Fourth, the voltage-dependent conformational change

of D1S4 (monitored by fluorescent probe tetramethylrhodamine-5-maleimide covalently bound to Cys-216) was kinetically very rapid compared with activation and deactivation of the fast Na^+ current (Cha et al., 1999), suggesting that the S4 segment as a whole moves outwardly upon membrane depolarization. Considering that the main pharmacological effects of GTX I on Na^+ channels are 1) a hyperpolarizing shift of the activation curve and 2) suppression of Na^+ inactivation, the site in the D1 S4-S5 linker that we have uncovered in this study can reasonably be suggested to have a connection with gating function. Hydrogen bonding at position 251 apparently is not essential for GTX binding, because GTX still interacts with the rH1 isoform, which lacks a hydrophilic residue at that position.

Pyrethroids exert pharmacological effects on Na^+ channels, which, in some respects, are similar to GTX I: pyrethroids prolong the open state of the Na^+ channel and slow the kinetics of both activation and inactivation. It has been reported that Na^+ channels with a Val-to-Met point mutation in D1S6 at position 421 (*Heliothis virescens*; Park et al., 1997) or an Ile-to-Asn point mutation in the intracellular loop of D1S4-S5 at position 265 (*Drosophila melanogaster*; Pittendrigh et al., 1997) gain pyrethroid resistance. It is intriguing to note that Val-421 in *H. virescens* and Ile-433 in μ1 are in coincident positions and that Ile-265 in *D. melanogaster* and Ser-251 in μ1 are in nearly corresponding positions.

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