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On site of action of grayanotoxin in domain 4 segment 6 of rat skeletal muscle sodium channel

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Abstract Grayanotoxin I (GTX I) is a diterpenoid extracted from the family of *Ericaceae* that binds to Na^+ channels and causes persistent activation. We investigated the interaction of GTX I with the amino acid residues I1575, F1579 and Y1586 in transmembrane segment D4S6 of μ1. In F1579A, GTX shifted the threshold potential about 50 mV in the hyperpolarizing direction and modified Na^+ channels twice as efficiently as that in wild-type. In contrast, these GTX-effects were eliminated completely in the I1575A mutant and were reduced substantially in mutant Y1586A. Lysine substitution for F1579 significantly reduced and for Y1586 completely eradicated the GTX-effect. Our data suggest that the GTX receptor site shares overlapping but non-identical molecular determinants with BTX in D4S6 and has common molecular determinants in D1S6.

Key words: Grayanotoxin; Binding site; Na^+ channel; Batrachotoxin; Domain 4 segment 6

1. Introduction

Modification of a certain function of Na^+ channels by biological toxins is so specific that it is utilized as a pharmacological probe for clarifying the functional structure of Na^+ channels. Among biological toxins, the so-called lipid soluble toxins, such as GTX, BTX, veratiridine and aconitine, are endowed with some characteristics in common: (1) they bind to the Na^+ channel in its open state; (2) the modified Na^+ channels lose the inactivation process; and (3) the activation voltage of the modified Na^+ channels shifts to the direction of hyperpolarization [1]. The effects of these toxins suggest that their binding site may contain amino acids that are crucial for both activation and inactivation gating mechanisms. Recent site-directed mutagenesis studies of μ1 showed that replacement of I433, N434 or L437 within D1S6 with the positively charged residue, lysine, abolishes the BTX- and GTX-effects, suggesting that these amino acid residues in D1S6 are critical components of both the GTX and BTX receptor site [2,3]. Furthermore, aware of the allostric interaction in RBIIA between local anesthetics and BTX, Linford et al. [4] replaced the amino acid residues of the binding sites for local anesthetics in D4S6 one by one with alanine, and showed that I1760 and F1764 are other possible binding sites for BTX. Wang and Wang [2] provided other supportive evidence for the BTX binding site in D4S6: replacement of F1579 in μ1 (corresponding to F1764 in RBIIA) with lysine also abolishes the BTX-effect, but the same replacement of Y1586 (homologous to Y1771 in RBIIA) does not.

In this study, three amino acids in I1575, F1579 and Y1586 in D4S6 in μ1 were converted to either alanine or lysine, and the resulting mutants were tested for the GTX-effect by transient expression in HEK 293. GTX failed to modify the I1575A and Y1586K mutants and was much less effective in the F1579K and Y1586A mutants than in WT, but it was twice as effective in F1579A, indicating that the GTX receptor sites do not coincide with the BTX sites but partially overlap them. The significance of the difference in the sensitivities of these mutants between GTX and BTX will be discussed.

2. Materials and methods

2.1. Construction of site-specific mutants

The α-subunit of rat skeletal muscle Na^+ channel, μ1 cDNA [5], was cloned into the *Eco*RI site of a mammalian expression vector pcDNA3.1(-) (Invitrogen) and pcDNAμ1 was constructed. To produce site-specific mutants of transmembrane segment D4S6 in μ1, firstly, a unique endonuclease restriction site, the *Hpa*I site, was created newly in the cDNA of μ1 by means of a transformer site-directed mutagenesis kit (Clontech). The mutagenesis primer containing the *Hpa*I site (underlined) was designed as 5′-CTTCCTCATCGTGGTTAACATGTACATTGCT, corresponding to 4734–4764 of μ1 cDNA. In this case, there is no change of the μ1 amino acid sequence by altered nucleotides. pcDNAμ1-I1575A, μ1-I1575K, μ1-F1579A and μ1-F1579K were produced using a PCR-based mutagenesis method. One forward primer F1 (5′-CCGCGGGGCCAAGGGCATCCG) and four mutagenic reverse primers (I1575A, I1575K, F1579A and F1579K) were designed for producing site-specific mutants. These mutagenic primers, with the nucleotides altered for creating a *Hpa*I site underlined and for producing a desired mutant set in bold, were as follows:

I1575A: 5′-GTTAACCACGATGAGGAAGGAGATGATG**GC**GTAGCT

I1575K: 5′-GTTAACCACGATGAGGAAGGAGATGAT**TTT**GTAGCT

F1579A: 5′-GTTAACCACGATGAGG**GC**GGAGAT

F1579K: 5′-GTTAACCACGATGAG**TTT**GGAGAT

These resulting PCR products correspond to nucleotides 4365–4752 of μ1 cDNA, and have *Sac*II and *Hpa*I sites at the 5′- and the 3′-ends, respectively. They were cloned by replacing the unique *Sac*II-*Hpa*I

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Abbreviations: BTX, batrachotoxin; D, domain; GTX, grayanotoxin; HEK 293, the tsA-201 subclone of human embryonic kidney cells; *I-V* curve, current-voltage relationship; μ1, α-subunit of rat skeletal muscle Na^+ channel; RBIIA, α-subunit of rat brain type II Na^+ channel; S, segment; WT, wild-type; each mutant channel will be referenced by the original amino acid followed by its number and introduced amino acid

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fragment of μ1 cDNA into pcDNAμ1 to produce the desired site-specific mutants. pcDNAμ1-Y1586A and μ1-Y1586K were also produced using a PCR-based mutagenesis method. One reverse primer R1 (5′-GAATTCGATTCGTCTTTGTCT) and two mutagenic forward primers (Y1586A and Y1586K) were designed for producing site-specific mutants. These mutagenic primers, with the nucleotides altered for creating a *Hpa*I site underlined and for producing a desired mutant set in bold, were as follows:

Y1586A: 5′-GTTAACATG**GC**CATTGCTATC

Y1586K: 5′-GTTAACATG**AAA**ATTGCTATC

These resulting PCR products correspond to nucleotides 4747–5827 of μ1 cDNA plus GAATTC (*Eco*RI site), and have *Hpa*I and *Eco*RI sites at the 5′- and the 3′-ends, respectively. They were cloned by replacing the unique *Hpa*I-*Eco*RI fragment of μ1 cDNA into pcDNAμ1 to produce the desired site-specific mutants.

On the site-specific mutants of transmembrane segment D1S6, pcDNAμ1-I433A, μ1-N434A and μ1-L437A were produced in the same manner as in our previous report [3]. Three mutagenic reverse primers (I433A, N434A and L437A) were designed for producing the desired mutants. These mutagenic primers, with the altered nucleotides set in bold, were as follows:

I433A: 5′-CGTACGCCATGGCCACCACGGCCAGGATCAGATTG**GC**GAGGT

N434A: 5′-CGTACGCCATGGCCACCACGGCCAGGATCAG**AGC**GATGAGGT

L437A: 5′-CGTACGCCATGGCCACCACGGCC**GC**GATCAGATTGATGAGGT

All of the resulting mutants were sequenced to confirm the nucleotide change using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems).

2.2. Transient transfection and cell culture

The constructed plasmids described above were co-transfected with CD8 cDNA transiently into HEK 293 using SuperFect Transfection Reagent (Qiagen). The cells were grown to 50% confluence in DMEM (Gibco) containing 10% fetal bovine serum (Bio-whittaker), 30 units/ml penicillin G (Gibco) and 30 μg/ml streptomycin (Gibco) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The transfected cells were used for electophysiological experiments as late as 3 to 4 days after replating in 35-mm tissue culture dishes. Transfection-positive cells were identified by CD8-Dynabeads (Dynal) before the Na^+ current recording.

2.3. Electrophysiological recording

Macroscopic sodium currents from the transfected cells were measured with a patch clamp method. The bath solution contained (in mM): 70 NaCl, 67 *N*-methyl-D-glucamine, 1 $CaCl_2$, 1.5 $MgCl_2$, 10 glucose and 5 HEPES (pH 7.4). The pipette solution contained (in mM): 70 CsF, 60 CsCl, 12 NaF, 5 ethylene-bis(oxonitrilo)tetraacetic acid and 5 HEPES (pH 7.4). To assess the effects of GTX on whole-cell sodium currents, 300 μM grayanotoxin I, a biologically active analogue, was added to the pipette solutions, because GTX is known to act intracellularly [6].

Data were presented as means ± S.D. (number of observations), unless otherwise stated.

3. Results

3.1. Comparison of kinetic parameters of gating mechanism among WT and mutant Na^+ channels

Because a mutation introduced into Na^+ channels may be able to change their gating properties of Na^+ channels, the electrophysiological properties of WT and all mutants transiently expressed in HEK 293 were analyzed by a whole-cell voltage clamp. Typical examples of Na^+ currents for WT and F1579A are displayed in Fig. 1A1 and A2. Depolarization of the transfected cells gave rise to a rapidly activating and inactivating inward current through the Na^+ channels. For WT and all mutants, the currents appeared at −60 mV and reached a maximum at around −20 mV, as shown in *I-V* curve in Figs. 2C and 3. The reversal potentials for peak transient currents in μ1 WT and mutants were consistent with the value calculated by the Nernst equation. From

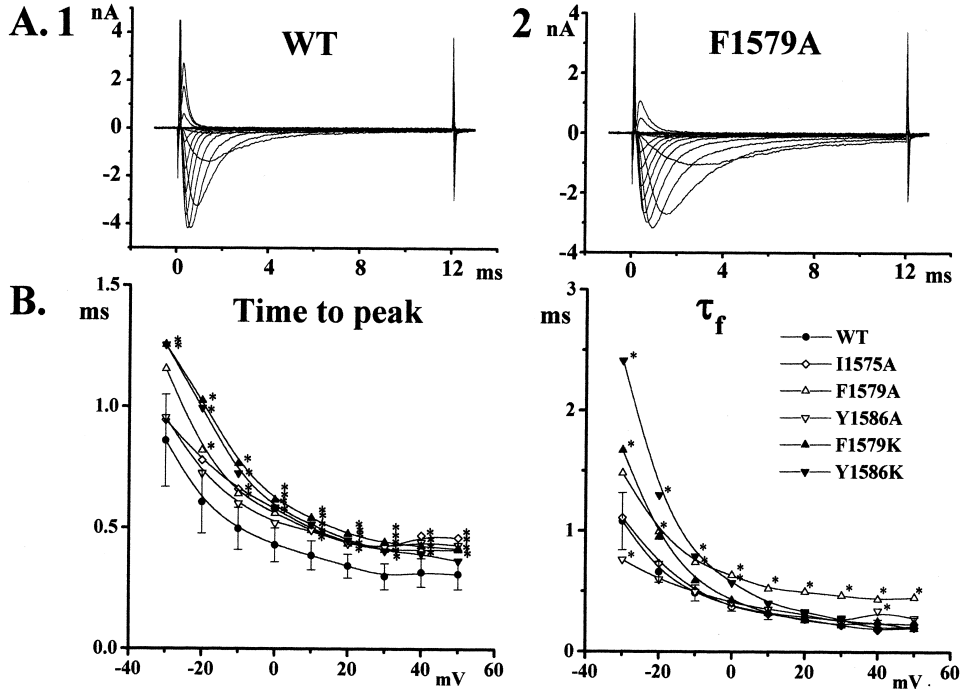

Fig. 1. Effect of the introduction of mutations into μ1 on the kinetic properties of Na^+ channels. A: Original traces of Na^+ currents from WT (1) and F1579A (2). Holding potential of −120 mV. Test pulses were given in 10 mV increments from −130 mV to 60 mV for WT and F1579A. B: (1) The relationship between the time to peak and the membrane potentials and (2) that between τ_f and the membrane potentials. Symbols used are listed in inset. For the sake of clarity, error bars are listed only in WT. Statistical significance for differences in the means ($P < 0.05$) is expressed as an asterisk. Number of observations was six for WT and four for the rest of them.

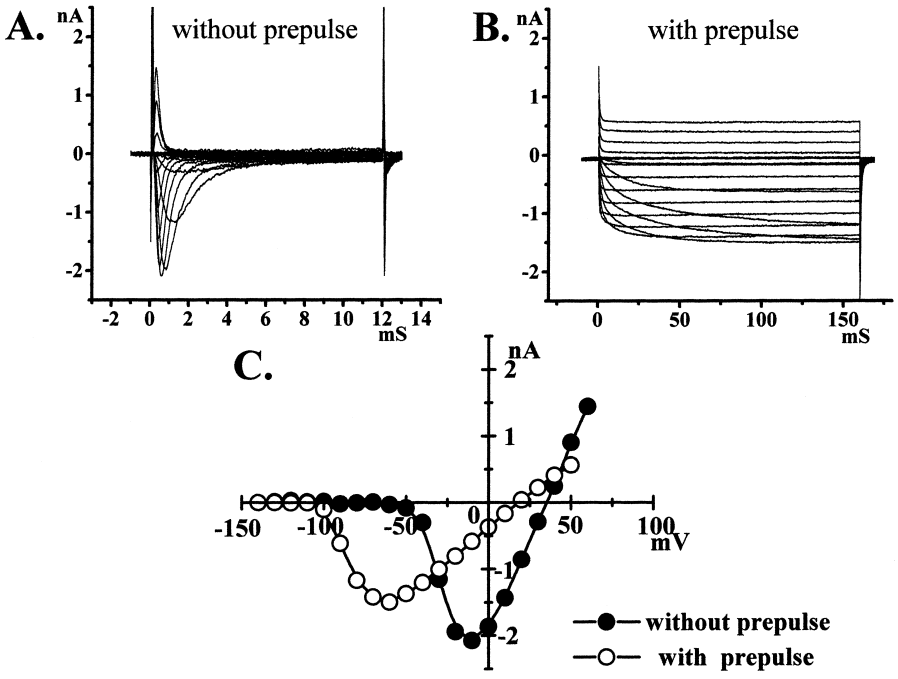

Fig. 2. GTX modification in WT. Original traces of Na^+ currents without (A) and with (B) conditioning pulses. Because GTX modifies Na^+ channels only in the open state, care was exercised to eliminate accumulated inactivation of sodium channels during repetitive conditioning pulses. Recovery from inactivation was measured using a two-pulse protocol, with conditioning and test pulses separated by a variable time interval. The time course of recovery from inactivation could be described as a single exponential, giving the time constant of 1.1 ± 0.3 ms ($n = 3$) for WT, 3.8 ± 1.1 ms ($n = 3$) for I1575A, 1.6 ± 0.3 ms ($n = 4$) for F1579A, 1.6 ± 0.2 ms ($n = 3$) for Y1586A, 1.9 ± 0.5 ms ($n = 5$) for 1579K and 114.9 ± 21.8 ms ($n = 4$) for Y1586K. As time constants for all mutants except Y1586K were similar, we set the time interval of 20 ms during repetitive pulsing. Because the recovery from inactivation in F1586K was unusually slow, giving a time constant of 114.9 ms, to induce GTX modification the time interval during repetitive conditioning pulses was set to be 500 ms in this particular mutant. Depolarizing pulses were triggered every 10 s. In A and B, holding potential of −120 mV. Test pulses were given in 10 mV increments from −130 mV to 50 mV. In C, amplitudes of transient current (closed circles) and of sustained current measured at the end of 160 ms test pulse (open circles) in 10 mV increments from −140 mV to 50 mV are plotted against membrane potentials.

Na^+ current records, the time to peak was measured as representative of the activation and the time constant for the falling phase (τ_f) as that of the inactivation process. As shown in Fig. 1B, the time to peak in most mutants is large, compared with those for WT channels and τ_f for F1579A and Y1586K is larger than that for WT channels.

3.2. Modification of Na^+ channels in WT and mutants by GTX

Including 300 μM GTX in the pipette had a marginal effect without repetitive stimulation. An impairment of fast inactivation in WT induced by GTX during a single test pulse appeared as a small persistent Na^+ current at the end of pulses of 12 ms (Fig. 2A). When 300 repetitive pulses to −20 mV of 6 ms in duration from a holding potential of −120 mV were applied, GTX drastically altered the functional properties of voltage-gated Na^+ channels (Fig. 2B). Modification by 300 μM GTX I was manifested by a loss of peak sodium currents and an increase in sustained currents which started to appear at a large negative membrane potential. The sustained currents reached a maximum at −50 mV and the reversal potentials for slow currents shifted a little extent to the hyperpolarizing direction, compared with that for peak transient currents. In moderately modified mutant channels such as Y1586A, persistent currents were accompanied by a decrease in peak current. In the case of mutants which completely lost their sensitivities to GTX, such as F1579K and Y1586K, the *I-V* curves before and after repetitive conditioning pulses remained unchanged in the presence of GTX.

Because the number of sodium channels expressed is different from cell to cell, it is necessary for GTX-modified Na^+ channels to be normalized to a representative factor of normal channel in an active state. We chose the maximum chord conductance for sodium channel without repetitive pulses, and measured it by adopting a straight line to data between 0 mV and 50 mV. Although GTX I was able to modify Na^+ channels during single depolarizing pulses to open channel, the modification of Na^+ channels was negligible, and was seen as an incomplete shutdown of Na^+ channels (Fig. 2A as compared with Fig. 1A1). Thus, it is reasonable to employ a family of Na^+ currents without repetitive stimulation as a reference. The chord conductance for GTX-modified sodium channel was obtained by adopting a straight line to the data for slow sustained current between −50 mV and 50 mV. The degree of GTX-evoked modification of Na^+ channels was estimated as the relative chord conductance for sustained Na^+ currents following conditioning prepulses, in relation to the maximum chord conductance. Results are summarized in Table 1.

4. Discussion

Through our site-directed mutagenesis study, three items of intriguing information were obtained: on the substitution with alanine of the three sites in μ1 homologous to those in RBIIA which Linford et al. [4] had manipulated, first, the mutants of I1575A and Y1586A, homologous to I1760A

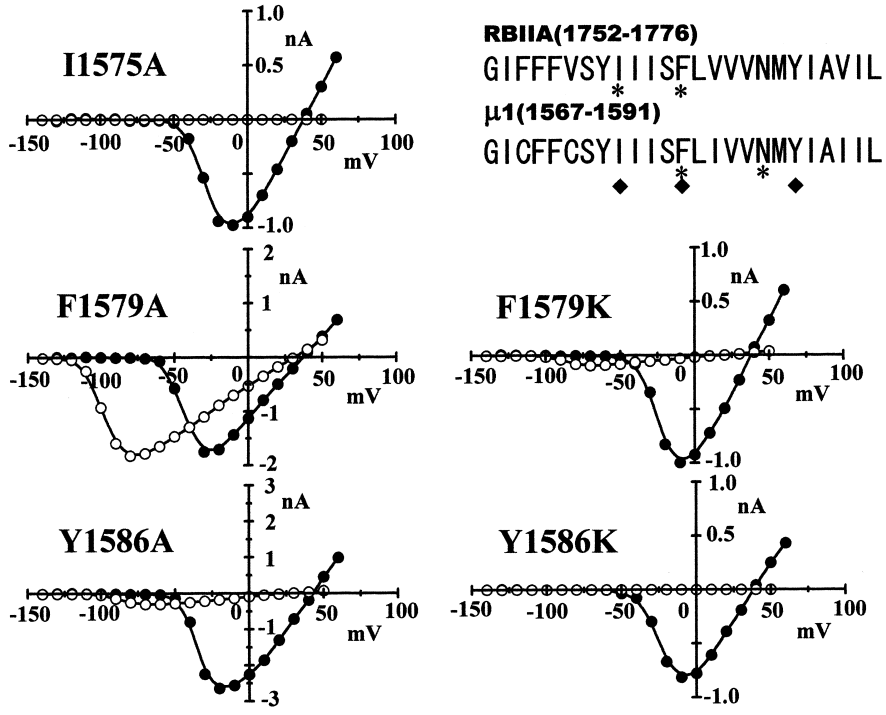

Fig. 3. *I-V* curves for peak current without conditioning pulses (closed circles) and steady currents with conditioning pulses (open circles) in various mutants. The pulse protocol for these records was the same as that given in Fig. 2. The mutants tested are specified in each graph abbreviated form. The inset figure shows the amino acid sequences for D4S6 of RBIIA and μ1 (the numbers represent amino acid sequence numbering from the beginning). Asterisks (*) for rBIIA and for μ1 indicate the sites of action for BTX claimed by Linford et al. [4] and by Wang and Wang [2], respectively. Diamonds (♦) for μ1 indicate the site for GTX.

and Y1771A in RBIIA, resulted in a mutant with a totally lost and with a substantially reduced sensitivity to GTX I, respectively, second, the mutant of F1579A, homologous to F1764A in RBIIA, was found to increase the GTX-effect significantly, and third, on the replacement of these sites by lysine, μ1-Y1586K became totally resistant to GTX I and GTX I was a less effective modifier of mutant μ1-F1579K than of WT channels. These observations imply that these three sites are involved in GTX binding. The loss and extensive reduction respectively of sensitivity to GTX on the introduction of alanine into I1575 and Y1586 indicate that these sites may be directly bound by GTX. Meanwhile, the enhancement of the GTX-effect seen in F1579A may well suggest the easy access of GTX to the binding site due to the reduction of steric hindrance by the side chain. McPhee et al. [7] reported that in mutant F1764A in RBIIA, homologous to mutant F1579A, the stability of the inactivated state is impaired, because substantial sustained Na^+ currents at the end of 30 ms depolarization were observed. As shown in Fig. 1, the kinetic parameters for both activation and inactivation in this mutant also belong to the most sluggish group. An alternative explanation for the augmented modification in F1579A is that the Na^+ channels, having an unstable inactivation state, might be transferred more easily to those in a GTX-modified state.

Because the structure-activity relation for GTX binding revealed that GTX requires a condition favorable for hydrophobic and hydrogen bonding for exerting pharmacological action [8–10], introduction of hydrophilic lysine into the sites critical for GTX binding is thought to disturb the hydrophobic microenvironment around these sites and lead to loss of the GTX-effect. A typical example is that, by changing the substituent from A to K in F1579, the relative conductance for F1579A of twice bigger than that for WT decreased to a marginal value. A similar trend, though to a lesser extent, was also recognized in the case of Y1586 (see Table 1). Taken altogether, point mutation experiments using two different substituents with distinctive chemical properties gave a consonant conclusion for GTX binding sites in D4S6.

Linford et al. [4] showed that, when amino acids falling on the same face of the predicted D4S6 α-helix, I1760, F1764 and Y1771 in RBIIA, were substituted by alanine, the BTX-effects were eliminated completely for the F1764A mutant, were reduced substantially for mutant I1760A, but remained unchanged in Y1771A. From these observations they suggested that the BTX receptor sites are localized in I1760 and F1764 in transmembrane segment D4S6. Wang and Wang [2] suggested that D4S6 has a possible involvement in BTX binding, using the lysine substitution method. Our results agree with

Table 1
Relative chord conductance in μ1 and its mutants

	μ1	I1575A	F1579A	Y1586A
mean ± S.D. (number)	0.35 ± 0.04 (n = 9)	0.00 (n = 3)	0.75 ± 0.16 (n = 5)	0.06 ± 0.16 (n = 3)
		I1575K	F1579K	Y1586K
mean ± S.D. (number)		not expressed	0.04 ± 0.01 (n = 3)	0.00 (n = 3)

the general scheme presented by Linford et al. [4], though with some minor differences in the crucial sites for BTX from those for GTX.

As for alanine substitution at the sites in which we have an interest, Wang and Wang [2] reported that F1579A and Y1586A in μ1 are BTX-sensitive. We independently clarified that μ1-I1575A, a mutant so far unexplored, is also as sensitive to 10 μM BTX as WT (data not shown). This finding indicates that the introduction of alanine into these sites in μ1 does not affect the BTX-effect. However, the substitution of lysine for F1579 eliminated the BTX-effect, but substitution of the same amino acid for Y1586 did not. The disparity of the conclusion on the role of F1579 in BTX binding in μ1 between the alanine and lysine substitution methods is problematic. A possible explanation is that BTX molecules fail to get access to the binding sites, which are located elsewhere, because of perturbation of the microenvironment around nearby binding sites. To confirm this supposition, we extended our study on the effect of GTX I to three mutants made by alanine substitution, I433, N434 and L437, which were thought to be the binding sites using lysine substitution [3]. The relative conductance was estimated to be 0.09 ± 0.02 ($n = 4$) for I433A, 0 ($n = 2$) for N434A and 0.33 ± 0.10 ($n = 4$) for L437A. An extensive reduction of sensitivity was recognized in I433A and N434A in accordance with the results previously obtained [3], but the relative conductance for L437 was as large (0.35) as for WT. As mentioned earlier, a similar change was recognized on switching the substituent from A to K in F1579 (see Table 1). As far as D1S6 and D4S6 are concerned, the possible binding sites of I433 and N434 in D1S6 and I1575A in D4S6 are aligned right next to the sites of L437 and F1579 under discussion, if the secondary structure of these segments is α-helical. Because the introduction of lysine into L437 and F1579 changes the local environment extensively, hydrophilic moiety lysine may well cover the binding sites and GTX fails to gain access. Although μ1-N1584 was claimed to be a site of action of BTX [2], the same rationale may account for the apparent unlikelihood of this claim, since it was also recognized that alanine substitution does not induce a complete BTX-resistant channel.

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