Distinct sites regulating grayanotoxin binding and unbinding to D4S6 of $Na_v1.4$ sodium channel as revealed by improved estimation of toxin sensitivity

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Running title: Differential roles of the sites affect GTX binding in $Na_v1.4$

SUMMARY

Grayanotoxin (GTX) exerts selective effects on voltage-dependent sodium channels by eliminating fast sodium inactivation and causing a hyperpolarizing shift in voltage-dependency of channel activation. In this study, we adopted a newly developed protocol that provides independent estimates of the binding and unbinding rate constants of GTX (k_{on} and k_{off}) to GTX-sites on the sodium channel protein, important in the molecular analysis of channel modification. Novel GTX-sites were determined in D2S6 (N784) and D3S6 (S1276) by means of site-directed mutagenesis; the results suggested that the GTX receptor consists of the S6 transmembrane segments of four quasi-homologous domains facing the ion-conducting pore. We systematically introduced at two sites in D4S6 ($Na_v1.4$-F1579 and $Na_v1.4$-Y1586) amino acid substituents with residues containing hydrophobic, aromatic, charged, or polar groups. Generally, substitutions at F1579 increased both k_{on} and k_{off}, resulting in no prominent change in dissociation constant (K_d). It seems that the smaller the molecular size of the residue at $Na_v1.4$-F1579, the larger the rates of k_{on} and k_{off}, indicating that this site acts as a gate regulating access of toxin molecules to a receptor site. Substitutions at Y1586 selectively increased k_{off} but had virtually no effect on k_{on}, thus causing a drastic increase in K_d. At position Y1586, a hydrophobic or aromatic amino acid side chain was required to maintain normal sensitivity to GTX. These results suggest that the residue at position Y1586 has a more critical role in mediating GTX binding than the one at position F1579. Here, we propose that the affinity of GTX to $Na_v1.4$ sodium channels might be regulated by two residues (Phe and Tyr) at positions F1579 and Y1586, which respectively control access and binding of GTX to its receptor.

INTRODUCTION

Biological toxins which act selectively on the voltage-dependent sodium channels have been used in order to elucidate molecular mechanisms of sodium channel gating processes. Batracotoxin (BTX), grayanotoxin (GTX), veratridine (VTD) and aconitine, classified as Site 2 toxins (1), are endowed with some characteristics in common (2-5): 1) they bind to the sodium channel in its open state; 2) the modified sodium channel lacks the inactivation process; and 3) the activation potential of the modified sodium channel is shifted in the direction of hyperpolarization. Among the well characterized toxins, GTX has distinct advantages in that the relevant interaction sites on the GTX molecule with the sodium channel are known as a result of extensive analyses of its structure-activity relationship (6-8). The methyl group of the molecule's β-surface at C-10 and the hydroxyl groups of its β-surface at C-3, C-5 and C-6 are essential to the pharmacological action of GTX, and the hydrophobic microenvironment of the α-surface, of which C-14*S* is most critical, greatly contribute to increased GTX potency (see Fig. 2*A*). Moreover, the GTX action has a unique dependency on the state of the sodium channel: although BTX binds to, and dissociates from the channel in its open state (9), GTX binds only to the open channel and dissociates from the closed channel (10).

The main structural component of sodium channels is the pore-forming α-subunit whose molecular mass is more than 250-kDa. The α-subunit consists of four quasi-homologous domains (D1-D4), each containing six α-helical transmembrane segments (S1-S6) (11, 12). The regions between S5 and S6 in each of the four domains form pore loops that dip into the membrane to create a narrow selectivity filter at the external end of the ion-conducting pore. The remainder of the pore is formed by each S6 segment of the four domains in close apposition to one another (13, 14). Recent site-directed mutagenesis studies have shown that specific amino acid residues within each of the four S6 segments are

important determinants of action of Site 2 toxins (15-23). Previously, we reported that D1S6 and D4S6 segments (but not D2S6 and D3S6) are required for GTX binding to the sodium channel (19-21). Unexplored GTX-sites in D2 and D3 have been examined in this study. Within these segments, a number of putative binding sites on the rat skeletal muscle sodium channel isoform, $Na_v1.4$ (I433, N434, L437, I1575, F1579 and Y1586) may interact with the α-surface of the GTX molecule, whose pharmacological action is effected within a hydrophobic microenvironment (8). Residue Y1586 in D4S6 has been found to provide a unique binding site for GTX, which excludes BTX and VTD. Moreover, we have attributed the difference in GTX sensitivity of the sodium channel isoforms, $Na_v1.4$ and the rat cardiac channel $Na_v1.5$, to a critical residue (S251) in the intracellular linker of D1S4-S5 (19). Kimura et al. (2001) deduced particular GTX binding sites by making several chimeras targeting the heterologous amino acid residues of $Na_v1.4$ and $Na_v1.5$; these chimeras showed reduced sensitivity to GTX (19). Most recently, Maejima et al. (2002) found novel binding sites both in D1S4-S5 and D4S4-S5 linkers of $Na_v1.4$ and $Na_v1.5$ through an alanine scanning method (24).

Recently, Yuki et al. (2001) have developed a novel method to evaluate the sensitivity of sodium channels to GTX; this method independently determines the binding and unbinding rate constant (k_{on} and k_{off}) of GTX to sodium channels (10). The sensitivity of sodium channels to GTX is indicated by the dissociation constant (K_d), i.e., the ratio of k_{off}:k_{on}. Here, using this new technique, we reevaluated already known GTX-sites, hoping to uncover novel roles of these sites in GTX binding. At the sites in D4S6, we made systematic amino acid substitutions to further clarify their roles in more detail.

EXPERIMENTAL PROCEDURES

Construction of Point Mutants of the Rat Skeletal Muscle Sodium Channel

Point mutants of the sodium channel were constructed using the cDNA clone encoding the rat skeletal muscle sodium channel α-subunit, $Na_v1.4$ (25). To construct the mutants by substitutions with various amino acids, we followed a PCR-based site-directed mutagenesis method as described previously (19-21, 24, 26). All of the resulting mutants were confirmed with restriction mapping and sequencing using an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Transient Transfection and Cell Culture

The constructed point-mutated cDNA clones were inserted into a mammalian expression vector pCI-neo (Promega Corp., Madison, WI, USA) or pcDNA3.1 (Invitrogen, Carlsbad, CA, USA), and were then transiently co-transfected with CD8 cDNA into HEK 293 cells using the SuperFect transfection reagent (Qiagen, Hilden, Germany). The cells were grown to 50% confluence in DMEM (Invitrogen), containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD, USA), 30 units/ml penicillin G (Meiji Corp., Tokyo, Japan) and 30 μg/ml streptomycin (Meiji Corp.), 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 by immunobeads (CD8-Dynabeads, Dynal, Oslo, Norway) before Na^+ current (I_{Na}) recording.

Electrophysiological Recording

Macroscopic I_{Na} from the transfected cells was measured using the whole-cell patch

clamp method. The bath solution contained 70 mM NaCl, 67 mM N-metyl-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, 5mM ethylene-bis (oxonitrilo) tetraacetic acid and 5 mM HEPES, pH 7.4. To assess the effects of GTX on whole-cell I_{Na}, 300 μM GTX I (a GTX analogue) was added to the pipette solution, because GTX is known to act intracellularly (27) and this concentration is sufficient to evaluate sensitivity of the mutated channels to GTX (19).

RESULTS

Comparison of the sensitivity to GTX among wild type $Na_v1.4$ and its mutant channels

Because GTX binds to sodium channels exclusively in their open state (10, 19-21, 24, 28), repetitive depolarizing pulses were applied to induce GTX modification. Following these repetitive conditioning pulses, test pulses to various membrane potentials induced a characteristic sustained current (Figs. 1*A*2, 1*B*2 and 1*C*2). Without repetitive prepulses, the majority of channels were unmodified by GTX so that the recorded currents in the presence of 300 μM GTX (in the internal medium) exhibited the typical behavior of unmodified channels. However, closer examination revealed a residual non-inactivating component, indicating that a very small fraction of the channels were modified during the test pulse itself without repetitive preconditioning pulses (Figs.1*A*1, 1*B*1 and 1*C*1).

In order to permit comparison of data from different cells, we standardized the currents by computing their maximum chord conductance from current-voltage curves (10, 19-21, 24, 28). The I-V relationships for unmodified I_{Na} through wild type $Na_v1.4$ channels and its mutant forms ($Na_v1.4$-F1579A and $Na_v1.4$-Y1586A) are given in Figs. 1*A*3, 1*B*3 and 1*C*3. The continuous line was fitted to peak I_{Na} at membrane potentials from 0 to +60 mV and the chord conductance was estimated from the slope. For GTX-modified currents, application of 100 conditioning pulses evoked an entirely different family of sustained currents through modified sodium channels (Figs. 1*A*2, 1*B*2 and 1*C*2). Modified I_{Na} at the end of a 160 msec test pulse was plotted against the membrane potential, and the chord conductance of the modified channel population was estimated from the slope of the I-V relationship between -50 and +50 mV (dashed line in Figs. 1*A*3, 1*B*3 and 1*C*3). We routinely used the ratio of maximal chord conductances of GTX-modified to unmodified channels as a measure of channel sensitivity to GTX modification, which is referred to as the relative chord

conductance (RCC). Our previously reported RCC values for wild type $Na_v1.4$ and various mutant channels have been provided for comparison with present results in Table 1. As for the F1579A mutant, our previously reported RCC value of 0.75 (20) was not confirmed in the present study, which yielded a value of only 0.22.

Novel binding sites of GTX in transmembrane segment D2S6 and D3S6 of $Na_v1.4$ sodium channel

In our previous studies, it was confirmed that I433, N434 and L437 in D1S6 and I1575, F1579 and Y1586 in D4S6 of sodium channel were critical sites for GTX modification (19-21). Recently, it was reported that N784 and L788 in D2S6 and S1276 and L1280 in D3S6 of $Na_v1.4$ were important in BTX action (15, 16). For this reason, we substituted N784 and L788 in D2S6 and S1276 and L1280 in D3S6 of $Na_v1.4$ with Ala or Lys. The resultant RCC values are shown in Table 1. RCC of $Na_v1.4$-N784K in D2S6 decreased from 0.35 to 0 compared with the wild type (Fig. 1*D* and Table 1). Similarly, in D3S6, RCC values of $Na_v1.4$-S1276A and $Na_v1.4$-S1276K also decreased to 0.19 and 0, respectively (Table 1). Mutant channels of $Na_v1.4$-N784A, -L788A, -L788K, -L1280A and -L1280K, did not exhibit measurable I_{Na}. Thus, we could confirm that N784 in D2S6 and S1276 in D3S6 are also critical in GTX action much like their role in BTX action, thus indicating that the sites in all S6 transmenbrane segments may cooperatively contribute to GTX binding. GTX-sites in $Na_v1.4$ identified in our studies are schematically summarized in Fig. 2*B*.

Protocol that derives k_{on} and k_{off} of GTX binding

In a previous communication, we presented a method that enables us to independently measure rate constants, k_{on} and k_{off}, for toxin binding and unbinding to sodium channels (10). GTX modifies sodium channels in proportion to the time integral of the

unmodified sodium channel conductance (To_{Na}; Fig. 3*B*). The I_{GTX}-To_{Na} relationship can be readily obtained since To_{Na} varies as a function of the potential of the three conditioning pulses employed to open unmodified sodium channels (Fig. 3*A*). Increment of I_{GTX} brought about by the three conditioning pulses is plotted against integrated currents evoked by the three conditioning pulses divided by the driving force (Fig. 3*B*). The slope of the observed linear relationship (Fig. 3*B*) gives k_{on} according to the equation 1 [see (10) for further details].

$$I_{GTX} \approx const \cdot k_{on} \cdot \int \frac{I_{Na}}{(E_{test} - E_{Na})}\ dt\ = const \cdot k_{on} \cdot To_{Na}, \quad \text{Eq. 1}$$

where I_{GTX} represents current through GTX-modified sodium channels; I_{Na}, current through unmodified sodium channels; E_{test}, the voltage applied during test pulses to evoke I_{GTX}; E_{Na}, sodium reversal potential; and *const*, constant of 1.61×10^{-3} which is invariant over sodium channel isoforms (19) and derived from sodium channel properties such as single channel conductance and open probability of unmodified or GTX-modified sodium channels. Briefly, numbers of GTX-modified sodium channels change according to Eq. 2, because GTX only binds to open state of the sodium channel.

$$\frac{dG^*}{dt} = [GTX] \cdot k_{on} \cdot O - k_{off} \cdot G^* \qquad \text{Eq. 2}$$

$$G^* = \frac{I_{GTX}}{(E_{test} - E_{Na}) \cdot g_{GTX} \cdot Po_{GTX}} \qquad \text{Eq. 3}$$

$$O = \frac{I_{Na}}{(E_{cond} - E_{Na}) \cdot g_{Na}}\ , \qquad \text{Eq. 4}$$

where O and G^* represent number of open unmodified and GTX-modified sodium channels, respectively; [*GTX*], concentration of GTX; g_{Na}, g_{GTX}, single channel conductances of unmodified and GTX-modified sodium channels, respectively; Po_{GTX}, open probability of GTX-modified sodium channels; E_{cond}, the voltage applied during conditioning pulses to induce GTX-modification. As G^* and O can be obtained from experimental data using Eq. 3

and Eq. 4, Eqs. 2, 3, and 4 can be combined and solved for I_{GTX} to yield Eq. 5.

$$I_{GTX} = const \cdot k_{on} \cdot To_{Na} - k_{off} \cdot \int I_{GTX}\, dt \qquad \text{Eq. 5}$$

However, since best-fitting values of k_{off} were negligible compared to the term $const \cdot k_{on}$ (i.e., k_{off} = 0.046 and $const \cdot k_{on}$ = 6.24 in the presence of 300 μM GTX (10)), we simplified Eq. 5 to Eq. 1 by setting k_{off} = 0, which thus provides an acceptable estimate of I_{GTX}. In fact, a linear relationship between To_{Na} and I_{GTX}, passing through the origin, is invariably obtained.

In order to obtain k_{off}, we measured the decay time constant of I_{GTX} at a potential of -80 mV (Fig. 3*C*). At this potential, unmodified sodium channels will not open, and hence no further channel modification can occur. At the same time, because GTX-modified channels do not inactivate and in fact maintain a constant open probability at this potential, decay of I_{GTX} can only represent dissociation of GTX from its binding site. Thus, the reciprocal of this time constant can be regarded as k_{off}.

Measurements of k_{on}, k_{off} and K_d of GTX binding to wild type $Na_v1.4$ and its mutant sodium channels

Technically, it is impossible to measure k_{on} and k_{off} in mutant channels that have no sensitivity to GTX. Thus, we measured k_{on} and k_{off} of the mutant channels whose sensitivity was decreased partially, and data are summarized in Table 1 and Fig. 4. As an overview, these data have four prominent features: 1) mutations at F1579 give the largest effect on k_{on}, 2) mutations at Y1586 give the largest effect on k_{off}, 3) the change in k_{off} produced by mutations at F1579 are balanced by the change in k_{on}, resulting in relatively constant K_d, and 4) mutations at Y1586 have relatively minor effects on k_{on}, resulting in a significant increase in K_d. These data indicate that association and dissociation of GTX with its binding site must be governed by different molecular entities.

Effects of various mutations at F1579 and Y1586 on k_{on}, k_{off} and K_d

As described above, we found that k_{on} was increased most markedly by mutations at F1579 and k_{off}, by mutations at Y1586. Thus, it is reasonable to assume that F1579 and Y1586 have particularly important roles in GTX binding. We introduced systematic mutations at these sites using substituent amino acids with hydrophobic, aromatic, charged, and polar side groups. Specifically, we substituted F1579 with Lys, Ser, Gly, Ala, His, Cys, Met, Val, Tyr and Trp, and substituted Y1586 with Glu, Lys, Ser, Gln, Ala, Cys, Met, Ile, Phe and Trp. We could not detect measurable I_{Na} in mutations F1579E, F1579Q, F1579I, F1579L, F1579P, Y1586D, Y1586R, Y1586N, Y1586T, Y1586H and Y1586P. Resultant RCC, k_{on}, k_{off} and K_d are summarized in Table 2. k_{on}, k_{off} and K_d values normalized to those of the wild type $Na_v1.4$ are also graphically dsiplayed in Fig. 4. The order of amino acids presented is based on degree of hydrophobicity (29-31). Only one mutant of Y1586K in the systematic substitution experiment, lost the sensitivity to GTX so that k_{on} and k_{off} could not be estimated.

In mutating F1579, we could not correlate the observed changes in k_{on} and k_{off} with side chain properties of amino acids such as their hydrophobic, aromatic, or polar nature or their ionic charge. However, we did find a prominent increase in k_{on} especially with amino acid substituents having small side chains (Gly, Ala, Ser and Cys). Large molecular dimension or ionic charge of side chains might be a hindrance to GTX binding at F1579. On the other hand, we found that substituents with extremely hydrophilic residues (Glu, Lys and Ser) at Y1586 significantly reduced the sensitivity to GTX. Hydrophobicity (Y1586A, Y1586C, Y1586M and Y1586I) may help to maintain sensitivity to GTX, but aromatic residues (Tyr, Phe and Trp) are most effective in minimizing the K_d value. One of neutral residues, Gln, at Y1586 could not be hindrance to GTX binding.

DISCUSSION

In this study, we utilized a new protocol that independently determines k_{on} and k_{off} values for GTX in sodium channel mutants. We thus uncovered new kinetic aspects of GTX-sites in $Na_v1.4$ channels. This protocol was remarkably effective in revealing the kinetic properties of GTX, because it detected changes in the response of sodium channel mutants to GTX when K_d of these mutants was not altered compared to the wild-type control. Accordingly, we found two functional sites for GTX action, F1579 and Y1586, which specifically affect GTX binding rate constants k_{on} and k_{off}, respectively. Systematic amino acid substitutions within these two sites revealed that hydrophobic or aromatic amino acids in $Na_v1.4$-Y1586 are required to maintain GTX binding, while mutations at $Na_v1.4$-F1579 do not alter the apparent GTX binding affinity (K_d), but in fact induce simultaneous changes in both k_{on} and k_{off}. In addition to previously identified GTX-sites, we have discovered novel GTX-sites in D2S6 (N784) and D3S6 (S1276), indicating that the GTX receptor consists of the S6 transmembrane segments of four quasi-homologous domains facing the ion-conducting pore.

Advantages of measuring k_{on} and k_{off} in the analysis of GTX responses of point mutants

Advantages of our newly developed method to independently measure k_{on} and k_{off} of GTX include the following: We could detect latent mutants that affect GTX binding. For example, many of the F1579 mutants having K_d values similar to the wild type (see Fig. 4 and Table 2) could be classified as GTX-sites with functions either to control access of toxin to receptor site or to serve as toxin binding site *per se*. The most prominent mutation-induced increases of k_{on} (F1579S or F1579G) and k_{off} (Y1586E) occurred at distinct sites. More precisely, systematic mutations at these two sites (Fig. 4*A* and 4*B*), revealed that mutations at

$Na_v1.4$-F1579 essentially increase k_{on} and those in $Na_v1.4$-Y1586 essentially increase k_{off}. This, for the first time, gives an indication that binding and dissociation of GTX might be regulated at two different sites within the sodium channel protein.

Assessment of newly derived parameters k_{on} and k_{off}

We previously adopted the convention of relative chord conductance (RCC) to assess the sensitivity of sodium channels to GTX (19-21, 24, 28). In the present study, we estimated K_d directly from k_{on} and k_{off} as an index of channel sensitivity to GTX. As shown in Fig. 5, a linear regression was found between K_d and RCC, indicating that both indices reliably report the GTX sensitivity. However, data points derived from mutants of the 1579 residue scattered well away from the regression line (Fig. 5). This is probably due to underestimation of RCC, because unmodified I_{Na} without preconditioning pulses in 1579 mutants was overestimated due to a residual non-inactivating current component; such a residual component was relatively large in 1579 mutants, and disappeared when GTX was depleted from internal solution (data not shown). Without preconditioning pulses, infrequent depolarizing pulses did not normally induce detectable GTX modification having the described non-inactivating component, but very large increases in k_{on} in these mutants enabled rapid channel modification to occur during the first pulse (see data from F1579A in Fig. 1*B*1). Indeed, the mutants having small molecule amino acids (Gly, Ser) at F1579 exhibited measurable non-inactivating component at the first depolarization (data not shown). On the other hand, F1579Y was exceptional: its behavior deviated in a direction markedly different from all other 1579 mutants. The F1579Y mutant exhibited a peculiar phenomenon, i.e., both peak current and GTX-modified sustained currents were spontaneously increased during the course of experiments by a factor of 1.5 relative to the unmodified peak current obtained at the beginning of experiments. By the RCC protocol, relative chord conductance

of the GTX-modified current (with prepulses) was obviously overestimated, because unmodified I_{Na} was only sampled at the beginning of experiments when the amplitude of unmodified I_{Na} was 1.5 times smaller than that of the unmodified I_{Na} that was actually present during subsequent measurement of GTX-modified I_{Na}. We do not know why F1579Y mutant exhibited this spontaneous increase in I_{Na} (Fig. 5). The newly developed method, which allows direct evaluation of k_{on} and k_{off}, has the virtue of being less sensitive than the RCC convention to perturbations such as spontaneous variations in I_{Na} or unexpectedly rapid development of GTX modification.

Specific amino acid requirements in the sites of $Na_v1.4$-F1579 and $Na_v1.4$-Y1586 in achieving interaction with GTX molecules

We introduced systematic amino acid substitutions for Phe with Lys, Ser, Gly, Ala, His, Cys, Met, Val, Tyr, Trp in $Na_v1.4$-F1579 and Tyr with Glu, Lys, Ser, Gln, Ala, Cys, Met, Ile, Phe, Trp in $Na_v1.4$-Y1586, consisting of hydrophilic, hydrophobic, aromatic, and polar amino acid side chains.

At the $Na_v1.4$-F1579 site, there was no clear indication that hydrophobic, polar, or aromatic properties of residue side chains play a critical role in increasing either k_{on} or k_{off}. It seems that larger molecular size of the amino acid side chains predicted smaller rate constants. Thus, the emerging picture is that the smaller the molecular size of the side chain, the greater the access of GTX molecules to the receptor site. As for the site at Y1586, it is distinct from the site at F1579 in that the GTX sensitivity ($K_d = k_{off}/k_{on}$) was extensively altered by the mutations. Furthermore, we could categorize the effect of mutations into three groups according to the resultant K_ds. Aromatic residues (Tyr, Phe, Trp) produce the highest sensitivity of the three groups, although an exception was found in the case of Gln. Hydrophilic residues (Glu, Lys, Ser) could not maintain a suitable microenvironment to

permit GTX binding. Hydrophobic residues (Ala, Cys, Met, Ile) improved the efficiency of GTX binding compared with hydrophilic residues.

It has been reported that specific amino acids are required in BTX or local anesthetic binding to a putative receptor site in D4S6 ($Na_v1.3$-F1710) corresponding to $Na_v1.4$ -F1579 (9, 32). Stable BTX binding to the channel protein requires a polar or aromatic residue at this receptor site to maintain the ion-dipole or cation-π interaction (9). Tetracaine requires an aromatic residue at its receptor site to maintain electrostatic interactions between the positive charge on the protonated tertiary amine of this local anesthetic and the electron rich-π face of the aromatic residue (32). As for GTX, Yakehiro et al. (2000) have shown that the hydrophobic moiety must be preserved on the α-surface of GTX molecules for effective GTX action, because the introduction of a hydroxyl group into C-14*S* facing the α-surface of the GTX analog markedly reduced GTX potency (8). Thus, it is supposed that the hydrophobic environment around the receptor site is important in GTX action. In this study, we argued that an aromatic or hydrophobic side chain at residue Y1586 in $Na_v1.4$ is required to preserve sensitivity to GTX. This is consistent with the requirement for hydrophobicity on the α-surface of GTX in maintaining GTX potency. Direct interaction between the α-surface of GTX and Y1586 may be necessary for normal GTX action.

While Y1586 has a determinant role in GTX action as discussed above, F1579 may have a rather regulatory function. Mutations at $Na_v1.4$-F1579 produced only a marginal change in K_ds. Furthermore, F1579K was the only mutant whose sensitivity was not totally eliminated by Lys-substitution (see Table 1). It is reasonable to assume that the charged side chain in F1579K interferes with access of the hydrophobic α-surface of GTX molecules, as we observed an exceptionally small k_{on} when Lys was substituted (see Fig. 4*A*).

Properties that differentiate GTX from other Site 2 toxins or local anesthetics

GTX shares common binding sites with the Site 2 toxins, BTX and VTD, namely, I433, N434 and L437 in D1S6, I1575 and F1579 in D4S6 of $Na_v1.4$ (17-23). Other GTX specific sites were further documented in D4S6 (Y1586) of $Na_v1.4$ (20). Several GTX-sites were also found in D1 and D4 (see Fig. 2*B*) (19-21, 24). This study has now added two novel sites in D2S6 (N784) and D3S6 (S1276) to the list of binding sites common to Site 2 toxins. However, Y1586 is GTX-specific, because mutations at this position did not affect BTX binding (17, 22). This might explain the difference in binding kinetics between GTX and BTX. BTX dissociates from the open state of the sodium channel (9), whereas GTX exclusively dissociates from the closed state (10). It is supposed that BTX dissociates from its receptor site in the ion-conducting pore and shifts to a cytoplasmic membrane site during the open state of the channel, as if it were reversing its itinerary (9). On the other hand, because Y1586 is GTX-specific among Site 2 toxins and mutation of this site dramatically increases unbinding (k_{off}) of GTX, it is possible that this site contributes to the GTX-specific binding-unbinding reaction, by trapping GTX in the open channel and releasing GTX from the closed channel.

Local anesthetics like lidocaine, tetracaine, QX314, and QX222 modify channel activation and block sodium current. These local anesthetics also share common binding sites at N434 and L437 in D1S6, S1276 in D3S6, I1575, F1579 and Y1586 in D4S6 of $Na_v1.4$ with GTX (16, 19-21, 33, 34). As in GTX binding, local anesthetic binding is affected by introduction of amino acid substitutions at both F1579 and Y1586. However, there are also differences in mode of reactions between GTX and local anesthetics. In contrast to GTX reactions, F1579 is thought to be the major local anesthetic binding site rather than Y1586, which merely regulates access of local anesthetics to the F1579 site (32).

Other critical sites for GTX action in S6 transmembrane segments

Much as we determined that sites F1579 and Y1586 in D4S6 are critical to GTX action on the basis of drastic changes in k_{on} or k_{off}, we are likely to find similarly important sites in other domains. However, in this study, mutants in other domains did not produce a prominent change in either k_{on} or k_{off}. There are two reasons for failure to detect these sites: 1) we cannot determine k_{on} or k_{off} in mutants that are completely insensitive to GTX, thus exhibiting RCC values of 0, as observed especially with Lys mutations at I433, N434, L437, N784 and S1276; 2) mutations such as I433V do not necessarily change the chemical properties of relevant amino acids, (i.e., both Ile and Val are hydrophobic residues). Thus, application of systematic mutation to these unexplored sites with measurement of k_{on} and k_{off} might be a productive avenue for future research. I433 of D1S6 could be one of the most promising sites, because mutations of this site affect binding not only of GTX and other Site 2 toxins, but also of pyrethroids (35). It is interesting to note the common functional effects of GTX and pyrethroids on sodium channel kinetics: both GTX and pyrethroids markedly prolong channel open time and remove or slow sodium inactivation (36-38).

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FOOTNOTES

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[1]The abbreviations used are: D, domain; GTX, grayanotoxin; HEK, human embryonic kidney; I_{Na}, Na^+ current; I-V, current-voltage; S, segment; Each mutant channel will be referenced by the original amino acid followed by its number and introduced amino acid.

FIGURE LEGEND

Fig. 1. GTX modification of sodium channels. Differences in sensitivity to GTX I between wild type $Na_v1.4$ and mutants (F1579A, Y1586A and N784K). ***A-D*:** I_{Na} families and I-V relations for unmodified peak I_{Na} and for GTX-modified steady-state I_{Na} in wild type $Na_v1.4$ (***A***), mutant F1579A ***(B***), Y1586A (***C***) and N784K (***D***). Control currents (unmodified currents) were taken in the presence of intracellular GTX. The currents were scarcely modified without preconditioning pulses even in the presence of 300 μM GTX, as shown in panels *A*1, *B*1 and *C*1. Modified currents (*A*2, *B*2 and *C*2) were induced by a train of 100 conditioning prepulses (pulse potential = -20 mV; pulse duration = 6 ms, interpulse interval = 100 ms; holding potential = -120 mV). I-V relationships (*A*3, *B*3, *C*3 and *D*3) of unmodified and modified currents were obtained using 160 msec test pulses to a variable potential (-140 mV to +50 mV in 10 mV steps); unmodified currents (filled squares) were measured as peak I_{Na} and modified currents (open circles), as the steady-state current at the end of the pulse.

Fig. 2. Structure of GTX and GTX-sites in $Na_v1.4$ sodium channel. ***A***. Structure of GTX I. Numerals indicate number of carbon atoms in GTX I molecule. ***B.*** Summary of GTX-sites in $Na_v1.4$ sodium channel. The indicated point mutations in $Na_v1.4$ caused moderate (squares) or marked suppression (squares with emboldened border) of GTX action. For reference, amino acid sequences (numbered from N terminus) for the S4-S5 linkers in domains D1 and D4, and GTX-sites in D1S6 and D4S6 (19-21, 24) are also displayed.

Fig. 3. Measurement of k_{on} and k_{off}. ***A***, protocol for the measurement of k_{on}. An initial pulse of 40 ms (from holding potential of -120 mV to -80 mV) evoked modified current (I_{First}) after various prepulses induced channel modification. Additional modification was produced by three subsequent conditioning pulses that opened unmodified sodium channels. The increment in modified current (I_{GTX}) caused by the three additional pulses (I_{Second}) was

assessed at -80 mV as $I_{GTX} = I_{Second} - I_{First}$. Increments in modified currents caused by trains of three conditioning prepulses to various potentials (-70 mV to 0 mV in 10 mV step; see the inset figure in ***A***) are shown in ***B*** as plot of To_{Na}, (time integral of unmodified currents during the three conditioning prepulses divided by the driving force) against I_{GTX}. k_{on} was measured as the slope of the fitted line (see Text for further details). ***C***, In order to measure $k_{off,}$ the time constant (τ) for decay of modified sodium currents were measured at -80 mV; for this experiment, channel modification was induced by 100 prepulses (pulse potential, 0mV; holding potential, -120mV). A single exponential curve was fitted to the decay of modified sodium current. The reciprocal of this time constant is equal to k_{off}; i.e., $k_{off} = 1/\tau$ (see text for additional details). The dissociation constant (K_d) is calculated from the ratio $k_{off} : k_{on}$, i.e., $K_d = k_{off} / k_{on}$.

Fig. 4. Normalized values of k_{on} (*A*), k_{off} (*B*) and K_d (*C*). Values of k_{on}, k_{off}, and K_d of mutant channels are normalized to those of wild type $Na_v1.4$ and displayed separately in ***A***, ***B***, ***C***, respectively.

Fig. 5. Correlation between RCC and binding constant (*K*). The parameter *K* is the reciprocal of K_d. Open circles indicate values (RCC, or relative chord conductance, and parameter *K)* obtained from mutants of position F1579; filled squares indicate those of wild type $Na_v1.4$ and the indicated mutants. Regression line is through filled squares, including wild type $Na_v1.4$. Note that the data points for F1579 mutants scatter away from this line.

Table 1 Relative chord conductance (RCC), k_{on}, k_{off} and K_d in wild type $Na_v1.4$ and its point mutants

	Wild type /mutant	Mean ± SD of RCC (number of reservation)	k_{on} ($\mu M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (μM)
	Wild type-$Na_v1.4$	0.35±0.04 (n=9) [a]	325	0.0046	14.2
D1S4-S5 linker	$Na_v1.4$-L243A	0.23±0.02 (n=3)[b]	N.A.	N.A.	N.A.
	$Na_v1.4$-S251A	0.26±0.003 (n=10)[c]	262	0.0054	20.7
	$Na_v1.4$-L243A&S251A	0.10±0.02 (n=3) [b]	231	0.0096	41.7
D1S6	$Na_v1.4$-I433A	0.09±0.02 (n=4) [a]	N.A.	N.A.	N.A.
	$Na_v1.4$-I433K	0 (n=4)[d]	N.A.	N.A.	N.A.
	$Na_v1.4$-I433V	0.24±0.05 (n=4) [c]	243	0.0059	24.1
	$Na_v1.4$-N434A	0 (n=4) [a]	N.A.	N.A.	N.A.
	$Na_v1.4$-N434K	0 (n=3) [d]	N.A.	N.A.	N.A.
	$Na_v1.4$-L437A	0.33±0.10 (n=4) [a]	-	-	-
	$Na_v1.4$-L437K	0 (n=3) [d]	N.A.	N.A.	N.A.
D2S6	$Na_v1.4$-N784A	N.E.	-	-	-
	$Na_v1.4$-N784K	0 (n=3)	N.A.	N.A.	N.A.
D3S6	$Na_v1.4$-S1276A	0.19±0.02 (n=4)	194	0.0061	31.3
	$Na_v1.4$-S1276K	0 (n=3)	N.A.	N.A.	N.A.
D4S6	$Na_v1.4$-I1575A	0 (n=3) [a]	N.A.	N.A.	N.A.
	$Na_v1.4$-I1575K	N.E.	-	-	-
	$Na_v1.4$-F1579A	0.22±0.02 (n=3)	1113	0.0164	14.8
	$Na_v1.4$-F1579K	0.04±0.01 (n=3) [a]	125	0.0063	50.5
	$Na_v1.4$-Y1586A	0.06±0.02 (n=3) [a]	244	0.0296	121.5
	$Na_v1.4$-Y1586K	0 (n=3) [a]	N.A.	N.A.	N.A.

[a] reported in Ref. (20), [b] in (24), [c] in (19), and [d] in (21).

N.A. means that GTX-modification was so small that it was not available to measure relative chord conductance, k_{on} or k_{off}.

N.E. means functional channels were not expressed in these mutants.

- denotes data are not available because experiments were not done in the mutant exhibited the same value of RCC as wild type or no functional mutants.

Table 2 Relative chord conductance (RCC), k_{on}, k_{off} and K_d in point mutants of $Na_v1.4$ at F1579 or Y1586

	Wild type /mutant	Mean ± SD of RCC (number of reservation)	k_{on} ($\mu M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_d (μM)
	Wild type-$Na_v1.4$	0.35±0.04 (n=9)[a]	325	0.0046	14.2
Mutant at F1579	$Na_v1.4$-F1579K	0.04±0.01 (n=3)[a]	125	0.0063	50.5
	$Na_v1.4$-F1579S	0.15±0.01 (n=3)	1366	0.0230	16.8
	$Na_v1.4$-F1579G	0.15±0.006(n=3)	1341	0.0217	16.2
	$Na_v1.4$-F1579A	0.22±0.02 (n=3)	1113	0.0164	14.8
	$Na_v1.4$-F1579H	0.14±0.004 (n=3)	619	0.0118	19.1
	$Na_v1.4$-F1579C	0.24±0.05(n=3)	927	0.0219	23.6
	$Na_v1.4$-F1579M	0.23±0.02 (n=6)	424	0.0293	69.0
	$Na_v1.4$-F1579V	0.15±0.01 (n=3)	529	0.0145	27.4
	$Na_v1.4$-F1579Y	0.53±0.11 (n=4)	526	0.0082	15.6
	$Na_v1.4$-F1579W	0.09±0.01 (n=3)	200	0.0062	30.9
Mutant at Y1586	$Na_v1.4$-Y1586E	0.05±0.01 (n=4)	234	0.0651	277.9
	$Na_v1.4$-Y1586K	0 (n=3)[a]	N.A	N.A.	N.A.
	$Na_v1.4$-Y1586S	0.05±0.02 (n=4)	78	0.0342	438.1
	$Na_v1.4$-Y1586Q	0.22±0.07 (n=3)	392	0.0092	23.4
	$Na_v1.4$-Y1586A	0.06±0.02 (n=3)[a]	244	0.0296	121.5
	$Na_v1.4$-Y1586C	0.08±0.02(n=3)	397	0.0418	105.2
	$Na_v1.4$-Y1586M	0.07±0.005 (n=3)	443	0.0455	98.4
	$Na_v1.4$-Y1586I	0.11±0.01 (n=3)	369	0.0458	124.2
	$Na_v1.4$-Y1586F	0.10±0.02 (n=3)	443	0.0263	59.2
	$Na_v1.4$-Y1586W	0.21±0.02 (n=3)	465	0.0162	34.9

[a] reported in Ref. (20)}

N.A. means that GTX-modification was so small that it was not available to measure relative chord conductance, k_{on} or k_{off}.

Fig. 1 Maejima et al.

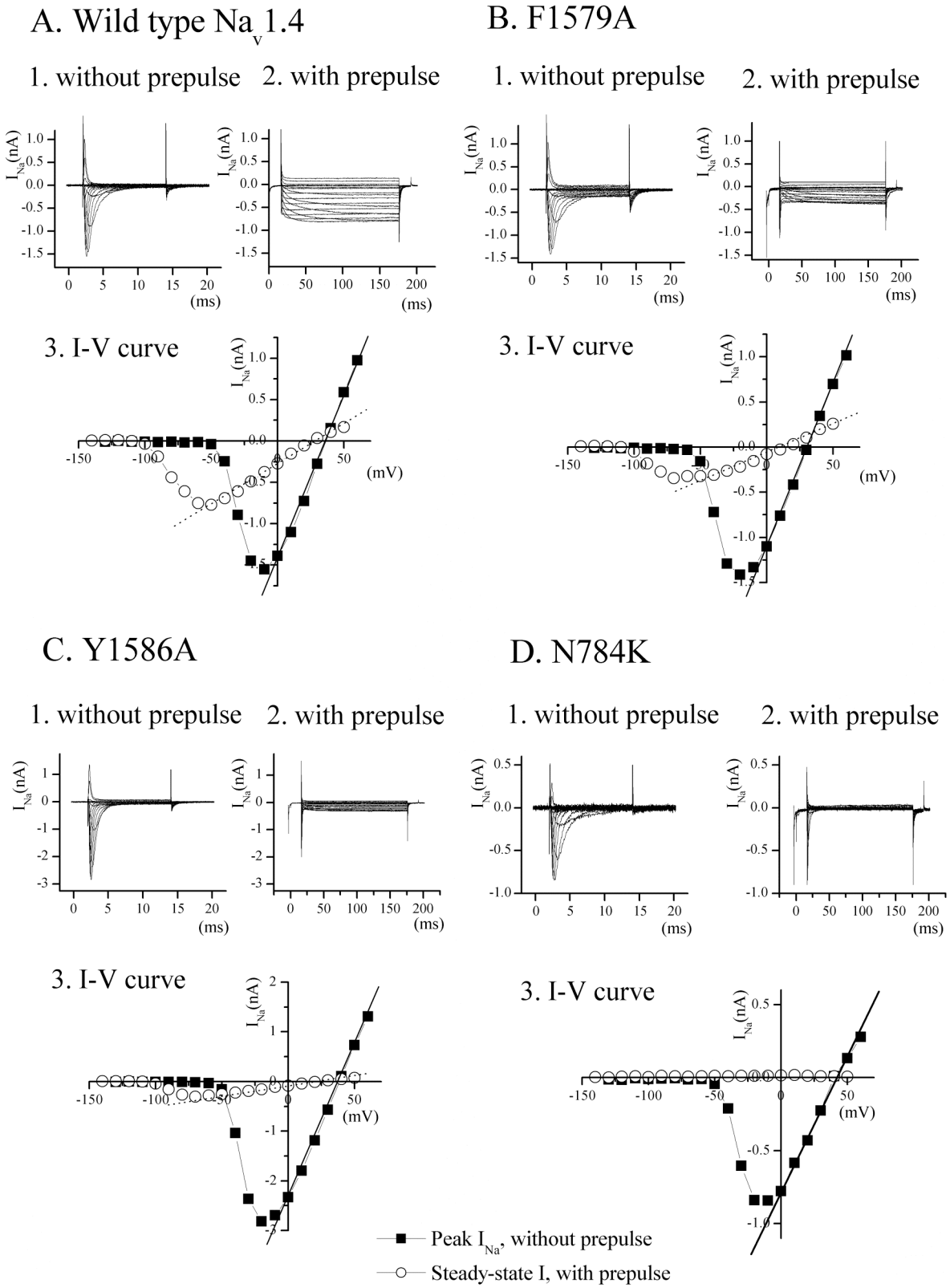
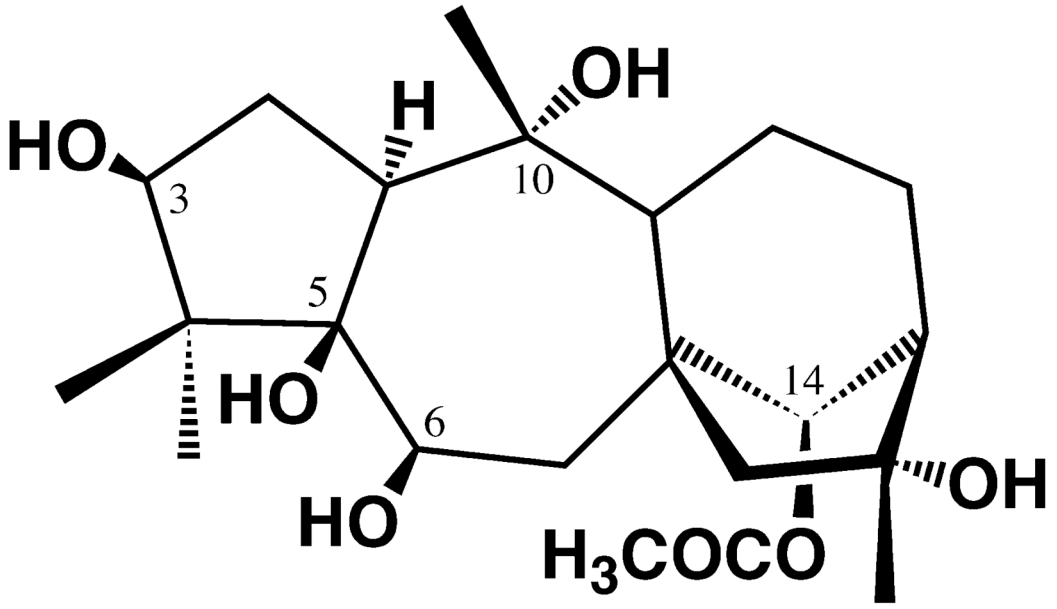


Fig. 2 Maejima et al.

A.



B.

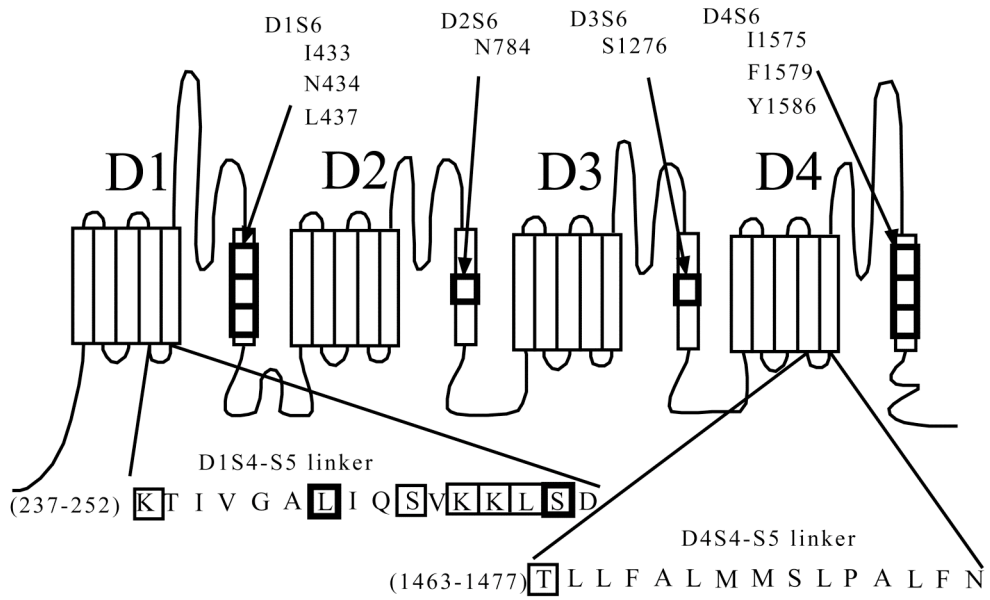


Fig. 3 Maejima et al.

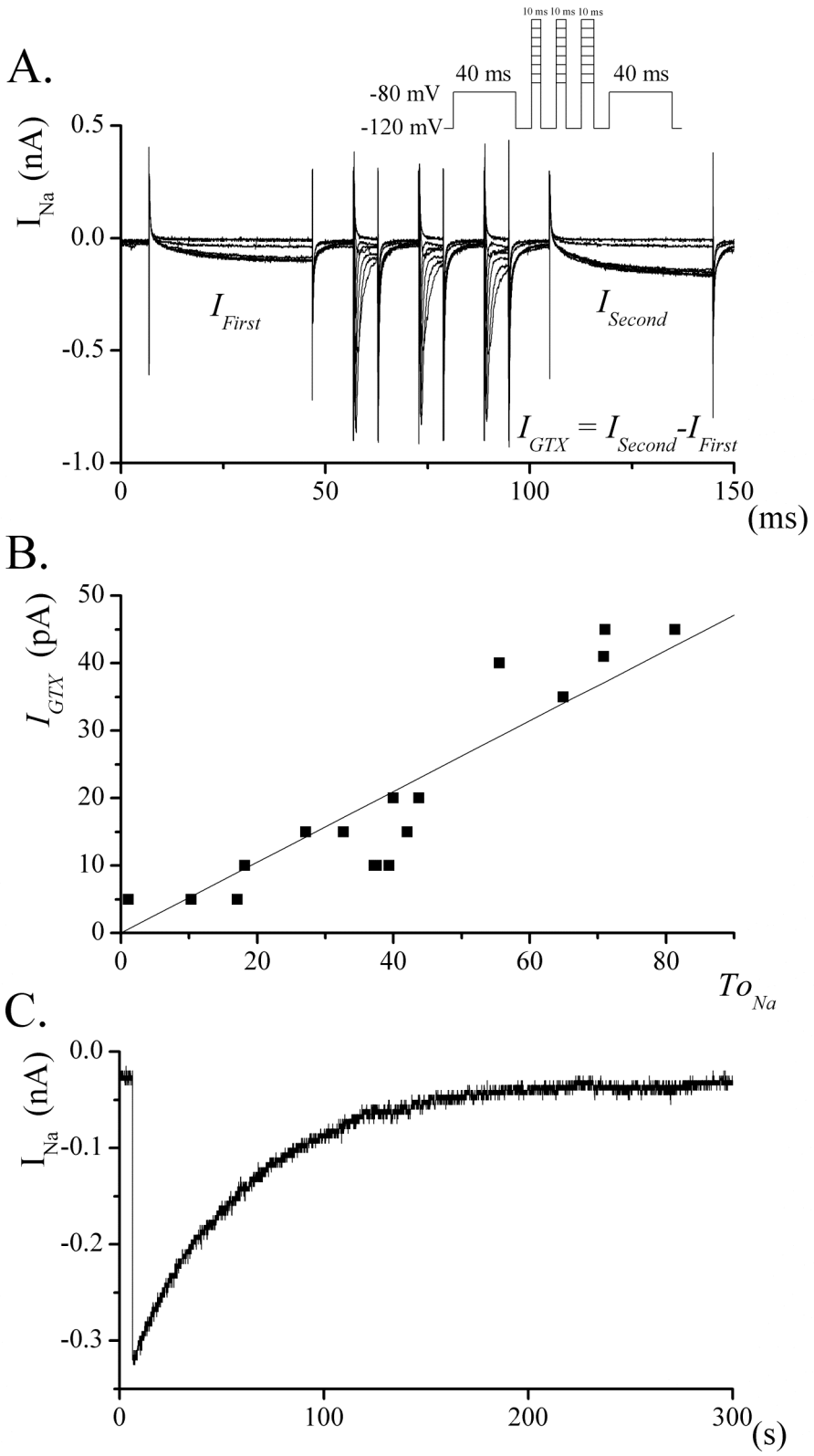


Fig. 4 Maejima et al.

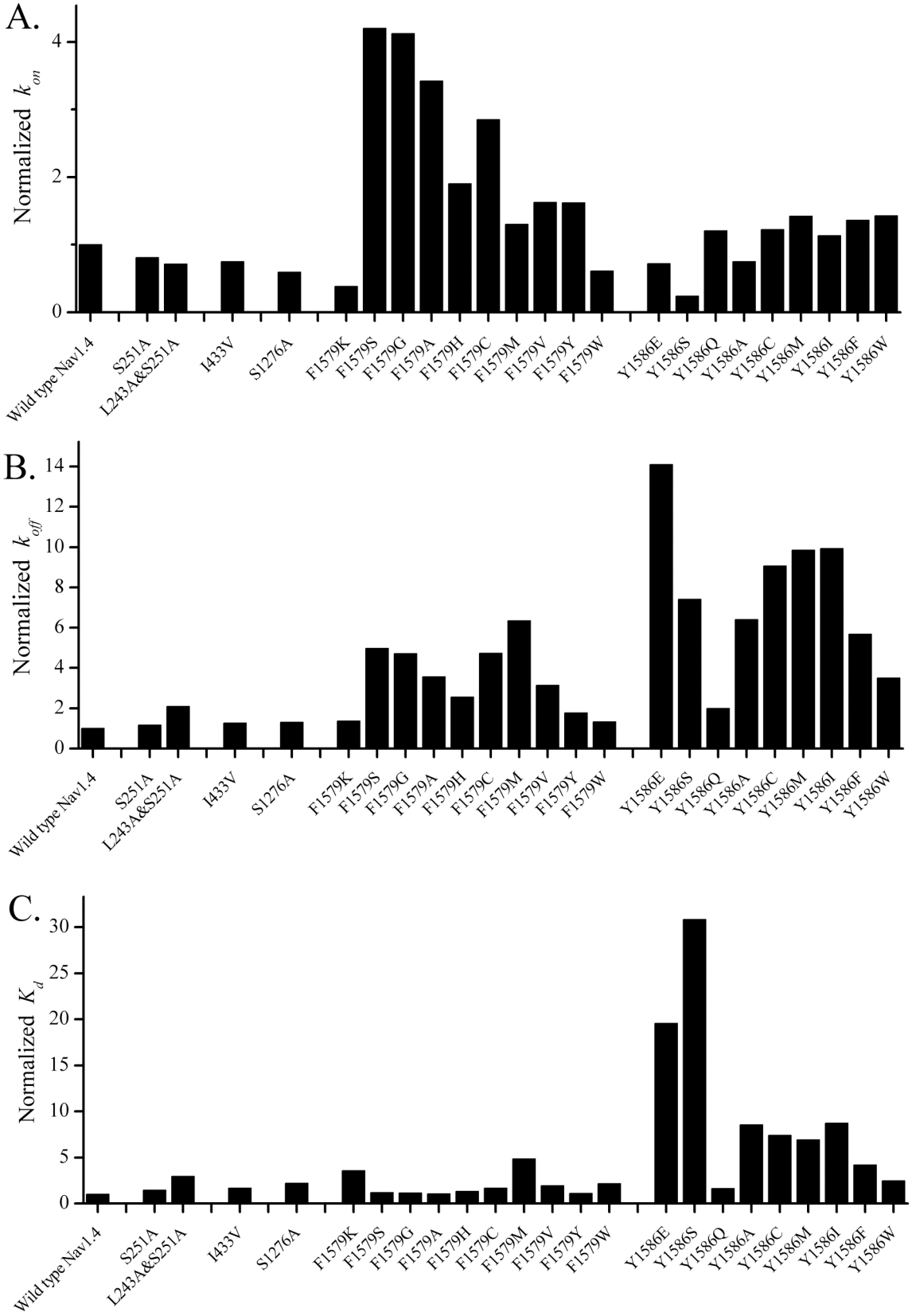


Fig. 5 Maejima et al.