Specificity for Block by Saxitoxin and Divalent Cations at a Residue Which Determines Sensitivity of Sodium Channel Subtypes to Guanidinium Toxins

Isabelle Favre,* Edward Moczydlowski,† and Laurent Schild*

From the *Institut de Pharmacologie et Toxicologie de l'Université, CH-1005 Lausanne, Switzerland; †Departments of Pharmacology and Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT 06520-8066

Abstract Tyrosine 401 of the skeletal muscle isoform (μ1) of the rat muscle Na channel is an important determinant of high affinity block by tetrodotoxin (TTX) and saxitoxin (STX) in Na-channel isoforms. In mammalian heart Na channels, this residue is substituted by cysteine, which results in low affinity for TTX/STX and enhanced sensitivity to block by Zn\(^{2+}\) and Cd\(^{2+}\). In this study, we investigated the molecular basis for high affinity block of Na channels by STX and divalent cations by measuring inhibition of macroscopic Na\(^{+}\) current for a series of point mutations at residue Tyr401 of the rat μ1 Na channel expressed in Xenopus oocytes. Substitution of Tyr401 by Gly, Ala, Ser, Cys, Asp, His, Trp, and Phe produced functional Na\(^{+}\) currents without major perturbation of gating or ionic selectivity. High affinity block by STX and neosaxitoxin (NEO) with \(K_I\) values in the range of 2.6-18 nM required Tyr, Phe, or Trp, suggestive of an interaction between an aromatic ring and a guanidinium group of the toxin. The Cys mutation resulted in a 7- and 23-fold enhancement of the dissociation rate of STX and NEO, respectively, corresponding to rapid toxin dissociation rates of cardiac Na channels. High affinity block by Zn\(^{2+}\) (\(K_I = 8-23 \mu M\)) required Cys, His, or Asp, three residues commonly found to coordinate directly with Zn\(^{2+}\) in metalloproteins. For the Cys mutant of μ1 and also for the cardiac isoform Na channel (rh1) expressed in the L6 rat muscle cell line, inhibition of macroscopic Na\(^{+}\) conductance by Zn\(^{2+}\) reached a plateau at 85-90% inhibition, suggesting the presence of a substate current. The Asp mutant of μ1 also displayed enhanced affinity for inhibition of conductance by Ca\(^{2+}\) (\(K_I = 0.3 \text{ mM vs } \sim 40 \text{ mM in wild type} \)), but block by Ca\(^{2+}\) was incomplete, saturating at \(\sim 69\%\) inhibition. In contrast, Cd\(^{2+}\) completely blocked macroscopic current in the Cys mutant and the L6 cell line. These results imply that the magnitude of substate current depends on the particular residue at
position 401 and the species of divalent cation. The His mutant also exhibited enhanced sensitivity to block by H⁺ with a pKₐ of ~7.5 for the His imidazole group. Our findings provide further evidence that residue 401 of α1 is located within the outer vestibule of the Na channel but external to the single-filling region for permeant ions.

INTRODUCTION

The blocking pharmacology of voltage-gated Na channels by toxins and divalent cations has played an important role in the identification of protein residues that form the outer entrance to the pore and participate in the mechanism of ionic selectivity for permeant cations. Multiple genes encode different isoforms of the α subunit of Na channels that can be distinguished pharmacologically by their sensitivity to tetrodotoxin (TTX), saxitoxin (STX), μ-conotoxin, and divalent metal cations such as Zn²⁺ and Cd²⁺ (reviewed in Catterall, 1992; Moczydlowski and Schild, 1994). For example, brain Na channels and the skeletal muscle isoform (α1) are sensitive to block by nanomolar concentrations of TTX and STX, but have millimolar affinity for block by external divalent cations (Worley, French, and Krueger, 1986; Guo, Uehara, Ravindran, Bryant, Hall, and Moczydlowski, 1987; Ravindran, Schild, and Moczydlowski, 1991). In contrast, the cardiac Na-channel isoform that is also expressed in noninnervated skeletal muscle is ~100-fold less sensitive to TTX and STX, and exhibits enhanced sensitivity in the micromolar range to block by the group IIB cations, Ca²⁺ and Zn²⁺ (Frelin, Cognotard, Vigne, and Lazdunski, 1986; Ravindran, Schild, and Moczydlowski, 1991). In contrast, the cardiac Na-channel isoform that is also expressed in noninnervated skeletal muscle is ~100-fold less sensitive to TTX and STX, and exhibits enhanced sensitivity in the micromolar range to block by the group IIB cations, Ca²⁺ and Zn²⁺ (Frelin, Cognotard, Vigne, and Lazdunski, 1986; Ravindran, Schild, and Moczydlowski, 1991).

In 1989, Noda, Suzuki, Numa, and Stühmer reported that mutation of a glutamate residue (E387) to glutamine in the S5–S6 linker of the first homologous domain (I) of rat brain II Na-channel isoform virtually abolished sensitivity to TTX and STX (Noda et al., 1989). This discovery led the way to the identification of critical structural determinants of the TTX/STX binding site, which has long been suspected to be intimately associated with pore entrance and "selectivity filter" (Hille, 1975). Mutations at positions neighboring E387 and in the corresponding S5–S6 linker regions of homologous domains II, III, and IV identified additional residues that are involved in TTX/STX binding (Terlau, Heinemann, Stühmer, Pusch, Conti, Imoto, and Numa, 1991). On the basis of sequence alignment of these four homologous "P-region" segments and conductance measurements by noise analysis, Terlau et al. (1991) proposed that two clusters of charged residues form ring structures at the outer pore entrance that electrostatically enhance unitary cation current. In particular, the inner ring identified as Asp/Glu/Lys/Ala in domains I/II/III/IV of Na channels is replaced by Glu/Glu/Glu/Glu in the homologous family of voltage-gated Ca channels, and these residues have been found to determine the selective permeability of monovalent versus divalent inorganic cations (Heinemann, Terlau, Stühmer, Imoto, and Numa, 1992b).

In previous studies on Na channels reconstituted into planar bilayers and activated with batrachotoxin to reveal direct interactions of divalent cations with the open channel conformation, we found that enhanced sensitivity of heart Na channels to Zn²⁺ is manifested as a discrete, but incomplete, block of the open channel
to a subconductance level at ~20% of the open-channel current (Schild, Ravindran, and Moczydlowski, 1991). Based on the observation that discrete Zn$^{2+}$ block of heart Na channels was abolished by the cysteine alkylation agent, iodoacetamide, we proposed that Cys374 of the rat heart Na-channel isoform (rhl) is directly involved in Zn$^{2+}$ coordination (Schild and Moczydlowski, 1991). This particular Cys residue was identified as a likely candidate, because this position is close to the Glu residue critical for TrX/STX block identified by Noda et al. (1989) and is uniquely present in Na-channel isoforms sensitive to Zn$^{2+}$/Cd$^{2+}$. This suggestion was verified by experiments showing that mutation of Cys374 to Tyr or Phe in rhl lowered sensitivity to block by Cd$^{2+}$ (Satin, Kyle, Chen, Bell, Cribbs, Fozzard, and Rogart, 1992). Conversely, the corresponding mutations of Tyr to Cys in the μ.l isoform and Phe to Cys in the rat brain II isoform enhanced sensitivity to Cd$^{2+}$ and Zn$^{2+}$ (Backx, Yue, Lawrence, Marban, and Tomaselli, 1992; Heinemann, Terlau, and Imoto, 1992a). Furthermore, in these latter mutagenesis studies, isoform-specific substitutions at this single position were also found to account for differences in TTX/STX sensitivity among native Na-channel isoforms.

Beyond this compelling evidence for the importance of Cys374 (rhl) in determining sensitivity to block by group IIB metal cations and insensitivity to guanidinium toxins, a number of questions remain regarding the intrinsic chemical specificity of the underlying molecular interactions. First, does this Cys residue specifically destabilize guanidinium toxin binding or do the corresponding Tyr/Phe substitutions specifically stabilize toxin binding? Secondly, since high affinity binding sites for Zn$^{2+}$ in proteins are generally formed by near-tetrahedral coordination by various combinations of Cys, His, Glu, and Asp residues (Christianson and Alexander, 1989; Vallee and Auld, 1990), can other functional group ligands substitute for Cys in this Zn$^{2+}$-binding site in Na channels? If so, what is the relative energetic contribution of such ligands in stabilizing Zn$^{2+}$ binding? Finally, as noted previously, we have observed a prominent subconductance state induced by Zn$^{2+}$ in heart Na channels activated by BTX (Schild et al., 1991; Schild and Moczydlowski, 1994). Interestingly, Backx et al. (1992) did not observe such a substate for high-affinity block by Cd$^{2+}$ in similar experiments. What is the basis of this discrepancy? Is this subconductance behavior a peculiarity of BTX-modified Na channels or does it depend on the divalent metal ion?

To address these questions, we characterized the inhibition of macroscopic Na$^{+}$ currents by STX and various divalent cations for a series of substitution mutations at position 401 of the μ.l Na-channel isoform. Five different classes of residue substitutions were examined at this position: neutral (Gly, Ala), sulfhydryl/hydroxyl (Cys, Ser), carboxylate (Asp), aromatic (Tyr, Phe, Trp), and imidazole (His). By replacing the native Tyr residue at this position with these different functional groups, the relative energetic contributions of amino acid ligands to the binding of guanidinium toxins and divalent cations can be directly assessed. The results provide new information on the chemical specificity of molecular interactions between STX/divalent metal ions and residue 401 in the outer vestibule of Na channels. Some of these results have been previously presented in abstract form (Schild, Favre, and Moczydlowski, 1993).
MATERIALS AND METHODS

Site-directed Mutagenesis and Expression in Xenopus laevis Oocytes

A recombinant plasmid (pM500) containing the entire coding region of the rat muscle $\mu$1 Na-channel (Trimmer, Cooperman, Tomiko, Zhou, Crean, Boyle, Kallen, Sheng, Barchi, Sigworth, Goodman, Agnew, and Mandel, 1989) cloned into the EcoRI site of pBluescript SK+ was obtained from Dr. W. A. Agnew (Department of Physiology, Johns Hopkins University, Baltimore, MD). Mutations were introduced in the cDNA of the $\alpha$ subunit of the $\mu$1 Na channel, according to the method of Nelson and Long (1989), using polymerase chain reaction (PCR). The protocol for site-directed mutagenesis included three steps. In the first step, a 199–205-bp DNA fragment was amplified by Taq DNA polymerase (Perkin-Elmer Corp., Norwalk, CT) between the sense mutation-directing primer (22–29 bp) and the inverse hybrid primer carrying an unrelated anchoring sequence, using the SacI-linearized $\mu$1 cDNA as template. In the second step, the inverse DNA strands obtained during the first PCR step were elongated. Finally, the mutated cDNA was selectively amplified with an anchoring primer, and a 5’-sense $\mu$1 primer. The unique BclI and BsiWII restriction sites were used to introduce the mutated DNA fragment into the wild-type cDNA amplified in a suitable Escherichia coli strain (dam-). Mutations were confirmed by dideoxy sequencing (Sanger, Nicklen, and Coulson, 1977). Complementary RNA was synthesized by in vitro transcription of linearized cDNA templates (in pBluescript SK+) with T7 polymerase. Stage V–VI oocytes were injected with 5–30 ng of cRNA. Macroscopic Na currents were measured after 1–4 d incubation at 19°C in standard Barth’s solution, containing (in millimolar): NaCl 85, KCl 1, NaHCO3 2.4, MgSO4 0.8, CaCl2 0.4, HEPES 16, and supplemented with penicillin and streptomycin.

Culture of L6 Muscle Cells

The rat skeletal muscle myoblast cell line, L6, was obtained from the American Type Culture Collection (Rockville, MD) and cultured according to the following procedure. Myoblasts were grown on plastic tissue culture dishes in Dulbecco’s modified Eagle medium containing 10% fetal calf serum at 37°C in a 95% O2/5% CO2 atmosphere. The growth medium was replaced at intervals of 2 d, and cells were maintained at low density by frequent subculture to prevent cell fusion and differentiation into multinucleated myotubes. Isolated myoblast cells were trypsinized and transferred into the recording chamber for electrophysiological studies.

Electrophysiology

Macroscopic Na+ current ($I_{Na}$) expressed in Xenopus oocytes was recorded by the two-electrode voltage clamp technique using a TEV2000 amplifier (Dagan Corp., Minneapolis, MN). Oocytes were bathed in a normal frog Ringer solution containing (in millimolar): 115 NaCl, 2.5 NKI, 1.8 CaCl2, 10 HEPES-NaOH, pH 7.2, at 20–22°C. Oocytes were continuously perfused, except during the $I$–$V$ pulse sequence for $I_{Na}$ measurements. In the recovery experiments from STX and NEO block, oocytes in a 200-μl chamber were continuously perfused at a 25 ml/min flow rate, and repetitive pulses were elicited at intervals of 0.5–15 s. Pipettes were filled with 0.2 M KCl. Electrode resistance was <1 MΩ when filled with 3 M KCl. Voltage protocols were computer controlled using an ATARI computer and the EPC9 AD/DA interface (HEKA Electronik, Lambrecht, Germany). Na+ currents were activated from a holding potential of −80 mV by a series of 20–25 depolarizing pulses in 5-mV increments from −50 mV, with a 2-s interval between each pulse. Leakage and capacitive currents were subtracted using a P/4 pulse protocol which was delivered at −120 mV. Current records were filtered at 1.8 kHz with an eight-pole Bessel filter and digitized at 10kHz for analysis using the REVIEW program (HEKA).

Macroscopic recording of $I_{Na}$ in L6 cells was performed by the patch clamp technique in the

on June 23, 2017 Downloaded from
Published August 1, 1995
FAVRE ET AL.  External Block of Na Channels by STX and Divalent Cations 207

whole-cell configuration using an EPC-9 amplifier (HEKA). Patch electrodes, pulled in two steps from Kimax 50 borosilicate capillaries, had a resistance of 2–4 MΩ with the standard filling solution (in millimolar): 100 CsF, 10 NaCl, 11 EGTA, 10 HEPES-NaOH salt, pH 7.3. The experimental bathing solution for L6 cells contained (in millimolar): 160 NaCl, 1.5 CaCl₂, 1 MgCl₂, 10 HEPES-NaOH salt, pH 7.4. Perfusion of the bathing solution was stopped during -V measurements. Holding potential was -20 mV, and pulse protocols consisted of 18 depolarizing steps of 10-mV increments starting at -60 mV with a 2s interval between pulses. Subtraction of leakage and capacitive currents was performed according to a P/4 protocol delivered at -40 mV. Digitizing and filtering of the current records were performed as for the two-electrode voltage-clamp.

For measurement of block by external divalent cations or toxins, paired experiments were performed, consisting of a complete -V pulse protocol as a control measurement of unblocked current, followed by the same pulse protocol in the presence of the blocking agent. The test pulse sequence was always followed by another control protocol after perfusion with bath solution to verify complete reversibility (>95%) of the blocking reaction. Three to four different concentrations of the same blocker could usually be tested in one oocyte. In blocking experiments CaCl₂ and ZnCl₂ (ultrapure, Alfa Corp., Danvers, MA) were added as a replacement for NaCl when tested at high concentration (>1 mM) to maintain constant osmolarity.

Data Analysis

Peak current-voltage (I₉₀-V) relations were fit to the following transform of a Boltzmann function:

\[ I_{\text{Na}} = C_m a \cdot \frac{(V - V_{rev})}{1 + \exp\left[-\frac{V - V_{0.5}}{k}\right]} \]  

where \( I_{\text{Na}} \) is the peak Na⁺ current elicited by the voltage pulse, \( V \) is the test potential, \( V_{rev} \) is the reversal potential, \( C_m \) is the maximal conductance, \( V_{0.5} \) is the voltage for half-maximal current activation, and \( k \) is equal to the slope factor (in millivolts).

Three different models were used to analyze titration curves for inhibition of macroscopic conductance, \( C_m \). For a channel blocking reaction due to occupancy of a single binding site, then the ratio, \( G/G_0 \), of the conductance measured in the presence of a particular blocker concentration ([B]) to that in the absence of the blocker is described by a Langmuir inhibition isotherm:

\[ \frac{G}{G_0} = \frac{K_i}{1 + [B]} \]  

where \( K_i \) is the inhibition constant of the blocker. Eq. 2 describes a blocking reaction where channel current is completely inhibited by the blocker. In the case of partial current inhibition (such as a subconductance state) due to binding of the blocker at a single site, then the following modification of Eq. 2 is applicable:

\[ \frac{G}{G_0} = 1 - \frac{R_{\text{max}}}{1 + K_i/[B]} \]  

where \( R_{\text{max}} \) is the maximal fraction of inhibited conductance. In cases where blocker titration curves lack sufficient data at high blocker concentration, Eq. 2 is inappropriate because the \( R_{\text{max}} \) parameter is poorly defined. This situation applies to the case of low affinity block by Zn²⁺, because ZnCl₂ concentrations >10 mM could not be tested due to formation of zinc hydroxide precipitate. In this case, deviation from strict Langmuir behavior (Eq. 2) was evaluated using the following empirical Hill equation, where \( n' \) is a pseudo-Hill coefficient:

\[ \frac{G}{G_0} = \frac{K_i^{n'}/[K_i^{n'} + [B]]}{1} \]
RESULTS

Macroscopic Current-Voltage Behavior of rh1, μ1, and Mutant μ1 Na-Channels

Macroscopic Na⁺ currents were recorded in two different cell types: the clonal L6 rat muscle cell line and Xenopus oocytes expressing the wild-type μ1 Na-channel isoform and various μ1 mutants. The former cell line was chosen because ion flux experiments (Lawrence and Catterall, 1981) and Northern blot analysis (Kallen, Sheng, Yang, Chen, Rogart, and Barchi, 1990) have previously shown that rat myoblast L5/L6 cells exclusively express TTX-insensitive Na channels corresponding to the rh1 isoform. Fig. 1 illustrates Na⁺ currents recorded from L6 myoblasts and Xenopus oocytes expressing wild-type μ1, Y401D, and Y401H mutants. In L6 myoblasts, peak whole-cell Na⁺ current was observed at a test voltage of ~-30 mV, with current amplitudes in the range of 1-6 nA from cell to cell (e.g., see Figs. 2D and 5A). In comparison with μ1 Na⁺ currents in oocytes, the inactivation time course in L6

![Figure 1](image-url)
cells was relatively fast, with a single-exponential time constant of 0.88 ± 0.23 ms at
-30 mV (mean ± SD); n = 7). In oocytes expressing μ1 Na channels, maximal
peak current in the range of 1-10 μA was observed at a test voltage of −15 mV
(e.g., see Figs. 1 A and 5 D). The inactivation time course of μ1 Na+ current in oo-
cytes measured at −10 mV was typically biphasic (Fig. 1), as described previously
(Zhou, Potts, Trimmer, Agnew, and Sigworth, 1991). This behavior has been attrib-
ted to multiple gating modes and is influenced by modulating factors (Zhou et al.,
1991) and holding potential (Ji, Sun, George, Horn, and Barchi, 1994). The rela-
tive amplitudes of the fast (τ = 1 ms) and slow (τ = 10−15 ms) components of inac-
tivation varied from oocyte to oocyte, but did not appear to be affected by the mu-
tations investigated in this study.

| TABLE I |

<table>
<thead>
<tr>
<th>Macroscopic Functional Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>rh1, μ1 wild-type, and μ1 mutants</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>rh1 (L6 cells)</td>
</tr>
<tr>
<td>μ1 wild-type</td>
</tr>
<tr>
<td>μ1 Y401A</td>
</tr>
<tr>
<td>μ1 Y401C</td>
</tr>
<tr>
<td>μ1 Y401C/N404R</td>
</tr>
<tr>
<td>μ1 Y401D*</td>
</tr>
<tr>
<td>μ1 Y401F</td>
</tr>
<tr>
<td>μ1 Y401G</td>
</tr>
<tr>
<td>μ1 Y401H</td>
</tr>
<tr>
<td>μ1 Y401S</td>
</tr>
<tr>
<td>μ1 Y401W</td>
</tr>
</tbody>
</table>

Values are mean ± SD as determined from 3 to 13 oocytes. Bath solution was standard frog Ringer containing 1.8 mM CaCl₂.
* Bath solution contained 0.2 mM CaCl₂.

Peak current-voltage relationships of wild-type and mutant Na channels were fit
to a simple Boltzmann function (Eq. 1), and the results are summarized in Table I.
All of the tested mutations of μ1 produced functional Na⁺-selective channels in oo-
cytes as shown by macroscopic conductances ranging from 20–80 μS and reversal
potentials (Vrev) that were higher than +45 mV. The extrapolated Vrev, generally de-
depended on the batch and condition of oocytes, suggesting that Vrev differences were
due to variations in intracellular ion activity, rather than changes in Na-channel se-
lectivity. The data in Table I argue that the tested mutations did not have major ef-
fects on channel function other than the alteration of toxin and divalent cation
sensitivity as described later. The observed voltage-activation midpoint (V0.5) was
~20 mV more positive for μ1 Na⁺ currents in oocytes compared to that in L6 cells.
To focus on the blocking effect of STX and divalent cations, we chose not to rely
on a simple measurement of peak-voltage Na\(^+\) current, but instead used the maximal conductance parameter, \(G_{\text{max}}\), obtained from a Boltzmann fit (Eq. 1) of peak \(I-V\) relationships. This latter parameter is a measure of channel conductance in the voltage range (\(-10\) mV) where all of the sampled Na channels are maximally activated. Measurement of \(G_{\text{max}}\) avoids the complication of current inhibition due to the well-known gate-shifting action of external divalent cations (Armstrong and Cota, 1991; Hanck and Sheets, 1992) such as that illustrated by the effect of 5 mM external Zn\(^{2+}\) on the \(V_{0.5}\) of voltage-activation of \(\mu 1\) Na\(^+\) current shown in Fig. 5 D.

**High Affinity Block by Saxitoxin and Neosaxitoxin Requires an Aromatic Residue at Position 401 (\(\mu 1\))**

The effect of STX on peak \(I-V\) relationships for \(rhl\) (L6 cells), \(\mu 1\) wild-type, Y401W, and Y401D mutants is illustrated in Fig. 2. In this assay, block by external STX is observed as a fully reversible reduction of macroscopic current with little effect on the midpoint voltage (\(V_{0.5}\)) of activation. The concentration dependence of STX block, as measured by inhibition of relative \(G_{\text{max}}\), for the two wild-type isoforms and various mutants is summarized in Fig. 3, with the corresponding \(K_i\) values presented in Table II. In cases where complete STX titration curves were obtained, STX block was well described by a Langmuir inhibition isotherm (Eq. 2), consistent with equilibrium binding of STX to a single class of sites and homogeneous expression of a single Na-channel isoform. The STX titration curves of wild-type \(\mu 1\) and the Y401F substitution that is naturally found in the rat brain II Na-channel isoform are practically superimposable with similar high affinity \(K_i\) values of 2.6 and 3.1 nM, respectively. The Y401W mutation produced a modest sixfold reduction in STX affinity.
FAVRE ET AL.  External Block of Na Channels by STX and Divalent Cations  211

\[ K_i = 17.5 \text{ nM}. \]

In contrast to the substitution of Y401 with aromatic Phe and Trp residues, mutations of the native Tyr residue to small neutral residues such as Ala and Gly and the small polar residue, Ser, decreased STX affinity by at least 1,000-fold. Assuming that the tested mutations do not cause significant rearrangement of protein structure, the contrasting effect of aromatic residues (Tyr, Phe, and Trp) and small neutral residues (Gly, Ala) at position 401 implies that an aromatic side chain provides a substantial free energy contribution to STX binding. To estimate the magnitude of this effect, we may use the Y401A mutation as a convenient reference, since Ala residues have weak molecular interactions with ligands in protein binding sites and generally do not perturb protein structure (Cunningham and Wells, 1989; Ward, Timms, and Fersht, 1990). Using the relationship, \( \Delta \Delta G = RT \ln \left( \frac{K_i}{K_i} \right) \), and the \( K_i \) data for STX in Table II, a value of \(-4.3 \text{ kcal/mol}\) is calculated for the apparent change in binding free energy attributable to the tyrosine side chain. As noted in the Discussion, this value is consistent with the expected free energy contribution of an amino-aromatic hydrogen bond (Levitt and Perutz, 1988).

Intermediate effects on STX affinity were observed for the Y401D and Y401C mutations, which exhibited \( K_i \)'s of 98 nM and 273 nM, respectively. In comparison to the reference mutation, Y401A, the side groups of Asp and Cys appear to enhance the binding of STX, but not as strongly as the aromatic substitutions. Asp and Cys residues could potentially interact with the polar cationic STX molecule by hydrogen bonding or via a weak electrostatic interaction between the dissociated anion \((-\text{COO}^-, \text{Asp}; -\text{S}^-, \text{Cys})\) and a positive guanidinium group of the toxin. The fact that the highest affinity for STX is specifically observed for aromatic residues supports the interpretation that these particular residues may participate in an interaction between the aromatic ring of the amino acid and a guanidinium group of the toxin as previously suggested by Satin et al. (1992).
In agreement with analogous findings (Satin et al., 1992; Backx et al., 1992; Heinemann, et al., 1992a), our results bolster the concept that natural differences in guanidinium toxin sensitivity between toxin-sensitive (wl and brain isoforms) and toxin-insensitive (rhl) Na-channel isoforms are primarily due to the substitution of a Cys residue for Tyr/Phe at the position homologous to Y401 of μ1. The N404R mutation, which also corresponds to a natural substitution in the P region of domain I that is present in cardiac Na-channels isoforms, decreased STX affinity by only 6.8-fold. This latter result is similar to the 4.7-fold reduction of STX affinity previously reported for the analogous N388R substitution in the brain Na-channel rbII (Heinemann et al., 1992a). In contrast to expectation from these results, Satin et al. (1992) found that the reverse substitution, R377N in rhl, decreased STX affinity by about twofold. When studied in conjunction with Y401C in the μ1 double mutant, Y401C/N404R, STX affinity was reduced to 3.05 μM (Table II), which is even lower than the STX affinity measured for rhl in L6 cells (95 nM). Taken together, these findings indicate that the effect of the Asn/Arg substitution is relatively small (0.5–10-fold change in STX affinity) and depends on the background sequence in which it occurs. Thus, although the position homologous to 404 in μ1 is not the basis of isoform-specific differences in guanidinium toxin sensitivity, it does modestly perturb toxin affinity, possible via electrostatic or steric effects of the Arg side chain.

Neosaxitoxin (NEO) is a naturally occurring derivative of STX that differs from STX by substitution of a single hydroxyl group for a hydrogen atom at the N-1 posi-

| Table II
Blocking Affinities for Saxitoxin and Neo-saxitoxin |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RH1, μ1 wild-type, and mutants</td>
<td>K&lt;sub&gt;c&lt;/sub&gt;</td>
<td>STX</td>
</tr>
<tr>
<td>μ1 wild-type</td>
<td>2.55 ± 0.31</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>μ1 Y401F</td>
<td>3.07 ± 0.23</td>
<td>(n = 4)</td>
</tr>
<tr>
<td>μ1 Y404R</td>
<td>17.3 ± 0.7</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>μ1 Y401R</td>
<td>17.5 ± 1.2</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>RH1</td>
<td>94.7 ± 6.8</td>
<td>(n = 6)</td>
</tr>
<tr>
<td>μ1 Y401D</td>
<td>98.2 ± 4.2</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>μ1 Y401C</td>
<td>273 ± 10</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>μ1 Y401C/N404R</td>
<td>3050 ± 695</td>
<td>(n = 2)</td>
</tr>
<tr>
<td>μ1 Y401A</td>
<td>3650 ± 750</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>μ1 Y401S</td>
<td>1500 ± 3100</td>
<td>(n = 8)</td>
</tr>
<tr>
<td>μ1 Y401H</td>
<td>ND</td>
<td>2760 ± 390</td>
</tr>
<tr>
<td>μ1 Y401G</td>
<td>&gt;2000</td>
<td>(n = 3)</td>
</tr>
</tbody>
</table>

Apparent K<sub>c</sub> are mean ± SE and were calculated according to Eq. 1. n represents the number of experiments done on different oocytes from the same batch, where a K<sub>c</sub> value was determined from three to five different concentrations of STX or NEO. ND = not determined.

finity by about twofold. When studied in conjunction with Y401C in the μ1 double mutant, Y401C/N404R, STX affinity was reduced to 3.05 μM (Table II), which is even lower than the STX affinity measured for rhl in L6 cells (95 nM). Taken together, these findings indicate that the effect of the Asn/Arg substitution is relatively small (0.5–10-fold change in STX affinity) and depends on the background sequence in which it occurs. Thus, although the position homologous to 404 in μ1 is not the basis of isoform-specific differences in guanidinium toxin sensitivity, it does modestly perturb toxin affinity, possible via electrostatic or steric effects of the Arg side chain.

Neosaxitoxin (NEO) is a naturally occurring derivative of STX that differs from STX by substitution of a single hydroxyl group for a hydrogen atom at the N-1 posi-
tion of the C-2 guanidinium group (Shimizu, Hsu, Fallon, Oshima, Miura, and Nakanishi, 1978). With respect to equilibrium blocking affinity at pH ~7, NEO has previously been found to be an equipotent or slightly more potent Na-channel blocker than STX (Hu and Kao, 1991). However, the blocking kinetics of STX and NEO differ markedly (Strichartz, 1984). By monitoring individual blocking events of single BTX-activated Na channels in planar bilayers, Guo et al. (1987) previously found that NEO exhibited a fivefold slower dissociation rate constant ($k_{off}$) than STX in toxin-sensitive Na channels from rat brain and skeletal muscle. In contrast, toxin-insensitive Na channels from denervated rat muscle or canine heart exhibited virtually the same $k_{off}$ for STX and NEO, which was about eight times faster than the $k_{off}$ of STX from toxin-sensitive isoforms (see Table III). These results suggested that the N1-OH group of NEO contributed to a stabilizing interaction (possibly a hydrogen bond) in toxin-sensitive Na-channel isoforms that is absent in toxin-insensitive isoforms. To examine the role of Y401 and N404 residues in this interaction, we measured the $K_r$ for conductance inhibition and the dissociation kinetics of NEO for $\mu l 1$ and several mutants. The $K_r$ data summarized in Table II indicates that NEO exhibited essentially the same equilibrium blocking affinity as STX for $\mu l 1$ Na channels and the mutants, Y401F, Y401C, Y401S, N404R, and Y401C/N404R.

Table III

| Dissociation Rate Constants for Saxitoxin and Neo-saxitoxin |
|---|---|
| $K_{off}$ | r$^{-1}$ |
| $\mu l$ wild-type and mutants | STX | NEO |
| $\mu l$ wild-type | 0.065 ± 0.002 (n = 8) | 0.011 ± 0.001 (n = 4) |
| $\mu l$ Y401F | 0.054 ± 0.002 (n = 7) | 0.0066 ± 0.0006 (n = 5) |
| $\mu l$ Y401C | 0.45 ± 0.08 (n = 6) | 0.26 ± 0.01 (n = 5) |
| $\mu l$ N404R | 0.058 ± 0.007 (n = 4) | 0.010 ± 0.002 (n = 4) |
| Rat muscle* | 0.064 ± 0.013 | 0.012 ± 0.002 |
| Denervated rat muscle* | 0.51 ± 0.002 | 0.46 ± 0.01 |
| Canine heart* | 0.045 ± 0.01 | 0.58 ± 0.03 |

$K_{off}$ in $\mu l$ and mutants were determined in oocytes from the monoexponential time course of current recovery after exchange with toxin-free solution. Sodium current was elicited by depolarizing pulses to ~10 mV from a holding potential of ~80 mV. Values are means ± SE. n represents the number of experiments.

* $K_{off}$ reported from single-channel blocking kinetics in rat skeletal or canine heart Na channels reconstituted in lipid bilayers by Guo et al. (1987).
hibited a 5.9-fold slower $k_{\text{eff}}$ for NEO as compared to STX (Fig. 4 A, Table III). The Y401C mutation resulted in a much faster time course of current relaxation from block by NEO and STX (Fig. 4). As compared to μ1, the $k_{\text{eff}}$ of Y401C for STX was increased by 6.9-fold and that for NEO was increased by 24-fold (Table III). Fig. 4 B also shows that the time course of recovery of block by STX and NEO was substantially slower than recovery from block induced by Zn$^{2+}$. Because it is known from single-channel analysis of Zn$^{2+}$ block that the dissociation rate of Zn$^{2+}$ is in the millisecond range (Ravindran et al., 1991), the recovery rate from Zn$^{2+}$ block provides a measure of the time resolution of the system as limited by the washout rate of the oocyte chamber. The rate of recovery of Y401C from block by STX and NEO is severalfold slower than the Zn$^{2+}$ control, which argues that the observed rate of toxin reversal actually reflects the rate of toxin dissociation from Na channels. Table III summarizes $k_{\text{eff}}$ values obtained in this manner for STX and NEO in μ1 and several mutants, and compares these values with those previously obtained for Na channels from adult (toxin sensitive) and denervated (toxin insensitive) rat muscle incorporated into planar lipid bilayers (Guo et al., 1987). Although the bilayer assay differs from the oocyte assay in that STX/NEO is primarily in equilibrium with the BTX-modified open state (bilayer) vs the closed resting state (oocyte) of the channel, there is good agreement for the $k_{\text{eff}}$ of STX and NEO from the wild-type μ1 channel as measured in both systems (Table III). Furthermore, the Y401C mutation closely simulates the enhancement of $k_{\text{eff}}$ for both STX and NEO that was previously observed for toxin-insensitive channel subtypes in the bilayer assay. Table III also

![Graph A](image1)

![Graph B](image2)

**Figure 4.** Determination of the time course for recovery from block by STX or NEO after toxin removal. Na$^+$ current was elicited by repetitive pulses to −10 mV from a holding potential of −100 mV. Initially, STX (●) or NEO (○) was added to the bath perfusion solution to set the fraction of unblocked current in the range of 0.3–0.5. Toxin was then removed by perfusion with toxin-free solution. The recovery of normalized current is plotted as a function of time. (A) Recovery time course for the wild-type μ1 channel. (B) Recovery time course for the Y401C mutant. Note the faster time scale in B vs A. Solid lines represent the best fit to the function, $1 - \exp(-kt)$, where $k$ is the rate constant for recovery (toxin dissociation). The dotted line shows an exponential fit to the similarly measured time course for recovery from block by Zn$^{2+}$ in the μ1 wt as an estimate of the rate of solution exchange in the oocyte recording chamber.
shows that the conservative Y401F substitution has little effect on the apparent toxin dissociation rates. The N404R substitution that naturally occurs in toxin-sensitive vs toxin-insensitive phenotypes also had no effect. These results thus demonstrate that previously measured differences in the dissociation kinetics of STX and NEO in muscle/brain vs cardiac subtypes are largely accounted for by the residue homologous to Y401 (μl). This finding further establishes the importance of this residue in determining isoform-specific differences in guanidinium-toxin sensitivity.

Substitution of Tyrosine 401 by Cysteine, Histidine or Aspartate Confers High Affinity Block by Zn²⁺

The effect of extracellular Zn²⁺ on two native Na-channel isoforms (μl and rh1) and two mutants of μl (Y401C and Y401S) are illustrated in Fig. 5. Sodium currents of the rh1 isoform assayed in L6 cells (Fig. 5 A) and the Y401C mutant of μl as-sayed in oocytes were both strongly blocked by Zn²⁺ at submillimolar concentrations, as indicated by a decrease in the slope of the peak I-V curve at positive voltages, corresponding to inhibition of the maximal conductance parameter, G_max. In contrast, millimolar concentrations of Zn²⁺ were required for a similar level of inhibition in wild-type μl and Y401S. High concentrations of external Zn²⁺ also resulted in a large depolarizing shift of the voltage activation midpoint, V_{0.5}, as illustrated by the effect of 5 mM Zn²⁺ in Fig. 5 D. For all of the mutants studied, external Zn²⁺ block was fully reversible and did not appear to affect ionic selectivity as monitored by the reversal potential.

Fig. 6 A shows corresponding titration curves for Zn²⁺ block of rh1 (L6 cells), μl,
Y401C, and Y401S. These data confirm the previous finding that high sensitivity for block by group IIB divalent cations metal ions is conferred by the presence of a Cys residue at position 401 ($\mu l$) (Backx et al., 1992; Heinemann et al., 1992$a$; Satin et al., 1992). However, the titration curves for Zn$^{2+}$ inhibition of maximal conductance ($G_{\text{max}}$) for rhl (L6 cells) and Y401C deviate significantly from a Langmuir inhibition isotherm (Eq. 2). This deviation is most evident at high Zn$^{2+}$ concentration where the relative conductance appears to approach a Zn$^{2+}$-resistant plateau rather than zero, as expected for complete block. Because this deviation occurs at <1 mM ZnCl$_2$, it is unlikely to result from surface charge screening as previously discussed by Ravindran et al. (1991). In the L6 cell line, one might argue that this phenomenon is due to a small population of Zn$^{2+}$-insensitive Na channels. However, this explanation is difficult to invoke for the Y401C mutant expressed from transcribed RNA injected into oocytes. As described in Materials and Methods, Zn$^{2+}$ titration curves for rhl and Y401C were fit to a modified one-site blocking model (Eq. 3) in which binding of the blocker produces a subconductance state rather than a complete block of the channel. A best fit to Eq. 3 was obtained with maximal Na$^+$ current inhibition of 85 ± 9% and 86 ± 2% (mean ± SD) for rhl and Y401C, respectively. This level of inhibition is close to that predicted, if normally gating Na channels exhibit a similar Zn$^{2+}$-induced subconductance as that previously described for single cardiac Na channels modified by batrachotoxin (Schild et al., 1991; Schild and Moczydlowski, 1994).

As shown in Fig. 6 B, the Y401H, Y401D, and Y401C/N404R mutations also exhibited high affinity for Zn$^{2+}$ block with $K_i$ values in the range of 15–23 $\mu$M (Table IV). The Zn$^{2+}$ titration curves for these mutants similarly appeared to exhibit a small
but significant Na\(^+\) current at 1 mM external Zn\(^{2+}\), suggestive of a Zn\(^{2+}\)-resistant substate current in the range of 5–10% of maximal conductance. Y401F, Y401S, Y401A, Y401G, and N404R mutants all exhibited low affinity for Zn\(^{2+}\) block, similar to that of wild-type \(\mu 1\) with \(K_i\) values in the range of 1.6–8.2 mM (Table IV). For these mutants, Zn\(^{2+}\) titration curves also departed from a simple Langmuir-type behavior as indicated by pseudo-Hill coefficients (Eq. 4) <1.0 (Fig. 6, legend). However, because of the limited solubility of ZnCl\(_2\), we could not distinguish whether this behavior results from a Zn\(^{2+}\)-insensitive plateau region (substate current) at high Zn\(^{2+}\) or is an effect of surface charge screening (Ravindran et al., 1991).

The apparent \(K_i\) values for Zn\(^{2+}\) summarized in Table IV show that a Cys, His, or Asp residue in place of Y401 results in a Na channel with a high affinity Zn\(^{2+}\) block-

\

<table>
<thead>
<tr>
<th>TABLE IV</th>
</tr>
</thead>
</table>
| \(\begin{array}{ll}
\text{Zn}^{2+} \text{ Blocking Affinities} \\
\hline
\text{mutants} & \text{\(K_i\) \text{\(\mu M\)}} \\
\text{\(\mu 1\) Y401C} & 8.0 \pm 0.6 \\
\text{\(\mu 1\) Y401H} & 22.9 \pm 1.6 \\
\text{\(\mu 1\) Y401C/N404R} & 20.8 \pm 1.5 \\
\text{\(rhl\)} & 25.4 \pm 1.1 \\
\text{\(\mu 1\) Y401D*} & 14.9 \pm 1.4 \\
\text{\(\mu 1\) Y401F} & 1550 \pm 150 \\
\text{\(\mu 1\) Y401S} & 1820 \pm 70 \\
\text{\(\mu 1\) Y401A} & 1700 \pm 230 \\
\text{\(\mu 1\) Y401G} & 4250 \pm 680 \\
\text{\(\mu 1\) wild-type} & 5310 \pm 620 \\
\text{\(\mu 1\) N404R} & 8180 \pm 520 \\
\hline
\end{array}\) |

Inhibitory constants \(K_i\) were obtained from the best fit of titration curves shown on Fig. 6. Values are means ± SE; \(n\) represents the number of experiments where a \(K_i\) value was determined from 3 to 10 different concentrations of Zn\(^{2+}\).

*Experiments performed in a bath solution containing 0.2 mM CaCl\(_2\).*

This finding is reminiscent of the generalization that structurally identified Zn\(^{2+}\) sites in proteins are formed by coordination of Zn\(^{2+}\) with Cys, His, Glu, or Asp residues (Vallee and Auld, 1990). Table IV also shows that the Y401C/N404R double mutant exhibited an approximately threefold higher \(K_i\) for Zn\(^{2+}\) compared with Y401C. This observation is suggestive of a weak, destabilizing electrostatic interaction between Zn\(^{2+}\)-coordinated at position 401 and a positively charged Arg residue at position 404. A similar effect of this neighboring Arg residue on Cd\(^{2+}\) and Zn\(^{2+}\) affinity was previously observed for analogous mutations in the rat brain II Na channel (Heinemann et al., 1992a).
Mutation of Tyrosine 401 to Aspartate Results in Enhanced Sensitivity to Block by Ca$^{2+}$

In preliminary experiments, we observed that the same amount of cRNA injected into oocytes yielded much less Na$^{+}$ current for the Y401D mutant vs wild-type $\mu l$ when the oocytes were assayed in normal frog Ringer containing 1.8 mM Ca$^{2+}$. In addition, the midpoint voltage of activation ($V_{0.5}$) for Y401D was shifted toward more depolarized potentials ($V_{0.5} = -12.2 \pm 2.3$ mV) in comparison with the typical value of $V_{0.5} \approx -20$ mV for $\mu l$. Because Asp residues are a common ligand in Ca$^{2+}$-binding sites, we tested the possibility that the Y401D mutation resulted in enhanced affinity for external Ca$^{2+}$. The effect of 320 $\mu$M external Ca$^{2+}$ on the peak $I$-$V$ relation for Y401D is shown on Fig. 7 A in comparison with a control $I$-$V$ curve obtained in the presence of 10 $\mu$M external Ca$^{2+}$. The decreased slope in the linear portion of the $I$-$V$ relationship indicates that Y401D is blocked by 320 $\mu$M Ca$^{2+}$, whereas >10 mM Ca$^{2+}$ is required to produce a similar decrease of $G_{\text{max}}$ for wild-type $\mu l$ (Fig. 7 C). Fig. 7 A also illustrates that 320 $\mu$M Ca$^{2+}$ produced a significant depolarizing shift in the activation midpoint voltage, $V_{0.5}$, suggesting that Y401D is also more sensitive to the gate-shifting action of Ca$^{2+}$.

Ca$^{2+}$ titration data for inhibition of macroscopic conductance of the Y401D mu-
tant and wild-type \( \mu l \) are compared in Fig. 7 C. Native Na-channels isoforms from brain, skeletal muscle, and heart have been previously found to be blocked by extracellular Ca\(^{2+}\) with an estimated \( K_i \) of \( \sim 40 \) mM at 0 mV (Ravindran et al., 1991; Sheets and Hanck, 1992). With the assay method used here, only a small (<10%) inhibition of \( G_{\text{max}} \) was observed over the range of 1.8–10 mM external Ca\(^{2+}\) for native \( \mu l \). In contrast, relative conductance of Y401D was inhibited by 65% over the range of 10 \( \mu M \) to 10 mM Ca\(^{2+}\). Furthermore, the Ca\(^{2+}\) titration curve for Y401D departed from a Langmuir inhibition isotherm in a manner similar to that previously described for the effect of Zn\(^{2+}\) on rh1 in L6 cells and the Y401C mutant of \( \mu l \). By fitting the data for Y401D in Fig. 7 C to a one-site model for incomplete block (Eq. 3), we obtained a \( K_i \) for Ca\(^{2+}\) of 290 ± 33 \( \mu M \) and a plateau value of 69 ± 1% for the maximal inhibition.

The residual current observed for Y401D at high Ca\(^{2+}\) could either be due to a subconductance block of Na\(^{+}\) current by Ca\(^{2+}\), such as that observed for Zn\(^{2+}\) in the cardiac Na\(^{+}\) channel (Schild et al., 1991; Schild and Moczydlowski, 1994), or it could be due to Ca\(^{2+}\) current if the Y401D mutation increased Ca\(^{2+}\) permeability through the channel. This latter possibility was considered since Heinemann et al. (1992b) found that substitutions of Glu for Lys at position K1422 in homologous domain III of a brain Na\(^{+}\) channel increased permeability to Ca\(^{2+}\) and Ba\(^{2+}\). To test if the Y401D mutation alters the relative permeability of monovalent vs divalent cations, inward current carried by Na\(^{+}\) and Ca\(^{2+}\) ions was compared in the experiment of Fig. 7 B. This data shows that when Na\(^{+}\) in the external bathing medium was completely substituted by Ca\(^{2+}\), there was no detectable inward current. The results of Fig. 7 B, together with the absence of a change in the normal reversal potential for \( \mu l \) (Fig. 7 A), indicate that the Y401D mutation increased the affinity for external Ca\(^{2+}\) block, but did not affect the ionic selectivity for permeation. Thus, the incomplete inhibition suggested by the Ca\(^{2+}\) titration of Y401D is compatible with the idea that Ca\(^{2+}\) binding at this site results in a subconductance state similar to that induced by Zn\(^{2+}\). Comparison with the best fit for the Zn\(^{2+}\) titration curve of Y401C (dotted line, Fig. 7 C) shows that Ca\(^{2+}\) binding in the Y401D mutant is lower in affinity and less efficient in blocking the channel than Zn\(^{2+}\) in the Cys-substituted channel.

Another possible interpretation of the results of Fig. 7 is that inhibition of Y401D current by Ca\(^{2+}\) is due to electrostatic screening of negative surface charge in the vestibule of the channel. However, the differing \( K_i \)'s of Y401D for block by Zn\(^{2+}\) (\( K_i = 15 \) \( \mu M \), Table IV) versus Ca\(^{2+}\) (\( K_i = 320 \) \( \mu M \); Fig. 7 C) indicate that ion-selective binding must be involved. If inhibition of Y401D current by Ca\(^{2+}\) is simply an effect of ionic strength, one would expect that further addition of micromolar Zn\(^{2+}\) in the presence of high Ca\(^{2+}\) would have little effect on the plateau level of Ca\(^{2+}\) inhibition. However, if both Ca\(^{2+}\) and Zn\(^{2+}\) reversibly bind to the substituted Asp residue at position 401 and produce different subconductance levels, then Zn\(^{2+}\) should further inhibit by binding Competition with Ca\(^{2+}\). Fig. 8 shows that addition of Zn\(^{2+}\) in the range of 5–80 \( \mu M \) further inhibits Y401D current in the presence of 1.8 mM Ca\(^{2+}\), a Ca\(^{2+}\) concentration that decreased \( G_{\text{max}} \) by 59% (illustrated by the fit of the Ca\(^{2+}\) titration curve, dotted line). At 1.8 mM Ca\(^{2+}\), the Zn\(^{2+}\) titration curve for inhibition of residual conductance was approximated by a Langmuir function.
with an apparent $K_i$ of $43.9 \pm 1.6 \mu M$. The increase in the apparent $K_i$ for Zn$^{2+}$ measured in the presence of 0.2 mM Ca$^{2+}$ (Table IV) 1.8 mM Ca$^{2+}$ (Fig. 8) is consistent with simple competitive binding between Ca$^{2+}$ and Zn$^{2+}$ at a site that exhibits an intrinsic $K_i = 320 \mu M$ for Ca$^{2+}$ and an intrinsic $K_i$ in the range of 6–9 $\mu M$ for Zn$^{2+}$, according to the binding competition relation: $K_{apparent,Zn} = K_{Zn,Zn} (1 + [Ca^{2+}]/K_D, Ca)$. The inhibition by Zn$^{2+}$ of residual Na$^+$ current in the presence of 1.8 mM Ca$^{2+}$ shown in Fig. 8 is also consistent with the idea that Zn$^{2+}$ and Ca$^{2+}$

bind to the same site in Y401D, but Ca$^{2+}$ binding produces a $\sim$90% substate current, whereas Zn$^{2+}$ binding produces a much smaller (<5%) substate current.

Incomplete Block Suggestive of Substate Current Depends on the Species of Divalent Cation

In contrast to the incomplete block observed here for Zn$^{2+}$ and Ca$^{2+}$, a previous study of flickering block by Cd$^{2+}$ in single Na channels of cardiac myocytes and the Y401C mutant of p1 did not find evidence for subconductance states induced by Ca$^{2+}$ (Backx et al., 1992). This discrepancy could be due to different experimental methods or the more interesting possibility that the level of substate current varies for the species of divalent cation. To address this question, we performed Cd$^{2+}$ titrations of macroscopic Na$^+$ current of rh1 in L6 cells and the Y401C mutant of p1 in oocytes. Fig. 9 summarizes the results of the Cd$^{2+}$ titration in comparison to titration curves for Zn$^{2+}$ inhibition of Y401C and Ca$^{2+}$ inhibition of Y401D. In contrast to the results for Zn$^{2+}$ and Ca$^{2+}$, external Cd$^{2+}$ completely blocked Na$^+$ current in Y401C and rh1, as demonstrated by Cd$^{2+}$–titration curves that are well described by a Langmuir inhibition isotherm. This comparison of channel block by different divalent cations reveals that different levels of current inhibition are observed for Cd$^{2+}$, Zn$^{2+}$, and Ca$^{2+}$, and implies that the level of substate current varies with the species of metal ion that coordinates with residue 401. The affinity for Cd$^{2+}$, as measured by the $K_i$ for inhibition of rh1 and Y401C, differs by $\sim$10-fold (Fig. 9), which is greater than the threefold difference in $K_i$ for Zn$^{2+}$ in rh1 vs Y401C (Fig. 6.

![Figure 8. Competitive interaction between Zn$^{2+}$ and Ca$^{2+}$ in the Y401D mutant. Data points (●) correspond to Zn$^{2+}$ inhibition of relative $G_{max}$ for the Y401D mutant in the presence of 1.8 mM external Ca$^{2+}$ (mean, n = 4). The data are fit (solid line) to a Langmuir isotherm (Eq. 2), normalized to the relative conductance measured in the presence of 1.8 mM Ca$^{2+}$ (59% inhibition). Fit parameters are given in the text. The dashed line represents the best fit of the Ca$^{2+}$ titration of Y401D, as shown in Fig. 7 C, to illustrate that Zn$^{2+}$ inhibits the "Ca$^{2+}$-resistant" fraction of conductance. Error bars are ± SE.](image-url)
A). As for Zn\(^{2+}\), the higher \(K_i\) for Cd\(^{2+}\) in rhl vs Y401C is probably related to the presence of the positively charged Arg 404 residue in rhl which appears to lower the affinity for Zn\(^{2+}\) by a repulsive electrostatic effect (Table IV, N404R mutation).

**Mutation of Tyrosine 401 to Histidine Results in Enhanced Sensitivity to Block by H\(^{+}\)**

In a mutagenesis study of the K-channel isoform Kv2.1, De Biasi, Drewe, Kirsch, and Brown (1993) found that introduction of a histidine residue in the P-region of the channel conferred high sensitivity for external block by external Zn\(^{2+}\) and H\(^{+}\). To explore whether the Y401H mutation in the \(\mu_1\) Na channel behaves in a similar fashion, we studied the effect of external pH on macroscopic current of this mutant channel. Many studies have previously shown that native Na-channel currents are inhibited by extracellular protons. For example, in guinea pig ventricular myocytes, the titration curve for reduction of unitary Na-channel current as a function of external pH exhibits half-maximal inhibition at pH ~5.04, but deviates from single-site behavior (Zhang and Siegelbaum, 1991). A similar titration of single rat brain Na channels activated by batrachotoxin yielded a \(pK_a\) of ~4.6 for external proton block under conditions of symmetrical 1 M NaCl (Daumas and Andersen, 1993). Macroscopic \(I-V\) curves at pH 7.2 and 6.5 for \(\mu_1\) and Y401H channels are compared in Fig. 10, A and B. For both channels, this decrease in external pH resulted in a reduction in conductance in the positive voltage range and a positive shift of the activation midpoint, \(V_{0.5}\). The effect of external pH on \(V_{0.5}\) was comparable for both channels. However, oocytes expressing Y401H Na channels generally exhibited a smaller current at pH 7.2 and a greater inhibition of \(G_{\text{max}}\) at pH 6.5. Titration curves of relative conductance as a function of pH show that Y401H is more sensitive to block by external H\(^{+}\) than \(\mu_1\) (Fig. 10 C). The Tyr to His mutation at position 401 resulted in a shift of the H\(^{+}\) titration curve by ~1.5 pH U more alkaline. The pH dependence of the \(\mu_1\) parent Na channel was fairly well described by a one-site titration curve with a \(pK_a = 5.88 \pm 0.03\), except for a possible deviation at pH 5, that may be interpreted as a surface charge effect (e.g., Zhang and Siegelbaum, 1991). In contrast, the pH titration curve of Y401H is distinctly biphasic. In view of the evidence for incomplete block by divalent cations described above, the biphasic pH titration curve of Y401 suggests that protonation of the His401 residue also results in an incomplete block of the channel. To estimate the underlying
pK_a's, the pH titration curve of Y401H was fit to a simple model based on two non-interacting sites of protonation: a high-pK_a site corresponding to His401 that results in incomplete block to a fractional (substate) conductance, S, and a low-pK_a site that results in complete block corresponding to that assumed to exist in the native μ1 channel. A three-parameter equation based on this model (see Fig. 10, legend) provided a good fit to the pH titration data for Y401H using the values: pK_2 = 7.5 ± 0.03 for the high-pK_a site, S = 0.16 ± 0.01 for the fractional substate conductance, and pK_1 = 4.5 ± 0.2 for the low-pK_a site.

**DISCUSSION**

The principal new findings with respect to external block of Na channels by STX and divalent cations can be summarized as follows: (a) Aromatic residues (Tyr, Phe, Trp) at position 401 (μ1) specifically stabilize the binding of STX relative to other amino acid residues at this position. (b) A Cys residue at position 401 (μ1) reduces the affinity of STX by ~100-fold relative to aromatic residues (Tyr, Phe) but enhances STX affinity by >10-fold relative to Ala and Ser. This Cys substitution is sufficient to account for the rapid dissociation rate of STX and NEO that is characteristic of cardiac Na-channel isoforms. (c) Introduction of any of the Zn²⁺-coordin...
nating residues, Cys, His, or Asp, at position 401 (μl) is sufficient to form a high affinity Zn²⁺-blocking site with a $K_i$ in the range of 10–20 μM. (d) Binding of a divalent cation or H⁺ to an appropriate residue at position 401 (μl) may result in conductance inhibition by complete block (Cd²⁺) or incomplete block (Zn²⁺, Ca²⁺, H⁺), depending on the particular blocking cation and amino acid residue at this position.

**General Considerations for Interpretation**

These conclusions were reached by using a relatively crude assay of Na-channel block, inhibition of macroscopic conductance as derived from peak $I$-$V$ relations. Our primary purpose in using this assay was to determine whether titrations of macroscopic conductance behave in a manner consistent with Zn²⁺-induced subconductance events previously observed at the single-channel level in batrachotoxin-modified cardiac Na channels in planar bilayers (Schild et al., 1991; Schild and Moczydlowski, 1994). This type of channel block by a small ion was first observed by Prod'hom, Pietrobon, and Hess (1987) for the effect of H⁺/D⁺ on L-type Ca channels with openings prolonged by a dihydropyridine agonist. These two examples of channel-blocking reactions are mechanistically distinctive because the discrete current interruptions induced by the blocking ion are incomplete closures to a well-resolved sublevel instead of complete channel closure typically associated with pore blocking events. Because such Zn²⁺-induced substate events have not yet been reported for normally gating Na channels in cell membrane patches, we wondered whether this phenomenon is a significant aspect of channel behavior under physiological conditions. To address this question, we specifically focused on defining the shape of blocker titration curves of macroscopic conductance. If ligand-induced subconductance behavior is an important phenomenon for particular blocking reactions of normally gating Na channels, we hypothesized that blocker titrations curves should deviate from strict Langmuir behavior (one-site, complete inhibition) and exhibit a blocker-resistant plateau conductance in the limit of high blocker concentration. Thus, we looked for evidence of a nonzero plateau region in the conductance-inhibition curves for Zn²⁺ in the cardiac isoform (rhl) and in the Y401C mutant of μl Na channels. As an internal control, we expected that macroscopic inhibition curves of high affinity block by STX should closely follow Langmuir behavior, because it is widely accepted that there is one STX binding site per Na-channel molecule and that occupation of this site results in complete channel block.

In reality, however, the possibility of observing and interpreting such a Zn²⁺-substate plateau by macroscopic conductance measurements faces certain difficulties. At the single-channel level, the Zn²⁺-induced substate is a very small fraction of the open channel current (~0.1–0.2), particularly at low Na⁺ concentration (Schild and Moczydlowski, 1994). Thus, this effect might not be observed macroscopically simply for lack of resolution. In addition, measurement of the $G_{max}$ parameter from peak $I$-$V$ curves according to Eq. 1 is based on a simple two-state Boltzmann fit to the voltage-activation process and the assumption of an ohmic conductance. This model is an extreme simplification of known Na-channel behavior, but it suffices for our purpose to discriminate the gate-shifting action of divalent cations (i.e.,
shift of $V_{0.5}$ from their blocking effect (i.e., reduction of $G_{\text{max}}$) (Armstrong and Cota, 1991; Hanck and Sheets, 1992; Sheets and Hanck, 1992). From the standpoint of quantitative interpretation, it must be recognized that relative $G_{\text{max}}$ as measured under the present conditions is not precisely proportional to the probability that Na channels are unblocked. In the presence of physiological Na$^+$ and K$^+$ gradients, current through open Na channels is known to rectify inwardly, as evident from close inspection of control $I-V$ data in the positive voltage range in Figs. 2 and 5. Also, the simple model of Eq. 1 does not take into account the possible voltage dependence of blocker binding. However, for the cases we are most interested in, high affinity block by Cd$^{2+}$ and Zn$^{2+}$, it is known that the blocking kinetics of these divalent cations are slow relative to the measured peak of macroscopic Na$^+$ current that occurs within the first few milliseconds after a voltage pulse (Schild et al., 1991; Backx et al., 1992; Hanck and Sheets, 1992). Thus, the voltage dependence for block by external divalent cations (apparent electrical distance of $\sim 0.2$) that is readily observed for steady state recordings of open single channels is underestimated by macroscopic $G_{\text{max}}$ measurements in the presence of Cd$^{2+}$ and Zn$^{2+}$ (Sheets and Hanck, 1992). While these aspects of Na-channel behavior undoubtedly affect the quantitative interpretation of our results, $G_{\text{max}}$ is nevertheless a useful parameter to evaluate the functional significance of substate conductance at the macroscopic level. In practical terms, because Eq. 1 provides good fits of peak macroscopic $I-V$ relations in the absence and presence of the various blockers (Figs. 2 and 5), the $G_{\text{max}}$ parameter may be viewed as an average measure of Na-channel conductance over a limited range of voltage, roughly from 0–40 mV.

The Molecular Interaction between Residue 401 (mus) and STX

It has been previously shown that the natural amino acid substitutions that occur in various isoforms (Tyr/Phe in muscle and brain, Cys in cardiac) at the position homologous to 401 (mus) determine whether native Na channels exhibit high or low affinity for the guanidinium toxins, STX and TTX (Backx et al., 1992; Heinemann et al., 1992a; Satin et al., 1992). Identifying the structural basis for such natural pharmacological differences in binding affinity among homologous members of a gene family has been recognized as a useful approach for locating residues that form or modulate ligand binding sites in proteins. However, by itself, the simple substitution of naturally occurring variant residues from one channel isoform to another is generally insufficient to determine the underlying energetic basis of ligand binding affinity (Ward et al., 1990). For example, if the replacement of Tyr401 by nonaromatic neutral residues was found to conserve high-affinity STX binding in the mus isoform, this would have implied that the side chain of Tyr401 is permissive for high-affinity STX binding, but does not participate in a specific molecular interaction with a toxin ligand in the STX-binding site. In this paper, we took the additional approach of referencing mutations of Tyr401 to the neutral functional groups of Gly ($R = -\text{H}$) and Ala ($R = -\text{CH}_3$). The functional groups of these two amino acids cannot form hydrogen bonds and generally only have small van der Waals interactions with ligands in protein binding sites. Because Ala is structurally the most suitable residue for this purpose, the concept of alanine-scanning mutagenesis has been introduced as a method of identifying the interaction
FAVRE ET AL.  External Block of Na Channels by STX and Divalent Cations

surface of peptide-ligands with protein receptor sites (Cunningham and Wells, 1989). The $K_i$ values for STX block of various substitution mutants at position 401 (Table II) reveal that all three aromatic residues (Tyr, Phe, and Trp) consistently enhance the affinity for STX and NEO by roughly a factor of 1,000 in comparison to Ala and Gly. As noted in Results, these data reinforce the interpretation that there is a specific interaction between the toxin molecule and an aromatic moiety in the binding site.

Two types of molecular interactions have been previously proposed to account for the effect of aromatic residues at position 401 ($\mu$1) in the outer vestibule of Na channels. One suggestion is a $\pi$-electron–cation interaction involving a positively guanidinium group of the toxin (Satin et al., 1992). Such an interaction is proposed to be analogous to the binding of acetylcholine to acetylcholinesterase, where aromatic residues that line the substrate binding pocket of this enzyme appear to provide favorable energetic interactions with the choline cation of the substrate (Sussman, Harel, Frolow, Oefner, Goldman, Toker, and Silman, 1991). Such $\pi$-electron–cation interactions have also been proposed to occur between the tetraethylammonium (TEA) cation and tyrosine side groups in the outer vestibule of TEA-sensitive K channels (Heginbotham and MacKinnon, 1992).

A second proposal is a hydrophobic interaction. In their recent structural model of TTX/STX binding to the four Ss–S6 Na-channel linker domains, Lipkind and Fozzard (1994) suggested that planar aromatic residues at position 401 ($\mu$1) interact favorably with a nonpolar surface of TTX that consists mainly of $-\text{CH}$ groups. While this latter idea is an interesting possibility, our results for the Y401D mutation suggest that the underlying interaction is electrostatic in nature rather than hydrophobic. Because the Asp substitution at position 401 enhances STX affinity by $\sim$30-fold relative to Ala, it appears that a carboxylate group can partially substitute for an aromatic ring in stabilizing STX binding. This is consistent with formation of a hydrogen bond or a weak electrostatic interaction with a toxin guanidinium group. Aromatic rings of Tyr, Phe, and Trp have previously been found to interact electrostatically with the amino groups of a number of amino acid side chains including Lys and Arg in protein crystal structures (Burley and Petsko, 1986; Levitt and Perutz, 1988). Therefore, we suggest that one of the six guanidinium amino groups of STX participates in an amino-aromatic hydrogen bond with Tyr 401 in the $\mu$1 Na channel (or the Phe/Trp substitution). This type of bonding interaction would be similar to the amino aromatic hydrogen bonds recently described between two Lys and two Tyr residues in the crystal structure of ovotransferrin (Dewan, Mikami, Hirose, and Sacchettini, 1993).

**Dual Role of Residue 401 as a Coordination Site for Divalent Cations**

The results of Table III show that Cys, His, or Asp substitutions at position 401 ($\mu$1) enhance the affinity for Zn$^{2+}$ block by a factor of at least 100-fold relative to Ala at this position. Because this effect is specific for side chains known to coordinate Zn$^{2+}$, the residue at position 401 appears to be located in a favorable orientation to provide a coordination site for Zn$^{2+}$ and other divalent cations in the outer vestibule of Na channels. The present results together with those of other laboratories (Backx et al., 1992; Heinemann et al., 1992a; Satin et al., 1992) demonstrate that a
variety of divalent cations can occupy this site. The Y401C mutation enhances the affinity for Cd\(^{2+}\) and Zn\(^{2+}\); the Y401D mutation also enhances the affinity for Ca\(^{2+}\) block. Regarding the question of whether a Zn\(^{2+}\)-induced substate current can be detected macroscopically, the shape of the Zn\(^{2+}\) titration curves for Y401C and rhl (Fig. 6 A) is strongly suggestive of a \(\sim15\%\) residual current when the Zn\(^{2+}\)-blocking site is fully occupied. Given the caveats mentioned above, this value is close to that predicted by the fractional current previously observed for Zn\(^{2+}\)-induced substates in single cardiac Na channels modified by batrachotoxin (Schild and Moczydlowski, 1994). The fact that similar titrations of macroscopic conductance by Cd\(^{2+}\) (Fig. 9) do not exhibit an obvious Cd\(^{2+}\)-resistant plateau is expected from the complete block by Cd\(^{2+}\) observed at the single-channel level (Backx et al., 1992).

Surprisingly, the Ca\(^{2+}\)-titration curve of Y401D (Fig. 7) and the H\(^{+}\)-titration curve of Y401H (Fig. 10) also exhibit plateau regions suggestive of subconductance block. Further studies with these mutants at the single-channel level are now needed to determine if this explanation is correct. If so, this would be compatible with our previous suggestion that residue 401 (\(\mu\)1) is located in a wide external vestibule but not within the single-filing region for ion permeation (Schild and Moczydlowski, 1994). However, these findings do not clearly support the idea that the substate current is a direct result of ion-ion repulsion between a blocking ion bound at a fixed location near residue 401 and a Na\(^{+}\) ion bound in the pore. For simple Coulombic repulsion at a fixed distance of interaction, one would expect to observe a similar substate level for blocking ions of the same charge valence (i.e., Zn\(^{2+}\), Cd\(^{2+}\), Ca\(^{2+}\)). To explain different subconductance levels for different residues and divalent cations, one would have to postulate that additional factors such as ion size, hydration, coordination geometry, involvement of other residues, and so forth play an important role. Regardless of the detailed mechanism of subconductance block by inorganic ions and H\(^{+}\), it is clear that such blocking interactions are important aspects of channel function. Recently, a proton blocking interaction has been described for a member of the cyclic nucleotide gated class of cation-selective channels that can be resolved as a two-level subconductance pattern at the unitary level (Root and MacKinnon, 1994). Further studies of the detailed mechanisms underlying such behavior may advance our knowledge of the structure of ion binding sites in protein pores.

Summary

The amino acid residue located at the position homologous to Tyr401 in the \(\mu\)1 Na channel plays a dual functional role. In Na-channel isoforms that are most sensitive to TTX/STX, aromatic residues at this position provide a favorable contribution to the toxin-binding energy that is consistent with an amino-aromatic hydrogen bond between a toxin guanidinium group and an aromatic ring of the amino acids, Tyr or Phe. In Na-channel isoforms that naturally have low affinity for TTX/STX, a Cys residue at this position serves as a coordination site for divalent cations such as Cd\(^{2+}\) and Zn\(^{2+}\). This residue can be mutated to alter the selectivity for block by external divalent cations and the pH dependence of block. The feasibility of such manipulations provides an attractive experimental system to investigate the mechanism of metal ion binding within a channel vestibule and its effect on the process
of ion permeation. The available results suggest that subconductance behavior is a physiologically important aspect of such interactions.

We would like to thank Dr. Bill Agnew for generously providing the µ1 Na-channel clone used for these experiments.

This work was supported by grants from Swiss National Science Foundation 31-39435.93 (Laurent Schild) and the United States National Institutes of Health grant GM51172 (Edward Moczydlowski).

Original version received 5 January 1995 and accepted version received 20 March 1995.

REFERENCES


