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Close Association of the N Terminus of Kv1.3 with the Pore Region*

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The Shaker superfamily encodes voltage-gated potassium (Kv) channels. The N termini of Shaker proteins are located intracellularly and contain several domains shown to regulate important aspects of channel function, such as speed of inactivation, channel assembly (T1 domain), and steady state protein level (T0 domain, amino acids 3–39 in rabbit). Mutations and/or deletion of certain amino acids in the T0 domain lead to a 13-fold amplification of Kv current as compared with wild type channels, primarily by increasing the absolute number of channel proteins present in the membrane (Segal, A. S., Yao, X., and Desir, G. V. (1999) *Biochem. Biophys. Res. Commun.* 254, 54–64). Although T0 mutants have kinetic properties virtually indistinguishable from wild type, they were noted to have a slightly larger single channel conductance, suggesting that the T0 domain might also interact with the pore region. In the present study we show that although T0 does not affect pore selectivity, it does modulate the binding affinity of the pore blocker, charybdotoxin. These results suggest that the N terminus of Kv1.3 is closely associated with the pore region.

The Shaker gene superfamily consists of at least 10 families (Kv1–Kv10) that encode voltage-gated potassium (Kv) channels. All Kv genes cloned so far code for proteins with identical secondary structures: intracellular localization of N and C termini, six transmembrane segments (S1–S6), a voltage sensor (S4), and a pore (P) region. The S4 segment senses changes in membrane voltage (1–4). Ion permeation occurs via the P region located between the fifth and sixth transmembrane segments (5–8). This is also the region that binds the channel blockers tetraethylammonium and charybdotoxin (9, 10). The structure of a potassium channel pore was recently determined from crystallographic data obtained from a potassium channel protein isolated from *Streptomyces lividans* (11). In brief, the channel is a homotetramer and its pore resembles an inverted cone with a narrow selectivity filter and a large, intracellular, water filled cavity. Because each Kv subfamily has distinctive kinetic properties, structure-function relationships of Kv proteins have been extensively studied using naturally occurring isoforms and mutants obtained by site-directed mutagenesis. The extreme N terminus mediates rapid inactivation (12, 13) in Kv proteins that inactivate quickly. A more distal N-terminal region known as the T1 domain regulates channel assembly (14–16) by serving as a recognition site for heteromultimeric channel assembly within a family and by preventing co-assembly between families.

Shaker Kv1.3 is expressed in brain, lymphocytes, and kidney, and there are excellent data showing that the Kv1.3 subfamily mediates the n type current of T lymphocytes (17, 18). The 20-fold rise in n type current that occurs upon T cell activation (19–21) is accompanied by an increase in Kv1.3 protein density at the plasma membrane, despite a decrease in steady state mRNA level (18). Moreover, the kinetics of activation/inactivation and voltage sensitivity of the n current do not change in activated T lymphocytes. Conversely, blockers of the n current inhibit mitogen-induced cell division and secretion of interleukin 2.

We recently identified an N-terminal region of Shaker Kv1.3 (T0 domain, amino acids 3–39 of rabbit Kv1.3) that modulates channel expression. Deletion of T0 increases whole cell current by more than 10-fold (22). Site-directed mutagenesis studies suggest that negatively charged amino acids are essential components of the T0 domain. Although the deletion amplifies the magnitude of expressed currents, it affects neither the time constants of activation and inactivation nor the voltage dependence. A slight increase in single channel conductance was noted in the mutant, but it could not account for the observed gain-of-function. Rather, the change in T0 mutant current was caused largely by a dramatic increase in channel protein at the plasma membrane (23). The molecular mechanisms mediating this change in steady state protein level are still being investigated but do not appear to involve clathrin-mediated endocytosis (22).

Because a small increase in single channel conductance was noted in T0 mutants, it was hypothesized the T0 domain might also interact with the pore region. We find that although T0 mutations do not affect pore selectivity, they do modulate the affinity of channel blockers binding to the extracellular surface of the pore region.

MATERIALS AND METHODS

Generation of Mutant Channels—The NT3–39 mutant was generated by deleting the DNA sequence coding for amino acids 3–39 from wild type rabbit Kv1.3 (GenBank™ accession number U38240) DNA using polymerase chain reaction (see Fig. 1). The sense primer (GGATCCTAATACGACTCACTATAGGGAGGAGCCACCATGACGGAGCAGGAGTGCTGCGGGGAG) contained a T7 polymerase site and the coding sequence for amino acids 1–2 and 40–51. The antisense primer (TTTTTTTTTTCCTGTCCTTGATGGATGGTCT) contained a stop codon and a poly(A) tail. NT3–39 was amplified by polymerase chain reaction using an Air Thermo Cycler 1605 (Idaho Technologies). The amplified product was cloned into pBluescript and sequenced by the method of Sanger *et al.* (24) to confirm that no other mutations besides the 3–39 deletion were introduced during the amplification process. Two other mutants, NT3–27 and NT3–37* were generated as described previously (22).

Expression in Xenopus Oocytes—Stage V-VI *Xenopus laevis* oocytes were dissected from ovarian lobes and stored in modified Barth's solu-

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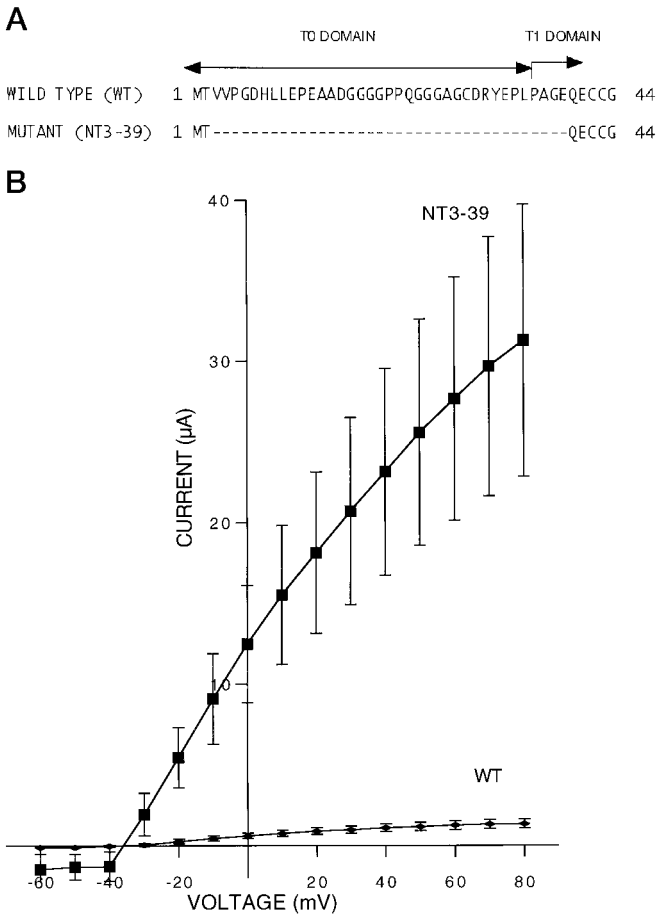

FIG. 1. *A*, T0 domain mutation. Shown are amino acids 1–44 of wild type rabKv1.3 and the deletion mutations NT3–39, NT3–27, and NT3–27*. The T1 domain mediates subunit interaction. *B*, peak current-voltage relationships for WT Kv1.3 (n = 19) and NT3–39 channels (n = 36). Current traces were elicited for wild type and NT3–39 mutant channels by a single-step command voltage protocol (voltage steps from −80 to +80 mV in 10-mV increments from a holding potential of −80 mV). Peak currents were measured and plotted against voltage.

tion. Oocytes were injected with 50 nl containing either 5 ng of *in vitro* transcribed, 5′-capped rabKv1.3 (WT)[1] RNA, 5 ng of NT3–39 (mutated) RNA, 5 ng of WT/5 ng of mutated RNA, or water as a negative control. Whole cell currents were recorded using a standard two-microelectrode voltage clamp (OC-725, Warner Inst.) 1–3 days after injection. Oocytes were impaled with microelectrodes filled with 0.5 M KCl (resistance, 0.5–5 MΩ). The bath contained 88 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM NaH_2CO_3, 5 mM HEPES, pH 7.4. In some experiments, the ionic strength of the bath solution was reduced while keeping osmolarity constant. This low ionic strength solution contained 40 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM $NaHCO_3$, 96 mM sucrose, and 5 mM HEPES. Amplified currents were filtered at 2 kHz then recorded and analyzed using PULSE (HEKA, Lambretch, Germany) and Igor-Pro (Wavemetrics). For ion substitution and tail current analysis, the chloride salt of the test cation was substituted for NaCl. Ion selectivity was measured by tail current analysis.

RESULTS AND DISCUSSION

Deletion of the T0 domain, mutant NT3–39 (Fig. 1*A*) was previously shown to cause a 13-fold increase in Kv1.3 current expressed in *Xenopus* oocytes (22). To confirm these results, cRNA of wild type or mutants was injected in *Xenopus* oocytes, and channel expression was analyzed using two-microelectrode voltage clamp and patch clamp. NT3–39 cRNA caused a large increase in whole cell current 3 days post-injection as compared with wild type cRNA as shown in the current-voltage (I-V) plot for peak currents (Fig. 1*B*). Peak current at +80 mV was 1.35 ± 0.28 μA (n = 8) for wild type as compared with 31.22 ± 8.4 μA (n = 8) for NT3–39 mutant ($p < 0.0001$).

Patch clamp recordings of wild type and NT3–39 single channels showed the unitary conductance was slightly increased in the mutant: between −40 mV and +40 mV: wild type = 8.7 pS and gNT3–39 mutant = 10.0 pS (22). Although such an increase could not explain the magnification in whole cell conductance, we were intrigued by the possibility the N-terminal T0 domain was interacting with the pore region. Because mutations in the S4-S5 loop of Shaker have been shown to alter rubidium selectivity, single channel conductance for potassium and rubidium, and the sensitivity of the channel to inhibition by intracellular cations (25), it is likely that other regions of Shaker channels also contribute to the overall structure of the pore.

To detect possible interactions of the T0 domain with the pore region, we first tested whether T0 domain mutations affected cation selectivity. To estimate the selectivity of WT and NT3–39 mutants with respect to potassium, rubidium, and sodium, tail currents were examined at voltages ranging from −100 to +20 mV, whereas external potassium or rubidium concentration was varied from 2 to 88 mM. Changes in reversal potentials were plotted against changes in potassium and rubidium concentrations. The zero current conductance ratios for potassium:sodium and rubidium:sodium were calculated based on the observed change in reversal potential per decade change in external cation concentration. The potassium permeability: sodium permeability ratio for both WT and NT3–39 was the same (ratio of 35). Similarly, the rubidium permeability:sodium permeability ratio for WT and NT3–39 was identical (ratio of 11). Substituting gluconate for chloride did not change the magnitude of the outward current. These results indicate that the T0 domain does not measurably affect channel selectivity.

We then asked whether the T0 domain could modulate the affinity of the channel blocker, charybdotoxin (CTX). CTX is a 37-amino acid peptide isolated from the venom of the scorpion *Leirus quinquestriatus*. It inhibits several types of potassium channels, including Kv1.3, at nanomolar concentration (9, 26) by binding to the extracellular face of the pore region, with a one-to-one stoichiometry and occluding the "mouth" of the channel. High affinity binding of CTX is critically dependent on electrostatic interactions of the positively charged toxin with negatively charged amino acid residues located at the mouth of the channel. Indeed, in *Drosophila* Shaker, binding affinity is reduced by a factor of 3.5 by mutating glutamate 422 to glutamine and by a factor of 12 by substituting lysine (9). We measured the effect of CTX on WT Kv1.3 and NT3–39 expressed in oocytes. Although 1 nM CTX inhibited WT current by 60%, in good agreement with published IC_{50} values ranging from 0.5 to 20 nM for human, mouse, and rat Kv1.3, NT3–39 currents were insensitive to inhibition by 1 nM CTX. The dose response curves for WT and NT3–39 were then determined (Fig. 2). Compared with WT, NT3–39 is 20-fold less sensitive to inhibition by CTX (K_i of 0.5 nM *versus* 9.3 nM).

It has been reported that the properties of Kv1.3 currents can vary depending on the cRNA concentration level injected in oocytes (27). For instance, at low Kv1.3 cRNA concentration (0.11 ng/oocyte), the expressed current inactivates and is fully inhibited by 10 nM CTX. In contrast, at high cRNA concentration (35 ng/oocyte), the current did not inactivate and was insensitive to 10 nM CTX. Honoré *et al.* (27) thought that at high RNA concentration, the channel interacted differently with the cytoskeleton. The RNA concentration used in our studies were the same (5 ng) for both WT and NT3–39. There

[1] The abbreviations used are: WT, wild type; CTX, charybdotoxin; i.s., ionic strength.

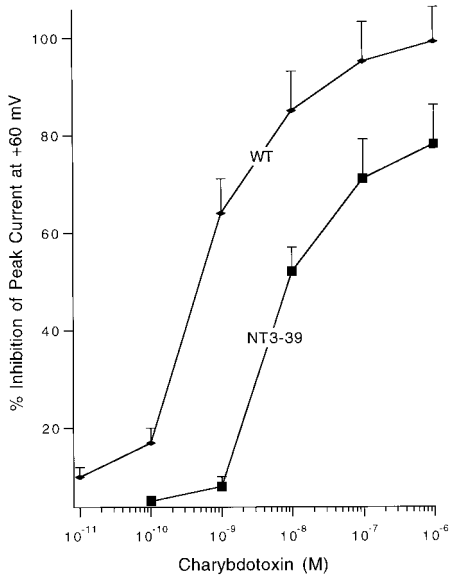

FIG. 2. **Dose response for CTX.** The percentages of inhibition of peak WT and NT3–39 currents by CTX are determined and plotted against [CTX]. As compared with WT, NT3–39 is significantly less sensitive to inhibition by CTX (K_i of 0.5 nM *versus* 9.3 nM).

TABLE I

Effect of T0 domain mutation on inhibitor profile of Kv1.3

The percentage of inhibition of peak WT and NT3–39 currents by various potassium channel inhibitors was determined.

	4AP (5 mM)	TEA (50 mM)	Glyb (250 μM)	CTX (1 nM)
Wild type	78 ± 5, n = 3	54 ± 3, n = 3	50 ± 2, n = 3	64 ± 7, n = 8
NT3–39	94 ± 5, n = 3	42 ± 4, n = 3	45 ± 2, n = 3	8 ± 2, n = 8
	NS	NS	NS	$p < 0.01$

were no differences in the activation and inactivation kinetics parameters of mutant and wild type. Furthermore, disruption of the cytoskeleton by cytochalasin D resulted in a similar degree of inhibition of both wild type and mutant channels currents (22).

Effect of Deleting the T0 Domain—It is possible that removal of the T0 domain causes large structural changes in the pore that lead to nonspecific alterations in the affinity of different and structurally unrelated inhibitors. To determine whether this was the case, other known inhibitors of Kv1.3 were tested. As shown in Table I, although NT3–39 displays relative resistance to the effect of CTX, it is inhibited to the same degree as wild type by other known blockers of the channel. These data strongly suggest that the T0 domain interacts specifically with the CTX-binding site.

This effect could be mediated by conformational changes in the pore that physically prevent CTX from reaching its binding site (steric hindrance), resulting in weaker CTX block. For instance, T0 could bind to the intracellular loop between S4, the voltage gate, and S5, the outer helix that form the pore and faces the lipid bilayer. As mentioned above, point mutations of that cytoplasmic loop can significantly alter pore function. Alternatively, T0 could interact to the carboxy end of S6, the inner helix lining the central pore. Although we have no data supporting such a mechanism, there is precedence for the N terminus of a Shaker protein binding to and blocking the intracellular mouth of the pore (13). Further support for the importance of conformational factors is given by the observation that replacing a glycine (60 $Å^3$) found at the mouth of Kv1.3 with larger residues such as glutamine and tyrosine (145–195 $Å^3$) confers resistance to inhibition by CTX. At present we cannot exclude the possibility that T0 modulates CTX binding by interacting with a site close to the pore and inducing a conformational change that strengthens the interaction of CTX to its binding site in the channel pore. Crystallographic information is needed to rigorously test this hypothesis.

Effect on Block by Other Inhibitors—Alternatively, the T0 domain that contains six negative charges and one positive charge (Fig. 1*A*) could affect the binding of CTX (net valence of +5) to the pore via through-space electrostatic interactions. Such a mechanism has been shown to operate over distances of 5–20 Å (9, 29, 30). As previously mentioned, high affinity binding of CTX depends on electrostatic interactions of the positively charged toxin with negatively charged amino acid residues located at the mouth of Shaker channels. The model predicts that T0 mutants should also be resistant to other toxins that bind to the pore and inhibit through-space electrostatic interactions. ShK, a 35-amino acid residue polypeptide isolated from the sea anemone *Stichodactyla helianthus*, also blocks Kv1.3. Its structure is different from that of scorpion toxins. However, it contains a set of conserved residues (a lysine and a tyrosine 7 Å apart) that have been shown to be critical for block of potassium channel by scorpion toxins (31). ShK-DAP22 (a mutant form of ShK) specifically inhibits Kv1.3 by binding to external mouth of pore region through electrostatic interactions. The potency of 50 pM ShK-DAP22 to inhibit WT and NT3–39 was tested. As shown in Fig. 3, NT3–39 was significantly less sensitive to inhibition by 50 pM ShK-DAP22 than WT (NT3–39, 31.4 ± 7%, n = 15; WT, 56.78 ± 6.5, n = 7; $p < 0.009$).

Effect of Ionic Strength—The electrostatic model also predicts that CTX binding should be sensitive to the ionic strength (i.s.) of the external solution. In WT, negatively charged amino acids located in the pore region and the T0 domain both contribute to the overall negative charge of the CTX-binding site. At low ionic strength the interaction of positively charged CTX with its negatively charged binding site should be stronger. One would then predict that, as compared with WT, the affinity CTX for T0 mutants will be less sensitive to changes in ionic strength. More specifically, as ionic strength decreases, the increase in affinity of CTX for NT3–39 will be less than that for WT. To test this hypothesis, inhibition of WT and NT3039 by 1 nM CTX was measured in external solutions of high and low ionic strength. The high i.s. solution contained 88 mM sodium and 2 mM potassium, whereas the low i.s. bath had 40 mM sodium, 2 mM potassium with sucrose added to keep tonicity constant. As shown in Fig. 4*A*, both WT and NT3–39 were more sensitive to inhibition by CTX at low ionic strength. However, the increase in CTX sensitivity was far greater for NT3–39 than for WT (Fig. 4*B*), suggesting that T0 does not mediate its action primarily via electrostatic interactions.

Mutation of Charged Amino Acids—If the T0 domain (net charge of −5) affects CTX sensitivity by electrostatic interactions, one would predict that progressive charge neutralization would lead to a measurable decrease in CTX sensitivity. NT3–27 is a mutant (net charge of −1) in which the T0 domain is partially deleted (Fig. 1*A*). Despite the significant reduction in overall charge of the T0 domain (from −5 to −1), NT3–27 is as sensitive to inhibition by 1 nM CTX as WT (53 ± 5%, n = 3). Furthermore, two point mutations (R31L and E33Q) that do not affect the overall charge were incorporated in NT3–27 (NT3–27*; Fig. 1*A*). Although NT3–27* carries the same net charge at the T0 domain as does NT3–27, the former exhibited a decrease in CTX sensitivity similar to that observed for NT3–39. Indeed, 1 nM CTX inhibited NT3–27* by only 9 ± 2%. (n = 3). Taken together, these data do not support the notion that the T0 domain interacts with the CTX-binding site located at the pore region via through-space electrostatic interactions.

Heteromultimers of WT and Mutated Channels—Because the

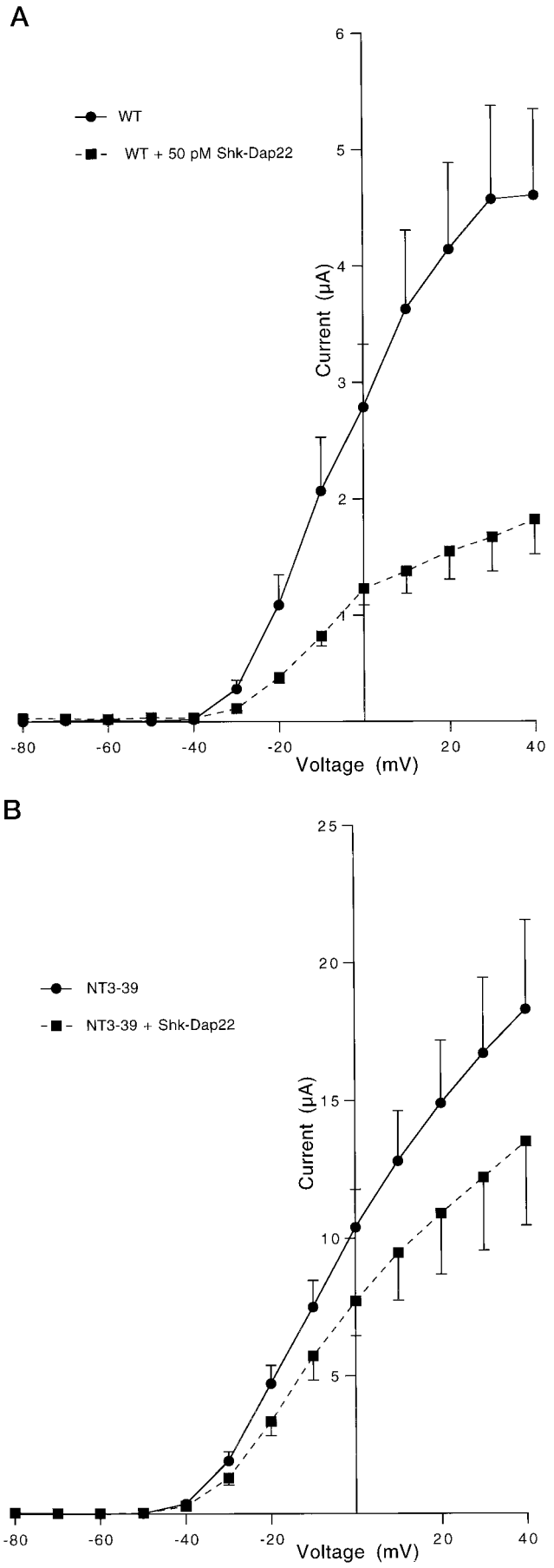

FIG. 3. **Inhibition of Kv1.3 currents by ShK-DAP22.** Current traces were elicited for WT (*A*) and NT3–39 mutant (*B*) channels by a single-step command voltage protocol (voltage steps from −80 to +80 mV in 10 mV increments from a holding potential of −80 mV). 50 pM ShK-DAP22, added to the bath, decreased base-line outward current significantly more for WT than for NT-3–39 mutant (56.78 ± 6.5% *versus* 31.42 ± 7, $p < 0.001$).

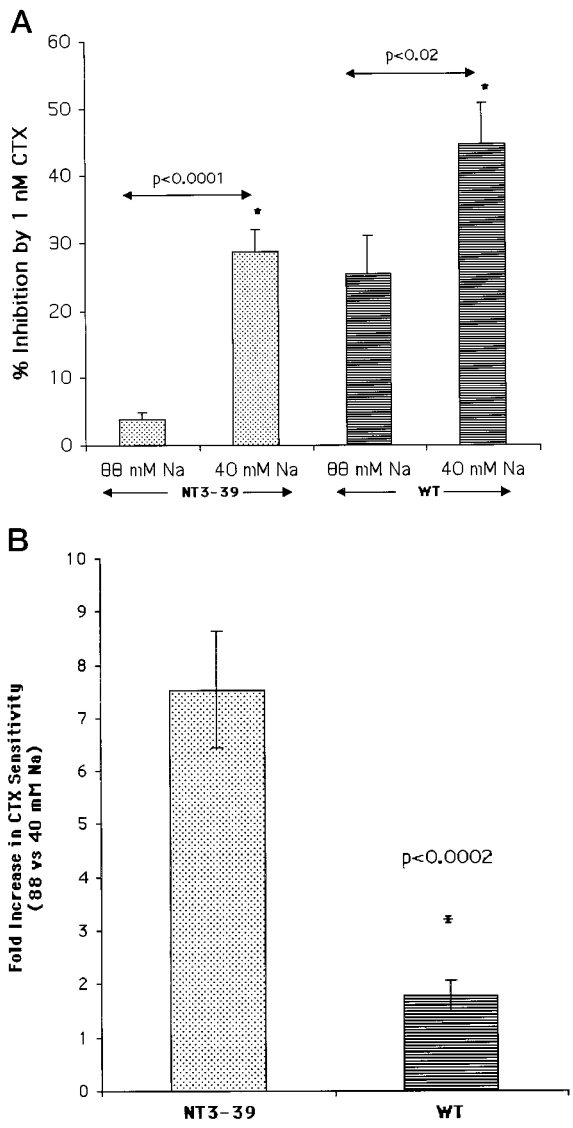

FIG. 4. **Effect of ionic strength on CTX inhibition of WT and NT3–39 currents.** *A*, current traces were elicited for wild type and NT3–39 mutant channels by a single-step command voltage protocol (a voltage step from a holding potential of −80 mV to +40 mV). The effect of 1 nM CTX on peak current at +40 mV for WT and NT3–39 mutant was determined in either a high i.s. solution (88 mM sodium) or in low i.s. (40 mM sodium). The osmolarity of both solutions was kept the same by adding 96 mM sucrose to the low i.s. solution. Low i.s. increases CTX sensitivity for both WT and NT3–39 mutant. *B*, the percentage of increase in CTX sensitivity for WT observed in the presence low i.s. is compared with that for NT3–39 mutant. The increase in CTX sensitivity was far greater for NT3–39 than for WT (7.53 ± 1.1-fold *versus* 1.75 ± 0.3-fold, $n = 8$, $p < 0.0002$).

functional Kv1.3 channel is a homotetramer, it contains four T0 domains that could potentially interact with the pore. A reduction in the number of T0 domains present in the functional channel should lead to the formation of a channel with intermediate CTX sensitivity. This prediction was tested by coinjecting WT and NT3–39 cRNAs in *Xenopus* oocytes and determining the K_i for CTX. We found that WT/NT3–39 current had a K_i for CTX of 4.8 nM. Heteromultimers of WT and

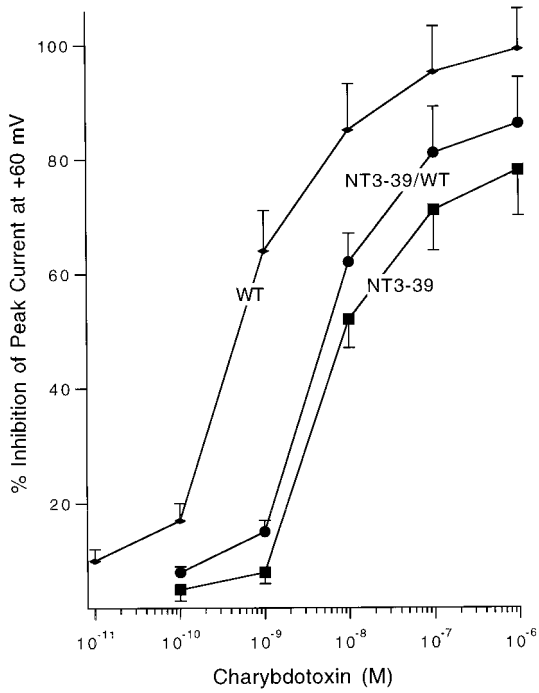

FIG. 5. **CTX sensitivity of heteromultimers of WT and NT3–39.** The percentage of inhibition of peak WT/NT3–39 currents by CTX is determined and plotted against [CTX]. WT/NT3–39 currents display an intermediate phenotype, being less sensitive to CTX than WT (K_i of 0.5 nM *versus* 4.8 nM) but more than NT3–39 (K_i of 4.8 nM *versus* 9.3 nM).

NT3–39 had, as predicted, an intermediate phenotype, being less sensitive to CTX inhibition than WT (K_i = 0.5 nM) but more than NT3–39 (K_i = 9.3 nM) (Fig. 5). Although it is clear that WT and NT3–39 form heteromultimers, the stoichiometry of each functional channel is unknown, and, therefore, the contribution of each T0 domain to the stabilization of CTX at its binding site cannot be estimated. However, we can definitively conclude that the CTX sensitivity is altered in the ensemble.

The crystal structure of the T1 domain of *Shaker* indicated that it forms a tetrameric structure, and this led to the suggestion that the complex might function as selectivity filter on the cytoplasmic side (32, 33). This hypothesis was not confirmed by recent studies examining the kinetic properties of mutant *Shaker* potassium channels lacking the entire T1 domain (28). Indeed, although channel expression was significantly reduced compared with wild type, both mutant and wild type had similar kinetic and pharmacologic properties. Of relevance to the present study, sensitivity to CTX was unchanged in the T1 deletion mutants. This discrepancy could be explained by several factors including different *Shaker* isoforms (Kv1.3 *versus* Shaker B), markedly different deletions (36 aa *versus* 205 aa), and use of a CTX variant (CTX-M29L) on *Shaker* B with a mutated CTX site.

Conclusion—We have previously shown that the T0 domain of *Shaker* Kv1.3 regulates steady state channel protein density in the plasma membrane. Compared with wild type, deleting the T0 domain leads to a 13-fold amplification of whole cell Kv current, largely by increasing the absolute number of channel proteins present in the membrane. Although the T0 domain is located at the N terminus (amino acids 3–39), the current data strongly suggest that it also interacts with the pore region. Indeed, although T0 does not affect pore selectivity, it does modulate the action of drugs whose binding to the pore depends in part on electrostatic interactions. Even though the T0 domain has a net negative charge of −5, our results do not support the notion that it modulates CTX sensitivity via through-space electrostatic interactions.

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