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Authors
Goldstein, SA
Miller, C

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A point mutation in a *Shaker* K^+ channel changes its charybdotoxin binding site from low to high affinity

Steve A. N. Goldstein and Christopher Miller
Howard Hughes Medical Institute, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254-9110 USA

INTRODUCTION

Shaker K^+ channels modified by point mutation were expressed in *Xenopus* oocytes and their block by recombinant charybdotoxin (CTX), a peptide inhibitor of K^+ channels, was studied. In particular, the effect of modifying residues in the pore-associated S5–S6 "linker" region of the channel was assessed. We found that changing just one *Shaker* residue from phenylalanine to glycine increased the affinity of the channel's CTX-receptor over 1,900-fold. Kinetic analysis revealed that the increase in binding affinity is due wholly to a decrease in toxin dissociation rate. This result demonstrates that a CTX receptor exists in the *Shaker* channel mouth, but that the presence of a phenylalanine in this position destabilizes the toxin on the binding site.

METHODS AND MATERIALS

Biochemical methods

The inactivation-removed *Shaker* channel variant used in this work was kindly provided by R. MacKinnon and was produced by deletion of amino acids 6–46 from *Shaker* H4 while in BluescriptKS+ (Stratagene). Site-directed mutagenesis was performed by the method of Kunkel (1). Mutations were confirmed by dideoxy sequencing and cRNA was prepared using T7 RNA polymerase (Promega) supplemented with capping nucleotide as previously described (2). Recombinant CTX was purified in fully active form after cleavage from a fusion protein produced in *E. coli,* as previously described (3).

Electrophysiology

Xenopus oocytes (NASCO) were prepared and injected with cRNA as previously described (2). Currents were recorded from outside-out macropatches without leak subtraction (Axopatch-1C, Axon Instruments, CA). The bath solution contained 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM Hepes (pH 7.1). Toxin was added to the bath solution with 25 μg/ml albumin. The pipette solution was 100 mM KCl, 1 mM $MgCl_2$, 5 mM EGTA, and 10 mM Hepes (pH 7.1). All experiments were performed at 21–23°C with the oocyte membrane potential held at −100 mV. During toxin perfusion and wash-out, currents were measured by periodic depolarizing pulses until equilibrium was reached. Calculations were made only from patches which persisted through both blocking and wash-out of toxin.

RESULTS AND DISCUSSION

Charybdotoxin (CTX) is a 37-residue peptide derived from scorpion venom which inhibits a variety of K^+ channels at nanomolar concentrations (3). Mechanistic studies on Ca^{2+}-activated K^+ channels have shown that one CTX molecule binds to a receptor site located in the channel's externally facing "mouth" and physically occludes its conduction pathway (4, 5). We are working to develop a picture of the CTX receptor of voltage-gated K^+ channels using recombinant CTX as a molecular probe.

CTX blocks *Shaker* K^+ channels with far lower affinity ($K_i \sim 200$ nM) than it blocks the mammalian Shaker homologue Kv3 ($K_i \sim 0.1$ nM) when studied using two-electrode voltage clamp of *Xenopus* oocytes expressing the channels (6; Goldstein, S. A. N. and C. Miller, unpublished data). Because the scorpion toxin receptor of *Shaker*-type K^+ channels appears to formed by residues in the pore-associated S5–S6 linker region (7), we compared the corresponding sequences of the two channels. *Shaker* differs from Kv3 in 10 of the 22 amino acid residues comprising the toxin receptor domain, Fig. 1. To assess if these differences influenced the toxin-receptor interaction, we probed the sites via mutagenesis.

In these studies we used a *Shaker* variant modified by an NH_2-terminal deletion which removes fast inactivation but does not alter toxin inhibition (8). Point mutations in the CTX-receptor region of *Shaker* did not alter the gating kinetics, K^+-selectivity, nor the expression level of these channels in *Xenopus* oocytes (data not shown). K^+ currents of 500 to 2,000 pA were typical in outside-out excised macropatches used for blocking studies, Fig. 2 *A*. When the phenylalanine residue at position 425 in the *Shaker* S5–S6 linker sequence was mutated to glycine (found at the equivalent position in Kv3) the affinity of the *Shaker* CTX-receptor increased nearly 2,000-fold, Fig. 2 *B*.

The kinetics of CTX binding and dissociation were studied by using outside-out macropatches in a rapid perfusion system (8) modified to exchange the bath solution with a rise time of 10 ms. The rate constants

FIGURE 1 Partial amino acid sequence of the S5–S6 linker region of *Shaker* and Kv3. Residues comprising the toxin receptor domains are displayed, the asterisk (*) indicates *Shaker* position 425 (11) which is equivalent to Kv3 position 377 (12). The channels are homologous through the 17 amino acid stretch thought to comprise the channel deep pore region, H5 (10).

were determined from the time-course of the transition from unblocked to equilibrium-blocked current levels. Fig. 3 shows the progressive block of K^+ currents through *Shaker*/425G channels in a patch abruptly exposed to 0.5 nM CTX, and a plot of the unblocked current as a function of time from which K_i, k_{on}, and k_{off} were determined.

Kinetic analysis revealed that the large change in the affinity of the CTX-receptor interaction was the result of an effect on toxin off-rate, Table 1. Mutation of *Shaker* position 425 from phenylalanine to glycine leaves the on-rate unchanged, whereas toxin off-rate was decreased over 1,800-fold. This result demonstrates that a CTX receptor structure exists in the *Shaker* channel mouth, but that a phenylalanine at position 425 destabilizes CTX in the site by ~4.5 kcal/mol.

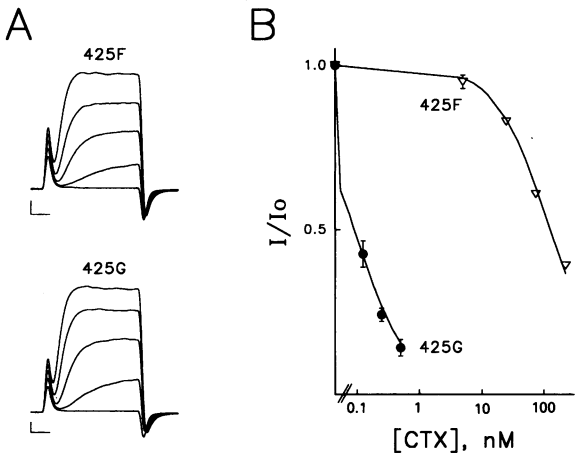

FIGURE 2 CTX blockade of *Shaker*/425F and *Shaker*/425G. (*A*) K^+ currents were measured in outside-out excised macropatches from *Xenopus* oocytes. The membrane potential was held at −100 mV and depolarized for 10 ms to test potentials from −40 to +40 in 20 mV increments. The scale bars represent 0.2 nA and 2 ms. (*B*) The fraction of unblocked current (I/Io) is plotted as a function of CTX concentration. Currents were measured during 10 ms test pulses to −20 or 0 mV from a holding potential of −100 mV once equilibrium blockade was achieved. Each point is the mean ± SEM for three or more determinations. The solid curves are fit to $I/Io = (1 + [CTX]/K_i)^{-1}$. Equilibrium dissociation constants (K_i) are reported in Table 1.

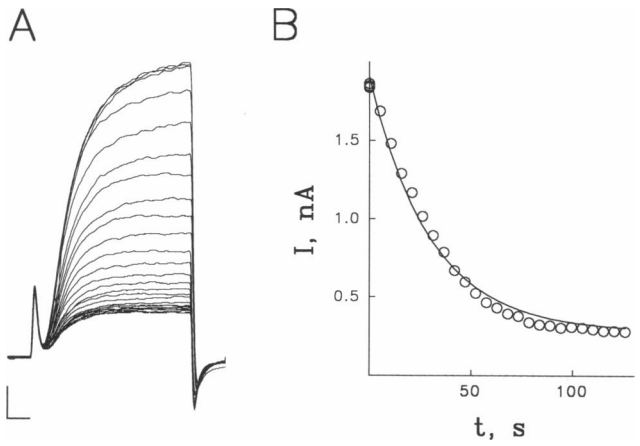

FIGURE 3 CTX blocking kinetics of *Shaker*/425G. (*A*) Currents were elicited by 15 ms depolarizing pulses to −20 mV from a holding potential of −100 mV every 5 s until equilibrium blockade was reached. After four control pulses the patch was exposed to 0.5 nM CTX. The scale bars represent 0.2 nA and 2 ms. (*B*) The raw macroscopic current is plotted as a function of time after solution change, t. The curve was fit according to $I(t) = I_\infty + (Io - I_\infty)e^{(-t/\tau)}$; τ = 30.3 s.

Because the structure of CTX is known (9), we hope to use this molecule as a caliper to measure physical distances between residues making up its receptor site in the *Shaker* channel mouth, a functionally important region of the outer conduction pathway (10). Both CTX and its binding site can be altered through site-directed mutation and we will seek complementary point mutations to map their interaction. *Shaker* position 425 appears to be a strong candidate for a local channel contact point with a specific toxin residue, in part, because isoforms of CTX with only a few amino acid differences tolerate a phenylalanine in position 425 and block *Shaker* with high affinity (8).

TABLE 1 Kinetic parameters for CTX block of *Shaker* K^+ channels

	k_{on}	k_{off}	K_i
	$M^{-1}s^{-1} \times 10^6$	s^{-1}	nM
Shaker/425F	50 ± 4	7.3 ± 0.6	152 ± 20
Shaker/425G	54 ± 1	0.004 ± 0.001	0.08 ± 0.01

CTX blockade of *Shaker*/425G was performed as described in Fig. 3. Blockade of *Shaker*/425F was evaluated by curve fitting raw data over a single 300 ms test sweep (data not shown). The equilibrium dissociation and rate constants are given in terms of the measured time constant, τ, and the fraction of unblocked current, f_{un}; $K_i = [CTX]f_{un}/(1\text{-}f_{un})$, $k_{off} = f_{un}/\tau$ and $k_{on} = (1\text{-}f_{un})/\tau[CTX]$. As expected for a bimolecular reaction, the on-rate for binding of CTX was found to be linearly dependent on toxin concentration, whereas the off-rate was independent of toxin concentration and was used as a confirmation of the value calculated from CTX wash-in data (data not shown).

Note added in proof: Mutation of *Shaker* position 425 from *F* to *G*, as described in this report, was recently also reported to increase CTX binding affinity in wild-type, inactivating *Shaker* H4 by Oliva, C., R. Wiedmann, C. Bennett, K. Folander, R. Swanson, and J. Smith (1991). Mutations in the S5-S6 of Kv3 affect charybdotoxin blockade and the rate of slow inactivation *(Soc. Neurosci.* 17:775*a. (Abstr.))*.

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