Electrostatic Interaction between Charybdotoxin and a Tetrameric Mutant of *Shaker* K^+ Channels

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ABSTRACT The scorpion toxin, Charybdotoxin (CTX), blocks homotetrameric, voltage-gated K^+ channels by binding near the outer entrance to the pore in one of four indistinguishable orientations. We have determined the pH-dependence of CTX block of a tetrameric *Shaker* potassium channel with a single copy of a histidine replacing the wild-type phenylalanine at position 425. We compared this pH-dependence with that from homotetrameric channels with four copies of the mutation. We found that protonation of a single amino acid at position 425 had a large effect on the affinity of the channel for CTX—much larger than expected if only one of the four CTX binding orientations was disrupted. The pK_a for the H^+-ion induced protection from CTX block indicates that the electrostatic environment near position 425 is likely basic in nature, perhaps because of the proximity of lysine 427. We also examined the pH-dependence of block of channels with one and four copies of the histidine mutation by CTX containing neutralizing mutations of four basic residues on the active face of the toxin. The results suggested an orientation of CTX on the channel that places three of the positively charged CTX residues very near three of the four *Shaker* 425 positions.

INTRODUCTION

The scorpion α-K-toxins, including Charybdotoxin (α-KTx1.1, CTX; Miller, 1995), bind to many types of K^+ channels with very high affinity. The small (37 amino acid) CTX peptide blocks ion movement by occluding the pore in these channels (Park and Miller, 1992a,b; Goldstein and Miller, 1993). These toxins bind to homotetrameric voltage-gated K^+ channels by binding to one of four independent, overlapping binding sites (MacKinnon, 1991).

The scorpion toxins have been shown to be useful probes of K^+ channel function and structure. The homotetrameric arrangement of voltage-gated K^+ channels was first revealed by an analysis of block of wild-type and mutant channels by CTX (MacKinnon, 1991). In addition, the α-K-toxins have been used to determine K^+ channel subunit composition in native tissues (see Garcia et al., 1998). Complementary mutations of the channel and the toxin provided a rough mapping of the outer vestibule of K^+ channels (Goldstein et al., 1994; Stampe et al., 1994; Hidalgo and MacKinnon, 1995; Aiyar et al., 1995) before the solving of the crystal structure of a bacterial K^+ channel (Doyle et al., 1998). Indeed, the properties of toxin block were exploited to establish the structural conservation of prokaryotic and eukaryotic K^+ channels (MacKinnon et al., 1998).

The α-K-toxins, including Charybdotoxin, contain several basic amino acids, and structural analysis shows that most of the positive charges on CTX are on one face of the molecule and exposed to solvent (Bontems et al., 1992). There are significant through-space electrostatic interactions between charges on the toxin and charges on the channel (Stocker and Miller, 1994; Naini and Miller, 1996). Detailed studies of these electrostatic interactions have provided new insights into the toxin-channel binding reaction and suggest that this is not a diffusion-limited mechanism (Escobar et al., 1993; Mullmann et al., 1999).

One important determinant of channel sensitivity for CTX is the amino acid at the position equivalent to 425 in *Shaker* channels (Goldstein and Miller, 1992). We have replaced the wild-type phenylalanine with a histidine (the wild-type amino acid in some K^+ channel types) and probed electrostatic interactions between CTX and the protonated imidazole ring of the introduced histidine (Perez-Cornejo et al., 1998). External H^+ ions inhibit CTX block of *Shaker* K^+ channels that contain four copies of the histidine at position 425. The inhibition of block is very sensitive to solution pH and cannot be accounted for by a single protonatable site in the channel. The homotetrameric channel structure provides four histidines for each mutant channel and the data are consistent with the presence of four titratable sites per channel.

Protonation of one of the four histidine side chains could prevent CTX from binding to only the protonated subunit or could disrupt the ability of CTX to bind to protonated and nonprotonated sites. This latter effect may seem unlikely because there are many examples of high toxin sensitivity of channels with only a single copy of an unfavorable mutation (e.g., MacKinnon, 1991 and Naranjo and Miller, 1996). To more directly determine if protonating only a single site is sufficient to render *Shaker* channels insensitive to CTX, we examined the pH dependence of CTX block of a tetrameric K^+ channel containing only a single copy of a histidine at one of the four 425 positions.

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We found that CTX block of *Shaker* K^+ channels with a single histidine at position 425 remained sensitive to external pH. The pH dependence of block was consistent with titration of a single protonatable site with an apparent pK value of 5.8—very similar to the value of 5.9 estimated from our study of channels with four copies of the histidine mutation (Perez-Cornejo et al., 1998). These results showed that protonating only a single histidine residue had a larger effect on CTX affinity than expected if only a single binding orientation was disrupted. The data are consistent with a mechanism in which protonation of a single site on the channel is sufficient to render the channel essentially insensitive to CTX.

We showed previously (Perez-Cornejo et al., 1998) that the inhibition of CTX block at low pH was not likely due to a steric interaction between the homotetrameric mutant channel and the CTX molecule. Rather, there is likely an electrostatic interaction between basic residues on the toxin and the protonated imidazole of the introduced histidine (Stocker and Miller, 1994; Naini and Miller, 1996). In our previous study (Perez-Cornejo et al., 1998), we attempted to determine which CTX charge interacts with the protonated histidine at position 425. The pH dependence of CTX block was little effected by the K11Q or K31Q neutralizing CTX mutations (Perez-Cornejo et al., 1998).

We extended these observations to include the neutralizing mutations R25Q and R34Q. The pH sensitivity of CTX block of homotetrameric F425H channels was only slightly affected by any single, neutralizing toxin mutation and moderately reduced by the double neutralizing mutation K11Q/K31Q. The pH dependence of CTX block of channels with a single copy of the histidine mutation was reduced by the K11Q mutation and very much reduced by the K11Q/K31Q double neutralization. These results suggest that no single basic residue on CTX interacts with the protonated imidazole on the histidine at position 425. Rather, the geometric arrangements of three of the charges on CTX may align with the channel such that each of these is in very close apposition to three of the four 425 positions in the channel.

MATERIALS AND METHODS

The experiments described here were done with methods similar to those we used in a previous study of the actions of CTX on *Shaker* K^+ channels (Perez-Cornejo et al., 1998). That work may be consulted for additional details.

Molecular biological methods

Several K^+ channel constructs were used in this study. The wild-type channel was the inactivation-deletion version of *Shaker* B, ShB Δ6–46 (Hoshi et al., 1990). The F425H mutation used in our previous study (Perez-Cornejo et al., 1998) was introduced into the ShB Δ6–46 clone using a two-step polymerase chain reaction protocol and the resulting mutant clone was analyzed by DNA sequencing.

We also designed a tetrameric channel construct of ShB Δ6–46. The four repeats were linked by eight amino acids (NNQQQNNQ) after the deletion of the stop codon. Each subunit was ligated to the next using compatible cohesive ends to allow control of subunit position within the tetramer. For this study, a single F425H mutation was produced in the subunit repeat nearest the amino terminal end of the protein. This construct is designated 1-F425H. These 1-F425H channels appeared to have assembled correctly because they displayed generally normal gating kinetics and K^+ selectivity (see Fig. 4). In addition, the CTX sensitivity at high pH is similar to homotetrameric F425H channels.

The molecular structures of Charybdotoxin (Figs. 1 and 7) and the KcsA channel (Fig. 7) were viewed with WebLab ViewerLite 3.2 (Molecular Simulations, Inc., www.msi.com).

Recombinant Charybdotoxin

CTX is a highly basic molecule with seven positively charged amino acids. Five of these lie essentially on one plane of the molecule that interacts with the channel (Park and Miller, 1992b; Stampe et al., 1992). These five residues, K11, R25, K27, K31, and R34 are identified in the two-dimensional view of CTX illustrated in Fig. 1. Toxin K27 is likely centered over the channel pore opening when CTX is sitting at its binding site (Park and Miller, 1992a). We examined several neutralizing mutations of these amino acids: K11Q, R25Q, K31Q, R34Q, and the double mutation K11Q/K31Q. A neutralizing mutant of K27 (K27N) did not appreciably block F425H channels even at a concentration of 20 μM.

The variants of CTX were produced by expressing a cleavable fusion protein in *Escherichia coli.* Sequence-specific proteases were used to cleave the fusion protein from the toxin. The recombinant CTX was purified using standard biochemical methods, oxidized to form the disulfide bonds, and the N-terminal end was cyclized to form pyroglutamate. For details, see Park et al. (1991) and Stampe et al. (1994).

Oocyte isolation and microinjection

Frogs, *Xenopus laevis*, were maintained as described by Goldin (1992). Isolated ovarian lobes were rinsed with Ca-free OR-2 solution (82.5 mM

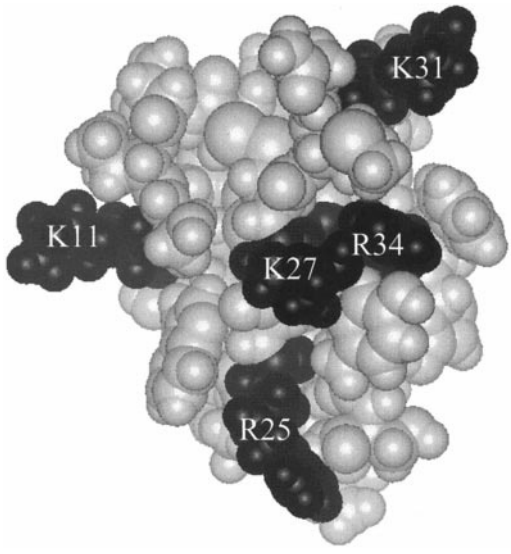

FIGURE 1 Interaction surface of Charybdotoxin. Two-dimensional representation of the face of CTX that binds to K^+ channels. The five basic amino acids on this face of the molecule are shown (*dark sections*) and labeled.

NaCl, 2.5 mM KCl, 1 mM $MgCl_2$ and 5 mM HEPES; pH 7.6 with NaOH) and then defolliculated by incubation for 60–90 min with 2 mg/ml collagenase Type IA (from Sigma). Cleaned oocytes were transferred and maintained for 2 h in ND-96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES and 2.5 mM Na-pyruvate; pH 7.6 with NaOH) before injection. Injected oocytes were transferred to multiwell tissue culture plates and incubated at 18°C in ND-96 solution supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin.

Electrophysiologic recordings

Potassium channel currents were assayed electrophysiologically 1–5 days after RNA injection. Electrophysiologic recordings were done at room temperature (20–22°C) using the cut-open oocyte voltage clamp apparatus (model CA-1B, DAGAN Corporation, Minneapolis, MN). The experimental chamber (ELV-1, Dagan Corporation; Costa et al., 1994) was modified to include a low volume (80 μl) insert to allow conservation and complete exchange of CTX solutions. Connections to the different compartments were made with glass capillaries containing 75-μm platinum wires and filled with a 1 M NaCl, 3% agar solution. No correction for the junction potential between the 1M NaCl solution and the experimental solution was made.

Electrodes for recording membrane voltage were made of 1 BBL glass with filament (1.5-mm outer diameter) from World Precision Instruments (Sarasota, FL). The resistance of the electrodes was less than 1 MΩ when filled with a 3M KCl solution. Data acquisition was performed using a 12-bit analog/digital converter controlled by a personal computer. Current records were filtered at 5 kHz. Series resistance compensation was used; the mean value was 1.4 $\pm$ 0.71 kΩ (SD; $N = 48$).

The composition of the external solution used for electrophysiologic recordings was (in mM): 100 NaCl, 2 KCl, 1 MgCl, and 1.8 $CaCl_2$. Solutions of pH 5.0, 5.5, 6.0, and 6.5 were buffered with 10 mM MES; solutions of pH 7.0 and 8.0 were buffered with 10 mM HEPES, and EPPS, respectively. These solutions and those with toxin also contained 30 ug/ml BSA.

CTX block was computed from the channel current recorded at the end of a 40-msec pulse to +40 mV compared to the average of current recorded before and after application of the toxin. As for F425H homotetrameric channels (Perez-Cornejo et al., 1998), block of 1-F425H channels was readily reversible: the current after the largest block (pH 7 and 8) recovered to 94 $\pm$ 1.4% (SEM, $N = 12$) of the control value.

RESULTS

pH dependence of CTX block of F425H homotetrameric channels

Shaker K^+ channels expressed from F425H subunits will have 4 protonatable imidazole groups. CTX block of these channels is pH-sensitive (Perez-Cornejo et al., 1998) as illustrated in Fig. 2. The top panel in this figure illustrates channel currents recorded before, during, and after application of 150 nM wild-type CTX in a pH 7.0 solution. In this example, block (at a membrane voltage of +40 mV) was 47% and recovery almost complete at 99% of control value. The lower panel contains similar data from the same oocyte at pH 6 and shows that, at pH 6.0, the channels were almost completely resistant to CTX.

Fig. 3 contains data (□) from our previous study (Perez-Cornejo et al., 1998) of the pH sensitivity of CTX block (at +40 mV) of homotetrameric *Shaker* channels that have four copies of the F425H mutation. The dotted line is the best fit

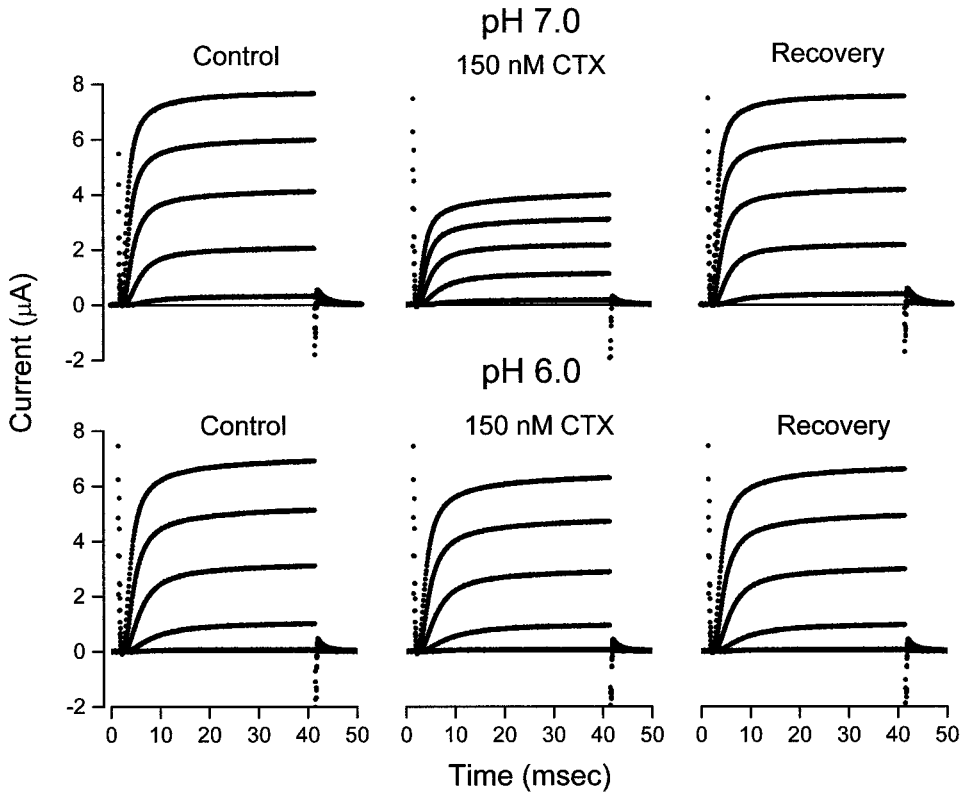

FIGURE 2 pH-Dependent block of homotetrameric *Shaker* K^+ channels with four copies of the F425H K^+ mutation. *Upper panel*, Currents before (control), during, and after (recovery) application of 150 nM CTX at pH 7.0. *Lower panel*, Currents from the same oocyte before, during, and after application of 150 nM CTX at pH 6.0. Currents in each panel are in response to 40-msec voltage clamp steps to −40, −20, 0, +20, and +40 mV from a holding potential of −70 mV.

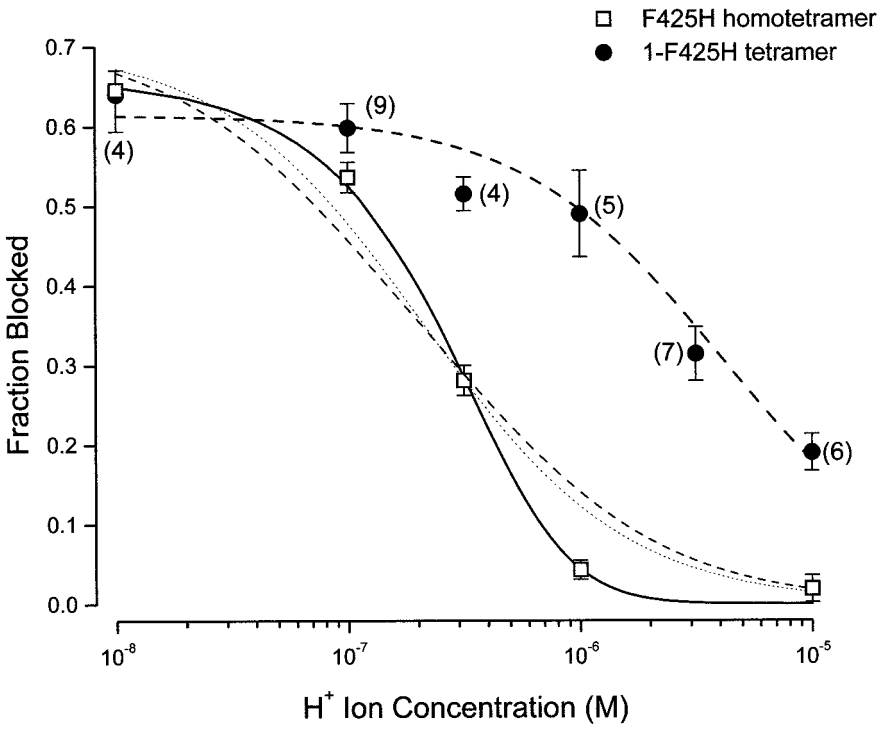

FIGURE 3 pH-Dependence of CTX block of F425H homotetrameric and 1-F425H tetrameric channels. Fraction of F425H homotetrameric channel current at +40 mV blocked by 100 nM CTX at the indicated hydrogen ion concentration (□, from Perez-Cornejo et al., 1998). *Dotted line*, fit of Eq. 1 to the F425H homotetrameric channel data; K_d and pK_H values are 42 nM and 7.2, respectively. *Solid line*, fit of Eq. 2 to the F425H homotetrameric channel data; K_d and pK_H values are 48 nM and 5.9, respectively. *Short-dashed line*, see text. (●), Fraction of 1-F425H tetrameric channel current at +40 mV blocked by 1 μM CTX at the indicated pH. Numbers of measurements are shown in parentheses. *Long-dashed line*, Fit of Eq. 1 to the 1-F425H tetrameric channel data; K_d and pK_H values are 630 nM and 5.8, respectively.

of simple, competitive inhibition between CTX and H^+ ions for binding to the channel:

$$\text{Fraction Blocked} = \frac{[\text{CTX}]}{[\text{CTX}] + K_d(1 + [H^+]/K_H)}, \quad (1)$$

where K_d is the dissociation constant for CTX interaction with the unprotonated channel, and K_H is the dissociation constant for channel protonation. The data are more sensitive to pH than can be accounted for by CTX competition with a single H^+ ion, likely because the homotetrameric F425H channel has four protonatable imidazole groups.

In our previous study, we considered the possibility that, as each of the four imidazole groups in the homotetrameric channel is protonated, one of the four available CTX binding orientations would be lost. That is, after one imidazole group was protonated, the effective CTX affinity would be reduced to ¾ of the value for the unprotonated channel; two protonated groups would reduce the affinity by ½, etc. The thin, dashed line in Fig. 3 is the best fit of this model to the data and provides a poor description of the results. Thus, protonating a single imidazole in the homotetrameric channel has a much larger effect on inhibiting CTX than simply eliminating one of the four overlapping CTX orientations.

The simplest model consistent with the data considers that protonation of any one of the four sites renders the channel entirely insensitive to CTX and the pH-dependence of block is given by

$$\text{Fraction Blocked} = \frac{[\text{CTX}]}{[\text{CTX}] + K_d(1 + [H^+]/K_H)^4}, \quad (2)$$

where K_d and K_H have the same meaning as in Eq. 1. The solid line in Fig. 3 is the best fit of this equation to the data and provides an accurate description of the results. The optimal CTX binding affinity (K_d in Eq. 2) for this fit was 48 nM and the inhibitory pK_H value was 5.9.

pH Dependence of CTX block of 1-F425H tetrameric channels

It appears that the presence of a positive charge at only one of the four 425 locations in the homotetrameric channel may be sufficient to eliminate CTX binding. This result is surprising because there are many examples of channels with only a single copy of a mutation unfavorable to CTX that retain fairly high toxin sensitivity (e.g., MacKinnon, 1991; Naranjo and Miller, 1996; Gross and MacKinnon, 1996). These studies were done with mixed RNA populations and relied on statistics (MacKinnon, 1991) or "gate-tagging" (Naranjo and Miller, 1996; Gross and MacKinnon, 1996) to determine the toxin sensitivity of channels with a single copy of an unfavorable mutation. To directly address the effect of a single positive charge at position 425, we tested the pH sensitivity of CTX block of tetrameric *Shaker* channels containing a single copy of a histidine at this position (see Methods). Examples of the action of CTX at pH 7 and 6.0 on 1-F425H tetrameric channels are illustrated in Fig. 4.

The upper panel of Fig. 4 shows 1-F425H channel currents recorded before, during, and after application of 1 μM CTX at a pH of 7.0. At this pH, 61% of the channels were reversibly inhibited by CTX at a potential of +40 mV. This

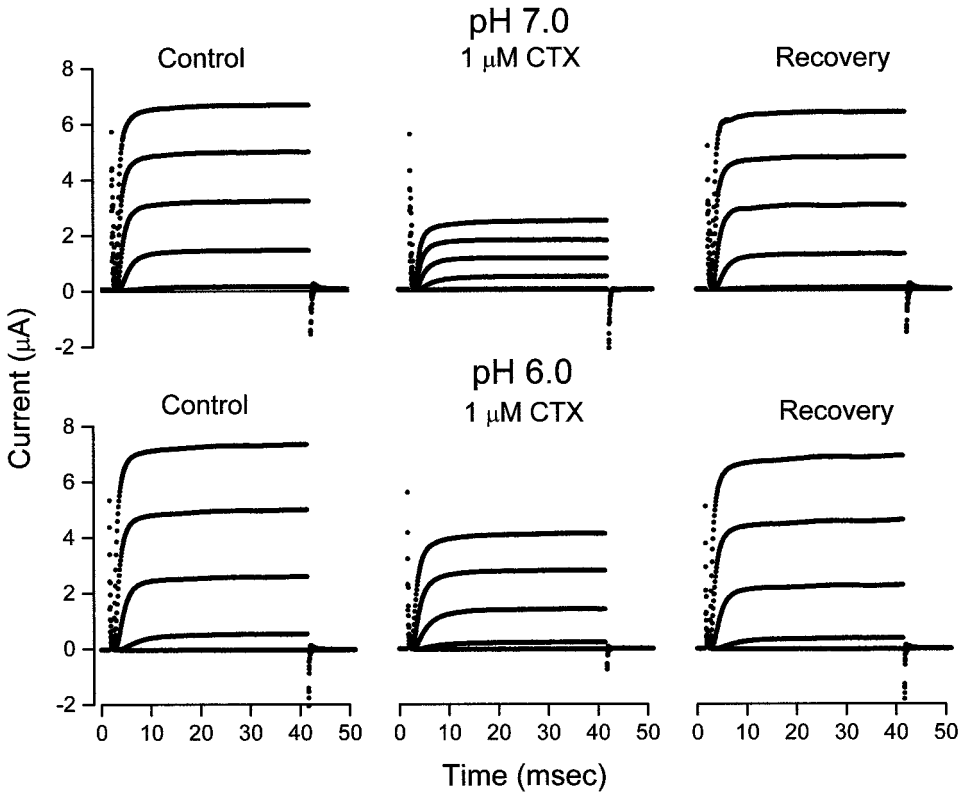

FIGURE 4 pH-Dependent block of tetrameric channels with a single copy of the F425H mutation. *Upper panel*, Currents before (control), during, and after (recovery) application of 1 μM CTX at pH 7.0. *Lower panel*, Currents from another oocyte before, during, and after application of 1 μM CTX at pH 6.0. Currents in each panel are in response to 40-msec voltage clamp steps to −40, −20, 0, +20, and +40 mV from a holding potential of −70 mV.

concentration of CTX blocked about the same fraction of 1-F425H channels as 100–150 nM CTX blocks F425H homotetrameric channels. At pH 7.0, wild-type *Shaker* channels are blocked (at +40 mV) by CTX with a K_d value of about 1 μM (Perez-Cornejo et al., 1998). Consequently, the lower affinity of CTX for 1-F425H tetramer channels is likely a result of the presence of three wild-type, low-affinity phenylalanine residues at position 425 and a single higher affinity histidine. As illustrated in the lower panel of Fig. 4, the 1-F425H channels remained sensitive to CTX at pH 6. In this oocyte, 42% of the channels were reversibly inhibited by 1 μM CTX at this pH.

The sensitivity of 1-F425H channels to block by CTX over the pH range 8–5 is illustrated by the filled circles in Fig. 3. The pH sensitivity of CTX block of channels with a single copy of the histidine mutation at position 425 was less than that of channels with four copies of the mutation. This is the expected difference between channels with one and four titratable residues. The heavy dashed line in the figure is the best fit of Eq. 1 to the data from the 1-F425H tetrameric channels with K_d and pK_H values of 630 nM and 5.8, respectively. The data are well described by this model, in which adding a single charge to position 425 renders the channel completely insensitive to CTX block.

Although H^+ ions were able to protect 1-F425H tetrameric channels from CTX block, a higher concentration was necessary than for protection of F425H homotetrameric channels. This difference likely reflects the difference between having four or one protonatable sites per channel. Protonating any one histidine protects from CTX block, but the probability of protonating at least one site is considerably higher if there are four such sites available. The fourth power of the H^+ ion term in Eq. 2 reflects this increased probability. Indeed, the fit of Eq. 2 to the F425H homotetrameric channel data (*solid line*) and Eq. 1 to the 1-F425H tetrameric channel data (*heavy dashed line*) yielded essentially the same estimate for the affinity of the introduced histidine for H^+ ions: 5.9 and 5.8, respectively. Thus, the differences between the pH dependence of toxin block of 1-F425H tetrameric and homotetrameric channels are entirely consistent with the difference between having one and four titratable groups per channel.

According to this analysis, at any fixed pH value, there should be two classes of channels: 1) channels with three wild-type phenylalanines and one uncharged histidine at 425; and 2) channels with three wild-type phenylalanines and one protonated histidine. The latter channels would be insensitive to CTX. The former class of channels would be blocked by CTX with a K_d value appropriate for the affinity of channels with three wild-type phenylalanine groups and one uncharged histidine. This value should be predictable from available knowledge of the affinity of pure wild-type channels and channels with four copies of histidine at position 425. As noted above, the apparent affinity of wild-type channels is about 1 μM. However, such channels have four orientations for CTX, so the microscopic affinity of

these channels for CTX is expected to be 4 μM. From a similar consideration, the affinity of CTX for a single uncharged histidine would be about 190 nM (4 $\times$ 48 nM). The macroscopic affinity of channels with three wild-type phenylalanines and one histidine at position 425 can be determined according to

$$\text{Effective } K_d = \frac{k_d \cdot k_d^*}{3k_d^* + k_d}, \quad (3)$$

where k_d is the microscopic affinity of the wild-type phenylalanine and k_d^* the affinity of the mutant neutral histidine. According to Eq. 3, the effective K_d for this class of channels should be about 170 nM.

The fraction of insensitive channels can be predicted from the pK_H for protonation of the single, introduced histidine. At pH 7.0, the pK_H value of 5.8 (Fig. 3) predicts an insensitive fraction of 0.06. We tested these predictions by measuring the block of 1-F425H channels at pH 7.0 and the results are illustrated in Fig. 5.

Each symbol in Fig. 5 represents data from a different oocyte. The amount of block appears to reach a maximum below 100%—the block by 5 μM CTX was similar to or less than the amount at 3 μM. The solid line is drawn with the values predicted above: an effective K_d value of 170 nM and an unblocked fraction of 0.06. The data appear to be consistent with this prediction.

pH Dependence of block by charge-neutralizing CTX mutants

As described in the Introduction, we have shown that protonating the histidine at position 425 inhibits CTX block by a mechanism that does not involve steric interference (Perez-Cornejo et al., 1998). The data and analysis in Figs. 3 and 5 indicate that protonating a single histidine does more to inhibit CTX binding than simply eliminating one of the four CTX binding orientations. There are significant through-space electrostatic interactions between charges on the toxin and charges on the channel (Stocker and Miller, 1994; Naini and Miller, 1996). Thus, it seems likely that a positive charge at position 425 eliminates CTX block through an electrostatic interaction with some basic residue(s) on the toxin. In an attempt to determine which toxin charges interacted with the protonated histidine at channel location 425, we tested several charge-neutralizing mutants of CTX on both F425H homotetrameric and 1-F425 tetrameric channels.

Neutralizing a toxin charge that interacts with the protonated channel histidine would be expected to remove the low pH protection of CTX block. We tested neutralization of toxin charged residues K11, K31, R25, and R34 (see Fig. 1) at pH 8 and 5.5. We used concentrations of the mutant toxins that produced block at pH 8 that was between ~40 and 75%. Neutralizing toxin lysine K27 (K27N) decreased the affinity for the channel to such a degree that even 20 μM of mutant toxin produced a negligible block at high pH. This is consistent with the almost 10^4-fold decrease in the affinity of this mutant toxin for *Shaker* channels with a glycine at position 425 (Goldstein and Miller, 1993). Thus, we were unable to directly assess the role of this charge on the pH dependence of block.

The estimated affinity of wild-type and mutant CTX for F425H homotetrameric and 1-F425H tetrameric channels is listed in Table 1. Block by these CTX molecules at pH 8.0

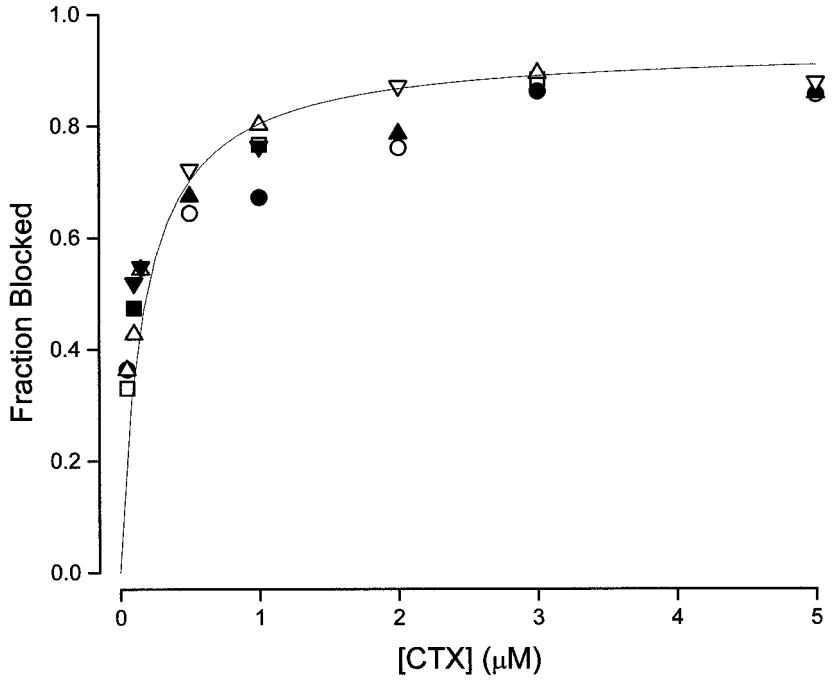

FIGURE 5 Block of 1-F425H tetrameric channels by CTX at pH 7.0 as the fraction of current blocked at the indicated concentration of CTX. Each symbol represents a different oocyte. *Line*, See text for details.

TABLE 1 Estimated affinity of mutant CTX for homotetrameric and tetrameric F425H channels

	F425H Homotetramer		1-F425H Tetramer	
CTX	pH 8.0	pH 5.5	pH 8.0	pH 5.5
		μM		
Wild-type	0.056 ± 0.01	21 ± 7.4	0.084 ± 0.016	0.33 ± 0.048
R25Q	0.55 ± .044	40 ± 13	—	—
R34Q	0.64 ± 0.082	16 ± 1.6	—	—
K31Q	0.37 ± 0.05	5.8 ± 0.87	—	—
K11Q	0.39 ± 0.009	8.9 ± 2.3	0.35 ± 0.067	0.9 ± 0.14
K11Q/K31Q	1.5 ± 0.13	8.3 ± 1.63	0.57	1.1

Estimated K_d values for channels with four (F425H homotetrameric) or one (1-F425H tetrameric) copy of a histidine at position 425. Wild-type, K11Q, and K31Q CTX data on F425H homotetrameric channels are from Perez-Cornejo et al. (1998). Wild-type CTX K_d for F425H homotetrameric channels at pH 5.5 was determined from four CTX concentrations on 4–5 oocytes; K11Q/K31Q values are from two CTX concentrations on two oocytes. The remaining K_d values were estimated from a single CTX concentration on 3–6 oocytes according to: $K_d = [CTX] \cdot (1/F_B - 1)$, where F_B is the fraction of current blocked by the applied concentration of CTX.

was near 50%, and so provides an accurate estimate of the affinity at this pH. Because, in some cases, block at low pH was quite small, the K_d values at these pH levels are less reliable, as reflected in the larger estimated errors.

These results show that, as expected, protonation of the imidazole group at low pH protected the channels from block by CTX. In general, there was less protection by the charge-neutralized CTX mutants. A quantitative description of the results can be made by considering the difference in binding free energy ($\Delta\Delta G$) between CTX block in pH 8.0 and pH 5.5 solutions:

$$\Delta\Delta G = RT \ln \frac{K_d(\text{pH5.5})}{K_d(\text{pH8.0})}. \quad (4)$$

Because the pK_H values for the introduced imidazole groups are near 5.8, almost all the sites will be unprotonated at pH 8.0. However, not all the sites will be protonated at pH 5.5. Thus, the computed free energy changes will underestimate the unfavorable energy provided by protonation of the histidines at position 425.

The free energy differences (in units of RT) are illustrated in graph form in Fig. 6. Figure 6 *A* shows the values for the indicated CTX mutants on F425H homotetrameric channels. Block by wild-type channels was very pH sensitive, as indicated by the large free energy change. Neutralizing any single positive charge on the toxin reduced the pH sensitivity as indicated by the smaller $\Delta\Delta G$ values. The free energy change associated with the pH 5.5 solution for the double

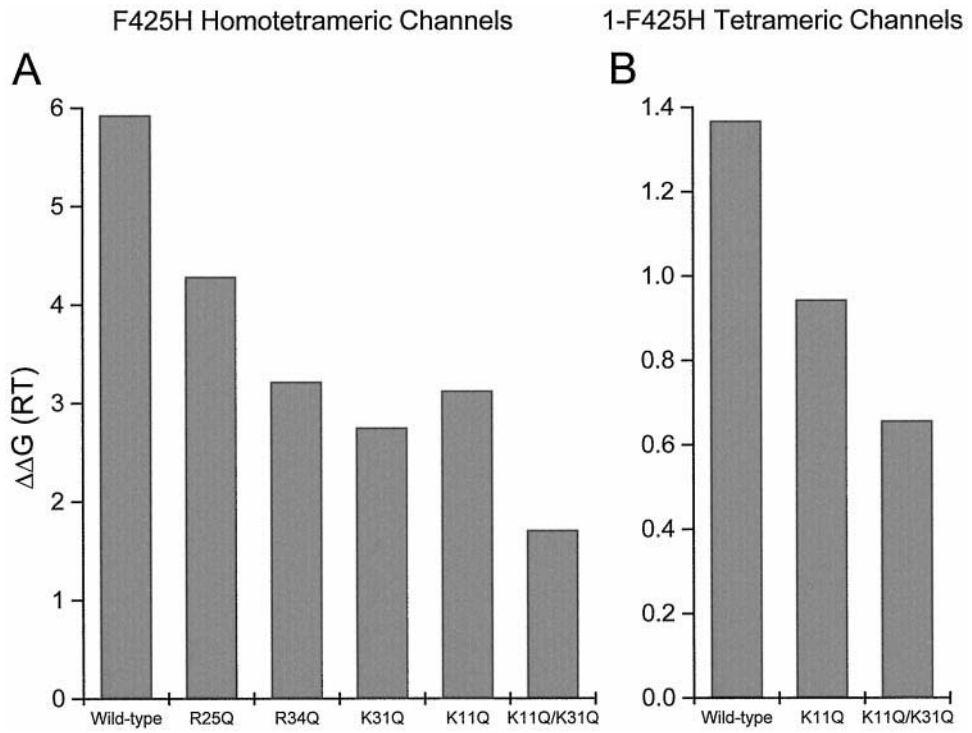

FIGURE 6 pH-Dependence of block of F425H homotetrameric and 1-F425H tetrameric channels by neutralizing mutants of CTX. The change in binding free energy at pH 5.5 compared to pH 8 determined for wild-type and the several charge-neutralized CTX mutants as described in text. (*A*) F425H homotetrameric channels. (*B*) 1-F425H tetrameric channels.

charge-neutralized mutant (K11Q/K31Q) was less than that of either single mutation alone. Thus, removing any single positive charge on the toxin reduced the ability of protonation of the introduced histidines to protect F425H homotetrameric channels from block. Removing two positive charges from the toxin reduced the protection ability more than removal of any single charge.

Figure 6 *B* illustrates the free energy change of toxin block associated with protonation of the introduced imidazole of 1-F425H channels. The values are all much less than those for the F425H homotetrameric channels consistent with the smaller number of protonatable histidines. Neutralizing one and two toxin charges produced a progressive decrease in the pH sensitivity of toxin block. Block of channels with a single copy of the histidine at position 425 by the toxin with two positive charges removed was almost insensitive to pH. That is, protonation of the single histidine at position 425 was hardly able to protect channels from block by toxins of reduced valence.

DISCUSSION

One result of this study is that protonating even a single histidine residue introduced at position 425 in *Shaker* K^+ channels protected the channel from block by CTX. This result is consistent with the finding of Gross and MacKinnon (1996) that a single lysine substitution at position 425 decreases *Shaker* channel affinity for a related scorpion toxin, Agitoxin, by at least 20-fold.

We found that neutralization of any single positive charge on the CTX molecule had little effect on the ability of low pH to protect homotetrameric F425H from block. The ability of a protonated histidine at position 425 to protect homotetrameric channels was reduced by a double neutralizing CTX mutant. CTX block of tetrameric 1-F425H channels was less sensitive to pH because of the presence of only a single, titratable, imidazole group. Block of these channels by toxin with one and, especially, two neutralized positive charges became almost independent of pH. That is, it was much more difficult to protect channels with a single histidine from block by toxin of reduced charge than channels with four copies of the histidine at 425. All these results may be reconciled by a model for CTX binding to *Shaker* channels that is illustrated in Fig. 7.

Shown in the right-hand side of Fig. 7 is the same view of CTX as in Fig. 1 with the important positively charged amino acids identified. As can be seen, K11, K31, and R25 form a near isosceles triangle with K27 and R34 approxi-

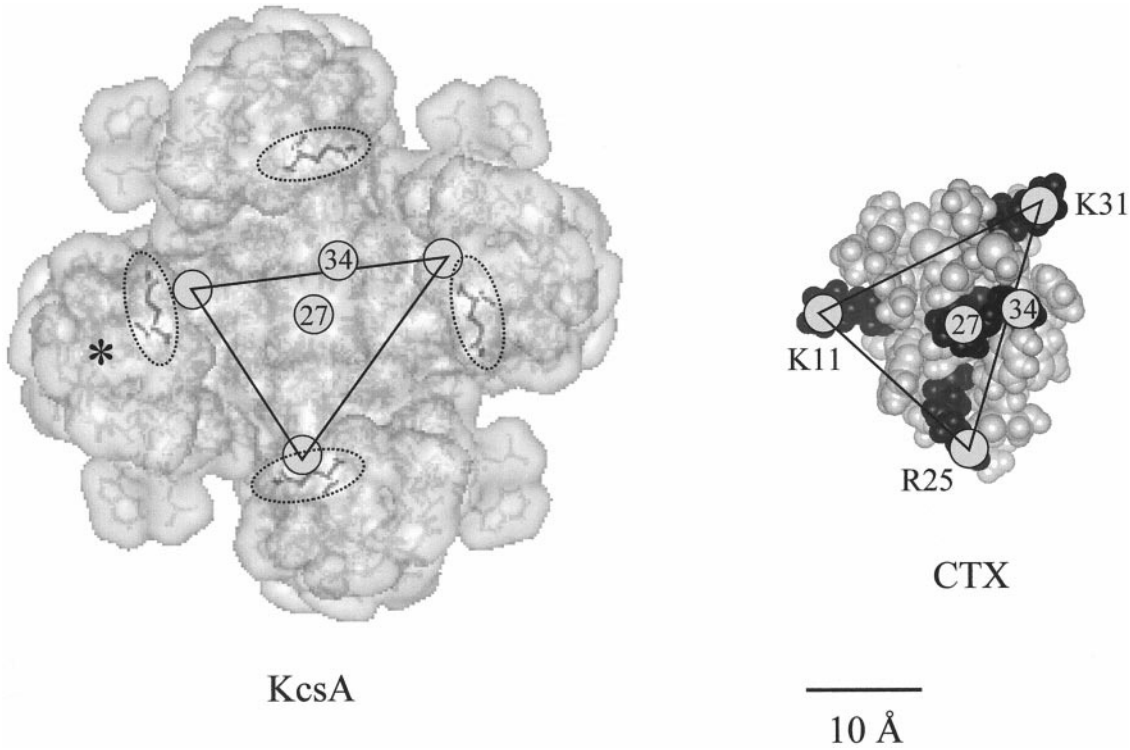

FIGURE 7 Proposed topology for CTX interaction with *Shaker* K^+ channel. *Right*, View of CTX as in Fig. 1 with the basic amino acids labeled. A triangle is drawn that connects K11, R25, and K31 with circles representing the approximate locations of the positive charges of the basic amino acids. *Left*, Two-dimensional representation of the outer surface of the KcsA bacterial K^+ channel as an approximation to the *Shaker* K^+ channel structure. The KscA residues equivalent to *Shaker* 425 are indicated by the dotted ellipses. The triangle and associated circles represent one of the proposed fourfold symmetric binding orientations of CTX on the *Shaker* K^+ channel. The approximate location of *Shaker* K427 is indicated by the ∗.

mately midway between the leg connecting R25 and K31. Shown on the left in the figure is a two-dimensional view of the outer entrance to the pore in the KcsA bacterial K^+ channel as determined by x-ray crystallographic methods (Doyle et al., 1998). The ion pore is in the middle of the diagram; the position of the four KcsA amino acids that are equivalent to those at *Shaker* 425 are outlined by dotted ellipses. The KcsA channel structure may be a good model for the scorpion toxin binding site on voltage-gated, including *Shaker*, K^+ channels (MacKinnon et al., 1998).

The triangle (with circular representations for the five basic amino acids) represents our view of CTX binding in one of the four possible orientations on the homotetrameric channel. This configuration places three toxin charges (K11, K31, and R25) within 2 Å or less of three of the four 425 locations. Thus, protonating any one of these three sites would be expected to substantially reduce the ability of CTX to bind to the channel. The fourth F425 site is approximately 14 Å from three charges (R25, K27, and K31) and 9 Å from R34. If the electrostatic potential from these charges follows a 1/distance law, this configuration is equivalent to a single charge 3 Å from the fourth 425 location. If the potential behaves as a Debye potential (see Stocker and Miller, 1994), the equivalent distance would be approximately 6 Å. In either case, the proximity of the four CTX charges would be expected to have a rather large unfavorable interaction with a protonated histidine at this fourth 425 position.

Thus, protonating any one of the four histidines in F425H homotetrameric channels would be expected to substantially destabilize binding of wild-type CTX in any of the four possible orientations. Neutralizing any single charge on the toxin would have only a relatively small effect on homotetrameric channels because the toxin would have only a single orientation that did not involve a very close apposition of toxin and channel charges. Neutralizing two toxin charges (e.g., K11 and K31) would simultaneously remove two unfavorable interactions with protonated 425 positions. This would result in less protection from CTX block at low pH in agreement with the data shown in Fig. 6.

Channels with a single histidine at position 425 are less protected from CTX block by low pH solutions because there is only a single protonatable group on the channel. Thus, at any single pH level, the probability of protonating the site is less in the channels with a single copy of the histidine. According to the binding scheme illustrated in Fig. 7, neutralizing a single charge on CTX will reduce the likelihood of an unfavorable interaction between the channel and CTX because only two of the four binding orientations would provide a close apposition of toxin and channel charges. Block by the double neutralizing mutant would be rather insensitive to pH because only one of four orientations would be unfavorable.

The data of Fig. 3 show that the pK_a of the histidine introduced at *Shaker* position 425 has a value of 5.8–5.9. This is considerably smaller than the value near 7 for histidine residues in a neutral protein environment (see, e.g., Thomas et al., 1985). Histidines that are involved in hydrogen bonds or located near basic amino acids have much lower pK_a values (e.g., Sancho et al., 1992; Oda et al., 1993). Thus, *Shaker* position 425 may be in a region of positive charge. Indeed, the KcsA crystal structure predicts that *Shaker* lysine 427 is located quite close to position 425, as indicated by the ∗ in Fig. 7.

Our proposed binding orientation for CTX on *Shaker* K^+ channels is similar to those previously described (e.g., Goldstein et al., 1994; Stocker and Miller, 1994). The introduction of four protonatable groups in homotetrameric channels and a single one in tetrameric channels allowed an assessment of the relationship between toxin charges and channel position 425. We propose that toxin position 31 is very close to *Shaker* position 425 in contrast to the view of Goldstein et al., (1994) that places toxin positions T8 and T9 near 425. MacKinnon et al., (1998) docked Agitoxin2, a close relative of CTX, on the KcsA crystal structure with toxin positions 24 and 25 partially overlapping KcsA position 58 (analogous to *Shaker* 425), an orientation similar to the one we propose for CTX and *Shaker* illustrated in Fig. 7.

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