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Inhibition of ClC-2 Chloride Channels by a Peptide Component or Components of Scorpion Venom

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Abstract. ClC chloride channels play essential roles in membrane excitability and maintenance of osmotic balance. Despite the recent crystallization of two bacterial ClC-like proteins, the gating mechanism for these channels remains unclear. In this study we tested scorpion venom for the presence of novel peptide inhibitors of ClC channels, which might be useful tools for dissecting the mechanisms underlying ClC channel gating. Recently, it has been shown that a peptide component of venom from the scorpion *L. quinquestriatus hebraeus* inhibits the CFTR chloride channel from the intracellular side. Using two-electrode voltage clamp we studied the effect of scorpion venom on ClC-0, -1, and -2, and found both dose- and voltage-dependent inhibition only of ClC-2. Comparison of voltage-dependence of inhibition by venom to that of known pore blockers revealed opposite voltage dependencies, suggesting different mechanisms of inhibition. Kinetic data show that venom induced slower activation kinetics compared to pre-venom records, suggesting that the active component(s) of venom may function as a gating modifier at ClC-2. Trypsinization abolished the inhibitory activity of venom, suggesting that the component(s) of scorpion venom that inhibits ClC-2 is a peptide.

Key words: Anion channel — Gating modifier — Ion channel inhibition — ClC-2 — Peptide toxin

Introduction

The phylogenetically ubiquitous Cl^- channel proteins within the ClC family are responsible for a multitude of physiological functions in organisms as varied as mammals, elasmobranchs, yeast, and green plants [3, 19, 20]. Represented by nine mammalian variants, ClC proteins show widespread differential tissue distribution and provide regulatory control over numerous cellular functions ranging from the electrical excitability of skeletal muscles and neurons [46] to volume regulation of epithelial cells [20]. ClC channels are voltage-gated, and in some cases pH- or swelling-activated, as in ClC-2 [21]. Mutations within these channels have been linked to such heritable human diseases as myotonia (ClC-1) [21], Dent's disease (ClC-5) [26] and Bartter's syndrome (ClC-Ka) [45]. ClC proteins such as ClC-2, ClC-5, and ClC-Kb are expressed in the kidney, where they play an important role in Cl^- transport in the ascending limb of the loop of Henle, as well as the collecting duct [9]. Knockout mutants suggest that ClC-3 has a role in the formation of the hippocampus [47], and mutations in ClC-2 may be responsible for certain forms of generalized epilepsy [18], although this has not been conclusively proven. ClC-2 is also proposed to play a role in cystic fibrosis (CF).

CF is the most common lethal, autosomal recessive disease among Caucasians, and is caused by a defect in the Cystic Fibrosis Transmembrane con-

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Abbreviations: 9-AC, anthracene-9-carboxylic acid; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CITx, chlorotoxin; CPB, 2-(p-chlorophenoxy) butyric acid; CPP, 2-(p- chlorophenoxy) proprionic acid; DIDS, 4′4′-Diisothiocyano-2,2′-Stilbene disulfonate; DMSO, dimethyl sulfoxide; DNDS, 4,4′-Nitrostilbene- 2,2′-disulfonate; DPC, diphenylamine 2-carboxylic Acid; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)- N,N,N′,N′-tetraacetic acid; VSTx, voltage sensing toxin.

ductance Regulator (CFTR). Both ClC-2 and CFTR are localized to epithelial cells in the lung and intestine [5, 17, 20, 25, 43, 50]. While CFTR is clearly localized to the apical membranes of these epithelia, there is disagreement concerning the membrane localization of ClC-2. Contradictory studies have shown it localized to either the basolateral membrane [25], or the apical membrane [17, 25, 34]. It has been proposed that ClC-2 may be a candidate channel for ameliorating the CF phenotype. It is possible that ClC-2 can provide the necessary Cl^- conductance at the apical membrane that is lacking when CFTR is defective. It has also been proposed, however, that disruption of ClC-2 can increase survivorship in CF patients [17, 50]. These contradictory studies make understanding ClC-2 very important, so that the role of ClC-2 in CF or treatment for CF can be better assessed.

Given the relative importance of ClCs, it is surprising that the fine structure and the underlying gating mechanisms of mammalian members of the ClC family remain unknown. In-depth structural analysis of membrane proteins is slow due to difficulty in successful formation of crystals for study. The recently solved structures of two bacterial ClC channel homologues have provided a roadmap for determination of pore structure of mammalian ClCs [12], but many questions remain unanswered. Despite considerable homology between bacterial and mammalian ClCs, the large cytoplasmic domains present in mammalian ClCs may have significant influence on both the function of those channels and their structure in comparison to the bacterial ClCs. A second problem is that crystal structures only capture a snapshot of molecular structure and may not describe the dynamic features of the gating process. ClC channels exhibit a fast gating process, which controls access to one of the two protopores, and a slow gating process, which controls access to both pores simultaneously [20]. Although the ClC crystal structure provides some hints about residues involved in fast gating, it confers little other information about the overall control of channel gating. Experiments utilizing structural probes in combination with site-directed mutagenesis are still necessary for the study of permeation and gating in ClC channels. Channel blockers such as DIDS and DNDS show little effect [15]; CPB and CPP have been used with some success in examining the gating processes of ClC-0 and ClC-1 [2, 38, 39], but they bind with relatively low affinity ($K_{1/2} \sim 15$ μM). DPC and NPPB ($K_{1/2} \sim 1$ mM) have been shown to be effective pore blockers of these channels, albeit with low affinity [15]. Heavy metal ions such as Zn^{2+} have been used in gating studies of ClC-0 [4]. However, high affinity probes of ClC channels have yet to be discovered. In order for detailed studies of ClC channel structure and dynamics to be performed, new tools are needed, which can address these issues.

Peptide toxins have been very useful for studying pore structure and gating mechanisms of voltage-gated cation channels [23, 24, 35]. They usually exhibit high affinity interactions and can act as either pore blockers or gating modifiers. These types of tools are lacking for chloride channel research. Recently, a component of scorpion venom has been shown to inhibit CFTR [14]. However, there are no known peptide inhibitors for the ClC family. Chlorotoxin (ClTx), a peptide toxin from the venom of *Leiurus quinquestriatus quinquestriatus*, has been shown to inhibit Cl^- current in channels reconstituted from rat intestinal cell membranes, although the molecular identity of this channel is not known [6, 7]. Studies have shown, however, that ClTx does not inhibit volume-regulated, calcium-activated, or cAMP- activated Cl^- channels from the extracellular side [29], or CFTR from the intracellular side [14]. Preliminary studies have shown that chlorotoxin has no inhibitory effect on ClC channels.

In this study, we asked whether scorpion venom contains a peptide component (or components) that may be an inhibitor of ClC Cl^- channels. The identification of a peptide inhibitor for ClC channels will provide an excellent tool for studying the structure and gating mechanisms of these complex proteins. We investigated the effects of partially-fractionated venom from the scorpion *L. quinquestriatus hebraeus* (Lqh) on the permeation and gating characteristics of ClC-0, ClC-1 and ClC-2. We show that one or more peptide components of venom interact in a voltage-dependent manner with ClC-2 from the extracellular side of the channel to inhibit Cl^- currents, while currents from ClC-0 and ClC-1 were not affected. Further, our data suggest that this peptide inhibitor acts as a gating modifier of ClC-2 channels.

Materials and Methods

Oocyte and cRNA Preparation

Xenopus oocytes were isolated as previously described [30] and incubated at 18°C in a modified Liebovitz's L-15 medium (pH 7.5) with a cocktail of antibiotics (gentamycin, penicillin, streptomycin) and HEPES. For electrophysiological recording, cRNA was prepared for *Torpedo* ClC-0 and human ClC-1 using constructs in the pTLN vector, kindly donated by T. Jentsch (Institut für Molekulare Neuropathobiologie, Hamburg, Germany), and for rabbit ClC-2, using a construct in pSportI [15], donated by H.C. Hartzell (Emory University, Atlanta, GA). For two-electrode voltage clamp, oocytes were injected with 2.5 to 25 ng of either ClC-0, ClC-1, or ClC-2 cRNA, and data were collected 2–5 days after injection. For macropatch recordings 100 ng ClC-2 cRNA were injected per oocyte. All recordings were performed at room temperature.

Preparation of Venom, Chlorotoxin, DPC, and NPPB

Dried whole venom (Latoxan, France; or Sigma Chemical Co., Chicago, IL) was prepared at a stock concentration of 2 mg/mL in the

appropriate bath solution (*see* below). The mucous portion of the venom was removed by processing the venom with four strokes in a Potter-Elvehjem tissue grinder, followed by centrifugation at 6000 $\times g$ for 30 minutes to pellet the mucous component. The supernatant was passed through a 10 kDa molecular weight cut-off filter (Millipore Corp; Bedford, MA) by centrifugation at 2000 $\times g$ for 40 minutes. The resulting partially fractionated venom "pf-venom" was then distributed into 1 mL aliquots and stored at −80°C. Native chlorotoxin (Latoxan, isolated from Lqh venom), was prepared in the appropriate bath solution at a concentration of 1.2 μM, and used immediately.

Trypsinization was performed by incubating pf-venom solution with 0.0028% trypsin (Sigma, from porcine pancreas) overnight at room temperature. The solution was then boiled for 1 hour to deactivate the trypsin, and stored at −80°C. For control experiments, undiluted pf-venom was boiled for 1 hour and allowed to cool before use. Boiled venom was then diluted to 0.1 mg/mL in recording solution.

DPC (Sigma) was dissolved directly in bath solution at a concentration of 1 mM, and stored at 4°C. NPPB (Sigma) was dissolved in DMSO to a stock concentration of 100 mM and stored at 4°C. Stock NPPB was diluted to 100 μM in bath solution for experiments and used immediately. The final concentration of DMSO in experimental solutions was no greater than 0.1%, which had no apparent effect on ClC-2 currents.

Two-Electrode Voltage Clamp Recordings

Standard two-electrode voltage clamp (TEVC) techniques were used to study whole-cell currents. Currents were acquired using a GeneClamp 500B amplifier and pClamp software (Version 8.4; Axon Instruments, Union City, CA) at a corner frequency of 500 Hz. Electrodes were pulled from borosilicate glass (Sutter Instruments; Novato, CA) for a resistance that ranged from 0.3–1.3 MΩ when filled with 3 M KCl. Bath solution for whole-cell experiments (ND96) was nominally Ca^{2+}-free and contained (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 1 $BaCl_2$ and 5 HEPES. For experiments with ClC-2, solutions also contained 20 mM mannitol to limit swelling-activated currents. For selectivity experiments, NaCl was replaced with the Na^+ salt of each of the anions studied, with 6 mM Cl^- remaining in the bath solution. Substitutions were always made in the same order (Br^-, I^-, aspartate) and bracketed with data for Cl^- (ND96 solution) before and after the substitute ion. Relative permeability (P_X/P_{Cl}) and relative conductance (G_X/G_{Cl}) for each substitute anion were calculated as previously shown, using the average data for the bracketed exposure to Cl^- [30]. Relative conductance was calculated as $I_{Erev\ +\ 25\ mV\ (X^-)}/\ I_{Erev\ +\ 25\ mv\ (Cl^-)}$, where $I_{Erev\ +\ 25\ mV}$ denotes the current at 25 mV more depolarizing than the reversal potential. Slope conductance for Cl^- used was the average of the Cl^- recordings bracketing the recordings with the substitute anion. This procedure allowed the comparison of several ions in each experiment while controlling for changes in cell conditions. All solutions were adjusted with NaOH to pH 7.5. All experiments were performed using an agar bridge, and corrected for changes in junction potentials measured using a flowing KCl electrode [30].

Macropatch Recordings

Outside-out macropatches were used to assess the activity of trypsinized venom on ClC-2. The oocyte was placed in hypertonic stripping solution to allow the cell to shrink, and then the vitelline membrane was manually removed. Macropatch pipettes with resistances of 1–5 MΩ were pulled from borosilicate glass, fire-polished, and filled with solution containing (in mM): 150 NMDG-Cl, 5 $MgCl_2$, 10 TES and 2 Tris-EGTA (pH 7.4). Extracellular bath solution contained (in mM): 150 NMDG-Cl, 5 $MgCl_2$ and 10 TES (pH 7.4). Experiments were performed on patches with seal resistance > 100 GΩ.

Inside-out macropatches were used to test the effect of boiled venom on ClC-2. Pipettes were backfilled with 0.1 mg/mL boiled venom in extracellular solution; the tip of the pipette was filled with extracellular solution lacking venom. Recordings were made every five minutes while venom diffused to the tip until current reached steady-state. Data were acquired using an Axopatch 200B amplifier (Axon) operated by pClamp software. Data were filtered at 500 Hz, and acquired at 1 kHz.

Voltage Protocols

To determine the specificity of the venom for the different plasma membrane ClC channels, we individually tested ClC-0, ClC-1 and ClC-2 using step protocols with 13 test pulses for ClC-0, and 12 test pulses for ClC-1 and -2 (*see* Fig. 1 *top*). For ClC-0, membrane potential was held at −140 mV for 5 seconds to activate the slow gate, and stepped to + 60 mV for 150 ms to activate the fast gate. Voltage was then stepped to potentials ranging from −160 to + 80 mV in 20 mV increments with a pulse duration of 300 ms, followed by a tail pulse to −100 mV for 100 ms. For ClC-1, V_M was held at −30 mV, and stepped to potentials ranging from −160 to + 60 mV in 20 mV steps of 160 ms duration, with a tail pulse at −120 mV for 40 ms. The ClC-2 12-pulse voltage protocol used for initial experiments held V_M at −30 mV, and then stepped to potentials ranging from −160 to + 60 mV in 20 mV steps, with a pulse duration of 3 s, and a tail pulse at + 40 mV for 100 or 500 ms. The interpulse duration was 30 s for ClC-0, 15 s for ClC-1, and 45 s for ClC-2.

Voltage- and concentration-dependence of inhibition of ClC-2 were examined using a 5-pulse protocol stepping from −160 mV to + 60 mV in + 55 mV increments. The pf-venom was applied at concentrations ranging from 0.01 mg/mL to 0.3 mg/mL for 5 minutes prior to testing (concentrations of pf-venom hereafter are expressed as equivalent to the stated concentration of whole, unprocessed venom). Concentration dependence was assessed by comparing maximal inhibition of quasi-steady-state currents at −160 mV or peak tail currents to pre-venom records for each concentration. Voltage-dependence was determined by comparing the *I–V* relationship for quasi-steady-state currents or peak tail currents to pre-venom records. For selectivity experiments cells were voltage-clamped at −30 mV, stepped to −160 mV for 6 s (to fully activate the channels) and rapidly ramped from −160 to + 80 mV over a 50 ms period.

We compared the macroscopic kinetics between control cells and venom-treated cells using a single voltage-pulse protocol of extended duration. V_M was held at −160 mV for 6 seconds, then stepped to + 40 mV for 1.2 seconds. Time constants (τ) were calculated by fitting the quasi-steady-state activation currents and the tail currents to the sum of two exponential functions, using pClamp software.

Voltage protocols for ClC-2 in outside-out macropatches were similar to those for TEVC, with a holding membrane potential of −30 mV. One pulse of 1.5 s duration at V_M = −160 mV with a tail pulse + 40 mV for 250 ms was used.

Data Analysis

Data were analyzed using pClamp and SigmaPlot 7.0 (Jandel Scientific; San Raphael, CA). The percent inhibition was determined from the quasi-steady-state activation currents at the end of the pulse to V_M = −160 mV (after subtracting instantaneous currents) and the peak tail currents as:

$$\%\text{Inhibition} = 100\left[\frac{I_c - I_v}{I_c}\right]$$

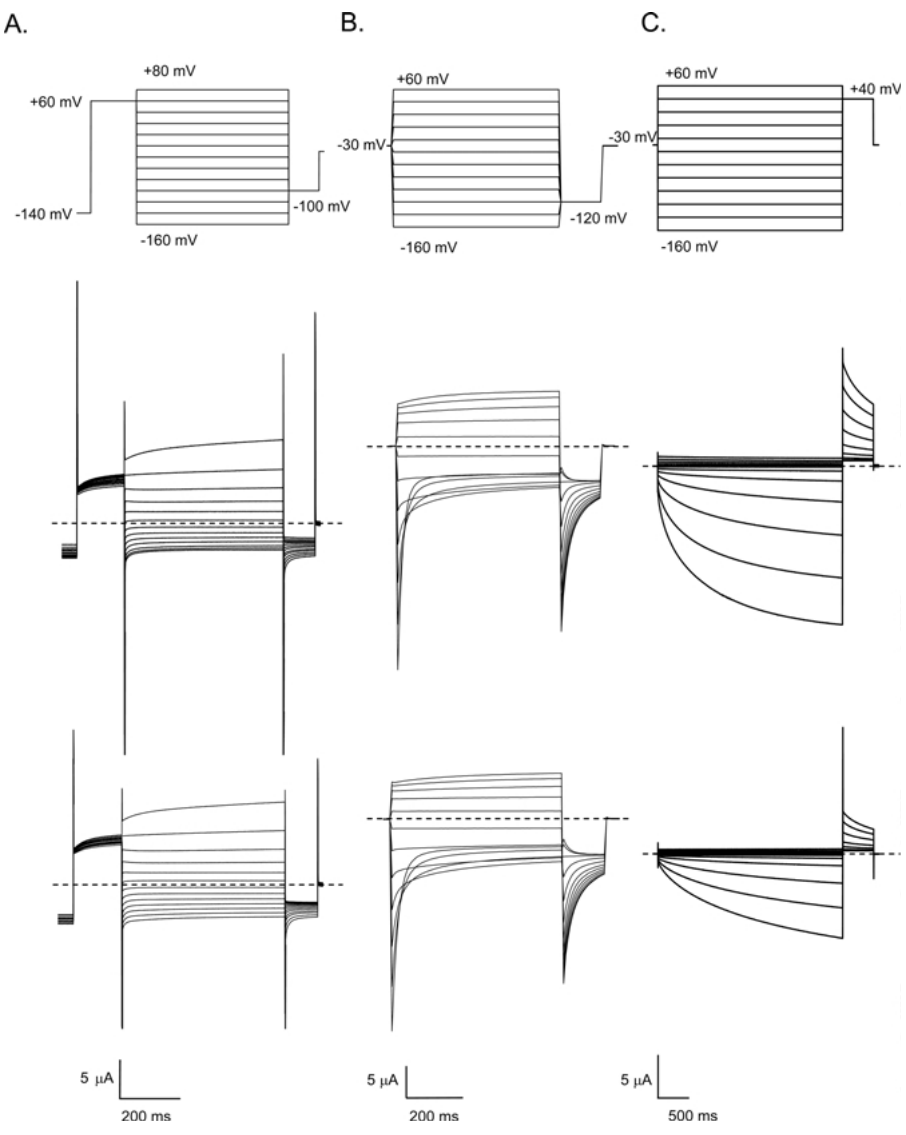

Fig. 1. Effects of scorpion venom on macroscopic ClC currents. *Upper panels* are the TEVC voltage protocols, *center panels* are representative currents in the absence of venom and the *lower panels* show representative currents from the same oocytes in the presence of venom for (*A*) ClC-0 (0.2 mg/mL pf-venom), (*B*) ClC-1 (0.2 mg/mL pf-venom), and (*C*) ClC-2 (0.1 mg/mL pf-venom). Note that inhibition occurred only for ClC-2 with maximum inhibition ~50% in this example. The dashed line indicates the zero-current level for all traces.

where I_c and I_v are the control currents and currents in the presence of venom, respectively. Values for percent inhibition given in the text, therefore, specifically reflect inhibition of quasi-steady-state currents at −160 mV, or tail currents at +40 mV.

Statistics

Results are expressed as mean ± SEM for n observations. Comparisons are by Student's t-test, with differences considered significant when $P \leq 0.05$. All statistical analysis was performed using SigmaStat 2.03 (Jandel Scientific; San Rafael, CA).

Results

Venom Selectively Inhibits ClC-2

The effect of partially fractionated scorpion venom (pf-venom) on ClC-0, human ClC-1, and rabbit ClC-2 expressed in *Xenopus* oocytes was examined using standard TEVC techniques (Fig. 1). While ClC-2 showed considerable inhibition both of the quasi-steady-state activation currents at hyperpolarizing potentials (58.0 ± 5.9% inhibition at 0.1 mg/mL and V_M = −160 mV, n = 16) and of the instantaneous tail currents (62.0 ± 6.7% inhibition), neither ClC-0 nor ClC-1 showed significant reduction in currents despite using up to two-fold higher concentrations than used for ClC-2 (20-fold higher than the apparent IC_{50} for ClC-2). Native, purified chlorotoxin showed no inhibition of ClC-2 Cl^- currents (6.3 ± 7.6% increase at 1.2 μM, n = 2). These data indicate that a component (or components) of scorpion venom other than chlorotoxin selectively inhibited ClC-2, suggesting that the active toxin, once isolated, would poten-

Table 1. Anion selectivity of ClC-2 currents in oocytes

	E_{rev} (mV)	P_X/P_{Cl}	G_X/G_{Cl}
Cl^-	−36.8 ± 2.52	1.0	1.0
Br^-	−30.0 ± 2.21*	0.76 ± 0.004*	0.85 ± 0.02*
I^-	−12.9 ± 2.78*	0.37 ± 0.02*	0.27 ± 0.03*
Aspartate	9.14 ± 3.62*	0.15 ± 0.01*	0.35 ± 0.02*

* indicates $P < 0.05$ compared to Cl^-.

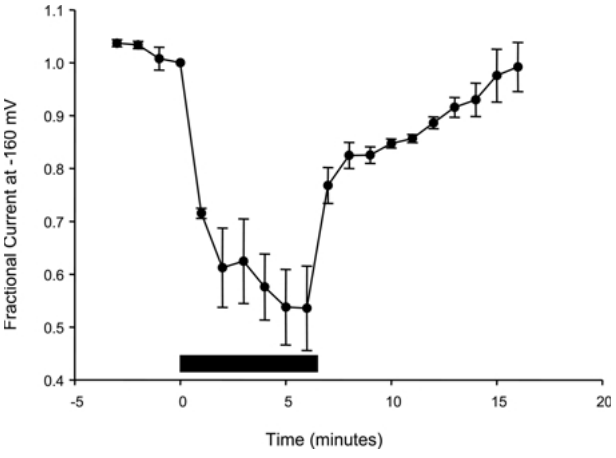

Fig. 2. Time series for the inhibitory effect of venom (0.1 mg/mL, $n = 3$) on ClC-2 currents using a single pulse voltage protocol (fractional activation currents at $V_M = -160$ mV) taken at one-minute intervals. Venom flow was initiated at $t = 0$ and continued for 6 minutes. Maximum inhibition occurred at ~5 minutes of exposure. Inhibition was reversible within ~10 minutes.

tially be a powerful tool for exploring structural aspects of this particular channel.

One potential problem with studying ClC-2 channels expressed in *Xenopus* oocytes arises from the fact that currents from endogenous oocyte channels may resemble currents from ClC-2 [27, 41], as endogenous channels are also activated by prolonged pulses to hyperpolarizing potentials. Because activity in these endogenous channels may be heightened when the cells are injected with large volumes of cRNA, it was important to determine whether the currents we identified as ClC-2 actually arose from ClC-2 channels. To address this problem, we periodically tested batches of ClC-2 cRNA-injected oocytes for permeability and conductance sequences by replacing Cl^- with Br^-, I^-, or aspartate. One distinguishing feature of these two channel types is that the endogenous channels show a selectivity sequence of $I^- > Cl^-$, while all ClCs have the selectivity sequence of $Cl^- > Br^- > I^-$ for both permeability and conductance [20]. The results of selectivity experiments (Table 1) showed that the currents elicited in ClC-2-injected oocytes had a permeability sequence of $Cl^- > Br^- > I^-$ > aspartate, and a conductivity sequence of $Cl^- > Br^-$ > aspartate > I^-, which is consistent with C1C channel currents [20].

Inhibition by Venom Is Reversible

To determine if inhibition of ClC-2 by venom is reversible, we measured the amount of inhibition over the duration of an experiment, including exposure to venom and subsequent washout. Time series of currents at $V_M = -160$ mV in one-minute intervals during exposure to 0.1 mg/mL pf-venom ($n = 3$) show the progression of inhibition to be relatively slow and completely reversible (Fig. 2). Maximum inhibition occurred after ~5 minutes of exposure and activity returned to pre-venom values after ~10 min of washout. However, in some cases washout took considerably longer than 10 minutes.

The Inhibitory Component (or Components) Is a Peptide

Many of the most potent selective inhibitors of voltage-gated channels are peptide toxins isolated from animal venom [31]. Peptide components of a wide variety of venoms have provided useful tools in the study of cation channels to determine aspects of both pore structure and gating processes [16, 48, 49]. We tested the hypothesis that the active component(s) of scorpion venom is a peptide by incubating pf-venom with the protease trypsin to digest any proteins within the venom, and then boiling the mixture to inactivate the enzyme. However, when applied to whole oocytes under TEVC, the trypsinized venom produced significant leak current. It is possible that the trypsinization process released bound calcium ions, which in turn activated calcium-activated chloride channels, although the addition of chelating agents such as EGTA into our solutions did not resolve this problem. In contrast, outside-out macropatches from oocytes expressing ClC-2 remained stable during exposure both to pf-venom and trypsinized pf-venom. Macropatch currents showed no significant inhibition by 0.1 mg/mL trypsinized pf-venom (Fig. 3*B, E*), while strong inhibition was observed in response to untreated pf-venom (Fig. 3*B, C*). As a control we asked whether boiled untrypsinized pf-venom inhibited ClC-2 currents using TEVC. Boiled pf-venom at 0.1 mg/mL inhibited ClC-2 quasi-steady-state currents in TEVC experiments to the same degree as unboiled pf-venom at the same concentration (62.0 ± 4.38% inhibition, $n = 3$;

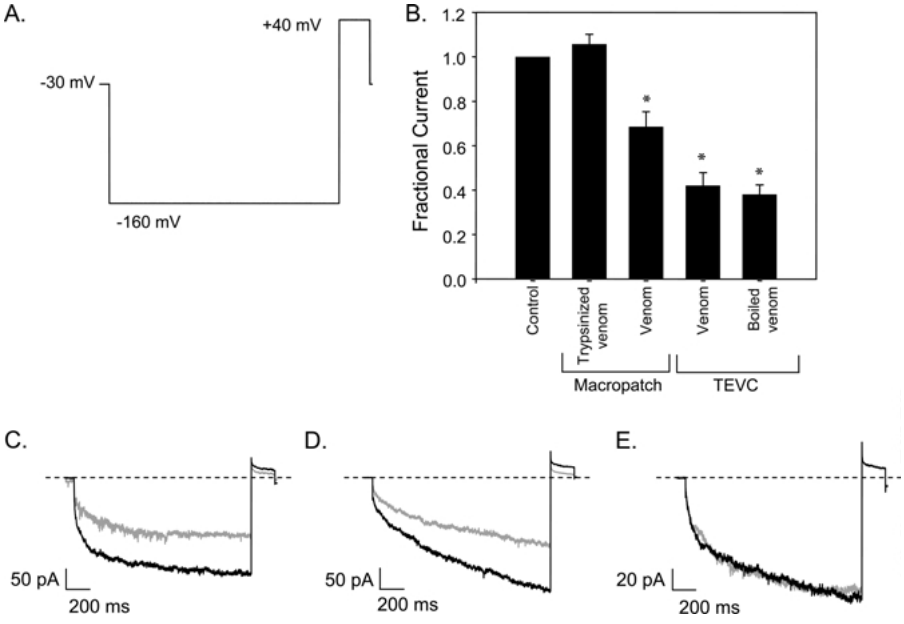

Fig. 3. Trypsinization destroys activity of pf-venom. (*A*) Voltage protocol for excised, outside-out macropatch recordings. (*B*) Normalized current remaining for currents at $V_M = -160$ mV for control, trypsinized venom (105.7 ± 10.0%, $P = 0.31$, $n = 5$), and pf-venom (68.5 ± 6.2%, $P < 0.015$, $n = 3$) in outside-out macropatches, as well as pf-venom (42.0 ± 4.9%, $P < 0.001$, $n = 16$) and boiled pf-venom (38.0 ± 4.38%, $P = 0.006$, $n = 3$) for TEVC recordings. Venom concentration was 0.1 mg/mL for all of these experiments. * indicates $P < 0.05$. (*C*) Macropatch currents at $V_M = -160$ mV in the absence (*black trace*) and presence (*gray trace*) of untreated pf-venom. (*D*) Inside-out macropatch current at $V_M = -160$ mV in the absence (*black trace*) and presence (*gray trace*) of boiled pf-venom that was backfilled in the patch pipette. (*E*) Macropatch current at $V_M = -160$ mV in the absence (*black trace*) and presence (*gray trace*) of trypsinized pf-venom. The dashed line indicates the zero-current level for all traces.

Fig. 3*B*). Boiled venom also inhibited ClC-2 in inside-out macropatches in experiments where the pipette solution contained 0.1 mg/mL boiled venom (36.9% inhibition; *see* Methods), as shown in Fig. 3*D*. Macropatch currents elicited by a pulse to $V_M = -160$ mV were allowed to reach steady-state in the presence of boiled venom, and currents showed no change over 20 minutes after maximal inhibition by boiled venom was reached. The difference in degree of inhibition between TEVC and macropatch recordings could be attributed to the differing cytoplasmic chloride concentrations. These data suggest that the lack of inhibition with trypsinized venom is due to the peptide being degraded by trypsin, and not denaturation by boiling. Since heat stability is a common feature of peptide toxins, which usually have multiple intrapeptide disulfide bridges [37], this supports the hypothesis that the active component(s) is a heat-stable peptide.

Venom Shifts Apparent Voltage Dependence of Activation

ClC-2 currents display a high degree of inward rectification due to poor activation at potentials more depolarizing than −30 mV. This form of current-voltage relationship is seen for both quasi-steady-state currents and tail currents at +40 mV. In the presence of venom the apparent voltage-dependence of activation is shifted to more hyperpolarizing potentials (Fig. 4*A*). This relationship was also seen for tail currents elicited when the voltage was stepped to +40 mV (Fig. 4*B*). This effect on voltage-dependence could either be due to a change in the voltage-dependence of gating, or due to a voltage-dependent block of the pore.

Inhibition Is Concentration-dependent

Peptide toxins typically inhibit their targets with very high affinity, usually in the nanomolar range [31–33], which makes them valuable tools for in-depth studies of channel structure and gating processes [16, 48, 49]. Inhibition of C1C-2 currents by scorpion venom was clearly concentration-dependent, for both the quasi-steady-state current (Fig. 5*A*), and the tail current (Fig. 5*B*). The dose-response curve for inhibition of quasi-steady-state current at $V_M = -160$ mV for each pf-venom concentration (Fig. 5*C*) was fit to a three-parameter Hill equation ($r^2 = 0.91$; $P = 0.08$). The data show clear concentration-dependent inhibition up to 0.1 mg/mL with saturation occurring at concentrations above 0.1 mg/mL. The apparent IC_{50} was 0.01 mg/mL, and the Hill coefficient was 0.97.

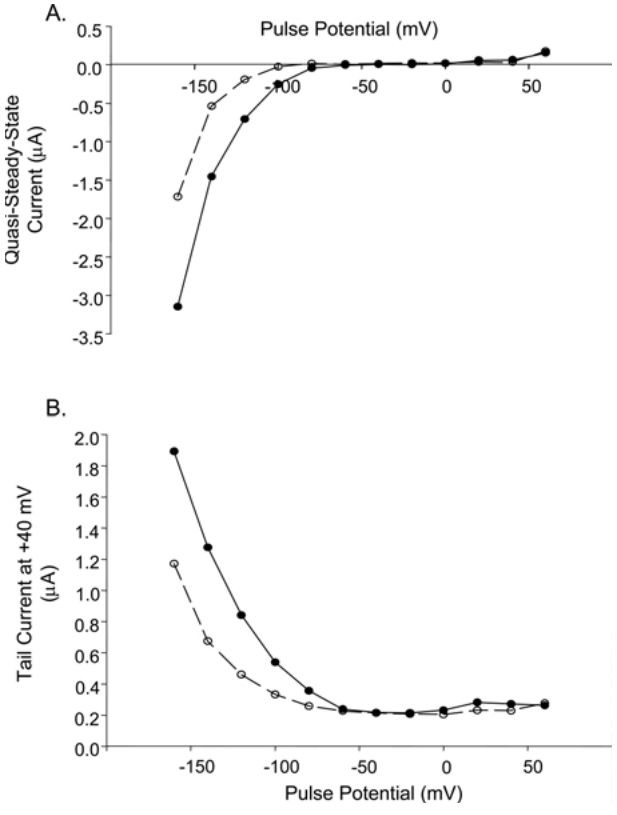

Fig. 4. Voltage dependence of ClC-2 whole-cell currents in the presence and absence of venom. Voltage-dependent inhibition by pf-venom (0.1 mg/mL) of the (*A*) quasi-steady-state and (*B*) tail currents of ClC-2 in a representative experiment using the 12-pulse protocol described in Fig. 1.

Voltage Dependence of Inhibition

We examined the voltage dependence of inhibition of quasi-steady-state currents with voltage-step protocols, as well as of tail currents at +40 mV (Fig. 6). Inhibition of quasi-steady-state current showed significant voltage dependence. There was increasing inhibition of quasi-steady-state currents as the membrane potential became more depolarizing (Fig. 6*A left*). Comparison of current remaining at −160 mV to that at −60 mV showed a significant difference ($P = 0.03$, $n = 8$). Plotting I/I_0 for tail currents, however, showed no voltage dependence when comparing the tail current remaining after steps to −160 mV versus that after steps to −60 mV ($P = 0.34$). This is likely due to small contaminating currents at positive membrane potentials that are due to activation of endogenous channels in the oocyte.

To further clarify the mechanism of inhibition, we compared inhibition of ClC-2 by pf-venom with inhibition by small organic molecules known to be pore blockers of a variety of Cl^- channels. Inhibition by DPC was voltage-dependent (Fig. 6*B*); however, as the I/I_0 plot indicates, inhibition decreased with more depolarizing potentials throughout the voltage protocol. Inhibition by NPPB followed the same trend as inhibition by DPC for both activation and tail currents (Fig. 6*C*). Indeed, both DPC and NPPB led to an increase in current at weakly hyperpolarizing potentials. These results were unlike the results for pf-venom, suggesting that venom inhibits ClC-2 channels via a mechanism different from that underlying inhibition by DPC or NPPB.

Kinetics of ClC-2 Gating

Gating modifiers generally influence the kinetics of channel opening or closing, while pore-blocking molecules typically do not. Channel kinetics were examined by using a prolonged protocol to obtain values for the time constant of activation ($\tau_{activation}$) at $V_M = -160$ mV, as well as the time constant for deactivation ($\tau_{deactivation}$) from the tail pulse at $V_M = +40$ mV (Fig. 7). Both of these relaxations were fit best by the sum of two exponential functions. In the presence of venom, inhibition of ClC-2 was exhibited as an increase (2.50 ± 0.35-fold, $n = 7$, $P = 0.008$, at 0.03 mg/mL venom) in the time constant for the fast component of $\tau_{activation}$ (Fig. 7*C*). At a higher concentration, both time constants of activation were significantly affected. The slow component showed a 1.92 ± 0.18-fold increase ($n = 11$, $P < 0.001$), while the fast component showed a 2.89 ± 0.49-fold increase ($n = 11$, $P < 0.001$) (Fig. 7*C*). There was no change in the time constants of deactivation for ClC-2 when exposed to venom at a concentration of 0.1 mg/mL (*data not shown*). This indicates that the activation of the channel is significantly slowed in the presence of venom, which is consistent with the action of a gating modifier. There were no significant changes in fractional area contributed by each time constant for activation (Fig. 7*D*) or deactivation (*data not shown*) in the presence of 0.03 or 0.1 mg/mL venom. This suggests that fast and slow gating may be highly coupled in ClC-2, as one would expect that the contribution of one time constant would change if it were affected more than the other. This coupling of fast and slow gating in ClC-2 has been described previously by Zuniga [51], and more recently by de Santiago et al. [8].

Discussion

Peptide toxins have proven to be indispensable tools in studying pore structures and gating mechanisms in a variety of voltage-gated channels [16, 31, 32, 44]. In this study, we investigated the inhibitory effects of venom isolated from the scorpion *L. quinquestriatus hebraeus* on three plasma membrane members of the ClC family. Of the three channels studied, the venom

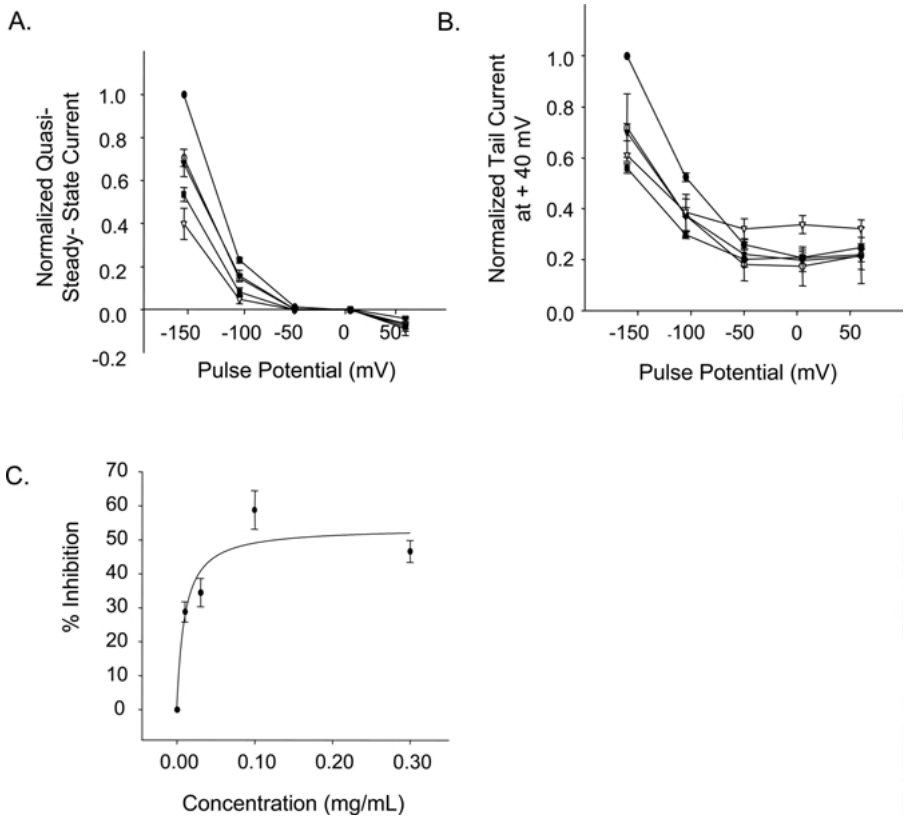

Fig. 5. Voltage- and dose-dependence of inhibition of ClC-2 by venom. Voltage dependence of the (*A*) quasi-steady-state activation currents and (*B*) tail currents of ClC-2 at zero (●), 0.01 (O), 0.03 (▼), 0.1 (∇) and 0.3 (■) mg/mL pf-venom using a 5-pulse voltage protocol. (*C*) Concentration dependence of inhibition at $V_M = -160$ mV. All concentrations include 3–16 replicates. The line was fit to the mean at each concentration using a three-parameter Hill equation ($r^2 = 0.91$; $P = 0.08$).

showed a strong inhibitory effect only on ClC-2. This suggests that the inhibition is not due to the action of heavy metal ions such as Zn^{2+} or Cd^{2+}, which could be contaminants carried in venom, that are known to inhibit ClC-0 and ClC-1, as well as ClC-2 [4]. Thus, unlike the inhibitory effects of other organic molecules such as DPC, 9-AC, and members of the clofibric acid family [15, 39], and despite the relatively high homology in the primary sequence of the three channels [13], the selective nature of the venom suggests that it could not only be a powerful tool for understanding the structure of ClC-2 but may also offer insight into the functional relevance of the structural differences between various ClC members.

Our results have shown that a peptide component (or components) of scorpion venom inhibits ClC-2. The toxin is slow to bind to the channel, taking approximately five minutes to show maximum inhibition, at 0.1 mg/mL, and is relatively tenacious once bound, taking approximately ten minutes to fully restore pre-venom current levels during washout. In addition, macropatch data have shown that trypsinization of the pf-venom removes the inhibitory effect, strongly suggesting that the active component(s) is a peptide. The possibility remains that multiple components of the venom are acting in concert, and that trypsinization of the partially fractionated venom disrupts the interaction of the components responsible for inhibition. Experiments have shown that native ClTx (Latoxan) does not inhibit ClC-2. Lqh venom contains several components that are similar to components of *L. quinquestriatus quinquestriatus* venom, the source of ClTx [1], and given the large number of peptide toxins that have been identified, it is possible that the active component(s) of this venom is a peptide that has already been characterized. However, it is also possible that the active component is a novel peptide. Once the toxin is isolated, sequence analysis will allow for an evaluation of evolutionary relationships to other toxins.

Determining the underlying mechanism of inhibition is complicated, since permeation and gating are tightly coupled in ClC family channels [20, 28]. However, two lines of evidence suggest that the venom acts as a gating modifier. First, ClC-2 channel activity is clearly voltage-dependent and venom shifts the voltage-dependence of activation to more hyperpolarizing potentials. However, at the test potentials used in this study, ClC-2 currents never apparently

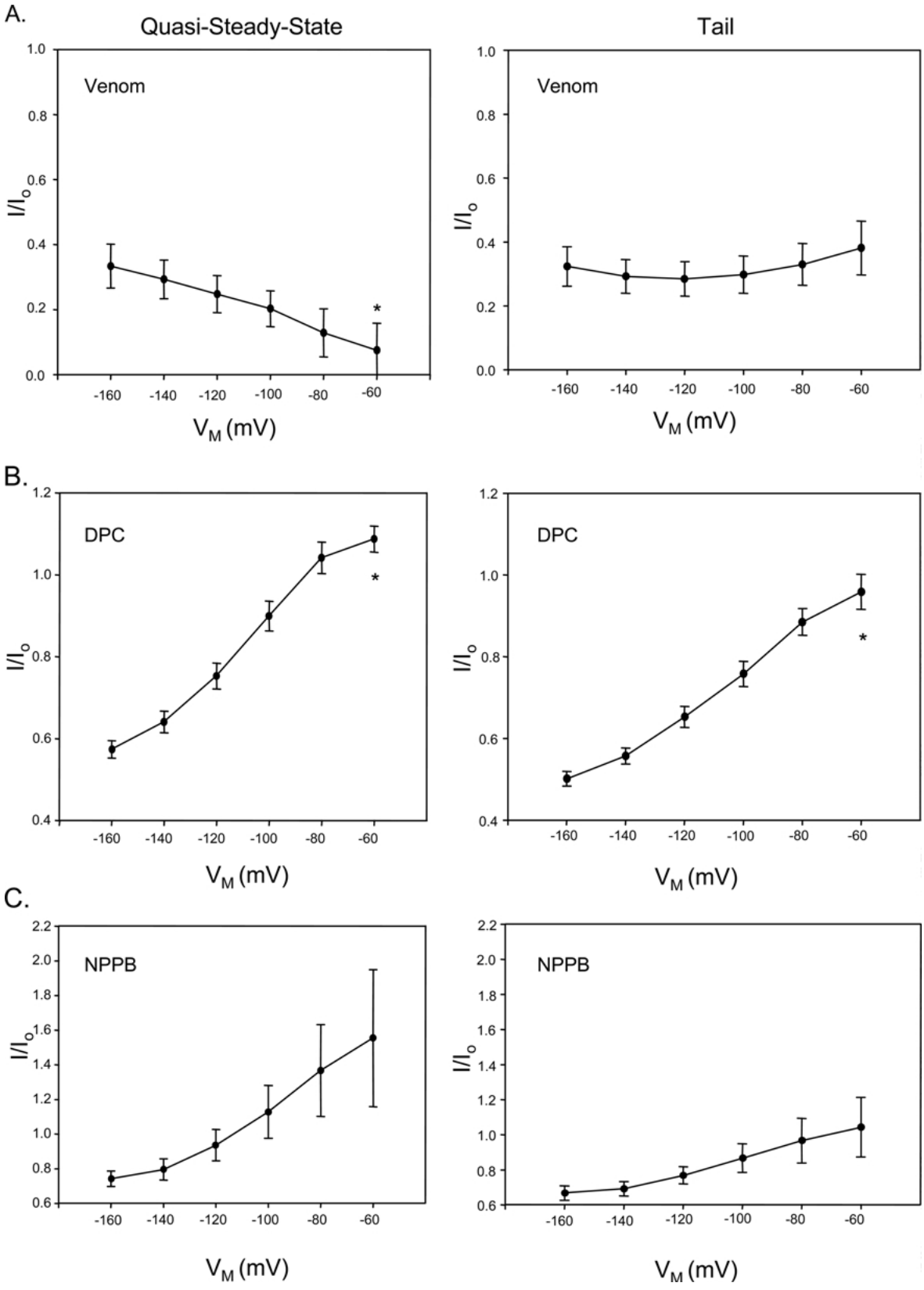

Fig. 6. Inhibition of ClC-2 by pf-venom and known pore blockers. Effect of (*A*) pf-venom (0.1 mg/mL, $n = 8$), (*B*) DPC (1.0 mM, $n = 6$), and (*C*) NPPB (100 μM, $n = 4$) on normalized ClC-2 activation currents (*left panels*) and tail currents (*right panels*). * indicates statistical difference between current remaining at $V_M = -60$ mV compared to current remaining at $V_M = -160$ mV.

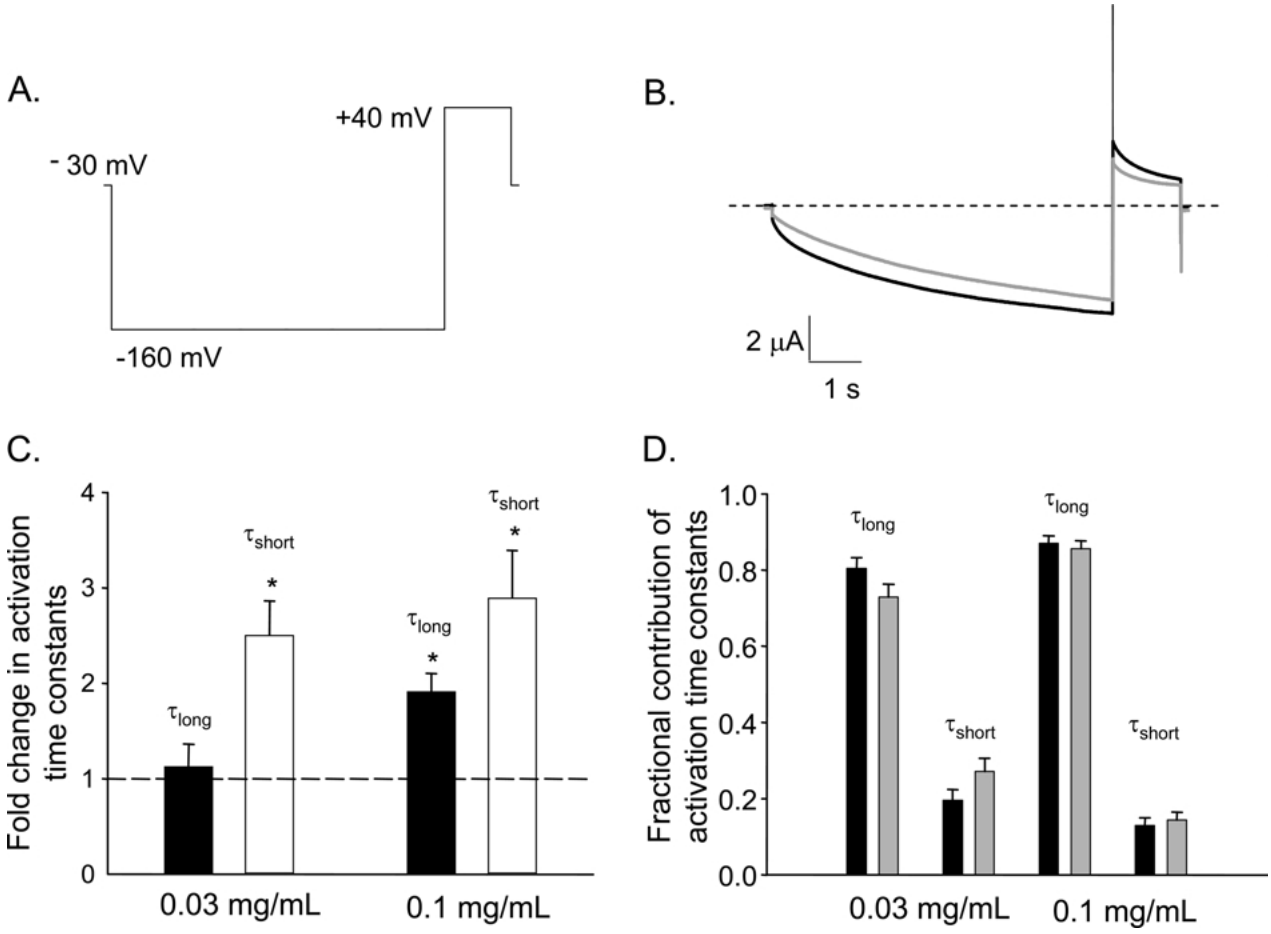

Fig. 7. Effect of pf-venom (0.03 mg/mL and 0.1 mg/mL) on the activation kinetics of ClC-2. (*A*) Extended TEVC voltage protocol. The step potential was held for six seconds; tail potential was held for 1.2 seconds. (*B*) Representative TEVC recording, at $V_M = -160$ mV, before pf-venom (*black trace*) and with 0.03 mg/mL pf-venom (*gray trace*). Data were fit with a second-order exponential and the time constants (τ) were, normalized to values before pf-venom. The dashed line indicates zero-current level. (*C*) Effect of pf-venom on the long (τ_1, *black bars*) and short (τ_2, *white bars*) time constants for activation. Values above the dashed line represent increased time constants while values below represent a decrease. The fractional contributions to the fit from τ_1 and τ_2 for channel activation are shown in panel (*D*). Pre-venom results are represented by the *black bars*, while results with pf-venom are represented by the *gray bars*. Statistical analysis was performed on non-transformed data. * indicates $P < 0.05$.

reach full activation, even at extremely hyperpolarizing potentials (e.g., −160 mV) that are barely tolerated by the cell. Hence, estimation of maximal P_0, and its potential decrement in the presence of venom, is difficult [21]. Secondly, inhibition by venom alters channel activation kinetics; this is observed as a slowing of both time constants of activation for ClC-2 in the presence of venom. This effect also appears to be dose-dependent, with more pronounced effects at higher concentrations of venom. This slowing of activation could represent a stabilization of the closed state, which is not uncommon for gating modifiers. Gating in ClC channels is complicated, in that there are both fast and slow gating processes, and in that gating and permeation are linked, with Cl^- acting as both the gating charge and the permeant ion [20]. It is possible that the active component(s) has some effect on permeation that is not evident in our assays, but these results taken together provide convincing evidence that the main effect of the venom is modification of channel gating. Whether the venom acts on the fast or slow gate presently is unknown. In ClC-2, it is difficult to separate the fast and slow gates at the macroscopic level. It may be that single-channel recordings, in association with the purified toxin, can differentiate between these possibilities, but the low single-channel conductance of these channels [21] will make those experiments difficult to perform.

The finding that the active component(s) of venom behaves as a gating modifier suggests that it must function to prevent some conformational change that is necessary for gating. In voltage-gated K^+ channels the S4 region responsible for the detection of voltage is linked to the movement of S5 and S6, which are the pore lining helices [10]. Binding of voltage-sensing toxin (VSTx) prevents the move-

ment of the S4 region, and thus inhibits gating [42]. Although the domain movements associated with gating of ClC channel proteins are not known, it is proposed that the movement of a conserved glutamate residue that lies within the pore regulates fast gating [8, 11, 36, 51]. The mechanism underlying the slow gate is still unknown, although the slower kinetics of this gate would suggest that large movements of the protein are necessary. Given that the ClC-2 activation rates are slowed by venom, it is possible that this peptide is directly interacting with one of the gates. The dose response indicates a Hill coefficient of 0.97, which would suggest that the toxin is interacting with the channel with a 1:1 stoichiometry.

This would indicate either an interaction with the single slow gate, or an allosteric interaction that may affect all gates. Interaction with the two fast gates would provide two binding sites, and possibly give a Hill coefficient significantly different from one, assuming binding of the toxin to each of the fast gates is an independent event. It has been shown that Cl^- can act as the principal gating charge in ClC channels; ClC-2 relies on intracellular Cl^- to gate the channel [40]. Since the venom works from the extracellular side of the channel, venom-induced disruption of intracellular Cl^- binding sites through a direct interaction is unlikely.

Purification of a single peptide that inhibits ClC-2 would provide a powerful tool in the study of gating for ClC channels. Once the active component or components are isolated, a functional map of the channel can be made, so that the protein movements necessary for gating can be identified. This report provides the basis for the identification of a tool that will lead to a better structural understanding of mammalian ClC channels.

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