G-32-6GU
#2

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"Identification of a Peptide Inhibitor of ClC Channels"
AHA Application ID: 0515272B
Final Report

A. Specific Aims

A.1 Specific Aim 1: To determine if a component of scorpion venom inhibits ClC channels, and determine the mechanism of inhibition.

Aim 1A: Using electrophysiological techniques we will assay for inhibitory activity of *Leiurus quinquestriatus hebraeus* (Lqh) scorpion venom on ClC Cl^- channels. *We hypothesize that venom contains peptide toxins that will inhibit current through ClC channels, and that trypsinization of whole venom will abolish toxin activity, showing that the active component is a peptide.* We will test this by examining whole cell currents in the presence and absence of venom for each channel, and will use trypsin to destroy peptide components of venom.

Aim 1B: We will determine the mechanism of inhibition by venom. *We hypothesize that Lqh venom will inhibit gating of ClC-2 channels, rather than blocking the pore.* To test this we will examine voltage-dependence of inhibition, as well as quantify any changes in the kinetics of activation and deactivation.

A.2 Specific Aim 2: Isolate the active component. *We hypothesize that the venom contains a single peptide that inhibits gating in the ClC-2 Cl^- channel.* A combination of HPLC and mass spectrometry will be used to isolate and characterize the active component of the venom. Using two-electrode voltage clamp (TEVC), we will be able to probe for activity in the fractions obtained from HPLC. With a purified toxin, true dose response curves can be obtained, and thus a true K_D can be measured.

B. Progress towards Aim 1

In the last update we showed that Lqh-venom contained a peptide component that slows ClC-2 channel activation. No further work has been done on Aim 1 as all experimental hypotheses in this aim have been completed. Details of the work performed towards the completion of this aim have been published in the *Journal of Membrane Biology*.

Thompson *et al.*, 2005, *J. Membr. Biol.* 208: 65-76.

C. Progress towards Aim 2

The goal of this aim was to isolate the component of Lqh-venom that inhibits the ClC-2 Cl- channel. In order to isolate the active component of this venom, we used reversed-phase HPLC (RP-HPLC) to separate the components of partially-fractionated venom, which contains only components smaller than 10 kDa. We tested each fraction for inhibition of ClC-2 currents using two-electrode voltage clamp (TEVC). With initial separation performed using a C3 column, we observed that the fraction collected from 0 - 10 minutes, Fraction A, retained activity similar to Lqh pf-venom (Fig. 1A, $45.7 \pm 6\%$ inhibition, fraction concentration 0.1 mg/mL equivalent, $n = 3$, $p = 0.01$). The brief

minute windows in both control and experimental conditions. When no toxin was backfilled into the pipette, we observed no change in average window current over the course of the experiment (Fig. 3A). However, when 2 nM synthetic GaTx2 was backfilled into the pipette average window currents were drastically reduced (Fig. 3A, B; 80.4 $\pm$ 2.0% decrease). We repeated these experiments with varying concentrations of GaTx2 to obtain a dose-response curve, which provided K_D = 11 pM and a Hill coefficient of ~1.

Published work as a result of this fellowship
Articles:

Selected peer-reviewed publications (in chronological order).

Refereed Papers:

1. Thompson, C.H., D.M. Fields, P.R. Olivetti, M.D. Fuller, Z.-R. Zhang, J. Kubanek, and N.A. McCarty (2005). Inhibition of ClC-2 chloride channels by a peptide component or components of scorpion venom, *J. Membr. Biol.* **208**(1): 65 – 76

2. Fuller, M.D., **C.H. Thompson**, Z.-R. Zhang, D. McMaster, R.J. French, J. Pohl, J. Kubanek, and N.A. McCarty. State-dependent inhibition of CFTR chloride channels by a novel peptide toxin. *Submitted* to PNAS.

3. C.H. Thompson, P.R. Olivetti, M.D. Fuller, D. McMaster, R.J. French, J. Pohl, J. Kubanek, and N.A. McCarty. Isolation of a novel peptide inhibitor of ClC-2 chloride channels. (*In preparation*).

Invited Papers, Reviews, Book Chapters (Peer-reviewed)

1. Cui, G., M.D. Fuller, **C.H. Thompson**, Z.-R. Zhang, and N.A. McCarty (2007). Recording currents from channels and transporters in macropatches. In: Patch-Clamp Analysis: Advanced Techniques, 2nd edition, W. Walz, editor, Human Press, Inc., Totowa, NJ.

Abstracts

1. Thompson, C.H., P.R. Olivetti, M.D. Fuller, C. Sullards, J. Kubanek, and N.A. McCarty (2006). Isolation of a peptide toxin active at ClC-2 chloride channels. Biophysical Society Abstracts. *Biophys. J.* **90**:536a.

2. Thompson, C.H., P.R. Olivetti, M.D. Fuller, J. Pohl, J. Kubanek, and N.A. McCarty (2007). GaTx2: A novel peptide inhibitor of ClC-2. Biophysical Society Abstracts. *Biophys. J.* **92**:272a.

3. Fuller, M.D., **C.H. Thompson**, J. Pohl, J. Kubanek, N.A. McCarty (2007). GaTx1: A novel peptide toxin inhibitor of CFTR. Biophysical Society Abstracts. *Biophys. J.* **92**:275a.

4. Thompson, C.H., N.A. McCarty (2007) GaTx2: A high affinity peptide toxin inhibitor of ClC-2 chloride channels. *Pediatric Pulmonology* (*in press*).

retention time of the active component on the C3 column suggests that the toxin is very hydrophilic.

Final isolation and purification of the active toxin was achieved through two successive rounds of RP-HPLC using a C18 column, as summarized in Figure 1B and 1C. The isolated toxin, peak #3, eluted at ~12 minutes and was sufficient to fully recapitulate the activity observed for Lqh pf-venom when diluted to the same equivalent concentration (64.2 $\pm$ 5.3% inhibition at 0.1 mg/mL equivalent, n = 4, p = 0.025). Amino acid analysis performed during protein sequencing (*see* Fig. 3) revealed that this toxin was present at very low abundance, with a concentration equivalent to 0.1 mg/mL containing isolated toxin at a concentration of only 10 nM. A dose-response curve constructed using native purified toxin showed that the K_D at V_M = -160 mV is only 80 pM.

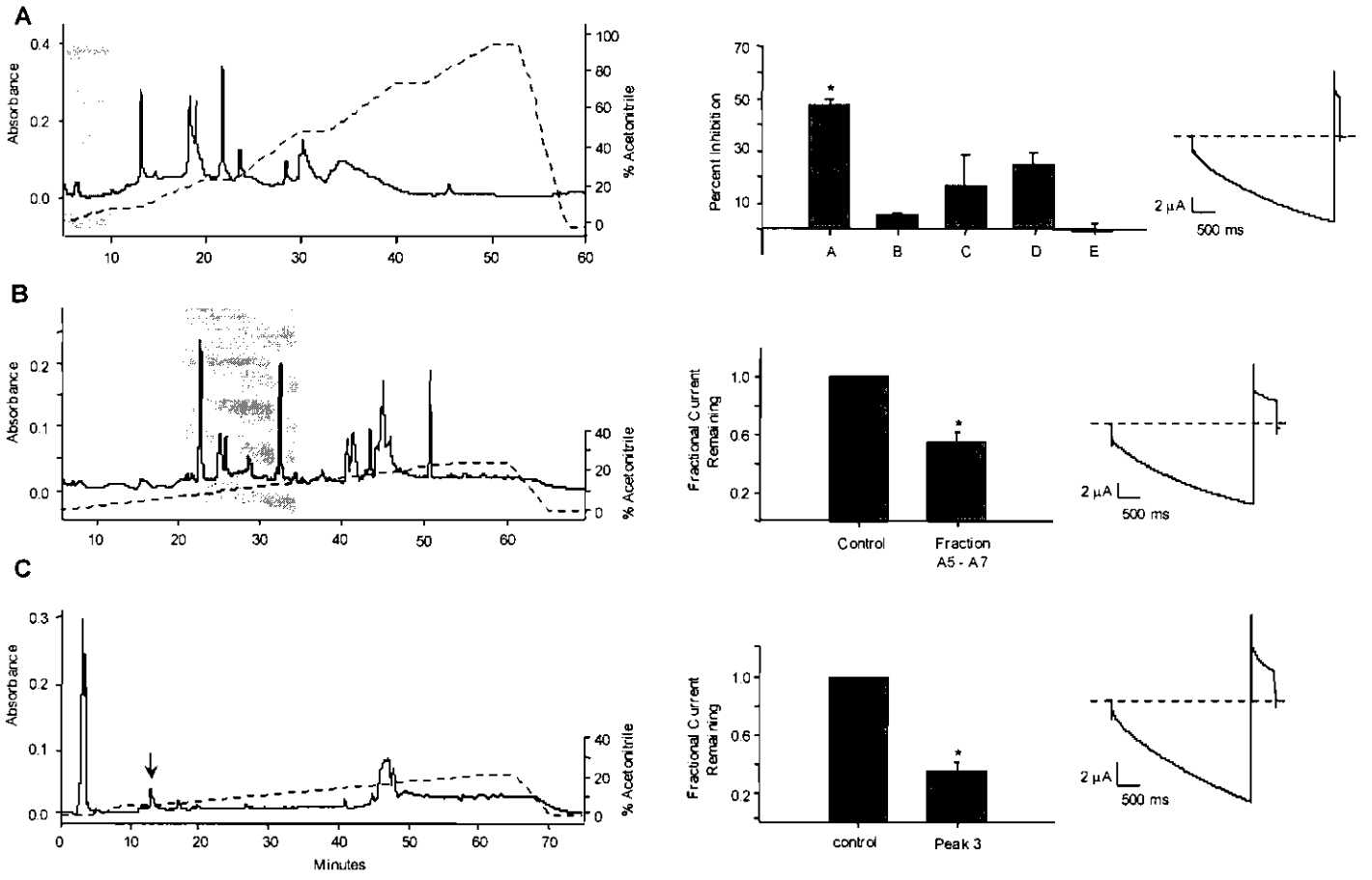
The toxin was then subjected to both MALDI-MS and Edman degradation. MS analysis revealed a single peptide component with a mass of 3191.5 Da; a double charged species was also apparent with m/z = 1.6 kDa (Fig. 2A). Edman degradation then revealed the peptide had a primary amino acid sequence of: **^{1}VSCEDCPDHCSTQKARAKCDNDKCVCEPI^{29}** (Fig. 2B). Comparison of this sequence with the primary sequence of other known toxins reveals that our peptide matches exactly with a previously identified peptide known a Leiuropeptide II (LPII) (Fig. 2B). However, no target has ever been identified for LPII, and it was in fact proposed to be a K^+ channel inhibitor, not a Cl^- channel inhibitor. Thus, to avoid confusion we have renamed this peptide GaTx2. We created a homology model of GaTx2 based on the NMR structure of Neurotoxin P01. GaTx2 maintains a fold very similar to that of other scorpion toxins, as is shown in Figure 2C, which compares the structures of Scyllatoxin, a Ca^{2+}-activated K^+ channel inhibitor, GaTx2, and Chlorotoxin, which is from a related scorpion. The alpha-helix of GaTx2 is connected to 2 beta-strands via 3 disulfide bridges, which are the only post-translational modification of GaTx2. With this data that we have obtained over the last year we have completed the goals set forth in Specific Aim 2.

Status: A manuscript describing this work is currently in preparation, with plans to submit to PNAS.

D. Progress beyond Aim 2

The next step in the characterization of GaTx2 is the production of the toxin in non-native setting in order for the production of large amounts of the toxin. Thus, we have synthesized GaTx2 via solid-phase chemical synthesis for this reason, and to confirm that the sequence associated with the native isolate toxin is indeed the active component from Lqh-venom. In order to test for activity of synthetic GaTx2, we recorded inside-out multi-channel patches, where the pipette was backfilled with varying concentrations of synthetic toxin. The pipette was backfilled in such a way as to allow 10 minutes of control recording (V_M = -100 mV), followed by 10 minutes of recording in the presence of GaTx2. We then calculated the average window current of five separate four-

Figure 1



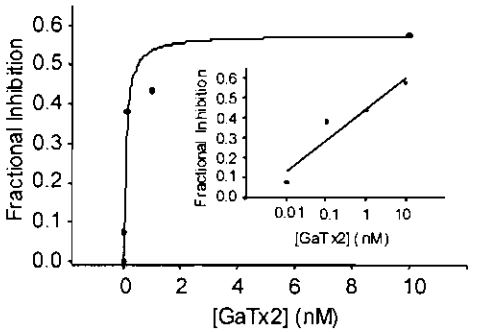


Figure 1: Isolation of the active toxin. (***A - C***). *Left:* Representative RP-HPLC chromatograms of Lqh pf-venom (***A***) or active fractions (***B, C***). The area included in the gray boxes contains the active fraction. The elution gradient is represented by a dashed line. The *middle* panel presents summary data for fraction activity. Bars show mean ± SEM for 3-10 observations. The *right* panel shows representative traces from TEVC experiments in the absence (*black* trace) or presence (*gray* trace) of the active fractions or isolated peak. (***D***). Dose-response curve from a single TEVC experiment for inhibition of CIC-2 at V_M = -160 mV by 0.01, 0.1, 1, or 10 nM native toxin. Inset shows dose-response plotted on a log scale.

Figure 2

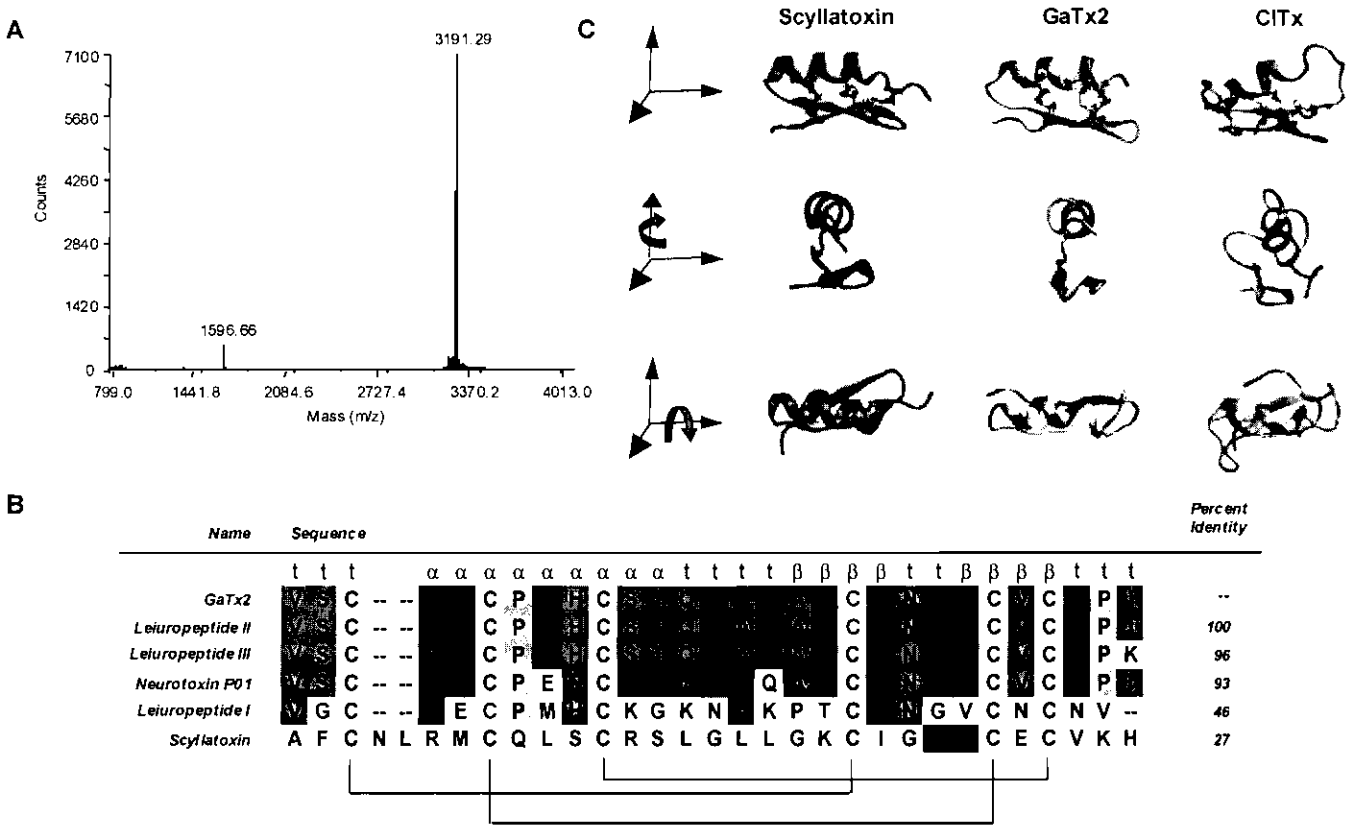


Figure 2: Proteomic characterization of GaTx2. (***A***) MALDI-MS analysis of peak 3. (***B***) Sequence alignment of GaTx2 with other previously identified, highly related toxins. Disulfide bridge connectivity is shown below the sequence alignment, while the predicted secondary structure is shown above the sequence. (**C**) Homology model of GaTx2 (*middle*) shown with the NMR structures of Scyllatoxin (*left*) and Chlorotoxin (*right*), in three orientations. The top panel shows disulfide bridges in bond representation.