State-dependent Inhibition of Cystic Fibrosis Transmembrane Conductance Regulator Chloride Channels by a Novel Peptide Toxin*§

Received for publication, September 27, 2007 Published, JBC Papers in Press, October 19, 2007, DOI 10.1074/jbc.M708079200

Matthew D. Fuller§*1, Christopher H. Thompson§, Zhi-Ren Zhang§2, Cody S. Freeman§, Eszter Schay§, Gergely Szakács§, Eva Bakos§, Balázs Sarkadi§, Denis McMaster**, Robert J. French***, Jan Pohl§§, Julia Kubanek¶¶, and Nael A. McCarty§¶¶

From the *Program in Molecular and Systems Pharmacology, Emory University, Atlanta, Georgia 30322, the §School of Biology, Georgia Institute of Technology, Atlanta, Georgia 30332, the ¶Institute of Enzymology, Hungarian Academy of Sciences, 1113 Budapest, Hungary, the **National Medical Center, Institute of Haematology and Immunology, Hungarian Academy of Sciences, 1113 Budapest, Hungary, the ***Peptide Synthesis Core Facility, University of Calgary, Calgary, Alberta T2N 4N1, Canada, the ¶¶Department of Physiology and Biophysics, University of Calgary, Calgary, Alberta T2N 4N1, Canada, the ¶¶¶Microchemical and Proteomics Facility, Emory University, Atlanta, Georgia 30322, and the ¶¶¶School of Chemistry & Biochemistry, Georgia Institute of Technology, Atlanta, Georgia 30332

Peptide toxins from animal venom have been used for many years for the identification and study of cation-permeable ion channels. However, no peptide toxins have been identified that interact with known anion-selective channels, including cystic fibrosis transmembrane conductance regulator (CFTR), the protein defective in cystic fibrosis and a member of the ABC transporter superfamily. Here, we describe the identification and initial characterization of a novel 3.7-kDa peptide toxin, GaTx1, which is a potent and reversible inhibitor of CFTR, acting from the cytoplasmic side of the membrane. Thus, GaTx1 is the first peptide toxin identified that inhibits a chloride channel of known molecular identity. GaTx1 exhibited high specificity, showing no effect on a panel of nine transport proteins, including Cl− and K+ channels, and ABC transporters. GaTx1-mediated inhibition of CFTR channel activity is strongly state-dependent; both potency and efficacy are reduced under conditions of elevated [ATP], suggesting that GaTx1 may function as a non-competitive inhibitor of ATP-dependent channel gating. This tool will allow the application of new quantitative approaches to study CFTR structure and function, particularly with respect to the conformational changes that underlie transitions between open and closed states.

Chloride is the predominant physiological anion; therefore, Cl− channels play critical roles in cell physiology. Plasma membrane Cl− channels are crucial to the process of secretion in many epithelial tissues such as the kidney, the intestine, and the airway (1). A Cl− channel gene is the locus of the primary defect in several human diseases, including Bartter syndrome, Dent disease, myotonia, and some forms of epilepsy (2, 3). Cl− channel proteins play important roles in a variety of other conditions, including cancer (4). Despite their central roles in many physiological processes, our understanding of the structures and mechanisms of anion-permeable channels has lagged far behind that of their cation-permeable peers. One clear reason for this discrepancy is a paucity of specific probes that may be useful as tools for studying the permeation pathways and/or gating mechanisms of Cl− channels. Indeed, most Cl− channel blockers available at present act with very low affinity and poor specificity.

Venoms from snakes, scorpions, marine snails, and spiders are rich sources of peptide toxins that have proven to be of great value in the functional exploration of cation channels (5–8). Peptide toxins have proven to be among the most potent and selective antagonists available for voltage-gated channels permeable to K+, Na+, and Ca2+, and have been very useful tools for detailed structural analysis of these proteins (9, 10). Pore-blocking toxins provide clues about the arrangement of channel domains, about the interactions between the permeant ions and the pore, and about the proximity and interactions of the gating machinery with the pore (8, 11). Gating modifiers provide tools to dissect the processes underlying the transitions between gating states. Peptide toxins have high potential as lead compounds for the development of therapeutics targeting pain, diabetes, multiple sclerosis, cardiovascular diseases, and cancer (4). Because peptide toxins have well-defined structures, constrained by disulfide bridges, and interact with their targets through multiple contacts, they bind with much higher affinity and specificity than most other blockers available to date and report the structures of their targets at molecular detail.
GaTx1: a State-dependent, Peptide Inhibitor of CFTR

The cystic fibrosis transmembrane conductance regulator (CFTR) protein forms a Cl⁻ channel, which is activated by protein kinase A-mediated phosphorylation plus ATP binding and hydrolysis (12), and is expressed predominantly in epithelial cells. Mutations in CFTR are associated with cystic fibrosis, the most common lethal autosomal recessive disease among Caucasians, affecting >60,000 individuals worldwide (13). Over-activity of CFTR plays a key role in secretory diarrhea, a worldwide health concern causing thousands of deaths per year, in diarrhea-predominant inflammatory bowel syndrome, and in autosomal dominant polycystic kidney disease, the most common inherited nephropathy and fourth leading cause of end-stage renal disease in the United States (1, 14).

The domain architecture of CFTR (Fig. 1A), with two sets of membrane-spanning domains, two nucleotide-binding domains, and a regulatory (R) domain, places CFTR in the superfamily of ABC transporters, although it is the sole member of this ancient family that forms an ion channel (15–17). Structure/function studies have led to improved understanding of which parts of the CFTR protein form the permeation pathway and of the mechanism of binding and hydrolysis of ATP at the nucleotide-binding domains that regulate channel gating (18). However, little is known about how binding and hydrolysis of ATP controls the conformation of the pore to regulate transitions between open and closed states. The availability of a peptide toxin that interacts with CFTR in a state-dependent manner would allow the application of quantitative approaches previously not accessible for answering these questions.

Unfortunately, no peptide inhibitor of CFTR, or of any other identified Cl⁻ channel, has been described, although several potential chloride channel toxins have been identified (10). In 1992, Strichartz and colleagues (19, 20) described the isolation from scorpion venom of chlorotoxin (CTx), a small basic peptide capable of inhibiting low conductance Cl⁻ channels from rat colon or brain reconstituted into lipid bilayers. However, neither recombinant CTx, nor native CTx isolated from venom, has been shown to block CFTR or any other Cl⁻ channel of known molecular identity (21). Native CTx also had no effect on Ca²⁺-activated Cl⁻ channels or volume-regulated Cl⁻ channels, when applied to the bath (22). Sontheimer and colleagues (23–25) have shown that CTx inhibits the migration of glioma cells by inhibition of matrix metalloproteinase-2. A recent study suggests that recombinant CTx inhibits an endogenous Ca²⁺-activated Cl⁻ channel in astrocytes (26), although the molecular identity of this channel is also unknown.

We previously determined that venom from the scorpion L. quinquestriatus hebraeus (Lqh) inhibits CFTR in a voltage-independent manner by binding to the channel in an inter-burst closed state when applied to the channel’s cytoplasmic surface (21, 27). In this study, we used bioassay-guided fractionation of scorpion venom to isolate a single peptide toxin, GaTx1, which selectively and reversibly inhibits CFTR. Initial characterization of the activity of GaTx1 using electrophysiological approaches indicates that it inhibits CFTR with higher affinity and higher specificity than most other known modulators of this channel; only GaTx1 exhibits state-dependence of inhibition at CFTR.

EXPERIMENTAL PROCEDURES

Oocyte Preparation and Electrophysiology—Adult female Xenopus were obtained from Xenopus One (Ann Arbor, MI); methods of animal handling are in accordance with the National Institutes of Health guidelines, and the protocol was approved by the Animal Use and Care Committee of the Georgia Institute of Technology (21, 27). cRNAs were prepared from high expression vectors encoding WT-CFTR (pGEMHE-WT) or FLAG-cut-CFTR (pGEMHE-FLAG3-633 plus pGEMHE-837–1480) (27). Oocytes were injected with 5–100 ng of cRNA.

Venom Preparation and Toxin Purification—Venom from the scorpion L. quinquestriatus hebraeus (Latoxan, France) was resuspended in recording solution, disrupted by a tissue grinder, and centrifuged to separate the mucous component (21, 27). The supernatant was then passed through a 10-kDa molecular mass cut-off spin filter (Biomax-10, Millipore) to remove high molecular weight components, resulting in “Lqh-pf venom.” Venom or fraction concentrations are stated as equivalent to venom dry weight before processing.

Reversed-phase HPLC (RP-HPLC) was used to separate components of Lqh-pf venom based on hydrophobicity, using a Waters 1525 binary HPLC and Waters 2487 dual wavelength absorbance detector, with a Zorbax 300SB-C3 silica column (4.6 × 250 mm, 5 μm, 300-Å pore size), and a gradient of water and acetonitrile. Size-exclusion chromatography used a 1.4-cm × 80-cm column (Bio-Rad) packed with Sephadex G50 Superfine (Sigma); toxins were eluted using a buffer containing 50 mM Tris-HCl and 100 mM KCl (pH 7.5). All fractions collected via RP-HPLC or size-exclusion chromatography were dried under vacuum, then resuspended in the appropriate recording solution, and stored at −80 °C until use. For all chromatograms, the solid line indicates absorbance at 220 nm. The dashed line in RP-HPLC chromatograms indicates the elution gradient.

Electrophysiology—Venom fractions were tested using either excised/inside-out macropatches or single channel patches (21, 27, 28). Oocytes were prepared for study by shrinking in hypertonic solution (in mM: 200 monopotassium aspartate, 20 KCl, 1 MgCl₂, 10 EGTA, and 10 HEPES-KOH, pH 7.2) followed by manual removal of the vitelline membrane. Pipettes were pulled from borosilicate glass (Sutter Instrument Co., Novato, CA) and had resistances averaging ~10 MΩ when filled with pipette solution containing (in mM): 150 N-methyl-d-glucamine-Cl, 5 MgCl₂, 10 TES (pH 7.5). Typical seal resistances were 200 GΩ or greater. Intracellular solution for CFTR single-channel and multi-channel patch experiments contained (in mM): 150 N-methyl-d-glucamine-Cl, 1.1 MgCl₂, 2–10 Tris-EGTA,
10 TES, 0–1 MgATP, and 0–50 units/ml protein kinase A (pH 7.5). CFTR currents were measured with an Axopatch 200B amplifier (Axon Instruments, Union City, CA), and were recorded at 10 kHz to DAT tape. For subsequent analysis, records were played back and filtered with a four-pole Bessel filter (Warner Instruments; Hamden, CT) at a corner frequency of 100 Hz and acquired using a Digidata 1322A interface (Axon) and computer sampling at 500 Hz with pClamp 9 (Axon). For inside-out macropatch recording, electrodes were filled with the same solution as for single-channel recording, with resistances averaging ~1–2 MΩ. Typical seal resistances were 100 GΩ or greater. Macropatch recordings were also performed with an Axopatch 200B amplifier operated with pClamp 9; data were filtered at 100 Hz and acquired at ≥2 kHz. The normal intracellular control solution contained (in mM): 150 NaCl, 1.1 MgCl₂, 2–10 tris-EGTA, 10 TES, and 0–1 MgATP (pH 7.45). For all CFTR patch experiments in Figs. 1 and 3, and supplemental Figs. S1–S3, seal resistances averaging ~3–4 MΩ were reported.

GTPy-cAMP (Bt2cAMP), with subsequent addition of toxin. Current at a voltage protocol was as described in the figure legends. For macropatch experiments, solutions containing venom, fraction, or toxin were applied to the intracellular face of the channel using a fast perfusion system (Warner instruments, model SF-77B) (27, 28). Dose-response curves were constructed using inside-out multichannel patches bearing 5–8 channels. To test for effects of GaTx1 on CFTR when applied extracellularly, CFTR also was studied using TEVC. Current at −60 mV was monitored during exposure to solution containing IBMX and dibutyryl-cAMP (B₄cAMP), with subsequent addition of toxin.

Human CIC-1, rabbit CIC-2, and rat CIC-3 voltage-gated chloride channels were expressed in oocytes and studied using TEVC or inside-out multichannel patches, as described in the legend to Fig. 4. Effects of toxin were determined after 5-min incubation. Ligand-gated chloride channels formed by human GABAₐ receptors (rho subunit) were expressed in oocytes and studied using TEVC. GABA receptor currents were activated by perfusing the cell with 10 μM GABA in the presence of toxin. Oocyte endogenous Ca²⁺-activated chloride channels were studied using inside-out macropatches. Channels were activated by exposure to 10 mM cytosolic Ca²⁺ using the fast perfusion system. For experiments with Shb-IR K⁺ channels, holding potential was −90 mV. Leak subtraction used a P/4 protocol with a conditioning pulse to −120 mV; corrected traces are shown. Voltage was stepped from −100 mV post-train to 0 mV. For all figures including electrophysiological recordings, the thick horizontal dashed line indicates the closed current level.

**ATP-dependent Transporter Activity of MRP Proteins**—The activity of the MRP1, MRP2, and MRP3 proteins was tested in transport assays using vesicles purified from insect SF9 cells expressing high levels of the respective MRP (29). Recombinant baculoviruses containing the cDNAs encoding MRP1–3 were prepared as previously described (29, 30). Virus-infected SF9 cells were harvested, their membranes were isolated and stored, and the membrane protein concentrations were determined. After dissolving and sonicating the isolated membranes in a disaggregation buffer, immunoblotting was performed using monoclonal antibodies developed against MRP1–3. Substrates [³H]leukotiene C₄ (0.05 μM), for MRP1, and [³H]E217βG (5 μM), for MRP2 and MRP3, were used at sub-saturating levels for transport measurements in isolated SF9 cell membrane vesicles, as described previously (31). In brief,inside-out vesicles were incubated in the presence of either 4 mM ATP or 4 mM AMP-PNP in a buffer containing 10 mM MgCl₂, 40 mM MOPS-Tris, and 50 mM KCl (pH 7.0) at 23 °C (for experiments with leukotriene C₄) or at 37 °C (for experiments with E217βG), in the presence or absence of 200 nM synthetic GaTx1. After incubation for 0.5 min for MRP1 or 5 min for MRP2 and MRP3, aliquots of this suspension were added to excess cold transport buffer and then rapidly filtered through nitrocellulose membranes with 0.25-μm pores. The filters were washed extensively, and radioactivity associated with the filters was measured by liquid scintillation counting. ATP-dependent transport leading to intravesicular accumulation of labeled substrate was calculated by subtracting the values obtained in the presence of AMP from those in the presence of ATP. The effect of the GaTx1 toxin was assessed at a single dose (200 nM). The mean results ± S.D. values from quadruplicate measurements are reported in Table 1.

**N-terminal Protein Sequencing**—The toxin isolated from venom, or the reduced/carboxamidomethyl-modified toxin, was purified by microbore RP-HPLC (Applied Biosystems, model 140A/785A system) on a Zorbax SB-C18 silica column (1 × 15 mm, 5 μm, 300-Å pore size) equilibrated in 0.1% aqueous trifluoroacetic acid and eluted at 25 °C using a linear gradient of acetonitrile in 0.08% aqueous trifluoroacetic acid. The toxin was eluted as a dominant peak absorbing at 214 nm. Peptide monoisotopic masses (supplemental Table S1) were obtained by MALDI-TOF/TOF MS analysis using the Applied Biosystems model 4700 Proteomics Analyzer system in the reflector mode.

| Substrate | Relative transport rate (percent control) |
|-----------|------------------------------------------|
| [³H]leukotriene C₄ | 105.91 ± 2.94 |
| [³H]E217βG | 96.78 ± 16.69 |
| [³H]E217βG | 106.06 ± 7.19 |

**Homology Modeling**—A homology model of GaTx1 was created using modeller 8.2, using the NMR structures of chloro-
**GaTx1: a State-dependent, Peptide Inhibitor of CFTR**

toxin and insectotoxin 5A as templates (32, 33). The GaTx1 homology model was then subjected to a 5-ps, 2500-step energy minimization in a solvated environment using NAMDv2 with the charmm22 force field. Disulfide bridges were patched to ensure that disulfide bonds remained intact during energy minimization. All graphics were rendered from VMD 1.8.4 software in combination with POV-Ray 3.6.

**Synthesis of GaTx1**—The general methods for peptide synthesis and purification, and disulfide bond formation, have been described previously (34–36). In brief, linear peptides were produced by solid phase synthesis using Fmoc (N-(9-fluorenylethoxycarbonyl) chemistry. Coupling of Fmoc amino acids was performed using an Applied Biosystems 431A synthesizer. The purified linear peptide was subjected to oxidative cyclization under equilibrating conditions to promote formation of the most stable disulfide bridges. Cyclized peptide was isolated from the acidified reaction mixture by reversed phase extraction, was purified by HPLC, and was characterized by MALDI-MS for molecular weight determination (supplemental Fig. S4) and by analytical HPLC (supplemental Fig. S5).

**Statistics**—Results are reported as mean ± S.E. for n observations, unless otherwise indicated. Statistical significance was assessed using paired and unpaired Student’s t-tests. Differences were considered statistically significant when p < 0.05.

**RESULTS**

**Inhibition of CFTR by Scorpion Venom**—We showed previously that partially fractionated scorpion venom (“Lqh-pf venom,” see “Experimental Procedures”) inhibits wild-type (WT) CFTR channels when applied to the cytoplasmic surface of the channel (21, 27). Lqh-pf venom contains all components <10 kDa in size (21). Rapid application of 0.1 mg/ml Lqh-pf venom to closed WT-CFTR channels in inside-out macropatches resulted in inhibition of macroscopic current by ~25% (supplemental Fig. S1A). Venom was applied to protein kinase A-phosphorylated CFTR channels that had been allowed to close upon removal of cytosolic ATP. When ATP was returned to the bath, the resulting current density was reduced. Similar results were seen when Lqh-pf venom was applied to FLAG-cut-ΔR-CFTR channels (supplemental Fig. S1A, right), which lack the R domain and therefore do not require protein kinase A-mediated phosphorylation (37), suggesting that the decrease in current observed in WT-CFTR is due to venom-induced inhibition rather than channel rundown. Application of 0.1 mg/ml equivalent concentration of Lqh-pf venom led to 21.2 ± 4.1% inhibition of FLAG-cut-ΔR-CFTR (n = 5). When the inhibitory activity was further characterized at the single channel level we found that the active component(s) of venom inhibited channel activity in a complex manner. Application of Lqh-pf venom onto single WT-CFTR channels resulted in a substantial reduction in channel open probability (P_o), as previously described (21). In the presence of 0.1 mg/ml Lqh-pf venom, P_o decreased 32 ± 9%. Application of venom to a patch containing 2–3 channels led to the apparent loss of some channels from the record for tens of seconds to minutes (supplemental Fig. S1B), consistent with the inhibition of channel opening. Our previous experiments indicated that this arose from binding of the active component(s) of venom to the channel during an interburst closed state, resulting in locking those channels closed (27). Application of venom also resulted in the introduction of toxin-induced intraburst blocked states in those channels that were able to open (supplemental Fig. S1C); these events were previously shown to arise from venom binding during very brief intraburst closings, leading to the prolongation of those intraburst closed states (27). These results suggest that Lqh-pf venom may contain two separate toxins that act to inhibit CFTR during different phases of the gating cycle, leading to interburst and intraburst inhibition, respectively, or one toxin that inhibits in two different ways. Our previous studies showed that inhibition of CFTR by Lqh-pf venom is reversible (21, 27).

**Separation of Active Components in Venom**—Previous experiments showing loss of activity upon trypsinization indicated that the active component of venom was a peptide (21). To isolate the active peptide, Lqh-pf venom was fractionated by means of RP-HPLC (Fig. 1B, left). Elution fractions were examined for activity against WT-CFTR in excised inside-out macropatches (Fig. 1C). The fraction collected from 20–30 min (fraction “C”) resulted in 23.7 ± 3.4% (n = 3) inhibition of WT-CFTR macroscopic current at a dilution equivalent to 0.1 mg/ml venom; fractions collected at other times also had some effect (21). To verify that this reduction in current did not reflect dephosphorylation-mediated rundown of WT-CFTR channels, we repeated these experiments using FLAG-cut-ΔR-CFTR. Fraction C resulted in 27.9 ± 7.2% (n = 4) inhibition of FLAG-cut-ΔR-CFTR (Fig. 1B, right).

To determine the mechanism of inhibition induced by venom fraction C, excised, inside-out patch recordings of single WT-CFTR channels were studied in the absence and presence of fraction C. Exposure to fraction C led to a decrease in channel activity due to an apparent prolongation of the interburst closed duration (supplemental Fig. S2A). In addition, application of fraction C also led to the introduction of intraburst closings (supplemental Fig. S2B). These results suggested that fraction C contains both of the activities previously described for venom.

Sequential purification using gel-filtration chromatography (supplemental Fig. S3) and additional RP-HPLC (Fig. 1D, left) resulted in the isolation of a single peptide whose biological activity was sufficient to recapitulate the interburst inhibitory activity of crude venom. The RP-HPLC chromatographic peak collected ~15 min into the run, peak #6, was found to inhibit WT-CFTR single channels by inducing long lived interburst closings (Fig. 1E). Peak #6 caused a 58.2 ± 11.3% decrease in P_o from 0.19 ± 0.06 in control conditions to 0.06 ± 0.004 (n = 3, p = 0.06) in the presence of peak #6 (Fig. 1D, middle), by a mechanism that involved an increase in interburst closed duration (Fig. 1D, right) (n = 3, p = 0.02). From these results we concluded that peak #6 contained a peptide toxin that inhibited CFTR. However, the venom-induced intraburst closings that were apparent during inhibition by fraction C were notably absent (Fig. 1E). These findings suggest that at least two toxins active at CFTR are contained in Lqh-pf venom and that the toxin responsible for inducing intraburst blocked states was not contained in peak #6.
GaTx1: a State-dependent, Peptide Inhibitor of CFTR

Characterization of the Isolated Peptide Toxin—Initial characterization of the active peptide contained in peak #6 was accomplished by subjecting an aliquot of the material to MALDI-TOF analysis. A single peptide was identified with a molecular mass of 3,674.6 Da (Fig. 2A). Additional, less abundant peaks with similar mass were also identified that likely represent different oxidative states of the same toxin; a doubly charged species also was observed ([m/z] = 1,838.8). The peptide was subjected to automated N-terminal sequencing following destruction of all putative disulfide bridges by reduction with dithiothreitol and alkylation of free cysteines by iodoacetamide. The reduction and alkylation of the native toxin resulted in an increase in molecular mass to 4,138.5 Da as determined by MALDI-TOF, which is in agreement with the modification of eight cysteines; expected mass = 4,138.6 Da (supplemental Table S1). The primary sequence was determined to be: \(^{1}CGPCFTT-DHQM\)E\(^{	ext{NBD}}\)K\(^{	ext{NBD}}\)AE\(^{	ext{NBD}}\)CG\(^{	ext{NBD}}\)GG\(^{	ext{NBD}}\)K\(^{	ext{NBD}}\)CYG\(^{	ext{NBD}}\)-P\(^{	ext{NBD}}\)Q\(^{	ext{NBD}}\)CL\(^{	ext{NBD}}\)C\(^{	ext{NBD}}\)NR\(^{	ext{NBD}}\)34, including C-terminal amidation as the only post-translational modification other than formation of disulfide bridges. The calculated mass of the isolated toxin based upon primary sequence and assuming post-translational C-terminal amidation was in close agreement with the mass determined by MS; these results suggest that the toxin does not include bound metal ions.

To confirm the primary sequence, an aliquot of the reduced/carboxamidomethyl-modified toxin was digested with Lys-C protease and analyzed by Edman degradation and electrospray ionization-MS/MS, resulting in the same primary sequence data. The same toxin has been purified from three different batches of scorpion venom.

Comparisons of the primary sequence with those of other known or putative peptide toxins suggested that the isolated toxin, which we named GaTx1 (for “Georgia anion toxin 1”), is novel, with C1x-d as its closest relative (Fig. 2B). We chose not to apply the chlorotoxin nomenclature to our new toxin because none of the chlorotoxins have been found to inhibit any channel of known molecular identity; hence, it is not clear that the sequences of chlorotoxins indicate specificity for chloride.

FIGURE 1. Isolation of the toxin active against CFTR. A, domain architecture of WT-CFTR and FLAG-cut-\(\Delta\)R-CFTR. B, representative RP-HPLC chromatogram of Lqh-pf venom (left). The average percent inhibition of FLAG-cut-\(\Delta\)R-CFTR macropatch currents by venom fractions at 0.3 mg/ml equivalent is shown on the right. C, representative macropatch traces of WT-CFTR (left) and FLAG-cut-\(\Delta\)R-CFTR (right); 0.1 mg/ml Lqh venom fraction C was applied 30 s prior to second exposure to 1 mM MgATP. D, representative RP-HPLC chromatogram of fraction C of Lqh venom RP-HPLC fraction C was applied 30 s prior to second exposure to 1 mM MgATP. D, representative RP-HPLC chromatogram of fraction C of Lqh venom RP-HPLC fraction C applied 30 s prior to second exposure to 1 mM MgATP. E, representative single channel trace in the absence and presence of peak #6. The isolated toxin inhibits CFTR by increasing the interburst closed duration. The bottom trace shows an expanded section of recording showing that peak #6 does not induce intraburst inhibition. Error bars show mean ± S.E. for \(n = 3–4\) observations at each condition.
channel targets. The amino acid sequences of ClTx-b, -c, and -d are speculative, being predicted from the sequences of cDNAs cloned from scorpion venom gland (38); hence, it is not clear that these sequences contribute to peptides found in scorpion venom. GaTx1 bears 75% sequence identity to ClTx, but is smaller by ~400 Da. Calculated pI values for GaTx1 and ClTx are 6.71 and 8.13, respectively. The primary sequence of GaTx1, the molecular mass, and the presence of four disulfide bridges between conserved cysteines place this toxin in the family of short chain insectotoxins active at K⁺ channels and putative toxins active at Cl⁻ channels (10).

A homology model of GaTx1 was created based on the known NMR structures of CITx and insectotoxin 5A (32, 33). Fig. 2C shows the NMR structure of CITx and the homology model of GaTx1, in three orientations. The two structures are quite similar in shape and secondary structure, although GaTx1, the molecular mass, and the presence of four disulfide bridges between conserved cysteines place this toxin in the family of short chain insectotoxins active at K⁺ channels and putative toxins active at Cl⁻ channels (10).

FIGURE 2. Proteomic characterization of GaTx1. A, MALDI-MS spectrum of peak #6. B, alignment of the GaTx1 sequence with other scorpion toxins shows that GaTx1 is a novel peptide. C, comparison of the CITx NMR structure (left) and the GaTx1 homology model (right). Disulfide bridges are shown in the upper panels. D, sequence alignment of GaTx1 with the sequences of CITx and ChTx. Predicted secondary structure is indicated above the sequences, whereas disulfide bridge linkage between conserved cysteines is indicated below.

Synthetic GaTx1 Inhibits CFTR in a State-dependent Manner—To confirm that the inhibitory activity in venom could be ascribed to the sequenced toxin, and did not arise from a contaminant, we prepared GaTx1 by solid-phase chemistry. After purification by HPLC, MS analysis indicated that the synthetic linear peptide has a mass in agreement with the theoretical mass for the linear form of the native toxin (i.e. all disulfides reduced to sulfhydryls) (supplemental Table S1). The mass of the folded synthetic peptide differed from the observed mass of the native toxin isolated from venom by only 0.5 atomic mass unit (supplemental Fig. S4); in RP-HPLC experiments, folded synthetic toxin co-eluted with the native toxin (supplemental Fig. S5).
GaTx1: a State-dependent, Peptide Inhibitor of CFTR

We used multichannel patch recordings from oocytes expressing FLAG-cut-ΔR-CFTR to test for activity of synthetic GaTx1 (Fig. 3A), taking advantage of the insensitivity of this CFTR variant to dephosphorylation-mediated rundown. Because GaTx1 appears to bind to channels in the closed state, we used intracellular solution containing 0.2 mM ATP to reduce $P_o$, thus increasing the likelihood of a toxin binding event. Fig. 3B shows a dose-response curve for inhibition of FLAG-cut-ΔR-CFTR under these conditions. The data were fit with a three-parameter Hill function providing a $K_D$ of 41.5 ± 21.5 nM; the apparent $IC_{50}$ under these conditions was 48 nM. In the presence of 0.2 mM ATP, maximum inhibition at 750 nM toxin, the highest concentration tested, was 96.5%. We previously established that venom was more effective when applied to CFTR channels in the absence of ATP, leading to strong state dependence of action (21, 27). We therefore predicted that under conditions that increase $P_o$, the apparent magnitude of inhibition should be reduced. In the continuous presence of 1 mM ATP, GaTx1 inhibited CFTR channel activity less effectively (Fig. 3B); maximal inhibition by 750 nM toxin under these conditions was only 60.4%, and the $K_D$ was increased to 84.6 ± 3 nM ($p = 0.0001$ at each toxin concentration); $IC_{50}$ under these conditions was 220 nM. The combined change in efficacy and in potency suggests that GaTx1 may be a non-competitive inhibitor of ATP-dependent channel activity. When CFTR channels were preincubated with 50 nM GaTx1 for 5 min in the absence of ATP, currents measured subsequently in the presence of 1 mM ATP plus toxin were inhibited by 80.2% ($p < 0.0001$ compared with toxin applied in the continuing presence of 1 mM ATP) (Fig. 3D). Hence, GaTx1 inhibits CFTR in a strongly state-dependent manner.

For comparison, we tested, under identical conditions, the sensitivity of CFTR to inhibition by cytoplasmic glibenclamide, a member of the sulfonylurea family of compounds. Glibenclamide blocks the channel pore in a voltage-dependent manner by interaction with multiple binding sites (39, 40); in contrast, our previous experiments indicated that inhibition by Lqh-pf venom is not voltage-dependent (21). In inside-out macropatches, glibenclamide inhibited FLAG-cut-ΔR-CFTR at $-80$ mV up to a maximum of 80% at 250 µM, with $K_D = 59 ± 25$ µM (Fig. 3, B and C). In contrast to our results with toxin, percent inhibition by 100 µM glibenclamide did not differ in the presence of 0.2 and 1 mM ATP (Fig. 3B and E) ($p = 0.64$), indicating that glibenclamide-mediated inhibition of CFTR is not state-dependent.

GaTx1 Selectively Inhibits CFTR—To verify the specificity of GaTx1-mediated inhibition, we tested the ability of the toxin to inhibit other channels and other ABC transporters. Our previous work showed that Lqh-pf venom or fraction C did not inhibit oocyte endogenous Ca$^{2+}$-activated Cl$^-$ channels (21), or CIC-0 or CIC-1 voltage-gated Cl$^-$ channels (41); Lqh-pf

5 min prior to subsequent reactivation in the presence of 1 mM ATP plus toxin. Note that inhibition by 50 nM GaTx1 was significantly increased when toxin was applied in the absence of ATP. $E$, comparison of percent inhibition by 100 µM glibenclamide when applied in the continuous presence of either 0.2 mM or 1 mM ATP. Inhibition of CFTR by 100 µM glibenclamide was not dependent on ATP concentration.
GaTx1: a State-dependent, Peptide Inhibitor of CFTR

A. CFTR extracellular

IBMX + db-cAMP

GaTx1

1 μA

5 min

Fractional current remaining

Control GaTx1

B. CIC-1

Fractional current remaining

GaTx1 +60 mV - 120 mV

C. CIC-2 extracellular

1 μA

500 ms

Fractional current remaining

Control GaTx1

D. CIC-2 intracellular

Fractional current remaining

+ 50 nM GaTx1

E. CIC-3

Fractional current remaining

GABA

GaTx1

0.4 μA

100 s

1 μA

1 min

1 μA

100 ms

F. GABAC Receptor

G. Cl(Ca) channels

20 s

50 pA

H. Shaker B-IR

2 μA

100 ms

% decay during pulse

2nd 3rd

0 10 20 30 40 50

Fractional current remaining

Peak Current Non-inactivated current

a vs. b a vs. c b vs. c

a vs. c

Shaker B-IR

20 s

10 mM Ca2+ 10 mM Ca2+ 10 mM Ca2+

50 pA

20 s

10 mM Ca2+ 10 mM Ca2+ 10 mM Ca2+

50 nM GaTx1

20 s

50 nM GaTx1

20 s

50 nM GaTx1

20 s

50 nM GaTx1

20 s
GaTx1: a State-dependent, Peptide Inhibitor of CFTR

Our previous experiments (21) indicated that Lqh-pf venom did not inhibit CFTR when applied to the extracellular side. To confirm that the isolated GaTx1 toxin exhibits this specificity as well, macroscopic currents from oocytes expressing WT-CFTR were recorded using two-electrode voltage clamp (TEVC) in the presence and absence of toxin. CFTR currents activated by 200 μM IBMX plus 25 μM Bt2cAMP were not affected by 5-min exposure to 20 nM synthetic GaTx1 (Fig. 4A). Synthetic GaTx1 also had no effect on CIC-1 or CIC-2 when applied to the extracellular side of those channels in TEVC experiments (Fig. 4, B and C), or on CIC-2 when applied to the cytoplasmic face of those channels in an inside-out patch (Fig. 4D). Similarly, GaTx1 had no effect on CIC-3 when applied to the extracellular side (Fig. 4E). GaTx1 had no effect on ligand-gated Cl− channels formed by GABA<sub>C</sub> receptors when applied to the extracellular face (Fig. 4F), or endogenous Ca<sup>2+</sup>-activated chloride Cl− channels when applied to the intracellular face (Fig. 4G). These experiments suggested that the GaTx1 toxin does not inhibit other known chloride channels at concentrations at which it effectively inhibits CFTR.

GaTx1 bears distant homology to the very well known ChTx, which inhibits a wide variety of voltage-gated and Ca<sup>2+</sup>-activated K<sup>+</sup> channels (42) (Fig. 2D). We therefore asked whether GaTx1 may also inhibit K<sup>+</sup> channel targets by assaying for effects on Shaker Kv1.1 K<sup>+</sup> channels. For these experiments, we made use of the ShB variant with inactivation-removed (ShB-IR) (43). As shown in Fig. 4H, GaTx1 had no effect on ShB-IR K<sup>+</sup> currents at a toxin concentration that effectively inhibits CFTR. As a positive control, we observed 15% inhibition of ShB-IR currents by 24 nM recombinant ChTx (data not shown), which is consistent with the reported <i>K<sub>i</sub></i> of ~200 nM for inhibition of this channel (44).

CFTR is a member of the ABC Transporter superfamily. We asked whether GaTx1 might inhibit other ABC Transporters by testing for effects on closely related proteins. These experiments focused on the MRPs, fellow members of the OAD/ABCC subfamily, with highest homology to CFTR (15). Using baculovirus-mediated expression of MRP1, MRP2, and MRP3, we asked whether GaTx1 could inhibit ATP-dependent transport in inside-out vesicles isolated from Sf9 cells expressing these proteins. Transport of [3H]leukotriene C4 (for MRP1) and [3H]E217βG (for MRP2 and MRP3) was measured in the presence and absence of 200 nM synthetic GaTx1, as described under “Experimental Procedures” (picomoles of substrate accumulated in the vesicles per milligram of vesicular protein per minute) and is expressed as percentage of control. As a positive control, we found that the addition of 50 μM benzbro- marone, a known inhibitor of MRPI–3 (45), resulted in 0–10% transport activity (data not shown). In contrast, 200 nM GaTx1 had no effect on transport activity for any MRP variant (Table 1). Given the nature of the GaTx1–CFTR interaction, we could not discount the possibility that the apparent lack of inhibition observed in the MRP transport assays is due to the high ATP concentration present in the reaction buffer (see “Experimental Procedures”). To rule out the possibility that ATP (or the test substrate) hinders the binding of the toxin to the MRPs, we exposed toxin to the vesicles prior to the initiation of the transport reaction. In these experiments, MRP-expressing membranes were preincubated for 5 min with the toxin prior to supplying the labeled test substrate and initiation of the reaction by the addition of ATP. Under these conditions, as well, transport rates were not different from controls (data not shown), suggesting that GaTx1 is specific for CFTR among members of the ABC Transporter superfamily.

**DISCUSSION**

Previous studies indicated that a toxin or toxins contained in Lqh venom preferentially inhibits CFTR activity in a state-dependent manner by binding to channels when they are in either the interburst or intraburst closed states (27). We have isolated a novel peptide toxin that selectively inhibits CFTR, making GaTx1 the first peptide toxin found to inhibit an anion channel of known molecular identity. Initial characterization of the inhibitory activity suggested that GaTx1-mediated inhibition of CFTR is strongly state-dependent: the toxin interacts with CFTR when the channel is in the interburst closed state, usually thought to represent disruption of the nucleotide-binding domain1-nucleotide-binding domain2 dimer (46), leading to inhibition of channel opening (27). These results suggest that there is a substantial change in the conformation of the cytoplasmic domains of CFTR during ATP-dependent channel gating. In patches bearing a few CFTR channels, application of GaTx1 resulted in channels that were locked in the interburst closed state for many tens of seconds to minutes, causing them to disappear from the record. However, the intraburst inhibition evident in experiments with either Lqh-pf venom or Lqh-pf venom does contain a separate toxin that inhibits CIC-2 channels (41), although this is not a component of fraction C.

**FIGURE 4. GaTx1 specifically inhibits CFTR.** A, effect of extracellular application of GaTx1 on WT-CFTR currents. TEVC currents were recorded at <i>V<sub>o</sub></i> = −60 mV (top). CFTR channels were activated by 200 μM IBMX plus 25 μM Bt2cAMP, then exposed to 20 nM GaTx1 for 5 min, which had no effect (bottom). B, effect of GaTx1 of CIC-1. Currents were activated with a pulse to <i>V<sub>i</sub></i> = +60 mV, followed by a tail pulse to −120 mV. Currents in the absence (solid trace) and presence (dotted trace) of 60 nM GaTx1 are shown (top). GaTx1 did not alter currents at <i>V<sub>i</sub></i> = +60 mV or −120 mV (bottom). C, effect of GaTx1 on CIC-2 when applied extracellularly. CIC-2 currents measured by TEVC were activated with a pulse to <i>V<sub>i</sub></i> = −160 mV, followed by a tail pulse to +40 mV. Currents in the absence (solid trace) and presence (dotted trace) of 60 nM GaTx1 are shown (top). GaTx1 had no effect on CIC-2 (bottom). D, effect of GaTx1 on CIC-3 when applied to the cytoplasmic face of the channel. CIC-3 currents from multichannel patches at <i>V<sub>i</sub></i> = −100 mV are shown (top). Analysis of window currents from control conditions and in the presence of 50 nM gaTx1 showed no significant difference (bottom). E, effect of GaTx1 on CIC-3 channels. CIC-3 currents measured by TEVC were activated with a pulse to <i>V<sub>i</sub></i> = +120 mV, followed by a tail pulse to −40 mV. Currents are shown in the absence (black trace) and presence of 60 nM GaTx1 (gray trace) (top). GaTx1 had no effect on CIC-3 currents (bottom). F, effect of GaTx1 on ligand-gated GABA<sub>C</sub> receptors at <i>V<sub>i</sub></i> = −60 mV. GABA<sub>C</sub> receptor currents (top) were activated by 10 μM GABA. Oocytes were then exposed to 20 nM GaTx1 for 5 min, and subsequently activated with 10 μM GABA in the continuing presence of GaTx1. Currents were then recorded from the bath solution, with GABA remaining. There were no differences in GABA-activated current magnitudes in the absence and presence of GaTx1 (bottom). G, effect of GaTx1 on endogenous Ca<sup>2+</sup>-activated chloride channels. Currents were activated 3 times, at <i>V<sub>i</sub></i> = −50 mV, by exposure to 10 mM Ca<sup>2+</sup> or Ca<sup>2+</sup> plus 50 nM GaTx1 (top). The decay rates between the first and second, or second and third pulses were measured for control patches, or patches in the presence of toxin (bottom). There was no significant change upon addition of toxin. H, effect of GaTx1 on Shaker B-IIR channels. Currents were activated by a pulse from <i>V<sub>i</sub></i> = −90 mV to 0 mV. Currents are shown in the absence (black trace) and presence (overlying gray trace) of 25 nM toxin (top). GaTx1 had no effect on peak currents or non-inactivating currents (bottom). All data are shown as mean ± S.E. for n = 3–5 observations.
GaTx1: a State-dependent, Peptide Inhibitor of CFTR

venom fraction C was not present when GaTx1 was used in isolation. This suggests that a second toxin exists in venom that inhibits CFTR through a mechanism different from that of GaTx1.

The discovery of GaTx1 provides a new tool that will be useful for investigation of CFTR structure and function; the high affinity, state-dependent character, and peptide nature of GaTx1 will allow the use of new approaches to identify conformational changes in the channel underlying transitions between the closed and open states. However, because GaTx1 inhibits CFTR only when applied to the cytoplasmic side, it is unlikely that CFTR represents the native target. It appears to be serendipitous that the cytoplasmic domains of CFTR form a motif that may mimic the extracellular toxin-binding motif of the natural target. Nonetheless, because GaTx1 binding to CFTR interferes with channel activity in an ATP-dependent manner, this peptide may be used as a probe of conformational changes in those cytoplasmic domains that participate in ATP-dependent channel gating.

Few inhibitors of gating in CFTR have been described with mechanistic detail (see Refs. 47–49). Because CFTR gating is an enzymatic process, including hydrolysis of nucleotide, substrate analogs that compete with ATP may be considered gating modifiers that interfere with channel opening (50–52). Similarly, ATP analogs that are non-hydrolyzable (or poorly hydrolyzed), or that exhibit higher binding affinities, may be considered gating modifiers that interfere with channel closing (53–55). Mg2+ is required for coordination of ATP during the hydrolytic cycle, so other divalent cations such as Ca2+ also serve as inhibitors (56, 57). Other categories of gating modifiers of CFTR are the xanthines (e.g. caffeine and IBMX (58–60)), flavones (e.g. querctin), isoflavones (e.g. genistein (61, 62)), and bipyridones (e.g. milrinone (49, 58)). However, many of these compounds act indirectly, by controlling phosphorylation of the R-domain of CFTR, or have dual actions. For example, genistein activates CFTR at low concentrations and inhibits it at high concentrations, suggestive of multiple binding sites (61).

Over the past several years, three new classes of compounds have been identified as high affinity inhibitors of CFTR as follows. (a) The glycine hydrazide GlyH-101 blocks the CFTR channel pore from the extracellular end; a lectin-conjugated derivative (MalH-lectin) inhibits CFTR at picomolar concentrations (63, 64). (b) High throughput screening approaches led to the discovery of the thiazolidinone CFTRinh172, which specifically inhibits CFTR channels with an apparent K_{i} of 0.3–0.6 μM by a mechanism that appears to involve inhibition of gating (62, 65). However, CFTRinh172-mediated inhibition does not exhibit state dependence. (c) Most recently, highly potent α-aminoazaheterocyclohexylglyoxal adducts have been reported, which inhibit CFTR activity in the picomolar to nanomolar concentration range, although specificity for inhibition of CFTR is less clear (66). However, the mechanism of action for this new class of inhibitors is not yet known.

Overall, the CFTR inhibitor compounds available to date have not provided much insight into the conformational changes underlying channel gating. We have shown that GaTx1 acts as a gating modifier but is clearly not acting as a nucleotide analog, binding at the same site as ATP or other nucleotides, because the efficacy of inhibition does not exhibit a simple relationship to [ATP] as one would expect based upon competition for the ATP binding site (Fig. 3B) (27).

Because both CFTR and other Cl− channels, including CIC-2, are expressed in many cell types, including airway and intestinal epithelial cells, whereas GaTx1 is selective for CFTR, GaTx1 also will facilitate distinguishing the functional roles of CFTR in a background of other potential chloride transport pathways. GaTx1 may also serve as a lead compound for the development of peptidomimetic drugs to inhibit CFTR; some scorpion peptide toxins of similar size, charge, and structure are able to permeate the plasma membranes of some cells (67). Furthermore, because GaTx1 appears to interact with CFTR with high affinity by locking the channel into one state, the toxin may be useful in promoting conformational stability in attempts to crystallize the channel. Finally, to our knowledge, GaTx1 is the first peptide toxin that has been shown to be active against any member of the ABC transporter superfamily; many human members of this protein family have clinical relevance.

Acknowledgments—We thank M. C. Sullards and J. C. Hardeman for experiments.

REFERENCES

1. Devuyst, O., and Guggino, W. B. (2002) Am. J. Physiol. 283, F1176–F1191
2. Jentsch, T. J., Stein, V., Weinreich, F., and Zdebik, A. A. (2002) Physiol. Rev. 82, 503–568
3. Haug, K., Warnstedt, M., Alekow, A. K., Sander, T., Ramirez, A., Poser, B., Maljevic, S., Hebeisen, S., Kubisch, C., Rebstock, J., Horvath, S., Hallman, S., Dullinger, J. S., Rau, B., Haverkamp, F., Beyenburg, S., Schulz, H., Janz, D., Geise, B., Muller-Newen, G., Propping, P., Elger, C. E., Fahlke, C., Lerche, H., and Heils, A. (2003) Nat. Genet. 33, 527–532
4. Lewis, R. I., and Garcia, M. L. (2003) Nat. Rev. Drug Disc. 2, 1–13
5. Gross, A., and MacKinnon, R. (1996) Neuron 16, 399–406
6. Stamep, P., Kolmakova-Partensky, L., and Miller, C. (1994) Biochemistry 33, 443–450
7. MacKinnon, R., Heginbotham, L., and Abramson, T. (1990) Neuron 5, 767–771
8. French, R. J., and Dudley, S. (1999) Meth. Enzymol. 294, 575–605
9. Castle, N. A., Haylett, D. G., and Jenkinson, D. H. (1989) Trends Neurosci. 12, 59–65
10. Possani, L. D., Merino, E., Corona, M., Bolivar, F., and Becerril, B. (2000) Biochimie 82, 861–868
11. Posson, D. J., Ge, P., Miller, C., Bezanilla, F., and Selvin, P. R. (2005) Nature 436, 848–851
12. Gadsby, D. C., and Nairn, A. C. (1997) Trends Biochem. Sci. 19, 512–518
13. MacKinnon, R., and Stanton, B. A. (2006) Nat. Rev. Mol. Cell Biol. 7, 426–436
14. Al-Awqati, Q. (2002) J. Clin. Invest. 110, 1599–1602
15. Dure, E. (2003) in ABC Proteins: From Bacteria to Man (Holland, I. B., Cole, S. P. C., Kuchler, K., and Higgins, C. F., eds) pp. 3–35, Academic Press, London
16. Higgins, C. F. (1992) Annu. Rev. Cell Biol. 8, 67–113
17. Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., and Tsui, L.-C. (1989) Science 245, 1066–1072
18. Gadsby, D. C., Vergani, P., and Csanda, L. (2006) Inhib, F1176–F1191
19. Mackinnon, R., and Stanton, B. A. (2006) Nature 436, 426–436
20. DeBin, J. A., and Strichartz, G. R. (1991) Toxicol 2, 1403–1408
21. DeBin, J. A., Maggio, J. E., and Strichartz, G. R. (1993) Am. J. Physiol. 264, C361–C369
22. Fuller, M. D., Zhang, Z.-R., Cui, G., Kubanek, J., and McCarty, N. A. (2004) Am. J. Physiol. 287, C1328–C1341
