Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion

JOHN A. DEBIN, JOHN E. MAGGIO, AND GARY R. STRICHARTZ
Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, and the Anesthesia Research Laboratories, Brigham and Women's Hospital, Boston, Massachusetts 02115

DeBin, John A., John E. Maggio, and Gary R. Strichartz. Purification and characterization of chlorotoxin, a chloride channel ligand from the venom of the scorpion. *Am. J. Physiol.* 264 (*Cell Physiol.* 33): C361-C369, 1993.—We have previously demonstrated that the venom of the scorpion *Leiurus quinquestriatus* blocks small-conductance Cl^- channels, derived from epithelial cells, when applied to the cytoplasmic surface. We have now purified to near homogeneity, and characterized, the component responsible for this blocking activity. It is a small basic peptide of 4,070 Da. The primary amino acid structure shows considerable homology to a class of previously described putative short insectotoxins. A brief characterization of the kinetics of Cl^- channel block as well as a demonstration of toxicity to arthropods is also presented.

anion channel; scorpion toxin; scorpion venom; insectotoxin; *Leiurus quinquestriatus quinquestriatus*

SCORPIONS COMPRISE an order (Scorpionidae) within the arthropod phylum so successful in nature that their basic anatomy and physiology have not changed for hundreds of thousands of millennia. Because scorpions prey primarily on other arthropods, they have developed the ability to produce and deliver small quantities of very potent toxins to their prey. These basic peptide toxins rapidly paralyze the scorpion's victim, primarily by disrupting normal ion channel function in the nervous system. From the Buthinae family, α- and β-neurotoxins, which alter fast Na^+ channel gating (8, 35, 41) and charybdotoxin (7) and leiurotoxin (6), blockers of Ca^{2+}-activated K^+ channels, have been described. The α- and β-toxins are a large class of toxins, 60–70 amino acids in length, that are generally effective against vertebrates. A structurally homologous group of peptides is known to be effective against either insects or crustaceans. The selectivity of these "long" neurotoxins is, however, not absolute. Some of the mammalian toxins also show considerable toxicity to arthropods (35; cf. Table 4 in Ref. 41), and many of the insect toxins are effective against crustaceans (12, 17). A series of short, 30–36 amino acid, putative insectotoxins (see DISCUSSION) from the *Buthus* and *Androctonus* genera have also been described (35, 40).

Previously we reported that crude venom extracted from the scorpion *Leiurus quinquestriatus quinquestriatus* inhibits reconstituted small-conductance Cl^- channnels isolated from rat epithelia and embryonic rat brain (10, 11). In this paper we describe the purification and characterization of the active component from crude *Leiurus* venom. We now know that this activity is due to a 4.1-kDa basic peptide with considerable primary sequence homology to the small insectotoxins. In recognition of the affinity of this previously undescribed peptide for Cl^--selective ion channels, we have named it "chlorotoxin."

MATERIALS AND METHODS

Rat colonic epithelial chloride channel reconstitution. Rat colonic enterocyte plasma membranes were prepared using the method described by Reinhardt et al. (34) with the following modifications. Male Sprague-Dawley rats, 250–300 g, six to eight at a time were used without exposure to dexamethasone. After decapitation, colons were dissected, washed with cold Ringer solution, everted onto a glass rod, and incubated at 37°C in a solution consisting of (in mM) 30 EDTA, 107 NaCl, 25 $NaHCO_3$, 4.5 KCl, 1.8 Na_2HPO_4, 0.2 NaH_2PO_4, and 12 glucose, pH 7.4. No attempt was made to remove the muscularis or submucosal layers. Over the next hour, the solution bathing the everted colons was bubbled with 5% CO_2, and each colon was gently scraped with a metal spatula every 10 min. After 60 min, the entire suspension of epithelial cells and sheets of cells were centrifuged at 600 *g* for 15 min at 4–6°C, and the pellet was resuspended in an equal volume of 8.5% sucrose (wt/vol), 1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), and 5 mM imidazole, pH 7.4.

The remainder of the protocol was carried out at 4–6°C and is as described in Ref. 34. In brief, this procedure entails washing the pellet two additional times in the 8.5% sucrose solution described above. After the third wash, the pellet was resuspended in approximately twice its volume of 8.5% sucrose solution and homogenized (20 strokes) with a tight-fitting (A) Dounce homogenizer. After centrifugation at 1,200 *g* for 15 min, the supernatent was saved and the pellet resuspended and rehomogenized as before. After four to five cycles of homogenization, the pooled supernatant fraction was centrifuged at 22,000 *g* for 75 min. Most of the pellet (≈3 ml total excluding the dense, yellow-white button present at the very bottom of the centrifuge tube) was then recovered and resuspended in 6 ml of 8.5% sucrose solution with the aid of a loose-fitting (B) Dounce homogenizer. This suspension of membranous particles was then layered on top of a discontinuous sucrose gradient consisting of 40% (wt/vol) sucrose, 1 mM EGTA, and 5 mM imidazole, pH 7.4, and 20% sucrose, 1 mM EGTA, and 5 mM imidazole, pH 7.4. After centrifugation at 130,000 *g* for 2 h, the material at the 20/40% interface was collected, and the entire volume (≈5 ml) was diluted to 30 ml with the 8.5% sucrose solution. After a final centrifugation at 27,000 *g* for 60 min, a small pellet (≈400 μl) was collected and resuspended in an equal volume (400 μl) of the 8.5% sucrose, 1 mM EGTA, and 5 mM imidazole, pH 7.4, solution. Subsequent to aliquoting, this suspension of membranes was stored at −80°C and used for up to 6 mo after the date of preparation.

Chloride channels were reconstituted from the rat enterocyte preparation into planar phospholipid bilayers using well-established methods (31). Lipid solutions were prepared each day from chloroform stock solutions. After removing the chloroform with a stream of N_2, 40 μg of 1-palmitoyl-2-oleoyl phosphatidylethanolamine and 10 μg of 1-palmitoyl-2-oleoyl phosphatidylcholine (nos. 850757 and 850457; Avanti Polar Lipids, Alabaster, AL) were redissolved in 20 μl *n*-decane. The sealed end of a glass capillary tube was used to apply a small amount of this solution across a 250-μm-diameter hole drilled through a 2-mm-thick polystyrene partition separating two (2.5 and 4 ml) chambers. The solution used in each chamber at the

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time of data acquisition consisted of 200 mM NaCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, and 0.2 mM EGTA, pH 7.4. A NaCl gradient, 200 mM *cis*-bath, 50 mM *trans*-bath, used to enhance channel incorporation, was collapsed before the beginning of an experiment by adding concentrated NaCl to the *trans*-bath. Chloride channels were usually seen within 30 min of pipetting 5 μl of a suspension of plasma membranes (~2 μg protein/μl) near the bilayer on the *cis*-side. All blocking experiments were conducted at ambient temperatures, 21–23°C.

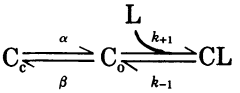
Single chloride channel recording. *Cis*- and *trans*-baths were connected through 1 M KCl-1% agar salt bridges and Ag-AgCl electrodes to a Warner Instruments (Hamden, CT) PC-501 amplifier. The analog signal was converted to digital data for storage on a videocassette recorder tape using a Sony PCM-501 pulse code modulator. These data were subsequently filtered at 50 Hz low pass using a Krohn-Hite model 3200 filter in the resistance-capacitance mode and then analyzed using a PC's Limited 286 computer and Axon Instruments TL-1 interface in conjunction with the single-channel programs found in PClamp software (Axon Instruments, Foster City, CA). Junction potentials measured at the end of each experiment were found to be <3 mV in all cases, and thus were ignored.

Ion channels were identified as being chloride selective by the direction of current flux at 0 mV applied potential in the presence of a NaCl gradient. Enterocyte Cl^- channels also displayed properties similar to those described by other investigators for reconstituted channels from the rat colon (4, 34): curvilinear current-voltage relationships with ≈50-pS conductance at +20 mV and ≈32 pS at −20 mV in symmetrical 200 mM NaCl, spontaneous gating with frequent rapid closures, and a reduction in current amplitude to one-half control by 5 μM 4,4'-dinitrostilbene-2,2'-disulfonic acid (DNDS) applied "extracellularly." Because the Cl^- channels were found to incorporate into bilayers in both of two possible orientations (cytoplasmic surface facing either the *cis*- or *trans*-bath), channel sidedness was determined from the current rectification properties of the individual channels, based on the assumption that the reconstituted enterocyte Cl^- channels are outwardly rectifying, like those described in intact cells using patch-clamp electrodes (14, 25). All voltages are, therefore, reported as the true membrane potential according to established conventions (i.e., outward rectification of an anion current would imply a greater flux of anions into a cell than in the reverse direction in response to applied potentials of equal magnitude but opposite polarity).

A current amplitude exactly halfway between the maximum steady-state open channel and zero-current levels was chosen as the threshold for the detection of an opening/unblocking or closing/blocking transition. Because at steady state the enterocyte Cl^- channels spontaneously close for brief periods and reopen (cf. controls, Figs. 2 and 3), channel block by ligand molecules must be distinguished from random channel gating. Because most of the spontaneous closures in the absence of ligand are of short duration (i.e., <500 ms), a cut-off time (t_c) of 1.5 s was chosen such that only events lasting longer were considered channel blocks. On the basis of the frequency of spontaneous closures of >1.5-s duration in control records, the potential maximum "contamination" of true channel blocks by spontaneous closures was determined to be <10% in all cases. In other words, for a given time interval, the number of zero-current events (i.e., closures/blocks) lasting longer than 1.5 s was at least nine times greater in the presence of ligand than in its absence. Moreover, the use of a t_c of 0.5 or 1.0 s resulted in less than a 10% change in the values obtained for mean open/unblocked (t_o) or mean blocked (t_b) times.

The use of a t_c for the detection of channel blocking events will, however, cause the exclusion of a number of brief channel blocks and thus result in artificially high determinations for t_b and t_o. The correction described by Moczydlowski et al. (32) was therefore used to arrive at "true" t_b and t_o values.

With the assumption that the binding of one ligand molecule to a Cl^- channel in the open state (see RESULTS) is sufficient to induce a channel block, and that each binding results in a channel block, the dissociation constant (K_d) and the rate constants for the binding (k_{+1}) and unbinding (k_{-1}) reactions can be obtained from t_b and t_o according to the following scheme



where L represents the ligand molecule and C_c and C_o the Cl^- channel in the closed and open states, respectively. The dissociation rate constant k_{-1} is simply equal to $(t_b)^{-1}$, while the bimolecular association rate constant k_{+1} is equal to $[\beta - (t_o)^{-1}]/[L]$, and K_d equals k_{-1}/k_{+1}. The intrinsic rate of channel closure at steady state (β) is equivalent to the reciprocal mean open time determined in the absence of ligand. For the case in which the ligand concentration is known only in terms of milligrams per milliliter, the inhibitory constant (K_I) (or $K_{0.5}$) can be defined as $\{L/[t_b/(t_b + t_o)]\} - L$. Values for blocking parameters and equilibrium constants are given as means ± SD.

The mass of purified peptide present in stock solutions was determined by integration of the chromatograms derived from the digests performed for the analysis of amino acid content as described below. We also performed Lowry protein assays on these same stock solutions to establish the ratio between protein concentration as determined by Lowry assay and true protein content as determined from the amino acid analyses. This ratio was determined to be 1.81, indicating that the Lowry method overestimates by nearly twofold the concentration of this particular peptide. In some instances, Lowry assays were performed to determine the protein concentration of various peptide fractions. In these instances the ratio determined above was used to correct the values obtained.

Crude venom source and processing. All venom for the experiments described in this paper was that of *Leiurus quinquestriatus quinquestriatus* obtained from Latoxan (Rosans, France). All venom was dispersed in deionized H_2O, at 20 mg dry venom/ml, using a Potter-Elvehjem tissue grinder. After pelleting of the ubiquitous tenacious mucous in a swinging bucket rotor at 10,000 *g* for 30 min, the upper, relatively mucous-free, solution was recovered with a Pasteur pipette. This was in turn filtered using an Amicon Micropartition System (Beverly, MA) with 10-kDa cut-off filters at 1,690 *g* in a fixed angle rotor. The filtrate was collected, vacuum centrifuged, and reconstituted in 10 mM trifluoroacetic acid (TFA) at a concentration of ~20 mg/ml and subsequently loaded, 1 ml at a time, onto a Waters C_{18} Sep-Pak cartridge. The fraction containing the active component was eluted with 25% acetonitrile in 10 mM TFA, vacuum centrifuged to dryness, and reconstituted in 10 mM TFA in preparation for reverse-phase high-pressure liquid chromatography.

Reverse-phase high-pressure liquid chromatography (RP-HPLC). All RP-HPLC was performed using linear gradients of acetonitrile in either of two ion pair reagents as indicated in text: 10 mM TFA or 5 mM heptafluorobutyric acid (HFBA). We employed a Vydac TP-54 C_{18} column (Nest Group, Southborough, MA) for all work. Flow rate was a constant 1 ml/min in all cases, and absorbance was monitored at 214 nm. For preparative work, loads of 100 μg processed venom in 0.3 ml of 10 mM TFA were used. Loads for analytical work were as indicated in the legend to Fig. 1.

Ion-exchange HPLC. Peptides purified by RP-HPLC were repurified by ion-exchange HPLC using a polysulfoethyl

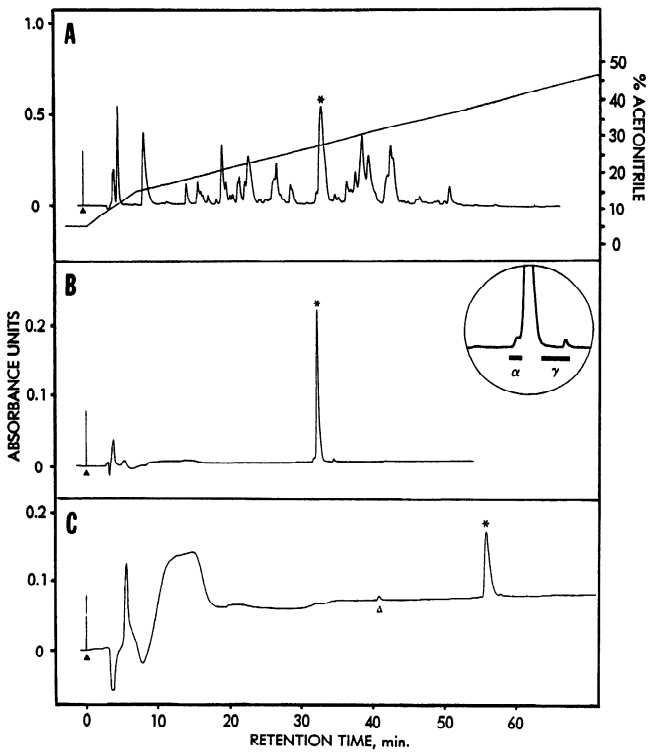


Fig. 1. Reverse-phase high-pressure liquid chromatography (RP-HPLC) chromatogram of processed *Leiurus quinquestriatus quinquestriatus* venom (*A*) and the purified ligand using 2 different ion pair reagents (*B* and *C*) are shown. A Vydac TP-54 C_{18} column was used for all separations. The same linear gradient of acetonitrile was used in each case, and this is shown in *A* only with percent acetonitrile indicated on *right axis*. Chromatograms have been drawn so that sample injection points (▲) are aligned vertically. Buffer flow rate was 1 ml/min in each case, and absorbance was continuously monitored at 214 nm. Note change in absorbance scales on *left axis*. In each panel, peak containing active component is indicated by an asterisk. *A*: crude scorpion venom was processed by filtration through 10-kDa cut-off filters as described in MATERIALS AND METHODS. Twenty micrograms of this processed venom were reconstituted in 0.3 ml of 10 mM trifluoroacetic acid (TFA) and loaded onto the column and eluted with a linear gradient of acetonitrile in 10 mM TFA. When the lag between elution profile and gradient is taken into account, active component is seen to emerge between 24 and 26% acetonitrile. *B*: 3 μg purified chlorotoxin were reconstituted in 0.3 ml of 10 mM TFA, loaded, and eluted with an acetonitrile gradient in 10 mM TFA as in *A*. *Inset*: enlargement of base of main peak. Areas collected as α- and γ-peak material are indicated by thick dark lines underneath chromatogram. *C*: 3 μg chlorotoxin were reconstituted in 0.3 ml of 5 mM heptafluorobutyric acid (HFBA) and eluted with 5 mM HFBA in acetonitrile. Injection artifact, small in *A* and *B*, encompasses nearly the first 20 min of this chromatogram. Small peak emerging at 40 min (△) was apparent also in blank (control) chromatogram.

aspartamide cation-exchange column (no. P1950-204; Nest Group). A linear gradient of NaCl (0–1.35 M) in 10 mM phosphate and 10% acetonitrile at constant pH 4.0 was used as indicated in Fig. 6. Flow rate was a constant 1 ml/min throughout, and the absorbance of the emerging peptides was continuously monitored at 280 nm.

Amino acid composition and primary sequence. Three separate amino acid analyses were performed. The first two analyses were performed by digesting 10 μg of purified peptide in 6 M HCl for 24 h at 110°C under argon. The amino acid composition of the hydrolysate was then determined following phenylisothiocyanate derivatization and chromatography using an Applied Biosystems 420A derivatizer and 130A separation system. A third analysis was performed by digesting the peptide in 6 N HCl for 22 h at 110°C under N_2. The amino acid composition was determined using a Beckman 6300 analysis system following conjugation with ninhydrin.

In preparation for sequencing, 40 μg of purified peptide were reduced and alkylated by first dissolving in 50 μl of 8 M urea, 0.4 M NH_4CO_3, pH 8.0. Dithiothreitol was added to a final concentration of 4 mM, and the solution was heated to 50°C for 30 min in a closed 1.5-ml polypropylene tube. Iodoacetamide was then added to a final concentration of 10 mM. After 15 min, the solution was diluted fourfold in 10 mM TFA and loaded onto a Waters C_{18} Sep-Pak. The reduced alkylated peptide was eluted with 100% acetonitrile, vacuum centrifuged to near dryness, and submitted for sequencing.

Sequencing was performed at the Harvard Microchemistry Facility, Cambridge, MA. In brief, an aliquot of alkylated peptide was applied to a polybrene precycled glass fiber filter and placed into the reaction chamber of an Applied Biosystems 477A Protein Sequencer equipped with an on-line phenylthiohydantoin (PTH) analyzer. Released PTH amino acids were subsequently identified by direct observation of the chromatogram. The sequence cycle, NORMAL-1, was modified using the manufacturer's recommendations for faster cycle time (37 min) by decreasing dry down times and increasing reaction cartridge temperatures to 50°C during coupling.

Arthropod toxicity experiments. All animals were obtained from Connecticut Valley Biological Supply (Southampton, MA). Crayfish (*Procambis clarkii*) weighing 4–6 g were injected with purified chlorotoxin (1.23–2.23 μg/g body wt) in 10 μl H_2O. Larger crayfish (9–10 g) received smaller doses of chlorotoxin (0.5 μg/g) as described in greater detail in RESULTS. In all cases the injections were made through the ventral surface of the thorax in the region of the subesophageal ganglion, using a 25-gauge needle on a 1-ml syringe. All crayfish served as their own controls and were first injected with 10 μl H_2O to which no response was observed for up to 3 min. Large (≈3 cm length) American cockroaches (*Periplaneta americana*) were similarly injected through the ventral surface at the thoracoabdominal junction using a 25-gauge needle. An equivalent volume of H_2O injected into control subjects was without effect as described in RESULTS.

The injection of small volumes of toxin was accomplished by first placing the 10-μl aliquot in a 1.5-ml test tube. The plunger of the syringe was then pulled back to draw roughly 50 μl of air into the syringe, and then the 10-μl aliquot of toxin or vehicle (H_2O) was drawn up. By injecting the solution first followed by the small bolus of air, we ensured the delivery of the entire amount of toxin.

RESULTS

Purification of the active component from Leiurus venom. Processed crude *Leiurus quinquestriatus quinquestriatus* venom was loaded onto a C_{18} RP-HPLC column and eluted with a linear gradient of acetonitrile in 10 mM TFA. The resulting chromatogram is shown in Fig. 1*A*. Individual peaks were collected by hand, taken to dryness, and reconstituted in 0.5 ml of 10 mM TFA. As an initial screen, small aliquots from five to six individual peaks were pooled and assayed for activity using reconstituted rat colonic enterocyte Cl^- channels, as described in MATERIALS AND METHODS. Once activity was localized to a general area of the HPLC chromatogram, the individual peak containing the active component (asterisk in Fig. 1) was identified using the same reconstituted Cl^- channel assay. A subsequent, large preparative purification was then accomplished in several separate runs, using the same HPLC conditions. The peaks containing the active component, easily identifiable by their size and

location in the elution profile, were collected, pooled, and reconstituted in 200–300 μl of TFA buffer. This partially purified component was subjected to a second round of chromatography under identical conditions in which only the center of the large peaks from several runs was collected, pooled, and reconstituted. This material was used for all blocking and arthropod toxicity experiments.

Effects of crude venom and purified ligand on reconstituted Cl⁻ channels. When crude *Leiurus* venom is applied to the cytoplasmic surface of a single reconstituted rat colonic enterocyte Cl^- channel, a potent inhibition results as seen in Fig. 2. This effect is seen only with application to the intracellular surface; no block is seen with application to the extracellular channel face. In the control record, open channel probability is high (P_o > 90%) with only occasional brief, reversible transitions from the open state to the closed or zero-current level. After exposure to 0.2 mg venom/ml, the Cl^- channel spends more time in the closed/blocked state than in the open state. There is no reduction in open-state current amplitude, indicative of a rapid open channel blocker, and there are no detectable subconductance states induced by the venom.

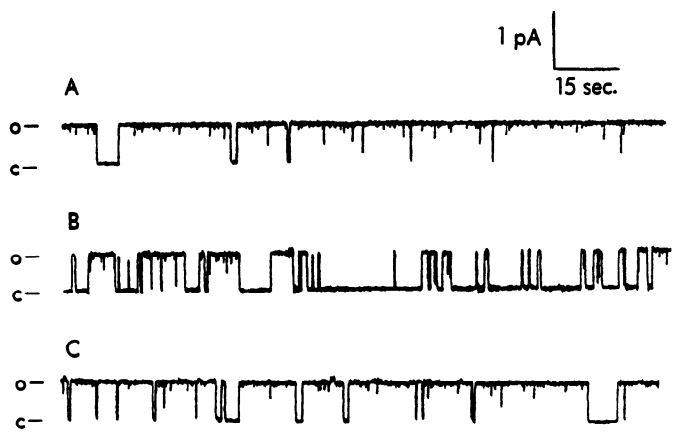


Fig. 2. Effects of crude venom on a reconstituted Cl^- channel are shown by 3 current traces from the same single rat enterocyte Cl− channel in a bilayer held at −20 mV. Methods of reconstitution were as described in MATERIALS AND METHODS. All records were filtered at 50-Hz low pass. o, Open/unblocked state; c, closed/blocked state. Scale bars represent 1 pA and 15 s. *A*: control situation before addition of venom [open channel probability (P_o) = 0.91]. *B*: immediately (<30 s) subsequent to addition of venom to 0.2 mg/ml final concentration in the "intracellular" bath (P_o = 0.31). *C*: washout: immediately after perfusion of intracellular bath with 4 vol of fresh buffer (P_o = 0.68).

Previously we reported that the blocking activity of crude venom on single enterocyte Cl^- channels was characterized by a dose-dependent shortening of open time accompanied by no change in t_b (11). These results, which are paralleled by the actions of purified ligand, are consistent with the selective binding of ligand to open channels. The inhibition by crude venom described here results from discrete blocking events lasting 5.23 ± 0.59 (SD) s at −20 mV (n = 4), on average, corresponding to a k_{-1} of 0.19 ± 0.02 s^{-1} (Table 1). From the values for mean open and closed times, a K_I for Cl^- channel inhibition of 0.11 mg venom dry wt/ml is obtained at −20 mV.

When 2.42 μg/ml of the purified ligand is applied to the cytoplasmic surface of a reconstituted enterocyte Cl^- channel, a reduction in P_o occurs that is similar to that induced by the crude venom (Fig. 3). Assuming that the binding of one ligand molecule is sufficient to induce channel block, we can calculate both k_{-1} and k_{+1} from mean block and mean open times, as described in the MATERIALS AND METHODS. Furthermore, from rate constants for the blocking reaction we can determine the true K_d for the ligand-channel interaction. From control records the intrinsic channel closing rate, β, was determined to be 0.016 s^{-1} and 0.015 s^{-1} at −20 and +20 mV, respectively.

Given a molecular mass of 4,070 Da from the amino acid sequence, a K_d of 1.15 μM (4.68 μg/ml) at −20 mV was calculated. When compared with the K_I for channel block by venom, this indicates that the active ligand is ~4.3% of crude venom dry weight. Given the affinity of this ligand for Cl^--selective ion channels, and its toxicity to arthropods (see below), we have elected to name it chlorotoxin.

A comparison of the blocking kinetics of an enterocyte channel at +20 mV to those at −20 mV (Fig. 3) indicates that chlorotoxin binding is voltage dependent.

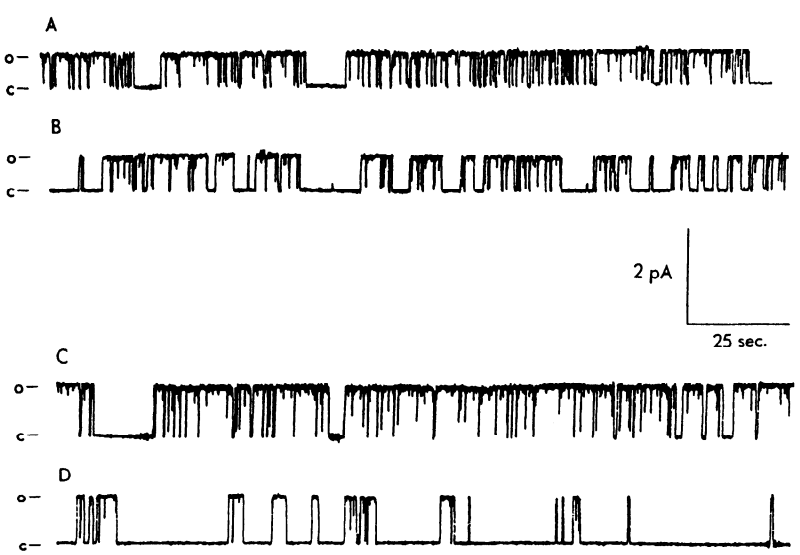


Fig. 3. Chlorotoxin block of a reconstituted Cl^- channel is shown. Current traces are from same reconstituted rat enterocyte Cl− channel before and after addition of purified chlorotoxin. As in Fig. 2, all records have been filtered at 50-Hz low pass. o, Open/unblocked current amplitude; c, closed/blocked state. Both control traces (*A*, −20 mV; *C*, +20 mV) were obtained before addition of any ligand. Scale bars represent 2 pA and 25 s. *A*: control at −20 mV; before addition of chlorotoxin (P_o = 0.80). Note that compared with Fig. 2*A*, there is considerably more intrinsic gating at −20 mV demonstrated by channel shown here. Such variations in channel behavior were found to be common. *B*: −20 mV; 7 min after addition of 594 nM chlorotoxin to the "intracellular" bath (P_o = 0.60). *C*: control at +20 mV; before adding ligand (P_o = 0.94). *D*: +20 mV; immediately after exposure to 594 nM chlorotoxin (P_o = 0.18).

Table 1. *Chloride channel blocking data for Leiurus venom and purified chlorotoxin*

	Membrane Potential, mV	t_b, s	k_{-1}, s^{-1}	k_{+1}, $s^{-1} \cdot M^{-1}$	K_I, K_d
Crude venom	−20	5.23±0.59	0.19±0.02		0.11±0.02 mg/ml
Chlorotoxin	−20	3.63±1.06	0.29±0.09	$(2.80\pm0.63)\times10^5$	1.15±0.61 μM (4.68 μg/ml)
Chlorotoxin	+20	11.85±2.76	0.09±0.02	$(5.07\pm0.84)\times10^5$	180.6±70.9 nM (0.74 μg/ml)

Values are means ± SD. Data represent a summary of kinetics of single Cl^- channel block by crude *Leiurus* venom and purified chlorotoxin. Methods of Cl^- channel reconstitution are described in MATERIALS AND METHODS, where a description of the various parameters is also given. All data are for block of single Cl^- channels held at either −20 or +20 mV membrane potential as indicated. Four individual Cl^- channels were analyzed to obtain the rate constants for block by crude venom or by chlorotoxin +20 mV, and 5 channels were analyzed for block by chlorotoxin at 20 mV (for a total of 13 channels). Two different concentrations of chlorotoxin were used, either 594 nM or 1.5 μM. Crude venom was used at 0.2 mg/ml in each case. The K_I for crude venom is given on the basis of the dry weight of whole venom used before extraction of mucous.

This voltage dependence seems to result primarily from a slowing of the dissociation rate, as evidenced by the approximate threefold decrease in k_{-1} during depolarization (0.29 ± 0.09 s^{-1} at −20 mV, n = 5; compared with 0.09 ± 0.02 s^{-1} at +20 mV, n = 4) in addition to a nearly twofold enhancement of the rate of binding (k_{+1} = 2.80 ± 0.63 × 10^5 $M^{-1} \cdot s^{-1}$ at −20 mV, n = 5; compared with 5.07 ± 0.84 × 10^5 $M^{-1} \cdot s^{-1}$ at +20 mV, n = 4). From the rates of channel block and unblock at +20 mV, a K_d of 180.6 ± 70.9 nM (0.74 μg/ml) can be determined. Thus the ligand's affinity increases more than fivefold between −20 and +20 mV membrane potential.

Open time (unblocked) and closed time (blocked) histograms for the data obtained at −20 mV are shown in Fig. 4. Both histograms could be fit by single exponential functions. The closed duration time constant (τ_b) was 2.84 s, which is close to the value for t_b (3.63 s) given in Table 1. The open duration time constant (τ_o) taken from the histogram was 3.62 s, which is close to the value of 4.92 ± 0.64 s determined for mean t_o for the three channels blocked by 594 nM chlorotoxin. Due to the long duration of the blocking events at +20 mV, we were unable to collect enough events to construct meaningful histograms at this potential.

Amino acid composition and primary structure. An analysis of the amino acid composition of the purified material was performed in triplicate using two different methods. The results (Table 2) indicate that chlorotoxin is a basic peptide of 30–35 amino acids. This conclusion is supported by Sephadex G-50 chromatography in which the active component eluted between markers microperoxidase (1,800 Da) and aprotinin (6,400 Da).

The primary amino acid sequence of the *S*-alkylated peptide was determined, and the results are shown in Fig. 5. The total yield of amino acid for the first cycle was 65% of the total mass loaded, indicating that the sequenced material was indeed the major component rather than a contaminating peptide.

The primary structure of chlorotoxin is shown in Fig. 5 along with the sequence of three other putative short insectotoxins. From the amino acid sequence a molecular mass of 4,070 Da can be determined for chlorotoxin (4,062 Da assuming four disulfide bonds).

Effects of purified chlorotoxin on arthropods. The effects of the purified ligand on crayfish were assessed as described in MATERIALS AND METHODS. Chlorotoxin at 1.23–2.23 μg/g body wt produced a loss of motor control begining at ≈20 s after injection, which progressed to a rigid paralysis of the walking and pincer legs that was complete within an additional ≈40 s. Within ≈90 s of injection, the tail musculature was immobilized. A total of four crayfish were injected this way with similar results from which no recovery was noted for 6 h, at which time the crayfish were destroyed. In four additional crayfish, chlorotoxin, at 0.5 μg/g, induced the same progressive paralysis with a slower onset, beginning at roughly 1 min postinjection. The paralysis was complete within 3–4

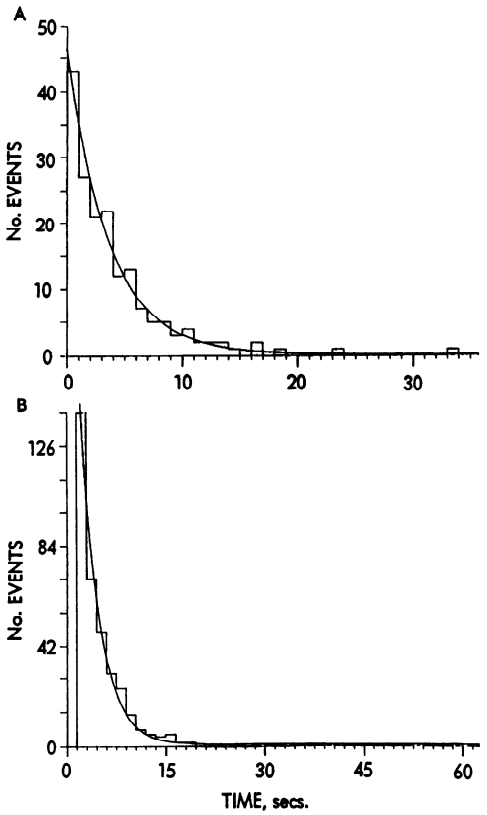


Fig. 4. Open and closed time histograms for chlorotoxin block of anion channels at −20 mV are shown. Histograms shown are probability density histograms generated by curve-fitting programs in PClamp software for the block of rat colonic enterocyte Cl^- channels by purified chlorotoxin. Membrane potential was −20 mV in each case, and a cut-off time of 1.5 s was used in the detection of channel closings/blocks, as described in MATERIALS AND METHODS. *A*: open time histogram; open/unblocked distribution for data from three channels blocked by 594 nM chlorotoxin. There were 174 total events fit from 0–35 s, yielding a τ_o of 3.62 s. *B*: closed time histogram, closed/blocked duration histogram for data from the 3 channels in *A*, plus 2 additional channels blocked by 1.55 μM chlorotoxin. There were 362 total events, and the exponential function was fit only from 1.5 to 60 s (because of the use of a cut-off time of 1.5 s) to yield a τ_b of 2.84 s.

Table 2. *Amino acid composition of chlorotoxin and other select Buthinae toxins*

Amino Acid	Mean Number of Amino Acids From Chlorotoxin Digests	Number of Amino Acids From Chlorotoxin Sequence	Mean Number of Amino Acids Reported for a Selection of Buthinae Toxins
Asx	3.03 (3)	3	9.2±0.8
Glx	2.02 (2)	2	2.9±1.6
Ser	0.03 (0)	0	3.1±1.4
Gly	4.96 (5)	5	6.2±0.9
His	1.00 (1)	1	0.9±0.8
Arg	2.82 (3)	3	2.5±1.0
Thr	1.82 (2)	2	2.0±1.0
Ala	1.01 (1)	1	3.7±1.6
Pro	1.91 (2)	2	3.4±1.4
Tyr	0.33 (0)	1	5.2±1.5
Val	0.26 (0)	0	3.3±1.5
Met	1.75 (2)	3	0
Ile	0.11 (0)	0	2.5±1.0
Leu	1.02 (1)	1	2.8±0.9
Phe	1.10 (1)	1	1.0±0.5
Lys	2.77 (3)	3	5.0±1.2

Values in *middle column* were obtained from primary sequence of chlorotoxin, and numbers in the *right column* are averages (±SD) of 19 different Buthinae toxins taken from Lazarovici and Zlotkin (29). Mean number of each amino acid was the average of 3 determinations obtained from digests of purified chlorotoxin as described in MATERIALS AND METHODS. For each individual determination, number of amino acids per molecule of chlorotoxin was calculated by dividing picomoles of each individual amino acid, recovered from digest and subsequent separation, by the average number of picomoles obtained for His, Ala, Leu, and Phe residues. Number in parentheses is the mean of the 3 determinations rounded to nearest whole number.

min. After 2 h, all crayfish had recovered from the effects of 0.5 μg/g chlorotoxin.

The injections of insects produced results similar to those observed in crayfish. Three large American cockroaches were each injected with 4.5 μg chlorotoxin. Within 30–60 s, each was immobilized with its legs contracted over its abdomen, a condition in which it remained for at least the next 2 h. After 20 h, two of the roaches had fully recovered, while the third showed a partial recovery of leg mobility. Three cockroaches injected with H_2O remained fully active throughout the ensuing 24 h.

Confirmation of chlorotoxin activity: repurification of chlorotoxin. The chlorotoxin used for all experiments described above was obtained from a two-step RP-HPLC purification protocol. This raises the possibility that the bioactivity reported for chlorotoxin may in fact be due to a contaminating peptide, present in small quantities, which copurifies with chlorotoxin. To exclude this possibility, purified chlorotoxin was repurified under different conditions to determine if it retains the bioactivity described above.

A small amount (≈500 μg) of the material from the second round of reverse-phase chromatography was subjected to yet a third round of purification using the same C_{18} column and a linear gradient of acetonitrile in 10 mM TFA (cf. Fig. 1*B*). This time not only was the central peak collected, but the small leading and trailing peaks (denoted by α and γ, respectively, in Fig. 1*B*, *inset*) were also collected. The fractions collected from several runs were pooled into three groups (α-peak, main peak, and γ-peak material), which were then taken to dryness and reconstituted in the same volume of H_2O. No inhibition was seen when the α- and γ fractions were simultaneously applied to the cytoplasmic surface of a Cl^- channel at twice the concentration that would normally be present along with the amount of chlorotoxin required to reduce P_o by >0.5. Thus these peripherally chromatographed proteins, the major contaminants of chlorotoxin, when used free of the main peak material at concentrations in excess of those that would normally contaminate purified chlorotoxin, appear to have no Cl^- channel blocking activity.

Two crayfish were used to test for toxic effects of the α- and γ-peak material. When a volume of each of the two fractions, equivalent to twice the volume of chlorotoxin solution subsequently to be used to induce paralysis, was injected (both fractions simultaneously) into the crayfish, no effects were noted for 10 min. The subsequent injection of exactly one-half this volume of chlorotoxin (to 0.77 μg/g) induced the paralysis described above in both crayfish.

Chlorotoxin was also repurified using the C_{18} RP-HPLC column described above except that HFBA was substituted for TFA as the ion pair reagent (Fig. 1*C*). No additional major peaks were observed, a result which confirms the near homogeneity of chlorotoxin. The main peak material (asterisk in Fig. 1*C*) was collected, vacuum centrifuged to dryness, and reconstituted as a concentrated stock solution for use in further testing. When applied to the cytoplasmic surface of a reconstituted Cl^- channel, this material proved to be fully active in inducing the prolonged blocks described for chlorotoxin (cf. Fig. 3). HFBA-purified chlorotoxin was also examined for arthropod toxicity. When injected into two crayfish at ≈1 μg/g, this material produced a progressive paralysis with

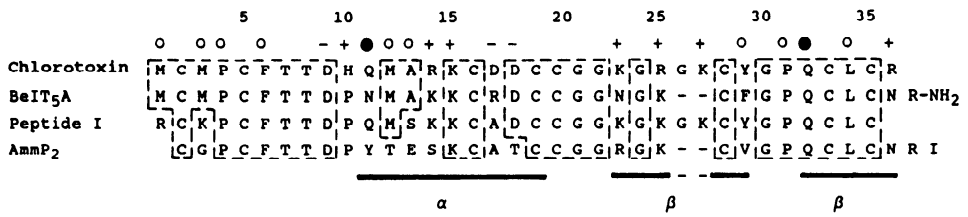


Fig. 5. Comparison of primary amino acid sequence of chlorotoxin with those of 3 small insectotoxins is shown. Primary sequence of chlorotoxin has been aligned with 3 small insectotoxins by matching cysteine residues. NH_2-terminus is on *left* in each case. Amino acids common to all 4 peptides are contained in boxed areas. Above the 4 sequences, positively charged amino acids are denoted by +, negatively charged amino acids by −, polar uncharged amino acids by closed circle, and apolar amino acids by open circle. Below the 4 sequences, extent of hypothesized α- and β-conformations of $BeIT_5A$ are indicated by thick lines. See DISCUSSION for further details. Peptide I is from *Buthus sindicus* (18), $BeIT_5A$ is from *Buthus eupeus* (1), and $AmmP_2$ is from *Androctonus mauretanicus* (35, 41).

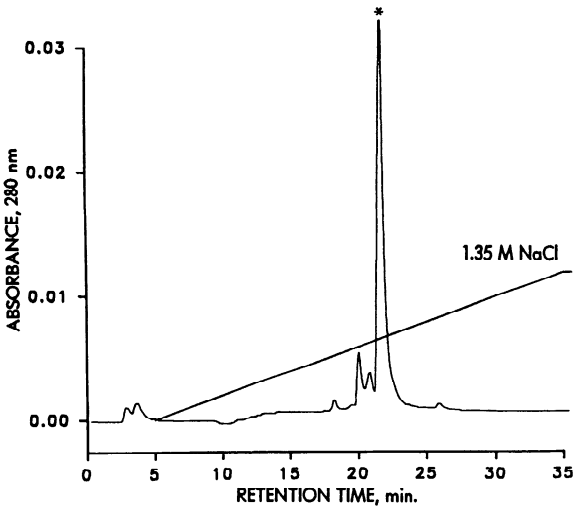


Fig. 6. Ion-exchange HPLC of purified chlorotoxin is shown. Chlorotoxin purified via RP-HPLC was repurified using a polysulfoethyl aspartamide ion-exchange column, and resulting chromatogram is depicted here. A linear gradient of NaCl (0–1.35 M, in 10 mM phosphate and 10% acetonitrile) was used to elute peptides. Injection of 60 μg purified chlorotoxin took place at *time 0*, and gradient was begun at 5 min postinjection. Peak containing active component is indicated by an asterisk. Flow rate was constant at 1 ml/min. Note that unlike chromatograms depicted in Fig. 1, absorbance was monitored at 280 nm.

the same time course described above for chlorotoxin used at 1.23 μg/g.

As a final test of the purity of chlorotoxin, 250 μg were rechromatographed using ion-exchange HPLC as described in MATERIALS AND METHODS. The resulting chromatogram is shown in Fig. 6, and again there is a single major peak (denoted by asterisk, representing chlorotoxin) surrounded by a few smaller satellite peaks. The major peak material was collected, reconstituted, and assayed for activity against Cl^- channels and crayfish. The application of this material to the cytoplasmic surface of a Cl^- channel to 2.5 μg/ml final concentration resulted in a reduction of P_o by >50%. Thus chlorotoxin retains its Cl^- channel blocking activity subsequent to repurification using ion-exchange HPLC. At 0.6 μg/g, this material was also effective in paralyzing two crayfish within 90 s of injection.

DISCUSSION

There are several properties of chlorotoxin that are typical of other Buthinae toxins (35, 41). The molecule is small and basic, being highly cationic at physiological pH. Charges are often paired in the primary sequence, as with Asp^9-His^{10}, Arg^{14}-Lys^{15}, and Asp^{17}-Asp^{18}. All the long Buthinae toxins are extensively cross-linked by disulfide bonds, with most containing four such links. The eight cysteine residues of chlorotoxin suggest the presence of four disulfide bonds that would make this molecule especially compact. Despite these similarities, the high methionine and glycine content, and the absence of serine, isoleucine, and valine, make chlorotoxin unique compared with previously described long neurotoxins (Table 2).

The primary structure does show considerable homology with the putative short insectotoxins (18, 35, 40). When cysteine residues are aligned as in Fig. 5, identical amino acids occur at 26 of 36 positions (72%) in chlorotoxin when compared with $BeIT_5A$ (from *Buthus epeus*; Ref. 1). Four additional positions show conservative replacement between chlorotoxin and $BeIT_5A$, resulting in 81% homology. Sequence identity between chlorotoxin and peptide I (from *Buthus sindicus*; Ref. 18) is even greater at 81%. Relative to $BeIT_5A$, major conserved sequences are Met^1-Asp^9, Asp^{18}-Gly^{22}, and Gly^{30}-Cys^{35}. We remark on this homology because $BeIT_5A$ is the only short insectotoxin whose secondary structure has been studied. Using high-resolution two-dimensional nuclear magnetic resonance, Arseniev et al. (1) measured interproton distances in this peptide in acidic aqueous solutions (pH 2.9 and 5.5) and subsequently proposed a three-dimensional conformation. They argued that a right-hand α-helix extended from Asn^{11} to Cys^{19} and that antiparallel β-structures occupied Asn^{23}-Phe^{27} and Gln^{30}-Asn^{34} (19). These regions in $BeIT_5A$ are indicated by the thick lines below the sequences in Fig. 5. Assuming an analogous secondary structure, the highly charged region of chlorotoxin would occur adjacent to and within the α-helical conformation. The remaining three positive residues occur in the next β-structure (Lys^{23}-Tyr^{29}).

Although experimental evidence is lacking, it has been proposed that the short insectotoxins might act on the "nerve system of insects" (33) at the level of the "glutamate receptor of the postsynaptic membrane" (1). However, other investigators believe that the small insectotoxin-like peptides found in several Buthinae venoms are not part of these scorpions' natural secretions but are found only in venom collected by electrical stimulation (36). Manual stimulation, that is stimulation by physically perturbing the scorpion, fails to elicit small insectotoxins. Furthermore, it has been reported that $AmmP_2$, a short insectotoxin, looses its toxicity to arthropods following treatment with antibodies raised against the long insect toxin of *Androctonus australis Hector* (36).

The venom used to isolate chlorotoxin was obtained by electrical stimulation of scorpion poison glands; we have not attempted to isolate chlorotoxin from manually elicited venom. Given that a few of the long insect toxins are effective against arthropods at concentrations as low as 10 ng/g body wt (12, 13), and that we have employed upwards of 500 ng chlorotoxin/g in our tests on live animals, it is conceivable that a contamination could explain the toxicity or Cl^- channel blocking activity of chlorotoxin. However, we consider this unlikely for the following reasons. First, the α- and γ-peak material, the major contaminants of chlorotoxin, when used at concentrations equal to or in excess of those that would normally contaminate chlorotoxin, were completely devoid of activity against either arthropods or reconstituted Cl^- channels. By switching to HFBA as the ion pair reagent for HPLC, no additional peaks were resolved, while the chlorotoxin peak was demonstrated to retain both Cl^- channel blocking activity and toxicity to arthropods. Moreover, after repurification using ion-exchange HPLC, chlorotoxin retains its channel blocking activity and its toxicity. Thus, if either of these two activities of chlorotoxin are in fact due to a contaminating peptide, this peptide would have to copurify with chlorotoxin under

three different HPLC conditions. Finally, in light of our previously published lack of effect of two purified α-toxins, or of charybdotoxin (the most likely types of contaminating peptides derived from *Leiurus* venom) on reconstituted Cl^- channels (11), we consider it unlikely that the Cl^- channel inhibition we see is due to a contaminant.

The Cl^- channels we describe here belong to the large group of neurotransmitter-insensitive "leak" channels found in a wide variety of cell types (2, 5, 9, 20, 23). In recent years these Cl^- channels have become the focus of intense interest because they have been shown to be the site of the defect in the common inherited disease cystic fibrosis (22, 30). The colonic enterocyte Cl^- channels also share several features with both the γ-aminobutyric acid (GABA)/glycine-activated channels prominent in the vertebrate central nervous system, and the extrajunctional glutamate/GABA activated Cl^- channels seen in both insect and crayfish muscle (16, 21). Apart from their selectivity for anions, all of these channels are of relatively small conductance ($\approx$25–75 pS), and at least the vertebrate GABA channels (3) and the colonic epithelium Cl^- channels (34) follow halide selectivity sequence I of Wright and Diamond (39): $I > Br > Cl > F$.

During our studies of Cl^- channel block by crude venom and purified chlorotoxin, we have consistently seen effects only with application to what appears to be the cytoplasmic-facing surface of neuronal growth cone or colonic enterocyte Cl^- channels (11). This intracellular site of action is difficult to reconcile with the observed toxicity of chlorotoxin to insects. However, it is unlikely that epithelial Cl^- channels are the natural target for chlorotoxin. The paralysis resulting from chlorotoxin injection into arthropods may be the result of the inhibition of some other structurally related anion channel such as the extrajunctional channels of arthropod muscle, noted above. Chlorotoxin may be effective when applied to the extracellular surface of these channels.

Studies of the small-conductance epithelial Cl^- channels have been hindered by the lack of adequate ligands. The classical anion transport inhibitors 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid, 4-acetamido-4′-isothiocyano-2,2′-disulfonic acid, and DNDS have been used but are effective only in the low micromolar range, at best. Additionally, the specificity of these inhibitors has been called into question (24, 26). Newer and more potent drugs, 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) (15, 23, 38) and 94-indanyloxyacetic acid (27, 28), have been developed, and the latter has even been used to purify a putative Cl^- channel. Unfortunately, both drugs induce a rapid channel block, and both are highly lipophilic, two features which limit their usefulness.

Chlorotoxin is the first reported high-affinity peptide ligand for Cl^- channels. The model we have chosen to describe chlorotoxin binding to anion channels is supported by our data. We have previously demonstrated that the block induced by crude *Leiurus* venom follows a first-order kinetic scheme (11). The open and closed duration histograms for chlorotoxin block of anion channels can also be fit by single exponential functions (Fig. 4), suggesting a first-order binding reaction. However, alternative kinetic schemes are not ruled out by our data. The model we present does allow us to estimate the affinity of chlorotoxin for Cl^- channels. In comparison with previously described ligands, chlorotoxin represents a significant advance in being water soluble and in producing channel inhibition as a result of what appear to be individual blocking events lasting 11–12 s, on average, at +20 mV and room temperature. With these properties, chlorotoxin holds promise for use in Cl^- channel purification or as a biophysical probe of channel structure.

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Address for reprint requests: G. R. Strichartz, Anesthesia Research Laboratories, Brigham and Women's Hospital, 75 Francis St., Boston, MA 02215.

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