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Chlorotoxin does not inhibit volume-regulated, calcium-activated and cyclic AMP-activated chloride channels

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1 It was the aim of this study to look for a high-affinity and selective polypeptide toxin, which could serve as a probe for the volume-regulated anion channel (VRAC) or the calcium-activated chloride channel (CaCC). We have partially purified chlorotoxin, including new and homologous short chain insectotoxins, from the crude venom of *Leiurus quinquestriatus quinquestriatus (Lqq)* by means of gel filtration chromatography. Material eluting between 280 and 420 min, corresponding to fractions 15–21, was lyophilized and tested on VRAC and CaCC, using the whole-cell patch-clamp technique. We have also tested the commercially available chlorotoxin on VRAC, CaCC, the cystic fibrosis transmembrane conductance regulator (CFTR) and on the glioma specific chloride channel (GCC).

2 VRAC and the correspondent current, $I_{Cl,swell}$, was activated in Cultured Pulmonary Artery Endothelial (CPAE) cells by a 25% hypotonic solution. Neither of the fractions 16–21 significantly inhibited $I_{Cl,swell}$ ($n=4-5$). Ca^{2+}-activated Cl^- currents, $I_{Cl,Ca}$, activated by loading T84 cells *via* the patch pipette with 1 μM free Ca^{2+}, were not inhibited by any of the tested fractions (15–21), ($n=2-5$).

3 Chlorotoxin (625 nM) did neither effect $I_{Cl,swell}$ nor $I_{Cl,Ca}$ ($n=4-5$). The CFTR channel, transiently transfected in COS cells and activated by a cocktail containing IBMX and forskolin, was not affected by 1.2 μM chlorotoxin ($n=5$). In addition, it did not affect currents through GCC.

4 We conclude that submicromolar concentrations of chlorotoxin do not block volume-regulated, Ca^{2+}-activated and CFTR chloride channels and that it can not be classified as a general chloride channel toxin.

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Abbreviations: CaCC, calcium-activated chloride channel; CFTR, cystic fibrosis transmembrane conductance regulator; ClTx, Chlorotoxin; CPAE, cultured pulmonary artery endothelial cells; DCDPC, 3′,5-dichlorodiphenylamine-2-carboxylic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid; EGTA, ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N,′,N′,-tetraacetic acid; FLX, fluoxetine; GLB, glibenclamide; GCC, glioma-specific chloride channel; GFP, green fluorescent protein; HEPES, N-(hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid); HTS, hypotonic solution; IBMX, 3-isobutyl-1-methylxanthine; $I_{Cl,Ca}$, Ca^{2+}-activated Cl^- current; $I_{Cl,cAMP}$, cAMP-activated chloride current through CFTR; $I_{Cl,swell}$, swelling activated chloride current; I_{GCC}, glioma specific chloride current ISO, isotonic solution; *Lqq, Leiurus quinquestriatus quinquestriatus*; NA, niflumic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid; TEA, tetraethylammonium; TEACl, tetraethylammoniumchloride; TRIS, Tris(hydroxymethyl)aminomethane; VRAC, volume-regulated anion channel; WT, wild type

Introduction

Anion channels play important roles in different aspects of cell physiology, including cell volume regulation, transepithelial transport and regulation of membrane potential (Jentsch & Günther, 1997). Our insights in the structure and function of anion channels have strongly grown during the last 10 years with the molecular identification of the first anion channel families and the discovery that a number of diseases result from anion channel defects (Jentsch & Günther, 1997). However, the molecular identity of two important functional classes, *sc.* the volume-regulated anion channel, VRAC, and the calcium-activated chloride channel, CaCC, is still unknown or is not yet unambiguously established. One of the cloning strategies that has proven its usefulness for the identification of several ion channel molecules, is the biochemical purification *via* specific high-affinity ligands. Unfortunately, except for ligand-gated anion channels, no high-affinity ligands for chloride channels are known.

It has previously been reported that the crude venom extracted from the scorpion *Leiurus quinquestriatus quinquestriatus (Lqq)* inhibits reconstituted small-conductance chloride channels isolated from rat epithelia and embryonic rat brain (DeBin & Strichartz, 1991). Chlorotoxin (ClTx), the active compound of this venom, has been purified and characterized as a 4.1 kDa basic peptide with considerable sequence homology to the class of small insectotoxins (DeBin *et al*., 1993; Tytgat *et al*., 1998). Recent studies have shown the existence of a glioma-specific chloride channel (GCC) in human astrocytoma cells that is sensitive to ClTx (~80% block at 600 nM) (Ullrich *et al*., 1998, 1995; Ullrich & Sontheimer, 1996; 1997). The data suggest that ClTx and ClTx-conjugated molecules may serve as glioma-specific markers with diagnostic and therapeutic potential (Soroceanu *et al*., 1998). As a consequence ClTx,

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which is also commercially available, is being used more and more frequently as a general chloride channel blocker.

Because, highly selective Cl^- channel blockers are still missing, the advent of a toxin which specifically binds to Cl^- channels would be of outstanding importance. It was therefore the purpose of the present experiments first, to purify chlorotoxin including new and homologous short chain insectotoxins, starting from the crude venom of *Lqq*, and second, to test the purified venom fractions on different well characterized Cl^- channels. Our aim was to find a high-affinity and selective polypeptide toxin, which could serve as a 'pharmacological' tool to probe especially the volume-regulated anion (VRAC) and/or the calcium-activated chloride channel (CaCC). We also focused on the commercially available ClTx, which was tested on VRAC, CaCC, the cystic fibrosis transmembrane conductance regulator (CFTR) and on the glioma-specific chloride channel.

Methods

Venom purification

Ten milligram of crude venom (ICN, USA) from *Lqq* was first lyophilized and then dissolved in 100 mM ammonium acetate (Merck, Germany), pH 7. After vortexing, the sample was clarified by centrifugation at 14,000 r.p.m for 15 min and its supernatant was then applied onto a 'Superdex 30 prep grade HiLoad 16/60' FPLC column (Pharmacia, Sweden) equilibrated with 100 mM ammonium acetate, pH 7. The material eluted with the same buffer at a flow rate of 0.2 ml min^{-1}. Absorbance of the eluate was monitored at 280 nm. Fractions were collected automatically and each fraction contained 4 ml from the eluate including potentially bioactive material (Figure 1A).

Commercially purchased chlorotoxin (Latoxan, France) was checked for purity and indicated concentration by means of HPLC. The lyophilized toxin was dissolved in 50 mM phosphate buffer, pH 6.8, and applied to a 'Mono S PC 1.6/5 cation exchange column' (Pharmacia, Sweden) equilibrated with 50 mM phosphate buffer, pH 6.8. A linear gradient of 0–100% 1 M NaCl (supplemented with 50 mM phosphate buffer, pH 6.8) was applied at a flow rate of 0.1 ml min^{-1}. The absorbance was simultaneously measured at 214, 254 and 280 nm (Figure 1B). Loading of 3 μg gave an absorbance of 0.035 as measured at 280 nm. Based on the law of Lambert-Beer, this absorbance is in accordance with the indicated amount of the commercially purchased chlorotoxin.

Gel filtration column calibration

Characterization of solute behaviour on our column was determined by $K_{av}=(V_e-V_o)\ /\ (V_t-V_o)$, with K_{av} the coefficient defining the proportions of pores occupied by the solutes, V_e the elution volume, V_o the void volume and V_t the total packed bed volume. For calibration, five standards were used (four obtained from Sigma, U.S.A.): cytochrome c (horse heart, 12,400 Da), aprotinin (bovine lung, 6500 Da), insulin chain B oxidized (bovine, 4050 Da), apamin (*Apis mellifera*, 2027 Da, Alomone Labs., Israel), and leucine-enkephalin (555 Da). The constructed calibration curve was used to derive the molecular weight of the bioactive material present in the different gel filtration fractions based on the respective K_{av}-values. Material eluting between 280 and 420 min was lyophilized for screening Cl^- channels and corresponds with fraction numbers 15–21 (20 min per fraction). Based on our calibration curve, fractions 16 and 17 contain material with an average molecular weight between 3500–7000 Da (Figure 1A). Therefore, we assumed that ClTx (and possible homologues of ClTx) was primarily present in fractions 16 and 17. Since the material in fractions 15–21 was not purified to homogeneity after this single gel filtration run and peaks clearly overlapped, we have screened the individual fractions on VRAC and CaCC, to ascertain that all potential peptidyl toxins in the range of 3500–7000 Da have been tested (see Table 1).

Vector construction

We used the pCINeo/IRES-GFP plasmid (Trouet *et al*., 1997) for expressing WT CFTR in COS cells. For insertion of WT CFTR the green fluorescent protein (GFP)-vector was cut with *Eco*RI, dephosphorylated and thereupon blunt-ended with T4 DNA-polymerase. The WT CFTR cDNA was obtained from a pcDNA/CFTR plasmid through *Sac*I digestion. The obtained

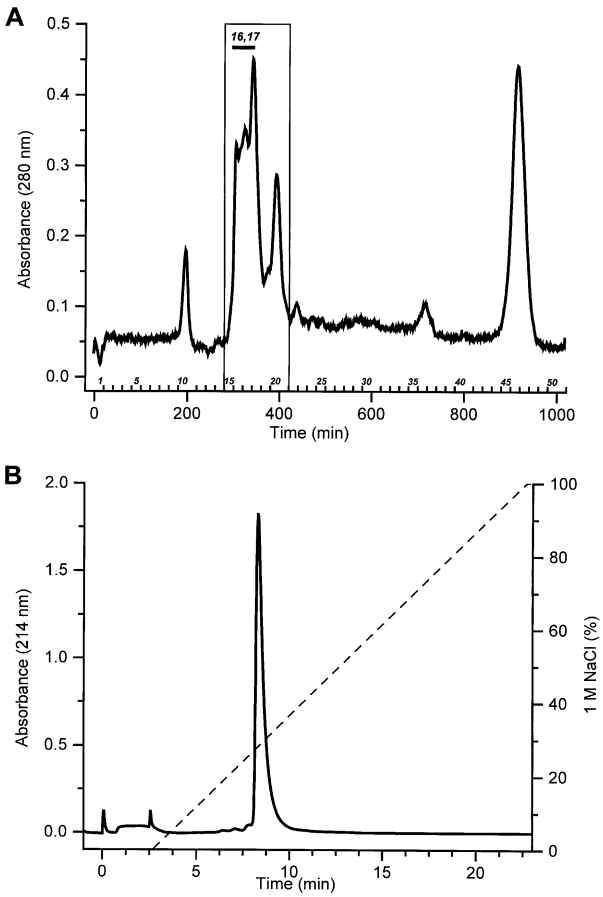

Figure 1 (A) Purification of ClTx and homologous short chain insectotoxins from *Lqq* venom. Crude venom was fractionated by FPLC gel filtration. Absorbance of the eluate was monitored at 280 nm and 4 ml fractions were collected automatically. Material eluting between 280 and 420 min, corresponding to fractions 15–21 (hollow rectangle), was lyophilized and tested on VRAC and CaCC. Based on a constructed gel filtration calibration curve, fractions 16 and 17 contain material with an average molecular weight between 3500–7000 Da. ClTx is assumed to be primarily present in fractions 16 and 17. (B) Commercially purchased chlorotoxin was checked for purity and indicated concentration by means of HPLC. A linear gradient of 0–100% 1 M NaCl was applied. The absorbance was simultaneously measured at 214, 254 and 280 nm (only shown for 214 nm).

Table 1 Currents (at +100 mV) expressed as percentage of the control current (for the toxins: measured after 3 min)

	VRAC		*CaCC*		*CFTR*	
	n	*% Current*	n	*% Current*	n	*% Current*
Fractions lqq						
15	–	–	4	95.6±3.6	–	–
16	4	105.3±1.4	5	95.2±2.3	–	–
17	5	96.6±8.7	5	94.4±6.4	–	–
18	5	98.3±2.5	5	103.5±9.9	–	–
19	5	100.8±1.2	4	108.9±16.3	–	–
20	5	100.3±10.1	4	97.0±5.8	–	–
21	5	100.3±4.9	2	94.1	–	–
Chlorotoxin						
625 nM	4	106.4±2.2	5	98.7±9.5	–	–
1200 nM	–	–	–	–	5	99.9±8.1
FLX 50 μM*	7	6.3±1.5	–	–	–	–
NA 100 μM	–	–	3	17.9±3.9	–	–
GLB 50 μM	–	–	–	–	3	21.2±2.0

*Maertens *et al.* (1999)

fragment was blunt-ended using T4 DNA-polymerase. Ligation was performed using standard procedures.

Cell culture and transfection

$I_{Cl,swell}$ (i.e. the current through VRAC) was investigated in cells from a cultured bovine pulmonary artery endothelial (CPAE) cell line (American Type Culture Collection, CCL 209) grown in DMEM medium, $I_{Cl,Ca}$ (i.e. the current through CaCC) in cells from a human colon carcinoma (T84) cell line (American Type Culture Collection, CCL 248) grown in DMEM/HamF12 medium, and I_{GCC} (i.e. the current through GCC) in cells from an established human astrocytoma cell line, CCF-STTG1 (American Type Culture Collection, CRL-1718) grown in RPMI 1640 medium. All media contained 10% (except for CPAE cells 20%) foetal calf serum, 2 mM L-glutamine, 2 U ml^{-1} penicillin and 2 mg ml^{-1} streptomycin. Cultures were maintained at 37°C in a fully humidified atmosphere of 5 (CPAE) or 10% (T84CCF-STTG1) CO_2 in air. Cells were detached by exposure to 0.05% trypsin in a Ca^{2+}- and Mg^{2+}-free solution, reseeded on gelatine coated cover slips, and kept in culture for 2–4 days before use. For electrophysiological experiments, only non-confluent single cells were used.

$I_{Cl,cAMP}$ (i.e. the current through CFTR) was investigated in COS cells transiently transfected with WT CFTR in the pCINeo/IRES-GFP vector (Trouet *et al.*, 1997), by means of electroporation. In short, $1.5-3\times10^7$ COS cells were transfected with 20 μg of the pCINeo/IRES-GFP-CFTR constructs and, 30 h later, seeded on gelatine coated cover slips in DMEM/HamF12 medium containing 10% foetal calf serum, 2 mM L-glutamine, 2 U ml^{-1} penicillin and 2 mg ml^{-1} streptomycin. Cultures were maintained at 37°C in a fully humidified atmosphere of 5% CO_2 in air. Electrophysiological measurements were done during 2–3 days after transfection. Incorporation of WT CFTR in the bicistronic unit allows coupled expression of the channels and GFP. Transfected cells, positive for GFP, could be identified in the patch-clamp set up. GFP was excited at a wavelength between 450 and 490 nm and the emitted light was passed through a 520 nm long pass filter.

Solutions and drugs

Except for the STTG1 cells, the standard extracellular solution was a modified Krebs solution containing (in mM): NaCl 150, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, N-(hydroxyethyl)piperazine-N′-(2-ethanesulphonic acid) (HEPES) 10, titrated with NaOH to pH 7.4. The osmolarity, as measured with a vapour pressure osmometer (Wescor 5500, Schlag, Gladbach, Germany), was 320±5 mOsm.

$I_{Cl,swell}$ At the beginning of the patch-clamp recording, the Krebs solution was replaced by an isotonic-Cs^+ solution (ISO, 320±5 mOsm) containing (in mM): NaCl 105, CsCl 6, $CaCl_2$ 1.5, $MgCl_2$ 1, D-mannitol 90, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH. Volume-sensitive Cl^- currents were activated by exposing the cells to a 25% hypotonic extracellular solution (HTS, 240±5 mOsm), containing (in mM): NaCl 105, CsCl 6, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 10, HEPES 10, adjusted to pH 7.4 with NaOH.

The standard pipette solution contained (in mM): CsCl 40, Cs-aspartate 100, $MgCl_2$ 1, $CaCl_2$ 1.93, ethylene glycol-O,O′-bis(2-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA) 5, Na_2ATP 4, HEPES 10, adjusted to pH 7.2 with CsOH (290±5 mOsm). The presence of Cs^+ in extra- and intracellular solutions blocked the inwardly rectifying K^+ currents, which are present in CPAE cells (Voets *et al.*, 1996). To suppress $I_{Cl,Ca}$, the free Ca^{2+} concentration in the pipette solution was buffered at 100 nM, which is below the threshold for activation of this current (Nilius *et al.*, 1997a), but which is sufficient for full activation of $I_{Cl,swell}$ during cell swelling in CPAE cells (Szucs *et al.*, 1996).

$I_{Cl,Ca}$ Krebs solution was replaced by a slightly hypertonic Krebs-Cs^+ solution (345±5 mOsm) containing (in mM): NaCl 150, CsCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, D-mannitol 25, HEPES 10, titrated with NaOH to pH 7.4. The slightly increased osmolarity prevented co-activation of $I_{Cl,swell}$. $I_{Cl,Ca}$, was activated by loading T84 cells *via* the patch pipette with 1000 nM free Ca^{2+} (calculated by the program CaBuf, G. Droogmans) as described previously (Nilius *et al.*, 1997a,b). The standard pipette solution contained (in mM): CsCl 40, Cs-aspartate 100, $MgCl_2$ 1, $CaCl_2$ 4.33, EGTA 5, Na_2ATP 4, HEPES 10, adjusted to pH 7.2 with CsOH (290±5 mOsm).

$I_{Cl,cAMP}$ To eliminate K^+ currents, the Krebs solution was replaced by a Krebs-Cs^+ solution (320±5 mOsm) containing (in mM): NaCl 150, CsCl 6, $MgCl_2$ 1, $CaCl_2$ 1.5, glucose 10, HEPES 10, titrated with NaOH to pH 7.4. The CFTR-channel was activated by a cocktail containing 100 μM 3-isobutyl-1-methylxanthine (IBMX) and 1 μM forskolin dissolved in the

Krebs-Cs^+ solution. The standard pipette solution contained (in mM): CsCl 40, Cs-aspartate 100, $MgCl_2$ 1, $CaCl_2$ 1.93, EGTA 5, Na_2ATP 4, HEPES 10, adjusted to pH 7.2 with CsOH (290±5 mOsm) (Wei *et al*., 1998).

I_{GCC} The standard bath solution contained (in mM): NaCl 125, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.0, Na_2HPO_4 1.6, NaH_2PO_4 0.4, HEPES 32.5, glucose 10.5 adjusted to pH 7.4 with NaOH. The osmolarity was 310±5 mOsm. To evaluate the effect of external Cl^-, the external Cl^- concentration was reduced to 10%, by replacing 121 mM NaCl with 121 mM Na-Aspartate in the standard bath solution ('low Cl^-' solution). In the external TEA (tetraethylammonium) solution, 10 mM NaCl was substituted by 10 mM TEACl to maintain the same osmolarity. To evaluate the effect of external K^+, all NaCl in the standard bath solution was replaced by KCl ('high K^+' solution). The standard pipette solution contained (in mM): KCl 145, $MgCl_2$ 1, $CaCl_2$ 0.2, EGTA 10, HEPES 10 adjusted to pH 7.4 with Tris(hydroxymethyl)aminomethane (TRIS). The osmolarity was 290±5 mOsm. The TEA pipette solution contained (in mM): TEACl 145, $MgCl_2$ 1, $CaCl_2$ 0.2, EGTA 10, HEPES 10 adjusted to pH 7.4 with TRIS.

ClTx was purchased from Latoxan (France) and the crude venom of *Lqq* from ICN (U.S.A.). Fluoxetine (FLX), glibenclamide (GLB), niflumic acid (NA), 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS), IBMX and forskolin were purchased from Sigma-Aldrich. TEA was from Fluka. 3′,5-dichlorodiphenylamine-2-carboxylic acid (DCDPC) was kindly provided by Dr H.J. Lang, HMR, Frankfurt, Germany. All the drugs were kept in stock solutions (DMSO), the toxins were dissolved directly in the bath solution.

Rapid solution exchange and extracellular application of drugs (except for the toxins, FLX and NA) occurred *via* a multi-barrelled pipette connected to solution reservoirs, and was controlled by a set of magnetic valves. The toxins were applied from an additional reservoir (1 ml syringe) connected to a small tube fixed onto the multi-barrelled pipette after stopping the above described solution exchange system. This delivery system was tested by applying FLX and niflumic acid *via* this system.

Current measurements and data analysis

Whole-cell membrane currents were measured in ruptured patches. All experiments were performed at room temperature (20–23°C). Currents were monitored with an EPC-7 patch clamp amplifier (List Electronic, Germany). Patch electrodes had a resistance between 3 and 5 MΩ. An Ag-AgCl wire was used as reference electrode. In Cl^- substitution experiments, an agar bridge was used.

The current voltage relation and time course of $I_{Cl,swell}$ and $I_{Cl,Ca}$ were obtained from a 'ramp' protocol, which consisted of: a step to −80 mV for 0.4 s, followed by a step to −150 mV for 0.1 s and a 1.3 s linear voltage ramp to +100 mV. This voltage protocol was repeated every 15 s from a holding potential of −20 mV. Currents were sampled at 2 ms intervals (1024 points per record, filtered at 200 Hz). Current-voltage relations were constructed from the ramp current, and time courses were obtained by averaging the current in a small voltage window around +100 and −150 mV. For $I_{Cl,Ca}$ we also used a protocol consisting of 1.5 s (or 1 s) voltage steps, applied every 10 s from a holding potential of −20 mV (or −50 mV) to test potentials from −100 to +100 mV with increments of 20 mV. Currents were sampled at 1 ms intervals and filtered at 200 Hz.

The I-V curves of $I_{Cl,cAMP}$ were obtained from 400 ms linear voltage ramps from −100 to +100 mV, repeated every 10 s from a holding potential of −20 mV. Currents were sampled at 0.5 ms intervals (1024 points per record, filtered at 5 kHz). The time course was obtained by averaging the current in a small voltage window around +100 and −100 mV.

For I_{GCC} we applied a step protocol similar to the 'original' (Ullrich & Sontheimer, 1996), consisting of 25 ms voltage steps, applied repetitively from a holding potential of 0 mV to test potentials from −105 to 195 mV with increments of 25 mV. Currents were sampled at 0.1 ms intervals (512 points per record, filtered at 1 kHz). The reversal potential was determined from tail currents recorded during the following protocol applied every 2 s: cells were held at 0 mV and pulsed to 180 mV for 25 ms (sampling interval 0.05 ms) followed by a 12.5 ms step to various voltages ranging from +120 to −120 mV in −20 mV increments. The amplitudes of the tail currents (sampled at 0.02 ms) were determined as the difference between the peak current measured between 1 and 1.5 ms from the beginning of the step and the current at the end of the voltage step. The reversal potential was obtained from the plot of these leak subtracted currents as a function of voltage.

Data were analysed in Winascd (G. Droogmans) and in Origin (MicroCal Software, Inc.). Pooled data are given as the mean±s.e.mean.

Results

Purified venom of Lqq *does not affect* $I_{Cl,swell}$ *or* $I_{Cl,Ca}$

Volume-activated chloride channels and the corresponding current, $I_{Cl,swell}$, were activated in CPAE cells by replacing the isotonic solution (ISO) with the hypotonic solution (HTS), as described in detail elsewhere (Nilius *et al*., 1994). Figure 2A shows a typical time course experiment for VRAC in which 50 μM FLX (as a positive control) was added to the external solution during a maintained superfusion with HTS. Current-voltage relations (Figure 2B) are obtained from voltage ramps before and during application of the drug. Application of 50 μM FLX induced a fast and reversible block (see Table 1) consistent with former experimental findings (Maertens *et al*., 1999). The current decays after returning to isotonic solution indicating that it is indeed activated by cell swelling. Figure 2C shows an analogous experiment, in which fraction 17 was applied, under exactly the same conditions and delivery system as for FLX. Fraction 17 did not significantly affect $I_{Cl,swell}$, ($n=5$). Figure 2D shows the corresponding current-voltage relations. Fractions 16, 18, 19, 20 and 21 were tested in analogous experiments ($n=4-5$). The results are summarized in Table 1. Neither of the six tested fractions caused significant inhibition of $I_{Cl,swell}$. In addition to this extracellular application, we also applied fraction 16 ($n=2$) and fraction 17 ($n=2$) which contain chlorotoxin, intracellularly *via* the patch pipette. The activation pattern and size of $I_{Cl,swell}$ activated with HTS after equilibration of the cell with this pipette solution did not differ from those observed under control conditions. Once fully activated, no significant run-down was observed during 15–20 min (data not shown).

Ca^{2+}-activated Cl^- currents, $I_{Cl,Ca}$, differ in many aspects from $I_{Cl,swell}$. The outward rectification of $I_{Cl,Ca}$ is much more

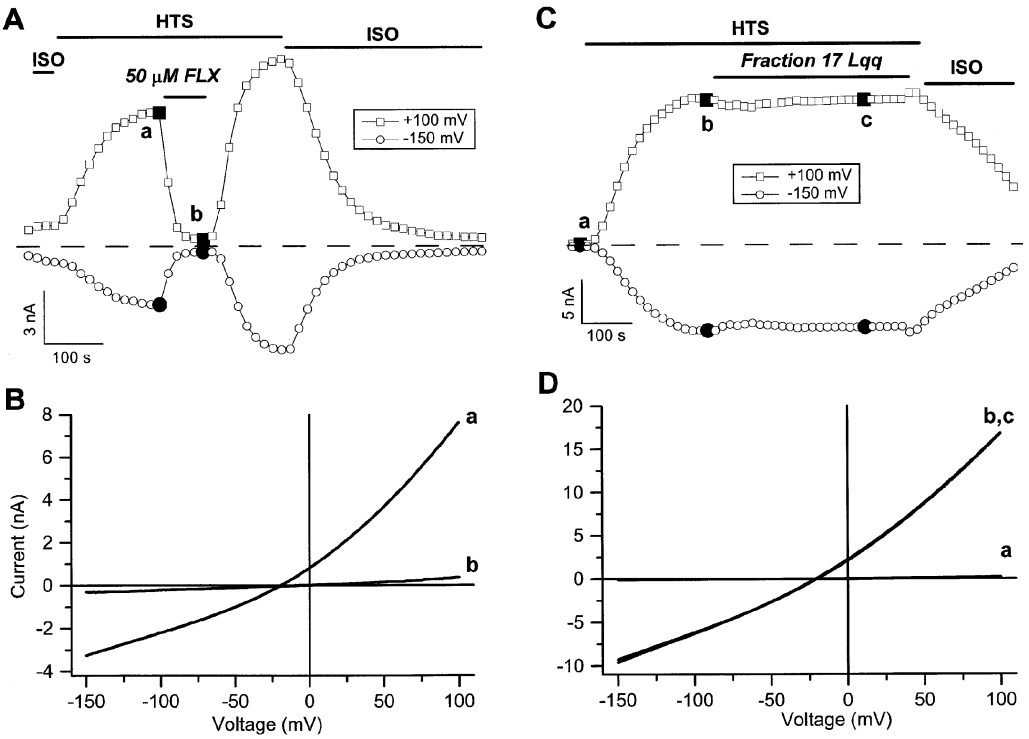

Figure 2 (A) Time course of activation of $I_{Cl,swell}$ during superfusion with hypotonic solution (HTS) (following superfusion with Krebs solution and isotonic-Cs^+ solution (ISO)), reversible inhibition of the current by 50 μM FLX and deactivation of the current after returning to isotonic-Cs^+ solution. The dashed line indicates zero current. Data were obtained from ramp protocols by averaging the current in small voltage windows around +100 and −150 mV. (B) Current-voltage relations obtained from voltage ramps at the times indicated in (A). (C) Time course of activation of $I_{Cl,swell}$ as described in (A) and application of fraction 17 through the same delivery system as FLX. The experiment was stopped before the current deactivation was completed. (D) Current-voltage relations obtained from voltage ramps at the times indicated in (C).

pronounced than that of $I_{Cl,swell}$ and the kinetic behaviour of both currents is completely different. (Nilius *et al*., 1997a,b; Pedersen *et al*., 1998). Typical Ca^{2+}-activated Cl^- currents were activated by loading T84 cells *via* the patch pipette with 1 μM free Ca^{2+} as described previously (Nilius *et al*., 1997b). In these cells, activation of $I_{Cl,Ca}$ was transient. After a peak, a stable plateau was reached. To avoid misinterpretations of the inhibitory effect due to the transient nature of current activation, we consciously added the various inhibitors and toxins during the plateau phase. Figure 3A shows a typical example of this current. NA (100 μM) was used as a positive control. Two representative current-voltage curves (control conditions and in the presence of 100 μM NA) are shown in Figure 3B. The almost complete block by 100 μM NA (see Table 1) is in agreement with previously published data (Nilius *et al*., 1997b; Pedersen *et al*., 1998). Figure 3C shows current traces in response to the voltage step protocol, performed directly after the last measured voltage ramp in Figure 3A. The phenotype of the current matches obviously $I_{Cl,Ca}$ indicating that this is the dominant current during the plateau phase. Another example of $I_{Cl,Ca}$ and the effect of adding fraction 16 to the external solution is demonstrated in Figure 3D. It is clear that fraction 16 did not significantly inhibit $I_{Cl,Ca}$ ($n=5$). Two representative voltage curves (control conditions and fraction 16) are shown in Figure 3E. Current traces in response to voltage steps applied after the last voltage ramp from Figure 3D are shown in Figure 3F. Fractions 15, 17, 18, 19, 20 and 21 were tested in analogous experiments ($n=2-5$), but neither of them exerted a significant inhibition. The results are summarized in Table 1.

The pure chlorotoxin peptide does neither inhibit $I_{Cl,swell}$, $I_{Cl,Ca}$ nor $I_{Cl,cAMP}$

Chlorotoxin, often depicted as a general chloride channel blocker, is known to be part of the *Lqq* venom and considered to be responsible for the alleged inhibitory effect on chloride channels (DeBin *et al*., 1993). Since we could not detect any inhibition of neither $I_{Cl,swell}$ nor $I_{Cl,Ca}$ by the venom, we investigated the possible effects of the pure chlorotoxin peptide on these two chloride currents. In addition we tested its effects on a third chloride current, *sc*. the cyclic AMP activated chloride current, $I_{Cl,cAMP}$, through CFTR.

Figure 4A shows a typical time course experiment for VRAC in which 625 nM ClTx was added to the external solution, during a maintained superfusion with HTS. Current-voltage relations before and during application of ClTx (Figure 4B) show that the peptide did not affect $I_{Cl,swell}$. In one experiment we applied 1.2 μM ClTx intracellularly *via* the pipette solution, but did not detect any significant differences with the current response under control conditions (data not shown). Figure 4C illustrates the effect of 625 nM ClTx on $I_{Cl,Ca}$ and Figure 4D the correspondent current-voltage relations. Also here no inhibition was observed. $I_{Cl,cAMP}$ was activated by a cocktail containing 100 μM IBMX and 1 μM forskolin dissolved in the Krebs-Cs^+ solution, as described in detail elsewhere (Cuppens *et al*., 1998). Figure 4E shows a typical time course experiment and the effect of adding 1.2 μM ClTx during stimulation with the IBMX-forskolin cocktail. Current-voltage relations before and during application of ClTx (Figure 4F) show that the toxin did not affect $I_{Cl,cAMP}$.

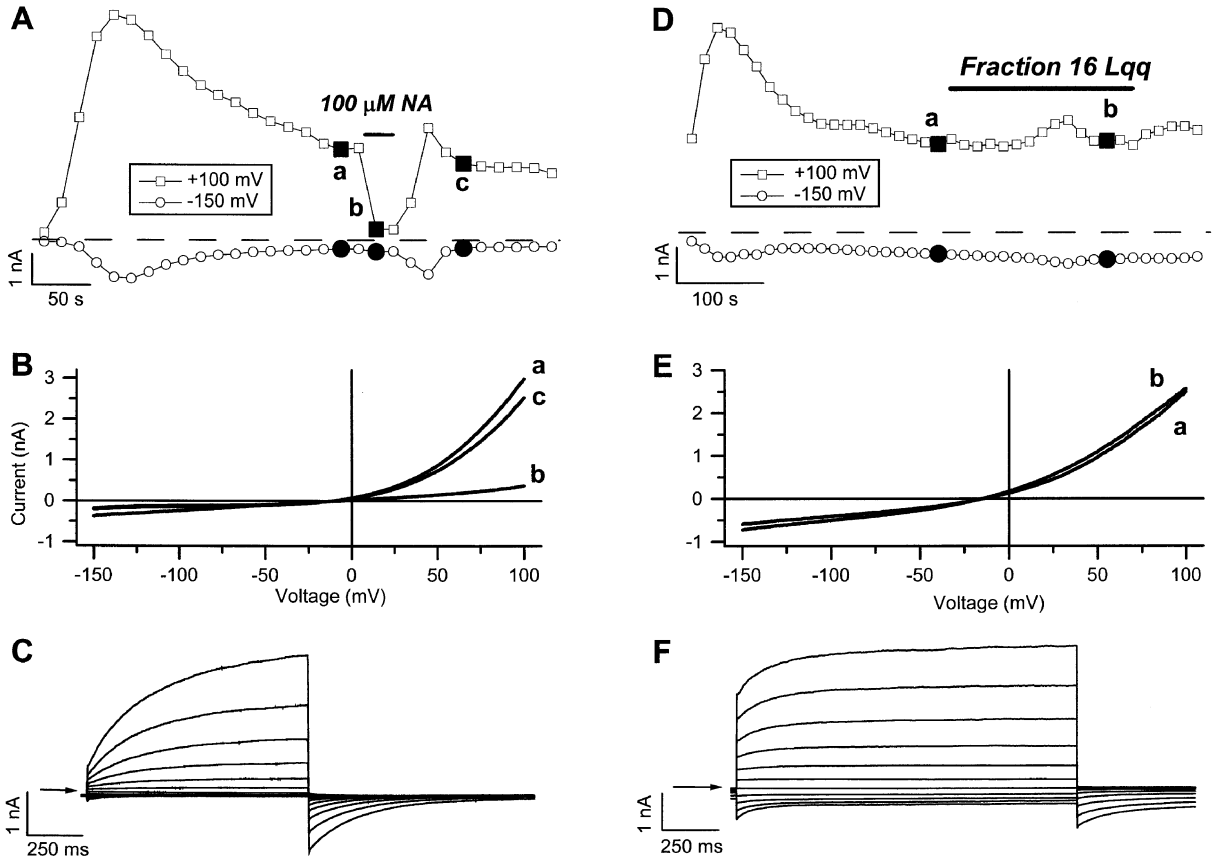

Figure 3 (A) Reversible inhibition of 100 μM NA on $I_{Cl,Ca}$, activated by loading the cell with a buffered 1000 nM Ca^{2+} pipette solution (after breaking the patch at the time indicated by the arrow). The bath solution is a slightly hypertonic Krebs-Cs^{+} solution. The dashed line indicates zero current. Data were obtained from ramp protocols by averaging the current in a small voltage window around +100 and −150 mV. (B) Current-voltage relations from voltage ramps at the times indicated in (A). (C) Current traces in response to the voltage steps protocol (1 s steps, holding potential −50 mV), performed directly after the last measured voltage ramp in (A). (D) Effect of fraction 16 on $I_{Cl,Ca}$, activated as described in (A) and applied through exactly the same delivery system as NA. Because of a little time difference between breaking into the cell and the first current recording, the time course does not start from zero current as in (A). (E) Current-voltage relations from voltage ramps at the times indicated in (D). (F) Current traces in response to the voltage steps protocol (1.5 s steps, holding potential −20 mV), performed directly after the last measured voltage ramp in (D).

In other cells from the same batch, 50 μM glibenclamide was used as a positive control and caused the predicted inhibition (Wei and Nilius, personal communication). The data are summarized in Table 1.

The pure chlorotoxin peptide does not inhibit I_{GCC}

Recent studies have shown an inhibitory effect of ClTx on chloride currents in human astrocytoma cells, including STTG1 cells. This current is activated under rather non-physiological conditions, *sc.* voltages more positive than +100 mV, and inhibited for 80% by 590 nM ClTx (Ullrich *et al.*, 1995; Ullrich & Sontheimer, 1996; 1997). Because of our negative results, we have repeated the measurements in STTG1 cells under the same experimental conditions as Ullrich *et al.* (1995) except for the P/4 leak subtraction procedure. Figure 5A,B show current traces in control conditions and after bath application of 1.2 μM ClTx during the voltage step protocol. We were not able to detect any inhibition of the current by 1.2 μM ClTx ($n=4$). This result urged us to test other chloride channel blockers, such as niflumic acid (500 μM), DIDS (500 μM), SITS (500 μM) and DCDPC (50 μM) on these currents, but also neither of these blockers caused inhibition. The results are summarized in Table 2.

Table 2 Currents (at +195 mV) measured after 2 min in the presence of the drug (except for chlorotoxin and 10% chloride, after 1 min; for TEA, within 1 min after exposure) and expressed as percentage of the control current

Drug	*GCC*	n	*% Current*
Chlorotoxin 1.2 μM		4	116.9±7.9
10% Chloride		4	101.4±2.4
Niflumic Acid 500 μM		3	135.4±52.1
DIDS 500 μM		3	193.1±41.5
SITS 500 μM		4	125.2±17.8
DCDPC 50 μM		3	234.3±15.2
TEA 10 mM		6	16.4±3.0

Within the frame of the above negative results, we investigated whether this current is a chloride current. Therefore we reduced the external Cl^{-} concentration to 10%, while keeping the pipette Cl^{-} concentration constant. Since we are dealing with a strongly outwardly rectifying 'chloride' current (i.e. influx of Cl^{-} ions), we would expect a decrease of current amplitude due to a shift of the reversal potential toward more positive values. Figure 5C,D show current traces in control conditions (100% Cl^{-}) and in the

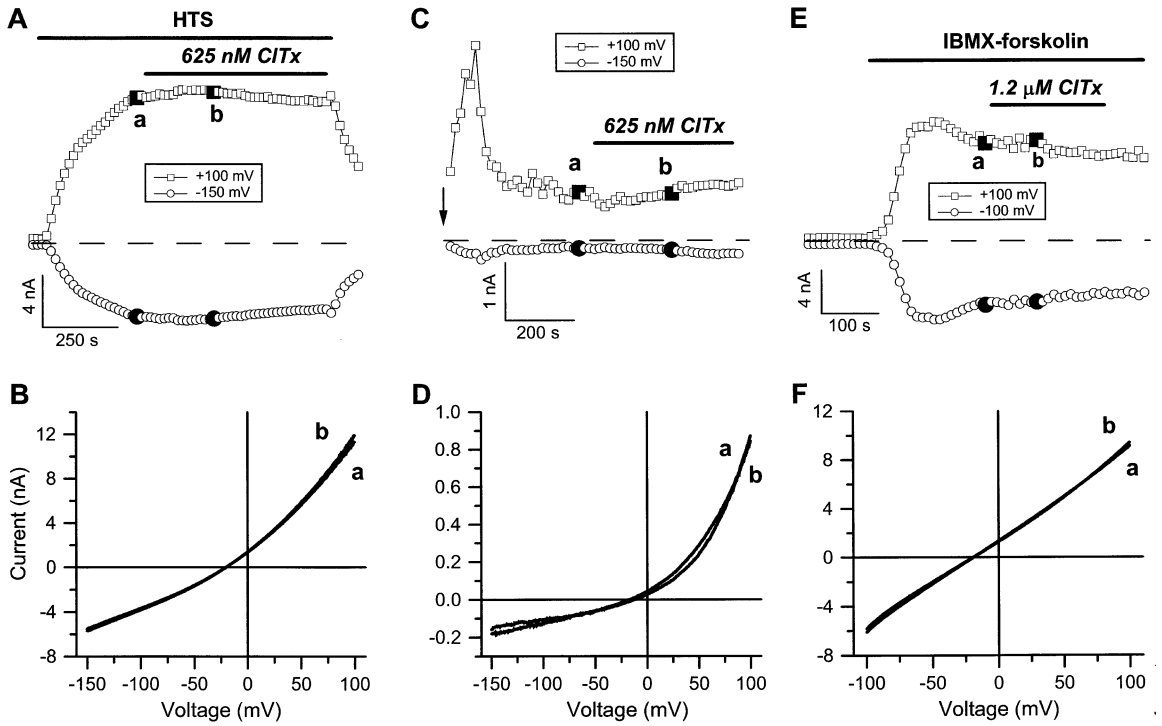

Figure 4 (A) Time course of activation of $I_{Cl,swell}$ during superfusion with hypotonic solution (HTS) (following superfusion with Krebs solution and isotonic-Cs^+ solution), application of 625 nM chlorotoxin and deactivation of the current after returning to isotonic-Cs^+ solution. The dashed line indicates zero current. Data were obtained from ramp protocols by averaging the current in a small voltage window around +100 and −150 mV. (B) Current-voltage relations obtained from voltage-ramps at the times indicated in (A). (C) Time course of $I_{Cl,Ca}$, activated by loading the cell with a buffered 1000 nM Ca^{2+} pipette solution (after breaking the patch at the time indicated by the arrow) and application of 625 nM chlorotoxin. The bath solution is a slightly hypertonic Krebs-Cs^+ solution. The dashed line indicates zero current. Data were obtained from ramp protocols by averaging the current in a small voltage window around +100 and −150 mV. (D) Current-voltage relations from voltage ramps at the times indicated in (C). (E) Effect of 1.2 μM chlorotoxin on $I_{Cl,cAMP}$ currents activated with a IBMX-forskolin cocktail. Bath solution is a Krebs-Cs^+ solution. The dashed line indicates zero current. Data were obtained from ramp protocols by averaging the current in a small voltage window around +100 and −100 mV. (F) Current-voltage relations from voltage ramps at the times indicated in (E).

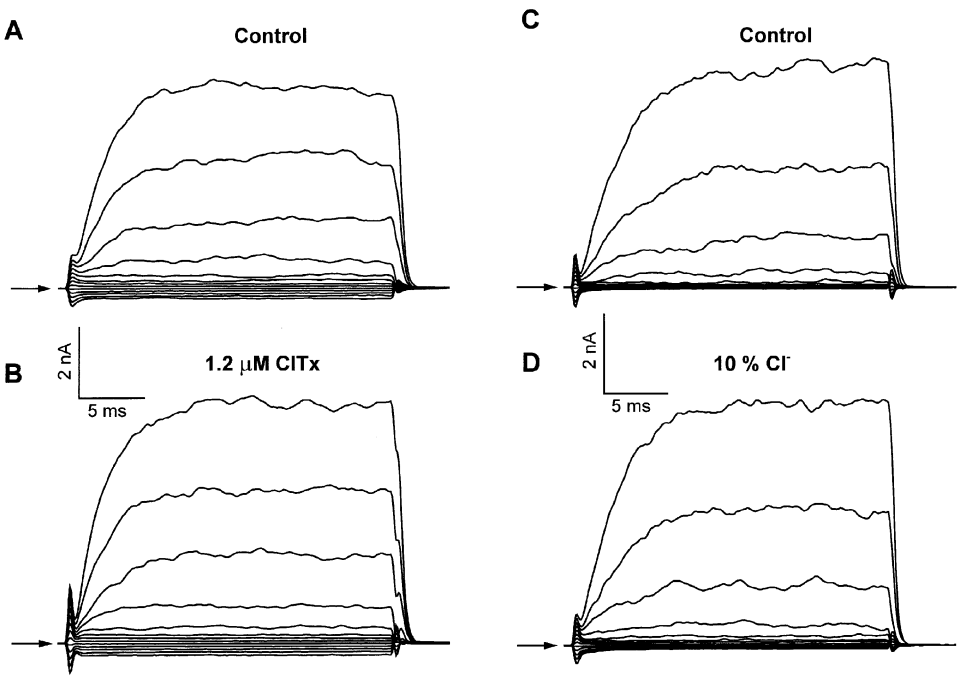

Figure 5 (A) I_{GCC} traces in control conditions in response to 25 ms voltage steps to potentials from −105 to +195 mV (spaced 25 mV), applied from a holding potential of 0 mV. Sampling interval was 0.1 ms. The arrow indicates zero current. (B) Currents recorded from the same cell as in (A) in the presence of 1.2 μM chlorotoxin, using the same protocol as in (A). (C) Current traces in control conditions (100% extracellular chloride), using the same protocol as in (A). (D) Currents recorded from the same cell as in (C) in the presence of 10% extracellular chloride, using the same protocol as in (A).

presence of 10% extracellular Cl^-. We could not observe a change in current amplitude ($n=4$, Table 2), indicating that the major component of this outward I_{GCC} is not a Cl^- influx. Since K^+ was the major cation in the pipette solution, we tested whether I_{GCC} has a K^+ component by applying the K^+ channel blocker TEA. Figure 6A,B show currents recorded in control conditions (for bath and pipette) and during bath application of 10 mM TEA. At this concentration, TEA induced a fast block to about 16% of the control value at +195 mV ($n=6$, Table 2). This block was fully reversible (Figure 6C) and recovery of the current upon wash out was fast. Figure 6D shows the TEA sensitive current, obtained as the difference between the currents in the absence and presence of the inhibitor, which shows the typical pattern of I_{GCC} as described by Ullrich *et al*. (1995). In a second series of experiments we replaced intracellular KCl with TEACl. From the current traces in Figure 6E it is obvious that this substitution dramatically reduced the amplitude of the current responses. Similar results were obtained in other cells ($n=4$). Replacing the control bath solution with the 10 mM TEA solution did not affect the remaining current (Figure 3F). In a third series of experiments we determined the reversal potential of I_{GCC} in three different bath solutions, i.e. control solution, a low Cl^- and a high K^+ solution from the fully activated I-V curve, i.e. the current was first fully activated by a voltage step to +180 mV, and the amplitude of the tail current was measured during subsequent voltage steps to various potentials. Figure 7A,B show these tail currents in control conditions and in the presence of 10% chloride. The corresponding tail current amplitudes plotted as a function of voltage (Figure 7C) does not reveal any significant difference between these two conditions. Figure 7D,E show tail currents from another cell in control conditions and in high K^+ solution and Figure 7F the corresponding voltage dependence of the tail current amplitude. In contrast with the low Cl^- solution, the high K^+ solution clearly shifts the curve to the right. The mean reversal potential was -35.38 ± 4.24 mV ($n=8$) for the control condition, -36.20 ± 5.95 mV ($n=5$) for the low Cl^- and $+2.67\pm0.88$ mV ($n=3$) for the high K^+ solution. Obviously, changing the extracellular Cl^- concentration does not affect the reversal potential, which is clearly different from the calculated chloride equilibrium potentials of +2 and +60 mV for the control condition and the 10% Cl^- solution, respectively. These results and the above-described pharmacological data are not compatible with a current through a Cl^- selective channel. The calculated potassium equilibrium potentials are −85 mV for the control and −3 mV for the high K^+ condition. Because the shift in reversal potential is much less than expected for a K^+ channel, this channel is apparently not highly selective for K^+.

Discussion

The molecular and functional analysis of volume-regulated and calcium-activated chloride channels is still hampered by the non-availability of sufficiently sensitive and selective pharmacological tools. The aim of this study was to find a high-affinity toxin for those channels and to gain a better insight into the toxicology of anion channels.

In previous studies it was shown that the crude venom extracted from the scorpion *Leiurus quinquestriatus quinquestriatus* inhibits reconstituted small-conductance chloride

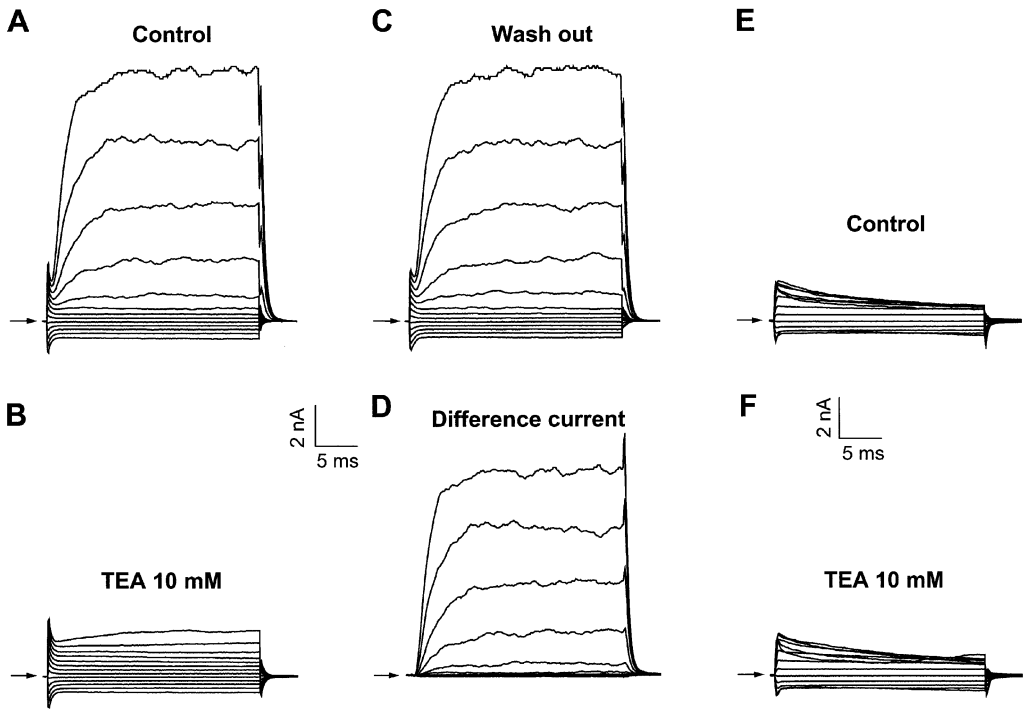

Figure 6 (A) I_{GCC} traces in control conditions in response to 25 ms voltage steps to potentials from −105 to +195 mV (spaced 25 mV), applied from a holding potential of 0 mV. Sampling interval was 0.1 ms. The arrow indicates zero current. (B) Currents recorded from the same cell as in (A) in the presence of 10 mM TEA, using the same protocol as in (A). (C) Currents after washing out the TEA effect, using the same protocol as in (A). (D) Current traces representing the difference current between control current and the current blocked by 10 mM TEA, obtained from the data shown in (A) and (B). (E) Current traces using the same protocol and bath solution as in (A), but with a pipette containing TEACl instead of KCl. (F) Currents recorded from the same cell as in (E) in the presence of 10 mM TEA, using the same protocol as in (A).

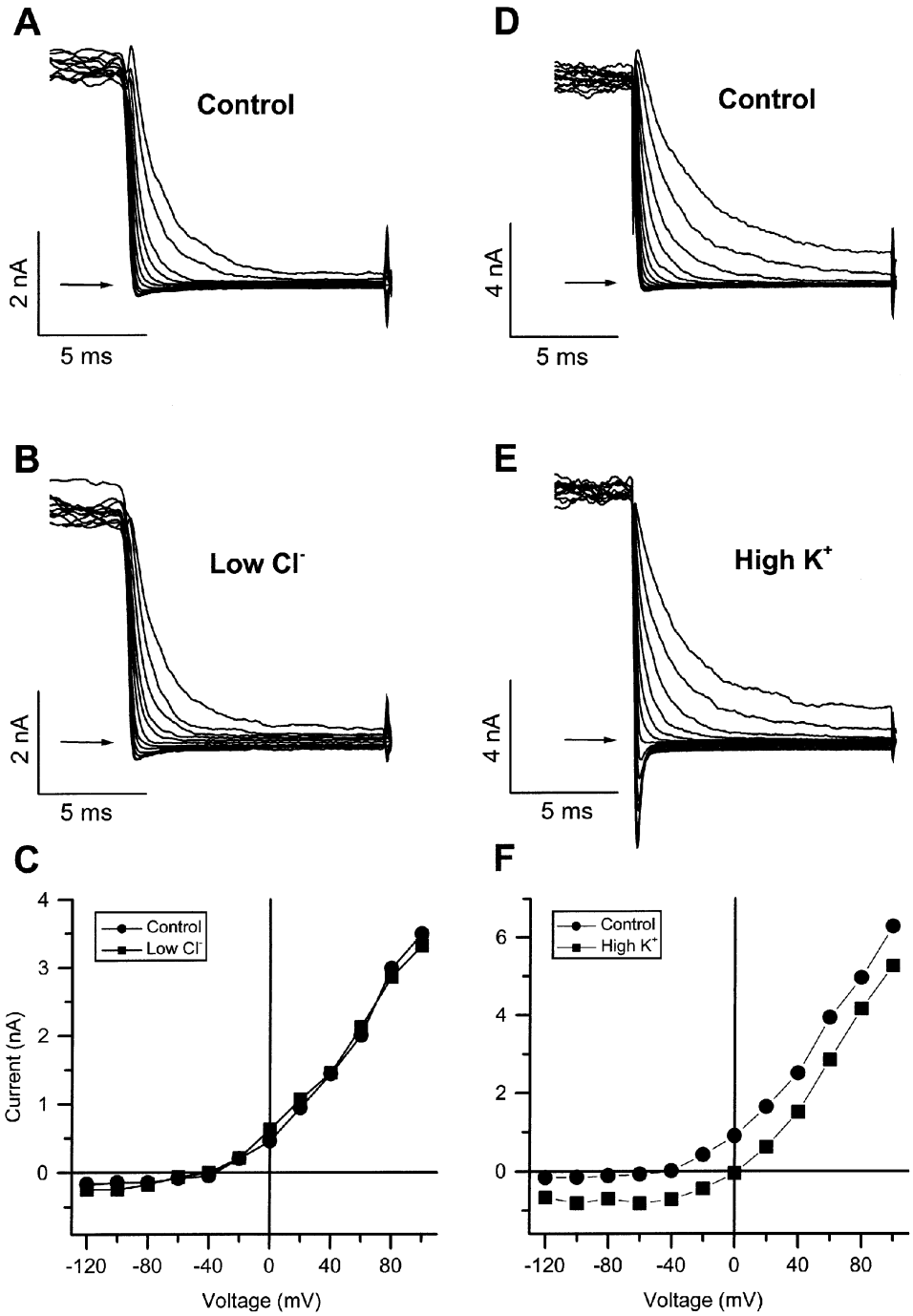

Figure 7 (A) Tail currents through GCC in control conditions (100% extracellular chloride), in response to the following protocol: the cell was held at 0 mV, pulsed every 2 s to 180 mV for 25 ms (not shown, sampling interval 0.05 ms) and then stepped (for 12.5 ms, sampling interval 0.02 ms) to various voltages ranging from +120 to −120 mV in −20 mV increments. (B) Tail currents from the same cell in 10% extracellular chloride, using the same protocol as described in (A). (C) Voltage dependence of the amplitude of the tail currents from (A) and (B), assessed from the peak currents measured between 1 and 1.5 ms from the beginning of the step, and corrected for leak current by subtracting the mean current at the end of the voltage step. (D) Tail currents through GCC from another cell in control conditions (5 mM KCl) in response to the voltage protocol described in (A). (E) GCC tail currents from the same cell in high K^+ conditions (130 mM). (F) I-V curve of the tail currents measured in control conditions (D) and in high K^+ (E), using the same procedure as in (C).

channels isolated from rat epithelia and embryonic rat brain (De Bin & Strichartz, 1991). Chlorotoxin, the active compound of this venom, was purified and characterized as a 4.1 kDa basic peptide with considerable sequence homology to the short insectotoxins (DeBin *et al.*, 1993; Tytgat *et al.*, 1998). The inhibition seen with chlorotoxin was only observed with application to what appears to be the cytoplasmic facing surface of neuronal growth cone or colonic enterocyte chloride channels. The authors admit that this intracellular site of action is difficult to reconcile with the observed toxicity to crayfish and cockroaches. Therefore they argue that the paralysis resulting from ClTx injection into arthropods may result from the inhibition of some other structurally related anion channels and that ClTx may be effective when applied to the extracellular surface of these channels (DeBin *et al.*, 1993). Since we were searching for a

high-affinity, rapidly acting toxin, we were interested in an inhibition after application to the extracellular side. After all, it is very unlikely that an ion channel is a natural target for a scorpion venom acting from the cytoplasmic side.

The experimental results in this study could not demonstrate any significant inhibition of $I_{Cl,swell}$ or $I_{Cl,Ca}$ by the different fractions of interest obtained from the purified *Lqq* venom. Neither could we demonstrate inhibition of $I_{Cl,swell}$ after cytoplasmic application of fraction 16 and 17, in which ClTx (and possible homologues of ClTx) is assumed to be primarily present. The stated inhibitory effect of the *Lqq* venom on chloride channels has been attributed to the 4.1 kDa basic peptide ClTx (DeBin *et al*., 1993). This peptide is now commercially available and often, in part thanks to its name, depicted as a general chloride channel blocker.

Since we could not detect inhibition of $I_{Cl,swell}$ and $I_{Cl,Ca}$ by the venom itself, we investigated the possible effects of the pure peptide ClTx on these two currents. High-affinity toxins purified from scorpion venoms, blocking ion channels in the nanomolar range, are well-documented for cation channels, among which e.g. charybdotoxin. This toxin, also isolated from the venom of the Israeli scorpion *Leiurus quinquestriatus* var. Hebraeus, inhibits high-conductance Ca^{2+}-activated K^+ channels with a K_d value of $\sim$ 3.5 nM (Miller *et al*., 1985; Smith *et al*., 1986). Ullrich & Sontheimer (1996) described an 80% inhibition of chloride currents in astrocytoma cells by 590 nM ClTx. In the present experiments we show here that 625 nM ClTx did neither affect $I_{Cl,swell}$ nor $I_{Cl,Ca}$. At a concentration of 1.2 μM, the toxin did also not affect $I_{Cl,cAMP}$. Moreover, intracellular dialysis with 1.2 μM ClTx did not inhibit $I_{Cl,swell}$. Toxins are designed by nature to affect their target in the nanomolar range, but even unusual high submicromolar concentrations used in this study completely failed to affect these well-defined Cl^- channels.

The only reported effects of ClTx on chloride currents since its discovery by De Bin & Strichartz (1991) and DeBin *et al*. (1993) are the inhibitory effects on a glioma-specific chloride current in human astrocytoma cells, including STTG1 cells. This current is activated under rather non-physiological conditions, *sc*. voltages more positive than +100 mV (Ullrich *et al*., 1995; Ullrich & Sontheimer, 1996; 1997). The authors also suggest that ClTx and ClTx-conjugated molecules may serve as glioma-specific markers with diagnostic and therapeutic potential (Soroceanu *et al*., 1998). To complete our study of ClTx, we repeated the ClTx experiments in STTG1 cells under identical conditions as described by Ullrich *et al*. (1995) but surprisingly we were unable to confirm their findings. Moreover, this current was not sensitive to other chloride channel blockers, such as niflumic acid, DIDS, SITS and DCDPC, a finding that is difficult to reconcile with this current being a chloride current. Also the observations that reducing the external chloride concentration to 10% did neither affect the outward current amplitude nor shift the reversal potential to more positive values is inconsistent with this contention.

Furthermore, some of the experimental results of Ullrich & Southeimer (1997) do not support the idea that this current is carried by chloride: (i) removal of 50 mM extracellular NaCl should have substantial effects on the amplitude of a chloride current, but such effects have not been observed; (ii) large outward current transients after stepping the potential back to 0 mV are unlikely if the reversal potential would be close to an E_{Cl} of +2.8 mV (Ullrich & Sontheimer, 1996). From a pharmacological point of view one could question the suggestion that this chloride channel might be involved in astrocytoma growth control, since some of the used chloride channel blockers (DIDS and DNDS) reduce cell proliferation, while ClTx enhances cell proliferation (Ullrich & Sontheimer, 1996). It should also be emphasised that Soroceanu *et al*. (1998) suggest that chlorotoxin may bind to an independent receptor and indirectly modulate the activity of GCC, rather than binding directly to GCC.

I_{GCC} is a strongly outwardly rectifying current and as a consequence mainly carried by the efflux of cations or influx of anions. Since the current was not affected by reducing the extracellular Cl^- concentration, we investigated a possible contribution of intracellular cations. I_{GCC} is inhibited by 10 mM TEA, and dramatically reduced when intracellular KCl is replaced by TEACl. In addition, its reversal potential shifted to more positive potentials when the extracellular control solution is replaced by a high K^+ solution. However, this shift of approximately 40 mV is much less than that of 80 mV expected for a highly K^+ selective channel. We therefore conclude that I_{GCC} is a current through K^+ channels that are not exclusively permeable for K^+. The physiological significance of a channel that activates at potentials more positive than +100 mV is however questionable.

Sanchez & Blatz (1992; 1994) demonstrated that external and internal TEA inhibits neuronal fast Cl^- channels. The biophysical and pharmacological profile of this channel is however completely different from that of GCC. It is activated in a physiological voltage range between −90 and +80 mV (Blatz, 1991), whereas GCC activates at potentials more positive than +100 mV. Sanchez & Blatz (1992) reported a voltage dependent TEA block with strong inhibition at negative but little inhibition at positive potentials, which is at variance with our results.

In conclusion, chlorotoxin does neither block the volume-regulated nor the calcium-activated and cyclic AMP-activated (CFTR) chloride channel. The natural target of this peptide toxin therefore remains elusive.

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