CSTX-1, a toxin from the venom of the hunting spider *Cupiennius salei*, is a selective blocker of L-type calcium channels in mammalian neurons

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Abstract

The inhibitor cystine-knot motif identified in the structure of CSTX-1 from *Cupiennius salei* venom suggests that this toxin may act as a blocker of ion channels. Whole-cell patch clamp experiments performed on cockroach neurons revealed that CSTX-1 produced a slow voltage-independent block of both mid/low- (M-LVA) and high-voltage-activated (HVA) insect Ca_v channels. Since *Cupiennius salei* venom affects both insect as well as rodent species, we investigated whether Ca_v channel currents of rat neurons are also inhibited by CSTX-1. CSTX-1 blocked rat neuronal L-type, but no other types of HVA Ca_v channels, and failed to modulate LVA Ca_v channel currents. Using neuroendocrine GH3 and GH4 cells, CSTX-1 produced a rapid voltage-independent block of L-type Ca_v channel currents. The concentration-response curve was biphasic in GH4 neurons and the subnanomolar IC_{50} values were at least 1000-fold lower than in GH3 cells. L-type Ca_v channel currents of skeletal muscle myoballs and other voltage-gated ion currents of rat neurons, such as $I_{Na(V)}$ or $I_{K(V)}$ were not affected by CSTX-1. The high potency and selectivity of CSTX-1 for a subset of L-type channels in mammalian neurons may enable the toxin to be used as a molecular tool for the investigation of this family of Ca_v channels.

Running Title: CSTX-1, a selective Ca_v channel blocker

Keywords: *Cupiennius salei* venom; CSTX-1; voltage-gated calcium channels; DUM neurons; rat neurons

Abbreviations: ATP, adenosine-5'-triphosphate; B_{max}, maximum binding capacity; Ca_v channel, voltage-gated Ca^{2+} channel; CSTX, peptide toxin from the venom of *Cupiennius*

salei; DMEM, Dulbecco's-modified Eagles Media; DRG, dorsal root ganglion; DUM, dorsal unpaired median; EDTA, ethylenediaminetetraacetic acid: EGTA, ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid; ESI-MS, electrospray ionisation-mass spectrometry; GTP, guanosine-5'-triphosphate; HVA, high-voltage-activated; IC_{50}, median inhibitory concentration; ICK, inhibitor cystine-knot; $I_{Ca(V)}$, voltage-gated calcium channel current; $I_{K(V)}$, voltage-gated potassium channel current; $I_{Na(V)}$, voltage-gated sodium channel current; *I–V* curve, current–voltage relation curve; K_v channel, voltage-gated K^+ channel; LD_{50}, median lethal dose; LVA, low-voltage-activated; M-LVA, mid/low-voltage-activated; Na_v channel, voltage-gated Na^+ channel; PBS, phosphate-buffered saline; r^2, regression coefficient; RP-HPLC, reverse phase high-pressure liquid chromatography; SCG, superior cervical ganglion; TAG, terminal abdominal ganglia; TEA, tetraethylammonium; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; TFA, trifluoroacetic acid; TTX, tetrodotoxin; V_h, holding potential.

1. Introduction

A multitude of applications make venom toxins important molecular tools. Cell biologists are employing a variety of animal, plant and microbial toxins to investigate the contribution of their molecular targets to various cell functions and as lead compounds for the development of pesticides and therapeutics (Bailey and Wilce, 2001). For example, phyla-specific spider neurotoxins that interact selectively with insect membrane proteins are being examined for their potential as biopesticides (Tedford et al., 2004a, b). More recently, the pharmaceutical industry has discovered the pre-optimised combinatorial peptide library that animal venoms represent, and are searching for toxins as templates for drug design or directly as therapeutics (Harvey, 2002; Bogin, 2005).

In neurobiology, specific antagonists are required to study the structure and role of voltage- and transmitter-gated ion channels. Venoms of different animals provide a rich but, as yet, largely unexplored source of ion channel modulators. Many of the toxins studied to date are selective blockers and discriminate between closely related ion channel subtypes. Families of Ca_v channels have been identified pharmacologically using specific peptide toxins from marine cone snail and spider venoms (Doering and Zamponi, 2003). However, at present, many of the Ca_v channel subtypes identified using molecular biology techniques cannot be isolated pharmacologically. This holds true for the L-type ($Ca_v1.x$) family of Ca_v channels, which, for mammalian neurons, are characterised by their sensitivity to dihydropyridines. The differences in the affinity of dihydropyridines for the four L-type Ca_v channel subtypes ($Ca_v1.1$ to 1.4), identified to date, were found to be too small to permit the study of the functional role of these channels in various cell types (Bourinet et al., 2004). It is therefore valuable to further isolate and characterise venom components in a search for novel toxins that may be used as pharmacological tools to further dissect the structure and function of Ca_v channels.

Cupiennius salei (Araneae: Ctenidae) is a nocturnal hunting spider inhabiting tropical rain forests from Central Mexico to northern South America (Kuhn-Nentwig et al., 2004). The venom of this spider is a complex mixture containing ions (Na^+ 8.9 mM, K^+ 215 mM, and Ca^{2+} 0.94 mM), low molecular mass substances as amino acids (mainly glycine 43.3 pmol/µl and taurine 70.0 pmol/µl), amines (histamine 5.7 nmol/µl) and polyamines in low quantities (Kuhn-Nentwig et al., 1994, 2004). Beside proteins (M_r 25-27 kDa) such as the enzyme hyaluronidase, peptides in the molecular range between 3-4 kDa were identified and therein two peptide families were named cupiennin 1 and cupiennin 2 (Kuhn-Nentwig et al., 2004). These cationic cupiennins exhibit a toxic activity towards insects, a strong cytolytic activity to erythrocytes and are bactericidal in the submicromolar range towards Gram positive as well as Gram negative bacteria (Kuhn-Nentwig et al., 2002). Synergistic toxic effects were

demonstrated between cupiennin 1a and the toxic peptides CSTX-1, 9 and CSTX-13 in a *Drosophila* bioassay (Wullschleger et al., 2005). Additionally, cupiennin 1a inhibits the formation of NO from neuronal nitric oxide synthase by involving complexation with the regulatory protein calcium calmodulin (Pukala et al., 2007). Most significant, though, are several neurotoxic peptides (CSTX), with molecular masses between 6 and 8 kDa, which are mainly responsible for the lethality of the venom. Interestingly, bioassays revealed that mice, despite not being usual prey species, are the most sensitive species to *Cupiennius salei* venom when compared to a wide range of arthropods (Kuhn-Nentwig et al., 1998). Among the 13 peptide toxins identified in *C. salei* venom (CSTX-1 to -13), the 74-residue peptide CSTX-1 is the most abundant and displays the highest toxicity in mammalian and insect bioassays (Kuhn-Nentwig et al., 1994). Despite the comprehensive knowledge of venom composition and peptide structure acquired within the last decade (Kuhn-Nentwig et al., 2004), evidence for the molecular targets of *Cupiennius salei* toxins remains obscure. CSTX-1, CSTX-9 and CSTX-13 are characterized by four disulfide bridges. Three of the four disulfide bridges form linkages between C1-C4, C2-C5 and C3-C8 and conform to an 'inhibitor cystine –knot' (ICK) motif (Norton and Pallaghy, 1998). This motif is characteristic for many ion channel blocking peptide toxins (Mouhat et al., 2004), especially from spider venoms (Nicholson, 2006). In this study we therefore tested the hypothesis that CSTX-1 may act as an antagonist of neuronal ion channels. We show that CSTX-1 blocks voltage-gated calcium (Ca_v) channel currents in neurons of the cockroach *Periplaneta americana*, but does not appear to discriminate between mid/low- (M-LVA) and high-voltage-activated (HVA) Ca_v channels. In rat neurons, CSTX-1 leaves LVA Ca_v channel currents unaffected but acts as a selective blocker of dihydropyridine-sensitive L-type HVA Ca_v channels. The potency of block appears to depend on the subtype of L-type Ca_v channels expressed in these neurons. Our data suggest that CSTX-1 has evolved as a blocker of insect Ca_v channels, and has a structure that permits a high affinity interaction with a subtype of L-type Ca_v channels expressed in mammalian

neurons. CSTX-1 may thus have a potential as a research tool to investigate the physiological role of L-type Ca_v channel subtypes. Some of these results have recently been presented in abstract form (Kubista et al., 2006).

2. Materials and methods

2.1. Toxin isolation

Spider maintenance, venom collection and purification of CSTX-1 were carried out as previously described (Kuhn-Nentwig *et al*., 1994) with the following modifications. Cation exchange liquid chromatography was performed on an Mono S HR 10/10 (10 cm x 1 cm, Pharmacia, Sweden) at a flow rate of 0.5 ml/min using a 200 mM ammonium acetate buffer, pH 5.5 and a gradient (B) of 200 mM ammonium acetate and 2 M NaCl, pH 5.5. The separation profile was 0% B for 20 min followed by a linear gradient from 0 to 100% from 20 to 74 min. The fraction containing CSTX-1, which eluted at 1 M NaCl (50% B), was desalted and separated by RP-HPLC using a Nucleosil 300-5 C_4 column (4.6 mm x 250 mm, Macherey & Nagel, Germany) with 0.1% v/v trifluoroacetic acid (TFA) in water for 0 to 15 min followed by a linear gradient of 0.1% TFA in acetonitrile from 0 to 10% from 15 to 30 min and from 10 to 50% from 30 to 125 min. Rechromatography of the peptide was performed on the same column using isocratic conditions with 32% acetonitrile in TFA for 30 minutes. Starting with 1.8 ml of crude venom we obtained 15 mg of highly purified CSTX-1, by a combination of gel filtration, cationic exchange chromatography and successive RP-HPLC. The purity of CSTX-1 was confirmed by RP-HPLC (Fig. 1), N-terminal amino acid sequencing, amino acid composition and electrospray ionisation-mass spectrometry (ESI-MS).

2.2. DUM neurons

Dorsal unpaired median (DUM) neurons from the terminal abdominal ganglion (TAG) of the nerve cord of the adult American cockroach, *Periplaneta americana* were isolated using methods modified from Grolleau and Lapied (1996) and Sinakevitch et al. (1996). Briefly, the sixth abdominal ganglia were excised, desheathed and incubated for 15 min at 37°C in insect saline of the following composition (in mM): 200 NaCl, 3.1 KCl, 5 $CaCl_2$, 4 $MgCl_2$, 10 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 40 D-glucose supplemented with 5% v/v foetal calf serum and 50 IU/l penicillin and streptomycin at pH 7.4, containing 1 mg/ml of collagenase and hyaluronidase. After washing in enzyme-free insect saline, DUM neurons were mechanically isolated from exogenous tissue by trituration, carefully passing the ganglia in and out of a sterile Pasteur pipette. The resulting suspension was then distributed into 8 wells of a 24-well cluster plate (Limbro, Ohio, USA). Each well contained a 12-mm diameter glass coverslip (Lomb Scientific, Taren Point, NSW, Australia), which had been previously coated with BD Cell-Tak™ (BD Biosciences, North Ryde, Sydney, Australia). Isolated cells were allowed to attach to the coverslips overnight in an incubator (5% CO_2, 95% O_2, 100% relative humidity, 28°C) in normal insect saline supplemented with 5% v/v foetal calf serum, prior to experimentation.

2.3. Rat SCG and DRG neurons

Primary cultures of dissociated superior cervical ganglia (SCG) and dorsal root ganglia (DRG) neurons from neonatal rats were prepared as described previously (Lechner et al., 2003). Briefly, ganglia were dissected from 2 to 6 day-old Sprague Dawley rat pups that had been killed by decapitation in accordance with the rules of the Animal Welfare Committee of

the Medical University of Vienna. Ganglia were cut into 3 to 4 pieces and incubated in 1.5 mg/ml collagenase and 3.0 mg/ml dispase (Sigma, Vienna, Austria) for 20 min at 36°C. Subsequently, they were trypsinised (2.5 mg/ml trypsin; Worthington, Lakewood, NJ, USA) for 15 min at 36°C, dissociated by trituration, and resuspended in Dulbecco's-modified Eagle's Medium (DMEM; Invitrogen, Lofer, Austria) containing 2.2 g/l D-glucose, 10 mg/l insulin, 50.000 IU/l penicillin and 50 mg/l streptomycin (Invitrogen, Lofer, Austria), 50 µg/l nerve growth factor (Invitrogen, Lofer, Austria), and 5% v/v foetal calf serum (Invitrogen, Lofer, Austria). Thereafter, the cells were plated onto 35 mm diameter culture dishes, previously coated with rat tail collagen, for electrophysiological experiments. DRG neurons were used on the day of plating, whereas SCG neurons were used for recordings within one week following plating. During that period SCG neurons were maintained in a humidified 5% CO_2 atmosphere at 37ºC. Media were exchanged on day one after plating and after another 4 to 5 days.

2.4. Cell lines

GH3 cells (American Type Culture Collection, Manassas, USA) were cultured in DMEM-HEPES modification (Sigma, USA) supplemented with 10% v/v foetal calf serum (Cultilab, Brazil). The cells were routinely grown as stocks in 75 cm^2 flasks (Costar, USA) at 37°C in a humidified 5% CO_2 atmosphere. The medium was changed three times a week. For electrophysiological recordings the cells were detached by trypsinisation, subcultured on glass coverslips (Corning #1, USA) without any pretreatment and plated in 47 mm diameter dishes. Trypsinisation was carried out with 0.1% w/v trypsin in the culture medium without serum and with 1 mM EDTA. Cells were used for electrophysiological recordings 1-2 days after plating.

GH4 cells (provided by T. Weiger, University of Salzburg, Austria) were grown in Nutrient Mixture F-10 Ham media supplemented with 15% v/v horse serum, 2.5% v/v foetal calf serum, 50.000 IU/l penicillin, 50 mg/l streptomycin and 2 mM L-glutamine. The cells were routinely grown as stocks in 10 cm diameter dishes (Iwaki, Japan) at 37°C in a humidified 5% CO_2 atmosphere. Cells were split once a week and growth media were replaced once between passages. For patch clamp experiments, GH4 cells were detached by trypsinisation and seeded at low density on poly-D-lysine-coated 35 mm diameter dishes.

Cell culture and differentiation of the C3H murine skeletal C2C12 cell line was carried out as described elsewhere (Zebedin et al., 2004). Briefly, cultures were maintained in serum containing 20% v/v fetal calf serum and differentiation of myoblasts was induced by serum reduction (2% v/v horse serum instead of 20% v/v fetal calf serum) on cells plated onto Matrigel. Electrophysiological experiments were performed 14 days after induction of differentiation on C2C12 myoballs as described previously (Zebedin et al., 2004).

2.5. Electrophysiology

Whole cell voltage-clamp recordings were performed at room temperature (20-24°C) using borosilicate glass patch pipettes of 2-3.5 MΩ tip resistance when filled with internal solution (see below) and patch clamp amplifiers from Axon Instruments (Axon Instruments, Foster City, CA, USA) or HEKA (HEKA Elektronik, Germany). Currents were elicited by voltage steps from a holding potential (V_h) of –80 mV in GH3 and GH4 cells, C2C12 myoballs and SCG neurons, from –100 mV in DRG neurons, and from –90 mV in DUM neurons, to the indicated depolarised potentials. Voltage steps were in most cases applied every 15 s, but pulse frequency was increased up to every 5 s in some experiments for a better resolution of the kinetics of current inhibition. Leakage and capacitive currents were digitally

subtracted on-line with *P-P*/4 procedures. Data were low-pass filtered at 2 to 5 kHz, digitised at 10 to 50 kHz and stored on computer for subsequent off-line analysis.

In whole-cell voltage-clamp recordings from DUM neurons, I_{Ba} were recorded using an Axopatch 200A patch-clamp amplifier and AxoData v3.0 software (Axon Instruments). Patch pipettes were filled with an internal solution of the following composition (in mM): 50 choline-Cl, 30 CsCl, 50 TEA-Br, 2 Na_2-ATP, 0.5 $CaCl_2$, 10 ethylene glycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), 10 HEPES and adjusted to pH 7.25 using CsOH. The external solution for recording I_{Ba} in DUM neurons contained (in mM): 160 choline-Cl, 50 TEA-Br, 3 $BaCl_2$, 10 HEPES, 500 nM tetrodotoxin (TTX), and the pH was adjusted to 7.4 using TEA-OH. 500 μM Cd^{2+}, a known insect Ca_v channel blocker, was used to abolish peak inward I_{Ba}, and confirmed the currents recorded were carried through this channel. Toxins and drugs were applied via a pressurised fast perfusion system (AutoMate Scientific, San Francisco CA, USA).

In whole-cell voltage-clamp recordings from GH3 cells, I_{Ba} were recorded using a HEKA EPC-9 patch-clamp amplifier. The internal pipette solution was of the following composition (in mM): 130 CsCl, 10 TEA-Cl, 2 Mg-ATP, 0.1 Li-GTP, 5 EGTA, 5 HEPES, and adjusted to pH 7.2 using CsOH. The cells were perfused continuously with an external solution containing (in mM): 140 NaCl, 25 $BaCl_2$, 0.5 $MgCl_2$, 5 CsCl, 5 HEPES, 0.1 μM TTX, and adjusted to pH 7.4 using NaOH.

Whole-cell ionic currents from SCG neurons, DRG neurons, GH4 cells and C2C12 myoballs were recorded as described previously (Vartian and Boehm, 2001), using an Axopatch 200B amplifier and pCLAMP v8.0 software. The internal pipette solution consisted of (in mM): (A) 140 KCl, 1.59 $CaCl_2$, 10 HEPES, 10 EGTA, 2 Mg-ATP, 2 Li-GTP, adjusted to pH 7.2 with KOH for measurement of outward $I_{K(V)}$, or (B) 130 CsCl, 20 tetraethylammonium (TEA)-Cl, 0.24 $CaCl_2$, 10 D-glucose, 10 HEPES, 5 EGTA, 2 Mg-ATP, and 2 Li-GTP, adjusted to pH 7.2 with CsOH, for measurement of I_{Ba} and $I_{Na(V)}$ currents,

respectively. The external bathing solution contained (in mM): (A) 120 NaCl, 3 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 20 D-glucose, 10 HEPES, adjusted to pH 7.4 with NaOH for the measurement of outward $I_{K(V)}$, or (B) 120 NaCl, 3 KCl, 2 $MgCl_2$, 5 $CaCl_2$ ($I_{Na(V)}$) or 5 $BaCl_2$ (I_{Ba}), 20 TEA-Cl, 20 D-glucose, 10 HEPES, adjusted to pH 7.4 with NaOH for the measurement of high-voltage activated I_{Ba} and $I_{Na(V)}$. In addition, TTX (0.5 μM) or $CdCl_2$ (0.5 mM) were added to the external solution to isolate either high-voltage activated I_{Ba} or $I_{Na(V)}$ currents, respectively. For the measurement of LVA Ca_v channel currents in DRG neurons, TTX-sensitive and -insensitive $I_{Na(V)}$ were eliminated by replacement of NaCl with choline-Cl using the following bathing solution (in mM): 120 choline-Cl, 3 KCl, 2 $MgCl_2$, 5 $BaCl_2$, 20 TEA-Cl, 20 D-glucose, 10 HEPES, adjusted to pH 7.4 with CsOH. Drugs and toxins were applied via a DAD-12 drug application device (Adams & List, Westbury, NY, USA).

2.6. Data Analysis

Traces were analysed off-line using Clampfit or AxoGraph v4.0 (Axon Instruments) or Pulse-Fit (HEKA). Mathematical curve fitting employed algorithms available in ORIGIN v5.0 software (Microcal Software, Northampton, MA), Sigma Plot v5.0 (SPSS Inc., Richmond, CA) or GraphPad Prism v4.0 for Macintosh (GraphPad Software, San Diego CA, USA) using non-linear least-squares methods. Concentration-response curves in GH3 and DUM neurons were fitted using the following form of the Langmuir equation:

$$y = B_{max} \bullet \frac{[C]}{IC_{50} + [C]} \qquad \text{(Equation 1)}$$

where y is the percentage current inhibition, B_{max} is the maximum response (typically 100), [C] is the toxin concentration, and IC_{50} is the concentration at half-maximal block.

Concentration-response curves in GH4 cells were fitted using a two-site binding model using the following equation:

$$y = B_{\max 1} \bullet \frac{[C]}{IC_{50(1)} + [C]} + B_{\max 2} \bullet \frac{[C]}{IC_{50(2)} + [C]} \qquad \text{(Equation 2)}$$

Current-voltage (*I–V*) curves were fitted using the following equation:

$$I = g_{\max}\left(1 - \left(\frac{1}{1 + \exp\left[\left(V - V_{1/2}\right)/s\right]}\right)\right)\left(V - V_{\text{rev}}\right) \qquad \text{(Equation 3)}$$

where I is the amplitude of the peak current at a given test potential V, $g_{\max}$ is the maximal conductance, $V_{1/2}$ is the voltage at half-maximal activation, s is the slope factor, and V_{rev} is the apparent reversal potential.

2.7. Chemicals

ATP-Mg, GTP-Li, TEA-Cl, Nutrient Mixture F-10 Ham, poly-D-lysine, nitrendipine, nicardipine hydrochloride and bulk chemicals were from Sigma (Vienna, Austria or St. Louis, USA). Unless otherwise stated all sera and antibiotics were from Life Technologies (Vienna, Austria). Matrigel was obtained from Becton Dickinson (Schwechat, Austria), rat tail collagen was from Biomedical Technologies (Stoughton, MA), TTX was purchased from Latoxan (Valence, France), and ω-conotoxin GVIA was obtained from Alomone Labs (Jerusalem, Israel).

3. Results

3.1. Effects of CSTX-1 on insect neurons

To test the hypothesis that ion channel antagonism may underlie the toxicity of CSTX-1 in typical prey animals of *Cupiennius salei* we investigated the blocking effect of CSTX-1 on insect Ca_v channels in DUM neurons of the cockroach *Periplaneta americana*. We focused our attention on two subtypes of Ca_v channels that are activated by suprathreshold voltages: a mid- to low- (M-LVA) and a high-voltage-activated (HVA) Ca_v channel (Wicher and Penzlin, 1994, 1997). To prevent Ca^{2+}-induced rundown of Ca^{2+} currents, Ba^{2+} was used as a charge carrier instead of Ca^{2+} (Wicher and Penzlin, 1997). Macroscopic I_{Ba} through Ca_v channels were elicited by 100-ms depolarising command pulses from a V_h of –90 mV. Inward I_{Ba} were evoked by depolarising pulses to –30 mV (M-LVA Ca_v channel currents dominating) and +30 mV (HVA Ca_v channel currents dominating). Depolarisations to –30 mV (Fig. 2A, C, E) caused a large inward current with slow decaying component, whereas depolarisations to +30 mV elicited a smaller current with a fast decaying component (Fig. 2B, D, F). The addition of 300 nM CSTX-1 resulted in a block of peak I_{Ba} of 34.2 ± 4% (n = 5) within 5 minutes at –30 mV (Fig. 2A), and 44.2 ± 2% block of peak I_{Ba} at depolarising pulses to +30 mV **(n=5)** (Fig. 2B). At a concentration of 900 nM CSTX-1, this block increased to 72.9 ± 5% and 90.0 ± 2% at –30 mV (Fig. 2C) and +30 mV (Fig. 2D) respectively (n = 4), with total block only being achieved at concentrations above 1 μM CSTX-1. The time course of toxin-induced I_{Ba} inhibition was relatively slow and described by a single exponential function with τ_{on} of 33.1 s. In addition, the recovery after prolonged washout with toxin-free external solution was incomplete. By fitting the concentration-response curve of the inhibition of peak I_{Ba} using a Langmuir equation (Equation 1; see Methods), the concentration of CSTX-1 at half-maximal

block (IC_{50}) at a –30 mV depolarising pulse was determined to be 467 nM, and 274 nM at +30 mV (Fig. 2G, H).

To determine if the block of Ca_v channels by CSTX-1 was due to a depolarising shift in the voltage-dependence of channel activation, families of I_{Ba} were generated by 100-ms test pulses from V_h (–90 mV) to a maximum of +40 mV in 10-mV increments, every 10 seconds. Barium currents were recorded before (Fig. 3A), and after (Fig. 3B) perfusion with 300 nM CSTX-1 **(n=3)**. The I_{Ba}–V relationship was determined from the maximal I_{Ba} values at each potential (Fig. 3C). Data were normalised against peak maximal control I_{Ba} and fitted with Equation 3 (see Methods). No significant shift was observed in the voltage at half-maximal activation, $V_{1/2}$, or slope factor, s. In addition, CSTX-1 at concentrations of 100 and 300 nM produced a voltage-independent block at all test potentials (Fig. 3D).

3.2. Effects of CSTX-1 on barium currents in SCG neurons

The experiments on cockroach DUM neurons indicated that CSTX-1 acts as a blocker of Ca_v channels. Since mice were found to be the most sensitive species to *Cupiennius salei* venom (Kuhn-Nentwig et al., 1998), we tested CSTX-1 on various Ca_v channel currents of rodent neurons. Initially we investigated the effects of CSTX-1 on currents flowing through Ca_v channels in primary cultures of rat SCG neurons, because these neurons express Ca_v channels of the L-, N- and R-type families (Lin et al., 1996). To avoid Ca^{2+}-dependent inactivation of Ca_v channels and to prevent Ca^{2+}-induced rundown of Ca^{2+} currents (Kepplinger et al., 2000; Soldatov, 2003), Ba^{2+} was substituted for Ca^{2+} as the charge carrier. Ba^{2+} currents were evoked by 30-ms voltage steps from a holding potential of –80 mV to 0 mV. At a concentration of 1 μM CSTX-1 inhibited, on average, 28.5 ± 6.4% (n = 11) of I_{Ba}. An example of this effect is shown in Fig. 4A. To identify which subtype(s) of Ca_v channels is affected by CSTX-1 we tested the additivity of CSTX-1 and blockers of L- and N-type Ca_v

channels. When the L-type component of I_{Ba} was blocked by 1 μM nitrendipine, CSTX-1 at 1 μM did not further reduce the current (n = 4, Fig. 4B). CSTX-1 did, however, block I_{Ba} when the N-type component was removed by 1 μM ω-conotoxin GVIA (n = 3, Fig. 4C, D). Our data indicate that CSTX-1 selectively blocks L-type Ca_v channels in SCG neurons, whilst sparing other Ca_v channel subtypes, for example N-type channels.

3.3. Effects of CSTX-1 on barium currents in GH3 and GH4 cells

To corroborate the finding that CSTX-1 may act as an inhibitor of L-type Ca_v channels, we tested CSTX-1 on GH3 cells, because $I_{Ca(V)}$ in these cells flows primarily via L-type Ca_v channels (Lievano et al., 1994; Leão et al., 2000; Glassmeier et al., 2001; Safa et al., 2001). Barium currents were elicited in GH3 cells by 50-ms voltage pulses to 0 mV from a holding potential of –80 mV. The dihydropyridine nicardipine (4 μM) inhibited I_{Ba} by more than 90% (typically 95%, **n=?**, data not shown). CSTX-1 at a concentration of 1 μM markedly and reversibly inhibited these currents **(n=?)**, as depicted in Figure 5A and B. The time course of inhibition could be described by a single exponential function with a time constant (τ_{on}) of 3.1 s, indicating that the rate of inhibition is very rapid (**n=?**, Fig. 5C). CSTX-1 caused an inhibition of I_{Ba} that, at the holding potential (V_h = –80 mV), was independent of the test potential (n = 3, Fig. 5D, E). To investigate the potency of CSTX-1 we tested the blocking effect at concentrations between 30 and 1000 nM. Inhibition was allowed to fully develop and amplitudes of inhibited currents were determined 30 to 60 seconds after toxin application Fig. 6A shows representative superimposed current traces obtained in the absence (control) and presence of 30 nM and 300 nM CSTX-1. The concentration-response graph determined from these experiments shows that CSTX-1 inhibits I_{Ba} in GH3 cells in a concentration-dependent manner (***n* = ?** for each concentration) (Fig. 6B). At the highest toxin concentration tested, the inhibition of I_{Ba} amounted to 91.5 ± 3.3% (n = 5). The concentration-response data were fitted

to a Langmuir isotherm, assuming a single-site inhibition model, and the parameters B_{max} and IC_{50} were estimated using Equation 1 (see Methods). B_{max} was constrained to a maximum of 100% inhibition, and the best fit was obtained with an IC_{50} value of 263 nM (continuous line on Fig. 6B, r^2=0.915). Little alteration occurred when B_{max} was constrained to 95%, the typical percentage of nicardipine-sensitive I_{Ba} (IC_{50} = 233 nM, r^2=0.900) or when the Hill coefficient (n) was treated as a variable (IC_{50} = 265 nM, n = 1.09, r^2=0.918). The data suggest that CSTX-1 can completely inhibit L-type Ca_v channels. We performed similar experiments on GH4 cells, a clonal isolate of GH3, because these cells lack Na_v channels and, in the presence of K_v channel blockers, depolarising voltage steps evoke only inward currents via Ca_v channels. In this cell line, I_{Ba} were also inhibited by CSTX-1 in a fully reversible (control current levels were restored at least within 30 s after toxin removal, data not shown) and concentration-dependent manner (Fig. 7A). Here, the apparent affinity of the toxin was considerably higher than in GH3 cells. Maximum current inhibition was observed at concentrations below 100 nM, because 30 and 100 nM CSTX-1 blocked the same percentage of total I_{Ba} (namely 80.0 ± 5.1%, and 76.7 ± 9.0%, respectively). This is illustrated in the concentration-response curve shown in Fig. 7B (n = 3 to 5 for each concentration) and in the sample traces in Fig. 7C and 7D. The concentration-response data in Fig. 7B could be best fitted (r^2=0.989) by employing a two-site binding model using Equation 2 (see Materials and Methods). With this fit, two plateaus with B_{max1} = 34.2% and B_{max2} = 45.2% with corresponding IC_{50} values of $IC_{50(1)}$ = 0.03 pM and $IC_{50(2)}$ = 393 pM were determined. To confirm that I_{Ba} was carried by L-type Ca_v channels in the GH4 cells we performed experiments using the dihydropyridine Ca_v channel antagonist nitrendipine. A concentration-response curve for the current block by nitrendipine was established to determine the contribution of the L-type Ca^{2+} current to I_{Ba} at a saturating concentration of the dihydropyridine. These experiments revealed an IC_{50} for nitrendipine of 267 nM and showed that on average 73.9 ± 6.1% (n = 5-9) of I_{Ba} in GH4 cells are carried via L-type Ca_v channels

(Fig. 8). This data also indicates clonal variation between GH3 and GH4 cells, since in the former cell line we observed up to a 95% contribution of the dihydropyridine-sensitive channel subtype to whole cell Ca^{2+} currents (see above).

3.4. CSTX-1 does not affect T-type and muscle L-type Ca_v channels

Having identified L-type Ca_v channels as a high affinity target of CSTX-1, we aimed to further address the subtype selectivity of this toxin. Because 100 nM CSTX-1 induced a saturating effect on I_{Ba} in GH4 cells and caused considerable inhibition in GH3 cells, we used this concentration to test for a specific effect of the spider toxin on other types of Ca_v channels. Since the effect of CSTX-1 on L-type Ca_v channels in GH3/GH4 cells was rapid, we evaluated current amplitudes after a 60 sec toxin application period, a duration during which maximum inhibition had always been reached in the experiments on GH3 and GH4 cells.

Dihydropyridine-insensitive I_{Ba} in SCG neurons were not affected by CSTX-1. While this current was suggested to flow via N-, R- and P/Q-type Ca_v channels (Lin et al., 1996), other types of Ca_v channels are not present in this neuronal cell type and thus need to be investigated separately in other preparations. We used dorsal root ganglion (DRG) neurons to test CSTX-1 on T-type Ca_v channels. Currents carried via these channels are characterized by their transient nature, low-voltage-activation (LVA channels) and by pronounced steady-state inactivation at depolarized potentials. When TTX-resistant $I_{Na(V)}$ were eliminated by replacement of sodium chloride with choline chloride in the bathing solution, a population of Ca_v channel currents that fitted these criteria were evoked in DRG neurons by voltage steps from a holding potential of -100 mV to a range of potentials between -55 mV and -25 mV. Voltage steps to more depolarized potentials also evoked long-lasting HVA Ca_v channel currents (Fig. 9A, B and data not shown). We chose -40 mV as a suitable test potential to

study the effect of CSTX-1 on isolated T-type Ca_V channel currents, because at this potential LVA currents appear to be maximally activated and minimally contaminated by HVA Ca_v channel currents. Following application of 100 nM CSTX-1, T-type Ca_v channel current amplitudes remained at 100.6 ± 10.0% (n = 14, Fig. 9C). Because CSTX-1 inhibited M-LVA Ca_v channel currents in DUM neurons with comparably low affinity (IC_{50} 282 nM, see section 3.1) we also tested 1 μM CSTX-1 on the rat LVA Ca_v channel currents: however, T-type Ca_v channel current amplitudes still remained unaffected and current amplitudes amounted to 96.1 ± 13.6% of control (n = 7, data not shown).

The data therefore demonstrate that CSTX-1 blocks vertebrate ion channels belonging to the L-type family of Ca_v channels, whereas channels of the N-, R- and T-type families appear to be insensitive to CSTX-1. To test for subtype-selectivity within the L-type family of Ca_v channels, we also addressed the question of whether Ca_v channels in skeletal muscle cells, which belong to the $Ca_v1.1$ subtype of the L-type Ca_v channel family (Catterall et al., 2003), could be inhibited by CSTX-1. We evoked I_{Ba} in C2C12 myoballs, using voltage-steps to +20 mV from a holding potential of –80 mV. In these muscle cells, I_{Ba} remained largely unaltered by 100 nM CSTX-1 with current amplitudes only decreased to 94.2 ± 0.6% of control (n = 3). The overlay of current traces obtained before and after toxin application revealed that neither current kinetics nor the amplitude is significantly affected by 100 nM CSTX-1 (Fig.9D).

3.5. CSTX-1 does not affect potassium and sodium currents

To determine whether CSTX-1 affects ionic currents other than those flowing via Ca_v channels, $I_{K(V)}$ were evoked in GH4 cells by 150-ms voltage steps from a V_h of –80 mV to +120 mV. Application of 20 mM TEA significantly reduced these outward currents. CSTX-1 did not alter the TEA-sensitive outward $I_{K(V)}$. With 100 nM CSTX-1 outward currents remained at 99.1 ± 2.5% (peak) and 99.4 ± 3.4% (at the end of the 150-ms voltage step, Fig.

10A, n = 5). For the investigation of $I_{Na(V)}$ we used primary cultures of SCG neurons. Sodium currents were evoked by 30-ms voltage steps from a holding potential of –80 mV to –10 mV. In the presence of 100 nM CSTX-1 $I_{Na(V)}$ remained largely unaltered with peak currents measuring 105.5 ± 8.6% of control (n = 5). These currents were, however, sensitive to TTX, which produced a complete block at a concentration of 0.5 μM (Fig. 10B). Additionally, we tested $I_{Na(V)}$ in GH3 cells for CSTX-1 sensitivity, but again observed no alterations of the currents upon toxin application (data not shown).

4. Discussion

CSTX-1 is the most important component of *Cupiennius salei* venom in terms of relative abundance and toxicity and therefore is likely to contribute significantly to the overall toxicity of the whole venom (Kuhn-Nentwig et al., 2004). The inhibitor cystine-knot structural motif identified for CSTX-1 indicated that this toxin may act as a modulator of voltage-gated ion channels. Moreover, the partial sequence homology with ω-toxins from *Phoneutria nigriventer* venom (another South American species also belonging to the Ctenidae family), namely ω-phonetoxin IIA and ω-PnTx3-6 (Cassola et al., 1998; Vieira et al., 2005) suggested that CSTX-1 may interact with Ca_v channels. This prompted us to investigate whether CSTX-1 toxicity may be caused by its putative action as an ion channel modulator. Using the whole-cell configuration of the patch-clamp technique in cockroach and a variety of rat neurons, we studied the effect of CSTX-1 on currents flowing through various voltage-gated ion channels.

In cockroach neurons, CSTX-1 inhibited Ba^{2+} currents evoked at -30 mV as well as +30 mV with similar potency. This indicates that both M-LVA and HVA Ca_v channels are blocked by this toxin in insects. Many spider toxins have evolved as effective blockers of Ca_v

channels expressed in prey animals. Considering the differences between insect and mammalian Ca_v channels (Wicher and Penzlin, 1997, and see below), it is not surprising that several of these spider toxins, namely the ω-atracotoxin-1 and -2 families, potently block Ca^{2+} currents in insects, but are ineffective on their mammalian counterpart (Wang et al., 1999; Wang et al., 2001; Tedford et al., 2004b). This phyla selectivity identifies several spider toxins as suitable lead compounds for the development of insecticides (Tedford et al., 2004a). On the other hand some spiders, for example *Agelenopsis aperta* (Adams, 2004), produce channel blockers that show potent actions in mammals, although animals from this phylum rarely contribute to the spider's prey. In particular, this has been observed in binding and electrophysiological experiments using mammalian vs. insect neurons with the δ-atracotoxin-1 family of Na_v channel modulators from Australian funnel-web spiders (Nicholson et al., 2004). Such toxins cannot be used as biopesticides but may become useful as molecular research tools in cell biology or as leads for drug development, with their therapeutic potential being dependent on ion channel subtype selectivity.

CSTX-1 appears to fall into the latter group of spider toxins, because it not only inhibits Ca_v channel currents of cockroach neurons but exerted the same effect on rat neurons. However, while in the insect preparation both M-LVA and HVA Ca_v channels were affected, CSTX-1 only inhibited L-type HVA Ca_v channel currents in neurons of the rat, leaving all other voltage-gated sodium, potassium and non-L-type Ca_v channel currents tested unaffected. Therefore we attempted to identify the molecular target of this toxin enabling it to block Ca_v channels in both invertebrates and vertebrates. To examine this issue we need to reconsider the currents that were blocked by CSTX-1. In SCG neurons, various Ca_v channel subtypes (L-, N-, and R-type) contribute to total Ca^{2+} currents (Lin et al., 1996). Several studies have shown that while N-type Ca^{2+} currents predominate, L-type Ca^{2+} currents contribute by up to 30% of the total current (Barrett and Rittenhouse, 2000; Liu et al., 2001; Martinez-Pinna et al., 2002). We found similar contributions of these Ca_v channel subtypes to I_{Ba} using the N-

type blocker ω-conotoxin GVIA and the L-type antagonist nitrendipine. CSTX-1 inhibited I_{Ba} in SCG neurons in a fully reversible manner. The lack of additivity of CSTX-1 and nitrendipine indicates that inhibition of I_{Ba} is due to a blocking effect of CSTX-1 on L-type Ca_v channels. Nevertheless, the contribution of L-type I_{Ba} to total macroscopic I_{Ba} was variable (15-40%) among different SCG neurons. Therefore we employed GH3/GH4 cells to further characterise the actions of CSTX-1 on Ca_v channels because these pituitary cell lines have been previously reported to exhibit a high proportion of L-type Ca_v channels (Leão et al., 2000). Experiments performed in this study revealed that, on average, 95% of HVA Ca_v channel currents flow via the dihydropyridine-sensitive L-type Ca_v channels in GH3 cells. Barium currents in GH3 cells were inhibited by CSTX-1 in a concentration-dependent and entirely reversible manner, with an IC_{50} of 260 nM and an inhibition of 92% ± 3% at the highest toxin concentration tested (1 μM). In GH4 cells (a clonal isolate of GH3 cells) a saturating effect of CSTX-1 on I_{Ba} was obtained with concentrations between 30 and 100 nM, which blocked 80% and 77% of total I_{Ba}, respectively. Using the dihydropyridine nitrendipine, we determined that a similar percentage of total I_{Ba} flows via L-type Ca_v channels in this cell line. Therefore, CSTX-1 appears capable of blocking up to 100% of this Ca_v channel subtype in GH3 and GH4 cells and can be considered as an effective antagonist of L-type Ca_v channels. The concentration-response curve established with GH4 cells appeared to be biphasic. The data could be best fitted using a two-site binding model yielding two IC_{50} values differing by ~1000-fold. This indicates that two high affinity targets for CSTX-1 are present in GH4 L-type Ca_v channels. The concentration-response data indicate that L-type Ca_v channels in GH3 cells have considerably lower affinity than corresponding channels in GH4 cells. The variation in affinities suggests that several different subtypes of L-type Ca_v channels may be affected by CSTX-1.

Native Ca_v channels are heteromultimers of a pore forming α_1-subunit and the auxiliary $\alpha_2\delta$, β and, in some tissues, γ subunits. These are encoded by four genes for β-

subunits (β_1 through β_4), four genes for the $\alpha_2\delta$ complex ($\alpha_2\delta$-1 through $\alpha_2\delta$-4), and eight genes for the γ-subunits (Doering and Zamponi, 2003). Available RT-PCR and electrophysiology data on GH3 cells suggest that L-type $I_{Ca(V)}$ in this cell line are mediated via the α_1-subunit of $Ca_v1.2$ (α_{1C}) and $Ca_v1.3$ (α_{1D}), with a predominance of the $Ca_v1.3$ subtype that is expressed as several splice variants (Safa et al., 2001). Considering this, the different L-type Ca_v channel targets suggested by the variation of CSTX-1 affinity in GH4 and GH3 cells may correspond to (i) different α_1-subunits, e.g. $Ca_v1.2$ and $Ca_v1.3$, (ii) splice variants of $Ca_v1.3$, or (iii) the presence of different isoforms of the various auxiliary subunits in the Ca_v channel heteromultimer, because the subunit composition has been found to significantly affect the interaction with ligands (Martin et al., 2002; Gurnett et al., 1997; Felix et al., 1997; Mould et al., 2004; Helton et al., 2002). The biphasic nature of the concentration-response curve obtained in GH4 cells may be caused by similar variations in channel subunits forming the Ca_v channel heteromer.

Considering the published channel expression data for GH3 cells, CSTX-1 appears to target vertebrate $Ca_v1.2$ and $Ca_v1.3$ channels. Our experiments on Ca_v channels in skeletal muscle cells suggest that this toxin, at least at submicromolar concentrations, does not inhibit $Ca_v1.1$ channels, which argues for subtype-selectivity within the L-type family of Ca_v channels.

In contrast to the apparent target-selectivity of CSTX-1 in rat neurons, the similar degree of current block at –30 mV (M-LVA currents dominating) and +30 mV (HVA currents dominating) in DUM neurons indicates that the molecular target of CSTX-1 maybe common to both M-LVA and HVA Ca_v channels in insects. The conclusion that M-LVA and HVA calcium currents were carried by different calcium channel populations was drawn based on pharmacological differences, such as sensitivity to cone snail and spider toxins (Wicher and Penzlin, 1997). Importantly, inhibition by these toxins often occurs with much slower kinetics than in vertebrate preparations (Wicher and Penzlin, 1997). It should be noted that

relatively slow association and dissociation kinetics of CSTX-1 on insect neurons as compared to rat neurons were also observed in this study as evidenced by a τ_{on} of 33.1 s for insect DUM neurons vs. a τ_{on} value of 3.1 s for GH3 cells. These observations indicate that Ca_v channels in cockroach neurons are only distantly related to vertebrate HVA Ca_V channels. In support of this view, antibodies directed against N- and P/Q-type Ca_v channels failed to immunostain membranes of cockroach neurons, although specific peptide toxin blockers of N- and P/Q-type channels were found capable of inhibiting $I_{Ca(V)}$ recorded from neurons of this arthropod (Wicher et al., 2001). With regard to L-type channels, Ca_v channel currents of DUM neurons were reported to show sensitivity to both phenylalkylamines and benzylalkylamines but, unlike vertebrates, not dihydropyridines such as nifedipine (Wicher and Penzlin, 1997). On the other hand, in *Drosophila* four genes have been reported to encode for the α_1-subunits of Ca_v channels based on homology to their mammalian counterparts. *Dmca1A* encodes for N-type Ca_v channels, *Dmca1D* for L-type, *Dmα_1G* for T-type while *Dmα_1U* is homologous to two Ca_v channel genes of *C. elegans* (Littleton and Ganetzky, 2000). *Dmca1D* is expressed in *Drosophila* muscle and throughout the nervous system, where alternative splicing causes substantial channel heterogeneity (Zheng et al., 1995; Ren et al., 1998). This gene is considered a candidate for encoding the phenylalkylamine-sensitive, dihydropyridine-insensitive Ca_v channel in *Drosophila* brain membranes (Zheng et al., 1995). Moreover, the Dmca1D Ca_v channel is thought to carry the dihydropyridine- and phenylalkylamine-sensitive Ca^{2+} current underlying action potentials in *Drosophila* muscle cells (Littleton and Ganetzky, 2000). In support of this suggestion, mutations in *Dmca1D* cause defects in muscle Ca^{2+} currents (Eberl et al., 1998). Thus, Dmca1D provides a perfect target for a spider toxin, since its blockade would cause muscular paralysis. The 78% sequence similarity between Dmca1D and rat brain $Ca_v1.3$ (α_{1D}) and the observation that *Drosophila* muscle HVA Ca^{2+} currents are sensitive to both phenylalkylamines and dihydropyridines (Gielow et al., 1995; Zheng et al., 1995; Morales et al., 1999) indicate that

Drosophila skeletal muscle Ca_v channels strongly resemble vertebrate L-type Ca_v channels. A similar situation may hold true for other insect species, because binding sites for both phenylalkylamines and dihydropyridines were identified in *Periplaneta* muscle cells but not in the nervous system, where only phenylalkylamines binding sites were found (Skeer and Sattelle, 1993). We therefore suggest that CSTX-1 has evolved as a blocker of muscle 'L-type-like' Ca_v channels in insects to assist the spider in capturing insect prey. Similarities between this channel and certain mammalian L-type channels of the $Ca_v1.3$ subfamily may explain the toxicity of CSTX-1 in rodent species. In support of this hypothesis, GH3 and GH4 cells preferentially express $Ca_v1.3$ α_1-subunits, and dihydropyridine-sensitive $I_{Ca(V)}$ in SCG neurons are mediated by $Ca_v1.3$, rather than $Ca_v1.2$, α_1-subunits (Lin et al., 1996). However, the degree of homology between L-type-like insect Ca_v channels and mammalian Ca_v channels of the $Ca_v1.3$ family varies among insect species. Using the ClustalW alignment program (European Informatics Institute) we found sequence identities of 47 to 66% between rodent $Ca_v1.3$ α_1-subunits and α_1-subunits of dipteran insects. This diversity may explain the differences in the susceptibility of various insect species to *C. salei* venom described previously (Kuhn-Nentwig *et al*., 1998). Flies, such as *Drosophila* and *Protophormia* spp. are the most sensitive arthropod species to *C. salei* venom (LD_{50} of < 0.02 nl/mg), whereas the LD_{50} of *C. salei* venom in *Periplaneta americana* was 10 nl/mg$^-$ (Wullschleger & Nentwig, 2002) some 10,000-fold less than in mice. This is borne out by the ~1000-fold reduction in IC_{50} values for CSTX-1 on rat GH4 cells compared to *P. americana* DUM neurons.

In conclusion, we postulate that a Ca_v channel heteromultimer containing an L-type pore-forming α_1-subunit is the most probable candidate for the molecular target of CSTX-1, especially given the potency in GH4 cells. Further experiments using heterologous expression of different combinations of L-type α_1- and auxiliary Ca_v channel subunits will be needed to unambiguously identify the high affinity molecular target of CSTX-1. Moreover, CSTX-1 appears to discriminate between channel subtypes within the L-type family of Ca_v channels in

terms of potency of channel block. Other voltage-gated ion channels appear to be insensitive to CSTX-1, including K_v and Na_v channels as well as N-type and P/Q type Ca_v channels, which are likely to be responsible for the non-L-type I_{Ba} in GH3/GH4 cells (Safa et al., 2001). Therefore our findings provide evidence that CSTX-1 may become a useful molecular tool that allows dissection of the functional role of various L-type Ca_v channel subtypes in cell biology.

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Legends

Figure 1 Purification and primary structure of CSTX-1. Purity control of CSTX-1 (10 µg) isolated from the venom of *Cupiennius salei* by RP-HPLC on an Atlantis dC_{18} 3 µm column (3.9 x 150 mm, Waters) under isocratic conditions (0.1% v/v TFA in 32% acetonitrile, Retention time [RT] 9.07 min). For complete details of isolation see the methods. The disulfide-bridge pattern for the cysteine residues (boxed in black) are indicated above the sequences as well as the positive charge, especially of the C-terminus of the peptide.

Figure 2 Concentration-dependent block of Ca_v channels in cockroach DUM neurons by CSTX-1. Whole-cell I_{Ba} were evoked by 100-ms depolarising test pulses from a holding potential of –90 mV to –30 mV (left-hand panels) and +30 mV (right-hand panels) respectively, in the absence, and presence, of CSTX-1. Panels show effects following a 10-min perfusion with 100 nM (A, B), 300 nM (C, D) and 900 nM CSTX-1 (E, F). Washout with toxin-free solution did not significantly reverse these effects. (G, H) Concentration-response curves for the inhibition of peak I_{Ba} by CSTX-1 at –30 mV (G) and +30 mV (H). Data were fitted with Eq. 1 in the Methods section. Numbers in brackets above each data point represent the number of experiments performed at that concentration.

Figure 3 Effects of CSTX-1 on the voltage-dependence of Ca_v channel activation in cockroach DUM neurons. Families of I_{Ba} were elicited by depolarising test pulses to +40 mV from a holding potential of –90 mV in 10-mV steps. Data shown represents superimposed current traces recorded before (A), and after (B), a 10-min perfusion with 300 nM CSTX-1. (C), Peak I_{Ba}–V relationship showing I_{Ba} recorded before (closed circles), and after (open circles), application of 300 nM CSTX-1 (n = 3). Data were fitted with Eq. 3 in the Materials and Methods section. (D) Voltage-independent block by CSTX-1. Percentage inhibition of

peak I_{Ba} by 100 nM (closed circles) and 300 nM (open circles) CSTX-1 at different depolarising test pulses (n = 3).

Figure 4 CSTX-1 blocks L-type Ba^{2+} currents in SCG neurons. I_{Ba} were elicited by 30-ms voltage steps from a holding potential of –80 mV to 0 mV. Currents are shown normalised to their respective control currents. (A) Current traces recorded before (control), during and after (wash) application of 1 µM CSTX-1. (B) Current traces recorded from the same neuron as in A in the presence of 1 µM nitrendipine and after the subsequent addition of 1 µM CSTX-1. In this neuron CSTX-1 blocked I_{Ba} by 33% (A) but did not further reduce the current in the presence of nitrendipine (B). (C) and (D) Currents recorded under control conditions, after addition of 1 µM ω-conotoxin GVIA (GVIA), after subsequent addition of 1 µM CSTX-1 and after washout of toxins (wash). Note that block by ω-conotoxin GVIA is irreversible. The current recorded in the presence of 0.5 mM Cd^{2+} is also shown (Cd^{2+}). In the experiment shown in (C), the conotoxin blocked I_{Ba} by 50%. CSTX-1 blocked 28% of the remaining non-N-type current, which equals 14% of total I_{Ba}. 36% of total I_{Ba} in this neurone were insensitive to conotoxin and CSTX-1. In the experiment shown in (D), 41% percent of I_{Ba} was blocked by the conotoxin, CSTX-1 inhibited the remaining current by 68%, which equals 40% of total I_{Ba} recorded in the absence of toxins. In (C) and (D) the current traces are shown truncated at the time where the voltage returned to the holding potential.

Figure 5 CSTX-1 inhibits Ba^{2+} currents in GH3 cells in a voltage-independent manner. Currents were induced in GH3 cells, voltage-clamped to –80 mV, by 50-ms voltage steps to 0 mV at a stimulation frequency of 0.2 Hz. (A) Plot of current density (pA/pF) determined before, during and after application of 1 µM CSTX-1. (B) Sample traces of I_{Ba} from the experiment shown in (A). (C) Time course of inhibition: I_{Ba} recorded before (50 s) and after

addition of 1 μM CSTX-1. Peak I_{Ba} amplitudes in the presence of the toxin were normalised to control peak I_{Ba}. The rate to inhibition is described by a single exponential function with a time constant (τ_{on}) of 3.1 s. Data represent the mean of 5 independent experiments. (D) Current-voltage curves for peak I_{Ba} elicited by 50-ms voltage steps from a holding potential of –80 mV to a series of depolarised potentials under control conditions (open circles) and in the presence of 1 μM CSTX-1 (closed circles). Data represent the mean of 3 independent experiments. (E) Evaluation of current inhibition at various depolarised potentials from the current-voltage relation shown in (D).

Figure 6 CSTX-1 inhibits Ba^{2+} currents in GH3 cells in a concentration-dependent manner. (A) Representative superimposed I_{Ba} traces obtained in the absence (control) and presence of 30 nM and 300 nM CSTX-1. (B) Concentration-response relationship for the inhibition of peak I_{Ba} by CSTX-1.

Figure 7 CSTX-1 blocks Ba^{2+} currents in GH4 cells in a concentration-dependent and reversible manner. (A) Ba^{2+} currents activated by 30-ms voltage steps from a holding potential of –80 mV to 0 mV in the absence (control), presence of the indicated concentration of CSTX-1 and after washout of the toxin (wash). (B) Concentration-response curve for the inhibition of peak I_{Ba} by CSTX-1. The curve was fitted to the data using a two-site binding model (see Eq. 2 in the Methods). (C) Ba^{2+} currents evoked by 30-ms voltage steps as in (A) in the absence, and presence (*), of the indicated concentration of CSTX-1. (D) Superimposed traces of I_{Ba} evoked as in (A) in the absence (control) or the presence of the indicated concentrations of CSTX-1. All currents are shown normalised to the current obtained under control conditions.

Figure 8 Ba^{2+} currents in GH4 cells are via L-type Ca_v channels. (A) Ba^{2+} currents induced in GH4 cells by 100-ms voltage steps from a holding potential of –80 mV to 0 mV in the absence (control) and presence of 0.1 to 10 μM nitrendipine, as well as after washout of the dihydropyridine (wash). (B) Concentration-response curve for the inhibition of peak I_{Ba} by nitrendipine derived from experiments (n = 5 to 9 for each concentration) carried out under the same conditions as shown in panel (A).

Figure 9 CSTX-1 neither inhibits T-type nor skeletal muscle L-type Ca_v channels. (A) Current-voltage relation from a DRG neuron of peak Ca_v channel currents evoked by 100-ms voltage steps from a holding potential of -100 mV to a range of potentials between –70 mV and +45 mV. (B) Superimposed currents from the current-voltage relation shown in (A) recorded at –55 mV, –45 mV, –35 mV, –25 mV and at –5 mV, + 5 mV as well as +15 mV. (LVA) and (HVA) in (A) and (B) denote transient LVA and sustained HVA I_{Ba}, respectively. The arrow in (A) indicates the test potential at which CSTX-1 was studied on LVA (T-type) Ca_v channel currents. (C) Superimposition of T-type I_{Ba} elicited in DRG neurons from a holding potential of –100 mV by 100-ms voltage steps to –40 mV before, and 60 sec after, application of 100 nM CSTX-1, as indicated. (D) Superimposed I_{Ba} elicited in C2C12 myoballs from a holding potential of –80 mV by 100-ms voltage steps to +20 mV before and 60 sec after application of 100 nM CSTX-1. Note that the fast current transients that occur at the beginning and at the end of the voltage pulse are shown truncated.

Figure 10 CSTX-1 does not modulate outward potassium or inward sodium currents. (A) Outward $I_{K(V)}$ were evoked in GH4 cells by 150-ms voltage steps from a holding potential of –80 mV to +120 mV. The traces show currents recorded before (control) and after addition of 100 nM CSTX-1, as well as in the presence of 20 mM TEA. (B) Inward $I_{Na(V)}$ were evoked in

SCG neurons by 30-ms voltage steps to -10 mV from a holding potential of –80 mV. The superimposed traces show currents recorded before (control), and after addition, of 100 nM CSTX-1, as well as after the addition of 0.5 μM TTX. The total overlap of the current trace recorded in the presence of CSTX-1 with the control current trace illustrates that neither amplitude nor inactivation kinetics are affected by the toxin. For clarity, parts of the control current and the current recorded during CSTX-1 application are also shown separately (shifted by 5 ms) and on an expanded time-scale (including the first 5 ms of the evoked current).