

The Janus-faced atracotoxins are specific blockers of invertebrate K_{Ca} channels

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Abbreviations: ACTX, atracotoxin; 4-AP, 4-aminopyridine; BK_{Ca} channel, large-conductance Ca^{2+} -activated K^+ channel; Ca_V channel, voltage-activated Ca^{2+} channel; ChTx, charybdotoxin; DRG, dorsal root ganglia; DUM, dorsal unpaired median; EGTA, ethylene glycol-bis(*b*-aminoethyl ether)-*N,N,N,N*-tetraacetic acid; IbTx, iberiotoxin; J-ACTX, Janus-faced atracotoxin; K_A channel, transient 'A-type' K^+ channel; K_{Ca} channel, Ca^{2+} -activated K^+ channel; K_{DR} channel, delayed-rectifier K^+ channel; K_V channel, voltage-activated K^+ channel; Na_V channel, voltage-activated Na^+ channel; *Slo*, *Slowpoke*; TEA, tetraethylammonium; TTX, tetrodotoxin.

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ABSTRACT

The Janus-faced atracotoxins are a unique family of excitatory peptide toxins that contain a rare vicinal disulfide bridge. Although lethal to a wide range of invertebrates, their molecular target has remained enigmatic for almost a decade. We demonstrate here that these toxins are selective, high-affinity blockers of invertebrate calcium-activated K^+ (K_{Ca}) channels. J-ACTX-Hv1c, the prototypic member of this toxin family, selectively blocked K_{Ca} channels in cockroach unpaired dorsal median neurons with an IC_{50} of 2 nM, but it did not significantly affect a wide range of other voltage-activated potassium (K_V), calcium (Ca_V), or sodium (Na_V) channel subtypes. J-ACTX-Hv1c blocked heterologously expressed cockroach BK_{Ca} (*pSlo*) channels without a significant shift in the voltage-dependence of activation. However, the block was voltage-dependent, indicating that the toxin likely acts as a pore blocker rather than a gating modifier. The molecular basis of the insect selectivity of J-ACTX-Hv1c was established by its failure to significantly inhibit mouse *mSlo* currents ($IC_{50} \sim 10 \mu M$) and its lack of activity on rat dorsal root ganglion neuron $I_{K(Ca)}$. This study establishes the Janus-faced atracotoxins as valuable tools for the study of invertebrate K_{Ca} channels and suggests that K_{Ca} channels might be a potential insecticide target.

INTRODUCTION

The Janus-faced atracotoxins (J-ACTXs) are a novel family of excitatory neurotoxins isolated from the venom of the deadly Australian funnel-web spider [1]. In addition to their unusual pharmacology, these peptide toxins are structurally unique: in addition to having an inhibitory cystine knot motif that is common to peptide toxins [2, 3], they contain a rare and functionally critical vicinal disulfide bridge between adjacent amino acid residues [1].

Although lethal to a wide range of invertebrates, including flies, crickets, mealworms, and budworms but inactive in mice, chickens and rats [1, 4-6], the molecular target of the J-ACTXs has remained elusive ever since their discovery. The insect-specificity and excitatory phenotype of J-ACTX-Hv1c are reminiscent of a subclass of scorpion β -toxins that target insect Na_V channels [7]. In addition, the 3D structure of J-ACTX-Hv1c resembles that of the excitatory Na_V channel modulator δ -ACTX-Hv1a from the funnel-web spider *Hadronyche versuta* [8]. However, Na_V channels cannot be the primary target of the J-ACTXs since they are active against the nematode *Caenorhabditis elegans* (G.F.K., unpublished data), which does not possess Na_V channels [9].

In this study, we used patch clamp analysis of cockroach dorsal unpaired median (DUM) neurons to determine the molecular target of the Janus-faced atracotoxins. We demonstrate that J-ACTX-Hv1c is a high-affinity blocker of insect large-conductance Ca^{2+} -activated K^+ (BK_{Ca}) channel currents, whereas it has minimal effect on mouse or rat BK_{Ca} channels. This work establishes the Janus-faced atracotoxins as valuable tools for the study of invertebrate BK_{Ca} channels, and it indicates that insect BK_{Ca} channels might be useful targets for development of novel insecticides.

RESULTS

Specificity of J-ACTX-Hv1c action

Because of its structural homology to δ -ACTX-Hv1a, the lethal toxin from Australian funnel-web spiders that delays inactivation of both vertebrate and invertebrate Na_v channels [8, 10], we examined whether J-ACTX-Hv1c modulates Na_v currents in cockroach DUM neuron. Test pulses to -10 mV elicited a fast activating and inactivating inward I_{Na} in DUM neurons that could be abolished by addition of 150 nM TTX. Subsequent exposure of isolated I_{Na} to 1 μ M J-ACTX-Hv1c failed to alter peak current amplitude, inactivation kinetics (Figure 2A), or the voltage dependence of activation (data not shown, $n = 5$). Subsequently, the actions of the toxin were assessed on global inward I_{Ca} in cockroach DUM neurons [11]. The elicited current was abolished by addition of 1 mM $CdCl_2$, confirming that currents were carried via Ca_v channels. Application of J-ACTX-Hv1c (1 μ M) failed to inhibit I_{Ca} elicited by a range of depolarizing test pulses from -80 to $+20$ mV (Figure 2B, $n = 5$), or alter the voltage dependence of Ca_v channel activation (data not shown, $n = 5$). This indicates that J-ACTX-Hv1c does not affect invertebrate Ca_v channels.

Effects of J-ACTX-Hv1c on K_v channel currents

Macroscopic I_K in DUM neurons were recorded in isolation from I_{Na} and I_{Ca} by using 200 nM TTX and 1 mM Cd^{2+} , respectively. Macroscopic I_K were elicited by 100-ms depolarizing pulses to $+40$ mV (Figure 2F inset) before, and 10 min after, perfusion with toxin. In contrast to the lack of overt modulation of Ca_v and Na_v channels, 1 μ M J-ACTX-Hv1c inhibited macroscopic outward I_K by $56 \pm 7\%$ ($n = 5$, Figure 2C). This block was not accompanied by a shift in the voltage-dependence of activation (data not shown). Block of macroscopic outward I_K indicates

that J-ACTX-Hv1c targets at least one of the four distinct K^+ channel subtypes identified in DUM neuron somata [12]. These include delayed-rectifier (K_{DR}), transient ‘A-type’ (K_A), Na^+ -activated (K_{Na}), as well as ‘late-sustained’ and ‘fast-transient’ Ca^{2+} -activated (K_{Ca}) K^+ channels. The fast-transient K_{Ca} channel differs from the late-sustained K_{Ca} channel in that it inactivates rapidly after activation and displays a voltage-dependent resting inactivation [13]. As a consequence of the inhibition of total I_K , all subtypes except K_{Na} channels were investigated as potential targets of the Janus-faced atracotoxins.

In order to isolate $I_{K(DR)}$ in DUM neurons, $I_{K(A)}$ were blocked with 5 mM 4-AP [13]. Additional experiments were required to determine the concentration of ChTx necessary to block $I_{K(Ca)}$ in DUM neurons. Initial tests using 1 mM $CdCl_2$ produced only $35 \pm 7\%$ ($n = 7$) inhibition of total outward I_K in the presence of 5 mM 4-AP. Increasing concentrations of ChTx in the presence of 1 mM $CdCl_2$ further inhibited total outward I_K in a concentration-dependent manner. Addition of ChTx revealed a steep dose-response relationship with inhibition of I_K to $46 \pm 5\%$ at 30 nM and $46 \pm 3\%$ at 100 nM ($n = 5$) indicating maximal inhibition of $I_{K(Ca)}$ at doses ≥ 30 nM (Figure 2D, E). This indicated that inhibition of Ca^{2+} entry using $CdCl_2$ alone was insufficient to block total $I_{K(Ca)}$. Experiments requiring complete inhibition of $I_{K(Ca)}$, such as those involving $I_{K(DR)}$ and $I_{K(A)}$, were therefore performed with both 1 mM $CdCl_2$ and 30 nM ChTx. Thus, outward $I_{K(DR)}$ could be recorded in isolation from other I_K channel subtypes by the addition of 1 mM $CdCl_2$, 5 mM 4-AP and 30 nM ChTx. J-ACTX-Hv1c (1 μM) did not inhibit $I_{K(DR)}$ (Figure 2F, $n = 5$), nor did it alter the voltage-dependence of activation ($n = 5$, data not shown).

Neither $I_{K(A)}$ nor $I_{K(Ca)}$ can be recorded in isolation from $I_{K(DR)}$ as they are no selective blockers of

insect K_{DR} channels [13]. Thus, $I_{K(A)}$ were isolated using a prepulse current subtraction routine in the presence of 1 mM $CdCl_2$ and 30 nM ChTx to block $I_{K(Ca)}$. $I_{K(DR)}$ were elicited in isolation from $I_{K(A)}$ by inactivating $I_{K(A)}$ using a one-second depolarizing prepulse to -40 mV followed by a 100-ms test pulse to $+40$ mV (Figure 2G inset). Currents recorded under these conditions were digitally subtracted off-line from $I_{K(DR)}$ and $I_{K(A)}$ recorded with a prepulse potential to -120 mV. This permitted isolation of $I_{K(DR)}$ from $I_{K(A)}$. J-ACTX-Hv1c ($1 \mu M$) produced a minor inhibition of $I_{K(A)}$ by $14 \pm 4\%$ ($p < 0.05$, $n = 5$) elicited by depolarizing pulses to $+40$ mV (Figure 2F). Again, J-ACTX-Hv1c failed to alter the voltage dependence of activation (data not shown, $n = 5$).

To record $I_{K(Ca)}$ in isolation from other K_V channel currents, a current-subtraction routine following perfusion with the K_{Ca} channel blockers $CdCl_2$ and ChTx was utilised. Control macroscopic $I_{K(DR)}$ and $I_{K(Ca)}$ were elicited in the presence of 5 mM 4-AP to block $I_{K(A)}$. J-ACTX-Hv1c was then perfused for a period of 10 min or until equilibrium was reached. $CdCl_2$ (1 mM) and ChTx (30 nM) were then added to block K_{Ca} channels. Residual K_{DR} channel currents recorded in the presence of the $I_{K(Ca)}$ blockers were then digitally subtracted from both controls and currents recorded in the presence of J-ACTX-Hv1c (see Fig. 2G) to isolate $I_{K(Ca)}$. This subtraction routine is valid given the distinct lack of activity of J-ACTX-Hv1c on $I_{K(DR)}$. Isolated $I_{K(Ca)}$ exhibited fast activation, but inactivated in two phases. Initial inactivation resulted in a fast-transient component, with a subsequent late-maintained phase that displayed much slower inactivation kinetics. The $I_{K(Ca)}$ also activated at membrane potentials greater than -50 mV. These characteristics are classical of BK_{Ca} currents recorded in DUM neurons [12, 13].

In contrast to the lack of overt actions on K_{DR} and K_A channels, J-ACTX-Hv1c produced a potent block of $I_{K(Ca)}$ that was only partially reversible following prolonged washout in toxin-free

solution (Fig. 3A). Inhibition of cockroach $I_{K(Ca)}$ was dose-dependent with IC_{50} values of 2.3 nM and 2.9 nM, at +40 mV, for the fast-transient and late-sustained $I_{K(Ca)}$, respectively (Fig. 3D). In order to further examine the hypothesis that the target of J-ACTX-Hv1c is an insect K_{Ca} channel we investigated if the toxin could produce an additional block in the presence of maximal concentrations of ChTx. Following inhibition of I_K with 30 nM ChTx, subsequent application of 1 μ M J-ACTX-Hv1c failed to produce any additional block (Fig. 3E). In the complementary experiment, 30 nM ChTx failed to produce any additional block of I_K following inhibition of the current with 1 μ M J-ACTX-Hv1c (Fig. 3F). These findings provide further evidence that these peptides act on the same molecular target in insect DUM neurons, namely K_{Ca} channels.

The effect of J-ACTX-Hv1c on $I_{K(Ca)}$ was invertebrate-selective as the toxin failed to block either macroscopic outward K_V currents in rat DRG neurons (Fig. 3B, $n = 4$) or $I_{K(Ca)}$ in these neurons (Fig. 3C, $n = 4$) isolated using the same current subtraction routine as described earlier. Block of $I_{K(Ca)}$ occurred without significant alteration of the voltage-dependence of K_{Ca} channel activation, including both the $I_{K(Ca)}$ threshold and $V_{1/2}$ (Figs 4A–D).

Effects on S/I_o channels.

The above findings suggest that J-ACTX-Hv1c selectively blocks cockroach BK_{Ca} channels rather than small- (SK_{Ca} , $K_{Ca2.x}$) and intermediate-conductance (IK_{Ca} , $K_{Ca3.x}$) K_{Ca} channels. First, the $I_{K(Ca)}$ in cockroach DUM neurons was voltage-activated, like all known BK_{Ca} currents, whereas SK_{Ca} and IK_{Ca} channel currents are voltage-insensitive. Second, no apamin-sensitive SK_{Ca} channels have been found in isolated cockroach DUM neurons [13]. Nevertheless, we confirmed that J-ACTX-Hv1c specifically blocks insect BK_{Ca} channels by examining its effect

on cockroach BK_{Ca} (*pSlo*) channels heterologously expressed in HEK293 cells. For these experiments we used the $AAAA\Delta$ splice variant that is strongly expressed in octopaminergic DUM neurons [14].

Consistent with previous reports [14], application of 10 mM TEA or 1 μ M ChTx produced an $84.1 \pm 1.5\%$ ($n = 31$) and $80.1 \pm 2.1\%$ ($n = 19$) block, respectively, of *pSlo* currents activated by depolarizing pulses to +40 mV. J-ACTX-Hv1c caused a concentration-dependent block of *pSlo* currents with an IC_{50} of 240 nM (Fig. 5A, C). This IC_{50} is 83-fold higher than that observed on DUM neuron $I_{K(Ca)}$ but similar to the IC_{50} of 150 nM previously reported for ChTx on *pSlo* [14]. The time constant (t_{on}) for block of *pSlo* currents by 300 nM J-ACTX-Hv1c was 102 s but the block was only partially reversible upon washout (Fig. 5D).

In contrast to its action on *pSlo* channels, J-ACTX-Hv1c only inhibited *mSlo* channels at much higher concentrations, with an estimated IC_{50} of $>9.7 \mu$ M (Fig. 5B, C). J-ACTX-Hv1c did not significantly shift the voltage-dependence of *Slo* channel activation (Figs 5E–G) nor did it alter the kinetics of channel activation (Fig. 5A, F). Similar to ChTx [15], the block of *pSlo* currents was voltage-dependent (Fig. 5G) suggesting that the blocker enters the electric field within the pore or interacts with permeant ions within the field. In this scenario, opening of the channel in response to large depolarizations would occur because the toxin dissociates from the pore. In support, alanine mutants of the pseudo-dyad (Arg⁸ and Tyr³¹) are inactive [4], consistent with Arg⁸ being important in binding to the pore region (see below) as is the case for Lys²⁷ in ChTx (see Fig. 1G and [16]).

Mapping the toxin pharmacophore

The functionally critical residues of J-ACTX-Hv1c were previously mapped using alanine-scanning mutagenesis [4, 5]. This revealed a bipartite epitope comprising residues Arg⁸, Pro⁹, and Tyr³¹ and the two residues that form the vicinal disulfide (Cys¹³ and Cys¹⁴). It was proposed that two additional residues, Ile² and Val²⁹, act as ‘gasket’ residues that exclude bulk solvent from the putative target binding site [4]. However, since toxin activity was examined using a fly lethality assay it is possible that some of these residues are not important for interaction with BK_{Ca} channels *per se* but rather are important for conferring resistance to proteases and/or the ability of the toxin to penetrate anatomical barriers. Thus, we decided to directly examine whether the functionally critical non-cysteine residues are critical for interaction with insect BK_{Ca} channels. Ile² was not investigated as it is not conserved in all J-ACTX-1 family members (Fig. 1A). CD spectra revealed that none of the mutations used in this study induced perturbations of the toxin structure [4].

The activity of the mutant toxins was examined using DUM neurons, rather than *pSlo*-expressing HEK293 cells, for two reasons. First, it is possible that an as yet unknown subunit modulates the pharmacology of BK_{Ca} blockers on insect *Slo* channels [17], evident from the higher potency of ChTx on native neurons [14]. Second, the lower potency of the wild-type toxin on *pSlo* channels would necessitate testing of relatively high concentrations of the mutants to determine their IC₅₀ values. Dose-response curves revealed that the IC₅₀ values for the block of DUM neuron $I_{K(Ca)}$ by the R8A, P9A and Y31A mutants was 1620-, 100-, and >10,000-fold higher, respectively, than the IC₅₀ value recorded for wild-type toxin (Fig. 6D–G), consistent with the critical roles identified for those residues in previous insect lethality assays [4]. The V29A mutation caused a 7.5-fold decrease in block of $I_{K(Ca)}$ (Fig. 6D, G, H), consistent with its less critical role in

insecticidal activity [4].

Chemical features of the toxin pharmacophore

To further probe the functional relevance of these residues and to investigate the role of individual chemical moieties in the toxin's interaction with insect BK_{Ca} channels, we designed a panel of additional mutants and determined their IC_{50} for inhibition of DUM neuron $I_{K(Ca)}$ as well as their LD_{50} when injected into house flies (*Musca domestica*). We first addressed the functional role of Arg⁸, the only charged residue in the pharmacophore, by construction of R8E, R8K, R8H and R8Q mutants. We previously showed that introducing a negative charge (R8E) results in a dramatic decrease in insecticidal activity, implying that the positively charged δ -guanido group contributes significantly to target binding [4]. If Arg⁸ makes an ionic interaction with a negatively charged group on the target, then an R8E mutation would be expected to reduce potency even more than an R8A mutation because it will introduce repulsive electrostatic interactions. While the R8E mutant exhibited a marked 2237-fold reduction in block of $I_{K(Ca)}$ relative to wild-type toxin (Fig. 6E, H), its IC_{50} and LD_{50} values were nevertheless only 1.4-fold and 2.8-fold higher, respectively, than the R8A mutant (Fig. 6H). Moreover, replacement of the Arg⁸ sidechain with the slightly shorter Lys sidechain caused a dramatic 226-fold reduction in IC_{50} (Fig. 6B, E, H) and 31-fold reduction in LD_{50} even though the positive charge on the sidechain is maintained.

In striking contrast, an R8H mutant was 28-fold more potent at blocking $I_{K(Ca)}$ than the R8K mutant. Indeed, this mutant is only 8.2-fold less potent than the native toxin (Fig. 6A, E, H). The His sidechain is much shorter than both Arg and Lys and is only slightly charged at physiological pH. These results therefore suggest that the capacity of the residue at position 8 to act as a

hydrogen bond donor/acceptor is as important as its ability to present a positive charge to the channel. Hydrogen-bonding capacity alone is not sufficient for a high-affinity interaction with insect BK_{Ca} channels since an R8Q mutant is much less potent than R8K and R8H mutants and only slightly more potent than an R8A mutant (Fig. 6E, H).

We next probed the critical features of Tyr³¹ by measuring the ability of mutants in which Tyr³¹ was replaced with Phe, Trp, Ile, Leu, Val or Ala to block $I_{K(Ca)}$ in cockroach DUM neurons (Fig. 6F). The Y31F and, to a lesser extent, Y31W mutants displayed almost wild-type activity (Fig. 6C, F, H), indicating that the hydroxyl group is relatively unimportant and that the aromatic ring is the more critical functional moiety of Tyr³¹ for interaction with insect K_{Ca} channels. Substitution of the aromatic ring with smaller hydrophobes produced mixed results. Y31I, tested only in the fly assay because of limited quantities, was almost fully active (Fig. 6H), while Y31L was significantly less active in both DUM neurons and flies (Fig. 6F, H). This suggests that the key requirement at this position in the toxin pharmacophore is a medium-sized hydrophobe since an aromatic residue is clearly not essential given the high toxicity of the Y31I mutant.

DISCUSSION

The Janus-faced atracotoxins specifically target insect BK_{Ca} channels

The Janus-faced atracotoxins are a unique family of excitatory peptide toxins that contain a rare vicinal disulfide bond. Despite significant interest in this class of peptides as bioinsecticides [18, 19], their molecular target has until now proven elusive. In the present study we have shown that J-ACTX-Hv1c, the prototypic member of this class of toxins, is a high-affinity blocker of insect BK_{Ca} channels. Notably, this block occurred in the absence of any significant changes in the

voltage-dependence of K_{Ca} channel activation. Thus, in contrast with other spider toxins that target K_V channels [20], J-ACTX-Hv1c appears to be a channel blocker, like charybdotoxin, rather than a gating modifier. Moreover, J-ACTX-Hv1c appears to have high molecular specificity, since other insect Na_V , Ca_V and K_V channel currents were unaffected by toxin concentrations that substantially reduced $I_{K(Ca)}$.

The specific action of J-ACTX-Hv1c on insect BK_{Ca} channels was confirmed by block of BK_{Ca} currents mediated by the α -subunit of the cockroach *pSlo* channel. While the IC_{50} for block by J-ACTX-Hv1c (240 nM) was higher than for the native BK_{Ca} channel in DUM neurons, the loss of potency parallels that seen with ChTx, with an increase in IC_{50} from 1.9 to 158 nM [14]. This may be due to the absence of a modulatory subunit since the β -subunit of human *hSlo* channels causes a 50-fold increase in the affinity of ChTx for these channels [21]. Consistent with this hypothesis, the activation kinetics of native $I_{K(Ca)}$ in DUM neurons were much more rapid than *pSlo* channel currents, as previously noted [14], similar to the more rapid onset and inactivation of currents when mammalian *Slo* channels are expressed in association with $\beta 2$ and $\beta 3$ subunits [22-24]. Homologs of mammalian β subunits have not been detected in the genomes of *Drosophila* or *C. elegans* [25], and *dSlo* currents are not functionally affected by coexpression with a mammalian $\beta 1$ subunit [26]. However, gating of *dSlo* channels is modulated by co-expression with *Slowpoke* binding protein (Slob) [27], indicating that insects may possess novel subunits not present in vertebrates for regulating the activity of BK_{Ca} channels. However, until the putative regulatory subunits associated with the *pSlo* channel have been identified, the native phenotype cannot be reconstituted and the influence of these subunits on the affinity of J-ACTX-Hv1c for *pSlo* channels cannot be determined.

Since we have demonstrated that J-ACTX-Hv1c is a specific, high-affinity blocker of insect BK_{Ca} channels, we propose that it be renamed κ -ACTX-Hv1c to be consistent with the rational nomenclature proposed earlier for naming spider toxins whose molecular target has been established [28].

Mode of interaction of J-ACTX-Hv1c with insect BK_{Ca} channels

Scorpion toxins from α -KTx subfamilies 1–3 block BK_{Ca} channels in the vicinity of the selectivity filter, mainly via residues in their C-terminal β -hairpin [16]. Despite its ability to block BK_{Ca} channels, J-ACTX-Hv1c has virtually no sequence homology with scorpion BK_{Ca} blockers, particularly in the functionally critical β -hairpin region (Fig. 1B). Moreover, superposition of the 3D structure of J-ACTX-Hv1c [1] with that of ChTx [29] demonstrates that the backbone folds of the two toxins are significantly different (Fig. 1F). This raises the question of whether the two toxins interact in fundamentally different ways with insect BK_{Ca} channels.

We previously speculated that the functional Lys-Tyr/Phe dyad that is largely conserved in toxins that target vertebrate K_v channels [30] might also be present in J-ACTX-Hv1c if Arg is considered a suitable substitute for Lys [4]. The "pseudo-dyad" of J-ACTX-Hv1c is topologically similar to that of ChTx (Fig. 1G) although the overlay is not as good as with the dyad of the K_v channel blockers BgK and agitoxin 2 [4]. However, since we demonstrated in the present study that Lys is a poor substitute for the functionally critical Arg⁸ residue in J-ACTX, then this apparent similarity to the dyad of vertebrate K_v channel toxins is likely to be coincidental and not predictive of the mode of binding of J-ACTX-Hv1c to insect BK_{Ca} channels.

Several lines of evidence suggest that J-ACTX-Hv1c and ChTx engage BK_{Ca} channels via quite different molecular mechanisms. First, the pharmacophore of J-ACTX-Hv1c is much smaller and involves far fewer residues than that of ChTx (see Fig. 1D, E). Second, in contrast to ChTx and other toxins that target K^+ channels [31, 32], the block of BK_{Ca} channels by J-ACTX-Hv1c is significantly less voltage-dependent (Fig. 5G). This suggests that J-ACTX-Hv1c does not bind as deeply into the extracellular mouth of the ion channel pore as these other toxins. This is likely due to the bifurcated δ -guanidinium group at the tip of the critical Arg⁸ residue, which is much bulkier than the single amine moiety at the tip of the linear sidechain of the key Lys²⁷ residue in ChTx. Consistent with this hypothesis, a K27R mutant of ChTx is 4-fold less potent on mammalian BK_{Ca} channels [33] and the voltage-dependency of block is significantly reduced compared with native toxin. Third, the ability of His, as opposed to Lys, to effectively substitute for Arg⁸ in J-ACTX-Hv1c suggests that factors other than electrostatic charge are also important at this position in the toxin pharmacophore. Hydrogen-bonding capacity might be critical, since the Arg guanido and His imidazole moieties contain two identically spaced nitrogens that can serve as hydrogen-bond donors/acceptors. It is possible that Arg⁸ forms hydrogen bonds with surface-exposed carbonyls in the pore region of the BK_{Ca} channel. The combined evidence therefore suggests that these two toxins, although both derived from arachnid venoms, have evolved to interact in quite different ways with invertebrate BK_{Ca} channels.

J-ACTX-Hv1c as a molecular tool

Large-conductance K_{Ca} channels, also termed BK_{Ca} ($K_{Ca1.1}$), Maxi-K or *Slo1* channels, are activated by an increase in intracellular Ca^{2+} and by depolarization [34]. These channels play an important role in controlling calcium homeostasis, excitability and action potential waveform,

and BK_{Ca} currents prevent excessive Ca^{2+} entry by contributing to action potential repolarization and membrane hyperpolarization [12]. It has been suggested that activators and blockers of BK_{Ca} channels may have application as neuroprotectants or as therapeutics in certain disease states including vascular dysfunction, urinary disease, and certain seizure conditions [35]

Study of invertebrate BK_{Ca} channels would be enhanced by the availability of a readily-available, high-affinity blocker that is devoid of activity on other ion channels. While ChTx and J-ACTX-Hv1c block cockroach BK_{Ca} channels with similar affinity, J-ACTX-Hv1c offers several potential advantages as a research tool for invertebrate studies. First, in addition to its block of BK_{Ca} channels, ChTx also blocks K_V channels with moderate affinity [36]. In contrast, even at very high concentrations, J-ACTX-Hv1c has very limited activity against K_V channels. Second, a bacterial expression system has been developed that allows recombinant J-ACTX-Hv1c to be produced cheaply and easily [4]. Third, since the binding epitope for J-ACTX-Hv1c has been mapped, point mutants that could be used for negative controls can be readily produced using this bacterial expression system.

BK_{Ca} channels: a potential insecticide target?

A major bottleneck in the development of new insecticides has been the difficulty in identifying new molecular targets. Indeed, the vast majority of chemical insecticides are directed against one of five targets (four of which are ion channels) in the insect nervous system [18, 37]. Although BK_{Ca} channels play important roles in the excitability of insect neurons and muscles [38], they have not been considered potential insecticide targets since no insect-selective ligands of these channels have previously been identified. However, our demonstration that the insect-selective spider toxin J-ACTX-Hv1c is a high-affinity blocker of insect BK_{Ca} channels has, for the first

time, identified this channel as a potential insecticide target.

Interestingly, paxilline, a well-known mammalian BK_{Ca} channel blocker [39], as well as several other structurally-related indole-diterpenes, are toxic to a wide range of insect genera [40-42]. In order to determine whether the insecticidal activity of these diterpenes might stem from their activity on BK_{Ca} channels, we examined their ability to block $I_{K(Ca)}$ in cockroach DUM neurons. Importantly, paxilline blocked both the fast-transient and late-sustained $I_{K(Ca)}$, with IC_{50} values of 17.1 and 16.0 nM ($n = 7-9$) respectively (data not shown). This supports our contention that inhibition of BK_{Ca} channels may contribute to their lethality to insects and that insect BK_{Ca} channels might therefore be potential insecticide targets.

‘Short-chain’ scorpion α -KTx 1 family toxins, such as ChTx (α -KTx 1.1) and iberiotoxin (IbTx, α -KTx 1.3), are frequently used as molecular tools to study BK_{Ca} channels. However, these toxins are poor leads for development of insecticides that block *invertebrate* BK_{Ca} channels since they have limited phyletic selectivity, with a tendency to be more active against vertebrate channels (Table 1). For example, ChTx blocks mammalian *Slo* channels (IC_{50} values of 36 and 7.4 nM for *hSlo* and *mSlo*, respectively) more potently than insect *Slo* channels (IC_{50} values of 158 nM and $>5 \mu M$ for *pSlo* and *dSlo*, respectively) [14, 43]. In contrast, J-ACTX-Hv1c is highly selective for insect BK_{Ca} channels: it blocks cockroach BK_{Ca} channels at low nanomolar concentrations and shows a 41-fold preference for *pSlo* over *mSlo* (Table 1). Since J-ACTX-Hv1c is a pore blocker, and the pore regions of *mSlo*, *rSlo*, and *hSlo* are identical (see Fig. 7), we predict that J-ACTX-Hv1c will also have little effect on *rSlo* and *hSlo* channels. Consistent with this hypothesis, J-ACTX-Hv1c failed to inhibit BK_{Ca} currents in rat DRG

neurons (which express *rSlo*) and it was previously shown that subcutaneous injection of J-ACTX-Hv1c into newborn mice, at five times the LD_{50} dose in insects, fails to produce any overt signs of toxicity [1]. Moreover, J-ACTX-Hv1c failed to alter neurotransmission in an isolated chick biventer cervicis nerve-muscle preparation [1].

BK_{Ca} channels have been highly conserved throughout evolution, and therefore it may seem surprising that toxins can discriminate between invertebrate BK_{Ca} channels and their vertebrate counterparts. However, insect and mammalian *Slo* channels display several important differences in the pore region between the S5 and S6 transmembrane helices (Fig. 7), which is believed to be the primary site of interaction with ChTx, IbTx, and most likely J-ACTX-Hv1c [36]. Remarkably, the phyletic selectivity of ChTx can be manipulated by a single point mutation in this region. For example, BK_{Ca} channels from fruit flies and cockroaches become significantly more sensitive to ChTx, a vertebrate-specific BK_{Ca} blocker, when individual pore residues are mutated to that found in the corresponding position in vertebrate *Slo* channels; these mutants include T290E in *Drosophila dslo* [43] and Q285K in cockroach *pslo* [14] (see Fig. 7). Thus, the amino acid variation in the pore region of the BK_{Ca} channel appears sufficient to explain the insect selectivity of J-ACTX-Hv1c.

J-ACTX-Hv1c is active against a diverse range of insect phyla [1, 4, 6] and therefore insecticides that target this channel might find wide application in the control of arthropod pests. The molecular epitope on this peptide toxin that mediates its interaction with insect BK_{Ca} channels comprises only five spatially proximal residues (this study and [4]). Since J-ACTX-Hv1c has 870-fold higher selectivity for insect BK_{Ca} channels than ChTx, this epitope should provide a convenient template for the rational design of small-molecule insecticides that selectively target

insect BK_{Ca} channels.

MATERIALS AND METHODS

Construction and Purification of J-ACTX-Hv1c

Mutants—Single point mutations were introduced using complementary mutagenic PCR primers using pFM1 as a template. This plasmid encodes a synthetic J-ACTX-Hv1c gene with codons optimised for expression in *Escherichia coli* [4]. Mutant gene sequences were amplified, digested with *Bam* HI and *Eco* RI, and subcloned into the pGEX-2T vector using standard methods. The resultant plasmids encode a synthetic toxin gene fused to the 3' end of the gene for glutathione *S*-transferase (GST), with an intervening thrombin cleavage site. Engineered plasmids were transformed into *E. coli* BL21 cells for protein expression. Cells were grown at 37°C in LB medium to an OD_{600} of 0.6–0.8 before induction with 300 μ M IPTG. Cells were harvested by centrifugation at an OD_{600} of 1.9–2.1 and lysed by sonication. GST-toxin fusion proteins were purified by affinity chromatography on a GSH-Sepharose (Amersham Biosciences) column, then cleaved on-column with thrombin to release recombinant J-ACTX-Hv1c. Toxin was eluted from the column with buffer and further purified by reverse-phase HPLC using a Vydac C18 analytical column (4.6 x 250 mm, 5- μ m pore size). HPLC was performed using a linear gradient of 15–22% acetonitrile over 15 min at a flow rate of 1 ml/min. Correctly folded toxin eluted as the major peak with a retention time of 9–12 min depending on the mutant purified. Toxin masses were verified by electrospray mass spectrometry.

Circular Dichroic Spectropolarimetry

Far-UV circular dichroic (CD) spectra (185–260 nm) were recorded on a Jasco J-715 spectropolarimeter at 4°C. Peptides were dissolved to 25 mM in sterile water and loaded into a

0.1-cm rectangular quartz cell for analysis. Final spectra were the average of eight scans using a scan rate of 20 nm/min and a response time of 4 s. A water blank run under identical conditions was subtracted from each toxin spectrum.

Lethality Assays

House flies (*Musca domestica*) weighing 9–20 mg were injected with 1–2 μ l of toxin diluted in insect saline [44] at a concentration range of 10–106 pmol/g. Each test dose was administered to 10 flies and performed in duplicate. Control flies received 2 μ l of insect saline. All injections were dispensed with a 29-gauge needle using an Arnold microapplicator (Burkhard Scientific Supply). Flies were injected in the dorsal thorax while immobilized at 4°C, then transferred to room temperature. The dose corresponding to 50% lethality of the test population 24 h after injection (LD_{50}) was calculated using the following equation.

$$Y = \frac{100}{1 + \left(\frac{LD_{50}}{x}\right)^{n_H}}$$

where Y is the percentage response at the dose (x), and n_H is the slope (Hill) coefficient.

Electrophysiology

Whole-cell recordings of ionic currents were made using an Axopatch 200A amplifier. Patch pipettes were pulled from borosilicate glass and had resistances of 1–2 M Ω for I_{Na} recordings and 2–4 M Ω for I_{Ca} , I_K and *Slo* channel current recordings. The holding potential was –80 mV, unless stated otherwise. External solution was administered via a continuous gravity-fed perfusion system at ~1.0 ml/min and a temperature of 20–23°C. Toxins were applied via a pressurized fast perfusion system (Automate Scientific). The osmolarity of all internal and external solutions was

adjusted to within ± 5 mOsmol/l with sucrose to reduce osmotic stress. Experiments were rejected if there were large leak currents or currents showed signs of poor space clamping. Stimulation and recording were controlled by AxoData or pClamp data acquisition systems (Axon Instruments). Data was filtered at 5 kHz (low-pass Bessel filter) with digital sampling rates between 15 and 25 kHz depending on voltage protocol length. Leakage and capacitive currents were digitally subtracted with *P-P/4* procedures and series resistance compensation was $>80\%$ for all cells. The liquid junction potential was determined using JPCalc [45], and all data were compensated for this value. Analysis parameters were as described [46].

DUM neuron Na_v , Ca_v and K_v channel currents

DUM neuron cell bodies were isolated from the terminal abdominal ganglion of the American cockroach *Periplaneta americana* as described [47]. The TAG was dissected and placed in sterile Ca^{2+} -free normal insect saline (NIS) containing (in mM): NaCl 200, KCl 3.1, $MgCl_2$ 4, N-hydroxyethylpiperazine-N-ethanesulphonic acid (HEPES) 10, sucrose 30, D-glucose 20, pH 7.4. The ganglia were then desheathed and incubated for 20 min in Ca^{2+} -free NIS containing Type IA collagenase (2 mg/ml). Subsequently, the ganglia were rinsed three times in NIS, containing 5 mM $CaCl_2$, 5% v/v bovine calf serum, penicillin (50 IU/ml) and streptomycin (50 μ g/ml) (Trace Biosciences). Single cells were mechanically isolated by trituration. The resulting suspension was then allowed to adhere overnight to glass coverslips which had been previously been coated with concanavalin-A (2 mg/ml) (Sigma Chemicals). Large tear-shaped DUM neurons with diameters of >45 μ m were selected for experiments.

To record voltage-activated Na^+ (Na_v) channel currents (I_{Na}), pipettes contained (in mM): NaCl

20, CsF 135, $MgCl_2$ 1, ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) 5, D-glucose 10, and HEPES 10, pH 7.4. The external solution contained (in mM): NaCl 130, CsCl 5, TEA-Cl 20, $CaCl_2$ 1.8, 4-aminopyridine (4-AP) 5, verapamil-HCl 0.01, $NiCl_2$ 0.1, $CdCl_2$ 1 and HEPES 10, pH 7.4. To record voltage-activated Ca^{2+} (I_{CaV}) channel currents (I_{Ca}), pipettes contained (in mM): CsCl 110, Na-acetate 10, ATP- Na_2 2, $CaCl_2$ 0.5, tetraethylammonium (TEA)-Br 50, EGTA 10, HEPES 10, pH 7.4. The external solution contained (in mM): Na-acetate 160, TEA-Br 30, $CaCl_2$ 5, HEPES 10, 150 nM TTX, pH 7.4. To record delayed-rectifier ($I_{K(DR)}$), transient 'A-type' ($I_{K(A)}$) and K_{Ca} ($I_{K(Ca)}$) channel currents, pipettes contained (in mM): KCl 135, KF 25, NaCl 9, ATP- Na_2 3, $CaCl_2$ 0.1, $MgCl_2$ 1, EGTA 1, HEPES 10, pH 7.4. External solutions contained (in mM): NaCl 100, KCl 30, $CaCl_2$ 5, $MgCl_2$ 1.5, D-glucose 10, HEPES 10, 150 nM TTX, pH 7.4. Since I_{Na} were blocked by TTX, any involvement of Na^+ -dependent I_K were eliminated. To record $I_{K(DR)}$ in isolation, 5 mM 4-AP, 1 mM $CdCl_2$ and 30 nM charybdotoxin (ChTx) were also added to the external solution to eliminate $I_{K(A)}$ [13] and $I_{K(Ca)}$ (see Results). $I_{K(A)}$ were elicited in the presence of 1 mM $CdCl_2$ and 30 nM ChTx and isolated using current subtraction routines following a two-pulse protocol to inactivate $I_{K(A)}$ (see Results). $I_{K(Ca)}$ were elicited in the presence of 5 mM 4-AP and isolated from $I_{K(DR)}$ using current subtraction routines following addition of 1 mM $CdCl_2$ and 30 nM ChTx (see Results).

DRG neuron K_V channel currents

Acutely dissociated dorsal root ganglion (DRG) neurons were prepared from 5 to 14-day-old Wistar rats and maintained in short-term primary culture as described [48]. Small- to medium-sized DRG neurons with diameters of 18–45 μm were selected for experiments as they have

previously been shown to express BK_{Ca} channels [49]. To record $I_{K(Ca)}$ pipettes contained (in mM): KCl 140, tetramethylammonium (TMA)-Cl 50, $CaCl_2$ 0.5, D-glucose 5, EGTA 1 and HEPES 5, pH 7.0. The external solution contained (in mM): TMA-Cl 120 KCl 5, NaCl 30, $MgCl_2$ 1, $CaCl_2$ 1.8, D-glucose 25, 4-AP 5, 300 nM TTX, HEPES 5, pH 7.2. Following completion of the experiment, the presence of $I_{K(Ca)}$ was confirmed by perfusion with 1 mM $CdCl_2$ and 100 nM ChTx. $I_{K(Ca)}$ was then isolated by subtraction from residual I_K .

***Slo* channel currents**

HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% v/v bovine calf serum and L-glutamine (1 mM). Expression of *pSlo* and *mSlo* was performed by transfection of HEK293 cells with a construct containing the coding region cloned into the expression vector pcDNA3.1, which also carries the G418 resistance gene. Stably transfected cells were then selected with 1 mg ml⁻¹ G418. These cells were maintained in the normal growth media described above and cultured on sterile glass coverslips. To record *Slo* channel currents from HEK293 cells pipettes contained (in mM): NaCl 4, KCl 140, ATP-Mg 2, $CaCl_2$ 0.1, HEPES 10, pH 7.25. The external solution contained (in mM): NaCl 135, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, NaH_2PO_4 0.33, D-glucose 10, HEPES 10, pH 7.4.

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FIGURE LEGENDS

Figure 1: Structure of the J-ACTX-Hv1c and comparison with other BK_{Ca} blockers. (A)

Primary structure of J-ACTX-1 family members. Identities are boxed in yellow. Green lines above the sequences represent the disulfide bonding pattern, while the arrowheads below highlight the pharmacophore (red) and proposed water-excluding gasket (pink) residues of J-ACTX-Hv1c. (B) Comparison of the primary structure of J-ACTX-Hv1c with known BK_{Ca} ($K_{Ca1.x}$) and SK_{Ca} ($K_{Ca2.x}$) channel blockers. Only toxins with nanomolar affinity for K_{Ca} channels are included. Toxins listed above the BmBKTx1 sequence are BK_{Ca} channel blockers while those below are SK_{Ca} channel blockers. (C) Schematic of the structure of J-ACTX-Hv1c (PDB code 1DL0) highlighting the sidechains of the key pharmacophore residues (green) as well as those proposed to serve as a water-excluding "gasket" (see text for details). Disulfide bonds and β strands are shown in red and cyan, respectively. (D and E) Surface representation of J-ACTX-Hv1c (D) and ChTx (E) highlighting the primary pharmacophore residues. In the case of ChTx (α -KTx 1.1), six of the eight residues crucial for activity on BK_{Ca} channels are located on the β strands. Pharmacophore and gasket residues are shown in green and yellow, respectively. (F) Overlay of the structure of J-ACTX-Hv1c (red) and ChTx (PDB code 2CRD, blue). (G) Stereoview of an overlay of the functional dyad of ChTx (green sidechains) with the "pseudo-dyad" of J-ACTX-Hv1c (red sidechains). Only the backbone of J-ACTX-Hv1c is shown for the sake of clarity.

Figure 2: Effect of J-ACTX-Hv1c on voltage-activated ion channels in cockroach neurons.

(A and B) Superimposed current traces showing typical lack of effect of 1 μ M J-ACTX-Hv1c on I_{Ca} (A) and I_{Na} (B). (C) Inhibition of macroscopic I_K by 1 μ M J-ACTX-Hv1c. (D) Typical block

of $I_{K(Ca)}$ by increasing concentrations of ChTx (in nM). Subsequent addition of TEA in the presence of 30 nM ChTx abolished remaining current, thus confirming that currents were carried by K_V channels. Data were recorded from the same cell. **(E)** Dose-response curve for ChTx inhibition of $I_{K(Ca)}$ recorded at the end of the pulse, in the presence of 1 mM Cd^{2+} ($n = 5$). **(F and G)** Typical effects of 1 μ M J-ACTX-Hv1c on $I_{K(DR)}$ **(F)** and $I_{K(A)}$ **(G)**. Superimposed $I_{K(A)}$ were obtained by current subtraction routines following prepulse potentials of -120 and -40 mV shown in the inset (see Methods). **(H)** Current subtraction routine employed to isolate $I_{K(Ca)}$ (see Methods). Currents in panels **C**, **D**, **F** and **H** were elicited by the test pulse protocol shown in the inset of panel **F**.

Figure 3: J-ACTX-Hv1c blocks K_{Ca} channels in cockroach DUM neurons. **(A)** Typical effects of 3 nM J-ACTX-Hv1c on $I_{K(Ca)}$ showing partial reversibility. **(B)** Typical effect of 1 μ M J-ACTX-Hv1c on rat DRG neuron macroscopic I_K . **(C)** J-ACTX-Hv1c (1 μ M) failed to inhibit rat DRG neuron $I_{K(Ca)}$ isolated by subtraction of the current remaining following addition of 100 nM ChTx and 1 mM Cd^{2+} shown in panel B. **(D)** Dose-response curve showing inhibition of $I_{K(Ca)}$ by J-ACTX-Hv1c in the presence of 1 mM Cd^{2+} ($n = 3$ at 1 μ M and $n = 5$ at all other concentrations). Currents in panels **A–D** were elicited by the test-pulse protocol shown in the inset of panel A. **(E and F)** J-ACTX-Hv1c and ChTx share the same target in cockroach DUM neurons. **(E)** Addition of 1 μ M J-ACTX-Hv1c fails to further inhibit I_K currents blocked by perfusion with 30 nM ChTx and 1 mM Cd^{2+} ($n = 5$). **(F)** In the complementary experiment, addition of 30 nM ChTx and 1 mM Ca^{2+} fails to further inhibit I_K currents blocked by perfusion with 1 μ M J-ACTX-Hv1c ($n = 5$). In both **E** and **F** currents were recorded in the presence of 4-AP to block $I_{K(A)}$.

Figure 4: Effects of J-ACTX-Hv1c on voltage-dependence of K_{Ca} channel activation in cockroach DUM neurons. (A and B) Typical families of $I_{K(Ca)}$ were elicited by 10-mV steps to +40 mV before (A) and after (B) the addition of 3 nM J-ACTX-Hv1c. (C–D) I/V curves for fast-transient (C) and late-sustained (D) $I_{K(Ca)}$ for controls (closed symbols), after 3 nM J-ACTX-Hv1c (open symbols), and following prolonged washout with toxin-free solution (gray symbols) ($n = 5$). Families of currents were elicited by the test pulse protocol shown in the inset of panel B.

Figure 5: Dose-dependent inhibition of Slo currents by J-ACTX-Hv1c (A and B) Typical effects of J-ACTX-Hv1c on $pSlo$ at 300 nM (A), and $mSlo$ at 3 μ M (B). (C) Dose-response curve for J-ACTX-Hv1c inhibition of Slo currents ($IC_{50} = 240$ nM, $n = 6$). For $mSlo$ currents, the IC_{50} was >9.7 μ M ($n = 4$). Currents in panels A–C were elicited by the upper test pulse protocol shown between panels A and B. (D) Timecourse of block of $pSlo$ currents by 300 nM J-ACTX-Hv1c and washout in toxin-free solution ($n = 5$). (E and F) Typical families of $I_{K(Ca)}$ were elicited by 10-mV steps from -90 to $+80$ mV before (E) and after (F) addition of 300 nM J-ACTX-Hv1c. Families of currents were elicited by the test-pulse protocol shown between panels E and F. (G) I/V curves for late $pSlo$ currents. Data corresponds to controls (closed symbols), after addition of 3 nM J-ACTX-Hv1c (open symbols), and following washout with toxin-free solution (gray symbols) ($n = 6$). (H) Voltage-dependence of the fractional block of $pSlo$ currents by 300 nM J-ACTX-Hv1c ($n = 6$).

Figure 6: Effect of J-ACTX-Hv1c mutants on cockroach DUM neuron $I_{K(Ca)}$. (A–D) Typical

effects of (A) 10 nM R8H, (B) 300 nM R8K, (C) 300 nM Y31F and (D) 30 nM V29A mutants on $I_{K(Ca)}$. Calibration bars represent 5 nA and 25 ms. (E–G) Dose-response curves for inhibition of peak $I_{K(Ca)}$ by Arg⁸ (E), Tyr³¹ (F), and Val²⁹ and Pro⁹ (G) mutants ($n = 3-4$). (H) Comparison of fold-reduction in DUM neuron $I_{K(Ca)}$ IC₅₀ (left y-axis, light bars) and house fly LD₅₀ (right y-axis, dark bars). For comparison, data for the fold-reduction in house fly LD₅₀ for R8A, R8E, P9A, Y31F and Y31A mutants are included [4]. *Mutant Y31A (gray symbols in panel F) has an estimated IC₅₀ value $\geq 10 \mu\text{M}$.

Figure 7: Alignment of the pore region of vertebrate and invertebrate *Slo* channels. This alignment is restricted to the pore region located between transmembrane segments S5 and S6. Sequences are from insects (*Periplaneta americana*, *p*; *Anopheles gambiae*, *a*; *Apis mellifera*, *Am*; *Tribolium castaneum*, *Tc*; *Manduca sexta*, *Ms*; *Drosophila melanogaster*, *d*; and *D. pseudoobscura*, *Dp*), vertebrates (chicken, *c*; mouse, *m*; rat, *r*; human, *h*; rabbit, *Rb*; bovine, *b*; *Canis familiaris*, *Cf*; and *Xenopus laevis*, *x*), marine invertebrates (*Cancer borealis*, *Cb*; and *Aplysia californica*, *Ac*) and *Caenorhabditis elegans* (*Ce*). Identical residues are boxed in gray while conservative substitutions are in gray italic text. Arrowheads denote residues important for ChTx binding (see text for details).

TABLE 1: Phyletic selectivity of J-ACTX-Hv1c and ChTx

BK_{Ca} channel		IC₅₀ (nM)	
		J-ACTX-Hv1c	ChTx ^a
Invertebrate	Native DUM neuron BK _{Ca}	2.3	1.9*, 1.4 ^b
	<i>pSlo</i>	240	158 ^b
Vertebrate	Native rat DRG neuron BK _{Ca}	>1 000	<100 ^a
	<i>hSlo</i>	N.D.	36 ^c
	<i>mSlo</i>	9776	7.4 ^c
<i>Phyletic selectivity (mSlo IC₅₀ / pSlo IC₅₀)</i>		41	0.047

ChTx data from ^aScholz *et al.* (1998), ^bDerst *et al.* (2003), ^cMyers *et al.* (2000), and *present study.

N.D., not determined.