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Crotamine Pharmacology Revisited: Novel Insights Based on the Inhibition of K_V Channels[S]

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ABSTRACT

Crotamine, a 5-kDa peptide, possesses a unique biological versatility. Not only has its cell-penetrating activity become of clinical interest but, moreover, its potential selective antitumor activity is of great pharmacological importance. In the past, several studies have attempted to elucidate the exact molecular target responsible for the crotamine-induced skeletal muscle spasm. The aim of this study was to investigate whether crotamine affects voltage-gated potassium (K_V) channels in an effort to explain its in vivo effects. Crotamine was studied on ion channel function using the two-electrode voltage clamp technique on 16 cloned ion channels (12 K_V channels and 4 Na_V channels), expressed in *Xenopus laevis* oocytes. Crotamine selectively inhibits $K_V1.1$, $K_V1.2$, and $K_V1.3$ channels with an IC_{50} of ~300 nM, and the key amino acids responsible for this molecular interaction are suggested. Our results demonstrate for the first time that the symptoms, which are observed in the typical crotamine syndrome, may result from the inhibition of K_V channels. The ability of crotamine to inhibit the potassium current through K_V channels unravels it as the first snake peptide with the unique multifunctionality of cell-penetrating and antitumoral activity combined with K_V channel-inhibiting properties. This new property of crotamine might explain some experimental observations and opens new perspectives on pharmacological uses.

Introduction

Crotamine is a 42-amino acid peptide present in the venom of *Crotalus durissus* terrificus rattlesnake (Laure, 1975). This molecule belongs to the small basic myotoxins family because of the high primary sequence identity, but also its fold and potential surface resemble the structural features of β-defensin-like peptides, including sea anemone Anthopleurin toxins, despite their different biological activities and low primary sequence similarity (~30%) (Siqueira and Nicolau, 2002; Nicastro et al., 2003). Nonetheless, it possesses a wide spectrum of biological activity, such as membrane penetration into different cell types and mouse blastocysts in vitro (Rádis-Baptista and Kerkis, 2011), antitumoral agent against several aggressive tumorigenic cell lineages but inactive against normal cells (Kerkis et al., 2010), irreversible membrane depolarization, and spontaneous repetitive firings of mammalian skeletal muscle (Chang and Tseng, 1978; Rizzi et al., 2007). When investigating the depolarizing action of crotamine in diaphragm muscle of mouse and rat, Chang and Tseng (1978) concluded that it was attributed to the effects on sodium channels because this depolarization could be reversed noncompetitively by tetrodotoxin (TTX), procaine, and high calcium and low sodium media (Chang and Tseng, 1978). After observing that TTX prevents and also restores the depolarization of membrane potential caused by crotamine but did not impede its irreversible binding, Hong and

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ABBREVIATIONS: TTX, tetrodotoxin; PDB, Protein Data Bank; r, rat; h, human; DTX, dendrotoxins; CS$\alpha\beta$, cysteine-stabilized α-helix and β-sheet.

Chang (1983) inferred that the interaction site of TTX is distinct from that of crotamine (Chang et al., 1983).

After several studies showing indirect evidence that crotamine acts on Na^+ channels, Rizzi et al. (2007) demonstrated that it actually did not affect directly mammalian voltage-dependent sodium channels. The authors used transfected human embryonic kidney cells expressing the α-subunits of Na_V 1.1 to 1.6 as well as dorsal root ganglion neurons (Rizzi et al., 2007). Those results were also supported by an envenoming behavior comparison between crotamine and toxins with proven sodium channel activity (tetrodotoxin, μ-conotoxin-GIIIa, BcIII, Tx2-6, and α- and β-pompilidotoxins), which were unable to mimic the hind-limb paralysis caused by crotamine. It was later proposed that crotamine could act as a voltage-dependent potassium channel blocker based on its three-dimensional structure resemblance with human antibacterial β-defensins using computational docking (Yount et al., 2009). These results suggested that crotamine interacts with specific residues of the channel pore.

To finally elucidate the exact target through which crotamine exerts its observed activity, we submitted it to a detailed and straightforward electrophysiological screening on a wide range of 16 ion channels expressed in *Xenopus laevis* oocytes. It was observed that the potent and selective K_V channel-inhibiting properties of crotamine confirmed the suggestion by Yount et al. (2009) and the inability of interaction with sodium channels as shown by Rizzi et al. (2007). Our findings do not contradict the pharmacological results with crotamine as discussed herein.

Materials and Methods

Purification and Biochemical Characterization of Crotamine. *C. durissus* terrificus venom was extracted from snakes maintained at the Faculdade de Medicina de Ribeirão Preto Serpentarium (Universidade de São Paulo) and dried under vacuum. Crude venom (600 mg) was dissolved in 5 ml of 0.25 M ammonium formate buffer, pH 3.5, and the bulk of crotoxin, the major venom component, was eliminated by slow-speed centrifugation as a heavy precipitate that formed upon the slow addition of 20 ml of ice-cold water to the solution. Tris-base (1 M) was then added dropwise to the supernatant to raise the pH to 8.8, and the solution was applied to a CM-Sepharose FF column (1.5 × 4.5 cm; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) equilibrated with 0.04 M Tris-HCl buffer, pH 8.8, containing 0.064 M NaCl. After the column was washed with 100 ml of equilibrating solution, crotamine was recovered as a narrow protein peak by raising the NaCl concentration of the diluting solution to 0.64 M. The material was thoroughly dialyzed against water (benzoylated membrane, cutoff molecular weight = 3000) and lyophilized, as described previously (Kerkis et al., 2004). Electrospray ionization-mass spectrometry analysis was done by using the LCQ Deca XP ion trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA). The crotamine peptide was subjected to Edman degradation using an automated peptide-sequencing instrument (PPSQ-33A; Shimadzu, Kyoto, Japan).

Expression of Voltage-Gated Ion Channels in *X. laevis* Oocytes. For the expression of the voltage-gated potassium channels ($rK_V1.1$, $rK_V1.2$, $hK_V1.3$, $rK_V1.4$, $rK_V1.5$, $rK_V1.6$, Shaker IR, $rK_V2.1$, $hK_V3.1$, $rK_V4.2$, $rK_V4.3$, and h*ERG*) and voltage-gated sodium channels ($rNa_V1.2$, $rNa_V1.3$, $hNa_V1.5$, and the insect channel $DmNa_V1$) in *X. laevis* oocytes, the linearized plasmids were transcribed using the T7 or SP6 mMESSAGE-mMACHINE transcription kit (Ambion, Austin, TX). The harvesting of stage V–VI oocytes from anesthetized female *X. laevis* frog was described previously (Liman et al., 1992). Oocytes were injected with 50 nl of complementary RNA at a concentration of 1 ng/nl using a microinjector (Drummond Scientific, Broomall, PA). The oocytes were incubated in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.4, supplemented with 50 mg/l gentamycin sulfate.

Electrophysiological Recordings. Two-electrode voltage-clamp recordings were performed at room temperature (18–22°C) using a Geneclamp 500 amplifier (Molecular Devices, Sunnyvale, CA) controlled by a pClamp data acquisition system (Molecular Devices). Whole-cell currents from oocytes were recorded 1 to 4 days after injection. Bath solution composition was ND96: 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES, pH 7.4, or HK (2 mM NaCl, 96 mM KCl, 1.8 mM $CaCl_2$, 2 mM $MgCl_2$, and 5 mM HEPES. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept between 0.5 and 1.5 MΩ. The elicited currents were filtered at 1 kHz and sampled at 500 Hz using a four-pole low-pass Bessel filter. Leak subtraction was performed using a −P/4 protocol. $K_V1.1$ to $K_V1.6$ and Shaker currents were evoked by 500-ms depolarizations to 0 mV followed by a 500-ms pulse to −50 mV from a holding potential of −90 mV. Current traces of h*ERG* channels were elicited by applying a +40-mV prepulse for 2 s followed by a step to −120 mV for 2 s. $K_V2.1$, $K_V3.1$, $K_V4.2$, and $K_V4.3$ currents were elicited by 500-ms pulses to +20 mV from a holding potential of −90 mV. Sodium current traces were, from a holding potential of −90 mV, evoked by 100-ms depolarizations to V_{max} (the voltage corresponding to maximal sodium current in control conditions). To investigate the current-voltage relationship, current traces were evoked by 10-mV depolarization steps from a holding potential of −90 mV. To assess the concentration dependence of the crotamine-induced inhibitory effects, a dose-response curve was constructed in which the percentage of current inhibition was plotted as a function of toxin concentration. All data represent at least three independent experiments ($n \geq 3$) and are presented as mean ± S.E.

Sequence Alignment, Molecular Visualization, and Dipole Moment Calculation. Protein sequences were aligned using Muscle in JalView 2.0 bioinformatic workbench and were shaded by similarity score using BLOSUM62. The following were visualized and graphically manipulated using the publicly available software Chimera (http://www.cgl.ucsf.edu/chimera/)[1] (Pettersen et al., 2004): crotamine [Protein Data Bank (PDB) code 1Z99]; ShK (PDB code 1ROO); BgK (PDB code 1BGK) from the sea anemones *Stichodactyla helianthus* and *Bunodosoma granulifera*, respectively; and κ-hefutoxin (PDB code 1HP9) and charybdotoxin (2CRD) from the scorpions *Heterometrus fulvipes* and *Leiurus quinquestriatus* hebraeus, respectively. The crotamine dipole moment was calculated using the default parameters with the Protein Dipole Moments Server available at http://bioinfo.weizmann.ac.il/dipol/.

Results

Purification and Biochemical Characterization of Crotamine. After purification, crotamine was evaluated to verify its purity. The purified peptide shows an electrospray ionization-mass spectrometry experimental mass of 4885.6 Da, which corresponds well with theoretical mass of 4886.32 Da. The N-terminal automated Edman sequencing showed the sequence YKQCHKKGGHCFPKEKICLP, the same residues described by Laure (1975).

Electrophysiological Recordings. Crotamine was subjected to a screening on a wide range of 16 ion channels. Its activity was investigated on 12 cloned voltage-gated potassium

[1] Molecular graphics and analyses were performed with the UCSF Chimera package. Chimera is developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from the National Institutes of Health National Center for Research Resources [Grant 2P41-RR001081] and the National Institutes of Health National Institute of General Medical Sciences [Grant 9P41-GM103311].

channels (K_V1.1–K_V1.6, K_V2.1, K_V3.1, K_V4.2–4.3, Shaker IR, and h*ERG*) (Fig. 1) and four cloned voltage-gated sodium channels (Na_V1.2, Na_V1.3, Na_V1.5, and the insect channel $DmNa_V1$) (data not shown). Crotamine showed no activity on Na_V channels at a 3 μM concentration ($n = 4$) (Supplemental Fig. 1). It is noteworthy that 3 μM toxin could block K_V1.1–1.3 channels, whereas the same concentration had no effect upon other K_V channel isoforms from the Shaker (K_V1.4–K_V1.6 and Shaker IR), Shab (K_V2.1), Shaw (K_V3.1), Shal (K_V4.3 and K_V4.3), and erg (K_V11.1) subfamilies. Because of its cytolytic effects, concentrations higher than 5 μM could not be tested. Concentration response curves were constructed to determine the values at which half of the channels were blocked by crotamine. The IC_{50} values yielded 369 ± 56, 386 ± 11, and 287 ± 92 nM for K_V1.1, K_V1.2, and K_V1.3, respectively (Fig. 2A). Because crotamine had the highest affinity for K_V1.3, this isoform was used to further investigate the characteristics of inhibition. The inhibition of K_V1.3 channels induced by the toxin was not voltage-dependent as in a range of test potentials from −20 to + 40 mV; no difference in the degree of block could be observed (Fig. 2B).

To investigate whether the observed current inhibition is attributed to pore blockage or rather to altered channel gating upon toxin binding, the IV curves in ND96 and HK solutions were constructed (Fig. 2, C and D). One micromolar concentration of crotamine caused 71 ± 3 and 77 ± 3% inhibition of the potassium current in ND96 and HK, respectively ($n = 4$). In ND96, the IV curves in control and in the presence of 1 μM toxin were characterized by $V_{1/2}$ values of 23 ± 2 and 27 ± 3 mV ($n = 4$), respectively. It can be concluded that no significant shift in the midpoint of activation occurred (*, $P > 0.05$). Crotamine does not significantly influence the reversal potential E_K, as can be concluded from the IV relationship in HK solution (*, $P > 0.05$; $n = 8$), showing that ion selectivity is not changed. E_K values yielded −10 ± 3 mV in control and −8 ± 3 mV after application of toxin. Altogether, these experiments imply that current inhibition upon crotamine binding does not result from changes in the voltage dependence of channel gating. The inhibition of K_V1.3 channels occurred rapidly ($\tau_{0n} = 35.2$ s), and its binding was reversible because the current recovered quickly ($\tau_{0ff} = 104.8$ s) and completely upon washout (data not shown).

Dipole Moment Calculation. The anisotropic moment of crotamine was calculated, and it is represented by the purple trace pointing from the most negative toward the most positive region of the molecule (Fig. 3A). On the basis of the proposal by Ferrat et al. (2002) and Jouirou et al. (2004) for scorpion toxins and Chagot et al. (2005a,b) that electrostatic anisotropy acts as an orientating force of the ligand within the electrostatic field of the membrane receptor (Ferrat et al., 2002; Jouirou et al., 2004; Chagot et al., 2005a,b), we can infer that the Tyr1 and Lys2 residues, together with Arg31 and Trp32, might be involved in the interaction surface of crotamine toward K_V1.1–3 channels, as these basic and hydrophobic patches are exposed in a surface in the vicinity of the dipole moment emergence (Fig. 3, A–B).

Discussion

Inhibition of K_V Channels by Crotamine. Here we report for the first time a thorough investigation of crotamine

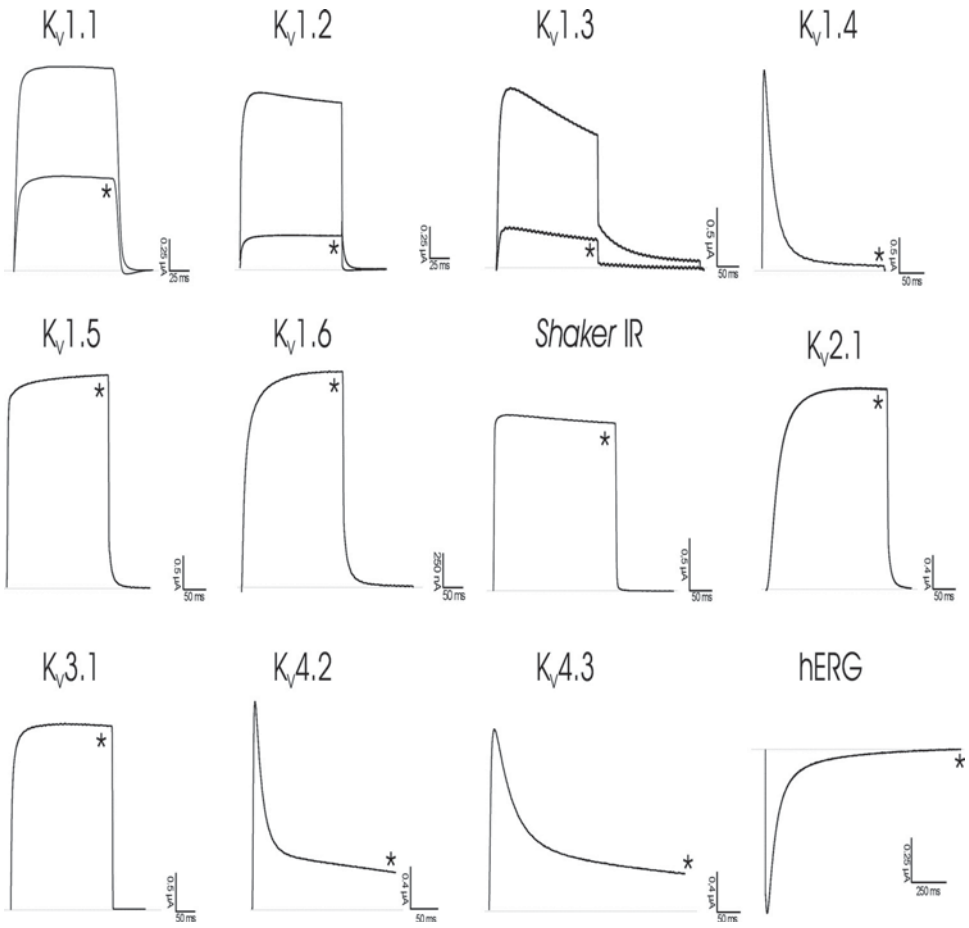

Fig. 1. Activity of crotamine on K_V channels. The current were measured in *X. laevis* oocytes expressing several cloned voltage-gated potassium channel isoforms. Traces shown are representative of at least three independent experiments ($n \geq 3$). The dotted line indicates the zero-current level. The asterisk (*) distinguishes the steady-state current after application of 3 μM crotamine.

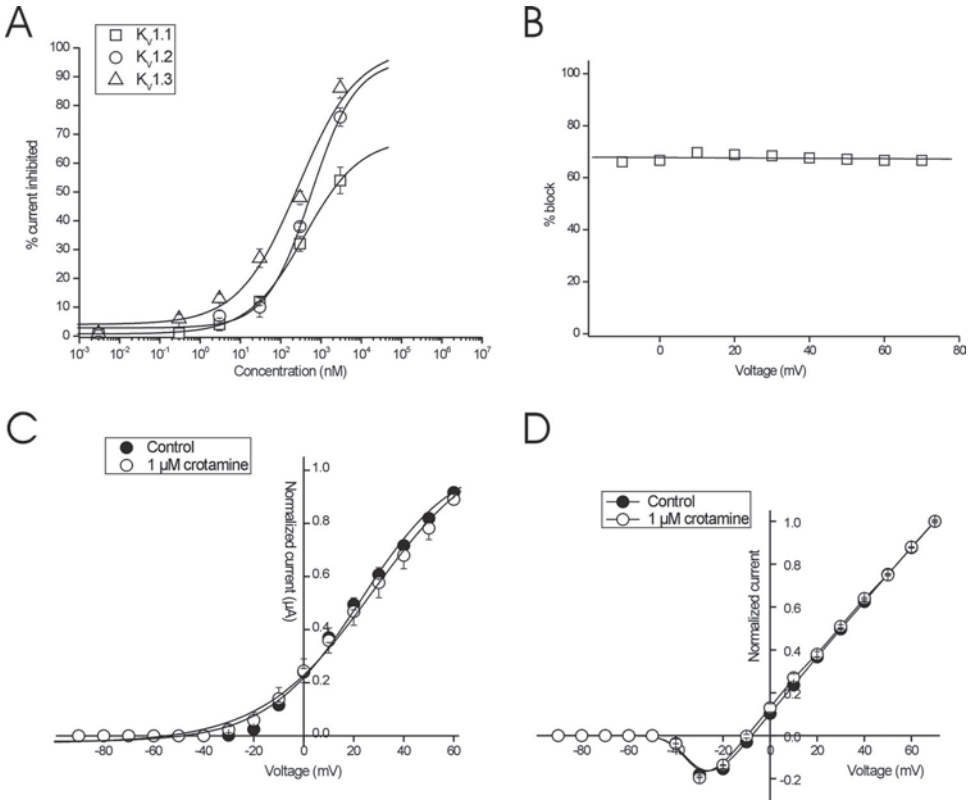

Fig. 2. Effects on K_V1.3 channel gating. A, concentration-response curve on K_V1.1, K_V1.2, and K_V1.3 channels obtained by plotting the percentage of blocked current as a function of increasing toxin concentrations. B, the plot shows the degree of inhibition at a broad range of potentials. No voltage dependence of inhibition was observed. C and D, IV curves in ND96 and HK, respectively, of K_V1.3. The potentials were tested in a range from −30 to +70 mV. Closed circles, the $V_{1/2}$ in control; open circles, the addition of 1 μM crotamine. All data represent at least three independent experiments ($n \geq 3$) and are presented as mean ± S.E.

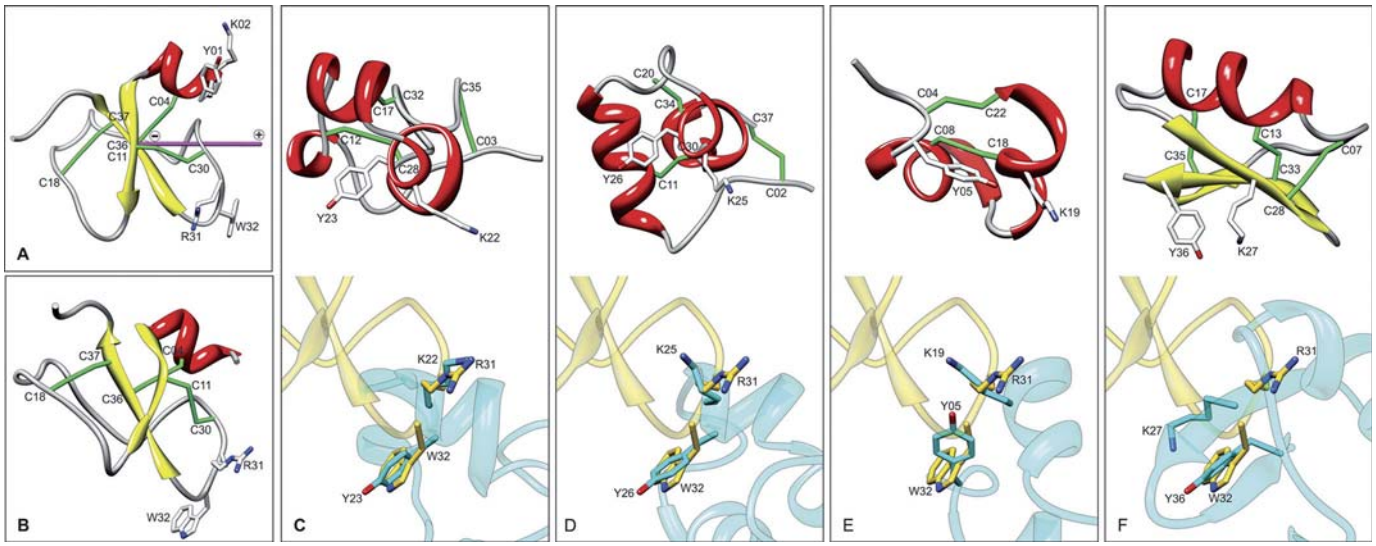

Fig. 3. Crotamine putative functional dyad and K-toxins from sea anemones and scorpions. Previous work has shown that, based on differences in topography of key residues, K_V channel toxin can be classified in distinct classes (Srinivasan et al., 2002). Therefore, crotamine putative functional dyad (Arg31-Trp32) was determined by superimposition for best fit with well described functional dyads of several sea anemone and scorpion toxins acting on voltage-gated potassium channel. Secondary structure, α-helix (Vargas et al., 2002), β-sheet (yellow), loops (Shon et al., 1998), and SS-bonds (green). A, crotamine dipole and presumed functional dyads (Tyr1-Lys2 and Arg31-Trp32); dipole momentum is depicted in purple. B, crotamine presumed functional dyad Arg31-Trp32. C–F, top, K-toxins structures and functional dyads: BgK from the sea anemone *B. granulifera* (Lys25-Tyr26); ShK from *S. helianthus* (Lys22-Tyr23); κ-Hefutoxin from the scorpion *H. fulvipes* (Tyr5-Lys19); charybdotoxin from *L. quinquestriatus* hebraeus (Lys27-Tyr36). C–F, bottom, close-up images of K-toxins functional dyad superimposition with crotamine functional dyad (Arg31-Trp32). Crotamine is depicted in gold, and K-toxins are shown in blue (ribbon representation with transparency, respectively). The functional dyads are also depicted in gold and blue, respectively.

on 16 different ion channels. This broad screening not only reveals significant activity against K_V1.1, K_V1.2, and K_V1.3 but also points out an interesting selectivity for these channels as none of the other isoforms tested was affected under the conditions of this study. Thus, this peptide represents the newest member of snake toxins acting on K_V channels.

Even though a wide variety of peptides targeting voltage-gated potassium channels has been isolated from scorpions and spiders, the number of comparable toxins identified in the venom of snakes remains scarce (Rodriguez de la Vega and Possani, 2005; Mouhat et al., 2008). Up to date, only the dendrotoxins (DTXs) have been very well studied and characterized in depth (Harvey and Robertson, 2004). α-DTX and δ-DTX, isolated from the venom of the green mamba, *Den-*

droaspis angusticeps and their respective homologous DTX-I and DTX-K from the black mamba, *Dendroaspis polylepis*, are all highly potent inhibitors of K_V channel isoforms of the Shaker subfamily (Harvey, 2001). Besides these dendrotoxins, only one other snake peptide capable of inhibiting K_V channels has been reported (Wang et al., 2006). Natrin is a cysteine-rich secretory protein isolated from the venom gland of the snake *Naja naja* atra that could block $K_V1.3$ channels within the nanomolar range.

Crotamine belongs to the β-defensin-like superfamily (Fig. 4), whose members exhibit relatively diverse biochemical and biological functions, although a first glance in the overall secondary structure also suggests a resemblance of crotamine as a small peptide stabilized by three disulfide bonds belonging to the structural cysteine-stabilized α-helix and β-sheet (CS$\alpha\beta$) superfamily. However, in most cases, these peptides share a common function in innate immunity of animals, plants, and microorganisms. The extensive distribution of this common motif throughout diverse organisms highlights that this relatively stable and versatile scaffold has the potential to tolerate insertions, deletions and substitutions within the structure (Zhu et al., 2005). It is noteworthy that the CS$\alpha\beta$ resemblance might suggest some similarity to scorpion toxins that block potassium channels.

Further electrophysiological characterization showed that crotamine does not modulate the voltage dependence of gating of $K_V1.3$ channels. The IV relationship in HK solution showed that ion selectivity was not changed after toxin binding. The observation that there is no difference on the percentage-induced block in ND96 or HK leads to the conclusion that channel blockage is independent of the direction of the potassium current flux and is not influenced by the extracellular concentration of K^+ ions. Moreover, this toxin did not show voltage dependence in its blockage of channels in a wide voltage range. The inhibition of current through $K_V1.3$ channels occurred rapidly, and the binding was reversible upon washout.

A Functional Dyad Contributes to K_V Channel Inhibition. All of the electrophysiological data suggest that the binding site of crotamine is presumably located at the extracellular side. It has been proposed that most toxins that block K_V channels possess a conserved functional core composed of a key basic residue (Lys or Arg) associated with a 6.6 ± 1-Å distant key hydrophobic or aromatic residue (Leu, Tyr, or Phe). Such a functional dyad can be found in a broad range of structurally unrelated peptides from various animals, such as scorpions, cone snails, snakes, and sea anemones (Dauplais et al., 1997; Jouirou et al., 2004). However, it has been reported that besides this dyad, other determinants are required for a high-affinity interaction between the toxin and its target (MacKinnon et al., 1998). Examples of toxins lacking a dyad but still capable of blocking K_V channels strongly suggest that the functional dyad on its own cannot represent the minimal pharmacophore or prerequisite for K_V1 binding (Shon et al., 1998). In general, it is assumed that toxins recognize the K_V1 subtypes through the interaction of their residues, among which the basic ring with certain residues of the K_V1 channel turret. These interactions can be sufficient to inhibit the potassium current. Moreover, these specific molecular contacts determine toxin selectivity toward particular K_V1 channel isoforms. The functional dyad can then be viewed as a secondary anchoring point, providing a higher toxin affinity without altering its selectivity. The side chain of the basic key residue enters the ion channel pore and is surrounded by four Asp residues of the P-loop selectivity filter. The key hydrophobic residue of the dyad will interact through both hydrophobic forces and hydrogen bonding with a cluster of aromatic residues in the P-loop.

Using the solution structure of crotamine and its electrostatic anisotropy represented by the dipole moment (Fig. 3A), a surface of basic and aromatic residues is displayed (Arg31-Trp32 and Tyr1-Lys2). The residues in the vicinity of the emerging dipole moment may be considered as involved in the direct contact surface of a toxin toward its ligand (Jouirou et al., 2004; Chagot et al., 2005a,b). In this way, it would be possible that the Arg31 and Tyr1 might fulfill the requirements to function as a possible dyad, which is in concordance with its previously reported docking model of crotamine with $K_V1.2$ (Yount et al., 2009).

However, the calculated distance between the Arg31 and Tyr1 amino acids is 9.5 Å, which is larger than the ideal 6.6 ± 1 Å between the two key residues of the functional dyad as described above. The other possible dyad would be formed by Arg31-Trp32, and their distances are 3.8 Å apart. Furthermore, the overall comparison of crotamine Arg31-Trp32 putative dyad fits well with other dyads from known potassium channel blockers, as represented by their superimpositions (Fig. 3, C–F). However, these crotamine possible dyads described here are only hypothesized, thus requiring further confirmation from site-directed mutagenesis studies and structure analysis to determine whether crotamine is exhibiting its K_V channel-inhibiting activity through a functional dyad and, if so, which residues are definitely composing it.

K_V Channel-Inhibiting Properties of Crotamine and Its Biological Versatility. Previous work has clearly demonstrated that crotamine will serve as a lead compound in the development of diagnostic probes and delivery systems in proliferative cells (Kerkis et al., 2004). Furthermore, crotamine is considered as a very promising cell-penetrating peptide-mediated delivery drug and anticancer agent (Kerkis et al., 2010). In fact, the potent inhibition of $K_V1.3$ channels, as demonstrated in this work, contributes to the antitumor

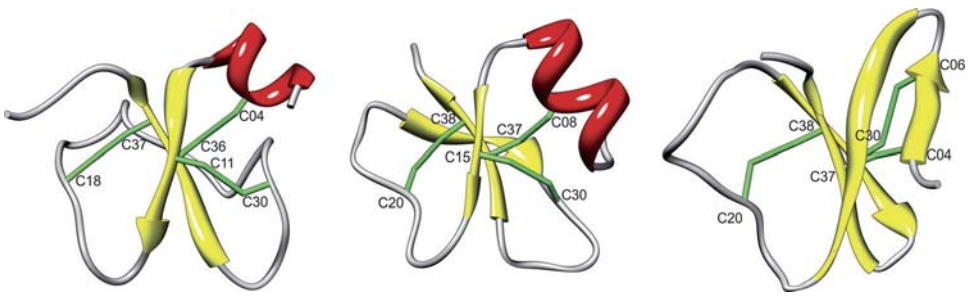

Fig. 4. Tridimensional scaffold of crotamine and β-defensin-like peptides. The orientation of β-defensin-like structures was determined by superposition for best fit with the cysteine heavy atoms involved in disulfide bonds. PDB models:1Z99, crotamine from *C. durissus* terrificus (left); 1FD3, human β-defensin (center); 1WQK, APETx-1 from the sea anemone *Anthopleura elegantissima* (right). Secondary structure, α-helix (Vargas et al., 2002), β-sheet (yellow), loops (Shon et al., 1998), and SS-bonds (green).

properties of crotamine. The enhanced expression of $K_V1.3$ channels and their critical role in the proliferation of several types of carcinogenic cell types has been well established (Bielanska et al., 2009). Overexpression of K_V channels has been reported for diverse cancers, such as breast cancer ($K_V1.1$ and $K_V1.3$), prostate cancer ($K_V1.3$), and melanoma ($K_V1.3$). Evidence indicates that K_V channel activity is a critical regulator of tumor cell proliferation by membrane polarization (Wang et al., 2004; Pardo et al., 2005). Thus, if crotamine acts on $K_V1.3$ in nontoxic concentration to humans, it might also be involved with the previously reported efficacy as an antitumoral agent against several aggressive tumorigenic cell lineages, such as murine melanoma cells (B16-F10), human skin melanoma cells (SK-MEL-28), and pancreatic carcinoma cell line (MIA PaCa-2) (Pereira et al., 2011).

Furthermore, crotamine should be evaluated as a potential "tool" for treatment of autoimmune diseases (e.g., multiple sclerosis, rheumatoid arthritis, and type 1 diabetes mellitus) (Beeton et al., 2001). Because $K_V1.3$ has been identified as the channel that sets the resting membrane potential of peripheral human T lymphocytes, they are responsible for activation of the cells involving an increase of cytosolic Ca^{2+} necessary for mitogen-induced activation that normally occurs after receptor-ligand coupling. Studies using selective blockers of $K_V1.3$ channel have proven that depolarization of the T-cell membrane potential attenuate Ca^{2+} entry and suppress the signaling cascade, leading to cytokine production and cell proliferation (Leonardi et al., 1992; Beeton et al., 2008; Chi et al., 2012).

Conclusions

We have demonstrated the unique multiple function of crotamine, which is not only a cell-penetrating peptide and antitumoral agent as reported before but also a potent K_V channel inhibitor. Moreover, several amino acid residues have been suggested to play a functional and critical role in the potent K_V channel inhibition of this toxin. In addition, the in vivo effects of crotamine and some of the previous contradictory electrophysiological results shown in the literature might be explained based on its novel K_V channel blockage activity.

Crotamine, together with the dendrotoxins, is one of the few snake K_V channel toxins known to date. Furthermore, to our knowledge, it is the first snake K_V channel toxin isolated from a non-mamba species. The fact that crotamine acts upon K_V channels as shown here, raises the interesting possibility that other defensin-like peptides present in the venom of snakes and other venomous animals might also exert some affinity toward voltage-gated potassium channel.

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Authorship Contributions

Participated in research design: Orts and Prieto da Silva.

Conducted experiments: Peigneur and Orts.

Contributed new reagents or analytic tools: Prieto da Silva, Oguiura, Boni-Mitake, de Oliveira, and Tytgat.

Performed data analysis: Peigneur, Orts, Prieto da Silva, and Zaharenko.

Wrote or contributed to the writing of the manuscript: Peigneur, Orts, Prieto da Silva, Oguiura, Boni-Mitake, de Oliveira, Zaharenko, de Freitas, and Tytgat.

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