

## Amino Acid Sequence Determination and Chemical Synthesis of CIIerg1 ( $\gamma$ -KTx1.5), a K<sup>+</sup> Channel Blocker Peptide Isolated from the Scorpion *Centruroides limpidus limpidus*

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Uma nova toxina denominada CIIerg1 (nomenclatura sistemática  $\gamma$ -KTx1.5) foi purificada do veneno do escorpião *Centruroides limpidus limpidus* e a sua seqüência de amino ácidos foi determinada. Ela tem 42 resíduos de amino ácidos entrecruzados por quatro pontes de dissulfetos e bloqueia especificamente um canal de potássio da família *ether-a-go-go* (ERG). O peptídeo completo foi quimicamente sintetizado e adequadamente enrolado, mostrando que é capaz de bloquear o canal-ERG humano (HERG) com uma afinidade idêntica ao peptídeo nativo. A toxina CIIerg1 sintética pode ser produzida em quantidades suficientes para compensar a sua baixa concentração encontrada no veneno natural. Isto prepara o caminho para conduzir estudos enfocando a identificação dos padrões estruturais do HERG que são críticos para o funcionamento adequado do canal iônico. Adicionalmente, outro peptídeo análogo CIIerg2 (nome sistemático  $\gamma$ -KTx4.1) foi purificado e a sua seqüência completa de amino ácidos foi determinada. Este contém 43 resíduos de amino ácidos, mantidos de forma compacta por quatro pontes de dissulfetos.

A novel toxin named CIIerg1 (systematic nomenclature  $\gamma$ -KTx1.5) was purified from the venom of the scorpion *Centruroides limpidus limpidus* and its amino acid sequence was determined. It has 42 amino-acid residues cross-linked by four disulfide bridges and blocks specifically a potassium channel of the family *ether-a-go-go* (ERG). The full peptide was chemically synthesized and properly folded, showing that it blocks the human ERG-channels (HERG) with identical affinity to that of the native peptide. Synthetic CIIerg1 can be produced in quantities enough to compensate its low concentration in the natural venom. It paves the way to conduct studies aimed at the identification of the structural motifs of HERG critical for proper channel function. Additionally, another analogous peptide CIIerg2 (systematic name  $\gamma$ -KTx4.1) was purified and had its full amino acid sequence determined. It contained 43 amino acid residues, maintained closely packed by four disulfide bridges.

**Keywords:** ERG, *Centruroides limpidus limpidus*, chemical synthesis, K<sup>+</sup>-channel, scorpion toxin

### Introduction

Excitable cells possess an intricate mechanism of communication based mainly on the selective permeability to ions, such as potassium (K<sup>+</sup>) and sodium (Na<sup>+</sup>).<sup>1</sup> The electric potential of these cells is maintained by active pumps and ion-channels.<sup>2</sup> One of these channels is the inward rectifier potassium channel (IKr).<sup>3</sup> The IKr voltage gated potassium ion current contributes to the repolarization phase of cardiac action potential and the non-specific blockade of cardiac IKr current is a significant contributor to fatal cardiac arrhythmias associated with prolongation of the QT interval.<sup>4</sup>

The human *ether-a-go-go* related gene (*h-erg*) encodes a potassium channel (HERG) that is responsible for the current Ikr.<sup>5,6</sup> Mutations in HERG predispose patients to long QT syndrome and sudden cardiac arrest.<sup>5,7,8</sup> Blockage of the channel by commonly used medications, such as class III antiarrhythmics, antihistaminics and antipsychotics can also lead to long QT. In most circumstances arrhythmias associated with long QT and sudden death is a result of a combination of genetic, environmental and pharmacological factors.<sup>9</sup> The HERG channels, are also expressed in a range of the others tissues including neurons,<sup>10</sup> neuroendocrine glands,<sup>11,12</sup> and smooth muscle.<sup>13</sup> In recent years comprehensive reviews of the physiology and pharmacology of HERG channel have been published.<sup>14,15</sup>

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Naturally occurring toxic peptides are present in the venom secretions of a variety of animals, such as snakes, scorpions, bees, sea anemone, spiders and marine snails.<sup>16</sup>

The venom of scorpions is a rich source of peptides that modify ion-channel function, either by blocking K<sup>+</sup> currents or modulating the Na<sup>+</sup>-channel gating mechanisms.<sup>17</sup> They have been essential tools for the purification, structural analyses, localization and identification of pore-forming regions of voltage-dependent K<sup>+</sup> channels.<sup>18-22</sup> In the venom of the Mexican scorpion *Centruroides noxius*, we have found Ergtoxin-1,<sup>23,24</sup> the first scorpion peptide capable of blocking specifically HERG channels. This was followed by the discovery of a large number of genes that code for similar peptides in the venomous glands of Mexican scorpions of the genus *Centruroides*. They constitute a novel family ( $\gamma$ -KTx) with 5 distinct subfamilies of putative peptides.<sup>25,26</sup> In the last publication,<sup>26</sup> results confirming the existence to these peptides in the corresponding venoms were performed and shown to block K<sup>+</sup>-currents using HERG-channels, heterologously expressed, but few of them have been isolated and directly characterized. A major problem is their low concentration in the whole venom. *Buthus eupeus*, another scorpion from central Asia was described to contain BeKm-1, a 36 amino acid long peptide with only three disulfide bridges, but capable of blocking the same kind of HERG channels.<sup>27</sup>

From the species *Centruroides sculpturatus* of USA, still another peptide named CSEKerg1 containing 43 amino acid residues and four disulfide bridges was characterized and shown to affect HERG channels.<sup>28</sup> From the large number of genes obtained from *Centruroides* scorpions reported by Corona et al 2002,<sup>26</sup> the peptide corresponding to CnErg2 was also isolated and its function assayed in lactotropic cells.<sup>29</sup> The first ergtoxin isolated (CnErg1) was recently used in various publications dealing with the possible sites of interaction of ergtoxin-1 (systematic nomenclature  $\gamma$ -KTx1.1) with the HERG channels.<sup>30,31</sup> More recently yet, the three-dimensional structure of synthetic CnErg1,<sup>32</sup> and native CnErg1,<sup>33</sup> as well as that of BeKm-1,<sup>34</sup> were determined by nuclear magnetic resonance (NMR) and the interactions of these peptides with HERG channels were studied.

Furthermore, structural studies on scorpion toxins that potently inhibit HERG are beginning to provide clues as to the structural differences between HERG and other voltage-gated K<sup>+</sup> channels.<sup>31,35,36</sup>

In this paper, we describe the isolation and sequencing of two novel peptides from the venom of scorpion *C. l. limpidus*. The first ergtoxin-like peptide isolated was named CIIErg1 and corresponds to the gene *CIIErg1* ( $\gamma$ -KTx1.5) initially reported.<sup>26</sup> Its full amino acid sequence was determined. Since this peptide occurs in small amounts in

the natural venom, here we report its chemical synthesis and correct folding *in vitro*. This synthetic analog displays functional properties identical to those of the authentic native CIIErg1. The design of a fully functional synthetic Ergtoxin analog should alleviate the problem associated with the scarcity of the toxin and accelerate its use as a peptide probe of the HERG function.

## Materials and Methods

### Toxin purification

Soluble venom of *C. l. limpidus* was separated by Sephadex G-50 and carboxymethyl-cellulose columns as described.<sup>37</sup> Fractions 8 and 9 from the CM-cellulose was subsequently separated in two steps of high-performance liquid chromatography (HPLC). From fraction 8, the HPLC separations allowed the purification of CIIErg1, and from fraction 9, CIIErg2 was obtained. The first HPLC fractionation used a C<sub>18</sub> analytical column (Vydac, Hisperia, CA) run in the system previously described,<sup>38</sup> with a linear gradient of solution A (0.12% TFA in water) to solution B (0.1% TFA in acetonitrile) run up to 60% B in 60 min. The second separation used the same column eluted with a linear gradient from 10% to 40% B in 50 min. Similar conditions were used for obtaining an homogeneous peptide CIIErg2.

### Sequence determination

A Beckman LF3000 Protein Sequencer (Fullerton, CA) was used for this study. Direct sequencing permitted the identification of the initial N-terminal sequences. The C-terminal segment and the overlapping sequences were unequivocally positioned after sequencing fragments of peptides, obtained after enzymatic digestion with *Staphylococcus aureus* protease V8 or mass spectrometry. Reduced and carboxymethylated toxin and peptide-fragments were submitted to direct sequencing in order to confirm the cysteinyl residues and to complete the full sequence, using the techniques described.<sup>39</sup> The last two amino acids of both peptides were obtained on the basis of amino acid composition and mass spectrometry data. Both sequences were confirmed by comparison with the nucleotide sequences, of the corresponding genes, as determined by Corona *et al.*<sup>26</sup>

### Chemical synthesis

A linear analog of CIIErg1 was synthesized by solid-phase methodology on Boc-amino acid-OCH<sub>2</sub>-Pam-Resin,<sup>40</sup>

using neutralization *in situ*.<sup>41</sup> Boc-amino acids were used with the following side chain protection: Arg(4-toluensulfonyl)-OH; Asp( $\beta$ -benzyl)-OH; Glu( $\gamma$ -benzyl)-OH; His( $N^{im}$ -dinitrophenyl)-OH; Lys ( $N^{\epsilon}$ -2-chlorobenzyl-oxy-carbonyl)-OH; Cys(4-methylbenzyl)-OH; Tyr(2,6-dichlorobenzyl)-OH; Ser(O-benzyl)-OH; Thr(O-benzyl)-OH; Asn, Gln and Met were used side chain unprotected.

The cesium salt of Boc-Ala was used to prepare Boc-Ala-Pam-resin.<sup>42</sup> Briefly, the cesium salt of Boc-Ala (1.3 mmol) was prepared, coupled to Bromo-methyl-phenylacetic-phenacyl ester and reduced with zinc (powder). Acetophenone excess was washed by repeated decantation over hexane. The Boc-Ala-phenylacetic acid was coupled to the amino-methyl-resin with dicyclohexylcarbodiimide in DCM in the presence of hydroxybenzotriazol neutralized with triethylamine. The  $N\alpha$ -Boc group was removed by treatment with 100% TFA for 2 X 1 min followed by 30 s flow wash with DMF. Boc-amino acids (0.8 mmol) were couple as active esters preformed in DMF with HBTU/DIEA (0.76 mmol/1.2 mmol, 2 min activation) as activating agents. Coupling times were 15 min. Unreacted or deblocked free amines were monitored through the ninhydrin test,<sup>43</sup> in every cycle of the peptide synthesis. During the entire synthesis, before coupling the next amino acid, the undesirable residual free amines were blocked by acetylation.<sup>42</sup> All the operations were performed manually in a 50 mL glass reaction vessel with a Teflon-lined screw cap. The peptide-resin was agitated by gentle inversion during the  $N\alpha$ -deprotection and coupling steps.

A total of 2 g of Boc-Ala-PAM-resin was prepared with a capacity of 0.22 mmol of Ala attached per gram of solid resin. At the end of the synthesis the peptide was treated with a solution of 20% mercaptoethanol, 10% DIEA in DMF for 2 X 30 min in order to remove the DNP group, prior to the removal of the Boc group.<sup>41</sup> The  $N\alpha$ -Boc-group was removed from the peptide-resin by treatment with neat TFA (2 X 1 min). The peptide-resin was washed with DMF and neutralized with 10% DIEA in DMF. After removal of DNP and the Boc group, the peptide-resin was dried under reduced pressure, washed with DMF and DCM. Side chain protecting groups were removed and, simultaneously, the peptide was cleaved from the resin by treatment with HF/anisole (9:1 v/v, 0 °C, 1 h) in a standard Peninsula Laboratories apparatus. The HF was removed under reduced pressure at 0 °C and the crude peptide was precipitated and washed with ice-cold diethyl ether, then dissolved in 5% aqueous acetic acid in presence of dithiothreitol to preserve the cysteine thiol group in its reduced form. The product was lyophilized and kept disseminated at -20 °C until used.

The cyclization reaction to make the corresponding disulfide bridges of the molecule was carried out in 0.1 mol L<sup>-1</sup> NaCl, 5 mmol L<sup>-1</sup> reduced glutathione, 0.5 mmol L<sup>-1</sup> oxidized glutathione, 20 mmol L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub> (pH 7.8) and 30  $\mu$ mol L<sup>-1</sup> of synthetic CIIerg1. The crude cyclized product was purified in two steps by HPLC. The first used a C<sub>4</sub> semi-preparative column, with a linear gradient of solution A (0.12% TFA in water) to solution B (0.1% TFA in acetonitrile) run up to 60% B in 60 min. The main component was finally purified using a C<sub>18</sub> column run with linear gradient from solvent A to 60% B in 60 min. The structure and the purity of the synthetic toxin were confirmed by analytical HPLC, amino acid analysis and mass spectrometry determination. For amino acid analysis, synthetic toxin was hydrolyzed in 350  $\mu$ L of 6 mol L<sup>-1</sup> HCl, 15  $\mu$ L of phenol at 120 °C for 24 h, and then analyzed in a Beckman analyzer. Mass spectrometry was carried in a Finnigan LCQ<sup>Duo</sup> spectrometer (San Jose, CA, USA).

#### Channel expression in oocytes

The oocytes were prepared following the technique described earlier.<sup>44</sup> In brief, female *Xenopus laevis* frogs were anesthetized by 15 min exposure to 0.15% of 3-aminobenzoic acid ethyl ester. The oocytes were surgically removed from the ovary, after which the frog was closed by suturing and placed in water to allow recovery from anesthesia. Defolliculation was performed by incubation for 1 h in 1.5 mg mL<sup>-1</sup> collagenase in Ca<sup>2+</sup>-free OR2 oocyte medium with gentle agitation. Oocytes were stored in ND96 solution (in mmol L<sup>-1</sup>): 96 NaCl, 2 KCl, 1 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 5 HEPES buffer adjusted to pH 7.4 with NaOH and supplemented with 10  $\mu$ g mL<sup>-1</sup> gentamicin, at 18 °C. Oocytes were injected with 50 nL of cRNA (0.3 ng mL<sup>-1</sup>) by using a micro-dispenser and a micropipet. Injected oocytes were incubated at 18 °C for 24-48 h in ND96 medium, before analysis.

#### Electrophysiological recordings

Channels were expressed to a level where 0.5-2.0  $\mu$ A of current was recorded during a depolarizing step from a holding potential of -80 mV to potentials between -80 and +60 mV, and repolarizing at -100 mV. Currents were recorded using the two-electrode voltage clamp method (CA-IB high performance oocyte clamp DAGAN). Electrodes were filled with 3 mol L<sup>-1</sup> KCl had resistance of 0.3-1.0 M $\Omega$ . The bath solution contained (mmol L<sup>-1</sup>): 95 NaCl, 5 KCl, 1 MgCl<sub>2</sub>, 0.3 CaCl<sub>2</sub>, 5 HEPES buffer adjusted to pH 7.4 with NaOH. Control recordings were taken prior to the addition of toxin. On the addition of toxin, the

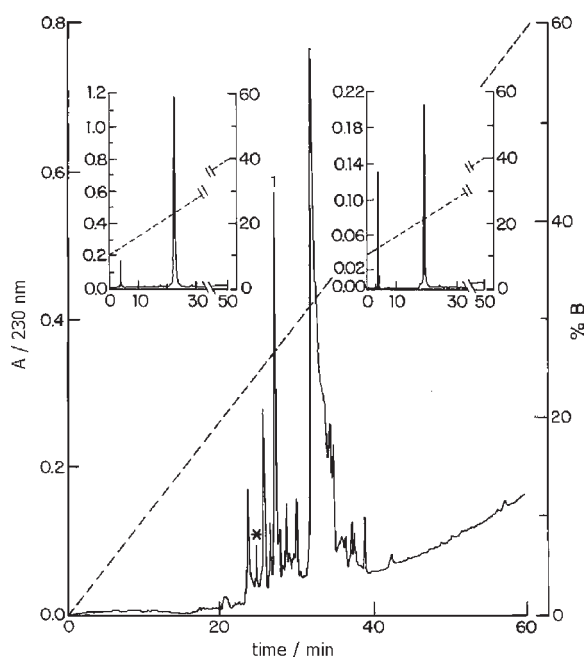
perfusion medium was stopped to allow homogeneous dispersion of the toxin. In most experiments, toxin was removed from the bath to demonstrate recovery.

## Results and Discussion

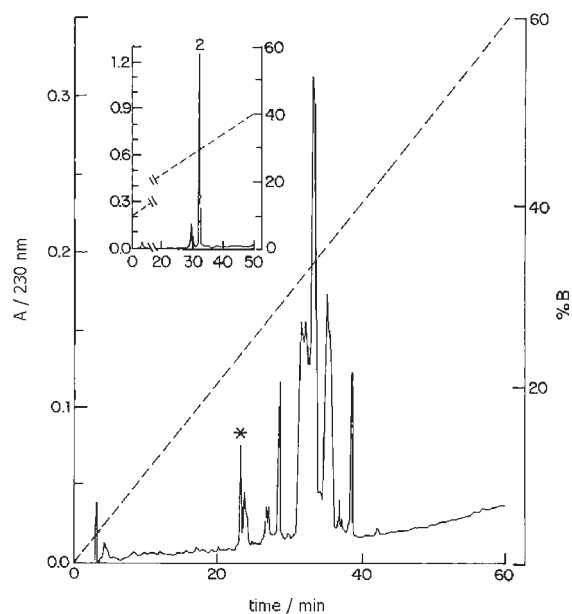
The presence of components that block HERG channel in the venom of *C. l. limpidus* was previously demonstrated by electrophysiological recordings, using mouse neuroblastoma cell line F11.<sup>26</sup> In the present study, we confirmed the existence and assessed the specificity of action on the HERG channels, of two peptides obtained from this venom. The alpha subunit of HERG channels was heterologously expressed in *Xenopus* oocytes for this study. The purification of two native peptides (CIIerg1 and CIIerg2) and an oxidized isoform of CIIerg1 were obtained by a combination of chromatographic steps of Sephadex G-50 gel filtration, carboxymethyl-cellulose ion exchange column chromatography and HPLC separations, as indicated in the section of Material and Methods. From the three main fractions of the Sephadex G-50 separation,<sup>37</sup> only fraction II showed significant blocking activity against HERG channels. From the 14 subfractions of the CM-cellulose column, obtained after separation of the Sephadex G-50 fraction II, as described earlier by Ramírez *et al.*,<sup>37</sup> fractions 8 and 9 proved active on HERG. In Figure 1 are the results of separating the active subfraction 8 on the HPLC system. The component labeled "1" contained the most active peptide and was further separated as indicated in the left-inset of the figure. The main component corresponds to CIIerg1, whereas small contaminants were eliminated (see shoulders to the right of the figure). A less active component, present in a very small quantity, was recovered from the HPLC, labeled with asterisk in Figure 1. This was fully isolated as indicated in the inset at the right-side. It corresponds to an oxidized form of CIIerg1, where the methionine in position 35 was shown to be oxidized. Figure 2 shows the profile of purification of component CIIerg2. It elutes at 23.19 min from the HPLC C<sub>18</sub> reverse phase column (labeled with asterisk), and under re-chromatography, using the same conditions of CIIerg1, gives an homogeneous component, as indicated in the inset of Figure 2, labeled 2, which corresponds to CIIerg2. The amount of CIIerg1 in the whole soluble venom was estimated to be 0.26%, whereas that of CIIerg2 was even much less, only 0.03%.

The complete amino acid sequences of CIIerg1 and CIIerg2 were determined by Edman degradation, mass spectrometry and confirmed by nucleotide sequencing, as indicated in Figure 3. For CIIerg1, residues D1 to N30 were unequivocally identified by direct sequencing. The

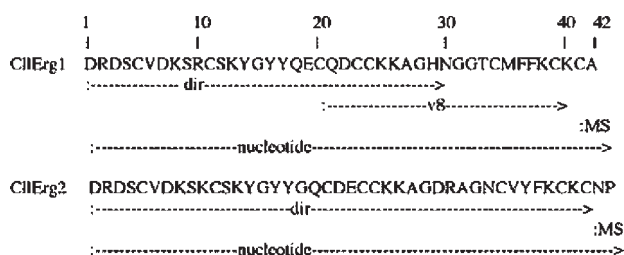
overlapping fragment from residues C20 to K40 were identified using a sub-peptide obtained from the HPLC



**Figure 1.** Purification of CIIerg1: Fraction 8 (700 microg of protein) from the CM-cellulose column of *C.l.limpidus* venom was applied to a C<sub>18</sub> analytical reverse-phase column in a Waters HPLC system and separated using a linear gradient from solution A (0.12% TFA in water) to solution B (0.1% TFA in acetonitrile) run up to 60% B in 60 min. The components labeled "1" and "\*" were further separated in an analytical C<sub>18</sub> reverse phase column (insets) to give pure peptides: CIIerg1 and its oxidized form, respectively. The second separation used the same column eluted with a linear gradient from 10% to 40% B in 50 min.



**Figure 2.** Purification of CIIerg2: Fraction 9 (1.3 mg protein) of the CM-cellulose column of *C.l.limpidus* venom was separated in the same conditions described in the legend of Figure 1 to give pure toxin CIIerg2 (see label 2 in the inset).

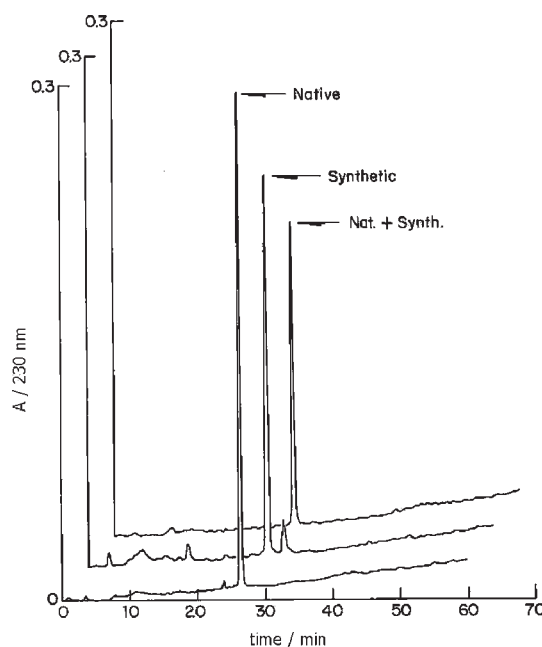


**Figure 3.** Amino acid sequence of CIIErg1 and CIIErg2: The first sequence corresponds to CIIErg1 and was obtained by direct sequencing 1 nmol of the native peptide (under-labeled "dir") It was confirmed by sequencing a reduced and carboximethylated sample of the same peptide. This provided an unequivocal identification of residues in position D1 to N30 (numbers on top of the sequence indicate amino acid position at the primary structure). An overlapping segment was obtained after enzymatic cleavage (under-labeled V8, from V8 endopeptidase), which allowed the identification of residues from C20 to K40. Residues C41 and A42 were determined by mass spectrometry. The same procedure was followed for CIIErg2, except that for this one, no enzymatic cleavage was required. The full sequence of both peptides was confirmed by nucleotide sequence obtained from the cloned genes that code for these toxins (see Corona *et al.*<sup>26</sup>).

separation (data not shown) of a sample of the toxin hydrolyzed with v8 endopeptidase. The last two residues were determined by mass spectrometry, as mentioned in Material and Methods. For CIIErg2 a similar protocol allowed the direct identification of residues from position D1 to C41. The two last residues in position N42 and P43 were determined by mass spectrometry. Thus, these peptides are composed by 42 and 43 amino acid residues, respectively. Their molecular masses experimentally determined were 4761 a.m.u. and 4833 a.m.u., and the theoretical expected masses were 4760.38 a.m.u. and 4832.40 a.m.u., respectively for CIIErg1 ( $\gamma$ -KTx1.5) and CIIErg2 ( $\gamma$ -KTx4.1), confirming the sequences found. These toxins are basic peptides containing eight cysteine residues that stabilize the three dimensional conformation by forming four disulfide bridges. The peptide eluted in Figure 1 (labeled with asterisk) when sequenced gave identical amino acid sequence as CIIErg1, except for the amino acid in position 35, which was not readily identified. By mass spectrometry this amino acid was shown to be an oxidized methionine, and its molecular weight contained 16 a.m.u. more than the unmodified CIIErg1, as expected.

Toxin CIIErg1 was assayed in the oocyte system described in Material and Methods, where the human ERG K<sup>+</sup>-channel was expressed heterologously and was shown to be a potent inhibitor of the channel activity, as it will be discussed below. However, due to the scarcity of sample we decided to synthesize the molecule. The amount of peptide obtained after chemical synthesis was in the order of 107 mg; when purified the component eluting at the expected time (approx. 35 min, data not shown) was about 11% of the

starting material. After the folding procedure, as described in Material and Methods, the amount of correctly folded peptide was again around 11%. This accounts for a relative low final yield of about 1.2% of the initial synthetic peptide. However, this peptide had the correct sequence, folding and the same physiological action as the native toxin. This was verified by the following criteria: amino acid sequence of the N-terminal region, molecular mass determination, elution profile of the HPLC system and electrophysiological action on HERG channels. The molecular mass obtained after folding was 4760.85. The chromatographic behavior on the HPLC is shown in Figure 4. After the two chromatographic separations described in the Material and Methods section, small contaminants were still present (see label "Synthetic" in Figure 4). The major component corresponded to the correctly folded peptide. This was mix with an equal amount of the native toxin and re-applied into the column in the same conditions (see label Nat + Synth in Figure 4). They both co-elute, indicating that the folding of the synthetic material is likely the correct one. Finally, the physiological results, as discussed below, show that the chemical synthesis was successful, otherwise the biological effect would have been compromised. It is also important to note that the correct folding of four disulfide bridges containing toxins has been

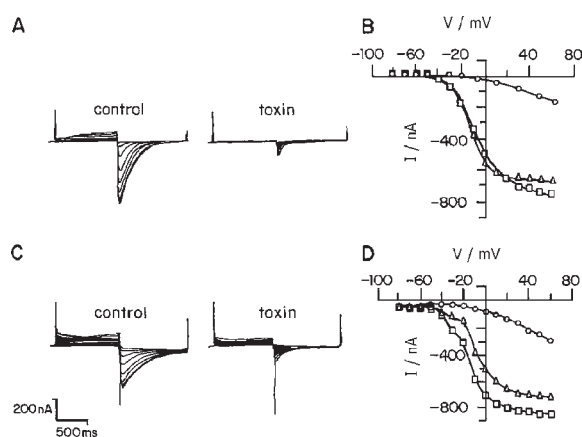


**Figure 4.** HPLC comparison of native and synthetic CIIErg1: Native and synthetic CIIErg1 (15microg each), as well as an equimolar mixture of them (8 microg each) were chromatographed on a C<sub>18</sub> reverse-phase analytical column. Peptides were eluted using 0.1% trifluoroacetic acid as solvent, applying a linear gradient from 0 to 60% (v/v) of acetonitrile in 60 min, at a flow rate of 1 ml min<sup>-1</sup>. The label Nat.+Synth shows the elution position, when both peptides were mix together. The time of elution for the three superimposed chromatogram is the same.

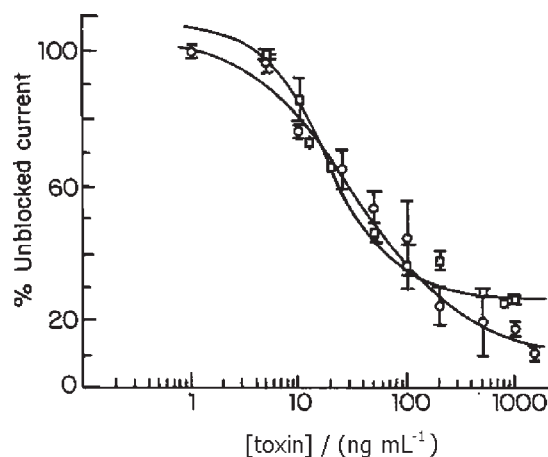
a difficult task in the field.<sup>45</sup> Thus, the results reported here, open the possibility of preparing much more material for future research related to the structure and function of these toxins.

Figure 5A and B shows that CIIerg1 ( $\gamma$ -KTx1.5) potently suppressed the HERG current amplitude. The toxin effect on HERG channels was almost fully reversible. A dose-response curve for the effect of CIIerg1 (Figure 6, circles) shows an  $IC_{50}$  value of 10 nmol L<sup>-1</sup>. The oxidized sample (methionine35) of CIIerg1, although containing an almost identical amino acid sequence, elutes in a different position of the HPLC (Figure 1, star) and has about two orders of magnitude less efficiency on the same channel (data not shown). This result is quite relevant, because it shows two possible situations: either Methionine-35 is involved in the proper folding of the molecule, as already discussed<sup>32,33</sup> or it plays a direct role in the interaction with the channel. In contrast with the oxidized form of CIIerg1, the synthetic and properly folded molecule, as shown in Figure 5C and D blocks HERG channel expressed in oocytes with the same potency and affinity as native CIIerg1. The dose-response curve (Figure 6) shows that the  $K_d$  for both the native and the synthetic CIIerg1 are super-imposable.

Additionally, it was found a concentration dependence of suppression of current like the wild type and a residual current not suppressed by high concentrations of CIIerg1. Pardo *et al.*,<sup>31</sup> suggest that this is possibly due to an incomplete occlusion of the HERG-channel pore. Both toxins (CnErg1, CIIerg1) lack the Lys-27 of other scorpion toxins specific for K<sup>+</sup>-channels, as earlier described by the group of C. Miller and collaborators for Charybotoxin and Agitoxin (see review).<sup>17</sup> Scanning cysteine mutagenesis



**Figure 5.** Effect of native and synthetic CIIerg1 on HERG channels: A) Left panel shows control recording, right panel is in the presence of 100 nmol L<sup>-1</sup> native CIIerg1, from a holding potential of -80mV to +60 mV by increments of 10 mV each, whereas the tail currents were elicited by repolarization to -100mV. B) Current versus voltage traces of A (squares for control; circles for toxin and triangles for recovery). C) and D) are the same recordings as A and B, but for the synthetic CIIerg1.



**Figure 6.** Dose-response curve for native and synthetic CIIerg1: The percentage of unblocked current was plotted against toxin concentration. Circles are results obtained with native toxin, whereas squares with the synthetic toxin. Estimated  $IC_{50}$  were 10 nmol L<sup>-1</sup> for native toxin and 9 nmol L<sup>-1</sup> for synthetic toxin.

of the S5-P linker in HERG has identified several residues that are important for the binding of CnErg1. These include three hydrophobic residues in a putative amphipathic helix in the S5-P linker (W585, L589, I593).<sup>31</sup> Also CnErg1 and CIIerg1 contain a large hydrophobic patch (Y14, F36, F37),<sup>32,33</sup> which can be involved in binding to the hydrophobic face of the amphipathic helix. The charged residues of these basic molecules form a basic ring that could also be implicated on the right fitness or binding of the toxin to the outer vestibule of the channels, as also discussed by Torres *et al.*<sup>32</sup> and Rodríguez de la Vega and Possani.<sup>17</sup> Concerning toxin CIIerg2, it was not obtained in sufficient amount to conduct physiological studies. The possibility of obtaining large amounts of these peptides by chemical synthesis should provide the opportunity to assay the natural variants of the ergotoxin-like peptides and to assess with more precision the interacting surfaces of these toxins with the HERG channel.

## Conclusion

This manuscript reports the isolation and primary structure determination of two novel ergotoxin-like peptides (systematic names  $\gamma$ -KTx1.5 and  $\gamma$ -KTx4.1, see Corona *et al.*<sup>26</sup>) isolated from the venom of the Mexican scorpion *C. l. limpidus*. The full chemical synthesis of CIIerg1 was successfully obtained. This paves the way for future studies on this highly competitive area of research.

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- Abbreviations: a.m.u., atomic mass units; *C.I. limpidus*, *Centruroides limpidus limpidus*; ERG, *ether-a-go-go* potassium channel family; HERG, human potassium ERG channel; IKr, rapid delayed rectifier potassium current; QT, time interval on the surface electrocardiogram; Boc, *ter*-butyloxycarbonyl; Boc-amino acid-Pam-Resin, Boc-aminoacyl-4-(oxymethyl)-phenacetamidomethyl-resin; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, N,N-dimethylformamide; HBTU, 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate; HF, hydrofluoric acid; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid.
- QT: the duration of QT interval (reviewed in Keating, M. T.; Sanguinetti, M. C.; *Cell* **2001**, *104*, 569) is a measure of the time required for depolarization and repolarisation of the heart. Prolongation of the QT interval significantly increases the risk of ventricular arrhythmia and in particular an arrhythmia known as "*torsade de pointes*", which causes syncope and, if it persists, sudden death.
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