A novel structural class of K^+-channel blocking toxin from the scorpion *Pandinus imperator*

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A novel peptide was purified and characterized from the venom of the scorpion *Pandinus imperator*. Analysis of its primary structure reveals that it belongs to a new structural class of K^+-channel blocking peptide, composed of only 35 amino acids, but cross-linked by four disulphide bridges. It is 40, 43 and 46% identical to noxiustoxin, margatoxin and toxin 1 of *Centruroides limpidus* respectively. However, it is less similar (26 to 37% identity) to toxins from scorpions of the geni *Leiurus*, *Androctonus* and *Buthus*. The disulphide pairing was determined by sequencing heterodimers produced by mild enzymic hydrolysis. They are formed between Cys-4–Cys-25, Cys-10–Cys-30, Cys-14–Cys-32 and Cys-20–Cys-35. Three-dimensional modelling, using the parameters determined for charybdotoxin, showed that is it possible to accommodate the four disulphide bridges in the same general structure of the other K^+-channel blocking peptides. The new peptide (Pi1) blocks *Shaker* B K^+ channels reversibly. It also displaces the binding of a known K^+-channel blocker, [^{125}I]noxiustoxin, from rat brain synaptosomal membranes with an IC_{50} of about 10 nM.

INTRODUCTION

Scorpion venoms are a well known source of different classes of peptides that affect the normal function of ion channels. The most widely known are those specific for Na^+ and K^+ channels (reviews [1,2]). However, peptides that recognize Ca^{2+} and Cl^- channels have also been recently reported [3,4].

Scorpion toxins against K^+ channels are short peptides of about 31 to 39 amino acids [1,2], whereas the toxins against Na^+ channels are longer peptides containing 61 to 70 amino acid residues [1,5]. Concerning their primary structure, both families of toxins are highly variable. Except for the constant relative positions of the cysteines, and a few other residues, such as for example Gly-39 of Na^+-channel toxins [1], most of the amino acids of the primary structure are variable. This variability could be an evolutionary attempt to meet with the diversity of ion channel molecules.

Although the toxins against Na^+ and K^+ channels differ greatly in their number of amino acids, the three-dimensional structure of both families of toxins presents a common motif: a short segment of α-helix and three strands of β-sheet structures, stabilized by four disulphide bridges, in the case of toxins specific for Na^+ channels, and three disulphide bridges, in the case of toxins specific for K^+ channels [6].

These structural features were probably evolved for the recognition of ion channels, which are the only known targets of both families of toxins, but with significant variations in the affinity of the toxins towards the members of their corresponding families of channels. For example, noxiustoxin (NTX) binds with picomolar affinity to the K^+ channels present in rat brain synaptosomes [2,7], whereas it binds with micromolar affinity to Ca^{2+}-dependent K^+ channels of both rabbit skeletal muscle [8] and epithelial cells [9]. Therefore the discovery and determination of the structure of new types of specific toxins adds an important tool for physiological studies where ion channels play a role.

In this paper, and to the best of our knowledge, we describe for the first time a new class of toxin against K^+ channels. The peptide isolated from the venom of the scorpion *Pandinus imperator* has a novel structural characteristic that departs from the classical scheme of the known toxins against K^+ channels. The peptide, which we propose to be called Pi1 (from *Pandinus imperator* toxin 1), has only 35 amino acid residues, but is cross-linked by four disulphide bridges (instead of the three commonly found). This peptide displaces the binding of [^{125}I]NTX, from rat brain synaptosomal membranes, and blocks efficiently and reversibly the *Shaker* B K^+ channels.

MATERIALS AND METHODS

Source of venom

Scorpions of the species *Pandinus imperator* (Gabon, Africa) were kept alive in the laboratory and once a month were anaesthetized with CO_2 and milked for venom by means of electrical stimulation. The venom was placed in double distilled water and centrifuged at 15000 ***g*** for 15 min. The supernatant was freeze-dried and stored at -20 °C until required for use.

Separation and bioassays

The soluble venom was first fractionated in a Sephadex G-50 column. Subfractions were further separated by HPLC, using a C18 reverse-phase column (Vydac, Hysperia, CA, U.S.A.) of a Waters 600E HPLC system, equipped with a variable wavelength detector and a WIPS 712 automatic sample injector.

Abbreviations used: NTX, noxiustoxin; Pi1, *P. imperator* toxin 1; RC-toxin, reduced and carboxymethylated toxin; Sf9, *Spodoptera frugiperda* cell line 9; TPCK-trypsin, tosylphenylalanylchloromethane-treated trypsin.

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The homogeneity of the purified peptides was confirmed by step-gradient HPLC and by direct Edman degradation using an automatic sequencer [10]. Amino acid analysis of peptides confirmed the molecular mass and the sequences found.

The bioassay used to identify these peptides was through binding and displacement experiments from brain synaptosomal membranes of previously bound [^{125}I]NTX [11]. NTX was the first K^+-channel-specific blocking peptide described [12,13]. With this procedure a peptide eluting initially in fraction III of the Sephadex G-50 column, and subsequently in peak 1 during HPLC, was isolated in homogeneous form and shown to contain an unusual number of cysteines (eight). This peptide was called Pi1, and was used for chemical characterization and electrophysiological experiments.

Amino acid analysis and sequencing

Samples (about 1 nmol each) of the pure native peptide Pi1, and their fragments generated by enzymic digestion, were analysed in a Beckman 6300E amino acid analyser, after acidic hydrolysis for 20 h in 6 M HCl at 110 °C. An aliquot of the pure peptide (100 μg) was reduced and alkylated with iodoacetic acid, as described [10]. An automatic ProSequencer (Millipore model 6400/6600) was used in order to determine the amino acid sequence of: (i) the native peptide, (ii) the reduced and carboxymethylated peptide (RC-toxin), and (iii) their HPLC-purified fragments produced by tryptic digestion. A sample of 50 μg of pure peptide in 0.25 ml of 0.1 M ammonium bicarbonate buffer, pH 7.8, was hydrolysed by 2 μg of tosylphenylalanylchloromethane-treated trypsin (TPCK-trypsin) (Boehringer, Mannheim, Germany), for 3 h at 37 °C. The products of this enzymic hydrolysis were separated by HPLC, and used for sequence and amino acid analysis.

Disulphide bridges determination

Native Pi1 (50 μg) was initially digested with trypsin, as described in the previous section, and separated by HPLC in a C18 reverse-phase column, using a linear gradient from solution A (0.12 % trifluoroacetic acid in water) to 40 % solution B (0.10 % trifluoroacetic acid in acetonitrile) over 60 min. The various peptides obtained were sequenced as described in the previous section, using the Millipore ProSequencer apparatus. The component eluting at 23.87 min was further digested with thermolysin for 20 h at 45 °C, in the presence of 100 mM ammonium bicarbonate buffer, pH 7.2, containing 1 mM $CaCl_2$. The products were separated under the same HPLC conditions as those described for trypsin, and sequenced. Peptides given double sequences with similar quantities were assumed to be heterodimers, in which the disulphide bridges were kept intact, and where used to reveal the complete covalent structure of Pi1.

Binding and displacement assays

NTX was radiolabelled with ^{125}I and used for binding and displacement assays, as described [11]. Rat brain synaptosomal membranes (fraction P3) were obtained by the method of Catterall and co-workers [14], and used for assessing the capability of Pi1 to displace the binding of [^{125}I]NTX.

Sf9 insect cells culture, and *Shaker* B K^+-channels expression

The culture of the insect cell line Sf9 from the army-worm caterpillar *Spodoptera frugiperda*, and the expression of *Shaker* B K^+ channels was done as previously reported [15]. In brief, the cells were kept at 27 °C in Grace's medium and infected with the recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus, containing the *Shaker* B K^+-channel cDNA [16]. The recombinant baculovirus was a gift from Dr. C. Armstrong.

Electrodes, electrical recordings and solutions

The electrodes for patch-clamp recordings were pulled from borosilicate glass (KIMAX 51), to a resistance of 1–1.8 MΩ, and used without further treatment.

Macroscopic currents through *Shaker* B K^+ channels were recorded under whole-cell patch-clamp [17] 2 days after the infection of the cells. Recordings were performed with an Axopatch 1D (Axon Instruments, Inc). The delivery of the pulses and the acquisition of the data was done through a TL-2 interface (Axon Instruments, Inc.) connected to a 486 PC computer (Gateway 2000), with the pClamp 5.5 software. Currents were sampled at 100 μs/point. Between 70 and 75 % of the series resistance was electronically compensated.

The holding potential was −80 mV. Pulses were delivered at a rate of one every 15 s, to allow full recovery from inactivation.

The internal solution was: 90 mM KF, 30 mM KCl, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM Hepes-K, pH 7.2. The external solution was: 145 mM NaCl, 10 mM $CaCl_2$, 10 mM Mes-Na, pH 6.4.

RESULTS AND DISCUSSION

Peptide purification and amino acid sequence

An unknown K^+-channel blocker peptide (Pi1) with a novel structure was isolated from the venom of the scorpion *Pandinus imperator*, by means of chromatographic procedures. The soluble venom was initially separated by gel-filtration chromatography in Sephadex G-50 (Figure 1a). The overall recovery was 94 %. Pi1 was found in fraction III of the chromatogram. This fraction corresponds to 18 % of the material absorbing at 280 nm. The components of fraction III were further separated by HPLC. Pi1 was recovered in the first peak of the chromatogram (labelled with an asterisk in Figure 1b). It corresponded to 10 % of the material. Step-gradient separation of this fraction gave a single component (inset, Figure 1b). Based on the recovery data, Pi1 is about 1.8 % of the total soluble venom.

The amino acid analysis of Pi1 (Table 1) showed the presence of 35 amino acid residues, with a calculated relative molecular mass of 3840 (that is: 4460 by composition, according to Table 1, minus 34 peptide bonds and four disulphide bridges, which is equal to 3840). In order to confirm the initial amino acid analysis and to determine the full amino acid sequence of Pi1, samples of native peptide, RC-toxin and the tryptic-hydrolysis products of RC-toxin, after HPLC fractionation (Figure 1c), were sequenced. In Figure 2 we show this sequence and indicate the corresponding overlapping segments. The last peptide, at the C-terminal region, was obtained from the tryptic digestion after Lys-31 (Figure 1c). This tetrapeptide was directly sequenced confirming the presence of two extra half-cystines in the molecule. Also this same peptide was hydrolysed in HCl (see the Materials and methods section), and the amino acid analysis confirmed the presence of only one Tyr, one Gly and two half-Cys. The presence of Tyr in this segment explains the high absorbance of this peptide in Figure 1(c) (labelled with an asterisk).

Disulphide bridges

The disulphide bridges were determined from the heterodimers obtained (see the Materials and methods section) and are shown in Table 2. Interestingly, peptide from tryptic digest eluting at

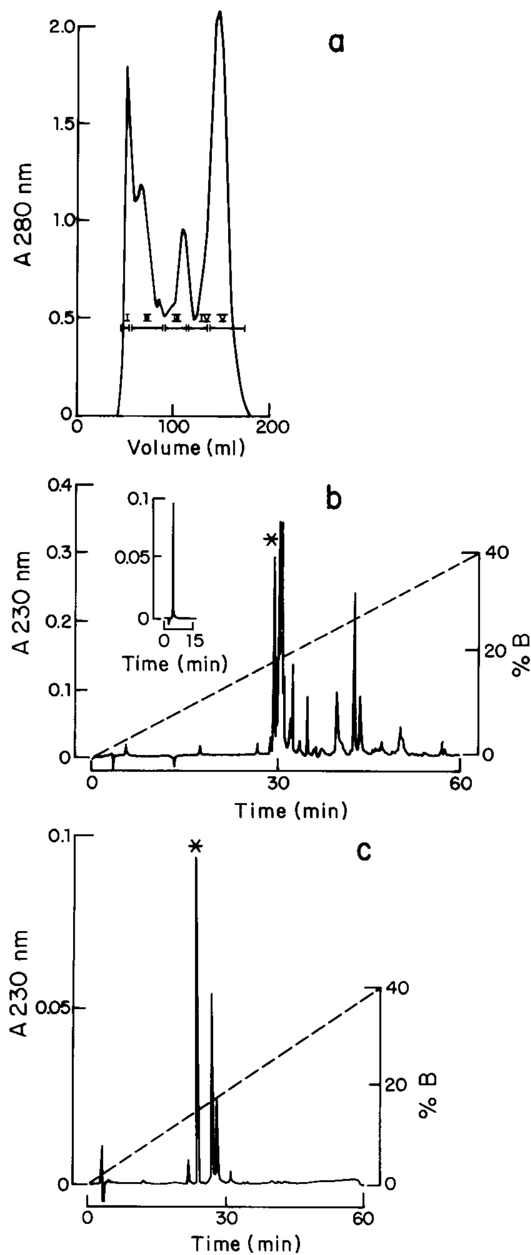

Figure 1 Chromatographic separation of Pi1

(**a**) Soluble venom from *Pandinus imperator* (120 mg) was applied to a Sephadex G-50 gel filtration column (0.9 cm × 200 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 30 ml/h. Five distinct subfractions (horizontal bars I–V) were obtained. (**b**) Subfraction III was further separated in a C18 reverse-phase column of an HPLC system, using a linear gradient from solution A (0.12% trifluoroacetic acid in water) to 40% B (solution containing 0.1% trifluoroacetic acid in acetonitrile). Fraction 1, labelled with asterisk, was obtained in pure form, as demonstrated in the inset of (**b**), and by Edman degradation. (**c**) RC-toxin (50 μg) was digested with trypsin and separated in the same HPLC system, from which the peptide labelled with an asterisk was used for final determination of the sequence.

23.87 min contained two disulphide pairings, which included half-cystines from positions 14, 20, 32 and 35. This fragment was further hydrolysed with thermolysin and finally separated and sequenced, given an unequivocal identification of the disulphide bridges. The pairs: Cys-4–Cys-25, Cys-10–Cys-30 and Cys-14–Cys-32 of Pi1 correspond to the equivalent positions: Cys-7–Cys-29, Cys-13–Cys-34 and Cys-17–Cys-36 of NTX [7], and

Table 1 Amino acid analysis of Pi1

Amino acid	Content (nmol found)	Nearest integer
Aspartic acid	3.39	3
Threonine	1.96	2
Serine	1.96	2
Glutamic acid	3.21	3
Proline	2.50	2–3[a]
Glycine	4.60	4–5[a]
Alanine	Traces	0
Valine	0.83	1
Methionine	0.48	1
Isoleucine	0.94	1
Leucine	0.91	1
Tyrosine	0.83	1
Phenylalanine	Traces	0
Histidine	Traces	0
Lysine	3.00	3
Arginine	2.30	2–3[a]
Tryptophan	–[b]	0
1/2 Cystine	–[b]	8
Total found		35
M_r		4460

[a] Proline, glycine and arginine were shown to be 2, 4 and 3, respectively.
[b] Determined by sequencing.

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1         10         20          30    35
LVKCRGTSDC GRPCQQQTGC PNSKCINRMC KCYGC
:--------------d--------------->
:...............rc................>
                                 :=t=>
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Figure 2 Total amino acid sequence of Pi1

The primary structure of Pi1 is shown using the one-letter code per amino acid residue. Numbers on top (first line) indicate the position of the amino acids. Underlining the sequence labelled with (-d-) shows the sequence obtained by direct Edman degradation of native toxin (cysteinyl residues were blanks). RC-toxin gave unequivocal sequence (.rc.) up to the residue at position 34, while the last overlapping segment 31 to 35 was obtained after tryptic digestion (=t=).

Table 2 Amino acid sequence of heterodimeric peptides corresponding to the disulphide bridges of Pi1

Amino acid sequences were obtained by microsequencing peptides purified by HPLC after cleavage with trypsin, and further digestion of peptide 23.70 with thermolysin (time between parentheses). Numbers on top of the amino acid mean positions in the sequence; Xxx, blank position corresponding to a cysteine; CYS, position of a cystine residue (usually in our machine we can identify a component that absorbs at 330 nm which correspond to this amino acid).

Elution time HPLC	Amino acid sequence	Corresponding disulfide
19.97	1 5 Gly-Thr-Ser-Asp-CYS-Gly Met-Xxx-Lys	Cys10-S-S-Cys30
21.23	1 CYS-Arg Xxx-Ile-Asn-Arg	Cys4-S-S-Cys25
23.70	1 5 10 Pro-CYS-Gln-Gln-Gln-Thr-Gly-CYS-Pro-Asn- Xxx-Tyr-Gly-Xxx	Cys14, Cys20 Cys32, Cys35
(21.09)	1 Pro-CYS Xxx-Tyr	Cys14-S-S-Cys32
(16.54)	1 5 Gln-Thr-Gly-CYS-Pro-Asn-Ser Gly-Xxx	Cys20-S-S-Cys35

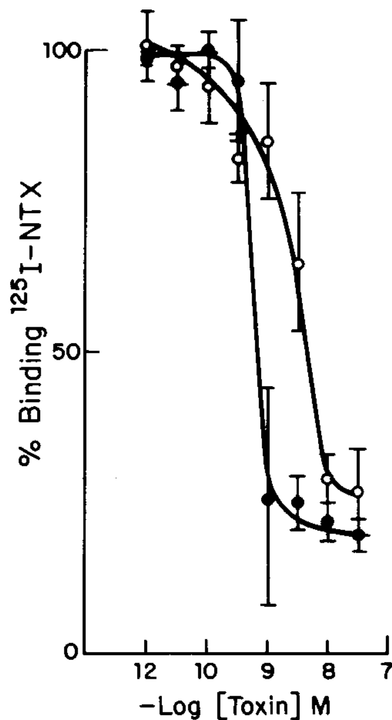

Figure 3 Binding inhibition of [^{125}I]NTX by Pi1 to synaptosome membranes

Rat brain synaptosome membranes (20 μg of protein) were incubated with [^{125}I]NTX at 50 pM, in a 500 μl final volume of a 20 mM Tris/HCl buffer, pH 7.8, containing 50 mM NaCl and 0.1 % BSA in the absence and presence of increasing concentrations of unlabelled NTX (●) and Pi1 (○), for 1 h at room temperature. The reaction mixtures were diluted and filtered immediately through glass-fibre filters, and the radioactivity measured. Values are mean of triplicate determinations ± S.E.M.

Cys-7–Cys-28, Cys-13–Cys-33 and Cys-17–Cys-35 of charybdotoxin [2]. The disulphide at positions Cys-20–Cys-35 is the novel structural characteristic of this Pi1 toxin. Molecular modelling, using the parameters obtained for charybdotoxin (see review by Ménez et al. [6]) showed that it is possible to accommodate the four disulphide bridges into the same general three-dimensional structure (results not shown) of the other known K$^+$-channel blocking peptides, extracted from other scorpion venoms. We are currently determining the three-dimensional structure of Pi1 by proton NMR (M. Delepierre, A. Prochnika-Chalufour and L. D. Possani, unpublished work) with results compatible with the computer modelling mentioned.

Binding assays

We were wondering what was the role, if any, of the two 'extra' half-Cys residues in terms of the physiological effects of this peptide on K$^+$ channels. We knew from our biossay experiments that it was capable of displacing [^{125}I]NTX from brain synaptosomal membranes. Thus, we proceeded in preparing a full binding-displacement curve in this system. Figure 3 shows that Pi1 can fully displace the binding of labelled NTX with an IC_{50} in the order of 10 nM.

Pi1 blocks *Shaker* B K$^+$ channels

The results of the displacement experiments prompted us to look directly at the effects of Pi1 on the activity of K$^+$ channels. For this purpose we express *Shaker* B K$^+$ channels in the insect cell line Sf9 (see the Materials and methods section). This system has the advantage that the *Shaker* channels are the only voltage-dependent macroscopic conductance in the membrane of the cells, thus making the results unambiguous [16]. Figure 4(a) shows macroscopic currents through *Shaker* B K$^+$ channels under whole-cell patch-clamp; addition of 70 nM of Pi1 to the external solution produced a reversible 75 % reduction of the current at all voltages, this is best seen by looking at the current–voltage curve (Figure 4b). We have observed the same result in at least 10 cells. Therefore, Pi1 reversibly blocks *Shaker* B K$^+$ channels at nanomolar concentrations.

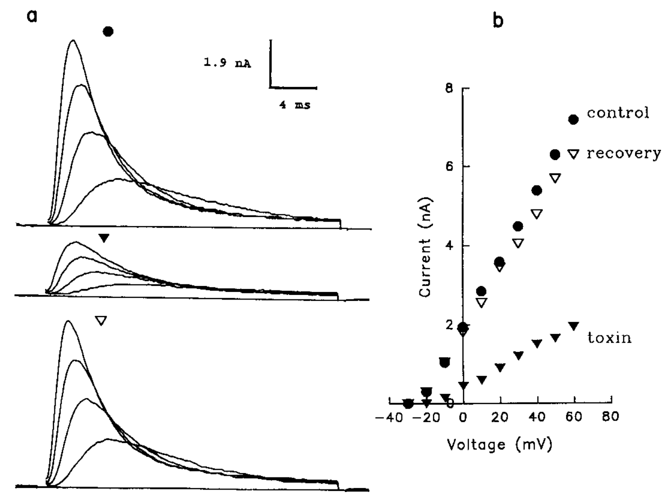

Figure 4 Pi1 blocks *Shaker* B K$^+$ channels

(**a**) K$^+$ currents through *Shaker* B channels were recorded under whole-cell patch-clamp. The channels were opened by depolarizing the membrane for 30 ms, from a holding potential of −80 mV. The upper panel shows K$^+$ currents in the control solution, for pulses of 0, 20, 40 and 60 mV. Addition of 70 nM of Pi1 to the external solution caused an approx. 75 % reduction in the amplitude of the current at all voltages (middle panel). The block by Pi1 is reversible; washing the cells with the control solution caused an almost complete recovery, as shown in the lower panel. (**b**) Current–voltage (IV) relationship of the currents in the left panel over the range −30 to +60 mV. The addition of Pi1 scaled down the control IV by approx. 75 % over all the voltage range; after washing the recovery was practically complete.

Table 3 Comparative amino acid sequence of K$^+$ channel toxins

Pi1, from this work; CllTX 1, toxin 1 from [10]; NTX, noxiustoxin from [13]; MgTX (margatoxin), ChTX (charybdotoxin), IbTX (iberiotoxin), Lq2 (*Leirus quinquestriatus* toxin 2) and AgTx1 to AgTX3 from *Leirus quinquestriatus* var. *hebraeus* (see reviews in [2, 18]); LeTX I leirutoxin 1 [19, 20]; KlTX, kaliotoxin [21]. Consensus means only positions in which amino acids are conserved in all sequences. Gaps (-) were introduced to enhance similarities. Cys in italic are new cysteines in Pi1.

Toxin	Amino acid sequence				Similarity (%)
	1 10	20	30	39	
Pi1	--L-VKCRGT	SDCGRPCQQQ	TG*C*PNS-KCI	NRMCKCYG*C*	100
CllTX 1	ITINVKCTSP	QQCLRPCKDR	FGQHAGGKCI	NGKCKCYP	46
MgTX	TIINVKCTSP	KQCLPPCKAQ	FGQSAGAKCM	NGKCKCYPH	43
NTX	TIINVKCTSP	KQCSKPCKEL	YGSSAGAKCM	NGKCKCYNN	40
KlTX	GVEINVKCSGS	PQCLKPCKDA	GMRF-G-KCM	NRKCHCTPK	37
AgTX1	GVPINVKCTGS	PQCLKPCKDA	GMRF-G-KCI	NGKCHCTPK	37
AgTX2	GVPINVSCTGS	PQCIKPCKDA	GMRF-G-KCM	NRKCHCTPK	34
AgTX3	GVPINVPCTGS	PQCIKPCKDA	GMRF-G-KCM	NRKCHCTPK	34
ChTX	pEFTNVSCTTS	KECWSVCQRL	HNTSRG-KCM	NKKCRCYS	31
IbTX	pEFTDVDCSVS	KECWSVCKDL	FGVDRG-KCM	GKKCRCYQ	29
Lq2	pEFTQESCTAS	NQCWSICKRL	HNTNRG-KCM	NKKCRCYS	26
LeTX I	AF----CNL-	RMCQLSCRSL	-GL-LG-KCI	GDKCECVKH	26
Consensus	-------C---	--C---C---	-------KC-	---C-C---	

Pi1 is the first class of K$^+$-channel blocking peptide isolated from scorpion venom to contain four disulphide bridges. If we

compare its primary structure with those of other known peptides that recognize K^+ channels, it is clear that it is quite different (Table 3). Apart from the six common cysteinyl residues the only other amino acid absolutely conserved in all these sequences is Lys-28, equivalent to that of Lys-27 shown to be essential for channel binding (reviewed in [2]). Toxin 1 from *Centruroides limpidus limpidus* [10] is the closest related one, with only 46 % similarity. Most of the Old World scorpion toxins have less than 37 % identity (Table 3). Thus, its unique structure makes Pi1 an interesting model peptide for structure–function relationship studies on K^+ channels.

The final analysis of the three-dimensional structure of Pi1 with that of other toxins (when it is finished), will certainly clarify the possible role of the fourth disulphide into the general folding, including possible structural modifications, of Pi1 in comparison with the other known toxins. Our present results suggest that these modifications are not fundamentally important for channel recognition or affinity. However, it might confer more stability to this peptide, by restraining mobility of the molecule through an extra disulphide pairing.

We thank Dr. Muriel Delepierre from the Pasteur Institute (Paris, France) for providing information on the initial three-dimensional structure of Pi1. This work was supported in part by grants from the Howard Hughes Medical Institute (No. 75191-527104), from the National University of Mexico (DGAPA No. IN-205893 and IN-206994), the National Council of Research and Technology of Mexico (CONACyT No.4734-N), and the European Commission (CI1*-CT 94-0045).

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Received 20 October 1995/11 December 1995; accepted 18 December 1995