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Two Novel Toxins from the Venom of the Scorpion *Pandinus imperator* Show that the N-terminal Amino Acid Sequence is Important for their Affinities towards *Shaker* B K^+ Channels

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Abstract. Two novel peptides were purified from the venom of the scorpion *Pandinus imperator*, and were named Pi2 and Pi3. Their complete primary structures were determined and their blocking effects on *Shaker* B K^+ channels were studied. Both peptides contain 35 amino acids residues, compacted by three disulfide bridges, and reversibly block the *Shaker* B K^+ channels. They have only one amino acid changed in their sequence, at position 7 (a proline for a glutamic acid). Whereas peptide Pi2, containing the Pro7, binds the *Shaker* B K^+ channels with a K_d of 8.2 nM, peptide Pi3 containing the Glu7 residue has a much lower affinity of 140 nM. Both peptides are capable of displacing the binding of ^{125}I-noxiustoxin to brain synaptosome membranes. Since these two novel peptides are about 50% identical to noxiustoxin, the present results support previous data published by our group showing that the amino-terminal region of noxiustoxin, and also the amino-terminal sequence of the newly purified homologues: Pi2, and Pi3, are important for the recognition of potassium channels.

Key words: K^+ channel — Scorpion toxin, — *Pandinus imperator* — *Shaker* B expression — Sf9 cells

Introduction

Blocking agents against ion channels have been instrumental in defining both structural and mechanistic properties of the channels. They also allow to discriminate, hence to study, the various different conductances normally present in most cells [12].

It is well known that among blocking agents of K^+ channels, the toxins from scorpion venoms are a particularly useful group. Most scorpion toxins against K^+ channels (K^+ toxins) are short peptides, composed of 31–39 amino acid residues, with a highly variable amino acid sequence [reviews 7,17]. Regardless of their great variability on the primary structure, the three-dimensional structure of K^+ toxins share a common motif: a short alpha helix and three strands of beta sheet structures, stabilized by three disulfide bridges [15]. Concerning their mode of action, previous work conducted with noxiustoxin (NTX), the first K^+ toxin ever described [3,18], suggested that part of the sequence that recognizes K^+ channels in NTX is located towards the amino terminal region. In fact, synthetic peptides with only nine and/or twenty amino acid residues corresponding to the amino terminal sequence of NTX are toxic to mice [9], and effectively block Ca^{2+}-activated K^+ channels [20]. Furthermore, while NTX needs the N-terminal segment for channel recognition, another K^+ toxin: charybdotoxin (ChTX) has the C-terminal segment as the most important segment for channel recognition [10,16], despite the fact that Ser10 and Trp14, at the N-terminal region are also considered to be ''crucial residues'' [19].

Since the affinity of the synthetically prepared N-terminal peptides, for the K^+ channels studied [10], are considerably lower than that of native NTX, it is quite possible that the C-terminal part is also necessary for full recognition and binding to the channels, either because the C-terminal segment also interacts with the receptors, or it is important for structural reasons, positioning the N-terminal region in its best conformation.

In this work, we describe for the first time two new K^+ toxins purified from the venom of the scorpion *P. imperator*. The toxins have identical primary structures except for position 7, at the N-terminal region, where a

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Pro substitute for a Glu. While Pi2 (Pro7) has a 8.2 nM K_d to *Shaker* B K^+ channels, expressed in Sf9 cells, Pi3 (Glu7) has a seventeenfold lower affinity (K_d) of about 140 nM). The differential affinity of these toxins, supports our previous suggestion that the amino terminal residues of NTX-like K^+ toxins are part of the domain that recognizes K^+ channels.

Materials and Methods

Source of Venom

Scorpions of the species *P. imperator* (Gabon, Africa) maintained alive in the laboratory were anesthetized monthly with CO_2 and milked for venom by electrical stimulation. The venom was placed in double distilled water and centrifuged at 15,000 × *g* for 15 min. The supernatant was freeze-dried and stored at −20°C until use.

Separation and Bioassays

The soluble venom was initially fractionated in a Sephadex G-50 column and their subfractions were further separated by HPLC, using a C18 reverse-phase column (Vydac, Hysperia, CA), of a Waters 600E high performance liquid chromographer (HPLC), equipped with a variable wavelength detector, and a WIPS 712 automatic sample injector.

The homogeneity of the purified peptides was confirmed by step-gradient HPLC and by direct Edman degradation using an automatic sequencer [14]. 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 synaptosome membranes of previously bounded ^{125}[I]-NTX [21]. With this procedure two peptides eluting initially in the fraction III of the Sephadex G-50 column, and subsequently in the peak 2 and 3 of the HPLC, respectively, were isolated in homogeneous form and used for chemical characterization and electrophysiological experiments.

Amino Acid Analysis and Sequencing

Samples (about one nmol each) of the pure native peptides Pi2, and Pi3, and their fragments generated by enzymatic digestion, were analyzed in a Beckman 6300E amino acid analyzer, after acid hydrolysis for 20 hr in 6 N HCl at 110°C. An aliquot of each one of the pure peptides (100 μg) was reduced and alkylated with iodoacetic acid as described [14]. An automatic ProSequencer (Millipore model 6400/6600) was used to determine the amino acid sequence of: (i) the individual native peptides, (ii) their reduced and carboxy-methylated derivatives (RC-peptides), and (iii) their HPLC-purified fragments produced by protease V8 endopeptidase (Bohering, Manheim, Germany) digestion. The cleavage of RC-peptides with protease V8 was performed with samples containing 50 μg peptide in 200 μl, buffered with 100 mM ammonium bicarbonate, pH 7.8; incubated for 4 hr at 37°C, in the presence of 2 μg digestive enzyme. For Pi2, an additional hydrolysis was performed with cyanogen bromide using a 25 μg sample in 400 μl of 10% formic acid at room temperature overnight. The corresponding fragments were also separated by HPLC, and used for sequence and amino acid analysis.

Protein Content

The concentration of peptides used for binding and electrophysiological measurements were estimated based on amino acid analysis. A solution containing 1.0 mg/ml peptide, for both toxins, absorbs 0.65 units at 280 nm, using a cuvette with 1 cm pathway.

Binding and Displacement Assays

NTX was radiolabeled with ^{125}Iodine and used for binding and displacement assays as described [21]. Rat brain synaptosomal membranes (fraction P3) were prepared by the method of Catterall, Morrow and Harfshorne [4], and used for assessing the capability of Pi2 and Pi3 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 were done as previously reported [8]. In brief: the cells were kept at 27°C in Grace's media, and infected with the recombinant baculovirus *Autographa californica* nuclear polyhedrosis virus, containing the *Shaker* B K^+ channel cDNA [13]. The recombinant baculovirus was a kind gift of 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 to 1.8 MΩ, and used without further treatment. Macroscopic currents through *Shaker* B K^+ channels were recorded under whole-cell patch-clamp [11], two days after the infection of the cells, with an Axopatch 1D (Axon Instruments). The delivery of the pulses and the acquisition of the data were done through a TL-2 interface (Axon Instruments) connected to a PC computer, with the pClamp 5.5 software. Currents were sampled at 100 μsec/point. Between 70-75% of the series resistance were electronically compensated.

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

The internal solution was (in mM): 90 KF, 30 KCl, 2 $MgCl_2$, 10 EGTA, 10 HEPES-K, pH 7.2. The external solution was: 145 NaCl, 10 $CaCl_2$, 10 MES-Na, pH 6.4.

Results and Discussion

Peptide Purification and Amino acid Sequence

Two unknown K^+- channel-blocking peptides (Pi2, and Pi3) were isolated from the venom of the scorpion *P. imperator*, by chromatographic procedures. Briefly, the soluble venom was initially separated by gel-filtration chromatography in Sephadex G-50 (Fig. 1*a*). The fraction III, which contained the peptides capable of displacing the binding of ^{125}I-NTX to synaptosome membranes, was subsequently applied to an HPLC column (Fig. 1*b*). Subfractions 2 and 3 from this column were rechromatographed in a step gradient, giving a homogeneous com-

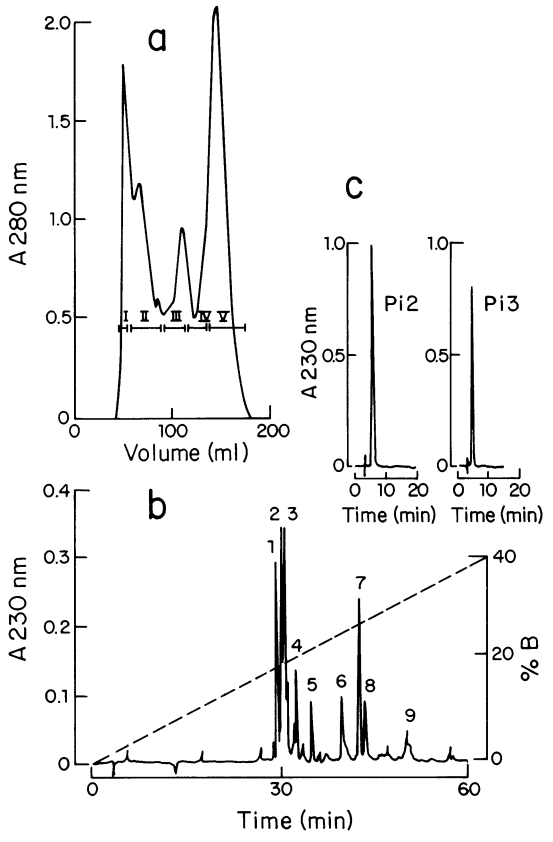

Fig. 1. Sephadex G-50 and HPLC purification of Pi2 and Pi3. (*a*) *P. imperator* soluble venom (120 mg) was applied to a Sephadex G-50 column (0.9 × 200 cm) equilibrated and run with 20 mM ammonium acetate buffer, pH 4.7, at a flow rate of 30 ml per hr. Five subfractions (horizontal bars *I-V*) were 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% solution B (0.1% trifluoroacetic acid in acetonitrile), for 60 min. (*c*) Fractions 2 and 3 were further separated by a step gradient (12% solution B for Pi2, and 13% B for Pi3) given pure toxins Pi2 and Pi3, as demonstrated by Edman degradation and amino acid analysis.

ponent (Fig. 1*c*). These peptides were named Pi2 and Pi3, which are the abbreviations of *P. imperator* toxin 2 and 3, respectively (the word toxin is not included). The total amount of these peptides, based on recovery values from the chromatographic separations, showed that Pi2 is about 2.1%, while Pi3 is 2.5% of the whole soluble venom.

Amino acid analysis (Table 1) of both peptides showed that they are composed of 35 amino acid residues each, with a calculated molecular weight of 4,036 for Pi2 and 4,068 for Pi3, respectively. To confirm the initial amino acid composition and to determine the full amino acid sequence of these toxins, samples of native peptides, RC-toxins and the endopeptidase V8 digestion products of RC-toxins, after HPLC fractionation (Fig. 2), were sequenced. The HPLC-separated fragments for both Pi2 and Pi3 (Fig. 2), were analyzed *de novo*, for their amino acid content (Table 1). Both peptides have confirmed the amino acid composition initially determined for the native peptides and also provided evidence for the presence of two arginines in both peptides.

In Fig. 3, we show the full amino acid sequence of Pi2 and Pi3, indicating the corresponding overlapping segments. The sequence of the C-terminal segment of Pi2 was confirmed after cyanogen bromide rupture of Met at position 26. Since the composition of Pi2 and Pi3 differs by only one amino acid, the sequence determination of Pi3 was conducted directly with RC-toxin. Pi2 and Pi3 are naturally occurring single amino acid mutants of each other. Their three dimensional structures are assumed to be closely compacted by three disulfide bridges (6 cysteines each), as demonstrated for the case of NTX and ChTx [7,10].

The Novel Peptides, Pi2 and Pi3, Block Voltage-dependent K^+ Channels

Two assays were used to determine the possible function of these peptides. By binding and displacement experiments, using radiolabeled noxiustoxin [21] it was found that both Pi2 and Pi3 were effective in competing for similar binding sites (K^+ channels) of membranes obtained from rat brain synaptosomes (*data not shown*). To confirm these data, we decided to verify their effects on the activity of well-defined K^+ channels. For this purpose we expressed *Shaker* B K^+ channels in the insect cell line Sf9, by infection with a recombinant baculovirus (*see Materials and Methods*). This system has the advantage that the *Shaker* B channels are the only voltage-dependent macroscopic conductance in the membrane of the cells, thus making the results unambiguous [13].

Addition of Pi2 to the external solution effectively reduces the current through *Shaker* B K^+ channels, in a reversible manner. This is shown in Fig. 4. The upper panel shows macroscopic currents through *Shaker* B K^+channels under whole-cell patch clamp. The channels were opened by depolarizing the membrane to the indicated voltages, from a holding potential of −80 mV (upper traces, control). Addition of 150 nM of Pi2 to the external solution, produced an almost 100% reduction of the current at all voltages (middle traces). The effect was easily reverted by washing the cells with the control external solution (lower traces, recovery). This is best seen by looking at the current *vs.* voltage curve (*IV*) in the bottom panel.

Using lower concentrations of Pi2, it is readily seen that the reduction in the current is not voltage dependent (*see below*), and does not change the kinetics of the macroscopic currents; therefore the simplest interpretation of the reduction of the current induced by Pi2, is that the peptide is blocking the K^+ channels.

We have shown that Pi2 is a highly effective blocker

Table 1. Amino acid composition of peptides

Amino acid	Pi2 nmol (i)[a]	Pi3 nmol (i)	Pi2N nmol (i)	Pi2C nmol (i)	Pi3N nmol (i)	Pi3M nmol (i)	Pi3C nmol (i)
Asp	3.4 (3)	3.2 (3)	1.2 (1)	2.0 (2)	1.1 (1)	t.a (0)[b]	2.0 (2)
Thr	2.6 (3)	2.6 (3)	1.7 (2)	1.0 (1)	1.7 (1)	t.a (0)	0.8 (1)
Ser	0.9 (1)	1.0 (1)	1.0 (1)	t.a (0)	0.7 (1)	t.a (0)	t.a (0)
Glu	2.4 (2)	3.2 (3)	2.1 (2)	t.a (0)	1.1 (1)	2.2 (2)	t.a (0)
Pro	2.6 (3)	1.8 (2)	1.7 (2)	0.9 (1)	t.a (0)	0.9 (1)	0.8 (1)
Gly	2.4 (2)	2.3 (2)	t.a (0)	2.0 (2)	t.a (0)	t.a (0)	2.1 (2)
Ala	1.1 (1)	1.1 (1)	t.a (0)	1.0 (1)	t.a (0)	t.a (0)	1.0 (1)
Val	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)
Met	1.1 (1)	0.9 (1)	t.a (0)	0.6 (1)	t.a (0)	t.a (0)	0.4 (1)
Ile	0.6 (1)	0.7 (1)	0.9 (1)	t.a (0)	1.0 (1)	t.a (0)	t.a (0)
Leu	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)	t.a (0)
Tyr	1.8 (2)	1.8 (2)	0.9 (1)	1.0 (1)	t.a (0)	0.7 (1)	0.8 (1)
Phe	1.0 (1)	1.0 (1)	t.a (0)	1.1 (1)	t.a (0)	t.a (0)	0.8 (1)
His	1.0 (1)	1.2 (1)	0.9 (1)	t.a (0)	t.a (0)	1.0 (1)	t.a (0)
Lys	6.2 (6)	6.3 (6)	2.9 (3)	3.1 (3)	t.a (0)	2.9 (3)	2.7 (3)
Arg	1.2 (1)[c]	1.2 (1)[c]	t.a (0)	1.9 (2)	t.a (0)	t.a (0)	2.1 (2)
Trp[d]	− (0)	− (0)	− (0)	− (0)	− (0)	− (0)	− (0)
½ Cys[d]	− (6)	− (6)	− (3)	− (3)	− (1)	− (2)	− (3)
Total	35	35	17	18	7	10	18

Pi2 and Pi3 are toxins 2 and 3, respectively. Pi2N and Pi3N, and Pi2C and Pi3C are N-terminal and C-terminal fragments generated by endopeptidase v8 hydrolysis of Pi2 and Pi3, respectively, while Pi3M means intermediate peptide from v8 cleavage of Pi3.

[a] (i), means nearest integer number of residues found per mol

[b] t.a, means trace amounts or none

[c] Arg were confirmed to be two per molecule

[d] −Trp and ½ Cys number determined by sequencing

of *Shaker* B K^+ channels. It was therefore of major interest to examine the effect of Pi3, a peptide considered to be a natural point mutation of Pi2, as mentioned before. Figure 5 shows the effect of 150 nM Pi3 over the activity of *Shaker* B. It is clear from Fig. 5 that Pi3 also blocks the channels, but whereas the addition of Pi2 caused an almost 100% reduction in the amplitude of the current, the addition of Pi3, at the same concentration, caused only approx. 48% reduction of the current. Therefore, the affinity of Pi3 for the channels seems to be significantly smaller than that of Pi2.

Neither the block by Pi2, nor that by Pi3 have a noticeable voltage dependence. This is shown in Fig. 6, where the fraction of blocked channels is plotted against the voltage. It is interesting to observe that even 7.5 nM of Pi2 produces a stronger block than 150 nM Pi3.

The above results clearly show that the change of Pro7 in Pi2, for Glu7 in Pi3 significantly reduces the affinity of Pi3 towards the *Shaker* B K^+ channels. To quantitate this point we measured the extent of the block produced by several concentrations of the peptides. The results in Fig. 7 show that: (i) the concentration dependence of the block, by both Pi2 and Pi3, follows a Michaelis-Menten saturation curve, meaning that each channel is blocked by a single peptide, (ii) with zero K^+ (*not added*) in the external solution, the K_d of Pi2 is 8.2 nM, and (iii) the change Pro7->Glu7 dramatically reduces the affinity of the peptide for the channels, K_d over 140 nM.

These results are not pH dependent in the range of 5.5 to 8.1 (*data not shown*). Since the binding of Pi2 and Pi3 is dependent on the concentration of external K^+, and for comparative purposes in identical conditions, these experiments were performed in absence of potassium. It is worth mentioning that K^+ is an effective competitor of the toxin binding after 5 mM. There is practically no difference between 0 and 5 mM external K^+ concentration (*data not shown*).

Structure-Function Relationship

Scorpion toxins against K^+ channels are short peptides of about 31 to 39 amino acids [7,17], whereas the toxins against Na^+ channels are longer peptides containing 61 to 70 amino acids residues [17,18]. Concerning their primary structure, both families of toxins are highly variable. Except for the constant relative positions of the cysteines, and a couple of the other residues, most of the amino acids of the primary structure are variable. This variability certainly explains the differential affinities of these peptides for their various subtypes of ion channels. For example, noxiustoxin (NTX) binds with 100 pM affinity to the K^+ channels present in rat brain synaptosomes [10], whereas it binds with 450 nM affinity to Ca^{2+}-dependent K^+ channels of rabbit skeletal muscle [22], and with 310 nM to Ca^{2+}-dependent K^+ channels of epithelial cells [20]. Its homologous peptide (about 50%

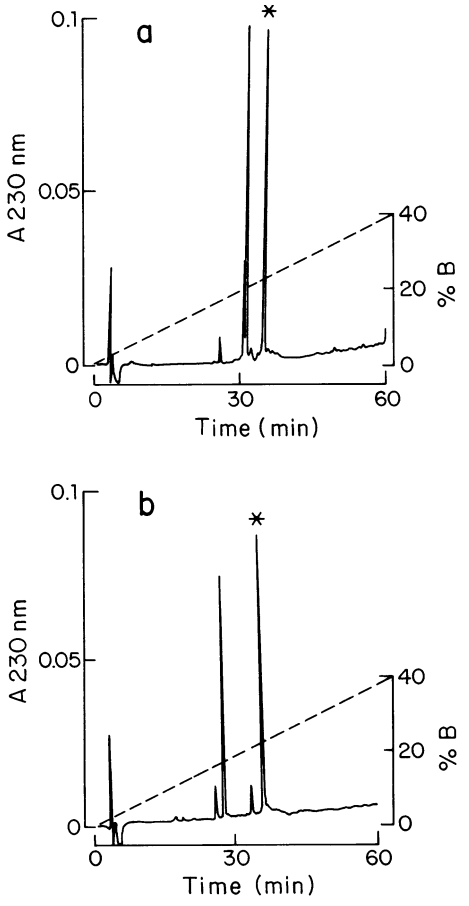

Fig. 2. HPLC separation of peptides from Pi2 and Pi3. Samples containing 50 μg each of Pi2 (Fig. 2*a*) and Pi3 (Fig. 2*b*) were hydrolyzed with endopeptidase v8 and separated in a C18 analytical reverse-phase column, using the same system as Fig. 1*b*. The stars in each chromatogram indicate the elution time of the C-terminal fragment.

```
     1         10         20         30    35
Pi2  TISCTNPKQC YPHCKKETGY PNAKCMNRKC KCFGR
     :--------- ----d----- ----->
     :......... .......rc. .......... >
                       :== =====v8=== ===>
                              :.-.-cb.->
                                      aa

     1         10         20         30    35
Pi3  TISCTNEKQC YPHCKKETGY PNAKCMNRKC KCFGR
     :......... .......rc. .......... >
                       :== =====v8=== ====>
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Fig. 3. Amino acid sequence of Pi2 and Pi3. Numbers on top of the sequences indicate the positions of the amino acids after ordering the final sequence, according to overlapping segments obtained by direct sequencing the native toxin (-d-); RC-toxin (.rc.); v8 endopeptidase cleavage (=v8=), cyanogen bromide (-.cb.-) and amino acid analysis (aa).

identical), charybdotoxin (ChTX) has a K_d of 25–30 pM to plasma K^+ channels of membranes prepared from rat brain synaptosomes [reviewed in 7] and 1.8 nM to Ca^{2+}-dependent K^+ channels from rabbit skeletal muscle [22].

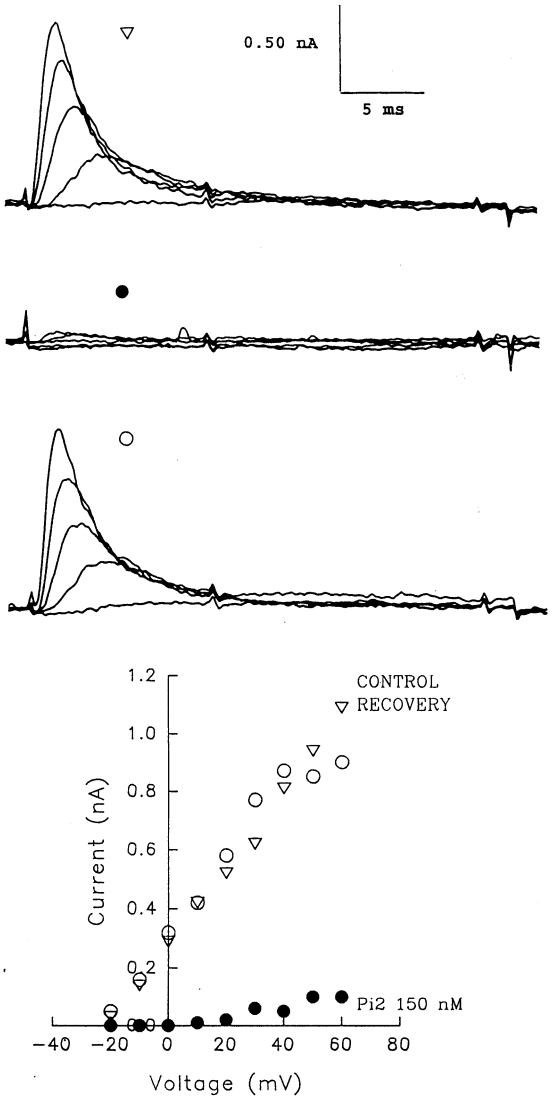

Fig. 4. Pi2 added to the external solution blocks *Shaker* B K^+ channels. The upper traces shows macroscopic currents under whole cell patch clamp, in the control external solution (*see* Materials and Methods). The channels were opened by 30 msec depolarizations from −10 to +60 mV in 20 mV increments, from the holding potential of −80 mV. Addition of 150 nM of Pi2 to the external solution produced an almost 100% reduction of the current (middle traces). The effect of Pi2 is easily and totally reversed by washing the cells with the control external solution (lower traces). These effects are best shown by the complete peak current *vs.* voltage curve (*IV*), at the bottom of the figure.

If we compare the primary structures of Pi2 and Pi3 with those of other known peptides that recognize K^+ channels, it is evident that they are quite different. As shown on Table 2, the toxins from scorpions of the New World, genus *Centruroides*, are most closely related to Pi2 and Pi3 (54 to 42% identity), than those of the Old World scorpions of the genera *Leiurus*, *Buthus* and *Androctonus* (only 38 to 29% identity). Apart from the six common cysteinyl residues, the only other amino acid

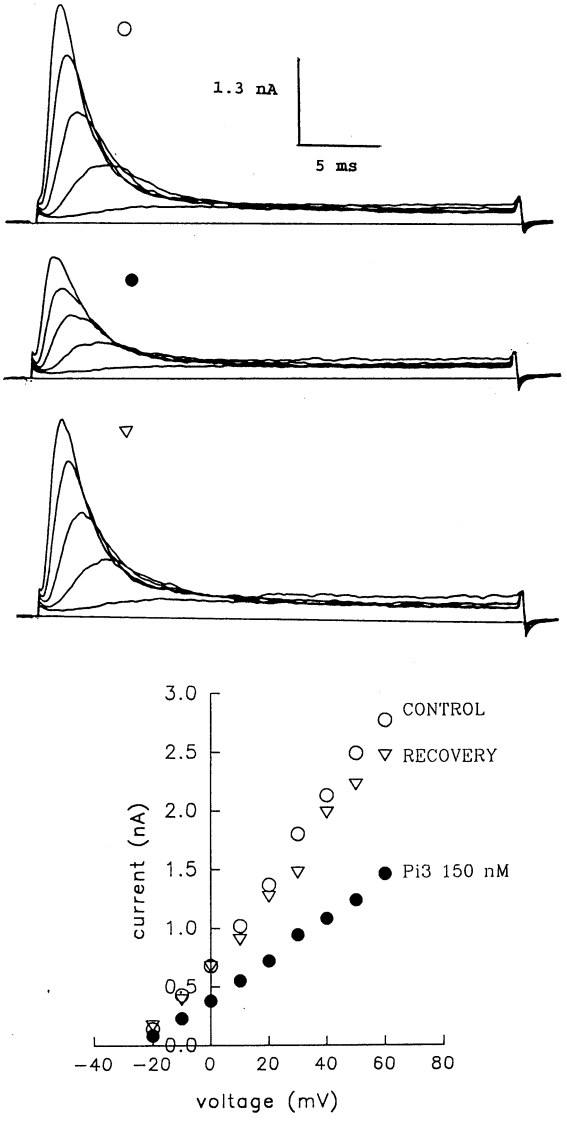

Fig. 5. Pi3 added to the external solution blocks *Shaker* B K^+ channels. The upper traces shows macroscopic currents under whole cell patch clamp, in the control external solution (*see* Materials and Methods). The channels were opened as indicated in Fig. 4. Addition of 150 nM of Pi3 to the external solution produced a moderate ~48% reduction of the current (middle traces). The effect of Pi3 is easily and totally reversed by washing the cells with the control external solution (lower traces). These effects are best shown by the complete *IV* at the bottom of the figure.

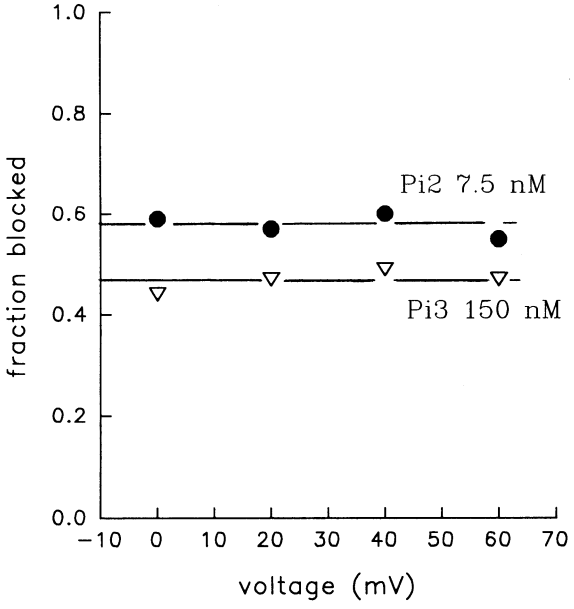

Fig. 6. Block by both Pi2 and Pi3 is not voltage dependent. The fraction of the channels blocked is plotted against the voltage, for two different cells. 7.5 nM of Pi2 caused an average 58% of block at all voltages, whereas 150 nM of Pi3 caused an average 49% of block. Thus, the extent of block by both toxins is not appreciably voltage dependent (the extent of block also does not depend on the holding potential—*not shown*). The fraction blocked at each voltage was calculated as 1-(*It*/*Ic*), where *Ic* is the peak current in the control and *It* is the peak current after the addition of the toxin.

absolutely conserved in all these sequences is lysine in position 28 of Table 2, which corresponds to Lys27 in charybdotoxin, shown to be essential for channel binding [7,16]. However, the most relevant information obtained by comparing these K^+-toxins (Table 2) is that Pi2 and Pi3 differs in only one position (number 7), and that is enough to change their affinities for the same type of *Shaker* B K^+ channels by at least seventeenfold.

This constitutes a clear indication that the N-terminal sequence of both toxins are important for channel recognition and/or affinity, in a fashion similar to that described for NTX [9,10]. The presence of a negatively charged amino acid (Glu) in position 7, substituting for the neutral proline, probably interacts unfavorably with the *Shaker* B K^+ channels. Nevertheless, the fact that the C-terminal regions have several cysteinyl residues clustered together in a highly conserved manner, including the constant Lys at position 28, is also suggestive that this part of the molecule must be important for maintaining the three-dimensional structure of the molecule and/or for channel recognition.

Additionally, the fact that in Table 2, at the N-terminal segment of the two newly purified toxins we added several gaps to enhance similarities between Pi2, Pi3 and NTX, it would suggest that the N-terminal region of these two newly purified peptides do admit some structural variations in their structures without loss of channel-binding capacity. These facts could again suggest an evolution of structural features to fit more properly with variations at the channel level.

In this same direction are the observations related to the amount of these peptides present in the various scorpion venoms. For example, the amount of Pi2 plus Pi3 (2.1% and 2.5, respectively) and Pi1 (also a K^+ toxin) which is approx. 1.5% [*unpublished results*], all together

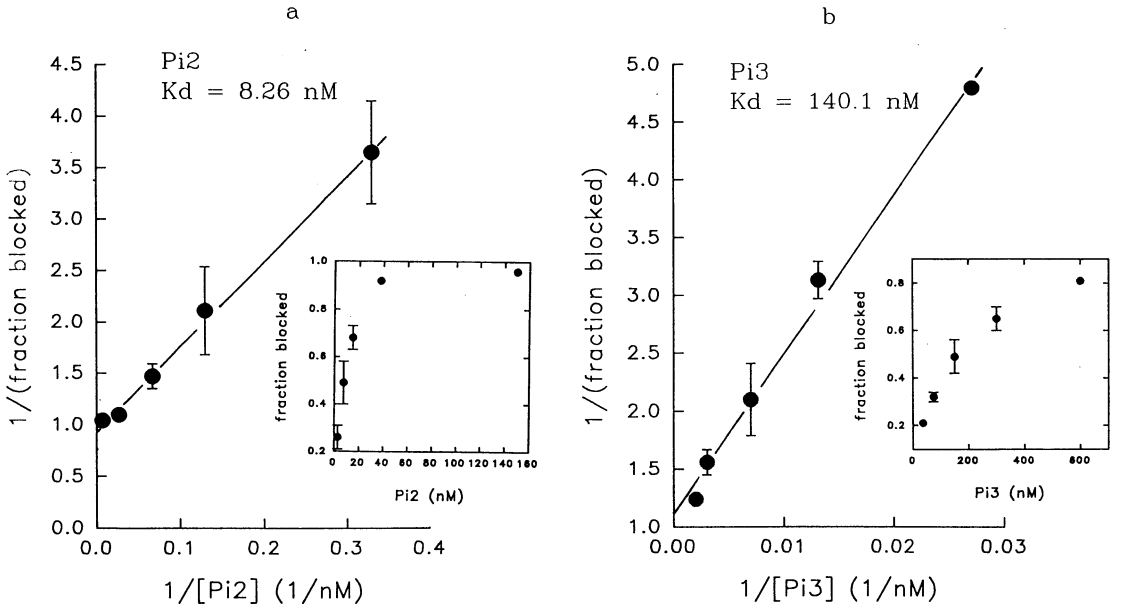

Fig. 7. The extent of block is a function of the toxin concentration. (*a*) The fraction blocked by Pi2 is shown in a double-reciprocal plot, each point represents the mean ± SD of at least three different cells. The block by Pi2 follows a Michaelis-Menten saturation curve with a K_d of only 8.2 nM ($r = 0.998$). The inset shows the direct (fraction blocked *vs.* concentration) curve. (*b*) Double-reciprocal plot of the fraction of channels blocked by Pi3. The block follows a Michaelis-Menten saturation curve with a K_d of 140 nM ($r = 0.995$). The inset shows the direct plot. The points represent the mean ± SD of at least 3 different cells.

Table 2. Comparative amino acid sequence of K^+ channel toxins

Toxin	Amino acid sequence				Identity (%)
	1 10	20	30	39	
Pi2	-TI---SCTNP	KQCYPHCKKE	TGYPN-AKCM	NRKCKCFGR	100
Pi3	-TI---SCTNE	KQCYPHCKKE	TGYPN-AKCM	NRKCKCFGR	97
MgTX	-TIINVKCTSP	KQCLPPCKAQ	FGQSAGAKCM	NGKCKCYPH	54
NTX	-TIINVKCTSP	KQCSKPCKEL	YGSSAGAKCM	NGKCKCYNN	51
Pi1	---L-VKCRGT	SDCGRPCQQQ	TGCPN-SKCI	NRMCKCYGC	43
C11TX 1	ITI-NVKCTSP	QQCLRPCKDR	FGQHAGGKCI	NGKCKCYP-	42
Lg2	pEFTQESCTAS	NQCWSICKRL	HNTNRG-KCM	NKKCRCYS-	38
AgTX2	GVPINVSCTGS	PQCIKPCKDA	GMRF-G-KCM	NRKCHCTPK	38
AgTX3	GVPINVPCTGS	PQCIKPCKDA	GMRF-G-KCM	NRKCHCTPK	36
ChTX	pEFTNVSCTTS	KECWSVCQRL	HNTSRG-KCM	NKKCRCYS-	33
K1TX	GVEINVKCSGS	PQCLKPCKDA	GMRF-G-KCM	NRKCHCTPK	33
IbTX	pEFTDVDCSVS	KECWSVCKDL	FGVDRG-KCM	GKKCRCYQ-	32
AgTX1	GVPINVKCTGS	PQCLKPCKDA	GMRF-G-KCI	NGKCHCTPK	30
LeTX I	AF----C-NL	RMCQLSCRSL	-GL-LG-KCI	GDKCECVKH	29
Consensus	------C---	--C---C---	-------KC-	---C-C---	

Pi2 and Pi3, from this work; Pi1 from T. Olamendi-Portugal et al., *unpublished results*; c11TX 1, toxin 1 from [14]; NTX, noxiustoxin from [18]; MgTX (margatoxin), ChTX (charybdotoxin), IbTX (iberiotoxin), Lq2 (*L. quinquestriatus* toxin 2) and AgTX1 to AgTX3 from *L. quinquestriatus* var. hebraeus [reviewed in 7] LeTX I, leirutoxin 1 [1,5]. K1TX, kaliotoxin [6]. Consensus means only positions in which amino acids are conserved in all sequences. Gaps (-) were introduced to enhance similarities.

comprises 6.1% of the whole soluble venom, which is a much greater amount in the venom of *P. imperator* than the related toxins, charybdotoxin and noxiustoxin, isolated respectively from *Leiurus quinquestriatus* and *Centruroides noxius.* The latter two occur in less than 0.5% in each of the corresponding venoms [2,17]. This fact probably indicates some evolutionary trait. While the first (*Pandinus*) is nontoxic to mammals, the two other species of scorpions *Centruroides* and *Leiurus* are. Thus, we can hypothesize that scorpions of the genus

Pandinus have compensated for the lack of typical Na^+-channel blocking toxins (rich in the two other genera) by producing a larger amount of K^+-channel specific toxins. Since *P. imperator* venom is toxic to insects and crustaceans (*unpublished results*), we are tempted to speculate that maybe these K^+ toxins are rather species specific. Hence, this variability could be, again, an evolutionary occurrence that meets with the diversity of ion channel molecules.

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