A Subfamily of Acidic \(\alpha\)-K\(^+\) Toxins*

Received for publication, October 7, 2003
Published, JBC Papers in Press, October 14, 2003, DOI 10.1074/jbc.M311029200

Isabelle Huys‡‡, Timoteo Olamendi-Portugal¶, Blanca Ines Garcia-Gómez¶, Isabel Van den Bergh†, Jozef Van Beeumen†, Karin Dyason**, Elke Clynen‡‡, Shunyi Zhu‡, Jurg van der Walt**, Lourival D. Possani¶, and Jan Tytgat†††

From the †Laboratory of Toxicology, University of Leuven, E. Van Evenstraat 4, 3000 Leuven, Belgium, ¶Department of Molecular Medicine and Bioprocesses, Institute of Biotechnology, Avenida Universidad, 2001, Cuernavaca, Mexico, ‡Laboratory of Protein Biochemistry and Protein Engineering, University of Gent, 9000 Gent, Belgium, **Department of Physiology, University of Potchefstroom, Potchefstroom, South Africa, and ‡‡Laboratory for Developmental Physiology and Molecular Biology, University of Leuven, Naamsestraat 59, 3000 Leuven, Belgium

Three homologous acidic peptides have been isolated from the venom of three different Parabuthus scorpion species, P. transvaalensis, P. villinosus, and P. granulatus. Analysis of the primary sequences reveals that they structurally belong to subfamily 11 of short chain \(\alpha\)-K\(^+\) blocking peptides (Tytgat, J., Chandy, K. G., Garcia, M. L., Gutman, G. A., Martin-Eucaulea, M. F., van der Walt, J. J., and Possani, L. D. (1999) Trends Pharmacol. Sci. 20, 444–447). These toxins are 36–37 amino acids in length and have six aligned cysteine residues, but they differ substantially from the other \(\alpha\)-K\(^+\) toxins because of the absence of the critical Lys\(^{27}\) and their total overall negative charge. Parabutoxin 1 (PBTx1), which has been expressed by recombinant methods, has been submitted to functional characterization. Despite the lack of the Lys\(^{27}\), this toxin blocks several Kv1-type channels heterologously expressed in Xenopus oocytes but with low affinities (micromolar range). Because a relationship between the biological activity and the acidic residue substitutions may exist, we set out to elucidate the relative impact of the acidic character of the toxin and the lack of the critical Lys\(^{27}\) on the weak activity of PBTx1 toward Kv1 channels. To achieve this, a specific mutant named rPBTx1 T24F/V26K was made recombinantly and fully characterized on Kv1-type channels heterologously expressed in Xenopus oocytes. Analysis of rPBTx1 T24F/V26K displaying an affinity toward Kv1.2 and Kv1.3 channels in the nanomolar range shows the importance of the functional dyad above the acidic character of this toxin.

Scorpion neurotoxins are known to inhibit several types of K\(^+\) channels (2, 3). Voltage-activated K\(^+\) channels (Kv channels)\(^1\) are ubiquitously present in almost all phylogenetic classes and are widely distributed in many cell types (4) where they are involved in the fundamental physiological processes.

Extensive progress in neurobiology and more specifically in the physiology of these large membrane-bound Kv channels was achieved by the use of specific peptide toxins produced by a variety of venomous species like snakes (Chordata) (5), sea anemones (Coelenterata), spiders (6), scorpions (Arthropoda) (7), honey bees (8), and snails of the genus Conus (Mollusca). Scorpion toxins that target K\(^+\) channels share a common globular scaffold, a cysteine-stabilized \(\alpha\)-helix-\(\beta\)-sheet structure (CS\(\alpha\beta\)), corresponding to the consensus sequence C...XXXCC...C...CXC in the primary structure of these toxins (9). More interestingly, most of the \(\alpha\)-KTx have a common functional dyad. The most critical residue of this dyad is the conserved positively charged lysine residue (Lys\(^{27}\), charybdotoxin numbering). Site-directed mutagenesis studies for several scorpion toxins have demonstrated that this lysine is indeed crucial for the interaction with the K\(^+\) channels (10) by inserting its side chain into the pore of the K\(^+\) channel (11–16). The second residue of the dyad is a hydrophobic residue (mostly Phe or Tyr) that is fully exposed from a flat surface (17, 18).

Studies on scorpion toxins have shown that almost all of these peptides are basic with an isoelectric point (pI) of >8. Only a few scorpion toxins have been reported to be neutral (pI = 7.2), like AaHIV (19, 20) and BmK M4 (21), or acidic (pI = 5.3), like the first identified \(\alpha\)-neurotoxin, BmK M8 (22). These toxins show an extremely weak toxicity in mice.

Based on the alignment of the cysteine residues and other highly related amino acids, K\(^+\) toxins have been classified into the three subfamilies \(\alpha\)-KTx, \(\beta\)-KTx, and \(\gamma\)-KTx peptides, and a general nomenclature for the \(\alpha\)-KTx has been proposed (1). The \(\alpha\)-KTx subfamilies are the best studied toxins and usually are small (up to 4 kDa) basic “short chain toxins.” To date, more than 50 different \(\alpha\)-KTx peptides have been reported and listed into 17 subfamilies (1, 23). Very recently, a new subfamily (\(\alpha\)-KTx18) has been reported (24).

In this paper, a subfamily of unusually acidic \(\alpha\)-KTx short chain scorpion toxins is described. These toxins, named parabutoxin 1 (PBTx1), parabutoxin 2 (PBTx2), and parabutoxin 10 (PBTx10), have extremely low pI values of 3.82 (for PBTx10), have extremely low pI values of 3.82 (for PBTx10), and 3.88 (for PBTx2) and 3.88 (for PBTx10). Interestingly, PBTx1, PBTx2, and PBTx10 lack the crucial pore-plugging Lys\(^{27}\) (charybdotoxin numbering). In addition, the second important residue of the dyad, the hydrophobic residue (Phe or Tyr) (17, 18), is also missing in these new toxins. At the equivalent positions, Val (PBTx1, PBTx2) and Ala (PBTx10) residues occur. Tytgat et al. (1) previously classified two of these toxins as members of the subfamilies \(\alpha\)-KTx11, \(\alpha\)-KTx11.1 (PBTx1), and \(\alpha\)-KTx11.2 (PBTx2), respectively, but a full characterization of the toxins was still lacking. Meanwhile, another similar
Acidic Potassium Channel Toxins

Experimental Procedures
Venom Fractionation and Bioassays
Venom obtained by electrical stimulation of scorpions of the species Parabuthus granulatus was dissolved in twice-distilled water and centrifuged at 10,000 × g for 10 min, and the supernatant was freeze-dried and kept at −20 °C until use. The first step of separation consisted of gel filtration through a Sephadex G-50 (medium) column equilibrated and run in the presence of 20 mM ammonium acetate buffer, pH 4.7, under the conditions described in Ref. 27. This chromatographic step resulted in at least four independent subfractions of which only fraction II was toxic to mice. This subfraction was further separated by high performance liquid chromatography (HPLC) using a C18 reverse column (Vydac, Hesperia, CA) eluted with a linear gradient composed of solution A (0.12% trifluoroacetic acid in water) to solution B (0.1% trifluoroacetic acid in acetonitrile). The gradient was run up to 45% solution B during 60 min. At least 16 components were separated and assayed for micromortality and for specific effects in various electrophysiological systems (27). Subfraction 5 of the HPLC chromatogram was found to be a K+ channel blocker (see below) and was further characterized.

Primary Structure Determination
Three independent strategies/procedures were used to fully determine the amino acid sequence of HPLC component 5: (i) direct amino acid sequence analysis using a Beckman LF 3000 protein sequencer (Palo Alto, CA) of the native product and/or of the reduced and alkylated derivative, as described earlier (27), (ii) sequence analysis of the fragments obtained from enzymatic hydrolysis of the toxin in conjunction with amino acid composition analysis of the C-terminal region by means of an automatic amino acid analyzer (Beckman, model 6300E) following protocols also described earlier (28), and (iii) mass spectrometric analysis using a Finnigan LCQ™ ion-trap mass spectrometer (San Jose, CA).

The enzymatic hydrolysis was performed using 50 μg of component 5 supplemented with 4 μg of Staphylococcus aureus endopeptidase V8 (Roche Applied Science) in 100 mM ammonium bicarbonate buffer, pH 7.2, for 4 h at 37 °C. The product was separated by HPLC under the same conditions described in the previous section resulting in several subpeptides of which the one eluting at 23 min and 39 s corresponded to the C-terminal peptide.

Gene Cloning
Cloning of the PBTx1 gene—RNA was isolated from the venomous gland (telson) of one scorpion of the species P. granulatus according to the specifications of the Promega Total RNA isolation system. Total RNA was used for cDNA synthesis using a polyT22NN oligonucleotide, a 22-mer of Ts having two terminal degenerate nucleotides (23), essentially following the experimental procedure described previously by our group (29). To perform the polymerase chain reaction (PCR) at 25 °C, a sample of the first reaction (2 μl) was added to 1× Vent DNA polymerase buffer (composed of 10 mM KCl, 10 mM (NH4)2SO4, 20 mM Tris-HCl, pH 9.8, 2 mM MgSO4, and 0.1% Triton X-100), 200 μM dNTPs, a 25-mer forward degenerated primer (GAY GAR GAR CCN AAR GAR GAGTAC GATGACGGACGGCCCGCAGAAGTGCAAGTG-3, corresponding to the same positions in PBTx1 (codon 29-36)), and 1 μl of Vent DNA polymerase (New England Biolabs, Beverly, MA) in a final volume of 50 μl. The reaction was performed using a PerkinElmer 9600 thermocycler according to the protocol described earlier. Plasmid DNA was sequenced from both strands using fluorescent nucleotides in an automatic apparatus (Applied Biosystems) as recommended by the manufacturer.

Production of PBTx1 and PBTx1 T24F/V26K by Recombinant Methods—A PBTx1 gene was synthesized in a way similar to that described in Ref. 30 using two oligonucleotide duplexes ligated into pMAL-p2X (New England Biolabs) using XmnI (5′) and BamHI (3′) restriction sites. The sense strand reads as follows: 5′-GAGCGAGCGGCGCCAGGG-AGTCGTCGCTCGACGGGCGTGTGCGACGGCCCGCAGAAGTGCAAGTG-TCGGAGCTGAG-3′, and the reverse primer was 5′-CCGCGGACGATCGAAGGCGGAGAAGGCT-3′. For the production of the mutant PBTx1 T24F/V-26K, the template used for PCR was the gene of PBTx1 inserted into the pMAL-p2X vector. Oligonucleotide primers were synthesized on an Applied Biosystems device, purified by PAGE, and phosphorylated at the 5′ end. The sequence of the forward primer was 5′-AAGGGCGGAGGATCAC/A-CTCGGCGAGGTGGGCGCGGCGGCGGCGCCAGAACAGTGCGCGTCGAG-3′, corresponding to positions 18–29 of the PBTx1 amino acid sequence, and the reverse primer was 5′-TTGCAAAACCCGCGTCCGCA-3′, corresponding to the same positions in PBTx1 (codon and complementary codon of the mutation are in bold). PCR was performed using the guidelines of the manufacturer, and PCR products were visualized by agarose gel electrophoresis. PCR products were transformed into DH5α competent cells. DNA was purified using the Wizard MiniPrep System (Promega) and analyzed with restriction enzymes (BstRI and XmnI, New England Biolabs) using standard techniques. Transformants containing the correctly constructed gene fusions were grown at 37 °C in Terrific Broth medium containing 50 μg/ml ampicillin. For both wild-type (WT) and mutant PBTx1, protein expression was carried out in a DH5α strain of E. coli in the presence of 0.2 mM isopropyl-1-thio-β-D-galactopyranoside (Sigma) at 37 °C. Periplasmic protein was harvested by osmotic shock and purified as described previously (30).

Electrophysiological Characterization
Isolation and micro-injection of the oocytes were performed as described previously (30). Whole-cell measurements were carried out 1 or
**A.**

PBTx1  
---DEEPKESCS-DEMCVIYCKGEE---YSTMVCDGPQKCKCS---

PBTx2  
---DEEPKETCS-DEMCVIYCKGEE---YSTMVCDGPQKCKCS---

PBTx10  
---DEEPKETCS-DEMCVIYCKGEE---FSTMVCDGPQKCKCS---

**B.**

ChTx  \(\alpha\)-KTx1  
---ZFTNVSTTTSKRCWVQRLH---TSRGQGCMN-KKCCRCY---

NTx  \(\alpha\)-KTx2  
---TITIVKCTSPKPGCNPCKELTYGSSAGCMN-GKCCYNN---

KTx  \(\alpha\)-KTx3  
---GVCNQKCGPSQCPKCKDA-G-MRFPGCMN-RKCHTPK---

TsII-9  \(\alpha\)-KTx4  
---VFINAKCRGSPCPCPKCEAI-GKACGMN-GKCCYCP---

LeTx  \(\alpha\)-KTx5  
---IAFCN-LRNCQLSGRL-G-L-DGCTG-DKCCVKH---

Pi1  \(\alpha\)-KTx6  
---LVKCRGTSDLCPQCPQQTG-CPNPSGCMN-RMCKVCY---

Pi2  \(\alpha\)-KTx7  
---TISCNTNPKCYPHKETG-YPNACGMN-RKCKCFGR---

P01  \(\alpha\)-KTx8  
---VSRG-DCPEHCSTQK-AGACCDN-DKCCYPFP---

BmpO2  \(\alpha\)-KTx9  
---VGB---ECVMCHCKGN---AKPTCDD-GVCN---V---

CoTx1  \(\alpha\)-KTx10  
---AVCV-YRTCDKCKRR-G-YRSGGCIN-NACKCYPY---

PBTx1  \(\alpha\)-KTx11  
---DEEPKESCS-DEMCVIYCKGEE---YSTMVCDGPQKCKCS---

TsTxIV  \(\alpha\)-KTx12  
---WCSCLDLACGASRCYDCPCKAFC-RHGCGCMN-NKCMCRYN---

Tc1  \(\alpha\)-KTx13  
---AC---GSCRKKCK---GSSCCTN---GRCKC---

Bmkk1  \(\alpha\)-KTx14  
---TPFAIKCATDADSRKCPQ---PSRIN-GPACCT---

Aa1  \(\alpha\)-KTx15  
---ZNTELLKCGQ-GSCASVCRVIG-VAGCMIN-GRCVCP---

Tampolin  \(\alpha\)-KTx16  
---AFCN-LRNCQLSRSL-G-L-DGCTG-EKCKVPY---

Bmkk4  \(\alpha\)-KTx17  
---AQCDQRQSMDCQOYQCLTP---DCSY-GTCYKTTG---

TsPepl  \(\alpha\)-KTx18  
---KPCKGLCR-YRCCSGCSS---GCVN-GACCSS---

**Fig. 2. Amino acid sequence alignments.** Primary sequence comparison between the three acidic \(\alpha\)-KTx11 scorpion toxins (A) and the representative members of the \(\alpha\)-KTx peptides known thus far (B). The arrows above the sequences indicate approximate positions for the \(\beta\)-sheet structure, whereas the rectangle shows the segments of primary structure involved in the \(\alpha\)-helix formation of the three-dimensional structure of these toxins.

**Fig. 3. Gene cloning and vector design for recombinant biosynthesis.** A. Nucleotide sequence of the cDNA encoding PBTx10. The deduced amino acid sequence is given below the nucleotide sequence. The 24-mer degenerated oligonucleotide used for the PCR amplification is underlined (5'-GAY GAR GAR CCC AAR GAR ACC TOY-3'). The 3'-nontranslated region is in lowercase letters, and the polyadenylation signal is bold. B. Schematic diagram of the pMAL-p2X vector. This vector contains the synthetic gene for PBTx1. Two ligations were performed using a 6,717-bp BamHI/Xmal pMAL-p fragment and a 111-bp fragment encoding PBTx1 immediately downstream of the factor Xa cleavage site in the vector. **Amp**, ampicillin resistance gene; ori, origin.
FIG. 4. Current traces of Kv 1-type channels. A, Kv1.1 current records evoked by depolarizing the oocytes to 0 mV from a holding potential of −90 mV. Tail currents were measured at −50 mV. Application of 1 μM native PBTx1, PBTx2, and PBTx10 reduced the currents by about 50%. B, records under control conditions were obtained by application of 500-ms test pulses ranging from −70 mV to 20 mV from a V_{hold} of −90 mV with 5-s episode intervals. After the test pulses, tail currents were recorded at a potential of −50 mV during 500 ms. For the records evoked after application of 20 μM (on Kv1.1), 1 μM (on Kv1.2), and 800 nM (on Kv1.3) rPBTx1 and rPBTx1 T24F/V26K, the same voltage-step protocol was used.
Acidic Potassium Channel Toxins 2785

**RESULTS**

**Purification and Sequencing of Novel Toxins**

The purification of the α-KTx scorpion toxins is exemplified by one member of this family of acidic peptides from the venom of the South African scorpion *P. granulatus* following the same strategy as used earlier for two novel toxins targeted to voltage-dependent Ca\(^{2+}\) and Na\(^{+}\) channels (27).

By this procedure, the peptide indicated as number 5 in Fig. 1A was homogeneous based on the unique symmetry of the HPLC peak and on the mass spectrometric amino acid sequence analysis. This peptide was given the name PBTx10 from *Parabuthus* toxin 10.

The first 35 amino acids were all unequivocally identified by direct sequencing (labeled by \(-d\)- underneath the sequence) as shown in Fig. 1B. The last residue, serine at position 36, was obtained by two independent methods. The C-terminal peptide obtained from endopeptidase hydrolysis with *S. aureus* V8, as described under “Experimental Procedures,” had the same amino acid composition as follows: (expected:found) Asp (1:1), Thr (1:0.90), Ser (1:1.90), Glu (1:1.30), Pro (1:0.77), Gly (2:2.30), Phe (1:0.88), Ala (1:1.00), Lys (2:1.77), Cys (not determined).

Thus, by this method, one serine residue was missing compared with the sequence (labeled by \(\ldots\) V8 \(\ldots\) underneath it) in Fig. 1B. To verify the complete sequence, a mass spectrometric analysis of the native peptide was performed giving a value of 3,931.3 Da, identical to the expected theoretical value. Thus, we concluded that the peptide has 36 amino acid residues including six cysteines that form three disulfide bridges (confirmed by the molecular mass found). The full sequence was further confirmed by the nucleotide sequence of the gene coding for this peptide as will be discussed below. The peptide corresponds to 3.9% of the total protein of soluble venom. PBTx1 and PBTx2 were purified from venoms of the scorpions *Parabuthus transvaalicus* and *Parabuthus villosus*, respectively (30, 32).

**Cloning and Expression of Genes Encoding Acidic Peptides**

**Gene Cloning of PBTx10**—The PCR strategy used to obtain the nucleotide sequence amplified a 197-bp fragment from the cDNA using the degenerated 24-mer and the T22 oligonucleotides. This fragment was inserted in the EcoRV site of pBluescript KS\(^{+}\). Six clones were sequenced, and in all of them the PCR inserted included the N-terminal part of the mature PBTx10 toxin up to the poly(A) tail. In the 3'-untranslated region, a putative polyadenylation site, AATAAA, was identified by two independent methods. The C-terminal peptide was further confirmed by the nucleotide sequence of the gene coding for this peptide as will be discussed below. The peptide corresponds to 3.9% of the total protein of soluble venom. PBTx1 and PBTx2 were purified from venoms of the scorpions *Parabuthus transvaalicus* and *Parabuthus villosus*, respectively (30, 32).

**Fig. 5. Steady-state activation curves.** Membrane conductances \((G/G_{\text{max}})\) were calculated, and curves in control (○) and in the presence of 20 µM, 700 nM, and 300 nM rPBTx1 (●), respectively, on Kv1.1, Kv1.2, and Kv1.3 channels were obtained after fitting with a Boltzmann function. In all cases, \(V\) is not shifted by toxin application as illustrated by the dashed lines.
Gene Design, Site-directed Mutation, and Recombinant Expression of PBTx1

The genes encoding PBTx1 and PBTx1 T24F/V26K mutants have successfully been prepared following the molecular techniques described in Ref. 33 (Fig. 3B). The plasmids containing the fusion constructs were transformed in E. coli DH5α cells, and expression was successfully induced with isopropyl-1-thio-D-galactopyranoside for 3 h. Longer incubation periods did not increase the yield of fusion proteins. As a part of the control, cells only containing the pMAL-p2X vector were simultaneously induced. Fusion proteins were directed to the periplasmic space of the E. coli cells. The yields of affinity-purified fusion proteins were 40–60 mg/liter of culture estimated by the absorbance at 280 nm, which after cleavage with factor Xa resulted in the production of 2–4 mg of recombinant (mutant) toxin (rPBTx1 or rPBTx1 T24F/V26K) per liter of culture. The periplasmic extract of the control cells contained the expected maltose-binding product molecule as was verified by mass spectrometry. The recombinant synthesis resulted in the production of rPBTx1 and rPBTx1 T24F/V26K with the expected molecular masses of 4,090 and 4,166 Da, respectively, taking into account the three disulfide bridges present in the polypeptide chain.

Physiological Effects of the New Subfamily α-KTx11

Preliminary studies indicated that native PBTx1 blocks Shaker-type Kv1.1 channels expressed in Xenopus laevis oocytes with a Kd value of ~150 nM (34). A more in-depth analysis has revealed the presence of a more potent contaminating peptide in this sample, PBTx3, recently characterized by our group (30). The Kv1 channel blockage induced by rPBTx1 was largely reversible at concentrations of <1 μM; at higher concentrations, however, relatively longer wash-out periods up to several minutes were required, and full recovery was usually not obtained. The affinity toward the Kv1.1 channel was very low (Kd ~21.1 μM). Therefore, two other Kv1-type channels (Kv1.2 and Kv1.3) were tested. The addition of 10 μM of rPBTx1 produced an almost complete reduction of the current through both channels at all voltages. Using lower concentrations of rPBTx1 (1 μM and 800 nM for Kv1.2 and Kv1.3, respectively), Kv1.2 and Kv1.3 currents were reduced by about 50% (Fig. 4).

To explore the role of a functional dyad in the structure of the acidic PBTx1, we produced a mutant of rPBTx1 under similar experimental conditions as described for rPBTx1. In this mutant, the residues Thr24 and Val26 of PBTx1 were substituted by a phenylalanine and a lysine residue, respectively. In Fig.
4A, identical concentrations (1 μM) of native PBTx1, native PBTx2, and native PBTx10 were applied on oocytes expressing Kv1.1 channels. All currents were reduced by about 50%. Fig. 4B shows superimposed current responses to different 500-ms step depolarizations from a holding potential of −90 mV recorded from the same oocyte before and after addition of 20 nM (Kv1.1), 1 μM (Kv1.2), and 80 nM (Kv1.3) rPBTx1 and rPBTx1 mutant (T24F/V26K) to the bath. All peak amplitudes were roughly reduced by a factor of 2 under WT rPBTx1 conditions corresponding to the Kd value of rPBTx1 blockage for these channels, without evidence of voltage-dependent inhibition and without any change in the kinetics of the macroscopic currents. At the same concentration, all the channels were fully blocked by the rPBTx1 T24F/V26K mutant (Fig. 4).

Conductance (G) values were calculated as $G = IV_m$. Dividing G values by $G_{\text{max}}$ normalized each experiment, where $G_{\text{max}}$ was defined as the largest G value obtained in each experiment. Data are presented as means ± S.E. and represent a minimum of three experiments. $G/G_{\text{max}}$ voltage curves for control and toxin experiments were fitted with a Boltzmann relationship of the form $G/G_{\text{max}} = (1 + \exp[(V - V_{1/2})/k])^{-1}$, using the Origin software. There was no shift in the $V_{1/2}$ value when rPBTx1 (Fig. 5) or rPBTx1 T24F/V26K (data not shown) were present.

Fig. 6 shows that the concentration dependence of the block by rPBTx1 and rPBTx1 T24F/V26K follows a Michaelis-Menten saturation curve, meaning that each channel is blocked by a single peptide. With ND-96 in the external solution, the $K_d$ values of rPBTx1 are 21.1 μM (Kv1.1), 1.0 μM (Kv1.2), and 0.8 μM (Kv1.3), whereas for rPBTx1 T24F/V26K, the $K_d$ values are 1.1 μM (Kv1.1), 137 nM (Kv1.2), and 138 nM (Kv1.3). Comparing the $K_d$ values of WT rPBTx1 and rPBTx1 T24F/V26K with the three different Kv1 channels, the most pronounced increase in affinity with the PBTx1 mutant was observed for the Kv1.1 channels.

The wash-in and wash-out kinetics of WT and mutant rPBTx1 on the three channels were also investigated as described previously (30). For WT rPBTx1, the calculated $k_{\text{on}}$ values for Kv1.1 (20 μM rPBTx1), Kv1.2, and Kv1.3 (both 1 μM rPBTx1) channels were 5.4 .10³ M⁻¹ s⁻¹, 0.2 .10⁵ M⁻¹ s⁻¹, and 0.5 .10⁵ M⁻¹ s⁻¹, respectively. The $k_{\text{off}}$ values with WT rPBTx1 were 0.121 s⁻¹, 0.032 s⁻¹, and 0.041 s⁻¹, respectively. For the mutant rPBTx1, we found values for $k_{\text{on}}$ of 5.8 .10³ M⁻¹ s⁻¹, 7.9 .10⁵ M⁻¹ s⁻¹, and 3.5 .10⁵ M⁻¹ s⁻¹ and $k_{\text{off}}$ values of 0.92 s⁻¹, 0.172 s⁻¹, and 0.041 s⁻¹, respectively, for Kv1.1 (390 nM rPBTx1 T24F/V26K), Kv1.2, and Kv1.3 (both with 100 nM rPBTx1 T24F/V26K). Using the formula $K_d = k_{\text{on}}/k_{\text{off}}$, we see that the calculated values for the dissociation constants correspond well to the values deduced.
from the dose-response curves in all cases; for WT rPBTx1 they are 22.2 μM (Kv1.1), 1.6 μM (Kv1.2), and 820 nM (Kv1.3), respectively, and for rPBTx1 T24F/V26K the values are 1.5 μM (Kv1.1), 217 nM (Kv1.2), and 117 nM (Kv1.3). These results support the 1:1 stoichiometry of both the WT and mutant toxin binding to the respective Kv1 channels.

Recombinant WT PBTx1 and PBTx1 T24F/V26K have also been tested on some other \( \mathbf{K} \)-channels, like the hERG channel and the KvLQT1 (+\( \text{minK} \)) channel. However, we could not detect any effect.

**DISCUSSION**

Three homologous acidic peptides (PBTx1, PBTx2, and PBTx10) were isolated from the venom of three scorpion species (\( P. \) transvaalicus, \( P. \) villosus, and \( P. \) granulatus, respectively) by chromatographic procedures (see “Experimental Procedures”). To investigate the function of the acidic toxins described in this study, recombinant analogs and mutants were produced. PBTx1, PBTx2, and PBTx10 are naturally occurring single amino acid mutants of each other. Their three-dimensional structures are proposed by homology modeling using scyllatoxin as a template (Fig. 7A) and are assumed to be closely compacted by three disulfide bridges (six cysteines each, as demonstrated for other short chain toxins) (9).

Binding sites of Kv1-blocking toxins contain a functional core or dyad composed of a lysine and a hydrophobic residue as has been reported for Kv1-blocking peptides from several origins (scorpions, sea anemones, snakes, conus) (17). In contrast, PBTx1, PBTx2, and PBTx10 lack the dyad including the most crucial residue for Kv1-type blocking activity, the pore-plugging Lys27 (ChTx numbering). At the equivalent position, there is a Val (PBTx1, PBTx2) or an Ala (PBTx10) residue. There are some other \( \alpha-K^+ \) toxins lacking the crucial lysine, such as the \( \alpha-KTx \) toxins BmP02 and BmP03 (35) and Lp1 (36), but for these members no blocking activity toward Kv1-type channels has been documented. In contrast, recently Batista et al. (37) reported a new toxin, Tc32 from \( Tityus cambridgei \), without a functional dyad displaying a very high affinity toward Kv1.3 channels in lymphocytes.

Therefore, we were interested in the importance and the consequences of the introduction of a specific dyad as a whole in the acidic toxins. We substituted the Val27 for lysine and the Thr24 for phenylalanine in PBTx1 corresponding to the conserved amino acid residues of the strong \( K^+ \) channel blockers of subgroup 3 of the \( \alpha-KTx \) scorpion toxins, like agitoxin 2. The substitutions indeed affect the binding of the rPBTx1 to the three Kv1-type channels; PBTx1 T24F/V26K displays a similar and strong increase in the affinity (10-fold increase). However, we did not observe that high increase in affinity as could be expected from the dyad hypothesis.

The absence of the crucial dyad seems to be more important than the overall negative charge on the toxin. Goldstein et al. (11) documented this phenomenon showing that electrostatic potentials fall off quickly near the protein surface, resulting in smaller effects of the global charge compared with the effect of the close contact residues. We compared the relative impact of the acidic character and the lack of the dyad of rPBTx1 using a specific mutant of rPBTx3. Wild-type PBTx3 is a basic toxin isolated from the same \( Parabuthus \) species as PBTx1, and the PBTx3 mutant contains a similar dyad as the PBTx1 mutant, as recently described by Huys and Tytgat (33). Both toxins displayed almost similar affinities to the three investigated channels (Fig. 8A).

In contrast, Fig. 8B shows that the toxin with the dyad represents a 5–15-fold higher activity compared with the toxin without dyad. In Fig. 8C, both characteristics are compared.

Considering the low affinity of the native and recombinant PBTx1 and native PBTx2 (lacking the dyad) for the Kv1-type channels, there should be another structural determinant playing a role in channel recognition (such as hydrophobicity). This observation has also been reported by Batista et al. (37) supporting the idea that the interaction of short chain \( \alpha-KTx \) toxins with different types of \( K^+ \) channels (Kv or Slo) is not necessarily governed by the same mechanisms (dyad).

Five residues recognized as “critical residues” in Kv-blocking activity (11) in ChTx, Lys27, Met29, Asn30, Arg34, and Tyr36, are well conserved among different \( \alpha-KTx \) peptides. However, none of them is conserved in PBTx1. The observed weak blocking effect of PBTx1 toward Kv channels can therefore be explained. In PBTx1, the negative charges are separated all over the toxin molecule (Fig. 8B), which could be interpreted to mean that not only one specific region with localized negative amino acid

**Fig. 8.** Comparison of the relative impact of the acidic character and the lack of the dyad. A, the affinities of two toxins with similar dyad, PBTx3 mutant (basic) and PBTx1 mutant (acidic), are almost similar. B, the affinity of the acidic toxin with a dyad is 5–15 \( \times \) higher compared with a similar acidic toxin without the dyad. C, comparison of both characteristics.
residues is responsible for the decreased bioactivity. Some very conserved residues throughout the other subfamilies (Gly for α-KTx2–7; Met for α-KTx1–3, α-KTx7, and α-KTx12; or Ile for α-KTx5–6, α-KTx10, α-KTx13, and α-KTx15–16, respectively) are mutated to some negative residues in PBTx1 (Glu⁴, Asp⁸) as can be seen from sequence alignments. Recently, Gly⁴⁰ in iberotoxin (IbTx, α-KTx1.3) has been shown to be unique among the α-KTx peptides in that all Kv-blocking peptides have an asparagine at this position. This Gly⁴⁰ (IbTx numbering) has been found to be a major determinant of the specificity of IbTx for maxi-K channels versus Kv channels (37) and is also present in the acidic Parabuthus toxins. However, further studies will be needed to rule out the functional activity of these toxins toward the maxi-K channels.

Studies of the effect of rPBTx1 on different K⁺ channels have shown that this peptide is a pore blocker not affecting the gating characteristics of the channels. The active surface of most Kv pore-blocking scorpion toxins is located in the β-sheet (like the dyad itself) in Fig. 7B, Face A, whereas the opposite α-helix (Fig. 7B, Face B) lies on the back site of the toxin molecule. We can clearly see that, compared with the prototype pore-blocker CbTx, PBTx1 displays prominently more acidic residues on its β-sheet. Because PBTx1 is a pore blocker, the interaction paratope is most probably the P region located between the transmembrane segments S5 and S6. This region is highly similar for the three Kv channels. We can also suppose that positively charged residues in the pore region are involved in the interaction of the negatively charged toxin with the channel (39, 40). Only Kv1.2 has a positively charged Arg residue (Arg¹⁷⁷) near the external mouth of the channel. Although this residue can make a direct intimate contact with the toxin, long-range electrostatic influences are known to play a substantial role in the toxin-channel binding as expressed in altered association rates (11, 41–44).

Electrostatic repulsion between negative residues on the channel surface and the negatively charged toxin also plays a role. Kv1.1 and Kv1.2 channels contain five negatively charged residues in their P region, whereas the Kv1.3 channel has only four such residues. PBTx1 shows the highest affinity toward this latter channel, whereas the PBTx1 mutant displays a small increase in activity. All the results suggest that the low activity of PBTx1 may be because of important differences in its electrostatic mapping compared with CbTx combined with the significant residue change Lys²⁰⁵–Val²⁰⁶ as shown for α-neurotoxins, the dramatic change in electrostatic property of the toxin can be produced by natural mutagenesis or gene divergence. From an evolutionary point of view, there is an important difference between the evolution of protein sequences of scorpion toxins and the morphological evolution of the scorpions themselves (45). Since their appearance on earth (400 million years ago), scorpions have experienced only a few changes. However, the toxins were subjected to several phases of evolutionary pressure to enhance the toxicity of the scorpion. Such an increase could be associated with an increase of the overall positive charge on the toxin surface as has been shown for other peptides where the in vivo toxicity changes are inversely proportional to the pI values (21). Therefore, the existence of acidic toxins in the venom scorpion venom could be considered an evolutionary process resulting in remnant peptides, although we still do not know exactly for which target molecules they have evolved.

Acknowledgments—We thank Olaf Pongs for providing the cDNA for the Kv1.2 channel and Fernando Z. Zamudio for technical assistance. The Kv1.3 clone was kindly provided by Maria L. Garcia.

REFERENCES

1. Tytgat, J., Chandy, K. G., Garcia, M. L., Gutman, G. A., Martin-Eauclaire, M. F., van der Walt, J. J., and Possani, L. D. (1999) Trends Pharmacol. Sci. 20, 444–447
2. Gimenez-Gallego, G., Navia, M. A., Reuben, J. F., Katz, G. M., Kaczorowski, G. J., and Garcia, M. L. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 3329–3333
3. Garcia, M. L., Garcia-Calvo, M., Hidalgo, P., Lee, A., and MacKinnon, R. (1994) Biochemistry 33, 6834–6839
4. Wei, A., Jegla, T., and Salkoff, L. (1996) Neuropharmacology 35, 805–829
5. Tsetlin, V. (1999) Eur. J. Biochem. 264, 811–816
6. Grishin, E. (1999) Eur. J. Biochem. 264, 276–280
7. Possani, L. D., Beereeir, B., Delepiere, M., and Tytgat, J. (1999) Eur. J. Biochem. 264, 287–300
8. Garcia, M. L., Hanner, M., Knaus, H. G., Koch, R., Schmalhofer, W., Slaughter, R. S., and Kaczorowski, G. J. (1997) Adv. Pharmacol. 39, 425–471
9. Boussema, F., Roumemont, C., Gilquin, B., Menez, A., and Toma, F. (1991) Science 254, 1521–1523
10. Park, C. S., and Miller, C. (1992) Nature 357, 307–313
11. Goldstein, S. A., Pheasant, D. J., and Miller, C. (1994) Nature 367, 1377–1388
12. Rangel-Penagos, R., Lewis, J. H., and MacKinnon, R. (1996) Nature 383, 151–158
13. Bednarek, M. A., Bugianski, R. M., Leonard, R. J., and Felix, J. P. (1994) Biochim. Biophys. Acta 1212, 61–1181
14. Buisine, E., Wieruszeski, J. M., Singleton, D. H., Andrews, G. C., Lin, W., Boyd, J., Hanson, D. C., Simon, M., and Dethlefs, B. (1995) Nature 375, 623–631
15. Luo, M. J., Xiong, Y. M., Wang, M., Wang, D. C., and Chi, C. W. (1997) Toxicon 35, 733–738
16. Guo, C., Chi, C. W., and Guo, J. (2002) Toxicon 40, 1229–1258
17. Pimenta, A. M., Legros, C. Marco Almeida, F., Mansuelle, P., De Lima, M. E., Bougis, P. E., and Martin-Eauclaire, M. F. (2003) Biochim. Biophys. Acta 1601, 69–760
18. Li, H. M., Wang, D. C. (1999) J. Gen. Physiol. 114, 188–198
19. Wang, D. C. (1999) Biochem. Biophys. Acta 141–144
20. Luo, M. J., Xiong, Y. M., Wang, M., Wang, D. C., and Chi, C. W. (1997) Toxicon 35, 723–731
21. Li, H., Jin, L., Zeng, Z., Wang, M., Zhang, Y., and Wang, D. (1996) Sci. China Ser. C Life Sci. 39, 373–384
22. Geuert, C., Chi, C. W., and Guo, J. (2002) Toxicon 40, 1229–1258
23. Armstrong, C. M., and Bezanilla, F. (1974) Science 183, 1377–1388
24. Schroeder, N., Mullmann, J. T., Schmalhofer, W. A., Gau, Y. D., Garcia, M. L., and Giangiacomo, K. M. (2002) FEBS Lett. 527, 298–302
25. Peter, M., Jr., Varga, Z., Hapdir, P., Gaspar, B., Jr., Damjanovitch, S., Horjales, E., Possani, L. D., and Panzy, G. (2001) J. Membr. Biol. 179, 13–25
26. Fu, W., Cui, M., Briggs, J. M., Huang, X., Xiong, B., Zhang, Y., Luo, X., Shen, X., J., J., Ji, R., Jiang, and Chen, K. (2002) Biochim. Biophys. Acta 1527, 2370–2385
27. MacKinnon, R., and Miller, C. (1989) Science 245, 655–655
28. Batista, C. V., Gomez-Lagunas, F., Rodriguez de la Vega, R. C., Hajdu, P., Porras, G., Gaspar, R., and Possani, L. D. (2002) Biochim. Biophys. Acta 1549, 125–131
29. Schroeder, N., Mullmann, J. T., Schmalhofer, W. A., Gau, Y. D., Garcia, M. L., and Giangiacomo, K. M. (2002) FEBS Lett. 527, 298–302
30. Peter, M., Jr., Varga, Z., Hapdir, P., Gaspar, B., Jr., Damjanovitch, S., Horjales, E., Possani, L. D., and Panzy, G. (2001) J. Membr. Biol. 179, 13–25
31. Fu, W., Cui, M., Briggs, J. M., Huang, X., Xiong, B., Zhang, Y., Luo, X., Shen, X., J., J., Ji, R., Jiang, and Chen, K. (2002) Biochim. Biophys. Acta 1527, 2370–2385
32. MacKinnon, R., and Miller, C. (1989) Science 245, 655–655
33. Escobar, L., Root, M. J., and MacKinnon, R. (1993) Biochemistry 32, 6982–6987
34. Wilson, A. C., Carlson, S. S., and White, T. J. (1977) Annu. Rev. Biochem. 46, 573–639

Acidic Potassium Channel Toxins 2789