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Purification of two depressant insect neurotoxins and their gene cloning from the scorpion *Buthus martensi* Karsch

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Abstract: Insect-specific neurotoxins are important components of scorpion venoms. In this study, two toxins from the scorpion *Buthus martensi* Karsch (BmK) were purified. They shared high sequence homology with other depressant insect toxins and were designated BmK ITa and BmK ITb, respectively. They were able to suppress the action potential of cockroach isolated axon, which is due to a decrease in the peak sodium current. Furthermore, the effect of BmK ITb was lower than that of BmK ITa, and some of the electrophysiological characteristics of BmK ITb even resemble that of excitatory insect toxins. Their primary structures were determined by N-terminal partial sequence determination and cDNA cloning. The differences in their structures, especially the 31st residues, may result in the unique activity of BmK ITb.

There is a diversity of neurotoxins in scorpion venom. These neurotoxins act specifically on different kinds of ion channels of nerve and muscle systems. According to their molecular size, these neurotoxins can be classified into two groups. The long-chain neurotoxins composed of 60–76 amino acids with 4 disulfide bonds affect mainly Na^+ channels (1–4), whereas the short-chain neurotoxins composed of only 28–41 amino acids with 3–4 disulfide bonds affect mainly K^+ or Cl^- channels (5–8). The long-chain neurotoxins have been more widely and intensively studied. According to their specificity, they can be further divided into two main subgroups: mammalian- and insect-specific neurotoxins.

Based on their modes of action, insect-specific neurotoxins are further divided into excitatory and depressant insect toxins (9,10). Excitatory insect toxins composed of

≈70 amino acids cause an immediate contraction paralysis in insect larvae (11–14), whereas depressant insect toxins induce a transient contraction paralysis followed by a progressive flaccid paralysis (9,15). Depressant insect toxins are composed of 61 amino acid residues and share great homology with each other (15,16). Because of their strict selectivity, insect-specific neurotoxins are of great potential value as candidates for biological pesticides and possibly for use in transgenic plants. It has been reported that the insecticidal properties of *Autographa california* nuclear polyhedrosis virus (AcNPV) were enhanced by introducing a gene encoding AaH IT1, an excitatory insect toxin (17,18), and transgenic tobacco introduced with either AaH IT or together with *Bacillus thuringiensis* toxin becomes more resistant to insects (19). Thus, it is important to first clone these insect toxin genes, and then try to make them useful as bioinsecticides by a genetic approach. However, only a few gene structures of insect neurotoxins, especially depressant insect toxins, have been reported (16,20–22).

An excitatory insect toxin, BmK IT, was first found (13) from the scorpion *Buthus martensi* Karsch (BmK), a species distributed widely in the east of China. Its cDNA has been cloned and the amino acid sequence determined (23). Meanwhile, another excitatory insect toxin with an analgesic effect on mice, designated BmK IT-AP, has also been purified, and its protein, cDNA and genomic DNA sequences elucidated (14). However, no gene structure for any depressant insect toxin from this scorpion has been reported to date.

In this study, two depressant insect toxins were purified, their electrophysiological effects studied and their N-terminal partial sequences determined. On the basis of their conserved sequences, their cDNAs were cloned successfully from the scorpion BmK using the rapid amplification of cDNA ends (RACE) method.

Experimental Procedures

Materials

Crude venom from BmK scorpion was obtained from a scorpion farm (Xichuang, Henan province, China). Sephadex G-50 and CM C-32 cellulose were purchased from Pharmacia (Uppsala, Sweden). Trifluoroacetic acid and acetonitrile for HPLC were from Merck (Darmstadt, Germany). The 3′- and 5′-RACE kits, TRIzol reagent and T4 DNA ligase were from Life Technology (Gaithersburg, MD, USA). Restriction endonucleases and *Taq* DNA polymerase were from MBI (Graiciuno8, Vilnius, Lithuania) and Sangon (Shanghai, China), respectively. Polymerase chain reaction (PCR) preps DNA purification system, Minipreps DNA purification system, DNA sequencing kit and pGEM-T vector were from Promega (Madison, WI, USA). [α-^{32}P]dATP was from Amersham (Cleveland, OH, USA) and 3,4-diaminopyridine was from Sigma (St. Louis, MO, USA). Acrylamide, bisacrylamide, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), isopropyl-thio-β-D-galactoside (IPTG) and other reagents were of analytical grade. *Escherichia coli* strain TG1 was used for transformation of phage M13mp18 and mp19, and *E. coli* strain DH5α for transformation of pGEM-T vector.

Purification of two BmK depressant insect toxins

The crude venom was dissolved in 0.05 N NH_4HCO_3 pH 8.0 buffer and then subjected to a column of Sephadex G-50 fine (1.2 × 155 cm) previously equilibrated with the same buffer. The flow rate was 0.4 mL/min. Two main peaks were eluted, of which the second peak was pooled and subjected to a CM-cellulose C32 column (2 × 11 cm). The buffer used for this column was 0.02 N Na_2HPO_4/NaH_2PO_4, pH 6.4; the flow rate was 0.8 mL/min. The effluent during sample loading was pooled and separated further using a C18 RP-HPLC column (0.46 × 25 cm, 5 μm particle size, Beckman) equilibrated with buffer A, 0.1% TFA in water. Elution was carried out with a linear gradient of 0–50% buffer B in 50 min and a flow rate of 1 mL/min. Buffer B was 70% acetonitrile in buffer A. The effluent was monitored by measuring the absorbance at 220 nm. Every peak obtained was then collected and rechromatographed on the RP–HPLC column to homogeneity.

Electrophysiological study

The experiments were carried out on isolated cockroach giant axons dissected from abdominal nerve cords (24). Electrophysiological recordings under current-clamp and voltage-clamp conditions were carried out using the double oil-gap-single-fiber technique (25). The methods of axon isolation and the recording techniques have been described in detail previously (24). Isolated axons were superfused with a physiological saline solution containing (in mM): 210 NaCl, 3.1 KCl, 5.4 $CaCl_2$, 5.2 $MgCl_2$. The saline was buffered with 1 mM HEPES, pH 7.2. When necessary, potassium currents were blocked using 3,4-diaminopyridine

(Sigma Chemical, France). The current and voltage traces were visualized on a digital oscilloscope (Tektronix) and digitized on 10 bits. The digitized traces were subsequently transferred to a desktop computer (Hewlett-Packard) and stored on floppy disks for further analysis. The current traces were usually corrected for nonspecific capacitive and leakage currents using an analog compensation circuit as described (26). Toxins were dissolved in the saline superfusing the axon.

Mass spectrometry

The mass spectra of toxins were obtained on a Finnigan LCQ ion trap mass spectrometer (ThermoQuest, San Jose, CA, USA) equipped with an electrospray ionization source with a spray voltage of 4.50 kV. The heated capillary was maintained at 200°C at a voltage of 30 V. The calculation was carried out using the program provided by the manufacturer.

Sequence determination

The N-terminal sequences of the depressant insect toxins were directly determined on a gas-phase sequencer PPSQ-10 (Shimadzu, Kyoto, Japan) based on automated Edman degradation.

3′-Race

Total RNA was extracted from 50 mg of scorpion venomous glands using TRIzol reagent kit. Then, 5 μg of total RNAs were taken to convert mRNA into cDNA using a 3′-RACE kit provided with Superscript II reverse transcriptase and a universal oligo(dT)-containing adapter primer with a *Sal*I restriction site (5′-GGCCACGCGTCGACTAGTACT$_{17}$-3′). The resulting first-strand cDNA was then used as the template for amplification. According to the conserved N-terminal sequence determined from the first residue (Asp-Gly-Tyr-Ile-Arg-Gly-Ser), a gene-specific primer GSP1 was designed (5′-CGGAATTCGATGGATATATTCGGGGCAGT-3′) and paired with an abridged universal amplification primer containing an additional *Eco*RI restriction site but devoid of the poly(dT) tail (5′-CGGAATTCGGCCACGCGTCGACTAGTAC-3′). The restriction sites in the primers are underlined. PCR was carried out through 30 thermal cycles of 94°C, 1 min; 45°C, 1 min; 72°C, 1 min. The PCR amplified product was then digested with *Eco*RI and cloned into M13mp19 for sequencing.

5′-Race

Based on the partial cDNA sequence determined using 3′-RACE, an antisense gene-specific primer GSP2 with a *Hin*dIII restriction site was designed for 5′-RACE. The GSP2 primer (5′-CGAAGCTTTTAACCGCATGTATTACTTTC-3′) corresponds to the conserved residues 61–56 of depressant insect toxins (Gly-Cys-Thr-Asn-Ser-Glu). Using 1 μg total RNAs as the template, the first-strand cDNA was then transcribed. Following purification of the cDNA on a Glassmax column, a homopolymeric (dC) tail was then added to its 3′-end using terminal deoxynucleotidyl transferase. The dC-tailed partial cDNAs of depressant insect toxins were first amplified by PCR with GSP2 and the anchor primer (5′-GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG-3′) complementary to the dC tails. The annealing temperature was 53°C. In order to obtain a higher yield of the specific cDNA of depressant insect toxins, the obtained PCR products were further amplified by PCR with GSP2 and the 5′-abridged universal amplification primer. The final PCR product was then digested with *Eco*RI and *Hin*dIII and cloned into M13mp18 for sequencing.

Results and Discussion

Purification and N-terminal sequencing of two BmK-depressant insect toxins

Depressant insect toxins, capable of inducing flaccid contraction on insects, are important components of scorpion venoms. In this study, two depressant insect toxins from scorpion BmK were purified and studied. The crude venom of scorpion BmK was first separated into two main peaks on the column of Sephadex G-50 fine (Fig. 1A). The first peak contained many components of molecular mass > 10 kDa, and the second comprised mainly the neurotoxins of molecular mass < 10 kDa. The neurotoxins were further fractionated according to their isoelectric points through a CM cellulose C32 column in 0.02 N phosphate buffer, pH 6.4. Only those acid toxins with low pI, ≈40% of the neurotoxin fractions, flowed through the CM column. The effluent was pooled and separated into > 10 peaks on the C18 RP–HPLC column (Fig. 1B). After their molecular

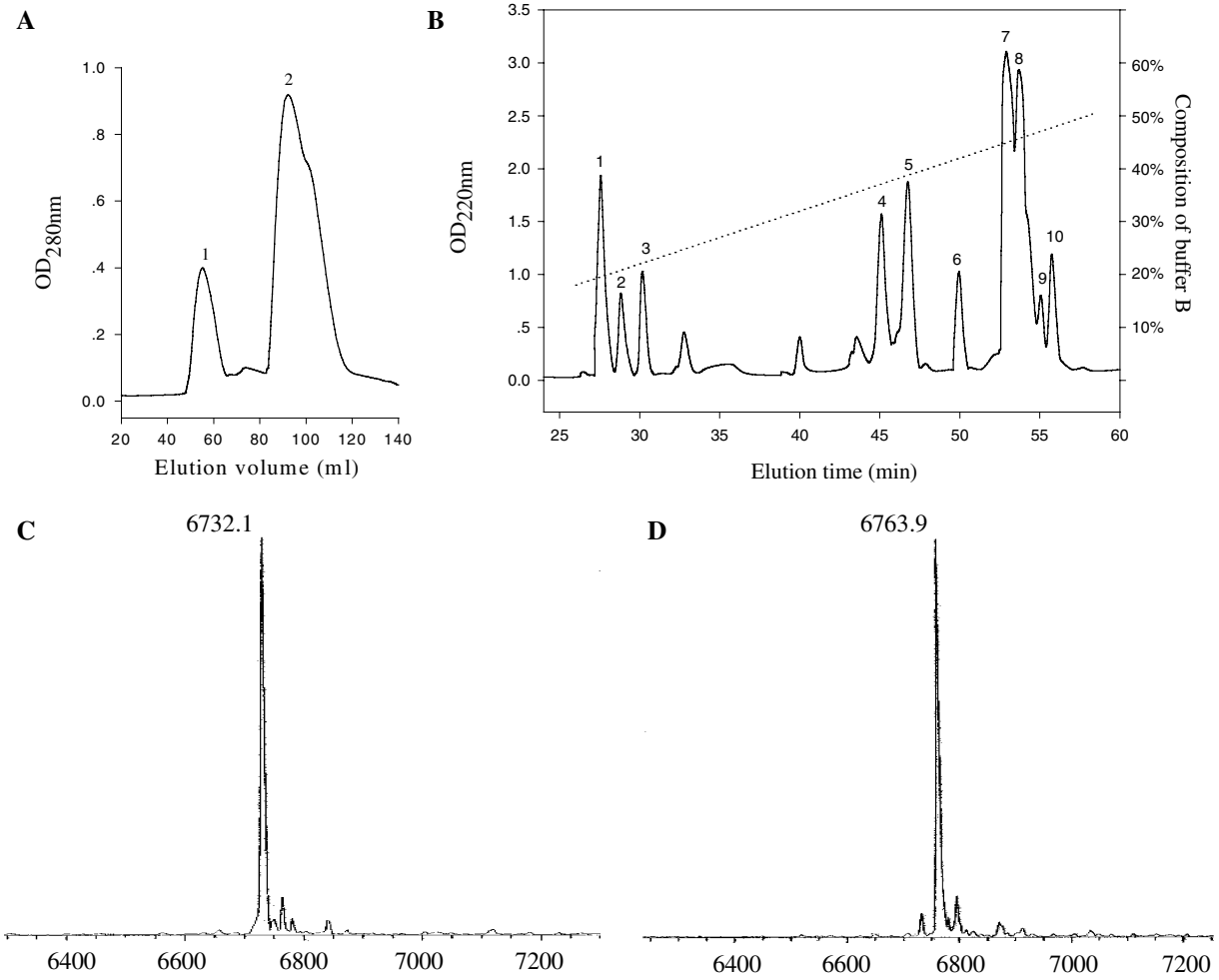

Figure 1. Purification of BmK ITa and BmK ITb. (A) Gel filtration of crude venom of the scorpion BmK on Sephadex G-50 fine (1.2 × 155 cm) equilibrated with 0.05 N NH_4HCO_3 (pH 8.0). Two main peaks were eluted. Neurotoxins in the second peak were pooled for further purification. (B) Neurotoxins in the effluent of the CM-cellulose column were separated on the HPLC C18 column (4.6 × 250 mm). Bufer A for HPLC is 0.1% trifluoroacetic acid in water; buffer B is 70% acetonitrile and 0.1% trifluoroacetic acid in water. The column was eluted with a linear gradient of 0–50% buffer B in 50 min. Peaks 7 and 8 are BmK ITa and BmK ITb. The molecular masses of BmK ITa (C) and BmK ITb (D) were determined on a Finnigan LCQ ion trap mass spectrometer. They coincided well with the calculated values of 6732.4 and 6764.5 Da for BmK ITa and ITb, respectively.

masses had been determined using mass spectroscopy (Fig. 1C, D), peaks 7 and 8 were directly subjected to sequencing. Their N-terminal partial sequences, differed in only five residues (I12V, RGY26–28KAF and S31Y), were highly homologous with other depressant insect toxins (Fig. 5). They were therefore named BmK ITa and BmK ITb, respectively.

Electrophysiological study

BmK ITa and BmK ITb toxins have been applied in 1 and 5 μM concentrations. BmK ITa was efficient at 1 μM and BmK ITb at 5 μM, so to compare the modifications of axonal bioelectrical activity induced by tested toxins, the effects of the 5 μM concentration are described.

Approximately 5 min after application of BmK ITa (5 μM), a progressive decrease in action potential amplitude was observed, together with the development of a resting depolarization (Fig. 2A). Within 7–10 min, action potentials were blocked and the axon was depolarized by ≈5–8 mV. Larger depolarizations were never observed. Artificial repolarization to normal resting potential level (– 60 mV) partially restored action potential amplitudes which did not exceed 70 mV (compared with 100 mV in control). Further artificial hyperpolarization to −70 mV permitted the regeneration of almost normal action potentials. However, in time, axons that had been repeatedly depolarized showed a marked decrease in action potential amplitude. These effects are typical of a flaccid toxin action. However, when the axon was hyperpolarized to −70 or −80 mV for 2–3 min, then suddenly depolarized to

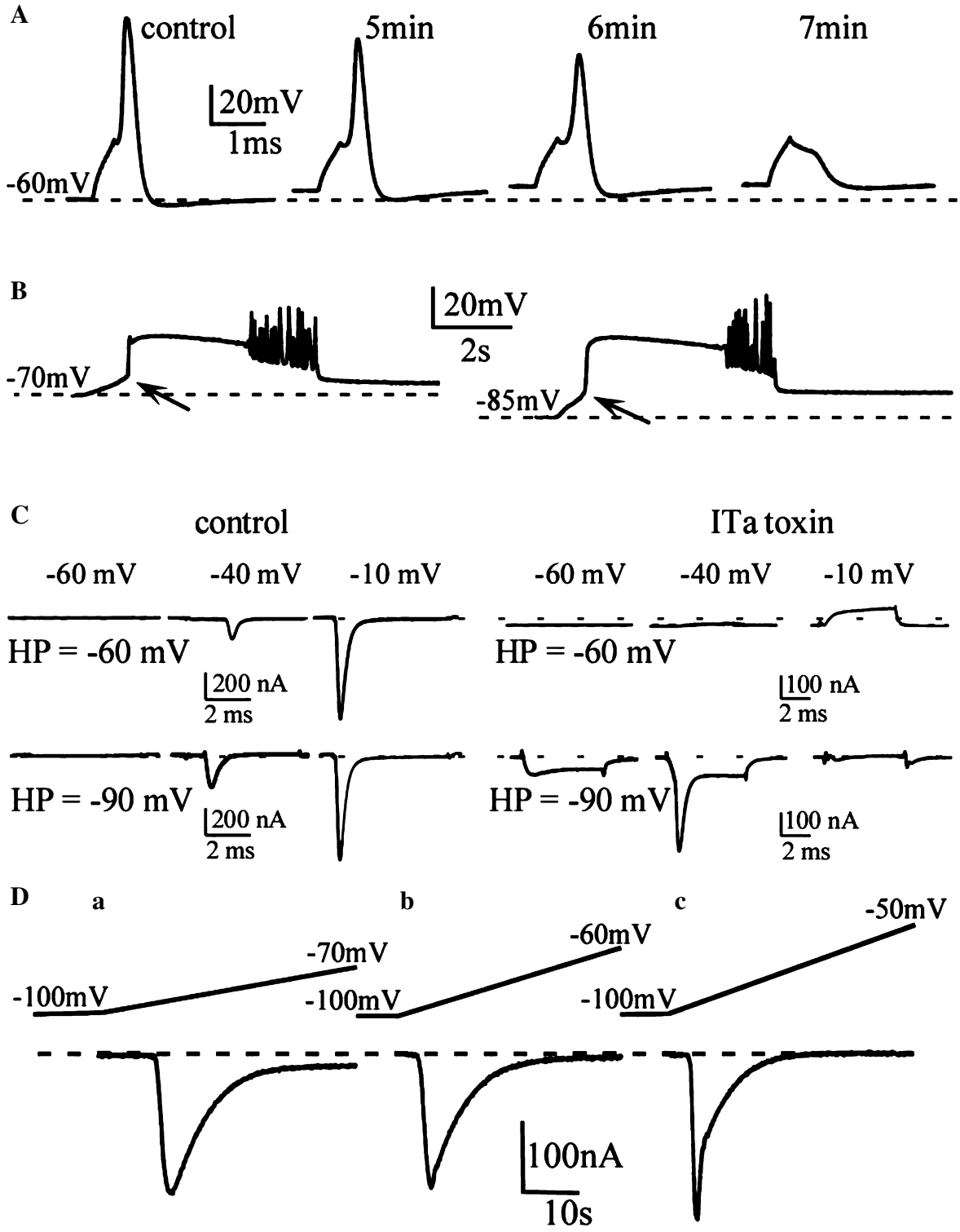

Figure 2. Action of BmK ITa on membrane potentials and sodium currents of the cockroach giant isolated axon. (A) Progressive decrease in amplitude of evoked action potential and development of resting depolarization observed after 5 μM BmK ITa application. (B) Plateau potentials with repetitive activity recorded when the axon was artificially hyperpolarized for some seconds to −70 or to −85 mV and then depolarized. Arrows indicate the action potential threshold corresponding to −63 and −70 mV, respectively. (C) Sodium currents at holding potentials (HP) of −60 and −90 mV for voltage pulses of −60, −40 and −10 mV under control conditions and in the presence of BmK ITa (5 μM). Dashed lines indicate the zero current level. Note the complete suppression of Na^+ current with toxin and the existence of a permanent inward holding current for HP = −60 mV. (D) Slow kinetic inward sodium current induced by BmK ITa (5 μM). Sodium current traces presented correspond to 50 s ramp pulses from HP = −100 mV to −70, −60 and −50 mV in a, b, c, respectively. Note the very slow time scale.

– 60 mV, plateau potentials (of only 40–50 mV in amplitude) with repetitive activity at the end were evoked (Fig. 2B). Under such conditions, the axon was able to generate plateau potentials with repetitive activity for at least 10–15 min. The threshold for plateau potential depended on the preceding artificial hyperpolarization. For example, in axon hyperpolarized to −70 mV, the fast depolarizing phase started at −63 mV and in axon hyperpolarized to −85 mV, it started at – 70 mV (Fig. 2B).

Under voltage-clamp conditions, BmK ITa modified sodium current, as a preliminary, potassium current was blocked by 3,4-diaminopiridine. The progressive effect of toxin was first observed on the sodium current induced from holding potential (HP) of −60 mV by depolarizing pulses to −10 mV. Within 5 min of toxin application, a marked decrease in current amplitude was seen and within about 10 min, the sodium current was suppressed completely (Fig. 2C). Steadiness of the leak current indicated that BmK ITa did not induce any other effect on axonal membrane (for instance the formation of ionophores). More than 10 min of the presence of the toxin eliminated almost completely the sodium current in each potential voltage pulse applied from HP −60 mV (Fig. 2C). However, when the HP was lowered to −70 mV or more negative values, the current was restored (Fig. 2C, HP = −90 mV) in a percentage depending on this HP value; for example the current developed by pulse to −27 mV from HP = – 80 mV measured – 380 nA and from HP = – 90 mV was – 460 nA. Under control conditions, changes of HP in the range of −60 to −90 mV did not alter the magnitude of current or its voltage dependence in a significant manner (Fig. 2C). Application of different depolarizing pulses from HP = – 90 mV showed an important shift in current–voltage dependence to more negative potentials. A slow change in holding potential from very negative to less negative values or application of ramp depolarizing pulses from HP = −100 mV revealed the development of a slow current having time constant of activation and inactivation in the range of several hundreds of milliseconds. This current did not inactivate completely at −70 mV or more negative potentials (Fig. 2D). Kinetics of the slow current activation and inactivation increased with the rate of potential changes during ramp pulses (compare Fig. 2D; a,b,c). Such slow currents were not observed under control conditions and were suppressed by TTX.

Similarly BmK ITa, toxin BmK ITb decreased the action potential amplitude, however, it was much less efficient in this range. The action potential amplitude was higher than 70% of control value (Fig. 3A) after 20 min of 5 μM BmK ITb action. During this time, resting depolarization did not exceed 10 mV. Artificial repolarization to – 60 mV restored almost normal action potential amplitudes, however, the last phase of repolarization was prolonged (not shown). More negative artificial hyperpolarization, for example to – 80, – 90 mV, followed by artificial return to – 60 mV induced bursts of repetitive activity generated at – 60 mV (Fig. 3B). Action potentials observed during bursts had amplitudes of only 60–80 mV and prolonged the last phase of repolarization (Fig. 3C). Plateau potentials in the presence of BmK ITb were never observed. In the voltage-clamp configuration, toxin BmK ITb decreased the peak sodium current amplitude, much more slowly than did BmK ITa, however. With 10 min of toxin action the sodium current was ≈ 60–70% of the control value (Fig. 3D) and last phase of inactivation was prolonged, giving a late, maintained current; such maintained inward sodium current was maximal for – 50 < Em < – 35 mV. BmK ITb, as opposed to BmK ITa, did not suppress the sodium current at HP −60 mV and a peak current could be recorded at each membrane potential above −55 mV. The voltage-dependence of BmK ITb-modified current was shifted to a more negative potential by ≈ 20 mV. Using a HP value more negative than 60 mV showed that only very rarely was the peak sodium current observed with a pulse to −60 mV. Experiments using ramp pulses revealed only a small, slow, inward sodium current, much smaller than that in the case of BmK ITa and with much faster kinetics of activation and inactivation (not shown).

Clearly, BmK ITa is a typical depressant insect toxin. Its potency is similar to that of other toxins studied previously on this axonal preparation (27–30). The suppression of action potentials is due to the decrease in the voltage-dependent peak sodium current. Further electrophysiological studies will be necessary to characterize this very slow toxin-induced conductance which probably accounts for insect flaccidity. BmK ITb is less potent than BmK ITa at suppressing axonal electrical activity. Some characteristics of toxin BmK ITb even resemble those of excitatory insect toxins (bursts of activity after the membrane has been hyperpolarized), however, its efficiency is several times lower than that of AaHIT from *Androctonus australis* (27; Pelhate & Zlotkin, personal observations), of LqqIT1 from *Leiurus quinquestriatus* (29), and of BjxtrIT from *Buthotus judaicus* (31). Moreover, the peak sodium current is decreased by BmK ITb when compared with the control, whereas it is increased with the contractive toxins. So BmK ITb could be considered, from the point of view of electrophysiology, as an intermediate between depressant

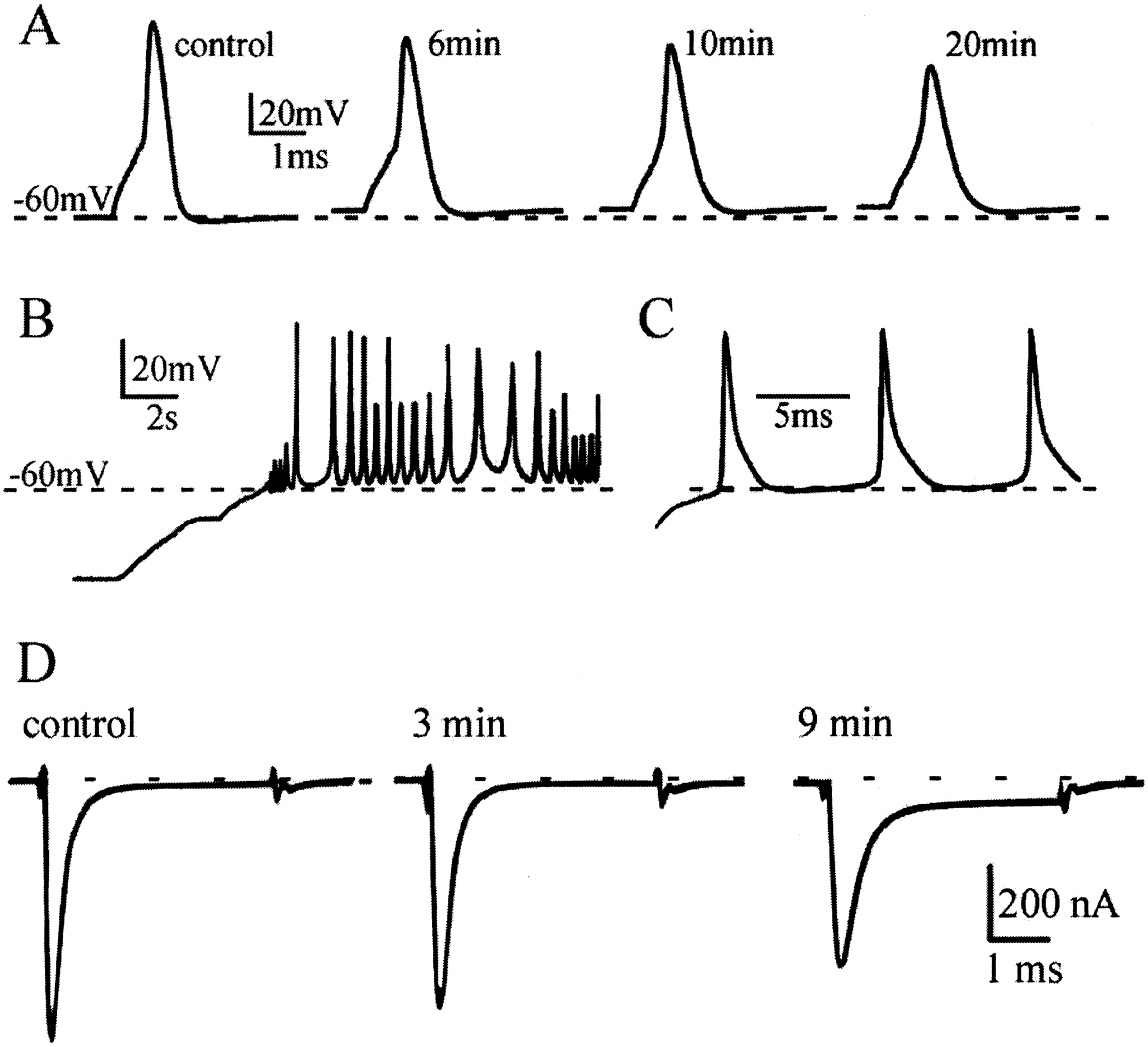

Figure 3. Actions of BmK ITb on membrane potentials and sodium currents of the cockroach giant isolated axon. (A) Slow decrease in action potential amplitude with minor resting depolarization in time (min) after toxin application. (B) Repetitive activity recorded within ≈ 10 min in the presence of BmK ITb when the membrane potential was artificially hyperpolarized to −90 mV for several seconds and then returned to −60 mV. (C) Shape of action potential during repetitive activity (as in panel B) showing prolonged last phase of action potential repolarization. (D) Changes in sodium current characteristics observed after 5 μM BmK ITb application: decrease in peak current amplitude together with a moderate slowing of the inactivation phase. The current was evoked by voltage pulses to −20 mV from a HP = − 60 mV.

and excitatory toxins and thus, details of its structure may therefore be informative.

Amplification of cDNAs of the depressant insect toxins

According to the N-terminal sequences of BmK ITa and ITb, using the 3′-RACE method, a DNA fragment of ≈ 300 bp was amplified from total RNAs of scorpion venomous glands. Their sequences contained an encoding region of 64 residues and the 3′-untranslated region down to the poly(A) site. According to the 3′-RACE sequences determined, an antisense primer corresponding to their conserved 56–61 residues was designed and used in 5′-RACE. The PCR products were also of ≈ 300 bp, the sequences included a 5′-untranslated region, a signal peptide of 21 residues and a mature peptide of 61 residues. Thus, the cDNAs of BmK ITa and -ITb were completed by overlapping sequences of the 3′ and 5′ products (Fig. 4). Each contained a 5′-untranslated region, a signal peptide of 21 residues, a mature peptide of 61 residues, three additional residues GKK at the C-terminus and a 3′-untranslated region down to the poly(A) site.

It is because of the high homology with other known depressant insect toxins and the determined molecular masses of BmK ITa and -ITb that their mature peptide was believed to be of 61 residues in size. Meanwhile, the three additional residues GKK meant amidation of the C-termini (32–34). This phenomenon is always found in the cDNAs of

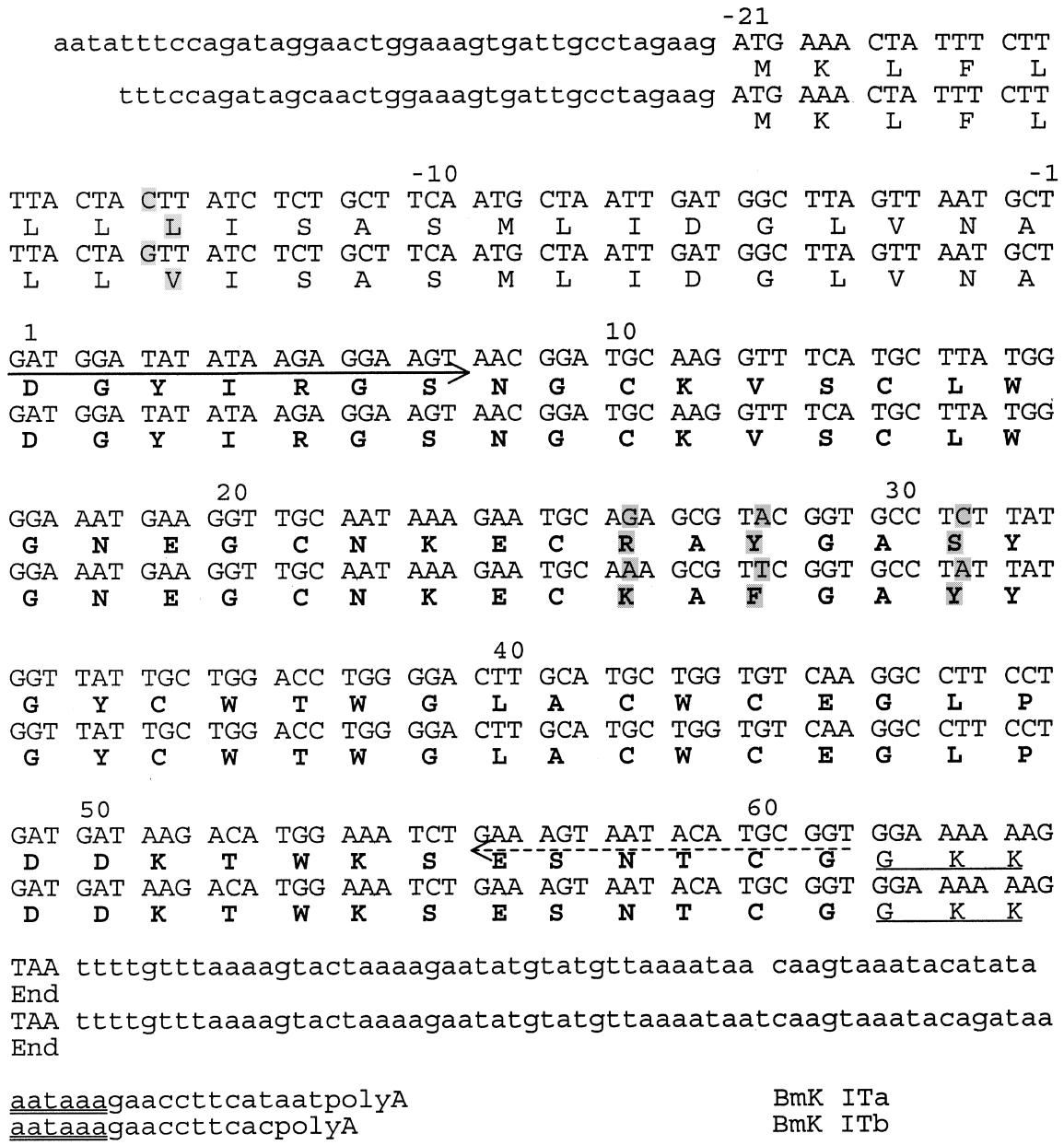

Figure 4. cDNA and deduced amino acid sequences of BmK ITa and ITb. Each of these cDNAs encodes a signal peptide of 21 residues (numbered −21 to −1), a mature peptide of 61 residues (in bold) and three additional residues (underlined). The primers for 3′- and 5′-RACE are indicated by arrows. Polyadenylation signals are indicated by a double underline. Amino acids are denoted using one-letter symbols.

other bioactive peptide and scorpion toxins (32,33,35–37). The amidation of BmK ITa and BmK ITb is further confirmed by the correspondence between their calculated and determined molecular masses (6732.4 and 6732.1 Da for BmK ITa, 6764.5 and 6763.9 Da for BmK ITb). Thus, the primary structures of BmK ITa and BmK ITb were determined using a combination of partial sequence determination and cDNA cloning.

We wish to point out that, in the case of BmK ITb, there happened to be a two-residue difference between the determined and cDNA-deduced sequences, Ile12Val and Gly27Ala. These two mismatches did not result in any change in molecular mass and have little effect on function. It is most likely that they were caused by polymorphism.

Sequence comparison

Obviously, the sequences of BmK ITa and BmK ITb share high homology with depressant insect toxins from other scorpions (Fig. 5). It is most likely that they evolved from the same ancestor into different forms with different

Figure 5. Sequence comparison. The determined partial sequences of BmK ITa and BmK ITb are compared with their corresponding cDNA-deduced sequences, marked as BmK ITa* and ITb*. The differences between their determined sequences are indicated with a gray background. Their sequences are also compared with those of other depressant insect toxins Lqh IT2 (35), Bot IT4 and Bot IT5 (15), Lqq IT2 (38) and Bj IT2 (22). Identical amino acids are indicated by a black background, homologous amino acids are indicated by a gray backgound. The asterisk at the C-terminus of sequences indicates amidation.

activities, which might be beneficial to survival of the scorpion. From the alignment of these sequences, one can easily find that most variances occur in the range of residues 6–31. Because no direct information about the three-dimensional structure or structure–function relationship of depressant insect toxins is available, it is deduced, by sequence comparison, that this highly variable region (residues 6–31) is probably activity related.

The uniqueness of the electrophysiological activity of BmK ITb makes its sequence worthy of more attention. However, there is only a five-residue difference between BmK ITb and -ITa. Furthermore, four of these five variations are between similar residues (V12I, R26K, A27G and Y28F). So, the last variation S31Y seems of more interest. Because Ser^{31} is highly conserved in all other depressant insect toxins (Fig. 5), it is tempting to hypothesize that Tyr^{31} gives BmK ITb its unique activity. Of course, more study is needed to clarify this. The gene cloning of these BmK depressant insect toxins makes it possible to study them further using protein engineering.

The sequences of these two depressant insect toxins have been deposited in the GenBank database under Accession numbers BmK ITa AF064821; BmK ITb AF272777.

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