Sequence and electrophysiological characterization of two insect-selective excitatory toxins from the venom of the Chinese scorpion *Buthus martensi*

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Abstract The two insecticidal peptides Bm32-VI and Bm33-I, isolated from the venom of the Chinese scorpion *Buthus martensi* induce paralytical symptoms typical of insect contractive toxins. They show, respectively, 74% and 77% homology with AaIT from *Androctonus australis*, comparable insecticidal activity and no vertebrate toxicity. Under voltage-clamp conditions, both toxins induced (1) an increased fast Na^+ current, (2) a shift in voltage dependence of Na^+ current activation, (3) the occurrence of a delayed current, and (4) a slow development of a holding current. Increased Na^+ conductance at negative potential values is responsible for axonal hyperexcitability and the contractive paralysis of insect prey.

Key words: Scorpion; Insecticidal toxin; Sequence; Pharmacology; Insect sodium channel

1. Introduction

As one possible alternative strategy to chemical pest control, genetically engineered microbial pesticides stand out, due to the possibility of improving their efficiency by expression of genes coding for proteins with deleterious effect in insects [1]. Strategies to increase pathogenicity are based on expression of enzymes or toxins, notably scorpion insect-specific sodium channel neurotoxins. Additional baculovirus enhancement strategies involve the expression of other peptide neurotoxins alone or in combination [2]. In addition, studies on the mode of action of insect-specific toxins brought a better understanding of the molecular determinants of vertebrate vs. insect specificity. The discovery of novel toxins and the elucidation of their molecular mode of action is thus of interest both academically and in agrochemical applications. As part of a systematic exploration of the peptide toxins of the Chinese scorpion *Buthus martensi*, we have undertaken a complete mapping of the insecticidal toxins contained in this venom, studying both short [3,4] and long toxins.

Venoms of scorpions in the family Buthidae are a rich source of insecticidal peptides and have been shown to contain three main categories of toxins: 'short toxins' (20–35 aa) cross-linked by three or four disulfide bridges; toxic for vertebrates and acting on different classes of potassium channels; 'short insectotoxins' (35–36 aa) linked by three disulfide bridges; and 'long toxins' (64–80 aa) cross-linked by four disulfide bridges which act on the voltage-dependent sodium channels of both vertebrates and insects. Among the latter, toxins have been classified as either vertebrate toxins (α and β) or insect toxins, according to their specificity, pharmacological and electrophysiological properties [5,6]. Insecticidal toxins are separated into excitatory and depressant toxins, which act together on sodium channel inactivation and activation, and resemble the mammalian β toxins in their mode of action. Insecticidal α toxins have also been described [7]. Their activity and complete insect specificity have been well characterized [8,9].

We report here the identification from the venom of *B. martensi* of two insecticidal peptides, Bm32-VI and Bm33-I, with high homology to the AaIT toxin, and the study of their electrophysiological properties against insect voltage-dependent sodium channels.

2. Materials and methods

2.1. Venom

B. martensi venom was collected every 20 to 30 days by manual or electrical stimulation of the telson of captive-bred scorpions (Chengdu, China) with an average yield of 0.2–0.3 mg. Eight to 9 g pooled venom was freeze-dried to give ca. 1 g of dry crude venom. Venom was extracted in 0.5 M acetic acid, centrifuged and filtered on 45 μm filters as previously described [4].

2.2. Activity bioassays

Gryllus bimaculatus crickets (3rd instar nymphs, average weight 65–70 mg) reared in a controlled environment (27°C, 70% relative humidity, 16:8 light/dark) were injected in the thorax with a 10 μl precision syringe (1 and 5 μl). Samples were dissolved in distilled water and controls were injected with water only. For calculation of median paralytic doses (ED_{50}), a range of doses was used, covering the range of toxic effects (0–100% effect) and three batches of five crickets per dose were treated. Observation of paralysis and/or death was made at 5 min, 15 min, 60 min, 24 h and 48 h post-injection. The assay endpoint for calculation of ED_{50}s was 15 min and ED_{50}s were calculated using probit analysis with the SoftTox program (SoftLabWare Inc.).

Activity against vertebrates was evaluated by intracerebroventricular injection (i.c.v.) in mice. C57/Bl6 5 weeks old male mice (ca. 20 g average weight) were anesthetized with diethylether, injected in the left cerebral ventricle with 5 μl of sample dissolved in a solution of bovine serum albumin (BSA) (20 g/l in 0.9% NaCl) and placed in glass jars for observation. Appearance of symptoms was noted continuously during the first hour post-injection and was monitored at regular intervals for 24 h or until death.

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2.3. Electrophysiological experiments

The experiments were performed on giant axons isolated from abdominal nerve cords of the cockroach *Periplaneta americana* using the double oil-gap method, in both current-clamp and voltage-clamp configurations [10]. Freeze-dried toxins were dissolved in physiological saline containing BSA (0.25 mg/ml) to a final concentration of 1 μM or 0.5 μM. Physiological saline composition was (mM): NaCl 200, KCl 3.1, $CaCl_2$ 5.4, $MgCl_2$ 5.0, HEPES buffer 1.0; pH 7.2. The potassium current was blocked by 1 mM 3,4-diaminopyridine (Sigma Co., USA) and sodium currents at the end of each experiment were completely suppressed with 0.5 μM tetrodotoxin (TTX) (Sigma Co., USA).

2.4. Peptide purification

Bulk venom fractionation (100 mg) was performed on a semi-preparative reversed-phase column (Shiseido C18, 10×300 mm) using an acetonitrile/water/0.1% TFA gradient (0–45% CH_3CN in 102 min, 3 ml/min) as previously described [4]. Fractions 32 and 33 were submitted to cation-exchange HPLC on a Tosoh SP5PW column (4.6×70 mm), with a gradient of NaCl (0–1 M NaCl in 88 min) in 10 mM phosphate buffer (pH 6.7). Final desalting of peptides Bm32-VI and Bm33-I was done on a Merck Lichrospher C18 column (4.6×120 mm) using a water/acetonitrile (0.1% TFA) gradient (0–60% in 60 min, 1 ml/min).

Capillary zone electrophoresis (CZE) analyses were performed on a Jasco CE800 system equipped with a UV detector and a 70 cm capillary (0.1 μm ID, 70 cm length, 50 cm to detector), with 20 mM sodium citrate buffer (pH 2.5) at 20 kV.

2.5. Sequence determination

Peptides were sequenced on a Shimadzu PPSQ-10 gas-phase sequencer either without prior derivatization or after reduction and 4-vinyl-pyridine alkylation [4]. Reduced alkylated toxins were submitted to proteolysis by (a) TPCK-treated trypsin (Sigma Co., USA), 2% w/w, 37°C, 14 h in 100 mM ammonium bicarbonate, 0.1 mM $CaCl_2$ pH 8.1 (for Bm32-VI, a second digestion was performed with 2% w/w of enzyme added after 4 h and a total reaction time of 24 h), (b) asparagine endopeptidase (Asp-N, Takara Shuzo, Japan), 5% w/w, 37°C, 14 h in 50 mM sodium phosphate buffer pH 8.0, and (c) *Achromobacter* protease I (API, Wako, Japan), 5% w/w, 37°C, 14 h in 100 mM Tris buffer, pH 8.0 and 12.5%, 37°C, 14 h in 100 mM ammonium bicarbonate pH 8.0.

For amino acid analysis, samples were hydrolyzed in a Waters Pico-Tag station, with 6 N HCl (0.6% phenol) at 110°C, under vacuum for 20 h, followed by phenylisothiocyanate derivatization and HPLC analysis.

2.6. Mass spectrometry

Mass spectra were recorded on a MALDI-TOF Perseptive Voyager Elite spectrometer (Perseptive Biosystems, USA), in positive ion linear or reflector mode using α-cyano-4-hydroxycinnamic acid matrix. Calibrations were done with a mixture of β-insulin (3495.94 Da) and bovine insulin (5733.5 Da). Data were analyzed using GRAMS 386 and theoretical molecular weights and p*I* values were calculated with the GPMAW software (Lighthouse software).

2.7. Database searches and multiple alignments

Sequence homologies were determined using the BLAST server (http://www.ncbi.nlm.nih.gov/). Multiple alignments and percentages of similarity were calculated with ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX/Top.html).

3. Results and discussion

3.1. Bioassay-guided fractionation

Fractionation of crude venom (100 mg) yielded 40 fractions numbered Bm1–Bm40 (Fig. 1A). Each fraction was analyzed by CZE and MALDI-TOF mass spectrometry to obtain a precise picture of complexity and mass range of the individual peptides. Since it has been well established that scorpion venoms contain a mixture of several structural types of toxins [5,6], we aimed at identifying the fractions where the insect-specific sodium channel toxins were located, using MS analysis to determine the distribution of molecular weights in relation to RP-HPLC retention times. Insecticidal activity was monitored with the cricket bioassay, and fractions Bm24–Bm34bis were selected for further fractionation work since they comprised mostly long toxins (MW > 6000 Da) and displayed strong insect paralytic activity. As those fractions were also toxic to mice, they were all submitted to a second separation by cation-exchange chromatography, yielding mostly pure compounds or simple mixtures as shown for fractions Bm32 (Fig. 1B, left-hand side) and Bm33 (Fig. 1B, right-hand side). A last reversed-phase chromatography step yielded pure peptides. A total of 53 active peptides was obtained. After quantification by amino acid analysis and MALDI-TOF mass analysis, all peptides were tested individually at a 1 μg dose against mice. A high dose was chosen for the purpose of quickly eliminating all compounds with vertebrate activity by testing well above the LD_{50} of known long toxins. This approach allowed elimination of the toxins with dual insecticidal and vertebrate activity and selection of insect-specific toxins. After elimination of toxins which induced neurotoxic symptoms in mice, 15 insect-specific peptide toxins were detected.

3.2. Structural analysis

The 15 insecticidal peptides were submitted to N-terminal sequencing without prior reduction alkylation, to determine homologies (data not shown). Toxins Bm32-VI and Bm33-I from fractions Bm32 and Bm33 (Fig. 1B) were selected for their N-terminal homology with the insect excitatory toxin AaIT from *Androctonus australis* and for their high activity against crickets. Sequence determination was achieved by reduction alkylation and sequencing of proteolytic cleavage peptides. Molecular weights of cleavage peptides were compared with known sequence data, to avoid sequencing redundancy and confirm results. Adequate fragments were generated by trypsin cleavage of Bm33-I (Fig. 2A). Peptide Bm32-VI was less sensitive to proteolytic cleavage and three different enzymes were used. A combination of fragment sequencing, and MALDI-TOF MS data allowed determination of the full sequence (Fig. 2A). Average MW values measured with internal standards were in good accordance with MW calculated from sequence data (Bm32-VI, calculated 7633.79 Da, measured 7632.83 Da; Bm33-I, calculated 8149.27 Da, measured 8149.39 Da). For Bm32-VI, a difference of one mass unit (−1 Da) between observed and calculated MW was observed in both the intact protein and a C-terminal fragment generated with trypsin and API (SYCDVQII). When including a C-terminal amidation and a pyridylethylated Cys residue, calculated average and monoisotopic MW (average 1044.22 Da, monoisotopic 1043.49 Da) match the corresponding observed MW (average 1044.56 Da, monoisotopic 1043.49 Da). In addition, MALDI-TOF MS data permitted confirmation of the Bm32-VI C-terminal sequence. The yield of the Edman degradation for the I_{68}–I_{69} couple was low, and MS data solved the ambiguity. For peptide Bm33-I, MS analysis of a C-terminal tryptic peptide (NYCDVQIIDLS) suggests a carboxylated C-terminal residue (calculated average 1387.57 Da, observed 1386.66; calculated monoisotopic 1386.64 Da, measured 1386.44). Bm32-VI is a basic protein (p*I* 7.43), similar to AaIT (p*I* 7.45) whereas Bm33-I is acidic (p*I* 4.60). Their yield was respectively 47.6 μg and 141.9 μg as determined by amino acid

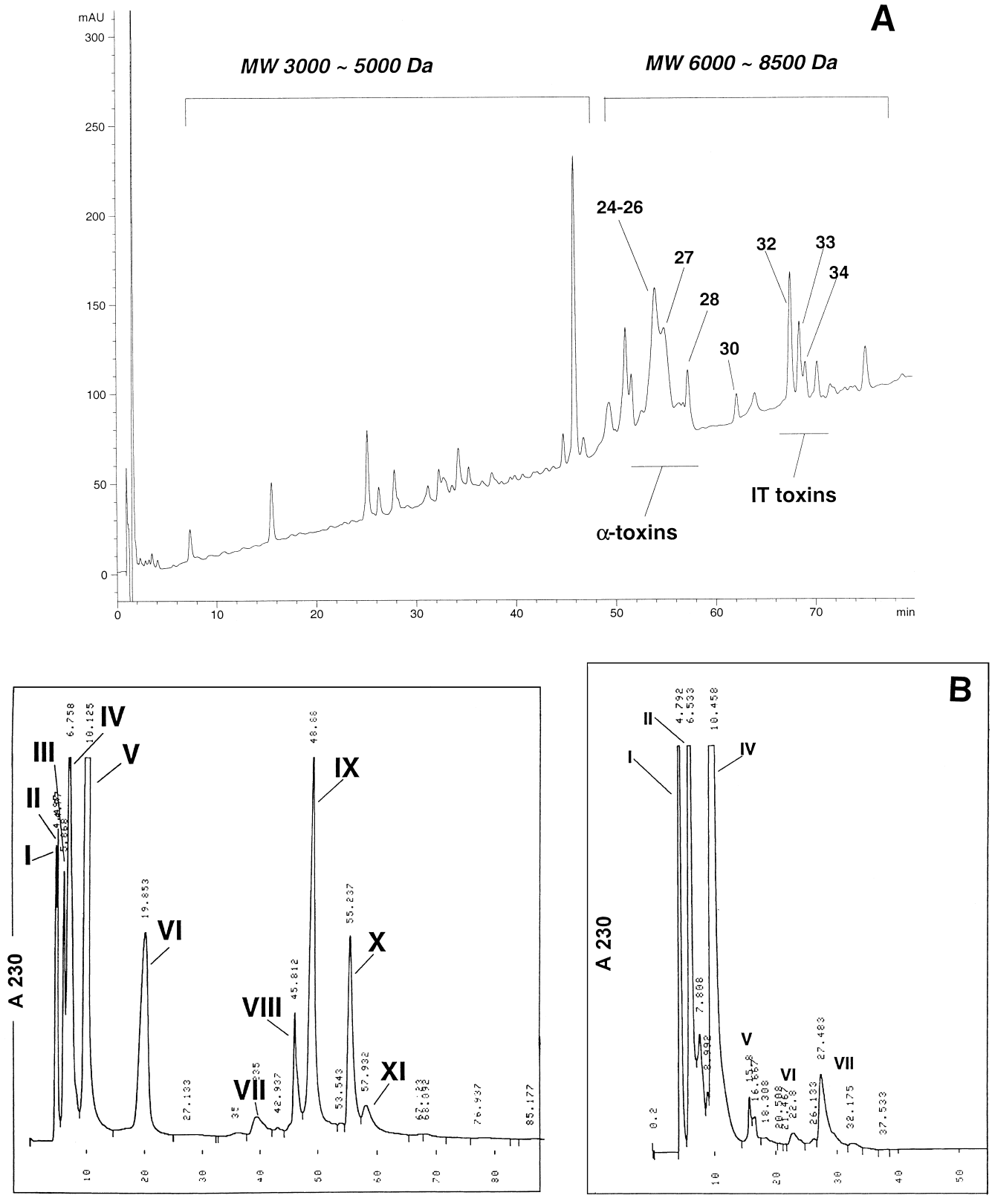

Fig. 1. Isolation of toxins Bm32-VI and Bm33-I. A: C18 RP-HPLC chromatogram of crude *B. martensi* venom, and molecular mass range of toxins measured by MALDI-TOF MS. N-terminal sequencing of peptides purified from fractions 24–28 showed homology with known α toxins. Insecticidal toxins were localized in fractions 32–34 and fractions 32 and 33 selected for further purification (see text). B (left): Cation-exchange chromatography of fraction 32 from A. B (right): Cation-exchange chromatography of fraction 33 from A. Roman numerals indicate peptide numbers. Peptides 32-VI and 33-I were selected according to bioassay results and were desalted and purified to homogeneity by C18 RP-HPLC.

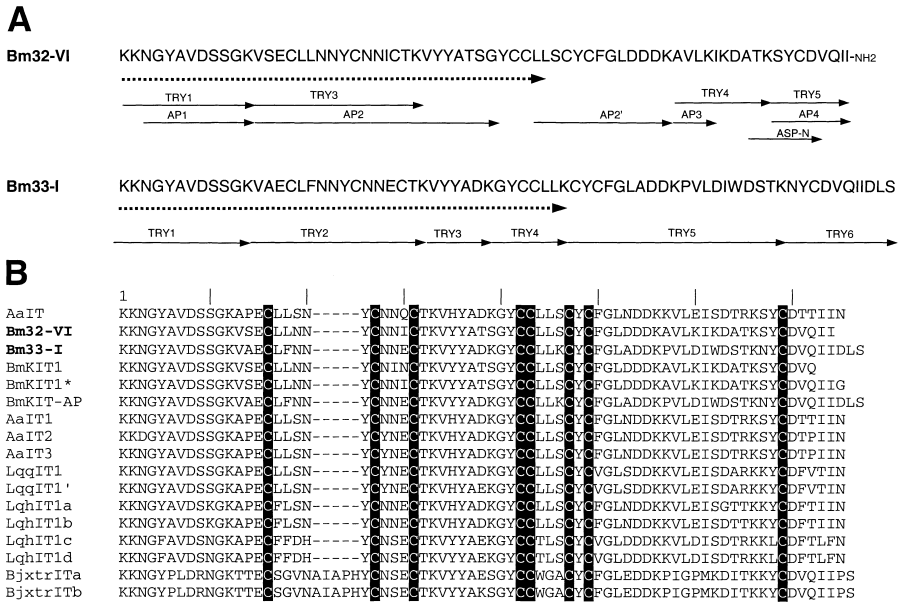

Fig. 2. Sequence determination of Bm32-VI and Bm33-I. A: Edman degradation of intact toxins (dotted line) and proteolytic fragments (Try = TPCK-trypsin, AP = *Achromobacter lyticus* protease I, AspN = Asp endoprotease). B: Sequence alignment of insect excitatory toxins. AaIT; AaIT1/AaIT2; AaIT3; LqqIT1/LqqIT1′; BmKIT1: [11]; BmKIT1*: [12]; BmKIT-AP: [15]; LqhIT1a-d; Bj-xtrITa-b.

analysis, representing ca. 0.05% and 0.14% of venom contents.

Homology of Bm33-I and Bm32-VI with AaIT was 73.6% and 78.6% respectively and places these in the group of insect-selective, contractive sodium channel toxins (Fig. 2B). Toxin Bm32-VI is highly homologous to toxin BmKIT previously isolated from the same venom [11]. The two peptides differ in the positions of residues Asn_{24} and $Ileu_{25}$ which are inverted in BmKIT ($Ileu_{24}Asn_{25}$), as well as in their C-terminal part where Bm32-VI has two additional isoleucine residues ($I_{68}I_{69}$). The exact molecular weight of BmKIT was not previously reported, and this result may suggest that BmKIT C-terminal sequencing was incomplete. The two C-terminal isoleucine residues are observed with a very low yield during the Edman degradation, as noted by the authors themselves following discrepancy with amino acid analysis results. In a recent paper, Xiong et al. [12] describe the cDNA sequence of a 70 residue mature excitatory toxin corresponding to BmKIT (also coded BmKIT1). The sequence deduced from the cDNA confirms the inversion of Asn_{24} and $Ileu_{25}$ as well as the double isoleucine at positions 68 and 69 and is in agreement with our data. However, deduced BmKIT1 sequence differs from that of Bm32-VI by a C-terminal Gly residue. As the measured molecular weight of purified Bm32-VI is not in agreement with this result, the toxin may undergo post-translational processing to yield an amidated C-terminal mature peptide, by oxidative deamination.

During the course of this work [13,14], a Chinese group described a novel insecticidal peptide with mouse analgesic properties named BMKIT-AP, which shares sequence homology with Bm33-I [15]. However, the pharmacology of this peptide has not been reported to date.

3.3. Biological activity

At doses of 1 μg, peptides Bm32-VI and Bm33-I did not demonstrate any toxicity to mice by i.c.v. injection. Higher doses could not be tested due to low toxin availability. In crickets, the ED_{50}s of Bm32-VI and Bm33-I were respectively 0.18 and 0.49 ng/mg of insect. These values are in the same toxicity range as an AaIT sample (0.099 ng/mg insect) tested under the same conditions, with Bm33-I being almost 5-fold less toxic. Structure–activity relationship studies on AaIT have not been done as extensively as for depressant long scorpion toxins [16,17]. It was previously suggested that the aromatic residues forming a solvent-exposed hydrophobic cluster are crucial for the binding of the protein to its receptor [18]. Those residues include Tyr_5, Tyr_{36}, Tyr_{43}, Phe_{45}, and Tyr_{63}, all of which are highly conserved in Bm toxins as well as other toxins of the group except LqqIT1/LqqIT1′ ($Phe_{45} \rightarrow Val_{45}$). The two C-terminal Ileu residues ($Ileu_{68}Ileu_{69}$) together with Leu_{47} and $Ileu_{56}$ are also thought to contribute to the formation of the hydrophobic cluster through their side chains and are also highly conserved in Bm32-VI and Bm33-I. Lys_{28} and Lys_{51} which are found in similar positions in the Bm peptides also play a crucial role in defining the insecticidal activity of these toxins [19]. Previous work has also emphasized the role of the C-terminal portion of AaIT (60–63 and 64–70 chains) in the insecticidal activity of the toxin [18]. As electrophysiological properties of the new peptides are altered, this suggests that the modified residues in the 64–70 chain of Bm32-VI and Bm33-I may play a role in modulating the neurophysiological activity of the toxins. The acidic character of the Bm33-I toxin may also be a factor in its diminished toxicity as observed with toxin BmKM8 when compared to more basic proteins from the same structural group [20].

3.4. Electrophysiology

Soon after toxin application, a minor resting depolarization was observed and the current necessary to evoke an action

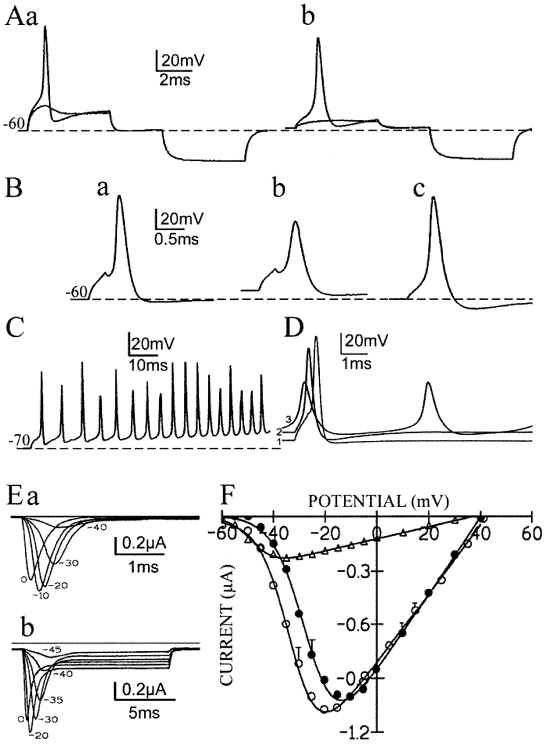

Fig. 3. Action of Bm33-I and Bm32-VI on cockroach axon electrical activity. A: Control (a) and Bm33-I (1 μM) modified (b) membrane responses to equal hyperpolarizing and threshold depolarizing pulses. In (Ab) a much smaller critical depolarization was necessary to evoke an action potential after toxin application. (Ba) Control action potential evoked by a short (0.5 ms) depolarizing current pulse (Bb). Action potential recorded after 8 min of Bm33-I superfusion. Late phase of toxin action. (Bc) Normal amplitude action potential restored by artificial repolarization to −60 mV. C: Spontaneous activity at a potential close to the resting level after previous artificial hyperpolarization to −70 mV in the presence of Bm33-I. D: Superimposed recordings illustrating a similar development of Bm32-VI effects (1 μM): 1, control action potential; 2, resting depolarization and decrease of action potential amplitude after 14 min of toxin application; 3, repetitive activity after 18 min of toxin action. E: Sodium current families recorded at different voltage pulse values from a holding potential of −60 mV in (Ea) control conditions and (Eb) after 7 min of Bm33-I (1 μM). F: Current–voltage relationships for the peak control current (filled circles), peak (empty circles) and late (empty triangles) currents after 7–10 min of Bm33-I superfusion. Mean values ($n = 5$, only examples of S.E. values are indicated for clarity) fitted with the Boltzmann distribution.

potential was decreased (e.g. from 3.75 nA in control to 1 nA after 1 μM Bm33-I). Effects of 4.5 ms hyperpolarizing and depolarizing current pulses (Fig. 3Aa,Ab) show that the passive resistance of axonal membrane remained unchanged while action potential could be generated at potentials close to the normal resting potential of the axon (ca. −55 mV). This early phase of toxin action, defined as an increase of axonal excitability, was followed by a progressive resting depolarization observed together with a decrease of action potential amplitude (Fig. 3Bb). At this stage, a single stimulus was often able to evoke several action potentials of small amplitude, and an artificial repolarization restored normal action potential amplitude (Fig. 3Ba,Bb,Bc). An artificial hyperpolarization to −70 mV for a few seconds followed by a return to −60 mV, induced a long train of action potentials with a lower frequency of firing at the beginning of a train, and a frequency increase up to 200–250 Hz 40 ms later (Fig. 3C). Bm32-VI induced very similar although slower effects (Fig. 3D). The resting depolarization reached 5–8 mV after 15 min of toxin application whereas it reached 10–12 mV after a 7–10 min application of Bm33-I. Repetitive firing of small action potentials was also observed during a longer time period after Bm32-VI application. However, repetitive activity following artificial hyperpolarization and the return to −60 mV, appeared with a lower frequency and was less sustained with Bm32-VI.

In voltage-clamp, no change in K^+ currents was detected after toxin application (data not shown) and in subsequent experiments only TTX-sensitive Na^+ currents were recorded. Application of step voltage pulses to different voltage values (Fig. 3Ea,Eb) showed that after Bm33-I application (1 μM), the current arised at more negative potentials, the maximal value of Na^+ current was higher and also occurred at more negative potentials than in control. The inactivation, rapid at the beginning of the pulses as in control, slowed after 2–3 ms in the presence of toxin, and a late current persisted throughout the voltage pulses (Fig. 3Ea,Eb). This current deactivated rapidly at the end of the pulses but increased in time after toxin application. After 7–10 min of toxin action, a progressive decrease of both peak and late currents was observed, which was correlated with the slow and progressive development of a holding, constant inward current reaching −40 to −80 nA at the end of the experiment. Very similar Na^+ current modifications were induced by Bm32-VI (1 μM) but the development of the holding current and the decrease of the peak current appeared later (in more than 15 min) when compared to Bm33-I.

Both toxins induced a shift in the voltage dependence curve of the sodium current. Under control conditions (Fig. 3F), measurable Na^+ peak inward current arose at −45 mV, reached a maximal amplitude at −10 mV and was annulled at +43 mV, determining equilibrium potential for Na^+ ions. In the presence of 1 μM Bm33-I, a current was already observed at the axon resting potential (−60 mV). At −45 mV it was more than twice the control. Maximal amplitude of peak current was reached for $E_m = -20$ mV and the reversal potential was +40 mV. Maximal amplitude of the late current was observed at −35 mV and decreased at less negative potentials (Fig. 3F). This shift ($V_{0.5} = -25$ mV in control to −34 mV after toxin, $n = 5$) of the voltage dependence of the sodium current to more negative potentials was observed 10–15 min after toxin application, and developed progressively. A smaller modification was noted upon application of Bm32-VI (not shown). The shift of $V_{0.5}$ induced by Bm32-IV (1 μM) after 10–15 min was only 6 mV ($n = 4$) and the current observed at −60 mV was twice smaller than with Bm33-I. The most important difference between the effect of Bm32-VI and Bm33-I was the magnitude of the late current which represents 20% of the peak current with Bm32-VI and up to 30% with Bm33-I.

Experiments where steady-state inactivation of sodium current was measured (not shown) indicate that in control conditions at −60 mV almost all Na^+ channels are free to open if activated by depolarizing pulses. At −34.6 mV ($V_{0.5}$) 50% of channels are inactivated and at −5 mV an absence of current after the test pulse indicates complete inactivation, which was never reached after toxin application. A 20–30% residual Na^+ current was always observed after application of Bm32-VI

and Bm33-I, even at +10 mV. With both toxins, $V_{0.5}$ was similarly shifted by about 10 mV to more negative potential values when compared to control.

Bm32-VI and Bm33-I effects were similar to that of AaIT action [7,8]. Effects developed in two steps: (1) an increase of axonal excitability and (2) a resting depolarization together with a decrease of action potential amplitude. The increase of axonal excitability results from a shift to more negative values in voltage dependence of sodium channel activation. This shift is more important after AaIT (10–12 mV) [7], smaller after Bm33-I (less than 10 mV), and the smallest after Bm32-VI. Similarly, repetitive activity is the most sustained after AaIT, less persistent with Bm33-I and still less with Bm32-VI (Stankiewicz and Pelhate, personal observations). However, the second step of toxin action appears later and is less pronounced for AaIT than for Bm33-I. In this respect, Bm32-VI is more similar to AaIT than to Bm33-I. Contractive characteristics of toxins observed in whole insect tests probably result mainly from the first step of toxin action and when the duration of this step is longer, contractive activity should be stronger. This idea seems to be confirmed in the toxicity tests which showed the highest potency for AaIT, followed by Bm32-VI, then Bm33-I. The second step of toxin action probably results from the development of the inward constant holding current observed later, mainly with Bm33-I.

The second additional current observed after application of contractive toxins is the late current present even at the end of long depolarizing pulses, which reflects an incomplete channel inactivation. Its size is correlated with the voltage dependence shift of channel activation (largest with AaHIT, followed by Bm33-I and Bm32-VI). This current probably facilitates the repetitive activity observed after application of the toxins.

4. Conclusions

In summary, Bm32-VI induces less modifications of axonal bioelectrical activity and axonal sodium current than Bm33-I however its lethal activity is closer to that of AaIT. Both toxins display pharmacological characteristics of excitatory toxins. The discovery of insecticidal peptides possessing highly homologous sequences and similar pharmacological properties will help in elucidating the involvement of specific residues in activity and the molecular interaction mechanisms of receptor–ligand interactions. Fine dissection of those mechanisms using toxins as molecular probes will lead to a better understanding of the spatial distribution of active residues on a conserved scaffold and will be of use in the development of more specific insecticidal peptides to be used in recombinant baculoviruses for insect control.

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