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Characterization of Na⁺ currents in isolated dorsal unpaired median neurons of *Locusta migratoria* and effect of the alpha-like scorpion toxin BmK M1

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Abstract

A primary cell culture was developed for efferent dorsal unpaired median (DUM) neurons of the locust. The isolated somata were able to generate Tetrodotoxin (TTX)-sensitive action potentials in vitro. The α -like scorpion toxin BmK M1, from the Asian scorpion *Buthus martensi* Karsch, prolonged the duration of the action potential up to 50 times. To investigate the mechanism of action of BmK M1, the TTX-sensitive voltage gated Na⁺ currents were studied in detail using the whole cell patch clamp technique. BmK M1 slowed down and partially inhibited the inactivation of the TTX-sensitive Na⁺ current in a dose dependent manner (EC₅₀ = 326.8 ± 34.5 nM). Voltage and time dependence of the Na⁺ current were described in terms of the Hodgkin–Huxley model and compared in control conditions and in the presence of 500 nM BmK M1. The BmK M1 shifted steady state inactivation by 10.8 mV to less negative potentials. The steady state activation was shifted by 5.5 mV to more negative potentials, making the activation window larger. Moreover, BmK M1 increased the fast time constant of inactivation, leaving the activation time constant unchanged. In summary, BmK M1 primarily affected the inactivation parameters of the voltage gated Na⁺ current in isolated locust DUM neurons.

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1. Introduction

The neuromodulatory, efferent dorsal unpaired median (DUM) neurons of the insect central nervous system play an important role in the locomotion of the locust. Since the DUM neurons were first described (Plotnikova, 1969) they have been the subject of extensive behavioural and physiological studies in insects. Several thoracic DUM neurons have been shown to innervate skeletal muscle. They secrete octopamine and change the basic tension and/or the use of different metabolic substrates (Evans and Siegler, 1982; Blau and Wegener, 1994; Stevenson and Spörhase-Eichmann,

1995). Most studies before 1996 indicate that thoracic DUM neurons of locusts are part of a general arousal system and are collectively activated (Burrows, 1996). However, in more recent studies subpopulations of efferent DUM neurons were shown to be specifically activated or inhibited in parallel with particular motor patterns. They ensure a well arranged neuromodulation in function of the selected motor programme (Pflüger, 1999).

In contrast to most other neurons in the insect central nervous system, the DUM neuron somata contain voltage gated Na⁺ channels and they are able to generate action potentials in the cell body (Crossman et al., 1971). The spontaneous electrical activity is probably involved in triggering the secretion of the octopamine. The presence of voltage gated Na⁺ channels in the soma was confirmed by immunostaining and whole cell patch clamp studies in short term cultured DUM neurons of the cock-

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roach, *Periplaneta americana* (Lapied et al., 1990; Amat et al., 1998). Until now the electrophysiological characteristics of the voltage gated Na^+ currents in isolated insect neurons have been subjected to detailed investigation in, e.g. *P. americana*, *Apis mellifera*, *Drosophila melanogaster* and *Heliothis virescens* (O'Dowd and Aldrich, 1988; Schäfer et al., 1994; Lee and Adams, 2000; for review see Wicher et al., 2001). However in locusts, most electrophysiological studies have been done in situ and to our knowledge, no voltage clamp studies on isolated locust DUM neurons have been performed.

Due to their key function in generating electrical signals, sodium channels are the target of various toxins present in natural venoms. Tetrodotoxin (TTX), probably the most used specific Na^+ channel blocker, was isolated from a pufferfish (Narahashi et al., 1964). Scorpion venoms are a rich source of toxic neuropeptides disturbing gating and permeation of ion channels. The long chain peptides (60–76 amino acid residues) affecting in particular Na^+ channels are traditionally divided into two major groups, α - and β -toxins, according to principal functional effect and binding characteristics (Possani et al., 2001). The α -toxins bind to receptor site 3 of the Na^+ channel, located at the extracellular loop connecting the transmembrane segments S3 and S4 of the fourth subunit, and slow down and/or inhibit the inactivation process of the Na^+ current (Cestele and Catterall, 2000).

Specificity of α -toxins varies considerably. The classical α -toxins are highly specific for mammalian Na^+ channels (e.g. AaH II from *Androctonus australis* Hector, Rochat et al., 1970), other α -toxins are highly active on insects (e.g. Lqq III from *Leiurus quinquestriatus quinquestriatus*, Kopeyan et al., 1993) and the third group, the α -like toxins, is active on both mammalian and insect Na^+ channels (e.g. LqhIII from *Leiurus quinquestriatus hebraeus*, Sautiere et al., 1998).

BmK M1 (also called BmK I), a 64 amino acid long member of the α -like toxins, was purified from the Asian scorpion *Buthus martensi* Karsch (BmK, Ji et al., 1996). The venom of this scorpion has been used for more than a thousand years in Chinese traditional medicine to treat neural diseases. BmK M1 is not only the most abundant toxin in the BmK venom (Wu et al., 1999) but probably also the best known toxin of this scorpion (for a review on BmK toxins see Goudet et al., 2002): the amino acid sequence and crystal structure are known (He et al., 1999; Li et al., 1999), the gene could be expressed and the binding characteristics to Na^+ channels were investigated (Shao et al., 1999; Li and Ji, 2000). Finally, the effect was characterized electrophysiologically on expressed human Na^+ channels (Goudet et al., 2001). Studies of the effect of BmK M1 on the native invertebrate voltage gated Na^+ currents were brief or showed no effect (Terakawa et al., 1989; Ji et al., 1996; Goudet et al., 2001). Although the inhibition of activation was

studied in detail on the human heart Na^+ channels ($\text{Na}_v1.5$ expressed in *Xenopus laevis* oocytes; Goldin, 2001), a thorough study of the effect of BmK M1 on invertebrate Na^+ currents is necessary to complete our knowledge of this toxin.

The aim of the present study was, first, to develop a method of isolation and bringing locust DUM neurons in primary cell culture; secondly, to give a detailed description of the electrophysiological properties of voltage gated Na^+ currents in isolated DUM neuron somata of the locust. These single cell properties contribute to a better understanding of the neurophysiology of locust DUM neurons. Thirdly, we used the isolated DUM neuron preparation to test the effect of the α -like scorpion toxins, BmK M1 and to complete our knowledge of its function and of its invertebrate/vertebrate or species specificity.

2. Materials and methods

2.1. Animals

Adult migratory locusts (*Locusta migratoria*) of both sexes were taken from the crowded laboratory colony 2–10 days after imaginal moult. Animals were reared at approximately 32 °C on a 14:10 h light/dark cycle on a diet of grass and oatmeal (Ashby, 1972).

2.2. Primary cell culture

Isolated DUM neuron cell bodies from the metathoracic ganglion were prepared under sterile conditions. The ventral parts of the thorax and abdomen were dissected together with the nerve cord attached. A group of ± 20 efferent DUM neurons is situated in the dorsal median region of the metathoracic ganglion, and these DUM neurons are surrounded by neuropil and smaller neurons (Leitch et al., 1993). The dorsal median region from ganglia of 6–7 animals was removed and subjected to a collagenase/dispase (2 mg/ml) treatment for 30 min at 37 °C in minimum essential medium with 25 mM HEPES and Hanks' salts. The cells were centrifuged at 900 rpm for 10 min and subsequently washed three times with culture medium consisting of equal parts of basal medium eagle with Hanks' salts and Grace's insect medium. The first time a penicillin/streptomycin mixture was added to the culture medium. The cells were dissociated by repetitive suction through a pipette tip and they were finally plated on Nunc petri dishes and incubated overnight at 28 °C and 5% CO_2 . All products were obtained from GibcoBRL (Invitrogen, Belgium) except the collagenase/dispase mixture (Boehringer, Roche Diagnostics, Belgium).

2.3. Electrophysiological experiments

The patch clamp technique was used in the whole cell configuration (Hamill et al., 1981) at room temperature. Petri dishes containing the cells were placed onto the stage of a Diaphod-TMD inverted microscope (Nikon corporation, Tokyo, Japan). The electrodes were pulled from borosilicate capillaries (Clark Electromedical Instruments, Harvard Apparatus Ltd, Kent, UK) with a programmable DMZ-universal puller (Zeitz-Instrumente, Munchen, Germany). They were mounted on and controlled by a WR-98 hydraulic micromanipulator (Narishige Scientific Instrument Lab., Tokyo, Japan). Healthy efferent DUM neurons were recognized by their bright appearance under phase contrast microscopy and by a smooth cell membrane. Moreover, the size of the DUM neurons allowed us to distinguish between DUM neurons and other smaller neurons.

Action potentials were recorded in current clamp conditions with a patch and cell clamp amplifier RK300 (Biologic, Echirrolles, France). Action potentials were generated spontaneously or were evoked by sending 0.1–0.5 nA current into the DUM neuron cell bodies. Electrodes with a maximal resistance of 1.5 m Ω were used. Data were recorded on a DTR-1200 digital audio tape recorder (Biologic, Echirrolles, France), visualized on a Trace 8708 digital oscilloscope (Trace, Vienna, Austria), filtered at 3 kHz by a VBF/8 filter (Kemo, Kent, UK) and digitized at 8 kHz via a CED 1401 A/D converter and the CED software version 6.41 (both Cambridge Electronic Design, Cambridge, UK).

Voltage clamp experiments and data acquisition were performed with a personal computer (Pentium III) controlled EPC9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). For voltage clamp experiments only neurons with a diameter smaller than 60 μ m were chosen in order to avoid space clamp problems. The resistance of the patch electrodes was in between 650 and 800 k Ω . Liquid junction potentials were taken into account at the start of the experiment, before forming a gigaseal (Barry and Lynch, 1991; Neher, 1992). The cells were clamped at a holding potential of -90 mV. Capacitive and leak currents were compensated for automatically by the Pulse program (HEKA Elektronik, Lambrecht, Germany) and residual capacitances and leak currents were eliminated by means of a P/6 protocol. The series resistance was compensated for in order to be lower than 1.5 m Ω , i.e. 1% of the input resistance of the cell. Data were filtered at 2.9 kHz and sampled at 50 kHz. Data were stored on the computer's hard disk for later offline analysis.

2.4. Solutions and toxins

The normal extracellular solution (ES) to record action potentials in current clamp condition and total

membrane currents in voltage clamp configuration contained (in mM) 172.5 NaCl, 6.5 KCl, 7.7 MgCl₂, 2 CaCl₂, 10 HEPES and 13 glucose. The pH was 6.80. The standard pipette solution (PS) contained (in mM) 160 potassium gluconate, 6.5 NaCl, 1 CaCl₂, 10 EGTA, 10 HEPES, 2 MgATP, and 45 glucose. The pH was 6.65.

In experiments where Na⁺ currents were isolated the ES (ES Na) contained (in mM) 40 NaCl, 90 CholineCl, 3 CaCl₂, 2 CdCl₂, 7 MgCl, 40 tetraethylammonium (TEA)-Cl, 10 HEPES and 5 4-aminopyridine (4-AP). The pH was 6.80. The PS for Na⁺ current isolation (PS Na) contained (in mM) 5 NaCl, 75 CsCl, 65 CsF, 1 CaCl₂, 1 MgCl₂, 2 MgATP, 10 EGTA, 10 HEPES and 5 glutathion. The pH was 6.65. The composition of the Na⁺-separating solutions was such that the Ca²⁺ and K⁺ currents were minimized. The Na⁺ concentration (40 mM) was low in order to reduce the inward Na⁺ current and to diminish voltage clamp errors.

The osmolality of all solutions was brought to \sim 380 mOsmol/kg with glucose, corresponding to the measured haemolymph osmolality in *L. migratoria*. Measuring the osmolality of locust haemolymph and experimental solutions was done by means of a Wescor 5100 C Vapor Pressure Osmometer (Wescor, Logan, USA). The haemolymph osmolality of adult locusts was 383 ± 17.2 mOsmol/kg ($n = 7$).

Since the contaminating K⁺ currents were not always completely abolished, TTX-sensitive Na⁺ currents were investigated. Note that the term 'TTX-sensitive currents' does not imply that TTX insensitive currents were found. The TTX-sensitive currents were obtained by subtracting current traces in the presence of 100 nM TTX (Alomone Labs, Jerusalem, Israel) from traces in the absence of TTX. Unless stated otherwise, all Na⁺ current properties described in this paper refer to the TTX-sensitive Na⁺ current. BmK M1 was prepared as previously described (Li et al., 1999).

2.5. Data analysis

Results are shown as means \pm standard error of the mean (SEM) and n is the number of DUM neurons. Significance of differences between two means was calculated with the Wilcoxon matched pairs test using the software INSTAT (demo version, graphpad Software, San Diego, USA). Data sets were fitted by mathematical expressions using ORIGIN 6.0 (Micoral Software, Northampton, USA). Significance of the difference between parameters obtained by curve fitting (mean \pm SEM) was calculated with a Student's t -test with Welch correction (Instat demo version, graphpad Software, San Diego, USA). Differences in the mean values were considered significant if $p \leq 0.05$.

The equation used for fitting dose–response relationships by means of the software ORIGIN 6.0 (Micoral Software, Northampton, USA) was

$$I_{\text{norm}} = \frac{I_{\text{min}} + I_{\text{max}}}{1 + ([X]/\text{EC50})^p} + I_{\text{max}}, \quad (1)$$

where I_{norm} was the normalized, measured current and $[X]$ is the concentration of the tested substance X . The parameters to be fitted were concentration of half maximal effect (EC50), the slope factor (p), the normalized minimal current (I_{min}) and the normalized maximal current increase (I_{max}).

Activation and inactivation kinetics of DUM neuron Na^+ currents were described in terms of the Hodgkin–Huxley (H–H) formalism using the Pulsefit program (HEKA elektronik, Lambrecht, Germany) to fit the Na^+ currents (cf. Wicher and Penzlin, 1998). If the Na current $I_{\text{Na}^+} = G(V - V_{\text{Na}^+})$, the conductance G was

$$G = G_{\text{max}} m^3 [a h_1 + (1-a) h_2], \quad (2)$$

where G_{max} is the maximum conductance, m the activation parameter and h_1 and h_2 are inactivation parameters, representing the fast component (time constant $0.5 \leq \tau_{h_1} \leq 6$ ms) and the slow component (time constant $15 \leq \tau_{h_2} \leq 25$ ms) of the inactivating phase of the current, respectively (Lapied et al., 1990). The parameter a defined the relative contribution of the inactivation parameters and was >0.95 in control conditions. Moreover, the reproducibility of fitting of the inactivating part by the H–H model was higher when τ_{h_2} was kept constant at values $15 \leq \tau_{h_2} \leq 20$ ms for control conditions and $20 \leq \tau_{h_2} \leq 25$ ms in the presence of BmK M1. Due to the minor contribution of the slow component and its fixed time constant τ_{h_2} , the effect of BmK M1 was shown on the fast time dependence (τ_{h_1}) only and the time constant was simply denoted as τ_h .

The activation and inactivation kinetics were both time and voltage dependent. Time dependence of m and h in control conditions was described as

$$m(t) = m_{\infty} - (m_{\infty} - m_0) \exp(-t/\tau_m), \quad (3)$$

$$h(t) = h_{\infty} - (h_{\infty} - h_0) \exp(-t/\tau_h), \quad (4)$$

where m_{∞} and h_{∞} are the steady state values of the activation and inactivation parameters and m_0 and h_0 are their values at time 0. At highly negative membrane potentials (e.g. $V_h = -90$ mV) m_0 is 0 because no Na^+ channels are activated and h_0 is 1 because no channels were inactivated.

The steady state parameters, m_{∞} and h_{∞} , are dependent on the membrane voltage in a sigmoidal fashion and can be described by the Boltzmann functions:

$$m_{\infty} = \frac{1}{1 + \exp\left(\frac{V_m - V}{K_m}\right)}, \quad (5)$$

$$h_{\infty} = \frac{h_{\text{max}} - h_{\text{min}}}{1 + \exp\left(\frac{V - V_h}{K_h}\right)} - h_{\text{min}}, \quad (6)$$

where V_m and V_h are potentials of half maximal activation and inactivation, respectively and K_m and K_h are slope parameters. Since inactivation was incomplete even in control condition it was necessary to include the minimum h value, h_{min} . The maximum h value (h_{max}) was 1.

3. Results

3.1. TTX-sensitive voltage gated Na^+ currents play a key role in repetitive firing of DUM neuron somata

The isolated locust DUM neuron cell bodies remained viable and functional after one night of incubation. The cell bodies often retained part of their primary neurite. In some cases cells regenerated their primary neurite that ramified into very fine branches. Although the outgrowth of branches showed that cells were in a good condition, only cells with a primary neurite stump were used to reduce space clamp problems. Upon switching from the voltage clamp ($V_{\text{holding}} = -80$ mV) to the current clamp mode a train of spontaneous action potentials generated by the cell body could be recorded (Fig. 1(A)). In some cases stimulating the cell by continuously injecting a 0.1–0.5 nA depolarizing current was necessary to evoke the repetitive firing (Fig. 1(B1)). In five experiments the mean threshold potential was -18.0 ± 3.7 mV, the amplitude and duration of the action potentials were 72.7 ± 2.22 mV and ~ 10 ms, respectively. Action potentials fired spontaneously at a frequency of 5.8 ± 0.7 Hz during 2.7 ± 0.8 s and after a train of spikes a resting membrane potential of -33.1 ± 1.9 mV was reached ($n = 5$).

To check whether the action potentials were Na^+ dependent, TTX, a specific Na^+ channel blocker, was added to the ES (not shown). Action potentials were completely abolished by adding 100 nM TTX to the ES. The effects of TTX on the action potentials were not reversible upon wash out.

The effect of TTX on the voltage gated Na^+ current, which seems to be responsible for generating the action potentials in isolated DUM neurons, was studied in more detail in voltage clamp experiments (solutions ES Na and PS Na were used). Fig. 2(A) shows that the fast activating and inactivating Na^+ current was inhibited by TTX in a concentration dependent manner. The normalized peak currents (control peak current $I_{\text{peak,control}} = 1$) in the dose–response relation could be fitted by eq. (1) (see Section 2). The concentration of half maximal inhibition was 5.6 ± 0.76 nM TTX and a complete block was achieved in the presence of 100 nM TTX as shown in the dose–response relationship in Fig. 2(C). According to Grolleau and Lapied (1994) we used the partial blockage of TTX to assess the quality of the voltage clamp. The time to peak was measured every 0.5 s while

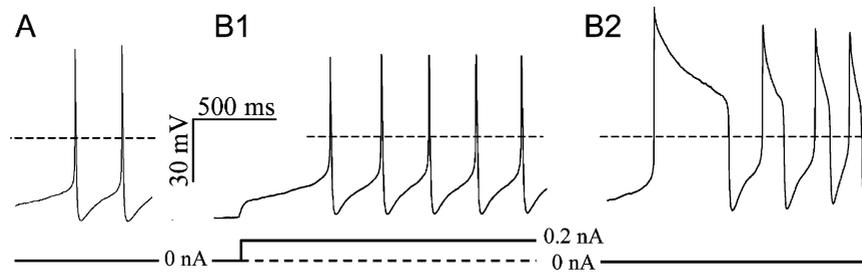


Fig. 1. Repetitive firing of action potentials in two different isolated DUM neurons ((A) and (B)), measured in current clamp mode. The action potentials are shown in the absence ((A) and (B1)) and in the presence (B2) of 500 nM BmK M1. Upper and lower traces are, respectively, membrane voltage and current. The dashed lines are zero voltage or zero current level. (A) Action potentials were generated spontaneously. (B1) In this cell a continuous, depolarizing current of 0.2 nA was needed to evoke a train of action potentials. Each action potential was followed by a typical afterhyperpolarization. (B2) In the presence of 500 nM BmK M1 the duration of the action potential was increased considerably. Action potentials were generated spontaneously without applying an artificial depolarizing current pulse and had higher amplitudes compared to control conditions. Recordings were made in normal bath and pipette solutions (ES and PS).

the peak current gradually decreased due to TTX blockage (100 nM). Fig. 2(B) shows that the reduction in the current amplitude did not produce a shift in the time to peak, as expected for good voltage clamp conditions.

A family of TTX-sensitive Na^+ currents, obtained by subtracting the current traces in the presence of TTX from the control currents, is shown in Fig. 3(A). The TTX-sensitive voltage gated Na^+ currents became gradually activated by subsequent voltage pulses of increasing amplitude (see inset of Fig. 3(A)) suggesting an adequate voltage clamp. The different current traces activated and inactivated quickly when DUM neurons were depolarized from a holding potential of -90 mV to membrane potentials more positive than -30 mV.

3.2. BmK M1 effect on action potentials and voltage gated Na^+ currents

As in control conditions, upon switching from voltage clamp to current clamp mode, action potentials were recorded. However, in the presence of the α -like scorpion toxin BmK M1 (500 nM) the duration of the first action potential in a train was prolonged from ~ 10 to ~ 500 ms (Fig. 1(B2)). The following action potentials became gradually shorter. In the presence of BmK M1 the spike amplitude was higher. Current injection to evoke this repetitive firing was not necessary due to a positive shift of the resting membrane potential induced by BmK M1. These effects were not completely reversible on wash out.

In whole cell voltage clamp experiments, 500 nM BmK M1 slowed down and partially inhibited the inactivation of the TTX-sensitive Na^+ current. This resulted in a maintained inward Na^+ current for all activating potentials, even at the end of a 100 ms depolarizing pulse. Peak Na^+ currents did not increase substantially. Peak currents (Fig. 3(C)) and maintained currents (Fig. 3(D)), normalized to the maximal inward current in control conditions, were plotted as a function of the different test potentials. Control currents and currents in the pres-

ence of 500 nM BmK M1 are overlaid in the I–V plots. Mean values were fitted by eq. (7) which expresses the dependence of the current on the driving force and on the conductance. The conductance itself changed in a sigmoidal fashion as a function of the membrane potential (Stühmer, 1988)

$$I = g \left(1 - \frac{1}{1 + \exp[(V - V_{0.5})/K]} \right) (V - V_{\text{rev}}), \quad (7)$$

where I is the peak or maintained Na^+ current, g the maximal Na^+ conductance, V the clamped membrane potential, $V_{0.5}$ the voltage of half maximal activation, K the slope factor and V_{rev} is the reversal potential for Na^+ . In control conditions (Fig. 3(C)) the TTX-sensitive Na^+ current activated at approximately -35 mV, the $V_{0.5}$ was -11.8 ± 0.2 mV and the slope was 4.5 ± 0.1 mV. The Na^+ current reached a maximum amplitude at approximately 0 mV. Fitting the data (measured up to clamp potential of $+35$ mV) resulted in an estimated reversal potential of 64.3 ± 1.6 mV. In the presence of the 500 nM BmK M1 the I–V plot shifted by 4.5 mV towards more negative potentials. The $V_{0.5}$ was -16.2 ± 0.2 mV and the slope 3.9 ± 0.1 mV. Consequently the maximum amplitude of the peak current occurred at approximately -5 mV (compared to 0 mV) and the maximal Na^+ conductance increased from 16.6 to 17.6 μS after BmK M1 treatment. The estimated reversal potential decreased from 64.3 to 55.7 ± 1.0 mV.

Fig. 3(D) shows that at least in control conditions the Na^+ current inactivated almost completely at the end of 100 ms voltage pulse. BmK M1 clearly inhibited inactivation, resulting in a maintained current of approximately 30% of the maximum amplitude of peak current. The current–voltage relationship of the maintained current in the presence of BmK M1 could be adequately fitted by eq. (7). The $V_{0.5}$ was -17.1 mV which is similar to the $V_{0.5}$ of the peak current. As expected by the lower amplitude, the conductance was smaller (5.9 μS) than for the peak current. Furthermore the estimated V_{rev} was 47.2 mV and the slope factor 5.3 mV.

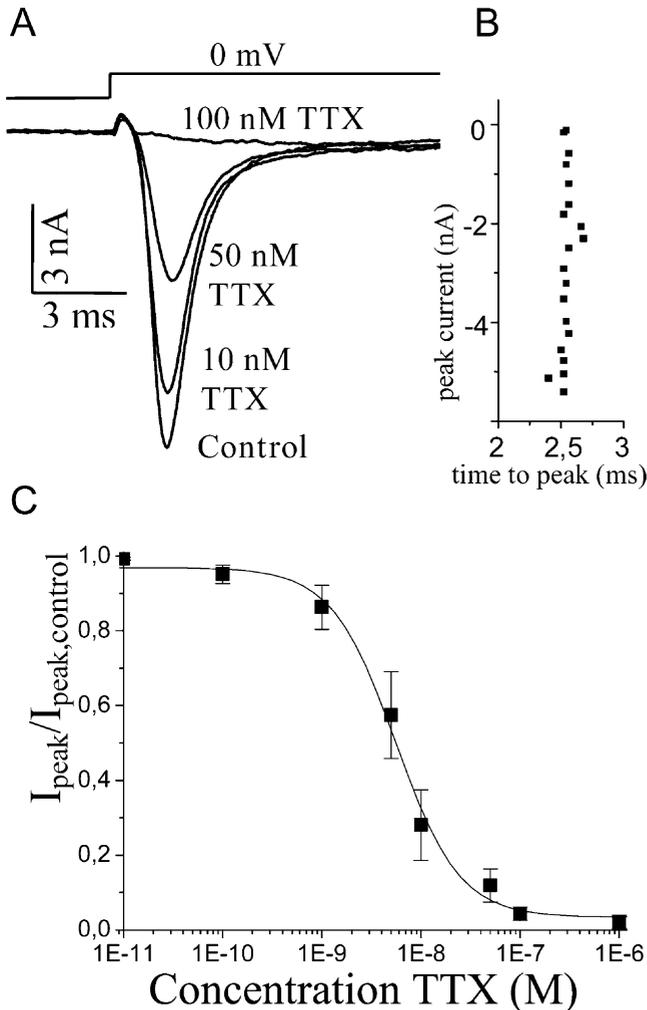


Fig. 2. TTX-sensitivity of Na^+ currents in whole cell voltage clamp experiments on isolated DUM neurons. Currents were measured in Na^+ current isolating solutions (ES Na and PS Na). Na^+ currents were evoked by a depolarizing pulse to 0 mV from a holding potential of -90 mV. (A) Effect of different concentrations of TTX on the peak Na^+ current of a DUM neuron. The time to peak remained unchanged. (B) The quality of the voltage clamp was tested by plotting the time to peak of the inward current as a function of the current amplitude that was partially blocked by 100 nM TTX. The gradually decreasing current, evoked by a depolarization from -90 to -10 mV, is measured every 0.5 s while TTX diffused in the solution. (C) Concentration dependent block of the peak Na^+ current by TTX. Normalized peak currents could be fitted by the dose–response curve $I_{\text{peak}}/I_{\text{peak,control}} = \{(I_{\text{min}} - I_{\text{max}})/[1 + ([\text{TTX}]/\text{EC50})^p]\} + I_{\text{max}}$ with $\text{IC50} = 5.6 \pm 0.76$ nM and slope factor $p = 1.35$ ($n = 12$).

3.3. Time and dose dependence of BmK M1 effect

The time dependence of the BmK M1 effect on the Na^+ current was tested by means of depolarizing potential steps of 40 ms (from -90 mV holding potential to 0 mV test potential; Fig. 4). TTX-sensitive current traces were recorded with an interval of 10 s (Fig. 4(A)).

In Fig. 4(B) the sustained current amplitude (before the application of TTX) is shown. BmK M1 treatment caused an immediate increase of the maintained Na^+ cur-

rent and a slowing down of inactivation while the peak current was hardly changed. In the presence of BmK M1 the maintained current reaches a plateau as a function of time. This current could be completely blocked by 100 nM TTX.

The effect of BmK M1 on the TTX-sensitive Na^+ currents of isolated DUM neurons is concentration dependent (Fig. 4(C)). The pulse protocol was the same as that described for Fig. 4(A) and it was applied in different concentrations of BmK M1. In Fig. 4(C) the effect on the maintained current is shown. The current amplitude at the end of the pulse at the highest concentration ($I_{\text{sus,max}}$) was taken as 1 and current amplitudes in other concentrations (I_{sus}) were normalized to this value. In between approximately 0.1 and 1 μM BmK M1 the maintained current rose in a sigmoidal fashion. The mean values could be fitted by the function (cf. eq. (1))

$$I_{\text{norm}} = \frac{I_{\text{sus}}}{I_{\text{sus,max}}} = \frac{I_{\text{min}} + I_{\text{max}}}{1 + ([\text{BmKM1}]/\text{EC50})^p} + I_{\text{max}}$$

The concentration of half maximal current increase was $\text{EC50} = 326.8 \pm 34.5$ nM and slope factor $p = 2.7 \pm 0.7$. The concentration of BmK M1 is $[\text{BmKM1}]$. I_{min} is the normalized minimal sustained current and I_{max} is the normalized maximal sustained current increase induced by BmK M1.

3.4. Kinetics of Na^+ currents and effect of BmK M1

A kinetic analysis was performed according to the H–H model (see Section 2) and the effects of BmK M1 on voltage and time dependent parameters were compared. The activation and inactivation kinetics of the TTX-sensitive Na^+ currents of isolated DUM neurons and their response to 500 nM BmK M1 are shown in Fig. 5. The voltage dependence of the steady state activation, m_{∞} , was taken as the cubic root of the normalized peak conductance, assuming that the inactivation process did not start yet. The activation curves in control conditions and in the presence of the toxin were fitted by a Boltzmann equation (eq. (5); Fig. 5(A)). In six cells the potential of half maximal activation, V_m , shifted by 5.5 mV from -18.5 ± 1.2 mV to -24.0 ± 0.6 mV in negative direction upon application of BmK M1. The slope factor, K_m , was 7.1 ± 0.5 mV (versus 6.5 ± 1.0 mV in control condition). The shift of the activation curve induced by BmK M1 was small but significant ($p = 0.0046$ for difference between V_m , Student's t -test). The effect of BmK M1 on the steady state inactivation, h_{∞} , was much more important however (Fig. 5(B)). A Boltzmann fit of the inactivation data sets in control conditions (cf. eq. (6), see Section 2) revealed a potential of half maximal inactivation, V_h , of -42.1 ± 0.2 mV, and $K_h = 7.4 \pm 0.2$ mV. The h_{min} was not zero (0.04) due to incomplete inactivation of the Na^+ current. BmK M1 induced a significant shift of V_h to -31.3 ± 0.4 mV ($p < 0.0001$,

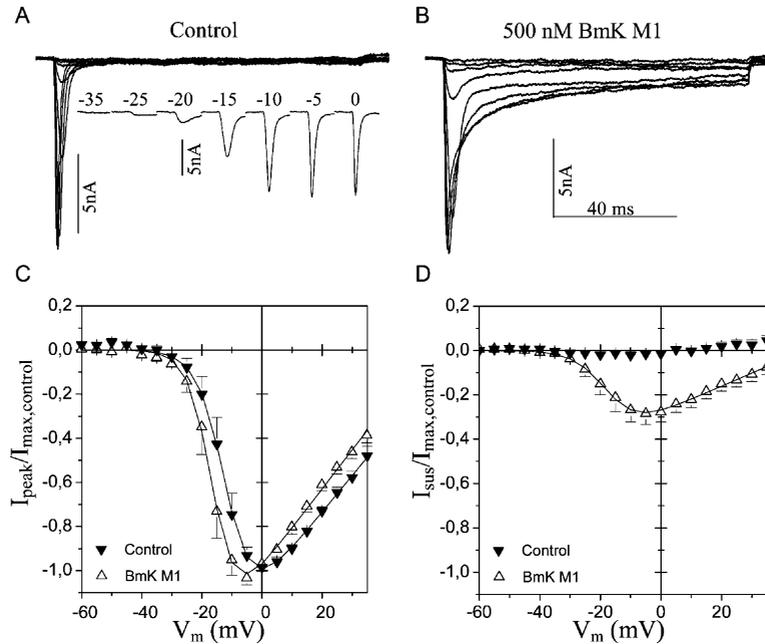


Fig. 3. The effect of 500 nM BmK M1 on TTX-sensitive Na^+ currents of isolated DUM neurons (ES Na and PS Na). (A) Control experiment: DUM neurons were depolarized from a holding potential of -90 mV to different test potentials (-35 to 0 mV in 5 mV steps) for 100 ms. The progressive activation of the individual current traces is shown in the inset, with the corresponding potential. (B) Effect of BmK M1: inactivation was reduced for all test potentials in the presence of the toxin. (C) The current–voltage relationship was plotted for normalized peak currents in control conditions and in the presence of 500 nM BmK M1. (D) The voltage dependence of the normalized sustained current (mean of last 20 ms of pulse) in the same conditions ($n = 6$). All I–V plots were fitted by $I = g\{1 - 1/[1 + \exp((V_m - V_{0.5})/K)]\}(V_m - V_{rev})$. The $V_{0.5}$ for the peak currents (in (C)) was -11.8 ± 0.2 mV in control and -16.2 ± 0.2 mV after BmK M1 application. In (D) only data in the presence of BmK M1 could be fitted adequately and $V_{0.5} = -17.1 \pm 0.3$ mV.

Student's t -test), K_h was 9.3 ± 0.3 mV and h_{min} increased considerably to 0.14 .

Fitting of the TTX-sensitive Na^+ current of DUM neurons according to the H–H formalism provided time constants of activation τ_m and inactivation τ_h (Fig. 5(C) and (D)). BmK M1 did not affect τ_m at any voltage while in the voltage range from -10 to 0 mV a significant increase of τ_h was induced by binding of BmK M1 ($p = 0.03$, Wilcoxon test).

The scorpion toxin caused a faster recovery from inactivation when DUM neurons were depolarized for a second time to 0 mV from a holding potential of -90 mV (Fig. 5(E)). Na^+ current recovery could be described as a single exponential function with a delay (the time constant of recovery is τ_r). The value obtained for τ_r was 1.16 ± 0.07 ms in control and 0.81 ± 0.14 ms 2 min after BmK M1 application. The time constants of recovery (τ_r) were similar in both conditions (Student's t -test). However, for short interpulse times (t_{IP}) a significant increase in the normalized, recovered current was seen ($p \leq 0.05$ if $t_{IP} \leq 3$, Wilcoxon test).

The voltage dependence of the time to peak of the Na^+ currents was not changed by application of 500 nM BmK M1 (Fig. 5(F)). The time to peak of 1.7 ms is relatively large and might indicate insufficient control of the voltage clamp. However, these relatively large time

to peak values seem to be common in insect neurons as noted by Wicher (2001).

4. Discussion

4.1. Action potentials in locust DUM neurons

The method developed to isolate efferent DUM neurons of *L. migratoria* was proven to be adequate. The neurons maintained their electrophysiological properties at least 36 h in vitro. The DUM neuron somata were able to generate a train of action potentials in vitro. The action potentials showed a 30 mV overshoot and a typical afterhyperpolarization, similar to the shape they have in situ (Hoyle, 1974; Goodman and Heitler, 1979; Burrows, 1996). Action potentials could be completely abolished by TTX suggesting that Na^+ currents through voltage gated channels were the predominant charge carrier for the overshooting spikes. In embryonic and adult DUM neurons of another locust *Schistocerca nitens* both Na^+ and Ca^{2+} currents were necessary to generate action potentials (Goodman and Heitler, 1979; Goodman and Spitzer, 1981). In other species it was shown that Ca^{2+} was less important: the Ca^{2+} current inhibitor ω -aga I of the funnel web spider could not block the action poten-

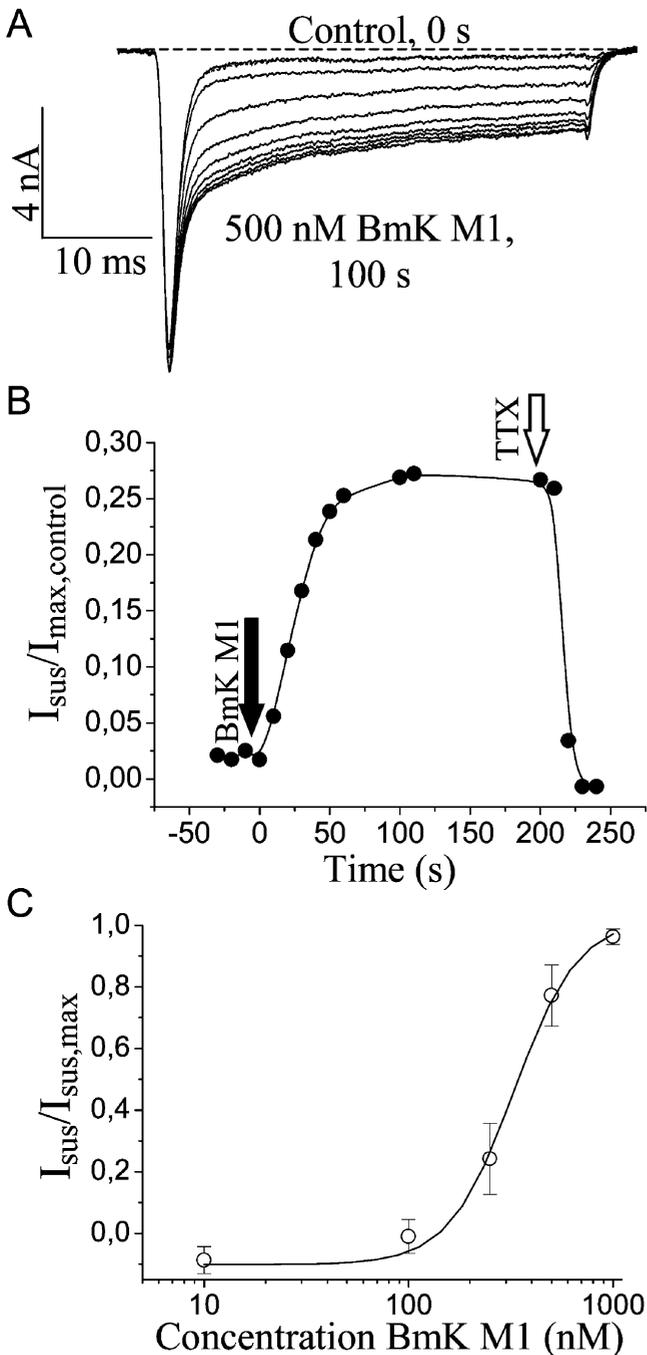


Fig. 4. Time and dose dependence of inhibition of Na⁺ current inactivation by BmK M1. (A) Whole cell voltage clamp experiments on isolated DUM neurons. Cells were depolarized from -90 to 0 mV for 40 ms. The upper trace was recorded in the absence of BmK M1. After application of 500 nM BmK M1 the pulse was repeated every 10 s during 2 min. (B) Time dependence: the data are the amplitude of the maintained current during the last 10 ms of the test pulse, normalized to the peak current in control conditions. The effect reached a plateau 100 s after BmK M1 application (black arrow). One hundred nanomolar TTX (white arrow) blocked the Na⁺ current completely. (C) Concentration dependence: means of normalized sustained currents were fitted by a dose-response curve $I_{\text{sus}}/I_{\text{sus,max}} = \{(I_{\text{min}} - I_{\text{max}})/[1 + ([\text{BmK M1}]/\text{EC50})^p]\} + I_{\text{max}}$, where EC50 = 326.8 ± 34.5 nM and the slope factor $p = 2.7 \pm 0.68$ ($n = 8$). All recordings are made in ES Na and PS Na.

tials in DUM neuron somata of adult *Schistocerca americana* (Bindokas and Adams, 1989). Furthermore Ca²⁺ channel blockers such as Co²⁺ and Mn²⁺ had no effect on action potentials in cockroach DUM neuron cell bodies (Lapied et al., 1989). Although the spikes in isolated DUM neurons of *L. migratoria* in this study show a clear Na⁺ dependence, we cannot rule out the possibility that the action potentials in *Locusta* DUM neurons switched from a mixed Na⁺-Ca²⁺ dependent spike in vivo to a solely Na⁺ dependent spike in vitro due to the isolation. This phenomenon has previously been described in other insect neurons (Grolleau and Lapied, 2000).

4.2. Na⁺ currents of locust DUM neurons and other species

The electrophysiological properties of voltage gated Na⁺ currents, responsible for the fast rising phase of the action potential in locust DUM neurons, were comparable to those in other insect species. Voltage dependence of steady state activation does not differ substantially in the different insect species (flies, cockroaches and bees) and this was confirmed for TTX-sensitive Na⁺ currents in locust DUM neurons in this study (Schäfer et al., 1994; Warmke et al., 1997; Wicher et al., 2001). The steady state inactivation seems to be less uniform in insects. Cockroach abdominal DUM neurons exhibit a slow or non-inactivating TTX-sensitive component in addition to the fast inactivating component of the TTX-sensitive Na⁺ current (Wicher and Penzlin, 1998). A similar TTX-sensitive, maintained current was present in *Drosophila* neurons and bee Kenyon cells (O'Dowd and Aldrich, 1988; Schäfer et al., 1994). When locust DUM neurons showed any maintained current at all, it was negligibly small.

4.3. Effect of BmK M1 on locust DUM neuron

4.3.1. Current clamp analysis

Five hundred nanomolar of the α-like scorpion toxin BmK M1 increased the duration of the first action potential in a train of spikes up to 50 times in locust DUM neurons. The prolongation of the action potential duration is similar but starts earlier in locusts compared to the BmK M1 effect in crayfish axon (Ji et al., 1996) and to other α and α-like toxin effects in different preparations (e.g. LqhIII in frog myelinated axon, Benoit and Gordon, 2001; LqhIII, Bom III and Bom IV in giant cockroach axon, Gordon et al., 1996; Cestele et al., 1999; Krimm et al., 1999). However, the duration of the subsequent action potentials decreased progressively, maybe due to the voltage dependence of the binding of α-toxins to Na⁺ channels. Indeed, some α-toxins, e.g. AahII and LqhII and α-like, e.g. LqhIII have been shown to dissociate from the receptor site 3 in rat brain Na⁺ channels in depolarizing conditions (Ray and Catterall,

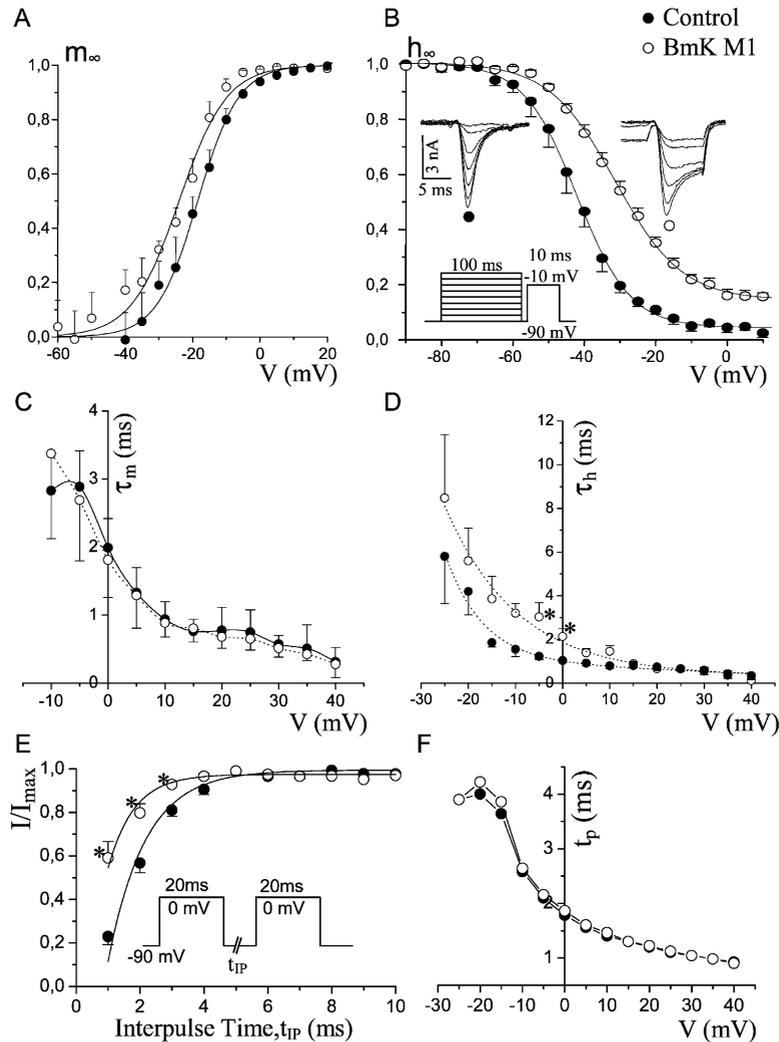


Fig. 5. Kinetics of the TTX-sensitive Na^+ currents in control (●) and in the presence of 500 nM BmK M1 (○). (A) Steady state activation, m_∞ : data were calculated from the cubic root of the normalized peak conductance ($n = 6$). The data could be fitted by the Boltzmann equation $m_\infty = 1 / \{1 + \exp[(V_m - V)/K_m]\}$, where V_m was -18.5 ± 1.2 mV and K_m was 6.5 ± 1.0 mV in control conditions. Five hundred nanomolar BmK M1 shifts V_m to -24.0 ± 0.6 mV and K_m to 7.1 ± 0.5 mV. (B) Steady state inactivation, h_∞ : values are means of normalized Na^+ currents evoked by the second pulse of the protocol (shown in the lower inset) and plotted against the potential of the prepulse (100 ms duration at various potentials). Examples of the currents in the last 20 ms of the protocol are shown in control conditions (●, the prepulse potentials were -70 , -55 , -50 , -45 , -40 , -30 and -5 mV) and in the presence of BmK M1 (○, the prepulse potentials were -70 , -50 , -40 , -30 , -15 and -5 mV). Means of seven cells were fitted by the Boltzmann function $h_\infty = \{(h_{\max} - h_{\min}) / \{1 + \exp[(V - V_h)/K_h]\} - h_{\min})$ and the values obtained were $V_h = -42.1 \pm 0.2$ mV, $K_h = 7.4 \pm 0.2$ mV and $h_{\min} = 0.04$ in control conditions and $V_h = -31.3 \pm 0.4$ mV, $K_h = 9.3 \pm 0.3$ mV and $h_{\min} = 0.14$ after BmK M1 application. BmK M1 induced a significant shift in V_h as well as in V_m (Student's t -test). (C) and (D) Time dependence: TTX-sensitive Na^+ currents could be fitted by the Hodgkin-Huxley model providing the time constants (see Section 2). (C) The time constant of activation, τ_m , did not change significantly in the presence of BmK M1 ($n = 4$) while this toxin caused a significant increase (indicated by *) of the time constant of inactivation τ_h at -5 and 0 mV as shown in (D) ($n = 6$, in both cases Wilcoxon test used). (E) The time course of recovery from inactivation of the TTX-sensitive Na^+ current was studied by means of a double pulse protocol with increasing interpulse time, t_{IP} (see inset, $n = 6$). Peak currents were normalized to the corresponding prepulse peak current. Smooth lines are single exponential functions with delayed onset fitted through the data. The parameters changed from $\tau = 1.16$ ms and delay = 0.63 ms in control to $\tau = 0.81$ ms and delay = 0.33 ms after application of BmK M1 (at least 2 min). Recovery was significantly different for small interpulse times (Wilcoxon test). (F) The time to peak as a function of the test potential for a representative cell.

1978; Jover et al., 1980; Cestele and Gordon, 1998; Gilles et al., 2000; Gilles et al., 2001). On the other hand, binding of α -toxins to locust Na^+ channels did not seem to be voltage dependent (Gordon and Zlotkin, 1993). Until now, the voltage dependence of the binding of BmK M1 has not been tested on insect Na^+ channels (Li

and Ji, 2000). Another possible cause of the decreasing duration of the action potentials in a train is the activation of Ca^{2+} - or Na^+ -activated K^+ currents, assuming that these currents are present in locust DUM neurons (for cockroach DUM neurons, see Wicher et al., 1994; Grolleau and Lapiéd, 1994). The influx of Ca^{2+} or Na^+

may have increased during the long depolarization of the previous action potential. In cockroach DUM neurons a 500 ms depolarization (comparable to the duration of the first action potential in the presence of BmK M1) increased the Ca^{2+} concentration via Ca^{2+} -induced Ca^{2+} release for several seconds (Messutat et al., 2001). This is long enough to activate, e.g. a maintained Ca^{2+} -activated K^+ current during the next action potentials (Grolleau and Lapied, 1995).

4.3.2. Voltage clamp analysis

The effect of the α -like scorpion toxin BmK M1 on the voltage and time dependent properties of the TTX-sensitive Na^+ currents has been studied in voltage clamp experiments. The effect of BmK M1 on locust DUM neurons was similar to the inhibition of inactivation it caused in Na^+ currents in crayfish axon. This study was much less detailed: only a single depolarizing voltage step in the presence of 300 nM BmK M1 was used (Ji et al., 1996).

BmK M1 did not have a significant effect on the voltage dependence of activation of the human cardiac Na^+ channels ($\text{Na}_v1.5$) expressed in *Xenopus* oocytes (Goudet et al., 2001). In locust DUM neurons BmK M1 shifted the activation curve (m_∞) by 5.5 mV to more negative potentials while the peak current amplitude did not change considerably. This is comparable to the effect of the α -like toxins BomIV on giant cockroach axon and LqhIII on expressed rat brain (rBII) Na^+ channels (Cestele et al., 1999; Gilles et al., 2000). This change in voltage dependence of activation reflected the toxin induced shift in the current–voltage relationship.

As expected for an α -toxin, the effect of BmK M1 on the voltage dependence of the steady state inactivation (h_∞) is more pronounced, i.e. shift of 10.8 mV in positive direction. In addition, the inactivation is incomplete in the voltage range more positive than 0 mV. In human $\text{Na}_v1.5$ channels 250 nM BmK M1 shifted the inactivation curve 3.6 mV to more negative potentials, i.e. in the hyperpolarizing direction (Goudet et al., 2001). Other α -toxins induced a shift of the voltage dependence of inactivation of Na^+ channels in depolarizing direction, e.g. the insect specific α -toxin Lqh α IT in adult *Heliothis virescens* neurons and in *Musca domestica* central neurons (Lee et al., 2000; Lee and Adams, 2000).

It is clear that the effect of BmK M1 on inactivation of the Na^+ current is the most important one. This idea is supported by changes in time dependence of the locust Na^+ current, induced by BmK M1. The fast time constant of inactivation (τ_h) is increased significantly while the activation time constant (τ_m) remains unchanged. The incomplete inactivation induced by BmK M1 probably caused the faster recovery from inactivation, suggesting that some Na^+ channels are only deactivated but not inactivated in between the two test pulses.

4.3.3. Selectivity of BmK M1

The α -like scorpion toxins do not have an insect or mammalian specific action. Indeed, the dose dependent slowing down of inactivation did not differ greatly in the locust Na^+ currents and in the human cardiac Na^+ channels expressed in *Xenopus* oocytes (Goudet et al., 2001). The concentration of half maximal effect EC50 is 326 nM BmK M1 in the locust DUM neurons and 195 nM human cardiac Na^+ current.

4.4. Conclusions

The many different ion currents, responsible for the specific shape of action potentials in isolated abdominal DUM neurons of the cockroach, were described in detail and reviewed by Grolleau and Lapied (2000). Since the action potentials of locust DUM neurons have a similar shape to the spikes in cockroach DUM neurons, we expect to find a similar composition and comparable properties of ion currents in the locust. Therefore, the isolated locust DUM neurons in combination with the patch clamp technique are very suitable as a model for screening or testing effects of substances on insect neurons. Together with the extensive in situ studies (Burrows, 1996) research on isolated locust DUM neurons may help in controlling the swarms of migratory locusts, that are causing serious damage to crops in Africa.

In the present study we started the research on isolated locust DUM neurons and investigated the effect of BmK M1. BmK M1 induced a shift in the voltage dependence of inactivation and (but to lesser extent) of activation in opposite directions, making the activation window larger and causing an increased electrical activity. This is a typical effect for an α -toxin in excitable cells (cf. BotIT₂, an α -toxin from another *Buthus* scorpion, on isolated cockroach axon and DUM neuron, Stankiewicz et al., 1996; AaHII and Lqh α IT are prototypes of mammalian or insect selective α -toxins, respectively, Possani et al., 1999; Eitan et al., 1990).

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