Electrophysiological characterization of *Bm*K M1, an α-like toxin from *Buthus martensi* Karsch venom

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Abstract The present study investigates the electrophysiological actions of *Bm*K M1, an α-like toxin purified from the venom of the scorpion *Buthus martensi* Karsch, on voltage-gated Na^+ channels. Using the voltage clamp technique, we assessed the *Bm*K M1 activity on the cardiac Na^+ channel (hH1) functionally expressed in *Xenopus* oocytes. The main actions of the toxin are a concentration-dependent slowing of the inactivation process and a hyperpolarizing shift of the steady-state inactivation. This work is the first electrophysiological characterization of *Bm*K M1 on a cloned Na^+ channel, demonstrating that this toxin belongs to the class of scorpion α-toxins. Our results also show that *Bm*K M1 can be considered as a cardiotoxin.

Key words: Voltage-gated sodium channel; Inactivation; Scorpion; α-Toxin; *Buthus*; *Buthidae*

1. Introduction

Voltage-gated sodium channels (VGSCs) are key players in the generation of action potentials in excitable tissues such as nerve, skeletal and cardiac muscles. These channels are heterotrimeric structures composed of a central, pore-forming α subunit and two auxiliary β subunits. The α subunit alone is sufficient to form a functional channel whereas the β subunits modulate the channel gating properties, level of expression and interaction with cytoskeleton proteins [1]. Sodium channels have been cloned in various species and tissues. They can be differentiated by their primary structure, kinetics and relative sensitivity to the neurotoxin tetrodotoxin (TTX). The cardiac isoform hH1 [2], $Na_V1.5$ according to the new nomenclature of VGSCs [3], is a TTX-resistant channel expressed in human cardiomyocytes where it plays a major role in the generation and propagation of the cardiac potential [4].

As key elements of signal transmission, sodium channels are the target of neurotoxins of diverse origins and chemical structures. These toxins have effects that range from pore blocker (e.g. TTX, μ-conotoxin) to modification of the gating and permeation (e.g. scorpion and sea anemone toxins, batrachotoxin) [5]. Toxins have proven to be invaluable tools for probing the structures of different ion channels and evaluating their physiological contribution to cell and organ behavior. Moreover, elucidation of the mechanisms of action of toxins, knowledge of their three-dimensional structures and the discovery of common scaffolds between toxins open wide perspectives in designing various drugs [6].

Scorpion venoms are rich sources for small, mainly neurotoxic proteins interacting specifically with various ionic channels in excitable membranes. Scorpion toxins acting on sodium channels have been divided in two major groups, α- and β-toxins, according to their pharmacological effects on Na^+ currents in electrophysiological preparations and their binding properties. The α-toxins are polypeptides of 62–67 amino acid residues long. Their major effect is to slow or inhibit the Na^+ current inactivation and thus induce prolongation of action potentials. They can be divided in three categories: (i) the classical α-toxins which are highly specific to mammals (e.g. *Aa*H II from *Androctonus australis* Hector [7]), (ii) the insect α-toxins, highly active on insects (e.g. *Lqq* III from *Leiurus quinquestriatus quinquestriatus* [8]) and (iii) the α-like toxins active on both mammals and insects (*Lqh* III from *Leiurus quinquestriatus hebraeus* [9]) (Fig. 1; see [10–12] for reviews). The receptor site 3 of Na^+ channels, located in the extracellular linker between segments S3 and S4, in the fourth domain, has been identified by binding studies as the specific binding site of α- and α-like toxins (see [13] for a review).

The Asian scorpion *Buthus martensi* Karsch (*Bm*K) is widely distributed in China where it has been one of the indispensable materials used in Chinese traditional medicine for thousands of years. Its venom has been extensively studied and several α-toxins have been isolated from it (see [12] for review). However, in most cases, electrophysiological characterizations are still lacking. In the present study, we focused on the α-like toxin *Bm*K M1 (also called *Bm*K I) [14,15], which is one of the most abundant long-chain toxins in *Bm*K venom [16]. It is also probably the most known: its site of interaction with Na^+ channels [17] and its structure [18] have been determined by binding and crystallographic studies while bioassay experiments defined its toxicity [14,17,19]. Ji et al. [14] also illustrated the effect of *Bm*K M1 on nerve excitability in the crayfish axon model. Intracellular recording showed that this neurotoxin prolonged the action potential and that, under voltage clamp, *Bm*K M1 also

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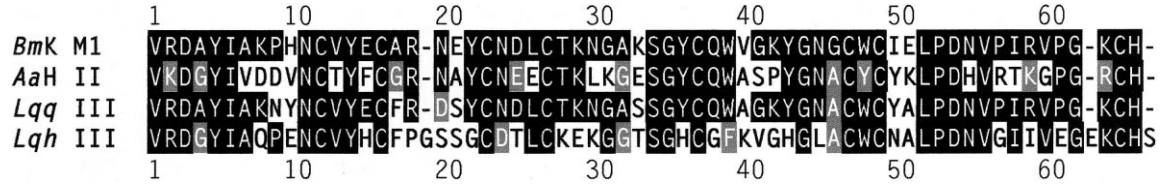

Fig. 1. Comparison of the amino acid sequence of *Bm*K M1 with other scorpion α-toxins. *Bm*K M1 has been purified from *B. martensi* Karsch [14], *Aa*H II from *A. australis* Hector [7], *Lqq* III from *L. quinquestriatus quinquestriatus* [8] and *Lqh* III from *L. quinquestriatus hebraeus* [9]. Identical amino acids are indicated with a black background, homologous amino acids are indicated with a gray background. Dashes represent gaps.

greatly prolonged the inward current. However no in-depth electrophysiological characterization was available until now. Moreover, the question whether *Bm*K M1 could affect cardiac Na^+ channels directly, and hence be considered as a cardiotoxin as well, was not answered. Consequently, in the present study, using the two-electrode voltage clamp technique on *Xenopus laevis* oocytes, we performed a more complete electrophysiological characterization of *Bm*K M1 on the cloned hH1 cardiac Na^+ channel isoform.

2. Materials and methods

2.1. Toxin

A fraction of the crude venom enriched in long-chain toxins of the scorpion *B. martensi* Karsch has been kindly provided by Dr. Da-Cheng Wang. Briefly, *Bm*K M1 was isolated and purified as follows. The material was applied on a monomeric 238TP54 C_{18} reversed phase high performance liquid chromatography (HPLC) column (Vydac, USA), equilibrated with 0.1% trifluoroacetic acid (TFA, Merck, Eurolab, Belgium) in distilled water. After 4 min, an immediate step to 5% acetonitrile (with 0.1% TFA) was applied, followed by a linear gradient to 30% acetonitrile for 5 min and a linear gradient to 60% for the last 15 min. The flow rate was 0.75 ml/min and the absorbance was simultaneously measured at 214, 254 and 280 nm. The fraction containing the toxin was recovered and applied to a polymeric 208TP5415 C_8 reversed phase HPLC column and the same separation procedure was followed. The material contained in the resulting peak was collected and dried (Speed Vac® Plus, Savant, USA). Verification of the identity of the final toxin material was carried out by a N-terminal sequencing of the first 22 amino acids and by mass determination using the matrix-assisted laser desorption ionization-time of flight technique (measured mass: 7227 Da; theoretical mass: 7226 Da). For the electrophysiological experiments, *Bm*K M1 was dissolved in ND96 (see Section 2.3) and stored at 4°C.

2.2. Expression in oocytes

The hH1 gene was subcloned into pSP64T [2]. For in vitro transcription, hH1/pSP64T was first linearized by *Xba*I. Then, capped cRNAs were synthesized from the linearized plasmids using the large-scale SP6 mMESSAGE-mMACHINE transcription kit (Ambion, USA). The in vitro synthesis of cRNA encoding hH1 and isolation of *Xenopus* oocytes were done as previously described [20]. Oocytes were injected with hH1 cRNA at a concentration of 1 ng/nl.

2.3. Electrophysiological recordings and analysis

Whole-cell currents from oocytes were recorded using the two-microelectrode voltage clamp technique between 1 and 3 days after injection. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept as low as possible (ca. 0.1–0.2 MΩ). Experiments were performed using a GeneClamp 500 amplifier (Axon instruments, USA) controlled by a pClamp data acquisition system (Axon instruments, USA). Using a four-pole low-pass Bessel filter, currents were filtered at 5 kHz. Digital leak subtraction of the current records was carried out using a P/4 protocol. The bath solution (ND96) composition was (in mmol/l): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4). This solution was supplemented with 50 mg/l gentamicin sulfate for incubation of the oocytes. All experiments were performed at room temperature (20–22°C).

3. Results and discussion

The amino acid sequence of *Bm*K M1, purified as detailed in Section 2, is shown in Fig. 1. An alignment was made with other scorpion α-toxins (*Aa*H II from *A. australis* Hector [7], *Lqq* III from *L. quinquestriatus quinquestriatus* [8] and *Lqh* III from *L. quinquestriatus hebraeus* [9]), using the program Clustal 1.8 (http://searchlauncher.bcm.tmc.edu:9331/multi-align/multi-align.html).

Fig. 2A displays the effect of 250 nM *Bm*K M1 on hH1 Na^+ channels. Currents were evoked by a step depolarization

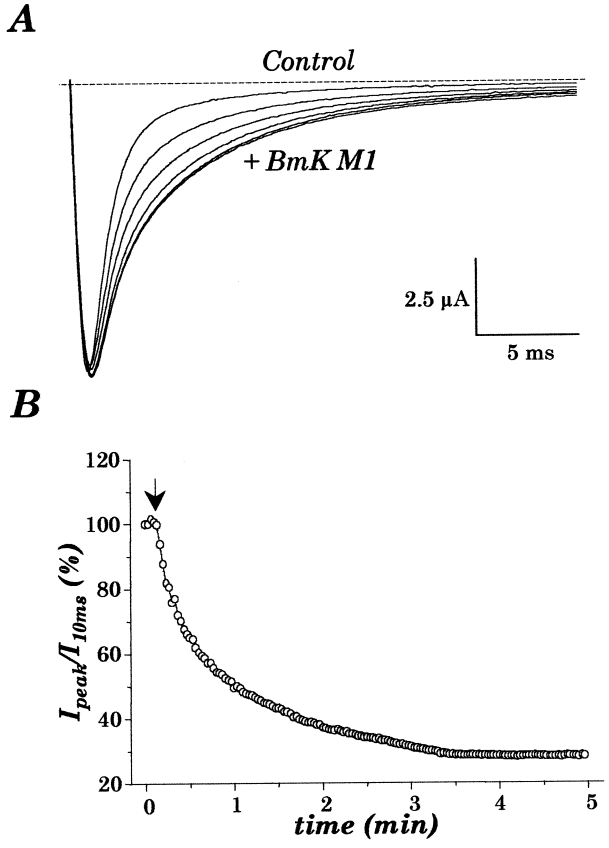

Fig. 2. Time dependence of *Bm*K M1 effect on the hH1 Na^+ channels. A: Time dependence of the slowing of inactivation induced by *Bm*K M1 on the hH1 Na^+ channel. Current traces were evoked by depolarizations to −20 mV during 25 ms from a holding potential of −90 mV, before and 1, 2, 3, 4 and 5 min after the addition of 250 nM *Bm*K M1 to the bath containing ND96 solution. B: Ratio of peak current (I_{peak}) relative to the current measured 10 ms after depolarization ($I_{10\,ms}$) plotted vs. time in control and after addition of *Bm*K M1 (from the same experiment as in A).

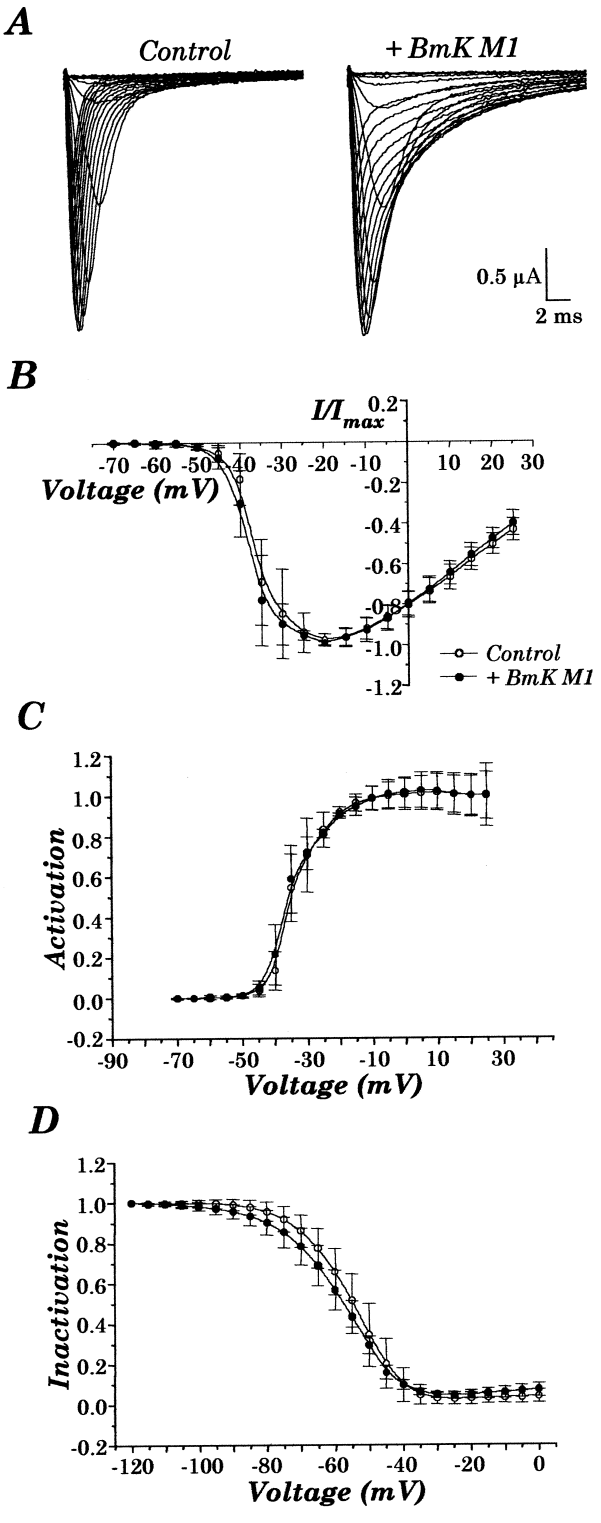

Fig. 3. Electrophysiological properties of hH1 Na^+ channel modified by *Bm*K M1. A: Representative currents evoked by depolarization ranging between −70 and +25 mV by 5 mV steps from a holding potential of −90 mV, in the absence (control, left traces) and in the presence of 250 nM *Bm*K M1 (+*Bm*K M1, right traces) in the same oocyte. B: Averaged and normalized *I–V* relationship of hH1 in the absence (○) and in the presence of 250 nM *Bm*K M1 (●). C: Averaged and normalized steady-state activation of hH1 in the absence (○) or in the presence of 250 nM *Bm*K M1 (●). D: Averaged and normalized steady-state inactivation of hH1 in the absence (○) or in the presence of 250 nM *Bm*K M1 (●). Data represent the mean ± S.E.M. of $n = 11$ and $n = 6$ experiments, respectively.

to −20 mV during 25 ms from a holding potential of −90 mV. Current traces recorded after the addition of the toxin reveal that *Bm*K M1 induces a slowing of the inactivation process of Na^+ currents. An estimate of the toxin-induced effect can be obtained from the degree of inactivation of the current 10 ms after the beginning of the step depolarization to −20 mV. Indeed, in control conditions, inactivation kinetics of hH1 channel Na^+ currents are rapid and almost no remaining currents are seen 10 ms after the beginning of the test pulse ($I_{10\,ms}$). In contrast, upon addition of *Bm*K M1, $I_{10\,ms}$ increases. The toxin-induced slowing of inactivation was thus evaluated by measuring the ratio of the peak current (I_{peak}) to $I_{10\,ms}$. Fractional inactivation as a function of time is shown in Fig. 2B. As can be seen, the increase of $I_{10\,ms}$ appears a few seconds after addition of the toxin and continues until reaching a steady-state after 4–5 min. Note that, at the same time, the peak amplitude of the Na^+ current and the time to reach the peak remain unchanged.

Fig. 3A displays families of fast inactivating Na^+ current generated by hH1 channel expressed in oocytes in control conditions and in the presence of 250 nM *Bm*K M1. Currents were evoked by step depolarizations of 25 ms ranging from −70 to +25 mV, with increments of 5 mV, from a holding potential of −90 mV. While Na^+ currents inactivate rapidly in control conditions, their inactivation kinetics are strongly slowed down at all tested voltages in the presence of *Bm*K M1. This is a hallmark of scorpion α-toxins [12].

The averaged and normalized current–voltage (*I–V*) relationship of hH1 in the absence or in the presence of 250 nM of *Bm*K M1 is displayed in Fig. 3B. In the presence of toxin, the *I–V* curve is slightly shifted to more hyperpolarized potentials but the voltage threshold of activation remains the same, approximately −50 mV. *I–V* curves were fitted according to the Goldman–Hodgkin–Katz equation. The voltage for half-maximal activation ($V_{1/2}$) and the slope factor (k) for the voltage dependence of activation determined by the fit are -41.4 ± 0.6 mV and 6.2 ± 0.6 mV, in control condition, and -42.8 ± 0.6 mV and 6.1 ± 0.6 mV in the presence of 250 nM *Bm*K M1, respectively.

Voltage dependence of steady-state activation is shown in Fig. 3C. In control conditions, the voltage for half-maximal activation ($V_{1/2}$) and the slope factor as measured by a sigmoidal fit of the activation curve are -34.2 ± 0.5 mV and 4.3 ± 0.4 mV ($n = 11$), respectively. In the presence of 250 nM *Bm*K M1, $V_{1/2}$ is -35 ± 0.6 mV and the slope factor is 5.1 ± 0.5 mV ($n = 6$). In contrast to *Bm*K M1, some α-toxins, like *Lqh* II and III [21], are able to shift the activation of Na^+ channels to more negative potentials. However, this property is still mainly observed with scorpion β-toxins [12].

Fig. 3D displays the voltage dependence of steady-state sodium channel inactivation in the absence and presence of 250 nM *Bm*K M1. Measurements of steady-state inactivation were made following a standard two-pulse protocol. A 50 ms conditioning pulse ranging from −120 mV to 30 mV was followed by a 50 ms test pulse at −20 mV. In control conditions, a prepulse of −120 mV forces all the channels in the resting state making them fully available for activation during the test pulse. In contrast to this, a prepulse of −30 mV moves all the channels in the inactivated state rendering them non-available for activation during the test pulse. In control conditions, the voltage at which half of the channels are inactivated (V_h) and the slope factor determined by a

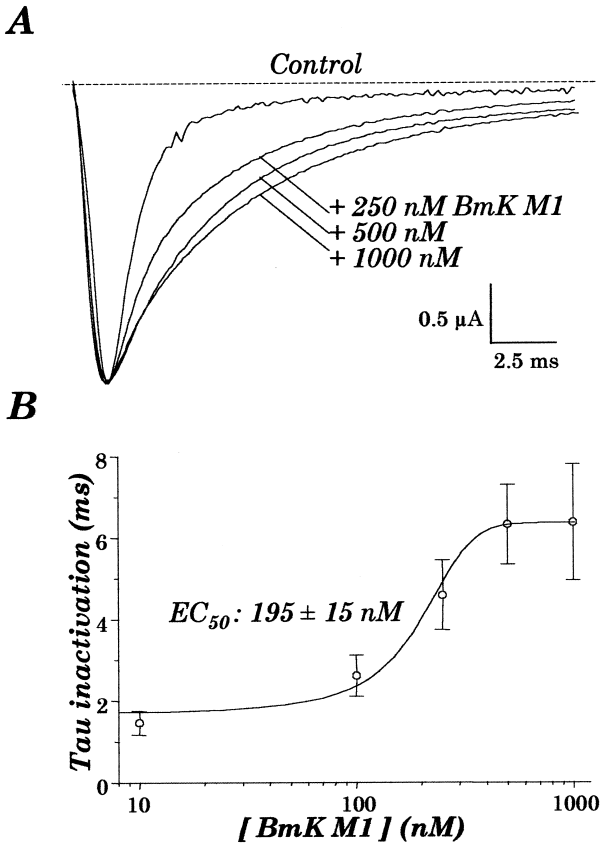

Fig. 4. Concentration dependence of *Bm*K M1 effect on hH1 Na^+ channels. A: Concentration dependence of the slowing of inactivation induced by *Bm*K M1 on hH1 Na^+ channel. Current traces were evoked by a depolarization to −20 mV during 20 ms from a holding potential of −90 mV, in the absence (control) and in the presence of increasing concentrations of *Bm*K M1 (as indicated), in the same oocyte. B: Averaged time constant of inactivation plotted vs. concentration of *Bm*K M1. The EC_{50} value determined by a sigmoidal fit is 195 ± 15 nM. Time constants of inactivation were calculated from a single exponential fit of current traces. Data represent the mean ± S.E.M. of at least four experiments at each concentration.

sigmoidal fit of the inactivation curve are −55.1 ± 0.4 mV and 7.6 ± 0.4 mV ($n = 11$), respectively. In the presence of 250 nM *Bm*K M1, V_h and the slope factor are −58.7 ± 0.4 mV and 8 ± 0.4 mV ($n = 6$), respectively. In the presence of 1 μM *Bm*K M1, V_h and the slope factor are −60.7 ± 0.3 mV and 7.6 ± 0.3 mV ($n = 4$), respectively. Thus, 250 nM and 1 μM *Bm*K M1 cause significant shifts of −3.6 mV and −5.6 mV in the voltage at which half the hH1 Na^+ channels are inactivated (Student's *t*-test: $P < 0.05$). Some other α-toxins are also able to shift the steady-state inactivation of Na^+ channels and this is the case, for example, for *Lqh*αIT from the venom of *L. quinquestriatus quinquestriatus* [21,22].

The slowing of inactivation induced by *Bm*K M1 is clearly concentration-dependent. The effect of different concentrations of *Bm*K M1 on hH1 Na^+ channels is shown in Fig. 4. Currents were evoked by a step depolarization to −20 mV during 25 ms from a holding potential of −90 mV. The inactivation time of hH1 currents increases with the concentration of toxin (Fig. 4A). The time constant of inactivation (τ) calculated by a single exponential fit increases from 1.5 ± 0.3 ms ($n = 11$) in control conditions to 4.6 ± 0.8 ms ($n = 6$) after the addition of 250 nM *Bm*K M1 and then to 6.4 ± 1.4 ms ($n = 4$) after addition of 1 μM *Bm*K M1. This represents an increase for the time constant of inactivation of approximately 300% and 450% in the presence of 250 nM and 1 μM *Bm*K M1, respectively. The EC_{50} value determined by a sigmoidal fit of the τ–V relationship is 195 ± 15 nM. In comparison, *Bm*K M1 is less potent than the α-like toxin *Lqh* III from the scorpion *L. quinquestriatus hebraeus* which has an EC_{50} of 5 nM on the cloned skeletal muscle Na^+ channel expressed in mammalian cells [21]. This difference may be explained based on the selectivity of each of the toxins, but may also reflect the pharmacological characteristics of the heterologous expression system used (i.e. mammalian cells vs. *Xenopus* oocytes).

In summary, we show here that *Bm*K M1 is able to modify the electrophysiological properties of the hH1 Na^+ channel. The main action is a marked slowing of the inactivation process of Na^+ currents, therefore confirming the classification of *Bm*K M1 into the α-toxin class. *Bm*K M1 also induces a significant hyperpolarizing shift of the steady-state inactivation of hH1. From a biological point of view, the slowing of inactivation of the hH1 Na^+ channel induced by *Bm*K M1 could result in vivo in a modification of the cardiac rhythm. Evidence for such a direct effect (among various other effects including pain at the site of the sting, vomiting, sweating, salivation and hypertension that can be seen after a scorpion sting) has also very recently been published for *Tityus serrulatus* venom [23]. We therefore conclude that *Bm*K M1 can be considered both as a cardiotoxin and a neurotoxin.

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References

[1] Catterall, W.A. (2000) Neuron 26, 13–25.
[2] Gellens, M.E., George Jr., A.L., Chen, L.Q., Chahine, M., Horn, R., Barchi, R.L. and Kallen, R.G. (1992) Proc. Natl. Acad. Sci. USA 89, 554–558.
[3] Goldin, A.L., Barchi, R.L., Caldwell, J.H., Hofmann, F., Howe, J.R., Hunter, J.C., Kallen, R.G., Mandel, G., Meisler, M.H., Netter, Y.B., Noda, M., Tamkun, M.M., Waxman, S.G., Wood, J.N. and Catterall, W.A. (2000) Neuron 28, 365–368.
[4] Balser, J.R. (1999) Cardiovasc. Res. 42, 327–338.
[5] Denac, H., Mevissen, M. and Scholtysik, G. (2000) Naunyn Schmiedebergs Arch. Pharmacol. 362, 453–479.
[6] Ménez, A. (1998) Toxicon 36, 1557–1572.
[7] Rochat, H., Rochat, C., Sampieri, F., Miranda, F. and Lissitzky, S. (1972) Eur. J. Biochem. 28, 381–388.
[8] Kopeyan, C., Mansuelle, P., Martin-Eauclaire, M.F., Rochat, H. and Miranda, F. (1993) Nat. Toxins 1, 308–312.
[9] Sautiere, P., Cestele, S., Kopeyan, C., Martinage, A., Drobecq, H., Doljansky, Y. and Gordon, D. (1998) Toxicon 36, 1141–1154.
[10] Gordon, D., Savarin, P., Gurevitz, M. and Zinn-Justin, S. (1998) J. Toxicol.-Toxin Rev. 17, 131–159.
[11] Pelhate, M., Stankiewicz, M. and Ben Khalifa, R. (1998) C.R. Seances Soc. Biol. Fil. 192, 463–484.
[12] Possani, L.D., Becerril, B., Delepierre, M. and Tytgat, J. (1999) Eur. J. Biochem. 264, 287–300.
[13] Cestele, S. and Catterall, W.A. (2000) Biochimie 82, 883–892.
[14] Ji, Y.H., Mansuelle, P., Terakawa, S., Kopeyan, C., Yanaihara, N., Hsu, K. and Rochat, H. (1996) Toxicon 34, 987–1001.
[15] Xiong, Y.M., Ling, M.H., Wang, D.C. and Chi, C.W. (1997) Toxicon 35, 1025–1031.

[16] Wu, H., Wu, G., Huang, X., He, F. and Jiang, S. (1999) Pure Appl. Chem. 71, 1157–1162.
[17] Li, Y.J. and Ji, Y.H. (2000) J. Pept. Res. 56, 195–200.
[18] He, X.L., Li, H.M., Zeng, Z.H., Liu, X.Q., Wang, M. and Wang, D.C. (1999) J. Mol. Biol. 292, 125–135.
[19] Shao, F., Xiong, Y.M., Zhu, R.H., Ling, M.H., Chi, C.W. and Wang, D.C. (1999) Protein Expr. Purif. 17, 358–365.
[20] Liman, E.R., Tytgat, J. and Hess, P. (1992) Neuron 9, 861–871.
[21] Chen, H., Gordon, D. and Heinemann, S.H. (2000) Pflug. Arch. 439, 423–432.
[22] Lee, D., Gurevitz, M. and Adams, M.E. (2000) J. Neurophysiol. 83, 1181–1187.
[23] Teixeira, A.L., Fontoura, B.F., Freire-Maia, L., Machado, C.R., Camargos, E.R. and Teixeira, M.M. (2001) Toxicon 39, 703–709.