The sea anemone *Bunodosoma granulifera* contains surprisingly efficacious and potent insect-selective toxins

Frank Bosmans[a], Abel Aneiros[b], Jan Tytgat[a,*]

[a] *Laboratory of Toxicology, University of Leuven, E. Van Evenstraat 4, B-3000 Leuven, Belgium*
[b] *Instituto de Oceanologica, Loma y 37 Alturas del Vedado, 10600 La Habana, Cuba*

Received 3 September 2002; revised 22 October 2002; accepted 22 October 2002

First published online 7 November 2002

Edited by Maurice Montal

Abstract **Two sodium channel toxins, *Bg*II and *Bg*III, isolated from the sea anemone *Bunodosoma granulifera*, have been subjected to an elaborate electrophysiological and pharmacological comparison between five different cloned sodium channels expressed in *Xenopus laevis* oocytes in order to determine their efficacy, potency and selectivity. Our results reveal large differences in toxin-induced effect between the different sodium channels. These toxins possess the highest efficacy for the insect sodium channel (para). Our data also show that *Bg*II, generally known as a neurotoxin, is especially potent on the insect sodium channel with an EC_{50} value of 5.5 ± 0.5 nM. Therefore, this toxin can be used as a template for further development of new insecticides. Based on our findings, an evolutionary relationship between crustaceans and insects is also discussed.**

Key words: Sea anemone toxin; Voltage-gated sodium channel; Neurotoxin; Insecticide; Evolution

1. Introduction

Voltage-gated sodium channels (VGSCs) are key elements in the signal transduction process of membranes of neurones and most electrically excitable cells. Therefore, they are the target of toxins of various origins and chemical structures. These toxins are powerful tools to study structure–function relationships and design new drugs and insecticides.

VGSCs are composed of a pore forming α subunit (260 kDa) and one to three auxiliary β subunits. The β_2 subunit (21 kDa) is disulphide linked to the α subunit, while the β_1 (23 kDa) and β_3 (22 kDa) subunits are non-covalently attached. In heterologous systems, expression of the α subunit alone is sufficient to form a voltage-gated sodium channel. The β subunits modulate the level of expression, channel-gating properties and interaction with cytoskeleton proteins [1–4].

It is known that sea anemones produce a class of cardio- and neurotoxins which mainly cause a slowing of the inactivation process of sodium currents and a prolongation of the action potential. These polypeptides bind to receptor site 3, located in the extracellular linker between segments S3 and S4 of domain IV. This site is also targeted by scorpion α-toxins and funnel web spider toxins [5–7]. The sodium channel inactivation process derives mainly from the voltage-dependent coupling of activation driven by the transmembrane movement of the voltage sensing S4 segment [3]. By binding to site 3, toxins prevent the normal gating movements of the S4 segment of domain IV and thus uncouple sodium channel activation from inactivation.

*Bg*II and *Bg*III are two toxins that have been purified and sequenced from the sea anemone *Bunodosoma granulifera*, a common anemone of Cuban sea shores [8]. Their sequence consists of 48 amino acids, each containing six cysteine residues that form three disulphide bridges. The only difference is an aspartic acid at position 16 in *Bg*III which replaces an asparagine in *Bg*II (Fig. 1).

Despite their high similarity, we show here through a detailed study of the inactivation process of five cloned sodium channels that the toxin concentration needed to produce an effect (potency) and the amplitude of this effect (efficacy) of these two toxins on sodium channels are remarkably different. To our knowledge, this is the first study comparing the effect of toxins on different types of sodium channels in a same expression system. Using the two-electrode voltage-clamp technique, our experiments show that *Bg*II especially affects insect sodium channels, thus being a model for the development of new insecticides. Due to the fact that large crustaceans are natural predators of sea anemones and small crustaceans serve as food, this work also points in the direction of an evolutionary relationship between insects on the one hand and crustaceans on the other.

2. Methods

2.1. Expression in Xenopus oocytes

For the expression in *Xenopus* oocytes, the hH1 ($Na_v1.5$) and β_1 genes were subcloned into pSP64T [9]. For in vitro transcription, hH1/pSP64T and SNS ($Na_v1.8$)/pSP64T [13] were first linearised with *Xba*I and β_1/pSP64T with *Eco*RI. Next, capped cRNA's were synthetised from the linearised plasmid using the large-scale SP6 mMESSAGE-mMACHINE transcription kit (Ambion, USA). The para/pGH19-13-5 vector [10], tipE/pGH19 vector [11–12], rBrainII($Na_v1.2$)/pLCT1 vector [14] and rSkMI($Na_v1.4$)/pUI-2 vector [15] were linearised with *Not*I and transcribed with the T7 mMESSAGE-mMACHINE kit (Ambion).

The harvesting of oocytes from anaesthetised female *Xenopus laevis* frogs was as previously described [16]. Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng nl^{-1} using a Drummond micro-injector (USA). The solution used for incubating the oocytes contained (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4), supplemented with 50 mg l^{-1} gentamicin sulphate.

2.2. Electrophysiological recordings in Xenopus oocytes

Two-electrode voltage-clamp recordings were performed at room

*Corresponding author. Fax: (32)-16-323 405; http://www.toxicology.be.
E-mail address: jan.tytgat@pharm.kuleuven.ac.be (J. Tytgat).

0014-5793/02/$22.00
PII: S0014-5793(02)03653-0

temperature (18°–22°C) using a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA, USA) controlled by a pClamp data acquisition system (Axon). Whole-cell currents from oocytes were recorded 1 to 4 days after injection. Voltage and current electrodes were filled with 3 M KCl. Resistances of both electrodes were kept as low as possible (<0.5 MΩ). Bath solution composition was (in nM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4). Using a four-pole low-pass Bessel filter, currents were filtered at 2 kHz and sampled at 10 kHz. Leak and capacitance subtraction were performed using a P/4 protocol. Current traces were evoked in an oocyte expressing the cloned sodium channels by depolarisations between −70 and 40 mV, using 10 mV increments, from a holding potential of −90 mV.

2.3. Fast inactivation

The degree of fast inactivation was assayed by measuring the $I_{5\,ms}/I_{peak}$ ratio which gives an estimate of the probability for the channels not to be inactivated after 5 ms [17]. Depending on the sodium channel, a test voltage was chosen so that $I_{5\,ms}/I_{peak}$ was close to zero under control conditions. $I_{5\,ms}/I_{peak}$ was measured at the same test voltage after addition of the toxin. Toxin-induced removal of fast inactivation was measured by plotting $I_{5\,ms}/I_{peak}$ as a function of toxin concentrations. The following equation was used:

$$I_{5\,ms}/I_{peak} = a_0 + \{a_1/[1 + (EC_{50}/[toxin])^h]\}$$

were h is the Hill coefficient, [toxin] the toxin concentration, a_0 the value of $I_{5\,ms}/I_{peak}$ obtained at a chosen test voltage under control conditions, the sum of a_0 and a_1 equals the maximum value of $I_{5\,ms}/I_{peak}$ at the chosen test voltage indicating the expected maximum effect of the toxin on fast inactivation. Curve manipulations were performed using pClamp8 (Axon) and Origin software (Microcal, USA).

2.4. Purification of BgII and BgIII toxins

Collection of the sea anemone *Bunodosoma granulifera*, toxin extraction and isolation were performed as previously described [18].

3. Results

Using the two-electrode voltage-clamp technique on *Xenopus* oocytes, a pharmacological comparison was made regarding the effects of *Bg*II and *Bg*III on five different cloned sodium channels (as shown in Fig. 2). Current traces were evoked using 25-ms-step depolarisations to a voltage range between −20 and 10 mV, depending on the sodium channel, from a holding potential of −90 mV. Both *Bg*II and *Bg*III caused the inactivation to slow down with every VGSC except $Na_v1.8$ which was not affected at any of the used concentrations. Both toxins showed the highest efficacy on para/tipE with an almost complete removal of the inactivation process. When the two toxins were compared, it was found that *Bg*II showed a higher efficacy than *Bg*III, except on para/tipE where *Bg*III showed a slight but significant higher efficacy. There was no significant difference in efficacy regarding *Bg*II between $Na_v1.5$/$Na_v1.4$ (*t*-test: $P < 0.05$).

Fig. 3 shows the concentration dependence of the slowing of inactivation induced by *Bg*II and *Bg*III toxins on the tested VGSCs. EC_{50} values were obtained after a sigmoidal fit of the data. For *Bg*II, the following EC_{50} values were obtained: para/tipE 5.5 ± 0.5 nM, $Na_v1.5/\beta_1$ 0.5 ± 0.1 μM, $Na_v1.4/\beta_1$ 1.0 ± 0.1 μM and $Na_v1.2/\beta_1$ 0.3 ± 0.2 μM. For *Bg*III, the following EC_{50} values were obtained: para/tipE 1.3 ± 0.2 μM, $Na_v1.5/\beta_1$ 5.1 ± 0.5 μM, $Na_v1.4/\beta_1$ 9.8 ± 0.9 μM and $Na_v1.2/\beta_1$ 18.9 ± 2.0 μM (see also Table 1). According to potency the following ranking is applicable for *Bg*II: para/tipE ≫ $Na_v1.2/\beta_1$ > $Na_v1.5/\beta_1$ > $Na_v1.4/\beta_1$ ⋙ $Na_v1.8/\beta_1$; for *Bg*III: para/tipE > $Na_v1.5/\beta_1$ > $Na_v1.4/\beta_1$ > $Na_v1.2/\beta_1$ ⋙ $Na_v1.8/\beta_1$. It should be stressed that the dose–response curve of *Bg*II on para/tipE is almost completely separated from the other curves, indicating its insect selectivity. *Bg*II is about one hundred times more potent on para/tipE as compared with $Na_v1.2/\beta_1$. Moreover, the efficacy of *Bg*II on $Na_v1.2/\beta_1$ is only half that of para/tipE. The EC_{50} values of *Bg*III indicate that this toxin is more than about two hundred times less potent than *Bg*II. The EC_{50} value of *Bg*III for para/tipE is almost 5 times lower than its next neighbour, $Na_v1.5/\beta_1$. *Bg*III shows a very low efficacy on $Na_v1.4/\beta_1$. The value of $I_{5\,ms}/I_{peak}$ of $Na_v1.8/\beta_1$ is the highest of all VGSCs because this channel is known to be a slowly inactivating channel, indicating that after 5 ms the inactivation is far from completed. Interestingly, the efficacy of *Bg*II and *Bg*III on $Na_v1.8/\beta_1$ is zero, making this channel completely resistant to these sea anemone toxins. A similar observation was made with several classical α-scorpion toxins (unpublished data). The EC_{50} values of $Na_v1.5/\beta$ confirm the earlier published values by Goudet et al. [18].

4. Discussion

The main effect induced by *Bg*II and *Bg*III is a concentration-dependent slowing of the inactivation process of sodium current. The electrophysiological effect of *Bg*II and *Bg*III can be compared with other VGSCs toxins from sea anemones, for example, ATX II from *Anemonia sulcata* [19,20] or ApA and ApB from *Anthopleura xanthogrammica* [21]. These toxins inhibit the inactivation process of sodium channels which causes cardiotoxic and neurotoxic effects. The EC_{50} values of the sodium influx increase induced by ATX II, ApA and ApB in rat cardiac tissues are 15, 3 and 2 nM, respectively [22]. Our pharmacological comparison clearly indicates that para/tipE is profoundly and preferentially affected by *Bg*II and *Bg*III. The difference in potency between these two toxins has already been reported by Loret et al. [8] and Goudet et al. [18]. It is a surprising observation that only one amino acid residue (Fig. 1) is responsible for drastically changing the potency while the efficacy remains practically unaffected.

Due to the fact that *Bg*II seems to be very active on the insect sodium channel, it could serve as a template for further development of new insecticides. Pyrethroids, for instance, are commonly used as insecticides in crop protection, animal health and the control of insects that endanger human health.

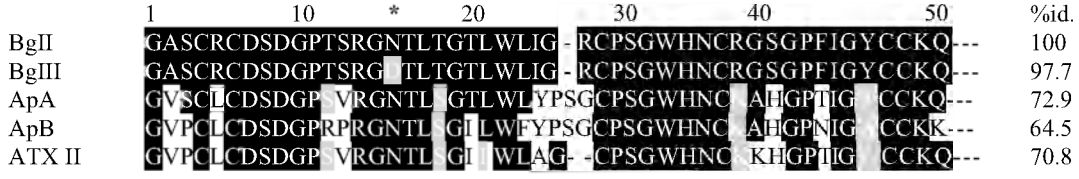

Fig. 1. Comparison of the amino acid sequences of *Bg*II and *Bg*III (*Bunodosoma granulifera*); ApA and ApB (*Anthopleura xanthogrammica*); ATX II (*Anemonia sulcata*). Identical amino acids have a black background, homologous amino acids have grey background. * indicates the amino acid on position 16; %id. indicates the percentage of identity in comparison to *Bg*II; dashes represent gaps.

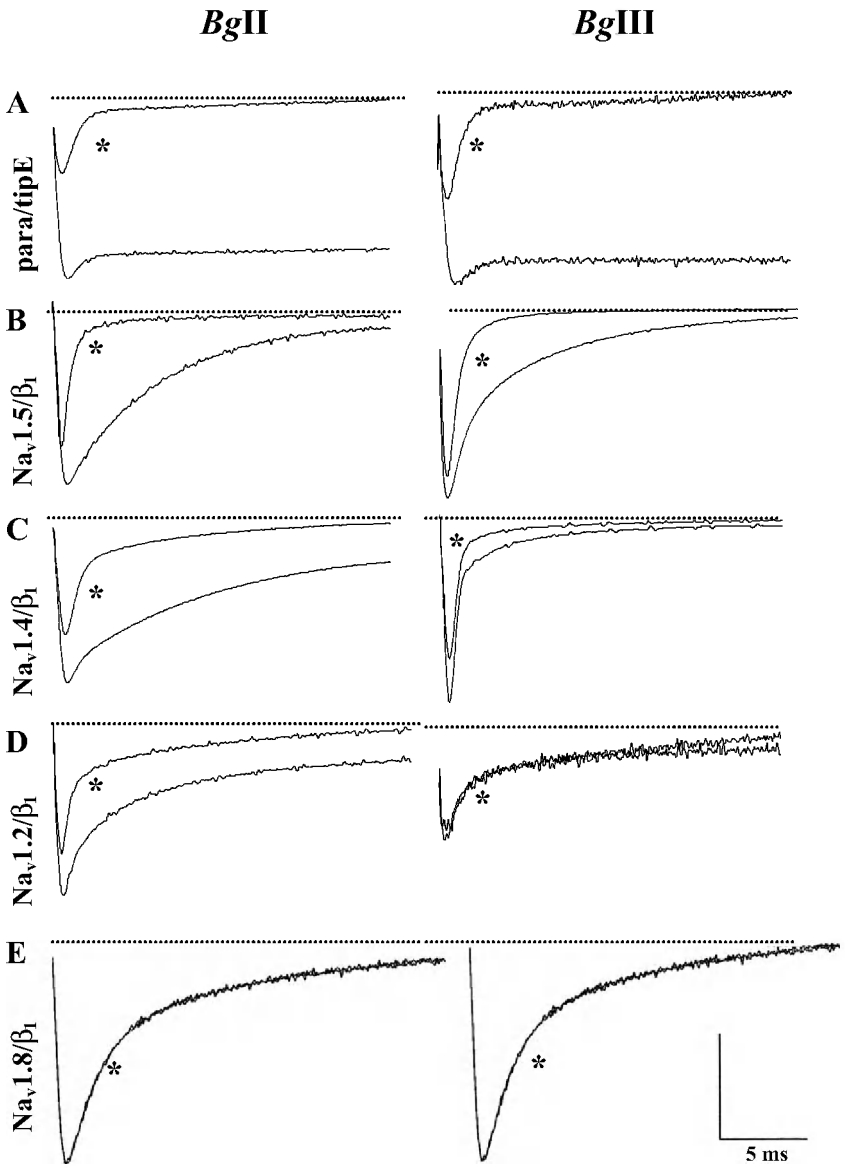

Fig. 2. Left column represents the effects of *Bg*II, right column the effects of *Bg*III on the cloned sodium channels expressed in *Xenopus* oocytes. * represents control conditions where no toxin was added. A: Para/*Bg*II (20 nM); para/*Bg*III (5 μM). B: $Na_v1.5$/*Bg*II (1 μM); $Na_v1.5$/*Bg*III (60 μM). C: $Na_v1.4$/*Bg*II (4 μM); $Na_v1.4$/*Bg*III (100 μM). D: $Na_v1.2$/*Bg*II (20 μM); $Na_v1.2$/*Bg*III (120 μM). E: $Na_v1.8$/*Bg*II (10 μM); $Na_v1.8$/*Bg*III (10 μM). Current traces were evoked by depolarisations ranging from −20 to 10 mV, depending on the sodium channel, from a holding potential of −90 mV. E: No effect was observed after the addition of *Bg*II and *Bg*III. A slowing of inactivation was observed for the other VGSCs. Scale bar: Y-axis scale for A and C = 1 μA; B = 3 μA, D = 0.5 μA; E = 0.75 μA.

Pyrethroids paralyse flying insects and, as such, are known as knockdown insecticides. The intensive use of these insecticides has led to the development of resistance in many insect species. One of the most important mechanisms of resistance in insects is the knockdown resistance (or kdr), caused by several mutations in the para gene (L1014F and M918T), which confers cross-resistance to the entire class of pyrethroids [23,24,12].

Table 1
Summary of the obtained EC_{50} values of *Bg*II and *Bg*III on the VGSCs

	EC_{50} *Bg*II	EC_{50} *Bg*III
Para/tipE	5.5 ± 0.5 nM	1.3 ± 0.2 μM
Na_v 1.2/β_1	0.3 ± 0.2 μM	18.9 ± 2.0 μM
Na_v 1.5/β_1	0.5 ± 0.1 μM	5.1 ± 0.5 μM
Na_v 1.4/β_1	1.0 ± 0.1 μM	9.8 ± 0.9 μM
Na_v 1.8/β_1	–	–

Data are mean ± S.E.M. of at least three experiments.

The natural predators of sea anemones are large crustaceans. Small crustaceans serve as food. One could assume that sea anemone toxins are directed against these organisms. We did not test *Bg*II and *Bg*III on crustaceans, but the fact that these toxins are very potent for insect sodium channels could be an argument to say that there is a link between insects and crustaceans. Both of them indeed belong to the family of the arthropods. Proof of the link between insects and crustaceans has already been found on a genetic level [25]. The toxins studied in this work could be considered proof on a pharmacological level.

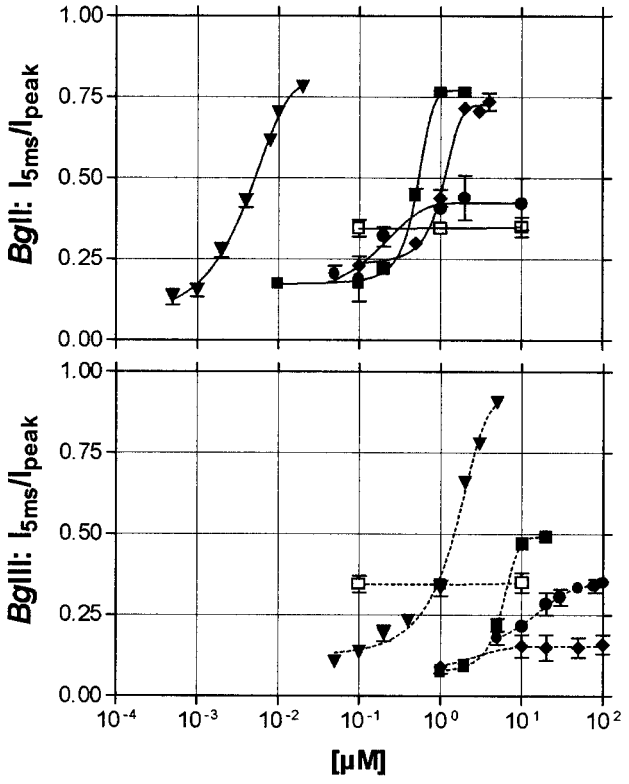

Fig. 3. Comparison of the dose–response curves of *Bg*II (upper panel) and *Bg*III (lower panel) on para/tipE (▼), $Na_v1.5/\beta$ (■), $Na_v1.2$ (●), $Na_v1.4$ (◆) and $Na_v1.8$ (□). The X-scale is the same for both panels in order to show the difference in potency between the two toxins. Y-axis is not normalised for better view of maximal efficacy of the toxins. Data are mean ± S.E.M. of at least three experiments. EC_{50} values are discussed in the text.

Acknowledgements: We would like to thank the following persons: Martin S. Williamson, IACR-Rothamsted, UK, for sharing the para and tipE clone; John N. Wood, University College London, UK, for sharing $Na_v1.8$; A.L. Goldin, University of California, Irvine, CA, USA, for sharing $Na_v1.2$; G. Mandel, State University of New York, NY, USA, for sharing $Na_v1.4$; R.G. Kallen, University of Pennsylvania, Philadelphia, PA, USA, for sharing $Na_v1.5$, and S.H. Heinemann, Friedrich-Schiller-Universität Jena, Germany, for sharing the β_1 subunit. We are grateful to C. Goudet for his comments and suggestions.

References

[1] Stevens, E.B., Cox, P.J., Shah, B.S., Dixon, A.K., Richardson, P.J., Pinnock, R.D. and Lee, K. Tissue distribution and functional expression of the human voltage-gated sodium channel β3 subunit, (2001) Eur. J. Physiol. 441, 481–488.

[2] Catteral, W.A. Structure and function of voltage-gated ion channels, (1995) Annu. Rev. Biochem. 64, 493–531.

[3] Catteral, W.A. From ionic currents to molecular mechanisms: the structure and function of voltage-gated sodium channels, (2000) Neuron 26, 13–25.

[4] Denac, H., Mevissen, M. and Scholtysik, G. Structure, function and pharmacology of voltage-gated sodium channels, (2000) Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 453–479.

[5] Gordon, D., Savarin, P., Gurevitz, M. and Zinn-Justin, S. Functional anatomy of scorpion toxins affecting sodium channels, (1998) J. Toxicol. Toxin Rev. 17, 131–159.

[6] Possani, L.D., Becerril, B., Delepierre, M. and Tytgat, J. Scorpion toxins specific for Na^+-channels, (1999) Eur. J. Biochem. 264, 287–300.

[7] Grolleau, F., Stankiewicz, M., Birinyi-Strachan, L., Wang, X., Nicholson, G.M., Pelhate, M. and Lapied, B. Electrophysiological analysis of the neurotoxic action of a funnel-web spider toxin, δ-atracotoxin-HV1a, on insect voltage-gated Na^+ channels, (2001) J. Exp. Biol. 204, 711–721.

[8] Loret, E.P., Del Valle, R.M., Mansuelle, P., Sampieri, F. and Rochat, H. Positively charged amino acid residues located similarly in sea anemone and scorpion toxins, (1994) J. Biol. Chem. 269, 16785–16788.

[9] Gellens, M.E., George Jr., A.L., Chen, L.Q., Chahine, M., Horn, R., Barchi, R.L. and Kallen, R.G. Primary structure and functional expression of the human cardiac tetrodotoxin-insensitive voltage-dependent sodium channel, (1992) Proc. Natl. Acad. Sci. USA 89, 554–558.

[10] Souslova, V.A., Fox, M., Wood, J.N. and Akopian, A.N. Cloning and characterization of a mouse sensory neuron tetrodotoxin-resistant voltage-gated sodium channel gene, Scn10a, (1997) Genomics 41, 201–209.

[11] Warmke, J.W., Reenan, R.A.G., Wang, P., Qian, S., Arena, J.P., Wang, J., Wunderler, D., Liu, K., Kaczorowski, J.P., Van Der Ploeg, L.H.T., Ganetzky, B. and Cohen, C.J. Functional expression of Drosophila para sodium channels: modulation by the membrane protein tipE and toxin pharmacology, (1997) J. Gen. Physiol. 110, 119–133.

[12] Feng, G., Deák, P., Chopra, M. and Hall, L. Cloning and functional analysis of tipE, a novel membrane protein that enhances Drosophila para sodium channel function, (1995) Cell 82, 1001–1011.

[13] Vais, H., Williamson, M.S., Goodson, S.J., Devonshire, A.L., Warmke, J.W., Usherwood, P.N.R. and Cohen, C.J. Activation of Drosophila sodium channels promotes modification by deltamethrin: reductions in affinity caused by knock-down resistance mutations, (2000) J. Gen. Physiol. 115, 305–318.

[14] Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. and Numa, S. Existence of distinct sodium channel messenger RNAs in rat brain, (1986) Nature 320, 188–192.

[15] Trimmer, J.S., Cooperman, S.S., Tomiko, S.A., Zhou, J.Y., Crean, S.M., Boyle, M.B., Kallen, R.G., Sheng, Z.H., Barchi, R.L. and Sigworth, F.J. Primary structure and functional expression of a mammalian skeletal muscle sodium channel, (1989) Neuron 3, 33–49.

[16] Liman, E.R., Tytgat, J. and Hess, P. Subunit stoichiometry of a mammalian K^+ channel determined by construction of multimeric cDNAs, (1992) Neuron 9, 861–871.

[17] Chen, H., Lu, S., Leipold, E., Gordon, D., Hansel, A. and Heinemann, S.H. Differential sensitivity of sodium channels from the central and peripheral nervous system to the scorpion toxins Lqh-2 and Lqh-3, (2002) Eur. J. Neurosci. 16, 767–770.

[18] Goudet, C., Ferrer, T., Galàn, L., Artiles, A., Batista, C.F.V., Possani, L.D., Alvarez, J., Aneiros, A. and Tytgat, J. Characterization of two Bunodosoma granulifera toxins active on cardiac sodium channels, (2001) Br. J. Pharm. 134, 1195–1206.

[19] Alsen, C., Harris, J.B. and Tesseraux, I. Mechanical and electrophysiological effects of sea anemone (Anemonia sulcata) toxins on rat innervated and denervated skeletal muscle, (1981) Br. J. Pharm. 74, 61–71.

[20] Chacine, M., Plante, E., Perez-Garcia, M.T., Tomaselli, G.F. and Marban, E. Sea anemone toxin (ATXII) modulation of heart and skeletal muscle sodium channel alpha-subunits expressed in tsA201 cells, (1996) J. Membr. Biol. 152, 39–48.

[21] Benzinger, G.R., Drum, C.L., Chen, L.Q., Kallen, R.G., Hanck, D.A. and Hanck, D. Differences in the binding sites of two site-3 sodium channel toxins, (1997) Pflug. Arch. 434, 742–749.

[22] Schweitz, H., Vincent, J.P., Barhanin, J., Frelin, C., Linden, G., Hugues, M. and Lazdunski, M. Purification and pharmacological properties of eight sea anemone toxins from Anemonia sulcata, Anthopleura Xanthogrammica, Stoichactis giganteus, and Actinodendron plumosum, (1981) Biochemistry 20, 5245–5252.

[23] Vais, H., Williamson, M.S., Devonshire, A.L. and Usherwood, P.N.R. The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels, (2001) Pest Manage. Sci. 57, 877–888.

[24] Inceoglu, A.B., Kamita, S.G., Hinton, A.C., Huang, Q., Severson, T.F., Kang, K. and Hammock, B.D. Recombinant baculoviruses for insect control, (2001) Pest Manage. Sci. 57, 981–987.

[25] Boore, J.L., Lavrov, D.V. and Brown, W.M. Gene translocation links insects and crustaceans, (1998) Nature 392, 667–668.