

**PHARMACOLOGICAL CHARACTERIZATION OF *Bunodosoma* TOXINS ON MAMMALIAN VOLTAGE DEPENDENT SODIUM CHANNELS.**

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**Summary**

Voltage dependent sodium channels represent an important target for different neurotoxins and there have been identified different binding sites according to these interactions. The so called site 3 toxins comprise a diverse group of peptides obtained from sea anemones and  $\alpha$ -scorpions that bind to voltage gated sodium channels slowing down the inactivation process. These polypeptides vary considerably in their affinities for the sodium channels in different excitable cells. In this work we studied the pharmacological action of three toxins: BcIII (isolated from *Bunodosoma caissarum*), BgII and BgIII (isolated from *Bunodosoma granulifera*) on isolated cultured neurons of rat dorsal root ganglia. The biophysical effects and the potency of these polypeptides were compared and their effects were studied using whole cell patch clamp techniques. These compounds considerably prolonged the action potential and selectively slowed down the inactivation process of tetrodotoxin-sensitive (TTX-S) sodium current. The potency of these compounds according to the IC<sub>50</sub> values was of: BcIII  $2.7 \pm 2$   $\mu$ M, BgII  $4.1 \pm 1.2$   $\mu$ M and BgIII  $11.9 \pm 1.4$   $\mu$ M. These differences could be determined for the slight variations in the amino acid composition of these peptides and the contribution of specific amino acids in the binding to the sodium channel.

Key words: sea anemone toxins, sodium channel toxins, inactivation process, *Bunodosoma* toxins.

Sea anemones represent a rich source of a variety of biologically active peptides. In particular, from few species of *Bunodosoma* genus have been obtained different compounds that show: cytolytic activity<sup>1,2</sup>, effect on Na<sup>+</sup> and K<sup>+</sup> voltage activated ionic channels<sup>3,4,5,6,7,8,9</sup> and others effects<sup>10,11,12</sup>.

The most studied toxins from *Bunodosoma* genus are polypeptides of about 5 kDa that act on the inactivation process on voltage gated sodium channels in different excitable cells. BcIII (*Bunodosoma caissarum*) was characterized in six different sodium channels (from Nav1.1 to Nav1.6)<sup>9</sup>. BgII and BgIII (*Bunodosoma granulifera*) were studied in rat dorsal root ganglion neurons (DRG)<sup>7</sup> and in five different cloned sodium channels expressed in *Xenopus laevis* oocytes<sup>9</sup>. In these preparations they produced a slowing of the Na<sup>+</sup> current inactivation with a different potency. They seem to bind to the so-called receptor site 3 of the Na<sup>+</sup> channel which is partially formed by amino acid residues located on IV/S3-S4 loop in cardiac or nervous alpha-subunit of the Na<sup>+</sup> channel<sup>13,14</sup>.

In this work we studied the pharmacological action of BcIII toxin in DRG and compare its effect and potency on the tetrodotoxin sensitive sodium currents (TTX-S) in relation with BgII and BgIII toxins using whole cell patch clamp techniques.

### Methods

BcIII, BgII and BgIII were isolated and purified from the sea anemones *Bunodosoma caissarum*<sup>9</sup> and *Bunodosoma granulifera*<sup>3,4</sup> respectively. Aliquots of stock solution in deionized water were prepared and stored in a freezer (-20 ° C). Prior to each experiment, they were dissolved in the perfusion solution.

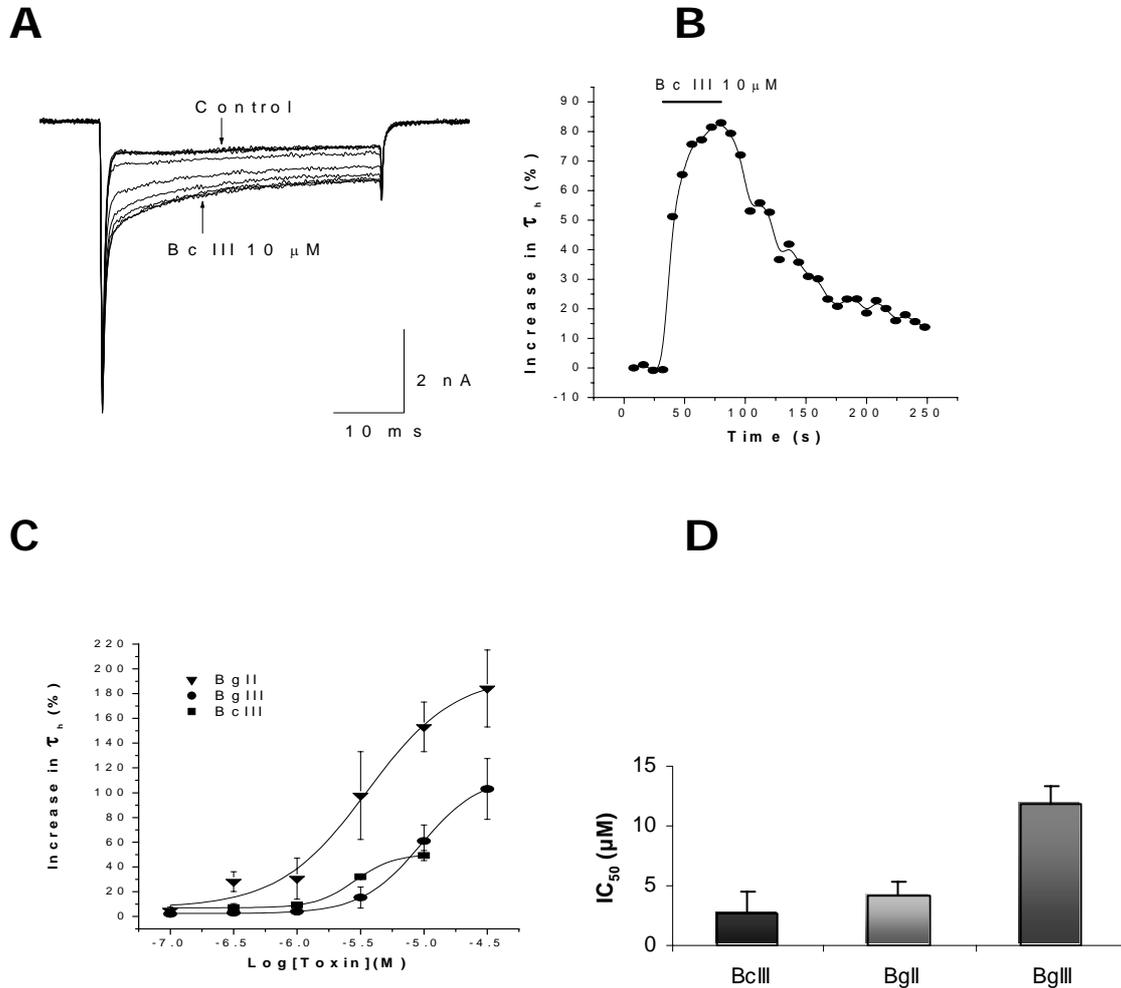
To study the effect of the three toxins on Na<sup>+</sup> currents the whole cell patch clamp technique was used. For this purpose DRG neurons were isolated and cultured from Wistar rats (P5-9) of either sex according to the procedure described by Salceda et al<sup>7</sup>.

Whole cell recording was carried out with an Axopatch-1D amplifier (Axon Instruments, Union City, CA). Command pulse generation and data sampling were controlled by the Pclamp 8.0 software (Axon Instruments) using a 16-bit data acquisition system (Digidata 1320A, Axon Instruments) Intracellular modified solution contained (in mM): 10 NaCl, 100 CsF, 30 CsCl, 10 tetraethylammonium chloride (TEA-Cl), 8 EGTA and 5 Hepes at pH=7,3). Extracellular modified solution contained (in mM): 20 NaCl, 1 MgCl<sub>2</sub>, 1,8 CaCl<sub>2</sub>, 45 TEA-Cl, 70 choline chloride, 10 4-aminopyridine and 5 Hepes at pH=7,4. A detailed description of methods and data analysis was shown by Salceda et al.<sup>7</sup>.

### Results

Sensory neurons of dorsal root ganglion are known to express tetrodotoxin-resistant (TTX-R) Na<sup>+</sup>-channels and tetrodotoxin-sensitive (TTX-S) Na<sup>+</sup>-channels. For this reason the type of I<sub>Na</sub> in the cell under study was determined before each experiment. There were selected for this study only those cells with < 10% TTX-R I<sub>Na</sub>, as derived from the steady-state inactivation profile following the criterion used by Strachan et

al<sup>15</sup>. The effects of the toxins on the Na<sup>+</sup>-currents were studied by a single step voltage protocol in which from a holding potential of -100 mV a 40 ms test pulse to -20 mV was applied with an interpulse interval of 8 s. The inactivation time constant was calculated adjusting the inactivation time course of the TTX-S sodium currents with an exponential function over the following 10 ms after the peak current. The inactivation process of TTX-S Na<sup>+</sup> was well fitted by a single exponential function.



**Figure 1-Effect of toxins on TTX-S Na<sup>+</sup> currents of rat DRG neurons.**  
**A**-Typical experiment showing the effect of BcIII (10 μM) under voltage clamp condition. The records represent superimposed traces before and after toxin application. Notice that the toxin produced a marked slowing of the inactivation process. **B**-Temporal course of BcIII action on the  $\tau_h$ . Bar indicates the time interval of toxin perfusion around the cell. **C**- Concentration-response curves of the effects of BcIII (n=24; squares), BgII (n=87; triangles) and BgIII (n= 22; circles). Points represent the mean  $\pm$  standard error of the mean. **D**-Comparison of IC<sub>50</sub> values of the three toxins.

As it is shown in the Fig. 1A that illustrates a typical experiment, 10  $\mu\text{M}$  BcIII slows down the inactivation process of TTX-S  $\text{Na}^+$ -current rendering it incomplete. The inactivation time constant ( $\tau_h$ ) changed from 0,4 ms in control condition to 0,87 ms after toxin application (last trace). In this experiment, the toxin application produced an increase in the current peak amplitude. The maximum effect was reached within 1-2 minutes depending on toxin concentration. This effect was reversible after repeated washing of the preparation. The temporal course of BcIII action on the  $\tau_h$  is depicted in figure 1B. Note that the maximum effect in this experiment was reached in the first minute. A qualitatively similar action on the TTX-S  $\text{Na}^+$  current was observed for BgII and BgIII. These toxins had no effect on TTX-R  $\text{Na}^+$  current (data not shown).

To construct the concentration-response curve the protocol described above was used. Data were fitted by a dose-response function as follows:

$y = A_1 + (A_2 - A_1) / (1 + 10^{-\text{LogIC}_{50} - x})^P$ , where  $A_1$  is the y value at the bottom plateau,  $A_2$  is the y value at the top plateau,  $\log \text{IC}_{50}$  is the concentration at which the response is halfway between  $A_1$  and  $A_2$  and  $P$  is the Hill slope. Concentration response effects of these toxins on the TTX-S  $\text{Na}^+$  current inactivation time course is depicted in figure 1C.  $\text{IC}_{50}$  rank order was the following: BcIII  $2.7 \pm 2 \mu\text{M}$ , BgII  $4.1 \pm 1.2 \mu\text{M}$  and BgIII  $11.9 \pm 1.4 \mu\text{M}$  (1D).

### Discussion

In this study we compare the electrophysiological action of three type I sea anemone toxins on the same preparation. It was shown that the inactivation time course of TTX-S  $\text{Na}^+$  current was delayed and became incomplete with significant current flow at the end of the pulse by the action of these peptides. It seems that these toxins slow the conformational changes that are required for fast inactivation possibly by binding to the receptor site 3 on the extracellular surface of the  $\text{Na}^+$  channel. In all the cases the effects of the toxins on the inactivation process take place very fast (within 1-2 min.) and these changes were reversible after washing with normal solution. The reversibility of sea anemone toxins action upon vertebrate  $\text{Na}^+$  channels are reported by many authors whereas toxin effect on crustacean  $\text{Na}^+$  channels have been described irreversible<sup>16</sup>.

Few characteristics in the mode of action of these compounds were also similar. As it was reported for BgII and BgIII<sup>7</sup> these peptides produced a leftward shift of the steady-state inactivation curve of the TTX-S  $\text{Na}^+$  current and they did not affect the activation process. Similarly, the perfusion of BcIII significantly shifted to the left (t de Student,  $p < 0.05$ ) the steady-state inactivation curve of the TTX-S  $\text{Na}^+$  current (the half maximal inactivation value [ $V_{1/2}$ ] were of  $-64 \pm 0.79 \text{ mV}$  and  $-72.6 \pm 0.52 \text{ mV}$  for control and toxin presence respectively, and the slope values were of  $-9.2 \pm 0.66 \text{ mV}$  in control and  $-10.6 \pm 0.48 \text{ mV}$  toxin presence).

Concerning to the effect on sodium conductance BcIII did not significantly affect (t de Student,  $p > 0.05$ ) the potential at which activation is 0.5 ( $V_{1/2 \text{ act}}$  were of  $30.4 \pm 0.62$  mV and  $-31.8 \pm 0.9$  mV for control and toxin action respectively) and the slope factor for this curve ( $7.7 \pm 0.53$  mV and  $7.9 \pm 0.77$  mV under control condition and after toxin application respectively). In addition a common characteristic for these compounds is that they did not affect TTX-R  $\text{Na}^+$  currents (data not shown).

In spite of the above mentioned similarities of the toxins there are few differences concerning to the action. BgII and BgIII perfusion did not significant affect current density however, perfusion with BcIII significantly increased the current density at  $40 \pm 18\%$  at -20, -10. 0 and 10 mV, without changes in the reversal potential of the current, which indicate that toxin does not disturb the ionic selectivity of sodium channels. In addition the peak of TTX-S sodium current did not significantly changed in presence of BgII and BgIII whereas a significant increase was produced by BcIII action. Moreover there are slight differences in their potencies that could be related with the specific amino acid composition and their participation in the toxin-channel binding. The magnitude of the effect of each toxin on the inactivation process vary and the order of potency according the  $\text{IC}_{50}$  was BcIII > BgII > BgIII.

The three studied toxins are structurally similar. BgII and BgIII differ in the presence of Asp at position 16 instead Asn in BgIII, and this only substitution may contribute to the less potency of this toxin. Concerning to BcIII, it shows a 91% identity in relation to BgII and differs in 4 amino acids. The presence of a basic amino acid (Lys) in position 47 could explain its higher affinity in comparison with the others *Bunodosoma* toxins. Our results show that the three toxins act on DRG-cells, and BcIII is the most potent in this model. Perhaps these differences in affinity could be determined for the slight variations in the amino acid composition of these peptides and the role of specific amino acid in the toxin-channel interaction. The differences in potency of these toxins in this model in comparison with previous results in others excitable cells reinforce the fact that the affinity of sea anemone toxins for the  $\text{Na}^+$  channel is highly dependent on the tissue or the species in study.

In addition, the presence of highly homologous  $\text{Na}^+$  channel toxins in two sea anemone species of the genus *Bunodosoma* seem to indicate that these peptides are essential in paralyzing preys and be employed as a defense. Furthermore, the highly similar peptides suggest that particular amino acid residues in each molecule may be involved in the discrimination of particular  $\text{Na}^+$  channel subtypes by the toxins.

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