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Identification of hemolytic and neuroactive fractions in the venom of the sea anemone *Bunodosoma cangicum*

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Abstract

Sea anemones are a rich source of biologically active substances. In crayfish muscle fibers, *Bunodosoma cangicum* whole venom selectively blocks the $I_{K(Ca)}$ currents. In the present study, we report for the first time powerful hemolytic and neuroactive effects present in two different fractions obtained by gel-filtration chromatography from whole venom of *B. cangicum*. A cytolytic fraction (Bcg-2) with components of molecular mass ranging from 8 to 18 kDa elicited hemolysis of mouse erythrocytes with an EC_{50} = 14 µg/ml and a maximum dose of 22 µg/ml. The effects of the neuroactive fraction, Bcg-3 (2 to 5 kDa), were studied on isolated crab nerves. This fraction prolonged the compound action potentials by increasing their duration and rise time in a dose-dependent manner. This effect was evident after the washout of the preparation, suggesting the existence of a reversible substance that was initially masking the effects of an irreversible one. In order to elucidate the target of Bcg-3 action, the fraction was applied to a tetraethylammonium-pretreated preparation. An additional increase in action potential duration was observed, suggesting a blockade of a different population of K^+ channels or of tetraethylammonium-insensitive channels. Also, tetrodotoxin could not block the action potentials in a Bcg-3-pretreated preparation, suggesting a possible interaction of Bcg-3 with Na^+ channels. The present data suggest that *B. cangicum* venom contains at least two bioactive fractions whose activity on cell membranes seems to differ from the $I_{K(Ca)}$ blockade described previously.

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- Sea anemone
- Hemolytic activity
- Neurotoxins
- Crab nerve

Introduction

Sea anemones contain a variety of biologically active substances including polypeptide toxins which affect sodium and potassium channels (1,2). Cytolysins that act on cell membranes (pore-forming toxins) have also been described (3,4). All these types of peptides have been isolated from different species such as *Anemonia sulcata* (5), *Stoichactis helianthus* (6), *Actinia equina* (7), *Bunodosoma granulifera* (2,8,9) and *Bunodosoma caissarum* (10,11). Sea anemone neurotoxins that affect sodium channels slow down the inactivation phase of the currents without affecting the activation process, so that the channels remain open for a longer period of time leading to a prolongation of the action potential duration (1,3). In contrast, neurotoxins that act on potassium channels behave as blockers of voltage-sensitive channels, similar to dendrotoxins (DTX) or to mast cell degranulating peptide isolated from mamba snakes (12,13) and bee venoms (14), respectively.

B. cangicum is a common sea anemone found along the Uruguayan and Brazilian seashores. Its venom selectively blocks the Ca^{2+}-dependent K^+ current ($I_{K(Ca)}$) present in crayfish muscle fibers in a reversible manner without affecting voltage-gated Ca^{2+} or K^+ currents (15). Furthermore, the venom reduces $I_{K(Ca)}$ in chromaffin cells without modifying voltage-gated Na^+, Ca^{2+} or K^+ currents (15). The venom also inhibits the binding of radiolabeled DTX to synaptosomal membranes (13). Although these studies (13,15) described interesting neuroactive effects of whole venom, no attempt was made to purify the toxin(s) responsible for such actions on ionic channels. Furthermore, the presence of cytolytic toxins in the venom has not been explored previously.

The aim of the present study was to describe the hemolytic and neuroactive properties of isolated fractions obtained after gel-filtration chromatography from the whole venom of *B. cangicum*, using different *in vitro* approaches.

Material and Methods

Venom extraction

The extraction method was a modification of that reported by Malpezzi and Freitas (10). *B. cangicum* specimens were collected on Cabo Polonio rocky shores (Rocha, Uruguay) and kept alive in laboratory aquaria for milking for several months. The isolation of the venom (probably including some other substances from the animal body) was performed by electrical stimulation of 20 animals immersed in 50 ml of 0.10 M ammonium acetate, pH 7.0. Each animal received an electric discharge (100 V, 10 ms and 20 Hz for 20 s) using two carbon electrodes, as described in the original method. This procedure allows most animals to recover and to be re-used to obtain more venom or to be returned to the sea. The solution obtained was freeze-dried and stored at -70°C.

Gel-filtration chromatography. The freeze-dried venom was dissolved in 10-12 ml of 0.10 M ammonium acetate, pH 7.0, and centrifuged at 2000 *g* for 15 min and the supernatant was submitted to gel filtration on a Sephadex G-50 column (1.9 x 131 cm; Pharmacia-LKB Biotechnology, Uppsala, Sweden) and eluted with the same solution. Fractions of 5 ml were collected at a flow rate of 20 ml/h, absorbance was measured at 280 nm and the effluent was then pooled as indicated in Figure 1. The molecular mass range of the components present in each group of fractions was estimated by the elution of a mixture of four standard proteins: bovine albumin (67 kDa), chymotrypsinogen (25 kDa), cytochrome *c* (12.5 kDa) and bacitracin (1.4 kDa) under the same elution conditions as used for the venom.

Hemolytic assay. The hemolytic assay used was a modification of the method of Galletis and Norton (16). To detect the he-

molytic activity in the whole venom and fractions obtained by gel filtration, a qualitative test was performed using multiwell plates. Each well received 50 µl of the test substance, plus 50 µl of a 4% mouse erythrocyte suspension in 0.85% saline, containing 10 mM $CaCl_2$. After gentle shaking at room temperature for 30-40 min, the lytic effect (positive) was detected visually and compared to the effect of Triton X-100 detergent. The fractions showing hemolytic properties were assayed quantitatively (10). Briefly, blood was diluted with 30 volumes of Krebs-Henseleit solution, pH 7.4, aerated with 95% O_2 and 5% CO_2 and maintained under conditions of constant gentle shaking without the addition of anticoagulant for 15 min. To reduce plasma contamination of the erythrocytes the blood suspension was washed three times by successive centrifugations (3000 *g*/10 min) and a 0.5% final dilution of the erythrocytes (v/v) was prepared. After incubation for 1 h at room temperature and centrifugation at 3000 *g* for 5 min, the percent hemolysis was estimated from the absorbance at 540 nm of the hemoglobin released. In both hemolytic assays, total hemolysis (100%) was obtained with Triton X-100 detergent.

Nerve sucrose gap assay. The nerve preparation was obtained from the crab leg sensory nerve. A walking leg was isolated from an adult blue *Callinectes danae* crab and its nerve exposed by cutting the membranes and articulations of the leg as described by Malpezzi et al. (11). The segments were then removed and the remaining nerve was placed in a groove of a Lucite chamber across eight interconnected compartments, each one isolated with vaseline plugs. The electrodes for stimulation (platinum-iridium) were connected to compartments 1 (positive) and 2 (negative), and the recording electrode (silver chloride) to compartments 5 and 8. Compartments 1-5 contained physiological solution for *C. danae*, 6 and 7 contained 1 M sucrose, and 8 contained isosmotic KCl (0.46 M). The test substance was added to compartment 5, which contained 100 µl of physiological solution. Compound action potentials were evoked by single supramaximal stimuli (20 V) at 0.1 Hz and lasting 0.05 ms (Grass SD-9 Stimulator). The resting membrane potential and the action potentials of the nerve were amplified with a DC pre-amplifier (NF-1, Bioelectrics Instruments, Hastings-on-Hudson, NY, USA) (cut-off frequency 0 to 10 kHz), displayed on an oscilloscope screen (Tektronix model 5103) and recorded with a polygraph (Beckman R411). Also, they were recorded and saved on a computer hard disk using an appropriate software (Whole Cell Electrophysiology Program (WCP), version 1.2) controlling an A/D board (Digidata 1200, Axon Instruments, Union City, CA, USA). The sampling interval was 0.2 ms and the record size was 1024 samples within each record. The following parameters were analyzed on the recorded samples: action potential duration (ms), postpotential duration (ms) and rise time (time taken for the signal to rise

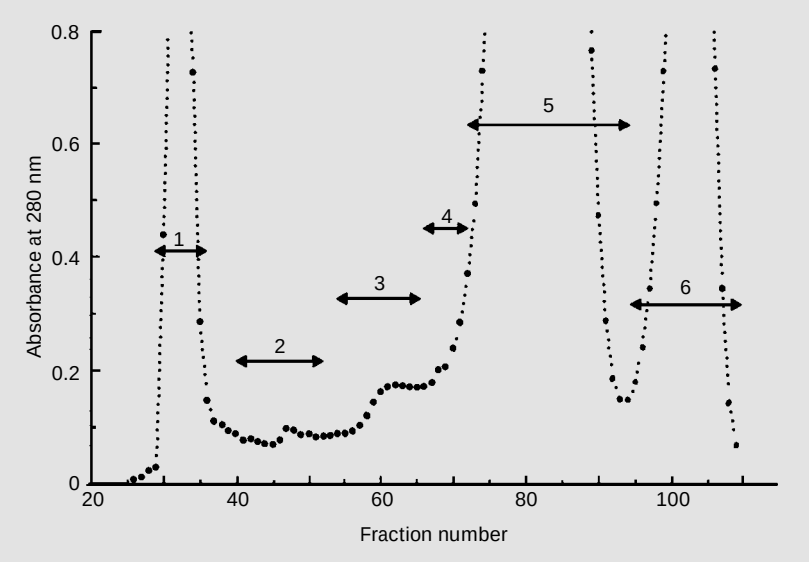


Figure 1. Gel filtration of 1 g of the venom of *Bunodosoma cangicum* on Sephadex G-50. The freeze-dried sample was applied to a Sephadex G-50 column (1.9 x 131 cm), equilibrated in 0.10 M ammonium acetate, pH 7.0. Fractions of 5 ml/15 min were collected and absorbance was measured at 280 nm. The fractions were pooled as shown in the figure (Bcg-1 to Bcg-6).

from 10 to 90% of peak amplitude, in ms). Action potentials measured before each treatment were used as controls. The effect of 15 mM tetraethylammonium (TEA) and 1 µM tetrodotoxin (TTX) on action potentials were studied in combination with the fractions in order to clarify their mechanism of action. The composition of the *C. danae* physiological solution was as follows: 470.4 mM NaCl, 8.0 mM KCl, 18.0 mM $CaCl_2$, 31.5 mM $MgCl_2$, 6.0 mM $NaCO_3$, and 5.6 mM glucose.

Statistical analysis

The results are reported as the mean ± SEM. Statistical significance ($P<0.05$) was assessed by one-way analysis of variance (ANOVA) followed by the Dunnett test.

Results

In the extraction procedure, 0.6 to 2 g of venom (dry weight) could be obtained from 20 electrically stimulated specimens.

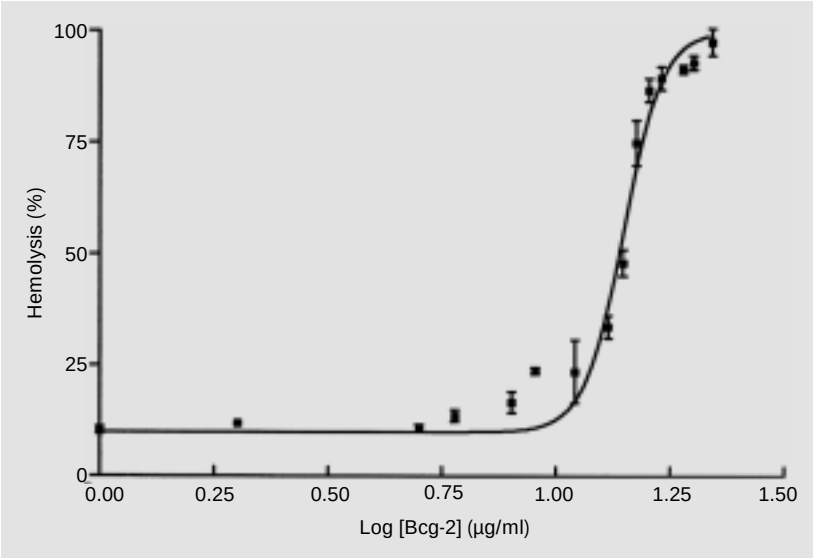


Figure 2. Cytolytic activity of fraction Bcg-2 on mouse erythrocytes. The data were fitted to a sigmoid dose-response curve (EC_{50} = 14 µg/ml; maximum dose = 22 µg/ml). Percent hemolysis was measured at 540 nm and compared with the hemolytic effect of Triton X-100. Data are reported as means ± SEM of percent hemolysis (N = 3).

Gel filtration of *B. cangicum* whole venom on Sephadex G-50 yielded six fractions (Figure 1) named Bcg-1 to Bcg-6. The estimated molecular mass for the components of each fraction were: 30 to 60 kDa for Bcg-1, 8 to 18 kDa for Bcg-2, 2 to 5 kDa for Bcg-3, and 1 to 2 kDa for Bcg-4. Fractions Bcg-5 and Bcg-6 contained components with molecular mass below 1.4 kDa.

A mild cytolytic activity on mouse erythrocytes was obtained with whole venom and some of the fractions (data not shown). One of the fractions, Bcg-2, produced the most potent effects and when tested in the qualitative hemolytic assay it elicited a dose-dependent effect. The data obtained were fitted to a sigmoid dose-response curve, the EC_{50} value obtained was 14 µg/ml and the maximum dose of Bcg-2 was 22 µg/ml (Figure 2).

All gel-filtration fractions were tested on the isolated crab leg sensory nerve using the sucrose-gap method. The Bcg-3 fraction elicited the most interesting neuroactive effects. First at all, it produced a rapid decrease in the amplitude of the action potentials (Figure 3A), as well as depolarization of the resting membrane potential (Figure 3B). After washout of the preparation, the amplitude increased and there was an increase of the action potential and postpotential duration with a concomitant increase in the rise time of the signal (Figure 3A and C). The effects that appeared after washout were irreversible (no alteration was obtained after a second washout) and dose-dependent, and became significant with 100 µg/ml of Bcg-3 (Figure 3C).

To clarify the mechanism of action of Bcg-3 on this nerve preparation, we combined the effects produced by TEA or TTX with those produced by Bcg-3. TEA (15 mM) application to the nerve produced a rapid and significant increase of the action potential duration, which was reversed after washout (Figure 4). When Bcg-3 (100 µg/ml) was applied to a TEA-pretreated preparation, an additional and significant increase

of the action potential duration was observed (Figure 4A,B), lasting as long as 1 h after washout of the preparation.

The blockade of Na^+ channels by TTX (1 µM) after Bcg-3 (100 µg/ml) application and washout of the preparation produced a decrease in the action potential amplitude (80%) that did not block nerve conduction. The increase of the duration of the action potential persisted, as observed before TTX (Figure 5). When the effects of TTX were reversed after a second washout, the effects of Bcg-3 soon appeared (data not shown).

Discussion

The present results show the presence of powerful hemolytic and neuroactive substances in two fractions obtained by gel-filtration chromatography of *B. cangicum* whole venom.

The extraction method was very simple, with the venom being less contaminated with other compounds from the sea anemone body, with consequent simplification of the purification procedures. Also, it is important to note that the animals stay alive and can therefore be re-used to obtain more venom or be returned to the sea.

Hemolytic activity on mouse erythrocytes was found in whole venom and some of its fractions. The Bcg-2 fraction elicited the most potent and marked hemolysis in a dose-dependent manner, with an EC_{50} of 14 µg/ml and a maximum dose of 22 µg/ml. The cytolytic toxins isolated from sea anemones had a molecular mass between 16 and 21 kDa (3,17). The components of the Bcg-2 fraction are in the same range, with a molecular mass between 8 and 18 kDa, and therefore may probably contain one or more cytolytic substances. Further purification will be necessary in order to test this hypothesis and to clarify the mechanism of action of the cytolytic components on cell membranes. It is interesting to note that Malpezzi and Freitas (10) isolated a hemolysin of 14 kDa from an equivalent G-50 fraction from *B. caissarum*, a sea anemone phylogenetically related to *B. cangicum*.

Bcg-3 elicited highly interesting neuroactive effects when tested on the isolated crab leg sensory nerve by the sucrose-gap method. After Bcg-3 was applied to the prepa-

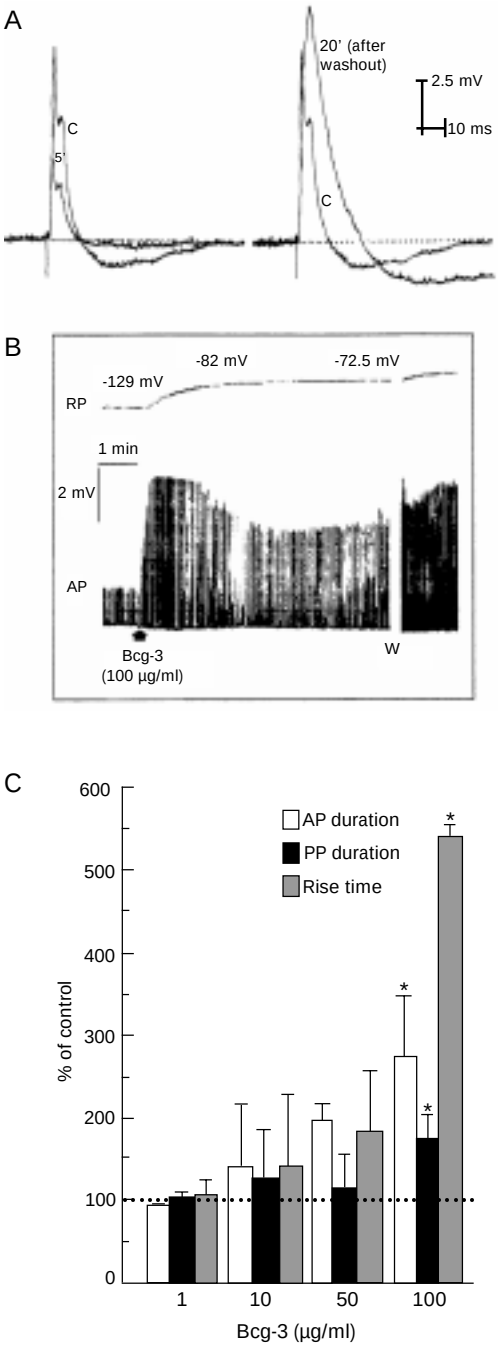
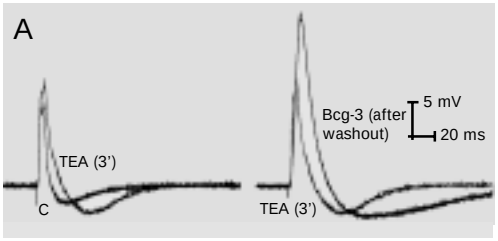


Figure 3. Effects of fraction Bcg-3 (100 µg/ml) on crab nerve. *A*, Representative traces showing control (C) action potential (AP) and traces obtained 5 min after Bcg-3 (left side traces). Right side traces compare control AP (C) and AP 20 min after Bcg-3 (after washout). *B*, Effects of Bcg-3 on resting membrane potential (RP) and AP duration; the effects were not washed out. W = washout. The value of RP obtained is indicated. *C*, Dose-response effect of Bcg-3 on AP, postpotential (PP) duration and rise time after washout of the preparation (20 min after the application). Absolute control values: AP duration = 14.8 ± 0.3 ms; PP duration = 63.2 ± 6.7 ms; rise time = 2.5 ± 1.2 ms (N = 3). *P<0.01 (ANOVA-Dunnett test).

ration, a rapid decrease of the action potential amplitude was observed. However, after washout Bcg-3 elicited an increase in the duration and rise time, resulting in a slowing down of the compound action potential. It seems that this fraction contains some substance that masks the effect of another, and only after the first is eliminated does the effect of the second appear, been irreversible (one or more substances may be responsible for the actions observed). The masking effect of one substance on another is also proposed for a unique substance like guanidine, which elicited two different effects before and after the washout of the preparation (18).



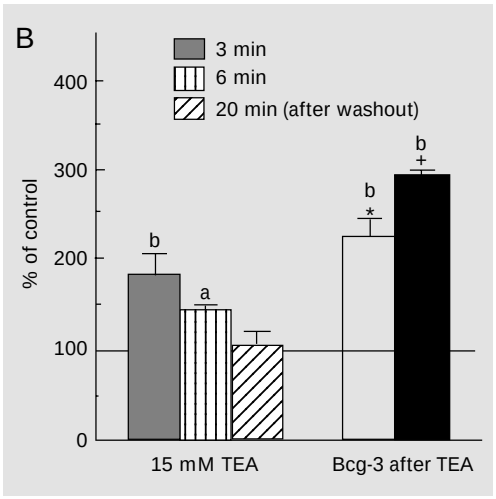


Figure 4. Effects of tetraethylammonium (TEA, 15 mM) and TEA plus Bcg-3 (100 µg/ml) on crab nerves. *A*, Left side traces show control (C) action potential (AP) and AP obtained 3 min after TEA application. Right side traces compare the effects of TEA (TEA, 3') with those elicited by Bcg-3 in a TEA-pretreated preparation (after washout). *B*, Left bars represent the effects of AP duration obtained at different times after TEA application. Right bars represent the effect of Bcg-3 on a TEA-pretreated preparation (open bar = 1 min after Bcg-3; black bar = 14 min after Bcg-3). The significance of the data as compared with their own controls are indicated by a and b (a = P<0.05, b = P<0.01); the significance obtained when comparing the two types of experiments at the same time (3, 6 and 20 min) is expressed as *P<0.05 and +P<0.01 (N = 4; ANOVA-Dunnett test).

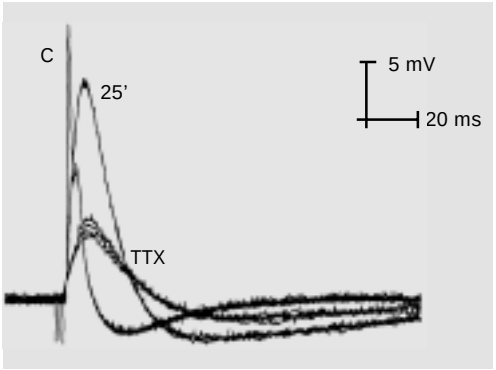


Figure 5. Effect of 1 µM tetrodotoxin (TTX) on action potential in a Bcg-3 (100 µg/ml)-pretreated nerve. C = control conditions (before TTX); 25' = 25 min after Bcg-3 application and washout of the preparation (and after TTX).

Since the effects produced by Bcg-3 on the duration of the action potential could be related to a blockade of the inactivation phase of Na^+ currents, or a blockade of K^+ currents (19), we studied the combined effects of Bcg-3 with those of the known toxins TEA and TTX.

When Bcg-3 (100 µg/ml) was added to a TEA-pretreated preparation, a synergistic effect on action potential duration appeared. This fact suggests an additional blockade of TEA-sensitive K^+ channels, or a blockade of a population of TEA-insensitive ones or a modulation of the Na^+ channels. The suggestion that Bcg-3 could be blocking a population of TEA-insensitive K^+ channels is probably valid since Bcg-3 application to this nerve preparation produced depolarization of the resting membrane potential, an effect that was not observed with TEA in this preparation (Figure 3B).

The hypothesis that another Bcg-3 component was probably modulating Na^+ channels is supported by the fact that 1 µM TTX failed to block completely the nerve conduction in a Bcg-3-pretreated and washed preparation. The neurotoxic effects of TTX may be reduced by the concentration of Na^+ ions in the solution of the Bcg-3 fraction obtained by gel filtration, thus preventing TTX binding to Na^+ channels. On the other hand, a possible interaction of Bcg-3 with these channels may prevent TTX binding to its site of action. The above results confirm the irreversible interaction of the component(s) of Bcg-3 with the nerve membrane, that also occurs in the presence of TTX, a fact that has been consistently observed with neuroactive substances isolated from sea anemones (20, 21).

Thus, the use of specific Na^+- and K^+-blocking agents in combination with Bcg-3

may suggest the co-existence of two types of neuroactive substances in this fraction, one modulating Na^+ channels and the other blocking K^+ channels, with an overall effect on the prolongation of the nerve action potential. Since the estimated molecular weight of Bcg-3 is of the same order as other Na^+ and K^+ channel toxins isolated from sea anemones, the co-existence of these two types of toxins cannot be ruled out. Data reported by Araque et al. (15) demonstrated that the venom obtained from the same anemone includes a toxin that selectively blocks the Ca^{2+}-dependent K^+ currents in a reversible manner without modifying the Na^+ currents. Moreover, Harvey et al. (13) demonstrated that the venom of the same anemone blocks the binding of labeled DTX (a known K^+ channel blocker) to synaptosomal preparations.

Similar pharmacological activities have been observed in other species of the same genus. BgK, isolated from *B. granulifera*, displaces DTX binding from rat brain synaptosomes and suppresses K^+ currents in cultured ganglion cells (8). Also, two neurotoxins, one of which interacts with Na^+ channels and the other with K^+ channels, have been isolated from *B. caissarum*, a co-habitant of *B. cangicum* along the Brazilian seashore (22).

It has been shown before that many sea anemone toxins that interact with Na^+ channels have a very small activity toward mammals but a very high activity toward crustacean (23,24). Araque et al. (15), using mammalian chromaffin cells, demonstrated that the whole venom of *B. cangicum* had no effects on Na^+ or K^+ currents. However, our results clearly show that the Bcg-3 fraction modified these currents in an invertebrate nerve preparation. These differences may be related to the species-specific activity of the sea anemone venoms described above.

The above data raise a number of questions about the role of different toxins found in the same organism. From a biological point of view, it makes sense that sea anemones should produce a diverse spectrum of toxins more oriented toward ionic channels normally found in their preys (fish, crustacean, jellyfish, etc.), a fact that gives predators a distinct evolutionary advantage. The mixture of cytotoxic and ion channel toxins found in sea anemone venom comprising pore-forming toxins (3,6,25), Na^+ channel toxins tending to "activate" these channels (1,5,23) and K^+ channel toxins tending to block K^+ currents (2,8,9,24), is expected to have devastating neurotoxic effects by producing a massive release of neurotransmitters and exerting a potent effect on heart, muscle and endocrine cells. As the number of anemone toxins continues to grow, more variability within a given species will undoubtedly be discovered.

We demonstrated the presence of powerful hemolytic and neuroactive substances in two fractions obtained by gel filtration of *B. cangicum* whole venom. Further purification of their components will permit us to clarify their mechanism of action on cell membranes and should provide interesting pharmacological tools isolated from marine organisms.

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