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Partial sequence and toxic effects of granulitoxin, a neurotoxic peptide from the sea anemone *Bunodosoma granulifera*

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Abstract

A neurotoxic peptide, granulitoxin (GRX), was isolated from the sea anemone *Bunodosoma granulifera*. The N-terminal amino acid sequence of GRX is AKTGILDSDGPTVAGNSLSGT and its molecular mass is 4958 Da by electrospray mass spectrometry. This sequence presents a partial degree of homology with other toxins from sea anemones such as *Bunodosoma caissarum*, *Anthopleura fuscoviridis* and *Anemonia sulcata*. However, important differences were found: the first six amino acids of the sequence are different, Arg-14 was replaced by Ala and no cysteine residues were present in the partial sequence, while two cysteine residues were present in the first 21 amino acids of other toxins described above. Purified GRX injected *ip* (800 µg/kg) into mice produced severe neurotoxic effects such as circular movements, aggressive behavior, dyspnea, tonic-clonic convulsion and death. The 2-h LD_{50} of GRX was 400 ± 83 µg/kg.

Key words
- *Bunodosoma granulifera*
- Sea anemones
- Toxic peptides

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Sea anemones possess tentacles which contain a variety of biologically active substances, including potent toxins, which are used for the capture of prey or for defense against predators. Toxic peptides have been isolated from several species of sea anemones, such as *Anthopleura fuscoviridis* (1), *Anemonia sulcata* (2), *Radianthus paumotensis* (3), *Radianthus macrodactylus* (4) and *Bunodosoma caissarum* (5,6). Several of these peptides act on the voltage-sensitive sodium channel, inhibiting the inactivating phase of sodium currents during depolarization and stabilizing channels in the open state without affecting the activation process (7). A peptide from *Bunodosoma granulifera* that acts as an inhibitor of potassium channel was recently isolated (8,9). These studies show that these toxic peptides may be important tools for investigating ionic channels.

In the present study, a new neurotoxic peptide, called granulitoxin (GRX), was isolated from *Bunodosoma granulifera*, its partial sequence was determined and its neurotoxic effects *in vivo* were investigated.

Ten sea anemones (100 g), *Bunodosoma*

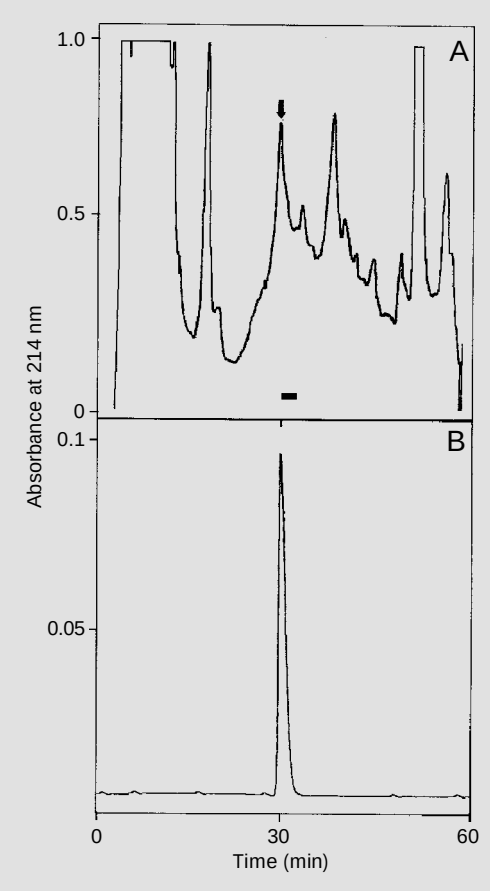

Figure 1 - *A*, HPLC elution profile of a crude sample from *Bunodosoma granulifera*. The sample was dissolved in TFA at a proportion of 1:4 (w:v). After centrifugation at 17,000 *g* for 60 min, the supernatant fraction was injected into an HPLC apparatus with a C_{18} column (Shim-pack 25 x 250 mm) eluted with a 0-40% gradient of acetonitrile containing 0.05% TFA, over a period of 30 min at a flow rate of 5 ml/min. The horizontal bar indicates the effluent containing neurotoxic activity that were combined. *B*, HPLC elution profile of an aliquot containing 10% of the purified toxin. The HPLC conditions are the same as used in panel A.

granulifera, collected along the north coast of Fortaleza city were homogenized in distilled water (1/4, w/v) at 4ºC. The resulting suspension was centrifuged at 17,000 *g* for 60 min. The supernatant was boiled (100ºC for 5 min) and acetic acid was added to obtain a final concentration of 5% (v/v). After 30 min, the suspension was centrifuged as described above, and the supernatant was lyophilized and kept at -80ºC.

For purification of the neurotoxic peptide, the lyophilized material was dissolved in 5 ml of 0.05% trifluoroacetic acid (TFA) and centrifuged as described above. The supernatant was fractionated by HPLC using a C_{18} column (Shim-pack PREP 25 x 250 mm) eluted with a 0-40% gradient of acetonitrile containing 0.05% TFA, over a period of 30 min at a flow rate of 5 ml/min. The effluent was lyophilized and stored at -80ºC.

Acute toxicity tests were carried out and 2-h LD_{50} was determined by injecting the purified toxin intraperitoneally (*ip*) into the cavity of the mice, and physical and observation parameters were evaluated as described previously (10). Two groups of six mice (control and test) were used for each experiment. The results are reported as means ± SEM.

The N-terminal sequence of GRX was determined using a Perkin Elmer-Applied Biosystems (Norwalk, CT) automated protein sequencer model 477A-120A modified for better recovery of PTH-Lys as described previously (11).

The mass of the native peptide was determined by mass spectrometry using a Perkin Elmer-Sciex API300 electrospray-triple quadrupole mass spectrometer according to Kalume et al. (12).

Figure 1A shows the fractionation of the crude sample of *Bunodosoma granulifera* by preparative HPLC. The neurotoxic activity was eluted between 60 and 70 ml, and 980 µg of the neurotoxic peptide was obtained. The purity of the peptide was demonstrated by further HPLC of this fraction under the same conditions as described above (Figure 1B).

Mice injected *ip* with 200 µg/kg of GRX presented increased locomotor activity, circular movements, aggressive behavior, dyspnea and increased sensitivity to touch and sound. These effects started 5 min after the injection and ended about 120 min later, with full animal recovery. The 2-h LD_{50} for GRX injected *ip* was 400 ± 83 µg/kg, and the animals died with tonic-clonic convulsions. The LD_{50} of other anemone peptides varied widely: it was only 8 µg/kg for the *Anthopleura xanthogrammica* peptide (AP-B) and 4000 µg/kg for the *Anemonia sulcata* peptide (ATXI) (13,14).

It is known that the 5000-Da anemone peptides present neurotoxic effects by acting on the voltage-sensitive sodium channel, inhibiting the inactivating phase of sodium

currents during depolarization, and stabilizing channels in the open state without affecting the activation process (7). Although the interaction of GRX with the voltage-gated sodium channel has not been studied, the *in vivo* neurotoxic effects described above suggest that it may act similarly to other anemone peptides.

The partial N-terminal amino acid sequence of GRX is shown in Figure 2. The peptide presented a molecular mass of 4958-Da by electrospray mass spectrometry.

Three classes of 5000-Da anemone peptides that act by binding to the voltage-gated sodium channel have been described according to amino acid sequences (7): the first comprises toxins from *Anemonia sulcata*, *Anthopleura xantogrammica*, *Anthopleura fuscoviridis* and *Bunodosoma caissarum*, the second comprises toxins from *Radianthus macrodactylus*, *Radianthus paumotensis* and *Stichodactyla helianthus*, and the third comprises the *Calliactis parasitica* toxin.

The N-terminal GRX sequence containing 21 amino acids only presents a partial degree of homology with the sequences of these three classes of toxic peptides (Figure 2), i.e., 57% with *Anthopleura fuscoviridis* toxin I (AfI), 35% with *Radianthus macrodactylus* toxin I (HmI) and 22% with *Calliactis parasitica* toxin (CLX). The sequence of the first six N-terminal amino acids is totally different from those of the three classes, with emphasis on the fact that no cysteine residues are present in the partial sequence. The sequence of residues 7 and 21 of GRX is similar to those of the three classes of anemone peptides.

There is general agreement that the flexible region between residues 7 and 14 of these peptides is important to make contact with the sodium channel and that Gly-10 and Pro-11 are important in maintaining the biologically active conformation of this region (7). Furthermore, it has been accepted that Asp-9 and Arg-14 are "functional", both playing an important role in binding and toxic action. However, the replacement of

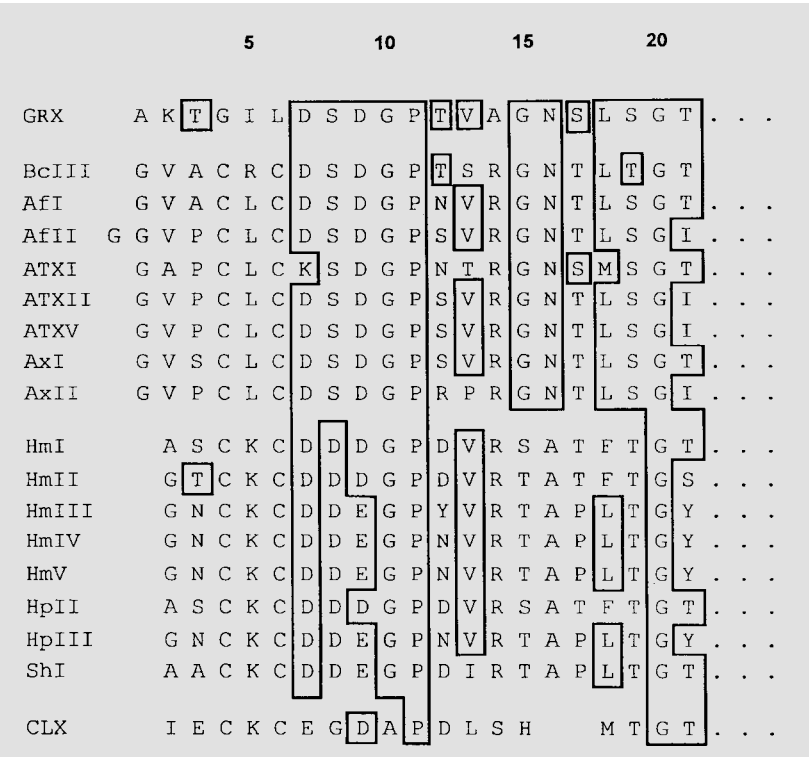

Figure 2 - Partial amino acid sequences of GRX, type 1, type 2 and type 3 long polypeptides from sea anemones. Identical residues are boxed. GRX was obtained from *Bunodosoma granulifera*, BcIII from *Bunodosoma caissarum*, AfI and AfII from *Anthopleura fuscoviridis*, ATXI, ATXII and ATXV from *Anemonia sulcata*, AxI and AxII from *Anthopleura xanthogrammica*, HmI, HmII, HmIII, HmIV and HmV from *Radianthus macrodactylus*, HpII and HpIII from *Radianthus paumotensis*, ShI from *Stichodactyla helianthus*, and CLX from *Calliactis parasitica* (7).

Arg-14 by Ser-14 in the amino acid sequence of the *Calliactis parasitica* toxin suggests that this residue is not absolutely essential for activity (15).

The sequence 7-14 of GRX is very similar to those of the three classes of anemone peptides, containing almost all of the residues described above, except for an important difference: similar to the *Calliactis parasitica* toxin, the Arg-14 of GRX is also replaced by another amino acid, Ala-14. Surprisingly, Ala at this position is not present in any class of these peptides.

In conclusion, the present results suggest that GRX may be a neurotoxic peptide representative of a new class of 5000-Da anemone peptides that act by binding to the voltage-gated sodium channel. However, further studies are necessary to determine the total sequence of this new peptide and the mechanism of its neurotoxic action.

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