A polypeptide toxin from the coral *Goniopora*

Purification and action on Ca^{2+} channels

Janti Qar, Hugues Schweitz, Annie Schmid and Michel Lazdunski

Centre de Biochimie, Centre National de la Recherche Scientifique, Parc Valrose, 06034 Nice Cedex, France

Received 25 April 1986

A polypeptide toxin has been isolated from *Goniopora* coral with an M_r of 19000. *Goniopora* toxin has the following properties: (i) it induces contraction of guinea pig ileum and this contraction is prevented by Ca^{2+}-channel blockers; (ii) it stimulates $^{45}Ca^{2+}$ influx in cardiac cells in culture and this stimulation is abolished by Ca^{2+}-channel blockers; (iii) it prevents binding of (+)-[^{3}H]PN 200–110 to the Ca^{2+}-channel protein of skeletal muscle T-tubule membranes. All these results taken together suggest that *Goniopora* toxin is a Ca^{2+}-channel activator.

(Goniopora) *Toxin* *Ca^{2+}-channel activator* *$^{45}Ca^{2+}$ influx* *1,4-Dihydropyridine*

1. INTRODUCTION

Marine toxins have been very useful as tools to analyze the properties of voltage-dependent ionic channels. The best known of all are tetrodotoxin and saxitoxin which have been and are still being used intensively in electrophysiological and biochemical studies of the Na^+ channel [1–3]. Sea anemone toxins have also been used with success for the study of the Na^+ channel [4,5]. Polypeptide toxins from *Conus geographus* have recently been isolated which seem to act at the same site as tetrodotoxin and saxitoxin [6]. The number of toxins known to be active on Ca^{2+} channels is very limited [7,8]. The only marine toxins which are well characterized are small polypeptides isolated from *C. geographus* [8,9].

Some coral species are known to be toxic. One polypeptide toxin with an M_r of 9000 has been isolated from *Goniopora* coral [10]. It seems to act on the voltage-sensitive Na^+ channel [11,12] in a way similar to that of sea anemone toxins [3].

This paper describes the purification and the properties of a coral toxin acting on Ca^{2+} channels.

2. MATERIALS AND METHODS

2.1. *Collection of* Goniopora *coral*

Goniopora coral was collected from the gulf of Aqaba on the Red Sea in Jordan in July 1984 at the marine science center of Yarmouk University. Coral tissue was shaved mechanically, put in dry ice, transferred by air to France and kept at −20°C until use.

2.2. *Purification of* Goniopora *toxin*

2.2.1. *Extraction*

Goniopora tissue (95 g) was homogenized in 100 ml of 150 mM NaCl, sonicated for 6 short bursts of 40 s each, and centrifuged for 1 h at 30000 × *g*. The pellet was discarded, and the supernatant was filtered on filter paper at 4°C.

2.2.2. Purification on C_{18} reversed-phase

Samples of the extract obtained as described above, were loaded on reversed-phase C_{18}-Sep-Pak (Waters Associates, Millipore) previously activated with methanol, washed with water, and equilibrated in 150 mM NaCl. 1 ml of extract was loaded per Sep-Pak. The active material was eluted

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

with 3 Sep-Pak vols of 150 mM NaCl, and concentrated to one third of the crude extract volume by filtration on an Amicon YM10 filter under nitrogen pressure.

2.2.3. Purification on gel filtration Spherogel HPLC

The active breakthrough fraction (detected by toxicity) from the reversed-phase C_{18} Sep-Pak was loaded on a Spherogel column (TSK 3000 of 0.75 × 30 cm from Toyosoda, Japan) equilibrated in 20 mM Tris-Cl at pH 7.5 containing 150 mM NaCl as described in fig.1. A Beckman 100A solvent delivery system was used to pump this buffer through the column at a flow rate of 0.5 ml/min. Samples of 200 μl were injected with an Altex 210 injector. The column effluent was monitored at 280 nm with a Beckman 160 absorbance detector and collected by hand.

2.3. *Polyacrylamide gel electrophoresis analysis*

Gel electrophoresis was performed according to Laemmli [13]. Samples were denatured for 5 min at 95°C in a buffer containing 2% SDS, 4% β-mercaptoethanol, 15% sucrose, and 50 mM Tris-Cl at pH 6.8. Protein samples (2–8 μg protein per gel lane) were applied to a gel containing 14% isocratic acrylamide. The gel was silver stained according to Merril [14]. M_r values of protein bands were estimated by comparison with the following proteins: phosphorylase (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000), soybean trypsin inhibitor (21500), and lysozyme (14400).

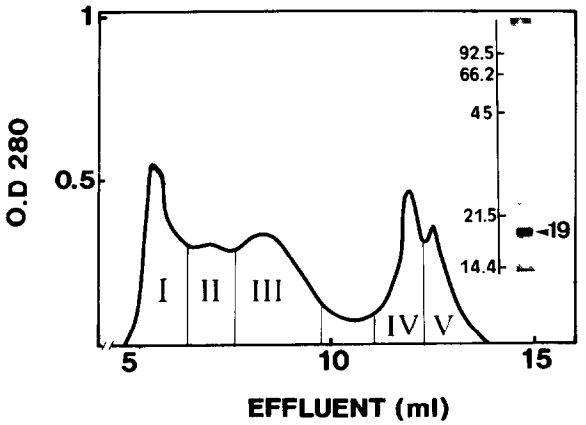

Fig.1. Spherogel-filtration chromatography of the breakthrough from C_{18} reversed-phase chromatography. Samples of 200 μl were loaded at a flow rate of 0.5 ml/min on a Spherogel TSK 3000 column 0.75 × 35 cm) equilibrated with 150 mM NaCl in 20 mM Tris-Cl at pH 7.5, eluted with the same buffer and at the same flow rate. Fractions I–V were collected by hand. Activity was found in fraction III. Inset: SDS gel electrophoresis on 14% polyacrylamide gel of fraction III from Spherogel-filtration chromatography (2 μg protein).

2.4. *Bioassay*

The crude extract and purified fractions of *Goniopora* tissue were assayed on guinea pig ileum for their contracting activity. A 1–1.5 cm long fragment of ileum was bathed in a 1 ml organ bath containing Ringer's Tyrode solution containing 140 mM NaCl, 12 mM $NaHCO_3$, 2.7 mM KCl, 5 mM glucose, 1 mM $MgSO_4$, 0.5 mM NaH_2PO_4, and 2.7 mM $CaCl_2$. The ileum was fixed to an Apeled transductor (Bagneux, France) connected to a Wheatstone bridge. The contraction was recorded on a PE1.10 Servotrace (Sefram, Paris, France). The ileum was contracted by the addition of 1 μM acetylcholine, and then washed with a Tyrode solution. A few minutes later, an aliquot of the toxin was added to the organ bath. The contraction was normalized with respect to the acetylcholine-induced contraction. The effect of the sodium-channel blocker, tetrodotoxin (TTX), and of calcium-channel blockers, nitrendipine and (−)-D_{888}, on the toxin-induced contraction were tested by adding to the bathing medium 1 μM TTX and 0.1 μM nitrendipine or 50 nM (−)-D_{888} 2 min before the addition of the toxin.

2.5. *Toxicity measurements*

Toxicity was measured on 10–20 g Swiss mice. Toxin solutions were geometrically diluted in 150 mM NaCl. Aliquots of 0.1 ml were injected in the tail lateral vein. One mouse unit (MU) was defined as the minimal amount of toxin which killed 10 g mouse.

2.6. *Effect of* Goniopora *toxin on (+)-[^{3}H]PN 200-110 binding to the transverse tubule membranes*

Transverse tubule membranes from rabbit skeletal muscle were prepared as described [15]. They (2 μg protein/ml) were incubated in 1 ml of 50 mM Tris-Cl at pH 7.5 with 70–80 pmol (+)-

[^{3}H]PN 200-110 and increasing concentrations of *Goniopora* toxin. Incubations lasted 45 min in the dark and were stopped by rapid filtration of 2 × 400 μl samples of the incubation mixture through Whatmann GF/C glass fiber filters, under reduced pressure. The filters were immediately washed twice with a cold solution of 100 mM Tris-Cl at pH 7.5. Filters were placed in 7 ml of the scintillation medium (NEN-Biofluor) and counted. The specific binding components of (+)-[^{3}H]PN 200-110 to the 1,4-dihydropyridine receptor of T-tubule membranes was determined as usual [15] by an incubation with an excess (10 μM) of nitrendipine in the absence of toxin.

2.7. *Chick cardiac cells culture preparation*

Hearts were removed under sterile conditions from 11-day-old chick embryos. Hearts were then dissociated by trypsinization as described [16] and cells were plated at a density of 4×10^5 cells/cm^2 and cultured as monolayers using both 24-well tissue culture plates (Nunc). The standard medium used was a mixture of Eagle's minimal essential medium and NCTC 135 medium (Gibco) (75:25, v/v) supplemented with penicillin at 100 units/ml and streptomycin at 50 μg/ml and with 5% fetal calf serum. Cultures were maintained at 37°C in a water-saturated atmosphere of air/CO_2 (95:5). The medium was changed once, two days after plating and monolayers were used at 5–7 days after plating for ^{45}Ca flux studies.

2.8. *^{45}Ca flux measurements*

The determination of ^{45}Ca uptake by chick cardiac cells in culture was carried out in 24-well tissue culture plates. The measurement of ^{45}Ca uptake first involved a preincubation of the cells for 15 min at 37°C with the desired concentration of the toxin in a medium containing 140 mM NaCl, 5 mM KCl, 5 mM glucose, and 25 mM Hepes-Tris at pH 7.4 in the presence or absence of calcium-channel antagonists. The medium was then removed by aspiration and the cells were incubated, for 20 s, in a new medium supplemented with 4 μCi/ml ^{45}Ca and 1.8 mM $CaCl_2$ in the presence of the same concentration of toxin and drugs that were used in the preincubation medium. At the end of incubation, cells were washed 3 times in less than 10 s with 3 ml of washing medium containing 100 mM $MgCl_2$. After the third wash, 2 ml of 0.1 N NaOH was added to each well. Cells were scraped and placed in 6 ml scintillation medium (Aquasure), 2 ml water was added, and radioactivity was determined.

3. RESULTS

After gel filtration on a Spherogel TSK 3000 column of the toxin fraction eluted in the breakthrough of the C_{18} reversed-phase (Sep-Pak) chromatography, the toxin appeared in fraction III (fig.1). After desalting and concentrating the toxic sample by ultrafiltration on Amicon YM10 membranes, SDS gel electrophoresis (fig.1) indicated that the toxin migrated in a single band of M_r 19000.

Goniopora toxin induced characteristic symptoms in mice before death. The first symptom was a loss of activity. Mice laid down on their abdomen with their limbs stretched away from their body with sudden bursts of movement. Just a few minutes before death, their limbs stretched backwards and became paralysed, their chest went into constriction and they gasped for breath. With high doses of toxin (0.4 mg) death occurred within 1 h but it took 1–2 days to kill mice with the minimal lethal dose.

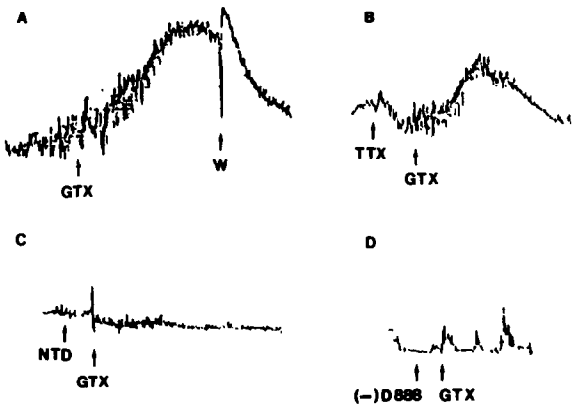

Fig.2. Effect of sodium channel and calcium-channel blockers on the guinea pig ileum contraction induced by *Goniopora* toxin (1.7 μM). Traces: (A) contraction induced by 1.7 μM *Goniopora* toxin in the absence of sodium or calcium-channel blockers; (B) in the presence of 1 μM tetrodotoxin (TTX); (C) in the presence of 100 nM nitrendipine (NTD); (D) in the presence of 50 nM (−)-D_{888}.

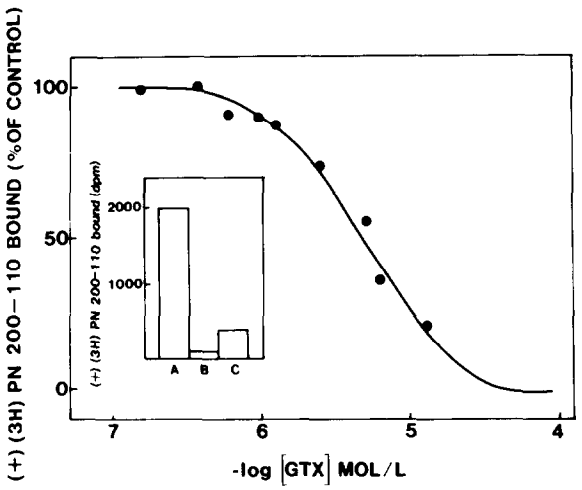

Fig.3. Effect of *Goniopora* toxin on the binding of (+)-[^{3}H]PN 200-110 to rabbit skeletal muscle transverse tubule membranes. Inset: (+)-[^{3}H]PN 200-110 binding to T-tubule membrane, (A) in the absence of *Goniopora* toxin or nitrendipine, (B) in the presence of 10 μM nitrendipine, (C) in the presence of 12 μM *Goniopora* toxin.

The pharmacological effects of *Goniopora* toxin were the following (i) contraction of guinea-pig ileum, (ii) an inhibition of (+)-[^{3}H]PN 200-110 binding to rabbit skeletal muscle transverse tubule membranes and (iii) activation of ^{45}Ca influx into chick cardiac cells in culture. *Goniopora* toxin induced ileum contraction at a concentration of 1.7 μM (fig.2). The toxin-induced contraction was inhibited by calcium-channel blocker such as nitrendipine (0.1 μM) and (−)-D_{888} (50 nM). TTX at 1 μM did not block the toxin-induced contraction (fig.2).

Goniopora toxin inhibited the specific binding component of (+)-[^{3}H]PN 200-110 to its receptor in rabbit skeletal muscle transverse tubule membranes with a IC_{50} value of 5.3 μM (fig.3).

Goniopora toxin also stimulated $^{45}Ca^{2+}$ influx in chick cardiac cells in culture. The concentration that provided a half-maximum stimulation of $^{45}Ca^{2+}$ influx was 5.3 μM (fig.4). This *Goniopora* toxin-stimulated influx of $^{45}Ca^{2+}$ was inhibited by nitrendipine with a half-maximum inhibition at 50 nM nitrendipine.

4. DISCUSSION

There are several types of voltage-dependent Ca^{2+} channels which differ in their voltage dependence, time course and pharmacological properties [17–20]. The best identified of all of them is the Ca^{2+} channel which is sensitive to 1,4-dihydropyridines. This class of drugs includes Ca^{2+}-channel antagonists such as nitrendipine, nifedipine or (+)-PN 200-110 [24]. It also includes Ca^{2+}-channel activators (agonists) such as Bay K8644, CGP 28392 and dihydropyridine (+)-202-791 [19–25].

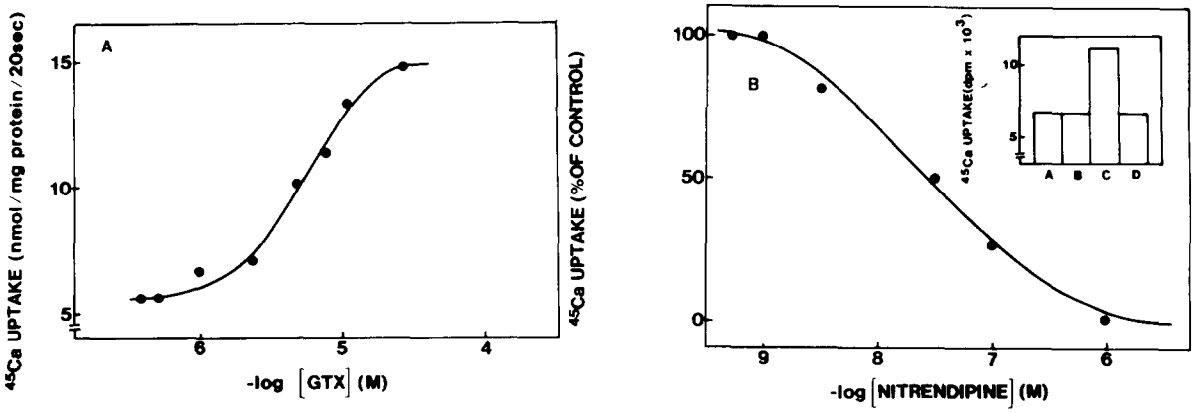

Fig.4. (A) Dose-response curve of the effect of *Goniopora* toxin on ^{45}Ca influx in chick cardiac cells cultures. (B) Inhibition of *Goniopora* toxin-induced ^{45}Ca uptake in chick cardiac cell cultures by nitrendipine Inset: ^{45}Ca uptake in 5 mM KCl, (A) in the absence of *Goniopora* toxin, (B) in the presence of 10 μM nitrendipine, (C) in the presence of 4.2 μM *Goniopora* toxin, (D) in the presence of 4.2 μM *Goniopora* toxin and 1 μM nitrendipine.

The *Goniopora* toxin which has been identified and purified in this work has an M_r of 19000. The observation of a *Goniopora* toxin-induced contraction of guinea pig ileum that was prevented in the presence of low concentrations of the Ca^{2+}-channel blockers nitrendipine and (−)-D_{888} (fig.2) suggested strongly that the polypeptide acted as a Ca^{2+}-channel activator. This interpretation of the physiological results was confirmed by the fact that *Goniopora* toxin stimulated $^{45}Ca^{2+}$ influx into chick cardiac cells in culture and that the toxin-activated component of Ca^{2+} influx was inhibited by low concentrations of nitrendipine (fig.4). Actions of *Goniopora* toxin on smooth muscle and on cardiac cells were very similar to those described previously for the Ca^{2+}-channel agonist Bay K8644 [26].

The receptor of 1,4-dihydropyridines is particularly well identified in T-tubule membranes of skeletal muscle which contains high amounts of this receptor [27]. The last interesting observation made in this study was that *Goniopora* toxin, similar to Bay K8644 (M. Fosset, personal communication), prevented the binding of the 1,4-dihydropyridine antagonist (+)-[^{3}H]PN 200-110 to its receptor in T-tubule membranes.

The effects of *Goniopora* toxin on smooth muscle contraction, $^{45}Ca^{2+}$ uptake by cardiac cells in culture, and (+)-[^{3}H]PN 200-110 binding to the skeletal muscle Ca^{2+} channel, were all seen in the same range of toxin concentration. The half-maximum effect of *Goniopora* toxin on the tissues investigated here was seen at a concentration of about 5 μM. It may be that the toxin is more active on Ca^{2+} channels of animals of marine origin. Affinities for other polypeptide toxins on Na^+ channels are also known to be very dependent on the animal species [28,29].

ACKNOWLEDGEMENTS

We thank Dr M. Van Praët of the Muséum National d'Histoire Naturelle (Paris) for histological studies of the *Goniopora* extracts and M. Zibada and Abu Othman from Marine Center of Aqaba for collecting the corals and Dr J. Jaubert for very useful advice. This work was supported by the Centre National de la Recherche Scientifique.

REFERENCES

[1] Lazdunski, M. and Renaud, J.F. (1982) Annu. Rev. Physiol. 44, 463–473.
[2] Agnew, S.W. (1984) Annu. Rev. Physiol. 46, 517–530.
[3] Ritchie, J.M. (1980) Trends Pharmacol. 1, 275–279.
[4] Romey, G., Abita, J.P., Schweitz, H., Wunderer, G. and Lazdunski, M. (1976) Proc. Natl. Acad. Sci. USA 73, 4055–4059.
[5] Vincent, J.P., Balerna, M., Barhanin, J., Fosset, M. and Lazdunski, M. (1980) Proc. Natl. Acad. Sci. USA 77, 1646–1650.
[6] Cruz, L.J., Gray, W.R., Olivera, B.M., Zeikus, R.D., Kerr, L., Yoshikami, D. and Moczydlowski, E. (1985) J. Biol. Chem. 260, 9280–9288.
[7] Kerr, L.M. and Yoshikami, D. (1984) Nature 308, 282–284.
[8] Olivera, B.M., McIntosh, J.M., Cruz, L.J., Luque, F.A. and Gray, W.R. (1984) Biochemistry 23, 5087–5090.
[9] Olivera, B.M., Gray, W.R., Zeikus, R., McIntosh, M.J., Varga, J., Rivier, J., De Santos, V. and Cruz, L.J. (1985) Science 230, 1338–1343.
[10] Hashimoto, Y. and Ashida, K. (1973) in: Proceeding of the Second International Symposium on Cnidaria, Publ. Seto. Mar. Biol. Lab. 20, 703–711.
[11] Fujiwara, M., Muramatsu, I., Hidaka, H., Ikushima, S. and Ashida, K. (1979) J. Pharmacol. Exp. Ther. 210, 153–157.
[12] Ikushima, S., Muramatsu, I., Fujiwara, M. and Ashida, K. (1981) Jap. J. Pharmacol. 31, 1051–1060.
[13] Laemmli, U. (1970) Nature 227, 680–685.
[14] Merril, C. (1981) Science 211, 1437–1438.
[15] Cognard, C., Romey, G., Galizzi, J.P., Fosset, M. and Lazdunski, M. (1986) Proc. Natl. Acad. Sci. USA 83, 1518–1522.
[16] Renaud, J.F. (1980) Biol. Cell 37, 97–104.
[17] Nowycky, M.C., Fox, A.P. and Tsien, R.W. (1985) Nature 316, 440–443.
[18] Nilius, B., Hess, P., Lansman, J.B. and Tsien, R.W. (1985) Nature 316, 443–446.
[19] Bean, B.P. (1985) J. Gen. Physiol. 86, 1–30.
[20] Carbone, E. and Lux, H.D. (1984) Nature 310, 501–502.
[21] Janis, R.J. and Triggle, D.J. (1984) Drug Dev. Res. 4, 257–274.
[22] Hess, P., Lansamn, J.B. and Tsien, R.W. (1984) Nature 311, 538–544.
[23] Kokuban, S. and Reuter, H. (1984) Proc. Natl. Acad. Sci. USA 81, 4824–4827.

[24] Hof, R.P., Ruegg, U.T., Hof, A. and Vogel, A. (1985) J. Cardiovasc. Pharmacol. 7, 689–693.
[25] Shramm, M., Thomas, G., Roward, R. and Franckowiak, G. (1983) Nature 303, 535–537.
[26] Renaud, J.F., Méaux, J.P., Romey, G., Schmid, A. and Lazdunski, M. (1984) Biochem. Biophys. Res. Commun. 125, 405–412.
[27] Fosset, M., Jaimovich, E., Delpont, E. and Lazdunski, M. (1983) J. Biol. Chem. 258, 6086–6092.
[28] Frelin, C., Vigne, P., Schweitz, H. and Lazdunski, M. (1984) Mol. Pharmacol. 26, 70–74.
[29] Schweitz, H., Vincent, J.P., Barhanin, J., Frelin, C., Linden, G., Hugues, M. and Lazdunski, M. (1981) Biochemistry 20, 5245–5252.