Effects of a Synthetic Analog of Polycavernoside A on Human Neuroblastoma Cells

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Key Words

Polycavernoside • Marine toxin • Human neuroblastoma • Membrane potential • Calcium • Actin • Cytoskeleton

gers an initial extracellular calcium entry neither produced across L-type voltage-gated calcium channels nor activation of muscarinic receptors ii) there is a depolarization induced by the toxin and due to the extracellular calcium entry.

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Abstract

Background: Polycavernoside A is a glycosidic marine toxin first extracted from the red alga *Polycavernosa tsudai* in 1991 when 3 people died after the ingestion of this food. Polycavernoside A is an interesting molecule because of its complex macrolide structure and strong bioactivity. However, the target site of this toxin has not been characterized. **Methods:** We studied the effects of a synthetic analog of polycavernoside A on human neuroblastoma cells by measuring changes in membrane potential with bis-oxonol and variations in intracellular calcium levels with fura-2. Fluorescent phalloidin was utilized for assaying activity on actin cytoskeleton. **Results:** Data showed that this polycavernoside A analog induced a membrane depolarization and an increase in cytosolic calcium levels. **Conclusion:** These results provide the first insight into the mode of action of polycavernoside A, suggesting that: i) this toxin trig-

Introduction

Polycavernoside A is a new marine toxin first discovered in 1991 in Guam by Yasumoto's group [1]. Yasumoto and co-workers were able to extract this toxin (as well as its analog polycavernoside B) from the red alga *Gracilaria edulis* (= *Polycavernosa tsudai*). This is a widely consumed alga that had no potential risk recorded before, although in this case 13 people were intoxicated, and three of them died [1]. More recently, along 2002-2003 in Philippines, 36 people got poisoned because of having eaten two red algae: *Acanthophora specifera* and *Gracilaria edulis*, and 8 of them died. Polycavernoside A was identified as the cause of poisoning from *G. edulis* [2].

This toxin is a structurally unique natural product, which becomes an interesting molecule for chemists and

biochemist. It is a glycosidic toxin representative from the group of macrolides because of its lactone macrocyclic core. Polycavernoside A has coupled a methylated disaccharide component and a trienyl side chain. This structure is similar to other toxins, as aplysiatoxins produced by cyanobacteriae (*Lyngbia majuscula*) and also found in some *Gracilaria* species (*G. coronopifolia*) [3, 4]. Elsemore, toxicity was detected in a filamentous cyanobacterium attaching *G. edulis*, so it may be possible that the biogenetic origin of polycavernoside A could be the cyanobacteria rather than the red alga [5].

Besides polycavernoside A, 4 new natural analogs were determined by NMR techniques from an extract of *Gracilaria* collected in Guam in 1992: polycavernoside A2, A3, B and B2 [6], with some little variations in the side chain and the disaccharide component. Later attempts of polycavernosides extraction from the alga were ineffective, so the occurrence of the toxin seems to be slightly occasional and transitory.

Due to the small quantity of extracted toxin, there is no much information about its toxic effects and possible mode of action. Nevertheless, its complete structure and intraperitoneal toxicity to mice have been previously described. Polycavernosides A and B induced gastrointestinal and neurological disorders, causing diarrhea, hypersalivation, lacrymation, muscle spasm, and cyanosis in mice [1], being these symptoms comparable to those observed in the human patients involved in the Guam case, according to Dr. R. L. Haddock [1]. LD99 values in mice by intraperitoneal injection were 200-400 µg/kg for both toxins. In spite of the scarcity of the toxin, great efforts were made to achieve the total synthesis of polycavernoside A, until it was finally got in 1998 [7], 1999 [8] and 2001 [9, 10]. In addition, Barriault et al. synthesized some analogs with different structures, in order to determine which parts of the molecule would be involved in the toxicity by testing them in a mouse bioassay [11]. They suggested that the macrocyclic core and the side chain are required for toxicity and that it could be maximum if the terminal group on the side chain was an isopropyl group. They also observed that the aglycone analogs have a faster and more serious effect. And this fact would agree with the high fatality to human, being the oral administration more toxic than the intraperitoneal injection to mice, probably because the sugars undergo hydrolysis to the aglycone in the stomach [11]. One of those synthetic analogs, with an enyne substitute in the C-15 side chain (named analog 5 in Barriault et al. 1999) was used for our experiments (see Scheme in materials and methods). This synthetic analog has a mini-

mal lethal dose very similar to that one of the natural toxin (380-770 µg/kg in mice by intraperitoneal injection). Also the symptoms were the same as those observed for polycavernoside A, with paralysis 30 minutes after injection and death occurring withing 24 h [11].

Despite the interest of polycavernoside A due to its great fatality, no pharmacological studies have been done in order to discover its molecular target or mechanism of action. The lack of material has really hampered this kind of studies, so this work tries to deep into the mode of action of this toxin, providing some clues that would help to understand its great fatality.

Materials and Methods

Materials

The synthetic analog of polycavernoside A, compound 5 was synthesized by Dr. Paquette. Fluorescent dyes bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3), bis-oxonol), used for membrane potential assays, and fura-2 acetoxymethyl ester (fura-2), used for the intracellular calcium experiments were purchased from Molecular Probes (Leiden, Netherlands). Oregon Green® 514 Phalloidin utilized to stain filamentous actin (F-actin) was also acquired from Molecular Probes.

EMEM, Ham's F12, glutamine, non-essential amino acids, gentamicine, and amphotericin B were from Biochrom AG (Berlin, Germany).

Nickel, nifedipine, acetylcholine, atropine, fetal bovine serum, bovine seroalbumine (BSA), trypsin-EDTA and all other chemicals, of reagent grade, were bought to Sigma (Madrid, Spain).

25 cm² culture flasks were purchased from Nunc (Roskilde, Denmark).

Cellular culture

We used human neuroblastoma cell line BE(2)M-17 (ATCC N° CRL-2267) as excitable cellular model. The cells were cultivated as described in Louzao et al., 2006 [12].

Solutions

Experimental standard solution contained (in mM): 137 NaCl, 5 KCl, 1 CaCl₂, 10 Glucose, 1.2 MgCl₂, 0.44 KH₂PO₄, and 4.2 NaHCO₃, pH 7.4. In the experiments to test the effect of polycavernoside A analog 5 in a Ca²⁺-free medium, CaCl₂ was omitted, and for fura-2 loading solution, 0.1% BSA was added.

[Ca²⁺]_i measurements

Neuroblastoma cells, previously plated onto glass coverslips, were loaded for 10 min with the Ca²⁺ sensitive dye fura-2 acetoxymethyl ester (0.5 µM) at 37 °C in darkness in a final volume of 2 mL salt solution containing 0.1% BSA. After dye loading, the cells were washed three times with the stand-

Scheme

Fig. 1. Effect of 12 μM polycavernoside A analog 5 (open symbols) in human neuroblastoma cells. Once stabilization of the baseline was obtained, cells were treated with the toxin (arrow), which increases bis-oxonol fluorescence indicating a membrane potential depolarization (A), and also involves an increment in cytosolic calcium levels (B). Mean $\pm$ S.E.M. of 3 experiments.

ard salt solution and resuspended in a Ca^{2+} -containing or Ca^{2+} -free solution, depending on the experimental conditions. These glass coverslips were inserted into a thermostated chamber at 37 $^{\circ}\text{C}$, and cells images were registered with a Nikon Diaphot microscope equipped with epifluorescence optics (Nikon 40x immersion fluor objective). The $[\text{Ca}^{2+}]_i$ was calculated from the fluorescent images registered by double excitation fluorescence: 340 and 380 nm, with emission at 530 nm. The calibration of the fluorescence was made by using the method of Grynkiewicz et al., 1985 [13].

Measurement of plasma membrane potential

The slow potential-sensitive fluorescent dye bis-(1,3-dibutylbarbituric acid) trimethine oxonol was used to detect changes in plasma membrane potential. 5 nM bis-oxonol, whose distribution across the membrane is potential-dependent, was added directly to neuroblastoma cells plated onto glass coverslips and were incubated with the dye for 10 min at 37 $^{\circ}\text{C}$. Fluorescence recordings were performed with the same equipment as previously described, at the excitation wavelength of 490 nm and emission wavelength of 530 nm. Downward or upward deflections of the fluorescence tracings represent hyperpolarization or depolarization, respectively.

The fluorescence intensities, measured in selected regions of interest, were analyzed off-line in the Metafluor software (Meta Imaging Series Environment).

Cytoskeleton assays and detection techniques

Neuroblastoma cells placed on glass supports, as previously, were incubated with polycavernoside A analog 5 and then fixed with 4% formaldehyde solution (10 min) and washed twice with PBS. After that, the cells were permeabilized and their actin cytoskeletons stained with Oregon Green[®] 514 Phalloidin as described in Ares et al., 2005 [14]. With the purpose to study variations in the fluorescence of the dye bound to F-actin, the green maximum pixel intensity was measured using a laser-scanning cytometer (LSC) (CompuCyte, Cambridge, MA). For the visual analysis of F-actin, a laser-scanning

Fig. 2. The cytosolic calcium increment evoked by 5 mM acetylcholine (first arrow in A, second arrow in C), a cholinergic receptors agonist, was blocked by 500 μ M atropine, a cholinergic antagonist (second arrow in A, first arrow in C). The effect of 12 μ M polycavernoside A analog 5 on the calcium increase was not blocked nor when 500 μ M atropine was added after (second arrow in B) nor when it was added before (first arrow in D) the toxin. Mean $\pm$ S.E.M. of 3 experiments.

confocal imaging system MRC-1024 (Bio-Rad; Hemel Hempstead, Herts, UK) and a Nikon X60 oil objective were utilized. In both techniques an argon laser light source (at 488 nm) was utilized for exciting the fluorescent dye.

Results

The symptoms observed in experimental animals and humans suggest that polycavernoside A effects could be related to neurotoxicity [1]. Our aim was to test the analog 5 of this toxin on human neuroblastoma cells, in order to detect any change in the membrane potential by using the sensitive dye bis-oxonol, which has been previously used for this kind of assays [15-17]. Bis-oxonol does not enter the mitochondria because of its negative charge, so the changes only refer to membrane potential [18]. Introduction of bis-oxonol to neuroblastoma cells plated onto glass coverslips shows an increase in fluorescence, which becomes stable in 10 min. In order to plot the results, we normalized the fluorescence values obtained in the imaging

microscope with respect to the baseline [12]. We first investigated the effect of polycavernoside A (the synthetic analog of polycavernoside A, compound 5) on membrane potential in the neuroblastoma cell line BE(2)-M17 by testing low concentrations of the toxin (4.8 μ M), and we found that it did not change the bis-oxonol fluorescence, even when preincubating with veratridine [19]. So we increased the concentration, testing 12 μ M polycavernoside A analog 5 alone, and it was able to produce an increment in the bis-oxonol fluorescence by itself, indicating a membrane depolarization (Figure 1A). Simultaneously, we studied the effect of polycavernoside A analog 5 on the basal state of intracellular calcium ($[Ca^{2+}]_i$) of neuroblastoma cells, since in reaction to hormonal, mechanical and electrical stimuli (as a depolarization may be) a temporary increment in the flow of calcium can be produced [20, 21]. In addition, among the symptoms of the poisoning evoked by polycavernoside A, there is secretory activity [1], an effect that is normally related to increments in $[Ca^{2+}]_i$ [22, 23]. The aim

Fig. 3. Influence of extracellular Ca^{2+} on the (A) depolarizing effect and on the (B) calcium increase induced by the toxin (arrow). Effect of 12 μM polycavernoside A analog 5 measured in a Ca^{2+} -free (open symbols) and Ca^{2+} -containing (filled symbols) solution. Cells were washed three times in a Ca^{2+} -free solution for the open symbols experiments. Mean $\pm$ S.E.M. of 3 experiments.

was to study calcium current alterations in relation to the action potential in order to correlate those symptoms with these parameters. It was observed (as shown in Figure 1B) that the toxin also stimulated a fast fluorescence raise in fura-loaded neuroblastoma cells, which was indicative of a cytosolic calcium increment.

Cytosolic calcium increment evoked by 12 μM polycavernoside A analog 5 was particularly interesting. Taking in account the symptoms observed in patients (diarrhea, hypersalivation, lacrymation, muscle spasms, and cyanosis), which seemed to be related to the Parasympathetic Nervous System, we tested if atropine (an antagonist of the muscarinic receptor) inhibits polycavernoside-induced calcium increase. We previously

Fig. 4. Time-course of the effect of nickel on the thapsigargin-induced Ca^{2+} increment (A) and on the calcium increase induced by polycavernoside A analog 5 (B). (A) Neuroblastoma cells were loaded with fura-2 and preincubated with 5 mM Ni^{2+} , an unspecific calcium entry blocker (first arrow). Cells were then exposed to 2 μM thapsigargin (second arrow), which depletes Ca^{2+} pools by preventing Ca^{2+} reuptake. (B) Preincubating with 5 mM nickel (first arrow), resulted in the abolishment of the calcium increase induced by 12 μM polycavernoside A analog 5 (second arrow). Mean $\pm$ S.E.M. of 3 experiments.

verified that acetylcholine increased cytosolic calcium concentration, and that this increment was abolished with atropine (see Figure 2A and 2C). Nevertheless, Figure 2B and 2D showed that the calcium influx induced by 12 μM polycavernoside A analog 5 was not affected by 500 μM atropine, when this inhibitor was added neither before nor after the toxin.

In order to study the influence of extracellular calcium in polycavernoside-induced depolarization and calcium increase we developed experiments where calcium was omitted from the salt solution. Thus, as it can be observed in Figure 3A, the depolarizing effect caused by 12 μM polycavernoside A analog 5 was abolished in this conditions, so depolarization induced by the toxin would

Fig. 5. Time course of the effect of nifedipine on the calcium increase induced by polycavernoside A analog 5. Fura-2 loaded neuroblastoma cells were preincubated with (open squares) or without (filled squares) 20 μ M nifedipine (first arrow), a L-type voltage-gated calcium channels blocker. Subsequently, 12 μ M polycavernoside A analog 5 was added to the cells (second arrow). Mean $\pm$ S.E.M. of 3 experiments.

Fig. 6. Evaluation of F-actin stained with fluorescent phalloidin. (A) The overlap between fluorescence profiles obtained using LSC shows a similar intensity between cells incubated without (grey colour) and with 4.8 μ M polycavernoside A analog 5 (black colour). (B) Quantification of fluorescence associated to F-actin in controls (=100%) and toxin-treated cells for 24 h (% fluorescence versus control). Representative photographs showing actin cytoskeletons of untreated cells (C) and cells incubated with polycavernoside A analog 5 (D). Mean $\pm$ S.E.M. of 3 experiments.

be dependent on calcium. In addition, in Figure 3B we observe that the increase of intracellular calcium in the neuroblastoma cells was annulled in the calcium-free medium. This result seems to indicate that polycavernoside A analog 5 increases the entry of extracellular calcium to neuroblastoma cells, and as a consequence promotes depolarization.

Next we incubated the neuroblastoma cells with 5 mM nickel (an unspecific calcium entry blocker) by nearly

10 min with the aim of deepening in the calcium increment evoked by polycavernoside A analog 5. As we plotted in Figure 4A, Ni^{2+} blocks the extracellular Ca^{2+} entry induced by 2 μ M thapsigargin. Thapsigargin depletes intracellular calcium stores by inhibiting the sarco-endoplasmic reticulum Ca^{2+} -ATPases, which brings as a consequence the activation of capacitative Ca^{2+} entry [24]. We can observe in Figure 4B that the increase in $[\text{Ca}^{2+}]_i$ induced by polycavernoside A analog 5 is completely abol-

ished when neuroblastoma cells are preincubated with 5 mM Ni^{2+} . Figure 5 shows that the cytosolic calcium increment evoked by polycavernoside A analog 5 is not affected by 20 μM nifedipine, a L-type voltage-gated calcium channels blocker.

Finally, knowing the influence of actin cytoskeleton in the maintenance of cellular integrity we also checked if this toxin exhibited activity toward this complex structure. Neuroblastoma cells were exposed to 4.8 μM polycavernoside A analog 5 for long time periods (12-24 h at 37 °C). Following incubation with the toxin, F-actin of cells was stained with Oregon Green® 514 Phalloidin before being analyzed. Measurement of cells fluorescence indicated no modifications in toxin-treated cells versus controls at any time assayed. Figure 6 shows data obtained in cells incubated with polycavernoside A analog 5 for 24 h. Considering that changes in fluorescence intensity of the dye indicate alterations in actin assembly, our results suggest that polycavernoside A analog 5 does not vary the F-actin level in neuroblastoma cells. Likewise, imaging of cells with confocal microscopy displayed that 4.8 μM of this toxic compound neither modified the actin cytoskeleton organization nor cytomorphology (Figure 6).

Discussion

Polycavernoside A is a new toxin recently synthesized [7-11] that has been classified because of its chemical structure, being a macrolide because of its lactone ring [25]. However the lack of information about its mechanism of action avoids any functional classification.

Toxicological data are very scarce because of the small quantity of toxin; so this work means the first approximation on the toxicity of polycavernoside A in cultured human cells.

Symptoms of intoxication induced by polycavernoside A comprise gastrointestinal disturbances (vomiting, diarrhea) as well as neurological alterations (scratching, muscle spasms, paralysis), which are the main ones, so neuroblastoma cultures, as excitable cells, represent an excellent model to study the mechanism of action of this toxin. Therefore, we evaluated the effects of polycavernoside A analog 5 on membrane potential and intracellular calcium, because control of cytosolic calcium concentration is a critical component of cellular homeostasis for all cell types, including excitation-contraction functions, cellular proliferation, secretion of fluids and neurotransmitters release [26]. In addition, since

other compounds inducers of neurological and/or gastrointestinal disorders cause alterations in the actin cytoskeleton [14, 27], polycavernoside A analog 5 was also assayed against this structure.

This study with neuroblastoma cells provides the first evidence that 12 μM polycavernoside A (the synthetic analog compound 5) depolarizes them and also enhances the cytosolic calcium level. Our results show that depolarization is a secondary effect induced by the calcium entry evoked by polycavernoside A analog 5. In a Ca^{2+} -free medium, the toxin-induced depolarization was completely abolished. These results indicate a calcium dependent depolarizing effect. Under this condition, a calcium-free medium, intracellular calcium increment is also annulled, which might indicate that the increase in $[\text{Ca}^{2+}]_i$ is the first effect induced by polycavernoside A analog 5.

Polycavernoside A has been linked to toxic episodes in humans following the consumption of the alga *Polycavernosa tsudai* [1, 2], but possibly the toxin has a cyanobacterial origin, basing on the sudden and transient occurrence of toxicity of the alga and its chemical structure [1, 28, 29]. Some of the toxins produced by cyanobacteria are neurotoxins that block neurotransmission by acting on cholinergic receptors. So we checked if acetylcholine, a cholinergic agonist, increased cytosolic calcium levels in our cellular model, as it was. But we did not find any inhibition of the increment in $[\text{Ca}^{2+}]_i$ evoked by polycavernoside A analog 5 when adding atropine, a muscarinic receptor antagonist, so acetylcholine muscarinic receptors are probably not the pharmacological target for the synthetic analog 5 of polycavernoside A.

As we pointed before, the intracellular calcium rise induced by polycavernoside A analog 5 seems to be due to the entry of this ion across the membrane. Nevertheless, the mechanism of this calcium entry activated by the toxin is still unknown. Deeping into its study, we used 5 mM nickel as an unspecific inhibitor of the calcium entry. Nickel is a specific blocker for T-type calcium channels at concentrations in the micromolar range (<50-100 μM) [30-33]. In the millimolar range nickel also blocks the activity of the Ca^{2+} release-activated and store operated calcium channels [34], voltage-gated calcium channels [35-37] and the $\text{Na}^{+}\text{-Ca}^{2+}$ exchanger [38-41]. We verified that Ni^{2+} abolished the calcium increase induced by polycavernoside A, which confirms that this toxin provokes a calcium influx from the external medium due to some of the previous mechanisms.

We also used a L-type voltage-gated calcium channel blocker, nifedipine [42, 43]. This antagonist would let

us establish a possible relation between both effects evoked by polycavernoside A analog 5: depolarization and calcium influx. We incubated neuroblastoma cells with nifedipine, but did not find a significant inhibition in cytosolic calcium increment induced by the toxin, so voltage-gated calcium channels (at least L-type) would not play an important role in the intracellular calcium rise evoked by the toxin.

As regards the cytoskeleton assays performed, in the literature there are some examples where calcium movements may alter actin cytoskeleton [44-46] and situations where microfilaments could modify calcium currents [47-49]. Elsewhere there are reports where a calcium influx induce variation on actin assembly [50] or do not modify it [51]. In our experiments, we have found that polycavernoside A analog 5 did not affect the F-actin quantity of neuroblastoma cells (after 12-24 h), and the cytomorphology was also maintained apparently unaltered. These findings seem to suggest that actin polymerization is not an early target for polycavernoside A analog 5.

As a summary, in human neuroblastoma BE(2)-M17 cell line, 12 μ M polycavernoside A analog 5 evokes a cytosolic calcium influx and a membrane potential depolarization that is dependent on the calcium entry. This calcium influx is neither due to the L-type voltage-gated

calcium channels nor muscarinic receptors blocked by atropine. This means a first approach to the effects of polycavernoside A by using the synthetic analog compound 5, even though more experiments are necessary to elucidate the mechanism by which this new phycotoxin alters neuronal activity.

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