

Regulatory volume decrease in isolated nematocytes is affected by crude venom from the jellyfish *Pelagia noctiluca*

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Abstract

Crude venom from nematocysts of the Scyphozoan *Pelagia noctiluca* possesses hemolytic and cytotoxic power on cultured cells and elicits local and systemic inflammation reactions *in vivo*. The ability of regulating their volume after exposure to an anisotonic solution is a fundamental feature common to cells from vertebrates and invertebrates, including Cnidarians. The aim of the present work is to assay whether crude venom from *Pelagia noctiluca* may affect the regulatory volume decrease (RVD) of nematocytes isolated from the Anthozoan *Aiptasia mutabilis*, here employed as a cell model. For this purpose, nematocytes were isolated by 605 mM NaSCN plus 0.01 mM Ca²⁺ application on acontia of *Aiptasia mutabilis*, while crude venom was obtained by sonication of a population of, respectively, 10, 25 and 50 nematocysts/ μ L (n/ μ L). Isolated nematocytes were pre-treated for 30 min with crude venom, submitted to hypotonic stress and their osmotic response and RVD were measured optically. Our results show that, after exposure to crude venom, nematocytes were morphologically intact, as shown by the Trypan blue exclusion

test, but did not exhibit RVD. This effect was dose-dependent and reversed by the ionophore gramicidin. The last observation suggests an inhibitory effect of venom on cell membrane ion transport mechanisms involved in RVD. Further studies are needed to verify this hypothesis and ascertain if a similar effect could be observed in human cells.

Introduction

Pelagia noctiluca (Cnidaria: Scyphozoa) is a jellyfish whose distribution in both temperate and cold seas, including north Atlantic and north Pacific, has been described, and is particularly abundant in the Mediterranean Sea.^{1,2} This jellyfish is provided with nematocytes, the stinging cells of Cnidaria used for prey capture, defense and locomotion. Nematocysts, produced by Golgi apparatus, possess a capsule wall containing an inverted tubule and a fluid matrix with different toxins. The application of an adequate chemico-mechanical stimulus³ elicits the rapid eversion of the tubule, adhering to or penetrating the prey integuments, thus injecting venom. This response, referred to as discharge, is one of the most rapid exocytotic phenomena known to date.⁴⁻⁷ The biological activity and toxicology of the compounds contained in the capsular fluid have been widely investigated.^{2,8,9} Specifically, the crude venom contained in *Pelagia noctiluca* nematocysts has been demonstrated to affect both cell and tissue functions,^{5,10,11} as demonstrated by different biological assays currently used to assess the toxicity of many other terrestrial and marine organisms.^{8,9,12} In particular, both hemolytic and cytotoxic properties of *Pelagia noctiluca* crude venom have been assessed,^{10,13-16} in line with other toxicological studies modeled on cultured cells⁸ and/or erythrocytes.¹⁶ In addition, *Pelagia noctiluca* crude venom can elicit both local¹⁷ and systemic¹¹ inflammation reactions in rats, as a consequence of oxidative stress.

The ability of regulate the cellular volume is a fundamental homeostatic response found both in vertebrates¹⁸ and invertebrates.^{19,20} When exposed to a hypotonic extracellular medium, cells initially undergo to osmotic swelling [the osmotic phase (OP)] and successively regulate their volume toward the resting values (RVD phase). The RVD phase is characterized by an obliged efflux of water, obtained by activating an efflux of ions and other osmotic substances.

On this basis, the aim of the present work is to verify the effect of *Pelagia noctiluca* crude venom, at non necrotic doses, on the homeostatic responses of nematocytes isolated from the Anthozoan *Aiptasia mutabilis*, chosen as a cell model. In particular, the response of nematocytes to hyposmotic shock (OP and RVD)^{19,21} has been monitored as a function of cell viability under venom treatment.

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Materials and Methods

Crude venom preparation

Nematocysts isolation

Specimens of the Scyphozoan *Pelagia noctiluca* were collected from the Strait of Messina (Sicily, Italy) and nematocysts were isolated as previously described.²² Shortly, oral arms were excised from each specimens and nematocysts were isolated by osmotic lysis of nematocytes in 4°C distilled water. The resulting suspension was filtered through plankton nets (100, 60 and 40 µm mesh, respectively) and spun (for 5 min at 4000× *g* at 4°C) to discard debris. Isolated nematocysts were then counted in a Bürker chamber and processed for venom extraction or stored at -20°C for later use.

Crude venom extraction

Samples containing 10, 25 or 50 nematocysts/µL were re-suspended in artificial sea water (ASW) and sonicated 30 times for 20 sec at 70 MHz on ice (Sonoplus; Bandelin, Berlin, Germany). Nematocyst debris was separated by centrifugation (for 10 min at 4000× *g* at 4°C) and the supernatant was used for the biological assay. The venom concentration was expressed as the number of nematocysts/µL (n/µL) in the sample prior sonication.

Regulatory volume decrease study

Specimens collection

Specimens of *Aiptasia mutabilis* (Anthozoa) were collected in the Strait of Messina at 50-90 cm depth, maintained in a closed-circuit aquarium at 18-24°C and weekly fed with shrimps (*Penaeus japonicus*).

Nematocytes isolation

Nematocytes, classified as microbasic-mastigophore,²³ were isolated from acontia of *Aiptasia mutabilis*, by treatment with an isosmotic solution of 605 mM NaSCN plus 0.01 mM Ca²⁺.²⁴ Acontia, once excised from the trunk of the specimens, were washed with low-Ca²⁺ ASW to remove mucus and then treated with an isosmotic solution of 605 mM NaSCN plus 0.01 mM Ca²⁺, for the nematocyte extrusion from the tissue. Substitution of the NaSCN solution first with a Ca²⁺-free ASW and then with complete ASW permitted cell isolation and restoration of physiological conditions. Isolated nematocytes were checked under a light microscope (Leica DMLS, 400× magnification; Leica Microsystems GmbH, Wetzlar, Germany) to ascertain their morphological integrity. They were kept at 10-12°C for 1 h and then used within 3 h from isolation for cell volume regulation tests. To establish the non-necrotic dose of crude venom, isolated nematocytes were treated with different venom concentrations and their morphological integrity was assessed by Trypan blue dye exclusion test.

Regulatory volume decrease tests

Control tests

One hour after nematocytes isolation, a make-shift perfusion chamber was assembled by placing double sided adhesive tape between a glass slide and coverslip containing isolated nematocytes, in order to allow for continuous perfusion during the entire test and substitution of experimental media. Cell volume experiments were performed on nematocytes chosen for their strong adhesion to the slide. To assess the cellular response to the anisomotic shock, RVD control test consisted of three periods: 1st period, isosmotic ASW ($\pi=1100$ mosm/kg_{H2O}) for 5 min; 2nd period, hyposmotic ASW ($\pi=710$ mosm/kg_{H2O}) for 20 min; 3rd period, isosmotic ASW for 5 min.

Crude venom effect on regulatory volume decrease response

To test crude venom effect on RVD capability, isolated nematocytes were pre-treated with 10, 25 or 50 n/µL crude venom for 30 min at room temperature in a damp room. After incubation with crude venom, nematocytes were quickly rinsed with ASW and submitted to RVD test with the protocol described above. Nematocytes treated for 30 min with 50 n/µL crude venom were also submitted to the following RVD test: period 1a (1st a), isosmotic ASW for 5 min; period 1b (1st b), isosmotic ASW plus 1 mM gramicidin for 6 min; period 2 (2nd), hyposmotic ASW for 20 min; period 3 (3rd), isosmotic ASW for 5 min. During each RVD test, about 30 images/nematocyte were taken with a phase contrast microscope (Leica DMLS, 400× magnification; Leica Microsystems GmbH) connected to a video camera (CCD camera) and to a computer equipped with suitable software (Movie Maker; Microsoft Co., Redmond, WA, USA). To assess cell volume changes as a function of time, the cross sectional area (as an indication of cell volume) of each recorded image was measured and A/A₀ ratio calculated. A represents the cross sectional area of a nematocyte at a given time and A₀ is the average of the cross sectional area of the same nematocyte in isosmotic ASW.

Experimental solution and reagents

Isosmotic ASW had the following composition (mM): NaCl 520, KCl 9.7, CaCl₂ 10, MgCl₂ 24, MgSO₄ 28, imidazole 5, pH 7.65, $\pi=1100$ mosm/kg_{H2O}. Low Ca²⁺ solution had the following composition (mM): NaCl 520, KCl 9.7, CaCl₂ 0.01, MgCl₂ 24, MgSO₄ 28, imidazole 5, pH 7.65, $\pi=1100$ mosm/kg_{H2O}. In the hyposmotic ASW NaCl concentration was reduced to obtain $\pi=710$ mosm/kg_{H2O} (~35% reduction of osmolality). All chemicals were purchased from Sigma (Sigma Aldrich, St. Louis, MO, USA).

Statistics

Data are shown as mean values±standard error of the mean. Each data set is derived from at least six individual nematocytes. The significance of the differences was tested using one- or two-way analysis of variance (ANOVA), followed by Dunnet's or Bonferroni's *post-hoc* test, as appropriated. P<0.05 was considered as statistically significant.

Results

Nematocytes isolated from acontia of *Aiptasia mutabilis* are depicted in Figure 1A. The cytoplasm is located around the organoid (nematocyst) and volume modifications, due to crude venom or hyposmotic shock application, are limited to this thin rim. Nematocytes isolated in ASW and treated with crude venom deriving from a population of at least 90 n/µL exhibited morphological changes of the cytoplasm within

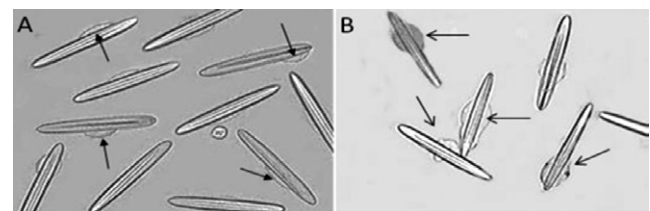


Figure 1. Morphological modifications and cell swelling in 90 n/mL crude venom-treated cells (B) with respect to the control (A). Arrows indicate the cytoplasm, 200× magnification.

10 min of treatment (Figure 1B). In particular, as depicted in Figure 1B, cell swelling was observed.

Viability of 90 n/μL crude venom-treated nematocytes within 10 min of treatment was confirmed by Trypan blue dye exclusion test. Nevertheless, after 30 min of treatment, cell necrosis was detected. On this basis, RVD assessment was performed on nematocytes treated with lower doses of crude venom, not leading to cell necrosis during 30 min of treatment.

Regulatory volume decrease control tests

Figure 2 shows the volume changes of isolated nematocytes in response to a reduction of extracellular osmolality from 1100 to 710 mosm/kg_{H₂O}. Following hyposmotic stress, cells rapidly swelled and after 10 min A/A_0 reached a peak value of 1.083 ± 0.005 . This value was significantly higher than the values measured in isotonic solution during the 1st period ($n=6$, $P<0.001$). Within 20 min, A/A_0 returned to a value (1.001 ± 0.003) significantly lower than the peak value and not different from the values measured during the 1st period, indicating that cells underwent complete RVD.

Regulatory volume decrease in crude venom-treated nematocytes

Figure 3 depicts nematocytes behavior following hyposmotic shock, after pre-treatment with different doses of crude venom (10, 25 or 50 n/μL).

In the first case, nematocytes were pre-treated with a dose of venom corresponding to 10 n/μL (Figure 3A). Following application of the hyposmotic challenge (2nd period), an increase in A/A_0 ratio was observed, corresponding to OP. A/A_0 reached a peak value of 1.080 ± 0.005 within 10 min, a value significantly higher ($P<0.001$) than that of the 1st period (incubation in isotonic solution). Through the 2nd period, A/A_0 ratio gradually fell to 1.005 ± 0.003 after 20 min, a value significantly lower than the peak value ($P<0.001$) but not different respect to that measured during the 1st period, denoting complete RVD. After returning to the isosmotic medium (3rd period), the cell volume was comparable to that observed during the 1st period (0.999 ± 0.022) without a post-RVD regulatory volume increase (RVI). A/A_0 values of treated

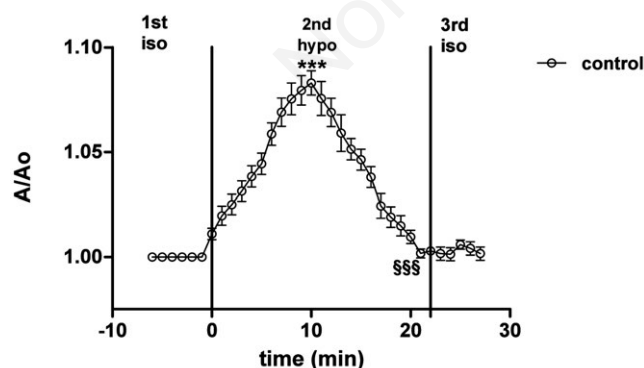


Figure 2. Effect of a hyposmotic challenge on isolated nematocytes. Cell volume, as A/A_0 , is plotted against time. Following exposure to hyposmotic artificial sea water (2nd period), nematocytes rapidly swell as expected for a perfect osmometer. After 10 min, regulatory volume decrease occurs despite the continued presence of a hyposmotic medium, and the initial volume is completely recovered after 20 min. *** $P<0.001$ with respect to A/A_0 in isosmotic artificial sea water (1st period); §§§ $P<0.001$ with respect to the peak A/A_0 value.

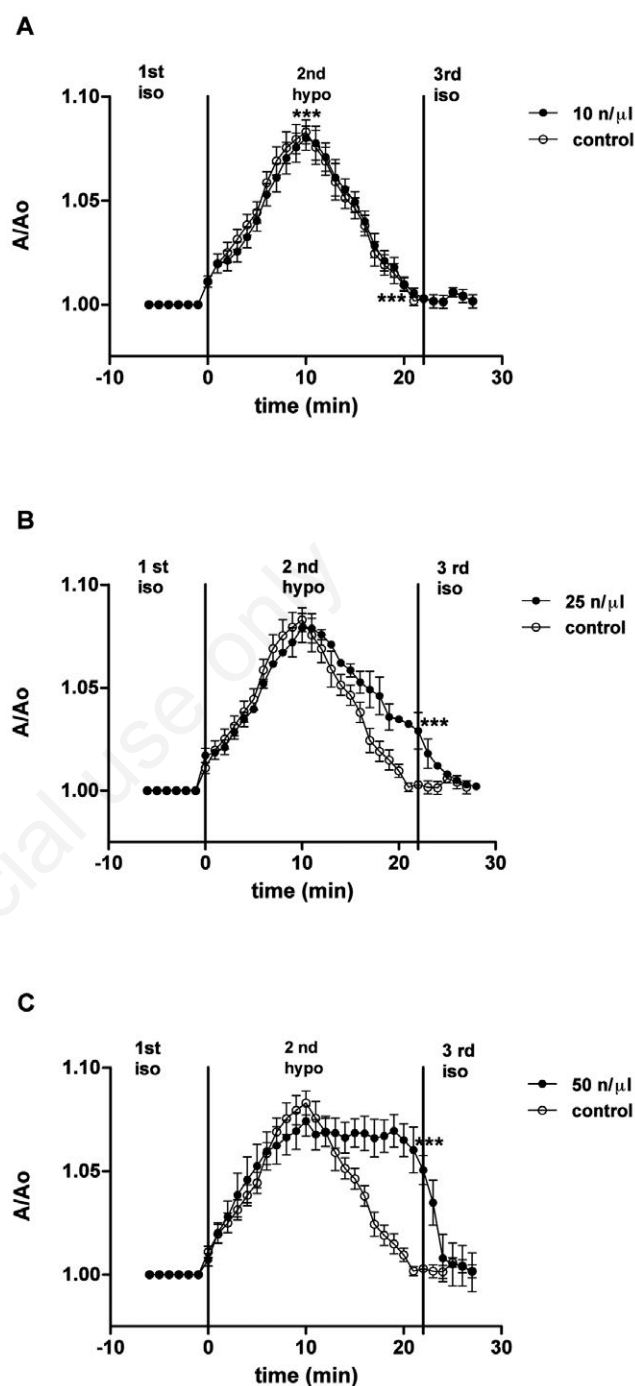


Figure 3. Effect of a hyposmotic challenge on isolated nematocytes pre-treated with different amounts of crude venom, from 10 (A), 25 (B) or 50 (C) n/μL respectively. Cell volume, as A/A_0 , is plotted against time. In each experimental condition cell volume reaches a peak value significantly higher than the value measured before hyposmotic challenge. A) At the end of the 2nd period, cell volume of treated cells decreases to a value significantly lower than the peak value (** $P<0.001$) and not significantly different with respect to the corresponding value of untreated cells. B) At the end of the 2nd period, cell volume of treated cells decreases to a value significantly lower than the peak value (** $P<0.001$) and significantly higher than the corresponding value of untreated cells (** $P<0.001$). C) At the end of the 2nd period, cell volume of treated cells is significantly higher than the value of untreated cells (** $P<0.001$), and not significantly different with respect to the peak value of both treated and untreated cells.

nematocytes were not different from those of control nematocytes, denoting that pre-treatment with 10 nematocysts/ μL crude venom did not affect OP or RVD.

In the second case (25 n/ μL crude venom; Figure 3B), the application of hyposmotic shock induced cell swelling and A/A_0 reached the peak after 10 min (1.079 ± 0.005 ; 2nd period). This value was significantly higher respect to the value observed before the hyposmotic challenge ($P < 0.001$), but not different respect to the corresponding value of control nematocytes, denoting that the OP was not affected by exposure to crude venom. A/A_0 then decreased to control values, being unchanged the hyposmotic external medium, reaching 1.029 ± 0.003 , at the end of the 2nd period. This value was statistically different respect to the peak value and respect to the corresponding A/A_0 value observed in untreated cells (1 ± 0.001 ; end of 2nd period), denoting that a partial RVD occurred. Substitution of hyposmotic solution with an isotonic one (3rd period) induced a further gradual decrease in A/A_0 .

With regard to the third case (50 n/ μL crude venom; Figure 3C), during the 2nd period cell volume significantly increased reaching a peak value of 1.074 ± 0.005 after 10 min of hyposmotic stress application. This value was significantly different respect to the value measured before the hyposmotic challenge ($P < 0.001$) but not different respect to control, indicating that OP was not affected. At the end of the 2nd period A/A_0 reached a value of 1.060 ± 0.003 , not statistically different respect to the peak value, denoting that RVD was completely abrogated. Cell volume then decreased towards control values once the hyposmotic solution was substituted with an isotonic one (3rd period).

In addition, RVD test was performed on 50 n/ μL crude venom-treated nematocytes in the presence of gramicidin as a ionophore (Figure 4).

Cells exposed to an isosmotic solution plus 1 μM gramicidin, did not exhibit significant changes in volume (period 1b) respect to control values. The application of a hyposmotic shock induced a notable increase in A/A_0 , reaching the peak after 10 min (1.085 ± 0.005 , a value significantly higher than that observed in isotonic conditions; $P < 0.001$). At the end of the hyposmotic stress, A/A_0 fell to 1.022 ± 0.003 , a value significantly different respect to both the peak value of treated cells and A/A_0 measured in untreated nematocytes at the end of the 2nd period. These observations denote that gramicidin significantly ameliorated but did not completely restored RVD capability. Upon substitution of the hyposmotic solution with an isotonic one, cell volume further decreased to 1 ± 0.001 (3rd period).

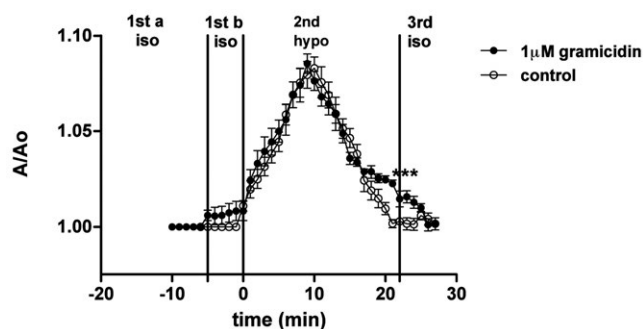


Figure 4. Effect of a hyposmotic challenge on isolated nematocytes pre-treated with 50 n/ μL crude venom, and then exposed to 1 μM gramicidin (period 1a). Cell volume, as A/A_0 , is plotted against time. *** $P < 0.001$ is compared to the peak value of gramicidin-treated cells and to the corresponding value of control cells.

Discussion

Venom extraction from isolated nematocysts is fine strategy for studying jellyfish toxins and is a needed step to learn more about their toxicological features, excluding other tissue-derived compounds.^{13,14} Investigations conducted on nematocysts isolated from *Pelagia noctiluca* have already shown that their venom elicits hemolytic activity on erythrocytes deriving from different sources, with a dose-dependent effect.¹³ Subsequently, it has been further demonstrated that the hemolytic power of this venom strictly depends on erythrocytes cell membrane damage.¹⁴ A pore formation onto cell membrane has been in fact supposed, and, subsequently confirmed using osmotic protectants, which impair the venom-induced hemolytic action.¹⁴

In an attempt to define more in detail the biological activity of *Pelagia noctiluca* crude venom on a cellular level, in the present work a functional parameter, rather than a morphological integrity assessment, has been adopted as a tool to verify the effect of crude venom. For this purpose, the capability of isolated nematocytes to regulate their volume in a hyposmotic medium following exposure to non necrotic doses of crude venom has been evaluated. In this regard, it has been already seen that isolated nematocytes of the Anthozoan *Aiptasia mutabilis* can regulate their volume after exposure to a hyposmotic medium, showing RVD within 20 min of hyposmotic shock.²⁵ Permeability for K^+ and Cl^- is crucial during RVD response, similarly to what already reported for other cell types.¹⁸

The findings of the present investigation show that the exposure of isolated nematocytes to non necrotic doses of *Pelagia noctiluca* crude venom did not impair the osmotic cell swelling (OP) when the cells were submitted to 35% hyposmotic shock. In fact, cell volume reached a peak value within a time frame comparable to that observed for untreated nematocytes. Nevertheless, venom treatment dramatically inhibited the RVD response with a dose-dependent effect. Such observations lead to two considerations: from one hand, aquaporins function, known to be involved in OP,²⁶ presumably was not affected by the venom, whereas, on the other hand, the toxic effect seems to target ion conductances reported to play a major role in RVD phase.¹⁸ That cell membrane transport systems, namely at level of voltage-gated Na^+ and K^+ channels and acid-sensing ion channels, may be affected by marine toxins has been already ascertained.²⁷ In this respect, palytoxin-group toxins (PITX) extracted from the tropical microalga *Ostreopsis ovata*, induces a massive intracellular Na^+ influx via modulation of the Na^+/K^+ ATPase.²⁸ The authors suggested that such Na^+ overload is the crucial step in oxidative stress-induced cell death in human HaCaT keratinocytes. More recently, *Pelagia noctiluca* crude venom has been demonstrated to interfere with the trans-membrane protein band 3 in human erythrocytes, by inhibiting the rate constant for anion transport.²⁹ In particular, the authors hypothesized that crude venom may affect membrane proteins and cytoskeleton, with consequent ionic imbalance, providing the first evidence for a non lytic action of the venom on red blood cells and for a modulation of ion transport.

Cytoskeleton has been previously demonstrated to be essential during RVD in nematocytes isolated from *Aiptasia mutabilis*.²⁵ Therefore, cytoskeleton re-arrangement, which normally follows the OP phase and is needed to restore the initial cellular volume, could be compromised as well by crude venom treatment. In this regard, other investigations have been attempted to link changes in cytoskeleton components with volume changes, and, more interestingly, to verify if drug-induced alterations of cytoskeleton affect cell volume regulation processes.³⁰

In addition, non-lytic doses of crude venom from *Pelagia noctiluca* directly induce mitochondrial trans-membrane potential collapse and generation of reactive oxygen species (ROS) in neuronal-like (SH-SY5Y) cells.¹⁵ Mitochondrial membrane alterations are possibly due to

a pore-forming mechanism, leading to oxidative damage evidenced by both ROS generation and Ca^{2+} decrease. Therefore, an oxidative damage by *Pelagia noctiluca* venom cannot be completely excluded, and is further supported by Marino and co-authors in a model of rat paw edema.¹⁷

The results of the present investigation lead to speculate that *Pelagia noctiluca* crude venom may affect membrane transport systems involved in RVD, rather than the cytoskeleton or the oxidative status of the cell. This hypothesis is supported by the finding that the ionophore gramicidin, associated to crude venom treatment, could at least partially restore RVD capability of isolated nematocytes, obscuring the inhibitory effect of the venom. Gramicidin has been already employed to restore RVD mechanisms when blocked in anthozoan nematocytes.³¹ Therefore, it is reasonable to hypothesize that the RVD inhibition observed here is the consequence of a blockage of ion conductances.

Conclusions

Pelagia noctiluca crude venom, at non-lytic concentrations, impairs RVD normally observed following hyposmotic shock in *Aiptasia mutabilis* nematocytes, without affecting the OP. Since RVD requires the activation of both K^+ and Cl^- conductive pathways along with KCl cotransporter (KCC) under Ca^{2+} control and an intact cytoskeleton,^{20,21,25,32} it is reasonable to suggest that venom treatment may have altered cell function at level of channels, transporters, cytoskeleton and/or signaling. This study suggests that the inhibition of RVD by crude venom could be the consequence of an inhibition of ion fluxes, that were effectively restored by the ionophore gramicidin. The comprehension of the molecular mechanism of action of *Pelagia noctiluca* venom is extremely useful to predict its impact on human health, and, on the other hand, may open the way to possible applications of its active components.

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