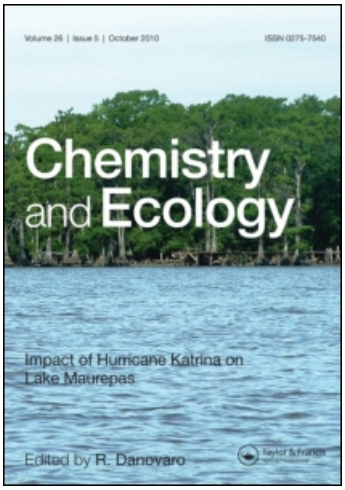

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Effect of crude venom from nematocysts of *Pelagia noctiluca* (Scyphozoa) on spread discharge of acontia of *Calliactis parasitica* (Anthozoa)

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The present investigation was aimed at assessing the toxic power of crude venom from *Pelagia noctiluca* nematocysts on spread discharge of *in situ* acontial nematocytes. Spread discharge, under 553 mM NaSCN plus 10 mM Ca^{2+}, consists of the protrusion and firing of the nematocytes all along the acontial filament. Acontia from *Calliactis parasitica* were treated with the discharging solution under a constant flow as a control condition. In a separate experiment acontia were firstly treated with *Pelagia noctiluca* crude venom aliquots and then with the discharging solution to prove spread discharge. Crude venom blocked the discharge with a dose-dependent irreversible effect. From these results arises that crude venom of *Pelagia noctiluca* nematocysts can affect the spread discharge with a mechanism that is still unknown. It could be hypothesized that crude venom can block gap junctions probably involved in this cellular communication. Nevertheless either a damage of cells involved in nematocyte activation or even the inhibition of Ca^{2+} influx, needed for discharge triggering, could be even suggested. Other studies will verify crude venom action mechanisms and may confirm the spread discharge as an interesting biological assay aimed at toxicological investigations.

Keywords: nematocyst; spread discharge; toxins; biological assay; Cnidaria

1. Introduction

Nematocysts are a secretory product of highly specialized stinging cells, termed nematocytes, that reside mainly in the tentacles, acontia and acrorhagi of Cnidaria specimens. Cnidaria obtain food and perform both defensive and aggressive strategies by means of nematocysts. Such peculiar organoid consists of a capsule wall that contains the capsule fluid and an inverted tubule. When exposed to an appropriate stimulus the tubule is explosively everted and releases the capsule fluid in which various toxins are stored. Such rapid process is called discharge and the underlying mechanisms are still not completely clarified. Discharge of *Hydra* isolated nematocysts has been investigated by Holstein and Tardent [1] capturing the discharge occurring within 3 msec by means of ultrahigh-speed cinematography. The discharge, known as one of the most rapid exocytosis processes so far discovered develops through some steps as the opening of the operculum, the

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evagination of the tubule with spines, the volume decrease of the capsule and then delivery of toxins contained in the capsular fluid.

Cnidarians possessing acontia are named Acontiaria. As described by Fautin and Mariscal [2] acontia are structures resembling mesenterial filaments, attached at one end to mesenteries while the other end is free. In addition they contain nematoblasts and several types of gland or secretory cells. Acontia function has not been completely established, although the nematocytes, located all along the filament, and the fact that they are often extruded when the sea anemone is disturbed, support a role in defense. Acontia may be extruded through cinclides or mouth upon contraction of the specimen. However they are semiautonomous organs, possessing muscle, nerve and sensory cells and can be withdrawn back inside the body after use.

Both chemical and mechanical stimuli concur in inducing the *in situ* discharge of nematocysts [3] with the contribution of supporting cells surrounding the nematocytes. In particular, in Anthozoans discharge mechanisms include the intervention of different types of chemoreceptors located in the supporting cells while the mechanoreception is supplied by specialized structures of the nematocyte itself and a process of transduction finally induces the discharge of the nematocyst [4,5]. Mechanoreception is performed by specialized nematocyte structures, such as the ciliary cone. Once chemically stimulated, the supporting cell modulates the sensitivity of the nematocytes to mechanical stimuli, so that nematocytes and supporting cells can be considered as a whole functional complex termed CSCC (Cnidocyte Supporting Cell Complex) [6].

The ciliary cone is located at the apex of the nematocyte and consists of a modified cilium surrounded by stereocilia. Mire-Thibodeaux and Watson [7] described two types of hair bundles on the tentacle surface of *Haliplanella luciae*: in the CSCC kinocilium arises from the nematocyte and while stereocilia from the adjacent supporting cells; in the sensory cell-supporting cell complex (SNSC) the small diameter stereocilia arise from several supporting cells and the kinocilium and large diameter stereocilia from the sensory cell. Successively La Spada and coworkers [8] showed that in nematocytes from the Anthozoan *Aiptasia diaphana* kinocilium and smaller stereocilia arise from the same cell apex.

As described by Santoro and Salleo [9,10] in acontia of *Calliactis parasitica* (Anthozoa) treated with isosmotic NaSCN plus 10 mM Ca^{2+}, the discharge response starts at one end of the filament and spreads sequentially along its entire length. The mechanism that underlies the spreading of the SCN^{-}-induced discharge along the acontium of *Calliactis parasitica* is completely unknown. The sequential spreading of cell responses suggests that some signal is transmitted from cell to cell thereby exciting sequentially the entire cell population. Furthermore Salleo et al. [11] demonstrated that the discharge of *in situ* acontial nematocytes from the Anthozoan *Aiptasia diaphana* is triggered by a Ca^{2+}-dependent release of NO from supporting and/or sensory cells. The discharge of acontial nematocytes is also thiol-induced as described by La Spada et al. [12].

An interesting feature linked to the nematocyst discharge is the toxicology of the capsular fluid. Cnidarians are known to contain a variety of chemical substances exhibiting biological activity on various cell types and tissues. In particular it has been reported that peptides from Cnidarians act as neurotoxins or cytolysins [13,14].

With the aim of verifying toxicological characteristics of venom compounds extracted from various specimens both terrestrial and aquatic, many biological assays have been set up. Amongst these the hemolytic and cytolytic tests are the most adopted [15–17]. Data from both such tests show that many toxins act in a pore-forming manner, as described by investigations concerning sea anemone toxins [18], *Physalia physalis* [19] and *Actinia equina* venom [20].

Amongst Cnidarians the Scyphozoan *Pelagia noctiluca* has become quite interesting in the latter years owing to its blooming in the Strait of Messina (Italy) and in general in the whole Mediterranean Sea. Such a blooming is well known, abundant and with no seasonality in the Strait of Messina, thus compromising both tourism and fishing activities. *Pelagia noctiluca* specimens,

measuring from 3 to 12 cm in diameter, possess 8 tentacles arising from the bell edge and 4 oral arms studded with warts of nematocysts.

With regard to toxicological aspects of nematocysts and on the basis of what has already been reported by Santoro and Salleo [9,10] about *in situ* nematocysts discharge, we aim to clarify the toxicological features of crude venom from isolated nematocysts of *Pelagia noctiluca* (Scyphozoa) by employing the spread discharge of *in situ* nematocytes in acontia of *Calliactis parasitica* (Anthozoa) as an original biological assay.

2. Materials and methods

2.1. *Venom preparation*

2.1.1. *Specimens collection*

Pelagia noctiluca specimens were collected daily in the Strait of Messina along the Sicilian coast during spring and summer 2006. Once collected the animals were immediately used for nematocysts isolation not being maintained in aquaria as Anthozoans.

2.1.2. *Nematocysts isolation*

Nematocysts from oral arms of *Pelagia noctiluca* were isolated according to Salleo and co-workers [21]. Briefly, the oral arms, once excised, were placed in cold distilled water for 2 hours to deliver undischarged nematocysts after osmotic lysis of nematocytes. Thus holotrichous-isorhiza nematocysts, classified according to Mariscal [22], were obtained. The nematocysts were repeatedly washed in distilled water (refrigerated centrifuge, ALC PK 120R, 4000 *g*, 5 min) and filtered through a plankton net of less than 0.1 mm mesh to discard debris.

2.1.3. *Extraction of crude venom*

Before use the nematocyst samples were thawed and filtered again. A drop of such obtained nematocyst suspension was placed on a glass and observed under a light microscope to verify morphological integrity. The nematocyst population was then counted by means of a Burker chamber and 80, 50, 25, 12.5 nematocysts/μl suspensions were considered. In order to extract the capsular fluid, aliquots of the above mentioned suspensions in ASW (artificial sea water) underwent sonication on ice (Sonoplus, 30 times, 20 sec at 20 kHz). The crude extract was then separated from crushed capsules by refrigerated centrifugation at 4000 *g* for 10 min. The obtained venom was kept at 4°C until use, generally within a few hours, or alternatively stored at −20°C or −80°C.

2.2. *Assay upon acontia*

Acontial filaments, approximately 15 mm in length, were excised from specimens of *Calliactis parasitica* collected in the Strait of Messina (Italy) at 20–30 m in depth, maintained in aquaria at 20–25°C and weekly fed with prawn meat. The tissue, repeatedly washed with low Ca^{2+}-concentrated ASW was placed in a glass channel (Figure 1) (4 mm wide, 75 mm long), on the bottom of which was a reticle that allowed an easy evaluation of the transmission speed of discharge by measuring the time interval between the discharge at the first and at the last line of the grid.

The acontial filament was straightened and fixed by penetrating its cut ends with two *Opuntia* spines. The tissue was neither stretched nor slackened. If some nematocysts discharged during

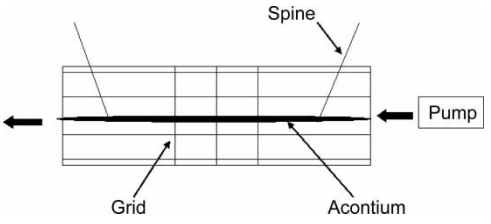

Figure 1. Scheme of the glass employed to detect the discharge of *in situ* nematocytes along the acontium. The latter is fixed in the centrally placed channel. The test solutions are perfused through the channel by means of a constant flow pump.

this procedure the acontium was discarded. The channel was filled with ASW. In this control condition both slow and coordinated contractile activity of the acontium and ciliary movement could be clearly observed. A constant flow pump (Sage Instruments, mod. 351) was used to keep the experimental solutions, flowing at a speed of 16 mm/sec, so that solution replacement occurred within a few seconds. The solution was not recirculated.

At least 10 acontia were treated with discharging solution containing 553 mM NaSCN plus 10mM $CaCl_2$ to assess spread discharge in control conditions. As a second step the fixed acontia were treated with crude extract from different suspensions (80, 50, 25, 12.5 nematocysts/μl respectively), at least 10 acontia for each test. Such treated filaments were incubated in a damp room at 21–24°C for 30 min. Successively, crude extract was replaced with ASW and the tissue could be adequately washed. Then the discharging solution could be applied and the time of discharge was measured.

Acontia discharge was checked under an inverted microscope (100 × magnification, Photozoom Cambridge Instrument). Only the discharge of microbasic-mastigophore nematocysts was taken into account since the smaller basitrichs are more difficult to detect at low magnification.

A solution was considered to be effective when it induced the discharge of almost all nematocysts along the tissue, performing total discharge within 2 min.

2.3. *Miscellaneous*

Aliquots of crude venom were employed to measure the protein content by BCA protein assay (Pierce). Isosmotic ASW (Artificial Sea Water) had the following composition in mM: 520 NaCl, 9.7 KCl, 10 $CaCl_2$, 24 $MgCl_2$, 28 $MgSO_4$, 5 imidazole, pH = 7.65, π = 1100 mOsm/kg_{H2O}. Low Ca^{2+}-concentrated ASW contained 0.01 mM $CaCl_2$. The osmotic pressure of all solutions was measured by an osmometer (Fiske OS). pH was measured by a pH-meter Orion. Chemicals were purchased from SIGMA (Milan, Italy).

2.4. *Statistics*

The data are presented as mean±SE (Standard Error). Each data set is derived from at least three separate days. The significance of the differences was tested using a Student's t test, $p < 0.05$ was considered statistically significant.

3. Results

A 553mM SCN^- solution containing 10 mM Ca^{2+} induced mass discharge in all tests within 180±8.4 sec and discharging speed was 9.6±0.5cm × 10^{-3} sec^{-1}. The first response of the

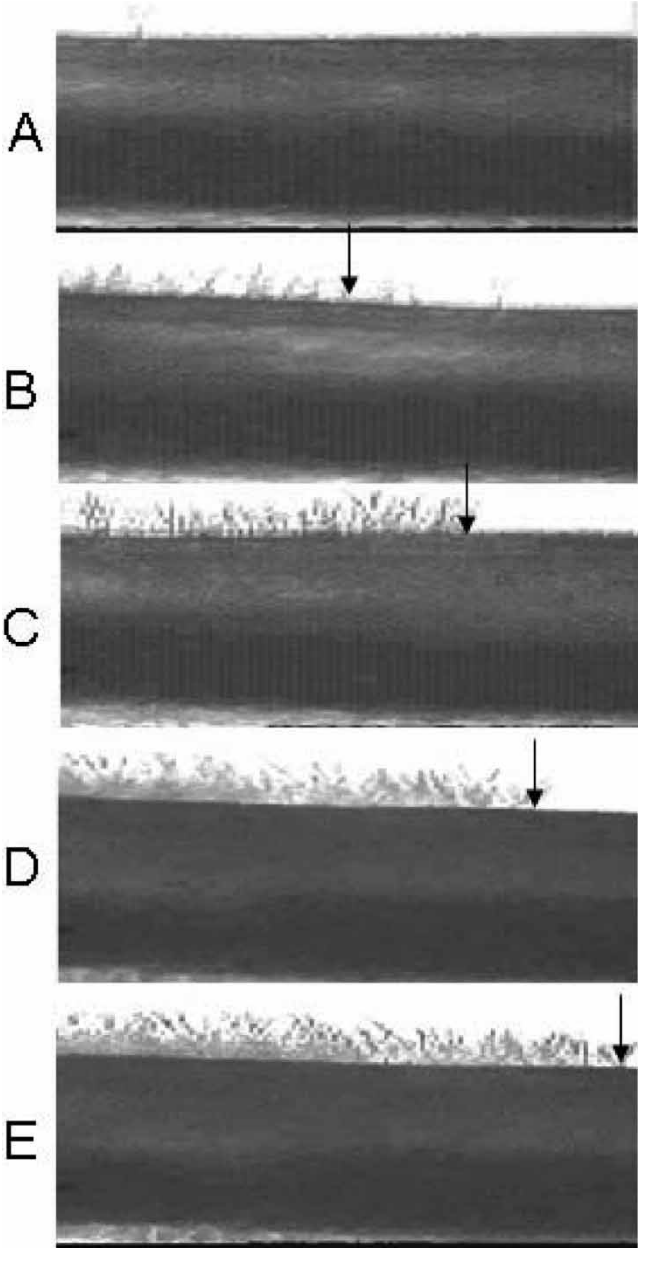

Figure 2. (A) untreated acontium observed under an inverted microscope (100× magnification); (B, C, D, E) discharge of *in situ* nematocytes along the acontium at, respectively, 100 sec (B), 130 sec (C), 160 sec (D) and 180 sec (E) after treatment with the discharging solution (553mM SCN^- plus 10mM Ca^{2+}). Once the discharge occurred, fully everted tubules were detected all along the acontium surface (arrows).

tissue to the discharging solution was the protrusion of nematocytes at the surface of the acontial filaments. The protrusion started at one cut end of the acontium and proceeded along its length toward the other end. Thereafter, the nematocytes began to fire at the end where protrusion had started and discharge of all nematocytes proceeded sequentially in the same direction without interruption (Figure 2).

Table 1. Spread discharge in both treated and untreated acontia.

Treatment	Time of discharge (sec)	Discharge after ASW	n
Control	180 ± 8.4		10
0.075 μg/μl crude extract	180 ± 12		8
0.15 μg/μl	400 ± 16.68	+	10
0.30 μg/μl	435 ± 6	+	10
0.48 μg/μl	No discharge	−	10
0.48 μg/μl MW < 30000Da	188 ± 10		8
0.48 μg/μl MW > 30000Da	No discharge		8

Crude venom doses are reported as protein content (μg/μl). Time of discharge is reported as mean±SE. + represents discharge detected under a second SCN^- application after washing of treated acontia with ASW.

Treatment of acontia with crude venom from 12.5 n/μl *Pelagia noctiluca* nematocysts (0.075 μg/μl protein) did not affect discharge mechanisms since the spread of nematocytes firing occurred within 180±12 sec as shown in Table 1.

After 30 min treatment with crude extract from 25 n/μl (0.15 μg/μl protein) and 50 n/μl *Pelagia noctiluca* nematocysts (0.30μg/μl protein) the acontia were not damaged and spread discharge occurred respectively within 400±16.68 sec and 435±6 sec of SCN^- application. No difference in speed of spread discharge was detected towards control conditions. Time discharge in these conditions was significantly higher than in the control treatment ($p < 0.05$) (Table 1).

The treatment of acontia with crude extract from 80 n/μl *Pelagia noctiluca* nematocysts (0.48 μg/μl protein), though not damaging the tissue, blocked the spread discharge at all, since neither nematocytes protrusion nor sequential firing was detected under microscope checking. The constant flow of a washing solution (ASW) upon treated acontia for at least 10 min did not restore spread discharge under a further SCN^- application. Discharging capability was instead recovered after washing acontia previously treated with crude extract from alternatively 12.5 n/μl, 25 n/μl and 50 n/μl. No difference in discharge speed was detected in thus treated acontia towards control conditions.

A first separation (microcon 30000Da cut-off) of crude venom from a 80 n/μl suspension let us obtain two different fractions successively tested upon acontia. Low PM fraction (PM<30000Da) did not block spread discharge, occurring within 188 sec of SCN^- application similarly to control conditions, while high MW fraction (PM>30000 Da) completely obscured discharge mechanism even not damaging the tissue.

Table 1 summarizes discharging time in acontia both in control conditions and under treatment with various crude venom concentrations.

4. Discussion

Discharge mechanism of both isolated and *in situ* nematocytes is still not completely clarified. However a transduction process involving both chemoreceptors and mechanoreceptors has been identified. An interesting study has been performed by Santoro and Salleo [9,10] and Salleo et al. [23] concerning the discharge of *in situ* acontial nematocytes of *Aiptasia diaphana* and *Calliactis parasitica.*

Nevertheless it has been proposed that in acontia the activation of spread discharge is triggered by an inflow of Ca^{2+}, since the treatment of the acontium with either Ca^{2+}-free media or with Ca^{2+} channel blockers such as La^{3+}, Co^{2+} or Cd^{2+} prevents the discharge. It is uncertain whether

Ca^{2+} acts directly on the nematocyte or, rather, on the supporting cell. In any case Ca^{2+} is not expected to act directly on the nematocyst since it has a well known inhibitory effect on the discharge of isolated nematocysts [24]. The Ca^{2+}-dependent discharge is induced experimentally by treating the tissue with NaSCN.

As concerns toxicological features about *Pelagia noctiluca* venom, interesting investigations have recently been performed by Marino and coworkers [25–27] showing the hemolytic power of crude extract from isolated nematocysts upon red blood cells from different sources.

In the present paper we have tested the possible crude venom effect on spread discharge investigated by Santoro and Salleo [10]. In particular from our results arises that spread discharge is slackened by treatment with both 0.15 μg /μl and 0.30 μg/μl crude extract while it is inhibited by treatment with 0.48 μg/μl crude venom.

As reported in a previous paper [10], 1-octanol prevented both K^+ induced-mass discharge and SCN^- induced-spread discharge. Octanol is known to uncouple the gap junctions or other permeable junctions as described in *Obelia geniculata* [28] where the reversible uncoupling of the gap junctions between photocytes and supporting cells has been observed. So octanol effect upon spread discharge along the acontia would suggest permeable junctions, possibly between supporting cells and nematocytes, may be involved in this process.

Gap junctions are the most common form of intercellular communication and their role in Invertebrates is strongly supported. Recent investigations show that gap junctions have similar physiological properties in different tissues from both Vertebrates and Invertebrates [29].

Gap junctions have been described in many mechanosensorial tissues and in ciliate cells in some Invertebrates [30,31]. Mire and colleagues [32] have described gap junctions on tentacles of *Haliplanella luciae* involved in discharge induced by vibration of kinocilium due to the prey movement. Thus activated supporting cells transmit signal to the sensorial cells and then to the nematocyte, leading to discharge. The same authors have identified connexin 43 (MW = 46kDa) and innexin has been described in other Invertebrates [33].

On the basis of what has been reported by Phelan and coworkers [33] the crude extract employed for our experiments could affect gap junctions with a mechanism different from that of the alcohols 1-octanol or heptanol. Crude venom, containing proteins, may bind to gap junctions thus inducing conformational changes. On the other hand the irreversible effect of crude venom upon acontia discharge, contrarily to the reversible effect observed in 1-octanol tests, may even support the different action mechanism.

Our observations suggest some hypothesis to explain the failure in spread discharge of crude venom treated acontia. The crude venom may: (i) block calcium influx in cells; (ii) affect gap junctions; (iii) damage cell membrane by a pore forming mechanism.

With regard to the first hypothesis, crude venom induced a complete inhibition of discharge probably due to $[Ca^{2+}]<0{,}01$mM not enough to trigger the discharge. Since the effect of Cnidarian toxins upon ion channels and/or transports at concentrations not inducing cell death has been already shown [13], it is reasonable that crude venom from *Pelagia noctiluca* nematocysts may affect ion channels thus leading to no detectable discharge. In treated acontia calcium channels blocking may occur in supporting cells rather than in nematocytes.

The second hypothesis concerns the role of gap junctions. In particular the absence of spread discharge under crude venom treatment is similar to what is observed under octanol treatment, supporting the involvement of gap junctions and revealing that crude venom components can act as uncouplers.

The third hypothesis finally suggests a cell membrane damage owing to the crude venom leading to an increase in Ca^{2+} permeability and then to cell death. It has been already described that toxins may act as pore forming proteins. The mechanism consists in binding and insertion of toxin molecules into the plasma membrane followed by oligomerization to form trans membrane pores [34]. Edwards et al. [19] have described that an increase in intracellular Ca^{2+} and the

damage of membrane, detected under electron microscope, is related to the different crude venom concentrations.

As shown in this paper the discharge blocking is induced by crude venom concentrations higher than 0.30 $\mu g/\mu l$. Similar doses were also effective in provoking lysis of red blood cells as recently reported by Marino et al. [27]. In particular thus treated erythrocytes undergo a notable swelling before lysis, suggesting a membrane damage.

On the other hand red blood cells treated with polyethylene glycol do not hemolyze after crude venom application (Marino et al., unpublished). Similar observations have been reported by other authors employing osmotic protectants to counteract venom effects. So it is reasonable that even in acontia of *Calliactis parasitica* crude venom from *Pelagia noctiluca* may act damaging supporting cell membrane, preventing discharge.

To support such hypothesis electron microscope observations are needed to possibly detect cell membrane changes due to crude venom treatment. Moreover, further immunohistochemical investigations could confirm gap junctions in acontia. Nevertheless, the spread discharge, as an interesting biological phenomenon, provides an original assay to perform toxicological investigations.

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