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Evidence for Aquaporin-mediated Water Transport in Nematocytes of the Jellyfish *Pelagia noctiluca*

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Key Words

Water transport • Aquaporins • Osmotic swelling • Volume regulation • Nematocytes • Jellyfish • *Pelagia noctiluca*

Abstract

Nematocytes, the stinging cells of Cnidarians, have a cytoplasm confined to a thin rim. The main cell body is occupied by an organoid, the nematocyst, containing the stinging tubule and venom. Exposed to hypotonic shock, nematocytes initially swell during an osmotic phase (OP) and then undergo regulatory volume decrease (RVD) driven by K^+, Cl^- and obligatory water extrusion mechanisms. The purpose of this report is to characterize the OP. Nematocytes were isolated by the NaSCN/Ca^{2+} method from tentacles of the jellyfish *Pelagia noctiluca*, collected in the Strait of Messina, Italy. Isolated nematocytes were subjected to hyposmotic shock in 65% artificial seawater (ASW) for 15 min. The selective aquaporin water channel inhibitor $HgCl_2$ (0.1-25 μM) applied prior to osmotic shock prevented the OP and thus RVD. These effects were attenuated in the presence of 1mM dithiothreitol (DTT), a mercaptide bond reducing agent. $AgNO_3$ (1 μM) and TEA (tetraethylammonium, 100 μM), also reported to inhibit water transport, did not alter the OP but significantly diminished RVD, suggesting different modes of action for the inhibitors tested. Based on estimates of the nematocyte surface area and volume, and OP duration, a relative water permeability of ~10^{-7} cm/sec was calculated and the number of putative aquaporin molecules mediating the OP was estimated. This water permeability is 3-4 orders of magnitude lower in comparison to higher order animals and may constitute an evolutionary advantage for Cnidarian survival.

Introduction

Fluid homeostasis is common to all animals and plants and can be altered by diverse factors including hydration, diet, environment and health. Trans-membrane water movement may be achieved by simple lipid bilayer diffusion or by water channels, referred to as aquaporins [1, 2], or by both. Osmotically driven water movements, along with ion fluxes, underly the physiological

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mechanisms regulating fluid homeostasis in most cells [3, 4]. Volume regulation under anisosmotic conditions is essential to cell survival and counteracts changes in medium osmolarity. When exposed to a hyposmotic medium, cells undergo osmotic swelling, known as osmotic phase (OP) involving water permeation mechanisms mentioned above. Depending on the species and cell types, cell volume returns either immediately or slowly to control values due to regulatory volume decrease (RVD). This homeostatic response has been mostly investigated in mammalian cells but also in cells of lower vertebrates and invertebrates. Cell volume regulation has been observed in numerous cell types, such as astrocytes [5], erythrocytes from different sources [6, 7], lymphocytes [8] and sperm [9]. RVD was also studied in invertebrate cells [10-16], although details of its mechanisms are still missing.

Among marine invertebrates, the stinging cells isolated from tentacles and mesenterial filaments (acontia) of jellyfish and sea anemones, respectively, are a suitable experimental model for cell volume regulation studies. These highly specialized cells produce an organoid called the nematocyst that occupies most of the interior of the cell, confining the cytoplasm to a thin rim. The nematocyst is encased in a three-layered capsule wall containing a tubule and venoms capable of being delivered under appropriate physicochemical stimuli (Fig. 1). La Spada and co-workers [12-15] have investigated the RVD mechanisms in isolated nematocytes, but studies on the OP after hyposmotic challenge are still missing.

The present study was designed to investigate the OP in *P. noctiluca* nematocytes subjected to hyposmotic shock in 65% ASW. The purpose of the study was to demonstrate the presence of putative water channels, not shown to be present in these cells thus far, by using a set of commonly known aquaporin inhibitors. Mercury chloride ($HgCl_2$), a selective inhibitor of aquaporins (AQP) [17-20] was tested and its effects compared with those of $AgNO_3$ [18, 21, 22] and TEA [18, 23-25]. Calculations based on coarse physical parameters of *P. noctiluca* nematocytes and the OP duration yielded a low relative water permeability of $\sim 10^{-7}$ cm/sec, compared to most species with $\sim 10^{-3}$ cm/sec, an apparent evolutionary adaptation for Cnidarian survival.

Materials and Methods

Specimens collection

Specimens of *P. noctiluca* (Anthozoa) were collected during Spring-Summer 2011 in the Strait of Messina (Italy) and immediately used for isolating nematocytes.

Nematocytes isolation

Nematocytes, classified as eurytele according to [26], based on the spiny tubule ejected upon discharge of the capsule, were isolated from tentacles of *P. noctiluca*, by treatment with an isosmotic solution of 605 mM NaSCN containing 0.01 mM Ca^{2+} as described elsewhere [27]. Briefly, tentacles, once excised from the umbrella of the jellyfish, were repeatedly washed with low Ca^{2+} ASW (see composition under Experimental Solutions) to remove mucus and then treated with isosmotic 605 mM SCN allowing nematocyte extrusion from the tissue. Substitution of the SCN- solution first with a Ca^{2+}-free ASW and then with complete ASW permitted cell isolation and restoration to physiological conditions. Isolated nematocytes, checked under a light microscope to ensure morphological integrity and exclude cell shape damage, were kept at 14-16 °C for 1 h and used within 3 h from isolation for cell volume regulation tests.

RVD tests

Isolated nematocytes, in isosmotic ASW, were placed on a glass slide and allowed to adhere for one h. Afterwards, double sided tape was placed between the slide and a coverslip for substitution of experimental media during the entire test. A flow system for medium replacement was used. Cell volume experiments were performed on a nematocyte chosen for its strong adhesion to the slide. To assess the response of nematocytes to anisosmotic shock, the RVD control consisted of three incubation periods: 1st period, isosmotic ASW (π=1,100 mosm/Kg_{H2O}) for 5 min; 2nd period, hyposmotic ASW (65 % ASW, π=710 mosm/Kg_{H2O}) for 15 min; 3rd period, isosmotic ASW for 5 min. In the experiments where the effect of a drug was studied, this was introduced in the second period and remained throughout the remainder periods. Thus the experimental protocol was performed as follows: 1st period, isosmotic ASW for 5 min; 2nd period, isosmotic ASW plus inhibitor for 5 min; 3rd period, hyposmotic ASW for 15 min; 4th period, isosmotic ASW for 5 min. About 30 images/nematocyte were taken with a phase contrast microscope (Leica DMLS, Milan, Italy) connected to a video camera (JVC model TK-1180E) and to a computer (Macintosh) equipped with suitable software (Apple Video Player, Adobe Photoshop). To assess cell volume changes as a function of time, the cross sectional area of each recorded image was successively measured (Aldus FreeHand, US) and the ratio of A/A_0 calculated, where A and A_0 represent the cross sectional areas at given time intervals and of the untreated nematocyte at t=0, respectively.

Experimental solutions and reagents

Isosmotic ASW had the following composition (mM): NaCl 520, KCl 9.7, $CaCl_2$ 10, $MgCl_2$ 24, $MgSO_4$ 28, imidazole 5, pH 7.65, π=1,100 mosm/Kg_{H_2O}. In the hyposmotic shock solution (65 % ASW), the NaCl concentration was reduced to π=710 mosm/Kg_{H_2O}. Stock solutions for drugs were dissolved in distilled water. Each reagent was then added to the experimental solution to yield the indicated final concentration. All chemicals were purchased from SIGMA (Milan, Italy).

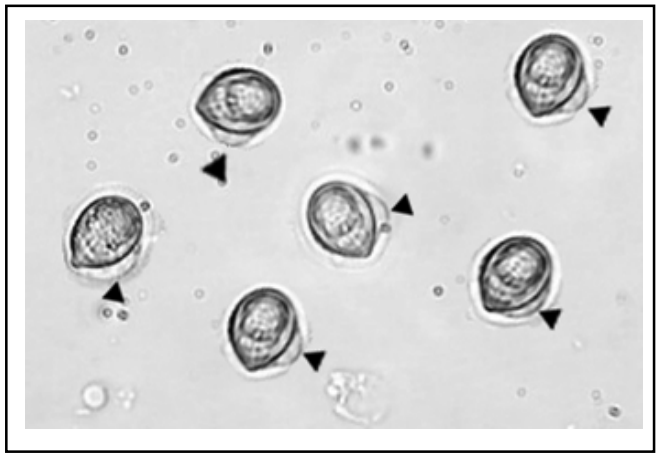

Fig. 1. Visualization of eurytele nematocytes from *P. noctiluca*. The figure shows a light microscopy picture of eurytele nematocytes isolated from tentacles of *P. noctiluca* by a chemical non-enzymatic method. The cytoplasm is located in a thin rim (arrowhead), while the nematocysts occupy most of the cell volume (200x magnification).

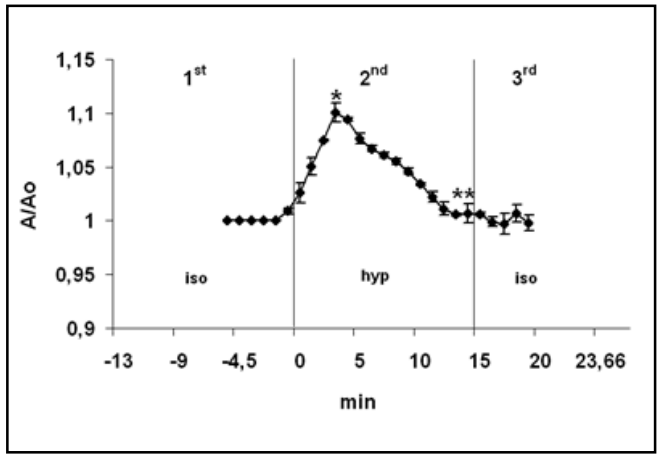

Fig. 2. Relative nematocytes volume when exposed to hyposmotic challenge. The ratio of A/A_0 of untreated nematocytes was measured as a function of time after exposure to isosmotic ASW (1st and 3rd periods) and to 35 % hyposmotic shock (65 % ASW, 2nd period).*($p<0.001$) Peak value significantly higher than that of the first phase of incubation in isosmotic solution. **($p<0.001$) data significantly different from the peak value.

Statistics

Data are shown as mean values ± standard error of the means (S.E.M.). Each data set is derived from at least five individual experiments performed on cells isolated from animals collected weekly. Significance of differences was tested by a Student's t-test. A probability of $p<0.05$ was considered statistically significant. Estimation of the cross sectional area changes (A/A_0) was calculated from the peak value of cell volume during the hyposmotic shock and the last value at the end of the same period.

Results

Control RVD tests

Treatment of tentacles with 605 mM NaSCN plus 0.01 mM Ca^{2+} produced a strong contraction with release of isolated nematocytes (Fig. 1). These cells, 10-12 μm long and with a cytoplasm located in a thin rim, were anatomically and functionally intact as assessed by trypsin-induced discharge of the nematocyst [28].When exposed to hypotonic ASW, only the cytoplasm rim expanded and not the nematocyst occupying 85% of the nematocyte's volume. Furthermore, no evidence has been found that the nematocyst's wall was altered by hyposmotic shock [12].

The control for all drug-treated samples shown in Fig. 2 provides mean values and is a typical response always observed with untreated nematocytes. Upon hyposmotic shock in 65% ASW (2nd phase in 65 % ASW, Fig. 2), an increase in the nematocyte A/A_0 ratio, i.e. the cell volume, was observed, with the osmotic phase (OP)

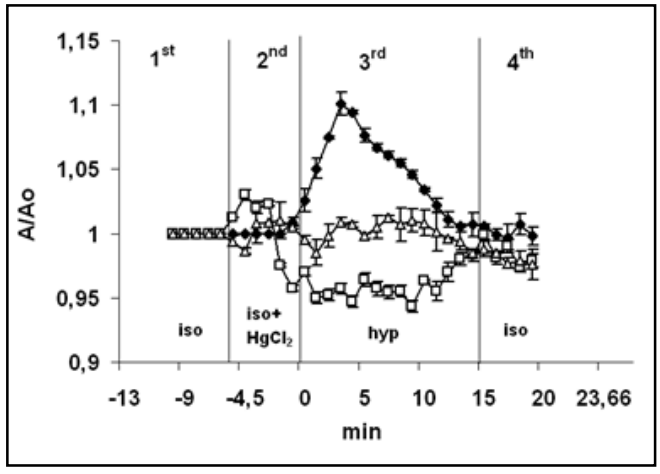

Fig. 3. Effect of $HgCl_2$ on *P. noctiluca* nematocyte's volume regulation**.** The effect of 25 μM (-□-) and 5 μM $HgCl_2$ (-△-) on the A/Ao ratio of isolated nematocytes were compared to that of untreated cells (-◆-). As specified in Materials and Methods, nematocytes were exposed to 25 or 5 μM $HgCl_2$ under continuous flow conditions, while individual A measurements were taken. Note also that in contrast to Fig. 2, in this experiment there are 4 periods: The 1st, 2nd and 4th periods were isosmotic flow, whereas the 3rd period corresponds to 65% ASW hyposmotic challenge. $HgCl_2$ was added at the beginning of the 2nd period and remained throughout.

reaching a peak value of 1.1008 ± 0.009 within 4 min, a value significantly higher than that of the 1st phase of incubation in 100 % ASW ($p<0.001$). Through the 2nd period, the A/A_0 ratio gradually fell, reaching 1.0068±

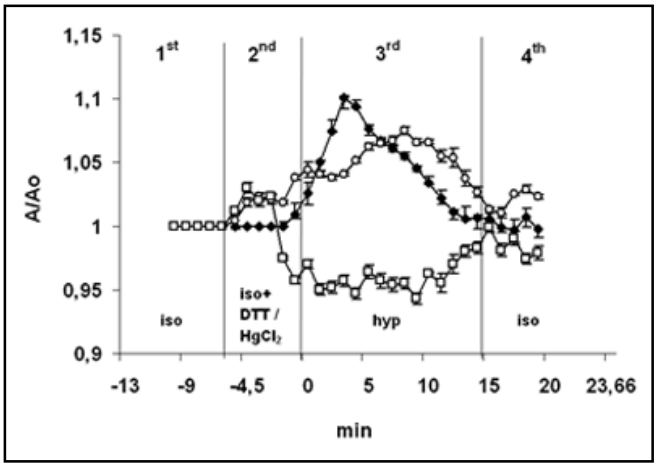

Fig. 4. Effect of a higher dose of $HgCl_2$ and DTT on *P. noctiluca* nematocyte's volume regulation**.** The time course of the A/A_0 ratio was determined in nematocytes simultaneously treated with 25 µM $HgCl_2$ and 1mM DTT (-○-). Comparison with data of Fig. 2 for untreated (-◆-) and of Fig. 3 for 25 µM $HgCl_2$-treated nematocytes (-□-). As specified in Materials and Methods, nematocytes were exposed to 25µM $HgCl_2$ ± 1 mM DTT under continuous flow conditions while individual A measurements were taken. Note also that like in Fig. 3, there were 4 periods: The 1st, 2nd and 4th periods were isosmotic flow, the 3rd period 35 % hyposmotic. Both 25 µM $HgCl_2$ and 1 mM DTT were added at the beginning of the 2nd period and remained throughout.

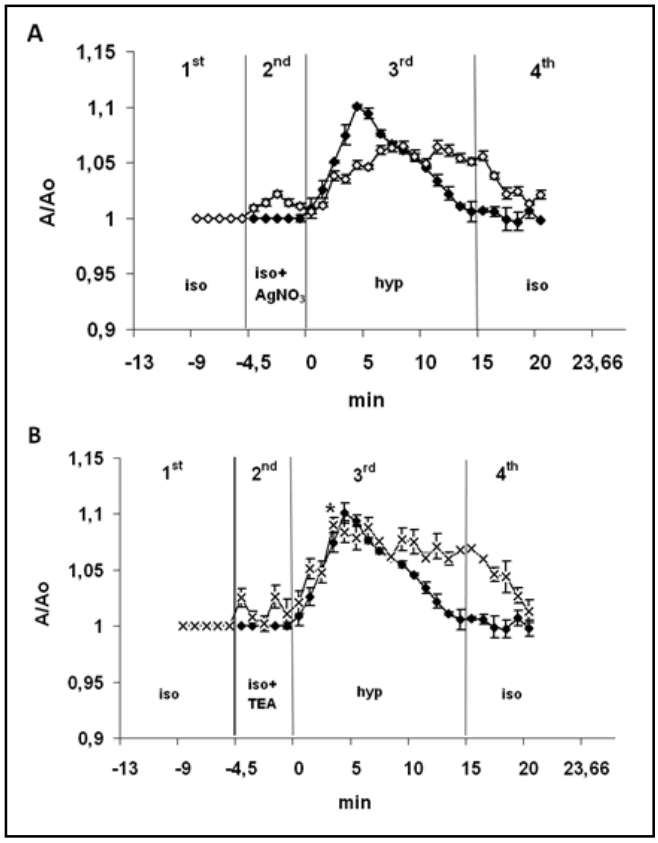

Fig. 5. Effect of $AgNO_3$ (A) and TEA (B) on *P. noctiluca* nematocyte's volume regulation. The time course of OP and RVD in 1 µM $AgNO_3$-treated (A) (-◇-) and in 100 µM TEA-treated nematocytes (B) (-◆-) was compared to data in Fig. 2 for untreated nematocytes (-x-). As specified in Materials and Methods, nematocytes were exposed to the inhibitors under continuous flow conditions while individual A measurements were taken. Like in Fig. 3, there were 4 periods: The 1st, 2nd and 4th periods were isosmotic flow, the 3rd period 35% hyposmotic. The inhibitors were added at the beginning of the 2nd period and remained throughout. *($p<0.001$) Peak value significantly higher than the last value at the end of the 2nd period.

0.009 at 15 min, a value significantly different from the peak value ($p<0.001$). After returning to the isosmotic medium (3rd period), the cell volume was completely restored without a post-RVD regulatory volume increase (RVI). Post-RVD RVI has not been seen in these experiments, probably because of the short time of observation after the replacement of the hyposmotic with isosmotic solution. Independently, RVI response to 45% hypertonic challenge of isolated nematocytes was shown elsewhere [13].

$HgCl_2$ is the classic inhibitor used as diagnostic for channel-mediated water transport [29, and ref therein] and thus is expected to inhibit the OP provided aquaporins are involved. Fig. 3 reveals that 25 µM $HgCl_2$ when applied at the beginning of the 2nd period led to a small and brief elevation of A/A_0 (1.03 ± 0.004) which was likely due to the technical perturbation introducing a drug (see also Fig. 4 and 5). The subsequent A/A_0 values fell to 0.95 by the end of the 2nd and beginning of the 3rd period and remained at that level for about 10 min before returning to values close to those of the control at the end of the 3rd and start of the 4th periods. Fig. 3 also shows that 5 µM $HgCl_2$ exerted a similar effect. The A/A_0 values, however, remained around unity throughout the 2nd, 3rd and 4th periods and both OP and RVD were absent. Similar observations were made at 0.1 µM $HgCl_2$ (not shown). These data unequivocally demonstrate that $HgCl_2$ is an inhibitor of the OP and thus prevents swelling and subsequent RVD suggesting the involvement of aquaporins.

The mercaptide bond of Hg with thiol groups can be easily dissociated by mercaptoethanol. Fig. 4 shows that the presence of 1 mM dithiothreitol (DTT) in addition to

25 μM $HgCl_2$ in the 2nd period led to a partial recovery of both the OP and RVD seen during the 3rd period, as compared to the control and the 25 μM $HgCl_2$-treated samples. The action of $HgCl_2$ thus was partially reversible.

It has been claimed that silver nitrate, $AgNO_3$, as well as tetraethylammonium (TEA) may inhibit water transport in certain model systems [18, 21]. Fig. 5A shows that continuous presence of 1 μM $AgNO_3$ from the 2nd to the 4th period of media flow-through attenuated the OP (peak value of 1.065±0.004 within 8 min) in comparison to the control in the absence of inhibitor and failed to elicit an RVD response during the 3rd period in 65 % ASW. However, upon return to the isosmotic medium in the 4th period, the A/A_0 values returned to values close to base line levels (1.051±0.003). These data suggest that $AgNO_3$ apparently did not impair the osmotically-induced inward and outward water shifts across the plasma membrane in the 3rd and 4th phase, and that silver only interfered with RVD which did not occur in the 3rd period. Similarly, and as shown in Fig. 5B, treatment of nematocytes with 100 μM TEA did not affect the OP after hyposmotic shock reaching a peak value of 1.084±0.001 within 5 min of the 3rd period, significantly higher than the value at the end of the 2nd period. At the end of the 3rd period the control volume was not restored, i.e. no RVD response occurred. However, nearly complete volume restoration was reached upon return to the isosmotic solution. Thus like silver, TEA, at the low dose applied, did not interfere with osmotically driven water movements but eliminated the RVD response.

Discussion

It is well established that nematocytes from *Cnidaria* (Fig. 1), when exposed to reduced salinity, display two phases of cell volume response: first, swelling of their cytoplasm, and second, shrinking to the original cell volume prior to returning these cells to isosmotic media. Whereas the second phase due to RVD has been characterized to some extent in these cells by this laboratory [12, 15] the mechanisms in charge of the first phase, termed osmotic phase (OP) are largely unknown. The novel finding of this study is that the OP appears to be mediated by a $HgCl_2$-sensitive transport mechanism: 0.1-25 μM $HgCl_2$ completely abrogated the OP and consequently, since no cell swelling occurred, inhibited RVD (Figs. 2-3). Since the thiol-reducing agent DTT reversed, at least in part, the $HgCl_2$ effect (Fig. 4), it is concluded that the formation of a mercaptide bond with a thiol residue crucial for commencement of OP led to its inhibition. This finding is consistent with abundant reports in mammalian cells on mercury-sensitive water transport [30] through aquaporins, originally discovered by Peter Agre and coworkers [31-32], which possess a critical cysteine (cys 189) unequivocally shown to be required in the reduced state to allow water transport [17]. A mechanism of action for aquaporins inhibited by $HgCl_2$ has been provided elsewhere [20], suggesting that the drug may either occlude the pore of water channel or may induce a conformational change through cysteine residues sensitive to $HgCl_2$. Thus it is inviting to conclude that, likewise, nematocytes from the most primitive marine animals, the Cnidaria, possess water channels as an evolutionary survival mechanism enabling them to respond to salinity changes with swelling and shrinking as the tides change the salinity in estuaries and junctions of rivers and oceans. The novel aspect of our study is further highlighted by the finding that other compounds like $AgNO_3$ (Fig. 5A) and TEA (Fig. 5B), claimed to affect water channels [2, 31, 32], failed to do so in nematocytes, underscoring the highly selective action of $HgCl_2$.

If indeed our conclusion that nematocytes possess $HgCl_2$-sensitive aquaporins is correct, it would be useful to calculate from the data in Fig. 2 of the present study, as well as from previous publications [15], the relative water permeability and the number of putative aquaporins mediating OP. Such a calculation would enhance our understanding of the physiology of osmoregulation and its molecular basis in the stinging cells of these some 600 millions year old animals. Our computations below are based on a number of assumptions, especially, that the nematocytes were considered as simple spheres ignoring the possibility of membrane folding.

Let us assume that the total area A_o of a nematocyte under isosmotic conditions is 0.0094 cm^2, and when maximally swollen, A = 0.014 cm^2. Assuming from Fig. 1 that the nematocytes of *P. noctiluca* are close to spheres, the corresponding volumes would be $V_o = 8.56 \times 10^{-7}$ cm^3 and, based on the ratio of A/A_o =1.489, then V = 12. 8 x 10^{-7} cm^3. Note that in all published Figures it takes some 4 min to swell the nematocytes in 65% ASW from V_o to V. The osmotically displaced volume then is: $\Delta V = V - V_o$ or 4.19 x 10^{-7} cm^3 or ml of water. Then, the flux of water will be: $J_{H20} = \Delta V/t$, where t= 4x 60 sec, equivalent to 1.667 x 10^{-9} cm^3 or ml/sec. Since the permeability $P_{H2O} = J_{H_2O}/A_o$ (assuming A_o does not change during osmotic swelling due to membrane folding), then P_{H_2O} = 1.667 x 10^{-7} cm/sec. This permeability

coefficient is some 3-4 orders of magnitude smaller than observed in mammalian red cell membranes (reviewed in [33]), and suggests that nematocytes are water "tight". Since the difference between the diffusional (lipid) and bulk osmotic (channel) water permeability of red blood cells is within an order of magnitude (see review [33]), it must be concluded that the membrane lipids of Cnidaria nematocytes may be tightly packed or chemically different from those found in plasma membranes of higher order animals, or both, not allowing much diffusional water flux across the plasma membrane. This may constitute, perhaps, an early crucial evolutionary adaptation to changes in marine salinities between open sea water and its dilution in estuaries and river deltas by fresh waters. If nematocytes would have a P_{H20} as found in mammalian cells, and would behave as osmometers, the archaic species of Cnidaria would not have survived for over 600 million years.

Taking these computations a step further, the mol fraction of water moving across the plasma membrane during the OP equals to 1.667×10^{-12} moles/sec, which based on N, the Avogadro's number equals 10^{12} molecules of water/sec. Given that, for example, aquaporin transports water at 10^{10} molecules/sec (reviewed in [33]), our conclusion is that nematocytes must have not more than about 100 aquaporin molecules over their entire surface area. Alternatively, if the nematocyte aquaporin is as water impermeable as the mammalian lens fiber cell aquaporin 0 [34, 35], then nematocytes may have a much larger number of an evolutionary primitive water-impermeable aquaporin. Whether the putative aquaporins are of type 3 or 9, which transport mainly glycerol, remains to be demonstrated. Indeed, preliminary attempts to find an aquaporin-type molecule in nematocyte extracts by immunochemical methods failed. This could be explained either by a low density of aquaporin molecules or by lack of cross reactivity of the antibodies used.

Aquaporins, with some 13 isoforms as of latest count [1, 2, 36], have been found in many living organisms, from bacteria to mammals [37, 38], determining the capability to regulate fluid homeostasis, especially in harsh thermal or osmotic stress, so that they are a physiological necessity to deal with osmotic pressure and volume changes. The role of aquaporins has been investigated in amphibians [37] shedding light on freeze tolerance mechanisms in these animals. With regard to invertebrates, like insects, only a few data have been published so far [38, 39]. Marine invertebrates include heterogeneous groups of animals inhabiting extremely variable environments and showing physiological adaptive mechanisms such as cell volume regulation. Marine invertebrates may undergo such osmolarity changes and adaptation strategies. Amado et al. [16] reported that intertidal species of anemones show morphological and behavioral adaptations to significant changes in the salinity of tidal pool water.

The $HgCl_2$ concentrations used in the present experiments were lower than those used by others [20, 40] who showed that 1 mM and 300 μM, respectively, prevented water permeability in an oocyte swelling assay and in aquaporin expression studies in *Caenorhabditis elegans*. This fact is compatible with the calculation of a low surface density of putative aquaporin molecules, i.e. a high ratio of free $HgCl_2$ /thiol groups available, and a mercaptide bond association constant, K_a, of $>10^{20}$. In our hands, concentrations of 50 μM $HgCl_2$, shown to alter osmoregulation in skate hepatocytes [41], caused cytoplasmic damage and cell lysis of isolated nematocytes. The present study indicates that $HgCl_2$ not only abolished the OP and consequently RVD, but may also have altered reversibly RVD which is known to involve a thiol-sensitive regulation of K channels and cotransporters [42]. Interestingly, at 25 μM $HgCl_2$, the A/A_o ratio fell below unity to 0.95 (Fig. 3). Although not readily explainable, it is quite possible that mercury exerted additional, most likely thiol-group-related, effects such as shape changes or decreasing the reflection coefficient for solutes that concomitantly may lead to additional water shifts, a phenomenon that disappeared upon addition of DTT or by lowering the mercury concentration to 5 μM.

$AgNO_3$ has been also proposed to be an even more effective water channel inhibitor than $HgCl_2$ with a similar mechanism of action of, implying the binding to –SH groups of water channels [18, 21]. In the present work, $AgNO_3$ treatment did not impair the OP of nematocytes under hyposmotic challenge with 65% ASW, but prevented the volume recovery during hyposmotic shock, suggesting a different mechanism of action than for $HgCl_2$, especially since the effect of $AgNO_3$ is not reversible by treatment with DTT [21].

TEA, a known K^+ channel inhibitor [43] appears to alter aquaporin function [23] and blocks water transport through AQP1 in Henle's loop and a renal cell line [44]. In a related study, TEA dose-dependently inhibited AQP1 overexpressed in oocytes. However, the possibility of an interaction between TEA and lipid components affecting AQP could not be excluded [43]. Based on this information, the effect of lower TEA concentrations than those required for K^+ channel inhibition were tested on nematocyte cell volume regulation. Indeed, 100 μM TEA failed to inhibit the OP, but abolished RVD, an effect

similar to that with $AgNO_3$. Thus rather than affecting the water transport mechanisms, both TEA and $AgNO_3$, as chemically different as they are, may target a machinery responsible for executing RVD in nematocytes, to be determined in future studies.

In conclusion, jellyfish nematocytes appear to possess aquaporin-type water transport proteins that, based on previous studies with the phosphatase inhibitor okadaic acid, are inactive in the phosphorylated state [15] and are inhibited by the thiol-selective $HgCl_2$. Based on theoretical considerations we propose here for the first time that a) nematocytes appear to express aquaporins and b) that they may have not more than a few hundred/cell. Alternatively, the estimates could also indicate that nematocytes may have a larger number of relatively water-impermeable aquaporins. It appears unlikely that the large difference in P_{H_2O} values of some 10^4 calculated for osmotically swollen nematocytes and reported for plasma membranes of higher order animals can be accounted for by similar orders of changes in membrane surface area. Both a low AQP number or a higher number of low permeability water channels may result from an adaptation to the ever changing marine salinities. Future studies are planned to address the nature of the aquaporin isoform/s present in *P. noctiluca* nematocytes.

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