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Mechanisms of Hyposmotic Volume Regulation in Isolated Nematocytes of the Anthozoan *Aiptasia diaphana*

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Key Words

Aiptasia diaphana • Nematocytes • Hyposmotic cell volume regulation • Potassium channels • Potassium-chloride cotransport • Water channels • N-ethylmaleimide • Okadaic acid

Abstract

The nature and role of potassium (K) and water transport mediating hyposmotically-induced regulatory volume decrease (RVD) were studied in nematocytes dissociated with 605 mM thiocyanate from acontia of the Anthozoan *Aiptasia diaphana*. Cell volume and hence RVD were calculated from the inverse ratios of the cross sectional areas of nematocytes (A/A_o) measured before (A_o) and after (A) challenge with 65% artificial sea water (ASW). To distinguish between K channels and K-Cl cotransport (KCC), external sodium (Na) and chloride (Cl) were replaced by K and nitrate (NO_3), respectively. Inhibitors were added to identify K channels (barium, Ba), and putative kinase (N-ethylmaleimide, NEM) and phosphatase (okadaic acid, OA) regulation of KCC. In 65% NaCl ASW, nematocytes displayed a biphasic change in A/A_o, peaking within 4 min due to osmotic water entry and thereafter declining within 6 min due to RVD. Changing NaCl to KCl or $NaNO_3$ ASW did not affect the osmotic phase but attenuated RVD, consistent with K channel and KCC mechanisms. Ba (3 mM) inhibited RVD. NEM and OA, applied separately, inhibited the osmotic phase and muted RVD suggesting primary action on water transport (aquaporins). NEM and OA together reduced the peak A/A_o ratio during the osmotic phase whereas RVD was inhibited when OA preceded NEM. Thus, both K channels and KCC partake in the nematocyte RVD, the extent of which is determined by functional thiols and dephosphorylation of putative aquaporins facilitating the preceding osmotic water shifts.

Introduction

Most cells are highly permeable to water, and, in the absence of significant hydrostatic pressure gradients, the water flow across the membrane is directed by the osmotic pressure gradient. When exposed to hyposmotic medium, cells initially behave almost as perfect osmometers swelling immediately thereafter and undergoing an active volume correction through extrusion of

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intracellular solutes together with osmotically obligated water, thereby normalizing cell volume. This adaptive mechanism, known as regulatory volume decrease (RVD) is essential for cell survival [1]. In most cells, RVD occurs within minutes and is the result of K and Cl loss by transient activation of their respective conductive channels [1], and of K-Cl cotransport (KCC), an electroneutral coupled movement of K and Cl ions which is ubiquitously distributed and affected by numerous activators and inhibitors [2-4]. In addition RVD may also involve K/H, Cl/HCO_3 exchange, Na/Ca exchange and a number of organic molecules, including amino acids, polyalchols, and amines [5, 6]. Thus, RVD is a complex chain of events requiring at least the following steps: 1) A sensor detecting transient changes in cell volume, 2) a signalling cascade transducing the volume change information into the activation of pathways for osmolyte extrusion, and 3) a "memory" of the original cell volume that sets the timing for inactivation of the regulatory processes.

Cell volume regulation mechanisms have been studied in several mammalian cell types, for example, Ehrlich ascites tumor cells (reviewed in [1]), lymphocytes [7], hepatocytes [8], vascular smooth muscle cells [9], renal [10, 11], intestinal [12, 13] and lens epithelial cells [14]. However, their occurrence in lower Metazoa is still far from being established. Amongst primitive Metazoa, Cnidarians have developed nematocytes, which are specialized secretory and sensorial cells used for predation and aggression strategies likely experiencing volume regulation as well. Nematocytes, with spirocytes and pthycocytes, are part of a larger group classified as cnidocytes and are localized in the tentacles and mesenterial filaments, termed acontia. A typical feature of the nematocyte is the presence of an inner organoid, the nematocyst, occupying more than 80% of its volume, which is surrounded by a girdle, a thin rim of cytoplasm (Fig. 1). Nematocysts consist of a three-layered capsule wall containing a long thread that, in the resting state (also called "unfired stinging cell") is coiled into the capsule fluid. Upon application of a proper chemical and mechanical stimulation, the tubule is rapidly ejected, thus becoming the "fired stinging cell", penetrating the target tissue and injecting toxins of the capsule fluid. This phenomenon is known as discharge, the most rapid process of exocytosis known today [15-18]. The presence of volume regulation following hyposmotic shock has also been recently demonstrated in nematocytes [19-22].

The aim of the present study was to focus on the putative electrogenic (K channels) and electroneutral mechanisms (KCC), as well as water movements, participating in RVD mechanism in nematocytes isolated from the Anthozoan *Aiptasia diaphana*.

Materials and Methods

Specimens collection

Specimens of *A. diaphana* (Anthozoa) were collected in the brackish pond Faro (Messina, Italy) at 50-90 cm depth, maintained in a closed-circuit aquarium at 18-24 °C and weekly fed with shrimp (*Penaeus japonicus*).

Nematocytes isolation

Nematocytes, classified as microbasic-mastigophore according to Mariscal [23] on the basis of the spined tubule contained inside the capsule, were isolated from acontia of *A. diaphana*, by treatment with an isosmotic solution of 605 mM NaSCN containing 0.01 mM Ca as described elsewhere [24]. Briefly, acontia, once excised from the trunk of the specimen, were repeatedly washed with low Ca-ASW (artificial sea water, composition see 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-free SCN and then with complete ASW permitted cell isolation and restoration to physiological conditions. Such isolated nematocytes, checked under a light microscope to ascertain morphological integrity and exclude any cell shape damage, were kept at 10-12 °C for 1 h and then used within 3 h from isolation for cell volume regulation tests.

RVD tests

One hour after nematocyte isolation, a coverslip was mounted with two strips of double-sided adhesive tape upon the slide containing the sedimented nematocytes, in order to allow proper substitution of experimental media during the entire test. Cell volume experiments were performed on a microbasic-mastigophore nematocyte chosen for its strong adhesion to the slide. To assess the cellular response to the applied anisosmotic shock, the RVD control test consisted of three periods: 1st period, isosmotic ASW (π=1100 mosm/KgH_2O) for 5 min; 2nd period, hyposmotic ASW (65% ASW, π=710 mosm/KgH_2O) for 10 min; 3rd period, isosmotic ASW for 8 min. Experimental protocols with different drugs were performed as follows: 1st period, isosmotic ASW for 5 min; 2nd period, isosmotic ASW plus inhibitor for 5 min; 3rd period, hyposmotic ASW plus inhibitor for 10 min; 4th period, isosmotic ASW plus inhibitor for 8 min. Ion substitution experiments were performed following this protocol: 1st period, isosmotic ASW for 5 min; 2nd period, isosmotic ASW with ion substitution for 5 min; 3rd period: hyposmotic ASW with ion substitution for 10 min; 4th period, isosmotic ASW with ion substitution for 8 min.

About 30 images/nematocyte were taken with a phase-contrast microscope (Leica DMLS) 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 suc-

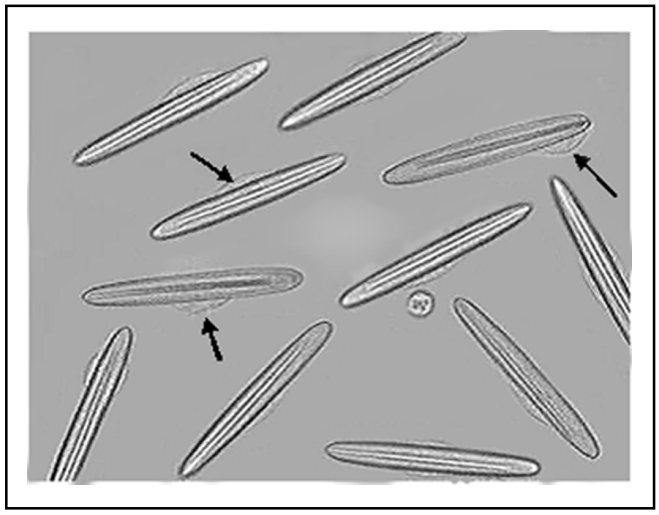

Fig. 1. Light microscope observation (200x magnification) of nematocytes isolated from acontia of *Aiptasia diaphana* by 605 mM SCN^- plus 0.01mM Ca^{2+} treatment. Note the cytoplasm located in either equatorial or apical zone (arrows).

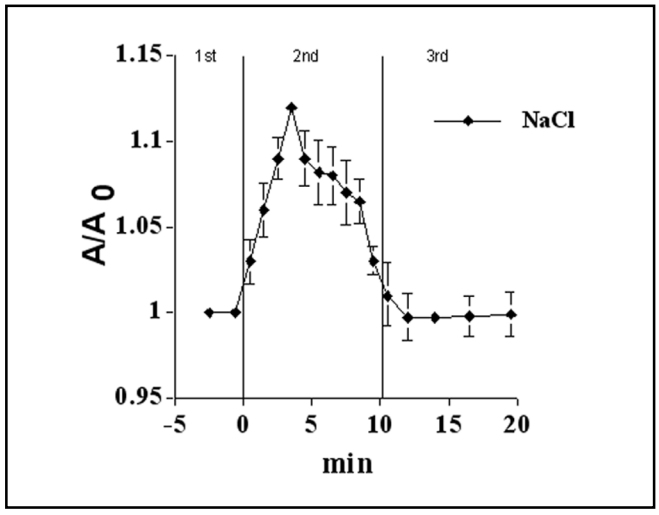

Fig. 2. Effect of hyposmotic challenge on isolated nematocytes. Cell volume, represented as A/A_0, is plotted against time. The test consists of three periods: 1st, 5 min isosmotic ASW; 2nd, hyposmotic ASW perfusion at time 0, for 10 min; 3rd, 8 min isosmotic ASW. Results are shown as the mean of 10 separate experiments ± standard error of the mean (S.E.M).

cessively measured (Aldus FreeHand) and the ratio of A/A_0 calculated, where A represents the cross sectional area of the cell at regular time intervals during the test and A_0 is the cross sectional area of the untreated nematocyte.

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=1100 mosm/KgH_2O. In the hyposmotic shock solution (65% ASW), the NaCl concentration was reduced to π=710 mosm/KgH_2O. Barium and N-ethylmaleimide (NEM) were dissolved in distilled water, and okadaic acid (OA) in 0.1% dimethylsulfoxide (DMSO). Each reagent was added to the experimental solution to yield the indicated final concentration. The thus achieved final DMSO concentration (0.1%) neither damaged cell integrity nor affected a nematocyte's osmotic behaviour. All chemicals were purchased from SIGMA (Milan, Italy). For ion substitution, $NaNO_3$, KNO_3 or KCl alternatively replaced NaCl in isosmotic and hyposmotic ASW.

Statistics

Data are shown as mean values ± standard error of the means (S.E.M.). Each data set is derived from cells originating from weekly collected animals and from at least three individual experiments performed on separate days. Significance of differences was tested using a Student's *t*-test. A probability of $p<0.05$ was considered statistically significant. Percentage of the cross sectional area changes in hyposmotic conditions were calculated from the peak value of cell volume during the anisosmotic shock and the last value at the end of the same period (2nd period).

Results

Effect of hyposmotic stress on the A/A_0 ratio

Marine invertebrates and thus *Cnidaria* nematocytes have intracellular osmotic concentrations like sea water (1100 mosm/KgH_2O) (see also [25]). Fig. 2 shows the A/A_0 ratio of nematocytes exposed to 710 mosm/KgH_2O extracellular osmolality. Under hyposmotic stress, the A/A_0 ratio increased significantly ($p<0.05$, N=10) from the unitary baseline value within 4 min of the 1st period in isosmotic ASW to 1.12±0.001 at the beginning of the 2nd period in hyposmotic ASW. Within the following 6 min, the A/A_0 ratio fell to 1.00±0.003 and did not change significantly once cells were returned into isosmotic ASW during the 3rd treatment period. We conclude that, because A/A_0 increased by 12% within 4 min exposure to hyposmotic ASW and fell to the basal value 6 min later, water had entered the nematocytes and left well before the cells were transferred back to isosmotic ASW. Whereas the increase in A/A_0 is easily attributed to osmotic equilibration, its fall still continued during the 2nd period. Thermodynamically, this phenomenon must require solute loss from the nematocytes and hence constitute RVD. Upon closer inspection, the RVD-associated decline of the A/A_0 ratio displayed a shoulder, with a more gradual decline followed by a steeper slope suggesting more than one solute efflux pathway mediating

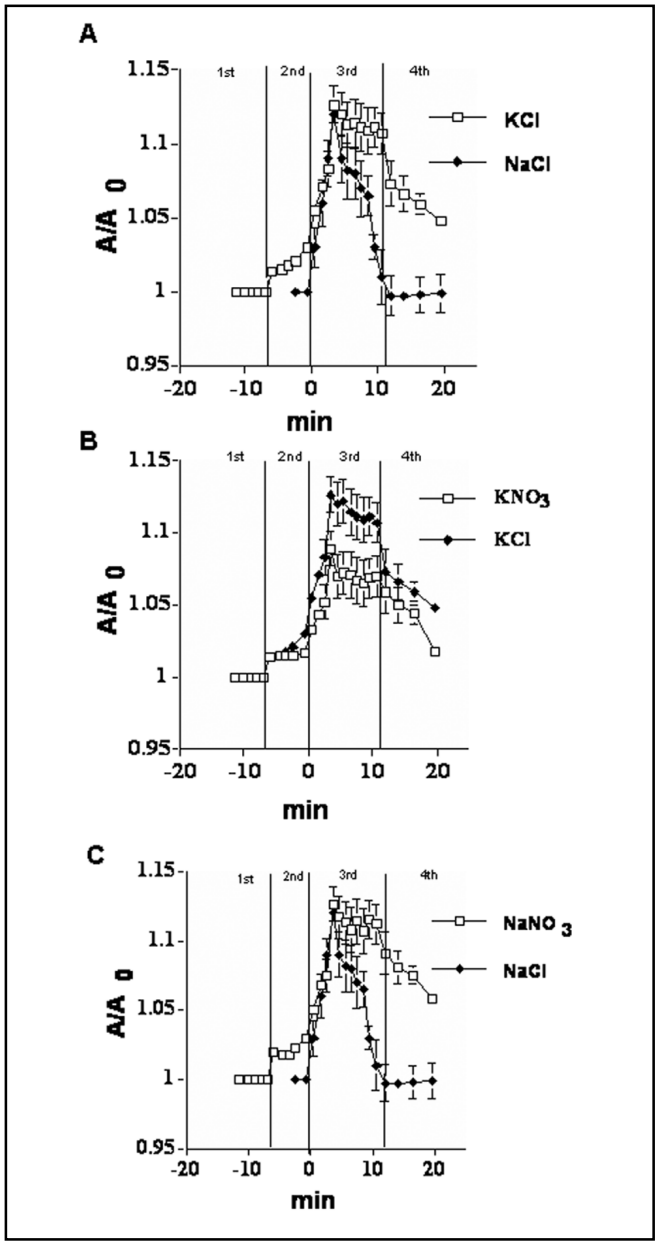

Fig. 3. Effect of ion substitution on nematocytes RVD under hyposmotic shock: NaCl was replaced by KCl (A), KNO_3 (B), and by $NaNO_3$ (C). Cell volume, represented as A/A_0, is plotted against time. The test consists of four periods: 1st, 5 min isosmotic ASW; 2nd, 5 min isosmotic ASW with salts indicated in A-C; 3rd, 10 min hyposmotic ASW perfusion, with salts as in the 2nd period, added at t=0; 4th, 8 min isosmotic ASW as in the 2nd period. Results: means of at least 8 separate experiments ± S.E.M.

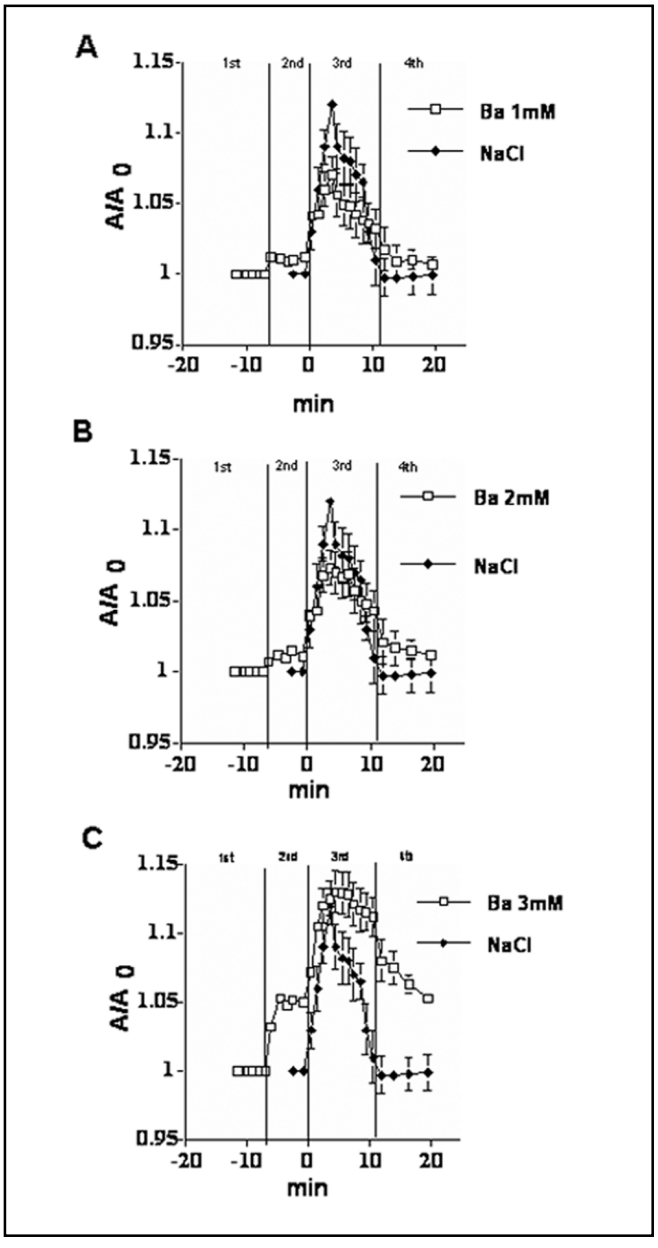

Fig. 4. Effect of Ba treatment on A/A_0 under hyposmotic shock in the presence of either 1 (A), 2 (B) or 3 (C) mM Ba. Cell volume, represented as A/A_0, is plotted against time. The test consists of four periods: 1st, 5 min isosmotic ASW; 2nd, 5 min isosmotic ASW; 3rd period, hyposmotic ASW perfused at time 0 for 10 min; 4th, 8 min isosmotic ASW. Results: means of at least 8 separate experiments ± S.E.M.

RVD. Many cell systems respond with a post-RVD regulatory volume increase (RVI) upon reintroduction to isosmotic media which may be mediated by NKCC or NHE (reviewed by [1]), however, such a secondary RVI apparently was absent in Cnidaria nematocytes.

In order to explore, whether RVD measured by the A/A_0 ratio might involve simple monovalent cations, such as K, the main cation in other invertebrates [26, 27], experiments were carried out with K replacing extracellular Na in the hyposmotic ASW solution. If indeed RVD

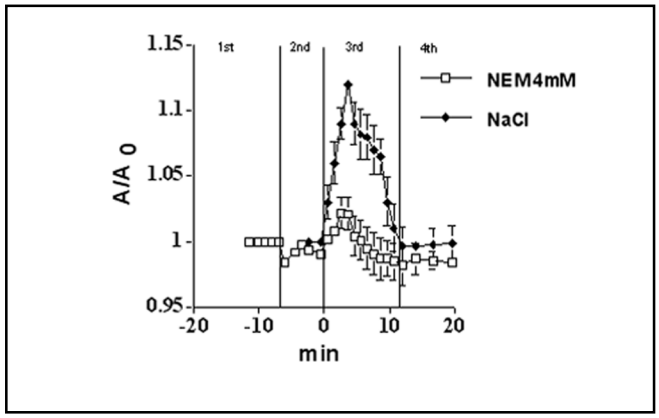

Fig. 5. Effect of 4 mM NEM treatment on nematocytes RVD under hyposmotic shock. Cell volume, represented as A/A_0 is plotted against time. The test consists of four periods: 1st, 5 min isosmotic ASW; 2nd, 5 min isosmotic ASW with 4 mM NEM; 3rd, hyposmotic ASW with 4 mM NEM perfused at time 0 for 10 min; 4th , 8 min isosmotic ASW with 4mM NEM. Results: means of at least 8 separate experiments ± S.E.M.

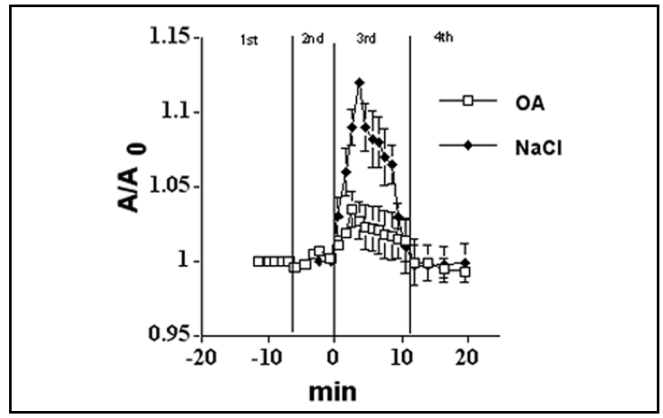

Fig. 6. Effect of 50 nM OA treatment on nematocytes RVD under hyposmotic shock. Cell volume, represented as A/A_0 is plotted against time. The test consists of four periods: 1st, 5 min isosmotic ASW; 2nd, 5 min isosmotic ASW with 50 nM OA; 3rd, hyposmotic ASW with 50 nM OA perfused at t=0, for 10 min; 4th, 8 min isosmotic ASW with 50 nM OA. Results: means of at least 8 separate experiments ± S.E.M.

involves K efflux, increasing the external K concentration should block the response. Fig. 3A compares the change in A/A_0 in high K ASW (open symbols) with that in high Na ASW (closed symbols, data from Fig. 2). It is readily visible that A/A_0 is higher in isosmotic high K ASW (2nd period) than in high Na ASW control, suggesting that some external K and water must have entered the nematocytes even before the hyposmotic challenge. At t=0, the nematocytes were exposed to hyposmotic high K ASW, their A/A_0 changed at a rate indistinguishable from the high Na ASW control to a peak value of 1.12±0.002, comparable to the peak value reported in Fig. 2. This finding means that there was no difference in the osmotically driven water entrance between high K and high Na ASW solutions. However, in contrast to the high Na ASW control, nematocytes in high K ASW failed to show a significant decrease of A/A_0 and hence RVD. Furthermore, once the hyposmotic solution was replaced with isosmotic high K ASW, A/A_0 returned only partially to basal values, remaining about 30% of the initial rise in hyposmotic K ASW above the high Na ASW control in the 4th period. These data show unequivocally that external K significantly attenuated and retarded RVD as measured by the A/A_0 ratio, implying that K played a major role in nematocytes' RVD.

Since loss of K requires, by electroneutrality, loss of an anion, Fig. 3B addresses the question whether in K ASW the replacement of Cl (closed symbols) by NO_3 (open symbols) would alter the observed shifts in A/A_0. The hyposmotically induced increase in the A/A_0 peak value was less in NO_3 (1.09±0.003) than in Cl-containing (1.12±0.002) K ASW. However, the rates of the subsequent decline in A/A_0 in the 3rd phase were similar in NO_3 and Cl containing K ASW, and also upon restitution to isosmotic K ASW (4th phase). One explanation for the difference in the rise of the A/A_0 ratios could be that K, in the presence of NO_3, is more permeable than in Cl blunting the full swelling effect due to an early fast K exit that cannot be measured precisely with our method. This conclusion would be consistent with the biphasic drop of the A/A_0 ratio in KCl between the 3rd and 4th phases. Thus, whereas nematocytes in hyposmotic K-NO_3 ASW still swell, their RVD mechanism remained muted as it was the case in KCl ASW consistent with a K efflux mechanism that was attenuated more by external K than by Cl replacement with NO_3.

The data in Figs. 3A and B revealed that RVD appears to be mediated by a K transporting mechanism and that removal of Cl and exchange with NO_3 interfered with RVD in the 3rd period, indicating evidence for a Cl-dependent K transport mechanism. Indeed, the replacement of Cl by other anions of the Hofmeister series is now a well established diagnostic for the presence of a Cl-dependent K transporter, and such a maneuver has been widely used to show the presence of KCC in many species and tissues either by determining K efflux or influx [3]. In the case of K efflux it is an implicit assumption that Cl reaches the cytosol to be cotransported from

the cytoplasmic or cis side to the external medium or trans-side, and that NO_3 exchanges with Cl but fails to be cotransported with K in the case of a tightly coupled electroneutral K-Cl cotransport system. Thus in order to demonstrate such a Cl dependence, nematocytes were exposed to isosmotic and hyposmotic $NaNO_3$ in the 2nd and 3rd periods (Fig. 3C) and the data superimposed onto those obtained in NaCl (closed symbols, from Fig. 2). That the A/A_0 ratios were elevated already in isosmotic NO_3 ASW, may suggest, together with similar observations in K media (Figs 3. A,B), the existence of non-selective cation channels [28, 29]. The rate of water entry and the peak values of swelling, as calculated from the A/A_0 ratios, were identical for the two media. However, the A/A_0 ratios failed to decrease in $NaNO_3$ with respect to the hyposmotic NaCl control solution and only did so partially ($A/A_0 = 1.05$) upon return to isosmotic NO_3 ASW (4th period). Since upon Cl replacement by NO_3 no change in the initial A/A_0 ratio occurred, we may assume that a complete exchange of the two anions occurred justifying the conclusion that in the absence of zero-trans K, cellular K had left the nematocytes as a Cl-dependent net efflux. Such a behavior suggests a major component of the RVD mechanism in the nematocyte was Cl-dependent, and possibly mediated by electroneutral KCC.

Pharmacological Interventions

In order to further distinguish between voltage-gated K channels and KCC-mediated RVD, experiments were carried out with Ba [30, 31], and with effectors of KCC activity [2, 3, 32]. Nematocytes treated with 1mM and 2mM Ba revealed smaller A/A_0 peak ratios (from 1.07±0.004 to 1.03±0.006) whilst the decline of A/A_0 during the 3rd period (Figs. 4 A, B) was not significantly different from the control in the absence of Ba. However, 3 mM Ba caused a rise of the A/A_0 values in the 2nd period with isosmotic ASW and inhibited their RVD-associated decline in the 3rd period with hyposmotic ASW (A/A_0 peak value of 1.13±0.004 *versus* 1.11±0.003) preventing their return to baseline values in the 4th period with isosmotic ASW (Fig. 4 C). These data mean that Ba *per se* appears to exert additional effects on nematocytes in isosmotic ASW (opening of non-selective cation channels? [28]) and inhibited RVD perhaps *via* voltage-gated K channels [29].

K-Cl cotransport is activated by dephosphorylation through okadaic acid (OA)-sensitive protein phosphatases and inhibited by phosphorylation involving N-ethylmaleimide (NEM)-modified serine protein kinases [2, 33]. The thiol reagent NEM, at concentrations from 0.5 to 2 mM, stimulates Cl-dependent K fluxes in almost all tissues tested and thus has become a diagnostic tool for the presence of KCC [2, 3, 33, 34]. NEM appears to inactivate inhibitory kinases and thus leading to dephosphorylation and consequent activation by an OA-sensitive protein phosphatase 1 (PP1) [3, 32, 33]. Thus NEM at ~2mM would be expected to activate K efflux through the KCC and accelerate RVD whereas OA should abolish RVD by this mechanism. However, NEM at concentrations >2 mM behaves as an inhibitor of KCC [2, 34]. Fig. 5 shows that 4 mM NEM had a dual effect: It reduced sharply the maximum hyposmotical increase of the A/A_0 ratio seen previously from 1.12±0.001 in the NEM-free controls to 1.02±0.003 ($p<0.05$), which was only slightly higher than the A/A_0 of 0.98±0.001 observed after restoration of isosmotic ASW. Since NEM targets functionally crucial thiols in aquaporins [35, 36], and blocks oxytocin-induced water transport in frog urinary bladder [37], it could well have inactivated the aquaporins responsible for the initial increase in A/A_0 ratio. Given that the concentration of NEM used here was higher than 2 mM [2], it is possible that its action was not only through thiol but also through amino groups. Future experiments are required to establish a dose-dependence of the NEM effect seen here. Nevertheless, by first interfering with the presumably aquaporin-mediated water permeability, the subsequent RVD response seen in Figure 5 was blunted as well, whether or not NEM had activated KCC.

As stated above, OA in nanomolar concentrations should have blocked dephosphorylation of the putative KCC mechanism and hence at least attenuated RVD. However, the effect of 50 nM OA on nematocyte volume behaviour was very similar to that seen with NEM. Fig. 6 reveals that the hyposmotically induced increase in the A/A_0 ratio observed in the 3rd period was blunted to 1.03±0.004 at 2.5 min, as opposed to the OA-free control. During the remainder of the 3rd period, A/A_0 decreased to 1.01±0.001 and thus pre-empted the RVD response. This novel effect is unexpected unless one postulates that aquaporins in nematocytes are only functional when dephosphorylated by a protein phosphatase 1, and dysfunctional when PP1 is blocked by OA and phosphorylation prevails. Future studies need dose-response testing of calyculin A, a selective PP1 inhibitor effective at even lower concentrations than OA.

Based on previous experiments [2, 3] and the bimolecular reaction kinetics theory of Jennings and Schulz [38], one would expect that applying both NEM and OA should have mutually exclusive effects on the KCC ac-

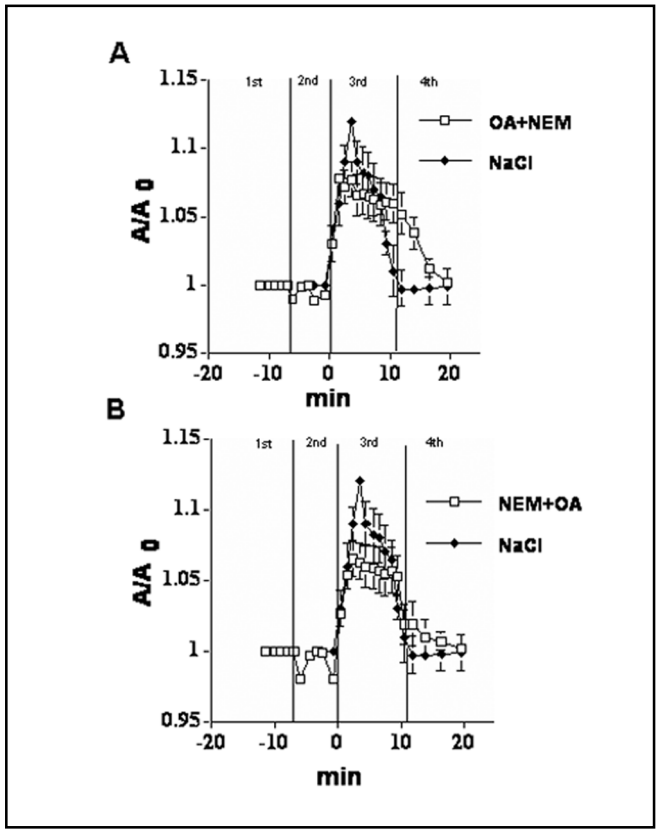

Fig. 7. Effect of 4 mM NEM and 50 nM OA treatment in alternate order on nematocyte RVD under hyposmotic shock. A: First 50 nM OA then 4 mM NEM, B: First 4 mM NEM then 50 nM OA. Cell volume, determined as A/A_0 is plotted against time. The test consists of four periods: 1st, 5 min isosmotic ASW; 2nd, 5 min isosmotic ASW with OA or NEM; 3rd, hyposmotic ASW with NEM or OA perfused at t=0, for 10 min; 4th, 8 min isosmotic ASW with both drugs. Results: means of at least 8 separate experiments ± SEM.

tivity, i.e. either stimulation through preventing kinase mediated phosphorylation (NEM effect) or inhibition by preventing dephosphorylation (OA effect). The combined treatment with 50 nM OA first for 5 min and then with 4 mM NEM for 10 min or in reversed sequence (Fig. 7A, B) did not affect the rate of increase but reduced the peak level of the A/A_0 ratio in the 3rd period from 1.12±0.001 to 1.08±0.002 and 1.06±0.002, at 1.5 and 2.5 min after hyposmotic shock, respectively. Furthermore, the return of the A/A_0 ratio to the basal level (about unity) in the 4th period of isosmotic ASW exposure was unaltered in the NEM-OA regime while clearly attenuated in the OA-NEM regime. This finding means that when added together NEM and OA, in any sequence, still reduced the water entry preceding RVD, and that OA prior NEM treatment attenuated the remainder of RVD possibly by KCC inhibition as discussed above.

Discussion

Cell volume regulation is a common feature essential to cell survival in mammalian and invertebrate cell types. By using a simple cell isolation method, cell volume regulation was previously assessed under both hyposmotic and hyperosmotic stress in nematocytes isolated from Cnidaria specimens such as *Pelagia noctiluca* (Scyphozoan) and *Aiptasia diaphana* (Anthozoan) [20, 24]. In particular, in the latter specimens, investigations have been more extensively carried out due to their availability. Therefore, the occurrence of RVD in nematocytes isolated from this Anthozoan has been well established. Tests performed with inhibitors and drugs, such as quinine, NPPB (5-nitro-2(3-phenylpropylamino) benzoic acid), and gramicidine, have shown K and Cl conductances are essential for nematocyte RVD. The aim of the present work was to clarify whether, in addition to such ion conductances, cotransport mechanisms, and in particular KCC, are involved in RVD.

Indeed, our study in nematocytes presents two pieces of novel findings: 1) the potential contribution of KCC to RVD and 2) the likely requirement of thiol-dependent and dephosphorylated aquaporins for the osmotically obliged swelling phase in hyposmotic ASW, in addition to confirming the previously observed role of K channels [19]. These conclusions are based on the following three main observations.

First, the RVD-associated decline of the A/A_0 ratio during the hyposmotic phase (third experimental phase in Figure 3) was significantly attenuated if not abolished by trans-K-ASW (Fig. 3A), an effect more pronounced in NO_3 than in Cl (Fig. 3B), and well in place before the return of the nematocytes to isosmotic ASW (fourth phase). Thus increasing trans-K concentrations reverse K efflux inwardly as expected for K channels such as the IK channel in human lens epithelial cells [14]. Indeed, higher than 2 mM concentrations of Ba, while already affecting nematocytes in isosmotic ASW, inhibited RVD as detected by an attenuated decline in the A/A_0 ratio (Fig. 4C) supporting the earlier claim by us [19] of voltage-gated K channels conducting RVD. Raising trans-K concentrations also reverses the KCC flux direction via the rate limiting step of the loaded carrier [39]. That RVD was less inhibited by trans-K in NO_3 than in Cl ASW (Fig. 3B) is consistent with an Eisenmann anion sequence that favours Cl [40], or a Cl-mediated K pathway, like the KCC. Alternatively, the first faster phase of the observed decline of the A/A_0 ratio could be the result of a Cl-rate-limited K channel, while the second more

gradual decline of the A/A_0 ratio in the 4th period is mediated by the KCC system. Indeed, the data of Figure 3C support this conclusion, as under zero-trans K ASW conditions, RVD, as expressed in the decline of the A/A_0 ratio in the 3rd and 4th period, was sharply reduced by the presence of $NaNO_3$, data characteristic of KCC zero-trans K efflux [2, 3].

Second, to further advance the hypothesis of KCC participation in nematocytes RVD, NEM and OA, both inhibitors of protein kinases and phosphatases regulating KCC activity, explicitly and separately muted RVD (Fig. 5 and 6). However, this effect was primarily due to abrogation of the fast hyposmotic swelling phase thus preventing water from entering the nematocytes which consequently failed to elicit a major RVD response. This novel finding makes sense because NEM is a known alkylant of a key thiol group (cysteine 189) in the primary structure of aquaporins crucial for its water transport function [36, 41] and was first shown by Adragna and Bourguet [37]. to block oxytocin-mediated water transport. That OA also inhibited the osmotic phase and thus most likely water transport, was unexpected because of a large body of data suggesting that vasopressin-induced water transport requires phosphorylation of aquaporins [42, 43]. More compatible with our findings is a report that the aquaporin channel in spinach leaf plasma membrane requires dephosphorylation to fully function [44]. Since OA, by inhibiting protein phosphatase 1, would augment phosphorylation and hence inactivation of the water channel, it is possible that OA acts instantaneously at the level of the transporter already present in the membrane, an effect perhaps separate from the necessity of vasopressin-induced insertion of the serine 264-phosphorylated protein required for cytosolic transport [43]. If the unknown isoform of the nematocyte aquaporin is "AQP" and P is the phosphorous group, the following bimolecular equation would define the membrane activity status of the unknown nematocyte "AQP" isoform where the bidirectional arrow defines the yet to be determined protein phosphokinases and phosphatases governing this equilibrium:

$$\left["AQP"-P\right]_{Inactive} \Leftrightarrow \left(\left["AQP"\right]_{Active} + P\right)$$

The novel inhibition by both NEM and OA of the swelling-enhanced A/A_0 ratio is notable in terms of the slow swelling rates of these cells relative to mammalian cells, where water entry is fast and occurs in seconds through both aquaporins and the lipid bilayer [1].

Third, the actions of NEM and OA, when applied together in alternate sequence (Fig. 7), on both the increase and the fall of the A/A_0 ratios during hyposmotic challenge are complex and presently most difficult to explain. When NEM was given after OA (Fig. 7A), a modest inhibition of the initial rise of the A/A_0 ratio was followed by an attenuation of RVD in the 4th episode which is commensurate with a predominant effect through OA-mediated inhibition of a presumable dephosphorylation and hence activation of KCC (open symbols). However, with NEM given prior to OA (Fig 7B), the A/A_0 ratio in the first part of the 3rd period was clearly lower than that of the NaCl control. As in Fig. 5, thiol modification of aquaporins is expected and hence water transport compromised, since the conserved cysteine 189 is required for its function [45]. Apparently NEM did block the OA action, since the subsequent RVD was not much different from the control, at least in its last part. Finally, it is possible that both OA and NEM chemically react with and inactivate each other in the ASW fluid phase or compete for the same membrane pathway to enter the nematocytes and hence their effective intracellular concentrations are too low.

Recently, Fels and co-authors [46] reported that permeabilized single substrate-attached cells responded to changes in osmolarity, pH and ionic composition by volume changes 3.7-fold greater than observed in non-permeabilized controls implying osmo-sensing properties of the cytoplams, as reported for non-living hydrogels (reviewed in [47]). Since both NEM and OA are membrane permeable, thiol alkylation by NEM or increased phosphorylation by OA-mediated PP1 inhibition may indeed have affected the properties of cytosolic components beyond the signalling cascades that control membrane channels and transporters. Future experiments will have to clarify the certainly multiple action mechanisms of these compounds both at the membrane and cytosol level.

The new data presented here on the potential molecular mechanisms of K and water movements in osmotically challenged nematocytes require molecular studies to complement the emerging picture of osmotically obliged swelling and secondary active cation + anion transport responsible for RVD and cell volume restoration to its basal levels in this interesting invertebrate cell model.

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