Poly-γ-glutamate synthesis during formation of nematocyst capsules in *Hydra*

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Summary

Nematocysts are explosive organelles found in all Cnidaria. Explosion of nematocyst capsules is driven by the high pressure within the capsule formed by the high concentration of poly-γ-glutamate in the capsule matrix. Poly-γ-glutamate is a polyanion that binds cations tightly, including the fluorescent cationic dyes acridine orange and DAPI (4′,6-diamidino-2-phenylindole). We have used acridine orange and DAPI staining to localize poly-γ-glutamate within capsules and to follow the biosynthesis of poly-γ-glutamate during capsule formation. The results indicate that poly-γ-glutamate biosynthesis occurs late in capsule formation after invagination of the tubule and that it is accompanied by swelling of the capsule due to increasing osmotic pressure. The matrix in all four capsule types is homogeneously filled with poly-γ-glutamate. In vivo this poly-γ-glutamate is complexed with monovalent cations. In addition, poly-γ-glutamate is formed within the tubule lumen of stenoteles. We argue that this poly-γ-glutamate is required to drive the two-step explosion process in stenotele nematocysts.

Key words: Hydra, Nematocyst, Poly-γ-glutamate, Acridine orange, DAPI

Introduction

Nematocysts are explosive organelles found in all members of the phylum Cnidaria (Hessinger and Lenhoff, 1988). They are used for capturing prey, for locomotion and for defence. Nematocysts are primarily localized in tentacles but smaller numbers are found mounted in ectodermal epithelial cells throughout the animal. A large number of different morphological types of nematocyst capsules are found within the Cnidaria. Individual species, however, have only a small number of different nematocyst types. For example, the common fresh water polyp *Hydra vulgaris* has four types: stenoteles, desmonemes, atrichous and holotrichous isorhizas.

Nematocyst capsules are formed in clusters of differentiating nematocytes (Lehn, 1951; David and Challoner, 1974; David and Gierer, 1974) in the body column of hydra polyps. Once capsule differentation is completed these clusters break up into single cells that migrate to tentacles and become mounted in specialized tentacle epithelial cells, called battery cells. One nematocyst capsule is formed per cell in a differentiating nematocyte. The capsule develops in a postgolgi vacuole that initially is very small but increases in size until it is almost as large as the cell itself. Extensive EM investigations have documented the morphology and differentiation of capsules (Slautterback and Fawcett, 1959; Mariscal, 1974; Holstein, 1981). Capsules have a strong outer wall surrounding an inverted tubule that is an extension of the wall structure. The tubule is formed initially outside the capsule and subsequently invaginates within the wall. Following invagination the wall structure ‘hardens’ (i.e. is no longer deformed when tissue is fixed) and the capsule swells to its final size. Wall ‘hardening’ appears to result from disulfide bond isomerization to form interchain S-S bridges between minicollagen peptides in the capsule wall (Engel et al., 2001).

The matrix of nematocysts contains a high concentration of poly-γ-glutamate (pG), which creates the osmotic pressure needed for the explosion process (Weber, 1989; Weber, 1990). Using specific antibodies, Weber has shown that pG appears in capsules late in the differentiation process after invagination has occurred but while capsules are still in nests in the body column (Weber, 1995). These results also showed that pG synthesis occurs within the capsule wall since pG could only be detected within capsules. However, the results did not resolve just when or where pG synthesis starts in capsules nor the precise localization of pG within capsules. The antibody studies also provided no information about the cations associated with pG within the matrix. This is an important feature influencing the osmotic pressure within capsules and hence the explosion process.

We have developed an alternative method to identify pG in differentiating nematocyst capsules using two cationic fluorescent dyes: 3,6-(dimethylamino)acridine (acridine orange, AO) and 4′,6-diamidino-2-phenylindole (DAPI). Both dyes bind tightly to pG. Binding is cooperative and leads to a metachromatic shift in the emission spectrum (Allan and Miller, 1980; Darzynkiewicz and Kapuscinski, 1990): AO bound to pG fluoresces red (monomer fluorescence green), and DAPI bound to pG fluoresces yellow (monomer fluorescence blue). Using these dyes we have shown that pG synthesis starts in differentiating capsules after invagination of the tubule and is correlated with an increase in capsule volume. Since dye binding depends on the nature of cations present in capsules, we have been able to show that capsules contain primarily monovalent cations in vivo. Our results also show,

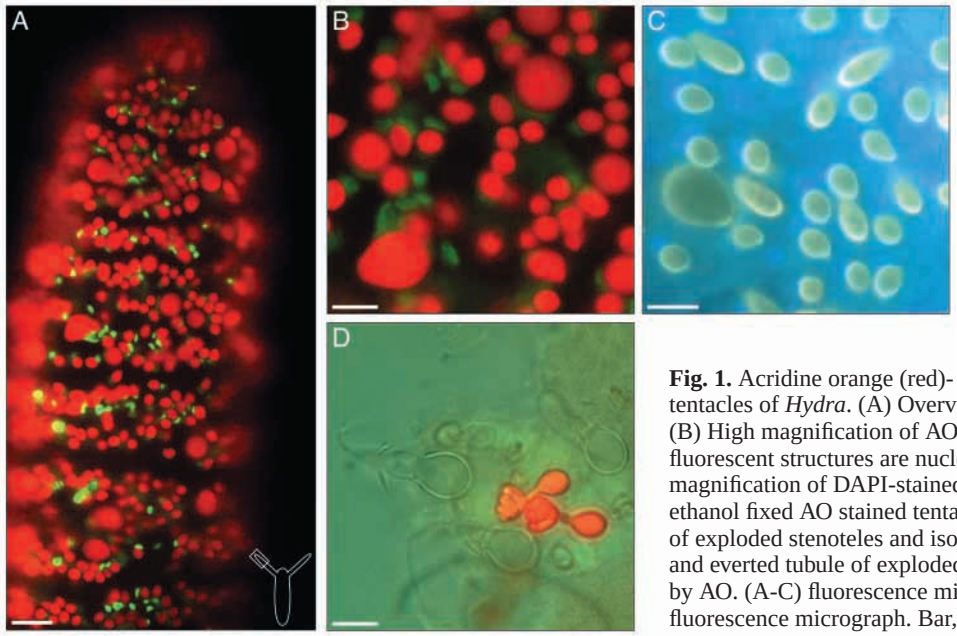

Fig. 1. Acridine orange (red)- and DAPI (yellow)-stained nematocysts in tentacles of *Hydra*. (A) Overview of distal tentacle stained with AO. (B) High magnification of AO stained tentacle. The small green fluorescent structures are nuclei of the nematocytes. (C) High magnification of DAPI-stained tentacle. (D) High magnification of ethanol fixed AO stained tentacle showing exploded capsules. The matrix of exploded stenoteles and isorhizas is empty and unstained; the matrix and everted tubule of exploded desmonemes is filled with pG and stained by AO. (A-C) fluorescence micrograph; (D) phase contrast and fluorescence micrograph. Bar, 20 μm (A); 10 μm (B-D).

unexpectedly, the presence of pG within the inverted tubule of stenoteles and provide a possible explanation for the complex two-step explosion process of stenoteles (Holstein and Tardent, 1984; Tardent and Holstein, 1982).

Materials and Methods

Animals

Hydra vulgaris polyps were mass cultured at 18±1°C in polyethylene trays as described (Loomis and Lenhoff, 1956). Modified hydra medium contained 1 mM $CaCl_2$, 1 mM NaCl, 0.1 mM $MgSO_4$, 0.1 mM KCl, 1 mM Tris, pH 7.6. *Hydra* were fed newly hatched *Artemia* larvae (washed in hydra medium) three to five times a week and were transferred to fresh hydra medium 4-6 hours after feeding.

Animals used for experiments were starved for 24 hours; they were relaxed in 4% urethane in hydra medium prior to fixation for 10 minutes. Fixation, washing and staining procedures were carried out with gentle shaking.

Acridine orange (AO) and DAPI staining

If not specified, fixation solutions contained 4% formaldehyde in Tris buffer (10 mM NaCl, 10 mM Tris pH 7.6), 10 mM EDTA pH 7.6, or 4% formaldehyde in PBS (125 mM NaCl, 40 mM K_2HPO_4, 15 mM NaH_2PO_4; pH was adjusted to 7.4 with conc. HCl). These conditions yielded brillantly stained capsules. When calcium chelators such as EDTA or phosphate buffer were not present during fixation, the staining was weak. To induce capsule explosion, 96% ethanol was used for fixation. After 45-60 minutes of fixation, animals were washed three times in Tris buffer or PBS. Fixed animals were stained in 140 μM DAPI or 110 μM AO in Tris buffer for 10-15 minutes and then washed three to five times in Tris buffer or PBS. Embedding medium contained two parts glycerol mixed with 1 part PBS or Tris buffer.

Influence of Na^+ and Ca^{2+} ions on AO and DAPI staining

Animals were fixed for 1 hour in 4% formaldehyde and 1, 10 or 100 mM NaCl or $CaCl_2$, then washed three times in 10 mM Tris pH 7.6 and the respective ion. Fixed animals were stained in 140 μM DAPI or 110 μM AO in 10 mM Tris and the respective ion, then washed over night in the same solution without dye. Embedding medium contained two parts glycerol mixed with 1 part 10 mM Tris and the respective ion.

Identification of poly-γ-glutamate by PAGE

Nematocysts were isolated as described (Weber et al., 1987). Capsule explosion was induced by resuspending capsules in sample buffer (200 mM Tris-HCl pH 6.8, 40% glycerol, 2% β-mercaptoethanol, 0.1% bromophenol blue). Capsule explosion was monitored microscopically in a sample without dye. Electrophoresis of the contents of lysed capsules was performed by native Tris-tricine-PAGE (Schägger and von Jagow, 1987) using a separating gel 16.5% T, 1.2% C, stacking gel 40% T, 3% C, without urea. Following electrophoresis, gels were stained in 90 μM AO, 10 mM Tris pH 7.6 for 10-15 minutes or 0.2% alcian blue 8GX in 40% methanol, 10% acetic acid for 15-30 minutes. The same solutions without dye were used for destaining.

Microscopy, photography and image processing

All preparations were analyzed using a Zeiss Axiovert microscope equipped with epifluorescence and a DAPI-filter (excitation 340-

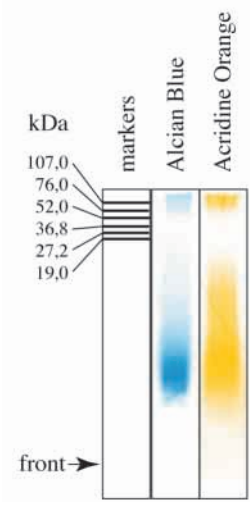

Fig. 2. Separation of poly-γ-glutamate (pG) from discharged nematocysts by native PAGE on 16% gel. Gel was fixed and stained with alcian blue (lane 1) and acridine orange (lane 2).

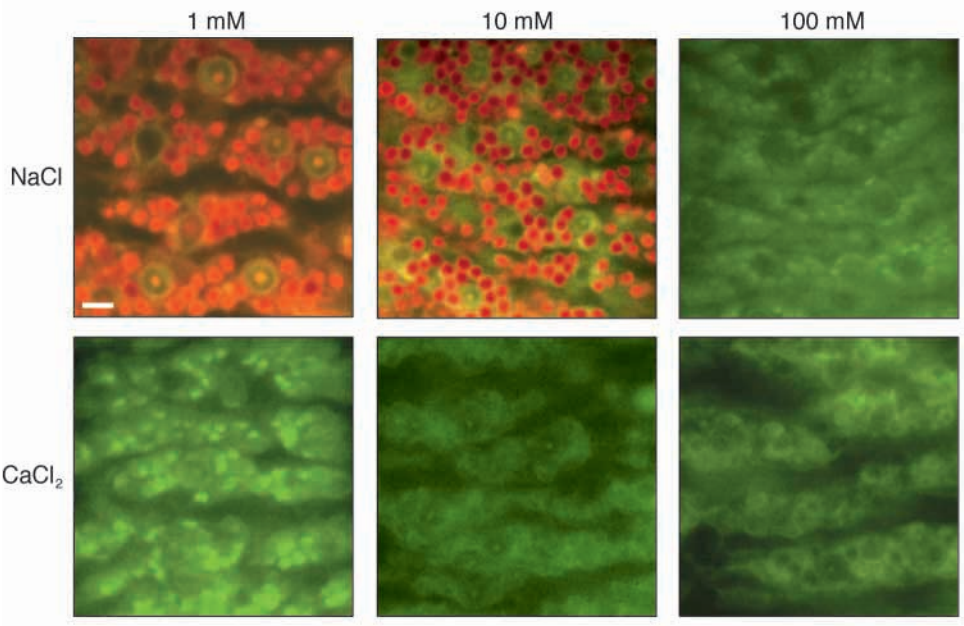

Fig. 3. Influence of Na^+ and Ca^{2+} ions on AO staining of nematocysts. Animals were fixed, stained and washed in the indicated ion concentrations. Bar, 10 μm.

380 nm, emission >425 nm) or a FITC-filter (excitation 450-490 nm, emission >515 nm). Photography was performed with Fuji Sensia II (100 or 200 ASA) and Fuji Provia (50 ASA) film.

Confocal laser scanning microscopy was performed on a Leica TCS NT confocal microscope. DAPI stained animals were scanned with a combination of 457 and 488 nm excitation and a 520-580 nm emission filter. Image analysis was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Results

Acridine orange and DAPI stain poly-γ-glutamate (pG) in the matrix of nematocyte capsules

Weber has shown that nematocyte capsules contain very high concentrations of pG (Weber, 1990). The pG is confined within the capsule wall and leads to the high osmotic pressure essential for capsule explosion. PG is a polyanion and binds cations to maintain electroneutrality. Addition of the fluorescent cations AO or DAPI to fixed hydra leads to strong binding to the matrix of nematocyte capsules and brillant red (AO) or yellow (DAPI) fluorescence (Fig. 1A-C). Both dyes emit at longer wavelengths when bound to pG than when monomers, probably due to aggregation of dye molecules on the pG substrate (Darzynkiewicz and Kapuscinski, 1990; Allan and Miller, 1980).

To confirm that AO and DAPI bind to the matrix we fixed tissue in ethanol to induce capsule explosion. Under these conditions pG is extruded from stenotele and isorhiza capsules, which have open-ended tubules, whereas the matrix material remains confined within exploded desmonemes, which have closed tubules. Fig. 1D shows that exploded stenoteles and isorhizas no longer stained with AO while both the capsule and the coiled tubule of exploded desmonemes stained brillantly with AO. When stained capsules were induced to explode under the microscope, it was possible to observe extrusion of the stained matrix material through the tubule of exploding stenoteles and isorhizas.

Acridine orange and DAPI bind to purified pG

To investigate whether AO and DAPI bind to pG or to some other component of the capsule matrix, we purified pG from capsules by gel electrophoresis (Weber, 1990). PG forms polymers of 10-50 residues depending on the capsule type (Weber, 1990). It does not stain with Coomassie blue or silver stain but can be visualized with alcian blue staining. Fig. 2 shows pG separated in a 16.5% native polyacrylamide gel. It formed a broad band of apparent molecular weight 2-15 kDa that stained strongly with alcian blue but did not stain with Coomassie or silver (not shown). This material also stained strongly with AO, confirming that AO binds to pG. Staining with AO was blocked by high concentrations of monovalent cations as expected if binding is ionic.

AO binding to pG depends strongly on the concentration and valency of competing ions

Weber showed that AO binding to isolated nematocyte capsules depends strongly on the concentration and valency of competing ions (Weber, 1989). The results in Fig. 3 confirm this observation on capsules in fixed tissue. Nematocyte capsules stained bright red in the presence of 1 and 10 mM Na^+ ions but lost all red fluorescence in 100 mM Na^+ ions. (Similar results were obtained with K^+ ions; data not shown.) By comparison, 1 mM Ca^{2+} ions was sufficient to completely inhibit cooperative

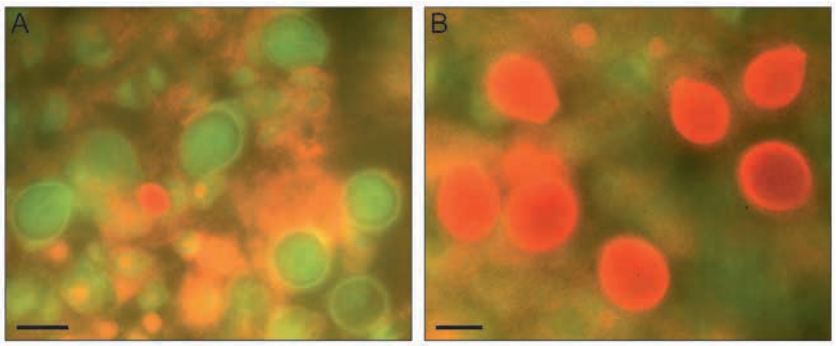

Fig. 4. Biosynthesis of pG in developing nematocysts stained with AO. (A) Nest of immature yellow/green stained stenoteles, a single red stained migrating desmoneme is also present; (B) nest of nearly mature red stained stenoteles. Bars, 10 μm.

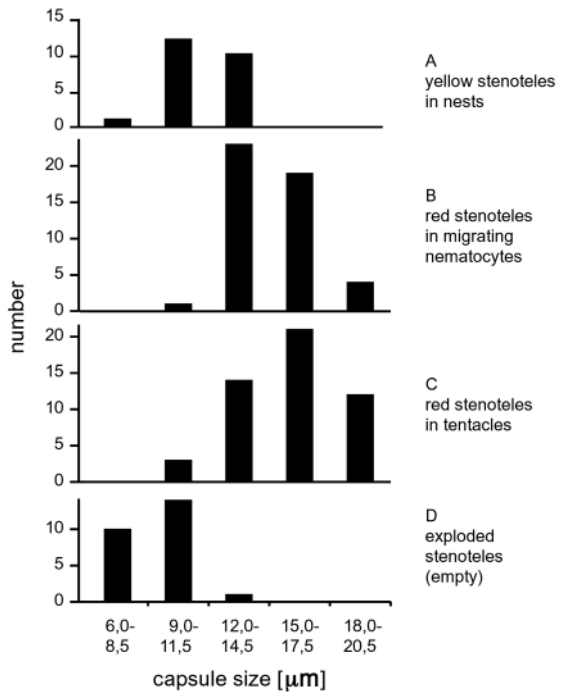

Fig. 5. Size distribution of AO-stained stenotele nematocysts in the body column and tentacles. (A) Nests of nematocytes with yellow fluorescent capsules in the body column. (B) Migrating nematocytes with red fluorescent capsules in the body column. (C) Stenotele nematocysts with red fluorescent capsules mounted in the tentacles. (D) Exploded stenotele nematocysts (empty) in tentacles.

binding of AO to capsules. These results demonstrate that divalent cations have roughly 100× higher affinity for pG than do the monovalent cations Na^+ and K^+, in agreement with Weber (Weber, 1989). AO, although a monovalent cation, also has a higher affinity for pG than Na^+ and K^+ ions, since 110 μM AO bound strongly to pG in the presence of 10 mM Na^+ ions (results for K^+ are not shown). This implies that some feature of AO other than charge contributes to the binding. Similar results were obtained with DAPI staining.

PG synthesis in differentiating nematocyte capsules

AO and DAPI staining have allowed us to follow pG synthesis in differentiating capsules. Because of their large size this was best seen in stenoteles but the results were the same for isorhizas and desmonemes. Fig. 4 shows the results for stenoteles stained with AO; similar results were obtained in DAPI stained animals (data not shown). PG synthesis started in a late stage after invagination of the external tubule. At this stage differentiating nematocytes were still in nests. Early nests showed homogeneous yellow/green staining, corresponding to the initially low concentration of pG (Fig. 4A). With increasing pG concentration, AO staining of nests changed to yellow-orange and finally to red (Fig. 4B). At this stage nests broke up into single nematocytes that migrated through the tissue toward the tentacles. These single migrating nematocysts fluoresced bright red as did mature nematocysts mounted in battery cells in tentacles (Fig. 1A). Fig. 4A shows a single migrating desmoneme stained bright red by AO.

Nematocyst capsules increased in size coincident with the change from yellow to red fluorescence associated with the increased concentration of pG within the capsule. Fig. 5 shows the size distribution of yellow capsules in stenotele nests, red capsules in single migrating stenoteles and red stenotele capsules in tentacles. Yellow capsules are significantly smaller than the red capsules as expected if pG is responsible for generating the osmotic pressure in capsules. There is, however, little difference in size between red stenotele capsules in migrating nematocytes and in tentacles. This indicates that pG biosynthesis is essentially completed prior to mounting of stenoteles in battery cells in the tentacles.

Following discharge, capsules no longer contain pG and do not stain with AO. Fig. 5 shows that such capsules have shrunk to 6-11.5 μm in diameter. This is smaller than the size of yellow capsules in nests and appears to represent the unstretched configuration in the absence of pG. Taken together our results clearly demonstrate that the capsule wall is elastic and stretches due to accumulation of pG in the matrix.

Localisation of pG in capsules

Stained capsules could only be examined at relatively low resolution with the fluorescence microscope and it was not possible to visualize internal structures, such as the tubule, in the capsules. In order to define the localisation of pG at a higher resolution we examined DAPI stained capsules in a confocal microscope. The results showed that DAPI stained pG was homogeneously distributed in the matrix of the capsules (Fig. 6), but absent from the tubule lumen of desmonemes and isorhizas (Fig. 6A,B). However, the confocal images revealed the presence of pG staining within the tubule lumen of stenoteles (Fig. 6C,D). Closer examination showed that this DAPI staining corresponded to three elongated regions within the inverted tubule (Fig. 6D). These regions lay between the spines at the base of the stylets. PG staining could not be observed in the other parts of the stenotele tubule lumen (Fig. 6C,D).

PG within tubule lumen of stenoteles could also be seen by fluorescence microscopy in stained animals that had been extensively washed following staining. Under these conditions, DAPI was washed out of the matrix but remained bound to pG in the tubule. This led to a ‘filament-like’ staining pattern in stenoteles (Fig. 6E,F). A similar staining pattern was also seen in well-washed, AO stained stenoteles (Fig. 3). To confirm that pG was still present in the matrix of stenoteles that exhibited the ‘filament-like’ staining pattern, tissue was restained with DAPI. Following restaining, stenotele capsules were homogeneously brightly stained.

The homogeneous DAPI and AO staining of the capsule matrix (Fig. 6) was interrupted by well-defined dark patches in confocal images. These are created by the inverted tubule that displaces the stained matrix. By tracing the dark patches from one optical section to another it has been possible to reconstruct the 3D folding pattern of the inverted tubule. This is shown in Fig. 6A-C for desmoneme, isorhiza and stenotele capsules. In these images the intensity scale has been inverted such that the unstained tubule is bright and the stained matrix is dark, thus making the inverted tubule more clearly visible. These images indicated a clear difference in the diameter of the inverted tubule between the different capsule types. The

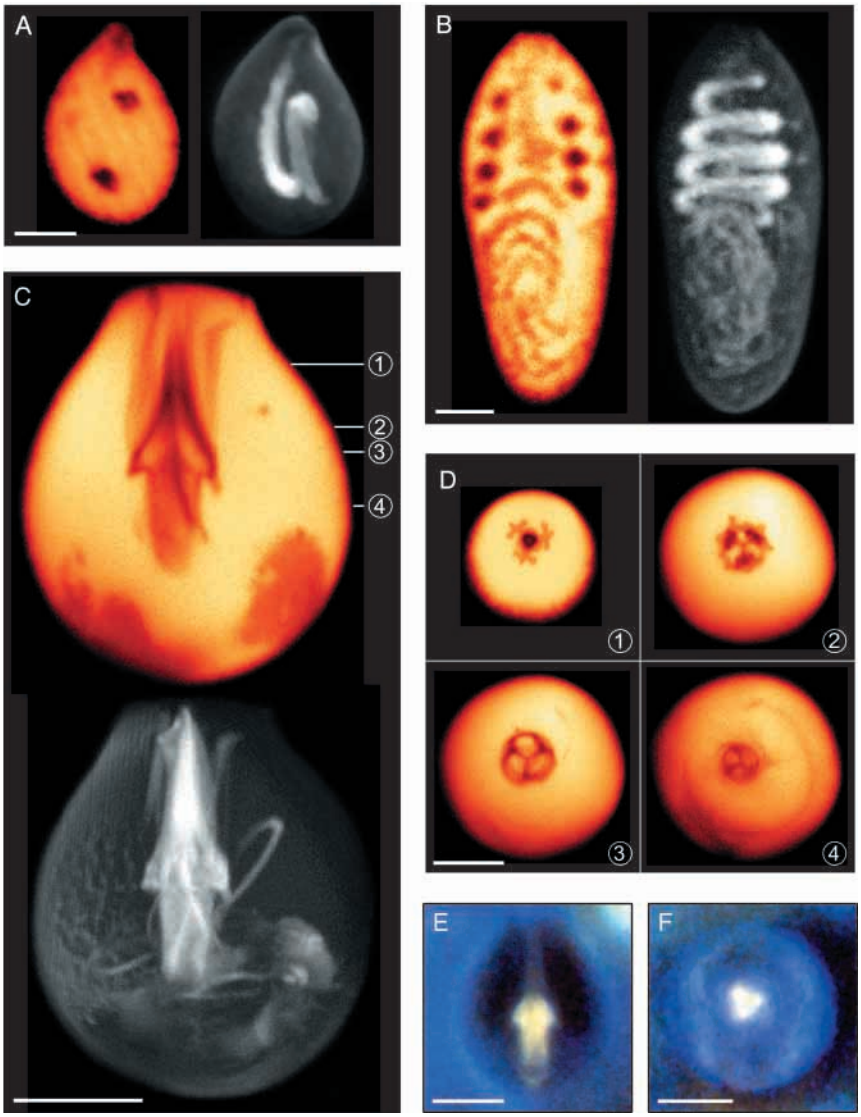

Fig. 6. Confocal images of DAPI-stained nematocysts (false colors). (A) Desmoneme, (B) isorhiza, (C,D) stenotele. The coloured images are single optical sections. The approximate positions of the sections shown in D are indicated by numbers next to the capsule in C. In A-C the black-and-white images are projections made from the full stack of optical sections. The colour scale has been inverted to permit better visualisation of the tubule within the capsule. (E,F) Fluorescence images of DAPI-stained stenoteles that have been extensively washed (see text for details). (E) View from the side of the capsule; (F) view down the long axis of the capsule. The three regions of DAPI-stained pG are located within the tubule lumen at the base of the stylets. Bars, 2 μm (A-B); 5 μm (C-F).

tubule in isorhiza and desmoneme capsules is thick due to the presence of spines in the lumen (Holstein, 1981). By comparison, the tubule of stenotele capsules is thick at the base due to the presence of very large spines and thin over most of its length due to the absence of spines (Holstein, 1981).

Cations in capsules in vivo

Weber (Weber. 1989) found primarily Mg^{2+} and Ca^{2+} ions in capsules that were isolated from homogenized tissue by density gradient centrifugation. By comparison, X-ray spectral analysis of nematocyst capsules in shock frozen polyps has indicated the presence of high concentrations of monovalent K^+ ions (Zierold et al., 1991) (I. Gerke, Characteristics of the capsular wall of stenoteles in *Hydra attenuata* and *H. vulgaris (Hydrozoa, Cnidaria)* in context with the discharge mechanism, PhD thesis, University of Zurich, 1989**)**. These results suggest that divalent cations found in isolated capsules may be the result of contamination during the isolation procedure. In an attempt to resolve this discrepancy, we examined AO and DAPI staining in tissue fixed in the presence and absence of chelators of divalent cations.

Fixation in 10 mM EDTA (in Tris buffer) or in PBS yielded brightly stained capsules (Fig. 7A), whereas fixation in the absence of chelators yielded weakly stained capsules (Fig. 7B). To investigate whether this effect was due to capture of divalent cations released during the fixation process or to extraction of divalent cations present in vivo in capsules, we attempted to improve the staining of weakly stained capsules by incubation in EDTA or PBS buffer after fixation. Animals were fixed in the absense of chelators and either stained immediately or post-treated with 10 mM EDTA (in Tris buffer) or with PBS. Animals stained immediately had weakly stained capsules as before. Animals post-treated with EDTA or PBS and then stained with AO also displayed weak staining of capsules (Fig. 7C) indicating that post-treatment with chelators was not sufficient to extract divalent cations from capsules.

We conclude from these results that the requirement for EDTA or PBS during fixation in order to achieve bright AO staining is due to the capture of divalent ions released during the fixation process and not to extraction of divalent ions already present in capsules in vivo prior to fixation. Our results are thus in agreement with the X-ray spectral analysis showing that capsules contain primarily monovalent cations.

Discussion

The present results demonstrate that pG can be visualized in nematocyte capsules by staining with the fluorescent dyes AO and DAPI. The staining is metachromatic and depends on the ability of the dyes to oligomerize on the pG substrate. Staining is sensitive to the concentration and valency of competing cations (Weber, 1989). Low concentrations of divalent cations and high concentrations of monovalent cations inhibit staining (Fig. 3). Strong staining requires the presence of EDTA or PBS during fixation to sequester divalent cations released in tissue by fixation (Fig. 7).

Our results show that pG synthesis starts in capsules after invagination of the tubule while differentiating nematocytes are

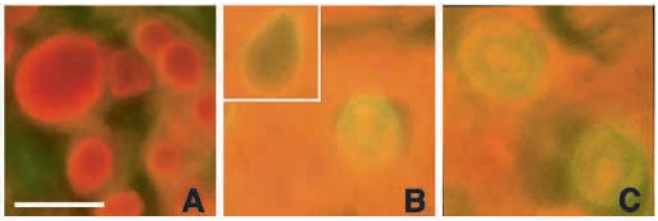

Fig. 7. AO staining of nematocysts after fixation in the presence and absence of chelators of divalent ions. Animals were fixed in 4% formaldehyde in the presence of PBS (A), Tris buffer (B), or Tris buffer followed by incubation in PBS (C). Capsules in A stain bright red with AO. Capsules in B stain weakly with AO; the insert shows an exploded capsule that is empty of pG for comparison. Capsules in C also stain weakly with AO indicating that post-treatment cannot improve AO staining. Identical results were obtained when 10 mM EDTA (in Tris buffer) was used in place of PBS. Bar, 10 μm.

still in nests in the body column. Initially, capsules fluoresce yellow/green when stained with AO due to the low concentration of pG (Fig. 4). As the concentration of pG increases capsules become red fluorescent and appear similar to mature capsules found in the tentacles. At this stage, nests break up into single cells that migrate to the tentacles. Measurements of stenotele size (Fig. 5) show that stenoteles swell as pG synthesis proceeds. Since the capsule wall is impermeable to pG, the increasing pG concentration in the matrix leads to increased osmotic pressure within the capsule and hence swelling.

In agreement with the results of Weber (Weber, 1995), we observed no AO or DAPI staining outside capsules. This implies that the enyzmes required for pG synthesis are imported into the capsule matrix during capsule formation. This behavior is similar to spinalin, a major constituent of the stylets and spines of nematocytes (Koch et al., 1998), and to the H22 antigen (Engel et al., 2001; Kurz et al., 1991), a major component of the outer surface of capsules. Both proteins accumulate within the matrix during capsule formation. After tubule invagination spinalin is transported across the tubule wall to form the spines that develop inside the inverted tubule. In a similar manner, H22 is transported through the capsule wall to the outer surface. The tubule and capsule wall at this stage are sufficiently permeable to permit passage of proteins. Morphologically this stage appears to correspond to the ‘soft’ wall stage (i.e. the stage at which formaldehyde fixation still leads to wall deformation). Subsequent wall ‘hardening’ caused by formation of interchain disulfide bonds in minicollagen peptides in the wall (Engel et al., 2001) blocks this transport process and allows accumulation of pG polymers within the capsule matrix and in the tubule lumen of stenoteles.

Since pG is also found within the tubule lumen in stenoteles, it is necessary to assume that in stenoteles the required enzymes enter the tubule lumen from the matrix, while the tubule wall still permits passage of proteins such as spinalin. PG synthesis then occurs within the tubule and is presumably independent of pG synthesis in the matrix. This ‘intratubule’ pG is different from matrix pG, since it remains stained with AO or DAPI under conditions in which these dyes have been washed off matrix pG (Figs 3, 6). Tubule pG cannot mix with matrix pG since the tubule wall at this stage has presumably become impermeable to the passage of macromolecules.

PG was only found in the tubule lumen in stenoteles but not in desmonemes and isorhizas. The reason for this difference is not immediately obvious but could be related to the complex explosion mechanism in stenoteles. Holstein and Tardent succeeded in filming the stenotele explosion process and showed that it consisted of two steps with very different kinetics (Fig. 8) (Holstein and Tardent, 1984). In the first, exceedingly fast step, the stylet apparatus is ejected from the capsule in less than 10 micro-seconds; in a second slower process the stylets open out and the tubule is everted through itself (3 milli-seconds). The unusual localisation of pG within the tubule at the base of the stylets (Fig. 6) suggests that it could contribute to the second phase of the explosion process by inducing the spreading of the stylets, as shown in Fig. 8.

Identification of the cations bound to pG in vivo

Determination of the cation content of capsules in vivo has been difficult because of the high affinity of pG for cations and hence the possibility of contamination with cations released from tissue during isolation or fixation procedures. The standard capsule isolation procedure (Weber et al., 1987) involves homogenization of polyp tissue followed by density gradient centrifugation. Capsules isolated by this procedure contain high levels of Mg^{2+} and Ca^{2+} ions. In an alternative approach, Gerke and Zierold et al. used shock freezing to preserve the in vivo status of capsules and avoid contamination with ions released from tissue during capsule isolation (Gerke, 1989; Zierold et al., 1991). These authors found high levels of K^{+}-ions in *Hydra* nematocysts using X-ray spectral analysis on EM sections and concluded that capsules contain monovalent cations in vivo.

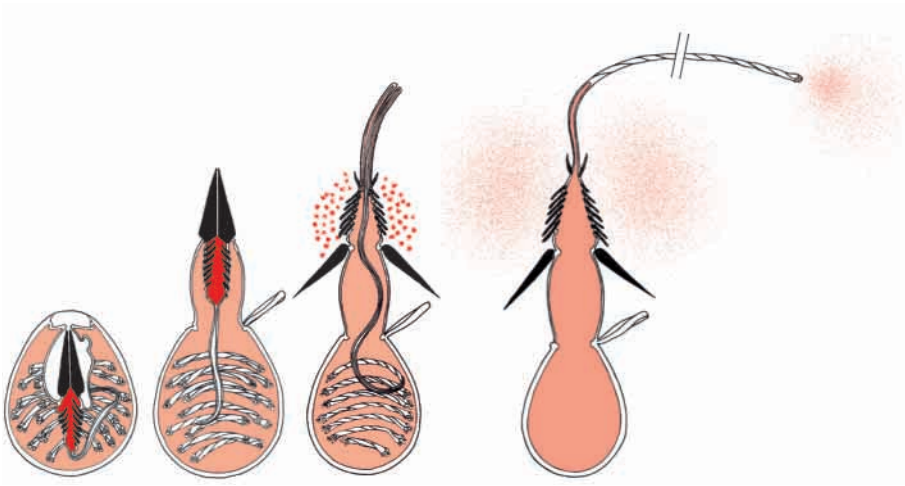

Fig. 8. The stenotele explosion process (modified from Tardent and Holstein, 1982). PG in the matrix is shown in pale red, pG in the lumen of the tubule in dark red. The tubule containing the stylets and spines is ejected in the first phase of the explosion process. In the second phase of the explosion, the pG in the lumen swells, spreading the stylets and permitting the continued ejection of the tubule.

The AO staining results described here provide further support for the monovalent cation hypothesis. Polyps fixed in buffer without chelators of divalent cations bound AO poorly. By comparison, polyps fixed in the same buffer plus EDTA or PBS had brightly stained nematocysts. Since these conditions were not sufficient to extract divalent cations from capsules after fixation (Fig. 7), we conclude that the bright staining observed when tissue is fixed in EDTA or in PBS is due to capture of divalent cations released during the fixation process. In the absence of chelators, these divalent cations bind to capsules and inhibit AO staining. Thus our results are in agreement with the X-ray spectral analysis (Gerke, 1989; Zierold et al., 1991) showing that capsules contain primarily monovalent cations in vivo.

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