Novel role of calcium in exocytosis: Mechanism of nematocyst discharge as shown by x-ray microanalysis

(Sea anemone/nematocyte excitation/calcium-binding protein/stimulus-secretion coupling)

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ABSTRACT Mature nematocysts of the sea anemones Rhodactis rhodostoma and Anthopleura elegantissima contain a fluid that has a high concentration of solutes and is extraordinarily rich in calcium (ca. 500-600 mmol/kg wet weight); this contrasts with the surrounding cytoplasm which is rich in potassium but poor in calcium. The undischarged capsule is surrounded by a membrane that probably acts as a selective permeability barrier between the cytoplasm and the nematocyst fluid. During discharge the nematocyst moves to the surface of the nematocyte and comes into contact with the external sea water medium. Calcium, which may be bound to proteins in the undischarged state, is rapidly lost from the fluid; at the same time, sea water enters the capsule. In vitro experiments have already shown that calcium loss increases the osmotic pressure of the capsular fluid, causing an influx of water from the external medium; this influx appears to increase the hydrostatic pressure inside the capsule to the point that the thread events explosively.

Nematocysts are stinging organelles found in cnidarians; each one consists of a closed capsule which contains an inverted harpoon-like thread that can be discharged explosively. Although the structural events during eversion of the thread have been described in detail (1), little is known about the mechanism of discharge (2–5). Recently, Lubbock and Amos (6) found that discharge could be initiated in vitro by the removal of bound calcium from the fluid inside the nematocyst; calcium removal increased the osmotic pressure of the nematocyst fluid, thus producing an influx of water from the external medium and an increase in the internal hydrostatic pressure of the capsule. It was notable that alterations in the permeability of the capsule wall to water did not seem to be involved in controlling discharge in vitro. At that time, the relevance of the in vitro experiments to nematocyst discharge in vivo was not clearly established because data were not available on the ionic changes occurring under natural conditions.

In the present study, we examined, by x-ray microanalysis of frozen sections, the elemental composition of both excited and unexcited nematocytes in the sea anemones Rhodactis rhodostoma and Anthopleura elegantissima. Our results enable us to present a generalized model of the events that take place during excitation.

MATERIALS AND METHODS

Mesenterial filaments from R. rhodostoma were placed in synthetic sea water (Tropic Marin Neu; specific gravity, 1.021) containing 16.8% (wt/wt) dextran (250,000 daltons; Sigma, clinical grade). To quench freeze, filaments with droplets of dextran/seawater were placed on the truncated conical tips of copper pins and rapidly immersed in a well-stirred slush of Freon-13 (monochlorodifluoromethane, m.p. −181°C) (7–9). In certain cases, filaments mounted on copper pins were electrically stimulated <3 sec before instant quenching in order to induce nematocyst discharge. Acrorhagial tissue was obtained from A. elegantissima by removing inflated acrorhagi with forceps and placed in sea water containing 20% (wt/wt) dextran and rubidium chloride (final concentration, 80 mmol/kg wet weight). Nematocyst discharge was induced by touching the tip of an inflated acrorhagus with a small piece of allogeneic columnar tissue. Acrorhagi were quenched as described above. All frozen samples were removed from Freon and stored in liquid nitrogen until required.

For electron microprobe x-ray analysis, frozen sections 1–3 μm thick were cut at −60 to −80°C in a cryomicrotome (SLEE, London) with steel knives, mounted, and examined in a JEOL JXA-50A microprobe analyzer. This instrument was fitted with a low-temperature transmission stage, an anticontamination cap, two x-ray diffracting spectrometers (used for sodium and calcium), a Kevex x-ray energy spectrometer, and a Link Systems multichannel analyzer backed with a computer and QUANTEM/FLS program (on loan from Link Systems, England) for deconvolution and background subtraction by least-squares fitting of prefiltered spectra (10). Quantification of x-ray data was performed by the continuum method (11) with dextran/seawater as the peripheral reference standard. Sections were analyzed first in a hydrated state and second after dehydration within the microanalyzer. Further details of the technique, quantification procedures, and criteria to assess full hydration of sections have been discussed elsewhere (7–9, 12, 13).

For conventional transmission electron microscopy, material was prepared by standard procedures of glutaraldehyde/osmium fixation, dehydrated in ethanol and embedded in Araldite. Thin sections stained with uranyl acetate and lead citrate were examined in a Philips EM 300 electron microscope.

RESULTS

The concentrations of elements detected in nematocytes of R. rhodostoma are shown in Table 1. The cytoplasm of unexcited nematocytes was found to contain relatively large amounts of potassium, chloride, and sulfur; sodium, magnesium, phosphorus, and calcium were also present but at lower concentrations. The composition of the cytoplasm contrasted strongly with that of the fluid inside undischarged nematocysts (Figs. 1–3) (nematocysts classified as holotrichous isorhizas; cf. ref. 5), the latter being particularly rich in calcium (606 mmol/kg wet weight) but poor in other elements detected. The nematocyst fluid contained about 32% dry matter; indicating an excess of almost 30% over sea water; this agrees with results obtained by interference microscopy (6). The wall of undischarged nematocysts, probably permeated with some fluid, contained 380 mmol of calcium per kg wet weight as well as moderate amounts of sodium, chloride, and sulfur; previous microprobe studies of air-dried empty cap-
tables (14) have shown sulfur to be an important constituent of the capsule wall. The thread, probably including some fluid, contained 212 mmol of calcium per kg wet weight as well as significant quantities of sodium, sulfur, and zinc; zinc is not shown in Table 1 because it was not quantified accurately, but it was present in the thread at about 50-100 mmol/kg wet weight. High calcium concentrations seemed to be restricted to mature nematocysts; the fluid contents of nematocysts in the process of formation (Fig. 1; Table 1) had an elemental composition similar to that of the surrounding cytoplasm.

Prior to discharge, the nematocyst is relatively deeply embedded in the nematocyte and can be seen in transmission electron micrographs to be surrounded by a unit membrane just exterior to the capsule wall. At an early stage in the discharge process the capsule seems to move toward the plasma membrane so that its tip is exposed to the external sea water medium; the rest of the capsule remains in the nematocyte but becomes surrounded by a narrow layer of fluid (Fig. 1 Inset). Eversion of the thread was only seen to begin after the capsule had come into contact with sea water.

As can be seen from Table 1, there is a marked change in the composition of the fluid inside the nematocyst during discharge. Previous work (6) on isolated capsules has shown that the fluid inside the nematocyst is progressively diluted until it has a refractive index similar to that of sea water. A decrease in the dry mass of the fluid inside discharging capsules was also noted in the present experiments; the extent of this decrease was probably not significantly affected by the presence of dextran (250,000 daltons) in the outside medium because the capsule wall is not permeable to substances above about 1000 daltons (6). Analysis of the major changes in elemental composition during discharge was carried out by plotting % dry mass of the fluid inside the nematocyst against the concentrations of calcium, sodium, and chloride (Fig. 4a). The results indicate that at the beginning of discharge there is a massive efflux of calcium from the nematocyst fluid that is disproportionate to the loss of dry mass; a 50% decrease in calcium concentration, for example, is accompanied by a decrease of about 15% in dry mass. As the calcium is being lost, there is an influx of sodium and chloride but not of potassium; this indicates that, during discharge in vivo, it is sea water that enters the capsule and not the cytosol from the nematocyte.

As pointed out above, the discharging capsule becomes surrounded by a narrow layer of fluid (Fig. 1 Inset); this fluid contains substantial quantities of sodium, chloride, and calcium (Table 1). Furthermore, the calcium concentration in the surrounding fluid frequently exceeds that of the internal fluid of the same nematocyst by more than 100-200 mmol/kg wet weight. The elemental composition of the surrounding fluid suggests that it may be sea water into which calcium has escaped from the capsule.

The concentrations of elements detected in nematocytes of A. elegantissima are shown in Table 2. The cytoplasm of unexcited nematocytes contained relatively large amounts of potassium and chloride; sodium, magnesium, phosphorus, sulfur, and calcium also were present but at lower concentrations. The relatively small size of the nematocytes (Fig. 5) (nematocysts classified as holotrichous isorhizas; cf. ref. 5) prevented proper resolution of the various components, and it is likely that mea-

![Fig. 1. Scanning transmission electron micrograph of frozen dried section (1-2 μm thick) of mesenterial filament from R. rhodostoma, showing external sea water medium (s), a mature undischarged nematocyst (n) (dark structures within the nematocyst are sections of the coiled thread), and a forming nematocyst (f). (Inset) Discharging nematocysts surrounded by a fluid layer (l). (Scale bar = 20 μm.)](image-url)
measurements of the fluid contents were in some cases affected by the unseen presence of thread, capsule wall, or even overlapping cytoplasm. Nevertheless, it is clear from Table 2 that the undischarged nematocysts contained a rather high proportion of dry mass and a very high calcium concentration (542 mmol/kg wet weight); magnesium was more concentrated than in the cytoplasm whereas potassium and chloride again were very low (see Table 1). Rubidium, included as a potassium analogue in the sea water, was found to enter the cytoplasm; the level of rubidium within the nematocyst was similar to that in the cytoplasm, showing that exchange of these ions must occur between the cytoplasm and the nematocyst fluid. The very high level of calcium in undischarged, mature nematocysts was ab-

FIG. 2. (a) Section of mesenterial filament from *R. rhodostoma* showing mature undischarged nematocysts; specimen prepared and photographed as in Fig. 1. (b) Calcium K$_\alpha$ x-ray intensity distribution over the same field, collected with 1-nA current over 500 sec. (Scale bar = 20 $\mu$m.)

FIG. 3. X-ray energy spectra from the internal fluid of a mature, undischarged nematocyst (a) and the adjacent cytoplasm within the nematocyte (b) of *R. rhodostoma*.

FIG. 4. Changes in the levels of calcium (solid circles, solid line), sodium (squares, broken line), and chloride (open circles, dotted line) in the internal fluid of discharging nematocysts from *R. rhodostoma* (a) and *A. elegantissima* (b). Each point is based on measurements from a single discharging nematocyst, except that the ringed points represent the condition before discharge and are based on 43 nematocysts in *R. rhodostoma* and 50 nematocysts in *A. elegantissima*. 
sent from those in the process of formation; the latter contained high levels of chloride and sulfur as well as moderate quantities of potassium and magnesium.

During discharge, the elemental composition of *A. elegansissima*'s nematocysts also changes markedly (Table 2). There is a massive initial efflux of calcium from the nematocyst fluid which, as in *R. rhodostoma*, is disproportionate to the loss of dry mass (Fig. 4b); for example, a 70% decrease in the calcium level is accompanied by an approximately 10% decrease in dry mass. As can be seen from Table 2, the efflux of calcium is accompanied by an influx of sodium, chloride, and rubidium but not potassium. This indicates that, as in *R. rhodostoma*, ions and fluid entering the discharging capsule are coming from the sea water medium rather than the cytoplasm.

**DISCUSSION**

The results from both *R. rhodostoma* and *A. elegansissima* indicate that the fluid contained within a mature but undischarged nematocyst is of a fundamentally different composition from the cytoplasm of the surrounding cell: the cytoplasm is rich in potassium but poor in calcium, whereas the nematocyst fluid is poor in potassium but very rich in calcium. High calcium concentrations seem to be found only in mature nematocysts, those in the process of formation having levels of calcium similar to those of the cytoplasm. The principal permeability barrier between the cytoplasm and the nematocyst is probably the unit membrane that surrounds the capsule wall rather than the capsule wall itself, the latter being permeable in *vitro* to charged substances of less than about 1000 daltons (6). The membrane appears to be selectively permeable because rubidium can enter undischarged nematocysts from the cytoplasm. At the beginning of discharge, the capsule moves toward the surface of the host cell and comes into contact with the external sea water medium. The fate of the membrane surrounding the capsule was not observed, although it seems likely that it fuses with the plasma membrane as in other exocytotic processes (15). Once the discharging capsule is in contact with the sea water, there is a massive loss of calcium from the internal fluid. Calcium loss has been shown in *vitro* (6) to increase the osmotic pressure of the nematocyst fluid, thus causing an influx of water and an increase in the internal hydrostatic pressure of the capsule; ultimately, the thread everts explosively.

The molecular events occurring during the liberation of calcium from the nematocyst are of considerable interest. In the undischarged state, the internal fluid of the nematocyst contains a substantial dry mass (about 32% in *R. rhodostoma* and 44% in *A. elegansissima*); data from other anemone species suggests that this dry mass may be primarily attributable to dissolved proteins (16, 17). The very large concentration of calcium inside the nematocyst is clearly not balanced by possible anionic elements detected with the microprobe; this indicates that the calcium may be associated either with hydroxyl ions or with organic anions. In this respect it is interesting to note that proteins in the nematocyst fluid are unusually rich in acidic amino acid residues, in particular glutamatic acid and aspartic acid (16, 17), calcium-binding proteins in other cells typically have high levels of aspartic, glutamic, or γ-carboxyglutamic acid residues which may be involved in the formation of high-affinity calcium-binding sites (18). This suggests that calcium within the nematocyst may be associated with glutamate or aspartate residues of dissolved proteins. The mechanism by which the osmotic pressure increases when calcium is lost (6) is not clear at present; one interesting possibility is that, in the resting state, dissolved proteins are bound by calcium into aggregates and that the removal of calcium causes dissociation of the aggregates into a greater number of smaller molecular particles.

The rapid loss of calcium from the nematocyst at the beginning of discharge is accompanied by only a small decrease in the dry mass of the capsular fluid. If the anions associated with calcium in the undischarged state are part of the bulk of the dry mass in the fluid, then clearly the loss of calcium must produce a marked cation deficiency. Although the sodium concentration in discharging capsules sometimes exceeds the chloride concentration, it seems clear that any net entry of cations from the sea is insufficient to balance the loss of calcium. This suggests that calcium within the capsule may be replaced by either organic cations or protons, thus maintaining the charge equilibrium. In *R. rhodostoma* there appears to be a narrow layer of fluid between the discharging capsule and the cytoplasm, into which the calcium passes. This fluid is partly derived from sea water but contains more chloride than sodium. The chloride...
excess is quite insufficient, however, to balance the large calcium content and it seems likely that again either organic anions or hydroxyl ions are also present.

Discharge does not appear to be attributable simply to the nematocyst coming into contact with sea water. Unpublished experiments show that isolated nematocysts brought into contact with sea water frequently do not discharge immediately unless a calcium chelating agent such as citrate is added to the medium (cf. ref. 6). This suggests that two simultaneous processes may be involved in vivo, the first being the translocation of the nematocyst into contact with the external medium by membrane fusion (15) and the second being the release of substances that unbind calcium.

The above results together with those of other recent studies suggest the following tentative model of the events occurring during nematocyte excitation. Specific cell surface receptors on the nematocyte or nearby cells are excited by contact with an appropriate substrate (19, 20), producing electrical activity (21). The nematocyst capsule moves to the surface of the nematocyte and comes into contact with sea water; the membrane that surrounds the undischarged nematocyst presumably fuses with the plasma membrane as in other exocytotic processes (15). Possibly at the same time, substances that unbind calcium are released from the nematocyte cytoplasm. Calcium, which is present at a very high concentration in the nematocyst fluid and may be bound to proteins, is rapidly lost from the capsule. The calcium loss increases the osmotic pressure of the capsular fluid (6), producing an influx of sea water. How the increase in osmotic pressure is produced is not clear, but it could involve the dissociation of protein aggregates bound by calcium. The water influx raises the internal hydrostatic pressure of the capsule to the point that the thread discharges explosively. Once the thread is fully everted, the hydrostatic pressure serves to inject the internal fluid, which is venomous (5), through the thread into the target.

In conclusion, it seems clear that calcium plays an important role in nematocyst discharge in vivo. A question of considerable interest is whether analogous discharge mechanisms exist in any other exocytotic processes; mucus granules in vertebrate goblet cells, for example, contain fairly high concentrations of calcium prior to release (8). The nematocyst is a specialized product of the Golgi apparatus (22), and it perhaps would not be surprising if its explosive discharge mechanism was modified from other less-striking secretion processes.

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