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VISUALIZATION OF CEMENT EXOCYTOSIS IN THE CYPRIS CEMENT GLAND OF THE BARNACLE *MEGABALANUS ROSA*

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Summary

Cementation to substrata during permanent attachment concludes the planktonic larval phase in many sessile marine invertebrates, including barnacles. However, the neural control and the mechanism of cement secretion from cement organs are poorly understood. In the present study, using isolated cement glands from cyprids of *Megabalanus rosa*, we have visualized cement secretion and demonstrated the stimulatory effect of dopamine and noradrenaline on such secretion. The abrupt disappearance of secretory granules and subsequent omega-figure formation indicated that exocytosis was the major mode of cement secretion. Exocytosis was localized at the apical surface of cement-secreting cells and lasted for over 30 min. Dopamine and noradrenaline also activated the directional transport of secretory granules to the sites of exocytosis. Glyoxylic acid staining provided histochemical evidence for catecholaminergic innervation to the cement glands. These results suggest that gradual, localized exocytotic secretion of cement triggered by catecholaminergic neurones is a key mechanism during permanent attachment by barnacle cyprids.

Key words: barnacle, cyprid, *Megabalanus rosa*, settlement, cement, exocytosis, secretion, dopamine, noradrenaline, catecholamine.

Introduction

The life cycle of the barnacle is split into two distinct phases; free-swimming larvae which eventually settle to become sessile adults (Darwin, 1854). The switch in life style occurs at larval permanent attachment, which is initiated by the release of special adhesive substances, the so-called cement. Following permanent attachment, barnacles become sedentary; therefore, this step by the cypris larvae decides their future and is also of special interest with respect to antifouling studies (Saroyan *et al.* 1970; Clare, 1995).

Cypris larvae do not feed but search for suitable substrata for settlement (Walker *et al.* 1987). The structure of cyprids and metamorphosing larvae as well as their exploratory behaviour and development to young barnacles have already been well described (Doochin, 1951; Walley, 1969).

A pair of kidney-shaped cement glands, ventrolaterally positioned, is the location of cement storage and secretion (Walker, 1971). Histochemical and electron microscopic studies in *Balanus balanoides* (=*Semibalanus balanoides*) (Walker, 1971) have shown that, when attachment occurs, cement in each gland is secreted into a median collecting duct, transported *via* a cement duct and released onto the surface of the antennule attachment disc. The muscular sac, located at the proximal portion of the cement duct, is believed to help expel cement by its pumping action (Walley, 1969). In the cement gland secretory cells, cement is stored in secretory granules, which are 3–4 μm in diameter in *B. balanoides* (Walker, 1971) and 1–4 μm in diameter in *Megabalanus rosa* (K. Okano, K. Shimizu, C. G. Satuito and N. Fusetani, in preparation).

An understanding of how cement is secreted from secretory cells and how this process is regulated is critical to understanding how permanent attachment occurs. There is, however, as yet no physiological study which addresses the control and mechanism of cement secretion. Furthermore, neural innervation of the cement glands has yet to be investigated. This is partly due to the small size and somewhat complex structure of these larvae (Anderson, 1994). Access to the cement glands is further hampered by their containment within the bivalved carapace (Walley, 1969).

Using isolated cement glands from cyprids of the barnacle *Megabalanus rosa* (Kado and Hirano, 1994), we sought to visualize cement secretion *in vitro*. We first assumed that, if exocytosis is the mode of secretion (Almers, 1990; Monck and Fernandez, 1992), it could be seen as a sudden disappearance of granules at the potential release sites because of the large size of the granules. We also expected that extensive exocytosis could cause morphological changes in the gland.

In the present study, we report that dopamine and noradrenaline induce exocytotic cement secretion which was localized at sites adjacent to the median collecting duct. We also demonstrate the stimulatory effect of these catecholamines

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on directional granule movement. The presence of catecholaminergic innervation of cement glands is then confirmed using glyoxylic acid fluorescence histochemistry. On the basis of these findings, a mechanism of cypris cementation is proposed.

Materials and methods

Larval culture

Adult *Megbalanus rosa* (Pilsbry) were collected regularly from a population growing on fish cages in Nagai Harbor on the Miura Peninsula (35°20′ N, 139°60′ E), Eastern Japan, during March–September 1995, and were maintained in cartridge-filtered (Microwynd II, 0.45 μm) natural sea water at 25 °C and fed daily on a mixed diet of *Artemia salina* (Nisshin Fine Chemical Co. Ltd) nauplii (cultured on *Isochrysis galbana*) and the diatom *Chaetoceros gracilis*. Under these conditions, the brood stock remained healthy and released nauplii constantly for up to 2 weeks, after which it was replaced with freshly collected adults. Nauplii were cultured at 25 °C with weak aeration at an initial density of 1.5 larvae ml^{-1} on a diet of *Chaetoceros gracilis*. Larvae were collected daily on 100 μm nylon plankton nets, washed with filtered sea water and transferred to newly prepared algal diet suspensions. Larvae reached the cypris stage in 7–8 days. The nauplius/cyprid moult is referred to as day 0. In all experiments, 4- to 11-day-old cyprids were used. During this period, *M. rosa* cyprids are known to be competent for settlement (C. G. Satuito, unpublished observations).

Dissection of cement glands

Cement glands of *M. rosa* cyprids were visible under the dissection microscope (Fig. 1A). Isolation of the glands was performed in Mg^{2+}-substituted nominally Ca^{2+}-free barnacle saline (Hayashi and Stuart, 1993) containing 462 mmol l^{-1} NaCl, 8 mmol l^{-1} KCl, 32 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} Hepes (buffered to pH 7.5–7.6 with TrisOH, osmolality 960–1000 mosmol kg^{-1}). The absence of Ca^{2+} assisted in immobilization and relaxation of the cyprids, which made the following dissection easier (Fig. 1A). This also minimized any transmitter release and, hence, cement secretion during the dissection. Using finely etched tungsten needles, the thorax bearing the six pairs of swimming appendages was removed from the larva, following which the adductor muscles and the conjunction of the bivalved carapace were severed to expose the internal cavity. The cement glands were then carefully dissected out, freeing them completely from the carapace, brain and attachment organ (Fig. 1B). Care was taken to avoid direct contact of needles with glands. Any damaged glands were discarded. Isolated glands (Fig. 1C) were thoroughly washed with normal barnacle saline (Hoyle and Smyth, 1963) containing 462 mmol l^{-1} NaCl, 8 mmol l^{-1} KCl, 20 mmol l^{-1} $CaCl_2$, 12 mmol l^{-1} $MgCl_2$ and 10 mmol l^{-1} Hepes (buffered to pH 7.5–7.6 with TrisOH, osmolality 960–1000 mosmol kg^{-1}). When the dissecting process had been successfully completed, there was little noticeable change in glandular appearance during isolation or solution changes.

Drug administration and video-microscopy

Test solutions were directly applied to the bath containing the isolated cement gland in saline. Test solutions were prepared immediately prior to experimentation by dilution of stock solutions with normal barnacle saline to the required experimental concentration. Each stock solution, containing 100 mmol l^{-1} biogenic amine dissolved in water (NANOpure, Barnstead), was stored at −30 °C until use. Exocytosis was observed and video-recorded under an inverted Nomarski microscope (Nikon TMD-300) equipped with a ×60 or ×40 differential interference contrast (DIC) objective lens. All experiments were carried out at room temperature (22–24 °C).

Glyoxylic acid fluorescence histochemistry

Freshly dissected cement glands were incubated for 10 min

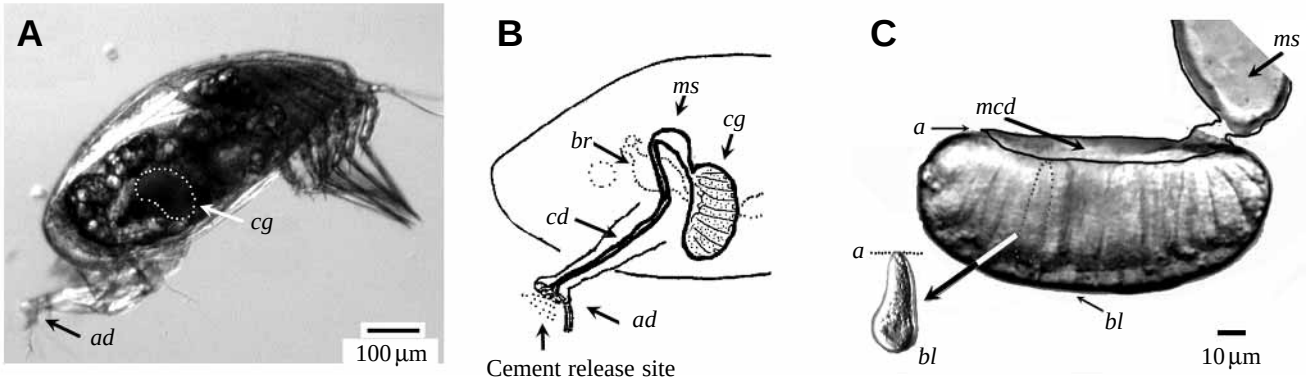

Fig. 1. Cement gland of *Megabalanus rosa*. (A) A cypris larva of *M. rosa* in Mg^{2+}-substituted nominally Ca^{2+}-free barnacle saline prior to dissection. A pair of kidney-shaped cement glands, ventrolaterally positioned, is visible (dotted line). *ad*, attachment disc, *cg*, cement gland. (B) Schematic representation of the organs involved in cement release. *br*, brain; *cd*, cement duct; *ms*, muscular sac. (C) Isolated cement gland. *mcd*, median collecting duct. Secretion occurs at the apical surface of cement-secreting cells facing the median collecting duct. The major part of the cement glands is occupied by elongated secretory cells with a basal nucleus and cytoplasm filled with large secretory granules (inset). *a*, apical. *bl*, basal.

with 30 % sucrose in 0.1 mol l^{-1} phosphate buffer (pH 7.4) containing 2 % glyoxylic acid (Lindvall and Bjorklund, 1974; Kurokawa *et al.* 1989). Specimens were then air-dried and heated at 95 °C for 3–4 min. Catecholamine staining was observed under a fluorescence microscope (Nikon, Optiphoto) using violet excitation and 430 nm emission.

Results

Isolated cement glands (Fig. 1C) perfused with barnacle saline demonstrated no change in appearance for over 1 h. The first question addressed was whether any known neurotransmitter could cause substantial morphological changes in the glands. Dopamine, noradrenaline, adrenaline, octopamine, 5-hydroxytryptamine, acetylcholine, L-glutamate, L-aspartate and γ-aminobutyric acid (GABA) (all at 10 μmol l^{-1}) were tested but only dopamine and noradrenaline were found to cause changes. These two amines were investigated further.

Visualization of cement exocytosis

The first visible change in apical surface morphology of an isolated gland was detected 20 s following application of 10 μmol l^{-1} noradrenaline and was characterized as increased curvature and subsequent expansion of the median collecting duct (Fig. 2). With time, the duct area expanded further and became concave, as shown by a focus change. Under the microscope, at the portion of the duct adjacent to the secretory cells, abrupt changes in brightness started approximately 25 s after noradrenaline application and were frequently observed thereafter (Fig. 2C). Each change in brightness is presumed to correspond to a single exocytotic event (Terakawa *et al.* 1991; Burgoyne and Morgan, 1993; Schweizer *et al.* 1995). At higher magnification, recorded sequences showed the disappearance of individual granules (Fig. 3; in response to the application of 1 μmol l^{-1} dopamine), characteristic of exocytosis. Exocytosis was rather slow (approximately 0.3 s) and was observed as an initial sudden fading of reflectance and gradual collapse of the granule, leaving a dimple (omega-shape) at the site it previously occupied. Exocytosis was never observed in the basal and lateral regions of the cement gland, indicating that exocytosis induced by catecholamine application was strictly localized at the apical region (Fig. 2C).

Granule recruitment

Time-lapse video recording further revealed that catecholamine application stimulated directional movements of mid and basal granules within the cells. Although these movements were erratic, the overall direction of movement was clearly towards the apical sites where exocytosis occurred (Fig. 2C).

Characteristics of catecholamine effects

Exocytotic responses were graded (Table 1). Under continuous exposure to the higher concentrations of catecholamines, exocytosis occurred at a fairly constant rate

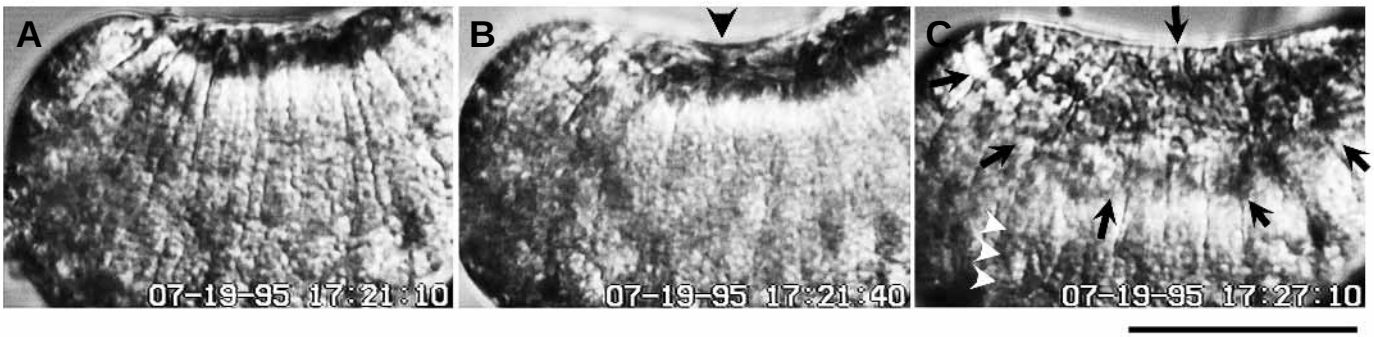

Fig. 2. Changes in morphology of a cement gland treated with 10 μmol l^{-1} noradrenaline. (A) Immediately before application; (B) 30 s after application. Note that the apical curvature is increased (arrowhead). (C) 6 min after application. Exocytoses, indicated by changes in brightness, were observed at the apical region (area surrounded by arrows). Directional granule movements (towards the apical region) were observed in the mid region (open arrowheads). Scale bar, 50 μm.

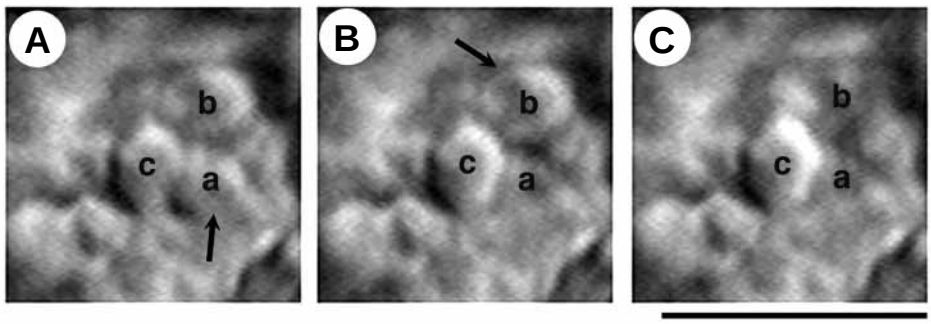

Fig. 3. Fate of individual granules. (A–C) Photomicrographs (1 s intervals) of the same apical surface of a cement gland stimulated with 1 μmol l^{-1} dopamine. Granule **a** disappeared between A and B, and granule **b** disappeared between B and C. Dimples were formed in the area previously occupied by these granules. The abrupt disappearance of granules followed by dimple formation (omega-shaped formation) is characteristic of exocytosis. Granule **c** did not exocytose during this sequence. Scale bar, 10 μm.

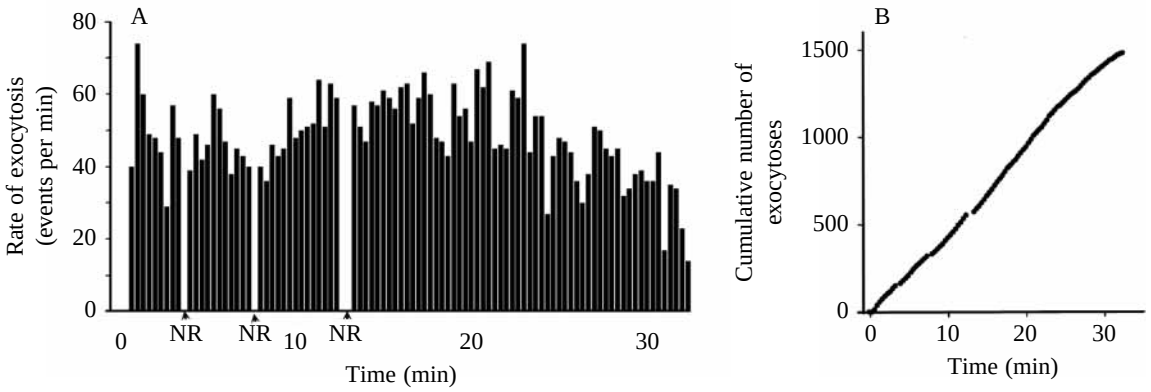

Fig. 4. Time course of exocytosis. A gland was stimulated with 10 μmol l^{-1} noradrenaline. (A) Every 20 s, the number of observed exocytoses (brightness changes at the apical region) was counted and plotted against time. NR, not recorded during this time span. (B) The cumulative number of exocytoses (data from A) *versus* time. Data are taken from the gland shown in Fig. 2.

for over 30 min (Fig. 4), with extensive granule recruitment. When lower doses of catecholamines were applied, only a short (approximately 5 min) and site-restricted change in gland appearance was observed (Table 1). In these cases, directional granule movements were not clearly observed. Dopamine was a more potent stimulant than noradrenaline (Table 1). Octopamine, the monohydroxylic analogue of noradrenaline, and adrenaline had only very weak effects at 10 μmol l^{-1}.

The catecholamine treatments, even at maximal concentrations, did not cause an increase in the volume of the muscular sac or cement release from the cut end of the cement duct, whereas the median collecting duct had the appearance of a translucent membrane-bound cavity, which indicated that the secreted cement had accumulated in it.

Table 1. *Effect of catecholamines on cement gland morphology*

	Concentration (μmol l^{-1})	Glands tested *N*	Changes in gland morphology			
			–	±	+	++
Control	–	11	10	1	–	–
Dopamine	0.1	10	4	5	1	
	1	15	–	–	4	11
	10	8	–	–	1	7
Noradrenaline	0.1	6	5	1	–	–
	1	6	–	2	4	–
	10	14	–	–	4	10
	100	8	–	–	–	8
Adrenaline	10	5	3	2	–	–
Octopamine	10	3	2	1	–	–

The appearance of glands treated with drugs for 30–40 min was compared with the original appearance prior to treatment.

–, no change; ±, a slight apical change without clear concavity formation (no exocytosis was observed); +, apical concavity formation (exocytosis was observed); ++, extensive change in overall gland morphology (with vesicular recruitment).

See text for further details.

The presence of catecholaminergic innervation

To test whether cement glands were innervated by catecholaminergic neurones, isolated glands were treated with glyoxylic acid (Lindvall and Bjorklund, 1974; Kurokawa *et al.* 1989). Bright fluorescence was observed at the apical region of the cement glands, indicating catecholaminergic innervation (Fig. 5). The innervation was characterized by a net-like structure with many varicosities present around the interface of the median collecting duct and the secretory cells. The number of varicosities (approximately 70) observed following glyoxilic acid staining was comparable to the number of large basal nuclei observed in this region when stained using 4′,6-diamidino-2-phenylindole (DAPI) (Russell *et al.* 1975). Since the number of these nuclei should correspond to the number of major secretory cells (Walker, 1971), the innervating nerve(s) may form a slow-acting synapse with each secretory cell (Burns and Augustine, 1995).

Discussion

The suggested mechanism for cement secretion from the cement glands is summarized as follows (Fig. 6). The brain activates the catecholaminergic neurones, which innervate the cement glands to release dopamine or noradrenaline. The catecholamines bind to catecholaminergic receptors on the plasma membrane of cement-secreting cells to induce three actions. The first action, initiated within 20 s, is characterized by changes in apical curvature and in the appearance of the median collecting duct. These changes may arise from cytoskeletal changes inside the secretory cells or duct cells (Walker, 1971), and prime the subsequent exocytotic secretion, since these changes precede the first exocytosis event. Alternatively, the change in curvature may be caused by fluid secretion into the median collecting duct, which may control the hydration of cement polymer, thus preventing cement hardening in the median collecting duct (Verdugo, 1990;

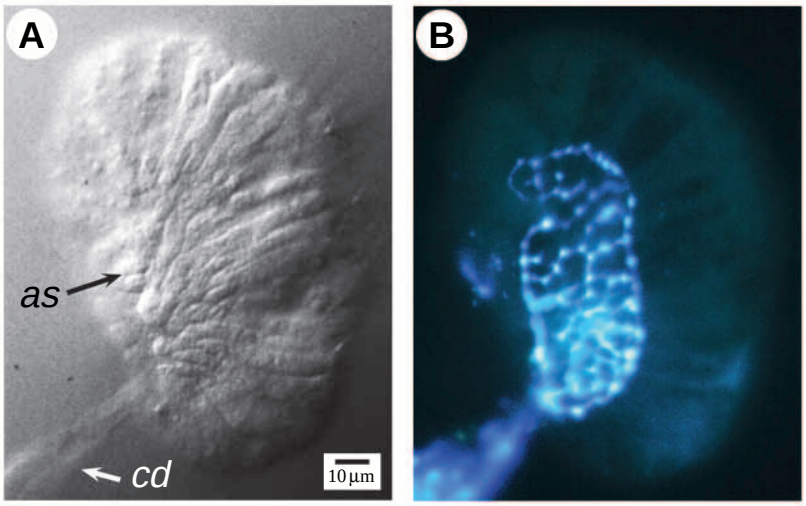

Fig. 5. The presence of catecholaminergic innervation at the apical region of the cement gland. Glyoxylic acid fluorescence histochemistry was used. (A) Apical view of a treated cement gland under Nomarski optics. *as*, apical surface of cement gland; *cd*, cement duct. (B) The same gland viewed under fluorescent microscopy. Blue fluorescence with many varicosities demonstrates the presence of catecholaminergic innervation.

Kamijo *et al*. 1993). The second action of catecholamines is to induce exocytosis, characterized by the disappearance of granules from the secretory cells. This process, which has a slow time course, could correspond to that of fusion pore expansion and the subsequent release of granule contents (Almers, 1990; Verdugo, 1990; Monck and Fernandez, 1992) (Fig. 6, inset). The third action is characterized by the directional transport of granules to the release sites (Burgoyne and Morgan, 1993), increasing their availability for release.

The mechanism of permanent attachment in barnacle cyprids has been described previously (Walker, 1971; Anderson, 1994). The distal part of the cement duct is thought to function as a stop valve (Nott and Foster, 1969), allowing cement to accumulate within the duct lumen. At the time of attachment, the duct valve presumably relaxes, resulting in explosive release of cement aided by the pumping action of the muscular sac (Walley, 1969). Our study now explains how cement is secreted from the cement glands. The accumulation of cement in the median collecting duct rather than in the cement duct, however, apparently contradicts the previous studies. This could have been due to malfunction of the muscular sac caused by isolation or, alternatively, may suggest a further physiological process involved in cypris permanent attachment. If it is assumed that the cement duct connecting the median collecting duct and the muscular sac functions as a stop valve to accumulate cement, then cement exocytosis

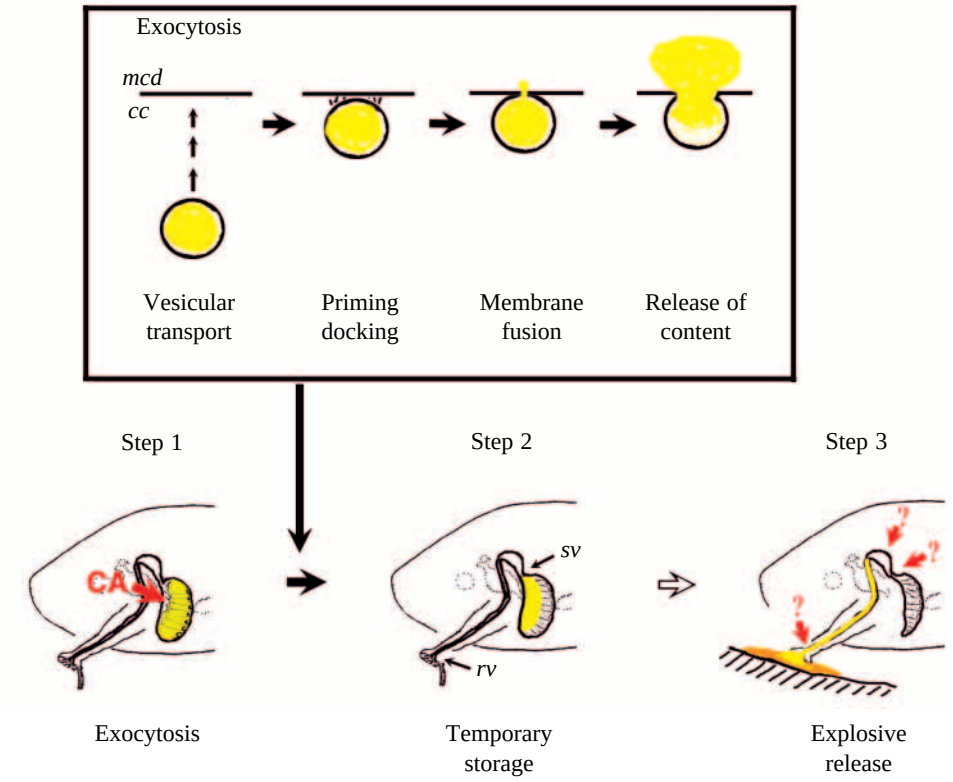

Fig. 6. Schematic representation of the proposed mechanism of cementation during the permanent attachment of barnacle cyprids. Catecholamine (CA) released from neurones innervating the cement glands induces cement granule transport and exocytosis by cement-secreting cells, resulting in cement secretion into the median collecting duct (step 1). The released cement accumulates in the median collecting duct and is stored temporarily (step 2). At the time of permanent attachment, an as yet undetermined system coordinates the actions of the valves and muscular sac contraction, resulting in explosive cement release (step 3). *mcd*, median collecting duct; *cc*, cement-secreting cells; *rv*, release valve (distal valve); *sv*, stop valve (proximal valve).

induced by a transmitter would precede permanent attachment and the median collecting duct would function as a reservoir (Fig. 6, step 2). The distal valve (Nott and Foster, 1969) would then function as a release valve, protecting the duct against the back-flow of sea water. In this case, the coordination of valve actions and muscular sac contractions would be necessary at the time of permanent attachment and would require an as yet unidentified control system.

Dopamine is the leading transmitter candidate for cement secretion because (1) dopamine showed a stronger and more consistent effect on cement secretion than did noradrenaline (Table 1); (2) glyoxylate staining has proved to be effective in dopaminergic neurones (Lindvall and Bjorklund, 1974); and (3) *Balanus amphitrite* cyprids contain substantial amounts of dopamine as detected electrochemically (S. Kawai and K. Shimizu, unpublished observations). The maximal secretory response was induced by $1\,\mu mol\,l^{-1}$ dopamine in 11 out of 15 cases (73 %, Table 1). This effective dopamine concentration is comparable to that found in other arthropod systems, including the salivary gland cell of *Nauphoeta cinerea* ($0.05–1\,\mu mol\,l^{-1}$, Ginsborg *et al.* 1976), the fast extensor musculature of *Macrobrachium rosenbergii* ($0.1–10\,\mu mol\,l^{-1}$, Miller *et al.* 1985), the cardiac muscle of *Limulus polyphemus* ($10\,\mu mol\,l^{-1}$, Groome and Watson, 1989) and *Drosophila melangaster* D1-like receptor expressed in Sf9 cells (EC_{50} approximately $0.3\,\mu mol\,l^{-1}$, Sugamori *et al.* 1995).

Although the involvement of many neurotransmitter candidates has been proposed for the process of attachment and metamorphosis in a range of marine invertebrate larvae, including the red abalone *Haliotis rufescens* (Morse *et al.* 1979), the Pacific oyster *Crassostrea gigas* (Coon and Bonar, 1985; Beiras and Widdows, 1995), the Japanese scallop *Patinopecten yessoensis* (Kingzett *et al.* 1990), the serpulid polychaetes *Hydroides ezoensis*, *Pomatoleios kraussii* and *Ficopomatus enigmaticus* (Okamoto *et al.* 1995), the sea urchin *Pseudocentrotus depressus* (Yazaki and Harashima, 1994) and the barnacle *Balanus amphitrite* (Kon-ya and Endo, 1995), most of these studies fail to specify the site of action. The neural control of cement secretion has not been addressed directly in any marine invertebrate larva. Many of these larvae are too small to manipulate using classical methodology, and seasonality can result in periodic availability (Anderson, 1994). The development of a laboratory culture of *M. rosa* larvae (C. G. Satuito, K. Shimizu, K. Natoyama, M. Yamazaki and N. Fusetani, in preparation) has enabled the stable production for extended periods of the relatively large cypris larvae (approximately 700 μm carapace length) essential for the completion of this study.

The use of isolated cement glands has several advantages: (1) the complexity related to the presence of a neural network is excluded by lesion from brain; (2) the application of transmitters is straightforward; (3) the site for exocytotic secretion can clearly be observed, allowing visualization of single granule fusion events (see Fig. 3); and (4) removal of the carapace allows successful staining of glands with glyoxylic acid. This report is the first direct evidence of neural control of cement exocytosis prior to the permanent attachment of a marine invertebrate larva.

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