The Journal of Experimental Biology 201, 1595–1613 (1998)
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JEB1377

DEFENSIVE INK PIGMENT PROCESSING AND SECRETION IN *APLYSIA CALIFORNICA*: CONCENTRATION AND STORAGE OF PHYCOERYTHROBILIN IN THE INK GLAND

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Accepted 16 February; published on WWW 27 April 1998

Summary

The marine snail *Aplysia californica* obtains its defensive ink exclusively from a diet of red seaweed. It stores the pigment (phycoerythrobilin, the red algal photosynthetic pigment, r-phycoerythrin, minus its protein) in muscular ink-release vesicles within the ink gland. Snails fed a diet of green seaweed or romaine lettuce do not secrete ink and their ink-release vesicles are largely devoid of ink. Successive activation of individual ink-release vesicles by ink motor neurons causes them to secrete approximately 55 % of their remaining ink (similar to the percentage of ink reserves released from the intact gland). The peripheral activation of vesicles appears to be cholinergic: 70 % of isolated vesicles were induced to squeeze ink from their valved end by solutions of acetylcholine at concentrations of 0.5 mmol l^{-1} or below.

Ultrastructural analysis commonly found three cell types in the ink gland. The RER cells, the most numerous, were characterized by an extensive rough endoplasmic reticulum with greatly distended cisternae. This cell type is probably the site for synthesis of the high molecular mass protein of secreted ink. The granulate cells, less common than RER cells, had nuclear and cell areas significantly larger than those of RER cells. In addition, granulate cells of red-algal-fed snails had 4–14 vacuoles that contained electron-dense material with staining characteristics similar to that of ink in mature ink-release vesicles. The granulate cell's plasma membrane was regularly modified into grated areas, which both localized and expanded the surface area for coated vesicle formation and provided a sieve structure that prevented large particles in the hemolymph either from being taken up by, or from occluding, the coated vesicles. Electron-dense particles within coated vesicles were similar in size to those in granulate vacuoles but larger (on average by approximately 1 nm) than those that make up the ink. In green-seaweed-fed snails, granulate cells and their vacuoles were present but the vacuoles were empty. The third cell type, the vesicle cell, expands markedly, with its nucleus enlarging concurrent with cell growth until it is on average 50 times larger in cross-sectional area than the nuclei of either RER or granulate cells; the cytoplasm eventually becomes filled with ink, which obscures the mitochondria, vacuoles and nucleus. Continued cell expansion ceases with the appearance of an encircling layer of muscle and 1–3 layers of cells of unknown origin, thereby becoming the ink-release vesicle itself. The absorption spectra of the soluble contents of mature ink-release vesicles from snails fed red algae had peaks characteristic of the red algal pigment r-phycoerythrin or/and phycoerythrobilin.

Immunogold localization of r-phycoerythrin showed no statistical difference in the amount of label within the ink-release vesicles, RER or granulate cell types. Furthermore, there was no localization of phycoerythrin immunoreactivity within the various cellular compartments of either the RER or granulate cells (nucleus, endoplasmic reticulum, mitochondria, vacuoles). Immunogold labeling in the ink gland ranged from 11 to 16 % of that for the digestive vacuoles of the rhodoplast digestive cells lining the tubules of the digestive gland. Our observations suggest (a) that the main form of the ink pigment in the gland is phycoerythrobilin or/and a non-antigenic form of phycoerythrin, and (b) that separation of the bilin from phycoerythrin (or its modification so that it is no longer antigenic) occurs before it reaches the ink gland, probably within the vacuoles of the rhodoplast digestive cells of the digestive gland.

We propose the following model. The ink pigment, phycoerythrobilin, is cleaved from its protein in rhodoplast digestive vacuoles in the digestive gland. Phycoerythrobilin is carried in the hemolymph to the ink gland, where granulate cells take it up and transport it *via* coated vesicles to membrane-bound vacuoles for long- or short-term storage; later, the ink is incorporated into developing ink-release vesicles. RER cells synthesize the high molecular mass, non-algal protein (whose function is unknown), which constitutes 35 % of the dry mass of secreted ink.

Key words: red alga, r-phycoerythrin, acetylcholine, anti-predator chemical defence, coated vesicle, scanning electron microscopy, transmission electron microscopy, invertebrate, neuroethology, *Aplysia californica*.

Introduction

The secretion of a purple ink provides *Aplysia* species with an active chemical defense against predators (Nolen *et al.* 1995). Of the 37 species of *Aplysia*, 30 have a purple gland that can release copious amounts of ink when the snail is disturbed or attacked by a predator (reviewed in Nolen *et al.* 1995). Ink acts as an antifeedant and causes sea anemones either to regurgitate or to drop captured *A. californica* (Nolen *et al.* 1995). Other predators, e.g. crabs and birds, may be similarly affected (DiMatteo 1981, 1982; Walters *et al.* 1993; Carlson and Nolen, 1997). Inking, therefore, has significant survival value for *Aplysia* sp. Little is known, however, about how this herbivore metabolizes, concentrates and secretes a plant pigment so that it serves as an effective active anti-predator defense. Moreover, the mechanisms of acquisition, processing and storage of these plant chemicals represent a highly elaborate collection of adaptations. Discovering the mechanistic underpinnings of pigment processing, storage and release has important implications for understanding the evolution of this defensive behavior as well as its ecological significance for this wide-ranging taxon.

Ink pigments are extracted from the phycobilisomes of red algal rhodoplasts (Chapman and Fox, 1969). MacColl *et al.* (1990) report that ink consists principally of a bilin chromophore, phycoerythrobilin, and that the protein in ink has a higher molecular mass than that associated with phycoerythrin – one of the main subunits of r-phycobilisomes. Chapman and Fox (1969) suggested that the cleavage of the protein from the bilin chromophore of phycoerythrin occurs in the ink gland itself, but our recent ultrastructural study (Coelho *et al.* 1998), using an immunogold tag for r-phycoerythrin, suggests that phycoerythrin is modified first in the rhodoplast digestive cells of the digestive gland; this product is then picked up by the hemolymph and circulated throughout the body.

The present study addresses the cellular mechanisms of ink pigment packaging, storage and release in the ink gland of *Aplysia californica.* In particular we ask (1) what are the important structural and functional characteristics of the cells involved in pigment accumulation, storage and secretion, (2) where is the algal protein split from phycoerythrin, and (3) what is the source of the high molecular mass protein of secreted ink?

Materials and methods

Animals

Laboratory-cultured *Aplysia californica* Cooper were raised from eggs at the NCRR National Resource for *Aplysia* at the University of Miami, Virginia Key, FL, USA. The young snails (stage 12 of juvenile development; Kriegstein, 1977) were fed a diet of red seaweed (*Gracilaria tikvahiae*), green seaweed (*Ulva lactuca*) or romaine lettuce. Before starting the snails on their various diets, they were mechanically de-inked (Nolen *et al.* 1995), so that each group's ink gland was at a comparable stage of maturation. *Gracilaria* was grown at the *Aplysia* Resource Facility; *Ulva* was obtained from Harbor Branch Oceanographic Institute, Fort Pierce, FL, USA; fresh romaine lettuce was purchased weekly from a local grocery store. We transferred snails from the *Aplysia* Resource Facility to our laboratory at least 1 week before the experiments and held them in 30 gallon (113.6 l) or 50 gallon (189.3 l) recirculating seawater aquaria at 18–22 °C under a 16 h:8 h L:D photoperiod. The animals were fed *ad libitum* every other day.

Observations of the whole gland in intact snails

Snails (100–200 g) were tethered by the parapodia using five pairs of hooks, and suspended in 8 l of aerated sea water at 22 °C. The lateral edge of the ink gland was glued (with cyanoacrylate adhesive) to a platform and reflected back so that its ventral surface could be positioned under a stereo microscope (T. G. Nolen and P. M. Johnson, unpublished observations). Ink secretion from the gland, therefore, could be observed and photographed, and individual ink-release vesicles could be identified and observed in successive inking episodes. Ink release was induced by anemone tentacle stimulation to the head or tail (Nolen *et al.* 1995). Photomicrographs of the gland were digitized using Photoshop 3.0 LE and analyzed using NIH Image for Macintosh. Dimensional changes of ink-release vesicles between inking episodes were quantified as a relative change in the vesicle's diameter as measured at its widest point.

Isolated vesicles

Spectral analysis of vesicle contents

Individual ink-release vesicles were dissected from the ink gland of chilled (at approximately 4 °C) snails that had been fed either red algae (*Gracilaria tikvahiae*) or romaine lettuce since stage 12 of juvenile development (Kriegstein, 1977). Animals ranged in mass from 250 to 500 g. Amber and dark red-purple vesicles ($N \geqslant 5$ vesicles of each) from red-algal-fed snails were placed in separate vials containing an isotonic solution of magnesium chloride to prevent vesicle activation. Amber and light red-purple vesicles (for the latter, only a few were found in the gland) from romaine-fed snails were isolated similarly. The vials were immediately frozen (–80 °C), subsequently defrosted (shattering the vesicles) and their contents centrifuged (1600 ***g*** for 30–60 s) to remove particulates. The optical absorption spectrum between 450 and 650 nm was then determined (Bausch & Lomb, Spectronic 2000).

Vesicle pharmacology

Individual ink-release vesicles were dissected from the chilled gland and placed in 100 µl of a control solution of high (3×) $[Mg^{2+}]+[Ca^{2+}]$ Ringer's solution (to minimize spontaneous ink secretion) or a solution of acetylcholine (ACh) in high-$[Mg^{2+}]+[Ca^{2+}]$ Ringer; ink release was monitored for 30 s. An ascending concentration series of ACh was used (0.1, 0.25, 0.5 or 1.0 mmol l^{-1}) with a 30 s wash in high-

$[Mg^{2+}]+[Ca^{2+}]$ Ringer between ACh tests. All test and control solutions were at pH 7.4 and room temperature (approximately 23 °C). The number of ink-release vesicles that released their contents at a given ACh concentration was noted. The high-$[Mg^{2+}]+[Ca^{2+}]$ Ringer consisted of 460 mmol l^{-1} NaCl, 10.4 mmol l^{-1} KCl, 33 mmol l^{-1} $CaCl_2$, 165 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris–HCl. Normal Ringer consists of 460 mmol l^{-1} NaCl, 10.4 mmol l^{-1} KCl, 11 mmol l^{-1} $CaCl_2$, 55 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} Tris–HCl. Acetylcholine chloride was obtained from Sigma.

Ultrastructure

The distal third of the ink gland was removed from 15 $MgCl_2$-anesthetized snails, 2.8–150 g wet mass, and prepared for transmission electron microscopy using methods described by Coelho *et al.* (1998). Thick sections (1 µm) stained with Toluidine Blue provided cell and nuclear size measurements for various cell types.

Scanning electron microscopy

For scanning electron microscopy of ink gland or isolated ink-release vesicles (see above for isolation of individual vesicles), tissue preparation was the same as for transmission electron microscopy except that, after the 100 % ethanol dehydration step, specimens were critical-point-dried in liquid CO_2, sputter-coated with gold and visualized with a Topcon, dual-scan, model 130 scanning electron microscope.

Immunogold localization of r-phycoerythrin

The techniques for immunogold localization of r-phycoerythrin are similar to those of Coelho *et al.* (1998) using Spurr's embedding medium except that: (1) a dilution of 1:15 was found to be optimal for the primary antiserum (mouse monoclonal anti-phycoerythrin antibody; =anti-PE; Sigma catalog no. P9669); (2) the cross-sectional areas of various cell types and their cellular compartments were determined for each treatment by digitizing 8–12 photomicrographic negatives (each at ×3900 magnification) using Photoshop 3.0 and then measuring areas using NIH Image for Macintosh (the area of the rough endoplasmic reticulum was estimated to be equivalent to the area of the cell remaining after subtracting the area of the mitochondria and nucleus; this leads to an overestimate for granulate cells since they have considerable 'free space'; see Fig. 8A,E); and (3) the number of gold grains in each cell compartment was counted directly from the negatives using a dissection microscope at 10× magnification. Control experiments using digestive gland tissue from *A. californica* (Coelho *et al.* 1998) showed that our technique of immunogold labeling is specific for phycoerythrin, but still produces a low background label. A single control (omission of the primary antiserum; =no anti-PE) was therefore incorporated in this study to allow calculations of specific anti-PE binding (see Coelho *et al.* 1998).

Statistical comparisons

Before analyzing experimental effects, we carried out tests of skewness and/or homogeneity of variances to determine whether parametric statistical tests were valid (Sokal and Rohlf, 1981). Where they were not, we employed appropriate non-parametric statistical tests (e.g. Kruskal–Wallis tests) instead (Krauth, 1988). When multiple comparisons were performed, significance levels were adjusted using a Bonferroni method (Krauth, 1988). Unless otherwise indicated, all significance levels reported are two-tailed, and values are reported as means ± standard errors of the mean. Statistical tests were performed with InStat 2.03 for Macintosh (GraphPad Software).

Results

Secretion of ink from the ink gland

The ink gland is situated on the roof of the mantle cavity, above the gill and just proximal to the mantle edge (Fig. 1A). The gland contains three types of vesicles (Figs 1B, 2A,C) embedded in a matrix of collagen, muscle and two different types of cells (described below). When the animal was fed red

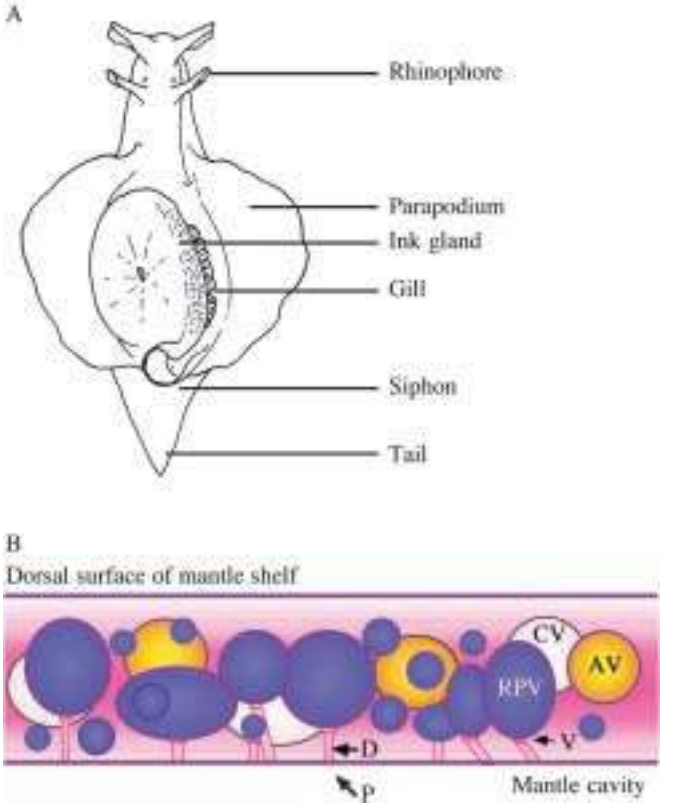

Fig. 1. The ink gland of *Aplysia californica*. (A) Diagram of a dorsal view of the mantle organs, showing the ink gland along the mantle edge (with ink-release vesicles located on the mantle's ventral surface), the gill and the mantle floor (below the gill). The mantle cavity is normally protected by the parapodia, which have been pulled back to reveal the mantle organs. (B) Diagram of a transverse section of the ink gland showing ink-release vesicles, ducts and pores. AV, amber vesicles; D, duct; P, pore; CV, clear vesicle; RPV, red-purple vesicle; V, vesicle valve.

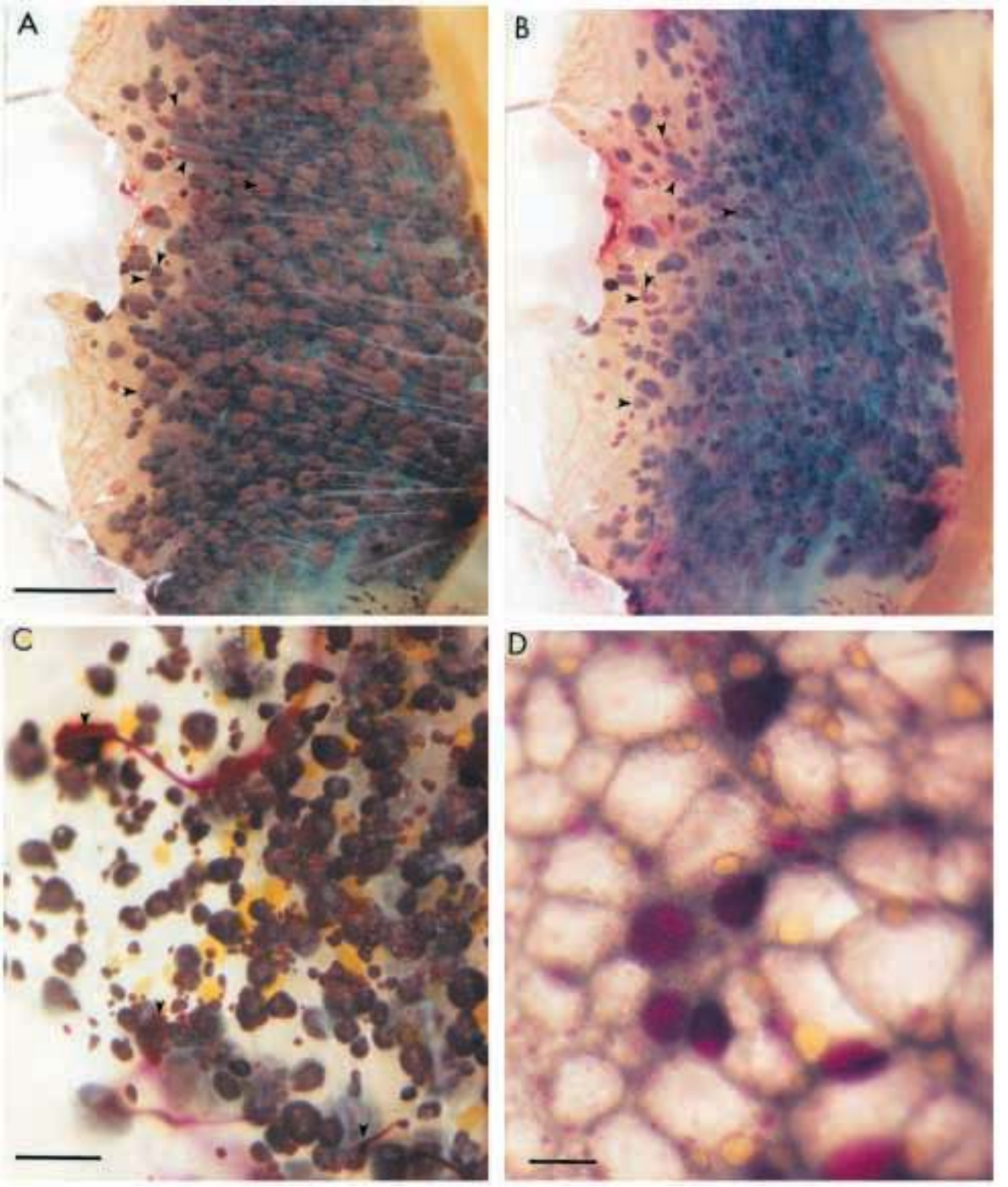

Fig. 2. View of the ventral surface of the ink gland before (A) and after (B) stimulated ink secretion. Anterior is at the top of the photograph; the base of the siphon is at the bottom. Note the change in size and density of the six ink-release vesicles (each labeled with a small arrowhead in A and B). (C) Ink secretion from individual dark red-purple ink-release vesicles (small arrowheads). Note that the ink is visible as it leaves the pore and streams through the bath. (D) View of the gland of a snail fed the green seaweed *Ulva lactuca* for 3 months; over that time, the snail grew from a mass of less than 1 g to more than 150 g. Note the dark red-purple, amber and clear vesicles (especially in C and D; in C, most of the white space consists of clear vesicles) and the light red-purple vesicles in D. Scale bars: A, B, 2 mm; C, 1 mm; D, 0.5 mm.

seaweed (e.g. *Gracilaria*), most of the vesicles were dark red-purple and ranged in diameter from 0.25 to 1.25 mm (Figs 1B, 2A). Other vesicles were either amber-colored or clear (devoid of ink and transparent on *trans*-illumination) and of similar dimensions to the dark red-purple vesicles (Figs 1B, 2C). Ink was secreted only from the dark red-purple vesicles (Fig. 2C). Ink glands that had recently secreted ink (from minutes to hours previously) also had light red-purple vesicles in addition to the three types described above (Fig. 2B; see Fig. 4B,C). When de-inked at an early age and then placed on a diet of green seaweed (e.g. *Ulva*) or romaine lettuce for several months, *Aplysia* sp. do not secrete ink when disturbed (MacColl *et al.* 1990; Nolen *et al.* 1995; Coelho *et al.* 1998). Romaine- or green-algal-fed snails had many clear vesicles as well as some amber and small light red-purple vesicles (similar to those of a recently de-inked gland) but no dark red-purple ones (Fig. 2D).

Ink-release vesicles are robust structures that survive dissection and mechanical isolation (e.g. see Fig. 5). The absorption spectrum of the contents of isolated dark red-purple vesicles from red-algal-fed snails had peaks characteristic of phycoerythrin and/or phycoerythrobilin. The light red-purple vesicles of romaine-fed snails contained smaller amounts of red algal pigment as indicated by their relatively weak optical absorbance compared with that of the dark red-purple vesicles of red-algal-fed snails (compare 'romaine-fed' with 'red-fed'; Fig. 3). Amber vesicles had no absorption peaks over the range 450–650 nm (Fig. 3). Clear vesicles were fragile and fell apart during dissection, so we did not collect enough to study.

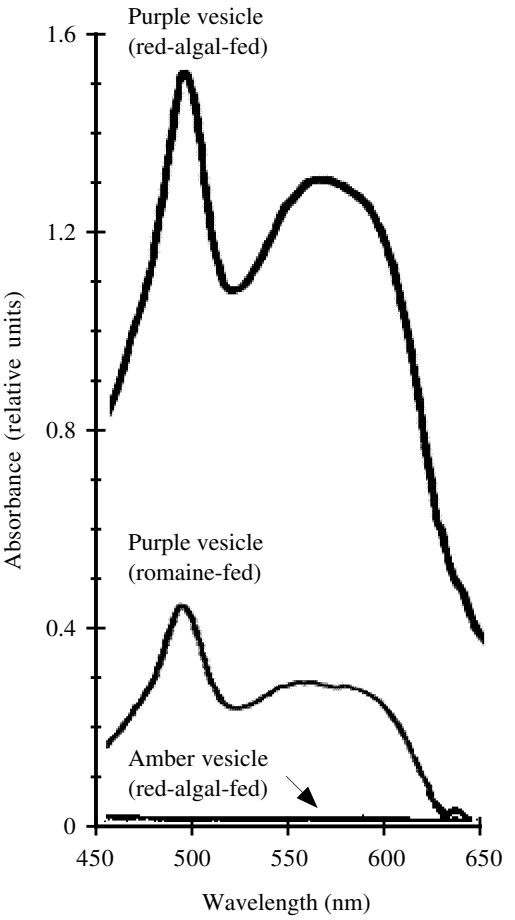

Fig. 3. Optical absorption spectra (in relative absorbance units) of dissected vesicles from the ink gland of *Aplysia californica* fed red algae (dark red-purple and amber vesicles) or romaine lettuce (light red-purple vesicles).

Ink release from vesicles

Examination of the gland of a restrained animal during ink secretion showed that ink was released from a subset (less than half) of dark red-purple vesicles throughout the gland (N=3 animals; see Fig. 4). Furthermore, an ink-release vesicle could be activated several times in successive inking episodes and usually released a portion of its contents each time (e.g. vesicles e and f in Fig. 4A–C; Table 1). The two-dimensional profile of ink-release vesicles decreased following secretion,

Table 1. *Relative size of vesicles a–f of Fig. 4A–C as seen under the dissection microscope*

Vesicle	Initial (A) (%)	Post 1 (B) (%)	%Diff in B	Post 2 (C) (%)	%Diff in C
a	100	73.7	−26.3	100.0	0
b	100	63.7	−36.4	100.0	0
c	100	87.5	−12.5	107.1	7.1
d	100	68.2	−31.8	106.7	6.7
e	100	75.0	−25.0	94.4	−5.6
f	100	92.9	−7.1	76.9	−23.1
Mean ± S.D.		76.8±11.3	−23.2±11.3	97.5±11.2	−2.5±11.2

Vesicle size: the longest dimension was measured and expressed relative to the initial size of the vesicle before inking (as seen in Fig. 4A).
Initial (A), the size of the vesicle prior to inking (Fig. 4A).
Post 1 (B), vesicle size (relative to the initial size) after the first inking episode (Fig. 4B).
%Diff in B, % difference in size of vesicle between Fig. 4B and Fig. 4A.
Post 2 (C), vesicle size (relative to its size in Fig. 4B after the first inking episode) after the second inking episode (Fig. 4C).
%Diff in C, % difference in size of vesicle between Fig. 4C and Fig. 4B.

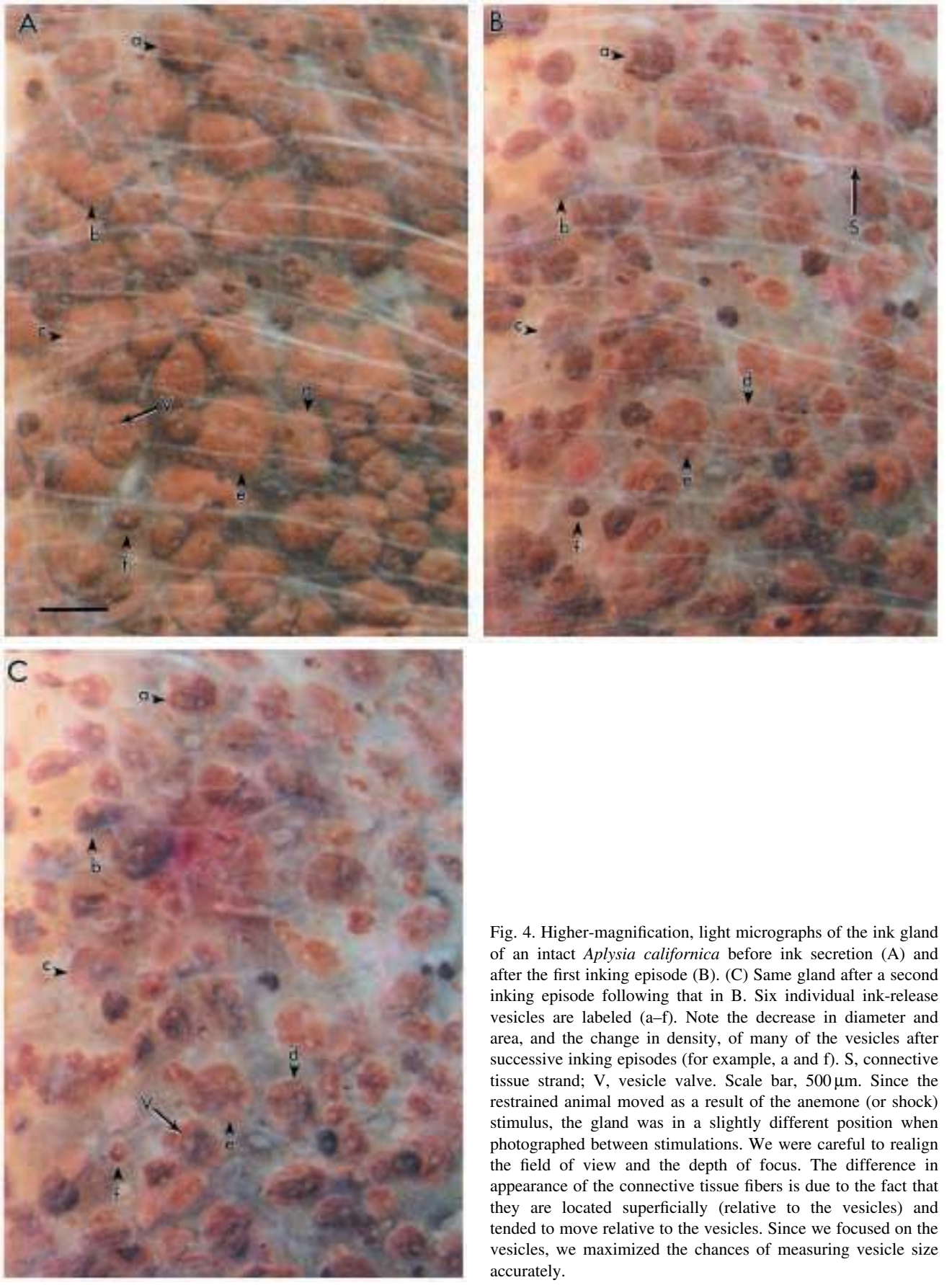

Fig. 4. Higher-magnification, light micrographs of the ink gland of an intact *Aplysia californica* before ink secretion (A) and after the first inking episode (B). (C) Same gland after a second inking episode following that in B. Six individual ink-release vesicles are labeled (a–f). Note the decrease in diameter and area, and the change in density, of many of the vesicles after successive inking episodes (for example, a and f). S, connective tissue strand; V, vesicle valve. Scale bar, 500 μm. Since the restrained animal moved as a result of the anemone (or shock) stimulus, the gland was in a slightly different position when photographed between stimulations. We were careful to realign the field of view and the depth of focus. The difference in appearance of the connective tissue fibers is due to the fact that they are located superficially (relative to the vesicles) and tended to move relative to the vesicles. Since we focused on the vesicles, we maximized the chances of measuring vesicle size accurately.

suggesting that vesicle volume decreased as part of the secretory process (Fig. 4; Table 1). The mean diameter of ink-release vesicles after ink secretion was 74.1±4.11 % of the diameter before secretion (t_{11}=6.29, P<0.0001, one-sample t-test). The observed decrease in profile diameter represents an approximately 55.1±16.5 % (mean ± 95 % CI, N=12 vesicles) decrease in spherical volume. (Fig. 4 and Table 1 show an additional preparation with a similar outcome.) Note also, in Fig. 4 (and Table 1) that some vesicles are approximately the same size after a second inking episode, although they contracted and released ink as indicated by the lighter color of the vesicles in Fig. 4C (compare the density of the vesicles labeled a and f in Fig. 4B,C).

Light microscopic study of intact ink glands suggests that ink is forced out of a valve at the apical (ventral) end of the ink-release vesicle (Figs 2C, 4A,C), through a duct and out of a pore on the surface of the gland and into the mantle cavity (Figs 1B, 5C; see also Fig. 12D). The valve structure was apparent in both scanning (Fig. 5A,B) and transmission (Fig. 6A) electron micrographs. Under transmission electron microscopy, the duct appeared to be lined by pie-shaped cells bearing numerous microvillae from their surface facing the duct lumen (Fig. 6B). The pore appeared in scanning electron micrographs of the ventral surface of the ink gland only after a vesicle had released some of its stored ink (Fig. 5C). Isolated ink-release vesicles retained their ink and released small amounts through the valve if gently squeezed with forceps or activated by neurotransmitter (ACh; see below).

Pilot studies showed that localized application of 5 mmol l^{-1} ACh to dissected glands caused ink secretion from nearby ink-release vesicles. To investigate the pharmacology of ink release, we minimized the chance that ACh was activating peripheral motor neurons (or presynaptic central motor neuron terminals) by removing individual ink-release vesicles from the gland, cleaning them of loose connective tissue and exposing them to varying concentrations of ACh. Ink was released from 24 of 29 vesicles exposed to ACh compared with only one of nine vesicles in control Ringer's (Fig. 7A; P=0.0002, Fisher's exact test). The secretion of ink from isolated vesicles occurred within a few seconds of exposure to ACh and involved contractions of the muscles of the vesicle wall, as seen under the dissection microscope. At least 70 % of the vesicles released ink at ACh concentrations of 0.5 mmol l^{-1} or below; 83 % secreted ink at ACh concentrations of 1.0 mmol l^{-1} or below (Fig. 7A,B).

Ultrastructure of the gland

In addition to the three types of vesicles described above, two principal classes of cells, the RER and granulate cell types, were observed in light microscopic and transmission electron microscopy studies to form the majority of the ground tissue between ink-release vesicles and vesicle cells (Fig. 8A). The RER cell, although significantly smaller than the granulate cell (Fig. 9A), was the most numerous of the two cell types. RER cells were characterized by an abundance of rough endoplasmic reticulum whose cisternae, frequently involving

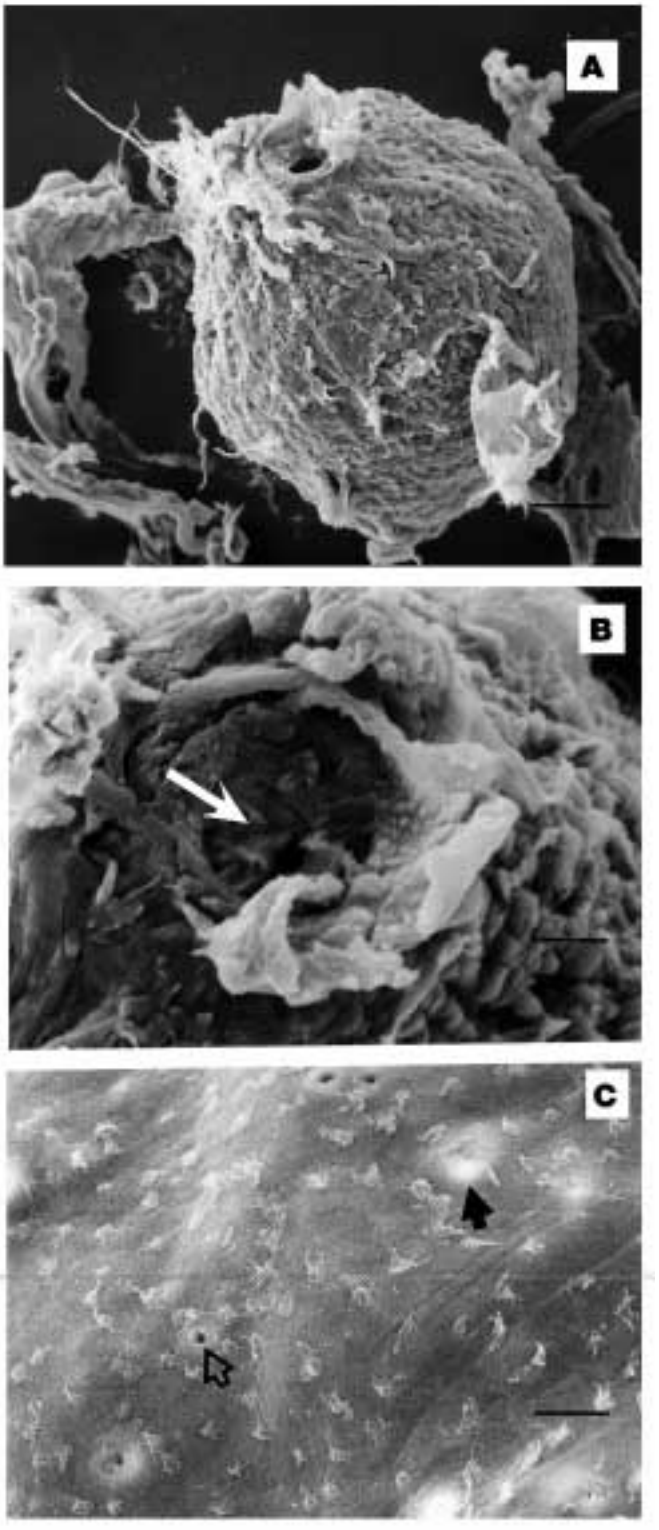

Fig. 5. Scanning electron micrographs of ink-release vesicles dissected from red-algal-fed *Aplysia californica*. (A) Whole ink-release vesicle, (B) the vesicle duct showing the basal valve (white arrow), and (C) the ink gland surface with open pores (open arrow) through which ink has already been released and closed pores (filled arrow) for vesicles that have not yet released ink. Scale bars: A, 50 μm; B, 15 μm; C, 35 μm.

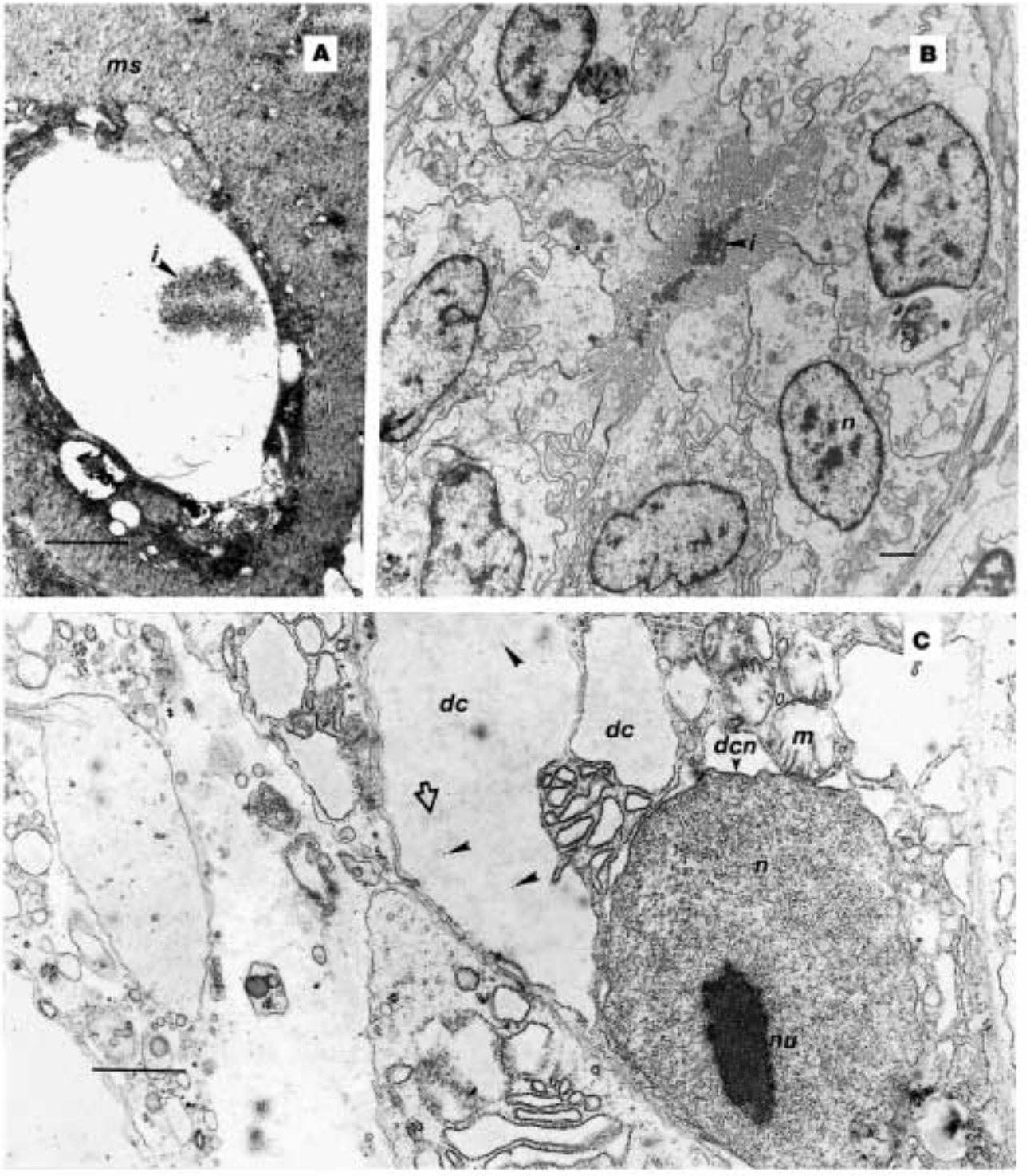

Fig. 6. Transmission electron micrographs of the ink-release vesicle valve (A) and duct (B). (C) RER cell with gold grains (arrowheads) that label r-phycoerythrin (approximately 1 grain μm^{-2} for this cell profile); these are found within distended endoplasmic retuculum cisternae (*dc*) which contain material of low electron density (open arrow). *dcn*, outer membrane of nucleus distended into an endoplasmic reticulum cisternum; *i*, ink; *m*, mitochondrion; *ms*, muscle; *n*, nucleus; *nu*, nucleolus. Scale bars, 1 μm.

the outer membrane of the nuclear envelope, were greatly distended and often contained material of low electron density (Figs 6C, 8A).

Granulate cells had 4–14 membrane-bound vacuoles, 1–4 μm in diameter. In red-algal-fed snails, these vacuoles contained electron-dense material (Fig. 8A,B) consisting of particles (3.5±0.12 nm in diameter) that were statistically similar in size to the electron-dense particles that characterized

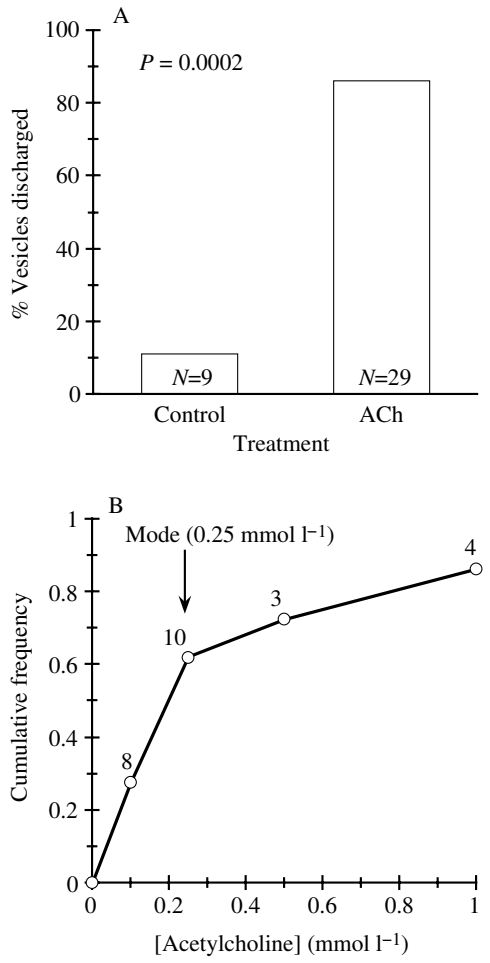

Fig. 7. (A) The proportion of isolated ink-release vesicles that secreted ink in response to acetylcholine (ACh) ($\leqslant$1 mmol l^{-1}) or control Ringer's solution. The outcome of a Fisher exact test is shown. (B) A cumulative frequency distribution for ACh-elicited release of ink from isolated ink-release vesicles for an ascending concentration series (see Materials and methods). The number of vesicles secreting ink at or below each concentration is shown. The modal active concentration was 0.25 mmol l^{-1}.

ink (3.2±0.1 nm in diameter) in mature ink-release vesicles [P=0.10, t_{38}=1.7062, N=20 particles each; the size of the electron-dense particles at 66 000× magnification making up a granule similar to that shown in Fig. 8B was compared with ink in an ink-release vesicle (the ink located as in Fig. 13A,B)]. Granulate cells had significantly less rough endoplasmic reticulum, as a percentage of sectional profile area, than RER cells (70±11 %, N=14 *versus* 87±6 %, N=11 mean ± S.D., P<0.0001, Student's t-test, t_{23}=4.5786) and a significantly larger nucleus (see Fig. 9B), but no nucleolus (the latter determined through analysis of thick sections).

Large portions of the granulate cell membrane were modified into grated areas (Figs 8C, 10B). In these regions, the plasma membrane invaginated and became an expanded surface for the formation of coated vesicles (Figs 8B–D, 10B,C). Fingers of cytoplasm extended over these areas, appearing in cross section as a series of small, membrane-bound, rectangular units of cytoplasm; fine filaments parallel to each other and spaced 15.5±1.0 nm (mean ± S.D.; N=10) apart crossed the space between adjacent fingers (Figs 8D, 10C). Electron-dense material was located inside the plasma membrane adjacent to the openings between the cytoplasmic fingers (Figs 8B,D, 10A). These grated areas (Fig. 11) permitted ample contact with the intercellular medium and provided areas of extensive coated vesicle formation (Figs 8C, 10B–D). Coated vesicles averaged 0.17±0.02 µm in diameter (N=5) and were not significantly different in size (P>0.05, Student's t-test, t_{15}=0.2105) from nearby vesicles that had no coat (N=12) but contained electron-dense particles (12.4± 2.1 nm, mean ± S.D., N=12, compare Figs 8C and 10A,B,D), which projected from the inner surface of the vesicle (Fig. 10A,D); these particles appeared to be composed of approximately three or more electron-dense subunits (4.15±0.92 nm; mean ± S.D., N=10). These subunits were the same size as the particles constituting the electron-dense material in granulate vacuoles (Kruskal–Wallis nonparametric analysis of variance, ANOVA, KW=10.979; Dunn's multiple-comparisons test, P>0.05) but slightly larger than the dense particles constituting ink in ink-release vesicles (P<0.01). In snails fed green algae, the grated areas, coated vesicles and storage vacuoles were present, but the vacuoles were devoid of electron-dense material (Fig. 8E).

All types of vesicles and cell types are seen in the glands of young and old snails. The ink-release vesicle appears to arise in both juvenile and mature sea snails by the growth of a single cell (the vesicle cell) whose nucleus increases in size concurrent with the expanding cell (Fig. 12A,D); the nucleus (1317±500 µm^2, N=8) of developing ink-release vesicles was 50–70 times the area of the nuclei of either RER or granulate cells (17±0.7 µm^2, N=37, and 26±1.8 µm^2, N=21, respectively) and had numerous, prominent nuclear pores which appeared to be exuding copious material (Fig. 12C). The growing vesicle cell became filled with mitochondria and small vacuoles (Fig. 12A,C) but eventually, in red seaweed-fed snails, electron-dense material (ink) collected and obscured both the nucleus and the other organelles, ultimately filling the cell itself (Fig. 13B–D). In contrast to those described above for red-seaweed-fed snails, in romaine-fed snails, mature ink-release vesicles, recognized by their size and morphology rather than by their dense ink content, were at most filled with a light, homogeneous material (Fig. 13E) and are probably equivalent to the clear vesicles seen under the light microscope (Fig. 2D). The expansion of the vesicle cell ceased with the appearance of smooth muscle bundles about its periphery. The vesicle cell was eventually enclosed by several layers of muscle (Fig. 13A,B); but before muscle completely encircled it, the cell continued to extend out between these bundles (see Figs 12B, 13D). The mature ink-release vesicle had, in addition, several strata of cells located just inside the muscle (Fig. 13A–C), while the cell membrane of the original vesicle cell became obscured. The origin and the mode of positioning

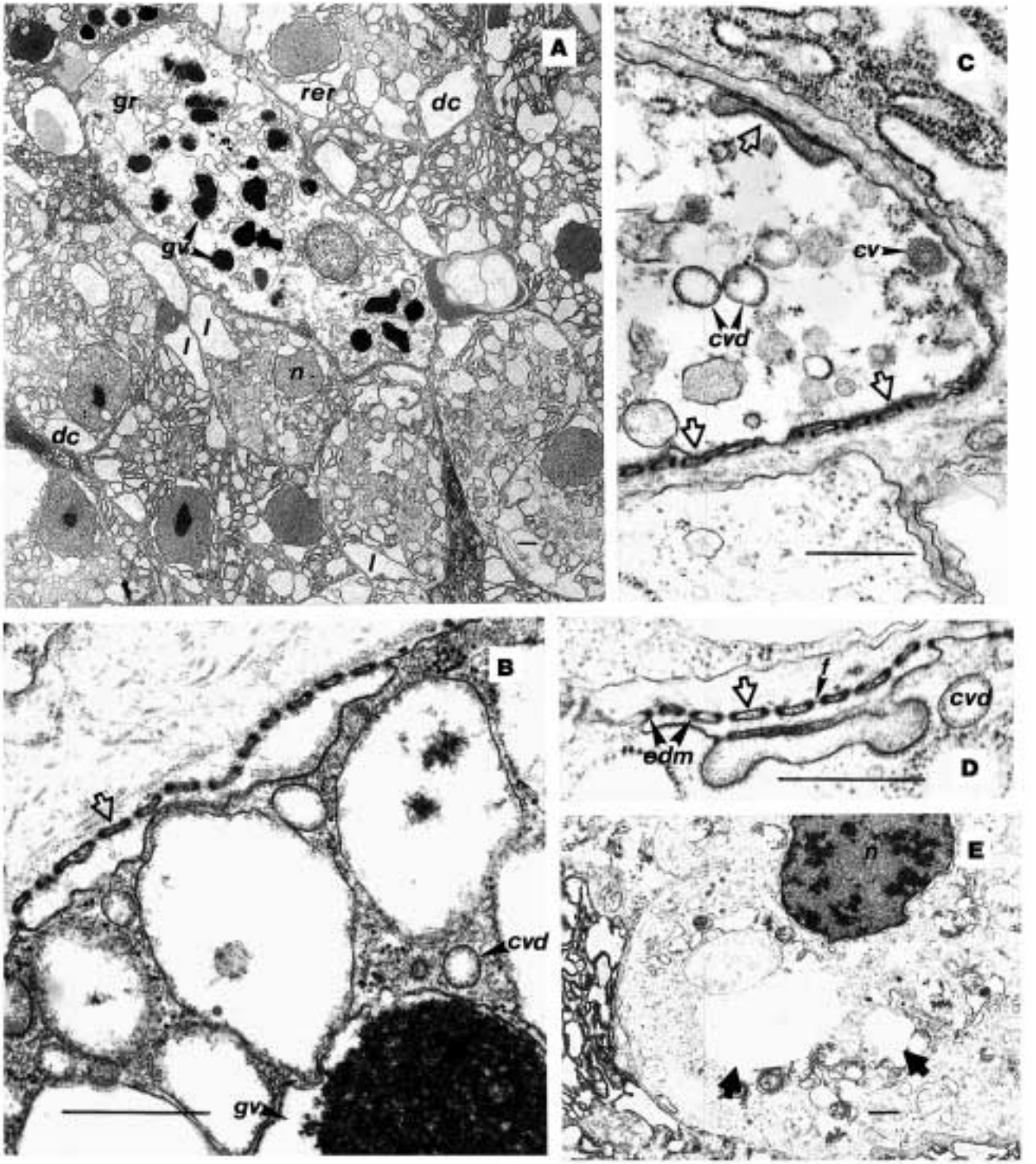

Fig. 8. Transmission electron micrographs of ink glands from red-algal-fed (A–D) and green-algal-fed (E) *Aplysia californica*. (A) RER cells, with numerous distended cisternae of rough endoplasmic reticulum (*dc*) containing material of low electron density (*l*), and granulate cell types (*gr*), the latter with several granule vacuoles (*gv*) containing electron-dense material. (B–D) Areas of the plasma membrane of the granulate cell modified into a grated area (open arrows) where (in cross section) a series of rectangular-shaped fingers of cytoplasm bound by plasma membrane extend over areas of coated vesicle formation. (E) Granulate cell from green-algal-fed *Aplysia californica* with empty granule vacuoles (filled arrows). *cv*, coated vesicle; *cvd*, coated vesicle whose clathrin coat has almost been entirely lost; *edm*, electron-dense material at grate edge; *f*, filament; *l*, material of low electron density; *n*, nucleus; *rer*, RER cell type. Scale bars: A, E, 1 μm; B–D, 0.5 μm.

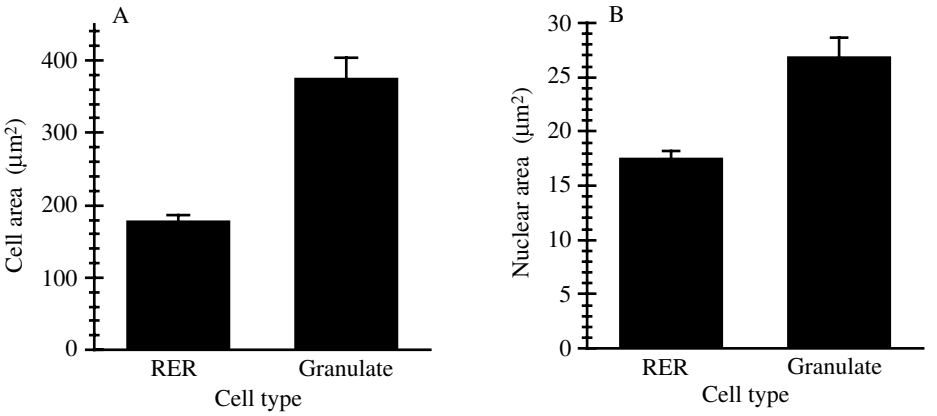

Fig. 9. Cell (A) and nuclear (B) areas (means + S.E.M.) for RER (N=37) and granulate (N=25) cells in the ink gland of red-algal-fed *Aplysia californica*. A significant difference occurs between RER and granulate cells for both cell and nuclear area (P<0.0001; alternate Welch t-test, t=6.5249, d.f.=27 for cell area; t=4.8889, d.f.=26 for nuclear area).

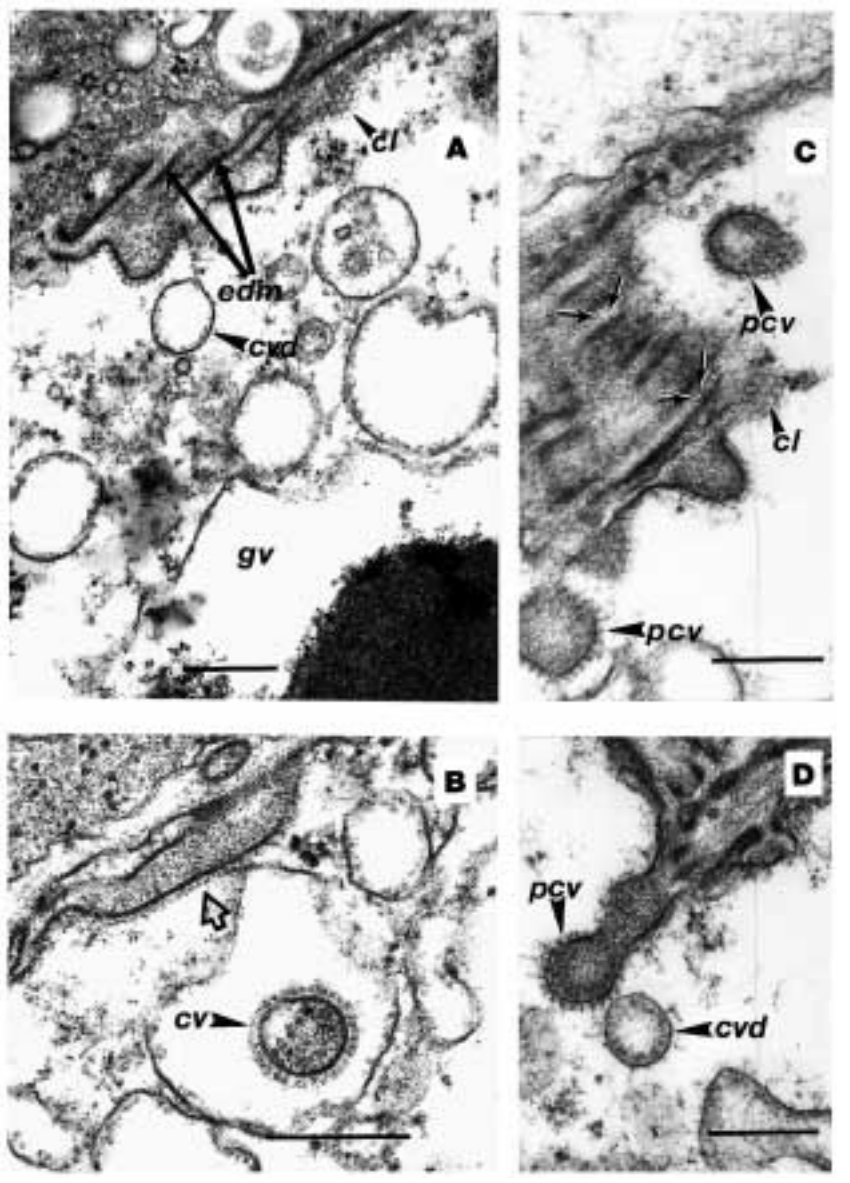

Fig. 10. Transmission electron micrographs of grated areas from granulate cells. (A,C) Tangential sections through grated areas showing electron-dense material (*edm*) lining cytoplasmic fingers (A) and regularly spaced filaments situated between cytoplasmic fingers (C, adjacent arrows). (B) Extensive area of plasma membrane modified for coated vesicle formation (open arrowhead). (D) A vesicle (*cvd*) whose clathrin coat has been almost entirely lost. *cl*, clathrin coat; *cv*, coated vesicle; *gv*, granulate vacuole; *pcv*, coated vesicle that has not pinched off from the plasma membrane. Scale bars, 0.25 mm.

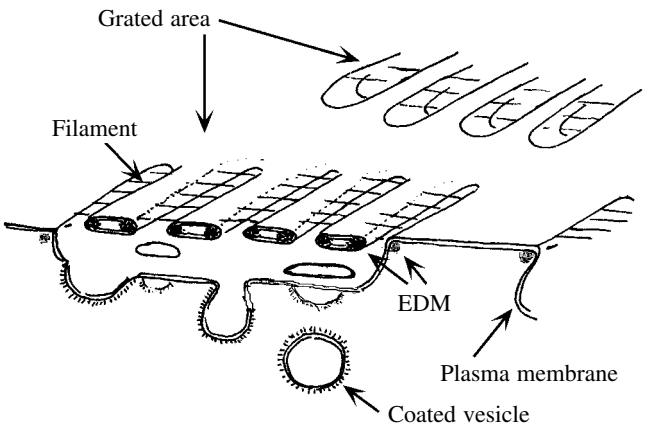

Fig. 11. Reconstruction of the sieve structure of a grated area of the granulate cell plasma membrane where coated vesicles are formed. EDM, electron-dense material.

of the cells layered against the muscle is unknown, but they could be RER and/or granulate cells that were able to infiltrate between the muscle bundles before the muscle completely encircled the vesicle cell, although this was never observed. Alternatively, these cells could originate through proliferation of cells lining the duct leading to the surface pore (Figs 6B, 12D), eventually coming to clothe the inside of the ink-release vesicle. The vesicle cell nucleus in mature ink-release vesicles was enclosed by ink, but otherwise appeared much like nuclei in less mature vesicles (Fig. 12A,D).

Immunogold localization of phycoerythrin

Immunogold localization of r-phycoerythrin showed significantly more label (see Fig. 14 for details of the statistics) in all cell types (i.e. the ink-release vesicle, granulate and RER cells) when anti-PE was used (Fig. 6C) than when it was omitted (Fig. 14 'no anti-PE'). Background labeling (i.e. control=no anti-PE) was greater in the ink-release vesicle than in granulate or RER cells (Fig. 14). Therefore, for each cell type, we used a randomization procedure to pair experimental and control observations and subtracted the non-PE-specific background label (='no anti-PE' control) from the observed anti-PE label. This analysis showed a statistically similar level of label for phycoerythrin in RER, granulate and ink-release vesicles (one-way ANOVA of means, P=0.62, d.f.=28). In addition, no statistically significant differences in localization of phycoerythrin occurred in any of the cellular compartments of either the RER or granulate cells (Fig. 15A,B). In both, the nucleus had the greatest amount of label (0.8–0.9 gold particles μm^{-2}), but this was not significantly different from that for the endoplasmic reticulum, mitochondria, granule cell vacuole or whole cell.

Discussion

The purple ink released by *Aplysia californica* and related snails appears to be distasteful, if not toxic, to potential predators (DiMatteo, 1981, 1982; Nolen *et al.* 1995; Walters *et al.* 1993), yet its major component originates from a non-toxic red algal pigment, phycoerythrin. As much as 65 % of ink's dry mass consists of the chromophores of phycoerythrin (primarily phycoerythrobilin with some phycourobilin; Chapman and Fox, 1969; MacColl *et al.* 1990; Rudiger, 1967; Troxler *et al.* 1981), while the remainder consists mostly of high molecular mass protein (M_r 32 000–78 000; Troxler *et al.* 1981). This protein is distinct from the low molecular mass chromopeptides of phycoerythrin (M_r 15 000–22 000; MacColl *et al.* 1990) and, therefore, is thought to be manufactured by the snail itself.

Generally, the use of the term 'ink' has referred to the purple secretion produced by the snail when disturbed (e.g. Kandel, 1979). But ink is a complex mixture of pigment, protein and small amounts of unknown, low molecular mass components (Troxler *et al.* 1981). Further, while a large percentage of the ink material is plant-derived, some is probably made by the snail. Red-algal-deprived snails do not secrete purple ink and do not escape from predators as successfully as those fed red algae (Nolen *et al.* 1995). So it appears that the plant-derived materials (probably the purple pigment, phycoerythrobilin, PEB) are of major importance for the anti-predator function of the ink. Still, the other snail-derived components found in secreted ink (Troxler *et al.* 1981) may play an as yet unknown role in avoiding predation.

Sites of processing of phycoerythrin and high molecular mass protein

Phycoerythrin

Where is phycoerythrin (PE) broken down to phycoerythrobilin (PEB) and phycourobilin? Secreted ink consists mainly of PEB, the chromophore of phycoerythrin, but 10 % of ink is chromophore still attached to cystein (Troxler *et al.* 1981) and therefore is probably undigested or partially digested phycoerythrin. Chapman and Fox (1969) reported that digestive gland extracts are capable of digesting PE to chromopeptides (PEB attached to remnants of protein specific to phycoerythrin), but they concluded that removal of the *majority* of the protein from phycoerythrin/chromopeptides may occur in the ink gland. Coelho *et al.* (1998), using an immunogold tag specific for phycoerythrin, also suggested that cleavage or modification of phycoerythrin occurs in the digestive gland, during digestion of the red algal chloroplasts in vacuoles of specialized rhodoplast digestive cells. But the issue of where PE is converted to PEB is unresolved: does the majority of the digestion of phycoerythrin occur in the ink gland (as suggested by Chapman and Fox (1969) or in the digestive gland (as proposed by Coelho *et al.* 1998)?

We found previously that the only location within the digestive gland of *Aplysia californica* which provides a high level of label for r-phycoerythrin is the digestive vacuoles of the rhodoplast digestive cells (mean 3.97 grains μm^{-2}; see Table 2), while significantly less label was found in the other compartments of the cell (Coelho *et al.* 1998). Using techniques similar to those of Coelho *et al.* (1998), we found

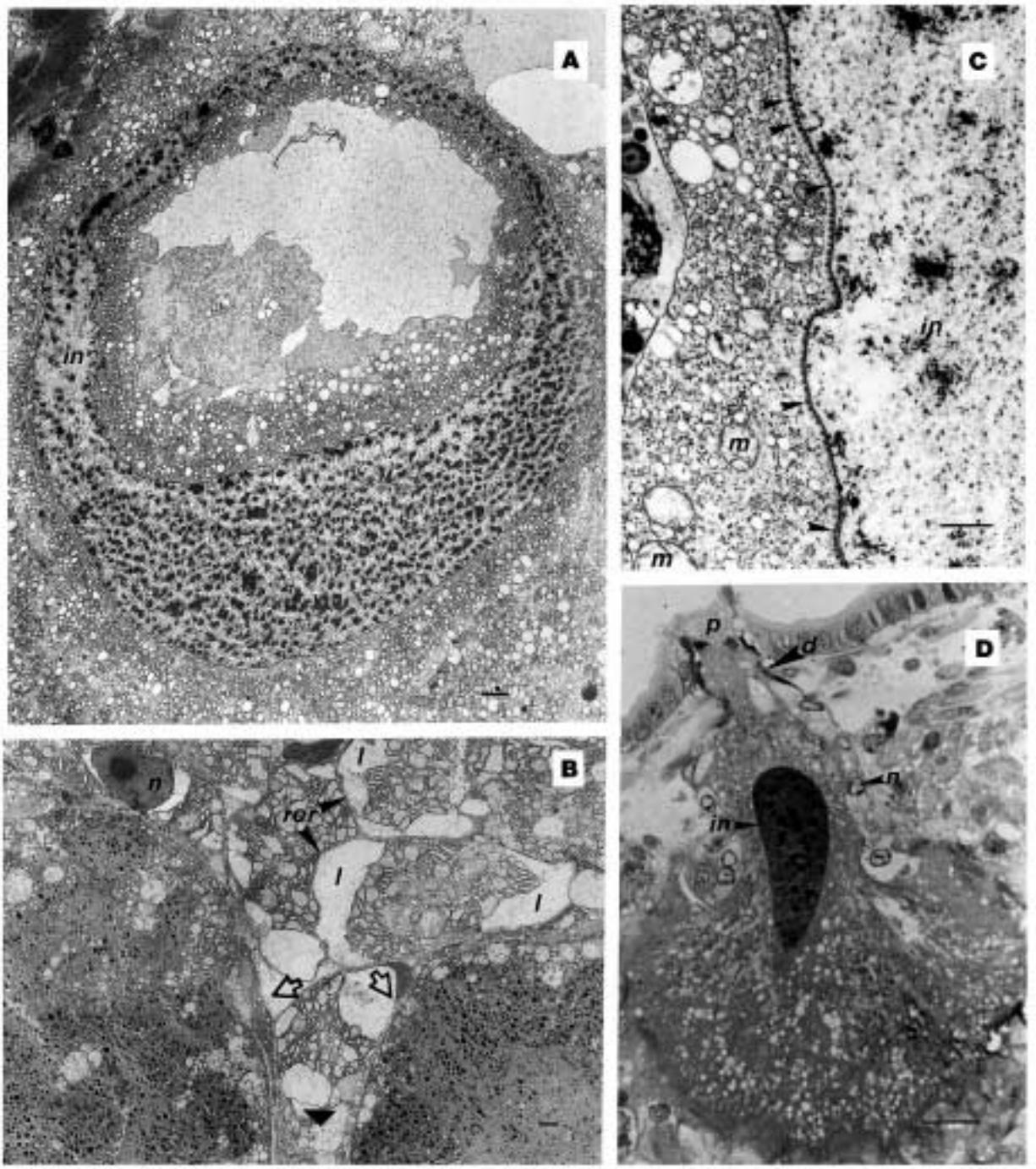

Fig. 12. Transmission electron micrographs (A–C) and a light micrograph (D) of developing ink-release vesicles in red-algal-fed *Aplysia californica*. (A,C) The vesicle cell nucleus (*in*) in A surrounds an island of cytoplasm, while in C its nuclear envelope has numerous, large, active nuclear pores (arrowheads). (B) Portions (open arrows) of the vesicle cell push out between muscle bundles (out of the plane of section but just distal to the black triangle and equivalent to the black triangle in Fig. 13D). (D) Ink vesicle cell with duct (*d*) and pore (*p*). *in*, nucleus of ink vesicle cell; *l*, material of low electron density; *m*, mitochondrion; *n*, nucleus; *rer*, RER cell type. Scale bars: A, 2 μm; B, C, 1 μm; D, 50 μm.

only low levels of label for PE in the ink gland: in the granulate cells, in the RER cells and in the ink-release vesicles themselves (Table 2). In fact, the amount of PE label was significantly greater than the amount found for resin only in the ink-release vesicles themselves.

While it is possible that some ink components (phycoerythrin) were spread uniformly throughout the ink gland tissue as a result of leaching during fixation (thereby masking any localized concentrations within or between cell types), the absolute level of labeling was still very low. For

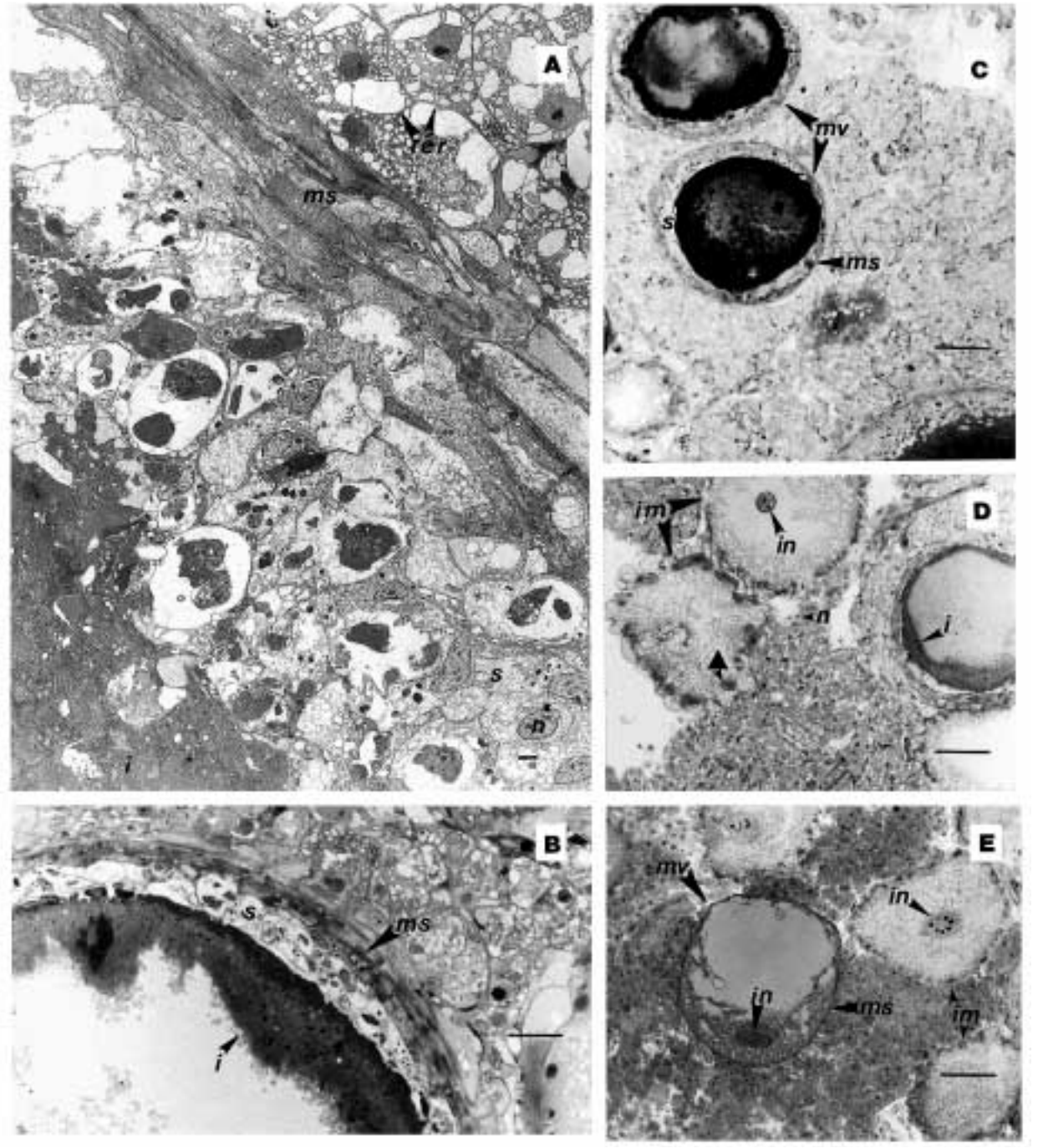

Fig. 13. Transmission electron micrograph (A) and light micrographs (B–E) of ink-release vesicles and vesicle cells from red-algal-fed (A–D) and romaine-lettuce-fed (E) *Aplysia californica*. Layers of muscle encircle mature vesicles (A–C), while several strata of cells (*s*) line the inside of the ink-release vesicle adjacent to the layer of muscle. Mature ink-release vesicles (C–E) and vesicle cells (D,E). The black triangle in D shows an immature, developing vesicle; the small arrowhead points to a muscle bundle with portions of the vesicle cell pushing outward on either side (as in Fig. 12B). *i*, ink; *im*, vesicle cell; *in*, nucleus of vesicle cell; *ms*, muscle; *mv*, mature ink-release vesicle; *n*, nucleus; *rer*, RER cell type. Scale bars: A, 2 μm; B, 50 μm; C–E, 200 μm.

example, the greatest immunogold label for r-phycoerythrin in the ink gland was found within the ink-release vesicles themselves, at a mean level of 1.1 grains μm^{-2}, but this value does not take into account the non-specific binding of both primary and secondary antibody. For ink-release vesicles, PE-specific labeling was calculated by subtracting from the above value the non-specific labeling by the secondary antibody (0.3 grains μm^{-2}, Fig. 14, 'no anti-PE') and the labeling due to the cross reactivity of the primary antibody [anti-PE, 0.23 grains μm^{-2}, see Coelho *et al.* (1998), although they used an anti-PE concentration that was 1.6 times lower (1:25 *versus* 1:15) than we used for the ink gland; this correction value is,

Table 2. *Immunogold label in RER cells, granulate cells and ink-release vesicles of the ink gland and in the digestive vacuoles of rhodoplast cells of the digestive gland and in resin*

Tissue - Cell type	Mean (grains μm^{-2})	S.D.	*N*
Ink gland			
RER	0.73	0.33	10
Granulate	0.60	0.32	10
Vesicle	1.12	0.35	10
Digestive gland*			
Rhodoplast vacuole*	3.97	2.20	13
Resin*	0.28	0.05	4

Cell type has a significant effect on the amount of label (P<0.0001, ANOVA, F=19.556, d.f.=3,39). The rhodoplast digestive cell has significantly more label (Bonferroni multiple-comparison test) than the RER cells (P<0.001, t=6.1569), granulate cells (P<0.001, t=6.4136) and ink-release vesicles (P<0.001, t=5.4248). The amount of label on resin is not significantly different from that in RER and granulate cells (P>0.05, t=2.4544, t=1.7297, respectively), but is significantly less than that in ink-release vesicles (P<0.001, t=4.5208).

*Values for the digestive gland and resin are taken from Coelho *et al.* (1998). These values do not take into account non-specific binding. Coelho *et al.* (1998) calculated a value of 3.57 grains μm^{-2} for PE-specific label in the rhodoplast vacuole, which we use in our discussion of PE-specific labeling in the ink gland (see Discussion).

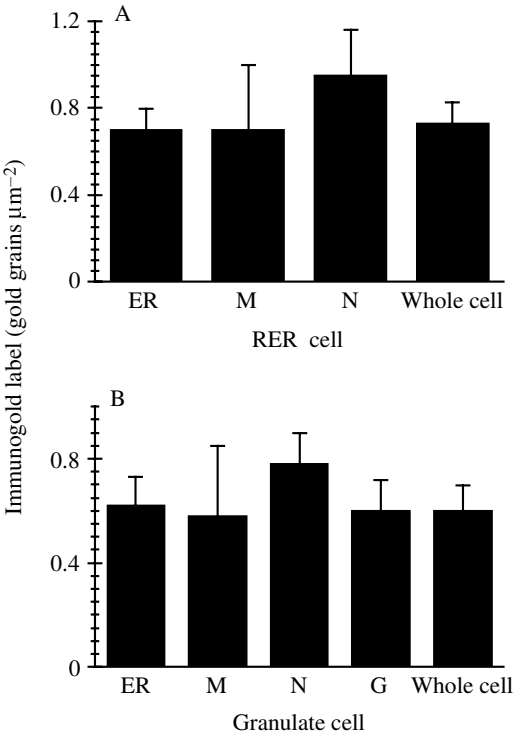

Fig. 15. Immunogold labeling (mean + S.E.M., N=10 for each compartment) for r-phycoerythrin in the compartments of RER (A) and granulate (B) cell types of the ink gland. The cell compartment had no significant effect on the amount of label (P=0.4569; Kruskal–Wallis nonparametric ANOVA, KW=2.6035). ER, endoplasmic reticulum; M, mitochondria; N, nucleus; G, granules.

therefore, conservative relative to the ink gland since the cross reactivity of the primary antibody with cellular components appears to be directly related to its concentration]. This gives an absolute PE-specific label of 0.57 grains μm^{-2} for ink-release vesicles. The same corrections for the RER and granulate cells gives absolute PE-specific labeling levels of 0.5 and 0.4 grains μm^{-2} respectively.

Anti-PE is specific for the protein component of phycoerythrin and not the chromophore, phycoerythrobilin (Coelho *et al.* 1998). Comparing the PE-specific labeling in the ink gland (see above) with the PE-specific labeling in the digestive gland (about 3.57 grains μm^{-2} for the rhodoplast digestive vacuoles as determined by Coelho *et al.* 1998) shows that the ink gland has no more than 16 % (=100×0.57/3.57) of the phycoerythrin of the digestive gland. Our data suggest, therefore, that there is little label specific for phycoerythrin in the ink gland and that 80 % or more of the label specific for PE occurs in the digestive gland which, therefore, is probably the site for most of the modification of PE. This does not exclude, however, the possibility that further modification/digestion might occur in the ink gland, as Chapman and Fox (1969) suggest.

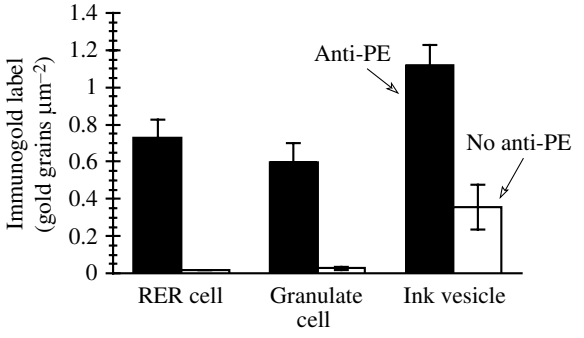

Fig. 14. Immunogold labeling (mean + S.E.M.; N=10) for r-phycoerythrin with and without anti-PE (see Materials and methods) in RER cells, granulate cells and ink-release vesicles. The presence or omission of anti-PE significantly affected labeling in the ink gland (Mann–Whitney U-test: for RER cells, P<0.0001, U=0; for granulate cells, P=0.0003, U=4; for ink vesicles, P=0.0007, U=8).

The absorption spectra of both dark red-purple vesicles (Fig. 3) and hemolymph from red-algal-fed snails indicated the presence of red algal bili-chromophores, but they were absent from the hemolymph of romaine-fed snails (Coelho *et al.* 1998). Peak absorbancies, however, cannot distinguish between the bilin chromophores (phycoerythrobilin, phycourobilin) and phycoerythrin itself (Coelho *et al.* 1998; MacColl and Guard-Friar, 1987). Nevertheless, the immunogold results suggest that the rhodoplast digestive vacuole is the site for most of the initial modification of

phycoerythrin, if not the site where the majority of protein is actually cleaved from phycoerythrin to produce PEB. The altered chromopeptides or chromophores then probably diffuse through the rhodoplast digestive cell to the hemolymph and circulate throughout the body until taken up by the ink gland.

Granulate vacuoles in red-algal-fed snails were filled with particles similar in size to those making up ink deposits in mature ink-release vesicles, suggesting that granulate cells take up ink pigment from the hemolymph and that this pigment eventually makes its way to the developing vesicle cell. Since no immunogold labeling was recorded over the grated areas of granulate cells, a site of coated vesicle formation, we suspect that coated vesicles may be involved with the uptake and storage of bilin chromophores, rather than phycoerythrin. However, no coated or pinocytotic vesicles or other modifications were observed (N=50) in association with the plasma membrane of the vesicle cell either before or during its encirclement by muscle. We do not know, therefore, how the chromophores enter the ink-release vesicle or whether the granule vacuole of granulate cells is simply a site of long-term storage of *excess* chromophore or a temporary storage site in its normal transport to the ink-release vesicle. Furthermore, it is possible that the RER and granulate cells and even the vesicle cell, which eventually gives rise to the ink-release vesicle, are different developmental or maturation stages of the same cell type, with pigment transfer occurring during its evolution.

The development of granule vacuoles of granulate cells and the ink-release vesicles themselves are not induced by the presence of either a particular food or a pigment constituent. The spacing of the filaments in the grated areas produces a sieve which should exclude particles greater than 16.5 nm in diameter from the intercellular medium/hemolymph; the electron-dense subunits composing the particles inside vesicles whose coat had disassembled were one-third of the size of the sieve. These subunits were the same size as, or slightly larger than, the electron-dense particles that made up the dense deposits in granule vacuoles and the ink in ink-release vesicles, respectively.

Green-algal- and romaine-fed snails had only a few, light red-purple vesicles and an abundance of non-pigmented vesicles (Fig. 2D). The small, light red-purple vesicles had an absorption spectrum similar to that of dark red-purple vesicles (Fig. 3). These small vesicles could be ink-release vesicles that had been partially depleted during the de-inking process just before the juvenile animal began growth on a diet of romaine lettuce. If so, these vesicles retained some pigment which did not degenerate as the animal grew over a 2 month period. However, these small, light-red vesicles may represent ink-release vesicles that were made immediately after de-inking and therefore utilized the limited pigment stores in granulate cells after the sea snail had been switched from a diet of red algae. Most of the rest of the vesicles in green-algal-fed and romaine-fed snails were non-pigmented (Fig. 2D). This suggests that vesicle production proceeded as the animal grew but that, after depletion of its limited pigment stores and in the absence of a supply of ink pigment, no more red-purple vesicles were manufactured to replace depleted ones. The relatively small size of the light red-purple vesicles in green-algal-fed snails is evidence for their synthesis just after de-inking when the snail – and its ink-release vesicles – was smaller.

High molecular mass protein in ink

Ink protein is probably not derived from the red algal phycobilisomes, as is the pigment: ink protein is of higher molecular mass than red algal biliproteins (MacColl *et al.* 1990). It is possible that the RER cell, the most common cell type in the ink gland, is the source of this high molecular mass protein. This is suggested by the abundant, greatly distended cisternae of rough endoplasmic reticulum in RER cells that frequently contained material of low electron density, consistent with the appearance of high molecular mass protein. If the RER cell manufactures the protein component of ink, then many questions remain, including (1) does the protein have an anti-predator function, (2) do red-purple ink vesicles contain this protein as well as pigment or is the protein packaged in other release vesicles, (3) how does the protein move from the RER cell to the developing release vesicle, and (4) what is the nature of the amber vesicles, which were found in both red-algal- and romaine-fed snails and lacked ink pigments (Fig. 3). They might represent immature vesicles prior to ink accumulation or mature vesicles unable to collect ink pigment or mature protein release vesicles: given their relatively large size (comparable to that of mature dark red-purple ink-release vesicles) and the presence of a valve structure as seen under the dissection microscope, the latter two possibilities seem more likely.

Animals fed green seaweed are eaten by predators more often than those fed red seaweed – as long as the red-algal-fed snails have ink to release (Pennings, 1990; Nolen *et al.* 1995). These observations suggest that, if the proteins have an anti-predator effect, they must either not be released by green-algal-fed snails or they must act synergistically with ink pigment. The RER cell's morphology was not affected by diet: green-algal-fed snails appear to be manufacturing protein. These observations suggest that, if an anti-predator protein component is synthesized and released by green-algal-fed snails, it is not as effective as whole ink. The anti-predator functions of the ink protein, if any, have yet to be determined. Our laboratory is currently conducting experiments to determine the identity of the distasteful component of the ink of *Aplysia*. Is it the chromophore or the protein that is distasteful or toxic, or is it both? Recent work on the aversive effect of ink on crab predator chemoreceptors suggests that a low molecular mass element of ink – probably phycoerythrobilin – is an active constituent (Carlson and Nolen, 1997).

Ink gland nomenclature and structure: a clarification

The literature is unclear about the nomenclature of the

purple-colored ink-release vesicles of *Aplysia* sp.: Hyman (1967) termed these dark red-purple structures 'Blochmann's glands', whereas Tarao (1934) referred to them as 'purple glands' and Kandel (1979) and Kriegstein (1977) called them ink vesicles. We have followed Kandel (1979) but adopted the term ink-release vesicle for the individual pigment-containing structures entirely enclosed by muscle, the term *vesicle cell* for developing vesicles prior to their complete enclosure by muscle, and the term *ink gland* for the whole structure along the mantle edge which contains many vesicle cells, ink-release vesicles as well as the other types of vesicles (see above).

Hyman [1967, citing Blochmann (1883) although no figures in Blochmann are comparable to those of Hyman] states that there are two types of ink-release vesicle: the unicellular type has a cytoplasm filled with droplets and a large nucleus, while the multicellular type has small cells filling the interior of the vesicle. Our study suggests that these two types are probably developmental stages in the normal formation and discharge of ink-release vesicles. Hyman's unicellular type is probably equivalent to vesicle cells (60–110 μm in diameter): they lack ink, have a large, obvious nucleus and the cytoplasm is filled with vacuoles and mitochondria; Tarao (1934) probably mistook these developing vesicle cells for nerve cells. Hyman's (1967) multicellular type probably represents mature ink-release vesicles (225–1150 μm in diameter) that have several cell layers inside the muscle wall while the nucleus and other cell organelles are obscured by accumulated ink. Alternatively, she could have been referring to discharged ink-release vesicles, in which the peripheral cells collapse into the empty interior of the vesicle.

Tarao (1934), working on a different species [although he reports on *Tethys* (=*Aplysia*) *punctata*; Eales (1921) and Carefoot (1987) suggested that it was unlikely to be *Aplysia punctata*], noted the relatively large size of granulate cells and their nuclei. But he also commented that they had a prominent nucleolus (we found that granulate cells are, indeed, significantly larger and have a significantly larger nucleus than RER cells but, at least in *A. californica*, granulate cell nuclei lack a nucleolus) and that their purplish-colored granules arose directly from transformed mitochondria. He suggested, furthermore, that the ink-release vesicle was filled by migration and collapse of granulate cells into the ink-release vesicle itself. We have never noted any developmental relationship between mitochondria and granule vacuoles. In addition, we have never observed granulate cells migrating or fusing with young or mature ink-release vesicles. Tarao (1934) may have interpreted the characteristic appearance of mature ink-release vesicles that have cells lining the inside of the muscle wall (see Fig. 13A–C) as the product of the fusion of granulate cells with ink-release vesicles.

The nucleus of the vesicle cell expands as the cell grows and reaches a cross-sectional area approximately 50 times that of RER and granulate cell nuclei. The opaline gland also consists of a number of unicellular vesicles in which the nucleus expands with each cell until enclosing muscle layers limit further enlargement (Rudman, 1972, and J. Prince, personal observation). Coggeshall (1967) reports the epitome in this trend of enlarging the size of a structure by increasing the size of a single cell and its nucleus rather than by repeated mitosis and cytokinesis: in the cell body of central neurons of mature *Aplysia californica*, nuclei expand by repeated chromosomal replication until the nuclei reach a diameter of 500 μm (or more).

Ink release mechanism

Carew and Kandel (1977*a*) showed that inking is triggered in an all-or-none fashion by a shock or noxious stimulus to the head or tail. In particular, they found that inking's stimulus–response function was not graded like a reflex, but was a step function requiring a high-intensity stimulus: above threshold, increasing the stimulus intensity did not cause any more ink to be secreted than did a stimulus of just-threshold intensity. Carew and Kandel (1977*b*,*c*) subsequently identified three electrically coupled neurons in the abdominal ganglion (the L14 neurons) that, when stimulated to a critical level, produced a high-speed burst of action potentials and caused ink secretion from the ink gland. They also showed that these cells could be brought to threshold by the electrical shock that elicited inking in the intact animal. They concluded that the L14 neurons were ink motoneurons and were responsible for triggering the behavior. They also suggested that the snail releases almost all of its stores of ink when stimulated by an electrical shock (Carew and Kandel, 1977*a*). However, T. G. Nolen and P. M. Johnson (unpublished observations), in a more extensive study of ink secretion, showed that the snail does not necessarily secrete all of its ink when brought to threshold: the amount secreted on each successive trial was approximately 30–50 % of the gland's remaining stores. Thus, while the triggering of ink secretion may be all-or-none, the amount secreted may be a fixed proportion of that left in the gland, but not necessarily all of the ink in the gland. In the present paper, we show that individual dark red-purple ink-release vesicles decrease in volume on average by 55 % and therefore probably release only approximately half of their contents upon stimulation. In addition, our observations of intact ink glands showed that only a proportion of the ink-release vesicles (less than half) were activated in any given trial. Thus, a fixed proportion of stored ink could be released if the relative number of ink-release vesicles activated is approximately the same on each trial and if each secreted a fixed proportion of its stores.

Our observations of the cellular mechanism of ink pigment secretion from the ink gland of *Aplysia californica* are consistent with the following scenario. An attack by a predator or a noxious electrical shock excites the all-or-none triggering mechanism of the three L14 neurons (Carew and Kandel, 1977*a–c*). The activation of ink motor neurons excites the smooth muscles surrounding a subset of ink-release vesicles in the ink gland, squeezing some of the ink through the vesicle's valve and into a duct that empties into the mantle cavity. The ink is forced out of the mantle cavity by parapodial and gill contractions and can be directed towards the eliciting stimulus

by coordination of the siphon and anterior parts of the parapodia (Walters and Erickson, 1986). A better understanding of the conditions/mechanisms involved with ink release will come only after we learn how vesicles are activated by the ink motor neurons (Carew and Kandel, 1977*b*). As yet, we have not identified any synapses on the muscle fibers of ink-release vesicles using transmission electron microscopy.

We have presented evidence that the peripheral control of vesicle activation is potentially cholinergic: individual ink-release vesicles, dissected from the ink gland, were activated by ACh to secrete ink through their valve, as was observed for ink-release vesicles *in situ* within the intact gland (see Figs 2C, 4A). At least 70 % of the isolated ink-release vesicles released ink at ACh concentrations of 0.5 mmol l^{-1} or below. Indeed, the vesicles are likely to be more sensitive to ACh than our assay suggests, since we had to use a high divalent cation concentration in the bath to prevent the excitation of the vesicle wall muscles by handling. Effective ACh concentrations that cause vesicle secretion in normal media are probably lower. However, Koester and Kandel (1977) report that the identified neurons in the abdominal ganglion thought to be responsible for triggering the inking response (L14s) are non-cholinergic. Therefore, our evidence for cholinergic activation of individual ink-release vesicles implies the existence of as yet unidentified cholinergic central and/or peripheral ink motor neurons. This is further indicated by pilot experiments (N=5) in which the peripheral nerves containing the axons of the L14 neurons were cut (Ross *et al.* 1995). Following recovery from surgery, some ink secretion was still elicited in response to mechanical stimulation as long as other peripheral nerves were left intact. These results suggest that other central neurons (potentially cholinergic), in addition to the L14 neurons, are sufficient for some inking function. We are currently employing intracellular staining, tract tracing, immuno-histochemical and pharmacological techniques to identify central and peripheral neurons of the ink circuit. These experiments should reveal the innervation pattern within the gland and should show us whether vesicles are directly activated by central and/or peripheral motor neuron synapses or indirectly by a diffuse neurohormonal mechanism.

In conclusion, we suggest that the ink chromophores, representing as much as 65 % of the dry mass of ink, are cleaved from phycoerythrin in the digestive gland and not in the ink gland. In the ink gland, the filter system composed of the grated areas of the granulate cell plasma membrane either excludes larger objects from being picked up from the hemolymph by coated vesicles or prevents the occlusion of coated vesicles by large objects. The coated vesicles contain particles similar in size to those in granulate vacuoles and they probably transport ink chromophore to these vacuoles for either long- or short-term storage. The pigment is then transported to the maturing vesicle cells. The high molecular mass protein, representing 35 % of the dry mass of the ink, appears to be synthesized in the RER cells of the ink gland. When attacked by a predator, central neurons, directly or indirectly, cause the muscle of a subset of the ink-release vesicles of the ink gland to contract (possibly *via* cholinergic activation), each vesicle releasing a portion of its ink into the mantle cavity, where it can be directed at the predator.

We would like to thank Linda Nolen for performing the scanning electron microscopy analysis of ink vesicles and the ink gland, Dr Dalton Dietrich for the use of his light photomicrographic system and Tanza Ross for help with the acetylcholine study. This research was supported in part by the NCRR *Aplysia* Resource Facility (RR10294) and by a pilot project grant from the NIEHS Marine and Freshwater Biomedical Sciences Center, Rosenstiel School of Marine and Atmospheric Sciences, University of Miami.

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