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REGULAR ARTICLE

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Rhogocytes (pore cells) as the site of hemocyanin biosynthesis in the marine gastropod *Haliotis tuberculata*

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Abstract Rhogocytes (pore cells) are specific molluscan cell types that are scattered throughout the connective tissues of diverse body parts. We have identified rhogocytes in large numbers in tissue taken from mantle, foot and midgut gland of the abalone *Haliotis tuberculata* (Vetigastropoda). Within cisternae of the endoplasmic reticulum, particles are visible that resemble, in shape and size, hemocyanin molecules, the respiratory protein of many molluscs. Immunohistochemical experiments using hemocyanin-specific antibodies demonstrated that these cells contain hemocyanin. In situ hybridization with a cDNA probe specific for *Haliotis* hemocyanin showed that hemocyanin-specific mRNA is present in rhogocytes, which confirmed that they are the site of hemocyanin biosynthesis in this gastropod. A possible path of hemocyanin release into the hemolymph is discussed. Also in the vetigastropod *Megathura crenulata*, many rhogocytes could be detected. However, they lacked hemocyanin molecules which, together with published data, indicates a seasonal expression of hemocyanin in this animal.

Keywords Hemocyanin biosynthesis · Rhogocytes · Pore cell · Keyhole limpet hemocyanin (KLH) · *Haliotis tuberculata* · *Megathura crenulata* (Mollusca)

Introduction

Molluscan hemocyanins are the largest respiratory proteins in nature and occur freely dissolved in the hemolymph. The structure, evolution and function of these oxygen-carrying copper proteins have been intensively studied in cephalopods and gastropods, and they are also found in chitons and some bivalves (for review, see van Holde and Miller 1995). Cephalopod and chiton hemocyanins are organized as so-called decamers. These are hollow cylinders consisting of ten identical subunits, with a molecular mass of 350 or 400 kDa and subdivided into seven or eight ca. 50-kDa functional units, depending on the species. In gastropods and bivalves, two decamers are assembled face-to-face to form cylindrical didecamers, and in some species tube-like multidecamers have also been observed.

Molluscan hemocyanin decamers, didecamers and multidecamers measure ca. 35 nm in diameter and are therefore easily identified under the electron microscope. Consequently, there is a wealth of electron-microscopic data, including dissociation-reassociation studies (e.g., Harris et al. 1997a, 1997b) and high-resolution computer reconstructions from cryospecimens (e.g., Orlova et al. 1997; Meissner et al. 2000). Moreover, the X-ray structure of functional unit "*g*" of *Octopus dofleini* hemocyanin is available (Cuff et al. 1998). The entire primary structure of isoform 1 (HtH1) of *Haliotis tuberculata* hemocyanin and of *O. dofleini* hemocyanin has been determined via cDNA sequencing (Miller et al. 1998; Lieb et al. 2000). In the cephalopods *O. dofleini*, *O. vulgaris* and *Eledone moschata*, the branchial glands have been identified as the site of hemocyanin biosynthesis (Messenger et al. 1974; Muzii 1981; Lang 1988). In *O. dofleini* and *Sepia officinalis*, this has been confirmed by successful isolation of hemocyanin-specific mRNA from branchial gland preparations (Miller et al. 1998). In addition, Beuerlein et al. (1998) identified ovoid cells of the branchial heart as the site of hemocyanin catabolism in *Sepia*. On the other hand, in *Nautilus pompilius* hemocyanin is apparently synthesized in the hepatopancreas (Ruth et al. 1988, 1996), and Ruth et al. (2000) reported that the four pericardial appendages of *Nautilus* are also involved in hemocyanin metabolism. When we started our experiments, the situation in gastropods was rather unclear. In the pulmonate gastropods *Lymnaea stagnalis*, *Helix aspersa* and *Arion hortensis*, "pore cells" (recently renamed "rhogocytes", for review, see Haszprunar 1996)

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scattered among the connective tissue have been proposed as candidates for the site of hemocyanin biosynthesis, from electron-microscopic images showing the presence of intracellular hemocyanin-like particles (Sminia 1972; Sminia and Boer 1973; Skelding and Newell 1975; Sminia and Vlugt van Daalen 1977). In contrast, similar experiments with tissues of opisthobranch species indicated the presence of intracellular hemocyanin in the so-called "blood gland" (Schmekel and Weischer 1973).

The problem with all these approaches is that electron-microscopic observation of intracellular hemocyanin does not exclude the possibility that these cells could possibly also serve for hemocyanin uptake from the hemolymph, for example, for transient hemocyanin storage or for metabolic reutilization of amino acids or copper ions. In the course of our structural studies on the hemocyanin of the vetigastropod *Megathura crenulata*, designated as "KLH" from "keyhole limpet hemocyanin" (Markl et al. 1991; Gebauer et al. 1994, 1999; Harris et al. 1997a, 1997b; Söhngen et al. 1997), we tried intensively to isolate hemocyanin-specific mRNA, in order to perform cDNA sequence analyses. However, we failed (Keller et al. 1999), and therefore started a search for the site of hemocyanin biosynthesis in *Megathura*. Although we found plenty of rhogocytes, they lacked hemocyanin molecules (see "Results"). However, in another member of the Vetigastropoda, the abalone *Haliotis tuberculata*, we found hemocyanin-rich rhogocytes (see "Results"), and consequently cDNAs encoding hemocyanin could be obtained from the relevant *Haliotis* tissues and sequenced (Keller et al. 1999; Lieb et al. 1999, 2000). We present here our data on the rhogocytes of *Haliotis* and *Megathura.*

Materials and methods

Animals

Individuals of the European abalone (*Haliotis tuberculata*) were donated by SMEL, Blainville sur Mer, France, and the Biosyn Company. Giant keyhole limpets (*Megathura crenulata*) were kindly provided by the Biosyn Company, Fellbach, Germany.

Tissue preparation and electron microscopy

Prior to tissue preparation, animals were anesthetized by cooling on ice. Small portions of excised tissues (1–6 mm^3) were fixed in 0.1 M sodium cacodylate, 2.5% glutaraldehyde, 10% sucrose, pH 7.3, for 2 h at room temperature. Tissues were then rinsed 3 times in 0.1 M sodium cacodylate, 10% sucrose, pH 7.3, and postfixed in 2% osmium tetroxide, 0.05 M sodium cacodylate, 5% sucrose, pH 7.3, for 2 h. After dehydration in a graded alcohol series (50%, 70%, 90%, 100% ethanol), tissues were incubated in propylene oxide (2×10 min) and embedded in Epon 812. Ultrathin sections (60–80 nm) cut on an OmU2 ultramicrotome (Reichert, Vienna, Austria) were stained with 0.8% uranyl acetate in ethanol/methanol (1:2) for 5 min and with 2% lead citrate in 0.16 M NaOH for 10 min, and studied under a Zeiss EM900 transmission electron microscope at 80 kV. Electron micrographs were recorded on Kodak electron microscope film type 4486.

Paraffin sections

Tissues were fixed overnight in a picric acid-formol-acetic acid mixture (Bouin's fluid 15:5:1) according to Langeron (1949) at room temperature followed by rinsing the specimens in 70% ethanol for 24–48 h. After dehydration in a graded alcohol series (see above), tissues were incubated in xylene (2×20 min and 1×45 min at 33°C) and embedded in paraffin (4×20 min at 58°C). Tissue sections (7 µm) were cut on a microtome (Leitz, Wetzlar, Germany) and transferred onto microscope slides covered with chrome alum/gelatin.

Immunohistochemistry

Paraffin sections were deparaffinized by immersion in xylene (3×10 min) and rehydrated in a graded alcohol series (100%, 96%, 80% 70% ethanol, 2×10 min each).

To block endogenous peroxidase activity, sections were incubated with 3% H_2O_2 for 5 min and rinsed in phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4). Detection of hemocyanin in tissue sections was performed using the Universal Immunostaining Kit (Dianova-Immunotech, Hamburg, Germany) with guinea pig anti-*Haliotis tuberculata* hemocyanin antiserum as the primary antibody. In brief, tissue sections were treated with protein-blocking agent for 10 min at room temperature followed by incubation with primary antibody (diluted 1:5000 in PBS) for 1 h at room temperature. After washing slides with PBS (3×5 min), tissues were covered with biotinylated polyvalent secondary antibody for 30 min at room temperature, washed again (3×5 min) and incubated with streptavidin-peroxidase reagent for 45 min at room temperature. Slides were washed with PBS (3×5 min) and incubated with freshly prepared chromogen (3,3-diaminobenzidine) working solution for 10 min. After washing slides in water for 2 min, sections were counterstained with hematoxylin for 10–30 s and washed in running water for 5 min.

In situ hybridization

A cDNA of 2.2 kbp coding for functional unit "*h*" and the C-terminal part of functional unit "*g*" of *H. tuberculata* hemocyanin isoform HtH1 (Keller et al. 1999) was labeled with digoxigenin (DIG-11-dUTP) using the DIG DNA-labeling kit (Boehringer, Mannheim, Germany) according to the protocol of Feinberg and Vogelstein (1983). Paraffin sections were deparaffinized and rehydrated as described above and incubated with 0.2% hydrochloric acid for 20 min at room temperature. To prevent non-specific binding of the probe to positively charged amino groups, sections were treated with 0.25% acetic anhydride, 0.1 M triethanolamine in 2 × SSC (20 × SSC stock solution: 3 M NaCl, 0.3 M sodium citrate, pH 7.0) for 10 min at room temperature. Slides were washed with distilled water. Tissue sections were dehydrated in a graded alcohol series (70%, 80%, 100% ethanol, 3 min each) and air dried.

The cDNA probe was diluted in hybridization medium containing 50% formamide, 7.5% dextran sulfate, 0.5% SDS and 2 mg/ml herring sperm DNA in 2 × SSC, denatured at 95°C for 5 min and cooled on ice. Final probe concentration was 50 ng/µl. The denatured probe (10–15 µl) was added to the tissue sections, covered with a coverslip and sealed with Fixogum. Hybridization was carried out overnight in a wet chamber at 39°C. Slides were then washed for 30 min in 2 × SSC at room temperature, 30 min in 0.5 × SSC at 45°C and 30 min in 0.1 × SSC at 45°C. Non-specific antibody-binding sites were blocked for 20 min with 0.3% bovine serum albumin, 0.1% Triton X-100 in 4 × SSC and washed with 0.1% Triton X-100 in 4 × SSC for 5 min at room temperature. Bound probe was detected with sheep anti-digoxigenin antibodies conjugated with fluorescein isothiocyanate (FITC) (diluted 1:200 in 0.1% Triton X-100/4 × SSC) for 1 h at 37°C in a wet chamber. Slides were then washed 3×5 min with 0.1% Triton X-100/4×SSC.

To prevent FITC-labeled sections from bleaching, they were embedded in Vectashield (Vector, CA). Negative controls were performed using a cDNA probe of equal length coding for a lamprey cytoskeleton protein.

Results

Electron-microscopic sections taken from foot, mantle and midgut gland tissue of the gastropods *M. crenulata* and *H. tuberculata* showed many areas with extracellular blood spaces containing hemocyanin molecules (Fig. 1). Such sections also revealed that rhogocytes are a characteristic element of the connective tissue (see Fig. 3). A typical feature of rhogocytes is their unique structure of the cell surface (Fig. 2). The plasma membrane of these cells shows many tubular and vesicular invaginations which are bridged by cytoplasmic bars that form the distinctive slits. The cells are entirely surrounded by an external basal lamina-like structure, and the most prominent cellular structure is the rough endoplasmic reticulum. Rhogocytes are up to 30 µm in size and can easily be recognized by their unique cell surface structure. In the abalone *H. tuberculata*, rhogocytes predominate in connective tissue taken from mantle and midgut gland. Within these rhogocytes, cisternae of the rough endoplasmic reticulum could be detected which contain particles that resemble intact single hemocyanin molecules in shape and size (Fig. 3a, b), sometimes organized into highly ordered paracrystalline structures (Fig. 3c). Hemocyanin accumulations could also be detected in the periphery of rhogocytes within invaginations of the plasma membrane beneath the cytoplasmic bars which form the slits (Fig. 4a, c). Occasionally an extracellular cisterna was found to communicate directly with the rough endoplasmic reticulum (Fig. 4b, d). To confirm that the particles in *Haliotis* rhogocytes indeed represent hemocyanin, immunohistochemical analysis was performed using rabbit antibodies against purified *Haliotis* hemocyanin (for the characterization of these antibodies, see Keller et al. 1999; Lieb et al. 1999). The antibodies specifically reacted with most if not all the rhogocytes in the respective tissue sections (Fig. 5a, a'), indicating a much higher hemocyanin concentration in these cells than in the surrounding hemolymph spaces, which reacted only weakly. Moreover, the signal was found to be unevenly distributed within the cytoplasm, with higher concentrations in the cell periphery and in distinct areas close to the nucleus (Fig. 5a').

In order to prove that rhogocytes are indeed the site of hemocyanin biosynthesis in *Haliotis*, in situ hybridization was performed using a cDNA probe specific for *Haliotis* hemocyanin. Figure 5b, b', b'' shows mantle tissue from *Haliotis*, with rhogocytes specifically labeled, indicating the presence of hemocyanin-specific mRNA. No other cell types of *Haliotis* foot, mantle and midgut gland tissue reacted.

Also in the keyhole limpet *M. crenulata*, rhogocytes were found throughout the connective tissue, with exceptionally large numbers in the connective tissue of the midgut gland. Representative examples of rhogocytes from the midgut gland of *M. crenulata* are shown in Fig. 6. However, in *Megathura*, neither rhogocytes nor any other cell type was found to contain hemocyanin-like particles, although the latter were abundant in the extracellular spaces. Instead, the cisternae of the rough endoplasmic reticulum of *Megathura* rhogocytes contained an amorphous flocculent material of unknown nature (Fig. 6b). This result was obtained from a number of individual limpets of different body size.

The rhogocytes of *Megathura* did not react with a number of rabbit antibodies raised against KLH (data not shown; for the specificity of these antibodies see Gebauer et al. 1994; Söhngen et al. 1997), and also in situ hybridization experiments showed no signals (using, how-

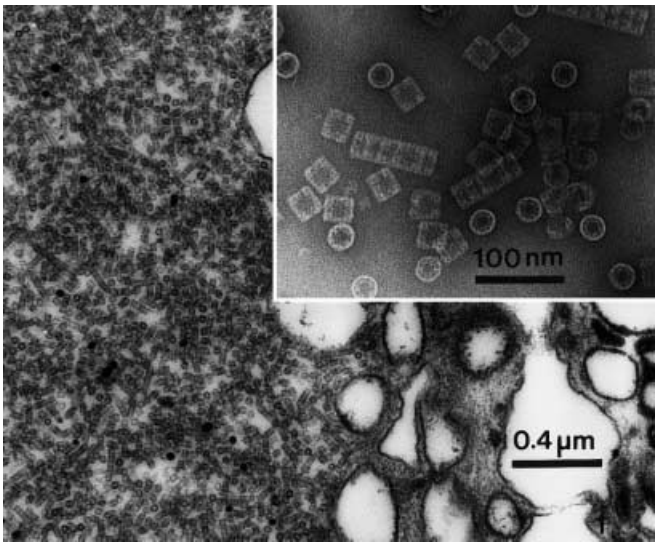

Fig. 1 Electron micrograph of a *Megathura crenulata* tissue section (mantle) showing a blood lacuna filled with hemocyanin molecules. *Insert*: Enlarged hemocyanin molecules purified from the hemolymph of *M. crenulata* and negatively stained as described by Harris et al. (1997a, 1997b)

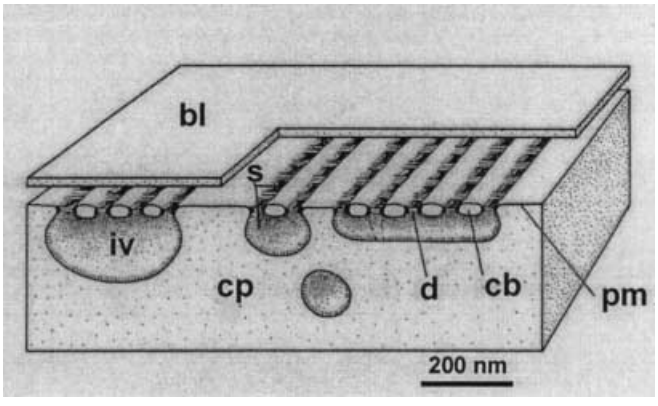

Fig. 2 Schematic drawing of the peripheral region of a rhogocyte according to Simkiss and Mason (1983) showing the distinct surface structure (*bl* basal lamina, *cb* cytoplasmic bar, *cp* cytoplasm, *d* diaphragm, *iv* invagination, *pm* plasma membrane, *s* slits)

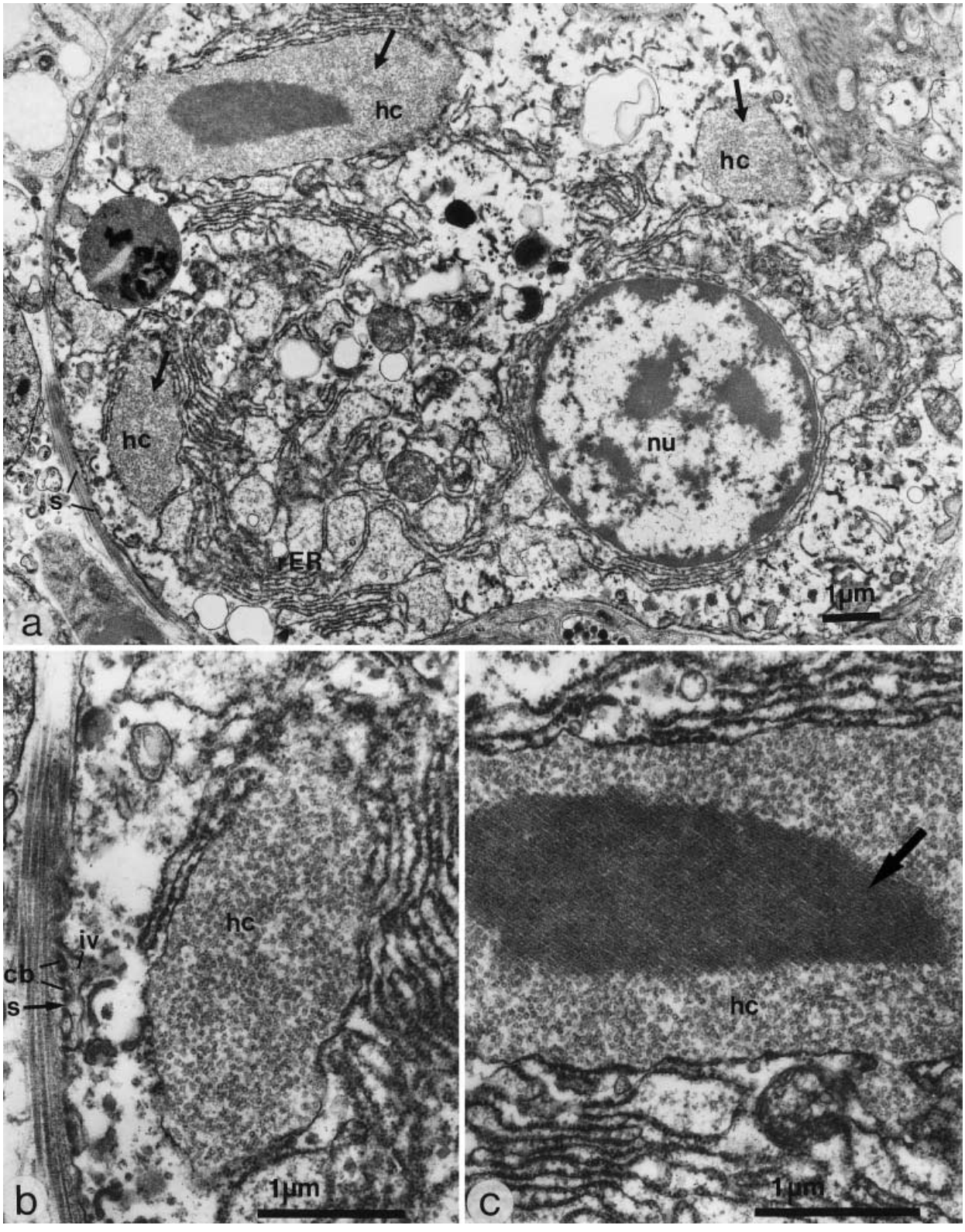

Fig. 3a–c Rhogocyte of *Haliotis tuberculata*. **a** Electron micrograph of a rhogocyte from the connective tissue of the midgut gland with enlarged cisternae of the rough ER, containing cylindrical structures that resemble hemocyanin molecules (*arrows*) (*hc* hemocyanin, *nu* nucleus, *s* slits, *rER* rough endoplasmic reticulum). **b** Enlarged detail of the rhogocyte shown in **a**. Note the invagination of the plasma membrane and an ER cisterna containing single hemocyanin-like molecules (*cb* cytoplasmic bar, *hc* hemocyanin, *iv* invagination, *s* slit). **c** Enlarged detail of the rhogocyte shown in **a**. Note the crystalline structure of hemocyanin molecules (*arrow*) inside a cisterna of the rough ER

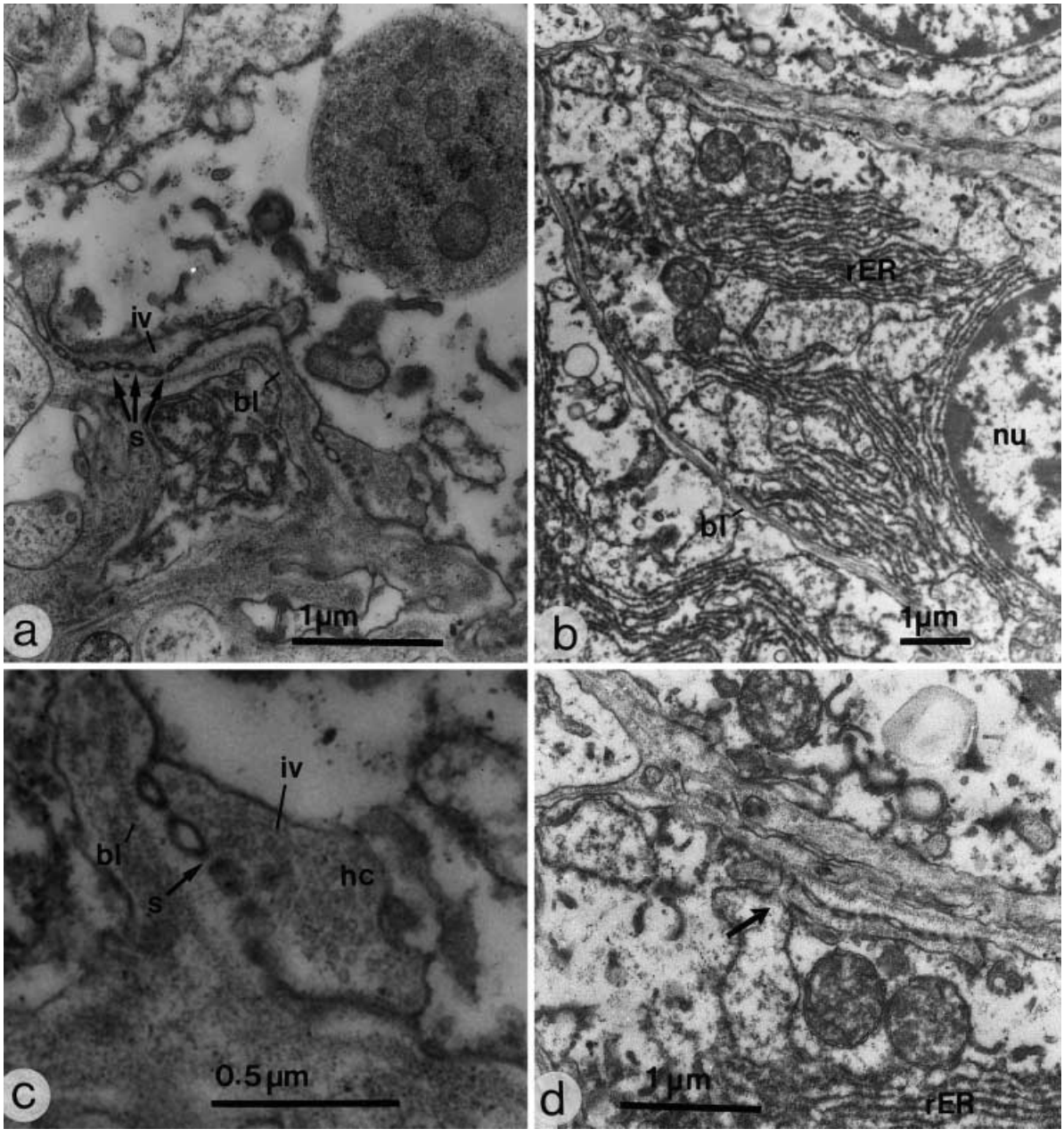

Fig. 4 **a, b** Peripheral regions of rhogocytes from the midgut gland of *Haliotis tuberculata* (*bl* basal lamina, *iv* invagination, *nu* nucleus, *s* slits, *rER* rough endoplasmic reticulum). **c** Enlarged detail of **a** showing an invagination of the plasma membrane filled with hemocyanin molecules (*hc*). **d** Enlarged detail of **b** showing a cisterna of the rough endoplasmic reticulum (*rER*) leading to the extracellular invagination (*arrow*)

ever, the probe specific for *Haliotis* because a cDNA encoding KLH is not yet available; data not shown).

Discussion

More than 30 years ago, a cell type showing unique invaginations of the plasma membrane was detected in the connective tissue of several pulmonate gastropods and described by Plummer (1966), Sminia (1972), and Sminia and Boer (1973). Later, this cell type was identified in many gastropod species and referred to a confusing number of terms, e.g., *Blasenzelle*, *Leydigsche Zelle*, *cellule nucale*, globular cell, fibrocyte, formative cell, pore cell, or rhogocyte; in this paper we use the term rhogocyte, as suggested by Haszprunar (1996). The biological function of rhogocytes is still a matter of debate. The accumulation of metal ions in the granules of rhogocytes suggests that these cells are involved in detoxification and metal ion metabolism (Haszprunar 1996). In previous studies rhogocytes have also been attributed to hemocyanin biosynthesis, as deduced from electron micrographs showing intracellular hemocyanin-like particles (Sminia and Vlugt van Daalen 1977) and from stud-

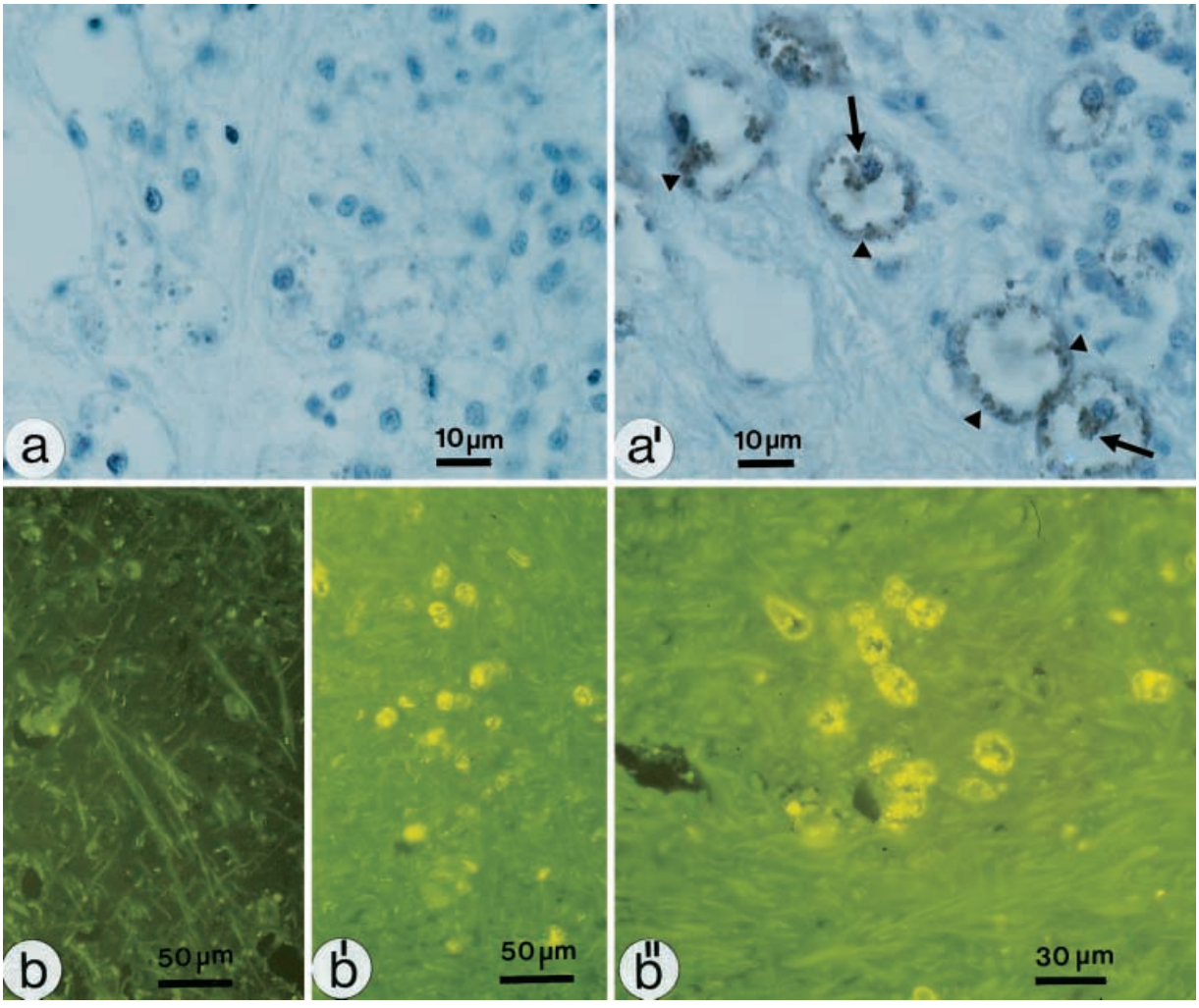

Fig. 5 **a, a'** Immunohistochemical localization of hemocyanin in paraffin sections of mantle tissue from *Haliotis tuberculata*. **a** Control sections processed in the absence of first antibody. **a'** Sections incubated with guinea pig anti-*Haliotis* hemocyanin antibodies (for specificity, see Keller et al. 1999; Lieb et al. 1999). Note labeling of rhogocytes at the cell periphery (*arrowheads*) and close to the nucleus (*arrows*). Sections were counterstained with hematoxylin. **b, b', b''** In situ hybridization of paraffin sections of mantle tissue from *H. tuberculata* using a hemocyanin-specific cDNA probe (Keller et al. 1999) labeled with DIG. **b', b''** The probe was detected with sheep anti-DIG antibodies conjugated with FITC. **b** Negative control section

ies on the copper content of these cells (Sminia 1972). However, other authors doubted that rhogocytes do indeed function as the site of hemocyanin biosynthesis (e.g., Haszprunar 1996). In the present study, electron microscopy revealed that rhogocytes are a characteristic element of the connective tissue from the abalone *H. tuberculata*. According to in situ hybridization experiments, the rhogocytes of *Haliotis* definitely contain hemocyanin-specific RNA and, therefore, are indeed a site of hemocyanin biosynthesis. Further support comes from the successful isolation of cDNA clones coding for functional units *a* to *h* of *Haliotis* hemocyanin isoform HtH1 (Lieb et al. 2000) and functional units *d*, *e*, *f*, *g* and *h* of isoform HtH2 (Lieb et al. 1999), derived from a cDNA library using mRNA of *Haliotis* mantle tissue.

We have also identified rhogocytes in several connective tissue preparations derived from *M. crenulata*. However, hemocyanin molecules could not be detected within these cells. This is in accord with our previous biochemical investigations, showing that hemocyanin concentration in the hemolymph of *M. crenulata* decreases with increasing time of captivity. Indeed, several months of captivity lead to a total lack of KLH1, one of the two hemocyanin isoforms (KLH1 and KLH2), in these animals (Gebauer et al. 1994). Moreover, it was routinely observed in our laboratory that bleeding of the limpets did not lead to a regeneration of the original hemocyanin level. Apparently hemocyanin synthesis is blocked in *M. crenulata* under our aquarium conditions. However, also from five different animals taken directly from the Pacific Ocean in November 1998, we failed to isolate hemocyanin-specific RNA (Keller et al. 1999; Lieb et al. 1999). In contrast, from *Haliotis*, hemocyanin-specific mRNA could be obtained, in several attempts, without difficulty (e.g., Lieb et al. 2000). Therefore, it appears that in *Megathura* hemocyanin biosynthesis is restricted

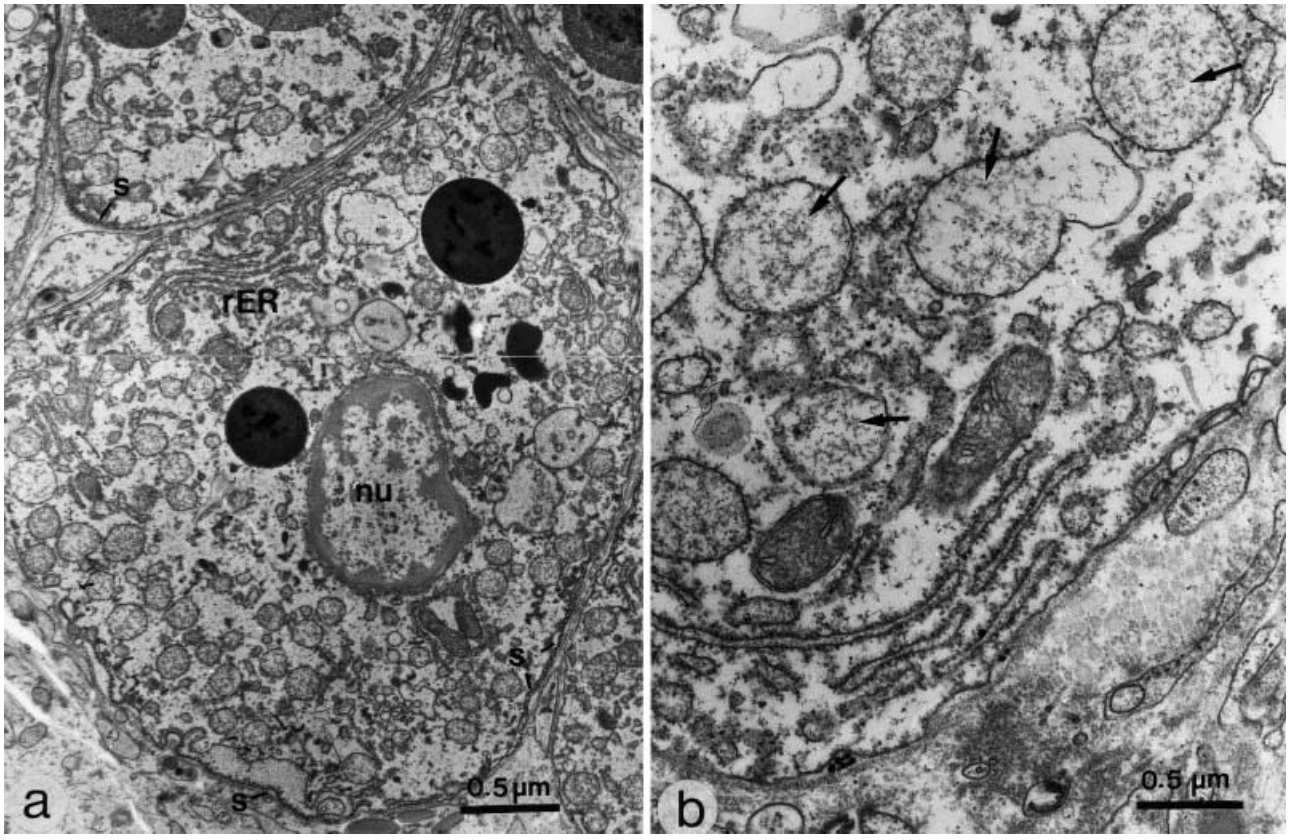

Fig. 6 Rhogocytes of *Megathura crenulata*. **a** Electron micrograph of a rhogocyte from the midgut gland (*nu* nucleus, *s* slits, *rER* rough endoplasmic reticulum). **b** Peripheral section of a rhogocyte showing cisternae of the rough ER containing amorphous flocculent material (*arrows*)

to special periods during the year. Sampling freshly collected animals over the year is necessary to prove this hypothesis of a seasonal hemocyanin biosynthesis in *Megathura*.

As mentioned above, two hemocyanin isoforms occur in the hemolymph of *Megathura* and *Haliotis*, termed KLH1/KLH2 and HtH1/HtH2, respectively (e.g., Markl et al. 1991). Immunological analysis of the hemocyanin molecules showed that no heteropolymers consisting of both type 1 and type 2 subunits exist (Gebauer et al. 1994; Keller et al. 1999). In this context the still unanswered question arises as to whether there are two different types of rhogocytes, each of them responsible for the synthesis of only one hemocyanin isoform, or whether the two isoforms are synthesized together in the same cells. Our earlier investigations on the specific reassociation behavior of hemocyanin subunits (Harris et al. 1997a, 1997b) suggest that both isoforms could be synthesized at the same time in the same rhogocyte. In these studies in vitro reassociation of a subunit mixture derived from both hemocyanin isoforms resulted in the exclusive formation of homo-oligomers. The presence of hetero-oligomers, constructed of both hemocyanin isoforms, could be experimentally excluded (Harris et al. 1997a, 1997b; 2000). However, this does not necessarily exclude the existence of two different cell types.

The mechanism of hemocyanin secretion into the hemolymph is still unclear. In *Helix aspersa*, vacuoles probably derived from the smooth ER, and filled with hemocyanin particles, were detected in the peripheral region of rhogocytes (Sminia and Vlugt van Daalen 1977). It was assumed by these authors that these vacuoles fuse with the plasma membrane, and the molecules are released by exocytosis. A similar mechanism of hemocyanin secretion has been proposed for rhogocytes from *Lymnaea stagnalis* (Sminia and Boer 1973). Hemocyanin-containing smooth exocytotic vesicles, as observed in pulmonates, were not found in *Haliotis*. Accumulations of hemocyanin molecules were nevertheless observed in invaginations of the plasma membrane. It could be shown that these cisternae sometimes communicate directly with the rough ER (Fig. 4), although a release of hemocyanin via the slits might be hindered by the diaphragms between the cytoplasmic bars and by the surrounding extracellular matrix. An alternative possibility might be a holocrine secretory mechanism, as discussed for the hemocyanin secretion in basal cells from the midgut gland of *Nautilus* (Ruth et al. 1988). However, this is rather hypothetical and more data are necessary to elucidate the exact pathway of hemocyanin molecules from the cisternae of the rough ER into the blood lacunae of the connective tissues.

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