**CALCIUM TRANSPORT MECHANISM IN CRAYFISH GASTROLITH
EPITHELIUM CORRELATED WITH THE MOLTING CYCLE**
(I) THE DEMONSTRATION OF CALCIUM-ACCUMULATING SITES

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Received for publication December 5, 1983

The transport of calcium ions in correlation with the molting cycle was studied in the columnar epithelium of the crayfish gastrolith disc tissue by three cytochemical methods. In material fixed by freeze-substitution or potassium oxalate-glutaraldehyde, electron-dense deposits were found only in the hypertrophied mitochondria of the epithelium. In material fixed with potassium antimonate-OsO₄, however, a number of fine deposits were observed throughout all membranous structures. The emission of calcium K line was demonstrated only in the deposits on the mitochondria in electron probe X-ray microanalysis with the material fixed with acrolein gas, oxalate-glutaraldehyde, and antimonate-OsO₄. These results may contribute to understanding the role of mitochondria in calcium transport when a large amount of calcium ions is moved from gastrolith epithelium to the outer molting epithelium or vice versa.

There are no reports on the mechanism of calcium transport by the biochemical procedure in the epithelium of crayfish, although there are a number of studies on calcium metabolism in mammals (4, 17, 18, 24). This is probably due to the difficulty in getting enough material from crayfish.

Histochemical and cytochemical methods are the only way to evaluate the mechanism of calcium transport without encountering the obstacle of quantity. An extensive investigation of the cytological changes and the process of gastrolith formation has been reported (21). Four types of epithelial cells (type I to type IV) are synchronized with the molting cycle and the formation of gastroliths was studied with the electron microscope (22). Quantitative inorganic detection and freeze-substitution are two methods which can be used to demonstrate the localization of calcium ions in gastrolith epithelium. In the present work we studied the distribution of calcium ions in the gastrolith epithelial cells by the potassium oxalate-glutaraldehyde method, the potassium antimonate-OsO₄ method, and the freeze-substitution method for fresh tissues (5). Some of the electron-dense deposits on unstained sections from these methods were also exposed to electron probe X-ray microanalysis (EDX) (13). The possible pathway of intracellular calcium ion will be discussed here from the results of these three methods.

MATERIALS AND METHODS

Animals

Adult male crayfish (*Procambarus clarkii*) of each molting stage were used. Both gastrolith epithelia were dissected out and separated if mineralized gastroliths had been formed.

Cytochemical methods

Potassium oxalate-glutaraldehyde method: The method was originally described by Popescu *et al.* (16) and modified by Mizuhira and Shiihashi (11) and Daimon *et al.* (2). Samples were preincubated in 170 mM potassium chloride with 40 mM potassium oxalate (pH adjusted to 7.4 with KOH) for 10 min at room temperature. They were then transferred into 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4 with 40 mM potassium oxalate for 2 hr at 4°C. Post-fixation with osmium tetroxide (OsO₄) was omitted in this experiment. The osmic fixative often removes the calcium oxalate deposits, so that it is difficult to detect calcium X-ray pulses, even though electron-dense deposits are precipitated in the tissues (11, 12).

Potassium antimonate-OsO₄ method: The method was described in earlier studies by Komnick (6), Shiina *et al.* (20), and Daimon *et al.* (1). Fresh samples were cut in small pieces and immersed for 2 hr in a mixture of equal volumes of 2% OsO₄ solution and 2% potassium antimonate dissolved in deionized water. The mixture was adjusted to pH 7.4 with a few droplets of 100 mM acetic acid. Fixed tissue blocks were dehydrated in an ethanol series and embedded in Epon 812 after immersion in propylene oxide. Ultrathin sections were cut on a LKB-4801A Ultratome with a glass knife, and were examined without staining under a Hitachi HU-11D-S electron microscope.

Freeze-substitution

The method of Harvey *et al.* (5) for keeping electrolytes in plant tissue was modified by Mizuhira and Shiihashi (11) for X-ray microanalysis and for obtaining a clear scanning transmission electron microscope (STEM) image. Fresh tissue was cut into small pieces, supported on a piece of aluminium foil, and frozen in liquid Freone-12 (CCl₂F₂) or propane after being exposed to acrolein gas for 3 min. Tissue blocks were stored in cold acetone with activated Molecular sieves 5A 1/16 at the temperature of acetone-dry ice mixture (−72°C) for one day, transferred to cold ether, and stored at −40°C for 20 days. Then they were moved to a 50% mixture of ether and vinyl cyclohexene dioxide (ERL 4206) at −40°C for 4 hr. Cold acetone also could be used instead of ether, and then the process of substitution and embedding could be finished within a week. The tissue blocks were moved to a pure resin mixture and gradually raised to room temperature. Finally they were polymerized at 60°C for one day (12).

Electron Probe X-ray Microanalysis

Relatively thick sections, approximately 100 to 150 nm, were transferred onto handmade carbon grids or standard copper grids. For freeze-substitution specimens, sections on the knife edge were picked up without water or floated on polyethylene glycol instead of water to prevent the elements leaching out into the water during the sectioning. The sections were observed and photographed without electron staining at 40 kV with an STEM-image system (ASID-4) fitted to a transmission

electron microscope (JEOL 100C). Selected points or areas in a STEM image were analyzed with an energy-dispersive type electron probe X-ray microanalyzer (EDAX-707B) and a microcomputer system (EDIT) at 40 KV accelerating voltage and with 5×10^{-10} amp illuminating current for a counting time of 100 to 1000 sec (12, 13). The data were analyzed by the utility programs (EDIT) of the strip elements, by smoothing peaks, and by memory subtract (9, 10, 12). Quantitative analyses were also performed with the manual calculation program for thin sections (19).

RESULTS

The oxalate-glutaraldehyde method

The distribution pattern of calcium oxalate, which appeared as electron dense deposits, varied from stage to stage of the molting. At the intermolt stage, no reaction product was seen in type I cells. A few deposits, however, were sometimes observed in the mitochondria of the connective tissue and of the granulocytes which invaded the open blood-vascular lumen. At the premolt stage, a number of deposits were clearly observed in all the hypertrophied mitochondria (M) of type II cells (Fig. 1). Most of the mitochondria of the subepithelial connective tissue also had fine deposits of calcium oxalate. However, no reaction product was observed anywhere in the cytoplasm or the membranous systems. The characteristic X-ray spectra of calcium (Ca-K), chlorine (Cl-K), and silicon (Si-K) were prominently observed in mitochondrial deposits (Fig. 2). The spectra of chlorine and silicon, however, may have originated from the resin base, since they were detected also in the Epoxy resin. The X-ray pulse images (mapping) of the calcium K line (white dots) were superimposed on the STEM-image (Fig. 3). The distribution pattern of the white dots coincided well with that of the deposits in a mitochondrion. On the other hand, type III and type IV cells had no reaction product. These cells are the major types of the large gastrolith period and the period just before molting.

The potassium antimonate- OsO_4 method

The preservation of the histology by this method was rather poor, since the fixative did not contain the conventional buffer solutions. The cytoplasmic membranes other than the mitochondria were also fragile in this fixative at the premolt stage, when the calcium transport was at the highest level. The reaction products appeared as large or small deposits in almost all cytoplasmic membranes of the epithelium at every stage. The distribution pattern of these reaction products was classified into two types. One had many non-specific deposits scattered throughout the epithelium, and the other had only a few deposits. Generally, the deposits

FIG. 1. Cytochemical demonstration of calcium ions by the potassium oxalate-glutaraldehyde method. The middle portion of a type II cell. Many vacuoles and hypertrophied mitochondria (M) are seen and electron-dense deposits are selectively formed in each mitochondrion. Unstained. Bar = 1 μm

FIG. 2. The spot-analyzing spectrum of X-ray energy analysis of the mitochondrial deposit and the background of Fig. 1. 40 kV accelerating voltage, 5×10^{-10} amp illuminating current. The spectrum from the mitochondrial deposit is displayed as vertical lines and the white spectrum is from the background. The superimposed figure clearly shows that the deposit is calcium.

FIG. 3. An X-ray mapping of Ca-K α energy pulses (white dots) is superimposed on the STEM image of a type II cell. The majority of the pulses overlap the mitochondria (M).

precipitated in the membranes were small, but those in the mitochondria were large (Fig. 4). The mitochondria always had clear deposits in epithelial cells of every type. Electron probe X-ray microanalysis revealed that these deposits were commonly composed, in large part, of osmium and antimony (Fig. 5), but contained little calcium (Fig. 6).

FIG. 4. Cytochemical demonstration of calcium ions by the potassium antimonate- OsO_4 method. Apical part of a type II cell. Large granule-like deposits are seen in the mitochondria (M) and fine deposits are distributed in the plasma membrane (pm). Unstained. Bar=0.5 μm

FIG. 5. The spot-analysing spectrum of X-ray energy analysis of the mitochondrial deposits of Fig. 4. Accelerating voltage, 40 kV; Illuminating current, 5×10^{-10} amp. Thick white lines show the theoretical central positions of Sb-L lines. The energy shift which shows the existence of overlapping peaks of the Sb-L line and Ca-K line is seen.

FIG. 6. Separating the combined spectrum of Fig. 5 with the computerized EDIT system. The computer identifies Ca from the Sb-L peak. Thin white vertical lines show the Sb-L line and the thick white lines show the Ca-K line.

The freeze-substitution method

The method had the defect of poor contrast, because the ultrathin sectioning had to be performed without water and observation had to be done on an unstained section. The improved acrolein vapor fixation gave better preservation of both the ultrastructures and the elements. Relatively large and uniform deposits were observed in the mitochondria of type II epithelium and subepithelial connective tissue (Fig. 7). The deposits were only observed in unstained sections, so that details of the distribution pattern of the deposits remained obscure. The results of X-ray microanalysis are shown in Figure 7, where X-ray energy spectra clearly demonstrate the presence of phosphorus, calcium, chlorine, sodium, potassium, sulphur, magnesium, and iron. Two typical intensity ratios of these elements are

FIG. 7. Demonstration of calcium ions by the freeze-substitution method. Many fine electron-dense deposits are seen in the mitochondria (M) of type II cells. The insert shows the spot-analysing spectrum of X-ray energy analysis of the mitochondrial deposit. In contrast with the results of the cytochemical methods, the emission spectra show the existence of many elements: sodium(Na), magnesium(Mg), phosphorus(P), sulphur(S), chlorine(Cl), potassium(K), and calcium(Ca). N: nucleus. Unstained. Bar=0.5 μ m

FIG. 8. Quantitative X-ray intensity ratios (%) of elements in two mitochondria of a type II cell visualized by freeze-substitution. Two types mitochondria are distinguished by their calcium and magnesium ratios. One type has a high calcium ratio and a low magnesium ratio (8a), and the other type has a high magnesium ratio and a low calcium ratio (8b). The ratios of other elements do not differ greatly between the two types of mitochondria.

summarized in a pair of circle graphs in figure 8. The intensity ratio of calcium K_{α} to magnesium K_{α} changed greatly from deposit to deposit, but the ratios of the other elements showed little variation.

DISCUSSION

Molting is a necessary process in the growth of crustacea, and the absorption of exoskeletal calcium occurs at the beginning of the molting cycle. After sloughing off the exoskeleton, calcium is secreted into the newly formed soft exoskeleton. The crayfish, which lives in freshwater, has a unique calcium-saving mechanism. It secretes the absorbed calcium into the gastric lumen as a pair of hard, calcified stones during the molting phase. The pair of stones are sloughed off in the stomach, and dissolve in the digestive duct after molting. The dissolved calcium is reabsorbed into the blood stream, and secreted to the newly formed soft exoskeleton after passing through the hypodermis. Gastrolith disc tissue, which secretes calcium during the molting stage, is composed of the cuticular lining of the stomach, columnar epithelium, and subepithelial connective tissue. These are located in the antero-lateral wall of the cardiac stomach. The hard calcified stones are called gastroliths and they develop in a sac between the epithelium and the cuticular lining. The existence of a drastic calcium transport mechanism in the gastrolith epithelium is worth considering, since the gastrolith secretion and reabsorption take place over a period of about a week respectively.

Absolutely clear results can not be obtained from cytochemistry alone, since each method has its own weak points. The fine location of calcium ions in mitochondria could not be shown because of the very large deposits in the oxalate-glutaraldehyde method. The separation of calcium K lines from superimposed

antimonate L lines was difficult in the antimonate-OsO₄ method. The ultrathin sections has poor contrast in the freeze-substitution method. The temporary accumulation of calcium ions in epithelial mitochondria is beyond doubt, since the results from the three methods matched each other. X-ray microanalysis with the three cytochemical methods demonstrated calcium exclusively in the mitochondria of type II cells, which are the major type of the period when a large amount of calcium ions move from the blood to the gastric lumen through the gastrolith epithelium. This fact strongly suggests that the mitochondria of type II cells store calcium during the molting stage. There is also the possibility that many calcium ions in the cytoplasm are lost during the normal chemical fixation and embedding processes, even with the oxalate method (11). X-ray microanalysis with the freeze-substitution method revealed that calcium was present only in the mitochondria. This may indicate that the mitochondria accumulate an extremely high concentration of calcium ions as compared with the low calcium accumulation of the other cytoplasmic elements. Isolated mitochondria of rat liver or kidney are capable of active accumulation of calcium ions (4, 17, 18, 24). Mitochondrial calcium-rich granules have also been reported in developing osteoblasts and in chondrocytes (3, 7, 8, 14, 15). Many of the investigators have considered these accumulations a protective mechanism in calcium-stressed cells. From these considerations, the main source of the calcium supply in calcification does not exist in the mitochondria, but the mitochondria performed a temporary buffering action on the excess calcium ions. The calcium ions clearly demonstrated by our experiments were limited to the mitochondria. This might result from the large amount of calcium ion transport and from the very short transport time. It is still unclear whether these calcium-containing mitochondria slough off to the gastric lumen with their cytoplasmic debris (22) or regain their inherent functions after discharging the accumulated calcium. The calcium in the mitochondria might be utilized as a major part of the calcium of the forming gastrolith, even though the calcium accumulation in the mitochondria is due to their protective function. This protective function might be specially developed in the mitochondria as a result of the anomalous massive influx of calcium ions to the epithelial cells. Therefore, mitochondria might accumulate calcium ions actively rather than protectively in the case of the gastrolith epithelium. This calcium ion accumulation by mitochondria may also exist in developing osteoblasts and chondrocytes, although these tissues utilize calcium phosphate and the crayfish utilizes calcium carbonate.

Although we have demonstrated calcium accumulation in mitochondria, the evaluation of the calcium transport mechanism may need other evidence. The calcium transport routes from cytoplasmic membrane to mitochondria and from mitochondria to the forming gastrolith are still unknown and the existence of another calcium transport route is conceivable in which the mitochondria accumulate calcium only as a "protective" function. Technical improvement of the freeze-substitution method may be required to clarify the whole mechanism of calcium transport, and other approaches are also needed, such as enzymocytochemical and immunocytochemical demonstrations of Mg²⁺-ATPase or Ca²⁺-ATPase (adenosine triphosphatase) and carbonic anhydrase activity. These problems will be dealt with in the following reports (23).

ACKNOWLEDGEMENTS

The authors wish to thank Michiko Ono for her expert technical assistance during this investigation. We also wish to thank Dr. Yutaka Futaesaku for his helpful discussions of the data.

The work is supported in part by a Grant-in-Aid for Co-operative Research, No. 56370002, from the Ministry of Education, Science and Culture, Japanese Government, to Professor V. Mizuhira.

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