Ultrastructural Studies on the Ca^{2+} Localization in the Dividing Cells of the Maize Root Tip

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ABSTRACT. The distribution of Ca^{2+} in dividing cells of the maize root tip was examined by potassium pyroantimonate precipitation and EGTA treatment methods. Ca^{2+} was found in most of the cell organelles, such as the matrix of the mitochondria, the thylakoid membrane of the proplastid and the Golgi vesicles, and on the plasma membrane. Ca^{2+} was also distributed throughout the cytoplasmic ground matrix and attractoplasm, inside the vacuoles, in the granular zone of the nucleolus in the interphasic nucleus and in the regenerated nucleolus in the telophasic nucleus. The amounts of Ca^{2+} distributed in the cytoplasmic ground matrix, the vacuole and the nucleolus varied during nuclear division. From the results of the present experiment, the following considerations on the role of Ca^{2+} and the regulation site of Ca^{2+} in dividing plant cells were drawn: 1) Ca^{2+} may play a role in the construction of the granular form of the ribosome. 2) Ca^{2+} may be an essential ion in the regeneration of nucleolus. 3) Vacuoles may act as the regulatory site of the Ca^{2+} concentration in the cytoplasm and attractoplasm in plant cells. Spindle microtubules and phragmo-microtubules are probably surrounded by other ions, such as Mg^{2+} .

The Ca^{2+} is necessary for many functions of animal and plant cells. Recent reports have shown that it plays important roles in the various intracellular events in plant cells, such as protoplasmic streaming (25, 27, 29), nuclear division (1, 5, 7, 9, 15, 30), cell division and cell plate formation (22, 23) and polarized growth such as in rhizoids and pollen tube (2, 8, 10, 13, 14, 18, 19). The Ca^{2+} may also act as a secondary messenger in stimulus transduction in the gravitropic response of roots (3, 12). Consequently, it is important to determine the precise kinetic localization of Ca^{2+} in plant cells.

Recently, some workers (1, 6, 7, 30) have demonstrated by means of fluorescent microscope that Ca^{2+} increases at the onset of anaphase and suggested that the phenomenon participates in the anaphase trigger during mitosis. Therefore, it is significant to clarify the precise distributions of Ca^{2+} in various cell stages during the nuclear division. However, there are few electron microscopic studies on the behavior of Ca^{2+} during nuclear division (23, 28). In the present study, we examined the precise localizations of Ca^{2+} in dividing cells by electron microscope, using the antimonate precipitation method (24), in plant root meristem.

MATERIALS AND METHODS

Maize (*Zea mays*) seeds were immersed in running tap water at 30°C overnight and germinated in a moist chamber at 26°C in the dark. Roots 17–20 mm in length were used.

Antimonate precipitation procedure. Root tip segments 3 mm long were fixed according to the microwave irradiation method by Mizuhira *et al.* (11) for 20 sec in a mixture solution of 5% glutaraldehyde and 4% paraformaldehyde adjusted to pH 7.6 with 1/10 M potassium phosphate buffer solution containing 2% potassium pyroantimonate ($\text{K}[\text{Sb}(\text{OH})_6]$) and 0.1% tannic acid. The materials were fixed then again with the same aldehyde fixative solution at room temperature for 3 h, and rinsed with the same buffer solution for 1 h. Post fixation was carried out overnight in 4°C in 1% OsO_4 adjusted to pH 7.6 with the same buffer solution containing 2% potassium pyroantimonate. After dehydration in a graded acetone series, the materials were embedded in epoxy resin via propylene oxide. Ultrathin sections were prepared and examined by electron microscope without electron staining.

Chelation of antimonate precipitates from thin sections. Grids with ultrathin sections obtained by the antimonate precipitation techniques were floated on 0.2 M EGTA (pH 8.0) at 60°C for 1 h. The grids were then rinsed with distilled water and examined without electron staining.

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RESULTS

The findings of the antimonate precipitation procedure are shown in Figs. 1, 3, 5, 6, 11, and 14. Electron opaque deposits of various sizes (10–100 nm) were distributed throughout the cells, but were not randomly distributed, as seen in Fig. 1. In the cell in interphase (Fig. 1), a large amount of deposits by potassium pyroantimonate were located in the granular zone of the nucleolus (Fig. 1, g), but the fibrillar zone of the nucleolus was free from them. Coarse deposits also were found throughout the nucleoplasm, while those on the chromatin strands were few and fine. The nuclear envelope was free from them. In the cytoplasm, deposits of antimonate were observed in the mitochondrial matrix (Fig. 1, M), and the thylakoid membrane of proplastids (Fig. 1, P), and the Golgi vesicles. On the plasma membrane (Fig. 1, PM), fine grains of deposits were also observed. Many deposits were distributed throughout the cytoplasmic ground matrix, whereas, the endoplasmic reticulum (ER) contained few deposits (Fig. 1, ER).

Potassium pyroantimonate reacts with Ca^{2+} , Mg^{2+} , K^+ and Na^+ , and produces electron opaque precipitates. Therefore, the preparations were treated with EGTA to identify Ca-antimonate deposits which should be removed by this treatment. The treatment conditions were according to the results of previous examination (21). Briefly, the minimal effective time of treatment with EGTA (pH 8.0) to remove Ca-antimonate precipitates was 1 h at 60°C. Fig. 2 showed part of a cell in interphase treated with EGTA. Deposits of antimonate were reduced as a whole. The granular zone of the nucleolus in the nucleus was free from deposits, but the deposits remained on the nucleoplasm. The remaining deposits were fine in size and somewhat reduced in number. This result shows that the granular zone of the nucleolus includes a large amount of Ca^{2+} and that the nucleoplasm contains a lot of other cations such as Mg^{2+} and K^+ in addition to Ca^{2+} . In the mitochondria, large sized deposits were removed, whereas small ones were remained. Since, generally accepted, mitochondria need certain cations, such as Ca^{2+} , Mg^{2+} and Mn^{2+} in order for their functions to proceed, so, it is likely that the residual deposits are Mg^{2+} and Mn^{2+} . The proplastids, the interior of vacuoles and the plasma membrane in the EGTA-treated preparation were almost free from deposits. Deposits throughout the ground matrix of the cytoplasm were almost removed after the treatment.

As shown in Fig. 3, the nucleolus of the prophase cell (Fig. 3, Nu), was almost completely occupied with fibrillar zone. The granular zone with antimonate deposits became small compared to that seen in interphase (Fig. 1, Nu), and located on the periphery of the fibrillar zone (Fig. 3, g). However, the deposits by antimonate could not be observed in the fibrillar zone. Fine deposits were distributed throughout the nucleoplasm. The ground matrix in the cytoplasm was almost free from deposits. The vacuoles were filled with deposits of antimonate (Fig. 3, V). Judging from the electron density a large amount of precipitates is contained in the vacuoles observed on the left side in Fig. 3. The distributions of precipitates in the mitochondria, the plastids and the plasma membrane were similar to those observed in interphasic cells. In the prophase nucleus after treatment with EGTA (Fig. 4, N), deposits in the granular zone of the nucleolus were removed but most deposits observed in the nucleoplasm remained. The deposits on the mitochondria, the plastids and the plasma membrane after EGTA treatment were similar to those observed in interphasic cells (Fig. 2). Precipitates in the vacuoles were almost completely removed by the treatment (Fig. 4, V).

In the metaphasic cell (Fig. 5), precipitates by antimonate were observed throughout the cytoplasmic ground matrix and the attractoplasm. Vacuoles localizing at the opposite poles of the spindle body contained a large amount of precipitates. The precipitates by antimonate in the mitochondria, the proplastids and the plasma membrane were similar to those observed in interphasic (Fig. 1) and prophase (Fig. 3) cells. A few and fine antimonate deposits were also observed on the metaphasic chromosomes (Fig. 6, Ch). In the metaphasic cells after EGTA treatment (Figs. 7–9), deposits distributed throughout the cytoplasmic ground matrix and the attractoplasm were severely reduced compared to those observed in untreated samples (Figs. 5 and 6). However, some deposits remained along the spindle microtubules (Figs. 8 and 9, arrow heads). The residual deposits along the spindle microtubules were removed by the EDTA treatment. The chromatin and kinetochore of chromosomes were entirely free from deposits after the EGTA treatment (Figs. 8 and 9, Ch and K). Along the ER localizing at the periphery of attractoplasm, deposits remained even after EGTA treatment (Fig. 7, ER). The precipitates inside the vacuole were conspicuously removed (Fig. 7, V). In addition, some deposits in the mitochondria and the proplastids were removed (Fig. 7), similar to those observed in the interphasic (Fig. 2)

Fig. 1. The preparations of the precipitation method with potassium pyroantimonate. Part of a cell in interphase. Symbols in figures (1–14): Ch; chromosome, CP; cell plate, CW; cell wall, ER; endoplasmic reticulum, G; Golgi body, g; granular zone of nucleolus, K; kinetochore, M; mitochondria, N; nucleus, NE; nuclear envelope, Nu; nucleolus, Ph; phragmosome, V; vacuole. Scale bar: 1 μm .

Fig. 2. EGTA treated preparations. Part of a cell in interphase. Scale bar: 1 μm .

Fig. 3. The preparations of the precipitation method with potassium pyroantimonate. Part of a cell in prophase. Scale bar: 1 μm .

Fig. 4. EGTA treated preparations. Part of a cell in prophase. Scale bar: 1 μm .

Figs. 1, 2.

Figs. 3, 4.

Figs. 5-9.

Fig. 10. The preparations of the precipitation method with potassium pyroantimonate. Many deposits are seen throughout the telophasic cell. Between the chromosomes many deposits are conspicuously present (arrows). Many precipitates are also contained inside the vacuoles. Scale bar: 1 μ m.

Fig. 11. The preparations of the precipitation method with potassium pyroantimonate. Enlargement of a part of the equatorial region in Fig. 10. Phragmosomes contain fine deposits arranged in an array at the equatorial plate. Around the phragmosomes, large sized deposits are distributed evenly. Scale bar: 0.5 μ m.

and prophasic cells (Fig. 4).

In telophase in which daughter chromosome groups reached the spindle poles and were encircled by the nuclear envelope, the nucleolus was regenerated between the chromosomes. Fig. 10 showed the feature that large amounts of coarse deposits localized on the regenerating nucleolar materials (Fig. 10, arrows), while fine deposits existed on the chromosomes (Fig. 10, Ch). Many deposits were also distributed throughout the phragmoplast and the cytoplasm. At the equatorial plane, phragmosomes filled with fine deposits were arranged side

by side (Fig. 11, Ph). The inside of the vacuole was filled with an extremely large amount of the precipitates (Fig. 10, V). The distributions of the deposits in the mitochondria, the proplastids and the plasma membrane were similar to those observed in earlier stages. After EGTA treatment with EGTA (Figs. 12 and 13), the deposits on the chromosomes and the regenerating nucleolar materials were removed (Fig. 12, N). The phragmosomes were also free from deposits (Fig. 13, Ph), however, considerable deposits were remained at the equatorial region of the phragmoplast. At the equa-

Fig. 5. The preparations of the precipitation method with potassium pyroantimonate. Low magnification of a metaphasic cell. Deposits are distributed throughout the cell. Many precipitates are conspicuously present inside the vacuoles. Scale bar: 1 μ m.

Fig. 6. The preparations of the precipitation method with potassium pyroantimonate. Enlargement of a part of the metaphasic cell in Fig. 5. On the chromosomes a few deposits are visible, while on the attractoplasm, most of the deposits are evenly distributed around the chromosomes. Scale bar: 1 μ m.

Fig. 7. EGTA treated preparations. Low magnification of a metaphasic cell. No deposits are seen inside the vacuole. Scale bar: 1 μ m.

Fig. 8. EGTA treated preparations. Enlargement of a part of the metaphasic cell of Fig. 7. Residual deposits after EGTA treatment are evident along the spindle microtubules (arrow head). Scale bar: 1 μ m.

Fig. 9. EGTA treated preparations. Another preparation of a metaphasic cell. The chromosome arm and kinetochore are free from deposits. Arrowhead indicates the spindle microtubules. Scale bar: 1 μ m.

Fig. 12. EGTA treated preparations. Low magnification of a telophasic cell. As a whole, deposits are reduced throughout the cell. Scale bar: 1 μ m.

Fig. 13. EGTA treated preparations. Enlargement of a part of the equatorial region in a telophasic cell after treatment with EGTA. Phragmosomes are free from deposits, but deposits around the phragmosomes remain. Scale bar: 0.5 μ m.

Fig. 14. The preparations of the precipitation method with potassium pyroantimonate. Telophasic cell in which the nucleus is in the reconstruction stage. Note that the small sized nucleolus in renewal is free from deposits (arrows). Scale bar: 1 μ m.

torial region, phragmo-microtubules were localized, hence these residual precipitates after EGTA treatment may play the same role as those observed along the metaphasic chromosome microtubules. The precipitates in the vacuoles were almost completely removed by the EGTA treatment (Fig. 12, V). The distributions of deposits in the mitochondria, the proplastids and the cytoplasmic ground matrix after EGTA treatment were similar to those observed in earlier stages.

Newly formed nucleolus seen as a small sized mass in

the telophase was free from any precipitates (Fig. 14, arrows).

DISCUSSION

The distribution of Ca²⁺ in dividing cells of the maize root tip was examined by potassium pyroantimonate precipitation and EGTA-treatment methods. Ca²⁺ was distributed in the granular zone of the nucleolus, throughout the cytoplasmic ground matrix and attracto-

plasm, and inside the vacuoles. Ca^{2+} was also found in most of the cell organelles, the mitochondrial matrix, the thylakoid membrane of the proplastid and the Golgi vesicles. Ca^{2+} was found on the plasma membrane, too. During nuclear and cell division, the amount of Ca^{2+} in the mitochondria, the proplastids, and the plasma membrane did not vary. However, conspicuous changes were noted on the nucleolus, the cytoplasmic ground matrix and the vacuoles, and some conclusions were drawn about the role of Ca^{2+} and its regulatory site in the plant cells. The first is that Ca^{2+} may play a role in the construction of the three dimensional form of the ribosome. Because, in the present observations, we showed that the Ca^{2+} was not contained in the fibrillar zone of nucleolus in interphasic nucleus, but that large amounts of the ion was contained in the granular zone. These fact indicates that the Ca^{2+} may be involved in the formation of the granular form of the ribosome by the bonding of fibrillar RNA and proteins. The second is that Ca^{2+} may be an essential ion in the regeneration process of the nucleolus in early telophase. Since, in the present observations, a large amount of Ca^{2+} was recognized between chromosome clusters where nucleolus regenerate in the early telophase. No information has been reported that the Ca^{2+} contributes to the construction of the three dimensional form of ribosome and on the regeneration of nucleolus. Present studies indicate that Ca^{2+} may be an important ion in the construction of subcellular structures, in addition to the physiological events in plant cells.

The present observations showed that the Ca^{2+} existing in the cytoplasm and attractoplasm may increase or decrease in quantity, during nuclear and cell division. Especially, in the prophasic cell, Ca^{2+} distributed throughout the cytoplasm decreased extremely, compared to that in other stages. Recently, Hepler (6) proposed that during mitosis, the transient increase of Ca^{2+} may act as a trigger for the progress of mitosis, although the opposite opinion was earlier expressed (26). Hepler (4) and Wick and Hepler (28) reported that ER associated closely with spindle microtubules acts to control the concentration of free Ca^{2+} in the mitotic apparatus. Their idea originates from the finding that the sarcoplasmic reticulum plays a role as the storage site of Ca^{2+} in muscle cell. However, ER are not always recognized in the spindle body of the all dividing cells. Moreover, their observations were made by the osmium-ferrocyanide fixation method, and it is unclear whether the fixation demonstrates the precise localization of Ca^{2+} in the cells or not. In the present materials, the maize root tip cells, the ER neither adhered to the spindle microtubules, nor existed in the spindle apparatus. Moreover, few precipitations of Ca-antimonate were recognized in ER. It is unclear whether or not ER acts as the control site of Ca^{2+} concentration in plant cells. As a final

presumption we propose that the vacuole may be suitable as a control site of Ca^{2+} rather than the ER in dividing plant cells, for the following three reasons: 1) The quantity of precipitates by Ca^{2+} in the vacuoles appears to vary in the different cell stages. 2) In the metaphasic and anaphasic cells, vacuoles filled with the precipitate of Ca-antimonate were located around the spindle body. 3) In animal cells, intracellular Ca^{2+} concentration is easily controlled by the existing of extracellular matrix. However, in the plant cells which do not have extracellular matrixes, the vacuole may play a role of the extracellular matrix in animal cells. Thus, the vacuole plays an important role in the regulation of various ions in plant cells, but, study on the functions except for those on the turgor pressure.

As mentioned above, the present observations showed that amount of Ca^{2+} decreased significantly in the prophasic cells. This evidence indicates that the Ca^{2+} concentration of cytoplasm in the prophasic cells might be necessary at low levels, prior to the contribution of the spindle body, especially the polymerization of the spindle microtubules. While the present observations showed that in the metaphasic cell, large amounts of Ca^{2+} were distributed throughout the attractoplasm, but only the spindle microtubules were surrounded by the other ions which were not removed by the EGTA treatment, but removed by the EDTA treatment. As generally accepted, microtubules are polymerized and preserve their tubular structure in the presence of Mg^{2+} and are depolymerized under the presence of Ca^{2+} . We reported previously that Mg-ATPase activity was recognized along the spindle microtubules in animal cells (20). Considering from these facts, spindle microtubules may be surrounded by Mg^{2+} , and the ion may play a role in the preservation and/or protection of microtubules from the Ca^{2+} which is distributed throughout the attractoplasm. In the present experiment, large amounts residual deposits of antimonate were confirmed around the equatorial region in the phragmoplasm of telophasic cell, after the EGTA treatment. The residual precipitates may originate from Mg^{2+} , and the ion may act to maintain the phragmo-microtubules as in the case of spindle microtubules.

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