The suitability of the lead precipitation method for the localization of active ATPases in the meristematic cells of *Allium cepa* L. roots

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Abstract

Demonstrated in this study is the suitability of the lead precipitation method for the localization of active ATPases bound with the mitochondrial membranes, ER and plasma membranes of meristematic cells of *Allium cepa* L. root apices. It was found that the presence of glutaraldehyde in the fixative has a significant influence on the fixation of ATPases and that a somewhat smaller influence is exhibited by formaldehyde. Also shown in this study is the relationship between the course of the reaction indicating the presence of active ATPases and the precipitating activity of Pb ions as well as the stimulatory effect of Ca^{2+} , Mg^{2+} and K on the transport ATPase bound with the plasma and ER membranes.

Key words: meristematic cells, active ATPases, lead precipitation method

INTRODUCTION

ATPases, which are phosphatases, constitute a diversified group of enzymes, localized in various cellular structures — the plasma membrane, mitochondria, ER, tonoplasts and nuclear membrane. They are capable of hydrolysing various triphosphates and exhibit different specificity to such stimulators as, e.g., Mg^{2+} , Ca^{2+} , Na, K and to inhibitors, such as ouabain and NaF (Ernst 1972).

The most commonly used reaction for the localization of active ATPases

is the precipitation reaction introduced by Wachstein and Meisel (1957) based on the assumption that the phosphate ions released by the enzymic hydrolysis of ATP bind with metal ions in the place where they were formed, giving insoluble precipitates. These precipitates are easily identified in the electron microscope since they are electron opaque. The Wachstein and Meisel method, initially used mainly for the localization of active phosphatases in animal tissues, has been modified many times, thanks to which it is possible to identify specific ATPases, localized in different cellular structures. The interpretation of the results obtained by the Wachstein and Meisel methods has, however, been for many years the subject of discussion (Novikoff 1967, Browning et al. 1980). The objections set forth by some authors in respect to the specificity of this method arise from the fact that lead ions can, to some degree, inhibit the activity of ATPases (Moses et al. 1966, Tormey 1966, Rosenthal et al. 1969a), bring about the un-enzymic hydrolysis of ATP (Rosenthal et al. 1966, 1969b, Moses and Rosenthal 1968), influence the effectivity of inhibitors (Tice 1969) and the distribution of the products (Rosenthal et al. 1969a). These objections, although disproven to a large extent by the results of studies by various authors (Novikoff 1967, Jacobsen and Joergensen 1969, Miętkiewski et al. 1970, Browning et al. 1980), are the reason why this method is used with a certain degree of caution.

The experiments conducted in this study were aimed at determining the suitability of the Wachstein and Meisel method for the localization of active ATPases in meristematic plant tissues. The influence of fixatives and Pb ion concentrations on the activity of ATPases and the specificity of certain stimulators (K^+ , Mg^{2+} , Ca^{2+}) were studied.

One of the basic conditions which must be met for the localization of active ATPases is proper fixing of material. From the experience gathered so far it is known that glutaraldehyde does not destroy the activity of ATPases, with the exception of mitochondrial ATPase (Essner et al. 1965, Ogawa and Mayahara 1969). Very important in the process of fixing tissues for ultrastructural studies is the composition and molarity of the buffer for the fixative. For the localization of ATPases in plant tissues, the most favorable buffer has turned out to be the cacodylic buffer (Winter-Sluiser et al. 1977, Bentwood and Cronshaw 1978, Browning et al. 1980, Hall et al. 1980). Experiments have shown that both the optimum strength of the buffer and the concentration of the components of the fixative are strictly dependent on the type of tissue being treated (Hall 1971, Winter-Sluiser et al. 1977, Bentwood and Cronshaw 1978, Browning et al. 1980, Hall et al. 1980, Belitser et al. 1982).

One of the most specific ATPases known is the Na^+ , K^+ -dependent transport ATPases present in the plasma membrane. The results presented

by Ganote et al. (1969) indicate that the Wachstein-Meisel method cannot be used to localize the activity of this transport ATPase because the concentration of lead ions used in this reaction completely inhibits it. The author of the method of identifying the specific transport ATPase is Ernst (1972a and b), who in modifying the method of Wachstein and Meisel, replaced lead ions by Sr ions which do not inhibit the activity of transport ATPase.

From the studies done in later years (Hall 1971, Winter-Sluiter et al. 1977, Bentwood and Cronshaw 1978, Browning et al. 1980, Hall et al. 1980, Belitser et al. 1982) it is seen that lead ions can be used as the factor precipitating the reaction product of the transport ATPase under the condition that their concentration in the reaction medium is not too high.

The Wachstein and Meisel method was also later used successfully to localize active mitochondrial ATPase. The course of this reaction was, however, dependent on the way in which the material was fixed. For observations in the electron microscope, Bouin's solution or 4% formaldehyde were used most often, since mitochondrial ATPase is characterized by a high sensitivity to glutaraldehyde.

MATERIAL AND METHODS

The experiments were conducted on the apical meristems of adventitious roots of edible onions, grown in aerated tap water. In order to localize active ATPases, 0.5 mm long apical meristem fragments were fixed according to one of the following variants:

1. 1% glutaraldehyde prepared in 50 μ M cacodylic buffer, pH 7.0, fixation for 1 hr, 0°C. Fixed tissues were rinsed in 48 μ M Tris buffer at pH 7.0 at 0°C for 4 hrs, then incubated for 2 hrs at 25°C in an incubation medium containing 48 μ M Tris buffer at pH 7.0 and 2 μ M $\text{Ca}(\text{NO}_3)_2$, 2 μ M ATP (sodium salt), 2 μ M $\text{Pb}(\text{NO}_3)_2$, 12 μ M K (in a solution of KNO_3). After 2 hrs of activity in the reaction medium, the tissues were rinsed in 48 μ M Tris buffer for 1 hr at 20°C and postfixed in 1% OsO_4 in 25 μ M cacodylic buffer at pH 7.2, for 12 hrs. The fixed material was rinsed in 50 μ M cacodylic buffer at pH 7.2, dehydrated in ethanol and propylene oxide. After embedding in epon, the material was sectioned into ultrathin sections and contrasted according to Reynolds' method (1963). The observations were conducted in a Tesla MB 500 microscope.

2. 5% glutaraldehyde + 4% formaldehyde in 50 μ M cacodylic buffer, fixation for 2 hrs at 0°C. The fixed tissues were rinsed in 50 μ M Tris buffer for 3 hrs at 0°C, then placed for 45 min at 30°C in a reaction medium

containing: 50 μM Tris buffer, pH 7.2 and 2 μM ATP (sodium salt), 2 μM $\text{Mg}(\text{NO}_3)_2$, 3.3 μM $\text{Pb}(\text{NO}_3)_2$. After washing for 30 min in 50 μM Tris buffer, pH 7.2, postfixation in 1% OsO_4 in cacodylic buffer for 12 hrs was carried out. Further steps were as described in variant 1.

3. 1% glutaraldehyde + 4% formaldehyde in 100 μM cacodylic buffer at pH 7.2. Fixation was for 2 hrs at 0°C. The fixed tissues were rinsed in 100 μM Tris buffer at pH 7.2 at 23°C for 2 hrs and subjected for 2 hrs at 23°C to the action of the incubation mixture which contained 100 μM Tris buffer at pH 7.2 and 2 μM ATP (sodium salt), 2 μM $\text{Mg}(\text{NO}_3)_2$ and 2 μM $\text{Pb}(\text{NO}_3)_2$. After rinsing in distilled water, postfixation was carried out in 1% OsO_4 in 20 μM cacodylic buffer, pH 7.2 at 4°C for 12 hrs. Further steps as described in variant 1.

4. 0.5% glutaraldehyde + 4% formaldehyde in 0.02 M cacodylic buffer, pH 7.2 for 2 hrs. The fixed material was rinsed in Tris buffer for 2 hrs at 22°C and incubated in the reaction medium containing: 0.2 M Tris buffer, pH 7.2 and 2 μM $\text{Mg}(\text{NO}_3)_2$, 2 μM $\text{Pb}(\text{NO}_3)_2$, 2 μM ATP (sodium salt). After a 3 hour period of washing in distilled water, postfixation was carried out in 1% OsO_4 in 0.02 M cacodylic buffer, pH 7.2 for 12 hrs at 4°C. Further steps were as in variant 1.

The compositions of the fixative solutions and incubation mediums used in this experiment were according to authors conducting experiments on various types of plant tissues — phloem, xylem, parenchyma (Winter-Sluiser et al. 1977, Bentwood and Cronshaw 1978, Browning et al. 1980, Hall et al. 1980).

The use of various fixatives containing different concentrations of glutaraldehyde and formaldehyde made it possible to analyse the relationship between the method of fixing tissues and the activity of ATPases. The use of different incubation mediums which, in addition to the ATP sodium salt as the substrate and Pb ions as the precipitating factor, contained Ca^{+2} , Mg^{+2} or K^{+} ions, enabled the role of mono- and bivalent ions in the activation of various ATPases to be determined.

For a more detailed analysis of the mechanism of change of ATPase activity in the studied tissues, part of the roots (along with the onion) which had been cultured in water, were transferred for 24 hrs to solutions of sodium azide (100 ppm), malonic acid (100 ppm) and ATP (200 ppm), fixed and used for ATPase assay according to variant 3.

In order to check the validity of the course of the reaction localizing active ATPases, in each experimental variant, some of the roots were incubated in a reaction mixture lacking the ATP which is the substrate for the enzyme or precipitating lead ions. In each such case, the result of the control reactions should be negative.

RESULTS

Shown on the basis of the experiments conducted in this study were the effects of the formaldehyde fixatives used herein, of mono- and bivalent activating ions and lead ions on the activity of several types of ATPases in the meristematic cells of onion roots. These were: transport ATPase, mitochondrial ATPase and cytoplasmic ATPase—localized in the ER.

In the tissues fixed in 1% glutaraldehyde (variant 1), distinct reactions indicating the presence of active ATPases in the plasma membrane (Figs. 1, 2, 3), mitochondria (Figs. 1, 2) and ER (Figs. 1, 2, 3) were seen. The transport ATPases exhibited exceptionally high activity, which could have been additionally brought about by the stimulatory effect of potassium ions contained in the reaction medium (variant 1).

High concentrations of glutaraldehyde (5%) and formaldehyde (4%) in the fixative (variant 2) caused a decrease in the activity of all of the ATPases in the studied tissues. This effect could also have been caused by the high concentration of lead ions in the reaction medium (3.6 μM), or was the result of both of these factors. In addition to this, it was also found that high concentrations of glutaraldehyde and formaldehyde in the fixative caused very serious vacuolization of the tissues, which interfered to a high degree in the evaluation of the conducted reactions.

In the meristematic cells fixed according to variant 3 (1% glutaraldehyde + 4% formaldehyde) a very intense reaction was found which indicated the presence of active ATPases in the plasma membrane (Figs. 4, 5, 7), as was a somewhat weaker reaction in the mitochondria (Fig. 7) and ER (Fig. 7). The reaction product was also sporadically found in the nuclear membrane. Analysis of the electronmicrographs shows that the intensity of the reaction was greater than in the tissues fixed according to variant 1 (1% glutaraldehyde). These results can, it seems, lead to the following conclusions:

1. The addition of 4% formaldehyde to the fixative does not destroy the activity of ATPases, especially of transport ATPase.
2. The activity of ATPases (transport, mitochondrial and cytoplasmic (ER)) can be stimulated both by monovalent potassium ions as well as bivalent calcium (variant 1) and magnesium (variant 3) ions. The increase in the intensity of the reaction in fixation variant 3 supports the latter (Mg^{2+} ions).

Significant differences in the course of the reaction indicating the presence of active ATPases were found in tissues fixed according to variant 4. In this fixative, next to the unchanged 4% concentration of formaldehyde, the glutaraldehyde concentration was reduced to one-half (0.5%). A distinct decrease in the intensity of the reaction was then noticed since it was

found only in the plasma membrane (Fig. 6). This result seems to point to the fact that glutaraldehyde is the reagent which fixes ATPases without destroying their activity.

The comparison of meristematic tissues fixed according to variants 1, 3 and 4 made it possible to scrutinize more closely the way in which the fixative components (GA and formaldehyde) act. It can be concluded on the basis of the conducted experiments that 4% formaldehyde does not inhibit the activity of ATPases. The course of the reaction in tissues fixed with pure glutaraldehyde (1) was approximately the same as that found when a fixative containing 4% formaldehyde was used (3). The concentration of glutaraldehyde, however, was shown to have significant meaning for the fixation of ATPases in their active form, which is demonstrated by the reaction carried out according to variants 3 (1%GA + 4% formaldehyde) and 4 (0.5%GA + 4% formaldehyde).

In order to check the mechanism of changes in the activity of the studied enzymes, which are dependent on the metabolism of the cell, were before fixation part of the roots subjected to the action of respiratory inhibitors. Used were malonic acid which inhibits Krebs' cycle and sodium azide which inhibits the reactions of the respiratory chain. The roots were also treated with ATP. An increase in the activity of ATPases was expected to be found in the presence of compounds which lower the ATP level in the cell. As is held by some authors (Bednarski et al. 1977), mitochondrial ATP deficit can be a factor stimulating the activity of ATPases. Therefore, the presence of exogenous ATP should cause the opposite effect.

It can be seen from the analysis of the electronograms that, in the presence of malonic acid, a slight increase in the activity indicating the presence of active ATPases in the ER and plasma membrane took place (Fig. 9). The presence of NaN_3 also did not cause significant changes in comparison with the course of the enzymatic reaction in tissues not treated with inhibitors (Fig. 8). In the presence of exogenous ATP, there was only a trace of the reaction indicating the presence of active ATPases (Fig. 12) and this only in the ER.

In order to check the proper course of the enzymatic reaction, part of the roots from each variant were incubated in a medium not containing Pb ions or ATP as the substrate. The results of the control reactions were negative, manifested by a complete lack of reaction products in the form of lead phosphate precipitates or only trace amounts being found (Figs. 10, 11).

DISCUSSION

Studies on the nature and distribution of ATPases are often conducted in order to explain the mechanism of transport across membranes. One

Fig. 1-3. *Allium cepa*, 1% glutaraldehyde. The reaction indicates the presence of an active ATPase in the plasma membrane, mitochondria and ER. Approx. 18000 $\times$

PLATE II

Figs. 4, 5. *Allium cepa*, 1% glutaraldehyde+4% formaldehyde. The reaction indicates the presence of active ATPases in the plasma membrane and ER. Approx. 18000 $\times$

Figs. 6, 7. *Allium cepa*. 0.5% glutaraldehyde+4% formaldehyde. The reaction indicates the presence of active ATPases in the plasma membrane. Approx. 18000 $\times$

PLATE IV

Fig. 8. *Allium cepa*, 1% glutaraldehyde + 4% formaldehyde; sodium azide 100 ppm; the reaction indicates the presence of active ATPases in the plasma membrane, nuclear membrane and mitochondria. Approx. 22000 $\times$.

Fig. 9. *Allium cepa*, 1% glutaraldehyde + 4% formaldehyde; malonic acid 100 ppm. The reaction indicates the presence of active ATPases in the plasma membrane and ER. Approx. 14000 $\times$.

Figs. 10, 11. *Allium cepa*, 1% glutaraldehyde+4% formaldehyde. Control sample; traces of the enzymatic reaction in the plasma membrane. Fig. 10 — approx. 18000 $\times$, Fig. 11 — approx. 22000 $\times$
 Fig. 12. *Allium cepa*, 1% glutaraldehyde+4% formaldehyde, ATP 200 ppm. Trace reaction in the plasma membrane. Approx. 22000 $\times$

form of transport is the translocation of a compound with the aid of a specific carrier and energy. Such ATPases as transport ATPase stimulated by monovalent ions (K^+ , Na^+) and cytoplasmic ATPase dependent on bivalent ions (Ca^{+2} , Mg^{+2}) take part in this process. The transport of ions with the aid of these enzymes takes place as the result of reversible phosphorylation of the carrier by ATP. The phosphorylated and unphosphorylated forms of the carrier exhibit different ion-binding properties. The necessary energy for the active transport, due to which the high energy state of the membrane is generated can be supplied from the oxidation of metabolites in the respiratory chain, hydrolysis of ATP or an ion gradient.

The observations carried out on the meristematic cells of *Allium cepa* L. root apices showed that active ATPases are found mainly in the plasma membrane, and therefore are transport ATPases, whose presence has also been found in various types of plant and animal cells. In the opinion of some authors (Price and Whitecross 1983), this type of ATPase, responsible for the transport of ions (Na^+ , K^+) is sensitive to the presence of H^+ ions. A very high level of transport-ATPase in the plasma membrane has been observed in some types of plant cells (Price and Whitecross 1983). Winter-Sluiter et al. (1977) have shown that the transport ATPase occurring in the oat stem xylem parenchyma, exhibits high activity in the presence of K^+ ions. The ATPase localized in the plasma membrane of plant and animal cells exhibits high activity when formaldehyde fixatives are used (Tatsuhisa et al. 1980, Beauge et al. 1981). It also results from the experiments done in this study that the ATPase found in the plasma membrane is sensitive to K^+ , Ca^{+2} , Mg^{+2} ions which stimulate its activity.

Another point in the cell where large amounts of active ATPases are found are the mitochondria. The ATPases present there most likely belong to the group of phosphatases catalysing both the degradation and synthesis of ATP. The presence of ATPases in the mitochondrial matrix and internal membrane of the mitochondria was first determined by Wachstein and Meisel (1957). Winter-Sluiter et al. (1977) in studies on barley xylem parenchyma cells found the presence of an ATPase also in the outer mitochondrial membrane. Its activity was especially evident when the reaction was conducted in the presence of the stimulating ions, K^+ and Mg^{+2} . The authors suggest the similarity of the mitochondrial membrane ATPase and chloroplast membrane ATPase, however, the role of both of these enzymes has not yet been sufficiently explained (Winter-Sluiter et al. 1977).

In the meristematic cells under study here, the reaction indicating the presence of active ATPases was also observed in mitochondria. The intensity of the reaction, however, was smaller in comparison with that identifying

transport ATPase. This may result from the fact that the mitochondrial ATPase exhibits a greater sensitivity to fixatives, including GA.

Another type of active ATPase has been found in the endoplasmic reticulum. It has been shown that in animal cells these ATPases are responsible for the transport of ions, especially of Ca^{2+} ions, and exhibit greater activity in the presence of Mg^{2+} and Ca^{2+} .

Our observations on meristematic cells have shown the presence of ATPases in the ER. The reactions identifying the enzyme gave a positive result both in the presence of Ca^{2+} and Mg^{2+} ions. In plant cells, as found by Winter-Sluiter et al. (1977), in the presence of Ca^{2+} and Mg^{2+} ions, an increase in the activity of ATPases in the ER and plastid membranes and in the outer mitochondrial membrane is observed. Our experiments have shown that the activity of all types of ATPases, with the exception of transport ATPase found in the plasma membrane, does not undergo significant changes in the presence of K^{+} , Mg^{2+} , Ca^{2+} ions.

As can be seen from our results, active ATPases can be localized rather precisely using the method of Wachstein and Meisel which is based on the assumption that the phosphate ions released from ATP by enzymic hydrolysis, bind with Pb in the same location where the reaction had taken place. This is supported by the results of the control reactions in which the tissues were incubated in mediums not containing Pb or ATP as the substrate for the enzymatic reaction. Our results are in agreement with those of Hall et al. (1980) who demonstrated that in the case of lack of ATP in the reaction medium, no reaction is observed in membranes and only a very weak reaction was noticeable in the cytoplasm. However, when Pb ions were lacking, the reaction for ATPases did not appear at all (Hall et al. 1980). Our experiments have also shown that glutaraldehyde does not destroy the activity of ATPases. The optimal concentration of glutaraldehyde in the fixative (in the case when meristematic tissues are the material used) is 1%. Under the conditions of our experiments, glutaraldehyde at a lower concentration (0.5%) does not fix ATPases, at a higher concentration (5%), destroys their activity. The addition of formaldehyde as a component of the fixative has a favorable effect on the course of the enzymatic reaction.

Slight differences were noted in the activity of ATPases in tissues treated before fixation with NaN_3 and malonic acid solutions. An increase in the intensity of the reaction indicating the presence of active ATPases was seen most clearly in the ER. This result, it seems, suggests that in the state of an energy deficit, the ATPases localized in ER cisterns and responsible for ion transport, are activated. The activity of the transport ATPase in the plasma membrane did not undergo significant changes in

the presence of respiratory inhibitors. The nature of the enzymatic reaction in the presence of exogenous ATP confirms the conclusion that the increase in the activity of ATPases may be induced by a deficit of ATP in the cell.

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Przydatność metody strącania ołowiem do lokalizacji aktywnych ATPaz w komórkach merystematycznych korzeni Allium cepa L.

Streszczenie

Wykazano przydatność metody strącania ołowiem (Wachstein i Meisel 1957) do lokalizacji różnego typu ATPaz, stymulowanych przez jony K⁺, Mg²⁺, Ca²⁺ w merystematycznych komórkach korzeni *Allium cepa* L. W doświadczeniu zastosowano różne utrwalacze i mieszaniny inkubacyjne dla wyeliminowania ewentualnych artefaktów i zanalizowania zależności przebiegu reakcji enzymatycznej od sposobu utrwalenia tkanek, działania precipitujących jonów Pb i stymulujących jonów Ca²⁺, Mg²⁺ i K⁺ na aktywność ATPaz.