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Ionophorous Functions of Phosphatidic Acid in the Plant Cell

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Abstract—Effects of phosphatidic acid (PA), a product of phospholipase D activity, on Ca^{2+} and H^+ transport were investigated in membrane vesicles obtained from roots and coleoptiles of maize (*Zea mays* L.). Calcium flows were measured with fluorescent probes indo-1 and chlorotetracycline loaded into the vesicles and added to the incubation medium, respectively. Phosphatidic acid (50–500 μM) was found to induce downhill flow of Ca^{2+} along the concentration gradient into the plasma membrane vesicles and endomembrane vesicles (tonoplast and endoplasmic reticulum). Protonophorous functions of PA were probed with acridine orange. First, the ionic H^+ gradient was created on the tonoplast vesicles by means of H^+-ATPase activation with Mg–ATP addition. Then, the vesicles were treated with 25–100 μM PA, which induced the release of protons from tonoplast vesicles and dissipation of the proton gradient. Thus, PA could function as an ionophore and was able to transfer Ca^{2+} and H^+ across plant cell membranes along concentration gradients of these ions. The role of PA in mechanisms of intracellular signaling in plants is discussed.

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Key words: Zea mays - phosphatidic acid - membrane vesicles - fluorescent probes - membrane transport of Ca^{2+} and H^+- ionophores - signal transduction

INTRODUCTION

Phosphatidic acid (PA) accounts for 1–2% of the total amount of membrane phospholipids in plant cells and plays a key role in lipid metabolism, as it is involved in the biosynthesis of structural phospho- and glycolipids [1, 2]. Moreover, current visions consider PA as a lipid secondary messenger that transmits signals concerning wounding, water deficit, salinity, and oxidative stresses and is involved in polar growth and in osmotic volume changes of stomatal guard cells [3–7]. The PA level in targeted cells can be transiently elevated under the action of pathogens, elicitors, ABA, and ethylene [4, 8–11].

Nevertheless, the mechanisms of PA-mediated signal transduction in plant cells are poorly investigated to date. Phosphatidic acid is thought to promote binding of some enzymes to the plasma membrane, thereby elevating the activities of NADPH oxidase, MAPK-cascade kinases engaged in ethylene signaling, and of calcium-dependent protein kinases (CDPK) [12, 13]. Changes in the PA level affect physical properties of cell membranes and their capacity of vesicle formation. Thus, PA may affect vesicle trafficking associated with exo- and endocytosis [4].

There is evidence that PA is able to transport Ca^{2+} across muscle and nerve cell membranes [14–16]. Experiments with guard cells of plant stomata revealed that changes in phospholipase D (PLD) activity during transduction of ABA signal are accompanied by the increase in cytosolic Ca^{2+} level [17]. However, it remains to be established whether changes in PLD activity and the increase in the cytosolic Ca^{2+} concentration are sequential events in an integrated signaling pathway or whether they represent independent processes.

The goal of this study was to investigate ionophorous functions of PA in membrane vesicles using spectrofluorometric methods.

Abbreviations: CDPK—calcium-dependent protein kinase; CTC—chlorotetracycline; DTT—dithiothreitol; FCCP—carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; MAPK—mitogen-activated protein kinase; PA—phosphatidic acid; PIP_2—phosphatidylinositol 4,5-diphosphate; PLD—phospholipase D; PLC—phospholipase C.

MATERIALS AND METHODS

Experiments were performed with 4-day-old etiolated maize seedlings (*Zea mays* L., Nart 150 F1 hybrid). We used root and coleoptile segments measuring 20 and 10 mm, respectively, with apices removed.

The vesicle preparations of the plasmalemma and endomembranes were obtained by differential centrifugation and subsequent separation of the crude microsomal fraction in the PEG–dextran aqueous polymer two-phase system (for isolation of the plasmalemma) [18] and in the sucrose density gradient (for isolation of endomembrane fraction comprising the tonoplast and endoplasmic reticulum).

The plant tissue (6 g per treatment) was ground in a mortar at 5°C in 12 ml of medium containing 50 mM Tris–HCl buffer (pH 7.8), 0.25 M sucrose, 5 mM EDTA, and 15 mM ascorbic acid. The homogenate was filtered and centrifuged at 8000 *g* for 5 min and at 13000 *g* for 10 min. The crude microsomal fraction was obtained by centrifugation of the supernatant for 1 h at 95000 *g*.

In order to obtain the endomembrane fraction, the pellet was resuspended with a glass homogenizer in the medium containing 5 mM Tris–Mes buffer (pH 7.2), 0.25 M sucrose, and 1 mM dithiothreitol (DTT). The suspension was layered onto a discontinuous sucrose density gradient (solutions with densities of 1.05 and 1.12 g/cm^3 containing 1 mM DTT and 5 mM Tris–Mes buffer, pH 7.2) and centrifuged for 2 h at 95000 *g*. The endomembrane fraction, comprising the tonoplast vesicles and the vesicles of the endoplasmic reticulum, was collected from the interface of sucrose layers with a Pasteur pipette. The membrane fraction was diluted with 0.3 M sucrose solution containing 5 mM Tris–Mes buffer (pH 7.2) and 1 mM DTT, and then it was precipitated by centrifugation (95000 *g*, 1 h).

In order to isolate the fraction of plasma membranes, the pellet of microsomes was resuspended in 5 ml of medium containing 0.33 M sucrose, 3 mM KCl, and 5 mM KH_2PO_4–KOH buffer (pH 7.8). This mixture was added to the phase system containing dextran T500, PEG 3350, 5 mM KH_2PO_4–KOH (pH 7.8), 3 mM KCl, and 0.33 M sucrose. The final concentration of dextran and PEG equaled 6.2% after mixing all components. The separation of phases was accomplished by centrifugation at 1500 *g* for 5 min. The upper phase enriched with PEG contained the plasma membrane vesicles. This phase was separated and mixed with a fresh portion of the dextran phase. The purification of the plasma membrane fraction was repeated two times. After purification, the upper phase was mixed with three volumes of medium containing 0.3 M sucrose, 10 mM Tris–Mes buffer (pH 7.2), and 1 mM DTT. The plasma membranes were precipitated by centrifugation at 95000 *g* for 1 h.

The Ca^{2+} transport in the vesicle fractions was assayed by spectrofluorometry using a Ca^{2+}-sensitive probe indo-1 and $Ca^{2+}(Mg^{2+})$-sensitive probe chlorotetracycline (CTC).

Indo-1 probe was loaded into the inner space of vesicles by means of osmotic shock [19]. The pellets of the plasmalemma and endomembranes were resuspended in the medium of the following composition: 10 μM indo-1, 100 μM EGTA, 150 mM K_2SO_4, 150 mM sucrose, 2 mM Tris–Mes buffer (pH 8.0), and 1 mM DTT. Then the membranes were precipitated and resuspended in 200 μl of the same medium lacking the probe and EGTA. The intensity of indo-1 fluorescence (excitation at 334 nm and emission detected at 405 nm) was determined after adding membrane preparations (10–20 μg protein) to 1 ml of incubation medium containing 150 mM Na_2SO_4, 150 mM sucrose, 2 mM Tris–Mes buffer (pH 7.2), and 200 μM EGTA. Measurements were made with a Cary Eclipse fluorescence spectrophotometer (Australia).

The concentration of Ca^{2+} ions in the incubation medium was calculated using WinMaxc computer program [20].

The internal Ca^{2+} concentrations in vesicles ($[Ca^{2+}]_{in}$) were calculated from indo-1 fluorescence using the formula:

$$[Ca^{2+}]_{in} = K_d(F - F_{min})/(F_{max} - F),$$

where K_d is the dissociation constant for indo-1–Ca complex (230 nM), F_{min} and F_{max} are minimal and maximal fluorescence values obtained during calibration, and F is the fluorescence response obtained in the sample examined [21].

In experiments with CTC, this agent was added directly to the incubation medium (150 mM Na_2SO_4, 150 mM sucrose, and 2 mM Tris–Mes buffer, pH 7.2, 200 mM EGTA) to a final concentration of 70 μM. The CTC fluorescence was excited at 470 nm and the emission was measured at 521 nm by means of a spectrofluorometer based on Lyumam I-3 fluorescence microscope (LOMO, Russia).

The proton gradient at the membranes of tonoplast vesicles was created by means of H^+-ATPase activation. The elevation of inner H^+ concentration in vesicles was detected spectrophotometrically from the decrease in optical density of ΔpH probe acridine orange at 495 nm. The incubation medium with a volume of 800 μl contained 10 μM acridine orange, 5 mM Tris–Mes buffer (pH 7.2), 1 mM Na_2-ATP, 1 mM $MgSO_4$, 0.2 M sucrose, 1 mM DTT, 0.1 mM EDTA, 50 mM KCl, and vesicles in the amount equivalent to 10–30 μg of membrane protein. The reaction was initiated by the addition of ATP.

The protein content in membrane preparations was assayed according to Bradford [22].

The preparation of phosphatidic acid was dissolved in the chloroform–methanol mixture (2 : 1), and solvents were evaporated in the stream of nitrogen. Then, the sample was combined with bidistilled water and

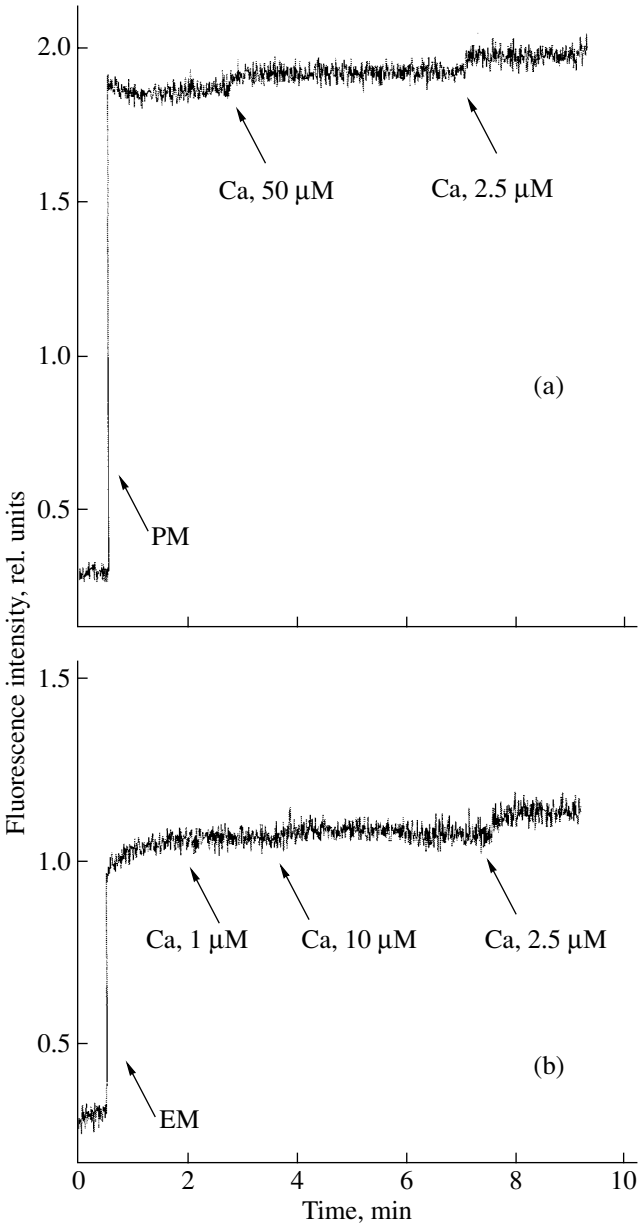

Fig. 1. Effect of Ca^{2+} added to the incubation medium on fluorescence of indo-1 loaded into (a) plasmalemma vesicles and (b) endomembrane vesicles from the roots of maize seedlings.

Arrows indicate the moments of adding Ca^{2+}, plasmalemma vesicles (PM), and endomembrane vesicles (EM).

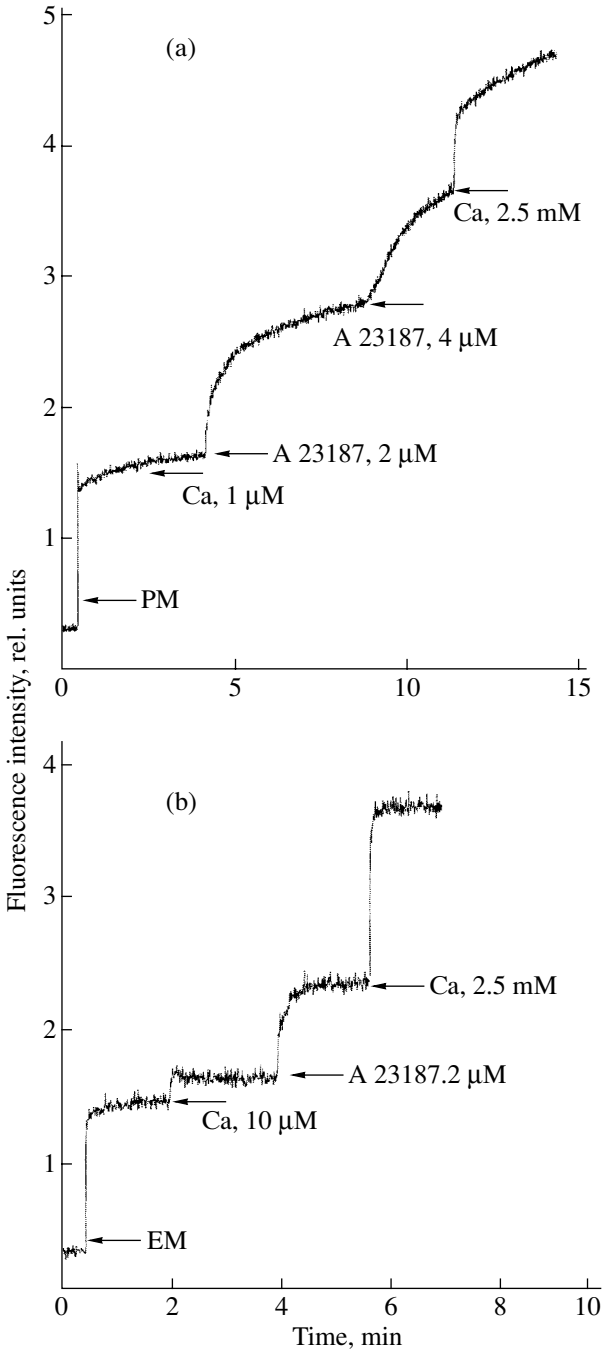

Fig. 2. Induction by ionophore A23187 of calcium permeability in isolated membranes from maize roots: (a) plasmalemma vesicles and (b) endomembrane vesicles loaded with a Ca-sensitive probe indo-1.

Designations are as in Fig. 1.

sonicated with an UZDN-2T sonicator (44 kHz) until the transparent emulsion was formed.

Ascorbic acid, EGTA, Mes, indo-1, sodium salt of phosphatidic acid, A23187, dextran T500, PEG 3350, and carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP) were obtained from Sigma (United States); Tris and DTT were from Reanal (Hungary); sucrose, CTC, EDTA, acridine orange, and other chemicals of the highest grade were produced in Russia.

At least five independent experiments were performed for each treatment. Figures 1–5 and 7 show representative changes of probe fluorescence. Figures 6 and 8 contain mean values. Only data significant at $P \geq 95\%$ are considered in the discussion.

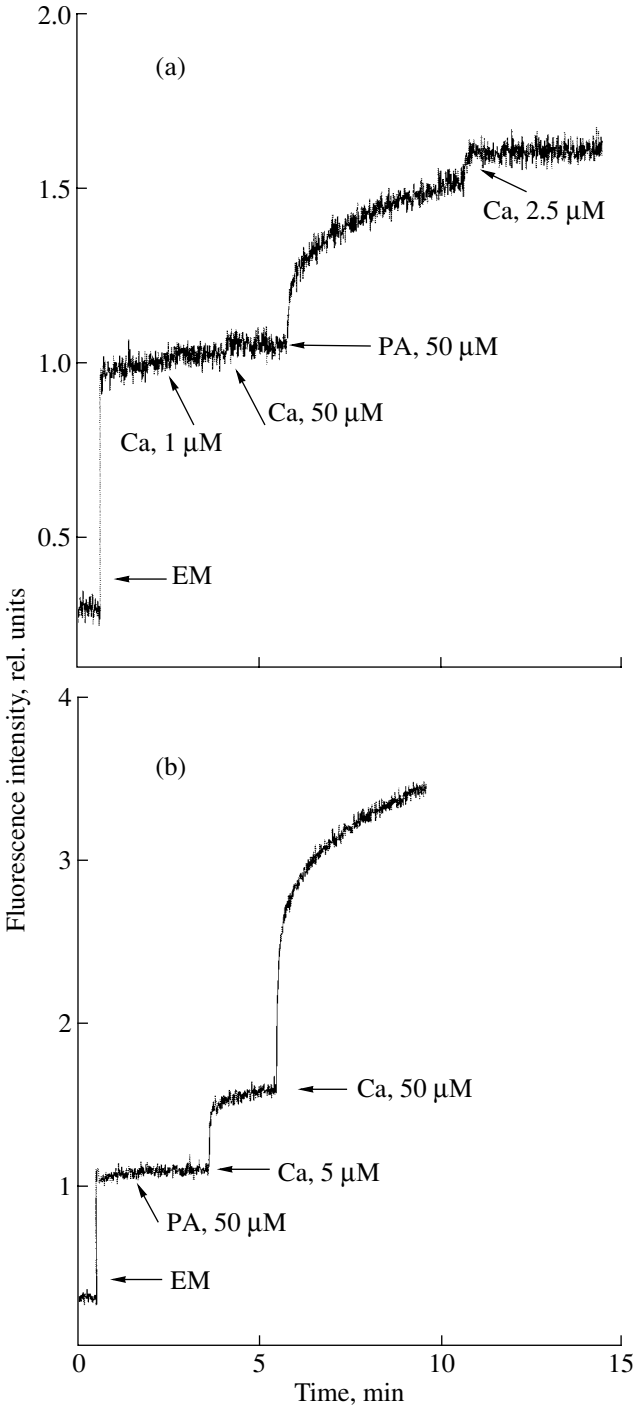

Fig. 3. Effect of phosphatidic acid (50 μM) on calcium permeability of endomembranes in maize root vesicles loaded with indo-1 probe.

(a) Endomembrane vesicles (EM) were treated with PA after the addition of Ca^{2+}; (b) endomembrane vesicles were first treated with PA and then Ca^{2+} was added. Other designations are as in Fig. 1.

RESULTS

On the first stage of our work, we assessed the integrity of plasma membrane and endomembrane vesicles isolated from maize seedlings; we also determined their permeability to Ca^{2+}. Figure 1 shows the effect of Ca^{2+} ions on fluorescence of indo-1 loaded into the vesicles. Changes in probe emission are indicative of changes in intravesicular Ca^{2+} concentration. It is seen that the addition of Ca^{2+} to the incubation medium had insignificant effect on probe fluorescence, which indicates low permeability of plasmalemma and endomembrane vesicles to Ca^{2+} ions (Figs. 1a, 1b). The increase in fluorescence was only observed after the addition of calcium ionophore A23187, which induced the Ca^{2+} flow into the vesicles (Fig. 2). Thus, we succeeded in obtaining plasmalemma and endomembrane vesicles featuring low passive permeability to calcium; this membrane integrity allowed us to measure inward Ca^{2+} flows driven by the concentration gradient.

In further experiments, we analyzed the capacity of PA to function as a calcium ionophore. It is seen from Figs. 3b and 4b that PA (50 μM) had no appreciable influence on fluorescence of indo-1 loaded into the plasmalemma and endomembrane vesicles in the absence of Ca^{2+} in the incubation medium. The addition of Ca^{2+} to the medium at a final concentration ranging from 5 to 100 μM resulted in the increase of indo-1 fluorescence, indicating that Ca^{2+} penetrated inside the plasma membrane and endomembrane vesicles. Similar results were obtained in experiments with plasmalemma vesicles isolated from coleoptiles of maize seedlings (Fig. 5). When the order of additions was reversed—calcium was added prior to the addition of PA—the increase in fluorescence of the probe loaded into vesicles was also observed (Figs. 3a, 4a).

The amplitude of the fluorescence response depended on the concentration of calcium and PA in the incubation mixture, as well as on the quantity of membranes added. Figure 6 shows the dependence of ionophorous effect of PA (0.5 mM) for Ca^{2+} on the concentration of Ca^{2+} in the medium. The ionophorous effect of PA was already observed upon the addition of 5 μM Ca^{2+}, and the maximum fluorescence responses were noticed in the presence of 500 μM Ca^{2+} in the incubation medium. The results suggest that PA initiates the permeation of Ca^{2+} into the plasmalemma and endomembrane vesicles along the concentration gradient; i.e., PA operates as a calcium ionophore.

It is well known that all phospholipids, including PA, can spontaneously aggregate and produce lipid micelles [23]. It is reasonable to assume that the induction of calcium permeability in our experiments was due to the incorporation of PA micelles into the membrane vesicles of plasmalemma and endomembranes.

The Ca^{2+} indicator CTC differs from other calcium probes in that it responds to calcium bound with cell organelles rather than to free calcium [16, 24]. The fluorescence of this divalent cation chelator significantly rises upon the increase in amount of membrane-associated calcium; i.e., upon the formation of tertiary complex Ca–CTC–membrane. Furthermore, the CTC fluo-

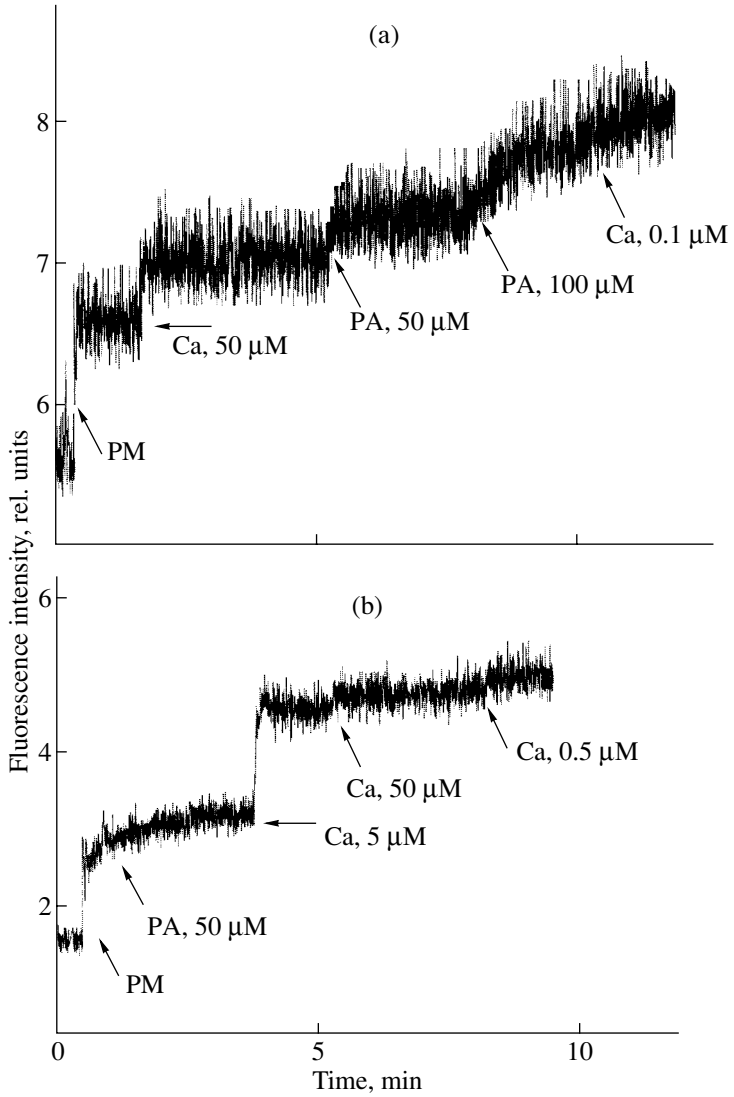

Fig. 4. Effect of PA on calcium permeability of plasma membranes in maize root vesicles loaded with a Ca^{2+} probe indo-1.

(a) Plasmalemma vesicles were treated with PA after the addition of Ca^{2+}; (b) plasmalemma vesicles were first treated with PA and then Ca^{2+} was added. Other designations are as in Fig. 1.

rescence provides a measure of lipid-bound calcium residing in equilibrium with aqueous phase rather than calcium bound to membrane proteins. Thus, changes in fluorescence of the Ca–CTC membrane complex reflect changes in the concentration of Ca^{2+} near the membrane. Therefore, fluorescence of the Ca–CTC complex provides the means for assessing Ca^{2+} fluxes across the membranes.

Figure 7 shows the result of an experiment in which the action of PA on calcium permeability of plasmalemma vesicles was assessed from CTC fluorescence. No appreciable changes in CTC fluorescence were noticed after adding PA without Ca^{2+}. The addition of PA and Ca^{2+} to the incubation medium containing CTC and plasmalemma vesicles resulted in the increase in the probe fluorescence. This phenomenon was probably due to the incorporation of PA micelles into the membrane vesicles, formation of the tertiary complex (Ca–CTC–PA), and the transport of Ca^{2+} into the plasmalemma vesicles. Thus, our data obtained with CTC fluorescent probe indicate the likelihood of direct involvement of PA in the Ca^{2+} transport across the plasmalemma in maize root cells.

In order to demonstrate possible participation of PA in the transmembrane transfer of H^+ ions, we used the pH gradient pregenerated by the membrane H^+-ATPase in tonoplast-containing vesicles from the coleoptiles of maize seedlings. Changes in the acidity of the inner space of vesicles were recorded with the use of acridine orange, a permeant monoamine. The optical density of this probe is known to decrease when the intravesicular pH is lowered [25]. First, the pH gradient was created

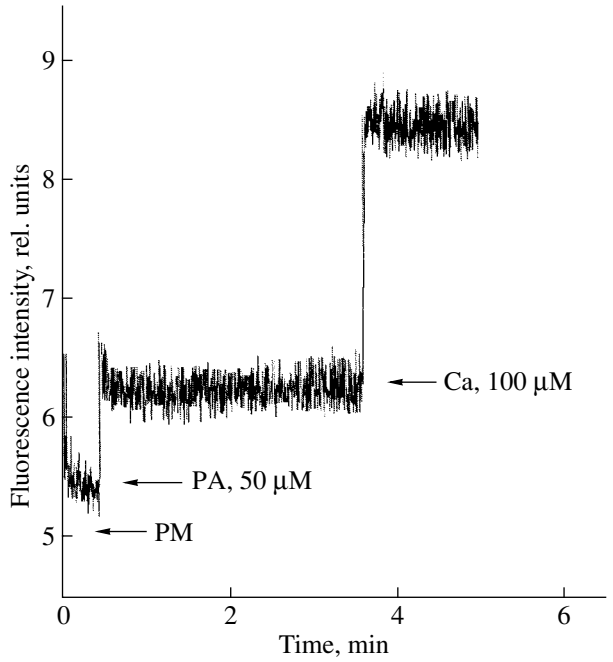

Fig. 5. Induction by PA of calcium permeability in plasma membrane preparations of maize coleoptile vesicles loaded with indo-1 probe.

The plasmalemma vesicles were first treated with PA and then Ca^{2+} was added. Designations are as in Fig. 1.

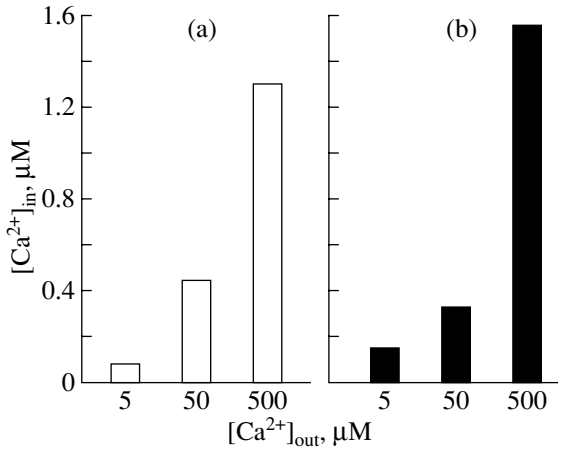

Fig. 6. Calcium influx induced by 0.5 mM PA in preparations of (a) plasmalemma vesicles and (b) endomembrane vesicles from maize roots as a function of Ca^{2+} concentration in the incubation medium ($[Ca^{2+}]_{out}$).

Concentration of Ca^{2+} was calculated from fluorescence of indo-1 probe loaded into vesicles [21].

at the tonoplast vesicles by means of H^+-ATPase reaction, and then the vesicles were treated with PA (Fig. 8). The addition of PA to vesicle preparations with preestablished pH gradient led to dissipation of this gradient. It is seen that the downhill transport of protons along the pH gradient was facilitated by PA as effectively as by FCCP, a well-known protonophore. Since the PA-induced H^+ efflux occurs concurrently with ATP-dependent H^+ uptake by the vesicles, the dynamics of H^+ movement should approach a balanced state; this was evident in experiments with the lowest PA concentration applied (25 μM). The results show that PA acts as a protonophore and may be directly involved in H^+ transport across the membranes.

DISCUSSION

Phosphatidic acid is one of the key metabolites of lipid metabolism. The main pathway of its biosynthesis consists in consecutive acylation of glycerophosphate and dihydroxyacetone phosphate [1, 2]. Phosphatidic acid is also produced by phospholipase D (PLD) during hydrolysis of membrane lipids, such as phosphatidylcholine and phosphatidylethanolamine; it also derives upon phosphorylation of diacylglyceride, one of the products of phospholipase C (PLC) [2, 4]. The PA molecules produced in different reactions are structurally distinct, because lipases utilize different lipids as the substrates.

The pathways reducing PA content in plant cells are related to PA phosphorylation and production of pyrophosphate diacylglyceride [3], as well as to dephosphorylation resulting in the formation of diacylglyceride. In addition, PA is an essential substrate for biosynthesis of phospholipids, and its level would depend on the rate of PA consumption in synthetic processes [1, 2].

Our experiments with plasmalemma and endomembrane vesicles from the roots and coleoptiles of maize seedlings provided evidence that PA can directly affect the Ca^{2+} concentration in the cytoplasm by promoting downhill Ca^{2+} movement into the cytoplasm from intracellular compartments and apoplast along the concentration gradient.

Short-term and prolonged modulations of the cytoplasmic Ca^{2+} concentration belong to main mechanisms of intracellular signaling, which are displayed under the action of various stimuli and are predominantly realized via activation of diverse Ca^{2+} channels in different locations [26].

The main intracellular targets for Ca^{2+} are different Ca^{2+}-binding proteins. Some of these proteins support ion transport; others serve as a kind of a buffer ensuring a low calcium level in the cytosol. The binding of Ca^{2+} to such proteins does not cause significant changes in their structure. However, when Ca^{2+} interacts with proteins performing regulatory functions, the formation of Ca–protein complex is followed by major structural rearrangement in the protein molecule, and this molecule acquires the capacity of transmitting the signal further on. These so-called sensor proteins are engaged in decoding the calcium signal, its transmission to operational mechanisms, and initiation of Ca^{2+}-dependent physiological processes [27]. These proteins include

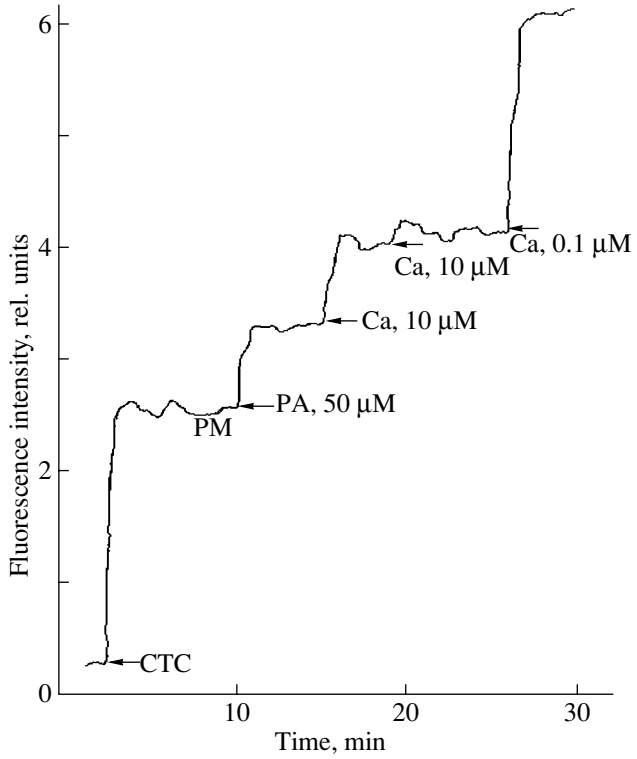

Fig. 7. Induction by PA of calcium permeability in plasmalemma vesicles from maize roots, visualized from CTC fluorescence.

CDPK, calmodulin, annexins, calreticulin, calnexin, as well as Ca-dependent forms of PLD and PLC [5].

Plants possess several PLD isoforms (PLDα, PLDβ, PLDγ, PLDδ, and PLDε) that differ in biochemical properties, substrate specificity, and physiological functions [8, 28, 29]. All PLD can be divided in two groups. The first group is characterized by the existence of Ca-binding C2-domain and includes PLDα, PLDβ, PLDγ, and PLDδ. The activation of these phospholipases depends on the presence of Ca^{2+}. For example, PLDα is activated by millimolar concentrations of Ca^{2+} and is insensitive to phosphatidyl 4,5-bisphosphate (PIP_2), whereas the activities of PLDβ and PLDγ depend on PIP_2 and are stimulated by micromolar concentrations of Ca^{2+}. The PLDδ isoform is activated by unsaturated fatty acids, such as oleic, linoleic, and linolenic acids. The isoforms PLDβ, PLDδ, and PLDγ utilize phosphatidylethanolamine as a substrate and, to lesser extent, phosphatidylcholine. The structure of PLDε contains no C2-domain; it utilizes phosphatidylcholine as the only substrate, and the activation of this isoform requires the presence of PIP_2 [3, 6, 8]. Phosphatidic acid produced by PLD is capable of binding Ca^{2+} ions. Physiological significance of this property can be considered in two aspects. On the one hand, PA elevates the membrane permeability to Ca^{2+} and, on the other hand, changes in the content of Ca^{2+} bound to membrane lipids would affect properties of membrane proteins [4].

The transport functions of PA in the membrane are determined by lipophilic properties of PA and by the presence of phosphate group with two hydroxyl residues ($pK_1 = 3.5$, $pK_2 = 9.0$) [30]. The dissociation of PA hydroxyl groups and, consequently, the capacity of PA to bind Ca^{2+} or proton should be pH-dependent. In alkaline media, both groups are dissociated, which produces two negative charges per each residue of phosphoric acid. In this case, PA would bind Ca^{2+} ions and transfer them across the membrane along the concen-

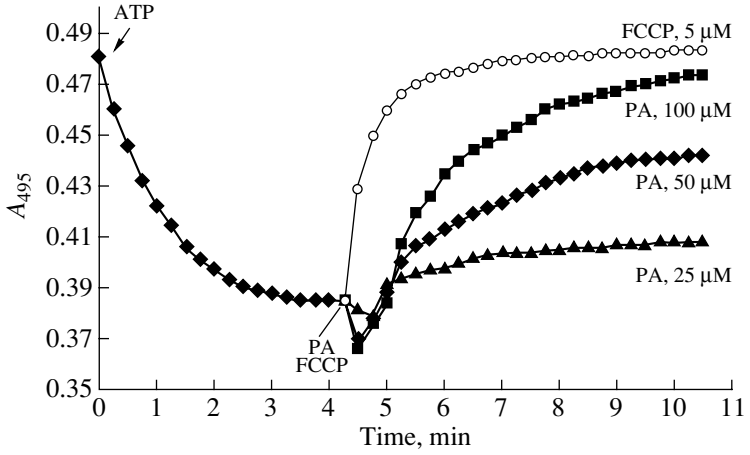

Fig. 8. Protonophorous effect of PA, visualized with acridine orange in tonoplast vesicles from maize coleoptiles.
The first arrow marks the ATP addition into incubation medium, which generated the proton gradient at the vesicle membrane through H^+-ATPase activity of the vacuolar membrane. Other arrows indicate the moments of eliminating the pH gradient at the tonoplast vesicles by adding FCCP or PA.

tration gradient. In the acidic media, PA would predominantly transfer protons.

If PA performs the function of calcium ionophore, it may participate in the calcium signaling system by initiating the Ca^{2+} transport along the concentration gradient into the cytoplasm. This would result in activation of Ca^{2+}-dependent processes. Functioning of PA as a protonophore may have consequences of two types. First, it may induce acid–base shifts in the cell compartments and cause, respectively, pH-dependent changes in the activity of enzymatic systems. Second, by shunting the electrochemical proton gradient at the energy-coupling membranes of mitochondria and chloroplasts, PA probably influences the ATP synthesis and affects the cell bioenergetics. Thus, owing to ionophorous properties, PA is apparently involved in the systems of calcium and proton signaling, as well as in plant cell bioenergetics.

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