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Identification of Phospholipase C Activated by GTPγS in Plasma Membrane of Oat Cell

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Abstract: In order to investigate whether phospholipase C (PLC) activity in oat cells is regulated by G-protein, we have characterized PLC in plasma membranes of oat tissues. To identify the purified plasma membrane, K^+-stimulated, Mg^{2+}-dependent ATPase activity was measured. The activity of ATPase was shown to be proportional to the concentration of membrane protein. To examine the PLC activity regulated by G-protein, we used the inside-out and outside-out plasma membrane mixture isolated from the oat cells. The plasma membrane mixture showed higher PLC activity than the one of the outside-out plasma mem brane. This suggests that PLC activity is located at the cytoplasmic surface of plasma membrane. PLC activity in plasma membrane mixture was dependent on Ca^{2+} with maximum activity at 100 μM Ca^{2+} and it was inhibited by 1 mM EGTA. Using Sep-pak Accell™ Plus QMA chromatography, we found that inositol 1,4,5-trisphosphate (IP_3) was produced in the presence of 10 μM Ca^{2+}. The PLC activity in the membrane was enhanced by an activator of G-protein (GTPγS) and not by an inhibitor (GDPβS). This indicates that a G-protein is involved in the activation of PLC in the plasma membrane of oat cells.

Key words: G-protein, oat cell, phospholipase C, plasma membrane mixture.

Signal transduction via metabolism of inositol phospholipids has well been understood in animal cells (Berridge, 1987). Phospholipase C is a key enzyme, which hydrolyzes inositol phospholipids to their corresponding inositol phosphates and diacylglycerol. IP_3 from PIP_2 hydrolysis by phospholipase C activity acts as a second messenger to stimulate calcium release from intracellular calcium pool and DAG is a well defined activator of protein kinase C (Nishizuka, 1992).

The activity of phospholipase C is regulated by a variety of hormones through G-protein (GTP-binding protein) pathways. Recently, G-protein (G_q) stimulated phospholipase C has been identified and purified by Smrcka *et al.* (1991). In plants, only a few reports describing transmembrane signaling via polyphosphoinositide metabolism have been published. Unlike phospholipase C in animal cells, the one of plant cells preferentially catalyzes hydrolysis of PIP and PIP_2. Previousely, we reported that PIP_2 breakdown was induced by red light but not by far-red light in oat cells (Chae *et al.*, 1992). This induction was controlled by red/far-red reversibility of phytochrome action. Drobak *et al.* (1985) reported that IP_3 stimulated Ca^{2+} release from its intracellular store in plant cells. These suggest that polyphosphoinositide metabolism is also involved in signal transduction in plant cells.

The presence of G-protein has also been identified in several plants (Hasunuma *et al.*, 1989; Miller, 1987; Romero *et al.*, 1991). Romero *et al.* (1991) described the relationship between phytochrome and G-protein in the light signal transduction. Although the existence of G-protein in plant cells has been suggested (Romero *et al.*, 1993), the roles of G-protein are largely unknown.

In this paper, we report the presence of phospholipase C in the plasma membrane of oat cells and the possibility that phospholipase C activity is regulated by G-protein.

Materials and Methods

Chemicals

Dextran T-500 for preparation of plasma membrane was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden) and PEG 3350 from Sigma (St. Louis, USA). Phosphatidyl [2-^{3}H] inositol 4,5-bisphosphate was purchased from Amersham (Amersham, UK) and phosphatidylinositol 4,5-bisphosphate from Sigma (St. Louis, USA). All other chemicals were standard enzyme grade.

Plant materials

Fifty grams of oat (*Avena sativa*, L. cv Garry) seed

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were soaked at 4℃ for 24 h in constant darkness. The seed was spread on wet vermiculite (100 g/tray) in aluminium trays (35×45 cm), and the trays were put in an oat-grown box made of wood. The oats were grown at 25℃ for 7 days in complete darkness.

Isolation of oat plasma membranes

Shoots of oat grown for 7 days were removed for isolation of plasma membranes. All procedures were performed at 4℃. Oat shoots were homogenized with a Blender (GoldStar, Korea) in homogenization buffer (10 mM Tris, pH 7.5, 330 mM sucrose, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin, and 70 mM 2-mercaptoethanol). The homogenate was filtered through nylon cloth (100 μm) and centrifuged at 10,000×*g* for 15 min. The resulting supernatant was centrifuged at 33,500×*g* for 90 min. The pellet (crude microsomal fraction) was resuspended in two phase buffer (5 mM K-phosphate buffer, pH 7.8, 330 mM sucrose, and 4 mM KCl) and the suspension was applied to a two-phase system with 6.5% (w/w) dextran T-500 and 6.5% (w/w) PEG 3350 in two phase buffer. Purification of plasma membrane from the crude microsomal fraction was performed by the method of Widell *et al.* (1982). The purified plasma membrane was resuspended in inside-out buffer (5 mM K-phosphate, pH 7.8, 330 mM sucrose, 50 mM KCl, 0.1 mM EDTA, and 1 mM DTT) for preparation of the outside-out and inside-out plasma membrane mixture.

Preparation of the outside-out and inside-out plasma membrane mixture

The mixture was prepared as described by Palmgren *et al.* (1990). After four cycles of freeze (−195℃)/thaw (25℃), the upper phase diluted with sample buffers was centrifuged at 100,000×*g* for 60 min. The pellet was resuspended in buffer for assay of K^+-stimulated, Mg^{2+}-dependent ATPase or phospholipase C activity.

K^+-stimulated, Mg^{2+}-dependent ATPase activity

ATPase activity was determined by the method of Perlin *et al.* (1981). The reaction mixture contained 50 mM Tris-Mes, pH 6.5, 330 mM sucrose, 1 mM NaN_3, 3 mM ATP, 0.1 mM EDTA, and 0.1 mM sodium molybdate and was incubated at 25℃ for 30 min. Colored products were detected by spectrophotometer (Beckman DU-70) at 660 nm.

Phospholipase C activity

Phospholipase C activity was determined as described by Tate *et al.* (1989). The reaction mixture (final volume of 100 μl) contained 30 μg membrane protein, 50 mM Trizma-maleate, pH 6.0, 100~200 μM PIP_2 (10,000~20,000 cpm). Reaction was started by addition of the substrate. After incubation at 37℃ for 20 min, reaction was stopped by adding 100 μl of 3.4 N HCl with 0.5% $CaCl_2$ and 750 μl of $CHCl_3$/MeOH (2 : 1, v/v). After centrifugation at 12,000×*g* for 30 sec, the upper phase was removed and counted by liquid scintillation counter (Beckman LS 5000TA). The upper phase was also applied to a Sep-Pak Accell™ Plus QMA cartridge for analysis of the reaction products.

Analysis of aqueous products by Sep-Pak Accell™ Plus QMA (Waters) chromatography

The aqueous (upper) phase was removed and dried under a N_2 (gas) stream. After resuspension in deionized water, the suspension was applied to a washed cartridge. The cartridges were washed with 4 ml of 0.02 M TEAB (Triethylammonium bicarbonate). IP_1, IP_2, and IP_3 were sequentially eluted with 4 ml of 0.1, 0.3, and 0.4 M TEAB, respectively. IP4 was finally eluted with 6 ml of 0.5 M TEAB. Radioactivity of eluates was measured by liquid scintillation counter.

Membrane protein determination

Membrane protein was determined by a modification of the method of Maxwell *et al.* (1978). Solution A (10 ml of 2 % Na_2CO_3 in 0.1 N NaOH+100 μl of 2% sodium potassium tartrate+100 μl of 1% $CuSO_4$) and SDS (final concentration of 1%) were sequentially added to the membrane sample. After mixing, 100 μl of 1 : 2 diluted phenol reagent was added and incubated at room temperature for 30 min. Optical density was measured at 660 nm.

Results and Discussion

In order to investigate whether or not phospholipase C is regulated by G-protein (GTP-binding protein) in plant cell, we first isolated plasma membrane from oat shoots by partition of microsomal fractions in a dextran-PEG two-phase system. This plasma membrane fraction consisted of outside-out vesicles. When we try to use these vesicles to determine the total activity of an enzyme whose active site is located on the cytoplasmic surface, a detergent is usually required. We have prepared an outside-out and inside-out plasma membrane mixture without using detergents. Using this mixture, the ATPase activity and the phospholipase C activity were determined.

As a marker for the plasma membrane mixture we used K^+-stimulated, Mg^{2+}-dependent ATPase. Fig. 1A shows ATPase activity as a function of membrane protein concentration. This activity was higher than that of plain outside-out plasma membrane (data not shown).

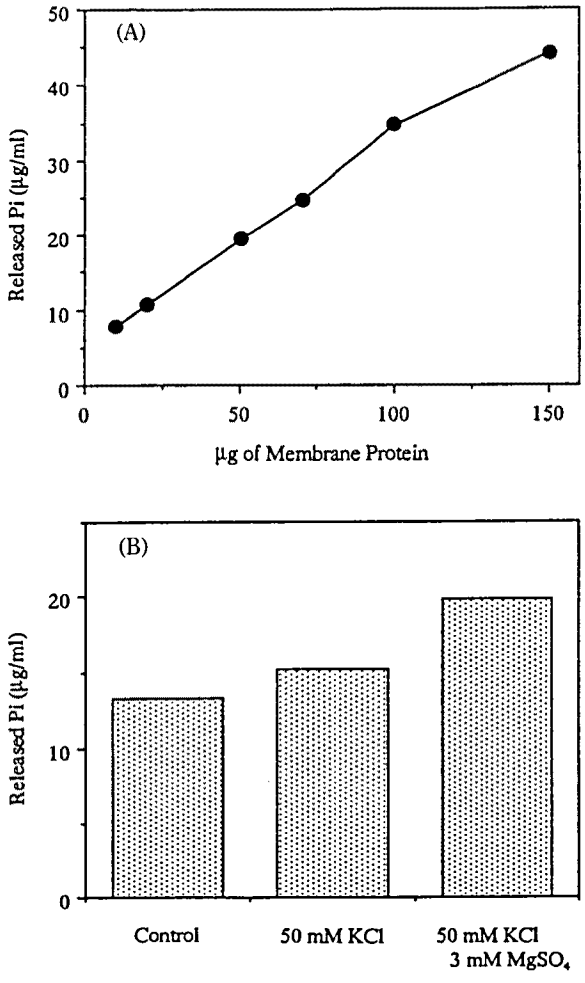


Fig. 1. (A) K^+-stimulated, Mg^{2+}-dependent ATPase Activity as a Function of Membrane Protein Concentration. ATPase activity was determined in a reaction mixture containing 50 mM KCl and 3 mM $MgSO_4$. The reaction mixture also contained 50 mM Tris-Mes (pH 6.5), 330 mM sucrose, 1 mM NaN_3, 3 mM ATP, 0.1 mM EDTA, 0.1 mM sodium molybdate, and a varying amount of membrane proteins. The ATPase activity was calculated from absorbance at 660 nm. (B) Effects of K^+ and K^+/Mg^{2+} on K^+-stimulated, Mg^{2+}-dependent ATPase Activity. ATPase activity was determined in a reaction mixture in the presence or absence of K^+ and K^+/Mg^{2+}.

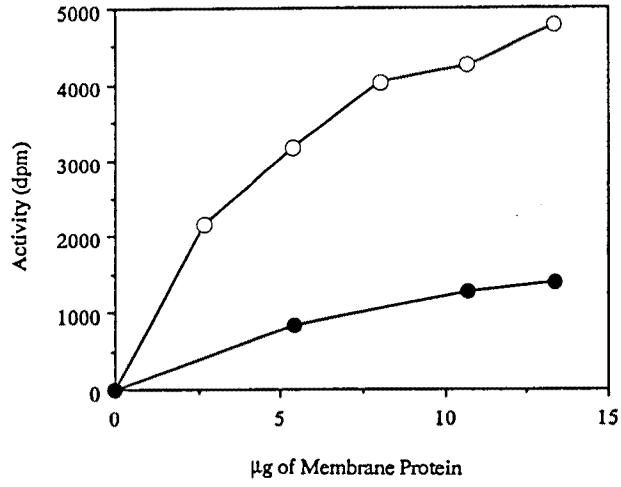


Fig. 2. Phospholipase C Activity as a Function of Membrane Protein Concentration. Phospholipase C activity was determined in a reaction mixture containing 10 μM of free calcium using Ca^{2+}-EGTA buffer. The reaction mixture also contained 50 mM Trizma-Maleate (pH 6.0), 200 M PIP_2 (10,000~20,000 cpm) and a varying amount of membrane proteins. ●–●: outside-out plasma membrane; ○–○: outside-out & inside-out plasma membrane mixture.

The activity increased linearly until the membrane protein concentration reached 150 μg in the reaction mixture. ATPase activity was dependent on K^+ and/or Mg^{2+} and the highest activity was shown in the presence of both ions (Fig. 1B). The ATPase obtained from oat shoots showed characteristics similar to that of various other plant tissues (Perlin *et al.*, 1981; Dupont *et al.*, 1981; Lurie *et al.*, 1979).

Since the GTP-binding site of G-protein in plasma membrane is located on the cytoplasmic surface, we prepared a plasma membrane mixture to test the effect of GTPγS and GDPβS on phospholipase C activity. First, we compared phospholipase C activity in outside-out plasma membrane with that of the plasma membrane mixture in the absence of detergent. Fig. 2 shows that phospholipase C activity changes as a function of the membrane protein concentration. The phospholipase C activity of the plasma membrane mixture is higher than that of the outside-out plasma membrane at the same concentration of membrane protein. An observation of higher activity in the plasma membrane mixture suggests that the active site of phospholipase C hydrolyzing PIP_2 is located on the inner cytoplasmic surface of the plasma membrane. Pical *et al.* (1992) obtained results similar to our result from the plasma membrane of wheat. The localization of polyphosphoinositide phospholipase C activity on the cytoplasmic surface of the plasma membrane has always been logically assumed, but not yet demonstrated in plant plasma membrane.

The phospholipase C activity in plasma membrane from photosynthetic cells showed a preference for PIP and PIP_2 over PI in contrast to the cytosolic fraction, in which the preference was for PI (Kregg *et al.*, 1990). In the present study, we used PIP_2 as an exogenous substrate of phospholipase C. When phospholipase C activity was measured as a function of PIP_2 concentration in the plasma membrane mixture, the activity increased until the concentration of PIP_2 reached 300 μM (Fig. 3).

In a previous report (Melin *et al.*, 1987), the optimal activity of phospholipase C in plasma membrane occurred at the micromolar level of Ca^{2+}, but in the cyto-

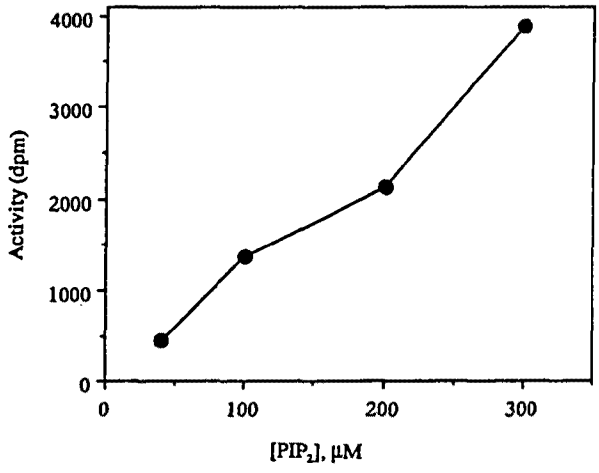
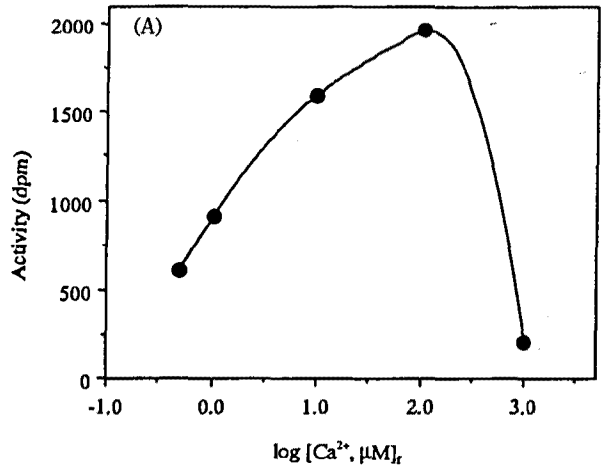


Fig. 3. Phospholipase C Activity as a Function of Substrate Concentration. The assay for phospholipase C activity was performed under the conditions as described in Fig. 2, except for using of a diverse amount of substrate.



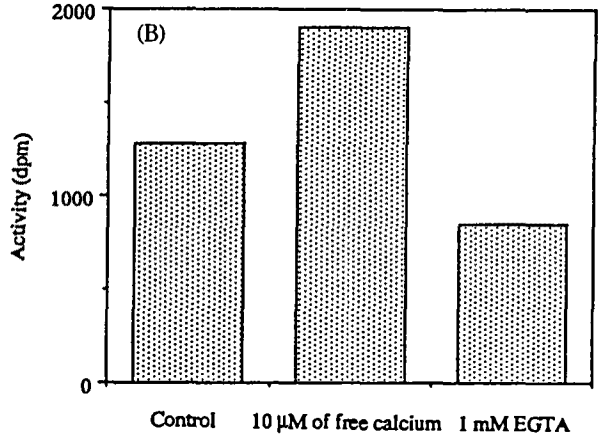


Fig. 4. (A) Calcium Dependency of Phospholipase C Activity. The assay for phospholipase C activity was performed as described in Fig. 2, using Ca^{2+}-EGTA buffer at free calcium values of 0.45 to 1000 μM. (B) Effects of Calcium and EGTA on Phospholipase C Activity. The assay for phospholipase C activity was performed as described in Fig. 2, in the presence of 10 μM of free calcium using Ca^{2+}-EGTA buffer and 1 mM EGTA.

solic fraction at the millimolar level. The dependency of phospholipase C activity on calcium concentration in oat plasma membrane mixture is shown in Fig. 4A: the maximal activity is obtaind at 100 μM Ca^{2+}. At millimolar concentrations of free calcium, phospholipase C activity was nearly completely inhibited. Addition of 10 μM free calcium increased the activity of the phospholipase C in hydrolyzing PIP_2, whereas addition of 1 mM EGTA caused a decrease in activity (Fig. 4B). In a manner similar to the phospholipase C from animals, the enzyme from the oat shoot plasma membrane mixture was dependent on Ca^{2+}. While Melin *et al.* (1987) found a markedly elevated phospholipase C activity in the presence of micromolar calcium from wheat seedlings, Pfaffmann *et al.* (1987) and McMurray *et al.* (1988) reported enzymes which require millimolar concentration of calcium. Tate *et al.* (1989) explained that the requirement for high levels of calcium observed by the latter two investigators might result from the presence of deoxycholate in their assay system or more likely, from contamination of their membrane fractions by a soluble phospholipase C specific for PI, which requires high calcium levels for activity.

To certify IP_3 formation from PIP_2 by phospholipase C, we performed Sep-Pak Accell™ Plus QMA chromatography. Fig. 5 shows the elution profile of aqueous products from the cartridges. Using [^{3}H]PIP_2 as an exogenous substrate, the product was eluted as a single peak of radioactivity. Total radioactivity of aqueous products was consistent with that of IP_3 formation. Thus, total radioactivity of aqueous products was measured in most cases.

Fig. 6 shows that phospholipase C activity in the membrane mixture is drastically enhanced by GTPγS, but not by GDPβS. GTPγS is a nonhydrolyzable analogue of GTP which can activate G-protein and GDPβS is known as an inhibitor of the binding of GTP to G-protein. When these effects were checked in the outside-out membrane vesicles, no effects by GTPγS and GDPβS were observed (data not shown). Since these two compounds are membrane impermeable, the membrane mixture containing inside-out membrane must be used. The best way to see the interaction between G-protein and its activator/inhibitor is to use the inside-out membrane. But the difficulty of obtaining the inside-out membrane led us to use the membrane mixture, which could be easily prepared.

Tate (1989) and Melin (1992) could not detect activity changes of PLC by GTP analogues in the isolated plasma membranes of either oat or wheat root cells. Tate (1989) explained the reasons as follows: phos-

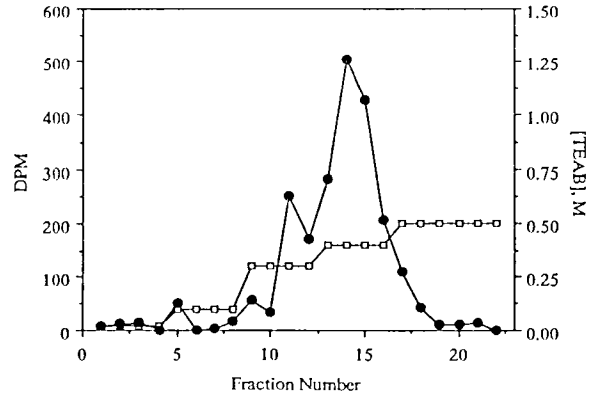


Fig. 5. Elution Profiles of Aqueous Products of Phospholipase C from Sep-Pak Accell™ Plus QMA Cartridges. Sep-Pak Accell™ Plus QMA chromatography was performed as described in "Materials and Methods". Before first use, the Sep-Pak cartridges was washed with 10 ml of deionized distilled water, 10 ml of 0.1 M TEAB (pH 8.4), and finally 10 ml of water again. The water-soluble products generated by phospholipase C were dissolved in 4 ml of deionized distilled water and then applied to the washed cartridge. The loaded cartridges was washed with 4 ml 0.02 M TEAB to elute free inositol. Inositol mono, bis and trisphosphates were then sequentially eluted by adding 4 ml each 0.1 M, 0.3 M and 0.4 M TEAB. Lastly, Ins 1,3,4,5-tetrakisphosphate was eluted by 6 ml of 0.5 M TEAB. Eluent was collected in 1 ml fractions and then radioactivity of a 200 μl aliquots from each fraction was measured by Liquid Scintillation Counter (Beckman LS 5000TA). The assay for phospholipase C activity was performed as described in Fig. 2, in the presence of 10 μM of free calcium using Ca^{2+}-EGTA buffer. ●—●: phospholipase C activity; □—□: [TEAB].

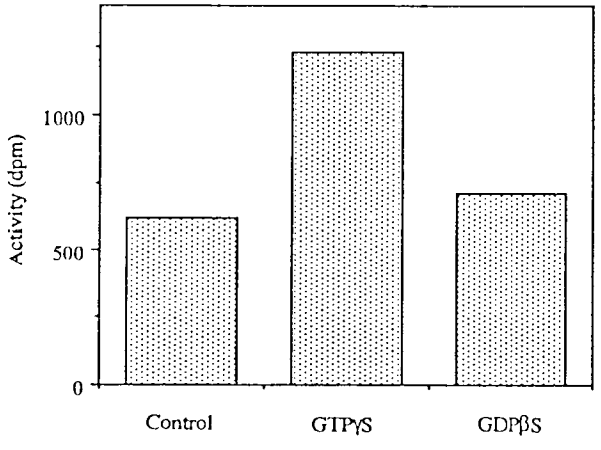


Fig. 6. Effects of Guanine Nucleotides on Phospholipase C Activity. Phospholipase C activity was determined in a reaction mixture containing 10 μM $MgCl_2$, 0.1 μM free calcium and 10^{-7} M GTPγS or GDPβS.

pholipase C was not stimulated by GTP analogues because of the loss of G-protein during membrane preparation or the sidedness of plasma membranes isolated by the two-phase polymer system.

In this paper we first report that phospholipase C in the plant system is also activated by G-protein. But further studies to understand whether phytochrome signal is connected with the G-protein-phospholipase C pathway or not, should be carried out soon.

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