Plant Physiol. (1991) 96, 340–343
0032-0889/91/96/0340/04/$01.00/0

Received for publication November 26, 1990
Accepted February 15, 1991

Communication

Neomycin Inhibits the Phosphatidylinositol Monophosphate and Phosphatidylinositol Bisphosphate Stimulation of Plasma Membrane ATPase Activity[1]

Qiuyun Chen[2] and Wendy F. Boss*

Department of Botany, North Carolina State University, Raleigh, North Carolina 27695-7612

ABSTRACT

The inositol phospholipids, phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP_2), have been shown to increase the vanadate-sensitive ATPase activity of plant plasma membranes (AR Memon, Q Chen, WF Boss [1989] Biochem Biophys Res Commun 162: 1295–1301). In this paper, we show the effect of various concentrations of phosphatidyinositol, PIP, and PIP_2 on the plasma membrane vanadate-sensitive ATPase activity. PIP and PIP_2 at concentrations of 10 nanomoles per 30 microgram membrane protein per milliliter of reaction mixture caused a twofold and 1.8-fold increase in the ATPase activity, respectively. The effect of these negatively charged phospholipids on the ATPase activity was inhibited by adding the positively charged aminoglycoside, neomycin. Neomycin did not affect the endogenous plasma membrane ATPase activity in the absence of exogenous lipids.

The plasma membrane H^+-ATPase is involved in the regulation of plant growth and development via controlling nutrient transport and the chemical potential of plant cells (for review see ref. 23). Physiological studies have shown that ATPase activity is modulated by light, plant growth regulators, phytotoxins, and mechanical or thermal shock (for review see ref. 20).

Plasma membrane ATPase activity is lipid dependent. Delipidation of the ATPase reduces the ATPase activity, and addition of lipids recovers the inhibition and increases the ATPase specific activity (6, 21). While lipids are needed to maintain membrane bilayer structure, changes in membrane lipid composition also can affect enzyme conformation and aggregation state in the lipid bilayer. For example, changes in the relative distribution of individual membrane lipids have been shown to affect the Na^+/K^+-ATPase activity (14). Addition of LPA,[3] LPC, fatty acids, or inositol phospholipids including PI, PIP, and PIP_2 to isolated plasma membrane vesicles results in an increase in the ATPase activity of yeast and higher plants (11, 13, 15–17).

Inositol phospholipids are of special interest because the enzymes that synthesize PIP and PIP_2 (the inositol phospholipid kinases) and the vanadate-sensitive ATPase activity responded rapidly (within 10 s) to stimuli (light, osmotic stress, and cell wall degrading enzymes) in two different plant systems (1, 2, 12), and there was a positive correlation in the response of both enzymes. These data suggested that an increase in the inositol phospholipids might result in an increase in the ATPase activity.

Neomycin, a positively charged aminoglycoside, can form electroneutral complexes with PIP and PIP_2 (4, 9, 18) and thereby block the binding of PIP and PIP_2 to the solubilized enzyme (8). If the effect of the inositol phospholipids is caused by interaction of the negatively charged, inositol phosphate head groups with the ATPase, then adding neomycin should decrease the effect of the lipids. In this paper we show that the ATPase activity of isolated plasma membrane vesicles is affected by exogenously added PIP and PIP_2 in a neomycin-sensitive manner.

MATERIALS AND METHODS

Plant Material

Wild carrot (*Daucus carota*) cells grown in suspension culture were transferred weekly as previously described (1). The cells were used on the fourth day after transfer.

Chemicals

PIP was purchased from Boehringer Mannheim Biochemicals, PA, PC, PI, and PIP_2 and neomycin sulfate were from Sigma, Triton X-100 from Pierce, and Driselase from Plenum Scientific Research, Inc. Vanadium oxide (gold label) was purchased from Aldrich Chemical Co., Inc., and a 5 mM stock solution was prepared according to the method of Gallagher and Leonard (5).

Cell Treatment and Plasma Membrane Isolation

The cells were collected on filter paper (Whatman No. 1), rinsed with conditioned medium, resuspended in 5 mL of

[1] This research was supported by grant number DE-FG05-87ER13693 from the U.S. Department of Energy and in part by the North Carolina Agricultural Research Service.

[2] Present address: Boyce Thompson Institute, Tower Road, Cornell University, Ithaca, NY 14853.

[3] Abbreviations: LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate.

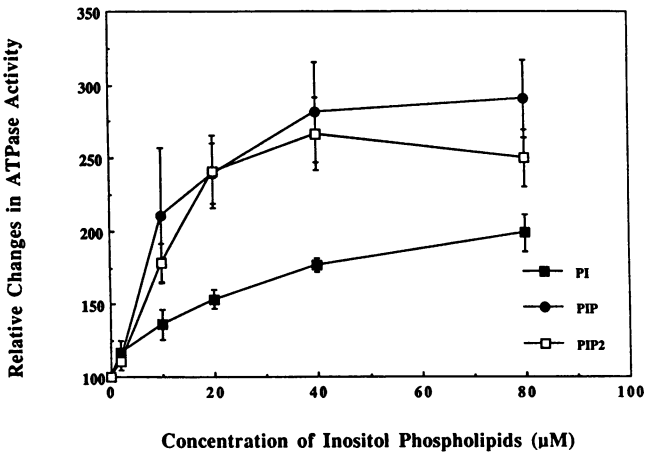

Figure 1. Effects of inositol phospholipids on the vanadate-sensitive ATPase of carrot plasma membranes. The plasma membranes (7.5 μg membrane protein for 250 μL reaction solution) were preincubated with different amounts of phospholipids for 15 min at 150 rpm and the ATPase assay was performed as described in "Materials and Methods." Data are presented as relative changes in the ATPase activity of lipid-treated plasma membranes compared to that in the control membrane in the absence of lipid ± SD of six values from three different experiments. The ATPase activity of the control was 164.37 ± 31.7 nmol·mg^{-1} protein·min^{-1}.

conditioned medium in a 25 mL Erlenmeyer flask, and placed on a shaker for 10 min at 110 rpm at a concentration of 0.1 g fresh weight/mL. For sorbitol and Driselase treatments, the cells were treated as described by Chen and Boss (1). The plasma membranes were isolated by aqueous two-phase partitioning as previously described (25). Protein was determined by the method of Lowry with BSA as a standard (10).

ATPase Assay

Vanadate-sensitive ATPase was assayed by adding 20 μL of the plasma membrane rich fraction (7.5 μg membrane protein) to 180 μL of reaction mixture to give a final concentration of 0.02% (v/v) Triton X-100, 3 mM $MgSO_4$, 30 mM Tris/Mes (pH 6.5), 50 mM KCl, 1 mM NaN_3 in the presence or absence of 500 μM vanadate. The membranes were incubated with the reaction mixture for 15 min at RT on a rotary shaker at 150 rpm. Controls were done without membranes in the presence and absence of vanadate. The reaction was started by adding 50 μL of a 15 mM ATP stock solution and continued for another 30 min at 200 rpm. The reaction was stopped by the addition of 125 μL 20% (w/v) ice-cold TCA. Pi was determined according to the method of Taussky and Shorr (24).

For treatments with inositol phospholipids, the lipids were dissolved in 1% (v/v) Triton X-100 and 5 μL of the lipid containing Triton was added to the reaction mixture before the addition of plasma membranes. For the treatment with PA, PA was dissolved in 95% ethanol, aliquots of PA were transferred to a test tube, dried under N_2 to form a thin film of PA, and resuspended in the reaction mixture containing Triton X-100 before the addition of plasma membranes. The final Triton concentration was the same in the reaction mixture in the presence or absence of lipids.

For neomycin treatment, a 5 mM neomycin sulfate stock solution was made in deionized water and titrated to pH 6.5 with Tris buffer. A 5 μL aliquot of the neomycin stock solution or water as a control was added to the 175 μL reaction mixture containing the added lipids. Plasma membrane was added followed by ATP to start the reaction. After 30 min at 200 rpm the reaction was terminated and the Pi assayed as described above. Vanadate was not present during the assay because when neomycin was added the vanadate solution turned yellow suggesting that a change in oxidation state of the vanadium had occurred; however, for all of the experiments, at least 90% of the ATPase activity in the isolated plasma membranes was vanadate-sensitive.

RESULTS

Effects of Inositol Phospholipids on Plasma Membrane Vanadate-Sensitive ATPase Activity

To understand to what extent the exogenously added inositol phospholipids could affect the ATPase, the concentration of each lipid was varied from 2 to 80 μM and the effect on the vanadate-sensitive ATPase was monitored (Fig. 1). Addition of 2 μM phospholipid to the plasma membrane did not change the ATPase activity significantly compared to the control values. The ATPase activity increased significantly when the phospholipid concentration was 10 μM or greater and reached the maximum activity level when the phospholipid concentration was 40 μM. Of the inositol phospholipids used, PIP had the greatest effect on the ATPase. PIP_2 and PI were less effective than PIP but more effective than PC, and PA did not affect the ATPase activity significantly (Fig. 2).

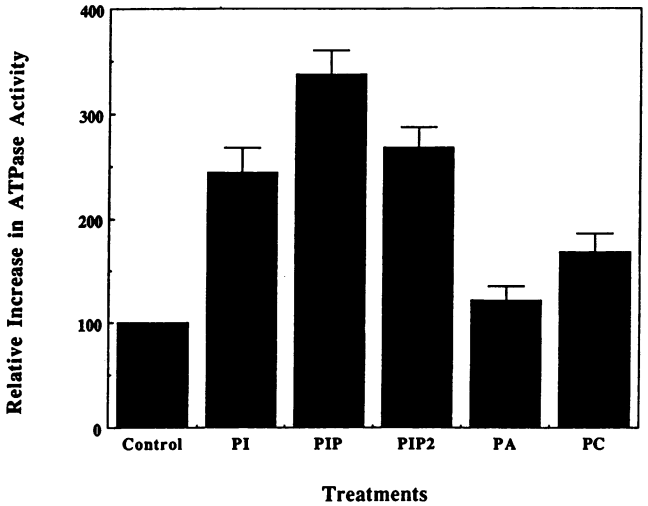

Figure 2. Effects of phospholipids on the vanadate-sensitive ATPase activity of plasma membrane isolated from sorbitol treated cells. The treatments were performed as described in Figure 1 except the lipid concentration was 40 μM. Data are the means of four values from two different experiments and are presented as in Figure 1. The ATPase activity of the control was 92.57 ± 19.0 nmol·mg^{-1} protein·min^{-1}.

Neomycin Reduces the Effect of Exogenous Polyphosphoinositides on Plasma Membrane ATPase Activity

PIP and PIP_2 are very negatively charged lipids. To determine whether or not the negatively charged inositol phosphate groups were involved in the increase in the ATPase activity, neomycin, a positively charged aminoglycoside which selectively binds PIP and PIP_2 at concentrations of 100 μM or less (18), was added. Neomycin (100 μM) decreased the effect of PIP and PIP_2 on the ATPase activity (Fig. 3). Although neomycin did not totally eliminate the effect of PIP and PIP_2, the ATPase activity of the PIP-treated membranes could be reduced 30% more by increasing neomycin concentration from 100 μM to 1 mM (data not shown). Neomycin did not significantly alter the effect of PI on the ATPase activity. The slight decrease seen when neomycin was added to PC-treated membranes may result from PC making the endogenous PIP or PIP_2 more available for neomycin binding because neomycin does not bind to PC (4).

To determine if the decrease in the ATPase activity by neomycin was caused by nonspecific interaction with components in the plasma membrane, neomycin was added to the plasma membrane in the absence of exogenously added lipids. The ATPase activity in the plasma membranes from either sorbitol-treated cells, or from Driselase (a mixture of cell wall degrading enzymes in sorbitol)-treated cells did not change when 100 μM (Table I) or up to 1 mM (data not shown) neomycin was added. In addition, neomycin was found not to inhibit the phosphorylation of endogenous PI and PIP (data not shown).

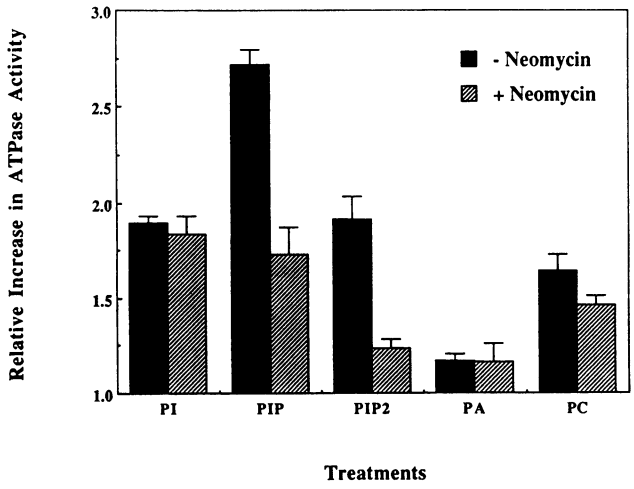

Figure 3. Effects of neomycin on the activation of ATPase by phospholipids. Carrot plasma membranes (7.5 μg membrane protein) were preincubated for 15 min at 150 rpm with 40 μM phospholipids in the presence or absence of 100 μM neomycin before ATPase activity was assayed in the absence of vanadate as described in "Materials and Methods." Data are presented as relative changes in the ATPase activity compared to that of the control which was assayed in the absence of phospholipid and neomycin. The error bar shows the standard deviation of six values from three different experiments. The vanadate-sensitive ATPase activity of the control was 129.36 ± 9.4 nmol·mg^{-1} protein·min^{-1} which was normalized to 1.0.

Table I. *Effects of Neomycin on Carrot Plasma Membrane ATPase Activity After the Cells were Treated with Sorbitol or Driselase*

The cells were treated for 10 min with 0.4 osmolal sorbitol containing 2 mM Mes (pH 4.8) or 2% (w/v) Driselase in the sorbitol. The plasma membranes were isolated as described in "Materials and Methods" and 7.5 μg of membrane protein was used in the ATPase assay. The assay was performed in the absence of vanadate and exogenous lipids. Data are presented as changes in the ATPase activity ± SD of four values from two different experiments.

Addition	ATPase Activity	
	Control	100 μM Neomycin
	nmol·mg^{-1} protein·min^{-1}	
Sorbitol	59.14 ± 15.2	65.71 ± 14.5
Driselase	392.55 ± 32.5	386.50 ± 34.0

DISCUSSION

Phospholipids have been shown to be essential in maintaining and regulating ATPase activity (1, 6, 8, 11, 15–17, 21). With carrot cell plasma membranes, 40 μM PIP and PIP_2 had the maximal effect on the vanadate-sensitive ATPase activity. The increase in plasma membrane ATPase activity caused by the addition of PIP and PIP_2 but not PI could be reduced by addition of 100 μM neomycin. The increase in Pi measured was not caused by dephosphorylation of PIP or PIP_2 because (a) increasing PIP and PIP_2 beyond 40 μM did not increase the Pi recovered; (b) the PIP and PIP_2 (10 nmol) were only a small fraction of the organic phosphate added; and (c) there is very little inositol phospholipid degradation in isolated plasma membranes of higher plants or algae (1, 3, 22).

The effect of inositol phospholipids on the relative ATPase activity varied somewhat with the cell culture (*cf.* Figs. 1, 2, 3 and Table 2 of ref. 11). Similar variation in the distribution of [^{3}H]inositol-labeled or ^{32}P-labeled inositol phospholipids was seen with the culture cells (1). For example, the [^{3}H] inositol-labeled PIP and PIP_2 in the carrot cells ranged from 1.63 to 3.65% and from 0.16 to 0.26% of total inositol-labeled lipids, respectively. If inositol phospholipids have a regulatory role, then the basal levels should affect the sensitivity of the ATPase to exogenous added lipids. PIP always had the largest effect on the ATPase activity and PA did not significantly affect the ATPase activity. In all instances, the effect of the inositol phospholipids was only on the vanadate-sensitive ATPase activity.

The increase in ATPase activity caused by PIP and PIP_2 but not that caused by the addition of PI was decreased by the addition of 100 μM neomycin. The order of neomycin sensitivity reflected the binding affinity of neomycin for the inositol phospholipids (PIP_2 > PIP ≫ PI) (4, 18). The fact that neomycin did not completely eliminate the effect of the exogenously added lipids might be attributed to noncharge-dependent effects of the lipids on the ATPase, to competitive binding of ATP (3 mM) to the neomycin, or to preferential binding of PIP and PIP_2 to the ATPase. Gabev *et al.* (4) showed that 10^{-6} M neomycin would bind >50% of the PIP_2 in artificial bilayers or monolayers consisting of 17 mole% PIP_2 in PC, and yet anywhere from 10^{-5} to 10^{-3} M neomycin was required to perturb that turnover of PIP_2 in animal cells.

They attributed the need for the higher concentration in the natural system to the fact that the lipids preferentially bound to membrane proteins and were not accessible to the neomycin. PI and PIP have been shown to be associated tightly with membrane proteins, such as the Ca^{2+}-ATPase of muscle sarcoplasmic reticulum (19) and the acetylcholine receptor of *Torpedo californica* electric organ (7). With the carrot membranes, increasing the neomycin concentration to 1 mM did decrease the response to PIP by 30% relative to that observed with 100 μM neomycin treatment suggesting that neomycin binding of the exogenous lipids was not complete at these concentrations.

Lipsky and Lietman (8) found that neomycin could inhibit more than 90% of the activity of partially purified canine renal ATPase, but only if the neomycin was added prior to PIP and PIP_2. If the inositol phospholipids were added first, neomycin had no effect, suggesting that the inositol phospholipids preferentially bound to the ATPase. A similar situation appears to occur with the isolated carrot membranes. With membranes from either Driselase-stimulated or -unstimulated cells, 1 mM neomycin did not affect endogenous ATPase activity. Thus, either the endogenous levels of PIP and PIP_2 do not affect the ATPase activity or PIP and PIP_2 present in the membrane preferentially bind to the membrane ATPase as was found with the solubilized enzyme. Preferential binding of PIP to plasma membrane proteins is suggested by the fact that neomycin did not inhibit phosphorylation of endogenous PIP to PIP_2.

Schäfer *et al.* (19) demonstrated that PIP regulated the Ca^{2+}-ATPase of muscle sarcoplasmic reticulum and proposed that PIP_2 did not serve as a source of second messengers in this system because the membranes did not synthesize appreciable PIP_2. The carrot cells also produced very little PIP_2 in the *in vitro* phosphorylation assay without exogenously added substrate (1, 2), and less than 0.5% of the [^{3}H]inositol-labeled lipid was PIP_2 even in stimulated cells (1). Since exogenously added PIP gave the largest and most consistent response with the carrot cells, PIP rather than PIP_2 may be the key regulator in this system as well.

In summary, we have shown that inositol phospholipids can serve as regulators of plasma membrane ATPase in culture carrot cells. The effects of inositol phospholipids on the ATPase may be caused by the structure of the inositol phosphate head group and its negative charge, and even though PIP is a relatively minor component of plasma membrane, a small increase in localized lipid concentration near the ATPase may have a dramatic effect on the ATPase.

LITERATURE CITED

1. **Chen Q, Boss WF** (1990) Short-term treatment with cell wall degrading enzymes increases the activity of the inositol phospholipid kinases and the vanadate-sensitive ATPase of carrot cells. Plant Physiol **94:** 1820–1829
2. **Chen Q, Brglez I, Boss WF** (1991) Inositol phospholipids as plant second messengers. *In* G Jenkins, W Schuch, eds, Molecular Biology of Plant Development, Company of Biologists, Ltd., Cambridge (in press)
3. **Einspahr KJ, Peeler TC, Thompson Jr, GA** (1989) Phosphatidylinositol 4,5-bisphosphate phospholipase C and phosphomonoesterase in *Dunaliella salina* membranes. Plant Physiol **90:** 1115–1120
4. **Gabev E, Kasianowicz J, Abbott T, McLaughlin S** (1989) Binding of neomycin to phosphatidylinositol 4,5-bisphosphate (PIP_2). Biochim Biophys Acta **979:** 105–112
5. **Gallagher SR, Leonard RT** (1982) Effect of vanadate, molybdate, and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots. Plant Physiol **70:** 1335–1340
6. **Imbrie CW, Murphy TM** (1984) Solubilization and partial purification of ATPase from a rose cell plasma membrane fraction. Plant Physiol **74:** 611–616
7. **Kiel R, Varsányi M, Neumann E** (1987) Phosphorylation of phosphatidylinositol associated with the nicotinic acetylcholine receptor of *Torpedo californica*. Biochem Biophys Res Commun **147:** 1251–1258
8. **Lipsky JJ, Lietman PS** (1980) Neomycin inhibition of adenosine triphosphatase: evidence for a neomycin-phospholipid interaction. Antimicrob Agents Chemother **18:** 532–535
9. **Lodhi S, Weiner ND, Schacht J** (1976) Interactions of neomycin and calcium in synaptosomal membranes and polyphosphoinositide monolayers. Biochim Biophys Acta **426:** 781–785
10. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ** (1951) Protein measurement with the folin phenol reagent. J Biol Chem **193:** 265–275
11. **Memon AR, Chen Q, Boss WF** (1989) Inositol phospholipids activate plasma membrane ATPase in plants. Biochem Biophys Res Commun **162:** 1295–1301
12. **Memon AR, Boss WF** (1990) Rapid light-induced changes in phosphoinositide kinases and H^+-ATPase in plasma membrane of sunflower hypocotyls. J Biol Chem **265:** 14817–14821
13. **Monk BC, Montesinos C, Leonard K, Serrano R** (1989) Sideness of yeast membrane vesicles and mechanisms of activation of the ATPase by detergents. Biochim Biophys Acta **981:** 226–234
14. **Palatini P, Dabbeni-Sala, Pitotti A, Bruni A, Madersloot JC** (1977) Activation of (Na^+ + K^+)-dependent ATPase by lipid vesicles of negative phospholipids. Biochim Biophys Acta **466:** 1–9
15. **Palmgren MG, Sommarin M** (1989) Lysophosphatidylcholine stimulates ATP dependent proton accumulation isolated oat root plasma membrane vesicles. Plant Physiol **90:** 1009–1014
16. **Palmgren MG, Sommarin M, Ulvskov P, Jørgensen PL** (1988) Modulation of plasma membrane H^+-ATPase from oat roots by lysophosphatidylcholine, free fatty acids and phospholipase A_2. Physiol Planta **74:** 11–19
17. **Sandstrom RP, DeBoer AH, Lomax TL, Cleland RE** (1987) Latency of plasma membrane H^+-ATPase in vesicles isolated by aqueous phase partitioning. Plant Physiol **85:** 693–698
18. **Schacht J, Lodhi S, Weiner ND** (1977) Effects of neomycin on polyphosphoinositides in inner ear tissues and monomolecular films. *In* Miller MW, Shamoo AE, eds, Membrane Toxicity. Plenum Press, New York, pp 191–208
19. **Schäfer M, Behle G, Varsányi M, Heilmeyer LMG Jr** (1987) Ca^{2+} regulation of 1-(3-*sn*-phosphatidyl)-1-D-*myo*-inositol 4-phosphate formation and hydrolysis on sarcoplasmic-reticular Ca^{2+}-transport ATPase. A new principle of phospholipid turnover regulation. Biochem J **247:** 579–587
20. **Serrano R** (1989) Structure and function of plasma membrane ATPase. Annu Rev Plant Physiol **40:** 61–94
21. **Serrano R, Montesinos C, Sanchez J** (1988) Lipid requirements of the plasma membrane ATPase from oat roots and yeast. Plant Sci **56:** 117–122
22. **Sommarin M, Sandelius AS** (1988) Phosphatidylinositol and phosphatidylinositol phosphate kinases in plant plasma membranes. Biochim Biophys Acta **958:** 268–278
23. **Sze H** (1985) H^+-translocating ATPase: Advances using membrane vesicles. Annu Rev Plant Physiol **36:** 175–208
24. **Taussky HH, Shorr E** (1953) A microcolorimetric method for the determination of inorganic phosphorus. J Biol Chem **202:** 675–685
25. **Wheeler JJ, Boss WF** (1987) Polyphosphoinositides are present in plasma membranes isolated from fusogenic carrot cells. Plant Physiol **85:** 389–392