Communication

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

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ABSTRACT

The inositol phospholipids, phosphatidylinositol monophosphate (PIP) and phosphatidylinositol bisphosphate (PIP2), 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 phosphatidylinositol, PIP, and PIP2 on the plasma membrane vanadate-sensitive ATPase activity. PIP and PIP2 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. De-lipidation 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, LPC, fatty acids, or inositol phospholipids

including PI, PIP, and PIP2 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 PIP2 (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 PIP2 (4, 9, 18) and thereby block the binding of PIP and PIP2 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 PIP2 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 PIP2 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
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. PIP2 and PI were less effective than PIP but more effective than PC, and PA did not affect the ATPase activity significantly (Fig. 2).

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 MgSO4, 30 mM Tris/Mes (pH 6.5), 50 mM KCl, 1 mM Na3VO4 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).

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 ± S.D. of six values from three different experiments. The ATPase activity of the control was 164.37 ± 31.7 nmol·mg⁻¹ protein·min⁻¹.

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 PIP₂ 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 PIP₂ at concentrations of 100 mM or less (18), was added. Neomycin (100 μM) decreased the effect of PIP and PIP₂ on the ATPase activity (Fig. 3). Although neomycin did not totally eliminate the effect of PIP and PIP₂, the ATPase activity of the PIP-treated membranes could be reduced 30% more by increasing neomycin concentration from 100 mM 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 PIP₂ 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).

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 PIP₂ had the maximal effect on the vanadate-sensitive ATPase activity. The increase in plasma membrane ATPase activity caused by the addition of PIP and PIP₂ but not PI could be reduced by addition of 100 μM neomycin. The increase in P_i measured was not caused by dephosphorylation of PIP or PIP₂, because (a) increasing PIP and PIP₂ beyond 40 μM did not increase the P_i recovered; (b) the PIP and PIP₂ (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 [³²P]inositol-labeled or [³H]inositol-labeled inositol phospholipids was seen with the culture cells (1). For example, the [³H]inositol-labeled PIP and PIP₂ 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 PIP₂ 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 > 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 PIP₂ to the ATPase. Gabev et al. (4) showed that 10⁻⁶ M neomycin would bind >50% of the PIP₂ in artificial bilayers or monolayers consisting of 17 mole% PIP₂ in PC, and yet anywhere from 10⁻⁵ to 10⁻³ M neomycin was required to perturb that turnover of PIP₂ in animal cells.

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

<table>
<thead>
<tr>
<th>Addition</th>
<th>ATPase Activity</th>
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<tbody>
<tr>
<td>Control</td>
<td>32.5 ± 2.78</td>
</tr>
<tr>
<td>100 μM Neomycin</td>
<td>38.6 ± 3.25</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>59.14 ± 15.2</td>
</tr>
<tr>
<td>Driselase</td>
<td>392.55 ± 32.5</td>
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DISCUSSION

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⁻¹·min⁻¹ which was normalized to 1.0.
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 \(\mu\)M 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 \textit{in vitro} phosphorylation assay without exogenously added substrate (1, 2), and less than 0.5% of the \([\text{H}]\)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**

3. Einspahr KJ, Peeler TC, Thompson Jr, GA (1989) Phosphatidylinositol 4,5-bisphosphate phospholipase C and phospho-
monooesterase in Dunaliella salina membranes. Plant Physiol 90: 1115–1120