The Phosphatidylinositol Species of Suspension Cultured Plant Cells

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Suspension cultured *Nicotiana tabacum* and *Catharanthus roseus* cells were labeled with [^{3}H]inositol, the phospholipid fraction extracted and separated by thin layer chromatography. Three different solvent systems and reference compounds were used to assign the different ^{3}H-labeled species by autoradiography. The ratio of [^{3}H]inositol incorporation into PI, PIP and PIP_2 was found to be 95:4:1; with some preparations a lyso-PI band was obtained which incorporated about a tenth of the label of the PIP band. With *Catharanthus roseus* cells a very faint band between PI and lyso-PI was detected which could not be assigned to a reference compound.

Introduction

With animal cells it has been well established that the high turnover of phosphatidylinositol (PI) is performed through the so-called PI cycle which involves phosphorylation of the inositol head group and cleavage by phospholipase C [1, 2]. The existence of phosphorylated PI species [3–6], PI phosphorylation activities [7–9] and cleavage products from phospholipase C action on PI [10–12] has recently been documented for the plant cell.

A further route of phospholipid turnover, initiated by the action of phospholipase A, would lead to lysophospholipid derivatives and free fatty acids [13]. Boss and Massel [3] reported significant amounts of lysophosphatidylinositol, besides the phosphorylated species, in their extracts from suspension cultured carrot cells. As our studies on the PI cycle were also performed with suspension cultured plant cells, a detailed analysis of [^{3}H]inositol labeled PI species is presented using three different thin layer chromatography systems.

Materials and Methods

Origin, media and growth conditions of suspension cultured *Nicotiana tabacum* W 38 [14] and *Catharanthus roseus* [4] cells have been described; both species were subcultured every two weeks. For [^{3}H]inositol labeling of the tobacco cells 80 ml medium containing 150 µCi [^{3}H]myo-inositol (Amersham Buchler, Braunschweig) were inoculated with 5 g two-week-old cells and grown to 15 g fresh weight at 25 °C with a 16 h/8 h light-dark cycle.

The original myo-inositol concentration (0.55 µM) of the *C. roseus* medium was reduced to one tenth, 50 µCi [^{3}H]inositol and 0.5 g two-week-old cells were added to 20 ml medium and incubation was performed in the dark at 27 °C; at harvest the fresh weight of these cells was 4.5 g. Extraction of the phospholipids was performed as described [4, 8]; all phospholipid reference compounds were purchased from Sigma Chemie, Deisenhofen, FRG.

Separation of the phospholipids was performed with three different solvent systems. Routinely [4, 8] we used system (a) which is a slight modification of the method described by Jolles *et al.* [15]. HPTLC plates from Merck, Darmstadt (silica gel 60), with a concentration zone were treated (10 sec) with a 1% solution of potassium oxalate in methanol/water/conc. acetic acid (4:2:3) and heated (15–20 min) at 115 °C. Separation was performed with chloroform/acetone/methanol/conc. acetic acid/H_2O (40:15:13:12:8). System (b) was the original procedure of Schacht [16]; silica gel 60 HPTLC plates with a concentration zone from Macherey & Nagel (without activation) and chloroform/methanol/3.3 M ammonia (43:38:12) were used. Separation according to system (c) was performed with chloroform/methanol/conc. ammonia/water (90:54:5.5:5.5) and the same plates as in (b) were used but after activation by spraying with a 0.2 M sodium tetraborate solution in methanol/water (1:1) and heating overnight at 125 °C [17].

The spots were visualized under UV light after spraying with a solution of 350 mg 6-*p*-toluidino-2-naphthalene sulfonic acid in 1 l of 50 mM Tris HCl (pH 7.4). Autoradiography was performed with Kodak-X-Omat AR and Agfa-Gevaert Curix RP 1 films using the EN^{3}HANCE spray from New England Nuclear.

Results and Discussion

Routinely we use the acidic solvent system (Fig. 1) to separate [^{3}H]inositol and ^{32}P-labeled phospholipids. Due to the high activities of diacylglycerol

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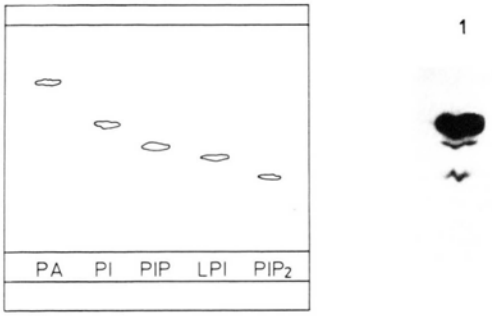

Fig. 1. Resolution of phospholipids with the acidic solvent system. Separation was performed with the acetic acid containing solvent [15] (solvent a) described in Materials and Methods on HPTLC silica gel 60 plates from Merck, Darmstadt, treated with potassium oxalate. Left side shows the separation of the reference compounds; the spots were stained with 6-*p*-toluidino-2-naphthalene sulfonic acid and thereafter marked with a pencil under UV light [4]. Lane 1 is from a 10 µl phospholipid extract of *Catharanthus roseus* cells (ca. 40 mg cells) labeled with [^{3}H]inositol (*cf.* Materials and Methods); autoradiography was performed with the Kodak X-Omat AR film after spraying the plate with EN^{3}HANCE from NEN; exposure time was 8 days.

kinase there is a strong labeling of PA by [γ-^{32}P]ATP with frozen and thawed cells [8, 9] or with (^{32}P) inorganic phosphate pulse feeded cells in culture (unpublished results). The acidic solvent gives a satisfactory resolution of PA and PI, whereas the basic solvent (Fig. 2) does not. On the other hand, separation of lysophosphatidylinositol (LPI) is worse in the acidic solvent than in the basic one (Fig. 1); in the former LPI occurs between PIP and PIP_2 and the distance between these was in most cases smaller than shown in the example of Fig. 1. Therefore, with the [^{3}H]inositol labeled lipid extract from *Catharanthus roseus* cells we could not detect LPI by this separation procedure.

Resolution of LPI is improved by the basic solvent system (Fig. 2); here it is placed between PI and PIP and the separation of these latter compounds is fairly large. Separation of the [^{3}H]inositol labeled phospholipid extract from *C. roseus* and *N. tabacum* cells resulted in labeled bands located between PI and PIP, whereas PIP_2 is scarcely visible. In contrast to animal cells labeled PIP_2 is only a minor fraction in plant cells [3–5] and is also relatively unstable especially under basic conditions.

There are two minor compounds visible in the separations of Fig. 2; however, only in lane 2 is the presence of the two compounds clearly demonstrated. According to the R_F value of the reference compound and the assignment of other authors [3, 5] we can assume that the band next to PIP is LPI, whereas the band next to PI obviously corresponds to the unknown compound which Boss and Massel [3] and Strasser *et al.* [5] also found in their extracts from suspension cultured carrot and parsley cells, respectively.

Boss and Massel [3] found twice as much labeled LPI as PIP from the suspension cultured carrot cells, whereas the extracts from our *C. roseus* and *N. tabacum* cells always revealed a minor fraction of labeled LPI which amounted to about 10% of the radioactivity of the PIP fraction and sometimes was scarcely visible.

Therefore we tried another basic solvent system which is a slight modification of the original procedure of Schacht [16]. A resolution of phospholipid extracts from *C. roseus* cells (Fig. 3) shows the clear presence of labeled LPI and the unknown compound

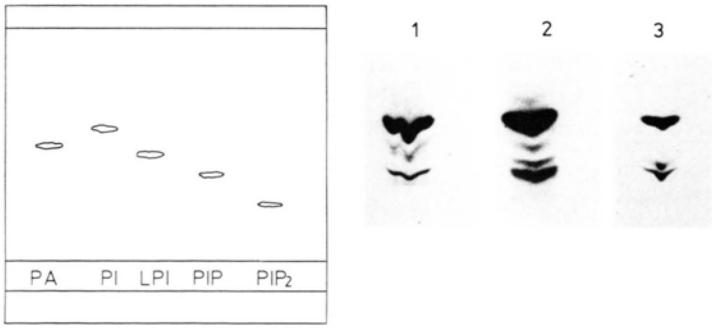

Fig. 2. Resolution of phospholipids with the basic solvent system described by Schacht [16] (solvent b) and in Materials and Methods; HPTLC plates were from Macherey & Nagel. Left side shows the separation of the reference compounds. Lane 1 and 2 shows the resolution of 5 µl (ca. 33 mg cells) and 10 µl (ca. 67 mg cells) phospholipid extracts from *Catharanthus roseus* cells, lane 3 of 10 µl (ca. 67 mg cells) extract from *N. tabacum* W 38 cells labeled with [^{3}H]inositol (*cf.* Materials and Methods). Autoradiography was performed with Agfa-Gevaert Curix RP1 film after spraying with EN^{3}HANCE from NEN; exposure time was 3 days.

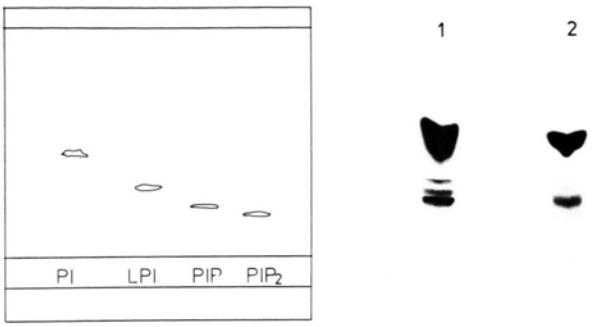

Fig. 3. Resolution of phospholipids with the basic solvent system described by Hohengasser *et al.* [17] (solvent c) and in Materials and Methods; HPTLC plates were from Macherey & Nagel. Left side shows the separation of the reference compounds. Lane 1 and 2 shows the resolution of 5 µl (ca. 33 mg cells) phospholipid extract from *Catharanthus roseus* cells labeled with [^{3}H]inositol. The HPTLC plate of lane 1 was used after (lane 2 without) activation with sodium tetraborate (*cf.* Materials and Methods). Autoradiography was performed as described in the legend of Fig. 2; exposure time was 4 days.

only with the sodium tetraborate pretreated plate, whereas ommission of this activation reveals only very faint bands between PI and PIP and also a faint band below PIP which is probably labeled PIP_2. The reason of this difference may be either a different resolution with the activated plates or some degree of degradation leading to higher amounts of labeled LPI and the unknown compound in the case of the pretreated plates.

In conclusion we can estimate from our data an incorporation of the [^{3}H]inositol into PI of about 95%, into PIP of about 4% and only about 1% or less into PIP_2. These data confirm our previous results [4] and those of other authors [3, 5] and reveal a different situation for proliferating plant cells relative to animal cells where larger amounts of labeled PIP and especially PIP_2 were found. Our data also confirm the labeling of lysophosphatidylinositol; however, the amounts demonstrated from our cells are much less than those reported from Boss and Massel [3].

Furthermore, the fact that the radioactivity of these compounds is variable and often very small may indicate that LPI is not a major intermediate and could represent an artifact of the preparation.

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