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The presence of *sn*-1-palmitoyl lysophosphatidylinositol monophosphate correlates positively with the fusion-permissive state of the plasma membrane of fusogenic carrot cells grown in suspension culture *

Jeffery J. Wheeler and Wendy F. Boss

Department of Botany, North Carolina State University, Raleigh, NC (U.S.A.)

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Two analogues of lysophosphatidylinositol monophosphate (LPIP) occur in fusogenic carrot cells and protoplasts and are distinguished from each other on thin-layer plates by differing R_f values. The lower R_f LPIP comigrates with *sn*-1-palmitoyl LPIP (16:0 LPIP) synthesized from *sn*-1-palmitoyl lysophosphatidylinositol. The upper R_f LPIP is presumed to be *sn*-2-linoleoyl LPIP (18:2 LPIP) based on fatty acid analysis of phosphatidylinositol monophosphate (PIP) and comigration with the upper R_f product resulting from the base-catalyzed hydrolysis of PIP. The 18:2 analogue is only a minor component of the LPIP recovered from the fusogenic cells and protoplasts, but it is the predominant form of LPIP in nonfusogenic cells and protoplasts. LPIP is found primarily in the plasma membranes of these cells representing from 10 to 20% of the total [^{3}H]inositol-labeled lipid recovered from isolated plasma membrane. Even though the fusogenic cells lose some of the LPIP as a result of cell wall digestion, if 2% or more of the total protoplast inositol lipid is LPIP, the protoplasts fuse spontaneously with a fusion frequency of greater than 60%. As the fusogenic protoplasts lose their fusion potential, they also lose the relative amount of 16:0 LPIP. Inhibiting the in vivo metabolism of phosphatidylinositol bisphosphate (PIP_2) and PIP in cells and protoplasts with the aminoglycoside, neomycin, does not inhibit fusion. These data indicate that the presence and metabolism of 16:0 LPIP but not the polyphosphoinositides, PIP and PIP_2, correlate positively with protoplast fusion.

Introduction

Many model systems have been used to study the mechanisms of cell fusion. Often liposomes are used, since specific lipid–lipid or lipid–protein combinations can be studied in a well-defined system. Naturally fusing biological systems, though necessarily more complex than liposomes, offer a valuable means of studying cell fusion. We have developed a model system of fusion-permissive or fusogenic wild carrot cells which fuse spontaneously when the cell wall is removed. The fusogenic cells incorporated [^{3}H]inositol into the phospholipids of the PI cycle at 5–7-times the rate of the nonfusogenic cells [1]; however, under the same conditions, no significant differences in the distribution of glycerol-labeled phospholipids were observed after labeling with [2-^{3}H]glycerol. These data suggested increased headgroup turnover of PI in the fusogenic cells.

The purpose of this work was to determine whether the metabolism of the lipids of the phosphatidylinositol cycle correlated with the fusion-permissive state of the carrot protoplasts. PI has been shown to hinder fusion in isolated membrane vesicles. This is presumably due to its large headgroup [2]; however, Wakelam and Pette [3] and Allan and Michell [4] have proposed that PIP_2 metabolism might be important in myoblast fusion. One hypothesis was that phospholipase C would cleave PIP_2 to yield inositol 1,4,5-trisphosphate and DAG and that

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Abbreviations: PI, phosphatidylinositol; LPI, lysophosphatidylinositol; PIP, phosphatidylinositol monophosphate; LPIP, lysophosphatidylinositol monophosphate; PIP_2, phosphatidylinositol bisphosphate; $LPIP_2$, lysophosphatidylinositol bisphosphate, DAG, 1,2-diacylglycerol; Mes, 4-morpholineethanesulfonic acid; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol.

Correspondence: W.F. Boss, Botany Department, Box 7612, North Carolina State University, Raleigh, NC 27695, U.S.A.

DAG would favor formation of a nonbilayer intermediate at the fusion site. Alternatively, it was proposed that the loss of the large, negatively charged PIP_2 headgroup itself might relieve a fusion block [4].

Other lipids have been implicated in a variety of fusion events. For example, lysophospholipids prefer the micelle configuration. Non-bilayer intermediates, such as inverted micelles or hexagonal II phases at the point of membrane contact, may be necessary for membrane fusion to occur. In general, lysophospholipids will enhance fusion either directly or indirectly when added to a naturally fusing system [5,6]. There are some exceptions, however, lysophosphatidylserine, when added to guinea pig sperm, inhibited acrosome formation, while lysophosphatidylcholine, lysophosphatidylethanolamine and LPI enhanced it [7]. Conway and Metz [8] have found a correlation between phospholipase A activity and sperm-egg fusion, suggesting a possible involvement of lysophospholipids.

In this study we show that with the fusogenic cells it is the presence of LPIP that correlates with the fusion potential of the protoplasts. PIP_2 and PIP appear not to be involved in protoplast fusion. Two 'putative' analogues of LPIP (*sn*-1-palmitoyl- and *sn*-2-linoleoyl LPIP) have been found. The *sn*-1 analogue (16:0 LPIP) is found predominantly in the fusogenic cells while the *sn*-2 analogue (18:2 LPIP) is found predominantly in the nonfusogenic cells. An increase in the percentage of 16:0 LPIP correlates with an increase in the fusion potential of the fusogenic protoplasts. In addition, when the fusogenic protoplasts begin to lose their ability to fuse, the 18:2 LPIP analogue becomes more predominant as the 16:0 LPIP analogue decreases.

Materials and Methods

Cell cultures. Fusogenic carrot cells were grown in suspension culture and serially transferred every 7 d [9]. Cells were used 4 d after transfer. Except where otherwise stated, cells were labeled 12 h with *myo*-[2-^{3}H]inositol (17 Ci/mmol, New England Nuclear) at a concentration of 5 μCi/0.35 g cells in 25 ml of medium. Nonfusogenic carrot cells were grown as previously described [10] and were also transferred every 7 d and used 4 d after transfer.

Lipid extraction and thin-layer chromatographic analysis. Lipids were extracted in $CHCl_3$/MeOH/2.4 M HCl/0.1 M EDTA (1:2:1:1, v/v) according to a modification of the procedure of Schacht [11,12], dried in vacuo and stored under N_2 at −15°C.

Lipids were reconstituted in $CHCl_3$/MeOH (3:1, v/v) and spotted on LK5D thin-layer chromatography plates (Whatman) which had been impregnated with 1% potassium oxalate and dried at 110°C for at least 2 h. The plates were developed for approx. 2 h in $CHCl_3$/MeOH/15 M NH_4OH/H_2O (90:90:7:22, v/v) and allowed to dry. Radioactivity was quantitated in each lane with a Bioscan System 500 Imaging Scanner.

Protoplast release. Protoplasts were released by digestion of 0.3 g fusogenic cells or 1.2 g nonfusogenic cells in a 20 ml solution containing the digestion enzymes, Driselase (2% w/v, Plenum Sci.), in 0.4 osmolal sorbitol, 2 mM Mes (pH 4.8). The cells were incubated 1.5–2 h at 25°C with constant shaking at 125 rpm. Protoplasts were collected by centrifugation at 40 × *g* and washed in 0.45 osmolal sorbitol 1 mM Mes (pH 6.0) as described elsewhere [9].

Formation of upper R_f and lower R_f LPIP. Nonfusogenic protoplasts (0.05 ml settled volume) were added to glass disposable test tubes containing 0.45 osmolal sorbitol, 2 mM Mes (pH 6.0) and shaken at 100 rpm for 10 min [^{32}P]P_i (10 μCi; 1 μCi/μl) was added to the protoplasts which were shaken at 100 rpm for 30 min. Phosphorylation was stopped by adding 1.5 ml $CHCl_3$/MeOH (1:2, v/v). Lipids were extracted and separated on thin-layer plates as described above. PIP was visualized via autoradiography; scraped from the thin-layer plate; eluted from the silica gel with 1 ml $CHCl_3$/MeOH/15 M NH_4OH/H_2O (90:90:7:22, v/v), and centrifuged at 1000 × *g* for 3 min to remove the silica gel. The supernatant was poured into a separate tube and the PIP was dried under vacuum to form a thin film. The PIP was hydrolyzed by adding 100 μl of 1 M $NaHCO_3$ (pH 10) (adjusted with 10 M KOH). The solution was vortexed and then allowed to shake at 100 rpm for 5 h at 25°C. The hydrolyzed PIP was spotted on an LK5D thin-layer plate as described above and the $^{32}P_i$-labeled lipids were visualized by autoradiography.

Plasma membrane isolation. Plasma membranes were isolated from both cell types by aqueous polymer two-phase partitioning. The details of the method have been published elsewhere [13,14]. Briefly, 0.7 g (fusogenic) or 1.2 g (nonfusogenic) cells were homogenized in 1.5 ml 50 mM Tris, 10 mM KCl, 1.0 mM $MgCl_2$, 1.0 mM EDTA, 95 mM LiCl, 2 mM EGTA, 0.05 g polyvinylpolypyrrolidone and 8% sucrose (pH 7.5). The homogenate was centrifuged at 1000 × *g* for 4 min to remove cell wall debris and nuclei, and the resulting supernatant was centrifuged at 40000 × *g* for 45 min. The pellet was resuspended in 500 μl H_2O and 0.5 g of the membrane suspension was added to 3.5 g of a two-phase system consisting of 1.26 g of 20% (w/w, assuming 12% water of hydration) Dextran T-500 (Pharmacia) and 0.63 g of 40% (w/w) PEG 3350 (Union Carbide), 875 μl 1 M sucrose, and 350 μl 50 mM potassium phosphate buffer (pH 7.5) brought to 3.5 g with deionized water in a 5 ml disposable test tube. The 6.3% polymer two-phase systems were inverted 70 times at 4°C. The separation was quickened by centrifuging at 600 × *g* for 10 min at 4°C. The upper (plasma membrane) and lower (intracellular membrane) phases were collected, diluted with 10 ml of 50 mM Tris, 10 mM KCl, 1.0 mM $MgCl_2$, 1.0

mM EDTA (pH 7.5) and centrifuged at 40000 × g for 45 min. The pellet was resuspended in 3 ml of the same buffer and centrifuged at 40000 × g for 20 min to remove the residual PEG and dextran. The resulting 40000 × g pellet was resuspended in 30 mM Tris-Mes (pH 6.5) and the lipids either were extracted immediately or equal aliquots were incubated in sorbitol (0.4 osmolal final concentration) with or without 2% (w/v) Driselase for 30 s prior to lipid extraction. Membrane purity was assessed by using marker enzymes as previously described [13,15].

Antibiotic treatment of cells. Fusogenic cells were labeled 12 h with *myo*-[2-^{3}H]inositol and preincubated for either 20 min prior to cell wall digestion or 2 h during cell wall digestion with 0.5 mM neomycin, kanamycin, or gentamycin sulfate. Fusion was assayed with a video camera attached to a microscope and linked to an Apple IIE computer. Diameters of protoplasts were measured with a VideoPlus + Image Measurement program (Dapple Systems, Inc.) and tabulated for five fields on three slides for each treatment including the control. The protoplasts were spherical in shape; therefore, doubling the volume caused the diameter to increase to 1.26-times the original diameter. Multinucleated protoplasts were considered fusion products if their diameters were greater than 1.26-times the diameter of the smaller, mononucleated protoplasts. The lipids from the cells and protoplasts were extracted and analyzed as described above.

Results

Identification and localization of phosphoinositides

The polyphosphoinositides PIP and PIP_2 were first reported in the fusogenic carrot cell cultures by Boss and Massel (Ref. 12, Table I). In addition, an unknown or 'mystery' lipid which incorporated [^{3}H]inositol was observed. When plasma membranes were isolated from the fusogenic cells, the polyphosphoinositides as well as the unknown lipid were found predominantly in these membranes [13]. It became apparent that the unknown lipid was actually two separate lipids which labeled with *myo*-[2-^{3}H]inositol, [1-^{14}C]myristate and [^{32}P]P_i and which comigrated with synthetic LPIP [13]. The R_f of the lipids suggested that they were two analogues of LPIP. While we were unable to recover enough of these lipids to analyze them by fast atom bombardment (FAB) mass spectrometry, we were able to recover LPI and to show that two analogues of LPI, the 16:0 and 18:2, were present. Furthermore, when *sn*-1-palmitoyl LPI (16:0 LPI) was phosphorylated in an in vitro phosphorylation system using [γ-^{32}P]ATP (Wheeler and Boss, manuscript in preparation) the compound formed comigrated with the lower R_f LPIP which was isolated from cells and with the lower R_f LPIP formed by enzymatic hydrolysis of PIP with phospholipase A_2 [13]. Alkaline hydrolysis of carrot [^{32}P]P_i-labeled PIP isolated from [^{32}P]P_i-labeled carrot protoplasts yielded two products. One comigrated with the upper R_f analog of LPIP (presumably 18:2 LPIP) and the other with the lower R_f analogue of LPIP (16:0 LPIP) (Fig. 1).

Photographs of the fusogenic and nonfusogenic cells and protoplasts [9] as well as a detailed account of the fusion process [16] have been published elsewhere. The fusogenic protoplasts contain only a few small vacuoles while the nonfusogenic protoplasts contain a large central vacuole which represents about 90% of the volume of the cell. The fusogenic protoplasts fuse spontaneously (i.e., exogenous fusogen is not required). In general, when the fusogenic protoplasts lose their fusion potential, they become more vacuolated and their morphology is similar to that of the nonfusogenic protoplasts.

The major difference in the inositol-labeled lipids of the two cell cultures was the LPIP. Although both analogues existed in both cell types, the lower R_f analogue was predominant in plasma membranes isolated from fusogenic cells and the upper R_f analogue was predominant in plasma membranes isolated from the nonfusogenic cells (Fig. 2). Note that the relative amounts of the two analogues of LPIP differed between the two cell types and that together they constituted a relatively large percentage (about 10–20%) of the [^{3}H]inositol-labeled lipids in the plasma membrane (Table II) [13]. Although LPI was about 5% of the inositol lipid in the plasma membrane, this represented about 2% of the total [^{3}H]inositol-labeled LPI recovered from both the upper and lower phase membranes and was

TABLE I

Percent distribution of [^{3}H]inositol-labeled lipids in fusogenic cells and protoplasts

Fusogenic cells (0.3 g) were labeled 12 h with 5 μCi of *myo*-[2-^{3}H]inositol (17 Ci/mmol). Cell lipids were extracted as described or protoplasts were released in Driselase in the absence of isotope and the lipids extracted. Data represent means ± S.D. from three experiments. The total counts integrated were 120000–180000.

	% Total counts [^{3}H]inositol recovered				
	PIP_2	LPIP	PIP	LPI	PI
Cells	0.46 ± 0.15	4.23 ± 1.11	4.87 ± 0.26	7.37 ± 1.10	80.30 ± 2.88
Protoplasts	0.67 ± 0.15	1.33 ± 0.58	4.87 ± 0.87	11.10 ± 2.69	80.43 ± 3.38

Fig. 1. Hydrolysis of [^{32}P]P_i-labeled PIP. [^{32}P]P_i-labeled PIP isolated from carrot protoplasts was base hydrolyzed in 1 M $NaHCO_3$ for 5 h shaking constantly at 100 rpm. The two products migrating below PIP observed by autoradiography (arrows) comigrated with the lower and upper R_f analogues of LPIP naturally occurring in the fusogenic cells.

indicative of contaminating intracellular membranes [13].

If LPIP is involved in fusion, then it should be present in the plasma membranes isolated from the fusogenic protoplasts. Fig. 3 shows the [^{3}H]inositol-labeled lipid profile from fusogenic protoplasts and plasma membrane isolated from fusogenic protoplasts. The plasma membranes were enriched in the polyphosphoinositides, including LPIP.

Since the cell wall is removed to generate a protoplast and since cell wall digestion can activate lipases [17], it became of interest to know what effect, if any,

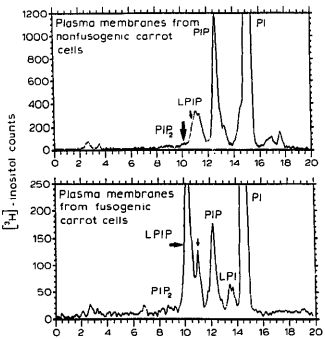

Fig. 2. Distribution of [^{3}H]inositol-labeled lipids from plasma membranes isolated from nonfusogenic (upper trace) and fusogenic carrot cells (lower trace). Cells were radiolabeled as described in Materials and Methods and plasma membranes were isolated after 12 h. Extracted lipids were spotted at approx. 2 cm. The small arrow denotes the upper R_f analogue of LPIP (18:2 LPIP). The lower R_f LPIP corresponds to 16:0 LPIP (large arrow). LPI is present as a tiny shoulder on PI in the upper trace. Note that the only qualitative difference between the two cell cultures is the LPIP.

TABLE II

The effects of the cell wall digestion enzymes, Driselase, on the [^{3}H]inositol-labeled lipids of plasma membranes isolated from fusogenic and nonfusogenic carrot cells

Plasma membranes from fusogenic and nonfusogenic carrot cells labeled as in Table I were isolated by aqueous polymer two-phase partitioning. The plasma membranes were incubated for 30 s in either 0.4 osmolal sorbitol pH 6.0 or 2% Driselase in 0.4 osmolal sorbitol (pH 6.0). Data are the average of duplicate samples from one experiment. The experiment was repeated three times and the trends were consistent. Total counts integrated were approximately 7000 for the fusogenic membranes and approximately 3000 for the nonfusogenic membranes.

Lipid	% Total [^{3}H]inositol recovered			
	fusogenic		nonfusogenic	
	control	Driselase	control	Driselase
PI	56.0±3.2	65.0±1.1	54.7±3.4	50.7±1.0
LPI	5.2±0.8	4.8±0.8	7.3±0.3	7.6±0.8
PIP	11.4±0.5	10.8±1.2	15.4±0.3	18.2±0.6
$LPIP_{upper\ R_f}$		4.9±1.3	16.4±2.0	15.2±2.0
LPIP [a]	12.9±0.9	..	–	–
PIP_2	1.2±0.4	1.0±0.3	2.4±0.6	2.4±0.1

[a] Although both analogues of LPIP are present, the lower R_f analogue was predominant, as depicted in Fig. 2B.

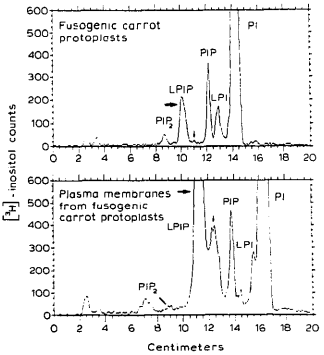

Fig. 3. Distribution of [^{3}H]inositol-labeled lipids from protoplasts (upper trace) and plasma membranes (lower trace) isolated from protoplasts from the fusogenic cell culture. Cells were radiolabeled 12 h prior to cell wall digestion. The legend is the same as in Fig. 2. Note that LPIP is located in the plasma membrane of these protoplasts.

cell wall digestion had on the plasma membrane lipids. Table I shows the effect of cell wall digestion on the phosphoinositides of fusogenic carrot cells. Prior to cell wall digestion, the 16:0 LPIP was $4.2 \pm 1.11\%$ of the total [^{3}H]inositol-labeled lipids recovered from the cells. After cell wall digestion, the 16:0 LPIP was $1.3 \pm 0.58\%$ of the total [^{3}H]inositol-labeled lipids recovered from the protoplasts isolated from the same cells. The total cpm of inositol lipids per g fresh weight were approximately the same for the cells and protoplasts. Although LPI tended to increase in the protoplasts, the only significant difference in the distribution of the inositol lipids was the loss of LPIP.

LPIP was lost not only when cells were treated in vivo with Driselase but also when isolated plasma membranes were treated with Driselase. When isolated plasma membranes were treated with sorbitol plus Driselase for 30 s, LPIP decreased about 50% compared to the sorbitol control (Table II). Importantly, there was no decrease in 18:2 LPIP, the predominant analogue in the nonfusogenic cells, PIP, or PIP_2 in response to the Driselase treatment.

Protoplasts isolated from the fusogenic cells vary in their ability to fuse. If the amount of LPIP present in the protoplast plasma membrane correlates with fusion, then it should decrease with decreasing fusion potential. Fig. 4 is a plot comparing the [^{3}H]inositol-labeled phosphoinositides LPI, PIP, LPIP, and PIP_2 from fusogenic protoplasts that were fusing well and from those that were fusing poorly. Among the 'good fusers,' LPIP was more abundant than in the 'poor fusers' and the ratio of LPIP to PIP was much closer to unity. Since the 18:2 LPIP was very low in the fusogenic protoplasts and did not appear to change with fusion potential and since the two analogues of LPIP were not always well-resolved in these studies, LPIP was reported as the sum of 16:0 LPIP and 18:2 LPIP. In later experiments where the two analogues of LPIP were resolved on the thin-layer plates, it was evident that only the lower R_f or 16:0 analogue of LPIP correlated with the fusion potential. As the cells began to lose their ability to fuse and became vacuolated, the 16:0 LPIP analogue decreased relative to 18:2 LPIP.

LPI also was higher in the 'good fusers' relative to the 'poor fusers'; however, the percentage of LPI recovered from 'good fusers' was comparable to that found in nonfusogenic protoplasts. In addition, more than 95% of the cellular LPI was found in the intracellular membranes [13]. These data suggested that the increase in LPI in the 'good fusers' relative to the 'poor fusers' might be indicative of differences in biogenesis of the intracellular membranes, rather than the fusogenic state of the plasma membrane.

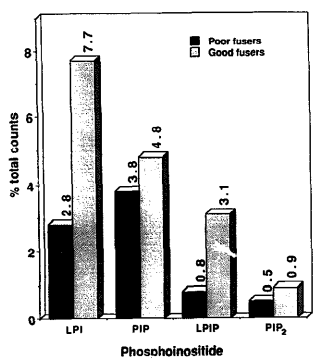

Fig. 4. Distribution of [^{3}H]inositol-labeled lipids from protoplasts isolated from the fusogenic cells at pH 4.8. Fusion was less than 10% for protoplasts denoted 'poor fusers' and was 60% or greater for protoplasts denoted 'good fusers'. LPIP includes with analogues of LPIP. Data are means of three separate experiments from both groups. Note that in the 'good fusers' LPIP is much higher than in the 'poor fusers'. Also, the ratio of LPIP to PIP approaches unity in the 'good fusers'. The difference in PIP_2 is not significant. The error for each lipid is within the limits indicated in Table I.

TABLE III

The effects of neomycin sulfate on fusogenic protoplasts

Fusogenic cells (0.3 g) were labeled 12 h with 5 μCi *myo*-[2-^{3}H]inositol (17 Ci/mmol). Neomycin-treated protoplasts were released in 0.5 mM neomycin sulfate for the 2 h during cell wall digestion at pH 4.8. Data are given as means ± S.D. from three separate experiments.

Treatment	% Total counts [^{3}H]inositol recovered					Fusion
	PIP_2	LPIP	PIP	LPI	PI	(%)
Control	0.56 ± 0.21	1.82 ± 0.51	3.75 ± 0.69	5.20 ± 1.45	84.68 ± 2.73	≈ 60
Neomycin	1.93 ± 0.05	1.55 ± 0.29	4.55 ± 0.21	7.43 ± 1.28	80.13 ± 0.87	> 60

PIP_2 and fusion

Because a role has been suggested for PIP and PIP_2 in myoblast fusion [3,4], the role(s) of these lipids in protoplast fusion was studied. Neomycin was used since it is known to bind to PIP_2 and PIP had groups [18–20]. Neomycin, however, is also a protein synthesis inhibitor; therefore, the aminoglycosides, kanamycin and gentamycin, which are structurally and functionally similar to neomycin but which have a lesser affinity for PIP_2 and PIP headgroups, were used as controls.

Table III shows the effect of releasing fusogenic protoplasts in the presence of 0.5 mM neomycin sulfate. Note that when protoplasts were released in the presence of neomycin, the percentage of PIP_2 and PIP increased significantly as percent total counts while the percentage of LPIP did not. If cells were pre-treated with neomycin prior to, but not during cell wall digestion, there was only a slight increase in PIP_2 and PIP and the percentage of LPIP was not affected or was decreased slightly (Table IV). In both instances, however, the percentage of LPI was significantly increased with neomycin. The antibiotics, gentamycin and kanamycin, did not significantly affect the lipids or fusion. Although neomycin binds to the inositol headgroups of PIP_2 and PIP and might thereby inhibit their metabolism [18–20], it did not inhibit fusion and, in fact, fusion products were larger than controls and multinucleated (Fig. 5).

TABLE IV

The effects of pretreatment of fusogenic cells with neomycin sulfate, kanamycin sulfate and gentamycin sulfate prior to cell wall digestion on the lipid composition of isolated fusogenic protoplasts

[^{3}H]Inositol-labeled fusogenic cells (0.3 g) were treated with 0.5 mM antibiotic for 20 min prior to cell wall digestion. Cells were incubated in Driselase for 2 h in the absence of aminoglycoside to release protoplasts. None of the treatments inhibited fusion, but treatment with neomycin produced significantly larger fusion products. These are representative data. The experiment has been repeated three times and the trends were consistent.

Treatment	% Total counts [^{3}H]recovered					Fusion
	PIP_2	LPIP	PIP	LPI	PI	(%)
Control	1.1	3.1	5.7	8.4	77.2	≈ 60
Neomycin	1.5	2.2	7.6	11.4	72.5	> 60
Kanamycin	0.9	2.4	6.9	7.9	78.5	≈ 60
Gentamycin	1.0	1.9	5.7	7.4	80.6	≈ 60

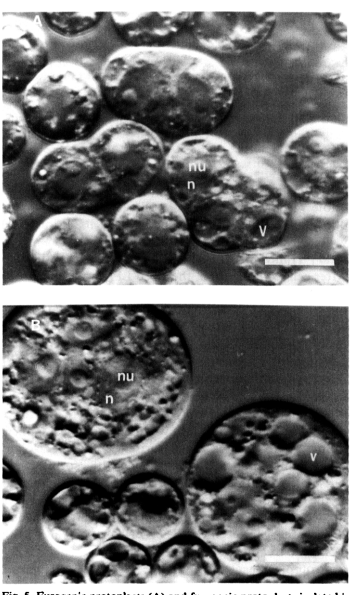

Fig. 5. Fusogenic protoplasts (A) and fusogenic protoplasts isolated in the presence of 0.5 mM neomycin sulfate (B). Note the numerous fusion products in (A). Treatment with neomycin produced large fusion products with many nuclei (B). Abbreviations: n, nucleus; nu, nucleolus; v, vacuole. Scale bar = 15 μm.

Discussion

The lysophospholipid, LPIP, can be formed by enzymatic hydrolysis of PIP via phospholipase A_2 or by phosphorylation of LPI. LPIP has been reported previously as being the product of in vitro phosphorylation of LPI [21]. It was thought, however, not to be of physiological significance since it had not been reported in natural systems. Recently, Kuroda et al. [22] have suggested that a new inositol lipid which was found after insulin stimulation of rat fat cells was LPIP. In this paper we have shown that LPIP is not only present in carrot cells, but its presence correlates positively with the fusion potential of the cells. This was true when comparing cells from the same culture as well as when comparing cells from fusogenic and nonfusogenic cultures. While the nonfusogenic cells and fusogenic cells with a low fusion potential contained LPIP, they contained predominantly the upper R_f analogue, *sn*-2-linoleoyl LPIP. Only the lower R_f analogue, *sn*-1-palmitoyl LPIP, correlated with the fusion potential.

Breakdown or metabolism of PIP_2 was proposed to be important in myoblast fusion [3,4]. The headgroup would be cleaved by the action of a phosphoinositide-specific phospholipase C yielding inositol 1,4,5-trisphosphate and DAG. DAG would then form a non-bilayer intermediate at the site of fusion. In addition, IP_3 could mobilize Ca^{2+} stores increasing intracellular Ca^{2+}, thereby enhancing fusion. If PIP_2 were involved in fusion in the fusogenic carrot protoplasts, then blocking the PIP_2 headgroup turnover would have blocked fusion. When headgroup turnover was blocked with the aminoglycoside neomycin sulfate, however, fusion was not inhibited. These data indicated that PIP_2 probably was not directly involved in the fusion of our carrot protoplasts.

Neomycin, but not kanamycin or gentamycin, increased LPI and seemed to enhance fusion significantly. The increase in LPI alone is not sufficient to make protoplasts fusion permissive since high levels of LPI are present in the nonfusogenic cells. Neomycin may have enhanced fusion by increasing cytosolic calcium; it has been shown to increase calcium mobilization, thereby activating phospholipase A and liberating arachidonic acid from PI in human platelets [23]. Increasing cytosolic calcium with the ionophore A23187 increased fusion of the fusogenic carrot protoplasts [24]. In addition, fusion was inhibited by EGTA and the calmodulin antagonists, trifluoperazine, chlorpromazine and W-13 [24].

Further evidence that PIP_2 metabolism is not involved in the fusion of fusogenic carrot protoplasts comes from studies of the inositol phosphates. Rincon et al. [25] have observed that although the fusogenic protoplasts release Ca^{2+} in response to exogenously added IP_3, no endogenous IP_2 or IP_3 could be detected in the fusogenic cells or protoplasts.

While the percentage of PIP and PIP_2 in the fusogenic cells did not correlate with fusion, the percentage of LPIP did correlate with the fusion potential of the protoplasts. Based on short-term [^{3}H]inositol and [^{32}P]P_i labeling studies (Wheeler et al., unpublished results), LPIP synthesis from PIP or LPI is not necessary during cell wall digestion but rather the level of LPIP in the cell prior to cell wall digestion is indicative of the final fusion potential. When the cell wall was removed from the fusogenic cells to form protoplasts, the 16:0 LPIP was preferentially lost. The fact that adding cell wall digestion enzymes to prelabeled membranes resulted in only the loss of the inositol-labeled 16:0 LPIP suggested that a lysolipid lipase was involved. In spite of the fact that there is a general increase in phospholipase A_2 activity using NBD-PC as a substrate [17], the effect of the cell wall digestion enzymes on the inositol lipids was specific and there was no evidence in vivo or in vitro for activation of PIP-specific phospholipase A_2 or A_1.

While the above data indicate that LPIP correlates positively with fusion, they bring to question the role of LPIP in fusion. Its mechanism of action may be explained by the following hypotheses. (i) The loss of LPIP during cell wall digestion would favor a nonbilayer intermediate and bilayer mixing; (ii) although 16:0 LPIP is lost during cell wall digestion, a minimal amount of residual LPIP (approx. 2% of the inositol-labeled lipid of the protoplasts) remains and this is sufficient to act as fusogen; (iii) LPIP enhances cation transport and thereby provides an osmotic driving force to enhance mixing of the bilayers; (iv) that LPIP is not involved in fusion but is only indicative of the fusion potential of the membrane.

The fact that the protoplasts with the higher 16:0 LPIP had the higher fusion potential favors hypothesis (ii) over (i). It is quite plausible, however, that LPIP plays no direct role in fusion and is only indicative of the fusion potential. $LPIP_2$ has been reported to enhance cation transport in liposomes [26]. Since fusion of the fusogenic protoplasts can be induced by osmotic stress alone [16], enhanced cation influx would enhance fusion. If, however, the role of LPIP is only to enhance ion transport, some other fusogen must be present, since osmotic stress alone will not cause fusion of the nonfusogenic protoplasts. In this regard, other lysophospholipids may be involved in fusion in this system. While LPI did not correlate with fusion potential, a role for lysophosphatidylcholine, which has been shown to facilitate fusion in fibroblast cells [27], has not been investigated.

Cell fusion appears to be developmentally regulated. Fusion of gametes occurs naturally. Protoplasts isolated from meiotic cells (e.g., pollen microsporocytes and pollen tetrads) also fuse spontaneously [27,28]. The

fusogenic carrot cells are derived from embryogenic cultures [9] and are maintained in a pre-embryogenic state. In contrast, the nonfusogenic carrot cells have completely lost the ability to differentiate. When the fusogenic cells become vacuolated or if they differentiate to form embryos, incorporation of [^{3}H]inositol into 16 : 0 LPIP decreases to the level of that in the nonfusogenic cells and fusion potential is lost. Furthermore, growing cells in the presence of the polyamine biosynthesis inhibitor, difluoromethylarginine, inhibits embryogenesis [30] and protoplast fusion [31]. These data suggested a correlation between development and the biogenesis or maintenance of the fusion-permissive plasma membrane. Whether the presence of 16 : 0 LPIP is associated with the developmental state of the cell and thereby is only indicative of the fusion potential of the cells, or whether it is directly involved in the fusion process, remains to be determined.

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