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PHOSPHOLIPIDS OF MEMBRANES OF CULTURED CELLS AND THE PRODUCTS OF PROTOPLAST FUSION

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Key Word Index—*Rauwolfia serpentina*; Apocynaceae; *Nicotiana tabacum*; *Atropa belladonna*; Solanaceae; *Bouvardia ternifolia*; Convolvulaceae; plant cell culture; phospholipids; phospholipid molecular species; protoplast fusion.

Abstract—The variety and distribution of phospholipids in the cell membranes of cultured cells and their fatty acid composition were analysed. Membranes of suspension cultured cells of *Rauwolfia serpentina* var. Bentham, *Nicotiana tabacum* var. Samsun, *Atropa belladonna* and *Bouvardia ternifolia* had almost the same composition of phosphatidylcholine, PC (*ca* 50%); phosphatidylethanolamine, PE (*ca* 25%); phosphatidylinositol, PI (*ca* 10%); phosphatidylglycerol, PG (several %); and phosphatidic acid, PA (several %). We determined the distribution of the molecular species of the three major phospholipid fractions (PC, PE and PI) in *R. serpentina* and *N. tabacum*. Membranes of both cell-types contained basically similar molecular species, 1-16:0/2-18:2 the main type, particularly in the PC- and PE-fractions; 1-18:2/2-18:2 and 1-18:0/2-18:2 for *R. serpentina*; and 1-16:0/2-18:3, 1-18:0/2-18:3 and 1-18:2/2-18:2 for *N. tabacum*. The influence of membrane fluidity on protoplast fusion as effected by the phase transition of the phospholipids, is discussed.

INTRODUCTION

Recent dramatic developments in protoplast fusion research suggest the possibility of the production of somatic hydrid cells. There are two questions associated with this study: how can we fuse protoplasts more easily and how can we separate useful, heterogenously fused protoplasts from homogenously fused protoplasts?

Protoplast fusion starts from the contact of membranes of two protoplasts; then, hopefully, fusion of the membranes occurs. Thus, the components of the cell membrane must be understood if we are to improve the process of protoplast fusion.

Fluidity of the membrane is probably important in the process of fusion. Fluidity is affected by the phase transition of phospholipids in the membrane; this transforms phospholipids from the gel to the liquid crystal phase, and *vice versa*. Even phospholipids that have the same polar head group vary in terms of their molecular species. Individual molecular species in phospholipids have inherent phase transition points; therefore, knowing the composition of the type of phospholipid and the relative distribution of the molecular species within the membrane is necessary in the study of protoplast fusion. There are no reports on this although a few papers have given the relative distributions of the phospholipid molecular species in *E. coli* and in some higher plants for other purposes [1–4]. Our data should aid future studies of protoplast fusion.

RESULTS

Suspension cultured cells were grown in the nutrient medium and subcultured as described in Experimental. Green cells of *N. tabacum* var. Samsun that had been cultured under light were used. They contained 2 mg/g dry wt of chlorophyll and had photosynthetic activity [5]. The other cells used had been cultured in the dark and were white or yellowish-white.

Table 1 shows the total content of phospholipids and

Table 1. Total phospholipids and relative distributions of the phospholipid types in cultured cells

Plant species	Total phospholipids mg/g wet wt	Percentage to total phospholipids (mol (%)) Type of phospholipid					
		PC	PE	PI	PG	PA	Unidentified
R. serpentina	1.53	55.0	20.1	10.2	2.0	2.5	10.3
N. tabacum	0.49	42.5	27.0	8.4	3.8	2.0	16.5
A. belladonna	0.65	51.1	28.1	9.6	7.8	tr*	3.5
B. ternifolia	0.83	53.6	26.2	8.6	7.3	0.5	3.9

Analytical details are described in Experimental.
*tr = trace.

Table 2. Relative distribution of the molecular species of suspension cultured cells of *R. serpentina*

Molecular species		Percentage to total molecular species		
		Type of phospholipid		
1-position	2-position	PC	PE	PI
18:2	18:2	12.1	32.7	tr
16:0	18:3	1.6	2.7	1.6
18:0	18:3	0.6	0.5	tr
16:0	18:2	73.7	59.1	92.3
18:0	18:2	9.9	4.6	5.4
18:1	18:1	2.1	0.4	0.6
18:0	18:1	tr	tr	0.1
$\bar{x}$*		2.26	2.69	2.02

Data represent mol (%), analytical details are described in Experimental.

Symbols for fatty acids represent: 16:0: palmitic acid, 18:0: stearic acid, 18:1: oleic acid, 18:2: linoleic acid, 18:3: linoleinic acid.

*Average of the degree of unsaturation (double bond index) in each type of phospholipid.

tr = trace.

the relative distribution of some types of phospholipids in the cultured cells. The phospholipid content of *R. serpentina* (1.53 mg/g wet wt) was high in contrast to that of *N. tabacum* (0.49 mg/g wet wt). These contents seem to reflect cell-size. In fact, the cells of *N. tabacum* were larger than those of *R. serpentina*.

In our plant materials, PC, PE and PI were the major types of phospholipid, and the percentage of each to the total phospholipids was *ca* 50, 25 and 10%, respectively. The contents of PG and PA were of the order of 2–7%. No trace of cardiolipin or phosphatidylserine was detected in any of our materials but unidentified lipids containing phosphorus were detected in all. Except for the low level of *N. tabacum* PC (42.5%) and *R. serpentina* PE (20.1%), the relative distributions of the individual phospholipid types in the four plant species were rather similar regardless of the culture conditions.

Table 3. Relative distribution of the molecular species of suspension cultured cells of *N. tabacum*

Molecular species		Percentage to total molecular species		
		Type of phospholipid		
1-position	2-position	PC	PE	PI
18:2	18:3	2.4	tr	tr
18:1	18:3	3.2	tr	tr
18:2	18:2	8.2	8.9	tr
16:0	18:3	15.6	13.2	16.9
18:0	18:3	8.1	10.5	tr
18:1	18:2	3.0	tr	tr
16:0	18:2	48.0	67.4	79.2
16:0	18:1	11.5	tr	3.9
$\bar{x}$*		2.45	2.42	2.13

Data represent mol(%), analytical details are described in Experimental.

Symbols for fatty acids are described in Table 2.

*Average of the degree of unsaturation (double bond index) in each type of phospholipid.

tr = trace.

We also analysed the phospholipid molecular species (which has an important role in changes in membrane fluidity) of the three major phospholipid types (PC, PE and PI), found in *R. serpentina* and *N. tabacum*. The lipids were extracted from the cells during the exponential growth phase. Tables 2 and 3 show the relative distribution of the molecular species. In the PC, PE and PI of both cultures, the 1-16:0/2-18:2-type was the major component; *ca* 70, 60 and 90% in the PC-, PE- and PI-fraction of *R. serpentina* and *ca* 50, 70 and 80%, respectively in *N. tabacum*. The next highest components were the 1-18:2/2-18:2-type and the 1-18:0/2-18:2-type for *R. serpentina*, and the 1-18:2/2-18:2-type, the 1-16:0/2-18:3-type and the 1-18:0/2-18:3-type for *N. tabacum*. Our data show that saturated fatty acids were generally combined at the 1-position of glycerol and that the more unsaturated fatty acids were at the 2-position. In the PI-fractions of both cultures, the 1-16:0/2-18:2-type predominated, but not in the PC- and PE-fractions. The degrees of unsaturation (double bond index) of the individual phospholipid fractions were 2.26, 2.69 and 2.02 in PC, PE and PI for *R. serpentina*, and 2.45, 2.42 and 2.13 for *N. tabacum*.

R. serpentina cells were cultured in the dark with 1 μM 2,4-dichlorophenoxyacetic acid and 1 μM kinetin as the growth regulators and *N. tabacum* cells were cultured with 10 μM α-naphthaleneacetic acid and 1 μM kinetin in light. In spite of the different growth conditions, both cell-types and basically comparable distributions of the molecular species in their PC-, PE- and PI-fractions.

DISCUSSION

We found that the distribution of the phospholipids in the cellular membranes of the four different types of cultured cells were almost equal, although the cells were cultured under various conditions. Furthermore, we determined the distribution of the phospholipid molecular species in the major phospholipid fractions of *R. serpentina* and *N. tabacum*. The molecular species can be classified according to the degree of unsaturation (number of total double bonds in fatty acyl residues) (Tables 2 and 3). These clarify the difference in the pattern of the molecular species between the two cultures. The pattern of *N. tabacum* showed more variety than that of *R. serpentina*. The patterns were basically similar, but differences in growth conditions (light, growth regulators, etc.) affected them.

In the two distributions of the molecular species, the predominance of the 1-16:0/2-18:2-type was more marked in the PI-fraction than in the PC- and PE-fractions. The distribution appears to control an activity of the cellular membrane; the change in membrane fluidity depending on the environmental temperature. Our data indicate that PI functions differently from PC and PE in the membrane.

Membrane fluidity is determined by the phase transition of phospholipids. Phase transition is deeply affected by temperature, the presence of such divalent cations as Ca^{2+}, the pH, and the relative distribution of the phospholipid molecular species. Each molecular species has its individual phase transition point. This point is

Table 4. Composition of the nutrient media for suspension cultured cells

Constituents	*R. serpentina*	*N. tabacum*	*A. belladonna*	*B. ternifolia*
Mineral salts	—#1	—#2	—#2	—#2
Organic substances				
2,4-D* (1 μM)	+	—	—	—
NAA† (10 μM)	—	+	+	+
Kinetin (1 μM)	+	+	—	—
6-Benzyladenine (μM)	—	—	1	0.01
Thiamine · HCl (mg/l)	0.4	0.8	0.4	0.4
Myoinositol (g/l.)	0.1	0.2	0.1	0.1

pH was adjusted to 5.7 with 0.2 N NaOH, sucrose 3% was present in all. #1 Modified Linsmaier and Skoog formula (nitrogen was the only nitrate). #2 Linsmaier and Skoog formula [8].

*2,4-Dichlorophenoxyacetic acid

†α-Naphthaleneacetic acid.

determined by the melting points of the fatty acids and the polar head group of the phospholipid.

Keller and Melchers [6], have reported that at a high pH and in the presence of a comparatively high concentration (50 mM) of Ca^{2+}, a rise in the incubation temperature was significantly effective for the protoplast fusion. Prives and Shinitzky [7] showed that a pre-fusional decrease in the microviscosity of the cell membrane caused by a rise in temperature triggered the transformation of mononucleated myoblasts to multinucleated myotubes.

These two phenomena and the information related to the phase transition of phospholipids led us to speculate that changes in the phase of phospholipids strongly affect the efficiency of protoplast fusion. Thus, we present the following hypothesis to account for a high frequency of protoplast fusion. When protoplasts fuse, the phospholipid molecules forming the bilayer seem to 'mix' and 'rearrange'. Clearly, the fluidity of the membrane (especially the plasma membrane), which is effected by the phase transition of phospholipids, is deeply involved in this process. The percentage of fused protoplasts may increase with an increase in membrane fluidity, and a concomitant decrease in the viscosity of the membrane. The most convenient method for increasing membrane fluidity is to raise the incubation temperature when the protoplasts fuse. This procedure may result in an increase in the amounts of phospholipids in the liquid crystal phase; if so, then the proportion of fused protoplasts would be greater. We may be able to create a new compound to increase the fluidity of membranes and to induce protoplast fusion in the higher frequencies under conditions harmless to the protoplasts.

In addition, the phospholipids that we analysed probably contained not only the phospholipids of the plasma membrane, but also those of the inner cellular membranes. However, Tables 2 and 3 show that the compositions of the molecular species were considerably specific, and that no cardiolipin (a marker for mitochondrial membrane) was present. We assume that our data in this method reflected the composition of the phospholipids and the distribution of the molecular species of the plasma membrane.

Proteins or glycoproteins in the plasma membrane also seem to play a significant role in protoplast fusion. Therefore, they also must be studied in future.

EXPERIMENTAL

Cell materials. An analysis of the phospholipids in the cellular membrane was conducted with cultured cells of *Rauwolfia serpentina* var. Bentham derived from the flower stalk, *Nicotiana tabacum* var. Samsun derived from pith, *Atropa belladonna* derived from seed and *Bouvardia ternifolia* derived from the flower stalk. Cells were maintained in liquid suspensions. The compositions of the nutrient medium are shown in Table 4. The basal constituents of the medium were those of the Linsmaier and Skoog formula [8]. Cultured cells of *R. serpentina*, *A. belladonna* and *B. ternifolia* were grown in the dark in 25 ml of nutrient medium in 100 ml Erlenmeyer flasks, which were shaken continuously. Cells of *N. tabacum* were grown in 75 ml of nutrient medium in 300 ml Erlenmeyer flasks, and were shaken under an illumination of 4000 lx from a fluorescent lamp. The temp. for all the cultures was a constant 25°. The cultures of *R. serpentina* and *A. belladonna* were subcultured every 7 days, and those of *N. tabacum* and *B. ternifolia* every 14 days. Cells were harvested by centrifugation; *B. ternifolia* cells 7 days after subculture, and the others 5 days after.

Lipid extraction and the distribution of some phospholipids. Lipids were extracted according to the method of Bligh and Dyer [9] after inactivation of the lipase in the cells with cold 5% TCA. The total content of phospholipids was quantified (via P) [10] and lipids were separated by 2 D TLC in $CHCl_3$–MeOH–7N NH_3 (200:120:15) and the second $CHCl_3$–MeOH–Me_2CO–HOAc–H_2O (40:30:20:3:2). Each spot was identified by comparing its R_f value with that of an authentic standard. Individual spots were scraped from the plates and the lipids were extracted with $CHCl_3$–MeOH (2:1). The distribution of the phospholipids was determined as before in the individual spots [10].

Analysis of phospholipid molecular species. Phospholipid molecular species were analysed by the method of Kito *et al.* [1, 2] with the following modification; 0.5–2.0 mg of isolated phospholipids (PC, PE and PI) were suspended in 1.0 ml of H_2O then dispersed by sonication. The suspensions were vigorously shaken with 50 μl of 210 mM Tris–HCl buffer (final concn 10 mM, pH 7.4) containing 10 U of phospholipase C (EC 3.1.4.3, from *Bacillus cereus*) and 2 ml of Et_2O at 30°. The solvent for $AgNO_3$-TLC was C_6H_6–$CHCl_3$–MeOH (98:2:1). The separated monoacetyldiglycerides (MADGs) were analysed by GLC on a 0.3 × 35 cm stainless steel column packed with 3% Dexsil 300 on Anachrome ABS (Analabs), 70–80 mesh, by increasing the temp. from 200° to 300° at 6°/min: N_2 flow rate: 60 ml/min. Each molecular species was identified by a comparison of its R_f value from $AgNO_3$–TLC and its RR_t from GLC with those of authentic standards. The MADGs were quantified with tricaprylin as the internal standard.

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