Plant Physiol. (1980) 65, 1099–1102
0032-0889/80/65/1099/04/$00.50/0

Protoplast Fusion

EFFECT OF LOW TEMPERATURE ON THE MEMBRANE FLUIDITY OF CULTURED CELLS

Received for publication September 11, 1979 and in revised form January 15, 1980

YASUYUKI YAMADA, YASUHIRO HARA, HIROAKI KATAGI, AND MITSUGI SENDA
Department of Agricultural Chemistry, Kyoto University, Kyoto 606, Japan

ABSTRACT

The relation between the composition of the phospholipid molecular species in a cell membrane and the velocity of protoplast fusion was studied using cells cultured at a low temperature (10 C). Cells cultured at a low temperature contained larger proportions of phospholipids of low phase transition point, the 1,2-dilinoleoyl-type, than those cultured at a normal temperature (25 C). When treated with polyethylene glycol 6000, protoplasts from cells cultured at 10 C fused and progressed to the fused sphere stage more rapidly than did those from cells cultured at 25 C.

The compositions of phospholipids and of the fatty acids in phospholipids, glycolipids, etc. have been analyzed in studies on frost resistance (15) and in the activity of photosynthesis (14) in plants. The composition of the fatty acids in the phospholipid fraction has been shown to be related to temperature (9, 12, 15). These studies also have shown that saturated fatty acids (16:0[1]) increased when plants were grown at a high temperature and that unsaturated fatty acids (18:2 and/or 18:3) increase when grown at a low temperature. Unsaturated fatty acids have lower Tm (18:2, −5 C; 18:3, −11 C) than saturated fatty acids (16:0, 63 C). Cells cultured at a low temperature require a more fluid membrane than those cultured at standard temperature; thus, the increase in unsaturated fatty acids means that molecular species with low Pc should increase.

The increase in membrane fluidity, as determined by the phase transition of phospholipids, was applied to the improvement of protoplast fusion in this research. Protoplast fusion is important in biochemical and genetic engineering studies, but many details regarding its mechanism are not known. Membrane fluidity may affect the rate of protoplast fusion.

We previously proposed that membrane fluidity is determined by the phase transition of the phospholipid molecular species (3, 17). In the present study we have analyzed the relative distributions of the phospholipid molecular species in cells cultured at two different temperatures (10 and 25 C) and found that protoplasts whose membranes have more unsaturated fatty acids increased the speed of protoplast fusion.

MATERIALS AND METHODS

Conditions for Cell Culture. Suspension-cultured cells of *Rauwolfia serpentina* var. Bentham were used. Growth conditions for the culture have been described in a previous paper (18). Stock cells were cultured at 25 C with subcultures every 7th day. To analyze the phospholipid molecular species and to measure the speed of protoplast fusion, the following types of cells were used: A, cells cultured at 25 C for 5 days; B, cells cultured at 25 C for 7 days; and C, cells cultured at 25 C for 5 days, then at 10 C for 2 days.

Analysis of Phospholipids. The relative distribution of the molecular species in the PC, PE, and PI fractions of A, B, and C cells were determined as described in previous papers (8, 11, 17).

Isolation of Protoplasts and Measurement of the Speed of Protoplast Fusion. Protoplasts were isolated from A, B, and C cells as described previously (18), but with some modifications (Cellulase Onozuka R-10 1% [w/v], Macerozyme R-10 0.2% [w/v], potassium dextran sulfate 0.5% [w/v], sorbitol 0.3 M, mannitol 0.3 M). After incubation for 40 min (35 C) of the cell-enzyme mixture, the resulting protoplasts were rinsed with a solution containing 0.3 M sorbitol, 0.3 M mannitol and 50 mM $CaCl_2$. The duration of the rinse was 60 min. Forty μl of a solution of 40% (w/v) PEG 6000 containing 10 mM $CaCl_2$ was added to 20 μl of a protoplast suspension whose density had been adjusted to 10^{-5} cells at room temperature (25 ± 2 C).

Protoplasts treated with PEG 6000 adhere to one another at one point (point adherence stage); then these point adherent protoplasts proceed to the next stage in which the protoplasts adhere closely face to face (face adherence stage). In time, these face adherent protoplasts fuse to form a single spherical or oval protoplast (fused sphere stage).

In this experiment, a total of about 500 protoplasts were counted under a microscope at 5, 30, 60, 90, and 120 min after the addition of PEG solution (15 replicated experiments).

RESULTS

Cell Growth and Composition of Phospholipids. The standard cultured cells at 25 C went into the exponential growth phase after about a 24-h lag phase (Fig. 1). To produce C cells, cultured cells were transferred from 25 to 10 C, on the 5th day at 25 C. These cells at 10 C stopped dividing, but protoplasmic streaming was observed as long as 1 week later.

Compositions of the phospholipids in A, B, and C cells are shown in Table I. All three cell types had similar compositions: major phospholipids, PC: 55–62%, PE: 22–26%, PI: 8–11%; and the minor phospholipid, phosphatidylglycerol: trace-3%. Phosphatidic acid, detected in a previous experiment (18), was not found in this analysis. Trace amounts of phosphatidylserine, lyso-PC, and lyso-PE were present.

Relative Distribution of Molecular Species. Relative distributions of the molecular species in the PC, PE, and PI fractions of A, B, and C cells are shown in Table II. The main molecular species were the 1-16:0/2-18:2-type and the 1-18:2/2-18:2-type in the PC and PE fractions. In the PI fraction, the 1-16:0/2-18:2-type predominated. The 1-18:3/2-18:3, 1-18:2/2-18:3 and 1-18:1/2-

[1] Abbreviations: 16:0: palmitic acid or palmitoyl group; 18:0: stearoyl; 18:1: oleoyl; 18:2: linoleic acid or linoleoyl group; 18:3: linolenic acid or linolenoyl group; Pc: phase transition point; PC: phosphatidylcholine; PE: phosphatidylethanolamine, PI: phosphatidylinositol; PG: phosphatidylglycerol; PEG: polyethylene glycol.

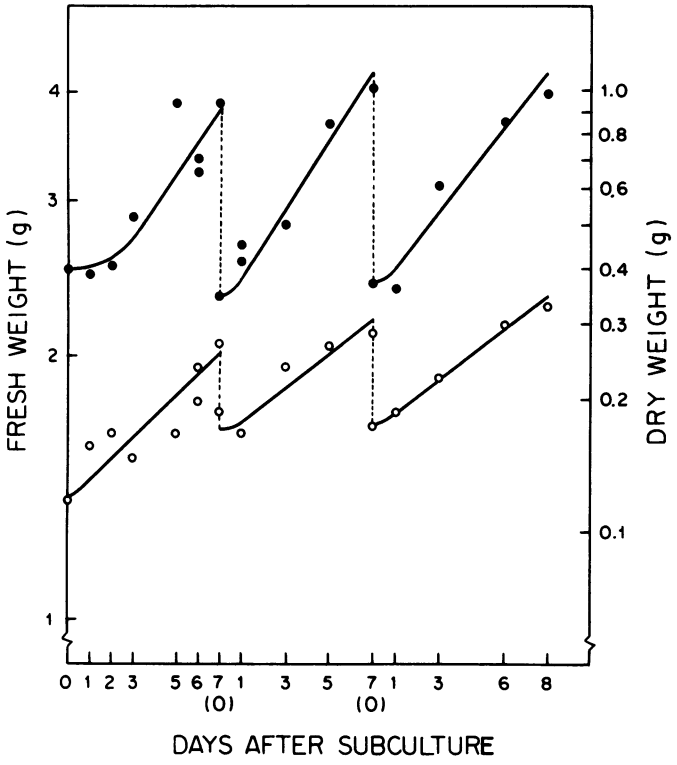

FIG. 1. Growth curves for the suspension culture of *R. serpentina*. Cells were subcultured every 7 days. Fresh and dry weights of the cells were indicated in (●) and (○), respectively.

Table I. *Total Cell Numbers and Compositions of Phospholipids in Rauwolfia Suspension-cultured Cells Cultured at Two Different Temperatures*

Lipids extracted from cultured cells were separated by two-dimensional TLC; phospholipids were quantified via phosphorus.

Type of Culture	Cell Numbers per Flask[b]	Total Phospholipids				
		PC	PE	PI	PG	Unidentified
	cells × 10^7	*mol %*				
A[a] (start)	7.5	54.9	26.3	10.8	2.8	5.2
B (control)	8.3	61.8	22.9	8.3	tr[c]	6.9
C (treated)	7.4	55.5	21.9	11.2	2.2	9.1

[a] A: cells cultured at 25 C for 5 days; B: cells cultured at 25 C for 7 days; C: cells cultured at 10 C for 2 days after a 5-day culture at 25 C.

[b] For every subculture, about 4.8 × 10^7 cells were inoculated into 25 ml of conditioned medium.

[c] tr: trace.

18:3-types in the PC, PE, and PI fractions of A, B, and C were all found as traces. In the 7-day culture (B cells), the 1-16:0/2-18:2-type in the PE fraction was slightly less than in the 5-day culture (A cells; from 79.1 to 72.8%) and the 1-18:2/2-18:2-type was slightly greater (from 10.1 to 14.9%) in the PC fraction of A and B cells. In C cells, the increase in the 1-18:2/2-18:2-type (PC, from 10.1 to 26.2%; PE, from 16.1 to 26.2%) and the decrease in the 1-16:0/2-18:2-type (PC, from 79.1 to 63.3%; PE, from 77.7 to 66.9%), were remarkable in the PC and PE fractions in comparison with the amounts found in A cells. No change in the proportions of the 1-16:0/2-18:2-type and the 1-18:2/2-18:2-type was found in the PI fraction. The average degree of unsaturation ($\bar{x}$) reflects this change in the proportions of the molecular species; for example,

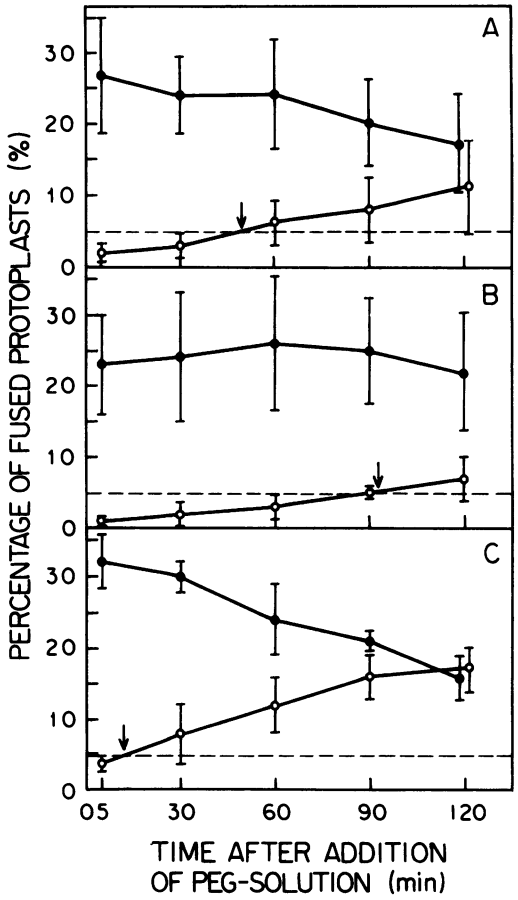

FIG. 2. Velocity of the fusion of protoplasts from a *R. serpentina* suspension culture. A (start): protoplasts from cells cultured at 25 C for 5 days; B (control): protoplasts from cells cultured at 25 C for 7 days; C (treated): protoplasts from cells cultured at 10 C for 2 days after a 5-day culture at 25 C. Face adherent (●) and fused sphere (○) protoplasts as a percentage of the total number of counted protoplasts (about 500). Arrows indicate the time needed to reach the fused sphere stage, for 5% of all the protoplasts treated with PEG solution.

$\bar{x}$ in the PC fraction increased from 2.20 (A) to 2.31 (B) or to 2.58 (C).

Velocity of Protoplast Fusion. More than 10% of the cells formed protoplasts in all the cultures (A, B, and C). Production of fused sphere protoplasts and face adherent protoplasts was higher in C cells than in A and B cells 5 min after the addition of PEG. The total percent of fused sphere protoplasts and face adherent protoplasts in A, B, and C cells at 5 min was about 29, 24, and 36%, respectively, with the majority being face adherent protoplasts in each treatment (Fig. 2). The time required to progress to the fused sphere stage, for 5% of all the protoplasts treated with the PEG solution was 10–15 min for C, about 50 min for A, and about 100 min for B (Fig. 2).

DISCUSSION

Phospholipid molecules are in the gel phase below and in the liquid crystal phase above their Pc. Although the Pc of the 1-18:2/2-18:2-type and the 1-16:0/2-18:2-type is not known, the 1-18:2/2-18:2-type surely would have a lower Pc than the 1-16:0/2-18:2-type in view of the Tm of the structural fatty acids. The membranes of cells cultured at 10 C for 2 days after being cultured at 25 C for 5 days contained more of the 1-18:2/2-18:2-type than did those whose culture continued at 25 C. The cell membranes of the 10 C-treated cells must have more fluidity because of the increase in

Table II. *Relative Distributions of Molecular Species in PC, PE, and PI Fractions with Three Types of Cells*

Monoacetyldiglycerides derived from phospholipids were separated with $AgNO_3$-TLC and were quantified with GLC in each phospholipid fraction.

Molecular Species		Total Molecular Species								
1-position	2-position	A[a] (start)			B (control)			C (treated)		
		PC	PE	PI	PC	PE	PI	PC	PE	PI
						mol %				
18:2	18:2	10.1	16.1	1.0	14.9	17.7	0.6	26.6	26.2	1.2
16:0	18:3	0.9	2.0	1.2	1.2	0.6	2.0	2.0	1.3	2.2
18:0	18:3	tr[c]	0.3	0.2	0.3	0.3	0.4	tr	0.4	0.5
18:1	18:2	1.8	1.4	tr	2.5	2.6	0.3	3.3	2.4	1.1
16:0	18:2	79.1	77.7	91.7	72.8	74.8	90.5	63.3	66.9	92.9
18:0	18:2	4.8	1.7	1.5	4.5	2.7	2.8	2.8	1.3	2.1
18:1	18:1	tr	0.4	0.2	0.6	0.3	0.6	0.4	0.3	tr
16:0	18:1	3.3	0.4	3.1	2.3	0.6	2.8	1.4	0.6	tr
18:0	18:1	tr	tr	1.1	1.0	0.5	tr	0.2	0.5	tr
$\bar{x}$[b]		2.20	2.36	1.99	2.31	2.38	2.01	2.58	2.55	2.06

[a] Samples were named as described in the footnote to Table I.

[b] Average of the degree of unsaturation; average number of double bonds in fatty acyl residues in each phospholipid fraction.

[c] tr: trace.

the molecular species with low Pc, *i.e.* the 1-18:2/2-18:2-type. No change in the PI fraction was found in the relative distributions of the molecular species in A, B, and C. This suggests that the function of PI in the cell membrane differs from that of PC or PE. Taking advantage of this change in the relative distribution of the fatty acids we succeeded in accelerating protoplast fusion.

Cultured *Rauwolfia* cells underwent few divisions at 10 C (Table I), but the relative distribution of the phospholipid molecular species in the C cells was distinct from that in the B cells (Table II). Therefore, at 10 C, cultured *Rauwolfia* cells at least underwent a turnover of their membrane phospholipids. The response rate of the changes in the phospholipid molecular species to temperature probably depends upon this turnover of phospholipids.

There are two methods of using temperature to fuse protoplasts effectively. The first is to raise the incubation temperature to increase membrane fluidity when the protoplasts are treated with fusogen, chemical and viral agents that cause membranes to fuse (7, 13). This rise in temperature causes the structural phospholipids to change from the gel to the liquid crystal phase; this increase in fluidity accelerates protoplast fusion. The second method is to culture plant cells at a low temperature before isolating the protoplasts in order to form a cell membrane that consists of more fluid components. Low temperature culture (*e.g.* 10 C) leads to an increase in the proportion of phospholipids of low Pc; the resulting phospholipids in the liquid crystal phase increase the fusion rate at a normal temperature (*e.g.* 25 C). We observed that the frequency of face adherent protoplasts was determined mainly by the density of the protoplasts treated with PEG solution. An increase in fluidity affected the transition from the face adherence stage to the fused sphere stage. Rivera and Penner (12) reported that the composition of fatty acids in soybean root cells responded to an alteration in temperature and changed within 2 days. Our study also indicates that the change in the plasma membrane takes place within 2 days.

The method used to extract phospholipids in this experiment (1), can be used for both the plasma membrane and part of the inner membrane (but not all of it because of the absence of cardiolipin as the mitochondrial marker). Kagawa *et al.* (5) and Morré (10) reported that the radioactive precursor of phospholipid first is incorporated in ER after which radioactivity appears in order in the Golgi body, mitochondria, and plasma membrane. Phospholipids of the isolated plasma membrane alone should be analyzed in the future.

Cultures of heterocaryocites obtained by treating protoplasts with fusogen PEG (4, 6, 16), $NaNO_3$ (2), or a high concentration of Ca^{2+} at a high temperature (7) have been reported. A shorter treatment period with low concentrations of fusogen is better for the subsequent culture of the fusion products because fusogens disrupt plant protoplasts. The method reported in this paper is advantageous because it enables protoplast fusion to proceed quickly.

Acknowledgment—We are most grateful to Dr. T. Suzuki of The Research Institute for Food Science Kyoto University, for his kind advice about the GLC analysis.

LITERATURE CITED

1. Bligh EG, WJ Dyer 1965 A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917
2. Carlson PS, HH Smith, RD Dearing 1972 Parasexual interspecific plant hybridization. Proc Nat Acad Sci USA 69: 2292–2294
3. Chapman D 1973 Physical chemistry of phospholipids. *In* GB Ansell, JN Hawthorne, RMC Dawson, eds, Form and Function of Phospholipids. Elsevier, New York, pp 117–141
4. Haydu Z, G Lázár, D Dudits 1977 Increased frequency of polyethylenglycol induced protoplast fusion by dimethylsulfoxide. Plant Sci Lett 10: 357–360
5. Kagawa J, JM Lord, H Beevers 1973 The origin and turnover of organelle membranes in castor bean endosperm. Plant Physiol 51: 61–65
6. Kao KN, MR Michayluk 1974 A method for high-frequency intergeneric fusion of plant protoplasts. Planta 115: 355–367
7. Keller WA, G Melchers 1973 The effect of high pH and calcium on tobacco leaf protoplast fusion. Z Naturforsch 28c: 737–741
8. Kito M, M Ishinaga, M Nishihara, K Kato, S Sawada, T Hata 1975 Metabolism of the phosphatidylglycerol molecular species in *Escherichia coli.* Eur J Biochem 54: 55–63
9. Mangold HK 1978 The common and unusual lipids of plant cell cultures. *In* W Barz, E Reinhard, MH Zenk, eds, Plant Tissue Culture and its Biotechnological Application. Springer-Verlag, Heidelberg, pp 55–65
10. Morré DJ 1970 *In vivo* incorporation of radioactive metabolites by Golgi apparatus and other cell fractions of onion stem. Plant Physiol 45: 791–799
11. Nishihara M, K Kimura, K Izui, M Ishinaga, K Kato, M Kito 1975 Phosphatidylethanolamine molecular species of fatty acid auxotroph of *Escherichia coli* grown with elaidate. Biochim Biophys Acta 409: 212–217
12. Rivera CM, D Penner 1978 Rapid changes in soybean root membrane lipids with altered temperature. Phytochemistry 17: 1269–1272
13. Senda M, H Morikawa, H Katagi, T Takada, Y Yamada 1980 Effect of temperature on membrane fluidity and protoplast fusion. Theor Appl Genet 56: In press
14. Siebertz HP, E Heinz, L Bergmann 1978 Acyl lipids in photosynthetically active tissue cultures of tobacco. Plant Sci Lett 12: 119–126
15. Smoleńska G, PJC Kuiper 1977 Effect of low temperature upon lipid and fatty acid composition of roots and leaves of winter rape plants. Physiol Plant 41:

29–35
16. WALLIN A, K GLIMELIUS, T ERIKSSON 1974 The induction of aggregation and fusion of *Daucus carota* protoplasts by polyethylene glycol. Z Pflanzenphysiol 74: 64–80
17. YAMADA Y, Y HARA, M SENDA, M NISHIHARA, M KITO 1979 Phospholipids of membranes of cultured cells and products of protoplast fusion. Phytochemistry 18: 423–426
18. YAMADA Y, M NAKAMINAMI 1973 The isolation and fusion of protoplasts from alkaloid plant cells cultured *in vitro*. *In* Protoplastes et Fusion de Cellules Somatiques Végetales. l'Institut National de la Recherche Agronomique, Paris, pp 373–383