

Fusion of Plant Protoplasts Induced by a Positively Charged Synthetic Phospholipid

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1,2-O-Dipentadecylmethylidene-glycerol-3-phosphoryl-(N-ethylamino)-ethanolamine, Dioxolane ring, ζ -Potential

A newly synthesized phospholipid, 1,2-O-Dipentadecylmethylidene-glycerol-3-phosphoryl-(N-ethylamino)-ethanolamine, having positive charge was found to exercise fusion activity on plant protoplasts. This phospholipid is not found in cells and has an unusual chemical structure with a dioxolane ring. Some properties of this compound are discussed in relation to phospholipid-induced cell fusion.

Introduction

Cell fusion of plant protoplasts has been achieved by various methods [1–4]. The possibility of developing new plant breeding methods by using cell fusion products has attracted much attention (see review of Melchers [15 and 5]). On the other hand, cell fusion of animal cells has been carried out since the introduction of inactivated Sendai virus (HVJ) [6, 7]. The fusion activity of this virus was shown to be located in the outer membrane of virions, and the reconstituted outer membrane, composed of phospholipid and polysaccharide, still showed fusion activity [8]. Furthermore, in some cases cell fusion of animal cells was performed using lipid vesicles (liposomes) [9]. Martin and MacDonald [10] utilized positively charged lipid vesicles to induce cell-fusion. These vesicles prepared from lecithin, lysolecithin and stearylamine were positively charged because of the presence of stearylamine. The increase in ζ -potential caused by the latter compound paralleled the increase in cell fusion activity.

We have attempted to change the negative surface charge of plant protoplasts by using positively charged phospholipids in order to establish a technique for inducing specific fusion between two different plant protoplasts [15] and (Nagata, Eibl and Melchers, in prep.). As reported below, we also found during these studies that protoplast fusion can be induced by a synthetic phospholipid which is not present in nature.

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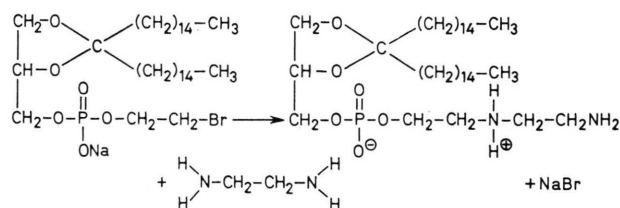
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Materials and Methods

Protoplasts were obtained from diploid (Xanthi nc) and haploid (s and v) tobacco (*Nicotiana tabacum* L.) by successive treatment of leaves with Macerozyme R 10 and Cellulase Onozuka R 10, essentially as reported by Takebe *et al.* [11].

The ζ -potential of protoplasts was determined with a cell electrophoresis apparatus according to Nagata and Melchers [12].

The phospholipid used in this experiments, 1,2-O-dipentadecylmethylidene-glycerol-3-phosphoryl-(N-ethylamino)-ethanolamine, was synthesized via the bromoethylester [13, 14] by amination with ethylenediamine.



1,2-O-Dipentadecylmethylidene-glycerol-3-phosphoric acid bromoethylester, 7.3 g (0.01 mol) was dissolved in 90 ml chloroform. After the addition of 150 ml 2-propanol and 200 ml of ethylenediamine (40% solution in water) the solution was heated to 50 °C for 24 h, when the starting bromoethylester was no longer detected by thin layer chromatography. The organic solvents were removed by evaporation. Acetonitrile was added to the residue and the crystals formed were collected. The reaction product was purified by column chromatography on silica gel Mallinckrodt Silic AR (100 to 200 mesh). Mixtures of chloroform, methanol and aqueous



ammonia (25%) were used for elution. Starting with $\text{CHCl}_3/\text{CH}_3\text{OH}/\text{ammonia}$ in a ratio of 200/15/1 (v/v) the polarity of the solvent mixture was raised by increasing the ratio stepwise up to 100/100/30 (v/v) to elute the product. The purity of the compound, 4.9 g (71% yield) of 1,2-O-dipentadecylmethylidene-glycerol-3-phosphoryl-(N-ethylamino)-ethanolamine, was determined by elemental analysis; mol wt calculated for $\text{C}_{38}\text{H}_{79}\text{N}_2\text{O}_6\text{P}$, 691.0; C 66.05%; H 11.52%; N 4.05%; P 4.48%; found: C 66.11%; H 11.63%; N 4.04%; P 4.70%.

Liposomes were prepared by sonication with Branson Sonifier at room temperature for 5 min.

The fusion medium contained 1 mM phospholipid, 0.05 M CaCl_2 and 0.7 M mannitol. This solution was filtered through Millipore filter GS (pore size $0.22 \mu\text{m}$). Protoplasts ($5 \times 10^5/\text{ml}$) were suspended in this medium at 30°C for 30 min.

Results

The newly synthesized phospholipid, 1,2-O-dipentadecylmethylidene-glycerol-3-phosphoryl-(N-ethylamine)-ethanolamine, was used throughout the experiments. This phospholipid is characterized by the presence of one negative charge (phosphate) and two positive charges (two ammonium groups) per molecule within the pH-region from 1 to 8 which corresponds to one positive net charge per phospholipid molecule.

Treatment of tobacco mesophyll protoplasts with phospholipids

Early indication of cell fusion in the mesophyll protoplasts was already observed after 5 minutes of incubation with the phospholipid vesicles, and cell fusion reached a plateau after 20 min incubation. We compared this cell fusion method to those reported before [1, 2, 4]. In the other methods cell fusion was observed at the earliest after 30 min of incubation, and where PEG or PVA was used, fusion occurred only after these compounds had been washed out. Thus the cell fusion reported here was very fast.

The cell fusion percentage was determined after 30 min of incubation and the values differed from one experiment to another, ranging from 10–30% for diploid tobacco and 5% for haploid. In contrast cell fusion induced by PEG or PVA was much higher and reached 40 to 50% (Table I). In the work of

Table I. Fusion percentage of different fusion methods of protoplasts.

Chemicals	%
Phospholipid used here	10–30
PEG # 4000 *	40
PVA # 203 **	45

* PEG # 4000 (Polysciences, Inc.) was utilized essentially according to Kao and Michayluk [2].

** PVA # 203 (Kurare Co. Ltd.) was utilized according to Nagata [4] with some improvements (to be published).

*, ** Fusion percentage was determined after washing out of the chemicals with a solution of Keller and Melchers [1].

Keller and Melchers [1] the cell fusion percentage was reported to be ca. 20–50%.

The ζ -potential of the phospholipid-treated protoplasts was determined using a cell electrophoresis apparatus according to Nagata and Melchers [12]. After treatment with the synthetic phospholipid, protoplasts were washed once with a solution containing 0.7 M mannitol and 0.05 M CaCl_2 . The decrease in the ζ -potential was accompanied by a large aggregation of protoplasts. Other phospholipids used to change the surface charges made the ζ -potential of protoplasts slightly positive ([15] and Nagata, Eibl and Melchers in prep.).

Culture of phospholipid-treated protoplasts

After phospholipid treatment protoplasts were washed once with a solution containing 0.7 M mannitol and 0.05 M CaCl_2 , and were cultured in the medium of Nagata and Takebe [16] under the illumination according to Enzmann-Becker [17]. After 1 day of culture, 70% of the lipid-treated protoplasts survived, as compared to 90% of the non-treated controls. However, the percentage of surviving cells did not decrease further on the second day or later. The fused cells were also able to divide at the same rate as the controls. Thus the toxicity of this phospholipid, if any, is not deleterious to tobacco protoplasts.

Factors affecting cell fusion

The fusion medium contained 0.05 M CaCl_2 . This concentration seemed to be optimal and removal of CaCl_2 damaged the protoplasts.

Filtration of the fusion medium through a membrane filter of pore size $0.22 \mu\text{m}$ was necessary not only for sterilization but also fusion itself. In unfiltered medium fusion was either absent or very

limited. This phenomenon suggests that sonication does not fully disrupt the multilamellar vesicles and that larger vesicles remaining in unfiltered medium perturb the cell fusion process.

Discussion

It was shown in this communication that a synthetic positively charged phospholipid with a dioxolane ring could induce cell fusion of protoplasts. The positive charges of this phospholipid induce adhesion of different protoplasts. The repulsion by negative charges on the protoplast surface was reduced by the addition of positively charged phospholipid, as was shown in our previous work [12]. The dioxolane ring of this phospholipid causes the adhered protoplasts to continue the fusion process. Lucy [18] utilized lysolecithin to induce cell fusion of animal cells. He stated that the membrane structure had to be disturbed to bring about cell fusion, and that this was accomplished by the use of lysolecithin. In our preliminary experiment we examined the effect of lysolecithin on fusion of

protoplasts but only found that they lysed. On the other hand, the phospholipid with the dioxolane ring induced cell fusion without deleterious effects, although it might have disturbed the membrane structure.

Thus, the positive charge and the dioxolane ring of this phospholipid are very important for cell fusion. It is highly desirable to synthesize derivatives of this phospholipid because they may induce fusion of protoplasts with much higher frequency than reported here.

Lastly it should be stressed that the phospholipid which induced fusion in these works was relatively simple in contrast to the mixtures of several phospholipids which were used previously for that purpose [9, 10]. By the use of a simple phospholipid the cell fusion mechanism could be elucidated more easily.

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