Aggregation of Plant Protoplasts by Artificial Lipid Vesicles

Received for publication July 21, 1981 and in revised form October 5, 1981

HIROFUMI UCHIMIYA, NAOI KUDO, TOSHIFUMI OHGAWARA, AND HIROSHI HARADA
Institute of Biological Sciences, University of Tsukuba, Sakura-mura, Ibaraki-ken 305, Japan

ABSTRACT

Sonicated unilamellar lipid vesicles, consisting of egg lecithin, stearylamine, and cholesterol in 7:2:1 molar ratios, promoted the aggregation of tobacco (Nicotiana glutinosa) protoplasts with the aid of mono- or divalent cations.

A reaction mixture containing liposomes (0.4 micromoles lipid per milliliter), 50 millimolar CaCl₂, 0.5 molar mannitol, and 5 x 10⁶ protoplasts per milliliter resulted in approximately 25% protoplast aggregation. To achieve the maximum protoplast aggregation, 1.6 x 10⁶ liposomes per protoplast per hour would be required.

The kind of liposomes effective in protoplast aggregation were positively charged, small-size vesicles which were obtained either by 60-minute sonication or by membrane filtration in conjunction with sonication.

RESULTS

Influence of Ions. As illustrated in Figure 1, Na⁺ was more effective than was K⁺ in inducing protoplast aggregation; with 100 mM NaCl, there was no protoplast survival. A similar relationship was observed for the divalent cations Ca²⁺ and Mg²⁺ (Fig. 2). A typical view of the aggregation of protoplasts in the presence of both liposomes and added salts is presented in Figure 3. Although the highest frequency of protoplast aggregation was in the neighborhood of 20 to 30% total protoplast population, Na⁺ and Mg²⁺ caused the formation of a cluster-like aggregation, whereas Ca²⁺ induced a few protoplasts to conjugate.

Liposome Concentration. Liposome-induced protoplast aggre-

Artificial lipid vesicles (liposomes) have been used as potential vehicles to transfer biologically active materials into living cells (4). Furthermore, liposomes were proven to induce fusion of cultured mammalian cells (3) and of plant protoplasts (2). In previous communications, we have demonstrated that multilamellar or reverse-phase evaporation lipid vesicles can be used as effective vehicles to insert materials such as fluorescein diacetate (5) or plasmid DNA (6) into plant protoplasts. In the latter case, we presented evidence suggesting that a combination of sodium ions and positively charged lipid vesicles might cause protoplast aggregation. This report describes the conditions favorable for liposome-mediated aggregation of plant protoplasts. It is hoped that the experimental data discussed in this communication provide useful information for the study of the interaction of artificial lipid membrane and plant protoplasts.

MATERIALS AND METHODS

Protoplasts. Nicotiana glutinosa protoplasts were prepared from suspension-cultured cells, according to a method reported elsewhere (7).

Liposomes. To maintain a model system to investigate the interaction of liposomes and plant protoplasts, we used small SUV liposomes throughout (3). In a standard procedure, egg lecithin, stearylamine, and cholesterol in 7:2:1 molar ratios were dissolved in 2 to 3 ml chloroform in a 10-ml round-bottom flask, which was then rotated under a vacuum. (The lecithin, stearylamine, and cholesterol were products of P. L. Biochemical.) The dried film from a flask was suspended in 1 ml 0.5 M mannitol solution and sonicated for 1 h at 4°C in a bath-type sonicator. The emulsion thus obtained was used as a source of liposomes.

Incubation of Protoplasts with Liposomes. One-tenth ml of protoplast suspension (10⁵-10⁶ protoplasts/ml) in 0.5 M mannitol was mixed with an equal volume of liposome suspension for 30 min at 25°C, then centrifuged at 150g for 1.5 min to pellet protoplasts. The protoplasts were then resuspended in 0.5 M mannitol solution, containing 50 mM CaCl₂, and centrifuged at 150g for 10 min. The resulting pellets were resuspended in a 0.5 M mannitol-50 mM CaCl₂ solution and used for investigation.

Aggregation and Survival of Protoplasts. Unlike the aggregation of protoplasts induced by polyethylene glycol treatments, liposome-mediated aggregation rarely resulted in complete fusion of protoplasts, which is designated 'fused sphere stage' by Yamada et al. (8). Thus, we use the term 'aggregation' to denote the coherence of protoplasts modulated by liposomes. Extent of protoplast aggregation was expressed as the percentage of the protoplasts participating in aggregation. To estimate protoplast aggregation, at least 200 protoplasts were counted. Using the same specimen, the number of protoplasts remaining intact after liposome treatment was counted and expressed as percentage of surviving protoplasts.

Fig. 1. Effects of KCl (a) or NaCl (b) on the aggregation and survival of N. glutinosa protoplasts. In each experiment, approximately 10⁶ protoplasts/ml were incubated in a solution containing liposomes (1.58 μmol lipid/ml) and 0.5 M mannitol for 30 min at 25°C. Handling and the composition of liposomes were as stated in "Materials and Methods."
Fig. 2. Effects of CaCl$_2$ (a) or MgCl$_2$ (b) on the aggregation and survival of *N. glutinosa* protoplasts treated with liposomes. The experimental conditions are described in the legend to Figure 1.

Fig. 3. Aggregation of *N. glutinosa* protoplasts induced with liposomes. Protoplast aggregation observed in a reaction mixture containing 50 mM CaCl$_2$ (a) and 50 mM MgCl$_2$ (b), respectively.

Aggregation was found to be a function of the liposome concentration (Fig. 4). In contrast, the higher the content of liposomes provided in the reaction mixture, the lower the protoplast survival.

Time Course. Time-course analysis of the liposome-mediated aggregation of protoplasts was done for a reaction mixture containing liposomes (0.4 μmol lipid/ml), 50 mM CaCl$_2$, and 0.5 M mannitol. As presented in Figure 5, protoplast aggregation appeared to be saturable during 15- to 60-min incubation periods. Protoplast survival was maintained at a high rate for up to 60-min incubation.

Effects of Liposome Charge. Table 1 summarizes the results of tests on the influence of surface charge of liposomes on protoplast

<table>
<thead>
<tr>
<th>Liposome Composition</th>
<th>Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lecithin (neutral)</td>
<td>10 (6-14)</td>
</tr>
<tr>
<td>Lecithin + dicetyl phosphate (negative)</td>
<td>7 (3-11)</td>
</tr>
<tr>
<td>Lecithin + stearylamine (positive)</td>
<td>17 (12-22)</td>
</tr>
<tr>
<td>Lecithin + stearylamine + cholesterol (positive)</td>
<td>19 (14-24)</td>
</tr>
</tbody>
</table>

* Molar ratio of lecithin, dicetyl phosphate, stearylamine, and cholesterol was 7:2:2:1.

* Numbers in parentheses are 95% confidence intervals by binominal distribution.
Table II. Influence of Liposome Size on the Aggregation of N. glutinosa Protoplasts

Protoplasts (1.9 × 10⁷) were incubated in a solution containing liposomes (1.58 μmol lipid/ml), 50 mM CaCl₂, and 0.5 M mannitol. Liposome composition was as described in "Materials and Methods."

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sonication Period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
</tr>
<tr>
<td>Sonication only</td>
<td>15 (10-20)*</td>
</tr>
<tr>
<td>Sonication and filtration</td>
<td>24 (19-29)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses are 95% confidence intervals by binomial distribution.

Effects of Liposome Size. As a standard procedure hitherto, 60-min-sonicated liposome preparations were used for each experiment. To determine the influence of liposome size on the protoplast aggregation, liposomes were prepared by sonication of the emulsion for various times and by membrane filtration. Liposomes sonicated for 60 min showed the highest protoplast aggregation activity (Table II). Furthermore, the Millipore filtrates of liposome preparations sonicated for 5 or 30 min possessed the aggregation potentials equal to those of the 60-min-sonicated preparation.

Other Observations. Of the other parameters examined in this investigation, neither temperature (15–45°C) nor pH (6.5–7.5) of the incubation medium affected the degree of protoplast aggregation caused by liposomes (data not shown).

DISCUSSION

The objective of the present investigation was to secure information concerning parameters which regulate liposome-mediated aggregation of plant protoplasts. One of the most critical factors for the induction of protoplast aggregation appeared to be the presence of specific ions in the liposome-protoplast mixture. Among the several mono- and divalent cations tested, calcium ion caused the least impairment of protoplasts while simultaneously promoting protoplast aggregation to the maximal extent. A similar phenomenon was reported by Nagata et al. (2). As expected, the frequency of protoplast aggregation induced with liposomes was a function of liposome concentration.

Upon sonication of lipid for 60 min, 1 μmol lecithin is capable of producing 2 × 10¹⁴ vesicles (3). There was a saturation of protoplast aggregation within a 1-h incubation period, when 0.4 μmol lecithin/ml (=8 × 10¹⁴ vesicles/ml) was mixed with 5 × 10⁵ protoplasts/ml (Fig. 5). Thus, 1.6 × 10⁶ liposomes/protoplast-h are necessary to achieve maximum aggregation. Assuming that the average diameter of a SUV liposome is 25 nm (3) and that of N. glutinosa protoplasts is 30 μm, 5.8 × 10⁴ vesicles would be needed to cover the entire surface of a protoplast. On the basis of the above calculation, it is estimated that approximately 27 times the number of liposomes which presumably can adhere to a protoplast surface are required to achieve maximum aggregation rate.

Inasmuch as the net charge of plant protoplasts has been reported to be negative (1), it was expected that positively charged liposomes might be more effective than neutral and negatively charged liposomes in inducing protoplast aggregation. Indeed, positively charged liposomes caused higher rates of protoplast aggregation than did other liposomes. A similar observation was made in our communication (5) on the transfer of liposome contents into carrot protoplasts.

Moreover, the present investigation suggests that small lipid vesicles obtained by 60-min sonication and Millipore filtration, in conjunction with sonication, may stimulate protoplast aggregation. The significance of liposome size in protoplast fusion also has been reported by Nagata et al. (2). Efforts are being made to establish conditions whereby complete fusion of protoplast aggregates induced by SUV liposomes can take place.

Acknowledgments—We are indebted to Drs. T. Fuji and K. Inoue for valuable advice.

LITERATURE CITED

2. **NAGATA T, H EIBL, G MELCHERS 1979** Fusion of plant protoplasts induced by a positively charged synthetic phospholipid, D Naturforsch 34c: 460–462