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STIMULATION OF QUIESCENT 3T3 CELLS BY PHOSPHATIDIC ACID-CONTAINING LIPOSOMES

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SUMMARY. Liposomes containing phosphatidic acid were capable of stimulating DNA synthesis in quiescent Swiss 3T3 cells while liposomes composed of other phospholipids were not. These results show that liposomes, which are usually employed to deliver non-lipid molecules into cells, can themselves have profound effects on cell growth. The possible mechanism of phosphatidic acid-mediated cell stimulation and its relation to other growth factors are discussed.

Liposomes have been employed to introduce many different types of molecules into cells for a wide variety of purposes (1-3). In this laboratory, liposomes have been used to encapsulate mRNA molecules from serum-stimulated Swiss 3T3 cells. These mRNAs were then delivered into quiescent cells in order to determine whether such mRNAs can stimulate cell growth. During these experiments, it was found that some liposomes were capable of stimulating quiescent cells even when they contained only buffer rather than mRNA. This stimulatory effect was specific for liposomes made from phosphatidic acid, indicating that this compound has a direct influence on cell growth. This result agrees with other recent work in which phosphatidic acid was found to act as a growth factor in Rat-1 cells (4).

MATERIALS AND METHODS

Cell Culture: Swiss 3T3 cells (ATCC 92) were routinely grown in Dulbecco's Modified Eagle Medium supplemented with 10% calf serum. The cells were maintained at 35°C in a 5% carbon dioxide atmosphere. To obtain quiescent cells, the cells were plated at 10^5 cells per 35 mm dish in 2.0 ml of medium containing 10% serum. The following day, the medium was replaced with medium containing 1% serum, and this medium replacement was repeated every two days. The cells were confluent after three days. One week after plating, the quiescent cells were used in an experiment.

The abbreviations used are: PA, phosphatidic acid: PS, phosphatidylserine; PC, phosphatidylcholine; PBS, phosphate-buffered saline.

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Liposome Preparation: Liposomes were prepared by the reverse-phase evaporation technique of Szoka and Papahadjopoulos (5). Various combinations of lipids were suspended in 0.6 ml of buffer (150 mM KCl, 10 mM NaCl, 5 mM Hepes, 1 mM EGTA, pH 7.0). After liposome formation, the mixture was dialyzed overnight at 4°C against Hanks' Balanced Salt Solution without Ca^{2+} and Mg^{2+}.

To test the encapsulation rate of liposomes prepared in this way, 20 mM carboxyfluorescein was included during the encapsulation (6). Following formation of the liposomes, the preparation was subjected to column chromatography using Sephadex G-75. Triton X-100 was added to the fractions to a final concentration of 0.1% to destroy the liposomes. The amount of carboxyfluorescein eluting in the void volume (encapsulated) and the amount eluting in the total volume (unencapsulated) were determined spectrophotometrically.

To test liposome fusion with cells, quiescent cells were exposed to liposomes containing carboxyfluorescein as described below, and the cells were then examined by fluorescent microscopy to detect the presence of the dye within the cells.

Exposure of Cells to Liposomes: The dialyzed liposome preparation (0.6 ml) was diluted with 1.5 ml of medium containing no serum. The conditioned medium containing 1% serum was removed from the quiescent cells and saved. The cells were washed once with PBS and then liposomes (1.0 ml per 35 mm dish) were added. The cells were incubated with the liposomes for 30 minutes at 35°C. The liposomes were then removed and the cells were washed once with PBS. To monitor cell stimulation, the original medium was added back to the cells, and 1.0 μCi (12 nmol) of [^{3}H] thymidine was added to each dish.

Quiescent control cells were treated in a similar manner. They were washed once with PBS, incubated for 30 minutes with 1.0 ml per dish of medium without serum, and washed again with PBS. The original medium and [^{3}H]thymidine were then added to the cells. Stimulated control cells were washed and incubated in the same way. Following the last PBS wash, fresh medium containing 10% serum and [^{3}H]thymidine was added to the cells.

Measurement of Cell Stimulation: At various times after exposure to liposomes or fresh serum, cells were analyzed for incorporation of [^{3}H]thymidine into DNA. The radioactive medium was removed, the cells were washed once with ice-cold PBS, and 2.0 ml of 5% trichloroacetic acid-1% sodium dodecyl sulfate was added to each dish to lyse the cells and precipitate the nucleic acids. The cells were scraped off the dish using a rubber policeman, and the precipitate was collected on a glass fiber filter. The amount of radioactivity was determined using an LKB 1219 liquid scintillation counter.

RESULTS

To ensure that the liposomes used in this study were functioning properly, the percent encapsulation and the fusion with cells were monitored. To measure the percent encapsulation, the liposomes were made containing the fluorescent dye, carboxyfluorescein. From a comparison of the amount of dye encapsulated into liposomes with the amount remaining outside the liposomes, an adequate percent encapsulation of 12-15% was found. To determine if the liposomes were fusing with cells, quiescent cells were exposed to liposomes containing carboxyfluorescein and the cells were then examined by fluorescent microscopy. Cells treated with liposomes showed distinct fluorescence in their cytoplasm. Cells treated with the same concentration of the dye in simple solution showed no fluorescence. Thus the liposomes were fusing with cells.

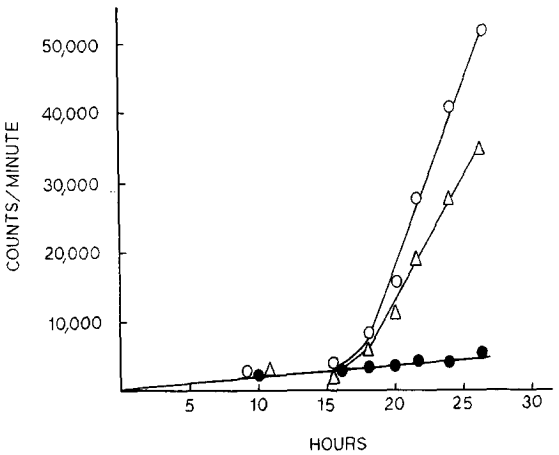

Figure 1. Stimulation of Quiescent 3T3 Cells by PA-Containing Liposomes. Quiescent cells were exposed to liposomes and the incorporation of [^{3}H]thymidine was measured. Results are shown for cells treated with liposomes made from 12 mg PA+ 4 mg PC (△). The incorporation into quiescent control cells (●) and serum-stimulated control cells (○) is also shown.

These results regarding encapsulation rate and cell fusion were obtained for liposomes made from all the various combinations of lipids used in these experiments.

Liposomes made from a combination of 12 mg PA + 4 mg PC were tested for their effect on quiescent Swiss 3T3 cells. As seen in Figure 1, these liposomes were capable of inducing cell growth as measured by the incorporation of [^{3}H]thymidine into DNA. Although the incorporation of thymidine was much greater in liposome-treated cells than in quiescent cells, it was not as great as that seen in serum-stimulated cells. The cell stimulation induced by the liposomes compared to stimulation by serum varied from 30-70% in different experiments. The extent of stimulation caused by the liposomes appeared to correlate with the age of the cultured cells. Cells cultured for less than a month after being thawed from storage in liquid nitrogen gave a high percentage of stimulation. After culture for 2-3 months, some cells lose the property of contact-inhibition, the culture appears less homogeneous, and the response to the liposomes is less. Thus the ability of the cells to be stimulated by phosphatidic acid-containing liposomes seems to vary with other growth control characteristics. It should be noted that the quiescent cells and serum-stimulated cells used as controls in these experiments were subjected to the same type of washing and incubation as the cells exposed to liposomes. However, comparisons of cells which had gone through the washings and incubation with cells which had not revealed that this procedure had no detectable effect on the cells.

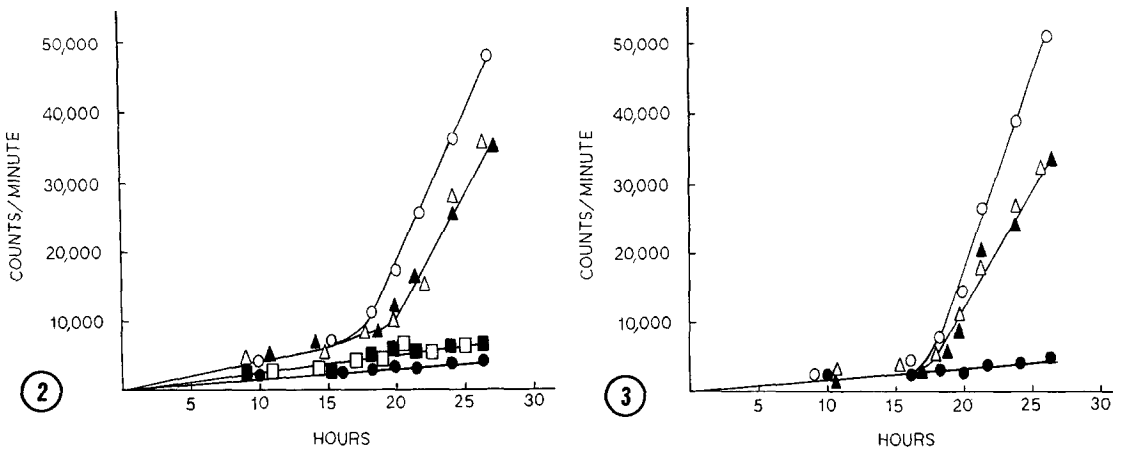

Figure 2. Effect of Different Types of Liposomes on Quiescent 3T3 Cells. The stimulation of quiescent 3T3 cells was measured after exposure to various types of liposomes. Results are shown for cells treated with liposomes made from 12 mg PA + 4 mg PC (△), liposomes made from 12 mg PA + 8 mg PC (▲), liposomes made from 12 mg PS + 4 mg PC (□), and liposomes made from 12 mg PS + 8 mg PC (■). Results are also shown for quiescent control cells (●) and for serum-stimulated control cells (○).

Figure 3. Stimulation of Quiescent 3T3 Cells after Exposure to a Lipid Suspension. The stimulation of quiescent cells was measured after exposure to liposomes made from 12 mg PA + 4 mg PC (△) or after exposure to 12 mg PA + 4 mg PC suspended in buffer (▲). Results are also shown for quiescent control cells (●) and for serum-stimulated control cells (○).

Studies with other combinations of lipids were performed, and the results are shown in Figure 2. Liposomes containing PA + PC were capable of stimulating quiescent cells even when the amount of PC was increased from 4 mg to 8 mg. Therefore, the relative amount of PA in the liposomes had no effect on the induction of cell growth, and quiescent cells treated with these liposomes again incorporated 30-70% of the thymidine incorporated by serum-stimulated cells. In contrast, liposomes composed of PS (12 mg) + PC (4 or 8 mg) had almost no ability to stimulate cells. Compared with serum-stimulated cells, cells treated with these liposomes showed 0-10% stimulation in various experiments. Thus the growth-inducing properties of some liposomes depend upon their specific lipid content and not merely upon fusion between liposomes and cells.

To determine whether phosphatidic acid had to be in the form of liposomes in order to stimulate growth in quiescent cells, an additional experiment was carried out. While some cells were exposed to liposomes made from 12 mg PA + 4 mg PC, other cells were exposed to the same lipid combination suspended in buffer rather than made into liposomes. As shown in Figure 3, both types of lipid preparation stimulated cells to about the same extent. Thus the presence of liposomes is not required in order for phosphatidic acid to induce cell growth.

DISCUSSION

These experiments show that liposomes containing PA are capable of stimulating DNA synthesis in quiescent 3T3 cells while other types of liposomes composed of PS and PC are not. The amount of cell stimulation produced was 30-70% of that found in serum-stimulated cells and was independent of the relative amount of PA in the liposomes. In addition, PA suspended in buffer was capable of stimulating cells to the same extent as PA-containing liposomes, so neither the liposome structure nor liposome-cell fusion is crucial in the growth-promoting process. However, since PA has such a profound effect on cells, it is important when using liposomes or any other form of phospholipid to take into account the possible influence the phospholipids themselves might have upon cells.

These results agree with other recent findings where treatment of cells with PA produced many of the same metabolic changes as growth factors, including stimulation of quiescent Rat-1 cells (4). In that study, a relatively low(50 μ g/ml) but continuous exposure to PA stimulated cells. In the current experiments, a somewhat greater amount of cell stimulation was achieved using a much higher PA concentration (5 mg/ml) for a brief period of time (30 minutes). However, since it is difficult to remove all traces of liposomes by washing the cells, some residual phospholipid probably remained with the cells throughout the experiment. Studies are underway to determine exactly what kind of cellular exposure to PA is required in order to elicit the growth response.

The mechanism whereby PA induces cell growth is not defined, but it seems to work by a pathway similar to that used by such growth factors as serum, vasopressin, platelet-derived growth factor, and fibroblast growth factor (7-11). These substances appear to trigger the breakdown of inositol phospholipids, resulting in activation of protein kinase C via diacylglycerol (12,13) and the mobilization of intracellular calcium via inositol-1,4,5-trisphosphate (14,15). There are several ways in which PA could participate in this pathway. The simplest explanation is that PA activates phospholipase C which in turn results in inositol phospholipid breakdown. An alternate suggestion (4) is that PA perturbs the cell membrane, rendering the phospholipids more susceptible to breakdown (16,17). In this case, however, the perturbation would have to be of a type specifically induced by PA since the membrane disturbances caused by liposome-cell fusion are not themselves sufficient to stimulate cell growth. Finally, PA might directly activate protein kinase C. Liposome-delivered PS and PC have been shown to change the relative amounts of protein kinase C in the membrane fraction and cytosol fraction of normal and dystrophic cells, with greater amounts of phosphorylation found in dystrophic cells where most of the enzyme is membrane-bound (18).

PA might exert a similar influence. However, the results of the current experiments showed that liposome-delivered PS and PC did not affect cell growth or PA-mediated cell stimulation. Since PS is a known activator of protein kinase C and PC is a known inhibitor (14), then these compounds should have produced some effect if the observed stimulation with PA resulted from its action on protein kinase C. In addition, a direct influence on protein kinase C would not account for PA-induced calcium mobilization (4). Further studies will be needed in order to determine which of these alternatives is the actual mechanism by which PA promotes cell growth.

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