EARLY EFFECTS OF SERUM ON PHOSPHOLIPID METABOLISM IN UNTRANSFORMED AND ONCOGENIC VIRUS-TRANSFORMED CULTURED FIBROBLASTS

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Received September 8,1976

SUMMARY

The addition of serum to previously serum-deprived 3T3 fibroblasts in culture caused a pronounced, rapid and selective stimulation of the incorporation of [^{32}P]phosphate into phosphatidyl inositol. Comparison of the content of radioactivity in phosphatidyl inositol after a short pulse with that obtained following a prolonged labeling period showed that serum accelerated the rate of the turnover (and not the net accumulation) of this substance. In cells transformed by SV-40 virus, the rate of labeling of phosphatidyl inositol was relatively high, and was not influenced significantly by the deprivation of serum or its resupplementation. It is suggested that the rate of phosphatidyl inositol turnover may be related to the state of the mobility of membrane constituents, and that this process escapes the control of serum factors in malignantly transformed cells.

INTRODUCTION

The multiplication of normal animal cells in culture requires the presence of serum growth factors (1, 2). At suboptimal serum concentrations, the cells enter a quiescent, non-growing state; upon the resupplementation of serum, a set of early "pleiotypic" metabolic changes are initiated (3) and these are followed by the resumption of DNA synthesis and cell multiplication. Malignantly transformed cells require less serum for growth (1, 4) and show much diminished pleiotypic responses during the removal of serum or its readdition (3). While the mode of action of serum growth factors is not understood, it appears reasonable to assume that the primary site of action of these macromolecular factors is at the plasma membrane(3). Among changes in membrane constituents elicited by stimuli that initiate cellular growth, it has been shown that treatment of lymphocytes with phytohemagglutinin elicits a rapid enhancement of the turnover of certain phospholipids (5, 6). We report here that serum accelerates markedly the turnover rate of phosphatidyl inositol in untransformed, but not in malignantly transformed cultured fibroblasts.

METHODS

BALB 3T3 and SV-40-transformed (SV3T3) fibroblasts (originally obtained from Dr. G. J. Todaro) were grown in Dulbecco's modified Eagle medium (Grand Island Biological Co.) supplemented with 10% calf serum (Bio-Lab, Israel). Cultures were grown and all experimental incubations were conducted in a humidified incubator at 37°C under 10% CO_2 atmosphere. Unless otherwise stated, the general experimental design for the examination of the effects of serum deprivation and its resupplementation was as follows. 10^6 cells were seeded onto 100 mm Falcon petri dishes containing 15 ml of complete growth medium and were allowed to grow for 24 hours. The media were then removed, the plates rinsed and the cultures incubated for 20 hours in a serum-free medium containing 10 µM inorganic phosphate (control experiments showed that cultures supplemented with serum grew at a normal rate for at least 3 days at the low concentration of phosphate used). The medium was then changed with 5 ml of fresh, low-phosphate medium and the cultures were incubated in the presence or absence of 10% dialyzed calf serum. At various time intervals following the addition of serum, 250 µCi of [^{32}P]phosphate (carrier-free, Nuclear Research Center, Israel) was added and incubation was continued for an additional 30 min period; the incubation times indicated in the Tables refer to the end of the labeling period. The cultures were immediately chilled on ice, washed 3 times with an ice-cold solution of phosphate-buffered saline (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4) and dissolved in 3 ml of 0.1% sodium dodecyl sulfate. An aliquot of this extract was withdrawn for the determination of protein concentration (7) and the rest of the sample was used for the extraction and separation of phospholipids.

Phospholipids were extracted with chloroform-methanol (2:1, v/v) (8) and the chloroform phase was extracted 3 times with a mixture of chloroform:methanol: 0.1 M KCl (6:96:94, by volume). The lower chloroform phase was evaporated to dryness under a stream of N_2, the residue dissolved in a small volume of chloroform and applied to Silica Gel-G thin layer plates (Merck) that had been previously activated by heating at 120°C for 1 hour. The phospholipids were separated by ascending chromatography with chloroform:methanol:H_2O (65:25:4) as the solvent system. Phospholipid spots were visualized with iodine vapours, scraped off and their radioactivity determined with 10 ml of a Toluene-Triton scintillation mixture (9).

The content of radioactivity in acid-soluble nucleotides was determined by washing labeled cultures with ice-cold phosphate-buffered saline, as described above, and then treating with 3 ml of 10% trichloroacetic acid. The plates were allowed to stay for 2 hours in the cold, the supernatant fluids were collected and the procedure was repeated. The combined trichloroacetic acid-soluble supernatants were mixed with 80 mg of Norit A, the charcoal was washed twice with water and the nucleotides were eluted with 50% (v/v) aqueous ethanol containing 2.5% (v/v) of conc. NH_4OH (10).

RESULTS

Table 1 shows the marked changes that occur in the pattern of the incorporation of [^{32}P]phosphate into cellular phospholipids following the addition of serum to previously serum-deprived 3T3 fibroblasts. Within 60 minutes following the resupplementation of serum, the incorporation of $^{32}P_i$ into the total phospholipid fraction increased by almost 8-fold as compared to the serum-deprived control culture. Examination of the

Table 1: Influence of serum on the incorporation of $^{32}P_i$ into phospholipids in previously serum-deprived 3T3 cells.

Phospholipid	$^{32}P_i$ incorporated (dpm/mg of protein x 10^{-3})		
	Control (no serum) 1 hr	Serum added	
		1 hr	5 hrs
Total phospholipids	438	3,380	-
Phosphatidyl inositol	89	2,120	2,421
Lecithin	197	617	801
Phosphatidyl ethanolamine	67	201	267
Phosphatidic acid	51	160	198

Experimental conditions were as described under "Methods". $^{32}P_i$ was added 30 min before the termination of the incubation. In the control ("no serum") cultures, identical results were obtained after 5 hours of incubation.

[^{32}P]phosphate content of various individual phospholipids revealed that a great part of this increase was due to the marked stimulation of the labeling of phosphatidyl inositol. Thus, the addition of serum stimulated the incorporation of $^{32}P_i$ into phosphatidyl inositol by 24-fold, whereas the labeling of lecithin, phosphatidyl ethanolamine and phosphatidic acid were increased by only about 3-fold. In other experiments it was found that the enhancement by serum of the labeling of phosphatidyl inositol was very rapid, since about 60% of the maximal stimulation was observed already at 15 minutes following the supplementation of serum (data not shown). Maximal stimulation of phosphatidyl inositol labeling was attained at around 60 minutes, and no further significant increase was observed until 5 hours of incubation following the addition of serum (Table 1). In repeated experiments, the extent of the effect of serum on the incorporation of $^{32}P_i$ into phosphatidyl inositol varied between 10 to 25-fold, as compared to serum-deprived controls.

That the observed changes in phospholipid labeling were not solely due to the acceleration of the transport of phosphate and its increased incorporation into cellular nucleotides (11, 12) was suggested by the markedly different pattern of the incorporation of the label into phosphatidyl inositol as compared to other phospholipids. In addition, we have found that the addition of serum caused only a 50% increase in the radioactivity of the total intracellular nucleotide fraction (as measured by the labeling of acid-soluble

Table 2: Effect of serum on the rate of turnover of phosphatidyl inositol in 3T3 cells: comparison of a short pulse with a prolonged labeling with $^{32}P_i$.

Fraction	[^{32}P]phosphate incorporated into phospholipid (pmoles/mg of protein)			
	30-min pulse		Long-term labeling	
	Minus serum	Plus serum	Minus serum	Plus serum
Total phospholipids	619	3,150	128,900	131,100
Phosphatidyl inositol	72	830	9,570	9,630

The general experimental design is described in the text. "Long-term labeling": 3T3 cells were seeded at 2.5×10^5 cells/plate and were allowed to grow for 3 days in the presence of [^{32}P]phosphate (10 μCi/ml, 10 μM). The cultures were then serum-starved and serum was resupplemented for 30 min at conditions as described under "Methods", except that $^{32}P_i$ was added during all treatments. "30-min pulse": Experimental treatments were identical to those described for long-term labeling, with the exception that labeled phosphate was not added until the final 30-min incubation. Results were calculated by dividing the radioactivity of isolated phospholipids by the specific radioactivity of $^{32}P_i$ in the medium.

material adsorbed to charcoal) under conditions identical to the experiment described in Table 1.

An additional problem is whether the increased incorporation of $^{32}P_i$ into phosphatidyl inositol reflects the net accumulation of this phospholipid, or whether it is due to the acceleration of the rate of its turnover. To examine this question, the effect of serum on phosphatidyl inositol was tested in cultures that had been previously equilibrated with labeled phosphate; under these conditions, the amount of the phospholipid can be calculated from its content of radioactivity. 3T3 cells were labeled with $^{32}P_i$ for 3 days (a sufficient time to obtain a complete equilibration of all phosphorylated cellular compounds (13)) and were then serum-deprived and subsequently treated with serum for a short period of time. During all these treatments, labeled phosphate was added to the medium at a constant specific radioactivity. The results (Table 2, "long-term labeling") show that the content of phosphatidyl inositol did not increase significantly following a 60-min treatment with serum. On the other hand, when the amount of $^{32}P_i$ incorporated into phosphatidyl inositol during a 30-min pulse (in previously unlabeled cells) was calculated by the specific activity of phosphate in the medium, as much as 800 picomoles/mg of protein were found to be incorporated in serum-treated cells. This is clearly an underestimation of the true rate of the incorporation, since it does not seem

Table 3: Comparison of the effects of serum deprivation and its reversal on the incorporation of $^{32}P_i$ into phospholipids in 3T3 and SV3T3 cell lines.

Additions	Relative incorporation of $^{32}P_i$			
	3T3		SV3T3	
	Phosphatidyl inositol	Lecithin	Phosphatidyl inositol	Lecithin
None (serum deprived)	0.20	0.21	0.92	0.91
Serum	2.76	0.35	1.17	1.06
Insulin	0.28	0.33	1.08	0.97

Experimental conditions are described under "Methods". Dialyzed calf serum (10%) or crystalline bovine insulin (5 µg/ml) were added for 30 min. The results are expressed as the fraction of the incorporation in logarithmically growing cell cultures that were labeled with $^{32}P_i$ of similar specific activity for 30 min. Incorporation values in growing cultures were (dpm/mg of protein x 10^{-3}): 3T3 cells phosphatidyl inositol 495, lecithin 891; SV3T3 cells - phosphatidyl inositol 7,830, lecithin 8,600.

likely that the specific activity of the immediate precursor of phosphatidyl inositol is completely equilibrated with extracellular phosphate in such a short period. It thus seems that the stimulation by serum of the incorporation of $^{32}P_i$ is much greater than could be accounted for by increased accumulation of this substance, and therefore it has to be mostly due to the acceleration of the rate of its turnover.

It may be asked whether the influence of serum on the metabolism of phosphatidyl inositol is related to its growth-promoting activity, or whether it is due to the effect of some entirely different substances present in dialyzed serum. We have therefore tested the influence of serum on phospholipid metabolism in malignantly transformed cells, that are much less dependent on serum for growth than are untransformed cells (1, 4). In the experiment shown in Table 3 it may be seen that in cells transformed by SV-40 virus (SV3T3 cells), serum deprivation had almost no influence on the high rate of incorporation of $^{32}P_i$ into phosphatidyl inositol, as opposed to the profound inhibition of this process in serum-deprived 3T3 cells. In accordance with these results, we also find that the addition of serum to previously serum-deprived SV3T3 cells caused only a slight stimulation of the labeling of this phospholipid. These data show that a

definite relationship exists between the actions of serum on cellular growth and on the turnover of phosphatidyl inositol.

Since phospholipids are constituents of cellular membranes and their turnove rate may reflect some kind of membrane activity, it was of interest to examine whether this phenomenon is related to some of the early membrane transport changes that occur when cells are stimulated to grow, such as the increased transport of nucleosides or phosphate (3, 11, 14). For this purpose, we have examined the influence of insulin since this hormone elicits many of the rapid transport (3) and some other pleiotypic (15) changes in a manner similar to serum, but cannot replace serum to support growth in 3T3 fibroblasts (3). As shown in Table 3, the addition of insulin to serum-deprived 3T3 cells caused only about a 50% stimulation of the incorporation of $^{32}P_i$ into both lecithin and phosphatidyl inositol, without any selective effect on the latter. Further experiments showed that most of this stimulation was due to the enhancement of the labeling of the intracellular nucleotide pool. In control experiments, it was found that under similar experimental conditions, the addition of insulin accelerated the uptake of uridine about 5-fold, in accordance with previous results (3). It appears, therefore, that the turnover of phosphatidyl inositol is not related to the membrane transport of these small molecular weight nutrients.

DISCUSSION

The present data are in agreement with previous reports concerning changes in phospholipid metabolism in lymphocytes stimulated by phytohemagglutinin (5, 6) or in chick fibroblasts relieved from contact inhibition (16), and thus lend support to the notion that this is a general phenomenon related to the stimulation of resting cells to multiplicate. Our further findings concerning phosphatidyl inositol metabolism in malignantly transformed cells strongly indicate that the observed effects of serum are indeed related to its growth-promoting action. The function of the increased turnover of phosphatidyl inositol is not known, but it should be noted that quite similar phenomena have been observed in a wide variety of rather different biological systems, such as during the stimulation of the secretion of proteins from exocrine glands (17), during the secretion of hormones (18), neurotransmitters (19) or in phagocytosis (20). A common feature of these processes appears to be that they all involve membrane relocation and the increased circulation of membrane constituents. Hokin has suggested that phosphatidyl inositol may link together membrane subunits, and that its increased turnover may allow the assembly of these subunits (17). In the present system, it appears that

this phenomenon is not related to increased membrane transport of small molecular weight nutrients but rather to some more drastic change in the properties of cellular membranes, such as the increased mobility of membrane constituents. This may be related to the commencement of cellular movement (16) or to some rearrangement of membrane structure that is required for the initiation of cellular growth. At any rate, it would seem that malignant transformation causes the escape of this membrane process from the control of serum growth-promoting factors.

REFERENCES

1. Temin, H.M., Pierson, R.W., and Dulak, N.C. (1972) Growth, Nutrition and Metabolism of Cells in Culture, pp. 50-81, Academic Press, New York.
2. Holley, R.W., and Kiernan, J.A. (1974) Proc. Nat. Acad. Sci. USA 71, 2942-2945.
3. Hershko, A., Mamont, P., Shields, R., and Tomkins, G.M. (1971) Nature New Biology 232, 206-211.
4. Dulbecco, R. (1970) Nature 227, 802-806.
5. Fisher, D.B., and Mueller, G.C. (1968) Proc. Nat. Acad. Sci. USA 60, 1396-1402.
6. Fisher, D.B., and Mueller, G.C. (1971) Biochim. Biophys. Acta 248, 434-448.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
8. Folch, J., Lees, M., and Sloane-Stanley, G.H. (1957) J. Biol. Chem. 226, 497-509.
9. Turner, J.C. (1969) Intern. J. Appl. Rad. Isotop. 20, 499-505.
10. Hershko, A., Wind, E., Razin, A., and Mager, J. (1963) Biochim. Biophys. Acta 71, 609-620.
11. Cunningham, D.D., and Pardee, A.B. (1969) Proc. Nat. Acad. Sci. USA 64, 1049-1056.
12. Weber, M.J., and Edlin, G. (1971) J. Biol. Chem. 246, 1828-1833.
13. Cunningham, D.D. (1972) J. Biol. Chem. 247, 2464-2470.
14. Jimenez de Asua, L., Rosengurt, E., and Dulbecco, R. (1974) Proc. Nat. Acad. Sci. USA 71, 96-98.
15. Epstein, D., Elias-Bishko, S., and Hershko, A. (1975) Biochemistry 14, 5199-5204.
16. Pasternak, C.A. (1972) J. Cell Biol. 53, 231-234.
17. Hokin, L.E. (1968) Intern. Rev. Cytol. 23, 187-208.
18. Trigano, M.J. (1969) Mol. Pharmacol. 5, 382-393.
19. Larrabee, M.G., and Leicht, W.S. (1965) J. Neurochem. 12, 1-13.
20. Karnovsky, M.L., and Wallach, D.F.H. (1961) J. Biol. Chem. 236, 1895-1901.