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ACID-SOLUBLE PRECURSORS AND DERIVATIVES OF PHOSPHOLIPIDS INCREASE AFTER STIMULATION OF QUIESCENT SWISS 3T3 MOUSE FIBROBLASTS WITH SERUM

C. H. Warden[1], M. Friedkin[1], and P. J. Geiger[2]

[1]Department of Biology, M-001, University of California, San Diego La Jolla, California 92093; [2]Department of Pharmacology and Nutrition University of Southern California, School of Medicine Los Angeles, California 90033

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SUMMARY

Automated phosphate analysis of acid-soluble pools of phosphate esters was employed to reveal possible biochemical changes during the transition of Swiss 3T3 mouse fibroblasts from quiescence to active replication of DNA. After 12 hours of stimulation with 10% fetal bovine serum the most notable were 3-fold increases in pools of phospholipid precursors and derivatives. These included glycerophosphocholine, glycerophosphoethanolamine, phosphocholine and phosphoethanolamine. Concurrent but less dramatic increases in pools of ATP, CTP and fructose 1,6-diphosphate were also obtained.

Biochemical responses to growth promoting factors have not been fully elucidated (1,2). When untransformed Swiss 3T3 mouse cells are brought to quiescence by serum deprivation, and then are stimulated with fresh fetal bovine serum or various peptide hormones a lag period of at least 10-14 hours precedes the start of S phase (3). A central question of concern is the nature of the biochemical events that occur before chromosomal replication. These have been shown to include increases in all of the following: uptake and phosphorylation of nucleosides (4), glycolysis (5), phosphofructokinase activity (6,7), ionic flux (8), phospholipase A_2 activity (9), and turnover of phospholipids (10-12).

Our first approach was to look for possible changes in acid-soluble pools of phosphate esters after serum stimulation of 3T3 cells. We made use of the Bessman automated phosphate analyzer to detect nanomolar amounts of these compounds (13-15). The general approach was to extract cold acid-soluble com-

Abbreviations: Fetal bovine serum, FBS; Dulbecco's modified Eagle's medium, DME; glycerophosphocholine, GPC; glycerophosphoethanolamine, GPE; fructose 1,6-diphosphate, FDP; glucose-1,6-diphosphate, G-1,6-P_2.

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pounds from quiescent and serum-stimulated mouse fibroblasts, and to subject samples to high pressure ion-exchange chromatography with an instrument that automatically ashes each eluted fraction to inorganic phosphate (13-15). This procedure does not depend on ultraviolet absorption and quantitates organic and inorganic phosphates. These include nucleotides, some of the common intermediates of glycolysis, and phospholipid precursors and derivatives.

MATERIALS AND METHODS

Cell culture. Swiss 3T3 mouse cells were routinely cultured in 100 mm Falcon tissue culture dishes in DME, 10% FBS, in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The cells were plated at 600 cells/cm^2 and switched to 0.25% FBS for 48 hours when they reached a density of 8000 cells/cm^2.

Extraction of acid-soluble phosphate esters. To begin a time course experiment cultures of quiescent cells were exposed to 10% FBS in DME. At various times after exposure to serum four 100 mm dishes were extracted for each point in the time course with 2 ml of ice cold 0.4 M perchloric acid. Extracts were transferred to screw-topped test tubes and neutralized by shaking with alamine-freon (16). The water phase, freed of perchlorate by this treatment, was separated by centrifugation and then removed and frozen until analysis.

Operation of the Bessman automated phosphate analyzer. Detailed descriptions of the phosphate analyzer are found in (13-15). We operated the Bessman phosphate analyzer as described except that we used an 0.3 x 50 cm precolumn to accomodate the 2 ml samples. Peaks were identified and quantitated by the methods of Bessman (13, 14) and Geiger and Roberts (15). The data was normalized for 100% recovery of glucose-1,6-diphosphate. We found that G-1,6-P_2 was undetectable in our samples and that it eluted from the phosphate analyzer distinct from any other phosphate compounds. By adding a known amount of G-1,6-P_2 to each sample during extraction we were able to determine the recovery of sample from extraction through analysis.

Other materials and methods. Protein was determined by the method of Lowry (17) using saline washed cells.

RESULTS AND DISCUSSION

The utility of automated chromatographic separation of acid-soluble phosphate esters from various cultures of 3T3 fibroblasts is displayed in Figure 1. Chromatographic profiles revealed marked differences between quiescent cells and cells stimulated with serum to begin the prereplicative transition. Already at 5 hours after serum stimulation of resting cultures, or 8 hours before the onset of DNA synthesis, one of the most obvious changes was an increase in the amount of ATP. Various acid-soluble phospholipid derivatives

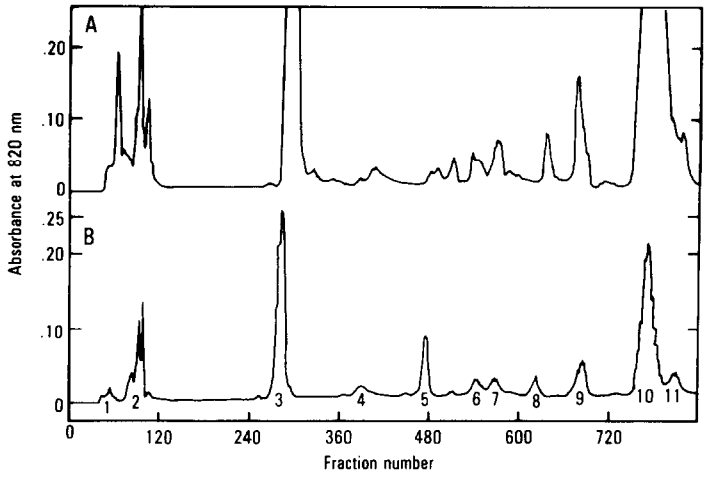

Figure 1. Chromatographic profiles of A) activated cells after 5 hours of stimulation (2.0 mg protein) and B) resting cells after 48 hours in 0.25% FBS (1.7 mg protein). Cultures were extracted and analyzed as described in Materials and Methods. Peak identities: 1, glycerophosphocholine plus glycerophosphoethanolamine; 2, phosphocholine plus phosphoethanolamine; 3, inorganic phosphate; 4, glucose 6-phosphate plus NAD; 5, glucose-1,6-diphosphate; 6, phosphoenolpyruvate plus AMP; 7, fructose 1,6-diphosphate; 8, CTP; 9, UTP plus ADP; 10, ATP; 11, GTP.

with only slight affinity for the anion-exchange columns, peaks 1 and 2, increased markedly after serum stimulation while CTP and fructose 1,6-diphosphate also increased significantly (15).

The results from 3 separate time course experiments are combined into Table 1. Several phospholipid precursors and derivatives show the largest percentage changes. GPC and GPE (Fig. 1, peak 1) increased approximately 3-fold. Peak 2 which includes both phosphocholine and phosphoethanolamine increased 3.3-fold by 12 hours after serum addition.

Several nucleotides were easily quantitated. CTP (Fig. 1, peak 8), important in phospholipid and RNA synthesis, showed a continuous and significant increase of 70% above control levels, while the amount of ATP (Fig. 1, peak 10) increased by 50% 12 hours after serum activation.

In the present study, we have used the Bessman automated phosphate analyzer as a means of directly measuring the major acid-soluble phosphate esters, UV absorbing as well as non-UV absorbing intermediates. Our preliminary findings have not only substantiated the results of others where the

Table 1

Peak Identity	Hours post stimulation (nmoles/mg protein ± SD) 0	.5	3	5	12
GPC+GPE	12 ± 3.8	17 ± 6.2	27 ± 3[d]	34 ± 23	38 ± 15[e]
PC+PE	43 ± 8.3	57 ± 10	80 ± 12[d]	95 ± 43	149 ± 22[b]
P_i	110 ± 37	100 ± 22	103 ± 38	112 ± 10	73 ± 8
PEP+AMP	11 ± 2.9	10 ± 2.9	6.6 ± .85	16 ± 7	12 ± 2.2
FDP	5.2 ± 1.2	5.3 ± 1.2	6.1 ± 0.9	7.6 ± 2.5	8.2 ± 0.5[d]
CTP	3.0 ± 0.2	3.8 ± 0.7	4.6	5.1 ± 1.2[d]	5.2 ± 0.2[a]
UTP+ADP	11 ± 1.0	14 ± 1.2[d]	16 ± 3.0[e]	15.3 ± 0.3[e]	17.5 ± 2.5[c]
ATP	40 ± 5.3	45 ± 4.5	48 ± 12	59 ± 10[d]	62 ± 14[e]
GTP	6.6 ± 2.5	6.1 ± .95	6.4 ± .35	6.9 ± 1.5	7.8 ± 1.2

Changes in phosphate ester concentrations following stimulation of quiescent 3T3 cells with fetal bovine serum. Quiescent cultures were prepared as described in Materials and Methods and the media then switched to fresh DME with 10% FBS. At the times indicated the cultures were extracted and analyzed as described in Materials and Methods. These data are the averages of 3 separate determinations and are expressed as nmoles/mg ± standard deviation. The data is adjusted for recovery of G-1,6-P_2 as described in Materials and Methods. Statistical significance was determined by comparing the amounts of each phosphate ester after stimulation to their respective amounts in quiescent cultures. Significance levels: a, $p < .001$; b, $p < .01$; c, $p < .02$; d, $p < .05$; e, $p < .10$.

main focus has been glycolytic and nucleotide compounds, but have revealed biochemical changes involved in phospholipid metabolism during the cell cycle.

In confirmation of earlier studies (18-20) the Bessman phosphate analyzer showed that levels of ATP increase during the G_1 phase of the cell cycle. In previous experiments most comparable to ours, Rapaport et al. (19) reported a 50% increase in ATP levels during the progression of 3T6 cells from early to late G_1.

The increased amount of FDP (Table 1) is consistent with several reports. Rubin (7) found that serum activation of chick embryo fibroblasts resulted in a 250 percent rise of FDP after just 30 minutes as well as an increase in phosphofructokinase activity. Schneider et al. (5) reported that phosphofructokinase activity in cellular homogenates is increased by 3 hours of stimulation of confluent 3T3 cells with 10% FBS.

The significant increases in CTP and phosphocholine (Table 1) are especially noteworthy in view of their role in the formation of CDP-choline, the direct precursor of phosphatidylcholine. If indeed CTP phosphocholine cytidyltransferase plays a central role in the regulation of phosphatidylcholine synthesis as suggested by Vance et al. (21), then the increases in cellular CTP and phosphocholine in response to serum stimulation may be critical pre-replicative events. Indeed Choy et al. (22) found that increased CTP levels were responsible for the activation of lecithin synthesis in poliovirus infected HeLa cells. However, they did not find increased phosphocholine levels after poliovirus infection. In studies with suspension cultures of Novikoff rat hepatoma cells Plagemann (23,24) found that choline kinase activity and levels of phosphocholine declined when the cultures became more dense. His results as well as ours suggest that choline kinase activity and phosphocholine concentrations may indeed be regulated during the cell cycle. A recent report by Kano-Sueoka et al. (25) is noteworthy because they found that phosphoethanolamine is a necessary growth factor for a rat mammary carcinoma cell line.

The marked increases in GPC and GPE (Table 1) were quite unexpected and remain unexplained. Several factors may be involved. Shier (9) has recently reported that stimulation of Swiss 3T3 cultures by serum resulted in increased phospholipase A_2 activity, particularly directed against arachidonic acid, the common prostaglandin precurser. There have been several reports (10-12) that phospholipid turnover of cultured cells increases in response to serum. Perhaps increased membrane turnover and increased phospholipase activity result in rising levels of GPC and GPE. Alternatively, Bailey (26,27) has shown that cultured cells rapidly take up serum lipids, so that the rise in GPC and GPE seen after serum activation may simply be an expression of increased lysosomal breakdown of internalized serum phospholipids. This remains to be investigated.

The most striking result of this investigation was the finding that upon serum stimulation of quiescent fibroblasts the pool sizes of several phosphate

esters, all precursors or derivatives of phospholipids, increased markedly. The remarkable increase of phosphocholine and phosphoethanolamine may be necessary for increased synthesis of phospholipids. Our novel and surprising discovery that GPC and GPE increase several fold requires further investigation.

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