Phospholipid turnover during cell-cycle traverse in synchronous Chinese-hamster ovary cells

Mitogenesis without phosphoinositide breakdown

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The turnover of phospholipids was investigated in quiescent serum-starved Chinese-hamster ovary (CHO-K1) cells stimulated to progress through the cell cycle by the addition of dialysed bovine serum. A variety of radiolabelling techniques were employed to study the rapid effects of serum on phospholipids and later events during G1 and S phases of the cell cycle. Pulse-labelling studies using [32 P] P_i revealed that there was a stimulation of the synthesis rate of all phospholipids investigated during the initial few hours after serum addition. The greatest stimulation (20-fold) was observed in phosphatidylcholine, and the smallest in the polyphosphoinositides (PPIs). Mock stimulation with serum-free medium caused a similar increase in PPI turnover, but little or no effect on turnover of other phospholipids. This effect could be accounted for by a stimulation of the turnover of cellular ATP pools increasing [32 P]ATP specific radioactivity. Late G1 and S phases were associated with a decrease in the rate of synthesis of all phospholipids. Phosphatidic acid was the only phospholipid whose labelling fell below that in mock-stimulated cells during the period of the cell cycle. Stimulation of serum-starved cells that had been prelabelled with *myo*-[2- 3 H]inositol caused no change in the amounts of inositol trisphosphate, but both serum-stimulated and mock-stimulated cells exhibited similar small decreases in both inositol bisphosphate and inositol monophosphate, of approx. 30% after 30 s. When cells were serum-stimulated in the presence of 10 mM-Li $^+$, there was no increase in the size of the total inositol phosphate pool. We conclude that mitogenic stimulation and cell-cycle traverse cause profound and complex effects on phospholipid turnover in CHO-K1 cells, but there is no evidence for a role of inositol lipid turnover in the proliferative response to serum in this cell line.

INTRODUCTION

In the absence of nutrients and/or appropriate hormonal stimuli, dividing mammalian cells accumulate in a quiescent non-cycling state known as the G0 phase of the cell cycle (Pardee *et al.*, 1978). Re-addition of nutrient or growth factor causes entry into G1 phase and the resumption of cycling, allowing biochemical studies to be performed on a synchronous population of cells (Ashihara & Baserga, 1979). The G0/G1 transition has received particular attention, as it is the primary point of control over the rate of cell division (Pardee *et al.*, 1978).

More recently, interest has focused on the role of inositol lipids, particularly the polyphosphoinositides (PPIs), in mediating the proliferative signal from cell-surface receptors to the interior of the cell, as is the case for a wide variety of peptide hormones and other stimuli (for reviews, see Berridge, 1984; Nishizuka, 1984). It is known that platelet-derived growth factor stimulates the breakdown of polyphosphoinositides in the Swiss-mouse 3T3 cell line, with consequent increases in water-soluble inositol phosphates (Berridge *et al.*, 1984; Hasegawa-Sasaki, 1985). Many mitogenic agents have been reported

to have some stimulatory effect on phosphatidylinositol (PtdIns) turnover, and it has been suggested that this is a characteristic of competence-type growth factors, which are exemplified by platelet-derived growth factor (Macara, 1985).

Studies measuring the incorporation of radioactive precursors have suggested that growth stimulation of fibroblasts leads to an increased rate of synthesis of PtdIns (Hoffmann *et al.*, 1974; Dubois & Rampini, 1979; Ristow *et al.*, 1980). Similar results have been obtained in other cell types, such as mouse thymocytes (Taylor *et al.*, 1984) and lymphocytes (Fisher & Mueller, 1971).

In addition to the rapid early effects of growth stimulation, there may be other points in the cell cycle at which marked changes in inositol lipid metabolism occur. Hoffmann *et al.* (1974) found a marked decrease in the rate of incorporation of [32 P] P_i into the PtdIns of embryonic-rat fibroblasts between 10 and 25 h after stimulation with a combination of mitogenic serum proteins. This was in contrast with other phospholipids, turnover of which remained relatively constant during the same period. Somewhat different results were described by Dubois & Rampini (1978), who reported that PtdIns turnover is stimulated during G1 and S

Abbreviations used: PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P $_2$, phosphatidylinositol 4,5-bisphosphate; PPI, polyphosphoinositide; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdOH, phosphatidic acid; InsP, inositol monophosphate; InsP $_2$, inositol bisphosphate; InsP $_3$, inositol trisphosphate; DMEM, Dulbecco's modification of Eagle's medium.

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phases of the cell cycle. This early work has been reviewed by Michell (1982).

Preliminary studies from our laboratory suggested that there may be specific events in CHO-K1-cell G1 and S phase which are linked to phosphoinositide turnover (Sharif *et al.*, 1985). However, that work was performed with undialysed serum as the mitogenic stimulus and without correction for the amount of cellular material extracted. As the composition of low- M_r components in serum is likely to be very different from that of the culture medium, we have performed some of the current experiments after prior dialysis of the bovine serum.

We report that stimulation of quiescent CHO-K1 cells does not cause phosphoinositide hydrolysis. On stimulation, dramatic changes occur in the turnover of several phospholipid species, including PtdIns, but little or no effect was observed in the PPIs. We conclude that, in this cell line, phosphoinositide turnover is unlikely to be a signalling event in the transition from quiescence to the dividing state, and we have observed no changes occurring during the cell cycle that were specific to inositol lipids.

MATERIALS AND METHODS

Cell culture and synchronization

CHO-K1 cells were initially purchased from Flow Laboratories and were routinely cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 5% (v/v) fetal-calf serum, 5% (v/v) newborn-calf serum, penicillin (50 i.u./ml), and streptomycin (50 μ g/ml) in 100 mm-diam. plastic culture dishes (Nunc).

For experiments, cells were plated out on 60 mm-diam. dishes (Corning), at a density of 0.35×10^6 – 1.0×10^6 cells per dish containing 3 ml of DMEM. After allowing 24 or 48 h (if initial plating density was lower than 0.6×10^6 cells/dish) for cell attachment, the medium was aspirated and replaced with DMEM containing no serum. In prelabelling experiments this contained *myo*-[2- 3 H]inositol at 2–4 μ Ci/ml (0.05–0.10 Ci/mmol). After 3 days in the absence of serum, cells were stimulated either by the addition of serum direct to the plates or by complete replacement of the medium with one containing 5% fetal-calf serum and 5% newborn-calf serum. Mock-stimulated cells were treated identically, except that serum-free DMEM was substituted in place of serum. For cell-cycle experiments, parallel cultures were pulse-labelled with [5- 3 H]thymidine (1 μ Ci/ml; 17 Ci/mmol) for 90 min at various points over the course of the experiment. To measure rates of phospholipid synthesis, replicate plates were pulse-labelled with [32 P]P_i (30 μ Ci/ml; 33.1 Ci/mol of P_i). All incubations were terminated by aspiration of the culture medium and addition of 2.5 ml of ice-cold 0.4 M-HClO₄ or 10% (v/v) trichloroacetic acid to each plate.

Initially we had found concentrations of *myo*-inositol in commercially supplied serum to be in the 0.5–0.6 mM range. This was determined by g.l.c. of the trimethylsilyl ether derivative by the method of Sweeley *et al.* (1963). To correct for this and the presence of other low- M_r factors in the serum, we routinely dialysed the serum against 15 vol. for 3 days with two changes of the dialysis medium. The dialysis buffer had the following composition (mM): NaCl (109.6), NaHCO₃ (44.05), KCl (5.36), CaCl₂ (1.8), NaH₂PO₄ (0.906), MgSO₄ (0.81),

glucose (25). This is identical with the ionic composition of DMEM. *myo*-Inositol was added back to the dialysed serum to a concentration of 38.9 μ M, equal to that in DMEM.

DNA synthesis

The acid-precipitated (10% trichloroacetic acid or 0.4 M-HClO₄) cells from plates labelled with [3 H]-thymidine were scraped and transferred to conical plastic tubes with a 1.5 ml wash with the same acid. The precipitates were washed twice with water and solubilized in 0.2 ml of formic acid before liquid-scintillation counting of radioactivity.

Lipid analysis

Precipitated cells were scraped from the culture dishes and transferred to a glass test tube with a 1.5 ml acid rinse. The precipitate was washed once with 1 mM-EDTA and once with water (4 ml of each). Lipids were extracted from the washed pellet by the method of Yagihara *et al.* (1973). Lipid classes were separated by t.l.c. The t.l.c. plates were prepared from a slurry of silica gel H in 1% potassium oxalate spread 0.4 mm thick. For one-dimensional separation, the solvent system was chloroform/acetone/methanol/acetic acid/water (40:15:13:12:7, by vol.). For two-dimensional separations, we used in the first dimension a solvent system consisting of chloroform/methanol/33% NH₃ (13:5:1, by vol.), followed by the acidic solvent mixture described above in the second dimension. Experimental samples were co-chromatographed with phospholipid standards. PPIs were prepared from ox brain by the method of Kiselev (1982), and all other lipid standards were purchased from Sigma. Lipids were detected by exposure to I₂ vapour, scraped from the plates, and radioactivity was measured by liquid scintillation counting after addition of 4 ml of scintillant (Packard Scintillator 199).

Total lipid phosphorus was measured by the Bartlett (1959) assay.

Inositol phosphate measurements

HClO₄-soluble cell extracts were neutralized with 1 M-KOH. After 10 min on ice, followed by centrifugation (2260 g for 5 min), the supernatant was decanted. This was then buffered by the addition of 2 ml of 50 mM-Hepes, pH 7.0, and diluted to 20 ml with water. Alternatively, trichloroacetic acid-soluble extracts were extracted with 5 $\times$ 2 vol. of diethyl ether, diluted, and Na₂B₄O₇ was added (final concn. 5 mM). Both procedures gave similar results. The diluted neutralized samples were applied to anion-exchange columns containing 1 ml of Dowex-1 (formate form). After washing of the columns to remove free inositol and glycerophosphoinositol, inositol phosphates were eluted separately with the increasing ammonium formate concentrations described by Berridge *et al.* (1983). Alternatively, the total inositol phosphate fraction was eluted with 0.1 M-formic acid/1.0 M-ammonium formate.

ATP measurements

[32 P]ATP specific radioactivity was measured by assaying neutralized acid extracts of labelled cells by the method of Schneider (1969). ATP radioactivity was measured in parallel samples after separation from other

phosphates on polyethyleneimine-cellulose t.l.c. plates in 1 M-formic acid/1 M-LiCl.

RESULTS AND DISCUSSION

Preliminary experiments showed that DNA synthesis takes 3 days to decline to a minimum in CHO-K1 cells deprived of serum. Therefore we have used this length of time to achieve a maximally quiescent state. After re-addition of serum to such cells, they entered the cell cycle and began to synthesize DNA after a lag period of 10 h (see Fig. 1 of Sharif *et al.*, 1985). The lag period represents the time taken for the cells to enter and traverse the G1 phase. The rate of DNA synthesis rose dramatically to a peak at around 20 h, indicating passage through S phase, and thereafter declined for the remaining period of the experiment as the cells entered and passed through G2 phase. This pattern of [³H]-thymidine incorporation was highly reproducible, and was established in parallel with lipid-labelling studies in each cell-cycle experiment.

Fig. 1 shows the pattern of [³²P]P_i incorporation into phosphoinositides after serum stimulation and subsequent cell-cycle traverse. During the first time period (the first 90 min after serum addition), there was a stimulation of radioactivity in the PPIs, of 39% and 90% for PtdIns(4,5)P₂ and PtdIns4P respectively. PtdIns(4,5)P₂ labelling increased to a maximum at 3–4.5 h (early to mid G1 phase), after which it declined, but to a value still greater than the zero-time incorporation. Similarly, PtdIns4P radioactivity remained above the pre-stimulated value. Surprisingly, mock-stimulated cells showed an almost identical stimulation of PPI labelling, but were not stimulated to divide. All other phospholipids investigated also responded to serum by an increased rate of synthesis, but to varying degrees (Fig. 2). The greatest stimulatory effect was observed in phosphatidylcholine, which showed a 20-fold increase in synthesis rate at 3–4.5 h, but no effect of mock stimulation. A small effect of mock stimulation was observed in

phosphatidylserine and PtdIns labelling, but this was much less than the effect of serum on the turnover of these lipids. Interestingly, phosphatidic acid radioactivity declined to below that of either unstimulated (i.e. zero time) or mock-stimulated cells after serum addition. This decrease in labelling was apparent by 3–4.5 h, when we estimate the cells to be in mid-G1 phase, and was persistent throughout S phase and G2 phase.

We have investigated the effect of serum and mock stimulation on the specific radioactivity of cellular ATP. Fig. 3 shows that both procedures cause a rapid increase in [³²P]ATP specific radioactivity to approximately the same degree. The magnitude and time course of the increase was comparable with that of the changes observed in PPI labelling in Fig. 1. We believe that it is most likely that the changes in PPI labelling simply reflect changes in [³²P]ATP specific radioactivity, owing to the rapid turnover of the phosphomonoester groups on the inositol ring (Hawkins *et al.*, 1984). This implies that the phosphate groups at positions 4 and 5 are turning over as rapidly in quiescent cells as in serum-stimulated cells. An alternative explanation is that the PPIs are turning over via a phospholipase-C-catalysed degradation, and that resynthesis is occurring via synthesis of PtdIns from free inositol and CDP-diacylglycerol. This is argued against by the fact that mock stimulation caused only a small effect on ³²P labelling of PtdIns. A possible explanation for the effect of mock stimulation on ATP turnover is that addition of serum-free DMEM and mixing causes the dilution of an inhibitory substance released by the cells during the preceding period of serum starvation. Such an inhibitory factor would exist in a concentration gradient from the cells on the bottom of the dish to the surface of the medium, in which case mixing alone would be sufficient to stimulate ATP turnover.

To look more directly for a role of the PPIs, we decided to measure inositol phosphate contents immediately after serum stimulation. Serum stimulation of cells which had been labelled with *myo*-[2-³H]inositol

Fig. 1. Phosphoinositide synthesis during cell-cycle traverse

Serum-starved CHO-K1 cells were stimulated by the addition of serum (▲) or serum-free DMEM (△) at zero time. Triplicate plates were pulse-labelled with [³²P]P_i (90 μCi/dish) for 90 min at various times thereafter. Incubations were terminated by the addition of 0.4 M-HClO₄, and lipids were extracted from the precipitated cells as described. Phospholipid species were separated by two-dimensional t.l.c. Points represent the mid-points of the pulse period, and are means ± S.E.M.

Fig. 2. Phospholipid synthesis during cell-cycle traverse

Details and symbols are as in Fig. 1 legend.

during the serum starvation period caused no change in the amount of $[\text{H}] \text{InsP}_3$ (Fig. 4), nor was there any effect of mock stimulation. However, both InsP_2 and InsP decreased by 30–50% in response to both serum and mock stimulation. The effect was evident after 15 s, the earliest time point employed in the study. Thereafter, amounts of InsP_2 and InsP remained relatively constant. In addition to these experiments, we have measured the accumulation of inositol phosphates in the presence of 10 mM-LiCl and found no change in total inositol phosphate pool size after serum stimulation (Fig. 5). The same negative result was obtained with undialysed serum. As most of the total inositol phosphate pool is in the form of InsP , these results suggest that, in the presence of Li^+ , InsP and InsP_2 amounts do not change when the cells are stimulated. Therefore we conclude that the decreases in InsP and InsP_2 seen in the experiment shown in Fig. 4 are due to the stimulation of the rate of breakdown of these phosphates, but there is no change in the rate of InsP_3 breakdown.

A wide variety of growth factors elicit characteristic ionic signals in their target cells, consisting of a rapid increase in intracellular Ca^{2+} and a slower but more persistent cytoplasmic alkalization (Burns & Rozen-gurt, 1983; Moolenaar *et al.*, 1983, 1984a; Hesketh *et al.*, 1985). It has been suggested that these ionic signals

may be the result of PPI breakdown, InsP_3 production and diacylglycerol activation of protein kinase C (Berridge, 1984). It is believed that the Na^+/H^+ exchanger is activated by protein kinase C (Moolenaar *et al.*, 1984b). The present results suggest that serum elicits a mitogenic response in CHO-K1 cells without a stimulation of PPI turnover. Besterman *et al.* (1986) have shown that dialysis removes almost 70% of the ability of serum to stimulate inositol-lipid turnover in BALB/c 3T3 fibroblasts, without affecting its mitogenic potential. However, this does not explain the lack of inositol phosphate release in the present work, since undialysed serum had no effect. The apparently higher labelling of PtdIns at 16–30 h in our preliminary study (Sharif *et al.*, 1985) may be due to differences in presentation. Total $[\text{P}] \text{PtdIns}$ in the dish of cells was recorded in that study. In the present study $[\text{P}] \text{PtdIns}$ per μg of total lipid P is presented, and cell growth will increase the lipid-P value at later times.

Pledger *et al.* (1978) have identified two plasma-dependent stages in the G1 phase of the cell cycle in addition to the G0/G1 transition point. These are the so-called V and W arrest points, which occur in mid and late G1 respectively. We were interested to see if these positions in the cell cycle were associated with changes in phosphoinositide turnover. Our results indicate no effects

Fig. 3. $[^{32}\text{P}]\text{ATP}$ specific radioactivity after stimulation

Serum-starved CHO-K1 cells were treated exactly as described in the legend to Fig. 1. The acid-soluble fraction was assayed for ATP and $[^{32}\text{P}]\text{ATP}$ radioactivity as described. Points are the means of triplicates.

that are specific to inositol lipid turnover, but some dramatic changes in phospholipid metabolism generally. It seems likely that the observed effects are related to membrane biosynthesis/turnover. As the effects of serum were not evident in the PPIs, which are largely localized in the plasma membrane of most cells (but see Downes & Michell, 1982), these phospholipid changes are probably occurring in the endoplasmic reticulum. G0/G1-phase transition and early G1 phase are associated with a dramatic increase in turnover of all phospholipids except the PPIs. The metabolic factors underlying these effects are unclear at present without further experimentation. We cannot exclude the possibility that phosphatidylcholine (and possibly other lipid species) are being broken down by a phospholipase C that is not specific for inositol-containing phospholipids, such as has been described by Wolf & Gross (1985). This could provide diacylglycerol for the activation of protein kinase C.

It seems likely that the decrease in phosphatidic acid labelling observed in serum-stimulated cells reflects the use of phosphatidic acid for the synthesis of phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, or indirectly for the synthesis of PtdIns after conversion into CDP-diacylglycerol.

Serum is a heterogeneous mixture of mitogens, and delineation of metabolic events necessary for CHO-K1 cell-cycle traverse would be greatly aided by the use of a better defined stimulus. It is unlikely that the cells

Fig. 4. Inositol phosphate amounts after serum stimulation

Serum-starved CHO-K1 cells which had been prelabelled with *myo*- $[2\text{-}^3\text{H}]\text{inositol}$ ($4\text{ }\mu\text{Ci/ml}$) for 3 days were stimulated by the addition of serum (▲) or serum-free DMEM (△) at zero time. At various times thereafter, incubations were terminated with ice-cold 10% trichloroacetic acid. Water-soluble inositol phosphates were separated as described in the Materials and methods section. Values shown are means $\pm$ S.E.M. for three to five replicates.

respond to epidermal growth factor, as they are known to lack the receptor for it (Livneh *et al.*, 1986). The present data suggest that they are also unresponsive to PDGF, which is present in the serum and would be expected to induce an effect on InsP_3 concentrations. We conclude that CHO-K1 cells do not use the phosphoinositide signalling system in their response to the mitogens in serum. The same is true of Nb2 node

Fig. 5. Total inositol phosphate production after serum stimulation

Serum-starved CHO-K1 cells were stimulated by the addition of serum. At various times thereafter, the cells were incubated with 10 mM-LiCl for 15 min, and then quenched with 0.4 M-HClO₄. The total water-soluble inositol phosphate pool was separated as described in the Materials and methods section. Values are means $\pm$ S.E.M. of triplicates, and are at the end of the 15 min Li⁺ incubation.

lymphoma cells (Gertler & Friesen, 1986), where the mitogenic effect of human growth hormone is not accompanied by increased phosphoinositide metabolism. In Chinese-hamster lung fibroblasts, there is evidence of two growth-factor signalling pathways, one of which is independent of PPI hydrolysis (Chambard *et al.*, 1987). The proliferative response of macrophages to interleukin 3 and colony-stimulation factor-1 is not accompanied by inositol lipid breakdown (Whetton *et al.*, 1986).

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