Requirement of phosphatidylcholine for normal progression through the cell cycle in C3H/10T1/2 fibroblasts

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Abstract We have investigated the possible requirement of phosphatidylcholine for normal progression through the cell cycle of C3H/10T1/2 fibroblasts. Incubation of the cells in a medium with 0.5% serum synchronized the cells in the G0 stage of the cell cycle. Supplementation of the cells with 10% dialyzed and delipidated serum ± choline resulted in normal cell division and growth for cells with choline, whereas cell division was markedly impaired in the absence of choline. Flow cytofluorometry analysis indicated that after 4 days in the absence of choline, 85% of the fibroblasts were in the G1 phase. Addition of choline resulted in synchronous synthesis of DNA with a peak occurring after 14 h. Incubation of cells with 0.5% serum had no effect on phosphatidylcholine (PC) levels in cells supplemented with 28 μM choline, but the concentration of PC was reduced from 32 to 20 nmol/10^6 cells after 1 day of incubation in the absence of choline. Supplementation with dialyzed serum, but not dialyzed and delipidated serum, allowed choline-deficient cells to replicate normally. This was attributed to the presence of lysophosphatidylcholine in dialyzed serum as this lipid, but not other lipids (e.g., phosphatidylcholine or mitogenic lipids) was able to replace the choline requirement. The choline-deficient effect was not complete; some DNA synthesis occurred in the absence of choline in the medium, and approximately 30% of the cells completed mitosis in 35 h compared to 100% in the presence of choline. The data suggest that phosphatidylcholine is required for normal progression of the cell cycle beyond the G1 phase and is unrelated to the induction of G0 to G1 transition. Choline deficiency should be a useful method for synchronizing cells in the G1 phase.—**Tercé, F., H. Brun, and D. E. Vance.** Requirement of phosphatidylcholine for normal progression through the cell cycle in C3H/10T1/2 fibroblasts. *J. Lipid Res.* 1994. **35:** 2130–2142.

Supplementary key words choline • lysophosphatidylcholine • G0 to G1 transition • flow cytofluorometry

In recent years, research on regulation of cell division in eukaryotes has focused mainly on the role of cdc2 kinase(s) and cyclin(s) for control of specific check-points in the cell cycle (1–3). A potential role for phospholipids in control of the cell cycle has been neglected. One potential role for phospholipids in cell proliferation is related to the generation of second messengers (mainly diacylglycerol, phosphatidic acid, and arachidonic acid) through the activation of phospholipases C, D, or A_2 upon stimulation of cells by growth factors (4, 5).

In addition, several lipids have been shown to be potent mitogens. These include lysophosphatidic acid, phosphatidic acid (6–8), or the sphingomyelin-derived compounds sphingosine (9), sphingosine-1-phosphate (10, 11), or sphingosylphosphocholine (12). The generation of phosphatidic acid and diacylglycerol by the action of phorbol ester or epidermal growth factor correlates with an inhibition of the G2 to M transition (13). These data indicate that catabolism of phospholipids can be part of the regulation of the cell cycle and growth.

Biosynthesis of phospholipids may also be an important element of the cell cycle because of the large increase in membrane biogenesis required for doubling of the cell components occurring at each cell division. Increased phospholipid biosynthesis occurs after induction of a G0 to G1 transition (14) or during S phase (15). But there is no report of a putative role for phospholipids as a part of the control of the cell cycle.

Phosphatidylcholine (PC) is the major phospholipid of mammalian cells, representing up to 50% of the membrane phospholipids. PC is synthesized mainly through the CDP-choline pathway, regulated by the enzyme

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; lysoPA, lysophosphatidic acid; lysoPC, lysophosphatidylcholine; lysoPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PDME, phosphatidyldimethylethanolamine; PE, phosphatidylethanolamine; PMME, phosphatidylmonomethylethanolamine; SM, sphingomyelin; sphingosyl-Pch, sphingosylphosphocholine; CT, cytidylyltransferase.

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CTP:phosphocholine cytidylyltransferase (16–18). The activity of the enzyme is regulated by a translocation process between a soluble reservoir of inactive enzyme and the membranes where it is activated by lipids. A nuclear localization of CT has also been recently proposed (19). Increased PC biosynthesis occurs when quiescent cells are triggered to enter the cell cycle by different types of stimuli (20–23). Stimulated PC synthesis was attributed to an increase in either the choline kinase activity (21) or in the activity and expression of cytidylyltransferase occurring in the first hours after the stimulation (22, 24). Recently, it has been demonstrated that PC accumulated during the S phase of the cell cycle in a macrophage cell line while CT activity was maximum during G1 (25). In fact, both biosynthesis and catabolism were occurring together in G1, but the degradative pathway(s) were inactivated during S phase, leading to a net increase in PC mass (25). Choline, the precursor for PC, is absolutely required for growth of cultured cells (26–28) and is an essential nutrient for mammals (29). Cells cultured in the absence of choline accumulate mainly in the G1 phase of the cell cycle (30) and a CHO mutant deficient in the cytidylyltransferase cannot grow at a non-permissive temperature (31, 32). Together, these results suggest a potential effect of the final product of the CDP-choline pathway, PC, on the control of cell growth and the cell cycle.

We have investigated the role of PC in the control of the S phase of the cell cycle by using a choline-deficient cell culture model. Choline deficiency is an effective and selective method to impair PC biosynthesis without affecting other metabolic pathways in cells (33). We demonstrate that choline-deficient fibroblasts are synchronized in late G1. In addition, cells that are both in G0 and choline-deficient have a dramatic decrease in the PC content which is correlated with a decrease in the number of cells progressing through the S phase. Normal cell replication can be restored by supplying choline or lysophosphatidylcholine (lysoPC), but no other phospholipid. We conclude that PC may be an important requirement for a normal progression through G1 and S phases of the cell cycle.

MATERIALS AND METHODS

Chemicals

[*Methyl*-^{3}H]thymidine (1.5–2.2 TBq/mmol) and [*methyl*-^{3}H]choline chloride (550 GBq/mmol) were supplied by Amersham Corp. 1,2 Dioleoyl-*sn*-glycero-3-phosphocholine (dioleoylPC), 1,2 dioleoyl-*sn*-glycero-3-phosphoethanolamine (dioleoylPE), egg yolk PC, egg yolk lysoPC, 1-oleoyl-lysophosphatidic acid (lysoPA), choline chloride, L-methionine, and sphingosylphosphocholine (sphingosyl-Pch) were from Sigma Chemical Co., St. Louis, MO. Lysophosphatidylethanolamine (lysoPE), phosphatidylmonomethylethanolamine (PMME), phosphatidyldimethylethanolamine (PDME), and phosphatidylethanolamine (PE) were obtained from Avanti Polar Lipids. Fetal bovine serum (FBS), streptomycin, penicillin, Dubelcco's modified Eagle's medium (DMEM cat. # 430-1600EL), and choline- and methionine-deficient medium (Formula # 79-5141EL) were purchased from Gibco.

Cell culture

C3H/10T1/2 mouse embryo fibroblasts, clone 8 (34) were kindly provided by Dr. W. J. Vaartjes, Utrecht, The Netherlands. These cells are highly sensitive to post-confluence inhibition of division. Cells were maintained in DMEM that contained 100 units of penicillin and 100 μg of streptomycin per ml, supplemented with 10% FBS in a 5% CO_2 humidified incubator. Subconfluent cells were passed every 7 days. Cell number was determined after trypsinization using a hemocytometer or a cell counter (Coulter Z1, Coultronic). Delipidation and dialysis of the serum were performed as described (35).

Cells were plated at a concentration of 1.25×10^3 cells per cm^2 for 2 days in 10% FBS, then shifted for 4 days to 0.5% FBS concentration to make the cells quiescent. Depending on the experiment, the incubation medium for quiescence was with 0.5% FBS, or was depleted of choline (sometimes choline and methionine) with 0.5% delipidated and dialyzed serum.

Induction of the cells into G1 phase was initiated by shifting the quiescent cells to a medium containing 10% FBS. Depending on the experiment, regular (28 μM choline), choline-supplemented (100 μM choline), or choline-deficient media were used with either regular, delipidated, or dialyzed serum.

Viability of the cells at the beginning or at the end of experiments was determined by two methods. Trypan blue exclusion to determine the ratio of dead to viable cells and an adhesion test to measure the number of cells able to re-adhere after trypsinization. Briefly, cells at time 0 or 32 h after addition of the serum were washed with phosphate-buffered saline, then trypsinized and counted in Trypan blue or with the cell counter. One third of the suspension was replated in complete medium and incubated for 6 h, a time required to obtain a complete adherence. Cells were washed two times with phosphate-buffered saline to eliminate nonadhesive cells, trypsinized, and counted again. The ratio between the second and the first counting gave the percentage of viable cells.

To analyze cell cycle parameters, cells were washed with phosphate-buffered saline, trypsinized, and fixed in 70% ethanol at −20°C. Analysis was performed using propidium iodide as a marker for DNA and analyzed by flow cytofluorometry on a Coulter Epics Elite (Coultronic) at wavelengths between 610 and 630 nm.

Pulse-chase experiments with choline

Cells were prelabeled with [^{3}H]choline (3 μCi/dish) in

a regular medium with 10% FBS. After 24 h, the medium was aspirated and the cells were washed with phosphate-buffered saline and further incubated in 0.5% FBS in the absence of choline. At each incubation time, the medium was harvested and the lipids were extracted from the cells according to Bligh and Dyer (36). The lower phase and upper phase were dried under nitrogen. The choline-containing compounds from each phase were separated by thin-layer chromatography on silica gel, scraped, and radioactivity was measured.

[^{3}H]thymidine incorporation into DNA and determination of entry into the S phase

For the analysis of DNA synthesis, [^{3}H]thymidine (0.5 μCi, 1 μM) was added to the Petri dish 1 h before cells were harvested. At each time point, the medium was aspirated, and the cells were washed with phosphate-buffered saline and treated with 10% ice-cold trichloroacetic acid for 10 min on ice to precipitate DNA. The dishes were washed twice with 10% trichloroacetic acid and the precipitated material was scraped into 1% sodium dodecyl sulfate–0.1 N NaOH. Radioactivity associated with the TCA-precipitate was determined by liquid scintillation spectrophotometry.

Entry into S phase was determined by measurement of [^{3}H]thymidine incorporated into DNA every 2 h after the induction of the G1 phase (addition of 10% FBS) and using a 1 h pulse with [^{3}H]thymidine (0.3 μCi, 1 μM). For each experiment, the time required to obtain the peak of radioactivity and the corresponding radioactivity (dpm) were used for the calculation of the data in Tables 1 and 2. Alternatively, entry into S phase was determined by flow cytofluorometry as previously described and results were correlated with [^{3}H]thymidine labeling.

In different experiments, a number of choline-containing or lipid-related compounds were added to the choline-deficient medium at the same time as the 10% delipidated FBS. Stock solutions of lysoPC, lysoPE, lysoPA, and sphingosyl-Pch (10 mM) were prepared in 100 mM KCl, 5 mM $MgCl_2$, 25 mM Tris-HCl (pH 7.4) (TKM buffer), sterilized by filtration, and added directly to the medium. All other phospholipids were prepared in ethanol (500 mM stock solution) and added to the medium prior to incubation with the cells.

Miscellaneous

Proteins were determined as described by Lowry et al. (37) in the presence of sodium dodecyl sulfate (0.07%, w/v) with bovine serum albumin as a standard. Radioactivity was analyzed in a Beckman scintillation counter (LS 3801) using Hionic Fluor (Packard Instrument Company Inc.) or ACS (Amersham) as scintillation fluids. Aqueous choline-containing compounds were separated from the aqueous phase of cell extracts by chromatography on silica gel G plates in the solvent ethanol–0.6% NaCl–NH_4OH 50:50:5 (v/v/v). Routine phospholipid analysis was performed by chromatography on silica gel G plates using the solvent $CHCl_3$–CH_3OH–CH_3COOH–H_2O 65:43:1:3 (by volume). Lipid phosphorus was determined as described (38). Lactate dehydrogenase activity was determined in the incubation medium as described (39). The photographs in Fig. 6 were obtained on a Nikon Diaphot-TMD inverted microscope with Kodak Tri X/400 ASA film.

RESULTS

Incubation at a low serum concentration (0.5%) rapidly synchronized C3H/10T1/2 cells at the G0/G1 stage of the cell cycle, which represented between 85% and 90% in 1 day (**Fig. 1-C**). Initiation of growth was achieved by addition of 10% FBS and indicated that the S phase (determined as a peak of radioactivity incorporated into DNA following a 1-h pulse with [^{3}H]thymidine) reached a maximum after 20 h (not shown). Analysis of the different phases of the cell cycle by flow cytofluorometry confirmed the results (not shown).

Choline deficiency accumulates C3H/10T1/2 cells in G1

In order to show that C3H/10T1/2 fibroblasts were sensitive to choline deficiency, several parameters for cell growth were analyzed in the presence or absence of choline during the log phase (Fig. 1). When growth was analyzed by measurement of the number of cells per dish (Fig. 1A), the cells in choline-supplemented medium reached confluence after 7 days. By contrast, in the absence of choline, a dramatically reduced growth curve was observed, indicating a potent inhibition of cell growth by choline deficiency. Normal cell growth correlated with an increased incorporation of [^{14}C]choline into PC, as well as with increased DNA synthesis (data not shown). In the absence of choline, DNA synthesis was attenuated and correlated with the low cell number indicating a potential effect of choline-deficiency on DNA replication.

Analysis of different phases of the cell cycle by flow cytofluorometry indicated that cells in the absence of choline progressively accumulated in G1-phase (Fig. 1-B), which represented up to 85% of total cells after 4 days of choline deficiency. In this experiment, the choline-deficient medium supplemented with 10% delipidated and dialyzed serum was changed daily, indicating that the observed accumulation in G1 was not due to the lack of serum as in Fig. 1-C. In addition, 4 days in choline-deficient medium versus 24 h in serum-deprived medium were necessary to reach the same percentage of G1 cells, strengthening the differences between both methods for growth arrest.

Addition of choline in the presence of serum induces a synchronous proliferation of the cells

The effect of addition of choline on cells previously accumulated in G1 upon choline deficiency (Fig. 1-B) was analyzed by flow cytofluorometry (**Fig. 2**). When 10% delipidated and dialyzed serum was added in the absence of choline (Fig. 2-A), a small increase in the number of cells in S-phase was observed, peaking at 20 h. At maximum, 33% of the cells were in S-phase. When choline alone was added (Fig. 2-B), about 18% of the cells were able to shift into the S-phase. By contrast, addition of both choline and delipidated and dialyzed serum induced a synchronous proliferation of 75% of the cells (Fig. 2-C). The peak of S-phase occurred at 14 h, indicating a net difference with serum or choline alone. Thus, the presence of both choline and serum lead to the synchronous proliferation of cells.

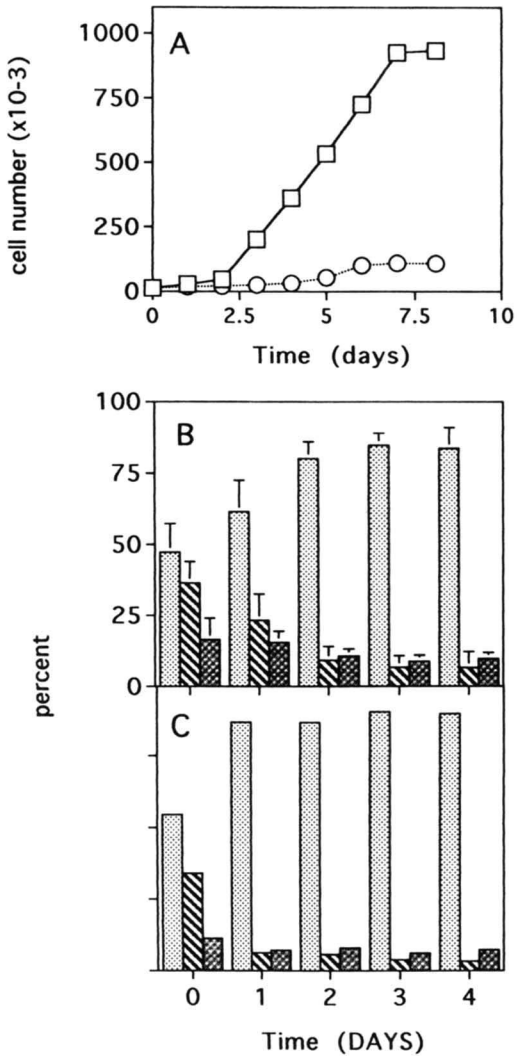

Fig. 1. Effect of choline deficiency on the growth of C3H/10T1/2 fibroblasts. Cells were incubated in DMEM containing 10% delipidated-FBS ± choline (28 μM) (panels A and B) or in 0.5% serum + choline (panel C). A: at the indicated times, one set of cells was harvested and the number of cells was determined (○, choline-deficient; □, choline-supplemented). The medium was changed to fresh medium every 3 days to maintain normal growth. The experiment was repeated twice with similar results. B: the different phases of the cell cycle were analyzed by flow cytofluorometry (see Experimental Procedures) on cells incubated in 10% delipidated and dialyzed serum in the absence of choline. The medium was changed daily. Results are given in percentage of cell in G1 (stippled), S (striped) or G2/M (shaded) and represent the average and standard deviation of five independent experiments. C: flow cytofluorometric analysis was applied on cells incubated in 0.5% serum in a complete medium; symbols as in B; results are the average of three experiments with standard deviation less than 1%.

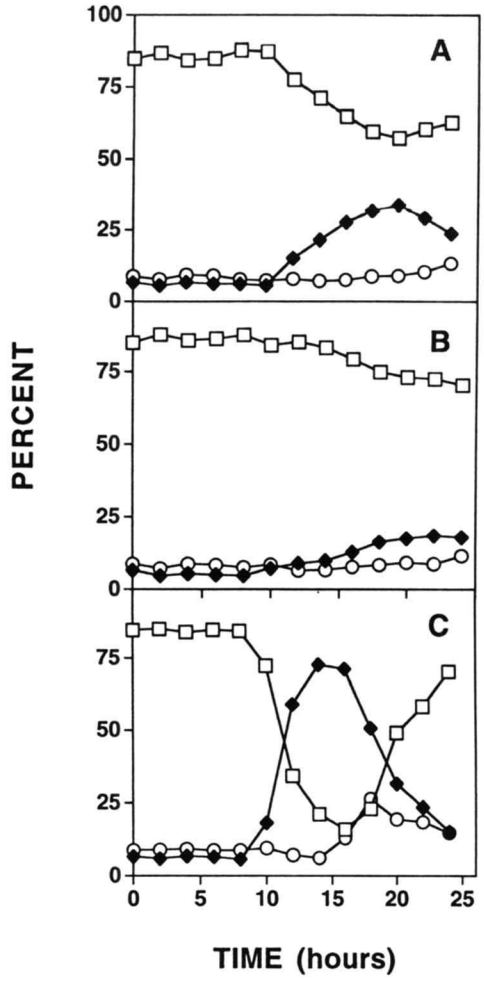

Fig. 2. Effect of addition of choline, serum or choline, and serum on cells starved for choline but not serum. Cells were incubated 4 days in the absence of choline plus 10% dialyzed and delipidated serum as in Fig. 1-B. The cells were then incubated under the same conditions (panel A), 100 μM choline (panel B) or 10% dialyzed and delipidated serum with 100 μM choline (panel C). Cell cycle parameters were determined every 2 h by flow cytofluorometry (see Experimental Procedures). Results are given as the percentage of G1 (○), S (♦) and G2/M (□) and are the average of four independent experiments.

Choline deficiency decreases the mass of PC and the phosphocholine pool in quiescent cells

We determined the effect of the absence of choline on different parameters of C3H/10T1/2 cells during the in-

duction of quiescence (**Fig. 3**). Cells were maintained for 1 to 4 days in choline-deficient or -supplemented medium, and in 0.5% delipidated and dialyzed FBS. Under choline-deficient conditions the activity of lactate dehydrogenase released into the medium increased modestly (not shown), probably due to a different permeability of the cell membrane. The protein content per cell (Fig. 3A) was lowered during quiescence as already described (40). The total viable cell number was slightly lower in the absence of choline, but did not change for up to 4 days (not shown). In addition, as will be discussed later, no morphological differences between choline-deficient and supplemented cells could be observed under the microscope (see Fig. 6-A and C). The quiescent cells became flat. Interestingly, the mass of PC per cell decreased dramatically, by more than 35%, upon choline deprivation (Fig. 3C), whereas no significant change could be observed in the mass of either SM (Fig. 3B) or PE (Fig. 3D).

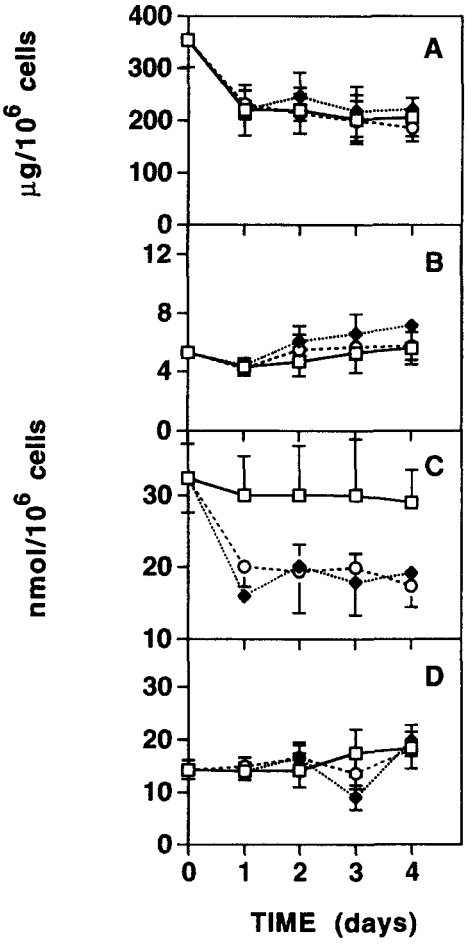

Fig. 3. Effect of choline, or choline and methionine deficiency on the lipid composition and protein content of quiescent cells. Forty-eight h after plating, the cells were incubated in 0.5% delipidated FBS in the presence of choline (28 μM, □), in the absence of choline (♦) or in the absence of choline and methionine (○). At the indicated times, the medium was harvested and the cells were washed with phosphate-buffered saline and extracted. The phospholipid composition, the protein content, and the number of cells were determined. (A), Protein content; (B), SM; (C), PC; (D), PE. The results are given in μg of protein or nmol of phospholipid per 10^6 cells and represent averages ± SD of at least three experiments.

Methionine is required for the control of DNA synthesis through the synthesis of polyamines, and can also be used by methyltransferases to supply PC in the liver (41) or by phosphoethanolamine methyltransferase to supply phosphocholine in the brain (42). The PE methyltransferase activity is very low in non-hepatic tissues (41). An enzyme assay performed on our cells indicated a low but detectable PE methyltransferase activity (20 pmol/min per mg). In order to ascertain whether or not methyltransferase activity might be attenuating the choline deficiency process, we performed the same experiments as above in a medium depleted of both choline and methionine (Fig. 3, A to D). The results were similar to those with choline deficiency only, indicating that quiescent fibroblasts did not obtain significant amounts of PC from methionine. These results clearly indicated that choline deficiency dramatically decreased the mass of PC in quiescent cells.

The amounts of radioactive choline-containing compounds were measured in a pulse-chase experiment. The cells were prelabeled for 24 h with [^{3}H]choline in 10% FBS followed by incubation for up to 72 h in 0.5% FBS in the absence of choline. The distribution of radioactivity in the water-soluble compounds at time 0 h was 4% in choline, 77% in phosphocholine, 1% in CDP-choline, and 18% in glycerophosphocholine (not shown). The amount of radiolabel in phosphocholine was reduced to 2.4% by 12 h after induction of quiescence in choline-deficient medium. No significant change was observed in the other aqueous choline-containing compounds. PC labeling increased in the first 12 h of the chase and then decreased by 50% in the subsequent 3 days of incubation in agreement with the decrease in mass of PC (Fig. 3C).

Choline deficiency retards and diminishes the S phase of the cell cycle

Choline deficiency was established during 4 days of quiescence in the absence of choline as described in the previous experiments (Fig. 3). Subsequently, the cell cycle was induced by addition of 10% FBS, in the presence or absence of choline (**Fig. 4; Table 1**). Entry of the cells into the S phase was monitored by the incorporation of [^{3}H]thymidine into replicating DNA or using flow cytofluorometric analysis. In the presence of choline and regular serum, (Fig. 4-A), the replication of DNA reached a maximum at 18 h after addition of serum. Comparison with quiescent cells obtained in the presence of choline (normal situation, not shown) indicated no difference in the timing of the S phase and in the amplitude of the peak when determined per cell number. When the cells were incubated in the presence of dialyzed serum and in the absence of choline (Fig. 4-A), the maximum amount of thymidine incorporated was slightly decreased but the

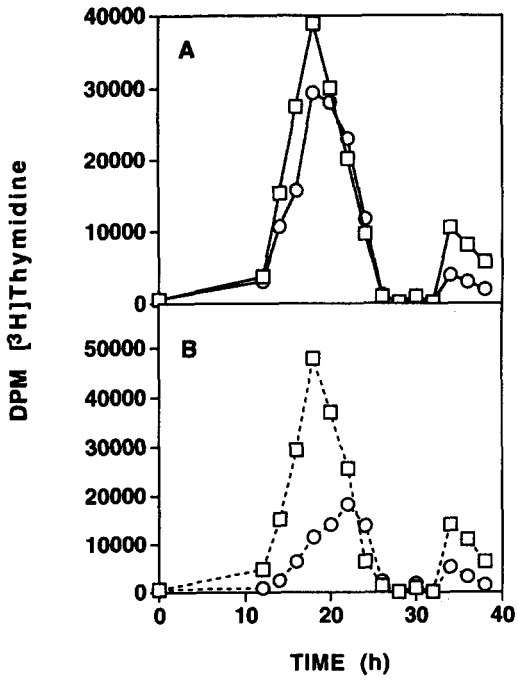

Fig. 4. Effect of choline deficiency on the S phase of the cell cycle in the presence of dialyzed or delipidated FBS. Cells were incubated 4 days in 0.5% FBS in a choline-deficient medium. At time 0, quiescent choline-deficient cells were incubated with 10% dialyzed FBS (panel A) or 10% delipidated FBS (panel B) in the absence (○) or presence (□) of choline (100 μM). At the indicated times, cells were labeled for 1 h with [^{3}H]thymidine (0.3 μCi, 1 μM) and the radioactivity incorporated into DNA was determined. The experiment was repeated many times as summarized in Table 1.

peak still occurred around 18 h. In contrast, when the cells were incubated with delipidated and dialyzed serum (Fig. 4-B), the S phase normally observed in the presence of choline (Fig. 4-A) was dramatically reduced in magnitude in the absence of choline. In addition, the peak for DNA synthesis was delayed by about 5 h. Thus, the absence of choline only had an effect on DNA replication in the absence of lipids from the serum.

The viability of the cells was checked at time 0 (addition of the serum) and 32 h after the experiment (Fig. 4) was begun (after the first mitosis has occurred). The percentage of cells excluding Trypan blue was between 90% and 95%. When the adhesion test was used, the percentage of viable cells was reduced only by a few percent, around 85% and 90% at time 0 and 32 h, respectively. No significant difference in viability was observed between choline-deficient and choline-supplemented experiments (not shown).

These results were confirmed repeatedly as summarized in Table 1 and the differences were highly significant in a paired *t* test. When the quiescence was established in the absence of both choline and methionine, the final result was very similar; thymidine incorporation was decreased and the peak of radioactivity occurred later in the choline-deficient cells than in the choline-supplemented cells. However, compared to choline deficiency alone, an additional delay of 4 h was observed when methionine was also absent from the medium. This probably reflected the time required for the quiescent methionine-deficient cells to recover normal quantities of compounds involving methionine for their synthesis: proteins, or, more specifically, polyamines (important for the control of

TABLE 1. Effect of choline deficiency on the parameters of the S phase under different conditions of induction of the G0 to G1 transition

		− Chol + Meth		− Chol − Meth	
10% Fetal Bovine Serum	Choline	Peak	dpm	Peak	dpm
		(n = 4)			
Regular	+	19.0 ± 2.2	31,000 ± 7,600	nd	nd
Dialyzed	−	20.0 ± 1.2 (ns)	31,000 ± 12,000 (ns)	nd	nd
		(n = 7)		(n = 4)	
Delipidated	+	19.7 ± 1.4	35,800 ± 9,400	23.5 ± 1	22,125 ± 7,700
Delipidated	−	24.9 ± 2.1[a]	15,900 ± 5,000[a]	29.0 ± 1.1[a]	11,800 ± 3,400[b]

Cells were incubated for 4 days in 0.5% delipidated FBS either without choline or without both choline (Chol) and methionine (Meth). The G0 to G1 transition was induced by addition of either 10% regular or delipidated FBS in the presence of 100 μM choline or 10% dialyzed or delipidated FBS in the absence of choline. The time in h to obtain the peak of [^{3}H]thymidine incorporation into DNA and the amount of maximum labeling of DNA (dpm/dish) were calculated for each experiment (as in Fig. 4). The values are averages ± SD calculated from the number of experiments indicated (n). The significance of differences was determined using a Student's paired *t* test; ns, not significant; nd, not determined.

[a] $P < 0.001$.

[b] $P < 0.01$.

DNA replication). These results clearly indicate a potent effect of choline deficiency on the replication of DNA.

Recovery of a normal S phase by addition of lysoPC but not mitogenic lipids

To determine which lipids in dialyzed serum might be responsible for the recovery of the normal S phase observed in Fig. 4, we analyzed the replication of DNA in medium containing delipidated serum and lacking choline but supplemented with phospholipids (**Fig. 5-A, Table 2**). When PC (50 μM) was added to the cells (Fig. 5-A), the same delay and decrease in the S phase were observed as for the cells incubated in the absence of choline. In contrast, when lysoPC (50 μM) was added, the delay and decrease in thymidine incorporation were suppressed and a normal S phase (similar to that in the presence of choline) was observed. Different concentrations of lysoPC and PC as well as lysoPE, PE, PMME, or PDME were also tested (Table 2-A). The recovery of normal cell division was highly specific for choline and lysoPC. The maximum amount of radiolabeled thymidine incorporated into DNA was, respectively, 1.93- and 2.02-fold higher in choline- or lysoPC-supplemented (50 μM) cells compared to control cells cultured in the absence of choline. The modest increase observed in the amplitude when PDME was added was not significant in a paired *t* test. Dioleoyl-PC or -PE caused a rapid lysis of the cells, in agreement with previous observations on the effect of polyunsaturated PC on the growth of CHO mutants defective in PC biosynthesis (32). Finally, the delay in the peak of S phase (not shown) strictly correlated with the amplitude of replication for every compound (as in Fig. 4-B and Table 1).

To determine whether the effect of the absence of choline on the S phase could be related to a deficiency in one/some of the characterized mitogenic lipids (7, 10–12), we incubated the choline-deficient cells in the presence of different mitogens (Table 2-B). Interestingly, not one of the tested compounds restored the S phase. The small increase in the maximum of S phase with sphingosyl-Pch was only significant at 50 μM, but the timing of the S phase was not significantly different from that in control cells incubated in the absence of choline (not shown). Sphingosine or lysoPA had no effect. These results suggested that the perturbation of the S phase in choline-deficient medium strongly correlated with the lack of PC.

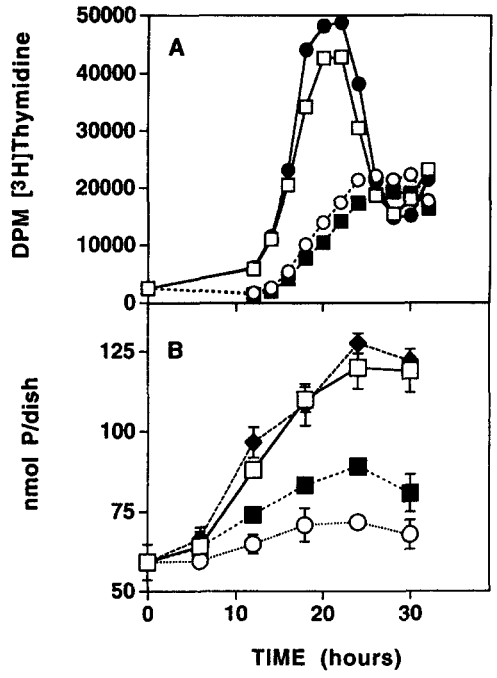

Fig. 5. Effect of addition of choline, PC, or lysoPC on the recovery of a normal S phase and PC content in C3H/10T1/2 cells. Panel A: quiescent choline-deficient cells were incubated in 10% delipidated FBS in the absence of choline (○) or in the presence of choline 100 μM (□), lysoPC 50 μM (●), or PC 50 μM (■). One h before harvesting, cells were incubated with [^{3}H]thymidine (0.3 μCi, 1 μM) and the radioactivity incorporated into DNA was determined. The experiment was repeated as summarized in Table 2. Panel B: the amount of PC was determined every 6 h on a second set of plates after lipid extraction. The results are averages ± SD (n = 3). No choline (○); +100 μM choline (□); +50 μM lysoPC (♦); +500 μM PC (■).

Characterization of the lysoPC-mediated recovery of the S phase

The phospholipid content of the cells was analyzed after addition of serum ± 100 μM choline, 500 μM PC, or 50 μM lysoPC (Fig. 5-B). No difference was observed for SM or PE upon addition of any of these compounds (not shown). However, in the presence of choline or lysoPC, a striking increase was observed in the mass of PC (Fig. 5-B), indicating recovery of the PC content during these incubations. When PC (500 μM) was added, the mass of PC increased slightly, but as a normal S phase was not recovered in the presence of PC, we assume that the increase in PC mass was insufficient to suppress the effect of choline deficiency. Alternatively, PC molecules might have been nonspecifically associated with the cell membrane and not efficiently incorporated into the cells. In addition, an increase in PC mass was observed when dialyzed, but not delipidated, serum was added to the cells grown in choline-deficient medium (data not shown), in agreement with the results from Fig. 4-A.

We also analyzed the morphology of the cells under different conditions of incubation (**Fig. 6**). The quiescent cells (Fig. 6-A, 0.5% FBS + choline) were flat and large when compared with the smaller and denser cells observed in synchronized growing cultures (Fig. 6-B, 10% FBS + choline). No difference could be observed between the quiescent choline-supplemented (Fig. 6-A) and choline-deficient (Fig. 6-C) cells. Addition of serum in a choline-deficient medium (Fig. 6-D) indicated that only a small percentage of the cells were similar in appearance to the growing cells observed in Fig. 6-B. On the other hand, addition of delipidated serum in the presence of

TABLE 2. Effect of phospholipids and mitogenic lipids on the parameters of the S phase in a choline-deficient medium

Variable	μM	n	Ratio Maximum dpm [^{3}H]Thymidine Incorporated
Control – choline			1
+ choline	100	12	1.93 ± 0.28[a]
A Phospholipids			
LysoPC	10	3	1.81 ± 0.07[a]
LysoPC	50	9	2.02 ± 0.12[a]
PC	50	2	0.86
PC	500	2	0.91
DioleoylPC	1000	2	0.00
LysoPE	50	2	1.06
PE	50	2	0.92
PE	500	2	0.85
DioleoylPE	1000	2	0.00
PMME	50	2	0.87
PMME	500	2	1.06
PDME	50	2	0.77
PDME	500	3	1.21 ± 0.10 (ns)
B Mitogenic lipids			
Sphingosine	20	2	1.12
Sphingosyl-Pch	10	3	1.22 ± 0.14 (ns)
Sphingosyl-Pch	50	3	1.38 ± 0.10[b]
LysoPA	10	4	0.66 ± 0.10[a]
LysoPA	50	4	0.55 ± 0.13[a]

Quiescent choline-deficient cells were incubated as in Fig. 5, with 10% delipidated FBS in the absence of choline (Control – choline) or in the presence of choline or different phospholipids (A) or mitogenic lipids (B). The maximum incorporation of [^{3}H]thymidine into DNA was determined; number of independent experiments (n). Results are expressed as the ratio of the maximum dpm [^{3}H]thymidine incorporated to the maximum dpm of the control cells (without choline). The significance of difference (P) between the treated cells and the control cells was calculated using a Student's paired t test; ns, not significant.

[a] $P < 0.01$.

[b] $P < 0.05$.

either choline (Fig. 6-E) or lysoPC (Fig. 6-F) indicated that almost all the cells displayed the morphology of growing cells. These results agree with the observations from Figs. 3 to 5 and Tables 1 and 2.

Only 30–40% of cells grown in choline-deficient medium undergo mitosis

To determine whether or not the cells going into the S phase in the previous conditions were able to proceed through a complete cell cycle, we counted the number of viable cells at the end of the time required to complete mitosis (32–35 h). The results (**Table 3**) clearly indicated that only 30–40% of the cells were dividing in the absence of choline or in the presence of PC, whereas almost all of the cells divided in the presence of choline or lysoPC. The results with PDME and sphingosyl-Pch were not significantly different from those with PC, while fewer cells treated with lysoPA acid were able to divide than in the absence of choline. These data strongly correlate with the recovery of the normal S phase only with choline and lysoPC, and the absence of an effect by PC and other lipids.

Effect of a delayed addition of choline on the S phase

Quiescent choline- (**Fig. 7-A**) or choline- and methionine-deficient cells (Fig. 7-B) were induced with delipidated serum, but choline was added later. The time required for the peak of thymidine incorporation, and the maximum amount of [^{3}H]thymidine incorporation, in the absence of choline, as well as the recovery of a normal S phase in the presence of choline added at time 0 h were in agreement with the data in Table 1. However, when choline was added 8 h after addition of the serum (Fig. 7-A), and 7 h or 13 h after addition of methionine and serum (Fig. 7-B), the amplitude of the S phase was as high as when choline was added at 0 h. Only a delay by 2 to 4 h was observed in the peak of S phase. By contrast, when choline was added at 28 h (corresponding to the end of a normal S phase, Fig. 7-A), no difference was seen up to 40 h when compared to the control minus choline. An intermediate situation could be observed upon addition of choline at 21 h (a time corresponding to the first part of a normal S phase, Fig. 7-B) where a partial recovery of the S phase was observed. These data indicated that upon addition of choline the cells rapidly compensated for the choline deficiency up to a defined point beyond which DNA replication was irreversibly affected. In the intermediate situation (21 h, Fig. 7-B), the partial recovery could be attributed to a number of cells still at the end of G1 and which could proceed through a normal (and delayed) replication upon choline addition.

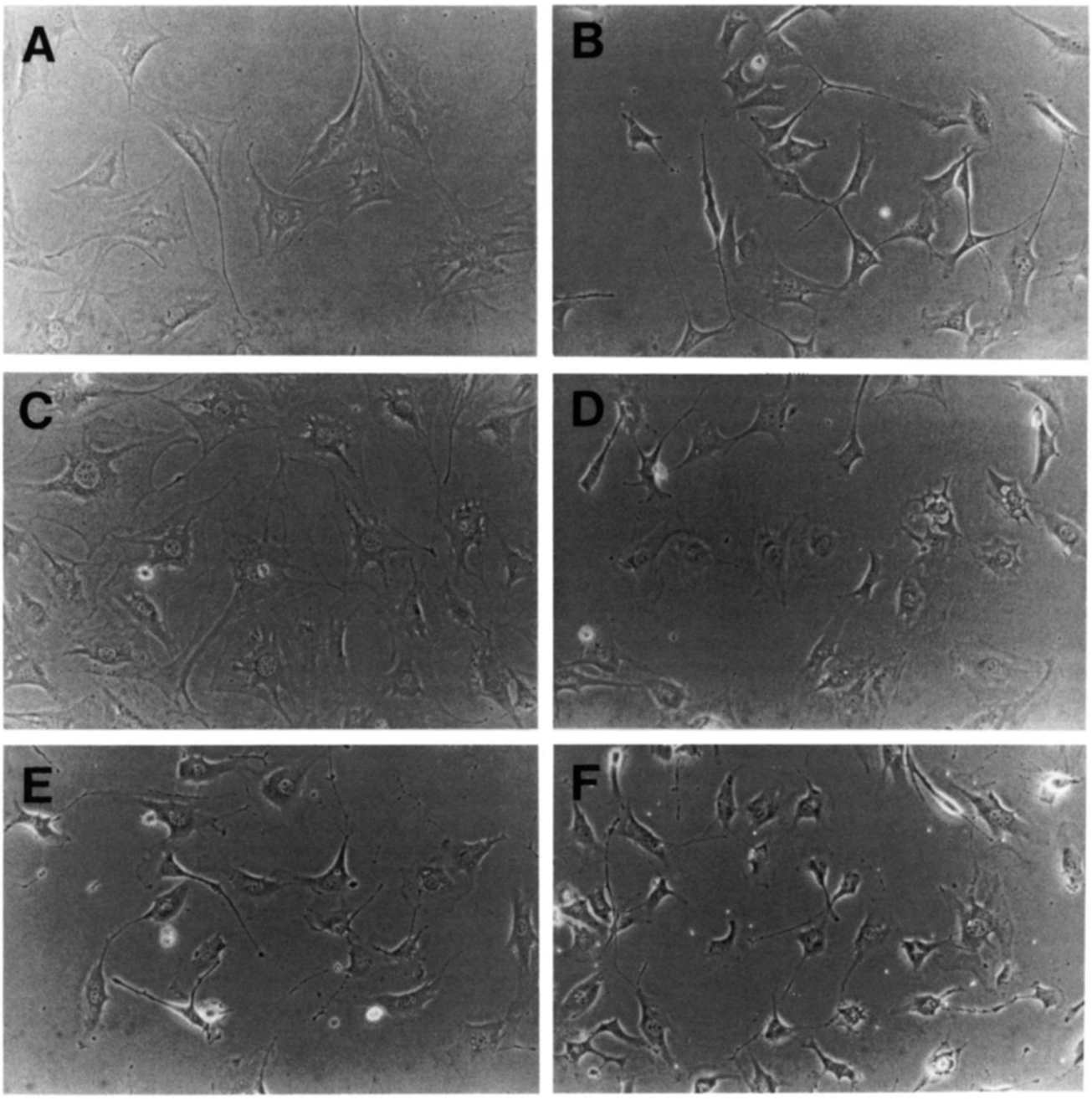

Fig. 6. Phase-contrast micrographs of cells incubated in the absence or presence of choline. Cells were incubated 4 days in 0.5% FBS under the presence (A-B) or in the absence (C-F) of choline, then further incubated under the following conditions: A, 0.5% FBS + choline; B, 10% delipidated FBS + choline; C, 0.5% FBS − choline; D, E, F, 10% delipidated FBS − choline (D), delipidated FBS + 100 μM choline (E) or delipidated FBS + 50 μM lysoPC (F). Micrographs were taken 22 h after addition of the FBS, i.e., at the time corresponding to the peak of the S phase.

DISCUSSION

To determine the requirement of PC for the cell cycle, we have used a system to perturb PC metabolism and further analyzed the effect on the progression of fibroblasts synchronized in G0 throughout the different phases of the cell cycle.

Choline deficiency is a useful model for studies on the role of PC in the cell cycle

The choline-deficient model has been a useful system with which to modify PC metabolism in animals (43). In cultured cells, choline deficiency increases the membrane-bound activity and decreases the cytosolic activity of

TABLE 3. Effect of phospholipids and mitogenic lipids on cell division in a choline-deficient medium

Condition	μM	n	Ratio between Cell Number after Mitosis to That at Time 0 h
Control (time 0 h)			1.00
− Choline		11	1.30 ± 0.22
+ Choline	100	8	1.97 ± 0.15
LysoPC	10	1	1.83
LysoPC	50	8	1.98 ± 0.36
PC	500	6	1.43 ± 0.32
PDME	500	3	1.37 ± 0.40
Sphingosyl-Pch	10	3	1.23 ± 0.13
Sphingosyl-Pch	50	3	1.46 ± 0.05
LysoPA	10	2	0.74
LysoPA	50	2	0.60

Quiescent choline-deficient cells were incubated as in Table 2 and supplemented with choline (100 μM), lysoPC (10 and 50 μM), PC (500 μM), PDME (500 μM), sphingosyl-Pch (10 and 50 μM), and lysoPA (10 and 50 μM). The number of cells in the dishes was determined after 32–35 h of incubation, the time required to complete mitosis in these synchronized cells. Results are expressed as averages ± SD of the ratio between the number of cells after mitosis and the number of cells at time 0 h.

cytidylyltransferase, the regulatory enzyme of PC biosynthesis (33, 44, 45). Binding of cytidylyltransferase is reversed by addition of choline (33, 44) or lysoPC (46). When we subjected the C3H/10T1/2 cells to choline deficiency during the induction of quiescence, we found a specific decrease in cellular PC content and phosphocholine pool, strongly affecting PC biosynthesis. However, cell viability, the content of other major phospholipids (PE, SM), as well as the cell morphology, were unaffected. The unchanged SM content is somewhat surprising because PC is the precursor for SM biosynthesis (47). However, the result is consistent with the previous observations on a CHO mutant defective in PC biosynthesis, in which the PC level decreased by 50% at a restrictive temperature, whereas the concentration of SM was unaffected (31). How the cells still survive with a loss of 35% of PC mass is not clearly explained. A decrease in PC mass up to 50% has already been observed with the CHO mutant (31) or in choline-deficient cells (30) without a modification in cell viability.

PC is required for normal replication of DNA

It is clear from our results that choline deficiency decreases the amplitude and delays the onset of DNA replication, which can be rescued by a lipid component present in the serum. Indeed, we found that lysoPC restores a normal S phase and PC content, and serum contains 1–3 mM phospholipids (32). Similarly, a regular PC content was restored after addition of choline or lysoPC to choline-deficient hepatocytes (46) or to the CHO mutant defective in PC biosynthesis (31).

In contrast to the experiments with the CHO mutant (32), we could not restore a normal S phase by addition of PC, although the effect of choline deficiency was reversed with dialyzed serum. The lack of an effect by PC can be attributed to the observation that in the quiescent state exogenous PC is not taken up by serum-deprived cells (32). In contrast, lysoPC is easily taken up by cells and is immediately acylated to PC (48, 49).

Among the checkpoints described in the cell cycle, there is a control point for DNA replication, at which the cells are arrested in G2 if a normal and complete DNA replication had not occurred (50). In the presence of choline or lysoPC virtually every cell present on the plate at time 0 could undergo a complete cell cycle. By contrast, only 30–40% of the cells still proceeded to mitosis in the absence of choline or after supplementation with lipids other than lysoPC. This indicates that the incorporation of thymidine monitored an abnormal or incomplete DNA replication. One or more of the following hypotheses might explain why the S phase is not inhibited by 100%. *1)* We can assume that in choline-deficient media, cells release choline-containing compounds into the medium which might be able to restore cellular PC content, allowing a normal S phase to occur in some of the cells. *2)* Possibly some residual lipids in the delipidated serum might be sufficient to replace the lack of PC molecules. *3)* We cannot exclude the possibility that there are two different pools of cells, some of which have their DNA synthesis less affected by the choline deficiency than others. This could also explain that 33% of the cells arrested in G1 in 10% serum without choline could proceed through a cell cycle (Fig. 2-A).

Requirement for PC to proceed through a normal S phase occurs in the late G1

A major point of our findings is in the timing of events related to the effect of choline deficiency on the progression through G1 and S. In the CHO mutant defective in PC biosynthesis (31, 32), an inhibition of cell growth was observed, but no relationship with the cell cycle events was described. Here we found that choline and PC

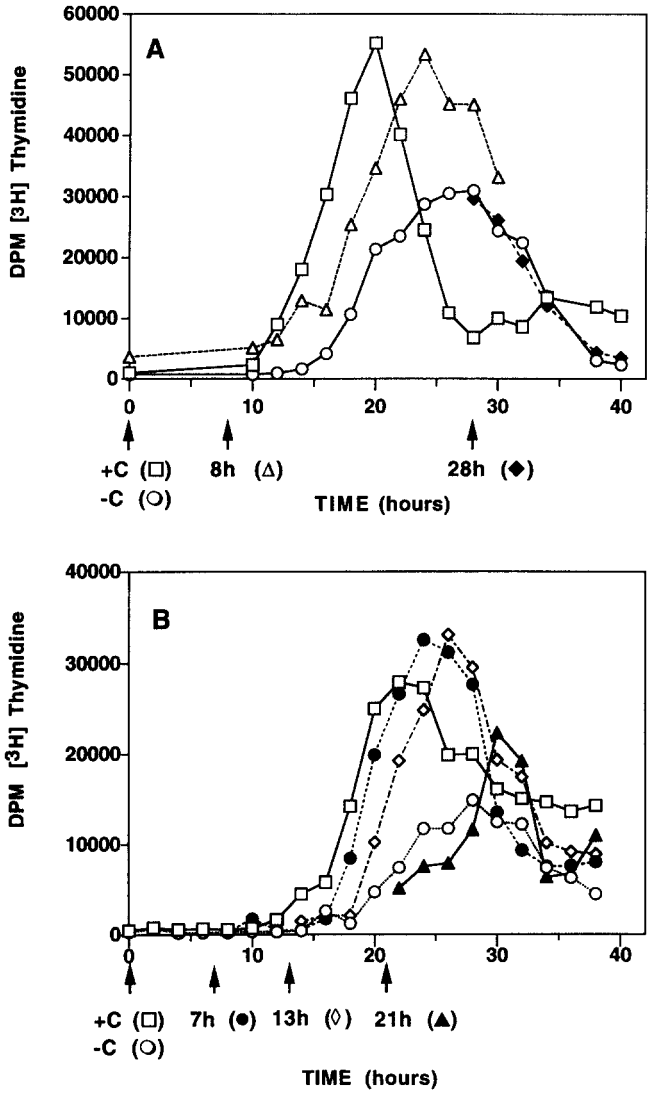

Fig. 7. Effect of addition of choline on the S phase at various times after the addition of delipidated FBS. Quiescent choline-deficient (panel A) or choline- and methionine-deficient cells (panel B) were incubated at time 0 h with 10% delipidated FBS containing methionine to induce the G0 to G1 transition. Choline (100 μM) was added at different times after the addition of serum as follows: no choline (○), choline added at time 0 h (□), 8 h (panel A, △), 28 h (panel A, ◆), 7 h (panel B, ●), 13 h (panel B, ◊), and 21 h (panel B, ▲). At the indicated times, cells were labeled for 1 h with [^{3}H]thymidine (0.3 μCi, 1 μM) and the radioactivity incorporated into DNA was determined. The results are the average of duplicate determinations and the experiment was repeated with similar results.

deficiency stop cell growth and cells accumulate in the middle to late G1. Indeed, after choline deficiency, the peak of S phase upon readdition of choline and serum is obtained around 6 h earlier than when cells are released from G0 (14 h versus 20 h, respectively). Moreover, choline is necessary just 2–4 h before the beginning of S phase in order to obtain a normal replication. If choline is added later during the S phase, DNA replication is not rescued. The need for PC just before S phase also explains the lack of effect of mitogenic lipids. LysoPA or the sphingomyelin-related lipids are potential second messengers inducing DNA replication and mitosis from a quiescent state (G0), but they are ineffective on choline-deficient cells. Finally, upon addition of lysoPC or choline, PC mass is increased by 40% as soon as 12 h after the induction of G1. Thus the cells have reached an almost normal level of PC before the beginning of S phase.

One intriguing point is that choline deficiency synchronizes the cells in G1, but both choline and serum are necessary to induce a synchronous proliferation of the cells in contrast to choline or serum alone. There is a restriction point for serum in G1 beyond which cells will proceed through a complete cell cycle even in the absence of growth factors. But it has been shown (51, 52) that when the cells at G1/S are prevented from synthesizing DNA, they return back to the restriction point and re-addition of serum is required in order to proceed through the S phase when the block is reversed. We propose that choline deficiency blocks the cells between this checkpoint and S phase, which could explain that new addition of serum is required to induce the synchronous proliferation of cells.

PC biosynthesis has been shown to occur in G1 and S phases, while PC catabolism is only active in G1 in a normal situation (25). From our results, there is also an increase in PC mass during the S phase upon addition of choline or lysoPC. The early accumulation of PC mass (between 6 and 12 h) probably reflects the need for the cells to restore a normal PC content rapidly. Indeed, choline deficiency increases CT activity in hepatocytes (33) and upon addition of serum on quiescent cells, CT expression is induced (22) and CT activity increases (25). Thus, in our situation, choline deficiency could strengthen the normal activation of CT in G1, leading to an accumulation of PC already in G1.

How does choline deficiency impede DNA replication?

We conclude that PC is an important component for progression of the cells through the cell cycle, while the deficiency of PC arrests cells just before the S phase. The reasons for these observations remain speculative. One possible explanation is that a perturbation of the structure and/or the function of the different membranes by the loss of 35% of the PC content could affect the replication of DNA. Indeed, the nuclear membrane is an important factor regulating DNA synthesis (53, 54) because the sites of replication are immobilized on a structural framework (54). Also, the "anchorage" requirement of the cells for DNA replication in fibroblasts mediates a G1/S control shortly before activation of the G1/S p34^{cdc2}-like kinase (55). Finally, transport of important components that regulate DNA replication into the nucleus might be impaired as a result of a deficiency of PC in the nuclear membrane. For example, there might be an effect on the transport of cyclin into the nucleus (56) which is required

for formation of the S phase-promoting factor (57).

Whether or not PC biosynthesis might directly affect DNA replication is not yet clarified. Although a block of DNA replication does not affect the increase in PC mass (25), a block of PC biosynthesis arrests the progression in late G1 and affects DNA synthesis.

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