Regulation of Choline Kinase Activity and Phosphatidylcholine Biosynthesis by Mitogenic Growth Factors in 3T3 Fibroblasts*

Craig H. Warden‡ and Morris Friedkin†

From the ‡Department of Medicine, Division of Cardiology, University of California, Los Angeles, Los Angeles, California 90024 and the ¶Department of Biology, University of California, San Diego, La Jolla, California 92039

The regulation of choline kinase activity by fetal bovine serum and the regulation of phosphatidylcholine biosynthesis by choline kinase have been investigated in 3T3 fibroblasts. Treatment of quiescent 3T3 cells with serum was shown in previous work to increase phosphocholine pool size and phosphatidylcholine biosynthesis. We now report that treatment of 3T3 cells with serum increased intracellular choline kinase activity by 2–3-fold, a concomitant 2–3-fold decrease of intracellular free choline concentrations. Initial rates of choline transport were the same in quiescent and serum-treated cells, whereas choline kinase activity was 2–3-fold higher in serum-treated cells. As a consequence, free choline concentrations were 2–3-fold lower in serum-stimulated cells than in control quiescent cells. Phosphocholine turnover rates were increased 2-fold by serum treatment both as a consequence of a serum-dependent increase of phosphocholine pools and as a result of a serum-dependent lowering of the phosphocholine half-life. Thus, the overall response of 3T3 cells to serum stimulation included decreased choline pools and increased choline kinase activity, phosphocholine pool size, phosphocholine turnover, and phosphatidylcholine biosynthesis.

Choline is incorporated into phosphatidylcholine (PC) by a three-step pathway first described by Kennedy and Weiss (1966). Choline kinase catalyzes the first committed step, phosphorylation of choline to form phosphocholine. Phosphocholine and CTP are then converted to CDP-choline by CTP:phosphocholine cytidylyltransferase, and finally CDP-choline is incorporated into PC by choline phosphotransferase.

Biosynthesis of phosphatidylcholine is clearly regulated by cytidylyltransferase, which catalyzes the rate-limiting step for PC synthesis (Vance and Choy, 1979). Activity of the cytidylyltransferase is regulated by phosphorylation (Pelech and Vance, 1982), translocation from cytosol to microsomes (Lim et al., 1983), increased cytoplasmic CTP concentrations (Choy et al., 1980), and increased concentrations of phosphocholine (Vigo and Vance, 1981; Paddon et al., 1982). In previous work, we have shown that stimulation of quiescent 3T3 fibroblasts with growth factors increased phosphorylation of choline, in vitro choline kinase activity, phosphocholine and phosphoethanolamine pools, and PC synthesis (Warden et al., 1980; Warden and Friedkin, 1984). These results suggested that choline kinase could regulate PC biosynthesis in 3T3 cells. If choline kinase is a regulatory enzyme for PC synthesis in 3T3 cells, then it should show several additional properties. First, it should be a crossover point. Enzymes whose intracellular substrate concentrations decrease while their product concentrations increase following an experimental manipulation are crossover points (Rolleston, 1972). Identification of crossover points gives a reliable indication of the in vivo sites of metabolic regulation. Thus, intracellular concentrations of choline and phosphocholine were studied to determine if choline kinase is at a crossover point for PC synthesis following serum stimulation of 3T3 cells. Second, phosphocholine turnover was measured to confirm that increased phosphocholine concentrations do in fact increase conversion of phosphocholine to CDP-choline. Finally, activation of intracellular choline kinase by serum was measured since in vitro and in vivo enzyme activities are not always correlated (Vance et al., 1980; Wohlhueter and Plagemann, 1981; Rozengurt et al., 1978).

EXPERIMENTAL PROCEDURES

Cell Culture—Swiss mouse 3T3 fibroblasts were cultured in 100-mm Falcon brand tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cells subcultured into 30-mm Nunc brand dishes in DMEM with 10% serum were utilized for experiments 7–14 days after reaching confluence.

Assay of Intracellular Choline Kinase Activity—In order to measure intracellular choline kinase activity it is necessary to: 1) know the specific activity of free intracellular choline, 2) separate choline from its labeled products; and 3) show that intracellular choline phosphorylation is linear with time. 1) When 3T3 cells are exposed to labeled choline, radioactivity in free intracellular choline increases for 5 min and then remains at a constant level for at least 3 h (Figs. 1A and 3A). Thus, after 5 min of exposure to labeled choline, the specific activity of intracellular choline can be approximated by the specific activity of labeled choline in the culture medium. This approximation will be accurate as long as 3T3 cells are not generating significant amounts of unlabeled choline by degradation of pre-existing PC. Intracellular phosphocholine specific activity was identical with the specific activity of choline in the labeling medium after exposing 3T3 cells to labeled choline for 60 min (Warden and Friedkin, 1984); thus, there is no dilution of intracellular labeled choline by unlabeled cell-derived choline. 2) 3T3 fibroblasts do not make betaine (Warden and Friedkin, 1984). Thus, the products of choline metabolism in 3T3 cells are confined to the products of the choline kinase reaction: phosphocholine, CDP-choline, PC, lysolecithin, and gleyrophosphorylcholine. To assay intracellular choline kinase activity, it is necessary to measure the radioactivity present in all of these products, since they will all be produced during exposures to labeled choline. Formation of choline kinase products can be quantitated from two assays. First, acid-soluble choline, phosphocholine, CDP-choline, and

* This work was supported by United States Public Health Service Grant CA 11440 from the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed.

The abbreviations used are: PC, phosphatidylcholine; DMEM, Dulbecco's modified Eagle's medium; MEM, Eagle's minimal essential medium.
RESULTS

Time Course of Choline Phosphorylation—Stimulation of PC synthesis by growth factors can be measured by exposing quiescent 3T3 cells to growth factors or DMEM for 6 h, washing, treating with labeled choline for 60 min at 37 °C, and then measuring uptake into acid-soluble and acid-insoluble material (Warden and Friedkin, 1984). In order to demonstrate that intracellular choline phosphorylation was linear with time, this protocol was altered for the experiment shown in Fig. 1 by labeling previously stimulated cells with [14C] choline for the times shown. Free intracellular labeled choline increased rapidly for 5 min and in all cases had reached maximal levels by 10 min (Fig. 1A). Control quiescent cells or cells exposed to DMEM for 6 h had 3-fold larger choline pools than cells stimulated with 10% fetal bovine serum or insulin (Fig. 1A). Incorporation of radioactivity into acid-soluble products of choline kinase was approximately linear over the 60-min exposure to [14C]choline (Fig. 1B). The data from Fig. 1, A and B, were utilized to calculate the percent of acid-soluble material which is phosphorylated (Fig. 1C). In cells exposed to serum or insulin, choline phosphorylation proceeded rapidly and was nearly maximal by 10 min, while quiescent and DMEM-treated cells showed a strikingly different pattern of linear time-dependent increases (Fig. 1C).

At the 60-min time point, uptake into acid-insoluble material was 242, 208, 552, and 727 pmol/mg of cell protein for quiescent cells or cells treated with DMEM, insulin, or serum, respectively. Uptake into acid-insoluble material measures the rate of choline transport and the initial rate of choline phosphorylation. When cells were exposed to 36 [14C]choline were clearly evident and virtually maximal after 60 min of stimulation with serum or insulin (Fig. 2). In another experiment, choline pools decreased most markedly after 15-30 min of exposure to serum (data not shown).

Choline Pools and Phosphorylation at a Concentration above the $K_m$ for Transport—The experiments shown in Figs. 1 and 2 were conducted with 7 or 8.4 μM choline in the labeling medium. When the initial rates of choline transport were measured by incubating quiescent 3T3 cells with several concentrations of labeled choline for 60 s, the $K_m$ for transport was found to be 16 ± 2 μM (n = three experiments, ±S.D., data not shown). When cells were exposed to 36 μM labeled choline, intracellular choline pools were decreased by 78% of the serum-stimulated pools (Fig. 3A). Amounts of phosphorylated acid-soluble material formed were 3-fold higher in serum-

...trichloroacetic acid or with 0.4 M glycerophosphorylcholine by extraction of labeled cells with 5% trichloroacetic acid and lysolecithin by extraction of labeled cells with 5% trichloroacetic acid or with 0.4 M glycerophosphorylcholine. The rate of formation of choline kinase products is linear with time. However, measurements of the rate of formation of choline kinase products, as described above, will lead to an underestimate of the true rate of intracellular choline kinase activity, since it has been reported that cells prelabeled with choline release label back into the culture medium during chase periods (Vance et al., 1980). Thus, the actual rate of intracellular choline kinase activity could be determined by adding together the combined choline kinase products which are cell-associated, measured as described above, and the choline kinase products which were released into the medium.

When 3T3 cells were prelabeled with [14C]choline for 3 h at 37 °C, 1% trichloroacetic acid or with 0.4 M glycerophosphorylcholine by extraction of labeled cells with 5% trichloroacetic acid or with 0.4 M glycerophosphorylcholine. The rate of formation of choline kinase products is linear with time. However, measurements of the rate of formation of choline kinase products, as described above, will lead to an underestimate of the true rate of intracellular choline kinase activity, since it has been reported that cells prelabeled with choline release label back into the culture medium during chase periods (Vance et al., 1980). Thus, the actual rate of intracellular choline kinase activity could be determined by adding together the combined choline kinase products which are cell-associated, measured as described above, and the choline kinase products which were released into the medium.

When 3T3 cells were prelabeled with [14C]choline for 3 h at 37 °C, the rate of release of label into medium, measured at 15-min intervals during a 90-min chase, was approximately 12, 14 or 26 pmol/mg of cell protein/min for cells exposed to culture medium, insulin, or fetal bovine serum, respectively (Fig. 6). In an analogous experiment, Vance et al. (1980) showed that phosphocholine accounted for 80% of the label lost from HeLa cells. Even if one assumes that all of the label lost from 3T3 cells is phosphocholine, rather than choline, then the rate of loss of label from 3T3 cells is at most 12-25% of the rate of formation of cell-associated choline kinase products at 36 μM choline (Figs. 1 and 3 and Table 1). Since we routinely measured cell-associated choline kinase products, true intracellular choline kinase activity was underestimated by approximately 12-25%; however, serum increased formation of all choline kinase products by 2-fold, both those which remain cell-associated and those released into the culture medium. Thus, use of cell-associated choline kinase products in our calculations has no qualitative effect on the results.

Other Materials and Methods—Measurement of uptake into acid-soluble and acid-insoluble material and thin-layer chromatography of acid-soluble material have been described by Warden and Friedkin (1984). Protein was determined by the method of Lowry et al. (1951) with saline-washed cells. Crystalline bovine insulin was a gift from Bill Bromer of Eli Lilly & Co. [14C]Choline was purchased from Amersham Corp. Dulbecco's modified Eagle's medium and Eagle's minimum essential medium were obtained from Grand Island Biological Co.

FIG. 1. Time course of choline uptake and phosphorylation at 7 μM choline. Confluent quiescent cultures of 3T3 fibroblasts, grown on 30-mm Nunc brand dishes, were washed twice with DMEM to remove residual serum and were then incubated in 2 ml of DMEM containing 10% fetal bovine serum (A), 5 μM insulin (B), or DMEM alone (C). Six hours later, these cultures and a fourth set of dishes of quiescent cells (D) were washed twice with DMEM and then incubated with [14C]choline (58 Ci/mol, 7 μM) in MEM at 37 °C. After 1, 2.5, 5, 10, or 60 min, culture dishes were washed, acid-soluble radioactivity was extracted with 0.4 M HClO₄, HClO₄, was neutralized with KOH, and choline was separated from phosphocholine, CDP-choline, and glycerophosphorylcholine, as described by Warden and Friedkin (1984). Acid-insoluble uptake was measured as reported by Warden and Friedkin (1984). All data reported are the averages of duplicate determinations. A, free intracellular labeled choline (nanomoles/milligram of cell protein); B, phosphorylated acid-soluble radioactivity, i.e. label in phosphocholine, CDP-choline, and glycerophosphorylcholine (nanomoles/milligram of cell protein); C, per cent of acid-insoluble radioactivity which is phosphorylated (per cent of acid-insoluble radioactivity in phosphocholine plus CDP-choline plus glycerophosphorylcholine); D, formation of combined choline kinase products. Acid-insoluble uptake after 60 min of exposure of [14C]choline was 242, 208, 552, and 727 pmol/mg of cell protein for quiescent cells or cells treated with DMEM, 5 μM insulin, or 10% serum, respectively. Formation of choline kinase products, in nanomoles/milligram of cell protein, was calculated as described under “Experimental Procedures.”
Increased Choline Phosphorylation Decreases Choline Pools in Serum-stimulated Cells—Figs. 1–3 clearly show that intracellular choline concentrations are reduced by serum. These data suggest that increased choline kinase activity is responsible for the decreased choline concentrations in serum-stimulated cells. Intracellular choline concentrations in 3T3 cells will depend on rates of choline influx (initial rate of transport) and on the rate at which choline is removed from the intracellular pool by efflux from the cell and phosphorylation by choline kinase. Initial rates of choline transport were linear with time from 15, 30, and 60 s and were approximately the same in quiescent and in serum-stimulated cells (Fig. 4); thus, any modulations of intracellular choline concentrations must depend on alterations of efflux or phosphorylation. Combined data from several experiments clearly show that the rate of intracellular choline kinase activity was 2-fold higher in serum-stimulated cells than in quiescent ones (Table I), which suggests that serum decreases choline pools by increasing removal of choline from the intracellular pool by choline kinase.

Half-life and Turnover Time of Phosphocholine—The half-life and turnover time of phosphocholine were measured to quantitate the consequences of increased phosphocholine pools for PC synthesis in serum-stimulated 3T3 cells (Fig. 5). Cells treated with serum had shorter half-lifes for \(^{14}C\) phosphocholine than cells exposed to DMEM or insulin (Table II). The radioactive half-life of phosphocholine can be used to calculate a turnover time, which is the amount of time required to synthesize one complete pool of phosphocholine (Table II). Dividing phosphocholine pool size (Warden and Friedkin, 1984) by the turnover time yields a turnover rate, which is the rate of less of radioactivity from phosphocholine (Table II). The phosphocholine turnover rate is increased more than 2-fold in serum-stimulated cells, as a result of increased phosphocholine pool size and a shorter phosphocholine half-life. Thus, increased phosphocholine pools, brought about by serum treatment, may contribute to an increased conversion of phosphocholine to CDP-choline by cytidylyltransferase, the rate-limiting step of PC synthesis. Fig. 6 shows the appearance of radioactivity in culture medium from cells prelabeled with \(^{14}C\) choline for the experiment shown in Fig. 5. Clearly, serum-stimulated cells lose radioactivity twice as fast as quiescent or insulin-stimulated cells. Thus, comparisons of in vivo choline kinase activity in quiescent or serum-stimulated cells (Figs. 1 and 3 and Table I) are not qualitatively affected by our reliance on the radioactivity present in intracellular choline kinase products for measurement of in vivo choline kinase activity.
The data presented are the average of five independent experiments with choline-free MEM and then further incubated in MEM containing choline (36 pM) at 37 °C. Washing, trichloroacetic acid extraction, and analysis were as described by Warden and Friedkin (1984). Data shown are the mean ± S.D. of three separate determinations.

To determine the initial rate of transport at 36 pM, cells or cells first exposed to 10% serum for 6 h were washed twice and trichloroacetic acid extractions were prepared as described (Warden and Friedkin, 1984). Three independent samples were used for both conditions. Intracellular choline kinase activity at 7 or 36 pM choline was calculated for quiescent cells and for cells pre-exposed to DMEM to 5 μg/ml insulin, or to 10% fetal bovine serum for 6 h at 37 °C. Cells were exposed to [3H]choline (7 μM, 60 Ci/mole) or to [14C]choline (36 μM, various specific activities) for 60 min at 37 °C, extracted, and analyzed for determination of intracellular choline kinase activity as described under “Experimental Procedures.” Statistical significance was determined by comparison to quiescent cells. The data presented are the average of five independent experiments at 7 μM choline and of two independent experiments at 36 μM choline, with duplicate determinations in each experiment.

### TABLE I

<table>
<thead>
<tr>
<th>Condition</th>
<th>Choline kinase Initial Intracellular choline kinase Initial Intracellular choline kinase transport activity transport activity (pM) (mg cell proteinfmin) transport activity (pM) (mg cell proteinfmin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7 μM choline</td>
<td></td>
</tr>
<tr>
<td>Quiescent</td>
<td>99 ± 11                                                               43 ± 5.5                                                          250 ± 30                                                             71 ± 14</td>
</tr>
<tr>
<td>DMEM</td>
<td>ND*                                                                  35 ± 7.2                                                          ND                                                                  62 ± 7.0</td>
</tr>
<tr>
<td>Insulin</td>
<td>ND                                                                    48 ± 6.6                                                          ND                                                                  94 ± 12</td>
</tr>
<tr>
<td>Serum</td>
<td>81 ± 10                                                              95 ± 12                                                          220 ± 5.0                                                          160 ± 20$^a$</td>
</tr>
</tbody>
</table>

a ND, not done.

p < 0.001.

### DISCUSSION

Choline kinase is clearly a regulated enzyme in 3T3 cells. Previous studies demonstrated that addition of serum to quiescent cultures of 3T3 fibroblasts resulted in enhanced choline phosphorylation, phosphocholine pools, in vitro choline kinase activity, and PC synthesis (Warden et al., 1980; Warden and Friedkin, 1984). The current studies strengthen the hypothesis that choline kinase is regulatory for PC biosynthesis in 3T3 cells. First, in serum-stimulated cells, choline kinase was at a crossover point for the intermediates of PC synthesis, since choline concentrations decrease (Figs. 1–3) while phosphocholine concentrations increase (Warden and Friedkin, 1984). Second, phosphocholine turnover was increased (Fig. 5 and Table II), in part because of increased phosphocholine concentrations. Third, intracellular choline kinase activity was increased (Table I) as one would expect if choline kinase activity were regulated in vivo by serum.

Choline kinase can regulate PC synthesis by modulating phosphocholine concentrations. Increased concentrations of phosphocholine could increase PC synthesis by two mechanisms. First, increased phosphocholine could raise cytidylyltransferase activity if the initial phosphocholine concentration in quiescent 3T3 cells is below or near the intracellular Km of cytidylyltransferase for phosphocholine. Rates of enzyme reactions in vivo may be different than rates predicted with Michaelis-Menten kinetics from in vitro measurements of kinetic constants (Wohlueter and Plagemann, 1981). Thus, comparison of intracellular phosphocholine concentrations with in vitro determined Km values of cytidylyltransferase for phosphocholine cannot be utilized to prove that changes of intracellular phosphocholine are regulatory for cytidylyltransferase; proof that increased phosphocholine increases PC synthesis would require that one have an experimental method for systematically changing intracellular concentrations of phosphocholine. Nevertheless, it is useful to
compare intracellular phosphocholine concentrations with in vitro determined $K_v$ values of cytidylyltransferase for phosphocholine since if they are approximately the same then this would be consistent with the hypothesis that modulations of phosphocholine can regulate cytidylyltransferase activity. While it is difficult to determine the exact intracellular concentration of phosphocholine, it is possible to make a rough estimate by assuming that protein accounts for 10% of the wet weight of a cell and that 80% of the wet weight is water. The estimated concentrations of phosphocholine are 240, 410, 270, and 630 μM in quiescent cells and in cells treated with DMEM, insulin, or serum, respectively (Warden and Friedkin, 1984). The reported $K_v$ of cytidylyltransferase for phosphocholine, under optimal conditions in vitro, ranges from 167 to 1000 μM (Ansell and Chojnacki, 1969; Choy et al., 1977; Feldman et al., 1978). Thus, increased phosphocholine concentrations might increase PC synthesis. Second, cytidylyltransferase is a reversible enzyme; under some conditions it catalyzes the net formation of CTP and phosphocholine (Feldman et al., 1978). Thus, increased phosphocholine could increase the rate of PC synthesis by shifting the equilibrium of the reversible reaction catalyzed by cytidylyltransferase toward the product, CDP-choline. In fact, serum increased the ratio of phosphocholine to CDP-choline by 2-fold (Warden and Friedkin, 1984). These results are consistent with the data in Table II in which the phosphocholine turnover rate was 2.5-fold higher in serum-stimulated cells than in control serum-stimulated cells than in control cells.

Nishijima et al. (1984) have found new evidence which suggests that choline kinase is a regulatory enzyme for PC synthesis. They found a mutant Chinese hamster ovary cell line with a 3-fold reduction of choline kinase activity and a corresponding 3-fold reduction of phosphorylcholine, CDP-choline, and PC synthesis. These results strongly suggest that choline kinase is regulatory for PC synthesis in Chinese hamster ovary cells.

Our measurements of intracellular choline concentrations implicitly assumed that there is only one pool of free choline in 3T3 cells since we assumed that the intracellular free choline pool which equilibrates with extracellular labeled choline by 5 min after exposure to radioactive choline (Figs. 1–3) is the only pool of free choline in 3T3 cells. In contrast, Sundler et al. (1972) have hypothesized that there are two choline pools in rat liver in vivo, a small one which rapidly equilibrates with extracellular choline and a 6–8-fold larger pool which does not equilibrate. Sundler’s two-choline-pool hypothesis does not change our conclusions for two reasons. First, Sundler et al. (1972) did not actually prove that there are two choline pools in rat liver; rather this hypothesis is just one of several explanations which are consistent with their labeling data. Second, we have previously shown that intracellular phosphocholine-specific activity is identical with the specific activity of extracellular choline after 60 min of exposure to labeled choline (Warden and Friedkin, 1984). Thus, there must be only one active pool of choline which acts as a substrate for choline kinase, and the presence or absence of a second inactive pool of free choline would not matter for labeling or metabolic studies in 3T3 cells.

Plasma choline concentrations may be important in vivo regulators of intracellular choline kinase activity and PC synthesis. In Figs. 1 and 3 and Table I it was shown that intracellular choline kinase activity was increased approximately 2-fold when $[^{14}C]$choline concentrations were raised from 0 to 36 μM. Since plasma choline concentrations range from 5 μM in rats to 16 μM in humans (Wang and Haubrich, 1975), then an increase of plasma choline concentrations could raise intracellular choline kinase activity. It is noteworthy that both Sundler and Åkesson (1975) and Pritchard and Vance (1981) have reported that increased choline concentrations at 7 μM external choline, estimated as described above for intracellular phosphocholine, were decreased from approximately 80 μM in quiescent cells to approximately 25 μM in serum-stimulated cells; thus, it is clear that intracellular choline concentrations are not the same as extracellular choline concentration and are also regulated.

Phosphocholine turnover rates provide the best single indication of the comparability of our experimental values with previously reported work, as turnover rates are dependent upon two independent experiments which determine first, phosphocholine pool size (Warden and Friedkin, 1984), and second, half-life of radioactivity in phosphocholine (Fig. 5 and Table II). In a study of PC biosynthesis in HeLa cells Vance et al. (1980) reported phosphocholine pools of 1820 nmol/g of cells or approximately 18 nmol/mg of protein, and a half-life for phosphocholine of 2-6 h. His calculated turnover rates translate to 1200–2000 pmol/mg of protein/h for mock-infected cells and 4200–6400 pmol/mg of protein/h for poliovirus-infected cells. Our results, which range from 1220 pmol/mg of cell protein/h for control cells in DMEM up to 2840 pmol/mg of cell protein/h for cells exposed to serum (Table II) are clearly comparable with those of Vance’s group.

In the current work, we have focused on the effects of serum and insulin upon choline phosphorylation and PC synthesis; however, in previous work (Warden and Friedkin, 1984), we showed that insulin-like growth factors I and II, epidermal and fibroblast growth factors, vasopressin, and 12-O-tetradecanoylphorbol 13-acetate all increase uptake into acid-insoluble material, apparently by activating different parts of the pathway for PC biosynthesis. The combination of insulin plus 12-O-tetradecanoylphorbol 13-acetate was particularly potent, stimulating uptake into acid-insoluble material to levels twice as high as seen in serum-stimulated cells. These results suggest that purified growth factors, and especially combinations of purified growth factors, may do everything that serum does: increase choline kinase activity, phosphocholine pools,
and PC synthesis. In the future, it should be possible to determine which mitogens affect which parts of the PC biosynthetic pathway.

REFERENCES