Phosphatidylethanolamine Biosynthesis in Rat Mammary Carcinoma Cells That Require and Do Not Require Ethanolamine for Proliferation*

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Epithelial cells and some of their transformed derivatives require ethanolamine to grow normally in defined culture medium. When these cells are cultured without ethanolamine, the amount of cellular phosphatidylethanolamine is considerably reduced. Using a set of rat mammary carcinoma cell lines whose growth is responsive (64-24 cells) and not responsive (22-1 cells) to ethanolamine, the biochemical mechanism of ethanolamine responsiveness was investigated.

The biosynthesis and metabolism of phospholipid, particularly of those involving phosphatidylethanolamine, were thus compared between the two types of cells. The incorporation of [3H]serine into phosphatidylserine and phosphatidylethanolamine in 64-24 cells was 60 and 37%, respectively, of those in 22-1 cells. However, the activity of phosphatidylserine decarboxylase was virtually the same in these cell lines. When these cells were cultured in the presence of [32P]phosphatidylethanolamine and [32P]phosphatidylserine from the radioactive phosphatidylethanolamine, the rate of accumulation of [32P]phosphatidylserine and phosphatidylethanolamine in 64-24 cells was considerably reduced in 64-24 cells compared to that in 22-1 cells, although the rate of synthesis of phosphatidylserine and phosphatidylethanolamine from the radioactive phosphatidylethanolamine was similar between the two cell lines. The rate of labeling phosphatidylethanolamine from the radioactive phosphatidylethanolamine was also reduced in 64-24 cells, although the difference was not as great as that of phosphatidylserine. Incorporation of [32P] into phosphatidylethanolamine was correlated with the concentration of ethanolamine in the culture medium in 64-24 cells, whereas in 22-1 cells the incorporation was not influenced by ethanolamine. Enzyme activities of the CDP-ethanolamine pathway were not significantly different between the two cell lines. The rate of degradation of phosphatidylethanolamine was also similar in these cell lines. These results show that ethanolamine responsiveness of 64-24 cells, and probably other epithelial cells, is due to a limited ability to synthesize phosphatidylserine from a limited base-exchange activity utilizing phosphatidylethanolamine.

A potent growth stimulator of a rat mammary carcinoma cell line purified from bovine pituitary extract has been identified as phosphoethanolamine (1). Ethanolamine functions in a manner essentially identical to that of phosphoethanolamine (2). Ethanolamine or phosphoethanolamine are structural components of the second most abundant phospholipid, phosphatidylethanolamine (PE), in mammalian cell membranes. Growth of many types of mammalian cells, particularly of epithelial origin, is stimulated by ethanolamine. In contrast, growth of mesenchymal cells such as fibroblasts and neuro cells are nonresponsive to ethanolamine (1; for a review, see Ref. 3). Although traditional tissue culture media do not contain ethanolamine, most mammalian cells do not exhibit the necessity of ethanolamine for growth if the medium contains serum. The growth response to ethanolamine is exhibited only when cells are cultured in defined culture medium or medium containing a low level of serum.

Using a set of rat mammary carcinoma cell lines whose growth is responsive and nonresponsive to ethanolamine, the mechanism of ethanolamine responsiveness has been investigated (2-4). When the responsive cells are grown in serum-free medium or medium containing 1 or 2% serum without supplemental ethanolamine, their growth slows down and eventually stops. These cells, however, continue to grow if the medium is supplemented with 5-10 μM ethanolamine or phosphoethanolamine. The growth of nonresponsive cells, on the other hand, is not affected at all by ethanolamine.

When the responsive cells are cultured without supplemental ethanolamine, their cellular phospholipid composition becomes altered (4). Namely, the cells grown in the presence of 5 μM ethanolamine possess a phospholipid composition similar to that of intact mammary tissues, whereas the cells starved for ethanolamine contain a significantly lower amount of PE. A similar alteration of phospholipid composition has also been observed in ethanolamine-responsive cells other than the rat mammary carcinoma line (4). Furthermore, the relative content of PE varies in a dose-dependent manner to the outside concentration of ethanolamine; however, it does not exceed the amount found in the intact mammary tissue. The phospholipid content of the nonresponsive cells, on the other hand, does not alter in response to the exogenous ethanolamine (2, 4). These results suggest that the external source of ethanolamine or phosphoethanolamine is important for proliferation of certain types of cells in animals. In this regard, the concentration of ethanolamine in rat serum is around 15 μM (5). Therefore, the phenomena described above occur at the physiological level.

The purpose of the present study was to compare the

* This work was supported by Grants CA30545 from the National Institutes of Health and PCM8313161 from the National Science Foundation. 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.
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1 The abbreviations used are: PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; BETA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; BME, basal medium Eagle; CHO cells, Chinese hamster ovary cells; MEM, minimum essential medium.
biosynthesis and metabolism of phospholipid, particularly of those involving PE, in ethanolamine-responsive and nonresponsive cells in order to understand the biochemical mechanism of ethanolamine responsiveness. The following three pathways exist for PE synthesis: 1) the incorporation of ethanolamine into pre-existing phospholipids by an energy independent, base-exchange reaction (6), 2) the transfer of ethanolamine via a CDP-ethanolamine intermediate to diacylglycerol by diacylglycerol ethanolamine phosphotransferase (7), and 3) the decarboxylation of phosphatidylserine (PS) (8, 9). Unless ethanolamine or PE is supplemented in the medium, a major pathway by which cultured cells produce PE should be the decarboxylation of PS. Although sphingomyelin can yield phosphoethanolamine and ethanolamine as degradation products, the contribution of sphingomyelin in supplying ethanolamine seems to be minimal in cultured cells (10, 11). The base-exchange reaction involving phosphatidylcholine (PC) or PE and free serine seems to be responsible for the synthesis of PS in animal cells (6). The results obtained in the present study show that the synthetic rates of PS and PE by decarboxylation were significantly lower in ethanolamine-nonresponsive cells as compared to those in ethanolamine-responsive cells. Accordingly, when an exogenous supply of ethanolamine is not available to ethanolamine-responsive cells, these cells cannot synthesize a sufficient amount of PE to sustain growth. Ethanolamine responsiveness of the cells we have studied is a naturally occurring phenomenon among many cultured cells and is not due to mutational events (11-13).

**MATERIALS AND METHODS**

**RESULTS**

_Synthetic Rate of Phospholipid Components Measured by [32P]Pi, Incorporation—64-24 and 22-1 cells were labeled with [32P]Pi, for 2 h or 2 days in order to compare the synthetic/turnover rate and the steady-state labeling of phospholipid components in these cells. The internal pool of free radioactive phosphate was in equilibrium 2 days after the addition of [32P]Pi. Therefore, the radioactivity associated with each phospholipid component after labeling for 2 days should be proportional to the total amount of these components in the cells, whereas the results of labeling for 2 h should indicate the synthetic rate of each component. The analysis of radioactive phospholipids from 2 h and 2 day labelings is shown in Table 1. The distribution of radioactivity among major phospholipid components was substantially different between the two cell lines. In 22-1 cells the distribution of the label after a 2-day pulse was similar to the relative content of each component (4), indicating that, in these cells, the steady-state labeling of phospholipid components, each having an equal specific activity, has indeed been attained after labeling for 2 days. The result of the 2-h pulse showed that the proportion of PE labeled was lower and that of phosphatidylinositol (PI) was higher. A high rate of labeling of PI must be the reflection of PI turnover triggered by extracellular signals during active growth (14). A lower rate of PE labeling is probably due to the fact that PE turns over significantly slower than PC or SM, sphingomyelin.

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**PI as shown below (see Table 2).**

The 2-h pulse in 64-24 cells showed an unusually high rate of PE synthesis, namely, about 50% of the label was associated with PE, whereas only 27% of the label was found in PC, although these results of PE and PC in 64-24 cells grown under the equivalent conditions were 25 and 55%, respectively. The rate of [32P]Pi incorporation into PI was also high in 64-24 cells, as in the case of 22-1 cells. After labeling for 2 days, however, the percent of labeled PE was reduced drastically so as to reflect the composition of endogenous phospholipid in the membranes. The unusually high rate of synthesis of PE found during the 2-h pulse in 64-24 cells may indeed be the result of a high synthetic/turnover rate of PE. However, it is also possible that 64-24 cells synthesize a large amount of PE during the 2-h pulse, simply because the rate of PE synthesis in these cells is proportional to the availability of ethanolamine in the medium, since a fresh supply of ethanolamine was given immediately prior to the addition of [32P].

In order to distinguish between the above two possibilities, 64-24 and 22-1 cells were plated in serum-free medium in the presence of ethanolamine, the percentage of PE labeled was reduced as compared to that of PC during the early period of culture. However, the amount of labeled PE decreased with time. In contrast, in the cells grown without supplemental ethanolamine, the percent of PE labeled remained constant throughout the culture period. By the 50th h the values of labeled PE or PC were the same in the cells grown with or without ethanolamine. In 22-1 cells the amount of labeled PE or PC was the same in the cells grown either with or without ethanolamine. In 22-1 cells the amount of labeled PE or PC was the same in the cells grown either with or without ethanolamine. In 22-1 cells the amount of labeled PE or PC was the same in the cells grown either with or without ethanolamine.

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**TABLE 1**

_Short- and long-term labels of phospholipid with [32P]Pi, 1 x 10⁶ cells (for 2-h pulse) and 5 x 10⁶ cells (for 2-day pulse) were plated in a 60-mm plate in serum-free medium containing 5 μM ethanolamine and labeled with [32P]Pi, 20 μCi/ml [32P]Pi was used for 2-h pulse and 5 μCi/ml for 2-day pulse. The distribution of radioactivity among phospholipid components was analyzed as described under "Materials and Methods." Values are means ± S.E.*

| Phospholipid | 64-24 | 22-1 | % Radioactive phospholipid |
|--------------|-------|------|---------------------------|
|              | 2 h   | 2 days | 2 h   | 2 days | %          |
| SM*          | 4.7 ± 2.0 | 4.7 ± 0.6 | 2.9 ± 1.2 | 4.8 ± 0.3 |  |
| PI           | 15.5 ± 2.1 | 7.6 ± 0.4 | 26.0 ± 2.7 | 58.5 ± 0.6 |  |
| PS           | 3.7 ± 1.3 | 5.7 ± 0.4 | 2.2 ± 1.2 | 4.6 ± 0.4 |  |
| PC           | 27.2 ± 0.7 | 28.4 ± 0.6 | 55.7 ± 0.3 | 59.0 ± 0.5 |  |
| PE           | 49.0 ± 2.2 | 13.9 ± 0.7 | 13.3 ± 0.3 | 25.4 ± 0.8 |  |

* SM, sphingomyelin.

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2 Portions of this paper (including "Materials and Methods," part of "Results," Tables 2, 4, 5, 9 and 10, and Figs. 2, 3, 6, and 7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9550 Rockville Pike, Bethesda, MD 20814. Request Document No. 9823-1925, cite the authors, and include a check or money order for $8.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

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In 22-1 cells, however, the synthesis of PE and PC seems to be independent of supplemental ethanolamine. Therefore, the considerable difference observed in the amount of labeled PE and PC in 64-24 cells between the short- and long-term label as described in the previous section is not likely to be due to a high turnover rate of PE, but due to a burst of PE synthesis as a result of a fresh supply of ethanolamine.

The ratio of the radioactivity associated with PE to the total trichloroacetic acid-precipitable radioactivity or to a unit amount of protein was also estimated from the above experiment in order to compare the synthetic activity of PE to the overall metabolic rate of the cells. In 64-24 cells, during the first 2-h pulse (1 h after the medium change) there was 8-fold more PE synthesis in the culture with ethanolamine than that without ethanolamine. After 24 h, the difference in the values was only 2-fold, and by 48 h the amount of PE labeled/mg of protein or trichloroacetic acid-precipitable count was virtually the same whether the cells were cultured with ethanolamine or not. The amount of labeled PC varied in a manner opposite to that of PE. Namely, the amount of labeled PC was higher in the culture without ethanolamine than with ethanolamine in the early culture samples. In 22-1 cells on the other hand, no significant variation in these values was observed throughout the duration of the experiment whether the culture contained ethanolamine or not (data not shown).

These results support the above notion that, in 64-24 cells, the synthetic rate of PE is proportional and that of PC inversely proportional to the outside concentration of ethanolamine, whereas those of 22-1 cells are independent of the outside concentration of ethanolamine.

In Vitro Enzyme Activities of Phosphatidylethanolamine Synthetic Pathway via CDP-Ethanolamine—Ethanolamine kinase, CTP:phosphoethanolamine cytidylyltransferase, and CDP-ethanolamine diacetylglcerol ethanolamine phosphotransferase were assayed in 64-24 and 22-1 cells using the supernatant or particulate fractions of the cell lysates as described under "Materials and Methods." The results are shown in Table 3. The activities of all three enzymes were not significantly different between the two cell lines, although that of ethanolamine kinase tended to be slightly higher in 22-1 cells. The activities of ethanolamine kinase in these cells were considerably lower than those of Chinese hamster ovary (CHO) cells (15) or Chinese hamster fibroblast V-79 (0.79 ± 0.01 nmol/min/mg of protein). The results previously obtained using intact cells indicated that 64-24 cells incorporated exogenously supplied ethanolamine more efficiently than 22-1 cells (4). Therefore, the CDP-ethanolamine pathway appears not to be involved in the ethanolamine responsiveness.

**Incorporation of Radioactive Serine into Phosphatidylserine and Phosphatidylethanolamine: Effect of Ethanolamine.** The cells were plated at 1.5 × 10⁵ cells/100-mm plate in Dulbecco's modified Eagle's medium containing 2% fetal calf serum and incubated overnight. The medium was then replaced with serum-free, serum-free medium with varied amounts of ethanolamine, and 2 μCi/ml [3H]serine (specific activity, 23.7 Ci/mmol) was added. The cells were harvested after labeling for 5 h, and the radioactive phospholipids were analyzed as described under "Materials and Methods." The amount of radioactive serine incorporated into phospholipids other than PS and PE was insignificant. O, PS; ●, PE.

**TABLE 3**

Activities of the CDP-ethanolamine pathway enzymes in 64-24 and 22-1 cells

| Enzyme                          | Activity (nmol/min/mg protein) |
|---------------------------------|---------------------------------|
| Ethanolamine kinase             | 0.27 ± 0.02                     |
| Phosphoethanolamine cytidylyltransferase | 2.11 ± 0.09                   |
| Diacylglycerol phosphoethanolamine transferase | 0.12 ± 0.01                   |

Fig. 4. Incorporation of radioactive serine into phosphatidyserine and phosphatidylethanolamine: Effect of ethanolamine. The cells were plated at 1.5 × 10⁵ cells/100-mm plate in Dulbecco's modified Eagle's medium containing 2% fetal calf serum and incubated overnight. The medium was then replaced with serum-free, serum-free medium with varied amounts of ethanolamine, and 2 μCi/ml [3H]serine (specific activity, 23.7 Ci/mmol) was added. The cells were harvested after labeling for 5 h, and the radioactive phospholipids were analyzed as described under "Materials and Methods."
TABLE 6
Incorporation of [3H]serine into phospholipids

The data obtained from the experiment shown in Fig. 4 were summarized. Values are the averages of 12 samples resulting from the cells grown with 0–50 μM ethanolamine in three separate experiments and are given as mean ± S.E. The mean values of each parameter are highly significantly different in two cell lines.

| Cell line | Radioactive phospholipid | cpm/mg protein | PE/PS |
|-----------|--------------------------|----------------|-------|
| 64-24     | PS                       | 4534 ± 401     | 0.31 ± 0.02 |
|           | PE                       | 7605 ± 212     | 0.49 ± 0.03 |
| 22-1      | PS                       | 1366 ± 143     |       |
|           | PE                       | 3702 ± 250     |       |

Fig. 5. Incorporation of radioactive serine into phosphatidylserine and phosphatidylethanolamine. Time course. 64-24 and 22-1 cells were plated at densities of 1.2 × 10^6 and 8 × 10^5 cells/60-mm plate and allowed to grow overnight in Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum. The next morning, medium was replaced with serum-free, serum-free medium with 6 μCi/ml [3H]serine (specific activity, 23.7 Ci/mmole). The culture medium contained 10 μM ethanolamine throughout the experiment. The cells were harvested, and processed as described under “Materials and Methods.” The amount of radioactive found in phospholipid components was divided by the total trichloroacetic acid-precipitable radioactivity and these values were plotted. O, PS; ●, PE; ×, PS plus PE.

cultured baby hamster kidney cells (16). 64-24 cells incorporated 60 and 37% of radioactive serine into PS and PE, respectively, as compared to those in 22-1 cells at all concentrations of ethanolamine tested. The ratio of the radioactive PE to PS, which is indicative of the rate of decarboxylation of PS, was significantly lower in 64-24 cells (Table 6). The activity of PS decarboxylase in 64-24 and 22-1 cells using crude cell lysate was, however, virtually the same (0.37 ± 0.01 nmol/min/mg of protein for 64-24 cells and 0.35 ± 0.01 nmol/min/mg of protein for 22-1 cells). Therefore, the lower rate of PS synthesis should be the reason for the low rate of PS to PE conversion.

Furthermore, the time course of the incorporation of [3H]serine into PE and PS was examined in the two cell lines. The cells grown in the presence of 10 μM ethanolamine were labeled with radioactive serine for 3, 6, 11, and 24 h, and the radioactivity in PE and PS was measured. In this experiment the radioactivity associated with phospholipids was compared with the total trichloroacetic acid-precipitable radioactivity in each sample in order to compare the synthetic activity of PS and PE to the overall cellular metabolic activity. The results are summarized in Fig. 5. Relative to trichloroacetic acid-precipitable radioactivity, the amount of radioactive PS and PE found in 64-24 cells was clearly less (3–4 fold) than 22-1 cells throughout the labeling period. Furthermore, the rate of PE synthesis seemed also to be lower in 64-24 cells. The above conclusion was also supported by the fact that,

when the cells were labeled with 32P, the percent of labeled PS per total labeled phospholipid was usually lower by 1.5–2 fold in 64-24 cells than 22-1 cells, although the results were not highly reliable since labeled PS consisted only of 0.2–0.5% of the total labeled phospholipid after 2-h pulse (data not shown). The results presented in this section provide convincing evidence that the synthetic rate of PS and PE utilizing exogenously supplied serine is significantly lower in 64-24 cells than 22-1 cells.

Utilization of Phosphatidylincholine and Phosphatidylethanolamine for the Synthesis of Phosphatidylserine—In animal cells PS is synthesized by means of energy independent, base-exchange reaction using pre-existing cellular phospholipids and free serine (11, 12), and two distinct serine-exchange enzymes, one that uses PC as a substrate and the other PE, have been described (12). The results obtained from the investigation of radioactive serine incorporation thus suggested that, in 64-24 cells, the activities of either one or both of these serine exchange enzymes must be limited. Accordingly, the ability of 64-24 and 22-1 cells to integrate exogenously supplied phospholipids and to use them to synthesize PS was compared. The cells were cultured for 12, 24, and 48 h with 32P-labeled PC or PE which were prepared from 64-24 cells grown in the presence of 32P, and the resulting radiolabeled phospholipids were analyzed. Table 7 and Fig. 6 (see Miniprint) show the results obtained from an experiment using [32P]PE and Table 8 and Fig. 7 (see Miniprint) show those of [32P]PC. The cells rapidly took up either radioactive PC or PE within the first 12 h, and the subsequent increase in the amount of radioactive phospholipid associated with the cells was very little as measured either by the total radioactivity taken up per cell or the amount of precursor phospholipid associated with the cells. Normally, 2–5% of the added phospholipids were utilized by these cells. However, the amount of metabolized phospholipids (e.g. PS and PE when PC was used as a precursor) kept increasing as the labeling period increased. 64-24 cells took up either phospholipid

TABLE 7
Incorporation of [32P]phosphatidylethanolamine into 64-24 and 22-1 cells

Values are averages of duplicate samples and are given as mean ± S.E. Duplicate extractions from the same sample usually gave essentially the same results. However, from experiment to experiment, the extent of radioactive PE converted to PS or PC varied a little as described in the text.

| Time  | PS/PE | PC/PE | PS/PE | PC/PE |
|-------|-------|-------|-------|-------|
| h     |       |       |       |       |
| 12    | 0.057 ± 0.004 | 0.072 ± 0.001 | 0.200 ± 0.007 | 0.107 ± 0.004 |
| 24    | 0.077 ± 0.001 | 0.074 ± 0.001 | 0.197 ± 0.006 | 0.115 ± 0.005 |
| 48    | 0.150 ± 0.004 | 0.112 ± 0.003 | 0.191 ± 0.004 | 0.153 ± 0.006 |

TABLE 8
Incorporation of [32P]phosphatidylcholine into 64-24 and 22-1 cells

Values are the averages of four independent samples and are given as mean ± S.E.

| Time  | PS/PC | PE/PC | PS/PC | PE/PC |
|-------|-------|-------|-------|-------|
| h     |       |       |       |       |
| 12    | 0.028 ± 0.007 | 0.015 ± 0.003 | 0.024 ± 0.003 | 0.022 ± 0.004 |
| 24    | 0.052 ± 0.013 | 0.038 ± 0.011 | 0.044 ± 0.004 | 0.044 ± 0.008 |
| 48    | 0.084 ± 0.015 | 0.084 ± 0.014 | 0.064 ± 0.008 | 0.088 ± 0.012 |
significantly more than 22-1 cells did per unit amount of protein. When \(^{32}\)P]PE was used as a precursor, the accumulation of radioactive PS was clearly less in 64-24 cells as compared to that found in 22-1 cells. Up to 24 h after the addition of radioactive PE, the fraction of incorporated PE which was converted to PS was less than 40% of that found in 22-1 cells. The synthesis of radioactive PC was also less in 64-24 cells as compared to 22-1 cells, although the difference between the two cell lines was not as great as that of PS. When \(^{32}\)P]PC was used as a precursor for the synthesis of other phospholipids, no obvious difference was observed between the two cell lines. Furthermore, the conversion of PE taken up to other phospholipid components was generally higher than that of PC, although the ratio of the product phospholipid to the precursor phospholipid varied a little from experiment to experiment. The variation in the rate of conversion was probably due to the variability in the properties of liposomes prepared from the radioactive phospholipids or the variable metabolic state of the cells used in individual experiments. These results suggest that PE may be utilized more for the base-exchange reaction than PC, and in 64-24 cells the level of biosynthesis of PS utilizing PE is lower than that in 22-1 cells. This conclusion agrees with the conclusion reached from the studies using \(^{3}H\)serine as a precursor for the synthesis of PS and PE.

**DISCUSSION**

If a thorough survey is made, many or maybe all types of normal epithelial cells and some of their transformed cells will require ethanolamine to proliferate normally in culture (1, 3). When these cells are grown in the absence of ethanolamine, their growth stops and the level of cellular phosphatidylethanolamine is reduced to a half or less than a half that of the level normally found in these cells. 5 \(\mu\)M ethanolamine is sufficient to maintain the normal level of PE. The above findings suggested that either the biosynthetic pathway of PE is defective or the rate of PE degradation is unusually high in these cells. The present study was focused on the elucidation of the biochemical mechanism of ethanolamine requirement.

The evidence obtained from the experiments using radioactive serine as a precursor for PE synthesis indicated that the level of biosynthesis of PS was significantly less in 64-24 cells (ethanolamine-responsive) as compared to that in 22-1 cells (ethanolamine-nonresponsive). In the same experiment it was shown that, in 64-24 cells, the labeling of PE was still lower than that of PS. PE is the major precursor for the synthesis of PE in mammalian cells, unless exogenous ethanolamine is available to the cells (6, 9, 16). The low rate of labeling of PE, therefore, must be the consequence of the limited supply of PS. This notion is also supported by the fact that PS decarboxylase activity in crude cell lysate of 64-24 and 22-1 cells is virtually the same.

PS in mammalian cells is synthesized by the base-exchange reaction. The above conclusion was further supported by experiments in which the cells were labeled with \(^{32}\)P]PC or \(^{3}\)P]PE, and the distribution of the radioactive phospholipids was examined. In these experiments, the level of PS synthesized by utilizing radioactive PE was significantly lower in 64-24 cells as compared to that of 22-1. Two serine-exchange enzymes have been described, one that catalyzes the base-exchange reaction using PC as a substrate and the other that uses PE (12). Our study indicates that the exchange enzyme which uses PE, but not the one that uses PC, is limited in 64-24 cells. In this connection, CHO mutants which are defective in PS biosynthesis are defective in the base-exchange enzyme that uses PC as a substrate (11, 12). Therefore, the defective-ness in PS biosynthesis in 64-24 cells and CHO mutants seems to be caused by two different enzymes. The CHO mutants grown without ethanolamine or PS exhibit the reduction in the amount of cellular PS (11, 12), whereas in 64-24 cells the content of PS does not alter, although that of PE reduces to 50% (Table 10 (see Miniprint)). 64-24 cells may have a mechanism to maintain a certain level of PS. Exogenously added PS may increase the cellular content of this phospholipid, causing the detrimental effect on cell growth.

Contrary to the results obtained using intact cells is the result on the base-exchange enzyme activities in crude cell homogenates (see "Results" in Miniprint). The \(^{32}\)P]PC was used as a precursor for the synthesis of the two cell lines. In \(^{32}\)P]PC was used as a precursor for the synthesis of the two cell lines. In \(^{32}\)P]PC was used as a precursor for the synthesis of the two cell lines. In \(^{32}\)P]PC was used as a precursor for the synthesis of the two cell lines. In vivo and in vitro studies using radioactive serine indicated that the amount of the intracellular radioactive serine (10% \(\times\)chloroform-soluble plus precipitable counts)/mg of protein was 50-200% higher in 64-24 cells than in 22-1 cells, and the rate of serine incorporation into protein was also considerably higher in 64-24 cells. The lower level of PS synthesis observed in intact 64-24 cells, therefore, cannot be due to poor active transport of serine. The soluble pool of free serine was also similar in the two cell lines (see "Results" in Miniprint). The discrepancy observed between the in vivo and in vitro studies could be explained as follows: 1) there is a difference in the availability of serine and/or PE where the exchange reaction takes place or 2) there is a regulatory system for PS biosynthesis in intact cells which is lost as a result of disrupting the cells.

With regard to the effect of exogenously supplied ethanolamine on PE synthesis, the synthetic rate of PE as measured by the incorporation of \(^{32}\)P was correlated with the outside concentration of ethanolamine in 64-24 cells, whereas in 22-1 cells the rate was constant. The rate of PE synthesis via decarboxylation of PS in either cell line seems not to be regulated by ethanolamine. The activities of biosynthetic enzymes of PE via CDP-ethanolamine were similar in the two cell types. Reactions that might affect the PE synthesis, such as degradation, conversion of PE to PC by methylations, or inhibition of PE synthesis by choline, were also found to be similar in both cell lines (see "Results" in Miniprint).

Mutants have been isolated from CHG cells which have defective PS synthase activities. These mutants require ethanolamine or PS to grow (11, 12). Another mutant of CHO cells having a temperature-sensitive choline exchange enzyme which contributes to the synthesis of PS has a reduced amount of PE when cultured in the absence of ethanolamine or PS (13), whereas a CHO mutant which has a reduced activity of CTP:phosphoethanolamine cytidylyltransferase grows normally without supplemental ethanolamine (15). As pointed out previously, the phenomenon discussed in this article is found in naturally occurring cells. All epithelial cells so far tested are ethanolamine-responsive, although transformation renders many of these cells ethanolamine-nonresponsive. The proliferation of epithelial cells may be regulated by the availability of ethanolamine or phosphoethanolamine in the body fluid.

**Acknowledgments**—We are grateful to Dr. D. Voelker, National Jewish Hospital, Denver, for his valuable advice and discussion throughout this study. PS decarboxylase activity in 64-24 and 22-1 cells was kindly assayed by Dr. Voelker. We also thank Drs. M. Nishijima and O. Kuge, Japanese National Institutes of Health, Tokyo, for their helpful suggestions on the use of \(^{32}\)P-labeled phospholipids. The base-exchange enzyme assays were carried out by Dr. Kuge in the laboratory of Drs. Y. Akamatsu and M. Nishijima. We are also grateful to Dr. H. Kasai in Dr. S. Nishimura's laboratory at...
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National Cancer Center Research Institute, Tokyo, for performing high pressure liquid chromatography to estimate the size of the serine pool.

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SUPPLEMENTARY MATERIAL

Phenothiazine-induced hypersensitivity in spontaneous rat mammary tumor cells that express and do not require 5-hydroxytryptamine for proliferation

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Intracellular Signal Transduction

Interpretation of 3HTP into phospholipid was carried out as follows. An appropriate number of cells were plated and grown for 3 days in 10% fetal calf serum in medium supplemented with 10% fetal calf serum, 10% newborn bovine serum albumin, and 100 U/ml of penicillin and streptomycin. The cells were relaxed at 80-90% confluence in 24-well plates (Costar) in medium supplemented with 10% fetal calf serum in 4.1 M CO2 and 5% O2 for 24 hr before the addition of medium supplemented with 10% fetal calf serum and 10% newborn bovine serum albumin. The assay was terminated by freezing the plates immediately at -80°C. The membranes were solubilized in 0.1% dodecylmaltoside (DDM) and stored frozen until ready to be analyzed.

For labeling phospholipids with radioactive sodium the cells were plated in DME containing 20% fetal calf serum and 10% newborn bovine serum albumin. Two days later, the cultures were washed with serum-free medium and then labeled with [3-3H]ethanolamine. The labeled cell membranes were subsequently extracted with chloroform/methanol (2:1, v/v) and subjected to thin layer chromatography in a solvent system comprised of chloroform/methanol/water [2:1:1, v/v]. The labeled phospholipids were separated on silica gel plates and then exposed to X-ray film. The ethanolamine was detected by autoradiography and quantified by densitometry.

Phosphatidylglycerol (PG) Analysis

Phospholipids were extracted from cell lines according to the method of Bligh and Dyer (1959). The lipids were solubilized in chloroform/methanol (2:1, v/v) and subjected to thin layer chromatography and autoradiography to identify PG as described above.

Saponin (100 μg/ml) was added to the culture medium and then cells were cultured for 24 hr. After washing with phosphate-buffered saline (PBS), the cells were incubated with 1 μCi/ml of [3-3H]ethanolamine for 24 hr. The cells were solubilized by sonication in chloroform/methanol (2:1, v/v) and then subjected to thin layer chromatography as described above.

For the preparation of dimercaptosuccinic acid (DMSA), the cells were exposed to 100 μg/ml of DMSA for 24 hr. After washing with phosphate-buffered saline, the cells were incubated with 1 μCi/ml of [3-3H]ethanolamine for 24 hr. The cells were solubilized by sonication in chloroform/methanol (2:1, v/v) and then subjected to thin layer chromatography as described above.

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Mechanism of Ethanolamine Responsiveness of Epithelial Cells

Table 1. Mobility of [3H]labeled phospholipid

| Cell line | Duration of chase (h) | SE | PE | PC | PS |
|-----------|----------------------|----|----|----|----|
| 60-24     | 24                   | 7.6| 7.0| 11.4| 9.7|
| 45        | 30                   | 11.7| 20.6| 9.0| 17.1|
| 24-72     | 48                   | 17.3| 24.7| 14.2| 15.6|

Figure 1. Stability of phosphatidylethanolamine labeled with [3H]ethanolamine

Table 2. Stability of phosphatidylethanolamine labeled with [3H]ethanolamine

| Hours | % radioactivity remaining |
|-------|--------------------------|
| 24    | 48                       |

Table 3. Inhibition of ethanolamine incorporation by choline

| Choline (mg/ml) | Ethanolamine incorporation (nmol/mg protein) |
|-----------------|---------------------------------------------|
| 0               | 100                                         |
| 5               | 90                                          |
| 10              | 80                                          |
| 25              | 70.5                                        |

Figure 2. Inhibition of ethanolamine incorporation by choline

Figure 3. Inhibition of ethanolamine incorporation by choline

Cells were plated at 1 x 10^5 cells/ml in 24-well dishes and allowed to adhere overnight. The medium was replaced with serum-free medium with varying amounts of ethanolamine and the cells were incubated for 48 h. The medium containing ethanolamine was replaced by fresh medium containing ethanolamine and chase for 24 h. The medium was then replaced with fresh medium containing ethanolamine and chase for 24 h. The medium was then replaced with fresh medium containing ethanolamine and chase for 24 h. The medium was then replaced with fresh medium containing ethanolamine and chase for 24 h.
Mechanism of Ethanolamine Responsiveness of Epithelial Cells

Effect of Phospholipids on Cell Growth

If our assumption that 46-24 cells have a limited capacity to synthesize PE, which leads to a limited synthesis of PC, is correct, exposure of 46-24 cells to PE should stimulate the growth of 46-24 cells. Accordingly, the growth stimulatory effect of PE and PC on 46-24 cells was determined. 5 to 15 times more cells were obtained after 4 to 5 days growth as compared to times without the addition of PE. The PE was often growth stimulatory. The stimulatory effect of PE was almost equivalent to that of ethanolamine. PE was later tested at low concentrations, but it was difficult to reproduce this effect. A higher concentration level of PE was consistently growth inhibitory to growth of 46-24 cells. PE from different sources gave essentially the same results. Growth of 22-1 cells was generally similar to growth of 46-24 cells. PE was often growth stimulatory. When PE was added to the previous section, PE can be efficiently taken up by the cells. Once taken up the PE must be maintained, converting the PE deficiency. Growth of mouse cells which were sensitive for PE biosynthesis is inhibited by ethanolamine added PE (11,12). The reasons for the growth inhibitory effect of ethanolamine on PE and 46-24 cells in our known at present.

Table 9. The effects of ethanolamine on the phospholipid composition of 46-24 cells

| Phospholipid | Ethanolamine | Ethanolamine |
|--------------|--------------|--------------|
| PE           | 5.3 ± 1.35   | 6.3 ± 0.7    |
| PS           | 9.3 ± 1.2    | 6.0 ± 1.5    |
| PE           | 6.8 ± 0.9    | 6.5 ± 1.1    |
| PC           | 53.5 ± 5.1   | 67.0 ± 1.4   |
| PE           | 25.3 ± 1.3   | 31.1 ± 2.8   |

The cells were grown in DM containing 5% fetal calf serum with or without ethanolamine for 5 days and used for the phospholipid analysis. The results are the summation of 7 separate experiments for the cells and 6 experiments for the same without ethanolamine. The abbreviations are the same as in Table 1.

*standard error*

Table 8. The effects of ethanolamine on the phospholipid composition of 46-24 cells

| Phospholipid | Ethanolamine | Ethanolamine |
|--------------|--------------|--------------|
| PE           | 5.3 ± 1.35   | 6.3 ± 0.7    |
| PS           | 9.3 ± 1.2    | 6.0 ± 1.5    |
| PE           | 6.8 ± 0.9    | 6.5 ± 1.1    |
| PC           | 53.5 ± 5.1   | 67.0 ± 1.4   |
| PE           | 25.3 ± 1.3   | 31.1 ± 2.8   |

The cells were grown in DM containing 5% fetal calf serum with or without ethanolamine for 5 days and used for the phospholipid analysis. The results are the summation of 7 separate experiments for the cells and 6 experiments for the same without ethanolamine. The abbreviations are the same as in Table 1.

*standard error*