Evidence for an ethanolamine cycle: differential recycling of the ethanolamine moiety of phosphatidylethanolamine derived from phosphatidylserine and ethanolamine

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Evidence is presented for the operation of an ethanolamine–phosphatidylethanolamine (PtdEtn) cycle in Chinese hamster ovary cells. PtdEtn was labelled with [^3H]ethanolamine and radioactivity was chased by incubation with 1 mM unlabelled ethanolamine. Radioactivity in [^3H]PtdEtn gradually declined over a 23 h time period. In contrast, when the cells were incubated in medium lacking unlabelled ethanolamine, radioactivity in PtdEtn remained constant for at least 23 h. These observations suggest that the ethanolamine moiety is continuously released from PtdEtn and recycled back into PtdEtn. In cells incubated without unlabelled ethanolamine, labelled ethanolamine released from PtdEtn is re-incorporated into PtdEtn without significant

dilution. In contrast, in cells incubated with unlabelled ethanolamine the specific radioactivity of the intracellular ethanolamine pool decreases as a result of dilution by the exogenous ethanolamine, hence radioactivity in PtdEtn gradually declines. Similar results were obtained for confluent and non-confluent cells. Our data also demonstrate that when PtdEtn is derived from phosphatidylserine decarboxylation, the ethanolamine cycle operates only in actively dividing, and not in confluent, cells, implying that PtdEtn derived from different biosynthetic origins [i.e. from decarboxylation of phosphatidylserine or from ethanolamine (most likely via the CDP-ethanolamine pathway)] is metabolized differently.

INTRODUCTION

Regulation of ethanolamine metabolism in mammalian cells is not well understood. Two major metabolic fates of ethanolamine are phosphatidylethanolamine (PtdEtn) biosynthesis and formation of the phosphatidylinositol–glycan linkage present in certain proteins [1]. PtdEtn is produced in animal cells by three different biosynthetic pathways: (i) from ethanolamine via CDP-ethanolamine [2], (ii) from the decarboxylation of phosphatidylserine (PtdSer) [3] and (iii) from a base-exchange reaction between ethanolamine and a pre-existing molecule of PtdSer or phosphatidylcholine [4,5]. The relative contribution of these three biosynthetic pathways is not precisely known but might be different among different cell types. For example, in cultured baby hamster kidney fibroblasts and Chinese hamster ovary (CHO) cells the majority of PtdEtn is derived from PtdSer decarboxylation, even when the cells are supplemented with ethanolamine [6–8]. However, in rat liver, the CDP-ethanolamine pathway appears to be quantitatively important [9]. In liver, the rate of synthesis of PtdEtn from CDP-ethanolamine has been calculated to be 0.3 $\mu\text{mol}/\text{min}$ per liver [10] whereas the rate of synthesis of PtdEtn from PtdSer decarboxylation has been estimated to be 10-fold lower (0.03 $\mu\text{mol}/\text{min}$ per liver [11]). It is generally accepted that the base-exchange pathway is not a quantitatively major pathway for PtdEtn synthesis [12,13].

Ethanolamine that is used for PtdEtn synthesis originates either from a base-exchange reaction between serine and PtdEtn [4,5] or as unbound ethanolamine from the circulation in intact animals. Media used for cell culture usually contain a very low concentration of free ethanolamine (most often in the range of 10% of that in serum), although certain cultured cells require ethanolamine as a growth factor [14,15]. The concentration of free ethanolamine in the circulation of most animals is normally in

the range 5–50 μM . For example, in rat serum, the ethanolamine concentration is approximately 20 μM [16,17], in fetal bovine serum the concentration is 23.4 μM [17] whereas in hamsters the plasma concentration of ethanolamine is much higher (0.9 mM) [18]. However, these levels can fluctuate depending on the metabolic status of the animal. For example, immediately after partial hepatectomy in rats the ethanolamine level of serum increases from 29 to 50 μM [19].

Extracellular ethanolamine is readily imported into cells (e.g. rabbit retina [20], hamster heart [18], Y79 retinoblastoma cells [21] and cultured bovine aortic endothelial cells [17]) by both high- and low-affinity transport systems. Addition of ethanolamine to the medium of cultured cells increases the sizes of the intracellular pools of ethanolamine and phosphoethanolamine [6,16,17], and over the long-term increases the content of PtdEtn in the cells [17,22,23].

In animal cells, there are five potential routes for PtdEtn degradation: (i) phospholipase A degradation with production of fatty acids and lysophosphatidylethanolamine and/or glycerophosphoethanolamine, (ii) phospholipase C degradation with generation of diacylglycerols and phosphoethanolamine, (iii) phospholipase D degradation with production of phosphatidic acid and ethanolamine, (iv) base-exchange reaction between PtdEtn and a molecule such as serine, with generation of ethanolamine and PtdSer, and (v) reversal of the CDP-ethanolamine pathway. The relative importance of these modes of PtdEtn degradation is not known, but might be different among different cell types. For example, in HL-60 leukaemia cells, 3T3 fibroblasts and baby hamster kidney cells both PtdEtn and phosphatidylcholine are hydrolysed by a protein kinase C-dependent phospholipase D [24], and in hypoxic myocytes PtdEtn is a substrate for phospholipase A [25]. Moreover, two base-exchange activities have been identified in CHO cells [7,26,27].

One of these enzymes, PtdSer synthase I, catalyses a base-exchange reaction with PtdSer, PtdEtn and phosphatidylcholine, whereas the other, PtdSer synthase II, apparently uses PtdSer and PtdEtn, but not phosphatidylcholine. To our knowledge, the existence of a mammalian phospholipase C activity that hydrolyses PtdEtn has not been reported.

In the present study we have investigated the recycling of the ethanolamine moiety of PtdEtn. We have compared the effect of cell growth and supplementation of the culture medium of CHO-k1 cells with exogenous ethanolamine on the metabolism of PtdEtn derived from its two biosynthetic origins: PtdSer decarboxylation and ethanolamine. We propose the existence of a PtdEtn-ethanolamine cycle in which ethanolamine is continually released from CDP-ethanolamine-derived PtdEtn, and recycled back into PtdEtn, in both confluent and dividing cells. In cells prelabelled with [^3H]ethanolamine, and subsequently supplemented with ethanolamine, the radioactive ethanolamine released from PtdEtn enters, and is diluted by, an intracellular pool of ethanolamine of low specific radioactivity, and consequently the radioactivity in PtdEtn gradually declines. In contrast, the PtdEtn-ethanolamine cycle is essentially dormant for PtdSer-derived PtdEtn in confluent cells, although the cycle is stimulated when the CHO-k1 cells are actively dividing.

MATERIALS AND METHODS

Materials

CHO cells (CHO-k1) were obtained from the American Type Culture Collection. The radiochemicals [$1\text{-}^3\text{H}$]ethanolamine and [$3\text{-}^3\text{H}$]serine were purchased from Amersham Canada, Oakville, Ontario, Canada, and [^{32}P]P_i was from ICN Pharmaceuticals Canada Ltd., Montreal, Canada. Silica gel G TLC plates (0.25 mm thickness) were from BDH Chemicals. Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM) and minimum essential medium (MEM) were from Life Technologies, Grand Island, NY, U.S.A. L-Serine and ethanolamine were purchased from Sigma, St. Louis, MO, U.S.A. Primaria culture dishes (100 mm) were from Becton-Dickinson, Oxnard, CA, U.S.A. Phospholipid standards were purchased from Avanti Polar Lipids, Birmingham, AL, U.S.A. All other reagents were from Sigma or Fisher Scientific.

Cell culture

CHO-k1 cells were routinely maintained in 100 mm culture dishes at 37 °C in an atmosphere containing 5 % CO₂, either with DMEM (without serum) or in Ham's F12 medium (containing 10 % fetal bovine serum), supplemented with 40 units/ml penicillin and 40 µg/ml streptomycin. Half-confluence was attained after 1 day and confluence after 3 days, as judged by visualization under the light microscope. Viability of the cells was estimated by their ability to exclude Trypan Blue and by the lack of release of lactate dehydrogenase into the medium [28].

Pulse-chase experiments

For labelling experiments with [^{32}P]P_i, CHO-k1 cells were washed twice with phosphate-free DMEM, then incubated with 3 ml/dish serum-free phosphate-free DMEM containing 5 µCi/ml [^{32}P]P_i for 5 h (pulse). For confluent cells, the medium was removed and replaced with 5 ml of DMEM containing 125 µg/ml sodium phosphate, with or without 1 mM ethanolamine. For non-confluent cells, after the pulse, the medium was replaced with Ham's F12 medium supplemented with 10 % fetal bovine serum, with or without 1 mM ethanolamine. For labelling

experiments with [$1\text{-}^3\text{H}$]ethanolamine, CHO-k1 cells were washed twice with DMEM, then incubated with 3 ml/dish serum-free DMEM containing 1 µCi/ml [$1\text{-}^3\text{H}$]ethanolamine for 1 h. For confluent cells, the medium was removed and replaced with 5 ml of DMEM containing 400 µM serine, with or without 1 mM ethanolamine. For non-confluent cells, after the pulse, the medium was replaced with Ham's F12 medium supplemented with 10 % fetal bovine serum, with or without 1 mM ethanolamine. For labelling experiments with [$3\text{-}^3\text{H}$]serine, CHO-k1 cells were washed twice with serine-free MEM, then incubated with 3 ml/dish serum-free serine-free MEM containing 5 µCi/ml [$3\text{-}^3\text{H}$]serine for 1 h. For confluent cells, the medium was removed and replaced with 5 ml of DMEM containing 1 mM serine, with or without 1 mM ethanolamine. For non-confluent cells, after the 1 h pulse, the medium was replaced with Ham's F12 medium supplemented with 10 % fetal bovine serum, with or without 1 mM ethanolamine.

For all labelling experiments, media were collected after defined chase periods and cells were harvested by scraping into PBS. Lipids were extracted by the addition of 3 vol. of chloroform/methanol (2:1, v/v). Unlabelled carrier phospholipids were added to each sample and individual phospholipids isolated by TLC in the solvent system chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:2, by vol.). Ethanolamine, phosphoethanolamine and CDP-ethanolamine were isolated by TLC in the solvent system ammonia/methanol/aq. 0.6 % NaCl (1:10:10, by vol.).

Other methods

Cellular protein content was determined by the method of Lowry et al. [29] with BSA as a standard. Lipid phosphorus was analysed by the method of Rouser and Fleischer [30].

RESULTS

Specific radioactivity of ethanolamine-derived PtdEtn decreases in the presence of exogenous ethanolamine

One major route for the synthesis of PtdEtn in mammalian cells is the CDP-ethanolamine pathway [2]. The metabolism of PtdEtn was investigated in confluent CHO-k1 cells which were first pulse-labelled for 1 h with [^3H]ethanolamine, then incubated for an additional 23 h in the presence or absence of 1 mM unlabelled ethanolamine. In the absence of ethanolamine in the chase medium, the specific radioactivity of PtdEtn remained essentially constant throughout the 23 h chase period (Figure 1a). In contrast, in cells to which 1 mM ethanolamine had been added during the chase period, the specific radioactivity of PtdEtn gradually declined, at the rate of approximately 2.3 % per h, over the 23 h period (Figure 1a). In actively dividing (non-confluent) CHO-k1 cells (Figure 1b) the specific radioactivity of PtdEtn decreased over the 23 h chase period irrespective of the presence or absence of 1 mM ethanolamine, most likely because of the increased mass of unlabelled PtdEtn synthesized in the cells during the chase period. In the presence of ethanolamine, the rate of decline in the specific radioactivity of PtdEtn in the non-confluent cells was slightly enhanced compared with that in cells incubated in the absence of ethanolamine (Figure 1b). In neither confluent nor non-confluent cells did the addition of 1 mM ethanolamine affect the cellular mass of phospholipids or protein.

Ethanolamine is a potential degradation product of PtdEtn that could be generated by the action of either phospholipase C or phospholipase D [31], or less likely by reversal of the CDP-ethanolamine pathway. Alternatively, ethanolamine could be released from PtdEtn by a base-exchange reaction [4,5]. The

Figure 1 Incorporation of [^3H]ethanolamine into PtdEtn of quiescent and non-confluent CHO-k1 cells

(a) Confluent monolayer cultures of CHO-k1 cells were incubated with $1\ \mu\text{Ci/ml}$ [^3H]ethanolamine for 1 h at 37°C . The pulse medium was removed and cells were incubated in chase medium with (■) or without (□) 1 mM unlabelled ethanolamine for an additional 2–23 h. After each time interval, lipids were extracted and the content of [^3H]PtdEtn was measured. Parallel dishes of cells were used to determine the phosphorus content of PtdEtn for calculation of specific radioactivity. (b) The experiment was as described in (a) except that half-confluent, instead of fully confluent, monolayer cultures of CHO-k1 cells were used and 10% fetal bovine serum was included in the chase medium. All data are means $\pm$ S.D. of three independent experiments. Some error bars are too small to be visible.

[^3H]ethanolamine content of cells and medium from the experiments depicted in Figure 1 was measured, as was the total radioactivity (d.p.m./dish) of PtdEtn (Figure 2). In both confluent (Figure 2a) and actively dividing (Figure 2b) cells, the [^3H]ethanolamine content of the cells and culture medium combined increased steadily over the 23 h of the chase period, only when unlabelled ethanolamine was added to the culture medium. The amount of [^3H]ethanolamine present in cells and medium combined corresponded quantitatively to the amount of radioactivity lost from PtdEtn for both confluent (Figure 2a) and non-confluent (Figure 2b) cells. The majority (approx. 97%) of radiolabelled ethanolamine was present in the medium. In the absence of unlabelled ethanolamine in the chase medium, the amount of [^3H]ethanolamine was low and remained constant throughout the experiment (Figures 2a and 2b).

The major radiolabelled metabolite generated from the degradation of [^3H]PtdEtn was ethanolamine. In confluent cells incubated in the presence of ethanolamine, after 23 h the radioactivity in phosphoethanolamine and CDP-ethanolamine was 1.24×10^5 and 2.64×10^5 d.p.m./dish, whereas the radioactivity in [^3H]ethanolamine was 9.33×10^5 d.p.m./dish. The radioactivity in CDP-ethanolamine is higher than expected from

Figure 2 Release of [^3H]ethanolamine from ethanolamine-derived PtdEtn in CHO-k1 cells

Cells were incubated as described in Figure 1. After each time interval, media and cells were harvested and ethanolamine was isolated by TLC. Data show total [^3H]ethanolamine content/dish of medium plus cells (diamonds) and radioactivity in [^3H]PtdEtn (squares) from (a) confluent and (b) non-confluent cultures. Open symbols represent incubations in the absence of exogenous unlabelled ethanolamine, and closed symbols represent incubations in the presence of 1 mM unlabelled ethanolamine. All data are means $\pm$ S.D. of three independent experiments. Some error bars are too small to be visible.

studies in other cell types, such as hepatocytes [16] and might be due to either contamination with a small amount of radiolabelled glycerophosphoethanolamine (from which CDP-ethanolamine is poorly separated on the TLC system used) or might reflect a reversal of the reaction catalysed by CDP-ethanolamine-diacylglycerol ethanolaminephosphotransferase. In the absence of exogenous unlabelled ethanolamine, the total radioactivity in CDP-ethanolamine, phosphoethanolamine and ethanolamine combined in non-confluent cells was less than 17% of that in PtdEtn. Similarly, in confluent cells incubated in either the presence or absence of 1 mM unlabelled ethanolamine the radioactivity in ethanolamine, CDP-ethanolamine and phosphoethanolamine was less than 15% of that in PtdEtn. In all cases, the amounts of radiolabelled CDP-ethanolamine and phosphoethanolamine remained unchanged throughout the 23 h incubation. The amounts of other [^3H]ethanolamine-labelled degradation products of PtdEtn (e.g. lysophosphatidylethanolamine and glycerophosphoethanolamine, which might have been generated by the action of phospholipases A) in the cells and culture medium were low and did not increase during the 23 h chase period, under any of the conditions examined.

One possible explanation for why the decline in radioactivity in PtdEtn was greater in the presence than in the absence of

Figure 3 Effect of exogenously added ethanolamine on the amount of $[^{32}\text{P}]\text{PtdEtn}$

(a) Confluent and (b) non-confluent CHO-k1 cells were labelled with $[^{32}\text{P}]\text{P}_i$ for 5 h and subsequently incubated for additional periods of up to 24 h in the absence ($\square$) or presence ($\blacksquare$) of 1 mM unlabelled ethanolamine. At the indicated times, cells were harvested and lipids extracted for measurement of radioactivity. Data are means $\pm$ S.D. of triplicate determinations.

ethanolamine was that the addition of 1 mM ethanolamine increased the rate of degradation of PtdEtn. However, in neither confluent nor non-confluent cells in which PtdEtn was prelabelled with $[^{32}\text{P}]\text{P}_i$ did exogenous ethanolamine accelerate the degradation of $[^{32}\text{P}]\text{PtdEtn}$ (Figure 3). The observation that in ethanolamine-treated non-confluent cells more $[^{32}\text{P}]\text{PtdEtn}$ was present in cells incubated without ethanolamine (Figure 3b) probably reflects a stimulation of ^{32}P incorporation into PtdEtn in the presence of ethanolamine [16]. Moreover, the mass of PtdEtn in the cells (13.6 ± 1.2 and 14.6 ± 1.5 nmol/mg of protein in the absence and presence respectively of ethanolamine) was not affected by incubation with exogenous ethanolamine for 23 h.

Specific radioactivity of PtdSer-derived PtdEtn decreases in the presence of exogenous ethanolamine, only in actively dividing cells

The second major route for synthesis of PtdEtn is by decarboxylation of PtdSer [3]. Therefore we studied the effect of addition of ethanolamine on the metabolism of $[^3\text{H}]\text{serine}$ -derived PtdSer and PtdEtn. Confluent CHO-k1 cells were pulse-labelled for 1 h with $[^3\text{H}]\text{serine}$, then incubated for an additional 23 h in the presence or absence of 1 mM ethanolamine. As shown in Figure 4(a), the specific radioactivity of PtdSer declined during the chase period with a concomitant increase in specific radioactivity of PtdEtn (Figure 4b). Unlike $[^3\text{H}]\text{ethanolamine}$ -derived PtdEtn, however, for which the specific radioactivity decreased on addition of ethanolamine (Figure 1a), the specific radioactivity

Figure 4 Incorporation of $[^3\text{H}]\text{serine}$ into PtdSer and PtdEtn of confluent CHO-k1 cells

Confluent monolayer cultures of CHO-k1 cells were incubated with $5 \mu\text{Ci/ml}$ $[^3\text{H}]\text{serine}$ for 1 h at 37°C . The pulse medium was removed and cells were incubated in medium containing 1 mM unlabelled serine with ($\blacksquare$) or without ($\square$) 1 mM unlabelled ethanolamine for an additional 2–23 h. At the indicated times, one dish of cells was harvested and lipids were extracted. The cellular content of $[^3\text{H}]\text{PtdSer}$ and $[^3\text{H}]\text{PtdEtn}$ was measured and parallel dishes were used to determine the phosphorus content for calculation of specific radioactivities of (a) PtdSer and (b) PtdEtn. Data are means $\pm$ S.D. of three independent experiments. In some instances error bars are too small to be visible.

of $[^3\text{H}]\text{serine}$ -derived PtdEtn in confluent cells was not significantly influenced by the addition of ethanolamine to the culture medium (Figure 4b). Nor did ethanolamine affect the specific radioactivity of PtdSer (Figure 4a). Moreover, exogenous unlabelled ethanolamine did not significantly alter the rate of decarboxylation of PtdSer as measured by the rate of loss of radioactivity (d.p.m./dish) from PtdSer, and the rate of appearance of radioactivity (d.p.m./dish) in PtdEtn (Figure 5a compared with Figure 5b). The $[^3\text{H}]\text{ethanolamine}$ content of the cells and medium was very low, and remained essentially constant throughout the 23 h chase period, in both the presence and absence of ethanolamine (Figures 5a and 5b).

Parallel experiments were performed with actively dividing CHO-k1 cells that had been labelled with $[^3\text{H}]\text{serine}$. In the absence of exogenous ethanolamine, the specific radioactivities of PtdSer (Figure 6a) and PtdEtn (Figure 6b) were similar to those in confluent cells (Figures 4a and 4b). The addition of ethanolamine to the chase medium neither altered the specific radioactivity of PtdSer (Figure 6a) nor affected the rate of loss of radioactivity from PtdSer (Figures 7a and 7b). However, the specific radioactivity of PtdEtn was lower in the presence of ethanolamine than in its absence (Figure 6b). This difference in specific radioactivity of PtdEtn in cells cultured in the presence

Figure 5 Effect of exogenous unlabelled ethanolamine on PtdSer decarboxylation and release of ethanolamine from PtdEtn in confluent CHO-k1 cells

Incorporation of [^3H]serine (d.p.m./dish) into PtdSer (circles), PtdEtn (squares) and ethanolamine (diamonds), and total radioactivity in PtdSer + PtdEtn + ethanolamine per dish (triangles), were determined from the experiment depicted in Figure 4 for cells incubated (■) in the absence and (□) in the presence of unlabelled ethanolamine (1 mM). Data are means $\pm$ S.D. of three independent experiments. Some error bars are obscured by symbols.

and absence of ethanolamine was not the result of a decreased rate of production of PtdEtn from PtdSer because the loss of radioactivity from PtdSer (d.p.m./dish) was not significantly influenced by ethanolamine (Figures 7a and 7b). Moreover, in non-confluent cells incubated without ethanolamine the radioactivity in PtdSer declined as the radioactivity in PtdEtn increased (Figure 7a), and essentially no [^3H]ethanolamine was detected (Figure 7c). In contrast, in non-confluent cells incubated in the presence of ethanolamine the radioactivity in PtdSer declined. However, after 6 h the radioactivity in PtdEtn no longer increased (Figure 7b), but the radioactivity in ethanolamine increased (Figure 7c), suggesting that PtdSer was converted into PtdEtn and that some PtdEtn had been degraded to ethanolamine. The results of these experiments with non-confluent cells are different from those in confluent cells (Figure 5), as in the latter no significant release of ethanolamine was detected either in the presence or absence of exogenous unlabelled ethanolamine.

DISCUSSION

Our data suggest that an active ethanolamine cycle exists in CHO-k1 cells, in which ethanolamine is continually released from, and re-incorporated into, PtdEtn as shown in the model depicted in Figure 8. According to this model, ethanolamine is released from PtdEtn and re-incorporated back into PtdEtn,

Figure 6 Incorporation of [^3H]serine into PtdSer and PtdEtn of non-confluent CHO-k1 cells

Half-confluent cultures of CHO-k1 cells were incubated with $5 \mu\text{Ci/ml}$ [^3H]serine for 1 h at 37°C . The pulse medium was removed and cells were incubated in chase medium containing 10% fetal bovine serum and 1 mM serine with (closed symbols) or without (open symbols) 1 mM unlabelled ethanolamine for an additional 2–23 h. At the indicated times, medium and cells from one dish were harvested and lipids extracted. Parallel dishes were used to determine the phosphorus content of PtdSer and PtdEtn for calculation of specific radioactivities of (■) PtdSer and (□) PtdEtn. Data are means $\pm$ S.D. of three independent experiments. In some instances error bars are too small to be visible.

most likely via CDP-ethanolamine, but from our data we cannot exclude a re-incorporation of some ethanolamine into PtdEtn via a base-exchange reaction.

We have investigated the existence of such a cycle by examining the release of [^3H]ethanolamine from [^3H]PtdEtn labelled from either [^3H]ethanolamine or [^3H]serine. For cells prelabelled with [^3H]ethanolamine, then cultured in medium lacking ethanolamine, the amount of radioactive PtdEtn remained constant for at least 23 h. In contrast, when ethanolamine (1 mM) was added to the culture medium, the radioactivity in PtdEtn gradually declined. A similar observation was made by Yorek et al. [21] with cultured bovine aortic endothelial cells supplemented with $25 \mu\text{M}$ ethanolamine. Our data suggest that the decline in radioactivity in PtdEtn is not due to stimulation of PtdEtn breakdown induced by ethanolamine. Rather, in the presence of exogenous ethanolamine, [^3H]ethanolamine is released from PtdEtn into a cellular pool of ethanolamine, the specific activity of which is greatly decreased by the high concentration (1 mM) of exogenous unlabelled ethanolamine (Figure 8a). Consequently, the specific radioactivity of ethanolamine used for PtdEtn synthesis is lower than that in cells incubated without 1 mM ethanolamine, and the specific radioactivity of the resulting PtdEtn gradually declines. In contrast, in the absence of exogenous unlabelled ethanolamine, the released [^3H]ethanolamine is not significantly diluted, is

Figure 7 Effect of exogenously added unlabelled ethanolamine on PtdSer decarboxylation in actively dividing CHO-k1 cells

Incorporation (d.p.m./dish) of $[^3\text{H}]$ serine into PtdSer (circles) and PtdEtn (squares), and total radioactivity in PtdSer + PtdEtn + ethanolamine (triangles), were determined from the experiment shown in Figure 6 in cells incubated (a) in the absence and (b) in the presence of 1 mM unlabelled ethanolamine. The release of ethanolamine from PtdSer-derived PtdEtn is shown in (c). Data are means $\pm$ S.D. of three independent experiments. Some error bars are too small to be visible.

rapidly recycled back into PtdEtn, and does not accumulate in cells or medium. The recycling of ethanolamine and PtdEtn occurs to a similar extent in both confluent and actively dividing cells. This explanation for the decline in specific radioactivity of PtdEtn is supported by the observations that (i) the amount of $[^3\text{H}]$ ethanolamine released corresponds quantitatively to the decrease in radioactivity in PtdEtn, and (ii) the addition of ethanolamine does not stimulate the breakdown of ^{32}P -labelled PtdEtn.

For PtdSer-derived PtdEtn we propose that a similar recycling of the ethanolamine moiety occurs (Figure 8b) only in cells that

Figure 8 The ethanolamine cycle: the putative turnover of $[^3\text{H}]$ ethanolamine- and $[^3\text{H}]$ serine-derived PtdEtn

(a) When CHO-k1 cells are pulse-labelled with $[^3\text{H}]$ ethanolamine ($^*\text{etn}$) the labelled products are phosphoethanolamine ($\text{p-}^*\text{etn}$), CDP-ethanolamine ($\text{CDP-}^*\text{etn}$) and $^*\text{PtdEtn}$, which are produced by ethanolamine kinase, CTP-phosphoethanolamine cytidyltransferase and CDP-ethanolamine-1,2-diacylglycerol ethanolaminephosphotransferase respectively. Ethanolamine ($^*\text{etn}$) is released from $^*\text{PtdEtn}$ by either a base-exchange reaction or the action of a phospholipase C or D. The four metabolites of ethanolamine participate in a turnover cycle in which the ethanolamine moiety is continuously recycled. When unlabelled ethanolamine (etn) is added to the culture medium, the specific radioactivity of the intracellular ethanolamine pool is reduced. The $^*\text{etn}$ released from $^*\text{PtdEtn}$ is therefore diluted on entry into the cellular ethanolamine pool, resulting in a gradual decrease in specific radioactivity of $^*\text{PtdEtn}$. (b) When CHO-k1 cells are pulse-labelled with $[3\text{-}^3\text{H}]$ serine ($^*\text{ser}$), radiolabelled $^*\text{PtdSer}$, $^*\text{PtdEtn}$, released ethanolamine ($^*\text{etn}$), phosphoethanolamine ($\text{p-}^*\text{etn}$) and CDP-ethanolamine ($\text{CDP-}^*\text{etn}$) are produced by the sequential actions of PtdSer synthase, PtdSer decarboxylase and the enzymes described in (a). When 1 mM unlabelled ethanolamine is included in the medium, the intracellular pool of $[^3\text{H}]$ ethanolamine is diluted, resulting in a gradual decrease in specific radioactivity of PtdEtn.

are actively dividing. In confluent cells, the ethanolamine cycle is apparently essentially dormant, but is stimulated during conditions of rapid cell growth. This statement is supported by the following observations. (i) Exogenously added unlabelled ethanolamine did not significantly affect the rate of decarboxylation of PtdSer to PtdEtn in either confluent or non-confluent cells. In cultured rat hepatocytes, Tijburg et al. [9] similarly observed that addition of ethanolamine to the culture medium did not affect the decarboxylation of PtdSer. (ii) In confluent cells, the specific radioactivity of PtdEtn was unaffected by the presence of exogenous ethanolamine, and ethanolamine liberated from PtdSer-derived PtdEtn did not accumulate. (iii) In actively dividing cells, the radioactivity, and specific radioactivity, of PtdSer-derived PtdEtn declined during the chase period, with a concomitant release of free ethanolamine into cells and culture medium. These findings are consistent with the idea that in confluent cells the ethanolamine cycle, as depicted in Figure 8(b), is not active with PtdSer-derived PtdEtn. In contrast, in non-confluent cells PtdSer-derived PtdEtn is continually catabolized, ethanolamine is released, and PtdEtn is subsequently resynthesized using an intracellular pool of ethanolamine of specific radioactivity that has been diluted by exogenously supplied unlabelled ethanolamine.

Lipton et al. [17] similarly observed that when actively dividing bovine aortic endothelial cells were cultured in the presence of exogenous ethanolamine (25 μ M) the amount of [3 H]serine-labelled PtdEtn was less than when ethanolamine was not added to the culture medium. These authors concluded, however, that exogenous unlabelled ethanolamine reduced the conversion of PtdSer into PtdEtn, although they did not explain why the amount of [3 H]PtdSer was unaffected by the presence of ethanolamine in the culture medium. In addition, these authors considered the alternative possibility that dilution of released [3 H]ethanolamine with the enlarged intracellular pool of free ethanolamine might have been an explanation for their observations.

Our data suggest that PtdEtn derived from the CDP-ethanolamine pathway and from PtdSer decarboxylation is differently utilized in the ethanolamine cycle. The compartmentalization of phospholipids into specific metabolic pools on the basis of their biosynthetic origin has been previously observed. For example, only a small newly synthesized pool of PtdSer is thought to be utilized for decarboxylation to PtdEtn [11,32,33]. Similarly, PtdEtn newly made from ethanolamine, rather than the bulk of cellular PtdEtn, is preferred for methylation to phosphatidylcholine in rat hepatocytes [34]. Furthermore, the methylation of PtdEtn derived from two alternative sources (ethanolamine and PtdSer) is differently affected by the inhibitor 3-deaza-adenosine [35,36]. Other studies have demonstrated that in rat hepatocytes ethanolamine-derived PtdEtn is not significantly remodelled by deacylation-reacylation [34], whereas PtdEtn derived from PtdSer is actively deacylated and reacylated [37]. In addition, pools of phosphatidylcholine and PtdEtn derived from PtdSer decarboxylation, rather than from CDP-ethanolamine, are preferred for secretion into lipoproteins by rat hepatocytes [38].

The reason why in confluent cells the ethanolamine cycle operates only for PtdEtn derived from CDP-ethanolamine, but not for PtdEtn derived from PtdSer, is not known. One possible explanation is that ethanolamine released from PtdSer-derived PtdEtn enters a pool different from that diluted by the exogenous ethanolamine. A similar compartmentalization of pools of aqueous precursors of phospholipids in rat glioma cells has been previously suggested [39]. However, this explanation does not appear to be likely because in non-confluent cells, ethanolamine released from PtdSer-derived PtdEtn is diluted by ethanolamine added to the culture medium. A more feasible explanation, and one that is consistent with our data, is that two different enzymes, which are differently regulated according to the growth status of the cells, are used for release of ethanolamine from the PtdEtn originating via the two different biosynthetic routes. One scenario might be that the ethanolamine-releasing enzyme that utilizes PtdSer-derived PtdEtn is only stimulated when the cells are actively dividing. On the other hand, the enzyme that liberates ethanolamine from ethanolamine-derived PtdEtn might be active in both confluent and non-confluent cells. Our data do not provide any information on which enzyme(s) is/are responsible for liberation of ethanolamine from PtdEtn. A likely candidate for the ethanolamine-releasing enzyme is a base-exchange enzyme, although ethanolamine might alternatively be released by the action of phospholipase C or D. However, the existence of two PtdSer synthases (base-exchange enzymes) has been implicated in CHO cells [26,27]; one, designated PtdSer synthase I, catalyses a base-exchange reaction with PtdSer, PtdEtn and phosphatidylcholine, whereas the other, PtdSer synthase II, uses PtdSer and PtdEtn, but not phosphatidylcholine. Our currently

favoured explanation for the data is that one of the PtdSer synthases releases ethanolamine from PtdSer-derived PtdEtn, and is active only during active cell growth, whereas the other PtdSer synthase releases ethanolamine from PtdEtn made from CDP-ethanolamine, and is active in both confluent and non-confluent cells. It would not be unreasonable that PtdSer synthase, which apparently operates in both a synthetic and a degradative capacity, would be stimulated during active cell growth. Confirmation of these speculations awaits further characterization of the two PtdSer synthases.

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