

Control of the CDPethanolamine pathway in mammalian cells: effect of CTP:phosphoethanolamine cytidyltransferase overexpression and the amount of intracellular diacylglycerol

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For an insight regarding the control of PtdEtn (phosphatidylethanolamine) synthesis via the CDPethanolamine pathway, rat liver cDNA encoding ECT (CTP:phosphoethanolamine cytidyltransferase) was transiently or stably transfected in Chinese-hamster ovary cells and a rat liver-derived cell line (McA-RH7777), resulting in a maximum of 26- and 4-fold increase in specific activity of ECT respectively. However, no effect of ECT overexpression on the rate of [³H]ethanolamine incorporation into PtdEtn was detected in both cell lines. This was explored further in cells overexpressing four times ECT activity (McA-ECT1). The rate of PtdEtn breakdown and PtdEtn mass were not changed in McA-ECT1 cells in comparison with control-transfected cells. Instead, an accumulation of CDPethanolamine (label and mass) was observed, suggesting that in McA-ECT1 cells the ethanolaminephosphotransferase-catalysed reaction became rate-limiting. However, overexpression of the human choline/ethanolaminephosphotransferase in McA-ECT1 and con-

trol-transfected cells had no effect on PtdEtn synthesis. To investigate whether the availability of DAG (diacylglycerol) limited PtdEtn synthesis in these cells, intracellular DAG levels were increased using PMA or phospholipase C. Exposure of cells to PMA or phospholipase C stimulated PtdEtn synthesis and this effect was much more pronounced in McA-ECT1 than in control-transfected cells. In line with this, the DAG produced after PMA exposure was consumed more rapidly in McA-ECT1 cells and the CDPethanolamine level decreased accordingly. In conclusion, our results suggest that the supply of CDPethanolamine, via the expression level of ECT, is an important factor governing the rate of PtdEtn biosynthesis in mammalian cells, under the condition that the amount of DAG is not limiting.

Key words: CTP:phosphoethanolamine cytidyltransferase, diacylglycerol, ethanolaminephosphotransferase, McArdle-RH7777, phorbol ester, phosphatidylethanolamine.

INTRODUCTION

PtdEtn (phosphatidylethanolamine) is a major membrane constituent, being the second most abundant phospholipid class in most mammalian tissues and cell types and often the predominant phospholipid in prokaryotic membranes, where PtdCho (phosphatidylcholine) is usually absent [1,2]. In mammalian cells, PtdEtn is synthesized *de novo* via the CDPethanolamine pathway, also known as the Kennedy pathway, in which ethanolamine is converted into PtdEtn by the subsequent actions of EKI (ethanolamine kinase), ECT (CTP:phosphoethanolamine cytidyltransferase) and EPT (CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase) [3]. Another important route to synthesize PtdEtn is the decarboxylation of PtdSer (phosphatidylserine) by PtdSer decarboxylase [4,5]. The contribution of each of these two pathways to overall PtdEtn synthesis is still a matter of debate.

Studies *in vivo* with labelled substrates [6] and experiments with freshly isolated rat hepatocytes [7] have shown that the CDP-ethanolamine pathway might be an important route for hepatic PtdEtn synthesis when physiological concentrations (30–50 μ M) of extracellular ethanolamine are present. On the other hand, most cells in culture do not require exogenous ethanolamine in the growth medium, which suggests that PtdEtn is synthesized mainly by PtdSer decarboxylation under 'normal' culture conditions.

Calcium-stimulated exchange of ethanolamine with pre-existing phospholipids is a third way of PtdEtn synthesis in mammalian cells, but it is generally considered that, under physiological conditions, base exchange represents only a minor contribution to the synthesis of most phospholipids except PtdSer [8,9].

The prevalent view is that ECT is the pace-setting enzyme of the Kennedy pathway for PtdEtn synthesis [10,11]. However, Lykidis et al. [12] have recently shown, by transiently overexpressing EKI activity in COS-7 cells, that EKI is also an important control point at physiological ethanolamine concentrations. The rate-controlling role of ECT is partially determined on the basis of experiments with freshly isolated hepatocytes. Sundler and Åkesson [13] and Houweling et al. [14] reported that, at physiological extracellular ethanolamine concentrations, phosphoethanolamine starts to accumulate with the level of CDPethanolamine remaining constant, implicating that ECT has become rate-limiting.

Little is known about the mechanism(s) that regulate ECT activity. Enzyme-release measurements from digitonin-permeabilized hepatocytes [15] and differential centrifugation studies [15,16] have revealed that ECT is a cytosolic enzyme. These findings make it unlikely that a reversible translocation mechanism similar to that proposed for CCT (CTP:phosphocholine cytidyltransferase), the rate-limiting enzyme in the CDPcholine pathway [1,17,18], is involved in controlling ECT activity. However, van Hellemond et al. [19] did observe a bimodal distribution

Abbreviations used: CCT, CTP:phosphocholine cytidyltransferase; CEPT, choline/ethanolaminephosphotransferase; CHO, Chinese-hamster ovary; DAG, diacylglycerol; DiC₈, 1,2-*sn*-dioctanoylglycerol; DMEM, Dulbecco's modified Eagle's medium; ECT, CTP:phosphoethanolamine cytidyltransferase; EKI, ethanolamine kinase; EPT, CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase; ER, endoplasmic reticulum; FBS, foetal bovine serum; HS, horse serum; PLC, phospholipase C; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; VC, vector control; WT, wild-type.

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of ECT between the rough ER (endoplasmic reticulum) and the cytosol. Further support for an association of ECT with the ER or with EPT, an integral ER protein [20], came from studies by Bladergroen et al. [21], showing metabolic 'channelling' of intermediates in PtdEtn biosynthesis in rat fibroblasts.

In the present study, we investigated the regulatory role of ECT in PtdEtn biosynthesis by determining the effect of ECT overexpression on the flux through the CDPethanolamine pathway. To our surprise, we found that a 4- to 27-fold ECT overexpression in McA-RH7777 and CHO (Chinese-hamster ovary)-K1 cells respectively did not have an effect on the rate of PtdEtn synthesis, but resulted in an accumulation of CDPethanolamine instead. This led us to investigate the possibility of post-ECT regulation of PtdEtn synthesis in these cells. Our results strongly suggest that, at physiological ethanolamine concentrations, the supply of CDPethanolamine, via the expression level of ECT, together with the amount of cellular DAG (diacylglycerol) available to EPT are major factors regulating the flux through the CDPethanolamine pathway.

MATERIALS AND METHODS

Materials

DMEM (Dulbecco's modified Eagle's medium), Ham's F12 nutrient mixture, FBS (foetal bovine serum), HS (horse serum), LIPOFECTAMINE Plus™ and the restriction enzymes were from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). The radiochemicals [³H-methyl]choline chloride (83 Ci · mmol⁻¹), [1-³H]ethanolamine hydrochloride (17.9 Ci · mmol⁻¹), [1,2-¹⁴C]-ethanolamine (4 mCi · mmol⁻¹), [γ -³²P]ATP (3000 Ci · mmol⁻¹) and the ECL[®] kit were from Amersham Biosciences (Bucks, U.K.). Phospho[1,2-¹⁴C]ethanolamine was prepared enzymically from [1,2-¹⁴C]ethanolamine with partially purified ethanolamine kinase as described previously [22]. *sn*-1,2-DAG kinase was from Calbiochem (La Jolla, CA, U.S.A.). PMA, dioctanoylglycerol, PLC (phospholipase C), 1,2-dioleoylglycerol, L-phosphatidic acid, octyl- β -D-glucopyranoside, cardiolipin and choline were from Sigma (St. Louis, MO, U.S.A.) and ethanolamine from Baker (Deventer, The Netherlands). All other chemicals, unless stated otherwise, were of analytical grade.

Cell culture

McArdle cells (McA-RH7777, A.T.C.C. no. CRL-1601) were cultured in DMEM supplemented with 10% (v/v) FBS and 10% (v/v) HS and maintained in 80 cm² culture flasks at 37 °C, in 5% CO₂ atmosphere with 90% humidity. WT (wild-type) CHO-K1 (A.T.C.C. no. CCL-61) and MT58 cells, kindly provided by Dr C. R. Raetz (Department of Biochemistry, Duke University Medical Center, Durham, NC, U.S.A.), were cultured in Ham's F12 containing 10% FBS. Cells were maintained in 80 cm² culture flasks at 33 °C, in 5% CO₂ atmosphere with 90% humidity.

Stable transfection of McA-RH7777 cells with ECT expression plasmid

A cDNA fragment harbouring the region coding for the entire ECT protein was excised from the pMOSBlue vector [23] with *EcoRI* and *XbaI*. The resulting 1.4 kb fragment was ligated between the *EcoRI* and *XbaI* sites of the mammalian expression vector pcDNA 3.1 (Invitrogen, Carlsbad, CA, U.S.A.). Plasmid DNA was amplified by transformation to JM109 cells and isolated with

Qiagen plasmid maxi kit. The expression plasmid (5 μ g) for ECT was transfected into McA-RH7777 cells, using the DOTAP liposomal method as described previously [24]. Individual neomycin-resistant colonies were selected with 0.6 mg/ml G418 in DMEM medium supplemented with 10% FBS and 10% HS. The colonies were picked and grown in the presence of 0.3 mg/ml G418. Each cell line was assayed for ECT activity to confirm expression.

Incorporation of radioactive precursors into PtdEtn and PtdCho

Cells were grown in 60 mm dishes to 60–80% confluence and labelled with either [³H]ethanolamine or [³H]choline for 1 h in pulse and 6 h in pulse–chase studies. Cells were washed three times with ice-cold PBS and scraped into methanol. Lipids and water-soluble precursors were extracted from the cells by the method of Bligh and Dyer [25]. Phospholipids were separated by TLC on prefab silica G60 plates (Merck, Darmstadt, Germany) in a solvent system of chloroform/methanol/water/acetic acid (65:43:2:2, by vol.). The aqueous phase was evaporated to dryness under nitrogen and the water-soluble ethanolamine-containing metabolites were separated on silica G60 plates in methanol/0.5% NaCl/NH₃ (10:10:1, by vol.). Lipids were visualized with iodine vapour and ethanolamine-containing metabolites by spraying with 0.1% ninhydrin in ethanol. Spots were identified by comparison with known standards. The silica was scraped off the plate and the amount of radioactivity incorporated was determined by liquid-scintillation counting.

Determination of *sn*-1,2-DAG and CDPethanolamine levels

sn-1,2-DAG was extracted from McA-RH7777 and MT58 cells incubated under different conditions, and the amount was determined enzymically with DAG kinase as described by Preiss et al. [26] except for the TLC. [³²P]Phosphatidic acid was separated on prefab silica G60 plates with chloroform/pyridine/formic acid (60:30:7, by vol.) as the developing solvent. Unlabelled phosphatidic acid was used as a standard. The silica containing [³²P]phosphatidic acid was scraped off the plates and the amount of radioactivity was measured by liquid-scintillation counting. To quantify the amount of CDPethanolamine in McA-RH7777 cells after various treatments, an aliquot of the cell homogenate was extracted as described above. A trace amount of labelled CDPethanolamine was routinely added to the extraction mixture to calculate recoveries. The water-soluble intermediates were collected, dried under a stream of nitrogen, dissolved in 20 mM Tris (pH 8.5) and applied to anion-exchange chromatography (Dowex 1 \times 8 formiate, Bio-Rad Laboratories; 8 cm \times 0.4 cm). The column was subsequently rinsed with 3 column vol. of water and 20 mM formic acid before the CDPethanolamine was collected in 10 fractions of 1 ml by elution with 20 mM formic acid. Fractions containing radioactivity were pooled and dried under nitrogen. Subsequently, CDPethanolamine was separated from other water-soluble compounds on a HPLC anion-exchange column (Macrosphere WAX 7u, 250 mm \times 4.6 mm; Alltech USA, Deerfield, IL, U.S.A.) using 10 mM sodium phosphate buffer (pH 4.0) at a flow rate of 0.6 ml/min. The CDPethanolamine, which was clearly separated in this system, was quantified spectrophotometrically at a wavelength of 280 nm.

Transfection of CHO cells with ECT cDNA and assay of ECT activity

CHO-K1 and MT58 cells were grown on 35 mm dishes till 30–40% confluency and transfected with 1 μ g of isolated pcDNA 3.1

containing the ECT cDNA insert (CHO-K1-ECT and MT58-ECT) using LIPOFECTAMINE Plus™, according to the manufacturer's instructions. Control cells were transfected with empty plasmid. At 48 h after transfection, cells were pulsed with radioactive precursors for 1 h to determine the rate of phospholipid synthesis as described above. In parallel dishes, cells were lysed in 300 μ l of ice-cold lysis buffer [25 mM Tris phosphate, pH 7.8/15% (v/v) glycerol/1% Triton X-100/8 mM MgCl₂/1 mM dithiothreitol]. After lysis for 10 min at 4 °C, cells were scraped off the dishes and the resulting cell homogenate was assayed for ECT activity. The activity of ECT was measured in 40 μ l of homogenate (approx. 100 μ g of protein), as described by Tijburg et al. [22]. Radioactive CDP[1,2-¹⁴C]ethanolamine and phospho[1,2-¹⁴C]ethanolamine were separated and quantified as described for the water-soluble ethanolamine-containing metabolites.

Transfection of McA-RH7777 cells with hCEPT1 cDNA, assay of cholinephosphotransferase activity and PtdEtn synthesis

McA-VC and McA-ECT1 cells were transiently transfected with the human choline/ethanolaminophosphotransferase 1 (hCEPT1; donated by Dr C. McMaster, The Atlantic Research Centre, Department of Pediatrics, IWK Health Centre, Dalhousie University, Halifax, NS, Canada) using the LIPOFECTAMINE™ procedure as described in the previous section. Samples were collected, 48 h after transfection, to confirm expression of hCEPT1 using the cholinephosphotransferase activity assay. The activity was determined in the presence of exogenous DAG, as described by Groener et al. [27]. At the same time point, the rate of PtdEtn synthesis was determined as described in the Incorporation of radioactive precursors into PtdEtn and PtdCho subsection.

Immunoblotting

Cell homogenate protein (50 μ g) from transfected McA-RH7777 and WT cells was resolved by SDS/PAGE (10% gel) and transferred on to Immobilon-P PVDF membranes by electrophoretic blotting [28]. Membranes probed with anti-ECT antiserum [19] were visualized with a chemiluminescence development reagent (ECL®, Amersham Biosciences) according to the manufacturer's instructions.

Other methods

Cell protein was measured as described by Lowry et al. [29], using BSA as a standard, and phospholipids were quantified by phosphorus analysis as described by Rouser et al. [30]. Results are expressed as means \pm S.D. All statistical analyses were performed using unpaired *t* test.

RESULTS

Stable overexpression of rat liver ECT does not accelerate PtdEtn biosynthesis in cultured rat hepatoma cells

McA-RH7777 cells were transfected with the VC (vector control) pcDNA3.1 or pcDNA3.1 containing the rat liver ECT cDNA insert, and colonies were selected for neomycin resistance. Several ECT-transfected and VC colonies were picked for screening. Cell lysates of each cell line were prepared and ECT overexpression was confirmed by Western-blot analysis and ECT activity assays. Figure 1 shows the results of four ECT-transfected cell lines (ECT1-4), a VC cell line and WT McA-RH7777 cells. ECT

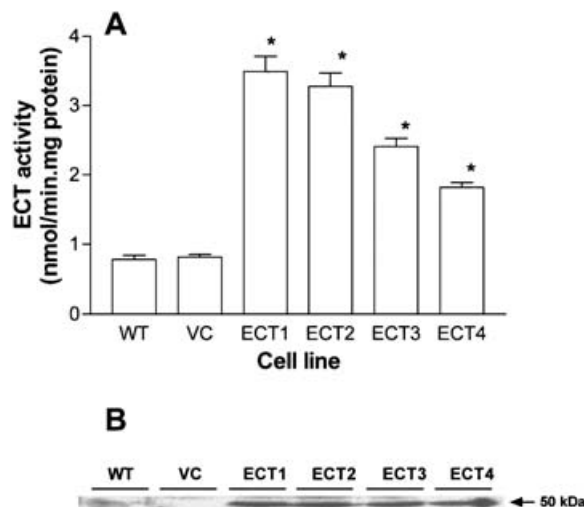


Figure 1 Stable overexpression of rat liver ECT in McA-RH7777 cells

Cells were grown to near confluency on 60 mm culture dishes in DMEM containing 10% FBS and 10% HS. The plates were rinsed twice with ice-cold PBS, and the cells were harvested. Aliquots were assayed for the activity of ECT (A) and subjected to immunoblot analysis of ECT protein (B). The experiment was repeated twice with similar results. WT, wild-type McA-RH7777 cell line; ECT-1-4, McA-RH7777 cell lines that have been stably transfected with the expression vector pcDNA 3.1, containing a 1.4 kb ECT cDNA insert; VC, vector control, a cell line stably transfected with the 'empty' pcDNA 3.1 plasmid. **P* < 0.001, significantly different from VC cells.

protein levels were increased in the ECT cell lines, compared with WT and VC cells (Figure 1B), and the increased amounts of ECT protein could be correlated to an enhanced activity of the enzyme (Figure 1A). The ECT cell lines notably contained a 2- to 4-fold higher enzyme activity compared with VC cells.

If ECT is the pace-setting enzyme of the Kennedy pathway for PtdEtn synthesis, the increased enzyme activity in the ECT transfectants should be mirrored by higher rates of PtdEtn synthesis in these cell lines. To study this, a high (ECT1) and a medium (ECT3) expressor, WT and control-transfected cells were pulsed with [³H]ethanolamine for 1 h in the presence of 50 μ M ethanolamine. It is clear from Figure 2(A) that label incorporation into PtdEtn was not increased in ECT1 and ECT3 when compared with VC and WT cells, suggesting that ECT overexpression does not accelerate the CDPethanolamine pathway. Similar results were obtained for the ECT2 and ECT4 cell lines (results not shown). The distribution of [³H]ethanolamine among the water-soluble intermediates of the CDPethanolamine route showed no significant difference in label incorporation into phosphoethanolamine between the ECT cell lines and control cells (Figure 2C). However, ECT overexpression did clearly result in an accumulation of label in CDPethanolamine: after the pulse, [³H]-ethanolamine incorporation in this intermediate was 1.5-fold higher in ECT3 and 3.5-fold in ECT1, compared with VC or WT cells (Figure 2B). Apparently, the increased amount of label shuttled from phosphoethanolamine to CDPethanolamine by ECT in the overexpressors, and was trapped in the CDPethanolamine pool instead of being further converted into PtdEtn. This implies that the reaction catalysed by EPT could not keep pace with the ECT-catalysed reaction. We therefore also measured the pool size of CDPethanolamine by HPLC analysis and found that the amount of this intermediate was approx. 14-fold higher in ECT1 cells (21.1 ± 2.0 nmol/mg of protein) than in VC cells (1.5 ± 0.2 nmol/mg of protein), cultured in the presence of 50 μ M ethanolamine (*n* = 3).

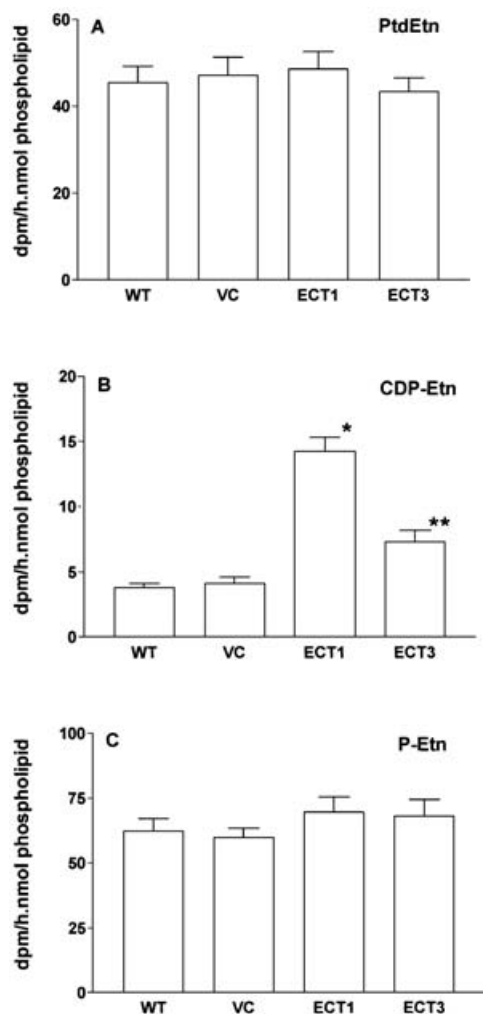


Figure 2 Effect of stable ECT overexpression on the incorporation of [³H]ethanolamine into PtdEtn and intermediates of the CDPethanolamine pathway in McA-RH7777 cells

Cells were grown in DMEM supplemented with 0.5% serum and 50 μ M ethanolamine for 4 h before the pulse with 1 μ Ci of [³H]ethanolamine. After 60 min, the pulse was stopped by rinsing the cells twice with ice-cold PBS and scraping the cells into methanol. Lipids were extracted and aliquots were assayed for phospholipid and subjected to TLC as described in the Materials and methods section. PtdEtn, phosphatidylethanolamine; CDP-Etn, CDPethanolamine; P-Etn, phosphoethanolamine. Results are expressed as means \pm S.D. for triplicate incubations of one representative experiment, which was repeated four times ($n = 5$). For the various cell lines, see the legend for Figure 1. * $P < 0.001$, ** $P < 0.01$, significantly different from VC cells.

Although overexpression of ECT did not seem to have an effect on the rate of PtdEtn synthesis via the Kennedy pathway, we could not rule out the possibility that this lack of effect was the result of adaptation of the transfected cells to the stable overexpression. For example, a possible increase in PtdEtn labelling in the stable ECT overexpressors could have been masked by a proportional increase in degradation of PtdEtn due to increased phospholipase activity as has been observed in maintaining cellular PtdCho homeostasis [31,32]. Therefore ECT1 and VC cells were pulse-chased with [³H]ethanolamine.

The slopes of the curves in Figure 3 are not significantly different for McA-ECT1 and VC cells, clearly showing that the rate of loss of radiolabel from PtdEtn was the same in ECT1 as in control cells. Thus the lack of effect of ECT overexpression on the rate of PtdEtn synthesis did not originate from increased PtdEtn breakdown. In line with the lack of effect of ECT overexpression

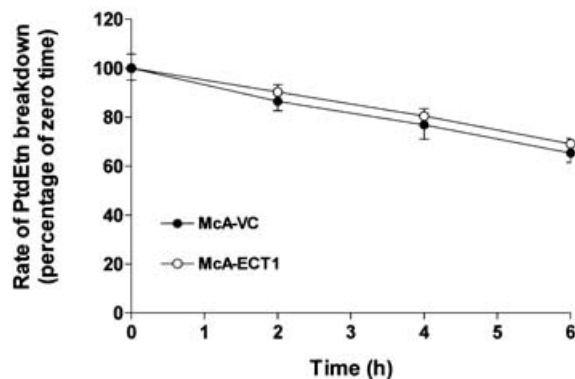


Figure 3 Effect of stable ECT overexpression on the rate of PtdEtn breakdown in McA-RH7777 cells

McA-VC (●) and McA-ECT1 (○) were grown in DMEM supplemented with 0.5% serum and 50 μ M ethanolamine for 4 h before the pulse with 2 μ Ci of [³H]ethanolamine. After 6 h, the pulse was stopped by rinsing the cells twice with DMEM supplemented with 0.5% serum and 50 μ M ethanolamine. The cells were subsequently incubated overnight (14 h) in the same medium without label containing 250 μ M ethanolamine. Next, the medium was aspirated and the chase was started ($t = 0$) in the presence of 500 μ M ethanolamine. Samples were collected at different periods of times after starting the chase, lipids extracted and aliquots were assayed for the amount of total phospholipid and label incorporated into PtdEtn as described in the Materials and methods section. Results are expressed as means \pm S.D. for triplicate incubations of one representative experiment ($n = 2$). The two cell lines are as explained in the legend for Figure 1.

on PtdEtn synthesis and breakdown, we observed no change in the amount of PtdEtn in ECT1 cells (36.1 ± 1.1 nmol/mg of protein) when compared with VC cells (35.7 ± 2.4 nmol/mg of protein) or in the PtdCho to PtdEtn ratio (2.13 ± 0.21 and 1.98 ± 0.24 in McA-ECT1 and VC, respectively).

Transient overexpression of human CEPT1 in McA-VC and McA-ECT1 cells and its effect on PtdEtn synthesis

One possibility to account for a rate limitation by the EPT-catalysed reaction after overexpression of ECT, is that the activity of EPT cannot keep up with the increased CDPethanolamine production due to the presence of an increased amount of ECT. On the other hand, the substrate availability may be insufficient to allow EPT to keep pace with the ECT-catalysed reaction. Since EPT uses DAG together with CDPethanolamine to produce PtdEtn, the availability of DAG could be limiting the flux through the Kennedy pathway after ECT overexpression. To study the first possibility, namely that the activity of EPT cannot keep pace with ECT overexpression, we transiently expressed the hCEPT1 cDNA in McA-VC and McA-ECT1 cells, resulting in a 2.8- and 3.0-fold increase in cholinephosphotransferase activity, respectively. However, no effect of hCEPT1 overexpression on the rate of [³H]ethanolamine incorporation into PtdEtn could be detected in McA-VC [9.5 ± 1.5 and 8.9 ± 1.3 d.p.m. $\times 10^{-3}$ h⁻¹ \cdot (mg of protein)⁻¹ in empty-vector and hCEPT1 respectively] and McA-ECT1 [10.1 ± 0.9 and 10.3 ± 1.0 d.p.m. $\times 10^{-3}$ h⁻¹ \cdot (mg of protein)⁻¹ in empty-vector and hCEPT1 respectively] cells.

Transient overexpression of rat liver ECT in CHO-K1 and MT58 cells: the effect of PtdCho depletion on DAG levels and on PtdEtn synthesis

To determine whether the amount of DAG available for the EPT reaction becomes limiting after ECT overexpression, we transiently transfected ECT in CHO-K1 and MT58 cells. These

Table 1 Transient overexpression of ECT in CHO-K1 and MT58 cells: effect on phosphatidylethanolamine and phosphatidylcholine synthesis at 40 °C

CHO-K1 and MT58 cells were transfected with the expression vector pcDNA 3.1 containing a 1.4 kb ECT cDNA insert (ECT) or with an empty plasmid (VC). Cells were disrupted 48 h after transfection and assayed for ECT activity, or pulsed with 1 μ Ci [3 H]choline or [3 H]ethanolamine for 60 min to measure the rate of PtdCho and PtdEtn synthesis respectively. Lipids were extracted and aliquots were assayed for phospholipid subjected to TLC as described in the Materials and methods section. Results are expressed as means \pm S.D. for triplicate incubations. The experiment was repeated once with similar results. * P < 0.01, significantly different from VC-cells; ** P < 0.001, significantly different from 33 °C control. N.D., not determined.

Cell line	ECT activity (nmol \cdot min $^{-1}$ \cdot mg of protein $^{-1}$)	Rate of phospholipid synthesis [d.p.m. \cdot h $^{-1}$ \cdot (nmol phospholipid) $^{-1}$]		
		PtdCho		PtdEtn
		33 °C	40 °C	40 °C
CHO-K1-VC	1.1 \pm 0.2	N.D.	29.4 \pm 2.3	103.0 \pm 5.8
CHO-K1-ECT	26.7 \pm 1.6*	N.D.	30.2 \pm 0.2	109.1 \pm 6.8
MT58-VC	1.2 \pm 0.2	9.8 \pm 1.0	3.0 \pm 0.4**	94.5 \pm 6.8
MT58-ECT	20.4 \pm 2.2*	9.2 \pm 0.9	2.8 \pm 0.3**	98.3 \pm 6.5

cell lines were chosen as they might offer the possibility to manipulate the DAG supply for the EPT-catalysed reaction. The MT58 cell line is a CHO-derived cell line carrying a thermo-sensitive mutation in CCT- α [33]. MT58 cells can grow and sustain normal PtdCho levels at the permissive temperature of 33 °C. However, shifting these cells to 40 °C results in a rapid inactivation of CCT- α and a decrease in the rate of PtdCho biosynthesis [33,34]. DAG is a central intermediate in the biosynthesis of triacylglycerols, PtdCho and PtdEtn [10]. Inhibition of PtdCho synthesis may therefore result in an increased availability of DAG for triacylglycerol and PtdEtn synthesis.

It is clear from Table 1 that transient transfection led to higher levels of ECT overexpression than in stable transfected McA-RH7777 cells (see Figure 1). The ECT activity in CHO-K1 and MT58 cells was increased 27- and 18-fold respectively when compared with controls 48 h after transfection. At this time point, cells were kept at 33 °C or shifted to 40 °C for 4 h before the 1 h pulse with [3 H]choline and [3 H]ethanolamine to determine the incorporation into PtdCho and PtdEtn respectively. As expected, the pulse-label experiments showed a clear effect of the 4 h temperature shift on the rate of PtdCho synthesis in the MT58 cells. [3 H]Choline incorporation into PtdCho was approx. 3-fold lower in MT58 grown at 40 °C compared with the cells grown at 33 °C (Table 1).

In line with the results obtained with McA-RH7777 cells stably overexpressing ECT (Figure 2A), transient overexpression of ECT did not lead to higher rates of PtdEtn synthesis in CHO-K1 cells, measured as incorporation of [3 H]ethanolamine into PtdEtn (Table 1). Similar results were obtained in MT58 cells, despite the shutdown of PtdCho synthesis in these cells at 40 °C. In agreement with this lack of effect, but in contrast with our prior expectations, measurement of the cellular DAG pool showed that the reduced rate of PtdCho synthesis at 40 °C did not significantly change the amount of DAG [0.28 \pm 0.02 and 0.26 \pm 0.01 nmol/mg of protein for MT58 cells incubated at 33 °C and 40 °C for 4 h respectively (n = 3)].

Stable overexpression of rat liver ECT in cultured rat hepatoma cells: the effect of DAG levels on PtdEtn synthesis

Since shutting down PtdCho biosynthesis in the MT58 cells failed to increase the amount of cellular DAG, several agonists were employed to augment the intracellular DAG level in the stable transfectants. Treatment of cells with DiC $_8$ (1,2-*sn*-dioctanoylglycerol) seemed the most obvious choice, as DiC $_8$ is a substrate for the cholinephosphotransferase-catalysed reaction and has been shown to increase PtdCho synthesis through

activation of CCT [35,36]. Treatment of McA-VC and McA-ECT1 cells with various concentrations of DiC $_8$ (up to 250 μ M) had no effect on the rate of labelled ethanolamine incorporation into PtdEtn (results not shown).

Next, we used PLC and PMA in pulse-label experiments. PMA has been shown to stimulate PtdEtn synthesis in freshly isolated hepatocytes [37] and to increase intracellular DAG levels via activation of the phospholipase D-catalysed hydrolysis of PtdCho and PtdEtn in combination with phosphatidate phosphohydrolase [38]. On the other hand, treatment of cells with PLC has been shown to increase intracellular DAG levels and stimulate PtdCho synthesis in several cell lines [35,39,40].

McA-ECT1 and control-transfected cells were pulsed with [3 H]ethanolamine and [3 H]choline for 1 h in the presence or absence of 0.05 unit/ml PLC or 1 μ M PMA, and label incorporation into phosphoethanolamine, CDPethanolamine, PtdEtn and PtdCho was determined. PLC treatment of McA-VC and McA-ECT1 cells showed a stimulation of [3 H]choline incorporation into PtdCho (Figure 4A; PLC-treated cells significantly different from untreated-controls; P < 0.05) and a significant 5- and 6-fold increase in DAG levels respectively (McA-VC: Cont, 0.50 \pm 0.07 and PLC, 2.50 \pm 0.32 nmol/mg of protein; McA-ECT1: Cont, 0.49 \pm 0.06 and PLC, 3.02 \pm 0.36 nmol/mg of protein). On the other hand, no effect on the incorporation of [3 H]ethanolamine into PtdEtn and its water-soluble intermediates, phosphoethanolamine and CDPethanolamine, was observed in McA-VC cells after PLC treatment (Figures 4B–4D). In contrast, exposure of the McA-ECT1 cells to PLC stimulated PtdEtn synthesis (155 % of non-treated cells), which was accompanied by a reduction of label in the CDPethanolamine pool.

Exposure to PMA stimulated the CDPcholine pathway (Figure 4A; PMA-treated cells significantly different from untreated-controls; P < 0.01) as well as the CDPethanolamine pathway (Figure 4B) in both cell lines and no difference in label incorporation into PtdEtn was observed between the non-stimulated ECT overexpressor and VC cells (Figure 4B). The percentage by which PMA stimulated PtdCho synthesis was not significantly different between McA-VC and McA-ECT1 cells (Figure 4A). However, PMA accelerated the CDPethanolamine pathway in the ECT-overexpressing cells to a significantly greater extent (290 % of non-treated) than in VC cells (145 % of non-treated) and this difference in stimulation was reflected in the amount of label associated with CDPethanolamine. Due to the action of PMA, label incorporation into this intermediate dropped 25 % in VC cells and 45 % in McA-ECT1 (Figure 4C). In McA-ECT1 cells transiently overexpressing hCEPT1, no additional increase in the rate of PtdEtn synthesis was observed after exposure to

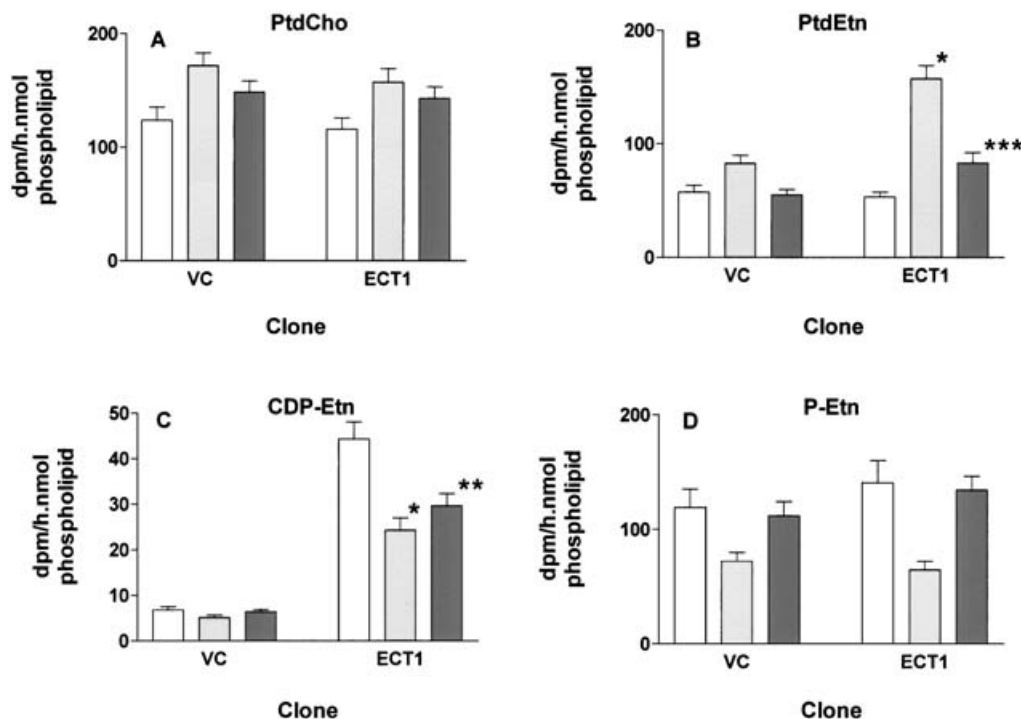


Figure 4 Effect of phorbol ester and phospholipase C on the incorporation of labelled ethanolamine and choline in control and ECT-overexpressing McA-RH7777 cell lines

The two McA-RH7777 cell lines were grown in DMEM supplemented with 0.5% serum and 50 μ M ethanolamine for 4 h. At this point of time, the cells were pulsed with 1 μ Ci of [3 H]ethanolamine and [3 H]choline in the absence (open bars) or presence of 10^{-6} M PMA (grey bars) or 0.05 unit/ml phospholipase C (dark-grey bars). After 60 min, the pulse was stopped by rinsing the cells twice with PBS and scraping the cells into methanol. Lipids were extracted and aliquots were assayed for phospholipid and subjected to TLC as described in the Materials and methods section. Results are expressed as means \pm S.D. for triplicate incubations of one representative experiment, which was repeated twice ($n=3$). The cell lines are as in the legend for Figure 1. * $P < 0.001$, significantly different from PMA-treated VC cells; ** $P < 0.001$, *** $P < 0.01$, significantly different from PLC-treated VC cells.

PMA when compared with McA-ECT1 cells transiently overexpressing the empty vector [33.5 ± 3.2 and 35.2 ± 3.8 d.p.m. $\times 10^{-3} \text{ h}^{-1} \cdot (\text{mg of protein})^{-1}$ respectively].

To determine whether this increased responsiveness of the CDPethanolamine pathway to PMA in ECT-overexpressing cells could be correlated to increased cellular DAG levels, the amount of DAG in both cell lines was determined after the addition of 1 μ M PMA. As depicted in Figure 5, PMA caused a fast increase in the amount of DAG in both ECT-overexpressing and VC cells, with a maximum reached within 20 min of incubation. In McA-ECT1 cells, however, the DAG level decreased to the initial steady-state value at a rate higher than that in the VC cells: after 60 min, the amount of DAG was even slightly below its initial level in McA-ECT1, whereas the level in VC was still increased. Table 2 shows that the difference in DAG consumption between VC and McA-ECT1 cells parallels CDPethanolamine usage. Whereas PMA treatment results in a transient reduction in the amount of CDPethanolamine in the McA-VC cells (0.5 nmol/mg of protein after 30 min PMA), it resulted in a more pronounced decrease in the McA-ECT1 cell line (3.3 and 5.2 nmol/mg of protein after 30 and 60 min PMA respectively).

Our results indicate that, after the stimulation of McA-RH7777 cells with PMA, the cellular DAG pools rapidly increase. These increased amounts of DAG are then, together with increased levels of CDPethanolamine, rapidly consumed by EPT to yield an acceleration of PtdEtn synthesis via the Kennedy pathway.

DISCUSSION

It is widely assumed that the ECT-catalysed reaction is the pace-setting step in *de novo* PtdEtn synthesis via the CDPethanolamine

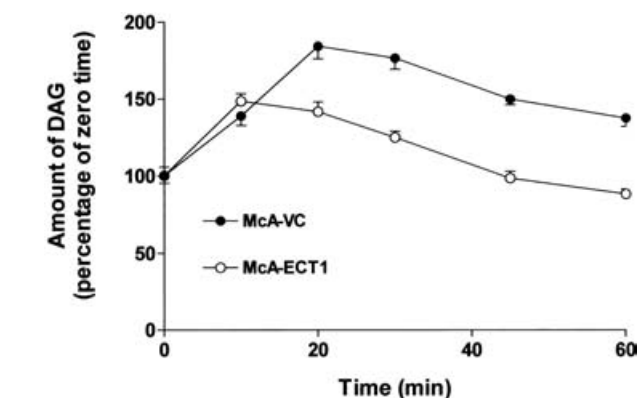


Figure 5 Effect of phorbol ester on the cellular amount of DAG in control and ECT-overexpressing McA-RH7777 cell lines

Cells were grown in DMEM supplemented with 0.5% serum and 50 μ M ethanolamine. After 4 h, McA-VC (●) and McA-ECT1 (○) cells were incubated in the presence of 10^{-6} M PMA for various time periods. Incubations were stopped by rinsing the cells twice with ice-cold PBS and scraping the cells into buffer. After homogenization, aliquots were assayed for protein and phospholipid, and lipids were extracted to determine the amount of DAG using the DAG-kinase assay as described in the Materials and methods section. Results are expressed as means \pm S.D. for triplicate incubations of one representative experiment, which was repeated twice ($n=3$), and expressed as percentage of DAG at $t=0$ (McA-VC: 0.64 ± 0.05 nmol/mg of protein and McA-ECT1: 0.72 ± 0.09 nmol/mg of protein). The cell lines are as in the legend for Figure 1.

pathway [10,11]. A key-regulatory role for ECT in the CDPethanolamine pathway is tempting in analogy with the Kennedy pathway for PtdCho synthesis, where ECT's counterpart CCT is generally acknowledged as the pace-setting enzyme, but a

Table 2 Effect of phorbol ester and phospholipase C on the pool size of CDPethanolamine in control and ECT-transfected McA-RH7777 cell lines

McA-VC and McA-ECT1 cells were kept in DMEM containing 0.5% serum and 50 μ M ethanolamine for 4 h prior to incubating the cells in the absence (Cont) or presence of 1 μ M PMA or 0.05 unit/ml PLC for various time periods. The amount of CDPethanolamine was determined as described in the Materials and methods section. Results are expressed as means \pm S.D. for triplicate incubations. The experiment was repeated once with similar results (significantly different from control; * P < 0.02, ** P < 0.01).

Cell line	Amount of CDPethanolamine (nmol/mg of protein)			
	Cont	PMA (30 min)	PMA (60 min)	PLC
McA-VC	1.37 \pm 0.18	0.88 \pm 0.12*	1.54 \pm 0.13	1.09 \pm 0.08
McA-ECT	21.12 \pm 1.51	17.85 \pm 0.42*	15.95 \pm 1.08**	19.18 \pm 1.12

rate-determining role for ECT is also supported by several pieces of evidence. Pulse-label studies demonstrated that exposure of freshly isolated hepatocytes to increasing concentrations of ethanolamine resulted in an enhanced incorporation of labelled glycerol and ethanolamine into PtdEtn. The enhanced PtdEtn synthesis was accompanied by a considerable increase in the pool size of phosphoethanolamine, whereas the amount of CDPethanolamine remained constant [13,14]. Furthermore, exposure of hepatocytes to phorbol ester has been shown to stimulate PtdEtn synthesis, to enhance the activity of ECT and to reduce phosphoethanolamine and CDPethanolamine pool sizes [37]. Recently, Houweling et al. [41] reported that AICAR (5-aminoimidazole-4-carboxamide ribonucleoside) inhibited PtdEtn synthesis in freshly isolated hepatocytes, partially by decreasing ECT activity. In the present study, we have used a molecular approach, overexpression of ECT activity, to gain insight into the regulatory role of this enzyme in PtdEtn biosynthesis.

We report that both transient and stable overexpression of ECT activity in CHO and McA-RH7777 cells respectively did not affect the rate of PtdEtn synthesis via the Kennedy pathway (Figure 2), but led to an accumulation of CDPethanolamine instead. This suggests that the reaction catalysed by EPT, the enzyme transferring the phosphoethanolamine moiety from CDPethanolamine to DAG to form PtdEtn, could not keep pace with the reaction catalysed by ECT. Although the last enzymes in the Kennedy pathways for PtdCho and PtdEtn synthesis do not normally determine the rate of phospholipid synthesis, earlier studies have shown that regulation at these steps can contribute to overall PtdCho and PtdEtn synthesis [3,17,42,43]. Two mammalian choline/ethanolaminephosphotransferases, encoding cDNAs CPT1 and CEPT1, have been cloned [44,45]. CPT1 codes for a CDPcholine-specific cholinephosphotransferase, whereas CEPT1 codes for an enzyme using both CDPcholine and CDPethanolamine as substrate. Transient overexpression of the hCEPT1 enzyme had no effect on the rate of PtdEtn synthesis in both McA-VC and McA-ECT1 cells, suggesting that the accumulation of CDPethanolamine in McA-ECT1 cells is due to a lack of DAG rather than EPT activity. Determination of the DAG specificity of CEPT1 showed that DiC₈ was a good substrate in combination with CDPcholine, but no activity was detectable when CDPethanolamine was used as the second substrate [44]. The latter is in agreement with earlier reports studying the substrate specificity of partially purified EPT, showing a preference for DAG species containing long-chain unsaturated fatty acids (e.g. C_{22:6}) in the sn-2 position [46]. Recently, it was suggested that, on the basis of the analysis of the human genome and those of other eukaryotic cells, these two genes (CPT1 and CEPT1) code for the entire set of CEPTs in mammalian cells [20]. This would agree with our results showing that DiC₈ was unable to increase the rate of PtdEtn synthesis

in McA-VC and McA-ECT1 cells, whereas the incorporation of labelled choline into PtdCho was enhanced (cf. [36]). To test the hypothesis of a rate-limiting role for the EPT-catalysed reaction after ECT overexpression, two other attempts were made to increase the levels of the second substrate, DAG, as well.

First, rat liver ECT was overexpressed in a clonal CHO cell line, MT58, with a conditionally defective CCT α activity, giving an up to 18-fold enhancement of ECT activity (Table 1). Shifting the MT58 cells to the non-permissive temperature would, as a result of diminished CDPcholine production, increase the amount of DAG available for the EPT-catalysed reaction. However, the rate of PtdEtn synthesis in control and transfected MT58 cells was the same 4 h after shifting the cells to 40 °C. At this point of time, we detected no further difference in the amount of intracellular DAG between control and transfected MT58 cells. This result can be interpreted in several ways: (i) shutting down PtdCho synthesis has no effect on the amount of DAG or (ii) more DAG becomes available, but this DAG is not preferentially used by EPT in the ECT-overexpressing MT58 cells. The latter seems the most probable explanation as it was shown that the rate of triacylglycerol synthesis, measured as labelled acetate incorporation into triacylglycerol and the amount of triacylglycerol, was increased in MT58 cells grown at 40 °C [47,48].

Secondly, McA-RH7777 cells stably overexpressing ECT (4-fold) were pulse-labelled with [³H]ethanolamine in the presence of the phorbol ester PMA or PLC. As a result of the PMA treatment, PtdEtn synthesis via the Kennedy pathway was stimulated (Figure 4B) in both control-transfected (145% of non-treatment) and ECT-overexpressing cells, but the ECT overexpressor was stimulated to a significantly greater extent (295% of non-treatment) than the VC cells. In agreement with this, the disappearance of label from CDPethanolamine after PMA treatment was significantly higher in the ECT expressor when compared with VC cells (Figure 4C). Cellular DAG concentrations were measured over the time course of the pulse-label experiments (Figure 5) and the results clearly showed that PMA causes a rapid but transient increase in cellular DAG levels. Interestingly, the increased amount of DAG was consumed faster in the ECT-overexpressing cells than in the control-transfected cells. Apparently, the increased cellular pools of CDPethanolamine (resulting from the ECT overexpression) and DAG (resulting from the PMA treatment) allow EPT to operate at a higher velocity when compared with the VC.

Exposure of McA-VC and McA-ECT1 cells to PLC resulted in a 2-fold higher DAG level in comparison with PMA treatment. However, the rate of PtdEtn synthesis was not significantly affected in VC cells and the effect of PLC in McA-ECT1 cells was less pronounced when compared with PMA (Figure 4B). One possible explanation might be that PMA, in addition to augmenting cellular DAG, is known to activate ECT via a yet unknown mechanism [37], whereas DAG formed after exposure to PLC activates CCT [36] without affecting ECT activity (results not shown). In agreement with this, treatment of VC cells with PLC had no effect on PtdEtn formation, whereas PtdCho synthesis was significantly increased in these cells. Altogether, this suggests that an increased supply of both substrates, DAG and the CDP-alcohol, is necessary to increase phospholipid synthesis. The discrepancy between the amount of DAG formed after exposure to PLC or PMA and its ability to stimulate PtdEtn synthesis might further be due to the fact that after exposure to PLC, the DAG, which is mainly produced from PtdCho at the outer leaflet of the plasma membrane: (i) is not as readily available to EPT as to CPT, enzymes that are located in the ER and Golgi respectively [20] or (ii) is composed of species that are a better substrate for CPT than for EPT.

Results from our experiments with PMA correlate well with the publication by Tijburg et al. [37]. They found an increase in the rate of PtdEtn synthesis via the Kennedy pathway in freshly isolated hepatocytes due to a transient increase in DAG levels and activation of ECT and EPT. The nature of the PMA-induced activation of ECT and EPT is, as yet, not clear. Phorbol esters have been shown to increase the rate of PtdCho biosynthesis, both *in vitro* [35] and in various cell types including GH₃ pituitary [35,49] and HeLa cells [50], by stimulating CCT. Activation of CCT involves a translocation of the enzyme from a soluble to a membrane-bound form, but the mechanism by which phorbol esters cause this translocation, is not completely understood.

Whether the effect of PMA on ECT activity (see Figure 4D) also involves translocation of the enzyme from cytosol to membranes, is yet to be determined, since no unambiguous conclusions have been drawn so far about the subcellular localization of ECT [3,15,19]. Kiss [51] reported that stimulation of PtdEtn and PtdCho synthesis by PMA involves separate protein kinase C isoenzymes, i.e. protein kinase C- β and - α , respectively. However, a direct phosphorylation of ECT by protein kinase C seems unlikely, as we could not detect phosphorylation of ECT under various conditions, e.g. by use of PMA and AICAR (M. Houweling and W. Klein, unpublished work).

Although Lykidis et al. [12] recently reported that a 170-fold overexpression of EKI in COS-7 cells, incubated in the presence of 32 μ M ethanolamine, led to an approx. 2.5-fold increase in [³H]ethanolamine incorporation into PtdEtn and despite the fact that the contribution of each step to overall regulation of the pathway may differ among tissues [52], the ECT-catalysed reaction is generally considered to be the pace-setting step in PtdEtn biosynthesis in mammalian cells [10,11]. Therefore it is interesting that both stable and transient overexpression of ECT in McA-RH7777 and CHO-K1 cells, cultured in the presence of physiological ethanolamine concentrations, does not have any effect on the rate of PtdEtn synthesis via the CDPethanolamine pathway. However, ECT overexpression does accelerate PtdEtn biosynthesis when cellular DAG levels are increased. The observation that PLC specifically stimulates PtdEtn synthesis in McA-ECT1 cells, in combination with the results showing no effect of hCEPT1 overexpression on the rate of PtdEtn synthesis, suggests that an increased availability of both DAG and CDPethanolamine is sufficient. Altogether, our results suggest that a simultaneous increase in the amount of CDPethanolamine and DAG is required to enhance the flux through the CDPethanolamine pathway, rather than that regulation of PtdEtn synthesis takes place at one particular enzymic step.

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