Channelling of intermediates in the biosynthesis of phosphatidylcholine and phosphatidylethanolamine in mammalian cells

Bellinda A. BLADERGROEN*, Math J. H. GEELEN*, A. Ch. Pulla REDDY*, Peter E. DECLERCQ† and Lambert M. G. VAN GOLDE¹

*Laboratory of Veterinary Biochemistry, Utrecht University, P.O. Box 80.176, Yalelaan 2, 3508 TD Utrecht, The Netherlands, and †Katholieke Universiteit Leuven, Instituut voor Farmaceutische Wetenschappen, Laboratorium voor Klinische Chemie, E. Van Evenstraat 4, B-3000 Leuven, Belgium

Previous studies with electroporabilized cells have suggested the occurrence of metabolic compartmentation and Ca^{2+} -dependent channelling of intermediates of phosphatidylcholine (PC) biosynthesis in C6 rat glioma cells. With a more accessible permeabilization technique, we investigated whether this is a more general phenomenon also occurring in other cell types and whether channelling is involved in phosphatidylethanolamine (PE) synthesis as well. C6 rat glioma cells, $\text{C}_3\text{H}_{10}\text{T}_2$ fibroblasts and rat hepatocytes were permeabilized with *Staphylococcus aureus* α -toxin, and the incorporation of the radiolabelled precursors choline, phosphocholine (P-choline), ethanolamine and phosphoethanolamine (P-EA) into PC and PE were measured both at high and low Ca^{2+} concentrations. In glioma cells, permeabilization at high Ca^{2+} concentration did not affect

$[^{14}\text{C}]$ choline or $[^{14}\text{C}]$ P-choline incorporation into PC. However, reduction of free Ca^{2+} in the medium from 1.8 mM to < 1 nM resulted in a dramatic increase in $[^{14}\text{C}]$ P-choline incorporation into permeabilized cells, whereas $[^{14}\text{C}]$ choline incorporation remained unaffected. Also, in fibroblasts, reduction of extracellular Ca^{2+} increased $[^{14}\text{C}]$ P-choline and $[^{14}\text{C}]$ P-EA incorporation into PC and PE respectively. In hepatocytes, a combination of α -toxin and low Ca^{2+} concentration severely impaired $[^{14}\text{C}]$ choline incorporation into PC. Therefore, α -toxin-permeabilized hepatocytes are not a good model in which to study channelling of intermediates in PC biosynthesis. In conclusion, our results indicate that channelling is involved in PC synthesis in glioma cells and fibroblasts. PE synthesis in fibroblasts is also at least partly dependent on channelling.

INTRODUCTION

In most mammalian tissues and cell types the major route of phosphatidylcholine (PC) synthesis is the CDP-choline pathway [1–3]. The corresponding pathway for phosphatidylethanolamine (PE) synthesis is the CDP-ethanolamine pathway. In culture, many animal cells do not require ethanolamine in the medium for growth and synthesize PE mainly by phosphatidylserine (PS) decarboxylation (reviewed in [1,3]). On the other hand, studies *in vivo* with labelled substrates [4] and experiments with freshly isolated hepatocytes [5] suggested that the CDP-ethanolamine pathway may be an important route for PE synthesis in eukaryotic cells *in vivo* when physiological concentrations of extracellular ethanolamine are present.

The enzymes involved in the cytidine pathways vary in their subcellular localization. The first enzyme, choline/ethanolamine kinase (EC 2.7.1.32), is a cytoplasmic protein, and the last enzymes, CDPcholine:1,2-diacylglycerol cholinephosphotransferase (EC 2.7.8.2) and CDPethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (EC 2.7.8.1), are membrane-bound enzymes embedded in the endoplasmic reticulum (ER). On the other hand, the key-regulatory enzymes CTP:choline-phosphate cytidylyltransferase (CT; EC 2.7.7.15) and CTP:ethanolamine-phosphate cytidylyltransferase (ET; EC 2.7.7.14) show a dual cellular distribution [1,2]. CT is present both as a soluble protein, acting as an inactive reservoir, and bound to cellular membranes, where it is active. One of the

mechanisms that regulates CT activity and the rate of PC biosynthesis is a reversible translocation of the enzyme between the soluble and particulate fractions of the cell [1,2].

From classical differential-centrifugation studies [6,7] and enzyme-release measurements from digitonin-permeabilized hepatocytes [6] ET has long been considered to be cytosolic. However, immunogold electron microscopy studies showed that ET is enriched in areas of the cell rich in rough ER and that it is located in the cytosol between the ER cisternae and in direct contact with the ER membrane [8]. This suggests that ET is able to associate weakly with subcellular organelles [9]. However, translocation between the cytoplasm and the ER membrane has never been reported for ET. It is not known how the intermediates of the cytidine pathways move from the cytoplasmic to the membrane-bound enzymes. The reaction intermediates are either free to diffuse between sequential enzymes or are channeled directly from one enzyme to the next through a functional cluster of metabolically related enzymes.

In this so-called channelling model, enzymes involved in a metabolic pathway are not randomly distributed throughout the cell, but are organized in a metabolon in which the reaction intermediates pass from one enzyme to the next without complete equilibration with other pools of those metabolites elsewhere in the cell [10,11]. Multienzyme complexes can be rather stable, but weaker specific interactions between soluble enzymes have been suggested too (reviewed in [10,12]). There are indications that multienzyme complexes, which had been regarded as soluble in

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; LDH, lactate dehydrogenase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PL, phospholipid; 3-GP, *sn*-glycerol 3-phosphate; p-NPP, *p*-nitrophenyl phosphate; ER, endoplasmic reticulum; CT, CTP: phosphocholine cytidylyltransferase; ET, CTP:ethanolamine-phosphate cytidylyltransferase; P-EA, phosphoethanolamine.

¹ To whom correspondence should be addressed (e-mail vangolde@pobox.accu.uu.nl).

differential-centrifugation studies, possibly associate with a variety of cellular components via weak and reversible interactions [13,14]. Evidence has been provided for compartmentation of intermediates in various metabolic pathways [10,15,16].

A functional organization of enzymes and substrates of the CDP-choline pathway has been described by George et al. [17]. In these studies, labelled phosphocholine (P-choline) and CDP-choline exogenously introduced into C6 rat glioma cells by electroporation did not incorporate into PC, whereas labelled choline was incorporated into PC in electroporated and control cells equally well. Addition of unlabelled P-choline or CDP-choline to the incubation mixture did not reduce incorporation of labelled choline into PC in permeabilized cells. Furthermore, hemicholinium-3, an inhibitor of choline transport, blocked incorporation of labelled choline into PC in control and electroporated cells. These findings indicated that P-choline and CDP-choline, made endogenously from choline that had entered the cell by carrier-mediated transport, did not equilibrate with exogenously added compounds, but were channelled directly towards PC. This view was supported by results suggesting a tight connection between specific carrier-mediated choline uptake and PC synthesis in Madin-Darby canine kidney cells [18]. Functional linkage between the individual steps of phospholipid (PL) synthesis has never been described for other PLs, including PE. However, the early observations by Sundler [19] that phosphoethanolamine (P-EA) formed from exogenous ethanolamine did not freely equilibrate with the endogenous liver P-EA pool already suggested that this metabolite may be specifically channelled towards ET.

Cellular structures, such as the cytoskeleton, may play a role in the formation of functional complexes of enzymes and substrates [13,20]. Because cytoskeletal structure and function are partly regulated by intracellular Ca^{2+} levels [21,22], the influence of Ca^{2+} on the functional organization of the CDP-choline pathway was investigated in C6 rat glioma cells [23]. Reduction of free Ca^{2+} in the medium resulted in a stimulation of the incorporation of exogenously added labelled P-choline and CDP-choline into PC. It was concluded that channeling of intermediates in the CDP-choline pathway and linkage of choline transport and PC synthesis were dependent on intracellular Ca^{2+} . The integrity of the channel was lost at low Ca^{2+} concentrations.

Since P-choline and CDP-choline are membrane-impermeant, cells have to be permeabilized to study incorporation of phosphorylated intermediates into PC. Permeabilization conditions should be used in which only the plasma membrane is permeabilized, leaving the structure and function of intracellular organelles and compartments intact. *Staphylococcus aureus* α -toxin, an amphiphilic polypeptide of 34 kDa, has been reported to permeabilize the plasma membrane without affecting the properties of intracellular membranes and associated enzymes [24,25]. α -Toxin forms a transmembrane channel through which small hydrophilic molecules with a molecular mass of less than 5000 Da can pass [24,26] and it has been used successfully for the study of lipid metabolism in hepatocytes [27–29].

The purpose of the present study was to investigate whether channeling of intermediates, as shown for PC synthesis [17,23] in C6 rat glioma cells, is a more general phenomenon also occurring in other cell types. For that reason, the incorporation of the labelled substrates P-choline and choline into PC was investigated in the presence of relatively high and low Ca^{2+} concentrations in C6 rat glioma cells, $\text{C}_3\text{H}_{10}\text{T}_2$ fibroblasts and hepatocytes. In order to study whether channeling is involved in PE synthesis as well, the incorporation of radiolabelled P-EA and ethanolamine into PE was measured in $\text{C}_3\text{H}_{10}\text{T}_2$ fibroblasts. We used α -toxin to permeabilize cells for the uptake of P-choline and P-EA.

MATERIALS AND METHODS

Materials

C6 rat glioma cells (CCL-107) and $\text{C}_3\text{H}_{10}\text{T}_2$ fibroblasts (CCL-226) were obtained from the American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were from Gibco. The radiochemicals [*methyl*- ^{14}C]choline chloride (55 mCi/mmol), phospho[*methyl*- ^{14}C]choline (56 mCi/mmol), L-[U- ^{14}C]glycerol 3-phosphate (3-GP) (152 mCi/mmol) and [2- ^{14}C]ethanolamine hydrochloride (57 mCi/mmol) were purchased from Amersham International, Amersham, Bucks., U.K. Phospho[2- ^{14}C]ethanolamine was enzymically prepared from [2- ^{14}C]ethanolamine with partially purified ethanolamine kinase as described earlier [30]. *S. aureus* α -toxin was prepared as described [31]. Ethanolamine was from Baker (Deventer, The Netherlands) and choline chloride, P-choline and P-EA from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade.

Cell culture of C6 rat glioma cells and $\text{C}_3\text{H}_{10}\text{T}_2$ fibroblasts

C6 rat glioma cells were grown in DMEM supplemented with 7% (v/v) FCS, 44 mM NaHCO_3 and penicillin (100 units/ml)/streptomycin (100 $\mu\text{g}/\text{ml}$). Cells were maintained under an atmosphere of 5% $\text{CO}_2/95\%$ air in a humidified incubator at 37 °C. $\text{C}_3\text{H}_{10}\text{T}_2$ fibroblasts were cultured in DMEM containing 7% (v/v) FCS, 10 mM Hepes, 2 mM NaHCO_3 and penicillin/streptomycin to a final concentration of 100 units/ml and 100 $\mu\text{g}/\text{ml}$ respectively. Cells were kept at a temperature of 37 °C.

All cells were subcultured once a week and the medium was changed every 2–3 days. For experiments, 2×10^6 glioma cells or 1×10^5 fibroblasts were seeded in 20 cm^2 dishes in 5 ml of the culture medium. Glioma cells were cultured 3 days before use and the medium was changed at day 2. Fibroblasts were cultured 7 days before use and the medium was changed at days 3 and 5.

Isolation and culture of rat hepatocytes

Hepatocytes were isolated from male Wistar rats according to the method of Seglen [32]. After isolation, cells were resuspended in DMEM supplemented with 7% (v/v) FCS, 10 mM Hepes, 2 mM NaHCO_3 and penicillin (100 units/ml)/streptomycin (100 $\mu\text{g}/\text{ml}$). A volume of 4.2 ml of a 0.5% (wet wt./v) cell suspension (2.5×10^6 cells) was seeded on 20 cm^2 collagen-coated dishes and incubated for 3.5–4 h at 37 °C before use.

Permeabilization and labelling of cultured cells

Labelling of glioma cells and hepatocytes with [^{14}C]choline or [^{14}C]P-choline

Monolayers of glioma cells or hepatocytes were rinsed with PBS and preincubated for 15 min at 37 °C in 2 ml of the above-mentioned culture medium without FCS, with or without 10 mM EGTA and supplemented with 10 mM *p*-nitrophenyl phosphate (p-NPP). With 10 mM EGTA the concentration of free Ca^{2+} in the medium was calculated to be $< 1 \text{ nM}$ [33]. After the preincubation, 0.5 $\mu\text{Ci}/\text{ml}$ [*methyl*- ^{14}C]choline (55 mCi/mmol) or 0.5 $\mu\text{Ci}/\text{ml}$ phospho[*methyl*- ^{14}C]choline (56 mCi/mmol) was added. The final concentration of either unlabelled choline or P-choline in the medium was 28 μM . In addition, 6.9 μg or 23 μg of α -toxin per dish was added to permeabilize hepatocytes or glioma cells respectively. Cells were incubated with the labelled substrates for 30 min (hepatocytes) or 60 min (glioma cells) at

37 °C. To stop the incubations the medium was removed and the cells were rinsed three times with ice-cold PBS containing 1 mM choline and 1 mM P-choline. Subsequently, 1 ml of methanol and 0.8 ml of water were added and cells were scraped off the dishes. Methanol and chloroform were added to make a mixture of chloroform/methanol/water of 1:2:0.8 (by vol.) and PLs were extracted and separated as described below.

Labelling of fibroblasts with [^{14}C]choline, [^{14}C]P-choline, [^{14}C]ethanolamine or [^{14}C]P-EA

Monolayers of fibroblasts were rinsed with PBS and subsequently incubated for 60 min at 37 °C in 2 ml of the aforementioned culture medium without FCS, with or without 10 mM EGTA and in the presence or absence of 23 μg of α -toxin. Incubations contained either [methyl- ^{14}C]choline (28 μM , 0.5 $\mu\text{Ci}/\text{ml}$), phospho[methyl- ^{14}C]choline (28 μM , 0.5 $\mu\text{Ci}/\text{ml}$) or a trace amount of [2- ^{14}C]ethanolamine or phospho[2- ^{14}C]ethanolamine (1 $\mu\text{Ci}/\text{ml}$, 57 mCi/mmol). At the end of the incubations the medium was removed and the cells were rinsed three times with PBS containing 1 mM choline plus 1 mM P-choline, in cases where cells had been incubated with radiolabelled PC precursors, and cells were rinsed with PBS containing 1 mM ethanolamine and 1 mM P-EA after incubation with radioactive PE precursors. Subsequently, the same procedure was followed as described for hepatocytes and glioma cells.

Labelling of glioma cells, hepatocytes and fibroblasts with [^{14}C]3-GP

Monolayers of cells were rinsed with PBS and incubated for 30 min (hepatocytes) or 60 min (glioma cells and fibroblasts) at 37 °C in 2 ml of an incubation medium consisting of the previously mentioned culture medium without FCS, supplemented with 3 % dextran T70, 2 % (w/v) purified BSA, 0.5 mM palmitate and 0.5 mM oleate complexed to the BSA, 4 mM MgCl_2 , 4 mM ATP and 0.1 mM coenzyme A. To the incubation medium was added 1 μCi (152 mCi/mmol) of L-[U- ^{14}C]3-GP per dish at a concentration of 1 mM. Furthermore, to permeabilize cells, 6.9 μg (hepatocytes) or 23 μg (glioma cells and fibroblasts) of α -toxin was added per dish.

The incubations were stopped by removal of the incubation medium and the cells were rinsed three times with ice-cold PBS. The cells were harvested by scraping into 1 ml of methanol together with 0.8 ml of water, and chloroform was added to make a chloroform/methanol/water mixture of 1:2:0.8 (by vol.), after which lipids were extracted.

Extraction of lipids and analysis by TLC

Lipids were extracted from the cells by the method of Bligh and Dyer [34]. PLs were separated by TLC on silica 60G plates in a solvent system of chloroform/methanol/water 65:25:4 (by vol.). For isolation of neutral lipids, light petroleum (boiling range, 40–60 °C): diethyl ether:acetic acid 80:20:2 (by vol.) was used as the solvent. Lipids were visualized with iodine vapour. Spots were identified by comparison with known standards. The silica was scraped off the plate and radioactivity was determined by liquid-scintillation counting.

Lactate dehydrogenase (LDH) assay

LDH activity was assayed in the media of control and permeabilized cells, as described by Vassault [35].

Protein determination

Protein was measured by the method of Lowry et al. [36] with BSA as a standard.

Statistical analysis

Although in cultured cells similar effects were observed in repeated experiments, the absolute values of incorporation of radiolabelled substrates into lipids, as well as the calculated percentages of effects, varied between experiments. Therefore, in the Figures and Tables, results from representative experiments are presented. These experiments have been reproduced one or two times and each individual experiment was performed in triplicate. Unless stated otherwise, results are given as the mean $\pm$ S. D. All statistical analyses were performed by unpaired Student's two-tailed *t*-test.

RESULTS

Permeabilization by α -toxin

Membrane-impermeant 3-GP has been shown to be taken up by hepatocytes after permeabilization with α -toxin [28,29]. To assess the amount of α -toxin necessary for optimal permeabilization of C6 rat glioma cells and $\text{C}_3\text{H}_{10}\text{T}_2$ fibroblasts, monolayer cultures of these cells were treated with increasing amounts of α -toxin for different incubation times and the incorporation of radioactive 3-GP into PLs and neutral lipids was measured. The incorporation of 3-GP into lipids increased both with the amount of α -toxin and with the incubation time. In the case of a 30 min incubation, maximal incorporation of 3-GP into lipids was achieved with 23 μg of α -toxin per dish, both for C6 rat glioma cells and fibroblasts (results not shown). Increasing the incubation time from 0 to 60 min clearly increased the incorporation of 3-GP into PLs and neutral lipids in both cell types (Table 1).

The viability of the cells was estimated by measuring leakage of the cytosolic protein (LDH) from the cells into the medium. Like the incorporation of 3-GP into lipids, LDH leakage also increased with increasing amount of α -toxin and with incubation time. Still, there was no substantial leakage of LDH from cells for up to 23 μg of α -toxin per dish, even after incubation for 60 min (results not shown). Hence, in subsequent experiments

Table 1 Permeabilization of glioma cells and fibroblasts with α -toxin

Glioma cells and fibroblasts were permeabilized with the indicated amounts of α -toxin and incubated with [^{14}C]3-GP (1 mM, 1 μCi per dish) for different incubation times. Incorporation of label into PLs and the neutral lipids diacylglycerol (DAG) and triacylglycerol (TAG) was determined as described in the Materials and methods section. All data are from a representative experiment performed in triplicate and each value represents the mean $\pm$ S.D.

Cells	Amount of α -toxin ($\mu\text{g}/\text{dish}$)	Incubation time (min)	Incorporation into lipid (pmol/mg of protein)		
			PL	DAG	TAG
Glioma cells	0	30	49 $\pm$ 5	15 $\pm$ 1	14 $\pm$ 4
	23	0	14 $\pm$ 0	9 $\pm$ 3	11 $\pm$ 4
	23	15	80 $\pm$ 14	39 $\pm$ 4	11 $\pm$ 1
	23	30	264 $\pm$ 72	104 $\pm$ 17	56 $\pm$ 20
	23	60	981 $\pm$ 121	384 $\pm$ 69	905 $\pm$ 204
Fibroblasts	0	60	400 $\pm$ 240	40 $\pm$ 11	189 $\pm$ 77
	23	0	64 $\pm$ 16	26 $\pm$ 3	43 $\pm$ 1
	23	15	679 $\pm$ 61	39 $\pm$ 6	34 $\pm$ 12
	23	30	1241 $\pm$ 20	176 $\pm$ 99	1409 $\pm$ 208
	23	60	2062 $\pm$ 154	423 $\pm$ 158	4926 $\pm$ 301

Figure 1 Incorporation of [^{14}C]choline into PC in control and α -toxin-permeabilized hepatocytes at high and low concentration of free Ca^{2+} in the medium

Hepatocytes were permeabilized with α -toxin and incubated for 30 min with [^{14}C]choline (28 μM , 1 μCi) in the absence or presence of 10 mM EGTA. This resulted in concentrations of free Ca^{2+} in the media of 1.8 mM and < 1 nM respectively. Control cells were not permeabilized. PLs were extracted, separated by TLC and the amount of radioactivity incorporated into PC was determined. Values are the means $\pm$ S.D. of one representative experiment performed in triplicate. This experiment has been repeated once giving similar results.

glioma cells and fibroblasts were permeabilized for 60 min with 23 μg of α -toxin per dish. Under these conditions maximal permeabilization was achieved and the viability of the cells was minimally affected.

Because incorporation of radioactive 3-GP into lipids was measured in the presence of fatty acids, BSA was added to the medium to obtain suitable molar ratios of fatty acid to BSA. BSA has been shown to be essential for the stability of permeabilized cells [28]. However, in the channeling experiments, cells were incubated in the absence of BSA. The stability of the permeabilized cells in these experiments was checked by measuring LDH leakage, which was found not to exceed 2.5 % of total cellular LDH activity.

Isolated rat hepatocytes are not suitable as model cells to study channeling of intermediates in PC and PE synthesis

One way to verify the hypothesis of George et al. [17], i.e. the channeling of intermediates in the CDP-pathways for PC and PE synthesis, is to measure the incorporation of labelled substrates into these PLs in the presence of relatively high and low Ca^{2+} concentrations. If channeling is involved, P-choline or P-EA added extracellularly should not be able to enter the channel and incorporate into PC or PE at high Ca^{2+} concentration, in contrast to choline and ethanolamine, which can be incorporated into these PLs under all conditions. Only upon reduction of Ca^{2+} levels, are P-choline and P-EA then expected to be incorporated into PC and PE, as it was found that the integrity of the channel was lost at low Ca^{2+} concentration [23].

Since the CDP-pathways are important routes for PC and PE biosynthesis, particularly in liver cells, we initiated our channeling studies in hepatocytes. However, isolated hepatocytes permeabilized with α -toxin turned out not to be a suitable model to study channeling of intermediates in PC and PE synthesis for two reasons. First, P-choline and P-EA should not be incorporated into PC and PE in intact cells. However, when

Figure 2 Incorporation of [^{14}C]choline and [^{14}C]P-choline into PC in control and α -toxin-permeabilized glioma cells at high and low concentrations of free Ca^{2+} in the medium

Glioma cells were permeabilized with α -toxin and incubated for 60 min with either [^{14}C]choline (28 μM , 1 μCi) or [^{14}C]P-choline (28 μM , 1 μCi) in the absence or presence of 10 mM EGTA. This resulted in concentrations of free Ca^{2+} in the media of 1.8 mM and < 1 nM respectively. Control cells were not permeabilized. PLs were extracted, separated by TLC and the amount of radioactivity incorporated into PC was determined. Values are expressed as the means $\pm$ S.D. of a representative experiment performed in triplicate. This experiment has been repeated once with comparable results.

hepatocytes were incubated with P-choline or P-EA, a significant amount of radioactivity was detected in PC and PE (results not shown), strongly suggesting that P-choline and P-EA had been degraded extracellularly to choline and ethanolamine, probably by an alkaline phosphatase present on the external side of the plasma membrane. Pretreatment of hepatocytes with p-NPP, a substrate of alkaline phosphatase, did in fact inhibit the incorporation of P-choline into PC almost completely (86 %), whereas the incorporation of P-EA into PE was much less reduced (45 %). p-NPP had no effect on the incorporation of choline or ethanolamine.

Second, and more importantly, the integrity of the metabolic pathway in these cells was seriously affected by use of α -toxin and EGTA. The results presented in Figure 1 demonstrate that, in hepatocytes, the incorporation of [^{14}C]choline into PC was markedly reduced (55 %) by treatment with α -toxin. Addition of EGTA to the medium to diminish the Ca^{2+} concentration also decreased the incorporation of radioactive choline into PC, and combined treatment with EGTA and α -toxin resulted in a severe impairment of hepatic PC synthesis from choline (Figure 1).

Incorporation of [^{14}C]choline and [^{14}C]P-choline into PC in control and permeabilized glioma cells at high and low Ca^{2+} concentrations

The results in Figure 2 show that, in glioma cells, [^{14}C]choline was readily incorporated into PC to the same extent in control and α -toxin-treated cells, both at high and low Ca^{2+} concentrations, indicating that the integrity of the metabolic pathway in this cell line was not disturbed by treatment with α -toxin and/or EGTA. The incorporation of [^{14}C]P-choline into PC in control cells was negligible. If channeling is involved in PC synthesis and the integrity of the channel is intact (at high Ca^{2+} concentration), [^{14}C]P-choline cannot enter the channel and should therefore not be incorporated into PC, even when it can enter the cell. Indeed, in permeabilized cells, the amount of [^{14}C]P-choline incorporation into PC at 1.8 mM Ca^{2+} was not significantly different ($P < 0.5$)

Figure 3 Incorporation of [¹⁴C]choline, [¹⁴C]P-choline, [¹⁴C]ethanolamine and [¹⁴C]P-EA into PC and PE in control and α -toxin-permeabilized fibroblasts at high and low concentrations of free Ca^{2+} in the media

Fibroblasts were permeabilized with α -toxin and incubated for 60 min with (A) [¹⁴C]choline (28 μM , 1 μCi) or [¹⁴C]P-choline (28 μM , 1 μCi) or (B) [¹⁴C]ethanolamine (2 μCi , 57 mCi/mmol) or [¹⁴C]P-EA (2 μCi , 57 mCi/mmol) in the absence or presence of 10 mM EGTA. This resulted in concentrations of free Ca^{2+} in the media of 1.8 mM and < 1 nM respectively. Control cells were not treated with α -toxin. PLs were extracted, separated by TLC and the amount of radioactivity incorporated into PC (A) or PE (B) was measured. Values are presented as the means $\pm$ S.D. of triplicate incubations of a representative experiment. This experiment has been repeated once for PC synthesis and twice for PE synthesis with similar results.

from the [¹⁴C]P-choline incorporation in control cells, and was still very low compared with the incorporation of [¹⁴C]choline. In contrast, when permeabilized cells were incubated with < 1 nM Ca^{2+} , incorporation of [¹⁴C]P-choline increased significantly to 24-fold the incorporation of [¹⁴C]P-choline at high Ca^{2+} concentration ($P < 0.001$).

Incorporation of radiolabelled precursors into PC and PE in control and permeabilized fibroblasts at high and low Ca^{2+} concentrations

It can be seen in Figure 3(A) that the incorporation of [¹⁴C]choline into PC in intact fibroblasts was not affected by changes in Ca^{2+} concentration, but was stimulated 1.5-fold when cells were permeabilized with α -toxin, both at high and low Ca^{2+} concentrations. There was hardly any incorporation of [¹⁴C]P-choline into PC in non-permeabilized fibroblasts. In permeabilized fibroblasts incubated at 1.8 mM Ca^{2+} , incorporation of [¹⁴C]P-choline into PC was enhanced to 811 % of that in control cells. Lowering the Ca^{2+} concentration resulted in a further 2.7-fold

increase to 2159 % of the amount of [¹⁴C]P-choline incorporated into PC ($P < 0.001$) in control cells.

As described above, degradation of P-EA by hepatocytes could only be partially inhibited by p-NPP. Also, in glioma cells, P-EA was incorporated into PE, and this could be inhibited only 12 % by p-NPP (results not shown). In intact fibroblasts, there was negligible incorporation of P-EA into PE (Figure 3). Because degradation of labelled P-EA resulted in a substantial amount of radioactivity being incorporated into PE in glioma cells and hepatocytes, but not into fibroblasts, involvement of channeling in PE synthesis could only be studied in fibroblasts. The incorporation pattern of labelled ethanolamine and P-EA into PE resembled that of radioactive choline and P-choline into PC in fibroblasts. Like the incorporation of [¹⁴C]choline into PC (Figure 3A), incorporation of [¹⁴C]ethanolamine into PE was also stimulated by treatment with α -toxin. This stimulation was 1.58-fold at high Ca^{2+} concentration and 1.85-fold at low Ca^{2+} concentration (Figure 3B). EGTA reduced the rate of PE synthesis from ethanolamine (29 %), but did not influence the incorporation of radioactive P-EA into PE. Similarly to [¹⁴C]ethanolamine incorporation into PE, the amount of [¹⁴C]P-EA incorporated into PE at high Ca^{2+} concentration was 1.9 times higher in permeabilized than in control cells. However, at low Ca^{2+} concentration there was an extra increase in [¹⁴C]P-EA incorporation from 14 ± 1 pmol/mg of protein at 1.8 mM Ca^{2+} to 39 ± 4 pmol/mg at < 1 nM Ca^{2+} , as can be seen in Figure 3(B).

DISCUSSION

Although channeling of substrates through functional complexes of enzymes has been proposed for various metabolic pathways (reviewed in [10]), only one example of channeling involved in phospholipid synthesis has been reported [17,23]. Our study supports the idea that the channeling process in PL biosynthesis is a more general phenomenon, occurring in different cell types. This process is not only involved in PC biosynthesis in glioma cells, but PC synthesis as well as PE synthesis in fibroblasts also appear to be, at least partly, dependent on channeling of endogenous intermediates from one enzyme of the pathway to the other. As channeling implies that different pools of substrates in a cell are used for different purposes and are not readily exchangeable, we postulate that the intermediates of PC and PE biosynthesis are not randomly distributed throughout the cell in glioma cells and fibroblasts. These conclusions are based on the following observations. (1) Incorporation of exogenous P-choline into PC is not significantly higher in permeabilized glioma cells than in control cells (Figure 2), indicating that in spite of the ability of P-choline to enter the cell, it was not available for PC synthesis. (2) Only after addition of EGTA, which lowers the Ca^{2+} concentration, and in that way abolishes the channeling process, did the incorporation of exogenous [¹⁴C]P-choline into PC in glioma cells increase dramatically (Figure 2). (3) Although incorporation of exogenous P-choline into PC was higher in permeabilized fibroblasts than in control cells, lowering the Ca^{2+} concentration by EGTA clearly increased further the incorporation of exogenously added [¹⁴C]P-choline into PC (Figure 3). This means that exogenous P-choline could only be maximally used for PC synthesis after destruction of the integrity of the putative channel, indicating that PC synthesis was at least partly dependent on channeling. (4) As for ethanolamine, incorporation of trace levels of exogenous P-EA into PE was somewhat higher in permeabilized fibroblasts than in control cells, but in contrast to ethanolamine incorporation into PE, in the absence of Ca^{2+} there was clearly an extra enhanced incorporation of exogenous

P-EA into PE in permeabilized cells when compared with control cells. So, also in this case, the incorporation pattern was as expected for involvement of channeling of intermediates.

The results in glioma cells and fibroblasts support and extend the results reported by George et al. [17,23]. Both in our studies and those of George et al. the flux of intermediates through the CDP-choline pathway appeared to be regulated by intracellular Ca^{2+} levels. The effect of removal of Ca^{2+} on [^{14}C]P-choline incorporation into PC was more pronounced in α -toxin-permeabilized glioma cells than in electroporated glioma cells.

Incorporation of choline and ethanolamine into PC and PE respectively, showed that the integrity of the metabolic pathways for PC and PE biosynthesis is still intact after permeabilization of glioma cells and fibroblasts with α -toxin. In hepatocytes, however, incorporation of choline into PC decreased by 55% after treatment with α -toxin. So, although these cells are still able to incorporate choline into PC, the functional organization of the pathway is affected by the permeabilization procedure. This may originate from changes in the organization of the pathway, which might have been induced during the isolation of the hepatocytes. Therefore, these cells are less suitable as a model system to study channeling of intermediates in PL metabolism.

Although permeabilization by α -toxin is a more accessible method than electroporation, both treatments appeared to be suitable to investigate the involvement of channeling in PL synthesis. In contrast, when HeLa cells were permeabilized with digitonin [37] no involvement of channeling could be measured, since CDP-choline was incorporated into PC. In addition to the possibility that no channeling is involved in the CDP-choline pathway in HeLa cells, digitonin might disturb the organization of the pathway for PC biosynthesis. It needs to be recognized that weak interactions between the members of multienzyme complexes that are not covalently linked or firmly attached to membranes can be easily disrupted [10,13].

Channeling of intermediates in PL biosynthesis is in line with an increasing amount of evidence for the existence of multienzyme complexes and topodynamic regulation of enzymes and substrates of various metabolic pathways (reviewed in [10,11,14]). α -Toxin has been used to study *in situ* channeling of intermediates of the urea cycle in hepatocytes [16]. The physiological significance of channeling is exemplified by an *in vivo* study about channeling of fumarate and succinate by Sumegi et al. [38]. In these systems channeling occurs by direct or indirect transfer of substrates between metabolically related enzymes [11]. The multienzyme complexes involved can be either stable or transient [12,15]. As a consequence of channeling, pools of substrates are compartmentalized and each pool is destined for a different metabolic pathway. For example, in the hamster heart [39] and Madin-Darby canine kidney cells [18] the existence of separate pools of choline has been suggested, and our studies confirmed those of George et al. [17] that P-choline formed in the CDP-choline pathway does not equilibrate with other P-choline pools in the cytoplasm.

Although compartmentation of substrates is a more general phenomenon, the underlying mechanism may differ from multienzyme complex formation. For example, in the case of lipid metabolism, PE derived from ethanolamine via the CDP-pathway and PE derived from PS decarboxylation are differently metabolized in the ethanolamine cycle [40], and even newly made PE is used differently from pre-existing PE [41]. PC synthesized by methylation of PE derived from PS decarboxylation, rather than from the CDP-ethanolamine pathway, is preferentially used for secretion into lipoproteins [42]. In these cases, compartmentalization of PL may be based on differences in fatty acid

molecular species, synthesis in different membranes or organelles, or metabolic activity of enzymes that preferentially recognize substrates from a specific biosynthetic origin. However, none of these mechanisms can fully explain compartmentation, and the exact mechanism responsible for distinguishing different pools of the same substrate remains to be elucidated.

The concept of channeling has implications for the regulation of PC and PE synthesis. With regard to the nuclear localization of CT [43,44] and the activation by translocation to membranes, the implication of channeling for the regulation of CT activity is not yet very clear. However, because of the ability to translocate, CT may function as the connection between the soluble enzyme choline kinase and the membrane-bound CDPcholine:1,2-diacylglycerol cholinephosphotransferase. Similarly, a potential key role in metabolic channeling has been suggested for ET [9].

As the pathways for PC and PE synthesis contain both soluble and membrane-bound enzymes, it can be hypothesized that the cytoskeleton may facilitate multienzyme complex formation and channeling of metabolites between cytoplasmic and membrane active sites.

In this respect, the association between actin and CT reported by Hunt et al. [45] may be in favour of this hypothesis. Since the organization of the cytoskeleton is mediated by Ca^{2+} , changes in Ca^{2+} levels might influence the organization of the pathways for PC and PE synthesis. Indeed, lowering the Ca^{2+} concentration resulted in a dramatic increase in incorporation of exogenous phosphorylated precursors into PC and PE in permeabilized glioma cells and fibroblasts, suggesting that under these circumstances no channeling was involved in PC and PE synthesis (Figures 2, 3A and 3B). But despite this Ca^{2+} dependence, the role of the cytoskeleton in the channeling process remains uncertain, since several agents that disrupt cytoskeletal structures do not influence PL synthesis [46]. Only cytochalasin B has been reported to inhibit PC and PE synthesis in glioma cells, but this effect was dependent on intracellular Ca^{2+} levels and was probably not exerted by perturbation of cytoskeletal filaments [46]. It cannot be excluded that the effects of Ca^{2+} on PC and PE synthesis are exerted via other mechanisms, e.g. inhibition of the activities of the enzymes of these pathways [33].

In conclusion, the findings from this study with α -toxin-permeabilized cells suggest that channeling of intermediates is involved in the CDP-choline pathway for PC biosynthesis in C6 rat glioma cells and $\text{C}_3\text{H}_{10}\text{T}_{\frac{1}{2}}$ fibroblasts and in the CDP-ethanolamine pathway for PE synthesis in $\text{C}_3\text{H}_{10}\text{T}_{\frac{1}{2}}$ fibroblasts. Although the channeling and compartmentation of intermediates appears to be dependent on the presence of intracellular Ca^{2+} , the precise mechanism responsible for these phenomena remains to be clarified. However, the widespread occurrence of compartmentation and channeling of intermediates between enzymes of metabolic pathways demonstrate that these processes are important for the regulation of cellular metabolism and deserve further research.

This study was supported by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organization for Scientific Research.

REFERENCES

- 1 Kent, C. (1995) *Annu. Rev. Biochem.* **64**, 315–343
- 2 Vance, D. E. (1996) in *Biochemistry of Lipids, Lipoproteins and Membranes* (Vance, D. E. and Vance, J., eds.), pp. 153–181, Elsevier Science Publishers B. V., Amsterdam
- 3 Tijburg, L. B. M., Geelen, M. J. H. and Van Golde, L. M. G. (1989) *Biochim. Biophys. Acta* **1004**, 1–19
- 4 Arthur, G. and Page, L. (1991) *Biochem. J.* **273**, 121–125
- 5 Tijburg, L. B. M., Geelen, M. J. H. and Van Golde, L. M. G. (1989) *Biochem. Biophys. Res. Commun.* **160**, 1275–1280

- 6 Vermeulen, P. S., Tijburg, L. B. M., Geelen, M. J. H. and Van Golde, L. M. G. (1993) *J. Biol. Chem.* **268**, 7458–7464
- 7 Schneider, W. C., Fiscus, W. G. and Lawler, J. A. B. (1966) *Anal. Biochem.* **14**, 121–134
- 8 Van Hellemond, J. J., Slot, J. W., Geelen, M. J. H., Van Golde, L. M. G. and Vermeulen, P. S. (1994) *J. Biol. Chem.* **269**, 15415–15418
- 9 Bladergroen, B. A. and Van Golde, L. M. G. (1997) *Biochim. Biophys. Acta* **1348**, 91–99
- 10 Srere, P. A. (1987) *Annu. Rev. Biochem.* **56**, 89–124
- 11 Spivey, H. O. and Merz, J. M. (1989) *BioEssays* **10**, 127–130
- 12 Srivastava, D. K. and Bernhard, S. A. (1986) *Science* **234**, 1081–1086
- 13 Masters, C. J. (1981) *CRC Crit. Rev. Biochem.* **11**, 105–143
- 14 Kaprelyants, A. S. (1988) *Trends Biochem. Sci.* **13**, 43–46
- 15 Ushiroyama, T., Fukushima, T., Styre, J. D. and Spivey, H. O. (1992) *Curr. Top. Cell Regul.* **33**, 291–307
- 16 Cheung, C.-W., Cohen, N. S. and Raijman, L. (1989) *J. Biol. Chem.* **264**, 4038–4044
- 17 George, T. P., Morash, S. C., Cook, H. W., Byers, D. M., Palmer, F. B. S. C. and Spence, M. W. (1989) *Biochim. Biophys. Acta* **1004**, 283–291
- 18 Zlatkine, P., Leroy, C., Moll, G. and Le Grimmellec, C. (1996) *Biochem. J.* **315**, 983–987
- 19 Sundler, R. (1973) *Biochim. Biophys. Acta* **306**, 218–226
- 20 Masters, C. J. (1984) *Trends Biochem. Sci.* **9**, 223
- 21 Weeds, A. (1982) *Nature (London)* **296**, 811–816
- 22 Bretscher, A. and Weber, K. (1980) *Cell* **20**, 839–847
- 23 George, T. P., Cook, H. W., Byers, D. M., Palmer, F. B. and Spence, M. W. (1991) *J. Biol. Chem.* **266**, 12419–12423
- 24 McEwen, B. F. and Arion, W. J. (1985) *J. Cell Biol.* **100**, 1922–1929
- 25 Bader, M.-F., Thiersé, D., Aunis, D., Ahnert-Hilger, G. and Gratzl, M. (1986) *J. Biol. Chem.* **261**, 5777–5783
- 26 Füssle, R., Bhakdi, S., Szegoleit, A., Tranumjensen, J., Kranz, T. and Wellensiek, H.-J. (1981) *J. Cell Biol.* **91**, 83–94
- 27 Mayorek, N. and Bar-Tana, J. (1989) *J. Biol. Chem.* **264**, 4450–4455
- 28 Stals, H. K., Mannaerts, G. P. and Declercq, P. E. (1992) *Biochem. J.* **283**, 719–725
- 29 Stals, H. K., Top, W. and Declercq, P. E. (1994) *FEBS Lett.* **343**, 99–102
- 30 Tijburg, L. B. M., Vermeulen, P. S. and Van Golde, L. M. G. (1992) *Methods Enzymol.* **209**, 258–263
- 31 Harshman, S., Sugg, N. and Cassidy, P. (1988) *Methods Enzymol.* **165**, 3–7
- 32 Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
- 33 De Groote, K. (1997) Ph.D. Thesis, University of Leuven, Belgium, pp. 57–66
- 34 Bligh, E. G. and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- 35 Vassault, A. (1983) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Bergmeyer, J. and Graßl, M., eds.), pp. 118–126, Verlag Chemie, Weinheim
- 36 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 37 Lim, P., Cornell, R. and Vance, D. E. (1986) *Biochem. Cell Biol.* **64**, 692–698
- 38 Sumegi, B., Sherry, A. D. and Malloy, C. R. (1990) *Biochemistry* **29**, 9106–9110
- 39 Zelinski, T. A., Savard, J. D., Man, R. Y. K. and Choy, P. C. (1980) *J. Biol. Chem.* **255**, 11423–11428
- 40 Shiao, Y.-J. and Vance, J. E. (1995) *Biochem. J.* **310**, 673–679
- 41 Samborski, R. W., Ridgway, N. D. and Vance, D. E. (1990) *J. Biol. Chem.* **265**, 18322–18329
- 42 Vance, J. E. (1988) *Biochim. Biophys. Acta* **963**, 70–81
- 43 Houweling, M., Cui, Z., Anfuso, C. D., Bussière, M., Chen, M. H. and Vance, D. E. (1996) *Eur. J. Cell Biol.* **69**, 55–63
- 44 Watkins, J. D. and Kent, C. (1992) *J. Biol. Chem.* **267**, 5686–5692
- 45 Hunt, A. N., Normand, C. S. and Postle, A. D. (1990) *Biochim. Biophys. Acta* **1043**, 19–26
- 46 George, T. P., Cook, H. W., Byers, D. M., Palmer, F. B. and Spence, M. W. (1991) *Biochim. Biophys. Acta* **1084**, 185–193