Cellular Responses to Excess Phospholipid*

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**Phosphatidylcholine (PtdCho) is the major membrane phospholipid in mammalian cells, and its synthesis is controlled by the activity of CDP:phosphocholine cytidylyltransferase (CCT). Enforced CCT expression accelerated the rate of PtdCho synthesis. However, the amount of cellular PtdCho did not increase as a result of the turnover of both the choline and glycerol components of PtdCho. Metabolic labeling experiments demonstrated that cells compensated for elevated CCT activity by the degradation of PtdCho to glycerophosphocholine (GPC). Phospholipase D-mediated PtdCho hydrolysis and phospholipase formation were unaffected. Most of the GPC produced in response to excess phospholipid production was secreted into the medium. Cells also degraded the excess membrane PtdCho to GPC when phospholipid formation was increased by exposure to exogenous lysophosphatidylcholine or lysophosphatidylethanolamine. The replacement of the acyl moiety at the 1-position of PtdCho with a non-hydrolyzable alkyl moiety prevented degradation to GPC. Accumulation of alkylacyl-PtdCho was associated with the inhibition of cell proliferation, demonstrating that alternative pathways of degradation will not substitute. GPC formation was blocked by bromoeno lactone, implicating the calcium-independent phospholipase A₂ as a key participant in the response to excess phospholipid. Owing to the fact that PtdCho is biosynthetically converted to PtdEtn, excess PtdCho resulted in overproduction and exit of GPE as well as GPC. Thus, general membrane phospholipid homeostasis is achieved by a balance between the opposing activities of CCT and phospholipase A₂.

PtdCho is the major membrane phospholipid in mammalian cells, and regulation of its biosynthesis and turnover is critical to maintaining membrane structure and function. CCT is a key regulatory step in PtdCho biosynthesis in mammalian cells (for reviews, see Refs. 1 and 2). CCT activity is regulated primarily through the interaction of lipid modulators with its amphipathic α-helical domain, which stimulate (1) or inhibit (3–5) activity by modulating the Kₘ for CTP (6). CCT is also a phosphoprotein (1) that is phosphorylated on multiple serine residues within its carboxyl-terminal domain (7). The presence of the highly phosphorylated carboxyl-terminal domain attenuates enzyme activation by lipid modulators (8).

Enforced CCTα (9) or CCTβ (10) expression results in an acceleration of PtdCho biosynthesis measured by the incorporation of radiolabeled Cho into PtdCho. However, transient overexpression of COS cells with CCTα did not lead to a significant increase in the amount of PtdCho per cell despite the increased biosynthetic rate (9). In transiently transfected COS cells, there was a concomitant increase in GPC and PCho, suggesting that elevated PtdCho degradation compensated for increased synthesis (9). An indication of a balanced relationship between PtdCho synthesis and degradation was also found in ras-transformed cell lines. Transformation with ras oncogenes accelerated both PtdCho synthesis (11–13) and PtdCho turnover (14–17). Elevated pools of two PtdCho breakdown products, PCho and GPC, were observed in these experiments (11–13, 15–18), although the activation of Cho kinase also makes a significant contribution to the PCho pool (12). Another example of balanced PtdCho synthesis and degradation was found during the G₁ stage of the cell cycle (19). PtdCho synthesis increased in G₁; however, the total phospholipid mass was maintained as a result of a concomitant increase in PtdCho degradation.

Taken together, these experiments suggest the existence of a system that coordinates PtdCho biosynthesis and degradation to maintain a constant membrane phospholipid content. The goals of this study were to determine if the coupling between CCT activity and PtdCho turnover is a manifestation of a general mechanism for phospholipid homeostasis and to investigate the phospholipase(s) responsible for regulated PtdCho degradation.

**EXPERIMENTAL PROCEDURES**

**Materials**—[5,6,8,9,11,12,14,15-3H]Arachidonic acid (specific activity 100 Ci/mmol), n-[4-3H]butanol (specific activity 10 Ci/mmol), [1-3H]ethanolamine (specific activity 30 Ci/mmol), [methyl-3H]LPC (specific activity 80 Ci/mmol), [methyl-3H]Cho (specific activity 85 Ci/mmol), and phosphomethyl-3H]Choline (specific activity 56 mCi/mmol) were from American Radiolabeled Chemicals. Lysophosphatidylcholine (PC) and phosphatidylcholine (PC) were from Cayman Chemical. [2-3H]Glycerol (specific activity 200 mCi/mmol) was from NEN Life Science Products. Doxycycline and choline metabolite standards were from Sigma. All other materials were reagent grade or better.

**Cell Lines and Growth Conditions**—The CCL.12 cell line was established in our laboratory by stable transfection of HeLa Tet-On™ cells (CLONTECH) using the pTRE vector carrying a CCTα cDNA (20). Vector control cells (CCT.00) were isolated following stable transfection of HeLa Tet-On™ cells using empty pTRE vector and selection in medium with hygromycin. Cells were routinely grown in Dulbecco’s modified Eagle’s medium (BioWhittaker) containing 10% fetal bovine serum (Summit Biotechnology) at 37 °C in 5% CO₂, 95% air. Induction
of CCTα overexpression in CCT.12 cells did not alter the proliferative rate of the cultures or the final cell density at 100% confluence. Cell density was determined using a hemocytometer, and viability was determined by trypan blue exclusion.

**CCT Activity**—Cells were washed twice with phosphate-buffered saline on ice and harvested by scraping into 1 ml of the same buffer followed by centrifugation. The cell pellets were resuspended in lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2% aprotinin, 1 μg/ml leupeptin, 50 mM NaF, 100 mM Na3VO4) and sonicated three times for 30 s in a cup-horn sonicator (model W225-K, Heat Systems-Ultrasonics, Inc.) at 50% duty cycle and maintained at 4 °C. The standard CCT activity assay contained an aliquot of the cell lysate mixed with 125 mM bis-Tris-HCl, pH 6.5, 0.5 μCi of phospho[3H]Cho, 1 mM phosphoethanolamine, 2 mM CTP, 20 mM MgCl2, 50 μM PtdCho/18:1 (1:1) in a final volume of 40 μl. The incubations were for 10 min at 37 °C and were stopped by placing the samples on ice and adding 5 μl of 0.5 M EDTA. CDP-[3H]Cho formation was determined by thin layer chromatography (21). CCT-specific activity was calculated from a series of assays that were linear with time and protein. Protein concentration was determined according to the method of Bradford (22) using γ-globulin as a standard.

**Phospholipase D Activity**—Cells were grown in 100-mm dishes to 20% confluence and either incubated with doxycycline (2 μg/ml) for 48 h or left untreated. At 48 h, phospholipase D activity was assayed by measuring phosphatidylbutanol formation (transphosphatidylation) following the addition of 0.5% [3H]butanol (10 μCi/ml) to the cultures and incubation for 30 min at 37 °C. After harvesting the cells, the phospholipids were extracted as described below and phosphatidylbutanol was quantitated following fractionation of the radiolabeled lipids by thin layer chromatography on Silica Gel H layers developed with chloroform/methanol/acetone. The distribution of the radiolabel among the PtdCho or PtdEtn precursors was determined by scintillation counting. The volume of the media samples was determined according to the method of Bradford using PtdCho to construct a standard curve. As a positive control, cells were treated with 100 μM phorbol myristate acetate 60 min prior to addition of [3H]butanol.

**Phospholipid Mass Determination**—Cells were grown to 80% confluence, washed twice with phosphate-buffered saline, and harvested by scraping followed by centrifugation. Aliquots of a cell suspension were counted with a hemocytometer, and the bulk of the cells were collected by centrifugation for quantitation of the phospholipid mass. Cell pellets were extracted using the method of Bligh and Dyer (23), and the phospholipid in the lower phase was quantitated as described by Charles and Stewart (24) using PtdCho to construct a standard curve.

**Metabolic Labeling**—Cells were grown in 60-mm dishes to 80% confluence and pretreated with 2 μg/ml doxycycline for 18 h. Labeled [3H]Cho was added, and the medium was changed at the start of the experiment with fresh medium. After 10 min, the label was removed from the cells, and unlabeled LPC (50 μM) was added, and the incubations were continued in the presence (●) or absence (○) of 2 μM phosphatidylserine. Neutral lipids (triacylglycerol, diacylglycerol, and water-soluble phospholipid precursors or metabolites) were confirmed with standards.

**Drug Treatments**—Brefeldin A (1 mg/ml) and monensin (1 μM) stocks were prepared in ethanol. Cells were incubated with doxycycline for 48 h and radiolabeled with 5 μCi/ml [methyll-3H]Cho for the last 2 h prior to addition of the indicated drug. The label remained in the cells for the duration of the drug treatment. Brefeldin A (5 μg/ml) was added, and both cells and media were harvested on ice at 15, 30, 60, 120, and 240 min. Alternatively, monensin (10 μM) was added, and both cells and media were harvested on ice at 1, 2, and 15 h. In a separate series of experiments, cells were prelabeled with 2 μCi/ml [methyll-3H]LPC for 2 h, the label was removed from the cells, and unlabeled LPC (50 μM) was added, and the incubations were continued in the presence (●) or absence (○) of 2 μM phosphatidylserine. Neutral lipids (triacylglycerol, diacylglycerol, and water-soluble phospholipid precursors or metabolites) were confirmed with standards.

**Confocal Fluorescent Microscopy**—Cells were grown on Lab-Tek®II Chamber Slides™ to 60% confluence and treated with 5 μg/ml brefeldin A for 15 min or left untreated. Cells were washed and fixed with 3.7% formaldehyde for 20 min and permeabilized with 0.2% Triton X-100 for 10 min. Nonspecific binding was blocked with 1% bovine serum albumin in phosphate-buffered saline for 60 min. Cells were treated with 50 μg/ml Texas Red™-conjugated wheat germ agglutinin as a marker for the Golgi apparatus (25). Cells were visualized after staining using a Leica DM-IRBE confocal microscope equipped with a Leica TCS NT scanning laser.

**RESULTS**

**PtdCho Biosynthesis in HeLa Cells Overexpressing CCTα**—The human HeLa cell line, CCT.12 (20), overexpressed the rodent CCTα in response to doxycycline (Fig. 1). The maximum level of CCTα overexpression was about 25-fold higher than the endogenous activity prior to addition of 2 μg/ml doxycycline. Both [methyll-3H]Cho (Fig. 2A) and [3H]glycerol (Fig. 2B) were incorporated into PtdCho at a higher rate following CCTα overexpression. The rate of [3H]glycerol incorporation into PtdCho was about 2.2 times higher when the CCTα level was maximal. These data showed that the rate of PtdCho biosynthesis increased in response to elevated CCT activity and verified the observations made in COS cells transiently trans-
Enforced CCTα expression stimulated the incorporation of Cho and glycerol into PtdCho but did not increase the cellular phospholipid mass. Panel A, enhanced radiolabeling of CDP-choline and PtdCho in cells overexpressing CCTα. CCT.12 or vector control cell cultures were grown to a final density of 1.5 x 10^6 cells/60-mm dish in the presence (+) or absence (−) of 2 μg/ml doxycycline for 48 h. [methyl-^3H]Cho (5 μCi/ml) was then added to duplicate cultures for 6 h, and the cells were harvested. Extraction and quantitation of the radiolabeled choline metabolites by thin layer chromatography were performed as described under “Experimental Procedures.” Panel B, overexpression of CCTα stimulates incorporation of [3H]glycerol into PtdCho. CCT.12 cells were incubated with (●) or without (○) 2 μg/ml doxycycline for 48 h. Cells reached a density of 5.4 x 10^6/dish, and [3H]glycerol (5 μCi/ml) was added to duplicate cultures. Cells were harvested at the indicated times thereafter. Extraction, fractionation, and quantitation of the radiolabeled glycerolipids were performed as described under “Experimental Procedures.” Panel C, cellular content of phospholipid remains constant following CCTα overexpression. CCT.12 cell cultures were incubated with (+) or without (−) 2 μg/ml doxycycline for 48 h and compared with vector control cultures. Phospholipid mass was quantitated in duplicate dishes as described under “Experimental Procedures” and normalized to cell number. The results from two independent experiments were combined. Standard errors were calculated from quadruplicate determinations.

Phosphatidylbutanol formation was linear over 60 min and increased as a function of increasing butanol concentration in the medium up to 0.7%. The data showed that phospholipid D activity did not increase in response to enhanced PtdCho biosynthesis following induction of CCTα activity with doxycycline. Phorbol myristate acetate at 100 μM was added as a positive control to stimulate phospholipase D activity under the same conditions (Fig. 3). These results demonstrated the existence of a typical phospholipase D response in the HeLa cells and confirmed the absence of elevated phospholipase D activity in CCT overexpression cells. Diacylglycerol and phosphocholine are the products of phospholipase C degradation, and diacylglycerol can be converted to phosphatidic acid or triacylglycerol in vivo. Overexpression of CCT in cells labeled with [3H]glycerol did not result in enhanced formation of diacylglycerol or triacylglycerol (data not shown). These experiments were consistent with the conclusion that neither phospholipase D nor C was involved in PtdCho turnover in response to accelerated PtdCho biosynthesis.

The route for PtdCho breakdown was determined by radiolabeling the PtdCho pool in cells with a tracer amount of [3H-methyl]LPC. This approach avoided the complications in the interpretation of results that arise from high background labeling of the PCho and Cho precursor pools when using [3H-methyl]Cho to prelabel PtdCho. [3H-methyl]LPC was readily acylated, and [3H]PtdCho constituted >98% of the total cellular radiolabel (data not shown). The [3H]PtdCho was subsequently degraded, and two radiolabeled metabolic products, PCho and GPC, were detected inside the cells (Fig. 4). GPC release from PtdCho was dramatically elevated in cells induced to overexpress CCTα (Fig. 4A). In contrast, PCho was not affected by CCTα overproduction (Fig. 4B). The lack of an effect on PCho release provided an internal control that underscored the specificity of the GPC response to enhanced PtdCho biosynthesis. The accumulation of GPC suggested that GPC was not readily metabolized to Cho or PCho.

GPC Is Released into the Medium—The amount of GPC
produced as a result of CCTα overexpression was measured in the cells and in the medium. HeLa CCT.12 cells (treated with doxycycline for 48 h) were labeled with [3H-methyl]Cho for the last 24 h, the label was removed, and the radiolabeled GPC was measured inside and outside the cells 24 h later in the continued absence or presence of doxycycline (Fig. 5). Almost all of the radiolabeled GPC (~90%) accumulated in the medium of cells under conditions of either CCTα overexpression or basal activity (Fig. 5). Radiolabeled Cho and PCho were also identified in the medium, but it is difficult to determine whether these metabolites were derived directly from PtdCho or from the degradation of a portion of the extracellular GPC. These data indicated that the GPC arising from the degradation of PtdCho accumulated in both inside and outside the cells.

The possibility that GPC was released by a secretion mechanism was investigated using either brefeldin A or monensin to block secretion. Brefeldin A block secretion by causing the redistribution of the Golgi apparatus into the endoplasmic reticulum (26), and monensin inhibits the function of the Golgi and lysosomes through the disruption of H⁺, Na⁺, and K⁺ gradients across cellular membranes (27). The efficacy of 5 μg/ml brefeldin A was verified by immunofluorescent staining of the cells using Texas Red™-conjugated wheat germ agglutinin. After 15 min of incubation with the drug, the juxtanuclear staining of the Golgi apparatus completely disappeared (data not shown). Cells were incubated with monensin (10 μM) for times ranging from 2 to 14 h prior to the measurement of GPC release. HeLa CCT.12 cells were induced to overexpress CCTα and prelabeled prior to the addition of either inhibitor. The accumulation of radiolabeled GPC in the medium was not reduced by the drugs (data not shown). These results showed that GPC did not exit the cells by a Golgi-mediated pathway.

**iPLA₂ Mediates the GPC Response**—The generation of GPC in response to phospholipid overproduction suggested the involvement of a regulated PLA activity. An earlier report from our laboratory concluded that the cytoplasmic PLA₂ was a growth factor-responsive activity and probably did not mediate the regulated phospholipid turnover observed during G₁ phase of the cell cycle (28). The group VI iPLA₂ enzymes are not responsive to extracellular stimuli and are speculated to have basal housekeeping activity that is visible through its involvement in phospholipid remodeling (29). Therefore, we tested whether the iPLA₂ was a participant in the pathway of GPC formation in response to PtdCho overproduction (Fig. 6). BEL is a specific inhibitor of iPLA₂ and is diagnostic for the identification of iPLA₂ in contrast to other cellular phospholipases A₂ (29). The efficacy of BEL was first examined in the HeLa cells by monitoring the incorporation of [3H]arachidonic acid into phospholipid by the remodeling pathway (Fig. 6A). BEL inhibition of arachidonic acid incorporation was dose-dependent, and incorporation was reduced to 50% of control cells without inhibitor. These data are in complete agreement with the results reported previously for the BEL inhibition of arachidonic acid incorporation mediated by iPLA₂ in intact cells (17). We then investigated the effect of 25 μM BEL on GPC production in...
cells by radiolabeling with \[^{3}H\]-methyl\[LPC. Overproduction of PtdCho was induced by doxycycline-dependent overexpression of CCTα in the CCT.12 cells 48 h prior to the start of the experiment. The formation of GPC in the doxycycline-treated cultures was dramatically reduced by BEL (Fig. 6B). In a separate experiment shown in Fig. 6C, BEL reduced the level of GPC produced in doxycycline-treated cultures and also reduced the GPC levels in control cultures in a dose-dependent manner. In these experiments total GPC (cells + medium) was quantitated. These results clearly pointed to a role for the iPLA₂ in regulating the membrane phospholipid content, either by initiating cleavage of the sn-2 acyl moiety of PtdCho, which would be further degraded by a lysophospholipase activity, or perhaps by hydrolyzing both the sn-1 and sn-2 acyl chains to yield GPC.

**Fig. 5. GPC exits from cells.** CCT.12 cultures were preincubated with or without doxycycline (2 \(\mu\)g/ml) as indicated for 48 h. [methyl\[^{3}H\]]Cho (5 \(\muCi/ml\)) was added for the latter 24 h of the preincubation. The radioactive medium was removed at 48 h, the cells were washed, fresh medium without radiolabel was added, and the cultures were incubated for an additional 24 h. Both cells and medium were harvested and extracted from duplicate cultures. Cho-derived metabolites were fractionated by thin layer chromatography and analyzed as described under "Experimental Procedures." Standard errors were calculated from quadruplicate determinations and from two independent experiments.

**Fig. 6. BEL inhibition of GPC formation.** Panel A, BEL was added to CCT.12 cultures at the indicated concentrations for 30 min prior to the addition of [5,6,8,9,11,12,14,15\[^{3}H\]]arachidonic acid (1 \(\muCi/ml\)) at the start of the experiment. The cells were washed, harvested, and extracted after 2 h of incubation with the radiolabel, and the incorporation of \[^{3}H\]arachidonic acid into phospholipids was determined as described under "Experimental Procedures." Panel B, CCT.12 cells were grown for 48 h with (●) or without (□) 2 \(\mu\)g/ml doxycycline. BEL (25 \(\mu\)M) was added to the indicated cultures (---) for 30 min prior to the addition of [methyl\[^{3}H\]]LPC (1 \(\muCi/ml\)) to start of the experiment. At the indicated times, duplicate cultures were harvested and the cells and media extracted. Radiolabeled GPC was quantitated following thin layer chromatography as described under "Experimental Procedures." Panel C. CCT.12 cells were grown for 48 h with (●) or without (□) 2 \(\mu\)g/ml doxycycline. BEL was added at the indicated concentrations to the cultures for 30 min prior to the addition of [methyl\[^{3}H\]]LPC (1 \(\muCi/ml\)) at the start of the experiment. Duplicate cultures were harvested and extracted after 2 h of further incubation. Radiolabeled GPC was isolated from cells and medium and quantitated following thin layer chromatography as described under "Experimental Procedures." Standard errors were calculated from quadruplicate determinations in a representative experiment, which was independently performed twice, except in panel B, where the error bars represent the range of duplicate determinations in a single experiment.
Fig. 7. Synthesis of excess PtdCho by acylation of LPC stimulates GPC production. CCT.12 cultures were incubated with [methyl-3H]LPC (2 μCi/ml) for 2 h. The medium containing the radiolabeled LPC was removed, the cells were washed, and fresh medium either with no additions (○) or with 50 μM unlabeled LPC (●) was added at the start of the experiment. Cells (8 × 10⁶/60-mm dish) and medium were harvested from duplicate dishes at the indicated times thereafter. Extraction and fractionation of radiolabeled LPC-derived metabolites was performed as described under “Experimental Procedures.” Standard errors were calculated from quadruplicate determinations and two independent experiments.

Fig. 8. Synthesis of excess PtdCho or PtdEtn stimulates GPC and GPE formation. Panel A, overloading with different phospholipids stimulates GPC levels. CCT.12 cultures were incubated with [methyl-3H]LPC (1 μCi/ml) for 2 h. The medium containing the radiolabeled LPC was removed, the cells were washed, and fresh medium either with no additions or with 50 μM unlabeled lysophospholipid was added as indicated. Cells (1 × 10⁶/60-mm dish) and medium were harvested 6 h after addition of the fresh medium. The radiolabeled GPC in the cells and medium was isolated and quantitated as described under “Experimental Procedures.” Panel B, PtdEtn degradation to GPE responds to phospholipid overload. CCT.12 cultures were incubated with [1-3H]ethanolamine (5 μCi/ml) for 2 h. The medium containing the radiolabeled ethanolamine was removed, the cells were washed, and fresh medium either without lysophospholipid or with 50 μM unlabeled lysophospholipid was added as indicated. Cells (8 × 10⁶/60-mm dish) and medium were harvested 6 h after addition of the fresh medium. The radiolabeled GPE in the cells and medium was isolated and quantitated as described under “Experimental Procedures.” Standard errors were calculated from quadruplicate determinations of representative experiments for each panel. Experiments in each panel were independently performed twice.

PtdCho and that PtdEtn degradation to GPE was a component of a general cellular response to excess membrane phospholipid.

Consequences of PtdCho Overload—The identification of GPC (or GPE) as the major breakdown product in response to excess phospholipid suggested that removal of the acyl moieties from PtdCho was an essential event in the cellular regulatory response. LysoPAF differs from LPC in that an alkyl group rather than an acyl group occupies the sn-1 position of the glycerol backbone, and the ether linkage is resistant to cleavage by phospholipases. HeLa CCT.12 cells were treated with doxycycline for 48 h to overexpress CCTα and synthesize PtdCho at a stimulated rate. Control, untreated cells and induced cells were incubated with a tracer amount of [3H-alkyl]lysoPAF alone or in the presence of 10 μM unlabeled lysoPAF for 3 days (Fig. 9A). Ninety percent of the radiolabel was incorporated into cellular lipids within 24 h and remained associated with the cellular lipids for the entire experiment. At 10 h, alklyacyl-PtdCho was the major metabolite and was converted to alklyacyl- or alklydiacylglycerol, indicating the loss of the phosphocholine headgroup. Radiolabeled water-soluble degradation products were not detected, and a very small level of lysoPAF (<1%) was detected throughout the experiment (data not shown). These results showed that the alklyacyl-phospholipids accumulated in the cell membranes and suggested that perhaps the cells would have a greater phospholipid content. However, when the cellular phospholipid mass was determined (described under “Experimental Procedures”) following incubation with up to 30 μM lysoPAF, the phospholipid did not significantly increase above the normal value of ~80 μg/10⁶ cells (data not shown).

Previous work suggested that lysoPAF (>20 μM) had a detrimental effect on cell growth (30). Therefore, we investigated whether the accumulation of alkyl phospholipids reduced the rate of cell proliferation during extended incubation. Cells were incubated for 48 h during exponential growth with two doses up to 50 μM of either LPC or lysoPAF at 24-h intervals (Fig. 9C). The total number of cells counted after treatments with lysoPAF were dramatically reduced compared with the control cultures treated with LPC. Viability was high (≥85%) at lyso-phospholipid concentrations <50 μM for both LPC and LPAF. Cell growth was only moderately affected by the 48-h incubation with 2 doses of 50 μM LPC (1.21 × 10⁶ total cells). Viability was slightly reduced but remained high (70%) following treatment at the 50 μM dosage of either LPC or LPAF. The growth rate and viability count for the 50 μM LPC condition after a single dose and 24 h of culture was identical to that for the control condition.

The data suggested that accumulation of the cellular alkyl phospholipids would correlate with the inhibition of cell proliferation. This point was tested directly by determining the growth rates of cultures incubated with lysoPAF up to 30 μM added daily (Fig. 9D). The number of viable cells was reduced as a function of increasing lysoPAF concentration; however, significant cell death (30%) was only evident after 72 h of treatment and the third dose of 30 μM lysoPAF. These data demonstrated that accumulation of the alkyl-phospholipids interfered with cell proliferation. Flow cytometric analysis of arrested cultures did not reveal that the cells accumulated at a specific phase of cell cycle progression (data not shown), and TdT-dependent TUNEL assays of arrested cultures did not indicate the presence of the specific DNA fragmentation associated with apoptosis (data not shown).

**DISCUSSION**

A large body of work has identified CCT as a key regulator of PtdCho synthesis. Both the positive and negative regulation of
CCT activity by lipid modulators and phosphorylation exert control over phospholipid formation (1, 2, 6, 8); however, regulation of biosynthesis at the CCT step is not the only mechanism to control PtdCho content. Enforced expression of CCTα significantly accelerates PtdCho biosynthesis, but does not increase cellular phospholipid content (9) (Fig. 2). This is surprising when one considers that PtdCho constitutes 72% of the phospholipid in HeLa cells (data not shown). Another method to deregulate PtdCho synthesis is to overload cells with exogenous LPC. LPC is a potent inhibitor of CCT but circumvents the de novo PtdCho biosynthetic pathway by being directly acylated to form PtdCho (3). Cells balance increased PtdCho production with an increased rate of phospholipid degradation to GPC, thereby maintaining the size of the PtdCho pool. The exit and accumulation of GPC in the culture medium suggests that the glycerol and choline moieties of PtdCho are not readily reutilized during exponential growth of cultured cells.

The perturbation of cell physiology with enforced CCT expression and overloading with exogenous LPC reveals the regulated decylation of PtdCho to GPC as a key process in membrane phospholipid homeostasis. PtdCho is the predominant membrane phospholipid and the precursor to the other two major phospholipids, sphingomyelin (31) and PtdEtn (32), in cultured animal cells. The metabolic interconnection between the major phospholipids means that the synthesis and degradation of PtdEtn (and likely sphingomyelin) reflects that of PtdCho. Overloading the PtdCho pool with exogenous lysoPAF (a molecular species of LPC) also increases the PtdEtn pool as alkylacyl-PtdCho is converted to alkylacyl-PtdEtn (Fig. 2B). Excess PtdCho can lead not only to stimulated GPC production but also to excess PtdEtn, and this is reflected by the increased production of GPE (Fig. 2B). Conversely, the stimulated production of GPC in response to overloading cells with LPE (Fig. 8A) can be explained if PtdCho accumulates as a result of a block in its conversion to PtdEtn. Cells maintain a specific profile of phospholipids when PtdCho synthesis is accelerated (data not shown) by degrading PtdEtn to GPE in addition to degrading PtdCho to GPC. Cells also maintain a specific phospholipid profile when PtdCho synthesis is inhibited by antineoplastic ether lipids such as ET-18-OCH₃ (5). The data reveal the general nature of the cellular response to excess phospholipid and suggest that the maintenance of the PtdCho pool is synonymous with the preservation of total membrane phospholipid content and composition in cultured cells. The rapid and virtually complete uptake of exogenous phospholipid (Fig. 9A) suggests that cells must have a physiological mechanism to adjust to variations in serum lipid composition while maintaining their typical membrane composition and function.

Our finding that loading cells with non-hydrolyzable alkyl-phospholipid analogs inhibits proliferation suggests that the regulated decylation of membrane phospholipids is critical to normal cell proliferation. These data also demonstrate that alternative pathways of degradation will not substitute. The doubling of membrane phospholipid mass is a periodic, cell cycle-regulated event that occurs during S phase (19). Phospholipid turnover is high during the G₁ phase, and the accumulation of phospholipid in S phase correlates with the abrupt cessation of phospholipid turnover at the G₂/M boundary (19). Thus, the down-regulation of the excess phospholipid response is likely instrumental in permitting the accumulation of phospholipid mass in preparation for cell division.

The inhibition of the excess phospholipid response by BEL implicates a calcium-independent (group VI) phospholipase A₂ as an initiator of the cellular response to excess phospholipid.

**FIG. 9. Inability to degrade excess PtdCho to GPC inhibits cellular proliferation.** Panel A, alkylacylphospholipids accumulate in cells. CCT.12 cultures were incubated with lysoPAF (1-O-[3H]octadecyl-glycerophosphocholine, 5 μCi/ml) with () or without (○) 10 μm unlabeled lysoPAF added at the start of the experiment. At indicated times, cells and medium were harvested and extracted, and the radioactivity in each compartment was quantitated as described under “Experimental Procedures.” Standard errors were calculated from quadruplicate determinations in a representative experiment. The experiments with or without 10 μm LPAF were performed independently. Panel B, cell-associated radiolabeled lysoPAF and its metabolites in the lipid fraction from the experiment described in panel A, were fractionated and quantitated by thin-layer chromatography as described under “Experimental Procedures.” Lipid profiles were calculated using the mean data points from panel A. Standard errors were not calculated. Panel C, lysoPAF, lysophospholipid at the indicated concentrations was added to cell cultures in two doses 24 h apart during exponential growth. Cells were harvested and counted 24 h after the second addition of lysoPAF. Viability was ≥95% in all cultures except following treatment with 50 μm lysoPAF. Standard errors were calculated from quadruplicate determinations in a representative experiment. The experiment was independently performed twice. Panel D, lysoPAF was added to duplicate cultures at the indicated concentrations daily on days 1, 2, and 3. Cells were harvested and counted on day 4. Viability was ≥90% except on day 4 following treatment with the 30 μm dose, where it dropped to 70%. The ranges of data from duplicate determinations of the cell number were smaller than the points on the graph. The data were from a representative experiment.
The identification of GPC and GPE as the primary products generated in response to phospholipid overproduction points to a phospholipase \( A_2 \) as the initiating event. There are several classes of phospholipase \( A_2 \) enzymes, and understanding their physiological roles and regulatory properties is an area of active investigation (29, 33, 34). BEL is an irreversible mechanism-based inhibitor of iPLA\(_2\) and is known not to effect the activities of other phospholipase \( A_2 \) enzymes (29, 35, 36). Thus, BEL inhibition of PtdCho deacylation points directly to iPLA\(_2\) as a phospholipase that is critical to the response of cells to excess phospholipid. BEL is not a completely selective iPLA\(_2\) inhibitor since it also blocks the activity of \( M_g \)-dependent phosphatidic acid phosphatase (37), some proteases (38), and perhaps other enzymes as well. However, the degradation of PtdCho to GPC in response to the acylation of exogenous LPC does not involve phosphatidic acid phosphatase. There are a number of lysophospholipases in cells (39), and one or more of these enzymes may participate in the removal of the second fatty acid to form GPC. BEL also decreased the formation of GPC in cells that were not stimulated to overproduce phospholipids, indicating that iPLA\(_2\)-dependent phospholipid breakdown via GPC formation occurs under normal physiological circumstances. However, BEL did not eliminate GPC formation in cells, indicating that there is another mechanism or process that impacts on the steady-state levels of this metabolite. Cellular GPC is an organic osmolyte that is modulated in response to changes in medium osmolality and the activity of a zinc-dependent phosphodiesterase (40–42). Thus, the portion of GPC metabolism that is not affected by BEL may be part of an osmotic regulatory system that operates independently of phospholipid homeostasis.

The ubiquitous occurrence of iPLA\(_2\) and the absence of regulation by phosphorylation or calcium are consistent with a fundamental, housekeeping role for this class of enzymes in cell physiology as opposed to an involvement in cell signaling and eicosanoid release (29). The iPLA\(_2\) enzymes are universally expressed, and iPLA\(_{2\beta}\) are being purified and characterized from an increasing number of sources (43–52). The participation of iPLA\(_2\) in the remodeling of membrane phospholipids is the most clearly defined function for this enzyme (29). This process is an ongoing cycle of phospholipid deacylation followed by the reacylation of the resulting lysophospholipid that generates a different phospholipid molecular species with new biophysical properties (17, 53). This remodeling cycle is a major route for the incorporation of arachidonic acid into phospholipids, and the inhibition of this process by BEL (17) and iPLA\(_2\) antisense RNA (54) support a role for iPLA\(_2\) in the deacylation component of this cycle. Experiments with BEL have also implicated iPLA\(_2\) as the phospholipase responsible for the stimulated release of fatty acids from glycerophospholipids during Fas-mediated apoptosis (55). Our results point to a determinant role for iPLA\(_2\) in the deacylation reactions involved in phospholipid homeostasis and reveal another manifestation of the housekeeping functions of iPLA\(_2\). One of the major challenges for the future is understanding how iPLA\(_2\) activity is modulated. The potential role of oligomerization of iPLA\(_2\) (43, 48) and the regulated expression of splice variants that act as dominant negative inhibitors (50) are two possible mechanisms that warrant evaluation.

The second step in GPC formation is the action of a lysophospholipase on the LPC generated by iPLA\(_2\). The lysophospholipases are grouped into two categories based on their molecular size, and representatives of both classes have been cloned. The low molecular mass (24.7 kDa) lysophospholipase is similar to bacterial esterases in that it has a GXSG motif associated with the active sites of serine esterases (39, 56). The high molecular mass (60 kDa) lysophospholipases are also widely distributed in tissues. Like the iPLA\(_2\), the 60-kDa lysophospholipase cloned from rat liver possesses ankyrin repeats (57). The ubiquitous occurrence of iPLA\(_2\) and the absence of regulation by phosphorylation or calcium are consistent with a fundamental, housekeeping role for this class of enzymes in cell physiology as opposed to an involvement in cell signaling and eicosanoid release (29). The iPLA\(_2\) enzymes are universally expressed, and iPLA\(_{2\beta}\) are being purified and characterized from an increasing number of sources (43–52). The participation of iPLA\(_2\) in the remodeling of membrane phospholipids is the most clearly defined function for this enzyme (29). This process is an ongoing cycle of phospholipid deacylation followed by the reacylation of the resulting lysophospholipid that generates a different phospholipid molecular species with new biophysical properties (17, 53). This remodeling cycle is a major route for the incorporation of arachidonic acid into phospholipids, and the inhibition of this process by BEL (17) and iPLA\(_2\) antisense RNA (54) support a role for iPLA\(_2\) in the deacylation component of this cycle. Experiments with BEL have also implicated iPLA\(_2\) as the phospholipase responsible for the stimulated release of fatty acids from glycerophospholipids during Fas-mediated apoptosis (55). Our results point to a determinant role for iPLA\(_2\) in the deacylation reactions involved in phospholipid homeostasis and reveal another manifestation of the housekeeping functions of iPLA\(_2\). One of the major challenges for the future is understanding how iPLA\(_2\) activity is modulated. The potential role of oligomerization of iPLA\(_2\) (43, 48) and the regulated expression of splice variants that act as dominant negative inhibitors (50) are two possible mechanisms that warrant evaluation.

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Cellular Responses to Excess Phospholipid
