CTP:phosphocholine cytidylyltransferase inhibition by ceramide via PKC-α, p38 MAPK, cPLA₂, and 5-lipoxygenase

SHANJANA AWASTHI, JEEVALATHA VIVEKANANDA, VIBHUDUTTA AWASTHI, DOLPHIN SMITH, and RICHARD J. KING

Department of Physiology, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78229-3900

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CPT:phosphocholine cytidylyltransferase inhibition by ceramide via PKC-α, p38 MAPK, cPLA₂, and 5-lipoxygenase. Am J Physiol Lung Cell Mol Physiol 281: L108–L118, 2001.—In a companion paper (Vivekananda J, Smith D, and King R). Am J Physiol Lung Cell Mol Physiol 281: L98–L107, 2001, we demonstrated that ceramide-induced inhibition of CT activity was dependent on 5-lipoxygenase but that its actions were likely exerted through a metabolite of sphingomyelin. In this paper, we explore the signaling pathway employed by TNF-α using C₂ ceramide as a cell-penetrating sphingolipid representative of the metabolites induced by TNF-α. We found that in H441 cells, as reported in other cell types, cytosolic phospholipase A₂ (cPLA₂) is activated by TNF-α. We also observed that the activating action of C₂ ceramide on CT requires protein kinase C-α, p38 mitogen-activated protein kinase, and cPLA₂. The actions of C₂ ceramide on CT activity can be duplicated by adding 2 μM lysoPC to these cells. Furthermore, we found that the effects of C₂ ceramide are dependent on 5-lipoxygenase but that cyclooxygenase II is unimportant. We hypothesize that CT activity is inhibited by the lysoPC generated as a consequence of the activation of cPLA₂ by protein kinase C-α and p38 mitogen-activated protein kinase. The product of the activation of cPLA₂, arachidonic acid, is a substrate for the synthesis of leukotrienes, which raise intracellular Ca²⁺ levels and complete the activation of cPLA₂.
were from Calbiochem (La Jolla, CA). [γ-32P]ATP, [32P]orthophosphoric acid, t-α-1-palmitoyl-2-arachidonyl-(arachidonyl-1-4)-PC, and [methy1-14C]phosphorylcholine were obtained from NEN (Boston, MA). NS-398 and nortrihydrylauric acid (NDGA) were from BIOMOL (Plymouth Meeting, PA).

**Plasmid constructs.** Kinase-inactive PKC-α (lysin in ATP-binding site mutated to methionine) (7) cloned into pTB701/HA was obtained from Dr. Robert V. Farese (University of South Florida College of Medicine, Tampa, FL). Anti-sense (dominant negative) and wild-type PKC-α cloned into pRSV (43) were obtained from Dr. Robert I. Glazer (Georgetown University Medical Center, Washington, DC) (2).

**Cell culture.** H441 cells (human lung adenocarcinoma cells) were obtained at the 50th passage from the ATCC. The cells were maintained in McCoy’s 5A medium containing 5% fetal bovine serum (FBS) and 50 μg/ml of gentamicin sulfate at 37°C in a 5% CO2-95% air humidified atmosphere. Before all experiments, the medium was changed to McCoy’s 5A medium without FBS and the cells were conditioned overnight. All experiments were conducted in medium without FBS.

**Dephosphorylation.** To dephosphorylate the transfected cells, the medium was overlaid on the cells. After 48 h of incubation, the transfected cells were trypsinized and selected with 500 μg/ml of Geneticin (G418, Life Technologies, Gaithersburg, MD). The cells were washed twice with serum- and gentamicin-free McCoy’s 5A medium, and the transfection selection with Geneticin (G418, Life Technologies). The medium (13). Briefly, cells were grown to 80–90% confluence and then treated with C2 ceramide (10 ng/ml) as described in Ref. 53. For investigating the effect of inhibitors of kinases, the cells were pretreated for 30 min to 1 h with 1 and 2 mM NS-398 [an inhibitor of COX-2 (18)]; 1 and 5 mM BEL [an inhibitor of Ca2+ influx (53)]; 0.1 and 1 μM BAFF (32); 5, 10, and 20 μM of Protein A agarose at 0–4°C. The immunoprecipitates were washed and resuspended in 25 μl of 50 mM Tris-HCl, pH 7.5, and 5 μM mercaptoethanol. To start the reaction, kinase assay buffer (see above) was added to the immunoprecipitated PKC, and the method thereafter was essentially identical to the one described above. The MBP 4–14 peptide was used for measuring activity.

**MAPK phosphorylation (activation).** Cells seeded in 60-mm tissue culture dishes were treated with TNF-α (10 ng/ml) for various time periods. The cells were washed twice with PBS and lysed in 200 μl of Tris-HEPES buffer (pH 7.4) containing 25 mM Tris, 1% Igepal, 150 mM NaCl, 50 mM NaF, 200 μM sodium orthovanadate, and 1 mM PMSF. The lysates were probe-sonicated for 5 s and centrifuged at 14,000 rpm for 10 min to remove the cell debris. Lysate protein was separated on 10% SDS-polyacrylamide, and the gels were detected with an enhanced chemiluminescence kit. The proteins of interest were detected with an enhanced chemiluminescence kit. The MAPK antibodies were obtained from New England BioLabs (Beverly, MA) and were used at 1:1,000 dilution in Tris-buffered saline containing 5% BSA and 0.2% Tween 20. The anti-rabbit IgG-horseradish peroxidase secondary antibody was from Santa Cruz Biotechnology and was used at 1:5,000 dilution with blocking buffer (10% milk in Tris-buffered saline-2% Tween 20).

**CT activity.** We used the method of Ansel and Chojnacki (3). Cells (0.2–0.3 × 10^6) were seeded on 60-mm petri dishes, grown to 40–50% confluence, and then treated with C2 ceramide (10 μM) as described in Ref. 53. For investigating the effect of inhibitors of kinases, the cells were pretreated for 30 min to 1 h with 1 and 2 μM NS-398 [an inhibitor of COX-2 (18)]; 5, 10, and 20 μM BEL [an inhibitor of Ca2+-insensitive PLA2 (2); type VI (1, 6)]; 0.1, 1.0, and 10 μM A23187 (22). Cells were washed twice with normal saline, harvested, and homogenized in 200 μl of homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol, 175 μg/ml of PMSF, 5 mM sodium fluoride, and 0.1 mM sodium orthovanadate). Homogenates were sonicated in a water bath sonicator for 20 min and centrifuged (14,000 rpm) for 5 min to isolate the membrane fraction. Membrane pellets were then homogenized in 200 μl of the homogenization buffer.
For the activity assay, 20 μg of membrane protein were mixed with assay buffer (400 mM Tris–HCl, pH 7.5, 30 mM magnesium acetate, 10 mM CTP, and 50,000 cpm of [14C]phosphocholine) and incubated at 37°C for 1 h. To stop the reaction, the tubes were immersed in a boiling water bath for 2 min. Radioactive CDPcholine was extracted on acid-washed charcoal, and the charcoal was washed three times with water before extraction with formic acid for scintillating counting.

**CT phosphorylation.** Cells were labeled with [32P]orthophosphate (100 μCi/60-mm dish) for 18 h in phosphate-free DMEM. The labeled cells were stimulated with TNF-α (100 ng/ml) or ceramide (10 μg/ml). After specified times (0, 1, 3, and 5 h), the cells were washed with ice-cold PBS and lysed in Tris–HCl buffer (pH 7.4) containing 25 mM Tris, 1% Igepal, 150 mM NaCl, 50 mM NaF, 200 μM sodium orthovanadate, and 1 mM PMSF. One hundred micrograms of lysate protein were mixed with 10 μl of a polyclonal antiserum against human CT and incubated overnight at 5°C. The antiserum was a mixture of antibodies developed to the peptide sequences acetyl-AKVNARKRKEAPGC-amide and acetyl-NEKKYHLQERVD-KVC-amide (QC8, Hopkinton, MA). The immune complex was captured on protein A agarose beads, and the bound radioactivity was eluted in SDS-PAGE loading buffer (75 μl). A portion of the elute was directly counted by scintillation counting, and the rest was loaded on a 10% polyacrylamide gel for further separation and autoradiography.

In some experiments, we measured the incorporation of [32P]orthophosphate into serine, threonine, and tyrosine after hydrolysis of the CT protein and the separation of amino acids by HPLC. Cells were labeled overnight with [32P]orthophosphate. The next morning, the cells were stimulated with TNF-α or the control vehicle. At varying times, the cells were washed, harvested, and homogenized. The lysate was immunoprecipitated with the antibody to CT, and the immunoprecipitate was hydrolyzed in a sealed, evacuated tube with 6 N HCl for 3 h. The samples were then lyophilized, mixed with carrier phosphotyrosine, phosphoserine, and phosphothreonine, and derivatized with 9-fluorenylmethoxycarbonyl (FMOC). Excess FMOC was removed by solvent partition with pentane, the sample was filtered, and the mixture was eluted on a column of Partisil 10 SAX (Phenomenex, Torrance, CA) with an isocratic solution of 55% methanol, 1% tetrahydrofuran, and 10 mM potassium phosphate, pH 3.9. The column was monitored by on-line fluorescence, and the peaks corresponding to phosphotyrosine, phosphoserine, and phosphothreonine were eluted, dried, and counted.

**PLA2 activity.** We measured PLA2 activity in the cytosol of the cells by the hydrolysis of a substrate of radioactive sn-2-arachidonoyl PC. Cells were seeded in 60-mm plates and allowed to attach for 4–6 h in 1.5 ml of 10% FBS-McCoy's 5A medium. The cells were then washed twice with serum-free McCoy's medium and equilibrated overnight (17–18 h) in 1.5 ml of serum-free McCoy's medium. After treatment with 10 μM C2 ceramide, the cells were washed twice with ice-cold PBS and harvested in 100 μl of a buffer containing 250 mM sucrose, 2 mM EDTA, 50 mM HEPES, pH 7.5, 20 μg/ml of leupeptin, and 1 mg/ml of PMSF. The cells were sonicated and centrifuged at 45,000 rpm for 30 min. The cytosolic fraction was used for the assay of cPLA2. The substrate, 1-α-1-palmitoyl-2-arachidonoyl-[arachidonoyl-1-14C]PC (NEN), was suspended in DMSO with vigorous vortexing. Five microliters of the substrate were mixed with 10 μl of CaCl2 solution and 10 μl of BSA solution in a glass tube (final assay concentration of the substrate was 6 μM). The assay was initiated by adding 30–40 μg of the protein and incubating for 30–60 min at 37°C. The products of the reaction were separated by silicic acid TLC, and the radioactive fatty acid was spotted-scraped and counted.
**RESULTS**

**TNF-α and C2 ceramide increase PKC-α activity in H441 cells.** TNF-α (38, 44) and ceramide (24, 40) have been shown to both activate and inhibit PKC depending on cell type and PKC isoform. In bronchial epithelial cells (56), TNF-α is an activator. We tested whether this TNF-α-induced stimulation holds in H441 cells when focusing on PKC-α. The results are shown in Fig. 1. Cells treated with 10 ng/ml of TNF-α showed PKC-α activity that was elevated threefold. Peak activity occurred within 30 min, the earliest time interval analyzed. There was no change in the activity of PKC-βII (data not shown). In one experiment, we also found that 10 μM C2 ceramide increased PKC-α activity by ~50% within 1 min, and this remained elevated through 3 h.

**TNF-α increases MAPK activity in H441 cells.** TNF-α and ceramide have been widely reported to activate MAPKs (see, for example, Ref. 47), and this was reproduced in H441 cells as shown in Fig. 2. TNF-α activates (phosphorylates) p38 MAPK maximally within 15 min, consistent with a report in other cell types (39). Similar results were found in separate experiments with 10 μM C2 ceramide (Fig. 2), with peak activation occurring after 5 min.

C2 ceramide inhibits CT activity; the effect is abolished in cells transfected with dominant negative clones of PKC-α. We first established that PKC-α activity was altered in the cells that we transfected with PKC-α constructs. The results are shown in Fig. 3. Activity of PKC-α in the two dominant negative transfected clones was reduced to ~60% of that in nontransfected control cells but was unchanged in the wild-type PKC-α transfectants.

We next measured CT activity in these transfected cells to determine if changing PKC-α activity affected the basal level of CT activity or the ability of ceramide to inhibit CT. Basal CT activity (activity in the absence of ceramide treatment) was not changed in any of the transfected cells. As expected [see companion paper (53)], 10 μM C2 ceramide reduced CT activity by 50–70% in nontransfected cells. However, the inhibition of CT activity by 10 μM C2 ceramide was abolished in cells transfected with two dominant negative constructs of PKC-α but not in cells transfected with wild-type PKC-α or the cloning vector (Fig. 4). These data suggest that PKC-α is a critical part of the sig-

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![Fig. 3. PKC-α activity in H441 cells stably transfected with constructs of PKC-α.](http://ajplung.physiology.org)
Ceramide-induced inhibition of CT activity requires a functional cPLA₂ but not iPLA₂. cPLA₂ has been reported to be activated in a number of cell types in response to TNF-α (19, 33, 41). We confirmed this activity in H441 cells with C₂ ceramide. cPLA₂ was rapidly activated, and activity remained elevated at least 150% of control value for at least 60 min (Fig. 5). Consistent with this response, we also found in one experiment that the phosphorylation of immunoprecipitated cPLA₂ was increased by 58% after cells were exposed to 10 μM C₂ ceramide for 15 min. This increase in phosphorylation was maintained through 30 min but reversed by 1 h. The results indicate that C₂ ceramide exerts an activation of cPLA₂ similar to that reported for TNF-α.

We used two inhibitors of PL₂: BEL, a relatively specific inhibitor of iPLA₂ (6), and AACOCF₃, a compound that inhibits cPLA₂ (1). Both compounds were administered to H441 cells at three concentrations, either with or separately from 10 μM C₂ ceramide. The results are shown in Fig. 6. AACOCF₃ at all concentrations effectively blocked the ceramide-induced inhibition of CT activity; BEL had no effect. Neither compound affected basal CT activity. The results indicate that a functioning cPLA₂ is required for the ceramide-
induced inhibition, whereas iPLA₂ does not appear to be involved.

SB-203580, a relatively specific inhibitor of p38 MAPK, abolished the ceramide inhibition of CT; it had no effect on basal CT activity.

We pretreated cells with 2 μM SB-203580 for 1 h and then added 10 μM C₂ ceramide (SB-203580 remained in the medium). We harvested cells after 1 and 3 h of ceramide and measured CT activity in the membrane fraction of the cell lysates. The results are shown in Fig. 7. As expected, ceramide reduced CT activity to ~60% of control values (P < 0.05). SB-203580 (2 μM) had no effect on basal CT activity (control vs. 2 μM SB-203580) but significantly reduced the ability of C₂ ceramide to inhibit CT activity (2 μM SB-203580 plus ceramide vs. control, not significant, P > 0.05). We also found in one experiment that the increase in phosphorylation of cPLA₂ induced by 10 μM C₂ ceramide was inhibited by SB-203580. This suggests that the ceramide signaling to CT involves both cPLA₂ and p38 MAPK, the latter possibly serving to activate cPLA₂ by phosphorylation.

The signaling pathway for ceramide-induced inhibition of CT involves a leukotriene generated through 5-lipoxygenase. We used NDGA (22), a compound that inhibits the lipoxygenases, principally, 5-lipoxygenase. We used two concentrations of NDGA, 1 and 5 μM, which we gave to the cells together with 10 μM C₂ ceramide. The results obtained with both concentrations were indistinguishable, and the data from the use of 1 μM NDGA are shown in Fig. 8. NDGA completely prevented the C₂ ceramide inhibition of CT.

Inhibition of CT does not involve an eicosanoid generated through COX-2. Two COXs have been described in the literature: a constitutively expressed enzyme, COX-1, and an induced enzyme, COX-2 (50). Most cytokine-induced actions involving COXs are through COX-2 (50). We used a relatively specific inhibitor for COX-2, NS-398 (18), and tested the effects of 10 μM C₂ ceramide on the activity of CT in the presence and absence of three concentrations of this compound, 0.1, 1, and 10 μM. Neither 0.1 nor 1 μM NS-398 had an effect on either basal CT activity or the ability of 10 μM C₂ ceramide to inhibit the activity of CT. At a concentration of 10 μM, NS-398 had no effect on the response to C₂ ceramide, but it did reduce basal activity (data not shown).

LysoPC inhibits CT activity just as effectively as C₂ ceramide. One explanation for the data presented is that cPLA₂ is activated by TNF-α or ceramide and that a metabolite of the reaction catalyzed by cPLA₂ is responsible for the effects on CT activity. We tested this hypothesis by treating cells with either 10 μM C₂ ceramide or 2 μM 1-palmitoyl-2-lysoPC and measuring the effects on CT activity after 1 h. The effects of both compounds were identical as shown in Fig. 9. The results indicate that the lysoPC generated through a ceramide-stimulated signaling pathway could be the compound actually responsible for the ceramide-induced inhibition of CT.

CT is not phosphorylated in response to TNF-α. Changes in the phosphorylation of CT have been observed in connection with certain physiological events such as mitosis (42a), but their significance is not clear. There is no proven cause and effect relationship between CT phosphorylation and its activity (42a). Consistent with that conclusion, we did not observe a strong correlation between the phosphorylation of CT and the changes in its activity induced by TNF-α.
by autoradiography the amount of \(^{32}\text{P}\)orthophosphate the immunoprecipitates in SDS-PAGE and quantified only at effects at 5 h were statistically significant and then were presented with 10 ng/ml of TNF-\(\alpha\).

We hydrolyzed the immunoprecipitated protein and separated the phosphorylated amino acids by HPLC. We conclude that TNF-\(\alpha\) and/or ceramide does not exert its inhibition on CT through changes in the phosphorylation of CT; if they did, we would expect significant increases in phosphorylation.

Neither PKC-\(\alpha\) nor p38 MAPK affect the inhibition by ceramide of orthophosphate incorporation into PC despite their effects on CT activity. CT is the rate-limiting enzymatic activity in the Kennedy pathway for PC synthesis, and changes in its activity should be reflected by differences in synthetic rates, which is consistent with the data presented in the companion paper (53). However, \(\text{C}_2\) ceramide may also inhibit other enzymes in the Kennedy pathway that could likewise affect PC synthesis. To investigate this possibility, we measured the rate of incorporation of \(^{32}\text{P}\)phosphate into cellular PC in cells transfected with dominant negative constructs of PKC-\(\alpha\) or in nontransfected H441 cells that had been pretreated with 1 and 2 \(\mu\text{M}\) SB-203580. The results are shown in Fig. 11. \(^{32}\text{P}\)phosphate incorporation was inhibited by 10 \(\mu\text{M}\) \(\text{C}_2\) ceramide just as effectively as in control H441 cells, even when PKC-\(\alpha\) or p38 MAPK was blocked, despite their apparent importance in the regulation of CT activity. The results suggest that ceramide inhibits multiple enzymes in the Kennedy pathway, only one of which is CT.

**DISCUSSION**

The activity of CT is controlled by its association with a lipid environment. CT is a lipid-dependent enzyme, the activity of which is regulated by its interaction with cytoplasmic and nuclear membranes (15). Diacylglycerol (DAG) and certain other lipids are potent activators (5). CT shuttles between nuclear and cytoplasmic sites during the course of physiological events, and changes in CT enzymatic activity correlate with its intracellular localization (42). The mechanisms by which CT is translocated between the cytoplasmic membranes (active) and nuclear membranes (inactive) are unknown, but they are likely to involve associated lipids. Jamil et al. (25) have presented intriguing evidence that activity (and translocation) may be related to the ratio of bilayer-forming to non-bilayer-forming associated lipids.

**The signaling pathway for ceramide-induced inhibition of CT involves cPLA2.** cPLA2 is an 85-kDa protein that is widely distributed, including in lung tissue (19). cPLA2 is an attractive candidate as a regulator of the TNF-\(\alpha\) or ceramide inhibition of CT. An inhibitor of cPLA2, but not of iPLA2, nullifies the ceramide-induced inhibition of CT activity (this study). TNF-\(\alpha\) stimulates cPLA2 activity, even when given in low concentrations (19). cPLA2 has a relatively high specificity for arachidonic acid (20:4) at the \(sn-2\) position (32), and arachidonic acid, in turn, may further activate sphingomyelins (26). LysoPC given to H441 cells (data reported here) and to BAC 1.2F5 cells, a macrophage-like cell line (11), inhibits CT activity. LysoPC also promotes bilayer formation, consistent with the hypothesis of Jamil et al. (25) for the regulation of CT activity, but...
and SB-20358, inhibitors of p42 and p38 MAPKs, respectively, inhibit phosphorylation and activity (12).

PKC is in the signaling sequence (40). There are now numerous studies that show that the activation of MAPKs frequently proceeds through a PKC intermediary (for example, Ref. 29). The role of PKC in this activation is known for one of the classes of MAPK, p42/p44 (extracellular signal-regulated kinase). PKC activates Raf-1 by phosphorylation (directly or possibly through an intermediate) (29), which functions as a MAPK kinase kinase (i.e., two levels upstream) for p42/p44. The mechanisms by which PKC functions in the other MAPK pathways are unknown; presumably, PKC also activates upstream kinases. In addition, PKC may directly phosphorylate cPLA2 on several serine sites, but the effect of this phosphorylation is not fully known (12).

PKCs are activated by TNF-α (14), and although the mechanisms of activation are uncertain, two are plausible. TNF-α activates PC-specific phospholipase C (PC-PLC) (54); this results in increased DAG and stimulation of conventional and novel classes of PKC. PKC is also activated by phosphorylation (42), but pertinent kinases have not been identified. Like many features of cell signaling, the results of differing studies can be confusing. Thus in some cells, PKC is downregulated by phosphorylation of tyrosines (17). In others, the phosphorylation of tyrosines (49) or threonines (52) results in its activation.

Leukotrienes may be involved in the ceramide inhibition. We have found that inhibiting the initial enzyme 5-lipoxygenase in the principal pathway of leukotriene synthesis abolishes the ability of C2 ceramide to inhibit CT. A similar observation has been reported by Arias-Diaz and coworkers (4). The mechanisms by which leukotrienes interact in this system have not been determined, and we can only speculate as to its pathways. Leukotrienes themselves stimulate the transcription of cytokines (48), which could amplify the overall effect. In addition, certain of the leukotrienes, LTB4 (46), LTD4 (23, 51), and LTE4 (51), increase concentrations of intracellular Ca2+. There are two mechanisms: activation of a phosphatidylinositol-specific PLC through pertussis toxin-sensitive G proteins (23, 51) to hydrolyze 1,4,5-trisphosphate and release intracellular stores of Ca2+ and the opening of receptor-operated Ca2+ channels through pertussis toxin-insensitive G proteins (51). Elevated Ca2+ could then activate a variety of calcium-dependent enzymes including cPLA2. This would, of course, result in a renewed synthesis of the arachidonic acid substrate, perpetuating the cycle. Although we consider it plausible that leukotrienes could interact in the signaling process through their effects on intracellular pools of calcium, the data in this study do not directly measure these concentrations. Confirmation of this purported effect will require further exploration in separate studies.

![Diagram](http://apjplung.physiology.org/)

**Fig. 11.** Inhibition of PKC-α and p38 MAPK does not affect the inhibition of [32P]orthophosphate incorporation into phosphatidylcholine (PC) despite their effects on CT activity. A: effects of PKC-α. B: effects of SB-203580. Cells were deprived of FBS overnight. After 1 h of exposure to 10 μM C2 ceramide in serum-free medium, cells were given [32P]orthophosphate for 5 h without removing the C2 ceramide (a total of 6 h of ceramide exposure). Cells were washed twice with PBS, harvested in 400 μl of deionized water, and homogenized. Total lipids from cell homogenates were extracted and were separated on silica gel TLC plates with chloroform-methanol-28% ammonia (65:25:5 vol/vol). PC synthesis was estimated from the amount of [32P]orthophosphate incorporated into PC. Values are means ± SE from 3 experiments. *P < 0.05 compared with time-matched controls.

this may be coincidental with the observed inhibition of CT rather than causative. LysoPC itself stimulates Ca2+ influx, thereby potentially activating cPLA2, reinforcing the elevation in lysoPC content (20).

The function of PKC and p38 MAPK may be to activate cPLA2. cPLA2 is regulated by phosphorylation (12). Activity is dependent on two serine phosphorylation sites: Ser228 in the lipase sequence and Ser505 in a proline-dependent kinase sequence. Micromolar concentrations of Ca2+ are required, but the Ca2+ is not involved in the catalytic action but rather serves to anchor the enzyme to lipid membranes. Activated cPLA2 is principally found at the nuclear membrane.

One of the phosphorylation sites, Ser505, is a consensus sequence for proline-dependent kinases. PD-98059

![Diagram](http://apjplung.physiology.org/)
Ceramide must also inhibit other enzymes in the pathway(s) for PC synthesis. We have shown that when either PKC-α or p38 MAPK is inhibited, C₂ ceramide does not inhibit CT activity. Despite this reversal of CT inhibition, however, C₂ ceramide inhibits PC synthesis. We think that this indicates that C₂ ceramide acts simultaneously on several enzymatic activities, only one of which is CT. We have not identified which of these enzymes are also affected by ceramide, although our previous results suggest that the last enzyme in the Kennedy pathway, CDPcholine:1,2-DAG cholinephosphotransferase, is not affected. An interesting candidate is phosphatidic acid phosphohydrolase (phosphatide phosphatase). Bleasdale and Johnston (9), in work that is nearly 20 years old, proposed that the activity of phosphatidic acid phosphohydrolase is critical in maintaining the balance between PC, phosphatidylglycerol, and phosphatidylinositol. An inhibition of phosphatidic acid phosphohydrolase should result (by their hypothesis) in a drop in PC, a decrease in phosphatidylglycerol, and an increase in phosphatidylinositol. This is exactly what happens in chronic lung injury (for example, see Ref. 27).

A hypothesis for the effects of ceramide on CT activity. We suggest that ceramide activates PKC and p38 MAPK and that these contribute to the activation of cPLA₂ by phosphorylation. Activated cPLA₂ hydrolyzes sn-2-arachidonoyl PC to lysoPC and arachidonic acid. LysoPC interacts with the cytoplasmic membrane lipids associated with CT and induces the translocation of CT to the nucleus where it is in an inactive state. Arachidonic acid, through 5-lipoxygenase, generates leukotrienes that further activate cPLA₂ through their elevation of intracellular Ca²⁺ pools. In addition, these leukotrienes stimulate the production of TNF-α. In this manner, the pathway is self-sustaining and reinforcing, consistent with the persistent effects of ceramide on CT activity. A signaling scheme consistent with our data is shown in Fig. 12. Although this scheme is plausible, alternative relationships among the intermediaries are also possible. For instance, our data do not distinguish whether the activation of p38 MAPK in response to ceramide is sequential through PKC-α or in parallel. Both PKC-α and p38 MAPK activate cPLA₂ (12). TNF-α has been shown to activate p38 MAPK in a variety of cell types (31), and more recently, Mallamalli and coworkers (35) have shown that it activates p42/p44 MAPK in type II cells. However, these studies do not distinguish whether the activation also involves PKC-α.

Our data support a central role for cPLA₂ in generating lysoPC and arachidonic acid, a substrate for the synthesis of leukotrienes. Other steps in this hypothesis, particularly the identity and functions of leukotrienes, remain speculative until addressed and confirmed by additional studies.

Multiple mechanisms may be involved in the regulation of CT. Mallampalli and coworkers (36), in a paper that appeared while this work was being completed, convincingly demonstrated that 500 ng/ml of TNF-α reduced CT protein after 24 h of exposure and that this phenomenon may involve proteasome-associated processing. However, there were modest or no changes at shorter times (4 and 12 h). This is in seeming contrast to our observations with 10 μM C₂ ceramide in which changes in CT activity were evident as early as 2 h. These differences could relate to differences in cell types, to the fact that C₂ ceramide rapidly traverses cell membranes and thereby acts more rapidly, or from some subtle and unrecognized procedural differences inherent in these two studies. We propose an addi-

Fig. 12. A hypothesis for the events that occur when ceramide inhibits CT activity. LT, leukotriene; PP_i, pyrophosphate; DAG, diacylglycerol; cPLA₂; cytosolic phospholipase A₂.

![EFFECTS OF TNF-α ON SPHINGOMYELIN/PC SYNTHESIS](https://example.com/fig12.png)
tional possibility, that signaling through cPLA₂ is a relatively rapid mechanism responsible for the earliest changes in CT activity, whereas protein processing and degradation require longer time intervals. This possibility seems to be hinted at in the study by Mallampalli et al. (36) as evident from comments in the discussion. An alternative explanation is that proteasome-associated processing requires cPLA₂, but a thorough literature search uncovered no evidence for this.

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L118 CERAMIDE INHIBITS CYTIDYLTRANSFERASE THROUGH cPLA₂