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Phospholipase D-catalyzed hydrolysis of phosphatidylcholine provides the choline precursor for acetylcholine synthesis in a human neuronal cell line

(neuroblastoma/LA-N-2 cells/Alzheimer disease)

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ABSTRACT **To identify the metabolic pathway that generates choline (Cho) for acetylcholine (AcCho) from its storage pool in membrane phosphatidylcholine (PtdCho), human neuronal cells (LA-N-2) were radioisotopically labeled with 1-*O*-hexadecyl-2-hydroxy-*sn*-glycero(3)phospho[^{14}C]choline. The compound was efficiently taken up by the cells and metabolically labeled PtdCho, Cho, AcCho, and phosphocholine pools. In pulse–chase experiments, the specific radioactivities of the metabolites of 1-*O*-hexadecyl-2-hydroxy-*sn*-glycero(3)phospho[^{14}C]choline indicated that it was rapidly acylated to PtdCho and then hydrolyzed first to free Cho and not to phosphocholine or glycerophosphocholine. This Cho was subsequently converted to AcCho and to phosphocholine. In the absence of exogenous Cho, at least 15% of the total cellular AcCho pool was synthesized by this pathway in 1 h. The data demonstrate that the liberation of the free Cho precursor for AcCho synthesis from PtdCho can be accomplished in a one-step process, indicating the involvement of a phospholipase D-type enzyme. In the presence of hemicholinium-3, which inhibits Cho transport, the amount of intracellular [^{14}C]Cho metabolites that accumulated during the chase period was higher than in control cells, indicating that PtdCho hydrolysis liberated Cho directly into the cytoplasm. These data show that cholinergic cells are characterized by an intracellular pathway, catalyzed by a phospholipase D, that generates Cho for AcCho synthesis from PtdCho. Abnormalities in the regulation of this pathway may contribute to selective vulnerability of cholinergic neurons in certain neurodegenerative diseases, e.g., Alzheimer disease.**

A supply of choline (Cho) for the synthesis of acetylcholine (AcCho) is essential for normal functioning of cholinergic neurons. Most of the Cho for this process derives from the diet, enters the nervous system via the circulation, and then is taken up into the nerve terminals of cholinergic neurons by a specialized high-affinity transporter that is also capable of salvaging a substantial proportion of Cho generated from AcCho intrasynaptically by acetylcholinesterase (for a review, see ref. 1). Additional Cho for AcCho synthesis is derived from membrane phosphatidylcholine (PtdCho) (2, 3), which might be considered a potential storage pool of Cho. This pool may be especially important when extracellular Cho is in short supply and/or when neuronal AcCho synthesis and release are accelerated by a high firing frequency (4–8). Although the enzymatic pathways of PtdCho turnover in the brain are reasonably well-characterized in general, the mechanisms responsible for liberating Cho from PtdCho in cholinergic neurons remain unknown.

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PtdCho can be hydrolyzed to Cho by a multienzymatic pathway (Fig. 1) consisting of phospholipase (PL) A_2 (EC 3.1.1.4) and lysophospholipase (EC 3.1.1.5), whose combined actions produce glycerophosphocholine (Gro*P*Cho), which is then degraded by Gro*P*Cho phosphodiesterase (EC 3.1.4.2). In human brain *in vitro* the latter enzyme has low activity and Gro*P*Cho tends to be converted to phosphocholine (*P*Cho) by Gro*P*Cho cholinephosphodiesterase (EC 3.1.4.38) (9). Alternatively, PtdCho may be degraded by a PLC (EC 3.1.4.3) also producing *P*Cho, which may be further broken down to free Cho by alkaline phosphatase (EC 3.1.3.1). In addition PtdCho can be hydrolyzed directly to Cho by a PLD (EC 3.1.4.4). The latter mechanism is especially interesting in view of the recent discoveries that PLD is likely to constitute a highly heterogeneous family of enzymes, some of which may be constitutively active, while the activities of others may be regulated by receptors (for reviews, see refs. 10–12) for hormones, growth factors, and neurotransmitters [including those of the muscarinic cholinergic type (13–18)]. Previous studies showed that the brain contains several types of PLD that differ in subcellular distribution, kinetic properties, and pH and ionic optima (16, 19–28). In addition to generating Cho, PLD produces phosphatidic acid, which may be further converted to 1,2-*sn*-diacylglycerol. The latter two compounds act as second messengers (29), and their production is regulated by PLD-catalyzed hydrolysis of glycerophospholipids in neuronal cell lines (17, 18, 30–37), including the human cholinergic cell line [LA-N-2 (13, 14)] used in the current study.

To determine how Cho is liberated from PtdCho in LA-N-2 cells, we labeled the cells with a [^{14}C]Cho-containing 1-*O*-hexadecyl-2-hydroxy-*sn*-glycero(3)phosphocholine (alkyl-lysoPtdCho), which, due to the presence of the ether bond in the *sn*-1 position, is resistant to hydrolysis by PLA_1 and/or lysophospholipase. Previously [^{3}H]alkyl-labeled lysoPtdCho has been successfully applied to studies of the turnover of the lipid backbone of PtdCho in a variety of systems (38–40). The use of [^{14}C]Cho-containing alkyl-lysoPtdCho permits the introduction of Cho label directly into cellular phospholipids prior to its incorporation into water-soluble metabolites. By using this design, we provide evidence that a PLD generates Cho from PtdCho for AcCho synthesis and that this Cho is released directly into the cytoplasm without first entering the extracellular space.

Abbreviations: Cho, choline; AcCho, acetylcholine; PtdCho, phosphatidylcholine; alkyl-lysoPtdCho, 1-*O*-hexadecyl-2-hydroxy-*sn*-glycero(3)phosphocholine; HC-3, hemicholinium-3; *P*Cho, phosphocholine; PL, phospholipase; SRA, specific radioactivity; Gro*P*Cho, glycerophosphocholine.

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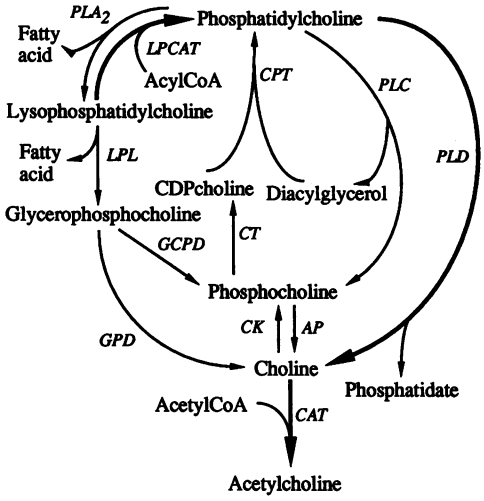

FIG. 1. Pathways of PtdCho turnover and AcCho synthesis. PtdCho synthesis is catalyzed in three enzymatic steps: Cho kinase (CK) catalyzes the formation of *P*Cho from Cho and ATP; *P*Cho cytidylyltransferase (CT) catalyzes the formation of cytidine diphosphocholine (CDPcholine) from *P*Cho and CTP; and cholinephosphotransferase (CPT) catalyzes the transfer of *P*Cho from CDPcholine to 1,2-*sn*-diacylglycerol, forming PtdCho. The latter can be degraded to Cho via several pathways, including hydrolysis by a PLD to free Cho and phosphatidate, or by PLC to *P*Cho and diacylglycerol. PLA_2 hydrolyses PtdCho to a fatty acid and lysoPtdCho, which can be reacylated by an acyltransferase (LPCAT) utilizing a fatty acyl-CoA. LysoPtdCho can be hydrolyzed to Gro*P*Cho by a lysophospholipase (LPL). Gro*P*Cho is then converted to *P*Cho by a cholinephosphodiesterase (GCPD) or to free Cho by a diesterase (GPD); and the *P*Cho is hydrolyzed to Cho by alkaline phosphatase (AP). The conversion of Cho to AcCho is catalyzed by Cho acetyltransferase (CAT). Some of the cofactors have been omitted for clarity. Thick arrows indicate the metabolic pathway characterized in this study.

MATERIALS AND METHODS

Preparation of Alkyl-[^{14}C]lysoPtdCho. The alkyl-[^{14}C]lysoPtdCho was prepared by the method of Stoffel (41). 1-*O*-Hexadecyl-2-hydroxy-*sn*-glycero(3)phosphodimethylethanolamine (Avanti Polar Lipids; 2 mg, 4.2 μmol) was combined with 20 μmol (1 mCi; 1 Ci = 37 GBq) of [^{14}C]methyl iodide (50 Ci/mol; Amersham) and 6 μl of cyclohexylamine in the final volume of 0.6 ml of methanol and incubated at room temperature for 24 h in the dark. The reaction mixture was dried under a vacuum, dissolved in methanol, and the alkyl-[^{14}C]lysoPtdCho product was purified by HPLC on a normal-phase silica column (Pecosphere-3C Si, 4.6 × 83 mm; Perkin–Elmer) using a linear gradient elution (1.5 ml/min) of the following two mobile phases: A, acetonitrile/water/ethanol/acetic acid/1.0 M ammonium acetate, 800:127:68:2:3 (vol/vol); B, same components, 400:400:68:53:79 (vol/vol). The mobile phase was varied from 0 to 20% buffer B over a 10-min period after sample injection. A commercial preparation of alkyl-[^{14}C]lysoPtdCho (American Radiolabeled Chemicals) was used as a standard for retention time. The product was radiochemically pure (by TLC and HPLC) and contained no detectable amounts of the 1-*O*-hexadecyl-2-hydroxy-*sn*-glycero(3)phosphodimethylethanolamine precursor.

Cell Culture and Experimental Protocol. LA-N-2 cells (generously provided by Robert Seeger, University of California, Los Angeles) were grown routinely in Leibowitz L-15 medium containing 5% (vol/vol) fetal bovine serum, 5% (vol/vol) horse serum (all GIBCO/BRL), and 0.1 mM Cho. For experiments, the cells were subcultured into 35-mm dishes and grown until 80–90% confluent. The cells were washed twice with 1 ml of Cho-free Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture, 1:1 (vol/vol) (GIBCO/BRL), without sodium bicarbonate, but buffered with 28 mM Hepes·NaOH (pH 7.4) and containing 15 μM neostigmine. The cells were labeled in 1 ml of this medium at 37°C, for various times as indicated, with 0.5 μCi of 10 μM alkyl-[^{14}C]lysoPtdCho. The alkyl-[^{14}C]lysoPtdCho was initially dissolved in 20 μl of dimethyl sulfoxide and then mixed with 4 ml of labeling medium containing 0.1% fatty acid-free bovine serum albumin (Sigma). This alkyl-[^{14}C]lysoPtdCho stock was diluted 1:5 to obtain the labeling medium. After labeling, the cells were washed twice with 1 ml of a physiological salt solution (135 mM NaCl/5 mM KCl/1 mM $CaCl_2$/0.75 mM $MgCl_2$/5 mM glucose/0.015 mM neostigmine/10 mM Hepes, pH 7.4) and incubated in this solution for an additional period at 37°C as indicated. After the experimental period, the media were collected, and the cells were extracted with 1 ml of methanol and scraped off the dishes. The methanolic suspensions were transferred to polypropylene tubes, 2 ml of chloroform was added, and the tubes were vortex mixed. Water (1 ml) was added, and the tubes were vortex mixed and centrifuged to separate the two phases. The aqueous and the organic phases and the protein interface were collected and dried under a vacuum.

Purification and Radioactivity Measurements of Cho Metabolites. The water-soluble Cho metabolites were purified by HPLC (42) on a normal-phase column (10 cm × 4.6 mm i.d.) containing 3-μm silica particles (Dynamax, Rainin, Woburn, MA), using a linear gradient elution (1.5 ml/min) with the following two mobile phases: A, acetonitrile/water/ethanol/acetic acid/1.0 M ammonium acetate/0.1 M sodium phosphate monobasic, 800:127:68:2:3:10 (vol/vol); B, same components, 400:400:68:53:79:10 (vol/vol). The mobile phase was varied from 0 to 100% of phase B with a slope of 5%/min, starting 6 min after sample injection. The radioactivity associated with AcCho, Cho, and *P*Cho was quantified by an on-line monitor using a solid-phase scintillation flow cell (model LB 507 A, Berthold, Nashua, NH). The phospholipids were purified by TLC on silica gel 60 (Merck) developed with chloroform/methanol/acetic acid/water, 25:15:4:2 (vol/vol). This chromatographic method permits the separation of lysoPtdCho (R_f = 0.12) from PtdCho (R_f = 0.45). However, the 1-*O*-alkyl- and the 1-*O*-acyl- compounds cannot be resolved by this system, and for this reason the prefix "alkyl" is not used whenever the discussion relates to those compounds subsequent to their purification by this system. In addition, this TLC system resolves platelet-activating factor [1-*O*-alkyl-2-acetyl-*sn*-glycero(3)phosphocholine; R_f = 0.17] from long-chain PtdCho and from lysoPtdCho. The plates were scanned using a System 200 radioactivity scanner (Bioscan, Washington, DC). Segments corresponding to lysoPtdCho and PtdCho were scraped off and hydrolyzed to free Cho using 6 M HCl at 80°C for 1 h. Radioactivity of an aliquot was determined by liquid scintillation spectrometry and the remainder was used for the determination of Cho mass.

Measurements of Mass of Cho Metabolites. AcCho and Cho was determined in the aqueous portion of cell extracts and in the medium by an HPLC kit (Bioanalytical Systems, West Lafayette, IN) consisting of a proprietary analytical column and an enzymatic reactor containing acetylcholinesterase and Cho oxidase and an electrochemical detector (43). This HPLC technique was also used to purify the radiolabeled Cho and AcCho from cell medium prior to the determination of the radioactivity of these compounds by liquid scintillation spectrometry. AcCho (retention time, 6 min) and Cho (retention time, 7.1 min) fractions were eluted at 1.5 ml/min with 25 mM sodium phosphate (pH 8.5) as a mobile phase and collected. Cellular *P*Cho was purified by the normal-phase HPLC as described above and hydrolyzed to free Cho by alkaline phosphatase (30 units/ml; type VII-NLA; Sigma) at 37°C for 45 min using 0.1 M glycine buffer (pH 10.5) containing 1 mM

$MgCl_2$. The free Cho liberated from *P*Cho, PtdCho, and lysoPtdCho was determined by HPLC as above.

Protein Assay. Protein was determined using bicinchoninic acid by the method of Smith *et al.* (44).

RESULTS

To label the Cho-containing cellular phospholipids, LA-N-2 cells were incubated with alkyl-[^{14}C]lysoPtdCho for various periods. The cells rapidly removed alkyl-[^{14}C]lysoPtdCho from the medium (Fig. 2 *Upper*) and incorporated it into cellular lipids. The amount of alkyl-[^{14}C]lysoPtdCho in the medium during the incubation declined with time; this process could be described by a single exponential decay curve (correlation coefficient, $r^2 = 0.99$), with a half-life of 167 min (Fig. 2 *Upper*). The alkyl-[^{14}C]lysoPtdCho was efficiently taken up by the cells and the radioactivity in [^{14}C]lysoPtdCho in cells apparently reached a steady-state level after 15 min of labeling. At this time the radioactivity found in PtdCho was already similar to that in lysoPtdCho, indicating that the acylation of [^{14}C]lysoPtdCho to [^{14}C]PtdCho was rapid. The accumulation of [^{14}C]PtdCho continued linearly with time for at least 2 h and then leveled off as the supply of alkyl-[^{14}C]lysoPtdCho in the medium declined. The rapid conversion of [^{14}C]lysoPtdCho to [^{14}C]PtdCho permitted extensive labeling of this phospholipid in LA-N-2 cells. In addition to being converted to long-chain fatty acyl-PtdCho, alkyl-[^{14}C]lysoPtdCho can also be acetylated to platelet-activating factor (for a review, see ref. 45). Radioactive material comigrating with authentic platelet-activating factor standards was observed on radiochromatographic scans of our TLC plates in a small minority of experiments and, when it was found, it constituted at most 5% of the radioactivity of [^{14}C]PtdCho, indicating that synthesis of platelet-activating factor is a minor metabolic pathway in LA-N-2 cells. The radiolabeled water-soluble metabolites of Cho (i.e., free Cho, AcCho, and *P*Cho) were undetectable at 15 min but could be observed by 45 min (Fig. 2 *Lower*). As expected, no [^{14}C]Gro*P*Cho was observed, indicating that [^{14}C]lysoPtdCho and [^{14}C]PtdCho were resistant to dealkylation at the *sn*-1 position. The delayed appearance of water-soluble metabolites of Cho suggests that [^{14}C]lysoPtdCho was not a good precursor for their formation but rather that the hydrolysis at the *sn*-3 position occurred subsequent to the formation of PtdCho.

Based on these data, a 45-min labeling period was chosen for the pulse–chase studies in which the specific radioactivity (SRA) of all of the [^{14}C]Cho-containing compounds was measured as a function of chase time. During the chase period the SRA of the cellular [^{14}C]lysoPtdCho declined exponentially with a half-life of 64 min (Fig. 3*A*). The SRA of [^{14}C]PtdCho increased with time (Fig. 3*B*) and remained higher than that of the water-soluble metabolites of Cho consistent with the notion that [^{14}C]lysoPtdCho was converted directly to [^{14}C]PtdCho. Among the water-soluble metabolites of Cho, the free [^{14}C]Cho had the highest SRA (Fig. 3*B*). This value rose steadily during the chase period, indicating the continual formation of Cho. It is worth noting that the cell content of PtdCho is 57 times larger than that of lysoPtdCho (Table 1), and thus, although the acylation of lysoPtdCho to PtdCho is rapid (Fig. 2), the rise in the SRA of PtdCho is less dramatic (Fig. 3). The SRAs of AcCho and of *P*Cho were similar to each other and lower than that of Cho throughout the chase period (Fig. 3*C*). These data indicate that free Cho was an intermediate both for the synthesis of AcCho and *P*Cho. Thus these observations are consistent with the hypothesis that membrane PtdCho was first hydrolyzed by a PLD, generating free Cho, which was subsequently acetylated to AcCho, and invalidate the alternative hypothesis that *P*Cho formed by a PLC was an intermediate *en route* to Cho and AcCho synthesis.

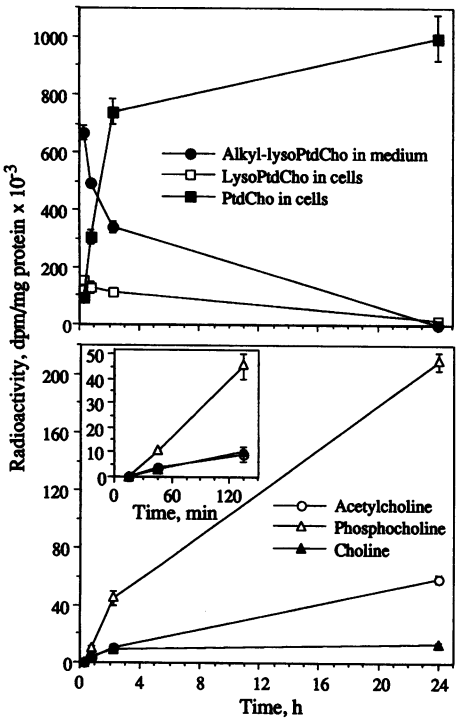

FIG. 2. Clearance of alkyl-[^{14}C]lysoPtdCho from the medium and its incorporation and turnover in LA-N-2 cells. The cells were incubated with alkyl-[^{14}C]lysoPtdCho as indicated. The labeled lipids (*Upper*) were purified by TLC and the labeled water-soluble metabolites of Cho (*Lower*) were purified by HPLC and their radioactivities were determined. (*Inset*) Incorporation of label into the water-soluble metabolites of Cho during the initial 135 min. The results are presented as the mean ± SD from a representative experiment.

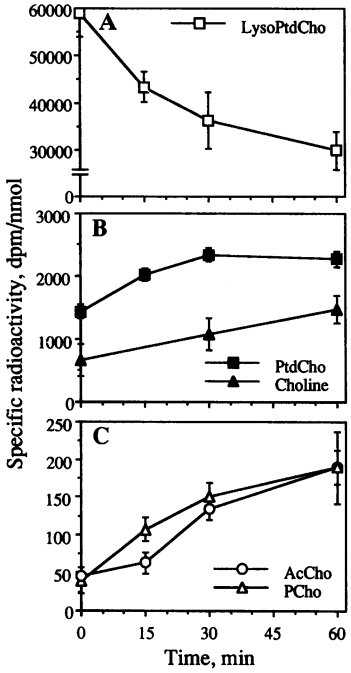

FIG. 3. Turnover of [^{14}C]lysoPtdCho in LA-N-2 cells. The cells were pulsed with alkyl-[^{14}C]lysoPtdCho for 45 min and then chased as indicated. The labeled lipids were purified by TLC and the labeled water-soluble metabolites of Cho were purified by HPLC and their radioactivities and mass were determined. Specific radioactivities were calculated and are presented as the mean ± SD from a representative experiment. (*A*) LysoPtdCho. (*B*) PtdCho and choline. (*C*) AcCho and *P*Cho.

Table 1. Quantitative analyses of the [^{14}C]lysoPtdCho turnover in LA-N-2 cells

	Cell content at 60 min, nmol/mg of protein	Radioactivity at 60 min, dpm/mg of protein	Specific radioactivity at 30 min, dpm/nmol	Synthesis, nmol per mg of protein per 60 min	Synthesis, % of total pool per 60 min
lysoPtdCho	1.6	77,285	36,217	—	—
PtdCho	84	203,936	2,338	5.6	7
Cho	1.9	2,642	1,075	1.1	59
*P*Cho	92	13,924	150	12.9	14
AcCho	29	5,071	134	4.7	16

Cells were incubated with alkyl-[^{14}C]lysoPtdCho for 45 min and then incubated for 60 min in the absence of radioactivity. The labeled lipids were purified by TLC, the labeled water-soluble metabolites of Cho were purified by HPLC, and their radioactivities and mass were determined. Specific radioactivities at 30 min were calculated and are presented as the mean from an experiment shown in Fig. 3. The amount of PtdCho, Cho, AcCho, and *P*Cho synthesized during the 60-min chase period was calculated using the cell content and radioactivity of each of the compounds at 60 min, and the average SRA of their immediate precursors was taken at 30 min. Errors are omitted for clarity; coefficients of variation ranged between 5 and 20% of the mean.

To estimate the contribution of this process to the pools of the Cho-containing compounds, we calculated the amount of PtdCho, Cho, AcCho, and *P*Cho synthesized during the 60-min chase period, using the cell content and radioactivity of each of the compounds at 60 min, and the average SRA of their immediate precursors taken at 30 min (Table 1). For example, the amount of AcCho synthesized in 60 min equals (5071 dpm/mg)/(1075 dpm/nmol) = 4.7 nmol/mg, and this constitutes 16% of the AcCho pool (4.7/29 = 0.16). These calculations represent conservative estimates because they do not account for further turnover and/or release into the medium of each of the products. In fact, assuming that all of the radioactivity present in the water-soluble metabolites of Cho derives from [^{14}C]PtdCho, it can be estimated that the amount of Cho liberated from PtdCho during the 60-min chase period is (2642 + 13924 + 5071 dpm)/(2338 dpm/nmol) = 9.3 nmol (i.e., 4.8 times the Cho pool size). These data show that the PLD-catalyzed turnover of PtdCho is rapid and that a substantial proportion of the AcCho and *P*Cho pools (16% and 14%, respectively, per 60 min) are synthesized from a Cho pool that is highly enriched by PtdCho breakdown.

To determine whether the Cho generated by PLD was first released into the cytoplasm or into the medium, LA-N-2 cells were labeled with alkyl-[^{14}C]lysoPtdCho for 45 min and, subsequently, incubated for 15 min in the presence or absence of hemicholinium-3 (HC-3). HC-3 inhibits the plasma membrane Cho transporter, preventing the influx of Cho into cells (46, 47) [including LA-N-2 (48)] as well as its efflux (49).

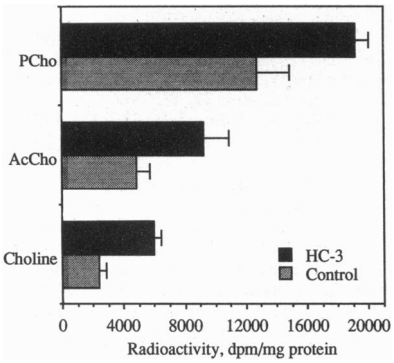

FIG. 4. Effect of HC-3 on the water-soluble metabolites of Cho in LA-N-2 cells. The cells were incubated with alkyl-[^{14}C]lysoPtdCho for 45 min and then incubated for 15 min in the absence (control) or presence of 100 μM HC-3. The labeled cellular water-soluble metabolites of Cho were purified by HPLC and their radioactivities were determined. The results are presented as the mean ± SD from a representative experiment. HC-3 significantly increased the radioactivity in Cho, *P*Cho, and AcCho ($P < 0.05$, *t* test).

HC-3 caused a marked elevation in the radioactivity of the cellular Cho, *P*Cho, and AcCho (Fig. 4). Concomitantly, HC-3 treatment reduced the radioactivity of the free Cho in the medium to 28% of control values (data not shown). These results indicate that HC-3 inhibited the efflux of the water-soluble Cho metabolites and demonstrate that PLD releases Cho into the cytoplasm and not to the extracellular space.

DISCUSSION

Cholinergic human neuroblastoma cells, LA-N-2, use the Cho moiety of PtdCho as a precursor of AcCho synthesis. The present results indicate that the liberation of free Cho from PtdCho can be accomplished in a one-step process without metabolic intermediates such as Gro*P*Cho and/or *P*Cho. Thus we conclude that a PLD-type enzyme is responsible for the hydrolysis of PtdCho to Cho in LA-N-2 cells. This enzyme is constitutively active and thus it is likely to be different from PLDs that are normally inhibited and that can be detected in cells only upon stimulation with a variety of pharmacologic agents including plasma membrane receptor agonists, phorbol esters, and certain amines (e.g., sphingosine) (12). The specificity for different molecular species of PtdCho of the PLD described here awaits further study. However, the PtdCho-specific PLD present in synaptic membranes hydrolyses both alkyl-acyl-PtdCho and diacyl-PtdCho at approximately the same rates (22). In addition to PLD, most cells [including LA-N-2 (50, 51)] possess a PLD-like activity called base-exchange that can liberate Cho from PtdCho in a one-step process, by catalyzing a transphosphatidylation reaction. The second substrate of this enzyme is not water (as in PLD-catalyzed reactions) but rather is serine or ethanolamine, which are incorporated into phospholipid concomitant with the liberation of Cho (52). The possibility that base exchange activity was responsible for the present results is not likely because the addition of 5 mM serine or 5 mM ethanolamine during the chase period had no effect on the formation of Cho, nor did it accelerate the PtdCho breakdown (data not shown). The observation that Cho was liberated directly into the cytoplasm suggests that the PtdCho substrate of PLD was present either on the inner leaflet of the plasma membrane or on one of the intracellular organelles. Such a system is more efficient than one in which Cho is first released into the extracellular space and then taken up into the cell.

The constitutive activity of this PLD (in contrast to the PLDs in other cells which are tonically inhibited) suggests that this activity may be specific for cholinergic neurons. If that were the case, the PLD might be regulated by the neuronal demand for Cho for AcCho synthesis. These contentions are consistent with the studies showing that basal turnover of PtdCho in nonneuronal cells is mediated by a PLC-type

enzyme (39, 53). It is important to note that our data do not exclude the possibility that PLA_2 and lysophospholipase may also contribute to Cho production via generation of Gro*P*Cho (Fig. 1). If that were the case, our results would tend to underestimate the contribution of PtdCho turnover to the generation of Cho and AcCho. The amount of AcCho synthesized from Cho produced by PLD during 60 min was 4.7 nmol/mg of protein or 16% of the total AcCho pool (i.e., 29.2 ± 4.0 nmol/mg of protein; mean ± SD; Table 1). This rate of synthesis of AcCho was apparently sufficient to maintain its cellular content, which was 31.8 ± 4.9 nmol/mg of protein at the start of the chase period, and to support its spontaneous release (2.8 ± 0.8 nmol per mg of protein per 60 min), which was determined in parallel experiments and was similar to that found in our previous studies (48). Thus the mechanisms of PtdCho breakdown are capable of generating sufficient amounts of Cho to maintain AcCho levels, at least for 1 h, in the absence of extracellular Cho and during resting periods of AcCho release. It remains to be determined whether PLD activity is regulated by the availability of extracellular Cho and/or by the rate of AcCho release.

Abnormal regulation of these pathways may result in (*i*) impaired cholinergic neurotransmission, when PtdCho hydrolysis is too slow and free Cho is in short supply, or (*ii*) perturbation in membrane function and viability, when PtdCho hydrolysis exceeds its resynthesis. Whereas these formulations remain hypothetical, evidence consistent with the idea that abnormal membrane phospholipid turnover may impart selective vulnerability to cholinergic neurons has been forthcoming from studies on Alzheimer disease. This disorder is characterized by the massive degeneration of cholinergic nerve terminals of neurons whose cell bodies reside in the basal forebrain and project to the cortex and hippocampus (54–56). Abnormal brain phospholipid metabolism is a feature of Alzheimer disease (9, 57–61). The observed abnormalities, detected in brain at autopsy, include reduced levels of PtdCho, phosphatidylethanolamine, Cho, and ethanolamine, concomitant with elevated levels of the phosphodiesters, Gro*P*Cho, and glycerophosphoethanolamine. Significantly, the increased concentrations of brain phosphodiesters in Alzheimer disease have been recently observed by noninvasive *in vivo* ^{31}P NMR measurements (62).

In addition to producing Cho, PLD also generates phosphatidic acid (Fig. 1). This compound is biologically active (29) and, if present in excess, might impair membrane functions. Thus it is likely that regulation of the removal of phosphatidic acid is an important process in cholinergic neurons, necessitated by the presence of a tonically active PLD catalyzing PtdCho hydrolysis to provide Cho for AcCho synthesis.

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1. Blusztajn, J. K. & Wurtman, R. J. (1983) *Science* **221**, 614–620.
2. Ulus, I. H., Wurtman, R. J., Mauron, C. & Blusztajn, J. K. (1989) *Brain Res.* **484**, 217–227.
3. Blusztajn, J. K., Liscovitch, M. & Richardson, U. I. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5474–5477.
4. Lefresne, P., Guyenet, P. & Glowinski, J. (1973) *J. Neurochem.* **20**, 1083–1097.
5. Collier, B., Poon, P. & Salehmoghaddam, S. (1972) *J. Neurochem.* **19**, 51–60.
6. Parducz, A., Joo, F. & Toldi, J. (1986) *Exp. Brain Res.* **63**, 221–224.
7. Parducz, A., Kiss, Z. & Joo, F. (1976) *Experientia* **32**, 1520–1521.
8. Quastel, J. H., Tennenbaum, M. & Wheatley, A. H. M. (1936) *Biochem. J.* **30**, 1668–1681.
9. Nitsch, R. M., Blusztajn, J. K., Pittas, A. G., Slack, B. E., Growdon, J. H. & Wurtman, R. J. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1671–1675.
10. Dennis, E. A., Rhee, S. G., Billah, M. M. & Hannun, Y. A. (1991) *FASEB J.* **5**, 2068–2077.
11. Exton, J. H. (1990) *J. Biol. Chem.* **265**, 1–4.
12. Liscovitch, M. (1991) *Biochem. Soc. Trans.* **19**, 402–407.
13. Sandmann, J. & Wurtman, R. J. (1991) *J. Neurochem.* **56**, 1312–1319.
14. Sandmann, J., Peralta, E. G. & Wurtman, R. J. (1991) *J. Biol. Chem.* **266**, 6031–6034.
15. Pepitoni, S., Mallon, R. G., Pai, J.-K., Borkowski, J. A., Buck, M. A. & McQuade, R. D. (1991) *Biochem. Biophys. Res. Commun.* **176**, 453–458.
16. Qian, Z. & Drewes, L. R. (1989) *J. Biol. Chem.* **264**, 21720–21724.
17. Kanoh, H., Kanaho, Y. & Nozawa, Y. (1992) *J. Neurochem.* **59**, 1786–1794.
18. Kanoh, H., Ohbayashi, H., Matsuda, Y., Nonomura, Y. & Nozawa, Y. (1992) *Biochem. Biophys. Res. Commun.* **188**, 510–515.
19. Qian, Z. & Drewes, L. R. (1991) *FASEB J.* **5**, 315–319.
20. Qian, Z. & Drewes, L. R. (1990) *J. Biol. Chem.* **265**, 3607–3610.
21. Qian, Z., Reddy, P. V. & Drewes, L. R. (1990) *J. Neurochem.* **54**, 1632–1638.
22. Möhn, H., Chalifa, V. & Liscovitch, M. (1992) *J. Biol. Chem.* **267**, 11131–11136.
23. Chalifa, V., Möhn, H. & Liscovitch, M. (1990) *J. Biol. Chem.* **265**, 17512–17519.
24. Saito, M. & Kanfer, J. M. (1975) *Arch. Biochem. Biophys.* **169**, 318–323.
25. Chalifour, R. J. & Kanfer, J. (1980) *J. Neurochem.* **39**, 299–305.
26. Hattori, H., Kanfer, J. N. & Massarelli, R. (1987) *Neurochem. Res.* **12**, 687–692.
27. Kobayashi, M. & Kanfer, J. N. (1991) *Methods Enzymol.* **197**, 575–582.
28. Taki, T. & Kanfer, J. N. (1979) *J. Biol. Chem.* **254**, 9761–9765.
29. Liscovitch, M. (1992) *Trends Biochem. Sci.* **17**, 393–399.
30. Liscovitch, M., Chalifa, V., Danin, M. & Eli, Y. (1991) *Biochem. J.* **279**, 319–321.
31. Liscovitch, M., Blusztajn, J. K., Freese, A. & Wurtman, R. J. (1987) *Biochem. J.* **241**, 81–86.
32. Liscovitch, M. (1989) *J. Biol. Chem.* **264**, 1450–1456.
33. Lavie, Y. & Liscovitch, M. (1990) *J. Biol. Chem.* **265**, 3868–3872.
34. Horwitz, J. (1991) *J. Neurochem.* **56**, 509–517.
35. Murrin, R. J. A. & Boarder, M. R. (1992) *Mol. Pharmacol.* **41**, 561–568.
36. Holbrook, P. G., Pannell, L. K., Murata, Y. & Daly, J. W. (1992) *J. Biol. Chem.* **267**, 16834–16840.
37. Horwitz, J. & Ricanati, S. (1992) *J. Neurochem.* **59**, 1474–1480.
38. Pai, J.-K., Siegel, M. I., Egan, R. W. & Billah, M. M. (1988) *J. Biol. Chem.* **263**, 12472–12477.
39. Strum, J. C., Emilsson, A., Wykle, R. L. & Daniel, L. W. (1992) *J. Biol. Chem.* **267**, 1576–1583.
40. Frenkel, R. A. & Johnston, J. M. (1992) *J. Biol. Chem.* **267**, 19186–19191.
41. Stoffel, W. (1975) *Methods Enzymol.* **35**, 533–541.
42. Liscovitch, M., Freese, A., Blusztajn, J. K. & Wurtman, R. J. (1985) *Anal. Biochem.* **151**, 182–187.
43. Potter, P. E., Meek, J. L. & Neff, N. H. (1983) *J. Neurochem.* **41**, 188–194.
44. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85.
45. Prescott, S. M., Zimmerman, G. A. & McIntyre, T. M. (1990) *J. Biol. Chem.* **265**, 17381–17384.
46. Haga, T. & Noda, H. (1973) *Biochim. Biophys. Acta* **291**, 564–575.
47. MacIntosh, F. C. (1961) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **20**, 562–568.
48. Richardson, U. I., Liscovitch, M. & Blusztajn, J. K. (1989) *Brain Res.* **476**, 323–331.
49. Marchbanks, R. M., Wonnacott, S. & Rubio, M. A. (1981) *J. Neurochem.* **36**, 379–393.
50. Singh, I. N., Sorrentino, G., Massarelli, R. & Kanfer, J. N. (1992) *FEBS Lett.* **296**, 166–168.
51. Singh, I. N., McCartney, D. G., Sorrentino, G., Massarelli, R. & Kanfer, J. N. (1992) *J. Neurosci. Res.* **32**, 583–592.
52. Kanfer, J. N. (1980) *Can. J. Biochem.* **58**, 1370–1380.
53. Hii, C. S. T., Edwards, Y. S. & Murray, A. W. (1991) *J. Biol. Chem.* **266**, 20238–20243.
54. Bowen, D. M., Smith, C. B., White, P. & Davison, A. N. (1976) *Brain* **99**, 459–496.
55. McGeer, P. L., McGeer, E. G., Suzuki, J., Dolman, C. E. & Nagai, T. (1984) *Neurology* **34**, 741–745.
56. Perry, E. K., Tomlinson, B. E., Blessed, G., Bergman, K., Gibson, P. H. & Perry, R. H. (1987) *Br. Med. J.* **11**, 1457–1459.
57. Blusztajn, J. K., Lopez Gonzalez-Coviella, I., Logue, M., Growdon, J. H. & Wurtman, R. J. (1990) *Brain Res.* **536**, 240–244.
58. Pettegrew, J. W., Panchalingam, K., Moossy, J., Martinez, J., Rao, G. & Boller, F. (1988) *Arch. Neurol.* **45**, 1093–1096.
59. Barany, M., Chang, Y. C., Arus, C., Rustan, T. & Frey, W. H. (1985) *Lancet* **i**, 517.
60. Pettegrew, J. W., Minshew, N. J., Cohen, M. M., Kopp, S. J. & Glonek, T. (1984) *Neurology* **34**, Suppl. 1, 281.
61. Pettegrew, J. W. (1989) *Ann. N.Y. Acad. Sci.* **568**, 5–28.
62. Klunk, W. E., Panchalingam, K., McClure, R. J. & Pettegrew, J. W. (1992) *Ann. Neurol.* **32**, 268.