Synthesis of acetylcholine from choline derived from phosphatidylcholine in a human neuronal cell line

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

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ABSTRACT   Cholinergic neurons are unique among cells since they alone utilize choline not only as a component of major membrane phospholipids, such as phosphatidylcholine (Ptd-Cho), but also as a precursor of their neurotransmitter acetylcholine (AcCho). It has been hypothesized that choline-phospholipids might serve as a storage pool of choline for AcCho synthesis. The selective vulnerability of cholinergic neurons in certain neurodegenerative diseases (e.g., Alzheimer disease, motor neuron disorders) might result from the abnormally accelerated liberation of choline (to be used as precursor of AcCho) from membrane phospholipids, resulting in altered membrane composition and function and compromised neuronal viability. However, the proposed metabolic link between membrane turnover and AcCho synthesis has been difficult to demonstrate because of the heterogeneity of the preparations used. Here we used a population of purely cholinergic cells (human neuroblastoma, LA-N-2), incubated in the presence of [methyl-^{3}H]methionine to selectively label PtdCho synthesized by methylation of phosphatidylethanolamine, the only pathway of de novo choline synthesis. PtdCho, purified by thin-layer chromatography, contained 90% of the label incorporated into lipids, demonstrating that LA-N-2 cells contained phosphatidylethanolamine N-methyltransferase. Three peaks of radioactive material that cochromatographed with authentic Ac-Cho, choline, and phosphocholine were observed when the water-soluble metabolites of the [^{3}H]PtdCho were purified by high-performance liquid chromatography. Their identities were ascertained by subjecting them to enzymatic modifications with acetylcholinesterase, choline oxidase, and alkaline phosphatase, respectively. The results demonstrate that AcCho can be synthesized from choline derived from the degradation of endogenous PtdCho formed de novo by methylation of phosphatidylethanolamine.

All cells use choline as the precursor of certain phospholipids [phosphatidylcholine (PtdCho), sphingomyelin, and choline plasmalogens], which are the major constituents of all biological membranes. Cholinergic neurons are unique, since they alone use choline for an additional purpose, synthesis of their neurotransmitter acetylcholine (AcCho). Therefore, the cholinephospholipids in these neurons constitute a large pool of choline that can potentially be used for AcCho synthesis when the demand for choline needed to sustain AcCho release is enhanced (for example, when particular cholinergic neurons fire frequently or when the supply of choline from the extracellular fluid is inadequate). The resulting depletion in the amount of cholinephospholipids within cell membranes might be expected to alter membrane functions, leading, perhaps, to compromised membrane viability. Selective vulnerability of certain cholinergic neurons in such neurodegenerative disorders as Alzheimer disease—in which the long

axon cholinergic neurons projecting from the nucleus basalis and septum to the cortex and hippocampus, respectively, degenerate early in the course of the illness (1–5)—or motor neuron disorders (amyotrophic lateral sclerosis, progressive muscular atrophy, primary lateral sclerosis) (degenerative disorders of motor neurons) (6), may be a reflection of the overutilization of membrane PtdCho to provide choline for AcCho synthesis. Indeed, AcCho synthesis in vitro can occur from an endogenous source of choline (7), derived from a "bound" pool (8, 9) (probably composed of membrane phospholipids) and a decrease in the amount of PtdCho has been described in repeatedly depolarized superior cervical ganglia (10) and in striatal slices (11, 12). However, these preparations contain only a small proportion of cholinergic terminals, and therefore it is possible that the reduction in their phospholipids might have occurred within noncholinergic neurons and that the choline liberated from those cells was used to sustain AcCho synthesis.

Membrane phospholipids are the only site at which choline is synthesized de novo (thus providing an additional potential choline source for AcCho synthesis). In this synthetic pathway, phosphatidylethanolamine (PtdEtn) is sequentially methylated to PtdCho by the enzymes phosphatidylethanolamine methyltransferase (S-adenosyl-L-methionine:phosphatidylethanolamine N-methyltransferase, EC 2.1.1.17) and phosphatidyl-N-methylethanolamine methyltransferase (S-adenosyl-L-methionine:phosphatidyl-N-methylethanolamine N-methyltransferase, EC 2.1.1.71), which utilize S-adenosylmethionine as the methyl donor (13). The presence of activity of PtdEtn methyltransferases in mammalian brain (14–16) and the liberation of free choline formed by this pathway in brain synaptosomes (17) have been demonstrated. However, it has remained uncertain whether or not PtdEtn methyltransferases are localized within cholinergic neurons.

Using pure cholinergic cells in culture—the human neuroblastoma line LA-N-2 (18)—we were able to radioisotopically label PtdCho synthesized by the PtdEtn methyltransferase pathway and to follow the transfer of this label from the phospholipid to AcCho, thus providing evidence that PtdCho is synthesized de novo by PtdEtn methyltransferases in cholinergic neurons and that the choline derived from this PtdCho is, in fact, used for AcCho synthesis.

MATERIALS AND METHODS

LA-N-2 cells, passage 66-81 (generously provided by Robert Seeger, University of California, Los Angeles), were grown routinely in Leibowitz L-15 medium containing 100 μM choline and 10% fetal calf serum. To increase the rate of PtdCho synthesis by PtdEtn methyltransferase, the cells

Abbreviations: AcCho, acetylcholine; CDP-choline, cytidinediphosphocholine; GroPCho, glycerophosphocholine; Me$_2$Etn, N,N-dimethylethanolamine; PCho, phosphocholine; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine.
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were incubated in L-15 medium containing 2 mM dimethylethanolamine (Me$_2$Etn) (in addition to 7 μM choline). Me$_2$Etn is incorporated into cellular phospholipids to form phosphatidyldimethylethanolamine, which is then rapidly methylated to PtdCho by PtdEtn methyltransferase (19, 20). After 24 hr, the medium was replaced with a serum-free N2 medium (21), containing 60 μM eserine and no Me$_2$Etn. The medium contained 10 μM [*methyl*-^{3}H]methionine (1 Ci/mmol; 1 Ci = 37 GBq) or 0.5 μM [*methyl*-^{3}H]choline (1 Ci/mmol) (New England Nuclear). After 20 hr, media were aspirated, and the cells were treated with methanol and scraped off the culture dishes. Two volumes of chloroform were added to the methanolic suspensions and the mixtures were washed with 1 vol of water. The organic phase (containing phospholipids) and the aqueous phase (containing water-soluble choline metabolites) were dried under a vacuum. Phospholipids were purified by TLC on silica gel G using chloroform/ethanol/triethylamine/water (30:34:30:8; vol/vol) as a mobile phase (22). The individual phospholipids were scraped off the plates; their radioactivities were determined by liquid scintillation spectrometry; and their amounts were determined by a phosphate assay (23). The labeled water-soluble metabolites of choline or of PtdCho formed by PtdEtn methyltransferase were purified by HPLC, using a normal phase silica column (5 μm spherical particles; 4.6 mm i.d.; 25 cm long) and a gradient elution based on increasing polarity and ionic strength (24). The mobile phase was composed of two buffers: buffer A, acetonitrile/water/ethanol/acetic acid/0.83 M ammonium acetate (800:127:68:2:3; vol/vol); buffer B, same components (400:400:68:53:79; vol/vol) (pH 3.6). A linear gradient from 0 to 100% buffer B, with a slope of 5%/min was started 15 min after injection. At a flow rate of 2.7 ml/min and a column temperature of 45°C, typical retention times for the following compounds were (in min): betaine, 11; AcCho, 18; choline, 22; glycerophosphocholine (GroPCho), 27; cytidinediphosphocholine (CDP-choline), 32; phosphocholine (PCho), 46. For routine quantitative analyses of the [^{3}H]methyl incorporation from labeled methionine, fractions corresponding to authentic choline, AcCho, and PCho were collected, dried under a vacuum, and subjected to repurification using identical chromatographic conditions. Fractions were collected each minute and radioactivities were determined by liquid scintillation spectrometry. The identities of choline, AcCho, and PCho were confirmed by treating, prior to the second chromatographic separation, half of each sample of putative choline, AcCho, or PCho with choline oxidase (choline:oxygen 1-oxidoreductase, EC 1.1.3.17), acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7), or alkaline phosphatase [orthophosphoric-monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1], respectively (see Fig. 4), and identifying the reaction products as betaine, choline, and choline, respectively. The amounts of choline, AcCho, and PCho (after hydrolysis with alkaline phosphatase) were determined by a radioenzymatic assay (25). Recoveries were estimated by subjecting radiolabeled standards, added briefly to cell-containing culture dishes, to the identical purification procedure.

RESULTS AND DISCUSSION

LA-N-2 cells incorporated exogenous [^{3}H]choline primarily into [^{3}H]AcCho (Fig. 1); [^{3}H]PCho, [^{3}H]GroPCho, [^{3}H]CDP-choline, and [^{3}H]betaine were also detected in the cell extracts (Fig. 1).

When LA-N-2 cells were incubated with [*methyl*-^{3}H]methionine, a large fraction of the label (2–3%) was recovered in [^{3}H]PtdCho, which was the main (>90%) labeled product of the [^{3}H]methyl incorporation into lipids (Fig. 2). Preincubation of cells with Me$_2$Etn increased by 10-fold the [^{3}H]PtdCho accumulation (Fig. 2). Since PtdEtn methyltransferase is the
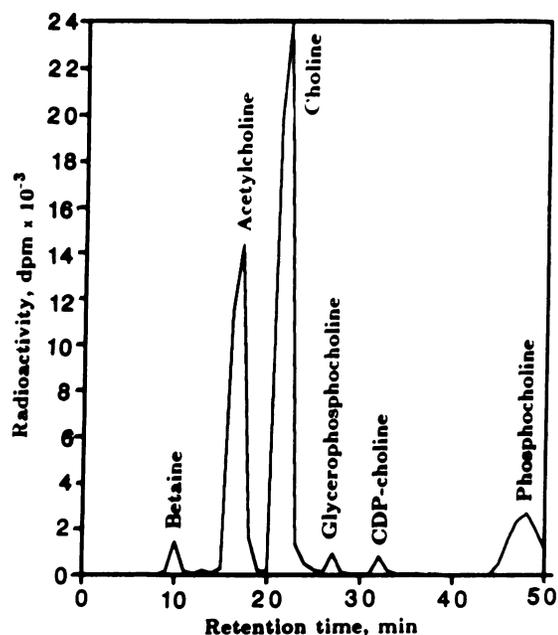

FIG. 1. Incorporation of [^{3}H]choline into LA-N-2 cells. Cells were incubated with [^{3}H]choline, and the aqueous extracts were subjected to HPLC. As indicated on the chromatogram, virtually all of the radioactivity was eluted at the retention times characteristic to betaine, AcCho, choline, GroPCho, CDP-choline, and PCho standards.

only known pathway that generates choline molecules *de novo*, any water-soluble form of labeled choline found in the cells must have been a product of degradation of membrane PtdCho formed by this pathway. Indeed, when the extracts of LA-N-2 cells that had been pretreated with Me$_2$Etn and then incubated with [*methyl*-^{3}H]methionine were subjected to our HPLC purification scheme, three small peaks of radioactivity that comigrated with the authentic choline, PCho, and AcCho were observed on the chromatograms
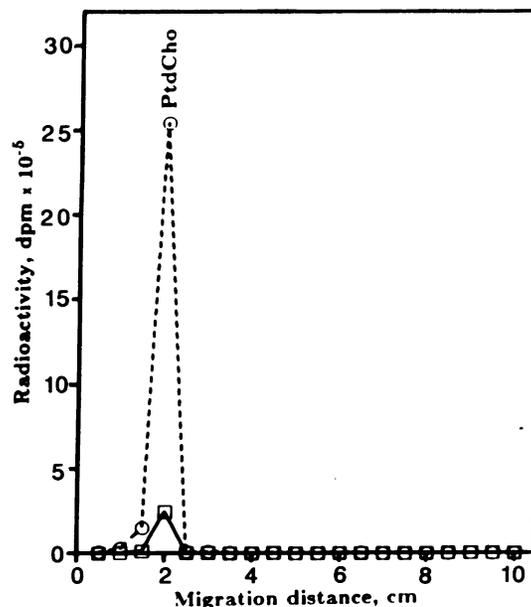

FIG. 2. Synthesis of PtdCho from [*methyl*-^{3}H]methionine in LA-N-2 cells. Radiochromatographic profile of labeled lipids purified by TLC. Cells, grown in the absence (solid line) or presence (broken line) of Me$_2$Etn prior to labeling, were incubated with [*methyl*-^{3}H]methionine, and the organic extracts were subjected to TLC.
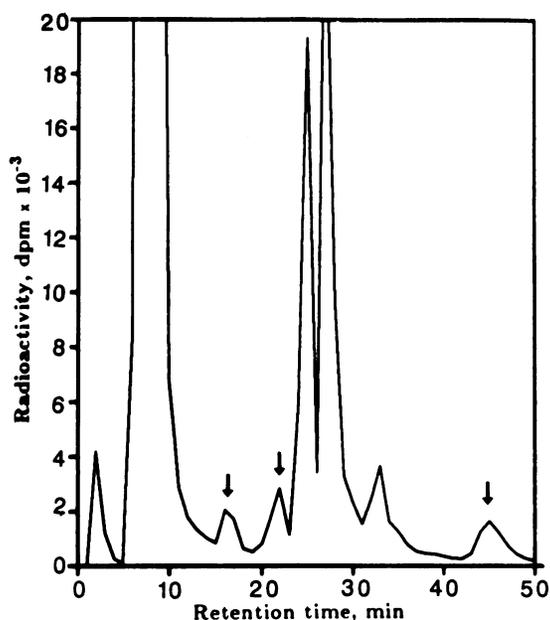
FIG. 3. Incorporation of [*methyl*-³H]methionine into LA-N-2 cells. Cells were pretreated with Me₂Etn, incubated with [*methyl*-³H]methionine, and the aqueous extracts were subjected to HPLC. A typical radiochromatogram is shown. Arrows indicate peaks of radioactivity eluting at the retention times of AcCho, choline, and *P*Cho.

(Fig. 3). When the putative [³H]choline, [³H]*P*Cho, and [³H]AcCho were treated with choline oxidase, alkaline phosphatase, and acetylcholinesterase, respectively, the radioactivities were quantitatively converted as expected to [³H]-betaine, [³H]choline, and [³H]choline, respectively (Fig. 4). Thus, the radiopurities of the labeled choline, *P*Cho, and AcCho were established and the amounts of radioactivity

incorporated into these compounds were used for quantitative analyses.

The amount of [³H]PtdCho synthesized during a 20-hr incubation period in the presence of [*methyl*-³H]methionine was 0.43 nmol per mg of protein or 0.6% of the total cellular PtdCho (assuming that the specific radioactivity of labeled S-adenosylmethionine was that of the [*methyl*-³H]methionine, and that most of the labeled PtdCho was synthesized from phosphatidyldimethylethanolamine) (Table 1). The amount of radioactivity found in choline, *P*Cho, and AcCho constituted 0.1%, 0.3%, and 0.3%, respectively, of that found in [³H]PtdCho. Although the free choline pool was small (0.13 nmol per mg of protein), its specific radioactivity was half that of PtdCho, indicating that PtdCho was the precursor of choline. These data suggest that a large proportion of free choline was derived from PtdCho and that the newly formed PtdCho probably entered the bulk pool of phospholipids. The amount of AcCho (1.9 nmol per mg of protein) in the cells was 14-fold greater than that of choline (Table 1); however, the specific radioactivity of AcCho was only 1/10th that of PtdCho (or 1/5th that of choline), suggesting that most of the cellular AcCho might have been present in a stable compartment that did not turn over during the labeling period. Assuming that the labeled AcCho was synthesized from choline, whose specific radioactivity was 6 dpm/pmol (Table 1), the total amount of newly formed AcCho would have been 2300/6 = 0.38 nmol per mg of protein or 20% of its pool.

These data indicate that cholinergic neurons form PtdCho by methylating PtdEtn and that this PtdCho can be hydrolyzed to free choline, which can then be acetylated to form AcCho. Preliminary observations suggest that this AcCho enters a releasable pool, inasmuch as radiolabeled AcCho was recovered from the growth media of LA-N-2 cells (data not shown). The choline liberated from PtdCho may enter the cytoplasm directly, or it may be released into the environment and then taken up by the high-affinity choline uptake. When in similar experiments the cells were incubated with
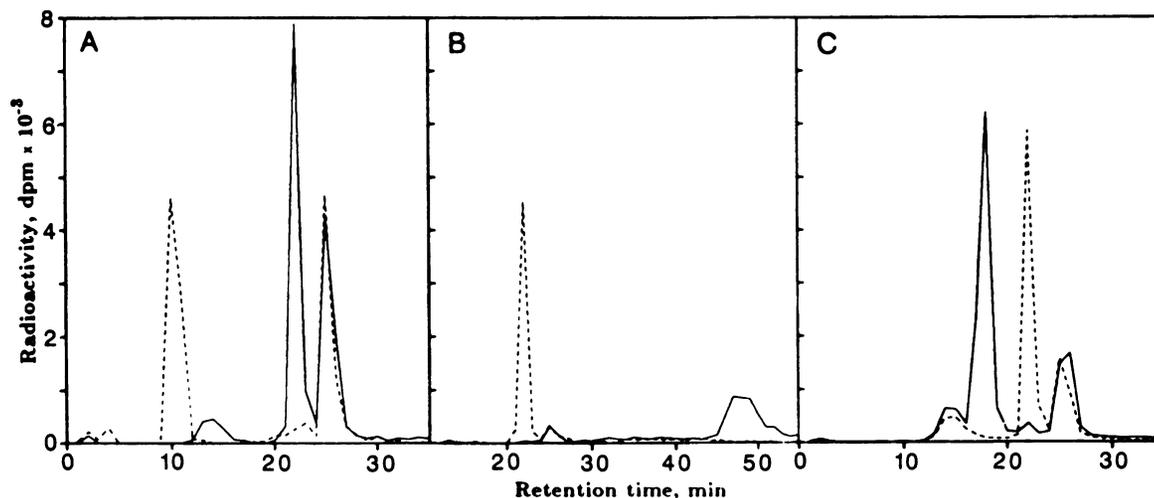

FIG. 4. Purification and identification of choline, *P*Cho, and AcCho synthesized from [*methyl*-³H]methionine in LA-N-2 cells. Cells were labeled with [*methyl*-³H]methionine and the aqueous extracts were subjected to HPLC as described in *Materials and Methods* and in Figs. 1 and 3. (*A*) Identification of radiolabeled choline. Cell extracts were subjected to HPLC and the fractions eluting at the retention time of choline (22–23 min) were collected and dried. The residues were reconstituted in 0.24 ml of 0.1 M sodium phosphate buffer (pH 7.8) and 0.1 ml of the samples was rechromatographed in the same HPLC system (solid line); 0.5 unit of choline oxidase (Sigma) was added to the remainder and the mixtures were incubated for 30 min at 30°C. Then, 0.1 ml was subjected to the HPLC (broken line). (*B*) Identification of radiolabeled *P*Cho. Cell extracts were treated as described in *A*, except that fractions eluting at the retention times of *P*Cho (43–55 min) were used. The dry residues were redissolved in 0.24 ml of buffer containing 0.1 M glycine, 1 mM MgCl₂, 1 mM ZnSO₄ (pH 10.7). This mixture (0.1 ml) was subjected to HPLC (solid line). Alkaline phosphatase (1 unit) (Sigma) was added to the remainder and the samples were incubated for 30 min at 37°C. This solution (0.1 ml) was subjected to HPLC (broken line). (*C*) Identification of radiolabeled AcCho. Cell extracts were treated as described in *A*, except that the fractions eluting at the retention time of AcCho (17–18 min) were used. The dry residues were redissolved in 0.24 ml of 0.1 M glycylglycine (pH 8.5). This solution (0.1 ml) was subjected to HPLC (solid line), and 10 units of acetylcholinesterase (from electric eel; Sigma) was added to the remainder and incubated for 30 min at 30°C. This solution (0.1 ml) was subjected to HPLC (broken line).

Table 1. Quantitative analyses of the incorporation of [³H]methyl from [*methyl*-³H]methionine into choline metabolites in LA-N-2 cells

	Cell content, nmol per mg of protein	Radioactivity, dpm per mg of protein	Specific radioactivity, dpm/pmol
PtdCho	70	8.7×10^5	12
Choline	0.13	0.8×10^3	6
PCho	1.5	2.9×10^3	1.9
AcCho	1.9	2.3×10^3	1.2

PtdCho was determined by a phosphate assay after purification by TLC. Choline, AcCho, and PCho [after hydrolysis to choline with alkaline phosphatase (see Fig. 4B)] were determined by a radioenzymatic assay in cell extracts. Radioactivities incorporated to each compound were determined after purification by TLC (for PtdCho) or HPLC (for choline, PCho, and AcCho). The data are means from a representative experiment repeated at least three times.

[*methyl*-³H]choline (0.5 μM), the amount of radioactivity that was incorporated into [³H]AcCho was 3-fold greater than that incorporated into [³H]PCho (Fig. 1). Since the same ratio was close to unity when the cells were labeled with [*methyl*-³H]methionine (Table 1), it is suggested that the newly formed choline was liberated directly into the cytoplasm where it served as a precursor of AcCho. This formulation is consistent with the results of experiments on the superior cervical ganglia (10), but it is at variance with those obtained from experiments on striatal slices (11, 12), which indicated that the endogenous choline first entered the extracellular fluid before it could be converted to AcCho. However, in these preparations, the choline might have derived from noncholinergic neurons, which constitute a majority of cells.

The specific radioactivity of choline was higher than that of PCho (Table 1), suggesting that PCho was not an intermediate of PtdCho degradation to choline. Thus, it is unlikely that PtdCho was hydrolyzed by a phospholipase C (phosphatidylcholine cholinephosphohydrolase, EC 3.1.4.3) or by a multienzymatic pathway consisting of phospholipase A₂ (phosphatidylcholine 2-acylhydrolase, EC 3.1.1.4), lysophospholipase (2-lysophosphatidylcholine acylhydrolase, EC 3.1.1.5), and glycerophosphocholine cholinephosphodiesterase (*sn*-glycero-3-phosphocholine cholinephosphohydrolase, EC 3.1.4.38) (26). Rather, if GroPCho were generated by phospholipase A₂ and lysophospholipase, it could then be hydrolyzed directly to choline by glycerophosphocholine phosphodiesterase (*sn*-glycero-3-phosphocholine glycerophosphohydrolase, EC 3.1.4.2) (27). Alternatively, PtdCho could be hydrolyzed directly to choline by a phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4), an enzyme activated by certain fatty acids (28).

It remains to be determined whether the choline used for AcCho synthesis is derived from a specific PtdCho pool [e.g., one synthesized by PtdEtn methyltransferase; or localized in a specific organelle, such as synaptic vesicles (10); or one containing a particular combination of fatty acids] or from the general catabolism of bulk PtdCho. In the former case, it is likely that a regulatory mechanism controls the relative rates of PtdCho synthesis and/or degradation, depending on the requirements of choline for AcCho synthesis, which, in turn, might be expected to depend on neuronal firing frequency.

The actual contribution of PtdCho hydrolysis in providing choline for AcCho synthesis may therefore vary according to AcCho output and exogenous choline supply.

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