Occurrence of an Unusual Phospholipid, Phosphatidyl-L-threonine, in Cultured Hippocampal Neurons

EXOGENOUS L-SERINE IS REQUIRED FOR THE SYNTHESIS OF NEURONAL PHOSPHATIDYL-L-SERINE AND SPHINGOLIPIDS*

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We have recently reported that L-serine released from astroglial cells supports the survival and neuritogenesis of hippocampal neurons under a serum- and glia-free culture condition (Mitoma, J., Furuya, S., and Hirabayashi, Y. (1998) Neurosci. Res. 30, 195–199). In this study, we show that exogenous L-serine is required for the synthesis of phosphatidyl-L-serine (PS) and sphingolipids in hippocampal neurons. When hippocampal neurons were maintained under an astroglial cell-free condition, the levels of sphingolipids and phosphatidyl-L-serine in the neurons were greatly reduced in the absence of external L-serine or glycine. Instead, a novel phospholipid appeared just ahead of PS on TLC. This novel lipid was determined to be phosphatidyl-L-threonine by TLC blotting/negative secondary ion mass spectrometry and amino acid analysis. Biochemical studies on rat brain microsomes have indicated that phosphatidyl-L-threonine is synthesized by the base exchange enzyme that is involved in PS synthesis with much lower affinity, that is, approximately 1/150 of L-serine. Addition of L-serine or glycine to the culture medium restored the synthesis of PS and sphingolipids in the neurons. These observations show that hippocampal neurons require exogenous L-serine for the synthesis of PS and sphingolipids in the absence of astroglial cells and suggested that astroglial cells contribute to neuronal lipid synthesis through the supply of L-serine.

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L-Serine is indispensable for the biosynthesis of phosphatidyl-L-serine (PS) and sphingolipids in cells. These serine-derived lipids play important roles in cellular functions. For example, PS is essential for cell growth (1), whereas the mutations that reduce sphingolipid synthesis also inhibit the growth of Chinese hamster ovary cells (2) and budding yeast (3). Furthermore, these lipids have been shown to be involved in intracellular signal transduction. A certain member of the protein kinase C family interacts with PS (for review see Ref. 4). Recent biochemical studies have demonstrated that sphingolipid metabolites such as ceramide and sphingosine-1-phosphate function as signaling molecules involved in many cellular processes including apoptosis, proliferation, differentiation, and response to stresses (for reviews see Refs. 5 and 6). In the case of neurons of the central nervous system, our studies using neuronal cultures have demonstrated that ceramide plays distinct and significant roles in survival and phenotypic growth of cerebellar Purkinje cells (7, 8), hippocampal neurons (9), and spinal motoneurons (10).

Although L-serine is known to be a nonessential amino acid and to be metabolically formed from 3-phosphoglycerate, a metabolite of glycolysis, in cells (for review see Ref. 11), we have recently demonstrated that hippocampal astroglial cells release a significant amount of L-serine with rapid kinetics and that the amino acid is trophic for hippocampal neurons (12). These observations suggest the possibility that L-serine released by astrocytes is taken up by neurons and utilized for the synthesis of various cellular compounds, which might underlie the trophic action of L-serine on hippocampal neurons. In this study, we investigated whether externally supplied L-serine contributed to the biosynthesis of L-serine-derived membrane lipids in hippocampal neurons. Sphingolipids and PS disappeared rapidly when the neurons were maintained in the absence of exogenous L-serine. In parallel with the disappearance of these serine-derived lipids, a novel aminophospholipid appeared in the neurons. We identified the structure of this lipid as phosphatidyl-L-threonine (PT). Biochemical analysis indicated that PT was synthesized by a base exchange enzyme that catalyzed the synthesis of PS with a different affinity. The biological significance of the glial cell-derived L-serine in the lipid biosynthesis and in the survival of the neurons is discussed.

EXPERIMENTAL PROCEDURES

Hippocampal Neuronal Culture—Primary cultures of dissociated hippocampal neurons were prepared from fetal rat (Wistar) of 18 days of gestation as described previously (9). A total of 2 × 10^6 cells were plated onto a dish 100 mm in diameter (Falcon 3003, Becton Dickinson and Co., Franklin Lakes, NJ). After 24 h, the medium was changed to a serum-free minimum essential medium supplemented with 25 mM HEPES, 30 mM selenium, 500 mM pyruvate, 3.9 mM glutamine, 16.7 mM glucose, 100 mM putrescine, 10 μg/ml gentamicin sulfate, 0.1 mg/ml bovine serum albumin, 10 μg/ml bovine insulin, 0.1 mg/ml human apo-transferrin, and 20 mM progesterone. To establish glial cell-free cultures, cytosine-β-D-arabinoside (1 μM) was added to the serum-free medium.

Lipid Analysis—Neurons cultured for 6 days were harvested from the 100-mm dishes with a cell scraper in Ca^2+-,Mg^2+-free phosphate-buffered saline.
buffered saline. Lipids were then extracted using the Bligh-Dyer method (13). Upper and lower phases were collected and dried under N2 gas and dissolved in chloroform/methanol (2/1, v/v). TLC was performed on a Silica Gel 60 HPTLC plate (Merek, Darmstadt, Germany) in chloroform/methanol/12 m MgCl2 (5/4/1, v/v/v) and chloroform/methanol/formic acid (80/20/0.5, v/v/v) for gangliosides and phospholipids, respectively. The lipids were detected with iodine vapor (14), ninhydrin (14), primuline reagent (15), and Ruy-MacCoss reagent (16). For sensitive detection of gangliosides, the HPTLC plate treated with sialiase was stained with horseradish peroxidase-labeled cholera toxin B-subunit as described previously (17).

**Assay for Base Exchange Enzyme Activity**—The microsomal fraction from the adult Wistar rat brain was obtained as follows. Whole brain was homogenized in a homogenization buffer consisting of 0.25 M sucrose, 20 mM HEPES-KOH (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, and 0.5 mM phenylmethanesulfonyl fluoride. The homogenate was centrifuged at 600 × g for 15 min, followed by sequential centrifugations of the supernatant at 10,000 × g for 15 min and 150,000 × g for 15 min. The resultant microsomal pellet was resuspended in the homogenization buffer and used directly for the base exchange assay. Protein was quantified using the BCA reagent (Pierce, Rockford, IL).

Base exchange assay followed the method of Kanfer (19). The reaction mixture consisted of 50 mM HEPES-KOH (pH 7.5), 15 mM CaCl2, 0.5 mM [U-C14]-serine (37 MBq/mmol, NEN Life Science Products, Boston, MA) or [U-C14]-threonine (148 MBq/mmol, NEN Life Science Products, Boston, MA), and 200 μg of microsomal protein in a total volume of 200 μL. After incubation at 37 °C for 15 min, the reaction was terminated by the addition of chloroform/methanol (1/2, v/v), followed by partitioning using the Bligh-Dyer method. The chloroform phase was removed, dried, and dissolved in chloroform/methanol (2/1, v/v). After TLC, the radioactivities of PS and PT were quantified using the BAS-2000 imaging analyzer (FujiFilm, Tokyo, Japan).

**RESULTS AND DISCUSSION**

**Exogenous L-Serine Requirement for the Synthesis of Gangliosides and Phosphatidyl-L-threonine in Cultured Hippocampal Neurons**—To investigate the role of exogenous L-serine in the biosynthesis of lipids in hippocampal neurons, we compared the composition of membrane lipids in neuronal cultures maintained in the presence or absence of external L-serine. Because L-serine and glycine are interconvertible in cells and glycine is also trophic for hippocampal neurons (12), we examined the effects of glycine also. Hippocampal neurons were cultured in a serum- and glial cell-free setting for 6 days, and then the total lipids were extracted. We first analyzed the composition of sphingolipids, particularly that of gangliosides, because gangliosides but not sphingomyelin constituted the dominant species of sphingolipids in cultured hippocampal neurons (9). To visualize complex gangliosides containing PS, GM1a structure, gangliosides recovered in the upper phase of the HPTLC partition were separated by TLC and converted to GM1a by chordial sialidase treatment, and the resultant GM1a was detected by staining with horseradish peroxidase-labeled cholera toxin B-subunit (17). As shown in Fig. 1A, GM1a, GD1a, and GT1b were the major gangliosides observed when hippocampal neurons were maintained in the presence of exogenous L-serine or glycine (lanes 2 and 3). The levels of these neuronal gangliosides were significantly reduced in the absence of external L-serine or glycine (Fig. 1A, compare lane 1 with lane 2 or 3). A mixture of other nonessential amino acids, L-alanine, L-asparagine, L-aspartic acid, and L-proline, did not restore the amount of gangliosides.

We next analyzed the phospholipids in the lower phases of the Bligh-Dyer partition by TLC. Fig. 1B shows hippocampal neuronal aminophospholipids visualized by ninhydrin staining. When the hippocampal neurons were cultured under L-serine-free conditions, PS was specifically depleted, and a novel band, designated as X1, appeared slightly above the position of PS (Fig. 1B, lanes 1 and 4). This compound was labeled with Ruy-MacCoss reagent (not shown), indicating that it belongs to the same class of aminophospholipids as PS and phosphatidylethanolamine (PE). The absence of external L-serine or glycine did not alter the amounts of phosphatidylcholine and phosphatidylserinol (not shown). Although PE is also an L-serine-derived lipid, only a slight decrease in the level of PE was observed in the absence of L-serine. Because our culture medium did not contain free ethanolamine, PE cannot be synthesized by the direct coupling of CDP-ethanolamine and diacylglycerol (20). Therefore, the subtle decrease in the level of PE may be explained by the difference in the turnover rates and/or the size of the metabolic pool between PS and PE. **Structural Characterization of a Novel Phospholipid X1**—We determined the structure of X1. Hippocampal neuronal lipids were developed on TLC plates, and X1 and two other aminophospholipids, X2 and X3, were purified by scraping out the corresponding bands (Fig. 1B). The amino acid compositions of these lipids were analyzed after acid hydrolysis. Interestingly,
that L-serine is not available in sufficient quantities for the
simultaneous reduction in the levels of gangliosides and PS implies
and with C18:0/C22:6, respectively. The ions at the
usual lipid remain to be elucidated.

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resulting in PT expression may not occur in hippocampal neu-

threonine-containing lipid.

X1 possessed only L-threonine (Fig. 2A). X2 contained both L-threonine and L-serine (Fig. 2B), and L-serine was the domi-
nant amino acid of X3, indicating that X3 corresponds to PS (Fig. 2C). These analyses revealed that the novel lipid X1 is an L-threonine-containing lipid.

To characterize the structure of X1 in detail, we carried out TLC blotting/SIMS analysis. The negative SIMS spectrum of X1 is shown in Fig. 2D. A pair of major molecule-related ions, [M + Na – 2H]–, was observed at m/z 846 and 870, indicative of the structures of phosphatidyl-L-threonine with C18:0/C20:4 and with C18:0/C22:6, respectively. The ions at the m/z 888 and 912 peaks are presumably the adduct of CH2=CH2 derived from triethanolamine as reported for the free amino-containing gangliosides GM3 (21) and GM1 (22). The characteristic fragment ions generated by sequential elimination of these constituents were at m/z 723 and 747 for phosphatidic acid (C18:0/ C20:4 and C18:0/C22:6, respectively), at m/z 560 and 582 for lysophosphatidylthreonine (–H and +Na – 2H, respectively), and at m/z 459 for lysophosphatidic acid (C18:0). We therefore conclude that X1 corresponds to PT. The most abundant unsaturated fatty acid on brain PS was reported to be C18:1 followed by C22:6 (23). In fact, the peak corresponding to PT with C18:0/18:1, [M + Na – 2H]–, was detected at m/z 824 in X2 (data not shown). Thus, the fatty acid composition of PT from cultured hippocampal neurons is almost identical to that of PS in mammalian brain. The existence of PT in neurons is being reported for the first time, although this lipid has been shown to exist in tuna muscle (24) and polyoma-transformed hamster embryo fibroblasts (25, 26). Because of the presence of astroglial cells, conditions of L-serine and glycine depletion resulting in PT expression may not occur in hippocampal neurons in vivo. Therefore, the physiological functions of this unusual lipid remain to be elucidated.

In Vitro Synthesis of Phosphatidyl-L-threonine—The simultaneous reduction in the levels of gangliosides and PS implies that L-serine is not available in sufficient quantities for the synthesis of these lipids in hippocampal neurons cultured in the absence of L-serine or glycine. The concomitant appearance of PT raises the possibility that neo-synthesis of PT is catalyzed by a base exchange enzyme that is involved in PS biosynthesis. To test this assumption, we examined whether the synthesis of PT was inhibited by L-threonine. We used microsomes from rat whole brain as an enzyme source, because only a small amount of microsomes were obtained from the cultured neurons. As shown in Fig. 3A, L-threonine certainly inhibited PS synthesis in vitro, although the amount of L-threonine required for such inhibition is rather high. The Lineweaver-Burk plot indicated that L-threonine is a competitive inhibitor with a Ki value of 23 mM, whereas the Km value for L-serine was 0.11 mM. We then examined whether PT was synthesized from L-threonine instead of L-serine as a substrate (Fig. 3B). The formation of PT was dependent on L-threonine concentration, and the Km value for L-threonine was determined to be 16 mM, which is much higher than that for L-serine. The synthesis of PT was greatly inhibited by the addition of lower concentrations of L-serine. The Lineweaver-Burk plot indicated that L-serine competitively inhibited the formation of PT with a Ki value of 0.10 mM. We also observed the synthesis of PT using rat liver micro-
somes (data not shown). These results indicate that PT is synthesized by the base exchange enzyme that catalyzes the synthesis of PS with much lower affinity.

Our present study demonstrates that in the absence of exogenous L-serine, hippocampal neurons synthesize PT instead of PS. The generation of PT implies that the intracellular level
of L-serine is insufficient for the synthesis of PS when the neurons are maintained in the absence of exogenous L-serine. This is supported by the observation that exogenous glycine also maintains the level of PS (Fig. 1B). Glycine is metabolically converted to L-serine by serine hydroxymethyltransferase (27). Furthermore, this assumption explains why ganglioside biosynthesis, which requires L-serine, is also inhibited when cultured in the absence of external L-serine or glycine. We have recently reported that significant amounts of L-serine are released from hippocampal astrocytes but not neurons and that this amino acid promotes the survival and morphological development of hippocampal neurons (12). Thus, we suggest that hippocampal neurons lose the ability to synthesize sufficient quantities of L-serine and consequently depend on astroglial cells for this amino acid. PT was not detected in lipids extracted from hippocampal regions of normal rat brains (data not shown), suggesting that L-serine was not depleted in normal brain tissues. Indeed, extracellular free L-serine has been reported to be present in the central nervous system (28). L-Serine released by astroglial cells probably contributes to the synthesis of L-serine-derived membrane lipids as well as other cellular components in the neurons, which are necessary for the survival of the neurons in vitro and also probably in vivo.

At present, the molecular basis of exogenous L-serine requirement in hippocampal neurons is unknown. However, our most recent work demonstrated that the expression of mRNA of 3-phosphoglycerate dehydrogenase, a rate-limiting enzyme for L-serine synthesis, is significantly reduced in hippocampal neurons. This down-regulation might underlie the dependence of hippocampal neurons on astroglial cells for L-serine. Neurons have to take up exogenous L-serine, which is presumably released from astroglial cells, through a specific transporter system. In this context, a cell membrane transporter for L-serine must be regarded as a key factor in terms of the regulation of lipid biosynthesis in neurons and, eventually, neuronal survival.

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