Journal of Neurochemistry, 2004, **89**, 142–151 doi:10.1111/j.1471-4159.2004.02308.x

Phospholipase D2 functions as a downstream signaling molecule of MAP kinase pathway in L1-stimulated neurite outgrowth of cerebellar granule neurons

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Abstract

Stimulation of the neuronal cell adhesion molecule L1 in cerebellar granule neurons (CGNs) enhances neurite outgrowth and this response is inhibited by the primary alcohol ethanol. Because primary alcohols suppress the formation of the signaling lipid phosphatidic acid (PA) by phospholipase D (PLD), this observation prompted us to investigate whether PLD plays a role in the L1-mediated neurite outgrowth in CGNs. In the cerebellum of postnatal day 8 mice, PLD2 protein was abundantly expressed, while PLD1 expression was not detected. The L1-stimulated neurite outgrowth was inhibited by primary alcohols and by overexpression of lipase-deficient PLD2. Increases in cellular PA levels by direct PA application or overexpression of wild-type PLD2 mimicked the L1-dependent stimulation of neurite outgrowth. Furthermore, it was found that L1 stimulation in CGNs increased PLD activity concomitantly with phosphorylation of extracellular signal-regulated kinase (ERK), both of which were inhibited by the MAP kinase-ERK kinase (MEK) inhibitor. These results provide evidence that PLD2 functions as a downstream signaling molecule of ERK to mediate the L1-dependent neurite outgrowth of CGNs, a mechanism that may be related to alcohol-related neurodevelopmental disorders.

Keywords: alcohol, cerebellar granule neurons, mitogen-activated protein kinase, neural cell adhesion molecule L1, neurite outgrowth, phospholipase D.

J. Neurochem. (2004) **89,** 142–151.

Fetal alcohol syndrome (FAS), which includes neurological abnormalities such as mental retardation, is provoked by excessive alcohol consumption during pregnancy (Jones and Smith 1973). Although the direct consequence of ethanol on the developing brain remains largely unknown, it has been proposed that they may be closely related to the effects seen after the disruption of L1 function (Charness *et al*. 1994), a member of the Ig superfamily of cell adhesion molecule implicated in a number of developmentally important processes including neuronal cell migration (Lindner *et al*. 1983), axonal outgrowth (Lagenaur and Lemmon 1987) and axonal fasciculation (Stallcup and Beasley 1985; Landmesser *et al*. 1988). Similar to the described neuropathological abnormalities observed in FAS, patients carrying mutations

Received August 19, 2003; revised manuscript received November 24, 2003; accepted November 28, 2003.

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Abbreviations used: ARF, ADP-ribosylation factor; BSA, bovine serum albumin; CGNs, cerebellar granule neurons; CNS, central nervous system; DAG, diacylglycerol; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular signal-regulated kinase; FAS, fetal alcohol syndrome; HRP, horseradish peroxidase; HS, horse sreum; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase-ERK kinase; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PEt, phosphatidylethanol; PLD, phospholipase D; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

in the L1 gene show abnormal brain development that is characterized by mental retardation and defects in central nervous system (CNS) axon tracts (Rosenthal *et al*. 1992 and for review see Kamiguchi *et al*. 1998). A putative link on a cellular level is indicated by the report that ethanol inhibits the L1-mediated neurite outgrowth of cerebellar granule neurons (CGNs) prepared from postnatal day 6 (P6) rat (Bearer *et al*. 1999). This report suggests that the toxicity of ethanol to the developing neurons is accountable for the inhibition of the L1-mediated neurite outgrowth. The molecular mechanism linking ethanol to the L1-mediated neurite outgrowth response, however, still remains unclear.

One of the known consequences of ethanol in cells is its antagonistic effect on phospholipase D (PLD) ability to produce the pleiotropic signaling lipid messenger phosphatidic acid (PA). PLD catalyzes the hydrolysis of the membrane phospholipid phosphatidylcholine (PC) to produce PA (Liscovitch *et al*. 2000). In the presence of primary alcohols, such as ethanol and butan-1-ol, PLD preferentially catalyzes a transphosphatidylation reaction, which transfers the phosphatidyl group of PC to the hydroxyl group of primary alcohols to yield the non-physiological phosphatidylalcohols, at the expense of PA. As this reaction is favored over the physiological hydrolysis reaction, primary alcohols suppress the PLD-catalyzed production of the physiological signaling lipid PA, thereby disturbing signaling pathways and cellular functions of PLD (Liscovitch *et al*. 2000). If PLD plays an essential role in neuronal development through its product PA, alcohol-related neurodevelopmental disorders can be in part interpreted by ethanol inhibition of the PLD-catalyzed PA production.

Mammalian PLD consists of two related gene products, PLD1 and PLD2 (Colley *et al*. 1997a; Hammond *et al*. 1997). PLD1 is directly regulated by classical protein kinase C, members of the ADP-ribosylation factor (ARF) and Rho family small GTPases in conjunction with phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] (Hammond *et al*. 1997), and contributes to a wide variety of cellular responses, including membrane trafficking and exocytosis (Ktistakis *et al*. 1996; Chen *et al*. 1997; Humeau *et al*. 2001; Vitale *et al*. 2001). In contrast, PLD2 is constitutively active in the presence of PI(4,5)P2 and insensitive to PLD1 activators *in vitro* (Colley *et al*. 1997a). PLD2 has been implicated in several cellular functions, such as receptor-mediated endocytosis (Shen *et al*. 2001) and anti-apoptotic signaling (Lee *et al*. 2000). In addition, we have previously suggested that PLD2 is involved in actin-based membrane dynamics such as membrane ruffling in non-neuronal cells (Honda *et al*. 1999). As actin-based membrane dynamics are critical for axonal outgrowth in response to extracellular guidance cues (Dickson 2001), we speculate that PLD2, of the two mammalian PLD isozymes, may be involved in axonal outgrowth. This idea is also supported by the report that PLD2 mRNA levels increase strikingly in the dentate gyrus of the prenatal and postnatal hippocampus and the granule cell layer of the cerebellum at early postnatal life (Colley *et al*. 1997b; Saito *et al*. 2000) during the critical period of neuronal differentiation and outgrowth (Jacobson 1991).

Here we provide the evidence of an involvement of PLD2 activity in the L1-mediated neurite outgrowth response in CGNs. Increases in the levels of the PLD product PA by direct application or overexpression of PLD2 in neurons mimicked the L1-dependent stimulation of neurite outgrowth. Moreover, PLD2 functions downstream of the MAP kinase pathway in the L1-mediated signaling pathway. These results provide novel insights into important aspects of the physiological function of PLD2 in neurons, and may indicate a feasible molecular mechanism affected in alcohol-related neurodevelopmental disorders.

Materials and methods

Materials and reagents

A rabbit polyclonal anti-PLD D4 antibody that detects both PLD1 and PLD2 (Fig. 1a) was raised against the bacterial recombinant peptide corresponding to amino acids 714–1074 of PLD1, and then affinity-purified. A rat monoclonal anti-mouse PLD2 411A antibody that specifically recognized PLD2 (Fig. 1a) was generated by rat medial iliac lymph node method (Kishiro *et al*. 1995). Briefly, WKY/NCrj rats were immunized with 0.2 mg of the bacterial recombinant peptide corresponding to amino acids 1–533 of mouse PLD2. After 3 weeks, the lymph node-derived lymphocytes were fused with SP2/O myeromas. The hybridoma clones that produce the anti-mouse PLD2 antibody were selected by ELISA and western blotting. Anti-L1 antiserum was a generous gift from Dr V. Lemmon (University of Miami School of Medicine, FL, USA). PA and PC prepared from egg were purchased from Avanti Polar Lipids (Alabaster, AL, USA). The following antibodies and probes were purchased from commercial sources: anti-actin antibody (Sigma, St Louis, MO, USA); anti-ERK2 D-2 and anti-phosphorylated ERK E-4 (anti-p-ERK E4) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA); horseradish peroxidase (HRP)-conjugated anti-mouse IgG antibody (Transduction Laboratory, Lexington, KY, USA); HRP-conjugated anti-rat IgG antibody (Dako, Glostrup, Denmark); HRP-conjugated anti-rabbit IgG antibody (Amersham, Arlington Heights, IL, USA); anti-GFP antibody (MBL, Nagoya, Japan); Alexa 488 phalloidin (Molecular Probes, Eugene, OR, USA); Cy3-conjugated secondary antibody (Jackson Immuno-Research Laboratory, West Grove, PA, USA).

Culture of CGNs

Primary cultured mouse CGNs were prepared by the method of Schnitzer and Schachner (1981) with minor modifications. Briefly, cerebella dissected from P8 ICR mice were treated with 10 U/mL papain and 100 U/mL DNase (Takara, Tokyo, Japan) in Dulbecco's modified Eagle's medium (DMEM) at 37°C for 20 min. The dissociated cells were then plated onto poly-L-lysine (PLL)-coated coverslips and cultured in DMEM supplemented with 10% horse serum (HS) and 25 mM KCl. Purity of CGNs thus prepared was greater than 95%.

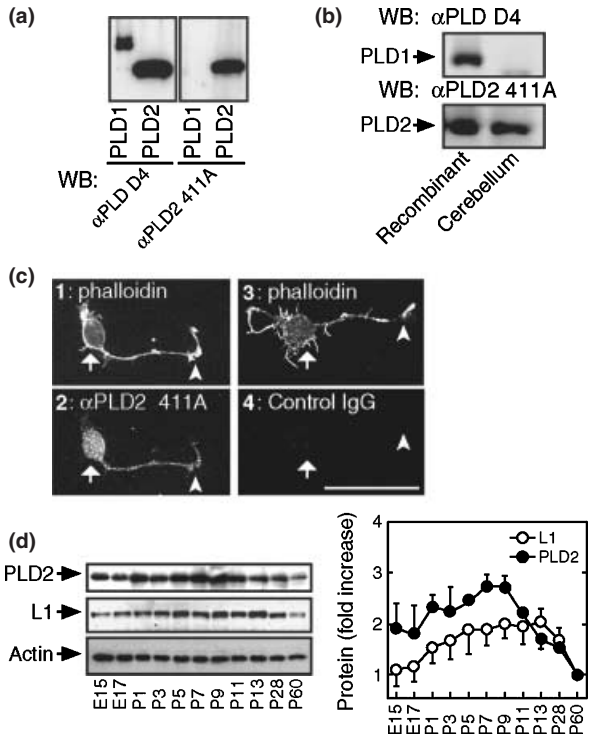

Fig. 1 Expression of PLD isozymes in the cerebellum and in isolated CGNs. (a) Specificity of PLD antibodies. Membrane fractions of COS7 cells expressing PLD1 or PLD2 were separated by SDS–PAGE, and specificity of the polyclonal anti-PLD D4 and the rat monoclonal anti-PLD2 411A antibodies for PLD isozymes were assessed by western blotting. Whilst the D4 antibody reacted with both isoforms, the 411A specifically recognized PLD2. (b) Expression of the PLD isozymes in the mouse cerebellum. Homogenate of P8 mice cerebella was immunoblotted with the anti-PLD D4 and the anti-PLD2 411A antibodies. Note the complete absence of PLD1 in the cerebellum. (c) Immunofluorescent staining of endogenous PLD2 in CGNs prepared from P8 mice. CGNs cultured for 24 h on PLL-coated coverslips were labeled with phalloidin (1 and 3) and the anti-PLD2 411A antibody (2). Background fluorescence was assessed using a control rat IgG (4). Punctate staining was seen in the neuronal growth cone-like structure (arrowheads), in the neurite and the cell body (arrows). Scale bar, 20 μm. (d) Developmental profiles of PLD2 and L1 expression in mouse cerebella. Homogenates (100 μg of protein) of mouse cerebella at the indicated stages in development were probed with the PLD2 411A and an L1-antibodies (left panel). As an internal standard, actin was immunoblotted with the anti-actin antibody. Expression levels of proteins were then quantified by the NIH image (right panel). The data, which were normalized with the internal standard actin, represent the mean ± SD of three independent experiments as a ratio of fold increase compared with the value on P60. E, embryonic days; P, postnatal days.

To observe the neurite outgrowth enhanced by L1 stimulation, CGNs (5.3×10^4 cells/cm^2) were plated on coverslips coated with the purified L1-Fc fusion protein (100 μg/mL), which consists of the Fc region of human IgG and the extracellular domain of human L1, over PLL, and cultured in DMEM supplemented with 10% HS and 25 mM KCl. In control experiments, a recombinant Fc-protein was coated on coverslips over PLL. For PA application to CGNs, PA in chloroform was dried under nitrogen, re-suspended in the culture medium by sonication and vortex, and immediately added to primary culture of CGNs.

Transient expression of PLD in primary cultured CGNs

cDNAs for wild-type (WT)-PLD1 and -PLD2 and their lipase-deficient (LD) mutants were constructed as described previously (Sung *et al*. 1997). For experiments of effects of LD-PLD mutants on the L1-stimulated neurite outgrowth, plasmids for LD-PLDs and EGFP (pEGFP-N3; Clontech Laboratories, Palo Alto, CA, USA) were cotransfected into primary cultured CGNs (1DIV) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). After 1-day culture, cells were harvested and re-plated at 5.3×10^4 cells/cm^2 on Fc- or L1-Fc-coated coverslips. In experiments for effects of PLDs on neurite outgrowth of non-stimulated CGNs, PLDs and EGFP were coexpressed by means of a sindbis virus expression system. For these experiments, cDNAs for PLDs were inserted into the *Xba*I/*Stu*I site in the pSinRep5-IRES-EGFP (Aizawa *et al*. 2001). The plasmids were linearized, and transcribed *in vitro*. These transcripts and the transcript of DH (26S) helper DNA (Invitrogen) were cotransfected into BHK cells by electroporation, and after 36 h the virus particles were harvested. Twenty μL of the virus solution was added to CGNs (1.3×10^6 cells/cm^2) cultured in 1 mL of medium on PLL-coated coverslips, and incubated for 30 h to express PLDs.

Estimation of neurite length of CGNs

The lengths of neurites were measured as the distance between the center of the cell body and the tip of neurite, and only neurites longer than 10 μm were analyzed. In experiments to examine the effects of L1 and PA on neurite outgrowth, neurite lengths of neurons that do not contact with other cells were measured. CGNs showing abnormal membrane blebbing and fragmentation, in addition to Purkinje cells characterized by their dendritic arbors and large somata (> 15 μm), were discarded from all analyses. To quantify the neurite lengths in each condition, several investigators blindly analyzed more than 100 CGNs in randomly selected fields. Statistical significance was evaluated using double-tailed Student's *t*-test.

Assay for PLD activity

PLD activity was assessed by measuring the formation of [^{32}P]phosphatidylethanol ([^{32}P]PEt), which is the unambiguous PLD marker produced in the presence of ethanol. CGNs were labeled with 25 μCi/mL [^{32}P]Pi at 37°C for 1.5 h in Pi-free MEM (Gibco BRL, Grand Island, NY, USA) supplemented with 25 mM Hepes, pH 7.5, and 1% bovine serum albumin (BSA), after being cultured for 3 days on PLL-coated dishes and subsequently starved for 4 h in Opti-MEM (Gibco BRL). After being washed, the labeled CGNs were incubated with control serum or 50 μL/mL of the anti-L1 antiserum in the presence of 1% ethanol at 37°C for 30 min. Lipids were extracted by the method of Bligh and Dyer (1959) and separated by two-dimensional TLC using a solvent system consisting of chloroform–methanol–ammonium hydroxide (65 : 35 : 5.5 v/v) and chloroform–methanol–acetic acid (65 : 15 : 2 v/v) in the first and the second dimensions, respectively. The [^{32}P]PEt produced

was analyzed by a BAS2000 Bio-imaging analyzer (Fuji Photo Film, Tokyo, Japan) and expressed as a percentage of total [^{32}P]phospholipids. The data are represented as the mean ± difference of duplicate determinations representative of at least three independent experiments with similar results.

Western blotting

Western blotting was performed as previously reported (Yamazaki *et al*. 1999). In brief, proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene difluoride membranes. The membrane was blocked with the basic buffer consisting of 50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$ and 80 mM NaCl supplemented with 0.2% Nonidet P-40 and 5% BSA, and then incubated with primary antibodies diluted in blocking buffer; anti-L1 antiserum (1 : 1000 dilution), anti-PLD D4 (0.5 μg/mL), anti-PLD2 411A (2 μg/mL), anti-actin (1 : 500 dilution), anti-ERK2 (1 : 1000 dilution) and anti-p-ERK E4 antibodies (1 : 1000 dilution). After incubation with the HRP-conjugated secondary antibodies in the basic buffer supplemented with 0.2% SDS and 2% Nonidet P-40, immunoreactive proteins were detected with an ECL immunoblotting detection reagent (Amersham Bioscience, Buckinghamshire, UK).

Confocal immunofluorescent microscopy

CGNs were fixed, permeabilized and blocked as previously reported (Honda *et al*. 1999). Briefly, cells were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) on ice for 30 min. After permeabilization in 0.1% Triton X-100 and 0.1% Tween 20, cells were blocked with 1 mg/mL BSA in PBS, and stained by sequential incubation with the anti-PLD2 411A (2 μg/mL) in PBS containing 0.05% Tween 20 and with the Cy3-conjugated secondary antibody (1 : 1000 dilution). F-actin was visualized by Alexa 488 phalloidin (1 : 1000 dilution). Cells were then imaged using an Axiovert S100 Zeiss fluorescent microscope (Zeiss, Gottingen, Germany) equipped with an ORCA-ER CCD camera (Hamamatsu Photonics, Shizuoka, Japan) and the confocal scanner unit CSU21 (Yokogawa Electric Co., Tokyo, Japan).

Results

Abundant expression of PLD2 protein in early postnatal cerebella and cerebellar granule neurons

In order to examine a possible involvement of PLD1 and/or PLD2 in neuronal function in the CNS, we first analyzed the expression levels of the different PLD isozymes by western blotting. In the membrane fraction prepared from COS7 cells expressing PLD1 or PLD2, the D4 antibody recognized both PLD isoforms, whilst another antibody, 411A, specifically reacted with PLD2 only (Fig. 1a). Using these antibodies, it was found that in mouse cerebellum at P8, PLD2 was abundantly expressed, whereas expression of PLD1 could not be detected (Fig. 1b). As CGNs outnumber the other types of cells in the cerebellum at P8-9 by a thousand-fold (Carlson *et al*. 1998), this result indicates that CGNs express only PLD2. Expectedly, expression of PLD2 protein was also detected by immunostaining at punctate structures in the growth cone-like structure, neurite shaft and cell body of CGNs prepared from P8 mouse (Fig. 1c). In agreement with the previous report that PLD2 mRNA level increases strikingly in the granule cell layer in cerebellum at early postnatal life (Saito *et al*. 2000), PLD2 protein level in cerebellum was steady higher during early postnatal days 1–13, concomitant with the time when maximal numbers of neurons are differentiating and pathfinding (Jacobson 1991), and thereafter decreased (Fig. 1d). Interestingly, this developmental profile of PLD2 expression was very similar to that of L1 expression (Fig. 1d), indicating a link of the enzyme in L1-dependent events during the period of the formation of neural connectivity. These results, taken together, suggest that PLD2, of two PLD isozymes, is involved in the L1-dependent development of CGNs.

Suppression of L1-mediated neurite outgrowth of CGNs by primary alcohols and LD-PLD2

In CGNs, stimulation of the neural cell adhesion molecule L1 enhances neurite outgrowth (Lagenaur and Lemmon 1987) and this response is inhibited by the primary alcohol ethanol (Bearer *et al*. 1999). As PLD preferentially catalyzes transphosphatidylation reaction over the hydrolysis reaction in the presence of primary alcohols at the expense of production of its physiological product PA, we reasoned that PLD2 might be involved in L1-mediated neurite outgrowth in CGNs through its product PA. If this is the case, butan-1-ol, in addition to ethanol, but not butan-2-ol (which is not the substrate for the PLD-catalyzed transphosphatidylation reaction), might also interfere with the L1-mediated neurite outgrowth. As was expected, the L1-mediated neurite outgrowth was fully inhibited by butan-1-ol as well as ethanol, whereas butan-2-ol was less effective (Fig. 2a,b). In contrast, the basal outgrowth on control substrate was not affected by any these alcohols (Fig. 2b, right panel). Furthermore, it was found that overexpression of LD-PLD2, but not that of LD-PLD1, completely inhibited the L1-mediated neurite outgrowth (Fig. 2c). These results strongly support the idea that PLD2 is specifically involved in the signaling pathway of the L1-mediated neurite outgrowth response.

Overexpression of WT-PLD2 and application of PA mimics the L1-dependent stimulation of neurite outgrowth of CGNs

In order to monitor directly the effects of PLD activity on the outgrowth rate of CGNs, we introduced WT- and LD-version of PLD1 or PLD2 into CGNs by the sindbis virus expression system. Localization of overexpressed WT-PLD2 was very similar to that of endogenous PLD2 shown in Fig. 1(c), whereas overexpressed WT-PLD1 exclusively located in the cell body (Fig. 3a). Overexpression of PLD2 significantly enhanced the neurite outgrowth of CGNs in a lipase activity-dependent manner (Fig. 3b,c, left panels), whilst introduction

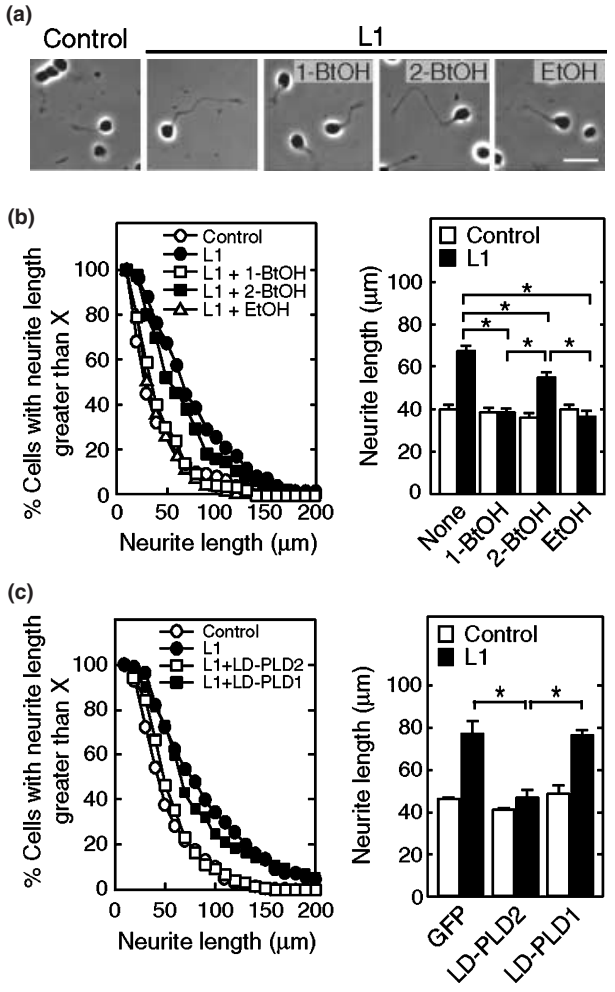

Fig. 2 Suppression of L1-stimulated neurite outgrowth of CGNs by primary alcohols and LD-PLD2. (a and b) CGNs were cultured for 22–26 h on recombinant Fc protein- (control) or L1-Fc fusion protein-coated coverslips (L1) in the presence or absence of 0.25% (27 mM) butan-1-ol (1-BtOH), 0.25% (27 mM) butan-2-ol (2-BtOH) or 0.6% (104 mM) ethanol (EtOH). After cells were fixed, neurites were observed by phase contrast microscopy (a). Neurite lengths were measured and plotted in a distribution curve of neurite lengths (b, left panel). The averages of neurite lengths were also calculated, and results were presented as the mean ± SEM of three independent experiments (b, right panel). (c) Primary cultured CGNs in which EGFP and LD-PLDs were transiently expressed were re-plated on Fc protein- (control) or L1-Fc fusion protein-coated coverslips (L1), and cultured for 24 h. Neurite lengths of CGNs expressing EGFP and LD-PLDs were plotted in a distribution curve of neurite lengths (left panel), and the averages of neurite lengths were presented as the mean ± SEM of three independent experiments (right panel). An asterisk denotes statistical significance (b and c, right panels; $p < 0.05$).

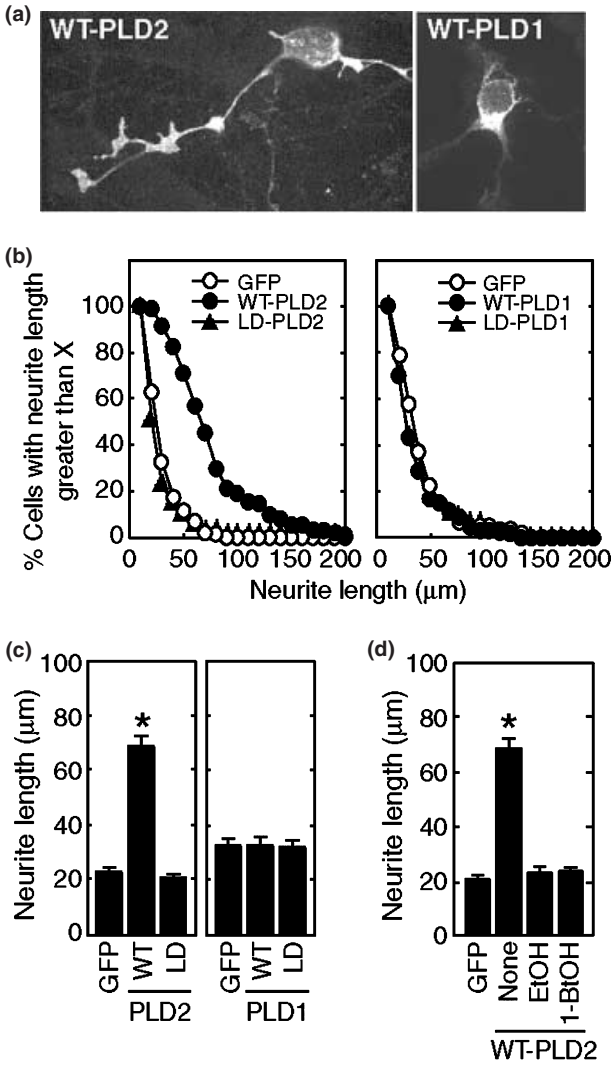

Fig. 3 Stimulation of neurite outgrowth of CGNs by overexpression of PLD2 and its inhibition by primary alcohols. CGNs were plated on PLL-coated coverslips, and then WT- and LD-PLD isozymes were overexpressed with EGFP using the sindbis virus expression system as described in the Materials and methods section. (a) After 30 h, cells were fixed, and transiently expressed PLDs were immunofluorescently stained. (b) The neurite lengths of CGNs expressing EGFP were plotted in a distribution curve of neurite lengths. (c) The averages of neurite lengths were also calculated, and results were presented as the mean ± SEM of three independent experiments. (d) CGNs coexpressed with EGFP and WT-PLD2 were incubated in the presence or absence of 0.6% (104 mM) ethanol (EtOH) and 0.25% (27 mM) butan-1-ol (1-BtOH) for 30 h. The averages of neurite lengths were determined, and results were presented as the mean ± SEM of three independent experiments. An asterisk denotes statistical significance (c and d; $p < 0.05$).

of WT- or LD-PLD1 had no effect in this system (Fig. 3b,c, right panels). Thus, overexpression of WT-PLD2 phenocopies the L1 stimulation of neurite outgrowth in CGNs, suggesting that the L1 stimulation of neurite outgrowth is mediated by PLD2. Again, primary alcohols, ethanol and butan-1-ol, almost completely inhibited the neurite outgrowth stimulated by WT-PLD2 overexpression (Fig. 3d). These results also supports the notion that PLD2, but not PLD1,

was selectively found to be expressed in CGNs and highlights that rather than a general PLD-dependent mechanism, PLD2 mediates L1-mediated neurite outgrowth through its product PA.

In general, PLD transduces signals to downstream effectors through its product PA. As described above, it appears that PLD2 functions as a key player in the L1-mediated neurite outgrowth through its product PA. In support of this idea, addition of PA to the CGN culture strongly enhanced neurite outgrowth, reaching a plateau at 1 μM, and 1 μM of PC was without effect (Fig. 4). In cells, PA can be metabolized to diacylglycerol (DAG) and lysoPA by PA phosphatase and phospholipase A2, respectively, indicating a possibility that these PA metabolites stimulate neurite outgrowth. However, direct application of DAG or lysoPA to CGN cultures did not alter the outgrowth rate (data not shown). These results, taken together with the findings that WT-PLD2, but not LD-PLD2, enhanced the neurite outgrowth (Fig. 3) and that LD-PLD2, but not LD-PLD1, inhibited the L1-stimulated neurite outgrowth (Fig. 2c), strongly suggest that PLD2 plays a crucial role in the L1-mediated neurite outgrowth though its product PA.

Activation of PLD2 through ERK in L1-stimulated CGNs

To substantiate the coupling of the L1 response to the activation of PLD2, we monitored PLD activity in L1-stimulated CGNs. L1-mediated responses can be activated by antibodies specifically recognizing L1 that are known to trigger signaling events indicative of homophilic binding

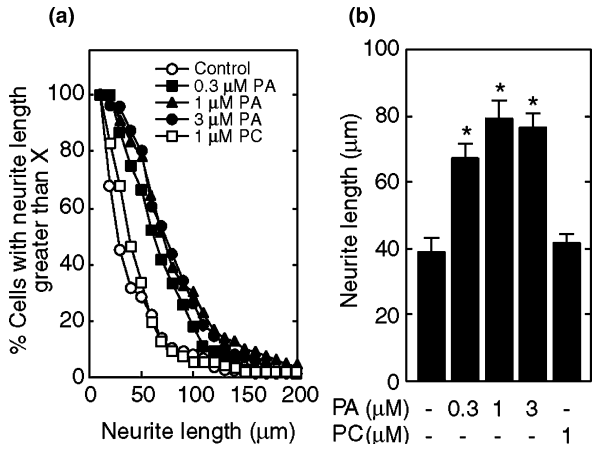

Fig. 4 Stimulation of neurite outgrowth of CGNs by the PLD product PA. CGNs were plated on PLL-coated coverslips and cultured for 24 h in the absence or presence of PA or PC at the indicated concentrations. (a) After cells were fixed, neurite lengths were plotted in a distribution curve of neurite lengths. (b) The averages of neurite lengths were also calculated, and results were presented as the mean ± SEM of three independent experiments; an asterisk denotes statistical significance ($p < 0.05$).

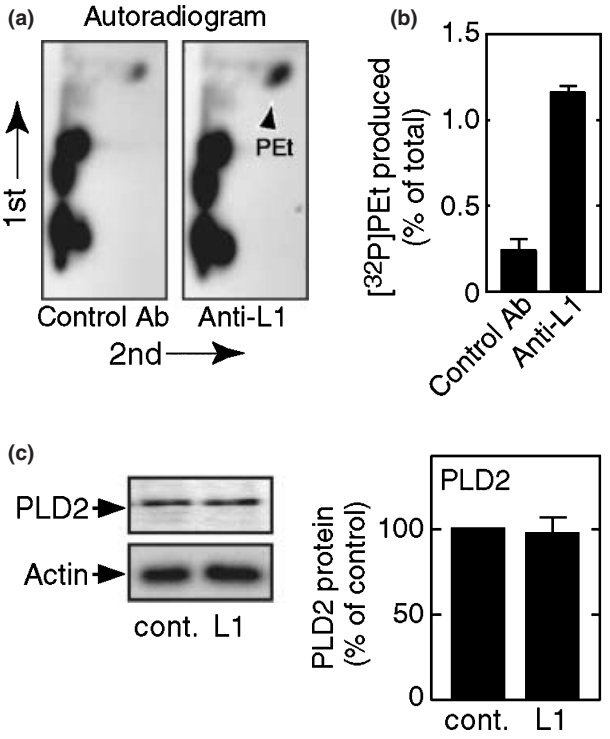

Fig. 5 Activation of PLD2 in CGNs by L1 stimulation. (a and b) CGNs plated on PLL-coated dishes were incubated for 1.5 h with [^{32}P]Pi (25 μCi/assay) to label membrane phospholipids, and then free [^{32}P]Pi was washed out. The ^{32}P-labeled CGNs were incubated with control serum (control Ab) or the anti-L1 antiserum (anti-L1) for 30 min in the presence of 1% ethanol. After lipids were extracted, [^{32}P]PEt produced was separated by TLC and visualized by autoradiography (a). [^{32}P]PEt separated by TLC was estimated as described in the Materials and methods section (b). The result is from a single experiment in duplicate and representative of three with similar results. The error bars represent the differences of duplicate determinations. (c) CGNs were cultured for 24 h on Fc protein- (cont.) or L1-Fc fusion protein-coated dishes (L1), and the cell lysate was immunoblotted with the anti-PLD2 411A and anti-actin antibodies (left panels). Expression levels of PLD2 were then quantified by the NIH image (right panel). The data, which were normalized with the internal standard actin, represent the relative expression levels of PLD2 protein as compared with the value of control. The error bar represents the SD of three independent experiments.

interactions (Schmid *et al.* 2000). As shown in Fig. 5(a,b), stimulation of L1 in CGNs activated PLD2 as assessed by production of the unambiguous marker of PLD activity phosphatidylethanol (PEt) in the presence of ethanol. In the absence of ethanol, increase in the physiological PLD2 product PA by L1 stimulation was also detected using ^{32}Pi-labeled CGNs (data not shown): [^{32}P]PA increased from 4.3% of total [^{32}P]lipids in non-stimulated control cells to 5.7% by L1 stimulation, and the L1-stimulated PA production was significantly suppressed when ethanol was included in the reaction. Increased production of PEt and PA by PLD2

in response to L1 stimulation was not due to increase in PLD2 protein expression, as L1 stimulation did not change the PLD2 protein level (Fig. 5c). These results, taken together with the results described above, confirm that the L1-mediated signaling links to PLD2 activation in CGNs.

It has recently been reported that L1 stimulation in rodent neuroblastoma B35 cells and CGNs phosphorylates and activates ERK, which is an essential step in order for the subsequent neurite outgrowth to occur (Schmid *et al*. 2000 and for review see Doherty *et al*. 2000). To analyze the relationship between ERK-dependent signaling pathway and PLD2 activation in the L1 response, we used a chemical inhibitor of MAP kinase-ERK kinase (MEK), U0126. As had been reported (Schmid *et al*. 2000), L1 stimulation by the L1 antibody increased the phosphorylation level of ERK2 significantly and ERK1 to a lesser extent, and their phosphorylation was markedly suppressed by U0126 (Fig. 6a). L1-stimulated PLD activity was concomitantly suppressed by treatment with U0126 (Fig. 6b). In non-stimulated state of cells, however, PLD activity was not inhibited by U0126 whereas the inhibitor evidently interfered with basal level of ERK phosphorylation. Although, at present, we cannot clearly elucidate this discrepancy observed in resting cells, there may be a threshold in the level of ERK phosphorylation to stimulate PLD2 activity in CGNs. Nonetheless, these results demonstrate that PLD2 is a downstream signaling molecule of ERK in the L1-mediated signaling pathway that triggers neurite outgrowth from CGNs.

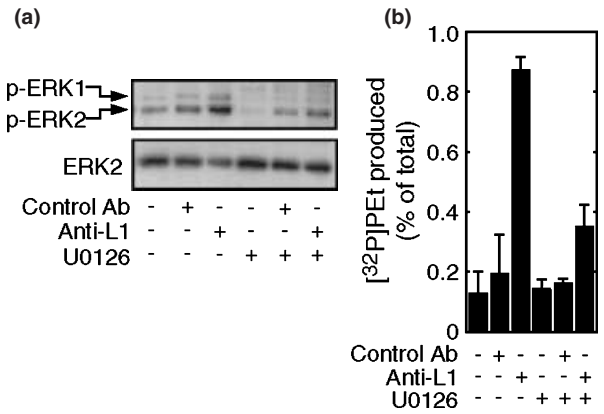

Fig. 6 Inhibition of L1-stimulated PLD2 activation by the MEK inhibitor U0126. (a) CGNs were incubated without or with control serum (control Ab) or the anti-L1 antiserum (anti-L1) in the presence or absence of the MEK inhibitor U0126 (10 μM) for 10 min. After proteins were separated by SDS–PAGE, the ERK2 protein and phosphorylation of ERK1 and 2 were detected by western blotting using the anti-ERK D2 and the anti-p-ERK E4 antibodies, respectively. (b) ^{32}P-labeled CGNs were incubated with control serum (control Ab) or the anti-L1 antiserum (anti-L1 in the presence or absence of 10 μM U0126 for 30 min, and [^{32}P]PEt was determined. Shown in (b) is the result from a single experiment in duplicate and representative of three with similar results. The error bars represent the differences of duplicate determinations.

Discussion

In the present study, we demonstrated that PLD plays a crucial role in the signaling pathway of the L1-mediated neurite outgrowth downstream of ERK MAP kinase activation. The PLD isozyme involved in this pathway is most likely to be PLD2, which was supported by the findings that only PLD2, but not PLD1, was expressed in cerebella, overexpression of WT-PLD2 in CGNs mimicked the effect of L1 stimulation in enhancement of neurite outgrowth, and LD-PLD2 inhibited the L1-stimulated neurite outgrowth.

Molecular mechanism for regulation of the L1-mediated neurite outgrowth by PLD2

In the L1-mediated neurite outgrowth of CGNs, the signaling from PLD2 to neurite outgrowth seemed to be mediated by the PLD2 product PA. This is supported by the findings that (i) primary alcohols that interfere with the PLD-catalyzed PA production and overexpression of LD-PLD2 completely inhibited the L1-stimulated neurite outgrowth (Fig. 2); (ii) stimulation of neurite outgrowth by overexpression of PLD2 was dependent upon the lipase activity (Fig. 3); and (iii) that direct PA application to CGNs significantly stimulated neurite outgrowth of non-stimulated control cells (Fig. 4). Although we cannot entirely exclude the possibility that the inhibition by primary alcohols is, in part, attributable to the formation of the non-physiological phospholipid phosphatidylalcohols in the plasma membrane, these results, taken all together, provide evidence that PA production by PLD2 is required for the L1-stimulated neurite outgrowth. Moreover, increase in cellular PA is sufficient to mimic this response. Although PLD1, as well as PLD2, catalyzes the hydrolysis of PC to produce PA, overexpression of PLD1 in CGNs failed to stimulate neurite outgrowth, while that of PLD2 did stimulate (Fig. 3b,c). This difference in their abilities may result from the distinct localization of overexpressed PLD1 and PLD2: the former localized only in the cell body, whereas the latter located at the growth cone-like structure, the shaft of neurite and the cell body. From these results, it seems that PA production at neurites is important to play a role in stimulation of neurite outgrowth.

A key question raised here is what is the molecular mechanism through which PA stimulates neurite outgrowth of CGNs. PA may regulate molecules involved in actin cytoskeleton reorganization, which is an essential event for neurite outgrowth (Dickson 2001). It has been reported that *in vitro* PA interacts directly with and regulates several types of proteins and enzymes, including phosphatidylinositol 4-phosphate 5-kinase (PI4P 5-kinase) (Jenkins *et al*. 1994), protein phosphatase 1 gamma (Jones and Hannun 2002), β-COP (Manifava *et al*. 2001) and PDE4D3 (Grange *et al*.

2000). Among these molecules, PI4P 5-kinase has been well documented to regulate actin cytoskeletal reorganization through actin-binding proteins that are direct effectors for the PI4P 5-kinase product PI(4,5)P_2 (Sechi and Wehland 2000). However, it is unlikely that PA stimulates neurite outgrowth through activation of PI4P 5-kinase, as we and others have recently demonstrated that PI4P 5-kinase functions as a downstream effector for Rho/ROCK in lysoPA-induced neurite retraction of mouse neuroblastoma N1E-115 cells (van Horck *et al*. 2002; Yamazaki *et al*. 2002). PA may reorganize differently the actin cytoskeleton by the mechanism(s) independent of PI4P 5-kinase to stimulate neurite outgrowth.

It is also conceivable that PA stimulates the anterograde vesicular transport, which is also essential for the neurite outgrowth in replenishment of membranes (Martinez-Arca *et al*. 2000, 2001). It has been demonstrated that in non-neuronal cells PA participates in membrane traffic: PA stimulates the budding from Golgi apparatus by recruiting the COP protein β-COP from cytosol to Golgi membrane (Ktistakis *et al*. 1996). Recent reports by Freyberg *et al*. (2001, 2002) have precisely identified the intracellular localization of endogenous PLD isozymes in non-neuronal cells: endogenous PLD2 are enriched in the Golgi apparatus, dense cytoplasmic puncta and plasma membrane. These reports suggest that the PLD2 product PA facilitates the anterograde vesicular transport to supply membrane components to neurites. It is of interest to investigate this point in the future.

Molecular mechanism for PLD2 activation by ERK

In addition to the involvement of PLD2 in the L1-mediated neurite outgrowth, we also suggested that PLD2 is up-regulated by ERK MAP kinase in the signaling pathway through L1 in CGNs, which provides a novel aspect into regulatory mechanisms for PLD2 activity. It has recently been reported that PLD2 in rabbit vascular smooth muscle cells is phosphorylated at tyrosine residue(s) in an ERK-dependent fashion in response to the norepinephrine (Parmentier *et al*. 2001). It is not likely in CGNs, however, that PLD2 is activated by an ERK-dependent tyrosine phosphorylation, as tyrosine phosphorylation of endogenous PLD2 was not observed in L1-stimulated CGNs (data not shown). Direct serine/threonine phosphorylation and activation of PLD2 by ERK can also be excluded: immunoprecipitated ERK2, which had been overexpressed in HeLa cells and activated by hydroxyperoxide stimulation, failed to phosphorylate and activate the purified recombinant PLD2 in the reconstituted system (data not shown). Mechanisms for ERK-dependent activation of PLD2 are now under investigation in our laboratory to be clarified.

Target molecule of ethanol in CNS neurons

Intrauterine exposure of the human fetus to ethanol causes FAS (Jones and Smith 1973). Because the brain is particularly sensitive to ethanol during the establishment of neural connectivity (Dobbing and Sands 1979), ethanol exposure during the perinatal period causes several neuronal dysfunctions including reduced proliferation, errors in migration, inappropriate connectivity, and excessive neuronal cell death (Miller 1995; Guerri 1998; Levitt 1998). The neuropathological abnormalities observed in FAS have been suggested to be attributable to the ethanol effect on the L1-dependent events of neurons from the observations that the neuropathological abnormalities overlap with those of patients with mutations of L1 (Charness *et al*. 1994) and that ethanol inhibits the L1-mediated neurite outgrowth of CGNs (Bearer *et al*. 1999).

It is well established that the L1-mediated neurite outgrowth absolutely requires activation of ERK MAP kinase (Schmid *et al*. 2000). Hallak *et al*. (2001) have recently reported that ethanol suppresses ERK activation in insulin-like growth factor I-stimulated CGNs. Based on this report and results obtained in this study, it is assumed that ERK is a target molecule of ethanol in the inhibition of the L1-mediated neurite outgrowth. However, this is unlikely, as, inconsistent with their reports, ethanol was without effect on the L1-mediated ERK activation in CGNs under conditions employed in this study (data not shown), while they completely inhibited the L1-mediated neurite outgrowth (Fig. 2). Although, at present, we cannot clearly explain these controversial results, distinct observations may result from the different experimental conditions.

In the present study, we demonstrate that, through its product PA, PLD2 plays a crucial role in the signaling pathway of the L1-mediated neurite outgrowth of CGNs, and that suppression of the PLD2-catalyzed PA production by ethanol inhibited the L1-mediated neurite outgrowth. These results provide evidence that a target molecule of ethanol in the signaling pathway of the L1-stimulated neurite outgrowth is PLD2. Ethanol concentrations that suppressed the L1-mediated neurite outgrowth were comparable to the range of the blood alcohol levels of pregnant mothers who drink heavily (Bearer *et al*. 1999; Ikonomidou *et al*. 2000). Furthermore, it has been reported that PEt is detected in hippocampus, cerebellum and cerebrum of rats after injection of ethanol (Lundqvist *et al*. 1994). Taken together, these results raise the possibility that the neuropathological abnormalities in FAS are, at least in part, attributable to the ethanol inhibition of PLD2-mediated PA production in the L1-dependent neurite outgrowth. This notion would provide insight into the pathophysiological aspect of FAS.

Acknowledgements

This work was supported by the research grants from the Ministry of Education, Science, Sports and Culture, Japan, Yamanouchi Foundation for Research on Metabolic Disorders, and Special Coordination Funds for Promoting Science and Technology from

the Ministry of Education, Science, Sports and Culture, Japan to YK and grants from the NIH to MAF (GM54813). We are grateful to Dr V. Lemmon for providing the anti-L1 antiserum. We thank to Dr Y. Sado for invaluable suggestions to prepare the rat monoclonal anti-PLD2 antibody. We also thank Dr Britta Eickholt for the revision of English of the manuscript.

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