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Lysophosphatidylcholine Drives Neuroblast Cell Fate

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Abstract Neuronal differentiation plays a key role during embryogenesis. However, based on the capacity of neuronal stem cells to either generate or regenerate neurons and because differentiation stops aberrant neuroblasts proliferation, neuronal differentiation is crucial during neuropathological conditions. Although phosphatidylcholine (PtdCho) has been proposed as an important molecule for neurite growth and neuronal regeneration, the identity of the molecular target has remained elusive. This study originally describes that lysophosphatidylcholine (LPtdCho), either exogenously supplied or generated by the imbalance of PtdCho metabolism through the enzymatic action of cytosolic phospholipase A_2, acts as a neurotrophic-like factor. We demonstrated that LPtdCho induces neuronal differentiation by activation of the small G protein Ras followed by the Raf/MEK/ERK signaling pathway. Accordingly, LPtdCho redirects neuroblasts gene expression leading to the generation of functional mature neurons expressing βIII-tubulin and having increased acetylcholinesterase activity and membrane biosynthesis required for neuritogenesis. These findings provide mechanistic details of the role of cytidine-5-diphosphocholine (CDP-choline) and PtdCho as neuroprotectors. Furthermore, as LPtdCho recapitulates the effect of the therapeutic agent retinoic acid, these results open new avenues for drug discovery for the treatment of neuropathological conditions.

Keywords Neuron · Cell differentiation · Neurotrophic factor · Phosphatidylcholine · MAPKK · Retinoic acid

Introduction

Neuritogenesis and neurite outgrowth are important for neuronal plasticity as well as for neuronal regeneration under neuropathological conditions. Both processes depend on membrane biogenesis, whereas phosphatidylcholine (PtdCho) is the most abundant phospholipid in neurons. PtdCho biosynthesis takes place by the Kennedy pathway in which phosphocholine (P-choline) and cytidine-5-diphosphocholine (CDP-choline) are the main intermediates [1]. It was demonstrated that CDP-choline offers a marked neuroprotection in several in vitro and in vivo models of acute and chronic brain ischemic and neurodegenerative diseases. Although the mechanism of this neuroprotection is far from being understood, the relevant hypothesis assumes that there is an acceleration in phospholipid synthesis or resynthesis necessary for membrane repair [2].

We have previously demonstrated that neuroblastoma Neuro-2a cells in which PtdCho biosynthesis is enhanced due to choline kinase (CK), CTP:phosphocholine cytidylyltransferase (CCT) overexpression undergo neuronal differentiation in the absence of retinoic acid (RA) stimulus, suggesting a role for PtdCho metabolism in regulating neuronal cell fate [3]. Our hypothesis proposes that PtdCho or its metabolites serve as signaling molecules driving neuritogenesis.

Although the factors that control the differentiation process are still emerging, considerable evidence now exists

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demonstrating the potent effect of lysophospholipids, lysophosphatidic acid (LPtdOH), and sphingosine-1-phosphate as regulators of a variety of cellular processes like proliferation, survival, migration, and differentiation [4]. Specifically, lysophospholipids are simple phospholipids with a single O-acyl chain that exert their functions as extracellular ligands for cell surface receptors [5], or as intracellular second messengers [6, 7]. Alternatively, lysophospholipids can change the membrane curve, shape, and thickness and thus regulate the activity of channels or enzymes [8]. Lysophospholipids are generated by the action of phospholipase A_2 (PLA_2) that cleave the sn-2 ester linkage of glycerophospholipids releasing free fatty acid and 2-lysophospholipids [9]. PLA_2 family enzymes participate in different processes such as membrane repair, phospholipids turnover, membrane trafficking, and fusion of vesicles [10, 11], or they are activated by different stimuli like neurotransmitters, neurotrophic factors, cytokines, membrane depolarization, and ion channel activators [12]. The generated signaling molecules could regulate and interact with multiple other signaling cascades contributing to the development, differentiation, function, protection, and repair of the cells in the nervous system [13]. It was proposed that the secretory PLA_2 ($sPLA_2$) induces neurite outgrowth in PC12 cells via the generation of lysophosphatidylcholine (LPtdCho) which mediate the cellular response by the G2A receptors [9, 14, 15]. In addition, it was shown that LPtdCho has a biological function by enhancing the nerve growth factor (NGF)-induced MAPK and Akt phosphorylation by stimulating the activation of TrkA in PC12 cells [16], and in cerebellar granule neurons by regulating the brain derivate neurotrophic factor (BDNF)-TrkB signaling and promoting cell survival [17].

Considering that the identification of molecules capable of promoting neuronal differentiation might help in many cases of neuronal damages or degeneration, we propose herein that either exogenous supplied LPtdCho or generated by the imbalance of PtdCho turnover act as a neurotrophin-like factor promoting neuronal differentiation. In agreement, current data demonstrate that exogenous treatment of neuroblasts with PtdCho or precursors of the PtdCho biosynthesis like choline, diacylglycerol (DAG), and phosphatidic acid (PtdOH) induce neuronal differentiation recapitulating the effect of RA [3]. Further experiments using pharmacological inhibition and molecular biology tools allowed us demonstrating that the activity of the cytosolic PLA_2 ($cPLA_2$) is essential for PtdCho and its precursors to promote neuronal differentiation. This enzyme generates LPtdCho from the newly synthesized or supplemented PtdCho which in turn acts as a signaling molecule activating the Ras/Raf/MEK/ERK pathway and promoting neuronal differentiation. We also demonstrated that neurons differentiated with LPtdCho express typical neuritogenesis-associated markers. Thus, we demonstrate and propose that LPtdCho treatment or any imbalance of Lands cycle [18] that raises the levels of LPtdCho would promote neuronal differentiation. This result might provide mechanistic details to the neuroprotective role of CDP-choline [2]. Finally, we demonstrated that LPtdCho and RA exert similar effect but acting by different mechanism. LPtdCho turns on differentiation by activation of the small G protein Ras that triggers the Raf/MEK/ERK pathway and without calcium mobilization; however, RA induces the same pathway by increasing the intracellular calcium. Accordingly, both molecules act as neurotrophic-like factors but with differences in the way of activation; considering that there are many neuroblastomas resistant to RA treatment, these results, perhaps, might provide sites for pharmacological design.

Materials and Methods

Cell Culture and Treatments

The mouse neuroblastoma cell line Neuro-2a (ATCC CCL-131) was cultured in modified Eagle's medium (MEM), 10 % fetal bovine serum (FBS) supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml) (proliferation conditions), and maintained in a 5 % CO_2 humidified incubator at 37 °C. In neuronal differentiation conditions, the medium was changed to Dulbecco's modified Eagle's medium (DMEM) plus 2 % FBS and RA (10 μM). The human neuroblastoma cell line SH-SY5Y (kindly provided by Dr. S. Quiroga U. of Córdoba) was cultured in a 1:1 mixture of DMEM and F12 Medium, 10 % fetal bovine serum (FBS) supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and maintained in a 5 % CO_2 humidified incubator at 37 °C. In neuronal differentiation conditions, the amount of serum was changed to 3 % and RA (10 μM) was added.

Liposomes Preparation and Lipids Supplementation

When phospholipids or lysophospholipids were added to the growth medium, concentrated lipid stocks were prepared following Esko [19]. Briefly, pure lipids in chloroform or chloroform/methanol (2:1 *v*/*v*) were dried in acid-washed glass centrifuge tubes under a stream of nitrogen. Phospholipid samples were resuspended at 2–6 mM in phosphate-buffered saline at pH 7.2 and sonicated twice for 5 min at power setting 0.2–0.5 % amplitude. All samples were sterilized with 0.22-μm pore filters (Millipore). The recovery of phospholipids after filtration was typically 90 % or more.

Metabolic Labeling

For the de novo PtdCho synthesis measurements, cells were incubated with RA (10 μM) (Sigma) or PtdCho (60 μM) or LPtdCho (10 μM) (Sigma) for 48 h. After that, cells were

incubated in medium supplemented with 2 μCi/ml [1-^{14}C]acetate (specific activity, 55 mCi/mmol), obtained from American Radiolabel Chemical. After labeling for 4 h, cells were harvested, counted, and subjected to extraction according to the method of Bligh and Dyer [20]. The [1-^{14}C]acetate incorporation into fractionated lipid molecular species was determined following 2D thin-layer chromatography of organic phase on Silica Gel H layers developed in chloroform methanol/water (75:25:2.5, *v*/*v*/*v*) and chloroform/methanol/acetic acid/water (80:9:12:2, *v*/*v*/*v*/*v*). Radioactivity on the plates was visualized using a Typhoon 9200 PhosphorImager screen and quantified using ImageQuant software (version 5.2).

Morphometric Analysis

Cells were plated at a density of 3×10^4/35-mm dish for 24 h, after which the medium was changed to DMEM supplemented with 2 % of FBS and supplemented with RA (10 μM), sonicated liposomes (60 μM for phospholipids; 10 μM for lysophospholipids), or phospholipids synthesis intermediates (60 μM). The concentration of each lipid analyzed was selected by MTT analysis [21]. When addition of inhibitors was necessary, they were added 30 min or 1 h prior to the treatment. The inhibitors utilized bromoenolactone (BEL) [22] (25 μM), arachidonyl trifluoromethyl ketone (ATK) [23] (5 μM), and 1,1′-[5-[3,4-Dihydro-7-hydroxy-2-(4-hydroxyphenyl)-2H-1-benzopyran-4-yl]-2,4,6-trihydroxy-1,3-phenylene]bis-1-dodecanone (YM) (5 μM) as specific inhibitors of iPLA2, cPLA2, and sPLA2, respectively, and (1R,2R)-N-([S]-1-{4-[5-bromo-2-oxo-2,3-dihydro-1H-benzo(d)imidazol-1-yl]piperidin-1-ylpropan-2-yl)-2-phenylcyclopropanecarboxamide (EVJ) (0.15 μM) and with N-{2-[4-oxo-1-phenyl-1,3,8-triazaspiro(4.5)decan-8-yl]ethyl}quinoline-3-carboxamide (APV) (0.15 μM) as specific inhibitors of PLD1 [24, 25] and PLD2 [25]. For lysophospholipid acyl transferase inhibitor, we used 2,2-methyl-N-(2,4,6,-trimethoxyphenyldodecanamide) (CI-976) (10 μM) [26, 27]. Control cells were maintained in MEM 10 % of FBS. After 48 h, cells were observed by phase contrast microscopy (Olympus CK2) and 15–20 random fields of view were sampled. Cells bearing at least one neurite equal or longer than the soma diameter were considered to be differentiated. To calculate the percent of cells bearing neurites, differentiated cell and total cell number were counted and/or measured in each field, using the "ImageJ" (NIH) software.

Western Blot and Immunofluorescence Analysis

For Western blot analysis, cells were plated at a density of 5×10^5/100-mm dish for 24 h, after which the medium was changed to DMEM supplemented with 2 % of FBS supplemented with RA (10 μM), PtdCho (60 μM), or LPtdCho (10 μM). Control cells were maintained in MEM 10 % of FBS. After 48 h of treatment, cells were collected, resuspended in 1× lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM KCl, 10 mM EDTA, 20 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 50 μM TPCK, and 1:1000 cocktail (Sigma)) and sonicated five times for 5 s at 5 % amplitude (Sonics and Materials Inc–Vibra Cell™). Proteins concentrations were determined using bovine serum albumine (BSA) as standard protein and "Sedmak and Grossberg" reagent [28]. 40 μg of cell lysate were resolved on 12 % SDS-polyacrilamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Amersham). After blocking overnight with 5 % nonfat milk in 0.1 % Tween TBS and washing, blots were incubated for 1 h with anti-CCTα (1:1000, provided by Dr. Suzanne Jackowski). As secondary antibody, peroxidise-conjugated anti-rabbit IgG was used (1:20000, Jackson Immuno Research). Loading protein control was demonstrated by measuring the levels of β-tubulin using anti-β-tubulin (1:1000, Sigma) and developed with secondary antibody peroxidise-conjugated anti-mouse IgG (1:10000, Jackson Immuno Research). For $ERK_{1/2}$ and p-$ERK_{1/2}$ immunoblotting, cells were incubated for 1 h after the administration of RA (10 μM), PtdCho (60 μM), PtdEtn (60 μM), or LPtdCho (10 μM). ATK, Edelfosine, or Hemicholineum-3 were preincubated 30 min prior to liposomes were added and Ras and Raf inhibitors, Farnesil thiosalicylic acid (FTS 30 μM), and BAY43-9006 (2.5 μM) respectively, and BAPTA (20 μM) were added 2 h prior to treatment with phospholipids. Then, cells were harvested and resuspended in 1× lysis buffer. 20 μg of cells lysated were resolved on 12 % SDS-polyacrilamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (Amersham). Primary antibodies were anti-ERK (1:500, Cell Signaling Technology) and anti-p-ERK (1:500, Cell Signaling Technology). For immunofluorescence, cells were plated at 3×10^4/35-mm dish in a cover slips and growth for 24 h, after which medium was changed to DMEM 2 % FBS supplemented with RA (10 μM), PtdCho (60 μM), PtdEtn (60 μM), LPtdCho (10 μM), or maintained in MEM 10 % FBS. Then, cells were fixed using 4 % of paraformaldehyde-sucrose, permeabilized with 0.2 % triton ×100 and incubated 1 h with 5 % BSA. The following antibodies were used: βIII-tubulin (1:1000, Sigma) during overnight at 4 °C and anti-rabbit Alexa Fluor® 488-labeled (1:1000, Invitrogen) for 1 h at room temperature. Slides were mounted with 10 mM Tris pH 7.6, 50 % glycerol, 0.1 % (*w*/*v*) phenylenediamine, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, 137 mM NaCl, and 2.7 mM KCl. Microscopy was carried out using a confocal microscope (Nikon Model Eclipse TE-2000-E2 C1 plus) equipped with Plan Apochromat ×20.0/0.75/1.00 dry objective; Nikon C1 Standard Detector and Nikon EZ-C1 3.60 Software. Images were adjusted for contrast and gamma using Nikon EZ-C1 3.70 FreeViewer Software.

Acetylcholinesterase Activity Assay

To measure acetylcholinesterase activity, cells were plated at 3×10^4/35-mm dish for 24 h, then the medium was replaced with DMEM 2 % FBS supplemented with RA (10 μM), PtdCho (60 μM), or LPtdCho (10 μM), and incubated for further 72 h. For control condition, cells were maintained in MEM 10 % FBS. After treatment, cells were harvested and lysed in 1× lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 20 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 50 μM TPCK, and 1:1000 cocktail (Sigma)). Proteins' concentrations were determined using BSA as standard protein and "Sedmak and Grossberg" reagent [28]. Reaction mixture consists in 40 μl of total protein extract (15–30 μg), 50 μl of 2 mM DTNB, and 10 μl of 5 mM acetylthiocholine iodide (Sigma). The reaction was incubated during 5–8 h at room temperature, and absorbance at 405 nm was measured each 30 min. To calculate acetylcholinesterase activity, absorbance versus time was plotted and the slope was calculated. This value was normalized to proteins' level of each sample and expressed as specific activity.

Ras-V12 Transfection

Neuro-2a cells were plated at 5×10^4 cells/35-mm dish. After 24 h, they were co-transfected with 0.5 μg of phr-GFP (Stratagene) and 0.5 μg of plasmid design to overexpress a Ras constitutive mutant (pLPC-Ras-V12) or empty vector (pLPC), using a cationic liposome method (Lipofectamine 3000, Invitrogen). pLPC-Ras-V12 plasmid was kindly provided by Dr. Girardini. After 6 h of transfection, medium was renewed. Cells were incubated for further 24 h, and then, medium was replaced for DMEM 2 % FBS alone or supplemented with RA (10 μM) or LPtdCho (10 μM). Control cells were maintained in MEM 10 % FBS. Forty-eight hours post-transfection, microscopy was carried out using an epifluorescence microscope (Nikon model Eclipse TE-2000-E2 C1 plus) equipped with Plan Apochromat ×20.0/0.75/1.00 dry objective; Nikon C1 standard detector, and Nikon EZ-C1 3.60 software. Images were adjusted for contrast and gamma using Nikon EZ-C1 3.70 Free Viewer software. Green cells bearing at least one neurite equal or longer than soma diameter were considered differentiated.

Calcium Influx

To measure the influx of calcium to cell cytoplasm, cells were washed, suspended in MEM 10 % FBS at 2×10^6 cells/ml, and then incubated with 3 μM Fluo-3/AM (Invitrogen) and 0.1 % Pluronic F-127 (Invitrogen) for 30 min at 37 °C and 5 % CO_2. After being washed twice with PBS, cells were resuspended in low calcium buffer (10 mM Hepes pH 7.9; 140 mM NaCl; 5 mM KCl; 2 mM $MgCl_2$; 10 mM Glucose) at 1×10^6 cells/ml. Fluorescence intensity was monitored at 526 nm with excitation at 506 nm using Cary Eclipse Fluorescence Spectrophotometer.

Analysis of Ras Activation

Ras activation analysis was done by the method of Downward et al. [29] and modified as described below. Cells were cultured in six-well plates as described earlier and incubated for 3 h in phosphate-free MEM 10 % FBS containing 300 μCi of [^{32}P]-orthophosphate. These cells were then stimulated with LPtdCho (10 μM) in DMEM 2 % FBS for various time periods and then washed with Tris-buffered saline and scraped in 0.5 ml of Ras lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 9 μg/ml PMSF, 1 % Triton X-100, 1 mg/ml BSA, and 10 mM benzamidine).

The cell lysate was first mixed with an equal volume of a solution of 1 M NaCl, 0.1 % SDS, and 1 % sodium deoxycholate. After a brief mixing and centrifugation, the supernatant was immunoprecipitated with anti-Ras antibody (0.5 μg, conjugated to protein A-Sepharose) by mixing ON at 4 °C. The beads were washed four times with Ras lysis buffer, and the guanine nucleotides were then eluted with potassium phosphate buffer (1 M, pH 3.4) by heating at 80 °C for 5 min. GTP and GDP were resolved by TLC on polyethyleneimine cellulose plates and were detected by autoradiography.

Statistical Analysis

Data were analyzed with GraphPad Prism 5.0d software. All normally distributed data were displayed as means±s.e.m and are representative of, at least, three experiments. Groups were analyzed by one-way ANOVA, with appropriate post hoc tests.

Results

Neuronal Differentiation is Induced by Exogenous PtdCho

We previously demonstrated that CCTα or CKα overexpression induces PtdCho biosynthesis and accumulation, driving neuroblastoma Neuro-2a cells to neuronal differentiation [3, 30]. To study the mechanism by which PtdCho regulates neuronal cell fate, we evaluated the effect on neuronal differentiation exerted by phospholipids supplemented in the culture media. Cells were grown in the presence or in the absence of sonicated liposomes of PtdCho and phosphatidylethanolamine (PtdEtn), both obtained from eggs source. We used RA as a differentiation control since it is a well-known

neurotrophic factor for neuroblastoma Neuro-2a cells [31]. Cells incubated under proliferating conditions were used as a control for basal differentiation level. The number of differentiated cells was measured by morphometric analysis after 48 h. As Fig. 1a shows, PtdCho but not PtdEtn promoted neuronal differentiation to a similar extent than RA. The observed effect of PtdCho on neuronal differentiation did not increased with the dose of the phospholipids in the range analyzed (60–120 μM) (data not shown).

As free fatty acids specially arachidonic acid (AA) and docosohexanoic acid (DHA) play important roles as signaling molecule, and have been previously implicated in neurite outgrowth [14, 32], we evaluated the effect of AA and sonicated liposomes of dioleyl-PtdCho (DO-PtdCho) on differentiation levels. We observed that DO-PtdCho (60 μM) induced the same rate of differentiation than the egg source-PtdCho, suggesting that the fatty acids composition is not crucial for the stimulus (Fig. 1a). Furthermore, when the medium was supplemented with AA (0.1 μM), no effect was observed (Fig. 1a). The neurotrophic effect of PtdCho was also demonstrated in human neuroblastoma cells SH5YSY confirming that it is not a cell line-specific effect (Fig. 1b).

RA induces neuronal differentiation of Neuro-2a cells by activating the MEK/ERK pathway [3]. To determine if the PtdCho-induced differentiation program entails this pathway, we evaluated the levels of phospho-ERK (p-ERK) by Western blot in cells treated with PtdCho, PtdEtn, or RA as a differentiation control. As Fig. 1c shows, PtdCho, like RA, induced ERK phosphorylation. However, PtdEtn failed to activate this kinase. As PtdCho activates the MAPK pathway, we assumed that its inhibition should affect the differentiation program. To validate this hypothesis, we determined the rate of differentiation induced by PtdCho in the presence of U0126, a MEK inhibitor [33]. As Fig. 1d shows, U0126 abolished the differentiation induced by PtdCho, confirming the involvement of the MEK/ERK signaling pathway.

The Imbalance in PtdCho Metabolism Affects Neuroblasts Cell Fate

PtdCho homeostasis is regulated by the balance between the opposing action of synthesis and hydrolysis [11]. PtdCho biosynthesis can be encouraged by P-choline, CDP-choline [34], and diacylglycerol (DAG), a direct intermediate for PtdOH to generate PtdCho [35] (Fig. 2a), and also by LPtdOH trough the LPtdOH acyltransferase (LPAT) [36, 37]. To evaluate the effect of these metabolites, we incubated Neuro-2a cells with the indicated concentration of each precursor during 48 h and measured neuronal differentiation by morphometric analysis. As Fig. 2b shows, all the metabolites tested except LPtdOH promoted neuronal differentiation.

Alternatively, PtdCho can be hydrolyzed by PtdCho-specific phospholipase C, phospholipase D (PLD), and PLA_2 (Fig. 2c). In mammalian cells, there are multiple isoforms of PLA_2 which are classified as calcium-dependent $cPLA_2$, calcium-independent cytosolic ($iPLA_2$), and $sPLA_2$ [12, 38–40]. To evaluate if the activity of any of these phospholipases (PLs) is required for PtdCho to exert its effect on neuronal differentiation, we treated cells with PtdCho plus specific PLs inhibitors. Cells were incubated with BEL (25 μM) [22], ATK (5 μM) [23], and YM (5 μM) as specific inhibitors of iPLA2, cPLA2, and sPLA2, respectively. Alternatively, they were incubated with EVJ (0.15 μM) and with APV (0.15 μM) as specific inhibitors of PLD1 [24, 25] and PLD2 [25], respectively. The involvement of PLD was also evaluated by treatment of the cells with the general antagonist (1-butanol) [41] (data not shown). The concentration of each inhibitor was selected by MTT analysis [21]. Neuronal differentiation was evaluated by morphometric analysis as previously described [3]. As Fig. 2d shows, among all inhibitors tested, only ATK clearly decreased the differentiation induced by PtdCho to the basal level. Considering that $cPLA_2$ activity is essential for PtdCho to promote neuritogenesis (Fig. 2d), and because it generates LPtdCho and free fatty acids from PtdCho hydrolysis, we evaluated if those products were capable of inducing neuronal differentiation. Cells were incubated with the indicated concentration of LPtdCho (10 μM) during 48 h, time in which we evaluated differentiation. As Fig. 2f shows, LPtdCho induced neuronal differentiation. However, the addition of RA together with LPtdCho did not significantly increase the rate of neuronal differentiation, further substantiating the role of LPtdCho as a neurotrophin. Moreover, this result shows clear differences with previous reports demonstrating that LPtdCho enhances the effect of the neurotrophic factors NGF and BDNF [16, 17].

LPtdCho-Induced Differentiation Depends on the ERK Pathway

We evaluated if differentiation induced by LPtdCho depends on ERK activation as was demonstrated for PtdCho (Fig. 1), as well as its precursors and RA [3]. Cells were incubated with U0126 during 1 h prior to the addition of LPtdCho, and neuronal differentiation was measured after 48 h. As Fig. 3a shows, the addition of U0126 blocked neuronal differentiation. We also measured the levels of p-ERK in cells incubated with PtdCho and LPtdCho in the presence or absence of the $cPLA_2$ inhibitor. As Fig. 3b shows, the MEK/ERK pathway was turned on by PtdCho only when the $cPLA_2$ was active. However, LPtdCho activated the pathway in both cases, when $cPLA_2$ was either active or inactive, confirming that LPtdCho generated by PtdCho hydrolysis is the molecule that drives neuritogenesis trough the MEK/ERK pathway.

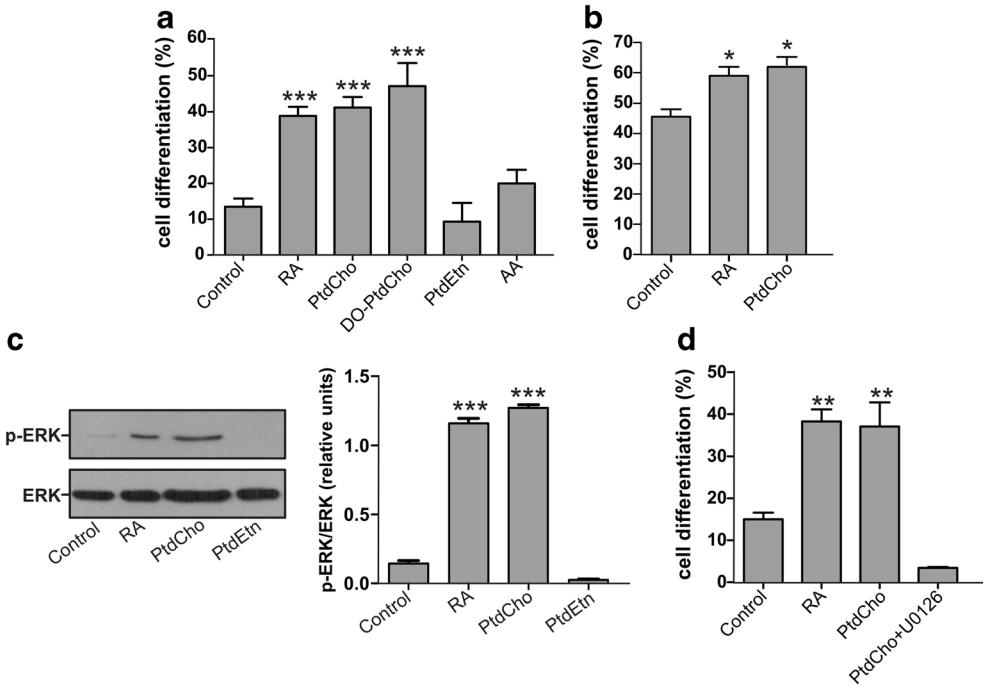

Fig. 1 PtdCho promotes neuronal differentiation. **a** Neuro-2a cells were incubated with RA (10 μM) as a control for differentiation or with sonicated liposomes (60 μM) of PtdCho, PtdEtn, DO-PtdCho (dioleyl-phosphatidylcholine), or AA (0.1 μM) during 48 h. Control cells were grown under proliferation conditions. *Graph* represents the percentage (%) of neurite-bearing cells for each culture condition from five independent experiments, ***$P<0.0001$. **b** SH5YSY cells were incubated with sonicated micelles (60 μM) of PtdCho or with RA (10 μM) as a control for differentiation with during 48 h. Control cells were grown under proliferation conditions. *Graph* represents the percentage (%) of neurite-bearing cells for each growth condition from two independent experiments, *$P<0.05$. **c** Western blot analysis was used to investigate the amount of p-ERK in Neuro-2a cells treated with the indicated phospholipids or RA as a control for differentiation. Control cells were grown under proliferation conditions. *Blot* is representative of three independent experiments. *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. ***$P<0.0001$. **d** Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM) or PtdCho (60 μM) in the presence or in the absence of U0126 (10 μM) during 48 h and analyzed morphometrically. *Graph* represents the percentage (%) of neurite-bearing cells for each culture condition and is representative of four independent experiments, **$P<0.001$

The Newly Synthetized PtdCho is Further Metabolized to Generate LPtdCho

Following the rationale that PtdOH and choline metabolites promote neuritogenesis by inducing PtdCho synthesis and the following degradation by $cPLA_2$, the incubation with ATK plus all these PtdCho precursors should block neuronal differentiation. To analyze this hypothesis, we incubated cells with the $cPLA_2$ inhibitor ATK plus the indicated concentration of PtdOH or choline, to later evaluate neuronal differentiation by morphometric analysis. As Fig. 4a shows, neither PtdOH nor choline were able to switch on differentiation when $cPLA_2$ was blocked. This result clearly suggests that after entry to the cells, choline and PtdOH are converted by the Kennedy pathway into PtdCho which is further hydrolyzed by the $cPLA_2$. Accordingly, choline treatment turned on the MEK/ERK pathway (increased levels of p-ERK) when the Kennedy pathway for PtdCho biosynthesis was active, but not when cells were preincubated with pharmacological inhibitors of either CK (Hemicholineum-3) or CCT (Edelfosine) [30]. Furthermore, choline was unable to activate the MEK/ERK pathway when the cPLA2 was inactivated by the ATK treatment (Fig. 4b).

LPtdCho could be further degraded by $cPLA_2$ or, alternatively, reacylated and converted into PtdCho by the Lands cycle [18]. To evaluate these two possibilities, we incubated cells with 1-O-palmityl-sn-glycero-3-phosphocholine (L-PAF) in which there is an ether bond in the 1-position that prevent it from being hydrolyzed by $cPLA_2$ [36]. As Fig. 4c shows, LPtdCho and L-PAF at two different concentrations exerted the same effect, suggesting that $cPLA_2$ acts as a PL rather than as a lysoPL. Lysophospholipids acyltransferase (LPAT) catalyzes the transfer of fatty acid from acyl-CoA donors to lysophospholipids [18, 37]. Thus, the inhibition of LPtdCho reacylation by LPAT would result in the accumulation of LPtdCho that might favor neuritogenesis. To inhibit the LPAT, we used the small molecule antagonist CI-976 [26, 27]. Cells incubated under differentiation condition in the absence of any neurotrophic stimulus or incubated with LPtdCho were treated with 10 μM of CI-976 and followed by morphometric analysis after 24 and 48 h. Fig. 4d shows that when LPAT was inhibited in cells incubated in the absence of neurotrophins,

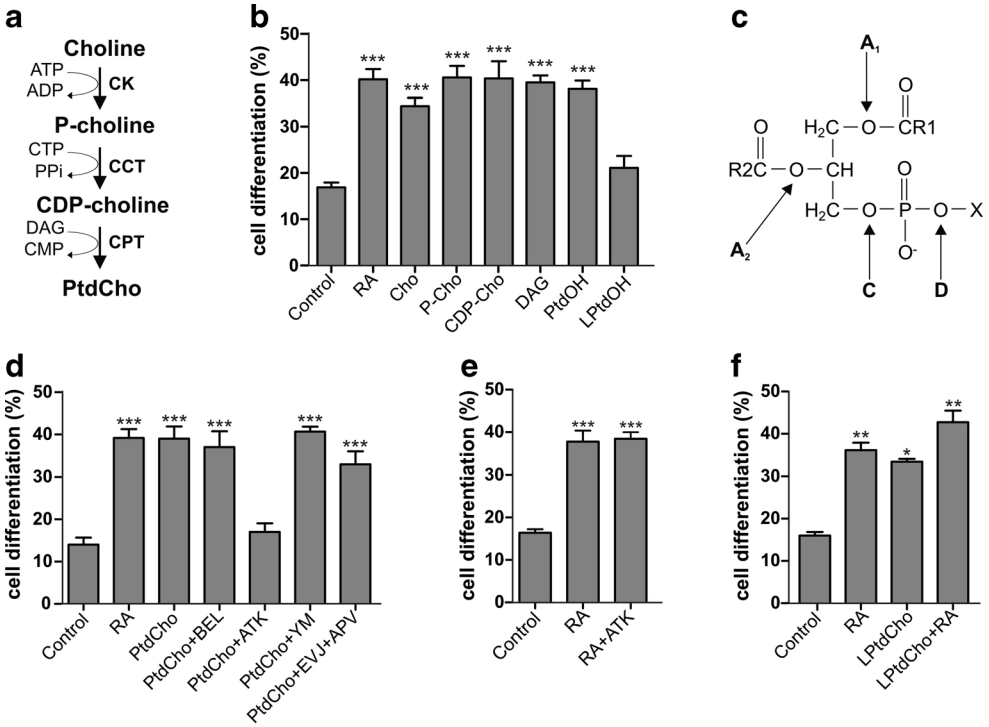

Fig. 2 The activity of cPLA2 is required for PtdCho-induced neuronal differentiation. **a** Schematic representation of the Kennedy pathway for PtdCho biosynthesis; choline kinase (*CK*), CTP:phosphocholine cytidylyltransferase (*CCT*), and choline phosphotransferase (*CPT*). **b** Neuro-2a cells were cultured in growing (control) or differentiating media in the presence of the indicated lipids (Cho (60 μM), P-Cho (60 μM), CDP-Cho (60 μM), DAG (60 μM), PtdOH (60 μM), LPtdCho (10 μM), and RA (10 μM)). **c** Schematic representation of phospholipases hydrolysis sites. **d** Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM) or PtdCho (60 μM) in the presence or in the absence of the indicated PLs inhibitors during 48 h and analyzed morphometrically. **e** Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM) in the presence or in the absence of ATK (5 μM) ($cPLA_2$ inhibitor). **f** Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM), LPtdCho (10 μM), and RA (10 μM) plus LPtdCho (10 μM) and analyzed morphometrically. *Graphs* represent the percentage (%) of neurite-bearing cells for each growing condition. and are representative of three independent experiments, ***$P<0.0001$; **$P<0.001$; *$P<0.05$

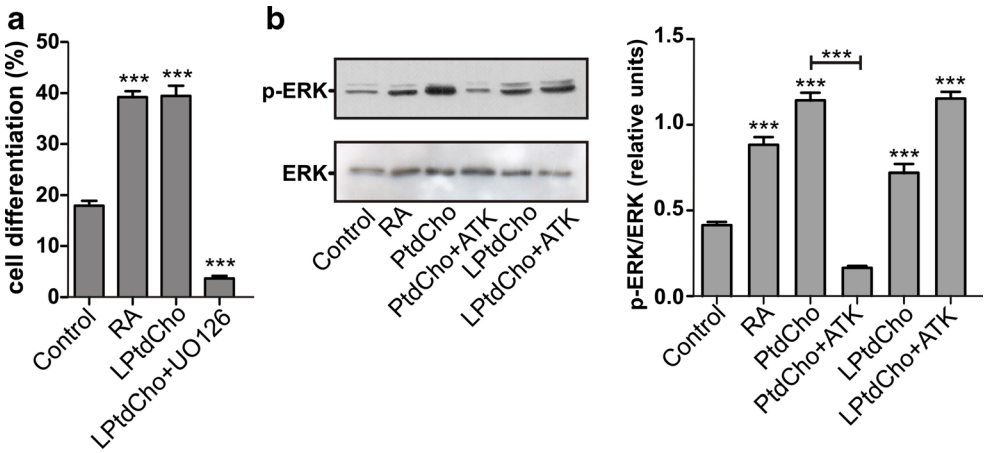

Fig. 3 LPtdCho activates the MEK/ERK pathway in Neuro-2a cells. **a** Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM) or LPtdCho (10 μM) in the presence or in the absence of U0126 (10 μM) during 48 h and analyzed morphometrically. *Graph* represents the percentage (%) of neurite-bearing cells for each growing condition and is representative of four independent experiments, ***$P<0.0001$. **b** Western blot analysis was used to investigate the amount of p-ERK in Neuro-2a treated with the indicated phospholipids with or without ATK (5 μM) treatment or RA as a control for differentiation. Control cells were grown under proliferation conditions. *Blot* is representative of three independent experiments. *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. ***$P<0.0001$

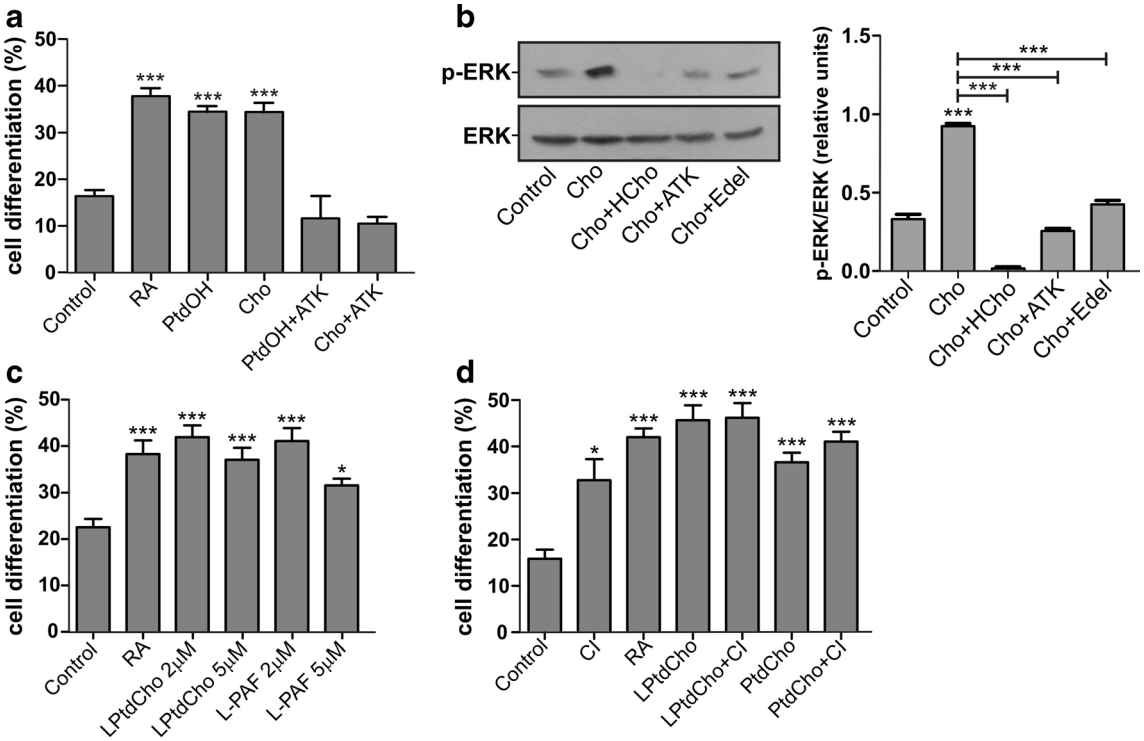

Fig. 4 Choline and PtdOH prompt differentiation by the generation of LPtdCho. **a** Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM), PtdOH (60 μM), and choline (*Cho*) (60 μM) in the presence or absence of ATK (5 μM) and analyzed morphometrically. *Graph* represents the percentage (%) of neurite-bearing cells for each growing condition and is representative of three independent experiments, ***$P<0.0001$. **b** Western blot analysis was used to investigate the amount of p-ERK in Neuro-2a treated with choline (*Cho*) and the indicated inhibitors hemicholineum-3 (*Hcho*, 75 μM) for CK, Edelfosine (*Edel*, 5 μM) for CCTα, and ATK (5 μM) for $cPLA_2$. Control cells were grown under proliferation conditions. *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. ***$P<0.0001$. **c** Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM), LPtdCho, or L-PAF at the indicated concentrations, and analyzed morphometrically. *Graph* represents the percentage (%) of neurite-bearing cells for each growing condition and is representative of three independent experiments. **d** Neuro-2a cells were incubated under control condition or in the presence of RA or liposomes of LPtdCho during 48 h in the presence or absence of the antagonist 2,2-methyl-N-(2,4,6,-trimethoxyphenyldodecanamide (CI, 10 μM). *Graph* represents the percentage (%) of neurite-bearing cells for each growing condition and is representative of three independent experiments, ***$P<0.0001$; *$P<0.05$

the percentage of neuronal differentiation increased. In the same direction, treatment of the cells with LPtdCho plus CI-976 did not affect the induction of neuronal differentiation promoted by the lysophospholipid (Fig. 4d). These results indicate that LPtdCho generated by the imbalance of the Lands cycle and not its recycling induces neuronal differentiation.

PtdCho and LPtdCho-Induced Differentiation Develop Mature Neurons

Neuronal differentiation is accompanied by a redirection in gene expression leading to generation of a functional mature neuron. βIII-tubulin is one of the earliest cytoskeletal proteins specifically associated with neuronal development [42–44], and it is considered as a neuronal differentiation marker [45]. We investigated whether Neuro-2a cells that were treated with PtdCho and LPtdCho express the neuritogenesis-associated marker βIII-tubulin. In the absence of RA, Neuro-2a progenitor cells did not express detectable levels of βIII-tubulin, as measured by immunocytochemistry; however, this protein was readily detectable after PtdCho or LPtdCho treatment. As expected, due to its inability to induce differentiation, PtdEtn failed to induce βIII-tubulin expression (Fig. 5a). We also analyzed different morphometric parameters like neurites lengths (Fig. 5b, c) and total number of neurites (Fig. 5d). As shown in Fig. 5e, Neuro-2a cells exhibited a significant increase in acetylcholinesterase activity when treated with PtdCho or LPtdCho for 72 h, suggesting that both lipids also induce a functional differentiation of the cholinergic neuroblastoma cells [46]. It was previously demonstrated that PtdCho biosynthesis increases coordinately with RA-induced differentiation due to the increased expression in CKα and CCTα [3, 30]. As PtdCho or LPtdCho exogenously supplied promotes neuronal differentiation, the expression of βIII-tubulin, and the activity of acetylcolinesterase, we investigated the expression of CCTα as the regulatory enzyme of the Kennedy pathway [1], and the rate of de novo

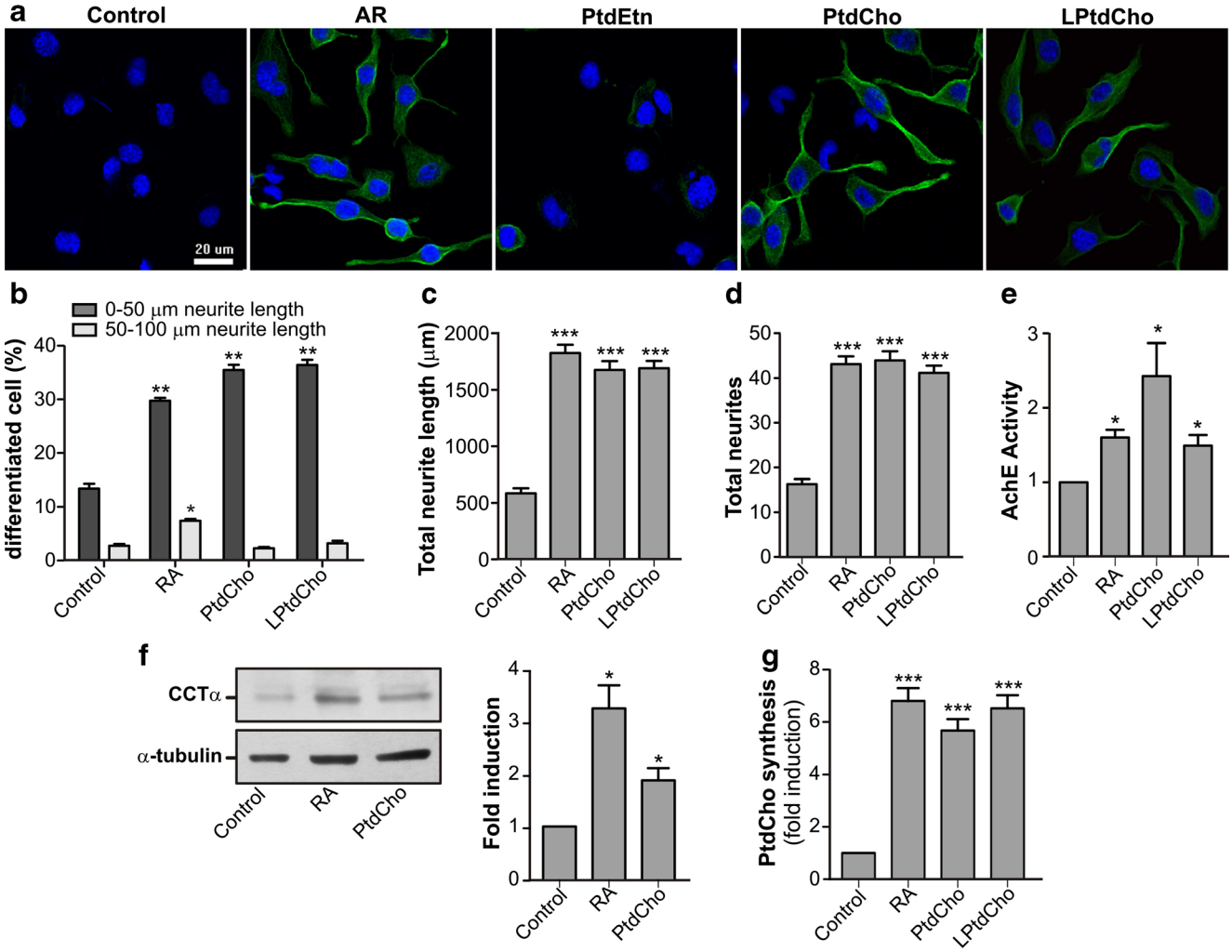

Fig. 5 Exogenous PtdCho like RA induces neurons associate markers. **a** Detection of β-III Tubulin (*green*) by immunocytochemistry in Neuro-2a cells treated without RA (control) or with RA (10 μM), PtdEtn (60 μM), PtdCho (60 μM), or LPtdCho (10 μM). *Bar*, 20 μm. Neuro-2a cells were cultured in growing (control) or differentiating media containing RA (10 μM), PtdCho (60 μM), or LPtdCho (10 μM) during 48 h and analyzed morphometrically. **b** *Graph* represents the percentage (%) of neurite-bearing cells harboring neurites of different lengths. $^{**}P<0.001$, $^{*}P<0.05$. **c** *Graph* represents total neurite length ($n=100$). $^{***}P<0.0001$. **d** Graph represents total number of neurites ($n=100$) $^{***}P<0.0001$. **e** Cells were treated with or without RA, PtdCho, or LPtdCho for 3 days. Total cell lysate was prepared for colorimetric assay of acetylcholinesterase activity. *Graph* is representative of four independent experiments and is given as Specific activity, $^{*}P<0.05$. **f** Western blot analysis was used to investigate the amount of CCTα in Neuro-2a treated with PtdCho and RA. *Graph* represents the densitometric analysis of the CCTα-specific band relative to β-actin from three independent experiments. *Blot* is representative of three independent experiments. $^{*}P<0.05$. **g** Cells were incubated with or without RA (10 μM), PtdCho (60 μM), or LptdCho (10 μM) and after 20 h cells were washed and pulsed with [1-^{14}C]acetate for 4 h, lipids were extracted and the radiolabel associated with PtdCho was quantified by scintillation counting following thin-layer chromatography. *Graph* is representative of three independent experiments

synthesis of PtdCho. Neuro-2a cells were incubated with sonicated liposomes of PtdCho or with LPtdCho during 20 h, collected for Western blot assay or washed and labeled during 4 h with [1-^{14}C]-acetate. Lipids were extracted, and the radiolabeled associated with PtdCho was counted. Similar assays were done with cells growing in proliferating (control) and differentiating (+RA) conditions. As Fig. 5 shows, there is a clear increase in the expression of CCTα (F) and in the de novo PtdCho biosynthesis (G) after PtdCho or LPtdCho treatment of neuroblastoma cells.

Calcium as a Second Messenger

The described results demonstrate that LPtdCho promotes neuronal differentiation mimicking the effect of RA as a well-known therapeutic agent for neuroblastoma treatment [47]. Both neurotrophic factors induce neuronal differentiation by activating the MEK/ERK pathway (Fig. 3) [3]. Considering that many neuroblastomas are RA-resistant, decoding the molecular mechanism by which both RA and LPtdCho exert their effect arises as a key question for drug design. To gain mechanistic insight, we first investigated the involvement

of calcium as a second messenger. Using Fluo-3-AM as a probe, we demonstrated that RA induced a massive increase in the levels of cytoplasmic calcium (Fig. 6a). As preincubation with EGTA prevented the calcium intake, we speculated that RA activates a membrane-associated channel (Fig. 6d). When cells were treated with PtdCho, regardless of treatment with EGTA, we detected an increase in the levels of cytoplasmic calcium, indicating the involvement of intracytoplasmic reservoirs (Fig. 6b, d). When calcium from the endoplasmic reticulum (ER) was depleted by thapsigargin pretreatment [48, 49], no further increase was observed upon PtdCho stimulus (Fig. 6e). Altogether, these data propose the ER as the source of calcium released upon PtdCho treatment. Interestingly, LPtdCho did not change cytoplasmic calcium levels (Fig. 6c).

Calcium is a versatile second messenger that affects many signaling process in the cell. Interestingly, elevated calcium concentration can activate the ERK cascade [50]. Having this information in mind, we next asked whether calcium mobilization is required for these neurotrophin-like factors to activate the ERK cascade. To answer this question, we measured the levels of p-ERK in cells treated with EGTA as an extracellular calcium chelator or BAPTA-AM as an intracellular calcium chelator [51]. We observed that when cells were

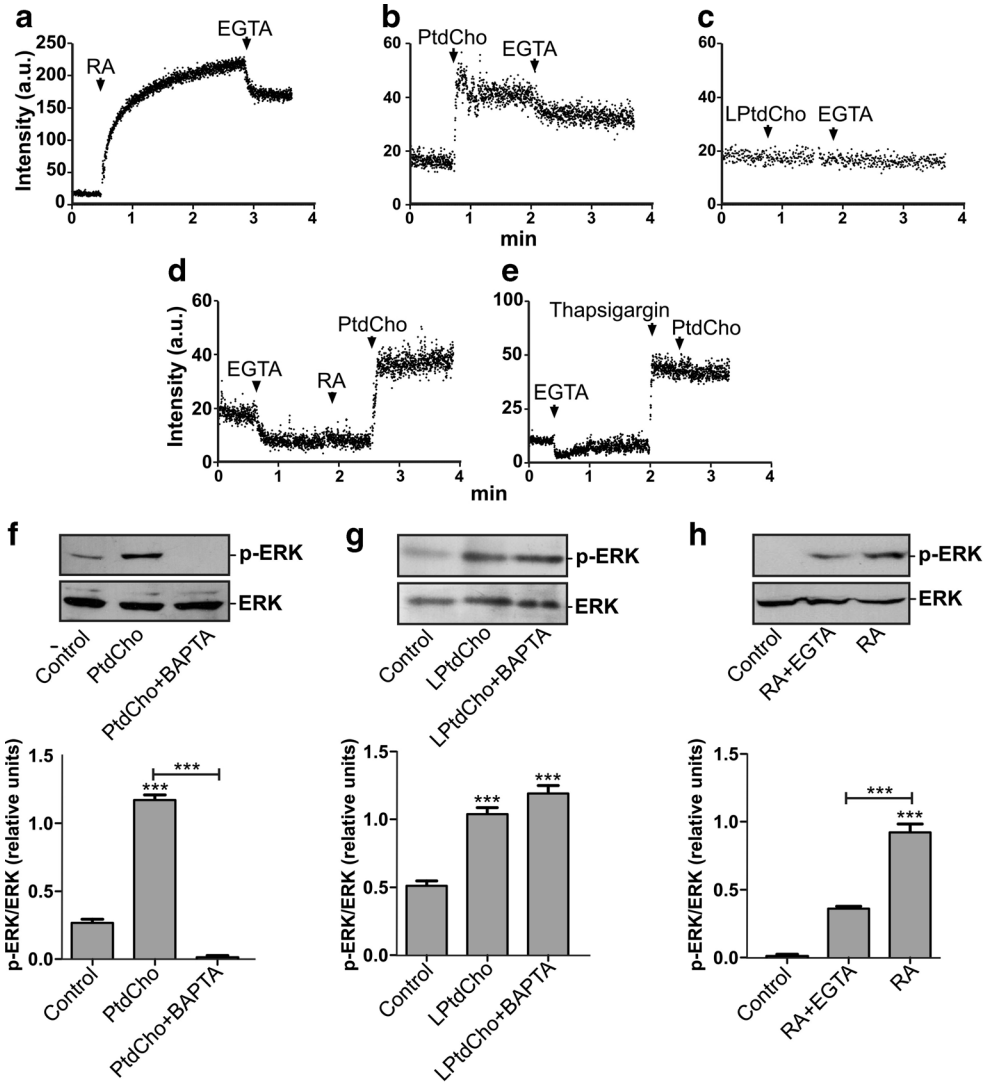

Fig. 6 LptdCho does not mobilize calcium but RA induces an increase in cytoplasmic calcium. **a**–**e** Cells were loaded with the fluorescent calcium indicator Flou3-AM (3 μM) and then treated with 10 μM RA (**a**), 60 μM PtdCho (**b**), and 10 μM LPtdCho (**c**), or preincubated with EGTA (2.5 mM) or thapsigargin (0.5 μM) before RA and PtdCho treatment (**d** and **e**). Changes in fluorescence were measured at 526 nm with excitation at 506 nm using Cary Eclipse Flourescence Spectrophotometer. *Graphs* are representative of three independent experiments. **f** and **g** Western blot analysis was used to investigate the amount of p-ERK in Neuro-2a treated with PtdCho (**f**) and LPtdCho (**g**), in the presence or absence of the cytoplasmic calcium chelator BAPTA (20 μM). As a control, cells were cultured in proliferating conditions. *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. ***$P<0.0001$. **h** Western blot analysis was used to investigate the amount of p-ERK in Neuro-2a treated with RA in the presence or absence of the extracellular calcium chelator EGTA (1 mM). As a control, cells were cultured in proliferating conditions. *Blot* is representative of three independent experiments. *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. ***$P<0.0001$

treated with BAPTA-AM, PtdCho failed in activating MEK/ERK (Fig. 6f); however, under the same experimental conditions, LPtdCho did activate the kinase (Fig. 6g). In addition, RA was unable to turn on the pathway in the absence of extracellular calcium (Fig. 6h).

LPtdCho Induces Neuronal Differentiation by Activation of Ras

We demonstrated that exogenous or newly generated LPtdCho recapitulate the effect of RA as neurotrophic-like factor promoting neuronal differentiation by turning on the MEK/ERK pathway. Activation of this pathway after different stimuli depends on the sequential activation of the membrane-associated small G protein Ras, followed by Raf recruitment to the membrane and finally by the activation of MEK, which phosphorylates and dissociates from $ERK_{1/2}$ [52]. The activation of this pathway is linked to differentiation in neurons [3, 53], and its temporal regulation depends on the activation of specific receptors, and also on the presence of modulators of the pathway. It was previously demonstrated that microinjection of Ras protein into PC12 cells induces morphological differentiation [54]. To get insight in the mechanism upstream of MEK, we co-transfected the plasmid pLPC-Ras-V12 designed to overexpress the constitutively active form of Ras (Ras-V12) together with phr-GFP (Stratagene) in order to visualize the transfected cells and measure the number of neurite-bearing cells. As a control, cells were transfected with the empty plasmid pLPC together with phr-GFP. We quantified neuronal differentiation by morphometric analysis of the fluorescent cells [30]. The results shown in Fig. 7a indicate that the constitutive active form of Ras promoted neuronal differentiation in the absence of any neurotrophic stimuli. Furthermore, analyses of the levels of p-ERK demonstrated that Ras-V12 alone was able to activate the MEK/ERK pathway (Fig. 7b). As RA and LPtdCho did not enhance differentiation in cells expressing Ras-V12, we propose that the effect of these neurotrophic-like factors converge in the Ras/Raf/MEK/ERK pathway. To evaluate this hypothesis, we measured the levels of p-ERK in cells induced with LPtdCho or RA that were preincubated during 1 h with specific inhibitors of Ras (FTS, 30 μM) and Raf (BAY43-9006, 2.5 μM). As Fig. 7c, d show, both inhibitors decreased the level of p-ERK, suggesting that LPtdCho and RA were unable to induce the MEK/ERK pathway when either Ras or Raf are pharmacologically inactivated.

This result, perhaps, suggests that LPtdCho could modulate the Ras activity. The biological activity of proteins encoded by the Ras family is dependent on whether they are bound to GTP or GDP, and Ras-GTP represents the activated form [29, 55]. To evaluate this hypothesis, we performed a Ras activation assay in cells that were first incubated with [^{32}P]-orthophosphate for 3 h and then treated with LPtdCho [29, 56, 57]. Proliferating cells were used as a control. As shown in Fig. 7e, LPtdCho markedly increased GTP binding to Ras within 2 min, resulting in Ras-GTP accumulation. This result indicates that Ras activation is an early step in the signaling by LPtdCho.

Discussion

In this report, we describe the involvement of PtdCho metabolism in the decision of neuroblastoma cells to either keep proliferating or to exit the cell cycle and differentiate. The present data reinforces the role of PtdCho in regulating neuronal cell fate [3], and proposes that LPtdCho acts as a neurotrophin-like factor. We demonstrated that exogenous PtdCho but not PtdEtn induces neuronal differentiation of Neuro-2a and SH5YS5 cells, mimicking the effect of the well-known therapeutic agent RA [47, 58] (Fig. 1). Furthermore, this finding recapitulates the effect shown by the stable cell lines enforced to synthesize PtdCho by the CCTα or CKα overexpression [3]. We also demonstrated that different precursors for PtdCho biosynthesis promote neuronal differentiation by inducing the de novo synthesis by the Kennedy pathway and the further PtdCho hydrolysis generating LPtdCho (Figs. 2b and 4). The lack of LPtdOH effect might reflect the absence of an active LPtdOH acyltransferase pathway in these conditions, or a low level of expression as was demonstrated in human brain tissues [59]. Furthermore, as LPtdOH acts as signal in different processes [60, 61], this result discards its participation in neuronal differentiation.

After PtdCho liposomes enter the cells, the lipids could be directly incorporated into the membrane as a building block [19], or be hydrolyzed by PLs [11]. We evaluated the second possibility as ample evidence demonstrates the role of lipids' second messengers in regulating cellular events [13, 62]. We utilized pharmacological inhibition to investigate if the activity of any PLs is required for PtdCho to promote neuronal differentiation, and demonstrated that $cPLA_2$ is essential (Fig. 2d). In the contrary, $cPLA_2$ is not required for RA-induced differentiation (Fig. 2e). We also showed that LPtdCho, but not AA, supplemented in the media induces neuronal differentiation in a similar extent than RA and PtdCho (Figs. 1a and 3).

LPtdCho could be further degraded by $cPLA_2$ or, alternatively, reacylated and converted into PtdCho by the Lands cycle [18]. As this cycle of acylation and reacylation was demonstrated to be essential for vesicle secretion [63], we analyzed its involvement for neuronal differentiation. We demonstrated that L-PAF promotes the same rate of differentiation than LPtdCho (Fig. 4c), and that the small molecule antagonist of LPAT (CI-976) [26, 27, 63] did not affect the differentiation induced by LPtdCho (Fig. 4d). These evidences confirm that LPtdCho per se and not its turnover

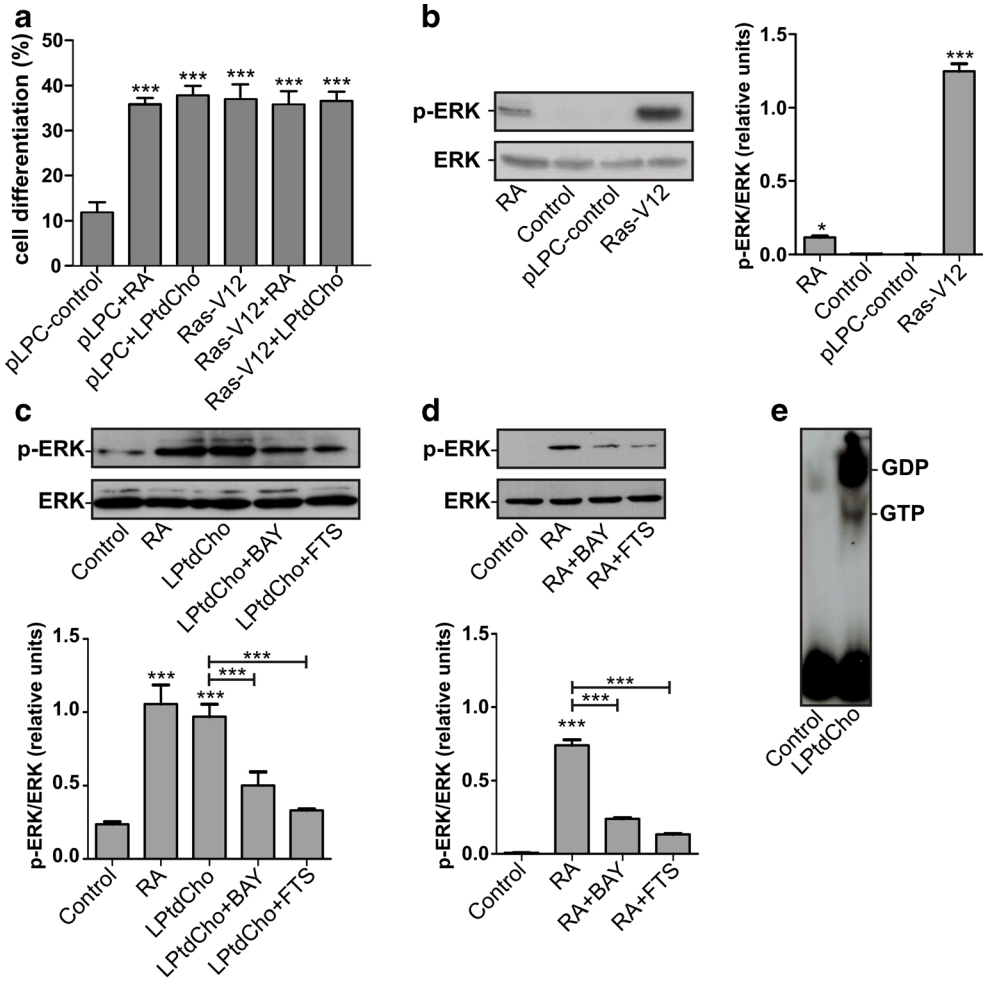

Fig. 7 LptdCho activates the small G protein Ras. **a** Neuro-2a cells were transfected with pLPC-Ras-V12 plus phr-GFP or the empty plasmid pLPC as a control and cultured in growing (control) or differentiating media containing RA or LPtdCho. Green cells were analyzed morphometrically. *Graph* is representative of three independent experiments, ***$P<0.0001$. **b** Western blot analysis of p-ERK in Neuro-2a transfected with Ras-V12 or pLPC as control, incubated with RA or under control proliferating conditions. *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. *$P<0.05$; ***$P<0.0001$. **c** Western blot analysis of p-ERK in Neuro-2a treated with RA (10 μM) or LPtdCho (10 μM) with or without the indicated inhibitors BAY (2.5 μM) and FTS (30 μM). *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. ***$P<0.0001$. **d** Western blot analysis of p-ERK in Neuro-2a treated with RA (10 μM), and the indicated inhibitors BAY (2.5 μM) and FTS (30 μM). *Graph* represents the densitometric analysis of the p-ERK/ERK ratio. ***$P<0.0001$. **e** Cells were incubated in phosphate-free MEM 10 % FBS containing 300 μCi of [^{32}P]-orthophosphate and stimulated with LPtdCho (10 μM) in DMEM 2 % FBS for 2 min. MEM 10 % FBS was used as a control for proliferation. The cells were then washed and lysated, and the supernatant was immunoprecipitated with anti-Ras antibody (conjugated to protein A-Sepharose). The beads were washed and the guanine nucleotides were then eluted by heating at 80 °C for 5 min. GTP and GDP were resolved by TLC on polyethyleneimine cellulose plates and were detected by autoradiography. *Blot* is representative of three independent experiments

triggers differentiation. Emphasizing this finding, we observed an induction of neuronal differentiation when cells growing without any lipids supplementation were treated with CI-976 (Fig. 4d). This result clearly suggests that any imbalance of the Lands cycle that may lead to an increase in LPtdCho levels could promote neuronal differentiation. Reinforcing this evidence, we demonstrated that the MEK/ERK activation promoted by LPtdCho is not affected by the $cPLA_2$ inhibitor ATK (Fig. 3b).

The neurotrophic-like effect exerted by LPtdCho was confirmed by morphometric analysis (Figs. 2f, 3a, and 5), by measuring the expression of neurons-associated markers (βIII-tubulin and acetylcholinesterase) and by demonstrating an increase in PtdCho biosynthesis for membrane expansion (Fig. 5) [3, 30].

There are many indications of specific cell signaling mediated by LPtdCho in accordance with the hypothesis of a specific receptor for LPtdCho which were implicated in regulation of chemotaxis [64], migration [65], induction of insulin receptor [66], proinflammatory activity [67], etc. However, the following evidence indicates that the neurotrophic effect demonstrated here for LPtdCho is not exerted by a direct

binding to the membrane-associated receptors like ORG1 and PAF [68, 69]. In fact, neuronal differentiation is stimulated by LPtdCho synthesized inside the cells after incubation with exogenous PtdCho, or PtdCho precursors (Figs. 1 and 2). The neurotrophic effect is independent of the fatty acid composition (Fig. 1a); however, the LPtdCho-receptor response depends on the physical properties of the phospholipid [70–74]. In addition, we demonstrated that LPtdCho signaling is independent of calcium influx (Fig. 6c, g) which was demonstrated for the effect of LPtdCho exerted trough the PAF-receptors [69].

The intriguing question was how LPtdCho turns on neuronal differentiation. For answering this, we first defined the still unknown mechanism by which RA promotes neuronal differentiation of neuroblastoma cells. The only clue we had at this point was that RA-induced differentiation depends on the MEK/ERK pathway [3, 30]. As $ERK_{1/2}$-MAPK cascade has a central and universal importance in synaptic plasticity and memory formation in many species [75], we first demonstrated that an active MEK/ERK is essential for the induction of neuronal differentiation by PtdCho, precursors for PtdCho biosynthesis or LPtdCho (Figs. 1 and 3). To evaluate the signaling events that may influence the ERK cascade, we focused in the free intracellular calcium concentration based in its versatile functions as second messenger [50]. We have demonstrated that RA treatment causes a rapid and potent increase in the intracellular calcium which depends on the extracellular calcium availability (Fig. 6a, d). More importantly, this calcium upload is essential for RA-induced activation of the ERK pathway (Fig. 6h). In addition, we have shown that PtdCho provokes a slighter increase in cytoplasmatic calcium due to its mobilization from intracellular compartments (Fig. 6b, d). Interestingly, LPtdCho does not change the levels of cytoplasmatic calcium (Fig. 6c). These two observations allow proposing the hypothesis that PtdCho-induced calcium mobilization is not involved in the ERK activation as no changes in calcium were observed with LPtdCho treatment. In support of this hypothesis, in the absence of intracellular calcium, LPtdCho but not PtdCho was able to activate the ERK pathway (Fig. 6f, g). Considering that we have demonstrated that $cPLA_2$ is essential for PtdCho to induce differentiation (Fig. 2d), together with the fact that the activity of this enzyme is calcium dependent [12], we propose that the mobilization of calcium caused by PtdCho is a requirement for the $cPLA_2$ activation and its further hydrolysis. Thus, LPtdCho induces ERK activation and neuronal differentiation in a calcium-independent way.

It is becoming apparent that the small G protein Ras and Raf represent potential integrators of the multiple signaling pathways that lead to the activation of ERKs. Furthermore, these signaling pathways appear to be important for neuronal differentiation [76]. The involvement of the small G protein Ras in Neuro-2a differentiation was confirmed by the experiment showing that the only overexpression of the constitutively active Ras (Ras-V12) promoted neuronal differentiation and ERK activation (Fig.7a, b). As RA and LPtdCho, together with the active form of Ras (Ras-V12), did not enhance the process (Fig. 7a), we propose that both neurotrophins promote neuronal differentiation by triggering the Ras/Raf/MEK/ERK pathway. Consistently, RA and LPtdCho were unable to turn on the MEK/ERK pathway in the presence of Ras or Raf inhibitors (Fig. 7c, d). In all, based in previous report [76], we propose a mechanistic model for RA in which calcium as a second messenger can control ERK signaling via the small G proteins Ras (Fig. 8). In the case of LPtdCho, which does not mobilize calcium, the question on how this lysophospholipid activates the MEK/ERK cascade was answered with an elegant experiment demonstrating that LPtdCho markedly increase GTP binding to Ras within 2 min (Fig. 7e). Thus, with this experiment, we demonstrate and conclude that LPtdCho, probably trough its biophysical properties, induces neuronal differentiation by a direct activation of Ras and in a calcium-independent manner (Fig. 7).

Finally, the demonstration that LPtdCho, either exogenously supplied or generated by the imbalance of the PtdCho turnover, acts as a neurotrophin-like factor is relevant for at least two reasons. First, it provides mechanistic details for the role of CDP-choline as a neuroprotective agent [2, 77–79]. In fact, we demonstrated that when the Kennedy pathway and the $cPLA_2$ are active, neurons treated with CDP-choline or other precursors for PtdCho biosynthesis undergo neuronal differentiation. Thus, the deleterious effect of LPtdCho

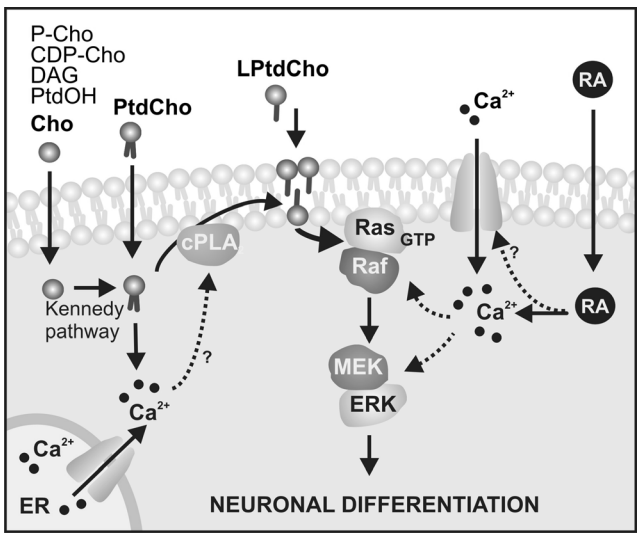

Fig. 8 Schematic model. PtdCho precursors enter to the cell and are converted into PtdCho. Exogenous PtdCho or the newly synthesized PtdCho is hydrolyzed by $cPLA_2$ generating LPtdCho. LPtdCho (exogenously supply or the one generated by $cPLA_2$) activates the small G protein Ras that triggers the Raf/MEK/ERK pathway and induces neuronal differentiation. RA promotes intracellular calcium upload which activates the Ras/Raf/MEK/ERK pathway and promotes neuronal differentiation

demonstrated at high concentration [80, 81] could be prevented by treating the cells with precursors and forcing the metabolism to the generation of LPtdCho. The proposed model is summarized in Fig. 8. Second, LPtdCho induces neuronal differentiation without the requirement of neither RA nor other neurothophic factors, and differs in the way that RA induces neuronal differentiation at least in calcium requirement and in the way of Ras activation (Fig. 7). Considering that there are many tumors resistant to RA [47, 58, 82, 83], this finding opens a door for future drugs discoveries.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no competing interests.

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