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Diacylglycerol kinase ζ generates dipalmitoyl-phosphatidic acid species during neuroblastoma cell differentiation

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ABSTRACT

Phosphatidic acid (PA) is one of the phospholipids composing the plasma membrane and acts as a second messenger to regulate a wide variety of important cellular events, including mitogenesis, migration and differentiation. PA consists of various molecular species with different acyl chains at the *sn*-1 and *sn*-2 positions. However, it has been poorly understood what PA molecular species are produced during such cellular events. Here we identified the PA molecular species generated during retinoic acid (RA)-induced neuroblastoma cell differentiation using a newly established liquid chromatography/mass spectrometry (LC/MS) method. Intriguingly, the amount of 32:0-PA species was dramatically and transiently increased in Neuro-2a neuroblastoma cells 24–48 h after RA-treatment. In addition, 30:0- and 34:0-PA species were also moderately increased. Moreover, similar results were obtained when Neuro-2a cells were differentiated for 24 h by serum starvation. MS/MS analysis revealed that 32:0-PA species contains two palmitic acids (16:0 s). RT-PCR analysis showed that diacylglycerol kinase (DGK) δ and DGKζ were highly expressed in Neuro-2a cells. The silencing of DGKζ expression significantly decreased the production of 32:0-PA species, whereas DGKδ-siRNA did not. Moreover, neurite outgrowth was also markedly attenuated by the deficiency of DGKζ. Taken together, these results indicate that DGKζ exclusively generates very restricted PA species, 16:0/16:0-PA, and up-regulates neurite outgrowth during the initial/early stage of neuroblastoma cell differentiation.

1. Introduction

Phosphatidic acid (PA) is one of the phospholipids composing the plasma membrane and acts as a second messenger to regulate a wide variety of cellular events, including mitogenesis [1], migration [2] and differentiation [3]. Previous reports have reported that PA regulates a number of signaling proteins such as phosphatidylinositol-4-phosphate 5-kinase [4,5], mammalian target of rapamycin [1], atypical protein kinase C [6], and p21 activated protein kinase 1 [7,8]. PA as an intracellular signaling lipid is generated by phosphorylation of diacylglycerol (DG) by DG kinase (DGK) [9–12] and hydrolysis of phosphatidylcholine (PC) by phospholipase D (PLD) [13–15].

PA consists of various molecular species with different acyl chains at the *sn*-1 and *sn*-2 positions, and mammalian cells contain at least 50 structurally distinct PA species. However, it has been poorly understood what PA species are produced during important cellular events until now. The main reasons for this are that PA species are minor components and it is difficult to quantify the amounts of PA molecular species using conventional liquid chromatography (LC)/mass spectrometry (MS) methods. To overcome this difficulty, we recently established an LC/MS method specialized for PA species [16]. Using this LC/MS method, we reported that a DGK inhibitor, R59949, attenuated the interleukin-2-dependent increases of 36:1-, 40:5- and 40:6-PA species in CTLL-2 cells [16]. Moreover, we revealed that DGKδ preferentially consumes palmitic acid (16:0)-containing DG species, but not arachidonic acid (20:4)-containing DG species derived from the phosphatidylinositol-turnover, in glucose-stimulated C2C12 myoblasts [17].

The sprouting of neurites, which will later become axons and dendrites, is an important event in early neuronal differentiation [18]. Some previous reports showed that the amount of PA was increased during neuronal differentiation [19,20]. However, it has not been revealed what kind of PA species (the lengths and degrees of unsaturation of the fatty acyl chains in PA species) are produced. In

Abbreviations: DG, diacylglycerol; DGK, diacylglycerol kinase; FBS, fetal bovine serum; FIPI, 5-fluoro-2-indolyl deschlorohalopemide; I.S., internal standard; LC, liquid chromatography; MS, mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PLD, phospholipase D; RA, retinoic acid

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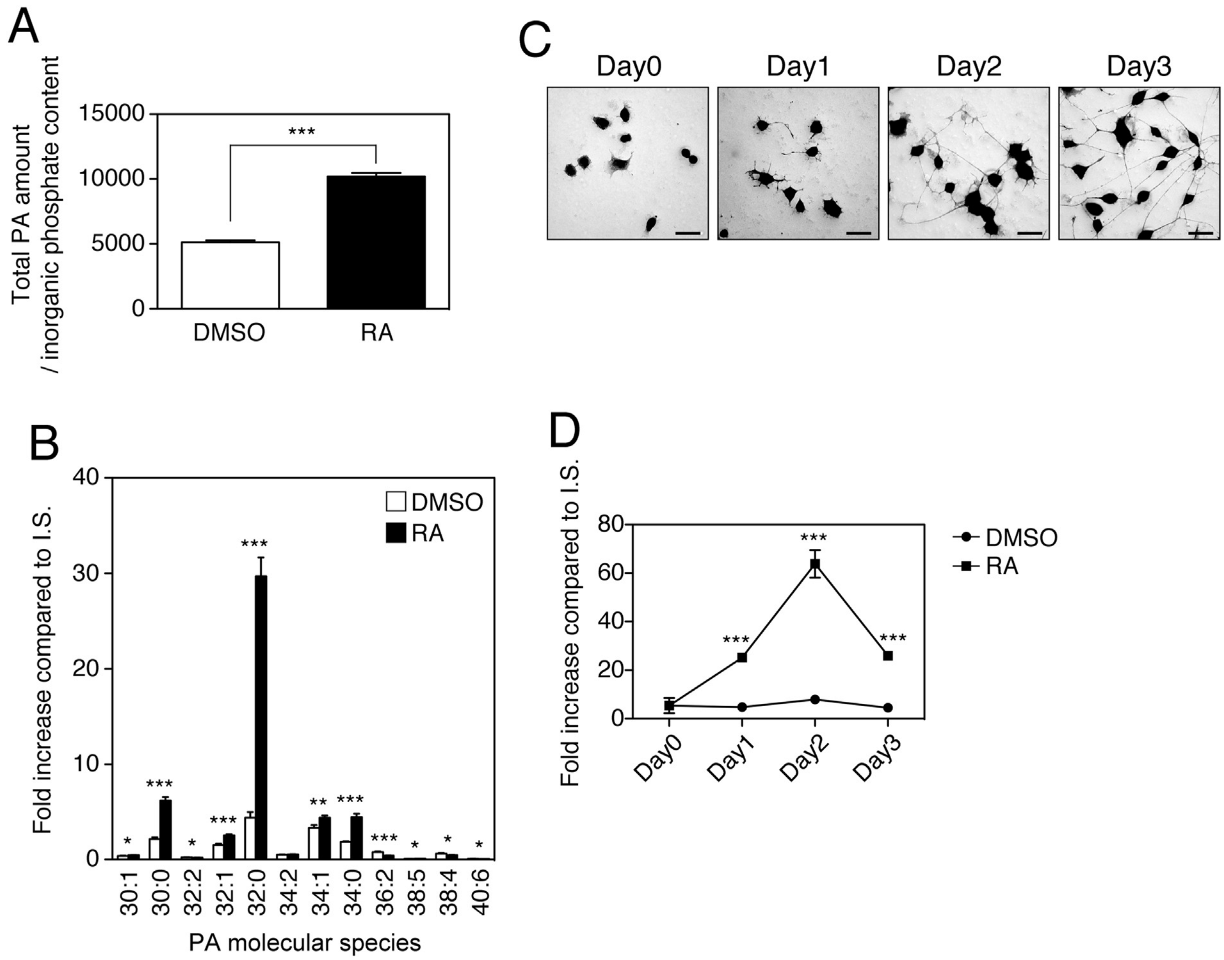

Fig. 1. Analysis of PA molecular species during RA-induced Neuro-2a cell differentiation. (**A**, **B**) Neuro-2a cells were differentiated with 20 μM RA for 24 h. The amounts of total PA **(A)** and major PA molecular species **(B)** in RA-treated (white bar) or RA-untreated (black bar) Neuro-2a cells were quantified using LC/MS (n=3). **(C, D)** Neuro-2a cells were differentiated with 20 μM RA treatment for 0–3 days. **(C)** Morphological changes in Neuro-2a cells were observed using a phase-contrast microscope. **(D)** Changes in the quantity of 32:0-PA species in a time dependent manner of RA treatment were analyzed using LC/MS (n=3). Values are presented as the mean ± S.D. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.005$. The scale bars represent 40 μm.

this study, we investigated the PA species production and its pathway during neuroblastoma cell differentiation using the newly developed LC/MS method [16]. We revealed that 16:0/16:0-PA species was dramatically increased in Neuro-2a neuroblastoma cells differentiated by retinoic acid (RA) treatment and serum starvation, and that ζ-isozyme of DGK generated the specific PA species, 16:0/16:0-PA, and up-regulated neurite outgrowth during neuroblastoma cell differentiation.

2. Materials and methods

2.1. Materials

5-fluoro-2-indolyl deschlorohalopemide (FIPI) was purchased from Calbiochem. RA was obtained from Wako Pure Chemical Industries. Standard lipids 14:0/14:0-PA and 12:0/12:0-DG were purchased from Sigma-Aldrich and Avanti Polar Lipids, respectively.

2.2. Cell culture and siRNA transfection

Differentiation of mouse neuroblastoma Neuro-2a cell (European Collection of Authenticated Cell Cultures) by serum withdrawal (0% fetal bovine serum (FBS)) and RA treatment (20 μM RA in 2% FBS-containing medium) presents a well-established model of neurite outgrowth in vitro [21–23]. Neuro-2a cells were maintained in Dulbecco's modified Eagle's medium (Wako Pure Chemical Industries) supplemented with 10% FBS (Biological Industries), 100 U/ml penicillin and 100 μg/ml streptomycin (Wako Pure Chemical Industries) at 37 °C in a humidified atmosphere containing 95% air and 5% CO_2. Neuro-2a cells were seeded in 100-mm dishes at a density of 5.0×10^5 cells/dish. To silence the expression of mouse DGKδ, we used Stealth RNAi duplexes (Invitrogen) described previously [17]. The following Stealth RNAi duplexes (Invitrogen) were used to silence the expression of mouse DGKζ: DGKζ-siRNA#1 (MSS200453), 3′-GAAUGUCCGUGAGCCAACCUUCGUA-5′ and 3′-UACGAAGGUUGGCUCACGGACAUUC-5′, DGKζ-siRNA#2 (MSS20-0454), 3′-CCAAGAUUCAGGACCUGAAACCGCA-5′ and 3′-UGCG-GUUUCAGGUCCUGAAUCUUGG-5′. Stealth RNAi™ siRNA Negative Control Med GC Duplex #2 (Invitrogen) was used as control siRNA. The duplexes were transfected into Neuro-2a cells by electroporation (at 350 V and 300 μF) using the Gene Pulser Xcell™ electroporation system (Bio-Rad Laboratories). The transfected cells were cultured in 10% FBS-containing medium for 24 h.

2.3. Lipid extraction and analysis of PA molecular species

Neuro-2a cells were harvested in ice-cold phosphate buffered saline. Total lipids were extracted according to the method of Bligh and Dyer [24]. An aliquot of the extracted lipids was used for measurement of the amount of inorganic phosphate in the phospholipid preparation [25]. PA species in extracted cellular lipids (10 μl), containing 65 pmol of the 14:0/14:0-PA internal standard (I.S.), were

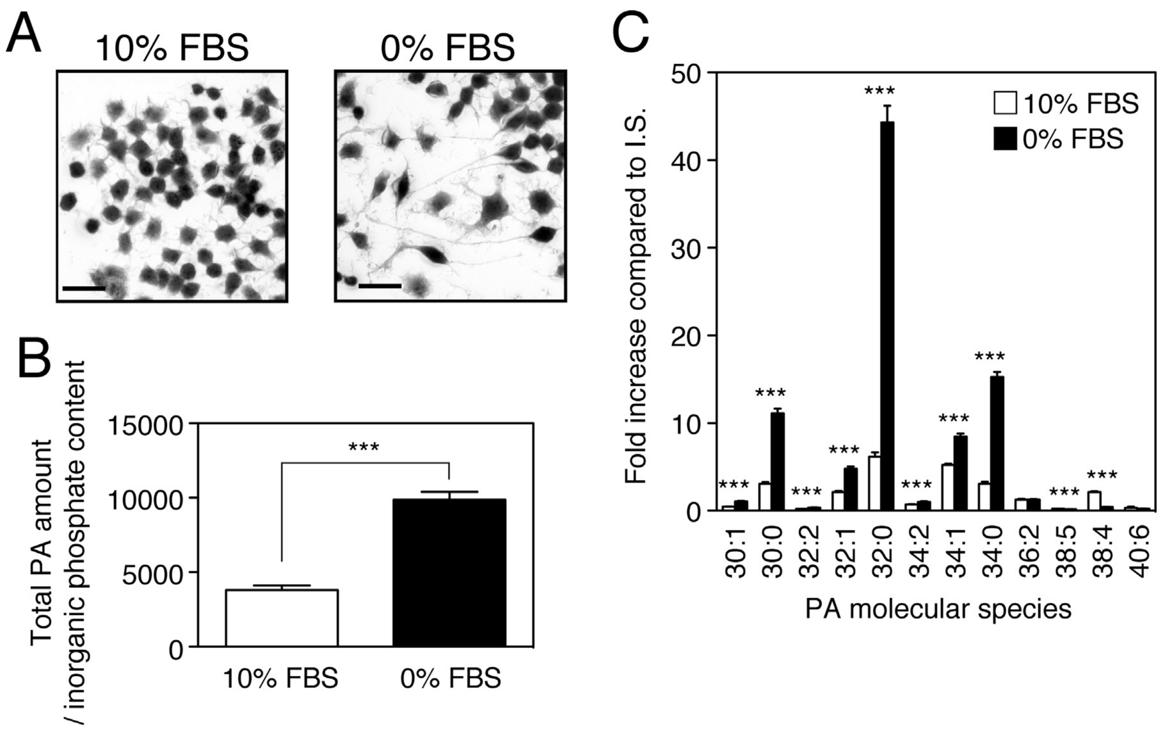

Fig. 2. Analysis of PA molecular species during serum starvation-induced Neuro-2a cell differentiation. Neuro-2a cells were differentiated by serum starvation for 24 h. (**A**) Morphological changes were observed using a phase-contrast microscope. The amounts of total PA **(B)** and major PA molecular species **(C)** in Neuro-2a cells incubated in 10% FBS containing medium (white bar) or FBS-free medium (black bar) for 24 h were analyzed using LC/MS (n=3). Values are presented as the mean ± S.D. ***, $p < 0.005$. The scale bars represent 40 µm.

Table 1
Identification of the acyl species in each PA molecular species.

PA species	Identified acyl chains	
30:0-PA	14:0/16:0 (100%)	
32:0-PA	16:0/16:0 (95.04%)	14:0/18:0 (4.96%)
34:0-PA	16:0/18:0 (100%)	

Neuro-2a cells differentiated with 20 µM RA for 24 h were used. The relative abundance (%) was based on the peak areas of the fragment ions (MS/MS) for each molecular ion.

separated by LC and detected by MS as described previously [16]. The MS peaks are presented in the form of X: Y, where X is the total number of carbon atoms and Y is the total number of double bonds in both acyl chains of PA. All LC/MS data were normalized based on the inorganic phosphate content (cellular phospholipid content) and the intensity of the internal standard. Identification of fatty acid residues in 30:0-, 32:0- and 34:0-PA species were performed by MS/MS analysis as described previously [17]. A collision energy of 35 eV was used to obtain fragment ions.

2.4. Reverse transcription polymerase chain reaction

The isolation of total RNA, reverse transcription and PCR amplification were performed as previously described [26]. The PCR amplification was performed using rTaq polymerase (TOYOBO) and the following mouse DGKα~θ-specific oligonucleotide primers. The DGKα primers were the following: forward primer (nucleotide positions 333–352, 5′-GATGGCCAAAGAGAAGGGCC-3′) and reverse primer (nucleotide positions 658–675, 5′-GTCTTCTGGCCGGCCACC-3′). The PCR conditions were as follows: 94 °C for 3 min, 32 cycles of 94 °C for 30 s, 64 °C for 30 s, and 72 °C for 0.5 min, and 72 °C for 15 min. The DGKβ primers were the following: forward primer (nucleotide positions 435–457, 5′-CCATGACAAACCAGGAAAAATGG-3′) and reverse primer (nucleotide positions 847–866, 5′-CCTCGGGT-CTTCCTCTTTCG-3′). The DGKγ primers were the following: forward primer (nucleotide positions 458–477, 5′-GATGAGCGAAGAA-CAATGGG-3′) and reverse primer (nucleotide positions 965–981, 5′-CCTGAGGTCGCCCGGTC-3′). The DGKδ [27], DGKκ [28], DGKη [28], DGKε [29], DGKζ [29], DGKι [29] and DGKθ [29] primers were previously described. The amplified PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide (Wako Pure Chemical, Osaka, Japan). A mouse whole brain was used for positive control.

2.5. Western blot analysis

Neuro-2a cells ($5{\times}10^5$ cells/100-mm dish) were lysed in 200 µl of lysis buffer (50 mM HEPES, pH 7.2; 150 mM NaCl; 5 mM $MgCl_2$; 1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; Complete protease inhibitor cocktail (Roche Applied Science) with 1% Nonidet P-40 (MP Biomedicals)). Cell lysates were separated using SDS-PAGE. The separated proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories) and blocked with 5% (w/w) skim milk. The membrane was incubated with polyclonal anti-DGKδ antibody [30,31], polyclonal anti-DGKζ antibody [32] or anti-β-actin antibody (Sigma Aldrich) in 5% (w/v) bovine serum albumin for overnight. The immunoreactive bands were visualized using peroxidase-conjugated goat anti-rabbit IgG antibody or goat anti-guinea pig IgG antibody (Jackson Immuno Research Laboratories), and the ECL Western-Blotting detection system (GE Healthcare). The relative band intensity was analyzed by Image J software. The expression levels of DGKδ or DGKζ were normalized with β-actin.

2.6. Assay of neurite outgrowth

To examine the effect of DGKζ-siRNA on RA- and serum starvation-induced neurite outgrowth of Neuro-2a cells, DGKζ-siRNA#2 transfected-cells were differentiated with 20 µM RA treatment or by serum starvation for 24 h. These cells were fixed with 3.7% paraformaldehyde for 15 min. The morphological changes were observed by a phase-

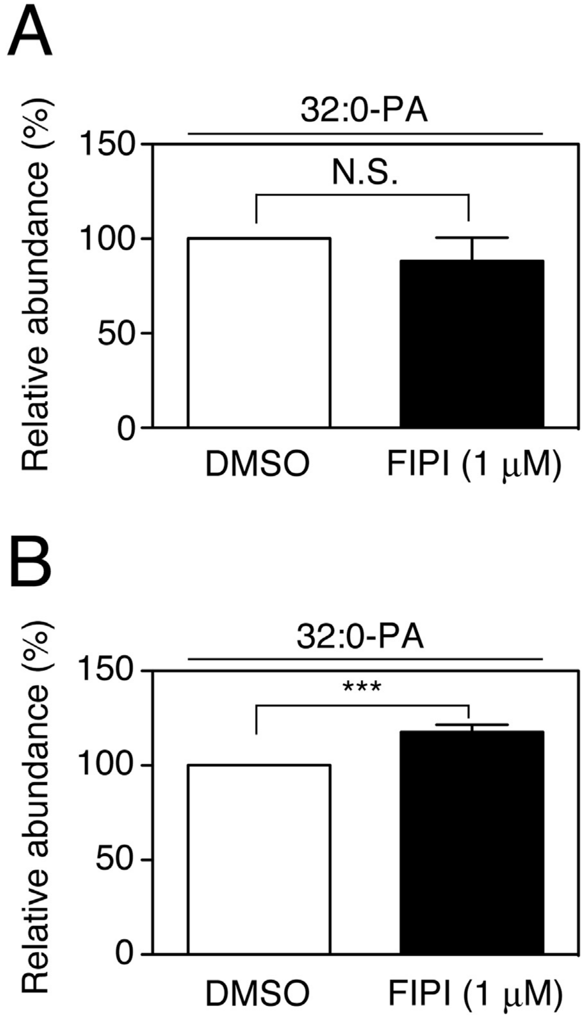

Fig. 3. Effect of PLD inhibitor on 32:0-PA species production during Neuro-2a cell differentiation. Neuro-2a cells were differentiated with 20 μM RA **(A)** or by serum starvation **(B)** in the presence or absence of 1 μM FIPI (dual PLD1/2 inhibitor) for 24 h. The amounts of 32:0-PA species were analyzed using LC/MS (n=3). Values are presented as the mean ± S.D. N.S., not significant.

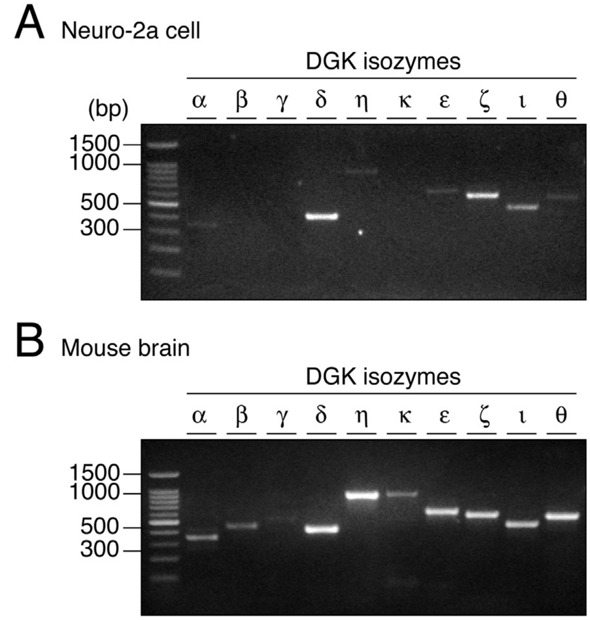

Fig. 4. Expression of DGK isozyme mRNAs in Neuro-2a cells. **(A)** mRNA expression of DGK isozymes in Neuro-2a cells was detected by RT-PCR. DGKα; 343 bp, DGKβ; 453 bp, DGKγ; 523 bp, DGKδ; 406 bp, DGKη; 828 bp, DGKκ; 843 bp, DGKε; 592 bp, DGKζ; 545 bp, DGKι; 451 bp, DGKθ; 533 bp. **(B)** As control, mRNA expression of DGK isozymes in mouse brain was also detected by RT-PCR.

contrast microscope (Olympus). The percentage of cells with neurites extending at least 2 diameters of the cell body was determined.

2.7. Statistics

Statistical analysis was performed by the two-tailed *t*-test.

3. Results

3.1. Analysis of PA molecular species during neuroblastoma cell differentiation

We first examined whether the amount of total PA was increased during Neuro-2a neuroblastoma cell differentiation. To induce neuronal differentiation, the cells were cultured in 2% FBS-containing medium with 20 μM RA for 24 h. Control cells were cultured in 2% FBS-containing medium with 0.1% (v/v) DMSO for 24 h. As shown in Fig. 1A, our LC/MS analysis revealed that the amount of total PA significantly increased 2.0-fold in RA-treated Neuro-2a cells. Notably, we observed 6.8-fold increase in the amounts of 32:0-PA molecular species in RA-treated Neuro-2a cells (Fig. 1B). Moreover, 30:0- and 34:0-PA species also moderately increased (Fig. 1B).

The time course of the production of 32:0-PA species was monitored during Neuro-2a cell differentiation with RA treatment. Neuro-2a cells were differentiated with RA treatment for 0–3 days. We confirmed that Neuro-2a cells gradually extended several neurites and differentiated (Fig. 1C). The amount of 32:0-PA species was significantly increased at day 1 and 2 after RA treatment, with maximal increases (11.9-fold) occurring at day 2 (Fig. 1D). The 32:0-PA species was then clearly decreased at day 3 after RA treatment (Fig. 1D). These results indicate that 32:0-PA species is transiently generated at the initial/early stage of RA-induced Neuro-2a cell differentiation.

We next tested whether the significant increase of 32:0-PA species was occurred by different differentiation stimulation, serum starvation. To induce Neuro-2a cell differentiation, the cells were cultured in FBS-free medium for 24 h. We confirmed that Neuro-2a cells actively extended several neurites and differentiated within 24 h after serum starvation (Fig. 2A). Control cells were cultured in 10% FBS-containing medium for 24 h. As shown in Fig. 2B, the amount of total PA was significantly increased by serum starvation (2.6-fold). The amount of 32:0-PA species dramatically increased by serum starvation (7.2-fold) (Fig. 2C) as observed for RA (Fig. 1). 30:0- and 34:0-PA species also moderately increased (3.6- and 5.0-fold, respectively). These results suggest that the production of specific PA species, 30:0-, 32:0- and 34:0-PA, is a common event during Neuro-2a cell differentiation.

Additionally, we analyzed the fatty acyl components of 30:0-, 32:0- and 34:0-PA species in Neuro-2a cells differentiated with 20 μM RA for 24 h by LC-MS/MS. Our analysis revealed that 32:0-PA is primarily dipalmitoyl-PA (16:0/16:0-PA) and that 30:0-, 32:0- and 34:0-PA species commonly contain palmitic acid (16:0) (Table 1).

3.2. Identification of the 32:0-PA species production enzyme

To gain an insight into the 32:0-PA production pathway, we examined whether PLD, which hydrolyzes PC to generate PA, produces 32:0-PA species during Neuro-2a cell differentiation. To determine the involvement of PLD activity, FIPI, which inhibits both PLD1 and PLD2 at a 10–100 nM concentration range *in vitro* and in intact cells [33,34], was used. Neuro-2a cells were differentiated with 20 μm RA or by serum starvation for 24 h in the presence of 1 μm FIPI. LC/MS analysis showed that, although 1 μm FIPI decreased several PA species such as

38:5- and 40:6-PA in undifferentiated cells (0.81-fold and 0.70-fold, respectively) (data not shown), RA-dependent 32:0-PA production was not decreased by FIPI (Fig. 3A). FIPI also failed to suppress serum starvation-dependent 32:0-PA production; rather it modestly enhanced the 32:0-PA production (Fig. 3B). These results indicate that FIPI-sensitive PLD does not substantially contribute to the production of 32:0-PA species during Neuro-2a cell differentiation.

DGK is another enzyme that is known to produce PA by phosphorylating DG. Although two DGK inhibitors, R59949 and R59022, are generally used, these inhibitors are non-specific and cannot inhibit all DGK isozymes, such as DGKα–θ [35]. Thus, we explored which DGK isozymes were strongly expressed in Neuro-2a cells using RT-PCR. RT-PCR analysis revealed that Neuro-2a cells expressed DGKα, δ, η, ε, ζ, ι and θ (Fig. 4). DGKβ, γ, and κ were undetectable in Neuro-2a cells. Because Neuro-2a cells highly expressed DGKδ and DGKζ (Fig. 4), we assessed the involvement of DGKδ and/or DGKζ in the neuronal differentiation-dependent production of 32:0-PA species.

We first examined the effect of DGKδ-siRNA on the production of 32:0-PA species. The suppression of DGKδ expression in Neuro-2a cells was confirmed by western blotting. Neuro-2a cells expressed DGKδ2, a splice variant of DGKδ gene [26]. DGKδ-siRNA efficiently suppressed DGKδ2 expression as shown in Fig. 5A. However, LC-MS analysis revealed that the RA-dependent production of 32:0-PA species was not attenuated by a deficiency of DGKδ expression (Fig. 5B). DGKδ-siRNA also failed to decrease the 32:0 PA production induced by serum starvation (Fig. 5C).

Neuro-2a cells expressed two alternative splicing products of DGKζ gene, ζ1 (104-kDa) and ζ2 (130-kDa) [36,37] (Fig. 6A). DGKζ-specific siRNA#1 and #2 efficiently suppressed DGKζ1 and DGKζ2 expression (Fig. 6A). Notably, DGKζ-siRNA#2 silenced DGKζ1 and DGKζ2 expression more effectively. Our LC/MS showed that the suppression of DGKζ expression markedly inhibited the production of 32:0-PA species with RA treatment (Fig. 6B and C). The treatment with these siRNAs also reduced 30:0- and 34:0-PA species (Fig. 6B). However, other PA species were not markedly affected (Fig. 6B). Furthermore, DGKζ-siRNA#1 and #2 also suppressed the production of 32:0-PA species by serum starvation (Fig. 6D and E). DGKζ-siRNA#2 more effectively inhibited the production of 32:0-PA species than siRNA#1 (Fig. 6B – E). The stronger effects of siRNA#2 are explainable by the stronger inhibition of DGKζ1/2 expression by siRNA#2 (Fig. 6A). These results suggest that 32:0-PA species is, at least in part, generated by DGKζ during Neuro-2a cell differentiation.

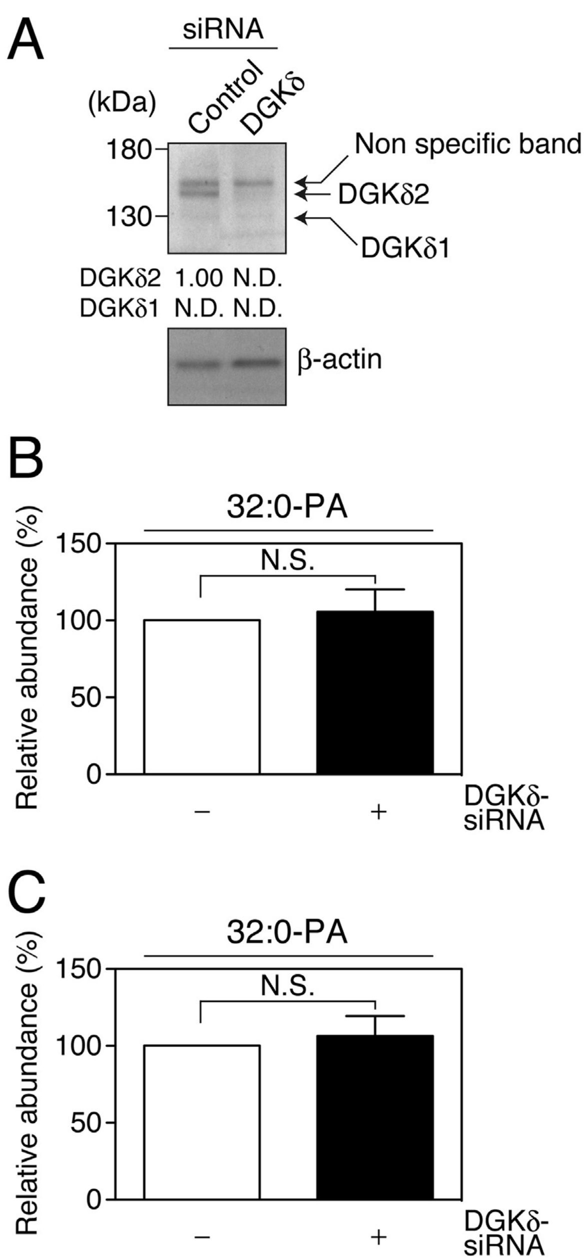

Fig. 5. Effect of DGKδ-siRNA on 32:0-PA species production during Neuro-2a cell differentiation. After 24 h of DGKδ-siRNA transfection, Neuro-2a cells were differentiated with 20 μM RA **(B)** or by serum starvation **(C)** for 24 h. **(A)** The suppression of DGKδ expression was confirmed by western blotting. **(B, C)** The amounts of 32:0-PA species were analyzed by LC/MS (n=3). The results are presented as the percentage of the value of 32:0-PA species in control siRNA-transfected cells. Values are presented as the mean ± S.D. N.D., not detectable. N.S., not significant.

3.3. Effect of DGKζ-siRNA on neurite outgrowth

We tested if the suppression of DGKζ expression by DGKζ-siRNA#2 attenuates neurite outgrowth in Neuro-2a cells. RA treatment and serum starvation markedly promoted neurite outgrowth (Fig. 7A and C). The suppression of DGKζ expression by DGKζ-siRNA#2 significantly decreased the number of Neuro-2a cells with RA-induced long neurites (Fig. 7A and B). Moreover, DGKζ-siRNA#2 strongly inhibited long neurite extension induced by serum starvation (Fig. 7C and D). Taken together, these results suggest that DGKζ promotes neurite outgrowth in Neuro-2a cells.

4. Discussion

It is known that total PA is increased during neuronal differentiation [19,20]. However, it has not been identified until now what PA species are increased. The main reasons for this are that PA species are minor components and it is difficult to quantify the amounts of individual PA species using conventional LC/MS methods. In this study, we revealed for the first time that the production of 32:0-PA (dipalmitoyl (16:0/16:0)-PA) was significantly enhanced during Neuro-2a cell differentiation induced by both retinoic acid (RA) and serum starvation (Figs. 1B and 2B) and DGKζ is involved in the production of 32:0-PA (Fig. 6). It should be noted that the 32:0-PA amount is greatly enhanced at the initial/early stage of RA-induced Neuro-2a cell differentiation (24–48 h after the RA addition) (Fig. 1D). Since budding, neurite formation, pathfinding, blanching and polarization occur at the initial/early stage of neuroblastoma differentiation [38], the 32:0-PA and its generating enzyme, DGKζ, may play important roles in those processes.

DGKδ and DGKζ were highly expressed in Neuro-2a cells (Fig. 4). Because the production of 32:0-PA species was unaffected by DGKδ-specific siRNA (Fig. 5B and C), DGKδ may be inactive during RA- and serum starvation-induced Neuro-2a cell differentiation. On the other hand, DGKζ-siRNA significantly reduced the production of 32:0-PA species induced by RA-treatment and serum starvation (Fig. 6C – E).

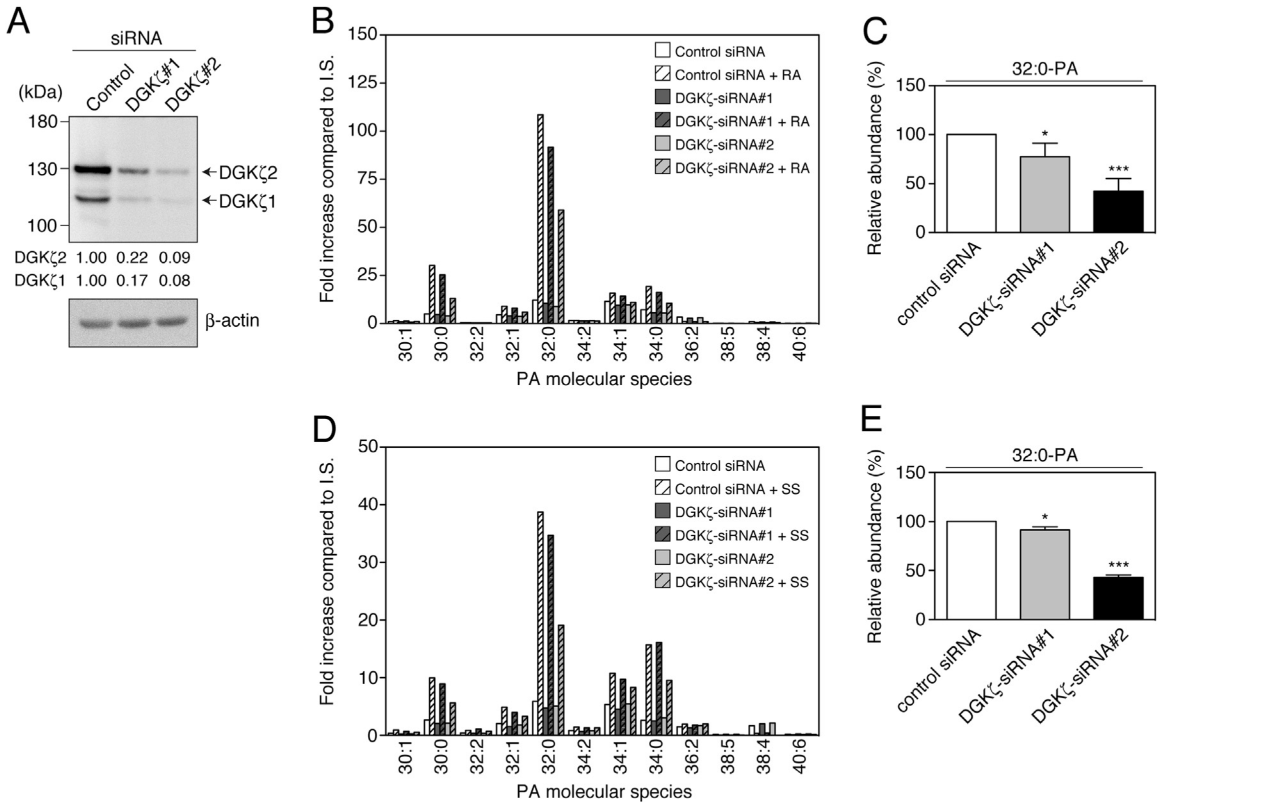

Fig. 6. Effects of DGKζ-siRNAs on 32:0-PA species production during Neuro-2a cell differentiation. After 24 h of DGKζ-siRNA#1 or DGKζ-siRNA#2 transfection, Neuro-2a cells were differentiated with 20 μM RA **(B, C)** or by serum starvation (SS) **(D, E)** for 24 h. **(A)** The suppression of DGKζ expression was confirmed by western blotting. **(B)** The amounts of major PA species were analyzed using LC/MS. Representative data from four independent experiments are shown. **(C)** The relative values of 32:0-PA species in RA-treated, DGKζ-siRNA#1- or DGKζ-siRNA#2-transfected Neuro-2a cells are shown. The amounts of 32:0-PA species were analyzed using LC/MS (n=4). **(D)** The amounts of major PA species were analyzed using LC/MS. Representative data from three independent experiments are shown. **(E)** The relative values of 32:0-PA species in serum starved, DGKζ-siRNA#1- or DGKζ-siRNA#2-transfected Neuro-2a cells are shown. The amounts of 32:0-PA species were analyzed using LC/MS (n=3). The results are presented as the percentage of the value of 32:0-PA species in control siRNA-transfected cells. Values are presented as the mean ± S.D. *, $p < 0.05$, ***, $p < 0.005$.

DGKζ was reported to promote neurite outgrowth in NIE-115 cells [39]. Rac1 is essential for RA-induced neurite extension of Neuro-2a cells [40]. DGKζ-derived PA activates p21 activated protein kinase 1, which initiates the release of Rac1 from Rho guanine nucleotide dissociation inhibitors [8]. We showed that RA-induced neurite outgrowth was attenuated by a deficiency of DGKζ (Fig. 7B). Therefore, it is possible that 32:0-PA species generated by DGKζ activates Rac1 and promote neurite extension. DGKζ-mediated synaptic conversion of DG to PA is required for the maintenance of dendritic spines [41]. In this study, we observed the role of DGKζ in the morphological changes at the initial/early stage of neuronal differentiation. Therefore, DGKζ would play important roles in both initial/early stage of neuronal differentiation and maintenance of dendritic spines through controlling PA contents. However, we could not analyze direct effects of 32:0-PA on neurite extension at present because introduction of PA into cells was technically difficult. Although we tried to stably and transiently express a kinase-dead DGKζ mutant in Neuro-2a cells many times, the expression levels were low. It was reported that overexpression of DGKζ in NIE-115 cells promoted neurite formation and this effect was independent of DGKζ kinase activity [39]. Thus, we cannot deny the possibility that DGKζ enhanced neurite extension in a kinase activity-independent manner. Further studies are needed to determine the role of 32:0-PA molecular species in neuronal differentiation. It is interesting to explore 32:0-PA-specific targets in neuronal cells and brain.

Even DGKζ-siRNA#2, which quite effectively silenced DGKζ1 and DGKζ2 expression (Fig. 6A), did not completely attenuated RA- and serum starvation-dependent 32:0-PA production (approximately 40% remains) (Fig. 6C and E), suggesting the involvement of other enzymes including other DGK isozymes and PLD. Although DGKδ was intensely expressed in Neuro-2a cells (Fig. 4A), the RA-dependent production of 32:0-PA species was not inhibited by a deficiency of DGKδ expression (Fig. 5B). Because DGKι, which is structurally highly similar to DGKζ [9–12], was also strongly expressed (Fig. 4A), this isozyme may be involved in the 32:0-PA production.

It was reported that PLD2-derived PA promotes NGF-induced neurite outgrowth [42,43]. However, Oliveira *et al.* demonstrated that, in PLD2 knock-out brains, 32:1- and 38:4-PA species were decreased but 32:0-PA, which was decreased by DGKζ-siRNA in RA-treated and serum starved Neuro-2a cells (Fig. 6), was increased [44]. In this study, FIPI failed to inhibit RA- and serum starvation-dependent 32:0-PA production (Fig. 3A and B). Thus, it is likely that PLD does not mainly participate in 32:0-PA production during Neuro-2a cell differentiation. Recently, protein arginine methyltransferase 8 was also reported to have PLD activity [45]. If protein arginine methyltransferase 8-PLD is a FIPI-insensitive enzyme, it is possible that this enzyme contributes to the DGKζ-independent PA production. Antonescu et al. [46] reported that cellular PA levels increased upon inhibition of PLD (750 nM FIPI) in epithelial BSC-1 monkey kidney cells and speculated that a DGK-dependent negative feedback regulation though PLD inhibition produced PA. Serum starvation-dependent 32:0-PA production also modestly increased with FIPI treatment (Fig. 3B). There might be a DGK-dependent negative feedback regulation to produce 32:0-PA.

The expression levels of DGKζ2 were only modestly increased (approximately 30% increase) and those of DGKζ1 were moderately decreased (approximately 20% decrease) with RA treatment for 24–48 h (data not shown). The expression changes cannot explain the drastic increase of 32:0-PA (approximately 7-fold increase) (Fig. 1). In addition, the membrane translocation of DGKζ was not observed

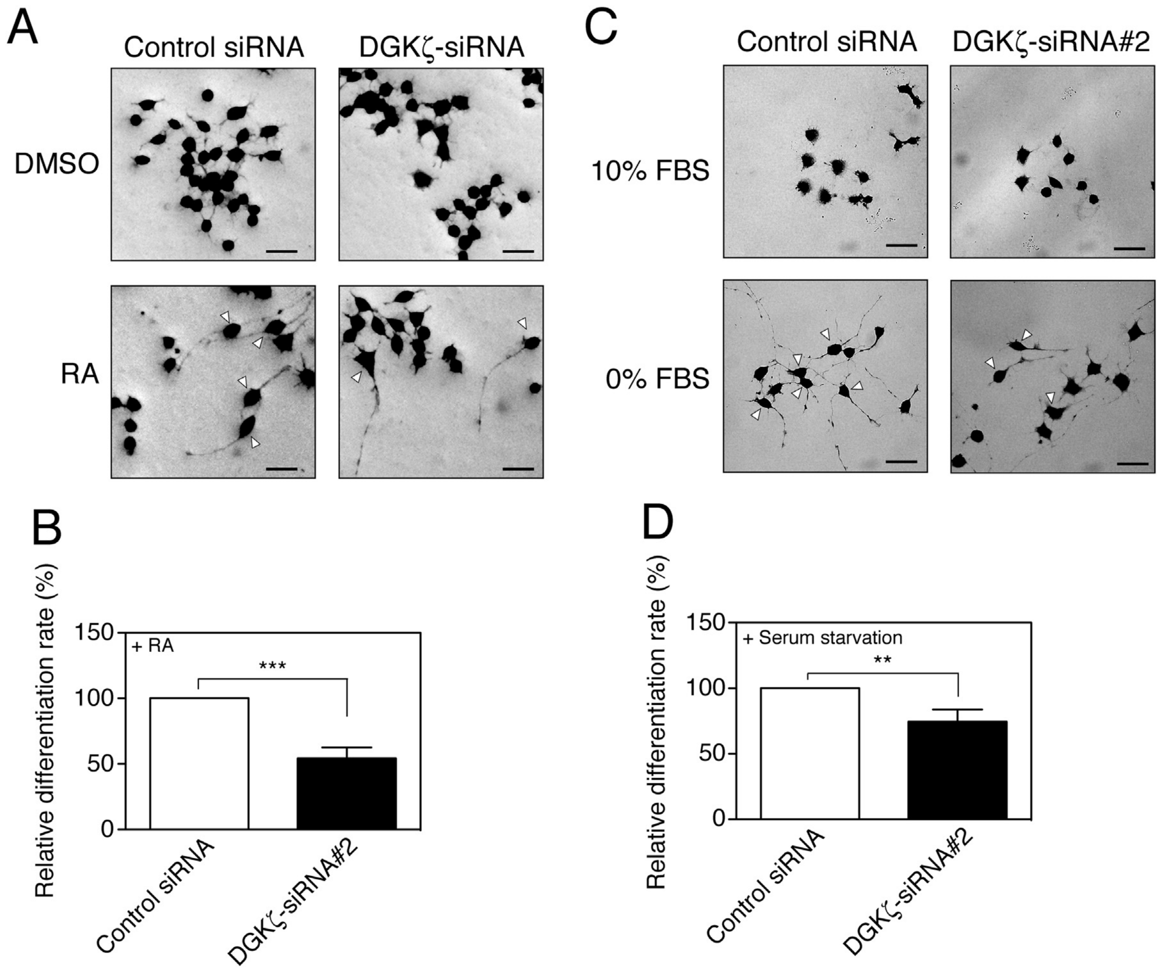

Fig. 7. Effect of DGKζ-siRNA on neurite outgrowth of Neuro-2a cells. After 24 h of DGKζ-siRNA#2 transfection, Neuro-2a cells were differentiated with RA treatment **(A and B)** or by serum starvation **(C, D)** for 24 h. **(A, C)** Morphological changes were observed using a phase-contrast microscope. **(B, D)** The percentage of cells with neurites extending at least 2 diameters of the cell body was measured (arrowhead). More than 100 cells were counted in each experiment. The average of relative RA- or serum starvation-dependent differentiation rates of DGKζ-siRNA#2 transfected cells (black bar) compared to control siRNA-transfected cells (white bar) are shown (n=3). Values are presented as the mean ± S.D. **, $p < 0.01$, ***, $p < 0.005$. The scale bars represent 40 µm.

during RA-induced differentiation. Intriguingly, 32:0-DG, substrate of DGKζ, was substantially increased at 24 h after the RA addition (data not shown). Therefore, it is possible that the production of 32:0-PA is regulated by the DG supply at the initial/early stage of RA-induced Neuro-2a cell differentiation.

Our recent report showed that R59949, a DGK inhibitor, attenuated the interleukin-2-dependent increases of 36:1-, 40:5- and 40:6-PA species in CTLL-2 cells [16]. Moreover, we also demonstrated that, compared to DGKζ, DGKδ generated relatively broad PA species such as 30:0-, 30:1-, 32:0-, 32:1-, 34:0- and 34:1-PA in glucose-stimulated C2C12 myoblasts [17]. These profiles are clearly different from that of DGKζ in Neuro-2a cells (Figs. 1B and 2B). These results further support the fact that DGK isozymes utilize a wide variety of DG molecular species as a substrate in different stimuli and cells.

In conclusion, the present study indicates that palmitic acid (16:0)-containing PA species, especially 16:0/16:0-PA species, were dramatically increased during RA- and serum starvation-induced Neuro-2a cell differentiation. Moreover, our results suggest that DGKζ is involved in the production of these PA species and promotes neurite outgrowth. These results provide a novel biochemical insight into the molecular mechanisms underlying neuroblastoma differentiation.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.10.004.

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