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The effect of cyclic phosphatidic acid on the proliferation and differentiation of mouse cerebellar granule precursor cells during cerebellar development

Misa Konakazawa[a], *Mari Gotoh*[b], *Kimiko Murakami-Murofushi*[b], *Ayana Hamano*[a], *Yasunori Miyamoto*[a,*]

[a]*Graduate School of Humanities Sciences, Ochanomizu University, Otsuka 2-1-1, Bunkyo-ku, Tokyo 112-8610, Japan*
[b]*Research Division of Human Welfare Science, Ochanomizu University, Otsuka 2-1-1, Bunkyo-ku, Tokyo 112-8610, Japan*

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ABSTRACT

The proliferation and differentiation of cerebellar granule cell precursors (GCPs) are highly regulated spatiotemporally during development. We focused on cyclic phosphatidic acid (cPA) as a lipid mediator with a cyclic phosphate group as a regulatory factor of GCPs. While its structure is similar to that of lysophosphatidic acid (LPA), its function is very unique. cPA is known to be present in the cerebellum at high levels, but its function has not been fully elucidated. In this study, we examined the role of cPA on the proliferation and differentiation of GCPs. A cell cycle analysis of GCPs revealed that cPA reduced the number of phospho-histone H3 (Phh3)-positive cells and bromodeoxy uridine (BrdU)-incorporated cells and increased an index of the cell cycle exit. We next analyzed the effect of cPA on GCP differentiation using Tuj1 as a neuronal marker of final differentiation. The results show that cPA increased the number of Tuj1-positive cells. Further analysis of the proliferation of GCPs showed that cPA suppressed Sonic hedgehog (Shh)-dependent proliferation, but did not suppress insulin-like growth factor-1 (IGF-1)-dependent proliferation. P2Y5 (LPA6), an LPA receptor, is highly expressed in GCPs. The knockdown of P2Y5 suppressed the inhibitory effect of cPA on the proliferation of GCPs, suggesting that P2Y5 is a candidate receptor for cPA. Thus, cPA suppresses the Shh-dependent proliferation of GCPs and promotes the differentiation of GCPs through P2Y5. These results demonstrate that cPA plays a critical role in the development of GCPs.

Abbreviations: BrdU, bromodeoxy uridine; cPA, cyclic phosphatidic acid; EGL, external granule layer; DAPI 4′, 6-diamidino-2-phenylindole dihydrochloride; GCP, granule cell precursor; GPCR, G-protein coupled receptor; HBSS, Hanks balanced salt solution; iEGL, inner external granule layer; IGF, insulin-like growth factor; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; PACAP, pituitary adenylate-cyclase activating polypeptide; PBS, phosphate buffered saline; Phh3, phospho-histone H3; PKA, protein kinase A; PFA, paraformaldehyde; Ptc, Patched; RT-PCR, reverse transcribed-polymerase chain reation; Shh, Sonic hedgehog; siRNA, small interfering RNA; Smo, smoothened

*Corresponding author.
E-mail address: miyamoto.yasunori@ocha.ac.jp (Y. Miyamoto).

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1. Introduction

Cyclic phosphatidic acid (cPA) is a lysophospholipid that has a unique structure consisting of a cyclic phosphate at the *sn*-2 and *sn*-3 positions of its glycerol backbone (Murakami-Murofushi et al., 1992, 2000). cPA was first discovered in the slime mold *Physarum polycephalum* (Murakami-Murofushi et al., 1992, 2000) and has since been isolated from a wide range of organisms, from myxomycetes to mammals, where it is abundant in both serum and brain tissue (Bandoh et al., 1999; Shan et al., 2008; Tsuda et al., 2006).

cPA has multiple biological functions including the regulation of cell proliferation and migration. cPA is a negative regulator of cell division via its inhibition of mitogen-activated protein kinase (MAPK) activity (Fischer et al., 1998; Murakami-Murofushi et al., 1993, 2002). Moreover, cPA inhibits the invasion of rat ascites hepatoma cells (MM1 calls) and HT-1080 human fibrosarcoma cells (Mukai et al., 2003; Murakami-Murofushi et al., 2002).

cPA is abundant in the central nervous system where it affects cell survival and neurite extension (Fujiwara et al., 2003; Fujiwara, 2008; Gotoh et al., 2010; Hotta et al., 2006). In addition, cPA exhibits neurotrophic effects in embryonic hippocampal neurons (Fujiwara et al., 2003). In vivo, cPA has a neuroprotective effect against both ischemia-induced delayed neuronal death in the hippocampal CA1 region and hypoxia-induced apoptosis in neuroblastoma Neuro2A cells (Gotoh et al., 2010, 2012).

Cerebellar granule cell precursors (GCPs), which are the most abundant neuronal precursors in the cerebellum, play an important role in the development of the cerebellum (Chizhikov and Millen, 2003; Hatten et al., 1982; Lander, 1987). Various extracellular factors regulate the proliferation and differentiation of GCPs; Sonic hedgehog (Shh) and insulin-like growth factor (IGF) are important for the proliferation of GCPs (Bondy and Cheng, 2004; Calikoglu et al., 2001; Dahmane and Ruiz i Altaba, 1999). Shh plays an important role in the proliferation of GCPs as well as in the occurrence of medulloblastoma, the most common cerebellar tumor (Browd et al., 2006; Cordeiro et al., 2014; Dahmane and Ruiz i Altaba, 1999; Roussel and Hatten, 2011; Vaillant and Monard, 2009). Several extracellular factors, such as the neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP), the cell adhesion molecule vitronectin, Wnt3, and the matricellular protein, NOV, are known to be suppressors of Shh-dependent cell proliferation in the cerebellum (Anne et al., 2013; Le Dreau et al., 2009; Nicot et al., 2002; Niewiadomski et al., 2013; Pons et al., 2001; Vaillant and Monard, 2009). Although cPA is abundant in the central nervous system (Bandoh et al., 1999; Shan et al., 2008), the effect of cPA on GCPs in the cerebellum remains unknown.

Here, we investigated the role of cPA in the proliferation of GCPs. Our results demonstrate that cPA suppresses Shh-dependent cell proliferation and promotes the differentiation of cerebellar granule cells. Furthermore, we revealed that P2Y5 acts as a receptor for cPA in GCPs and plays an important role in the regulation of the proliferation and differentiation of GCPs.

2. Results

2.1. The effect of cPA on the proliferation of GCPs

First, we investigated the effect of cPA on the proliferation of cerebellar GCPs. To address this question, we cultured GCPs derived from the cerebellum of postnatal day 6 (P6) mice, at the height of proliferation in the cerebellum. Next, we checked for glial cell incorporation in the culture. The incorporation of glial cells was less than 3%, indicating that this culture system was almost entirely composed of GCPs and granule cells (data not shown).

To analyze the effect of cPA on the proliferation of GCPs, we examined the number of cells in the S-phase using 2 h-bromodeoxy uridine (BrdU)-labeling. cPA reduced the number of BrdU positive cells, compared with vehicle treated cultures (63% decreased) (Fig. 1A, B, and G). Next, we examined the number of cells in the M-phase using the M-phase marker phospho-histone H3 (Phh3). cPA significantly reduced the number of Phh3-positive cells by 46% (Fig. 1C, D, and H). To investigate the effect of cPA on the efficiency of cell cycle exit, GCPs were labeled with BrdU for 24 h and stained with BrdU and Ki67, a marker of cell cycling. We then examined the index of cell cycle exit (Ki67−;BrdU+/Ki67+;BrdU+). Compared to the vehicle control, cPA increased the index from 1.46 to 2.43 (Fig. 1E, F, and I). Taken together, these results show that cPA suppresses the proliferation of GCPs and promotes cell cycle exit.

2.2. The effect of cPA on the differentiation of GCPs

As mentioned above, our results show that cPA suppresses the proliferation of GCPs and promotes cell cycle exit, suggesting that cPA may promote the differentiation of GCPs. To examine whether cPA affects the differentiation of GCPs, we used Tuj-1, a neuronal marker of cell differentiation, and 24 h BrdU labeling. cPA significantly increased the number of Tuj-1 and BrdU double positive cells by 1.4-fold (Fig. 2A, B, and E). Moreover, the number of cells positive for BrdU and NeuN, a marker of neuronal differentiation, was also increased by cPA (1.7-fold increased) (Fig. 2C, D, and F). These results are similar to those using Tuj-1.

Next, we verified whether cPA promotes the progression of differentiation using TAG-1, a marker of early differentiation in GCPs, combined with 24 h BrdU labeling. Treatment with cPA did not significantly affect the number of BrdU and TAG-1 double positive cells (Fig. 2G). This result suggests that cPA does not affect the progression of the early differentiation of GCPs. These results combined demonstrate that cPA suppresses cell proliferation and promotes the final stages of differentiation of GCPs.

2.3. The effect of cPA on Shh-and IGF-dependent cell proliferation

The above experiments were performed in culture media containing Shh. We next confirmed whether the effect of cPA on the proliferation of GCPs was Shh-dependent. To address

this question, we examined the effect of cPA on the number of 24 h-labeled BrdU-positive cells with Shh or IGF-1. The results showed that Shh treatment increased the number of BrdU-positive cells, and the Shh-dependently increased BrdU-positive cell number was reduced to baseline levels by the cPA treatment (Fig. 3A), whereas cPA did not affect the IGF-dependently increased BrdU positive cell number (Fig. 3B). These results indicate that cPA suppresses the Shh-dependent proliferation of GCPs, but not the IGF-dependent proliferation.

Patched and Smoothened (Smo) play important roles in the Shh-signaling pathway (Stanton and Peng, 2009; Vaillant and Monard, 2009). Patched (Ptc) not only is a receptor for Shh but also suppresses the activity of Smo. The binding of Shh to

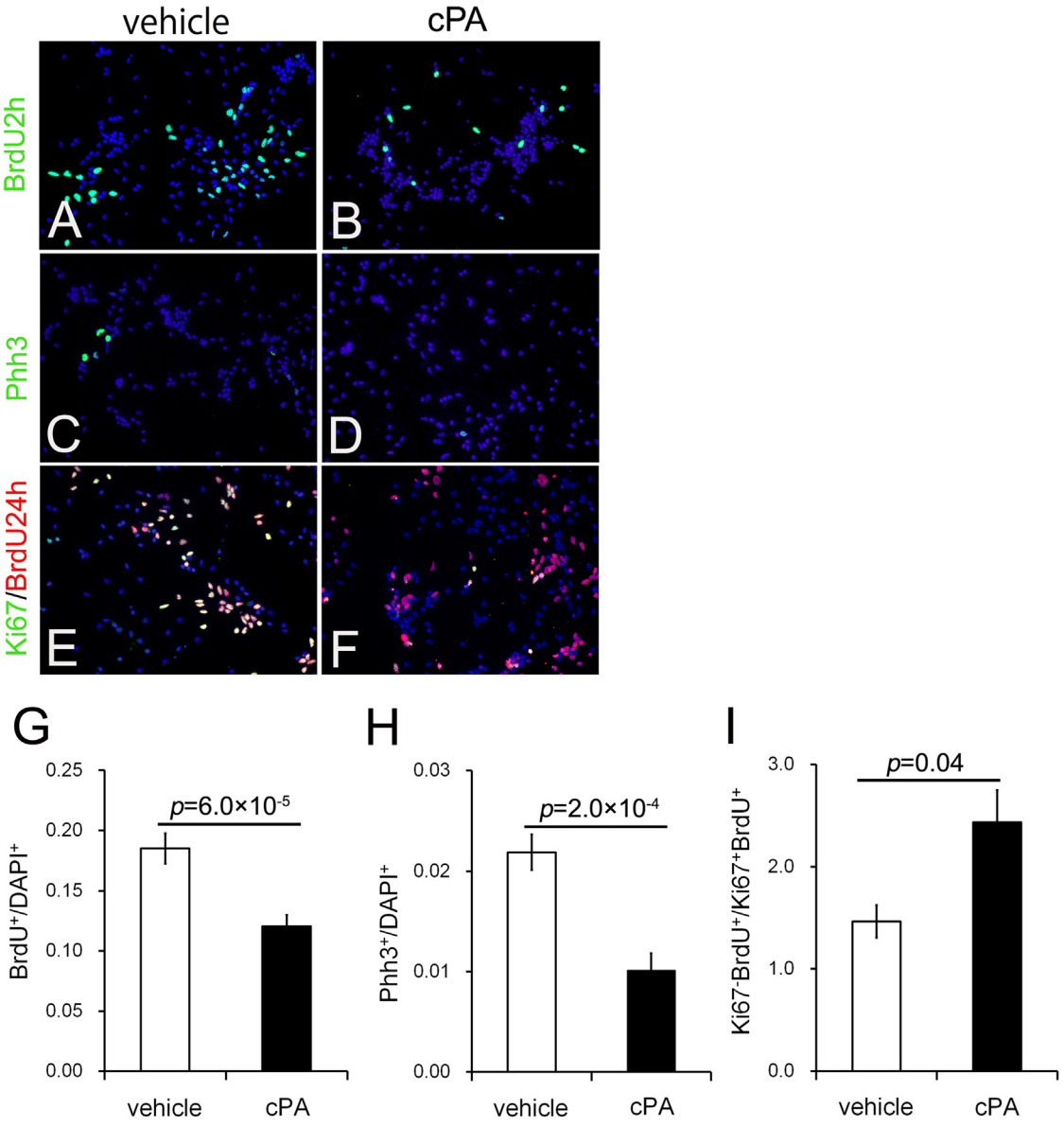

Fig. 1 – The effect of cPA on the proliferation of GCPs. In the primary cultured cells derived from the cerebellum of ICR mice at postnatal day 6 (P6), cPA (1 μM) (cPA) or 0.1% bovine serum albumin (BSA) (vehicle) was added at 1 day after plating. The cells were fixed after 4 days in culture. (A, B, G) The effect of cPA on GCPs in S-phase. GCPs were labeled with bromodeoxy uridine (BrdU) for 2 h. The GCPs were stained with anti-BrdU (green) and 4′6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue) (A, B). cPA significantly reduced the number of BrdU-positive cells (normalized to the total number of cells) compared with the vehicle control (G) ($p=6\times10^{-5}$, $n=3$). (C, D, H) The effect of cPA on GCPs in M-phase. GCPs were stained with anti-phosphohistone-H3 (Phh3) (green), a marker of M-phase, and DAPI (blue) (C, D). cPA significantly reduced the number of Phh3-positive cells compared with the vehicle control (H) ($p=2.0\times10^{-4}$, $n=3$). (E, F, I) Analysis of the index of cell cycle exit using Ki67, a maker for cell cycling, and BrdU. The GCPs were labeled with BrdU for 24 h and stained with anti-Ki67 (green) and anti-BrdU (red) and DAPI (blue) (E, F). The index of cell cycle exit was calculated from the ratio of the number of BrdU-positive and Ki67-negative cells to the number of Ki67 and BrdU double-positive cells. cPA significantly increased the index of cell cycle exit, compared to the vehicle control (I) ($p=0.04$, $n=3$).

Ptc disinhibits the activity of Smo, and thereby activates downstream signaling of the pathway (Stanton and Peng, 2009; Vaillant and Monard, 2009). To examine which part of the Shh-signaling pathway is affected by cPA, the effect of cPA on the Smo agonist-dependent proliferation of GCPs was investigated using 24 h-BrdU labeling. First, it was observed that a Smo-agonist (4, 8, or 10 nM) increased the number of BrdU-positive cells in a dose-dependent manner (Fig. 3C). At 8 nM of Smo-agonist, the Smo-agonist dependent proliferation of GCP was suppressed by cPA dose-dependently (Fig. 3D). These results demonstrate that cPA suppresses the Shh-signaling pathway downstream of Smo.

2.4. *The role of P2Y5 in the suppression of GCP proliferation by cPA*

Next, we examined which receptor was responsible for the cPA-induced suppression of GCP proliferation and promotion of differentiation. cPA is a lysophospholipid that has a cyclic phosphate and is similar in structure to LPA. LPA receptors have also been reported to bind cPA (Murakami-Murofushi et al., 2002). As cPA specific receptors have not yet been identified, we examined the expression level of different LPA receptors using reverse transcribed-polymerase chain reaction (RT-PCR), and found that only P2Y5 (LPA6) was highly expressed (Fig. 4A). Therefore, we examined neurite retraction in B103-p2y5 cells, a neuroblastoma cell line that stably expresses P2Y5. These cells indeed exhibited neurite retraction upon LPA and cPA stimulation. LPA was a more potent activator than cPA; nonetheless, the results indicated that cPA can function as a ligand for P2Y5 (Fig. 4B). To verify whether cPA affects the proliferation and differentiation of GCPs through P2Y5, we knocked down P2Y5 expression using small interfering RNAs (siRNA). The efficiency of the P2Y5 knockdown was 32.4% at the mRNA

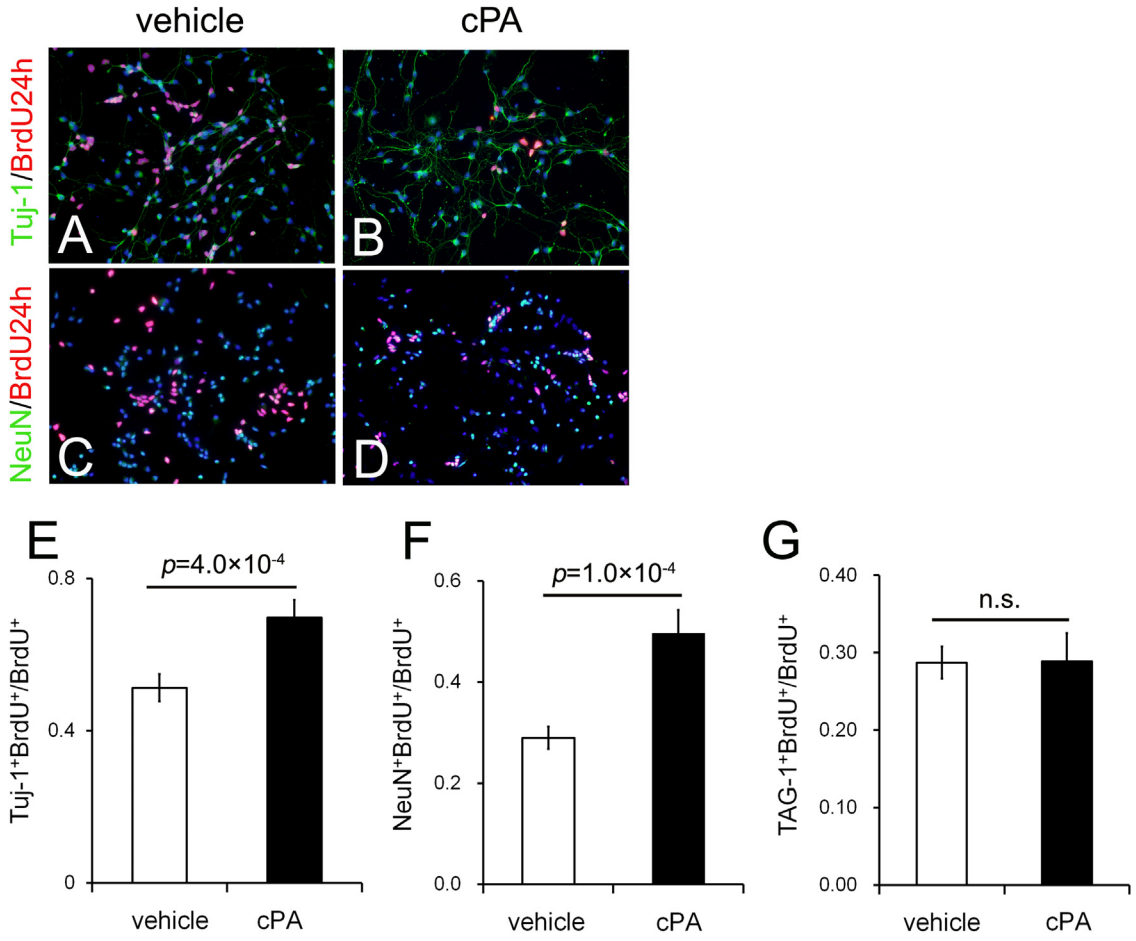

Fig. 2 – The effect of cPA on the differentiation of GCPs. cPA (1 μM) (cPA) or 0.1% BSA (vehicle) was added to the GCPs at 1 day after plating. (A, B, E) The effect of cPA on the differentiation of GCPs using a Tuj-1 antibody. The GCPs were labeled with BrdU for 24 h and stained with anti-Tuj-1 (green), a neuronal marker of final differentiation, anti-BrdU (red), and DAPI (blue) (A, B). cPA significantly increased the efficiency of Tuj-1 positive differentiation in the last 24 h (E) ($p=4.0\times10^{-4}$, $n=4$). (C, D, F) The effect of cPA on the differentiation of GCPs using a NeuN antibody. The GCPs were stained with anti-NeuN (green), a neuronal marker of differentiation, anti-BrdU (red), and DAPI (blue) (C, D). A comparison of the differentiation efficiency showed that cPA significantly increased the ratio (F); similar results were obtained with the Tuj-1 antibody ($p=1.0\times10^{-4}$, $n=3$). (G) The effect of cPA on the early differentiation of GCPs using a TAG-1 antibody. GCPs were stained with anti-TAG-1, a marker of early differentiation, and anti-BrdU. This result shows that cPA did not affect the progress of early differentiation (G) ($p=0.48$, $n=3$).

level (data not shown). With the knockdown of P2Y5, the suppression of cell proliferation by cPA was prevented to some extent (Fig. 4C). Additionally, P2Y5 knockdown using a second siRNA for P2Y5 showed a similar effect (data not shown). These results demonstrate that P2Y5 is responsible for at least part of the inhibitory growth effect on GCPs, acting as a receptor for cPA.

3. Discussion

In this study, we investigated the effect of cPA on developing cerebellar GCPs. We examined primary cultures derived from mouse P6 cerebellar GCPs. Our results reveal that cPA suppresses Shh-dependent cell proliferation and promotes the

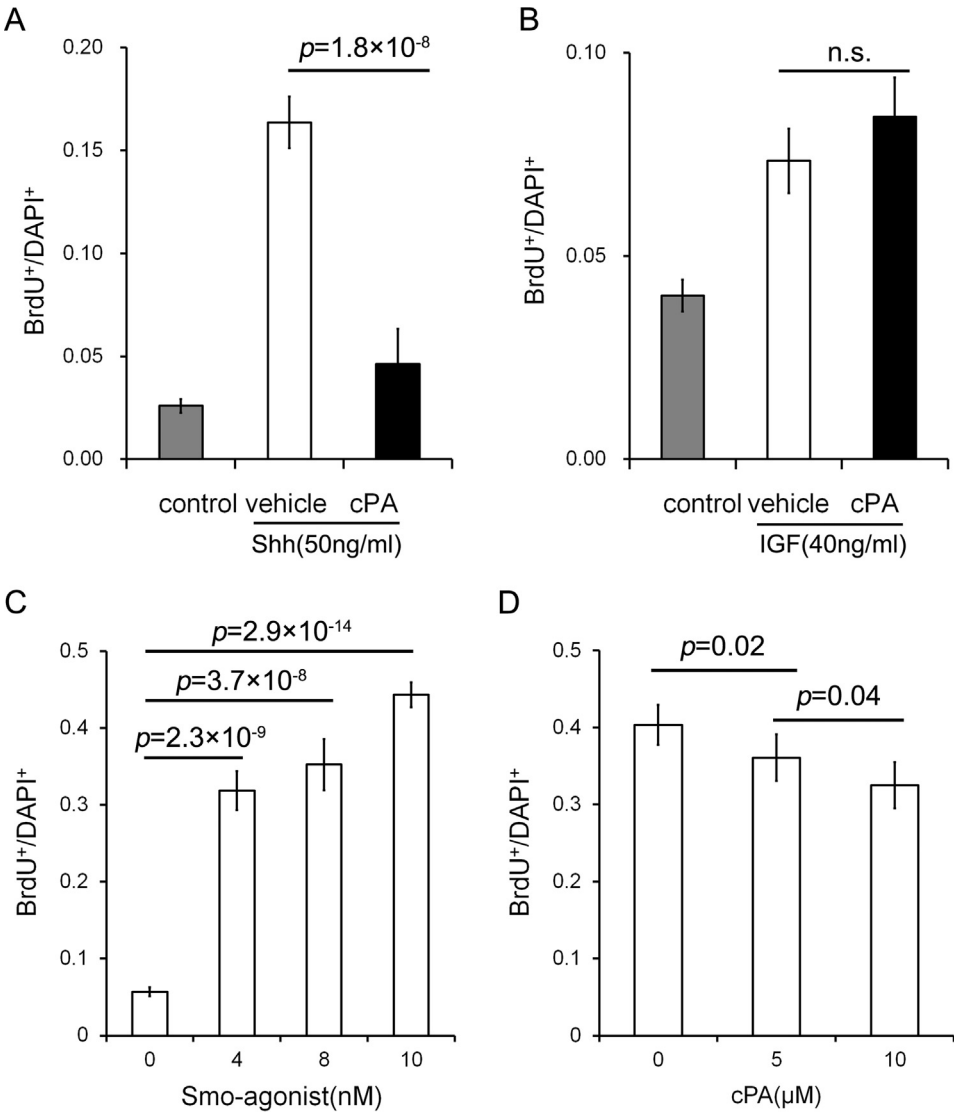

Fig. 3 – The mechanism by which cPA regulates cell proliferation. We investigated the effect of cPA on Shh-, IGF-, and Smo-agonist-dependent proliferation. (A) The effect of cPA on the Shh-dependent proliferation of GCPs. GPCs were cultured with Shh protein (50 ng/ml) in 0.1% BSA (Shh) or 0.1% BSA (control). After 1-day of culture, cPA (1 μM) (cPA) or 0.1% BSA (vehicle) was added to the culture with Shh, and the cells were labeled with BrdU for 24 h. While Shh increased the number of BrdU-positive cells, the cPA treatment significantly reduced the number of BrdU-positive cells back to baseline levels (i.e., without Shh (control)) ($p=1.8\times10^{-8}$, $n=7$). (B) The effect of cPA on IGF-1-dependent proliferation. GPCs were cultured with IGF-1 protein (40 ng/ml) in 0.1% BSA (IGF) or 0.1% BSA (control). After 1-day of culture, cPA (1 μM) (cPA) or 0.1% BSA (vehicle) was added to the culture with IGF-1. The results showed that cPA did not significantly affect the number of BrdU-positive cells with IGF-1 (cPA and vehicle) ($p=0.074$, $n=6$). (C) The effect of Smo-agonist on GCPs. GPCs were cultured with Smo-agonist (0, 4, 8, or 10 nM) and then labeled with BrdU for 24 h. Smo-agonist increased the number of BrdU-positive cells. ($p=2.3\times10^{-9}$ (Smo-agonist 0 nM/4 nM), $p=3.7\times10^{-8}$ (0 nM/8 nM), $p=2.9\times10^{-14}$ (0 nM/10 nM)) (D) The effect of cPA on the Smo-agonist-dependent proliferation of GPCs. GPCs were cultured with Smo-agonist (8 nM). After 1-day of culture, cPA (0, 5, or 10 μM) was added and the GPCs were labeled with BrdU for 24 h. The results showed that the Smo-agonist-dependent increase in cell number was reduced by cPA ($p=0.02$ (0 μM/5 μM), $p=0.04$ (5 μM/10 μM)).

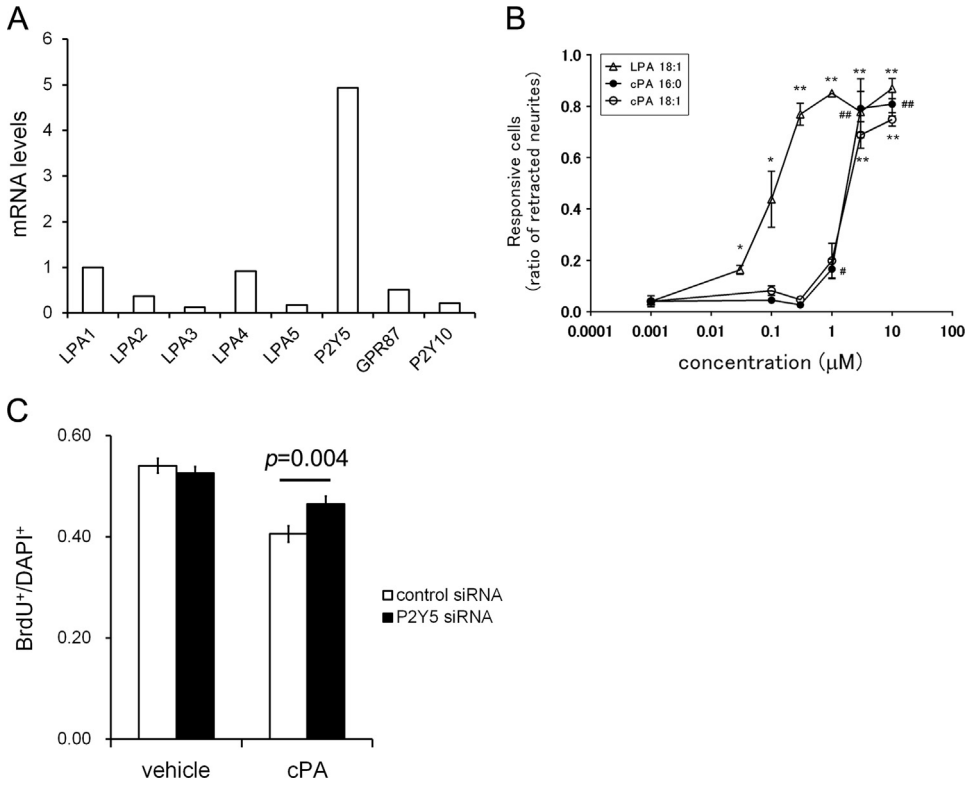

Fig. 4 – The characterization of cPA receptor candidates in GCPs. (A) Quantification of the mRNA levels of the cPA receptor candidates in GCPs. Total RNA was isolated from 4-day cultured GCPs. Each LPA receptor mRNA level was relatively quantified using the ΔΔCt method for real-time reverse transcribed-polymerase chain reaction (RT-PCR). The amount of LPA1 was regarded as 1, and the amount of each receptor candidate for cPA was normalized to LPA1. (B) Neurite retraction of B103-p2y5 cells induced by LPA and cPA. B103-p2y5 cells were serum-starved and stimulated with various concentrations of LPA 18:1, cPA 16:0 and cPA 18:1 for 10 min. The cells were then fixed. The ratio of responsive cells was quantified as the number of rounded cells over the total number of cells. The ratio of responsive cells was expressed as the mean ± standard error. The data were analyzed using a Student's t-test ($^{*}p<0.05$ and $^{**}p<0.01$ vs. vehicle-treated group for LPA 18:1, cPA 18:1, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ vs. vehicle-treated group for cPA 16:0). (C) Knockdown of the P2Y5 receptor. GCPs were transfected with small interfering RNAs (siRNA) (si-P2Y5) or negative-control siRNA at 1-day after plating and cultured with or without cPA (2.5 μM) for 2 additional days. The GCPs were labeled with BrdU for 24 h before fixation. That results show that the number of BrdU-positive cells that was reduced by cPA was recovered by si-P2Y5 treatment ($p=0.004$).

differentiation of GCPs. In addition, the experiments using a Smo agonist suggest that cPA suppresses the Shh signaling pathway downstream of Smo. Moreover, we propose that the cPA receptor responsible for the suppression of GCP proliferation is P2Y5 (LPA6).

3.1. *The role of cPA in cerebellar development*

cPA suppresses the Shh-dependent cell proliferation of GCPs. The effect of cPA appears to be similar to that of PACAP and NOV, both known suppressors of the Shh-dependent cell proliferation in cerebellar GCPs (Le Dreau et al., 2009; Nicot et al., 2002). We also detected the presence of autotaxin mRNA, a synthetic enzyme of cPA, in GCPs (data not shown). These results suggest that cPA is secreted from GCPs and diffuses throughout the external granular layer (EGL) in vivo. GCPs proliferate in the EGL, exit the cell cycle, and then initiate their differentiation into granule cells in the inner EGL (iEGL) (Alder et al., 1996; Lee et al., 2011). Our results suggest that cPA secreted from GCPs diffuses throughout the EGL; cPA ceases the proliferation of GCPs and initiates the differentiation of GCPs in the iEGL during the development of the cerebellum (Behesti and Marino, 2009; Hatten et al., 1982; Miale and Sidman, 1961; Wey et al., 2010).

3.2. *The mechanism underlying cPA-mediated suppression of Shh-dependent cell proliferation*

We analyzed the mechanism by which cPA suppresses GCP proliferation. In our experiments, cPA did not suppress the IGF-dependent cell proliferation of GCPs, but specifically suppressed Shh-dependent cell proliferation. The signaling pathway of Shh-dependent proliferation is as follows. In the absence of Shh, Ptc, a receptor for Shh, suppresses the activity of Smo. However, the binding of Shh to Ptc inhibits the suppression of Smo, thereby activating the Shh-signaling pathway through Smo (Stanton and Peng, 2009; Vaillant and Monard, 2009). Shh signaling activates the transcription of N-myc. Subsequently, N-myc up-regulates the expression of cyclin D and promotes cell proliferation (Browd et al., 2006).

Conversely, IGF-1 suppresses the activity of GSK3β, which results in the degradation of N-myc (Browd et al., 2006). In this way, each signaling pathway (i.e., Shh and IGF) is different. Our results indicate that cPA specifically affects the Shh-signaling pathway as it suppressed the proliferation of GCPs induced by a Smo agonist. Taken together, our results support the notion that cPA suppresses the downstream pathway of Smo in GCPs.

3.3. *cPA receptor candidates in GCPs*

We identified a candidate receptor through which cPA regulates the proliferation and differentiation of GCPs. cPA and LPA are lysophospholipids that are similar in structure, and the same enzyme is responsible for their synthesis. So far, several receptors have been identified for LPA, and LPA1-5 have also been shown to bind cPA (Baker et al., 2006; Fujiwara et al., 2005; Fujiwara, 2008). Therefore, we examined which LPA receptors were expressed in GCPs. Our results revealed that the mRNA level of P2Y5 (LPA6) was the highest among the LPA receptors. P2Y5 belongs to the non-Edg subfamily of the LPA receptor family (Ishii et al., 2009). Human P2Y5 is a G-protein coupled receptor (GPCR) encoded within an intron of the retinoblastoma (Rb) gene (Herzog et al., 1996) and is recognized as a LPA receptor family member because human P2Y9/GPR23 (LPA4) is closely related to human P2Y5 (Janssens et al., 1997). It has been reported that mutations in the P2Y5 gene cause woolly hair/hypotrichosis (Pasternack et al., 2008), and it is suggested that P2Y5 regulates the development of follicle cells and hair growth. Our results show that P2Y5 is a functional receptor for cPA and is responsible for cPA signaling in GCPs. This is the first report identifying P2Y5 as a receptor for cPA in GCPs.

3.4. *The mechanism underlying the P2Y5-dependent suppression of GCP proliferation by cPA*

Our results suggest that P2Y5-mediated signaling is responsible for the suppression of GCP proliferation by cPA. A number of pathways through P2Y5 have been reported (Lee et al., 2009; Pasternack et al., 2008; Yanagida et al., 2009). P2Y5 is known to couple to G12/13 protein signaling (Ishii et al., 2009). LPA activates the G13-Rho signaling pathway in P2Y5-expressing rat neuroblastoma B103 cells (Yanagida et al., 2009). In addition, P2Y5 reduces cell adhesion in the intestine by LPA and this reduction is mediated by G12/13 (Lee et al., 2009). One study using P2Y5-overexpression in CHO cells has suggested that P2Y5 is coupled to Gsα, resulting in an increase in cAMP levels. Thus far, however, a function for P2Y5 stimulated by cPA in the nervous system has yet to be reported.

The complete mechanism underlying the suppression of GCP cell proliferation is not yet clear. We propose two possibilities: one being that protein kinase A (PKA) phosphorylates Gli, a component of the Shh-signaling pathway, and thereby induces the degradation of Gli through the proteasomal pathway (Jiang, 2006; Vaillant and Monard, 2009). The activity of PKA is known to be upregulated by Gsα-coupled receptors such as the PACAP receptor via cAMP (Miyamoto et al., 1994). As mentioned above, P2Y5 could potentially be coupled to Gsα. The other possibility is that P2Y5 couples with G12/13 to activate PI-3 kinase and thus inactivate GSK3β, such as occurs with NOV. However, as mentioned above, cPA does not inhibit the IGF-dependent proliferation of GCPs, which makes this possibility less likely.

The cell proliferative signaling caused by Shh is maintained by a balance between Shh and its various suppressors (Roussel and Hatten, 2011). An unbalance causes aberrations in cerebellar formation (Behesti and Marino, 2009; Roussel and Hatten, 2011). For example, the overexpression of Shh induces tumorigenesis and medulloblastoma, a childhood cancer (Behesti and Marino, 2009; Dahmane and Ruiz i Altaba, 1999). In this study, we revealed that cPA suppresses the Shh-dependent cell proliferation of GCPs and suggest that cPA acts in concert with PACAP and NOV to suppress this proliferation. These results could potentially contribute to the development of novel treatments for medulloblastoma.

3.5. *Conclusion*

We revealed that cPA suppresses the proliferation of GCPs while promoting the final stage of GCP differentiation. Furthermore, we found that cPA specifically suppresses the proliferation induced by Shh but not IGF signaling and that P2Y5 (LPA6) mediates this suppression effect of cPA. In conclusion, we have demonstrated that cPA suppresses the Shh-dependent proliferation of GCPs through P2Y5.

4. Experimental procedures

4.1. *Animals*

Pregnant ICR mice were obtained from Charles River laboratories, Japan (Yokohama, Japan). The mice were maintained at a constant temperature (22 °C) with a 12 h light–dark cycle (8:00–20:00). Postnatal day-animal care was approved by the Institutional Animal Care and Use Committee of Ochanomizu University (animal study protocols 12010, 13002, and 14006) and followed the guidelines of the Ministry of Education, Science and Culture in Japan.

4.2. *Pharmacological agents*

cPA 16:0 was chemically synthesized as previously described (Kobayashi et al., 1993). Bovine serum albumin (BSA; fraction V, fatty acid free) was purchased from Sigma-Aldrich (St. Louis, MO). Smo agonist (Sonic Hh Ag 1.5) was purchased from Cellagen Technology (San Diego, CA). DNase I was purchased from Roche Diagnostics (Bazel, Switzerland).

4.3. *Cell culture*

The culture of GCPs was performed using a modification of the procedure described by Weber and Schachner (1984). Postnatal day-6 wild-type ICR mouse cerebella were washed in Hanks balanced salt solution (HBSS: 136 mM NaCl, 5.36 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1 mg/ml glucose, 4.17 mM $NaHCO_3$) and digested using 1% trypsin and DNase I (0.5 mg/ml) in HBSS for 13 min at room temperature.

The digested cerebella were resuspended in 0.5 mg/ml DNase I in HBSS, dispersed by pipetting, and passed through a nylon net with a mesh size of 70 μm. The dispersed cells were resuspended in serum-free media (Neurobasal Medium (Life technologies, Carlsbad, CA), supplemented with 500 μM L-glutamine, 2% B-27 Supplement (Life technologies), 20 mM KCl, 50 unit/ml penicillin and 50 μl/ml streptomycin) with 50 ng/ml Shh protein or the indicated concentration of Smo agonist. The cell density was adjusted to $4.0–6.0 \times 10^5$ cells/ml. The cell suspensions were plated on poly-L-lysine (PLL)-coated 24-well plates and cultured at 5% CO_2 and 37 °C. After 1-day of culture, the indicated concentration of cPA or 0.1% BSA was added to the culture media. For immunostaining of the cells, hydrophilic treated glass cover slips were added to the 24-well plates prior to the PLL treatment. During the culture period, one-half of the volume of culture media was exchanged with fresh media at 2 and 3 days after plating, and the cells were fixed at 4 days after plating. To label the cultured cells with BrdU, BrdU (20 μM) was added to the culture media at 24 h or 2 h before fixation.

4.4. *Immunofluorescence staining*

Immunostaining of the cultured cells was performed as follows. The 3-day cultured cells were washed in phosphate buffered saline (PBS: 8 g/l NaCl, 2.9 g/l $Na_2HPO_4/12H_2O$, 0.2 g/l KCl, 0.2 g/l KH_2PO_4); the cells were then fixed using 4% paraformaldehyde (PFA) in 0.2 M PBS for 20 min at room temperature. The fixed cells were washed with PBS and then blocked with Tris-buffered saline (TBS)++++ (10% calf serum, 3% BSA, 130 nM glycine, 0.4% Triton-X100; in TBS). The cells were incubated with primary antibody overnight and then with secondary antibodies for 1 h in dark (Table 1). To stain with anti-BrdU and anti-Ki67 antibodies, the cells were boiled in 10 mM citrate buffer (pH 6.0) prior to blocking. Following antibody staining, the cells were stained with 0.5 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Roche Diagnostics) in PBS. Images were captured using a microscope (FSX100, Olympus, Tokyo, Japan).

4.5. *Introduction of siRNA against P2Y5 in cultured GCPs*

To knockdown P2Y5, cultured GCPs were transfected with a siRNA (Stealth RNAi™ siRNA, Life Technologies) against P2Y5 or a control siRNA (Negative Control Hi GC, Med GC, or LoGC). To transfect the siRNAs, a mixture of 0.5 μl Lipofectamine 2000 (Life Technologies) and 1.5 μl of each siRNA (20 μM) in 100 μl of OptiMEM (Life Technologies) was added into each well of a 24-well plate at 1 day after plating, and incubated for 5 h. The GCPs were cultured with or without 2.5 μM cPA for 2 additional days.

4.6. *Real-time RT-PCR*

To examine the expression level of each LPA receptor, total RNA was extracted from the cultured GCPs. The total RNA was reverse-transcribed and subjected to real-time RT-PCR using Platinum SYBR Green qPCR SuperMix-UGD (Life Technologies). mRNA levels were quantified using an ABI 7300 real-time PCR machine (Life Technologies). Gene-specific primer sets were used as previously reported (Gotoh et al., 2012). To confirm the efficiency of knockdown, the following primer pair set was used: 5′-CCAGTGCCCTTATGACGACT-3′ (F) and 5′-CACAGCAATGCAAACGATCT-3′ (R). The data were analyzed using the delta Ct method. The expression level of each LPA receptor was normalized to β-actin expression.

4.7. *Analysis of neurite retraction*

B103-p2y5 cells, rat neuroblastoma cells that stably express P2Y5, were kindly provided by Prof. Takao Shimizu (University of Tokyo, Tokyo, Japan) and maintained as described previously (Yanagida et al., 2009). The morphological changes of B103-p2y5 cells induced by LPA 18:1, cPA 16:0 or cPA 18:1 were measured as follows. Briefly, the cells were seeded at 1×10^4 cells/well in a 24-well plate and cultured overnight. Then, the cells were serum-starved for 8 h. After serum-starvation, the cells were stimulated with various concentrations of the indicated compounds for 10 min, and then fixed with 2% PFA. To quantify the ratio of responsive cells, the number of rounded cells and total cells were counted (Lee et al., 2007).

4.8. *Statistical analysis*

At least three independent experiments were performed, and the data were analyzed using a Student's *t*-test. The results

Table 1 – Kind of the antibody which it used in this experiment.

Primary antibodies (dilution)	
Anti-GFAP (1:200)	SIGMA-Aldrich
Anti-BrdU (1:500)	Millipore
Anti-Ki67 (1:200)	Thermo SCIENTIFIC
Anti-TAG1 (1:200)	Developmental Studies Hybridoma Bank
Anti-Tuj1 (1:200)	Covance
Secondary antibodies (dilution)	
Alexa Fluor 488 goat anti-mouse IgG (1:200)	Life technologies
Alexa Fluor 488 goat anti-rabbit IgG (1:200)	Life technologies
Alexa Fluor 488 goat anti-mouse IgM (1:200)	Life technologies
Alexa Fluor 568 goat anti-mouse IgG (1:200)	Life technologies
Alexa Fluor 568 goat anti-rabbit IgG (1:200)	Life technologies
Alexa Fluor 568 goat anti-mouse IgM (1:200)	Life technologies

are expressed as the mean ± standard error of the mean and p values <0.05 were considered significant.

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