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

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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

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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