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Role of phospholipases D1 and 2 in astroglial proliferation: effects of specific inhibitors and genetic deletion

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ABSTRACT

Phospholipase D (PLD) activity has been linked to proliferation in many cell types including tumor cells. In the present study, we investigated the effects of genetic deletion of PLD1 and PLD2 and of specific PLD1 and PLD2 inhibitors on PLD activity and cell proliferation in primary mouse astrocytes. Basal and stimulated PLD activity was negligible in PLD1/2 double knockouts. PLD activity was significantly reduced in PLD1-deficient cells when fetal calf serum (FCS), insulin-like growth factor 1 (IGF-1) or phorbol ester was used as a stimulant. The specificity of PLD inhibitors VU0359595 and VU0285655-1 at 500 nM was confirmed in phorbol ester-stimulated cells. Significant reductions of cell proliferation were observed in PLD-deficient cell lines under basal and stimulated conditions. At 500 nM, the PLD1 inhibitor VU0359595 reduced proliferation in PLD2-deficient cells, but also in PLD1-deficient cells stimulated by IGF-1 or phorbol ester. Vice versa, at 500 nM, the PLD2 inhibitor VU0285655-1 reduced proliferation in PLD1-deficient cells, but also in PLD2-deficient cells exposed to IGF-1. At 5 μM, both inhibitors showed non-specific effects because they inhibited cell proliferation even in PLD1/2 double knockouts. Summarizing, inhibition of PLD occurs in parallel with reduced cell proliferation in astrocytes which are deficient in PLD1 or PLD2. Synthetic PLD inhibitors show high specificity for PLD in low (nanomolar) concentrations, but have additional, non-specific effects on cell proliferation when used at high (micromolar) concentrations.

1. Introduction

The phospholipases D (PLD) are ubiquitous enzymes which catalyze the hydrolysis of phosphatidylcholine to choline and phosphatidic acid, a lipid second messenger which is involved in cell proliferation (Bruntz et al., 2014a, 2014b). The two major mammalian isoforms of PLD are PLD1 and PLD2. PLD1 is located in the perinuclear region, is activated by small GTPases such as ARF and Rho and participates in budding and fusion of secretory vesicles and in stress fiber formation. In contrast, PLD2 is located at the cellular membrane, shows high basal activity, is regulated by tyrosine kinases and protein kinase C and participates in receptor endocytosis (Jang et al., 2012; Peng and Frohman, 2012).

A large number of mitogenic signals such as hormones, growth factors and certain lipids can activate PLD (Foster and Xu, 2003; Klein, 2005), and PLD and its product, phosphatidic acid, have been found to mediate cell survival and proliferation and to prevent apoptosis. Accordingly, a role for PLD in tumorigenesis has been postulated (Gomez-Cambronero, 2014; Zhang and Frohman, 2014). For instance, PLD activity is increased in gastric carcinomas (Ye et al., 2013), and ablation of PLD2 compromised tumor growth and metastasis in a breast cancer model (Henkels et al., 2013). These findings suggest that specific inhibitors of PLD isoforms may be useful as cancer therapeutics (Selvy et al., 2011). After many previous attempts had failed, a potent, PLD1-specific inhibitor VU0359595 was reported in 2009 which was 1700-fold selective over PLD2 in a cellular PLD1 assay (IC_{50} for PLD1: 3.7 nM) (Lewis et al., 2009). The PLD2-specific inhibitor VU285655-1 was described in 2010; it had an IC_{50} of 90 nM for PLD2 and was 21-fold selective over PLD1 (Lavieri et al., 2010). Concomitantly, Scott et al. (2009) reported that these PLD inhibitors blocked invasiveness in a metastatic breast cancer model. Further work focused on glioblastoma cells in which novel PLD1 and PLD2 specific inhibitors decreased migration (O'Reilly et al., 2013) and reduced autophagic flux and cell viability (Bruntz et al., 2014a, 2014b). Moreover, Chen et al. (2013) demonstrated that migration and tumor angiogenesis were reduced in several cancer cell lines exposed to PLD inhibitors, however, high concentrations of PLD inhibitors (up to 20 μM *in vitro*) were used in most of these studies.

An alternative way to test the cellular function of PLDs is genetic ablation. The first PLD1-deficient mice were published in

Abbreviations: DMSO, dimethylsulfoxide; FCS, fetal calf serum; IGF-1, insulin-like growth factor 1; PA, phosphatidic acid; PC, phosphatidylcholine; PDB, phorbol 12,13-dibutyrate; PEth, phosphatidylethanol; PLD, phospholipase D

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2010 (Dall'Armi et al., 2010; Elvers et al., 2010), and in 2012 the PLD2-deficient mouse and the Pld1$^{-/-}$/Pld2$^{-/-}$ double knockout mouse followed (Thielmann et al., 2012). Importantly, all PLD-deficient mice developed normally, were fertile and showed no obvious behavioral phenotype when compared to wild-type littermates; however, PLD-deficient mice showed deficits in thrombocyte function (Thielmann et al., 2012).

In the present study, we combined the availability of PLD-deficient mice and specific PLD inhibitors to investigate the relationship between PLD activity and cellular proliferation in primary mouse astrocytes. Our results corroborate the hypothesis that PLD activity and cellular proliferation are closely linked.

2. Material and methods

2.1. Materials

[^{3}H]-Glycerol and [^{3}H]-thymidine were from Biotrend (Koln, Germany), fetal calf serum (FCS) was from Sigma-Aldrich (Deisenhofen, Germany), recombinant IGF-1 was from BioVision (Wehrheim, Germany) and cell culture materials were from Greiner (Kremsmunster, Austria). PLD1 inhibitor VU0359595 and PLD2 inhibitor VU0285655-1 were from Avanti Lipids (Hamburg, Germany).

2.2. Animals

Transgenic mice were obtained from the lab of Prof. Nieswandt (Würzburg, Germany) (Elvers et al., 2010; Thielmann et al., 2012). All mice were housed in a facility with controlled temperature and humidity and a day/night cycle of 12/12 h. They had free access to food and water. All animal experiments were performed in agreement with EU directive 2010/63 and were registered with the local animal committee (Regierungspräsidium Darmstadt, Germany).

2.3. Cell culture

Newborn mouse pups from Pld1$^{-/-}$ and Pld2$^{-/-}$ mice, Pld1$^{-/-}$/Pld2$^{-/-}$ double knockout mice and wild-type controls were used for preparation of mouse astrocyte cultures (McCarthy and De Vellis, 1980). Briefly, cortices of 1-day old pups were collected and meninges and blood vessels were removed. Brain tissue was dissociated by passage through a 40 μm cell strainer (BD, Heidelberg), and cells were seeded into plastic culture flasks (30,000 cells per cm^2). The growth medium was DMEM containing 10% FCS, glucose (1 g/l), and antibiotics. Cells were incubated at 37 °C in a 95:5% mixture of air and CO_2. Experiments were carried out after 2 weeks in nearly (60–80%) confluent cell cultures of passages 2–4. As judged by GFAP immunostaining, these cultures contained $>$98% astrocytes, $<$1% other glial cells, and no neurons (Kötter and Klein, 1999).

2.4. Cell proliferation assay

Astroglial cell proliferation was measured by incorporation of [^{3}H]-thymidine into DNA (Freshney et al., 1980). Briefly, cells were seeded in 12-well-plates (100,000 cells per mL). Once cells were up to 80% confluent, medium was changed to serum-free medium for 24 h, then growth factors were added for an additional 24 h. 0.5 μCi of [methyl-^{3}H]-thymidine was added per well for the last 6 h of incubation. PLD inhibitors were added 30 min before the growth factors. After 24 h, cells were washed, fixed with methanol, and DNA was precipitated in three steps using 10% trichloroacetic acid, 0.5% trichloroacetic acid and 1 N NaOH. The solution was then neutralized with HCl, and DNA synthesis was determined by measuring the radioactivity in a scintillation counter.

2.5. Phospholipase D activity assay

PLD activity was measured by formation of [^{3}H]-phosphatidylethanol (PEth) as described before (Morris et al., 1997; Kötter and Klein, 1999). Astrocytes were seeded in 6-well-plates (100,000 cells per mL). To label phospholipids, cells were kept in serum-free medium containing [^{3}H]-glycerol (1 μCi per mL) for 24 h. Subsequently, the cells were washed and exposed to medium containing growth factors and 2% ethanol (v/v). PLD inhibitors were added 30 minutes prior to addition of growth factors. After 10 min of incubation, cells were extracted, phospholipids were separated by thin layer chromatography (TLC), and spots corresponding to phosphatidylcholine (PC), phosphatidic acid (PA) and phosphatidylethanol (PEth) were isolated and counted in a scintillation counter.

2.6. Statistics

All experiments were done in duplicate using at least three different preparations of astrocytes taken from different groups of newborn mice. The data from each proliferation assay were normalized defining basal cell proliferation as 100%. Data of the PLD activity assay is given as the ratio of [^{3}H]-PEth over [^{3}H]-PC. Statistical comparisons were made by ANOVA for paired or unpaired data using the GraphPad Prism program. Details are given in the figure legends.

3. Results

3.1. PLD-deficient cells have reduced PLD activity and cell proliferation

PLD1 and 2 are uniquely capable of transphosphatidylation, a reaction in which PC is transformed into phosphatidylalcohols (e.g., phosphatidylethanol) when alcohols (e.g. ethanol) are present. This reaction was used in the present study as a specific assay for total (PLD1 plus PLD2) enzymatic activity (Morris et al., 1997). Primary astrocytes were isolated from PLD-deficient mice and had measurable PLD activity under basal (unstimulated) conditions when measured by the PLD-specific transphosphatidylation assay (Fig. 1A). Surprisingly, when compared to wild-type animals, astrocytes from PLD1- and PLD2-deficient mice had only 22% and 31% lower PLD activity, respectively, than astrocytes from wild-type mice. In contrast, astrocytes from PLD1/2-deficient, "double-knockout" mice had almost undetectable PLD activity; the value for PEth formation (0.02 ± 0.02%) was not significantly different from zero.

To measure basal cell proliferation, astrocytes were kept in serum-free medium for 24 h, then [^{3}H]-thymidine incorporation into DNA was used to estimate cell proliferation. In PLD1- and PLD2-deficient cells, cell proliferation measured by DNA synthesis was reduced by 39% and 53%, respectively (Fig. 1B). In PLD1/2-deficient cells, the basal proliferation rate was only 30% of wild-type cells. These findings corroborate a role of PLD activity for normal glial proliferation and support our earlier data from rat astrocytes (Burkhardt et al., 2014a).

3.2. Effects of mitogenic stimulation on PLD activity and astroglial proliferation

In this experiment, astrocyte cultures were exposed to three mitogenic stimuli: fetal calf serum (FCS, 1% in medium), insulin-like growth factor-1 (IGF-1, 0.5 μg/ml) and phorbol-12,13-dibutyrate (PDB, 1 μM). All three mitogenic factors increased PLD activity in wild-type cells (Fig. 2A) and, in parallel experiments, also