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

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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.

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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 (IC50 for PLD1: 3.7 nM) (Lewis et al., 2009). The PLD2-specific inhibitor VU285655-1 was described in 2010; it had an IC50 of 90 nM for PLD2 and was 21-fold selective over PLD1 (Lavieri et al., 2010). Comitantly, 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 (Brunz 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
2. Material and methods

2.1. Materials

[^3H]-Glycerol and [^3H]-thymidine were from Biotrend (Koln, Germany), fetal calf serum (FCS) was from Sigma-Aldrich (Deisenhofen, Germany), recombinant IGF-1 was from BioVision (Weehrm, 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 CO2. 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 [^3H]-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-[^3H]-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 [^3H]-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 [^3H]-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 phosphatidylethanol (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 [^3H]-PEth over [^3H]-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 transphosphatidylating, a reaction in which PC is transformed into phosphatidalcohols (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 transphosphatidylating 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 [^3H]-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
increased DNA synthesis by 3-5 fold (Fig. 2B). In FCS-treated cells, both PLD isoforms were stimulated as shown by the responses of PLD1- and PLD2-deficient cells (Fig. 2A); however, only PLD2 seems to contribute to cell proliferation, because PLD1-deficient cells yielded similar proliferation rates as wild-type cells (Fig. 2B). IGF-1 stimulated PLD1 more strongly than PLD2, but cell proliferation was significantly reduced in all three PLD-deficient cell types. PDB caused a very prominent stimulation of PLD1 activity, because PLD1-deficient cells showed only 41% of PLD activity with PDB, compared to wild-type cells (Fig. 2A). PLD1 was also involved in PDB-induced cell proliferation, because PLD1-deficient cells had only 55% of the proliferation rate of wild-type cells (Fig. 2B). As expected, PLD activities of PLD1/2-deficient cells were at the detection limit, even after mitogenic stimulation (Fig. 2A).

3.3. Specificity of PLD inhibitors

In addition to genetic deletion of PLD isoforms, we tested the effects of subtype-specific inhibitors of PLD. To test their specificity, we measured PLD activity in the presence of the strongest PLD activator, PDB (cf. Fig. 2A). At 500 nM, the PLD1 inhibitor VU0359595 caused a highly significant, 58% reduction of stimulated PLD activity in astroglial cultures from wild-type mice (Fig. 3). In comparison, the PLD2 inhibitor VU0285655-1 reduced total PLD activity by only 17% (p > 0.2). PLD1-deficient cells had 50% reduced PLD activity, and the PLD1 inhibitor had no further, significant inhibitory effects. In this situation, the PLD2 inhibitor caused nearly complete inhibition of PLD activity (Fig. 3). PLD2-deficient mice had slightly, but not significantly reduced PLD activity. In these cells, the PLD1 inhibitor completely inhibited PLD activity, whereas the PLD2-specific inhibitor was inactive. These results demonstrate the high specificity of the two inhibitors used in this study, at least at concentrations of up to 500 nM.

3.4. Effects of PLD isoform-specific inhibitors on astroglial cell proliferation

Both PLD inhibitors were tested for their effects on cell proliferation in murine astrocytes stimulated by FCS, IGF-1, or PDB. In cells stimulated by 1% FCS, the PLD1 inhibitor VU0359595 decreased cell proliferation in wild-type and PLD2-deficient cells, but only at a concentration of 500 nM (Fig. 4A). Surprisingly, at this concentration, the inhibitor was also active in PLD1-deficient cells, indicating additional inhibitory effects of this compound on mitogenic pathways that do not involve PLD1. Similar observations were made when IGF-1 (Fig. 4B) or PDB (Fig. 4C) were used as stimuli.
mitogenic agents. In both cases, compound VU0359595 reduced cell proliferation in wild-type and PLD2-deficient cells, with significant effects observed at 500 nM; however, at this concentration, the substance was also active in PLD1-deficient cells (Fig. 4B and C).

The PLD2-specific inhibitor VU0285655-1 reduced DNA synthesis in wild-type and PLD1-deficient cells at 500 nM while being inactive in PLD2-deficient cells stimulated with FCS (Fig. 5A) or PDB (Fig. 5C). In cells stimulated with IGF-1, however, this compound inhibited cell proliferation in PLD1- as well as in PLD2-deficient cells, even in the lower concentration of 50 nM. Therefore, unspecific effects of VU0285655-1 on components of the IGF-1 signaling pathway have to be postulated.

3.5. Unspecific effects of PLD inhibitors

As the previous results (Figs. 4 and 5) suggested non-specific effects of PLD inhibitors, we decided to test these compounds in PLD1/2-deficient cells prepared from PLD double knockout mice. First, however, we had to test DMSO, the vehicle used to dissolve the lipophilic inhibitors, for its effect on cell proliferation. To dissolve the PLD inhibitors, we diluted saturated stock solutions so that 0.01%, 0.1% and 1% DMSO were present in the media for final inhibitor concentrations of 50, 500 and 5,000 nM (Figs. 3–5). As shown in Fig. 6A, up to 0.1% DMSO did not affect DNA synthesis in stimulated astrocytes. However, 1% DMSO in the medium, the amount required to dissolve and test 5000 nM (5 μM) concentrations of the two PLD inhibitors, caused a highly significant inhibition of glial proliferation when astrocytes were stimulated by FCS or IGF-1, respectively (Fig. 6A). Stimulation by PDB, in contrast, was insensitive to DMSO.

With this in mind, 500 nM and 5 μM of the PLD inhibitors were tested in PLD1/2-deficient cells. The high concentration of 5 μM was included because previous studies (see Section 4) had shown inhibitory effects of PLD inhibitors on tumor growth when inhibitors were used in the micromolar range. As shown in Fig. 6B, 0.1% DMSO as well as 500 nM of inhibitors dissolved in DMSO did not affect cell growth. However, Fig. 6C demonstrates that the PLD inhibitors caused further reductions of cell proliferation if even the inhibitory action of 1% DMSO is taken into account. Specifically, the PLD1 inhibitor VU0359595 caused significant reductions of cell growth in cells stimulated by either mitogen. The PLD2 inhibitor also reduced cell growth beyond the DMSO effect, but significant data was only obtained after stimulation with PDB (Fig. 6C). Nevertheless, the data shows that PLD inhibitors affect mitogenic pathways even in cells that express neither PLD1 nor PLD2.
4. Discussion

Phospholipase D activity has been linked to cell proliferation in cancer (Bruntz et al., 2014a, 2014b; Gomez-Cambronero, 2014). In addition to tumorigenesis, however, PLD activity has also been linked to brain development, in particular neurite outgrowth (Kanaho et al., 2009; Giordano et al., 2011). Dysfunction of the PLD pathway evidently contributes to impaired neuritogenesis in Alzheimer’s disease (Cai et al., 2006), whereas ablation of PLD2 was reported to improve synaptic dysfunction in an Alzheimer mouse.
model (Oliveira et al., 2010). Our group has a long-standing interest in signal-induced PLD activation (Klein, 2005) because the PLD signaling pathway is suppressed in the presence of alcohols such as ethanol which reduce phosphatidic acid formation and cellular proliferation, e.g. in glial cells. Initially, we found that the proliferation of astrocytes is disrupted by ethanol due to interference with the PLD pathway (Kötter and Klein, 1999; Schatter et al., 2003); this effect occurs in parallel to the formation of pro-apoptotic ceramide (Schatter et al., 2005). More recently, we reported that inhibitors of the PLD pathway, as well as down-regulation of PLD1, impair rat astroglial development and proliferation (Burkhardt et al., 2014a). Furthermore, in PLD-deficient mice, we found delayed brain development, impaired cognitive function, and reduced neurotransmitter release in vivo (Burkhardt et al., 2014b). Based on these findings, we postulated that inhibition of the PLD signaling pathway by ethanol contributes to the development of fetal alcohol syndrome, a major cause of microcephaly and cognitive impairment in humans. The main goal of the present study, therefore, was to study the relevance of PLD1 and 2 activities for astroglial proliferation. Here, we used murine astrocytes isolated from control and PLD-deficient mice as well as novel, specific PLD inhibitors to test the influence of reduced PLD activity on DNA synthesis and cell proliferation.

4.1. Phospholipase D activities

Total PLD activity was measured using the transphosphatidylation assay, and our data shows that both PLD1 and 2 contribute significantly to total PLD activity, and may be capable of substituting for each other (Fig. 1A), whereas no significant activity was left after depletion of both PLD1 and 2. When PLD activity was stimulated by mitogenic factors, fetal calf serum (FCS), a mixture of many mitogenic factors, was less active in the absence of either PLD isoform (Fig. 2A). In contrast, PLD activity stimulated by IGF-1 or by phorbol ester was reduced in PLD1-deficient cells, but not significantly in PLD2-deficient cells. PLD activity remained very low in PLD1/2-double knockouts. The data indicate that mitogenic stimuli preferably activate the PLD1 isoform in astrocytes, especially when protein kinase C, the target of phorbol esters, is involved. This finding is in agreement with previous data obtained with rat astrocytes (Burkhardt et al., 2014a).

In addition to PLD-deficient cells, we used isofrom-specific PLD inhibitors which had been introduced in recent years by the Vanderbilt group (Lewis et al., 2009; Lavieri et al., 2010). In our hands, the inhibitors were very effective in interfering with PLD activity. When used at 500 nM, the PLD1 inhibitor VU0359595 significantly reduced PLD activity in wild-type cells (by 50%, Fig. 3) and completely abrogated PLD activity in PLD2-deficient cells but was not significantly active in PLD1-deficient cells. In contrast, the PLD2 inhibitor VU0285655-1 had minor effects in wild-type and PLD2-deficient cells, but completely blocked PLD activity in PLD1-deficient cells (Fig. 3). This data corroborate the isofrom specificity of these two PLD inhibitors, at least in the concentration used (500 nM) (Fig. 3).

4.2. Astroglial proliferation

The majority of the data obtained for astroglial proliferation supports our concept that PLD activity is required for mitogenic signaling in this cell type. Astrocytes deficient for PLD1, PLD2, or both isoforms show reduced basal proliferation (Fig. 1B) and reduced proliferation after mitogenic stimulation (Fig. 2B). In PLD1-2-deficient cells, basal proliferation (measured as DNA synthesis) is reduced by 70% which clearly indicates a requirement for PLD in mitogenic signaling (Fig. 1B). PLD2 deficiency significantly reduces serum stimulation whereas PLD1 is required for phorbol ester stimulation. With IGF-1 as a mitogen, both PLD1 and 2 deficiency impairs astroglial proliferation, a finding that is particularly relevant because IGF-1 is an important growth factor during early brain development (Fernandez et al., 2012).

A somewhat more complex picture was obtained when different concentrations of PLD inhibitors were used in the proliferation experiments. With FCS as a stimulator, significant inhibitions were seen with both PLD1 and 2 inhibitors at concentrations of 500 nM (Figs. 4A and 5A). Serum is a mixture of many mitogens, therefore a multitude of signaling pathways will contribute to glial proliferation, and some of these pathways may involve PLD activation. Nevertheless, both inhibitors were also active when IGF-1, a specific mitogen for astrocytes, was used (Fig. 4B); here, the PLD2-specific inhibitor gave significant results already at 50 nM (Fig. 5B). PLD1, however, also seems to contribute to IGF-1 signaling because IGF-1 was clearly less active in PLD1-deficient cells (Figs. 4B and 5B). With phorbol ester as a stimulator of proliferation, weaker actions were again seen in PLD1-deficient cells. Moreover, the PLD1 inhibitor significantly reduced proliferation in wild-type and PLD2-deficient cells, respectively (Fig. 4C). Vice versa, the PLD2 inhibitor was poorly active in wild-type cells but significantly reduced proliferation in PLD1-deficient cells (Fig. 5C). This finding again indicates that PLD isoforms may, to some extent, substitute for each other, i.e. one isoform may more strongly contribute to signaling when the other isoform is absent.

While these data underscore a contribution of PLD signaling to mitogenic pathways, there were some peculiar findings with PLD inhibitors (Figs. 4 and 5). In particular, the PLD1 inhibitor VU0359595 reduced proliferation in PLD1-deficient cells when used at 500 nM (Fig. 4). Moreover, the PLD2 inhibitor VU0285655-1 reduced glial proliferation in IGF-1 stimulated cells, even at the low concentration of 50 nM (Fig. 5B). These results suggested that the two compounds, VU0359595 and VU0285655-1, may have additional inhibitory mechanisms on cell proliferation that go beyond inhibition of PLD1 and 2, respectively. To test this hypothesis, we used PLD1/2-deficient cells exposed to PLD inhibitors. For these experiments, we had to correct for the inhibitory properties of DMSO (which was required to dissolve the inhibitors) on cell growth (Fig. 6A). It should be noted that anti-mitogenic effects of DMSO were reported before (Lyman et al., 1976; Chakravarthy et al., 1992; Notman et al., 2006). Our data shows that 0.1% DMSO was inactive in the proliferation assay, but 1% DMSO was strongly inhibitory (Fig. 6A). PLD inhibitors did not significantly affect cell proliferation in double-knockout cells at 500 nM (Fig. 6B), but our data (Fig. 6C) show clearly that, at 5 μM concentrations, both PLD inhibitors affect mitogenic signaling irrespective of PLD inhibition. PLD1 inhibitor VU0359595, in particular, decreased mitogenic stimulation by more than 50% in stimulated astrocytes, independent of the stimulus, while PLD2 inhibitor VU0285655-1 was less active and only caused significant inhibition after phorbol ester stimulation. Nevertheless, we have to conclude that both inhibitors lose selectivity for PLD isoforms when used in micromolar concentrations.

These findings have consequences for the hypothesis that PLD inhibitors may be useful to suppress tumor growth. In several previous studies, cytostatic properties of PLD inhibitors were observed with micromolar concentrations of inhibitors. These effects, however, may have been due to unspecific effects on cell signaling that are unrelated to PLD inhibition (e.g., Scott et al., 2009; Norton et al., 2011; Bruntz et al., 2014a, 2014b) whereas the compounds have high selectivity when used in nanomolar concentrations. While the precise targets of these effects remain to be elucidated, it is important to note that the availability of other methods to reduce PLD activity, e.g. siRNA and knockout mice, are required to link PLD activity to biological outcomes in a reliable manner.
4.3. Conclusion

Taken together, our data confirm that PLD signaling pathways are involved in mitogenic signaling in astrocytes because PLD inhibitors (in nanomolar concentrations) as well as PLD deficiency strongly and significantly reduce astroglial cell proliferation. Fetal calf serum, a mixture of mitogens, can activate both PLD isoforms. IGF-1, an important growth factor for brain development, stimulates mainly PLD1 activity, but both PLD isoforms seem to contribute to mitogenic signaling. The phosphor ester PDB, another strong mitogen for astrocytes and the strongest stimulator of PLD activity, mainly activates and involves the PLD1 isoform in mitogenic signaling, although PLD2 can substitute to some extent in PLD1-deficient cells. In all experiments with mitogenic stimulation, disruption of PLD signaling reduced astroglial cell proliferation.

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