Retinoic acid specifically activates an oleate-dependent phospholipase D in the nuclei of LA-N-1 neuroblastoma cells

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Abstract Earlier studies showed that treatment of LA-N-1 cells with TPA, a tumoral promoter, leads to the stimulation of a G protein-regulated phospholipase D (PLD) in the nuclei. Now we demonstrate that retinoic acid, a cellular differentiation inducing agent, activates a nuclear oleate-dependent PLD in LA-N-1 cells. Treatment of the nuclei with retinoic acid induces the breakdown of phosphatidylcholine (PtdCho). Our results indicate that PLD is regulated differentially depending on the nature of the stimulatory agent. These results strongly suggest the existence of two nuclear PLD isoforms in LA-N-1 nuclei that hydrolyze PtdCho.

Key words: Phospholipase D; LA-N-1 nucleus; Retinoic acid; Retinoic acid receptor; Cellular differentiation

1. Introduction

All-*trans*-retinoic acid (atRA), the active metabolite from vitamin A, plays a key role in development and induces long-term cellular responses such as differentiation or apoptosis [1]. In the human LA-N-1 neuroblastoma cell line, atRA induces concentration- and time-dependent differentiation resulting in complete growth arrest and neuritic phenotype-like extensions [2]. These effects of RA are mediated through the activation of two types of nuclear receptors, retinoic acid receptors (RARs) and retinoic X receptors (RXRα, β and γ) [3]. Both types of receptors belong to the steroid/thyroid receptor superfamily [4,5]. RAR binds either atRA or *cis*-RA with high affinity, heterodimerizes with RXR and acts as ligand-dependent transcription factor [6].

Phospholipases C and D (PLC, PLD) hydrolyze phospholipids to produce diacylglycerol (DAG) and phosphatidic acid (PtdOH), respectively [7]. Prior studies have reported that PLC or PLD activation is dependent on the physiological state of LA-N-1 cells [8]. Furthermore, our recent data indicate that TPA, a tumoral promoter, stimulates a G protein-dependent PLD in nuclei of LA-N-1 proliferative cells [9]. In order to gain insights into the regulation of phospholipases in the nuclei of differentiating cells, we investigated the effect of atRA on LA-N-1 nuclear PLD activity using a transphosphatidylation assay [10]. In this, a primary alcohol, like ethanol or butanol-1, acts as a preferential nucleophilic acceptor for the phosphatidyl moiety resulting from mediating PLD breakdown, to produce essentially the non-metabolizable product phosphatidylalcohol, instead of PtdOH. Our results reveal that the treatment of whole cells or intact nuclei with atRA in the presence of ethanol leads to the production of nuclear DAG and phosphatidylethanol (PtdEtOH) suggesting the activation of PLC and demonstrating that of PLD. In nuclei of atRA-differentiated LA-N-1 cells, the production of PtdEtOH is not affected by the addition of the non-hydrolyzable nucleotide guanosine triphosphate (GTP)γS. However, that of oleate induces a strong enhancement of PtdEtOH, demonstrating that a nuclear oleate-regulated PLD is specifically stimulated. Moreover, in atRA-treated nuclei, an increase in the production of free choline is observed indicating that phosphatidylcholine (PtdCho) was the main substrate for the LA-N-1 oleate-dependent PLD.

2. Materials and methods

2.1. Cells and reagents

Human neuroblastoma LA-N-1 cells were obtained from Dr. Seeger, University of California at Los Angeles. Leibovitz's L-15 medium and fetal calf serum were supplied by Life Technologies (Eragny, France). Streptomycin, penicillin, trypsin inhibitor, phenylmethylsulfonylfluoride, atRA, adenosine triphosphate (ATP), GTPγS, PtdOH, dipalmitin and Dowex 50×8 WH^+ were purchased from Sigma Chemical (St. Louis, MO, USA); PtdEtOH from Tebu S.A. (Le Parray en Yvelines, France); silica gel plates G 60 (Kieselgel 60, 20×20 cm; 250 μM) from Merck (Darmstadt, Germany); [^{3}H]choline, [^{3}H]palmitic acid from NEN Life Sciences Products (Paris, France) and 1-2-di-[1-^{14}C]oleyl PtdCho from Amersham Life Sciences (Oakville, Canada).

2.2. LA-N-1 cell cultures and preparation of nuclei

LA-N-1 cells were maintained in culture with Leibovitz's L-15 medium containing 15% fetal calf serum. The medium was renewed at days 2 and 5. At day 5, the cells were prelabeled with [^{3}H]palmitic acid or with [^{3}H]choline (specific activity 60 Ci/mmol each) for 48 h. At day 7, the medium was removed and [^{3}H]palmitic acid labeled cells were either untreated or incubated with dimethyl sulfoxide (DMSO 0.01%) or 10 μM RA in DMSO (0.01%) respectively for 30 min in the presence of 1% ethanol. Differentiation of LA-N-1 cells was per-

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Abbreviations: PLC, phospholipase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtOH, phosphatidylethanol; DAG, diacylglycerol; PtdOH, phosphatidic acid; RA, retinoic acid; atRA, all-*trans*-retinoic acid

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formed by adding to the culture medium 10 μM RA in DMSO (0.01%) at day 2 for 5 days. LA-N-1 nuclei were isolated according to the method of Antony et al. [9]. Isolated nuclei were suspended in 50 mM Tris–HCl, 4.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 0.25 M saccharose, pH 7.5. Whole cells or intact nuclei were incubated with atRA according to experimental requirements. Cell culture and incubations were conducted at 37°C.

2.3. Separation of nuclear PtdOH, PtdEtOH and DAG

Nuclear lipids were extracted by the method of Kiss and Crilly [11] and lipid carrier standards were added. PtdOH, PtdEtOH and DAG were separated by thin layer chromatography on silica gel G plates using a double development system and the radioactivity of products quantified as reported [9].

2.4. Determination of nuclear PLD activities

The determination of the activation of nuclear PLD was performed as previously described [9]. In vitro determination of either GTPγS/phosphatidylinositol 4,5-diphosphate (PIP_2) or oleate-dependent PLD specific activities was achieved on nuclei of control or of 5 days atRA-treated LA-N-1 cells. The incubations for the PLD activity measurements contained 50 mM dimethylglutarate pH 6.5, a substrate cosonicate of 0.75 mM 1-2-di-[1-^{14}C]oleyl PtdCho (specific activity 114 mCi/mmol), 10 mM ethylenediamine tetraacetic acid (EDTA), 25 mM NaF and 20 μg of nuclear protein either with 100 μM of GTPγS/PIP_2 or 1.2 mM oleate in the presence of 1% ethanol for 30 min [12]. PtdOH and PtdEtOH were separated from other lipids and their radioactivity quantified as reported above.

2.5. Quantification of PtdCho hydrolysis in atRA-treated nuclei

Nuclei of [^{3}H]choline prelabeled cells were directly incubated with DMSO or atRA in DMSO for 1 h. Water-soluble choline-containing compounds were separated by ion exchange chromatography on Dowex-50-WH$^+$ [13] and the radioactivity on aliquots of each fraction measured by scintillation counting.

2.6. Protein determination

The protein content was determined by the method of Lowry et al. [14] using bovine serum albumin as the standard.

2.7. Statistics

The data represent the mean of at least three experiments performed in triplicate and analyzed utilizing Student's test.

3. Results

3.1. Production of DAG, PtdOH and PtdEtOH in LA-N-1 nuclei

The treatment of the [^{3}H]palmitic acid labeled cells with atRA in the presence of ethanol leads to an increase in the production of DAG and PtdEtOH in the nuclei, but not PtdOH (Fig. 1A). A similar enhancement of both compounds is observed when isolated nuclei are incubated with atRA (Fig. 1B). However, the level of PtdEtOH produced in atRA-treated nuclei is much lower than in stimulated cells suggesting that an essential cofactor may be lost during the preparation of nuclei. These results indicate that the binding of atRA with nuclear RARs activates a nuclear PLD. DAG production may be the consequence of stimulation of a PLC and/or the sequential activation of a PLD and a phosphatidate phosphohydrolase.

3.2. PtdCho hydrolysis in atRA-treated nuclei

Prior studies have shown that PLD acts preferentially on PtdCho [15]. We therefore investigated whether this phospholipid is a substrate for the nuclear PLD. The stimulation with atRA of [^{3}H]choline prelabeled nuclei induces a 30% increase in the production of water-soluble choline compounds, phosphocholine and free choline (Fig. 2), suggesting that PtdCho is a target for the nuclear PLC and PLD.

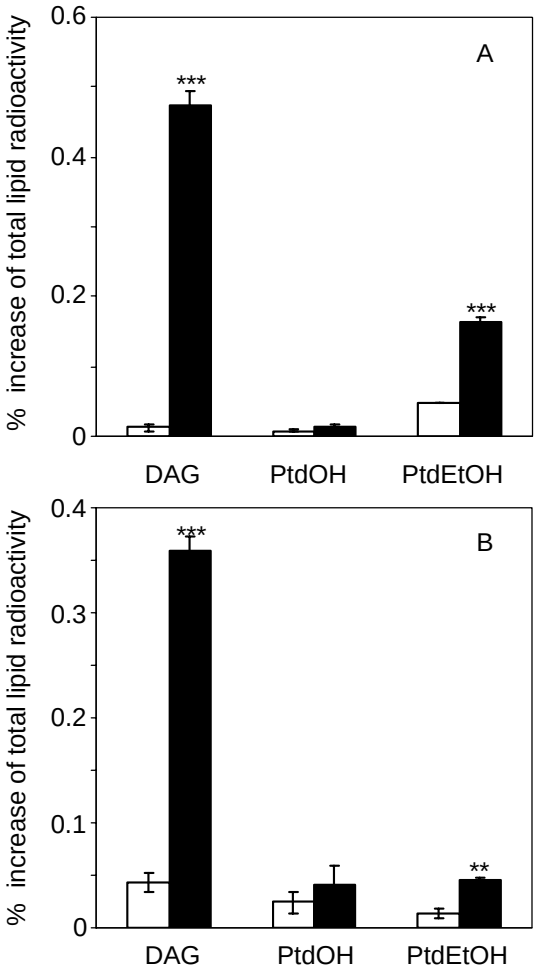

Fig. 1. Effect of atRA on the production of DAG, PtdOH and PtdEtOH in LA-N-1 nuclei. A represents the formation of nuclear lipid compounds resulting from the incubations of whole cells whereas B depicts that of isolated nuclei. The experimental details are described in Section 2. Results are the means ± S.E.M. of three experiments and represent the percentage increase of radioactivity of each lipid relative to total nuclear lipids. The amount of the total radioactivity in the nuclear fraction is 140 000 cpm (A) and 100 000 cpm (B). The open bars represent the control incubations and the filled ones that of the atRA-treated cells or nuclei. ***$P < 0.001$; **$P < 0.01$.

3.3. Regulation of the nuclear PLD

Two essential modes of regulation of PLD (protein G- and oleate-dependent enzymes) have been observed in nuclei [15]. We searched for the in vitro effect of GTPγS/PIP_2 and oleate on the PLD activity in the nuclei of control and atRA-treated cells in the presence of ethanol and inhibitor of PtdOH phosphatase activity to carry out the experiment at optimal conditions. We monitored the production of the sum of PtdOH and PtdEtOH as index of PLD activity. Results show that the specific activity of the oleate-dependent PLD in nuclei of control cells is about 20-fold higher than that of GTPγS/PIP_2. The treatment of the cells with atRA for 5 days provokes a two-fold increase in the specific activity of the nuclear oleate-dependent PLD whereas no effect is observed on that of the GTPγS/PIP_2-dependent one (Fig. 3). In the present cellular

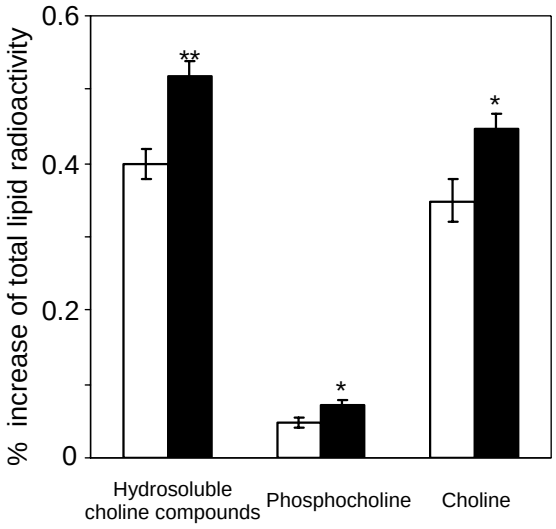

Fig. 2. Effect of atRA on the hydrolysis of PtdCho in LA-N-1 nuclei. The experimental details are described in Section 2. Results are the means ± S.E.M. of three experiments and represent the percentage increase of radioactivity of each water-soluble choline compound relative to total nuclear lipids. The amount of the total radioactivity in the nuclear fraction is 100 000 cpm. The open bars represent the control incubations and the filled ones the atRA incubations. $^{**}P < 0.01$; $^{*}P < 0.05$.

model, the oleate-dependent PLD preferentially catalyzes the formation of PtdEtOH as compared to PtdOH in nuclei (ratio 3/1, data not shown). Thus LA-N-1 cellular differentiation with atRA induces specifically the activation of an oleate-dependent PLD in the nuclei.

4. Discussion

The regulation of the PLD isoform activities is controlled by multiple compounds such as protein kinases C, G proteins and various lipids [15,16]. The signaling pathway involved in the activation of G protein-dependent PLDs, particularly the PLD1 isoform, has been extensively studied. However, little is known about the molecular mechanisms that control the oleate-dependent PLD [17]. Since neuroblastoma cell lines represent a well-established model for deciphering the molecular mechanisms implicated in neural cell differentiation [18], we investigated the regulation of PLD activity in the nuclei of LA-N-1 cells induced to differentiate with atRA.

Our results of transphosphatidylation assays indicate the presence of a PLD activated by atRA in nuclei (Fig. 1), because PtdEtOH is not dephosphorylated into DAG by a phosphatidate phosphohydrolase, and remains a stable molecule. The molecular mechanisms leading to DAG formation may result in the stimulation of a PLC and/or a dephosphorylation of PtdOH by a PtdOH phosphohydrolase activity. This latter may explain the absence of PtdOH formation. The activity of PLD may be regulated by either small G proteins like RhoA [19–21] or by oleate as reported in rat brain neuronal nuclei [22]. We provided compelling evidence that the differentiation of LA-N-1 cells induced by atRA specifically stimulates a nuclear oleate-dependent PLD (Fig. 3). Incubations of intact nuclei with atRA, ATP and GTPγS in the presence of ethanol do not provoke an increase of PtdEtOH as compared to atRA alone (data not shown) [9]. Therefore we believe that this PLD isoform is different from the one stimulated by phorbol esters [9] and the one present in HL60 cells because it is insensitive to GTPγS/PIP_2 [23]. Furthermore, two forms of PLD have been described in rat hepatocyte nuclei [24]. One is activated by the nuclear adenosine diphosphate (ADP) ribosylation (ARF), GTP and PIP_2, and is associated with cell proliferation, whereas the activity of the oleate-dependent PLD remained constant during the cell cycle progression in liver regeneration. Together these observations suggest the presence in LA-N-1 nuclei of two PLD isoforms, a G protein-dependent one that is activated by TPA during cellular proliferation and an oleate-dependent PLD stimulated by atRA during cellular differentiation. It should be emphasized that no increase of PtdOH is detected when [^{14}C]PtdCho labeled LA-N-1 cells are incubated in the presence of oleate, but without ethanol [25]. Therefore, this experimental procedure employed by Singh et al. does not permit any conclusion about an in vitro oleate-dependent PLD activity involvement in whole cell, because the level of PtdEtOH is not measured. Whether our finding is a specific phenomenon to the LA-N-1 nucleus, remains to be clarified.

We showed that the treatment of LA-N-1 nuclei with atRA leads to an enhancement of choline and phosphocholine production (Fig. 2). Similar results are obtained with TPA as agonist [26] indicating that PtdCho is a substrate for both oleate- and G protein-dependent PLDs. However, the specific activity of the nuclear oleate-regulated PLD increases during atRA cellular treatment but not that of the GTPγS/PIP_2-dependent one (Fig. 3). We showed that the oleate-dependent PLD catalyzes preferentially the formation of PtdEtOH as compared to PtdOH in nuclei of atRA-differentiated LA-N-1 cells. However, the formation of PtdOH may be explained in part by a stimulation of a PLC activity followed by that of a DAG kinase [27].

This is the first report indicating that an oleate-dependent

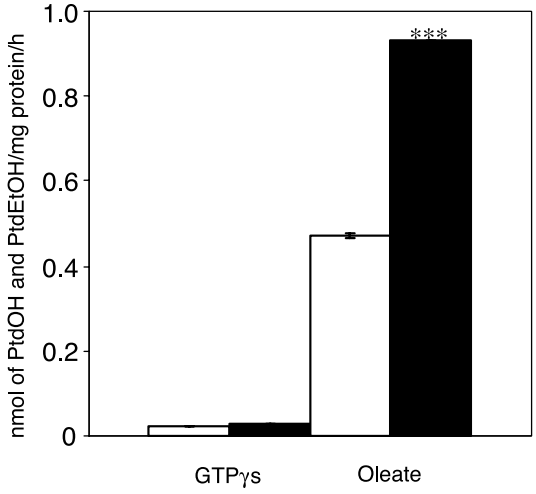

Fig. 3. Effect of GTPγS/PIP_2 and oleate on the specific activity of PLD in LA-N-1 nuclei of control or atRA-treated cells. The experimental details are described in Section 2. Results are the means ± S.E.M. of three experiments and represent the specific activity of nuclear PLD expressed in nmol of PtdOH+PtdEtOH/mg protein/h. The amount of the total radioactivity in the nuclear fraction is 150 000 cpm. The open bars represent the control incubations and the filled ones the atRA incubations. The amount of PtdOH produced in nuclei of control and atRA-treated cells represents about 30% of the total production of PtdOH+PtdEtOH. $^{***}P < 0.001$.

PLD is specifically activated in nuclei of human-differentiated neuroblastoma cells. We hypothesize that distinct nuclear PLDs may produce different molecular species of PtdOH and consequently DAG by a phosphatidate phosphohydrolase activity during cellular proliferation or differentiation [8]. This is in accordance with the recent observation of two distinct sources of DAG in nuclei of proliferative and DMSO-differentiated HL60 cells [28]. The production of different molecular species of PtdOH or DAG may activate different isoforms of protein kinase C [29] leading to cellular proliferation or differentiation. Further investigations are required to confirm this hypothesis.

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