Effect of D609 on phosphatidylcholine metabolism in the nuclei of LA-N-1 neuroblastoma cells: a key role for diacylglycerol

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Abstract In our previous studies, TPA treatment of LA-N-1 cells stimulated the production of diacylglycerol in nuclei, probably through the activation of a phospholipase C. Stimulation of the synthesis of nuclear phosphatidylcholine by the activation of CTP:phosphocholine cytidylyltransferase was also observed. The present data show that both effects were inhibited by the pretreatment of the cells with D609, a selective phosphatidylcholine-phospholipase C inhibitor, indicating that the diacylglycerol produced through the hydrolysis of phosphatidylcholine in the nuclei is reutilized for the synthesis of nuclear phosphatidylcholine and is required for the activation of CTP:phosphocholine cytidylyltransferase. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Phospholipase C; Diacylglycerol; D609 (tricyclodecan-9-yl xanthogenate); LA-N-1 nuclei; CTP:phosphocholine cytidylyltransferase; Phosphatidylcholine cycle

1. Introduction

Phosphatidylcholine (PtdCho) represents the major phospholipid in eukaryotic membranes where it is a fundamental structure element as well as a reservoir for lipid mediators generated upon appropriate cellular stimuli [1]. Its hydrolysis by a specific phospholipase C (PtdCho-PLC) [2,3] or by the sequential activation of a phospholipase D and phosphatidate phosphohydrolase (PLD-PPH) [4] gives rise to diacylglycerols (DAG). DAG may stimulate the translocation and the activation of protein kinase C within the nucleus [5] leading to the induction of long term cellular responses, such as mitosis. The enzymic mechanisms involved in nuclear DAG production and their possible involvement in the regulation of cellular proliferation are the topic of only a few reports [6–8]. Our previous data showed that the treatment of LA-N-1 cells or nuclei with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter, led to the production of nuclear DAG through the activation of a PLC and/or PLD/PPH hydrolyzing preferentially PtdCho. Moreover, an increase of the synthesis of PtdCho was observed suggesting the presence of a PtdCho cycle in LA-N-1 nuclei [9]. Among the three enzymes involved in the de novo synthesis of PtdCho [10,11], only the CTP:phosphocholine cytidylyltransferase (CCT) is activated by TPA [12]. In order to gain insights into the mechanisms involved in the regulation of the PtdCho cycle in the nuclei of LA-N-1 cells when stimulated by TPA, we investigated the effect of D609 (tricyclodecan-9-yl xanthogenate), a selective PtdCho-PLC inhibitor [13], on the production of DAG and the synthesis of nuclear PtdCho. The pretreatment of LA-N-1 cells with D609 inhibited the production of DAG, but not phosphatidylethanol (PtdEtOH), in the nuclei of TPA treated LA-N-1 cells in the presence of ethanol. Moreover, D609 also inhibited the TPA stimulation of nuclear PtdCho synthesis and the activation of the specific CCT. These observations indicate that the DAG produced by the activation of the specific PtdCho-PLC plays a key role in the regulation of the LA-N-1 nuclear PtdCho cycle.

2. Materials and methods

2.1. Materials

Leibovitz's L-15 medium and fetal calf serum were supplied by Life Technologies (Eragny, France). Streptomycin, penicillin, trypsin inhibitor, phenylmethylsulfonylfluoride, TPA, phosphatidic acid and Dowex 50×8 WH^+ were obtained from Sigma Chemical (St. Louis, MO, USA); PtdEtOH and D609 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); [^{14}C]choline, phospho[^{14}C]choline and [^{3}H]palmitic acid from NEN Life Science Products (Paris, France).

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

Human neuroblastoma LA-N-1 cells obtained from Dr. Seeger, University of California at Los Angeles, 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 (specific activity 60 Ci/mmol) for 48 h. At day 7, the medium was removed and the cells pretreated with D609 (50 μg/ml) or water (vehicle) for 15 min and then incubated with 160 nM TPA in DMSO (0.01%) or with DMSO (control) for 30 min in the presence of 1% ethanol. LA-N-1 nuclei were isolated according to the method of Antony et al. [9].

2.3. Quantification of nuclear DAG

Nuclear lipids were extracted by the method of Kiss and Crilly [14] and lipid carrier standards were added. DAG and PtdEtOH were separated by thin layer chromatography on silica gel G plates using a double development system and quantified as previously reported [9].

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Abbreviations: PLC, phospholipase C; PLD, phospholipase D; PtdCho, phosphatidylcholine; PtdEtOH, phosphatidylethanol; PtdIns, phosphatidylinositol; CCT, CTP:phosphocholine cytidylyltransferase; DAG, diacylglycerol; TPA, 12-*O*-tetradecanoylphorbol-13-acetate

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2.4. Incorporation of choline into PtdCho

Incorporation of [^{14}C]choline into PtdCho of nuclei from treated or untreated cells was carried out according to Soto et al. [15] in a medium containing 20 mM K_2HPO_4-KH_2PO_4 buffer pH 7.4, 20 mM Na pyruvate, 1.3 mM Na malate, 5.0 mM $MgCl_2$, 1.7 mM ATP, 0.25 mM CTP, 0.1 mM CoA, 1.7 mM CMP, 2.0 mM creatine phosphate, 125 μg/ml of creatine phosphokinase, 17.0 μM [^{14}C]choline (specific activity 55 μCi/μmol) and 0.5–1 mg protein in a final volume of 1.0 ml. Samples were incubated at 37°C with continuous shaking for 2 h. The reaction was stopped by the addition of 3.75 ml chloroform/methanol 1:2 (v/v) and the lipids extracted according to the procedure of Bligh and Dyer [16]. The radioactivity of PtdCho was determined by scintillation counting.

2.5. In vitro determination of nuclear CCT

The determination of nuclear CCT activity in LA-N-1 nuclei was performed on vehicle and D609 treated cells as described by Tronchère et al. [17] in a final volume of 100 μl. The incubation mixture contained 20 mM Tris–succinate pH 7.8, 6 mM $MgCl_2$, 8 mM CTP, 4 mM phospho[^{14}C]choline (specific activity 0.5 μCi/μmol) and up to 0.3 mg of nuclear protein. The reaction was stopped by addition of 50 μl perchloric acid 1.5 M. After centrifugation at $3000\times g$, the supernatant was neutralized with 150 μl NaOH 1 M and the CDP-[^{14}C]choline separated by column chromatography on Dowex-50-WH$^+$ and the radioactivity measured by scintillation counting.

2.6. Protein determination

The protein content was determined by the method of Lowry et al. [18] using bovine serum albumin as the standard.

2.7. Statistics

The data represent the mean of three experiments performed in triplicate and analyzed utilizing Student's *t*-test.

3. Results

3.1. Production of DAG in nuclei

The treatment of the cells with TPA in the presence of ethanol led to a strong enhancement of DAG in nuclei after 5 min. The level of DAG remained constant between 5 and 15 min and increased thereafter up to 30 min (Fig. 1). As expected, the production of nuclear PtdEtOH was higher in TPA treated cells as compared to control, but did not change whatever the time of incubation (not shown).

3.2. Effect of D609 on the production of DAG in nuclei

The signaling pathway involved in the production of nuclear DAG was investigated with D609. Preincubation of the cells with D609 had no effect on the basal level of nuclear DAG of control cells. Nevertheless, this pretreatment led to inhibition by about 75% of the DAG production in nuclei of TPA stimulated cells in the presence of ethanol (Fig. 2).

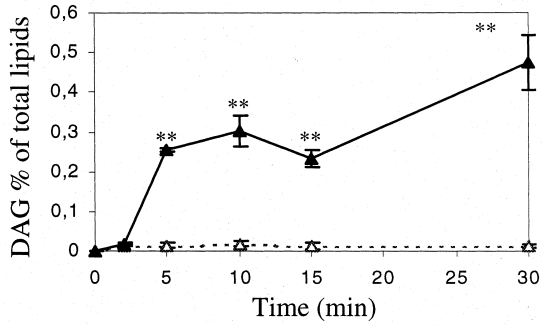

Fig. 1. Effect of TPA stimulation of LA-N-1 cells on the production of DAG in nuclei. The experimental details are described in Section 2. Dashed lines represent the control cells and the solid lines the TPA treated cells. $^{**}P<0.01$.

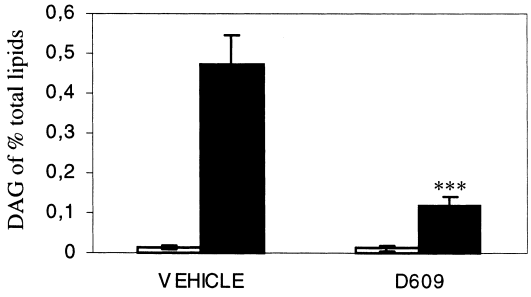

Fig. 2. Effect of D609 on the production of DAG in nuclei of TPA stimulated LA-N-1 cells. The experimental details are described in Section 2. The open bars represent the control incubations and the filled bars the TPA treated cells. $^{***}P<0.001$ against vehicle.

3.3. Effect of D609 on the PtdCho synthesis in nuclei

Treatment of the cells with TPA stimulated the incorporation of choline into nuclear PtdCho (Fig. 3A), suggesting that the DAG produced may be reutilized as substrate. Preincubation of the cells with D609 switched off the stimulation of the incorporation of choline into the nuclear PtdCho.

Since the de novo synthesis of nuclear PtdCho seems to be regulated by the activation of the CCT and not that of choline kinase and CDP-choline DAG phosphocholine transferase [9], it was important to determine if the nuclear DAG produced in TPA stimulated cells were necessary for the activation of CCT in nuclei. Pretreatment of the cells with D609 completely abolished the activation of nuclear CCT induced by the treatment of cells with TPA (Fig. 3B).

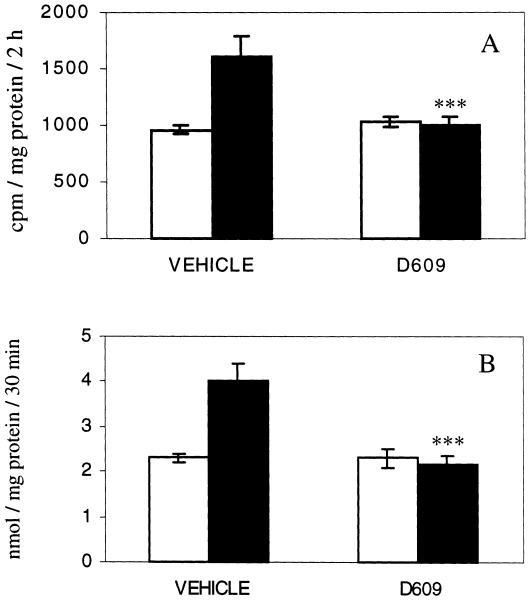

Fig. 3. Effect of D609 on the synthesis of PtdCho in nuclei of TPA treated LA-N-1 cells. A represents the incorporation of choline into PtdCho and B the specific activity of nuclear CCT. The experimental details are described in Section 2. The open bars represent the control incubations and the filled bars the TPA incubations. $^{***}P<0.001$ against vehicle.

4. Discussion

This study was focussed on the production of nuclear DAG in order to determine which phospholipases and substrates are involved in long term cellular responses such as proliferation. Classically, the hydrolysis of phosphoinositides by a phosphatidylinositol (PtdIns)-PLC is considered to be the major mechanism for rapid DAG generation, whereas PtdCho breakdown contributes mainly to DAG production during a longer time after stimulation [19,20]. Thus, PtdCho may be the main substrate utilized for the production of DAG in the nuclei of LA-N-1 cells stimulated with TPA for 5 and 30 min. Previous results have shown that TPA treated LA-N-1 nuclei led to the increase of water soluble choline compounds, especially phosphocholine. Moreover the same stimulation of nuclei prelabeled with palmitate also induced the production of labeled DAG, suggesting the activation of a PLC catalyzing the hydrolysis of PtdCho [9]. Although the same stimulation produced an activation of a nuclear PLD, its involvement in the production of DAG has been excluded because the experiments were conducted in the presence of ethanol; PLD catalyzes the formation of [^{3}H]PtdEtOH, which is not dephosphorylated into DAG [21]. To confirm this hypothesis, the effect of D609, a selective PtdCho-PLC inhibitor, on the production of DAG in nuclei of TPA treated cells has been investigated. The dramatic decrease in the DAG generation in the nuclei of D609 pretreated cells stimulated by TPA indicates the activation of a PtdCho-PLC by TPA [22]. The observation that D609 treatment of the cells did not inhibit completely the nuclear DAG production suggests that the hydrolysis of phospholipids like PtdEtn may contribute a small amount of DAG [23].

Earlier experiments also have shown that TPA induced a stimulation of the PtdCho synthesis in LA-N-1 nuclei as has been reported in SK-N-SH human neuroblastoma cells [24] and that DAG produced by the treatment of LA-N-2 cells by exogenous PLC activated the CCT [25]. The observation suggests that the nuclear DAG generated upon the degradation of PtdCho may be of prime importance in the activation of PtdCho synthesis.

The data showing that the pretreatment of LA-N-1 cells by D609 completely inhibited the TPA stimulation of the choline incorporation into nuclear PtdCho confirm this hypothesis and indicate the existence of a correlation between the production of DAG through the activation of PtdCho-PLC and the stimulation of PtdCho synthesis. The DAG produced upon PtdCho-PLC activation may be utilized as substrate by the CDP:choline DAG phosphocholine transferase catalyzing the last step of PtdCho de novo synthesis and/or as activator of the CCT induced by cellular TPA. The fact that the D609 pretreatment of LA-N-1 cells completely inhibited the activation of CCT induced by the DAG generated by cellular TPA stimulation indicates that the newly produced DAG molecules are required for the activation of this enzyme. Three isoforms of CCT have been described. CCTα is an ubiquitous enzyme that contains at its N-terminal region a signal peptide of 21 amino acids sufficient for nuclear localization [26], whereas CCTβ_1 and CCTβ_2 lack this signal [27]. The nuclear localization of CCTα and its close relationship with the activation of synthesis of PtdCho in mammalian cells is widely debated [28,29]. Our results suggest that a nuclear CCTα is activated by the DAG generated upon TPA treatment of LA-N-1 cells, which enhances in turn the de novo synthesis of PtdCho in nuclei. Chromatin from human IRM-32 neuroblastoma cells possesses the three enzymes of the Kennedy pathway necessary for endonuclear synthesis of PtdCho, which may then be involved in cell proliferation [30].

Whereas several studies have elicited the enzymic mechanisms of a nuclear PtdIns cycle [31,32], the existence and the regulation of nuclear PtdCho cycle have not been well defined. Our results show for the first time that TPA treatment of LA-N-1 cells activates in the nuclei a PtdCho-PLC, leading to the production of DAG, which in turn induces the stimulation of PtdCho synthesis by the specific activation of CCT. Such a mechanism may equilibrate the nuclear pool of PtdCho for cellular homeostasis. Altogether these results indicate the existence of a nuclear PtdCho cycle in LA-N-1 neuroblastoma cells that may be involved in nuclear functions such as DNA synthesis and cell proliferation.

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