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Mechanisms whereby nerve growth factor increases diacylglycerol levels in differentiating PC12 cells

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Abstract

We previously showed indirectly that the increase in diacylglycerol (DAG) levels caused by exposing differentiating PC12 cells to nerve growth factor (NGF) must derive mainly from de novo synthesis and, to a lesser and transient extent, from the hydrolysis of [^{3}H]phosphatidylinositol (PI). To explore further the biochemical mechanisms of this increase, we measured, in PC12 cells, DAG synthesis from glycerol or various fatty acids; its liberation from phosphatidylcholine (PC); and the activities of various enzymes involved in DAG production and metabolism. Among cells exposed to NGF (0–116 h), the labeling of DAG from [^{3}H]glycerol peaked earlier than that of [^{3}H]PC, and the specific radioactivity of [^{3}H]glycerol-labeled DAG was much higher than those of the [^{3}H]phospholipids, indicating that [^{3}H]DAG synthesis precedes [^{3}H]phospholipid synthesis. NGF treatment also increased (by 50–330%) the incorporation of monounsaturated ([^{3}H]oleic acid) and polyunsaturated ([^{14}C]linoleic acid or [^{3}H]arachidonic acid) fatty acids into DAG, and, by 15–70%, into PC. NGF treatment increased the activities of long chain acyl-CoA synthetases (LCASs), including oleoyl-CoA synthetase and arachidonoyl-CoA synthetase, by 150–580% over control, but cholinephosphotransferase activity rose by only 60%, suggesting that the synthesis of DAG in the cells was increased to a greater extent than its utilization. NGF did not promote the breakdown of newly formed [^{3}H]PC to [^{3}H]DAG, nor did it consistently affect the activities of phospholipase C or D. NGF did increase phospholipase A_2 activity, however the hydrolysis catalyzed by this enzyme does not liberate DAG. Hence the major source of the increased DAG levels in PC12 cells exposed to NGF appears to be enhanced de novo DAG synthesis, probably initiated by the activation of LCASs, rather than the breakdown of PC or PI.

Keywords: Diacylglycerol; Phosphatidylcholine; Long chain acyl-CoA synthetase; Phospholipase; Nerve growth factor; PC12 cell

1. Introduction

The induction of neurite outgrowth when PC12 cells are treated with NGF is associated with enhanced biosynthesis of membrane phosphatidylcholine (PC). This is largely mediated by the activation of cholinephosphotransferase (CPT) and an increase in its substrate-saturation with diacylglycerol (DAG) [1]. DAG is a common intermediate and a possible regulatory locus in the biosyntheses of both triacylglycerol (TAG) and phospholipids. The choline- and ethanolamine-phosphotransferases have higher affinities for DAG than does diacylglycerol acyltransferase, suggesting that, in situations of low fatty acid availability, the major flux from DAG is directed to phospholipid synthesis and maintenance of membrane phospholipid turnover. DAG released from the hydrolyses of phosphatidylinositol (PI) or PC, as catalyzed by phospholipase C (PLC), phospholipase D (PLD), or phosphatidate phosphohydrolase [8,9,12,13,16–18,20], also serves as a second messenger in intracellular signal transduction. It is assumed that the biological roles of DAG are related to its origins [2], e.g., DAG from PI temporarily activates protein kinase C (PKC), inducing glandular secretion and metabolic changes, while DAG formed from PC permanently activates PKC, leading to cellular proliferation and differentiation. Little information is available concerning the role of DAG produced via other metabolic pathways, e.g., the de novo syntheses of glycerolipids [3].

In previous studies designed to identify the major source of the increased DAG levels in PC12 cells exposed to NGF, we examined the short-term effects of NGF treatment on the conversion of monounsaturated fatty acid (oleic acid, OA) to glycerolipids; the blockage of this conversion by triacsin C (an inhibitor of de novo DAG

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Table 1
Effects of NGF on incorporation of radioactive fatty acids into DAG and PC in differentiating PC12 cells

Lipids	Control cells	NGF-treated cells
[^{3}H]OA	1.10 ± 0.07	0.49 ± 0.06[b]
[^{3}H]OA-labeled DAG	2.83 ± 0.21	9.94 ± 0.77[b]
[^{3}H]OA-labeled PC	74.4 ± 3.32	101 ± 5.10[b]
[^{14}C]LA	1.13 ± 0.12	0.28 ± 0.01[b]
[^{14}C]LA-labeled DAG	4.80 ± 0.43	7.04 ± 0.94[b]
[^{14}C]LA-labeled PC	95.4 ± 7.58	110 ± 8.75[a]
[^{3}H]AA	1.62 ± 0.11	0.70 ± 0.08[b]
[^{3}H]AA-labeled DAG	1.35 ± 0.17	2.10 ± 0.11[b]
[^{3}H]AA-labeled PC	19.9 ± 1.67	29.1 ± 2.03[b]

PC12 cells pre-treated with NGF for 24 h were exposed to NGF and [^{3}H]OA, [^{14}C]LA or [^{3}H]AA (0.5 μCi/ml, 10 μM) for an additional 24 h. The cells were harvested and assayed for the levels of radioactive DAG and PC as described in Ref. [11]. Values represent means ± S.D. of 4 determinations (pmol/μg DNA). Compared with control values, a: $P < 0.05$, b: $P < 0.01$.

synthesis); the turnover of [^{3}H]OA-labeled products; and the incorporation of [^{3}H]glycerol into the cells briefly exposed to NGF. Those studies suggested that the increase in DAG levels derives mainly from its de novo synthesis and, to a lesser and transient extent, from the hydrolysis of PI [11]. The present studies were designed to extend these findings by examining the duration of NGF's effect on cellular levels of [^{3}H]glycerol-labeled DAG; the effects of NGF on the incorporation of polyunsaturated fatty acids into glycerolipids; the particular enzymatic steps in de novo DAG synthesis that are affected by NGF; and the possibility that PC hydrolysis could also be a source of this increased DAG.

2. Materials and methods

2.1. Cell culture and treatment

PC12 cells (ATCC, Rockville, MD, USA) were cultured according to the method described previously [11]. The experimental cells were treated with NGF (2.5S, 50 ng/ml; GIBCO BRL, Grand Island, NY, USA) and labeled with [^{3}H]glycerol (8 μCi/ml, 0.1 μM), [^{3}H]OA (0.5 μCi/ml, 10 μM), [^{3}H]arachidonic acid (AA; 0.5 μCi/ml, 10 μM) or [^{14}C]linoleic acid (LA; 0.5 μCi/ml, 10 μM) for various periods. All radioactive chemicals were obtained from New England Nuclear (Boston, MA, USA).

2.2. Assay of radioactive or non-radioactive lipids

Cell collection and lipid measurements were performed by our methods described previously [11].

2.3. Enzymatic assay of LCASs

PC12 cells grown in 100 mm dishes were collected and sonicated in a cold Tris–HCl buffer (20 μmol/ml, pH 7.2;

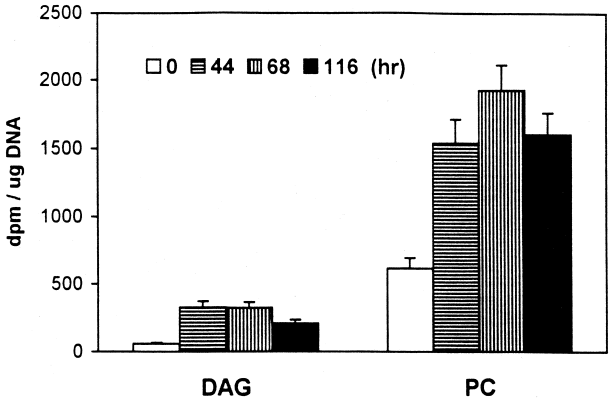

Fig. 1. Effects of NGF treatment on levels of [^{3}H]glycerol-labeled DAG and PC. After incubation in medium B for 24 h, PC12 cells were pre-treated with 50 ng NGF/ml medium B for 24, 48 or 96 h, and then simultaneously treated with NGF and labeled with 8 μCi [^{3}H]glycerol/ml medium B for an additional 20 h to give final times of 44, 68 and 116 h; control cells were never treated with NGF. The cells were extracted with methanol/chloroform/water (1:2:1), and [^{3}H]glycerol-labeled lipids were purified and measured by TLC and liquid scintillation spectrometry as described in [11]. Values represent means ± S.D. of [^{3}H]glycerol-labeled lipid levels (dpm/μg DNA; $n = 6$). Data (44 h) are from our previous study [11].

containing 1 μmol/ml of EGTA and 0.1 μmol/ml of phenylmethylsulfonyl fluoride), and measured for protein and DNA contents. The cellular homogenates were stored at −80°C.

Assay of LCASs, i.e., oleoyl-CoA synthetase (OCS) and arachidonoyl-CoA synthetase (ACS) relies on heptane extraction of unreacted free fatty acid and the insolubility of long chain acyl-CoA esters in heptane [22]. The reaction mixtures contained 15 μmol of Tris–HCl (pH 8.0), 3 μmol of $MgCl_2$, 1 μmol of ATP, 100 nmol of CoA, 150 nmol of 2-mercaptoethanol, 0.1% of Triton X-100, 20 nmol of [^{3}H]OA or [^{3}H]AA (5 nCi/nmol) in 50 mM $NaHCO_3$, and an aliquot of cellular homogenates (enzyme), in a total volume of 0.15 ml. The reaction was initiated by the addition of enzyme and, after incubation at 37°C for 0, 5, 10 and 30 min, terminated by the addition of 2.25 ml of

Table 2
Increases in specific radioactivities of [^{3}H]glycerol-labeled DAG and phospholipids in NGF-treated PC12 cells

Lipids	Control	NGF
DAG	8210 ± 1230	15205 ± 1430[b]
PC	412 ± 52	728 ± 82[b]
PE	140 ± 16	324 ± 39[b]
PS	701 ± 83	1542 ± 163[b]
PI	1466 ± 159	2441 ± 278[b]

PC12 cells pre-treated with NGF for 24 h were then treated with NGF and labeled with [^{3}H]glycerol for an additional 20 h. DAG and individual phospholipids were purified with TLC. Specific radioactivities were calculated based on levels of these compounds as well as of their [^{3}H]glycerol-labeled analogs. Values represent means ± S.D. of DAG and phospholipids (dpm/nmol individual lipids; $n = 6$). Compared with control values, b: $P < 0.01$.

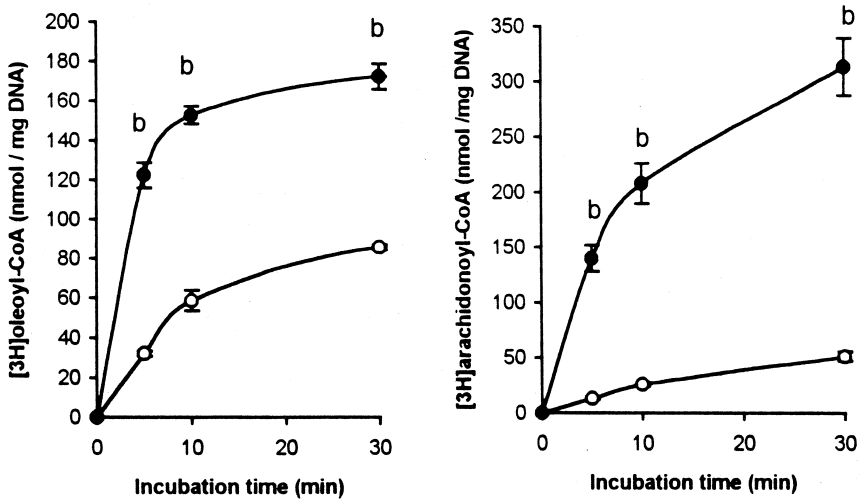

Fig. 2. Effects of NGF treatment on the activities of OCS (left) and ACS (right). PC12 cells treated with 50 ng NGF/ml medium B for 0 or 96 h were collected, sonicated and then incubated at 37°C for 0–30 min in a 150 μl of reaction mixture containing Tris–HCl (pH 8.0), $MgCl_2$, ATP, CoA, 2-mercaptoethanol, Triton X-100, 20 nmol of [^{3}H]OA or [^{3}H]AA and 125 μg cellular protein. [^{3}H]Oleoyl-CoA and [^{3}H] arachidonoyl-CoA were extracted, measured and identified as described in Section 2. Contents of [^{3}H]oleoyl-CoA and [^{3}H]arachidonoyl-CoA were calculated based on dpm and specific radioactivity. Values represent means ± S.D. of [^{3}H]oleoyl-CoA (left) or [^{3}H]arachidonoyl-CoA (right) contents (nmol/mg DNA; $n = 3$). Comparisons between groups: b: $P < 0.01$. Closed circles represent NGF-treated groups; open circles represent control groups.

isopropanol/heptane/2 M sulfuric acid (40:10:1, by volume). 1.5 ml of heptane and 1 ml of water were added, and the mixture was then vortexed vigorously. The upper phase was discarded and the aqueous phase was extracted twice with 2 ml of heptane containing 4 mg/ml of palmitic acid. A 1 ml sample of the aqueous phase was counted in 5 ml of ultrafluor scintillation fluid for [^{3}H]oleoyl-CoA or [^{3}H]arachidonoyl-CoA content. Blanks included in each experiment were treated identically but the additions of enzyme and incubation at 37°C were omitted. The products of OCS or ACS were identified by TLC [14] with isopropanol/pyridine/acetic acid/water (60:15:1:25, by volume; $R_f = 0.30$).

2.4. Enzymatic assay of phospholipases

For phospholipase assays, incubation mixtures consisted of 180 nmol of Tris–HCl (pH 7.2), 90 nmol of EGTA, 9 nmol of phenylmethylsulfonyl fluoride, 0.1 mg of octyl glucopyranoside, 0.5% of ethanol, 0.25 μCi of L-α-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]phosphatidylcholine (2.8 pmol of [^{3}H]PC; New England Nuclear, Boston, MA, USA) supplemented with 1–300 nmol of nonradioactive PC (from bovine brain; Sigma, St. Louis, MO, USA), and an aliquot of cellular homogenates, in a final volume of 0.1 ml. The reaction was started by adding the substrate, i.e., the PC suspension. After incubation for 10, 30, 60 or 120 min at 37°C, the reaction was stopped by adding 4 ml of methanol/chloroform/0.5 N HCl (1:2:1, by volume). Samples were mixed thoroughly, and then centrifuged to separate the phases. The organic phase was dried in a speed vacuum concentrator and reconstituted in 50 μl of chloroform. The three kinds of products of phospholipases, i.e., the [^{3}H]palmitic acid (PaA, $R_f = 0.65$) formed by phospholipase A_2 (PLA_2), the [^{3}H]DAG formed by PLC and the [^{3}H]phosphatidylethanol (PEt, $R_f = 0.58$) formed by PLD, were purified and measured by TLC and liquid scintillation spectrometry as described above and previously [15,21]. Blanks included in each experiment were treated identically, however incubation at 37°C was omitted.

2.5. Data presentation

Assays were done in duplicate or in triplicate. Each set of experiments was performed at least three times, and representative experiments are shown. Data are expressed as averages or means ± S.D./μg or mg DNA, in order to

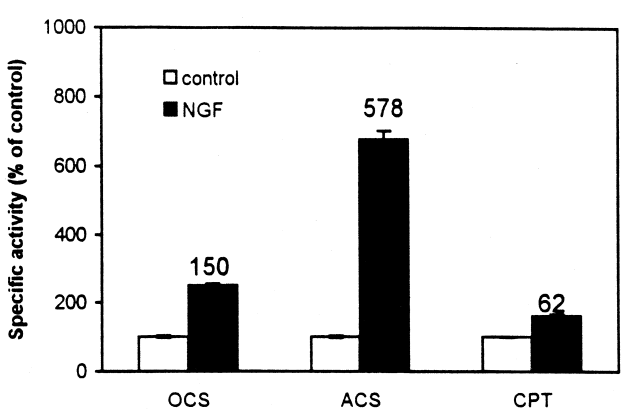

Fig. 3. Comparison between effects of NGF on activities of OCS, ACS and CPT. PC12 cells were cultured with or without 50 ng/ml of NGF for 96 h. OCS and ACS were assayed in total homogenates, and CPT in membrane fractions. Values are expressed as percentages of control, and represent means ± S.D. of 6 determinations. Numbers above bars are percent increase over control. CPT data are from a previous study [1].

consider the amounts of each assayed substance per cell. Student's *t*-test, two-way analysis of variance, or multiple comparison tests were used for comparisons between groups. Kinetics and regression analyses of the data were carried out according to linear and nonlinear models.

3. Results

3.1. De novo synthesis of DAG from fatty acid and glycerol is accelerated by NGF treatment

Studies involving cells labeled with radioactive glycerol or fatty acids were designed to explore further the effects of NGF treatment on de novo syntheses of glycerolipids. We previously reported that NGF treatment significantly increased the levels of DAG and PC labeled with [^{3}H]OA (7140 nCi/nmol; labeling for one hour) by 3.3- and 0.7-fold, respectively, over those of control cells [11]. To rule out possible artifacts resulting from labeling the cells for only a brief period with high specific-activity [^{3}H]OA, and to explore the effects of NGF treatment on the incorporation of polyunsaturated fatty acids (LA and AA) into glycerolipids, we labeled cells in the present study with lower specific-activity fatty acids (50 nCi/nmol) and for a longer period (24 h). NGF greatly promoted the incorporation of the lower-specific-activity [^{3}H]OA, [^{3}H]AA or

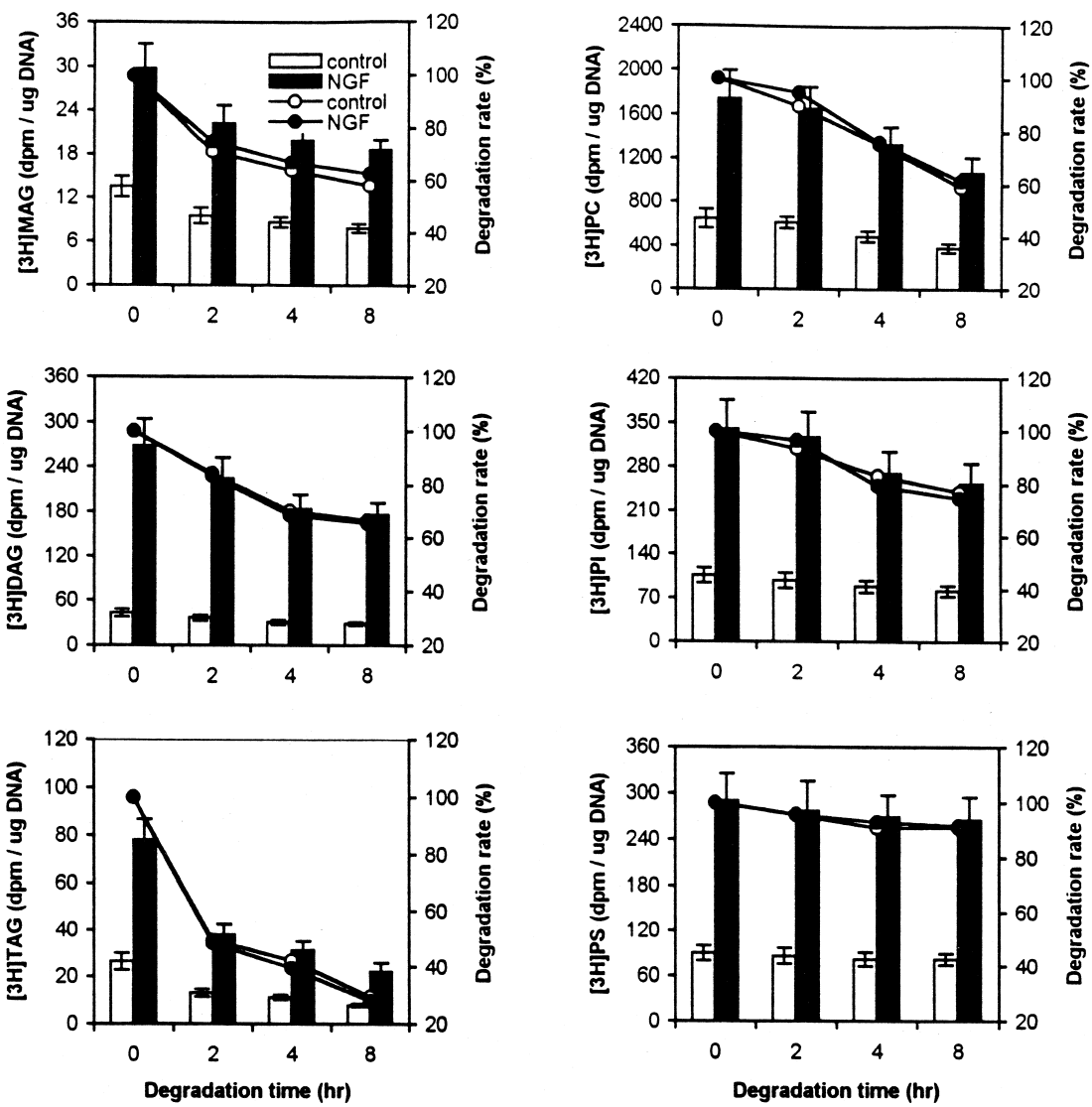

Fig. 4. Data from pulse-chase studies on degradation of [^{3}H]glycerol-labeled lipids. PC12 cells were pre-treated with 50 ng NGF/ml medium B for 24 h and then simultaneously treated with NGF and labeled with 8 μCi [^{3}H]glycerol/ml medium B for an additional 20 h. After cessation of [^{3}H]glycerol labeling, the cells were washed twice with non-radioactive medium B and subsequently maintained with NGF but without [^{3}H]glycerol for an additional 2–8 h. Control cells were treated identically but exposure to NGF was omitted. The cells were extracted, and [^{3}H]glycerol-labeled lipids and DNA were measured as described in [11]. Values are means ± S.D. of 6 determinations, and represent both the absolute changes of [^{3}H]lipid contents (Bar, dpm/μg DNA) and the degradation rates of newly formed [^{3}H]lipids (Curve, percent of baseline after cessation of [^{3}H]glycerol labeling) in control cells and NGF-treated cells.

[^{14}C]LA into DAG (50–330% over control) and into PC (15–70% over control) (Table 1).

Our previous data showed that levels of [^{3}H]glycerol-labeled DAG or PC increased time-dependently when cells were pre-treated with NGF for 24 h [11]. The present study examined the persistence of these effects with longer exposure to NGF (48 or 96 h) followed by exposure to both NGF and [^{3}H]glycerol for an additional 20 h (Control cells were never exposed to the NGF). [^{3}H]glycerol-labeled DAG levels peaked within 44 h of NGF treatment and [^{3}H]glycerol-labeled PC peaked after 68 h of NGF treatment (Fig. 1).

We measured the cellular levels of DAG and phospholipids, and then used this information to estimate their specific activities in cells exposed to [^{3}H]glycerol. NGF treatment increased the proportions of radioactivity in these pools (Table 2), suggesting that the increases in levels of [^{3}H]glycerol-labeled DAG and phospholipids (Fig. 1) truly reflected accelerations in their de novo syntheses. The fact that [^{3}H]DAG levels peaked earlier than those of [^{3}H]PC, and that specific radioactivities of [^{3}H]DAG were much higher than those of [^{3}H]phospholipids (Table 2), also indicates that [^{3}H]DAG synthesis was greater than those of [^{3}H]phospholipids.

3.2. Activation of LCASs by NGF is involved in accelerated de novo synthesis of DAG

We hypothesized that the accelerated de novo synthesis of DAG during NGF-stimulated neurite outgrowth was initiated by the first enzymatic step in the acylation of glycerol, i.e., the activation of fatty acid by LCASs. Hence we measured NGF-induced changes in the activities of two kinds of LCASs in the PC12 cells: OCS, which is a substrate-nonspecific LCAS, and ACS, which is specific for arachidonic acid. OCS catalyzes the reaction of OA or other fatty acids with CoA to form oleoyl-CoA or other acyl-CoAs; while ACS specifically forms arachidonoyl-CoA. The apparent K_m values of OCS (for OA) and ACS (for AA) in the PC12 cells were both about 50 μM. NGF treatment for 96 h did not change these K_m values, but did significantly enhance the specific activities of OCS and ACS (Fig. 2). The levels of [^{3}H]oleoyl-CoA and [^{3}H]arachidonoyl-CoA (nmol/mg DNA) produced by the differentiating PC12 cells were about 1.5 and 5.8 times higher than those produced by control cells (Fig. 3).

3.3. Hydrolysis of PC is not promoted by NGF treatment

Pulse-chase studies on the degradation of [^{3}H]glycerol-labeled lipids were conducted to look for NGF effects on the conversion of [^{3}H]phospholipids to [^{3}H]DAG. PC12 cells pre-treated with NGF for 24 h and then exposed to [^{3}H]glycerol for an additional 20 h were washed twice with fresh medium B to terminate the labeling. By the end of the 8 h chase period the proportions of [^{3}H]DAG and [^{3}H]PC that had disappeared from both control and NGF-treated cells were on the order of 35% and 38%, respectively (Fig. 4). The fact that NGF treatment had no obvious effects on these degradation rates suggests that NGF did not stimulate the conversion of [^{3}H]PC to [^{3}H]DAG under these conditions.

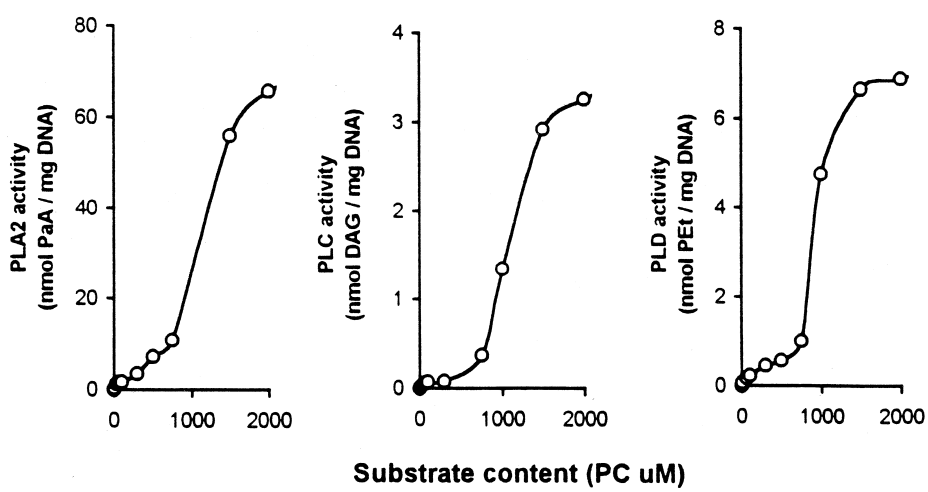

Fig. 5. Dependence of phospholipase activity on substrate concentration. PC12 cells scraped from 100 mm dishes were collected, sonicated and then incubated for 60 min in a 100 μl of reaction mixture containing Tris–HCl, EGTA, phenylmethylsulfonyl fluoride, octyl glucopyranoside, ethanol, 200 μg cellular protein, 0.25 μCi of [^{3}H]PC and 10–3000 nmol/ml of non-radioactive PC. Three products of [2-palmitoyl-9,10-^{3}H]PC hydrolyzed by PLA_2, PLD and PLC, i.e., [^{3}H]PaA, [^{3}H]PEt and [^{3}H]DAG, were separated, purified and then measured by TLC and liquid scintillation spectrometry as described in Section 2. Two types of K_m values were calculated based on double reciprocal models. K_{m1} values (substrate concentration below 500 μM) of PLA_2, PLD or PLC were in below 200 μM of exogenous PC, and K_{m2} values (substrate concentration over 500 μM) of them, from 900 to 1200 μM. Values represent the contents of [^{3}H]PaA, [^{3}H]DAG and [^{3}H]PEt (nmol/mg DNA) produced from exogenous PC, and are averages of 3 determinations.

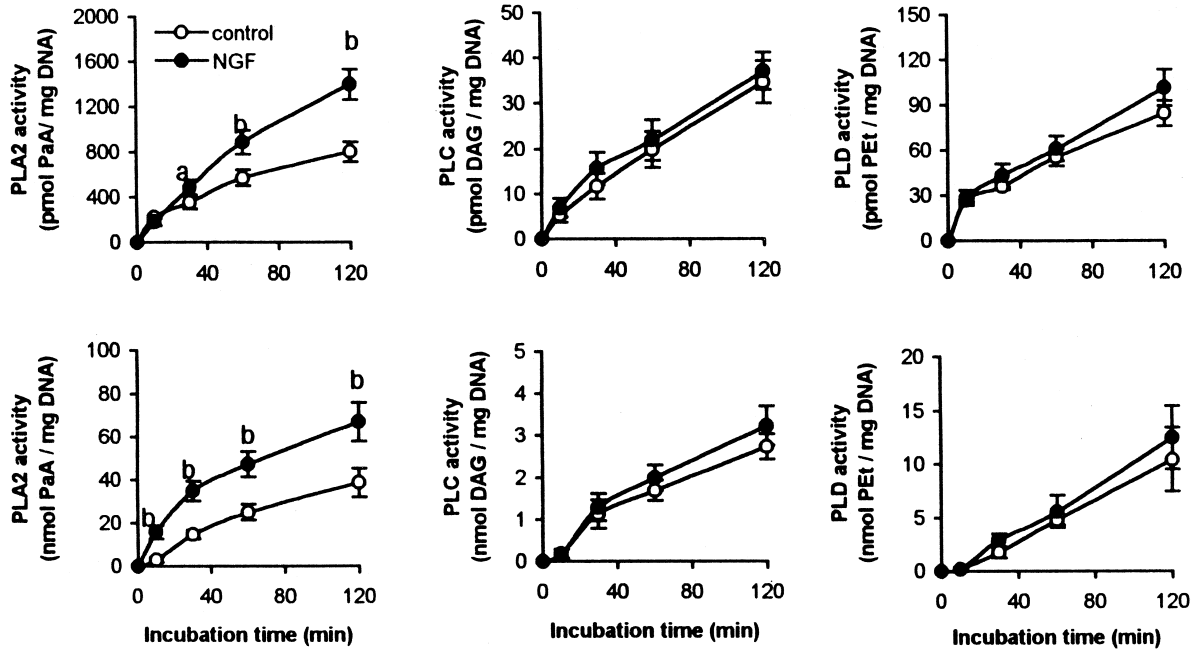

Fig. 6. Effects of NGF treatment on the activities of phospholipases. PC12 cells treated with 50 ng NGF/ml medium B for 96 h were collected, sonicated and then incubated in either 30 (upper) or 1000 (lower) nmol/ml of exogenous PC for 0–120 min. [^{3}H]PaA, [^{3}H]PEt and [^{3}H]DAG were purified and measured as described in Fig. 5 and in Section 2. The contents of [^{3}H]PaA, [^{3}H]DAG and [^{3}H]PEt were calculated according to dpm and specific radioactivity. Values (pmol/mg DNA or nmol/mg DNA) represent means ± S.D. of 6 determinations and statistical analyses were performed for comparison between groups. Compared with control cells, a: $P < 0.05$, b: $P < 0.01$.

Because levels of [^{3}H]glycerol-labeled PC were much higher than those of [^{3}H]DAG (Fig. 1), we examined the activities of three kinds of PC-directed phospholipases, i.e., PLA_2, PLD and PLC, in the PC12 cells. The metabolites of PC produced in our assays by PLA_2, PLD and PLC are PaA, PEt and DAG, respectively; their levels reflect the activities of the phospholipases. The activities of the phospholipases were dependent upon PC concentrations (Fig. 5), and each enzyme had two apparent K_m values, K_{m1} and K_{m2}. When the PC concentration in the reactive mixture was below 500 μM, the activities of PLA_2, PLD and PLC increased slowly with increasing substrate concentrations, and their K_{m1} values were below 200 μM. When PC concentrations were above 500 μM, the phospholipase activities peaked sharply, and their K_{m2} values were around 1000 μM. PC hydrolysis was most extensively catalyzed by PLA_2 (Fig. 5).

Based on this observation of two apparent K_m values for the phospholipases, we examined the effects of NGF treatment on phospholipase activities using both 30 and 1000 μM PC. NGF treatment for 96 h did not change the apparent K_m's for the three phospholipases, nor did it increase the specific activities of PLC or PLD in the PC12 cells at any substrate concentration or incubation time point (Fig. 6), affirming that the NGF-induced increase in DAG does not principally reflect hydrolysis of PC. On the other hand, levels of PaA (the product of PLA_2) in NGF-treated cells were about twice those of control cells after 60 min of incubation (Fig. 6), indicating that NGF treatment for 96 h stimulates PLA_2 activities (at either 30 or 1000 μM PC) and promotes the hydrolysis of PC to lysophospholipids and fatty acids.

4. Discussion

These data show that NGF treatment, which induces the differentiation of PC12 cells, concurrently promotes the incorporation of monounsaturated and polyunsaturated fatty acids of low specific-radioactivity into DAG and PC (Table 1). The fact that the level of [^{3}H]glycerol-labeled DAG level peaks earlier than that of [^{3}H]PC level (Fig. 1), and that the specific radioactivity of [^{3}H]glycerol-labeled DAG is much higher than those of [^{3}H]phospholipids (Table 2), suggests that the acceleration of [^{3}H]DAG synthesis occurs prior to those of the [^{3}H]phospholipids. Our data also indicate that the enhancement by NGF of de novo glycerolipid synthesis is initiated via activation of LCASs (OCS and ACS) (Fig. 2), and that the tendency of DAG to accumulate in differentiating PC12 cells results because LCAS activities are stimulated to a greater extent than CPT activity (Fig. 3). The similarity of the degradation rates of newly formed glycerolipids in both control and NGF-treated cells (Fig. 4), and the failure of NGF treatment to stimulate PLC and PLD activities (Fig. 6), indicate that neither PC nor PI hydrolysis is a major source of the increased DAG in NGF-treated PC12 cells.

In mammalian cells de novo glycerophospholipid synthesis involves two major steps: the reaction of long chain

acyl-CoA (activated fatty acid) with glycerol-3-phosphate or, successively, with lysophosphatidate to form phosphatidate or DAG; and the combination of DAG with CDP-choline or CDP-ethanolamine (or of CDP-DAG with inositol or serine) to form membrane phospholipids [1]. Thus, [^{3}H]glycerol-labeled metabolites on the pathway for de novo [^{3}H]glycerophospholipid synthesis should appear successively in the following order: [^{3}H]glycerol → [^{3}H]glycerol phosphate → [^{3}H]lysophosphatidate → [^{3}H]phosphatidate → [^{3}H]DAG → [^{3}H]phospholipids ([^{3}H]PC, [^{3}H]PE, [^{3}H]PS and [^{3}H]PI). [^{3}H]glycerol-labeled DAG should thus be produced mainly via its de novo synthesis, and only secondarily from the hydrolysis of newly formed [^{3}H]phospholipids or [^{3}H]TAG, and it should be impossible for [^{3}H]glycerol to label pre-existing neutral lipids and phospholipids.

Based on our previous findings [11] we conducted two additional experiments on [^{3}H]glycerol incorporation into DAG and PC. In the first, we extended NGF pre-treatment from 24 h to 96 h, to explore when the increases in [^{3}H]glycerol-labeled DAG and PC levels peaked; in the other, we measured the specific radioactivities of [^{3}H]glycerol-labeled lipids. The level of [^{3}H]glycerol-labeled DAG peaked earlier than that of [^{3}H]glycerol-labeled PC, and the specific radioactivity of [^{3}H]glycerol-labeled DAG was much higher than that of [^{3}H]glycerol-labeled PC. These findings further support the conclusion that the rate at which DAG is converted to PC exceeds the reverse when PC12 cells are exposed to NGF.

The present data further show that NGF treatment also promotes the incorporation of polyunsaturated LA or AA into glycerolipids by increasing the activities of the LCAS enzymes which catalyze the initial and indispensable steps in fatty acid utilization by mammalian cells [6]. Comparing our present data on LCASs with those we previously obtained on CPT (which controls the flux from DAG to PC by catalyzing the reaction of DAG with CDP-choline), it appeared that NGF treatment of the PC12 cells (96 h) enhanced LCAS activities by about 150–580%, but that of CPT by only 60% (Fig. 3). CPT activity appears to be rate-limiting in the conversion of DAG to PC when membrane phospholipid synthesis is enhanced by NGF in association with neurite outgrowth [1]. Hence, the different degrees of activation of LCASs and CPT could explain why DAG accumulates during PC12 cell differentiation.

Since at all times studied the levels of [^{3}H]glycerol-labeled PC in the PC12 cells were much higher than those of [^{3}H]DAG, the possibility arises that PC hydrolysis is a source of the increased DAG in NGF-treated cells. However, data from our pulse-chase studies on the disappearance of newly formed [^{3}H]glycerolipids (Fig. 4) show that the degradation rates for [^{3}H]DAG and [^{3}H]PC in control and NGF-treated cells did not differ, indicating that NGF does not promote the conversion of [^{3}H]PC to [^{3}H]DAG. Moreover, at no time were [^{3}H]glycerol-labeled TAG levels higher than those of [^{3}H]DAG, nor did NGF change the rate at which [^{3}H]TAG was degraded to [^{3}H]DAG; hence TAG is not a major source of the increase in DAG after NGF treatment.

PLA_2 hydrolyzes the acyl group on the *sn*-2 position of glycerophospholipids, generating free fatty acid and lysophospholipid [5,10]; PLC catalyzes the cleavage of the glycerol–phosphate bond in glycerophospholipid, producing DAG and the phosphate base group [7]; while PLD not only removes the base group from glycerophospholipid, but also transfers the phosphatidyl moiety of the substrate to nucleophiles like ethanol, thereby forming PEt [4]. Using L-α-dipalmitoyl-[2-palmitoyl-9,10-^{3}H(N)]PC (diluted with non-radiolabeled natural PC) as a common substrate of PLA_2, PLC and PLD, we were able simultaneously to measure the three radioactive products ([^{3}H]PaA, [^{3}H]DAG and [^{3}H]PEt), and thus estimate the specific activities of PLA_2, PLC and PLD. The method also provides additional advantages: prelabeling incubations were not required; differential labeling of metabolic compartments was not encountered; and the concentrations and specific activities of substrates were known [19].

Because the substrates hydrolyzed by phospholipases normally exist in an aggregated state, these enzymes must be able to act at a water–lipid interface; this property distinguishes them from the general class of esterases that exhibit normal saturation kinetics. We found that PLA_2, PLC and PLD each have two apparent K_m values for PC: the K_{m1} values are below 200 μM, while K_{m2} values are about 1000 μM. However, using either 30 or 1000 μM of PC as substrate, we found no effects of NGF on the activities of PLC or PLD, the phospholipases that can liberate DAG. This tends to affirm that hydrolysis of PC is not a source of the increased DAG levels in NGF-treated PC12 cells. On the other hand, NGF treatment did stimulate PLA_2 activity, and induced increases in the formation of free fatty acids and lysophospholipids from the breakdown of PC. However, such changes in PLA_2 activity could not be a mechanism for DAG accumulation.

In summary, our findings suggest that the mechanisms whereby NGF increases DAG levels in neuronally-differentiated PC12 cells involve the activation of LCASs, accompanied by a relatively weaker activation of CPT for converting DAG to PC. The breakdown of PC does not seem to be an important source for the DAG in this special circumstance.

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