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Nuclear diacylglycerol kinase-θ is activated in response to nerve growth factor stimulation of PC12 cells

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Abstract

Previous evidence from independent laboratories has shown that the nucleus contains diacylglycerol kinase (DGK) isoforms, i.e., the enzymes, which yield phosphatidic acid from diacylglycerol, thus terminating protein kinase C-mediated signaling events. A DGK isoform, which resides in the nucleus of PC12 cells, is DGK-θ. Here, we show that nerve growth factor (NGF) treatment of serum-starved PC12 cells results in the stimulation of both a cytoplasmic and a nuclear DGK activity. However, time course analysis shows that cytoplasmic DGK activity peaked earlier than its nuclear counterpart. While nuclear DGK activity was dramatically down-regulated by a monoclonal antibody known for selectively inhibiting DGK-θ, cytoplasmic DGK activity was not. Moreover, nuclear DGK activity was stimulated by phosphatidylserine, an anionic phospholipid that had no effect on cytoplasmic DGK activity. Upon NGF stimulation, the amount and the activity of DGK-θ, which was bound to the insoluble nuclear matrix fraction, substantially increased. Epidermal growth factor up-regulated a nuclear DGK activity insensitive to anti-DGK-θ monoclonal antibody. Overall, our findings identify nuclear DGK-θ as a down-stream target of NGF signaling in PC12 cells.
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Keywords: Nucleus; Diacylglycerol kinase-θ; Phosphatidic acid; Phosphatidylserine; Inositol lipid cycle; Nuclear matrix

1. Introduction

Over the last 15 years, several investigations have focused on the study of nuclear lipid-metabolizing enzymes [1–3]. Nuclear lipid metabolism is stimulated by various types of agonists and, remarkably, is operationally separated by its counterpart residing in the plasma membrane. Indeed, many agonists that stimulate the membrane cycle do not activate the nuclear cycle and vice versa. In other cases, if an agonist stimulates both cycles, it does so in a temporally distinct manner [4].

The activation of lipid metabolism within the nucleus leads to the generation of second messengers such as diacylglycerol (DG), which can derive from either inositol lipids or phosphatidylcholine (PC) [5]. The role played by DG in attracting to the nucleus some DG-dependent protein kinase C (PKC) isoforms (conventional PKC-α, -β_I, -β_{II}, -γ, and novel PKC -δ, -ε, -η, -θ) is now widely accepted [1,6], however, this lipid second messenger also modulates other signaling proteins, such as the guanine nucleotide exchanger factor vav, whose presence within the nucleus has been reported [7,8].

One of the major routes for terminating DG signaling is by its phosphorylation to phosphatidic acid (PA) by diacylglycerol kinase (DGK), which, in mammals, comprises a

Abbreviations: BSA, bovine serum albumin; CLSM, confocal laser scanning microscope; DG, diacylglycerol; DGK, diacylglycerol kinase; D-MEM, Dulbecco's modified minimum essential medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; NGF, nerve growth factor; NGS, normal goat serum; OG, octylglucoside; PA, phosphatidic acid; PC, phosphatidylcholine; PKC, protein kinase C; PI(4)P 5-kinase, phosphatidylinositol-4-phosphate 5-kinase; PI(4,5)P_2, phosphatidylinositol-4,5-bisphosphate; PI(3,4,5)P_3, phosphatidylinositol-3,4,5-trisphosphate; PLD, phospholipase D; PMSF, phenylmethylsulphonyl fluoride; PS, phosphatidylserine; SDS-PAGE, sodium dodecylsulphate polyacrylamide-gel electrophoresis; TLC, thin layer chromatography.

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family of 9 isozymes. Mammalian DGK isoforms are further organized into five subfamilies: class I comprises the α, β, and γ isozymes; class II the δ and η; class III the ε isoform; class IV the ζ and ι; class V the θ [9,10]. All of the mammalian DGKs share a conserved catalytic domain in the COOH-terminal region and at least a pair of cysteine-rich motifs (DGK-θ has three) similar to the C1A and C1B motifs of PKC, but lacking certain consensus residues present in phorbol ester-binding proteins. DGK isotypes can be distinguished by the presence of additional domains that conceivably confer to each isozyme specific functions in biological processes, sensitivity to different regulatory mechanisms, and a differential intracellular localization [9,10].

Interestingly, PA also has signaling functions, as it can modulate the activity of several enzymes including PKC-ζ, Ras-GTPase activating protein, and phosphatidylinositol-4-phosphate 5-kinase (PI(4)P 5-kinase) [11–13]. Moreover, PA has a role in actin polymerization [14]. Since they can attenuate local accumulation of signaling DG, DGKs play a pivotal role in many biological responses including cell proliferation, differentiation, survival, and apoptosis [9,10].

Several DGK isoforms (-α, -γ, -δ, -ζ, -θ) localize to the nucleus (reviewed in Ref. [15]). It has been demonstrated that DGK-ζ plays a key role in the control of intranuclear DG levels and, as a consequence, it regulates cell proliferation [16]. Regarding nuclear DGK-θ, it is activated in response to α-thrombin stimulation of IIC9 fibroblasts, an experimental model in which there is an increase in nuclear PC-derived DG mass due to an up-regulation of nuclear phospholipase D (PLD) [17]. We recently reported that in PC12 cells, nuclear DGK-θ is concentrated in distinct subnuclear domains, referred to as nuclear speckles [18]. Interestingly, components of the nuclear inositide metabolism, including phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$) and PI(4)P 5-kinase, are localized within the speckles [19,20].

Previous results have indicated that in response to nerve growth factor (NGF) treatment of PC12 cells, there is an increase in the amount of nuclear DG [21]. Thus, it could be hypothesized that a nuclear DGK isozyme is activated in response to NGF treatment of PC12 cells, as it happens in the case of insulin-like growth factor-1 (IGF-1)-treated Swiss 3T3 cells, i.e., another experimental system in which a rise in nuclear DG has been reported [22–24]. With the above in mind, we decided to investigate whether or not NGF up-regulates nuclear DGK-θ activity of PC12 cells. We have found that nuclear DGK activity increases in response to NGF exposure. This activity was inhibited by a monoclonal antibody, which has been previously shown to be capable of selectively down-regulating DGK-θ [17]. In contrast, epidermal growth factor (EGF), which has a proliferative effect on PC12 cells, activated a nuclear DGK activity insensitive to the anti-DGK-θ antibody. Taken together, our results identify nuclear DGK-θ as a specific down-stream target of NGF-elicited signaling pathways.

2. Materials and methods

2.1. Source of materials

Tissue culture media components, protease and phosphatase inhibitors, dithiothreitol, detergents, normal goat serum (NGS), bovine serum albumin (BSA), octylglucoside (OG), phosphatidylserine (PS), DG (dioleoyl), L-α-PA (dioleoyl), anti-β-tubulin monoclonal antibody, Cy3-conjugated anti-mouse IgG, and peroxidase conjugated anti-mouse IgG were from Sigma Chemical company, (St. Louis, MO).) DGK inhibitor R59949 was obtained from Calbiochem, (La Jolla, CA). Anti-DGK-θ monoclonal antibody (IgG_1) was from BD Transduction Laboratories, (Milan, Italy). Anti-lamin B monoclonal antibody was from Chemicon International, (Temecula, California). Mouse IgG_1 was purchased from DakoCytomation, (Glostrup, Denmark). NGF was from Upstate Biotechnology Incorporated, (Lake Placid, NY). Mouse EGF, the Lumi-Light^{Plus} enhanced chemiluminescence (ECL) detection kit, and RNase-free DNase I were from Roche Applied Science, (Milan, Italy). [γ-^{32}P] ATP was from Amersham Biosciences, (Milan, Italy). Silica Gel G-60 thin layer chromatography (TLC) plates was from Whatman (Maidstone, United Kingdom). The Protein Assay kit (detergent compatible) was from Bio-Rad, (Hercules, CA).

2.2. Cell culture

Rat pheocromocytoma PC12 cells were cultured in Dulbecco’s modified minimum essential medium (D-MEM) containing 10% heat-inactivated horse serum and 5% heat-inactivated fetal calf serum. Prior to stimulation with 100 ng/ml NGF for the indicated times, 50% confluent cells were switched to serum-free medium (D-MEM plus 0.5% BSA) overnight. EGF was used at 40 ng/ml.

2.3. Preparation of cytoplasmic fraction

Cells were resuspended in 10 mM Tris–Cl, pH 7.4, 10 mM NaCl, 2 mM $MgCl_2$, 1.0 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml soybean trypsin inhibitor, 1 μg/ml of leupeptin and aprotinin, 1.0 mM Na_3VO_4, 20 nM okadaic acid. They were incubated at room temperature for 2 min, cooled in ice water for 5 min, and homogenized on ice in a Dounce homogenizer with a type A pestle. Crude nuclear fraction was removed from the total homogenate by centrifugation at 400 × *g* for 15 min. The supernatant was used as cytoplasmic fraction.

2.4. Isolation of nuclei

This was accomplished as previously reported, with minor changes [21]. Briefly, cells were resuspended in 10 mM Tris–Cl, pH 7.4, 10 mM NaCl, 2 mM $MgCl_2$, 1.0 mM PMSF, 10 μg/ml soybean trypsin inhibitor, 1 μg/ml of

leupeptin and aprotinin, 1.0 mM Na_3VO_4, 20 nM okadaic acid. They were incubated at room temperature for 2 min, cooled in ice water for 5 min, then Nonidet P-40 was added to a final concentration of 1%. After a passage through a 22-gauge needle, the concentration of $MgCl_2$ was adjusted to 5 mM. The samples were centrifuged at 600 × g for 5 min. and washed once in 10 mM Tris-–Cl, pH 7.4, 5 mM $MgCl_2$, plus protease and phosphatase inhibitors as above. The purity of nuclear preparations was evaluated by Western blot analysis using a monoclonal antibody to β-tubulin. The absence of immunoreactivity to the cytoskeletal protein in isolated nuclear preparations confirmed that the isolation procedure yielded nuclei of high purity that were free of cytoplasmic contaminants.

2.5. Preparation of nuclear matrix

Nuclear matrices were isolated according to Belgrader et al. [25], with modifications. Briefly, isolated nuclei from PC12 cells were digested with 40 IU/mg DNA of RNase-free DNase I for 30 min at 4 °C. Subsequently, the chromatin-associated proteins were released by adding dropwise 2 M $(NH_4)_2SO_4$ to a final concentration of 0.6 M $(NH_4)_2SO_4$. After 15 min of incubation on ice, the nuclear matrices were pelleted at 1,500 × g for 15 min and washed once in 10 mM Tris–HCl, pH 7.4, 0.2 mM $MgCl_2$.

2.6. Protein assay

This was performed according to the instruction of the manufacturer using the Bio-Rad Protein Assay (detergent-compatible).

2.7. DGK in vitro activity assay

This was accomplished essentially as reported elsewhere [17,24], using OG/DG mixed micelles. OG/DG mixed micelles were prepared as follows: a mixture of 0.25 mM DG, 55 mM OG, and PS (either 1 mM resulting in 1.8% in micelles or 5 mM resulting in 8.3 mol% in micelles) was resuspended in 1 mM diethylenetriamine pentaacetic acid, pH 7.4, by vortexing and sonicating until the suspension appeared clear. Twenty microliters of mixed micelles were added to 70 μl of the reaction mixture (final concentration: 100 μM diethylenetriamine pentaacetic acid, pH 7.4, 50 mM imidazole–HCl, 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 5 μM ATP, 1 μCi of [γ-^{32}P] ATP). Fifty micrograms μg of protein/assay (cytoplasmic fraction, isolated nuclei, nuclear matrix) was used. Reaction volume was 100 μl. After a 30-min incubation at 25 °C, the reaction was stopped by the addition of chloroform/methanol/1% perchloric acid (1:2:0.75, v/v) and then vortexed. After addition of 10 μg unlabeled PA and 1% perchloric acid/chloroform (1:1, v/v), the mixture was briefly microfuged. The organic phase was washed twice in 1% perchloric acid, dried under a stream of nitrogen, and spotted on Silica Gel G-60 TLC plates, which were developed with chloroform/methanol/H_2O/ammonia (45:35:8:2). Spots of interest were scraped and the amount of [γ-^{32}P] PA was measured in a liquid scintillation counter. DGK activity was found to be linear to time (15–60 min) and protein concentration (20–100 μg) (data not shown).

2.8. Western blot analysis

Protein (80 μg/lane) was separated on sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose sheets using a semi-dry blotting apparatus. Sheets were saturated in PBS containing 5% NGS and 4% BSA for 60 min at 37 °C (blocking buffer), then incubated overnight at 4 °C in blocking buffer containing primary antibodies. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with peroxidase-conjugated anti-mouse IgG, diluted 1:5,000 in PBS–Tween-20, and washed as above. Immunoreactive bands were visualized by the ECL method.

2.9. In situ immunofluorescence

Cultures of PC12 cells (control and NGF-treated), growing on coverslips coated with rat tail collagen, were washed twice in cold PBS, pH 7.2, fixed with freshly prepared 4% paraformaldehyde (30 min at room temperature) and permeabilized with 0.2% Triton X-100 in PBS (10 min). Samples were reacted (overnight at 4 °C) with the monoclonal antibody to DGK-θ diluted 1:10 in 2% BSA, 3% NGS in PBS. The secondary antibody (1 hour at 37 °C) was a Cy3-conjugated anti-mouse IgG, diluted 1:500 in 2% BSA, 3% NGS in PBS. Finally, the coverslips were mounted in glycerol containing 1,4-diazabicyclo [2.2.2] octane to retard fading.

2.10. CLSM and image processing analysis

Samples were imaged by a LSM410 confocal laser scanning microscope (CLSM) (Zeiss, Oberckochen, Germany). This confocal system was coupled with a 1-mW HeNe ion laser as light source, used to reveal Cy3 signal with whith a 543-nm wavelength. Samples were observed with a × 100, 1.3 numerical aperture, PlanNeofluar objective lens. The image processing was performed using the ImageSpace software (Molecular Dynamics, Sunnyvale, CA).

3. Results

3.1. NGF increases DGK activity in PC12 cell subfractions

We first assayed in vitro DGK activity in cytoplasmic fraction or isolated nuclei prepared from NGF-stimulated

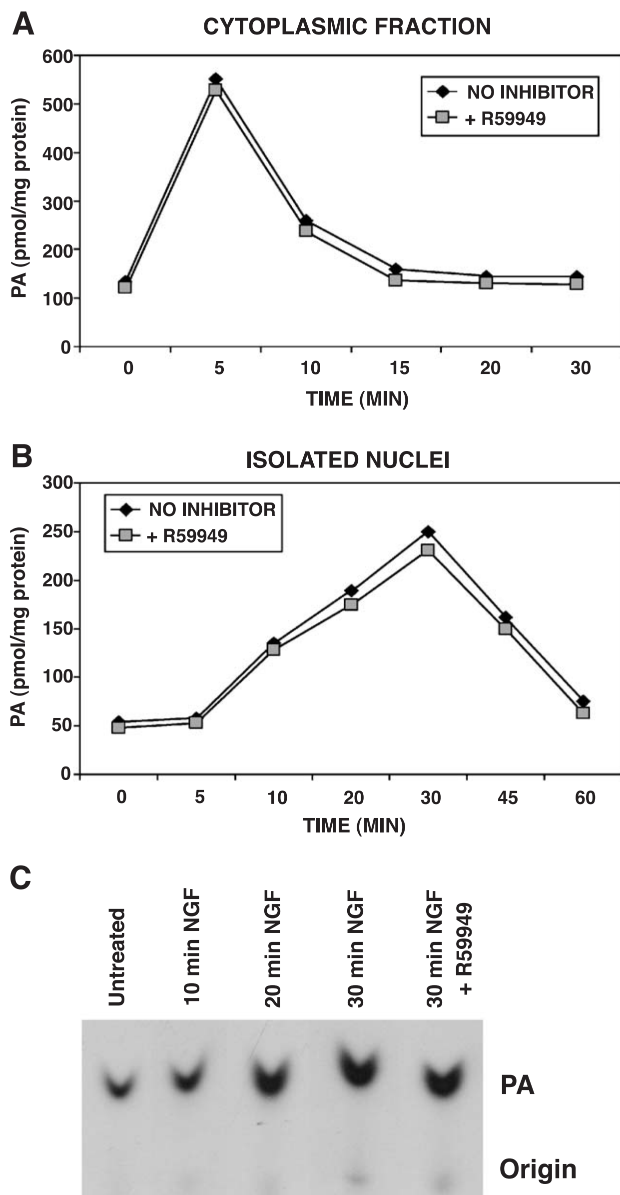

Fig. 1. DGK activity is increased in response to NGF treatment of PC12 cells. (A) Time course analysis of DGK activity in cytoplasmic fraction and (B) isolated nuclei. DGK activity was measured in 50 μg of protein for 30 min. The mixed micelles contained OG (55 mM), DG (0.25 mM), and PS (1 mM). Data are from a single experiment performed in duplicate and are representative of at least three experiments. (C) A TLC autoradiograph representative of the results reported in panel (B). DGK inhibitor R59949 (final concentration: 1 μM) was added to the reaction mixture 10 min prior to adding the mixed micelles.

PC12 cells. A time course analysis of cytoplasmic fraction DGK activity showed that the activity maximally increased at 5 min after stimulation. However, by 20 min, the activity has returned to basal levels. This DGK activity was insensitive to the DGK inhibitor R59949 (Fig. 1A).

In isolated nuclei, in vitro DKG activity started to increase 10 min after NGF stimulation, peaked at 30 min, and has returned to basal levels by 60 min. Also nuclear DGK activity was insensitive to R59949 (Fig. 1B and C).

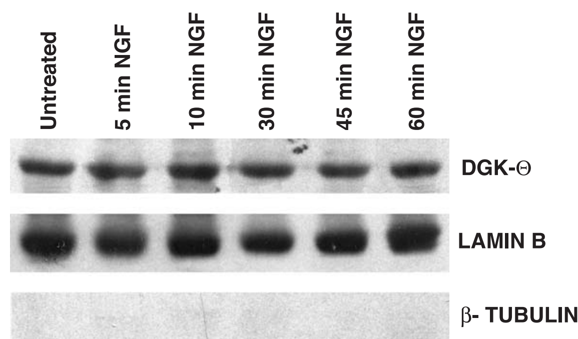

Fig. 2. NGF treatment does not increase the amount of nuclear DGK-θ. Western blot analysis for DGK-θ, lamin B, and β-tubulin in isolated nuclei prepared from PC12 cells stimulated for increasing periods of time with NGF (100 ng/ml). Nuclear protein (80 μg/lane) was separated on SDS-PAGE and blotted to nitrocellulose sheets. Immunoreactive bands were revealed by ECL. The blots are representative of three separate experiments.

3.2. NGF treatment neither increases nuclear amount of DGK-θ nor affects its intranuclear spatial distribution

Since our previous results indicated that DGK-θ is mainly concentrated in the nuclear speckle domains of PC12 cells [18], we investigated whether or not in response to NGF there was a change either in the nuclear amount or in the intranuclear distribution of DGK-θ. Western blot analysis demonstrated that the quantity of DGK-θ present

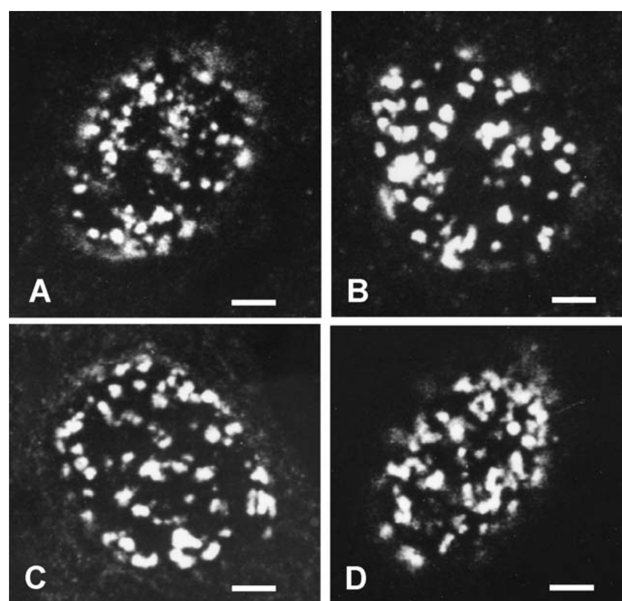

Fig. 3. NGF treatment does not change the intranuclear spatial distribution of DGK-θ. (A) Untreated cells; (B) cells treated with NGF for 10 min; (C) cells treated with NGF for 30 min; (D) cells treated with NGF for 60 min. PC12 cells growing on glass cover slips, were stimulated with 100 ng/ml NGF for the indicated times. They were then fixed, permeabilized, and fluorescently immunolabeled for DGK-θ. A Cy3-conjugated secondary antibody was employed. Samples were analyzed with a CLSM. Scale bar: 1 μm.

in isolated nuclei did not change upon stimulation with NGF (Fig. 2). Immunocytochemical staining coupled with CLSM analysis showed that in untreated, serum-starved cells DGK-θ was mainly located in 25–50 irregular, punctate domains, corresponding to speckles, as expected (Fig. 3A). However, the spatial distribution of DGK-θ did not change in response to NGF challenging (Fig. 3B–D).

3.3. Anti-DGK-θ antibody blocks the NGF-stimulated nuclear DGK activity in vitro

Previous results from another group have demonstrated that the monoclonal antibody to DGK-θ employed in this study could be used to inhibit the in vitro activity of nuclear DGK-θ, because it binds to this DGK isozyme in its catalytic domain [17]. Therefore, we decided to employ this antibody to establish whether or not DGK-θ was responsible

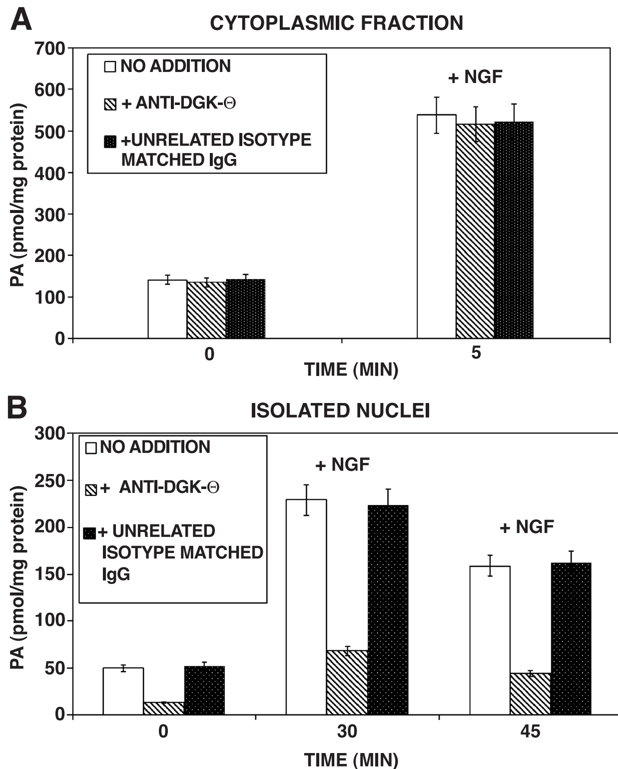

Fig. 4. Anti-DGK-θ antibody blocks NGF-elicited DGK activity in isolated nuclei. Cells were stimulated with NGF (100 ng/ml) for the indicated times. Subcellular fractions [(A) cytoplasmic fraction; (B) isolated nuclei] were then prepared. To disrupt nuclei, they were resuspended in 5 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, 100 IU/ml RNase-free DNase I, plus the protease and phosphatase inhibitor cocktail (see Materials and methods). After swelling on ice for 10 min, disruption was achieved by 60 passages through a 25-gauge hypodermic needle [17]. Either monoclonal antibody to DGK-θ (50 ng/ml) or an unrelated isotype matched mouse IgG (50 ng/ml) was added to the samples (50 μg protein), which were then incubated overnight at 4 °C with constant agitation. DGK activity assay was performed with mixed micelles containing OG (55 mM), DG (0.25 mM), and PS (1 mM). Results are the mean of three different experiments ± S.D.

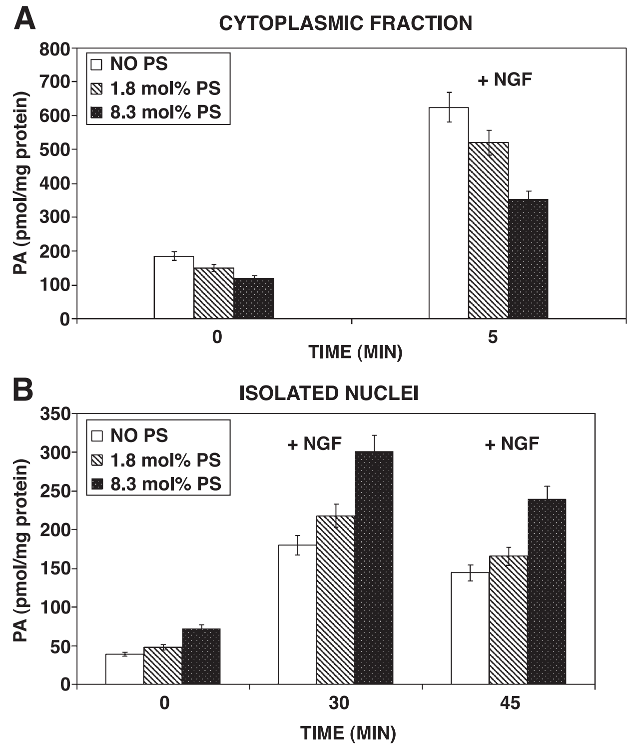

Fig. 5. PS stimulates nuclear DGK activity. PC12 cells were challenged with NGF (100 ng/ml) for the indicated times. Subcellular fractions [(A) cytoplasmic fraction; (B) isolated nuclei] were then prepared. DGK activity assay was performed with mixed micelles containing OG (55 mM), DG (0.25 mM), and PS (either 1 mM: 1.8 mol%; or 5 mM: 8.3 mol%). Results are the mean of three different experiments ± S.D.

for the increase of DGK activity measured in isolated nuclei prepared from NGF-stimulated PC12 cells.

Either cytoplasmic fraction or disrupted isolated nuclei from untreated or NGF-treated cells were incubated overnight (4 °C) with constant agitation in the presence or absence of the monoclonal antibody to DGK-θ. As a control, we employed an unrelated, isotype matched mouse monoclonal antibody. As shown in Fig. 4A, neither the anti-DGK-θ antibody nor the unrelated isotype mouse monoclonal antibody was capable of affecting DGK activity of the cytoplasmic fraction of untreated or 5 min NGF-treated cells. In contrast, when this type of experiment was performed using isolated nuclei, it was evident that the anti-DGK-θ antibody dramatically inhibited nuclear DGK activity measured in response to NGF treatment (30 or 45 min), whereas the control antibody did not. Also, in nuclei prepared from untreated cells, the anti-DGK-θ antibody significantly affected basal DGK activity (Fig. 4B). Overall, the anti-DGK-θ antibody inhibited approximately 75% of nuclear DGK activity at all the investigated times.

3.4. Nuclear DGK activity is stimulated by PS

Since DGK-θ is known for be activated by PS in IIC9 cells [17], we decided to analyze whether or not this was

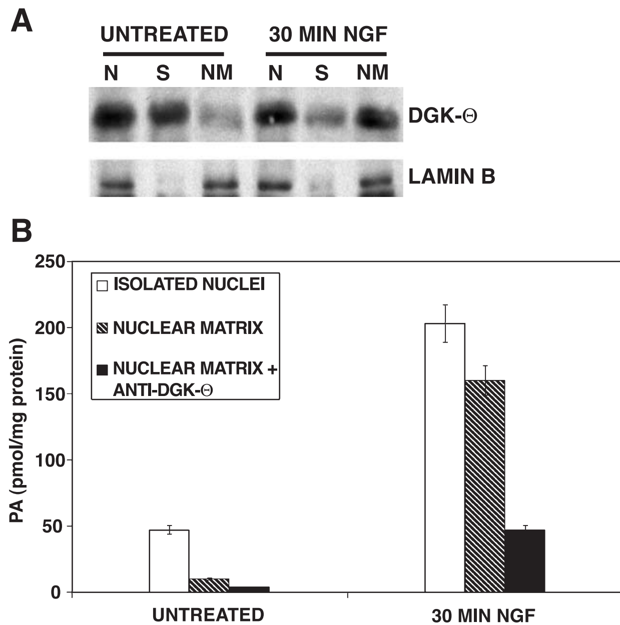

Fig. 6. NGF stimulation increases the amount of nuclear matrix-bound DGK-θ. PC12 cells were incubated with NGF (100 ng/ml). The nuclear matrix was then prepared from isolated nuclei by DNase I digestion and 0.6 M $(NH_4)_2SO_4$ extraction. (A) Western blot analysis for DGK-θ and lamin B. N, isolated nuclei; S, supernatant after $(NH_4)_2SO_4$ extraction; NM, nuclear matrix. Protein (80 μg/lane) was separated on SDS-PAGE and blotted to nitrocellulose sheets. Immunoreactive bands were revealed by ECL. The blots are representative of three separate experiments. (B) DGK activity assay. Nuclear matrix was disrupted as for nuclei, except that RNase-free DNase I was not employed. Either monoclonal antibody to DGK-θ (50 ng/ml) or an unrelated isotype matched mouse IgG (50 ng/ml) was added to the samples (50 μg protein), which were then incubated overnight at 4 °C with constant agitation. DGK activity assay was performed with mixed micelles containing OG (55 mM), DG (0.25 mM), and PS (1 mM). Results are the mean of three different experiments ± S.D.

also true of PC12 cell DGK activity. As shown in Fig. 5A, both the basal and the NGF-evoked DGK activity measured in cytoplasmic fraction was actually slightly inhibited by PS. In contrast, in isolated nuclei PS had a marked stimulatory effect on DGK activity (Fig. 5B).

3.5. NGF treatment increases nuclear matrix-bound DGK-θ

Our own previous results have shown that in exponentially growing MDA-MB-453 cells, DGK-θ is a component of the nuclear matrix [18]. The nuclear matrix is viewed by several investigators as the fundamental organizing principle of the nucleus where many functions take place, including DNA replication and transcription, and protein phosphorylation [26,27]. Several enzymes involved in lipid metabolism have been found associated with the nuclear matrix (reviewed in Refs. [8,28]), an indication that the matrix may also be involved in intranuclear signal transduction pathways.

We therefore investigated whether or not DGK-θ protein and activity were associated with the nuclear matrix prepared from PC12 cells. Western blot analysis indicated that in untreated cells, most of nuclear DGK-θ was extracted by the high ionic strength buffer (0.6 M $(NH_4)_2SO_4$) used to remove chromatin (Fig. 6A). iIn contrast, most of DGK-θ was bound to the nuclear matrix prepared from cells stimulated for 30 min with NGF. As a control, we analyzed the behaviour of another nuclear matrix protein, i.e., lamin B [29]. The blots revealed that all of this protein was associated with the nuclear matrix independently from the state of the cells (either untreated or NGF-stimulated) (Fig. 6A).

As far as nuclear DGK activity was concerned, in nuclei prepared from control cells, about 21% of the activity was matrix-bound. This activity was inhibited by the monoclonal antibody to DGK-θ (Fig. 6B). However, when the nuclear matrix was prepared from 30 min NGF-stimulated PC12 cells, the percentage of nuclear DGK activity recovered in the matrix fraction rose to about 79%. The antibody to DGK-θ inhibited 70% of the matrix-associated DGK activity (Fig. 6B).

3.6. EGF increases a nuclear DGK activity insensitive to anti-DGK-θ antibody

While NGF triggers neuronal differentiation of PC12 cells, EGF elicits a proliferative response (e. g., Ref. [30]). Therefore, we investigated whether or not also in response to EGF treatment, there was an increase in DKG activity. As shown in Table 1, we detected an up-regulation of nuclear DGK activity in response to EGF on a time scale similar to that observed with NGF. Also, the amplitude of the activity increase was the same. However, while at 0 min, 5 min, and 60 min approximately 75% of nuclear DGK activity was inhibited by the blocking anti-DGK-θ monoclonal antibody,, the EGF-elicited DGK activity was insensitive to it. In fact, at 10 min of stimulation, only about 20% of the activity was inhibited, while at 30 min inhibition was only 14%.

Table 1
EGF increases nuclear DGK activity

Time (min)	Without antibody	With anti-DGK-θ antibody
0	53.2 ± 7.3	18.8 ± 2.4
5	55.4 ± 8.1	19.4 ± 2.7
10	161.9 ± 18.0	128.4 ± 16.4
30	240.3 ± 27.7	207.7 ± 23.5
60	59.9 ± 8.4	20.1 ± 3.3

Serum-starved PC12 cells were stimulated with EGF (40 ng/ml) for the indicated times. Nuclei were then isolated and assayed for DGK activity. DGK activity assay was performed with mixed micelles containing OG (55 mM), DG (0.25 mM), and PS (1 mM). Monoclonal antibody to DGK-θ (50 ng/ml) was added to samples of disrupted nuclei (50 μg protein), which were then incubated overnight at 4 °C with constant agitation. Results are the mean of three different experiments ± S.D. and are expressed as pmol PA produced/mg protein.

4. Discussion

Recent studies have indicated that DGK isoforms are important components of the nuclear lipid metabolism (e.g., Refs. [15–18,24,31]).

In this report, we show that in response to NGF treatment of serum-starved PC12 cells, there was an increase in DGK activity both in the cytoplasmic and in the nuclear fraction. However, activation of DGK in the cytoplasmic fraction differed in many respects from that of nuclear DGK. Cytoplasmic DGK activity peaked much earlier (5 min) than nuclear activity (30 min), and was not inhibited by a monoclonal antibody, which has been previously demonstrated to selectively down–regulate DGK-θ [17]. In contrast, this antibody dramatically inhibited both basal and NGF-elicited nuclear DGK activity.

This finding strongly suggested that nuclear DGK-θ is a down-stream target of NGF-evoked signaling pathways. Moreover, while cytoplasmic DGK activity was inhibited by PS, nuclear DGK activity was stimulated by it. As far as the effect of PS on DGK isozymes is concerned, available data indicate that DGK-δ and DGK-ϵ isoforms are inhibited by PS [32,33], whereas the DGK-θ, -ζ, and -α isoforms are stimulated by this anionic phospholipid [17,33,34].

Regarding the sensitivity of cytoplasmic and nuclear DGK activity to the well-established DGK pharmacological inhibitor R59949, both activities were markedly inhibited when this chemical was included in the in vitro kinase assay. R59949 is a selective and powerful inhibitor of DGK-α, while other tested isoforms are either not or poorly inhibited [35,36].

Taken together, these findings suggest that NGF activates nuclear DGK-θ, whereas the cytoplasmic DGK isoform, whose activity is also up-regulated by NGF, remains to be identified, even though it could not be DGK-α.

Recently, it has been shown that nuclear DGK-θ is activated in response to α-thrombin stimulation of IIC9 cells [17]. Overall, our results are in agreement with such reports, even though there are some differences, which most likely depends on the cell type being investigated. In IIC9 cell nuclei, there is a basal DGK activity that is completely insensitive to anti-DGK-θ. In contrast, in PC12 cells basal nuclear DGK activity is also sensitive to the antibody inhibition. Moreover, in α-thrombin-stimulated IIC9 cells, there was a translocation of DGK-θ from cytoplasm to the nucleus, so that the intranuclear amount of DGK-θ increased upon α-thrombin incubation. This nuclear migration was at least partially responsible for the increased nuclear DGK-θ activity [17]. At variance, using both Western blot and immunofluorescence staining, we have demonstrated that there are no changes in the intranuclear amount of DGK-θ in NGF-treated PC12 cells. Also, the spatial distribution of DGK-θ within the nucleus was not affected by the cytokine. Indeed, in serum-starved or NGF-exposed cells, DGK-θ was concentrated in discrete domains which correspond to speckles, as reported previously [18].

However, in response to NGF, there was a marked increase in both the quantity and the enzymatic activity of DGK-θ, which remained associated with the nuclear matrix. DGK-α has also been demonstrated to become associated with the nuclear matrix of rat thymocytes and peripheral T-lymphocytes in response to either concanavalin A or anti-T-cell receptor antibody [37].

Association with the matrix may be responsible for the increase in the activity of nuclear DGK-θ measured after NGF stimulation. Other enzymes, such as DNA primase and polymerase-α, have been reported to become more active upon binding to the nuclear matrix [38]. Among the molecular mechanisms, which may control DGK activity, there is phosphorylation/dephosphorylation. Both DGK-α and –DGK-ζ can be phosphorylated by PKC-α. DGK-ζ is negatively regulated by this phosphorylation [39], while the effect on DGK-α is still unclear [40]. Furthermore, DGK-α, when activated, is phosphorylated on tyrosine residue [36]. As to DGK-θ, it can be phosphorylated in vitro by PKC isoforms, but also in this case, the outcome on enzymatic activity is yet to be established [41]. In this connection, it is worth emphasizing that the nuclear matrix is a site for protein phosphorylation [42] and that nuclear speckles contain protein kinases and phosphatases [43].

Activation of nuclear DGK-θ seems to be peculiar to NGF, because EGF up-regulated a nuclear DGK activity, which was not inhibited by the anti-DGK-θ antibody. The EGF-evoked nuclear DGK activity might correspond to the DGK-ζ isozyme, which has been observed to be a target of EGF signaling in other cell types [16].

The function of intranuclear DGK isozymes has generally been related to attenuating DG-dependent signaling events, such as attraction and activation of PKC isoforms [15,17,24]. Consistently, in NGF-treated PC12 cells, there is a nuclear migration of several DG-dependent PKC isozymes, including PKC-α and PKC-β_{II} [44,45].

However, it might be that nuclear DGK-θ fulfills other roles. Indeed, in nuclear speckles, it associates with phosphatidylinositol-4-phosphate 5-kinase [18], whose activity can be stimulated by PA [46]. Considering that NGF stimulates nuclear inositide metabolism by promoting phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P_3) synthesis from PI(4,5)P_2 [21,47], it might be that the enhanced activity of DGK-θ would produce PA necessary for stimulating phosphatidylinositol-4-phosphate 5-kinase, which, in turn, would synthesize additional PI(4,5)P_2. It should be emphasized here that PI(4,5)P_2 also localizes to nuclear speckles [18,48].

PA produced by DGK-θ might also be involved in regulating nuclear matrix structure and/or function, because actin seems to be a critical component of the nuclear skeleton [49]. In this connection, it seems interesting that

the spatial distribution of small nuclear ribonucleoproteins and intranuclear actin changed as early as 4 hour after exposure of PC12 cells to NGF [50] and that these changes might be related to the up-regulation of gene expression [51]. However, PI(4,5)P_2 by itself might also influence actin and, by as a consequence, nuclear structure and organization. In fact, a relationship between the chromatin remodelling SWI/SNF-related complex BAF and PI(4,5)P_2 signaling has recently begun to emerge recently. PI(4,5)P_2 enhances the binding of the BAF complex to the nuclear matrix [52]; PI(4,5)P_2 binds BAF at one molecule per complex, which, in turn, increases actin polymerization in a PI(4,5)P_2-sensitive manner, suggesting that PI(4,5)P_2 can induce the uncapping of actin, leading to nucleation or filament assembly [53,54]. Therefore, multiple functions may be envisaged for nuclear DGK-θ and its product PA.

Further investigations should shed more light on the multiple emerging roles played by lipids and lipid second messengers generated within the nucleus, as well as on the mechanisms which regulate the enzymes involved in the synthesis of these highly bioactive molecules.

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