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Proliferating or Differentiating Stimuli Act on Different Lipid-dependent Signaling Pathways in Nuclei of Human Leukemia Cells

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Previous results have shown that the human promyelocytic leukemia HL-60 cell line responds to either proliferating or differentiating stimuli. When these cells are induced to proliferate, protein kinase C (PKC)-βII migrates toward the nucleus, whereas when they are exposed to differentiating agents, there is a nuclear translocation of the α isoform of PKC. As a step toward the elucidation of the early intranuclear events that regulate the proliferation or the differentiation process, we show that in the HL-60 cells, a proliferating stimulus (i.e., insulin-like growth factor-I [IGF-I]) increased nuclear diacylglycerol (DAG) production derived from phosphatidylinositol (4,5) bisphosphate, as indicated by the inhibition exerted by 1-*O*-octadeyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine and U-73122 (1-[6((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione), which are pharmacological inhibitors of phosphoinositide-specific phospholipase C. In contrast, when HL-60 cells were induced to differentiate along the granulocytic lineage by dimethyl sulfoxide, we observed a rise in the nuclear DAG mass, which was sensitive to either neomycin or propranolol, two compounds with inhibitory effect on phospholipase D (PLD)-mediated DAG generation. In nuclei of dimethyl sulfoxide-treated HL-60 cells, we observed a rise in the amount of a 90-kDa PLD, distinct from PLD1 or PLD2. When a phosphatidylinositol (4,5) bisphosphate-derived DAG pool was generated in the nucleus, a selective translocation of PKC-βII occurred. On the other hand, nuclear DAG derived through PLD, recruited PKC-α to the nucleus. Both of these PKC isoforms were phosphorylated on serine residues. These results provide support for the proposal that in the HL-60 cell nucleus there are two independently regulated sources of DAG, both of which are capable of acting as the driving force that attracts to this organelle distinct, DAG-dependent PKC isozymes. Our results assume a particular significance in light of the proposed use of pharmacological inhibitors of PKC-dependent biochemical pathways for the therapy of cancer disease.

INTRODUCTION

The human promyelocytic cell line HL-60 can be induced, in culture, either to proliferate with growth factors such as insulin growth factor-I (IGF-I) (Li *et al.*, 1997) or to differentiate toward a granulocyte-like phenotype by a variety of chemicals, including dimethyl sulfoxide (DMSO) (Collins, 1987). One of the earliest events that follows exposure to proliferating or differentiating stimuli is the intranuclear migration of diacylglycerol (DAG)-dependent protein kinase C (PKC) isozymes. In particular, although PKC-βII is recruited to the nucleus in response to proliferating stimuli, a nuclear translocation of α isozyme of PKC typically occurs when HL-60 cells are exposed to differentiating agents (Ho-

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Abbreviations used: BSA, bovine serum albumin; CLSM, confocal laser scanning microscope; DAG, diacylglycerol; DMSO, dimethyl sulfoxide; EPO, erythropoietin; ET-18-OCH_3, 1-*O*-octadeyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; HPLC, high-performance liquid chromatography; IGF-I, insulin-like growth factor-I; NGS, normal goat serum; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PC-PLC, phosphatidylcholine-specific phospholipase C; PKC, protein kinase C; PI-PLC, phosphoinositide-specific phospholipase C; PLD, phospholipase D; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; PtdIns (4,5) P_2, phosphatidylinositol (4,5) bisphosphate.

cevar and Fields, 1991; Murray *et al.*, 1993; Zauli *et al.*, 1996; reviewed by Martelli *et al.*, 1999c). Once in the nucleus, PKC isozymes phosphorylate proteins, such as lamins, which are likely to play an important role during either proliferation or differentiation (Fields *et al.*, 1988).

DAG is a biologically active lipid second messenger that is produced in response to cell stimulation with a bewildering variety of agonists, including polypeptide growth factors, hormones, and neurotransmitters (Wakelam, 1998). It was initially thought that DAG derived exclusively from phosphatidylinositol (4,5) bisphosphate [PtdIns (4,5) P_2] hydrolysis through the action of a phosphoinositide-specific phospholipase C (PI-PLC). However, it has subsequently become evident that DAG can derive from other sources: 1) phosphatidylcholine (PC) is hydrolyzed by a phospholipase D (PLD), yielding phosphatidic acid (PA), which in turn is converted to DAG by a specific PA phosphohydrolase; or 2) PC is hydrolyzed by a PC-PLC, which produces DAG (Wakelam, 1998). Nevertheless, the two most common pathways that give rise to DAG are those controlled through PI-PLC and PLD. The interest that surrounds DAG is due to the fact that this molecule is a physiological activator of some PKC isoforms, both conventional and novel (Ron and Kazanietz, 1999). Other than at the plasma membrane, DAG is generated at the nuclear level (D'Santos *et al.*, 1998). In this context, it should be recalled that the existence of several signaling pathways leading to the generation of lipid second messengers in the nucleus has been demonstrated by independent laboratories (Divecha *et al.*, 1991; Jarpe *et al.*, 1994; York and Majerus, 1994; Mallia *et al.*, 1997; Sun *et al.*, 1997; Neri *et al.*, 1998, 1999a; reviewed by D'Santos *et al.*, 1998; Martelli *et al.*, 1999b; Cocco *et al.*, 2001). These nuclear lipid-signaling pathways are involved in the control of both cell proliferation and differentiation (Manzoli *et al.*, 1997; Matteucci *et al.*, 1998; Avazeri *et al.*, 2000; Martelli *et al.*, 2000). Also, in the nucleus DAG has been shown to derive from either PtdIns (4,5) P_2 hydrolysis (Sun *et al.*, 1997; Neri *et al.*, 1998) or PLD-mediated PC hydrolysis (Martelli *et al.*, 1999a). In addition, D'Santos *et al.* (1999) recently showed that nuclei contain two distinct pools of DAG, one highly disaturated and mono-unsaturated and one highly polyunsaturated. The former derives from PC hydrolysis (conceivably through the action of a PC-PLC), whereas the latter from the hydrolysis of PtdIns (4,5) P_2. Whether it derives from PtdIns (4,5) P_2 or PC, the function of DAG seems to be the attraction of PKC isoforms to the nuclear compartment (Divecha *et al.*, 1991; Leach *et al.*, 1992; Sun *et al.*, 1997; Neri *et al.*, 1998). The existence of two separate pools of nuclear DAG suggests that this lipid second messenger might be involved in distinct pathways that lead to different cell responses. However, a conclusive demonstration that in the same cell line different stimuli activate distinct phospholipases present in the nucleus and that this differential activation is responsible for attracting to the organelle-specific, DAG-dependent PKC isoforms, is still lacking.

In this article, we provide evidence that, in the HL-60 cell line, nuclear PI-PLC activity causes changes in DAG levels after a proliferating stimulus represented by IGF-I, and that this increase in DAG mass is responsible for PKC-βII translocation to the nucleus. In contrast, in response to DMSO administration, we observed a rise in nuclear DAG levels and a translocation of PKC-α to the nucleus that were blocked by inhibitors selective for PLD-mediated DAG generation. Therefore, we postulate the existence in HL-60 cells of two independently regulated nuclear DAG sources that are related to distinct stimuli and that recruit to the nucleus different PKC isozymes.

MATERIALS AND METHODS

Materials

RPMI-1640, fetal calf serum, DMSO, dioleylglycerol, oleate, PtdIns (4,5) P_2, guanosine-5′-*O*-(3-thio)triphosphate (GTPγS), 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, PS, normal goat serum (NGS), peroxidase-conjugated anti-goat, anti-rabbit, and anti-mouse IgG, Cy3-conjugated anti-rabbit IgG, histone H1, leupeptin, aprotinin, phenylmethylsulfonyl fluoride (PMSF), benzamidine, polyclonal antibodies to PKC-α and -βII, and bovine serum albumin (BSA) were from Sigma Chemical (St. Louis, MO). Phosphatidylethanol was from ICN Pharmaceuticals (Costa Mesa, CA). YO-PRO-1 nucleic acid staining was from Molecular Probes (Eugene, OR). 1-*O*-octadeyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine (ET-18-OCH_3), propranolol, U-73122 (1-[6((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione), U-73343 (1-[6((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]2,5-pyrrolidinedione), D-609 (tricyclodecan-9-yl-xanthogenate), and monoclonal antibody (mAb) to lamin B1 were from Calbiochem (La Jolla, CA). IGF-I, the Lumi-Light^{Plus} enhanced chemiluminescence detection kit, and NP-40 were from Roche Applied Sciences (Milan, Italy). mAb to histone H1 and polyclonal antibody to PLD were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies to PI-PLC isoforms and to phospho-PKCs (PKC-β phosphorylated on Ser-660 and PKC-α phosphorylated on Ser-657) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). p81 paper was from Whatman (Maidstone, United Kingdom). [^{3}H]PdIns (4,5) P_2, [^{3}H]palmitic acid, phosphatidyl-[*methyl*-[^{3}H]]choline, and [γ-^{32}P]ATP were from Amersham Biosciences AB (Uppsala, Sweden). The Protein Assay kit (detergent compatible) was from Bio-Rad (Hercules, CA).

Cell Culture, Proliferation, and Differentiation

HL-60 human leukemia cells were grown in RPMI-1640 medium supplemented with 10% fetal calf serum at 37°C. For experiments with IGF-I, cells were washed three times and incubated in serum-free medium for 24 h before each assay. Cells were then stimulated with 50 ng/ml IGF-I (Li *et al.*, 1997). To induce differentiation into granulocytic-like cells, cells were plated at a density of 2×10^5/ml in complete medium in the presence of 1.25% DMSO. When used, the various phospholipase inhibitors were present starting 5 min before simulation, at the following concentrations: ET-18-OCH_3, 100 μM; U-73122 and U-73343, 30 μM; D-609, 30 μM; propranolol, 100 μM; and neomycin, 1 mM.

Isolation of Nuclei and Cytoplasmic Fraction from HL-60 Cells

This was accomplished essentially as reported by Fields *et al.* (1989) and Martelli *et al.* (1999a). All steps were executed at 4°C in buffers containing 0.1 mM Na_3VO_4, 10 μM aprotinin, 10 μM benzamidine, and 1 mM PMSF. Cells were washed three times with phosphate-buffered saline (PBS) and incubated in 50 mm Tris-HCl pH 7.4, 250 mM sucrose, 5 mM $MgSO_4$ containing 1% (vol/vol) 2-mercaptoethanol for 10 min at 10^7 cells/ml. Then 10% (wt/vol) NP-40 was added to a final concentration of 0.02% (wt/vol), and the cells were lysed with 50 strokes of a Dounce homogenizer by using a B-type pestle. The lysate was layered over a cushion of 2.1 M sucrose, 50 mm Tris-HCl pH 7.4, 5 mM $MgSO_4$, 1% 2-mercaptoethanol, and the nuclei were pelleted at 70,000 $\times$ *g* for 60 min in a Beckman SW28 rotor. It is worth remembering here that this isolation protocol

yields nuclear preparations that were free from plasma membrane contamination, as exemplified by the absence of the IGF-I receptor (Martelli *et al.*, 1999a). The cytoplasmic fraction was prepared according to Martelli *et al.* (1999a).

Protein Assay

This was performed according to the instruction of the manufacturer by using the Bio-Rad protein assay (detergent compatible).

Measurement of DAG Produced In Vivo

The assay was performed according to Divecha *et al.* (1991) by using DAG kinase enzyme purified from rat brain. DAG was extracted from nuclei, dissolved in 20 μl of 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate (9.2 mg/ml), and sonicated at room temperature for 15 s. After the addition of 80 μl of reaction buffer (50 mM Tris acetate pH 7.4, 80 mM KCl, 10 mM Mg-acetate, 2 mM EGTA), the assay was started by the addition of 20 μl of DAG kinase enzyme followed by 80 μl of reaction buffer containing 5 μM ATP and 1 μCi of [γ-^{32}P]ATP. Incubation was for 1 h at room temperature, and then PA was extracted, chromatographed, autoradiographed, and its radioactivity counted in a liquid scintillation system.

PI-PLC Activity Assay

The procedure outlined by Martelli *et al.* (1992) was followed. Assays (100 μl) contained 100 mM 2-[*N*-morpholino]ethanesulfonic acid buffer pH 6.7, 150 mM NaCl, 0.06% Na-deoxycholate, 3 nmol of [^{3}H]PtdIns (4,5) P_2 (specific activity 30,000 dpm/nmol), and 10 μg of nuclear protein. Incubation was for 30 min at 37°C. Hydrolysis was stopped by adding chloroform/methanol/HCl, and inositol phosphates recovered in the aqueous phase were analyzed by high-performance liquid chromatography (HPLC) by using a Partisil 10 SAX column eluted with a linear gradient from distilled water to 2 M ammonium formate (pH 3.7, adjusted with phosphoric acid). Fractions (1 ml) were collected and counted by liquid scintillation.

Solubilization of Nuclear PI-PLC Isoforms and Immunoprecipitation

Nuclei were resuspended at 1 mg of DNA/ml in 5 mM Tris-HCl pH 8.0, 0.1 mM EDTA plus protease inhibitors as described above, and allowed to swell at 4°C for 10 min before rupturing by 40 passages through a 25-gauge hypodermic needle. The lysate was centrifuged for 10 min at 48,000 $\times$ *g*. Protein (5 μg) from nuclear lysates was incubated under constant agitation for 1 h at 4°C in the presence of 1.25 μg of antibody to various PI-PLC isoforms. Protein A-Agarose was the added to 10% (wt/vol) and incubation proceeded for an additional 60 min. Immunocomplexes were collected by centrifugation, and the supernatant was assayed for residual PI-PLC activity (Marmiroli *et al.*, 1994; Martelli *et al.*, 2000a).

PLD In Vitro Activity Assay

This was accomplished as follows (Martelli *et al.*, 1999a): cells were labeled for 20 h in the presence of [^{3}H]palmitic acid (5 μCi/ml). Nuclei were isolated and incubated (50 μg/assay in 200-μl final volume) for 30 min at 37°C in 25 mM HEPES-NaOH pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM $MgCl_2$, 1 μM $CaCl_2$, 1 mM PMSF, 10 μM benzamidine and leupeptin, and 1.5% ethanol. Total lipids were extracted and the incorporated radioactivity was quantified at this time by scintillation counting. Phosphatidylethanol was resolved from nuclear lipids by thin layer chromatography on silica gel plates by using a system consisting of chloroform/methanol/ammonia/water (45:35:2:8, by volume). After chromatography, the plates were dried and the location of material was revealed by staining with iodine vapors. Phosphatidylethanol was identified by comparison of Rf values with those from authentic standards. Spots were scraped from the plates and counted by scintillation counting. Values were expressed as percentage of radioactivity in phosphatidylethanol with respect to total nuclear phospholipid. In some experiments nuclear lysates (150 μg of nuclear protein) were incubated with Triton X-100 (6.25 mM), phosphatidyl-[methyl-[^{3}H]]choline (2.25 mM at 29 μCi/μmol) mixed micelle (3:1, Triton X-100/PC). The reaction mixture was incubated at 37°C for 1 h, and the released water-soluble head groups were separated by ion pairing with tetraphenylboron and quantified by liquid scintillation counting (Martelli *et al.*, 1999a).

Western-blotting Analysis

Nuclear proteins (80 μg), separated on SDS-PAGE, were transferred to nitrocellulose sheets. Sheets were saturated in PBS containing 5% NGS and 4% BSA for 60 min at 37°C (blocking buffer), and then incubated overnight at 4°C in blocking buffer containing the primary antibody. After four washes in PBS containing 0.1% Tween 20, they were incubated for 30 min at room temperature with the appropriate peroxidase-conjugated secondary antibody, diluted 1:5000 in PBS-Tween 20, and washed as described above. Bands were visualized by enhanced chemiluminescence. In some cases, to normalize the amount of the loaded protein, blots were stripped and reprobed with mAb to either lamin B1 or histone H1.

Densitometric analysis was performed on the Molecular Analyst GS670 (Bio-Rad) as previously described (Martelli *et al.*, 2000b).

Preparation of Nuclear Extracts and Immunoprecipitation

Nuclear extracts were prepared essentially as reported elsewhere (Neri *et al.*, 1999b), with some modifications. Nuclei were resuspended in 5 mM Tris-HCl pH 8.0, 1 mM EGTA, 1 mM EDTA, 0.1 mM Na_3VO_4, 10 μM aprotinin, 10 μM benzamidine, 1 mM PMSF, 0.3% Triton X-100, and then ruptured by 50 passages through a 25-gauge hypodermic needle, and centrifuged at 5000 $\times$ *g* to remove insoluble material. Nuclear extracts (1 ml, containing 500 μg of protein) were precleared by adding 5 μg of normal rabbit IgG and 10 μg of 50% protein A-Agarose, followed by incubation for 1 h at 4°C and centrifugation at 12,000 $\times$ *g* for 10 min at 4°C. The samples were incubated for 4 h at 4°C under constant agitation with 3 μg of the primary antibody; 10 μg of 50% protein A-Agarose was added, and incubation proceeded for 1 h at 4°C under constant agitation. Samples were then centrifuged. The beads were washed once with lysis buffer and twice with kinase buffer (50 mM Tris-HCl pH 7.4, 1 mM Na_3VO_4, 0.5 mM EGTA, 0.5 mM EDTA, 2 mM $MgCl_2$, 5 μg/ml leupeptin, 1 mM PMSF).

In Vitro Assay for Nuclear PKC Activity

Immunoprecipitates were incubated at 30°C for 10 min in 20 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 μM ATP, 0.4 μg/ml histone H1, 10 μCi of [γ-^{32}P]ATP, in the presence of 1.2 mM $CaCl_2$, 40 μg/ml PS, and 3.3 μM dioleylglycerol. The reactions were terminated with 15 μl of acetic acid, and spotted on to Whatman p81 paper, followed by washing with 0.75 mM H_3PO_4. Radioactivity was measured by Cerenkov counting.

Detection of PKC by In Situ Immunofluorescence

Cells in PBS were plated onto 0.1% poly-L-lysine–coated glass slides and adhesion was allowed to proceed for 30 min at 37°C. Cells were then fixed with freshly made 4% paraformaldehyde (30 min at room temperature) and permeabilized with 0.2% Triton X-100 in PBS (10 min). Antibodies to PKC isoforms were used at a dilution of 1:100 in 2% BSA, 3% NGS in PBS. The secondary antibody was a Cy3-conjugated anti-rabbit IgG, diluted 1:100. All incubations were carried out at 37°C. Samples were counterstained for DNA with YO-

Table 1. Generation of DAG in HL-60 cells in response to either sphingosylphosphorylcholine or PMA

Agonist	No pretreatment	ET-18-OCH_3	U-73122	U-73343	D-609	Propranolol	Neomycin
None	194.5 ± 21.7	199.7 ± 22.6	191.3 ± 23.3	198.5 ± 20.4	189.6 ± 19.3	188.0 ± 19.5	191.5 ± 22.1
Sphingosylph osphorylcholine	466.8 ± 46.4	227.5 ± 26.9	234.6 ± 25.8	479.7 ± 50.6	460.5 ± 48.9	456.7 ± 46.4	459.0 ± 46.6
PMA	933.6 ± 97.3	925.1 ± 94.9	919.5 ± 90.6	938.5 ± 96.4	929.9 ± 96.2	239.4 ± 26.8	225.5 ± 27.3

HL-60 cells were exposed to either sphingosylphosphorylcholine (30 μM for 30 s) or PMA (100 nM for 2.5 min). The DAG in the cytoplasmic fraction was then assayed. The results are expressed as picomoles of DAG per milligram of protein. When the DAG generation inhibitors were present, cells were preincubated for 5 min before exposure to agonists. The data are the mean of three different experiments ± SD.

PRO-1 (1 μM for 10 min). Finally, the coverslips were mounted in glycerol containing 1,4-diazabicyclo [2.2.2] octane to retard fading, by using additional coverslips as spacers to preserve the three-dimensional structure of cells.

CLSM and Image-processing Analysis

Samples were imaged by an LSM410 confocal laser scanning microscope (CLSM) (Zeiss, Oberckochen, Germany). This confocal system was coupled with a 1-mW HeNe and a 25-mW Argon ion laser as light source, which were used for detection of Cy3 and YO-PRO-1, respectively. In the detection path the emitted fluorescent light was focused, in front of each detector, on a back pinhole aperture that was set at a value of 20 (in a scale ranging from 0 to 250), which corresponds to a diameter of ~50 μm. Samples were observed with a 100×, 1.4 numerical aperture Planneofluar objective lens. Images were acquired, frame by frame, with a scanning mode format of 512 × 512 pixels. The fluorochromes were acquired on two different channels and separately. Cy3 was acquired first using a 590-nm long pass filter (channel 1); YO-PRO-1 was acquired immediately after using a 560-nm dichroic mirror and a 525 ± 15 nm band pass filter (channel 2). Digitalized optical sections, i.e., Z series of confocal data ("stacks"), were transferred from the CLSM to the graphics workstation Indy (Silicon Graphics, Mountain View, CA) and stored on the graphics workstation with a scanning mode format of 512 × 512 pixels and 256 gray levels. The image processing was performed using the ImageSpace software (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Selectivity of Phospholipase Inhibitors

A critical issue during this investigation was represented by the selectivity of the phospholipase inhibitors we used. To address this issue, we took advantage of previous studies (mostly carried out in HL-60 cells) that have demonstrated that these pharmacological inhibitors may be indeed considered selective. The drugs we used were ET-18-OCH_3 and U-73122, that inhibit PI-PLC (Okajima and Kondo, 1995; Sun *et al.*, 1997; Neri *et al.*, 1998; Cabaner *et al.*, 1999); U-73343, an inactive analog of U-73122 (Tatrai *et al.*, 1994; Stam *et al.*, 1998); D-609, a purported PC-PLC inhibitor (Machleidt *et al.*, 1996, Sun *et al.*, 1997); propranolol, an inhibitor of PLD-mediated DAG generation (Ohguchi *et al.*, 1997; Sun *et al.*, 1997; Tool *et al.*, 1999); and neomycin, an inhibitor of PLD (Ohguchi *et al.*, 1996; Guillemain and Exton, 1998). In HL-60 cells it is well established that sphingosylphosphorylcholine activates a PI-PLC activity (most likely a member of the β family of PI-PLC; Okajima *et al.*, 1995; Baek *et al.*, 1996), whereas phorbol 12-myristate 13-acetate (PMA) is a powerful stimulator of PLD activity (Ohguchi *et al.*, 1996; Houle *et al.*, 1999). Therefore, we measured DAG levels in the cytoplasmic fraction of HL-60 cells stimulated with either sphingosylphosphorylcholine or PMA in the presence of the above-listed pharmacological inhibitors. The results from these experiments are presented in Table 1. As expected, sphingosylphosphorylcholine-evoked DAG rise was inhibited by both ET-18-OCH_3 and U-73122, but not by U-73343, D-609, propranolol, or neomycin. In contrast, the PMA-dependent DAG increase was sensitive to both propranolol and neomycin, but not to ET-18-OCH_3, U-73122, U-73343, or D-609. Therefore, these results indicated that the inhibitors we used for the subsequent experiments were selective.

Changes in Cytoplasmic or Nuclear Fraction DAG Levels after a Proliferating Stimulus

We first assayed DAG levels in the cytoplasmic fraction. At all the examined times, the mass of DAG in this cell fraction did not change in response to IGF-I stimulation (Figure 1A). Next, DAG levels were measured in isolated nuclei. Control nuclei contained ~38 ± 4.7 pmol/mg protein of DAG, but already after 10 min of mitogenic stimulation this value rose to 82.6 ± 7.5 pmol/mg protein, i.e., a nearly twofold increase (Figure 1A). This value was essentially maintained for the following 20 min, as shown in Figure 2A. However, after 60 min it had returned to basal levels.

To assess the phospholipase activity responsible for the nuclear DAG production that follows IGF-I stimulation of HL-60 cells, we used a panel of pharmacological inhibitors. As presented in Figure 1B, when cells pretreated with either ET-18-OCH_3 or U-73122 were challenged with IGF-I, we observed a dramatic inhibition of the DAG rise at any time investigated. U-73343, an inactive analog of U-73122, did not inhibit the DAG increase (Figure 1B). Also, D-609, neomycin, or propranolol did not affect the IGF-I–dependent rise in nuclear DAG mass (Figure 1C).

Changes in Cytoplasmic or Nuclear DAG Levels after a Differentiating Stimulus

To investigate DAG metabolism during DMSO-induced differentiation of HL-60 cells, DAG levels were examined at

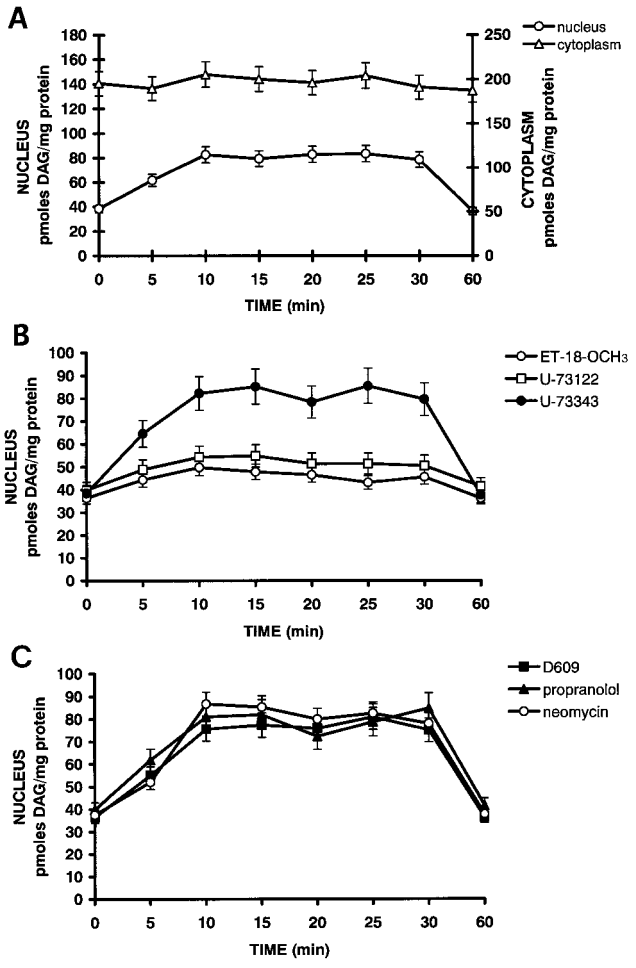

Figure 1. Effect of IGF-I stimulation on the genesis of DAG. (A) Time course of changes in DAG concentration in the cytoplasmic fraction and in nuclei obtained from IGF-I–stimulated HL-60 cells. Quiescent cells were stimulated with 50 ng/ml IGF-I for the indicated times. (B and C) Effect of different inhibitors on IGF-I–elicited nuclear DAG production. Cells were preincubated with the chemicals for 5 min before mitogenic stimulation. Each point represents the mean of three different experiments ± SD.

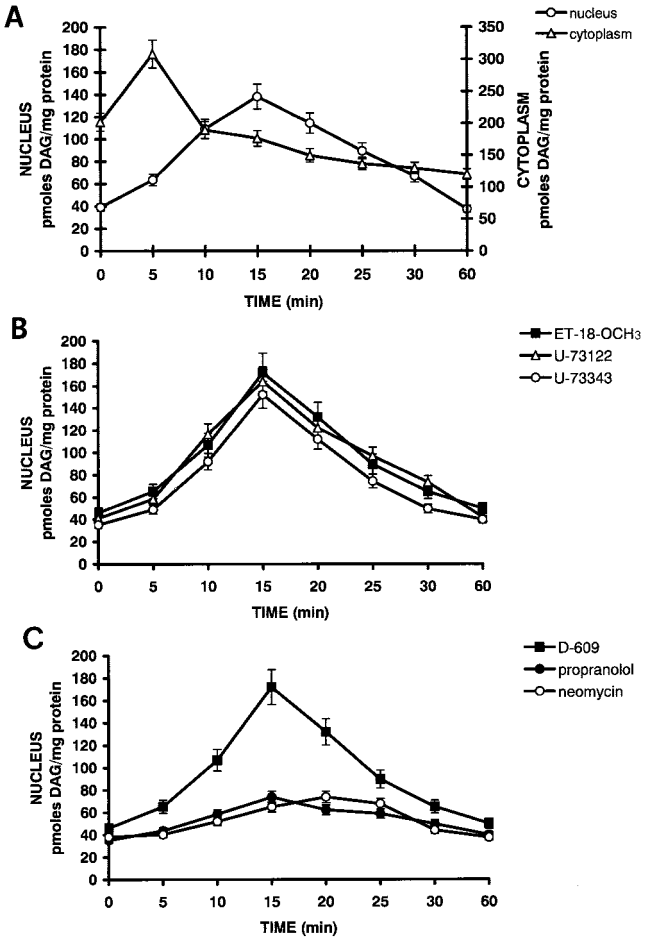

Figure 2. Effect of DMSO stimulation on the genesis of DAG. (A) Time course of changes in DAG concentration in the cytoplasmic fraction and in isolated nuclei prepared from HL-60 cells exposed to DMSO. (B and C) Effect of different inhibitors on DMSO-elicited nuclear DAG production. Cells were preincubated with the chemicals for 5 min before mitogenic stimulation. Each point represents the mean of three different experiments ± SD.

different times after DMSO administration. In the cytoplasmic fraction, the DAG levels increased significantly from a mean basal level of 201 ± 18.9 to 308 ± 32.5 pmol/mg protein by 5 min. Thereafter, the DAG mass fell progressively (Figure 2A). The response observed in isolated nuclei was similar, except for the fact that the maximal increase was higher (~4-fold) and delayed in time (at 15 min of stimulation) (Figure 2A). To identify the phospholipase activity involved in the nuclear DAG production after DMSO treatment of HL-60 cells, we used the same panel of inhibitors as described above. As presented in Figure 2B, ET-18-OCH_3, U-73122, and U-73343 were ineffective. On the contrary, either neomycin or propranolol, but not D-609, almost completely blocked the DMSO-dependent rise in nuclear DAG mass (Figure 2C).

In Vitro Nuclear Phospholipase Activities in Response to Either IGF-I or DMSO

Because the use of inhibitors strongly suggested the involvement of a PI-PLC in the nuclear DAG generation that follows exposure of HL-60 cells to IGF-I and of a PLD in the nuclear DAG rise measured after DMSO incubation, we next assayed these activities in nuclei isolated from cells at various times after either IGF-I or DMSO stimulation. For PI-PLC activity, we used HPLC analysis of the production of radiolabeled inositol (1,4,5) trisphosphate derived from [^{3}H]PtdIns (4,5) P_2, whereas for PLD we took advantage of an assay in which isolated nuclei, prepared from [^{3}H]palmitic acid-labeled cells, are incubated in vitro in the presence of ethanol. Under these conditions, detection of PLD activity is based on the formation of phosphatidylethanol, a product that is generated from PLD by a transphosphatidylation reaction when ethanol is present (Balboa *et al.*, 1995; Balboa and Insel, 1995). The

Table 2. In vitro activity of nuclear PLD and PI-PLC activities in response to either IGF-I or DMSO

Stimulus	PLD activity	PI-PLC activity
None	0.501 ± 0.061	18.5 ± 2.4
IGF-I 10 min	0.487 ± 0.059	44.4 ± 5.5
IGF-I 20 min	0.521 ± 0.065	43.1 ± 5.8
IGF-I 30 min	0.565 ± 0.071	42.4 ± 4.7
DMSO 10 min	1.23 ± 0.15	18.2 ± 2.1
DMSO 20 min	1.97 ± 0.22	19.5 ± 2.8
DMSO 30 min	1.75 ± 0.20	18.7 ± 1.9

HL-60 cells were exposed to either IGF-I or DMSO for the indicated times. For PLD the data are expressed as the percentage of labeled nuclear phosphatidylethanol relative to total nuclear lipid radioactivity, whereas for PI-PLC they are expressed as nanomoles of radiolabeled inositol (1,4,5) triphosphate per milligram of nuclear protein. The data are the mean of three different experiments ± SD.

results are presented in Table 2. It is evident that IGF-I activated only PI-PLC activity, whereas DMSO stimulated only PLD activity.

Influence of Ethanol on DMSO-evoked Cytoplasmic or Nuclear DAG Mass Rise

As a further control, we assayed DAG mass in cytoplasmic or nuclear fraction of cells treated with a combination of DMSO plus ethanol. Ethanol acts as an alternate substrate in place of water for PLD and then inhibits DAG production (Burke *et al.*, 1999). As shown in Table 3, the presence of ethanol blocked the increase in both cytoplasmic and nuclear DAG mass that occurs in response to DMSO.

Identification of Nuclear PI-PLC Isoform Activated by IGF-I

Because isolated nuclei have been shown to contain a variety of PI-PLC isoforms (Bertagnolo *et al.*, 1997; D'Santos *et al.*, 1998; Martelli *et al.*, 1999b) we designed a series of experiments aimed at identifying the isozyme that is activated in response to IGF-I stimulation. In preliminary experiments we found, by Western blotting, that HL-60 cell nuclei contain the following PI-PLC isoforms: β1, β3, γ1, and γ2 (our unpublished data). To determine which (if any) of these isoforms was activated in response to IGF-I, nuclei were lysed and the lysates were subjected to immunoprecipitation with polyclonal antibodies specific for the various PI-PLC isozymes. Then residual PI-PLC activity was assayed in the supernatant of the immunoprecipitates. As shown in Figure 3, only the antibody to PI-PLC-β1 was capable of reducing to a significant extent the PI-PLC activity that was present in the supernatant of the nuclear lysates, in samples prepared from growth factor-stimulated cells [from 38.1 ± 3.7 to 9.6 ± 1.1 nmol of inositol (1,4,5) trisphosphate/mg protein/30 min of incubation]. Western-blotting analysis showed that each of the nuclear the PI-PLC isoforms was completely recovered in the respective immunoprecipitates (our unpublished data).

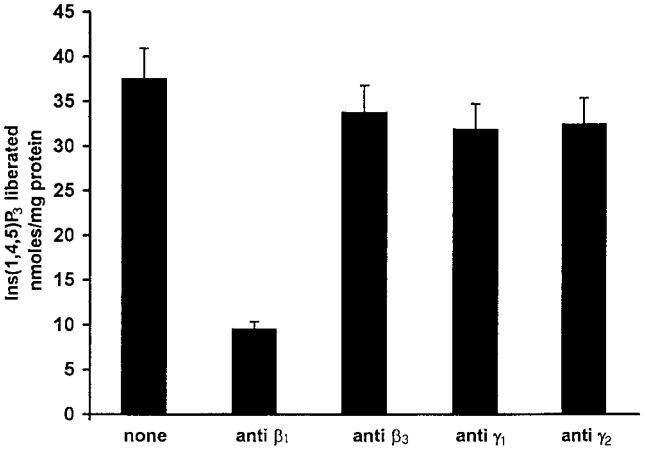

Figure 3. Residual soluble nuclear PI-PLC activity after immunoprecipitation with antibodies to the different isozymes in IGF-I–stimulated samples. Solubilized nuclear protein was incubated with affinity-purified antibodies to the various PI-PLC isoforms. Then the immunocomplexes were precipitated with protein A-Agarose and the supernatant, containing residual isoforms, was assayed for PI-PLC activity as described. Data are expressed as mean of three different experiments ± SD.

Table 3. Mass assay of cytoplasmic and nuclear DAG in response to DMSO plus 1.5% ethanol

HL-60 cells were exposed to a combination of 1.25% DMSO and 1.5% ethanol for the indicated times. DAG mass was then assayed in both the cytoplasmic and nuclear fraction. The data are expressed as picomoles of DAG per milligram of protein. The data are the mean of three different experiments ± SD.

	PLD activity	
Stimulus	Cytoplasm	Nucleus
None	127.6 ± 14.9	41.2 ± 5.7
5 min	117.8 ± 15.3	39.6 ± 6.1
10 min	123.5 ± 15.5	45.1 ± 5.6
15 min	NA	42.4 ± 4.5
20 min	NA	46.6 ± 4.7

NA, not assayed.

Mechanisms of Nuclear PLD Activation

We next moved to investigating the mechanism(s) by which DMSO could stimulate a PLD activity in the nucleus of HL-60 cells. We first performed Western-blotting analysis on nuclear immunoprecipitates. For both the immunoprecipitation and the detection we used an anti-pan PLD polyclonal antibody that recognizes the conserved sequence CIIGSANINERS (Horn *et al.*, 2001). With this technique it was possible to see that nuclei from unstimulated cells contained a protein with an apparent molecular mass of 90 kDa (Figure 4A, lane 1). The amount of this protein increased in response to a 15-min stimulation with DMSO and then decreased to basal levels (Figure 4A, lanes 2 and 3). Therefore, the increased nuclear PLD activity that follows HL-60 cell stimu-

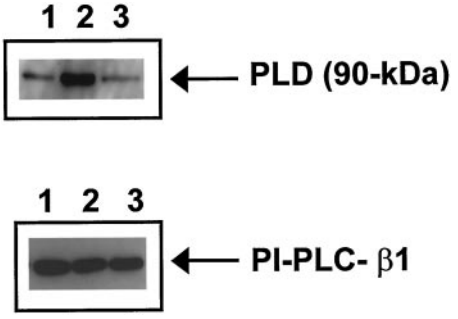

Figure 4. Western blot analysis of PLD and PI-PLC-βI in HL-60 cell nuclei. PLD: lane 1, control cells; lane 2, cells treated with DMSO for 15 min; and lane 3, cells treated with DMSO for 60 min. PI-PLC-βI: lane 1, control cells; lane 2, cells treated with IGF-I for 15 min; and lane 3, cells treated with IGF-I for 60 min.

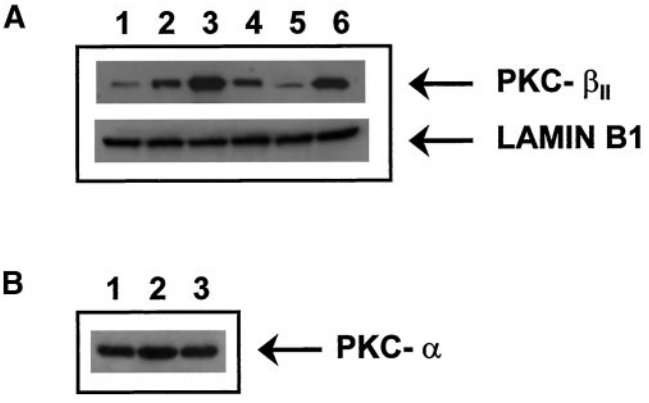

Figure 5. Western blot analysis of PKC-βII (A) and -α (B) in isolated nuclei prepared from IGF-I–stimulated HL-60 cells. Nuclear protein (80 μg) was separated on a 7% SDS-PAGE and transferred to nitrocellulose paper, which was then probed with antibodies to either PKC-βII or -α, and lamin B1. (A) Lane 1, control nuclei; lane 2, 15 min of IGF-I stimulation; lane 3, 30 min of IGF-I stimulation; lane 4, 60 min of IGF-I stimulation; lane 5, 30 min of IGF-I stimulation plus ET-18-OCH_3; and lane 6, 30 min of IGF-I stimulation plus propranolol. (B) Lane 1, control nuclei; lane 2, 15 min of IGF-I stimulation; and lane 3, 30 min of IGF-I stimulation. The data are representative of three separate experiments.

lation with DMSO is due to an increased amount of nuclear PLD protein. As an additional control, we verified whether the amount of nuclear PI-PLC-β1 also increased in response to IGF-I stimulation. However, as shown in Figure 4B, this was not the case. To further characterize the PLD activity in vitro we tested a series of molecules that are known to enhance or inhibit 90-kDa PLD. As shown in Table 4 the PLD activity was stimulated by the presence of PtdIns (4,5) P_2 and ATP, whereas it was inhibited by oleate and GTPγS, whereas guanosine-5′-*O*-(2-thio)diphosphate (GDPβS) was ineffective. In some cases, nuclei were lysed and in vitro PLD activity was assayed by measuring the levels of water-soluble head groups released from phosphatidyl-[*methyl*-[^{3}H]]choline. As presented in Table 4, also with this technique we determined that ATP was stimulatory, whereas GTPγS was inhibitory and GDPβS had no effect.

Behavior of PKC-βII after a Proliferating Stimulus

Because previous reports indicated that in HL-60 cells the PKC-βII isoform is selectively involved in nuclear events related to proliferation (Hocevar and Fields, 1991), we investigated whether such an isoform translocated to the nucleus after IGF-I exposure. Western blotting with an anti-PKC-βII-specific antibody demonstrated, in nuclei prepared from control cells, the constitutive presence of the kinase, as a band migrating at 78/80-kDa (Figure 5A, lane 1). IGF-I treatment of HL-60 cells caused a marked increase in the amount of nuclear PKC-βII protein, which was evident after 15 min and maximal after 30 min of stimulation. After 60 min, the amount of nuclear PKC-βII returned to control levels (Figure 5A, lane 4). ET-18-OCH_3 but not propranolol was capable of blocking the growth factor-dependent nuclear translocation of the enzyme (Figure 5A, lanes 5 and 6). As a control, we investigated the behavior of PKC-α. Also, this isoform was expressed in the nucleus of serum-starved cells, but its amount did not increase after stimulation with IGF-I both at 15 and 30 min (Figure 5B, lanes 1–3). Results obtained by means of visual inspections of the blots were corroborated by densitometric analysis (Table 5).

Changes in nuclear PKC-βII activity were monitored by immunoprecipitation of the enzyme and in vitro phosphorylation of exogenous histone H1. Again, Western-blotting analysis of the immunoprecipitate and of the supernatant showed PKC-βII to be exclusively present in the former. Moreover, no other PKC isoforms known to be present in the nucleus of HL-60 cells (PKC-α and -ζ; Zauli *et al.*, 1996) were detected in the immunoprecipitates (our unpublished data). After IGF-I stimulation, nuclear PKC-βII activity increased progressively, reaching its peak after 30 min with a nearly fivefold increase (Table 6). When the cells had been pretreated with ET-18-OCH_3 before stimulation, no increase in nuclear activity was seen in response to incubation with IGF-I. In contrast, both D-609 and propranolol did not affect nuclear PKC-βII activity.

Table 4. In vitro activity of nuclear PLD in the presence of various activators or inhibitors

HL-60 cells were exposed to DMSO for 20 min and the nuclei where then isolated and assayed for PLD activity. The data are expressed as either the percentage of labeled nuclear phosphatidylethanol relative to total nuclear lipid radioactivity or picomoles per minute per milligram of protein of choline release (Martelli *et al.*, 1999c). The data are the mean of three different experiments ± SD.

	PLD activity	
Addition	[^{3}H]Palmitic acid labeling	Choline release
None (basal activity)	2.08 ± 0.18	3,257 ± 376
PtdIns (4,5) P_2 20 μM	3.88 ± 0.39	NA
ATP 30 μM	2.57 ± 0.35	4124 ± 489
Oleate 1 mM	0.86 ± 0.092	NA
GTP-γ-S 50 μM	0.93 ± 0.012	1590 ± 256
GDP-β-S 50 μM	2.01 ± 0.19	3044 ± 338

NA, not assayed.

Table 5. Densitometric analysis of immunoblots showing intranuclear translocation of PKCβII or PKCα
Data are expressed in arbitrary units as a representative of three separate experiments ± SD.

Condition	PKC-βII	Condition	PKC-α
Control cells	2.1 ± 0.4	Control cells	3.5 ± 0.3
IGF-I 15 min	4.8 ± 0.7	DMSO for 10 min	7.9 ± 0.7
IGF-I 30 min	8.3 ± 1.1	DMSO for 15 min	10.5 ± 1.3
IGF-I 60 min	3.4 ± 0.5	DMSO for 60 min	3.8 ± 0.4
IGF-I 30 min + ET-18-OCH_3	2.4 ± 0.5	DMSO for 15 min + ET-18-OCH_3	10.8 ± 1.4
IGF-I 30 min + propranolol	7.8 ± 0.9	DMSO for 15 min + propranolol	3.4 ± 0.5

If PS, DAG, and Ca^{2+} were omitted from the reaction buffer we still measured a similar increase in PKC-βII activity, even although the absolute value of counts per minute were lower. This suggests that the increase in nuclear PKC activity was mostly due to the increase in the amount of PKC protein in the nucleus.

The distribution pattern of PKC-βII in response to IGF-I was analyzed by in situ immunocytochemistry followed by CLSM analysis performed using optical sections taken at the equatorial plane of nuclei. The nuclear compartment was identified by YO-PRO-1 staining of DNA. Immunostaining with anti-PKC-βII antibody in control cells showed the protein to be predominantly located in the cytoplasm, with tiny dots in the nucleus (Figure 6A). After 30 min, the immunoreactivity redistributed to the nuclear periphery and, to a lesser extent, to the nuclear interior (Figure 6, D–F), as demonstrated by merging of PKC with DNA labeling, which gave an orange-yellow color (Figure 6F). The amount of PKC immunofluorescence labeling in the cytoplasm was reduced and appeared as a very narrow red rim (Figure 6, D and F). Cells pretreated with ET-18-OCH_3, and then exposed to IGF-I up to 30 min, did not show any intranuclear migration and the bulk of PKC immunoreactivity remained in the cytoplasm (Figure 6, G–I). On the contrary, pretreatment with propranolol did not block PKC-βII translocation to the nucleus. In this case, the pattern of the merged signals was corresponding to that of control cells (Figure 6, L–N).

In addition, we performed a Z series of sections through both unstimulated and IGF-I–stimulated cells, to further demonstrate that the immunofluorescence signals were truly intranuclear. These results are presented in Figure 7, A and B. Whereas in control cells the immunofluorescence signal was mainly cytoplasmic (Figure 7A, a–f), in stimulated cells the labeling mainly accumulated in a ring corresponding to the nuclear periphery, which was visible at different levels of the sections through the nucleus (Figure 7B, a–f).

Behavior of PKC-α after a Differentiating Stimulus

The involvement of nuclear PKC-α during all-*trans*-retinoic acid-dependent differentiation of HL-60 cells has previously been described (Zauli *et al.*, 1996). Therefore, we investigated, by means of Western blotting, the behavior of PKC-α in DMSO-exposed HL-60 cells. In nuclei prepared from control cells, a band with an M_r of ~80/82 kDa was seen (Figure 8A, lane 1). After treatment with 1.25% DMSO there was a progressive increase in the amount of nuclear PKC-α, which was evident after 10 min and peaked after 15 min of stimulation (Figure 8A, lanes 2 and 3). Then the intranuclear amount of the isoform declined and it returned to control level by 60 min (Figure 8A, lane 4). This translocation still occurred if ET-18-OCH_3 was administered to the cells before DMSO stimulation, but was blocked by propranolol (Figure 8A, lanes 5 and 6). In contrast, the amount of nuclear PKC-βII was unaffected by incubation of HL-60 cells with DMSO at both 10 and 15 min (Figure 8B, lanes 1–3). Also, the densitometric analysis confirmed the impression resulting from the visual inspection of the blots (Table 5).

The PKC-α activity present in isolated nuclei was immunoprecipitated and assayed using exogenous histone H1. Also, Western-blotting analysis of the immunoprecipitate and of the supernatant showed PKC-α to be exclusively present in the former. Moreover, neither PKC-βII nor -ζ was

Table 6. Activity of immunoprecipitable nuclear PKC-βII after exposure of serum-starved HL-60 cells to IGF-I
Nuclei were isolated at the given times from HL-60 cells. The inhibitors were present in the tissue culture media starting from 5 min before exposure to IGF-I. For minus exogenous cofactors, PS, DAG, and Ca^{2+} were absent from the reaction buffer. Data are expressed as counts per minute per reaction and are the mean of three different experiments ± SD.

Time (min)	No inhibitor addition plus exogenous cofactors	No inhibitor addition minus exogenous cofactors	ET-18-OCH_3 plus exogenous cofactors	D-609 plus exogenous cofactors	Propranolol plus exogenous cofactors
0	1,254 ± 235	379 ± 38	1,073 ± 213	1,371 ± 288	1,117 ± 222
5	2,827 ± 367	903 ± 87	1,612 ± 287	2,777 ± 391	2,955 ± 393
15	4,688 ± 533	1,344 ± 141	1,744 ± 307	4,744 ± 547	4,571 ± 607
20	5,117 ± 569	1,632 ± 156	1,799 ± 301	5,233 ± 587	5,239 ± 620
30	5,895 ± 521	1,649 ± 163	1,713 ± 345	5,729 ± 612	5,957 ± 588
60	1,415 ± 258	477 ± 51	1,119 ± 256	1,516 ± 290	1,497 ± 244

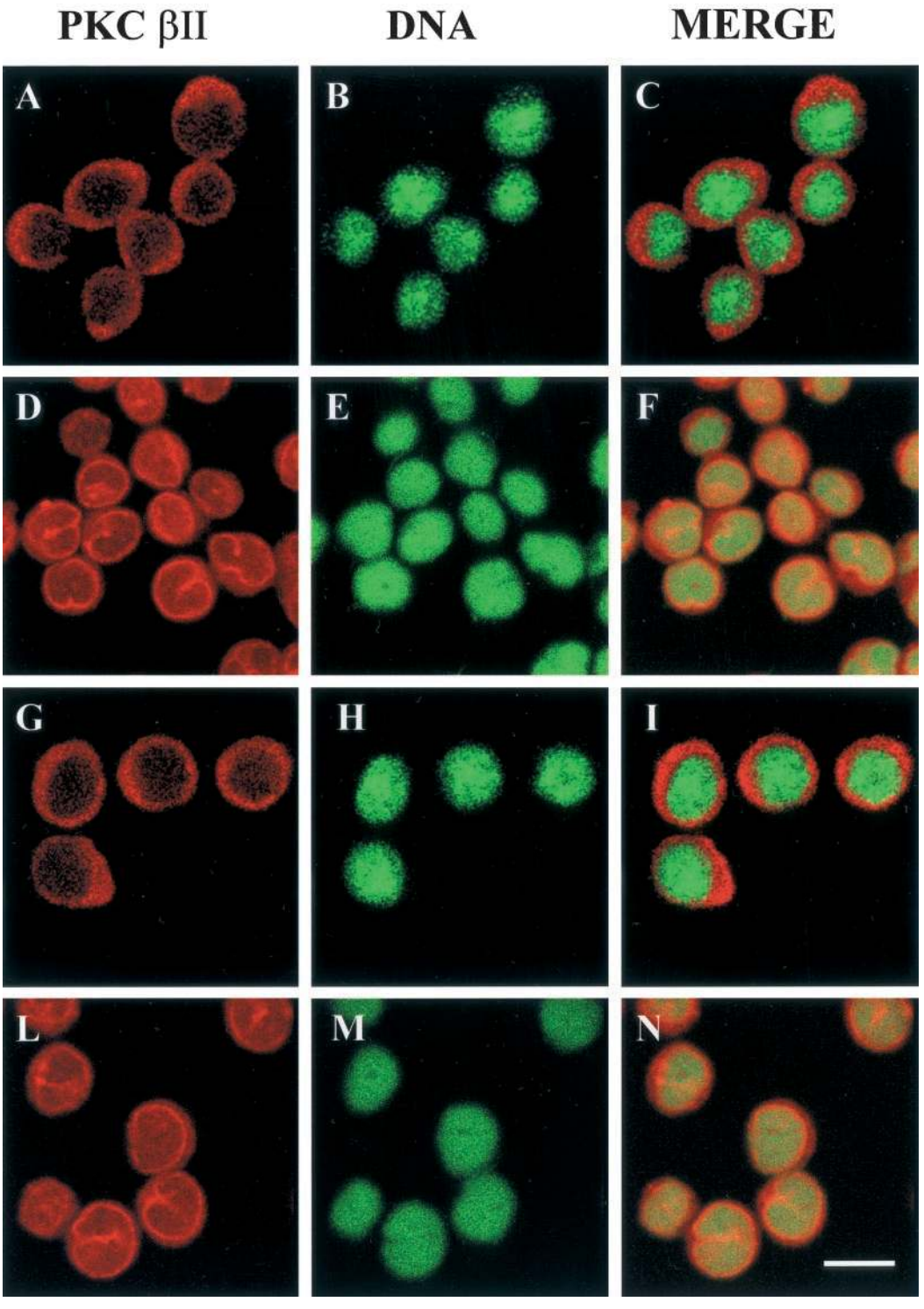

Figure 6. CLSM analysis of PKC-βII distribution in HL-60 cells. A single optical section through the equatorial plane of the nucleus is shown. (A, D, G, and L) Cy3 fluorescence identifies PKC-βII. (B, E, H, and M) YO-PRO-1 fluorescence stains the nucleus. (C, F, I, and N) Merging of the two signals. (A—C) Unstimulated cells. (D–F) Cells exposed to IGF-I for 30 min. (G–I) Cells pretreated for 5 min with ET-18-OCH_3 and then exposed to IGF-I for 30 min. (L–N) Cells pretreated for 5 min with propranolol and then exposed to IGF-I for 30 min. The data are representative of three separate experiments. In C and I, the faint PKC labeling of the nucleus is masked by the strong signal of YO-PRO-1. In F and N, the coincidence of Cy3 and YO-PRO-1 signals is shown by orange-yellow color. Bar, 10 μm.

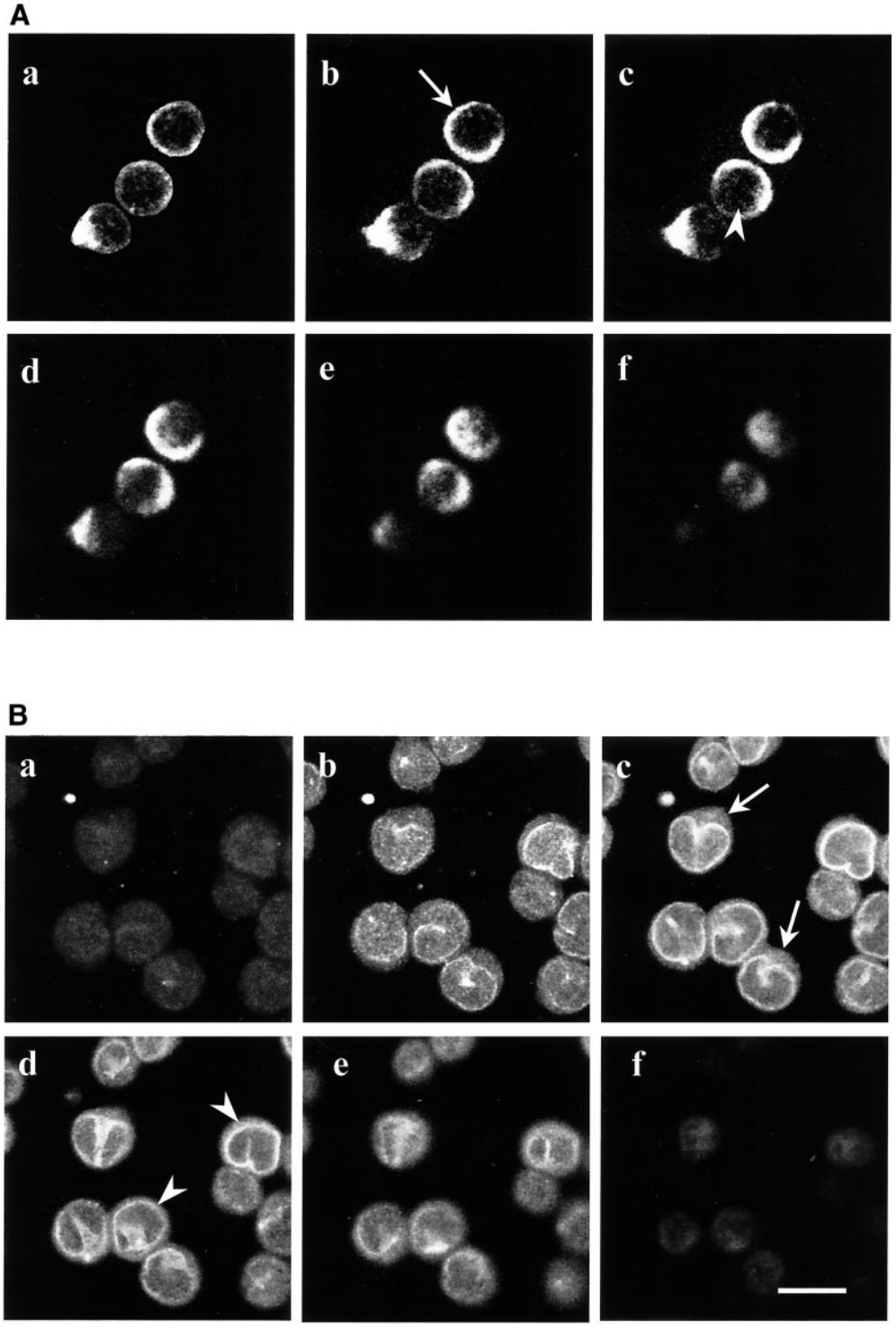

Figure 7. Section series through unstimulated (A) and IFG-I–exposed (B) HL-60 cells immunostained for PKC-βII. Sections were taken from bottom (a) to top (f), 1.2 μm apart. Note that in unstimulated cells cytoplasm is intensely immunostained (A, b, see arrow). Some tiny dots are visible within the nucleus (A, c, see arrowhead). In stimulated cells residual cytoplasmic staining is visible (B, c, see arrows) and it is evident the presence of a strong labeling at the nuclear periphery and of a weaker immunoreactivity in the nuclear interior (B, d, arrowheads). The first section of each series was taken at the lowest available level to avoid glass reflectance. Bar, 10 μm.

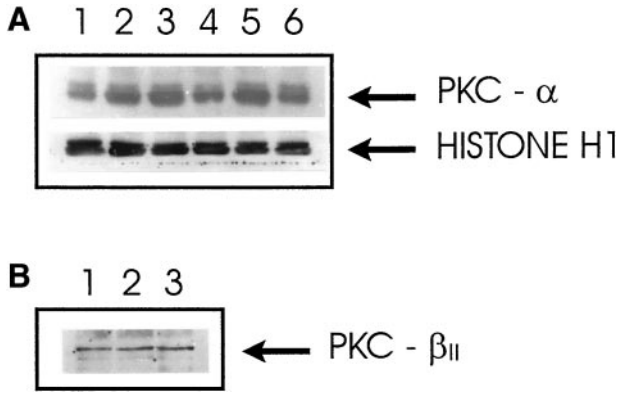

Figure 8. Western blot analysis of PKC-α (A) and -βII (B) in isolated nuclei prepared from DMSO-stimulated HL-60 cells. Nuclear protein (80 μg) was separated on a 10% SDS-PAGE and transferred to nitrocellulose paper, which was then probed with antibodies to either PKC-α or -βII, and histone H1. (A) Lane 1, control nuclei; lane 2, 10 min of DMSO stimulation; lane 3, 15 min of DMSO stimulation; lane 4, 60 min of DMSO stimulation; lane 5, 15 min of DMSO stimulation plus ET-18-OCH_3; and lane 6, 15 min of DMSO stimulation plus propranolol. (B) Lane 1, control nuclei; lane 2, 10 min of DMSO stimulation; and lane 3, 15 min of DMSO stimulation. The data are representative of three separate experiments.

detected in the immunoprecipitates (our unpublished data). Low levels of activity were detected in nuclei obtained from unstimulated cells (Table 7), in agreement with the results of the immunochemical experiments. However, in nuclei prepared from cells treated for 15 min with DMSO, a >3.5-fold increase in PKC-α activity was measured. This increase was markedly inhibited by propranolol but not by either ET-18-OCH_3 or D-609. If PS, DAG, and Ca^{2+} were not included in the reaction buffer, we still measured a comparable increase in PKC-βII activity, even although the absolute value of counts per minute was lower. This suggests that the increase in nuclear PKC-α activity was mostly due to the increase in the amount of PKC enzyme in the nucleus.

As far as immunocytochemical analysis by CLSM was concerned, PKC-α in control cells was also predominantly cytoplasmic, with some faint labeling within the nucleus (Figure 9, A–C). In DMSO-stimulated cells there was a translocation of the PKC to the nuclear interior (Figure 9D), as evidenced by the orange-yellow color in Figure 9F. Some immunoreactivity was still present in the cytoplasm and appeared as a red staining (Figure 9, D and F). Exposure of cells to ET-18-OCH_3 for 5 min before DMSO administration did not block nuclear translocation of PKC-α, which displayed a pattern similar to that seen in cells exposed to DMSO alone (Figure 9, G–I). On the contrary, pretreatment with propranolol abolished the recruitment of PKC-α to the nucleus (Figure 9, L–N).

Also, for PKC-α, a Z series of sections carried out through both unstimulated and DMSO-stimulated cells demonstrated an immunofluorescence signal really present within the nucleus. The results presented in Figure 10A showed the kinase mainly located in the cytoplasm (Figure 10A, a–f). At variance with PKC-βII, the staining of stimulated cells was homogeneously distributed in the nuclear region and was detectable at various levels of the sections through the nucleus (Figure 10B, a–f). Nevertheless, residual cytoplasmic immunoreactivity was evident.

Nuclear PKC-βII and -α Are Phosphorylated

Finally, we investigated whether nuclear PKC-βII and -α are phosphorylated on serine residues. To this end, we used polyclonal antibodies specific for PKC-βII phosphorylated on Ser-660 or for PKC-α phosphorylated on Ser-657. By immunoblotting analysis (Figure 11), it was possible to see that the amount of the nuclear phospho-PKCs rose in a dramatic way in response to stimulation with IGF-I (for PKC-βII) or DMSO (for PKC-α). Nevertheless, by reprobing the immunoblots with antibodies against total PKC-βII and -α, we also determined an increase in the total amounts of these isoforms.

DISCUSSION

The present study was designed to analyze some of the early events that may control proliferation or differentiation in HL-60 cells through the intranuclear translocation of different DAG-sensitive PKC isoforms. To this end, we used chemicals that act as selective inhibitors of PI-PLC or PC-PLC or interfere with the PLD-dependent pathway that yields DAG, because it is not possible to selectively radiolabel nuclear phospholipids (Raben *et al.*, 1994).

Table 7. Activity of immunoprecipitable nuclear PKC-α after exposure of HL-60 cells to DMSO

Nuclei were isolated at the given times from HL-60 cells. The inhibitors were present in the tissue culture media starting from 5 min before exposure to DMSO. For minus exogenous cofactors, PS, DAG, and Ca^{2+} were absent from the reaction buffer. Data are expressed as counts per minute per reaction and are the mean of three different experiments ± SD.

Time (min)	No inhibitor addition plus exogenous cofactors	No inhibitor addition minus exogenous cofactors	ET-18-OCH_3 plus exogenous cofactors	D-609 plus exogenous cofactors	Propranolol plus exogenous cofactors
0	3,315 ± 423	1,125 ± 127	3,201 ± 398	3,273 ± 377	3,015 ± 355
5	3,612 ± 444	1,290 ± 143	3,497 ± 435	3,377 ± 405	3,089 ± 400
10	8,446 ± 989	2,733 ± 321	8,519 ± 867	8,717 ± 1,009	3,984 ± 511
15	11,979 ± 1,234	4121 ± 396	11,003 ± 1276	11,345 ± 1,112	4,124 ± 524
30	6,239 ± 803	2134 ± 209	5,998 ± 756	6,099 ± 861	3353 ± 466
60	3,217 ± 456	997 ± 101	3,421 ± 407	3,555 ± 423	3,129 ± 338

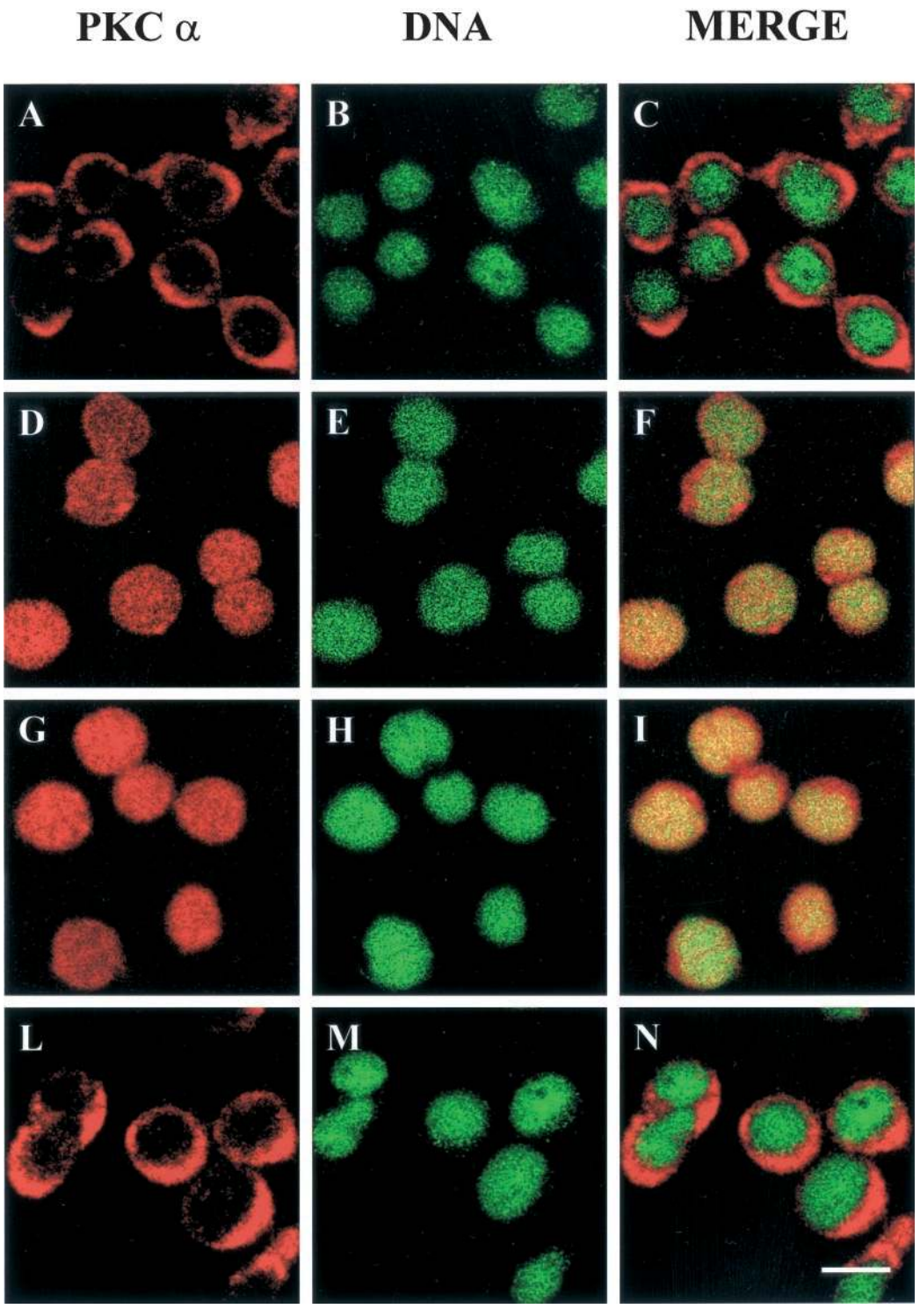

Figure 9. CLSM analysis of PKC-α distribution in HL-60 cells. A single optical section through the equatorial plane of the nucleus is shown. (A, D, G, and L) Cy3 fluorescence corresponds to PKC-α. (B, E, H, and M) YO-PRO-1 fluorescence identifies the nucleus. (C, F, I, and N) Merging of the two signals. (A–C) Unstimulated cells. (D–F) Cells exposed to DMSO for 15 min. (G–I) Cells pretreated for 5 min with ET-18-OCH_3 and then exposed to DMSO for 15 min. (L–N) Cells pretreated for 5 min with propranolol and then exposed to DMSO for 15 min. The data are representative of three separate experiments. In C and N, the faint labeling of the nucleus is masked by the strong signal of YO-PRO-1. In F and I, the coincidence of Cy3 and YO-PRO-1 signals is shown by orange-yellow color. Bar, 10 μm.

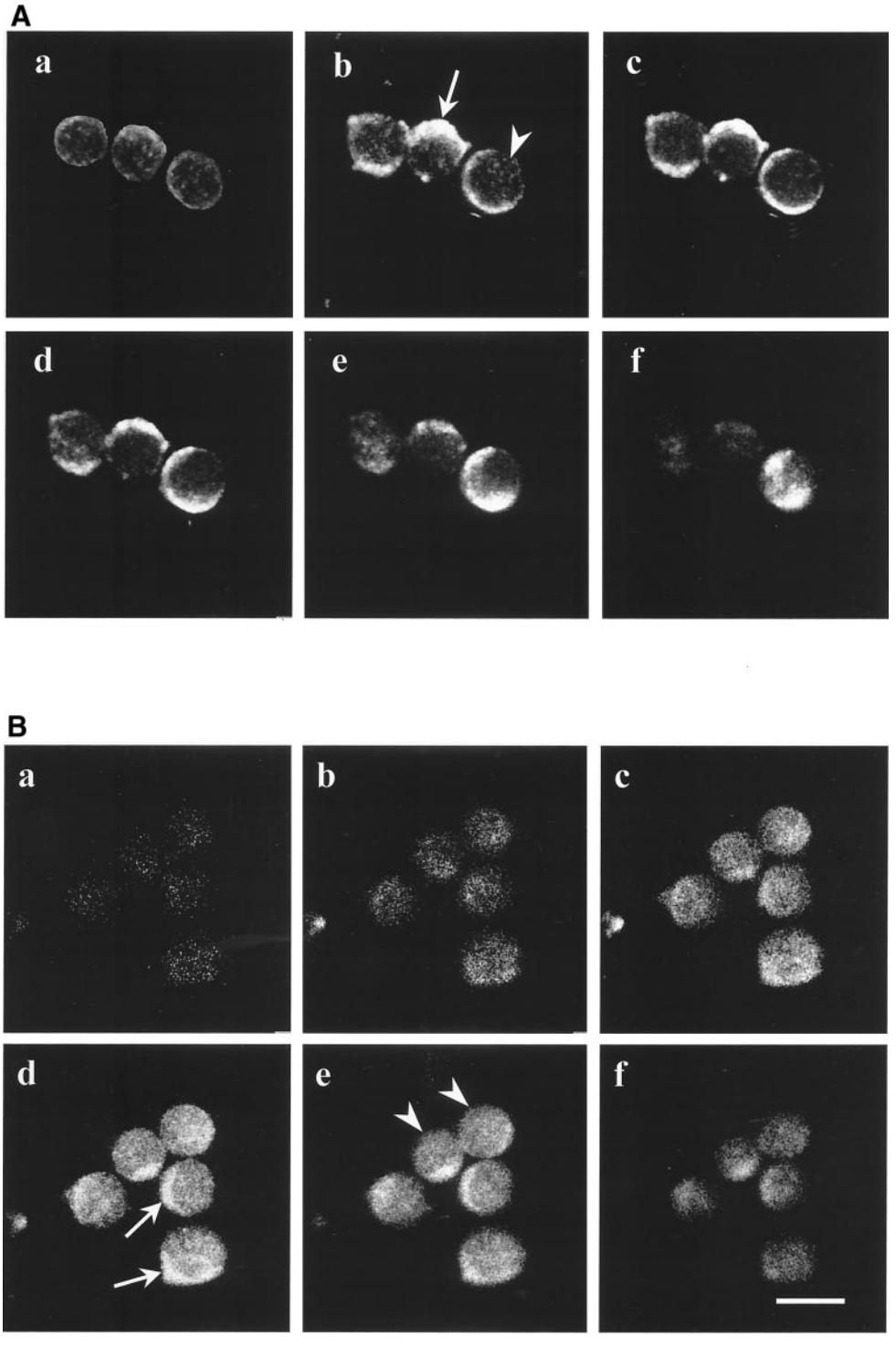

Figure 10. Section series through unstimulated (A) and DMSO-exposed (B) HL-60 cells immunostained for PKC-α. Sections were taken from bottom (a) to top (f), 1.2 μm apart. Note that in control cells a bright PKC immunoreactivity is mainly confined to the cytoplasm (A, b, see arrow). In the nuclear compartment some faint tiny dots are visible (A, b, see arrowhead). In DMSO-stimulated cells a residual immunostaining is present in the cytoplasm (B, d, see arrows), whereas the nuclear interior appears to be much more intensely decorated than in unstimulated cells (B, e, see arrowheads). The first section of each series was taken at the lowest available level to avoid glass reflectance. Bar, 10 μm.

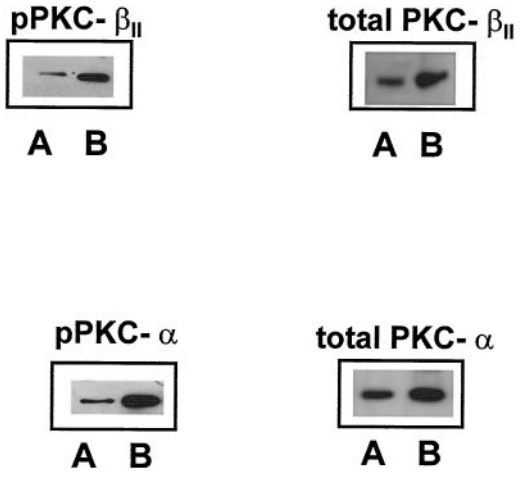

Figure 11. Western blotting analysis showing the presence of either phosphorylated PKC-βII (pPKC-βII) and -α (pPKC-α) or total PKC-βII and -α in nuclei from HL-60 cells. pPKC-βII and total PKC-βII: lane A, control cells; lane B, cells treated with IGF-I for 15 min. pPKC-α and total PKC-α: lane A, control cells; lane B, cells treated with DMSO for 15 min.

Our results demonstrated that two inhibitors of PI-PLC led to inhibition of the increase in the mass of nuclear DAG that follows IGF-I stimulation of quiescent, serum-starved HL-60 cells. On the other hand, treatment of cells with D-609, neomycin, or propranolol did not result in any appreciable increase in the mass of nuclear DAG, thus suggesting that PC-PLC or PLD are not responsible for the measured rise. No changes in DAG levels were detected in the cytoplasmic fraction. The results provided by DAG mass assays are in agreement with the data provided by in vitro measurement of PI-PLC activity in isolated nuclei. Indeed, the rise in the production of nuclear DAG in vivo was paralleled by an increase in nuclear PI-PLC activity in vitro. These findings fit well with our own previous findings showing that IGF-I is capable of inducing a PtdIns (4,5) P_2-derived DAG increase in quiescent 3T3 cells (Neri *et al.*, 1998). In this model system, several lines of evidence indicate that the β1 isoform of PI-PLC is activated in response to IGF-I treatment (Martelli *et al.*, 1992; Billi *et al.*, 1997). We established that also in HL-60 cells, IGF-I stimulation results in the activation of nuclear PI-PLC-β1. Exposure of HL-60 cells to the differentiating chemical DMSO caused a significant, rapid, and transient increase in cytoplasmic DAG, which was followed on a minute time scale by a more pronounced DAG rise at the nuclear level. Using the same panel of pharmacological inhibitors, we could assess that in this case nuclear DAG derived exclusively through the action of a PLD. Measurement of in vitro activity of nuclear PLD corroborated these results. We established that DMSO treatment increased the amount of a 90-kDa PLD. The existence of this PLD form has recently been demonstrated by others (Horn *et al.*, 2001). Consistent with their results, we found that nuclear basal PLD activity was enhanced by PtdIns (4,5) P_2 and ATP, whereas it was inhibited by oleate and GTPγS. GDPβS was ineffective. These results were somehow unexpected, because GTPγS has been described as an activator of PLD activity (Liscovitch *et al.*, 2000). Because this effect was also seen if nuclei were lysed and incubated with an exogenous substrate and considering also that GDPβS had no effect, we feel it may be due to a direct interaction of GTPγS with PLD and not mediated by a G protein. The interaction appeared very specific because GDPβS was not inhibitory. Conceivably, the effect of both ATP and GTPγS is related to their stereochemical configuration. This 90-kDa PLD is distinct from both PLD1 and PLD2. Indeed, the activity of PLD1 is known to be stimulated by PKC-α, the small GTPases Rho and ARF-1, as well as PtdIns (4,5) P_2, whereas PLD2 activation depends in part on PtdIns (4,5) P_2 (Liscovitch *et al.*, 2000). Another major mammalian PLD form, still awaiting cloning, is oleate-dependent PLD (Exton, 1999; Liscovitch *et al.*, 1999). Previous investigations, exclusively based on the sensitivity to stimulatory or inhibitory cofactors, have indicated that conceivably the nucleus may contain different types of PLD, including PLD1, PLD2, and oleate-dependent PLD (reviewed by Martelli *et al.*, 1999b). Very recently, Freyberg *et al.* (2001) have shown by immunological techniques that in GH_3 or NRK cells PLD1 was localized in the nucleus. However, we did not find PLD1 or PLD2 associated with the nuclear fraction of HL-60 cells (our unpublished data). It is likely that these differences are dependent on the cell line being investigated.

The more marked increase in nuclear DAG after a differentiating rather than a proliferating stimulus could be related to the fact that PC is much more abundantly represented in the nucleus than phosphoinositides (D'Santos *et al.*, 1999). The rapid increase in the cytoplasmic DAG mass elicited by DMSO is consistent with the findings obtained by other investigators in tumor cell lines. For example, a rapid rise (within 5 min) in DAG content was induced in mouse erythroleukemia cells by hexamethylene bisacetamide, a polar/planar compound with properties similar to DMSO (Michaeli *et al.*, 1992). Moreover, Clejan *et al.* (1996), who studied N1E-115 rat neuroblastoma cells, reported an early rise in DAG production derived from inositol lipids, subsequently followed by DAG originating from PC. There are also conflicting reports suggesting that DMSO caused an early decrease in inositol lipid turnover and DAG production in mouse erythroleukemia cells (Faletto *et al.*, 1985; Kuramochi *et al.*, 1990). However, it should be underlined that the first time point examined by Faletto *et al.* (1985) was already 30 min after the beginning of incubation with DMSO. Therefore, our data, showing a rapid rise in DAG, are not necessarily in contrast with theirs.

As far as nuclear DAG in hematopoietic cell lines is concerned, it should be noted that in the murine target cell line B6Sut.EP, erythropoietin (EPO) induced a fivefold increase in the mass of nuclear DAG as well as translocation of PKC-βII (Mallia *et al.*, 1997). At present, no information is available regarding the source of nuclear DAG in this model system, although when DAG species extracted from whole B6Sut.EP cells were analyzed, EPO appeared to stimulate both PI-PLC and PLD (Beckman *et al.*, 1996). The issue of nuclear DAG during DMSO-induced differentiation of mouse erythroleukemia cells has been examined by Divecha and coworkers. In Divecha *et al.* (1995), the analysis was started at 24 h from the induction and they claimed that differentiation led to a progressive decrease in the mass of nuclear DAG, detected first after 48 h. This decrease was

accompanied by a drop in the activity of nuclear PI-PLC. Subsequently, however, they showed that the activity of this nuclear PI-PLC did not change along erythroid differentiation. Thus, they interpreted the drop in DAG mass as a consequence of a down-regulation of nuclear PC turnover (D'Santos *et al.*, 1999). Our present results and the above-cited literature demonstrate that either PtdIns (4,5) P_2- or PC-derived DAG is capable of attracting specific PKC isozymes to the nucleus. One example is represented by the stimulation of IIC9 fibroblasts with α-thrombin, which led to a fourfold increase in nuclear DAG levels and a 10-fold increase in total nuclear PKC-α activity (Jarpe *et al.*, 1994). By a technique involving an acute labeling of the cells with [^{3}H] myristic acid, it was concluded that DAG conceivably derived, at least in part, from PC hydrolysis (Jarpe *et al.*, 1994). However, others (Pettitt *et al.*, 1997) have shown that in porcine aortic endothelial cells DAG, produced as a result of PLD activation, does not appear to act as a regulator of PKC at the plasma membrane level. These controversial results point to the likelihood of a different regulation of lipid-dependent signaling pathways in the nuclear compartment versus the plasma membrane. DAG derived from PtdIns (4,5) P_2 is capable of recruiting to the nucleus either PKC-α (Neri *et al.*, 1998) or -βII (Sun *et al.*, 1997). Nevertheless, DAG, derived through PLD activation, has been demonstrated to drive to the nuclear compartment mostly the PKC-α isozyme (Jarpe *et al.*, 1994; Martelli *et al.*, 1999a). Therefore, other investigations are necessary to clarify is the fatty acid composition of nuclear DAG may have a selective effect on specific isoforms of the PKC family.

A growing body of evidence suggests that translocation to the nucleus of different PKC isoforms plays an important role in the mechanisms that regulate cell differentiation and proliferation. In particular, several studies have indicated in PKC-βII or -α, the isozymes responsible for mediating the nuclear response of hematolymphopoietic cells stimulated to proliferate or differentiate, respectively (reviewed by Martelli *et al.*, 1999c). A selective PKC-βII translocation to the nucleus of HL-60 and K562 leukemia cells treated with bryostatin1, a compound that stimulates continuous proliferation, was described by Fields and coworkers (Hocevar and Fields, 1991; Hocevar *et al.*, 1992). The levels of PKC-βII also correlated with the proliferating state of K562 cells, being lost when cells underwent megakaryocytic differentiation in response to phorbol esters. Furthermore, cell proliferation was blocked when PKC-βII expression was inhibited by specific antisense oligonucleotides (Murray *et al.*, 1993).

On the other hand, a variety of differentiating stimuli have been reported to induce nuclear translocation of PKC-α. One of the first biochemical events for a developmental decision in primary cultures of granulocyte/macrophage colony-forming cells is represented by a stimulation of nuclear translocation of PKC-α, after exposure to either macrophage colony-stimulating factor or interleukin-4 (Whetton *et al.* 1994; Nicholls *et al.*, 1995). In response to vitamin D_3, a rapid nuclear translocation of PKC-α was seen in human acute promyelocytic leukemia NB4 cells, a cell line that differentiates into monocytes when exposed to various inducers (Berry *et al.*, 1996). Immunochemical and immunocytochemical investigations indicated that the PKC-α isozyme accumulated within the nucleus of HL-60 cells committed to granulocytic differentiation by all-*trans*-retinoic acid (Zauli *et al.*, 1996). In mouse erythroleukemia cells induced to hemoglobin synthesis in response to hexamethylene bisacetamide, PKC-α was found associated to the nucleus after 24 h of treatment and was absent at 96 h. When cells were transfected with PKC-α cDNA in antisense orientation, differentiation was blocked, suggesting an important role for nuclear PKC-α localization in this process (Mallia *et al.*, 1999). Interestingly, $CD34^+$ cells from human bone marrow, stimulated with EPO, showed a rapid and transient nuclear translocation of both PKC-α and PKC-βII, but not of PKC-ε (Myklebust *et al.*, 2000). This result is not in contrast with our findings and the literature, because EPO is known to be a hormone with both proliferating and differentiating effects on hematopoietic progenitor cells (Muta *et al.*, 1994). A novel finding we report in this article is that both nuclear PKC-βII and PKC-α were phosphorylated on serine residues. It is now established that serine phosphorylation at a conserved carboxyl-terminal motif of some PKC isoforms is very important to transform the kinase into the mature, cofactor-responsive enzyme. PKC must first be processed by three distinct phosphorylation events before it is competent to respond to second messengers (Keranen *et al.*, 1995; Tsutakawa *et al.*, 1995). As far as PKC-α is concerned, its autophosphorylation at Ser-657 controls the accumulation of phosphate at other sites on the kinase, as well as contributes to the maintenance of the phosphatase-resistant conformation (Bornancin and Parker, 1997). Regarding PKC-βII, Ser-660 phosphorylation causes a 10-fold increase in the enzyme's affinity for PS and Ca^{2+} (Edwards and Newton, 1997). As far as we know, this is the first report showing that PKC isoforms migrated to the nucleus are phosphorylated. In any case, the increase in in vitro PKC activity that we measured in the nuclei of HL-60 cells treated with IGF-I or DMSO is due to an increase in the amount of PKC protein in the nucleus, because a similar increase was measured even if PKC activity was assayed without exogenously added activators. However, because these PKC isoforms were phosphorylated, it is likely that in vivo they are active and capable of regulating functions critical for cell proliferation or differentiation.

Overall, we feel that we have defined some of very early intranuclear events that are critical for the attraction of DAG-dependent PKC isoforms in HL-60 cells subjected to either proliferating or differentiating stimuli. This knowledge might prove to be of great interest also for cancer therapy, given the fact that PKC-dependent signaling pathways are increasingly being seen as a pharmacological target in some forms of neoplastic disease (Parker, 1999; Watters and Parsons, 1999).

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