THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 45, Issue of November 6, pp. 29738–29744, 1998
Printed in U.S.A.

Nuclear Diacylglycerol Produced by Phosphoinositide-specific Phospholipase C Is Responsible for Nuclear Translocation of Protein Kinase C-α*

(Received for publication, June 15, 1998, and in revised form, August 6, 1998)

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It is well established that an independent inositide cycle is present within the nucleus, where it is involved in the control of cell proliferation and differentiation. Previous results have shown that when Swiss 3T3 cells are treated with insulin-like growth factor-I (IGF-I) a rapid and sustained increase in mass of diacylglycerol (DAG) occurs within the nuclei, accompanied by a decrease in the levels of both phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5-bisphosphate. However, it is unclear whether or not other lipids could contribute to this prolonged rise in DAG levels. We now report that the IGF-I-dependent increase in nuclear DAG production can be inhibited by the specific phosphatidylinositol phospholipase C inhibitor 1-*O*-octadeyl-2-O-methyl-*sn*-glycero-3-phosphocholine or by neomycin sulfate but not by the purported phosphatidylcholine-phospholipase C specific inhibitor D609 or by inhibitors of phospholipase D-mediated DAG generation. Treatment of cells with 1-*O*-octadeyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine or neomycin sulfate inhibited translocation of protein kinase C-α to the nucleus. Moreover, exposure of cells to 1-*O*-octadeyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine, but not to D609, dramatically reduced the number of cells entering S-phase upon stimulation with IGF-I.

These results suggest that the only phospholipase responsible for generation of nuclear DAG after IGF-I stimulation of 3T3 cells is PI-PLC. When this activity is inhibited, neither DAG rise is seen nor PKC-α translocation to the nucleus occurs. Furthermore, this PI-PLC activity appears to be essential for the G_0/G_1 to S-phase transition.

Upon stimulation by more than one hundred signaling molecules, the hydrolysis of a minor membrane phospholipid, PIP_2[1] by a specific PI-PLC rapidly occurs (1). PIP_2 breakdown leads to the generation of two well characterized second messengers: IP_3, which is responsible for the release of Ca^{2+} from intracellular stores (2), and DAG, which activates some PKC isoforms (3). However, DAG can also derive from PC, through the action of PLC or PLD (4). In fact, PLC is reactive with selective PC to produce DAG and *p*-choline (5). Nevertheless, it should be emphasized that the evidence for the stimulation of this pathway in mammalian cells remains mostly circumstantial and controversial, because so far no PC-PLC activity has been identified *in vitro* (6). In this connection, it should be pointed out that recent work by Luberto and Hannun (7) raises the possibility that sphingomyelin synthase at least in some cell types accounts for the putative mammalian PC-PLC. On the other hand, PLD catalyzes the hydrolysis of PC, thus generating phosphatidic acid, which can be dephosphorylated by phosphatidic acid phosphohydrolase to produce DAG (8, 9). Whereas DAG production deriving from PIP_2 hydrolysis occurs in an early and transient manner, DAG production from PC can be delayed and prolonged, thereby allowing a sustained activation of PKC. A prolonged elevation of DAG is frequently observed with long-acting signals, such as growth factors, cytokines, and phorbol esters (10).

Additional evidence has shown that a sustained rise in DAG levels is a prerequisite for long term cellular responses, such as proliferation and differentiation. However, it is not known whether early or continuous PKC activation is needed for a certain period of time in specific cell compartments during the cell cycle (4).

The enzymatic machinery that hydrolyzes PIP_2 has been shown to be present in the nucleus also, suggesting that it may be involved in second messenger production within this cell compartment (11–15). Treatment of Swiss 3T3 cells with IGF-I led to a sustained increase in intranuclear DAG and a decrease in the levels of PIP and PIP_2, whereas whole-cell levels of DAG showed no changes. This effect of IGF-I was opposite that of bombesin, because the latter had no effect on nuclear DAG and caused a rise in DAG measured in whole-cell extracts (16). Such an increase in nuclear DAG mass has been related to the translocation of PKC-α to this cell compartment (16–18). Subsequently, other investigations showed the existence of a relationship between mitogenic stimuli and changes in nuclear DAG mass: indeed, during compensatory hepatic growth, a peak in nuclear DAG levels was observed at 20 h following hepatectomy (19), and Sun *et al.* (20) demonstrated that nuclear DAG rises particularly at the G_2/M transition

* This work was supported by Associazione Italiana per la Ricerca sul Cancro 1996 and 1997 grants (to A. M. M. and S. C.) and by Italian Ministero Universita è Ricerca Scientifica 40% and 60% grants to the Universities of Trieste and Ferrara. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PI-PLC, phosphoinositide-specific PLC; PC-PLC, phosphatidylcholine-specific PLC; IP_3: inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; PC, phosphatidylcholine; PLD, phospholipase D; IGF-I, insulin-like growth factor-I; BrdUrd, bromodeoxyuridine; ET-18-OCH_3, 1-*O*-octadeyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate; NGS, normal goat serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MES, 2-(*N*-morpholino)ethanesulfonic acid; CLSM, confocal laser scanning microscope.

of the cell cycle.

Nevertheless, extracellular ligands, such as α-thrombin, could increase nuclear DAG derived, at least in part, from PC hydrolysis (21), which was followed by translocation of PKC-α to the nucleus in IIC9 cells (22). Moreover, the presence at the nuclear level of PLD has recently been demonstrated in different cell lines, such as Madin-Darby canine kidney and IIC9 cells (23–25).

In Swiss 3T3 cells treated with IGF-I, Divecha *et al.* (16) noted that the mass increase in DAG is greater than the combined loss in PIP and PIP_2, and they could not rule out a contribution to DAG from other lipids. Moreover, the rise in the nuclear DAG levels was sustained, hinting at the possibility that a phospholipase other than PI-PLC could be involved.

Therefore, we decided to analyze whether in Swiss 3T3 fibroblasts the nuclear elevation of DAG seen after stimulation with IGF-I derives from either PIP_2 or PC. In addition, we wanted to clarify whether or not PKC translocation to the nucleus may depend on DAG derived from either inositol lipids or PC because both DAG species have been shown to be capable of activating PKC *in vitro* (26).

Our results demonstrate that exclusively nuclear PI-PLC activity is responsible for a rise in DAG levels that is followed by PKC-α translocation to the nucleus. Indeed, DAG production and PKC-α translocation were blocked by a specific PI-PLC inhibitor, whereas chemicals that inhibit formation of DAG from PC were ineffective.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified minimum essential medium, fetal calf serum, 1,2-dioleyl-3-palmitoyl-glycerol, dioleylglycerol, CHAPS, phosphatidylserine, neomycin, NGS, peroxidase-conjugated anti-rabbit IgG, Cy3-conjugated anti-mouse and anti-rabbit IgG, 5′-BrdUrd, histone H1, leupeptin, aprotinin, propranolol, phenylmethylsulfonyl fluoride, polyclonal antibody to PKC-α, and BSA were from Sigma. Monoclonal antibody to 5′-BrdUrd was from Becton Dickinson (Milan, Italy). ET-18-OCH_3 and D609 were from Calbiochem (La Jolla, CA). IGF-I and the enhanced chemiluminescence detection kit were from Boehringer Mannheim. p81 paper was from Whatman (Maidstone, United Kingdom). [^{3}H]PIP_2 and [γ-^{32}P]ATP were from Amersham Pharmacia Biotech (Uppsala, Sweden). The protein assay kit (detergent compatible) was from Bio-Rad. The synthetic peptide RFARKGSLRQKNVHEVKN was from Upstate Biotechnology Inc. (Lake Placid, NY).

Cell Culture—Swiss 3T3 mouse fibroblasts were cultured in Dulbecco's modified minimum essential medium containing 10% fetal calf serum. Prior to stimulation, cells were subcultured at a density of 10^4 cells/cm and incubated until they became confluent (6 days). They were then cultured for an additional 24 h in serum-free medium containing 0.5% BSA. Quiescent cultures were washed twice with serum-free medium containing 0.2% BSA and then incubated in the same medium for the indicated times in the presence of 50 ng/ml of IGF-I. The various phospholipase inhibitors were present starting 5 min prior to simulation, at the following concentrations: ET-18-OCH_3, 100 μM; D609, 30 μM; neomycin, 1.2 mM; propranolol, 100 μM; ethanol, 1,3% (250 mM). For cell viability, the trypan blue exclusion test was performed. Briefly, cells were incubated for 15 min at room temperature in the presence of 0.2% Trypan Blue in Hanks' balanced salt solution. Positive cells were then scored .

Isolation of Nuclei—This was accomplished as previously reported (11). Briefly, cells (5×10^6) were suspended in 500 μl of 10 mM Tris-Cl, pH 7.8, 1% Nonidet P-40, 10 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin and aprotinin for 2 min at 0 °C. Then 500 μl of double-distilled H_20 was added, and the cells were allowed to swell for 2 min. Cells were sheared by 10 passages through a 22-gauge needle. Nuclei were recovered by centrifugation at 400 × *g* for 6 min and washed once in 10 mM Tris-Cl, pH 7.4, 2 mM $MgCl_2$, plus protease inhibitors as above.

Protein Assay—This was performed according to the manufacturer's instructions using the Bio-Rad protein assay (detergent-compatible).

Measurement of DAG Produced in Vivo—The assay was performed according to Divecha *et al.* (16), using DAG kinase enzyme purified from rat brain. DAG was extracted from nuclei, dissolved in 20 μl of CHAPS (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 magnesium 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; then, phosphatidic acid was extracted, chromatographed, and autoradiographed, and its radioactivity was counted in a liquid scintillation system (Betamatic IV, Kontron, Milan, Italy). Standard curves were obtained as reported by Divecha *et al.* (16), using 1,2-dioleyl-3-palmitoyl-glycerol as substrate.

PLC Activity Assay—The procedure outlined by Martelli *et al.* (11) was followed. Assays (100 μl) contained 100 mM MES buffer, pH 6.7, 150 mM NaCl, 0.06% sodium deoxycholate, 3 nmol [^{3}H]PIP_2 (specific activity 30,000 dpm $nmol^{-1}$), 10 μg of nuclear protein. Incubation was for 30 min at 37 °C. Hydrolysis was stopped by adding chloroform-methanol-HCl, and inositol phosphates were recovered by the aqueous phase were analyzed by high performance liquid chromatography, 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. For experiments in the presence of inhibitors, isolated nuclei were preincubated with the chemicals for 5 min on ice.

Detection of PKC-α by in Situ Immunofluorescence—Quiescent cultures of Swiss 3T3 cells were washed twice with serum-free medium containing 0.2% BSA and incubated in the same medium containing 50 ng/ml of IGF-I. Cells were then 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). The anti-PKC-α polyclonal antibody was 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 1:500. All incubations were carried out at 37 °C. Finally, the coverslips were mounted in glycerol containing 1,4-diazabicyclo[2.2.2]octane to retard fading, using additional coverslips as spacers to preserve the three-dimensional structure of cells.

CLSM and Image Processing Analysis—Samples were imaged by a LSM410 CLSM (Zeiss, Oberckochen, Germany). This confocal system was coupled with a 1 mW HeNe ion laser as light source, used to reveal the Cy3 signal with a 543-nm wavelength. 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.

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). To reduce the unwanted background noise generated by the photomultiplier signal amplification, all the image stacks were treated with a three-dimensional filter (Gaussian filtering) that was carried out on each voxel, with a mask of 3 pixels in the *x, y,* and *z* directions (3 × 3 × 3).

Photographs were taken by a digital video recorder Focus ImageCorder Plus (Focus Graphics, Foster City, CA) using 100 ASA TMax black and white film (Eastman Kodak Co.).

Western Blotting Analysis—Nuclear proteins separated by 8.0% SDS-polyacrylamide gel electrophoresis (27) were transferred to nitrocellulose sheets using a semidry blotting apparatus (Amersham Pharmacia Biotech, Uppsala, Sweden). 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 affinity-purified rabbit polyclonal antibody to PKC-α, diluted 1:2,000. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with peroxidase-conjugated anti-rabbit IgG, diluted 1:3,000 in PBS-Tween-20, and washed as above. Bands were visualized by the enhanced chemiluminescence method.

In Vitro Assay for Nuclear PKC Activity—Isolated nuclei (10 μg of protein) were incubated at 30 °C for 10 min in 20 mM Tris-Cl, pH 7.4, 10 mM $MgCl_2$, 10 μM ATP, 0.4 μg/ml histone H1, 5 μCi of [γ-^{32}P]ATP, in the presence of 1.2 mM $CaCl_2$, 40 μg/ml phosphatidylserine, and 3.3 μM dioleylglycerol. The reactions were terminated with 15 μl of acetic acid and spotted onto Whatman p81 paper, followed by washing with 0.75 mM H_3PO_4. Radioactivity was measured by Cerenkov counting. In some cases, instead of histone H1, 10 μg of the synthetic peptide RFARKGSLRQKNVHEVKN (corresponding to amino acids 19–36 of PKC) was employed as substrate. An equal volume of Laemmli's sample buffer was added, and proteins were separated using 18% SDS-polyacrylamide gel electrophoresis. Gels were then stained with Coomassie Blue R-250, dried, and autoradiographed. The radioactive bands were excised and counted using a liquid scintillation counter.

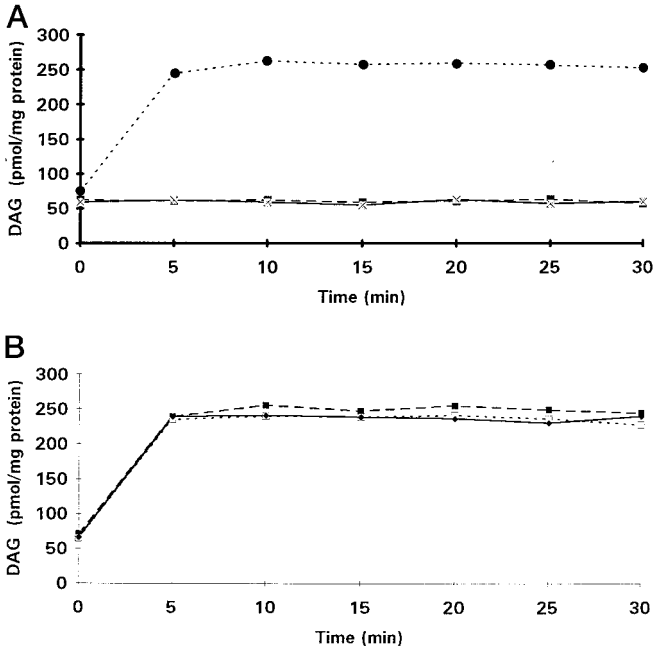

FIG. 1. **Time course of changes in DAG concentration in nuclei obtained from Swiss 3T3 cells.** Each point represents the mean from three different experiments ± an S.D. of 10%. Quiescent cells were stimulated with 50 ng/ml IGF-I for the indicated times. Cells were incubated with the chemicals for 5 min prior to mitogenic stimulation. *A,* ET-18-OCH_3 was employed at 100 μM, neomycin (*NEO*) at 1.2 mM. ●, C; ■, ET-18-OCH_3; ⊠, NEO. *B,* D609 was used at 30 μM, propranolol at 100 μM, and ethanol at 1.3%. ■, D609; □, propranolol; ♦, ethanol.

5′-BrdUrd Fluorescent Immunolabeling—Cells at 15 h after IGF-I stimulation were labeled with 100 μM 5′-BrdUrd for 10 min as described previously (28). Samples were fixed in freshly prepared 4% paraformaldehyde in PBS for 30 min at room temperature, and then DNA was denatured in 4 N HCl for 30 min and fixed in a −20 °C graded ethanol series to prevent reannealing. Slides were then air-dried and incubated for 3 h at 37 °C with a monoclonal antibody to 5′-BrdUrd diluted 1:10 in PBS, 2% BSA, and 3% NGS. Slides were then washed three times in PBS and reacted with a Cy3-conjugated anti-mouse IgG diluted 1:400 in PBS, 2% BSA, 5% NGS for 1 h at 37 °C. Samples were subsequently washed three times in PBS and mounted as detailed above.

Statistical Analysis—Data are the mean from three different experiments and are expressed in the tables as mean ± S.D. Significant differences ($p < 0.01$) in a Student's paired t test are indicated in the tables by an asterisk. All of the other differences were found to be not significant with $p > 0.01$.

RESULTS

Effect of Different Chemicals on Nuclear DAG Levels and PLC Activity after IGF-I Treatment—Nuclei were isolated from cells after different times of IGF-I stimulation and assayed for DAG levels. Control nuclei contained about 75 pmol/mg protein of DAG, but after only 5 min of mitogenic stimulation, this value rose to 245 pmol/mg protein, *i.e.* a 3.25-fold increase (Fig. 1*A*). This value was essentially maintained for the following 30 min, as shown in Fig. 1, in agreement with Divecha *et al.* (16), even though the method we have employed to isolate nuclei is different from theirs. However, after 60 min, nuclear DAG mass returned to basal levels (data not shown). To assess the nuclear phospholipase activity responsible for the DAG production that follows IGF-I stimulation of 3T3 cells, we employed inhibitors that interfere with several enzymes that may be involved in DAG formation. First, the specific PI-PLC inhibitor, ET-18-OCH_3 (100 μm) was used. As presented in Fig. 1*A*, exposure of intact cells for 5 min to this chemical resulted in a slight decrease in nuclear DAG levels (about 15%), to 63 pmol/mg protein. When cells exposed to this chemical were stimulated with IGF-I, no DAG rise was observed at any time (Fig. 1*A*). Similar results were obtained when the nonselective phospholipase inhibitor neomycin (1.2 mM) was employed (Fig. 1*A*). In contrast, 30 μM D609 (which is thought to be a PC-PLC specific inhibitor, even though so far no such mammalian enzyme has been shown to be sensitive *in vitro* to this chemical), as well as propranolol (a phosphatidic acid phosphohydrolase inhibitor, 100 μM) and 1.3% ethanol (which causes PLD to generate phosphatidylethanol rather than phosphatidic acid), did not affect the basal levels of nuclear DAG (Fig. 1*B*). Moreover, in these cases, the rise in nuclear DAG that followed IGF-I stimulation was comparable to that seen in controls (Fig. 1*B*). Next, we wanted to analyze whether changes in the nuclear DAG mass could also be abolished by different concentrations of the chemicals. Thus, cells were pretreated with the chemicals for 5 min and then stimulated for 15 min with IGF-I, the nuclei were isolated, and the nuclear DAG mass was measured. The results are presented in Table I. When used at 10 μM, ET-18-OCH_3 resulted in an approximately 45% decrease in the production of DAG, in good agreement with the IC_{50} of this chemical (20). When employed at 50 μM, the inhibition was 60%. No significant inhibitory effect of D609 was observed in a range of 10–30 μM.

TABLE I
Effect of different concentrations of PI-PLC and PC-PLC inhibitors on the production of nuclear DAG in vivo after 15 min of IGF-I stimulation.

Quiescent cells were treated for 5 min with the chemicals, and then stimulated with IGF-I. Nuclei were isolated, and DAG mass was assayed. The data are the mean ± S.D. from three separate experiments. An asterisk indicates values that are significantly different ($p < 0.01$) from control.

Inhibitor	DAG mass
	pmol/mg of nuclear protein
None (control)	255 ± 19
10 μM ET-18-OCH_3	141 ± 12*
50 μM ET-18-OCH_3	102 ± 9.6*
100 μM ET-18-OCH_3	60 ± 5.5*
10 μM D609	245 ± 21
20 μM D609	241 ± 20
30 μM D609	238 ± 20

We next assayed *in vitro* PLC activity in nuclei isolated from cells at various times after IGF-I stimulation using high performance liquid chromatography analysis of radiolabeled IP_3 derived from [^{3}H]PIP_2 hydrolysis.

As shown in Fig. 2*A*, the rate of IP_3 generation corresponded well with that of nuclear DAG. In fact, in control nuclei a nearly 4-fold increase was observed after 5 min of stimulation. Again, a plateau was present up to 30 min. On the contrary, when either ET-18-OCH_3 or neomycin sulfate were used, this PLC activity was reduced to negligible levels (Fig. 2*A*) for all the examined times, resulting in less than 10% of the original activity. The addition of either D609 or propranolol did not block IP_3 formation, which was similar to control nuclei (Fig. 2*B*).

Nuclear Translocation of PKC-α Is Dependent on DAG Levels—The increase in nuclear DAG described above has already been suggested to be the driving force that attracts PKC to the nucleus (16). However, no clear evidence has been presented thus far. Therefore, we investigated this issue using a PKC-α-specific antibody, because it has previously been demonstrated that IGF-I induces in Swiss 3T3 cells the selective nuclear translocation of this isoform (18). By means of CLSM, in quiescent cells PKC-α, was mainly detected in the cytoplasm and in the perinuclear region. Nuclei, identified by means of phase-contrast microscopy (data not shown) were immunolabeled to a much lower extent (Fig. 3*A*). Incubation of cells with IGF-I

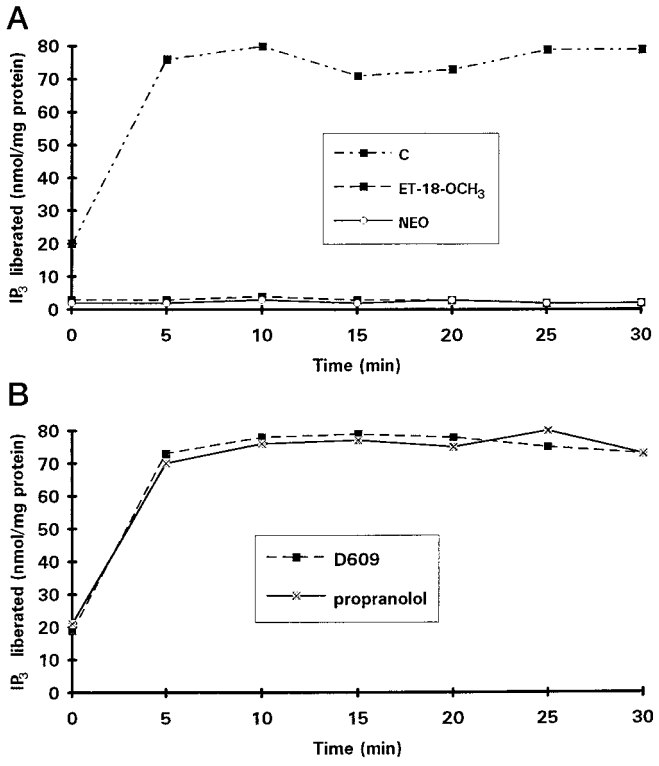

FIG. 2. **Time course of changes of IP_3 production in isolated nuclei obtained from Swiss 3T3 cells following stimulation with IGF-I.** Each *point* represents the mean from three different experiments ± an S.D. of 9%. Isolated nuclei were incubated with the chemicals for 5 min at 0 °C prior to [^{3}H]PIP_2 addition. *A,* ET-18-OCH_3 was employed at 100 μM and neomycin (*NEO*) at 1.2 mM. - - -■- - -, C; – – –■– – –, ET-18-OCH_3; —○—, NEO. *B,* D609 was used at 30 μM and propranolol at 100 μM. ■, D609; ☒, propranolol.

showed a progressive increase of PKC-α labeling in the nuclear interior starting from 5 min after IGF-I stimulation (data not shown) and reaching a maximum after 30 min (Fig. 3*B*). When Swiss 3T3 fibroblasts were preincubated with either ET-18-OCH_3 or neomycin, no nuclear translocation of PKC-α was observed (Fig. 3, *C* and *D*, respectively). On the other hand, if D609 or propranolol were administered to the cells 5 min before mitogenic stimulation with IGF-I, nuclear translocation of PKC-α was similar to that of control cells after 30 min of stimulation (see Fig. 3, *E* and *F*, and compare with Fig. 3*B*).

In Table II we report the percentage of cells displaying a nuclear translocation of PKC-α following IGF-I stimulation under the various conditions listed above. It is evident that exposure to either ET-18-OCH_3 or neomycin sulfate completely blocked nuclear translocation of the kinase. In contrast, chemicals interfering with DAG production from PC did not affect the accumulation of PKC within the nucleus.

We next investigated nuclear translocation of PKC-α by means of Western blotting analysis. In nuclei prepared from unstimulated cells, a band with a molecular mass of 80 kDa was seen (Fig. 4, *lane a*). After 30 min of treatment of Swiss 3T3 cells with IGF-I, there was a clear increase in the amount of nuclear PKC-α (Fig. 4, *lane b*). This effect was not evident when ET-18-OCH_3 or neomycin were administered to the cells prior to stimulation (Fig. 4, *lanes c* and *d*). The translocation of PKC-α to the nucleus induced by IGF-I was clearly visible when D609, propranolol, or ethanol were used prior to IGF-I exposure (Fig. 4, *lanes e–g*).

PKC Activity Assays in Isolated Nuclei—The PKC-α activity present in isolated nuclei was first assayed using histone H1 as substrate. Some activity was detected in nuclei from quiescent cells (Fig. 5*A*), in agreement with the results of both immunocytochemical and immunochemical experiments. However, in nuclei prepared from cells treated for 30 min with IGF-I, a more than 5-fold increase in PKC activity was measured. This increase was almost completely abolished by either ET-18-OCH_3 or neomycin sulfate, whereas D609, propranolol, and ethanol were ineffective (Fig. 5*A*). Similar results were obtained when the synthetic peptide RFARKGSLRQKNVHEVKN (corresponding to amino acids 19–36 of PKC) was employed as substrate instead of histone H1, as presented in Fig. 5*B*.

5′-BrdUrd Labeling Index—To assess the percentage of cells that entered S-phase of the cell cycle after treatment with IGF-I in the absence or presence of various chemicals, we used labeling with 5′-BrdUrd followed by fluorescent immunostaining. The results from these experiments are presented in Table III. We checked cell proliferation at 15 h after mitogenic stimulation because it has previously been reported that at this time after a mitogenic stimulus one can detect a higher number of cells that have entered S-phase than at previous or successive times (29). In quiescent cultures, no more than 2% of cells positive for 5′-BrdUrd were detected. IGF-I stimulation raised this value to 18%. The inhibition of PI-PLC activity by means of 50 μM ET-18-OCH_3 resulted in a marked decrease in the number of positive cells (6%). When D609 (20 μM) was employed, no effects on cell proliferation were evident: indeed, 17% of cells were 5′-BrdUrd-positive. It is important to emphasize that the chemicals we used did not significantly affect viability of 3T3 cells, as presented in Table III. Indeed, we found that 3T3 cells, at variance with HL-60 cells, are more resistant to the proapoptotic effect of ET-18-OCH_3, so that we could use it at 50 μM, whereas Sun *et al.* (20) were forced to employ it at 10 μM, during their prolonged incubations (12 h).

DISCUSSION

To clarify the mechanisms responsible for the increase in the nuclear DAG mass that follows IGF-I stimulation of Swiss 3T3 cells, it is important to identify the enzyme(s) responsible for this phenomenon. In a previous study, a small decrease in PIP and PIP_2 levels was observed, thus suggesting that the hydrolysis of PIs could not fully explain the increase in nuclear DAG levels caused by IGF-I (16) and supporting the idea of another phospholipid source. Because it is not possible to selectively radiolabel nuclear phospholipids, potential sources of nuclear DAG cannot be identified by analysis of the release of water-soluble radiolabeled headgroups from metabolically labeled cultures (30). Therefore, we took advantage of chemicals that either act as inhibitors of PI-PLC or PC-PLC or interfere with the PLD pathways that lead to the formation of DAG (20, 31–34).

Our data demonstrate that a specific inhibitor of PI-PLC blocked the increase in the mass of nuclear DAG that characteristically follows IGF-I stimulation of confluent 3T3 fibroblasts. It should be pointed out that in nuclei of quiescent cells exposed for 5 min to ET-18-OCH_3 or neomycin, we detected a small but reproducible decrease in the DAG levels. In our opinion, this suggests that PI-PLC is active in the nucleus even if no stimulus is given to the cells. On the other hand, treatment of cells with D609, propranolol, and ethanol did not result in any appreciable decrease in the mass of nuclear DAG, thus suggesting that PC-PLC and PLD are either not present or completely inactive in nuclei of these cells. Although so far, no evidence at all has been presented concerning the existence of a PC-PLC in the cell nucleus, several recent reports have demonstrated that nuclei prepared from either Madin-Darby canine kidney or IIC9 cells contain a PLD activity (21–25). However, it should be pointed out that this PLD activity was

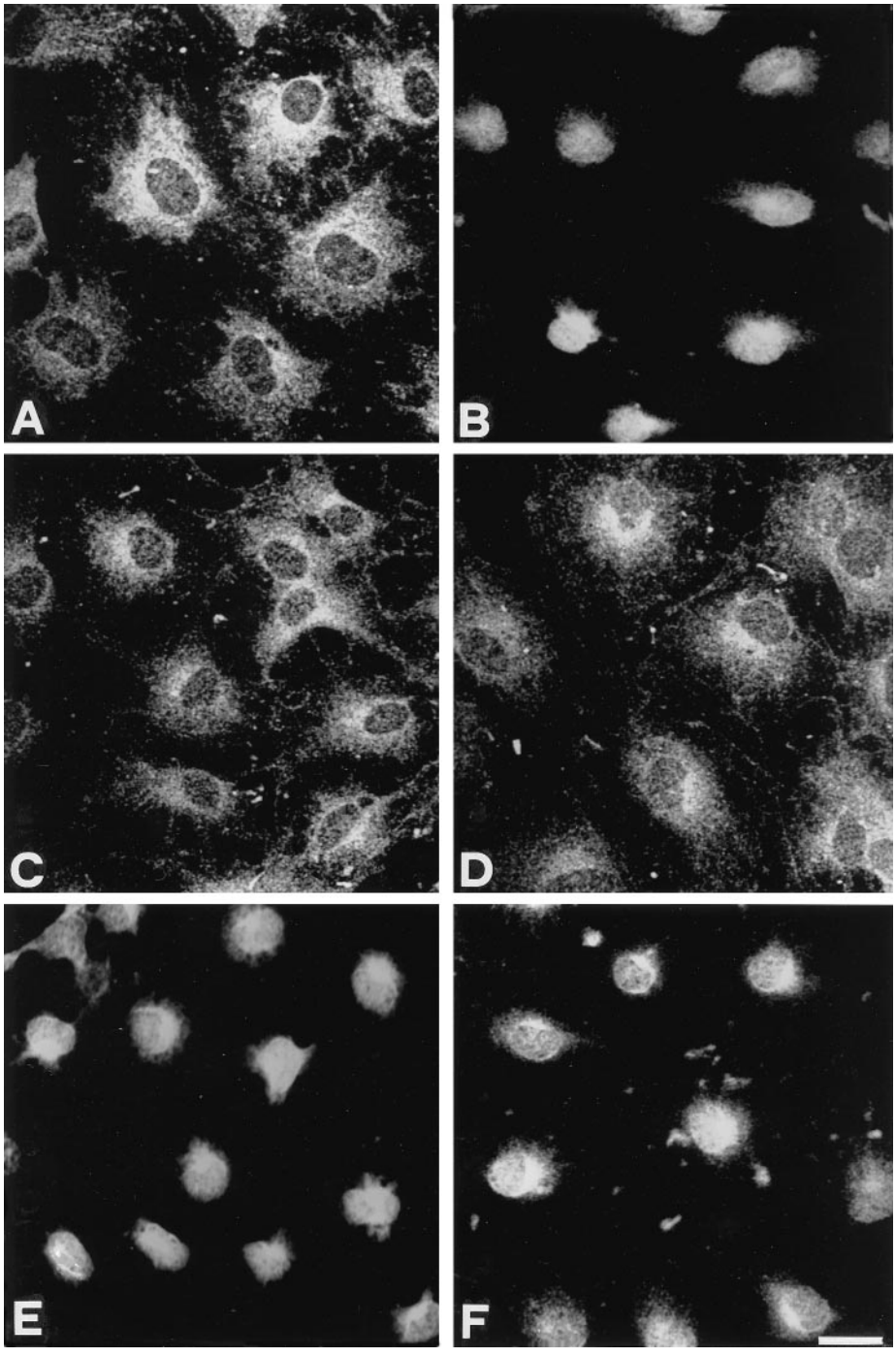

FIG. 3. **Single CLSM optical sections showing the subcellular distribution of PKC-α in Swiss 3T3 cells.** *A,* quiescent cells; *B,* 30 min of IGF-I stimulation; *C,* 30 min of IGF-I stimulation plus ET-18-OCH_3 (100 μM); *D,* 30 min of IGF-I stimulation plus neomycin (1.2 mM); *E,* 30 min of IGF-I stimulation plus D609 (30 μM); *F,* 30 min of IGF-I stimulation plus propranolol (100 μM). In *C* and *D,* it can be observed that chemicals inhibited nuclear translocation of PKC-α. *Bar,* 10 μm.

TABLE II

Percentage of cells showing nuclear translocation of PKC-α

For each experiment, 300 cells were counted. The data are the mean ± S.D. from three separate experiments. An asterisk indicates values that are significantly different ($p < 0.01$) from control.

Cell treatment	Cells with nuclear translocation
None (quiescent)	2 ± 1
IGF-I	38 ± 5*
IGF-I + ET-18-OCH_3 (100 μM)	5 ± 2
IGF-I + neomycin (1.2 mM)	4 ± 2
IGF-I + D609 (30 μM)	36 ± 4*
IGF-I + propranolol (100 mM)	35 ± 4*

observed in nuclei that retained their envelope, whereas our nuclear preparations did not, because they were obtained in the presence of Nonidet P-40. Thus, it may be that PLD activity is not present in detergent-treated nuclei, because it is located in the nuclear envelope. In contrast, several lines of evidence indicate that PI-PLC is present in the nuclear interior, where it associates with the nuclear matrix (35).

The results provided by DAG mass assays are in agreement with the data provided by *in vitro* measurements of PLC activity in isolated nuclei. Indeed, the rise in the production of nuclear DAG *in vivo* was paralleled by an increase of PI-PLC activity *in vitro*, which is due to the β1 isoform (11). The intranuclear amount of PLC-β1 did not increase following IGF-I treatment of 3T3 cells (11). PLC-β1 has also been detected in the nucleus of Swiss 3T3 cells by other investigators (36). Interestingly, its intranuclear levels did not change significantly through the cell cycle. This is at variance with another isoform, PLC-δ4, the expression of which in the nucleus is induced by mitogens and reaches a maximum at the S-phase (36).

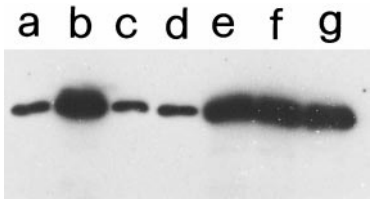

FIG. 4. **Western blot analysis of PKC-α in isolated nuclei prepared from 3T3 cells.** Protein from 1×10^6 nuclei was separated by 8% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper, which was then probed with a polyclonal antibody to PKC-α. *Lane a,* quiescent cells; *lane b,* 30 min of IGF-I stimulation; *lane c,* 30 min of IGF-I stimulation plus ET-18-OCH_3 (100 μM); *lane d,* 30 min of IGF-I stimulation plus neomycin (1.2 mM); *lane e,* 30 min of IGF-I stimulation plus D609 (30 μM); *lane f,* 30 min of IGF-I stimulation plus propranolol (100 μM); *lane g,* 30 min of IGF-I stimulation plus ethanol (1.3%). The band detected by enhanced chemiluminescence migrated with a molecular mass of 80 kDa.

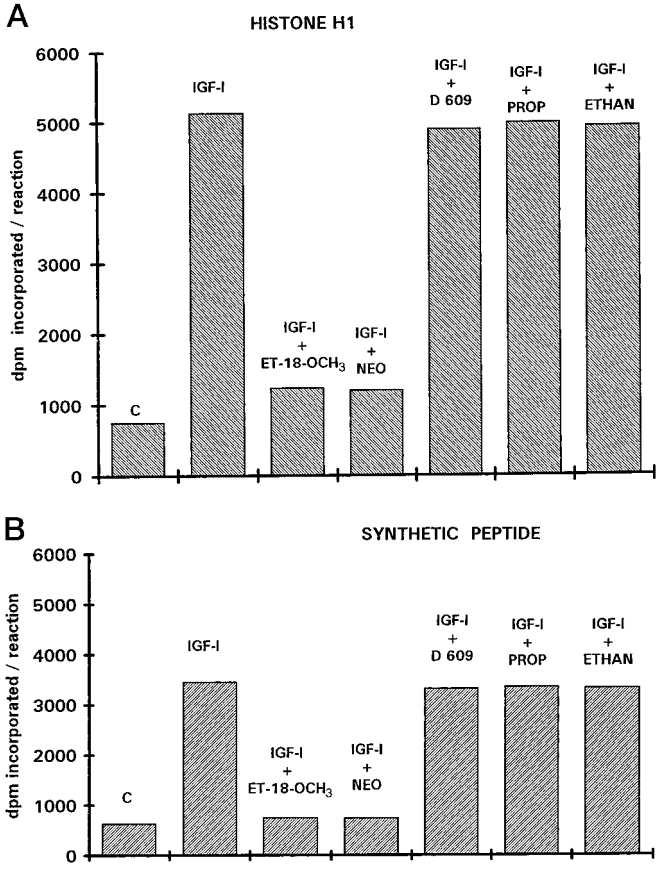

FIG. 5. **PKC activity assays in isolated nuclei using as substrate either histone H1 (*A*) or a synthetic peptide (*B*).** *C,* quiescent cells. *NEO,* neomycin; *PROP,* propranolol; *ETHAN,* ethanol. The chemicals were employed at the concentrations indicated under "Experimental Procedures." IGF-I stimulation was for 30 min. Each value represents the mean from three different experiments ± an S.D. of 12%.

The *in vitro* PLC activity was sensitive to ET-18-OCH_3 or neomycin but not to D609 or propranolol. Therefore, even if the observed decrease in PIP and PIP_2 mass did not account for all the increase in the amount of DAG (16), our results indicate that nuclear DAG produced in Swiss 3T3 cells in response to stimulation with IGF-I derived exclusively from inositol lipid hydrolysis. It might be that the methods employed by Divecha *et al.* (16) to measure the mass of nuclear inositol lipids led them to underestimate the decrease that follows IGF-I stimulation. PIP mass was assayed by incorporation of radioactivity from [γ-^{32}P]ATP using PIP-5-kinase partially purified from rat brain, whereas PIP_2 levels were measured by a chemical procedure whereby the fatty acids and glycerol are sequentially removed from the lipid and then the resulting IP_3 is assayed by a radioreceptor assay, as reported by Palmer and Wakelam (37).

TABLE III
Percentage of cells viable or positive to immunostaining for 5′-BrdUrd after 15 h of stimulation with IGF-I.

For each experiment, 300 cells were counted. The data are the mean ± S.D. from three separate experiments. An asterisk indicates values that are significantly different ($p < 0.01$) from control.

Cell treatment	Viable cells	5′-BrdUrd-positive cells
None (quiescent)	92 ± 5	2 ± 1
IGF-I	94 ± 3	18 ± 3*
IGF-I + 50 μM ET-18-OCH_3	89 ± 4	6 ± 1
IGF-I + 20 μM D609	88 ± 7	17 ± 3*

Our findings also strongly support the hypothesis that DAG generated by PI-PLC activity is the driving force for PKC-α translocation to the nucleus, in agreement with the proposal of Divecha *et al.* (38). These authors argued that PKC may continuously cycle in and out of the nucleus and may become "fixed" in this cell compartment by an increase in DAG. We demonstrated PKC localization to the nuclear fraction in three ways. First, *in situ* CLSM analysis of intact cells showed an increased staining within nuclei of IGF-I-treated cells, whereas translocation was not evident in cells exposed to either ET-18-OCH_3 or neomycin sulfate. Second, Western blot analysis also demonstrated a marked increase in PKC-α in purified nuclei prepared from IGF-I-treated cells. Again, the increase was blocked only by ET-18-OCH_3 or neomycin sulfate. Third, biochemical assays for PKC enzymic activity demonstrated a low specific activity in nuclei from control cells that increased more than 5-fold in nuclei obtained from IGF-I treated cells. As also demonstrated by immunocytochemical and immunochemical analysis, the rise in enzymic activity was inhibited only by ET-18-OCH_3 or neomycin sulfate but not by other chemicals. It should be emphasized that already after 5 min of IGF-I exposure, a nearly maximal nuclear PI-PLC activation could be observed by DAG mass assay. Although PKC-α started to translocate to the nucleus 5 min after the beginning of IGF-I stimulation (data not shown), 30 min was required to obtain an almost complete migration from cytoplasm to the nucleus, showing an ordered sequence in nuclear PI-PLC activation, DAG production, and PKC translocation.

Our results fit well with the recent observations by Pettitt *et al.* (39). Indeed, these authors were able to demonstrate that in Swiss 3T3 cells treated with bombesin, PI-PLC activation generated DAG with primarily polyunsaturated fatty acid species, whereas PLD activation resulted in saturated/monounsaturated species. Treatment of porcine aortic endothelial cells with lysophosphatidic acid resulted in activation of PLD independently of PI-PLC. Whereas in 3T3 cells there was an activation of PKC, no similar results were seen in porcine aortic endothelial cells. Thus, the authors concluded that DAG produced as a result of PLD activation does not appear to act as a regulator of PKC, at least in this cell line.

It should be pointed out, however, that in other systems, such as IIC9 cells, the nuclear translocation of PKC-α caused by α-thrombin has been shown to rely on DAG derived predominantly, if not exclusively, from PC (21). Thus, differences due to the cell type and/or agonist employed appear to be likely.

Moreover, recent evidence hinted at phosphatidylglycerol as another possible physiological activator of PKC-βII at the nuclear level in HL-60 cells (40).

Our results also demonstrate that PI-PLC activation and PKC-α translocation to the nucleus are essential steps to elicit mitogenic response after IGF-I stimulation, in agreement with

the results by Manzoli *et al.* (41). Therefore, it seems that PLC-β1 is important to regulate the transition from G_0 to G_1, whereas PLC-δ4 is likely to be involved in phenomena occurring during S-phase.

Up to now it has not been clarified what targets are downstream of PKC-α translocation to the nucleus in 3T3 cells, even if nuclear proteins are certainly phosphorylated by PKC following its migration to this cell compartment elicited by IGF-I (42). Future investigations in this field should provide important information about the exact mechanisms that lead to cell proliferation.

Acknowledgments—We thank Sonia Lach and Giovanna Baldini for the illustrations.

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Nuclear Diacylglycerol Produced by Phosphoinositide-specific Phospholipase C Is Responsible for Nuclear Translocation of Protein Kinase C- α

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J. Biol. Chem. 1998, 273:29738-29744.
doi: 10.1074/jbc.273.45.29738