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

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It is well established that an independent inositol 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-octadecyl-2-O-methyl-sn-glycero-3-phosphocholine or by neomycin sulfate but not by the purported phosphatidylycholine-phospholipase C specific inhibitor D609 or by inhibitors of phospholipase D-mediated DAG production. Treatment of cells with 1-O-octadecyl-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-octadecyl-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 G1/G0 to S-phase transition.

Upon stimulation by more than one hundred signaling molecules, the hydrolysis of a minor membrane phospholipid, PIP₂, by a specific PI-PLC rapidly occurs (1). PIP₂ breakdown leads to the generation of two well characterized second messengers: IP₃, which is responsible for the release of Ca²⁺ 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 ρ-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 phosphatic acid, which can be dephosphorylated by phosphatidic acid phosphohydrolase to produce DAG (8, 9). Whereas DAG production deriving from PIP₂ 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 PIP₂ 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 PIP₂ 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 G1/M transition.

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of the cell cycle.

Nevertheless, extracellular ligands, such as α-thrombin, could increase DAG delivery at, 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, Dicheva et al. (16) noted that the mass increase in DAG is greater than the combined loss in PIP and PIP₂, 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 PIP₂ 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-dioleoyl-3-palmitoyl-glycerol, dioleoyleoylglycerol, CHAPS, phosphatidylserine, neomycin, NGS, peroxidase-conjugated anti-rabbit IgG, anti-mouse and anti-rabbit IgG (cat. no. H-7035), and BSA were from Sigma. Mono- chloride to PKC-α, and BSA were from Sigma. Monoclonal antibody to 5′-BrUrd was from Becton Dickinson (Milan, Italy). ET-18-OCH₃ 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). α- and γ-32P-ATP were from Amersham Pharmacia Biotech (Uppsala, Sweden). The protein assay kit (detergent compatible) was from Bio-Rad. The synthetic peptide RFARKGSLRQKNVHVKN 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 10⁴ cells/cm² 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 stimulation, at the following concentrations: ET-18-OCH₃, 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 counted, and the cell viability was noted that the mass increase in DAG is greater than the combined loss in PIP and PIP₂, 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.

**Isolation of Nuclei—**This was accomplished as previously reported (11). Briefly, cells (5 × 10⁶) 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 μM leupeptin and aprotinin for 2 min at 0 °C. Then 500 μl of double-distilled H₂O 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 MgCl₂, 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 DAGProduced in Vivo—**The assay was performed according to Dicheva 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 μl ATP, and 1 μCi of [γ-32P]ATP. Incubation was for 1 h at room temperature; then, phosphatidic acid was extracted, was followed by washing, and was digested, and acidified, and [3H]PAP was counted in a liquid scintillation system (Betanorm IV, Kontron, Milan, Italy). Standard curves were obtained as reported by Dicheva et al. (16), using 1,2-dioleoyl-3-palmitoyl-glycerol as substrate.

**PLC Activity Assay—**The procedure outlined by Martelli et al. (11) was followed. Assays (100 μl) contained 100 mM MESS buffer, pH 6.7, 150 mM NaCl, 0.06% sodium deoxycholate, 3 nmol [3H]PIP₂ 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 chlorormethanol-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 ET-18-OCH₃. Cells were then washed twice in 1 × 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, Oberkochen, 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 at an ×100, 1.4 numerical aperture, Plannefluar 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. It was performed using the ImageSpace software (Molecular Dynamics, Sunnyvalle, 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 Image-Card2 (Fokus Graphics, Foster City, CA) using 100 ASA TMax black and white film (Eastman Kodak Co.).

**Western Blotting Assay—**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 μl of protein) were incubated at 30 °C for 10 min in 20 mM Tris-Cl, pH 7.4, 10 mM MgCl₂, 10 μl ATP, 0.4 μg/ml histone H1, 5 μl of [γ-32P]ATP, in the presence of 1.2 mM CaCl₂, 40 μg/ml phosphatidylserine, and 3.3 μM dioleoylglycerol. The reactions were terminated with 15 μl of acetic acid and spotted onto Whatman p81 paper, followed by washing with 0.75 M H₃PO₄. Radioactivity was measured by Cerenkov counting of some aliquots of the supernatant using a RFARKGSLRQKNVHVKN (corresponding to amino acids 19–36 of PKC) was employed as a substrate. An equal volume of Lammli’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.
DNA was denatured in 4N HCl for 30 min and fixed in a paraformaldehyde in PBS for 30 min at room temperature, and then treated previously (28). Samples were fixed in freshly prepared 4% ethanol series to prevent reannealing. Slides were then air-dried and incubated for 3 h at 7 °C with a monoclonal antibody to \( \text{BrdUrd} \). Samples were subsequently washed three times in PBS and reacted with a Cy3-conjugated anti-mouse IgG. Following a further wash in PBS, the slides were mounted as detailed below.

**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, with 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. 1A). Similar results were obtained when the nonselective phospholipase inhibitor neomycin (1.2 mM) was employed (Fig. 1A). In contrast, 30 \( \mu \text{M} \) D609 (which is thought to be a 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 propanolol (a phosphatidic acid phosphohydrolase inhibitor, 100 \( \mu \text{M} \)) and 1.3% ethanol (which causes PLD to generate phosphatidylethanol rather than phosphatidic acid), did not affect the basal levels of nuclear DAG (Fig. 1B). Moreover, in these cases, the rise in nuclear DAG that followed IGF-I stimulation was comparable to that seen in controls (Fig. 1B). 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 \( \mu \text{M} \), 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 \( \mu \text{M} \), the inhibition was 60%. No significant inhibitory effect of D609 was observed in a range of 10–30 \( \mu \text{M} \). 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. 2A, 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. 2A) for all the examined times, resulting in less than 10% of the original activity. The addition of either D609 or propanolol did not block IP\(_3\) formation, which was similar to control nuclei (Fig. 2B).

Nuclear Translocation of PKC-\(\alpha\) 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-\(\alpha\)-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-\(\alpha\), 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. 3A). Incubation of cells with IGF-I...
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. 3B). When Swiss 3T3 fibroblasts were preincubated with either ET-18-OCH₃ 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. 3B).

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-OCH₃ 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-OCH₃ 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 was 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. 5A), in agreement with the results of both immunocytochemical and immunocytochemical 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-OCH₃ or neomycin sulfate, whereas D609, propranolol, and ethanol were ineffective (Fig. 5A). Similar results were obtained when the synthetic peptide RFARKGLRQKN-VHEVKI (corresponding to amino acids 19–36 of PKC) was employed as substrate instead of histone H1, as presented in Fig. 5B.

**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 PL-PLC activity by means of 50 μM ET-18-OCH₃ 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-OCH₃, 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 inPIP and PIP₂ 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 PL-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-OCH₃ 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...
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).

### Table II

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Cells with nuclear translocation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (quiescent)</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IGF-I</td>
<td>38 ± 5*</td>
</tr>
<tr>
<td>IGF-I + ET-18-OCH₃ (100 μM)</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>IGF-I + neomycin (1.2 mM)</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>IGF-I + D609 (30 μM)</td>
<td>36 ± 4*</td>
</tr>
<tr>
<td>IGF-I + propranolol (100 mM)</td>
<td>35 ± 4*</td>
</tr>
</tbody>
</table>

*An asterisk indicates values that are significantly different (p < 0.01) from control*.

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**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-OCH₃ (100 μM); **D**, 30 min of IGF-I stimulation plus D609 (30 μM); **E**, 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.
represents the mean from three different experiments

Experimental Procedures.” IGF-I stimulation was for 30 min. Each value

chemicals were employed at the concentrations indicated under “Ex-

brain, whereas PIP 2 levels were measured by a chemical pro-

stimulation plus D609 (30 μM); lane e, 30 min of IGF-I stimulation plus

neomycin (1.2 mM); 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.

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 \[^{32}P\]ATP using PIP-5-kinase partially purified from rat brain, whereas PIP 2 levels were measured by a chemical pro-

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 nitrocel-
lulose 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 sub-
strate either histone H1 (A) or a synthetic peptide (B). C, quies-
cent cells. NEO, neomycin; PROP, propranolol; ETHAN, ethanol. The chemicals were employed at the concentrations indicated under “Ex-
perimental Procedures.” IGF-I stimulation was for 30 min. Each value

represents the mean from three different experiments ± an S.D. of 12%.

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 con-
tinuously 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, bio-
chemical 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 expo-
sure, 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 lysophosphatic acid resulted in activation of PLD inden-
dependently of PI-PLC. Whereas in 3T3 cells there was an acti-

viation of PKC, no similar results were seen in porcine aortic
endothelial cells. Thus, the authors concluded that DAG pro-
duced 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 predom-

inantly, 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 nu-
clear 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

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.

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>Viable cells</th>
<th>5'-BrdUrd-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (quiescent)</td>
<td>92 ± 5</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>IGF-I</td>
<td>94 ± 3</td>
<td>18 ± 3*</td>
</tr>
<tr>
<td>IGF-I + 50 μM ET-18-OCH 3</td>
<td>89 ± 4</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>IGF-I + 20 μM D609</td>
<td>88 ± 7</td>
<td>17 ± 3*</td>
</tr>
</tbody>
</table>
the results by Manzoli et al. (41). Therefore, it seems that PLC-β1 is important to regulate the transition from G₀ to G₁, whereas PLC-δ1 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.

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REFERENCES

Nuclear Diacylglycerol Produced by Phosphoinositide-specific Phospholipase C Is Responsible for Nuclear Translocation of Protein Kinase C-α
Luca M. Neri, Paola Borgatti, Silvano Capitani and Alberto M. Martelli

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