α-Thrombin-Induced Nuclear *sn*-1,2-Diacylglycerols Are Derived from Phosphatidylcholine Hydrolysis in Cultured Fibroblasts†

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ABSTRACT: Diglycerides play an important role in a number of agonist-induced signal transduction pathways. We have recently demonstrated that α-thrombin induces a rapid increase in the level of diglyceride mass in the nucleus and a selective increase in nuclear PKC-α [Leach, K. L., Ruff, V. A., Jarpe, M. B., Fabbro, D., Adams, L. D., & Raben, D. M. (1992) *J. Biol. Chem. 267*, 21816–21822]. In the present report, we examined the potential source of the induced nuclear diglycerides by examining the molecular species profiles of both the induced diglycerides and nuclear phospholipids by capillary gas chromatography. The molecular species profiles of the nuclear diglycerides generated resemble the species profiles of PC, and not PI species, at all times. In addition, while our previous data indicated that the molecular species of whole-cell phospholipids did not change in response to α-thrombin, nuclear PE was altered in a dramatic and selective manner in response to this agonist. These results demonstrate that PC hydrolysis is the predominant, if not exclusive, source of the α-thrombin-induced nuclear diglycerides in these fibroblasts.

It is now well recognized that many agonist-induced responses are mediated by the elevation of cellular diglyceride levels (Nishizuka, 1992; Liscovitch, 1992). These functions include the activation of protein kinase C (PKC),[1] modulation of membrane architecture, and regulation of enzymatic activities (Siegel et al., 1989; Badwey et al., 1989; Nishizuka, 1992; Liscovitch, 1992; Zidovetzki et al., 1992). As a result of the diverse roles played by diglycerides, there has been considerable interest in establishing the events regulating the generation and metabolism of these lipids. We and other investigators have demonstrated that agonist-induced diglycerides are primarily derived from the hydrolysis of either phosphoinositides (PIs) or phosphatidylcholines (PCs) [Wright et al., 1988; Pessin & Raben, 1989; Pessin et al., 1990; and reviewed in Exton (1990)]. These studies, however, have been largely confined to analyses of whole-cell lipid changes and did not address the subcellular compartments in which these changes were occurring.

Mitogens induce a number of nuclear responses including the transcription of certain genes [reviewed in Karin and Smeal (1992)] and the phosphorylation of specific nuclear proteins [reviewed in Meek and Street (1992)]. It is clear, therefore, that these signal transduction pathways must include a mechanism which would allow signals generated at the plasma membrane to impinge upon the nucleus. In this regard, a number of studies have provided compelling evidence implicating a role for the activation of nuclear PKC in agonist-induced changes in nuclear functions (Macfarlane, 1986; Butler et al., 1986; Cambier et al., 1987; Hornbeck et al., 1988; Martelli et al., 1989; Samuels et al., 1989; Leach et al., 1989, 1992; Fields et al., 1989, 1990; Divecha et al., 1991; Hocevar & Fields, 1991). Irvine and co-workers have reported an IGF-1 induced increase in nuclear diglycerides in stimulated 3T3 cells accompanied by changes in nuclear PKC levels (Divecha et al., 1991). In a recent study, we reported an α-thrombin-induced elevation of nuclear diglycerides and suggested that these lipids are involved in the activation of nuclear PKC (Leach et al., 1992).

The mechanism(s) responsible for the elevation of nuclear diglycerides remain(s) obscure. In the 3T3 cell studies, a small decrease in PIP and PIP_2 levels was observed which did not quantitatively account for all of the induced diglycerides (Cocco et al., 1988, 1989, 1992; Cataldi et al., 1990). While these studies suggested PIs as one potential source, other sources were not investigated.

In view of the above, and the potential important physiological role of the nuclear diglycerides, identification of the source of the induced nuclear diglycerides is imperative. In a recent attempt to address this question, we took advantage of our previous observation indicating that [^{3}H]myristate is preferentially incorporated into PC when intact IIC9s are acutely labeled (Wright et al., 1992). While an increase in radiolabeled nuclear diglycerides was observed after α-thrombin stimulation, differences in the temporal increases in diglyceride mass versus radiolabeled diglycerides precluded a definitive identification of the phospholipid source (Leach et al., 1992). As a result, the source of these diglycerides remains uncertain.

Since it is not possible to selectively radiolabel nuclear phospholipids, potential sources of the induced nuclear diglycerides cannot be obtained by analysis of the release of water-soluble radiolabeled headgroups from metabolically labeled cultures. We have previously reported a method

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[1] Abbreviations: PKC, protein kinase C; PLC, phospholipase C; PC, phosphatidylcholine; PIs, phosphoinositides; PS, phosphatidylserine; PE, phosphatidylethanolamine; NCDC, 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate; BHT, butylated hydroxytoluene; RIA, radioimmunoassay; MEM, minimal essential medium; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; NADH, β-nicotinamide adenine dinucleotide, reduced; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced; HPLC, high-pressure liquid chromatography; GC, gas chromatography.

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whereby the source of induced diglyceride may be ascertained by comparing the molecular species of the induced diglycerides with the molecular species of the potential phospholipid sources (Pessin & Raben, 1989). This analysis has proven useful in establishing the source of diglycerides generated in response to mitogens in fibroblasts (Pessin & Raben, 1989; Pessin et al., 1990) and in response to neurotransmitters and neurotrophic factors in PC12 cells (Pessin et al., 1991). In addition, similar analyses have been used to identify PC as the source of induced diglycerides and PA in a variety of systems (Augert et al., 1989; Lee et al., 1991; Holbrook et al., 1992).

In the experiments presented here, we apply the same molecular species analysis to diglycerides generated in the nucleus of α-thrombin-stimulated IIC9 cells. We previously demonstrated that diglycerides are generated in intact IIC9 cells from two sources in response to a high concentration of α-thrombin (Wright et al., 1988; Pessin & Raben, 1989; Pessin et al., 1990). Phosphoinositides are the primary source during the first 15–60 s of stimulation while PC is the primary, if not exclusive, source after 5 min (Pessin & Raben, 1989; Pessin et al., 1990). In contrast, we show here an analysis of the induced nuclear diglycerides that indicates PC hydrolysis is the source of the induced diglycerides at all times. Interestingly, while the molecular species of whole-cell phospholipids did not appear to change significantly in response to α-thrombin (Pessin & Raben, 1989; Pessin et al., 1990), the molecular species nuclear PE is dramatically altered.

MATERIALS AND METHODS

Materials. Cell culture medium was from Gibco. Tissue culture dishes were from Falcon. Bovine serum albumin, highly purified human α-thrombin, BHT, EGTA, EDTA, quinacrine, 2-nitro-4-carboxyphenyl *N,N*-diphenylcarbamate (NCDC), and trizma base were obtained from Sigma Chemical. Human transferrin was from Behring Diagnostics. Phospholipase C (*Bacillus cereus*), aprotinin, and leupeptin were from Boehringer Mannheim. *tert*-Butyldimethylchlorosilane/imidazole was from Alltech Associates. Silica gel G TLC plates were from Analtech. Diglyceride standards were purchased in the form of phosphatidylcholine from Avanti Polar Lipids. Acetonitrile (HPLC grade) was from J. T. Baker. Isopropyl ether was from Aldrich. Diethyl ether (high purity) and chloroform, methanol, acetone, and hexane (all GC[2]) were from Burdick and Jackson. All organic solvents contained 50 μg/mL BHT.

The SP-2380 capillary column was from Supelco. The Zorbax PRO-10 Sil HPLC column (4.6 mm i.d. × 23 cm) was from DuPont.

Cell Culture. Cell culture was performed as previously described (Wright et al., 1988; Leach et al., 1992). Briefly, IIC9 cells were grown in 150-mm dishes for 3 days in α-MEM/Ham's F-12 containing 5% fetal calf serum. The medium was removed, and cells were washed twice with serum-free Dulbecco's modified Eagle's medium containing 1 mg/mL RIA-grade BSA and supplemented with 5 μg/mL human transferrin (serum-free medium). The cells were incubated in serum-free medium for 2 days and then washed twice with fresh serum-free medium. They were incubated at 37 °C in fresh serum-free medium for at least 30 min before beginning the experiment.

Stimulation and Isolation of Nuclei. The cells were incubated in 150-mm dishes at 37 °C either in serum-free media alone or in serum-free media containing 500 ng/mL α-thrombin. At the end of the incubation time, the medium was removed, the dishes were immediately transferred to an ice bath, and 4 mL of fractionation buffer (10 mM Tris, 10 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, 10 μM leupeptin, 10 μg/mL aprotinin, 20 μM quinacrine, and 200 μM NCDC, pH 7.5 at 4 °C) was added. All further steps were performed at 4 °C until the extraction of lipids. The cells were scraped from the dishes and subjected to 15 passes in a Potter-type Teflon-on-glass homogenizer. Homogenates from 15–20 dishes were pooled for each time point for molecular species analysis, and homogenates from 4 dishes were used for quantification of diglyceride mass levels using *Escherichia coli* diglyceride kinase as previously described (Wright et al., 1988).

Nuclei were isolated by centrifugation of the homogenate at 2000 rpm (700*g*) for 7 min in an RT6000B centrifuge with a swinging-bucket rotor. The pellet was resuspended in 5 mL of fractionation buffer and dounced with a tight-fitting (type B) pestle for 20 passes. The resulting nuclear suspension was layered over a 5-mL cushion of 45% sucrose in fractionation buffer and centrifuged at 2800 rpm (1660*g*) for 30 min. The pellet was resuspended in 0.8 mL of water, and a small aliquot was quickly assessed for gross contamination by light microscopy.

The isolated nuclei in water were transferred into 1 mL of chloroform. The centrifuge tube was washed twice with 1 mL of methanol, and the wash was added to the water and chloroform. Nuclear lipids were extracted (Bligh & Dyer, 1959) and dried under a stream of dry nitrogen.

In some experiments, nonnuclear membranes were isolated by centrifugation of the postnuclear supernatant, obtained from the 700*g* spin, at 100000*g* for 1 h and resuspension in fractionation buffer.

Molecular Species Analysis of Nuclear Lipids. Diglycerides and phospholipids were isolated from the nuclear lipid extract by thin-layer chromatography on a silica gel G plate developed in isopropyl ether/acetic acid (96:4). Standard diglycerides and phospholipids were visualized by iodine staining, and the corresponding regions on the plate containing nuclear lipids were scraped. Diglycerides were extracted from the silica with chloroform/methanol (90:10) and dried under N_2. Phospholipids were extracted from the silica according to Bligh and Dyer and dried under N_2 as previously described (Pessin & Raben, 1989). The diglycerides were converted to *tert*-butyldimethylsilyl ethers and extracted into hexane as previously described (Pessin & Raben, 1989).

Individual phospholipids were separated by HPLC on a Zorbax PRO-10 Sil column with acetonitrile/phosphoric acid (99:1) as previously described (Pessin & Raben, 1989). Fractions containing the individual phospholipids, identified by their relative retention times, were collected and dried under a vacuum in a Savant Speed Vac concentrator. The dried lipid was then extracted according to Bligh and Dyer and dried under N_2.

Phospholipid headgroups were removed by hydrolyzing each phospholipid with *Bacillus cereus* PLC, and the resulting diglycerides were isolated, extracted, and dried under N_2 as previously described (Pessin & Raben, 1989). The diglycerides were then converted to the *tert*-butyldimethylsilyl ethers as previously described.

The derivatized nuclear and phospholipid-derived diglycerides were analyzed and quantified by capillary gas chromatography (GC), and diglyceride profiles were calculated as described previously (Pessin & Raben, 1989). Table 1 lists the molecular species of IIC9 diglycerides as previously identified. In all the figures presented in this paper, the

Table 1: Identification of 1,2-Diglycerides Separated by Capillary Gas Chromatography[a]

peak no.	relative retention time	diglyceride molecular species
1	0.833	16:0–16:0*
2	0.862	16:0–16:1ω9
3	0.871	16:0–16:1ω7
4	1.000	16:0–18:1ω9*
5	1.011	16:0–18:1ω7
6	1.030	16:1ω9–18:1ω9
7	1.048	16:1ω7(9)–18:1ω9(7)
8	1.160	16:1ω7–18:1ω7
9	1.218	18:0–18:1ω9*
10	1.235	18:0–18:1ω7
11	1.273	18:1ω9–18:1ω9*
12	1.288	18:1ω7–18:1ω7
13	1.301	18:0–18:2ω6*
14	1.341	16:0–20:4ω6*
15	1.355	18:1ω9(7)–18:2ω6
16	1.517	18:0–20:3ω6
17	1.568	18:1ω9–20:3ω6
18	1.620	18:1ω7–20:3ω6
19	1.655	16:0–20:5ω6
20	1.703	18:0–20:4ω6*
21	1.791	18:1ω9–20:4ω6
22	1.818	18:1ω7–20:4ω6
23	1.863	18:0–20:5ω3
24	1.925	18:1ω9–20:5ω3
25	1.985	18:1ω7–20:5ω3

molecular species profile is shown as the contribution of each individual species to the total makeup of the profile. Subtraction profiles show the differences between two profiles at the level of fatty acid composition.

Other Methods. The protein contents of the homogenate, nuclear fraction, and nonnuclear membranes were determined by the method of Bradford (1976). NADH/NADPH cytochrome *c* reductase activity, an endoplasmic reticulum enzyme, was quantified as described by Kreibich et al. (1973).

RESULTS

Assessment of Purity and Recovery. Since the present studies were designed to examine the mitogen-induced changes in nuclear lipids, it was important to isolate nuclei in the absence of detergents as described under Materials and Methods. The purity and recovery of nuclei in this procedure were therefore evaluated. The presence of endoplasmic reticulum was assessed by quantification of the level of NADH/NADPH cytochrome *c* reductase activity. The results indicate that approximately 3.3% of the total cellular activity of this enzyme was present in the nuclei. Plasma membrane contamination, assessed by the presence of adenylate cyclase activity, was determined previously and found to be negligible (Leach et al., 1992). The nuclei are not contaminated with cytoskeletal components as assessed by immunofluorescence and Western blots using antibodies directed against vimentin and tubulin (Leach et al., 1992). In addition, examination of the nuclear fraction by both light and electron microscopy indicated that the fraction was free of contaminating organelles and possessed intact inner and outer membranes (Leach et al., 1992). These results demonstrate that the nuclei isolated in our procedure are highly pure and structurally intact.

The contribution of nuclear diglyceride to the whole-cell levels quantified in previous work was determined in the following manner. Nuclei were prepared as described above except that an aliquot of whole-cell homogenate was reserved before the first centrifugation. The number of nuclei in the final nuclear pellet and in the whole-cell homogenate was determined by counting in a hemocytometer. The mass levels of diglycerides in the nuclear pellet and whole-cell homogenate were determined as described above.

Table 2: Contribution of Nuclear Diglyceride to Resting and Thrombin-Induced Levels of Whole-Cell Diglyceride[a]

sample	whole-cell homogenate (fmol/cell)	nuclei (fmol/nucleus)	% in nucleus
control	6.5 (±3.5)	2.4 (±0.62)	38
α-thrombin (30 s)	12.6 (±1.2)	5.5 (±2.1)	44
30-s induced	6.1	3.1	50
α-thrombin (5 min)	21.3 (±8.3)	10.7 (±2.4)	50
5-min induced	14.8	8.3	56

[a] IIC9 cells from four 150-mm dishes were stimulated with α-thrombin and homogenized, and an aliquot of this homogenate (one-fourth of the total) was reserved as the whole-cell homogenate. The remainder was fractionated to obtain nuclei as described under Materials and Methods. The number of nuclei in each sample was determined by counting in a hemocytometer. The number of cells was also quantified by counting nuclei. Diglyceride mass levels were determined by the *E. coli* diglyceride kinase assay as described (Wright et al., 1988). Values shown are average ± SD (n = 3).

As shown in Table 2, the mass of nuclear diglyceride contributes a significant portion of both resting and α-thrombin-induced levels of cellular diglycerides. In quiescent cells, nuclear diglycerides represent 38% of the whole-cell diglyceride mass while α-thrombin-induced diglyceride in the nucleus makes up roughly half that seen in whole cells. Also, as previously reported, the diglyceride mass increase in the nuclear membrane (from 0.3 mol % in quiescent cells to 1.1 mol % in stimulated cells) is capable of mediating the activation of PKC (Leach et al., 1991).

Assessment of the Redistribution of Lipids. Two potential problems exist which could seriously affect the interpretation of the results obtained in the present study. First, redistribution of the diglycerides between various membranes during the isolation would prevent any meaningful interpretation of the results. For example, if lipid from nonnuclear membranes were redistributing to nuclear membranes during the isolation, these lipids would be incorrectly analyzed as nuclear lipids. Second, the metabolism of the diglycerides during the isolation would also seriously compromise any interpretations.

The potential problem of lipid redistribution was assessed by quantifying the amount of radiolabel present in nuclei isolated in the presence of nonnuclear membranes containing radiolabeled lipids essentially as described by Cocco et al. (1985). Briefly, cellular lipids were radiolabeled by incubating cells with 1 μCi/mL [^{3}H]glycerol for 2 days in serum-free media (Raben et al., 1987). Nonnuclear membranes were isolated from these cells by centrifuging the 700*g* postnuclear supernatant at 100000*g* for 1 h as described under Materials and Methods. These nonnuclear membranes were resuspended in fractionation buffer and added to unlabeled cells from which nuclei and nonnuclear membranes were isolated. The amount of radiolabel present in the isolated nuclei and nonnuclear membranes was quantified by scintillation counting in Cytoscint.

When nuclei are isolated from cells in the presence of exogenously added labeled nonnuclear membranes, virtually no radiolabeled lipid is found in the nuclear fraction (data not shown). These results are not only consistent with our previous findings demonstrating that our nuclei are free of nonnuclear membranes (Leach et al., 1992) but also indicate that lipids from other organelles do not redistribute to the nuclear fraction upon homogenization. When radiolabeled nuclei were added to unlabeled cells, one-third of the label appeared in the nuclear

fraction, one-third was in the nonnuclear membranes, and one-third was lost (data not shown). In order to determine if this loss of label was due to loss of whole organelles or loss of lipid material, we double-labeled cells with [^{35}S]methionine and [^{3}H]glycerol. The ratio of $^{35}S/^{3}H$ in the nonnuclear fractions equalled the ratio present in isolated nuclei (data not shown). These results indicate that these losses are due to loss of whole nuclei and not lipids alone.

Assessment of Lipid Metabolism during Nuclear Isolation. The next important problem to address was the possible metabolism of lipids during isolation of nuclei. While this was unlikely given the presence of lipase inhibitors (see Materials and Methods), we felt it was necessary to directly assess this possibility. Clearly, nuclear lipids cannot be examined prior to isolation. We can, however, examine the effect of fractionation on diglyceride metabolism by comparing the whole-cell-induced diglyceride profile before and after fractionation. If these profiles are significantly different, this would indicate that metabolism was occurring during the fractionation. If these profiles are similar, this would strongly indicate that lipid metabolism was not occurring during the nuclear isolation.

This comparison was accomplished by fractionating quiescent cells or cells incubated in the presence of α-thromin for 5 min at 37 °C as described under Materials and Methods. All pellets and supernatants were pooled together, and lipids were extracted. Diglycerides were isolated, derivatized, and analyzed by capillary GC. The profile of α-thrombin-induced diglycerides was generated by subtracting the profile obtained from stimulated cells from the profile obtained from quiescent cells as previously described (Pessin & Raben, 1989; Pessin et al., 1990, 1991). The profile of induced diglycerides in intact cells after a 5-min incubation with α-thrombin was generated as previously described (Pessin & Raben, 1989; Pessin et al., 1990). These two profiles were then compared by subtraction as previously described (Pessin & Raben, 1989; Pessin et al., 1990, 1991).

As shown in Figure 1, there are only minor differences between the molecular species profile of induced diglycerides obtained from the "reconstituted" membranes and the profile of induced diglycerides present in intact cells. These results demonstrate that lipid metabolism is not occurring during our nuclear isolation procedure and is therefore not compromising our results. It is important to note that this was only true when cells were fractionated in the presence of protease and lipase inhibitors. When these inhibitors were not present, the induced diglyceride profiles obtained from "reconstituted" membranes were dramatically different from the profile obtained from whole cells. This may be due to the action of catabolic enzymes released from lysosomes or activated by proteolysis when the cells are broken open, or by bringing phospholipases into contact with compartments they do not normally act upon. While the exact reason for this is not clear, the data indicate that the presence of the inhibitors is necessary during the isolation of the nuclei.

Molecular Species of Nuclear Phospholipids. A major goal of the present study was to identify potential phospholipid sources of α-thrombin-induced nuclear diglycerides. As we previously described, this can be accomplished by comparing the molecular species profile of induced nuclear diglycerides to the profile of nuclear phospholipids. This requires an analysis of nuclear phospholipids present in quiescent and α-thrombin-stimulated cells. As shown in Figure 2, the molecular species profiles of nuclear PC, PS, and phosphoinositides are very similar, although not identical, to the

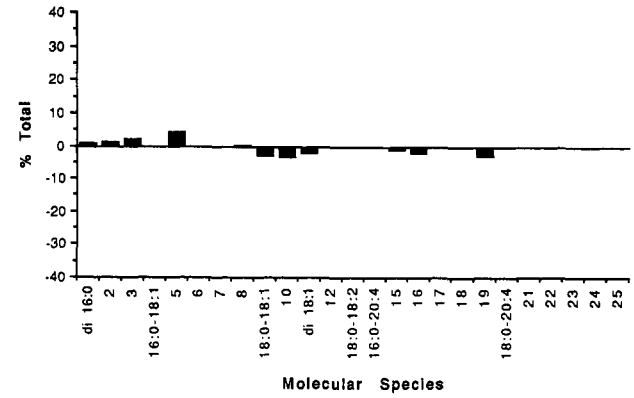

FIGURE 1: Assessment of lipid metabolism during isolation of nuclei. The profile of diglycerides induced after a 5-min incubation with 500 ng/mL α-thrombin in intact cells was determined as previously described (Pessin & Raben, 1988; Pessin et al., 1990, 1991). The profile of diglycerides induced after 5-min incubation with 500 ng/mL α-thrombin was also determined from "reconstituted" cells as follows: nuclei, nonnuclear membranes, and also all supernatants were isolated from quiescent cells or cells that had been incubated with α-thrombin for 5 m in and pooled into a "reconstituted" mixture. The profiles of the diglycerides in these reconstituted fractions were determined, and the induced diglyceride profile was calculated as previously described. The molecular species shown on the x axis are identified in Table 1. This induced diglyceride profile was subtracted from the profile of induced diglycerides isolated from intact cells. Results shown represent one of two experiments. Individual molecular species did not vary by more than ±5% between experiments.

molecular species of these phospholipids isolated from whole cells (Pessin & Raben, 1989; Pessin et al., 1990). In general, nuclear phospholipids are composed of fewer distinct species than whole-cell phospholipids. This is most likely due to the fact that (a) whole-cell species profiles describe a heterogeneous mixture of membranes and (b) low recovery of nuclear lipids prevents less abundant species from being quantified.

The predominant species in nuclear PC are 16:0–18:1ω9, 18:0–18:1ω9, and 18:1ω9–18:1ω9 (Figure 2, top). The predominant nuclear PS species is 18:1ω9–18:1ω9 (Figure 2, bottom). These profiles are similar to whole-cell PC and PS profiles (Pessin & Raben, 1989; Pessin et al., 1990). A more significant difference between nuclear and whole-cell phospholipid profiles is seen with PI. The predominant nuclear PI species are 18:0–20:4ω6, 18:1ω9–18:1ω9, and 18:1ω7–18:1ω7 (Figure 2, middle). Whole cell PIs also contain significant amounts of 18:0–18:1ω9 as well as several other species (Pessin & Raben, 1989; Pessin et al., 1990) not found in nuclear PIs. With the exception of PE, the addition of α-thrombin for any length of time examined had no effect on the molecular species of the phospholipids (data not shown).

Interestingly, the molecular species profile of nuclear PE changed following α-thrombin stimulation (Figure 3). Nuclear PE profiles from quiescent cells had very little resemblance to whole-cell PE profiles (Pessin & Raben, 1989; Pessin et al., 1990). Quiescent nuclear PE is composed predominantly of 16:0–18:1ω9 and 16:0–16:1ω7 (Figure 3, top). Five minutes after the addition of α-thrombin, several species with later retention times were found, with a corresponding loss of the above-mentioned species with earlier retention times (Figure 3, bottom). In addition, a complex of peaks was consistently seen on chromatograms of PE that could not be identified with a known diglyceride molecular species. These peaks are not shown in Figure 3. We are currently attempting to identify them. The peaks make up a significant portion of the total area of the chromatogram, and they also change upon stimulation with α-thrombin.

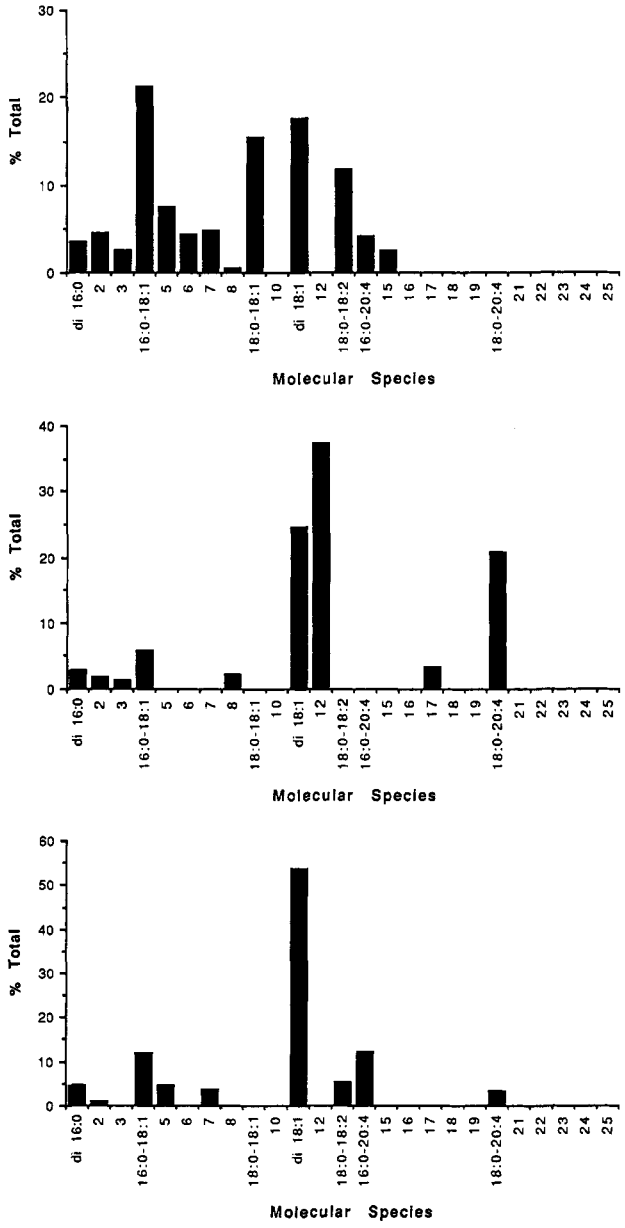

FIGURE 2: Molecular species profiles of nuclear PC, PS, and PI. Nuclei were isolated from quiescent cells. Phospholipids were isolated and headgroups were removed as described under Materials and Methods. Resulting diglycerides were derivatized and analyzed by capillary gas chromatography. The molecular species shown on the x axis are identified in Table 1. Normal variation in the percent total values in these analyses is ±5%. Representatives of three independently generated profiles are presented. (Top) Nuclear PC; (middle) nuclear PI; (bottom) nuclear PS.

Molecular Species Profile of Nuclear Diglycerides and Comparison to Nuclear Phospholipid Profiles. The molecular species of diglycerides from nuclei of quiescent cells and cells stimulated with α-thrombin for 1 and 5 min are shown in Figure 4. The induced species profiles shown in the middle and bottom panels of Figure 4 show the species of only those diglycerides that appear as a result of thrombin stimulation. They were generated by subtracting the control profile from the species profile obtained from the nuclei of stimulated cells at the given time points (Pessin & Raben, 1989; Pessin et al., 1990, 1991). These profiles contain some differences with respect to the profile of diglycerides isolated from whole cells (Pessin & Raben, 1989; Pessin et al., 1990). In particular, resolution of species 1–3 was possible in these experiments, and no species was seen with an acyl chain containing more

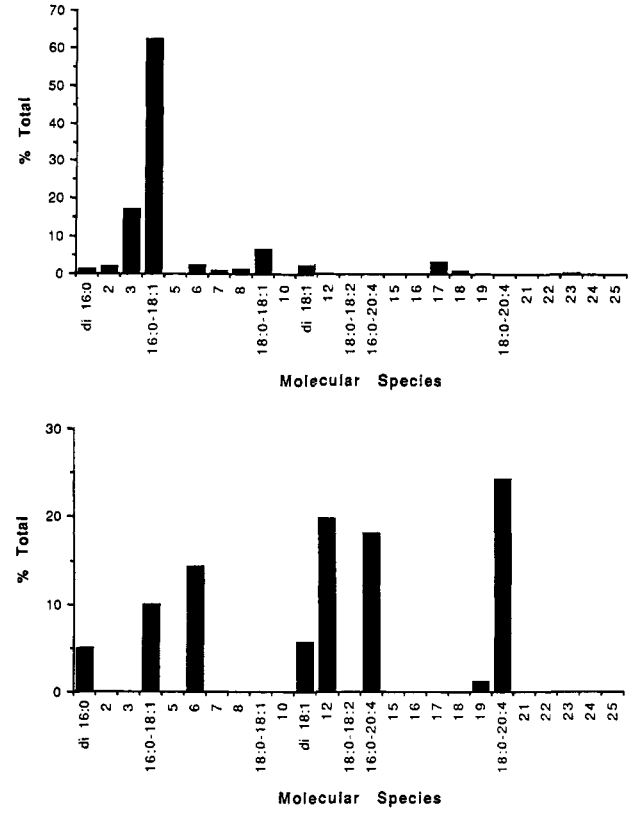

FIGURE 3: Molecular species profile of nuclear PE from (top) quiescent cells and (bottom) cells stimulated with α-thrombin for 5 min. PE was isolated, derivatized, and analyzed as described for the other phospholipids. The results are representative of two independently generated profiles. The molecular species shown on the x axis are identified in Table 1. Normal variation in molecular species profiles is ±5% of the total diglyceride.

than 18 carbons. Nuclear diglyceride generated after a 30-s incubation with α-thrombin were essentially identical to those generated after a 1-min incubation (Figure 4, middle) (data not shown).

In order to identify potential phospholipid sources of the induced diglycerides, we compared the molecular species profile of these induced diglycerides to the molecular species profile of the endogenous phospholipids as previously described (Pessin & Raben, 1989; Pessin et al., 1990). As shown in Figure 5, a comparison of the induced diglyceride profile obtained at 1 min with nuclear phospholipid profiles indicates that diglycerides generated at this early time are derived primarily, if not exclusively, from PC (Figure 5, top panel) and are very different from other nuclear phospholipids (Figure 5, three lower panels). The diglyceride species with the greatest deviation from PC is 16:0/18:1 (Figure 5, top, and compare top panel of Figure 2 with middle panel of Figure 4). The reason for this is unclear at this time but may represent slight differences in diglyceride metabolism or source pools at different times following stimulation.

The same comparison was carried out between the nuclear phospholipids and induced diglycerides at 5 min (Figure 6). In this comparison, it is clear that diglycerides appearing 5 min after α-thrombin also resemble PC (Figure 6, top panel). This indicates that the nuclear diglycerides are again derived primarily, if not exclusively, from an induced hydrolysis of nuclear PC at 5 min. We should note that the molecular species profiles of PC, PI, and PS used here were from quiescent cells but that the species from cells stimulated for 30 s or 5 min with α-thrombin were identical. Since the profile of PE changes when cells are incubated with α-thrombin for 5 min,

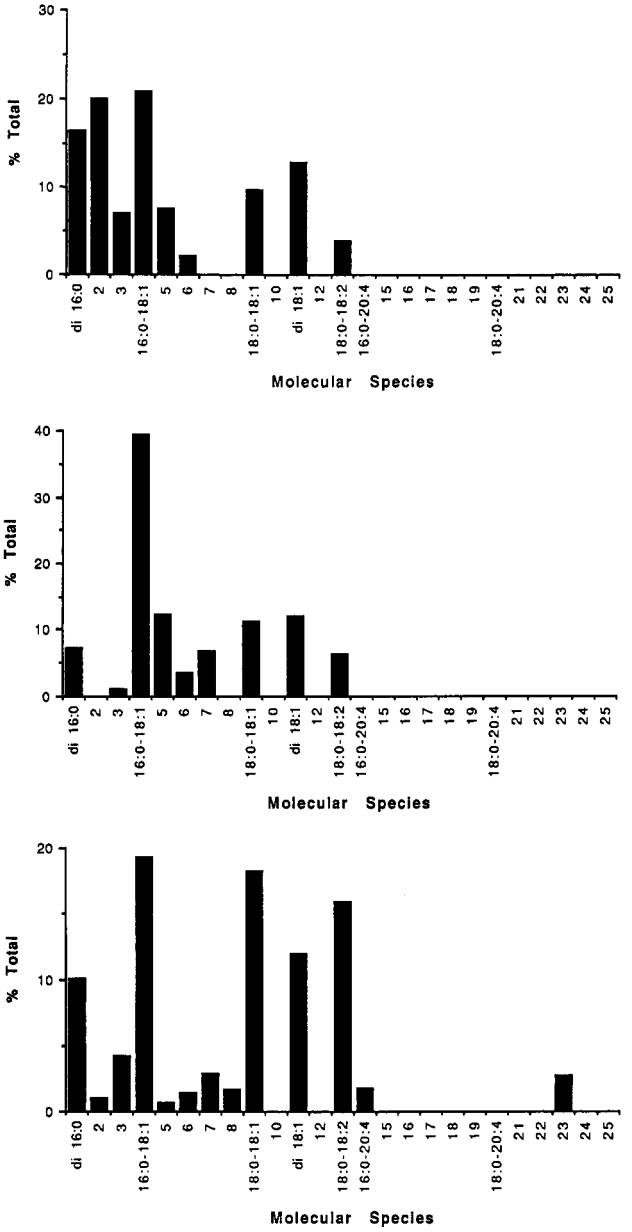

FIGURE 4: Molecular species profile of nuclear diglyceride. Nuclear diglycerides were obtained as described under Materials and Methods. Species profiles were generated from (top) quiescent cells, and induced profiles were generated from cells stimulated with α-thrombin for (middle) 1 min or (bottom) 5 min. The induced profiles were generated by subtracting the species profiles of the quiescent cells from those of the stimulated cells. These results are representative of (top) five, (middle) two, and (bottom) three independent experiments. The molecular species shown on the x axis are identified in Table 1. Normal variation in molecular species profiles is ±5% of the total diglyceride.

we compared induced diglycerides to PE from both quiescent cells and induced cells. The data indicate that the molecular species profile of nuclear diglycerides induced by α-thrombin at all times is significantly different from the profile of PE isolated from quiescent cells (bottom panels of Figures 5 and 6) or cells incubated with α-thrombin (data not shown).

DISCUSSION

The mechanism by which signals initiated at the plasma membrane modulate nuclear activities remains a pivotal question in signal transduction research. It is now clear that phospholipid metabolism and the generation of diglycerides play central roles in these mechanisms. In support of this

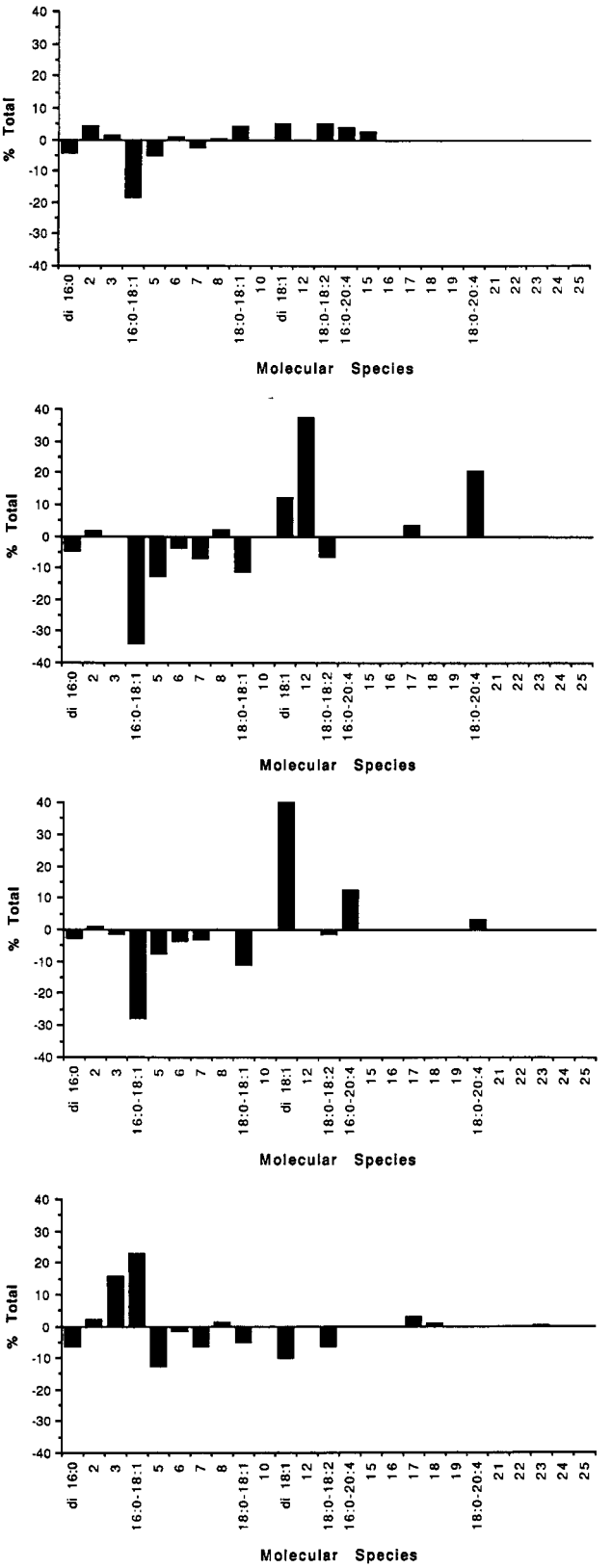

FIGURE 5: Comparison of induced nuclear diglycerides at 1 min with nuclear phospholipids. The induced species profile shown in the middle panel of Figure 4 was subtracted from the profiles of (top) PC, (second from top) PI, (third from top) PS, and (bottom) PE from quiescent cells. The values are shown as a percent of the total diglyceride in the phospholipid profile. The molecular species shown on the x axis are identified in Table 1. Normal variation in molecular species profiles is ±5%.

notion, cell-permeant diglycerides, and diglyceride "analogues", have been shown to stimulate a number of nuclear

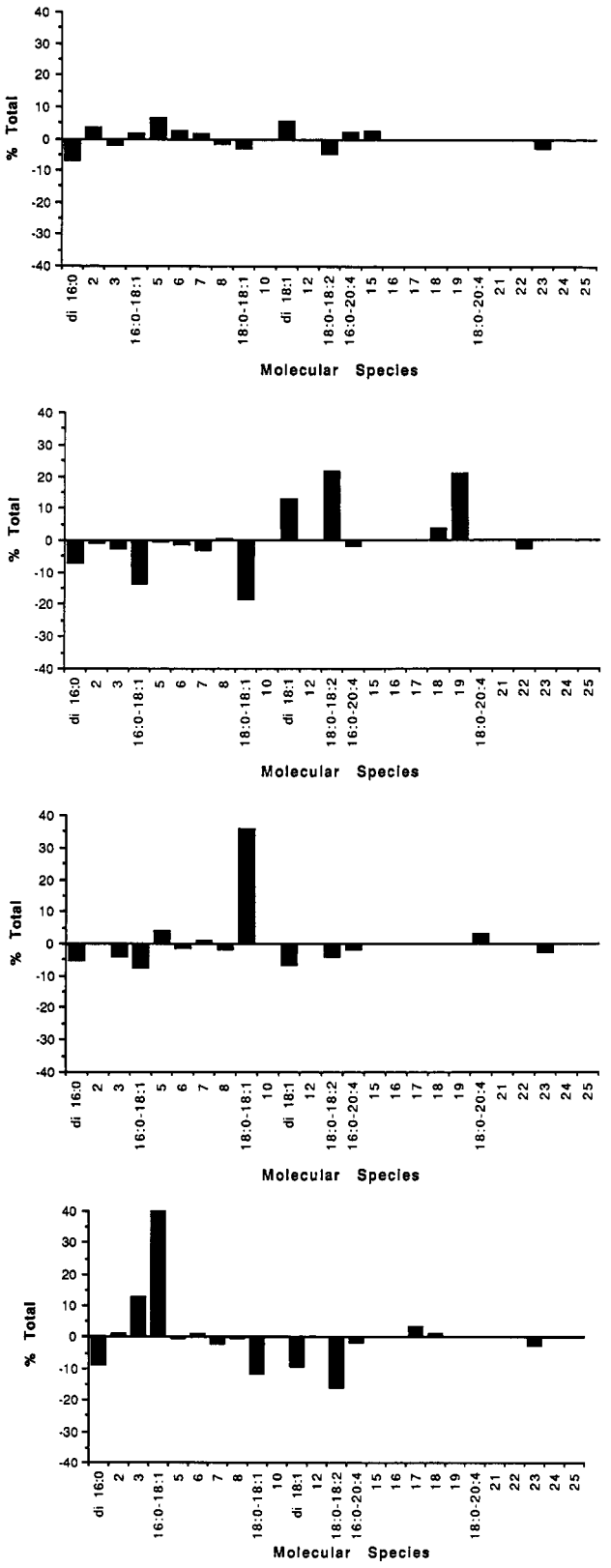

FIGURE 6: Comparison of induced nuclear diglycerides at 5 min with nuclear phospholipids. The induced species profile shown in the bottom panel of Figure 4 was subtracted from the profiles shown in (top) PC, (second from top) PI, (third from top) PS, and (bottom) PE from quiescent cells. The values are shown as a percent of the total diglyceride in the phospholipid profile. The molecular species shown on the x axis are identified in Table 1. Normal variation in molecular species profiles is ±5%.

activities including the expression of specific genes and DNA synthesis (Rozengurt et al., 1984; Davis et al., 1985; Karin & Smeal, 1992). It is becoming clear that second messengers may be generated in the nucleus. For example, releasable calcium pools in rat liver nuclei (Nicotera et al., 1990) and IP_3 receptor in *Xenopus* nuclear envelope vessicles (Sullivan et al., 1993) have been identified. Further, a number of laboratories have reported agonist-induced activation of PKC at the nucleus. In addition to our own studies (Leach et al., 1992), nuclear PKC activation has been seen in lymphocytes treated with PMA and 3T3 cells treated with IGF-1, and PDGF (Hornbeck et al., 1988; Fields et al., 1990; Hocevar & Fields, 1991). This activation of nuclear PKC has been implicated in modulating important nuclear activities such as the phosphorylation of transcription factors and factors involved in DNA replication (Karin & Smeal, 1992).

There is increasing evidence which indicates that the activation of nuclear PKC is correlated with an increase in nuclear diglycerides. In our recent study, we demonstrated that the addition of α-thrombin to quiescent IIC9 cells results in a rapid increase in nuclear diglycerides with a concomitant increase in the level of nuclear PKC-α (Leach et al., 1992). This is consistent with other reports demonstrating an agonist-induced increase in nuclear diglycerides and a concomitant increase in nuclear PKC activity (Cocco et al., 1988, 1989; Martelli et al., 1989; Cataldi et al., 1990; Divecha et al., 1991). However, it is important to note that it is difficult to compare these other studies with our present investigation. In the other studies, nuclei were prepared in the presence of detergents while in our study nuclei were isolated without detergents. The nuclei isolated in the other studies lacked at least a outer nuclear envelope which could result in a loss of nuclear diglycerides. Examination of the nuclei isolated by our procedure shows that they possess intact inner and outer membranes. Interestingly, if IIC9 nuclei are isolated in the presence of detergent, diglycerides are undetectable at any time at our level of sensitivity (data not shown). The control experiments we present in this paper show that when we analyze nuclear lipids by this method we are examining the lipids present in the nuclear membrane following stimulation of the cells. Contaminating membranes are not present, and redistribution and metabolism of lipids do not contribute to these results.

As mentioned, we have shown previously that α-thrombin induces a rapid increase in nuclear diglycerides with a concomitant increase in nuclear PKC-α (Leach et al., 1992). We have also shown that in whole cells, the activation of PKC was temporally related to the hydrolysis of PI and not PC (Leach et al., 1991). In this paper, we report a molecular species analysis of the nuclear diglycerides and phospholipids and demonstrate that the source of the diglycerides induced by a high concentration of α-thrombin is PC hydrolysis at all times. These data indicate that the diglycerides derived from PC in the nucleus under these conditions likely mediate the immediate activation of nuclear PKC-α, and may be involved in the activation of PKC-ϵ at later times (Ha & Exton, 1993). This is consistent with our observation that PC-derived diglycerides are capable of activating the endogenous PKC *in vitro* (Leach et al., 1992).

Our finding that nuclear diglycerides are not derived from nuclear PIs appears to be in contrast to reports implicating a turnover of nuclear PIs in modulation of nuclear PKC in 3T3 or (Divecha et al., 1991). These authors hypothesized that the PI being hydrolyzed was associated with chromatin, as the nuclear envelope in their preparation was removed by detergents. In these studies, however, diglyceride increased approximately 100 pmol/mg of protein above control levels

while PIP and PIP_2 decreased about 23 pmol/mg of protein. This suggests that approximately 23% of the diglyceride in these detergent-stripped nuclei was derived from PI hydrolysis. If a similar amount of PI hydrolysis occurred in our system, it would likely account for much less than 23% of the total diglyceride and may, therefore, escape detection. Together, these reports suggest that PI and PC both contribute to the formation of nuclear diglycerides, but the PI being hydrolyzed exists as a proteolipid component of chromatin (Rose & Frenster, 1965; Cocco et al., 1987; Manzoli et al., 1989) while the PC being hydrolyzed exists as a component of the two bilayers surrounding the nucleus.

In our preparation, PKC translocated to the nuclear fraction soon after DAG increased. Divechia et al. (1991) saw PKC translocation only in nuclei prepared in the absence of detergent. This supports the hypothesis that PC-derived diglyceride in the nucleus supports PKC activation.

The molecular species profile of nuclear PC is not distinct enough from the molecular species of PC present in intact cells to determine if nuclear diglyceride is derived from nuclear PC. Consequently, there are two possibilities for the source of nuclear diglyceride: either it is generated at the nuclear membrane from nuclear PC or it is generated in some other cellular compartment and is translocated to the nucleus *in vivo*.

If the first possibility is true, an increase in PC hydrolyzing activity at the nucleus would be responsible for the generation of the diglycerides. Indeed, consistent with an agonist-induced phosphoinositide turnover in some cells, PI-PLC-β1 has been identified in isolated rat liver nuclei (Divecha et al., 1993). PC hydrolyzing activity in the IIC9s may be increased by activation of an enzyme(s) localized in the nucleus, or by the induced translocation of the enzyme(s) to the nucleus in response to α-thrombin. It is interesting to note that we have found a PC hydrolyzing activity in our nuclear preparations.[2]

The second possibility, that diglycerides are generated in another compartment and are translocated to the nucleus *in vivo*, can occur in several ways. Diglycerides could be actively transported to the nucleus by an intracellular carrier protein. Such an event cannot be completely ruled out at this time. In this regard, however, we do not detect any lipid redistribution using fluorescent diglycerides,[3] rendering this possibility unlikely. Diglycerides could also simply diffuse into the nuclear membrane from the topologically contiguous ER membrane. A definitive answer to these questions requires further study.

One of the unexpected findings of this work was the dramatic change in PE molecular species after a 5-min incubation with α-thrombin (compare top and bottom panels of Figure 3). Species with later retention times on polar columns, such as the SP2380 used in this study, are generally either larger or less saturated. Nuclear PE may be remodeled upon α-thrombin stimulation, with smaller or more saturated fatty acids replaced with larger or less saturated fatty acids. Another possibility is, of course, *de novo* synthesis of larger or less saturated PE. It is interesting to speculate, that since the molecular species analysis indicates that the induced diglycerides are not derived from PE, present in quiescent or induced cells, this agonist-induced event may represent another important lipid-mediated signal transduction process. Further speculation on changes in nuclear PE would be unwarranted until the structural differences caused by mitogen stimulation are determined. We have begun GC/MS analysis of these lipids to further study this question.

The data in this report represent the first to our knowledge demonstrating an agonist-induced increase in diglycerides in intact nuclei and to identify PC as their source. It is interesting to speculate that a regulated metabolism of nuclear PC exists which serves an important role in signal transduction. Consistent with this notion, Kent and co-workers have recently reported the nuclear localization of CTP:phosphocholine cytidylyltransferase (Wang et al., 1993) which may be induced by diglycerides (Kent, 1990; Kolesnick & Hemer, 1990). In addition, the remodeled PE may serve other important signal transduction roles. Future experiments to define the role of these novel aspects of lipid metabolism are currently in progress.

[2] M. J. Jarpe and D. M. Raben, unpublished observation.

[3] L. A. Rangan and D. M. Raben, unpublished observation.

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