Metabolic Fate of Plasma Membrane Diacylglycerols in NIH 3T3 Fibroblasts*

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biochem (San Diego, CA) and phospholipases A from Crotalus durissus, C from Bacillus cereus, and D from Streptomyces cromofuscus were from Boehringer (Mannheim, Federal Republic of Germany). Polylysine (M, 30,000–70,000), 1-stearoyl-lyso-PC, oleic anhydride, thin layer chromatography (TLC) lipid standards, dioleoyl phosphatidylethanolamine, molybdenum blue, phosphatidylcholine, Dulbecco’s modified Eagle’s minimal essential medium (DMEM), fetal calf serum, and glutamine were from Sigma. Phosphate-buffered saline, CaCl2 and MgCl2 free (PBS) was from Gibco (Gatheringsburg, MD). RG 82067 (also designated RHC 80267 or RC 80267) was donated by Rorer Central (Horsham, PA). Rexyn 1–300 ion exchange resin and purified by blue or charring.

mM Tris-HCl, 30 mM sodium borate, 0.04% NaN3, pH 7.4, containing compound exactly co-chromatographs with the dioleoyl phosphatidic acid, [14C]PA) was synthesized by acylation of anhydride as described before. After thin layer chromatography of a mixture of the compound, no chemical or radioactive impurities were found. The product was purified by TLC.

The DGs were hydrolyzed to DG under these conditions as shown by TLC. No radioactive or chemical impurities were found. The cells were then centrifuged for 10 min at 10,000 rpm in the SS34 rotor of a high speed Sorvall centrifuge to remove any metal particles originating from the sonicator probe. To monitor the interaction of the lipid vesicles with the cells, we used two procedures: (a) N-Rh-PE, a nonexchangeable fluorescent phospholipid analog, incorporated into the liposomes (1 mol %) and (b) 5(6)-carboxyfluorescein (CF). In this case, we prepared the liposomes containing 0.15 M CF dissolved in 10 mM Tris-HCl, pH 7.4. These liposomes were then passed through a Sephadex G-50 column (1 × 20 cm), equilibrated, and eluted with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, to separate untrapped CF. At the concentration used, CF is self-quenched and, upon dilution, fluorescence increases markedly. Fluorescence microscopy revealed that the N-Rh-PE-labeled liposomes rapidly attach to the surface of the cells pretreated with polylsine. Diffuse fluorescence over the whole cells indicated fusion between plasma membranes and vesicles. There was, however, substantial punctate fluorescence, which remained cell-associated despite extensive washing. This suggests that part of the lipids associated with the cells are not incorporated to the membranes and are not available for metabolic processing. This possibility is supported by the observation that part of the lipids delivered by the liposome system remain unmetabolized after 2 h of incubation. When we used the CF-labeled liposomes, the cells showed green fluorescence, which indicates dilution of the CF in the cytoplasm and therefore, fusion of liposomes and PM.

In addition to the PS/PC liposomes, we used two alternative systems for delivering radioactive DGs: cardiolipin (CL) and DDB-PE liposomes. They were prepared as described above for the PS/PC liposomes using 2 µmol of CL or 1 mg of PE + 0.4 mg of DDB, respectively, for every 4 µCi of the radiolabeled DGs employed. In the experiments with CL liposomes, we took advantage of the induction of fusion of this kind of liposomes by acidic conditions (9). In this case, a two-step procedure was followed. First, the cells were washed twice with cold PBS and incubated in 0.9 ml of PBS + 0.1 ml of liposome suspension at 4 °C for 1 h. The cells were then washed twice with DMEM and incubated at 37 °C for 30 and 60 min, after which the lipids were extracted as described below. A mixture of PE and a cationic detergent has been shown to form liposomes that fuse with the plasma membrane and are an efficient means for cell transfection with exogenous DNA (10). In this work we have used PE and DDB as cationic lipid. This mixture is also an efficient DNA carrier for transfection of cells.2 We employed these liposomes as described for the PS/PC ones, except that the step of exposure to polylsine was omitted.

Preparation of DGs

The DGs 1-stearoyl-2-[1-14C]myristoyl-sn-glycerol ([14C]-SMG), 1,2-[1-14C]dioleoyl-sn-glycerol (14C]DAG), and 1-stearoyl-2-[1-14C]arachidonoyl-sn-glycerol ([14C]SAG) were prepared by phospholipase C hydrolysis of the corresponding phosphatidylcholines. Two to 4 µCi (0.036–0.07 µmol) of the pure radiolabeled phospholipids (without addition of unlabeled compound) were resuspended in 0.5 ml of 50 mM Tris-HCl, 30 mM sodium borate, 0.04% NaN3, pH 7.4, containing 25 µl of phospholipid C from Bacillus cereus, commercial preparation. After 30 min of incubation at 37 °C, the DG formed was extracted with 4 × 2 ml of water-saturated diethyl ether. The solvent was evaporated under nitrogen and the DGs were used directly in the experiments as described below. The phospholipids were completely hydrolyzed to DG under these conditions as shown by TLC.

Labeling Procedures

We tested a number of liposome systems for delivering the radiolabeled lipids to the PM of cultured NIH 3T3 fibroblasts. The protocol finally selected involved phosphatidylserine/phosphatidylcholine (PS/PC) liposomes which were induced to associate with the PM by brief pretreatment of the cells with polylsine (for the principles involved, see Stegmann et al. (7)). The procedure comprised three steps: 1) treatment of the cells for 1 min with 25 µg/ml of polylsine in PBS at room temperature; exposure to negatively charged PS/PC liposomes carrying the radioactive lipids (0.1 ml of liposome suspension + 0.9 ml of PBS) for 5 min at room temperature; and removal of the liposome suspension, washing twice with DMEM, and incubation for the indicated time periods at 37 °C, after which the lipids were extracted as described below. The liposome suspension was prepared as follows: aliquots of CHCl3 solutions containing 0.5 µmol of bovine heart PS and 0.5 µmol of egg PC were mixed with 4 µCi, with the microtip of an Ultrasonic W-385 sonicator at setting 4. The solvent was evaporated under nitrogen and further in vacuo for 30 min and 1 ml of 20 mM Tris-HCl, 150 mM NaCl, 0.1 mM EDTA, pH 7.4, was added. The lipids were suspended by vigorous vortexing with the aid of a glass bead and finally sonicated (2 × 1 min), under N2, with the microtip of an Ultrasonic W-385 sonicator at setting 4. The liposomes were then centrifuged for 10 min at 10,000 rpm in the SS34 rotor of a high speed Sorvall centrifuge to remove any metal particles originating from the sonicator probe. To monitor the interaction of the lipid vesicles with the cells, we used two procedures: (a) N-Rh-PE, a nonexchangeable fluorescent phospholipid analog, incorporated into the liposomes (1 mol %) and (b) 5(6)-carboxyfluorescein (CF). In this case, we prepared the liposomes containing 0.15 M CF dissolved in 10 mM Tris-HCl, pH 7.4. These liposomes were then passed through a Sephadex G-50 column (1 × 20 cm), equilibrated, and eluted with 0.15 M NaCl, 10 mM Tris-HCl, pH 7.4, to separate untrapped CF. At the concentration used, CF is self-quenched and, upon dilution, fluorescence increases markedly. Fluorescence microscopy revealed that the N-Rh-PE-labeled liposomes rapidly attach to the surface of the cells pretreated with polylsine. Diffuse fluorescence over the whole cells indicated fusion between plasma membranes and vesicles. There was, however, substantial punctate fluorescence, which remained cell-associated despite extensive washing. This suggests that part of the lipids associated with the cells are not incorporated to the membranes and are not available for metabolic processing. This possibility is supported by the observation that part of the lipids delivered by the liposome system remain unmetabolized after 2 h of incubation. When we used the CF-labeled liposomes, the cells showed green fluorescence, which indicates dilution of the CF in the cytoplasm and therefore, fusion of liposomes and PM.

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Cell Treatments

The cells were incubated with these agents for 30 min prior to the addition of liposomes. A mixture of PE and a cationic detergent has been shown to form liposomes that fuse with the plasma membrane and are an efficient means for cell transfection with exogenous DNA (10). In this work we have used PE and DDB as cationic lipid. This mixture is also an efficient DNA carrier for transfection of cells.2 We employed these liposomes as described for the PS/PC ones, except that the step of exposure to polylsine was omitted.

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Thin Layer Chromatographic Separation of Lipids

Two procedures were used for lipid analysis: (a) samples of labeled cellular lipids were separated on Silica Gel 60 or LK60D plates,
developed in two steps in the same direction: first, with CHCl₃/
CH₃OH/H₂O (65:35:2, v/v) to a height of 12 cm (for separation of
polar lipids) and then, with petroleum ether (30–60 °C)/diethyl ether/
acetic acid (90:20:2, v/v) to the top (to separate neutral lipids).
Radioactive lipids were located by autoradiography, exposing Kodak
XAR films for 48 h at -70 °C. (b) We also analyzed samples of lipids
on separate plates for neutral or polar lipids using the same solvent
mixtures as described above. For quantification, unlabeled TLC
standards were added to the samples and lipid spots were detected by
exposure to iodine vapor, marked, and scraped into scintillation vials
after allowing evaporation of the iodine. The samples were counted
in a Packard Tri-Carb Liquid Scintillation Analyzer 1900 CA, using
Ecoscint scintillation fluid.

RESULTS

Metabolic Fate of [1-¹⁴C]Dioleoyl Glycerol ([¹⁴C]DOG) and
[1-¹⁴C]Oleic Acid ([¹⁴C]18:1)—We employed the PS/PC liposome
system to label the plasma membrane of NIH 3T3 fibroblasts. The cells were briefly exposed to polylysine im-
nediately before a short incubation with the liposomes containing
either [¹⁴C]DOG or [¹⁴C]18:1. These were then re-
moved and the cells washed as described under "Experimental
Procedures." The labeled cells were incubated at 37 °C for 30
min, at the end of which their lipids were extracted and
analyzed by TLC. In Fig. 1 we show autoradiograms of TLC
plates developed in two steps for separation of both polar and
neutral lipids. Quantitative results are shown in the diagrams
of Fig. 2. The most important cellular lipids labeled as a result of the metabolism of [¹⁴C]DOG are PC and TG, followed by
FA, MG, and PA. There are striking differences when the precursor is the fatty acid [¹⁴C]18:1. These include the labeling

Fig. 1. Labeling patterns of NIH 3T3 fibroblasts obtained
using different precursors. Autoradiograms of thin layer chro-
nomatographic separations of lipids from cells incubated for 30 min
after labeling with precursors and then extracted for the fatty acids using PS/PC liposomes, as described under "Experimental
Procedures." Lanes designated DOG, SMG, and SAG correspond to
cells labeled with the diacylglycerols [¹⁴C]DOG, [¹⁴C]SMG, and [¹⁴C]
SAG, respectively. Lanes 18:1, 14:0, and 20:4 correspond to cells labeled with the fatty acids [¹⁴C]18:1 (oleic acid), [¹⁴C]14:0 (myristic
acid), and [¹⁴C]20:4 (arachidonic acid). The DGs appear in two spots
corresponding to the positional isomers sn-1,2 (2, 3) and sn-1,3,
originated by acyl migration upon addition to the culture. The differ-
ent DGs and fatty acids originate different patterns of labeling, indicating distinct metabolic pathways in each case.

Effects of R59022 and RG 80267 on the Metabolism of [¹⁴C]
DOG—Cells were preincubated with 10 μM R59022, a DG
kinase inhibitor (11), 30 μM RG 80267, inhibitor of DG lipase
(12), or the same amount of solvent (0.5% dimethyl sulfoxide),
for 30 min and then they were labeled with PS/PC liposomes carrying [¹⁴C]DOG. After incubation of the labeled cells for
60 min, the cellular lipids were extracted and separated as
described under "Experimental Procedures." The results in
Table I show that R59022 does not affect the conversion of
[¹⁴C]DOG to PC and TG but strongly reduces the incorpora-
tion to PA and PI. In the presence of RG 80267, the amount
of [¹⁴C]DOG converted into FA and MG is markedly reduced,
but the amounts converted to PC and TG are not altered.

Metabolism of [¹⁴C]Myristate- and [¹⁴C]Arachidonate-la-

FIG. 2. Distribution of radioactivity in cellular lipids after
labeling with [¹⁴C]DOG (left panel) or [¹⁴C]18:1 (right panel).
Results are expressed as percentages of the total radioactivity incor-
porated into cellular lipids other than the precursor used, marked with an asterisk (*). In the case of [¹⁴C]DOG, the total radioactivity in all lipids except DG averaged 24,708 cpm which corresponds to
22.5% of the total radioactivity associated with the cells. The remain-
der of the radioactivity is probably present in nonfused liposomes
that account for the punctate fluorescence in N-Rh-PE-labeled lipo-
somes (see "Experimental Procedures"). In the case of [¹⁴C]18:1, total
radioactivity in all lipids other than FA was 57,390 cpm. Data are
means ± S.E. of three parallel cultures and are representative of three
similar experiments. Abbreviations are as described in the legend to
Fig. 1.

Table I

| Control | R59022 | RG 80267 |
|---------|--------|----------|
| cpm     |        |          |
| PC      | 11,782 ± 1,296 | 11,577 ± 1,575 | 11,025 ± 1,488 |
| PI      | 831 ± 124 | 111 ± 20 | 887 ± 133 |
| PA      | 962 ± 144 | 327 ± 52 | 1,230 ± 170 |
| MG      | 5,101 ± 637 | 5,652 ± 661 | 1,541 ± 231 |
| FA      | 6,047 ± 786 | 6,566 ± 821 | 2,078 ± 307 |
| TG      | 6,848 ± 712 | 7,331 ± 799 | 6,955 ± 835 |
| CE      | 398 ± 59 | 422 ± 67 | 366 ± 60 |

of PE only when [¹⁴C]18:1 acts as precursor. Likewise, PI
labeling is much stronger in this case. Among the neutral lipids, alkyl-acylglycerol (AG), migrating between DG and FA is
labeled with [¹⁴C]18:1 but not with [¹⁴C]DOG. These results
indicate that different sets of reactions account for the
metabolism of DOG and 18:1. When the incubation of the
cells labeled with [¹⁴C]DOG is carried out at 18 °C instead of
37 °C, the same basic pattern arises, although relatively higher
amounts of MG and fatty acids (FA) accumulate in the cells
(not shown). We also investigated two alternative liposome
carriers: cardiolipin liposomes, which fuse with the plasma
membrane at acidic pH (9), and DDAB-PE liposomes, that fuse
at neutral pH values (10). We found that the PS/PC
system was the most efficient in labeling the cells, followed
by the cardiolipin liposomes. Parallel experiments using the
three systems showed the same basic patterns of cellular lipid
labeling irrespective of the system used (data not shown).

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Metabolism of [¹⁴C]Myristate- and [¹⁴C]Arachidonate-la-
In this investigation, we have used a new methodology to associate [14C]DOG with the plasma membrane of NIH 3T3 cells. In Fig. 5 quantitative information on the time course of [14C]SMG metabolism is shown. The results shown in Table II emphasize the differences discussed between the fates of the two DG molecular species and also between labeling the cells with either DGs or free fatty acids. In Fig. 4, the time course of conversion of [14C]SMG and [14C]SAG to other lipids is shown separately for polar and neutral lipids. Examination of the conversion to polar lipids indicates that PC is a major end product for both DGs. PA is formed notably only in the case of [14C]SAG and seems to have an early peak at or before 15 min. Labeling of PI increases steadily with time, but the amounts formed do not account for the decrease observed in PA after 15 min. Thus, PA is being converted to other lipids in addition to PI. The neutral lipid panel shows the striking conversion to TG in the case of [14C]SMG. This lipid also becomes labeled from [14C]SAG but to a lesser extent and in a transient fashion. TG labeled from [14C]SAG peaks at 30 min and thereafter decreases, whereas that labeled from SMG as the precursor appears to be a more stable cellular lipid. Both DGs are subject to lipolytic attack, indicated by the appearance of FA and MG.

**Metabolism of [14C]Glycerol Backbone-labeled Dioleoyl Phosphatidic Acid**—We have prepared 1,2-dioleoyl-sn-[[1-14C]glycerol-3-phosphate ([14C]PA) from [U-14C]-sn-3-glycerol phosphate by acylation with oleic anhydride. The metabolism of this compound delivered via the PS/PC liposomes was examined in NIH 3T3 fibroblasts. Initially, all the radioactivity is present exclusively as PA. After 15 min of incubation, considerable conversion to DG and PC is evident. The only other radioactive compounds detected are TG and minor amounts of MG and PI.

**DISCUSSION**

In this investigation, we have used a new methodology to associate [14C]DOG with the plasma membrane of NIH 3T3 cells. The predominant species labeled as a result of SMG metabolism is TG, with a smaller but significant amount of PC labeled, but not PE or PI, and only a barely detectable amount of PA. In contrast, in the case of SAG, the major species labeled is PC, with much smaller labeling of TG but significant labeling of PA and PI.

In Fig. 3a the patterns obtained using free [14C]14:0 or [14C]20:4, delivered by the same liposome procedure are plotted. In both cases, the labeling is markedly different from that obtained with the corresponding DGs. A noteworthy difference in the case of [14C]SMG versus [14C]14:0 is the appearance of AG and PE only when the fatty acid is used as precursor. In the case of [14C]SAG versus [14C]20:4, also PE labels only with the fatty acid. Moreover, free arachidonate is a poor precursor for TG while sizeable amounts of TG are labeled from [14C]SAG. In Table II, we compare the ratios of TG and PI to PC for the two DGs and the corresponding fatty acids. The results shown in Table II emphasize the differences discussed between the fates of the two DG molecular species and also between labeling the cells with either DGs or free fatty acids.

**TABLE II**

|       | TG/PC | PI/PC |
|-------|-------|-------|
| [14C]SMG | 2.565 | 0.008 |
| [14C]SAG | 0.495 | 0.074 |
| [14C]14:0 | 1.490 | 0.031 |
| [14C]20:4 | 0.044 | 0.392 |

Plasma Membrane Diacylglycerols

**FIG. 4.** Time course of labeling of cellular lipids with either \(^{14}C\)SMG or \(^{14}C\)SAG. The results are expressed in percentages of radioactivity recovered in each lipid spot with respect to the total radioactivity recovered from all lipids other than the precursor diacylglycerols at each time point. Data are means of two parallel cultures and are representative of three separate experiments.

**FIG. 5.** Time course of labeling of cellular lipids from \(^{14}C\)PA. Results are percentages of radioactivity measured in the different lipids with respect to the total radioactivity recovered from all lipids other than PA. Data are means of two parallel cultures and are representative of three similar experiments.

fibroblasts. Upon incubation of the labeled cells, the radioactivity is recovered mainly in PC and TG and, to a lesser extent, in other lipids, like MG, FA, PA, and PI. The pattern observed is markedly different from the one resulting from labeling with \(^{14}C\)18:1 under the same conditions. These results indicate that incorporation of \(^{14}C\)DOG into cellular lipids does not proceed to any significant extent through prior lipolytic breakdown to free fatty acids, and imply that the DGs supplied directly enter the biosynthetic pathways leading to PC and TG. Since the enzymes catalyzing these conversions are known to reside in the endoplasmic reticulum (14), our finding provides the first experimental evidence for a transport process conveying naturally occurring DGs from the plasma membrane (PM) to internal membranes. Previously, Pagano and co-workers (15-17) had obtained evidence of translocation of fluorescent DG analogs and their conversion to TG and PC. As is the case with our own previous studies with \(^{14}C\)diC6,3 the physiological relevance of their findings remained to be determined. Indeed, the relatively polar nature of the lipids utilized makes them significantly different in their physicochemical properties compared to longer chain natural lipids. The importance of chain length was emphasized by studies of Nichols and Pagano (18). Thus, when these authors compared the spontaneous diffusion rates of C12-NBD-PC and C6-NBD-PC, they found that the latter transfers spontaneously more than 2 orders of magnitude faster than the first. For this reason, our experiments utilizing radioactively labeled DGs that occur naturally, like DOG, SMG, and SAG, are critical in providing convincing evidence of DG transporting mechanisms in intact cells. They also help to clarify how PM DGs are metabolized.

We found similar results using three alternative and unrelated liposome systems for delivery of radiolabeled DOG. The three systems have different efficiency in labeling the cells, but the patterns obtained are closely similar. This strongly suggests that any alterations of the PM lipid composition induced by the liposomes do not affect the processes studied. Furthermore, in our previous investigation on the metabolism of cell permeant diC6,3 no liposome system was used, ruling out membrane alterations, and the results were essentially the same as those found here. Incubation of \(^{14}C\)DOG-labeled cells at 18 °C, a temperature that blocks fusion of pinocytic vesicles with lysosomes (19), also results in labeling of PC and TG predominantly. This supports the notion that lysosomal degradation of the radiolabeled lipids plays no significant role in the processes observed.

The results of our experiments using the inhibitor of DG lipase RG 80267 are consistent with the notion that release of FA from DG does not precede the incorporation of label from \(^{14}C\)DOG into cellular lipids, as strong inhibition of the appearance of MG and FA does not result in decreased amounts of PC and TG. This further supports the view that the DGs are incorporated as intact molecules. The inhibitor of DG kinase had strong inhibitory effects on PI formation, which suggests that this compound effectively inhibits a DG

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\(^2\) J. Florin-Christensen, M. Florin-Christensen, J. M. Delfino, and H. Rasmussen, submitted for publication.
kinase involved in the PI cycle. More importantly, the observation that PC and TG labeling is unaffected by R59022 suggests that the transfer of DG molecules to the sites of biosynthesis of these two cellular lipids does not require conversion to PA, i.e. DGs are likely to be translocated as such within the cells. The mechanism by which such transfer can take place deserves further study. The fact that lysosomal degradation appears not to be involved suggests that transport on endosomal membranes is not a prevailing process for DG transfer. Our analysis of the lipids associated with the cells shows that substantial amounts of DGs remain unmetabolized even after prolonged incubation and our fluorescence microscopy observations indicate that, in addition to fusion, liposomes can become rapidly attached to the cells in a punctate pattern. These results are consistent with the notion that part of the liposomes associate with the cells without fusion and that DGs contained in these liposomes remain excluded from cellular metabolic processes. They emphasize that close apposition of lipid bilayers is not sufficient for the exchange of DGs. Translocation of DGs within the cells, thus, calls for specific DG transfer proteins. This translocation raises the question as to how such transfer can take place deserves further study. The facts that lysosomal degradation is not a prevailing process for DGs and that DGs contained in these liposomes remain unmetabolized from cellular metabolic processes suggest that the mechanism by which such DGs are transferred to the cells may involve specific DG transfer proteins that has not yet been reported.

In this study, we examined the fate of different DG molecular species. We found that two naturally occurring DGs, SAG and SMG, are metabolized at different rates. We have identified three kinds of metabolic reactions affecting these DGs. In order of importance they are: (i) conversion to either PC or TG, which involves the action of two enzymes: CDP-choline:DG cholinephosphotransferase and acyl-CoA:DG acyltransferase, respectively; (ii) lipolytic breakdown to yield MG and FA, and (iii) phosphorylation to PA by DG kinase. The resulting PA can be further metabolized to PI. It can be also hydrolyzed back to DG (see below). The rates at which these different processes take place are markedly dependent on the DG molecular species examined. Both [14C]SMG and [14C]SAG are good precursors for cellular PC, but they differ in the rates at which they are converted to TG and PA/PI. [14C]SMG is a good precursor for TG and a poor precursor for PA/PI. The opposite is true for [14C]SAG. These observations most probably reflect the substrate specificities of the different enzymes involved. Our findings agree well with a recent report showing that DG kinase can show marked substrate specificity towards arachidonate-containing DGs in Swiss 3T3 cells (22).

Recently, Simpson et al. (23), studied the metabolism of exogenous plasma membrane DGs radiolabeled on their glycerol moieties in Swiss 3T3 fibroblasts. They reported that a number of enzymes prefer arachidonoyl- to oleoyl-MGs. Their results led them to propose that an initial phosphorylation of the monoarachidonoylglycerol channels the glycerol backbone to PA and PI, whereas acylation to DG directs the labeled glycerol backbone to PC and TG. Our present findings are in excellent agreement with this proposal.

We studied the labeling patterns obtained using two very different fatty acids, [14C]14:0 and [14C]20:4. These experiments provide useful information regarding two aspects. First, we found that markedly different patterns are observed when the labeled fatty acid is provided as a free compound or esterifying position sn-2 of a DG. Second, they show that labeling of cellular PI with the free fatty acids is much stronger than that observed with the corresponding DGs, as reflected in the ratios of labeling of PI to PC shown in Table II. These findings indicate that PI biosynthesis is more closely connected to de novo synthesis of phospholipids than to recycling of DGs from the PM, as compared to PC.

We examined the fate of the radiolabeled glycerol backbone when supplied to the PM as [14C]glycerol-dioleoyl PA via PS/PC liposomes. The results have two noteworthy aspects. First, the PA is rapidly converted to DG, strongly indicating the presence of PA phosphohydrolase activity at the PM. This is in agreement with results reported previously using fluorescent phospholipid analogs (24). The DG formed is thereafter rapidly cleared from the cells. Second, the glycerol backbone labels cellular lipids, precisely as expected if conversions to PC and TG were the major processes withdrawing DGs, since these are almost the only lipids in which the radioactivity is recovered in these experiments. Formation of PI is, indeed, a very minor event. These results considerably strengthen the conclusions reached using fatty acyl-labeled DGs discussed above.

It is now generally accepted that, in addition to phosphoinositides, PC is an important source of second messenger DGs (25, 26). It should be noted, therefore, that if the PI cycle were the predominant cellular means of DG removal, its operation would lead to an expansion of the PI pool at the expense of PC. This is against available experimental evidence (27). It has also been shown that phorbol esters stimulate both PC breakdown and biosynthesis (28-30). Thus, it seems reasonable to propose that a PC cycle could be activated in connection with signaling events (31). Our finding, that naturally occurring DG molecular species associated with the PM are precursors of cellular PC, substantiates the notion that such a cycle may indeed take place.

From this work, a new picture for the metabolic fate of plasma membrane DGs arises. In it, new processes emerge as key components of the cellular mechanism for DG signal termination. We recognized that the conversions catalyzed by transferases that act on DG molecules to yield PC or TG are the most important metabolic processes affecting cellular DGs initially associated with the PM. On the other hand, breakdown by DG lipase and phosphorylation to PA by DG kinase, two pathways demonstrated in platelets, seem to play a less important role in 3T3 fibroblasts. The relative rates of these possible conversions appear to be dictated by the fatty acyl composition of the particular molecular species. Indeed, differential channeling of the diverse molecular species may explain how, upon cell stimulation by DG-enhancing agonists, some of the DG species represent only brief signals, while others, lasting longer, can support sustained cell activation (32).

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