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New patterns of diacylglycerol metabolism in intact cells

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The metabolism of di[1-^{14}C]octanoylglycerol metabolism was examined in four cell lines: NIH 3T3 fibroblasts, BHK cells, Pam 212 keratinocytes and WEHI 3BD+ cells. We found the direct conversion of 1,2-di[1-^{14}C]octanoyl-*sn*-glycerol ([^{14}C]diC_8) into dioctanoylphosphatidylcholine and dioctanoylacylglycerol, but no formation of phosphatidylinositol. The [^{14}C]diC_8 also underwent lipolytic breakdown. In contrast, 1-[1-^{14}C]oleoyl-2-acetyl-*sn*-glycerol was metabolized exclusively by lipolysis. Our findings support a new scheme for the metabolic termination of diacylglycerol signals.

INTRODUCTION

Diacylglycerols (DGs) are now recognized to be important second-messenger molecules that stimulate protein kinase C (PKC), an enzyme that appears to be implicated in the regulation of a number of cellular processes [1–3]. One kind of evidence that provides support for this role of DGs has been provided by research in which cell-permeant DGs, such as dioctanoylglycerol (diC_8) and oleoylacetylglycerol (OAG), have been employed. These types of compounds can stimulate PKC activity in intact cells [4] and also in platelets [5]. Other support for the messenger role of DGs comes from the observation that stimulation by diverse agonists in many cell types induces an increase in DG content and activation of PKC [1–3].

Second-messenger DGs, initially thought to originate from phosphoinositide (PI) turnover, are now believed to derive also from phosphatidylcholine (PC), upon hydrolysis by phospholipase C or by the sequential action of phospholipase D and phosphatidic acid (PA) hydrolase. In many cases, PC appears to be quantitatively the most important source of DG signals [6,7].

For DGs to function as second messengers, once generated they should undergo rapid elimination in order to terminate the cellular response. The mechanisms involved in the removal of DGs have been investigated in human platelets [8–12]. These naturally occurring cellular fragments respond to thrombin with phospholipase C-mediated PI breakdown [13]. Majerus and co-workers have proposed that lipolysis of the DGs to monoacylglycerol (MG) and fatty acids is an important pathway for signal termination [10]. Other authors suggest that phosphorylation of DGs by DG kinase plays a major role in the metabolism of these compounds. This viewpoint is supported by studies in which inhibitors of this activity have been employed [11,14]. The PA generated by DG kinase can then be converted into PI, replenishing the PI pool, a process known as the PI cycle [15]. Bishop and Bell [12] investigated the metabolism of diC_8 in platelets and found considerable conversion of this compound into PA and PI in this system. Operation of a PI cycle for removal of DGs may appear adequate in systems in which PI is the only source of DGs. However, in view of the recently disclosed role of PC as an important source of messenger DGs [6,7], this pathway may not account for messenger termination, and a PC cycle would seem a more plausible possibility. In the present work, the metabolism of diC_8 and OAG in several cell types was studied. Our results show that intact whole cells in culture metabolize DGs in a different manner from that observed in platelets, and support a new view of how DG signals may be terminated.

EXPERIMENTAL

Materials

The radiochemicals 1,2-di[1-^{14}C]oleoyl-*sn*-3-phosphatidylcholine (105 μCi/μmol) and [1-^{14}C]oleic acid ([^{14}C]18:1; 58 μCi/μmol) were purchased from Amersham (Arlington Heights, IL, U.S.A.). [1-^{14}C]Octanoic acid ([^{14}C]8:0; 58 μCi/μmol) was from NEN (Boston, MA, U.S.A.). Dicyclohexylcarbodi-imide and 4-dimethylaminopyridine (DMAP) were from Aldrich (Milwaukee, WI, U.S.A.); R59022 was from Calbiochem (San Diego, CA, U.S.A.); and phospholipases A_2, C and D were from Boehringer (Mannheim, Germany). Lipid standard mixtures (mono-oleoylglycerol/dioleoylglycerol/trioleoylglycerol; cholesterol/fatty acid/trioleoylglycerol/cholesteryl oleate and sphingomyelin (SM)/PC), lysoPC, PC, PI, phosphatidylethanolamine (PE), PA, dioctanoyl-PC, diC_8, 1,2-dioleoyl-*sn*-glycerol, 1-oleoyl-2-acetyl-*sn*-3-PC, glycerophosphocholine ($CdCl_2$ complex), dioctanoylethyleneglycol (diC_8EG), octanoic anhydride and oleic anhydride were from Sigma (St. Louis, MO, U.S.A.). Rexyn I-300 was from Fisher Scientific (Pittsburgh, PA, U.S.A.), and LK60D plates were from Whatman (Hillsboro, OR, U.S.A.). Silica gel H60 thin-layer plates and analytical-grade solvents were from Merck (Darmstadt, Germany). Phosphorus pentoxide and acetic anhydride were from Baker (Phillipsburg, NJ, U.S.A.), and Ecoscint scintillation liquid was from National Diagnostics (Manville, NJ, U.S.A.). RG 80267 (also designated RHC 80267 and RC 80267) was donated by Rorer Central (Horsham, PA, U.S.A.).

Cells and cultures

NIH 3T3 fibroblasts and WEHI 3BD+ murine myelomonocyte cells were obtained from the Yale Cancer Comprehensive Center collection. BHK (baby-hamster kidney) cells were a gift from Dr. Ari Helenius, Department of Cell Biology, Yale School of

Abbreviations used: DG, diacylglycerol; MG, monoacylglycerol; TG, triacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; diC_8, dioctanoylglycerol; [^{14}C]diC_8, 1,2-di[1-^{14}C]octanoyl-*sn*-glycerol; [^{14}C]8:0, [1-^{14}C]octanoic acid; [^{14}C]18:1, [1-^{14}C]oleic acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; [^{14}C]OAG, 1-[1-^{14}C]oleoyl-2-acetyl-*sn*-glycerol; DMAP, 4-dimethylaminopyridine; PKC, protein kinase C; DMEM, Dulbecco's modified minimal essential medium; diC_8EG, dioctanoylethyleneglycol.

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Medicine. Pam 212 cells were a gift from Dr. G. P. Dotto, Department of Pathology, Yale School of Medicine. NIH 3T3, BHK and Pam 212 cells were cultured in 60 mm Petri dishes in Dulbecco's modified minimal essential medium (DMEM), supplemented with 2 mM glutamine and 10% fetal-calf serum. Confluent cultures were then transferred to DMEM without serum for 24 h before the experiments. WEHI 3BD+ murine leukaemia cells were cultured with stirring in spinner flasks with RPMI 1640 medium supplemented with 10% fetal-calf serum. Cells were washed in fresh RPMI 1640 medium without serum immediately before the experiments.

Synthesis of 1,2-di[1-^{14}C]octanoyl-*sn*-3-PC ([^{14}C]diC$_8$PC)

This compound was synthesized as described by Gupta et al. [16] by reaction of [1-^{14}C]octanoic anhydride with dry glycerophosphocholine–$CdCl_2$ complex in the presence of DMAP. The final radiolabelled product was free from radioactive impurities, as shown by t.l.c. and autoradiography, and no other products were detected by exposure to iodine vapours, staining with Molybdenum Blue and charring. It co-chromatographed exactly with commercial dioctanoyl-PC, and yielded the expected reaction products on treatment with phospholipases C, D and A$_2$.

Synthesis of 1-[1-^{14}C]oleoyl-2-acetyl-*sn*-3-PC

For the preparation of this phospholipid, we started from 1-[1-^{14}C]oleoyl-lysoPC, prepared by hydrolysis of di[1-^{14}C]oleoyl-*sn*-3-PC with phospholipase A$_2$ from *Crotalus durissus*. The radiolabelled lysoPC was purified by t.l.c. thoroughly dried and acetylated by reaction with acetic anhydride in the presence of DMAP. The 1-[1-^{14}C]oleoyl-2-acetyl-*sn*-3-PC was purified by t.l.c. using commercial 1-oleoyl-2-acetyl-*sn*-3-PC as standard.

Preparation of [^{14}C]diC$_8$ and [^{14}C]OAG

These DGs were prepared by hydrolysis of the corresponding phospholipids with phospholipase C from *Bacillus cereus*, as described previously [17]. Reaction variables were adjusted so as to minimize isomerization of the DGs. However, partial conversion into the *sn*-1,3 isomers was observed on addition to the cell culture medium.

Synthesis of unlabelled triacylglycerols (TGs)

Dioctanoyloleoylglycerol was prepared by acylation of diC$_8$ with oleic anhydride in the presence of DMAP, in anhydrous chloroform/pyridine (4:1, v/v). The conditions for acylation were as those previously described [16]. Similarly, dioleoyloctanoylglycerol was prepared by reaction of 1,2-dioleoyl-*sn*-glycerol with octanoic anhydride. In both cases, acylation was allowed to proceed at room temperature for 30 h in the dark, at the end of which the mixtures were analysed by t.l.c. in hexane/diethyl ether/formic acid (40:10:1, by vol.) as solvent system. The mixtures only showed the expected TGs, non-esterified fatty acid and non-migrating material, including DMAP. The TGs were eluted from the plates with chloroform and stored under nitrogen at −20 °C. The R_F values of the different TGs in this system were 0.65 for trioleoylglycerol, 0.55 for octanoyldioleoylglycerol and 0.50 for oleoyldioctanoylglycerol.

Preparation of unlabelled dioctanoyl-PA and mono-octanoylglycerol

The first of these lipids was prepared by phospholipase D (*Streptomyces chromofuscus*) hydrolysis of commercial dioctanoyl-PC, whereas mono-octanoylglycerol was obtained by sequential hydrolysis of dioctanoyl-PC with phospholipase A$_2$ (*Crotalus adamanteus*) and phospholipase C (*Bacillus cereus*).

Labelling procedures

(a) [^{14}C]diC$_8$ and [^{14}C]8:0. After evaporation of the solvents under nitrogen, these two lipids were resuspended in DMEM for NIH 3T3, BHK and Pam 212 cells, or in RPMI 1640 for WEHI 3BD+ cells, supplemented with 1% BSA, and 0.1 ml of the suspensions were added to 2 ml of culture media. Preliminary experiments showed that octanoic acid is poorly taken up by the cells, compared with diC$_8$. To obtain comparable labelling, about 20 times more radioactivity had to be used with the fatty acid. The final concentration of [^{14}C]diC$_8$ was 10^5 c.p.m./ml, or about 0.4 μM, and that of [^{14}C]8:0, 2×10^6 c.p.m./ml, or about 16 μM.

(b) [^{14}C]OAG and [^{14}C]18:1. These lipids were also freed from organic solvent and resuspended in DMEM containing 1% BSA. A 0.1 ml portion of the suspension was added to 2 ml of culture medium in each dish. The final concentration was 10^5 c.p.m./ml, equivalent to about 0.75 μM for both [^{14}C]OAG and [^{14}C]18:1.

Cell treatments

The cells were preincubated with the different agents (the inhibitors of DG kinase R59022 [14] and diC$_8$EG [11] and the DG lipase inhibitor RG 80267 [18]) for 30 min before the experiments. All substances were added from 200× concentrated stock solutions in dimethyl sulphoxide. Control samples received the same amount of dimethyl sulphoxide (0.5% final concn.).

Lipid extraction

After incubation for the selected time periods, lipids were extracted by a modification of the method of Bligh and Dyer [19]. The medium from the dishes containing NIH 3T3, BHK or Pam 212 cells was aspirated, the cell monolayers were washed twice with cold phosphate-buffered saline (PBS), pH 7.4, and 1.9 ml of methanol/water (10:9, v/v) was added. The cells were then scraped into glass tubes containing 1 ml of chloroform. After mixing and centrifugation, the lower phase was separated and evaporated under nitrogen and the lipids were dissolved in chloroform. For WEHI 3BD+ cells, samples of the cell suspensions were sedimented by centrifugation, washed once with cold PBS and resuspended in 400 μl of this buffer, and the lipids were extracted as described by Bligh and Dyer [19].

T.l.c. separation of lipids

Samples of cellular lipids labelled with [^{14}C]diC$_8$ and [^{14}C]8:0 were analysed by t.l.c. on 20 cm × 20 cm silica gel 60 or LK60D plates, developed in two steps in the same direction: first, with chloroform/methanol/water (26:14:1, by vol.) for a distance of 12 cm (for separation of polar lipids) and then with light petroleum (b.p. 30–60 °C)/diethyl ether/acetic acid (45:10:1, by vol.) to the top (to separate neutral lipids). Spots of radioactive lipids were located by autoradiography, upon exposure of Kodak XAR films for 48 h at −70 °C. The autoradiograms were used to locate the lipid spots which were removed from the plates and quantified by liquid-scintillation counting in a Packard Tri-Carb liquid scintillation analyser 1900 CA, using Ecoscint as scintillation fluid. A similar method was used for the autoradiographic examination of lipids labelled with [^{14}C]OAG and

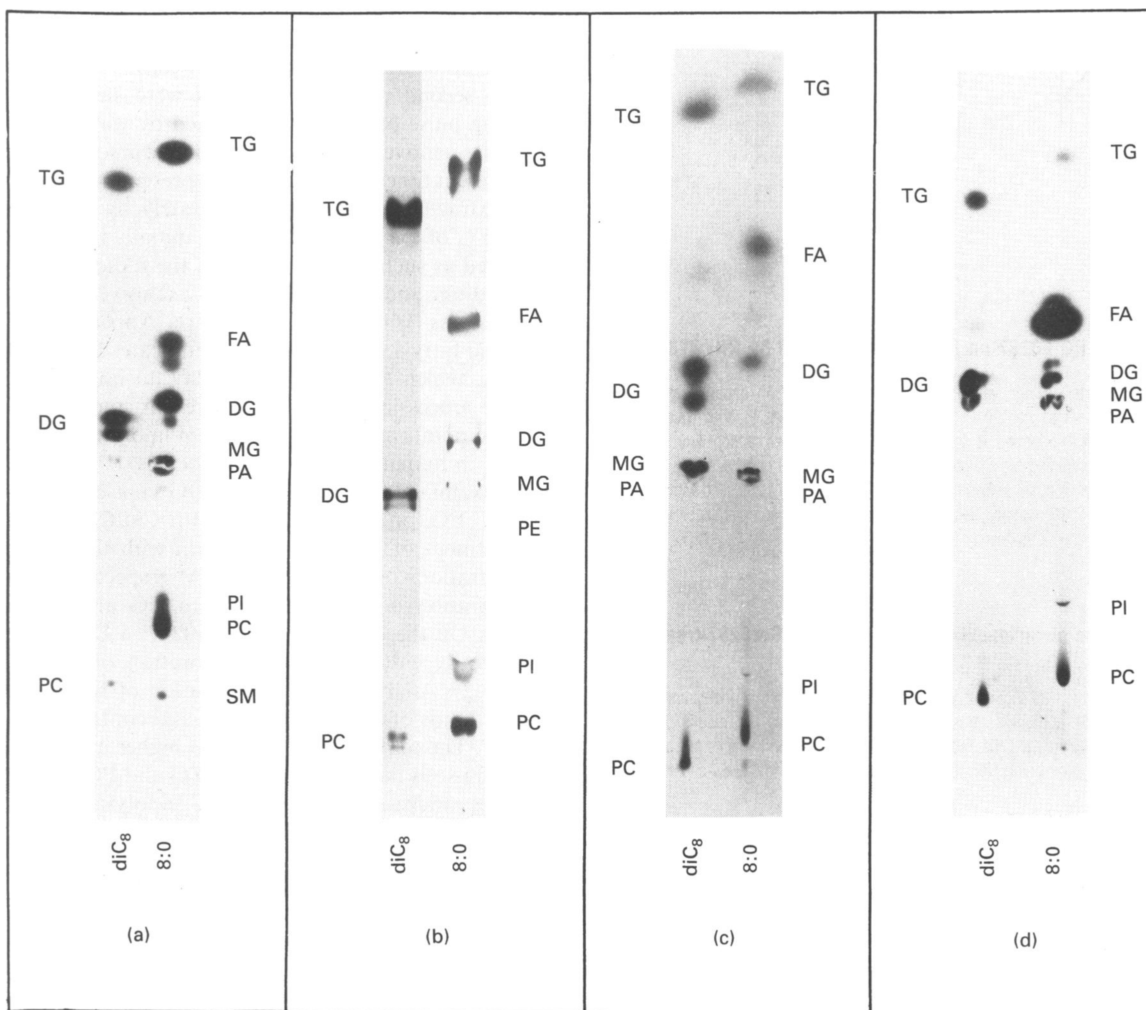

Figure 1 Patterns of labelling with either [^{14}C]diC$_8$ or [^{14}C]8:0 of four different cell types

Panels (**a**)–(**d**) show autoradiograms of t.l.c. analysis of lipids extracted from cells labelled with either of these precursors for 5 min as described in the Experimental section. (**a**) NIH 3T3 fibroblasts; (**b**) WEHI 3BD+ cells; (**c**) BHK cells; (**d**) Pam 212 keratinocytes. Lanes indicate cells labelled with [^{14}C]diC$_8$ or with [^{14}C]8:0. Abbreviation: FA, non-esterified fatty acids. Two DG spots can be discerned, which correspond to the positional isomers *sn*-1,2 (which co-migrates with *sn*-2,3) and *sn*-1,3, which are formed by acyl-group migration in the aqueous media.

[^{14}C]18:1. A second procedure was also used in this case. Marker unlabelled lipids were added to the samples, which were then analysed on separate LK60D plates for neutral and polar lipids by using the same solvent systems. Lipid spots were detected by exposure to iodine vapours, marked and scraped into scintillation vials. After allowing evaporation of the iodine, quantification was performed by liquid-scintillation counting as described above.

RESULTS AND DISCUSSION

We have compared the patterns of cellular lipid labelling obtained by exposing NIH 3T3 cells to radiolabelled synthetic dioctanoylglycerol ([^{14}C]diC$_8$) and octanoic acid ([^{14}C]8:0). In Figure 1(a) a t.l.c. separation of cellular lipids extracted after a short incubation period (5 min) with each of the labelled precursors is shown. Qualitative differences are evident. In these cells, the main product of labelling with [^{14}C]diC$_8$ is TG. The TG formed is dioctanoylacylglycerol, which migrates behind longer-chain TGs. DiC$_8$ is converted to a lesser extent in these cells into PC, migrating as dioctanoyl-PC, MG and small amounts of PA. No PI formation was observed. The identity of these labelled lipids was ascertained by co-migration with unlabelled synthetic compounds. The radiolabelled cellular TG co-migrated with synthetic oleoyldioctanoylglycerol in two additional systems on t.l.c. on silica gel G: hexane/diethyl ether/formic acid (40:10:1, by vol.) and benzene/ethyl acetate/diethyl ether/acetic acid (400:50:50:1, by vol.). The labelled cellular PC, PA and MG co-migrated with unlabelled dioctanoyl-PC, dioctanoyl-PA and mono-octanoylglycerol also in two systems in addition to that described in the Experimental section: chloroform/methanol/conc. ammonia, (45:45:1, by vol.) and chloroform/methanol/acetic acid (26:14:1, by vol.).

Upon incubation with [^{14}C]8:0, a strikingly different pattern of labelling is seen. Radioactivity is found in PC, PI, MG, PA, DG and TG, which migrate ahead of the corresponding compounds labelled with [^{14}C]diC$_8$, strongly suggesting that they are octanoyl-acyl-lipids. Indeed, the labelled cellular TG in this case co-chromatographs with octanoyldioleoylglycerol in the systems mentioned above. Another lipid, migrating between DG and non-esterified fatty acid, presumably alkylacylglycerol, is also conspicuously labelled. Trace amounts of radioactivity are also

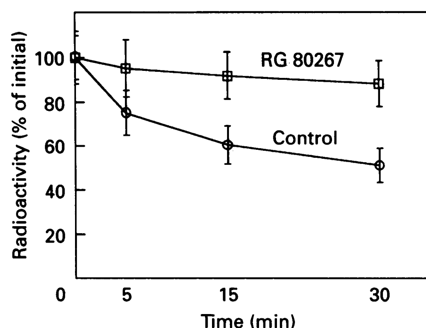

Figure 2 Effect of RG 80267 on [^{14}C]diC$_8$ metabolism by NIH 3T3 cells

Cells were preincubated for 30 min with either 30 μM RG 80267 in dimethyl sulphoxide (Me_2SO) solution or Me_2SO as control (final concn. of Me_2SO 0.5% in both cases). Then labelling with [^{14}C] diC$_8$ was performed as described in the Experimental section. After washing, new media were added containing Me_2SO alone or RG 80267 and the cells were incubated for the indicated time intervals. The plot shows the radioactivity remaining associated with the cells after 5, 15 and 30 min of incubation. Results are expressed as percentages of the initial radioactivity in the cells. The remainder is lost to the medium.

Table 1 Distribution of radioactivity in control and RG 80267-treated cells

The cells from the experiment of Figure 2 were extracted after 30 min of incubation with or without 30 μM RG 80267 and their lipids were separated on t.l.c. The results show the percentages (mean ± S.E.M.) of the initial radioactivity incorporated into the cells present in TG, diC$_8$ (DG) and PC at this time point. Note the increased recovery of diC$_8$, TG and PC in RG 80267-treated cells.

	Radioactivity (% of initial)		
	TG	DG	PC
Control	19.7 ± 2.8	25.8 ± 3.2	5.3 ± 0.8
RG 80267	36.2 ± 4.8	43.1 ± 4.0	9.0 ± 1.2

found in PE and SM. These results allow us to draw the following conclusions. First, our finding that dioctanoyl-lipids are formed from diC$_8$ demonstrates that this exogenous DG is directly incorporated into the pathways leading to PC and TG biosynthesis. The different labelling patterns between diC$_8$ and 8:0 further support the notion that lipid labelling from diC$_8$ does not proceed by prior degradation to free 8:0, followed by its incorporation into TG and PC. Second, although the free 8:0 distinctly serves as a precursor for PI, this phospholipid is not being formed from diC$_8$ by NIH 3T3 cells under the same conditions. Thus the PI cycle does not contribute to any significant extent to the metabolism of this DG. Fatty acid incorporation into cell lipids proceeds by synthesis *de novo* or remodelling through deacylation–reacylation cycles [20–22]. The present results link PI biosynthesis to these processes, rather than to recycling of DG into PI. This is in contrast with previous observations made in human platelets [12]. Thus platelets do not appear to be a suitable model for whole cells.

In addition to the NIH 3T3 fibroblasts, we investigated the metabolism of [^{14}C]diC$_8$ and [^{14}C]8:0 in three other cell types, WEHI 3BD+ murine leukaemia cells, BHK cells and Pam 212 keratinocytes (Figures 1b–1d). In all cases, TG and PC were the major products formed from diC$_8$. None of the cells formed labelled PI from this DG, despite stronger labelling of PA from the DG in some of them. Incubation with [^{14}C]8:0 generated again different patterns. As the cells used represent widely divergent cell types, we can conclude that the conversions that we observed reflect a general pattern for diC$_8$ incorporation into cellular lipids by intact mammalian cells.

In a second protocol, the cells were subjected to a 2 min labelling pulse of [^{14}C]diC$_8$, followed by washing of the monolayers to remove non-incorporated precursor. After incubation for different time periods, the lipids present in the media and cells were extracted and analysed separately by t.l.c. After 15 min, only 45% of the radiolabelled diC$_8$ initially taken up by the cells remained as such, whereas 38% of the radioactivity was lost to the medium and consisted almost exclusively of [^{14}C]8:0. The balance was TG and PC, as before. Appearance of the non-esterified fatty acid in the medium indicates lipolytic breakdown of diC$_8$, which thus appears to play an important role in the cellular processing of this DG. A recent study by Asaoka et al. [23] also points out the importance of lipolysis in the metabolism of diC$_8$ in human resting T lymphocytes.

We examined the effects of two DG kinase inhibitors, R59022 and diC$_8$EG, on the metabolism of [^{14}C]diC$_8$ in NIH 3T3 cells. Pretreatment of the cells for 30 min with these compounds at concentrations of 10 μM and 50 μM respectively did not block incorporation of labelled diC$_8$ into TG and PC (results not shown). On the other hand, RG 80267, a DG lipase inhibitor, significantly enhanced the incorporation of diC$_8$ by the cells (Figure 2). Analysis of the distribution of radioactivity among cellular lipids of RG 80267-treated and control cells is shown in Table 1. The cellular diC$_8$ content is higher in RG 80267-treated cells, and so is its conversion into TG and PC. This observation provides additional evidence that lipolysis is an important pathway by which cells eliminate the [^{14}C]diC$_8$ entering them. When lipolysis is decreased by the inhibitor, the competing processes of PC and TG formation are favoured.

Our results compare well with those of Pagano and co-workers, who used a fluorescent PA analogue instead of a DG [24,25] and found conversion into TG and PC, but not into PI, in CHO cells. They observed translocation of the fluorescence from the plasma membrane to intracellular sites where TG and PC biosyntheses are known to take place [26]. From indirect evidence [27], they proposed that, after insertion of the fluorescent PA into the plasma membrane, hydrolysis to DG by a PA phosphohydrolase takes place. Our studies show that a DG that spontaneously partitions into the plasma membrane is converted into TG and PC. Since we used directly DG, and not PA, no assumptions are needed with respect to the presence of plasma-membrane PA phosphohydrolase. Interestingly, Pagano and Longmuir [27] observed rephosphorylation of the DG analogues, raising the possibility that this process could be required for translocation of the fluorescent lipid moiety. Our finding that DG kinase inhibitors do not affect the conversions observed, however, strongly suggests that phosphorylation to PA is not a prerequisite for translocation. Therefore, our study provides evidence of translocation of DGs across cytoplasmic compartments in intact cells, which suggests the occurrence of cytoplasmic DG carrier proteins in mammalian cells.

In their study on radiolabelled diC$_8$ metabolism in resting T lymphocytes, Asaoka et al. [23] observed the formation of a number of metabolites, which they did not characterize. They attributed the appearance of these labelled lipids to the incorporation of radiolabelled octanoic acid previously released by lipolysis of diC$_8$. However, no experimental support for this view was offered. As mentioned above, octanoic acid is poorly incorporated into cellular lipids as compared with diC$_8$. We suggest that the uncharacterized metabolites observed by Asaoka et al. [23] represent lipids derived directly from diC$_8$ rather than from 8:0. Our observation that the short-chain 8:0 is a poor

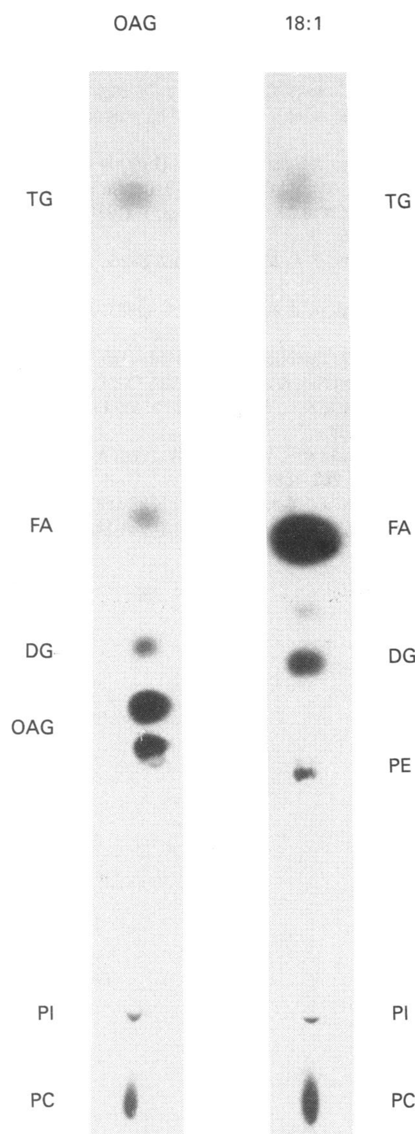

Figure 3 Autoradiogram of a t.l.c. separation of the lipids of NIH 3T3 cells labelled with either [^{14}C]OAG or [^{14}C]18:1

In both cases the precursors were added to the cells as a suspension in DMEM containing 1% BSA, the cells were thereafter incubated for 3 min, after which lipids were extracted and separated as described in the Experimental section. Abbreviations are as in Figure 1. The lanes are labelled OAG and 18:1, corresponding to incubation with [^{14}C]OAG and [^{14}C]18:1 respectively. In the left lane, OAG designates two spots. They correspond to the *sn*-1,2 (2,3) and *sn*-1,3 positional isomers resulting from acyl-group migration upon addition to the cultures. Synthetic 1-oleoyl-2-acetyl-*sn*-glycero-3-phosphocholine migrates distinctly below the cellular PCs that become labelled from [^{14}C]OAG (results not shown). Note that both the PC and TG formed from either [^{14}C]OAG or [^{14}C]18:1 as precursors have similar R_F values and cannot be distinguished chromatographically from one another. Compare with the case of diC$_8$ in Figure 1.

precursor of cellular lipids agrees well with that by Pagano and co-workers [25], who showed that the short-chain fatty acid analogue 2-(*N*-4-nitrobenzo-2-oxa-1,3-diazolyl)aminohexanoic acid ('C$_6$-NBD') is not incorporated into cellular lipids. In our study with radiolabelled 8:0, we found that the total radioactivity in cells + medium remains unchanged throughout the incubation period, indicating that selective oxidation of this short-chain fatty acid does not take place (results not shown).

Asaoka et al. [23] observed only small formation of PA, coinciding with our own observations. MacDonald et al. [28,29] have shown the occurrence of a membrane DG kinase activity with high selectivity for 1-stearoyl-2-arachidonoyl-*sn*-glycerol. This is the main DG derived from phospholipase C hydrolysis of phosphoinositides. Another DG kinase present in the cytosol does not show this selectivity [29]. Different DG kinase isoenzymes were also reported and characterized by Kanoh and co-workers [30,31]. In a previous study [17] we found that, although the main product of 1-stearoyl-2-arachidonoyl-*sn*-glycerol metabolism in intact NIH 3T3 fibroblasts was PC, formation of PA and PI was also important. It therefore seems that a special metabolic path may operate for this DG, supporting the validity of a PI cycle for this specific case. For molecular species other than 1-stearoyl-2-arachidonoyl-*sn*-glycerol, like those originating from PC breakdown [7], the events observed with diC$_8$ may be more representative.

We studied the metabolism of a second cell-permeant DG, 1-[1-^{14}C]oleoyl-2-acetyl-*sn*-glycerol ([^{14}C]OAG). We compared the labelling patterns obtained in NIH 3T3 cellular lipids with either [^{14}C]OAG or [1-^{14}C]oleic acid ([^{14}C]18:1). T.l.c. separation of the lipids extracted from the cells after a 3 min incubation period with the two radiolabelled compounds shows very similar patterns (Figure 3). No radioactivity was found to be associated with 1-oleoyl-2-acetyl-*sn*-PC, which migrates behind cellular PC, and also the TGs formed in both cases migrated the same distances, against what should be expected if 1-oleoyl-2-acetyl-3-acyl-*sn*-glycerol had been formed. Thus OAG, in contrast with diC$_8$, does not appear to be a good substrate for CDP-choline:1,2-DG cholinephosphotransferase and acyl-CoA:1,2-DG acyltransferase, which catalyse the formation of PC and TG respectively. Labelling of cellular PC and TG seems to be a consequence of the release of the radiolabelled oleic acid by lipolytic action. Measurement of radioactivity associated with unmetabolized OAG shows that only half of the OAG taken up by the cells remains as such after 40 min. By contrast, in cells treated with RG 80267, 80% of the OAG remains unmetabolized after the same time period (results not shown). Thus RG 80267 partially protects this DG from lipolytic breakdown.

This study complements our previous investigation in which longer-chain DGs were delivered via liposomes to NIH 3T3 cells [17]. In contrast with the latter, the present study has the advantage that any possible perturbations induced by liposomes are ruled out. Yet the main metabolic conversions observed with diC$_8$ are similar to those with longer-chain DGs, particularly concerning the relevance of transferase activities in eliminating plasma-membrane DGs.

In conclusion, we have here provided new data on the metabolism of diC$_8$ and OAG in intact cells, two synthetic compounds that have played a significant role in the demonstration of the second-messenger activity of DGs [4,5] and which continue to be used as important research tools in this area. The emerging scheme emphasizes the importance of lipolysis and conversion into PC and TG and does not support any role of the PI cycle in the attenuation of these DG signals. Finally, comparison of the two DG molecules examined, diC$_8$ and OAG, reveals drastic differences in the paths along which they are metabolized. This demonstrates that the fatty acyl composition of the DGs is a key determinant of their fate within intact cells.

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