

Diacylglycerol generated in CHO cell plasma membrane by phospholipase C is used for triacylglycerol synthesis

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Abstract The diacylglycerol (DAG) signal generated from membrane phospholipids by hormone-activated phospholipases is attenuated by mechanisms that include lipolysis or phospholipid resynthesis. To determine whether the DAG signal might also be terminated by incorporation of DAG into triacylglycerol (TAG), we studied the direct formation of TAG from endogenous DAG generated by bacterial phospholipase C (PLC). When Chinese hamster ovary (CHO) cells prelabeled with [¹⁴C]oleate were treated with PLC from *Clostridium perfringens* for 6 h, [¹⁴C]phospholipid decreased 15% and labeled TAG increased 60%. This transfer of ¹⁴C label was even greater when the cells were simultaneously exposed to PLC and 100 μM oleic acid. PLC as well as oleate treatment concomitantly increased the TAG mass within the cell. Moreover, when phospholipids were prelabeled with [³H]glycerol, a subsequent increase in [³H]TAG indicated that an intact DAG moiety was channeled into the TAG structure. Incubating CHO cells with the diacylglycerol kinase inhibitor R59022 enhanced the formation of TAG from phospholipids hydrolyzed by PLC or by PLC in the presence of 100 μM oleate, but not by incubation with oleate alone, indicating that the DAG released from plasma membrane phospholipids does not require the formation of a phosphatidic acid precursor for TAG synthesis. Similarly, the diacylglycerol lipase inhibitor RHC 80267 did not alter TAG synthesis from plasma membrane DAG, further supporting direct incorporation of DAG into TAG. These studies indicate that DAG derived from plasma membrane phospholipid is largely used for TAG formation, and support the view that this mechanism can terminate DAG signals. The studies also suggest that a transport mechanism exists to move plasma membrane-derived DAG to the endoplasmic reticulum.—Igal, R. A., J. M. Caviglia, I. N. T. de Gómez Dumm, and R. A. Coleman. **Diacylglycerol generated in CHO cell plasma membrane by phospholipase C is used for triacylglycerol synthesis.** *J. Lipid Res.* 2001. 42: 88–95.

Supplementary key words diacylglycerol lipase inhibitor • diacylglycerol kinase inhibitor • phospholipids

Diacylglycerol (DAG) synthesized de novo from glycerol 3-phosphate is a precursor for the quantitatively most important phospholipids, phosphatidylcholine (PC) and

phosphatidylethanolamine (PE), and the principal energy depot, triacylglycerol (TAG). Via the action of DAG kinase to form phosphatidic acid (PA), DAG is a precursor of the anionic phospholipids.

In addition to its role in the synthesis of glycerolipids, DAG is an important second messenger in signal transduction pathways generated by the hydrolysis of phosphatidylinositol (PI) and PC by phospholipase C (PLC). As a second messenger, DAG mediates the actions of a large number of hormones and cytokines by activating protein kinase C (PKC). Although products of PI hydrolysis play a central role in signaling cascades (1), intracellular release of DAG derived from cytokine-mediated PC hydrolysis is required to establish a complete proliferative response (2), and a sustained elevation in intracellular DAG is believed to be responsible for neoplastic transformation. NIH 3T3 cells transfected with the bacterial PC-PLC gene produce excess intracellular DAG and concomitantly develop a transformed phenotype (3). Moreover, ablation of the G₀α subunit in Chinese hamster embryo fibroblasts constitutively activates PC-PLC and causes overproduction of DAG and a transformed phenotype (2). Intracellular DAG levels are also consistently elevated during neoplastic transformation by simian virus 40 (SV40), and by the oncogenes *ras*, *fps*, and *src* (4–6). The increase in DAG content in *ras*- and *fps*-transformed fibroblasts was accompanied by an increased synthesis of PA and TAG (5). These lipid changes were attributed to accelerated phospholipid turnover, presumed to be caused by a high PLC activity.

The DAG signal can be attenuated via lipolysis by DAG and monoacylglycerol (MAG) lipases, by phosphorylation by DAG kinase followed by synthesis of PI, or by synthesis

Abbreviations: BSA, bovine serum albumin; CE, cholesteryl esters; CHO, Chinese hamster ovary; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; DMSO, dimethyl sulfoxide; MAG, monoacylglycerol; PA, phosphatidic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase C; PI, phosphatidylinositol; PLC, phospholipase C; TAG, triacylglycerol; TLC, thin-layer chromatography.

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of PC by DAG choline phosphotransferase. The metabolic fate of DAG varies in different cells. In human skin fibroblasts and Jurkat cells, DAG kinase acts only on DAG that has been generated by receptor-activated PLC, suggesting a physical association between DAG kinase and cytokine-stimulated PLC participating in tandem to attenuate the DAG signal (7). In human fibroblasts, most of the DAG substrate for DAG kinase is provided from PI rather than from PC (8). In smooth muscle cells, the main destiny of both endogenous and exogenous DAG appears to be the conversion of DAG into MAG and fatty acid by DAG lipase (9, 10). In addition, the lipase pathway also acts on DAG that has been incorporated into the plasma membrane; in NIH 3T3 Swiss fibroblasts, DAG that is added to cells by liposome fusion or as soluble dioctanoyl glycerol can be incorporated into both PC and TAG (11), but about 15% and 20% of the DAG-derived products are MAG and fatty acid, respectively.

Apart from the study using dioctanoyl glycerol, little information is available on the formation of TAG from DAG second messengers. To determine whether the DAG signal is physiologically attenuated in this manner, we examined the recycling of plasma membrane-derived DAG. Our findings strongly suggest the presence of a channeling route that involves the direct transfer of the DAG molecule from its site of release in the plasma membrane to the endoplasmic reticulum, where it is acylated by diacylglycerol acyltransferase (DGAT) to form TAG.

MATERIALS AND METHODS

Materials

Silica gel G plates were from Whatman (Clifton, NJ). [^3H]glycerol and [^{14}C]oleic acid were from New England Nuclear (Boston, MA). Essentially fatty acid-free bovine serum albumin (BSA) was from Sigma (St. Louis, MO). Lipid standards were from Doosan Serdary Research Laboratories (Yongin, Korea). The DAG lipase inhibitor RHC 80267 and the DAG kinase inhibitor R59022 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). Tissue culture supplies were from Corning Costar (Acton, MA), and culture medium was from GIBCO (Grand Island, NY). Ultrafiltered fetal bovine serum (FBS) was purchased from Nutrientes Celulares (Buenos Aires, Argentina).

Cell culture

Chinese hamster ovary (CHO-K1) cells (American Type Culture Collection [ATCC], Manassas, VA) were routinely cultured in F-12 Ham's with 10% heat-inactivated FBS plus penicillin (100 U/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$) (normal medium) at 34°C, 5% CO_2 , and 100% humidity. The medium was changed every other day.

Cell radiolabeling and PLC treatment

CHO cells were seeded in 60-mm dishes and grown to near confluence. Cells were incubated with either trace concentrations of [^{14}C]oleic acid (0.25 $\mu\text{Ci}/\text{dish}$) or [^3H]glycerol (4 $\mu\text{Ci}/\text{dish}$) in normal medium supplemented with 0.5% BSA for 48 h. After labeling, the radioactive medium was removed and monolayers were washed twice with 0.1% BSA in 37°C phosphate-buffered saline (PBS), in order to eliminate residual label. Next, cells were treated with PLC (2 U/dish) from *Clostridium perfrin-*

gens and/or 100 μM sodium oleate in normal medium plus 1% BSA for up to 6 h. Sodium oleate was dissolved in distilled water at 65°C and added to either dry [^{14}C]oleic acid or [^3H]glycerol. Then normal medium plus 1% BSA was added to make a 100 μM oleate solution. Identical experiments were performed with addition of the DAG kinase inhibitor R59022 (10 μM) or the DAG lipase inhibitor RHC 80267 (25 μM). Control cells received an equal volume of the vehicle dimethyl sulfoxide (DMSO) (maximum concentration, 0.05%). Each experiment was performed two or three times in duplicate and duplicate values differed by less than 7%. Cell viability measured by trypan blue exclusion test remained at >90% at the end of the different treatments.

After these treatments, medium was removed, cell monolayers were washed with 0.1% BSA in ice-cold PBS, and cells were scraped in ice-cold methanol and H_2O . Total lipids were extracted (12) and then concentrated in a Savant (Hicksville, NY) Speed-Vac concentrator.

Lipid analysis

Neutral and total polar lipids were quantified by thin-layer chromatography (TLC) and fluorescence emission of 1,6-diphenylhexatriene (DPH) as described by Igal et al. (13) with modifications. Briefly, the total cell lipids were chromatographed with a solvent system consisting of hexane-ethyl ether-acetic acid 80:20:1 (v/v/v) plus 100 μM DPH. After development, the TLC plate was dried for 5 min in the dark under a N_2 stream. Next, the fluorescent lipid spots were photographed and analyzed with a DS120 Kodak (Rochester, NY) Digital Science image system coupled to a UV transilluminator. The lipid species were quantified by comparison with known amounts of pure lipid standards run on the same plate. As a control for the fluorescence method, the lipid were also stained with iodine vapor and the spot images were processed in the same way as for the fluorescent spots, with no essential differences between both methods.

For radioactivity measurements, neutral lipids were separated by TLC as stated above. Polar lipid species were resolved on TLC plates with chloroform-methanol-acetic acid-water 50:37.5:3.5:1.5 (v/v/v/v). All samples were chromatographed in parallel with pure lipid standards. ^3H - and ^{14}C -labeled lipids were detected with a Berthold (Bad Wildbad, Germany) Scanner II. The TLC plates were also exposed to iodine vapor in a glass chamber. Radioactive spots were scraped into vials and counted in a liquid scintillation counter.

Statistics

Data are presented as means \pm SD. Differences were analyzed by two-way analysis of variance at the $P < 0.05$ level.

RESULTS

DAG released from plasma membrane is a substrate for TAG synthesis in the endoplasmic reticulum: [^{14}C]oleic acid labeling

To label all membrane phospholipids in CHO cells, we used tracer amounts of [^{14}C]oleic acid. After labeling to steady state for 48 h, cells were exposed to bacterial PLC to generate endogenous DAG from phospholipids in the plasma membrane. During this chase period we also added unlabeled oleate to some dishes, which besides inducing TAG synthesis (14), increases phospholipid turnover by either stimulating PLD activity (15) or by exchanging acyl groups from phospholipids (16). At the start of the chase, 83% of the total label was in polar lipids, 12%

in TAG (Fig. 1A and B), and the remaining 5% was in cholesteryl ester (CE) (data not shown). No other labeled lipids were detected at any time point. Among the different phospholipid species, PC and PE accounted for approximately 60% and 20%, respectively, of all labeled polar lipids (data not shown). After 3 and 6 h of chase, labeled TAG from control cells decreased 10%. However, in untreated cells neither phospholipid nor CE fractions were altered during the chase period. At the end of the 6-h incubation, more than 95% of the label was recovered in the lipid extract, indicating that fatty acid oxidation was negligible.

Incubating the CHO cells for 3 h with medium that contained either PLC or oleate prevented the decay of the TAG label, and after 6 h increased the TAG values 30% and 35%, respectively, over controls at the beginning of chase (Fig. 1A). The increase in the TAG label was paralleled by a decrease in labeled phospholipid caused by addition of either PLC or oleate (Fig. 1B). Proportional

losses of ^{14}C label were observed equally in all phospholipid species (data not shown). Labeled CE was unaffected by the PLC or oleate additions (data not shown).

After a 6-h chase, the combination of both PLC and oleate resulted in a net gain in labeling of TAG (100% increase at 6 h) compared with control TAG at the beginning of the chase period (Fig. 1A). When compared with untreated control groups at 3 and 6 h, the TAG values for PLC plus oleate-treated cells were approximately 45% and 1.25-fold higher, respectively. PLC and oleate added together resulted in a decrease in [^{14}C]phospholipid label of 15% at 3 h and 35% at 6 h (Fig. 1B).

Net mass formation of TAG after stimulation with PLC

As shown in Table 1, the incorporation of membrane-derived DAG molecules into TAG promotes the expansion of the TAG pool. The incubation of CHO cells with bacterial PLC (2 U/dish) increased the cellular TAG by 54% with respect to the 6-h incubation control group. The net formation of TAG was greater (1.85-fold) in the cells chased into 100 μM oleate. The presence of PLC plus oleate in the incubation medium led to a 3.5-fold increase in the TAG pool compared with the nontreated controls. Small or minor changes were seen in either phospholipid, cholesterol, or CE content with the various treatments. However, the presence of other lipids, such as free fatty acids or monoacylglycerol, was not detected.

The entire phospholipid-derived DAG molecule is channeled into newly formed TAG:

[^3H]glycerol labeling

To establish whether the phospholipid-derived glycerol backbone can be transferred intact into TAG molecules, we labeled CHO cells with [^3H]glycerol for 48 h. As was observed for [^{14}C]oleate-labeled lipids, the percentage of [^3H]glycerol-labeled TAG and phospholipid was about 20% and 80%, respectively (Fig. 2A and B). The cell monolayers were then incubated for up to 6 h in the presence or absence of either PLC and/or oleate. With no addition, the TAG pool again lost approximately 20% of its label at 3 and 6 h in control cells. Adding PLC, however, increased [^3H]TAG at 6 h to levels present in controls at the start of the chase (Fig. 2A). Adding oleate to the chase medium stimulated the incorporation of the ^3H -labeled backbone into TAG by 35%, and when PLC was added together with oleate, labeled TAG increased 35% and 60% at 3 and 6 h, respectively, compared with untreated cells.

In contrast, although control cells did not show any loss of labeled total phospholipid, cells chased in medium that contained PLC or oleate lost about 10% of their phospholipid label (Fig. 2B). However, the decrease in [^3H]phospholipid was 25% at 6 h when the cells were chased in the presence of both PLC and oleate.

Phosphorylation of membrane-derived DAG is not required before incorporation into TAG: use of a DAG kinase inhibitor

After acute stimulation of PLC in Jurkat cells by bradykinin or endothelin, DAG kinase immediately transforms

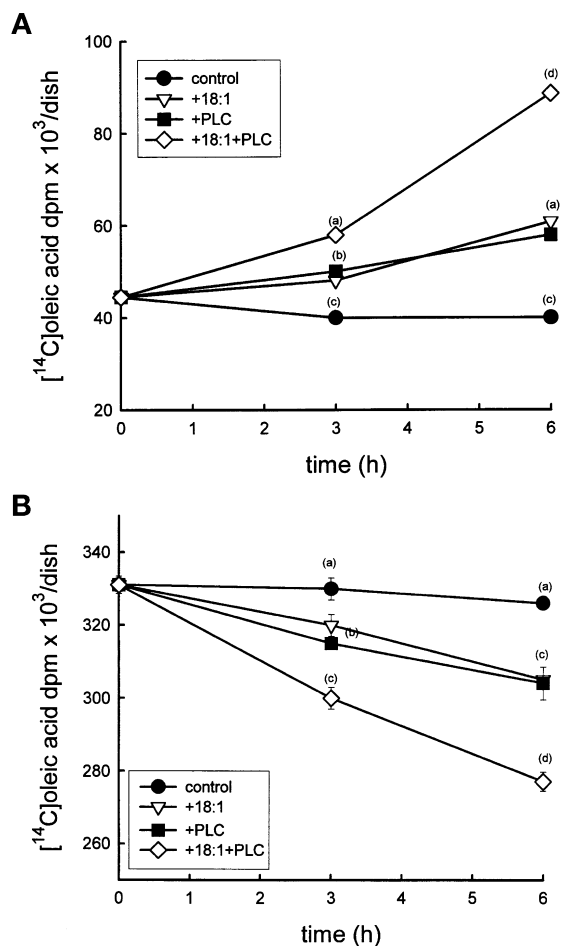


Fig. 1. Endogenous DAG generated by bacterial PLC in the plasma membrane is directly channeled into cellular TAG. Preconfluent CHO cells were incubated with 0.25 μCi of [^{14}C]oleic acid per dish for 48 h. Cells were then chased into growing medium for up to 6 h in the presence or absence of PLC (2 U/dish) and/or sodium oleate (100 μM). Values represent means \pm SD of three separate determinations. Some error bars are hidden by the symbols. Values not bearing the same superscript letter are significantly different at $P < 0.05$ or less. (A) [^{14}C]TAG; (B) [^{14}C]phospholipids.

TABLE 1. Lipid content of CHO cells treated with 100 μ M oleate and/or PLC (μ g lipid per mg protein)

	Triacylglycerol	Phospholipids	Cholesterol	Cholestery Esters
Control 0 h	17.0 \pm 1.3 ^a	75.8 \pm 1.8 ^{a,b}	11.0 \pm 0.5 ^a	10.8 \pm 0.1 ^a
Control 6 h	18.5 \pm 1.2 ^a	72.5 \pm 2.0 ^{a,c}	11.3 \pm 0.3 ^a	10.8 \pm 0.3 ^a
Oleate	52.8 \pm 1.5 ^b	80.0 \pm 1.8 ^b	10.0 \pm 0.5 ^{a,c}	10.5 \pm 0.5 ^a
PLC	28.5 \pm 0.8 ^c	79.0 \pm 1.8 ^b	10.3 \pm 0.3 ^{b,c}	9.8 \pm 0.8 ^a
Oleate + PLC	64.0 \pm 8.8 ^d	68.8 \pm 4.5 ^c	11.3 \pm 0.5 ^a	10.5 \pm 1.0 ^a

Cells were grown to near confluence in 60-mm petri dishes. Monolayers were treated with 100 μ M sodium oleate, bacterial PLC, or both for 6 h. Nontreated cells were considered as the control group. Values represent means \pm SD of three separate determinations. Values not bearing the same superscript letter are significantly different at $P < 0.05$ or less.

DAG to PA (7). In smooth muscle cells DAG generated by the addition of bacterial PLC is attacked by DAG lipases and converted into MAG and fatty acids (9). In our system the presence of oleate and/or PLC appears to promote a

sustained turnover of phospholipids. As shown in Fig. 3, with no addition, CHO cells decreased the levels of [¹⁴C]TAG by 30% after 6 h incubation. When the DAG kinase inhibitor R59022 was present to block PA synthesis (17), control cells did not modify their distribution of labeled lipids. However, when PLC alone was present, the formation of [¹⁴C]TAG from [¹⁴C]DAG increased 30% compared with untreated controls at 6 h. When the DAG kinase inhibitor was also present [¹⁴C]TAG formation increased 50%. Enhanced TAG synthesis in the presence of the inhibitor suggests that the inhibitor blocks the portion of DAG released by PLC that is normally phosphorylated to PA and converted back to phospholipid, thereby increasing DAG substrate for TAG synthesis. When the inhibitor was present, [¹⁴C]CE production increased 35%, suggesting that its synthesis is triggered by a high rate of TAG synthesis. [¹⁴C]phospholipid decreased 10%, probably indicating a lower resynthesis of phospholipids caused by a reduced formation of PA.

Adding oleate alone to the medium produced a different effect from that observed with PLC. The oleate addition decreased [¹⁴C]phospholipid 10%, and concomitantly increased incorporation of label into TAG by 50% (Fig. 3A and B). When the DAG kinase inhibitor was also present, loss of [¹⁴C]phospholipid was only 7% and incorporation of label into TAG decreased from 50% to 30%, compared with cells treated with oleate alone. If DAG were the immediate precursor for TAG, one would expect that blocking the formation of PA would enhance movement to TAG. Because, in fact, less TAG is formed when both oleate and the inhibitor are present, oleate-enhanced lipid recycling from phospholipid to TAG may occur via a PA intermediate, unlike recycling of DAG produced by PLC.

The greatest recycling of [¹⁴C]oleate from phospholipid to TAG (80% increase compared with controls) occurred when both PLC and oleate were present in the chase medium (Fig. 3A and B). Under these conditions, when phosphorylation of DAG was blocked by R59022, there was a 25% loss of [¹⁴C]phospholipid together with a massive incorporation of [¹⁴C]oleate into TAG that was 2.7-fold higher than in control cells and 57% higher than in cells incubated with PLC and oleate alone. In addition, incorporation of [¹⁴C]oleate into CE increased 64% during the chase compared with untreated cells (Fig. 3A),

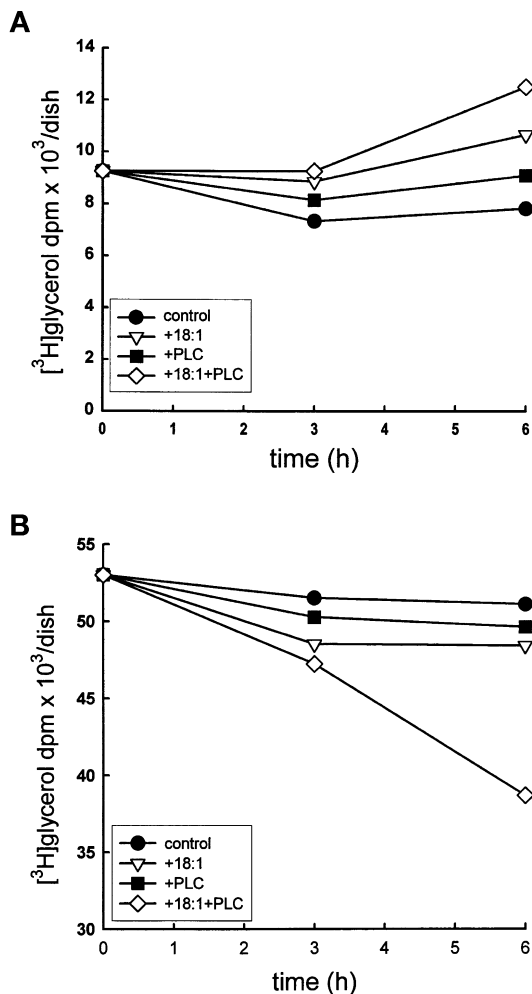


Fig. 2. The intact molecule of DAG released from phospholipids is incorporated into newly formed TAG. Nearly confluent CHO cells were labeled to steady state (48 h) with 4 μ Ci of [³H]glycerol per dish. Next, cell monolayers were treated with PLC (2 U/dish) and/or 100 μ M sodium oleate for up to 6 h. Values represent two or three independent determinations, each performed in duplicate. Variability between duplicates was 5% to 7%. (A) [¹⁴C]TAG; (B) [¹⁴C]phospholipids.

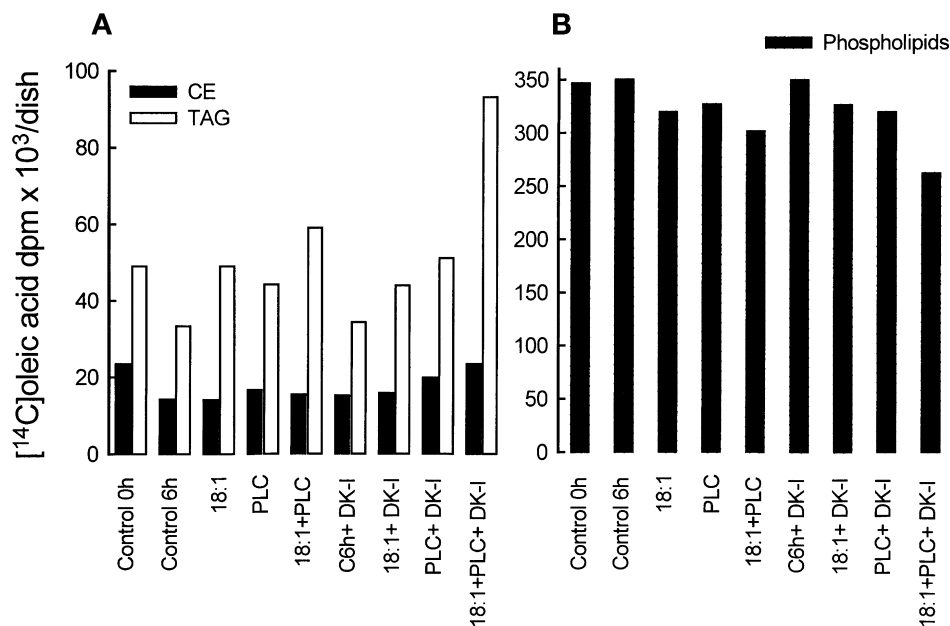


Fig. 3. Inhibiting DAG kinase stimulates DAG conversion into cellular TAG when excess fatty acid is present. Nearly confluent CHO cells were labeled with 0.25 μCi of [^{14}C]oleic acid per dish for 48 h. Cells were then incubated for 6 h with medium alone or with medium containing PLC (2 U/dish) and/or sodium oleate (100 μM), in the presence or absence of 10 μM R59022, the DAG kinase inhibitor (DK-I). Control cells received an equal volume of the DMSO vehicle. Data points correspond to two or three independent experiments, each performed in duplicate. Variability between duplicates was 5% to 7%. (A) [^{14}C]TAG and [^{14}C]CE; (B) [^{14}C]phospholipid.

suggesting that the inhibition of PA synthesis also increased the amount of DAG available for hydrolysis and that more [^{14}C]oleate was thereby available for esterification to cholesterol.

DAG is not hydrolyzed before its glycerol backbone or acyl groups are available for TAG synthesis: use of a DAG lipase inhibitor

To determine whether DAG lipolysis occurred during the transfer of DAG from phospholipid to TAG, we used the DAG lipase inhibitor RHC 80267 to block DAG degradation (18) (**Fig. 4**). When no additions were present, [^{14}C]oleate-labeled TAG decreased 30% (**Fig. 4A**). When 100 μM oleate was added, the amount of label incorporated into TAG increased 60%. The presence of the DAG lipase inhibitor produced a further increase of 17% in [^{14}C]TAG compared with nontreated controls. As was shown previously (**Fig. 3**), adding 100 μM oleate plus PLC to the medium induced a 2.5-fold increase in oleate-labeled TAG, compared with cells with no addition.

When the DAG lipase inhibitor alone was present, labeled TAG and CE increased 17% and 22%, respectively, compared with controls. When CHO cells were treated with PLC, oleate, or both given together in the presence of RHC 80267, TAG labeling was 15%, 25%, and 10% higher, respectively, than in cells incubated with PLC or oleate without the inhibitor. In all the groups treated with the DAG lipase inhibitor, the incorporation of phospholipid-derived [^{14}C]oleate into CE increased almost 50%.

Phospholipid labeling decreased in the presence of oleate, PLC, or both compounds together, but the treatment with RHC 80267 did not produce any further change (**Fig. 4B**). In addition, the release of ^{14}C label into the chase medium was reduced 50% by the lipase inhibitor in all groups (data not shown), indicating that the target of the inhibitor was TAG hydrolysis.

To determine whether these labeling changes were the result of movement of the acyl groups or of the glycerol backbone to TAG, we labeled CHO cells with [^3H]glycerol (**Fig. 5**). The initial labeling pattern was similar to that observed with [^{14}C]oleate, with 15% of the label in TAG and 85% in phospholipid. During a chase, the fate of the glycerol label derived from phospholipid was similar to that of oleate-labeled lipids. After a 6-h incubation, [^3H]TAG decreased 30% in control cells. When oleate, PLC, or both were present together, TAG formation increased 70%, 50%, or 180%, respectively, compared with control cells incubated for 6 h with medium alone. In the presence of the DAG lipase inhibitor RHC 80267, only 15% and 25% increases in TAG labeling were observed in control and PLC-treated cells, respectively. After treating CHO cells with the DAG lipase inhibitor plus either PLC, oleate, or PLC plus oleate, no major changes were detected in the labeled phospholipid distribution. Again, the presence of a higher TAG formation without a corresponding decrease in phospholipid labeling in those cells treated with the lipase inhibitor would indicate that an inhibition in TAG hydrolysis is taking place rather than an incorporation of DAG into that glycerolipid.

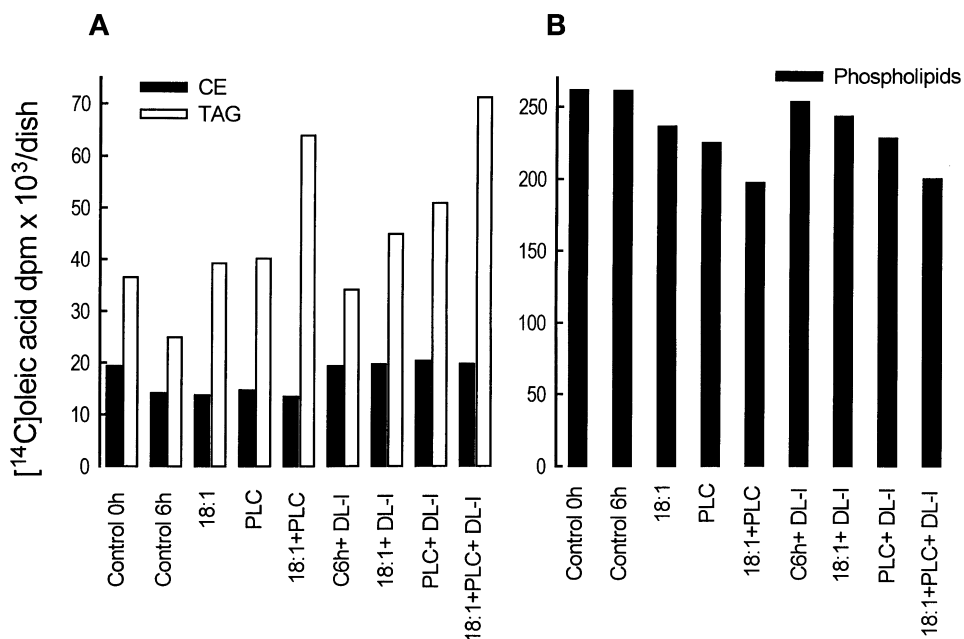


Fig. 4. Inhibition of DAG lipolysis does not affect the transfer of oleate-labeled DAG from phospholipids to newly synthesized TAG. Cells were labeled with 0.25 μCi of [^{14}C]oleic acid per dish for 48 h. Next, cells were treated for 6 h with medium alone or with medium containing PLC (2 U/dish) and/or 100 μM sodium oleate, in the presence or absence of 25 μM RHC80267, the DAG lipase inhibitor (DL-I). Control cells received an equal volume of the DMSO vehicle. Values correspond to two or three experiments, each performed in duplicate. Variability between duplicates was less than 7%. (A) [^{14}C]TAG and [^{14}C]CE; (B) [^{14}C]phospholipid.

DISCUSSION

DAG is a central molecule in glycerolipid metabolism, acting as a common intermediate in the biosynthesis of both TAG and phospholipids (Fig. 6). During normal phospholipid turnover, the main flux of DAG seems to be directed toward cytidine diphosphocholine and cytidine diphosphoethanolamine phosphotransferases in order to maintain the homeostatic equilibrium of membrane composition. A direct link between phospholipid turnover and synthesis of TAG was exposed in neutral lipid storage disease fibroblasts, in which the excess of glycerolipid intermediates generated by rapid phospholipid degradation and resynthesis seems to be directed toward TAG synthesis (19). In addition to being the main energy store within the cell, TAG is also a source of DAG molecules for phospholipid synthesis in human fibroblasts (20).

Because the characteristics and relevance of phospholipid-TAG recycling have received little attention, the main goal of the present work was to determine whether endogenous DAG generated in plasma membrane could be used as substrate for TAG synthesis in the endoplasmic reticulum. This potential metabolic fate of DAG would link attenuation of signaling DAG molecules and the synthesis of neutral lipids.

Our data demonstrate that DAG derived by action of PLC on endogenously labeled phospholipids in the plasma membrane can be channeled into TAG. In addition, incubating cells with 100 μM oleate appears to in-

duce phospholipid turnover with a concomitant incorporation of the recycled acyl groups and glycerol backbone into TAG stores. This transfer of lipid molecules from plasma membrane to TAG stores seems greater than the basal neutral lipid turnover, thereby promoting the accumulation of cellular TAG mass. However, in both the PLC and/or oleate treatments, no trace of labeled DAG was detected at any time point, indicating that this molecule does not accumulate but, instead, is quickly converted into either TAG or phospholipids. The fact that the effects of PLC and oleate were additive suggests that increasing the endogenous production of DAG or adding excess fatty acid to cells promotes lipid recycling from phospholipid to TAG by different mechanisms. This discrepancy raises an additional question: under cytokine stimulation, are the fatty acids released by PLA₂ and the DAG released by PLC used within the cell for TAG synthesis as a mechanism for removing both bioactive lipids?

Both *fps* and *ras*-transformed fibroblasts contain higher contents of DAG and TAG, possibly as a consequence of an elevated phospholipid turnover (5). These authors attributed the finding of greater neutral lipid synthesis to an enhanced PLC activity, because they observed that normal cells react to exogenous PC-PLC by raising the levels of DAG. Our data demonstrate direct incorporation of membrane-derived DAG into TAG in the endoplasmic reticulum. Both [^{14}C]oleate and [^3H]glycerol-labeled lipids participate in the formation of TAG, strongly suggesting that DAG is the primary intermediate in this phospholipid-TAG recycling process.

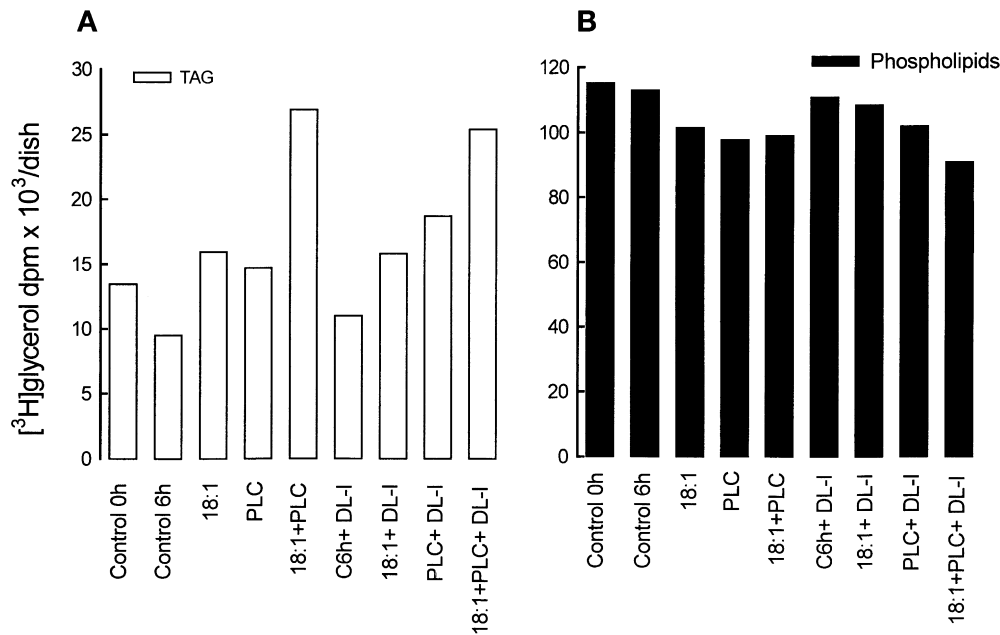


Fig. 5. Inhibiting DAG lipolysis does not alter the transfer of glycerol-labeled DAG from phospholipids to TAG. Cells were incubated with 4 μ Ci of [³H]glycerol per dish for 48 h. Cells were then treated for 6 h with medium alone or with medium containing PLC (2 U/dish) and/or 100 μ M sodium oleate, in the presence or absence of 25 μ M RHC 80267, the DAG lipase inhibitor (DL-I). Control cells received an equal volume of the DMSO vehicle. Values correspond to duplicates of two or three experiments. Variability between duplicates was 5% and 7%. (A) [³H]TAG; (B) [³H]phospholipid.

Recycling of plasma membrane-derived DAG must involve the transport of intact DAG from the plasma membrane to the endoplasmic reticulum where DGAT resides (21). In a similar fashion, the fluorescent precursor 1-acyl-2-[*N*-(4-nitrobenzo-2-oxa-1,3-diazole)aminocaproyl]phosphatidic acid (C₆-NBD-PA) is converted to NBD-DAG, which moves to intracellular membranes, where it is used for glycerolipid synthesis (22, 23). Internalization and metabolism of DAG to phospholipids and TAG was also shown in NIH Swiss 3T3 fibroblasts that received [¹⁴C]DAG via

liposome fusion (24). Our experimental approach using endogenous labeled phospholipids as a source of the DAG avoids possible alterations in membrane physiology caused by liposome fusion or by the different behavior of a synthetic soluble DAG such as dioctanoylglycerol.

We wondered whether the route of DAG exchange between phospholipids and TAG could be a one-step mechanism, via direct acylation of DAG. In our studies, we observed that approximately 30% of DAG generated by PLC is phosphorylated to PA. Because inhibiting DAG kinase with R59022 increased the amount of TAG formed, we hypothesize that PA is used mainly for resynthesis of phospholipids and not TAG. Thus, when DAG phosphorylation is inhibited, more DAG is directed to neutral lipid synthesis. Differential channeling of DAG has been shown indirectly: PLD-derived DAG does not activate PKC, whereas DAG derived from PI-specific PLC does promote PKC translocation from cytosol to membranes (25). Thus, the DAG generated by PLD (followed by the action of PA phosphohydrolase) or by PLC must make up separate pools that are individually channeled. We hypothesize that the use of the DAG substrate by DGAT in the endoplasmic reticulum to produce TAG could enable the cell to decrease the number of signaling DAG molecules once PKC has been targeted.

DAG created in the plasma membrane can be metabolized to form other lipid intermediates such as free fatty acids and monoacylglycerol. In most of the studies that examine the DAG released from the plasma membrane, lipolysis seems to be a major fate (10, 11). Because we used bacterial PLC to generate endogenous DAG, we won-

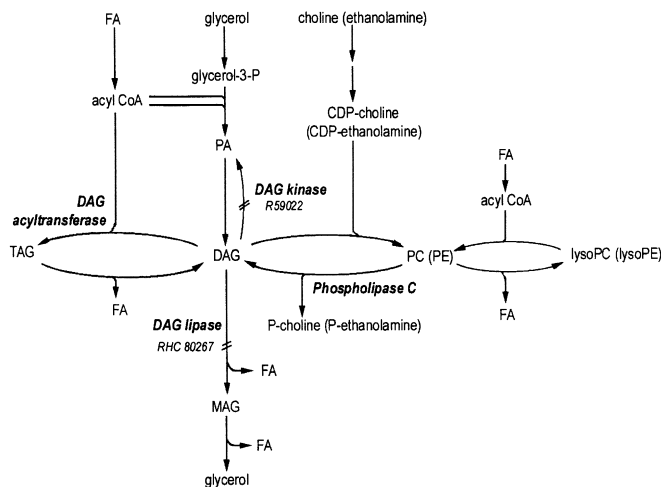


Fig. 6. Scheme of glycerolipid synthesis in mammalian cells. FA, fatty acid. Enzymes are written in bold letters, inhibitors are written in italic letters.

dered whether DAG lipase could be playing a role in the recycling of DAG from phospholipids to TAG. Exposing PLC-treated cells to the DAG lipase inhibitor RHC 80267 had only a small effect on the rate of incorporation of phospholipid-derived lipids into TAG, indicating that DAG generated in CHO plasma membrane is not a major target for lipases. These observations agree with studies that show that the presence of DAG lipase altered the amount of MAG and fatty acid produced by lipolysis of DAG exogenously added to fibroblasts, but that no effect was seen in subsequent PC and TAG labeling (11, 24). In our study the minor increases in TAG levels occurring in control and PLC-treated cells incubated with RHC 80267 can be attributed to the inhibition of TAG hydrolysis rather than to actual enhancement of DAG incorporation into TAG stores. We have observed a strong correlation between level of TAG hydrolysis and the release of fatty acid into the incubation medium by cultured cells (20). The 50% decrease in [¹⁴C]oleate release into the medium when the DAG lipase inhibitor was present indicates a lower TAG lipolysis in CHO cells, reinforcing the aforementioned suggestion that the lipase inhibitor affects TAG accumulation via the recycling of neutral lipid stores.

Taken as a whole, the present studies suggest that CHO cells convert endogenously generated plasma membrane DAG directly into cellular TAG. In addition to other pathways that deactivate the DAG signal, this process constitutes a one-step, low energy-consuming method of terminating a DAG signal during chronic stimulation. Further, our studies also suggest that a mechanism exists to transport plasma membrane-derived DAG to the endoplasmic reticulum. ■■

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