Swiss 3T3 Cells Preferentially Incorporate sn-2-Arachidonoyl Monoacylglycerol into sn-1-Stearoyl-2-arachidonoyl Phosphatidylinositol*

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The sn-1-stearoyl-2-arachidonoyl phospholipids of animal cells appear to be formed by special mechanisms. To determine whether monoacylglycerol (MG) incorporation pathways are involved we incubated quiescent Swiss 3T3 cells with [3H]glycerol-labeled sn-2-arachidonoyl MG, then analyzed the radioactive cell lipids that accumulated. We also examined cell homogenates to identify enzyme activities that might promote the incorporation of sn-2-arachidonoyl MG into other cell lipids. The cell incubation experiments demonstrated rapid labeling of several lipids, including diacylglycerol, lysophosphatidic acid, phosphatidic acid, and phosphatidylinositol. They also demonstrated selective labeling of sn-1-stearoyl-2-arachidonoyl species of phosphatidylinositol, phosphatidylethanolamine, and phosphatidylserine. The cell homogenate experiments identified an sn-2-acyl MG acyltransferase activity, an MG kinase activity that phosphorylates sn-2-arachidonoyl MG in preference to sn-2-oleoyl MG, and a stearoyl-specific acyl transferase activity that converts sn-2-arachidonoyl lysophosphatidic acid into sn-1-stearoyl-2-arachidonoyl phosphatidic acid. The results also showed that this stearoyl transferase could act with other enzymes to convert sn-2-arachidonoyl lysophosphatidic acid into sn-1-stearoyl-2-arachidonoyl phosphatidylinositol. The combined results indicate that Swiss 3T3 cells incorporate sn-2-arachidonoyl MG into phospholipids by at least two different pathways, including one that specifically forms sn-1-stearoyl-2-arachidonoyl phosphatidylinositol.

Labeling experiments with [3H]glycerol and other radioactive precursors have shown that animal cells rapidly form phospholipids that contain palmitic acid and monoenoic, dienoic, or hexaenoic fatty acids, but only slowly form phospholipids that contain stearic acid and arachidonic acid (for reviews, see Refs. 1–4). For example, this differential formation has been demonstrated for several different lipids in rat liver, including phosphatidylcholine (PC),1 phosphatidylethanolamine (PE), phosphatidic acid (PA), diacylglycerol (DG), and phosphatidylinositol (PI) (5–8). To account for these results it has been proposed that sn-1-stearoyl-2-arachidonoyl phospholipids may be formed by a multistep pathway. First, phospholipids that contain palmitic acid in the sn-1 position and a monoenoic, dienoic, or hexaenoic fatty acid in the sn-2 position are formed de novo by the classical pathways of phospholipid biosynthesis. Then these phospholipids are "remodeled" by reactions that selectively replace the sn-1 palmitoyl group with a stearoyl group and replace the sn-2 fatty acyl group with an arachidonoyl group. In support of this possibility, phospholipase A and acyltransferase activities that might catalyze this type of phospholipid "remodeling" have been identified (see, for example, 9–12). Nevertheless, alternative possibilities remain to be excluded. For example, one could imagine that sn-2-arachidonoyl monoacylglycerol (MG), formed from intracellular triacylglycerol (TG) by the action of a TG lipase or formed from sn-1-palmitoyl-2-arachidonoyl PC by the successive action of a phospholipase C and a neutral lipase, might be "recycled" into phospholipids by a stearoyl-specific pathway. We addressed this possibility in the study of quiescent Swiss 3T3 cells that is described below. A preliminary report of some of the results has appeared (13).

EXPERIMENTAL PROCEDURES

Materials—[1,2,3-3H]Glycerol-labeled sn-2-arachidonoyl monoacylglycerol ([3H]MG) (37 Ci/mmol), [1,2,3-3H]glycerol (37 Ci/mmol), [γ-32P]ATP (3000 Ci/mmol), and [1-14C]palmitoyl-CoA (54 mCi/mm), were purchased from Du Pont-New England Nuclear. sn-1-Stearoyl-2-arachidonoyl DG, octyl glucoside, phosphatidylase D (Strep-tomyces chromofuscus), TG lipase from Rhizopus arrhizus, CTP, dithiothreitol (DTT), MOPS, myristoyl-CoA, palmitoyl-CoA, and stearyl-CoA were obtained from Sigma. Carrier lipids, PC, PI, phosphatidylserine (PS), PE, PA, phosphatidylglycerol, cytidine diphosphodiacylglycerol (CDP-DG), cardiolipin, lysophosphatidylcholine, MG, DG, TG, cholesteryl ester, and free fatty acid were obtained either from Serdary (London, Ontario, Canada) or Sigma. Thesit (polyoxyethylene(9) lauryl ether) was obtained from Boehringer Mannheim. Glass plates for thin layer chromatography (TLC), precoated with 250 μm of Silica Gel-H or Silica Gel-G, were from Analabs, Inc. (North Haven, CT), and plastic-backed and aluminum-backed Silica Gel-60 plates were from EM Science (Gibbstown, NJ). Aquamix and Ecolume scintillants were from ICN Radiochemicals (Irvine, CA).

1 The abbreviations used are: PC, phosphatidylcholine; MG, monoacylglycerol; [3H]MG, [3H]glycerol-labeled MG; DG, diacylglycerol; TG, triacylglycerol; PA, phosphatidic acid; lysophosphatidic acid; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; CDP-DG, cytidine diphosphodiacylglycerol; MOPS, 3-[N-morpholino]propanesulfonic acid; BSA, bovine serum albumin; DTT, dithiothreitol; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.
Cell Culture and Labeling—Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. They were made quiescent by plating in medium containing 5% human plasma-derived serum (14). On day 5 the quiescent cells (2.5 × 10⁶ cells/dish) were incubated with either 25 µCi/dish of [H]MG (in methanol-EDTA saline) or 25 µCi/dish of [14C]glycerol under argon at room temperature. The medium was decanted, the cells were frozen at −70 °C in 1.5 ml of 0.15 M HCl in phosphate-buffered saline. Lipids were extracted from the cells with the method of Bligh and Dyer (15). All solvent mixtures contained 0.005% butyric acid rather than HCI in the second wash. It was then dried down and redissolved in toluene/ethanol/water (30:40:10:10:5, v/v). The lipids were visualized on a silica SEP-PAK cartridge (Waters) by using chloroform/methanol (90:20:3:2, v/v) and chloroform/methanol/acetic acid/water (59:4:2, v/v) as eluents. Stearic acid and DG were eluted in the first step and DG kinase activity was determined by measuring the formation of DG from sn-2-arachidonoyl MG and [1-'4C]palmitoyl-CoA according to the method of Coleman and Haynes (24). Activity was expressed as picomoles of [14C]palmitate incorporated into DG/milligram of protein/minute. (With this assay protocol we were able to detect specific activity similar to that of Coleman and Haynes for MG acyltransferase in liver microsomal preparation from 6-day-old rats.)

Activity of MG Kinase Activity—MG kinase activity was determined by measuring the formation of 32P-labeled lysophosphatidic acid from 32P-labeled sn-2-arachidonoyl MG and unlabeled sn-2-arachidonoyl MG. The latter was prepared as follows: sn-2-stearoyl-2-arachidonoyl DG was suspended in 2% (w/v) BSA in 50 mM Tris HCl, pH 7.5, by sonication for 3 min under argon at room temperature, then hydrolyzed for 2.5 min at 37 °C with TG lipase from R. arrhizus (2.4 × 10⁶ unit/10 mg of substrate). The reaction was quenched with chloroform/methanol (1:2, v/v), sn-2-Arachidonoyl MG was separated from undigested DG and free stearic acid and glycerol by a silica SEP-PAK cartridge (Waters). This was then dried under argon and dissolved in toluene/hexane (1:1, v/v, solvent A) for loading onto the cartridge. Stearic acid and DG were eluted with a mixture of 75% solvent B in solvent A (where solvent B was toluene/ethylacetate (3:1, v/v) containing 1.2% (v/v) formic acid). sn-2-Arachidonoyl MG was eluted with chloroform/methanol (1:2, v/v), dried under argon, and redissolved in chloroform for storage. The overall yield of sn-2-arachidonoyl MG from this procedure was ~30%. The final product contained <5% of the sn-1-isomer.

Optimal MG kinase activity was attained in a reaction mixture (35 µl) containing 20 µg of sn-2-arachidonoyl MG and 12 µg of PS (dried under argon) in 25 µl of 2% (w/v) BSA (pH 7.25) in MOPS, pH 7.25, by sonication for 3 min under argon at room temperature, 150 mM MOPS, pH 7.25, 3 mM DTT, 54 mM MgCl₂, 0.5 mM [γ-32P]ATP (700-1200 cpn/pmol and 5-15 µg of cell protein. Assays were performed at room temperature for 4 or 5 min. Under these conditions, about 80% of the MG substrate was maintained as the sn-2-isomer. The assays were quenched with 0.6 ml of chloroform/methanol/HCl (66:33:1, v/v, carrier) and lysoPA and PA were added, and the phases were separated by addition of 0.5 ml of chloroform/methanol/water (3:4:8:7, v/v). The lower phase was dried under argon or in a Speed Vac (model 111R, Savant Instruments, Farmingdale, NY), and the reaction products, 32P-labeled lysophosphatidic acid (lysoPA) and 32P-labeled PA, were resolved by TLC on plastic-backed Silica Gel-60 plates with chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5, v/v). The lipids were visualized by exposure to I₂ vapor and by autoradiography. Enzyme-specific activity was expressed as picomoles of 32P incorporated into product/milligram of protein/minute. Protein concentrations were measured with the methods of Bradford (25) and Lowry et al. (26).

Assay of DG Kinase Activity—DG kinase was determined at room temperature, in a final volume of 35 µl, by a modification of a previously reported method (27). The reaction mixtures contained 50 mM MOPS, pH 7.25, 1 mM DTT, 18 mM MgCl₂, 73 mM octyl glucoside, 3.3 mM PS, 2 mM substrate DG, and 0.5 mM [γ-32P]ATP (700-1200 cpn/pmol). 5-20 µg of protein were used for each assay. After 2 min, the reaction was quenched and the reaction mixture was treated as described in the assay protocol for MG kinase.

Assay of Lipolytic Activity—Lipolytic activity was determined by measuring the formation of 32P-labeled lysophosphatidic acid from 32P-labeled sn-2-arachidonoyl MG. The latter was prepared as follows: sn-2-arachidonoyl MG was suspended with 2% (w/v) BSA in 375 mM MOPS, pH 7.2, by sonication for 3 min under argon at room temperature. The MG was hydrolyzed for 2 min at room temperature, then hydrolyzed for 15 min (PC or PE) or 4 h (PI) at room temperature (21). The resulting PAs were purified by TLC and methylated with diazomethane (29). To verify the positional distribution of sn-1-acyl groups, PC, PE, and PI that had been labeled during a 1-h cell incubation experiment with radiolabeled sn-2-arachidonoyl MG were isolated and purified as described above. In the presence of 10 mM MgCl₂ and 0.2 µM DAG (14), the resulting PAs were hydrolyzed with phospholipase D for 15 min (PC or PE) or 4 h (PI) at room temperature (21). The resulting PAs were purified by TLC and methylated with diazomethane (29). The assay of lysoPA acyltransferase activity was performed in a modification of a method previously described. Several synthetic phospholipids were treated with the same method and used as standards for the HPLC analysis. Each yielded only one peak, which corresponded to that predicted on the basis of the positional specificity of the lipase reaction (22).

Statistical testing was performed with the Student's t test (Bold, Barone, and Newman, Cambridge, MA). Means and standard deviations reported in the tables and shown on the graphs were calculated for replicate assays from original Prophet data tables using the "compute stats" command available in the Prophet applications package (23).

Assay of MG Acyltransferase Activity—MG acyltransferase was determined by measuring the formation of DG from sn-2-arachidonoyl MGDG and [1-32P]palmitoyl-CoA according to the method of Bold, Barone, and Newman (24). Activity was expressed as picomoles of [32P]palmitate incorporated into DG/milligram of protein/minute.

Glomset, manuscript in preparation.
4 min and quenched with 2.4 ml of chloroform/methanol/HCl (66:33:1, v/v). Then 2 ml of chloroform/methanol/water (3:48:47, v/v) were added, the phases separated, and the lower phase dried under argon. LysopaPA in the extracted material was purified on a silica-based cation exchange column (Bond Elut-PRS cartridge column, Analytichem International, Harbor City, CA). The extract was loaded onto the column in chloroform; neutral lipid and a small amount of PA were removed by washing the column with 8 ml each of chloroform/methanol (98:2, v/v) and chloroform/methanol (97:5:2, v/v). LysopaPA was then eluted with 7 ml of chloroform/methanol (95:5, v/v). The purity of lysopaPA prepared by this method was determined to be >96%.

Membrane-associated enzyme activity was measured as follows. The 32P-labeled sn-2-arachidonoyl lysopaPA in a polypropylene tube was dried down under argon, then resuspended at an apparent concentration of 10 μM in 100 mM Tris-HCl buffer, pH 7.5, containing 2% BSA. The reaction mixture (50 μl) containing 50 pmol 32P-labeled sn-2-arachidonoyl lysopaPA, 1% BSA, 50 mM NaF, 2 mM Na3VO4, 2.5 mM DTT, 0.5 mM EDTA, and a 10-μl suspension of Swiss 3T3 cell membranes was incubated for up to 30 min at 37 °C. The reaction was stopped by addition of 0.9 ml of chloroform/methanol/HCl (66:33:1, v/v). After addition of carrier PA and lysopaPA the lipids were extracted by addition of 0.75 ml of chloroform/methanol/water (5:48:47, v/v). The lower phase was dried under argon, and the PA was separated from lysopaPA by TLC as described for the MG kinase assay.

The activity of acyl-CoA:lysopaPA acyltransferase was measured as follows. Optimal activity was attained in a reaction mixture (50 μl) containing 60 mM Tris-HCl, pH 7.5, 3 mM DTT, 50 mM NaF, 2 mM Na3VO4, 0.75 μM unlabeled glycerol, then measured the radioactivity in the incubation medium and was partially hydrolyzed in the presence of cells (see "Experimental Procedures"). The cells, incorporated about 5% of the added [3H]MG into lipids was noteworthy because the MG isomerized rapidly in the incubation medium and was partially hydrolyzed in the conditions of our experiments ("Experimental Procedures"), glycerol) incorporated only 0.1-0.2% of the label into lipids revealed a major difference in the relative distribution of radioactivity among the cellular species of the lipids that were labeled during the time.

RESULTS
Cell Incubation Experiments with [3H]MG—To investigate the ability of quiescent Swiss 3T3 cells to incorporate sn-2-arachidonoyl MG into PI and other phospholipids, we incubated the cells with exogenous [3H]MG in the presence of a large excess of unlabeled glycerol, then measured the radioactivity that accumulated in several lipid classes. Under the conditions of our experiments ("Experimental Procedures"), the cells incorporated about 5% of the added [3H]MG into cellular lipids (other than MG) during a 1-h incubation. By contrast, cells that were incubated with free [3H]glycerol of the same specific activity (in the absence of unlabeled free glycerol) incorporated only 0.1-0.2% of the label into lipids (not shown). The cells' ability to incorporate [3H]MG into lipids was noteworthy because the MG isomerized rapidly in the incubation medium and was partially hydrolyzed in the presence of cells (see "Experimental Procedures").

A comparison of the cell lipids that were labeled with [3H]MG with those that were labeled with free [3H]glycerol revealed a major difference in the relative distribution of radioactivity (Fig. 1). The PI, PE, and PC of the [3H]MG-labeled cells respectively accounted for 35, 22, and 28% of the combined radioactivity in eight different lipid classes, whereas the values for the corresponding lipids of the [3H]glycerol-labeled cells were 3, 12, and 63%. These results provided evidence that the cells preferentially incorporated the [3H]MG into PI and PE.

In further studies of the cells' ability to incorporate this MG into lipids, we incubated the cells with [3H]MG for 0, 0.5, and 1 h, then changed the medium on some of the cells that had been labeled for 1 h to medium that contained no label, and continued the incubation for an additional 24 h. Upon measuring the radioactivity in the cells that had been exposed to [3H]MG for no more than a few seconds, i.e. after 0 h of incubation, we observed significant labeling of DG, TG, lysopaPA, PA, and PI (Table 1). The fact that both DG and lysopaPA were labeled at this early time point raised the possibility that the cells might have contained two different MG incorporation pathways, one initiated by an MG acyltransferase and one initiated by an MG kinase.

Very little PS was labeled during the 1 h of incubation with [3H]MG, but the labeling of PS increased 8-fold during the subsequent 24 h chase (Fig. 2). The net increase in radioactive PS was comparable with the increase in radioactive PE, which suggested that the PS might have been formed by a head group exchange reaction involving PE (8, 9).

Analysis of the distribution of radioactivity among molecular species of the lipids that were labeled during the time
Monoacylglycerol Incorporation Pathways in Swiss 3T3 Cells

In the absence of added cofactors or in the presence of added GOA (Fig. 3), we confirmed the identity of this species of PA by hydrolyzing the membranes formed even more PA when they were incubated with 32P-labeled lysoPA in the presence of added CoA and/or ATP (Fig. 3B). However, the distribution of radioactivity among molecular species of the PA differed under these incubation conditions. Selective formation of sn-1-stearoyl-2-arnachidonoyl PA occurred in the absence of added cofactors or in the presence of added CoA. We confirmed the identity of this species of PA by hydrolyzing the PA with lipase and analyzing an sn-1-acetyl-2-acyl derivative of the resulting lysoPA by HPLC (data not shown).

The membranes of Swiss 3T3 cells also contain a DG kinase that preferentially phosphorylates arachidonic acid-containing substrates (27). To examine the relationship between this enzyme and the MG kinase, we extracted cell membranes with 0.3 M KCl, then measured the activities of the two enzymes in the extract and residue. The KCl extract and the residue each contained about one-half of the recovered MG kinase activity, and in each case the MG kinase showed an approximately 2-fold preference for sn-2-arnachidonoyl MG as compared with sn-2-oleoyl MG (Table IV). In contrast, the residue contained essentially all of the arachidonoyl-specific DG kinase activity, and this activity showed a 10–11-fold preference for sn-1-stearoyl-2-arnachidonoyl DG as compared with sn-1-stearoyl-2-oleoyl DG (Table IV). These results showed that the MG kinase activity differed from the arachidonoyl-specific DG kinase activity.

To search for an enzyme activity that could catalyze the conversion of sn-2-arnachidonoyl lysoPA into sn-1-stearoyl-2-arnachidonoyl PA, we incubated cell homogenate fractions with 32P-labeled sn-2-arnachidonoyl lysoPA in the presence or absence of potential cofactors. The cell membranes converted the radioactive sn-2-arnachidonoyl lysoPA into PA in the absence of added cofactors, whereas the high speed supernatant fraction showed little or no activity (Fig. 3A). Moreover, the membranes formed even more PA when they were incubated with 32P-labeled lysoPA in the presence of added CoA and/or ATP (Fig. 3B). However, the distribution of radioactivity among molecular species of the PA differed under these different incubation conditions. Selective formation of sn-1-stearoyl-2-arnachidonoyl PA occurred in the presence of added cofactors or in the presence of added CoA (Fig. 4, A and B). We confirmed the identity of this species of PA by hydrolyzing the PA with lipase and analyzing an sn-1-acetyl-2-acyl derivative of the resulting lysoPA by HPLC (data not shown).

Because we had also observed rapid labeling of lysoPA in these experiments we next measured the MG kinase activity of the cell homogenates. The cells did indeed contain this type of activity, and more than 90% of it was associated with the cell membranes, as had been found for the MG acyltransferase. A comparison of the membrane-associated enzyme’s activities toward sn-2-arnachidonoyl MG and sn-2-oleoyl MG provided little evidence of enzyme specificity. In three experiments the mean ratio of the enzyme’s activity toward the two substrates (arnachidonoyl MG/oleoyl MG) was 0.8. Furthermore, both substrates were converted into TG (not shown), presumably by the coupled action of the MG acyltransferase and a DG acyltransferase. This suggested that the MG acyltransferase might have initiated the incorporation of [3H]MG into DG and TG in the incubation experiments with intact cells.

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Monoacylglycerol Incorporation Pathways in Swiss 3T3 Cells

### Table II

**Distribution of radioactivity in molecular species of lipids from 3T3 cells labeled with \[^{32}P\]HMG**

Quiescent 3T3 cells were incubated with \[^{32}P\]HMG (25 μCi/dish in the presence of 7000-fold excess of unlabeled glycerol) for 0, 0.5, and 1 h. The medium on some of the cells that had been labeled for 1 h was subsequently changed to one containing no radiolabeled precursor, and the cells were incubated for a further 24 h. After the appropriate pulse or chase times, cells were harvested and the lipids extracted and analyzed as described under "Experimental Procedures." Molecular species of the individual lipid classes were resolved by reverse-phase HPLC. The percentage distribution of radioactivity in the species was calculated from the total radioactivity recovered in arachidonoyl and non-arachidonoyl species. The data represent the means ± standard deviation of four replicates within a single experiment. Similar results were obtained in three separate experiments, though in one of the experiments somewhat lower percentages were obtained for sn-1-stearyl-2-arachidonoyl PA. 14:0-20:4, sn-1-myristoyl-2-arachidonoyl phospholipid species; 16:0-20:4, sn-1-palmitoyl-2-arachidonoyl phospholipid species; 16:0-20:4, sn-1-stearyl-2-arachidonoyl phospholipid species.

| Incubation time | 14:0-20:4 species | 16:0-20:4 species | 18:0-20:4 species |
|-----------------|------------------|------------------|------------------|
| h               | dpm/dish | % of total | dpm/dish | % of total | dpm/dish | % of total |
| PI              |          |            |          |            |          |            |
| 0               | ND*      |            | 417 ± 54 | 8.1 ± 1.1  | 4,660 ± 742 | 87.9 ± 2.8 |
| 0.5             | ND       |            | 24,357 ± 1,490 | 8.2 ± 0.4 | 262,043 ± 22,877 | 88.1 ± 1.4 |
| 1               | ND       |            | 26,931 ± 1,516 | 6.9 ± 0.5 | 334,154 ± 53,753 | 87.2 ± 2.5 |
| 24a             | ND       |            | 12,679 ± 2,558 | 5.0 ± 0.5 | 224,186 ± 33,113 | 89.7 ± 2.3 |
| PA              |          |            |          |            |          |            |
| 0               | 131 ± 18 | 1.5 ± 0.0  | 901 ± 98 | 11.8 ± 0.5 | 5,984 ± 452 | 78.9 ± 4.0 |
| 0.5             | 686 ± 38 | 5.0 ± 0.2  | 2,918 ± 307 | 21.2 ± 0.9 | 9,025 ± 135 | 65.9 ± 4.1 |
| 1               | 537 ± 65 | 5.8 ± 0.8  | 1,775 ± 21 | 18.9 ± 0.5 | 5,871 ± 204 | 62.6 ± 3.0 |
| 24a             | ND       |            | 288 ± 39 | 7.0 ± 1.3  | 2,801 ± 633 | 66.9 ± 2.6 |
| PS              |          |            |          |            |          |            |
| 0               | ND       |            | 24       | 8.9        | 196       | 73.0 ± 0.0 |
| 0.5             | ND       |            | 402 ± 91 | 9.5 ± 0.8  | 2,987 ± 449 | 70.4 ± 3.0 |
| 1               | ND       |            | 593 ± 132 | 9.7 ± 1.2  | 4,593 ± 525 | 76.3 ± 6.1 |
| 24a             | ND       |            | 1,773 ± 279 | 3.6 ± 0.4 | 35,789 ± 5,271 | 71.3 ± 3.5 |
| PE              |          |            |          |            |          |            |
| 0               | 187 ± 12 | 6.9 ± 1.1  | 603 ± 102 | 21.4 ± 1.7 | 1,408 ± 322 | 49.7 ± 8.0 |
| 0.5             | 6,330 ± 270 | 3.6 ± 0.1 | 29,733 ± 1,683 | 17.0 ± 1.7 | 122,024 ± 6,779 | 68.4 ± 2.1 |
| 1               | 6,705 ± 526 | 3.0 ± 0.3 | 36,103 ± 2,120 | 16.3 ± 1.1 | 150,813 ± 6,015 | 68.1 ± 1.4 |
| 24a             | 2,361 ± 395 | 1.5 ± 0.3 | 19,304 ± 1,612 | 12.7 ± 0.6 | 103,232 ± 3,889 | 67.8 ± 0.8 |
| PC              |          |            |          |            |          |            |
| 0               | 128 ± 31 | 6.1 ± 1.6  | 439 ± 94 | 20.6 ± 2.4 | 895 ± 157 | 42.5 ± 6.4 |
| 0.5             | 12,597 ± 859 | 8.7 ± 0.9 | 46,306 ± 3,470 | 31.7 ± 1.5 | 63,517 ± 5,017 | 43.5 ± 2.3 |
| 1               | 24,355 ± 1,519 | 9.7 ± 1.1 | 68,792 ± 6,162 | 27.2 ± 0.7 | 107,162 ± 12,080 | 42.3 ± 0.8 |
| 24a             | 7,310 ± 911 | 3.1 ± 0.3 | 43,971 ± 3,822 | 18.7 ± 0.9 | 92,764 ± 4,360 | 39.5 ± 1.1 |
| DG              |          |            |          |            |          |            |
| 0               | 989 ± 194 | 8.2 ± 1.0  | 2,933 ± 381 | 24.3 ± 1.0 | 6,356 ± 481 | 53.3 ± 3.7 |
| 0.5             | 4,371 ± 722 | 9.7 ± 1.6 | 13,742 ± 2,106 | 30.4 ± 1.1 | 20,698 ± 1,231 | 46.0 ± 2.4 |
| 1               | 3,938 ± 1,272 | 11.2 ± 2.0 | 9,834 ± 2,009 | 25.5 ± 1.8 | 14,828 ± 4,665 | 41.9 ± 3.8 |
| 24a             | 346 ± 98  | 7.3 ± 0.7  | 527 ± 158 | 11.1 ± 1.4 | 2,463 ± 1,018 | 50.4 ± 8.0 |

* ND, not detected.

a Chase time.

**Note that small amounts of sn-1-stearyl-2-linoleoyl PA and sn-1-stearyl-2-oleoyl PA also seemed to be formed in the presence of CoA. We assume that these species were products of secondary transacylation reactions because they did not appear at early time points (data not shown). Both sn-1-stearyl-2-arachidonoyl PA and sn-1-palmitoyl-2-arachidonoyl PA were formed when the membranes were incubated with \[^{32}P\]labeled lysoPA in the presence of ATP or ATP + CoA (Fig. 4, C and D). Thus, the membranes appeared to contain both a CoA-dependent stearyl transerase activity and a second, ATP-dependent activity that was probably an acyl-CoA-dependent acyltransferase activity. In support of this possibility, extraction of the membranes with the neutral detergent, Triton, yielded a soluble acyl-CoAlysoPA acyltransferase activity that catalyzed the transfer of myristoyl, palmitoyl, and stearyl groups to sn-2-arachidonoyl lysoPA. In three experiments the mean (±S.D.) rates of transfer of these respective acyl groups were 351 ± 92, 772 ± 147, and**

### Table III

**Distribution and substrate specificity of MG acyltransferase activity in 3T3 cell subfractions**

3T3 cells from 30 (150 cm²) dishes were subfractionated into a low speed pellet, high speed pellet, and high speed supernatant. The MG acyltransferase activity toward sn-2-arachidonoyl MG and sn-2-oleoyl MG was measured as described under "Experimental Procedures." Assays were performed at 23 °C at a protein concentration of 4 μg/200 μl reaction mixture. 20:4, arachidonic acid; 18:1, oleic acid.

| Specific activity | Protein |
|-------------------|---------|
| sn-2:20:4 MG      | sn-2:18:1 MG |
| pmol/min/mg protein | mg/fraction |

| Homogenate  | 163.0 ± 42.0 | 163.8 ± 45.7 | 9.86 ± 1.85 |
| Low speed pellet | 258.2 ± 66.5 | 310.6 ± 85.7 | 7.11 ± 1.80 |
| High speed pellet | 164.3 ± 63.9 | 244.1 ± 145 | 1.05 ± 0.35 |
| High speed supernatant | 10.7 ± 2.7 | 27.6 ± 1.8 | 1.25 ± 0.43 |

* Average of three independent preparations ± standard deviation.
The presence or absence of added CoA demonstrated that all of the radioactivity was present in the sn-1-stearoyl-2-arachidonoyl lysoPA into PI (Table I). 2) The sn-1-stearoyl-2-arachidonoyl species of PI accounted for about 90% of the radioactivity that was present in this phospholipid class at all incubation times. 3) Examination of cell homogenates revealed the presence of an MG kinase activity that phosphorylated sn-2-arachidonoyl MG in preference to sn-1-oleoyl MG (Table IV). 4) When cell membrane preparations were incubated with 32P-labeled sn-2-arachidonoyl lysoPA in the presence or absence of added CoA, radioactive sn-1-stearoyl-2-arachidonoyl PI was formed selectively (Fig. 4). 5) The membrane preparations apparently converted this PA into sn-1-stearoyl-2-arachidonoyl PI in the presence of CTP and inositol (Table V), although we cannot exclude the possibility that an sn-2-arachidonoyl CDP-MG intermediate may have been involved. It is interesting to compare those results with those of Nakagawa and colleagues (31), who incubated alveolar macrophage microsomes in the presence of [14C]glycerol 3-phosphate, CTP, and inositol and found that only 2–3% of the label that was converted into PI was present in the sn-1-stearoyl-2-arachidonoyl species. It appears that the results of Nakagawa and colleagues reflect the de novo pathway of PI synthesis.

The MG kinase-initiated pathway appears to branch after the first step because we found that the membranes of Swiss 3T3 cells contained both a CoA-dependent stearyl transferase and an ATP-dependent acyltransferase that could catalyze the conversion of 32P-labeled sn-2-arachidonoyl lysoPA into PA. The ATP-dependent acyltransferase reaction formed a mixture of sn-1-palmitoyl-2-arachidonoyl and sn-1-stearoyl-2-arachidonoyl PA (Fig. 4), which suggests that the acyl-CoA-dependent acyltransferase that we found in detergent extracts of the membranes was probably involved.

The fate of the PA formed by the ATP-dependent acyltransferase in intact cells remains to be determined, but one possibility is that the PA may be converted (via DG) into PE. Formation of PE by this branch of the MG kinase-initiated pathway might account for the preferential labeling of PE that occurred in the intact cell incubation experiments (Fig.

**TABLE IV**

| Distribution of MG kinase and DG kinase activities in KCl-extracted Swiss 3T3 cell membranes |
|---------------------------------------------------|---------------------------------------------------|
| 3T3 cell total membrane fraction was isolated, extracted with 0.3 M KCl and subfractionated into supernatant and pellet fractions. All fractions were adjusted to the same volume. The MG kinase activity toward sn-2-arachidonoyl MG or sn-2-oleoyl MG was measured in BSA, and the DG kinase activity toward sn-1-stearoyl-2-arachidonoyl (18:0–20:4) DG or sn-1-stearoyl-2-oleoyl (18:0–18:1) DG was measured in octyl glucoside. The assays were performed as described under “Experimental Procedures.” Similar results were obtained in two other experiments. | |
| MG kinase, LysoPA formed | DG kinase, PA formed | Protein |
|--------------------------|-----------------------|---------|
| sn-2-20:4 MG | sn-2-18:1 MG | 18:0–20:4 DG | 18:0–18:1 DG | mg/sample |
| Membranes | 0.83 | 0.30 | 46.5 | 6.6 | 24.5 |
| KCl-extracted supernatant | 1.07 | 0.63 | 18.8 | 11.0 | 7.27 |
| KCl-extracted pellet | 0.57 | 0.24 | 63.9 | 5.7 | 15.0 |

**DISCUSSION**

The results of this study show that quiescent Swiss 3T3 cells incorporate radioactive sn-2-arachidonoyl MG into several cell lipids including sn-1-stearoyl-2-arachidonoyl PI. Furthermore, they suggest that two or more MG incorporation pathways are involved and that the pathway that forms PI is initiated by the successive action of an MG kinase and a CoA-dependent stearoyl transferase. The evidence for the participation of these enzymes in PI formation can be summarized as follows. 1) When quiescent Swiss 3T3 were incubated for a few seconds with radioactive sn-2-arachidonoyl MG, significant amounts of radioactive lysoPA, sn-1-stearoyl-2-arachidonoyl PA, and sn-1-stearoyl-2-arachidonoyl PI accumulated (Table I). 2) The sn-1-stearoyl-2-arachidonoyl species of PI accounted for about 90% of the radioactivity that was present in this phospholipid class at all incubation times. 3) Examination of cell homogenates revealed the presence of an MG kinase activity that phosphorylated sn-2-arachidonoyl MG in preference to sn-1-oleoyl MG (Table IV). 4) When cell membrane preparations were incubated with 32P-labeled sn-2-arachidonoyl lysoPA in the presence or absence of added CoA, radioactive sn-1-stearoyl-2-arachidonoyl PA was formed selectively (Fig. 4). 5) The membrane preparations apparently converted this PA into sn-1-stearoyl-2-arachidonoyl PI in the presence of CTP and inositol (Table V), although we cannot exclude the possibility that an sn-2-arachidonoyl CDP-MG intermediate may have been involved. It is interesting to compare those results with those of Nakagawa and colleagues (31), who incubated alveolar macrophage microsomes in the presence of [14C]glycerol 3-phosphate, CTP, and inositol and found that only 2–3% of the label that was converted into PI was present in the sn-1-stearoyl-2-arachidonoyl species. It appears that the results of Nakagawa and colleagues reflect the de novo pathway of PI synthesis.

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The fate of the PA formed by the ATP-dependent acyltransferase in intact cells remains to be determined, but one possibility is that the PA may be converted (via DG) into PE. Formation of PE by this branch of the MG kinase-initiated pathway might account for the preferential labeling of PE that occurred in the intact cell incubation experiments (Fig.
Monoacylglycerol Incorporation Pathways in Swiss 3T3 Cells

Fig. 4. Molecular species of $^{32}$P-labeled PA formed by Swiss 3T3 cell membranes. $^{32}$P-Labeled sn-2-arachidonoyl lysoPA was incubated with Swiss 3T3 cell membranes for 20 min at 37°C in the presence or absence of cofactors. The incubations were performed as follows. A, without cofactors; B, with 0.5 mM CoA; C, with 2.5 mM ATP + 4 mM magnesium acetate; D, with CoA + ATP + magnesium acetate. The PA that was formed was methylated and analyzed by reverse-phase HPLC as described under "Experimental Procedures." In the case of the incubation performed in the absence of cofactors (panel A) the PA from two separate incubation replicates was pooled in order to obtain a sufficient amount of radioactivity for HPLC analysis. As a blank, a parallel incubation was done without a source of enzyme, and the radioactivity in the effluent from the HPLC column was determined. The values obtained were then subtracted from those obtained for the test samples. The arrows in the figure indicate the retention times of standards; dashed lines indicate the absorbance of carrier lipids at 206 nm; solid lines indicate radioactivity. Similar results were obtained in a second experiment.

Table V
Production of PI from sn-2-arachidonoyl lysoPA by 3T3 cell membranes in the presence of CTP and myoinositol

| Cofactors added | Without CoA | With CoA |
|----------------|-------------|----------|
|               | PA          | CDP-DG   | PI      | PA          | CDP-DG   | PI      |
| None          | 19.9        | 0.0      | 0.6    | 134.6       | 1.3      | 0.5    |
| CTP           | 4.3         | 8.6      | 5.1    | 14.4        | 81.8     | 37.4   |
| Inositol      | 22.6        | 0.0      | 0.3    | 125.9       | 2.8      | 0.3    |
| CTP and inositol | 2.1   | 6.1      | 16.1   | 7.5         | 54.8     | 54.9    |

1). However, the relatively high content of labeled sn-1-stearoyl-2-arachidonoyl PE observed (Table II) suggests that a stearoyl-specific pathway, such as the stearoyl-specific branch of the MG kinase-initiated pathway, may also have been involved. Furthermore, the stearic acid-containing PE that was formed may have been converted into PS.

An MG acyltransferase reaction may have formed most of the radioactive DG that accumulated in the cell incubation experiments. The cells clearly contained such an activity (Table III), and studies by previous investigators have shown that sn-2-acyl MG acyltransferase activities in other cells can initiate MG incorporation pathways that form DG and TG (30, 32). However, the specificity of the Swiss 3T3 cell enzyme remains to be fully characterized. We demonstrated that it shows no preference for sn-2-arachidonoyl MG over sn-2-oleoyl MG, but have yet to examine its preference for different acyl-CoAs. Thus, we do not know whether the distribution of labeled DG species that we observed in the cell incubation experiments would be consistent with its activity.

The MG acyltransferase reaction that formed most of the cell DG may also have formed most of the cell PC. The major reason for believing this is that the distribution of radioactive species of DG was very similar to the corresponding distribution found for PC (Table II). The simplest way to account for this similarity would be to postulate that the MG acyltransferase formed sn-1,2-DG and that a DG:phosphorylcholine transferase reaction subsequently converted this DG into PC (Fig. 5). However, direct evidence for this remains to be obtained, and we cannot exclude the possibility that some sn-2,3-DG also may have been formed.

Much more work will be needed to clarify the precise nature of these MG incorporation pathways. None of the enzymes involved has been isolated or fully characterized, so it is not clear how many enzymes or enzyme isoforms may be present or how they may relate to other enzymes that have been described. For example, we presume that the MG kinase in Swiss 3T3 cell membranes is related in some way to the soluble MG kinase that was recently found in bovine brain (33) because both enzymes phosphorylate sn-2-arachidonoyl MG in preference to sn-2-oleoyl MG. But the relation between the two enzymes remains to be determined.

Both the CoA-dependent stearoyl transferase and the ATP-dependent acyltransferase that convert sn-2-arachidonoyl lysoPA into PA also remain to be characterized. We are currently examining the CoA-dependent enzyme in an effort to identify the stearoyl donor, establish the basis for the CoA requirement, determine the enzyme's specificity for different sn-2-acyl lysophospholipids, and relate its activity to that of a recently discovered but still uncharacterized CoA-dependent stearoyl transferase activity in brain membranes (34).
Fig. 5. Proposed pathways of sn-2-arachidonoyl MG incorporation in Swiss 3T3 cells. Our data suggest that Swiss 3T3 cells metabolize sn-2-arachidonoyl MG by at least two different pathways. Reactions in these pathways for which we have direct evidence are indicated by solid arrows; other possible reactions are indicated by open arrows. One of the pathways is initiated by an MG kinase activity that phosphorylates sn-2-arachidonoyl MG in preference to sn-2-oleoyl MG. The sn-2-arachidonoyl lysoPA that is formed may then be acylated by a CoA-dependent stearyl transferase to form sn-1-stearyl-2-arachidonoyl PA, which can be converted into CDP-DG and PI. It is possible that this pathway may also form PE and PS. Alternatively, the sn-2-arachidonoyl PA may be acylated by an acyl-CoA-dependent acyltransferase to form a mixture of sn-1-acyl-2-arachidonoyl PAs. A second MG incorporation pathway is initiated by an acyl-CoA:MG acyltransferase which may show little acyl group specificity. We suggest that this may form sn-1,2-DG which may subsequently be converted into TG, PC, and possibly PE.

Other questions concern the cellular role of the MG incorporation pathways. This role is likely to depend on the source of sn-2-arachidonoyl MG, which may well differ under different metabolic conditions and in different cells. The results of the present study demonstrate that Swiss 3T3 cells readily incorporate exogenous sn-2-arachidonoyl MG into PI and other phospholipids. Thus, the MG incorporation pathways in these cells clearly represent an alternative to the type of phospholipid remodeling pathways that have been postulated by others. However, the quantitative significance of the MG incorporation pathways has yet to be determined. Moreover, their major cellular role may well be to recycle or remodel cell phospholipids. For example, Swiss 3T3 cells that are stimulated by platelet-derived growth factor both convert PI and other phospholipids into MG and resynthesize PI (13, 14). Therefore, it is possible that some of this MG is recycled into phospholipids. Further research concerning this possibility is needed because much remains to be learned about the metabolism and function of sn-1-stearyl-2-arachidonoyl phospholipids in animal cells.

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