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# Heterogeneous Long Chain Acyl-CoA Synthetases Control Distribution of Individual Fatty Acids in Newly-Formed Glycerolipids of Neuronal Cells Undergoing Neurite Outgrowth

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Using PC12 cells undergoing neurite outgrowth, we studied the activation of various fatty acids, of different chain lengths and degrees of saturation, by long chain acyl-CoA synthetases (LCASs). Cells treated with nerve growth factor (NGF) were labeled with [$^3$H]glycerol, [$^3$H]oleic acid (OA) or [$^3$H]arachidonic acid (AA) in the presence of other unlabeled fatty acids of endogenous or exogenous origin. Triacsin C (4.8 μM), an inhibitor of acyl-CoA synthetase, decreased the incorporation of exogenous [$^3$H]OA into glycerolipids by 30–90%, and increased by about 60% the accumulation of free [$^3$H]OA in the cells. However it did not affect the incorporation of endogenous fatty acids nor of exogenous [$^3$H]AA into phospholipids, suggesting that LCASs which activate exogenous AA and at least some endogenous fatty acids are relatively insensitive to this drug. Activities of the LCAS that is specific for AA (ACS), or of the non-specific LCAS which activates OA and other fatty acids (OCS), were much higher in microsomal and cytoplasmic fractions than in mitochondria or nuclei. The *Vmax* and *Km* values of ACS and OCS in microsomes were 12 and 0.7 nmol/min/mg protein and 70 and 37 μM, respectively; and in cytoplasm, 6 and 0.6 nmol/min/mg protein and 38 and 60 μM, respectively. Triacsin C (2–33 μM) did not affect ACS activity in microsomal or cytoplasmal fractions, but inhibited OCS activities dose-dependently and competitively: $IC_{50}$ and apparent *Ki* values were 13.5 μM and 14 μM in microsomes, and 3.8 μM and 4 μM in cytoplasm. NGF stimulated the activities of the LCASs, and, consistently, the incorporation of the various fatty acids into glycerolipids. These data indicate that LCASs are heterogeneous with respect to their intracellular locations, substrate specificities, kinetic characteristics and sensitivities to triacsin C; and that this heterogeneity affects the extents to which individual fatty acids are utilized to form glycerolipids.

**KEY WORDS:** Long chain acyl-CoA synthetase; fatty acid; glycerolipid; nerve growth factor; triacsin C; PC12 cells.

## INTRODUCTION

Membrane glycerophospholipids obtain their fatty acid constituents via two mechanisms: *de novo* synthesis via acylation of glycerol, and re-acylation of lysophospholipid by long chain acyl-CoA. That a given phospholipid can be highly heterogeneous with reference to its fatty acid contents is well known; however, the relationships between a phospholipid's fatty acids, its distinct functions, and its mode of synthesis are unclear (1).

Activation of fatty acids, catalyzed by long chain acyl-CoA synthetase (LCAS; EC 6.2.1.3), is the initial reaction in fatty acid utilization within mammalian cells;

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thus, the LCAS enzyme or enzymes play a key role in lipid metabolism. The long chain acyl-CoAs produced by LCAS act as precursors for lipid synthesis and for fatty acid elongation and desaturation reactions, as well as for degradation via the β-oxidation system. Long chain acyl-CoAs also play regulatory roles in numerous reactions, including for example protein modification (2), intracellular protein transport (3), protein kinase C activation (4), nuclear thyroid hormone receptor modulation (5), and cell proliferation (6). These functions presumably are performed by different long chain acyl-CoAs, existing in different cellular compartments; however, little is known about the partitioning of LCAS enzymes within cells, nor about their heterogeneity.

We previously examined the sources of the increased DAG levels observed in differentiating PC12 cells exposed to nerve growth factor (NGF) (7), and found that triacsin C, a potent inhibitor of LCAS, partially blocked the incorporation of exogenous oleic acid (OA) into glycerolipids. Triacsin C [1-hydroxyl-3-(*E,E,E*-2′,4′, 7′-undecatrienylidine) triazene], one of the few known naturally occurring compounds that competitively inhibit LCAS (8), is widely used in studies on fatty acid metabolism. In the present study we further use this drug to explore the utilization of different fatty acids to form glycerolipids, and the control of this process by heterogeneous LCASs in cells undergoing neurite outgrowth.

## EXPERIMENTAL PROCEDURE

*Cell Culture.* PC12 cells (ATCC) were cultured according to the method of Greene and Tischler (9). Growth medium (medium A) was RPMI 1640 (GIBCO BRL) supplemented with 10% (v/v) heat-inactivated horse serum and 5% (v/v) fetal bovine serum (GIBCO BRL). Cells at a density of $5 \times 10^5$/ml in 12 ml medium A were routinely maintained in 75 $cm^2$ tissue culture flasks at 37°C and an atmosphere of 95% air/5% $CO^2$, and the medium was changed every 2 days. Cells used for experiments had undergone 5–10 passages.

*Measurement of Fatty Acid Composition in Phospholipids.* PC12 cells at a density of $2 \times 10^5$/ml in 2 ml medium A were plated on 35-mm tissue culture dishes coated with mouse collagen IV (Fisher) for at least 1 day. At 24 hours prior to an experiment, the medium A bathing the cells was replaced with a differentiation medium (Medium B), i.e., RPMI 1640 medium containing only 1% horse serum. After 4 days, purification of the non-radiolabeled neutral lipids and phospholipids extracted from the cells was performed by one dimensional thin layer chromatography (TLC) on silica gel G plates (Analtech) as described below (extraction and assay of lipids). Their fatty acids were analyzed by gas chromatography (10). The powders were scraped from the related bands, and methylated directly by alkaline methanolysis. Each sample was mixed thoroughly with 1 ml of saturated NaOH in chloroform and methanol (2:1, by volume), and stirred for 10 minutes. 1 ml of 1 N HCl in saline was added and the sample was mixed and centrifuged at 3,000 *g* for 5 minutes. Approximately 0.5 ml of the lower phase was transferred into a clean tube, dried in the speed vacuum concentrator (Savant), and resuspended in 20 μl hexane, 1 μl of which was injected in a gas chromatography apparatus (Hewlett-Packard 5880A, equipped with a flame ionization detector and an electronic integrator). A fused capillary column (SP2330, 30 m long × 0.25 mm internal diameter; Supelco) was used; the carrier gas was helium; its flow rate was 1 ml/min, the split ratio was 1:30. The initial column temperature was 190°C; after 10 minutes, it was increased to 210°C at a rate of 2°C/min. Injector and detector temperatures were both 250°C. The fatty acid methyl esters were identified by comparing their retention times with those of standard solutions run under identical conditions. Measurements of peak areas were made using an automatic integrator attached to the gas chromatography apparatus. Fatty acid compositions of phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) or phosphatidylinositol (PI) were expressed both as individual concentrations (pmol/μg DNA) and proportional amounts of total fatty acids (%).

*Preparation of Subcellular Fractions.* PC12 cells cultured in the growth medium (medium A in flasks; without NGF treatment) or in the differentiation medium (medium B in collagen-coated dishes; pre-treated with 50 ng/ ml of NGF for 4 days) were collected and sonicated with a cell disrupter in chilled phosphate buffered solution (PBS) containing 0.25 M sucrose (PBS-sucrose). The whole suspensions were centrifuged at 900 *g* for 10 minutes at 4°C to yield nuclear fractions, and pellets were washed twice with PBS-sucrose by re-centrifugation as above. Supernatants and washings were combined and subjected to successive centrifugation at 15,000 *g* for 20 minutes and at 230,000 *g* for 60 minutes to yield mitochondrial (including peroxisomes and lysosomes) and microsomal fractions, respectively; each fraction was washed once with a small volume of PBS-sucrose, and the washing was combined with the supernatant (11). The last supernatant was the soluble cytoplasm. All fractions were adjusted with PBS-sucrose to a protein level of 5 mg/ml and stored at −80°C for enzymatic assay.

*Assay of LCAS Activity.* The isotopic assay of arachidonoyl-CoA synthetase (ACS) or oleoyl-CoA synthetase (OCS) relies on heptane extraction of non-reacted free fatty acid, and the insolubility of long chain acyl-CoA esters in heptane (12). The final composition of each reaction mixture (0.15 ml) was: 15 μmol Tris-HCl (pH 8.0), 3 μmol $MgCl_2$, 1 μmol ATP, 100 nmol Coenzyme A, 150 nmol 2-mercaptoethanol, 0.1% Triton X-100, 20 nmol [$^3$H]AA or [$^3$H]OA (5 nCi/nmol) in 50 mM $NaHCO_3$ and 200 μg of protein from a subcellular fraction as a source of enzyme. The reaction was initiated by addition of the enzyme. After incubation at 37°C with or without inhibitors (various fatty acids and triacsin C) for 0, 5, 10, 20, 30, or 40 minutes, the reaction was stopped by adding 2.25 ml of isopropanol/heptane/2 M sulfuric acid (40:10:1, by volume). 1.5 ml of heptane and 1 ml of water were added to the reaction mixture, which was then vortexed vigorously. The organic phases were discarded and the aqueous phase was extracted twice with 2 ml of heptane containing 4 mg/ml of palmitic acid (PA). A 1 ml sample of the aqueous phase in 5 ml of ultrafluor scintillation fluid was counted for [$^3$H]arachidonoyl-CoA or [$^3$H]oleoyl-CoA levels. The products of LCASs were identified by TLC (13) on silica gel G plates, using isopropanol/pyridine/acetic acid/water (60:15:1:25, by volume) as a mobile phase. Over 80% of the radioactivity co-chromatographed with arachidonoyl-CoA or oleoyl-CoA standard ($R_F$ = 0.30; Sigma).

*Incorporation of Radioactive Glycerol, OA or Arachidonic acid (AA) into Cellular Lipids.* PC12 cells at a density of $2 \times 10^5$/ml in 2 ml medium A were plated on 35-mm collagen-coated dishes for at least 1 day, and then the medium A bathing the cells was replaced

with medium B, in which the final content of free OA was less than 0.04 μM.

To examine the utilization of endogenous fatty acids, cells were pretreated with 50 ng/ml of NGF (2.5 S; GIBCO BRL) in medium B for 1 day, and then exposed to fresh medium B supplemented with 50 ng/ml NGF, 4.8 μM triacsin C (Biomol) and 8 μCi/ml [1,2,3-$^3$H]glycerol ([$^3$H]glycerol, 80 Ci/mmol; New England Nuclear) for an additional 20 hours.

To examine the utilization of exogenous OA, cells were pretreated with NGF for 1 day, and then exposed to fresh medium B supplemented with 50 ng/ml of NGF, 4.8 μM triacsin C, and 5 μCi/ml [$^3$H]OA (7.4 Ci/mmol; New England Nuclear), in the presence of 100 μM non-radioactive OA, for an additional 1 hour.

To compare the incorporation of exogenous OA and AA into phospholipids, cells were pre-treated with NGF for 1 day, and then exposed to fresh medium B supplemented with 50 ng/ ml NGF, 4.8 μM triacsin C, and 0.5 μCi/ml of [$^3$H]OA (10 μM or [$^3$H]AA (10 μM, New England Nuclear) for an additional day.

To examine recycling of endogenous OA from pre-labeled neutral lipids to phospholipids, cells pre-labeled with 5 μCi/ml of [$^3$H]OA for 1 hour were washed twice with medium B, at 37°C, to remove residual radiolabel, and then chased in fresh medium B, in the absence or presence of 4.8 μM triacsin C, for 0.5–12 hours.

*Extraction of Lipids.* Media were aspirated from the 35-mm coated dishes; the cells were then washed once with 2 ml of PBS (4°C), and harvested by being scraped in 1 ml of ice-cold methanol (–20°C) and transferred into a test tube. After sonication with a cell disrupter (Ultrasonic Inc.), 0.1 ml of suspension was taken for analyses of protein and DNA; 1.8 ml of chloroform and 0.9 ml of distilled water were then successively added to 0.9 ml of the remaining suspension for extracting lipids by the method of Van Veldhoven and Bell (14). The suspensions were vortexed and then centrifuged at 3,000 × *g* for 5 minutes at 4 °C. The aqueous phase was aspirated and the organic phase was dried using a speed vacuum concentrator.

*Assay of [$^3$H]Glycerol- or [$^3$H]Fatty Acid-Labeled Lipids.* The residue from the organic phase was reconstituted in a 50 μl of chloroform and methanol (1:1, by volume). A 20 μl aliquot of the labeled neutral lipid extract was then purified by one dimensional thin layer chromatography (TLC) for about 45 minutes on a pre-adsorbent silica gel G plate (Analtech), using petroleum ether/diethyl ether/acetic acid glacial (70:30:2, by volume) as the mobile phase (15). OA ($R_F$ = 0.64), monoacylglycerol (MAG, $R_F$ = 0.12), DAG ($R_F$ = 0.39) and triacylglycerol (TAG, $R_F$ = 0.88) standards (Sigma) were used to identify the corresponding bands alter staining the plate with iodine vapor. Another 20 μl aliquot of the labeled phospholipid extract was purified by TLC (16) for about 150 minutes on silica gel G plates, using a mobile phase containing chloroform/triethylamine/ ethanol/ water (30:30:34:8, by volume). Phosphatidylcholine (PC, $R_F$ = 0.09), phosphatidylethanolamine (PE, $R_F$ = 0.54), phosphatidylserine (PS, $R_F$ = 0.30) and phosphatidylinositol (PI, $R_F$ = 0.42) standards (Sigma) were used to identify the corresponding bands under long wave ultraviolet light, after spraying the plate with 0.1% diphenyl-hexatriene in petroleum ether. The lipid bands were scraped from the plate and collected into vials containing 15 ml of ultrafluor (National Diagnostics). The associated radioactivities were counted by liquid scintillation spectrometry (Beckman LS 6500).

*Other Methods.* To measure phospholipid masses, cellular PC, PE, PS and PI were extracted and purified by TLC as described above; the amounts of various phospholipids were determined by phosphate assay (17). Protein was assayed according to the method of Smith et al. (18). DNA was measured by the method of Labarca and Paigen (19) using calf thymus DNA as the standard (Sigma).

## RESULTS

*Properties of LCASs.* Using the subcellular fractions as enzyme sources we measured the intracellular distribution of ACS and OCS, their substrate specificities and kinetic properties, and also examined the effects of NGF and/or triacsin C on these parameters.

*(1) Intracellular Distribution of ACS and OCS.* Highest activities of ACS (Fig. 1A) and OCS (Fig. 1B) in the PC12 cells were found in microsomes, and lowest activities in nuclei. ACS activities in microsomes and cytoplasm were 6.5 and 4.4 times higher, respectively, than those of OCS (Fig. 1).

*(2) Substrate Specificity of ACS and OCS.* Seventeen individual long chain fatty acids were measured in glycerophospholipids of PC12 cells by gas chromatography; these included 5 saturated fatty acids and 12 unsaturated fatty acids (Table I). Four prominent fatty acids, i.e., palmitic acid (PA, 16:0; 3.3 nmol/μg DNA), stearic acid (SA, 18:0; 2.1 nmol/μg DNA), OA (18:1; 5.7 nmol/μg DNA) and AA (20:4; 0.7 nmol/μg DNA), were further used as substrates to explore the substrate specificities of LCASs.

Competition experiments were performed to examine the fatty acid specificity of ACS or OCS in microsomes and cytoplasm (Fig. 2). When 133.3 μM [$^3$H]AA was used as a substrate for ACS, the further addition of 66.7 μM of non-radioactive AA inhibited ACS activities by about 25–30% in cytoplasm and microsomes (Fig. 2A), however the addition of identical amounts of non-radioactive PA, SA or OA did not change the ACS activities (Fig. 2A). Non-radioactive AA competitively inhibited the formation of [$^3$H]arachidonoyl-CoA, with a *Ki* (47.8 μM) that approximated its *Km* (38 μM) (Fig. 2C). On the other hand, when [$^3$H]OA was used as a substrate for OCS, the addition of non-radioactive PA SA, OA and AA inhibited OCS activities (Fig. 2B) by 52, 24, 40 and 29% in cytoplasm, or by 30, 18, 48 and 22% in microsomes, respectively; non-radioactive PA, OA and AA competitively inhibited OCS activities, with apparent *Ki* values of 49.3, 64.0 and 137 μM (Fig. 2D), respectively. These data (Fig. 2) indicate that the two kinds of LCASs differ in their substrate specificities: ACS utilizes AA as its specific substrate, while OCS can activate several fatty acids; this suggests that ACS and OCS have distinct roles in the utilization of particular fatty acids.

*(3) Sensitivities of ACS and OCS to Inhibition of Triacsin C.* Triacsin (33 μM) slightly decreased the formation of [$^3$H]arachidonoyl-CoA, i.e., by about 10% in microsomes and 20% in cytoplasm (Fig. 3A). ACS catalyzed the formation of [$^3$H]arachidonoyl-CoA in micro-

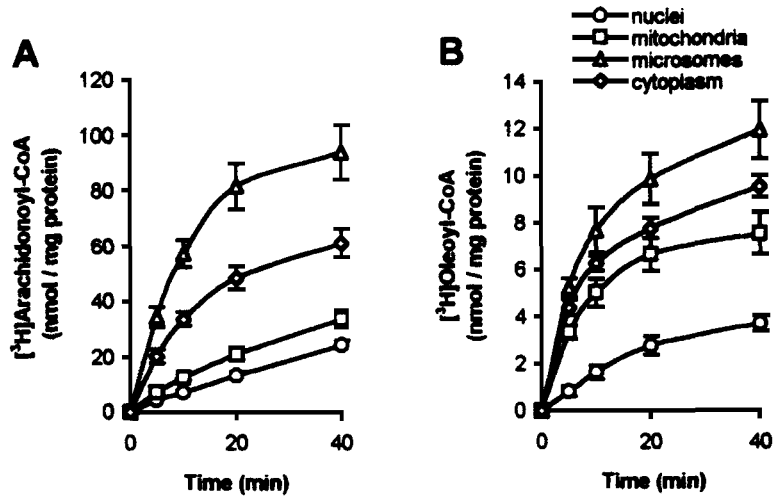


**Fig. 1.** Activities of LCASs in subcellular fractions. PC 12 cells cultured in the growth medium (medium A) were collected and sonicated with a cell disrupter in chilled PBS containing 0.25 M sucrose (PBS-sucrose). The subcellular fractions of nuclei, mitochondria, microsomes and cytoplasm were prepared by successive centrifugation as described in Experimental Procedure. For enzymatic assay, the total volume of 0.15 ml of the standard reaction mixture contained finally 15 μmol Tris-HCl (pH 8.0), 3 μmol $MgCl_2$, 1 μmol ATP, 100 nmol Coenzyme A, 150 nmol 2-mercaptoethanol, 0.1% Triton X-100, 20 nmol [$^3$H]AA or [$^3$H]OA (5 nCi/nmol) in 50 mM $NaHCO_3$, and 200 μg of protein (as a enzyme source) from the subcellular fraction.. The reaction was initiated by the addition of enzyme and, after incubation at 37 °C for 5, 10, 20, or 40 minutes, stopped by the addition of 2.25 ml of isopropanol/heptane/2 M sulfuric acid (40:10:1, by volume). [$^3$H]arachidonoyl-CoA or [$^3$H]oleoyl-CoA was extracted and measured as described in Experimental Procedures. Activities of LCASs were expressed as the levels of arachidonoyl-CoA or oleoyl-CoA formed in the above reaction mixtures. Values represent the means ± SD of [$^3$H]arachidonoyl-CoA (**A**) or [$^3$H]oleoyl-CoA (**B**) levels (nmol/mg protein; n = 3).

**Table I.** Fatty Acid Content (pmol/μg DNA) and Percent Composition (% Total Fatty Acids)

| Carbon atoms: double bonds | PC content | % | PE content | % | PS content | % | PI content | % | Phospholipids content | % |
|---|---|---|---|---|---|---|---|---|---|---|
| 14:0 | 123 | 1.4 | ND | ND | ND | ND | ND | ND | 123 | 0.8 |
| 16:0 | 2869 | 32.6 | 308 | 8.1 | 73 | 5.2 | 17 | 2.1 | 3267 | 22 |
| 16:1 | 739 | 8.4 | 80 | 2.1 | 59 | 4.2 | 3 | 4 | 881 | 5.9 |
| 18:0 | 458 | 5.2 | 783 | 20.6 | 547 | 39.1 | 340 | 42.5 | 2128 | 14.3 |
| 18:1 | 3995 | 45.4 | 1163 | 30.6 | 493 | 35.2 | 75 | 9.4 | 5726 | 38.6 |
| 18:2 | 308 | 3.5 | 137 | 3.6 | 62 | 4.4 | ND | ND | 507 | 3.4 |
| 20:0 | ND | ND | ND | ND | 13 | 0.9 | ND | ND | 13 | 0.1 |
| 20:1 | 141 | 1.6 | ND | ND | 18 | 1.3 | ND | ND | 159 | 1.1 |
| 20:2 | ND | ND | 65 | 1.7 | 6 | 0.4 | 154 | 19.3 | 225 | 1.5 |
| 20:3 | 44 | 0.5 | 30 | 0.8 | 29 | 2.1 | 22 | 2.7 | 125 | 0.8 |
| 20:4 | 44 | 0.5 | 467 | 12.3 | 13 | 0.9 | 172 | 21.5 | 696 | 4.7 |
| 20:5 | 18 | 0.2 | 179 | 4.7 | 10 | 0.7 | 13 | 1.6 | 220 | 1.5 |
| 22:1 | 26 | 0.3 | ND | ND | 8 | 0.6 | ND | ND | 34 | 0.2 |
| 22:4 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| 22:5 | ND | ND | 179 | 4.7 | 32 | 2.3 | 4 | 0.5 | 215 | 1.4 |
| 22:6 | 26 | 0.3 | 422 | 11.1 | 60 | 4.3 | 6 | 0.8 | 514 | 3.5 |
| 24:0 | ND | ND | ND | ND | ND | ND | ND | ND | ND | ND |
| Total | 8791 | | 3813 | | 1423 | | 806 | | 14841 | |

The non-radiolabeled phospholipids (PC, PE, PS and PI) from PC12 cells cultured with medium B in collagen-coated dishes for 4 days were extracted and purified by TLC. The cellular levels of individual phospholipids were measured by phosphate assay, and their fatty acids were analysed by gas chromatography as described in Experimental Procedure. Fatty acid composition of PC, PE, PS or PI was expressed by both fatty acid content (pmol / μg DNA) and percentage (% of total fatty acid). Values represent the average of two determinations, and coefficients of variation were about 15–35%. ND: not detectable.

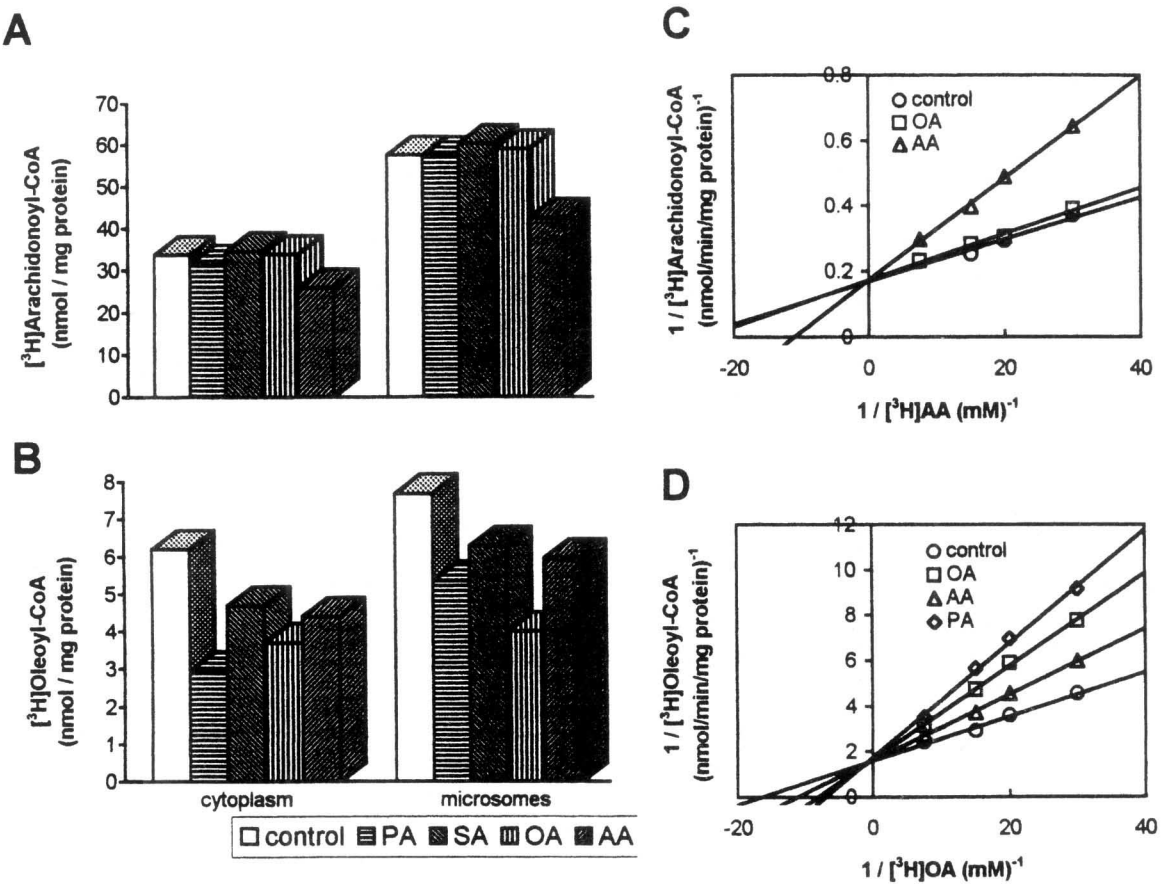


**Fig. 2.** Substrate specificity of LCASs. Microsomes and cytoplasm prepared from PC12 cells were used as enzyme sources as described in Fig. 1. To observe the substrate specificity of ACS (**A**) or OCS (**B**), an additional 10 nmol of non-radioactive fatty acid (PA, SA, OA or AA) was added into the standard reaction mixture containing 20 nmol of either [$^3$H]AA or [$^3$H]OA (5 nCi/nmol) and 200 μg of protein from microsomes or cytoplasm. To explore the inhibition kinetics of ACS (**C**) or OCS (**D**), an additional 10 nmol of non-radioactive fatty acid was added into the reaction mixture containing varying amounts of [$^3$H]AA or [$^3$H]OA (5 nCi/nmol) and 200 μg of protein from cytoplasm. After 10 minutes (**A** and **B**) or 5 minutes (**C** and **D**) of incubation at 37 °C, [$^3$H]long chain acyl-CoA was extracted and measured as described in Experimental Procedure. Activities of LCASs were expressed as the levels of arachidonoyl-CoA (**A** and **C**) or oleoyl-CoA (**B** and **D**) formed in the above reaction mixtures. Values represent the average of three experiments.

somes with a *Vmax* of 12 nmol/min/mg protein and a *Km* of 70 μM (Fig. 3C), and in cytoplasm, with a *Vmax* of 5.9 nmol/min/mg protein and a *Km* of 38 μM (Fig. 3E); triacsin (33 μM) slightly and uncompetitively inhibited ACS activity only in the cytoplasm (Fig. 3E).

On the other hand, triacsin (2.1–33.3 μM) dose-dependently inhibited the formation of [$^3$H]oleoyl-CoA, in both microsomes and cytoplasm (Fig. 3B); the concentrations of triacsin required for 50% inhibition ($IC_{50}$ values) on OCS were 13.5 μM in microsome, and 3.8 μM in cytoplasm. OCS catalyzed the formation of [$^3$H]oleoyl-CoA in microsomes with a *Vmax* of 0.68 nmol/min/mg protein and a *Km* of 37.6 μM (Fig. 3D), and in cytoplasm, with a *Vmax* of 0.62 nmol/min/mg protein and a *Km* of 60.2 μM (Fig. 3F); triacsin (8.3 μM) greatly and competitively inhibited the activities of OCS, with apparent *Ki* values of 14.2 μM in microsomes (Fig. 3D) and 3.8 μM in cytoplasm (Fig. 3F). The data for the enzymatic kinetics of ACS and OCS are summarized in Table II.

*(4) Effects of NGF on Activities of ACS and OCS.* In PC12 cells treated with 50 ng NGF/ml for 4 days, [$^3$H]arachidonoyl-CoA levels were 49 nmol/mg protein (after 10 minutes of incubation), or about 4.5 fold those of control cells (Fig. 4A); the addition of 16.7 μM of triacsin into the reaction mixture did not affect the stimulation of ACS by NGF in whole cells or subcellular fractions (Fig. 4C). As for OCS activities, the levels of [$^3$H]oleoyl-CoA (Fig. 4B) were 26.1 and 36.2 nmol/mg protein in control cells and NGF-treated cells, respec-

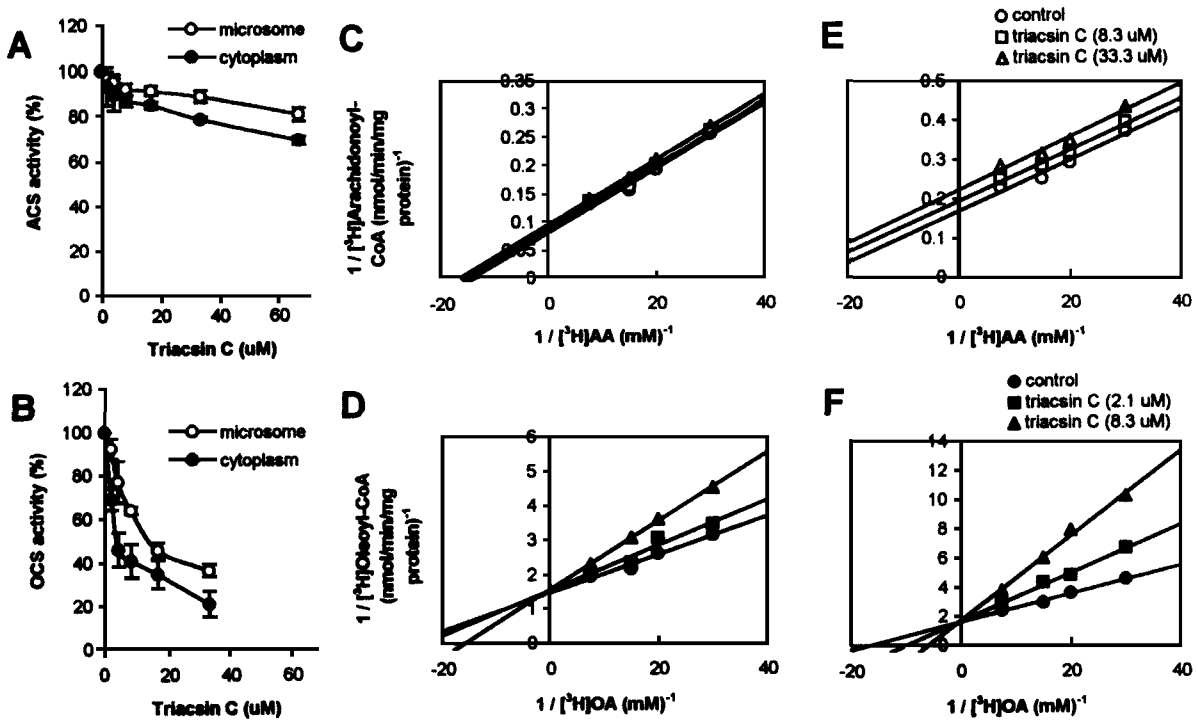


**Fig. 3.** Dose-dependent inhibition of LCAS activities. Microsomes and cytoplasm prepared from PC12 cells were used as enzyme sources as described in Fig. 1. To observe dose-related inhibition on LCAS activities, 2.1 - 66.7 μM of triacsin C were added into the standard reaction mixtures containing 20 nmol of either [$^3$H]AA (**A**) or [$^3$H]OA (**B**) (5 nCi/nmol) and 200 μg of protein from microsome or cytoplasm. To explore the inhibition types, triacsin C (2.1, 8.3 or 33.3 μM) was added into the standard reaction mixture containing varying amounts of [$^3$H]AA (**C** and **E**) or [$^3$H]OA (**D** and **F**) (5 nCi/nmol) and 200 μg of protein from microsomes (**C** and **D**) or cytoplasm (**E** and **F**). After 10 minutes (**A** and **B**) or 5 minutes (**C, E, D** and **F**) of incubation at 37 °C, [$^3$H]long chain acyl-CoA was extracted and measured as described in Experimental Procedure. Activities of LCASs were expressed as the levels of arachidonoyl-CoA (**A, C** and **E**) or oleoyl-CoA (**B, D** and **F**) formed in the above reaction mixtures. Values represent the average of three experiments.

tively; 8.3 μM triacsin significantly inhibited the NGF-stimulated activities of OCS by 28% in whole cells, 27% in microsomes and 65% in cytoplasm (Fig. 4D).

*Utilization of Different Fatty Acids to Form Glycerolipids.* Our previous studies showed that NGF increased *de novo* glycerolipid synthesis from exogenous [$^3$H]OA, and that triacsin C partially inhibited this effect (7). In the present study we further explored the effects of NGF and/or triacsin C on the utilization of particular fatty acids to form glycerolipids, when PC12 cells were exposed to various radioactive and/or non-radioactive precursors.

**Table II.** Summary of Enzymatic Kinetics of ACS and OCS

| Parameter | | ACS | | OCS | |
|---|---|---|---|---|---|
| | | microsomes | cytoplasm | microsomes | cytoplasm |
| *Vmax* | (nmol/min/mg protein) | 12 | 6 | 0.7 | 0.6 |
| *Km* | (μM) | 70 | 38 | 37 | 60 |
| *Ki* | (μM) | N/D | N/D | 14 | 4 |
| $I_{50}$ | (μM, 10 min) | >>70 | >70 | 13.5 | 3.8 |

Microsomes and cytoplasm prepared from PC12 cells were used for measuring *Vmax* and *Km* values of ACS and OCS, and for measuring *Ki* and $I_{50}$ values of triacsin C on these enzymes, as described in Fig. 1, 2, and 3. The *Vmax*, *Km*, and *Ki* values were measured depending on a double-reciprocal plot of enzymatic kinetics, and the $I_{50}$ values depending on an exponential curve. Values represent the average of three experiments. N/D: not detected.

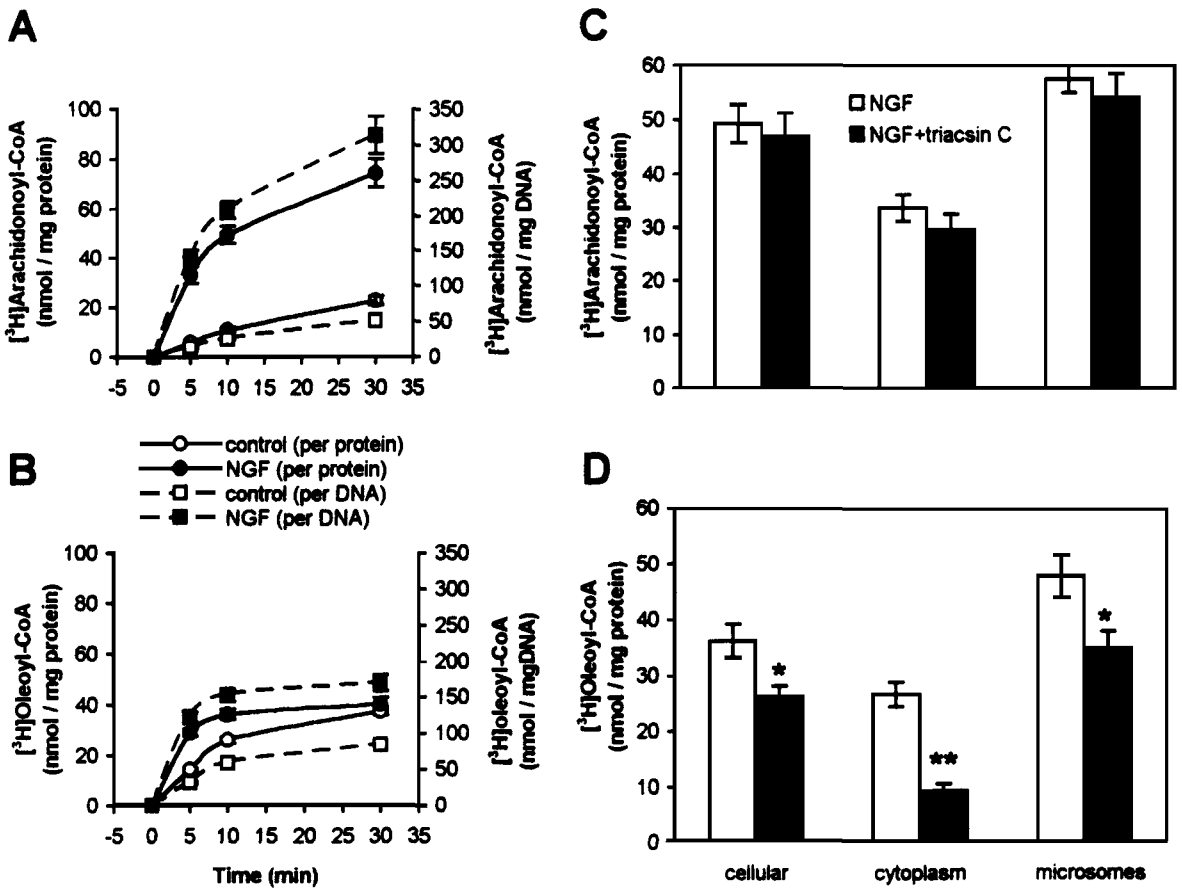


**Fig. 4.** Increases in activities of LCASs by NGF treatment. PC12 cells treated with NGF (50 ng/ml medium B) for 4 days were collected and sonicated in PBS-sucrose solution. Microsomes and cytoplasm were prepared by successive centrifugation as described in Experimental Procedure. The standard reaction mixture containing 20 nmol of either [$^3$H]AA (**A**) or [$^3$H]OA (**B**) (5 nCi/nmol) and 200 μg of cellular protein from control cells or NGF-treated cells was incubated at 37°C for 5, 10 and 30 minutes. To observe the effects of triacsin C on NGF-stimulated activities of LCASs, 16.7 μM (**C**) or 8.3 μM (**D**) of triacsin C was added into the standard reaction mixture containing 20 nmol of either [$^3$H]AA (**C**) or [$^3$H]OA (**D**) (5 nCi/nmol) and 200 μg of cellular protein or proteins from microsomes or cytoplasm. The reaction mixtures were then incubated at 37 °C for 10 minutes. [$^3$H]long chain acyl-CoA was extracted and measured as described in Experimental Procedure. Activities of LCASs were expressed as the levels of arachidonoyl-CoA (**A** and **C**) or oleoyl-CoA (**B** and **D**) formed in the above reaction mixtures. Values represent the means ± SD of [$^3$H]arachidonoyl-CoA or [$^3$H]oleoyl-CoA levels (nmol/mg protein; n = 3). Compared with NGF group, *: $P < 0.05$; **: $P < 0.01$.

NGF (50 ng/ml), an activator of LCASs, greatly stimulated the utilization of endogenous fatty acids (Fig. 5): the levels of [$^3$H]glycerol-labeled MAG, DAG and TAG were 1–3 times, and those of [$^3$H]PC, PE, PS and PI 1–2 times, higher in NGF-treated cells than in control cells. NGF treatment also significantly promoted the incorporation of exogenous [$^3$H]OA (10 or 100 μM in medium) or [$^3$H]AA (10 μM in medium) into glycerolipids (Figs. 7 and 8).

Triacsin C (4.8 μM), an inhibitor of OCS, significantly blocked the incorporation of exogenous OA into [$^3$H]OA-labeled neutral lipids by 30–50%, and into [$^3$H]OA-labeled phospholipids by 20–30%, as compared with those in control cells (Figs. 7 and 8), inducing by about 60% the accumulation of free [$^3$H]OA in the cells (Fig. 7).

On the other hand, triacsin did not change the utilization of endogenous fatty acids to form [$^3$H]glycerol-labeled lipids (Fig. 5); did not decrease the recycling of endogenous [$^3$H]OA from [$^3$H]DAG and TAG to [$^3$H]PC and PE (Fig. 6); and did not block the incorporation of exogenous [$^3$H]AA into phospholipids (Fig. 8).

## DISCUSSION

These data show that LCASs (ACS and OCS) are heterogeneous with respect to intracellular location

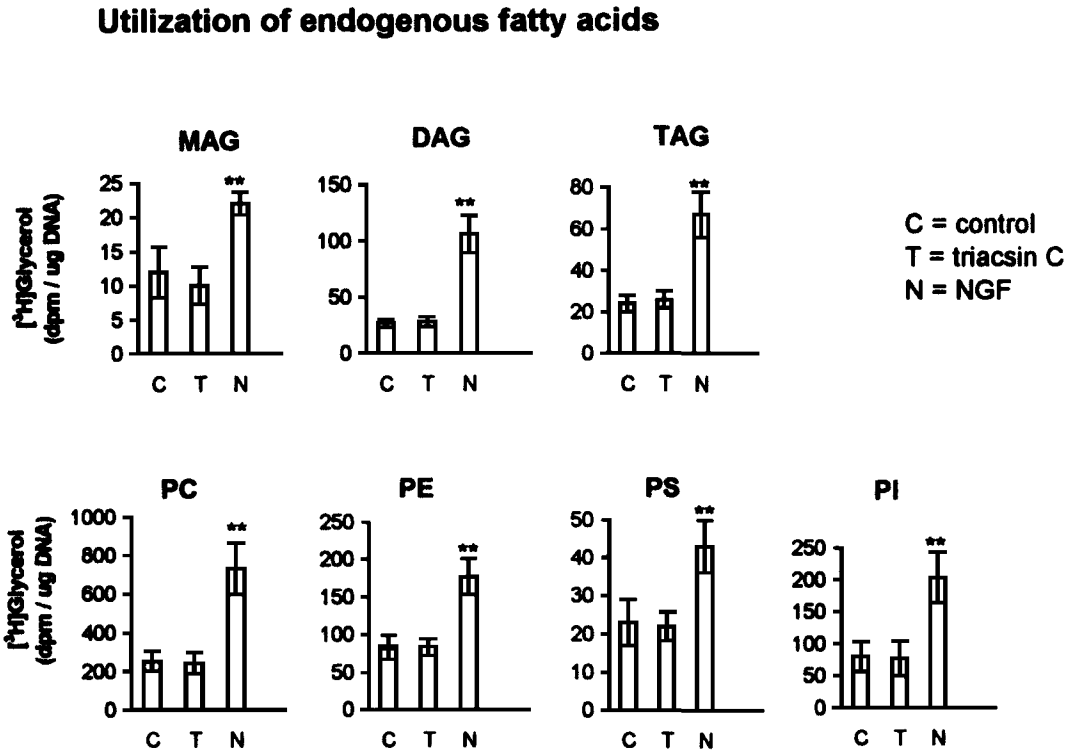


**Fig. 5.** Effects of NGF and triacsin C on the utilization of endogenous fatty acids to form [$^3$H]glycerol-labeled lipids. PC12 cells were pre-treated with NGF for 1 day, and then exposed to NGF, triacsin C and [$^3$H]glycerol for an additional 20 hours. The cells were extracted and [$^3$H]glycerol-labeled glycerolipids were purified by TLC and measured by liquid scintillation spectrometry as described in Experimental Procedure. Values represent the means ± SD of [$^3$H]glycerolipid levels (dpm/μg DNA; n = 4). Compared with control group, **: $P < 0.01$.

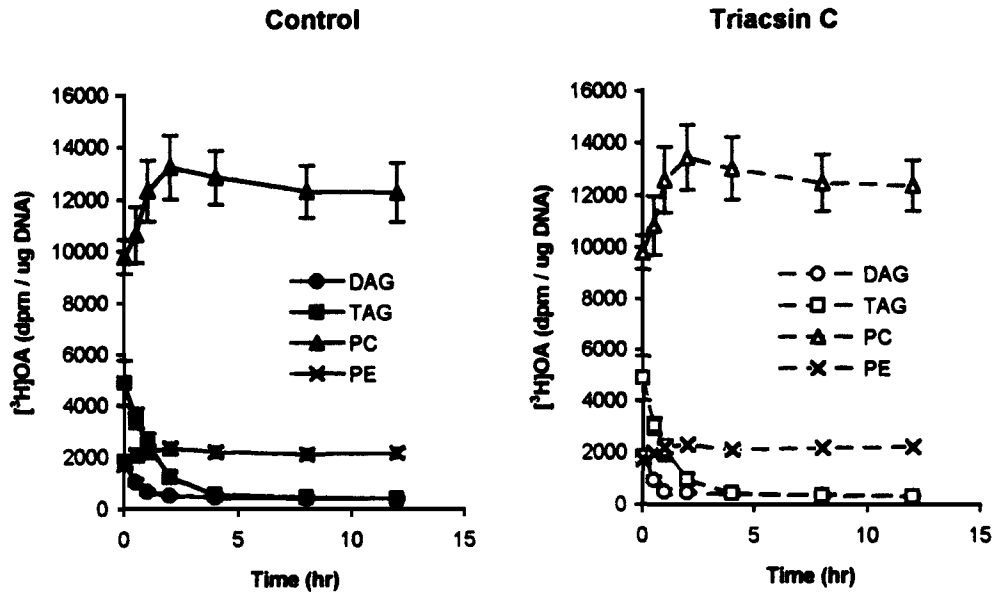


**Fig. 6.** Recycling of endogenous [$^3$H]OA from pre-labeled neutral lipids to phospholipids. PC12 cells pre-labeled with [$^3$H]OA for 1 hour (until 96% of intracellular radioactivity is found in glycerolipids) were washed twice with warm medium B to remove residual radiolabel, and then chased in fresh medium B in the presence or absence of 4.8 μM triacsin C. After 0, 0.5, 1, 2, 4, 8 or 12 hours of chase, the cells were harvested and assayed for the levels of [$^3$H]OA-labeled neutral lipids and phospholipids as described in Experimental Procedure. Values represent the means ± SD of the levels of [$^3$H]OA-labeled lipids (dpm/μg DNA; n = 4).

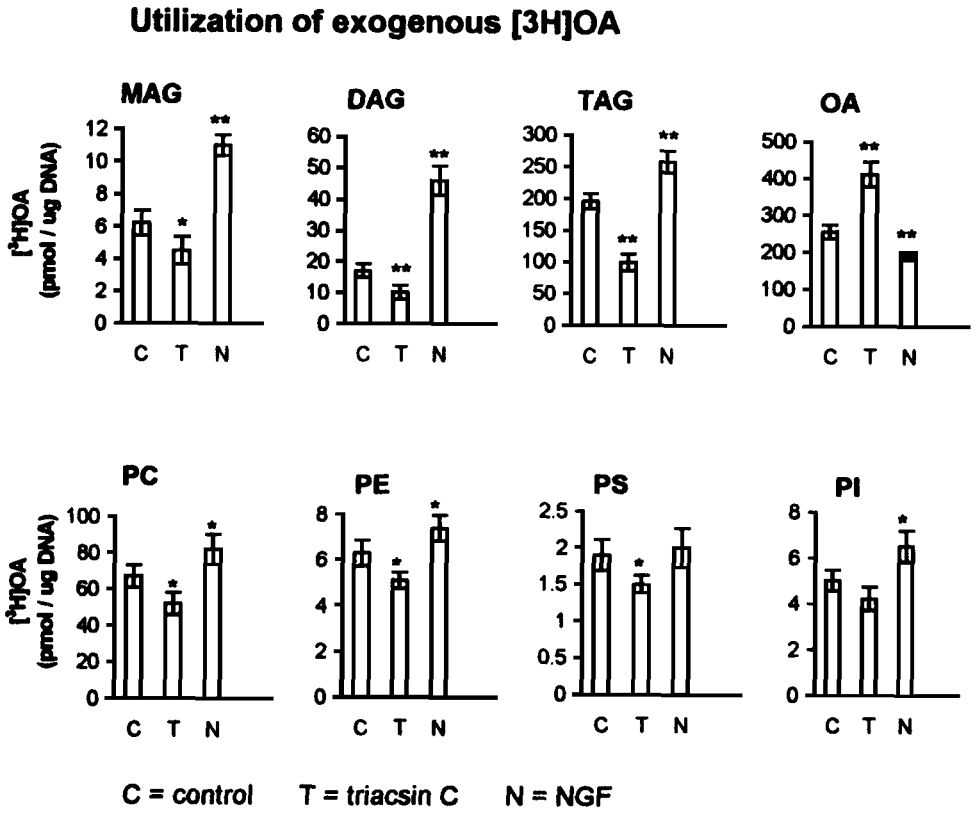


**Fig. 7.** Effects of NGF and triacsin C on the utilization of exogenous OA to form [$^3$H]OA-labeled lipids. PC12 cells were pre-treated with NGF for 1 day, and then exposed to NGF, triacsin C and [$^3$H]OA for an additional hour. The cells were extracted and [$^3$H]glycerol-labeled glycerolipids were purified by TLC and measured by liquid scintillation spectrometry as described in Experimental Procedure. Values represent the means ± SD of [$^3$H]glycerolipid levels (dpm/μg DNA; n = 4). Compared with control group, *: $P < 0.05$, **: $P < 0.01$.

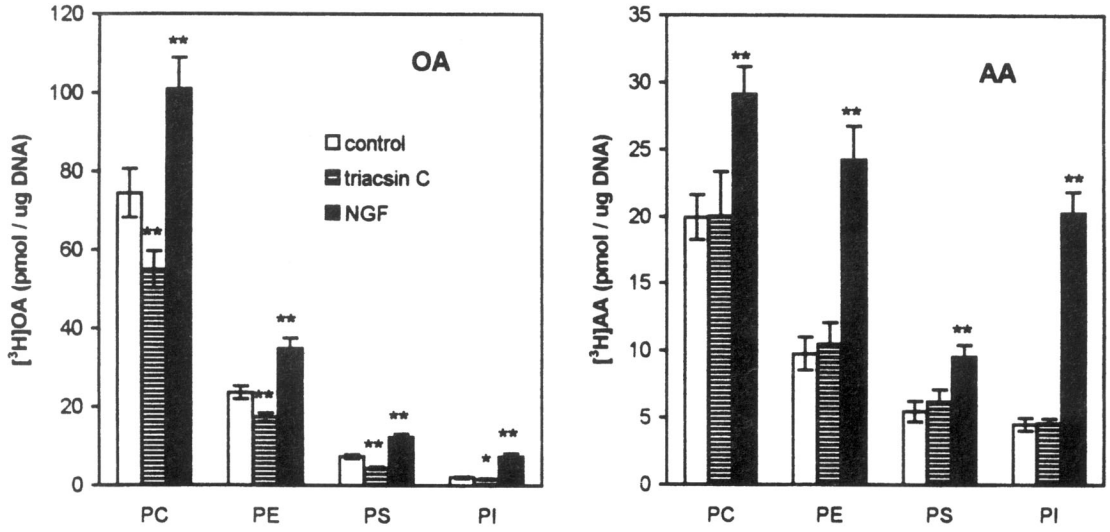


**Fig. 8.** Comparison between incorporation of exogenous OA and AA into glycerophospholipids. PC12 cells pre-treated with NGF for 1 day were exposed to NGF, triacsin C and [$^3$H]OA or [$^3$H]AA for an additional day. [$^3$H]OA- or [$^3$H]AA-labeled glycerophospholipids were purified and measured as described in Experimental Procedure. Values represent the means ± SD of [$^3$H]phospholipids (pmol/μg DNA; n = 4–6). Compared with control group, *: $P < 0.05$, **: $P < 0.01$.

(Fig. 1), substrate specificity (Fig. 2), kinetic characteristics (Figs. 2 and 3, Table II) and sensitivity to the inhibitor triacsin (Figs. 3 and 4, Table II). They also demonstrate that NGF greatly stimulates the activities of both ACS and OCS in all subcellular fractions (Fig. 4), while triacsin C inhibits the activity of OCS, particularly in the cytoplasm (Figs. 3 and 4). NGF greatly promotes the utilization of various fatty acids to form glycerolipids (Figs. 5, 7 and 8); triacsin C blocks the incorporation of exogenous [$^3$H]OA into glycerolipids (Figs. 7 and 8), but does not affect the utilization of endogenous fatty acids (Fig. 5), endogenous [$^3$H]OA (Fig. 6) or exogenous [$^3$H] AA (Fig. 8) for this purpose.

Studies on LCASs, the most important fatty acid activating enzymes in mammalian cells, have been conducted for about two decades. Tanaka found that the LCAS enzymes isolated from endoplasmic reti-culum and the outer mitochondrial membrane of rat liver were identical in molecular weight and their effects on saturated (C10–18) or unsaturated (C16–20) fatty acids (11). The mitochondrial enzyme synthesizes fatty acyl-CoA thioesters for oxidation, whereas the enzyme in the endoplasmic reticulum provides substrates for glycerolipid synthesis. Wilson discovered that platelets contained two LCASs: one showed activity with a range of different fatty acids, while the other enzyme was specific for the prostaglandin precursor AA (14). Sleeman reported an association of LCAS-1 with GLUT4 (glucose transporter)-containing vesicles. The insulin-sensitive membrane compartment that sequesters GLUT4 in fat cells contains LCAS-1, and its product, fatty acyl-CoA, is required for budding and fusion in the membrane trafficking processes (20). Hurtado de Catalfo, investigating LCAS activity in rat testicular microsomes, found broadly-specific activating enzymes in testis which are subject to hormonal regulation (21). Kono recently identified two distinct LCASs, with different kinetic properties, in the guinea pig Harderian gland. One was localized in microsomes and the other in mitochondria. The substrate specificity and catalytic rate of the mitochondrial but not the microsomal enzyme were similar to those of liver enzyme (22). The heterogeneity of LCASs seems to be a plausible explanation for the heterogeneity of the phospholipids in brain, but unfortunately, few data have been available characterizing LCASs in nervous system. We examined four fatty acids that are abundant in the phospholipids of PC12 cells, i.e., PA 16:0, SA 18:0, OA 18:1 and AA 20:4, as substrates of LCASs, and isolated two kinds of LCASs: the substrate non-specific enzyme (OCS) which activates PA, OA, AA or SA in different degrees, and the substrate specific enzyme (ACS) which activates only AA. Both were found mainly in microsomes, but significant amounts of OCS also existed in mitochondria, suggesting that OCS synthesizes fatty acyl-CoA esters for both glycerolipid synthesis in microsomes and β-oxidation in mitochondria. Furthermore we found differences in the *Vmax* and *Km* values of ACS and OCS in various subcellular fractions, and also observed that these LCASs have different sensitivities to triacsin C, a competitive inhibitor of LCAS with respect to its fatty acid substrate (7,8, 23–27).

The syntheses of glycerolipids begin with the activation of long chain fatty acids to long chain acyl-CoA esters. The main acceptor for long chain acyl-CoA in most tissues is thought to be the *sn*-glycerol-3-phos-

**Table III.** Summary of Effects of NGF and Triacsin C on the Activity of LCASs and the Utilization of Fatty Acids to Form Glycerolipids

| | *Activity of LCASs* | | | |
|---|---|---|---|---|
| | *cytoplasm* | | *microsomes* | |
| Treatment | OCS | ACS | OCS | ACS |
| NGF (50 ng/ml) | + | + | + | + |
| triacsin C (8.2 μM) | −/− | +/− | − | +/− |

| | *Utilization of fatty acids* | | | |
|---|---|---|---|---|
| | *exogenous* | | *endogenous* | |
| Treatment | OA | AA | OA | fatty acids |
| NGF (50 ng/ml) | + | + | + | + |
| triacsin C (4.8 μM) | − | +/− | +/− | +/− |

The subcellular fractions of PC12 cells were used for exploring the effects of NGF or triacsin C on the activities of ACS and OCS in microsomes and cytoplasm, and on the utilization of various fatty acids to form glycerolipids. The methods were as described in Experimental Procedure, and in the above Figures and Tables. +: increase; −: decrease; −/−: intensive decrease; +/−: no change.

phate that is formed by the phosphorylation of glycerol. When PC12 cells were labeled with radioactive glycerol, in the absence of exogenous fatty acids, the newly-formed [$^3$H]glycerol-labeled lipids contained both radioactive glycerol and endogenous, nonradioactive fatty acids. We observed that triacsin C unexpectedly failed to block the synthesis of [$^3$H]glycerol-labeled lipids. This suggested that other, distinct LCASs might be involved in the utilization of endogenous fatty acids. When we compared the effects of triacsin C on the utilization of an exogenous, mono-unsaturated fatty acid (OA) and a polyunsaturated fatty acid (AA), we again observed that triacsin C blocked the incorporation of exogenous OA into phospholipids, but failed to affect the utilization of exogenous AA, which confirmed that ACS can selectively activate polyunsaturated fatty acids. When we used pulse-chase experiments, we found that triacsin C did not inhibit the recycling of endogenous OA from newly-formed TAG into phospholipids, suggesting again that different enzymes activate endogenous and exogenous OA.

Taken together (Table III), our data suggest that the effects of NGF and triacsin C on LCAS activities are highly consistent with their effects on the utilization of individual fatty acids to form glycerolipids; and that a variety of LCASs, with individual intracellular distributions, substrate specificities, kinetic characteristics and sensitivities to inhibitors, control the utilization of individual fatty acids.

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## REFERENCES

1. Igal, R. A., Wang, P., and Coleman, R. A. 1997. Triacsin C blocks *de novo* synthesis of glycerolipids and cholesterol esters but not recycling of fatty acid into phospholipids: evidence for functionally separate pools of acyl-CoA. Biochem. J. 324:529–534.
2. Grand, R. J. 1989. Acylation of viral and eukaryotic proteins. Biochem. J. 258:625–638.
3. Glick, B. S., and Rothman, J. E. 1987. Possible role for fatty acyl-coenzyme A in intracellular protein transport. Nature 326: 309–312.
4. Prentki, M., and Corkey, B. E. 1996. Are the beta-cell signaling molecules malonyl-CoA and cystolic long-chain acyl-CoA implicated in multiple tissue defects of obesity and NIDDM? Diabetes 45:273–283.
5. Li, Q. L., Yamamoto, N., Inoue, A., and Morisawa, S. 1990. Fatty acyl-CoAs are potent inhibitors of the nuclear thyroid hormone receptor *in vitro*. J. Biochem. (Tokyo) 107:699–702.
6. Tomoda, H., Igarashi, K., Cyong, J-C., and Omura, S. 1991. Evidence for an essential role of long chain acyl-CoA synthetase in animal cell proliferation: inhibition of long chain acyl-CoA synthetase by triacsins caused inhibition of Raji cell proliferation. J. Biol. Chem. 266:4214–4219.
7. Li. J., and Wurtman, R. J. 1998. Nerve growth factor stimulates diacylglycerol *de novo* synthesis and phosphatidylinositol hydrolysis in pheochromocytoma cells. Brain Res. 803: 44–53.
8. Tomoda, H., Igarashi, K., and Omura, S. 1987. Inhibition of acyl-CoA synthetase by triacsins. Biochim. Biophys. Acta 921: 595–598.
9. Greene, L. A., and Tischler, A. S. 1976. Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which responds to nerve growth factor. Proc. Natl. Acad. Sci. USA 73:2424–2428.
10. Tacconi, M., and Wurtman, R. J. 1985. Phosphatidylcholine produced in rat synaptosomes by *N*-methylation is enriched in polyunsaturated fatty acids. Proc. Natl. Acad. Sci. USA 82: 4828–4831.
11. Tanaka, T., Hosaka, K., Hoshimaru,. M., and Numa, S. 1979. Purification and properties of long-chain acyl-coenzyme-A synthetase from rat liver. Eur. J. Biochem. 98:165–172.
12. Wilson, D. B., Prescott, S. M., and Majerus, P. W. 1982. Discovery of an arachidonoyl coenzyme A synthetase in human platelets. J.Biol.Chem 257:3510–3515.
13. Mishina, M., Kamiryo, T., Tashiro, S., and Numa, S. 1978. Separation and characterization of two long-chain acyl-CoA synthetases from *Candida lipolytica*. Eur. J. Biochem. 82: 347–354.
14. Van Veldhoven, P. P., and Bell, R. M. 1988. Effect of harvesting methods, growth conditions, and growth phase on diacylglycerol levels in cultured human adherent cells. Biochim. Biophys. Acta 959:185–196.
15. Saltiel, A. R., Fox, J. A., Sherline, P., and Cuatrecasas, P. 1986. Insulin-stimulated hydrolysis of a novel glycolipid generates modulators of cAMP phosphodiesterase. Science 233:967–972.
16. Touchstone, J.C., Chen, J.C., and Beaver, K.M. 1980. Improved separation of phospholipids on thin layer chromatography. Lipids 15:61–62.
17. Svanborg, A., and Svennerholm, L. 1961. Plasma total lipids, cholesterol, triglycerides, phospholipids and free fatty acids in a healthy Scandinavian population. Acta Med. Scand. 168: 43–49.
18. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76–85.
19. Labarca, C., and Paigen, K. 1980. A simple, rapid and sensitive DNA assay procedure. Anal. Biochem. 102:344–352.
20. Sleeman, M. W., Donegan, N. P., Heller-Harrison, R., Lane, W.S., and Czech, M.P. 1998. Association of acyl-CoA synthetase-1 with GLUT4-containing vesicles. J. Biol. Chem. 273: 3132–3135.
21. Hurtado de Catalfo, G. E., de Gomez Dumm, I. N., and Mandon, E. C. 1993. Long-chain acyl-CoA synthetase of rat testis microsomes: substrate specificity and hormonal regulation. Biochem. Mol. Biol. Int. 31:643–649.
22. Kono, M., Hori, C., Hashimoto, T., Hori, S., and Seyama, Y. 1996. Two distinct long-chain acyl-CoA synthetases in guinea pig Harderian gland. Eur. J. Biochem. 238:104–111.
23. Hundley, T. R., Marshall, L. A., Hubbard, W. C., and MacGlashan Jr. D. W. 1998. Characteristics of arachidonic acid generation in human basophils: relationship between the effects of inhibitors

of secretory phospholipase $A_2$ activity and leukotriene $C_4$ release. J. Pharmacol. Exp.Ther. 284:847–857.
24. Noel, R. J., Antinozzi, P. A., McGarry, J. D., and Newgard, C. B. 1997. Engineering of glycerol-stimulated insulin secretion in islet beta cells: differential metabolic fates of glucose and glycerol provide insight into mechanisms of stimulus-secretion coupling. J. Biol. Chem. 272:18621–18627.
25. Arai, M., Imai, H., Metori, A., and Nakagawa, Y. 1997 Preferential esterification of endogenously formed 5-hydroxyeicosatetraenoic acid to phospholipids in activated polymorphonuclear leukocytes. Eur. J. Biochem. 244:513–519.
26. Igal, R. A., and Coleman, R. A. 1996. Acylglycerol recycling from triacylglycerol to phospholipid, not lipase activity, is defective in neutral lipid storage disease fibroblasts. J. Biol. Chem. 271: 16644–16651.
27. Hartman, E. J., Omura, S., and Laposata, M. 1989. Triacsin C: a differential inhibitor of arachidonoyl-CoA synthetase and nonspecific long chain acyl-CoA synthetase. Prostaglandins 37:655–671.