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Differential Metabolism of Diradyl Glycerol Molecular Subclasses and Molecular Species by Rabbit Brain Diglyceride Kinase*

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Elevations in the mass of ether-linked diglycerides (*i.e.* 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol (AAG) and 1-*O*-alkyl-2-acyl-*sn*-glycerol (Alkyl AG)) during cellular activation are prolonged in comparison to their 1,2-diacyl-*sn*-glycerol (DAG) counterparts. Since the metabolic removal of DAG is determined, in large part, by the rate of its phosphorylation by diglyceride kinase, we quantified differences in the activity of diglyceride kinase utilizing individual subclasses of diradyl glycerols as substrate. Rabbit brain microsomal diglyceride kinase activity was over 30-fold greater utilizing DAG as substrate (25.8 nmol·mg^{-1}·min^{-1}) in comparison to AAG (0.8 nmol·mg^{-1}·min^{-1}). No alterations in the affinity of microsomal diglyceride kinase for ATP were present ($K_m \approx 0.5$ mM) utilizing each diradyl glycerol subclass. Similar subclass specificities for diglyceride kinase (*i.e.* DAG > Alkyl AG >> AAG) were present in brain and liver cytosol as well as in liver microsomes utilizing multiple assay conditions. In sharp contrast, *Escherichia coli* diglyceride kinase phosphorylated DAG, Alkyl AG, or AAG diradyl glycerol molecular subclasses at identical rates. Furthermore, although DAG was rapidly hydrolyzed by diglyceride lipase, catabolism of AAG or Alkyl AG by plasmalogenase, alkyl ether hydrolase, or diglyceride/monoglyceride lipase was undetectable. Collectively, these results demonstrate the importance of the differential catabolism of each diradyl glycerol molecular subclass as a primary determinant of their biologic half-lives. Since individual subclasses of diglycerides have distinct physical properties and physiologic functions, these results underscore the importance of lipid subclass specific metabolism in tailoring individual cellular responses during activation.

Diradyl glycerols are key branch point intermediates in lipid synthetic and catabolic processes which have gained widespread acceptance as important second messengers of signal transduction (1-3). Although the synergistic activation of protein kinase C by 1,2-diacyl-*sn*-glycerol (DAG)[1] and calcium is well documented, only recently has evidence been accrued which suggests that naturally occurring ether-linked diglycerides (*i.e.* 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol (AAG) and 1-*O*-alkyl-2-acyl-*sn*-glycerol (Alkyl AG)) may also be specific mediators of signal transduction (4-8). For example, the kinetics of ether-linked diglyceride accumulation during neutrophil activation as well as during myocardial ischemia are markedly different from the kinetics of accumulation of DAG molecular species (6, 7). Furthermore, ether-linked diglycerides activate protein kinase C with separate and distinct calcium requirements in comparison to activation of protein kinase C by DAG (8). Accordingly, we have suggested that specific diradyl glycerol molecular subclasses may act as mediators of signal transduction which target protein phosphorylation in specific subcellular loci with a different temporal course and calcium requirement than protein kinase C-mediated protein phosphorylation elicited by DAG (8).

The duration of protein phosphorylation elicited by diradyl glycerols during cell activation is dependent upon the longevity of elevations in each diradyl glycerol molecular species. Although several studies have demonstrated markedly disparate rates of accumulation of DAG, Alkyl AG, and AAG, the biochemical mechanisms underlying their differential biologic half-lives have not been delineated. Since the majority of DAG released during cellular stimulation is rapidly converted to phosphatidic acid (9, 10), we hypothesized that the differential biologic half-lives of each diradyl glycerol molecular subclass were intimately related to the kinetics of their phosphorylation by diglyceride kinase. We now report that rabbit brain and liver diglyceride kinase selectively phosphorylate DAG with only diminutive amounts of activity utilizing AAG as substrate.

EXPERIMENTAL PROCEDURES

Preparation of Brain and Liver Microsomes and Cytosol—Microsomes and cytosol were prepared from cerebrums and livers of New Zealand rabbits (1-2 kg body weight). Tissues were homogenized in 100 mM Tris-HCl buffer (pH = 7.4) consisting of 0.25 M sucrose, 2 mM EGTA, 2 mM EDTA, and 1 mM dithiothreitol (homogenization buffer) utilizing a Teflon-glass Potter-Elvehjem homogenizer. Microsomes and cytosol from either cerebrum or liver were prepared by the sequential centrifugation of homogenate at 10,000 × *g* for 20 min, centrifugation of the 10,000 × *g* supernatant at 100,000 × *g* for 60 min, collection of the 100,000 × *g* supernatant (cytosol) and resuspension of the 100,000 × *g* pellet (microsomes) in homogenization buffer (5 mg/ml).

Diglyceride Kinase Assay—Diglyceride kinase was assayed by minor modifications of previously described assay systems (11, 12). Briefly, selected concentrations of diradyl glycerols and microsomal (20-50 μg) or cytosolic protein (20-50 μg) were co-solubilized into mixed micelles of octyl glucoside (final octyl glucoside concentration = 51 mM) by co-incubation at 24 °C for 2 min. Diglyceride kinase activity was assessed by incubating these mixed micelles in assay buffer (50 mM Tris-HCl, pH = 7.4, 50 mM NaCl, 12.5 mM $MgCl_2$, 0.5 mM EDTA, and 1 mM dithiothreitol) with selected concentrations of [γ-^{32}P]ATP at 30 °C for 4 min (final reaction volume = 0.1 ml). Reactions were terminated by the addition of 0.4 ml of chloroform/methanol (1:1, v/v), and extraction of phosphatidic acid was facili-

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[1] The abbreviations used are: DAG, 1,2-diacyl-*sn*-glycerol; AAG, 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol; Alkyl AG, 1-*O*-alkyl-2-acyl-*sn*-glycerol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; HPLC, high performance liquid chromatography.

tated by inclusion of 0.1 ml of 200 mM $CaCl_2$ (13). Radiolabeled phosphatidic acid was quantitated by liquid scintillation spectrometry after purification by thin layer chromatography utilizing Silica Gel G plates and a mobile phase consisting of chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1, v/v/v/v/v). This method resolved phosphatidic acid (R_f = 0.86) and lysophosphatidic acid (R_f = 0.46). Diglyceride kinase reaction rates were linear with respect to time and protein. In diglyceride kinase assays employing mixtures of DAG and AAG as substrate, the phosphorylation of each individual diradyl glycerol was assessed by treating extracted reaction products for 10 min with HCl fumes and subsequently determining the acid-catalyzed production of ^{32}P-labeled lysophosphatidic acid (originating from AAG phosphorylation) by TLC. *Escherichia coli* diglyceride kinase activity was assayed as described above except that 3 mol% cardiolipin was included in the assays, incubations were 1 min in duration, and 5 μg of *E. coli* membranes containing diglyceride kinase was utilized. The assay system of Kanoh *et al.* (14) was utilized to assess cytosolic diglyceride kinase activity in the absence of detergent by separately sonicating phosphatidylcholine and diradyl glycerols prior to mixing with brain cytosol and reaction buffer. To assess brain microsomal diglyceride kinase activity in the absence of detergent, phosphatidylserine and selected diradyl glycerols were co-sonicated in 10 mM Tris-HCl (pH 7.4) prior to mixing with brain microsomes and reaction buffer (10).

Diglyceride/Monoglyceride Lipase Assay—Diglyceride/monoglyceride lipase activity was assessed utilizing the assay system described by Majerus and Prescott (15). Radiolabeled DAG (0.5 mM 1-hexadecanoyl-2-[5,6,8,9,11,12,14,15-^{3}H]eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol, Alkyl AG (0.5 mM 1-*O*-hexadecyl-2-[5,6,8,9,11,12,14,15-^{3}H]eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol, or AAG (0.5 mM 1-*O*-hexadec-1′-enyl-2-[5,6,8,9,11,12,14,15-^{3}H]eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol) was co-sonicated with brain microsomes in 0.1 ml of assay buffer consisting of 42.5 mM HEPES (pH = 7.0), 85 mM NaCl, 4.25 mM $CaCl_2$, 15 mM glutathione (reduced), and 0.001% Triton X-100. The diradyl glycerol/microsome co-sonicate was immediately incubated for 30 min at 37 °C, and reactions were terminated by the addition of 0.4 ml of chloroform/methanol (1:1, v/v) and 0.1 ml of 200 mM $CaCl_2$. Reaction products were purified by thin layer chromatography utilizing Silica Gel G plates in an initial mobile phase consisting of petroleum ether/ethyl ether/acetic acid (60:40:1, v/v). After full development in this mobile phase, TLC plates were dried and then subjected to partial development utilizing a second mobile phase consisting of petroleum ether/ethyl ether/acetic acid (25:75:1, v/v). This method resolved monoglycerides (R_f = 0.19), DAG (R_f = 0.35), AAG (R_f = 0.43), Alkyl AG (R_f = 0.43), and fatty acid (R_f = 0.59). The purified reaction products of diglyceride/monoglyceride lipase activity (radiolabeled monoglycerides and fatty acids) were subsequently quantitated by liquid scintillation spectrometry.

Choline and Ethanolamine Phosphotransferase Assay—Brain microsomal choline phosphotransferase activity was assayed as previously described (16). In brief, 100–200 μg of microsomal protein was incubated with 100 μM [*methyl*-^{14}C]CDP-choline (specific activity, 2 μCi/μmol) in a total volume of 0.1 ml of assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$ (pH 7.4)) for 30 min at 37 °C. In these experiments, specific molecular species of DAG, AAG, or Alkyl AG were suspended in 20 μl of assay buffer containing 4 μg of Tween 20 by sonication for 3 min prior to the addition of microsomal protein and [*methyl*-^{14}C]CDP-choline. Reactions were terminated by the addition of butanol (150 μl). Brain microsomal ethanolamine phosphotransferase activity was assayed similarly except that 10 mM $MnCl_2$ was included in the assay buffer, and 100 μM [*ethanolamine*-1,2-^{14}C]CDP-ethanolamine was utilized. Reaction products from choline or ethanolamine phosphotransferase assays were separated by TLC utilizing Silica Gel G plates and a mobile phase consisting of chloroform/methanol/ammonium hydroxide (65:35:5, v/v/v). Radioactivity in choline glycerophospholipids (R_f = 0.25) or ethanolamine glycerophospholipids (R_f = 0.34) was quantitated by liquid scintillation spectrometry.

Preparation of Synthetic Diradyl Glycerols—Specific diradyl glycerol molecular subclasses were prepared by phospholipase C (*Bacillus cereus*) treatment of their respective choline glycerophospholipid precursors. Synthetic choline glycerophospholipids utilized for phospholipase C treatment were prepared as previously described (17) utilizing either 1-*O*-alk-1′-enyl-2-lyso-*sn*-glycero-3-phosphocholine or 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine and either oleoyl chloride, arachidonoyl chloride, or [5,6,8,9,11,12,14,15-^{3}H]arachidonic anhydride (generated from [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid utilizing dicyclohexylcarbodiimide) as precursors. Each diradyl glycerol molecular species was purified by HPLC utilizing a silica stationary phase and a mobile phase comprised of hexane/isopropyl alcohol/water (100:1:0.01, v/v) at a flow rate of 2 ml/min (16). Utilizing this HPLC system, DAG (R_t = 9 min), AAG (R_t = 6 min), and Alkyl AG (R_t = 6 min) were resolved from other neutral lipids. Diradyl glycerols were subsequently quantitated by capillary gas chromatography after methanolic HCl derivatization utilizing arachidic acid (20:0 fatty acid) as internal standard. HPLC and capillary gas chromatographic analyses demonstrated that each synthetic diradyl glycerol molecular species was greater than 99% pure.

Materials—Specific molecular species of phosphatidylcholine, phosphatidic acid, and phosphatidylserine, as well as cardiolipin, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine, and bovine heart lecithin were purchased from Avanti Polar Lipids. Fatty acyl chlorides were purchased from NuCheck Prep. 1-Hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol was obtained from Serdary Chemicals. [γ-^{32}P]ATP, 1-hexadecanoyl-2-[5,6,8,9,11,12,14,15-^{3}H]eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine,1-O-hexadecyl-2-[5,6,8,9,11,12,14,-15-^{3}H]eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycero-3-phosphocholine, [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid, and [*methyl*-^{14}C]CDP-choline were purchased from Du Pont New England Nuclear. [*ethanolamine*-1,2-^{14}C]CDP-ethanolamine was purchased from ICN Radiochemicals. *Bacillus cereus* phospholipase C and ATP were obtained from Boehringer Mannheim. *E. coli* diglyceride kinase was purchased from Lipidex Inc. Silica Gel G thin layer chromatography plates were purchased from Analabs. All other reagents were purchased from Sigma.

RESULTS

Differential Utilization of Diradyl Glycerol Molecular Subclasses and Individual Molecular Species by Rabbit Brain Microsomal Diglyceride Kinase—The diradyl glycerol molecular subclass specificity of rabbit brain microsomal diglyceride kinase was assessed with synthetically prepared and HPLC-purified diradyl glycerol subclasses utilizing a mixed micelle assay system (11, 12). Rabbit brain diglyceride kinase selectively utilized DAG in comparison to Alkyl AG (Fig. 1). Remarkably, AAG molecular species were ineffective substrates for diglyceride kinase (Fig. 1). Since phosphatidic acid, cardiolipin, and phosphatidylserine each augment diglyceride kinase activity utilizing DAG substrate (12), the effects of these lipid activators in augmenting phosphorylation of individual diradyl glycerol molecular subclasses was assessed. As expected, phosphatidic acid and phosphatidylserine activated diglyceride kinase phosphorylation of DAG and Alkyl AG molecular subclasses (Fig. 2). However, no significant activation of diglyceride kinase by these anionic phospholipids was present utilizing AAG as substrate.

Modification of the *sn*-2 aliphatic constituents of individual diradyl glycerol molecular subclasses did not alter the observed rank order of subclass specificity of diglyceride kinase (*i.e.* phosphorylation of DAG > Alkyl AG >> AAG was present when the *sn*-2 constituent was either arachidonic or oleic acid) (Fig. 1). As anticipated from previous studies utilizing diglyceride kinase from Swiss 3T3 cells (12), rabbit brain diglyceride kinase preferentially utilized DAG molecular species containing arachidonic acid at the *sn*-2 carbon (*i.e.* 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol) in comparison to DAG molecular species containing oleic acid (*i.e.* 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol) (Fig. 1). Similar selectivities for Alkyl AG or AAG containing arachidonic acid at the *sn*-2 aliphatic constituent were present (Fig. 1). Taken together, these results demonstrate that constituents at both the *sn*-1 and *sn*-2 positions of diradyl glycerols are important determinants of diglyceride kinase reaction velocity.

To further characterize the differential utilization of diradyl glycerol molecular subclasses and species by diglyceride kinase, the affinity of rabbit brain diglyceride kinase activity for ATP utilizing each diradyl glycerol molecular subclass was

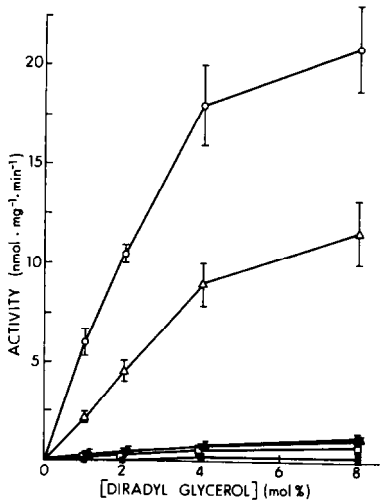

FIG. 1. **Brain diglyceride kinase activity utilizing selected diradyl glycerol molecular subclasses and species.** Rabbit brain microsomes (20–50 μg) were incubated with [γ-^{32}P]ATP (1 mM) and selected concentrations of DAG molecular species (1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (●) or 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (○)), Alkyl AG molecular species (1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (▲) or 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (△)), or AAG molecular species (1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (■) or 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (□)) in 100 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 51 mM octyl glucoside at 30 °C for 4 min. Incorporation of [γ-^{32}P]ATP into phosphatidic acid was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction of reaction products utilizing a $CaCl_2$ aqueous phase and TLC purification of phosphatidic acid as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least three independent determinations obtained from separate microsomal preparations.

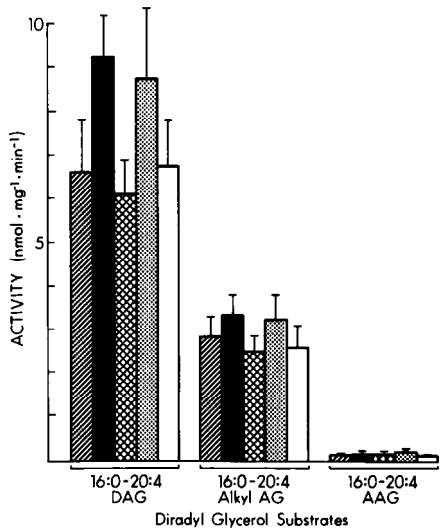

FIG. 2. **Phospholipid modulation of brain diglyceride kinase activity utilizing exogenous diradyl glycerol molecular subclasses.** Rabbit brain microsomes (20–50 μg) were incubated with [γ-^{32}P]ATP (1 mM) with either 1 mol% 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 DAG), 1 mol% 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 Alkyl AG) or 1 mol% 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 AAG) as shown. Incubations contained either no added phospholipid (▨), 4 mol% phosphatidylserine (■), 4 mol% cardiolipin (▩), 4 mol% phosphatidic acid (⊡), or 4 mol% phosphatidylcholine (□). Reactions were performed in 100 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 51 mM octyl glucoside at 30 °C for 4 min. Incorporation of [γ-^{32}P]ATP into phosphatidic acid was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction of reaction products utilizing a $CaCl_2$ aqueous phase and TLC purification of phosphatidic acid as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least four independent determinations obtained from separate microsomal preparations.

determined. The K_m for ATP utilization by diglyceride kinase activity was indistinguishable utilizing DAG, Alkyl AG, and AAG molecular subclasses with arachidonic acid at the *sn*-2 carbon ($K_m \approx 0.5$ mM) (Fig. 3 and Table I). The calculated kinetic constants of diglyceride kinase for ATP utilizing DAG and Alkyl AG containing oleic acid at the *sn*-2 carbon were similar (Figure 3 and Table I). Kinetic constants for 1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (*i.e.* AAG) phosphorylation could not be accurately determined due to the ineffective utilization of AAG by diglyceride kinase.

Since DAG molecular species are both activators of, and substrates for, diglyceride kinase (12), additional experiments were performed. Co-incubation of mixtures of DAG (2 mol%) and AAG (1–8 mol%) demonstrated that AAG did not inhibit DAG phosphorylation (Fig. 4). Analysis of the phosphatidic acid produced from co-incubation of DAG and AAG demonstrated the predominance of diacyl phosphatidic acid (>95%). In contrast, co-incubation of DAG (2 mol%) and Alkyl AG (1–8 mol%) resulted in stepwise increases in diglyceride kinase activity as the mol% of Alkyl AG was increased (Fig. 4). Thus, DAG does not activate AAG phosphorylation by diglyceride kinase, AAG does not activate DAG phosphorylation by diglyceride kinase, and Alkyl AG and DAG are both utilized by diglyceride kinase in an additive manner.

Utilization of Diradyl Glycerol Molecular Subclasses as Substrates for Diglyceride Kinase from Brain Cytosol, Liver Microsomes, Liver Cytosol, and E. coli Membranes—Diglyceride kinase from rabbit brain cytosol, liver microsomes, liver cytosol, and *E. coli* membranes was utilized to determine if the observed rank order of diradyl glycerol subclass selectivity is unique to brain microsomal diglyceride kinase or is present in other tissues and subcellular fractions as well. In direct comparisons, brain microsomal, brain cytosolic, liver microsomal, and liver cytosolic diglyceride kinase each selectively phosphorylated DAG substrate but had virtually no activity utilizing AAG substrate (Fig. 1 and Table II). Furthermore, each of these diglyceride kinases preferentially utilized DAG and Alkyl AG containing arachidonic acid at the *sn*-2 carbon (Table II). In sharp contrast, *E. coli* diglyceride kinase indiscriminantly phosphorylated each diradyl glycerol molecular subclass and species tested (*i.e.* it does not distinguish between molecular subclasses containing ester, alkyl ether, or vinyl ether bonds at the *sn*-1 carbon or between molecular species containing arachidonic acid or oleic acid at the *sn*-2 carbon) (Fig. 5). Thus, although specific diradyl glycerol molecular subclasses and species are differentially phosphorylated by mammalian diglyceride kinase, *E. coli* diglyceride kinase is more promiscuous.

Substrate Selectivity of Brain Microsomal and Cytosolic Diglyceride Kinases in the Absence of Detergent—Since detergents affect the substrate selectivity of some enzymes, additional experiments were performed to examine rabbit brain diglyceride kinase substrate selectivity in systems which do not employ detergent (10, 14). Sonicated DAG molecular species co-incubated with phosphatidylcholine bilayers were rapidly phosphorylated by rabbit brain cytosolic diglyceride kinase in comparison to Alkyl AG and AAG (Fig. 6). Since microsomal diglyceride kinase activity could not be detected by this assay system (*i.e.* co-incubating diradyl glycerol sonicates and phosphatidylcholine sonicates), the substrate selectivity of microsomal diglyceride kinase was assessed using

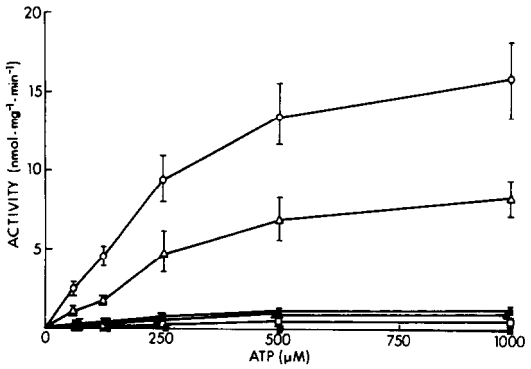

FIG. 3. **ATP dependence of brain diglyceride kinase activity utilizing diradyl glycerol molecular subclasses.** Rabbit brain microsomes (20–50 μg) were incubated with selected concentrations of [γ-^{32}P]ATP (2-4 μCi/μmol) and either 8 mol% DAG molecular species (1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (●) or 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (○)), Alkyl AG molecular species (1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (▲), or 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (△)) or AAG molecular species (1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (■) or 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (□)). Incubations were performed at 30 °C for 4 min in 100 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 51 mM octyl glucoside. Incorporation of [γ-^{32}P]ATP into phosphatidic acid was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction of reaction products utilizing a saturated $CaCl_2$ aqueous phase and TLC purification of phosphatidic acid as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least three independent determinations obtained from separate microsomal preparations.

TABLE I

Kinetic constants for rabbit brain diglyceride kinase

Kinetic constants for diglyceride kinase activity utilizing the data depicted in Fig. 3 were calculated by linear regression analyses following Eadie-Hofstee transformation of the data.

Diradyl glycerol substrate (molecular subclass)	K_m	V_{max}
	mM	*nmol/mg·min*
1-Hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (DAG)	0.52	25.8
1-*O*-Hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (Alkyl AG)	0.49	11.7
1-*O*-Hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (AAG)	0.45	0.8
1-Hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (DAG)	0.52	2.1
1-*O*-Hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (Alkyl AG)	0.74	2.2
1-*O*-Hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (AAG)	—[a]	—[a]

[a] — indicates that kinetic constants for 1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol could not be determined since this AAG molecular species is not readily phosphorylated by diglyceride kinase.

another previously described assay system (10). Incubation of diradyl glycerol/phosphatidylserine co-sonicates with brain microsomal diglyceride kinase demonstrated rank order substrate specificities that were qualitatively similar to that observed in the presence of octylglucoside (*i.e.* DAG > Alkyl AG >> AAG) (Fig. 7). Thus both in the absence and presence of detergent both the cytosolic and microsomal brain diglyceride kinases preferentially utilized DAG molecular species.

Utilization of Diradyl Glycerol Molecular Subclasses by Brain Diglyceride/Monoglyceride Lipase, Ethanolamine Phosphotransferase, and Choline Phosphotransferase—Since diglyceride lipase, ethanolamine phosphotransferase, and choline phosphotransferase also mediate the metabolic removal of

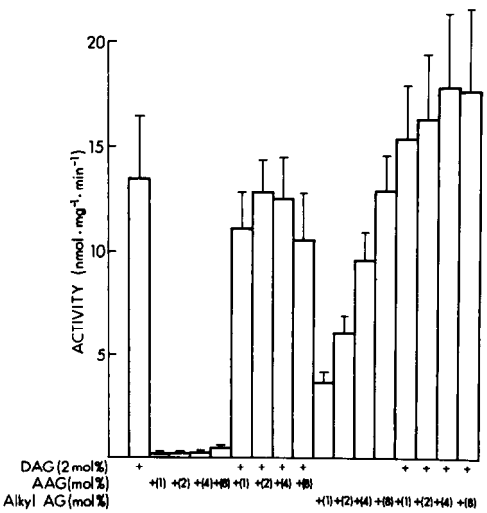

FIG. 4. **Brain diglyceride kinase activity utilizing selected concentrations of ether-linked diradyl glycerols in the presence of subsaturating concentrations of diacyl glycerol.** Rabbit brain microsomes (20–50 μg) were incubated with [γ-^{32}P]ATP (1 mM) and the indicated concentrations of DAG (1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol), Alkyl AG (1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol), or AAG (1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol). Reactions were performed in 100 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 51 mM octyl glucoside for 4 min at 30 °C. Incorporation of [γ-^{32}P]ATP into phosphatidic acid was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction of reaction products utilizing a $CaCl_2$ aqueous phase and TLC purification of phosphatidic acid as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least four independent determinations obtained from separate microsomal preparations.

diradyl glycerols, their activities utilizing AAG and DAG were determined. Brain diglyceride/monoglyceride lipase was measured utilizing synthetically prepared and HPLC-purified diradyl glycerol molecular subclasses containing radiolabeled arachidonic acid at the *sn*-2 carbon. Incubation of brain microsomes with DAG resulted in the accumulation of both arachidonic acid (0.2 $nmol \cdot min^{-1} \cdot mg^{-1}$) as well as radiolabeled monoglyceride (0.5 $nmol \cdot min^{-1} \cdot mg^{-1}$). No plasmalogenase activity, alkyl ether hydrolase activity, or *sn*-2 diglyceride lipase activity were detected in brain cytosol or microsomes utilizing AAG or Alkyl AG substrates in this assay system (*i.e.* no radiolabeled 2-monoacylglycerol or fatty acid was produced).

Ethanolamine phosphotransferase possessed a substrate preference for utilization of AAG in comparison to Alkyl AG or DAG (Fig. 8). Furthermore, ethanolamine phosphotransferase activity demonstrated a preference for molecular species with arachidonic acid at the *sn*-2 position in each subclass. In contrast, brain choline phosphotransferase did not demonstrate either subclass or species selectivity for diradyl glycerol substrates (Fig. 9).

DISCUSSION

The present study demonstrates a remarkable disparity in the rates of phosphorylation of DAG, Alkyl AG, and AAG substrates by both rabbit brain and liver diglyceride kinase from both cytosol and microsomes in the presence or absence of detergent. Accumulation of a metabolite during cellular perturbation reflects the combined amounts of total enzymic activity mediating its production in comparison to that mediating its metabolic removal. Since the predominant pathway for DAG metabolism is its phosphorylation by diglyceride

TABLE II

Utilization of diradyl glycerol molecular subclasses as substrates for diglyceride kinase from brain cytosol, liver microsomes, and liver cytosol

Rabbit brain cytosol (20–50 μg), liver microsomes (100–250 μg), or liver cytosol (300–500 μg) were incubated with [γ-^{32}P]ATP (1 mM) and 8 mol% of selected diradyl glycerols. Incubations were performed at 30 °C for 4 min (10 min for liver cytosol) in 100 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, and 51 mM octyl glucoside. Incorporation of [γ-^{32}P]ATP into phosphatidic acid was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction of reaction products utilizing a $CaCl_2$ aqueous phase and TLC purification of phosphatidic acid as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least four independent determinations obtained from separate preparations.

Diradyl glycerol substrate (molecular subclass)	Diglyceride kinase activity		
	Brain cytosol	Liver microsomes	Liver cytosol
	pmol·mg^{-1}·min^{-1}		
1-Hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (DAG)	1941 ± 158	154 ± 12	6.6 ± 1.0
1-*O*-Hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (Alkyl AG)	1042 ± 28	57 ± 16	2.8 ± 0.4
1-*O*-Hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (AAG)	143 ± 13	6 ± 3	0.2 ± 0.1
1-Hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (DAG)	465 ± 22	58 ± 10	2.2 ± 0.1
1-*O*-Hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (Alkyl AG)	329 ± 24	36 ± 7	1.6 ± 0.3
1-*O*-Hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (AAG)	61 ± 7	11 ± 6	0.4 ± 0.1

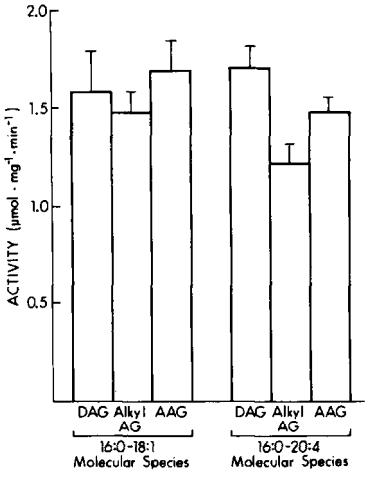

FIG. 5. ***E. coli* diglyceride kinase activity utilizing selected diradyl glycerol molecular subclasses and species.** *E. coli* membranes enriched with diglyceride kinase (5 μg) were incubated with [γ-^{32}P]ATP (1 mM) and 8 mol% DAG molecular species (1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 DAG) or 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 DAG)), Alkyl AG molecular species (1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 Alkyl AG) or 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 Alkyl AG)), or AAG molecular species (1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 AAG) or 1-O-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 AAG)). Incubations were performed at 30 °C for 1 min in 100 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 3 mol% cardiolipin, and 51 mM octyl glucoside. Incorporation of [γ-^{32}P] ATP into phosphatidic acid was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction of reaction products utilizing a $CaCl_2$ aqueous phase and TLC purification of phosphatidic acid as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least four independent determinations.

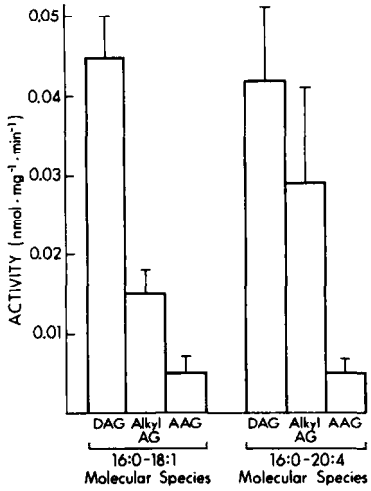

FIG. 6. **Substrate selectivity of rabbit brain cytosolic diglyceride kinase in the absence of detergent.** Rabbit brain cytosol (20–50 μg) was incubated with [γ-^{32}P]ATP (1 mM) and 1 mM concentration of DAG molecular species (1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 DAG) or 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 DAG)), Alkyl AG molecular species (1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 Alkyl AG) or 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 Alkyl AG)), or AAG molecular species (1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 AAG) or 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 AAG)). Individual sonicates of dioleoyl phosphatidylcholine (2 mM) and diradyl glycerol molecular species were incubated at 30 °C for 10 min in 100 mM Tris-HCl (pH 7.4) containing 20 mM NaF, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Phosphatidic acid synthesis was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction and TLC purification of phosphatidic acid as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least four independent determinations obtained from separate cytosolic preparations.

kinase (9, 10), the dramatic subclass specificity of mammalian diglyceride kinase likely contributes to the markedly disparate biologic half-lives of each diradyl glycerol molecular subclass produced during cellular activation. Differences in the metabolic flux of DAG *versus* AAG are likely to be further amplified by the virtual absence of demonstrable AAG catabolism by plasmalogenase or *sn*-2 diglyceride lipase. Although the substrate selectivities of diglyceride kinase, choline phosphotransferase, ethanolamine phosphotransferase, plasmalogenase, and diglyceride/monoglyceride lipase utilizing these broken cell preparations are compatible with the observed accumulation of the mass of each diradyl glycerol subclass in stimulated cells (6, 7), the subclass selectivities of other enzymes during cellular activation (*e.g.* phospholipase C, phospholipase D, and phosphatidate phosphohydrolase) are likely also of considerable importance.

The differential substrate selectivity of diglyceride kinase and ethanolamine phosphotransferase for AAG and DAG suggests that each diradyl glycerol subclass has disparate

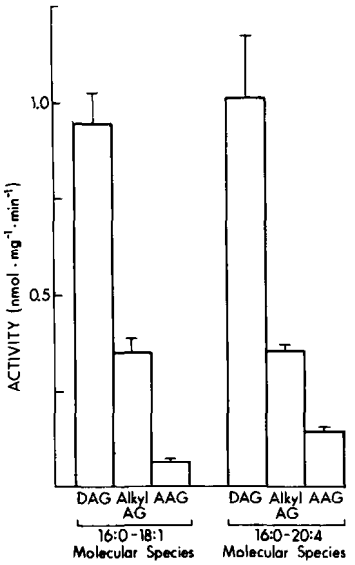

FIG. 7. **Substrate selectivity of rabbit brain microsomal diglyceride kinase in the absence of detergent.** Rabbit brain microsomes (50–100 μg) were incubated with [γ-^{32}P]ATP (1 mM) and 1 mM concentration of DAG molecular species (1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 DAG) or 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 DAG)), Alkyl AG molecular species (1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 Alkyl AG) or 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 Alkyl AG)), or AAG molecular species (1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (16:0–18:1 AAG) or 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (16:0–20:4 AAG)). Co-sonicates of each diradyl glycerol subclass and phosphatidylserine (0.5 mM) were incubated at 30 °C for 10 min in 100 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EGTA, and 1 mM dithiothreitol. Incorporation of [γ-^{32}P]ATP into phosphatidic acid was quantified by liquid scintillation spectrometry following Bligh and Dyer extraction and TLC purification as described under "Experimental Procedures." Each value represents the mean ± S.E. of at least four independent determinations obtained from separate microsomal preparations.

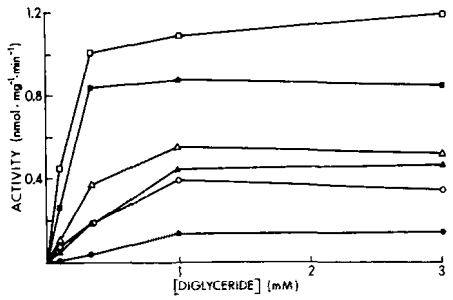

FIG. 8. **Rabbit brain microsomal ethanolamine phosphotransferase diradyl glycerol subclass selectivity.** Rabbit brain microsomes (100–200 μg) were incubated with [^{14}C]CDP ethanolamine (100 μM) and selected concentrations of DAG molecular species (1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (●) or 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (○)), Alkyl AG molecular species (1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (▲) or 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (△)), or AAG molecular species (1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (■) or 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (□)) in 50 mM Tris-HCl containing 10 mM $MgCl_2$ and 10 mM $MnCl_2$ (pH 7.4) for 30 min at 37 °C. Incorporation of [^{14}C]CDP-ethanolamine into ethanolamine glycerophospholipids was quantitated following extraction of reaction products with butanol, TLC purification, and liquid scintillation spectrometry as described under "Experimental Procedures." Each value represents the mean of at least four independent determinations from separate microsomal preparations. S.E. for each value was less than 10% of the mean.

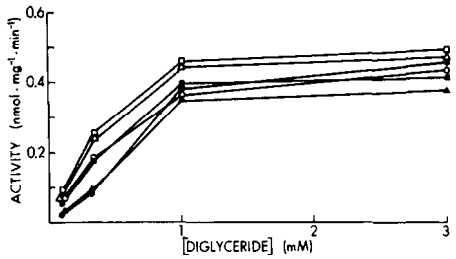

FIG. 9. **Utilization of diradyl glycerol molecular subclasses by rabbit brain microsomal choline phosphotransferase.** Rabbit brain microsomes (100–200 μg) were incubated with [^{14}C]CDP-choline (100 μM) and selected concentrations of DAG molecular species (1-hexadecanoyl-2-octadec-9′-enoyl-*sn*-glycerol (●) or 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (○)), Alkyl AG molecular species (1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-glycerol (▲) or 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (△)), or AAG molecular species (1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-glycerol (■) or 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-glycerol (□)) in 50 mM Tris-HCl containing 10 mM $MgCl_2$ (pH 7.4) for 30 min at 37 °C. Incorporation of [^{14}C]CDP-choline into choline glycerophospholipids was quantitated following extraction of the reaction products with butanol, TLC purification, and liquid scintillation spectrometry as described under "Experimental Procedures." Each value represents the mean of at least four independent determinations from separate microsomal preparations. S.E. for each value was less than 10% of the mean.

metabolic fates during cellular stimulation. Since the maximal rate of DAG phosphorylation is over 10-fold greater than the combined rates of AAG phosphorylation and AAG incorporation into ethanolamine and choline glycerophospholipids, it is likely that DAG will be metabolically removed more efficiently than AAG during cellular stimulation. Furthermore, since the velocity of DAG phosphorylation by diglyceride kinase is over 10-fold greater than the velocity of its incorporation into ethanolamine or choline glycerophospholipids, the majority of DAG produced during cellular stimulation is anticipated to be rapidly converted into phosphatidic acid as demonstrated in *in vivo* systems (*e.g.* platelets (18)). In contrast, AAG incorporation into ethanolamine glycerophospholipids is greater than its phosphorylation by diglyceride kinase suggesting that the primary metabolic fate of AAG is its metabolic salvage resulting in the reincorporation of the vinyl ether linkage into plasmenylethanolamine. Collectively, the results contained herein demonstrate that the type of covalent linkage at the *sn*-1 carbon in diradyl glycerol molecular species likely contributes to the biological half-life of each subclass and that the nature of the covalent linkage at the *sn*-1 position is a primary structural determinant of its ultimate metabolic fate.

Since both brain and liver diglyceride kinases preferentially utilize DAG molecular subclasses, the observed subclass specificity is not tissue-specific. However, since *E. coli* diglyceride kinase displayed no diradyl glycerol subclass selectivity, it is apparent that this phenomenon is not ubiquitous but is dependent on as yet unidentified characteristics of diglyceride kinase activity in each cell type. The effective utilization of each diradyl glycerol molecular subclass as substrates for *E. coli* diglyceride kinase substantiates the efficacy of accurately quantitating DAG, Alkyl AG, and AAG mass utilizing the *E. coli* diglyceride kinase method previously described by Preiss *et al.* (19). The robust phosphorylation of synthetic AAG molecular species by *E. coli* diglyceride kinase documents the structural integrity of the vinyl ether containing substrates and products (*i.e.* AAG and plasmalogen phosphatidic acid) in our assay system and underscores the disparity of subclass substrate specificity in rabbit and *E. coli* diglyceride kinases.

The present findings demonstrate that the differential sub-

class specificity of mammalian diglyceride kinase likely contributes to the disparate biological half-lives of ether-linked diglycerides and DAG during cellular perturbation. Accordingly, it is anticipated that the attenuated metabolism of ether-linked diglycerides will result in extended protein phosphorylation in critical subcellular loci specifically enriched in ether-linked diglycerides.

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