The Discordant Rates of *sn*-1 Aliphatic Chain and Polar Head Group Incorporation into Plasmalogen Molecular Species Demonstrate the Fundamental Importance of Polar Head Group Remodeling in Plasmalogen Metabolism in Rabbit Myocardium†

David A. Ford* and Richard W. Gross

Division of Bioorganic Chemistry and Molecular Pharmacology and the Departments of Internal Medicine, Chemistry, and Molecular Biology & Pharmacology, Washington University School of Medicine, St. Louis, Missouri 63110

Received August 5, 1993; Revised Manuscript Received October 29, 1993®

ABSTRACT: Although recent studies have demonstrated the existence of the requisite enzymic machinery necessary for the shuttling of vinyl ether linkages through polar head group remodeling, the relative rates of plasmalogen *de novo* synthesis and polar head group remodeling are unknown. Pulse–chase radiolabeling of perfused rabbit hearts with [1-^{3}H]hexadecanol demonstrated the rapid and progressive incorporation of radiolabel into plasmenylethanolamine (e.g., after 0.5 h of radiolabeling, 10% of [1-^{3}H]hexadecanol incorporated into ethanolamine glycerophospholipid was in plasmenylethanolamine, and after 1.5 h, 21% was in plasmenylethanolamine) with no detectable radiolabeling of plasmenylcholine until 3 h after the pulse. Furthermore, perfusion of hearts with [1′,2′-*alkyl*-$^3H_2$]1-*O*-alkyl-GPC resulted in the rapid incorporation of radiolabel into plasmanylcholine, but not plasmenylcholine, even after extended perfusion intervals. In contrast, both radiolabeled choline and ethanolamine were rapidly incorporated into plasmalogens through polar head group remodeling at rates that were over 300-fold greater than that of plasmalogen *de novo* synthesis (e.g., an incorporation rate of 31 nmol/g_{dry}·h for ethanolamine but only 93 pmol/g_{dry}·h for hexadecanol into plasmenylethanolamine was manifest). Similarly, *sn*-2 remodeling of plasmalogen molecular species with arachidonic or oleic acid also occurred at rates that were over 100-fold greater than that of *de novo* plasmalogen biosynthesis. Collectively, these results underscore the fundamental importance of rapid polar head group remodeling of plasmalogen molecular species in the synthesis and maintenance of plasmenylcholine and plasmenylethanolamine pools in intact contracting myocardium.

Recent studies have demonstrated the predominance of plasmalogen molecular species in specific subcellular membrane compartments and emphasized their role as the major intracellular phospholipid storage depot of arachidonic acid in many cell types (Mueller et al., 1983; Gross, 1984; Ford & Gross, 1989a; Ramanadham et al., 1993a). The unique molecular conformation and dynamics of plasmalogens in conjunction with the identification of the activation of a plasmalogen-selective phospholipase A_2 during signal transduction have focused attention on the biological role of plasmalogens in mammalian tissues (Wolf & Gross, 1985; Pak et al., 1987; Han & Gross, 1990; Gross et al., 1993; Ramanadham et al., 1993b). Despite the importance of plasmalogen molecular species as the major source of arachidonic acid released during signal transduction in many cell types (Mueller et al., 1983; Chilton & Connell, 1988; Ford & Gross, 1989a; Ramanadham et al., 1993a), a paucity of information is available on the relative rates of metabolic turnover of the aliphatic and polar head group constituents in plasmalogen molecular species compared to their diacyl phospholipid counterparts.

The demonstration of enzyme-catalyzed transfer of an aliphatic alcohol into the *sn*-1 position of dihydroxyacetone acyl phosphate resulted in the initial mechanistic insight into the biological strategy exploited for *de novo* synthesis of ether lipids (Hajra, 1970). Subsequently, the importance of a pyridine-dependent mixed-function oxidase in the oxidation of plasmanylethanolamine to plasmenylethanolamine demonstrated a formal route to natural products containing *sn*-1 vinyl ether linkages (Wykle et al., 1972; Wykle & Schremmer, 1979). However, the detailed characterization of the substrate specificity of this oxidase demonstrated that plasmanylcholine is not a substrate for this enzyme (Schmid et al., 1972), and thus, considerable uncertainty regarding the pathways, dynamics, and metabolic regulation of choline and ethanolamine plasmalogen synthesis in mammalian cells remained.

We and others have demonstrated the presence of the enzymic machinery necessary for polar head group remodeling of plasmalogen substrate, the selectivity of both choline and ethanolamine phosphotransferases for diradyl glycerol acceptors containing a vinyl ether linkage at the *sn*-1 position, and the potential importance of polar head group remodeling in the dynamic maintenance of myocardial plasmalogen pools under a variety of physiological and pathophysiological perturbations (Kiyasu & Kennedy, 1960; Ford & Gross, 1988, 1989b; Ford et al., 1992). Despite the demonstration of the requisite enzymic machinery for this remodeling pathway in broken cell preparations, many questions regarding the quantitative significance of polar head group remodeling in the dynamic maintenance of plasmalogen pools in myocardium remained. Perhaps foremost among these was the relative rate of plasmalogen polar head group remodeling compared to the rate of plasmalogen *de novo* synthesis. We now report that the incorporation of hexadecanol into plasmenylethanolamine substantially precedes its incorporation into plasmenylcholine in intact tissue and that the rate of plasmalogen *sn*-2 fatty acid remodeling and polar head group remodeling

† This research was supported by NIH Grant No. 42665.

* Author to whom correspondence should be addressed at the Division of Bioorganic Chemistry and Molecular Pharmacology. Telephone: 314-362-2690. FAX: 314-362-1402.

® Abstract published in *Advance ACS Abstracts,* January 15, 1994.

0006-2960/94/0433-1216$04.50/0

exceeds, by over 300-fold, the rate of plasmalogen *de novo* synthesis. Collectively, these results underscore the fundamental importance of polar head group and *sn*-2 aliphatic chain remodeling in the synthesis and maintenance of plasmalogen pools in functioning myocardium.

MATERIALS AND METHODS

Langendorff Perfusion of Rabbit Myocardium with Radiolabeled Precursors of Ether-Linked Phospholipids. Hearts were removed from New Zealand white rabbits (1 kg body weight) after cervical dislocation and were perfused retrograde through the aorta in a Langendorff mode utilizing a modified Krebs–Henseleit buffer (137 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM NaEDTA, 15 mM $NaHCO_3$, and 11 mM glucose). After a 10-min equilibration period of control perfusion, hearts were perfused with modified Krebs–Henseleit buffer containing 0.4 mM octanoate, 2 mM pyruvate, 100 nM epinephrine, 0.3% BSA, and either 50 μCi of [1-^{3}H]hexadecanol (11.2 Ci/mmol, 40 nM), 25 μCi of [^{3}H]ethanolamine (2 Ci/mmol, 100 nM), 25 μCi of [^{3}H]choline (2 Ci/mmol, 100 nM), 25 μCi of [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC[1] ([^{3}H]lyso platelet activating factor) (60 Ci/mmol, 4 nM), 50 μCi of [9,10-$^3H_2$]palmitic acid (60 Ci/mmol, 8.3 nM), 50 μCi of [9,10-$^3H_2$]oleic acid (10 Ci/mmol, 1.6 nM), or 50 μCi of [5,6,8,9,11,12,14,15-$^3H_8$]-arachidonic acid (60 Ci/mmol, 8.3 nM) in a volume of 100 mL, utilizing a recirculation mode for the indicated intervals.

At the end of the labeling period, hearts were either freeze-clamped or further perfused with fresh buffer in a non-recirculating mode for selected intervals of up to 21 h. During this postlabeling perfusion interval, the remaining endogenous radiolabel was diluted by perfusion with its respective unlabeled counterpart (i.e., chase at concentrations of either 20 μM hexadecanol, 100 μM ethanolamine, 1 mM choline chloride, or 400 nM lyso platelet activating factor). At the end of each experimental interval, hearts were freeze-clamped at the temperature of liquid nitrogen, ventricular tissue was pulverized and weighed, and an aliquot was taken for the determination of wet/dry weight ratios. Next, lipids were extracted from pulverized ventricular tissue employing a modified method of Bligh and Dyer (1959) that included 100 mM $CaCl_2$ in the aqueous phase.

Purification and Analysis of Phospholipid Classes, Subclasses, and Individual Molecular Species. Phospholipid classes were purified from lipid extracts by straight-phase HPLC as previously described (Ford & Gross, 1989a). In some experiments, the incorporation of radiolabel into phospholipid classes was also determined by two-dimensional TLC of lipid extracts, utilizing silica gel G plates and a solvent system comprised of chloroform/methanol/NH_4OH (65/35/5, v/v) for the first dimension and chloroform/acetone/methanol/acetic acid/water (3/4/1/1/0.5, v/v) for the second dimension. After two-dimensional TLC, plates were sprayed with Enhance, and fluorography was performed utilizing Kodak X-AR X-ray film. Quantification of radiolabel incorporation into individual molecular species of choline and ethanolamine glycerophospholipids of rabbit myocardium was determined by reverse-phase HPLC of straight-phase HPLC-purified choline and ethanolamine glycerophospholipids, as previously described (Ford & Gross, 1988). The assignment of individual molecular species to peaks of radioactivity in column eluents was determined by their cochromatography with UV-absorbing peaks from authentic standards obtained from bovine or rabbit heart choline or ethanolamine glycerophospholipids. Molecular species in these standard samples were identified by their known elution profiles and by capillary gas chromatography of their fatty acid methyl esters or aliphatic dimethyl acetal derivatives after acid methanolysis. The identity of plasmalogen molecular species resolved by reverse-phase HPLC was further confirmed by the specific removal of plasmalogen molecular species by brief pretreatment with HCl fumes.

Similarly, the plasmalogen content in lipid extracts from rabbit myocardium radiolabeled with choline or ethanolamine was also analyzed by determining their acid lability. The separation of neutral lipid classes, as well as the determination of their vinyl ether content, was assessed utilizing a two-dimensional TLC method, with the exposure of TLC plates to HCl fumes employed following first-dimension elution but prior to elution in the second dimension. Briefly, lipid extracts were applied to silica gel G plates and developed utilizing a mobile phase of petroleum ether/ethyl ether/acetic acid (70/30/1, v/v). Plates were then dried, placed in a chamber containing HCl fumes for 10 min, and subsequently air-dried for 2–3 min to remove residual HCl fumes. Finally, the plates were developed in a second dimension utilizing the same mobile phase, and the radioactivity in each neutral lipid class as well as the radioactivity in vinyl ether moieties of each neutral lipid class (e.g., radiolabels present in vinyl ether moieties of each neutral lipid class migrated as fatty aldehydes after treatment with HCl fumes) was visualized by fluorography of the TLC plates after treatment with Enhance spray.

Quantitation of Myocardial Hexadecanol. Myocardial lipids were extracted from Langendorff perfused rabbit myocardium after the addition of 30 μg of the internal standard, eicosanol [30 μg of eicosanol overwhelms the trace amounts of endogenous eicosanol present in myocardium (Takahashi & Schmid, 1970)]. Hexadecanol was partially purified by TLC utilizing silica gel G plates and a mobile phase comprising petroleum ether/ethyl ether/acetic acid (70/30/1, v/v). The region corresponding to hexadecanol (R_f = 0.38) was scraped into test tubes and extracted with chloroform/methanol (95/5, v/v). Myocardial fatty alcohol as well as contaminating 1,3-diacyl-*sn*-glycerol, 1-*O*-alkyl-2-acyl-*sn*-glycerol, and 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol were then benzoylated, utilizing benzoyl anhydride with (dimethylamino)pyridine as catalyst, as previously described (Ford & Gross, 1988). Fatty alcohol benzoates were then purified by TLC utilizing silica gel G TLC plates and a mobile phase comprising hexane/diethyl ether/NH_4OH (85/15/1, v/v) (R_f of fatty alcohol benzoates = 0.7). Individual molecular species of fatty alcohol benzoates were resolved utilizing an octadecyl silica HPLC column and a mobile phase comprising acetonitrile/isopropyl alcohol (95/5, v/v) at a flow rate of 2 mL/

[1] Abbreviations: CDP, cytidine diphospho-; GPC, *sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; 16:0-18:1, 1-*O*-hexadec-1′-enyl-2-octadec-9′-enoyl-*sn*-, 1-*O*-hexadecyl-2-octadec-9′-enoyl-*sn*-, and 1-hexadecanoyl-2-octadec-9′-enoyl-*sn*- for plasmalogen, alkyl ether, and diacyl molecular species, respectively; 16:0-20:4, 1-*O*-hexadec-1′-enyl-2-eicosatetra-5′,8′,-11′,14′-enoyl-*sn*-, 1-*O*-hexadecyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*-, and 1-hexadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*- for plasmalogen, alkyl ether, and diacyl molecular species, respectively; 16:0-18:2, 1-*O*-hexadec-1′-enyl-2-octadecadi-9′,12′-enoyl-*sn*-, 1-*O*-hexadecyl-2-octadecadi-9′,12′-enoyl-*sn*-, and 1-hexadecanoyl-2-octadecadi-9′,12′-enoyl-*sn*- for plasmalogen, alkyl ether, and diacyl molecular species, respectively; 18:1-18:2, 1-*O*-octadecadi-1′,9′-enyl-2-octadecadi-9′,12′-enoyl-*sn*- for plasmalogen molecular species; 18:0-18:2, 1-octadecanoyl-2-octadecadi-9′,12′-enoyl-*sn*- for diacyl molecular species; 18:0-20:4, 1-*O*-octadec-1′-enyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*- and 1-octadecanoyl-2-eicosatetra-5′,8′,11′,14′-enoyl-*sn*- for plasmalogen and diacyl molecular species, respectively.

min, with UV detection at 231 nm. The mass of hexadecanol was then determined by comparisons of the UV absorbances of the hexadecanol benzoate with that of the internal standard, derived eicosanol benzoate.

Synthesis of [1-^{3}H]Hexadecanol. [1-^{3}H]Hexadecanol was synthesized by the reduction of hexadecanal with tritiated sodium borohydride ([^{3}H]$NaBH_4$). Hexadecanal was synthesized by oxidation of hexadecanol utilizing oxaloyl chloride and dimethyl sulfoxide, as previously described (Mancuso et al., 1978). After purification by HPLC utilizing isocratic elution with 100% hexane, hexadecanal was reduced with [^{3}H]-$NaBH_4$ (Hajra, 1968), and crude [1-^{3}H]hexadecanol was purified by thin-layer chromatography on silica gel G TLC plates employing a mobile phase of petroleum ether/ethyl ether/acetic acid (70/30/1, v/v). TLC-purified [1-^{3}H]-hexadecanol was further purified by straight-phase HPLC utilizing a silica HPLC column (4.6 × 250 mm, 5-μm particle size) and a mobile phase comprising hexane/isopropyl alcohol (100/1, v/v) at a flow rate of 2 mL/min with UV detection at 205 nm (t_R of hexadecanol = 6 min) (Ford & Gross, 1988).

Materials. Hexadecanol was obtained from Nu Chek Prep, Inc. Bovine heart lecithin, bovine heart ethanolamine glycerophospholipid, and lyso platelet activating factor were obtained from Avanti Polar Lipids, Inc. [^{3}H]Ethanolamine, [^{3}H]choline, [^{3}H]sodium borohydride, and [^{3}H]lyso platelet activating factor were obtained from DuPont NEN. Whatman silica gel G TLC plates and Ultrasphere Si and ODS HPLC columns were purchased from Baxter Scientific and PJ Cobert, respectively. All other chemicals were purchased from Sigma Chemical Company.

RESULTS

Quantitation of Plasmalogen de Novo Synthesis by Pulse–Chase Radiolabeling with [1-^{3}H]Hexadecanol. The obligatory utilization of hexadecanol in *de novo* ether lipid synthetic pathways facilitates the direct quantification of plasmalogen biosynthesis. Since hexadecanol contains no charged functionality, it is anticipated that it will have facile access to all salient intracellular membrane compartments. Oxidation of [1-^{3}H]hexadecanol prevents the incorporation of radiolabel into lipid pools (in contrast to the oxidation of [9,10-$^3H_2$]-hexadecanol to [9,10-$^3H_2$]hexadecanoic acid, which results in the incorporation of radiolabeled probe into diacyl phospholipids) (Lumb & Snyder, 1971; Rizzo et al., 1987). Accordingly, ether lipids in isolated perfused rabbit hearts were labeled with [1-^{3}H]hexadecanol for either 0.5 or 1.5 h. During these experimental perfusion periods, the predominantly radiolabeled neutral lipids were alkyl ether triradyl glycerols and diradyl glycerols, with virtually no radiolabeled nonpolar plasmalogen molecular species present (i.e., <0.5% in each pool). Similarly, during the initial perfusion interval, the predominantly radiolabeled polar lipids were plasmanylcholine and plasmanylethanolamine (Figure 1). No detectable amounts of radiolabel were present in any other polar lipid class (e.g., <0.1% label was found in phosphatidylinositol, phosphatidylserine, or phosphatidic acid pools). Substantial amounts of radiolabel also were present in plasmenylethanolamine molecular species (10 and 21% of total radioactivity in ethanolamine glycerophospholipids were present in plasmenylethanolamine after 0.5- and 1.5-h pulse radiolabeling intervals, respectively), but no plasmenylcholine molecular species were radiolabeled during this interval (Figure 1). Radiolabeled plasmenylcholine was not detected until after 3 h of chase with unlabeled hexadecanol and continued to increase throughout a 21-h chase interval (Figure 1). Thus,

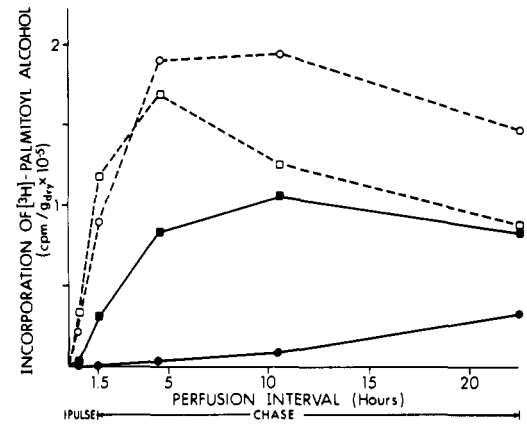

FIGURE 1: Temporal course of [1-^{3}H]hexadecanol incorporation into ether-linked choline and ethanolamine glycerophospholipids. Rabbit hearts were perfused in a Langendorff recirculating mode with modified Krebs–Henseleit buffer containing [1-^{3}H]hexadecanol (40 nM) for either 30 min or 1.5 h. Following the radiolabeling intervals, hearts were subsequently perfused in a non-recirculating mode with modified Krebs–Henseleit buffer containing unlabeled hexadecanol (20 μM) for the indicated intervals. Perfusions were terminated by rapid freeze-clamping of myocardium, and myocardial lipids were extracted (Bligh & Dyer, 1959). Choline (○, ●) and ethanolamine (□, ■) glycerophospholipids in the extracts were purified by straight-phase HPLC, as described in Materials and Methods. The incorporation of radiolabel into plasmalogens (●, ■) was determined by treating the purified phospholipids with acid fumes, TLC purification of the resultant radiolabeled fatty aldehydes, and subsequent liquid scintillation spectrometry. Acid-stable glycerophospholipids (○, □) were alkyl ether molecular species, as determined by reverse-phase HPLC.

incorporation of radiolabel into plasmenylethanolamine lagged the incorporation into plasmanylethanolamine, and the appearance of radiolabeled plasmenylethanolamine molecular species substantially preceded the appearance of plasmenylcholine molecular species.

To gain further insight into the relative rates of synthesis and flux between subclasses and individual molecular species, choline and ethanolamine glycerophospholipids radiolabeled during pulse–chase experiments were resolved by reverse-phase HPLC, and the flux of radiolabel into individual molecular species was quantified by scintillation spectrometry. Reverse-phase HPLC analysis of ethanolamine glycerophospholipids from hearts pulse-radiolabeled for 1.5 h and subsequently chased for 21 h with cold hexadecanol demonstrated radiolabel in 16:0-20:4, 16:0-18:2, and 16:0-18:1 plasmenylethanolamine molecular species (Figure 2, top). Furthermore, analysis of the choline glycerophospholipids from hearts that were subjected to a 21-h chase with cold hexadecanol revealed appreciable amounts of plasmalogen molecular species with 16:0-20:4 > 16:0-18:2 > 16:0-18:1 (Figure 2, bottom). Collectively, these experiments utilizing [1-^{3}H]hexadecanol as a precursor for plasmalogen molecular species clearly demonstrate that alkyl ether lipid synthesis precedes plasmalogen synthesis and that either plasmanylcholine or plasmenylethanolamine represents the likely precursor for plasmenylcholine biosynthesis (*vide infra*).

Pulse–Chase Radiolabeling of Choline Glycerophospholipid Pools with [1′,2′-alkyl-$^3H_2$]-1-O-Alkyl-GPC. To determine whether plasmanylcholine or plasmenylethanolamine serves as the predominant biosynthetic precursor for plasmenylcholine, isolated perfused rabbit hearts were pulse–chase-radiolabeled with [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC ([^{3}H]-lyso platelet activating factor). Since lysophospholipids are amphiphilic constituents that have rapid access to virtually all intracellular myocytic membrane compartments (Gross et al., 1982), if plasmanylcholine were the chemical precursor

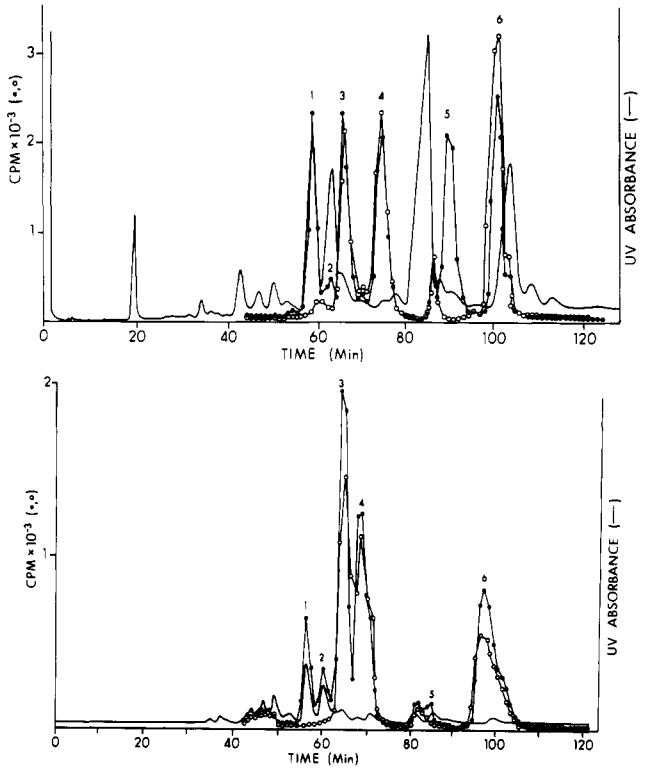

FIGURE 2: Identification of radiolabeled rabbit myocardial choline and ethanolamine glycerophospholipid molecular species produced in isolated rabbit heart perfused with [1-^{3}H]hexadecanol. Ethanolamine (top) and choline (bottom) glycerophospholipids were purified by straight-phase HPLC from hearts that were radiolabeled with [1-^{3}H]hexadecanol for 1.5 h followed by a 21-h perfusion with modified Krebs–Henseleit buffer containing 20 μM hexadecanol, as described in Materials and Methods. Individual molecular species of choline glycerophospholipids were resolved on an octadecyl silica HPLC column with a mobile phase of methanol/acetonitrile/water (90.5/2.5/7, v/v) containing 20 mM choline chloride, at a flow rate of 2 mL/min with UV detection at 203 nm. Column eluents were collected every minute, and the radioactivity (●) in each fraction was quantitated by liquid scintillation spectrometry. The identities of plasmalogen molecular species were confirmed both by their coelution with UV-absorbing peaks of known plasmalogen molecular species at 203 nm and by the removal of these peaks by pretreatment of the HPLC injectate with HCl fumes (○). Peak 1, 16:0-20:4 plasmalogen; peak 2, 16:0-18:2 plasmalogen; peak 3, 16:0-24:4 alkyl ether; peak 4 16:0-18:2 alkyl ether; peak 5, 16:0-18:1 plasmalogen; and peak 6, 16:0-18:1 alkyl ether were identified by their coelution with authentic standards.

of plasmenylcholine, then substantial amounts of acylation of the radiolabeled lyso platelet activating factor and subsequent desaturation would be anticipated in this system. However, after a 1.5-h pulse radiolabeling interval of rabbit myocardium with [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC ([^{3}H]lyso platelet activating factor), >99% of the incorporated radiolabel was present in either the plasmanylcholine or 1-*O*-alkyl-GPC pools with no detectable radiolabel in plasmenylcholine or plasmenylethanolamine (Figure 3). During all chase intervals studied (3, 8, and 21 h), radiolabel decreased substantially from the 1-*O*-alkyl-GPC pool to less than 5% of the pulse level, while that present in plasmanylcholine declined to a lesser degree (Figure 3). Reverse-phase HPLC analysis confirmed that, during the pulse radiolabeling interval with [^{3}H]lyso platelet activating factor as well as during the chase intervals, radiolabel was incorporated only into plasmanylcholine molecular species, and no label was detectable in plasmalogen molecular species (Figure 4).

Since the plasmanylcholine molecular species present in the hearts that were pulse-labeled with [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC and chased for 21 h with cold 1-*O*-alkyl-GPC were similar to those found after hexadecanol labeling (e.g., 16:0-20:4, 16:0-18:2, and 16:0-18:1), and since no radiolabeled plasmenylcholine molecular species were observed in the [^{3}H]-lyso platelet activating factor labeled hearts (Figures 3 and 4), it seems unlikely that plasmanylcholine represents a *bona fide* metabolic precursor for plasmenylcholine biosynthesis through an undiscovered desaturase activity. Collectively, these data, in conjunction with the [1-^{3}H]hexadecanol pulse–chase radiolabeling experiments, strongly suggest that plasmenylethanolamine represents the predominant biosynthetic

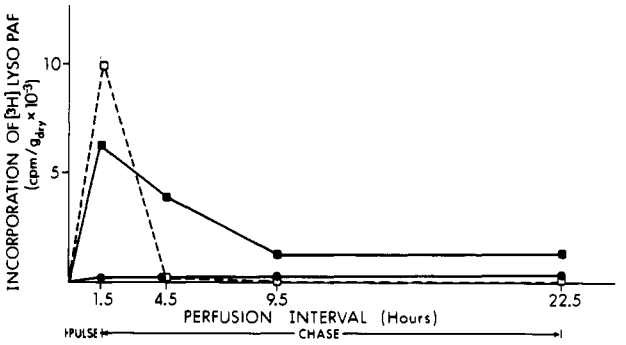

FIGURE 3: Temporal course of [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC incorporation into myocardial lysoplasmanylcholine and plasmanylcholine pools. Rabbit myocardium was perfused with modified Krebs–Henseleit buffer containing [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC for 1.5 h in a recirculating Langendorff perfusion mode. Following the radiolabeling interval, hearts were perfused with modified Krebs–Henseleit buffer containing unlabeled 1-*O*-alkyl-GPC (400 nM) in a non-recirculating mode for the indicated intervals. Perfusions were terminated by freeze-clamping myocardial tissue, and lipids were immediately extracted (Bligh & Dyer, 1959). Radiolabeled 1-*O*-alkyl-GPC (□), 1-*O*-alkyl-2-acyl-GPC (■), and 1-*O*-alkyl-2-acyl-GPE (●) were purified by straight-phase HPLC, and the absence of plasmalogen molecular species in each of these phospholipid pools was documented by the inability of acid fumes to liberate radiolabeled fatty aldehydes from each pool, as described in Materials and Methods.

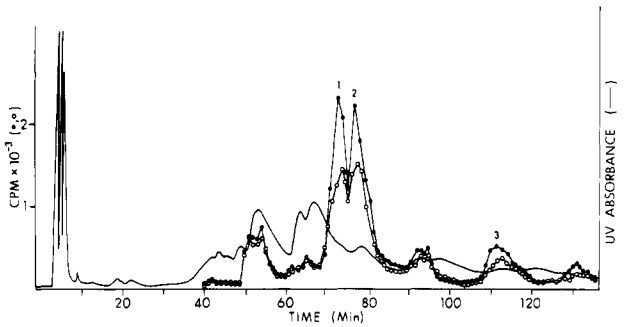

FIGURE 4: Reverse-phase HPLC purification of radiolabeled plasmanylcholine from isolated rabbit heart perfused with [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC. Rabbit myocardium was perfused with [1′,2′-*alkyl*-$^3H_2$]-1-*O*-alkyl-GPC in a recirculating Langendorff mode for 1.5 h followed by a perfusion interval of 21 h with modified Krebs–Henseleit buffer containing unlabeled 1-*O*-alkyl-GPC (400 nM) in a non-recirculating Langendorff mode, as described in Materials and Methods. Perfusions were terminated by freeze-clamping of myocardial tissue, and lipids were extracted (Bligh & Dyer, 1959). Plasmanylcholines were purified by straight-phase HPLC as described in Materials and Methods. Individual molecular species of plasmanylcholine were resolved on an octadecyl silica HPLC column utilizing a mobile phase of methanol/acetonitrile/water (90.5/2.5/7, v/v) containing 20 mM choline chloride, at a flow rate of 2 mL/min with UV detection at 203 nm. Column eluents were collected every minute, and the radioactivity (●) in each fraction was quantitated by liquid scintillation spectrometry. An identical HPLC injectate was pretreated with HCl fumes (○) to demonstrate the absence of plasmalogen molecular species in these samples. Peak 1, 16:0-20:4 plasmanylcholine; peak 2, 16:0-18:2 plasmanylcholine; peak 3, 16:0-18:1 plasmanylcholine.

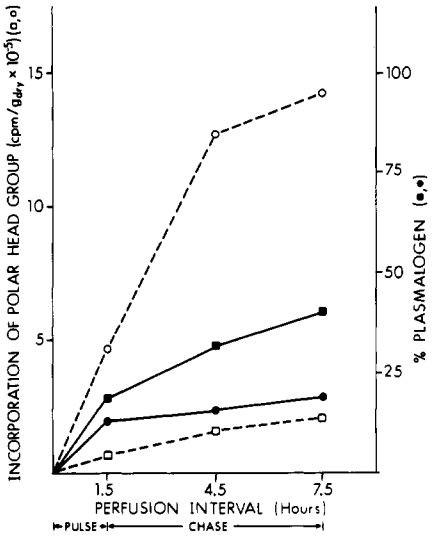

FIGURE 5: Temporal course of radiolabeled choline and radiolabeled ethanolamine incorporation into choline and ethanolamine glycerophospholipids of isolated perfused rabbit myocardium. Rabbit hearts were perfused in a recirculating mode with modified Krebs–Henseleit buffer containing either [^{3}H]ethanolamine or [^{3}H]choline for 1.5 h followed by selected perfusion intervals with modified Krebs–Henseleit buffer containing either ethanolamine (100 μM) or choline (1 mM), respectively, in a non-recirculating Langendorff mode. Perfusions were terminated by rapid freeze-clamping of myocardial tissue, and lipids were extracted (Bligh & Dyer, 1959). Radiolabeled choline (●, ○) and ethanolamine (■, □) glycerophospholipids were purified by straight-phase HPLC, as described in Materials and Methods, and radiolabel incorporation into choline glycerophospholipids (○) or ethanolamine glycerophospholipids (□) was quantitated by liquid scintillation spectrometry. Incorporation of [^{3}H]choline into plasmenylcholine (●) or incorporation of [^{3}H]ethanolamine into plasmenylethanolamine (■) was determined by the sequential treatment of purified phospholipid with acid fumes, TLC purification, and liquid scintillation spectrometry.

precursor of plasmenylcholine in contracting rabbit myocardium. It should also be noted that, in intact myocardium under these conditions, 1-*O*-alkyl-2-acyl-GPC is not a precursor of plasmenylethanolamine, as has been described for Madin Darby kidney cells (Strum et al., 1992).

Pulse–Chase Radiolabeling of Myocardial Choline and Ethanolamine Glycerophospholipids with Precursors of Their Polar Head Groups, sn-1 Fatty Acid Constituents, and sn-2 Fatty Acid Constituents. To compare the rate of *de novo* synthesis to that of polar head group remodeling, the incorporation of radiolabel into the ether-linked aliphatic moieties of plasmalogens was compared to the rate of polar head group incorporation in isolated perfused rabbit hearts pulse–chase-radiolabeled with either [^{3}H]choline or [^{3}H]-ethanolamine. Both radiolabeled [^{3}H]choline and [^{3}H]-ethanolamine were rapidly incorporated into plasmenylcholine and plasmenylethanolamine during a 1.5-h pulse perfusion interval. Incorporation of radiolabeled choline and ethanolamine into both plasmenylcholine and plasmenylethanolamine continued to increase during 3- and 6-h chases with unlabeled choline and ethanolamine following the initial 1.5-h pulse with radiolabeled choline and ethanolamine, demonstrating the relative longevity of the CDP-choline and CDP-ethanolamine pools (Figure 5). The plasmenylcholine molecular species synthesized in the choline glycerophospholipid pool were 16:0-20:4 > 16:0-18:2 > 16:0-18:1 (Figure 6, top). The diacylcholine glycerophospholipid molecular species produced under these conditions were present in similar amounts and largely comprised 16:0-20:4, 16:0-18:2, 16:0-18:1, and 18:0-20:4 (Figure 6, top). In contrast, ethanolamine radiolabeling of perfused myocardium resulted in the incorporation

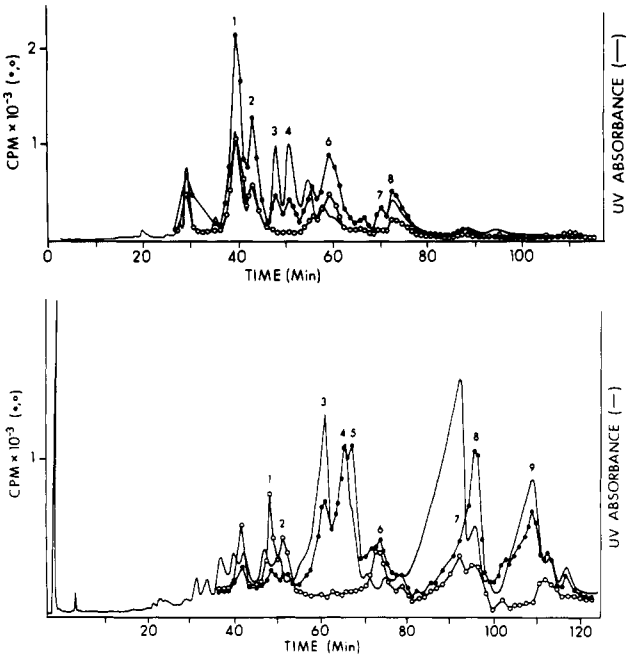

FIGURE 6: Reverse-phase HPLC purification of ethanolamine and choline glycerophospholipid molecular species from isolated rabbit myocardium perfused with [^{3}H]ethanolamine or [^{3}H]choline. Rabbit myocardium was perfused in a recirculating Langendorff mode with modified Krebs–Henseleit buffer containing either [^{3}H]choline (top) or [^{3}H]ethanolamine (bottom) for 1.5 h followed by a 6-h perfusion interval (in a non-recirculating Langendorff mode) with modified Krebs–Henseleit buffer containing either cold choline (1 mM) or ethanolamine (100 μM), respectively. Perfusions were terminated by freeze-clamping myocardial tissue, and lipids were extracted (Bligh & Dyer, 1959). Ethanolamine and choline glycerophospholipids were purified by straight-phase HPLC as described in Materials and Methods. Individual molecular species of ethanolamine and choline glycerophospholipids were resolved on an octadecyl silica HPLC column utilizing a mobile phase of methanol/acetonitrile/water (90.5/2.5/7, v/v) containing 20 mM choline chloride, at a flow rate of 2 mL/min with UV detection at 203 nm. Plasmalogen molecular species were identified both by their coelution with authentic standards and by comparisons with samples pretreated with HCl fumes. Column eluents were collected every minute, and radioactivity from native glycerophospholipids (●) and from acid-pretreated glycerophospholipids (○) was quantitated by liquid scintillation spectrometry. Peak 1, 16:0-20:4 diacyl glycerophospholipid; peak 2, 16:0-18:2 diacyl glycerophospholipid; peak 3, 16:0-20:4 plasmalogen glycerophospholipid; peak 4, 16:0-18:2 plasmalogen glycerophospholipid; peak 5, 18:1-18:2 plasmalogen glycerophospholipid; peak 6, 16:0-18:1 glycerophospholipid; peak 7, 18:0-20:4 diacyl glycerophospholipid; peak 8, 18:0-18:2 diacyl glycerophospholipid; and peak 9, 18:0-20:4 plasmalogen glycerophospholipid.

of ethanolamine into 16:0-18:2 and 18:1-18:2 > 16:0-18:1 > 16:0-20:4 and 18:0-20:4 plasmalogen molecular species (Figure 6, bottom). The predominant diacyl molecular species of ethanolamine glycerophospholipid produced in intact heart under these conditions were 18:0-20:4 and 16:0-18:1 (Figure 6, bottom).

The incorporation rates of radiolabeled hexadecanol, choline, ethanolamine, palmitic acid, oleic acid, and arachidonic acid into plasmenylcholine, plasmanylcholine, phosphatidylcholine, plasmenylethanolamine, plasmanylethanolamine, and phosphatidylethanolamine during pulse radiolabeling conditions were estimated by the utilization of previously determined pool sizes of myocardial choline and ethanolamine (Zelinski et al., 1980; McMaster & Choy, 1992) and previously determined rabbit myocardial palmitic acid, oleic acid, and arachidonic acid mass (Ford & Gross, 1989), and by the quantitation of rabbit myocardial hexadecanol in this study (2 nmol/g$_{dry}$). Quantitation of the incorporation of [1-^{3}H]-

Table 1: Incorporation Rates of Polar Head Group and Aliphatic Precursors of Ether-Linked Glycerophospholipids[a]

	precursors					
product	hexadecanol ($pmol/g_{dry}·h$)	choline ($nmol/g_{dry}·h$)	ethanolamine ($nmol/g_{dry}·h$)	palmitic acid ($nmol/g_{dry}·h$)	oleic acid ($nmol/g_{dry}·h$)	arachidonic acid ($nmol/g_{dry}·h$)
plasmenylcholine		21			11	35
1-*O*-alkyl-2-acyl-GPC	256					
phosphatidylcholine		131		134	92	113
plasmenylethanolamine	93		31		4	12
1-*O*-alkyl-2-acyl-GPE	338					
phosphatidylethanolamine			139	15	19	16

[a] Rabbit hearts were perfused in a recirculating Langendorff mode with modified Krebs–Henseleit buffer containing either [1-^{3}H]hexadecanol, [^{3}H]choline, or [^{3}H]ethanolamine for 1.5 h or alternatively with modified Krebs–Henseleit buffer containing either [^{3}H]palmitic acid, [^{3}H]oleic acid, or [^{3}H]arachidonic acid for 20 min. Perfusions were terminated by freeze-clamping myocardial tissue, lipids were extracted, and radiolabeled choline and ethanolamine glycerophospholipids were purified by straight-phase HPLC as described in Materials and Methods. Incorporation of each radiolabel in each glycerophospholipid pool was quantitated by liquid scintillation spectrometry. Plasmalogen content in choline and ethanolamine glycerophospholipids was determined by treatment of the purified phospholipids with acid fumes, TLC purification of the resultant acid-generated products, and liquid scintillation spectrometry. The specific activity of each radiolabel in each lipid pool was determined from the amount of radiolabel in each precursor pool and either the measured mass of myocardial hexadecanol or the previously determined mass of myocardial choline and ethanolamine pools by others (Zelinski et al., 1980; McMaster & Choy, 1992), as well as our previous determination of palmitic acid, oleic acid, and arachidonic acid content in rabbit myocardium (Ford & Gross, 1989b).

hexadecanol was calculated from the specific activity of radiolabeled hexadecanol in myocardium, and the observed flux was multiplied by a factor of 2 in the case of plasmalogen synthesis to account for the loss of one of the tritiated hydrogens during oxidation. Furthermore, incorporation rates were determined with the assumptions that choline and ethanolamine pools in myocardial cytosol are not highly compartmentalized and that choline and ethanolamine in cytosol can rapidly equilibrate by diffusion (i.e., incorporated choline and ethanolamine radiolabel have access to a common cytosolic pool). Utilizing these conservative assumptions, the incorporation of polar head group precursors (choline and ethanolamine) into plasmalogen glycerophospholipid pools occurs at rates (i.e., 21 and 31 $nmol/g_{dry}·h$, respectively) that are over 2 orders of magnitude greater than the rate of *de novo* synthesis for plasmenylethanolamine in intact hearts (93 $pmol/g_{dry}·h$) (Table 1). Similarly, the incorporation of oleic acid or arachidonic acid into the *sn*-2 position of plasmalogens was over 2 orders of magnitude greater than the *de novo* synthesis of plasmalogens (Table 1). Additionally, incorporation of palmitic acid into diacyl phospholipids was over 3 orders of magnitude greater than the rate of *de novo* plasmalogen synthesis (Table 1).

DISCUSSION

Despite the known abundance of plasmenylcholine molecular species in myocardium (Rapport & Alonzo, 1955; Gross, 1984), the biosynthetic pathways responsible for their synthesis are poorly understood. In fact, the only available information on the biosynthetic pathways for plasmenylcholine has been deduced from broken cell preparations and/or cell culture studies where the relative abundance of plasmenylcholine is less than one-third of that present in adult myocardial tissue (Suga et al., 1990; Lee et al., 1991; Blank et al., 1993). No information on the relative rates of plasmalogen synthesis, degradation, or remodeling in intact myocardial tissue has previously been available. In the present study, several salient issues regarding plasmenylcholine biosynthesis and degradation have been addressed, including (1) the demonstration that the relative rates of polar head group remodeling of plasmalogens in contracting myocardium are over 300-fold more rapid than that of *de novo* plasmalogen synthesis; (2) the demonstration that the pathways mediating *de novo* synthesis, polar head group remodeling, and *sn*-2 aliphatic chain remodeling of plasmenylcholine in functioning myocardium possess distinctive and differential enzymic selectivities for vinyl ether and diacyl phospholipid constituents; and (3) the flux of hexadecanol into plasmenylethanolamine precedes its incorporation into plasmenylcholine, compatible with a precursor–product relationship between plasmenylcholine and plasmenylethanolamine in intact myocardium.

Previous studies have demonstrated and characterized the presence of choline and ethanolamine phosphotransferase activities in myocardium, which can utilize 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol as cosubstrate, resulting in the synthesis of plasmenylcholine and plasmenylethanolamine (Kiyasu & Kennedy, 1960; Ford & Gross, 1988; Ford et al., 1992). After the identification of phospholipase C in myocardium and the demonstration of substantive amounts of its direct reaction product, 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycerol, in rabbit myocardium, the formal demonstration of a pathway capable of synthesizing plasmenylcholine was realized (Wolf & Gross, 1985; Ford & Gross, 1988). One experimentally verifiable prediction of this scheme for plasmenylcholine biosynthesis was the sequential temporal flux of hexadecanol into the plasmanylethanolamine pool, its subsequent appearance in the plasmenylethanolamine pool, and finally its incorporation into the plasmenylcholine pool. The present results clearly demonstrate that, in intact functioning myocardium, hexadecanol first enters the alkyl ether choline and ethanolamine glycerophospholipid pools, subsequently appears in plasmenylethanolamine, and lastly becomes incorporated into the plasmenylcholine pool. Furthermore, the independent demonstrations that lysoplasmanylcholine is acylated to form plasmanylcholine and that this plasmanylcholine is not desaturated to form plasmenylcholine also strongly argue that plasmanylcholine is not a direct chemical precursor of plasmenylcholine. Collectively, these results demonstrate that plasmenylcholine in myocardium is synthesized by the sequential shuttling of the vinyl ether linkage from the ethanolamine glycerophospholipid pool to the choline glycerophospholipid pool and that plasmenylcholine synthesis does not occur by the action of a previously unrecognized alkyl ether desaturase activity on plasmanylcholine in intact functioning myocardium.

Although the reasons underlying the high plasmenylcholine content of myocardium remain enigmatic, it is clear that the synthesis of the *cis*-vinyl ether linkage is an arduous process involving multiple sequential enzymic activities in different subcellular compartments. Whatever the reasons underlying the predominance of plasmenylcholine in myocardium, it seems likely that given the chemical complexity inherent in the

generation of the *cis*-vinyl ether linkage, once generated, the cell would have substantial enzymic machinery to facilitate the preservation of this moiety. Accordingly, we hypothesized that the relative rates of polar head group cleavage and reincorporation of the derivatized polar head group (i.e., CDP-choline or CDP-ethanolamine) of plasmalogen molecular species would greatly exceed the rate of *de novo* vinyl ether synthesis (i.e., *sn*-2 and *sn*-3 remodeling is rapid in comparison to the rate of *sn*-1 vinyl ether synthesis). Through comparisons of the flux of hexadecanol (an obligatory constituent in *de novo* synthesis) into ether glycerophospholipids to the flux of either the choline or ethanolamine polar head groups into plasmenylcholine or plasmenylethanolamine, we demonstrated that the rate of polar head group remodeling was over 300-fold more rapid than the rate of *de novo* synthesis in intact functioning myocardium. Of course, some compartmentation and nonequilibrium of metabolites are inevitable in these systems, but modest compartmentation seems unlikely to account for the over 300-fold difference in the rates of *de novo* synthesis and polar head group remodeling observed in intact tissue in this study.

In addition to polar head group remodeling, the combined action of a plasmalogen-selective phospholipase A_2 (present in myocardium and selective for *sn*-2 arachidonic acid) in conjunction with lysophospholipid acyltransferase activity can result in the *sn*-2 aliphatic chain remodeling of plasmalogen molecular species [e.g., Dennis et al. (1991)]. Alternatively, plasmenylethanolamine *sn*-2 remodeling may occur through acyl transfer from other phospholipids rather than acyl-CoA (Kuwae et al., 1990). We have previously documented the preponderance of arachidonic acid located at the *sn*-2 position of plasmenylcholine and plasmenylethanolamine molecular species in myocardium from several species (Gross, 1984; Hazen et al., 1993). In addition, we have demonstrated that choline and ethanolamine phosphotransferase activities possess substantial subclass, but not individual molecular species, selectivity (Ford & Gross, 1988; Ford et al., 1992). The results of the present study demonstrate that the selectivity inherent in *sn*-2 group remodeling of plasmalogen molecular species likely contributes to their enrichment in arachidonic acid. Specifically, the rate of incorporation of arachidonic acid was demonstrated to be 3-fold greater than that of oleic acid in plasmalogen, but not diacyl phospholipid molecular species. These results suggest that the enrichment of arachidonic acid in plasmalogen molecular species is facilitated by *sn*-2 remodeling in intact tissue. Since *de novo* synthesized plasmenylethanolamines contain a disproportionate amount of individual molecular species containing oleic acid at the *sn*-2 position (in comparison to the mass of this molecular species in rabbit myocardial plasmenylethanolamines), these results further underscore the importance of *sn*-2 aliphatic remodeling in generating the high content of arachidonic acid in plasmenylethanolamines.

Collectively, these results demonstrate the temporal flux of individual constituents (i.e., *sn*-1, *sn*-2, and polar head groups) into plasmalogens and emphasize the importance of remodeling in the manufacture of phospholipid classes, subclasses, and individual molecular species in intact contracting myocardium. Recent studies on plasmalogen structure, dynamics, and conformation have demonstrated unforeseen differences between plasmalogen and diacyl phospholipids engendered by the *sn*-1 vinyl ether linkage. The demonstration that diacyl phospholipids undergo *de novo* synthesis and remodeling at similar rates, while plasmalogen molecular species are remodeled over 300-fold more rapidly than they are synthesized, further underscores the profound differences between mammalian phospholipid subclasses.

REFERENCES

Blank, M. L., Fitzgerald, V., Lee, T., & Snyder, F. (1993) *Biochim. Biophys. Acta 1166*, 309–312.

Bligh, E. G., & Dyer, W. J. (1959) *Can. J. Biochem. Physiol. 37*, 911–917.

Chilton, F. H., & Connell, T. R. (1988) *J. Biol. Chem. 263*, 5260–5265.

Dennis, E. A., Rhee, S. G., Billah, M. M., & Hannun, Y. A. (1991) *FASEB J. 5*, 2068–2077.

Ford, D. A., & Gross, R. W. (1988) *J. Biol. Chem. 263*, 2644–2650.

Ford, D. A., & Gross, R. W. (1989a) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3479–3483.

Ford, D. A., & Gross, R. W. (1989b) *Circ. Res. 64*, 173–177.

Ford, D. A., Rosenbloom, K. B., & Gross, R. W. (1992) *J. Biol. Chem. 267*, 11222–11228.

Gross, R. W. (1984) *Biochemistry 23*, 158–165.

Gross, R. W., Corr, P. B., Lee, B. I., Saffitz, J. E., Crafford, W. A., Jr., & Sobel, B. E. (1982) *Circ. Res. 51*, 27–36.

Gross, R. W., Ramanadham, S., Kruszka, K. K., Han, X., & Turk, J. (1993) *Biochemistry 32*, 327–336.

Hajra, A. K. (1968) *J. Biol. Chem. 243*, 1617–1622.

Hajra, A. K. (1970) *Biochem. Biophys. Res. Commun. 39*, 1037–1044.

Han, X., & Gross, R. W. (1990) *Biochemistry 29*, 4992–4996.

Hazen, S. L., Hall, C., Ford, D. A., & Gross, R. W. (1993) *J. Clin. Invest. 91*, 2513–2522.

Kiyasu, J. Y., & Kennedy, E. P. (1960) *J. Biol. Chem. 235*, 2590–2594.

Kuwae, T., Schmid, P. C., Johnson, S. B., & Schmid, H. H. O. (1990) *J. Biol. Chem. 265*, 5002–5007.

Lee, T. C., Qian, C., & Snyder, F. (1991) *Arch. Biochem. Biophys. 286*, 498–503.

Lumb, R. H., & Snyder, F. (1971) *Biochim. Biophys. Acta 244*, 217–221.

Mancuso, A. J., Huang, S. L., & Swern, D. (1978) *J. Org. Chem. 43*, 2480–2482.

McMaster, C. R., & Choy, P. C. (1992) *Biochim. Biophys. Acta 1124*, 13–16.

Mueller, H. W., Purdon, A. D., Smith, J. B., & Wykle, R. L. (1983) *Lipids 18*, 814–819.

Pak, J. H., Bork, V. P., Norberg, R. E., Creer, M. H., Wolf, R. A., & Gross, R. W. (1987) *Biochemistry 26*, 4824–4830.

Ramanadham, S., Bohrer, A., Mueller, M., Jett, P., Gross, R. W., & Turk, J. (1993a) *Biochemistry 32*, 5339–5351.

Ramanadham, S., Gross, R. W., Han, X., & Turk, J. (1993b) *Biochemistry 32*, 337–346.

Rapport, M. M., & Alonzo, N. (1955) *J. Biol. Chem. 217*, 199–204.

Rizzo, W. B., Craft, D. A., Dammann, A. L., & Philips, M. W. (1987) *J. Biol. Chem. 262*, 17412–17419.

Schmid, H. H. O., Muramatsu, T., & Su, K. L. (1972) *Biochim. Biophys. Acta 270*, 317–323.

Strum, J. C., Emilsson, A., Wykle, R. L., & Daniel, L. W. (1992) *J. Biol. Chem. 267*, 1576–1583.

Suga, K., Kawasaki, T., Blank, M. L., & Snyder, F. (1990) *J. Biol. Chem. 265*, 12363–12371.

Takahashi, T., & Schmid, H. H. O. (1970) *Chem. Phys. Lipids 4*, 243–246.

Wolf, R. A., & Gross, R. W. (1985) *J. Biol. Chem. 260*, 7295–7303.

Wykle, R. L., & Schremmer, J. M. (1979) *Biochemistry 18*, 3512–3517.

Wykle, R. L., Blank, M. L., Malone, B., & Snyder, F. (1972) *J. Biol. Chem. 247*, 5442–5447.

Zelinski, T. A., Savard, J. D., Man, R. Y. K., & Choy, P. C. (1980) *J. Biol. Chem. 255*, 11423–11428.