The appearance, disappearance and reappearance of plasmalogens in evolution

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Abstract

Plasmalogens, 1-O-alk-1'-enyl 2-acyl glycerol phospholipids and glycolipids, seem to have evolved first in anaerobic bacteria, but they did not persist when facultative and aerobic species appeared after the concentration of oxygen increased in the early earth's history. Later, when aerobic animal cells appeared with their mitochondria and other intracellular organelles, plasmalogen biosynthesis requiring molecular oxygen, reappeared. The possible reasons for the disappearance and reappearance of plasmalogens in the evolution of life on earth are discussed. The sensitivity of plasmalogens to reactive oxygen species may have caused their disappearance when respiration first evolved. Special features of plasmalogen structure and the resulting lipid packing may account for their reappearance.

Keywords:
Phospholipid
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Abbreviations: DHAP, dihydroxyacetone phosphate; Gro, glycerol; PlaEtn, Plasmenylethanolamine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; ROS, reactive oxygen species.

Contents

1. Introduction

Plasmalogens differ from the more common diacyl polar lipids in having an alk-1'-enyl ether-linked chain at the glycerol sn-1 position. The alk-1'-enyl ether linkage occurs in both glycerophospholipids and glyceroglycolipids (Fig. 1). The most commonly found phospholipids are phosphatidylethanolamine (PtdEtn), phosphatidylglycerol (PtdGro) and cardiolipin and their plasmalogen forms. Phosphatidyl-N-methylethanolamine and its plasmalogen have been found in *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum* [1] and aminoacyl-PtdGro are present in *Clostridium perfringens* [2,3] and *Clostridium novyi* (Guan, Z. et al. unpublished). In the latter species both alanyl- and lysyl-PtdGro are present in both the diacyl and plasmalogen forms. Glycosyldiaradyl glycerols have been found in a number of species [1].

Plasmalogens have a very unusual, if not unique, distribution in living organisms, as they are found in many strictly anaerobic bacteria including most species of *Clostridium* that have been examined [1], but are not present in aerobic or facultatively anaerobic bacteria. With few exceptions, they are also not found in fungi [4] and their presence in plants has been reported [5,6], but not confirmed [7]. Conversely, they are widely distributed in invertebrate and vertebrate animal species [4]. In mammalian species the highest concentrations are found in brain, heart, and circulating cells of the immune system. A significant portion of the ethanolamine phosphoglycerides in kidney, lung and skeletal muscle is also in the plasmalogen form [4,8]. The best known pathway for their biosynthesis is that existing in animal tissues which requires molecular oxygen and is distinct from the bacterial pathway.

2. Plasmalogen biosynthesis

The biosynthetic pathway in higher organisms begins with the reduction of long chain acyl-CoAs to long chain alcohols by an acyl-CoA reductase, a peroxisomal enzyme [9]. The first step in ether lipid biosynthesis is the acylation of dihydroxyacetone phosphate (DHAP) by another peroxisomal enzyme, dihydroxyacetone phosphate acyltransferase leading to the formation of acyl DHAP [9] (Fig. 2, step 1). This enzyme is obligatory for plasmalogen biosynthesis, but is not required for cell viability. Through the action of alkyl-DHAP synthase, acyl-DHAP is converted to 1-*O*-alkyl DHAP by substitution of a fatty alcohol for the acyl ester (Fig. 2, step 2). Alky-DHAP is converted to 1-*O*-alkyl-2-lyso-*sn*-glycero-3-P through reduction of the keto function to a hydroxyl by NADPH:alkyl-DHAP oxidoreductase (Fig. 2, step 3) [9].

After acylation of the sn-2 position by an acyltransferase and dephosphorylation (Fig. 2, steps 4 and 5), the stage is set for the introduction of ethanolamine using CDP-ethanolamine (Fig. 2, step 6). The final step in plasmalogen biosynthesis is catalyzed by a 1'-alkyl desaturase which stereospecifically abstracts hydrogen atoms from the C-1' and C-2' of the *O*-alkyl chain of the ethanolamine lipid to form the *cis* double bond of the plasmalogen (Fig. 2, step 7). This reaction requires NADPH and molecular oxygen. It appears that the desaturase does not use 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine. Rather the choline plasmalogen is formed from the ethanolamine plasmalogen by either a base exchange or by removal of the ethanolamine phosphate by a phospholipase C and a cholinephosphotransferase [9].

Although the presence of plasmalogens in anaerobic bacteria has been known for almost 50 years [10-12] and a number of studies have been reported on the biosynthesis of these lipids in bacteria [13-20], the pathway and mechanism for their biosynthesis, which differs from that in animals, is not known. Several lines of evidence differentiate it from that found in higher organisms. The most compelling finding is the fact that DHAP has been ruled out as an intermediate in plasmalogen biosynthesis in *C. beijerinckii* ATCC 6015 (formerly *C. butyricum*) [21]. Upon addition of [2-^{3}H] glycerol and [1-^{14}C] glycerol to the growth medium, the ^{3}H / ^{14}C ratios of the resulting diacylphospholipids and the plasmalogens were found to be almost identical. If DHAP had served as an intermediate, the hydrogens on C-2 of glycerol would have been lost. Similar experiments were carried out with the Gram-negative anaerobes *Megasphaera elsdenii* and *Veillonella parvulla* and with the anaerobic protozoa *Isotricha prostoma* and *Dasytricha ruminantium* with similar results, suggesting that the dividing line between the organisms that utilize DHAP for plasmalogen biosynthesis and those that do not is not eukaryote-prokaryote but

rather aerobic-anaerobic organisms [22]. A common conclusion from many of the studies on bacterial plasmalogen biosynthesis cited above is that the diacyl phospholipids, PtdGro and PtdEtn serve as precursors for their plasmalogens [20]. Recent studies have shown that in clostridia the precursor lipids, phosphatidic acid and phosphatidylserine have very low levels of plasmalogens, but the end products, phosphatidylglycerol, cardiolipin and phosphatidylethanolamine, do ([23] and unpublished studies).

Long-chain alcohols are utilized for plasmalogen biosynthesis in eukaryotes, but only limited success in their incorporation into plasmalogens by bacteria has been reported [20, 24]. In contrast, long-chain aldehydes and fatty acids were readily incorporated into the alk-1'-enyl chains of plasmalogens in *C. beijerinckii* ATCC 6015 [13, 14]. With doubly labeled [1-^{3}H,1-^{14}C] palmitaldehyde as precursor, approximately 15% of the tritium was retained in the alk-1'-enyl chains indicating that aldehydes do not have to undergo oxidation to fatty acids prior to incorporation [14]. These findings lead to an incomplete and still somewhat speculative pathway for plasmalogen biosynthesis in anaerobes, which is shown in figure 3. All the steps leading to PtdGro and PtdEtn in clostridia were shown to occur *in vitro* [17, 25, 26]. Thus an anaerobic pathway to plasmalogen evolved first in bacteria and was later replaced by an aerobic pathway in animals, but not in aerobic bacteria, fungi and plants.

3. Why did the anaerobic pathway to plasmalogens not persist in aerobic bacteria?

It is evident that plasmalogen biosynthesis has undergone a switch from an anaerobic mechanism that still exists in contemporary anaerobic bacteria and even in some anaerobic protozoa [22] to an aerobic mechanism found in oxygen-tolerant eukaryotes. It is widely

accepted that life on earth first evolved in an anaerobic environment [27,28]. The essential prokaryotic biosynthetic mechanisms are all anaerobic and aerobic bacteria retain almost all of these pathways [29,30]. A classic example is the biosynthesis of monounsaturated fatty acids in bacteria in which the double bond is inserted by elimination of water from a β-hydroxy acid intermediate during the process of chain elongation [31-33]. Although some bacteria have gained the capacity to desaturate fatty acids, the 'anaerobic' pathway is usually retained [33]. In plants, fungi and animals unsaturated fatty acids are formed by an oxidative mechanism requiring molecular oxygen, NADPH and cytochrome b_5 in which two hydrogens are abstracted in a positionally-sensitive manner from long-chain saturated fatty acids [34,35]. Thus stearic acid, C_{18}, is converted to oleic acid, 18:1Δ9.

The biosynthesis of plasmalogens mirrors that of unsaturated fatty acids. Anaerobes accomplish this without molecular oxygen whereas most eukaryotes that make plasmalogens use an oxidative mechanism that has similar requirements to the aerobic desaturation of fatty acids. Why did the anaerobic pathway not survive when aerobic bacteria evolved? The answer may lie in the sensitivity of plasmalogens to reactive oxygen species (ROS). In the presence of ROS plasmalogens are rapidly degraded with scission at the alk-1'-enyl ether bond [36]. Oxygen sensitivity of plasmalogens was described as early as 1972 [37-40]. From this it would appear that plasmalogens could be a target for damage by ROS and that cells containing these lipids would be damaged as a result of their breakdown [8].

If so, how does one explain the abundance of plasmalogens in animal cells which produce ROS? Zoeller *et al.* proposed that plasmalogens could serve to limit oxidative damage to cells by

serving as antioxidants [36,41]. They found that peroxisome/plasmalogen-deficient variants of CHO cells were more sensitive to killing when exposed to a pyrene-labeled fatty acid (PLFA) which generates singlet oxygen and/or other ROS upon exposure of the cells to long-wavelength (>300 nm) UV light. They further observed the selective breakdown of plasmalogens in the membranes of wild-type CHO cells subjected to treatment with PLFA and UV light. A mutant that had a plasmalogen deficiency with normal peroxisomal function also was hypersensitive to PLFA/UV exposure [42]. Similar results were obtained with plasmalogen-deficient variants isolated from the murine macrophage-like cell line RAW 264.7. In these studies ROS was generated by exposure to electron transport inhibitors such as antimycin-A and cyanide in glucose-deficient buffers, which results in ROS generation and cell death. The plasmalogen-deficient variants were much more sensitive to this treatment than wild-type cells. Supplementation of the growth medium of the variant cells with 1-alk-1'-enyl-*sn*-glycerol restored plasmalogen levels and produced wild-type resistance to PLFA/UV and other ROS generators [43]. The ability of animal cells to withstand the damage caused by breakage of the alk-1-enyl ether bond may result from their ability to acylate the resulting 2-monoacylglycerophosphoethanolamine or to deacylate the resulting lyso phospholipid [36]. Upon reacylation, a plasmalogen would be replaced by the corresponding diacyl lipid.

As the concentration of oxygen in the earth's atmosphere increased and early life forms gained the capacity to use oxygen for respiration and the concomitant benefits of the greatly increased amounts of energy that can be obtained from organic substrates through oxidative phosphorylation, a side effect was the formation of ROS. The production of superoxide radicals occurs in mitochondria at two points in the electron transport chain, at complex I, NADH

dehydrogenase, and at complex III, ubiquinone-cytochrome *c* reductase [44]. A similar pathway exists in bacteria. Aerobic and aerotolerant bacteria have superoxide dismutase and catalase which detoxifies the superoxide free radical, but these are missing in strict anaerobes [45]. The appearance of ROS would result in plasmalogen breakdown at some level, resulting in the generation of *sn*-1-lyso-phospholipids. Bacteria rich in plasmalogens probably did not have the eukaryotic mechanisms for acylation of the resulting free hydroxyl group at the sn-1 position and the accumulation of these lysophospholipids would be toxic to cells. This would have provided a selective advantage to anaerobes that had lost the capacity to produce plasmalogens. Since all contemporary anaerobes that have been examined have polar lipids with acyl chains at the sn-1 position analogous to their plasmalogens, for example PtdEtn, PtdGro and cardiolipin, when plasmalogens are lost membranes spaces are filled by these lipids. This has been observed to occur in several anaerobes. For example, from *Megasphaera elsdenii*, which normally has about 80% of its phospholipids in the plasmalogen form, strains were isolated several times, which had reduced their plasmalogen content over a period of about 40 weeks to less than 1% [46]. This occurred during serial passage and storage at room temperature under anaerobic conditions. Similar results were obtained with *C. beijerinckii* ATCC 6015. This strain, normally rich in plasmalogens [13], appears to have lost the capacity to synthesize them during serial passage (P-O. Hagen, personal communication). We have recently found that *C. tetani* ATCC 10779 has no plasmalogens in contrast to five other strains of *C. tetani* that are rich in plasmalogens [23]. *C. tetani* ATCC 10779, also called strain Massachusetts, was used in vaccine production [47] and it is reasonable to assume that it was subjected to serial passage. As noted above, no plasmalogens have been found in existing aerobic or facultatively anaerobic bacteria [48,49].

4. The special properties of plasmalogens

Why, then do plasmalogens continue to exist in many anaerobes? Several differences in the physical properties of plasmalogens and the corresponding diacyl lipids may account for their persistence. Some of these differences probably result from significant differences in their conformation. In diacylphosphoglycerides the sn-2 acyl chain is bent so that the first and second carbon atoms are approximately parallel to the plane of the bilayer and the rest of the chain is perpendicular to that plane extending into the bilayer core [50]. The resulting inequivalence of the two protons on carbon-2 of the sn-2 chain of diacyl phospholipids can be detected by deuterium NMR. Plasmenylethanolamine (PlaEtn) labeled with deuterium at carbon 2 of the *sn*-2 acyl chain produces a single quadrupole splitting which suggested that the sn-2 acyl chain is perpendicular to the bilayer surface at all chain segments [51,52]. Deuterium NMR studies of the *sn*-1-alk-1'-enyl chain of plasmalogens indicated that this chain is also perpendicular to the bilayer plane at all segments [51]. Similar conclusions were reached employing truncated driven nuclear Overhauser enhancement which allows measurement of internuclear distances [53]. The resulting dynamic properties of the two types of phospholipids also differ significantly [54-56]. The decreased mobility of the lipids within a bilayer results from the closer packing of plasmalogens than diacyl phospholipids attributed to the lack of a kink at the top end of the sn-2 acyl chain [57]

These chemical and structural differences lead to differences in the thermotropic phase behavior of the lipids. When lipids such as PtdEtn are heated from a low temperature, at which they are in an ordered gel phase, they undergo a transition to the liquid crystalline phase. Results obtained with biosynthetic and semisynthetic plasmalogens indicated that they undergo this G →

L transition 4 to 6°C below the temperature for the corresponding diacyl PE [58, 59]. Upon further heating PtdEtn, especially when it contains unsaturated fatty acids, undergoes a transition from the liquid crystalline phase to the non-lamellar reversed hexagonal (H_{II}) phase. The L → H transition for semisynthetic PlaEtn with a mostly saturated sn-1 chains and oleate at the sn-2 position was 30°C compared to 68°C for the diacyl form [59]. In some clostridia, glycerol acetals of the plasmalogens are also present (Fig. 1). The presence of these lipids raises the L → H transition temperature. Addition of oleate-enriched glycerol acetal of PlaEtn (Fig. 1) to dioleoyl PE (1:1) elevated the L → H transition temperature to 35°C compared to below 10°C for dioleoyl PE alone [60]. Thus the presence of plasmalogens and their glycerol acetals provides considerable plasticity to bacterial membranes especially for cells that have to grow at a wide range of temperatures, salinity and the presence of solutes such as hydrocarbons and solvents that have the potential of perturbing the bilayer arrangement of the cell membrane [61]. So far the glycerol acetals of plasmalogens have only been found in the solventogenic clostridia, consistent with a special, bilayer-stabilizing role for these unusual lipids in these species, e.g. *C. acetobutylicum* and *C. beijerinckii* [1].

5. The reappearance of plasmalogens in eukaryotes

At some time, early during the evolution of eukaryotic organisms, only in the animal lineage, a new pathway for aerobic synthesis of plasmalogens appeared (Fig. 2). This pathway involves a saturated ether lipid intermediate and both saturated (*sn*-1-alkyl) and unsaturated (*sn*-1-alk-1'-enyl) ether lipids are abundant in animal species. Orthologs of the enzyme that produces alkyl DHAP have been found in protozoa, placozoa, sponges, sea anemones, flatworms, sea urchins, insects, and vertebrates. It appears that the tree of life was rapidly populated with species

that had the capacity to synthesize 1-*O*-alkyl lipids. It is more difficult to identify species that can make plasmalogens based on bioinformatics, since no specific gene has been identified for the Δ1'-alkyl desaturase. However, plasmalogens are also distributed widely in the animal kingdom. They exist in amoeba Chaos [62], slime molds, insects and in a large number of other invertebrates including jellyfish, *Cnidaria*, *Mollusca,* and *Echinodermata* and vertebrates (reviewed in [4])**.**

Thus, the reemergence of plasmalogens occurred in very deep evolutionary time. The earliest multicellular fossils are found in the proterozoic eon, about one billion years ago, long after the rise of oxygen concentrations in the air to present-day levels, which is estimated to have occurred about two billion years ago [27,28]. As noted above, plasmalogen biosynthesis occurs in peroxisomes, an oxidative organelle found in virtually all eukaryotic cells, which appears to be derived from the endoplasmic reticulum rather than from an endosymbiont [63]. An important function of this organelle is detoxification by means of peroxidative reactions.

There are a number of special roles that plasmalogens may play in animal cells. Eukaryotes have evolved several intracellular signaling mechanisms that involve lipids. Diacylglycerol formed through the actions of phospholipases C and D serves to activate protein kinase C. Phospholipases and lipases release arachidonic acid from phospholipids and diacylglycerol, respectively, when exposed to a multiplicity of stimuli. Arachidonic acid is a source of prostaglandins, thromboxanes and leukotrienes, which can act in an autocrine fashion on the parent cell or on neighboring cells [64]. Plasmalogens can serve as a source of arachidonic acid in several tissues and cell types [8]. A plasmalogen-selective, calcium-independent phospholipase A_2 ($iPLA_2$) has been described in canine myocardium [65] and this activity is

activated in the ischemic rabbit myocardium [66]. Other plasmalogen-selective PLA_2 have been described in bovine brain [67,68] and rabbit kidney cortex [69]. Thus, the presence of plasmalogens and selective phospholipases may permit regulation of signaling depending on the source of arachidonate.

The abundance of plasmalogens in conductive tissues in animals suggests that they may serve to modify the permeability of these membranes. Studies on ion fluxes in artificial membrane vesicles composed of diacyl phosphatidylcholine or the corresponding plasmalogen species both containing arachidonic acid at the sn-2 position show that the rate of passive K^+ flux was an order of magnitude higher for the diacyl species [70]. Similar conclusions on both passive and carrier-mediated ion transport through plasmalogen-rich artificial membranes were reached by Chen and Gross [57]. Thus the presence of plasmalogens containing arachidonic acid needed for signal transduction pathways permits the preservation of transmembrane ion gradients while providing arachidonic acid released through the action of phospholipase A_2. The reemergence of plasmalogens, synthesized by an oxygen-dependent pathway may have served to provide unique functions that emerged in the evolution of eukaryotes and especially those of multicellular organisms.

6. Conclusions and perspectives

Admittedly, examination of the genomes and lipids of living organisms, can only lead to conjecture about the origins of contemporary biosynthetic pathways. The genes for plasmalogen biosynthesis in anaerobic bacteria have not been discovered. When they are, it is possible that remnants of them may be found in aerobic and facultatively anaerobic bacteria. When oxygen appeared on earth it was used for the biosynthesis of complex molecules such as cholesterol and

the many important compounds of higher organisms within the sterol family, not found in bacteria and for polyunsaturated fatty acids, which have unique physical properties. In addition, one of these, arachidonic acid, serves as a precursor for hormones such as prostaglandins and leukotrienes, important lipid messengers found in animals. Konrad Bloch, has characterized these and other oxygen-dependent biosynthetic pathways as a "driving force behind evolutionary innovations"[30]. In the case of plasmalogens, nature appears to have rediscovered an ancient molecule and molded it to the purposes of protozoan and metazoan life.

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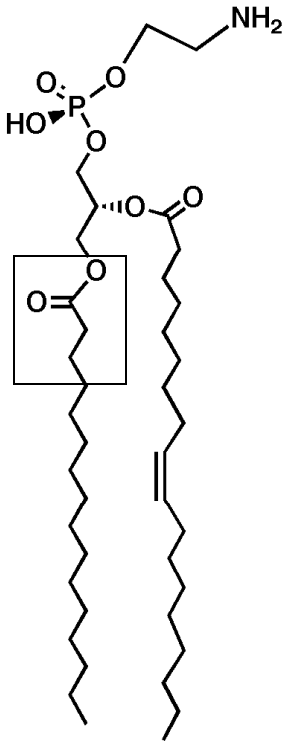
Figure legends.

Figure 1. Subclasses of glycerol lipids.

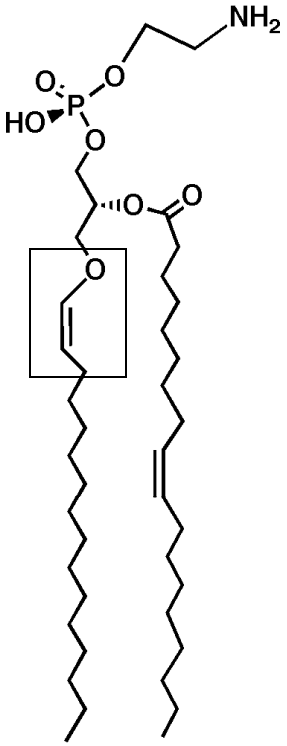
Figure 2. The oxygen-dependent pathway for plasmalogen biosynthesis in animal tissues.

Figure 3. A proposed anaerobic pathway for plasmalogen biosynthesis in bacteria. The number of steps leading from the diacyl phospholipids to the plasmalogens is unknown.

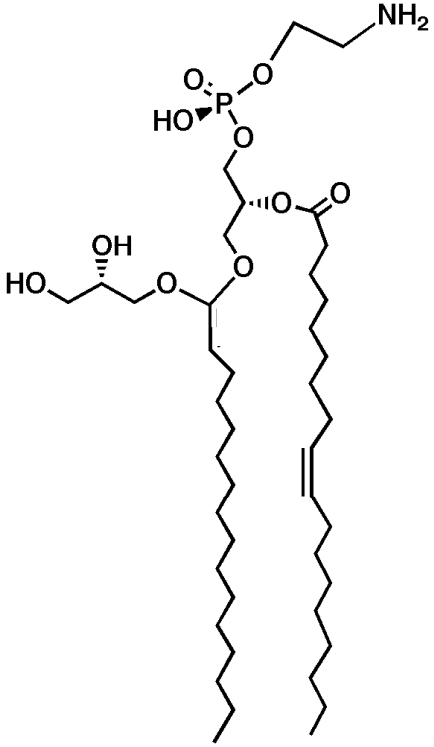
Figure 1



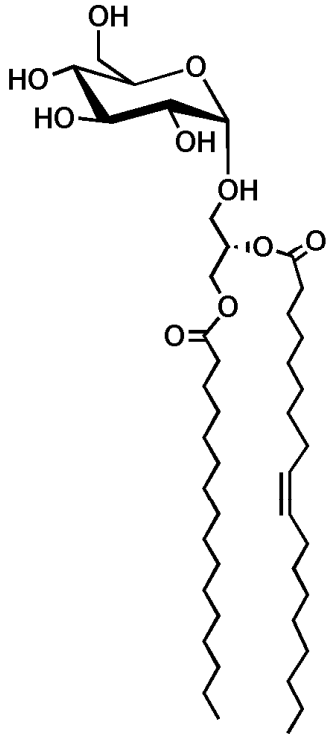
PtdEtn



Plasmalogen
(PlaEtn)

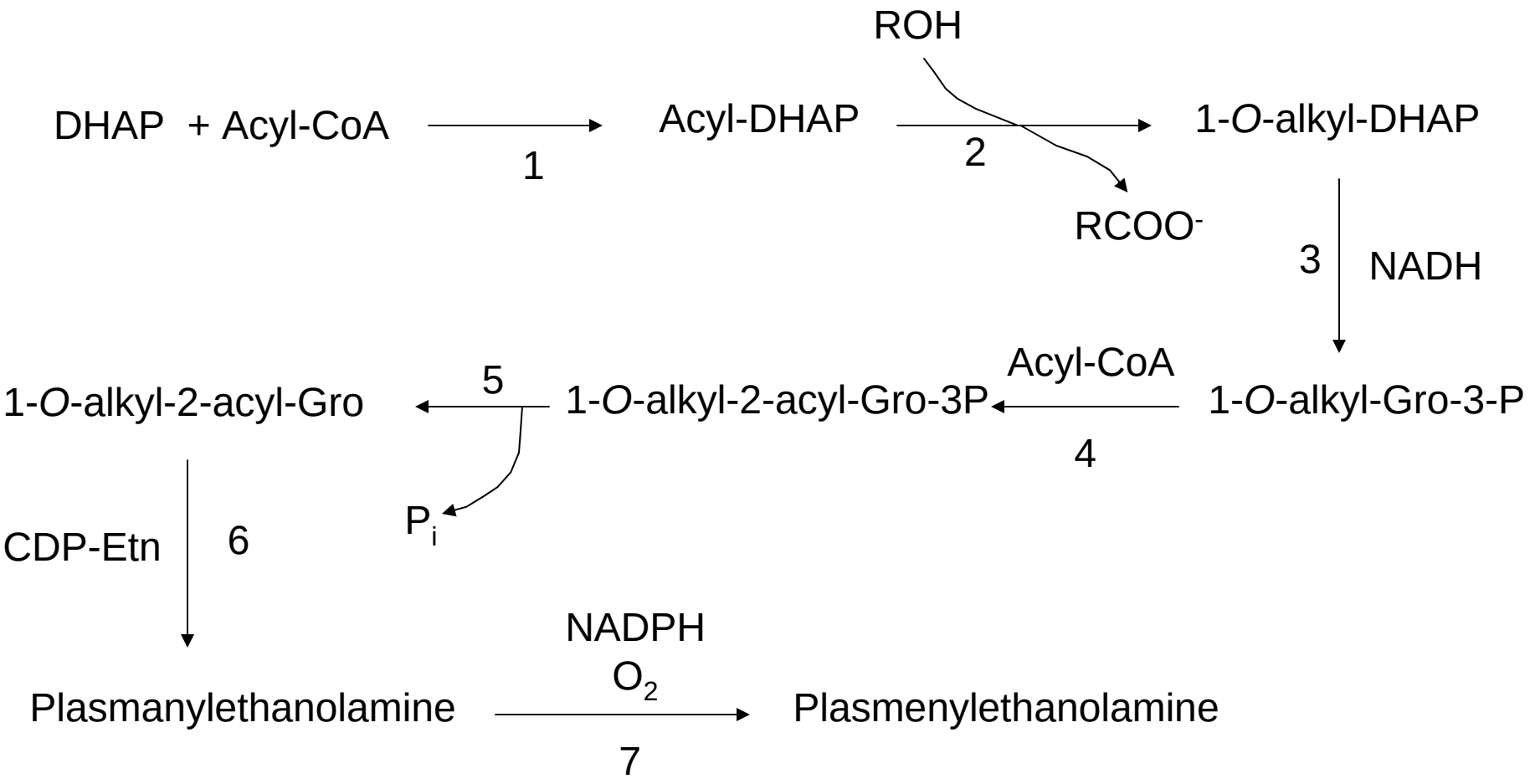


Glycerolacetal
of PlaEtn



Glucosyl
diacylglycerol

Figure 2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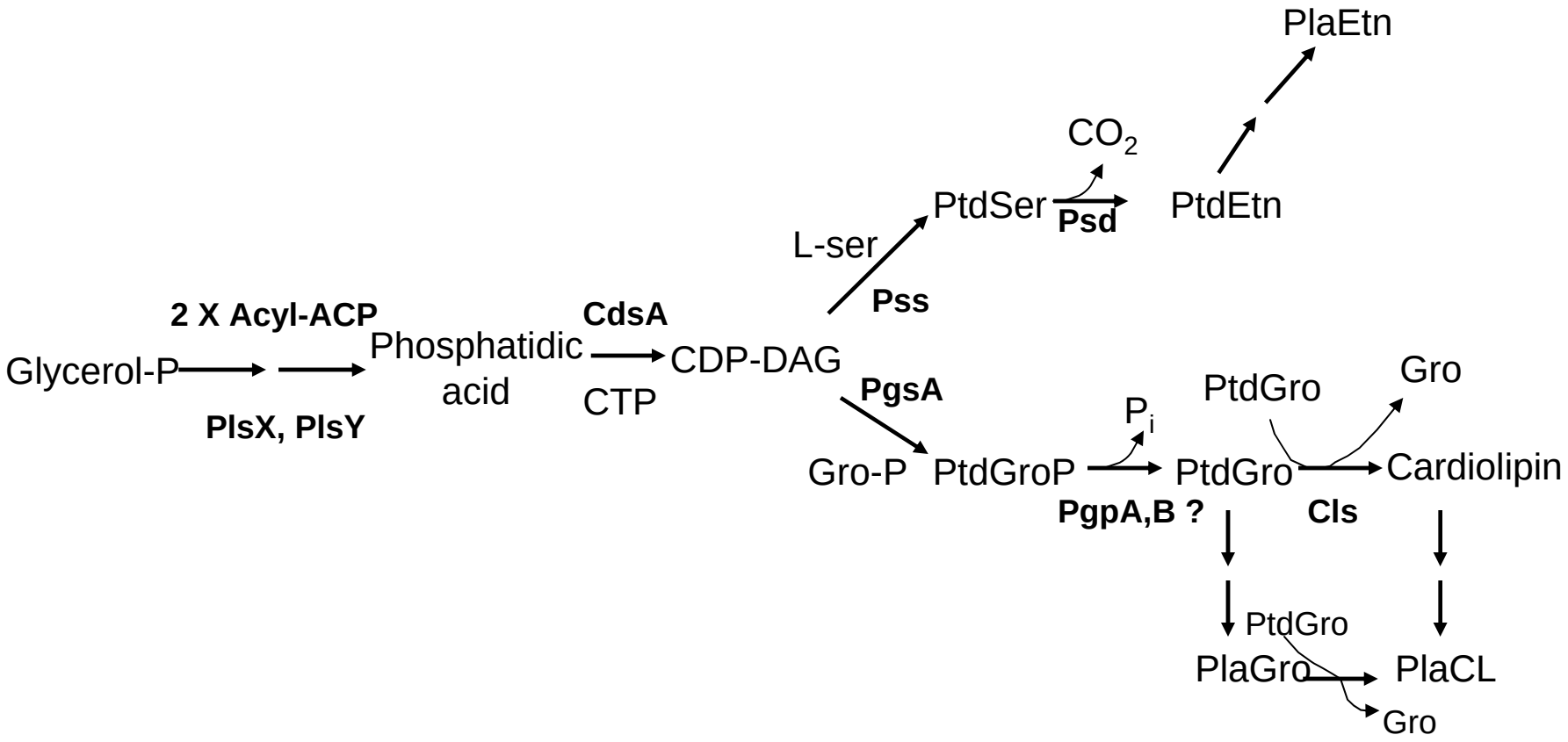
2
1-O-alkyl-DHAP
RCOO-
3
NADH
Acyl-CoA
1-O-alkyl-2-acyl-Gro
5
1-O-alkyl-2-acyl-Gro-3P
4
1-O-alkyl-Gro-3-P
Pi
CDP-Etn
6
NADPH
O2
Plasmanylethanolamine
7
Plasmenylethanolamine

Figure 3


PlaEtn
CO_2
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Psd
PtdEtn
L-ser
Pss
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CdsA
Glycerol-P
Phosphatidic acid
CDP-DAG
CTP
PlsX, PlsY
PgsA
PtdGro
Gro
P_i
Gro-P
PtdGroP
PtdGro
Cardiolipin
PgpA,B ?
Cls
PtdGro
PlaGro
PlaCL
Gro