Published in final edited form as:

Biochim Biophys Acta. 2011 March ; 1811(3): 186–193. doi:10.1016/j.bbalip.2010.12.010.

Structural Characterization of the Polar Lipids of *Clostridium novyi* NT. Further Evidence for a Novel Anaerobic Biosynthetic Pathway to Plasmalogens

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Abstract

A study of the polar lipids of *Clostridium novyi* NT has revealed the presence of phosphatidylethanolamine (PE) and cardiolipin as major phospholipids with smaller amounts of phosphatidylglycerol (PG), lysyl-PG and alanyl-PG. Other minor phospholipids included phosphatidic acid, CDP-diacylglycerol, phosphatidylserine (PS) and phosphatidylthreonine (PT). PE, PG and amino acyl PG were present in both the diacyl and alk-1'-enyl acyl (plasmalogen) forms and cardiolipin plasmalogens were found to contain one or two alk-1'-enyl chains. In contrast, the precursor lipids phosphatidic acid, CDP-diacylglycerol and PS were present almost exclusively as diacyl phospholipids. These findings are consistent with the hypothesis that plasmalogens are formed from diacylated phospholipids at a late stage of phospholipid formation in *Clostridium* species. This novel pathway contrasts with the route in animals in which a saturated ether bond is formed at an early stage of plasmalogen biosynthesis and the alk-1-enyl bond is formed by an aerobic mechanism.

1. Introduction

Despite the vast accumulation of knowledge about of bacterial lipids, information on the polar lipids of the more than one-hundred species of *Clostridium* has been limited [1,2]. Wild type *Clostridium novyi* (*C. novyi*) is a pathogen that produces a number of toxins, the presence of which varies among the different groups designated A, B and C. The clinical

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Research highlights

- The lipidome of *Clostridium novyi* is presented for the first time
- The end-products of lipid biosynthesis are found in all acyl or plasmalogen species
- The intermediates of phospholipid biosynthesis contain very little plasmalogen form
- Plasmalogens in clostridia are formed from the corresponding all acyl lipids

manifestations vary depending on the location of the infection and the *C. novyi* type. Anaerobic wound infections with this organism can lead to gas gangrene, which can be fatal.

As part of an on-going project to study the polar lipids of clostridia for which genome sequences are available, we have used TLC and highly sensitive HPLC coupled with ESI/MS to examine the lipids of *Clostridium novyi* NT [3], an attenuated strain that has been developed as an adjunct to cancer therapies. Since the interior of some cancers can be necrotic and lacking in blood supply, these areas are difficult to treat with ionizing radiation and chemotherapeutic agents. As a result, cells in the hypoxic tumor core often survive treatment and become a source for subsequent cancer recurrence and spread. Several studies have shown strong anti-tumor effects of intravenously injected *C. novyi* NT spores, although the mechanisms by which the tumors are destroyed are not known [4-6].

Our studies on the polar lipids of *C. novyi* NT have shown that it contains a number of lipids that could be predicted from its genome including phosphatidylethanolamine (PE) synthesized by the products of the *pss* and *psd* genes, phosphatidylglycerol (PG) presumably synthesized by the product of *pgsA*, cardiolipin synthesized by the product of the *cls* gene and lysyl-PG and alanyl-PG produced by amino acyltransferases designated MprF, encoded by *mprF*. The precursors of these lipids, phosphatidic acid, CDP-diacylglycerol and phosphatidylserine (PS) were also detected, but these lipids have either no or very low levels of plasmalogens, consistent with the hypothesis that plasmalogens are formed at late stages in phospholipid biosynthesis in clostridia [7-10]. This is in contrast to the pathway found in animal cells, in which a saturated ether bond is formed at an early stage of plasmalogen biosynthesis, is retained in the subsequent intermediate, plasmanic acid (*1-O*-alkyl, 2-acylglycerophosphate), and the alk-1-enyl bond is formed subsequently by an aerobic mechanism [11,12]. Thus, it is possible that once the genes and enzymes have been discovered, the enzymes required for this pathway could potentially become the targets of novel antibiotics.

2. Materials and methods

2.1. Bacterial strains and growth conditions

C. novyi NT was obtained from Drs. Shubin Zhou and Bert Vogelstein, Ludwig Center at Johns Hopkins University, Baltimore, MD. *C. novyi* strain ATCC 27606 was obtained from the American Type Culture Collection, Manassas, VA. For lipid isolation, *C. novyi* strains were grown overnight on reinforced clostridial medium without agar, which contained per liter: yeast extract, 10.5 g; peptone, 12.5 g; glucose, 5 g; soluble starch, 1 g; NaCl, 5 g; sodium acetate, 3 g; and cysteine HCl•H₂O, 0.6 g. The pH was adjusted to approx. 7.1 with NaOH. The glucose solution was autoclaved separately.

2.2. Lipid extraction

Cells were harvested by centrifugation at 2900 × g for 10 min and washed twice in 20 mM MOPS buffer, pH 7.2. The wet cell pellets were extracted with chloroform/methanol/water by the method of Bligh and Dyer [13], as modified [14]. The dried lipids were dissolved in chloroform, and stored at -20 °C.

2.3. Thin layer chromatography

Two dimensional thin layer chromatography (2D-TLC) was performed on silica gel 60, 10 × 10 cm, thin-layer plates. The solvents were chloroform/methanol/concentrated ammonia/water, 65:30:2.5:2.5 (by vol.) in the first dimension and chloroform/methanol/acetic acid/water, 80:18:12:5 (by vol.) in the second dimension. Acid hydrolysis of lipids on TLC plates for detection and quantification of plasmalogens has been described [15]. Phospholipids or

amine-containing lipids were detected using 0.3% (w/v) molybdenum blue or 0.3% ninhydrin in ethanol, followed by heating at 120 °C for 10 min, respectively. For quantification of the polar lipids the cells were labeled during growth for 24 h in 10 ml reinforced clostridial medium with 10 μ Ci [1- 14 C] acetate (60 mCi/mmol $^{-1}$, Perkin Elmer Life Sciences, Waltham, Massachusetts 02451, USA) at 37 °C. Cells were harvested by centrifugation, washed and the lipids extracted as described above. As needed, carrier lipid extracted from *C. novyi* grown without [1- 14 C] acetate, was added. 2D-TLC was performed using 14 C-labeled material (7,500 cpm). Radiolabeled lipids were visualized and quantified with a PhosphorImager (Typhoon 9410, Amersham Biosciences, Arlington Heights, IL), equipped with ImageQuant software.

2.4. Liquid chromatography/mass spectrometry (LC/MS)

Normal phase LC-ESI/MS of lipids was performed using an Agilent 1200 Quaternary LC system (Santa Clara, CA), coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA). An Ascentis® Si HPLC column (5 μ m, 25 cm $\times$ 2.1 mm) was used. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide (800:195:5, by vol.). Mobile phase B consisted of chloroform/methanol/water/ aqueous ammonium hydroxide (600:340:50:5, by vol.). Mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, by vol.). The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 μ l/min. The post-column splitter diverted ~10% of the LC flow to the ESI source of the Q-Star XL mass spectrometer, with MS settings as follows: IS = -4500 V, CUR = 20 psi, GS1 = 20 psi, DP = -55 V, and FP = -150 V. Nitrogen was used as the collision gas for MS/MS experiments. Data acquisition and analysis were performed using the Analyst QS software (Applied Biosystems, Foster City, CA).

3. Results

3.1. Preliminary identification of *C. novyi* NT polar lipids by thin layer chromatography

Two dimensional TLC of the total lipids of *C. novyi* NT revealed two major polar lipids, which were tentatively identified as PE (Fig. 1A, spot 4) and cardiolipin (Fig. 1A, spot 1) based on their relative mobilities and staining with phosphomolybdate and/or ninhydrin. Two of the minor lipids, PG (Fig. 1A, spot 2) and phosphatidic acid (Fig. 1A, spot 9) were also tentatively identified by their positions relative to other lipids and standards, and the absence of ninhydrin staining. Two ninhydrin and phosphomolybdate-positive minor lipids were noted and later identified by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as alanyl-PG (Fig. 1A, spot 3) and lysyl-PG (Fig. 1A, spot 7). PS was tentatively identified by its mobility and staining with ninhydrin and phosphomolybdate. It was later found that this spot may also contain phosphatidylthreonine (PT) (Fig. 1A, spot 8).

Because plasmalogens are commonly found in anaerobic bacteria, to detect these acid-labile lipids we exposed the lipids, to hydrochloric acid (HCl) fumes between the first and second dimension in 2D-TLC [16]. In addition to production of the lyso compounds formed by hydrolysis of the alk-1'-enyl ether bond, aldehydes are also released and observed at the solvent front of the second run (Fig. 1B). In this chromatogram it can be seen that cardiolipin, PG and PE were partially hydrolyzed by the acid treatment. The aldehydes resulting from acid hydrolysis of cardiolipin and PG are spots 1 and 2 and the lyso compounds resulting from hydrolysis are spots 6 (lysocardiolipin), 10 (lyso-PG) and 11

(dilyso-cardiolipin). Lysyl-PG was also partially hydrolyzed producing aldehydes and lyso-lysyl-PG, (Fig. 1B, spots 19 and 18, respectively). Some of the minor lipids were either not hydrolyzed or the amounts of the hydrolysis products were too small to be visualized by the phosphorimager (see below). 2D-TLC of the total lipids extracted from *C. novyi* ATCC 27606 was also performed and the same set of major lipids was observed. No phosphatidic acid was observed. Acid hydrolysis between the first and second TLC dimensions showed that the major lipids consisted of both diacyl and plasmalogen species (data not shown). The lipids of strain ATCC 27606 were not quantified.

3.2 Quantification of *C. novyi* NT polar lipids

The polar lipids of *C. novyi* NT before (Fig. 1A) and after treatment with HCl fumes (Fig. 1B) were quantified isotopically from cells that had been grown for 24 h with [^{14}C] acetate (Table 1). The two predominant lipids in these stationary phase cells are PE and cardiolipin accounting for 85.6% of the lipid mass. The plasmalogen form of PE accounted for 25% of the total PE. Since the aldehydes resulting from hydrolysis of the cardiolipin and PG plasmalogens were not separated, we could not determine the amounts of plasmalogens associated with each of these phospholipids, but the average amount of plasmalogen in the two lipids was 43% (Table 1).

3.3 LC/ESI-MS of total *C. novyi* NT polar lipids

In order to confirm and extend the TLC analysis of the polar lipids of *C. novyi*, we subjected the total lipids to LC/ESI-MS. All major lipids assigned by TLC were confirmed, and the minor lipids were identified by tandem mass spectrometry (MS/MS) in conjunction with exact mass measurement. PG and PE were present as both the diacyl form and the plasmalogen with 14:0 as the predominant acyl and alk-1'-enyl chains with some 14:0/16:0 species (Figure 2, Table 2, and Supplementary material, Figs. 1 and 2). The spectra for PGp show a somewhat larger $M + 2$ signal at m/z 651.44 than the corresponding isotopomer seen for PG. This suggests the possibility of the presence of saturated ether forms. Whether there is some saturated ether or its possible role as an intermediate in plasmalogen biosynthesis, have not been investigated. Cardiolipin was present as the tetra-acyl, tri-acyl, alk-1'-enyl, and the diacyl, di-alk-1'-enyl forms which were observed at m/z 1239.84, 1223.75, and 1207.84, respectively (Fig. 3, Table 2).

Minor lipids tentatively identified by TLC were confirmed by MS/MS. Alanyl-PG, (Fig. 1A, spot 3) had similar mobility to alanyl-PG from *Clostridium perfringens* in the same solvent systems[17]. It too was present in diacyl and plasmalogen forms with two 14:0 chains (Table 2, Fig. 4A), and its identity was confirmed by MS/MS (Supplementary material, Fig. 3). Lysyl-PG, (Fig. 1A, spot 7) had similar mobility to a standard (Avanti Polar Lipids). It was predominantly present in both the diacyl and plasmalogen forms with two 14:0 chains (Table 2, Fig. 4B). MS/MS confirmed its identity (Supplementary material, Fig. 4).

Three of the minor lipids that serve as precursors to the major lipids were found to be present almost exclusively in the diacyl form. The mobility of phosphatidic acid (Fig. 1A, spot 9) in this 2D-TLC system was similar to that of a standard (Sigma Life Sciences). The $[\text{M-H}]^-$ ion of the predominant 14:0/14:0 species was detected at m/z 591.37, and a trace amount corresponding to the plasmalogen form was detected at m/z 575.41 (Fig. 5). Some phosphatidic acid 30:0 was also observed, with its $[\text{M-H}]^-$ ion at m/z 619.42. The lipid migrating as spot 8 on 2D-TLC (Fig. 1A) was ninhydrin- and molybdate-positive and was presumed to contain PS, based on the mobility of PS in this system. We observed that PS and PT eluted very close to one another upon normal phase liquid chromatography and they were present almost exclusively in the diacyl forms (Fig. 5B). On TLC they appear to separate partially after acid treatment (Fig. 1B, spot 14). The neutral losses of 87 and 101

amu in the MS/MS spectra are characteristic of the fragmentation of PS and PT ions, respectively (Supplementary material, Fig. 5). As expected from the predominance of the diacyl forms, neither Spot 8 nor phosphatidic acid (Spot 9) produced detectable hydrolysis products after HCl treatment on the TLC plate (Fig. 1B, spots 14 and 17). Predictably, CDP-diacylglycerol, a precursor to PS contained no detectable plasmalogen (Fig. 6A). Its identity was confirmed by MS/MS (Supplementary material, Fig. 6). A trace of PG-P, an intermediate between CDP-DAG and PG was also detected by MS, with its $[M-H]^-$ ion being observed at m/z 745.41 (Fig. 6B). There was no detectable signal for the plasmalogen form at m/z 729.4. Representative structures of plasmalogens identified in the polar lipid extract of *C. novyi* are shown in Fig. 7.

4. Discussion

As expected from the sequenced genome [3] which contains genes annotated as responsible for PE, PG and cardiolipin synthesis, these lipids were found in *C. novyi* NT (Tables 1 and 2). It is possible that the ratios of these lipids would change during the logarithmic phase of growth, and changes in environmental factors such as growth temperature, but this was not studied. All of these lipids were present in the all acyl and the plasmalogen form which contains an *O*-alk-1'-enyl ether linked chain.

Amino acyl-PG lipids are found in a number of Gram-positive species including *Staphylococcus aureus* and *Listeria monocytogenes* [18]. The genes encoding the enzymes that transfer the amino acid from an aminoacyl-tRNA have been designated *mprF* (multiple peptide resistance factor) which confers resistance to cationic peptides. Among clostridia, amino acyl PG had up to now been found only in *C. perfringens*, which has both alanyl-PG and lysyl-PG [17,19]. The amino acid sequence of MprF of *C. novyi* (YP_877589.1) is 45% identical and 65% positive compared to MprF1 of *C. perfringens* (YP_69880.1), which has been characterized as an alanyl transferase [18]. Protein blast of *C. perfringens* MprF2 (YP_69850), which has been characterized as a lysyl transferase [18], produces no closely related *C. novyi* protein. *C. novyi* YP_877589.1 is 33% identical and 55% positive to MprF2 of *C. perfringens*. Since both alanyl-PG and lysyl-PG are present in *C. novyi*, we tentatively conclude that the single amino acyl transferase is responsible for both of these lipids. This conclusion needs to be confirmed by expression of this protein in another organism such as *E. coli* which has no MprF activity [18].

A long-standing problem in lipid biochemistry concerns the anaerobic biosynthesis of plasmalogens. An oxygen-dependent pathway to plasmalogens in animal tissues was described about 40 years ago [20,21]. In this pathway saturated ether phosphoglycerides are formed initially with dihydroxyacetone phosphate and a long-chain alcohol as precursors. The resulting alkyldihydroxyacetone-P is converted to 1-*O*-alkyl-2-lyso-*sn*-glycerol-3-P and then to plasmanic acid by acylation at the *sn*-2 carbon. In the final step the saturated ether analog of PE is converted to the plasmalogen by an oxygen-dependent desaturase. Aside from the need for oxygen, this pathway was ruled out in anaerobes by isotopic experiments which showed that dihydroxyacetone phosphate could not be an intermediate in the plasmalogen pathway [22,23]. Earlier work in one of our laboratories provided evidence that plasmalogens in clostridia are synthesized from the corresponding diacyl phospholipids [7,9,24,25]. In the current study we have shown that the precursors of the diacyl-glycerophospholipids, phosphatidic acid, CDP-DAG and PS are present almost exclusively in the diacyl form, consistent with our earlier work. In studies on *Clostridium butyricum*, we showed that when PS decarboxylase was inhibited with hydroxylamine in cells labeled with $^{32}P_i$, label accumulated mainly in the diacyl form of PS, and when the block was released, label appeared first in PE and then in the plasmalogen form of PE [9]. The finding

of PS with very little plasmalogen in *C. novyi* is consistent with the hypothesis that diacyl PE is the precursor to the plasmalogen form.

This work combined with previous work cited above on the biosynthesis of plasmalogens in clostridia leads to a hypothetical pathway to plasmalogens in these anaerobes (Fig. 8). In this pathway the intermediates, phosphatidic acid, CDP-DAG, PGP and PS do not accumulate plasmalogens, but the end products do. Some intermediates, phosphatidic acid and PS in *C. novyi* only contain trace amounts of plasmalogen. Therefore, the mechanism of conversion of the diacyl phospholipids may apply to them, but their existence is so transient, that plasmalogen forms of these precursors do not accumulate. Alternatively, the trace of plasmalogens in PA and subsequent intermediates could arise from recycling of the fully formed plasmalogen end products by unknown mechanisms. We have previously shown that the PS decarboxylase of *C. butyricum* is approximately equally active on PS and the plasmalogen form of PS [26]. We have observed the presence of alk-1-enyl, acyl glycerol in addition to DAG by mass spectrometry (data not shown). Whether this lipid results from turnover of the plasmalogens or is involved in their formation is presently unknown. Previous studies on the formation of the neutral and phospholipids of *Clostridium beijerinckii* ATCC 6015 (formerly *C. butyricum*) after labeling with ¹⁴C-acetate indicated that there was no precursor-product relationship between the aldehydes of the neutral glycerol ethers and the aldehydes of the phospholipids [8]. The pathway shown in Fig. 8 is consistent with all the available information concerning clostridial plasmalogen formation. Further studies are needed to elucidate the molecular mechanisms of this enigmatic pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Drs. Shubin Zhou and Bert Vogelstein for the gift of *C. novyi* NT. The mass spectrometry facility in the Department of Biochemistry of the Duke University Medical Center and Z.G. are supported by the LIPID MAPS Large Scale Collaborative Grant number GM-069338 from NIH. S. A.-S. was supported by NIH R01 GM38237 and DOEER91-20053 to F.D. This work was supported by a research grant from the University of Pennsylvania Research Foundation.

Abbreviations

LC	liquid chromatography
MS	mass spectrometry
PA	phosphatidic acid
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PS	phosphatidylserine
PT	phosphatidylthreonine
TLC	thin layer chromatography

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A

Figure 1. Analysis by 2D-TLC of [^{14}C]-labeled lipids from *C. novyi* NT

Panel A. Polar lipids of *C. novyi* NT as visualized by the phosphoimager. Based on their positions and staining, the major lipids were tentatively identified as: 1. Cardiolipin; 2. PG; 3. alanyl-PG; 4. PE; 5. and 6. Unknown; 7. lysyl-PG; 8. PS and PT; 9. phosphatidic acid; X, unknown, probably not a lipid as it is not seen on charred plates. **Panel B.** The lipids were subjected to hydrolysis with HCl fumes between the first and second chromatographies, as described in Material and Methods. 1 and 2. Aldehydes from PG and cardiolipin plasmalogens; 3 and 4. aldehydes from PE plasmalogen; 5. cardiolipin; 6. lysocardiolipin from cardiolipin plasmalogen; 7. PG; 8. PE; 9. alanyl-PG; 10. lyso-PG from PG plasmalogen; 11. di-lyso-cardiolipin from cardiolipin plasmalogen; 12. unknown; 13. lyso-PE from PE plasmalogen; 14. PS and PT; 15. unknown; 16. lysyl-PG; 17. phosphatidic acid; 18. lyso-lysyl-PG from lysyl-PG plasmalogen; 19. aldehyde from lysyl-PG plasmalogen; X, unknown, probably not a lipid. The arrows indicate the first and second dimensions of the chromatograms. The material in the upper left hand corners is presumed to be neutral lipids.

Figure 2. LC/MS identification of PG and PE and their plasmalogens in *C. novyi* NT
 A) Mass spectrum showing the $[M-H]^-$ ions of PG and PG plasmalogen. B) Mass spectrum showing the $[M-H]^-$ ions of PE (PE) and PE plasmalogen (PEp). Each mass spectrum was averaged from the spectra acquired during the retention times indicated in the figures.

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Figure 4. LC/MS identification of alanyl-PG and lysyl-PG and their plasmalogens in *C. novyi* NT
 A) Mass spectrum showing the $[M-H]^-$ ions of of alanyl-PG and alanyl-PG plasmalogen (alanyl-PGp). B) Mass spectrum showing the $[M-H]^-$ ions of lysyl-PG and lysyl-PG plasmalogen (lysyl-PGp).

Figure 5. LC/MS identification of phosphatidic acid (PA), phosphatidylserine (PS) and phosphatidylthreonine (PT) in *C. novyi* NT

A) Mass spectrum showing the $[M-H]^-$ ions of PA species. A minor $[M-H]^-$ ion corresponding to the plasmalogen form of PA (PAp) was detected at m/z 575.41. B) Mass spectrum showing the $[M-H]^-$ ions of PS and PT species. Minor $[M-H]^-$ ions for the plasmalogen forms of PS (PSP) and PT (PTp) were detected at m/z 662.44 and 676.43, respectively.

Figure 6. LC/MS identification of CDP-diradylglycerol (CDP-DAG) and PG-P (PGP) in *C. novyi* NT

A) Mass spectrum showing the $[M-H]^-$ ion of CDP-DAG at m/z 896.45. B) Mass spectrum showing the $[M-H]^-$ ion of PG-P at m/z 745.41.

Representative Structures of Major Plasmalogens in *C. novyi*

Figure 7. Representative structures of major plasmalogens in *C. novyi* NT

The abbreviations for the plasmalogens are: PGp, plasmenylglycerol; PEp, plasmenylethanolamine; alanyl-PGp, the plasmalogen form of alanyl-PG; lysyl-PGp, the plasmalogen form of lysyl-PG, and CLp, plasmalogen form of cardiolipin with a single alk-1-enyl chain.

Figure 8. Proposed pathway for the formation of plasmalogens in clostridia

To simplify the figure, the formation of the minor lipids, alanyl-PG and lysyl-PG and their plasmalogens is not shown. For abbreviations, see legend for figure 7.

Table 1

Polar lipid composition of *Clostridium novyi* NT as judged by radiolabeling.

	PtdEtn	PtdGro	Cardiolipin	Ala-PtdGro	Lysyl-PtdGro	PtdSer	Phosphatidic acid
Percent of polar lipid ¹⁴ C ^a	54.0 ± 2.9	3.74 ± 0.30	31.6 ± 2.6	0.88 ± 0.11	2.26 ± 0.48	2.61 ± 0.61	3.59 ± 0.17
Plasmalogen, % of each species ^b	25	43 ^c		n.d. ^d	n.d. ^d	trace	trace

^aMean % ¹⁴C, n = 2

^bPercent diacyl PtdEtn was calculated using the formula: % diacylPtdEtn = % (of total cpm) in diacylPtdEtn after acid hydrolysis/Σ % diacylPtdEtn + %LysoPtdEtn + % aldehydes. A similar formula was used for the other lipids. The % plasmalogen = 100 – % diacyl lipid. This analysis was done twice.

^cAlthough these lipids are present in both the diacyl and plasmalogen forms as determined by mass spectrometry (see Fig. 4), the percent plasmalogen could not be determined by TLC separation of the products because of the small amounts of labeled lipids. Since they are derived from PtdGro, we would expect there to be similar amounts of plasmalogen as seen in PtdGro and cardiolipin. The results of the MS analysis support this, but since we do not know that the relative ionization efficiencies of the diacyl forms and the plasmalogens, we cannot determine the exact ratios.

^dPresent, but amount was not determined (see text).

Table 2

Molecular species of *C. novyi* polar lipids detected by normal phase LC- ESI/MS operated in the negative ion mode.

	Major polar lipids, acyl forms	Plasmalogen Forms
Major molecular species	[M – H] ^{-a}	[M – H] ^{-a}
PtdEtn, 14:0/14:0	634.40	618.40
PtdGro, 14:0/14:1	663.43	
PtdGro, 14:0/14:0	665.42	649.43
PtdGro, 14:0/16:0	693.45	677.46
Cardiolipin, 56:0	1239.84	1223.75, 1207.84 ^b
Lysyl-PtdGro, 14:0/14:0	793.52	777.55
Alanyl-PtdGro, 14:0/14:0	736.43	720.45
PtdSer, 14:0/14:0	678.38	Trace (662.44)
PtdThr, 14:0/14:0	692.40	Trace (676.41)
Phosphatidic acid, 14:0/14:0	591.37	Trace (575.40)
CDP-Diradylglycerol, 14:0/14:0	896.46	880.47

^a The species listed in Table 2 were identified by tandem mass spectrometry in conjunction with exact mass measurement. For examples, the collision-induced dissociation mass spectra of PtdEtn (28:0) with m/z 634.40 and the plasmalogen form with m/z 618.40 are shown in Supplementary material Fig. 2. The presence of C14:0 fatty acyl chains is supported by its carboxylic anion (m/z 227.19) in the MS/MS spectra.

^b Dialkenyl species