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New cardiolipin analogs synthesized by phospholipase D-catalyzed transphosphatidylation

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ABSTRACT

Cardiolipin (CL) and related diphosphatidyl lipids are hardly accessible because of the complexity of their chemical synthesis. In the present paper, the transphosphatidylation reaction catalyzed by phospholipase D (PLD) from *Streptomyces* sp. has been proven as an alternative enzyme-assisted strategy for the synthesis of new CL analogs. The formation of this type of compounds from phosphatidylcholine was compared for a series of N- and C2-substituted ethanolamine derivatives as well as non-charged alcohols such as glycerol and ethylene glycol. The rapid exchange of the choline head group by ethanolamine derivatives having a low molecular volume (diethanolamine and serinol) gave rise to an efficient production of the corresponding CL analogs. In contrast, the yields were comparably low in the reaction with bulky nitrogenous acceptor alcohols (triethanolamine, tris(hydroxymethyl)aminomethane, tetrakis(hydroxyethyl)ammonium) or the non-charged alcohols. Therefore, a strong dependence of the conversion of the monophosphatidyl to the diphosphatidyl compound on steric parameters and the head group charge was concluded. The enzyme-assisted strategy was used for the preparation of purified diphosphatidyldiethanolamine and diphosphatidylserinol.

1. Introduction

Unlike the common membrane phospholipids, cardiolipin (CL) contains two phosphatidyl residues linked to the external hydroxyl groups of glycerol. This dimeric phospholipid specifically occurs in the membranes of bacteria and mitochondria harboring the respiratory complexes. Therefore, the role of CL in membrane organization and cellular energy metabolism has attracted much attention (Schlame, 2008). For example, this unique phospholipid is involved in the maintenance of the characteristic shape of the inner membrane (Khalifat et al., 2008). CL is crucial for the stabilization of the complexes of the respiratory chain (Arias-Cartin et al., 2012) and of mitochondrial carriers (Klingenberg, 2009). Besides its structural function, CL serves as a reservoir for the electrochemical gradient due to protonation of its head group at low pH (Haines and Dencher, 2002) and thus contributes to proton conduction (Lange et al., 2001). Additionally, it is involved in cell signaling related to oxidative stress (Gohil and Greenberg, 2009) and apoptosis (Wright et al., 2004; Joshi et al., 2009).

Because of its special structural features, the physicochemical properties of CL significantly differ from the corresponding monophosphatidyl compounds (Lewis and McElhaney, 2009). Since those properties are beneficial for the stabilization of lipid vesicles, CLs and structurally similar anionic compounds are of great interest for the preparation of liposomes used for DNA packing (Resina et al., 2009) and HIV prophylaxis (Malavia et al., 2011). Positively charged CL analogs, which mediate cellular nucleic acid uptake are attractive as components of liposome-based gene-delivery systems (Zhang et al., 2006).

However, the chemical synthesis of CL or structurally related compounds is complicated and involves various reaction steps (Paltauf and Hermetter, 1994). Phospholipase D (PLD)-catalyzed transesterification of the better accessible glycerophospholipids seems to be a useful alternative strategy to produce CL compounds. PLD is a well-known biocatalyst due to its capability to

Abbreviations: br, broad; dd, doubled doublet; dm, doubled multiplet; CL, cardiolipin; HPLC, high performance liquid chromatography; COSY, homonuclear correlation spectroscopy; DSS, 4,4-dimethyl-4-silapentane sodium sulfonate; FT-ICR, Fourier transform ion cyclotron resonance; HPTLC, high performance thin layer chromatography; m, multiplet; PA, phosphatidic acid; PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; PE, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine; PG, phosphatidylglycerol; PLD, phospholipase D; q, quartet; t, triplet; THA, tetrakis(2-hydroxyethyl)ammonium bromide; TOCSY, total correlation spectroscopy; Tris, tris(hydroxymethyl)aminomethane.

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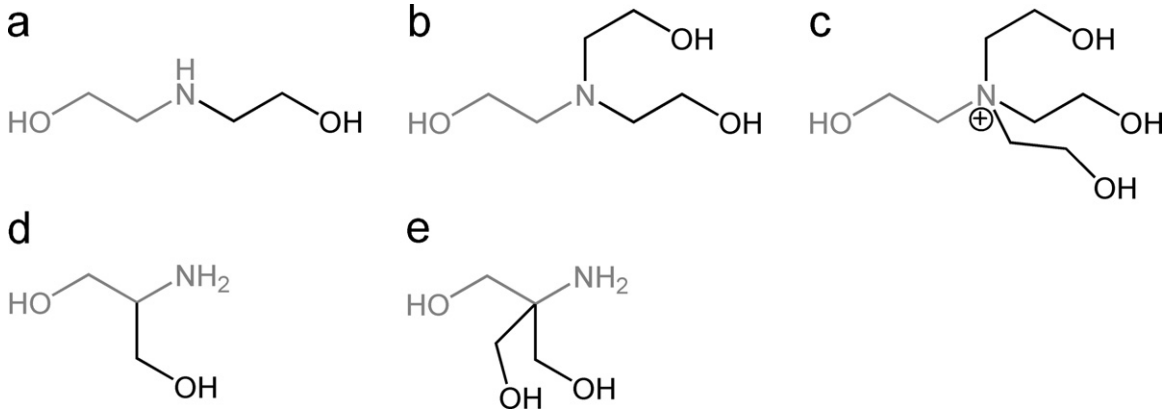

Fig. 1. Ethanolamine derivatives assayed for the enzymatic production of CL analogs. The ethanolamine moiety of the N-substituted derivatives diethanolamine (a), triethanolamine (b) and THA (c) and the C2-substituted derivatives serinol (d) and Tris (e) are drawn in gray.

exert a head group exchange reaction called transphosphatidylation. In phospholipid synthesis, especially bacterial PLDs such as the enzyme from *Streptomyces* sp. are used because they strongly favor transphosphatidylation of the acceptor alcohol over the undesired hydrolytic cleavage of the phospholipid substrate (Ulbrich-Hofmann et al., 2005). In contrast to other PLDs, this enzyme also mediates the conversion of phosphatidylglycerol (PG) to CL (D'Arrigo et al., 1996; Piazza and Marmer, 2007).

Interestingly, a similar reaction was observed in the enzymatic synthesis of phosphatidylethanolamine derivatives with multiple hydroxylated head groups (Dippe et al., 2008) leading to the formation of side products which were identified as dimeric phospholipids with a positively charged hydrophilic linker. In the present paper, we evaluate the applicability of this reaction for the synthesis of dimeric phospholipids from phosphatidylcholine and a series of N- and C2-substituted derivatives of ethanolamine or non-nitrogenous acceptors (glycerol and ethylene glycol). The reactions were compared with respect to chemical structure and charge of the used acceptor alcohols. Two of the CL analogs (diphosphatidylserinol and diphosphatidyldiethanolamine) are shown to be accessible in preparative scale.

2. Materials and methods

2.1. Materials

1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (PC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-*rac*-glycerol, 1,2-dioleoyl-*sn*-glycero-3-phospho-*rac*-glycerol, CL from bovine heart, PLD from *Streptomyces* sp. (Type VII), and 2-bromoethanol were products from Sigma (USA). The phosphatidic acids (PAs) 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate and 1,2-dioleoyl-*sn*-glycero-3-phosphate were from Avanti Polar (USA), and phosphatidyl-4-nitrophenol was synthesized from egg yolk phosphatidylcholine according to D'Arrigo et al. (1995). 1,2-Dioleoyl-*sn*-glycero-3-phosphoethylene glycol was produced by transphosphatidylation of the corresponding phosphatidylcholine by recombinant PLD from white cabbage according to the procedure described by Dippe et al. (2008) Its molecular mass was checked as described in Section 2.2.5. Ethylene glycol, water-free glycerol, tris(hydroxymethyl)aminomethane (Tris) and serinol were from Merck, Germany. All other reagents and solvents were purchased from Roth, Germany.

2.2. Methods

2.2.1. Determination of PLD activity

PLD activity was assayed in adaption to D'Arrigo et al. (1995) toward a micellar solution of the substrate phosphatidyl-4-nitrophenol (0.42 mM) in 0.21 mM sodium dodecyl sulfate, 3.3 mM Triton X-100, 120 mM $CaCl_2$ and 300 mM acetate buffer, pH 5.6. One unit (U) of enzyme activity represents the amount of enzyme releasing 1 μmol of 4-nitrophenol per minute.

2.2.2. Synthesis of tetrakis(2-hydroxyethyl)ammonium bromide (THA)

48 mmol of triethanolamine and 2-bromoethanol, respectively, were dissolved in 15 ml of nitromethane and heated under reflux at 95 °C for 10 h. Upon cooling, white crystals of THA were obtained, which were collected by filtration and washed with acetone. The crude product was re-crystallized from methanol/acetone/water (15:10:1, v/v/v) and dried in vacuum in the presence of P_4O_{10}, yielding 1.20 g (4.4 mmol) of pure THA. ^{1}H NMR (according to Section 2.2.6, in D_2O-DSS-external): δ 3.41 ppm (t, 8H, —CH_2—NR_3^+); δ 3.89 ppm (t, 8H, —CH_2—OH).

2.2.3. Kinetic measurements

PLD-catalyzed reactions were performed in an aqueous-organic two-phase system according to Hirche and Ulbrich-Hofmann (2000). 620 μl of substrate solution (1.24 μmol PC, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol or 1,2-dioleoyl-*sn*-glycero-3-phosphoethylene glycol in diethyl ether) were mixed with 80 μl of enzyme solution (61.6 mU PLD in 300 mM sodium acetate buffer, 120 mM $CaCl_2$, pH 5.6). In the reactions containing PC, 96 μmol of acceptor alcohol was added to the aqueous phase. Before usage, stock solutions of amino alcohols were adjusted to pH 5.6 by addition of HCl. Reaction mixtures were shaken (400 rpm) in HPTLC screw flasks (Roth, Germany) at 30 °C. After a distinct reaction time (0–17 h), samples (25 μl) were taken from the organic phase which exclusively contained the phospholipids. The solvent was removed by air-drying and the residuals were re-dissolved in 25 μl of toluene, applied to silica 60 plates (Merck, Germany) and analyzed by HPTLC according to Dippe et al. (2008). After staining of the phospholipids with $CuSO_4/H_3PO_4$ (Touchstone et al., 1983), the resulting bands were evaluated by densitometry (densitometer CD60, Desaga, Germany). The concentrations of the individual phospholipids, which were identified according to their R_f values (Dippe et al., 2008), were calculated using standards of the corresponding PC, PA, PG, PE (for phospholipids having