Incorporation of serine into Paramecium ethanolamine phosphonolipid and phosphonolipid head groups

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Ethanolamine phospholipid head groups in Paramecium were synthesized directly from ethanolamine. As in other cell types, radioactivity from ethanolamine failed to incorporate significantly into head groups of ethanolamine phosphonolipids, indicating that the phosphonolipids are not derived from their phospholipid analogues. Unlike other systems previously examined, radioactivity from serine is incorporated into both ethanolamine phospholipid and phosphonolipid head groups of glycerolipids and sphingolipids in this ciliate. These observations suggest that synthesis of ethanolamine phosphonolipids involves synthesis de novo of free phosphonoserine, which is then incorporated into lipids, and then lipid-bound phosphonoserine intermediates (glycerolipids or sphingolipids) undergo decarboxylation, forming lipid-bound phosphonoethanolamine compounds.

Phosphonolipids have been isolated from various invertebrates, protozoa and mycobacteria (Kittredge & Roberts, 1969; Rosenberg, 1973; Hori & Nozawa, 1982). Formation of free aminoethylphosphonate (phosphonoethylamine, AEP) involves the decarboxylation and amination of phosphoenolpyruvate (Rosenberg, 1973; Hori & Nozawa, 1982).

Although free AEP was found in the ciliate Tetrahymena, it was demonstrated that this was not a major precursor of lipid-bound AEP since it was synthesized after lipid-bound AEP (Liang & Rosenberg, 1968; Rosenberg, 1973; Hori & Nozawa, 1982). The 'Kennedy pathway', in which free CMP–AEP and diacylglycerol participate in an exchange reaction, was considered a salvage pathway (Rosenberg, 1973; Hori & Nozawa, 1982). Re-arrangement of phosphoenolpyruvate to phosphonopyruvate, as CMP–AEP, or as phosphatidylphosphonopyruvate, was suggested as the major step de novo to lipid-bound 2-amino-3-phosphonopropanate (alanine-3-phosphonate, 3-phosphonoalanine. phosphonoserine, APnP) followed by the formation of ethanolamine phosphonolipids by decarboxylation (Rosenberg, 1973). Smith & Law (1970) showed that APnP was incorporated into Tetrahymena phosphonolipids more efficiently than was AEP. These workers, however, concluded that APnP was converted into AEP as the free phosphate. Formation of ethanolamine phosphonolipids by a mechanism involving decarboxylation of a lipid-bound APnP intermediate has been dismissed by a number of investigators because the lipid intermediate has not been isolated, phosphatidylserine was not detected in Tetrahymena, and incorporation of serine into ethanolamine phosphonolipids was not demonstrated (Warren, 1968; Rosenberg, 1973). No unified scheme for explaining the formation of phosphonolipids that receives general acceptance has yet been postulated (Hori & Nozawa, 1982). The processes leading to the formation of phosphonyl bonds in sphingolipids are totally unknown (Hori & Nozawa, 1982). In the present paper we describe metabolic radiolabelling studies with Paramecium that implicate the existence of previously hypothesized serine pathways for the synthesis of ethanolamine phosphonolipid head groups (Rosenberg, 1973). This ciliate has three ethanolamine sphingophospholipids and three ethanolamine sphingophosphonolipids, in addition to the ethanolamine glycerocephospholipids and glycerocephosphonolipids (Rhoads & Kaneshiro, 1979; Andrews & Nelson, 1979).

Experimental

Paramecium tetraurelia 51s was grown in an axenic medium in the presence of 0.02–3.0 μCi of radiolabelled precursor/ml as previously described (Rhoads & Kaneshiro, 1979; Kaneshiro et al.,
1979). Unless otherwise indicated, all radiolabelled compounds were added at day 3 or 4 of culture growth, and cells were extracted for lipids on day 5 (late exponential phase). Lipids were extracted, purified and fractionated by TEAE- (triethylaminoethyliccellose column chromatography as previously described (Rhoads & Kaneshiro, 1979; Kaneshiro et al., 1979). Individual sphingolipids and glycerolipids in the ethanolamine lipid fraction eluted from TEAE-cellulose columns were separated by two-dimensional t.l.c. on 0.25mm-thick pre-coated Silica Gel 60 plates (E. Merck, Darmstadt, Germany) with the solvent systems previously described (Rhoads & Kaneshiro, 1979).

Fatty acids and water-soluble products from glycerolipids were isolated after hydrolysis under alkaline conditions (Rhoads & Kaneshiro, 1979) or phospholipase C (from Bacillus cereus; Sigma Chemical Co., St. Louis, MO, U.S.A.) digestion (Kaneshiro, 1980). Fatty acids, long-chain bases and water-soluble groups were isolated from sphingolipids after acid hydrolysis (Carter & Gaver, 1967) or phospholipase C digestion.

Lipids were radiolabelled in vitro with 0.6µCi of phosphoenol[1-14C]pyruvic acid/ml and a crude sonicated Paramecium whole-cell preparation. Cells were centrifuged as described previously (Rhoads & Kaneshiro, 1979; Kaneshiro et al., 1979) and washed in a solution containing 150mm sucrose and 20mm-Tris/HCl buffer, pH 7.4. A cell suspension of 106 cells in 8ml of buffer solution containing either 10mm-CaCl2 or 10mm-MgCl2 was maintained at 4°C. The cells were sonicated with three 10s pulses. The radiolabelled compound was added to this sonicated whole-cell preparation and the reaction was left to proceed at room temperature for 30min. The reaction was terminated by the extraction of lipids with 30ml of chloroform/methanol (1:2, v/v).

Results

Incubation of Paramecium cells with 0.7–1.0µCi of H332PO4/ml for 5 days under isotope-equilibrium conditions resulted in the distribution of radioactivity in lipids that reflected the quantity of the various phospholipid classes present in the cell (Rhoads & Kaneshiro, 1979). However, when cells were grown with 0.6–3.0µCi of H332PO4/ml for shorter periods, the rate of 32P incorporation into phospholipids was different from that of the incorporation into phospholipids. Ethanolamine phospholipids from cells grown for 9h (at day 5) with the [32P]phosphate were more radioactive than the ethanolamine phospholipids. The relative specific radioactivities (% of radioactivity of ethanolamine lipid fraction% of mass of ethanolamine lipid fraction±s.e.m., n=9) were: N-acetyl-trans-4-hydroxyxphipanogamine-1-phosphonothanolamine (N-acetylphosphadinosine-1-phosphonoethanolamine, PPnE), 0.58±0.08; N-acetylsphingosine-1-phosphonoethanolamine (N-acetylsphingosine-1-phosphonoethanolamine, SPnE), 0.89±0.11; N-acetylsphingosine-1-phosphonoethanolamine (N-acetyldihydrophosphadinosine-1-phosphonoethanolamine, DPnE), 4.47±0.82; 1,2-dia-
cyl-sn-glycerol-3-(2-aminoethyl)phosphonate and 1-
alkyl-2-acyl-sn-glycerol-3-(2-aminoethyl)phosphonate (PnE), 0.86±0.04; N-acetyl-trans-4-hydroxyxphipanogamine-1-phosphonoethanolamine (N-acetylphosphadinosine-1-phosphonoethanolamine, PPsE), 0.99±0.20; N-acetylsphingosine-1-phosphonoethanolamine (N-acetylsphingosine-1-phosphonoethanolamine, SPsE), 1.66±0.45; N-acetylsphingosine-1-phosphonoethanolamine (N-acetyldihydrophosphadinosine-1-phosphonoethanolamine, DPsE), 8.68±2.68; 1,2-dia-
cyl-sn-glycerol-3-(2-aminoethyl) phosphate and 1-
alkyl-2-acyl-sn-glycerol-3-(2-aminoethyl) phosphate (PsE), 1.09±0.03. Radioactivity appeared faster in head groups of phospholipids compared with the respective phospholipid analogues. Furthermore, in these relatively short exposures to H332PO4 in vitro, the relative specific radioactivities of sphingolipids were greatest for those containing the long-chain base, dihydro-
sphingosine > sphingosine > phytosphingosine.

Over 75% of the radioactivity from [2-14C]ethanol-1-ol-2-amine hydrochloride (0.2µCi/ml for 24h) that was incorporated into whole-cell lipids was associated with the ethanolamine phospholipids. Of the fraction isolated by TEAE-cellulose column chromatography, 0.4% of the label was in the neutral lipids and free ceramides, 12% was in the choline lipid fraction and 20% was associated with the serine lipids (n=2). [14C]Ethanolamine was incorporated readily into both ethanolamine sphingophospholipids and glycerophospholipids and only slightly into the phospholipid analogues (Table 1). These results were confirmed by experiments performed in vitro (results not shown). This suggests that the head group of ethanolamine glycerophospholipids and sphingophospholipids in Paramecium can be synthesized de novo by direct incorporation of ethanolamine.

Phosphoenol[1-14C]pyruvic acid (0.4µCi/ml of culture for 48h) that was tested as a precursor of phospholipids did not label cellular lipids, probably owing to decarboxylation of phosphoenol[1-14C]pyruvic acid, although 10% of the label was taken up by the cells after 24h. However, when crude sonicated whole-cell preparations were used for assays of incorporation in vitro radioactivity from phosphoenol[14C]pyruvate (0.6µCi/ml of incubation mixture) was incorporated into the neutral (14%), choline (21%), ethanolamine (53%) and minor (12%) lipid frac-

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Table 1. Incorporation of radiolabelled precursors into water-soluble head-group fractions of Paramecium ethanolamine lipids

Radiolabelled compounds were added to cultures for 1–2 days (day 3 or 4 to day 5 of culture growth) in the studies performed in vivo, and in experiments performed in vitro phosphoenol[14C]pyruvate was added to the incubation mixtures for 30 min at room temperature. Water-soluble products after phospholipase C, acid (sphingolipids) or alkaline (glycerolipids) hydrolysis were isolated and the radioactivities determined. For experimental details see the text. Polar head groups are water-soluble products from sphingolipids; polar head groups and glycerol backbones are water-soluble products from glycerolipids. Relative specific radioactivities (% of radioactivity of the total ethanolamine lipid fraction/ % of mass of the total ethanolamine lipid fraction) are given in square brackets. The composition of the ethanolamine lipid fraction at day 5 was 4.9% sphingophosphonolipids, 1.6% sphingophospholipids, 34.2% PnE and 59.3% PsE (n = 9). For definition of abbreviations see the text.

<table>
<thead>
<tr>
<th>Incorporation in vivo</th>
<th>Sphingophosphonolipids</th>
<th>Sphingophospholipids</th>
<th>PnE</th>
<th>PsE</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2,14C]Ethan-1-ol-2-amine hydrochloride (in vivo; n = 3)</td>
<td>Trace [-]</td>
<td>10.3 ± 3.3 [2.10]</td>
<td>20.6, 28.3 [4.99]</td>
<td>9.6, 15.1</td>
</tr>
<tr>
<td>L-[U-14C]Serine (in vivo; n = 3)</td>
<td>5.0 ± 2.2 [3.13]</td>
<td>6.3 ± 1.8 [3.94]</td>
<td>0.0, 0.0 [-]</td>
<td>36.4 ± 6.3</td>
</tr>
<tr>
<td>PsE</td>
<td>1.5 ± 1.0 [0.04]</td>
<td>19.7 ± 4.8 [0.58]</td>
<td>0.0</td>
<td>42.9 ± 2.5</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>92.4 ± 5.8 [1.56]</td>
<td>64.5 ± 5.7 [1.09]</td>
<td>57.2 ± 5.5</td>
<td>70.0, 58.1</td>
</tr>
</tbody>
</table>

Table 2. Incorporation of radiolabelled precursors into Paramecium sphingolipid moieties

Sphingolipid moieties were isolated after phospholipase C digestion or acid hydrolysis, and the radioactivities were determined. For experimental details see the text.

<table>
<thead>
<tr>
<th>Incorporation in vivo</th>
<th>Sphingolipid fraction</th>
<th>Fatty acid</th>
<th>Long-chain base</th>
<th>Polar head group</th>
</tr>
</thead>
<tbody>
<tr>
<td>[2-14C]Ethan-1-ol-2-amine hydrochloride (3)</td>
<td>Total</td>
<td>0.0, 0.0</td>
<td>0.0, 0.0</td>
<td>100, 100</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.0, 0.0</td>
<td>0.0, 0.0</td>
<td>93.6, 96.1</td>
<td></td>
</tr>
<tr>
<td>Phosphonolipids</td>
<td>0.0, 0.0</td>
<td>0.0, 0.0</td>
<td>3.1, 7.5</td>
<td></td>
</tr>
<tr>
<td>L-[U-14C]Serine (3)</td>
<td>Total</td>
<td>Trace</td>
<td>43.7 ± 5.0</td>
<td>57.3 ± 7.1</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>0.0</td>
<td>36.4 ± 6.3</td>
<td>63.6 ± 2.8</td>
<td></td>
</tr>
<tr>
<td>Phosphonolipids</td>
<td>Trace</td>
<td>42.9 ± 2.5</td>
<td>57.2 ± 5.5</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Incorporation in vitro</th>
<th>Sphingolipid fraction</th>
<th>Fatty acid</th>
<th>Long-chain base</th>
<th>Polar head group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphoenol[1-14C]pyruvic acid (2)</td>
<td>Total*</td>
<td>9.6, 15.1</td>
<td>20.1, 24.5</td>
<td>70.0, 58.1</td>
</tr>
</tbody>
</table>

* Radioactivity was not detected in sphingophospholipids, and therefore values represent radioactivities in sphingophospholipids.

Radioactivity was detected in the water-soluble fraction of hydrolysed sphingolipids (Table 2), and all label in the ethanolamine lipid fraction was associated with the phospholipids (Table 1). In these experiments performed in vitro the incorporation of radiolabel from phosphoenol-[14C]pyruvate into lipids in the presence of Mg2+ was only 20–25% of that obtained with Ca2+.

Radiolabel from L-[U-14C]serine (0.02–0.05 μCi/ml of culture for 1–2 days) was incorporated into all lipid fractions; 5% was associated with neutral lipids and free ceramides, 30% with choline lipids, 52% with ethanolamine lipids and 18% with serine lipids (n = 5). Furthermore, radioactivity from [14C]serine was incorporated into PsE, PnE and all six ethanolamine sphingolipids (Fig. 1). Incorporation of label from [14C]serine into long-chain bases of all sphingolipids is consistent with the known initial condensation reaction of serine and palmitoyl-CoA, forming 3-dehydrophosphinganine (Stoffel, 1977). More than half of the radioactivity in all sphingolipids was in the water-soluble fraction isolated after acid hydrolysis or phospholipase C digestion (Table 2). Radiolabel from [14C]serine was also readily detected in the water-soluble products of the glycerolipids, PsE and PnE (Table 1). It was considered unlikely that 14CO2 derived from decarboxylation of [U-14C]serine was subsequently incorporated into glucose and glycerol and thereby labelled the water-soluble products of glycerolipids. In other studies, 14CO2 was not detected when Paramecium cells were grown with 0.034 μCi of [U-14C]serine/ml for 48 h of expo-
ternal growth (E. S. Kaneshiro, S. F. Reuter & D. F. Matesic, unpublished work). The results of the present studies on incorporation in vivo of radiolabel from $[^{14}\text{C}]$serine into ethanolamine lipids of Paramecium indicate that serine was a precursor of ethanolamine glycerophospholipid, glycerophospholipid, sphingophospholipid and sphingophospholipid head groups.

Discussion

Biosynthesis of nitrogen bases, or polar head groups, of ethanolamine glycerolipids and sphingolipids in Paramecium involves a de novo pathway in which ethanolamine is directly utilized for the formation of phospholipids (Scheme 1). Only small amounts of label from ethanolamine were detected in ethanolamine phospholipid fractions, which was similar to observations on Tetrahymena (Thompson, 1969), indicating that phosphonolipids are not directly derived from ethanolamine phospholipids and that synthesis of the P–C bond involves slower or more reaction steps than does the addition of phosphorus to phospholipid head groups. The observation that $^{32}\text{P}$ incorporation into ethanolamine phospholipids appeared earlier than into the phosphonolipids is in agreement with this conclusion. The observations that the relative specific radioactivities were highest in sphingolipids containing dihydro- sphingosine and lowest in those containing phytosphingosine during the 9th $^{32}\text{P}$-incorporation studies suggest that (1) the head groups, if not the entire molecules, of dihydrophingosine-containing sphingolipids have turnover rates greater than those of sphingolipids containing sphingosine, which in turn are greater than those of sphingolipids containing phytosphingosine, or (2) long-chain base moieties can be modified after dihydrophingosine is incorporated into phospholipids and that the conversion of the long-chain base moiety in Paramecium is sequential from dihydrophingosine to sphingosine to phytosphingosine.

The possibility of synthesis of phosphonolipids via alternative phosphoenolpyruvate pathways in Paramecium, either by CMP-phosphoenolpyruvate or through lipid-bound APhN intermediates (Scheme 1), was suggested by experiments performed in vitro. We are presently unable to conclude whether or not these are major or minor pathways, or that both of these pathways are present in this organism. Furthermore, the results with phosphoenol$[^{14}\text{C}]$pyruvate indicate that biosynthetic reactions leading from phosphoenolpyruvate to the formation of phosphonolipids ‘may be more complex than originally envisaged’ (Rosenberg, 1973). The inability to detect incorporation of radiolabel from phosphoenol$[^{14}\text{C}]$pyruvate into phosphonolipids in experiments performed in vitro is interpreted as either the result of a loss of radiolabel from the C-1 position and/or strict cellular compartmentalization of pools that do not exchange. This interpretation is supported by other studies of lipid metabolism in Paramecium, e.g. radiolabel from exogenously supplied $[^{14}\text{C}]$acetate, $[^{14}\text{C}]$malonate, $[^{14}\text{C}]$pyruvate and $[^{14}\text{C}]$glucose was not incorporated into fatty acids of the cell (Rhoads & Kaneshiro, 1980). The presence of enzymes for fatty acid synthesis de novo was demonstrated by experiments performed in vitro with acetyl-CoA and malonyl-CoA.

The results of the present study provide evidence that pathways for synthesis de novo from serine to ethanolamine phospholipids as well as phosphonolipids occur in Paramecium. One pathway, as proposed by Dennis & Kennedy (1970) for Tetrahymena, is the direct incorporation of serine into phosphatidylserine followed by decarboxylation to phosphatidylethanolamine (Scheme 1). The incorporation of label from $[^{14}\text{C}]$serine into ethanolamine, choline and serine lipids indicated that pathways for synthesis de novo from serine are
Paramecium ethanolamine phospholipids and phosphonolipids

important in phospholipid synthesis in Paramecium. Recovery of label from water-soluble headgroup fractions from ethanolamine glycerolipids and sphingolipids further suggested the presence of these pathways. Clearly, Paramecium differs from Tetrahymena, and other cell types examined (Warren, 1968; Rosenberg, 1973) in that label from [14C]serine was efficiently incorporated into ethanolamine phosphonolipid head groups of this ciliate. The biosynthetic pathways leading to phosphonolipids in Paramecium appear to be similar for both glycerolipids and sphingolipids.

Our results support the hypothesis that major pathways in ethanolamine phospholipid and phosphonolipid metabolism in Paramecium involve serine lipids.

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References


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Scheme 1. Proposed pathways for the biosynthesis of ethanolamine lipid head groups in Paramecium (→)

Pathway 1 is the de novo pathway known for the synthesis of PsE from ethanolamine. Paramecium ethanolamine glycerophospholipids and sphingolipidphospholipids can be synthesized by this pathway. Pathways 2 and 3 are those that have been proposed for the synthesis of Tetrahymena glycerophospholipids from phosphoenolpyruvate (PEP). One or both exist in Paramecium. Pathways 4 and 5 involve serine and include the decarboxylation of phosphatidylserine to PsE, as proposed for Tetrahymena. The proposed serine pathways leading to the formation of ethanolamine glycerophosphonolipids and sphingophosphonolipids involve the lipid-bound intermediates that contain ApnP. For definition of abbreviations see the text.