Formation of Ceramide Phosphorylethanolamine from Phosphatidylethanolamine in the Rumen Protozoon *Entodinium caudatum*

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From 'pulse'-labelling experiments of *Entodinium caudatum* with [^{14}C]ethanolamine and by incubating the organism with [^{32}P]phosphatidylethanolamine it is concluded that phosphatidylethanolamine can act as a direct precursor of the phosphorylethanolamine moiety of ceramide phosphorylethanolamine. The phosphorylethanolamine is probably never liberated in the free form but is transferred directly to a ceramide or ceramide-containing acceptor. The results are also in agreement with previous conclusions that phosphatidylethanolamine is the direct lipid precursor of *N*-(1-carboxyethyl)phosphatidylethanolamine.

The ethanolamine analogue of sphingomyelin, namely ceramide phosphorylethanolamine, has been reported in the rumen protozoon *Entodinium caudatum* (Dawson & Kemp, 1967), in certain marine invertebrates (Hori *et al.*, 1968), in the blowfly *Calliphora erythrocephala* (Dawson & Kemp, 1968) and housefly *Musca domestica* (Hildenbrandt *et al.*, 1971) and in some anaerobic bacteria (Dawson & Kemp, 1969; Labach & White, 1969). Often it is found associated with the phosphonolipid ceramide aminoethylphosphonate, which is of similar structure except that the oxygen atom is missing in the bonding between the ethanolamine and phosphate moieties. Nothing is known about the biosynthesis of ceramide phosphorylethanolamine, although by analogy with the accepted scheme for the synthesis of sphingomyelin in animal tissues it might be expected that phosphorylethanolamine would be synthesized *de novo*, converted into CDP-ethanolamine and transferred to a ceramide acceptor. The following experiments, however, indicate that phosphatidylethanolamine may act as a direct donor of the phosphorylethanolamine moiety of ceramide phosphorylethanolamine.

Materials and methods

E. caudatum cultures were obtained from Dr. G. S. Coleman and maintained as described by him (Coleman, 1962). They contained a flourishing growth of bacteria, the ingestion of which appears to be essential for the viability of the culture. The protozoa were recovered by centrifuging at 1000g for 2 min followed by washing in a protozoal culture buffer (Coleman, 1962). After suspension in protozoal buffer solution (approx. 10^7 cells), either [$2\text{-}^{14}\text{C}$]ethanolamine (final volume 3 ml) (The Radiochemical Centre, Amersham, Bucks., U.K.) or [^{32}P]phosphatidylethanolamine (final volume 2 ml) was added and the incubation at

39°C was commenced under $\text{N}_2 + \text{CO}_2$ (95:5). The [^{32}P]phosphatidylethanolamine was isolated from *Saccharomyces cerevisiae* grown in a medium in which [^{32}P]phosphate was limiting (Dawson & Bangham, 1959). In 'pulse-chase' experiments with [^{14}C]ethanolamine, after a labelling incubation, the cells were washed thrice with protozoal buffer and resuspended in 3 ml of buffer to which had been added 0.1 mg of non-radioactive ethanolamine/ml (i.e. 300-fold excess) for further incubation. After incubation the cells were spun down and washed thrice with 5 ml of buffer solution. They were treated with 5 ml of chloroform-methanol (2:1, v/v) for 2 h at room temperature, and the suspension was then heated at 60°C for 10 min. After centrifuging, the extract was shaken with 1 ml of 0.9% NaCl solution, and the lower phase was collected and washed three times with an equal volume of theoretical upper phase (chloroform-methanol-0.9% NaCl, 3:45:47, by vol.) containing 0.1% (w/v) of ethanolamine. The lower phase was evaporated to dryness *in vacuo*, the lipids were dissolved in 0.5 ml of chloroform and 0.1–0.4 ml was subjected to two-dimensional t.l.c. [Merck Silica Gel F₂₄₀: first dimension, chloroform-methanol-water, 19:7:1, by vol.; second dimension, chloroform-methanol-aq. NH_3 (sp.gr. 0.880)-water, 110:50:8:5, by vol.]. After radioautography for 2–7 days, the spots, identified by the use of phospholipid markers, were scraped off the plates, the phospholipids eluted with chloroform-methanol-water (25:25:1, by vol.), and the extracts assayed for total phosphorus, for ^{14}C by counting in a toluene scintillator fluid and for ^{32}P by Čerenkov counting.

Results

The uptake of [^{14}C]ethanolamine into *E. caudatum* phospholipids over 10 h is shown in Fig. 1(a). Clearly the fastest incorporation of the ethanolamine moiety

occurred into phosphatidylethanolamine. The uptake into ceramide phosphorylethanolamine was appreciably slower, but was much faster than that into *N*-(1-carboxyethyl)phosphatidylethanolamine. In *E. caudatum* phosphatidylethanolamine is the immediate lipid precursor of the latter phospholipid (Coleman *et al.*, 1971). No incorporation of radioactivity into phosphatidylcholine was observed, and so the methylation pathway for the conversion of phosphatidylethanolamine into phosphatidylcholine observed in the aerobic protozoon *Tetrahymena pyriformis* (Smith & Law, 1970) is inoperative in *E. caudatum*.

Although the protozoa were well washed before incubation with the labelled ethanolamine, it is likely that they would still be contaminated with viable bacteria (White, 1969), which are a necessary prerequisite for their successful culture. However, when the bacteria-containing supernatant, obtained by spinning down the original culture fluid, was tested for its ability to incorporate [14 C]ethanolamine into phospholipids, this was insignificant compared with that of the protozoa (2% of that incorporated into protozoa after a 10h incubation). In these experiments on ethanolamine incorporation the ceramide phosphorylethanolamine and phosphatidylethanolamine separated were contaminated with their aminoethylphosphonate-containing analogues (Dawson & Kemp, 1968). However, no evidence could be obtained of any significant incorporation of radio-

activity from [14 C]ethanolamine into these analogues, which agrees with previous results on the synthesis of phosphonolipids within the organism (Coleman *et al.*, 1971).

If the protozoa were 'pulse'-labelled with [14 C]ethanolamine for 4h and then washed and incubated in the presence of non-radioactive ethanolamine, the phospholipid fractions behaved quite differently. Whereas the total radioactivity and specific radioactivity in the phosphatidylethanolamine fell somewhat during the 'chase', the incorporation of total radioactivity into ceramide phosphorylethanolamine contained to increase and its specific radioactivity rose (Fig. 1b). A similar relationship was observed when the ethanolamine-containing phospholipids were separated from their phosphonolipid analogues. Acid hydrolysis of the ceramide phosphorylethanolamine fraction to split off the phosphorylethanolamine moiety (Dawson & Kemp, 1967) showed that the incorporation had been exclusively into the ethanolamine moiety and not into the hydrophobic components, i.e. long-chain bases and fatty acids.

In further experiments the protozoa were incubated with [32 P]phosphatidylethanolamine. Being a particulate substrate, it is likely that some of the phospholipid would be ingested and digested in the food vacuoles. This is probably the origin of the high-specific-radioactivity lysophosphatidylethanolamine that accumulates in the organism (Fig. 2). Examination of the water-soluble fraction also revealed the

Fig. 1. Incorporation of [14 C]ethanolamine into the phospholipids of *E. caudatum*

(a) Continuous labelling; (b) 'pulse'-labelling for 4h. Experimental details are given in the text. $\circ$, Phosphatidylethanolamine; Δ , ceramide phosphorylethanolamine; $\square$, *N*-(1-carboxyethyl)phosphatidylethanolamine; $\bullet$, phosphatidylcholine.

Fig. 2. Phospholipid labelling on incubating *E. caudatum* cells with [32 P]phosphatidylethanolamine

Experimental details are given in the text. ▲, Lyso-phosphatidylethanolamine; ○, phosphatidylethanolamine; △, ceramide phosphorylethanolamine; □, *N*-(1-carboxyethyl)phosphatidylethanolamine.

presence of glyceryl[32 P]phosphorylethanolamine and glycerol[32 P]phosphate, in confirmation of results obtained in previous studies (Coleman *et al.*, 1971). However, examination of the ceramide phosphorylethanolamine and *N*-(1-carboxyethyl)phosphatidylethanolamine fractions reveals a steady increase in radioactivity during the experimental period. Since the incubations were performed in a medium containing phosphate buffer, it seems highly unlikely that this incorporation represents a total breakdown to inorganic [32 P]phosphate followed by synthesis of the phospholipids *de novo*. In fact, experiments with [32 P]phosphate confirmed that under such incubation conditions the incorporation into ceramide phosphorylethanolamine and *N*-(1-carboxyethyl)phosphatidylethanolamine would be negligible.

Discussion

The 'pulse-chase' experiments with [14 C]ethanolamine indicate that the ceramide phosphorylethanolamine is not being labelled by synthesis *de novo* through a CDP-ethanolamine pathway, as presumably occurs during phosphatidylethanolamine synthesis, but from a pre-labelled pool. Phosphatidylethanolamine is the only obvious candidate for the latter pool, and it would certainly fulfill the necessary

isotopically labelled precursor requirements. Further, [32 P]phosphatidylethanolamine transfers radioactivity to ceramide phosphorylethanolamine by a process that is apparently not dependent on complete degradation followed by synthesis *de novo*. Presumably this process occurs after some of the [32 P]phosphatidylethanolamine escaping digestion in the food vacuoles has been incorporated as a molecule into the cellular membranes, as envisaged previously (Coleman *et al.*, 1971).

If phosphorylethanolamine is transferred as a unit from phosphatidylethanolamine, it seems likely that this must be through some type of direct exchange of a ceramide with the diglyceride unit. Thus there is no evidence of any substantial release of [32 P]phosphorylethanolamine from [32 P]phosphatidylethanolamine sufficient to account for ceramide phosphorylethanolamine synthesis. The main route of degradation is through lysophosphatidylethanolamine, glycerylphosphorylethanolamine and glycerophosphoric acid. The small amounts of 32 P-labelled phosphorylethanolamine released by *E. caudatum* from [32 P]phosphatidylethanolamine observed in a previous investigation are probably derived by catabolism of the ceramide [32 P]phosphorylethanolamine produced (Hildenbrandt *et al.*, 1971). The conclusions reached about the synthesis of ceramide phosphorylethanolamine after 'pulse'-labelling with [14 C]ethanolamine and incubation with [32 P]phosphatidylethanolamine would equally apply to the formation of *N*-(1-carboxyethyl)phosphatidylethanolamine. This confirms previous results obtained with *E. caudatum*, when it was concluded that the latter phospholipid was formed by a reaction between phosphatidylethanolamine and a C_3 intermediary of glycolysis (Coleman *et al.*, 1971).

Recently evidence has been obtained for a possible transfer of a phosphorylcholine moiety from phosphatidylcholine to form sphingomyelin in transformed mouse fibroblasts (Diringer *et al.*, 1972). Again the radioactivity in the sphingomyelin fraction continued to increase after a 'pulse'-labelling with radioactive choline or inorganic phosphate, whereas that in the phosphatidylcholine fraction decreased. It could be that pathways of this type are widespread for forming ceramide-containing phospholipids throughout the animal kingdom.

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