Regulation of Phospholipid Metabolism in Differentiating Cells from Rat Brain Cerebral Hemispheres in Culture

II. INCORPORATION OF [U-14C]ETHANOLAMINE INTO 1-ALKENYL, 2-ACYL- AND 1,2-DIACYL-ETHANOLAMINE PHOSPHOGLYCERIDES*

(Received for publication, June 10, 1974)

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SUMMARY

Cultured dissociated cells from rat embryo cerebral hemisphere incorporate [3H]- and [U-14C]ethanolamine into cellular lipids. Nearly all radioactivity in the lipid fractions is incorporated into 1,2-diacetylethanolamine phosphoglycerides and 1-alkenyl, 2-acylethanolamine phosphoglycerides (plasmalogens). Kinetic data suggest that the rate of labeling of both ethanolamine phospholipids from the phosphoethanolamine is similar.

A relative increase of the plasmalogen labeling is observed when free ethanolamine is continually present in the medium. The rate of incorporation of label from ethanolamine and phosphorylethanolamine into lipids was measured using a double label technique. Based upon these studies, an independent labeling pattern of the ethanolamine moiety of plasmalogens is suggested. A relative delay for the incorporation of label in plasmalogens could be explained by the presence of a variety of cell types which may differ in their capacity for phospholipid biosynthesis.

The rate of incorporation of phosphorylethanolamine into the phosphatidylethanolamine was not affected by the presence of high concentrations of either choline or serine.

Mammalian brain tissue contains relatively high levels of phospholipids which are synthesized at a high rate during the period of brain development (1, 2). Alkenyl-ether phospholipids (plasmalogens) are characteristic lipid components of brain tissue and are actively deposited during the myelination period (3-6). The biosynthetic steps responsible for alkenyl-ether lipids formation are reviewed in a recent monograph (7). Some of these individual reactions have also been demonstrated in brain tissue (8-11). Studies by Horrocks et al. (12) suggested that the alkenyl-ether

* This work was supported by United States Public Health Service Grants HD 05515, HD 04147, and NS 10330, and by Grant 906 from the Maternal and Child Health Project.
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1 The abbreviations used are: alk-1-enyl-EPG, 1-alkenyl, 2-acylethanolamine phosphoglyceride; EPG, 1,2-diacetylethanolamine phosphoglyceride; TLC, thin layer chromatography.
ml of ethyl formate and the solvents were evaporated to dryness under nitrogen. The residue was dissolved in 2 ml of methanol-water-chloroform (48:46:3 by volume) and after the addition of 2 ml of chloroform, the tubes were mixed vigorously for 2 min at 55°. The layers were cleared by a brief centrifugation and separated. The upper phase contained glycerophosphorylethanolamine obtained by two-dimensional TLC were subjected to alkaline hydrolysis as described. Aliquots of the chloroform phase after partition, and the radioactive content was below the level of accurate quantification. This fraction might represent the alkyl derivative of ethanolamine phosphoglycerides.

**Method 2: Alkaline Hydrolysis**—The ethanolamine-containing phospholipids obtained by two-dimensional TLC were subjected to alkaline hydrolysis as described. Aliquots of the chloroform layer containing labeled nonasaponifiable lipids were chromatographed on precoated Silica Gel G plates on two dimensions. Approximately 88 to 90% of the total radioactivity migrated in the first solvent system with an Rf corresponding to lyso-alkenylethanolamine phosphoglyceride. Approximately 10 to 15% had an Rf value slightly below the EPG standard. The plate was exposed to HCl fumes and was chromatographed in the second direction. The major radioactive spot corresponding to the lysoalkenyl derivative remained at the origin of the second direction, indicating that the vinyl-ether linkage had been cleaved, the label remaining in the glycerophosphorylethanolamine moiety. One minor unidentified radioactive compound migrated behind the lyso derivative of alk-1-enyl-EPG. The remaining radioactivity was equally distributed between sphingomyelin and choline phosphoglyceride.

**Identification of Ethanolamine Phosphoglycerides**

Alk-1-enyl-EPG and EPG were identified by selective mild acidic or alkaline hydrolysis combined with TLC separation.

**Method 1: Acid Hydrolysis**—Aliquots of the total lipid extract were chromatographed on precoated Silica Gel G plates (10 x 10 cm) and lipids were separated (19) in order to cleave the alkenylether bonds. The plates were exposed to HCl fumes between the first and the second solvent system (20). A brief exposure to iodine vapors was employed to locate the areas containing lipid which were then scraped from the plate and counted.

For further identification, the area corresponding to standard EPG, after acid hydrolysis and subsequent TLC, was scraped from the plate and was eluted and subjected to alkaline hydrolysis as described. Approximately 5 to 8% of the radioactivity was detected in the lower chloroform phase after partition, and the phosphate content was below the level of accurate quantification. This fraction might represent the alkyl derivative of ethanolamine phosphoglycerides.

**RESULTS**

**Distribution of [U-14C]Ethanolamine Label into Phospholipids**

Cultured brain cells incubated in the presence of [U-14C]ethanolamine incorporate label into the ethanolamine phosphoglycerides. Two-dimensional thin-layer chromatographic separation of the alkyl-acyl (alk-1-enyl-EPG) and of the diacyl (EPG) type ethanolamine phospholipids was achieved by exposure to HCl fumes. Approximately 92 to 96% of the radioactivity in the lipid fraction was recovered in those two compounds. Cellular protein was determined according to Lowry et al. (21).

**Table I**

| Culture age | Alk-1-enyl-EPG | EPG | Alk-1-enyl-EPG/EPG (mass ratio) |
|-------------|---------------|-----|-------------------------------|
| days        | mmol phosphate/mg protein |       |                               |
| 2           | 15.8 (2)      | 32.0 (2) | 0.49                           |
| 4           | 21.5 (1)      | 39.5 (1) | 0.54                           |
| 6           | 23.6 (0)      | 48.0 (0) | 0.49                           |
| 9           | 35.1 (1)      | 68.5 (1) | 0.60                           |
| 12          | 39.2 (2)      | 60.5 (2) | 0.65                           |
| 16          | 42.5 (4)      | 62.7 (4) | 0.68                           |
| 19          | 51.4 (1)      | 61.5 (1) | 0.84                           |
| 27          | 46.3 (2)      | 59.2 (2) | 0.78                           |
| 37          | 50.8 (2)      | 62.5 (2) | 0.81                           |

A minor, unidentified radioactive compound migrated behind the lyso derivative of alk-1-enyl-EPG. The remaining radioactivity was equally distributed between sphingomyelin and choline phosphoglyceride.

**Alk-1-enyl-EPG and EPG Levels in Growing Brain Cultures**

Most of the label present in the lipid fraction from the [U-14C]ethanolamine was incorporated in the alk-1-enyl-EPG and EPG. Since the developmental patterns of rat brain lipids indicated a considerable increase in the content of alk-1-enyl-EPG (3-5), the levels of these compounds were determined in cultured rat brain cells. At 2 days in culture, when the cells were at their initial stage of morphological differentiation as judged microscopically by the presence of neuritic extensions, the levels of alk-1-enyl-EPG and EPG are 15.8 and 32.0 nmol/mg of protein, respectively (Table I). The EPG approaches maximum levels by the second week in culture, while alk-1-enyl-EPG levels are still increasing. Microscopic observations of the brain cells at this time revealed the presence of long and thick neuritic processes which may indicate a state of differentiation (23).

**Time Course for [U-14C]Ethanolamine Incorporation in Alk-1-enyl-EPG and EPG**

When 7-day-old cultures were incubated with [U-14C]ethanolamine for various periods of time, the specific radioactivities of both alk-1-enyl-EPG and EPG were constantly increasing (Table II). The specific radioactivities were low at the initial times examined; however, that of EPG was greater than that of alk-1-enyl-EPG. The specific activity of the alk-1-enyl-EPG was greater than that of EPG after 6 hours and at all subsequent times examined. The ratio of specific radioactivity of alk-1-enyl-EPG to EPG at 24 hours was about 4-fold higher than that observed after 5 min.

**Labeling Patterns of Ethanolamine-containing Phospholipids from Ethanolamine and Phosphorylethanolamine**

The incorporation of [U-14C]ethanolamine into lipids was greatly impaired when the incubation temperature was reduced from 37 to 15 or 22° as previously demonstrated (15). After 6 hours of incubation at 15°, the incorporation of labeled ethanolamine into alk-1-enyl-EPG and EPG was reduced more than 50-fold and 20-fold, respectively (Table III). The ratio of specific activity of alk-1-enyl-EPG to EPG was greater at 37 than at 15°. At 22°, the incorporation of
Brain cultures were incubated with [U-14C]ethanolamine (5 µM, 1.81 X 10^6 cpm/petri dish) for various periods of time. Determination of the specific activities of alk-1-enyl-EPG and EPG is described under "Materials and Methods." Values expressed as counts per min/nmol of phosphorus represent pooled lipid extracts of 2 petri dishes.

| Incubation time | Specific radioactivity | Alk-1-enyl-EPG:EPG ratio |
|-----------------|------------------------|--------------------------|
| hours           | cpm/nmol lipid phosphorus |                          |
| 0.08            | 15                      | 41                       | 0.30 |
| 0.25            | 20                      | 49                       | 0.41 |
| 0.75            | 71                      | 103                      | 0.69 |
| 1.50            | 220                     | 286                      | 0.77 |
| 3.0             | 743                     | 786                      | 0.94 |
| 6.0             | 3194                    | 2790                     | 1.14 |
| 8.0             | 4452                    | 3540                     | 1.26 |
| 24.0            | 7569                    | 5079                     | 1.49 |

Table III

Effect of incubation temperature on [U-14C]ethanolamine uptake into ethanolamine phosphoglycerides

Cultures were incubated with [U-14C]ethanolamine (5 µM, 1.81 X 10^6 cpm) for 6 hours at various temperatures as indicated and the specific radioactivities were determined as described under "Materials and Methods." For Experiment II, cells were further incubated for 3 hours at 37° after removal of the radioactive medium. Values expressed as counts per min/nmol of phosphorus represent pooled lipid extracts of 3 petri dishes.

| Incubation conditions | Specific radioactivity | Alk-1-enyl-EPG:EPG ratio |
|-----------------------|------------------------|--------------------------|
| hours                 | cpm/nmol P_i          |                          |
| I. 6 hours at 15°     | 50                     | 122                      | 0.41 |
| II. 6 hours at 22°    | 101                    | 238                      | 0.43 |
| III. 6 hours at 37°   | 265                    | 643                      | 0.41 |
|                       | 2813                   | 2597                     | 1.08 |

This observation prompted us to examine the metabolic turnover of phosphorylethanolamine and free ethanolamine in relation to alk-1-enyl-EPG and EPG. Brain cultures were incubated with [U-14C]ethanolamine for a period of 3 hours. This procedure enabled us to label the endogenous pool of phosphorylethanolamine since preliminary studies indicated that phosphorylethanolamine could not be taken up directly by the cells. The 14C-containing medium was removed and fresh medium containing a small quantity of [3H]ethanolamine (0.75 µM, 3.98 X 10^4 cpm/petri dish) was then added. Cultures were incubated at 37° and at the times indicated they were harvested and the per cent distribution of label in the medium (m, 3H; p, 14C) in the water-soluble compounds (0, H; o, 14C) and in the lipids (A, 3H; A, 14C) was determined as described (15). Determination of 14C and 3H was performed by the channel ratio method using a Packard model 3058B instrument. Under those conditions no tritium was detected in the 14C channel while 7.5% of the 14C detected in the 3H channel was corrected accordingly. Values are expressed as per cent of tracer present in the medium, in the water-soluble compounds, and in the lipids at the beginning of the second incubation period. The values represent mean analysis of two to three cultures. E, ethanolamine; PEa, phosphorylethanolamine.
bation in the presence of [3H]ethanolamine, the specific radioactivities of alk-1-enyl-EPG and EPG were 146 and 215, respectively (Table IV). Between the 1st and the 5th hour, the incorporation of alk-1-enyl-EPG and EPG compounds was constant as described under "Materials and Methods." Values are expressed as per cent of total radioactivity and represent 3 pooled dishes. The counts in the medium are expressed as per cent S.E.M. determined as described under "Materials and Methods."'

**TABLE IV**

| Experimental conditions | Radioactivity distribution | Specific radioactivity |
|-------------------------|---------------------------|-----------------------|
|                         | Medium | Water-soluble fractions | Lipids | Alk-1-enyl-EPG | EPG | Alk-1-enyl-EPG:EPG ratio |
| Exposed to 5 μM [14C]ethanolamine for 6 hours. Chase in the presence of: |          | %                        | cpm/μmol lipid phosphorus |   |   |                      |
| 2 mM choline             | 12.5 ± 1.8 | 49.9 | 44.6 | 3884 | 3588 | 1.08 |
| 2 mM ethanolamine        | 41.3 ± 6.6 | 37.7 | 21.0 | 2151 | 1342 | 1.60 |
| 2 mM serine              | 11.9 ± 0.8 | 40.0 | 48.1 | 3285 | 3426 | 1.11 |
| No base addition         | 9.4 ± 1.1  | 44.2 | 46.4 | 4238 | 3888 | 1.09 |

**FIG. 2.** Changes of the specific activities in the major ethanolamine labeled compounds after consecutive incubation of brain cells with [14C] and [3H]ethanolamine. Experimental conditions were the same as described for Fig. 1. The specific activities expressed as counts per min/μmol of lipid phosphorus represent the material obtained from pooled extracts of two to three cultures. PEs, phosphorylethanolamine; PEPG, alk-1-enyl-EPG.

This report provides further evidence for the role of phosphorylethanolamine as the major precursor for the ethanolamine moiety of phospholipids in cultured brain cells. During a 24-hour exposure to [U-14C]ethanolamine there is a greater incorporation into alk-1-enyl-EPG than EPG (Table II). This is concluded from results of studies in which cells prelabeled with [U-14C]ethanolamine continued to produce labeled alk-1-enyl-EPG and EPG even after the removal of labeled ethanolamine (Tables III and IV, Fig. 2). In the absence of free ethanolamine, there was no subsequent increase of the alk-1-enyl-EPG specific radioactivity over that of EPG. When a small dose of [3H]ethanolamine is added to the medium the specific activity of [3H]alk-1-enyl-EPG was greater than that of [3H]EPG (from 0.68 to 0.92). The incorporation of label from the [14C]phosphorylethanolamine precursor into both ethanolamine phospholipids under those conditions proceeded at the same rate (0.7) (Fig. 2). By employing this double label approach it seems possible to differentiate between the metabolic roles of phosphorylethanolamine and ethanolamine as precursors of phospholipids. In order to explain the relative increase in the alk-1-enyl-EPG labeling, an additional sequence of reactions which is dependent on the availability of free ethanolamine is suggested. This is summarized in the following scheme where PEA is phosphorylethanolamine and PEPG is alk-1-enyl-EPG.

The direct conversion of [3H]phosphorylethanolamine to [3H]alk-1-enyl-EPG (Reaction Sequence I) seems to lag in its capacity of labeling this compound. This is seen from the precursor product-time relationship (Table II and Fig. 2). The lag period could be explained by a compartmentalization of Reaction Sequences I and II. It has already been pointed out that the disso-
certain types of cells will rapidly take up ethanolamine and 
neuroglia, and ependymal-like cells (23). Thus, it is possible that 
associated brain cell cultures contain a mixed population of neurons, 
EPG and EPG at the same rate, as suggested by the data pre-
phorylethanolamine would then be incorporated into alk-1-enyl-
cumulate it as a phosphorylated compound. The resulting phos-
rate ethanolamine more efficiently in alk-1-enyl-EPG than in 

time cannot be excluded.

reflect a different site of synthesis of certain species of alk-1-enyl-
alk-1-enyl-EPG labeling at the initial time points studied may 
ations, such a possibility has to be considered. The slow rate of 
may exhibit a greater lipid synthesizing capacity might incorpo-
rate ethanolamine more efficiently in alk-1-enyl-EPG than in 

brain cultures, in some cases up to 2 mm, has been docu-
mented (23). Studies employing labeled precursors indicated an 
active transport of a variety of metabolites along the axons (25). 
The possibility that phosphorylethanolamine is such a metabo-
lite cannot be excluded.

The physiological significance of the plasmalogen accumulation 
in the growing brain cells is as yet not clear (Table I). Histological 

related brain cell cultures contain a mixed population of neurons, 
neuroglia, and ependymal-like cells (23). Thus, it is possible that 
certain types of cells will rapidly take up ethanolamine and accu-
cumulate it as a phosphorylated compound. The resulting phos-
phorylethanolamine would then be incorporated into alk-1-enyl-
cumulate it as a phosphorylated compound. The resulting phos-
rate ethanolamine more efficiently in alk-1-enyl-EPG than in 

EPG. In view of the recent in vitro study by Binaglia et al. (24) on 
the phospholipid metabolism of neuronal and glial cells prepara-
tions, such a possibility has to be considered. The slow rate of 
alk-1-enyl-EPG labeling at the initial time points studied may 
reflect a different site of synthesis of certain species of alk-1-enyl-
EPG within the cell. The presence of long neuritic extensions in 
the brain cultures, in some cases up to 2 mm, has been docu-
mented (23). Studies employing labeled precursors indicated an 
active transport of a variety of metabolites along the axons (25). 
The possibility that phosphorylethanolamine is such a metabo-
lite cannot be excluded.

The physiological significance of the plasmalogen accumulation 
in the growing brain cells is as yet not clear (Table I). Histological 

stainings employing luxol dye techniques (26) of 3- to 4-week-old 
brain cultures did not reveal the presence of myelin such as those 
observed in cerebellar explants (27). Biochemical parameters 
such as activities of synthetic enzymes and accumulation of 
“characteristic myelin lipids” predates the histological appear-
ance of myelin in the brain (1, 2, 28-31). It is possible that in 
this culture system, which is cerebral in origin, alk-1-enyl-EPG 
is synthesized and accumulated in certain cell types, and initia-
tion (microscopically undetectable) of myelin formation may 
indeed occur.

Further analysis of the various cell types and their metabolic 
capacity to utilize the free bases for phospholipid biosynthesis is 
in order.

Acknowledgments—The authors wish to express their apprecia-
tion to Dr. Z. Yavin for providing the brain cultures.

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J. Biol. Chem. 1975, 250:2891-2895.

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