THE SYNTHESIS OF CHOLINE PLASMALOGEN BY THE METHYLATION PATHWAY IN RAT BRAIN

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1. Introduction

The *N*-methylation of phosphatidylethanolamine (ptd-ethanolamine) to phosphatidylcholine (ptd-choline) has been demonstrated in rat brain in vitro [1–4]. Experimental evidence has been also obtained that synthesis of choline plasmalogen may take place in brain microsomes by a similar pathway [4]. Labelled lysophosphatidylcholine was in fact isolated after incubation of rat brain microsomal membranes with *S*-adenosyl-L-[*methyl*-^{3}H]methionine with a maximal incorporation at pH 8.2 [4].

Although the action of phospholipases A on the ptd-choline synthesized from ptd-ethanolamine could be responsible for this finding, we have considered the possibility that the lysocompound originates from choline plasmalogen, thus indicating a methylation of ethanolamine plasmalogen. In fact, because of the acidic extraction procedure [4] the cleavage of the vinyl-ether linkage of plasmalogen occurred, giving rise to the formation of the corresponding labelled lysocompound.

Experimental data are here reported which indicate, with the aim of a suitable extraction procedure, the direct transfer of methyl groups from labelled *S*-adenosyl-L-methionine (SAM) into ethanolamine plasmalogen, thus indicating a possible pathway for choline plasmalogen synthesis both in whole brain homogenate and in brain microsomes in vitro.

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2. Materials and methods

2.1. *Purification of choline and ethanolamine plasmalogen*

Choline-containing lipids and ethanolamine phosphoglycerides were purified on aluminum oxide from a Folch extract obtained from beef heart [5]. Choline phosphoglycerides, obtained by further purification with silicic acid columns [5], and ethanolamine phosphoglycerides were then incubated with *Rhizopus delemar lipase* (Seikagaku Kogio, Tokyo) and the corresponding plasmalogen purified [6].

2.2. *Synthesis of choline plasmalogen in brain homogenate*

Wistar male rats, of 28–30 days of age, were sacrificed by decapitation and the whole brain was quickly homogenized in 0.32 M sucrose. The homogenate (0.3–0.4 mg protein) was incubated at 37°C for different time intervals in a medium (total vol. 80 μl) containing 60 mM Tris–HCl buffer (pH 8.2) and 1.8 μM of a freshly prepared solution of *S*-adenosyl-L-[*methyl*-^{3}H]methionine (The Radiochemical Centre, Amersham, spec. act. 68 Ci/mmol). At the end of incubation, given amounts of authentic choline plasmalogen were added to the samples and lipid extracted with hexane–isopropanol (3:2, v/v) [7]. The organic phase was dried under a stream of nitrogen and stored in *n*-hexane.

In another series of experiments, 54–160 nmol ethanolamine plasmalogen were added to the incubation medium, by sonicating for 15 min at 37°C under a continuous nitrogen stream the lipid in the incubation buffer using a 100 W MK-2 ultrasonic disintegrator (MSE, England). Incubation was then carried out as

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above.

Lipids, resuspended in chloroform–methanol (2:1, v/v), were applied after incubation on silica gel-G thin-layer chromatographic (TLC) plates and chromatographed [8] to separate choline plasmalogen from the corresponding diacylglycerophospholipid. After exposure to iodine vapors, lipids were detected and the spots scraped off from the plates. The radioactivity was measured in 1 ml methanol–water (1:1, v/v)–10 ml Packard 299 Emulsifier Scintillator, on a Tri-Carb 460 C Packard scintillation spectrometer. Protein was determined by the Lowry method [9].

2.3. *Synthesis of choline plasmalogen in brain microsomes.*

Microsomes obtained from rat cerebral cortex [10] in 0.32 M sucrose–2 mM dithioerythritol (DTE) were resuspended in 0.32 M sucrose–2 mM DTE and incubated in a medium similar to that used for the homogenates. Lipids were extracted and analyzed, as described. In an additional series of experiments, exogenous authentic ethanolamine plasmalogen was added to the incubation mixture.

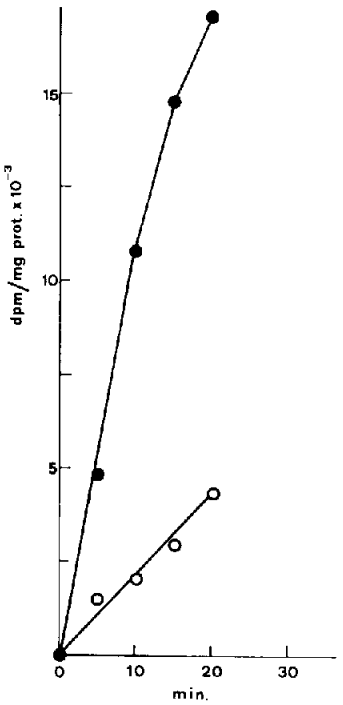

Fig.1. Incorporation of methyl groups into ptd-choline (•) and choline plasmalogen (○). Brain homogenates were incubated as described in the text with labelled SAM for various time intervals. Results are expressed as dpm . mg protein^{-1} . 10^{-3}.

3. Results

In a first series of experiments, whole brain homogenates were incubated with radioactive SAM for various time intervals. Fig.1 shows the incorporation of methyl groups into ptd-choline and choline plasmalogen. It is apparent that choline plasmalogen incorporates labelled methyl groups, with a linear time–activity relationship, although the degree of incorporation is lower than into ptd-choline.

In other experiments ethanolamine plasmalogen prepared from beef heart was added to the incubation mixture (see section 2). At shorter times of incubation no clear effect was obtained, possibly because of the physical state of the exogenous substrate, which hindered a close contact with SAM. After 20 min incubation, however, an increase of the rate of incorporation of labelled methyl groups into choline plasmalogen takes place, depending on the amount of the ethanolamine plasmalogen added to the incubation medium. No effect, or even a small decrease, is observed for the incorporation of SAM into ptd-choline (table 1).

Although the time–activity relationship and the effect of adding exogenous ethanolamine plasmalogen were similar in all the experiments, the absolute val-

Table 1
The effect of the addition of beef heart ethanolamine plasmalogen on the incorporation of labelled methyl groups into choline lipids

Ethanolamine plasmalogen added (μmol)	Incorporation ratios[a]	
	A	B
0.054	1.15	0.88
0.100	1.30	0.86
0.143	1.58	0.82
0.160	–	0.82

[a] A = incorporation into choline plasmalogen
B = incorporation into ptd-choline

Data represent the ratios between the incorporation in brain homogenates obtained in the presence of added ethanolamine plasmalogen and in its absence. Additional experiments have given comparable results

ues of incorporation of methyl groups into choline plasmalogen were different. In two experiments, 28-day-old rats, weighing less than normal, were used and in this case the rate of incorporation was very low.

This TLC system, useful for the analysis of choline plasmalogen, did not allow a good separation of ptd-ethanolamine, phosphatidyl-monomethylethanolamine (PME) and phosphatidyl-dimethylethanolamine (PDE), as demonstrated with the use of authentic pure standards. Accordingly, radioactivity into the ptd-ethanolamine spots was always found after incubation, indicating a possible contamination by PME. In addition, radioactivity into ethanolamine plasmalogen spot was always found after incubation, indicating the involvement in the methylation pathway of the mono- and/or dimethyl-ethanolamine plasmalogens. The radioactive ethanolamine plasmalogen will be here indicated as 'methylated' ethanolamine plasmalogen.

Table 2 shows the degree of incorporation of methyl groups into ptd-choline, choline plasmalogen and the 'methylated' ethanolamine plasmalogens. It is clear that the incorporation of methyl groups into choline plasmalogen varies much more than into the other phospholipids.

The synthesis of choline plasmalogen by the *N*-methylation pathway has been analyzed also in brain microsomes. Results are presented in fig.2. It is apparent that ethanolamine plasmalogen incorporates methyl groups from SAM although at a lesser extent than ptd-ethanolamine. Fig.2 shows the incorporation obtained when exogenous ethanolamine plasmalogen is added to the incubation mixture. The increase due to the addition of exogenous substrate starts only after 15–20 min incubation.

Table 2
The incorporation of methyl groups into phosphatidylcholine, 'methylated' ethanolamine plasmalogen and choline plasmalogen of rat brain homogenates

Ptd-choline	'Methylated' ethanolamine plasmalogen	Choline plasmalogen
11 407	10 543	7744
10 021	8206	8543
8726	6178	1414
10 089	6834	1082
13 000	10 000	3313
17 000	7000	4358

Incubations were carried out for 20 min, as described in the text. Data are reported as dpm . mg protein^{-1} . 20 min^{-1}

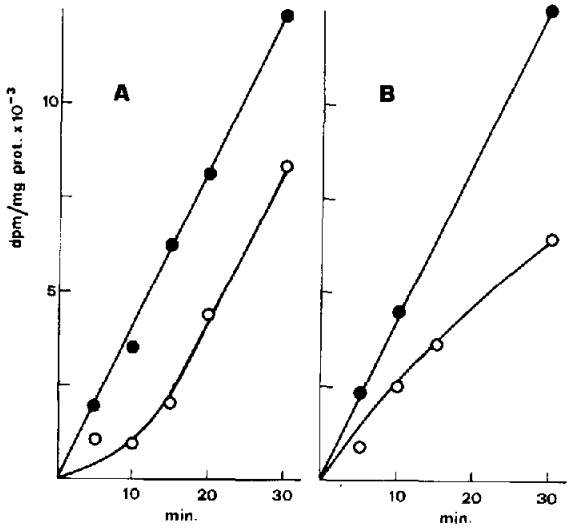

Fig.2. Incorporation of methyl groups into ptd-choline (•) and choline plasmalogen (○). Brain cortex microsomes were incubated as in the text with labelled SAM for various time intervals. In expt (A), 0.074 μmol ethanolamine plasmalogen were added to the incubation medium. Results are expressed as dpm . mg protein^{-1} . 10^{-3}.

4. Discussion

The mechanisms for the synthesis of choline plasmalogen in brain and other tissues are largely unknown. The results of this work provide evidence indicating that the *N*-methylation of ethanolamine plasmalogen represents in brain a pathway for the synthesis of this lipid. To better evaluate these results, it must be observed that although choline plasmalogens represent only a small amount of brain phospholipid, their turnover is very high in this tissue [11]. Choline plasmalogens are also differently distributed throughout the brain [12], and the results from this laboratory on the different distribution of methyl transferase activity in brain areas [13] may be relevant in this connection.

These results indicate a linear incorporation of methyl groups into choline plasmalogen both in homogenate and in brain microsomes up to 20 min of incubation. In all experiments a noticeable increase of the rate of incorporation was found when ethanolamine plasmalogen was added to the incubation mix-

ture. The increase was evident only after 10–20 min of incubation, probably due to the physical state of ethanolamine plasmalogen, the methyl acceptor. In all experiments cold choline plasmalogen, added after incubation, was cochromatographed with the newly formed labelled product. All these results clearly indicate a synthesis of choline plasmalogen by methylation in brain.

The rate of synthesis of choline plasmalogen seems to depend on the nutritional state and on the health of the animals. These factors presumably affect more choline plasmalogen synthesis than the incorporation of the first and second methyl groups into ethanolamine plasmalogen. A definite answer to this problem can be given only by preparing pure monomethyl- and dimethyl-ethanolamine plasmalogens and by carrying out separation of these partially methylated forms after incubation.

Choline plasmalogens are differently distributed in cerebral areas [12]. A study of the distribution of ethanolamine plasmalogen methyl transferase activities in different brain areas, similar to the investigation carried out for the synthesis of ptd-choline [13], seems therefore interesting in this regard.

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