CONVERSION OF PHOSPHATIDYLETHANOLAMINE TO PHOSPHATIDYLCHOLINE IN RAT BRAIN BY THE METHYLATION PATHWAY

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1. Introduction

Phosphatidylcholine (Ptd-choline) is synthesized in animal tissues chiefly by the cytidine pathway, although the stepwise methylation of endogenous phosphatidylethanolamine (Ptd-ethanolamine) and the base-exchange reaction also contribute to its production [1–7]. The *N*-methylation pathway was first demonstrated in vivo in the liver [3]. However, in vivo experiments [8] excluded the occurrence in brain of this pathway, although now contrary evidence has been presented [9,10].

Methyltransferase activity for Ptd-choline synthesis has been observed in liver and brain microsomes [10], using phosphatidyl-*N,N*-dimethylethanolamine as substrate. The authors found a very low activity in brain as compared to liver. The presence of two different enzymes has been reported [11,12] in adrenal medulla and erythrocyte membrane acting at different pH and involved in the methylation of Ptd-ethanolamine to Ptd-choline. The first enzyme catalyzes the synthesis of phosphatidyl-*N*-monomethylethanolamine from *S*-adenosylmethionine (SAM); the second the other two methylations. The aim of this work is to investigate the *N*-methylation pathway in brain at two different pH values to establish the activity of both enzymes. The transfer of the last methyl group was examined by adding phosphatidyl-*N,N*-dimethylethanolamine to the incubation medium. All experiments were carried out with rat brain homogenate prepared after prolonged cardiac perfusion to avoid red cell contamination.

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2. Materials and methods

Wistar male rats (day 28–30) were sacrificed by prolonged cardiac perfusion after light ether anesthesia. Brain cortex was removed and homogenized in 0.32 M sucrose. The hemoglobin content showed no erythrocyte contamination in the tissue [13]. The incubation mixture (total vol. 80 μl) contained, unless otherwise stated, 1.79 μM *S*-adenosyl-L-[*methyl*-^{3}H]methionine from The Radiochemical Center, Amersham (1.72 μCi), 10 mM $MgCl_2$, 60 mM sodium phosphate (pH 6.8) or Tris–HCl (pH 8.0) and brain homogenate protein (0.4–0.6 mg). Incubation was carried out at 37°C for different time intervals.

The reaction was terminated with 80 μl cold 10% trichloroacetic acid, and the precipitates washed 3 times after centrifugation with 4 ml cold H_2O. Extraction of the pellet was performed with 4 ml chloroform–methanol (2:1, v/v). After filtration and washing of the pellet with 1 ml chloroform–methanol as above, 0.2 ml 0.9% NaCl was added and the 2 phases separated. The organic phase was dried under a stream of nitrogen and stored in *n*-hexane. Controls were made throughout the work, denaturing the protein by heat (100°C) before the addition of labelled SAM. In all control experiments no detectable radioactivity in extract or separated lipid was ever detected.

In a different series of experiments 100 μg phosphatidyl-*N,N*-dimethylethanolamine (Mann Res. Labs., NY) were added to the incubation medium, by sonicating for 3 min the lipid in the incubation buffer with a 100 W MK2 ultrasonic disintegrator (MSE, England).

Phospholipids were analyzed by resuspending the sample in 300 μl chloroform–methanol (2:1, v/v);

an aliquot of the extract was used assaying total lipid radioactivity and the remainder, applied on silica gel G thin-layer chromatographic plates, was chromatographed with the following solvent systems, which were found suitable for Ptd-choline separation:

(a) Chloroform–methanol–water (65:25:4, v/v/v);
(b) Chloroform–propionic acid–*n*-propyl alcohol–water (2:2:3:1, v/v/v/v), chloroform–methanol–acetic acid–water (25:15:4:2, v/v/v/v).

The sample lipids and authentic lipid standards (Mann Res. Labs., NY) were detected on the same thin-layer chromatographic plate by exposure to iodine vapors or by spraying with 1% ninhydrin in ethanol; spots were then scraped off from plates and radioactivity measured in 3 ml water–10 ml Lumagel (Supelchem, s.r.l.) in a model 3330 Packard scintillation spectrometer. Protein was determined by the Lowry method [14].

3. Results

In a first series of experiments the brain homogenate was incubated with labelled SAM at pH 6.8 and 8.0 for different time intervals. Figure 1 shows the incorporation of [*methyl*-^{3}H]groups into the total lipid fraction following incubation without (expt A) and with (expt B) the addition of phosphatidyl-*N,N*-dimethylethanolamine. It is apparent that in expt A incorporation was higher at pH 8.0 than at pH 6.8 at any time of incubation. Moreover, the reaction levelled off after a few minutes. The addition of the lipid substrate for the second methyltransferase as in [11] increases noticeably the rate of incorporation at both pH values (fig.1B) with linearity up to 20 min of incubation. Although radioactivity was clearly distributed also in phosphatidyl-*N*-monomethylethanolamine and phosphatidyl-*N,N*-dimethylethanolamine, as observed by direct thin-layer chromatography of the extract (see section 2), 'the complete' Ptd-ethanolamine methylation is indicated in fig.2, where the labelling in the isolated, chromatographically pure Ptd-choline is shown. Very small methylation to Ptd-choline takes place at pH 6.8 as compared to pH 8.0 (fig.2A); it is noticeably increased by the addition of exogenous phosphatidyl-*N,N*-dimethylethanolamine (fig.2B). The incorporation rate of the methyl-groups is evident at pH 8.0. A linear time–activity relationship is also evident.

To identify by other means the final product of methylation, the Ptd-choline spot after incubation was eluted several times with chloroform, dried under a stream of nitrogen and treated with phospholipase D as in [15]. Radioactivity measurements on the aqueous phase indicated that almost all label was found in the choline moiety of the lipid.

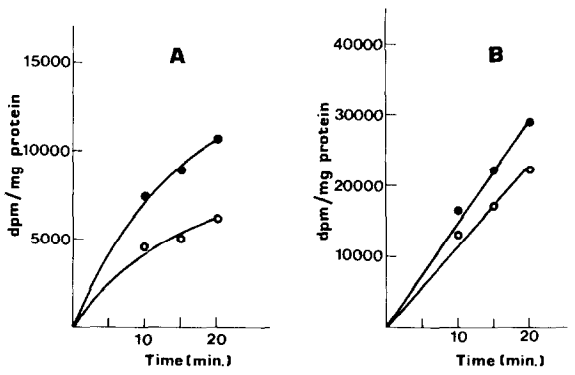

Fig.1. Time course of SAM incorporation into the total lipid fraction of rat brain. SAM was 1.79 μM in the medium. Incubation was carried out at pH 6.8 (○–○–○) or 8.0 (●–●–●) for the indicated time intervals. Experiments A and B were carried out respectively without and with the addition of phosphatidyl-*N,N*-dimethylethanolamine to the incubation medium. Activity is expressed as dpm/mg homogenate protein. Each point represents mean values from 6 experiments with 5–10% SD. See text for additional information.

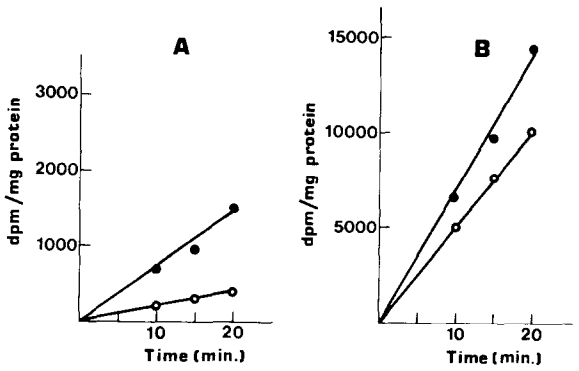

Fig.2. Time course of SAM incorporation into rat brain phosphatidylcholine. See fig.1 for explanation of symbols and further information. Mean values from 6 experiments with 5–10% SD.

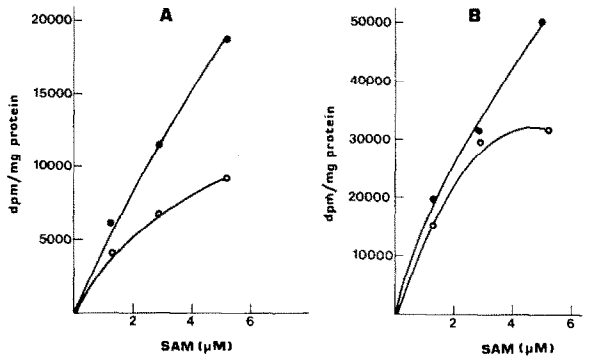

Fig.3. Incorporation of labelled *S*-adenosyl-L-methionine (SAM) into the total lipid fraction of rat brain at different substrate concentration. SAM concentration (μM) is plotted against dpm/mg homogenate protein. Incubation time, 15 min. Each point represents mean values from 5 experiments with 5–10% SD. See fig.1 for explanation of symbols and additional information.

The dependence of the rate of methylation versus SAM concentration is shown in fig.3, where different amounts of methyl donor have been incubated under standard conditions. The incorporation rate into the total lipid fraction was higher at pH 8.0 than at pH 6.8, and this difference was more striking at 5 μM substrate. The addition of the dimethylated Ptd-ethanolamine increased noticeably the rate of incorporation into lipid (fig.3B). Figure 4 indicates that at the highest SAM concentration a complete methylation of Ptd-ethanolamine to Ptd-choline is detectable also at pH 6.8, though lower than at 8.0 (fig.4A). The addition of phosphatidyl-*N,N*-dimethylethanolamine brings about an increase of methyl-group incorporation into Ptd-choline, which is no more linear with SAM concentration, particularly at pH 6.8.

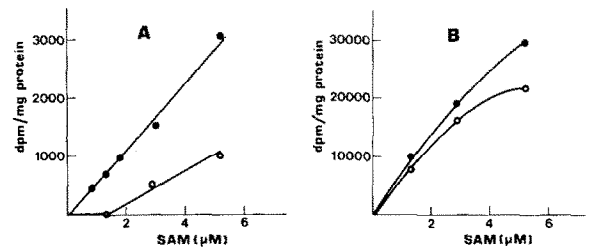

Fig.4. Incorporation of labelled *S*-adenosyl-L-methionine into rat brain phosphatidylcholine at different substrate concentration. Incubation time, 15 min. Each point represents mean values from 5 experiments with 5–10% SD. See fig.1 for explanation of symbols and additional information.

4. Discussion

The present data indicate that brain tissue is able to convert endogenous Ptd-ethanolamine to Ptd-choline by a stepwise methylation pathway. Complete methylation to Ptd-choline is poor at pH 6.8, and only higher concentrations of methyl donor allow the process. On the contrary, a complete synthesis of Ptd-choline takes place at pH 8.0 with linear relationships either versus time or substrate concentration. Higher rates of incorporation of labelled SAM into Ptd-choline are evident on adding sonicated phosphatidyl-*N,N*-dimethylethanolamine to the incubation medium. This result may either be due to simple activations of enzymes by the added lipid or to higher substrate availability for methyl transfer. The fact that almost similar levels of radioactivity of mono- and di-methylated forms of Ptd-ethanolamine are obtained with or without the addition of phosphatidyl-*N,N*-dimethylethanolamine favors the second hypothesis. The transfer of the third methyl group takes place efficiently either at pH 6.8 or 8.0, with higher activity at the second pH value.

Synthesis of Ptd-choline by the methylation pathway is rather low in the brain. The heterogeneity and complexity of this tissue may not exclude, however, that particular brain area or cell types be enriched in this metabolic pathway. The occurrence in brain of the *N*-methylation pathway might be of some importance due to the possibility for the nerve cell to enrich its Ptd-choline fraction of unsaturated fatty acids [16].

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References

[1] Kennedy, E. P. and Weiss, S. B. (1956) J. Biol. Chem. 222, 193–197.
[2] Weiss, S. B., Smith, S. W. and Kennedy, E. P. (1958) J. Biol. Chem. 231, 53–64.
[3] Bremer, J. and Greenberg, D. M. (1960) Biochim. Biophys. Acta 43, 477–488.
[4] Rehbinder, R. and Greenberg, D. M. (1965) Arch. Biochem. Biophys. 109, 110–115.
[5] Scarborough, G. A. and Nyc, J. F. (1967) J. Biol. Chem. 242, 238–242.
[6] Porcellati, G., Arienti, G., Pirotta, M. and Giorgini, D. (1971) J. Neurochem. 18, 1395–1417.
[7] Kanfer, J. N. (1972) J. Lipid Res. 13, 468–474.
[8] Ansell, G. B. and Spanner, S. (1967) J. Neurochem. 14, 873–885.
[9] Morganstern, R. and Abdel-Latif, A. (1974) J. Neurobiol. 5, 393–411.
[10] Skurdal, D. N. and Cornatzer, W. E. (1975) Int. J. Biochem. 6, 579–583.
[11] Hirata, F., Viveros, O. H., Diliberto, E. J. and Axelrod, J. (1978) Proc. Natl. Acad. Sci. USA 75, 1718–1721.
[12] Hirata, F. and Axelrod, J. (1978) Proc. Natl. Acad. Sci. USA 75, 2348–2352.
[13] Antonini, E. (1965) Physiol. Rev. 45, 123–170.
[14] Lowry, O. H., Rosenbrough, N. J., Farr, A. L., Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[15] Kates, M. and Sastry, P. S. (1969) Methods Enzymol. 14, 197–203.
[16] MacDonald, G. and Thompson, W. (1975) Biochim. Biophys. Acta 398, 424–432.